DEVELOPMENT AND CHARACTERIZATION OF SNEDDS OF *TERMINALIA ARJUNA* LINN. AND ITS ANTIHYPERTENSIVE ACTIVITY IN EXPERIMENTAL ANIMALS

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Ayurvedic Pharmacy

By

Pankaj

Registration Number: 12208250

Supervised By

Dr. Manish Vyas (17410)

Department of Pharmaceutical Science

(Professor)

Lovely Professional University

Co-Supervised by

Dr. Palwinder Kaur (28572)

Department of Pharmaceutical Science

(Assistance Professor)

Lovely Professional University

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2025

DECLARATION

The research work embodies in this thesis "**Development And Characterization Of SNEDDS Of *T. arjuna* Linn. And Its Antihypertensive Activity In Experimental Animals**" for the fulfilment of the requirement for the award of the degree of Doctor of Philosophy in Ayurvedic Pharmacy has been accomplished under the supervision of **Dr Manish Vyas, Professor, Department of Ayurvedic Pharmacy, School of Pharmaceutical Sciences, Lovely Professional University, Punjab, India**, and co-supervision of **Dr. Palwinder Kaur, Assistant Professor, Department of Pharmacology, School of Pharmaceutical Sciences, Lovely Professional University, Punjab, India**

The extent of information derived from the existing literature has been indicated in the body of the thesis at appropriate places along with the source of information. The work is original and has not been submitted in part or full any degree or diploma of this or any other University.

Date:

Place: Lovely Professional University,
Phagwara, Punjab, India

Pankaj

Reg. No: 12208250

CERTIFICATE

This is to certify that the thesis entitled "**Development And Characterization Of SNEDDS Of *Terminalia arjuna* Linn. And Its Antihypertensive Activity In Experimental Animals**" submitted for the award of the degree of **Doctor of Philosophy in Ayurvedic Pharmacy** to **Lovely Professional University, Punjab**, India is a record of bonafide research work carried out by **Pankaj** under my guidance/ co-guidance. To the best of my knowledge, the thesis has not been previously submitted elsewhere for the award of any other degree, diploma or dissertation of any kind anywhere before.

Supervisor

Dr Manish Vyas
Professor
Department of Ayurvedic Pharmacy
School of Pharmaceutical Sciences
Lovely Professional University, Punjab, India

Co-Supervisor

Dr. Palwinder Kaur
Professor,
Department of Pharmacology,
School of Pharmaceutical Sciences,

ACKNOWLEDGEMENT

At this pivotal moment, as nearly three years of hard work culminate in this dissertation, I am overjoyed to express my deepest gratitude to everyone who contributed to making this significant achievement possible.

It is in the presence and with the blessings of Lord Rama, Lord Shiva and Lord Krishna that the work actually happened successfully.

And in that order the first to come is my Dadaji Late. Shri Banwari Lal, Dadi ji Smt. Shanti Devi, my Tauji Shri Rajkumar & Mosi ji Mrs. Nirmla Devi who always wish to see me in this position. Then comes my Parents Mummy -Mrs. Neelam Devi, Papa – Mr. Rajpal the best affectionate and care taking people who made me understand how to enjoy the work and responsibilities. Constantly bestowing confidence and love by my dear sisters Usha Rani, Rajni and elder brother Mr. Sanjeev Verma & his wife Mrs. Sarita Devi, My lovely brother Inspector. Deepak Verma & his wife Mrs. Ritu Bedi have always galvanized me with their ever-inspiring attitude.

To my beloved wife, Mrs. Neha Verma, thank you for being a constant source of happiness and encouragement.

I am very thankful to the person, who made me understand the importance of my work and my profession better, the most creditable source of success in this work, my guide Dr. Manish Vyas, his parental guidance and affectionate nature throughout the work kept me motivated, without his guidance and continuous support and encouragement, this work could not be completed.

I extend my cordial thanks to my co-guide, Dr. Palwinder Kaur for his prized guidance and positive attitude.

I am extremely thankful to Dr. Sanjeev Sahu, Dr, Pranav Kumar Prabhakar for their responsible attitude, sharing of practical experiences and supportive nature.

I am extremely thankful to Dr. Navneet Khurana for his support in my pharmacological study.

I acknowledge Dr. Amrinder Kaur, Dr. Davinder Pandey, Dr. Vijay, for guiding me in various aspects.

Words are short to express my feelings for Miss. Kamlesh Kaur who constantly guided me for the betterment of my future.

I would also extend my gratitude towards the Dean, Dr. Monica Gulati for providing me an opportunity to study in this Institution.

I would like to extend my thanks to Dr Isha Agrawal who helped me for the preparation of this dissertation, gave me considerable impetuous in achieving the milestone, is worth remembering.

I am very thankful to my senior Dr Amritpal Singh Sir for their friendly behaviour and encouragement.

I would extend my thanks to Jagan, for his unwavering support and understanding, Madhur, for cheering me up every time. Each of you have been the balm to my weary soul, my source of strength and camaraderie, reminding me of the power of friendship in navigating life's challenges.

I am grateful to my friends Aveek Gupta for their timely motivation, encouragement and genuine support.

Furthermore, I extend my thanks to my seniors and colleagues, administrative staff, technical support teams, all the fellow researchers, juniors and Department Pharmaceutical sciences and Department of research and development whose collective efforts create the fertile ground upon which academic pursuits can thrive.

Central Instrumentation Facility, LPU needs special acknowledgement specially for their Kind cooperation.

I sincerely pay my salute to those sacred creatures, who suffered for the Pharmacological study for the better future of entire humanity.

I express my gratitude to all those, who co-operated me directly or indirectly in this venture.

Content

CHAPTER 1
INTRODUCTION

Chapter 1

Introduction

Cardiovascular diseases (CVDs) encompass a broad range of disorders affecting the heart and blood vessels. Severe cardiovascular diseases include “congenital heart disease, coronary heart disease, cerebrovascular disease, peripheral arterial disease, and rheumatic heart disease”. In 2019, cardiovascular diseases (CVDs) accounted for an estimated 17.9 million deaths globally, representing approximately 32% of total mortality, according to data published by the World Health Organization (WHO).[1-2]. Heart attacks and strokes accounted for 85% of cardiovascular-related deaths, with coronary heart disease (CHD) being the leading cause across most populations, except among females in Sub-Saharan Africa and both sexes in South Asia, where different mortality patterns are observed. Hypertension, commonly referred to as high blood pressure, is a persistent medical condition marked by consistently elevated arterial pressure. It serves as a significant risk factor for cardiovascular diseases, including heart attacks and strokes, as well as other related health issues. [2]. Hypertension, a key risk factor for cardiovascular disease, is frequently manageable through lifestyle interventions such as dietary adjustments and regular physical activity, alongside pharmacological treatment. According to data from the Centers for Disease Control and Prevention (CDC), approximately 47% of adults in the United States are afflicted with elevated blood pressure, highlighting its critical contribution to early mortality and the escalating worldwide incidence of cardiovascular disorders.[3]. Consistent medical assessments are vital for accurate monitoring and control of blood pressure.[4]. The prevalence of hypertension has been steadily rising, particularly in low- and middle-income nations, where limited access to healthcare infrastructure and preventive interventions poses significant challenges to effective management and control [5]. In modern therapeutic approaches, cardiovascular diseases are managed using a broad spectrum of medicinal agents such as natriuretic drugs, beta-adrenergic blockers, renin-angiotensin system inhibitors (e.g., ACE inhibitors and angiotensin II receptor antagonists), and calcium influx inhibitors. These pharmacological interventions exert their effects through multiple molecular pathways to ensure optimal cardiovascular function and disease control. However, these drugs are associated with side effects such as elevated potassium levels in the blood (hyperkalaemia), dizziness, and swelling of the skin due to fluid accumulation (angioedema)[6]. The fundamental philosophy of the Ayurvedic system of medicine emphasizes prevention over cure. A key advantage of Ayurveda compared to allopathic medicine is the use of medicinal herbs and plants, which are generally associated with minimal adverse effects[7].*T. arjuna*

(Linn.), commonly known as Arjuna, has been traditionally employed in Ayurvedic medicine for centuries due to its reputed efficacy in managing various cardiac disorders.[8]. This traditional Ayurvedic herb has been recognized since the Vedic era and is extensively documented in classical Indian medical treatises such as the *Charaka Samhita*, *Sushruta Samhita*, and *Ashtanga Hridayam* for its therapeutic applications, particularly in the management of cardiovascular ailments. [9]. *Vagbhata* initially endorsed the recommendation of stem bark powder for managing cardiovascular ailments. According to Ayurvedic principles, *Vyan Vayu* regulates blood circulation, and its dysfunction is chiefly associated with the development of elevated blood pressure [10]. Natural products are gaining growing interest from the scientific and medicinal areas due to their chemical diversity. *Terminalia arjuna* Linn. (*T. arjuna)*, Family: *Combretaceae* is widely incorporated into pharmaceutical formulations due to its broad therapeutic potential and is commonly utilized in the management of various pathological conditions [11]. *T. arjuna* is employed in the treatment of ulcers and to enhance wound healing, owing to its diverse pharmacological properties, including antibacterial, antioxidant, antimutagenic/anticarcinogenic, and hypocholesterolemic activities. [12]. The stem bark of *T. arjuna* has long been employed in the treatment of various cardiovascular conditions. Historically, its decoction has been extensively utilized across the Indian subcontinent for managing anginal pain, hypertension, congestive heart failure, and dyslipidemia. These traditional applications are supported by centuries of empirical use documented by ancient medical practitioners [13].

While plant-based medicines offer natural healing benefits, they also have some notable downsides. One big challenge is the lack of consistency, since plants grow under different conditions and are processed in various ways, the potency of herbal remedies can vary from batch to batch[14].Another concern is that many of these therapies have not been extensively evaluated through scientific research, resulting in limited evidence regarding their safety, efficacy, and appropriate dosage. In contrast to synthetic medications, which generally produce rapid effects, plant-based medicines often require a longer duration to demonstrate therapeutic benefits and may need prolonged use[15] There is a potential risk of contamination by pesticides, heavy metals, or microbial agents, particularly in cases where stringent quality control measures are not adequately implemented. [16]. Nanotechnology has recently emerged as a promising approach in the development of innovative drug delivery systems, aiming to overcome the limitations commonly associated with conventional dosage forms. These include solubility, bioavailability, toxicity, repeated dose administration and stability related issues.

Nanotechnology aid in increasing targetability and efficiency of drug molecules, thereby reducing side effects, toxicity related problems. They can also provide controlled release of active constituent making this approach even more attractive [17].

A range of techniques exists to enhance drug solubility, including the application of surfactants, particle size reduction through micronization, salt formation, pH modulation, nanoformulations, solid dispersions, and the use of permeation enhancers. These strategies have been extensively studied to improve the solubility, absorption, and consequently the bioavailability of hydrophobic drugs with poor aqueous solubility.

