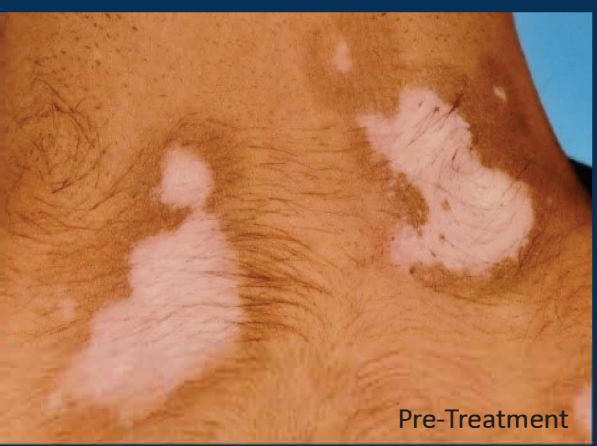
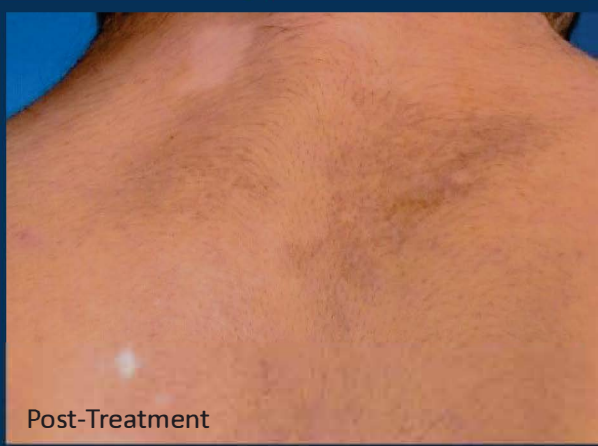
SAFETY AND EFFICACY OF

UNIM-001 and UNIM-003 in *BARAṢ*

(VITILIGO)





Central Council for Research in Unani Medicine
Ministry of Ayush, Government of India

Safety and Efficacy of

UNIM-001 and UNIM-003 in BARAṢ (VITILIGO)

A Technical Report

CENTRAL COUNCIL FOR RESEARCH IN UNANI MEDICINE
Ministry of Ayush, Government of India

Safety and Efficacy of UNIM-001 and UNIM-003 in *Baraṣ* (Vitiligo)
A Technical Report

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Safety and Efficacy of
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A Technical Report

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PREFACE

Baraṣ, commonly known as vitiligo, presents a significant depigmentary challenge affecting both skin and mucous membranes. Its prominence in India raises concerns not just in the medical sphere but also in social and cosmetic contexts, manifesting as depigmented macules of varied sizes and shapes. Despite its prevalence, the aetiology of this disorder remains elusive, posing a substantial challenge for medical researchers. However, the profound insights provided by ancient luminaries of Unani Medicine such as *Buqrāṭ* (Hippocrates) (460-377 BC), *Rabban Ṭabarī* (810-895 AD), *Zakariyā al-Rāzī* (850-925 AD), and *Ibn Sīnā* (Avicenna) (980-1037 AD) have laid a comprehensive foundation for understanding this issue.

Statistics vary, suggesting the incidence of *Baraṣ* (vitiligo) at 1 to 2% across different global regions, yet a consensus aligns around its impact on approximately 1% of the world's population. In India, reports indicate an incidence of around 3%. Despite extensive global research efforts, achieving a satisfactory and safe treatment for vitiligo continues to challenge the modern medical world.

Baraṣ (vitiligo) transcends being merely a dermatological concern; it embodies a social stigma, prompting an intensified quest for viable medical solutions. The Central Council for Research in Unani Medicine (CCRUM) has led the charge in this pursuit, having treated approximately 150,000 vitiligo cases from diverse corners of the globe over four decades. Notably, the National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD) in Hyderabad has garnered international recognition for its successful treatment of various skin disorders.

This document, titled 'Safety and Efficacy of UNIM-001 and UNIM-003 in *Baraṣ* (vitiligo) - A Technical Report,' comprises three parts. The first part outlines the gist of preclinical trials for the study drugs, the second part delves into clinical trials, and the third part scrutinizes biomarkers.

Preclinical toxicological studies conducted on Wistar rats for both coded drugs revealed no toxic effects upon oral administration of UNIM-001 and topical application of UNIM-003 in experimental animals. These findings establish the safety of both drugs for oral and topical use.

A randomized controlled trial involving 518 clinically confirmed vitiligo cases demonstrated the effectiveness of Unani formulations UNIM-001 (oral) and UNIM-003 (local) compared to the control group treated with Psoralen. Safety assessments showcased consistent reduction of VASI score in both groups, affirming the efficacy of the coded Unani formulations without eliciting any side effects.

The third section encompasses the impact of Unani formulations UNIM-001 and UNIM-003 on various biomarkers in vitiligo patients, shedding light on the involvement of specific genes and protein biomarkers, thereby aiding in monitoring immune events, predicting vitiligo progression, and assessing therapeutic responses.

These studies offer a robust foundation for further research endeavours aiming to discover safe and effective treatments for *Baraṣ* (vitiligo). The CCRUM enthusiastically welcomes suggestions for future research in this domain.

I extend my heartfelt acknowledgment to the scientists and esteemed experts whose dedication and diligence have propelled these studies at various levels.

Dr. N. Zaheer Ahmed
Director General, CCRUM

CONTENTS

ABBREVIATIONS

AD	= Anno Domini
ADRs	= Adverse Drug Reactions
ADEs	= Adverse Drug Events
ALP	= Alkaline Phosphatase
ALT	= Alanine Aminotransferase
AST	= Aspartate Aminotransferase
BC	= Before Christ
bw	= Body Weight
BUN	= Blood Urea Nitrogen
CRE	= Serum Creatinine
CBC	= Complete Blood Count
DLC	= Differential Leukocyte Count
ESR	= Erythrocyte Sedimentation Rate
ECG	= Electrocardiogram
FU	= Follow-up
g	= Gram
g/dL	= Gram per decilitre
Hb	= Haemoglobin
HE	= Hematoxylin and Eosin staining
IgA	= Immunoglobulin A
IgG	= Immunoglobulin G
ID	= Initial Date of Experiment
IU/L	= International Units per Litre
IEC	= Institutional Ethics Committee
KFTs	= Kidney Function Tests
Kg	= Kilogram
LFTs	= Liver Function Tests
NSV	= Non-Segmental Vitiligo

NRIUMSD = National Research Institute of Unani Medicine for Skin Disorders
NUMC = National Unani Morbidity Codes
mL = Millilitre
mg = Milligram
RBC Count = Red Blood Cell Count
SD = Sacrificed Date of Experiment
SGOT = Serum Glutamic Oxaloacetic Transaminase
SGPT = Serum Glutamic Pyruvic Transaminase
SV = Segmental Vitiligo
SEM = Standard Error of Mean
TLC = Total Leukocyte Count
UVB = Ultra-violet B
UNIM = Unani Coded Drug
VASI = Vitiligo Area Severity Index
VGICC = Vitiligo Global Issues Consensus Conference
VU = Vitiligo Universalis
WBC Count = White Blood Cell Count

INTRODUCTION

Baraṣ (vitiligo) presents as whitish discoloration affecting the skin and mucous membranes, often bearing a heavy societal stigma. Its underlying cause remains elusive, posing a considerable challenge for medical researchers. Ancient Unani texts abound with insights into the aetiology and treatments for Baraṣ. Notably, eminent scholars such as *Rabban Ṭabarī* (810-895 AD) in '*Firdaus al-Ḥikmat*', *Zakariyā al-Rāzī* (850-925 AD) in '*Kitāb al-Ḥāwī*', and *Ibn Sīnā* (980-1037 AD) in '*Al-Qānūn fil-Ṭib*' provided comprehensive descriptions of this condition.

Baraṣ (vitiligo) is increasingly prevalent, affecting 1-2% of the global population. It manifests as acquired depigmentation caused by melanocyte destruction, resulting in starkly contrasting colours on the skin. While seemingly cosmetic, its psychological impact is profound, distorting body image and evoking fear, anxiety, and distress. Initial signs emerge as white spots on the skin, potentially evolving into localized or extensive hypo-melanosis, although the exact cause remains ambiguous. Proposed hypotheses include auto-immune responses, self-destruction, and neural factors, with the auto-immune hypothesis widely accepted, while the "melanocyte destruction" hypothesis persists.[1, 2]

Strong indications of genetic predisposition to vitiligo arise from studies involving monozygotic twins and families. The disease emerges from a complex interplay of environmental and genetic factors culminating in melanocyte destruction and characteristic depigmented patches. Societal prejudice often associates vitiligo with social, marital, and economic discrimination. Onset can occur at any age or gender, with approximately 50% of cases developing lesions before the second decade and 70% by the third decade of life. Its prevalence is equitable across genders.[1, 2]

Genetic Pre-Disposition

There is an undeniable genetic predisposition towards the occurrence of vitiligo. Approximately 20-30 percent of patients have affected relatives, though the mode of inheritance is not fully understood. Reports of monozygotic twins both having vitiligo, with repigmentation or depigmentation occurring at similar sites simultaneously, suggest that the location and sequence of vitiligo lesion development may be genetically

determined. The mode of transformation remains unclear, possibly involving polygenic factors, environmental influences, or other genetic factors, such as multi-factorial inheritance. Another possibility is that the occurrence, distribution, and extent of the lesion are determined by an unstable genetic system.[3, 4, 5]

The level of melanin pigment in human skin is influenced by genes, sunlight exposure, and pituitary hormones (melanocyte-stimulating hormone or adreno-corticotrophic hormone).[6]

Various factors contribute to the development of vitiligo, including food allergies, gastrointestinal disorders, chronic dyspepsia, protozoal and worm infestations, prolonged use of antibiotics (especially oral antibiotics that disrupt intestinal flora), mental stress, and contact with chemicals like monobenzyl ether, hydroquinone, and pure tetrabutyl phenol.[8]

Vitiligo is a progressive disease that may evolve slowly or rapidly. Contact vitiligo, the fastest emerging type, develops due to contact with poor-quality recycled plastic ware, *bindi*, cosmetics, condoms, gloves, and photographic chemicals. This contact leads to abnormal antigen secretions, resulting in melanocyte destruction and subsequent depigmentation.[9,10,1]

Aetiology

Various hypotheses surround the aetiology of vitiligo, with autoimmunization leading to the formation of antibodies against the melanogenic system being a compelling theory. Additionally, neural control and melanocyte self-destruction hypotheses have been considered.[12, 13,14,15,16]

The coloration of vitiligo patches may vary, appearing milky white, pinkish, white, or slightly hypo-pigmented compared to the surrounding skin. The disorder can extend beyond the skin, affecting melanocytes in the leptomeninges, retinal epithelium, uveal tract, and inner ear. Predominantly affected areas include exposed regions (face, upper chest, dorsal hands), body folds (axilla, groin), orifices (eyes, nose, mouth, ears, nipples, umbilicus, genitals, anus), trauma-prone areas (elbows, knees), pilous regions, and segmentally distributed areas supplied by nerves such as those by the facial, trigeminal, intercostal, and ulnar nerves. The expanding borders of depigmented areas are typically sharp and may exhibit hyperpigmentation. In rare instances, vitiligo may become universal, leaving melanin pigment only in the eyes. Linear or zosteriform patterns, as well as a tendency to localize in areas of trauma and scars, are observed. The onset

and periods of active pigment loss sometimes correlate with severe physical or emotional stress.[18]

Vitiligo is usually asymptomatic, except for sensitivity to solar irradiation due to the absence of the protective melanin pigment screen.[19] Some authors note an increase in local temperature in pigmented areas, heightened sweating, and longer bleeding times compared to normal adjacent skin.[20] While the majority of individuals with vitiligo are otherwise healthy, there is an associated higher incidence of vitiligo with various disorders, often speculative of autoimmune pathogenesis. Examples include hyperthyroidism, hypothyroidism, Addison's disease, pernicious anaemia, hyperparathyroidism, and rheumatic disease. Among skin diseases, alopecia areata, atopic dermatitis, psoriasis, connective tissue diseases (especially scleroderma), and lichen planus are frequently linked to vitiligo. Clinically associated disorders extend to ocular syndromes featuring uveitis, such as sympathetic ophthalmic, and an elevated incidence of vitiligo among malignant melanoma patients. Gastritis, gastric carcinoma, and IgA deficiency are also reported associations.[21]

Incidence

The incidence of vitiligo exhibits considerable variation across countries, with reported rates as low as 0.14% in Russia, 0.24% in London, contrasting with higher rates of 1-2% in India. Earlier surveys suggested an approximate incidence of 1% in the USA, 1.64% in Japan, 0.39% in Switzerland, and 1% in Egypt. While systematic nationwide surveys were not conducted in India, localized surveys indicated a diverse incidence.[3]

In India, reports from different cities vary, ranging from 2.9% in Goa to 8.8% in Delhi. Despite this variation, most authors agree that the incidence hovers around 4%, significantly higher than the global average of 1%.[3,22,23,24,25,26] The prevalence of vitiligo in specific regions, such as Hyderabad and Secunderabad, has been reported at 1.2%.[27] According to a study, the prevalence of vitiligo in India spans a range of 0.46% to 8.8%.[28, 29]

Classification

According to the Vitiligo Global Issues Consensus Conference (VGICC) review conducted during 2011–2012, vitiligo can be categorized into the following clinical forms:

Non-Segmental Vitiligo (NSV)

Common Vitiligo (Vitiligo Vulgaris): This prevalent form is characterized by asymptomatic, well-defined, milky-white macules affecting various body parts symmetrically. While it can initiate at any body site, the fingers, hands, and face are commonly the initial sites.

Acrofacial Vitiligo: Involves limited areas on the face, head, hands, and feet, with depigmentation of the distal fingers and facial orifices. It may later progress to typical generalized vitiligo.

Vitiligo Universalis (VU): The most extensive form, typically occurring in adulthood, where depigmentation is nearly universal (80–90% of body surface). Some pigmentation may persist, and hair may be partially spared. While this diagnosis is straightforward in dark-skinned individuals, it may pose challenges in very fair-skinned individuals. Typically, VU is preceded by generalized vitiligo, gradually progressing to complete or near-complete depigmentation of the skin and hair.

Mucosal Vitiligo: Involves the oral and/or genital mucosae, either as part of generalized vitiligo or as an isolated condition, which remains in the 'unclassified' category per VGICC nomenclature until at least 2 years of follow-up.

Mixed Vitiligo: Concomitant occurrence of Segmental Vitiligo (SV) and Vitiligo/NSV. Criteria include absence of depigmented areas in a segmental distribution at birth, SV preceding Vitiligo/NSV, specific distribution patterns, and response to narrow-band Ultra-violet B (UVB) treatment.

Focal Vitiligo: Focal vitiligo refers to a small isolated patch that does not fit a segmental distribution, and which has not evolved into vitiligo /NSV after a period of at least 2 years. This form of vitiligo may evolve into SV or vitiligo /NSV.

Other Unclassified Conditions

Vitiligo Punctata: Manifesting as sharply demarcated depigmented punctiform macules measuring 1 to 1.5 mm, these lesions can appear on any area of the body.

Vitiligo Minor: This rare form of Non-Segmental Vitiligo (NSV) is predominantly observed in dark-skinned individuals. The term 'minor' does not strictly imply restriction to a limited surface area but rather signifies a partial defect in pigmentation. Its connection to true vitiligo is established through pathology and coexistence with conventional vitiligo macules.

Differential diagnosis from early-stage cutaneous lymphoma is crucial, often necessitating repeated biopsies with molecular studies of clonality.

Follicular Vitiligo: Representing a subtype of generalized vitiligo, this condition is observed in young black patients and primarily involves the follicular reservoir, contrasting with marked generalized hair whitening and melanocyte loss in hair follicles. Further cases are required to determine whether follicular vitiligo should be recognized as a distinct form of vitiligo.

Segmental Vitiligo

Mono-Segmental Vitiligo: The most common form of SV, characterized by white depigmented macules on one side of the body, usually respecting the midline, with early follicular involvement and a protracted course.

Multiple Segmental Vitiligo: Rarely, multiple segmental lesions distributed unilaterally or bilaterally, distinguished by a clear segmental distribution with midline demarcation.[30,31,32,33]

History

The term "vitiligo" finds its roots in the Latin word "Vitellus," meaning calf, referring to the characteristic white patches resembling a spotted calf. Celsus, a Roman physician of the 2nd Century AD, first used this term.[34] The earliest documented information about vitiligo dates back to the period of *Āshūriyān* (2200 BC) and is found in *Tārīkh Ṭibb-i-Irān*.[35]

Further insights into the disease were gleaned from the Ebers Papyrus (1550 BC)[36], where two types of pigmentary dilutions were distinguished. One type, associated with tumours and mutations, represented leprosy, while the other, characterized by a mere change of colour, was identified as vitiligo. According to the *Ebers Papyrus*, vitiligo was considered treatable. In the sacred Indian text *Atharva Veda*, dating back to 1400 B.C., the condition known as "*Shweta Kustha*" was mentioned, referring to vitiligo. Greek literature also described white spots, with Herodotus (484-425 B.C.) noting in Clio in 449 B.C. the presence of such pigmentation changes.[37]

Concept of *Baraṣ* (Vitiligo) in Unani Medicine

Baraṣ, or vitiligo, is intricately defined in ancient Unani Medicine as a skin disease associated with discoloration. The aetiology and treatment of *Baraṣ* have been extensively elaborated in the classic texts of Unani Medicine.[38, 39, 40, 41]

Jālīnūs (Galen) (130–200 AD), as cited in the manuscript *Muʿālajāt al-Buqrāṭiyya* (10th century AD), attributes *Baraṣ* to the weakness of *Quwwat Mughayyirah wa Mushabbiḥa* (transformative faculty) in the organs.[38]

Rabban Ṭabarī (810-895 AD), in his renowned work *Firdaus al-Ḥikmat*, identifies '*Fasād al-Dam*' (impairment of blood) and '*Burūdat al-Dam*' (coldness of blood) as the primary causes of *Baraṣ*. He emphasizes that *Baraṣ* arises when impurity in the blood, caused by an inadequately functioning digestive faculty, is influenced by *Balgham* (phlegm) or coldness.[39]

Zakariyā al-Rāzī (Rhazes) (850–925 AD) offers a comprehensive description of *Baraṣ* in his esteemed work, *Al-Ḥāwī*. He discusses the examination of affected areas and the importance of colour changes. Here are few excerpts from his most esteemed work:[40]

"Sometimes *Bahaq Abyaḍ* (Pityriasis alba) reaches a stage when greyish hairs grow on the patches. For examining whether it is curable or not, rub the affected areas; if the patches do not turn red then prick the lesion, if the whitish fluid comes out, then the possibility of recovery is remote and vice versa."

"At the site of *Baraṣ*, the flesh becomes phlegmatic. Thus, the blood reaching this flesh also turns phlegmatic and this (phlegmatic) flesh becomes (so soft) as that of molluscs. So, the area getting such blood cannot be nourished properly."

Shamūn, as quoted in *Rāzī's al-Ḥāwī*, attributes *Baraṣ* to the frequent consumption of water-rich food articles.

Ibn Sarābiyūn (as quoted in *Rāzī's al-Ḥāwī*) says: "If *Baraṣ* spreads over a large portion of the body or when it becomes highly chronic or when milky fluid comes out on pricking the *Baraṣ* patch, it is not curable and vice versa."[43]

Ibn Sīnā (Avicenna), in *Al-Qānūn fiʾl-Ṭib*, describes *Baraṣ* as a condition where the *Quwwat Dāfiʿa* (expulsive power) of the affected organ becomes sluggish, leading to the accumulation of *Balghamī Mādda* (phlegmatic substance). This substance alters the skin colour to white, affecting underlying structures like muscles and bones. Avicenna also highlights the role of *Tashbīḥ*, the power shaping nutrients into tissue, and identifies derangements caused by *Balghamī Mādda*, resulting in depigmentation.[42]

According to Unani physicians, the perfection of tissue metabolism depends on four factors: *Quwwat Jādhiba* (Absorptive faculty), *Quwwat Māsika*

(Retentive faculty), *Quwwat Mughayyira* and *Mushabbiḥa* (Transformative faculty), and *Quwwat Dāfiʿa* (Expulsive faculty).[43, 45, 46]

Avicenna suggests that defects lie in the function of *Quwwat Mushabbiha* at the tissue level, leading to depigmentation. He also notes that certain diseases, including *Baraṣ*, can be transmitted from generation to generation.

Akbar Arzānī (17th century AD) provides further insights into *Baraṣ* in his book *Ṭib Akbar*.[44] He distinguishes between curable and incurable cases based on the involvement of hairs and colour changes upon rubbing.

Rāzī adds that extensive, spreading *Baraṣ* with bloodless areas and cloudy-coloured patches is generally incurable, and certain areas like the feet and head respond less favourably to treatment.[43]

Treatment Module & Regimens in Unani Medicine

In the management of *Baraṣ*, a comprehensive approach employing both oral and local drug therapies is recommended. *Baraṣ*, characterized by the accumulation of *Ghalīẓ Fāsid Mādda* (thick morbid matter), necessitates the initiation of treatment with *Tanqiya-i-Badan* (removal of harmful matter from the body) through *Munḍij* and *Mushil* therapy. The following principles may be adopted:

Istifrāgh-o-Tanqiya-i-Balgham **(Evacuation of morbid matter - Phlegm):** Prioritize the elimination of accumulated morbid matter, specifically phlegm, through targeted therapies aimed at cleansing the body.

Taʿdīl-i-Mizāj **(Correction of morbid temperament):** Address the underlying imbalance in the body's temperament to restore equilibrium and promote overall health.

Produce heat at the affected part using *Mukhaddirāt* (Anaesthetics): Apply localized heat therapy using anaesthetics to alleviate symptoms and enhance the healing process in the affected area.

Iṣlāḥ-i-Ghidhāʾ **(Dietary recommendation):** Implement dietary interventions based on sound Unani principles to support the body's natural healing mechanisms.[12,42,43]

This integrative approach, encompassing *Tanqiya-i-Badan*, correction of temperament, localized heat therapy, and dietary adjustments, reflects the holistic nature of Unani Medicine in addressing *Baraṣ*.

ʿIlāj biʾl-Dawāʾ (Pharmacotherapy)

Munḍij-Mushil therapy can be done by using *Mā' al-Uṣūl* and suitable *Munḍij-i-Balgham Adwiya* for *Nuḍj*. An appropriate dose of *Munḍij-i-Balgham* may be administered till *Nuḍj* appears. Then *Mushil* (purgatives) alternated with *Tabrīd* (cooling) agents can be given. After completion of *Munḍij* and *Mushil* treatment, *Maʿjūnāt Ḥārra* and *Iṭrifalāt* are prescribed, and the specific medicines including local applications are advised. When *Nuḍj* appears, 5 g of the following formulation (*Maʿjūn*) may be used orally for *Ishāl*. The formulation contains *Halela* (*Terminalia chebula* Retz.) one part, *Āmla* (*Phyllanthus emblica* L.) one part, *Turbud* (*Operculina tuerpethum* (L.) Silva Manso) three parts, and *Qand Safaid* (sugar) in sufficient quantity.[12, 42, 43]

Oral administration of 4.5 g of the following *Maʿjūn* followed by exposure to sun rays may be done:[12, 42, 43]

- *ʿĀqarqarḥā* (*Anacyclus pyrethrum* (L.) Lag.), *Atrīlāl* (*Ammi majus* L.), *Post-i-Bīkh-i-Kibr* (Root bark of *Capparis spinosa* L.), *Shītraj Hindī* (*Plumbago zeylanica* L.) each 7 g, honey Q.S., vinegar Q.S.

The following formulation may be used for local application:

- *Qust* (*Saussurea costus* (Falc.) Lipsch.), *Shitraj Hindī* (*Plumbago zeylanica* L.), *Zarnīkh Surkh* (Arsenic sulphide), *Filfil Siyāh* (*Piper nigrum* L.), *Zangar* (*Copper rust*), vinegar.

The above drugs to be ground along with vinegar in a copper vessel and used after exposing the vessel to sun light for a week.[12, 42, 43]

The following formulation may be used for local application:

- *Bhujāyā Huwā Chūnā* (Lime treated with water) Q.S.[12, 42, 43]

To improve the hotness and blood circulation over the affected area, powder of any of the following drugs mixed with water may be applied:

- *Zarāvand* (*Aristolochia longa* L.)
- *Qust* (*Sassurea costus* (Falc.) Lipsch.)
- *Khulanjān* (*Alpinia galanga* (L.) Willd.)
- *Rāʾī* (*Brassica juncea* (L.) Czern.)
- *Junṭiyānā* (*Gentiana lutea* L.)
- *Ḥanẓal* (*Citrullus colocynthis* (L.) Schrader)
- *Būzīdān* (*Pyrethrum indicum* L.)[12, 42,43]

Compound Formulations

- *Ḥabb-i-Ayārij* in a dose of 3–9 g can be taken orally for evacuation of morbid matter.
- *Safūf-i-Baraṣ*: 10 g of powder is soaked in 50 ml of water overnight. The infusion so obtained is decanted and orally administered the next morning.
- Any of the following formulations may be used as local application:
  - *Ḍimād-i-Baraṣ*
  - *Rowghan-i-Balsāṉ*
  - *Rowghan-i-Sudāb*
  - *Rowghan-i-Behroza*
  - *Rowghan-i-Bābchī*
  - *Rowghan-i-Bayḍa-i-Murgh*[12, 42, 43]

Buqrāṭ (Hippocrates) recommended post-*Tanqiya* intervention to correct the digestive system. He advocated for an easily digestible diet that promotes the production of more pure blood. Emphasizing adherence to age-appropriate customs and traditions, he advised against consuming food without a genuine appetite. Additionally, he proposed taking specific digestive tonics two hours after meals to enhance the digestion process.[43]

In certain cases, both oral and topical medicines, including *Adwiya Mukhaddira* (anaesthetic drugs), are prescribed concurrently to boost overall metabolism. Notably, Unani physicians observed that exposure to sunlight activates the pigmentation process.

Al-Rāzi, while discussing external application of medicine, recommended treating *Baraṣ* and *Bahaq* in their early stages by applying the medicine to the affected areas and exposing the patient to sunlight.[43]

Table 1: List of single Unani drugs considered effective in the treatment of *Baraṣ* [43]

S. N.	Unani Name	Botanical Name	Part Used	Chemical Constituents
1.	*Aftīmūn*	*Cuscuta chinensis* Lam.	Stem	Phenols, Steroids, tannins
2.	*Amaltās*	*Cassia fistula* L.	Fruit pulp	Anthraquinones, volatile oils
3.	*Āmla*	*Phyllanthus emblica* L.	Fruit	Tannins, phenolic compounds

S. N.	Unani Name	Botanical Name	Part Used	Chemical Constituents
4.	*Anjīr*	*Ficus carica* L.	Fruit	Glycosides, resins, phenolic compounds
5.	*Asārūn*	*Asarum europaeum* L.	Rhizome	Alkaloids, volatile oils, fixed oils
6.	*Bābchī*	*Psoralea corylifolia* L.	Seeds	Essential oils, fixed oils, psoralen, isopsoralen
7.	*Bādām*	*Prunus dulcis* (Mill.) D.A.Webb.	Seed's Kernels	Thiamine, nicotinic acid, phosphorous, calcium, riboflavin, folic acid, tocopherol
8.	*Bādiyān*	*Foeniculum vulgare* Mill.	Fruit & Root	Volatile oil, Anethol, pentosan, iodine, thiamine, riboflavin, niacin, ascorbic acid
9.	*Balādur*	*Semecarpus anacardium* L.f.	Seed oil (Asl)	Flavonoids, Resins, Glycosides
10.	*Balsāṉ*	*Commiphora gileadensis* (L.) C.Chr.	Root & aerial shoots	Essential oils, gum, resins
11.	*Bihīdāna*	*Cydonia oblonga* Mill.	Seed mucilage	Steroids, glycosides, tannins, volatile oils, fixed oils
12.	*Bhāngra*	*Eclipta prostrata* (L.) L.	Whole plant	Alkaloids - ecliptine and nicotine
13.	*Bisfāʾij*	*Polypodium vulgare* L.	Leaves and rhizomes	Essential oils, glucoside-samambain, and saponins
14.	*Chirāʾita Talkh*	*Swertia chirayita* (Roxb.) H.Karst.	Whole plant	Bitter principles
15.	*Dār Chīnī*	*Cinnamomum verum* J.Presl	Bark	Essential oil – eugenol
16.	*Fodnaj*	*Mentha piperita* L.	Whole plant	Essential oil – menthol
17.	*Gandhak Amlasar*	Sulphur	Crystals	Sulphur
18.	*Gerū*	Silicate of alumina/ Oxide of iron	Crystal	Oxide of iron
19.	*Gulnār Fārsī*	*Punica granatum* L.	Flower	Tannins
20.	*Ḥabb al-Nīl*	*Ipomoea hederacea* L.	Seeds	Resins and glucoside
21.	*Halela Siyāh*	*Terminalia chebula* Retz.	Dried Fruit	Tannins, tannic acid, ellagic acid, gallic acid

S. N.	Unani Name	Botanical Name	Part Used	Chemical Constituents
22.	*Halela Zard*	*Terminalia chebula* Retz.	Dried mature fruit	Tannins, tannic acid, ellagic acid, gallic acid
23.	*Ḥanẓal*	*Citrullus colocynthis* (L.) Scharder	Fruit	Bitter principles- colocynthin, colocynthetin.
24.	*Idhkhār*	*Cymbopogon jawarancusa* (Jones) Schult.	Root	Essential oils- citral, citronella glycosides, phenolic compounds, tannins
25.	*Kalonjī*	*Nigella sativa* L.	Seeds	Alkaloids, glycosides, terpinoids
26.	*Karafs*	*Apium graveolens* L.	Root	Glucoside, traces of Copper
27.	*Kharbaq*	*Helleborus niger* L.	Rhizome	Helleborin and Helleborein
28.	*Khaṭmī*	*Althaea officinalis* L.	Seeds	Alkaloids, saponins, resins and volatile oils
29.	*Khubāzī*	*Malva sylvestris* L.	Seeds	Resins, tannins, glycosides, alkaloids
30.	*Kundūr*	*Boswellia serrata* Roxb.ex Colebr.	Stem exudate	Oleo - gum-resin
31.	*Mawīz*	*Vitis vinifera* L.	Fruits	Tartaric acid, tannins
32.	*Mulethī*	*Glycyrrhiza glabra* L.	Root	Glycyrrhizin, Glycyrrhizinic acid, resins
33.	*Mundī*	*Sphaeranthus indicus* L.	Flower	Alkaloids, glycosides, tannins
34.	*Panwad*	*Cassia tora* L.	Seed	Plant sterol
35.	*Parsiyāʾoshān*	*Adiantum capillus-veneris* L.	Leaves and branches	Glycosides, tannins, resins
36.	*Sanā Makkī*	*Senna alexandrina* Mill.	Leaves and pods	Glycoside, Kaempherol, anthraquinone, essential oils, Crysophanic acid, Isorhamnetin, Flavanols
37.	*Ṣandal Surkh*	*Pterocarpus santalinus* L.f.	Stem	Santalin, pterostilbene, pterocarpin
38.	*Sarphokā*	*Tephrosia purpurea* (L.) Pers.	Whole plant	Tephrosin, deguelin, Isotephrosin, rotenone, glucoside, rutin

S. N.	Unani Name	Botanical Name	Part Used	Chemical Constituents
39.	*Sazij Hindī*	*Cinnamomum tamala* (Buch.-Ham.) T.Nees& Eberm.	Leaves	Essential oils
40.	*Shāhitara*	*Fumaria officinalis* L.	Whole plant	Alkaloids
41.	*Shītraj*	*Plumbago zeylanica* L.	Bark	Alkaloid – plumbogin
42.	*Ṣibr*	*Aloe vera* (L.) Burm.f.	Leaves	Aloin, isobarbaloin, emodin, gum, resin
43.	*Sumbul al-Ṭīb*	*Nardostachys jatamansi* (D.Don) DC.	Fibrous Root	Essential oil and jatamansic acid
44.	*Turb*	*Raphanus sativus* L.	Seeds	Essential oil
45.	*Turbud*	*Operculina turpethum* (L.) Silva Manso	Stem	Glycosidic resin, trupenthein, turpentheine
46.	*ʻUnnāb*	*Zizyphus sativa* Gaertn.	Fruits	Phenolic compounds, alkaloids, aglycone, gum, mucilage
47.	*Usṭūkhūdūs*	*Lavandula stoechas* L.	Flowers	Glycosides, lavandol, phenolics, steroids, terenes and tannins
48.	*Waj*	*Acorus calamus* L.	Root stock	Glucoside acorin and essential oil
49.	*Ward (Gul-i-Surkh)*	*Rosa damascena* Mill.	Flower	Aromatic volatile oil, phenolics, tannins
50.	*Zaʿfrān*	*Crocus sativus* L.	Flowers/ styles and stigmas	Essential oil
51.	*Zanjabīl*	*Zingiber officinale* Roscoe	Rhizome	Volatile oil, glutamic acid, aspartic acid, serin, glycine, threonine, alanine, glutamine, argenine, zingiberene, zingiberol

Table 2: List of Unani formulations considered effective in the treatment of *Baraṣ*

S. N.	Formulation	Dosage Form	Source
1.	*Safūf-i-Baraṣ*	Powder	National Formulary of Unani Medicine, part-I, p. 233
2.	*Ḥabb-i-Hindī*	Tablet	*Hakīm Kabīruddin*, *Al-Qarābādīn*, CCRUM, New Delhi, 2006, p. 231.
3.	*Ḥabb-i-Baraṣ*	Tablet	*Hakīm Kabīruddin*, *Al-Qarābādīn*, CCRUM, New Delhi, 2006, p. 231.
4.	*Sufūf-i-Atrīlāl*	Powder	Standardisation of Single Drugs of Unani Medicine, CCRUM, New Delhi, Govt. of India, Part-1, pp. 1–7.
5.	*Iṭrīfal Mushil*	Semi-solid	National Formulary of Unani Medicine, Part-II, Vol-I, p. 128
6.	*Ḍimād-i-Baraṣ*	Semi-solid (paste)	National Formulary of Unani Medicine, Part II, Vol-I. p. 160

ʿIlāj biʾl-Tadbīr (Regimenal Therapy) [12, 42, 43]

- *Ḥammām Muʿarriq* may be performed to produce sweating after *Tanqiya-i-Balgham.*
- *Dalk Khashin* (rough massage) can be done on affected area with a rough cloth to draw blood towards the skin and to improve hotness.

Dietary Restrictions [12, 42, 43]

- Milk and dairy products
- *Aghdhiya Ghalīẓa*
- *Muwallid-i-Balgham Aghdhiya*
- All the sour and citrus substances
- Sea food
- Cold and moist substances
- Moist vegetable and fruits

Dietary Recommendations [12, 42, 43]

- *Sarīʿ al-Haḍm* (easily digested) dietary substances
- Diet of hot temperament producing *Dam* (sanguine)

Taḥaffuẓ (Prevention) [12, 42, 43]

- *Faṣd* (bloodletting) to be avoided.
- *Kayy* (cautery) to be avoided
- *Ḥammām* (*Turkish* bath) to be avoided but *Ḥammām Muʿarriq* may be performed in some cases for sweating.
- *Adwiya Mashrūba* should be stopped when *Taʿdīl-i-Mizāj* (correction of morbid temperament) is achieved.
- Avoid excessive coitus.
- Avoid excessive use of *Sharāb* (alcohol).

PART – A

Preclinical Safety Studies of Coded Unani Formulation UNIM-001 (Oral)

Preclinical safety studies of coded Unani formulations were undertaken before initiation of clinical trial involving human subjects. Two coded Unani formulations, i.e. UNIM-001 (Oral) & UNIM-003 (Topical) were studied on animals and the safety data was generated which is as follows:

Acute Toxicity Study on UNIM-001 in Wistar Rats

The acute toxicity study of UNIM-001, a coded Unani formulation, was conducted on Albino Wistar rats to assess its impact on morphological features, gross behaviours, and body weight changes.

Material & Methods: UNIM-001 was suspended in double-distilled water, with a volume not exceeding 2 mL/100 g body weight. A dose of 2000 mg/kg was administered via intra-gastric intubation to ten female albino Wistar rats, divided into control and test groups. Observations on morphological changes, behaviour, and body weight were recorded daily and weekly, adhering to established guidelines.

Table 3: Brief description of study protocol for UNIM-001

Name of the Study	Acute Toxicity Study of UNIM-001
Test material	UNIM-001
Animal model	Albino *Wistar* rats
Age	11–12 weeks
Weight	180 ± 20 g
Animal procured from	Central Animal Facility, Jamia Hamdard (173/CPCSEA)
Sex	Female
Number of animal per group	05 per group
Groups	2 group
Route of administration	Intra-gastric administration
Dose volume	Not more than 2 mL/100 g body weight per animal
Number of administration	Single dose
Concentration of dose	2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	14 days

Observations

All animals were inspected for signs of morbidity and mortality at least twice daily, usually at the beginning and at the end of each day. Signs noted including changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions.

Body Weight

Body weights were recorded prior to dosing and on 7^{th} and 14^{th} day. Vital organs of the sacrificed animals were observed macroscopically.

Table 4: Effect of UNIM-001 on body weight and organ weight

Body & Organ Weight	Control	Dose (2000 mg/kg)
Body weight (g)	142 ± 14.832	146 ± 6.519
Heart (g)	0.511 ± 0.018	0.531 ± 0.047
Stomach (g)	1.108 ± 0.105	1.22 ± 0.160
Liver (g)	4.673 ± 0.506	4.862 ± 0.394
Left kidney (g)	0.529 ± 0.063	0.555 ± 0.081

Mean values of 05 animals ± standard deviation

Results

Prior to dosing (day 0) and every week following dosing, the body weight of individual animals was measured. At the end of the 14-day observation period, all the animals were subjected to necropsy. No visible signs of toxicity after treatment such as change in respiratory, circulatory, autonomic and central nervous system, behavioural pattern were observed in the study. No mortality was observed in any of the animals. Body weight measurement in the test drug-treated animals showed increasing trend. Gross pathology examination conducted on the animals at the end of 14-day observation did not reveal any lesion that could be attributed to the toxicity of the test substance.

Conclusion

The acute oral toxicity study was conducted on female *Wistar* rats to evaluate the potential of UNIM-001 to produce toxicity from a single dose via oral route. No toxic effect was found as evidenced from the above observation.

