Exploring the therapeutic potential of the ayurvedic formulation *Vilwadi Gulika* through an integrated approach employing network pharmacology and microbiome analysis

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BY

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UNDER THE GUIDANCE OF

DR PAVITHRA N

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THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

Private University Established in Karnataka by ACT 35 of 2013

BENGALURU - 560064

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DECLARATION BY THE CANDIDATE

I declare that this thesis entitled "**Exploring the therapeutic Potential of Ayurvedic Formulation *Vilwadi Gulika* through an Integrated Approach Employing Network Pharmacology and Microbiome Analysis"** submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide Dr Pavithra N, Associate Professor, Functional Genomics and Bioinformatics (TDU). I also wish to inform that no part of the research has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged.

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CERTIFICATE

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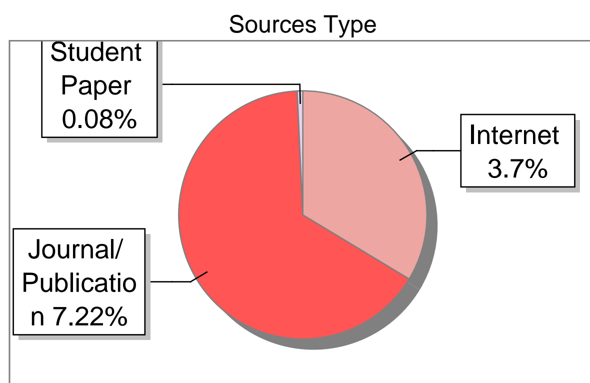

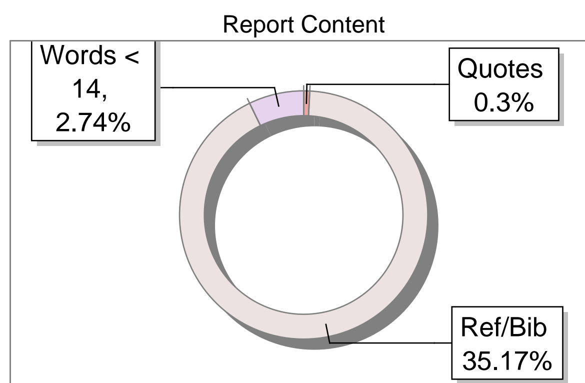

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Synopsis

Title

Exploring the Therapeutic Potential of Ayurvedic Formulation Vilwadi Gulika through an Integrated Approach Employing Network Pharmacology and Microbiome Analysis

Background and Rationale

Vilwadi Gulika is a classical Ayurvedic polyherbal formulation traditionally indicated in toxico-pathological and gastrointestinal conditions such as *Ajeerna* (indigestion due to impaired digestive function), *Visuchika* (acute gastrointestinal disturbance), *Jwara* (fever), *Sarpa Dansha* (snake bite envenomation), and disorders arising from impaired *Agni* (digestive and metabolic fire) and *Ama* (metabolic toxins) accumulation. Although widely used in clinical practice, the mechanistic basis of its therapeutic action remains inadequately explored at the molecular and microbial levels. Gastrointestinal disorders including colitis, Crohn's disease, ileocolitis, gastroesophageal reflux disease, and duodenal ulcer are complex conditions involving inflammatory signalling pathways, immune dysregulation, and gut microbiome imbalance. Integrating network pharmacology with microbiome analysis offers a systems-level approach to understand the multi-component and multi-target nature of classical Ayurvedic formulations.

Objectives

1. To investigate the molecular targets, pathways, and disease associations of Vilwadi Gulika using a network pharmacology framework.
2. To characterize the microbial composition and dynamics of Vilwadi Gulika prepared using in-house, Vaidya-made, and commercial methods through long-read 16S rRNA sequencing.

Methodology

Phytochemicals corresponding to the specific plant parts of thirteen medicinal plants constituting Vilwadi Gulika were curated from IMPPAT, Dr. Duke's Phytochemical and Ethnobotanical Database, and KNApSAck. Target prediction was performed using Binding DB and SwissTargetPrediction, followed by disease and pathway enrichment analysis using DAVID. Interaction networks were constructed in Cytoscape with a focus on gastrointestinal disorders.

For microbiome analysis, Vilwadi Gulika was prepared in-house using classical *Bhavana* with goat urine and sampled across preparation stages. Commercial and Vaidya-prepared formulations were also included. Metagenomic DNA was isolated using an optimized Qiagen DNeasy Power Food Microbial Kit protocol. Full-length 16S rRNA gene amplicons were sequenced on the Oxford Nanopore MinION platform. Reads were processed using a modified long-read bioinformatics pipeline with stringent quality and length filtering prior to taxonomic classification.

Results

Network pharmacology analysis identified 3,004 phytochemicals and highlighted key gastrointestinal disease-associated targets including TNF, PTGS2, JAK2, NOS2, SRC,

TGFB1, and CXCL8, implicating inflammatory and cytokine signalling pathways. Colitis-related targets (NOS2, SRC, PTGS2) were associated with ellagic acid, eugenol, elemicin, and ethyl gallate. Crohn's disease was linked to JAK2-associated terpenoids and alkaloids, while duodenal ulcer and ileocolitis were associated with TGFB1- and TNF-modulating phytochemicals.

Microbiome profiling revealed *Firmicutes* dominance across all formulations. In-house Vilwadi Gulika showed enrichment of *Aerococcus*, with *Aerococcus viridans* as the dominant species. Vaidya-made and commercial formulations were dominated by *Bacillus*, with the *Bacillus cereus* group prevalent at the species level. Fermentation stages exhibited increased microbial richness, evenness, and stabilization of community structure.

Conclusion

This study demonstrates that Vilwadi Gulika exerts its therapeutic potential through a dual mechanism involving multi-target phytochemical interactions and structured microbial communities. The integration of network pharmacology and microbiome analysis provides a mechanistic framework supporting its traditional use in gastrointestinal disorders and establishes a foundation for future functional and clinical investigations.

1.Abstract

Vilwadi Gulika is a classical Ayurvedic polyherbal formulation traditionally used for snake bites, insect bites, gastrointestinal disorders, toxicological conditions, and inflammatory states. Despite its long use, systematic scientific evaluation of its molecular mechanisms and microbial characteristics remains limited. In this study, the therapeutic potential of Vilwadi Gulika was investigated using an integrated approach combining network pharmacology and microbiome analysis.

Network pharmacology analysis was performed using phytochemicals derived from 13 medicinal plants, comprising of 3004 phytochemicals, curated based on the traditionally prescribed plant parts. Phytochemical data were retrieved from 3 databases (IMPPAT, Dr. Duke's Phytochemical and Ethnobotanical Database, and KNApSAck), followed by molecular target prediction using Binding DB and SwissTargetPrediction accumulating 68,635 targets. 1,347 Disease and 238 pathway annotation led to the construction of interaction networks with 1,047 genes. 6 gastrointestinal diseases were identified yielding 7 key genes, TNF, PTGS2, JAK2, NOS2, SRC, TGFB1, and CXCL8. Colitis-related targets (NOS2, SRC, PTGS2) were associated with ellagic acid, eugenol, elemicin, and ethyl gallate. Crohn's disease–associated JAK2 showed interactions with multiple terpenoids and alkaloids, while TGFB1 and TNF were linked to phytochemicals relevant to duodenal ulcer, ileocolitis, and regional enteritis.

Microbiome profiling was carried out across different stages of Vilwadi Gulika preparation, including in-house, Vaidya-prepared, and commercial formulations, using 16S rRNA gene sequencing. The in-house formulation was dominated by Firmicutes (97.37%), with Aerococcus (94.15%) and Aerococcus viridans (71.65%) as the predominant genus and species, respectively.

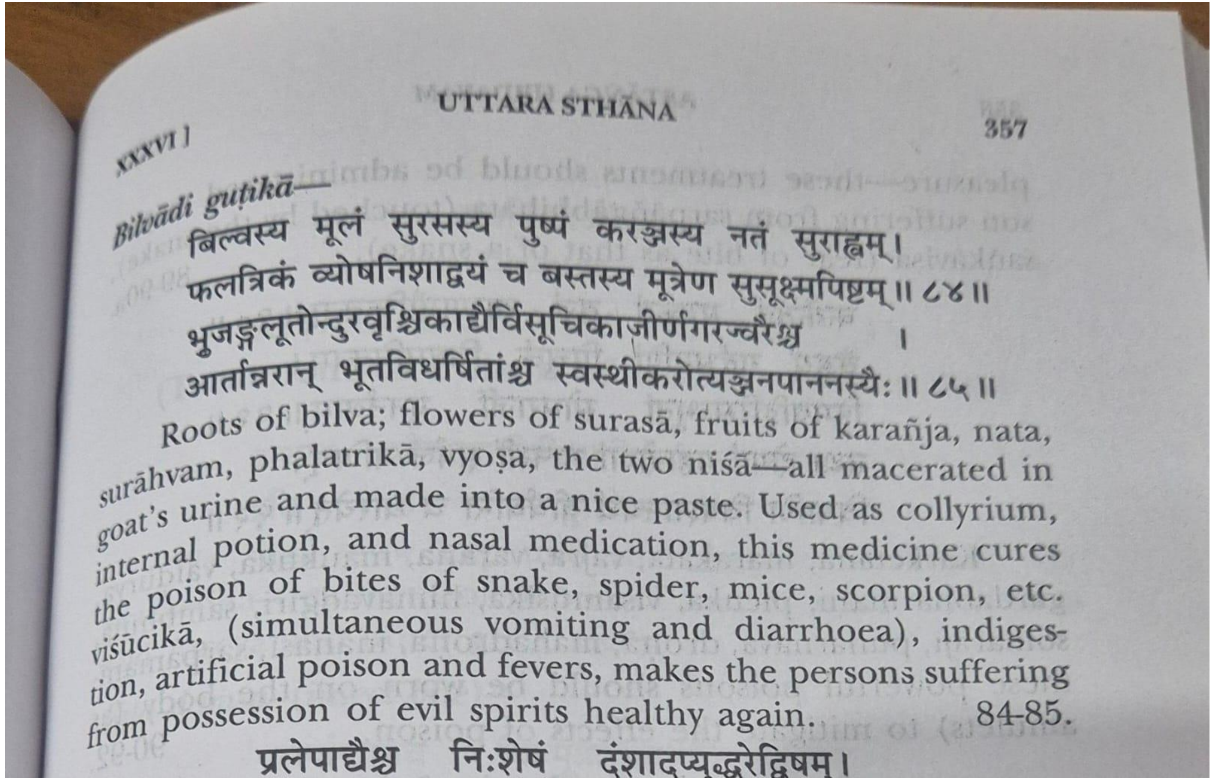
Overall, the findings indicate that Vilwadi Gulika exhibits a layered therapeutic profile involving inflammatory gene modulation and a structured microbial community, supporting its traditional use in gastrointestinal disorders.

1.1 Introduction

Ayurveda, which was originated approximately 5000-year ago in the Indus Valley region. The word Ayurveda is derived from the Sanskrit words Ayush, meaning life, and Veda meaning knowledge or science". The literal meaning of Ayurveda is "the science of life". "Ayurveda is a natural and holistic system of health care that focuses on maintaining balance in the body, mind, and spirit. The remedies offered by Ayurveda for illness are designed as preventive medicine that supports health and longevity[1]. In Ayurveda, various treatment approaches are used, including herbs, spices, special diets, polyherbal formulations, yoga meditation, massage therapy. These treatments are described in classical Ayurvedic texts such as the *Sushruta Samhita and Charaka Samhita"*[2].Oils and common spices are often used in Ayurvedic formulations, and polyherbal formulations typically contain multiple herbs. The Ayurvedic text *Sarangdhar Samhita* highlights the concept of polyherbalism in Ayurveda. Due to synergistic effects, polyherbal formulations provide greater therapeutic benefits compared to single-herb formulations. Ayurvedic medicine is divided into eight core divisions [3].

- Kayachikitsa: General medicine, medicine of the body.
- Kaumara-bhṛtya (Pediatrics): Discussions about prenatal and postnatal care of the baby and mother.
- Shalyatantra: Surgical techniques and the extraction of foreign objects.
- Shalakya Tantra: Treatment of ailments affecting openings or cavities in the upper body: ears, eyes, nose, mouth, etc.
- Bhuta Vidya: pacification of possessing spirits, and the people whose minds are affected by such possession.
- Agadatantra/Vishagara-vairodh Tantra (Toxicology): Includes epidemics; toxins in animals, vegetables, and minerals; and keys for recognizing those anomalies and their antidotes.
- Rasayana Tantra: Rejuvenation and tonics for increasing lifespan, intellect, and strength.
- Vajikaraṇatantra: aphrodisiacs; treatments for infertility problems.

The study of poisons is described in Agadtantra, which includes the effects, origins, traits, characteristics, types and management of toxins. Agadatantra also explains the symptoms, classification of poisons, and their treatments, including various Vishaghna Yogas (antitoxic formulations) that are useful in managing several pathological conditions such as toxic exposures and certain infectious diseases. Among the significant Agada formulations described in classical Ayurvedic texts, Vilwadi Agada is one of the most important. [4,5]. The classical description of Vilwadi Gulika as an Agada formulation is documented in Ayurvedic texts as illustrated in Figure 1.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Bilvādi guṭikā—

बिल्वस्य मूलं सुरसस्य पुष्पं करञ्जस्य नतं सुराह्वम्।
फलत्रिकं व्योषनिशाद्वयं च बस्तस्य मूत्रेण सुसूक्ष्मपिष्टम्॥ ८४॥
भुजङ्गलूतोन्दुरवृश्चिकाद्यैर्विसूचिकाजीर्णगरज्वरैश्च ।
आर्तान्नरान् भूतविधर्षितांश्च स्वस्थीकरोत्यञ्जनपाननस्यैः॥ ८५॥

Roots of bilva; flowers of surasā, fruits of karañja, nata, surāhvam, phalatrikā, vyoṣa, the two niśā—all macerated in goat's urine and made into a nice paste. Used as collyrium, internal potion, and nasal medication, this medicine cures the poison of bites of snake, spider, mice, scorpion, etc. viśūcikā, (simultaneous vomiting and diarrhoea), indigestion, artificial poison and fevers, makes the persons suffering from possession of evil spirits healthy again. 84-85.

प्रलेपाद्यैश्च निःशेषं दंशादप्युद्धरेद्विषम्।

Figure 1. Classical reference of Vilwadi Gulika as an Agada formulation is documented in Ayurvedic texts

Vilwadi Gulika, also known as Vilwadi Agada, Bilwadi Vati, and Bilwadi Gutika, is a multi-herbal Ayurvedic formulation traditionally used for the treatment of poisonous bites and various systemic illnesses. It is commonly used to manage toxic conditions that arise in the human body due to various underlying causes, including infections because of its wide availability and therapeutic effectiveness. It is considered a highly potent antitoxic formulation [6,7]. Vilwadi Gulika is prepared by grinding 13 ingredients, taken in equal measures along with goat's urine. The ingredients are thoroughly mixed using the bhavana (trituration) method. The 13 constituents are *Aegle marmelos* (Bilwa), *Ocimum tenuiflorum* (Surasa), *Pongamia Pinnata* (Karanja), *Valeriana jatamansi* (Nata), *Cedrus deodara* (Surahva), *Terminalia chebula* (Haritaki), *Phyllanthus emblica* (Amalaki), *Terminalia bellirica* (Vibhitaki), *Zingiber officinale Roscoe* (shunti), *Piper nigrum* (Maricha), *Curcuma longa* (Haridra), *Berberis aristata* (daruharidra), and goats' urine/bhasta mutra. Vilwadi Gulika is prepared using specific plant parts of thirteen medicinal plants, as summarized in **Table 1** [8].

Plant Name	Plant part used
Aegle marmelos	Root
Ocimum sanctum	Flower
Pongamia pinnata	Seed/bark
Valeriana Jatamansi	Root
Cedrus deodara	Bark/wood
Terminalia chebula	Fruit
Terminalia bellirica	Fruit
Phyllanthus emblica	Fruit
Zingiber officinalis	Rhizome
Piper nigrum	Fruit
Piper longum	Fruit
Curcuma longa	Rhizome
Berberis aristata	Stem

Table 1. Plant Sources and Parts Used in the Preparation of *Vilwadi Gulika.* This table lists the botanical ingredients and specific plant parts used in the formulation of *Vilwadi Gulika*, a classical Ayurvedic medicine. The fine powder of the above ingredient is triturated with goat urine and tablets are prepared.

The duration and medium used for triturating this formulation remain a subject of debate. Commercially available samples are often ground for varying durations whereas traditional toxicologists recommend hand grinding for six months at a rate of six hours per day. An alternative view suggests grinding for three hours per day for 108 days or twenty-four hours a day for 15 days. Traditional Ayurveda toxicologists also believe that trituration, or bhavana, should be carried out for six Yamas (one Yama equals ~~to~~ three hours) each day for six months[6]. Traditional Ayurvedic formulations are often prepared using diverse processing practices that vary across practitioners, classical texts, and commercial manufacturers. Such variations in the duration of trituration, liquid media, and processing conditions significantly influence the physicochemical characteristics, biological activity of the final formulation, and microbial composition. Therefore, a systematic scientific comparison of formulations prepared using traditional in-house methods and commercially available preparations is essential to understand formulation - dependent differences [9].

Vilwadi Gulika formulation has been traditionally used for the treatment of fever, psychiatric disorders, dyspepsia, and venom intoxication caused by snakes, spiders, rodents, scorpions, and other similar creatures. It is also known for its strong antitoxic properties, and is recommended for managing vomiting, parasite infestations, and infections attributed to invisible microorganisms (bhuta). The formulation can be administered orally, topically, as

collyrium or through the nasal route. This ayurvedic medicine is believed to balance *kapha* and *Vata* (individual's body type or nature), enhance digestion (Dipana), promote intestinal absorption (Grahi), promote intestinal absorption (Grahi), clear blockages in the body's channels (Srotho shodhana), and exhibit a scraping effect (Lekhana) [6].

Despite its long history of use and well – documented therapeutic benefits, the precise mechanisms underlying the effects of Vilwadi Gulika remain unclear. The application of in-silico approaches to study this formulation has been limited, resulting in an incomplete understanding of its mechanism of action. Furthermore, the potential association between Vilwadi Gulika and the human microbiome has largely been overlooked. The microbiome, a complex community of microorganisms, plays a crucial role in modulating the effects of herbal medicines. However, little is known about the interaction between Vilwadi Gulika and the human microbiome[10]. Although Vilwadi Gulika is one of the most frequently prescribed Agada formulations due to its broad therapeutic indications, studies employing modern integrative scientific approaches remain limited. In particular, investigations that combine microbial profiling with systems - level mechanistic analysis are lacking, highlighting a significant gap in scientific validation of this formulation.

To better understand the microbial composition of Vilwadi Gulika, both culture-dependent and culture-independent methods are essential. Culture-dependent methods involve growing microorganisms in a laboratory conditions, allowing detailed analysis of cultivable species. However, these methods are limited because many microorganisms cannot be easily cultured, resulting in a significant portion of the microbiome remaining undetected using traditional techniques.

To overcome these limitations, culture-independent approaches, particularly those based on Next-Generation Sequencing (NGS), have become increasingly important. NGS techniques enable the identification and characterization of microbial communities without the need for culturing. By directly analysing the genetic material of microorganisms present in a sample, these methods provide a comprehensive overview of the microbiome, including both culturable and non-culturable species. Several NGS platforms are widely used for microbiome studies, including Illumina, PacBio, and Oxford Nanopore Technologies.

The 16S rRNA gene plays a crucial role in bacterial identification in microbiome studies. Although this gene is highly conserved among bacterial species, it contains hypervariable regions that enable differentiation between species. By targeting these regions, researchers can distinguish among bacterial taxa and construct detailed microbial community profiles. Among these regions, the V1–V3 and V4–V6 segments of the 16S rRNA gene are commonly sequenced, particularly using Illumina platforms.

Illumina sequencing technology is known for its short-read capabilities, typically producing reads of 150–300 base pairs. This approach usually targets specific hypervariable regions such as V1–V3 or V4 of the 16S rRNA gene, enabling high-throughput sequencing with high accuracy. However, due to the short-read length, it often limits microbial identification to the genus level, as shorter sequences may not capture sufficient variation to distinguish closely related species. While Illumina is widely used for microbial community analysis and can generate large volumes of data rapidly, resolving species-level differences can be challenging, especially in studies requiring precise microbial identification.

In contrast, Oxford Nanopore Technology provides long-read sequencing that can cover the entire 16S rRNA gene, including the V1–V9 regions. This allows for more comprehensive sequencing and enables species-level identification. Additionally, Nanopore sequencing offers real-time analysis and greater flexibility, making it a cost-effective and powerful tool for studying complex microbiome samples[11].

Moreover, the metabolites present in the constituent herbs, which are likely responsible for their therapeutic properties, have not been adequately investigated. Network pharmacology provides a powerful approach to identify these metabolites, map their interactions with biological targets, and elucidate the pathways through which they exert their effects. Integrating microbiome analysis with network pharmacology is therefore essential for achieving a comprehensive understanding of the mechanisms of action of Vilwadi Gulika. This integrative approach not only helps to uncover the underlying molecular mechanisms but also provides insights into how the formulation's bioactive compounds interact with the microbiome to enhance or modulate its therapeutic effects. Exploring these aspects is crucial for scientifically validating the traditional uses of Vilwadi Gulika and for facilitating its potential application in modern medicine[12,13,14].

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2. Review of literature

2.1 Phytochemical and Analytical Studies on Vilwadi Gulika

High performance thin layer chromatography (HPTLC) of Vilwadi Gulika gave the presence of Scopoletin, Umbelliferon, Gallic acid, Ferulic acid, Palmatine, Curcumin, Berberine and Caffeic acid which are known for their immunomodulatory, antiviral and anti-inflammatory effects suggesting VG's potential in enhancing immunity against SARS-CoV-2 infection [15].

A comparative evaluation of three marketed Vilwadi Gulika revealed acceptance of herbal formulation and its standards overall, which highlighted the uniformity of formulation and components integrity across the brands. Therefore, emphasizing on establishing robust quality control protocols to maintain the patient safety and clinical efficacy [16].

A review by Jesney Rodrigues N. C. et al. emphasized the role of Vilwadi Agada as a potent formulation in Ayurvedic toxicology, attributing its effectiveness in infectious and toxic conditions to its balanced polyherbal composition, antimicrobial properties, and digestive stabilizing actions [17].

While these studies confirm the presence of bioactive metabolites, they do not elucidate how these compounds interact with disease - associated genes or signalling pathways.

2.2 Network Pharmacology and in silico Approaches in Polyherbal Formulations

To study the multi – component and multi target nature of traditional herbal medicines Network pharmacology has emerged as an important in – silico approach. This methodology enables mapping of phytochemicals to molecular targets and biological pathways, providing insights into complex herbal formulations that cannot be explained by single target pharmacology.

Many studies have applied network pharmacology and molecular docking approaches to investigate individual medicinal plants that are constituents of Vilwadi Gulika. For example, network pharmacology studies on *Aegle marmelos* (Bilwa) have explored its bioactive phytochemicals and mapped their interactions with inflammation and inflammatory bowel disease related molecular targets, supporting its traditional use in inflammatory conditions and gastrointestinal conditions [18].

Ocimum tenuiflorum (Tulsi/Surasa) has been studied using network pharmacology and molecular modelling approaches, where its phytocompounds were analysed against the (Crohn's disease) disease associated genes linked to gut related, inflammatory, and neuroinflammatory pathways [19].

Network pharmacology has been used to predict active compounds, targets, pathways of *Valeriana jatamansi* for post-traumatic stress disorder (PTSD), showing associations with neurotransmitter systems [20]. For investigating potential mechanism of *Valeriana jatamansi* in spinal cord injury network pharmacology combined with molecular docking has been applied, identifying key hubs such as PI3K/Akt signalling [21].

Terminalia bellirica (Vibhitaki), *Terminalia chebula* (Haritaki), *Phyllanthus emblica* (Amalaki), network pharmacology papers on these alone are less common, while studies on Triphala (a classical polyherbal Ayurvedic formulation comprising these three fruits), have used network pharmacology and molecular docking approach to identify bioactive compounds

and construct protein – compound – disease networks in metabolic disorders such as obesity [22].

Network pharmacology approach used to investigate the mechanisms of *Zingiber officinale* for the treatment of neurodegenerative disorders [23].

Piper nigrum using network pharmacology approaches showed that it improves Cognitive Impairment and mood in sleep deprived mice through JAK1/STAT3 signalling pathways [24].

A study done on bioactive of *Curcuma longa L* at molecular level network pharmacology approach to see the protein - protein interaction [25].

Another study where network pharmacology approach is use to study the mechanism of *Berberis aristata* on Diabetes Mellitus [26].

All together, these studies show that network pharmacology and molecular docking approaches are widely applied to individual medicinal plants used in Vilwadi Gulika formulation. Despite the availability of network pharmacology and in silico studies on the individual plant ingredients of Vilwadi Gulika, no comprehensive network-based analysis has been reported for this formulation as an integrated polyherbal formulation. In specific, the combined phytochemical – target – pathway interactions arising from all the thirteen plant components has not been explored. This lack of mechanistic understanding at systems level highlights the significant research gap and gives a strong rationale for applying network pharmacology approaches to elucidate the molecular mechanism of Vilwadi Gulika in the present study.

2.3 Gut – Related and Microbiome – Associated Studies Involving Vilwadi Gulika

In managing dysbiosis associated with autism spectrum disorders, Ayurvedic polyherbal interventions specifically Vilwadi Gutika and Rajanyadi Churna when administered along with this dietary and lifestyle modifications significantly enhanced Bifidobacterium abundance in children with ASD was seen [27].

The trial by Balakrishnan et al. (2022) demonstrated that giving Vilwadi Gulika along with Rajanyadi Churna in protocols of Ayurvedic polyherbal protocols along with modifications in lifestyle significantly decreased Shigella and E. coli levels in the gut microbiota of autistic children. As a part of integrated management studies Vilwadi Gulika modulated dysbiosis and restored the microbial homeostasis [28].

Soumyasree & Aniruddha (2024) mentioned that using Vilwadi Gulika along with classical treatments and panchakarma modalities in management of chronic Crohn's disease the patient developed symptoms like weight gain and discontinue long term immunosuppressants. Therefore, highlighting gut integrity and reduction in inflammation [29].

In a case study where Vilwadi Gulika was given to patients for treating Visuchika, a kapha dominant toxic gastrointestinal condition, which alleviated vomiting, diarrhea, abdominal pain and weakness within 15 days of treatment. This highlights Vilwadi Gulika's multifaceted gut regulators (Anulomana, Grahi), digestive stimulants (Deepana -Pachana) and antitoxic agents [30]. Gaikwad Sayali Rajendra et al., 2022 highlights Bilwadi Agada as an effective drug for acute gastrointestinal toxicity [31].

A review article reported that Vilwadi Gulika contributes to normalization of gut pathophysiology through its combined actions of moisture absorption, digestive stimulation,

and gentle bowel regulation, making it effective in managing acute gastrointestinal distress along with traditional dosha-based clinical applications [32].

Although these studies indicate beneficial effects on gut health and microbial balance, most rely on indirect clinical outcomes or limited microbial observations. Comprehensive profiling of microbial diversity using next generation sequencing and evaluation of formulation associated microbiota remains unexplored.

2.4 Role of Vilwadi Gulika in Immune and Inflammatory Disorders

From the literature a study on Immune related disorders and their treatment Vilwadi Gulika was one compound administered to the patients suffering from allergic rhinitis an immune related disorder. Its known anti-toxic and immune-modulatory qualities justify its inclusion in the therapy plan [33].

In the management of herpes zoster with agada yogas Vilwadi Gulika is known to act on the pain relief part and reduce the burning sensation along with this Vilwadi Gulika indicated all envenomation's which includes microbial infection and Luta visha [34].

In COVID -19 associated inflammatory conditions, Vilwadi Gulika was associated with reduction of inflammatory markers such as IL-6 and NLR indicating immunomodulatory potential [35]

Vilwadi Gulika as a part of Ayurvedic intervention along with rasayana and sodhana therapies was given as a dose of two tablets daily with ginger juice, which acted as Grahi, Deepana pachana and detoxifier contributing to improved reproductive health, eliminating toxins. This integrated approach resulted in a successful live birth in women with early recurrent pregnancy loss [36].

In the study by Asha Sreedhar et al. (2022) reports that Vilwadi Gulika given along with Phala Sarpis and other therapies contributed to 46.2% live birth rate over a 15 month follow period, which underscores the potential role in enhancing uterine health, balancing Pitta-Vata and also supporting pregnancy across recurrent miscarriage cases [37].

In the management of psoriasis (Kitibha kushta) Vilwadi Gulika was prescribed two tablets twice daily after food for addressing scaling, pigmentation and itching. The classical properties of the drug heating potency, pungent bitter flavour, kapha-Vata neutralizing anti-inflammatory, supporting antitoxic efficacy in skin pathology management [38].

In a randomized controlled trial involving patients with mild to moderate depressive disorder, nasya using Vilwadi Gulika showed significant improvement in depressive symptoms when compared with Hingvadi Yoga and baseline nasya control. Although both interventions were statistically significant ($p < 0.001$), Vilwadi Gulika demonstrated superior efficacy, indicating its potential role as an adjunct Ayurvedic intervention in mood disorders, which are increasingly associated with immune-inflammatory dysregulation[39].

In a case series on immune-related disorders, Kurunhikattil et al. (2021) reported the use of Vilwadi Gulika in conditions such as allergic rhinitis and psoriasis, where its antitoxic and dosha-balancing properties (Ushna Veerya, Tikta-Katu Rasa) contributed to statistically significant improvement in clinical symptoms and immune modulation [40].

Overall, these studies suggest Vilwadi Gulika exhibits anti – inflammatory and immunomodulatory effects across diverse clinical contexts. These findings do not explain molecular interactions between individual phytochemical constituents and immune related targets.

2.5 Clinical Applications of Vilwadi Gulika in Toxicological and systemic conditions

In case study on Ayurvedic management of herpes zoster, Vilwadi Gulika was given to the patient as internal medication because of its detoxifying, anti-inflammatory properties which helped in relieving pain, burning and vesicular eruptions. Its use aligned with the treatment of Pitta-Vata related conditions [41].

Similarly, a review by Divya K M et al. highlights the therapeutic potential of Vilwadi Gulika as an antitoxic formulation, which is not only effective in spider, snake and scorpion bite but also in infectious conditions, skin disorders and analgesic properties. Altogether these findings give diverse clinical applications in managing systemic inflammatory and acute toxicity disorders [42].

From the paper conceptual study of action of Bilwadi Gulika in chemotherapy induced hepatotoxicity Bilwadi gutika could act as toxic pathologies by chemotherapeutic drugs as the drugs present in the formulation possess Hepatoprotective action and the formulation as a whole is well known anti toxic formulation. It may act as remedy for hepatotoxicity caused secondary to chemotherapy [43].

In a clinical study on high-risk COVID-19 patients with type 2 diabetes, Vilwadi Gulika was selectively administered to 6 out of 24 patients in Ayurveda add on group, which indicates its use in personalized therapeutic interventions [44].

From a clinical study of Evaluating personalized add on ayurveda therapy in oxygen dependent diabetic COVID-19 where along with other medication Vilwadi Gulika was prescribed to add on ayurveda group which showed a better symptomatic response and faster normalization in inflammatory markers, such as NLR and IL-6 by 14 days and cardiac markers by 28 days.

In case of chronic healing ulcer due to viper bite, which had no response towards the modern treatment, Vilwadi Gulika when given internally during the early phase for its anti-inflammatory, antitoxic actions along with Vishahara chikitsa, complete healing is seen within eight weeks including Vrana ropana and Vrana sodhana which demonstrated the efficacy of integrative herbal therapy in managing toxic wounds [45]**.**

In severe bone fractures Vilwadi Gulika acted as anti-inflammatory, Kapha- Vata pacifying capable of aiding injury and supporting trauma related contexts [46]. Satpute & Pakhmode (2024) mentioned about Vilwadi Gulika as the part of classical antitoxic in the management of Keeta Visha (insect envenomation) using approach of external and internal detoxifying therapies [47].

In clinical case report where a 42-year-old female patient with fungal sinusitis opted Ayurveda treatment instead of surgery., where Vilwadi Gulika along with other churnas was given in the form of dhuma nasya (intranasal therapy)[48].

In a case involving a two-month-old infant with a haemangiomatous ulcer unresponsive to antibiotic therapy, Ayurvedic management using Bilwadi Agada administered orally along with Jatyadi Taila for topical dressing and Triphala Kwatha for wound lavage resulted in

complete healing within two months, highlighting the holistic and safe application of antitoxic formulations even in pediatric wound management[49].

A conceptual review by Ajeet Singh et al. (2024) described Bilwadi Agada as a classical polyherbal toxin-neutralizing formulation in Ayurveda, emphasizing its broad applicability in toxicological conditions [50].

From a conceptual study on Luta Visha (spider envenomation) and its management, Bilwadi Gulika was highlighted as a dosha-balancing formulation with anti-inflammatory and detoxifying properties, reinforcing its relevance in venom-related diseases [50].

Collectively these studies demonstrate the extensive clinical use of Vilwadi Gulika across toxicological, traumatic, infectious, systemic conditions. However, the majority of evidence is derived from observational studies and case reports, with limited investigation into molecular or microbial mechanisms responsible for observed therapeutic outcomes.

Taken together, the reviewed literature highlights the extensive traditional use and clinical relevance of Vilwadi Gulika, while also revealing the critical gaps in the understanding formulation – dependent microbial composition and molecular mechanisms. Addressing these gaps requires an integrative experimental and computational approach, which forms the basis of the present study.