Some of the commonly used approaches include forming inclusion complexes with cyclodextrins, developing lipid-based formulations, reducing particle size, and preparing solid dispersions. Other effective techniques involve salt formation, supercritical fluid processing, pH modification, the use of co-solvents, hydrotropic agents, as well as incorporating surfactants and solubilizers. Each of these methods aims to improve how the drug dissolves and is absorbed in the body, ultimately enhancing its therapeutic effectiveness[18-23]. Among the array of formulation strategies, lipid-based delivery systems, particularly self-emulsifying platforms such as Self-Emulsifying Drug Delivery Systems (SEDDS) and Self-Nanoemulsifying Drug Delivery Systems (SNEDDS), have demonstrated considerable potential. These formulations are notably effective in enhancing the dissolution, absorption, and bioavailability of lipophilic drugs, thereby representing a preferred approach to overcoming the limitations posed by poor aqueous solubility [24-26]. Self-nanoemulsifying drug delivery systems (SNEDDS) offer a highly efficient method to surmount complex dissolution limitations and augment the stability of natural extracts with low water solubility[27].

SNEDDS differs from microemulsions in that it is more transparent, fully disperses in water, and has ultra-low interfacial tension, making it a more stable system. It is composed of isotropic mixtures, including oil, surfactants, and co-surfactants[28]. While surfactants alone help reduce surface tension, they are not sufficient to achieve optimal stability. This is where co-surfactants improve the fluidity of the interface, increasing system entropy and ensuring better dispersion[29].The primary objective of this investigation is to develop a Self-Nanoemulsifying Drug Delivery System (SNEDDS) aimed at addressing the intrinsic limitations associated with phyto therapeutic agents.[30].

CHAPTER 2

REVIEW OF LITERATURE

Chapter 2

Review of Literature

2.1. Review of literature

Hypertension is a clinical condition marked by consistently elevated arterial blood pressure. It is measured as the ratio of systolic pressure—the force applied to the arterial walls during ventricular contraction—to diastolic pressure, which corresponds to the pressure in the arteries during the cardiac relaxation phase between beats. The specific values used to identify hypertension can vary depending on how the blood pressure is measured.[31].

There are several possible causes of hypertension. In most cases, about 90 to 95% individuals are diagnosed with essential or primary hypertension[32]. The condition is not attributed to a single known cause but is considered to develop through a complex combination of genetic factors and environmental influences [33].Arterial tone itself is influenced by intravascular volume and various neurohumoral mechanisms. These interconnected mechanisms work synergistically to regulate blood pressure, maintaining it within a normal physiological range [34].

A finely tuned neurohumoral network plays a central role in this regulation. This includes the renin-angiotensin-aldosterone system (RAAS), natriuretic peptides, endothelial function, the sympathetic nervous system (SNS), and the immune system. These systems are interconnected and work in harmony to ensure that blood pressure remains stable under different physiological conditions[35].

T. arjuna Linn. is widely distributed in the forested areas and along dry riverbeds of India, Sri Lanka, and several Southeast Asian regions. The species flourishes under hot, dry climatic conditions and exhibits the ability to grow across a range of soil types, including sandy, loam, and clay soils. [36]. The species can reach a height of approximately 25 meters and is distinguished by a broad, spreading crown. Its trunk is generally straight and cylindrical, with a maximum diameter of about 2 meters [36].The tree's bark is smooth with a grayish-white hue, characterized by horizontal bands and a rough, scaly texture. The leaves are large, oval-shaped, and leathery, with a glossy green color and a slightly curved tip[11], [37]. The Arjuna tree bears small, white, and aromatic flowers that typically blossom during the summer season. The fruit is a woody, ellipsoid capsule that contains several seeds. The fruit ripens in the winter months and falls from the tree, where it is often

collected and used for its medicinal properties[38]. For centuries, the bark and leaves of *T. arjuna* have been employed in Ayurvedic medicine to address a variety of health disorders, especially those affecting the heart and cardiovascular system[39]. The bark is harvested from mature trees and dried and then used to make various herbal remedies and supplements[40].

Arjuna, commonly referred to as "White Murdah," is a large, virtually evergreen tree that may be found over most of India. The term "Arjuna" denotes that it strengthens the heart and aids in the treatment of cardiac problems[41]. The term *Terminalia* denotes the typical leaf arrangement observed at the tips of branchlets in some species, and the name *Arjuna* originates from the Sanskrit language. This plant is commonly employed in the Ayurvedic medical system for managing a variety of diseases [42].

T. arjuna bark exhibits potent cardioprotective and anti-inflammatory activities supported by both traditional and modern studies. Research studies highlighted its antioxidant, hypolipidemic, and myocardial-protective effects, validating its long-standing use in cardiovascular ailments [43]. Bharatraj further demonstrated that the Ayurvedic formulation *Arjuna Kṣhīra Pāka* (bark powder boiled in milk) showed significant in-vivo anti-inflammatory efficacy, supporting the Ayurvedic concept that preparation in milk enhances solubility and bioavailability of active constituents [44].

Ayurvedic physicians frequently use the medicinal plant of the genus *Terminalia* called *T. arjuna* for its ability to treat structural and functional cardiac issues like angina, hypertension, and artery deposits[45]. Fever, fractures, and contusions can all be treated with its bark. The powdered bark functions as a diuretic in the treatment of liver cirrhosis and exhibits styptic, tonic, febrifuge, and anti-dysenteric properties. The fruits are nutritive and possess decongestant effects. Additionally, leaf juice is applied in the management of earaches. Supplementation with *Terminalia arjuna* bark powder significantly reduced oxidative stress and enhanced antioxidant enzyme activities. The study suggested its protective role against oxidative tissue damage. [46], [47].

2.2. Habit and habitat

T. arjuna, a prominent perennial evergreen species endemic to the Indian subcontinent, is extensively dispersed throughout the central and northern territories of India. This species is adaptable to a wide range of ecological niches, predominantly colonizing both xeric and mesic deciduous forest biomes.[46]. The species favors tropical climates with average temperatures of 25 to 30°C and is also present in Sri Lanka, Myanmar, and Pakistan [47]. It grows best

in well-drained soils with a pH range of 6.5 to 8.5 and is frequently found along riverbanks and rocky terrains. The tree can attain heights of 20 to 25 meters.

It has a thick, light grayish-brown bark, which is smooth in younger trees and becomes rough and cracked in older trees. The leaves of *T. arjuna* are simple, alternate, and oblong with a pointed tip[48]. The tree produces white to yellow flowers in clusters, which appear in March to June. The tree is deciduous, common in dry hill regions of the Indian peninsula, and grows by the sides of streams in utter Pradesh, south Bihar, and Madhya Pradesh. It is also very common in the Chotta Nagpur region. The shrub is predominantly found near rivers and ponds and has additionally been recorded within the forested habitats of Sri Lanka, Myanmar, and Mauritius [49, 50].

Fig2.1. *T. arjuna* (stem bark)

2.3. Taxonomical classification of *T. arjuna*[46]

Kingdom	Plantae
Order	Myrtales
Family	*Combretaceae*
Division	Magnoliopsida
Genus	*Terminalia*
Species	*Arjuna*

2.4. Macroscopic characteristics of the *T. arjuna*

Table 2.1: Macroscopic characteristics of the *T. arjuna*

Habit	An evergreen tree, *T. arjuna* typically grows new leaves from February

	to April before the leaves fall [51].
Stem-bark	Its appearance is smooth, simple, and somewhat grey. The bark is characterized by a thick, soft texture with a crimson-colored inner surface and exhibits a bitter taste [52].
Leaves	The leaves of *T. arjuna* are sub-oppositely arranged, glabrous, and asymmetrical, bearing a morphological resemblance to guava foliage; they are oblong in shape, typically measuring 4–6 inches in length and 2–3 inches in width, with a crenulate apex that is acute or subacute, a base that may be cordate or rounded, two prominent basal glands at the petiole junction, and a petiole length ranging from 0.5 to 1.3 cm. [52, 53]
Flowers	White or yellowish blooms grow in clusters. In summer flowers occurring, while winter or spring is when fruit arrives[53].
Fruits	The fruit of *T. arjuna* is a drupe, typically 1–1.5 inches in diameter, characterized by 5–7 smooth, woody, fibrous wings forming longitudinal lobes, and often displays an apical notch with obliquely ascending striations.[53].

2.5. Reference found in *Vedic Kala*

In the Vedas, Arjuna is mentioned. The *Krirru* (microbes) keep themselves away from *Arjuna*, according to the *Atharva Veda*. In the Vedic era, the drug would be widely used as the *KrimiNashaka*, Vayu mandala *Shodhaka, KshetriyaRogaNashaka*, *Bala Vardhaka*, and *Hridya*[54].

2.5.1. Reference found in various *Samhitas*[54]

Table 2.2: Reference found in various *Samhitas*

Sr. No.	Samhita	Description
1.	Charaka Samhita	The medicinal plants was categorised in Charaka Samhita (3rd century B.C.D.) based on Karma (activity) and Prabhava (specific action). The Kashayaskandhagana refers to Arjuna as Udardaprashamana (anti urticarials).
2.	Sushruta Samhita	Sushruta (6th century B.C. 13) categorised

		medications based on their effects, odour, and taste. Salsaradigana has a description of the Arjuna. These medications treat pandu, meha (diabetes), and kustha (Anaemia). These medicines also calm down the kapha and medas (fat).
3.	Ashtanga Hridaya	Arjuna was referred to as partha and stated in Nyagrodadhigana by Vagbhtah II (4th century AD), the author of Ashtanqa Hridaya, the well-known work of highest authority and essence of Charaka and Sushruta Sarnhita. He was be the first to recommend Rasa ghrita, or ground bark with milk and treacle water, as a tonic and to elucidate in great detail its medicinal benefits.
4.	Bhaishajya Ratnavali	Arjuna is referenced in the following formulations by Bhaishajya Ratnavali author Govinda Dasa, in the 19th century A.D. (Cardiac disorders)

2.6. Vernacular names of *T. arjuna*[55]

Table 2.3: Vernacular names of *T. arjuna*

English	White Murdah
Hindi	Arjuna, Arjun
Assamese	Arjun
Irula	Mathi
Kannada	Nirmatti, Atumaruthu, Neermaruthu
Malayalam	Vellamathi, Nirmarutu, Aatumaruthu
Manipuri	Maiyokpha
Tamil	Vella Maruda, Vella Maruthu, Marutu

2.7. Ayurvedic properties of *T. arjuna*[56]

Rasa: Kashaya

Guna: Laghu, Ruksha

Veerya: Shita

Vipaka: Katu

Doshakarma: Kaphapittashamak

Prabhav: Hridya

2.8. Phytochemical characters of *T. arjuna*

The *T. arjuna* is known to contain various phytochemicals that contribute to its therapeutic properties. Tannin content in *T. arjuna* is 15%. Triterpenoids, polyphenols, flavonoids, tannins, sterol, and minerals are the main phytoconstituents. Arjunolic acid, arjunic acid, and arjungenin are important triterpenoid saponins that are found. Beta-sitosterol, ellagic acid, and arjunic acid are also present[57]. The crystallisable compound reported are Arjunine and Arjunetin. The bark of *T. arjuna* is enriched with diverse phytoconstituents, including chromogenic compounds (coloring agents), saccharides, cardiac glycosides, and inorganic elements such as calcium and sodium carbonates, along with trace quantities of alkali metal halides. Glycosides have the ability to increase the heart's contraction force[58]. For isolating various chemical ingredients, a variety of organic solvents like ethanol, hexane, chloroform, benzene, etc. are used. The bark of *T. arjuna* is rich in flavonoids, including arjunolone, flavones, baicalein, quercetin, and kaempferol. Luteolin, a key flavonoid present, demonstrates potent antibacterial and antimutagenic activities. *Terminalia arjuna* possesses cardioprotective, antimicrobial activities due to the presence of flavonoids, tannins, and polyphenolic compounds. Recent studies has shown that these phytochemicals found in *T. arjuna* have a range of therapeutic properties, including antioxidant, anti-inflammatory, cardioprotective, hepatoprotective, and antitumor effect[59], [60].