Sub-acute Toxicity Study on UNIM-001 in Wistar Rats

Repeated dose 28-day oral toxicity study of UNIM-001 was carried out in *Wistar* rats to assess its effects on morphological, gross behaviour, body

weight change as well as to find out the haematological, biochemical, and histopathological changes.

Material & Methods

UNIM-001 was supplied by the Central Council for Research in Unani Medicine (CCRUM), New Delhi. The test substance was suspended in water and administered in the volume not exceeding 2 mL/100 g body weight. Dose levels tested were 0 (control), 500, 1000 and 2000 mg/kg body weight. Five female and five male *Wistar* rats were used. The test substances were administered in three dose levels, i.e. 500, 1000, 2000 mg/kg of body weight once daily for 28 days by gavage using a suitable intubation cannula. Under cage-side observations, i.e., change in morphology and behaviour were noted daily and body weight was noted weekly. At the end of the study, i.e., 29th day, haematological, biochemical and pathological changes (macroscopic and microscopic) were examined.

Table 5: Brief description of study protocol for UNIM-001

Name of the Study	Sub-acute Study on UNIM-001
Test material	UNIM-001
Animal model	*Wistar* rats
Age	11–12 weeks
Weight	180 g average
Animal procured from	Central Animal Facility, Jamia Hamdard (173/CPCSEA)
Sex	Male and female
Number of animal per group	05 per each group
Groups	04
Route of administration	Intra-gastric administration
Dose volume	Not more than 2 mL per 100 g body weight per animal
Number of administration	Once daily for 28 days
Dose levels	500, 1000, 2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	28 days

Observations

The study spanned a 28-day period, during which all animals underwent a thorough examination for signs of morbidity and mortality, conducted at least twice daily. Noted observations encompassed changes in skin, fur, eyes, and mucous membranes, as well as the occurrence of secretions, excretions, and autonomic activity.

Body weight

Weight changes were meticulously calculated and documented throughout the study. Upon the conclusion of the testing period, surviving animals were weighed and subsequently humanely sacrificed.

Haematology and Clinical Biochemistry

Towards the conclusion of the testing period, blood samples were collected immediately before the animals were sacrificed. Haematological and clinical biochemistry determinations were then performed to identify potential toxic effects in both blood and tissues, with a specific focus on evaluating impacts on the kidney and liver.

Results

Table 6: Effect of oral administration of UNIM-001 on body weight and organ weight of female rats

Body & Organ Weight	Control	UNIM- 001 (500 mg/kg)	UNIM- 001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Body weight (g)	281 ± 15.16	257 ± 4.472**	253 ± 13.038**	240 ± 22.361**
Stomach (g)	2.769 ± 0.233	2.0992 ± 0.3349	2.237 ± 0.3176	2.311 ± 0.1884
Liver (g)	8.696 ± 0.965	8.172 ± 1.870	7.237 ± 1.021	9.6378 ± 0.3122
Heart (g)	0.883 ± 0.061	0.825 0 ± .1681	0.76980 ± .08410	0.8384 ± 0.04345
Left kidney (g)	0.864 ± 0.065	0.9180 ± 0.1285	0.86960 ± 0.1246	0.9092 ± 0.1076

Mean values of 05 female rats ± Standard deviation; *P < 0.05, **P < 0.01 vs. control group (Dunnett's test). Control group was given water.

Table 7: Effect of oral administration of UNIM-001 on body weight and organ weight of male rats

Body & Organ Weight	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Body weight (g)	142 ± 16.808	168 ± 21.966**	166 ± 18.16**	157 ± 14.832*
Stomach (g)	1.412 ± 0.2640	1.732 ± 0.1951	1.552 ± 0.1272	1.5576 ± 0.1148
Liver (g)	6.176 ± 0.2176	6.9172 ± 0.9772	4.812 ± 0.2347	4.456 ± 0.6851
Heart (g)	0.6326 ± 0.03607	0.6746 ± 0.08921	0.564 ± 0.0312	0.447 ± 0.027
Left kidney (g)	0.6918 ± 0.05823	0.7718 ± 0.06465	0.6538 ± 0.03419	0.732 ± 0.080

Mean values of 05 male rats ± Standard deviation; *p < 0.05, **P < 0.01 vs. control group (Dunnett's test). Control group was given water.

Table 8: Effect of oral administration of UNIM-001 on biochemical parameters of female rats

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
ALT (IU/L)	35.855 ± 4.678	16.442 ± 5.509	27.686 ± 4.110	35.501 ± 8.194
AST (IU/L)	64.532 ± 10.644	38.224 ± 8.159**	31.399 ± 4.875**	36.951 ± 5.733**
BUN (mg/dl)	39.640 ± 8.593	31.131 ± 5.519	41.791 ± 4.996	44.089 ± 5.743
CRE (mg/dl)	0.617 ± 0.140	0.939 ± 0.543	1.562 ± 2.070	0.902 ± 0.204

Mean values of 05 female rats ± Standard deviation; **P < 0.01 vs. control group (Dunnett's test). Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 9: Effect of oral administration of UNIM-001 on biochemical parameters of male rats

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
ALT (IU/L)	20.756 ± 7.630	25.494 ± 6.056	21.371 ± 10.371	28.818 ± 6.454
AST (IU/L)	67.360 ± 12.788	55.196 ± 8.982	66.476 ± 15.040	72.028 ± 7.354
BUN (mg/dl)	32.136 ± 6.937	28.183 ± 1.059	22.668 ± 13.033	25.159 ± 8.31
CRE (mg/dl)	0.658 ± 0.170	0.610 ± 0.124	0.914 ± 0.309	0.645 ± 0.164

Mean values of 05 male rats ± Standard deviation; Dunnett's test. No significant difference was observed in any parameter. Control group given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 10: Complete blood cell count (CBC) at the conclusion of experimental duration with UNIM-001 (Female)

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
RBC Count (mil/ µL)	6.974 ± 0.380	6.9151 ± 0.524	7.977 ± 0.2636	6.686 ± 0.525
HB (g/dL)	14.26 ± 1.226	13.075 ± 3.020	15.975 ± 0.125	13.2 ± 0.806
Haematocrit (%)	39.4 ± 3.543	40.05 ± 2.252	44.675 ± 0.4500	36.66 ± 2.535
Mean Corpuscular Volume (fL)	56.46 ± 3.543	58 ± 1.281	56.025 ± 1.325	54.88 ± 1.770
Mean Corpuscular Haemoglobin (pg)	20.44 ± 0.654	18.8 ± 3.407	20.025 ± 0.6292	19.76 ± 0.7701
Mean Corpuscular Haemoglobin Concentration (g/dL)	36.2 ± 0.692	32.425 ± 6.160	35.775 ± 0.4573	35.98 ± 0.609

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Red Cell Distribution Width (%)	15.26 ± 1.258	19.525 ± 3.963	14.775 ± 0.4113	16.68 ± 0.5891
Platelet Count (thou/µL)	816.8 ± 53.467	1032 ± 407.48	941 ± 136.39	796.6 ± 183.90
Mean Platelet Volume (fL)	5.82 ± 0.327	5.755 ± 0.6702	5.75 ± 0.1291	5.94 ± 0.288
WBC Count (thou/µL)	3.48 ± 2.337	3.875 ± 2.579	3.85 ± 1.245	4.44 ± 3.974
Segmented Neutrophils (%)	16.6 ± 15.323	27.75 ± 4.924	12.5 ± 5.000	13.2 ± 7.190
Lymphocytes (%)	82.2 ± 16.438	69.25 ± 8.421	84.5 ± 4.726	81.8 ± 8.379
Monocytes (%)	1.2 ± 1.789	4.75 ± 2.872	3 ± 2.000	5 ± 1.225

Mean values of 05 female rats ± Standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Table 11: Complete blood cell count (CBC) at the conclusion of experimental duration with UNIM-001 (Male)

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
RBC Count (mil/ µL)	7.74 ± 0.8692	6.727 ± 0.354	5.19 ± 0.422	6.44 ± 1.33
HB (g/dL)	15.22 ± 1.616	12.952 ± 0.708	10.3 ± 0.583	12.7 ± 2.426
Haematocrit (%)	42.08 ± 5.274	36.875 ± 1.565	28.475 ± 1.875	36.35 ± 7.11
Mean Corpuscular Volume (fL)	54.26 ± 0.978	54.9 ± 2.008	54.875 ± 0.987	56.525 ± 1.921
Mean Corpuscular Haemoglobin (pg)	19.68 ± 0.311	19.225 ± 0.846	19.875 ± 0.842	19.775 ± 0.670
Mean Corpuscular Haemoglobin concentration (g/dL)	36.24 ± 0.757	35.025 ± 2.341	36.175 ± 1.391	34.975 ± 0.377

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Red Cell Distribution Width (%)	14.64 ± 0.983	13.95 ± 1.930	13.575 ± 1.014	14.975 ± 0.838
Platelet Count (thou/µL)	834.4 ± 44.579	430.75 ± 248.08	754.75 ± 104.75	898.5 ± 75.884
Mean platelet volume (fL)	5.84 ± 0.472	6.425 ± 0.419	6.175 ± 0.1708	6.175 ± 0.359
WBC Count(thou/µL)	1.22 ± 0.540	8.65 ± 1.330	17.625 ± 6.901	4.925 ± 0.797
Eosinophils (%)	1 ± 1.00	2 ± 0.000	2 ± 0.000	3 ± 2.000
Segmented Neutrophils (%)	15.8 ± 17.094	26 ± 4.967	32.25 ± 7.762	30.5 ± 11.818
Lymphocytes (%)	81.6 ± 18.33	66.25 ± 10.563	52.25 ± 12.971	61.5 ± 7.895
Monocytes (%)	1.6 ± 3.578	3.25 ± 1.500	13.5 ± 19.807	5 ± 5.292

Mean values of 05 male rats ± Standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Histopathology Reports of Sub-acute Toxicity Studies

At the end of study, the rats were sacrificed and histopathological studies were performed on vital organs.

Liver: In a normal rat, the liver exhibits hexagonal or pentagonal lobules featuring central veins and peripheral hepatic triads or tetrads, ensconced in connective tissue. Hepatocytes form trabeculae radiating from the central vein, separated by sinusoids housing Kupffer cells. These hepatocytes are orderly, characterized by large spheroidal nuclei with distinct nucleoli and peripheral chromatin distribution. Some cells may present with dual nuclei.

Upon administration of UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of body weight, the liver architecture in the control rats remains unaffected. The histological examination reveals a sustained normalcy in the liver structure, indicating no discernible influence from the administered doses of UNIM-001.

Heart: In the Haematoxylin and Eosin (HE) staining, the normal group exhibits well-organized rat myocardial cells with a tidy, compact, and clear

structure. There is a minimal extracellular matrix, and fibroblasts are present in small numbers. The myocardium in the normal group displays meticulously arranged myofibrils within long cylindrical mononucleated cells. Striations in the cytoplasm and elongate nuclei characterize the muscle fibres.

Post-administration of UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg, the sections of the rat heart reveal no irregularities. The myocardial architecture remains unaffected across all three respective doses.

Kidney: The kidney of control rats exhibits a normal structure in both the cortex and medulla. Collecting tubules are lined with relatively low simple cubic epithelium. Cross-sections reveal thick descending and ascending parts of Henle's loops, collecting coils of small calibre, and a limited amount of interstitial tissue.

UNIM-001, administered to Wistar rats at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg in separate groups, does not induce any changes in the normal architecture of the kidney. The renal structure post-administration remains comparable to the control group, indicating the absence of adverse effects.

Stomach: The gastric mucosa, constituting the innermost lining of the stomach, forms the largest and widest portion of the stomach wall. Internally covered by a simple layer of columnar epithelial cells with prominent nuclei, the gastric mucosa is predominantly occupied by gastric glands at the luminal surface of the epithelial lining. These glands, opening singly or in groups with certain apertures, consist of chief (zymogenic) cells at the basal portions, occasional parietal cells, and mucous neck cells along the neck of each gland.

Surface Epithelial (Mucous) Cells: These cells make up the lining epithelium covering the inner surface of the stomach in direct contact with the lumen. They are irregular in shape, often pyramidal, with an ovoid nucleus located basally and surrounded by clear cytoplasmic mass. The apical portions of these cells contain dense discrete granules.

Chief (Zymogenic) Cells: Primarily located at the bases of the gastric glands, these cells are responsible for secreting pro-pepsinogen. They exhibit a conical or pyramidal outline, with a basophilic cytoplasm and basally situated spherical nuclei.

Parietal (Oxyntic) Cells: Known as acid-forming cells, parietal cells are the principal secretors of hydrochloric acid in the stomach. They are scattered

among other cell types, being large in size with a spherical or pyramidal outline, acidophilic cytoplasm, and centrally situated spherical nucleus.

Argentaffin (Enteroendocrine) Cells: Found at the bases of the gastric glands, these small-sized cells are conical or pyramidal in shape. The cytoplasm contains secretory granules, and spherical nuclei are located in the basal regions.

UNIM-001 was administered to Wistar rats in doses of 500, 1000, and 2000 mg/kg in separate groups, as described above. Post-administration, there were no alterations observed in the normal architecture of the stomach, as compared to the control group (as described above).

In the photomicrograph of the stomach wall from the treatment (test) group, animals displayed an intact surface lining epithelium and superficial glands in the stomach mucosa, similar to the control group.

Testes: The evaluation of the testes included an assessment for the presence of haemorrhage, edema, vascular congestion, polymorphonuclear leukocyte infiltration, interstitial fibrosis, basal membrane thickening, Leydig cell proliferation, degeneration of seminiferous epithelium, tubular atrophy, and necrosis.

Results for the Wistar rats in the group where only distilled water was administered revealed normal features in the histology of the testes. The sections displayed a typical arrangement of seminiferous tubules, sperms, and interstitial cells of Leydig.

UNIM-001 was administered to the Wistar rats in doses of 500, 1000, and 2000 mg/kg in separate groups, as described above. Following the administration of UNIM-001, no alterations were observed in the normal architecture of the testes in comparison to the control group (as described above).

Ovary: The normal section of the rat ovary exhibited unilaminar primary follicles, antral follicles, a previously formed corpus luteum with foamy eosinophilic cells, primordial follicles, stromal fibroblasts, and smooth muscle cells. Additionally, the section displayed numerous irregular, tortuous, and wide-lumen endometrial glands, along with an extensive fold of the luminal epithelium. The luminal epithelium consisted of a single layer of columnar epithelial cells with large nuclei at the basal aspect.

The ovary of the control rat maintained a normal architecture, unaffected by the administration of UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of body weight. Photomicrographs of ovary sections from both

the control and treatment group animals exhibited normal lumen endometrial glands and luminal epithelium.

Conclusion

The sub-acute study of UNIM-001 was conducted to assess the safety and toxicity of the test drug. No toxic effects were identified, as evidenced by biochemical parameters, haematological parameters, and histopathological studies. The results confirm that UNIM-001 is safe for oral administration in both sexes of Wistar rats.

Sub-chronic Toxicity Study on UNIM-001 in Wistar Rats

Repeated dose 90-day oral toxicity study of UNIM-001 was carried out in Wistar rats to assess its effects on morphological, gross behaviour, body weight change as well as to find out the haematological, biochemical, and histopathological changes.

Materials & Methods

UNIM-001 was supplied by the Central Council for Research in Unani Medicine, New Delhi. The test substance was suspended in water and administered in the volume not exceeding 2 mL/100 g body weight. Dose levels tested were 0 (control), 500, 1000 and 2000 mg/kg b.w. According to the guidelines, ten female and ten male *Wistar* rats were used in each group. The test substances were administered in three dose levels, i.e. 500, 1000, 2000 mg/kg b.w. Cage-side observations, i.e., change in morphology, behaviour were noted daily and body weights were noted weekly. At the end of study, haematological, biochemical and pathological changes (macroscopic and microscopic) were examined.

Table 12: Brief description of study protocol for UNIM-001

Name of the Study	Sub-chronic Toxicity Study of UNIM-001
Test material	UNIM-001
Animal model	*Wistar* rats
Age	11 – 12 weeks
Weight	180 g (average)
Animal procured from	Central Animal Facility, Jamia Hamdard (173/CPCSEA)
Sex	Male and female
Number of animal per group	10 animals per group
Groups	04
Route of administration	Intra-gastric administration

Name of the Study	Sub-chronic Toxicity Study of UNIM-001
Dose volume	2 mL per 100 g body weight per animal
Number of administration	Once daily for 90 days
Dose levels	500, 1000, 2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	90 days

Observations

The study spanned a duration of 13 weeks. A comprehensive examination of all animals was conducted twice daily to identify signs of morbidity and mortality. This assessment encompassed observations on skin, fur, eyes, mucous membranes, the presence of secretions and excretions, as well as autonomic activity.

Body Weight

Changes in body weight were meticulously calculated and documented throughout the study period. Upon conclusion of the test, surviving animals were weighed and subsequently humanely sacrificed.

Haematology and Clinical Biochemistry

Towards the conclusion of the study, blood samples were obtained just before euthanizing the animals. Haematological and clinical biochemistry determinations were then performed to assess potential toxic effects on blood and tissues, with a specific focus on evaluating impacts on kidney and liver functions.

Results

Table 13: Effect of oral administration of UNIM-001 on body weight and organ weight in female rats

Body & Organ Weight	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Body weight (g)	245 ± 26.874	237.5 ± 28.30	232.5 ± 27.10	220.5 ± 36.39
Heart (g)	0.33 ± 0.038	0.37 ± 0.04	0.33 ± 0.05	0.35 ± 0.019
Left kidney (g)	0.30 ± 0.027	0.38 ± 0.04	0.32 ± 0.05	0.37 ± 0.037
Liver (g)	3.12 ± 0.133	3.45 ± 0.45	3.06 ± 0.49	3.651 ± 0.34
Stomach (g)	0.786 ± 0.100	0.95 ± 0.14	0.76 ± 0.20	0.792 ± 0.131
Ovary (g)	1.55 ± 0.259	0.57 ± 0.20	0.54 ± 0.21	0.469 ± 0.112

Mean values of 10 female rats ± Standard deviation; No significant difference was observed in any parameters; Dunnett's test. Control group was given water.

Table 14: Effect of oral administration of UNIM-001 on body weight and organ weight in male rats

Body & Organ Weight	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Body weight (g)	271.5 ± 25.06	261 ± 33.73	286.5 ± 36.29	262 ± 43.34
Heart (g)	0.34 ± 0.05	0.28 ± .04	0.37 ± 0.06	0.34 ± 0.06
Left kidney (g)	0.33 ± 0.04	0.32 ± 0.04	0.38 ± 0.07	0.34 ± 0.05
Liver (g)	3.19 ± 0.38	2.70 ± 0.32	3.38 ± 0.40	2.84 ± 0.54
Stomach (g)	0.83 ± 0.27	0.86 ± 0.14	0.60 ± 0.16	0.64 ± 0.19
Testes (g)	1.80 ± 0.18	1.65 ± 0.24	1.38 ± 0.25	1.87 ± 0.28

Mean values of 10 male rats ± standard deviation; No significant difference was observed in any parameters; Dunnett's test. Control group was given water.

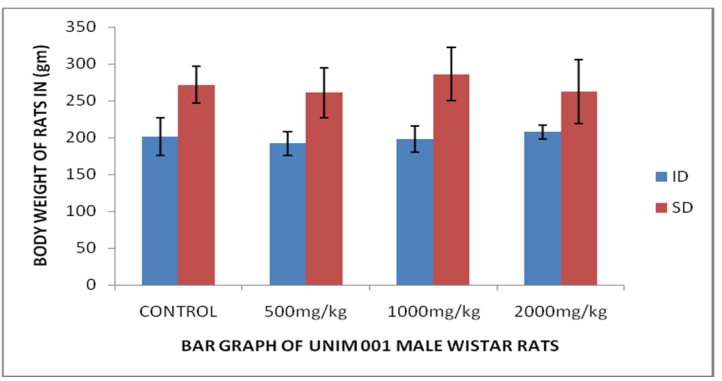


Figure 1: *Change in body weight of male rats*

Comparison of the body weight of Albino *Wistar* rats from the date of starting and termination of the experimental schedules. Body weights are shown as mean value ± standard deviation; ID = Initial date of experiment, SD = Sacrificed date of experiment (male group)

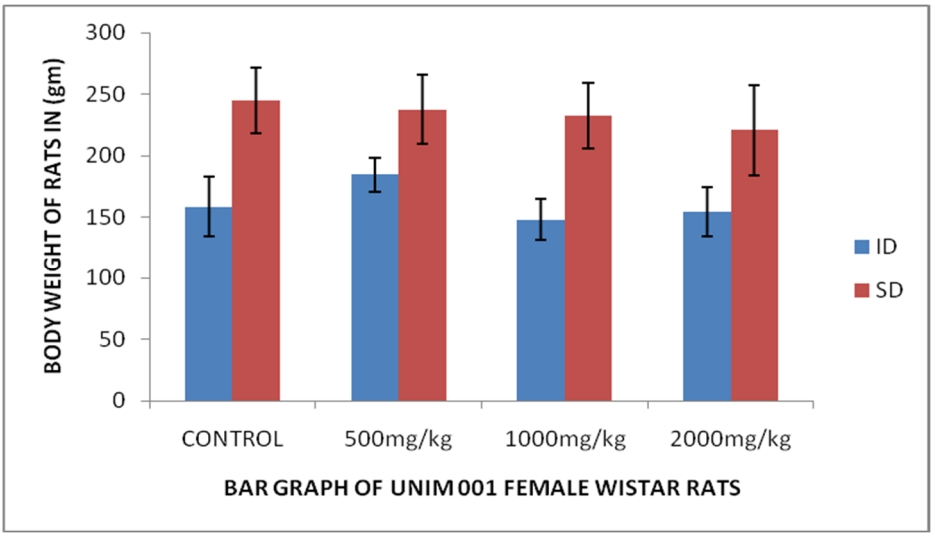


Figure 2: *Change in body weight of female rats*

Comparison of the body weight of Albino *Wistar* rats from the date of starting and termination of the experimental schedules. Body weights are shown as mean value ± standard deviation; ID = Initial date of experiment, SD = Sacrificed date of experiment (female group)

Table 15: Effect of oral administration of UNIM-001 on biochemical parameters in female rats

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
ALT (IU/L)	21.79 ± 5.65	123.77 ± 144.77	140.83 ± 145.72*	146.85 ± 174.03*
AST (IU/L)	65.06 ± 10.82	71.39 ± 40.87	59.91 ± 18.81	71.90 ± 13.77
BUN (mg/dl)	18.41 ± 9.39	18.94 ± 12.76	17.15 ± 12.91	23.76 ± 10.17
CRE (mg/dl)	0.82 ± 0.28	1.01 ± 0.29	1.26 ± 0.21	1.42 ± 0.30

Mean values of 10 female rats ± Standard Deviation; *p<0.05vs. control group (Dunnett's test). Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 16: Effect of oral administration of UNIM-001 on biochemical parameters in male rats

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
ALT (IU/L)	23.76 ± 6.63	112.9 ± 136.08	122.55 ± 153.84	109.45 ± 115.80
AST (IU/L)	57.51 ± 9.55	58.16 ± 17.87	58.09 ± 11.25	72.52 ± 11.04
BUN (mg/dl)	17.45 ± 18.56	15.71 ± 7.33	13.21 ± 6.44	19.72 ± 6.25
CRE (mg/dl)	0.91 ± 0.27	0.96 ± 0.24	1.24 ± 0.24	1.43 ± 0.36

Mean values of 10 male rats ± Standard deviation; No significant difference was observed in any parameters; Dunnett's test. Control group given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 17: Complete blood cell count (CBC) at the conclusion of experimental duration with UNIM-001 in female rats

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
RBC Count (mil/ µL)	6.441 ± 0.913	7.20 ± 0.708	6.146 ± 0.593	6.293 ± 0.231
HB (g/dL)	11.7 ± 1.25	14.12 ± 1.52	11.48 ± 1.026	12.21 ± 0.566
Haematocrit (%)	37.55 ± 4.709	45.36 ± 4.76	36.51 ± 3.438	39.14 ± 2.312
Mean Corpuscular Volume (fL)	58.46 ± 1.194	62.9 ± 1.380	59.5 ± 3.067	62.18 ± 2.014
Mean Corpuscular Haemoglobin (pg)	18.33 ± 2.07	19.53 ± 0.374	18.71 ± 0.566	19.41 ± 0.680
Mean Corpuscular Haemoglobin Concentration (g/dL)	31.37 ± 3.33	31.08 ± 0.345	31.49 ± 0.691	31.26 ± 1.203

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Red Cell Distribution Width (%)	11.73 ± 4.07	15.04 ± 1.190	15.08 ± 0.405	14.87 ± 0.711
Platelet Count (thou/µL)	426.87 ± 109.02	543.3 ± 54.73	654.9 ± 174.41	474.1 ± 247.60
Mean platelet volume (fL)	5.96 ± 0.232	6.61 ± 0.826	5.98 ± 0.405	5.88 ± 0.209
WBC Count (thou/µL)	3.062 ± 1.78	4.73 ± 1.138	8.68 ± 9.281	7.38 ± 4.769
Segmented Neutrophils (%)	47.25 ± 29.090	29.9 ± 16.670	33.8 ± 21.390	26 ± 14.780
Lymphocytes (%)	30 ± 20.438	65.9 ± 17.842	63.3 ± 21.172	70.9 ± 15.466
Monocytes (%)	4.875 ± 4.422	3.5 ± 2.173	2.1 ± 1.197	2.5 ± 1.780

Mean values of 10 female rats ± Standard deviation; No significant difference was observed in any parameters; Dunnett's test. Control group was given water.

Table 18: Complete blood cell count (CBC) at the conclusion of experimental duration with UNIM-001 in male rats

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
RBC Count (mil/ µL)	7.12 ± 0.274	7.79 ± 0.461	6.837 ± 0.521	7.198 ± 0.614
HB (g/dL)	12.857 ± 0.528	14.51 ± 0.817	12.487 ± 0.596	13.05 ± 1.011
Haematocrit (%)	38.54 ± 1.152	44 ± 3.741	36.875 ± 2.375	39.762 ± 2.766
Mean Corpuscular Volume (fL)	53.457 ± 2.074	56.571 ± 3.590	53.962 ± 0.918	55.325 ± 1.93
Mean Corpuscular Haemoglobin (pg)	17.857 ± 0.713	18.671 ± 0.502	18.25 ± 0.819	18.2 ± 0.960

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Mean Corpuscular Haemoglobin Concentration (g/dL)	33.385 ± 0.926	33.014 ± 1.384	33.86 ± 1.286	32.912 ± 0.83
Red Cell Distribution Width (%)	15.68 ± 0.79	12.87 ± 0.49	15.4 ± 0.41	15.33 ± 1.99
Platelet Count (thou/µL)	636.01 ± 324.40	751.14 ± 144.93	724.5 ± 86.01	878.87 ± 91.59
Mean Platelet Volume (fL)	6.00 ± 0.20	6.12 ± 0.48	6.56 ± 0.26	6.06 ± 0.32
WBC Count (thou/µ)	3.05 ± 1.05	8.52 ± 3.77	7.35 ± 1.34	8.51 ± 4.20
Segmented Neutrophils (%)	20.42 ± 16.67	14.42 ± 12.86	15.37 ± 11.68	11.75 ± 12.62
Lymphocytes (%)	56.14 ± 35.26	82.57 ± 12.19	83.13 ± 13.314	85.87 ± 14.49
Monocytes (%)	1.42 ± 2.07	2.71 ± 2.13	1.00 ± 1.14	2.12 ± 2.35

Mean values of 10 male rats ± Standard Deviation; No significant difference was observed in any parameters; Dunnett's test. Control group was given water.

Histopathology Reports of Sub-chronic Toxicity Study

At the conclusion of the study, the rats were euthanized, and histopathological examinations of vital organs were conducted.

Liver: The liver in the control group exhibited a typical hexagonal or pentagonal lobular structure with central veins and peripheral hepatic triads or tetrads. Hepatocytes were arranged in trabeculae radiating from the central vein, and Kupffer cells were present in sinusoids. The architecture remained unchanged even with the administration of UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg.

Heart: In the normal group, myocardial cells displayed a well-organized structure with clear cytoplasm, less extracellular matrix, and elongated

nuclei. The myocardium retained its normal features after the administration of UNIM-001 at doses of 500, 1000, and 2000 mg/kg

Kidney: The kidney in the control rats showed a normal cortex and medulla structure, with collecting tubules lined by low simple cubic epithelium. Following UNIM-001 administration at various doses, the kidney's normal architecture remained unaffected.

Stomach: The gastric mucosa, which constitutes the innermost lining of the stomach, forms the largest and widest portion of the stomach wall. Internally covered by a simple layer of columnar epithelial cells with prominent nuclei, the gastric mucosa is predominantly occupied by gastric glands at the luminal surface of the epithelial lining. These glands, opening singly or in groups with certain apertures, consist of chief (zymogenic) cells at the basal portions, occasional parietal cells, and mucous neck cells along the neck of each gland.

Surface Epithelial (Mucous) Cells: These cells make up the lining epithelium covering the inner surface of the stomach in direct contact with the lumen. They are irregular in shape, often pyramidal, with an ovoid nucleus located basally and surrounded by clear cytoplasmic mass. The apical portions of these cells contain dense discrete granules.

Chief (Zymogenic) Cells: Primarily located at the bases of the gastric glands, these cells are responsible for secreting pro-pepsinogen. They exhibit a conical or pyramidal outline, with a basophilic cytoplasm and basally situated spherical nuclei.

Parietal (Oxyntic) Cells: Known as acid-forming cells, parietal cells are the principal secretors of hydrochloric acid in the stomach. They are scattered among other cell types, being large in size with a spherical or pyramidal outline, acidophilic cytoplasm, and centrally situated spherical nucleus.

Argentaffin (Enteroendocrine) Cells: Found at the bases of the gastric glands, these small-sized cells are conical or pyramidal in shape. The cytoplasm contains secretory granules, and spherical nuclei are located in the basal regions.

UNIM 001 was administered to Wistar rats in doses of 500, 1000, and 2000 mg/kg in separate groups, as described above. Post-administration, there were no alterations observed in the normal architecture of the stomach, as compared to the control group (as described above).

In the photomicrograph of the stomach wall from the treatment (test) group, animals displayed an intact surface lining epithelium and superficial glands in the stomach mucosa, similar to the control group.

Testes: The evaluation of the testes included an assessment for the presence of haemorrhage, edema, vascular congestion, polymorphonuclear leukocyte infiltration, interstitial fibrosis, basal membrane thickening, Leydig cell proliferation, degeneration of seminiferous epithelium, tubular atrophy, and necrosis.

Results for the Wistar rats in the group where only distilled water was administered revealed normal features in the histology of the testes. The sections displayed a typical arrangement of seminiferous tubules, sperms, and interstitial cells of Leydig.

UNIM-001 was administered to the Wistar rats in doses of 500, 1000, and 2000 mg/kg in separate groups, as described above. Following the administration of UNIM 001, no alterations were observed in the normal architecture of the testes in comparison to the control group (as described above).

Ovary: The normal section of the rat ovary exhibited unilaminar primary follicles, antral follicles, a previously formed corpus luteum with foamy eosinophilic cells, primordial follicles, stromal fibroblasts, and smooth muscle cells. Additionally, the section displayed numerous irregular, tortuous, and wide-lumen endometrial glands, along with an extensive fold of the luminal epithelium. The luminal epithelium consisted of a single layer of columnar epithelial cells with large nuclei at the basal aspect.

The ovary of the control rat maintained a normal architecture, unaffected by the administration of UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of body weight. Photomicrographs of ovary sections from both the control and treatment group animals exhibited normal lumen endometrial glands and luminal epithelium.

Conclusion

The sub-chronic toxicity study of UNIM-001 demonstrated its safety for oral administration in Wistar rats. No toxic effects were observed based on biochemical parameters, haematological parameters, and histopathological examinations, affirming the safety profile of UNIM-001 in both male and female rats.

Chronic Toxicity Study on UNIM-001 in Wistar Rats

A 6-month repeated dose oral toxicity study of UNIM-001 was conducted in Wistar rats to assess its impact on morphological aspects, gross behaviour, body weight changes, as well as haematological, biochemical, blood clotting factors, and histopathological alterations.

Material & Methods

UNIM-001, supplied by the Central Council for Research in Unani Medicine, New Delhi, was suspended in water and administered at three dose levels: 500, 1000, and 2000 mg/kg body weight. The study included ten female and ten male rats for the 500 mg/kg and 1000 mg/kg doses, and twenty male and twenty female rats for the 2000 mg/kg dose. Daily administration for 180 days via intra-gastric intubation was conducted. General observations, body weight changes, haematological, biochemical, blood clotting factors, and pathological examinations were performed at the end of the study.

Table 19: Brief description of study protocol for UNIM-001

Name of the study	Chronic toxicity study of UNIM-001
Test material	UNIM-001
Animal model	Wistar rats
Age	11–12 weeks
Weight	200–180 g (average)
Animal procured from	Central animal facility, Jamia Hamdard (173/CPCSEA)
Sex	Male and female
Number of animal per group	10 per group (control, 500mg/kg, 1000mg/kg) 20 per group (2000 mg/kg)
Groups	04
Route of administration	Intra-gastric administration
Dose volume	2 mL per 100 g body weight per animal
Number of administration	Once daily for 180 days
Dose levels	500, 1000, 2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	180 days

Observations

During the 180-day study period, daily general clinical observations included monitoring for signs of morbidity and mortality, changes in skin, fur, eyes, mucous membranes, secretions, excretions, and autonomic activity.

Body Weight

Body weight changes were calculated and recorded, and surviving animals were weighed and humanely sacrificed.

Haematology and Clinical Biochemistry

Blood samples collected at the end of the study allowed for haematological and clinical biochemistry determinations to identify major toxic effects on blood and tissues, with a focus on kidney and liver function.

Results

Table 20: Effect of oral administration of UNIM-001 on body weight and organ weight in female rats

Body & Organ Weight	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Body weight (g)	219.44 ± 15.50	209.28 ± 8.86	211.66 ± 11.25	207.72 ± 21.72
Heart (g)	0.303 ± 0.02	0.319 ± 0.01	0.305 ± 0.044	0.338 ± 0.037
kidney (g)	0.274 ± 0.03	0.287 ± 0.015	0.304 ± 0.038	0.291 ± 0.038
Liver (g)	2.540 ± 0.48	2.484 ± 0.403	1.947 ± 0.374	2.367 ± 0.563
Stomach (g)	0.692 ± 0.11	1.046 ± 0.186	0.838 ± 0.096	0.650 ± 0.095
Ovary (g)	0.114 ± 0.03	0.118 ± 0.043	0.125 ± 0.023	0.141 ± 0.035

Parameters are shown as mean ± Standard deviation; No significant difference was observed in any parameters; Dunnett's test. All treatment groups contain 10 animals and highest dose treated group contains 20 animals. Control group was given water.

Table 21: Effect of oral administration of UNIM-001 on body weight and organ weight in male rats

Body & Organ Weight	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
Body weight (g)	293.75 ± 25.17	267.85 ± 34.01	271.42 ± 44.32	270 ± 35.64
Heart (g)	0.345 ± 0.079	0.325 ± 0.046	0.46 ± 0.07	0.368 ± 0.079
Kidney (g)	0.314 ± 0.057	0.340 ± 0.051	0.479 ± 0.192	0.361 ± 0.73
Liver (g)	2.979 ± 0.335	2.805 ± 0.429	3.420 ± 0.77	2.90 ± 0.35
Stomach (g)	0.671 ± 0.228	0.790 ± 0.160	0.643 ± 0.106	0.616 ± 0.11
Testes (g)	1.658 ± 0.400	1.953 ± 0.272	1.732 ± 0.399	1.806 ± 0.349

Parameters are shown as mean ± Standard deviation; No significant difference was observed in any parameters; Dunnett's test. All treatment groups contain 10 animals and highest treated group contain 20 animals. Control group was given water.