2.6 Research Gap

- Most existing studies on Vilwadi Gulika are clinical, conceptual, or observational, with limited molecular-level validation of its therapeutic mechanisms.
- The specific phytochemicals, molecular targets, and signalling pathways responsible for the therapeutic effects of Vilwadi Gulika remain largely unexplored.
- The microbial diversity associated with Vilwadi Gulika formulations and the influence of preparation methods on microbial composition have not been systematically investigated.
- Although network pharmacology studies exist for individual plant ingredients, no integrated network pharmacology analysis has been performed for Vilwadi Gulika as a complete polyherbal formulation.
- Integrated studies combining microbiome profiling and network pharmacology to understand the mechanistic basis of Vilwadi Gulika are currently lacking.

2.7 Objectives of the Study

1. To identify key molecular targets and pathways associated with the therapeutic effects of Vilwadi Gulika through network pharmacology.
2. To analyse the microbiome composition of Vilwadi Gulika formulation and understand its role in enhancing health benefits.

2.8 Significance of the study

- The study gives scientific evidence on microbial diversity associated with Vilwadi Gulika, which contributes to understanding of formulation associated with microbiota in traditional Ayurvedic medicines.

- By comparing in house prepared Vilwadi Gulika and commercially available Vilwadi Gulika the study highlights impact of preparation methods on microbial load and composition, supporting the need to standardize the formulation.
- The study offers mechanistic insights into the therapeutic potential of Vilwadi Gulika by mapping the specific phytochemicals to disease associated genes and signalling pathways using network pharmacology approach, thereby showing how connected phytochemical – target interactions are and may contribute to modulation of disease related biological processes.
- Integration of computational and microbiome analysis strengthens the scientific validation of traditional formulations and bridges classical Ayurvedic knowledge with modern research.
- The findings of this study provide a foundation for future experimental validation, including molecular docking, in vivo and in vitro studies.
- Overall, the study contributes to evidence-based evaluation and development of Ayurvedic polyherbal formulations, supporting acceptance in integrative and modern healthcare systems.

CHAPTER 3

3.Materials and Methods

3.1 Network Pharmacology Analysis of Vilwadi Gulika

This chapter describes the workflow used to investigate the molecular mechanisms of Vilwadi Gulika using network pharmacology approach. The overview of the methodology is shown in Figure 2. The methodology includes retrieval of phytochemicals based on the formulation of specific plant parts, molecular target prediction, functional annotation for disease and pathway enrichment and construction of a network to elucidate phytochemical – target – disease – pathway interactions.

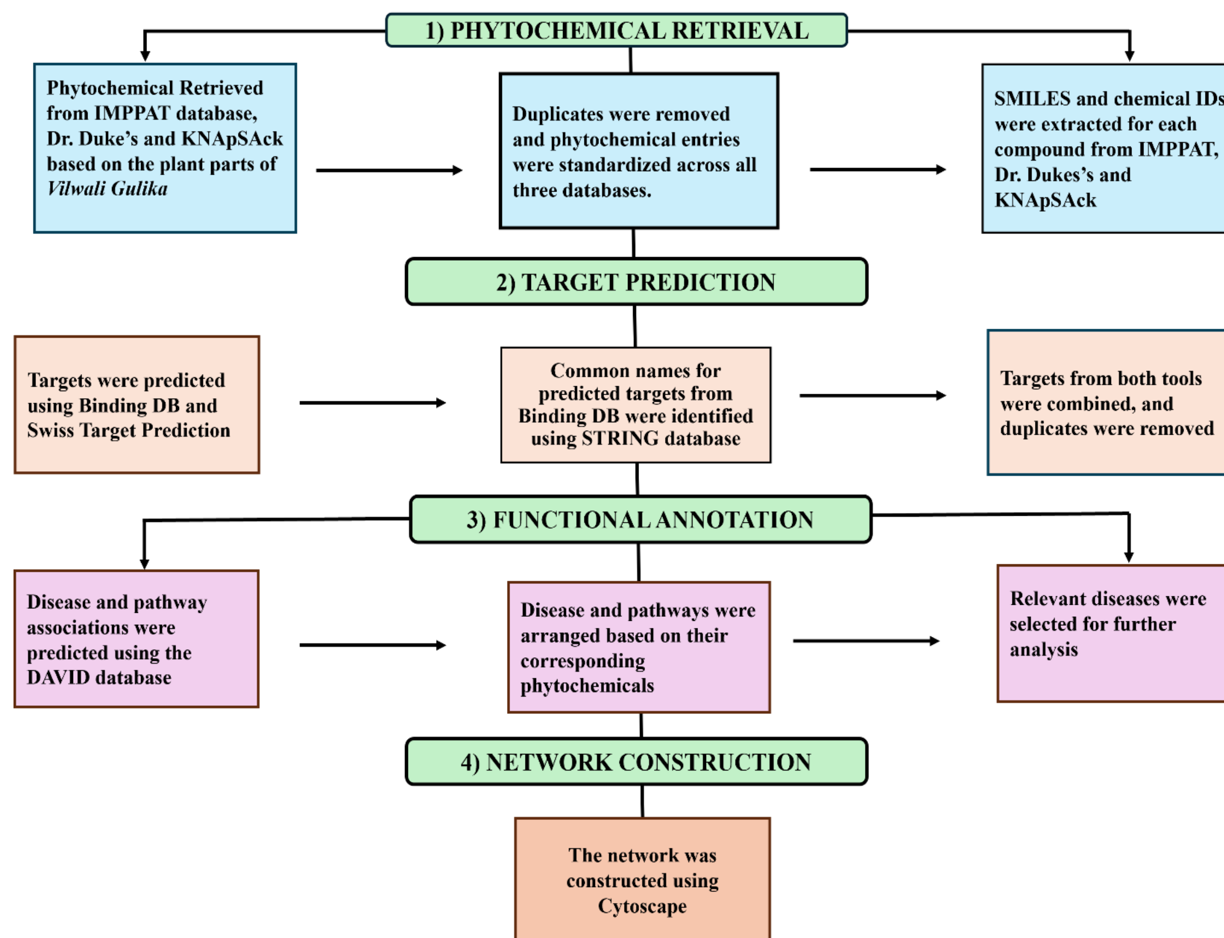

Figure 2. Workflow for the Retrieval of Phytochemicals, Target Prediction, Disease and Pathway Mapping and Network Construction of Vilwadi Gulika. Stepwise pipeline used to identify phytochemicals, predict molecular targets and map associated disease and pathways for the thirteen ingredients of Vilwadi Gulika is represented in this figure.

3.1.1 Phytochemical Retrieval

The phytochemical constituents of *Vilwadi Gulika,* derived from thirteen plants namely *Aegle marmelos* (root/bark)*, Ocimum sanctum* (flower)*, Pongamia pinnata* (bark)*, Valeriana jatamansi* (root)*, Cedrus deodara* (wood)*, Terminalia chebula* (fruit)*, Terminalia bellerica* (fruit)*, Phyllanthus emblica* (fruit)*, Zingiber officinalis* (rhizome)*, Piper longum* (fruit)*, Piper nigrum* (fruit)*, Curcuma longa* (Rhizome)*, Berberis aristata* (stem)*,* were retrieved based on specific plant parts used in the formulation. Phytochemical for each plant was collected from IMPPAT, Dr. Duke's Phytochemical and Ethnobotanical Database and KNApSAck (all accessed on 18th October 2023).

Phytochemicals obtained from these databases were compiled and duplicate entries were removed carefully to ensure a non-redundant phytochemical list. For each compound Chemical ID and SMILES (Simplified Molecular input line Entry System) notations were retrieved for the target prediction.

3.1.2 Target Prediction

Molecular targets were predicted using SMILES structures of each phytochemical constituent through two different databases: Binding DB and SwissTargetPrediction. In Binding DB, the workflow followed a sequence in which *Special Tools* were selected, my Compound's Targets was opened, the SMILES structure was pasted into the designated input field and OK was clicked to generate the compound structure, and the GO option was selected to obtain the predicted targets. For SwissTargetPrediction, the species was first set to *Homo Sapiens* and the SMILES structure was pasted into the input box and the Predict targets option was selected to generate the complete list of predicted molecular targets. The outputs from both databases were used for further analysis. Detailed protocol is given in the Annexure (Annexure 1).

3.1.3 Retrieval of Common Names of Predicted Targets

The SwissTargetPrediction database provides the common names of the predicted targets, whereas the Binding Database does not. Therefore, the targets obtained from the Binding Database were processed through the STRING database to retrieve their common names. In STRING, the target list was entered in the "List of Names" field, the organism was set to *Homo sapiens*, and the search was executed. The "Mapping" option was selected, and the output file was opened to extract the corresponding common names. After retrieving the common names, the targets from both databases were combined, and duplicate entries were removed, to generate a non – redundant list of predicted molecular targets. Detailed protocol is given in the Annexure (Annexure 1).

3.1.4 Functional Annotation

A unique, non-redundant list of predicted gene targets associated with all identified phytochemicals was prepared. The complete target list was uploaded to the DAVID database by pasting the genes into the *Gene List* input field. *Official_Gene_Symbol* was chosen under Select Identifier, the List Type was set to *Gene List*, and *Homo sapiens* was selected as the reference species. The analysis was initiated by submitting the gene list. From the Annotation Summary, the DISGENET option under the disease category was selected to generate the disease enrichment results, which were subsequently downloaded. Within the same annotation interface, the KEGG PATHWAY module was selected to obtain pathway enrichment results,

and the output files were downloaded. The disease and pathway output files corresponding to each plant were merged into a single consolidated dataset using Merger.exe. Detailed protocol is given in Annexure 1**.**

3.1.5 Network Construction and Visualization

The consolidated lists of phytochemicals, predicted targets, associated diseases, and enriched pathways for each plant were used to construct interaction networks using Cytoscape v3.10.4 software. The merged datasets were imported into Cytoscape as network tables, networks representing compound – target – disease – pathway interactions were generated.

Topological network parameters, including degree centrality and betweenness centrality, were computed using Network Analyzer plugin in Cytoscape to identify the key hub targets and pathways. Individual plant – specific networks corresponding to six selected gastrointestinal and inflammatory diseases were generated and are provided in Annexure 1.

3.2 Microbiome Analysis of Vilwadi Gulika

3.2.1 Overview of Experimental design

Objective 2 was designed to characterize and compare the microbial diversity associated with commercially available, traditionally prepared and in house prepared Vilwadi Gulika formulations using culture independent metagenomic approaches. The workflow of the experiment included sample collection, preparation, method validation for metagenomic DNA extraction, 16S rRNA gene amplification, Oxford Nanopore sequencing and data analysis. The overall workflow adopted for microbiome analysis is given the Figure 3.

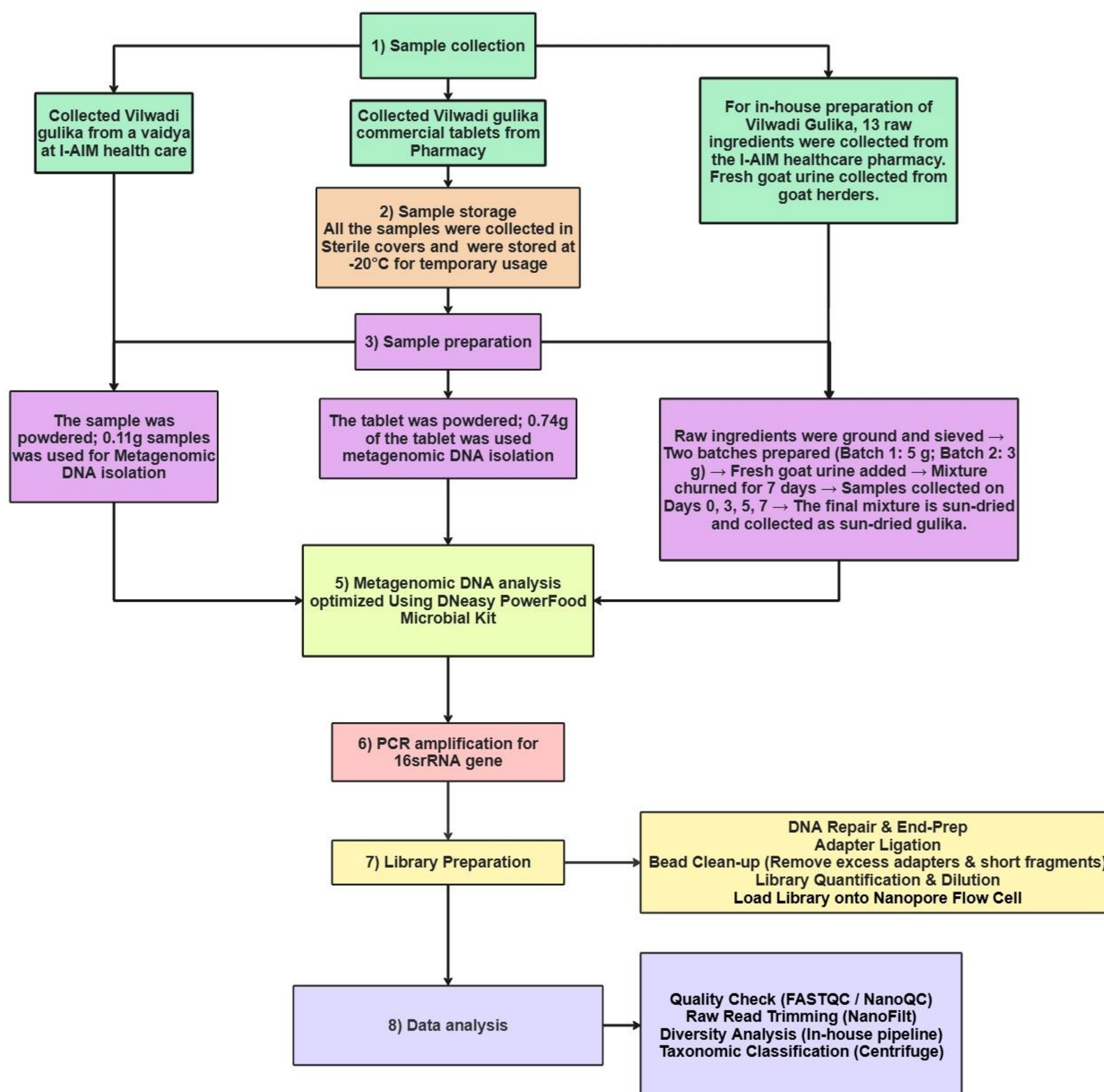

Figure 3. Schematic workflow for metagenomic analysis of Vilwadi Gulika formulations. The workflow shows the sample collection, preparation, culture dependent validation, metagenomic DNA extraction, 16S rRNA gene amplification, Oxford Nanopore sequencing and data analysis.

3.2.2 Sample Collection

Sample collection was carried out for three different types of Vilwadi Gulika. The first was prepared by a traditional Vaidya and named as Formulation 1. The second was a commercially available tablet named Formulation 2. The third was prepared by procuring 13 Polyherbs ingredients and is referred as in house Vilwadi Gulika. All the three formulations were procured from the Institute of Ayurveda and Integrative Medicine (I-AIM), Bengaluru, Karnataka – 560064. For in house preparation of Vilwadi Gulika fresh goat urine, was procured daily from the nearby village of Mylappanahalli, Bengaluru, Karnataka – 560064.

3.2.3 Sample Preparation and Processing

A) Preparation of Formulation 1 and Formulation 2

Formulation 1 was powdered using an autoclaved mortar and pestle. A portion of the powdered sample (0.11g) was further used for genomic DNA isolation. Formulation 2 was similarly powdered and 0.19g of the powdered tablet was taken for genomic DNA isolation.

B) In house Preparation of Vilwadi Gulika

For in house preparation, polyherbal ingredients were collected, ground using a sterile grinder and sieved with a sterile tea infuser. The raw ingredients used are shown in the Figure 4.

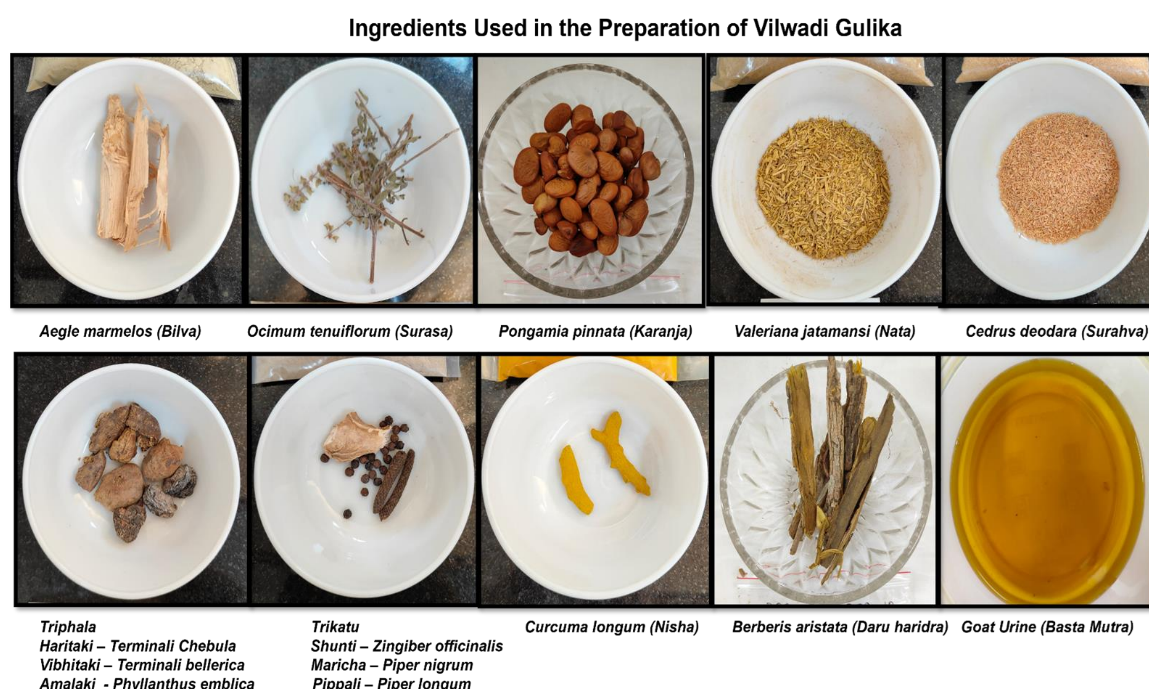

Figure 4. Raw plant ingredients used for in - house preparation of Vilwadi Gulika, including selected plant parts and goat urine. Thirteen raw ingredients used in preparation of Vilwadi Gulika, a traditional ayurvedic polyherbal formulation along with goat's urine This formulation comprises plant parts including barks, seeds, roots, rhizomes and fruit traditionally churned with goat's urine.

The polyherbal mixture was divided into two batches, batch 1 had 5g of the mixture and batch 2 had 3g. The mixture was churned continuously for three hours each day with daily addition of fresh goat's urine for seven consecutive days. On the 7th day, the formulation was rolled into small round pills (Gulika's) and sun dried. During the trituration process, distinct and progressive physical changes were observed. In the initial stages, the polyherbal mixture appeared to be uneven, partially dry, showing incomplete absorption of the liquid medium. As daily trituration continued, gradual softening was observed and uniform darkening of colour and improvement in the consistency was observed. Reduction in cracks and lumps were seen at the intermediate stage, showing incorporation of goat's urine into the herbal matrix. At the final stage the formulation showed a dark, smooth and homogenous paste, demonstrating the integration of the goat's urine and formation of the stable mass suitable for shaping into Gulika.

These sequential transformations were observed during Batch1 and Batch 2 preparation and are shown in the Figure 5.

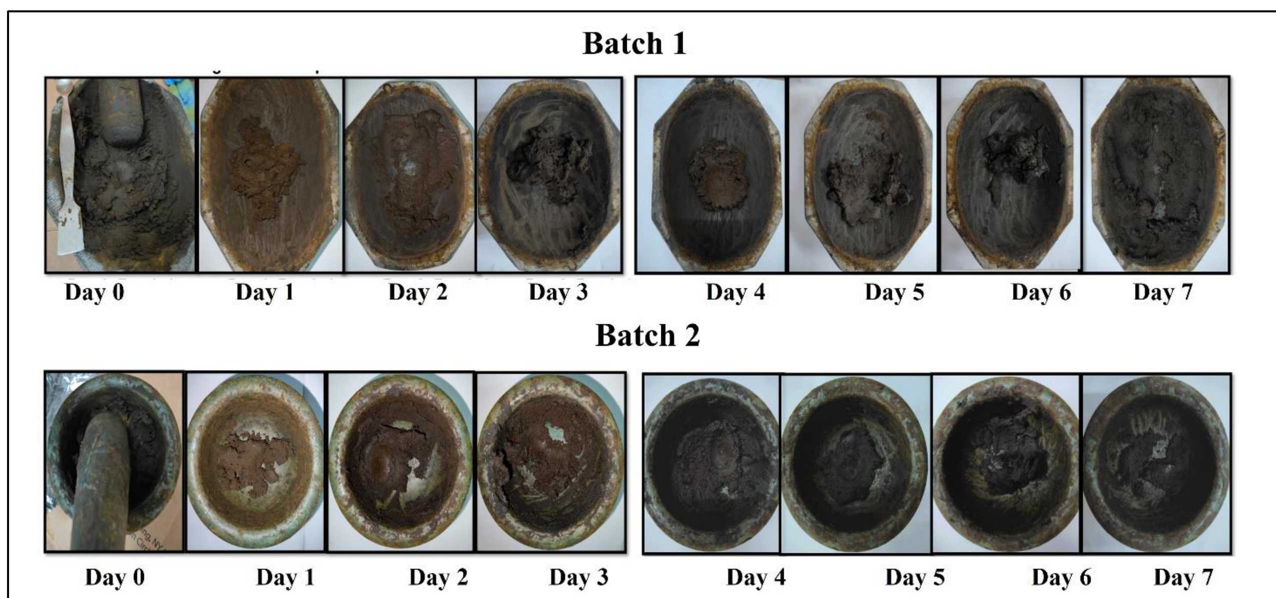

Figure 5. Comparative stepwise preparation of Vilwadi Gulika (Batch 1 and Batch 2)

Samples were collected at Day 0, Day 3, Day 5, Day 7 and after sun drying and stored in sterile zip lock bags at -20°C until further processing.

The quantities of polyherbal material, fermentation time points and volume of goat urine used in the in-house preparation were systematically recorded and are summarized below in Table 2.

Day	Total Goat Urine Added		No of hours triturated	Weight of the sample collected at different time points	
	Batch 1	Batch 2		Batch 1	Batch 2
0	106ml	70 ml	3 hours	W1=1.55g W2=3.97g W3 = 2.42g	W1=1.5g W2=4.04g W3 = 2.45g
1	150ml	68ml	3 hours		
2	30ml	70ml	3 hours		
3	35ml	21ml	3 hours	W1=1.67g W2=4.90g W3 = 3.23g	W1=1.65g W2= 4.21g W3 = 2.56g
4	30ml	30ml	3 hours		
5	12ml	2ml	3 hours	W1=1.66g W2=9.13g W3=7.47g	W1=1.66g W2=9.10g W3=7.44g
6	22ml	12ml	3 hours		
7	5ml	4ml	3 hours	W1=1.61g W2=7.52g W3=5.91g	W1=1.57g W2=4.70g W3=3.13g

Table 2. Sample weights and volume of goat urine used during in house preparation of Vilwadi Gulika across different fermentation time points.

3.2.4 Gravimetric Determination of Sample weight

The weight of each sample was determined gravimetrically. The weight of the empty vial (**W_1**) was recorded, followed by the weight of vial containing the sample (**W_2**). The net sample weight (**W_3**) was calculated using formula **$W_3 = W_2 – W_1$.**

For batch 1 the net weights were 0.24g (day 0), 0.24g (day 3), 0.19g (day 5), 0.21g (day 7), and 0.10g (sun dried). For batch 2, corresponding values were 0.17g (day 0), 0.25g (day 3), 0.31g (day 5), 0.16g (day 7) and 0.14g (sun dried). These measured samples were further proceeded for genomic DNA extraction. Additionally, the polyherbal mixture (0.13 g) and goat urine (200 ml) were taken forward separately for genomic DNA extraction.

3.2.5 Challenges and Validation in Metagenomic DNA Isolation

Metagenomic DNA isolation from Vilwadi Gulika showed methodological challenges due to its complex polyherbal matrix, binders, presence of inhibitory secondary metabolites, and low microbial biomass. Initial attempts of isolating metagenomic DNA from commercial Vilwadi

Gulika tablets showed inconsistent DNA yields, low purity ratio and repeated failures in PCR amplifications.

For the validation and optimization of DNA extraction procedure, multiple extraction strategies were applied which included commercial column - based kits, modified CTAB protocols, PBS pre-treatment, enzymatic lysis, extended incubation conditions and mechanical homogenization. Several early extraction attempts gave unsuitable DNA for PCR amplification, showing interference from formulation derived inhibitors.

Failed DNA extractions and unsuccessful PCR amplifications were documented as part of a validation method to ensure the transparency and reproducibility. Detailed records of the optimization attempts are given in Annexure 2.

3.2.6 Culture - Dependent Validation of Microbial Presence

After the multiple failed attempts of isolating the metagenomic DNA from the Vilwadi Gulika samples, to confirm the presence of microbial communities within Vilwadi Gulika samples culture dependent validation was performed to check the presence of microbes. Inoculation of Vilwadi Gulika samples were done into nutrient media under sterile conditions and were incubated at appropriate temperatures.

Initially the growth of microbes in Vilwadi Gulika samples was not evident immediately, upon the extension of the incubation under controlled conditions, showed turbidity and appearance of discrete colonies on the media plates. Systematic variation in the duration and temperature showed differences in the time required for observable microbial growth across the samples. As summarized in Table 3.

	Sample	Time of Incubation	No of hours took to observe colonies	Plating Technique
1	Commercial Vilwadi Gulika – Manufacturer 1	2 hours at 37°C,121rpm	72 hours at 37°C	Pour plate, spread plate
2		24 hours at 37°C (121 rpm)3 hours at 4°C1½ hours at room temperature	48 hours at 37°C	Pour plate, spread plate
3		5 days at 37°C (121 rpm)	9 hours at 37°C	Pour plate, spread plate
4	Manufacturer 1, Manufacturer 2, Manufacturer 3	5 days 37°C (121rpm)	11 hours	Pour plate, spread plate, Zigzag

Table 3. Overview of media preparation, sterilization, and streaking procedures used for culturing microbes from Vilwadi Gulika samples

This culture - dependent validation showed that Vilwadi Gulika harbours low abundance microbial communities. The culture plates and broth enrichment results are given in Annexure 3.

3.2.7 Genomic DNA Isolation (Commercial kit)

Following the culture dependent validation, genomic DNA extraction was performed using DNeasy Power food Microbial Kit (QIAGEN, Germany) with protocol modifications.

Sample Details and Weights

Formulation 1 (Vaidya prepared Vilwadi Gulika)]

Weight of the Gulika used for Metagenomic DNA Isolation: 0.11g

Formulation 2 (Commercial Vilwadi Gulika)

Weight of one tablet: 0.76g

Weight after powdering the tablet: 0.74g

Weight of powdered tablet taken for DNA isolation: 0.74g

In house prepared Vilwadi Gulika

Batch 1: Formulation

Day-0:**0.24g**
Day-3:**0.24g**
Day-5:**0.19g**
Day-7:**0.21g**
Sun-dried: **0.10 g**

Batch 2: Formulation

Day-0:**0.17g**
Day-3:**0.25g**
Day-5:**0.31g**
Day-7:**0.16g**
Sun-dried: **0.14 g**

Polyherbal mixture: 0.13g

Goat Urine: 200 ml

All the above samples were taken for Metagenomic DNA extraction.

Metagenomic DNA Extraction Protocol

- The weighed samples were finely powdered using autoclaved mortar and pestle.
- To the powdered sample 1000 µL of pre-warmed MBL buffer was added from the Qiagen kit
- To resuspend the pellet sample was vortexed
- To dissolve the pellet, the tube was incubated in a water bath at 60 °C for 5 minutes.
- The supernatant was transferred to the Power Bead tube.

- The tube was incubated at 60 °C for 10–15 minutes.
- Horizontal vertexing was performed for 10 minutes.
- The tube was centrifuged for 14,000 rpm for 2 minutes.
- The supernatant was carefully collected into a fresh microcentrifuge tube.
- 100 µL of Inhibitor Removal (IR) solution was added.
- The mixture was incubated at 4 °C for 10 minutes.
- The sample was centrifuged at 14,000 rpm for 2 minutes.
- The pellet was discarded, and the supernatant was transferred to a new tube.
- 900 µL of MR solution was added and vortexed briefly for 1 second.
- The mixture was transferred to a spin column and centrifuged at 14,000 rpm for 2 minutes.
- Since the total volume exceeded the column capacity (650 µL), loading and centrifugation were repeated until the entire volume was processed.
- The flow-through was discarded.
- 650 µL of PW wash solution was added and centrifuged at 14,000 rpm for 2 minutes.
- The flow-through was discarded.
- 650 µL of 100% ethanol was added and centrifuged at 14,000 rpm for 2 minutes.
- A dry spin was performed at 14,000 rpm to remove residual ethanol.
- The spin column was transferred to a new collection tube.
- 20 µL of elution buffer was added and incubated at room temperature for 5 minutes.
- Centrifugation was performed at 14,000 rpm for 1 minute at 4 °C.
- A second elution was carried out by adding 20 µL of the elution buffer, incubating at room temperature for 2 minutes, and centrifuging at 12,000 rpm for 2 minutes at 4 °C.
- The eluted genomic DNA was collected in sterile microcentrifuge tubes and stored at –20 °C for downstream analysis.

The same protocol was applied to all Vilwadi Gulika formulations, In house, commercially, Vaidya prepared goat urine and polyherbal mixtures.

3.2.8 Quality Check of Extracted Genomic DNA

The DNA sample was quantitated using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) by measuring the UV absorption at wavelengths of 260/280 nm. Based on A260/A280 nm the DNA purity was calculated. All extracted samples were stored at -20°C for further analysis.

3.2.9 Bacterial Identification

Genomic DNA from *Vilwadi Gulika* samples was amplified for sequencing. The samples served as templates for the amplification of the bacterial 16S rRNA gene using PCR. The amplification targeted the V1–V9 regions of the 16S rRNA gene. Each PCR reaction was performed in a final volume of 20 µL containing 10 µL of KAPA master mix, 6 µL of template DNA, 2 µL of forward primer (5'-AGAGTTTGATCCTGGCTCAG-3'), and 2 µL of reverse primer (5'-GGTTACCTTGTTACGACTT-3').The PCR amplification consisted of 35 cycles with the given temperature profile - initial denaturation at 95°C for 3 minutes; denaturation at 98°C for 20 seconds; primer annealing at 61.5°C for 15 seconds; and elongation at 72°C for 1 minute 30 seconds. A final extension was carried out at 72°C for 5 minutes. The reactions were performed in a BIO-RAD T100 gradient 96-well thermal cycler.

3.2.10 Quality control of PCR product

The PCR amplicons were analysed using 1 % agarose gel. 1 kb (kilobase) DNA ladder was also loaded as a molecular size marker to verify the amplification of the 16S rRNA gene. The gel was visualized under ultraviolet (UV) transillumination using a Gel Doc imaging system (Bio – Rad Universal hood II Gel Doc Imaging system).

3.2.11 Oxford Nanopore Sequencing

The DNA amplicons generated from the PCR reactions were subjected to library preparation using the Native Barcoding Kit 24 V14 (SQK-NBD114.24, Oxford Nanopore Technologies, UK), which facilitates the multiplexing of up to 24 samples in a single sequencing run.

DNA Quantification and Input

The concentration and quality of DNA were determined using a Qubit fluorometer ds DNA HS assay (Thermo fisher, USA) and agarose gel electrophoresis. 130 ng of DNA per sample was used as an input for library preparation.

End-Repair and dA-Tailing

DNA amplicons were subjected to end-repair and dA-tailing using the NEBNext End Repair/dA-Tailing Module following the manufacturer’s protocol. This step ensures that DNA fragments have blunt ends and a 3′ adenine tail, to enable efficient ligation of the native barcodes.

Native Barcode Ligation

Each DNA sample was ligated to a unique native barcode provided in the kit. Barcodes were handled carefully, and each well was used only once to prevent cross-contamination between the samples. The ligation reaction was performed under optimal conditions as specified in the protocol, ensuring efficient attachment of barcodes to the DNA fragments.

Adapter Ligation and Library Purification

Following barcode ligation, sequencing adapters were attached to the barcoded DNA fragments. The library was purified using (AMPure beads) magnetic beads based clean up to remove excess adapters, enzymes, and other reaction components. The purified libraries were stored temporarily at 4°C if used immediately, or at –20°C for longer-term storage.

Flow Cell Preparation and Sequencing

Sequencing was performed on a MinION Mk1C platform using R10.4.1 flow cells (FLO-MIN114). Prior to loading the library, the flow cells were checked and primed according to the manufacturer’s protocol. Libraries were then loaded, and sequencing was initiated and monitored using MinKNOW software (Oxford Nanopore Technologies), which controls the sequencing run and performs real-time base calling and run management. Optionally, downstream analysis was performed using the Kraken2 platform.

3.2.12 Bioinformatics Processing and Quality Control of Sequencing

Raw sequencing reads generated from the Oxford Nanopore MinION platform were processed using a bioinformatics workflow adapted from previously published long-read 16S rRNA gene analysis pipelines, with minor modifications to suit the present dataset and experimental design. Similar approaches for Nanopore-based full-length 16S rRNA analysis have been reported in recent studies focusing on quality control, length-based filtering, and reliable taxonomic assignment of microbial communities[51,52,53,54].