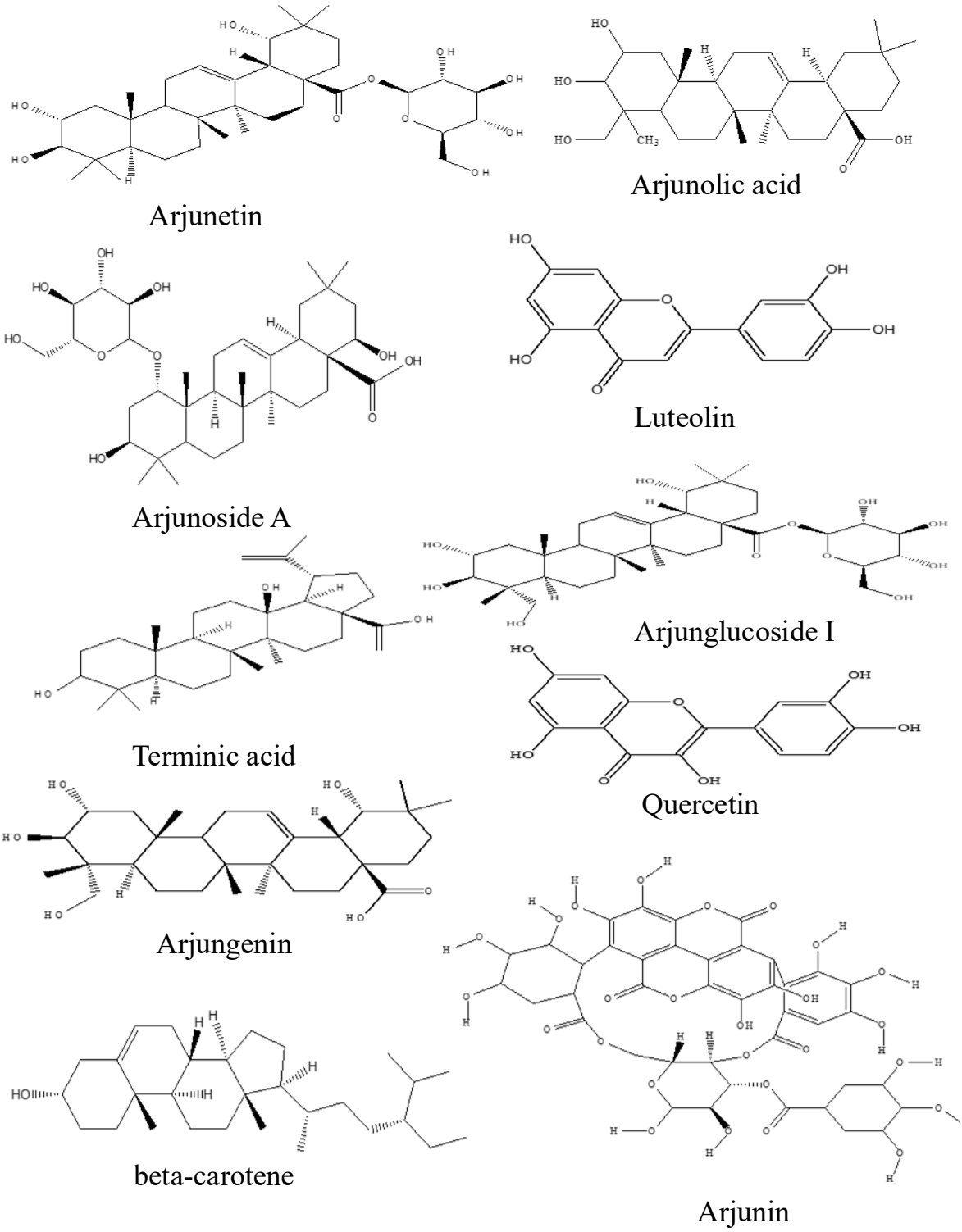


Fig.2.2. Chemical constituents of *T. arjuna*

2.9. Pharmacological properties of *T. arjuna*

Table 2.4. Pharmacological properties of *T. arjuna*[61]

Sr. No	Pharmacological properties	Extract	Part used
1.	Antioxidant	Methonolic	Stem Bark
2.	Antimutagenic activity	Aqueous and ethanolic	Stem Bark
3.	Inflammatory activity	Ethanolic	Stem Bark
4.	Immunomodulatory activity	Aqueous	Stem bark
5.	Antimicrobial activity	Acetone	Leaves
6.	Cardioprotective activity	Aqueous	Stem bark
7.	Gastro-productive effect	Methanolic	Stem bark

2.10. Self -Nanoemulsifying Drug Delivery System (SNEDDS):

Self-Nanoemulsifying Drug Delivery Systems (SNEDDS) are a distinct category of lipid-based formulations that have gained significant interest owing to their capability to address challenges related to drugs with poor aqueous solubility. These systems offer multiple advantages over conventional delivery methods, such as increased oral bioavailability, enhanced drug solubility and dissolution rates, and improved permeation through biological membranes. [62]. Self-Nanoemulsifying Drug Delivery Systems (SNEDDS) involve the dissolution of the drug in a carefully optimized blend of oil, surfactant, and co-surfactant. Upon oral administration, when this isotropic mixture comes into contact with the aqueous environment of the gastrointestinal (GI) tract, it undergoes spontaneous emulsification, leading to the formation of a fine oil-in-water nanoemulsion. This process enhances drug solubilization and facilitates improved absorption. [63]. SNEDDS produce a nanoemulsion that is readily absorbed by the body, which can significantly enhance the drug's therapeutic effectiveness. Compared to traditional drug delivery methods, SNEDDS offer several notable benefits: they increase the solubility and bioavailability of poorly

water-soluble drugs, improve formulation stability, and may allow for less frequent dosing—ultimately making treatment more effective and easier for patients to manage.[64]. A mild agitation produces an ultrafine droplet of an oil in water (o/w) nanoemulsion from this isotropic combination of medication, oil, surfactant, and co-surfactant. This delivery system is uniquely designed to spontaneously emulsify upon exposure to gastrointestinal (GI) fluids. The natural motility of the GI tract provides mild agitation, which facilitates the formation of a fine oil-in-water emulsion, thereby enhancing the dispersion and absorption of the drug.[65]. SNEDDS offer a significant benefit by improving the bioavailability and stability of poorly water-soluble drugs. This enhanced absorption may also allow for reduced dosing frequency, which is particularly advantageous in supporting patient adherence to prescribed therapies. *Terminalia arjuna* contains bioactive phytoconstituents with therapeutic potential; however, poor aqueous solubility may limit bioavailability. Lipid-based systems such as SNEDDS can improve their oral delivery. [66]. The oils typically used in SNEDDS formulations include medium- and long-chain triglycerides or vegetable-based oils. Non-ionic surfactants and co-surfactants, such as polyethylene glycols and glycerol derivatives, are selected based on their ability to stabilize the emulsion and improve performance. SNEDDS have been studied for their application in a range of drug classes, including anticancer drugs, antibiotics, antifungal agents, and peptides[67]. SNEDDS have been widely studied for delivering a broad range of drugs, including anticancer agents, antibiotics, antifungals, and peptide-based treatments. Although primarily designed for oral delivery, their applications are expanding to include transdermal, ocular, and nasal routes. In summary, SNEDDS present a versatile and promising strategy for drug delivery, as they improve drug solubility, stability, and absorption, thereby enhancing the efficacy of medications that are otherwise constrained by poor water solubility [68], [69].

2.10.1. Types of SNEDDS (nano emulsion):

Table 2.5. Different types of SNEDDS [70], [71]

S.no.	Types of nanoemulsion (SNEEDS)	Detail
1.	W/O	'Droplets of water disperse in continuous phase oil'

2.	O/W	'Droplets of oil disperse in continuous phase water'
3.	Bi-continuous	'Surfactant soluble in both oil and water phase in this type'

2.10.2. Selection of excipients for SNEDDS:

The performance of a self-emulsifying system is primarily determined by the type and concentration of oil used, along with the presence of co-surfactants. These constituents are essential for promoting efficient nanoemulsion formation and ensuring its long-term stability. Oils act as drug solubilizers and enhance lymphatic absorption, while surfactants reduce interfacial tension for efficient nanoemulsion formation. Co-surfactants improve interfacial flexibility and facilitate the self-emulsification process. [72], [73].

2.10.2.1.Oil:

Oil plays an important role in the emulsification of SNEDDS. It aids in emulsification and enhances lipophilicity through the intestinal lymphatic system. In SNEDDS formulation, selecting an oily phase with a superior solubilizing ability for the target drug is essential to achieve optimal drug incorporation and maximize delivery efficiency.It is responsible for the maximum drug loading in nanoemulsion (SNEDDS). Some synthetically or chemically modified oils, such as hydrolysed vegetable oils, are often used to prepare self nanoemulsions because they have a higher capacity to perform self-emulsification because they also contain surfactant properties.

2.10.2.1.1. Oils used in the preparation of SNEDDS[74], [75]

Table 2.6. Various oils used in the preparation of SNEDDS

S.No	Class	Commercial names	Example
1.	Vitamins	-	Vitamin E
2.	Fatty acids	"Crossential 094"	Oleic acid and caprylic acid
3.	Fixed oils	-	Soybean oil, castor oil
4.	MCTs	"Miglyol 1 810,812, Labrafac CC, Crodamol	Triglycerides of caprylic acid

		GTCC, Captex 300,355"	
5.	Medium chain monodiglycerides	"Capmul MCM, Imwitor 742, Akoline MC"	Mono and diglycerides of caprylic acids
6.	PG fatty acid esters	"Capryol 90, Capmul PG 8, Sefsol 218"	PG monocaprylate

2.10.2.2. Surfactants:

Surfactants are the molecules or ions that has been adsorbed at interface. It can reduce interfacial tension. It enhances the self-emulsification ability of SNEDDS, which further improves the bioavailability of partial adsorable drug or poorly water-soluble drugs.