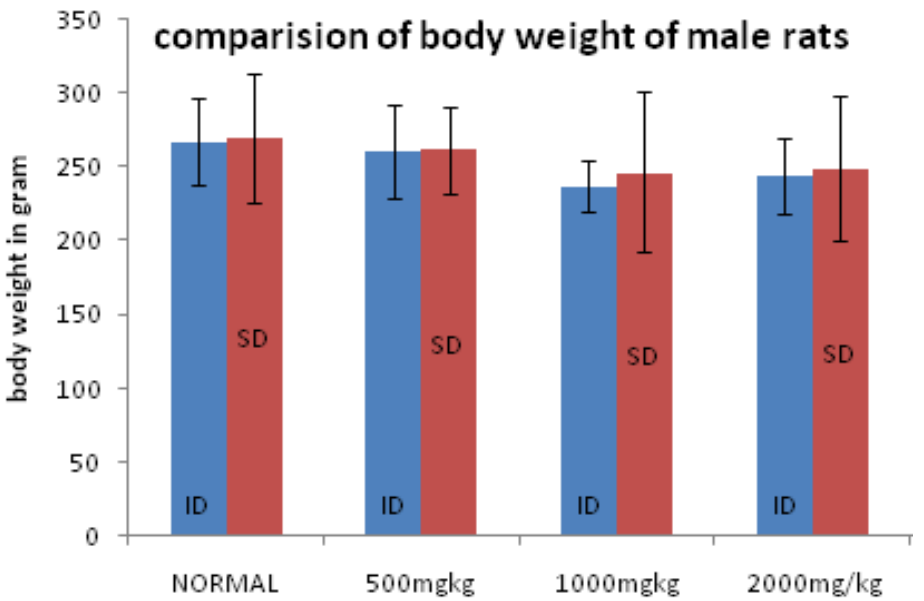


Figure 3: *Change in body weight of male rats*

Comparison of the body weight of Albino *Wistar* rats from the date of starting and termination of the experimental schedules. Body weights are shown as mean value ± standard deviation; ID = Initial date of experiment, SD = Sacrificed date of experiment (male group)

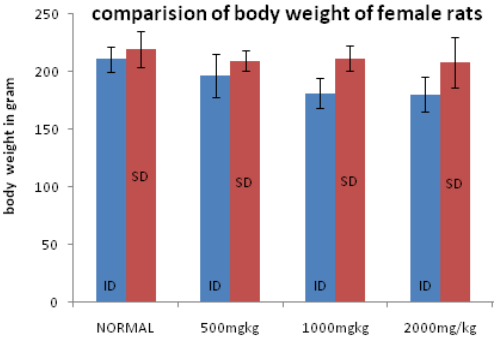


Figure 4*: Change in body weight of female rats*

Comparison of the body weight of Albino *Wistar* rats from the date of starting and termination of the experimental schedules. Body weights are shown as mean value ± standard deviation; ID = Initial date of experiment, SD = Sacrificed date of experiment (Female Group)

Table 22: Mortality detail of male and female rats during experimental schedule

Gender	Control	UNIM-001 500 (mg/kg)	UNIM-001 1000 (mg/kg)	UNIM-001 2000 (mg/kg)
Male	2/10	3/10	3/10	7/20
Female	1/10	3/10	4/10	7/20

Table 23: Effect of oral administration of UNIM-001 on biochemical parameters in female rats

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
ALT (IU/L)	30.09 ± 10.03	29.10 ± 5.30	26.16 ± 2.92	22.27 ± 5.07
AST (IU/L)	49.84 ± 14.32	39.49 ± 8.95*	43.54 ± 8.57	41.14 ± 8.34
BUN (mg/dl)	17.80 ± 5.25	17.36 ± 3.64	17.89 ± 4.85	17.06 ± 3.37
CRE (mg/dl)	1.27 ± 0.57	1.72 ± 1.07	0.96 ± 0.57	0.88 ± 0.39

Parameters are shown as mean ± Standard deviation; *P < 0.05 vs control group (Dunnett's test). All treatment groups contain 10 animals and highest dose treated group contains 20 animals. Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 24: Effect of oral administration of UNIM-001 on biochemical parameters in male rats

Parameters	Control	UNIM-001 500 (mg/kg)	UNIM-001 1000 (mg/kg)	UNIM-001 2000 (mg/kg)
ALT (IU/L)	28.158 ± 3.112	27.782 ± 2.832	33.391 ± 4.492	32.267 ± 3.002
AST (IU/L)	35.547 ± 3.121	34.768 ± 4.410	41.839 ± 3.471	38.856 ± 2.621
BUN (mg/dl)	16.211 ± 1.059	17.781 ± 1.488	20.971 ± 2.424	23.339 ± 1.208
CRE (mg/dl)	0.7986 ± 0.131	0.7399 ± 0.1678	0.7052 ± 0.1324	0.7623 ± 0.1025

Parameters are shown as mean ± Standard deviation; No significant difference was observed in any parameters; Dunnett's test. All treatment groups contain 10 animals and highest dose treated group contains 20 animals. Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 25: Complete blood cell count (CBC) in female rats at the conclusion of experimental duration with UNIM-001

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
RBC Count (mil/ cumm)	7.06 ± 0.63	7.28 ± 0.40	7.35 ± 1.32	6.98 ± 0.73
Hb (gm/dl)	11.94 ± 1.09	12.64 ± 0.62	12.31 ± 1.85	11.48 ± 1.11
Mean Corpuscular Volume (fL)	61.30 ± 2.33	61.57 ± 2.03	62.07 ± 3.38	61.95 ± 2.28
Mean Corpuscular Haemoglobin (pg)	16.88 ± 0.37	16.80 ± 0.36	16.76 ± 0.59	16.34 ± 0.81
Mean Corpuscular Haemoglobin Concentration (%)	27.56 ± 1.04	27.27 ± 0.74	27.02 ± 0.87	26.38 ± 0.81
Platelet Count (La./cumm)	8.04 ± 2.30	5.18 ± 1.30	7.44 ± 1.45	8.46 ± 0.82
PCV (%)	43.43 ± 4.80	45.33 ± 4.12	45.51 ± 6.45	48.06 ± 6.67

WBC Count (cells/cumm)	4611.11 ± 1238.4	6737.5 ± 1634.4	6250 ± 2840.5	4550 ± 1571.8
Polymorph (%)	36.22 ± 7.69	45.5 ± 19.16	44.25 ± 44.25	39.18 ± 10.42
Lymphocytes (%)	61.77.12	56.28 ± 16.71	53 ± 20.51	60.86 ± 6.56

Parameters are shown as mean ± Standard deviation; No significant difference was observed in any parameters; Dunnett's test. All treatment groups contain 10 animals and highest dose treated group contains 20 animals. Control group was given water.

Table 26: Complete blood cells count (CBC) in male rats at the conclusion of experimental duration with UNIM-001

Parameters	Control	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
RBC Count (mil/ cumm)	7.75 ± 0.0755	6.78 ± 0.08	7.714 ± 0.07	6.39 ± 2.1
Hb (gm/dl)	13.4 ± 0.28	10.686 ± 0.37	12.714 ± 0.25	9.854 ± 1.12
Mean Corpuscular Volume (fL)	60.313 ± 0.711	57.614 ± 0.87	57.77 ± 0.99	64.70 ± 1.63
Mean Corpuscular Haemoglobin (pg)	17.38 ± 0.29	15.7 ± 0.59	16.414 ± 0.206	16.946 ± 0.37
Mean Corpuscular Haemoglobin Concentration (%)	28.43 ± 0.2755	27.27 ± 0.307	28.2 ± 0.19	26.185 ± 0.45
Platelet Count (La./cumm)	8.390 ± 0.2	7.9 ± 0.43	9.14 ± 0.3	7.99 ± 0.46
PCV (%)	46.9 ± 0.9	35.04 ± 4.8	44.67 ± 1.18	37.108 ± 3.73
TLC (cells/cumm)	9641 ± 422	7542.9 ± 684	8614.3 ± 369	6838 ± 376
Polymorph (%)	19.7 ± 2.3	39.85 ± 3.18	22.42 ± 0.48	37 ± 4.306
Lymphocytes (%)	65.5 ± 2.27	57.7 ± 3.3	75.28 ± 0.47	59.46 ± 4.289

Parameters are shown as mean ± Standard deviation; No significant difference was observed in any parameters; Dunnett's test. All treatment groups contain 10 animals and highest treated group contain 20 animals. Control group was given water.

Table 27: Effect of oral administration of UNIM-001 on blood clotting factors in male rats

Clotting Factors	Normal	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
PT (sec)	9.91 ± 2.16	8.87 ± 2.26	8.85 ± 1.66	8.92 ± 2.48
APTT (sec)	15.83 ± 2.70	17.95 ± 3.24	18.92 ± 5.46	18.95 ± 3.53

Parameters are shown as mean ± standard deviation; No significant difference was observed in any parameters; Dunnett's test. All treatment groups contain 10 animals and highest treated group contain 20 animals. Control group was given water.

Table 28: Effect of oral administration of UNIM-001 in blood clotting factors in female rats

Clotting Factors	Normal	UNIM-001 (500 mg/kg)	UNIM-001 (1000 mg/kg)	UNIM-001 (2000 mg/kg)
PT (sec)	18.51 ± 4.68	18.94 ± 2.93	16.8 ± 4.51	19.94 ± 4.86
APTT (sec)	7.5 ± 1.26	8.72 ± 1.62	7.68 ± 1.29	8.03 ± 1.34

Parameters are shown as mean ± standard deviation; No significant difference was observed in any parameters; Dunnett's test. All treatment groups contain 10 animals and highest treated group contain 20 animals. Control group was given water.

Histopathology Reports of Chronic Toxicity Study

At the conclusion of the study, the rats were euthanized, and histopathological examinations of vital organs were conducted.

Liver: The liver in the control group exhibited a typical hexagonal or pentagonal lobular structure with central veins and peripheral hepatic triads or tetrads. Hepatocytes were arranged in trabeculae radiating from the central vein, and Kupffer cells were present in sinusoids. The architecture remained unchanged even with the administration of UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg.

Heart: In the HE staining, the normal group exhibits well-organized rat myocardial cells with a tidy, compact, and clear structure. There is a minimal extracellular matrix, and fibroblasts are present in small numbers. The

myocardium in the normal group displays meticulously arranged myofibrils within long cylindrical mononucleated cells. Striations in the cytoplasm and elongate nuclei characterize the muscle fibres.

Post-administration of UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg, the sections of the rat heart reveal no irregularities. The myocardial architecture remains unaffected across all three respective doses.

Kidney: The kidney of control rats exhibits a normal structure in both the cortex and medulla. Collecting tubules are lined with relatively low simple cubic epithelium. Cross-sections reveal thick descending and ascending parts of Henle's loops, collecting coils of small calibre, and a limited amount of interstitial tissue.

UNIM-001, administered to Wistar rats at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg in separate groups, does not induce any changes in the normal architecture of the kidney. The renal structure post-administration remains comparable to the control group, indicating the absence of adverse effects.

Stomach: The gastric mucosa, constituting the innermost lining of the stomach, forms the largest and widest portion of the stomach wall. Internally covered by a simple layer of columnar epithelial cells with prominent nuclei, the gastric mucosa is predominantly occupied by gastric glands at the luminal surface of the epithelial lining. These glands, opening singly or in groups with certain apertures, consist of chief (zymogenic) cells at the basal portions, occasional parietal cells, and mucous neck cells along the neck of each gland.

Surface Epithelial (Mucous) Cells: These cells make up the lining epithelium covering the inner surface of the stomach in direct contact with the lumen. They are irregular in shape, often pyramidal, with an ovoid nucleus located basally and surrounded by clear cytoplasmic mass. The apical portions of these cells contain dense discrete granules.

Chief (Zymogenic) Cells: Primarily located at the bases of the gastric glands, these cells are responsible for secreting pro-pepsinogen. They exhibit a conical or pyramidal outline, with a basophilic cytoplasm and basally situated spherical nuclei.

Parietal (Oxyntic) Cells: Known as acid-forming cells, parietal cells are the principal secretors of hydrochloric acid in the stomach. They are scattered among other cell types, being large in size with a spherical or pyramidal outline, acidophilic cytoplasm, and centrally situated spherical nucleus.

Argentaffin (Enteroendocrine) Cells: Found at the bases of the gastric glands, these small-sized cells are conical or pyramidal in shape. The cytoplasm contains secretory granules, and spherical nuclei are located in the basal regions.

UNIM-001 was administered to Wistar rats in doses of 500, 1000, and 2000 mg/kg in separate groups, as described above. Post-administration, there were no alterations observed in the normal architecture of the stomach, as compared to the control group (as described above).

In the photomicrograph of the stomach wall from the treatment (test) group, animals displayed an intact surface lining epithelium and superficial glands in the stomach mucosa, similar to the control group.

Testes: The evaluation of the testes included an assessment for the presence of haemorrhage, edema, vascular congestion, polymorphonuclear leukocyte infiltration, interstitial fibrosis, basal membrane thickening, Leydig cell proliferation, degeneration of seminiferous epithelium, tubular atrophy, and necrosis.

Results for the Wistar rats in the group where only distilled water was administered revealed normal features in the histology of the testes. The sections displayed a typical arrangement of seminiferous tubules, sperms, and interstitial cells of Leydig.

UNIM-001 was administered to the Wistar rats in doses of 500, 1000, and 2000 mg/kg in separate groups, as described above. Following the administration of UNIM-001, no alterations were observed in the normal architecture of the testes in comparison to the control group (as described above).

Ovary: The normal section of the rat ovary exhibited unilaminar primary follicles, antral follicles, a previously formed corpus luteum with foamy eosinophilic cells, primordial follicles, stromal fibroblasts, and smooth muscle cells. Additionally, the section displayed numerous irregular, tortuous, and wide-lumen endometrial glands, along with an extensive fold of the luminal epithelium. The luminal epithelium consisted of a single layer of columnar epithelial cells with large nuclei at the basal aspect.

The ovary of the control rat maintained a normal architecture, unaffected by the administration of UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of body weight. Photomicrographs of ovary sections from both

the control and treatment group animals exhibited normal lumen endometrial glands and luminal epithelium.

Conclusion

The chronic toxicity study of UNIM-001 was conducted to assess the safety and toxicity of the test drug. No toxic effects were identified, as evidenced by biochemical parameters, haematological parameters, and histopathological studies. The results confirm that UNIM-001 is safe for oral administration in both sexes of Wistar rats.

Acute Toxicity Study of Hydroalcoholic Extract of UNIM-001 in Wistar Rats

An acute toxicity study was conducted on the hydroalcoholic extract of UNIM-001 in Albino Wistar rats to evaluate its impact on morphological aspects, gross behaviour, and body weight changes.

Materials & Methods

The test material, UNIM-001 (supplied by the CCRUM), underwent drying and grinding, followed by extraction in a Soxhlet apparatus using a hydroalcoholic solution (20:80). The resulting hydroalcoholic extract (HA-UNIM-001) was concentrated to obtain a dark viscous residue. For animal treatment, the extract was suspended in distilled water. Ten female Albino Wistar rats were divided into two groups, with dose levels tested at 0 (control) and 2000 mg/kg body weight. A single dose of HA-UNIM-001 was administered via intra-gastric intubation for 14 days.

Table 29: Brief description of study protocol for HA-UNIM-001

Name of the Study	Acute Toxicity Study of HA-UNIM-001
Test material	HA-UNIM-001
Animal model	Albino Wistar rats
Age	11-12 weeks
Weight	180 ± 20 g
Animal procured from	Central animal facility, Jamia Hamdard (173/CPCSEA)
Sex	Female
Number of animal per group	05 per group
Groups	2 groups
Route of administration	Intra-gastric administration

Name of the Study	Acute Toxicity Study of HA-UNIM-001
Dose volume	Not more than 2 mL/100 g body weight per animal
Number of administration	Single dose
Concentration of dose	2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	14 days

Observations

The observation period of 14 days involved daily inspection for morbidity and mortality, focusing on changes in skin, fur, eyes, mucous membranes, secretions, and excretions.

Body Weight

Body weights were recorded before dosing and on the 7th and 14th days. Macroscopic observations were made on vital organs of sacrificed animals. Both total body weight and the weight of specific organs, including the heart, stomach, liver, and left kidney, were statistically analyzed in comparison to the normal control group. No significant differences were observed in the total body weights between the control and HA-UNIM-001 groups. Additionally, the weights of specific organs in the control group did not exhibit any significant variations compared to the HA-UNIM-001 group.

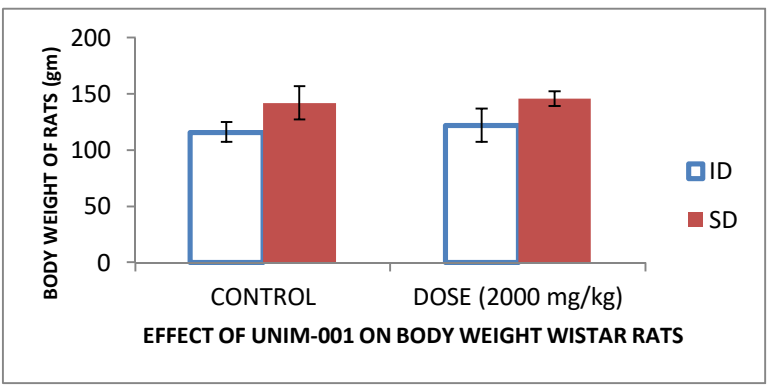


Figure 5: *Change in body weight of Wistar rats*

Comparison of Wistar rat body weights from the experiment's start to termination, presented as mean value ± standard deviation. ID = Initial date of the experiment, SD = Sacrificed date of the experiment.

Results

Prior to dosing (day 0) and weekly thereafter, individual animal body weights were measured. At the end of the 14-day observation period, all animals underwent necropsy. No visible signs of toxicity, such as changes in respiratory, circulatory, autonomic, and central nervous systems or behavioural patterns, were observed. No mortality occurred among the animals.

Body weight measurements in the test drug-treated animals showed an increasing trend. Gross pathology examination at the 14-day observation's conclusion revealed no lesions attributable to the test substance's toxicity.

Conclusion

The acute oral toxicity study, conducted with female Wistar rats, aimed to assess the potential of HA-UNIM-001 to induce toxicity from a single oral dose. The results indicate no toxic effects, supporting the safety of HA-UNIM-001 under the tested conditions.

Sub-acute Toxicity Study of Hydroalcoholic Extract of UNIM-001 in Wistar Rats

A repeated dose 28-day oral toxicity study of HA-UNIM-001 was conducted in Wistar rats to evaluate its impact on morphological characteristics, gross behaviour, body weight changes, and to investigate haematological, biochemical, and histopathological alterations.

Materials & Methods

The test material, UNIM-001, provided by the CCRUM, underwent drying and grinding to a coarse powder. Subsequently, it was extracted in a Soxhlet apparatus using a hydroalcoholic solution (20:80). The hydroalcoholic solution was concentrated, yielding a dark viscous residue named HA-UNIM-001. For animal treatment, the hydroalcoholic extract was suspended in distilled water.

Five female and five male Wistar rats were included in the study. Dose levels tested were 0 (control), 500, 1000, and 2000 mg/kg body weight. The animals were administered the test substance daily for seven days each week over a period of 28 days. Dosages were adjusted according to the body weight of the animals and administered via gavage using a suitable intubation cannula in a single dose.

Cage-side observations, including changes in morphology and behaviour, were recorded daily, and body weight was noted weekly. At the conclusion of the study on the 29th day, haematological, biochemical, and pathological changes (both macroscopic and microscopic) were examined.

Table 30: Brief description of study protocol for HA-UNIM-001

Test Material	HA-UNIM-001
Animal model	Wistar rats
Age	11–12 weeks
Weight	180 g (average)
Animals procured from	Central Animal Facility, Jamia Hamdard (173/CPCSEA)
Sex	Male and female
Number of animals per group	05 per sex per group
Groups	04
Route of administration	Intra-gastric administration
Dose volume	Not more than 2 mL per 100 g body weight per animal
Number of administration	Once daily for 28-days
Dose levels	500, 1000, 2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	28 days

Observations

During the study period, which spanned 28 days, all animals underwent thorough inspection for signs of morbidity and mortality. This included a meticulous examination of skin, fur, eyes, mucous membranes, as well as the monitoring of secretions, excretions, and autonomic activity. Observations were conducted twice daily to ensure comprehensive monitoring.

Body Weight

Weight changes were diligently calculated and recorded throughout the study. At the conclusion of the test, surviving animals were weighed and subsequently humanely sacrificed.

Haematology and Clinical Biochemistry

Blood samples were collected just before euthanizing the animals at the end of the test period. Haematological and clinical biochemistry determinations

were conducted to assess major toxic effects in both blood and tissues, with a specific focus on evaluating the impact on kidney and liver functions.

Results

Effect of Oral Administration of HA-UNIM-001 on Body Weight and Organ Weight in Female Rats: The administration of HA-UNIM-001 at doses of 500, 1000, and 2000 mg/kg to female rats exhibited significant (**P < 0.01) decreases in the total weight of animals compared to the control group. However, internal organ weights, including the stomach, liver, heart, and left kidney, at different doses of HA-UNIM-001 showed non-significant differences compared to the control group administered with water only.

Effect of Oral Administration of HA-UNIM-001 on Body Weight and Organ Weight in Male Rats: The total body weight of HA-UNIM-001 showed significant (**P < 0.01) differences at 500 mg/kg and 1000 mg/kg. At a higher dose of 2000 mg/kg, it also showed significant (*P < 0.05) differences compared to the control group. Additionally, internal organs, mainly the stomach, liver, heart, and left kidney at doses of 500, 1000, and 2000 mg/kg, showed non-significant differences compared to the control group administered with water only.

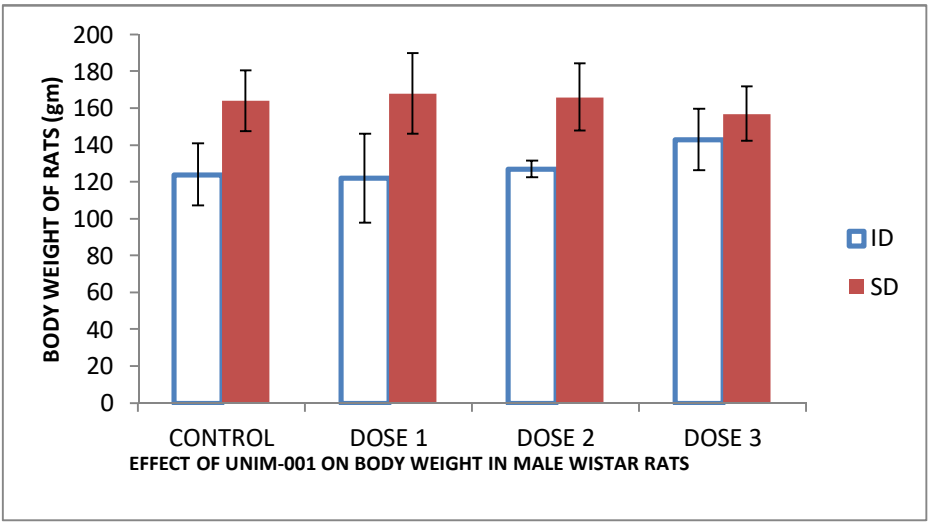


Figure 6: *Change in body weight of the rats (male)*

The body weights of albino Wistar rats were assessed at the initiation (ID) and termination (SD) of the experimental schedules. Body weights are presented as mean values ± standard deviation.

Table 31: Effect of oral administration of HA-UNIM-001 on biochemical parameters in female rats

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
ALT (IU/L)	21.10 ± 6.58	20.75 ± 7.78	25.31 ± 13.90	32.31 ± 12.93
AST (IU/L)	56.75 ± 13.25	28.57 ± 11.37*	21.46 ± 5.17*	32.24 ± 12.03
BUN (mg/dl)	31.25 ± 3.97	30.19 ± 5.79	30.86 ± 4.54	34.84 ± 8.02
CRE (mg/dl)	0.45 ± 0.27*	0.79 ± 0.34	0.76 ± 0.39	0.81 ± 0.21

Mean values of 05 female rats ± standard deviation; *p < 0.05, vs. control group (Dunnett's test). Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 32: Effect of oral administration of HA-UNIM-001 on biochemical parameters in male rats

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
ALT (IU/L)	30.83 ± 8.78	24.46 ± 2.75	23.05 ± 8.61	34.51 ± 14.83
AST (IU/L)	59.54 ± 17.51	45.15 ± 7.8	60.96 ± 17.08	87.76 ± 31.87*
BUN (mg/dl)	34.69 ± 5.47	29.88 ± 5.79	23.01 ± 9.96	21.88 ± 11.47
CRE (mg/dl)	0.704 ± 0.17	0.710 ± 0.13	0.99 ± 0.22	0.75 ± 0.18

Mean values of 05 male rats ± standard deviation; *p < 0.05, vs. control group (Dunnett's test). Control group given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 33: Complete blood cell count (CBC) in female rats after treatment with HA-UNIM-001

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
RBC Count (mil/ µL)	6.704 ± 0.41	6.56 ± 0.64	6.73 ± 0.43	6.628 ± 0.58
HB (g/dL)	13.56 ± 0.78	13.1 ± 2.19	14.86 ± 1.40	12.94 ± 0.66

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
Haematocrit (%)	36.3 ± 5.06	35.84 ± 5.55	41.88 ± 5.15	36.06 ± 5.80
Mean Corpuscular Volume (fL)	54.66 ± 2.23	54.8 ± 3.56	54.82 ± 1.35	52.98 ± 4.18
Mean Corpuscular Haemoglobin (pg)	20.44 ± 2.43	18.8 ± 2.30	19 ± 1.41	20.72 ± 2.28
Mean Corpuscular Haemoglobin concentration (g/dL)	32.92 ± 2.24	31.54 ± 2.01	33.7 ± 0.64	32.94 ± 1.33
Red Cell Distribution Width (%)	14.43 ± 0.71	15.64 ± 3.99	12.74 ± 1.41	15.64 ± 1.13
Platelet Count (thou/µL)	933.16 ± 50.52	867.6 ± 158.22	796.2 ± 143.62	910 ± 85.82
Mean platelet volume (fL)	6.38 ± 0.49	6.14 ± 0.59	6.66 ± 0.55	5.96 ± 0.60
WBC Count (thou/µL)	4.4 ± 1.96	4.28 ± 0.55	4.4 ± 1.99	6.94 ± 4.48
Segmented Neutrophils (%)	12.33 ± 7.50	11.2 ± 8.58	19.8 ± 5.84	15.4 ± 5.41
Lymphocytes (%)	66.66 ± 24.08	64 ± 13.98	77.8 ± 10.06	73.6 ± 10.87
Monocytes (%)	2.8 ± 1.09	4 ± 1.58	2.4 ± 1.34	2.4 ± 1.51

Mean values of 05 female rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Table 34: Complete blood cell count (CBC) in male rats after treatment with HA-UNIM-001

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
RBC Count (mil/ µL)	8.098 ± 1.04	7.384 ± 0.88	7.5 ± 0.78	7.328 ± 0.82
HB (g/dL)	15.02 ± 2.18	13.24 ± 1.17	12 ± 1.14	12.32 ± 2.38
Haematocrit (%)	41.14 ± 4.68	37.14 ± 1.21	34.88 ± 5.81	30.84 ± 5.57
Mean Corpuscular Volume (fL)	53.48 ± 1.64	53.64 ± 1.93	54.22 ± 1.32	54.62 ± 1.38
Mean Corpuscular Haemoglobin (pg)	17.82 ± 2.39	17.36 ± 2.52	16.02 ± 1.97	17.56 ± 1.04
Mean Corpuscular Haemoglobin concentration (g/dL)	33.86 ± 2.72	32.58 ± 1.21	32.4 ± 0.9	31.56 ± 2.17
Red Cell Distribution Width (%)	13.72 ± 1.48	14.12 ± 2.28	11.98 ± 1.71	12.92 ± 0.71
Platelet Count (thou/µL)	884.8 ± 71.11	757 ± 208.66	904.2 ± 143.11	810.6 ± 116.28
Mean Platelet Volume (fL)	7.64 ± 1.80	7.22 ± 1.67	7.4 ± 0.75	7.92 ± 1.33
WBC Count (thou/µL)	5.56 ± 3.68	5.3 ± 3.89	8.44 ± 5.08	5.5 ± 1.84
Eosinophils (%)	17.8 ± 11.1	26.4 ± 5.03	18.08 ± 9.07	22.6 ± 1.34
Segmented Neutrophils (%)	65 ± 15.7	74.6 ± 14.46	67.2 ± 21.53	66.4 ± 18.77
Lymphocytes (%)	4.4 ± 3.28	4 ± 2.55	5.2 ± 2.58	4.4 ± 1.67
Monocytes (%)	3 ± 2.33	3.2 ± 2.71	3.1 ± 1.70	2.77 ± 3.11

Mean values of 05 male rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Histopathology Reports of Sub-acute Toxicity Study

At the conclusion of the study, rats were sacrificed, and histopathological examinations of vital organs were conducted.

Liver: The liver of the control rat displayed a normal architecture, composed of hexagonal or pentagonal lobules with central veins and peripheral hepatic triads. Hepatocytes were arranged in trabeculae, with regular morphology.

Heart: Histological examination of the heart in the normal group revealed well-organized myocardial cells with clear structures. After the administration of HA-UNIM-001 at doses of 500, 1000, and 2000 mg/kg, no irregularities were observed in the myocardium.

Kidney: Normal structure of the cortex and medulla was maintained in the kidneys of control rats. After HA-UNIM-001 administration, there were no alterations in the normal architecture of the kidney.

Stomach: The gastric mucosa in both the control and treatment groups exhibited a normal lining coat with well-organized epithelial cells, chief cells, parietal cells, and argentaffin cells. Photomicrographs of stomach sections from the treatment group resembled those of the control group, indicating an intact surface lining epithelium.

Testes: Histological examination of the testes in rats administered HA-UNIM-001 showed normal features with no evidence of haemorrhage, edema, or degeneration. The arrangement of seminiferous tubules and interstitial cells of Leydig remained unaffected.

Ovary: Normal ovarian sections displayed unilaminar primary follicles, antral follicles, and a well-formed corpus luteum. Sections from both control and treatment groups showed normal lumen endometrial glands and luminal epithelium.

Conclusion

The sub-acute study of HA-UNIM-001 revealed no toxic effects based on biochemical parameters, haematological parameters, and histopathological studies. The results confirm the safety of HA-UNIM-001 for oral administration in both sexes of Wistar rats.

Sub-chronic Toxicity Study of Hydroalcoholic Extract of UNIM-001 in Wistar Rats

A repeated 90-day oral toxicity study of HA-UNIM-001 was conducted on Wistar rats to assess its impact on morphological, gross behaviour, body

weight changes, as well as to identify haematological, biochemical, and histopathological alterations.

Materials & Methods

The test material, UNIM-001 (supplied by CCRUM), was dried and ground to a coarse powder. It was then extracted in a Soxhlet apparatus using a hydroalcoholic solution (20:80). The resulting hydroalcoholic solution was concentrated, yielding a dark viscous residue (HA-UNIM-001). For animal treatment, the hydroalcoholic extract was suspended in distilled water. Following guidelines, ten female and ten male Wistar rats were used. Dose levels tested were 0 (control), 500, 1000, and 2000 mg/kg b.w. The test substance was administered in a single dose by gavage using a suitable intubation cannula.

Cage-side observations, such as changes in morphology and behaviour, were recorded daily, and body weights were noted weekly. At the study's conclusion, haematological, biochemical, and pathological changes (macroscopic and microscopic) were examined.

Table 35: Brief description of study protocol for HA-UNIM-001

Name of the Study	Sub-chronic Study of HA-UNIM-001
Test material	HA-UNIM-001
Animal model	Wistar rats
Age	11–12 weeks
Weight	180 g (Average)
Animal procured from	Central animal facility, Jamia Hamdard (173/CPCSEA)
Sex	Male and female
Number of animal per group	10 per sex per group
Groups	04
Route of administration	Intra-gastric administration
Dose volume	2 mL per 100 g body weight per animal
Number of administration	Once daily for 90 days
Dose levels	500, 1000, 2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	90 days

Observations

The 90-day study period involved monitoring animals twice daily for signs of morbidity and mortality, including changes in skin, fur, eyes, mucous membranes, occurrence of secretions & excretions, and autonomic activity.

Body Weight

Weight changes were calculated and recorded. Surviving animals were weighed and then humanely sacrificed at the end of the test.

Haematology and Clinical Biochemistry

Blood samples collected just prior to animal sacrifice were used for haematological and clinical biochemistry determinations to identify major toxic effects in blood & tissues, specifically focusing on the kidney and liver.

Results

Table 36: Effect of oral administration of HA-UNIM-001 on body weight and organ weight in female rats

Body & Organ Weight	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
Body weight (g)	252.5 ± 21.11	237.5 ± 28.30	250.5 ± 22.29	274.5 ± 10.12
Heart	0.31 ± 0.31	0.36 ± 0.04	0.315 ± 0.035	0.315 ± 0.05
Left kidney	0.30 ± 0.16	0.38 ± 0.03	0.30 ± 0.01	0.30 ± 0.03
Liver	3.01 ± 0.23	3.44 ± 0.39	2.97 ± 0.42	2.82 ± 0.15
Stomach	0.75 ± 0.10	0.95 ± 0.13	0.71 ± 0.17	0.63 ± 0.08
Ovary	1.53 ± 0.15	0.50 ± 0.12	0.55 ± 0.13	0.36 ± 0.05

Mean values of 10 female rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Table 37: Effect of oral administration of HA-UNIM-001 on body weight and organ weight in male rats

Body & Organ Weight	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
Body weight (g)	262.5 ± 11.84	263.5 ± 12.70	296.5 ± 19.86	275 ± 25.52
Stomach	0.35 ± 0.03	0.29 ± 0.05	0.35 ± 0.03	0.32 ± 0.05
Liver	0.32 ± 0.04	0.31 ± 0.03	0.36 ± 0.05	0.32 ± 0.04
Heart	3.10 ± 0.41	2.69 ± 0.18	3.25 ± 0.25	2.69 ± 0.54
Left kidney	0.63 ± 0.16	0.85 ± 0.17	0.57 ± 0.12	0.60 ± 0.11
Testes	1.83 ± 0.15	1.65 ± 0.26	1.29 ± 0.23	1.75 ± 0.11

Mean values of 10 male rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

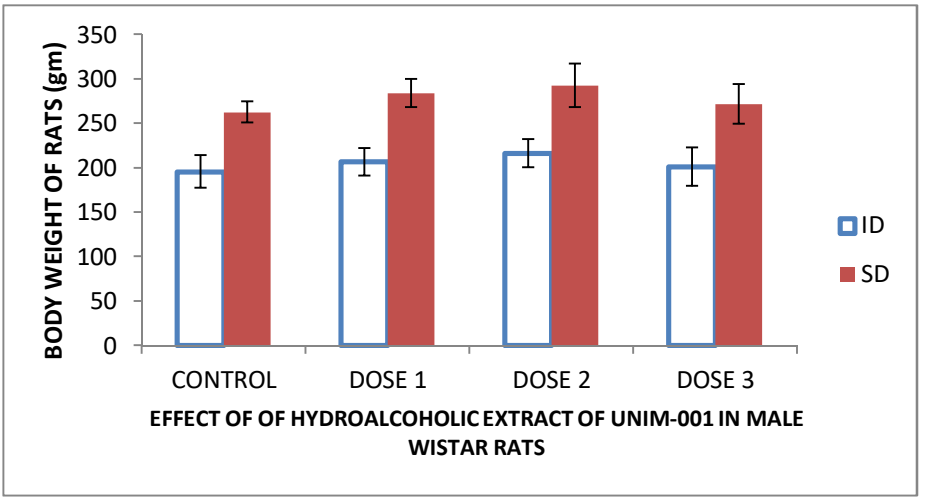


Figure 7: *Change in body weight of male rats*

Comparison of the body weight of Albino Wistar rats from the date of starting till termination of the experimental schedules. Body weights are shown as mean value ± standard deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment (Male Group)

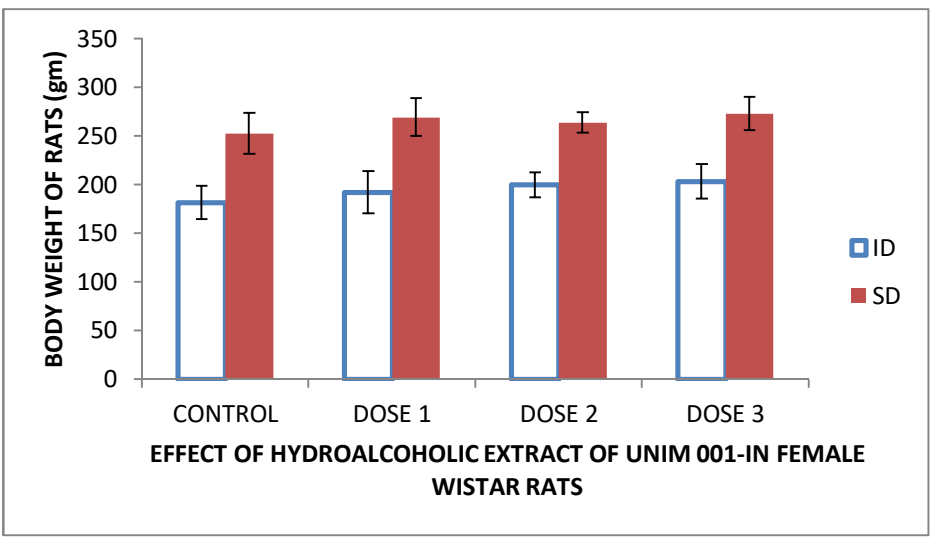


Figure 8: *Change in body weight of female rats*

Comparison of the body weight of Albino Wistar rats from the date of starting till termination of the experimental schedules. Body weights are shown as mean value ± standard deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment (Female Group)

Table 38: Effect of oral administration of HA-UNIM-001 on biochemical parameters in female rats

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
ALT (IU/L)	65.41 ± 64.54	46.92 ± 47.41	54.77 ± 46.92	55.69 ± 40.73
AST (IU/L)	62.51 ± 40.87	62.76 ± 61.40	56.53 ± 44.93	79.33 ± 68.46
BUN (mg/dl)	14.62 ± 9.36	20.04 ± 8.46	15.09 ± 11.413	19.73 ± 12.73
CRE (mg/dl)	2.01 ± 1.58	4.31 ± 3.91	3.02 ± 1.74	4.40 ± 3.45

Mean values of 10 female rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 39: Effect of oral administration of HA-UNIM-001 on biochemical parameters in male rats

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
ALT (IU/L)	22.54 ± 7.28	43.68 ± 19.32	48.73 ± 16.24	41.63 ± 20.68
AST (IU/L)	46.28 ± 18.03	37.51 ± 10.02	54.03 ± 20.42	37.49 ± 16.21
BUN (mg/dl)	15.43 ± 2.78	11.71 ± 6.59	8.17 ± 4.45	12.54 ± 7.72
CRE (mg/dl)	1.22 ± 0.42	1.10 ± 0.44	1.24 ± 0.49	1.30 ± 0.56

Mean values of 10 male rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 40: Complete blood cell count (CBC) in female rats after treatment with HA-UNIM-001

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
RBC Count (mil/ µL)	6.64 ± 1.52	7.36 ± 0.54	6.27 ± 0.53	6.27 ± 0.53
Hb (g/dL)	11.75 ± 0.61	13.71 ± 0.961	11.97 ± 0.47	11.97 ± 0.47
Haematocrit (%)	41.43 ± 3.2	46.18 ± 2.24	41.8 ± 3.17	41.8 ± 3.17
Mean Corpuscular Volume (fL)	53.32 ± 0.78	53.36 ± 1.48	54.6 ± 1.90	54.6 ± 1.90
Mean Corpuscular Haemoglobin (pg)	18.13 ± 0.65	18.62 ± 0.58	18.1 ± 0.59	18.1 ± 0.59
Mean Corpuscular Haemoglobin concentration (g/dL)	33.2 ± 1.01	32.87 ± 0.87	33.03 ± 1.26	33.03 ± 1.26
Red Cell Distribution Width (%)	15.48 ± 0.47	14.48 ± 0.86	15.23 ± 0.19	14.96 ± 0.73
Platelet Count (thou/µL)	732.625 ± 81.15	721.88 ± 79.69	737.33 ± 78.24	848.4 ± 51.20
Mean Platelet Volume (fL)	6.075 ± 0.23	6.06 ± 0.57	6.46 ± 0.19	5.98 ± 0.32
WBC Count (thou/µL)	5.875 ± 2.88	8.85 ± 3.07	7.87 ± 1.04	9.89 ± 2.48
Segmented Neutrophils (%)	17.75 ± 11.90	24.44 ± 12.69	18.22 ± 10.08	18.4 ± 8.95
Lymphocytes (%)	52.625 ± 18.04	71.22 ± 24.05	79 ± 13.60	74 ± 20.67
Monocytes (%)	2.12 ± 2.10	2.66 ± 2.73	1.22 ± 1.48	1.9 ± 1.66

Mean values of 10 female rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Table 41: Complete blood cell count (CBC) in male rats after treatment with HA-UNIM-001

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
RBC Count (mil/ µL)	5.872 ± 0.81	7.128 ± 0.83	6.209 ± 0.63	6.269 ± 0.26
HB (g/dL)	12.43 ± 1.16	14.16 ± 0.61	13.09 ± 1.25	12.53 ± 0.68
Haematocrit (%)	40.01 ± 7.63	45.52 ± 4.2	37.81 ± 2.87	38.812.41
Mean Corpuscular Volume (fL)	56.39 ± 2.21	60.72 ± 1.30	58.38 ± 2.90	61.06 ± 3.38
Mean Corpuscular Haemoglobin (pg)	17.76 ± 1.95	19.43 ± 0.59	18.51 ± 0.76	18.84 ± 0.99
Mean Corpuscular Haemoglobin concentration (g/dL)	31.55 ± 2.99	31.16 ± 0.57	31.53 ± 0.81	31.42 ± 1.10
Red Cell Distribution Width (%)	12.79 ± 0.83	14.37 ± 0.88	14.23 ± 1.58	14.73 ± 0.82
Platelet Count (thou/µL)	413.2 ± 73.44	492.6 ± 39.30	582.5 ± 101.21	502.4 ± 94.67
Mean platelet volume (fL)	5.66 ± 0.86	5.8 ± 0.63	5.95 ± 0.46	6.29 ± 0.63
WBC Count(thou/µ L)	3.8 ± 1.49	4.36 ± 0.51	4.3 ± 1.05	6.23 ± 1.42
Segmented Neutrophils (%)	47.2 ± 17.88	44.7 ± 8.85	41.9 ± 11.47	31.8 ± 15.61
Lymphocytes (%)	35.6 ± 9.54	44.8 ± 20.64	49.9 ± 21.33	56.5 ± 18.69
Monocytes (%)	4.2 ± 4.34	3.2 ± 1.47	2.4 ± 1.43	3.2 ± 1.61

Mean values of 10 male rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Histopathology Reports of Subchronic Toxicity Study

At the conclusion of the study, rats were euthanized, and histopathological examinations of vital organs were conducted.