Initial quality assessment of the raw FASTQ files was performed using NanoQC software to evaluate sequencing yield, read length distribution, and quality score profiles for each sample, which was used to evaluate sequencing yield, read length distribution, and quality score profiles for each sample. This step enabled an overview of sequencing performance and identification of samples requiring further quality scrutiny, consistent with established Nanopore microbiome workflows.

Subsequently, reads were subjected to quality and length filtering using NanoFilt software based on defined length and quality thresholds. Following criteria reported in earlier long-read 16S rRNA studies. Reads shorter than 1200 bp or longer than 1700 bp were excluded to retain near full-length 16S rRNA gene sequences, while a minimum Phred quality score threshold of $Q \geq 9$ was applied to remove low-quality reads. These thresholds were selected based on published evidence indicating that near full-length Nanopore reads within this size range improve taxonomic resolution while minimizing sequencing artefacts and misclassification.

Post-filtering statistics, including total read counts, mean read lengths, and quality metrics, were generated using NanoStat tool. To evaluate the effectiveness of the filtering process and ensure consistency across samples. Filtered reads were then demultiplexed based on native barcode sequences to segregate individual samples for downstream analysis.

Samples exhibiting very low sequencing depth or suboptimal read quality after filtering were critically evaluated prior to taxonomic classification. Based on quality control metrics, Batch 2 Day 0 (VGB2D00) and goat urine (VGGU02) samples showed mean processed read lengths below - 402 bp and low processed read counts (115 and 144 reads, respectively). Such read depths fall below commonly accepted thresholds for robust microbial community profiling in Nanopore-based 16S rRNA studies, where insufficient sequencing depth can lead to biased diversity estimates and unreliable taxonomic assignments. Therefore, these samples were excluded from downstream taxonomic classification and diversity analyses to ensure analytical rigor and avoid overinterpretation.

The final dataset, comprising high-quality, length-filtered, and demultiplexed reads, was taken forward for taxonomic classification and microbial community analysis.

3.2.13 Taxonomic Classification and Microbial Diversity Analysis

Taxonomic classification of the quality-filtered reads was performed using the Centrifuge database, a k-mer based classifier optimized for metagenomic datasets. Classification was carried out against a pre-indexed microbial reference database to assign reads to bacterial taxa across multiple taxonomic levels, including phylum, class, order, family, genus, and species. The Centrifuge output files were generated in text format and used as the primary input for downstream visualization and analysis.

The taxonomic classification outputs were imported into the Pavian metagenomic data browser software for interactive visualization and exploration of microbial community profiles[55]. From Pavian, raw abundance tables were downloaded for further analysis. To minimize batch-specific variation and improve robustness of the results, corresponding samples from Batch 1 and Batch 2 were averaged for each preparation stage (for example, Batch 1 Day 3 and Batch 2 Day 3). These averaged values were used for all subsequent taxonomic and diversity analyses.

To focus on biologically meaningful taxa and reduce the influence of low-abundance noise, a relative abundance filter of >2% was applied prior to graphical representation. Only taxa exceeding this threshold were included in bar plots, upset plots, and diversity analyses presented in the Results section. This filtering approach enabled clearer visualization of dominant microbial groups and facilitated comparison across different stages of Vilwadi Gulika preparation.

Microbial diversity was assessed at multiple levels, including taxonomic richness, evenness, and overall community structure. Alpha diversity indices, including species richness and Shannon diversity index, were calculated to evaluate within-sample diversity across preparation stages and sample types. Beta diversity analysis was performed using Principal Coordinates Analysis (PCoA) to assess differences in microbial community composition between the polyherbal mixture, goat urine, fermentation stages (Day 0 to Day 7), sun-dried formulation, and tablet forms.

Together, this integrated bioinformatics workflow enabled high-resolution characterization of microbial dynamics during the preparation of Vilwadi Gulika and provided a robust framework for comparative microbiome analysis across different formulations and processing stages.

Although multiple stages of Vilwadi Gulika preparation were analysed, the overall number of biological samples included in this study was limited due to constraints in sample availability and sequencing resources. The microbiome analysis included commercially available tablets, Vaidya-prepared formulations, and in-house preparations across fermentation stages. Therefore, the observed microbial diversity patterns should be interpreted as exploratory findings, and future studies with larger sample sizes and additional biological replicates are recommended to further validate the microbial dynamics associated with Vilwadi Gulika preparation.

4.Results

4.1 Network Pharmacology Analysis of Vilwadi Gulika

4.1.1 Phytochemical Identification Across Databases

A total of 3424 phytochemicals were retrieved for the thirteen medicinal plants from the IMPPAT, Dr. Dukes' and KNApSAck phytochemical databases. After data cleaning and removal of duplicate entries, 3004 phytochemicals were retained **(Table 4)**. Among the plants, *Zingiber officinale* contributed the highest number of phytochemicals (1148), followed by *Piper nigrum (461)* and *Curcuma longa* (404).

To examine the distribution of phytochemicals across 13 plants, an Upset plot was constructed **(Figure 6).** The analysis revealed that *Zingiber officinale, Curcuma longa*, and *Piper nigrum*, contained the largest sets of unique phytochemicals, reflecting chemical diversity within these species. No single phytochemical was found to be common across all thirteen plants, indicating that each plant contributes a distinct set of phytochemicals to the formulation.

SL NO	Polyherbs	Plant part	IMMPAT	Dr. Dukes	KNApSAck	Total (unfiltered)	Filtered metabolites after duplicate removal
1	*Aegle marmelos*	Root/bark	27	2	60	89	68
2	*Ocimum tenuiflorum*	Flower	94	0	50	144	141
3	*Pongamia pinnata*	bark	51	1	33	85	74
4	*Valeriana jatamansi*	Root	153	29	47	229	212
5	*Cedrus deodara*	Bark/wood	43	20	28	91	70
6	*Terminalia chebula*	Fruit	33	37	29	99	84
7	*Phyllanthus emblica*	Fruit	26	55	109	190	176
8	*Terminalia bellirica*	Fruit	25	21	3	49	42
9	*Piper longum L*	Fruit	73	0	35	108	100
10	*Piper nigrum L*	Fruit	187	156	184	527	461
11	*Curcuma longa L*	Rhizome	169	146	167	482	404
12	*Berberis aristata DC*	Stem	10	0	15	25	24
13	*Zingiber officinale Roscoe*	Rhizome	283	393	630	1306	1148
	TOTAL		**1174**	**860**	**1390**	**3424**	**3004**

Table 4. Phytochemicals identified for the 13 ingredients of Vilwadi Gulika Across IMPPAT, Dr. Duke's and KNApSAck databases.

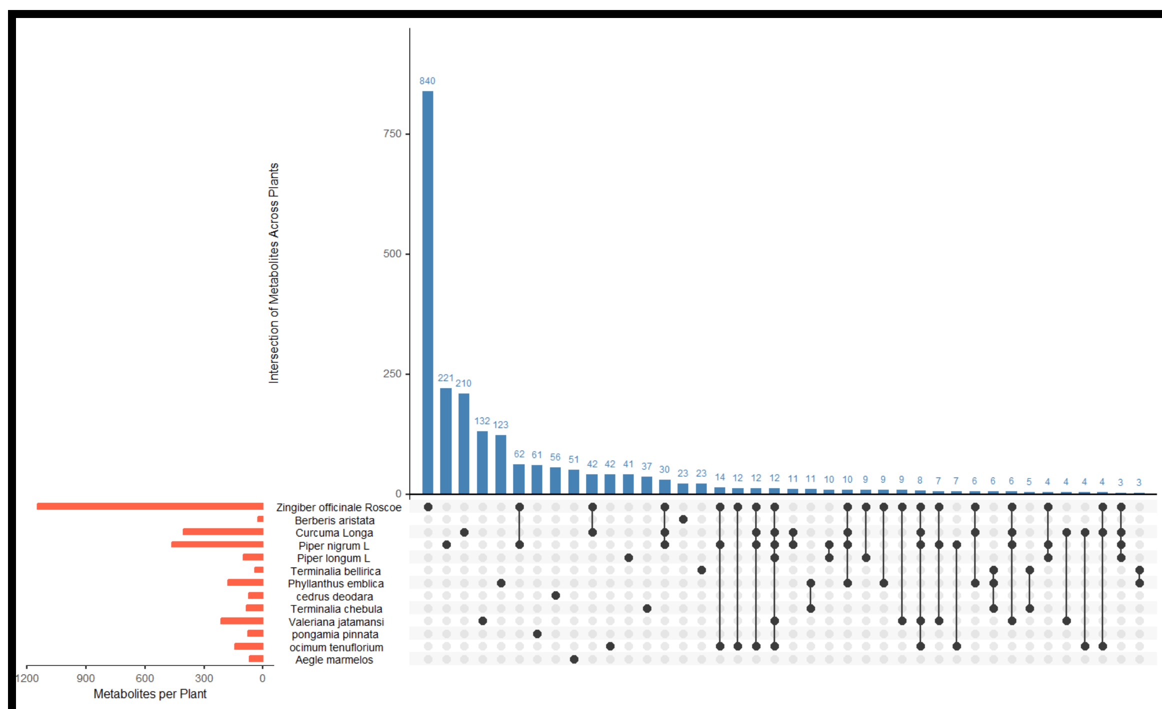

Figure 6. Intersection of Phytochemicals Among the Thirteen Medicinal Plants in the Vilwadi Gulika Formulation. The Upset plot illustrates the overlap of phytochemicals identified across the thirteen medicinal plants used in the Vilwadi Gulika formulation. Each bar on the top represents the number of shared phytochemicals among different plants, while the connected dots below indicate which plants share those phytochemicals.

4.1.2 Target prediction and Standardization

A total of 72,367 molecular targets were retrieved for the phytochemicals present in the thirteen plants of *Vilwadi Gulika* using Binding DB and SwissTargetPrediction. After removing duplicates between the two databases 68,635 targets were retained for further analysis (Table 5). Among the individual plants *Zingiber officinale, Curcuma longa* and *Phyllanthus emblica* contribute the highest number of predicted targets reflecting their rich and diverse chemical profiles. Overall, the filtered dataset provided a comprehensive and non-redundant pool of gene targets for further disease and pathway analysis.

Plants	Targets Retrieved from Binding DB	Common Names Mapped via STRING	Targets Retrieved from SwissTargetPrediction	Total Targets (Before Filtering)	Overlapping Targets Between Databases	Final Filtered Targets
Aegle marmelos	189	154	3155	3309	100	3209
Ocimum tenuiflorum	468	409	3786	4195	31	4164
Pongamia pinnata	754	10	5008	5018	5	5013
Valeriana jatamansi	469	395	5086	5481	115	5366
Cedrus deodara	151	120	2459	2579	20	2559
Terminalia chebula	703	605	1317	1922	300	1622
Phyllanthus emblica	1269	1123	2269	3392	500	2892
Terminalia bellirica	419	356	768	1124	150	974
Piper longum	134	126	2946	3072	50	3022
Piper nigrum	34	25	989	1,014	5	1009
Zingiber officinale	4122	3385	26,633	30,018	2,079	27,939
Curcuma longa	560	467	8523	8990	200	8790
Berberis aristata	469	373	1880	2253	150	2103
Total				**72,367**		**68,635**

Table 5. Summary of Predicted Molecular Targets for the 13 herbs in Vilwadi Gulika. This table presents the number of molecular targets predicted for each of the thirteen plants in Vilwadi Gulika using Binding DB and SwissTargetPrediction. After merging and removing duplicates, a refined list of unique targets was generated for each plant. These filtered targets form the basis for understanding the biological mechanisms and therapeutic relevance of Vilwadi Gulika.

4.1.3 Target, Disease, Pathway and Gene prioritization

From the list of filtered molecular targets, disease and pathway annotations were generated across 13 plants, resulting in 1,347 disease entries, 238 KEGG pathways and 1,047 genes. Based on established clinical studies, pharmacological relevance, and supportive evidence from literature, six gastrointestinal disorders were identified as the most pertinent: colitis, Crohn's disease, gastroesophageal reflux disease (GERD), regional enteritis, ileocolitis, and duodenal ulcer.

Comparison of disease – associated genes across all the plants showed clear disease specific patterns. A total of 23 genes were associated with gastrointestinal disorders across the formulation. Among these **NOS2, SRC, PTGS2** were associated with colitis across the thirteen plants. **JAK2** linked to **Crohn's disease** appeared in the ten plants. **TGFB1** relevant to duodenal ulcer was identified in the three plants and **CXCL8** associated with GERD (gaster esophageal reflux disease).

This frequency-based evaluation enabled identification of genes most consistently associated with gastrointestinal condition.

Further mapping of phytochemicals to disease specific gene shows recurrent phytochemicals interacting with key molecular targets. For colitis **Ellagic acid, Eugenol, Elemicin and Ethyl gallate** were associated with **NOS2, SRC, PTGS2.** In **Crohn's disease JAK2** was linked to phytochemicals including **Geranyl acetate, citronellal, citronellol, (E, Z) – farnesol, nerol, linalool, linalyl acetate, turmerol, nerolidol, piperine, and neryl acetate.** For duodenal ulcer **TGFB1** was associated with **Geranyl diphosphate, Aristolactam, geranylgeranyl diphosphate, and allocryptopine,** while **TNF** in ileocolitis and regional enteritis was linked to **Curlone and Arjungenin**.

4.1.4 Network Construction and Topological Analysis

A comprehensive interaction network integrating all phytochemicals from the thirteen medicinal plants of *Vilwadi Gulika* with their predicted gene targets and biological pathways and the gastrointestinal diseases were constructed (Figure 7). In addition to disease specific networks, individual plant wise interaction network was constructed to examine the contribution of each medicinal plant to gastrointestinal disease – associated genes and pathways. These plant specific networks show how distinct phytochemical overlap inflammatory and immune related targets. The detailed interaction maps for each of the thirteen plants are given in the Annexure 4.

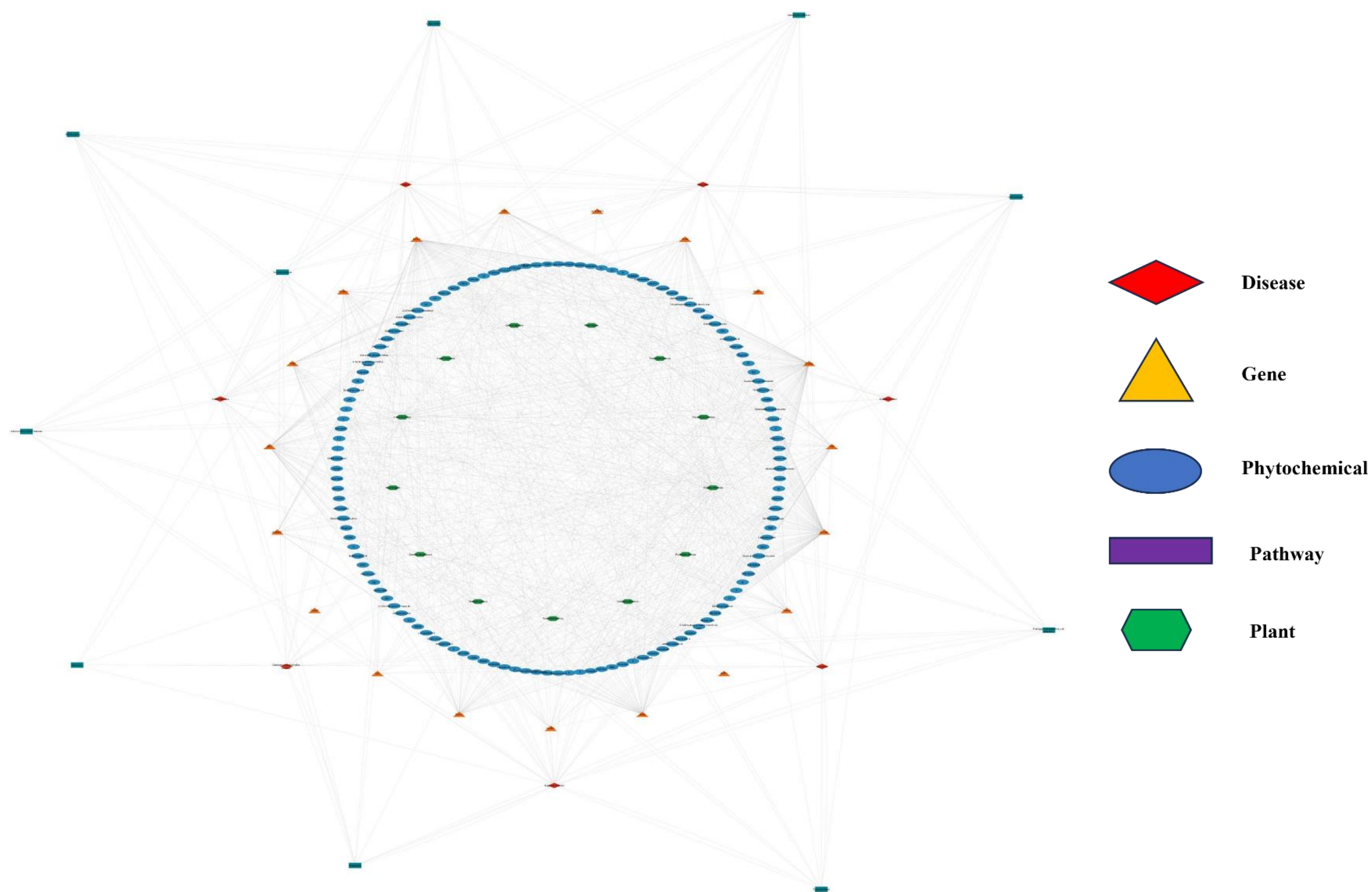

Figure 7. Integrated phytochemical-gene-pathway and disease network of *Vilwadi Gulika.* The network represents the complete set of phytochemicals, their associated gene, targets, KEGG pathways and the six selected gastrointestinal diseases, illustrating multi component and multi target nature of the formulation.

Central hub genes including IL6, TNF, PTGS2, IL1B, RELA and PPARG and several MAPK-related gene showed high connectivity, indicating their roles in mediating the potential therapeutic effects. Key pathways relevant to inflammatory and gastrointestinal regulation, NF-κB signalling, cytokine signalling, Toll-like receptor signalling, TNF signalling, and epithelial barrier regulation, were prominently represented.

Disease specific subnetwork was generated for the six gastrointestinal conditions: Regional enteritis, ileocolitis, Colitis, Crohn’s disease, gastroesophageal reflux disease (GERD) and duodenal ulcer **(Figure 8)**. These networks demonstrated that a substantial proportion of Vilwadi Gulika’s phytochemicals interacted with targets directly implicated in gastrointestinal pathology.

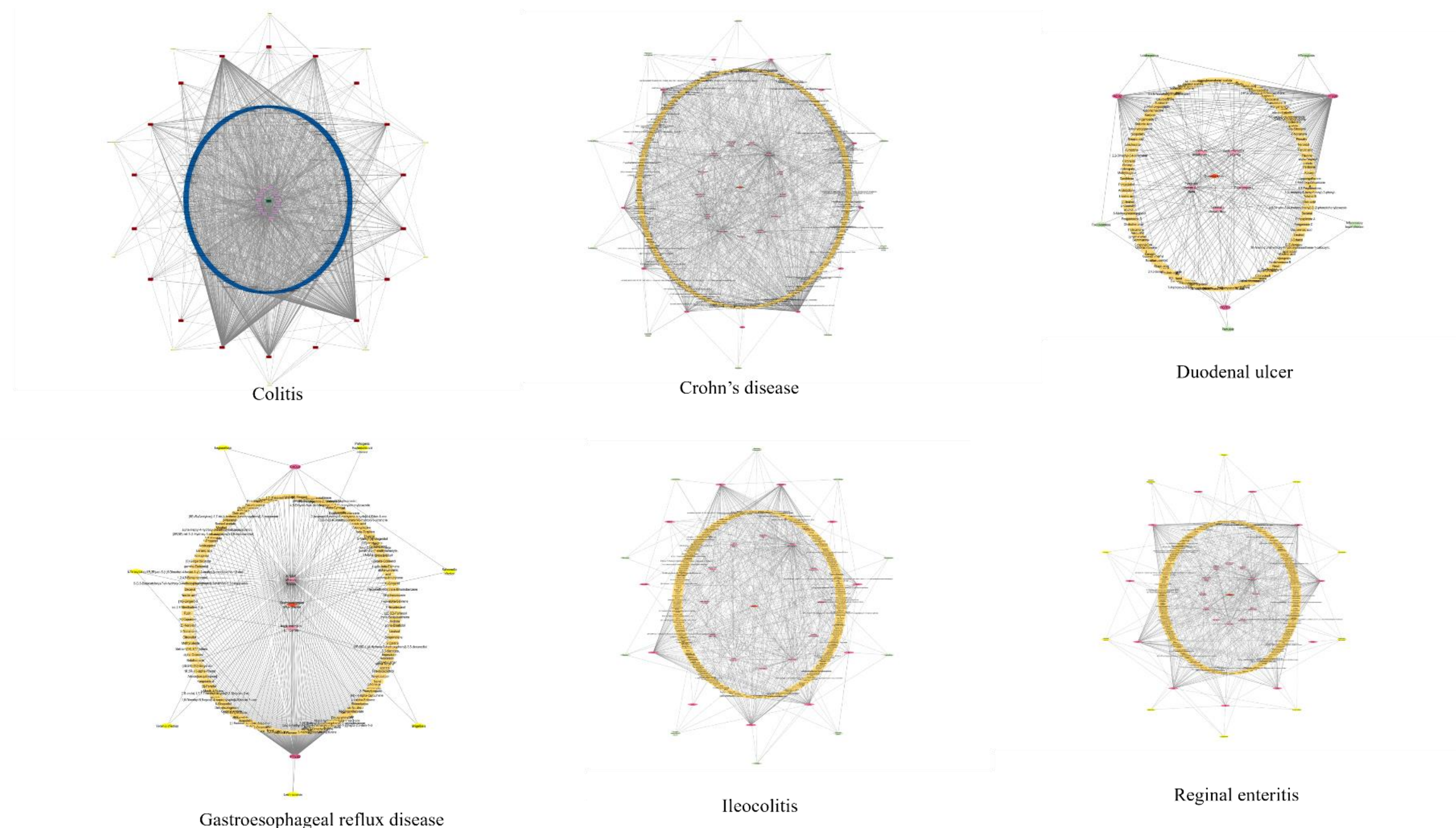

Figure 8. Disease – specific interactions networks of Vilwadi Gulika phytochemicals with gastrointestinal disease related genes and pathways. These networks illustrate the interactions between Vilwadi Gulika's phytochemicals, the genes they target, and the pathways associated with six major gastrointestinal diseases. Each disease-specific network shows how multiple phytochemicals converge on key inflammatory and immune-regulatory genes. The connectivity patterns highlight the multi-component and multi-target therapeutic potential of the formulation.

Among the 3,004 retrieved phytochemicals, a refined subset displayed strong, direct interactions with the main gene targets. Notable molecules included eugenol, ellagic acid, ethyl gallate, linalool, nerolidol, geranyl formate, β-sitosterol, allocryptopine, geranyl diphosphate, geranylgeranyl diphosphate, aristolactam, auraptene, geranic acid, nerolic acid, arjungenin, and curlone. These compounds collectively interacted with 23 key genes, several of which SRC, JAK2, NOS2, PTGS2, TGFB1, CXCL8, and TNF served as central regulatory nodes involved in inflammatory signalling, cytokine modulation, and immune pathways.

4.2 Microbiome Profiling of Vilwadi Gulika

4.2.1 Quality and Yield of Genomic DNA

Genomic DNA was isolated successfully from in house prepared Vilwadi Gulika samples, polyherbal mixture, goat urine, Vaidya prepared formulation, and commercial tablet. The purity and DNA concentration were assessed using Nanodrop spectrophotometry (Table 6).

Sample description	Sample ID	ng/µl	A260/280	A260/230
Vilwadi Gulika Batch 1, Day 0	VGB1D00	18.1	1.01	0.23
Vilwadi Gulika Batch 1, Day 03	VGB1D03	9.3	1.34	0.09
Vilwadi Gulika Batch 1, Day 05	VGB1D05	6.2	1.36	0.06
Vilwadi Gulika Batch 1, Day 07	VGB1D07	3.7	1.15	0.01
Vilwadi Gulika Batch 1, Sun dried	VGB1SD	17.9	0.71	0.33
Vilwadi Gulika Batch 2, Day 0	VGB2D00	6.4	1.19	0.11
Vilwadi Gulika Batch 2, Day 03	VGB2D03	0.4	0.66	0.01
Vilwadi Gulika Batch 2, Day 05	VGB2D05	22.4	1.39	0.33
Vilwadi Gulika Batch 2, Day 07	VGB2D07	7.6	1.24	0.17
Vilwadi Gulika Batch 2, Sun dried	VGB2SD	7.7	1.28	0.08
Vilwadi Gulika, Polyherbs	VGPH1	9.2	0.81	0.19
Vilwadi Gulika, Goat Urine 01	VGGU01	6.6	0.74	0.2
Vilwadi Gulika Goat Urine 02	VGGU02	61	1.58	0.51
Vilwadi Gulika Commercial Tablet	VGCT	10.1	1.10	0.04
Vilwadi Gulika by Vaidya	VGBV	13.6	1.34	0.09

Table 6. Quality check of genomic DNA using Nanodrop, showing concentration and purity levels in Vilwadi Gulika samples across two batches.

The purity of DNA was evaluated using the ratio A260/280 and A260/230 ratios. Ideally an A260/280 ratio of approximately 1.8 and an A260/230 ratio in the range of 2.0 – 2.2 are considered of pure DNA. In several samples, these ratios were lower than the ideal values, suggesting presence of impurities such as phenolic compounds, polysaccharides, plant derived secondary metabolites, or any other inhibitors.

Despite these challenges, sufficient quantities of meta genomic DNA were obtained from the Vilwadi Gulika samples and the quality was adequate for successful PCR amplification and further microbiome analysis.

4.2.2 Bacterial Identification - Quality Assessment of PCR – Amplified 16S rRNA Gene Products

The yield and the quality of PCR amplified 16S rRNA gene products from the Vilwadi Gulika samples were assessed prior to sequencing to confirm successful amplification and suitability for further analysis. The nanodrop spectrophotometric measurements showed strong DNA concentrations across samples, ranging from 500ng/µL to >1400 ng/µL, showing efficient amplification of microbial DNA from all the preparation stages, including Batch 1 and Batch 2 samples, sun dried formulation, polyherbal, goat urine, commercial tablet and Vaidya prepared Vilwadi Gulika **(Table 7).**

Sample description	Sample ID	ng/µl	A260/280	A260/230
Vilwadi Gulika Batch 1, Day 0	VGB1D00	547.7	1.81	1.73
Vilwadi Gulika Batch 1, Day 03	VGB1D03	1203.6	1.81	1.8
Vilwadi Gulika Batch 1, Day 05	VGB1D05	816.8	1.84	1.7
Vilwadi Gulika Batch 1, Day 07	VGB1D07	961.1	1.84	1.59
Vilwadi Gulika Batch1, Sun dried	VGB1SD	1060.4	1.78	1.7
Vilwadi Gulika Batch 2, Day 0	VGB2D00	1035.3	1.83	1.78
Vilwadi Gulika Batch 2, Day 03	VGB2D03	800.6	1.82	1.82
Vilwadi Gulika Batch 2, Day 05	VGB2D05	909.5	1.82	1.85
Vilwadi Gulika Batch 2, Day 07	VGB2D07	1482.5	1.7	1.39
Vilwadi Gulika Batch 2, Sun dried	VGB2SD	1173.9	1.81	1.78
Vilwadi Gulika, Polyherbs	VGPH1	1116.6	1.77	1.73
Vilwadi Gulika, Goat Urine 01	VGGU01	1039.1	1.75	1.68
Vilwadi Gulika Goat Urine 02	VGGU02	904.2	1.85	1.82
Vilwadi Gulika Commercial Tablet	VGCT	608.6	1.82	1.53
Vilwadi Gulika by Vaidya	VGBV	796.5	1.85	1.77

Table 7. Quality check of PCR product from Vilwadi Gulika samples (two independent batches across different preparation days, sun-dried samples, polyherbal mix, and goat urine), showing DNA concentration and purity ratios.

The A260/280 ratios were generally close to the ideal value of 1.8, suggesting minimal protein contamination, while A260/230 ratios showed moderate variation, which is commonly observed in PCR products derived from polyherbal and fermented matrices due to the presence of residual salts or amplification reagents. Overall, the nanodrop results confirms the PCR products were of adequate concentration and acceptable purity for sequencing.

Further validation was done using agarose gel electrophoresis. Clear and distinct bands corresponding to expected 1500 bp fragment of the full length bacterial 16S rRNA gene were observed across the samples of Vilwadi Gulika (**Figure 9).** The presence of uniform bands across the different samples confirms the successful PCR amplification.

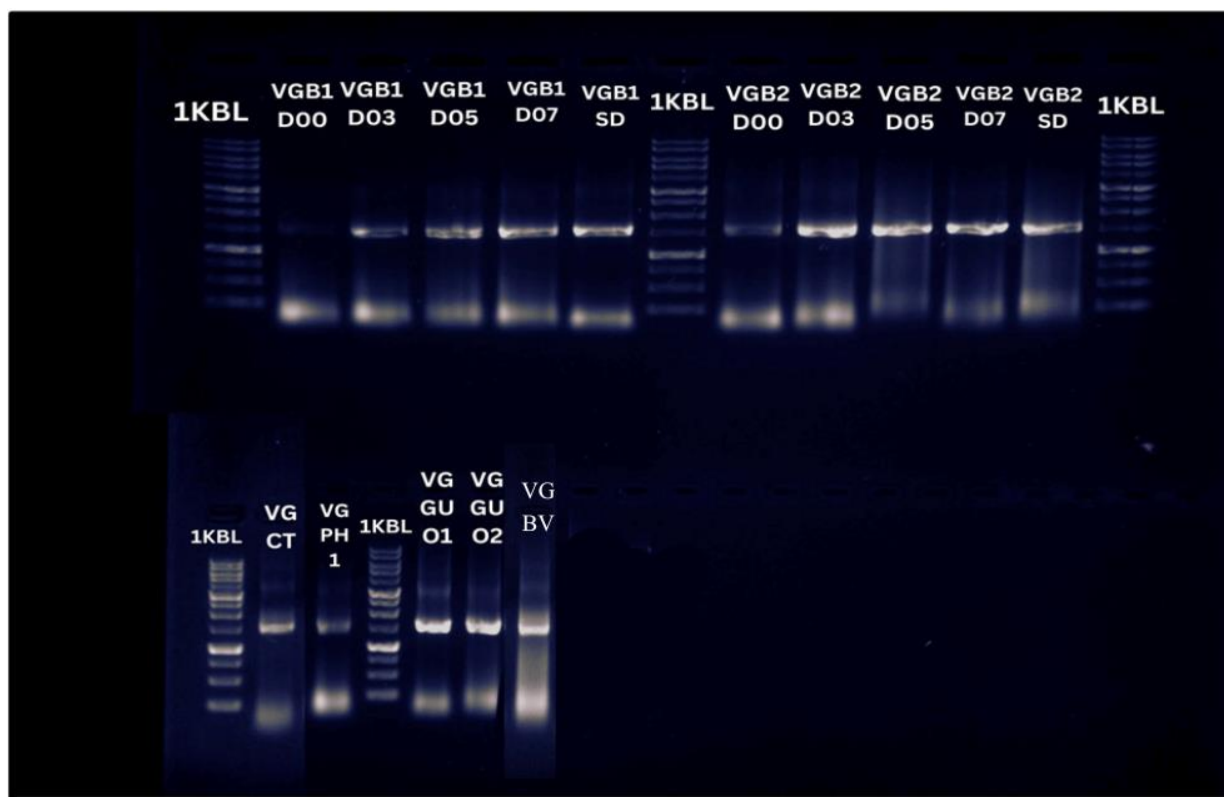

Figure 9. Agarose gel image of Vilwadi Gulika showing 16S rRNA gene amplification (~1500 bp) at different preparation stages (Day 0, 3, 5, 7, Sun-dried), with 1 kb DNA ladder used as molecular marker

4.2.3 Qubit Based Quantification and Library Preparation

Qubit fluorometric quantification showed high concentration of PCR products across the samples, confirming the efficient amplification (Table 8)

Sample Description	Sample ID	PCR product Qubit Reading	Qubit Reading-End Prep library ng/ul
Vilwadi Gulika Batch 1, Day 0	VGB1D00	96.8	11.6
Vilwadi Gulika Batch 1, Day 3	VGB1D03	19.3	5.68
Vilwadi Gulika Batch 1, Day 5	VGB1D05	56.8	6.54
Vilwadi Gulika Batch 1, Day 7	VGB1D07	94.6	4.88
Vilwadi Gulika Batch 1, Sun dried	VGB1SD	35.6	7.8
Vilwadi Gulika Batch 2, Day 0	VGB2D00	19	4.7
Vilwadi Gulika Batch 2, Day 3	VGB2D03	27.2	7.78
Vilwadi Gulika Batch 2, Day 5	VGB2D05	25.8	10.7
Vilwadi Gulika Batch 2, Day 7	VGB2D07	34	7.7
Vilwadi Gulika Batch 2, Sun dried	VGB2SD	34.2	10.1
Vilwadi Gulika Goats urine 1	VGGU01	79.0	1.71
Vilwadi Gulika Goats urine 2	VGGU02	34.4	4.6
Vilwadi Gulika poly herbs 1	VGPH1	15.2	9.26
Vilwadi Gulika Commercial Tablet	VGCT	160	7.06
Vilwadi Gulika by Vaidya	VGBV	102	8.04

Table 8. Qubit-based Quantification of PCR Products and Prepared Libraries from Vilwadi Gulika Samples

Following library preparation, DNA concentrations decreased to expected levels due to fragmentation and adapter ligation steps. The final concentrations fell within the recommended range for Oxford Nanopore sequencing, showing the successful library preparation suitable for sequencing.