2.10.2.2.1. Surfactants used in preparation of SNEDDS:

Table 2.7. Different surfactants used in the preparation of SNEDDS[75]

S. No	Class	Commercial name	Example
1.	Polysorbates	"Tween 80, Crillet 4" Tween 20, Crillet 4	POE-20-sorbitan monoleate POE-20-sorbitan monolaurate
2.	POE castrol oil	Cremphor EI, Etocas 35 HV	POE-35- Catrol oil
3.	Sorbitan esters	Span 80, crill 4 Span 20, crill 1	Sorbitan monoleate Sorbitan monolaureate
4.	POE-Vitamin E	Vitamin E TPGS	POE- Vitamin E
5.	POE- PPO- Block copolymers	Pluronic/Lutrol F 68	Poloxamer 188
6.	POE-sterate	Solutol HS 15	PEG-660-12-hydroxystearate

2.10.2.3. Co-surfactants:

An effective self- emulsify formulation need high concentration of surfactant. These can improve the solubility of hydrophilic surfactants or hydrophobic drugs in lipid or oil bases.

2.10.2.3.1. Co surfactants used in the preparation of SNEDDS

Table 2.8.Types of Co surfactants used in the preparation of SNEDDS

S. No	Class	Example
1.	Short-chain alcohols	Ethanol, benzyl alcohol
2.	Glycofurol	Tetrahydrofurfuryl alcohol Poly ethylene glycol ether
3.	Polyethylene glycol	PEG 40
4.	Alkanes, diols and triols	P.G.
5.	Glycol ethers	Diethylene-glycol-monoethyl-ether (Transcutol)

2.10.3. Self-emulsification mechanism:

The energy dynamics involved in the emulsification process can be described using a specific equation

$\boldsymbol{G} = \sum N\pi r2$

Where:

- **G** denotes the total energy linked to the formation of the emulsion,
- **N** stands for the total number of droplets formed
- **r** represents the radius of each droplet.

The equation makes it clear that the oil phase-to-aqueous phase interface formation is not the optimal energy configuration. The thermodynamic impulsive emulsification of the system that is generally referred to as SNEEDS has not yet been shown. Water may readily enter the oil-water interface as part of the emulsification process, and this may cause the interface to swell as a liquid crystalline phase form, making emulsification easier. The developed SNEDDS produced stable nano-sized droplets and demonstrated efficient self-emulsification behavior. Optimization through experimental design improved formulation characteristics and stability. [76].

2.10.4. Characterization of SNEEDS:

SNEEDS are characterized by several parameters. These parameters optimize the result. These results may be helpful in the successful commercialization of the product [77].

2.10.4.1.Parameters of Characterization:

Table 2.9. Different parameters of Characterization

S. No	Parameters	Description

1.	Entrapment efficiency	Total drug loading is determining in the formulation
2.	Particle size	Determine droplet size of formulation
3.	Poly- disperse index	Measure of droplet size homogeneity
4.	Zeta potential	Determine charge of the oil droplet
5.	TEM	Transmission electron microscope (TEM) analysis determines the shape of dispersed oil droplets

2.10.5. Advantages of SNEDDS[78], [79], [80]

2.10.5.1.Stability or entrapment efficiency

It provides improved physicochemical stability profile over long-term storage and having advantage of 100% entrapment capacity.

2.10.5.2.Reduction in the dose of drug

SNEDDS offer enhanced bioavailability, better drug-loading capacity, and improved therapeutic outcomes for many water-insoluble drugs. Increased drug loading and bioavailability can result in reduced drug dosage and related side effects.

2.10.5.3.Ease of manufacture and scale- up

These two key factors have played a major role in the successful use of SNEDDS in the pharmaceutical industry. The preparation process, such as mixing with an agitator and a volumetric liquid-filling device, makes it simple to manufacture in large quantities while also providing economic advantages.

2.10.5.4.Pharmaceutical formulations

A wide range of formulations can be developed using SNEDDS, including foams, sprays, creams, ointments, and gels. In the pharmaceutical field, SNEDDS-based nanoemulsions

have been effectively applied across various routes of administration, such as oral and topical, offering flexibility in drug delivery approaches.

2.10.6. Commercially available nanoemulsion (SNEDDS) formulations

Table 2.10. Commercial SNEDDS formulations

S.No	Active compound	Brand Name	Therapeutic indication
1.	Vit.A, D, E, K [21]	Vitalipid	Parenteral nutrition
2.	Propofol [21]	Diprivan	Anaesthetic
3.	Palmitate alprostadil [22]	Liple	Platelet inhibitor
4.	Dexamethasone [22]	Limethasone	Steroid
5.	Flubiprofenaxtil [22]	Ropovin	NSAID

CHAPTER 3

AIMS AND OBJECTIVES

Chapter 3
Research Gap and Objective

3.1 Research Gap

Although several nanoformulations of *Terminalia arjuna* have been reported, limited research has focused on developing an optimized Self-Nanoemulsifying Drug Delivery System (SNEDDS) specifically for improving the stability, bioavailability, and therapeutic efficiency of its bark extract. Existing studies mainly emphasize conventional formulations and do not adequately address issues such as poor solubility, low absorption, dose variability, and short shelf life of phytoconstituents. Therefore, the novelty of the present study lies in designing a SNEDDS-based nanoformulation for T. arjuna that may enhance dissolution, intestinal absorption, physicochemical stability, and antihypertensive efficacy while reducing the required dosage, thereby offering a more effective and advanced herbal drug delivery approach.

3.2 Objectives

- Development and optimization of SNEDDS loaded with the extract of *T. arjuna*.
- Characterization of the optimized batch of SNEDDS.
- Stability study of the optimized batch of SNEEDS.
- In-vivo evaluation of hypertensive activity of optimized batch of SNEDDS and its comparison with extract of *T. arjuna.*

CHAPTER 4

PROPOSED METHODOLOGY

Chapter 4

Proposed Methodology

4.1 Raw drug collection

T. arjuna bark was collected from the Ayush Vatika, Lovely professional university.

4.2 Raw drug authentication

The collected sample of *T. arjuna* was authenticated from the government accredited laboratory.

4.3 Organoleptic study

The *T. arjuna* bark was observed for morphological characters like colour, odour, taste, texture etc

4.4 Physicochemical analysis of raw material[81], [82]

Table 4.1. IDENTITY, PURITY AND STRENGTH (A.P.I. Standards)

Parameters	Standard (%)	Reference
Foreign matter	NMT 2	Appendix 2.2.2
Total Ash	NMT 25	Appendix 2.2.3
Acid-insoluble ash	NMT 1	Appendix 2.2.4
Alcohol-soluble extractive	NLT 20	Appendix 2.2.6
Water-soluble extractive	NLT 20	Appendix 2.2.7

***NMT**-Not more than **NLT-** Not less than

4.4.1. Foreign matter

A sample of 100 g was obtained and spread out on a stainless-steel tray. The foreign substance was spotted without the use of a magnifying glass. The remaining sample quantity was weighed, and the proportion of foreign matter was calculated.

$$\boldsymbol{Foreign\ matter = \left(weight\ of \frac{foreignmatter}{weight} of\ drug\right) X100}$$

4.4.2. Loss on drying

5-10 gm of sample was taken (without preliminary drying) in the accurately weighed dry petri dish and was kept into the dry oven at 105°C for 5 hours. Then, the petri dish was removed from the oven and placed into desiccator under vacuum till shelf cooling and weighed the reduced moisture content from the sample.

4.4.3. Total ash

A 2.5-gram portion of the sample was placed in a crucible and incinerated at 450°C for five

hours. Once the process was complete, the crucible was allowed to cool on a shelf and then stored in a vacuum-sealed desiccator. The resulting ash was carefully weighed, and the percentage of ash content was calculated based on the original sample weight.

$$\boldsymbol{Total\ Ash = \left(weight\ of \frac{ash}{weight} of\ sample\right) X100}$$

4.4.4. Acid-insoluble ash

The ash obtained from the preceding step was combined with 25 ml of dilute hydrochloric acid and gently boiled for approximately five minutes, followed by filtration through ashless filter paper; the residue retained on the filter was then extensively washed with hot water until chloride-free, subsequently ignited to a constant weight, and the percentage of acid-insoluble ash was calculated based on the final residue mass.

$$\boldsymbol{Acid\ insoluble\ ash\ = \frac{(Weight\ of\ residue \times Volume\ made)}{(Weight\ of\ sample\ \times\ Volume\ taken) \times\ 100}}$$

4.4.5. Alcohol soluble extractive

A total of 5 grams of the coarse powdered sample was added to a closed conical flask containing 100 ml of alcohol. The mixture was shaken frequently for the first 6 hours and then allowed to stand undisturbed for the next 18 hours. After this period, the contents were filtered using standard filter paper. From the resulting filtrate, 25 ml was measured and transferred into a china dish, where it was left to evaporate completely. The remaining residue was weighed, and the percentage of alcohol-soluble extractives was calculated accordingly.

$$\boldsymbol{Alcohol\ soluble\ extractive\ value\ = \frac{Weight\ of\ residue \times Volume\ made}{(Weight\ of\ sample\ \times\ Volume\ taken) \times\ 100}}$$

4.4.6. Water soluble extractive

5 grams of the coarse powdered sample were placed in a sealed conical flask containing 100 ml of water, shaken intermittently for 6 hours, and then left undisturbed for an additional 18 hours; the mixture was subsequently filtered through standard filter paper, from which 25 ml of the filtrate was transferred to a china dish, evaporated to dryness, and the residue weighed to determine the percentage of water-soluble extractives.

$$\boldsymbol{Water\ soluble\ extractive\ value\ = \frac{Weight\ of\ residue \times Volume\ made}{(Weight\ of\ sample\ \times\ Volume\ taken) \times 100}}$$

4.5 Qualitative analysis of *T. arjuna*[83]

4.5.1. Test for flavonoids

4.5.1.1. Lead acetate test

The presence of flavonoids in the extract was verified by adding several drops of lead acetate solution, which induced the formation of a yellow precipitate, thereby confirming a positive qualitative test.

4.5.1.2. Shinoda test

The extract was dissolved in 1–2 ml of 50% methanol by gentle heating. Flavonoid identification was conducted by introducing three magnesium chips into the alcoholic solution of each extract, followed by the addition of concentrated hydrochloric acid. The appearance of a distinctive orange, pink, or reddish-purple coloration signified the presence of flavonoid constituents within the sample.

4.5.2. Test for Alkaloids

4.5.2.1. Mayers's Test

For the qualitative detection of alkaloids, 1 mL of the aqueous plant extract was initially acidified with 2–3 drops of 1M hydrochloric acid. Subsequently, 4–5 drops of Mayer's reagent (potassium mercuric iodide solution) were added. The development of turbidity or the formation of yellow or white precipitates served as an indicator of alkaloid presence in the extract.

4.5.2.2. Dragendroff test

Each extract was diluted with diluted hydrochloric acid and filtered to yield a clear solution. Dragendorff's reagent, containing potassium bismuth iodide, was then added to the filtration. The formation of an orange or red precipitate indicated a positive presence of alkaloid compounds.