Liver: The liver of a normal rat exhibited hexagonal or pentagonal lobules, central veins, and peripheral hepatic triads or tetrads within connective tissue. Hepatocytes were arranged in trabeculae radiating from the central vein, separated by sinusoids containing Kupffer cells. Cells were generally regular, containing large spheroidal nuclei with distinct nucleoli and peripheral chromatin distribution. Some cells had two nuclei.

The liver of a control rat maintained a normal architecture, unaffected by the administration of HA-UNIM-001 at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of body weight.

Heart: In HE staining, the normal group exhibited well-organized myocardial cells with a neat, compact, clear structure, minimal extracellular matrix, and a small amount of fibroblasts. The myocardium in the normal group also displayed organized myofibrils with long cylindrical mononucleated cells, featuring muscle fibers with striations in cytoplasm and elongate nuclei.

The rat heart sections did not reveal any irregularities following the administration of HA-UNIM-001 at doses of 500, 1000, and 2000 mg/kg.

Kidney: The kidney of control rats showed a normal structure in both cortex and medulla, with collecting tubules lined by relatively low simple cubic epithelium. Cross-sections displayed thick descending and ascending parts of Henle's loops, collecting coils of small caliber, and a small amount of interstitial tissue.

Following the administration of HA-UNIM-001 at doses of 500, 1000, and 2000 mg/kg, the normal architecture of the kidney remained unchanged in comparison to the control group.

Stomach: The gastric mucosa, the innermost lining coat of the stomach, featured a simple layer of columnar epithelial cells with prominent nuclei. Gastric glands, occupying the bulk of the mucosa, were situated at the luminal surface, opening singly or in groups. The mucosa consisted of various cell types, including Surface Epithelial (Mucous) Cells, Chief (Zymogenic) Cells, Parietal (Oxyntic) Cells, and Argentaffin (Enteroendocrine) Cells.

Following the administration of HA-UNIM-001, the normal architecture of the stomach remained unchanged in comparison to the control group, as confirmed by photomicrographs.

Testes: The evaluation of testes for various parameters showed normal features in the group where only distilled water was administered. The testes sections displayed a normal arrangement of seminiferous tubules, sperms, and interstitial cells of Leydig.

After the administration of HA-UNIM-001 at doses of 500, 1000, and 2000 mg/kg, there was no change in the normal architecture of the testes in relation to the control group.

Ovary: The normal section of a rat ovary displayed unilaminar primary follicles, antral follicles, a previously formed corpus luteum with foamy eosinophilic cells, primordial follicles, stromal fibroblasts, and smooth muscle cells. The control rat's ovary showed a normal architecture, and photomicrographs of ovary sections from both control and treatment group animals confirmed the normalcy of lumen endometrial glands and luminal epithelium.

Conclusion

In the subchronic study of HA-UNIM-001, no toxic effects were observed based on biochemical parameters, hematological parameters, and histopathological studies. The results confirm the safety of HA-UNIM-001 for oral administration in both sexes of Wistar rats.

Chronic Toxicity Study on Hydroalcoholic UNIM-001 in Wistar Rats

A six-month oral toxicity study of HA-UNIM-001 was conducted in Wistar rats to assess its impact on morphological features, gross behavior, body weight changes, as well as to investigate hematological, biochemical, blood clotting factors, and histopathological alterations.

Materials & Methods

The test material UNIM-001 (supplied by CCRUM) was dried and ground to coarse powder and then was extracted in a Soxhlet apparatus using hydro alcoholic solution (20:80). The hydroalcoholic extract was concentrated and a dark viscous residue (HA-UNIM 001) was collected. For animal treatment, the hydroalcoholic extract was suspended in distilled water. According to guidelines, ten female and ten male Wistar rats were administered water (control), 500, 1000 and 20 female and 20 male were administered 2000 mg/kg b.w. The animals were challenged with the test substance daily for a period of 180 days. The animals were weighed and the dose was calculated according to the body weight. The test substance was administered in a single dose by gavage using a suitable intubation cannula.

Cage-side observations, i.e., change in morphology, behaviour were noted daily and body weights were noted weekly. At the end of study, hematological, biochemical, blood clotting factor and pathological changes (macroscopic and microscopic) were examined.

Table 42: Brief description of Study Protocol for HA-UNIM-001

Name of the Study	Chronic Study of HA-UNIM-001
Test material	HA-UNIM-001
Animal model	Wistar rats
Age	11–12 weeks
Weight	200–220 g (Average)
Animal procured from	Central Animal Facility, Jamia Hamdard (173/CPCSEA)
Sex	Male and female
Number of animal per group	10 per sex group (control, 500 mg/kg, 1000 mg/kg) and 20 per sex per group (2000 mg/kg).
Groups	04
Route of administration	Intra-gastric administration
Dose volume	2 mL per 100 g body weight per animal
Number of administration	Once daily for 180 days
Dose levels	500, 1000, 2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	180 days

Observations

The duration of the study spanned 180 days. Daily observations were conducted twice to assess signs of morbidity and mortality in all animals. This involved monitoring changes in skin, fur, eyes, mucous membranes, occurrences of secretions and excretions, as well as autonomic activity.

Body weight

Weight changes were recorded, and surviving animals were weighed and then humanely sacrificed at the end of the test.

Haematology and Clinical Biochemistry

Blood samples were collected before euthanizing the animals, and haematological and clinical biochemistry determinations were conducted to identify major toxic effects on blood, tissues, kidneys, and liver.

Results

Table 43: Effect of oral administration of HA-UNIM-001 on body weight and organ weight in female rats

Body & Organ Weight	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
Body weight (g)	269.44 ± 12.1	267.85 ± 12.1	265 ± 15.1	263.3 ± 15.42
Heart (g)	1.05 ± 0.14	1.03 ± 0.104	1.09 ± 0.16	1.01 ± 0.122
Left kidney (g)	0.96 ± 0.124	0.89 ± 0.18	1.05 ± 0.15	0.89 ± 0.11
Liver (g)	7.54 ± 0.94	7.04 ± 0.575	7.25 ± 2.62	7.31 ± 2.37
Stomach (g)	1.98 ± 0.28	1.79 ± 0.21	1.69 ± 0.35	1.7 ± 0.97
Ovary (g)	1.57 ± 0.38	1.75 ± 0.42	2.3 ± 0.34	2.12 ± 0.12

Parameters are shown as mean ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Table 44: Effect of oral administration of HA-UNIM-001 on body weight and organ weight in male rats

Body & Organ Weight	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
Body weight (g)	290 ± 16.84	273 ± 20.18	267.5 ± 27.47	265.5 ± 24.19
Heart (g)	1.05 ± 0.165	0.95 ± 0.13	1.18 ± 0.12	1.01 ± 0.17
Left kidney (g)	0.911 ± 0.12	0.82 ± 0.16	0.98 ± 0.11	0.87 ± 0.12
Liver (g)	8.24 ± 0.97	7.12 ± 0.42	7.89 ± 1.03	7.6 ± 1.07
Stomach (g)	2.09 ± 0.37	1.97 ± 0.39	2.02 ± 0.33	1.95 ± 0.28
Testes (g)	4.57 ± 0.9	4.9 ± 0.6	3.17 ± 0.66	4.2 ± 0.7

Parameters are shown as mean ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

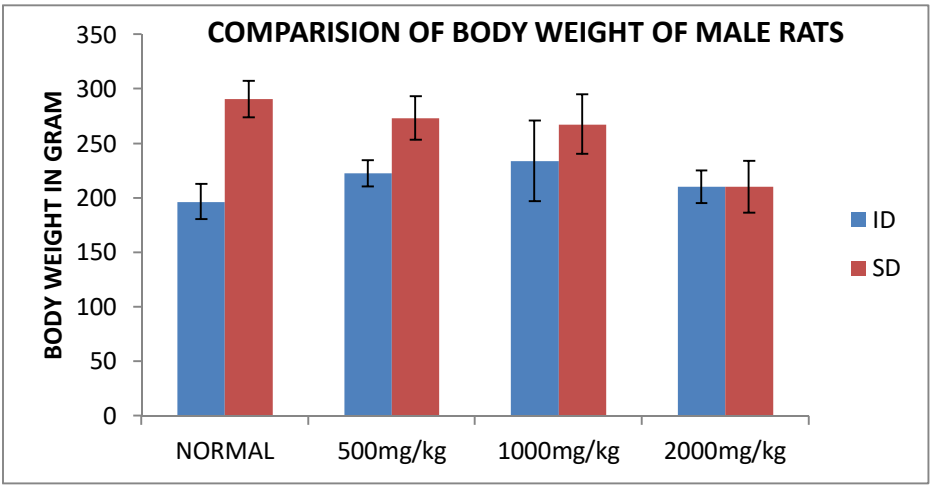


Figure 9: *Change in body weight of male rats*

Comparison of the body weight of Albino Wistar rat from the date of starting till termination of the experimental schedules. Body weights are shown as mean value ± standard deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment (Male Group)

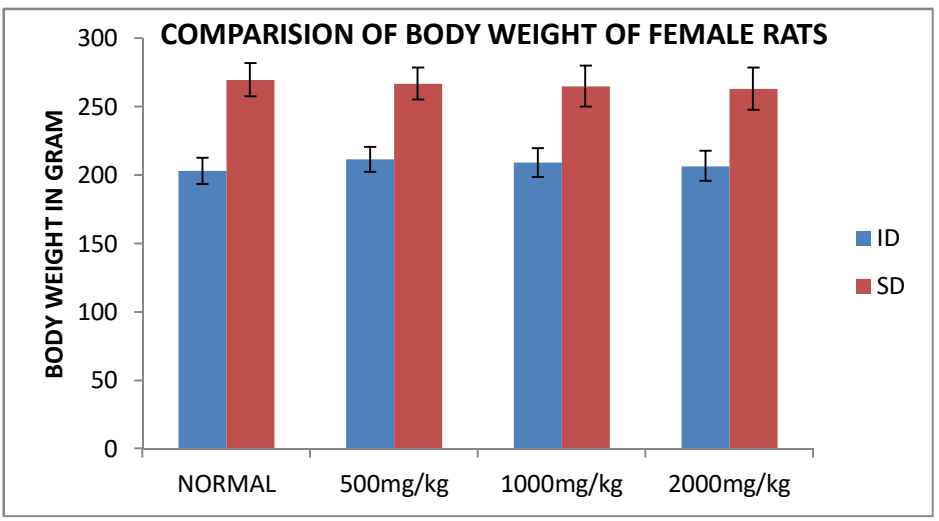


Figure 10: *Change in body weight of female rats*

Comparison of the body weight of Albino Wistar rats from the date of starting till termination of the experimental schedules. Body weights are shown as mean ± standard deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment (Female Group)

Table 45: Effect of oral administration of HA-UNIM-001 on biochemical parameters in female rats

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
ALT (IU/L)	36.43 ± 12.94	25.73 ± 5.2 *	26.03 ± 7.97	28.01 ± 7.2
AST (IU/L)	48.85 ± 14.8	34.7 ± 17.35	46.67 ± 19.2	36.1 ± 13.8
BUN (mg/dl)	21.43 ± 4.49	17.02 ± 1.488	19.1 ± 9.5	19.2 ± 4.34
CRE (mg/dl)	0.83 ± 0.22	1.092 ± 0.38	0.985 ± 0.304	1.4 ± 0.54

Parameters are shown as mean ± standard deviation; *P < 0.05 vs. control group (Dunnett's test). Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 46: Effect of oral administration of HA-UNIM-001 on biochemical parameters in male rats

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
ALT (IU/L)	30.56 ± 14.24	27.73 ± 9.32	27.03 ± 7.37	25.85 ± 9.1
AST (IU/L)	36.3 ± 18.5	30.17 ± 20.5	43.8 ± 14.7	39.41 ± 13.7
BUN (mg/dl)	21.87 ± 2.87	18.5 ± 2.9	19.72 ± 5.7	20.7 ± 3.4
CRE (mg/dl)	0.936 ± 0.69	1.076 ± 0.62	1.02 ± 0.443	1.087 ± 0.418

Parameters are shown as mean ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 47: Complete blood cell count (CBC) in female rats after treatment with HA-UNIM-001

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
RBC Count (mil/ cumm)	6.87 ± 1.15	7.08 ± 0.58	7.42 ± 0.28	7.47 ± 1.6
HB (gm/dl)	12.38 ± 1.10	11.486 ± 1.37	12.54 ± 0.81	11.16 ± 1.9
Mean Corpuscular Volume (fL)	65.28 ± 4.34	61.8 ± 3.05	61.61 ± 4.7	65.10 ± 5.9
Mean Corpuscular Haemoglobin (pg)	17.07 ± 1.04	14.07 ± 1.26	14.99 ± 1.46	14.56 ± 2.03
Mean Corpuscular Haemoglobin Concentration (%)	26.78 ± 2.04	23.67 ± 1.3	25.21 ± 0.99	24.31 ± 1.9
Platelet Count (La./cumm)	9.04 ± 1.47	8.2 ± 1.13	8.7 ± 1.5	8.19 ± 1.26
PCV (%)	53.45 ± 12.65	39.18 ± 4.8	44.67 ± 3.4	39.68 ± 11.43
WBC Count (cells/cumm)	5866.66 ± 906.2	8463 ± 1883**	8685.7 ± 947**	8156.9 ± 1731**
Polymorph (%)	45.55 ± 3.94	39.75 ± 4.9	23.75 ± 1.21	34.25 ± 11.6
Lymphocytes (%)	61.88 ± 5.48	65.6 ± 8.7	73.2 ± 1.37*	66.2 ± 7.6

Parameters are shown as mean ± standard deviation; *P < 0.05, **P < 0.01 vs. control group (Dunnett's test). Control group was given water.

Table 48: Complete blood cell count (CBC) in male rats after treatment with HA-UNIM-001

Parameters	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
RBC Count (mil/ cumm)	6.79 ± 0.744	9.78 ± 1.38	7.54 ± 0.38	7.05 ± 1.4
Hb (gm/dl)	11.87 ± 1.08	10.486 ± 0.94	12.714 ± 0.96	10.6 ± 3.6
Mean Corpuscular Volume (fL)	63.88 ± 5.034	61.8 ± 2.35	62.43 ± 6.7	64.80 ± 4.7
Mean Corpuscular Haemoglobin (pg)	17.1 ± 1.374	15.7 ± 1.16	16.11 ± 1.506	16.946 ± 1.03
Mean Corpuscular Haemoglobin concentration (%)	27.25 ± 1.839	24.27 ± 1.9	23.9 ± 6.19	25.91 ± 1.82
Platelet Count (La./cumm)	8.51 ± 1.154	7.9 ± 1.23	9.2 ± 0.65	7.99 ± 1.36
PCV (%)	50.76 ± 6.461	35.04 ± 13.8	46.07 ± 2.64	40.78 ± 13.53
TLC (cells/cumm)	5022.22 ± 1239.7	7793.9 ± 1684	9237.3 ± 902	7473 ± 1657
Polymorph (%)	42.11 ± 5.689	41.14 ± 5.8	23.875 ± 2.08	39 ± 11.2
Lymphocytes (%)	58.77 ± 6.016	59.7 ± 3.3	74 ± 2.37	55.8 ± 12.6

Parameters are shown as mean ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Table 49: Prothrombin time (PT) and activated partial thromboplastin time (APTT) following treatment with HA-UNIM-001 in male rats

Clotting Factors	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
PT(sec)	10.08 ± 1.82	10.15 ± 1.57	8.44 ± 1.40	8.47 ± 1.47
APTT (sec)	16.55 ± 1.71	20.9 ± 1.94	18.51 ± 2.66	20.83 ± 4.34

Parameters are shown as mean ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Table 50: Prothrombin time (PT) and activated partial thromboplastin time (APTT) following treatment with HA-UNIM-001 in female rats

Clotting factors	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
PT(sec)	17.64 ± 1.21	18.58 ± 2.38	18.07 ± 1.27	19.73 ± 1.72
APTT (sec)	7.41 ± 1.52	8.33 ± 1.299	8.08 ± 1.46	8.63 ± 1.42

Parameters are shown as mean ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given water.

Table 51: Mortality detail of both sexes during experimental schedule

Gender	Control	HA-UNIM-001 (500 mg/kg)	HA-UNIM-001 (1000 mg/kg)	HA-UNIM-001 (2000 mg/kg)
Male	1/10	3/10	2/10	4/20
Female	1/10	2/10	3/10	4/20

Histopathology Reports of Chronic Toxicity Study

Upon completion of the study, the rats underwent sacrifice, and comprehensive histopathological examinations of vital organs were conducted.

Liver: The liver of a normal rat displayed hexagonal or pentagonal lobules with central veins and peripheral hepatic triads or tetrads embedded in connective tissue. Hepatocytes were arranged in trabeculae radiating from

the central vein, separated by sinusoids containing Kupffer cells. They exhibited regularity, featuring a large spheroidal nucleus with a distinctly marked nucleolus and peripheral chromatin distribution. Some cells had two nuclei. The liver of the control rat maintained a normal architecture.

Heart: In the HE staining, the normal group showcased well-arranged myocardial cells with a neat, compact, clear structure, less extracellular matrix, and a small amount of fibroblasts. The myocardium also displayed well-organized myofibrils with long cylindrical mononucleated cells. Sections revealed muscle fibers with striations in the cytoplasm and elongated nuclei. No irregularities were observed in the rat heart after the administration of HA-UNIM-001 at doses of 500, 1000, and 2000 mg/kg.

Kidney: A normal structure of the cortex and medulla was observed in the kidneys of control rats. Collecting tubules were lined with relatively low simple cubic epithelium. The cross-sections showed thick descending and ascending parts of Henle's loops, collecting coils of small calibre, and a small amount of interstitial tissue. Following the administration of HA-UNIM-001 at doses of 500, 1000, and 2000 mg/kg, there was no change in the normal architecture of the kidney in relation to the control group.

Stomach: The gastric mucosa, forming the innermost lining of the stomach, consisted of a simple layer of columnar epithelial cells with prominent nuclei. Gastric glands occupied the bulk of the mucosa, situated at the luminal surface of the epithelial lining. The basal portions of these glands were composed of chief (zymogenic) cells with occasional parietal cells, while the neck contained both mucous neck cells and parietal cells. Cells types included Surface Epithelial (Mucous) Cells, Chief (Zymogenic) Cells, Parietal (Oxyntic) Cells, and Argentaffin (Enteroendocrine) Cells. Following HA-UNIM-001 administration, the normal stomach architecture remained unchanged compared to the control group.

Photomicrographs of the stomach wall from the treatment (test) group animals depicted an intact surface lining epithelium and superficial glands in the stomach mucosa, similar to the control group.

Testes: Evaluation of the testes included the assessment of various parameters. In the group where only distilled water was administered, Wistar rats showed normal features in testes histology, with a normal arrangement of seminiferous tubules, sperms, and interstitial cells of Leydig. After the administration of HA-UNIM-001 at doses of 500, 1000, and 2000 mg/kg, there was no change in the normal architecture of the testes in relation to the control group.

Ovary: A normal section of a rat ovary showcased unilaminar primary follicles, antral follicles, a previously formed corpus luteum with foamy eosinophilic cells, primordial follicles, stromal fibroblasts, and smooth muscle cells. Numerous, irregular, tortuous, and wide lumen endometrial glands and an extensive fold of the luminal epithelium were observed. The luminal epithelium consisted of a single layer of columnar epithelial cells with large nuclei at the basal aspect of the cells. The ovary of the control rat displayed a normal architecture. Photomicrographs of ovary sections from both control and treatment group animals showed normal lumen endometrial glands and luminal epithelium.

Conclusion

In the chronic study of HA-UNIM-001, no toxic effects were identified, as evidenced by biochemical parameters, hematological parameters, and histopathological studies. The results affirm the safety of HA-UNIM-001 for oral administration in both sexes of Wistar rats.

Preclinical Safety Studies of Coded Unani Formulation UNIM-003 (Topical)

Acute Dermal Toxicity Study of UNIM-003 in Wistar Rats

An evaluation of acute dermal toxicity was conducted for UNIM-003 using Albino Wistar rats of both sexes as the experimental model to assess its impact on morphological, gross behaviour, and body weight changes.

Materials & Methods

UNIM-003, provided by the Central Council for Research in Unani Medicine, New Delhi, was administered based on the body weight of the animals (2000 mg/kg bw) and suspended in double-distilled water for topical application. The fur on the back of each animal was removed 24 hours before treatment, and UNIM-003 was directly applied to a small area (4×5 cm) of the skin. After application, the test area was covered with a non-occlusive dressing (a gauze patch) and a semi-occlusive bandage for 24 hours.

Table 52: Brief description of study protocol for UNIM-003

Name of the Study	Acute Dermal Toxicity Study
Test material	UNIM-003
Animal model	Albino Wistar rats

Name of the Study	Acute Dermal Toxicity Study
Age	11–12 weeks
Weight	180 ± 20 g
Animal procured from	Central Animal Facility, Jamia Hamdard (173/CPCSEA)
Sex	Female and male
Number of animal per group	06 per group (3 animal each sex)
Groups	2 groups (control & test)
Route of administration	Topical
Number of administration	Single dose (topical application)
Concentration of doses	2000 mg/kg bw
Vehicle for administration	Water (double distilled)
Study duration	21 days

Observations

At the end of the exposure period, any residual test substance was removed by distilled water. Animals were observed for signs of toxicity at the application site, changes in fur, eyes mucous membrane, and any other overt sign of toxicity, including behavioural changes. Observations were made daily for the next 21 days following treatment. Mortality and morbidity checks were performed daily. On the 21st day, surviving rats were sacrificed, and necropsies were performed. All the above-listed gross observations were recorded.

Body Weight

Individual weights of animals were determined before the test substance was administered. Body weight was recorded before treatment, on day 0, 7th day, 14th day, and 21st day following administration. Weight changes were calculated and recorded. At the end of the test, surviving animals were weighed and then humanely sacrificed.

Result

Table 53: Effect of oral administration of UNIM-003 on body weight and organ weight

Body & Organ Weight	Control	Test (2000 mg/kg bw)
Body weight (g)	215 ± 17.321	214.166 ± 15.943
Heart (g)	0.716 ± 0.046	0.746 ± 0.074
Stomach (g)	2.111 ± 0.284	2.061 ± 0.188
Liver (g)	6.687 ± 0.7616	6.429 ± 0.642
Left kidney (g)	0.746 ± 0.036	0.74 ± 0.041

Mean values of 06 animals ± standard deviation

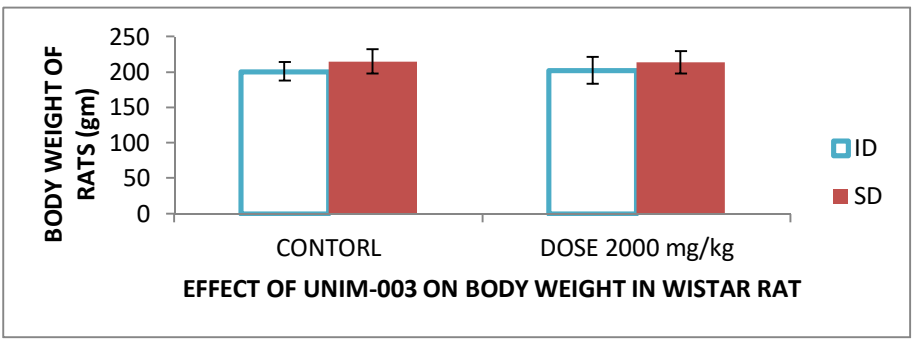


Figure 11: *Change in body weight of rat*

Comparison of the body weight of the Wistar rats from the date of starting and termination of the experimental schedules. Body weight is shown as the mean value ± standard deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment

Conclusion

The dermal toxicity study aimed to evaluate the potential effects of the drug after topical application in a single dose. Macroscopic observation of the skin and vital organs of the sacrificed animals did not reveal any visible signs of toxicity, such as changes in behaviour. No mortality was observed. The body weight of test substance-treated animals showed an increasing trend. Gross pathological examination did not indicate any lesions attributable to the toxicity of the test substance. In conclusion, the dermal toxicity study of the drug did not show any evidence of toxicity. This study supports the safe topical use of UNIM-003.

Sub-acute Dermal Toxicity Study of UNIM-003 in Wistar Rats

A repeated dose 28-day dermal toxicity study of UNIM-003 was conducted in Wistar rats as an experimental model to assess its effects on morphological features, gross behaviour, body weight changes, and to investigate haematological, biochemical, and histopathological alterations.

Materials & Methods

UNIM-003 was provided by the Central Council for Research in Unani Medicine, New Delhi. Five female and five male Wistar rats were utilized for the study. The tested dose levels included 0 (control), 500, 1000, and 2000 mg/kg body weight. Daily cage-side observations, noting changes in morphology and behaviour, were conducted, and body weight was recorded weekly. At the conclusion of the 28-day study (29th day), haematological, biochemical, and pathological changes (macroscopic and microscopic) were examined.

The test drug was administered to each group at doses corresponding to the control, 500, 1000, and 2000 mg/kg of body weight. The fur on the back of each animal was closely clipped 24 hours before treatment. The test substance was directly applied to a small area (4x5 cm) of the skin. Subsequently, the treated area was covered with a non-occlusive dressing (a gauze patch) and then a semi-occlusive bandage for 24 hours.

Table 53: Brief Description of Study Protocol for UNIM-003

Name of the Study	Sub-acute Study of UNIM-003
Test material	UNIM-003
Animal model	Wistar rats
Age	11–12 weeks
Weight	180 ± 20g
Animal procured from	Central Animal Facility, Jamia Hamdard (173/CPCSEA)
Sex	Male and female
Number of animal per group	05 male and 5 female in each group
Groups	04
Route of administration	Topical application
Number of administration	Single (Topical application) per day for 28 days
Dose levels	500, 1000 and 2000 mg/kg bw
Vehicle for administration	Water (double distilled)
Study duration	28 days

Observations

The study spanned 28 days, during which general clinical observations were conducted at least once daily, consistently at the same time each day. The animals' clinical conditions were documented, and comprehensive inspections for signs of morbidity and mortality were performed at least twice daily—typically at the commencement and conclusion of each day.

Signs encompassed changes in skin, fur, eyes, mucous membranes, as well as the presence of secretions and excretions. Additionally, autonomic activities such as lacrimation, piloerection, pupil size, and any unusual respiratory patterns were carefully monitored throughout the study period.

Body weight

Individual weights of animals were determined before the test substance was administered. Body weight was recorded before treatment, on day 0, 7^{th} day, 14^{th} day, 21^{st} day and 28^{th} day following the administration. Weight changes were calculated and recorded. At the end of the test, surviving animals were weighed and then humanely sacrificed.

Haematology and Clinical Biochemistry

At the end of the test period, blood samples were collected just prior to killing the animals and the haematological & clinical biochemistry determinations were done to find out the major toxic effects in blood & tissues and, specifically, effects on kidney and liver.

Results

Effect of UNIM-003 on body weight and organ weight in female rats: The body weight of HA-UNIM-003 showed significant difference (**$p < 0.01$) at doses 500 mg/kg and 1000 mg/kg, whereas there was less significant variation (*$p < 0.05$) at higher dose of 2000 mg/kg bw as compared to control group. Weights of stomach, liver, heart and left kidney at doses 500, 1000, and 2000 mg/kg showed non-significant variation as compared to organ weights of control group administered with water.

Effect of UNIM-003 on body weight and organ weight in male rats: The body weight of HA-UNIM -003 treated group showed significant difference (**$p < 0.01$) in male rats at doses 500 mg/kg and 1000 mg/kg, whereas less significant (*$p < 0.05$) at higher dose of 2000 mg/kg as compared to control group. Further, organs mainly stomach, liver, heart and left kidney at doses 500, 1000, and 2000 mg/kg showed non-significant variation in weight of different organs as compared to organ weight of control group administered with water only.

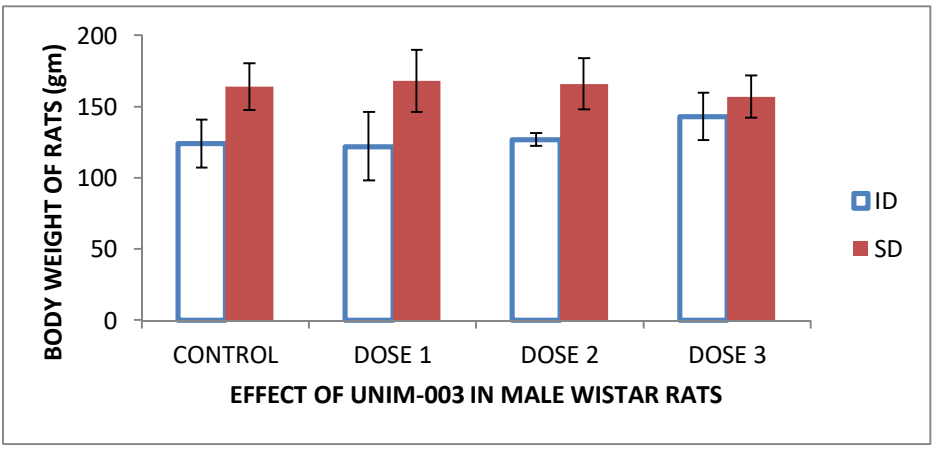


Figure 12: *Change in body weight of rats (male)*

Comparison of the body weight of the albino Wistar rats from the date of starting and termination of the experimental schedules. Body weights are shown as mean value ± standard deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment (male group)

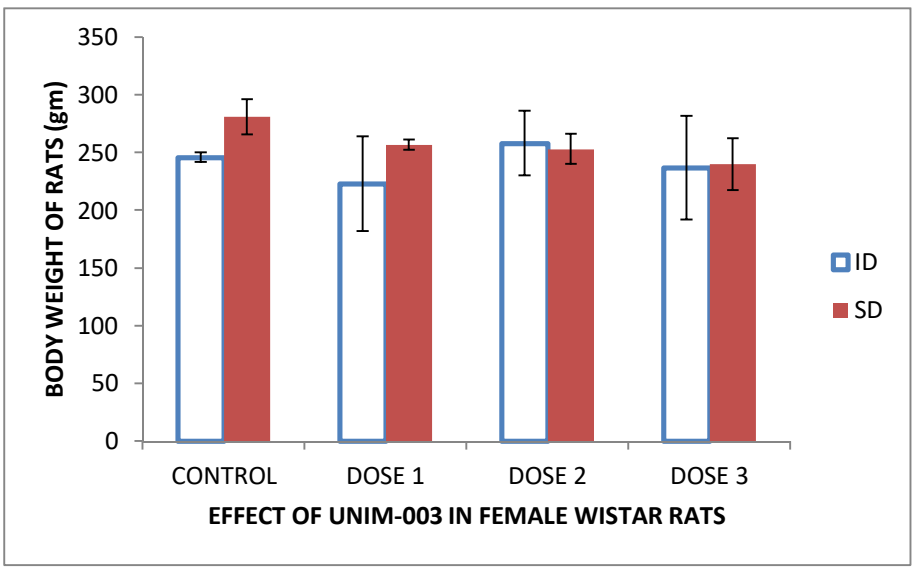


Figure 13: *Change in body weight of rats (female)*

Comparison of the body weight of albino Wistar rat from the date of starting and termination of the experimental schedules. Body weights are shown as mean value ± standrad deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment (Female Group)

Effect of topical application of UNIM-003 on biochemical parameters in female rats: In female rats, UNIM-003 at different doses of 500, 1000 and 2000 mg/kg showed non-significant difference in levels of ALT (IU/L) as compared to control group whereas the levels of AST (IU/L) at different doses showed significant decrease (**$p < 0.01$) when compared with control group. Further, at doses 500, 1000 and 2000 mg/kg, levels of BUN (mg/dL) as well as CRE (mg/dL) showed no significant difference as compared to control group BUN and CRE levels.

Effect of topical application of UNIM-003 on biochemical parameters in male rats: In male rats, UNIM-003 at different doses 500, 1000 and 2000 mg/kg showed non-significant difference in levels of ALT (IU/L) as compared to control group. Similarly, the levels of AST (IU/L) at different doses showed no significant difference in animals as compared to AST (IU/L) of control group. Further, at doses 500, 1000 and 2000 mg/kg, levels of BUN (mg/dL) as well as CRE (mg/dL) showed no significant difference as compared to control group in both BUN as well as CRE levels.

Complete blood cells count (CBC) in female rats after treatment with UNIM 003 formulation: UNIM-003 was administered in female rats at doses of 500, 1000 and 2000 mg/kg and CBC has been recorded. At different doses, RBC count (mil/ µL), HB (g/dL), Haematocrit (%), Mean Corpuscular Volume (fL), Mean Corpuscular Haemoglobin (pg), Mean Corpuscular Haemoglobin Concentration (g/dL), Red Cell Distribution Width (%), Platelet Count (thou/µL), Mean Platelet Volume (fL),WBC Count (thou/µL), Eosinophils (%), Segmented Neutrophils (%), Lymphocytes (%) and Monocytes (%) showed no significant variation as compared to control group which was administered with water.

Complete blood cell count (CBC) in male rats after treatment with UNIM- 003: UNIM-003 was administered in male rats at doses of 500, 1000 and 2000 mg/kg and CBC has been recorded. At different doses, RBC count (mil/ µL), HB (g/dL), Haematocrit (%), Mean Corpuscular Volume (fL), Mean Corpuscular Haemoglobin (pg), Mean Corpuscular Haemoglobin Concentration (g/dL), Red Cell Distribution Width (%), Platelet Count (thou/µL), Mean Platelet Volume (fL),WBC Count (thou/µL), Eosinophils (%), Segmented Neutrophils (%), Lymphocytes (%) and Monocytes (%) showed no significant variation as compared to control group which was administered with water.

Histopathology Reports of Subacute Toxicity Study

At the conclusion of the study, the rats were euthanized, and histopathological examinations were conducted on vital organs.

Liver: In normal rats, the liver exhibited hexagonal or pentagonal lobules with central veins and peripheral hepatic triads or tetrads embedded in connective tissue. Hepatocytes were organized in radiating trabeculae from the central vein, separated by sinusoids containing Kupffer cells. These hepatocytes displayed regularity, featuring a large spheroidal nucleus with a distinctly marked nucleolus and peripheral chromatin distribution. Some cells possessed two nuclei each. The liver of control rats displayed a normal architecture.

Heart: In the HE staining, the normal group shows arranged rat myocardial cells having neat, compact, clear structure, less extracellular matrix, and a small amount of fibroblasts. The myocardium in normal group also showed well- organized myofibrils with long cylindrical mononucleated cells. The section showed muscle fibres with striations in cytoplasm and elongated nuclei. The section of rat heart showed no irregularities after the administration of UNIM-003 in all the three doses of 500, 1000 and 2000 mg/kg bw.

Kidney: In control rats, the kidney displayed a normal structure in both the cortex and medulla. Collecting tubules were lined with relatively low simple cubic epithelium. Cross-sections revealed the thick descending and ascending parts of Henle's loops, collecting coils of small caliber, and a small amount of interstitial tissue. Following the administration of UNIM-003 at doses of 500, 1000, and 2000 mg/kg, the kidney sections showed no discernible change in the normal architecture in comparison to the control group.

Stomach: The gastric mucosa forms the innermost lining of the stomach, constituting the largest and widest portion of the stomach wall. Internally, it is covered by a simple layer of columnar epithelial cells with prominent nuclei. The bulk of the gastric mucosa is occupied by gastric glands situated at the luminal surface of the epithelial lining, opening singly or in groups. The basal portions of these glands consist of chief (zymogenic) cells, occasionally accompanied by parietal cells, while the neck of each gland contains both mucous neck cells and parietal cells.

Surface Epithelial (Mucous) Cells: These cells form the lining epithelium in direct contact with the stomach lumen. They are irregular in shape, tend to be pyramidal with an ovoid nucleus located basally, surrounded by a clear cytoplasmic mass. The apical portions of these cells contain dense discrete granules.

Chief (Zymogenic) Cells: Mainly located at the bases of gastric glands, these cells are responsible for secreting pro-pepsinogen. They have a conical or pyramidal outline, with basophilic cytoplasm and basally situated spherical nuclei.

Parietal (Oxyntic) Cells: Known as acid-forming cells, these cells are the primary secretors of hydrochloric acid in the stomach. They are scattered among other cell types and are large in size, with a spherical or pyramidal outline, acidophilic cytoplasm, and centrally spherical nucleus.

Argentaffin (Enteroendocrine) Cells: Found at the bases of gastric glands, these small-sized cells have conical or pyramidal shapes. The cytoplasm contains secretory granules at the basal regions with spherical nuclei in the basal regions.