4.2.4 Sequencing Output and Read Statistics

Variable number of raw reads were generated across the samples of the 16S rRNA gene (Table 9).

Sample Description	Sample ID	Total Raw Reads	Mean Raw Read length (bp)	Total Processed Reads	Mean Processed Read length (bp)
Vilwadi Gulika Batch 1 Day 0	VGB1D00	66169	807	3094	1497
Vilwadi Gulika Batch 1 Day 03	VGB1D03	168265	524	14549	1590
Vilwadi Gulika Batch 1 Day 05	VGB1D05	779027	463	36272	1596
Vilwadi Gulika Batch 1 Day 07	VGB1D07	555669	595	67077	1599
Vilwadi Gulika Batch 1 Day Sun dried	VGB1SD	104000	393	6486	1595
Vilwadi Gulika Batch 2 Day 03	VGB2D03	168265	524	14549	1590
Vilwadi Gulika Batch 2 Day 05	VGB2D05	341436	476	23958	1588
Vilwadi Gulika Batch 2 Day 07	VGB2D07	140000	476	11651	1600
Vilwadi Gulika Batch 2 Day Sun dried	VGB2SD	688876	461	42694	1594
Vilwadi Gulika Goat Urine	VGGU01	344888	543	38048	1602
Vilwadi Gulika Polyherbs	VGPH1	332237	535	7211	1535
Vilwadi Gulika Commercial Tablet	VGCT	25275	493	400	1570.3
Vilwadi Gulika by Vaidya	VGBV	9889	692.8	757	1601

Table 9. Sequencing Read Statistics in Vilwadi Gulika Samples.

Total raw reads ranged from 9,889 reads in the Vaidya-prepared formulation to 779,027 reads in Batch 1 Day 05, with mean raw read lengths varying between 393 bp and 807 bp. After quality filtering and processing, the number of retained reads ranged from 400 reads in the commercial tablet to 67,077 reads in Batch 1 Day 07. The mean processed read length across samples consistently remained within the range of approximately 1500–1600 bp, indicating successful retention of near full-length 16S rRNA gene sequences suitable for reliable taxonomic classification. Samples yielding fewer than 1,000 quality-filtered reads were excluded for the taxonomic and diversity analyses, as very low sequencing depth is known to result in unstable and unreliable microbial community profiles. Accordingly, Vilwadi Gulika Batch 2 Day 0 (VGB2D00) and the goat urine sample (VGUU02), which retained only 115 and 144 processed reads respectively after quality filtering, were not included in further microbiome analyses.4.11 Taxonomic Diversity Across Vilwadi Gulika samples. At different preparation stages taxonomic classification showed diverse microbial communities across Vilwadi Gulika samples (Table 10)

Sample Description	Sample ID	Phylum	Class	Order	Family	Genus	Species
Vilwadi Gulika Day 0	VGBD00	16	34	67	125	215	349
Vilwadi Gulika Day 03	VGBD03	16	25	59	104	164	275
Vilwadi Gulika Day 05	VGBD05	16	28	61	98	164	285
Vilwadi Gulika Day 07	VGBD07	18	29	66	117	214	354
Vilwadi Gulika Sun dried	VGBSD	16	30	63	112	201	**382**
Vilwadi Gulika Goat Urine	VGGU01	**21**	**40**	**83**	**139**	**226**	379
Vilwadi Gulika Polyherbs	VGPH1	20	29	67	110	198	352
Vilwadi Gulika Commercial Tablet	VGCT	12	19	34	53	64	106
Vilwadi Gulika by Vaidya	VGBV	12	19	34	59	81	151

Table 10. Taxonomic Distribution Across Vilwadi Gulika Samples. During the fermentation (day 0 to day 7), number of phyla remained (16 – 18), The sun-dried formulation exhibited the dominant microbial community at species level.

Goat urine showed highest microbial communities at phylum, class, order, family genus. The polyherbal mixture also showed highest taxonomic diversity. The commercial and Vaidya prepared formulation exhibited lower microbial communities mainly at species level.

4.2.5 Taxonomic Diversity Across Vilwadi Gulika samples

Phylum level distribution during the preparation of in house Vilwadi Gulika

At phylum level (Figure 10), polyherbal mixture showed a diverse microbial profile dominated by *Proteobacteria*, along with *Firmicutes, Actinobacteria, Cyanobacteria* and *Bacteroidetes.* Goat urine was showed *Firmicutes* as dominant one. Further mixing and during the initial stage of fermentation (Day 0), *Firmicutes* became dominant phylum. From day 3, *Firmicutes* consistently dominated the microbial community through day 5, 7 and sun-dried stages, showing the selective enrichment and stabilization during the processing of formulation.

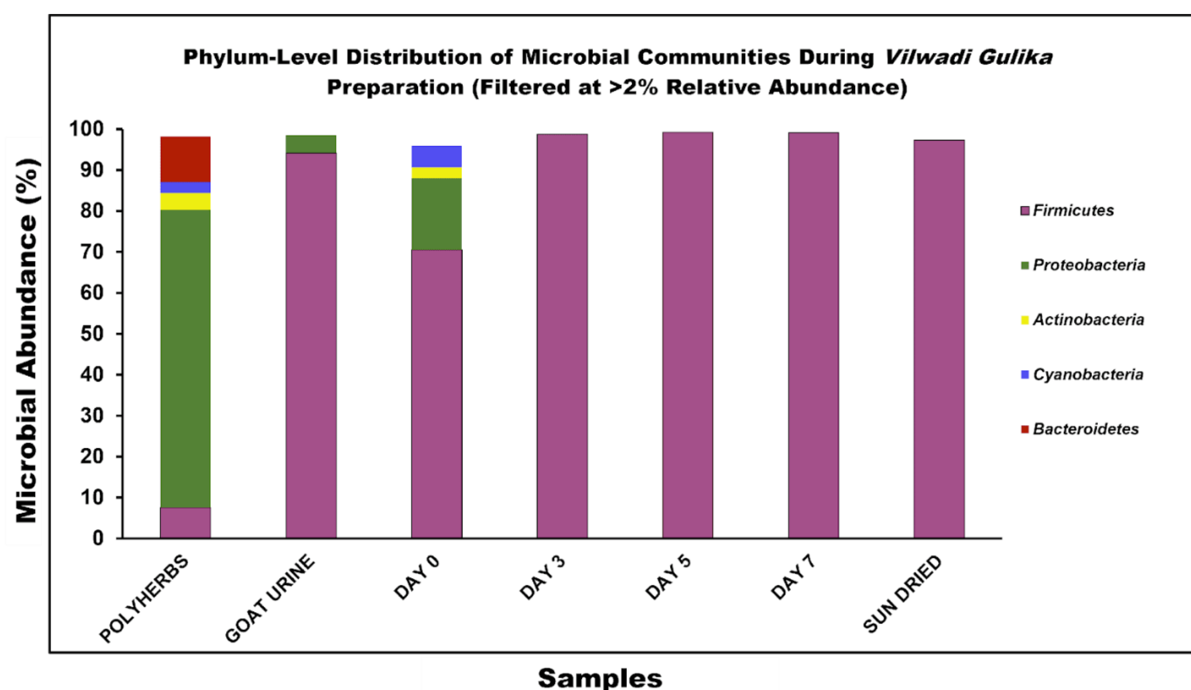

Figure 10. Distribution of Microbial Communities at the Phylum Level Found During Vilwadi Gulika Preparation. The relative abundance of prominent bacterial phyla (>2% prevalence) at various stages of Vilwadi Gulika processing, including raw Polyherbs and goat urine, is depicted in this stacked bar figure. By Day 3, the formulation exhibits a swift transition toward Firmicutes dominance, which continues through Days 5, 7, and the sun-dried stage. Higher diversity is seen in early-stage samples (Polyherbs, goat urine, Day 0), especially in Proteobacteria, Actinobacteria, and Bacteroidetes, which decrease as fermentation stabilizes the microbial profile.

Genus and Species level microbial shifts

At the genus level, poly herbs and goat urine showed diverse microbial populations. However, from day 3 onwards reduction of diversity was observed, with dominance of *Aerococcus.* (Figure 11).

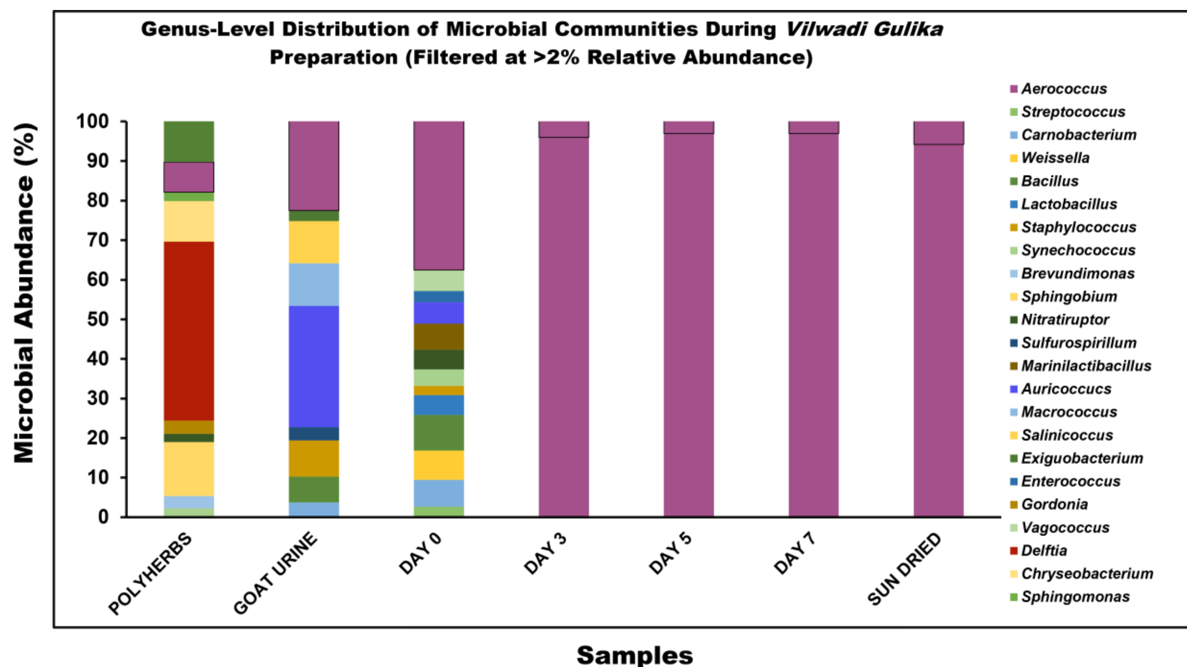

Figure 11. Genus-Level Distribution of Microbial Communities During Vilwadi Gulika Preparation (Filtered at >2% Relative Abundance).

The predominant bacterial genera found at various Vilwadi Gulika production phases are depicted in this figure. *Staphylococcus*, *Carnobacterium, Weissella, Bacillus, Brevundimonas, Sphingobium*, and other species contribute to the high microbial heterogeneity of the raw ingredients (Polyherbs and goat urine). By Day 0, the microbial composition starts to change in favour of taxa linked to fermentation, such as *Weissella, Lactobacillus, Streptococcus*, and *Carnobacterium*. *Aerococcus* virtually completely dominates the microbial community from Day 3 to Day 7 and in the sun-dried product, suggesting a high selective enrichment of this genus during the fermentation and churning stages. This noticeable change indicates the stabilization of a microbiome driven by fermentation, which was probably influenced by the availability of nutrients, pH variations, and competing microbial interactions during conventional preparation.

Species-level analysis revealed a strong selection toward *Aerococcus viridans* during later stages of preparation, including Day 3, Day 5, Day 7, and sun-dried Vilwadi Gulika (Figure 12). Other species persisted at low relative abundance, indicating microbial stabilization and dominance of specific taxa during fermentation.

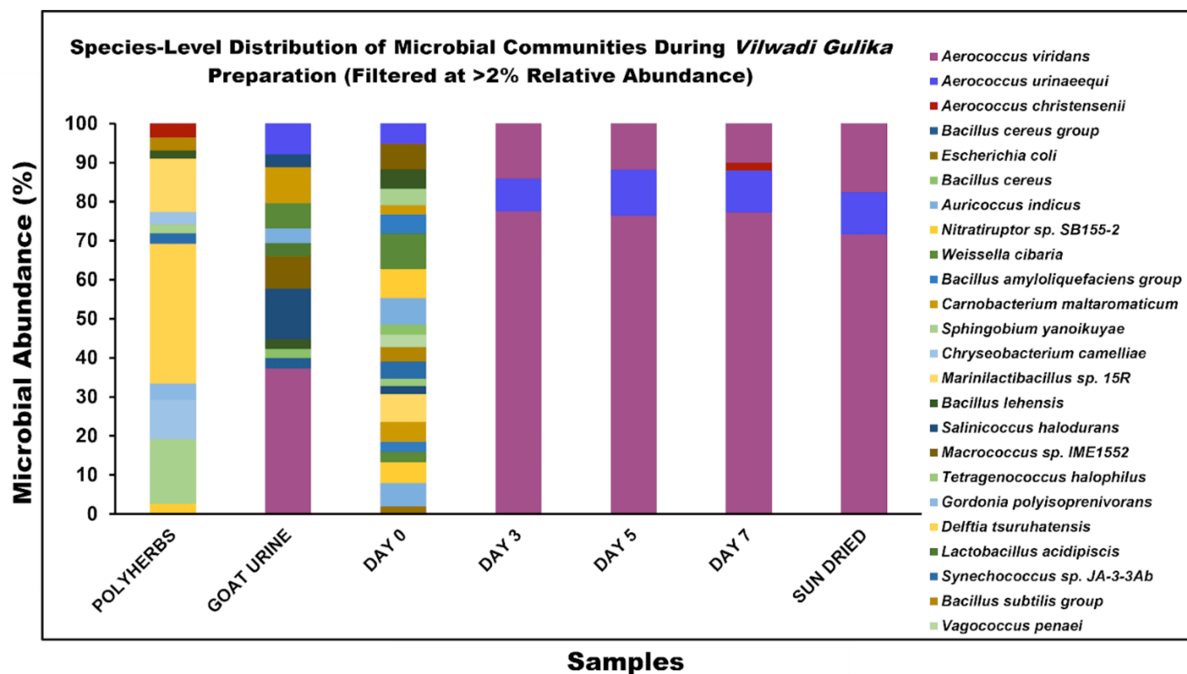

Figure 12. Microbial Communities' Species-Level Distribution During Vilwadi Gulika Preparation (Filtered at >2% Relative Abundance).

The predominant bacterial species found at each stage of Vilwadi Gulika preparation are depicted in this image. *Weissella cibaria, Bacillus cereus group, Escherichia coli, Chryseobacterium camellae, Sphingobium yanoikuyae*, and a number of environmental and plant-associated bacteria are among the extremely varied species profiles found in the raw materials (Polyherbs and goat urine). Day 0 still exhibits this variability, which is a direct result of bacteria from goat urine and herbs. *Aerococcus viridans* exhibits a sharp and steady shift toward near-total dominance starting on Day 3, although *A. urinaeequi* is present in lower amounts. Day 5, Day 7, and the finished sun-dried product all exhibit this species-level stabilization, suggesting a significant selective enrichment of Aerococcus species throughout fermentation. The establishment of a single dominating species and the gradual decline in microbial diversity point to a microbiome homogenization process that is fuelled by interspecies competition, pH fluctuations, churning, and selective enrichment during conventional preparation.

Shared Species and Microbial Overlap Analysis

An Upset plot analysis (Figure 13) demonstrated shared and unique microbial species across different sample sets, including Polyherbs, goat urine, fermentation stages, and final formulations. The analysis showed partial overlap between raw materials and formulated samples, while fermentation stages shared a higher number of common species, reflecting microbial succession and stabilization during preparation.

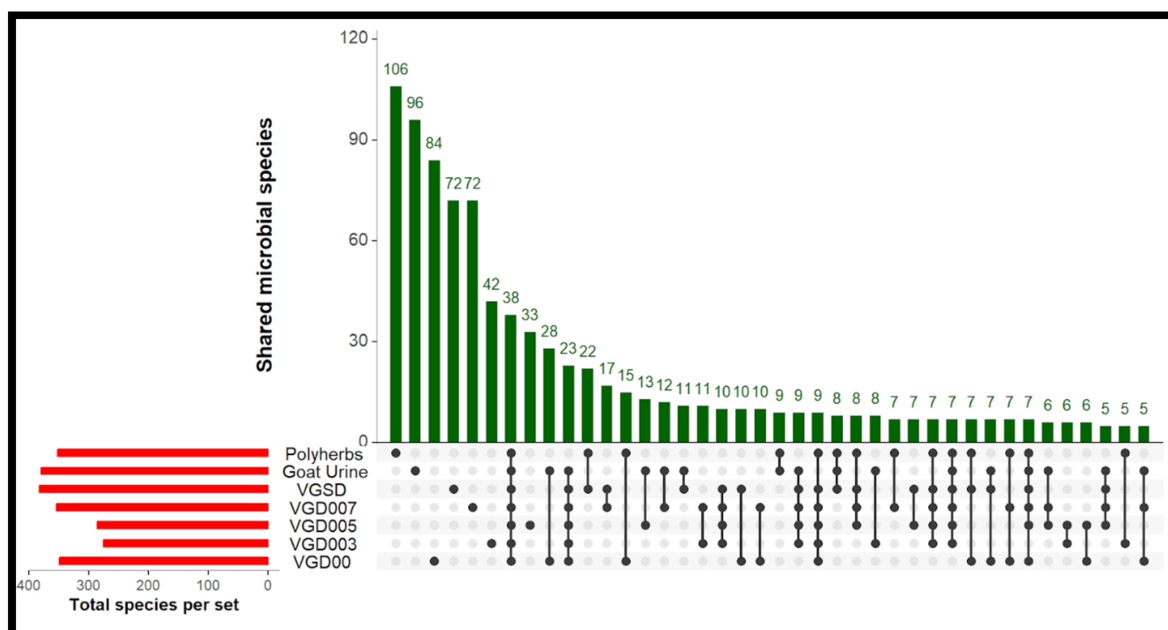

Figure 13. Shared Microbial Species Across *Vilwadi Gulika* Samples Identified Through Upset plot.

The overlap of microbial species between various Vilwadi Gulika preparation components and stages is seen in this Upset figure. The total species richness in each sample (Polyherbs, Goat Urine, VGSD, VGD07, VGD05, VGD03, VGD00) is shown by the horizontal red bars on the left. Because of their naturally varied microbial inputs, Polyherbs and Goat Urine have the highest total species counts among these. The number of species shared by particular sample combinations is shown by the vertical green bars, with the biggest intersections between the main stages of formulation. Microbial stability during churning and fermentation is highlighted by the tallest bars (106, 96, and 84 shared species), which show a sizable core microbiome consistently maintained across several stages of preparation. The number of shared species rises and becomes more consistent as the formulation advances (Day 0 → Day 7 → Sun-dried), indicating the selection and enrichment of a stable microbial population within the Ayurvedic product. This illustrates how the preparation procedure stabilizes and refines the microbial assemblage while the raw ingredients add a variety of microbial characteristics

Comparative Microbial Profiles of Vaidya-Prepared and Commercial Formulations

Comparative analysis of Vaidya-prepared and commercial Vilwadi Gulika revealed differences in microbial composition at phylum, genus, and species levels (Figure 14). While both formulations were dominated by *Firmicutes*, the Vaidya-prepared formulation exhibited greater taxonomic diversity compared to the commercial tablet, which showed reduced microbial richness.

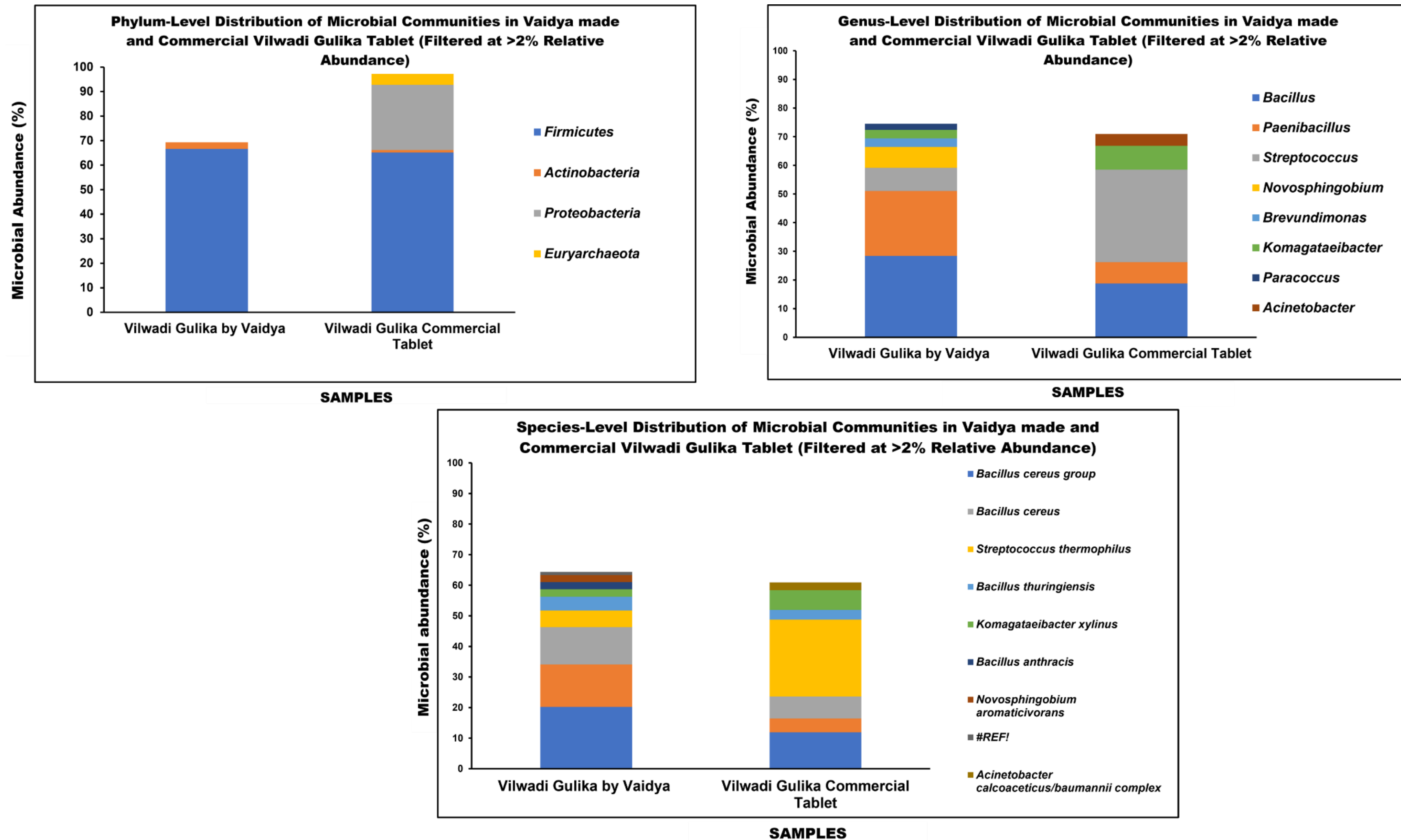

Figure 14. Phylum, Genus, and Species-Level Microbial Composition of Vaidya-Prepared and Commercial Vilwadi Gulika

Alpha and Beta Diversity Analysis of Microbial Communities in Vilwadi Gulika

Microbial diversity across different samples of Vilwadi Gulika was evaluated using alpha diversity indices, including species richness, evenness, Shannon diversity index, and Simpson diversity index, along with beta diversity analysis using Principal Coordinates Analysis (PCoA).

Species richness analysis (Figure 15) revealed lower richness in the raw materials, namely Polyherbs (VGPH) and goat urine (VGGU), compared to the mixed and fermented formulations. Following the initiation of formulation and churning, microbial richness increased markedly and remained consistently high across all fermentation stages (Day 0 to Day 7) as well as in the sun-dried formulation. This trend indicates an expansion and stabilization of microbial populations during the preparation process of Vilwadi Gulika.

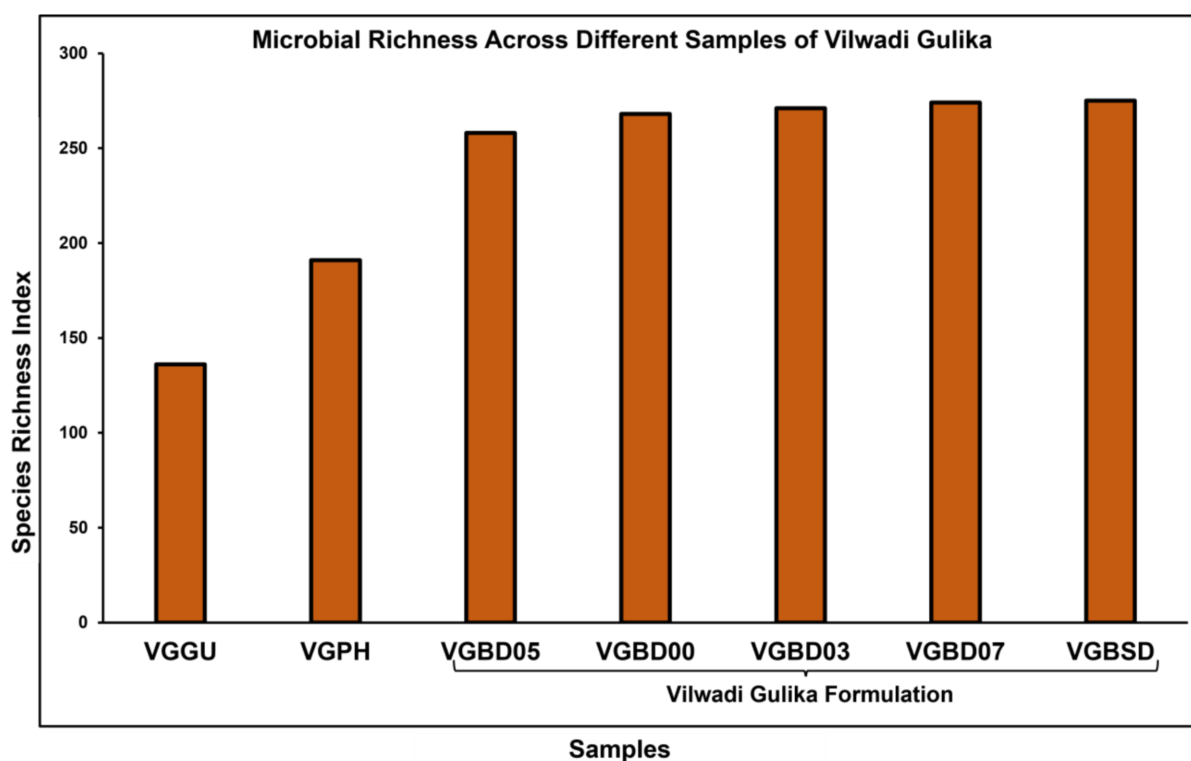

Figure 15. Microbial Species Richness in Various Vilwadi Gulika Components and Preparation Stages.

The dynamic expansion of various microbial taxa made possible by fermentation is reflected in the microbial richness peaks that occur between days 3 and 7. The sun-dried sample (VGBSD) has the highest richness. The formulation that has been sun dried has the highest species richness (276). Sun drying probably concentrates microbial DNA without significantly decreasing variety, showing the product's ultimate stabilized microbiome.

Microbial evenness analysis (Figure 16) showed lower evenness values in Polyherbs and goat urine, suggesting dominance of a limited number of microbial taxa in the raw materials. In contrast, Vilwadi Gulika samples collected after mixing and during fermentation exhibited higher evenness indices, indicating a more balanced distribution of microbial taxa. The

evenness remained stable from Day 0 through Day 7 and in the sun-dried formulation, reflecting the establishment of a structured microbial community.

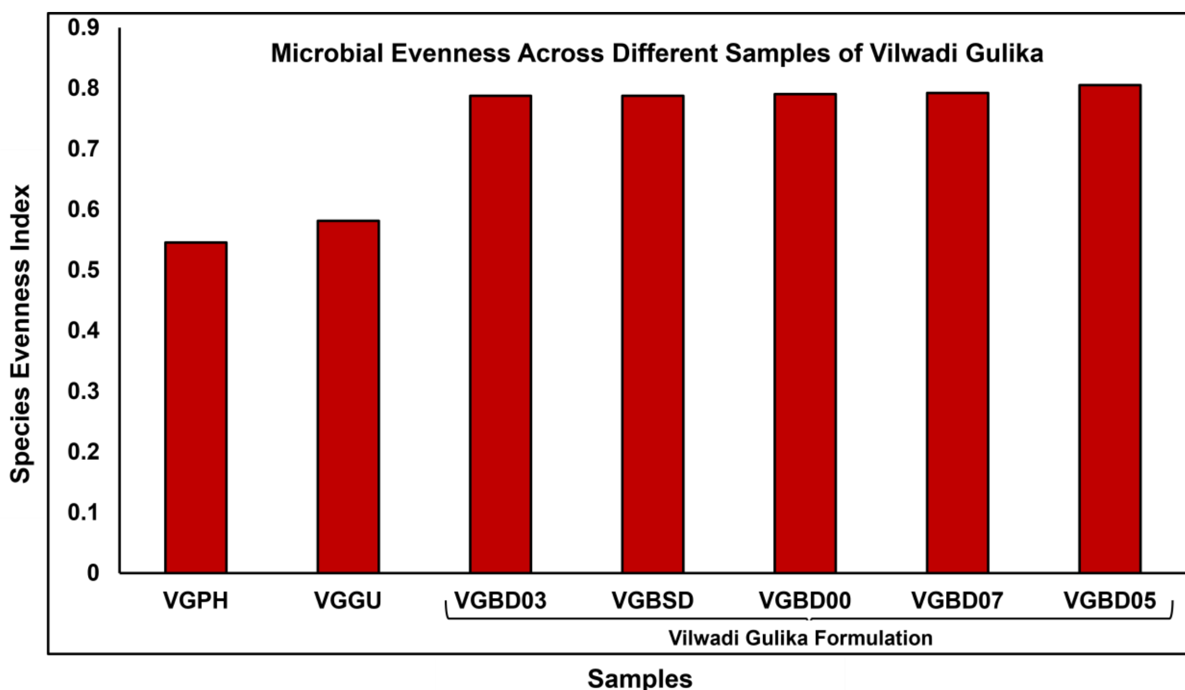

Figure 16. Microbial Evenness Across Different Samples of Vilwadi Gulika Samples.

The species evenness index for each stage and component of Vilwadi Gulika preparation is displayed in this figure. Goat urine (VGGU) and Polyherbs (VGPH) had lower evenness scores, suggesting that a small number of bacterial species predominate in the raw materials. The Vilwadi Gulika formulation samples (VGBD00, VGBD03, VGBD05, VGBD07, and VGBSD), on the other hand, show noticeably greater evenness values (0.78-0.80), indicating a more balanced distribution of species both during and after the preparation procedure. A more homogeneous microbial structure is promoted by mixing, churning, and fermentation-like processes, which lessen dominance by any one taxon while enabling community stabilization, as indicated by the improvement in evenness from raw inputs to final formulations. This lends credence to the notion that conventional preparation techniques aid in the final product's microbial homogeneity and balance.

Shannon diversity index values (Figure 17) further supported these observations. Lower diversity was observed in the raw materials, whereas a significant increase in Shannon diversity was noted following the initiation of fermentation. The elevated Shannon index values persisted across all fermentation stages and in the final sun-dried formulation, indicating sustained microbial diversity throughout the preparation process.

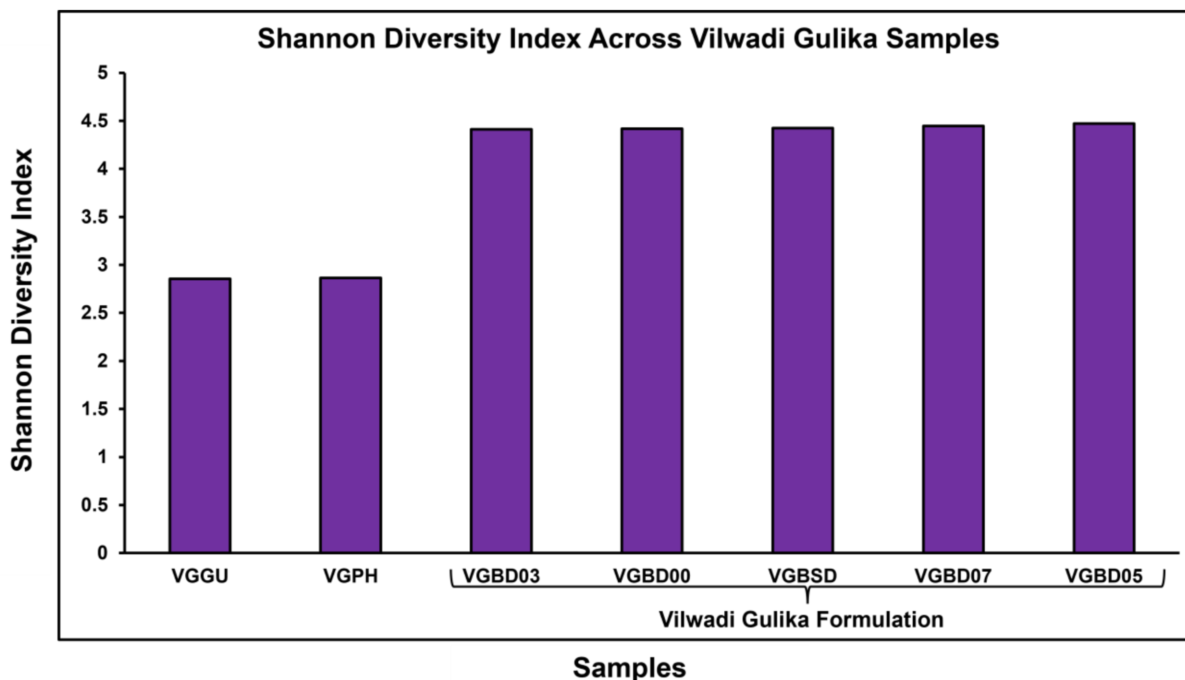

Figure 17. **Shannon Diversity Index Across *Vilwadi Gulika* Samples**.