4.5.2.3. Wagner reagent test

Two drops of Wagner's reagent were added to 2 mL of the extract and mixed carefully. The formation of a brownish color indicated the presence of alkaloid compounds.

4.5.3. Test for Tannin

A specific volume of the extract was heated with water and filtered to produce a clear filtration. The addition of two drops of ferric chloride solution to this filtrate resulted in the formation of blackish-green precipitates, indicating the presence of tannins.

4.5.4. Test for Phenol (Ferric chloride test)

To each extract, 3–4 drops of ferric chloride solution were added. The development of a blue-black coloration indicated the presence of phenolic compounds.

4.5.5. Test for reducing sugar (Fehling's test)

Equal volumes (1 mL each) of Fehling's solutions A and B were mixed thoroughly in a test

tube. Subsequently, 2 mL of the plant extract was introduced, and the mixture was heated in a boiling water bath for 10 minutes. The formation of a brick-red or orange precipitate confirmed the presence of reducing sugars or carbohydrates.

4.5.6. Test for saponins (Foam test)

An aqueous mixture was prepared by adding 2 mL of water to 0.5 g of the extract. The formation of stable foam persisting for 10 minutes indicated the presence of saponins.

4.5.7. Test for proteins (Xanthoproteic test)

A few drops of concentrated nitric acid were added to the extract. The presence of proteins is shown by the formation of a yellow color.

4.5.8. Test for Glycosides (Keller-killani-test)

The extract was mixed with 1 mL of glacial acetic acid and a few drops of ferric chloride solution. Concentrated sulfuric acid was then slowly added down the side of the test tube to create a layered interface. The appearance of a reddish-brown ring at this junction indicated the presence of deoxysugars.

4.5.9. Test for Phytosterols (Salkowski test)

The extracts were treated with chloroform and filtered to obtain clear solutions. These filtrates were then mixed with a few drops of concentrated sulfuric acid, gently shaken, and left undisturbed. The formation of a golden yellow coloration indicated the presence of triterpenes.

4.6 Microbial test [84], [85]

4.6.1. Total microbial plate count

Method: Aseptic techniques must be employed throughout the analysis.

Procedure: Take accurately about 2 g or 2 mL of the sample into 18 mL of the sterile Tryptic Soya broth and shake well. Pipette 1 mL of the well-mixed broth in sterile petri dish in duplicate and add approximately 20-25 mL of Tryptic Soya agar to the plates at the temperature of 43°C to 45°C. Keep the plates for solidification of agar. Then put it into the incubator in inverted position and incubate for 48 hours at 37°C ±2°C. After completion of incubation, count colonies appeared on the plates with the help of colony counter. Express the result in terms of colony forming units per gram (cfu/g) or colony forming units per mL (cfu/mL), taking into account the dilution factor. Count below 30 or above 300 per plate is considered invalid and the analysis must be repeated at a suitable dilution as described above.

4.6.2. Total yeast and mould count

Method: Aseptic techniques must be employed throughout the analysis.

Procedure: An accurately weighed quantity of 2 grams (for solid samples) or 2 milliliters (for liquid samples) of the test specimen was transferred into 18 mL of sterile Tryptic Soy Broth (TSB) and mixed thoroughly to ensure uniform suspension. From this preparation, 1 mL was aseptically pipetted into each of two sterile Petri dishes. Subsequently, 20–25 mL of molten Potato Dextrose Agar (PDA), maintained at 43°C–45°C, was poured into each dish.

The plates were gently swirled to facilitate even distribution of the inoculum throughout the agar medium, then allowed to solidify at room temperature. Once solidified, the dishes were incubated in an inverted position at 30 ± 1°C for five days.

After the incubation period, the developed fungal colonies, including both yeasts and molds, were counted using a calibrated colony counter. Results were expressed in terms of colony-forming units per gram (cfu/g) of the original sample, with appropriate adjustments based on the dilution factor. If no microbial growth was observed from the 1:10 dilution, the fungal count was reported as <10 cfu/g, indicating negligible contamination.

4.7 Pathogen test [85], [86]

4.7.1. *Escherichia coli*

Method: Aseptic techniques must be employed throughout the analysis.

Cognitive Test: Put 10 mL Mac Conkey broth in test tube, put one Durham's tube in inverted position. Cap the tube by cotton. Autoclave the same at 15 Ib psi / 121°C for 20 minutes. Cool down and put 2mL of enrichment broth medium in the tubes and shake well and put it into the incubator for 48 hours ± 3 hours at 37°C±2°C. After due time observe the tube. If there is acidity marked by maroonish colour and gas production in Durham's tube, then *E. coli* may be present. Proceed further for confirmatory test as follows.

Confirmatory Test: Pour 20 – 25 mL of sterile E.M.B. agar in the petri dish and solidify. Streak the Mac Conkey broth (showing positive for *E. coli* in cognative test) by sterile nichron wire on E.M.B. agar plate.

Incubate at 37°C±2°C for 48 hours. Examine any growth on the E.M.B. agar. *E. coli* colonies are dark in colour having greenish metallic sheen.

4.7.2. *Staphylococcus aureus*

Method: Aseptic techniques must be employed throughout the analysis.

Cognitive test: Put 2 mL of enrichment broth medium into 10 mL of Giolitti Cantoni broth tube containing 0.15 mL of potassium tellurite (1%w/v) and shake well and put in the incubator for 48 ± 3 hours at 37°C±2°C. After due time observe the tube. If there is blackening in the solution, then *Staphylococcus aureus* may be present. Proceed further for confirmatory test.

Confirmatory Test: Streak from the incubated Giolitti Cantoni broth (showing positive for *Staphylococcus aureus*) by sterile nichron wire on Vogel Johnson's Agar or on Baird Parker's Agar and incubate at 48 ± 3 hours. Visually examine. Typical *Staphylococcus aureus* colonies are round convex black in colour with yellow zone in Vogel Johnson's Agar and black colonies with haloes on Baird Parker's Agar.

4.7.3. *Pseudomonas aeruginosa*

Method: Aseptic techniques must be employed throughout the analysis.

Cognitive test: Put 2 mL of the enrichment broth medium into 10 mL of cetrimide broth tube and shake well and put in the incubator for 48 ± 3 hours at 37°C±2°C. After due time observe the tube, if there is turbidity and greenish blue or greenish or greenish- yellow color in the upper part of the tube then *Pseudomonas* may be present. Proceed further for confirmatory test.

Confirmatory test: Streak from the incubated cetrimide broth (showing positive for *Pseudomonas aeruginosa*) by nichron wire on pseudomonas isolation agar. Incubate at 37°C±2°C for 48 hours ± 3 hours. Visually examine typical *Pseudomonas aeruginosa* colonies, which give greenish fluorescence in UVlight.

4.7.4. *Salmonella*

Method: Aseptic techniques must be employed throughout the analysis.

Cognitive Test: Put 2 mL of enrichment broth medium into 10 mL of selenite broth tube and shake well and put in the incubator for 48 ± 3 hours at 37°C±2°C. After due time observe the tube. If there is turbidity and little higher consistency with little brownish tinge, then *salmonella spp / shigella* may be present. Proceed further for the confirmatory test as follows:

Confirmatory Test: Streak from the incubated selenite broth (showing positive for *salmonella spp / shigella*) by sterile nichron wire on XLD agar and incubate at 48hours ± 3 hours. Examine any growth on XLD plate. *Salmonella* colonies are red with dark black center and *shigella* colonies are red.

4.8 Test for Aflatoxins (by TLC)[87]

Aflatoxin Analysis Procedure

(Caution: Aflatoxins are highly toxic and should be handled with proper safety precautions.)

Prepare the aflatoxin standard solution by dissolving aflatoxins B1, B2, G1, and G2 in chloroform: acetonitrile (9.8:0.2) to obtain concentrations of 0.5 µg/mL for B1 and G1, and 0.1 µg/mL for B2 and G2. Apply 2.5, 5, 7.5, and 10 µL of the standard solution on a silica

gel TLC plate (0.25 mm thickness). Also, spot three 10 µL aliquots of Test Solution 1 or 2, with one spot additionally mixed with 5 µL of the standard solution. Dry the spots completely.

Develop the plate in an unsaturated chamber using chloroform:acetone:isopropyl alcohol (85:10:5) as the mobile phase until the solvent front reaches at least 15 cm. Remove the plate, mark the solvent front, and air-dry.

Observe the plate under UV light at 365 nm. The standard should show four blue fluorescent spots corresponding to aflatoxins B1, B2, G1, and G2. Compare the Rf values and fluorescence intensity of test spots with the standards for identification and semi-quantitative estimation of aflatoxins.

4.9 Heavy metal

Atomic absorption spectrophotometry is commonly used to detect heavy metals and certain non-metal elements in their atomic form. In this method, light at a specific wavelength, emitted by a cathode lamp, passes through a vaporized sample containing the element of interest. When the element is in its ground (atomic) state, it absorbs some of this light. The amount of light absorbedreflected by the reduction in light intensityis measured to determine the presence and concentration of the element. This technique is based on the fundamental principles of absorption spectrophotometry. The final assay is carried out by comparing the absorbance of the test sample with that of a known reference standard.

4.9.1. Lead (as Pb)

Operating Conditions:

Drying Temperature: 100–120°C, held for 20 seconds

Ashing Temperature: 400–750°C, held for 20–25 seconds

Atomization Temperature: 1700–2100°C, held for 4–5 seconds

Measurement Wavelength: 283.3 nm

Background Correction: Deuterium lamp (D lamp) or Zeeman effect

Preparation of Lead Nitrate Stock Solution:

Weigh 159.8 mg of analytical reagent (AR/GR grade) lead nitrate and dissolve it in 100 ml of distilled water. Add 1 ml of nitric acid, then dilute the mixture to a final volume of 1000 ml with distilled water. Store the prepared solution in a clean glass container.

Preparation of Standard Lead Solution:

On the day of testing, dilute 10.0 ml of the lead nitrate stock solution to 100 ml using distilled water. This yields a standard solution where each milliliter contains 10 µg of lead.

Testing Procedure:

Use a sample quantity (in grams) calculated with the formula:

$$\boldsymbol{Sample\ weight = 2.\frac{0}{1000XL}}$$

Where **L** is the heavy metal limit expressed as a percentage. This calculation ensures the correct test quantity for accurate lead detection and quantification

Test sample preparation

Accurately weigh the sample into a crucible and moisten it with sulfuric acid. Gently heat the mixture until charring occurs. After cooling, add 2 mL of nitric acid followed by a few drops of sulfuric acid, then heat until the emission of white fumes ceases. Transfer the crucible to a muffle furnace and ignite at 500–600°C until complete removal of carbonaceous material is achieved. After cooling, add 4 mL of dilute hydrochloric acid, cover the crucible, and heat in a water bath for 15 minutes. Subsequently, uncover and evaporate the mixture to dryness. Moisten the residue with one drop of concentrated hydrochloric acid and 10 mL of hot water, then digest for 2 minutes. Filter the solution into a Nessler tube and dilute to a final volume of 35 mL with distilled water.