Following the administration of UNIM-003 to Wistar rats at doses of 500, 1000, and 2000 mg/kg, there was no discernible change in the normal architecture of the stomach in comparison to the control group. Photomicrographs of the stomach wall from the treatment (test) group animals showed an intact surface lining epithelium and superficial glands in the stomach mucosa, similar to the control group.

Testes: The testes were evaluated for various parameters, including haemorrhage, edema, vascular congestion, polymorphonuclear leukocyte infiltration, interstitial fibrosis, basal membrane thickening, Leydig cell proliferation, degeneration of seminiferous epithelium, tubular atrophy, and necrosis. In the control group, where only distilled water was administered, the testes exhibited normal histology, including the arrangement of seminiferous tubules, sperms, and interstitial cells of Leydig.

After the administration of UNIM-003 at doses of 500, 1000, and 2000 mg/kg, there was no observed change in the normal architecture of the testes in relation to the control group.

Ovary: In the normal rat ovary section, features such as unilaminar primary follicles, antral follicles, a previously formed corpus luteum with foamy eosinophilic cells, primordial follicles, stromal fibroblasts, and smooth

muscle cells were observed. The section also revealed numerous, irregular, tortuous, and wide-lumen endometrial glands, along with an extensive fold of the luminal epithelium. The luminal epithelium consisted of a single layer of columnar epithelial cells with large nuclei at the basal aspect.

The ovary of the control rat exhibited a normal architecture. Photomicrographs of ovary sections from both the control and treatment group animals showed normal lumen endometrial glands and luminal epithelium.

Conclusion

Topical application of UNIM-003 did not result in any significant change in organ weight when compared with the control group. No toxic effects were observed, as evidenced by biochemical, hematological parameters, and histopathological studies. The results confirm that UNIM-003 is safe for topical application in both sexes of Wistar rats.

Sub-chronic Dermal Toxicity Study of UNIM-003 in Wistar Rats

A repeated dose 90-day dermal toxicity study of UNIM-003 was conducted in Wistar rats to assess its effects on morphological features, gross behaviour, body weight changes, as well as to investigate haematological, biochemical, and histopathological changes.

Materials & Methods

UNIM-003 was provided by the Central Council for Research in Unani Medicine, New Delhi. Ten female and ten male Wistar rats were utilized, with dose levels tested at 0 (control), 500, 1000, and 2000 mg/kg body weight. Daily cage-side observations, noting changes in morphology and behavioural changes, were recorded, and body weights were noted weekly. At the end of the study, haematological, biochemical, and pathological changes (macroscopic and microscopic) were examined.

The test substance was suspended in water and applied at three dose levels (500, 1000, 2000 mg/kg body weight) on the shaved skin. The fur on the dermal surface of each animal was closely clipped 24 hours before treatment. The test substance was directly applied to a small area (4x5 cm) of skin. Following application, the test area was covered first with a non-occlusive dressing (a gauze patch) and then a semi-occlusive bandage for 24 hours.

Table 54: Brief description of study protocol for UNIM-003

Name of the Study	Sub-chronic Study on UNIM-003
Test material	UNIM-003
Animal model	Wistar rats
Age	11–12 weeks
Weight	250 g (average)
Animal procured from	Central Animal Facility, Jamia Hamdard (173/CPCSEA)
Sex	Male and female
Number of animal per group	10 per sex in each group
Groups	04
Route of administration	Topical application
Dose volume	2 mL per 100 g body weight per animal
Number of administration	Single dose per day
Concentration of dose	500, 1000, 2000 mg per kg body weight
Vehicle for administration	Water (double distilled)
Study duration	90 days

Observations

At the end of the exposure period, any residual test substance was removed by distilled water, and each animal was observed daily for signs of toxicity at the application site, changes in fur, eyes, mucous membrane, and any other overt signs of toxicity, including behavioural changes. Mortality and morbidity checks were performed daily.

On the 91st day, surviving rats were sacrificed, and necropsies were performed. All the gross observations were recorded. Individual weights of animals were determined before the test substance was administered and at weekly intervals. Weight changes were calculated and recorded. At the end of the study, surviving animals were weighed and then humanely sacrificed.

Haematology and Clinical Biochemistry

At the end of the test period, blood samples were collected just prior to killing the animals, and haematological and clinical biochemistry determinations were conducted to identify major toxic effects in blood and tissues, specifically focusing on effects on the kidney and liver.

Results

Table 55: Effect of topical application of UNIM-003 on body weight and organ weight in male rats

Body & Organ Weight	Control	UNIM-003 (500 mg/kg)	UNIM-003 (1000 mg/kg)	UNIM-003 (2000 mg/kg)
Body weight (g)	288.5 ± 17.803	277 ± 18.28	290 ± 37.93	278.5 ± 15.28
Heart	0.358 ± 0.045	0.359 ± 0.029	0.398 ± 0.096	0.322 ± 0.020
Left kidney	0.311 ± 0.040	0.327 ± 0.020	0.292 ± 0.032	0.300 ± 0.396
Liver	3.29 ± 0.28	2.64 ± 0.159	3.04 ± 0.24	3.00 ± 0.396
Stomach	0.856 ± 0.151	0.846 ± 0.154	0.565 ± 0.141	0.628 ± 0.139
Testes	1.77 ± 0.153	1.68 ± 0.173	1.429 ± 0.16	1.64 ± 0.20

Mean values of 10 male rats ± standard deviation; No significant difference was observed in any parameter; Dunnett's test. Control group was given water.

Table 56: Effect of topical application of UNIM-003 on body weight and organ weight in female rats

Body & Organ Weight	Control	UNIM-003 500 (mg/kg)	UNIM-003 1000 (mg/kg)	UNIM-003 2000 (mg/kg)
Body weight (g)	285 ± 14.33	267 ± 15.12	272.5 ± 13.99	275.5 ± 17.07
Heart (g)	0.33 ± 0.03	0.31 ± 0.02	0.29 ± 0.01	0.34 ± 0.09
Left kidney (g)	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.28 ± 0.03
Liver (g)	3.15 ± 0.26	3.11 ± 0.29	2.85 ± 0.19	3.04 ± 0.26
Stomach (g)	0.72 ± 0.18	0.72 ± 0.20	0.54 ± 0.15	0.45 ± 0.06
Ovary (g)	0.41 ± 0.09	0.40 ± 0.10	0.44 ± 0.10	0.37 ± 0.06

Mean values of 10 female rats ± standard deviation; No significant difference was observed in any parameter; Dunnett's test. Control group was given water.

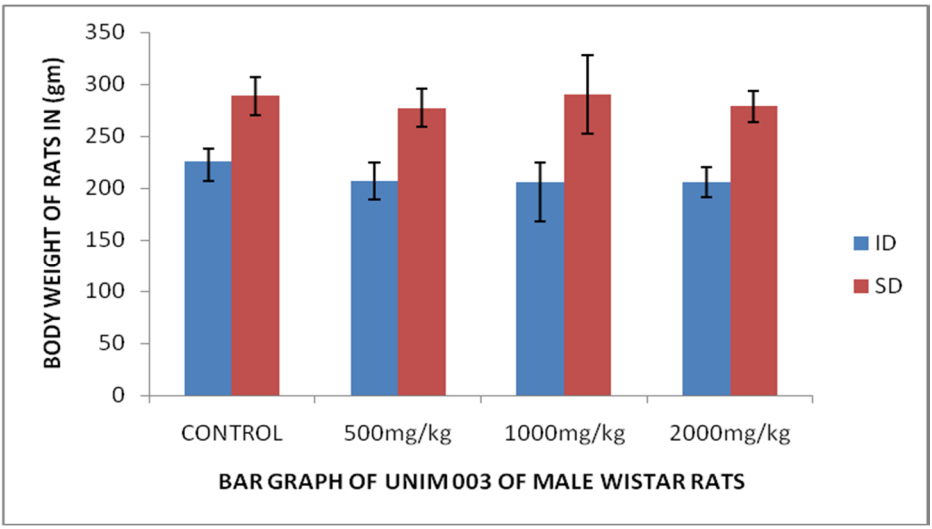


Figure 14: *Change in body Weight of Rats*

Comparison of the body weight of the Albino Wistar rat from the date of starting and termination of the experimental schedules. Body weights were shown as mean value ± standard deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment (male group)

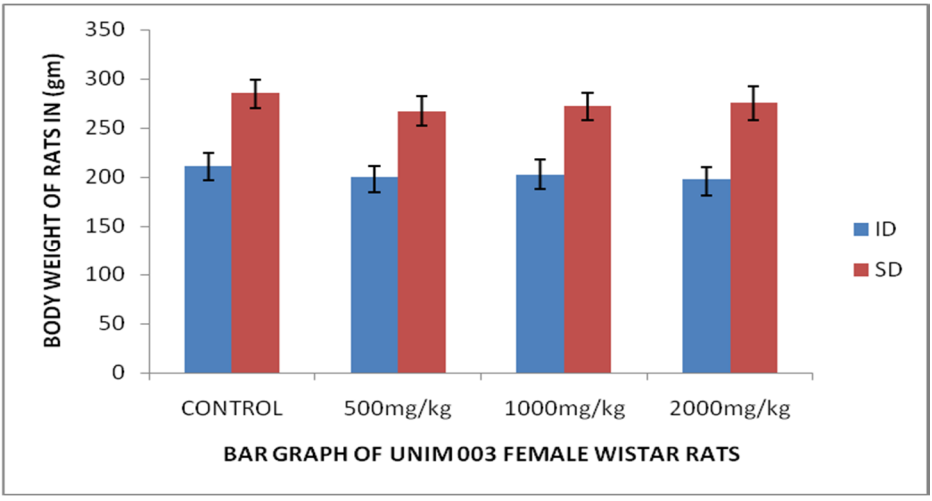


Figure 15: *Change in body weight of rats*

Comparison of the body weight of the Albino Wistar rats from the date of starting and termination of the experimental schedules. Body weights were showed as mean value ± standard deviation. ID = Initial date of experiment, SD = Sacrificed date of experiment (Female Group)

Table 57: Effect of topical application of UNIM-003 on biochemical parameters in female rats

Parameters	Control	UNIM-003 (500 mg/kg)	UNIM-003 (1000 mg/kg)	UNIM-003 (2000 mg/kg)
ALT (IU/L)	48.84 ± 28.56	62.53 ± 51.85	90.80 ± 51.85	65.52 ± 49.58
AST (IU/L)	47.43 ± 29.79	59.89 ± 36.00	58.32 ± 21.77	60.65 ± 36.02
BUN (mg/dl)	20.31 ± 23.53	10.21 ± 8.93	12.64 ± 8.19	11.01 ± 8.19
CRE (mg/dl)	2.15 ± 1.99	1.40 ± 1.62	0.89 ± 0.43	0.59 ± 0.26

Mean values of 10 female rats ± standard deviation; No significant difference was observed in any parameter; Dunnett's test. Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 58: Effect of topical application of UNIM-003 on biochemical parameters in male rats

Parameters	Control	UNIM-003 (500 mg/kg)	UNIM-003 (1000 mg/kg)	UNIM-003 2000 (mg/kg)
ALT (IU/L)	50.29 ± 31.47	46.51 ± 29.25	55.93 ± 40.91	78.87 ± 33.41
AST (IU/L)	68.81 ± 50.13	77.26 ± 47.71	64.63 ± 44.96	73.93 ± 49.84
BUN (mg/dl)	14.91 ± 6.34	10.37 ± 7.57	12.90 ± 6.00	9.89 ± 6.56
CRE (mg/dl)	0.79 ± 0.34	0.70 ± 0.64	1.00 ± 0.53	1.01 ± 0.62

Mean values of 10 male rats ± standard deviation; No significant difference was observed in any parameter; Dunnett's test. Control group was given water. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, BUN = Blood urea nitrogen, CRE = Creatinine

Table 59: Complete blood cell count (CBC) in male rats after treatment with UNIM-003

Parameters	Control	UNIM-003 (500 mg/kg)	UNIM-003 (1000 mg/kg)	UNIM-003 (2000 mg/kg)
RBC Count (mil/µL)	7.323 ± 0.763	7.33 ± 0.76	7.32 ± 1.00	7.21 ± 0.75
HB (g/dL)	12.18 ± 1.34	13.36 ± 1.28	12.88 ± 1.47	13.25 ± 0.56
Haematocrit (%)	34.79 ± 1.69	37.62 ± 6.15	37.37 ± 6.20	37.18 ± 6.53
Mean Corpuscular Volume (fL)	58.31 ± 7.39	57.94 ± 9.04	59.17 ± 4.90	57.21 ± 10.44
Mean Corpuscular Haemoglobin (pg)	17.75 ± 2.01	17.57 ± 1.36	17.01 ± 2.73	18.4 ± 2.64
Mean Corpuscular Haemoglobin Concentration (g/dL)	34.18 ± 4.77	33.9 ± 2.56	36.77 ± 3.43	36.73 ± 2.80
Red Cell Distribution Width (%)	16.15 ± 1.26	16.1 ± 1.74	16.92 ± 1.75	16.35 ± 1.89
Platelet Count (thou/µL)	745.1 ± 127.54	763.1 ± 112.67	728.6 ± 87.00	853.33 ± 52.66**
Mean Platelet Volume (fL)	6.57 ± 0.89	7.14 ± 1.28	6.96 ± 1.70	7.06 ± 0.86
WBC Count (thou/µL)	4.12 ± 0.73	5.17 ± 3.58	6.37 ± 1.40	7.98 ± 3.05
Segmented Neutrophils (%)	26 ± 9.91	32.8 ± 8.02	31.8 ± 10.92	25.88 ± 11.98
Lymphocytes (%)	68.5 ± 21.15	73.1 ± 15.17	70 ± 18.87	69.77 ± 16.05
Monocytes (%)	3 ± 2.21	3.4 ± 2.71	3.1 ± 1.72	2.77 ± 3.11

Mean values of 10 male rats ± standard deviation; **P < 0.01 vs. control group (Dunnett's test). Control group was given water.

Table 60: Complete blood cell count (CBC) in female rats after treatment with UNIM-003

Parameters	Control	UNIM-003 (500 mg/kg)	UNIM-003 (1000 mg/kg)	UNIM-003 (2000 mg/kg)
RBC Count (mil/ µL)	6.89 ± 1.18	6.89 ± 0.74	6.756 ± 0.62	7.364 ± 1.08
HB (g/dL)	12.1 ± 1.11	13.62 ± 2.02	11.97 ± 1.08	12.17 ± 1.44
Haematocrit (%)	39.94 ± 7.12	43.97 ± 8.51	42.3 ± 7.20	40.87 ± 5.09
Mean Corpuscular Volume (fL)	59.01 ± 5.82	61.34 ± 6.86	60 ± 6.40	59.89 ± 7.81
Mean Corpuscular Haemoglobin (pg)	16.33 ± 2.19	18.87 ± 3.11	17.4 ± 0.79	18.25 ± 1.88
Mean Corpuscular Haemoglobin Concentration (g/dL)	33.01 ± 1.35	33.58 ± 2.64	31.18 ± 2.17	34.84 ± 2.15
Red Cell Distribution Width (%)	14.36 ± 0.91	14.78 ± 0.76	15.44 ± 1.91	15.31 ± 1.31
Platelet Count (thou/µL)	455.7 ± 115.44	615.7 ± 51.5	662.6 ± 110.5	594.1 ± 62.82
Mean Platelet Volume (fL)	6.31 ± 0.57	6.84 ± 1.27	7.43 ± 1.10	6.98 ± 0.93
WBC Count (thou/µL)	3.67 ± 1.58	4.26 ± 1.45	4.43 ± 1.81	4.78 ± 1.51
Segmented Neutrophils (%)	53.2 ± 18.47	42.9 ± 16.65	51.3 ± 14.53	53.9 ± 10.85
Lymphocytes (%)	38.7 ± 9.71	44.1 ± 14.33	36.3 ± 7.54	40.1 ± 13.79
Monocytes (%)	8.7 ± 3.88	6 ± 2.2	6.5 ± 3.20	7.3 ± 3.43

Mean values of 10 female rats ± standard deviation; Dunnett's test. No significant difference was observed in any parameters. Control group was given normal saline water.

Histopathology Reports of Sub-chronic Toxicity Study

At the end of study, the rats were sacrificed and histopathological studies of vital organs were performed.

Liver: In the normal rat, the liver is comprised of hexagonal or pentagonal lobules featuring central veins and peripheral hepatic triads or tetrads embedded in connective tissue. Hepatocytes are organized in trabeculae radiating from the central vein and are separated by sinusoids containing Kupffer cells. These hepatocytes exhibit regularity, with a large spheroidal nucleus, a distinctly marked nucleolus, and peripheral chromatin distribution. Some cells may contain two nuclei each. The liver of the control rat demonstrated a normal architecture.

Heart: Under H&E staining, the normal group exhibited well-arranged rat myocardial cells with a neat, compact, and clear structure, minimal extracellular matrix, and a small number of fibroblasts. The myocardium in the normal group displayed well-organized myofibrils within long cylindrical mononucleated cells. The section revealed muscle fibres with cytoplasmic striations and elongated nuclei.

Following the administration of UNIM-003 at doses of 500, 1000, and 2000 mg/kg, the sections of rat hearts did not manifest any irregularities. The structural integrity and characteristics of the myocardium remained unchanged in all three respective doses.

Kidney: Normal structure of the cortex and medulla was observed in the kidney of control rats. Collecting tubules are lined with the relatively low simple cubic epithelium. The thick descending and ascending parts of Henle's loops and collecting coils of small calibre, and a small amount of interstitial tissue were seen in the cross-sections

UNIM-003 was administered to the Wistar rats in the dose of 500, 1000 and 2000 mg/kg in each separate group as described above. After the administration of UNIM-003, there was no change in normal architecture of kidney in relation to the control group.

Stomach: The gastric mucosa constitutes the innermost lining of the stomach, forming the largest and widest portion of the stomach wall. Internally, the gastric mucosa is covered by a simple layer of columnar epithelial cells with prominent nuclei. Gastric glands, situated at the luminal surface of the epithelial lining, occupy the bulk of the gastric mucosa, opening singly or in groups with specific apertures. The basal portions of these glands consist of chief (zymogenic) cells, occasionally accompanied

by parietal cells, while the neck of each gland contains both mucous neck cells and parietal cells.

Surface Epithelial (Mucous) Cells: These cells make up the lining epithelium in direct contact with the stomach lumen. Irregular in shape, they tend to be pyramidal with an ovoid nucleus located basally, surrounded by a clear cytoplasmic mass. The apical portions of these cells contain dense discrete granules.

Chief (Zymogenic) Cells: Mainly located at the bases of gastric glands, these cells are responsible for secreting pro-pepsinogen. They have a conical or pyramidal outline, with basophilic cytoplasm and basally situated spherical nuclei.

Parietal (Oxyntic) Cells: Known as acid-forming cells, these cells are the primary secretors of hydrochloric acid in the stomach, scattered among other cell types. Parietal cells are large, with a spherical or pyramidal outline, acidophilic cytoplasm, and centrally situated spherical nuclei.

Argentaffin (Enteroendocrine) Cells: Found at the bases of gastric glands, these small-sized cells have conical or pyramidal shapes. The cytoplasm contains secretory granules at the basal regions, with spherical nuclei in the basal regions.

UNIM-003 was administered to Wistar rats at doses of 500, 1000, and 2000 mg/kg in separate groups as described above. Following the administration of UNIM-003, there was no discernible change in the normal architecture of the stomach in relation to the control group. Photomicrographs of the stomach wall from the treatment (test) group animals showed an intact surface lining epithelium and superficial glands in the stomach mucosa, similar to the control group.

Testes: The evaluation of the testes included an assessment for the presence of haemorrhage, edema, vascular congestion, polymorphonuclear leukocyte infiltration, interstitial fibrosis, basal membrane thickening, Leydig cell proliferation, degeneration of seminiferous epithelium, tubular atrophy, and necrosis.

In the Wistar rat group, where only distilled water was administered, the results revealed normal histological features of the testes. The testes section exhibited a typical arrangement of seminiferous tubules, sperm, and interstitial cells of Leydig.

UNIM-003 was administered to separate Wistar rat groups at doses of 500, 1000, and 2000 mg/kg, as described above. Following the administration of UNIM-003, there were no discernible changes in the normal architecture of the testes in comparison to the control group.

Ovary: In the normal rat ovary section, unilaminar primary follicles, antral follicles, a previously formed corpus luteum with foamy eosinophilic cells, primordial follicles, stromal fibroblasts, and smooth muscle cells were observed. Additionally, the normal section revealed numerous, irregular, tortuous, and wide-lumen endometrial glands, along with an extensive fold of the luminal epithelium. The luminal epithelium consisted of a single layer of columnar epithelial cells with large nuclei at the basal aspect.

The ovary of the control rat exhibited a typical architecture. Photomicrographs of ovarian sections from both the control and treatment group animals depicted normal lumen endometrial glands and luminal epithelium.

Conclusion

The topical application of UNIM-003 in both male and female Wistar rats demonstrated no significant alterations in haematological and biochemical parameters when compared to the control group. No discernible toxic effects were observed, as supported by biochemical, haematological, and histopathological studies. The findings affirm the safety of UNIM-003 for topical application on the skin in both sexes of Wistar rats.

PART – B

CLINICAL TRIAL

Study Rationale

Vitiligo poses a significant and persistent cosmetic challenge, often labelled as a social stigma, warranting increased attention and comprehensive exploration for effective solutions. This condition reaches a substantial magnitude as individuals afflicted by vitiligo encounter obstacles in securing matrimonial alliances and frontline jobs that necessitate frequent public interactions, resulting in their social isolation.

Compounding the issue, there is a notable absence of a universally recognized safe and effective treatment for vitiligo within the prevailing medical systems practiced in India. Consequently, there is a compelling need to scientifically investigate the treatment principles outlined in the classical texts of Unani Medicine.

While Psoralen stands out as the primary treatment in modern medicine, its effectiveness, safety, and tolerability have been extensively documented in numerous studies. Recognizing both the safety considerations associated with Psoralen and the inherent limitations of re-pigmentation, this study was devised to assess the safety and efficacy of Unani compound formulations in comparison to the standard Psoralen treatment for promoting re-pigmentation in depigmented maculae.

Study Objectives

Primary

- To assess the safety of Unani formulations in comparison with Psoralen (hepato-renal and cardiac), recording the incidence of adverse effects, both systemic and topical.
- To investigate the efficacy of Unani formulations in re-pigmenting depigmented maculae compared to the standard treatment Psoralen.

Secondary

- To examine the impact of clinical factors on the course of treatment.

Study Design

Randomized, single-blind, parallel-group, comparative study.

Overview of Study Design

The multi-centric study, conducted at various centres under the CCRUM, adopted a randomized, single-blind design, where the assessor remained unaware of the treatment allocation. It was a comparative study involving two active formulations—oral and topical. Patients were randomly assigned to one of these two treatment groups.

Selection Criteria

Inclusion Criteria

- Patients clinically diagnosed with segmental and non-dermatomal vitiligo.
- Subjects aged 12-50 years.
- Either gender.
- Any duration, site, extension, and distribution of lesions.
- Willingness to provide written informed consent.

Exclusion Criteria

- Subjects undergoing active vitiligo treatment with other drugs/systems.
- Unwilling participants.
- History of drug or alcohol abuse, chronic smokers not willing to abstain during the study.
- Clinically significant abnormalities identified in physical examination or laboratory tests.
- Subjects with systemic or other skin diseases.
- Known allergies.
- Impaired cardiac, hepatic, and renal function.
- History of malignancy.
- Concomitant use of other antioxidants.
- Hypersensitivity to investigational drugs/herbal medicine.
- Receipt of any other investigational product within the past 4 weeks.
- Medical conditions where participation could be detrimental.
- Uncontrolled infection.
- Pregnant and lactating women.

Endpoints

- Evaluate cosmetically acceptable re-pigmentation with a 40%-50% decrease in VASI score.
- Assess overall safety, toxicity, and acceptability of Unani drugs.
- Determine the percentage of study participants responding to Unani treatment and Psoralen.
- Identify the time of initial re-pigmentation and subsequent re-pigmentation rate.
- Study post-treatment retention of re-pigmentation for at least 3 months.

Materials & Methods

The study was conducted at various CCRUM centres, enrolling clinically diagnosed vitiligo patients of different ages, chronicities, and genders. A total of 518 participants (262 in the test group, 256 in the control group) were included. Informed consent was obtained, and a block randomization technique was used for treatment allocation.

Patients of either gender with 12–50 years of age and clinically diagnosed segmental and non-dermatomal vitiligo were enrolled in the study. In order to ascertain and avoid bias, it was ensured that no patient had received either the topical or systemic anti-vitiligo therapy for a period of 4 weeks, prior to the entry into the trial. Pregnant women, lactating mothers, patients with cardio-pulmonary, hepato-renal malfunctions were excluded from the study. Study participants were asked not to take any anti-vitiligo therapy during the trial period. At the outset, study participants were diagnosed and classified as per their somatic appearance as localized and generalized vitiligo. Further they were sub classified as segmental, mucosal, focal, acrofacial, and non-dermatomal mixed vitiligo.

Informed consent was obtained from the participants after informing them about the study trial and its possible outcome.

Block randomization technique was used to allocate the treatment schedule to the participants. Pre-randomization was done by using statistical software Graph pad. Participants were allocated to the treatment group by pre-randomized schedule. The study was carried out in two groups. One group received UNIM-001 (oral) and UNIM-003 (local) and another group received Psoralen (oral & local).

All the parameters were recorded at baseline, and clinical follow up was done once in a month for evaluation of re-pigmentation. Digital photographs of de-pigmented parts were taken and recorded. Response was evaluated as follicular and perilesional re-pigmentation over the de-pigmented areas. Rate of re-pigmentation was assessed. Assessment of *Mizāj* and dermatology life quality index (DLQI) was recorded initially and at the end of the study. Safety of the intervention was assessed through monitoring of haematological, biochemical parameters and ECG recording.

Study participants were instructed to expose themselves to sunlight for half an hour to two hours after ingestion of oral drugs. The exposure time was adjusted according to the skin sensitivity of an individual which could range from 3–30 minutes. The systemic and topical treatments were coded as UNIM-001+UNIM-003 and Psoralen. Investigational product UNIM-001 and Psoralen were in tablet form given orally in the dose of two to four tablets, two-three times a day; each tablet was of 800 mg and 10 mg respectively. Whereas UNIM-003 and topical Psoralen were in lotion form given for topical application for a duration of 8 months.

Study Sites

The study was conducted at the following centres:

- National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad (formerly known as CRIUM, Hyderabad)
- Regional Research Institute of Unani Medicine (RRIUM), New Delhi
- RRIUM, Srinagar
- RRIUM, Aligarh
- RRIUM, Chennai

Investigational Drugs

- UNIM-001 and Psoralen tablet were given orally in the dose of one to four tablets, two-three times a day; each tablet of 800 mg and 10 mg respectively.
- UNIM-003 and topical Psoralen were in lotion form, given for topical application.

The Unani formulations were prepared at the GMP-certified pharmacy of NRIUMSD, Hyderabad.

Table 61: Composition of UNIM-001 (Tablet)

S. No.	Ingredients	Part used	Botanical name
1.	*Bābchī*	Seed	*Psoralea corylifolia* L.
2.	*Zanjabīl*	Rhizome	*Zingiber officinale* Roscoe
Total weight = 800 mg			

Table 62: Composition of UNIM-003 (Lotion)

S. No.	Ingredients	Part used	Botanical /Scientific name
1.	*Bābchī*	Seed	*Psoralea corylifolia* L.
2.	*Gerū*	-	Silicate of alumina, oxides of iron
3.	*Gandhak*	-	Sulphur
4.	*Gulnār*	Flower	*Punica granatum* L.

Table 63: Treatment regimen for investigational drug UNIM-001

Age (yr)	Weight (kg)	Dose (mg)	Dose (mg*) with frequency
12–18	30–50 ± 5%	1600	1 b.i.d.
>18–30	50–60 ± 5%	2400	1 t.i.d.
>30–50	60–70 ± 5%	3200	2 b.i.d

**Each tablet of 800 mg

Table 64: Treatment regimen for comparator oral Psoralen

Age (yrs)	Weight (kg)	Dose (mg)**	Frequency
12–18	30–50 ± 5%	20	(2 tablets) Single dose
>18–30	50–60 ± 5%	30	(3 tablets) Single dose
>30–50	60–70 ± 5%	40	(4 tablets) Single dose

**Each tablet of 10 mg

For Topical Application

To be applied every alternate day followed by exposure to sun light. The exposure time was fixed according to an individual's sensitivity. Initially, study participants were advised to apply on one patch and expose to sunlight at least for 3–5 minutes in order to ascertain the sensitivity of an individual. Based on that, further applications were planned and advised.

Photographs of Ingredients of UNIM-001 & UNIM-003

Figure 16: *Bābchī* (*Psoralea corylifolia* L.)

Figure 17: *Zanjabīl* (*Zingiber officinale* Roscoe)

Figure 18: *Gulnār* (*Punica granatum* L.)

Figure 19: *Gandhak* (Sulphur)

Figure 20: *Gerū* (Oxides of iron)

Follow-up Evaluation

The participants were assessed clinically every month for 8 months. The clinical observations were recorded in the follow-up sheets and digital photographs were taken at each visit. Post-treatment follow-up was done for 3 months.

Efficacy Evaluation

By clinical observation, digital photography and Vitiligo Area Severity Index (VASI) score.

Primary efficacy variable: Re-pigmentation was evaluated in relation to age at treatment, sex, chronicity of disease, type of vitiligo, site, extension, and distribution of lesions.

Safety Evaluation

ADR/ADE if any was reported. Safety parameters (Haemogram, LFT, KFT, Urine & stool examinations) were recorded once in a month, i.e. at every follow-up, to monitor the safety of the investigation product. ECG was also recorded.

Data and Statistical Analyses

Baseline and follow-up values of clinical subjective parameters, pathological and biochemical parameters were statistically analysed using unpaired t-test, repeated measures of ANOVA and mixed ANOVA model. The result was expressed as the Mean ± SEM. $p<0.05$ has been considered as statistically significant and $p<0.01$ and $p<0.001$ have been considered as statistically highly significant.

Observations and Results

The primary objective of this study was to investigate the efficacy and safety of Unani formulations in re-pigmenting depigmented macules, comparing them with a standard treatment.

Out of the total screened patients, 518 participants successfully completed the trial. The randomization process led to the formation of a test group (n=256) and a control group (n=262). The test group received UNIM-001 orally (in tablet form, 800 mg) and UNIM-003 topically (in lotion form), while the control group was administered Psoralen orally (in tablet form, 10 mg) and topically (in lotion form). Both groups underwent treatment for a duration of 8 months.

Demographic Details, Personal History, Chronicity

In this trial, a total of 518 patients successfully completed the study. The age range of the participating population spanned from 12 to 50 years, with a mean age of 25.42 ± 0.46 years. The median chronicity revealed that half of the patients had been enduring the condition for more than 4 years (Table 65).

Table 65: General distribution of patients

S. No.	Characteristics	Number of Cases
1.	Male	235 (45%)
	Female	283 (55%)
	Total cases	518
2.	Age (Mean ± SEM)	25.42 ± 0.46
	Age (Range) in years	12–50
3.	Chronicity (Median) in years	4.00

The majority of patients (42%) were in the 12–20 age group, followed by 21–30 years (32%) and 31–40 years (15%). The 41–50 age group had the least number of patients (10%) (Table 66).

Table 66: Age-wise distribution of patients

Age Group (Years)	Total	Percentage (%)
12–20	218	42.08
21–30	166	32.05
31–40	81	15.64
41–50	53	10.23
Total	518	100
Mean ± SEM	25.42 ± 0.46	

Females (55%) dominated, with a mean age of 24.4 ± 0.58 years. Males constituted 45%, with a mean age of 26.66 ± 0.72 years (Table 67, Fig. 21).

Table 67: Gender-wise distribution of patients

Gender	Number of Cases	Percentage (%)	Age in Year (Mean ± SEM)
Male	235	45.37	26.66 ± 0.72
Female	283	54.63	24.4 ± 0.58
Total	518	100	25.42 ± 0.46

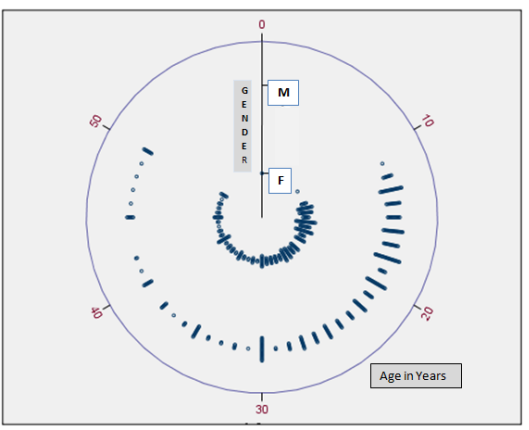


Figure 21

Only 2% were smokers, 1.7% tobacco users, and 1.3% alcoholics (Table 68).

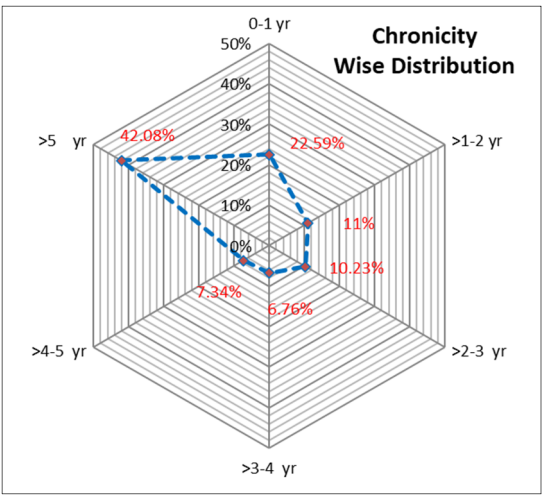
Table 68: Habit-wise distribution of patients

Social & Occupational History		Number of Cases	Percentage (%)
Smoking habit	No	507	97.88
	Yes	11	2.12
Tobacco chewing habit	No	509	98.26
	Yes	9	1.74
Alcoholic	No	511	98.65
	Yes	7	1.35
Drug addict	No	518	100
	Yes	-	-

Maximum patients (42.8%) had vitiligo for more than 5 years, and the mean chronicity was 6.33 ± 0.29 years. The average age at onset was 19.05 ± 0.6 years (Table 69, Fig. 22).

Table 69: Chronicity and age at onset

Chronicity of Disease (Year)	Number of Cases	Percentage (%)
0–1	117	22.59
>1–2	57	11
>2–3	53	10.23
>3–4	35	6.76
>4–5	38	7.34
>5	218	42.08
Total	518	100
Chronicity of Disease (Year) (Mean ± SEM)	6.33 ± 0.29	
Age at onset (in years) (Mean ± SEM)	19.05 ± 0.6	



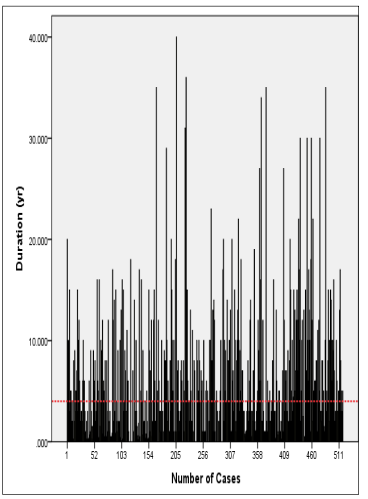


Figure 22

Assessment based on classical Unani parameters revealed 64.48% had *Balghamī Mizāj* followed by 22.39% having *Damawī*, 11% *Ṣafrāwī* and 2.12% *Sawdāwī Mizāj* (Table 70, Fig. 23).

Table 70: Distribution of patients according to Temperament

Mizāj (Temperament)	Number of Cases	Percentage (%)
Damawī (Sanguine)	116	22.39
Balghamī (Phlegmatic)	334	64.48
Ṣafrāwī (Bilious)	57	11
Sawdāwī (Melancholic)	11	2.12
Total	518	100

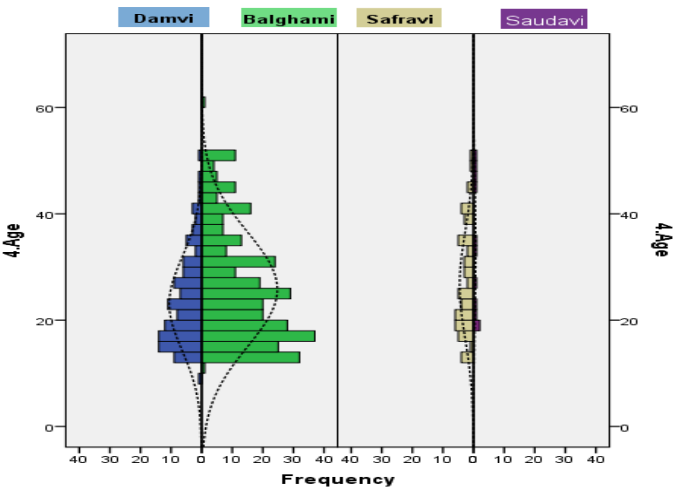


Figure 23

Distribution According to Vitiligo Associated Parameters

Out of total 518 patients, 291 (56%) patients belonged to the non-dermatomal type, whereas 227 (44%) belonged to the dermatomal type. While 63% had non-extensive lesions, 37% had extensive lesions. Total 167 (32%) patients presented with unilateral distribution, and 351 (68%) with bilateral distribution, of which 215 were having bilaterally symmetrical type distribution and 136 were having bilaterally asymmetrical distribution of lesions (Table 71).

In terms of skin colour, 28% had fair skin, 26% whitish, 26% dark, and 21% very fair skin. Maximum number of patients, i.e. 133 (26%) were having grade-5 skin sensitivity whereas minimum 27 (5%) were having grade-6 skin sensitivity (Table 71).

A positive history of spontaneous re-pigmentation was observed in 75 (14%) cases only, while. 443 (86%) patients did not give any such history. Total 436 (84%) patients gave negative family history while only 82 (16%) patients gave a positive family history (Table 71).

In 17 (3.2%) cases, pregnancy was found to be an aggravating factor whereas only 4 (0.7%) cases were found associated with certain auto-immune disorders. In 2 (0.3%) cases, stress was found to be an aggravating factor. Traumatic injury was an aggravating factor in 1.7%, whereas 78% had no identifiable aggravating factor. Six patients (1%) had a positive history of exposure to certain chemicals (Table 71).