The Shannon Diversity Index, which shows the richness and uniformity of microbial populations throughout Vilwadi Gulika's components and preparation phases, is shown in this figure. Goat urine (VGGU) and Polyherbs (VGPH), the raw materials, show comparatively reduced diversity (2.8), suggesting unequal microbial distribution dominated by a handful of species. On the other hand, Shannon diversity (4.4 - 4.5) is much higher in all Vilwadi Gulika formulation samples (VGBD00, VGBD03, VGBD05, VGBD07, and VGBSD). This steady rise implies that a more intricate and stable community structure is encouraged by the conventional preparation processes, such as churning, mixing, and time-dependent microbial interactions. Selection pressures, nutrient redistribution, and microbial cross-feeding during preparation are probably the driving forces behind the microbial diversification process that occurs from raw materials to the finished formulation. A strong, balanced microbial ecology is growing in the finished product, as evidenced by the high and consistent Shannon index across the Day 3–7 and sun-dried samples.

Similarly, Simpson diversity index analysis (Figure 18) demonstrated lower diversity in Polyherbs and goat urine, with substantially higher values observed in all fermented and final Vilwadi Gulika samples. The consistently high Simpson index values across Day 0 to Day 7 and sun-dried samples suggest reduced dominance by single taxa and increased community complexity during formulation.

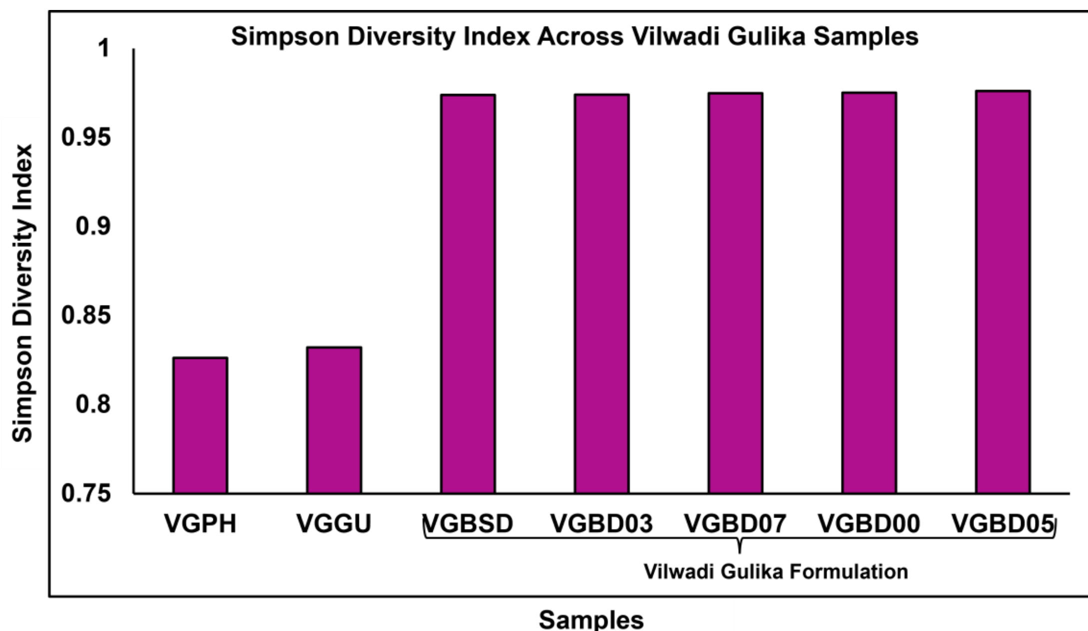

Figure 18. Simpson Diversity Index Across Different Vilwadi Gulika Samples.

Both species richness and evenness are reflected in the Simpson Diversity Index, which ranges from 0 to 1. Values nearer 1 indicate greater diversity and less dominance by any one species. The findings indicate that: Goat urine and Polyherbs, the initial constituents, have an inconsistent species distribution and a moderate level of diversity. Microbial evenness and diversity are greatly increased by Vilwadi Gulika preparation. A highly stable, diversified, and balanced microbial ecology is produced by the churning/fermentation process, which may enhance the formulation's medicinal potential.

Beta diversity analysis using Principal Coordinates Analysis (PCoA) (Figure 19) revealed distinct clustering patterns between raw materials and fermented Vilwadi Gulika samples. Polyherbs and goat urine formed separate clusters, while samples from Day 0, Day 3, Day 5, Day 7, and the sun-dried formulation clustered closely together. This clear separation indicates that fermentation and processing lead to the development of a stable and distinct microbial community structure in Vilwadi Gulika, differentiating it from the initial raw ingredients.

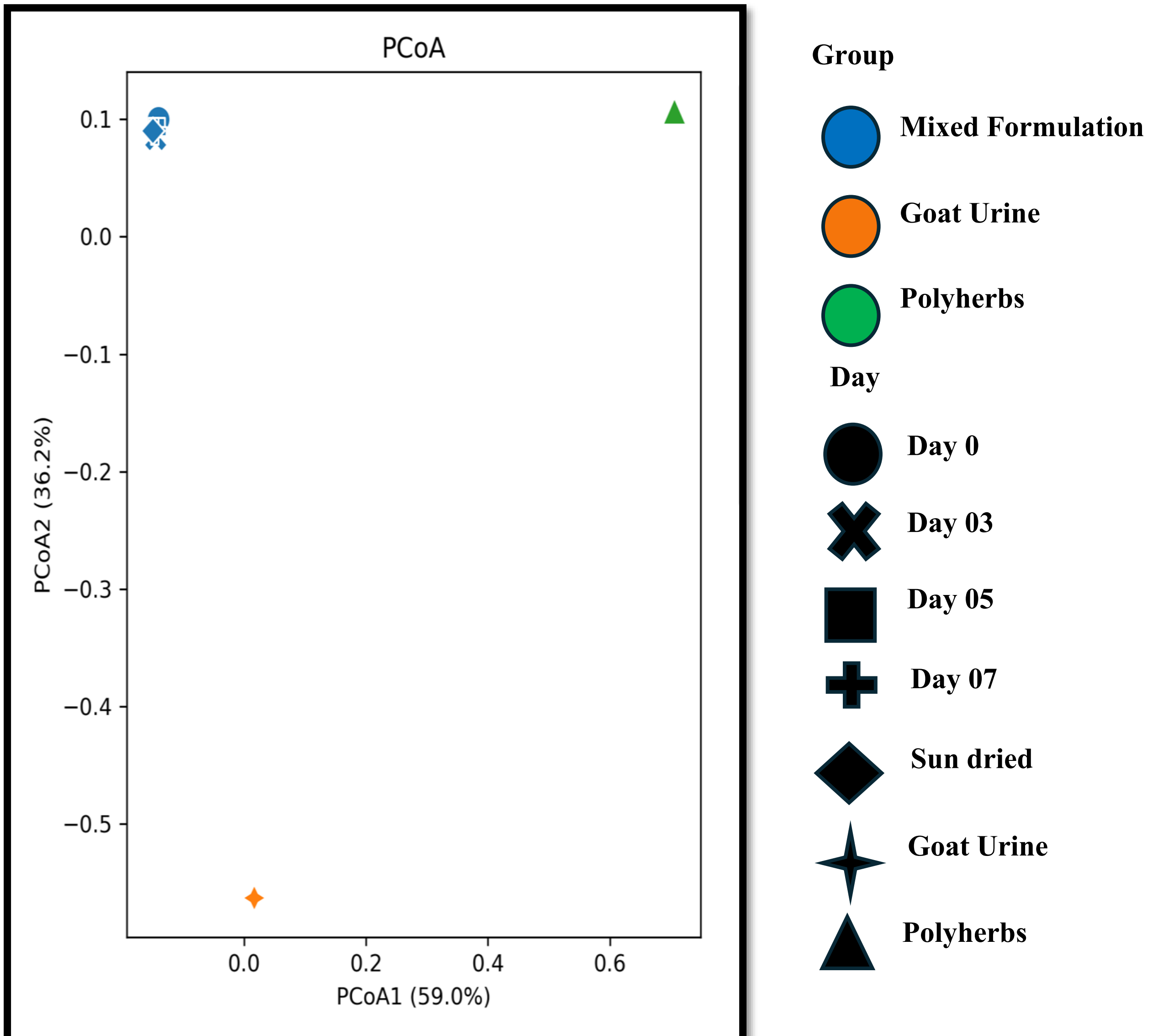

Figure 19. Principal Coordinates Analysis (PCoA) plot showing the clustering of microbial communities across different groups of Vilwadi Gulika.

Mixed formulation day 0,3,5,7 and Sun dried, Goat Urine, and polyherbal mixture are represented by different colours, while individual time points or sample types are indicated by distinct shapes. Overall, alpha and beta diversity analyses collectively demonstrate that the preparation and fermentation of Vilwadi Gulika promote increased microbial richness, diversity, and evenness, resulting in a stable and structured microbial ecosystem in the final formulation.

5.Discussion

5.1 Integrative Evaluation of Vilwadi Gulika Using Network Pharmacology and Microbiome Analysis

The present study evaluated the therapeutic potential of the classical Ayurvedic formulation *Vilwadi Gulika* through an integrative framework combining network pharmacology and microbiome profiling. Vilwadi Gulika is traditionally prescribed for acute toxico-pathological and gastrointestinal conditions, including *Sarpa Daṃśa*, *Visuchiks*, *Ajirna*, *Jvara*, and disorders associated with deranged *Agni* and *Ama* accumulation. Classical Ayurvedic texts attribute its actions to *Dipana*, *Pacana*, *Krimighna*, *Sulahara*, *Anulomana*, *Jvaraghna*, and *Viṣaghna* properties, with predominant action on *Kapha–Vata* doṣas. These traditional claims are supported by emerging clinical and experimental evidence [56].

Previous clinical observations indicate that Vilwadi Gulika, either alone or in combination with other Ayurvedic interventions, improves gastrointestinal health by modulating gut microbial composition and inflammatory status. Reduction in pathogenic taxa such as *Escherichia coli* and *Shigella* and improvement in beneficial microbial populations have been reported in autistic children receiving Vilwadi Gulika-based interventions [57]. Its therapeutic relevance in Crohn’s disease and acute gastroenteritis further suggests an immunomodulatory and gut-protective role [58,59].

5.2 Network Pharmacology Reveals Multi-Target Anti-Inflammatory Mechanisms

Network pharmacology analysis identified 3004 phytochemicals across the thirteen constituent herbs of Vilwadi Gulika, with 23 genes prioritized for gastrointestinal disorders. Core inflammatory targets included SRC, NOS2, PTGS2, JAK2, TGFB1, CXCL8, and TNF, mapping to conditions such as colitis, Crohn’s disease, GERD, duodenal ulcer, ileocolitis, and regional enteritis.

This absence of a single phytochemical common across all herbs highlights the synergistic, multi-component nature of the formulation rather than reliance on a dominant active principle. Such a pharmacological architecture aligns with Ayurvedic formulation philosophy and modern systems biology concepts of polypharmacology.

5.3 Modulation of Colitis-Associated Targets (SRC, NOS2, PTGS2)

Inflammatory bowel diseases (IBD), including colitis and Crohn’s disease, are characterized by chronic mucosal inflammation driven by oxidative stress and sustained cytokine production [60].The colitis-specific subnetwork identified SRC, NOS2, and PTGS2 as central regulatory nodes modulated by phytochemicals such as eugenol, ellagic acid, ethyl gallate, linalool, nerolidol, and β-sitosterol.

SRC family proteins act as key signal integrators in inflammatory cascades, influencing NF-κB and MAPK pathways [61,62]. Compounds targeting SRC have demonstrated efficacy in suppressing downstream inflammatory signaling. Notably, eugenol—identified in this study as

an SRC-interacting phytochemical—has been shown to restore gut microbial balance and reduce dysbiosis while attenuating inflammatory signalling [63].

Ellagic acid further contributes to anti-colitic effects by inhibiting NF-κB activation and suppressing PTGS2 (COX-2) expression, a key mediator of prostaglandin-driven inflammation in IBD [64,65]. Additionally, microbial conversion of ellagic acid into urolithins suppresses NOS2 and pro-inflammatory cytokines, highlighting a microbiome-assisted therapeutic mechanism [66].

5.4 Microbiome–Phytochemical Synergy in Colitis

Microbiome dysbiosis, characterized by depletion of Firmicutes and beneficial anaerobes, plays a critical role in IBD pathogenesis [67]. In the present study, Firmicutes dominance and consistent detection of Lactobacillus spp. during fermentation stages suggest that Vilwadi Gulika supports a microbial environment conducive to gut homeostasis.

Lactic acid bacteria are known to inhibit NF-κB activation, increase IL-10, and restore epithelial barrier integrity. Even at low abundance, these taxa can exert significant immunomodulatory effects via SCFA production and cytokine regulation. These microbial functions complement the anti-inflammatory actions of phytochemicals identified through network analysis, reinforcing the formulation's dual mechanism of action.

5.5 Targeting CXCL8-Mediated Inflammation in GERD

CXCL8 (IL-8) is a central mediator of neutrophil recruitment and inflammation in gastroesophageal reflux disease (GERD), with elevated expression observed even in non-erosive reflux conditions [68,69]. Network analysis identified auraptene, geranic acid, and nerolic acid as key phytochemicals interacting with CXCL8-associated pathways.

Auraptene has demonstrated robust inhibition of NF-κB translocation and suppression of IL-6 and IL-8 in gastrointestinal inflammation models [70,71]. Geraniol and its metabolite geranic acid suppress TNF-α, IL-1β, and IL-6 via NF-κB and MAPK inhibition [71,72]Nerol and nerolic acid further contribute through antioxidant and anti-ulcerogenic activities [73].

Microbiome profiling revealed Firmicutes dominance with detectable Lactobacillus and Actinobacteria, taxa associated with SCFA production and mucosal protection. SCFAs are known to inhibit CXCL8 secretion and reinforce epithelial integrity, suggesting that microbial metabolism may complement phytochemical inhibition of GERD-associated inflammation.

5.6 JAK2-Centered Modulation in Crohn's Disease

JAK2-mediated signalling is central to cytokine-driven inflammation in Crohn's disease, and interruption of the JAK–STAT axis is a validated therapeutic strategy [74]. The Crohn's disease subnetwork highlighted monoterpenes and sesquiterpenes - such as farnesol, linalool, nerolidol, geranyl derivatives, and piperine, as modulators of JAK2-associated pathways.

These phytochemicals reduce upstream cytokines (TNF-α, IL-6) and inhibit NF-κB and MAPK signalling, thereby indirectly suppressing JAK2 activation. Concurrently, the Firmicutes-enriched microbiome observed during fermentation supports SCFA production, which strengthens epithelial barriers and suppresses pro-inflammatory cytokine cascades that feed into JAK-STAT signalling.

5.7 TNF-Centered Regulation in Ileocolitis and Regional Enteritis

TNF-α is a pivotal cytokine in ileocolitis and regional enteritis, with anti-TNF therapies forming the backbone of current clinical management [75]. Arjungenin and curlone emerged as key phytochemicals interacting with TNF-associated network nodes.

Arjungenin has demonstrated suppression of LPS-induced TNF-α and oxidative stress in colitis models [76,77]. Curlone inhibits NF-κB signalling and enhances mucosal repair[78].The Firmicutes-dominant microbiome further supports TNF suppression via SCFA-mediated NF-κB inhibition.

5.8 TGF-β1-Mediated Repair in Duodenal Ulcer

TGF-β1 is a key mediator of mucosal repair in peptic ulcers, promoting epithelial restitution and angiogenesis [79,80]. Network analysis linked TGF-β1 to allocryptopine, aristolactam, and geranyl/geranylgeranyl diphosphate derivatives.

Allocryptopine attenuates intestinal inflammation and preserves barrier integrity [81], while aristolactam suppress pro-inflammatory cytokines [82]. SCFA-producing Firmicutes detected during fermentation are known to induce epithelial TGF-β1 expression, reinforcing mucosal healing pathways[83].

5.9 Study Limitations

While the present study provides an integrative perspective on the therapeutic potential of Vilwadi Gulika, certain limitations should be acknowledged. The network pharmacology analysis was primarily based on publicly available databases and predicted target interactions, which may not fully capture the complete pharmacological behaviour of the formulation in biological systems. Experimental validation of the predicted phytochemical–target interactions would further strengthen these findings. In addition, the microbiome analysis was conducted on a limited number of samples representing different stages of formulation preparation. Although these observations provide useful insights into microbial dynamics during fermentation, the results should be interpreted cautiously. Future studies incorporating larger sample sizes, biological replicates, and functional metagenomic analyses would help to better understand the microbial contributions to the therapeutic properties of Vilwadi Gulika.

5.10 Integrative Interpretation

Taken together, the findings support a dual-layered therapeutic model for Vilwadi Gulika. Network pharmacology reveals coordinated modulation of inflammatory, immune, and repair pathways, while microbiome profiling demonstrates fermentation-driven enrichment of taxa capable of reinforcing epithelial integrity, producing bioactive metabolites, and enhancing phytochemical efficacy through biotransformation.

This convergence of molecular and microbial mechanisms provides a strong scientific rationale for the traditional use of Vilwadi Gulika in gastrointestinal and inflammatory disorders and underscores the value of integrating Ayurvedic principles with systems biology approaches.

Chapter 6

6. Conclusion, Limitations and Future Directions

6.1 Conclusion

The present study aimed to scientifically investigate the classical Ayurvedic formulation Vilwadi Gulika using an integrative approach combining network pharmacology and microbiome analysis. This strategy was adopted to explore both the molecular interactions of phytochemical constituents and the microbial changes occurring during formulation preparation, areas that have remained largely unexplored for this formulation.

Network pharmacology analysis revealed that Vilwadi Gulika contains a wide range of phytochemicals contributed by its thirteen plant ingredients. Target prediction and disease association analysis showed that these phytochemicals collectively interact with multiple genes involved in gastrointestinal inflammation and immune regulation. Key targets identified in this study included SRC, NOS2, PTGS2, JAK2, TNF, CXCL8, and TGFB1, which are known to play important roles in inflammatory signalling pathways, cytokine production, oxidative stress, epithelial integrity, and tissue repair. The presence of multiple phytochemicals acting on overlapping targets supports the multi-component and multi-target nature of the formulation, which is consistent with the traditional principles of Ayurvedic polyherbal therapy.

Microbiome analysis demonstrated that the preparation of Vilwadi Gulika is accompanied by progressive changes in microbial composition. During the churning and fermentation stages, microbial richness and diversity increased, followed by stabilization in the final sun-dried formulation. A consistent enrichment of Firmicutes was observed across later stages of preparation, along with the presence of fermentative and lactic acid–associated genera. These microbial shifts indicate that the formulation process supports the development of a structured microbial community rather than random contamination.

When considered together, the findings suggest that the therapeutic relevance of Vilwadi Gulika may arise from the combined effects of phytochemical-mediated modulation of inflammatory pathways and microbiome-associated contributions to gut homeostasis. The convergence of predicted molecular targets with microbial patterns provides a plausible biological basis for the traditional use of Vilwadi Gulika in gastrointestinal and inflammatory conditions.

Overall, this study contributes to the scientific understanding of Vilwadi Gulika by providing systems-level evidence supporting its multi-target pharmacological nature and its association with microbial dynamics during preparation. The integrated analytical framework applied in this work demonstrates how traditional Ayurvedic formulations can be examined using modern computational and microbiome-based approaches. These findings provide a preliminary scientific basis for the therapeutic relevance of Vilwadi Gulika in gastrointestinal inflammatory conditions.

6.2 Limitations of the Study

Despite the comprehensive approach adopted in this study, certain limitations need to be acknowledged.

First, the network pharmacology analysis was based on in-silico target prediction, which relies on existing databases and computational models. While these predictions are useful for identifying potential molecular interactions, they do not confirm direct binding or functional effects at the protein or cellular level.

Second, the study did not include experimental validation of gene or protein expression, such as in vitro assays, enzyme inhibition studies, or pathway activation analyses. As a result, the functional relevance of the predicted phytochemical–target interactions remain to be experimentally verified.

Third, the microbiome analysis was restricted to 16S rRNA gene-based taxonomic profiling. This approach provides information on microbial composition but does not directly assess functional metabolic activity or microbial gene expression.

Fourth, although beneficial microbial taxa were detected consistently during formulation preparation, some were present at low relative abundance, and their precise contribution to biological activity could not be quantitatively assessed within the scope of this study.

Finally, the study focused on selected preparation batches and conditions. Variability arising from differences in raw material sources, preparation duration, and traditional practices across regions was not examined.

6.3 Future Directions

Based on the findings and limitations of the present study, several future research directions are suggested.

Experimental validation studies using cell-based or molecular assays should be conducted to confirm the predicted interactions between key phytochemicals and molecular targets such as JAK2, PTGS2, NOS2, TNF, and TGFB1.

Functional microbiome studies employing shotgun metagenomics, meta transcriptomics, or metabolomics would provide deeper insight into microbial metabolic pathways, short-chain fatty acid production, and biotransformation of herbal compounds.

Further work is also needed to examine phytochemical–microbiome interactions, particularly the role of microbial fermentation in modifying herbal metabolites into bioactive forms.

Comparative studies involving traditionally prepared, commercially available, and modified formulations could help clarify the influence of preparation methods on microbial composition and biological activity.

In addition, future studies may focus on identifying key phytochemical markers and microbial signatures associated with different stages of formulation preparation. Such investigations may help establish quality control parameters and improve standardization of the formulation across different preparation conditions.

Finally, in vivo studies and controlled clinical investigations would be essential to establish the translational relevance of the mechanistic insights generated in this study and to evaluate the therapeutic potential of Vilwadi Gulika in gastrointestinal inflammatory disorders. Such studies could also help determine dosage parameters, safety profiles, and long-term effects of

the formulation in clinical settings. This study does not claim direct clinical efficacy but provides mechanistic and exploratory evidence that supports traditional knowledge using modern scientific tools. The results form a foundation for future hypothesis-driven experimental validation.

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Target prediction

A. Target Prediction Using Binding DB

1) Prepare the SMILES input: The SMILES structures of all phytochemical constituents were compiled for target prediction.
2) Open Binding DB → select "Special Tools."
3) Choose "My Compound's Targets"
4) Paste the SMILES string into the input field → press ENTER / click OK.
5) Select the "GO" option to generate the list of predicted molecular targets.
6) Download and store the predicted target list for further analysis.

B. Target Prediction Using Swiss Target Prediction

1) Open Swiss Target Prediction database
2) Set the organism to *Homo sapiens*.
3) Paste the SMILES structure into the input box → press ENTER.
4) Click "Predict Targets" to initiate the prediction process.
5) Download the predicted targets generated by the data base
6) Combine the predicted targets obtained from both databases.
7) Remove duplicate entries to create a unique target list for further analysis.

C. Retrieval of Common Names of Targets

1) Note that Swiss Target Prediction already provides common names for predicted targets
2) The Binding DB database do not include the common names for the derived targets, so STRING database is used for retrieving the targets.
3) Open the string database, go to Multiple proteins, paste the Binding DB target list into the "List of Names", set Organisms to "*Homo Sapiens"* → press SEARCH.
4) Select the "Mapping option" to retrieve common names.
5) Download and open the mapped output file.
6) Extract the common names from the STRING results.
7) Merge the common names from STRING (Binding DB) with those from Swiss Target Prediction.
8) Remove duplicates again to obtain the final, non-redundant list of unique molecular targets. Prepare this unique target list for disease and pathway analysis using DAVID database

D. Functional Annotation

1) Open DAVID database → go to "Enter Gene List" input field.
2) Paste the common names of the targets into the input field.
3) Select "Official_Gene_Symbol" under "Select Identifier."
4) Select *Homo sapiens* as the species under "Select Species"
5) Choose "Gene List" under "List Type" → click Submit list.
6) Analysis Wizard window appears click on "Step 2. Analyse above gene list with one of DAVID tools"
7) Annotation Summary Results dialogue window appears, go to "DISGENET" and click on chart, download the file.

8) Return to annotation summary results, click on Pathways, go to KEGG pathway and click on chart, download the file.
9) Use Merger.exe → upload the disease file + pathway file → click Merge → save the consolidated output for each plant.

E. Network Construction and Visualization

1) Prepare the consolidates disease- pathway – target files for network construction.
2) Open Cytoscape software, import the merged data set → click *File* → *Import* → *Network from Table*.
3) Define node identifiers (targets, diseases, pathways) and set interaction edges.
4) Generate the compound–target–disease–pathway network → press ENTER to render.
5) Apply layout algorithms (e.g., Perfuse Force Directed, files Organic) to optimize network visualization.
6) Use *Style* settings to assign: Different node shapes (e.g., compounds = circles, targets = diamonds, diseases = triangles, pathways = hexagons)
7) Colour coding for better interpretation
8) Perform network analysis using Cytoscape tools (Network Analyzer):
    i. Degree centrality
    ii. Betweenness centrality
    iii. Clustering coefficients
9) Identify key nodes (hub targets, hub diseases, enriched pathways).
10) Export the final network as a high-resolution image (PNG/SVG) for the manuscript.
11) Save the session file for reproducibility.

Microbial DNA Extraction from Vilwadi Gulika
Date: 20 November 2023

A.DNA Extraction Using DNeasy Power Food Microbial Kit

1.Preparatory Steps

MBL and MR solutions were preheated to a warm temperature prior to use.
All centrifugation steps were carried out in RPM. Conversion from relative centrifugal force (g) to RPM was calculated using the formula:

$$g = (1.118 \times 10^{-5}) \times R \times S^2$$

where:
g = relative centrifugal force
R = radius of the centrifuge rotor
S = speed in RPM

2.Sample Preparation

One tablet of Vilwadi Gulika was powdered using a sterile mortar and pestle.
Approximately 0.1–0.2 g of the powdered sample was weighed and used for DNA extraction.

3.DNA Extraction Protocol

1. The powdered sample was transferred into a tube and 800 µL of MBL buffer was added. The mixture was vortexed for 1 minute.
2. The tube was incubated in a water bath at 61°C for 5 minutes.
3. The tube was removed from the water bath and incubated at room temperature for 5 minutes.
4. The entire sample was transferred into a Power Bead tube.
5. The tube was vortexed horizontally for 8–10 minutes.
6. The sample was centrifuged at 14,000 g for 2 minutes.
7. A minimum of 400 µL of supernatant was transferred into a new tube.
8. 100 µL of IRS solution was added and vortexed for 1 minute.
9. The tube was incubated at 4°C for 5 minutes.
10. The sample was centrifuged at 13,000 g for 2 minutes.
11. The supernatant was carefully transferred to a new tube without disturbing the pellet.
12. 900 µL of MR solution was added, briefly vortexed, and the mixture was transferred to spin column tubes.
13. The column was centrifuged at 13,000 g for 1 minute.
14. The flow-through was discarded and the column was placed back into the collection tube.
15. The column was centrifuged again at 13,000 g for 1 minute, flow-through discarded, and 650 µL of PW solution was added.
16. The column was centrifuged at 13,000 g for 1 minute, flow-through discarded, and washed with 650 µL of ethanol.
17. The column was centrifuged again at 13,000 g for 1 minute, and the flow-through was discarded.
18. A dry spin was performed at 13,000 g for 2 minutes.
19. The filter was removed from the column and placed onto a clean collection tube.
20. 200 µL of elution buffer was added directly to the filter.
21. The column was incubated at room temperature for 5 minutes and centrifuged at 13,000 g for 1 minute.
22. An additional 10–30 µL of elution buffer was added, incubated for 5 minutes, and centrifuged at 13,000 g for 2 minutes.
23. The filter was discarded and the eluted DNA was stored at 4°C.

4.Agarose Gel Electrophoresis
Genomic DNA extracted from Vilwadi Gulika and other samples was analysed by agarose gel electrophoresis to assess the presence and integrity of DNA.

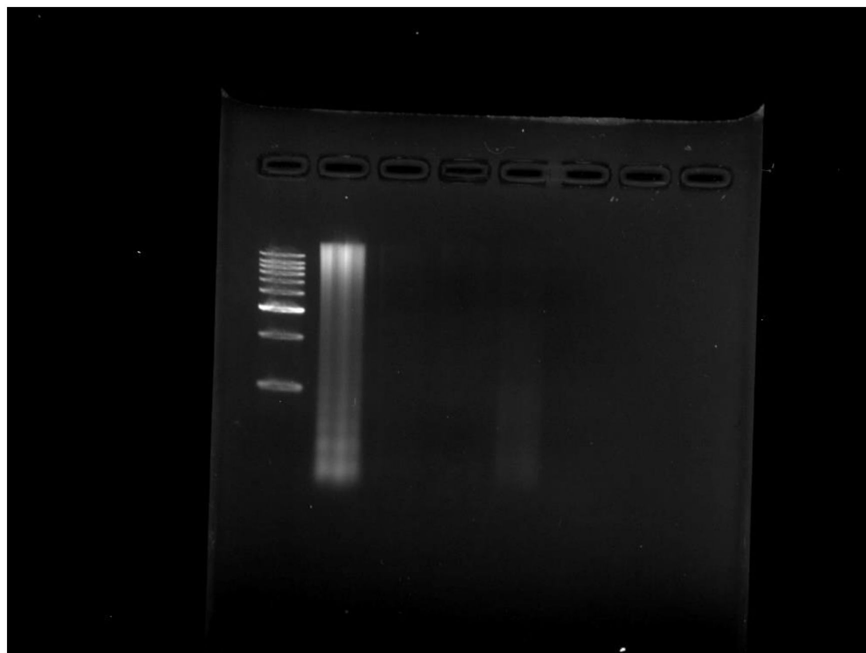

Figure 2.1: Agarose gel electrophoresis image of DNA isolation from Vilwadi Gulika and other samples.
W1: DNA ladder
W4: Vilwadi Gulika sample

5.Interpretation
No visible DNA bands were observed for the Vilwadi Gulika sample on the agarose gel, indicating either low DNA yield or the presence of inhibitors affecting DNA visualization.

B. Isolation of DNA from Vilwadi Gulika Using CTAB Method
Date: 21 November 2023

1.DNA Isolation Using CTAB Method
This method was performed as an alternative approach following unsuccessful DNA recovery using the commercial kit-based protocol.

2.Sample Preparation
One tablet of Vilwadi Gulika was powdered using a sterile mortar and pestle.
Approximately **0.1–0.2 g** of the powdered sample was weighed for DNA extraction.
3.Stock Solutions Used
The following stock solutions were prepared and used in the CTAB-based DNA extraction protocol:
CTAB (Cetyltrimethylammonium bromide) – **12%**
Sodium chloride (NaCl) – **5 M**
EDTA (Ethylenediaminetetraacetic acid) – **0.5 M**
Tris-HCl (Tris base + hydrochloric acid) – **1 M**

PVP (Polyvinylpyrrolidone) – **2%**
Chloroform: Isoamyl alcohol – **24:1 (v/v)**
SDS (Sodium dodecyl sulphate) – **20%**

4.DNA Isolation Procedure

- 1 mL of lysis buffer was added to a tube, and the weighed powdered sample was transferred into the tube.
- 40–60 µL of SDS and 40 µL of β-mercaptoethanol (2%) were added, followed by thorough vertexing.
- The tube was incubated at 60–65°C for 1 hour, with vertexing performed once after 30 minutes of incubation.
- After incubation, 0.6–0.8 mL of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed gently by inverting the tubes.
- The emulsion was centrifuged at 12,000 rpm for 15 minutes, and the aqueous phase was carefully transferred to a fresh tube.
- If the aqueous phase appeared cloudy, steps 4 and 5 were repeated.
- 0.5 mL of cold ethanol was added to the aqueous phase and mixed gently. The tubes were refrigerated for 1 hour or overnight at −20°C.
- After refrigeration, the tubes were centrifuged at 10,000 rpm for 6 minutes.
- The supernatant was discarded, and the pellet was washed with 200 µL of 70% ethanol.
- The tubes were centrifuged at 8,000 rpm, ethanol was discarded, and the ethanol wash step was repeated twice.
- The tubes were placed in an inverted position and allowed to air-dry completely.
- 20 µL of TE buffer was added to dissolve the DNA pellet and incubated for 10 minutes, followed by the addition of another 20 µL of TE buffer and further incubation for 10 minutes.
- The DNA samples were stored at −20°C until further use.

5.Agarose Gel Electrophoresis

Extracted DNA samples were subjected to agarose gel electrophoresis to assess the presence of genomic DNA.

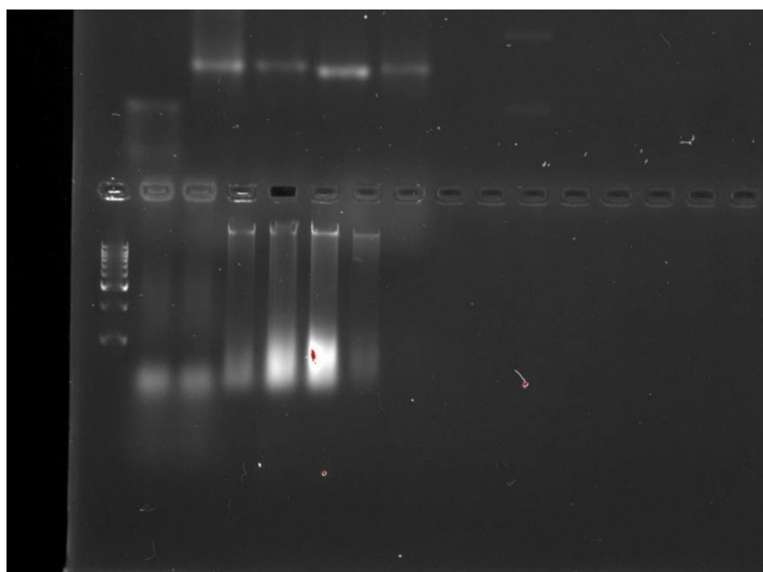

Figure 2.2: Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

W1: DNA ladder
W2, W3: Vilwadi Gulika samples (wells 2 and 3)

6.Interpretation
No visible DNA bands corresponding to Vilwadi Gulika samples were observed on the agarose gel following the CTAB-based extraction method.