Control Preparation:

Take 2 ml of standard lead solution and dilute to 35 ml with distilled water in a Nessler tube.

Final Step:

Add 10 ml of hydrogen sulfide solution to both test and control tubes, dilute to 50 ml with water, and let stand for 5 minutes. Compare the color intensity on a white background. The test solution should not be darker than the control.

4.9.2. Cadmium

Determination conditions: The measurement wavelength was set at 228.8 nm, with background correction using either the Zeeman effect or a deuterium lamp (D lamp). The atomic temperature was maintained between 1500–1900°C for 4–5 seconds, the ashing temperature ranged from 300–500°C for 20–25 seconds, and the drying temperature was held at 100–120°C for 20 seconds.

Preparation of Standard Stock Solution:

Accurately measure the required volume of a cadmium single-element standard solution and dilute it with 2% nitric acid (HNO_3) to prepare a stock solution containing 0.4 µg/mL of cadmium. Store this solution at a temperature between 0–5°C.

Calibration Curve Preparation:

From the cadmium stock solution, prepare a series of dilutions with 2% HNO_3 to obtain standard solutions with concentrations of 1.6, 3.2, 4.8, 6.4, and 8.0 ng/mL. Accurately pipette 10 µL of each standard into the graphite furnace. Measure the absorbance of each and construct a calibration curve by plotting absorbance values on the Y-axis and cadmium concentrations on the X-axis.

Test Solution Preparation:

Prepare the test solution using the same procedure outlined for lead (Pb) determination.

Measurement:

Precisely pipette 10–20 µL of the test sample along with its corresponding reagent blank and measure their absorbance using the same analytical procedure employed for the calibration standards. In cases where interference is detected, accurately transfer 1 mL each of the standard, blank, and test solutions, and add 1 mL of a mixed reagent solution composed of 1% ammonium dihydrogen phosphate ($NH_4H_2PO_4$) and 0.2% magnesium nitrate [$Mg(NO_3)_2$]. Thoroughly mix the solutions before re-measuring their absorbance. Quantify the cadmium concentration in the test sample by comparison with the established calibration curve.

4.9.3. Mercury (Hg)

Instrument Conditions:

- **Hydride generator** system with AAS
- **Reducing agent:**0.5% sodium borohydride in 0.1% NaOH
- **Carrier liquid:**1% HCl
- **Carrier gas:** Nitrogen
- **Wavelength:**253.6 nm
- **Background correction:** Deuterium lamp or Zeeman effect

Standard Stock Solution:

Prepare a 1.0 µg/mL mercury stock solution in 2% nitric acid. Store at 0–5°C.

Calibration Curve:

Pipette 0–0.9 mL of the stock solution into 50 mL volumetric flasks. Add 40 mL of 4% sulfuric acid and 0.5 mL of 5% potassium permanganate. Mix, then add 5% hydroxylamine hydrochloride dropwise until the violet-red color disappears. Dilute to volume with 4%

sulfuric acid. Measure absorbance and plot the calibration curve.

Test Solution:

Weigh 1 g of powdered sample into a Casparian flask. Add 5–10 mL of a 4:1 nitric and perchloric acid mix. Let stand overnight, then heat at 120–140°C for 4–8 hours. Cool, add 4% sulfuric acid and 0.5 mL of 5% potassium permanganate, and decolorize with 5% hydroxylamine hydrochloride. Dilute to 25 mL with 4% sulfuric acid. Centrifuge if needed. Prepare a blank using the same method without the sample.

Determination:

Inject the test and blank solutions into the hydride generator. Measure absorbance and determine mercury content using the calibration curve.

4.9.4. Arsenic (As)

Suitable Instrument Setup and Conditions:

The analysis is performed using an atomic absorption spectrophotometer equipped with a hydride generation system.

- **Reducing Agent:** 1% sodium borohydride in 0.3% sodium hydroxide
- **Carrier Liquid:** 1% hydrochloric acid
- **Carrier Gas:** Nitrogen
- **Measurement Wavelength:** 193.7 nm
- **Background Correction:** Deuterium lamp or Zeeman effect

Preparation of Arsenic Standard Stock Solution:

An appropriate volume of a single-element arsenic standard is accurately measured and diluted with 2% nitric acid to prepare a stock solution containing 1.0 µg/mL of arsenic. The solution is stored at a temperature between 0–5°C to preserve stability.

Calibration Curve Preparation:

To prepare calibration standards, dilute the arsenic stock solution with 2% nitric acid to obtain concentrations of 2, 4, 8, 12, and 16 ng/mL. Transfer 10 mL of each diluted solution into separate 25 mL volumetric flasks. Add 1 mL of freshly prepared 25% potassium iodide solution and mix well. Then, add 1 mL of freshly prepared ascorbic acid solution, shake again, and dilute to volume with hydrochloric acid (1:100). After sealing, immerse the flasks in an 80°C water bath for 3 minutes. Cool the solutions, then transfer appropriate volumes to the hydride generation system and record their absorbance. Use the results to construct a calibration curve by plotting absorbance (Y-axis) against arsenic concentration (X-axis).

Preparation of Test Solution:

Prepare the test solution following the same procedure used for lead (Pb) determination, as previously described.

Measurement and Calculation:

Accurately pipette 10 mL each of the test solution and its reagent blank. Proceed with the same steps used for calibration standards, starting from the addition of 1 mL potassium iodide. Measure absorbance and calculate the arsenic content in the sample using the previously plotted calibration curve.

4.10 Pesticide residue[87]

In accordance with pharmacopoeial criteria, a pesticide is defined as any chemical compound or formulation intended to prevent, control, or eradicate pests, encompassing plant or animal species that may adversely affect the cultivation, harvesting, processing, storage, transport, or commercialization of herbal raw materials. This definition also extends to substances employed as plant growth regulators, defoliants, and desiccants, as well as chemicals applied pre- or post-harvest to safeguard crops against deterioration during storage or distribution.

Regulatory Limits:

Unless a specific monograph states otherwise, the herbal drug being tested must comply with the maximum residue limits outlined in Table 1. For any pesticides not listed but suspected to be present, the permissible levels must align with the European Union directives 76/895 and 90/642, along with any amendments or updates to these regulations.

Analytical Methods for Pesticide Detection

Common Techniques:

Gas Chromatography (GC) is one of the most widely used techniques for detecting pesticide residues. GC is especially effective for analyzing mid-polar to non-polar substances. In contrast, High-Performance Liquid Chromatography (HPLC) is more suitable for detecting polar compounds. Advanced methods like GC-MS (Gas Chromatography-Mass Spectrometry) provide enhanced sensitivity and specificity.

Multi-Residue Methods (MRMs):

MRMs are developed to simultaneously detect and quantify multiple pesticide residues in a variety of plant or food matrices. These methods typically follow a structured sequence:

1. Sample Preparation
2. Extraction
3. Clean-up

4. Chromatographic Separation

Currently, among the 10 MRMs used by regulatory agencies like the FDA and USDA, 8 rely on gas chromatography and 2 utilize HPLC. However, no single MRM is capable of identifying all pesticide residues across all crop types.

Quantitative Analysis Workflow

A comprehensive pesticide residue analysis includes the following steps:

a. **Sample Preparation:**
   Plant materials are divided into edible and non-edible parts, followed by grinding, chopping, or macerating to create a uniform sample.

b. **Extraction:**
   Pesticide residues are isolated from the sample by dissolving them in an appropriate solvent. This usually involves blending the material in a homogenizer and filtering the mixture to collect the liquid extract.

c. **Clean-up:**
   The raw extract is further purified to eliminate unwanted substances that could interfere with the detection process.

d. **Separation:**
   The cleaned extract is subjected to chromatographic techniques where the target analytes are separated based on their interaction with a stationary and a mobile phase.

Detection and Quantification:

As each compound passes through the detector, its signal is measured and compared against known standards to determine its concentration.

4.11 Preparation of *T. arjuna* Extract

The extraction method for *T. arjuna* depends on factors such as the desired phytochemicals, solvent polarity, and the intended application of the extract. Different analytical techniques help identify and characterize bioactive compounds like flavonoids, tannins, saponins, and glycosides, providing a detailed phytochemical profile. Common extraction methods include aqueous, hydroalcoholic, alcoholic (ethanolic or methanolic), Soxhlet, cold maceration, and supercritical fluid extraction (SFE). Techniques such as decoction, reflux extraction using a Soxhlet apparatus, maceration, cold percolation, and supercritical CO_2 extraction with co-solvents like ethanol are commonly employed [88].

Various solvents, including water, ethanol, methanol, chloroform, acetone, hexane, and ethyl acetate, are used depending on their polarity and affinity for specific phytochemicals

to enhance extraction efficiency[89].

Among these methods, Soxhlet extraction is considered highly efficient and reliable because it allows continuous and exhaustive extraction with reproducible results. It is especially suitable for heat-stable bioactive compounds and is widely used in laboratory studies [90].

Methanol is frequently preferred as an extraction solvent for *T. arjuna* due to its broad polarity range, high extraction efficiency, and ability to maintain the stability of bioactive compounds. It is extensively used in both *in vitro* and *in vivo* pharmacological studies and often demonstrates stronger antibacterial and antifungal activity than aqueous or ethanolic extracts because of its superior ability to extract antimicrobial constituents [91].

Process of Extraction: A total of 100 g of oven-dried, finely powdered bark material was accurately weighed and loaded into the extraction chamber of a Soxhlet apparatus. The chamber was positioned between a round-bottom flask containing 500 mL of methanol and a condenser mounted above. The system was subjected to continuous heating for 18 hours. During this process, methanol was vaporized in the boiling flask, condensed in the condenser, and subsequently dripped into the extraction chamber, thoroughly permeating the plant material.

The Soxhlet system was designed to allow the solvent to accumulate until a specific level was reached, at which point it automatically siphoned back into the boiling flask, enabling repeated solvent circulation and efficient extraction. Upon completion of the extraction cycle, the methanolic extract was collected from the flask.

To concentrate the extract, methanol was evaporated using a digital water bath maintained at 65 °C, corresponding to the boiling point of methanol (64.7 °C). The remaining dried extract was weighed, and the percentage yield was calculated based on the initial mass of plant material [87].

Extract yield % = W1/W2 X100

Where,

W1 refers to the net weight (in grams) of the dried extract obtained after complete evaporation of the solvent,

while **W2** corresponds to the initial weight (in grams) of the powdered bark material used for the extraction process.

4.11.1 Characterization of Extract

4.11.1.1 Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a widely used technique for both the qualitative and quantitative analysis of chemical constituents and herbal medicines. It operates on two

distinct phases—a stationary phase and a mobile phase. In this study ,TLC was performed to analyze the *T. arjuna* extract, employing an appropriate solvent system to achieve efficient separation of its constituents.

Sample preparation

The extract of *T. arjuna* was filtered, concentrated using a water bath, and then stored in a sealed container for later use in chromatographic analysis.