Table 71: Distribution according to vitiligo associated parameters

Parameters		Number of Cases	Percentage (%)
Type of vitiligo	Dermatomal	227	43.82
	Non-Dermatomal	291	56.18
Extension of lesion(s)	Extensive	190	36.68
	Non-extensive	328	63.32
Distribution	Unilateral	167	32.24
	Bilateral	351	67.76
	Bilateral symmetrical	215	
	Bilateral asymmetrical	136	
Type of skin	Very fair	107	20.66
	Fair	143	27.61
	Whitish	135	26.06
	Dark	133	25.68
Skin sensitivity	Grade-1	95	18.34
	Grade-2	35	6.76
	Grade-3	96	18.53
	Grade-4	132	25.48
	Grade-5	133	25.68
	Grade-6	27	5.21
H/o Spontaneous re-pigmentation	No	443	85.52
	Yes	75	14.48
Family history	No	436	84.17
	Yes	82	15.83
	Pregnancy	17	3.28

Parameters		Number of Cases	Percentage (%)
Aggravating factors (if any)	Associated autoimmune disease	4	0.77
	Stress	2	0.39
	Traumatic injury	9	1.74
	any other	81	15.64
	No any	405	78.19
Working environment: Exposure to chemicals	No	512	98.84
	Yes	6	1.16
Working environment: Direct contact with industrial chemicals	No	518	100
	Yes	-	-

Impact on Clinical Parameters: Vitiligo Area Severity Index (VASI)

Group-1 (UNIM-001 and UNIM-003): At baseline, the mean VASI score was recorded as 5.82 ± 0.57.

Subsequent reductions were observed at each follow-up: 5.78 ± 0.53 (1st FU), 5.61 ± 0.52 (2nd FU), 5.5 ± 0.54 (3rd FU), 5.85 ± 0.62 (4th FU), 4.81 ± 0.43 (5th FU), 4.9 ± 0.52 (6th FU), 4.47 ± 0.4 (7th FU), and 4.96 ± 0.45 at the end (8th FU) of the treatment. This reduction demonstrated high significance with $p<0.001$ (Table 72, Fig. 24).

Group-2 (Psoralen): The baseline mean VASI score was recorded as 4.91 ± 0.4. Sequential reductions were observed during follow-ups: 4.63 ± 0.35 (1st FU), 4.39 ± 0.34 (2nd FU), 4.01 ± 0.3 (3rd FU), 4 ± 0.31 (4th FU), 3.73 ± 0.29 (5th FU), 3.55 ± 0.29 (6th FU), 3.44 ± 0.28 (7th FU), and 3.29 ± 0.27 at the end (8th FU) of the treatment. Similar to Group-1, this reduction was highly significant with $p<0.001$. (Table 72, Fig. 24)

Table 72: Effect on clinical parameters

Parameter	Group	Mean ± SEM									F-Statistic for	P-value	F-Statistic for	P-value
		Baseline	**1FU**	**2FU**	**3FU**	**4FU**	**5FU**	**6FU**	**7FU**	**8FU**				
Vitiligo Area Severity Index (VASI)	Gr-1 (n=262)	5.82 ± 0.57	5.78 ± 0.53	5.61 ± 0.52	5.5 ± 0.54	5.85 ± 0.62	4.81 ± 0.43	4.9 ± 0.52	4.47 ± 0.4	4.96 ± 0.45	3.99	<0.001	1.05	0.39
	Gr-2 (n=256)	4.91 ± 0.4	4.63 ± 0.35	4.39 ± 0.34	4.01 ± 0.31	4 ± 0.31	3.73 ± 0.29	3.55 ± 0.29	3.44 ± 0.28	3.29 ± 0.27	4.33	<0.001		

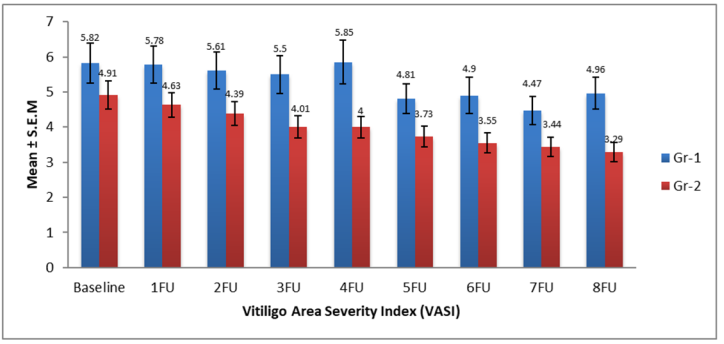


Figure 24

Assessment of Safety

For evaluation of the safety of the test drug, all the patients were assessed on clinical, haematological and biochemical parameters before, in the middle, and after completion of the treatment.

Clinical Evaluation

During the protocol therapy, no adverse events or drug reactions were observed in any of the patients.

Effect on Laboratory Parameters

Haemogram: In group-1 (n=262), baseline mean Hb of 13.06 ± 0.12, reduced to 12.79 ± 0.16 ($p<0.01$, within the normal range). In group-2 (n=256), the mean value of Hb at the baseline was 12.89 ± 0.13 which reduced to 12.66 ± 0.14. This reduction was statistically non-significant with p=0.68 (Table 73, Fig. 25).

Table 73: Effect on haemogram

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
Haemoglobin (gm%)	Gr-1	13.06 ± 0.12	12.79 ± 0.16	7.9 - 17.7	6 - 18.4	2.55	<0.01
	Gr-2	12.89 ± 0.13	12.66 ± 0.14	7-20	7.3 - 20.1	0.71	0.68

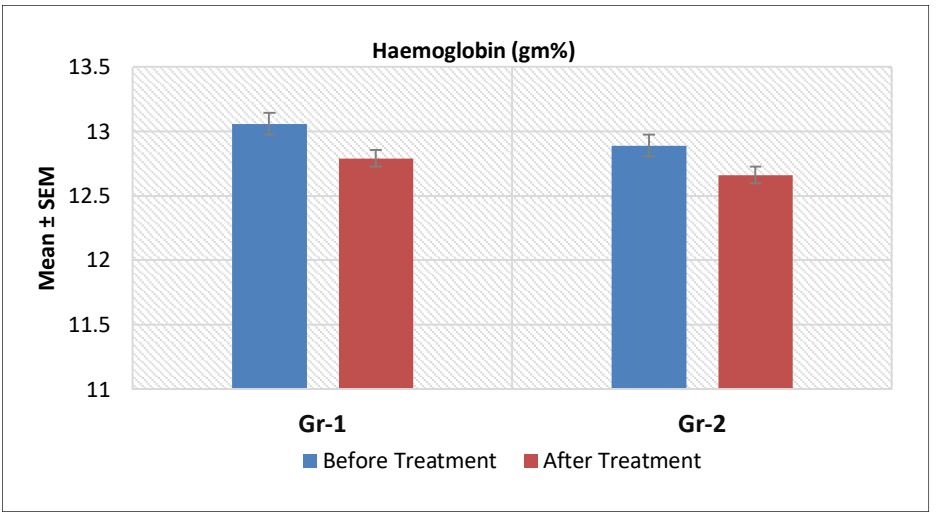


Figure 25

Red Blood Cells (RBCs): In group-1 (n=262), the mean value of RBC at baseline was 4.48 ± 0.04 which reduced to 4.41 ± 0.04 after treatment. This reduction was statistically non-significant with p = 0.8. In group-2 (n=256), the mean value of RBC at baseline was 4.47 ± 0.04 which reduced to 4.46 ± 0.05 after treatment. This reduction was statistically non-significant with p= 0.59 (Table 74, Fig. 26).

Table 74: Effect on RBC

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
RBC (Cells/cmm)	Gr-1	4.48 ± 0.04	4.41 ± 0.04	1 - 5.94	1.72 - 5.9	0.57	0.8
	Gr-2	4.47 ± 0.04	4.46 ± 0.05	2.3 - 5.97	2.8 - 8.8	0.81	0.59

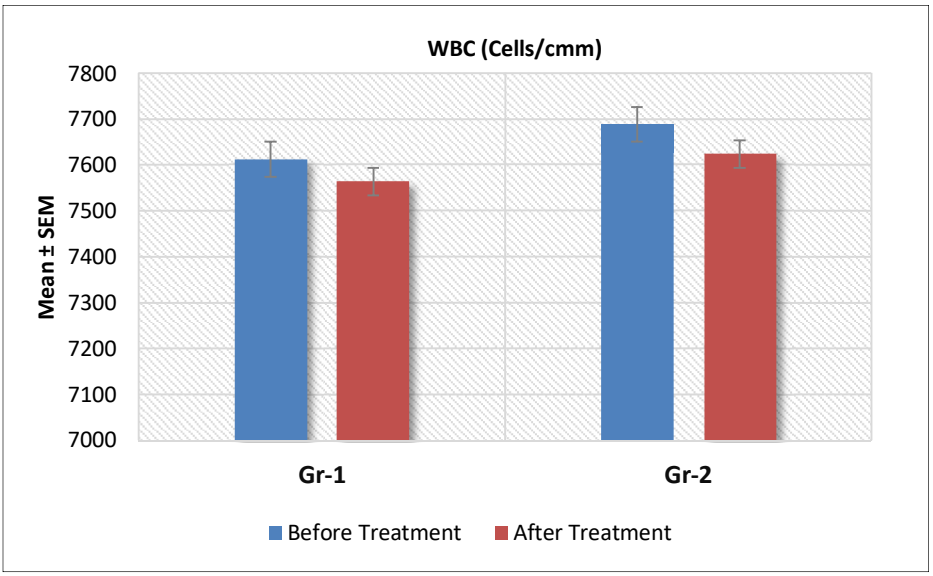


Figure 26

White Blood Cells (WBC): In group-1 (n=262), the mean value of WBC was 7612.39 ± 124.25 before treatment which decreased to 7563.25 ± 121.37 after treatment. This reduction was statistically non-significant (p=0.47). The mean value of WBC in group-2 (n=256) was 7688.68 ± 124.31 before treatment which decreased to 7623.47 ± 121.06 after treatment. This reduction was also statistically non-significant (p=0.85) (Table 75, Fig. 27).

Table 75: Effect on WBC

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
WBC (Cells/cmm)	Gr-1	7612.39 ± 124.25	7563.25 ± 121.37	3180 - 15000	4000 - 11910	-0.94	0.47
	Gr-2	7688.68 ± 124.31	7623.47 ± 121.06	2500 - 14120	4190 - 11310	-0.51	0.85

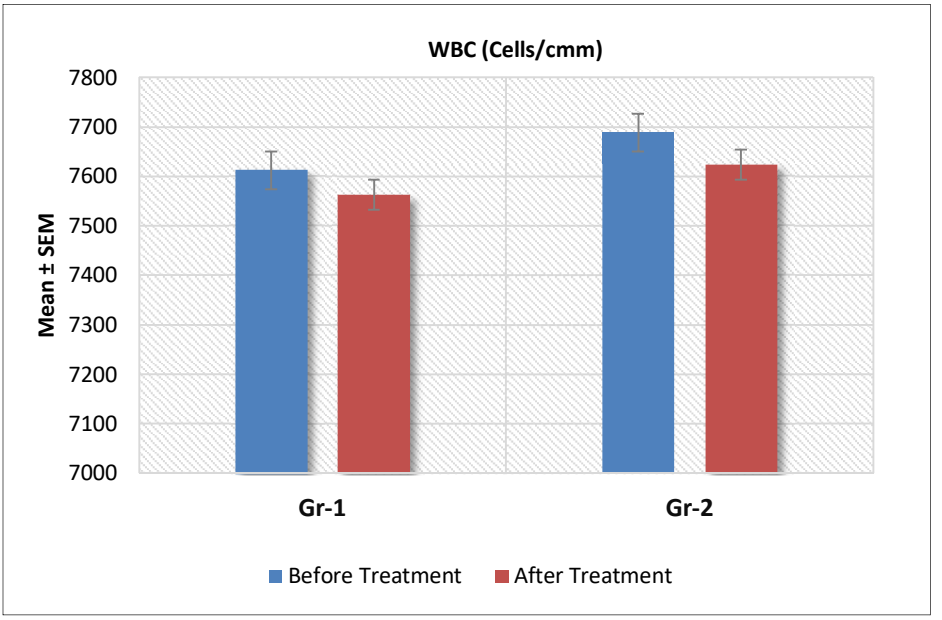


Figure 27

Platelet Count: In group-1 (n=262), the mean value of platelets at baseline was 2.57 ± 0.05 which reduced to 2.53 ± 0.05 at the end of treatment. This reduction was statistically non-significant (p=0.46). The mean value of platelets in group-2 (n=256) at baseline was 2.64 ± 0.05 which reduced to 2.58 ± 0.05 at the end of treatment. This reduction was also statistically non-significant (p=0.17) (Table 76, Fig. 28).

Table 76: Effect on platelet count

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
Platelet (Cells/cmm)	Gr-1	2.57 ± 0.05	2.53 ± 0.05	1.1 - 7.2	0.79 - 5.05	0.96	0.46
	Gr-2	2.64 ± 0.05	2.58 ± 0.05	0.64 - 7.23	0.76 - 5.05	1.44	0.17

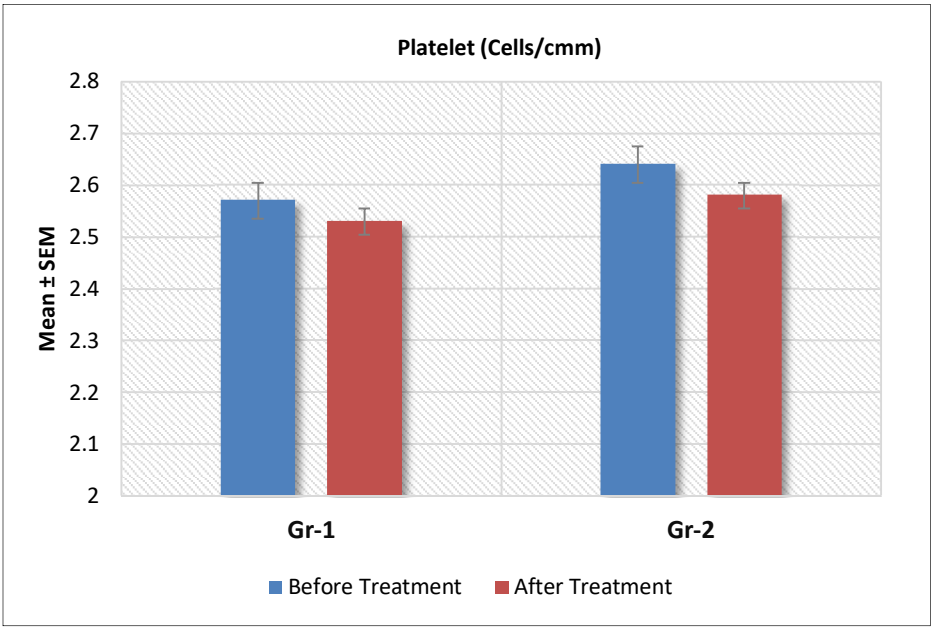


Figure 28

Effect on Differential Leucocyte Count (DLC)

Neutrophils (N): In group-1 (n=262), the mean value of N at the baseline was 60.23 ± 0.51 which slightly increased to 60.91 ± 0.62 after treatment. This increment was statistically non-significant with p=0.84. In group-2 (n=256), the mean value of N at baseline was 59.49 ± 0.54 which slightly increased to 60.05 ± 0.62 after treatment. This increment was also statistically non-significant (with p=0.4) (Table 77, Fig. 29).

Table 77: Effect on neutrophils (N)

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
Neutrophils (%)	Gr-1	60.23 ± 0.51	60.91 ± 0.62	30 - 85	20 - 85	0.51	0.84
	Gr-2	59.49 ± 0.54	60.05 ± 0.62	25 - 83	20 - 87	1.04	0.4

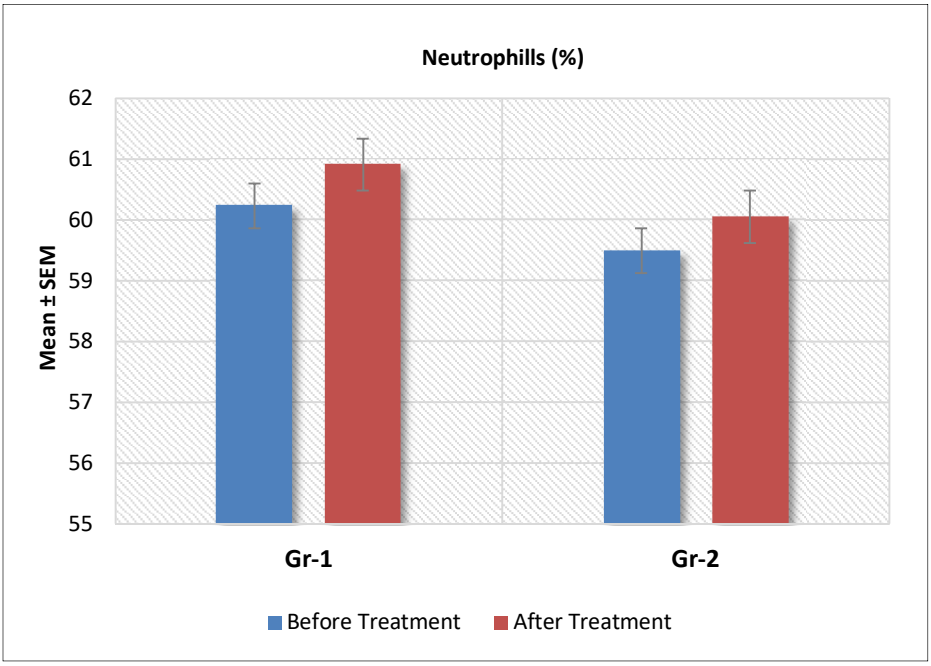


Figure 29

Lymphocytes (L): In group-1(n=262), the mean value of L was 33.66 ± 0.45 at baseline which decreased to 32.45 ± 0.53 after treatment. This change was statistically non-significant with p=0.62. In group-2 (n=256), the mean value of L at baseline was 34.32 ± 0.49 which was reduced to 33.75 ± 0.56 after treatment. This reduction was statistically non-significant with p=0.17 (Table 78, Fig. 30).

Table 78: Effect on lymphocytes

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
Lymphocytes (%)	Gr-1	33.66 ± 0.45	32.45 ± 0.53	11-67	12-50	0.77	0.62
	Gr-2	34.32 ± 0.49	33.75 ± 0.56	15 - 60	11-60	1.45	0.17

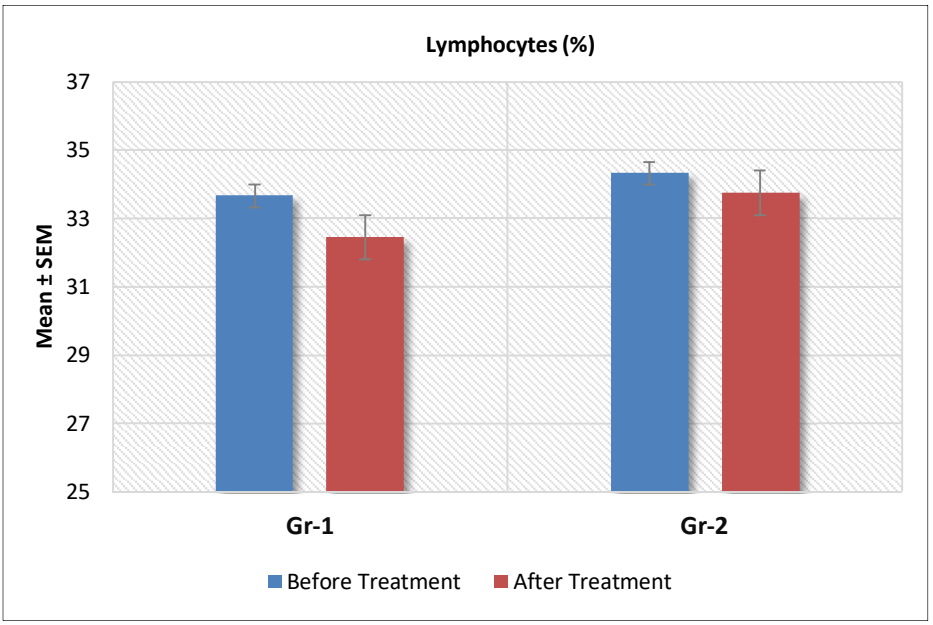


Figure 30

Eosinophils (E): In group-1(n=262), the mean value of E was 3.94 ± 0.2 at baseline which increased to 4.21 ± 0.26 after treatment. This increment was statistically non-significant with p=0.68. In group-2 (n=256), the mean value of E was 3.69 ± 0.14 at baseline which increased to 3.71 ± 0.17 after treatment. This increment was statistically significant with p<0.05 (Table 79, Fig. 31).

Table 79: Effect on eosinophils

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
Eosinophils (%)	Gr-1	3.94 ± 0.2	4.21 ± 0.26	0 - 40	0 - 40	0.71	0.68
	Gr-2	3.69 ± 0.14	3.71 ± 0.17	0 - 16	0 - 22	2.14	<0.05

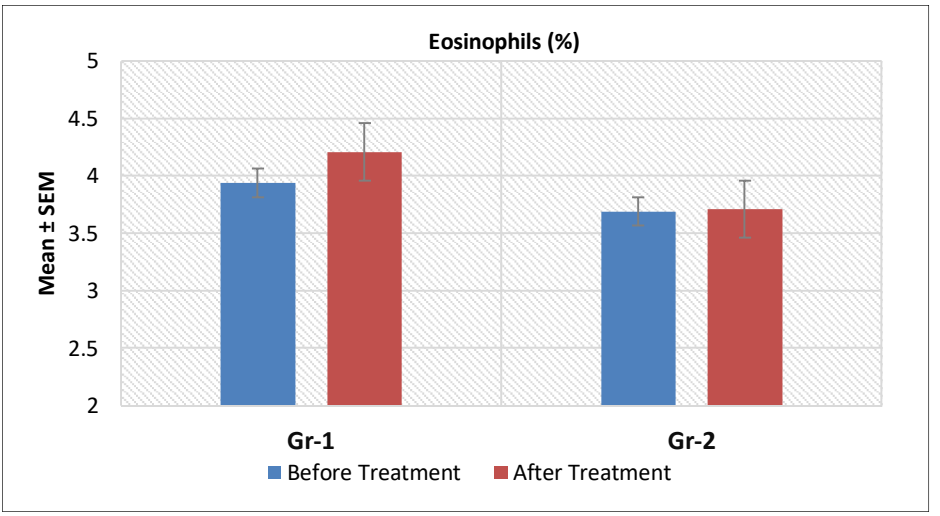


Figure 31

Monocytes (M): In group-1 (n=262), the mean value of M was 2.23 ± 0.11 at baseline which increased to 2.35 ± 0.15 after treatment. This increment was statistically non-significant with p=0.07. In group-2 (n=256), the mean value of M was 2.26 ± 0.12 at baseline which was reduced to 2.17 ± 0.11 after treatment. This reduction was statistically non-significant with p=0.06. No effect was observed on basophils in both the groups (Table 80, Fig. 32).

Table 80: Effect on monocytes

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
Monocytes (%)	Gr-1	2.23 ± 0.11	2.35 ± 0.15	0 - 8	0 - 7	1.78	0.07
	Gr-2	2.26 ± 0.12	2.17 ± 0.11	0 - 10	0 - 8	1.87	0.06
Basophils (%)	Gr-1	0 ± 0	0 ± 0	0 - 0	0 - 0	0.99	0.43
	Gr-2	0 ± 0	0 ± 0	0 - 0	0 - 1	-	-

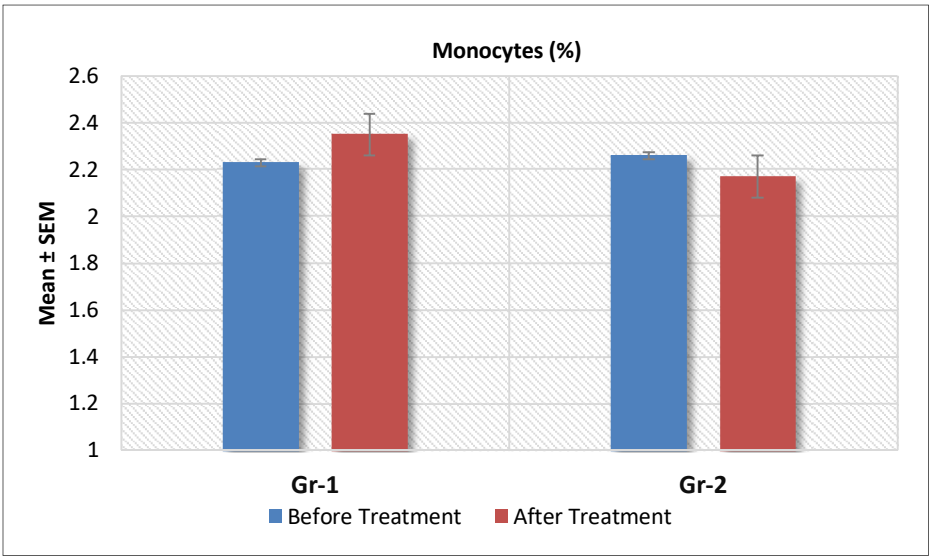


Figure 32

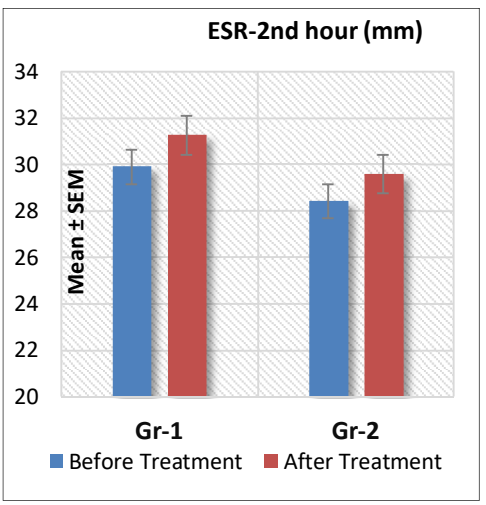
Erythrocyte Sedimentation Rate (ESR): In group-1 (n=262), the mean value of ESR at 1st hour was 17.95 ± 0.89 at baseline which increased to 20.46 ± 1.12 after treatment. Similarly, the mean ESR at 2nd hour was 29.9 ± 1.16 at baseline, which increased to 31.26 ± 1.29 at the end of treatment. However, the increment was statistically non-significant with p=0.56 and p=0.18 respectively.

In group-2 (n=256), the mean value of ESR at 1st hour was 17.13 ± 0.84 at baseline which increased to 18.73 ± 1 after treatment. Similarly, the mean of ESR at 2nd hour was 28.42 ± 1.15 at baseline, which increased to 29.59 ± 1.28 at the end of treatment. However, the increment was statistically non-significant with p=0.57 and p=0.96 respectively (Table 81, Fig. 33).

Table 81: Effect on erythrocyte sedimentation rate (ESR)

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
ESR-1st	Gr-1	17.95 ± 0.89	20.46 ± 1.12	2-62	2-57	0.84	0.56

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
hour (mm)	Gr-2	17.13 ± 0.84	18.73 ± 1	1-60	0 - 60	0.82	0.57
ESR-2nd hour (mm)	Gr-1	29.9 ± 1.16	31.26 ± 1.29	4-96	4-80	1.42	0.18
	Gr-2	28.42 ± 1.15	29.59 ± 1.28	4-92	2-92	0.31	0.96



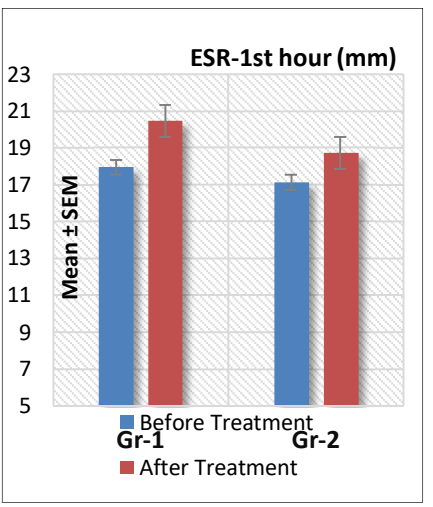


Figure 33

Effect on Liver Function Test

Serum bilirubin: In group-1(n=262), the mean value of S. Bilirubin was 0.72 ± 0.02 at baseline, which slightly changed to 0.7 ± 0.03 at the end of treatment showing statistically non-significant (p=0.17) change. In group-2 (n=256), the mean value of S. Bilirubin was 0.71 ± 0.02 at baseline, which was slightly reduced to 0.67 ± 0.02 at the end of treatment showing statistically non-significant (p=0.19) change (Table 82, Fig. 34).

Table 82: Effect on serum bilirubin

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
Serum bilirubin (mg%)	Gr-1	0.72 ± 0.02	0.7 ± 0.03	0.25 - 2.18	0.03 - 2.36	1.44	0.17
	Gr-2	0.71 ± 0.02	0.67 ± 0.02	0.2 - 3.792	0.22 - 2.55	1.4	0.19

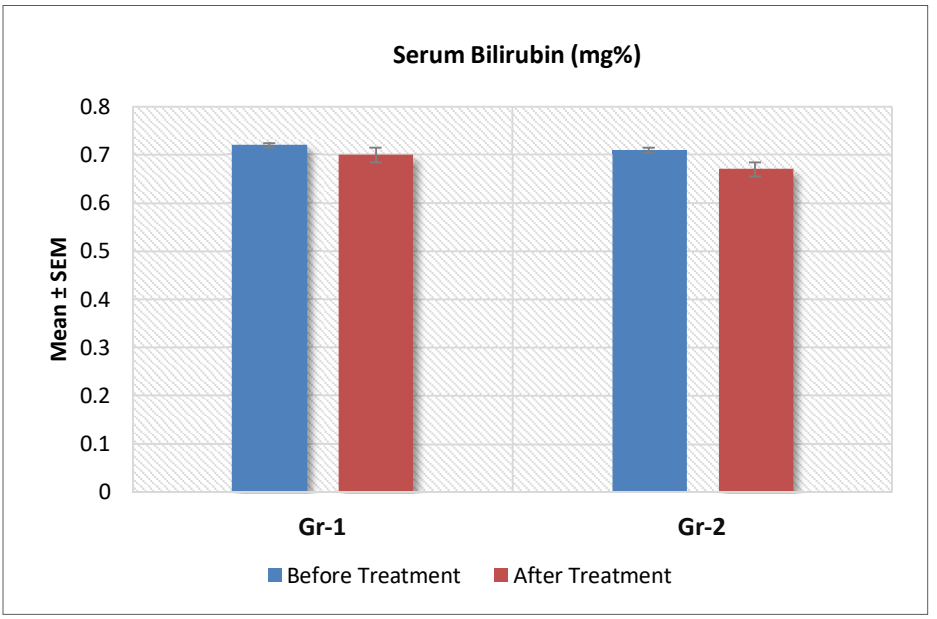


Figure 34

SGOT: In group-1 (n=262), the baseline mean SGOT was 23.52 ± 0.54, which reduced to 22.59 ± 0.66 at the end of treatment, indicating a statistically non-significant reduction (p=0.28). For group-2 (n=256), the baseline mean SGOT was 23.42 ± 0.58, and it significantly reduced to 21.55 ± 0.5 at the end of treatment ($p<0.05$) (Table 83, Fig. 35).

Table 83: Effect on SGOT

Parameter	Group	Mean ± S.E.M		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
SGOT (IU/L)	Gr-1	23.52 ± 0.54	22.59 ± 0.66	4.6 - 72.23	0.89 - 60.11	1.22	0.28
	Gr-2	23.42 ± 0.58	21.55 ± 0.5	7.07 - 89.72	8.84 - 56.14	2.47	<0.05

Figure 35

SGPT: In group-1 (n=262), the baseline mean SGPT was 23.59 ± 0.83, and it showed a slight change to 23.49 ± 1.15 at the end of treatment, indicating a statistically non-significant (p=0.85) change. For group-2 (n=256), the baseline mean SGPT was 25.59 ± 1.06, and it significantly reduced to 22.58 ± 0.88 at the end of treatment, with a statistically non-significant (p=0.055) change (Table 84, Fig. 36).

Table 84: Effect on SGPT

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
SGPT (IU/L)	Gr-1	23.59 ± 0.83	23.49 ± 1.15	5.3 - 112.29	7 - 102.5	0.5	0.85
	Gr-2	25.59 ± 1.06	22.58 ± 0.88	0.56 - 182.77	0.7 - 97.97	1.9	0.055

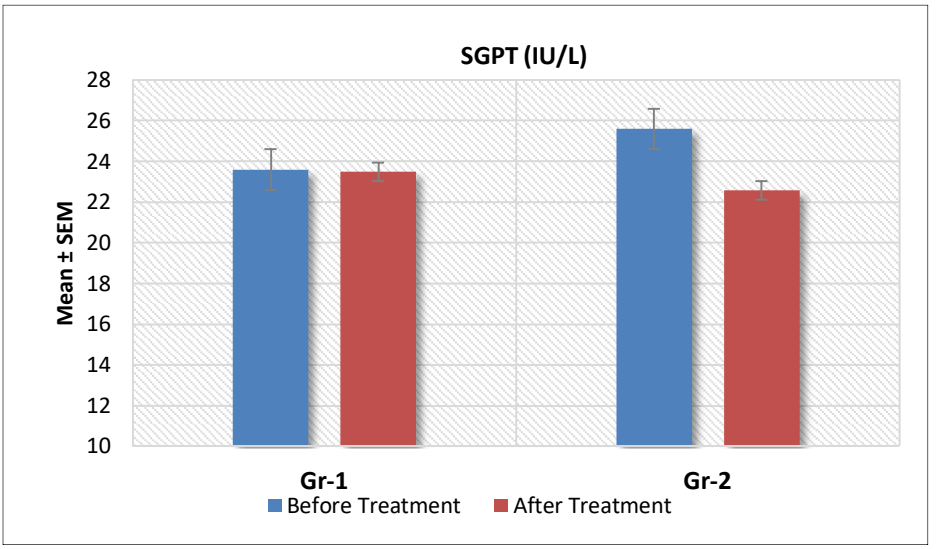


Figure 36

Effect on Kidney Function Test

Blood Urea: In group-1 (n=262), the mean value of blood urea was 23.51 ± 0.42 at baseline, which reduced to 22.63 ± 0.41 at the end of treatment. This reduction was statistically non-significant with p=0.99. In group-2 (n=256), the mean value of blood urea was 22.36 ± 0.4 at baseline, which increased to 23.08 ± 0.46 at the end of treatment. This increment was statistically non-significant p=0.43 (Table 85, Fig. 37).

Table 85: Effect on blood urea

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
Blood urea (mg%)	Gr-1	23.51 ± 0.42	22.63 ± 0.41	10-57	6 - 38.19	0.18	0.99
	Gr-2	22.36 ± 0.4	23.08 ± 0.46	7.81 - 40.9	6 - 58.25	1	0.43

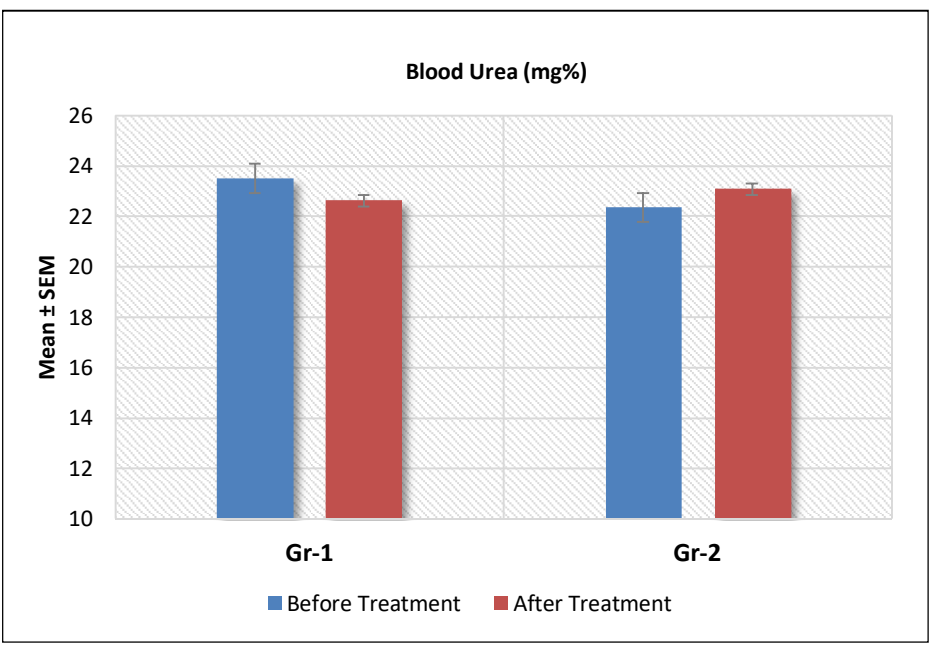


Figure 37

Serum Creatinine: In group-1 (n=262), the baseline mean serum creatinine was 0.85 ± 0.01, and there was no change observed after treatment. The p-value was 0.51, indicating a statistically non-significant result. For group-2 (n=256), the baseline mean serum creatinine was 0.84 ± 0.01, and it slightly reduced to 0.83 ± 0.01 after treatment. The p-value was 0.57, showing a statistically non-significant change (Table 86, Fig. 38).