C. ISOLATION OF DNA FROM VILWADI GULIKA USING HUMAN SALIVA METHOD
Date: 23/11/2023
1.Preparatory Steps Proteinase K preparation was carried out by weighing approximately 20.5 mg of Proteinase K and dissolving it in 1 mL of nuclease-free water. The solution was mixed thoroughly until completely dissolved.
2.SamplePreparation
One Vilwadi Gulika tablet was powdered using a sterile mortar and pestle. Approximately 0.05 g of the powdered sample was weighed and transferred into a sterile microcentrifuge tube.
3. DNA Isolation Procedure
One millilitre of H1 buffer was added to the tube containing the powdered sample.

- The tube was incubated in a water bath at 60°C for 1 hour.
- After 30 minutes of incubation, 20 µL of Proteinase K was added to the sample, and incubation was continued.
- Subsequently, 150 µL of SDS was added, followed by incubation for 30 minutes.
- Then, 450 µL of H4 solution was added, and the sample was incubated at 4°C for 20 minutes.
- The tube was centrifuged at 13,000 rpm for 10 minutes at room temperature.
- The supernatant was transferred into a fresh tube, and 500 µL of isopropanol was added. The mixture was kept at room temperature for 15 minutes and then stored at −20°C overnight.

Date:24/11/2023
8. The sample was centrifuged at 13,000 rpm for 15 minutes, and the supernatant was discarded.
9. The DNA pellet was washed by adding 500 µL of 70% ethanol, gently inverting the tube several times, and centrifuging at 8,000 rpm for 10 minutes at room temperature.
10. The supernatant was discarded, and the DNA pellet was air-dried until all traces of ethanol were removed.
11. The DNA pellet was dissolved by adding 80 µL of 1X TE buffer, mixed gently by slow tapping, and stored at −20°C until further analysis.

Result
No visible DNA bands were observed upon agarose gel electrophoresis of the extracted samples.

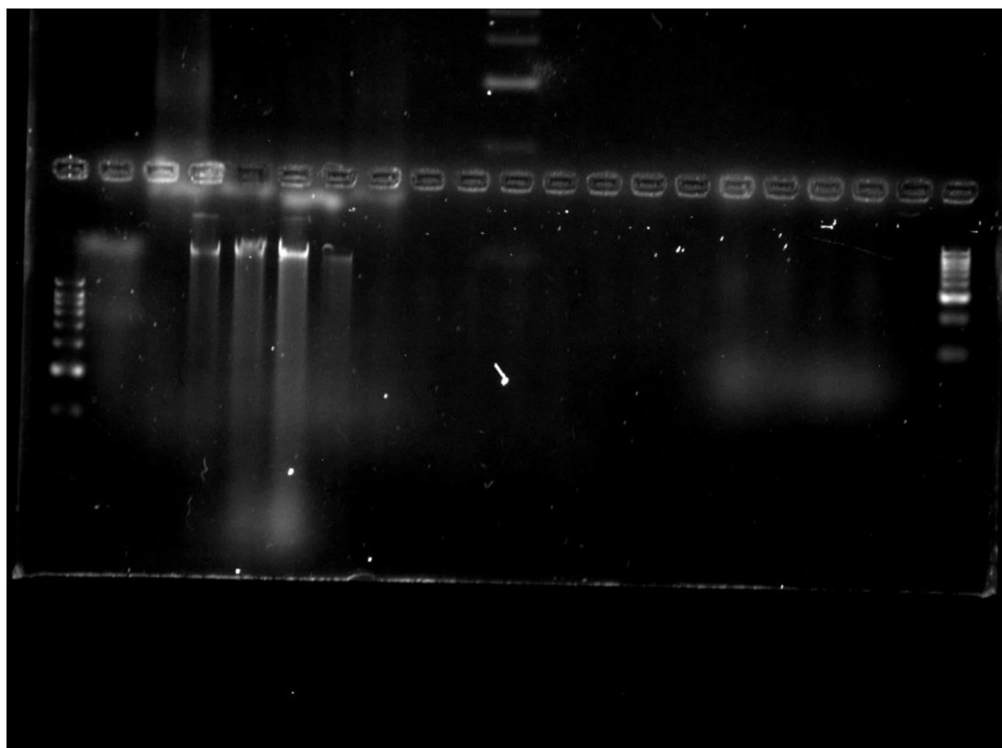

Figure 2.3 Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

Interpretation
No DNA bands corresponding to Vilwadi Gulika samples (W2 and W3) were detected, indicating unsuccessful genomic DNA isolation using the human saliva-based method.

D.DNA ISOLATION WITH PBS BUFFER AND CTAB METHOD
Date: 27/11/2023

Preparation of Phosphate Buffered Saline (PBS)
10× PBS (100 mL)
Components and quantities
NaCl – 8 g
KCl – 0.2 g
KH_2PO_4 – 0.2 g
Na_2HPO_4 – 1.44 g
Distilled water – 80 mL
Finalvolume:100mL
pH: Adjusted to 7.2–7.4
Procedure
All components were weighed and added sequentially to a beaker containing 80 mL distilled water
The solution was mixed using a magnetic stirrer until complete dissolution
The pH meter electrode was rinsed with standard pH solution and immersed in the PBS solution
Concentrated HCl was added dropwise until the pH reached 7.4
The solution was transferred to a measuring cylinder and the final volume was adjusted to 100 mL using distilled water

Sample Preparation – 1 (PBS Pre-treatment)
- Approximately 0.05 g of Vilwadi Gulika sample was weighed into a sterile tube

- 1000 μL of PBS was added
- The sample was incubated overnight at 37–40°C in a water bath

E. DNA Isolation (PBS + Kit-based Method)

1.The sample was centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant was discarded
2.450 μL of MBL buffer was added and vortexed for 1 minute
3.The tube was incubated in a water bath at 61°C for 5 minutes, followed by incubation at room temperature for 5 minutes
4.The entire sample was transferred to a Power Bead tube
5.The tube was incubated at 60°C for 15 minutes
6.Horizontal vertexing was performed for 8–10 minutes
7.Centrifugation was carried out at 14,000 rpm for 2 minutes at 4°C
8.A minimum of 400 μL supernatant was transferred to a new tube
9.100 of Inhibitor Removal Solution (IRS) was added and vortexed for 1 minute
10.The tube was incubated at 4°C for 5 minutes
11.Centrifugation was performed at 14,000 rpm for 2 minutes
12.The supernatant was transferred to a fresh tube without disturbing the pellet
13.900 of MR solution was added, vortexed briefly, and transferred to spin columns
14.Centrifugation was performed at 14,000 rpm for 1 minute (repeated if required)
15.Flow-through was discarded and 650 μL PW solution was added
16.The column was centrifuged at 14,000 rpm for 1 minute and flow-through discarded
17.A wash with 650 μL ethanol was performed and centrifuged at 14,000 rpm for 1 minute
18.A dry spin was carried out at 14,000 rpm for 2 minutes
19.The filter was transferred to a clean collection tube
20.20L elution buffer was added directly to the filter
21.The column was incubated for 5 minutes at room temperature and centrifuged at 14,000 rpm for 1 minute
22.An additional 10–30 μL elution buffer was added, incubated for 5 minutes, and centrifuged at 14,000 rpm for 2 minutes
23.The filter was discarded and the eluate was stored at 4°C

Nanodrop Analysis

Sample I.1: 4.5 ng/μL, A260/280 – 1.35, A260/230 – 0.33
Sample I.2: 8.4 ng/μL, A260/280 – 1.02, A260/230 – 0.40

Sample Preparation – 2 (PBS + CTAB Pre-treatment)

Approximately 0.05 g of sample was weighed into a sterile tube
1000 μL of PBS was added
1000 μL of CTAB lysis buffer was added
20 μL of Proteinase K was added
The sample was incubated overnight at 37–40°C in a water bath

F.DNA Isolation (PBS + CTAB + Kit-based Method)

1.The sample was centrifuged at 14,000 rpm for 10 minutes at 4°C
2.450 μL of MBL buffer was added and vortexed
3.Incubation was performed at 61°C for 5 minutes followed by 5 minutes at room temperature
4.The sample was transferred to a Power Bead tube and incubated at 60°C for 15 minutes
5.Horizontal vertexing was carried out for 8–10 minutes
6.Centrifugation was performed at 14,000 rpm for 2 minutes at 4°C
7.A minimum of 400 μL supernatant was transferred to a new tube
8.100 μL IRS was added and vortexed

9.Incubation was carried out at 4°C for 5 minutes
10.Centrifugation was performed at 14,000 rpm for 2 minutes
11.Supernatant was transferred carefully to a new tube
12.900 MR solution was added and transferred to spin columns
13.Centrifugation was carried out at 14,000 rpm for 1 minute
14.PW wash and ethanol wash steps were performed as described above
15.Elution was carried out using 20 μL elution buffer

Sample Preparation – 3 (PBS + CTAB + Classical CTAB Method)
Approximately 0.05 g of sample was weighed into a tube
1000 μL PBS and 1000 μL CTAB lysis buffer were added
20 μL Proteinase K was added
The sample was incubated overnight at 37–40°C

G.DNA Isolation (CTAB Classical Method)
1.Lysis buffer was added and the sample was mixed thoroughly
2.40–60 μL SDS and 40 μL β-mercaptoethanol (2%) were added and vortexed
3.The tube was incubated at 60–65°C for 1 hour with intermittent mixing
4.Chloroform: isoamyl alcohol (24:1 v/v) was added and mixed gently
5.Centrifugation was performed at 12,000 rpm for 15 minutes
6.The aqueous phase was transferred to a fresh tube
7.Cold ethanol was added and the tube was incubated at −20°C
8.Centrifugation was performed at 10,000 rpm for 6 minutes
9.The pellet was washed twice with 70% ethanol
10.The pellet was air-dried and resuspended in TE buffer
11.DNA was stored at −20°C
Nanodrop Analysis
Sample I.3: 655.2 ng/μL, A260/280 – 1.07, A260/230 – 0.63
Sample I.4: 389.5 ng/μL, A260/280 – 1.04, A260/230 – 0.60

PCR Amplification
Date: 29/11/2023
PCR Conditions
Initial denaturation: 95°C for 5 minutes
Denaturation: 95°C for 30 seconds
Annealing: 59.5°C for 30 seconds
Extension: 72°C for 1 minute 30 seconds
Final extension: 72°C for 5 minutes
Hold: 4°C
Total cycles: 34
PCR Reaction Components
Green Master Mix – 25 μL
Forward primer – 5 μL
Reverse primer – 5 μL
Template DNA – 3 μL

Result
No amplification was observed for Vilwadi Gulika samples upon agarose gel electrophoresis.

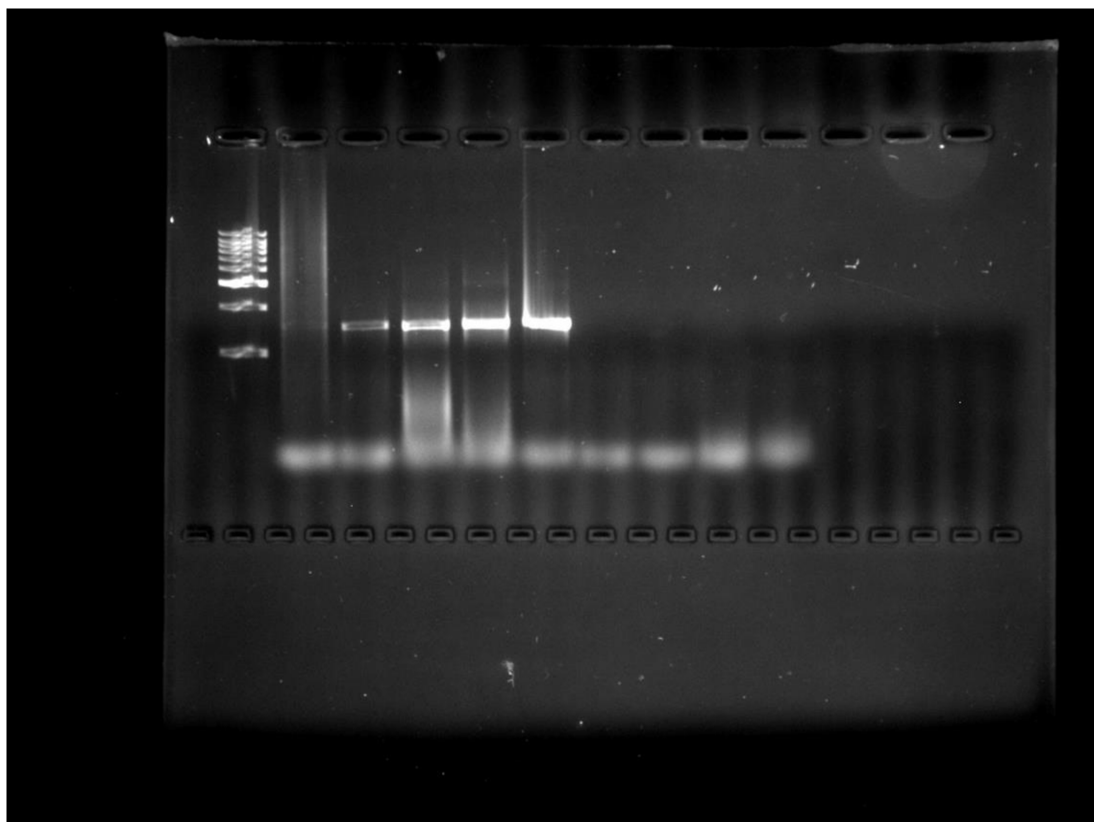

Figure 2.4 Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

H. Isolation of DNA from Vilwadi Gulika Using QIAGEN DNeasy Power Food Microbial Kit

Sample-details

Vilwadi Gulika enriched in nutrient broth on **05/01/2024** was used for DNA isolation.

Sample volume

Total volume: 1 mL

Both supernatant and pellet fractions were processed

DNA Extraction Procedure

1.The enriched sample was centrifuged at **14,000 rpm for 5 minutes at 4°C** and the supernatant was discarded

2.The MBL solution was preheated, and the pellet was resuspended thoroughly by vertexing

3.The tube was incubated in a water bath at **70°C for 5 minutes** to dissolve the pellet

4.The supernatant was transferred to a PowerBead tube

5.The tube was incubated at **70°C for 10–15 minutes**

6.Horizontal vortexing was performed for **10 minutes**

7.Centrifugation was carried out at **14,000 rpm for 2 minutes at 4°C**

8.The supernatant was collected into a fresh vial

9.100 µL of Inhibitor Removal (IR) solution was added and the mixture was incubated at **4°C for 10 minutes**

10.The sample was centrifuged at **14,000 rpm for 2 minutes at 4°C**

11.The pellet was discarded and the supernatant was transferred into a fresh tube

12.900 of MR solution was added and mixed briefly by vortexing for 1 second

13.The mixture was transferred into a spin column and centrifuged at **14,000 rpm for 2 minutes**
14.As the spin column capacity is **650 µL**, this step was repeated twice to accommodate the total sample volume
15.The flow-through was discarded and **650 µL of PW solution** was added using the same collection tube
16.The column was centrifuged at **14,000 rpm for 2 minutes**
17.The flow-through was discarded and **650 µL of ethanol** was added, followed by centrifugation at **14,000 rpm for 2 minutes**
18.A dry spin was performed at **14,000 rpm for 2 minutes** to remove residual ethanol
19.The column was transferred to a new collection tube
20.20 µL of elution buffer was added directly onto the column and incubated at room temperature for **5 minutes**
21.Centrifugation was performed at **14,000 rpm for 1 minute at 4°C**
22.A second elution was carried out using **10 µL of elution buffer**, incubated for **2 minutes** at room temperature
23.Final centrifugation was performed for **1 minute**
24.The eluted DNA was collected in respective tubes

Nanodrop Analysis

Parameter	Value
DNA concentration	13.7 ng/µL
A260/A280	1.24
A260/A230	0.16

PCR Amplification
PCR reaction components (10 µL total volume)
Master mix: 5 µL
16S Forward primer: 1 µL
16S Reverse primer: 1 µL
DNA template: 3 µL

PCR Cycling Conditions
Initial denaturation: **95°C for 5 minutes**
Denaturation: **95°C for 30 seconds**
Annealing: **59.6°C for 30 seconds**
Extension: **72°C for 1 minute 30 seconds**
Final extension: **72°C for 5 minutes**
Hold: **4°C (infinite)**

Result
PCR amplification did not yield detectable bands for the Vilwadi Gulika sample when visualized under UV illumination. A **1 kb ladder** was used as the molecular size marker. The gel image showed **no amplification in the Vilwadi Gulika PCR product lane**.

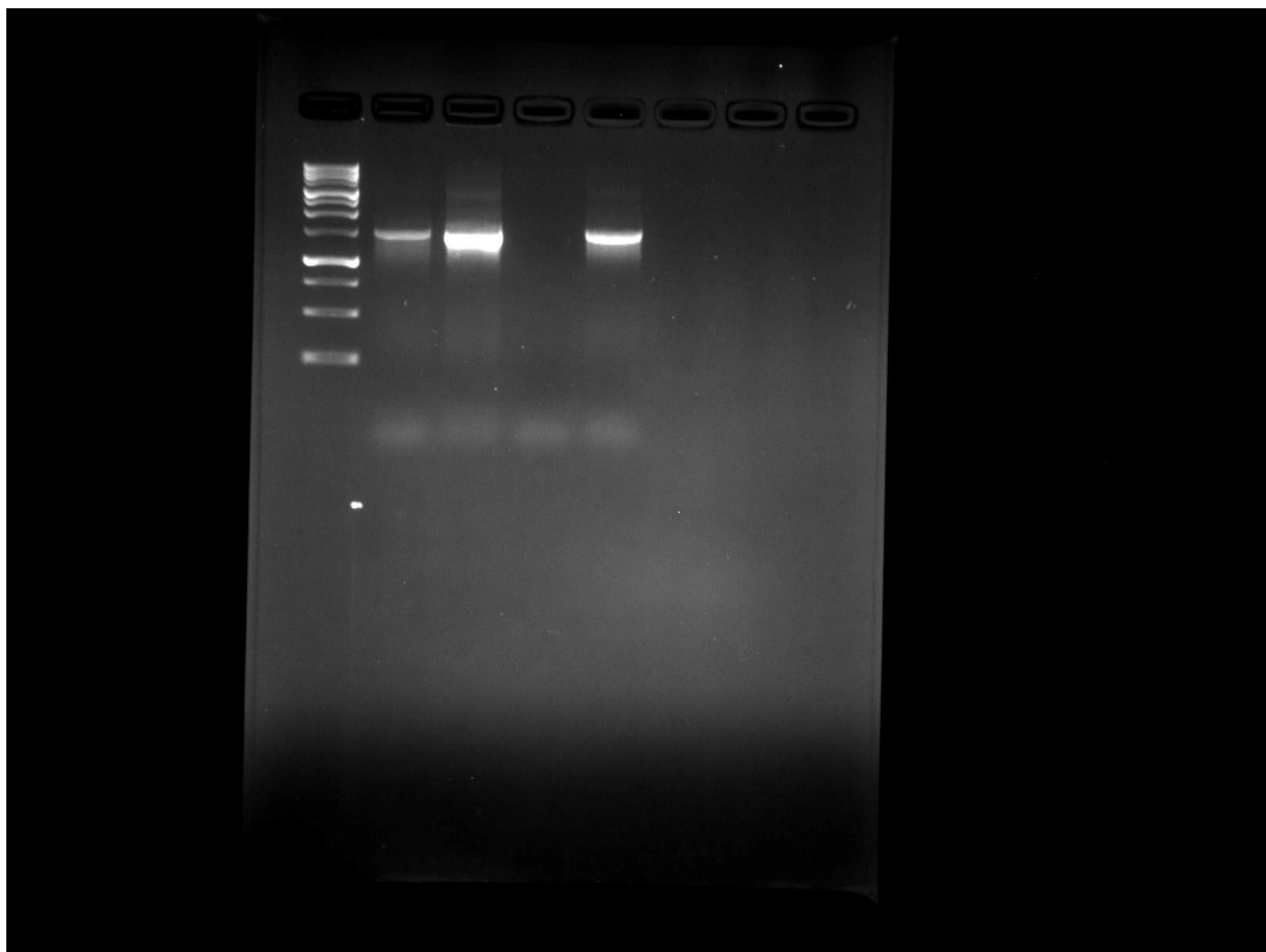

Figure 2.5 Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

I.DNA Isolation of Vilwadi Gulika Using Homogenizer and Arigna Lysis Buffer

Control sample

Saraca ashoka leaf tissue was used as a positive control.

Sample Preparation

A **5× homogenizer solution** was diluted to **1× working concentration** before use

Fresh **Saraca ashoka leaves** were washed thoroughly and ground using mortar and pestle with **750 µL of homogenizer solution**

Vilwadi Gulika tablet, previously enriched in nutrient broth and incubated at **37.2°C at 121 rpm on 09/01/2024**, was processed

700 µL of the enriched Vilwadi Gulika sample was ground using mortar and pestle with **150 µL of homogenizer solution** and **150 µL of sterile water**

Both samples were incubated at **55°C for 1 hour**

DNA Extraction Procedure

1.After incubation, **1 mL of lysis buffer (Arigna)** was added to each sample

2.Samples were further incubated at **55°C for 30 minutes**

3.Centrifugation was performed at **13,000 rpm for 5 minutes at room temperature**

4.The supernatant was carefully collected into fresh tubes

5.Chilled **isopropanol** was added to the supernatant for DNA precipitation

6.Samples were incubated **overnight at −20°C**

DNA Recovery and Washing

7.On **12/01/2024**, samples were centrifuged at **13,000 rpm for 5 minutes at room temperature**
8.The supernatant was discarded
9.The pellet was washed with **200 µL of 70% ethanol**
10.Centrifugation was carried out at **8,000 rpm for 10 minutes**
11.The pellet was air-dried
12.DNA was eluted by adding **20 µL of elution buffer** and incubating for **10 minutes**
13.A second elution was performed using an additional **20 µL of elution buffer**

Agarose Gel Electrophoresis

1% agarose gel was prepared in **1× TAE buffer**
For 100 mL gel preparation:
2 mL of 50× TAE buffer was diluted in **98 mL of distilled water**
1 g of agarose was added and dissolved by heating
1–2 µL of ethidium bromide was added for DNA visualization
Samples were loaded and electrophoresis was performed under standard conditions

Result

No visible DNA bands were observed in **wells 7 and 8** corresponding to the Vilwadi Gulika samples. The method did not yield detectable genomic DNA under the conditions tested.

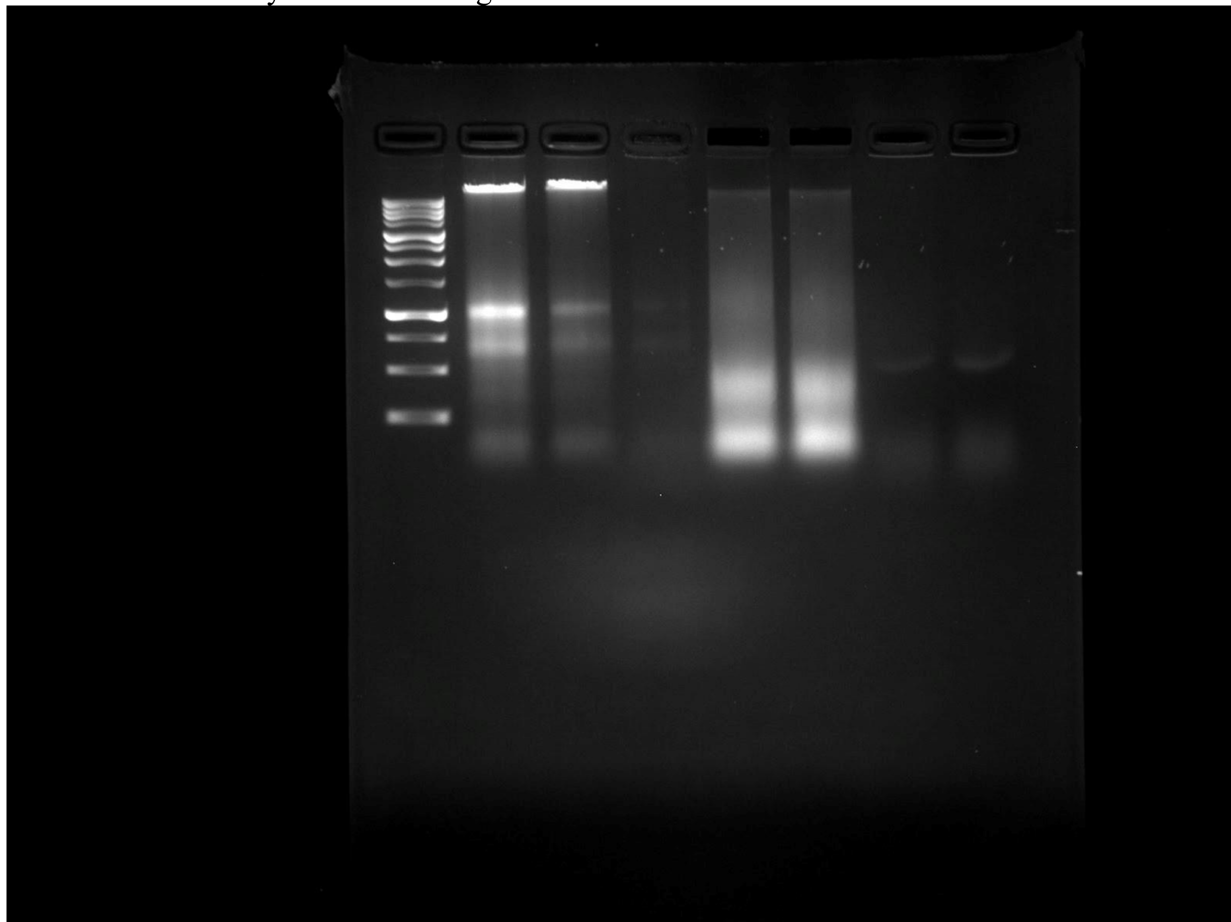

Figure 2.6 Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

J.DNA Isolation from Vilwadi Gulika Using QIAGEN DNeasy PowerFood Microbial Kit
(Isolation from Nutrient Broth Supernatant)
Sample details
Vilwadi Gulika sample incubated in nutrient broth on **18/01/2024** was used for DNA isolation. The **broth supernatant** obtained after incubation was processed for DNA extraction.

Sample Processing
The nutrient broth **supernatant** was transferred into a **2 mL microcentrifuge tube**
The sample was centrifuged at **14,000 rpm for 5 minutes at 4°C**
The supernatant was transferred into a fresh tube
A second centrifugation was performed at **14,000 rpm for 5 minutes at 4°C** to concentrate residual material
500 µL of pre-warmed MBL buffer was added to the pellet and mixed by vortexing
The sample was incubated in a water bath at **70°C for 5 minutes** to dissolve the pellet

DNA Extraction Procedure
1.The lysate was transferred to a **PowerBead tube**
2.The tube was incubated at **70°C for 10–15 minutes**
3.Horizontal vortexing was performed for **10 minutes**
4.The sample was centrifuged at **14,000 rpm for 2 minutes at 4°C**
5.The supernatant was transferred to a fresh tube
6.100 µL of Inhibitor Removal (IR) solution was added and mixed gently
7.The mixture was incubated at **4°C for 10 minutes**
8.Centrifugation was carried out at **14,000 rpm for 2 minutes at 4°C**
9.The pellet was discarded, and the supernatant was transferred to a new tube

Column Binding and Washing
10.900 µL of MR solution was added and vortexed briefly for **1 second**
11.The solution was transferred to a spin column and centrifuged at **14,000 rpm for 2 minutes**
12.Since the sample volume exceeded column capacity, loading and centrifugation were repeated as required
13.The flow-through was discarded
14.650 µL of PW wash buffer was added and centrifuged at **14,000 rpm for 2 minutes**
15.The flow-through was discarded
16.650 µL of ethanol was added and centrifuged at **14,000 rpm for 2 minutes**
17.A **dry spin** was performed at **14,000 rpm for 2 minutes**, and the collection tube was replaced

DNA Elution

18.20µL of elution buffer (EB) was added directly onto the spin column membrane
19.The column was incubated at **room temperature for 5 minutes**
20.Centrifugation was performed at **14,000 rpm for 1 minute at 4°C**
21.A second elution was carried out using **10 µL of EB**, incubated for **2 minutes at room temperature**
22.Centrifugation was performed for **1 minute**
23.Eluted DNA was collected in sterile tubes and stored at **4°C**

Nanodrop Analysis

Sample Source	DNA Concentration (ng/µL)	A260/A280	A260/A230
Nagarjuna	−78	−12.80	−0.06
Arya Vaidyasala	−43.4	0.96	−0.09
Vaidyaratnam	36.3	0.88	0.21

PCR Amplification
Reaction Components (Total Volume: 10 µL)

- Master mix: 5 µL
- 16S rRNA forward primer: 1 µL
- 16S rRNA reverse primer: 1 µL
- Template DNA: 3 µL

PCR Cycling Conditions
Initial denaturation: **95°C for 5 minutes**
Denaturation: **95°C for 30 seconds**
Annealing: **59.6°C for 30 seconds**
Extension: **72°C for 1 minute 30 seconds**
Final extension: **72°C for 5 minutes**
Hold: **4°C (infinite)**

Result
No amplification was observed in **well number 8**, immediately after the DNA ladder, indicating unsuccessful PCR amplification of DNA isolated from the nutrient broth supernatant of Vilwadi Gulika.

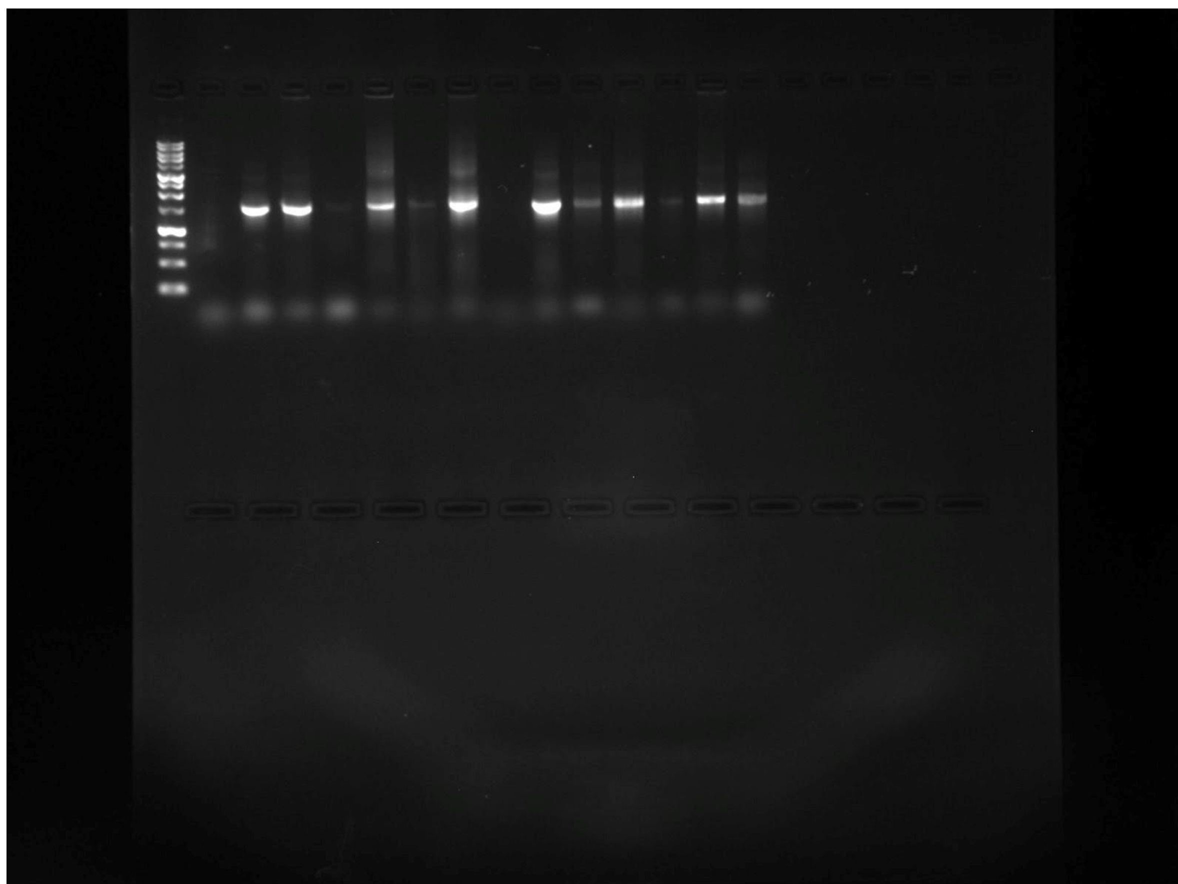

Figure 2.7 Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

K.DNA ISOLATION FROM DNEASY FOOD KIT

Sampleweight:0.14g(Vaidya-made-sample)

Powdered sample weight: 0.11 g

•Added 1000 µL of warm MBL buffer to powdered sample and resuspended the pellet by vortexing.