4.11.1.1.1 Conditions for Chromatography

Solvent system : Chloroform: methanol (80:20) [57]

Extract : Methanol extract

Chamber Saturation: 30 minutes

4.11.1.2 UV Spectrophotometer Analysis

A UV spectrophotometer measures light absorption to accurately quantify the concentration of a drug in solution. This is often accomplished using the calibration curve approach, which gives an accurate approach for determining how much of the drug is actually present.

4.11.1.2.1 Calibration curve for U.V.

A UV spectrophotometer is used to detect light absorption, which aids in determining the real concentration of a medicine in solution. This is often accomplished using the calibration curve approach, which gives an accurate approach for determining how much of the drug is actually present**.**

4.11.1.2.2 Stock Solution Preparation

A Stock solution had been prepared by adding 50 mg of drug extract in 50 ml of methanol.

4.11.1.2.3 Sample Dilution

Serial dilutions of the sample were prepared at concentrations of 20, 40, 60, 80, 100, 120, and 140 µg/mL for the calibration curve. The absorbance of each dilution was measured at 258 nm, and the resulting data were used to construct the calibration curve.

4.12 Preparation of SNEEDS

4.12.1 Solubility studies of *T. arjuna* extract in oils, co-surfactants, and surfactant [92], [93]

A Solubility study was performed to check the solubility of extract in different – different oils, surfactants, and co-surfactant before formulating SNEDDS.

Table 4.2. List of various oils, surfactants, co-surfactants and oils utilized in the formulation

Oil	Surfactant	Co- Surfactant
Castor oil	Cremophor EL	Span 20
Soybean oil	Labrasol	Span80
Sunflower oil	Labrafac CC	Capryol 90
Sesame oil	Polysorbate 20 (Tween 20)	PEG 400
Labrafil	Polysorbate 80 (Tween 80)	Transcutol P

The solubility evaluation for the SNEDDS formulation was conducted by individually combining a precise amount of *T. arjuna* extract with selected oils, surfactants, and co-surfactants. Each mixture was subjected to vortexing for 15 minutes, followed by incubation in a water bath at 25 °C for 72 hours under continuous agitation. Post-incubation, the samples were centrifuged at 3500 rpm for 15 minutes, and the resulting supernatant was analyzed using UV-Visible spectrophotometry at 275 nm. All experiments were performed in triplicate, and mean values were calculated. Among the excipients tested, Labrafil M 1994 CS exhibited the highest solubility with the extract in the oil category, Tween 80 demonstrated superior solubilization among surfactants, and Transcutol P showed notable solubility as a co-surfactant. Based on these findings, these excipients were selected for the formulation of the *T. arjuna* SNEDDS.

4.12.2 SNEDDS preparations[94]

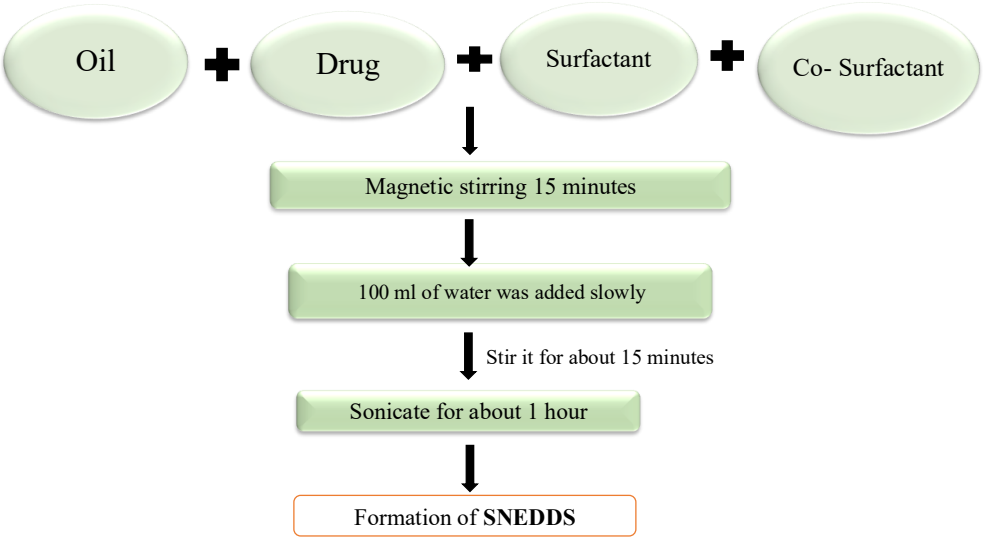


Fig. 4.1. Procedure of SNEDDS formulation

The Self-Nanoemulsifying Drug Delivery System (SNEDDS) was formulated by blending *T. arjuna* extract with Labrafil (oil), Tween 80 (surfactant), and Transcutol-P (co-surfactant) in varying ratios. The components were mixed thoroughly using a magnetic stirrer at 600–750 rpm, maintained at 37 °C to facilitate the formation of a uniform preconcentrate. The resultant mixture was then diluted with distilled water to a final volume of 100 mL and further stirred under the same conditions to ensure complete dispersion and the development of a stable nanoemulsion.

4.12.3 Construction of Ternary phase diagram

The construction of a ternary phase diagram incorporating the surfactant, oil, and co-surfactant is a critical step in identifying the optimal concentration ranges capable of producing stable and spontaneous nanoemulsions.

This diagram helps clearly highlight the specific regions where self-emulsification occurs. Determination of the self-nanoemulsifying region on the ternary phase diagram necessitates precise measurement of the droplet size of the prepared nanoemulsions.

This is typically done by mixing different compositions taken from the diagram with an equal amount of waterThe self-nanoemulsification region is characterized by the spontaneous formation of nanoemulsions with droplet sizes smaller than 200 nm.

4.13 Optimization of *T. arjuna* extract loaded SNEDDS with Design of experiment

4.13.1 QbD-based formulation optimization studies

Formulation optimization was carried out utilizing Response Surface Methodology (RSM), specifically employing a Central Composite Design (CCD), which is essential for the development of an optimized SNEDDS formulation. The CCD approach was implemented to evaluate the influence of varying concentrations of oil, surfactant, and co-surfactant on the physicochemical characteristics of the SNEDDS. The concentration ranges for these variables were determined based on the ternary phase diagram, with the lower and upper boundaries outlined in Table 4.3. This three-factor CCD comprised four axial points, each positioned at an axial distance (α) of 1.414 from the central point of the design space. The quantity of *T. arjuna* extract was maintained constant throughout the study as specified in Table 4.3.

Table 4.3. Experimental variables of CCD for SNEDDS of *T. arjuna*

Variables		Design level	
Uncoded	**Coded**	**Low**	**High**
Oil	A	0.1	0.2

Surfactant	B	0.1	0.3
Cosurfactant	C	0.6	0.8

Using Design-Expert® software (version 11), a central composite design (CCD) matrix comprising twenty experimental runs—including five replicates at the center point—was developed based on the selected coded factors. As presented in Table 4.4, the resulting formulations were systematically evaluated for critical quality attributes (CQAs), including globule size, polydispersity index (PDI), and zeta potential.

Table 4.4 Summary of all the SNEDDS of *T. arjuna* formulations as per CCD

	Factor 1	**Factor 2**	**Factor 3**
Run	**A: Oil**	**B: Surfactant**	**C: Cosurfactant**
1	0.1	0.3	0.6
2	0.15	0.03	0.7
3	0.15	0.2	0.86
4	0.2	0.1	0.8
5	0.15	0.2	0.7
6	0.15	0.2	0.7
7	0.1	0.3	0.8
8	0.06	0.2	0.7
9	0.15	0.2	0.7
10	0.1	0.1	0.6
11	0.23	0.2	0.7
12	0.15	0.2	0.53
13	0.15	0.2	0.7
14	0.15	0.2	0.7
15	0.2	0.1	0.6
16	0.15	0.2	0.7
17	0.2	0.3	0.8

18	0.2	0.3	0.6
19	0.15	0.36	0.7
20	0.1	0.1	0.8

Design-Expert® software was utilized to conduct Multiple Linear Regression Analysis (MLRA), enabling the determination of polynomial equation coefficients that describe the relationship between formulation variables and critical quality attributes (CQAs). Statistical model parameters, including the P-value, correlation coefficient (R), and predicted residual error sum of squares (PRESS), were evaluated, alongside the generation of three-dimensional response surface plots and two-dimensional contour diagrams to visualize the interactions between variables.The final optimized formulation was determined using numerical optimization, with the objective of maximizing the desirability function value toward unity, thereby ensuring the formulation closely met the predefined criteria.

4.13.2 Optimized formulation and validation studies

The optimization of the formulation aimed to identify the appropriate levels of factors A, B, and C to achieve vesicle sizes between 311.2 nm and 924.8 nm, zeta potential values ranging from –28.7 mV to –58.2 mV, and polydispersity index (PDI) values between 0.225 and 0.866, ensuring desirable physicochemical characteristics.

4.8.2.1 Globule size

4.8.2.2 Zeta potential

4.8.2.3 PDI

4.14 *In vitro* Drug Release Study

The in vitro drug release evaluation was conducted using a dialysis membrane with a molecular weight cutoff of 12 kDa (Sigma Chemical Co., USA), which was immersed in simulated intestinal fluid (SIF) at pH 6.8. Prior to use, the membrane was conditioned by soaking overnight in SIF, followed by thorough rinsing under running water for several hours to eliminate residual glycerin [91]. The nanoemulsion formulation, equivalent to 80 mg of the active drug, was loaded into the dialysis membrane and subsequently placed in 150 mL of 1N SIF (pH 6.8). The assembly was maintained in a shaking incubator at 50 rpm and a controlled temperature of 37 ± 0.5°C. At specified time intervals, aliquots were withdrawn and immediately replenished with an equal volume of fresh medium to preserve sink conditions. The collected samples were filtered and analyzed for drug content at 210 nm using a UV-visible spectrophotometer, employing suitable blanks as controls [95].

4.15 Characterization of SNEEDS

4.15.1 Entrapment efficiency

The drug encapsulation percentage was assessed by centrifuging the SNEDDS formulation at 15,000 rpm for three hours at ambient temperature. Post-centrifugation, the supernatant was carefully extracted using a micropipette and dissolved in ethanol to lyse the vesicles. The drug concentration was then quantified via UV spectrophotometry at 275 nm following appropriate dilution [96]. The entrapment efficiency was subsequently calculated using the formula below

$$\%\ \boldsymbol{Entrapment\ efficiency} = (\boldsymbol{Amount\ of\ total\ drug} - \boldsymbol{amount\ of\ encapsulated\ drug / amount\ of\ total\ drug}) \times \mathbf{100}$$

4.15.2 Polydispersity index (PDI)- Particle size analysis [95]

The polydispersity index (PDI) is used to assess the molecular weight distribution (MWD) of polymers. Experimentally obtained MWD data for various polymers are analyzed to allow for accurate interpretation and comparison. For the optimized SNEDDS batch, the mean droplet size and polydispersity index (PDI) was determined using a Malvern Nano Zetasizer (DTS Ver. 5.10). A 0.1 ml aliquot of the optimized formulation was diluted in 100 ml of distilled water, and 1 ml of this dilution was transferred into a cuvette for analysis. Measurements was conducted at 25 °C with a fixed scattering angle of 90°.