Table 86: Effect on serum creatinine

Parameter	Group	Mean ± SEM		Range		Paired 't' test	
		Before Treatment	After Treatment	Before Treatment	After Treatment	Statistic value	P-value
S. Creatinine (mg %)	Gr-1	0.85 ± 0.01	0.85 ± 0.02	0.24 - 1.82	0.4 - 1.97	0.89	0.51
	Gr-2	0.84 ± 0.01	0.83 ± 0.01	0.41 - 1.35	0.4 - 1.3	0.83	0.57

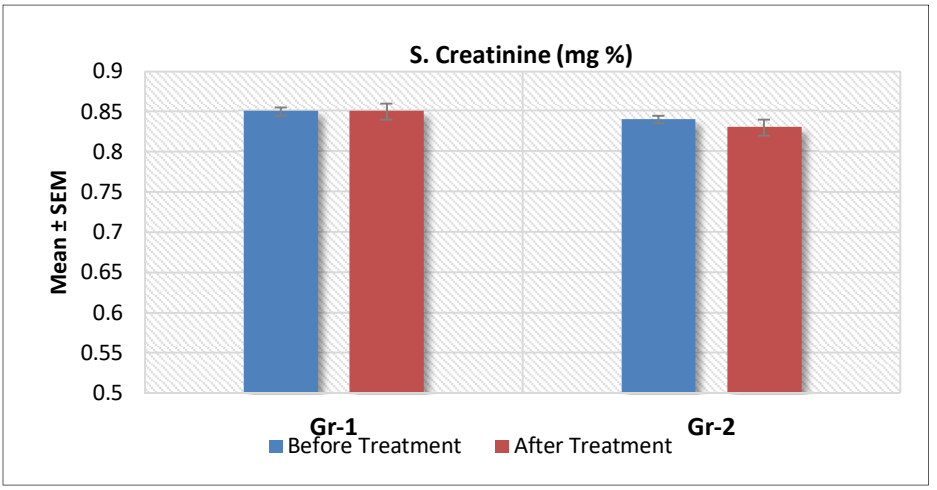
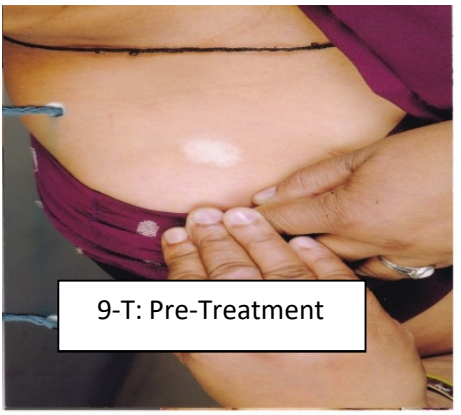
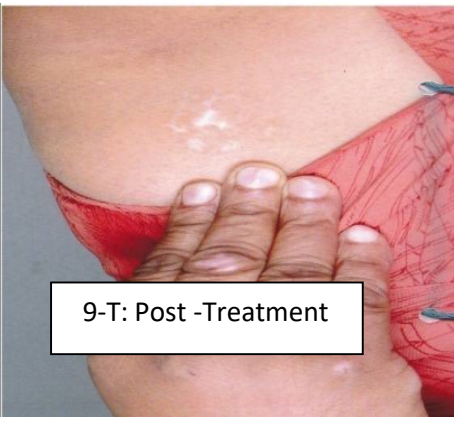
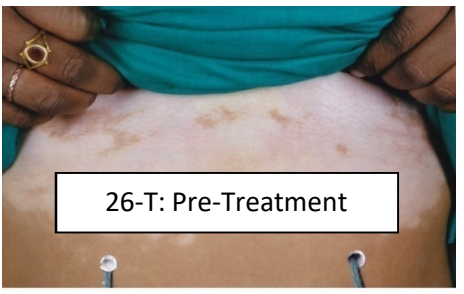


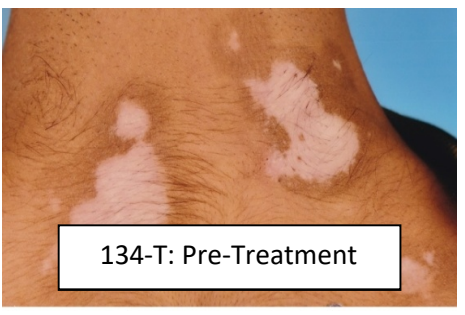
Figure 38

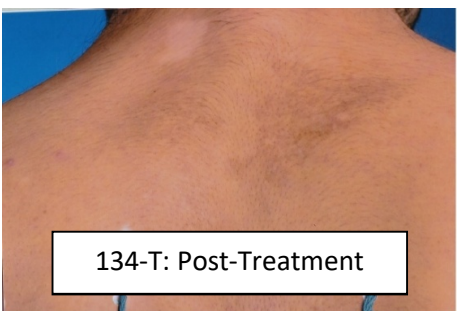
Test Group Photographs

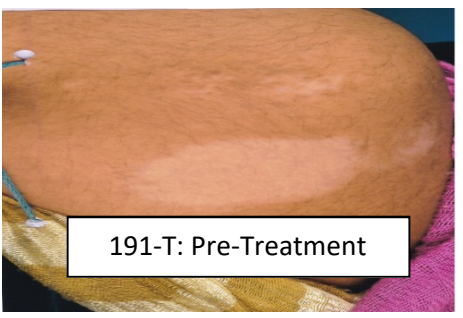


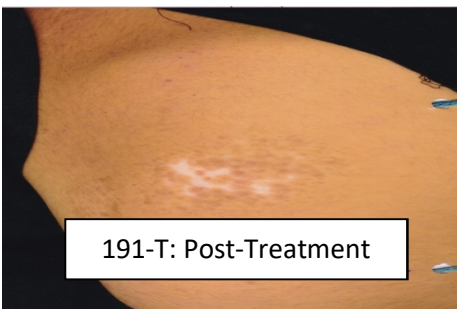












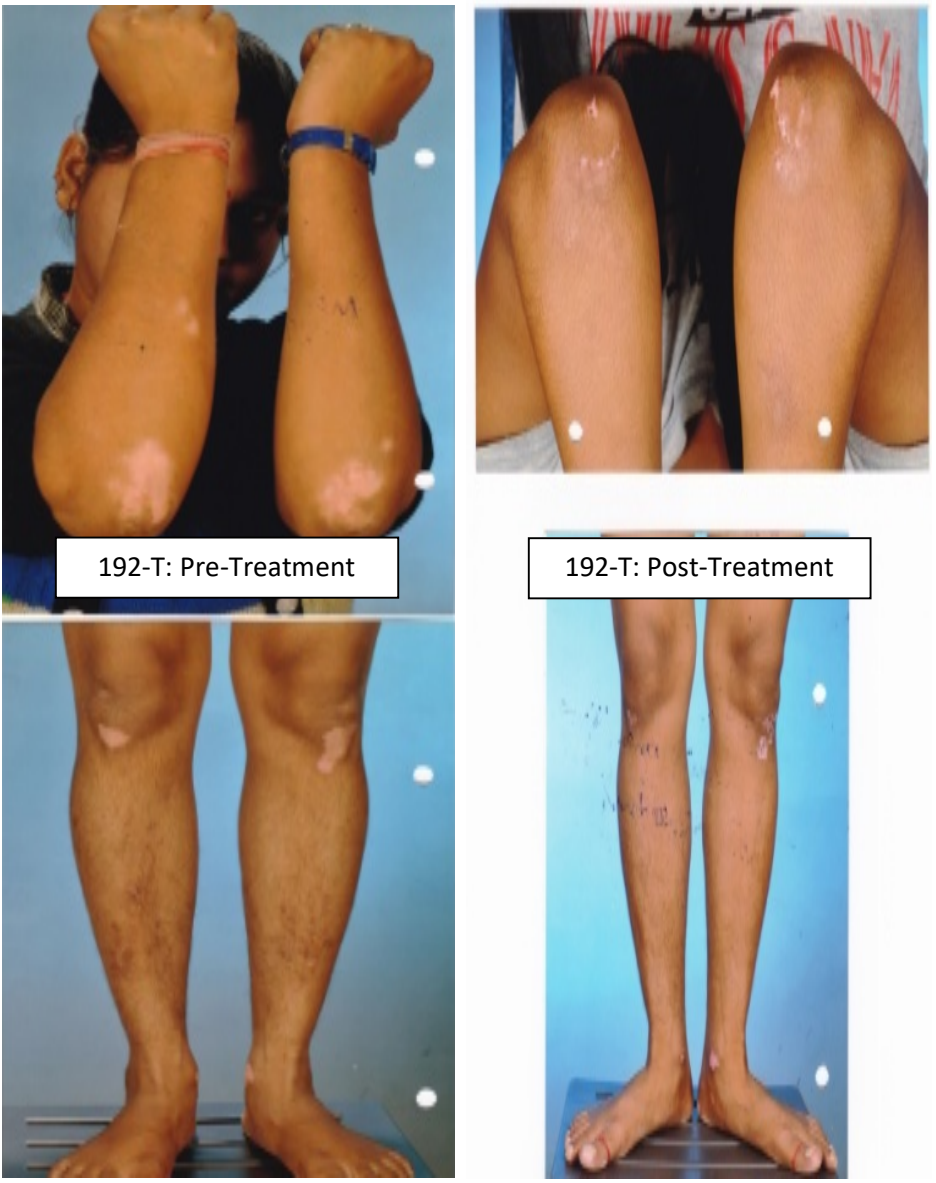


Figure 39: *Photographs of trial group showing response to Unani coded drugs UNIM-001 tablet and UNIM-003 topical in vitiligo patients.*

Summary and Conclusion

Baraṣ (vitiligo), a prevalent dermatological and social concern affecting 1% of the global population, manifests as acquired depigmentation of the skin due to melanocyte destruction. Despite its cosmetic nature, vitiligo profoundly impacts patients psychologically, causing fear, anxiety, and body image distortion. The disease, characterized by hypo-melanosis, can be localized or generalized, and various hypotheses, including auto-immune, self-destruction, and neural factors, contribute to its causation. While auto-immune involvement is widely accepted, the hypothesis of "melanocyte destruction" persists.

With a frequency of 0.1-2.0% globally, vitiligo exhibits a strong genetic predisposition, often leading to social stigma, matrimonial challenges, and economic discrimination. It can emerge at any age, affecting both genders equally, with a significant proportion developing lesions before the second decade. While modern medicine, particularly PUVA therapy, is the current standard, it has limitations, including reported hepatotoxicity and carcinogenicity with prolonged use.

Given the psychological impact and recent reports on the efficacy and safety of polyherbal formulations, a clinical trial was designed to assess a Unani regimen's safety and efficacy against Psoralen, a widely accepted modern medicine treatment.

In the randomized controlled trial, 518 vitiligo patients completed the 8-month trial. The test group (Gr-2) received coded Unani formulations (UNIM-001 Oral + UNIM-003 Local), while the control group (Gr-1) received Psoralen (oral & local). Temperament analysis revealed a predominance of *Balghamī* temperament (64%), aligning with Unani claims associating vitiligo with phlegm dominance.

In the randomized controlled trial, a total of 518 patients of *Baraṣ* (vitiligo) had completed the trial. The patients were randomized into test (n=256) and control (n=262) groups. The test group was treated with coded Unani formulations UNIM-001 (oral) + UNIM 003 (local) while control group was treated with Psoralen (oral & local). The clinical and laboratory findings in both groups were analysed and compared. It was observed that highest percentage of patients of *Baraṣ* (vitiligo) were of *Balghamī* temperament (64%), followed by *Damawī* (22%), *Ṣafrāwī* (11%) and *Sawdāwī* (2%). This is in consonance with the claims of Unani physicians that vitiligo or *Baraṣ* is a disease caused by predominance of *Balgham* i.e. phlegm.

Comparative clinical efficacy analysis demonstrated consistent VASI score reduction in both groups, suggesting the efficacy of Unani drugs was not inferior to Psoralen. Safety profiles, analyzed through haematological and biochemical parameters, showed no toxicity, with minor alterations within the normal range. The coded Unani formulations proved effective in vitiligo treatment without side effects, making them suitable for long-term therapy, essential for maintaining an individual's normal homeostasis due to the absence of drug toxicity or side effects.

PART-C

FUNDAMENTAL RESEARCH

Study Rationale

Baraṣ or vitiligo is one of the research priorities of the Central Council for Research in Unani Medicine. Although a rich treasure of therapeutically active Unani products is available for treatment of vitiligo, there is lack of any established potential biomarker for the disease. Therefore, the study on the effect of Unani formulation(s) on various biomarkers in patients with *Baraṣ* or vitiligo was designed to provide scientific relevance to these tested Unani formulations which have been used for centuries. This study aimed to provide a reliable substitute for clinical response with potential to improve the efficiency of the clinical trial/drug development program. This study may also contribute in understanding the pharmacology of drug, may provide information on mechanism of action and characteristic of disease, may act as diagnostic tool for classifying stages of disease and most importantly will help in monitoring of clinical response to an intervention.

The scientific validation in terms of this biomarker study for Unani drug will also help to create evidence-based data and will pave the way for acceptability of these drugs globally. On the basis of available information on vitiligo, battery of biomarkers including the molecular biomarkers, which are being expressed in blood as well as in the skin cells have been selected. The potential biomarkers studied in this study are as follows:

Molecular Biomarkers (Genes)

1. Macrophage Migration Inhibitory Factor (MIF)

MIF has been originally identified as a lymphokine, which can concentrate macrophages at inflammation loci. MIF is also one of the immuno-regulatory cytokines involved in macrophage and T-cell activation and, conversely, macrophages and T cells are the primary source of MIF. MIF is known to be involved in immune-mediated diseases, and may also play a pivotal role in many autoimmune skin diseases, such as systemic lupus erythematosus, systemic sclerosis, atopic dermatitis, psoriasis vulgaris, bullous pemphigoid and vitiligo.[47] It has been reported recently that MIF participates in the pathogenesis of vitiligo vulgaris and may be useful as an index of disease severity and activity.[48] To elucidate the involvement of MIF

in the pathogenesis of vitiligo, MIF mRNA levels were measured in Peripheral Blood Mononuclear Cells (PBMCs) and serum MIF concentrations in vitiligo patients and normal controls. Further, the possible relationship between MIF expressions and the severity and activity of disease in vitiligo patients was also analysed.

2. Interleukin 22 (IL22)

Studies have suggested the skin to be a potential target for IL10 family interleukins (IL10, IL19, IL20, IL22 and IL24) because skin has a high expression level of different receptor subunits forming respective receptor complexes for these interleukins.[49] Despite homology and sharing receptor complexes, interleukins of the IL10 family have distinct physiological roles. Among various IL10 family interleukins, IL22 has been reported to be significantly associated with vitiligo, especially with the active stage of vitiligo.[50]

3. Nucleotide Oligomerization Domain (NOD)-like receptor P1 (NLRP1)

The NLRP1 gene is a leucine-rich repeat protein 1 (a member of the NLR family/nucleotide oligomerization domain like receptor). It contains a caspase recruitment domain that is known to be the key mediator of apoptosis. High levels of NLRP1 expression in immune cells, particularly T cells and Langerhans cells emphasize the role of NLRP1 in regulation of the immune system. Genome wide association studies revealed a highly significant association of familial cases of generalized vitiligo with polymorphic variants of the gene encoding NLRP1.[51] The upregulation of NLRP1 mRNA in patients with susceptible genotypes advocates the crucial role of NLRP1 in generalized vitiligo.[52]

4. Forkhead Box P3 (FOXP3)

FOXP3 (Forkhead Box P3) also known as Scurfin, a member of FOX protein family is a protein involved in immune system responses. FOXP3 appears to function as a master regulator (transcription factor) in the development and function of regulatory T cells.[53] In regulatory T cell model systems, the FOXP3 transcription factor occupies the promoters for genes involved in regulatory T-cell function, and may repress transcription of key genes following stimulation of T cell receptors.[54] To investigate expression of FOXP3 with other parameters in patients with vitiligo after effective therapy, the expression of FOXP3 mRNA in CD4+CD25+ Treg cells has been reported to increase significantly.[55]

Protein Biomarkers

5. Interleukin 2 (sIL2)

The role of IL-2 includes the regulation of antibody production by controlling the proliferation of B lymphocytes as well as the activation of T lymphocytes. It has been reported that several auto-antibodies to numerous organs in addition to anti-melanocyte antibody are the common occurrence in vitiligo patients. It has also been reported that lymphocytes are directly involved in the destruction of melanocytes.[56] Interleukin-2 (IL-2) is lymphokine synthesized and secreted primarily by T helper lymphocytes. IL-2 stimulates the production of IL-2Rα on the T cell surface. IL-2Rα is then released to the serum as a measurable protein (sIL-2R). The amount of sIL-2R is proportionally related with the amount of IL-2Rα expressed on the T cell surface.[57] Since the release of sIL-2R is correlated with the amount of IL-2R expressed on the surface of activated T cell lymphocytes, many studies have shown that serum sIL-2R levels are raised in patients with vitiligo[58,59] along with others, viz. chronic plaque psoriasis, atopic dermatitis, cutaneous T cell lymphoma and systemic sclerosis. Thus serum levels of sIL-2R can be used to monitor in vivo immune activation as its elevation has been shown to be correlated with T cell mediated immune disease. Vitiligo may be related to activate T cell immunity and may be the consequence of an autoimmune response of cytotoxic T lymphocytes against melanocyte antigens carried by normal or abnormal melanocytes.[60]

6. Tumour Necrosis Factor Alpha (TNFα)

TNF-α plays important role in apoptosis through activation of the receptor-mediated apoptosis pathway in numerous cell types[61]. It is produced by many different cell types, including activated T cells, fibroblasts, adipocytes, smooth muscle cells and keratinocytes. In the epidermal melanin unit of epidermis, a melanocyte is in close interaction with keratinocytes. The keratinocytes synthesize cytokines, such as TNF-α, Interleukin (IL) 1a, IL-6, and Transforming Growth Factor-b (TGF-b), which are paracrine inhibitors of human melanocyte proliferation and melanogenesis. TNF-α also affects the apoptotic pathway of melanocytes and its level may play an important role in vitiligo pathogenesis. Moreover, TNF-α can inhibit melanocyte stem cell differentiation.[62] Further it has been also reported recently that the up-regulation of TNF- α transcript and protein levels in individuals with susceptible haplotypes advocates the crucial role of TNF-α in autoimmune pathogenesis of vitiligo.[63]

7. Anti-Thyroid Auto Antibodies (Anti TPO)

This antibody has been reported as sensitive biomarker for autoimmune thyroid disorder including vitiligo. Thyroid Peroxidase (TPO) is an enzyme expressed in thyroid involved in metabolism of thyroid hormones. TPO is frequent epitope of auto-antibodies in auto immune thyroid disease called as anti-TPO antibodies. Anti-TPO has been shown to mediate thyroid cell destruction in *in-vitro* studies. Its increased levels have been seen as more common in vitiligo patients especially in young women.

8. Malondialdehyde (MDA)

Oxidative stress is a major form of assault on the skin and has been implicated as the initial triggering event in vitiligo pathogenesis leading to melanocyte destruction.[64] Some studies have shown antioxidant systems to play a role in the pathogenesis of generalized vitiligo.[65] Malondialdehyde (MDA), the end product of Lipid Peroxidation (LPO), arising from the free radical degradation of polyunsaturated fatty acids, can cause cross-linking in lipids, proteins and nucleic acids. A defective antioxidant defense is also postulated to lead to the unhindered cytotoxic action of reactive oxygen species such as superoxide anion, hydroxyl radical, etc. After formation, these highly reactive free radicals can start a chain reaction and bring about lipid peroxidation producing lipid peroxides and lipoxidase, whose further decomposition yields a variety of end products, including Malondialdehyde (MDA).[66] These decomposition products can cause damage to cell membrane or DNA leading to cytotoxicity, mutagenicity and cell death. These reactive oxygen species are generated following ultraviolet rays induced damage to the epidermis. They are also cytotoxic to melanocytes and can inhibit tyrosinase.[67] Imbalances in the oxidant/antioxidant system, such as the accumulation of hydrogen peroxide (H_2O_2), increased level of Malondialdehyde (MDA) and low catalase (CAT) levels, have recently been reported in the epidermis and blood of vitiligo patients.[68,69]

9. Total Antioxidant Status (TAS)

Oxidative stress is a major form of assault on the skin and has been implicated as the initial triggering event in vitiligo pathogenesis leading to melanocyte destruction.[64] Some studies have shown antioxidant systems to play a role in the pathogenesis of generalized vitiligo[65]. The purpose of analyzing TAS was to evaluate the role of antioxidants in the pathogenesis of vitiligo and also to support the proposed aetiology which suggests that the destruction of melanocytes in vitiligo is induced by increased oxidative stress which in turn activates an autoimmune response.

Study Objectives

- To evaluate the biomarkers of blood among vitiligo patients and healthy controls
- To evaluate the effect of Unani formulation(s) on biomarkers in vitiligo patients

Materials & Methods

The Ethics Committee of NRIUMSD, Hyderabad approved the study and the study was registered at Clinical Trials Registry-India (CTRI) with registration number CTRI/2015/10/006275. All the participants enrolled in the study gave written informed consent for the study prior to their enrolment. All vitiligo and control subjects were enrolled at the Research OPD of NRIUMSD, Hyderabad. Total 34 vitiligo patients (with a mean age of 30.13 ± 8.3 years) were enrolled in the study. All the vitiligo subjects were of non-segmental generalized vitiligo type with progressive (non-segmental vitiligo, in which new areas of depigmentation, enlargement of the area of depigmentation or both were observed during the previous month) state of disease, i.e. with VIDA score of V1 (progressive vitiligo). The vitiligo subjects were excluded if they had any clinically significant abnormality identified or any systemic disease or any other skin disease. Pregnant or lactating women, patients who had received any other investigational product within last 4 weeks were also excluded. The vitiligo patients enrolled in the study were treated with coded Unani formulations UNIM-001 (oral) and UNIM 003 (local). This was an eight month long study with first follow-up at 4^{th} month and final follow-up at 8^{th} month. The control group also consisted of 34 healthy subjects (with a mean age of 32.42 ± 6.3 years). The age and gender ratio were well-matched in the two groups. Blood was taken from all subjects (vitiligo and healthy) after an overnight fast in the morning.

Isolation of RNA and Expression Study

For isolation of RNA, 2.5 ml collected blood was transferred to Pax gene blood RNA tubes and was stored at -20^0 C in the refrigerator till further use. Total RNA was isolated using Pax gene blood RNA isolation kit (PreAnalytix, Qiagen/BD Company) following manufacturer instructions. The quality of the RNA samples was assessed by inspecting the 28S and 18S bands on agarose gel electrophoresis. Further, 1.9 to 2.0, 260/280 absorbance ratio or each RNA sample was observed. Extracted RNA was

dissolved in RNase-free water and stored at -80^0 C until cDNA synthesis. A reverse transcriptase kit (Quantitect® Reverse transcription kit, from Qiagen India (P) Ltd) was used for complementary DNA (cDNA) synthesis.

Real-time RT-PCR was performed using a CFX96 Real Time System (C1000™ Thermal cycler, Bio-Rad) and mRNA levels were quantified using KAPA SYBR® FAST qPCR master mix (Kappa Bio Systems).

The primers (Table 1) were designed by the Internet Primer Design Software and synthesized by Bio Serve Company (India). All the reactions were carried out in 20 µl reaction volumes in triplicates. Gene expression studies of NLRP1, MIF, IL22 and FOXP3 genes were carried out in non-segmental vitiligo subjects (n=34) and 34 healthy controls by using GAPDH as an internal control gene.

Table 87: Primers sequence of MIF, NLRP1, FOXP3, IL22 and GAPDH

Genes	Sense	Antisense	Size (bp)
MIF	5′-ACCAGCTCATGGCCTTCG-3′	5′-CTTGCTGTAGGAGCGGTT-3′	110
NLRP1	5′-GGCAGCACAGATCAACATGGA-3′	5′-CAGGTTTCTGGTGACCTTGAGGA-3′	112
FOXP3	5′-GAGAAGCTGAGTGCCATGC-3′	5′-AGCCCTTGTCGGATGATG-3′	85
IL22	5′-CAACAGGCTAAGCACATGTCA-3′	5′-ACTGTGTCCTTCAGCTTTTGC-3′	78
GAPDH	5′-ACCCACTCCTCCACCTTTGA-3′	5′-CATACCAGGAAATGAGCTTGACAA-3′	75

Estimation of Serum TNF-α Levels

Serum levels of TNF-α in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the ImmunoTech Human TNF-α ELISA kit. The kit was supplied by Bio LegendR-

/9727PacificHeightsBlvd./San Diego, CA92121U.S.A.; Human TNFα ELISA MAXTM.

Estimation of Serum Lipid Peroxidase (MDA) Levels

Serum levels of lipid peroxidation marker, Malondialdehyde (an oxidant) in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the lipid peroxidation (MDA) assay kit. The kit was supplied by Sigma-Aldrich, USA.

Estimation of Serum Total Antioxidant (TAS) Levels

Serum levels of Total Anti-oxidant in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the Total antioxidant (TAS) assay kit. The kit was supplied by Sigma-Aldrich, USA.

Estimation of Anti -TPO

Serum levels of Anti TPO in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the Human TPO/ Thyroid peroxidase ELISA kit. The kit was supplied by Sigma-Aldrich, USA.

Estimation of IL2

Serum levels of IL2 in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the Human IL2 kit. The kit was supplied by Bio LegendR-/9727PacificHeightsBlvd./San Diego, CA92121U.S.A.

Estimation of IL22

Serum levels of IL22 in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the Human IL22 kit. The kit was supplied by Bio LegendR-/9727PacificHeightsBlvd./San Diego, CA92121U.S.A.

Estimation of Biochemical Parameters

All the study subjects were subjected to Liver Function Tests (Bilirubin, ALT, AST and ALP) and Renal Function Tests (Urea, Creatinine) which were analyzed by using an Erba Auto Analyzer. About 5 ml venous blood samples were collected from the vitiligo patients and control subjects in a metal-free sterile tube, between 8 to 9 am after an overnight fasting. The

blood was then allowed to clot at room temperature for 30 minutes and centrifuged for 15 minutes at 5000 rpm to extract the serum. The serum was taken in Eppendorf tube and stored at −80°C for further analysis. Results including follow-ups 1st and 2nd have been presented in Tables 3, 4 & 5.

Statistical Analysis

Statistical analysis was performed using openEpi software (Version 3.01, April 2013; http://www.openepi.com). Univariate statistical analysis was performed using t-test for biochemical parameters. 2- tailed p value of equal variance <0.05 was considered as significant. $\chi 2$ test was used to compare the differences in each genotype, allele, and combined genotypes frequencies. The risk analysis was performed by calculating odds ratio (OR) at 95 % confidence intervals (CIs). A two-tailed *p* value of <0.05 was considered to be significant.

Results

Real-time PCR Validation

The specificity of the MIF, NLRP1, FOXP3, IL22 and internal control gene GAPDH products were checked by melting curve analysis and agarose gel electrophoresis. The PCR products size of MIF, NLRP1 FOXP3 and IL22 was 110bp, 112bp, 85bp and 78bp with melting temperatures of 79° C, 78.5° C and 81° C respectively. Results have been evaluated for the 34 baseline, 34 first follow-up (4th month) and 34 second follow up (8th month) vitiligo patients and 34 healthy volunteers.

Relative Gene Expression of NLRP1 in Non-Segmental Vitiligo Subjects and Healthy Controls

Expression of NLRP1 mRNA levels after normalization with GAPDH expression as internal control gene in blood samples of non-segmental vitiligo subjects as represented by mean $2^{-\Delta\Delta Cp}$ were 0.54 folds difference at the baseline in relation to control, 0.28 folds difference at the first follow-up and 0.35 folds difference at the second follow-up (Fig. 40).

Relative gene expression of MIF in Non- Segmental vitiligo subjects and healthy controls

Expression of MIF mRNA levels after normalization with GAPDH expression as internal control gene in blood samples of non-segmental vitiligo subjects as represented by mean $2^{-\Delta\Delta Cp}$ were 0.02 folds difference at

the baseline in relation to control, 0.03 difference folds at the first follow-up and 0.05 folds difference at the second follow-up (Fig. 40).

Relative gene expression of IL22 in vitiligo subjects and healthy controls

Expression of IL22 mRNA levels after normalization with GAPDH expression as internal control gene in blood samples of non-segmental vitiligo subjects as represented by mean $2^{-\Delta\Delta Cp}$ were 0.95 folds difference at the baseline in relation to control, 0.67 difference folds at the first follow-up and 0.87 folds difference at the second follow-up (Fig. 40).

Relative gene expression of FOXP3 in vitiligo subjects and healthy controls

Expression of FOXP3 mRNA levels after normalization with GAPDH expression as internal control gene in blood samples of non-segmental vitiligo subjects as represented by mean $2^{-\Delta\Delta Cp}$ were 0.41 folds difference at the baseline in relation to control, 0.31 difference folds at the first follow-up and 0.42 folds difference at the second follow-up (Fig. 40).

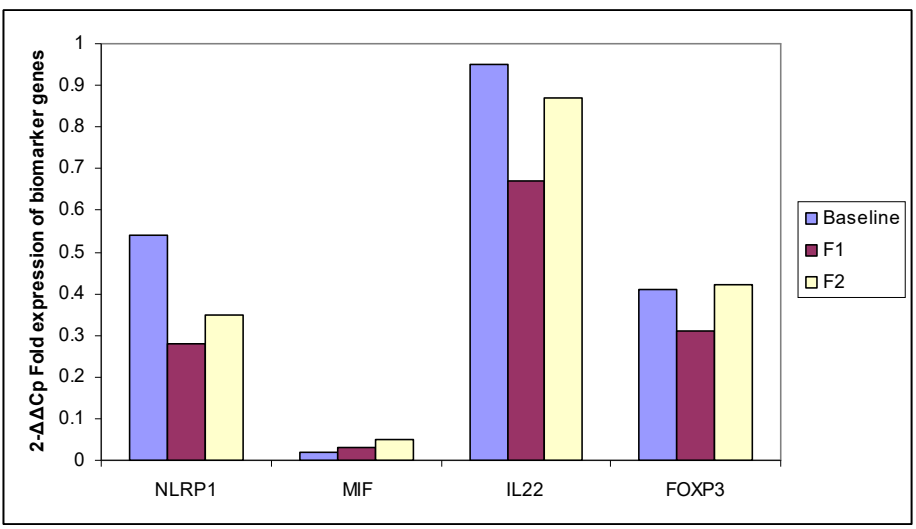
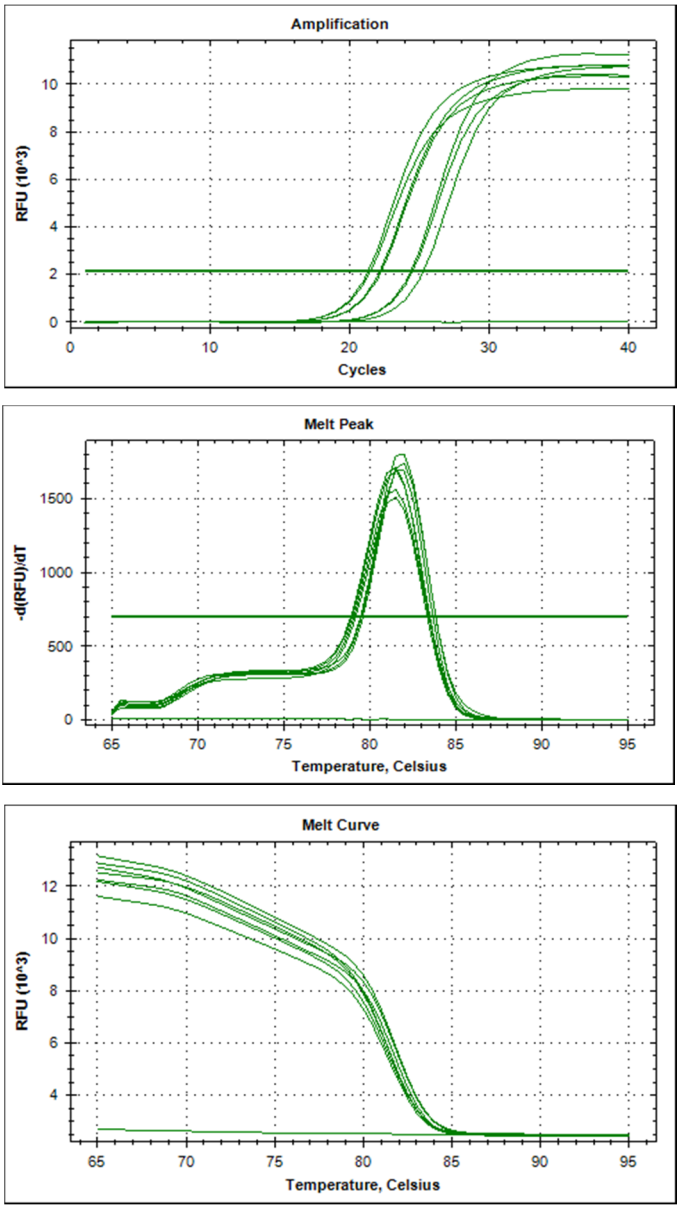


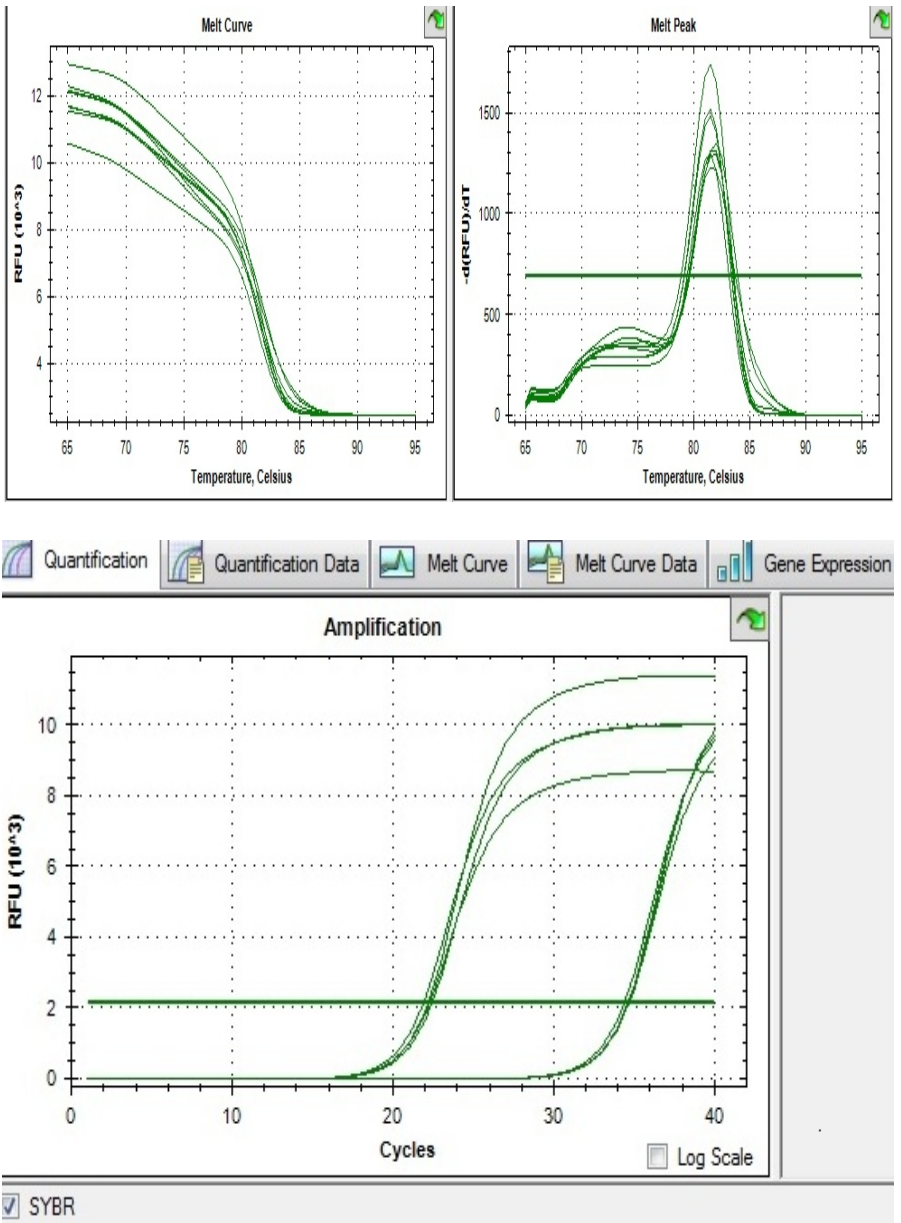
Figure 40: *Fold expression determination of NLRP1, MIF, IL22 and FOXP3 genes mRNA levels by the 2¬¬-ΔΔCp method;* F1 = 1st follow-up; F2 = 2nd follow-up

RT-PCR of NLRP-1 Gene



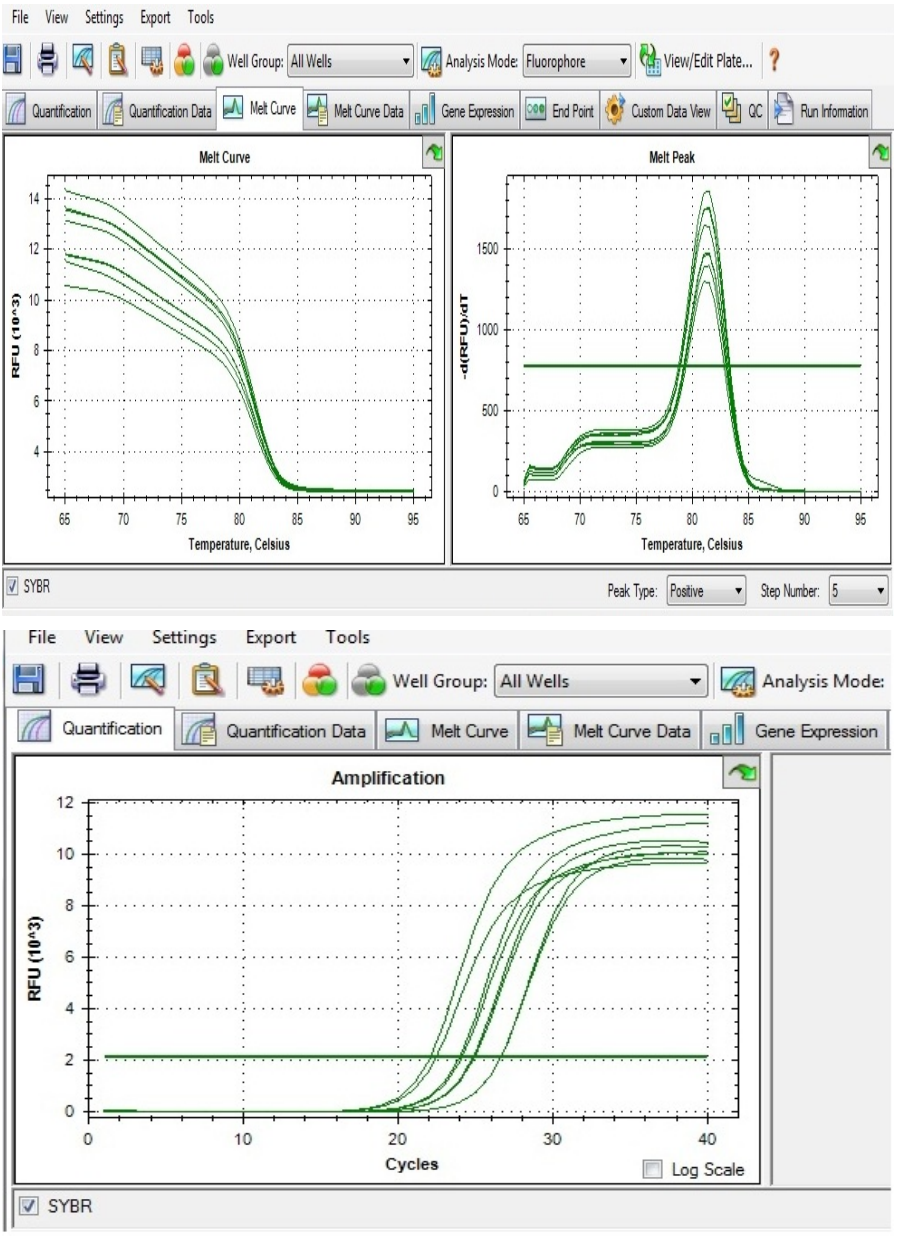
Comparative expression by RT-PCR of NLRP-1 gene along with internal control gene GAPDH in vitiligo (Baraș) subjects and healthy volunteers

RT-PCR of IL – 22 Gene



Comparative expression by RT-PCR of IL-22 gene along with internal control gene GAPDH in vitiligo (*Baraş*) subjects and healthy volunteers

RT-PCR of MIF gene



Comparative expression by RT-PCR of MIF gene along with internal control gene GAPDH in vitiligo (Baraș) subjects and healthy volunteers

Estimation of Biomarkers

TNFα: During the course of investigations on 34 vitiligo patients and 34 healthy controls, it was observed that the level of TNF**α** was significantly higher at the baseline in relation to controls. But, during the 1st follow-up (after 4th month), the level of TNFα significantly decreased to the control level with Unani medicines (UNIM-003 and UNIM-001). But the level significantly increased at the 2nd follow-up (8th month) again – this may probably be due to the fact that the therapeutic response decreased on 8th month of follow-up whereas the therapeutic response was significantly better at the 4th month of follow-up (Table 2).