•Incubated in a water bath for 5 minutes to dissolve the pellet.

•Transferred the supernatant to the PowerBead tube

•Incubated at 60°C for 10–15 minutes

•Vortexed horizontally for 10 minutes

• Centrifuged at 14,000 rpm for 2 minutes

• Collected the supernatant and added 100 µL of IR solution, incubated at 4°C for 10 minutes

• Centrifuged at 14,000 rpm for 2 minutes

• Discarded the pellet and transferred the supernatant to a fresh tube

• Added 900 µL of MR solution and vortexed briefly for 1 second

• Transferred the solution into the spin column and centrifuged at 14,000 rpm for 2 minutes; repeated twice as sample volume exceeded column capacity (650 µL)

• Discarded the flow-through and added 650 µL of PW solution using the same collection tube

• Centrifuged at 14,000 rpm for 2 minutes

• Discarded the flow-through and centrifuged after adding 650 µL ethanol at 14,000 rpm

• Discarded the flow-through and performed dry spin at 14,000 rpm

• Added 20 µL elution buffer and incubated at room temperature for 5 minutes

• Centrifuged for 1 minute at 14,000 rpm at 4°C

- Added another 20 µL elution buffer and incubated at room temperature for 2 minutes
- Centrifuged for 2 minutes at 12,000 rpm at 4°C
- DNA isolated in respective tubes

NANODROP READING
ng/µL: 13.6
A260/A280: 1.34
A260/A230: 0.09
PCR (16S rRNA)
Master mix: 4.5 µL
16S rRNA Forward Primer: 1 µL
16S rRNA Reverse Primer: 1 µL
DNA: 3.5 µL
Total reaction volume: 10 µL
PCR (ITS)
Master mix: 4.5 µL
ITS Forward Primer: 1 µL
ITS Reverse Primer: 1 µL
DNA: 3.5 µL
Total reaction volume: 10 µL
PCR PROFILE
95°C – 5 minutes
95°C – 30 seconds
59.6°C – 30 seconds
72°C – 1 minute 30 seconds
72°C – 5 minutes
4°C – Hold

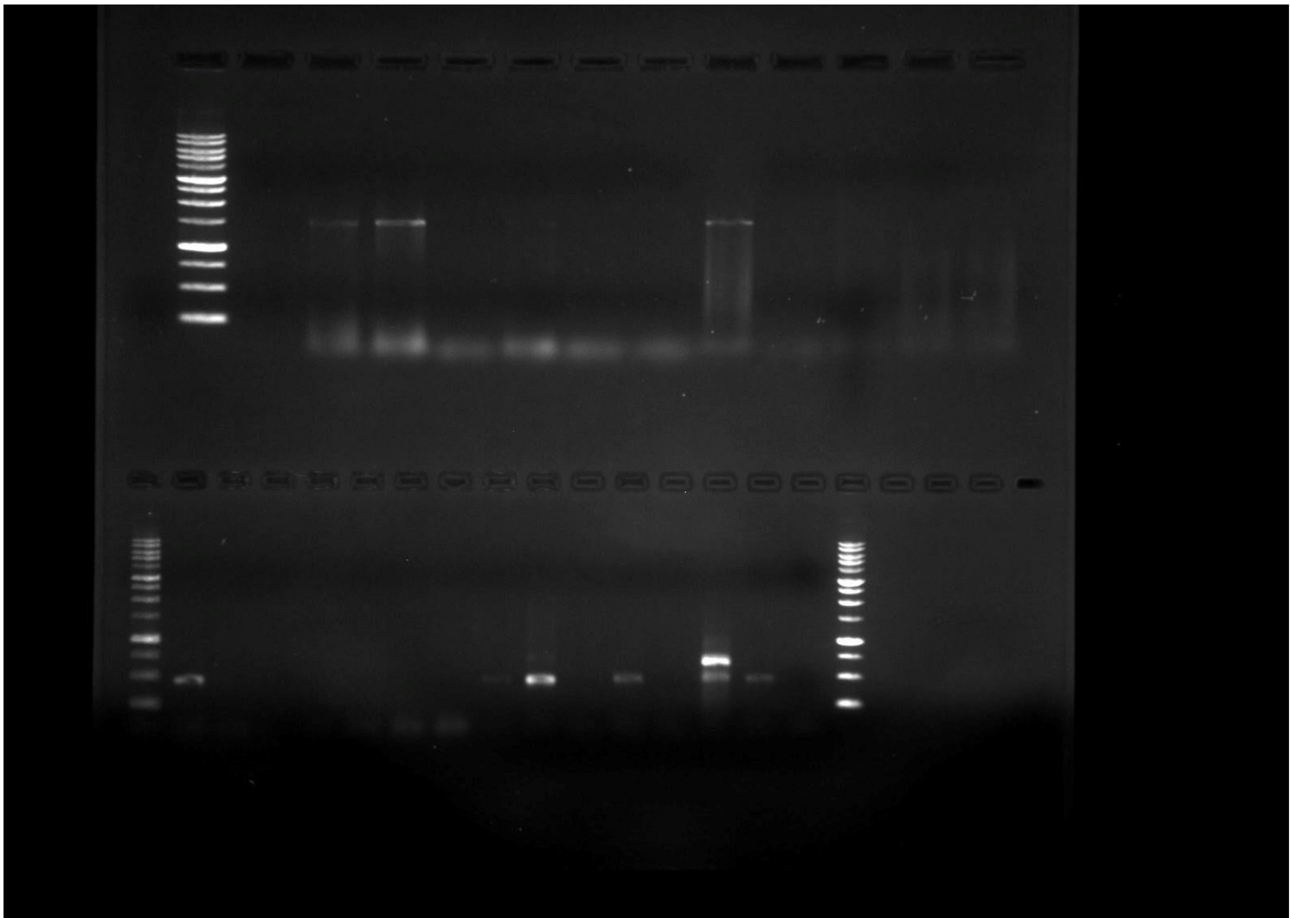

Figure 2.9 Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

RESULT: No amplification observed (well no. 9 and well no. 24, second row excluding ladder)
1/2/24 (Morning)
Sample: Same DNA isolated on 31/1/24
Master mix used: High-fidelity KAPA
Master mix (KAPA): 10 µL
16S rRNA Forward Primer: 2 µL
16S rRNA Reverse Primer: 2 µL
DNA: 6 µL
Total reaction volume: 20 µL
PCR PROFILE
95°C – 3 minutes
95°C – 20 seconds
61.5°C – 15 seconds
72°C – 1 minute 30 seconds
72°C – 5 minutes
4°C – Hold
Nanodrop reading
ng/µL: 796.5
A260/A280: 1.85
A260/A230: 1.77
Tank buffer preparation

50X TAE diluted to 1X
$50 \times V1 = 1 \times 500$
$V1 = 10$ mL
10 mL TAE added to 490 mL distilled water
PCR product agarose gel (1.2%)
0.6 g agarose in 50 mL
1 mL TAE + 49 mL water
RESULT: Well no. 2 showed amplification; amplicon size ~1500 bp (16S rRNA)
1/2/24 (Evening)
Master mix (KAPA): 5 µL
16S rRNA Forward Primer: 1 µL
16S rRNA Reverse Primer: 1 µL
DNA: 3 µL
Total reaction volume: 10 µL
PCR PROFILE
95°C – 3 minutes
98°C – 20 seconds
61.5°C – 15 seconds
72°C – 1 minute 30 seconds
72°C – 5 minutes
4°C – Hold
Nanodrop reading
IV1: 779.1 ng/µL, A260/A280: 1.84, A260/A230: 1.71
IV2: 761.0 ng/µL, A260/A280: 1.87, A260/A230: 1.72

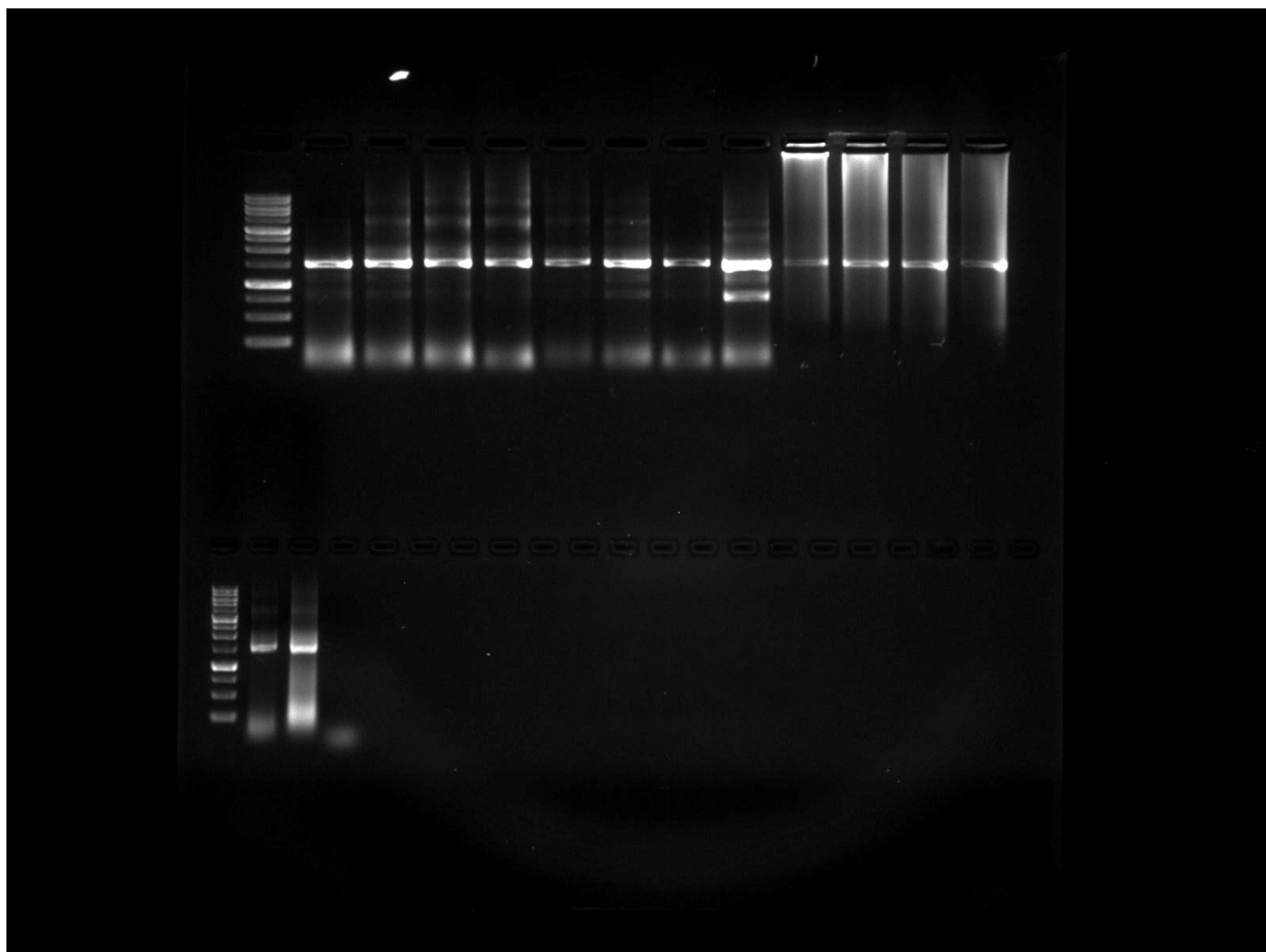

Figure 2.10 Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

RESULT: Well no. 2 (IV1) and well no. 3 (IV2) amplified; amplicon size ~1500 bp (including ladder count)
2/2/24
DNA ISOLATION FROM DNEASY QIAGEN KIT
Sample: Vaidyaratnam Vilwadi Gulika
Weight of one tablet: 0.76 g
Weight after powdering: 0.74 g
Weight used for isolation: 0.19 g
Uday sir Vilwadi Gulika weight: 0.19 g

- Added 1000 µL of warm MBL buffer and resuspended pellet by vortexing
- Incubated in water bath for 5 minutes
- Transferred supernatant to PowerBead tube
- Incubated at 60°C for 10–15 minutes
- Vortexed horizontally for 10 minutes
- Centrifuged at 14,000 rpm for 2 minutes
- Added 100 µL IR solution and incubated at 4°C for 10 minutes
- Centrifuged and discarded pellet
- Added 900 µL MR solution and loaded onto spin column (repeated due to volume)
- Washed with PW solution and ethanol

- Performed dry spin
- Eluted twice using 20 µL elution buffer

NANODROP READING FOR GENOMIC DNA
Tablet: 10.1 ng/µL, A260/A280: 1.10, A260/A230: 0.04
Uday sir tablet: 6.1 ng/µL, A260/A280: 0.91, A260/A230: 0.07
PCR
Master mix (KAPA): 10 µL
16S rRNA Forward Primer: 2 µL
16S rRNA Reverse Primer: 2 µL
DNA: 6 µL
Total reaction volume: 20 µL
PCR PROFILE
95°C – 3 minutes
98°C – 20 seconds
61.5°C – 15 seconds
72°C – 1 minute 30 seconds
72°C – 5 minutes
4°C – Hold
PCR PRODUCT NANODROP READING
Formulation 2: 608.6 ng/µL, A260/A280: 1.82, A260/A230: 1.53
Formulation 1: 620.1 ng/µL, A260/A280: 1.82, A260/A230: 1.77

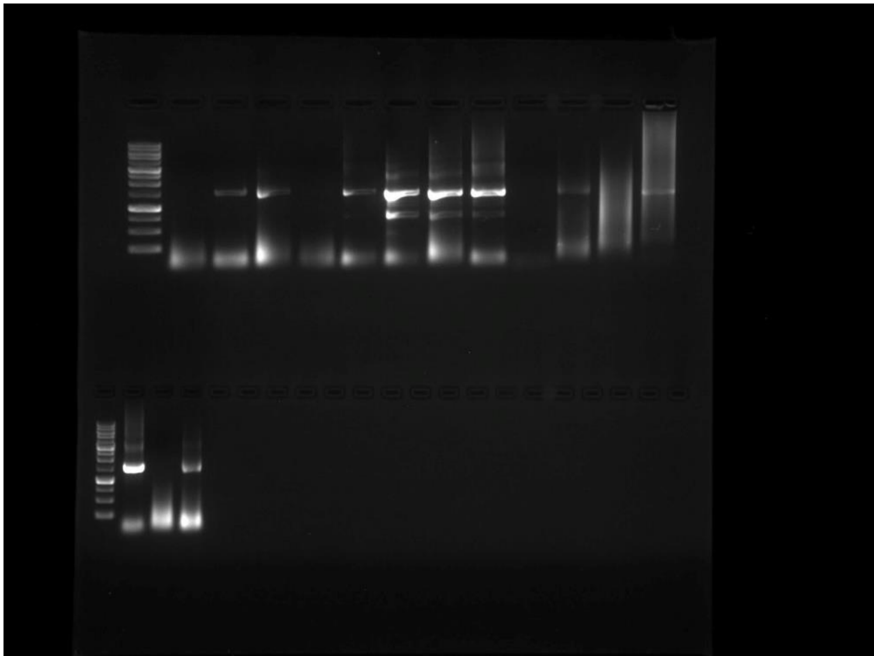

Figure 2.11 Agarose gel electrophoresis image showing DNA isolation attempts from Vilwadi Gulika.

RESULT: Well no. 10 (excluding ladder) showed amplification; amplicon size ~1500 bp
3/2/24

Culture-Dependent Validation of Microbial Presence in Vilwadi Gulika

This annexure documents the culture-dependent experiments carried out to confirm the presence of viable microbial communities in Vilwadi Gulika following repeated challenges in metagenomic DNA isolation. All calculations, incubation conditions, and observations are presented as recorded during experimentation.

A. Preparation of Nutrient Agar (First Experiment)

Number of plates prepared: 3
Volume of nutrient agar per plate: 20 mL
Total nutrient agar required:20 mL × 3 plates = 60 mL

Calculation of Nutrient Broth
Standard concentration: 13 g per 1000 mL
Required volume: 60 mL

$$x = \frac{13 \times 60}{1000} = 0.78 \text{ g}$$

Nutrient broth used: 0.78 g in 60 mL distilled water
Agar Concentration
Agar concentration maintained at 1.5–2%
Agar added: 1.5 g in 60 mL distilled water

B. Sample Preparation (Vaidya Ratnam Vilwadi Gulika – First Trial)

Empty 5 mL test tube weight: 19.23 g
Test tube with crushed tablet: 19.93 g
Weight of crushed tablet:19.93 − 19.23 = 0.70 g

Broth Enrichment
3 mL nutrient broth added to the crushed tablet
Incubated in a shaker incubator at:
Temperature: 37 °C
Shaking speed: 121 rpm
Duration: 2 hours (2:15 pm – 4:15 pm)

Annexure 3

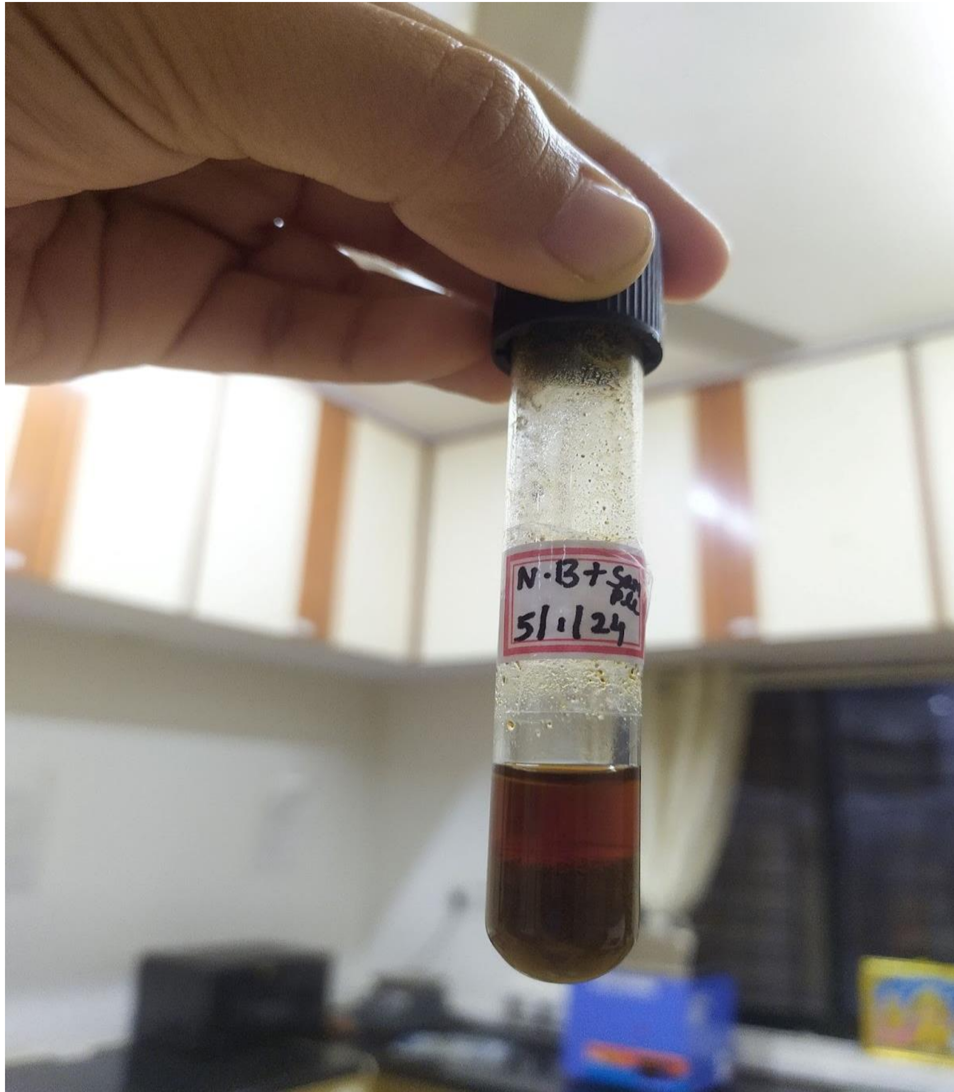

Figure 3.1: Vilwadi Gulika suspension after 2 hours of incubation

C. Inoculation and Plating (First Trial)

Nutrient agar media was autoclaved and poured into sterile Petri plates
Media allowed to solidify
One plate maintained as control

Plating Techniques
Pour plate:100 µL of sample added to molten media, mixed, poured, and allowed to solidify

Spread plate:100 µL of sample spread using a sterile L-rod spreader (alcohol dipped and flame sterilized)

Incubation
Plates incubated at 37 °C overnight
Observations
06/01/2024: No growth observed
07/01/2024: No growth observed
08/01/2024 (9:00 am): Minute colonies observed

08/01/2024 (5:00 pm): Colonies increased in size

Pre-inoculation duration	Growth duration
2 hours	72 ours

Result
Large white colonies appeared on the pour plate
Crowded colony formation observed on the spread plate

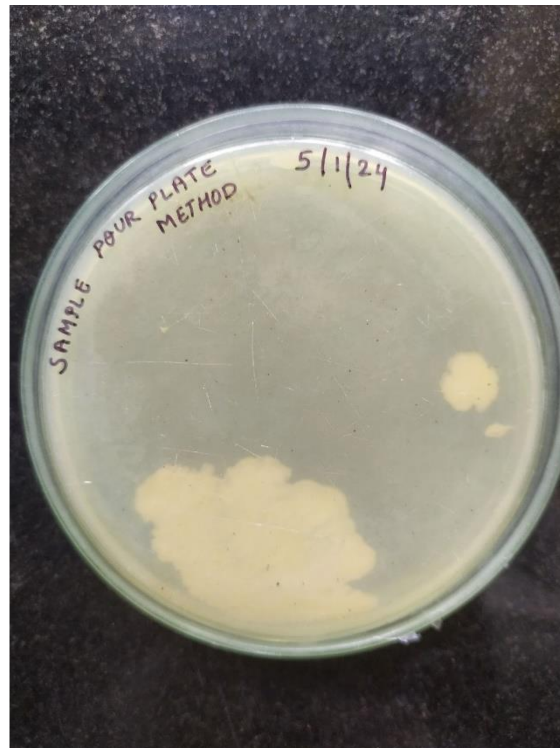

Figure 3.2: Pour plate showing colony formation

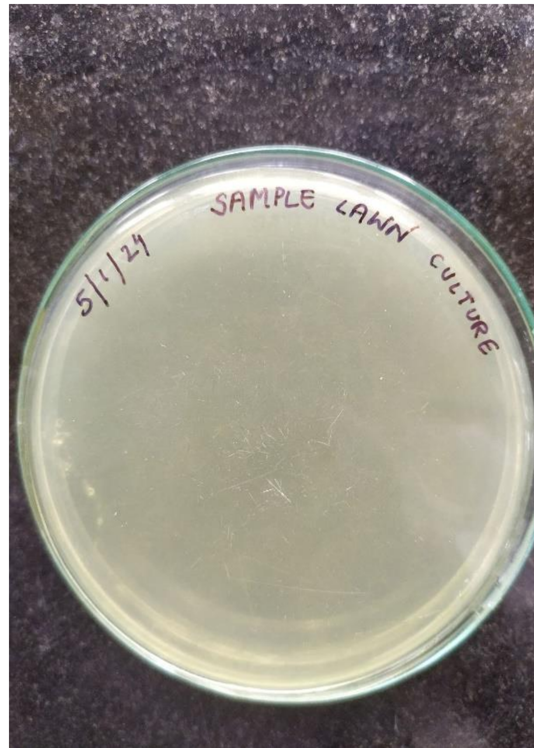

Figure 3.3: Spread plate showing lawn-like growth

Figure 3.4: Control plate showing no growth

D. Preparation of Nutrient Agar (Second Experiment)
Number of plates prepared: 4
Volume of nutrient agar per plate: 25 mL
Total nutrient agar required: 100 mL

Calculation of Nutrient Broth
Required volume: 100 mL

$$x = \frac{13 \times 100}{1000} = 1.3 \text{ g}$$

Nutrient broth used: 1.3 g in 100 mL distilled water

Agar Concentration
Agar added: 1.5 g in 100 mL distilled water

E. Sample Preparation (Vaidyaratnam Vilwadi Gulika – Second Trial)
Empty 5 mL test tube weight: 21.94 g
Test tube with crushed tablet: 22.60 g
Weight of crushed tablet-22.60 − 21.94 = 0.66 g
Broth Enrichment and Incubation Timeline
08/01/2024:
4 mL nutrient broth added to crushed tablet
Incubated at 37 °C, 121 rpm overnight
09/01/2024 (12:46 pm):
Sample stored at 4 °C
09/01/2024 (3:03 pm – 4:50 pm):
Sample equilibrated to room temperature

F. Inoculation and Plating (Second Trial)
Media poured and allowed to solidify
Plates prepared:
Control plate
Quadrant streak plate (loopful of sample)
Spread plate (100 µL)
Pour plate (100 µL)
Incubation
Plates incubated at 37.2 °C overnight
Observations
10/01/2024: No growth observed
11/01/2024: Tiny colonies observed; plates retained for extended incubation
Subsequent incubation showed visible colony growth

Pre-inoculation duration	Growth duration
24 h at 37 °C	
3 h at 4 °C	48 hours
1.5hour at room temp	

Result
White colonies observed on both pour plate and spread plate
Control plate remained sterile

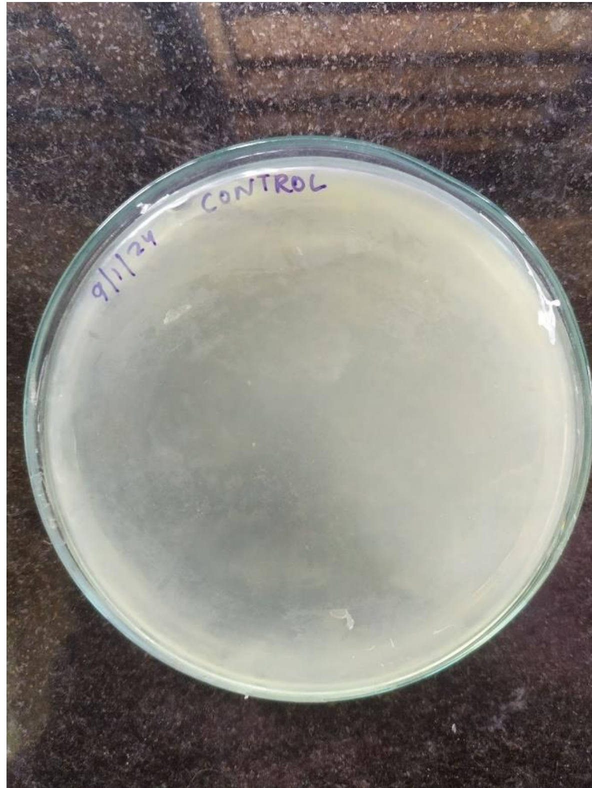

Figure 3.5: Control plate (no growth)

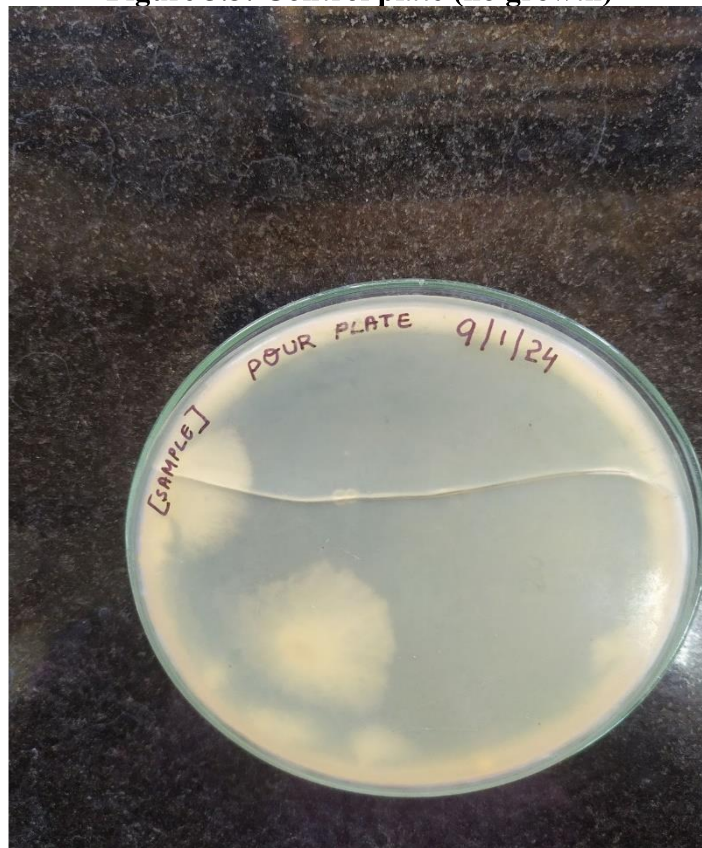

Figure 3.6: Pour plate showing colony formation

Figure 3.7: Spread plate showing microbial growth

G.Summary of Culture-Dependent Validation

The culture-dependent experiments confirmed the presence of viable microbial populations in Vilwadi Gulika. Delayed colony appearance indicated low microbial abundance and possible stress adaptation. These findings validated the biological presence of microbes in the formulation and justified further optimization of metagenomic DNA isolation and sequencing-based microbiome analysis.

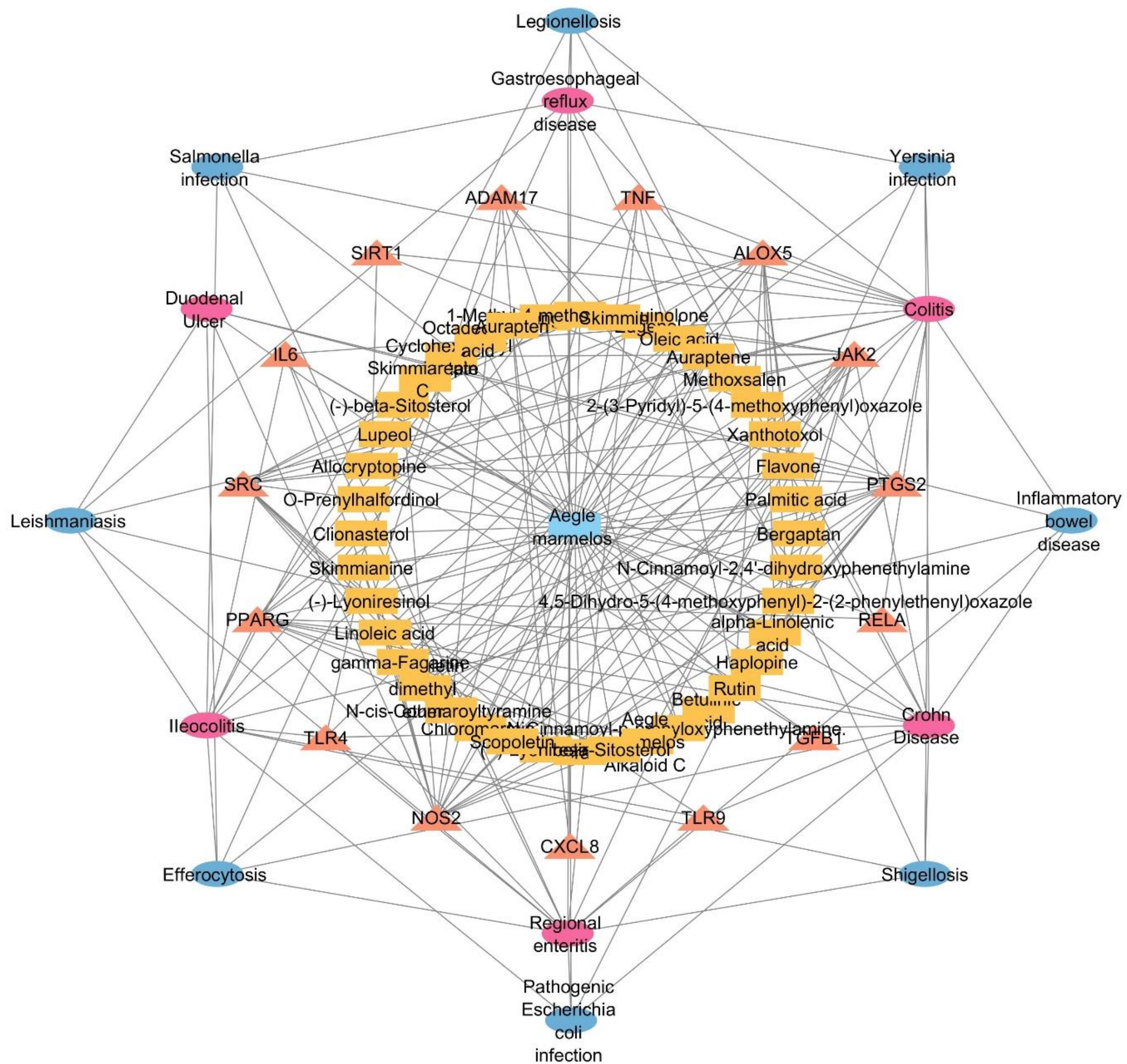

Fig no.1 Network showing the interactions between phytochemicals, disease-associated genes, and pathways for *Aegle marmelos*. The network shows connection between the phytochemicals to disease – associated genes and KEGG pathways for six gastrointestinal conditions. Nodes represent compounds, genes, pathways and diseases. Edges indicate reported or predicted compound – target or target – pathway/disease associations. Network statistics (70 nodes, 206 edges, average degree = 5.9).