MDA: There were no changes in MDA levels at baseline in relation to controls. The level remained same on 1st follow-up as well as 2nd follow-up, i.e. 8th month of treatment with UNIM-001 and UNIM-003. Though there were changes in therapeutic efficiency during the follow-ups but the MDA levels did not show any significant alteration (Table 2).

TAS: The TAS levels were significantly lower in non-segmental vitiligo patients at baseline level in relation to controls. Interestingly, the significant lower level continued throughout the 1st follow-up whereas it increased during 2nd follow-up which may add some increment to the therapeutic response, i.e. eight months of treatment (Table 2).

Anti -TPO: The Anti-TPO levels were significantly increased at the baseline level in vitiligo patients in relation to healthy controls. The increase continued significantly even during the 1st follow-up (4th month) and 2nd follow-up (8th month) on treatment with Unani medicines UNIM-001 and UNIM-003. According to our study in NSV patients, anti-TPO was shown to be significantly higher in vitiligo patients who continued during follow-ups on treatment with coded Unani regimes UNIM-001 and UNIM-003 (Table 2).

IL2: During our experiments, increased serum IL-2 concentrations were evident in relation to control values (healthy subjects) at the baseline level. During the 1st follow-up (at 4th month) with Unani medicines, the level significantly decreased and it continued till the 2nd follow-up at the 8th month (Table 2).

IL-22: Expression studies in the blood of vitiligo patients in relation to controls revealed that the folds were significantly higher in vitiligo baseline in relation to controls – which decreased during the 1st follow-up and increased again during the 2nd follow-up (Table 2).

Table 88: Estimation of biomarker levels in vitiligo patients at baseline, follow-up 1 and follow-up 2 in relation to control (healthy volunteers)

Parameters	Control n=34	Patients n=34 (Baseline)	Patients n=34 (1st Follow-up)	Patients n=34 (2nd Follow-up)	P-value
	A	B	C	D	
TNF-α pg/ml	3.82 ± 10.32	8.719 ± 4.532	5.847 ± 2.84	15.775 ± 2.81	A-B → 0.01 B-C → 0.0005 C-D → 0.0001 B-D→ 0.0001
Lipid per oxidation (MDA) n mole/µL	9.77 ± 1.116	9.891 ± 2.353	8.749 ± 1.24	7.77 ± 2.06	A-B → NS B-C → 0.01 C-D → 0.02 B-D→ 0.0001
TAS activity mM/ml	0.099 ± 0.030	0.058 ± 0.047	0.03 ± 0.02	0.17 ± 0.141	A-B → 0.0005 B-C → 0.004 C-D → 0.0001 B-D→ 0.0001
IL22pg/ml	489.43 ± 75	924 ± 11	960 ± 14	984 ± 23	A-B → 0.0001 B-C → 0.0001 C-D → 0.0001 B-D→ 0.0001
IL2pg/ml	54.97 ± 11.45	70.02 ± 14.96	51.32 ± 14.41	43.87 ± 10.24	A-B → 0.0001 B-C → 0.0001 C-D → 0.02 B-D→ 0.0001
Anti – TPO g/ml	8.10 ± 4.85	15.19 ± 5.86	25.30 ± 6.0	28.20 ± 4.8	A-B → 0.0001 B-C → 0.0001 C-D → 0.043 B-D→ 0.0001

Biochemical Parameters

Results of estimation of various biochemical parameters are as follows:

Table 89: Parameters at baseline of vitiligo patients and control subjects

Parameters	Reference Values	Controls	Vitiligo patients	T-value	P-value
Age		31.73 ± 5.9	32.8 ± 8.7	0.593	0.5691
BMI		23.5 ± 4.3	24 ± 8.0		
Serum bilirubin (mg/dl)	0.1-1.2	0.72 ± 0.37	0.79 ± 0.35	0.801	0.45
ALT(IU/L)	0.5-40	22.6 ± 11.5	23.5 ± 6.5	0.397	0.710
AST (IU/L)	0.5-42	24 ± 8.1	25 ± 7.1	0.541	0.544
ALP (IU/L)	30-111	67.6 ± 21.7	77.4 ± 12.5	2.281	0.0374
Urea (mg/dl)	13-45	22.2 ± 6.6	26 ± 5.5	2.57	0.0187
Creatinine (mg/dl)	0.7-1.4	1.06 ± 0.15	1.09 ± 0.18	0.74	0.45

$p<0.05$ is considered significant

Table 90: Parameters of vitiligo patients at baseline and follow-up 1

Parameters	Reference Values	Vitiligo patients (Baseline)	Vitiligo patients (Follow-up 1)	T value	p Value
Serum bilirubin (mg/dl)	0.1-1.2	0.79 ± 0.35	0.69 ± 0.2	1.446	0.1528
ALT(IU/L)	0.5-40	23.5 ± 6.5	28.4 ± 13.9	1.861	0.0877
AST (IU/L)	0.5-42	25 ± 7.1	27.9 ± 11.7	1.23	0.2516
ALP (IU/L)	30-111	77.4 ± 12.5	81.2 ± 17.2	1.03	0.3321
Urea (mg/dl)	13-45	26 ± 5.5	24.9 ± 5.4	0.832	0.4376
Creatinine (mg/dl)	0.7 – 1.4	1.09 ± 0.18	1.1 ± 0.1	0.283	0.77

$p<0.05$ is considered significant

Table 91: Parameters of vitiligo patients at follow-up 1 and follow-up 2

Parameters	Reference Values	Vitiligo Patients (Follow- up 1)	Vitiligo Patients (Follow- up 2)	T - Value	P-Value
Serum bilirubin (mg/dl)	0.1-1.2	0.69 ± 0.2	0.73 ± 0.4	0.521	0.6267
ALT(IU/L)	0.5-40	28.4 ± 13.9	27.4 ± 11.6	0.322	0.7484
AST (IU/L)	0.5-42	27.9 ± 11.7	26.4 ± 8.8	0.597	0.577
ALP (IU/L)	30-111	81.2 ± 17.2	78.5 ± 19.0	0.614	0.5662
Urea (mg/dl)	13-45	24.9 ± 5.4	22.8 ± 4.6	1.72	0.1105
Creatinine (mg/dl)	0.7 – 1.4	1.1 ± 0.1	1.1 ± 0.1		0.999

$p<0.05$ is considered significant

Discussion

The basis of vitiligo especially non-segmental vitiligo (90% of total vitiligo), is primarily functional loss of melanocytes. Currently vitiligo is viewed as multifactorial process where different phenomena can lead to loss of functional melanocytes. The puzzled vitiligo pathogenesis mirrors the wide range of methodological approaches to the study of its biological basis.[70] Though asymptomatic, the psychosocial impact of vitiligo can be devastating, and affected persons are often desperate for effective therapy. Clinically, treatment modalities for vitiligo require extended treatment plans that may last many months to years and may still result in disappointing outcomes. This lack of success in vitiligo treatment indicates that in addition to present clinical selection criteria, suitable biomarkers are urgently needed for monitoring harmful immune events, predicting vitiligo progression, as well as monitoring therapeutic responses. Herein, the single disease type of vitiligo, i.e., non-segmental generalized vitiligo type with progressive state of disease was studied for evaluation. During the International Pigment Cell Conference (IPCC) 2011, it was decided that utmost consideration should be taken while studying this type of skin disease.[31] Expression studies of different genes including MIF, FOXP3, NLRP1 and IL22 have showed their possible role in many diseases including vitiligo. Although independent studies have been conducted on these genes but as per our knowledge this is the first study wherein the relative expression of four different genes has

been done on same group of non-segmental generalized vitiligo subjects with progressive / active state of disease. The gene studied here is NLRP1, known to be involved in inflammation, apoptosis and the melanocyte death mediated by apoptosis most likely through cytokines.[71] No significant difference in the expression of NLRP1 could be found in the study. However, one of the earlier study had shown reduction in NALP1 (now known as NLRP1) gene expression in PBMCs of vitiligo subjects when compared to normal although expression was measured semi-quantitatively with less sample size.[72,73] Whereas, one of the study had shown significant increase in NLRP1 mRNA expression in patients with GV when compared to healthy controls.[66] Although the above study has been done on Indian subjects, more studies may be proposed with large sample size to establish the difference, if any, in relative expression of NLRP1 in blood of vitiligo patients compared to healthy controls, as any correlation between active NSV and NLRP1 expression could not be established.

In the present study, the relative expression of second gene, MIF mRNA, in blood cells of active NSV subjects was significantly high when compared with healthy controls. During earlier studies, MIF has been shown to play a role in many skin diseases including atopic dermatitis, systemic sclerosis, bullous pemphigoid, psoriasis and vitiligo[61,74]. The present study was in line with one of the reported study which suggested that MIF may act as an index of disease severity and activity in Vitiligo Vulgaris. It has been reported that MIF may induce the local inflammatory and immunological responses of depigmentation associated with Vitiligo Vulgaris in cooperation with other cytokines.[62] Further it has been reported that macrophages are involved in melanocytes clearance as macrophage infiltration and their increased number are observed in perilesional skin in vitiligo.[75,76] Macrophages and MIF loop may contribute the pathogenesis of vitiligo as the macrophages are important source of MIF, whereby MIF exerts a variety of biological functions such as macrophage activation, phagocytes and tumoricidal activity as reported earlier.[62] MIF has also been shown to induce up-regulation of many inflammatory cytokines like IL-6, IL-8, and TNF-α, which in turn play important role in melanocyte cytotoxicity and have inhibitory effect on pigmentation.[77]

The third gene which has been studied herein is Interleukin (IL)-22, which is a member of the IL-10 cytokine family that also includes IL-10, IL-19, IL-20, IL-24, IL26, and the cell surface IL-22 receptor complex is a hetero-dimer composed of IL-22R1 and IL-10R2.[78] IL-22 is produced by immune cells such as CD4+ T cells, γδ-T cells, NK cells, NKT cells, and lymphoid

tissue inducers. The IL-22 receptor complex is highly expressed within the gastrointestinal tract, lung, and kidney in the skin.[78,79,80] In the literature, one report has described the relationship between IL-22 and vitiligo. They observed higher IL-22 expression in the skin of vitiligo patients compared with normal skin.[81]

The fourth gene which has been studied herein is FOXP3, a member of FOX protein family which appears to function as master regulator in the development and function of regulatory T cells.[82] FOXP3 is the defective gene in the X-linked recessive immune dysregulation, poly-endocrinopathy, and enteropathy multiple autoimmune disease syndrome which include generalized vitiligo. In the present study, no significant difference ($P > 0.05$) in relative expression of FOXP3 mRNA in blood cells of active NSV subjects could be found when compared with healthy controls. Some genome wide studies have shown the association of FOXP3 gene polymorphism with vitiligo[83] including the one from Indian subcontinent.[84] In one of the earlier studies a negative correlation between FOXP3 expression in vitiligo patients has been observed.[85]However, no correlation between FOXP3 expressions in blood cells with active NSV could be established.

For the first time it is being reported that NSV has significantly higher TNF-α biomarker levels as compared to the controls, which indicate involvement of autoimmunity in participation of NSV. Investigations on cytokines like TNF-α shows that these inflammatory markers play an important role in apoptosis through activation of the receptor-mediated apoptosis pathway in numerous cell types.[86] It has been reported that cytokines such as IFN-γ and TNF-α can initiate apoptosis and thus lead to melanocyte death in the context of autoimmunity. TNF-α also has the capacity to inhibit melanogenesis through an inhibitory effect on tyrosinase and tyrosinase related proteins.[87,88]

During the 1st follow up (after 4th month) the therapeutic efficiency was nearly 40%which led to decrease in TNFα levels which may have led to the inhibition of melanogenesis by abolishing cytotoxic T-cell-mediated melanocyte destruction. Whereas, on 8th month (2nd follow up) therapeutic efficiency decreased without any known cause. It may probably be due to the fact that the increase in TNFαmay have led to the inhibition of melanogenesis by acting on tyrosinase enzyme and tyrosinase related proteins. It may be interpreted it as indirectly proportional to the process of melanogenesis.

MDA, another biomarker, is an end-product of lipid peroxidation induced by reactive oxygen species (ROS). It is well correlated with the degree of

lipid peroxidation and is an indicator of oxidative stress.[89] The present study revealed no significant differences in the level of MDA when measured in serum between NSV patients and control group. In agreement with this result, Picardo *et al*[90]found normal serum MDA level in combined types of vitiligo. However other studies showed higher serum MDA in patients compared to control as Yousry *et al*[91] and Yildirim *et al*[89] and Dammak *et al*[92] found statistically significant high levels of MDA in tissues of vitiligo patients compared to control group and they explained this that it is a condition of oxidative stress. Moreover, Dammak *et al* added that lipid peroxidation in the cellular membrane of melanocytes may play an important role in the rate of depigmentation observed in the skin of patients with Non segmental vitiligo.

TAS biomarker levels were found lower in NSV patients as compared to controls. This implies that either an increased oxidative stress in vitiligo leads to exhaustion of TAS reserves, or those vitiligo patients have inherently inadequate TAS levels which are unable to effectively neutralize the melanocyte damaging oxidants. It may be concluded that there is impairment in the antioxidant system in vitiligo, leading to free radical mediated destruction of melanocytes or dysregulation of melanogenesis which may activate an autoimmune response. Thus antioxidants may be beneficial as therapeutic agents in vitiligo. Similar findings have been observed by researchers in different parts of the world.[93,94,95]

According to the present study in NSV patients, anti-TPO was shown to be significantly higher in vitiligo patients which continued during follow ups on treatment with coded Unani regimes UNIM 003 and UNIM-001.

This work is in line with the works of Morgan *et al* [96]who also found higher prevalence of thyroid antibodies in vitiligo patients especially in generalized vitiligo, compared with healthy people.

The work of Dave *et al* [97] showed antibody positive in 31.4% of their cases against 10% of controls. The findings in the present study are concordant with the findings of Betterle *et al*, Korkij *et al*[98]and Kurtev *et al.*[99]

IL-2 biomarker is a cytokine that seems to play an important role in vitiligo patients. A limited number of studies in the literature have evaluated the serum levels of IL-2 in patients with vitiligo. In the present study it has been demonstrated that the serum levels of IL-2 were significantly elevated in vitiligo patients at baseline but decreased significantly to the control level within 4 months of follow up with Unani medicine and continued till the 8th month.

During the experiments, increased serum IL-2 concentrations were evident in relation to control values (healthy subjects) at the baseline level. During the 1st follow up with Unani Medicines at 4th month the level significantly decreased and it continued till the 2nd follow up at the 8th month. Although the initiating event in vitiligo has not been defined, a growing body of evidence indicates that cytokines may help the development and the perpetuation of the chronic inflammatory state.[100,101,102,103] Elevated levels were observed at baseline and significantly decreased on treatment which relates to the fact that the study is in line with that of other researchers. The alterations observed in the cytokine IL-2 concentration aimed in the current study suggest their involvement in the pathogenesis of vitiligo.

Levels of IL-22 biomarker in the peripheral blood were higher in vitiligo patients in relation to controls and it persistently remained significantly higher even during follow ups at 4th& 8th months i.e. till completion of the trial.

To our knowledge, this study is the first study to compare IL-22 expression with IL-22 levels in peripheral blood in vitiligo patients and healthy volunteers.

Conclusion

The study investigated the gene expression of MIF, NLRP1, IL22, and FOXP3 in the blood cells of individuals with active non-segmental vitiligo (NSV). A notable alteration in the expression level of MIF mRNA was observed in active NSV subjects compared to healthy controls among the four studied genes. The findings suggest that MIF likely plays a role in the pathogenesis of the non-segmental generalized active form of vitiligo. However, the study did not establish a significant role for NLRP1 and FOXP3. TNF-α was identified as influencing the apoptotic pathway of melanocytes, indicating its potential importance in vitiligo pathogenesis. Additionally, there was an evidence of impaired antioxidant systems in vitiligo, leading to elevated free radicals, which contribute to melanocyte destruction or dysregulation of melanogenesis, triggering an autoimmune response. Therefore, antioxidants may hold therapeutic potential in vitiligo.

The study's strength lies in conducting four different gene expression analyses within the same set of samples from individuals with the active form of non-segmental vitiligo. However, to further establish the role of these genes in disease pathogenesis, larger studies with consistent vitiligo types and more extensive gene coverage in the same set of samples are recommended.

This pioneering study in non-segmental vitiligo patients treated with Unani regimes includes relative expression studies on four genes (MIF, NLRP1, IL22, and FOXP3) and six biomarkers (TNFα, MDA, TAS, IL22, IL2, and Anti TPO). Scientific validation of these biomarkers with Unani regimes contributes evidence-based data that may enhance the global acceptance of these treatments.

REFERENCES

1. Shajil, Chatterjee EM, Agrawal S, Bagchi D, Begum T, Rasheedunnisa. Vitiligo: Pathomechanisms and Genetic Polymorphism of Susceptible Genes. *Indian Journal of Experimental Biology*, 2006.44 (07): 526-539.
2. Ongenae K, Beelaert L, Van Geel N, Naeyaert JM. Psychosocial effects of vitiligo. *Journal European Academy of Dermatology and Venereology*, 2006. 20(1):1-8.
3. Sehgal VN andSrivastava G (2007) Vitiligo: Compendium of Clinico-epidemilogical features. Indian J Dermatol Venereol Leprol. 73(3): 149-156.
4. Das SK, Majumdar PP, Majundar TK and Helder F. Studies on Vitiligo familial aggregation and genetics. *Genet. Epidemial*.1985 2(3): 255-262.
5. Gouide RB, Goudie DR, Dick HM and Ferguson Smith MA. Unstable mutation in Vitiligo. *Lancet*1980:8198, 863.
6. Sinnot, Denn & Dobzhansky. Principles of Genetics, 1992. TMH, Edi. Tata Me. Craw-Hill Publishing Company Ltd, New Delhi. pp. 103-104
7. Anonymous. Annual Report2002-03. Central Research Institute of Unani Medicine, Hyderabad. India
8. Fisher AA. Vitiligo due to contactants. *Cutis*1976.(17): 431-439.
9. Pasricha JS. National survey of contact dermatitis in India, 2nd edition. Ministry of Science and Technology, Govt. of India, New Delhi.1988 p. 42.
10. Chenco Turner ML. and Lerner AB. Physiological changes in Vitiligo. *ArchDermatol.*1965 (91): 390.
11. Rone M and Mackie. Current Perspective Immunodermatology, 2nd Churchill Livingstone, New York. 1984 pp. 293- 294
12. Waheed MA, Mateenuddin K, Khan MMA, Ali MI. Effects of solar eclipse on Vitiligo - An observation. Second Scientific Seminar of CCRUM. 29- 30 March, 1980, Mumbai.
13. Bahauddin & Rabia B. The Munzij and Mushil Therapy in Bars: A Clinical and therapeutic study. Second Scientific Seminar of CCRUM. 29-30 March, 1980, Mumbai
14. Khursheed B, Ali M I. Unani research formulae are more effective in the treatment of Bars: A comparative study. Second Scientific Seminar of CCRUM. 29-30 March, 1980, Mumbai
15. Bahauddin & Khursheed B. Susceptibility of the Bars lesion in any part of the body to Unani treatment: A clinical and therapeutic study. Second Scientific Seminar of CCRUM. 29-30 March, 1980, Mumbai.

16. Hussain S.J. & Taiyab M. Application of statistical designs in the clinical trials with coded Unani formulae. II Scientific Seminar of CCRUM. 29-30 March, 1980. Mumbai.

17. Kenney J A Jr. Vitiligo. *Dermatol Clin.*1988. 6(3): 425-434.

18. Silvan M. The psychological aspect of vitiligo. *Cutis*, 2004.73(3): 163-170.

19. Matsumura Y. and Ananthaswamy H N. Toxic effects of ultraviolet radiation on the skin. *Toxicol Appl Pharmacal.*2004. 195(3): 298-308.

20. Datta AK, Maiti AK and Banerjee BN. Skin temperature graded of Vitiligo patches under local and general heat and cold exposures. *Ind. J. Dermatol.*1963. (8): 97.

21. Dawber RPR. Clinical association of vitiligo. *Postgrad. Med. Journal.*1970. (46): 276.

22. Sabina Mansoor. Biochemical and cytogenetic studies on Vitiligo (in human beings). Thesis submitted for PhD Bio-chemistry. 1989. Institute of Genetics, Hospital for Genetic Diseases, Osmania University, Hyderabad.

23. Seghal VN and Dube B. ABO Blood groups in vitiligo. *J. Med. Genet.*1968. (5):308- 369.

24. Behl PN and Bhatia RK. 400 cases of Vitiligo a clinical therapeutic analysis. *Ind. J. Dermatol.*1972 (17): 51-56.

25. Datta K and Mandai SB. A Clinical study of 650 Vitiligo cases and their classification. *Ind. J. Dermatol.*1969 (14): 103-111.

26. Levai M. A study of certain contributory factors in the development of Vitiligo in South Indian patients. *Arch. Dermatol.*1958. (7) 8: 364-370.

27. Kugelman TP and Lerber AB. Albinism, partial albinism and vitiligo. *Yale J. Biol. Med.*1961 (33): 407-414.

28. Krupa Shankar DS, Shashikala K, Madala R. Clinical patterns of vitiligo and its associated co-morbidities: A prospective controlled cross-sectional study in South India. *Indian Dermatology* (May-Aug.) 2012. 3 (2):114-118

29. Shah H, Mehta A, Astik B. Clinical and sociodemographic study of vitiligo. *Indian J Dermatol Venereol Leprol* 2008 (74) :701

30. Reichert-Faria A, Tarle RG, Dellatorre G, Mira MT, Silva de Castro CC Vitiligo - Part 2 - Classification, Histopathology and Treatment. *An Bras Dermatol*. 2014; 89(5):784-90.

31. Ezzedine K, Lim HW, Suzuki T, Katayama I, Hamzavi I, Lan CC, et al. Revised classification/nomenclature of vitiligo and related issues: the Vitiligo Global Issues Consensus Conference. *Pigment Cell Melanoma Res.* 2012;25:E1-13.

32. Silva de Castro CC, de Nascimento LM, Olandoski M, Mira MT. A pattern of association between clinical form of vitiligo and disease-related variables in a Brazilian population. *J Dermatol Sci.* 2012;65:63-7.

33. Ezzedine K, Gauthier Y, Leaute-Labreze C, Marquez S, Bouchtnei S, Jouary T, Taieb A. Segmental vitiligo associated with generalized vitiligo (mixed vitiligo): a retrospective case series of 19 patients. *J Am Acad Dermatol*. 2011; 65:965–971.

34. Khan PJ, Khan MM.A. Duration of treatment and response to Unani formulae, Workshop on Bars, Jan, 18-20, 1986, Hyderabad.

35. Shareef MA, Lateef A, Hussain A, Parveen S, Hussain S J &Taiyat M. Effect of Aatrilal *(Ammi majus* Linn) on different colours of vitiliginous lesion with relation to chronicity of disease. 1st International Seminar on Unani medicine. 13-15 Feb., 1987 New Delhi.

36. TaheraSS, Reddy KRK, Ansari S, Ali Khan MM. Toxicological studies of Unani formulae in the treatment of Bars. First International Seminar on Unani Medicine, CCRUM. 13-15 Feb. 1987. New Delhi.

37. TaheraSS, Goud PV, Reddy KRK, Ahmed M, Noor A, Ali Khan MM. Unani concoctives and purgatives action and effects in clinical trials and experimental studies. First International Seminar on Unani Medicine, CCRUM. 13-15 Feb. 1987. New Delhi.

38. Goud PV, Haq SM. Reappearance of Pigmented melanocytes in Leucoderma, patches after Unani treatment - A cytological evidence. First International Seminar.Feb.13-15, 1987. New Delhi.

39. Tabri ABM. *Moalijat-e-Buqratiya* (Urdu Translation), Vol-2. Central Council of Research in Unani Medicine, Ministry of AYUSH, Govt. of India, 1997; pp.199-200.

40. Tabri Ali Bin Sahal Rabban. *Firdaus-ul-Hikmat* (Urdu Translation), Vol-1; Idara Tarjuman Tibb, Karachi, Pakistan, 1996 p.825.

41. Al Razi ABZ. *Kitab-ul-Havi Fit Tib* (Arabic version). Vol-23. Dairatul Marif Osmania University, Hyderabad, 1955, pp.72-75.

42. Ibn-e-Sina. *Al Qanoon Fit Tibb*. Vol 4 (Urdu Translation). Idara Matbuat Sulaimani, Lahor. 1998; pp.351-356.

43. Anonymous. *Monograph on Bars (Vitiligo)*. Central Council for Research in Unani Medicine, Dept. of AYUSH, Ministry of Health and Family Welfare, Govt. of India,2006. pp. 45-119,125- 139.

44. Arzani MA. *Tibb-e-Akbar*.Vol-2, Matba Islamia, Lahore,1915: pp.731

45. Khan HA. *Ikseer-e-Azam*, vol no.4, Lucknow, Nawal Kishore Press.1885.

46. Anonymous. *Standard Treatment Guidelines for Common Diseases*, vol-I published by CCRUM, Ministry of AYUSH, Govt. of India, 2014; pp.180-85.

47. Shimizu T. Role of macrophage migration inhibitory factor (MIF) in the skin. *J Dermatol Sci.* 2005; 37: 65-73.

48. Ma L et al., A Relationship of macrophage migration inhibitory factor levels in PBMCs, lesional skin and serum with disease severity and activity in vitiligo vulgaris. Brazilian Journal of Medical and Biological Research; 2013. (46): 460-464.

49. Renauld JC. Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators. *Nat Rev Immunol,* 2003(3):667 76.

50. Ratsep R et al., Gene expression study of IL10 family genes in vitiligo skin biopsies, peripheral blood mononuclear cells and sera. *British Journal of Dermatology*. 2008(159), pp. 1275-1281.

51. Jin Y et al. NALP1 in vitiligo-associated multiple autoimmune disease. *N Engl J Med* (2007) 356:1216- 25.

52. Dwivedi M et al., Association ofNLRP1 genetic variants and mRNA overexpression with generalized vitiligo and disease activity in a Gujarat population. *British Journal of Dermatology*2013. (169), pp.1114 -1125.

53. Zhang L and Zhao Y. The regulation of Foxp3 expression in regulatory CD4(+) CD25(+) T cells: multiple pathways on the road. *J. Cell. Physiol.* (2007). 211 (3): 590 597.

54. Jump Marson A et al., Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* (2007). 445 (7130): 931 5.

55. Ting JP, Willingham SB, Bergstralh DT. NLRs at the intersection of cell death and immunity. *Nat Rev Immunol*; (2008) 8:372–9.

56. Abdel-Naser MB, et al., Further evidence for involvement of both cell mediated and humoral immunity in generalized vitiligo. *Pigment Cell Res* (1994) 7:1 8.

57. Rubin LA et al., The soluble interleukin-2 receptor: biology, function, and clinical application. *Ann Intern Med* (1990) 113:619 27.

58. Honda Y et al., Relationship between levels of soluble interleukin-2 receptors and the types and activity of vitiligo. *J Dermatol* (1997). 24:561 3.

59. Yeo UC, et al., Serum concentration of the soluble interleukin-2 receptor in vitiligo patients. *Journal of Dermatological Science* (1999) 19: 182-188.

60. Wolkentein P et al., Autoimmune disorders and interleukin-2 therapy: a step toward unanswered questions. *Arch Dermatol* (1995) 131:615.

61. Gupta S, and Gollapudi S. Molecular mechanisms of TNF-α-induced apoptosis in 136 naive and memory Tcell subsets. *Autoimmun Rev.* (2006) 5: 264 268.

62. Alghamdi KM et al., Treatment of generalized vitiligo with anti-TNF-α Agents. *J Drugs Dermatol* (2012) 11: 534 539.

63. Laddha NC, et al., Increased Tumor Necrosis Factor (TNF)-α and Its Promoter Polymorphisms Correlate with Disease Progression and Higher Susceptibility towards Vitiligo. *PLoS ONE* (2012). 7(12): e52298.

64. Naresh C et al., Involvement of superoxide dismutase isoenzymes and their genetic variants in progression of and higher susceptibility to vitiligo. *Free Radical Biology and Medicine* (2003);65: 1110-1125.

65. Koca R et al., Oxidant-antioxidant enzymes and lipid peroxidation in generalized vitiligo. *Clin Exp Dermatol*. (2004), 29:406 9.

66. Cross CC, Halliwell B, Borish et al. Davis conference: oxygen radicals and human disease. *Ann. Intern. Med* (1987). 107, 536-545.

67. Jaynath DP, Pai, BS, Shenoi SD and Balachandran C. Efficacy of antioxidants as an adjunct to photochemotherapy in vitiligo. A case study of 30 patients. *Indian J. Dermatol. Venereol. Leprol* (2002). 68, 202-205.

68. Schallreuter KU, Moore J, Wood JM, et al. Epidermal H_2O_2 accumulation alters tetrahydrobiopterin (6BH4) recycling in vitiligo: Identification of general mechanism in regulation of all 6BH4-dependent processes. *J. Invest. Dermatol* (2001). 116: 167-74.

69. Schallreuter KU, Moore J, Wood JM et al. In vivo and in vitro evidence for hydrogen peroxide (H_2O_2) accumulation in the epidermis of patients with vitiligo and its successful removal by a UVB-activated pseudo catalase. *J. Invest. Dermatol*1999 (4) 91-96.

70. Dell Anna ML, Cario-Andre M, Bellei B, Taieb A, Picardo M. In vitro research on vitiligo: strategies, principles, methodological options and common pitfalls. *Exp Dermatol*; 2012.21(7):490-6.

71. Tschopp J, Martinon F, Burns K. NALPs. Anovel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 2003(4):95–104.

72. Huang CL, Nordlund JJ, Boissy R. Vitiligo: a manifestation of apoptosis. *Am J Clin Dermatol;*2002(3):301–8

73. Deo SS, BhagatAR, Shah RN. Genetic variations in NALP1 mRNA expressions in human vitiligo. *Indian J Dermatol*; 2011(56):266–71.

74. Asano Y, Makino T, Norisugi O, Watanabe H, Abe R, Shimizu H, et al. Macrophage migration inhibitory factor (MIF) in bullous pemphigoid. *J Dermatol Sci*.2008. (49): 95-97.

75. Kim JY, Kwok SK, Hur KH, Kim HJ, Kim NS, Yoo SA, et al. Up-regulated macrophage migration inhibitory factor protects apoptosis of dermal fibroblasts in patients with systemic sclerosis. *Clin Exp Immunol*.2008 (152):328-35.

76. Serarslan G, Yonden Z, Sogut S, Savas N, Celik E, Arpaci A. Macrophage migration inhibitory factor in patients with vitiligo and relationship between duration and clinical type of disease. *Clin Exp Dermatol*. 2010. (35):487-90.

77. Yu HS, Chang KL, Yu CL, Li HF, Wu MT, Wu CS, et al. Alterations in IL-6, IL-8, GM-CSF, TNF-alpha, and IFN gamma release by peripheral mononuclear cells in patients with Non segmental Vitiligo. *J Invest Dermatol*.1997. (108):527-29.

78. Sonnenberg GF, Fouser LA, Artis D. Border patrol regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol*.2011 (12):383–90.

79. Zenewicz LA, Flavell RA. Recent advances in IL-22 biology.*Int Immunol*.2011 (23): 159–163.

80. Sonnenberg GF, Fouser LA, Artis D. Functional biology of the IL-22R pathway in regulating immunity and inflammation at barrier surfaces. *Adv Immunol*.2010 (107):1–29.

81. Basak PY, Adiloglu AK, Ceyhan AM, Tas T, Akkaya VB. The role of helper and regulatory T cells in the pathogenesis of vitiligo. *J Am Acad Dermatol.*2009; 60(2):256-60.

82. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*. 2007; 445(7130):936-40.

83. Birlea SA, Jin Y, Bennett DC, Herbstman DM, Wallace MR, McCormack WT, et al. Comprehensive Association Analysis of Candidate Genes for Generalized Vitiligo Supports XBP1, FOXP3, and TSLP. *J Invest Dermatol*2011; 131(2):371–81.

84. Jahan P, Cheruvu R, Tippisetty S, Komaravalli PL, Valluri V, Ishaq M. Association of FOXP3 (rs3761548) promoter polymorphism with nondermatomal vitiligo: A study from India.*J Am Acad Dermatol*. 2013; 69(2):262-6.

85. Elela MA, Hegazy RA, Fawzy MM, Rashed LA, Rasheed H. Interleukin 17, interleukin 22 and FoxP3 expression in tissue and serum of non-segmental vitiligo: a case- controlled study on eighty-four patients. *Eur J Dermatol.* 2013. 23(3):350-5.

86. Venters HD, Tang Q, Liu Q, VanHoy RW, Dantzer R, Kelley KW. A new mechanism of neurodegeneration: a proinflammatory cytokine inhibits receptor signaling by a survival peptide. *Proc Natl Acad Sci* USA; 1999; 96:9879-84.

87. Huang CL, Nordlund JJ, Boissy R. Vitiligo. A manifestation of apoptosis. *Am J Clin Dermatol*. (2002) 3: 301–308.

88. Martinez-Esparza M, Jimenez-Cervantes C, Solano F, Lozano JA, Garcia-Borron JC. Mechanisms of melanogenesis inhibition by tumour necrosis factor-α in B16–F10 mouse melanoma cells. *Eur J Biochem*. (1998). 255: 139–146.

89. Yildirim M, Baysal V, Inaloz HS, *etal;*The role of oxidants and antioxidants in generalized vitiligo at tissue level. *Europ Acad Dermatol and Venereol*.2004. 18: 683- 686.

90. Picardo M, Passi S, Morrone A, *et al;*Antioxidant status in the blood of patients with Non segmental Vitiligo. *Pig Cell Res*.1994. (7): 110– 115.

91. Yousry MM, Hanan F, MohamedAS, *et al*. Evaluation of Some Indices of Oxidative Stress in Patients with Vitiligo, *Pan-Arab League of Dermatologists*; 2007. 18 (3):43-53.

92. Dammak I, Boudaya S, Ben Abdallah F, *et al.*Antioxidant enzymes and lipid peroxidation at the tissue level in patients with stable and Non segmental Vitiligo. *Int J Dermatol*; 2009. 48: 476- 480.

93. Jalel A, Hamdaoui MH. Study of total antioxidant status and glutathione peroxidase activity in Tunisian vitiligo patients. *Indian J Dermatol;* 2009. 54:13–6.

94. Khan R, Satyam A, Gupta S, Sharma VK, and Sharma A. Circulatory levels of antioxidants and lipid peroxidation in Indian patients with generalized and localized vitiligo. *Arch Dermatol Res*. 2009. (301):731–7.

95. Akoglu G, Emre S, Metin A, Akbas A, Yorulmaz A, Isikoglu S, *et al.* Evaluation of total oxidant and antioxidant status in localized and generalized vitiligo. *ClinExp Dermatol.* 2013. (38):701–6.

96. Morgan M, Castells A, Ramirez A. Auto antibodies in vitiligo: Clinical Significance. *Med Cutan Ibero Lat Am*, 1986 (14):139-42.

97. Dave S, D'Souza M, Thappa DM, Reddy KS, Bobby Z. High frequency of thyroid dysfunction in Indian patients with vitiligo. *Indian J Dermatol*, 2003 (48):68-72.

98. Korkij W, Soltani K, Simjee S, Marcincin PG, Chuang TY. Tissue-specific autoantibodies and autoimmune disorders in vitiligo and alopecia areata: a retrospective study. *J Cutan Pathol,* 1984 (11):522-30.

99. Kurtev A, Dourmishev AL. Thyroid function and autoimmunity in children and adolescents with vitiligo. *J Eur Acad Dermatol Venereol*, 2004. (18):109-11

100. Kasumagic-Halilovic, Emina & ovcina-kurtovic, Nermina & Soskic, Samra &Helppikangas, Hana & Ridic, Oggi. Serum Levels of Interleukin-2 and Interleukin-2 Soluble Receptor in Patients with Vitiligo. *B J of Med and Medi Res*. (2016); 13. 1-7. 10.9734/BJMMR/2016/23959.

101. Singh S, Singh U, Pandey SS. Serum concentration of IL-6, IL-2, TNF-α, and IFN-γ in vitiligo patients. *Indian J Dermatol*; 2012. 57(1):12-4.

102. Khan R, Gupta S, Sharma A. Circulatory levels of T-cell cytokines (interleukin [IL]-2, IL-4, IL-17, and transforming growth factor-β) in patients with vitiligo. *J Am Acad Dermatol*,2012. 66(3):510-11.

103. Laddha NC, Dwivedi M, Mansuri MS, GaniAR, Ansarullah M, Ramachandran AV, *etal*; Vitiligo: Interplay between oxidative stress and immune system. *Experimental Dermatology*; 2013. 22(4):245-50

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