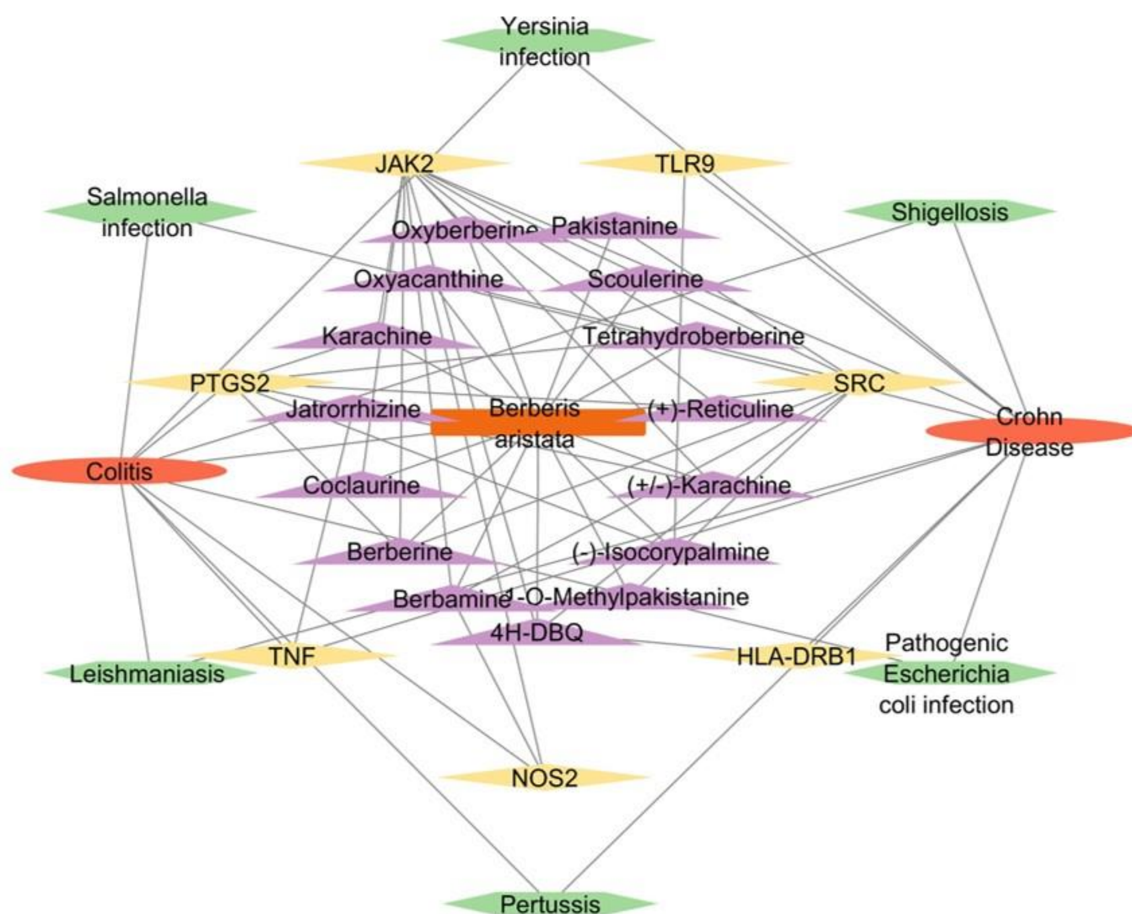

Fig no.2 Network Analysis of Berberis aristata Phytochemicals and their Target Genes in Gastrointestinal diseases. This network depicts the connections between *Berberis aristata* phytochemicals and the genes and pathways associated with colitis and Crohn’s disease. Nodes represent compounds, genes, pathways and diseases, while edges denote documented or predicted interactions. The network forms a single connected component (31 nodes, 63 edges), reflecting multi-target activity.

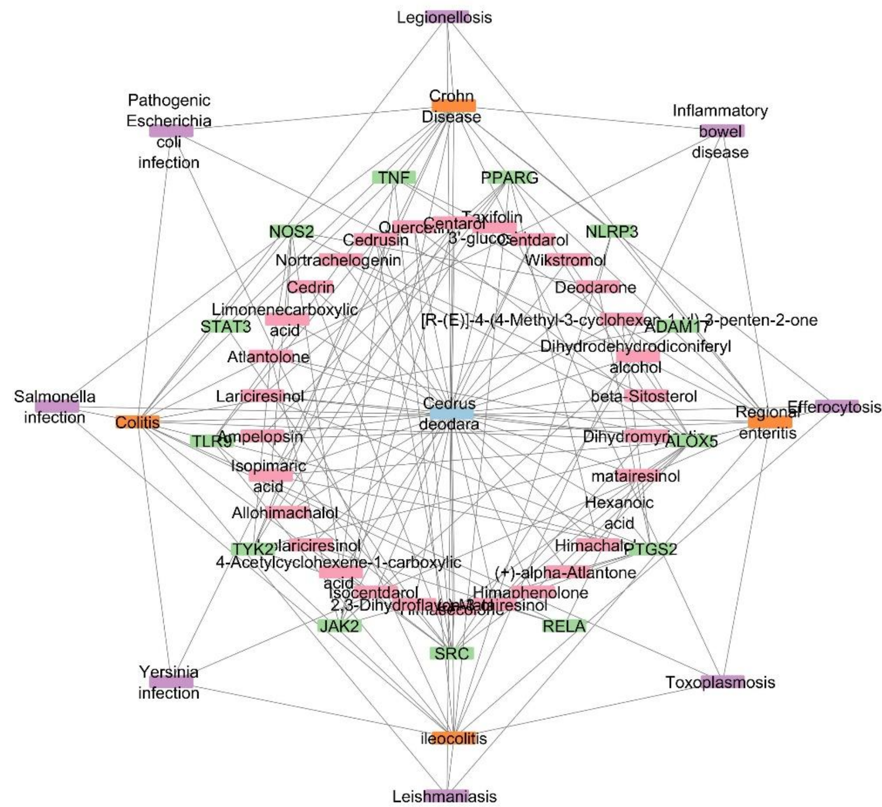

Fig no.3 Interaction network showing the connections between *Cedrus deodara* phytochemicals, gastrointestinal disease–associated genes, and their pathways. This network illustrates how *Cedrus deodara* phytochemicals interact with genes and pathways involved in six gastrointestinal diseases.

Fig no 4. Curcuma longa interaction network highlighting the Phytochemicals with gastrointestinal disease associated genes and their pathways. The network shows how the phytochemicals of *Curcuma longa* associated with the six gasterointestinal diseases, their gene and pathways. The dense central region shows the mutiple compounds converging on the shared disese related targets. The peripheral nodes represent specific pathway and disease associations.

Fig no.5 Interaction network of *Phyllanthus emblica* phytochemicals with gastrointestinal disease–associated genes and pathways. This network shows the connection between the phytochemicals of *Phyllanthus emblica* to genes, KEGG pathways and six gastrointestinal disorders. Nodes represent compounds, genes, pathways and diseases.

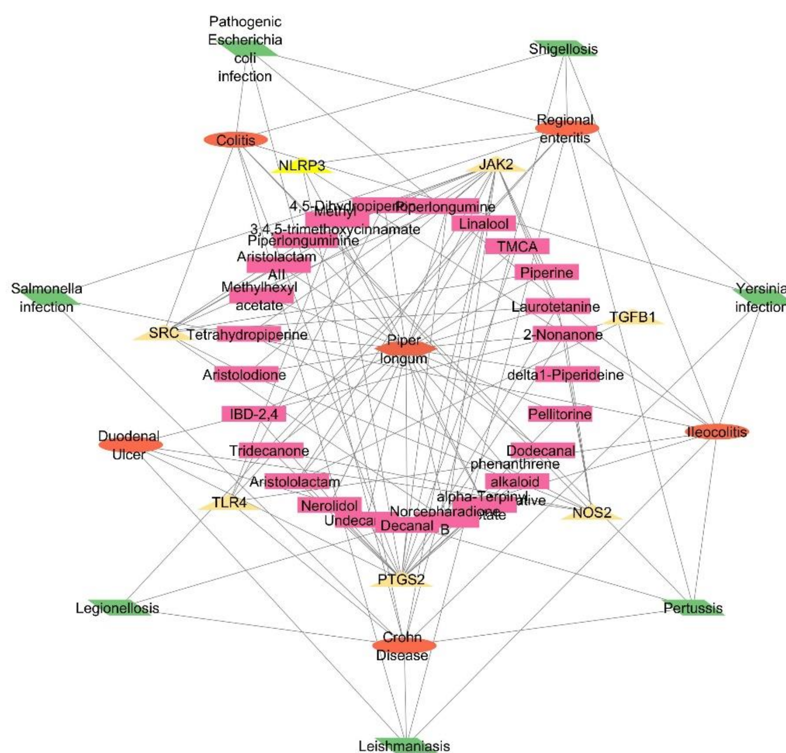

Fig no.6 Interaction network of *Piper longum* phytochemicals with gastrointestinal disease associated genes and pathways. Network connecting phytochemicals, disease – associated genes and KEGG pathways for six gastrointestinal conditions. Nodes denote compounds, genes, pathways and diseases. Edges represent interactions. Summary statistics (45 nodes, 114 edges).

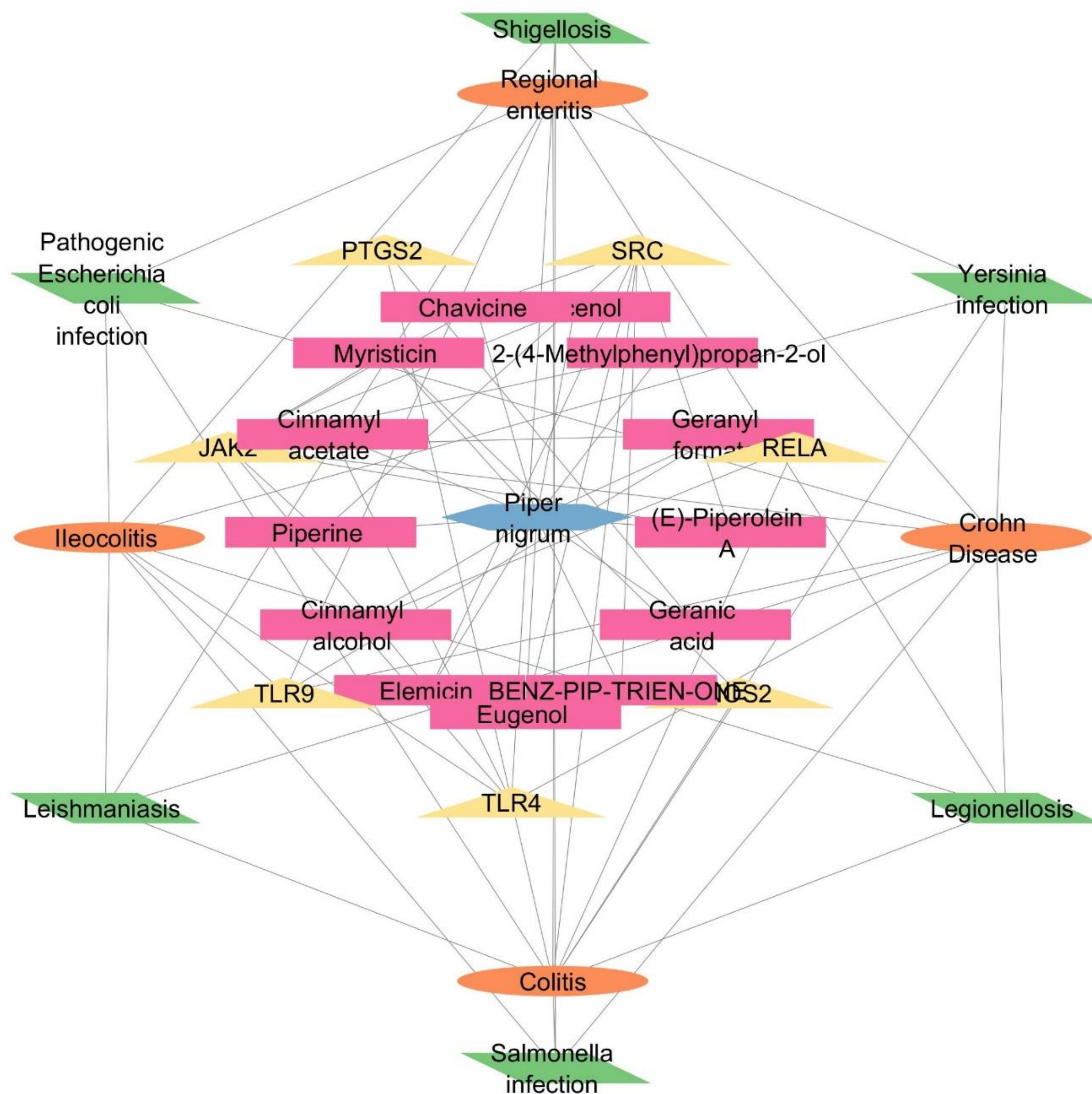

Fig no.7 Interaction network of *Piper nigrum* phytochemicals with gastrointestinal disease–associated genes and pathways. Network linking *Piper nigrum* phytochemicals to genes and pathways in six gastrointestinal conditions.

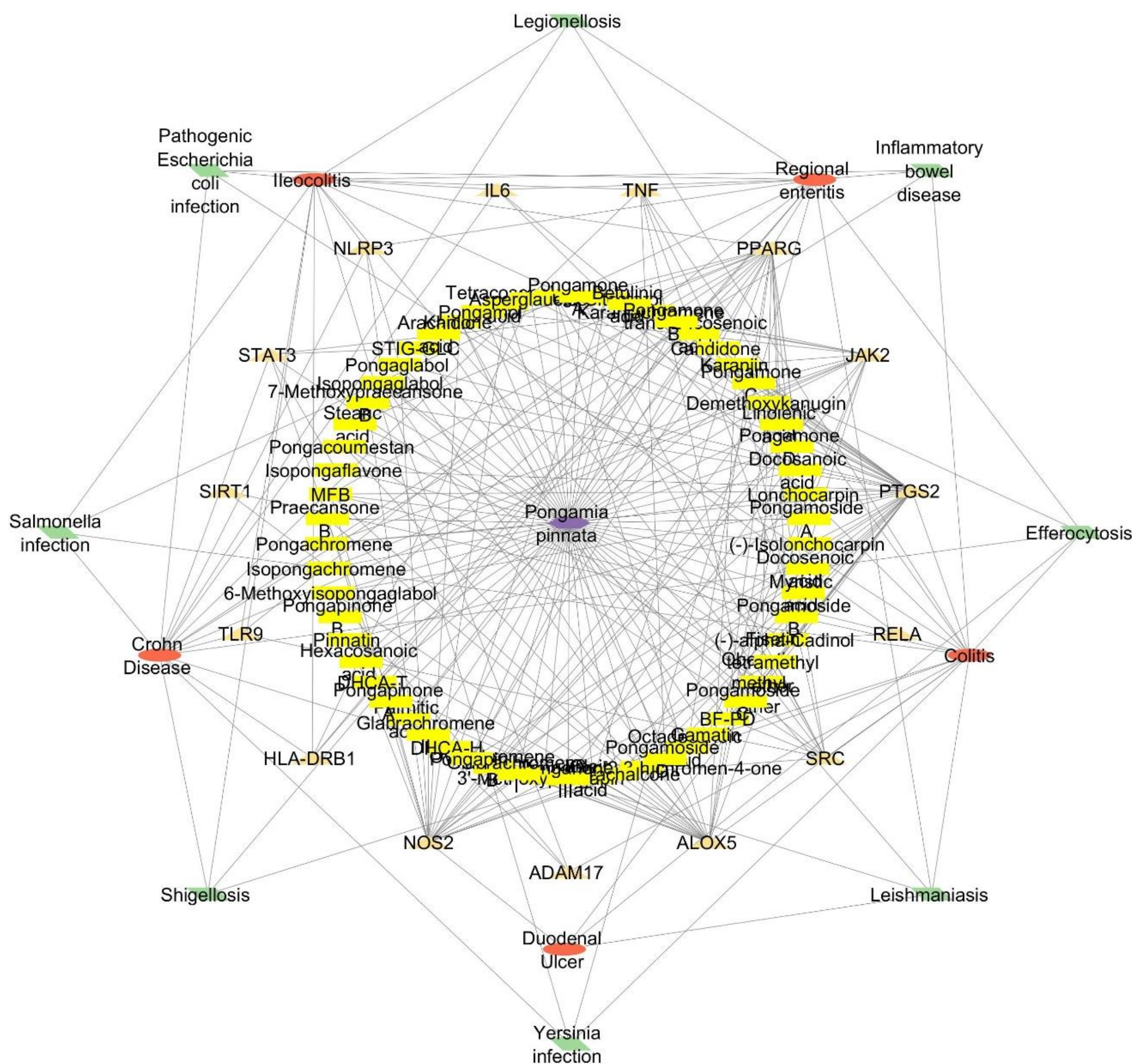

Fig no. 8 Interaction network linking *Pongamia pinnata* phytochemicals with gastrointestinal disease – associated genes and pathways. Network showing connections between *Pongamia pinnata* phytochemicals, disease related genes and KEGG pathways and diseases.

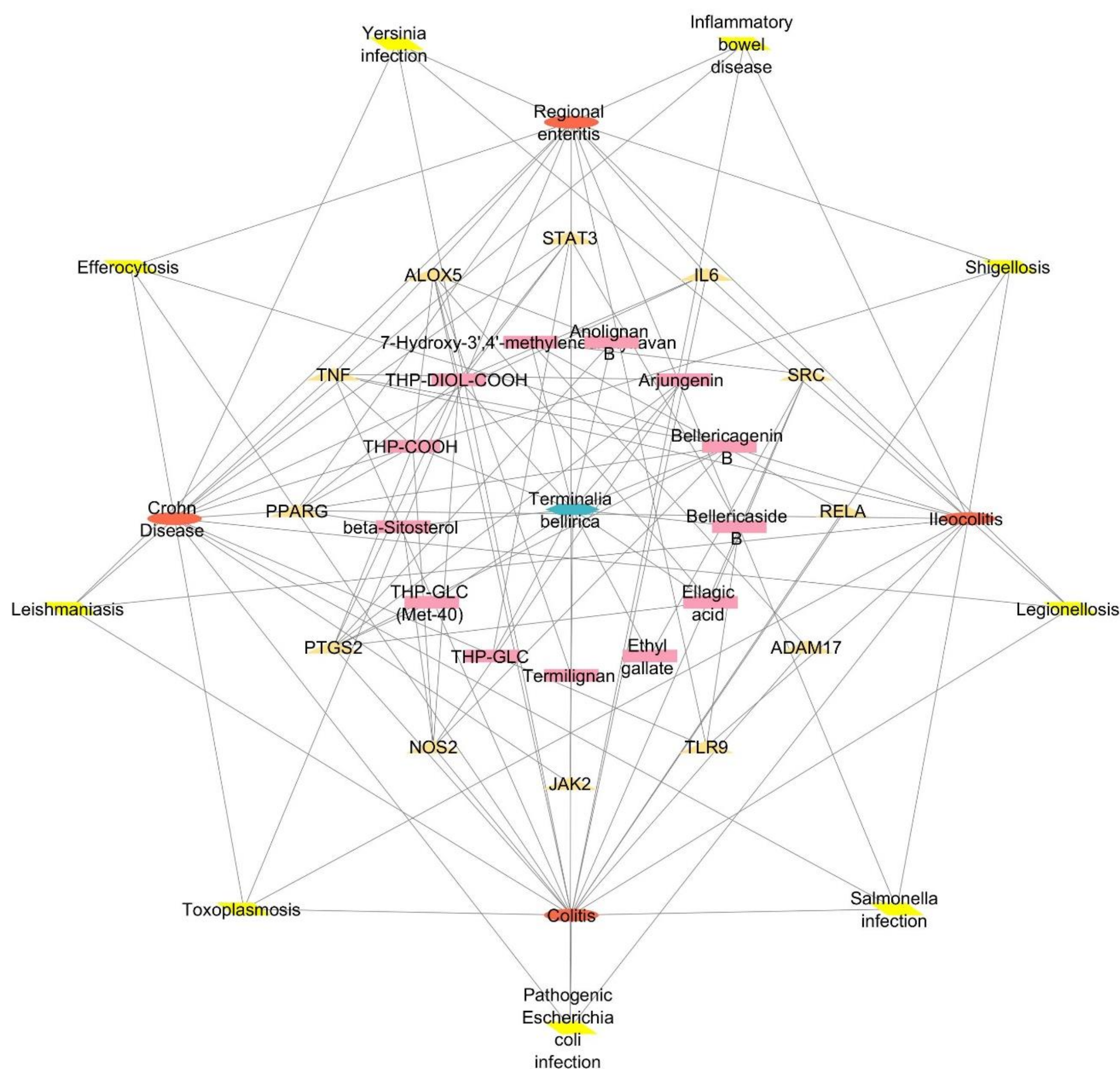

Fig no.9 Interaction network of *Terminalia bellerica* phytochemicals with gastrointestinal disease–associated genes and pathways. Network linking *Terminalia bellerica* phytochemicals to genes and KEGG pathways in six gastrointestinal conditions. Nodes represent phytochemicals, genes, pathways and disease. Edges indicate predicted interactions. Summary statistics (39 nodes, 107 edges) are shown in the inset.

Fig no. 10 Interaction network of *Terminalia chebula* phytochemicals with gastrointestinal disease–associated genes and pathways. This network shows *Terminalia chebula* phytochemicals connect with genes and KEGG pathways involved in six gastrointestinal diseases. Nodes represent compounds, genes and pathways and diseases, while edges indicate predicted interactions.

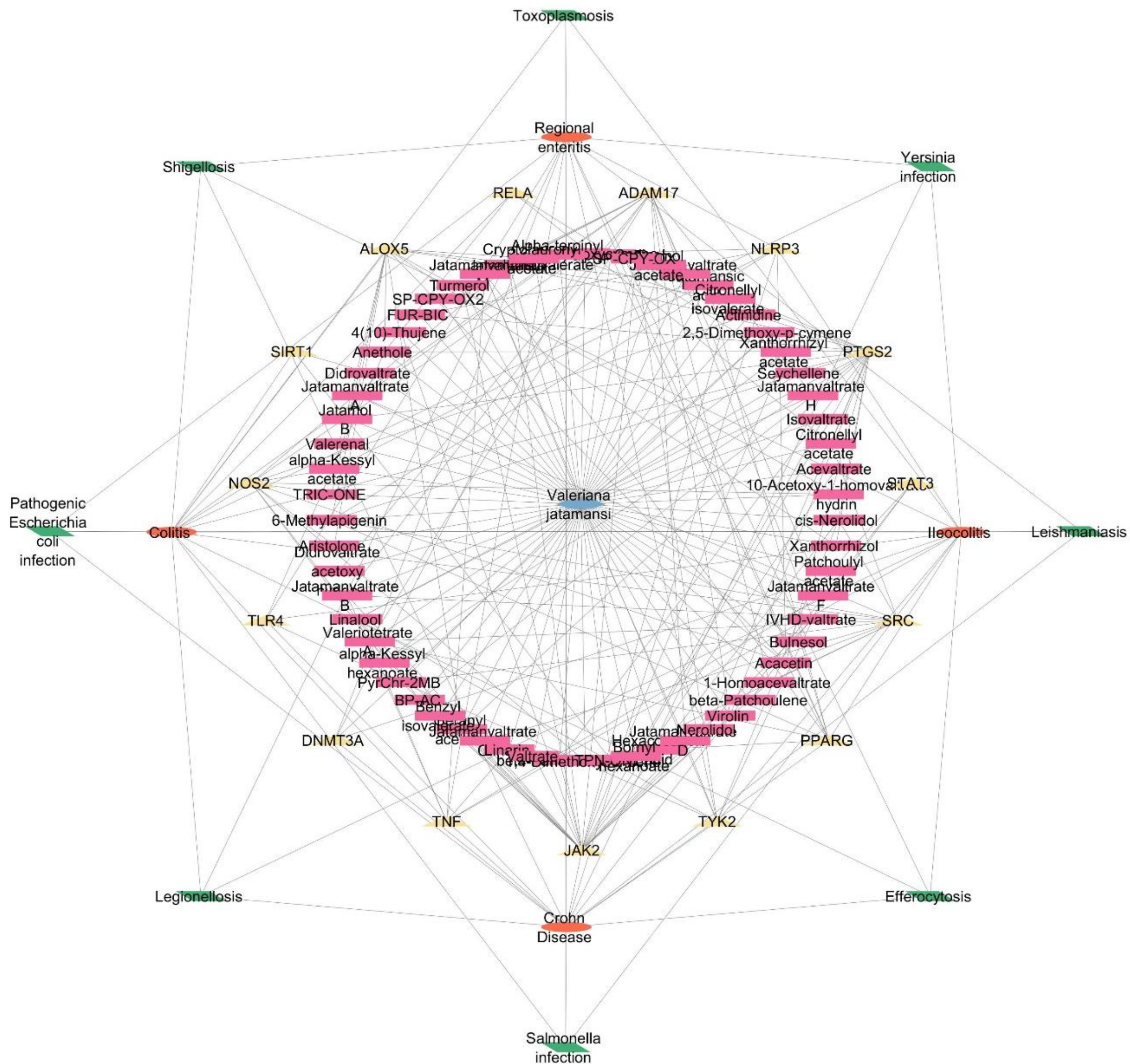

Fig no. 11 Valeriana jatamansi's Metabolite – Disease - Gene Interaction Network in the Vilwadi Gulika Formulation. This network shows compounds from *Valeriana jatamansi* linked to genes, KEGG pathways asscociated with six gasterointestinal. The phytochmeicals form dense central cluster, connecting to several inflammation and immunity related targets.

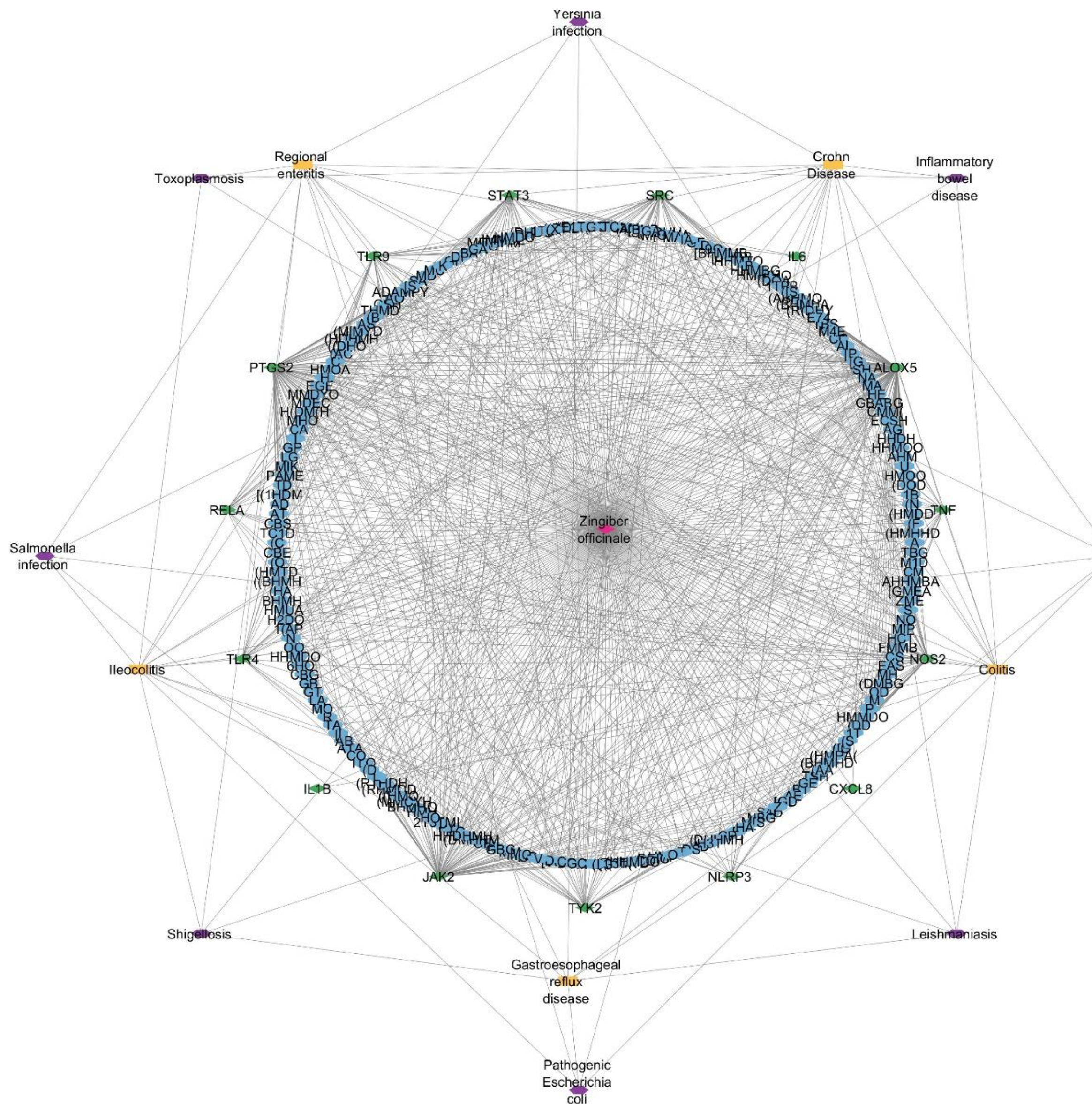

Fig no 12. Interaction network linking *Zingiber officinale* phytochemicals with gastrointestinal disease–associated genes and pathways. Nodes represent phytochmeicals, genes,pathways and six gastrointestinal diseases, edges indicate reported interactions. The network shows a large central cluster of phytochmeicals connected to many targets, with several genes acting as major connection points between phytochemicals and dises pathways.

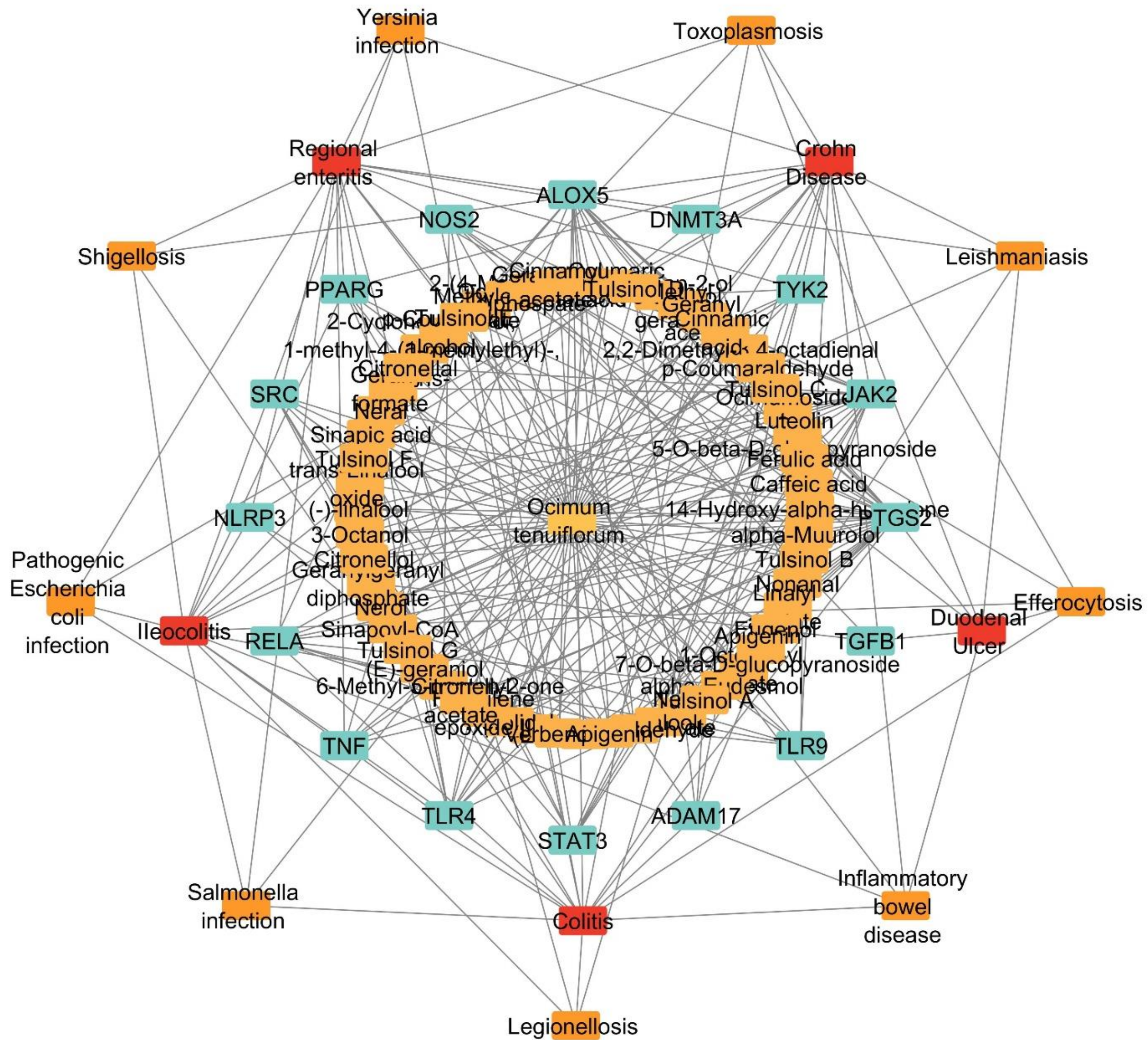

Fig no 13. Interaction network linking *Ocimum tenuiflorum* phytochemicals with gastrointestinal disease–associated genes and pathways. Network connecting *O. tenuiflorum* compounds to genes and KEGG pathways implicated in six gastrointestinal disorders. Nodes represent phytochemicals, genes, pathways and diseases; edges indicate documented or predicted associations. Summary statistics (87 nodes, 259 edges) are shown in the inset.