4.15.3 Zeta potential

Zeta potential measurements were performed using a Malvern Zetasizer to evaluate the surface charge of the oil droplets in the SNEDDS formulation. The oil droplets generally possess a negative charge in conventional SNEDDS, attributable to the presence of free fatty acids.[97]. A high zeta potential of droplets in SNEDDS emulsions signifies enhanced stability and an extended shelf life.

4.16 Scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) serves as a highly valuable and extensively utilized characterization technique, especially within the domains of material science and pharmaceutical research.It provides detailed insights into a sample's topography, offering crucial information about particle surface properties, texture, and overall structural integrity. Beyond surface analysis, SEM also helps in developing a deeper understanding of the sample's morphology, covering aspects like particle size, shape, distribution, and arrangement. By generating high-resolution, three-dimensional images, SEM allows researchers to closely examine the micro- and nanostructural features of materials with great precision. This level of detail is essential for interpreting material behavior, optimizing

formulations, and maintaining strict quality control during product development[98]. The rich visual and dimensional data obtained through SEM play a pivotal role across various fields, including pharmaceuticals, nanotechnology, and advanced material research.

4.17 Accelerated stability study

Accelerated shelf-life study for SNEDDS was be checked for stability without any kind of changes in their physicochemical properties[73]. The optimized SNEDDS formulation was placed in a stability chamber (Remi Electro Technique, India) and evaluated under accelerated storage conditions of 40 ± 2°C and 75 ± 5% relative humidity for a period of six months, in accordance with standard stability testing protocols. The aged samples was be analyzed for drug precipitation and change in droplet size.

4.17.1 Preparation of Test solution:

Sample was evaporated, and residue was dissolved in 1 ml methanol. The collected solution was then filtered with 0.45 μm membrane filter. Obtained test solution was then used for HPTLC fingerprinting.

4.17.2 Preparation of spray reagent:

A reagent solution was prepared by sequentially mixing 10 mL of glacial acetic acid with 0.5 mL of anisaldehyde, followed by the addition of 85 mL of methanol and 5 mL of concentrated sulfuric acid (98%). For Chromatography Toluene:Methyl acetate and methanol in the ratio 8:2:0.5 v/v was used. Chamber saturation time set for 30 min. and test solution was visualised at 254nm, 366nm, 540nm absorbance.

4.17.3 Sample preparation

Samples were prepared at concentrations of 1 g/mL or 10 g/mL by dissolving the respective amounts in 10 mL or 100 mL of sterile Soybean Casein Digest Broth. The solutions were then thoroughly mixed using a cyclomixer to achieve uniform homogenization.

Method: Pour –plate method

4.17.4 Total microbial plate count

From the prepared sample, 1 mL was aseptically pipetted into two separate sterile Petri dishes, each distinctly labeled. Approximately 25 mL of autoclaved Soybean Casein Digest Agar medium, cooled to around 40°C, was poured into each plate. The plates were gently rotated to ensure even distribution and thorough mixing of the inoculum with the agar. After solidification, the plates were incubated at 30–35°C for 3 to 5 days. Post-incubation, colonies on both plates were counted, ensuring counts did not exceed 250 colonies. The mean colony count was calculated, and the colony-forming units (CFU) were determined

using the following formula:

$$\textbf{Colony forming unit } (cfu): \frac{\textbf{No. of colonies x dilution}}{\textbf{Weight of sample}}$$

4.17.5 Total yeast and mould count

From the prepared sample, 1 mL was aseptically pipetted into two separate sterile Petri plates, each properly labeled. Autoclaved Soybean Casein Digest Agar medium, cooled to approximately 40°C, was poured into each plate (approximately 25 mL per plate). The plates were gently rotated to ensure uniform mixing of the sample with the medium. After the agar solidified, the plates were incubated at 20–25°C for 5 days. Following incubation, colonies were counted on both plates, selecting counts within the range of 20 to 25 colonies. The average colony count was calculated, and the colony-forming units (CFU) were determined using the previously described formula.

4.17.6 Test for Specified Organisms:

4.17.6.1 *Escherichia coli*

A 0.1 mL aliquot of the sample was pipetted and transferred into 10 mL of MacConkey Broth, followed by incubation at 42–44°C for 24 to 48 hours. Subsequently, the broth culture was subcultured onto MacConkey Agar plates, which were then incubated at 30–35°C for 18 to 72 hours.

The appearance of pink, non-mucilaginous colonies indicates a probable presence of *Escherichia coli*, warranting further confirmation via specific identification tests.

a. Identification Test:

streaked suspected colony on Eosin Methylene Blue agar (EMB). Incubate the plate *at* 30° to 35°C for 18 to 24h. If growth with greenish metallic sheen observed it confirms the presence of *E.coli*.

Indol Test:

Take o.1 mL sample from MacConkey broth into 10 mL peptone broth. Mixed well. Incubate the tube at 30° to 35°C for 18 to 24h. Next day add Kovach's reagent into peptone broth. If pink ring is observed, it ***Salmonella***

- A 0.1 mL of the sample was transferred into 10 mL of Rappaport Vassilidis Salmonella enrichment broth and incubated at 30–35°C for 24 to 48 hours.
- The enriched culture was then subcultured onto Wilson and Blair's Bismuth Sulfite (BBS) agar plates, which were incubated at 30–35°C for 24 to 48 hours. Typical green colonies with black centers develop initially, and after 48 hours, colonies

become uniformly black. Colonies exhibiting a dark halo and metallic sheen are indicative of potential Salmonella presence.

- Additionally, subculturing on Xylose Lysine Deoxycholate Agar (XLDA) plates incubated at 30–35°C for 24 to 48 hours results in well-developed red colonies, with or without black centers, further suggesting Salmonella contamination.
- Confirmation of *Salmonella* presence should be performed using appropriate identification tests.
- For *Escherichia coli* detection, growth on agar plates incubated at 18 to 24 hours displaying a greenish metallic sheen confirms its presence.

b. Identification Test:

The suspected colony was streaked onto a Triple Sugar Iron (TSI) agar slant and incubated at 30–35°C for 24 to 48 hours. Confirmation of Salmonella presence is indicated by acid and gas production, with or without blackening of the agar butt, accompanied by a lack of acid formation in the surface growth.

4.17.6.2 Pseudomonas aeruginosa

A loopful of the test sample was aseptically streaked onto Cetrimide Agar and incubated at 30 ± 2°C for a period of 18 to 72 hours. The development of greenish pigmented colonies suggests the possible presence of *Pseudomonas aeruginosa*. Confirmation of the organism's identity should be performed using standard microbiological identification procedures.

a. Identification Test :

Streak suspected colony on Pseudomonas agar with fluroscens& Pseudomonas agar with pyocyanin media containing plates. The plates were incubated at 30 to 35°C for 18 to 24 hours. If greenish colonies observed on Pseudomonas agar with fluroscens& colorless to yellow colony observed on Pseudomonas agar with pyocyanin, it confirms the presence of *pseudomonas.*

- **Oxidase test:**

Picked suspected colony & put it on Oxidase disc, if it turns white to blue, confirms the presence of pseudomonas.

4.17.6.3 Staphylococcus aureus

To assess the presence of *Staphylococcus aureus*, a loopful of the test sample was inoculated onto Mannitol Salt Agar. The plates were incubated at a temperature range of 30 to 35°C for a period of 18 to 72 hours. The development of white or yellow colonies accompanied by yellow coloration in the surrounding medium was indicative of possible *S.*

aureus growth..

- **Identification test:**

Catalase test: Picked suspected colony on sterile slide add 1-2 drop of Hydrogen peroxide. If bubbles are formed it indicates presence of *S.aureus.*

Positive Control:

A positive Control is used with a known amount of the organism to check the viability of the organism and also to check the property of the medium used for its detection. There should be characteristic growth on all the different types of medium.

Negative Control:

Negative control, prepared without the addition of microorganisms, is used to evaluate the sterility of the medium and diluent. The absence of microbial growth in this control confirms the sterility and integrity of the test components.

Note: The samples will be checked for globule size, zeta potential, and entrapment efficiency. It is then compared with the characterization test. The values will be significant if the p-value is less than 0.05.

4.18 HPTLC[99]

HPTLC analysis was carried out using an extract prepared through the Soxhlet extraction technique. HPTLC fingerprint profile was utilized to authenticate and confirm the botanical identity of the plant material.

Quantitative analysis of the hyperspectral data was performed using densitograms and image profiles. HPTLC, as an analytical method, relies on the use of advanced instrumentation, standardized and well-documented procedures, and established protocols to ensure accuracy and reproducibility. The reliability of HPTLC is highlighted by its ability to produce consistent results even when the same samples and plates are used for repeated detections. Marker compounds play a critical role in this process, helping to authenticate botanicals, detect adulteration, and assess product quality. These markers are chemically defined constituents of an herbal product, selected for quality testing purposes rather than therapeutic action. During the HPTLC analysis, the extract's Rf value was determined and subsequently compared to the standard Rf to confirm the authenticity of the plant material and ensure the quality of the sample.

Preparation of standard solution

An accurately weighed 2 mg of standard Gallic acid, Quercetin was transferred into two different 2 mL volumetric flask and 1 mg of standard Arjunolic acid was transferred in 1mL Volumetric flask. Approximately 1 mL of methanol was added to both Gallic acid and

Quercetin, while 0.5 mL of methanol was added to Arjunolic acid. Each mixture was sonicated until the respective standard was completely dissolved. Following dissolution, the volume was adjusted to 2 mL with methanol for Gallic acid and Quercetin, and to 1 mL for Arjunolic acid. These prepared standard solutions were subsequently utilized for HPTLC fingerprinting.

Preparation of Test Solution for SNEDDS

Precisely weighed 1 g of the sample was transferred into an iodine flask, followed by the addition of 5 mL methanol. The mixture was refluxed in a water bath for 10 minutes and then allowed to cool. The resulting solution was initially filtered through Whatman No. 1 filter paper and subsequently passed through a 0.22 µm syringe filter into a 5 mL volumetric flask, where the volume was adjusted to 5 mL with methanol. The prepared test solution was then employed for High-Performance Thin-Layer Chromatography (HPTLC) fingerprint analysis.

4.18.1 Preparation of Test Solution for *T. arjuna* Bark Extract

Precisely weigh 100 mg of the sample into an iodine flask and add 5 mL of methanol. Reflux the mixture in a water bath for 10 minutes. After cooling, filter the solution through Whatman No. 1 filter paper, then further filter it using a 0.22 µm syringe filter into a 5 mL volumetric flask. Finally, adjust the volume to 5 mL with methanol. The resulting test solution is then utilized for HPTLC fingerprinting.

4.18.2 Preparation of Spray reagent [Vaniline – sulphuric acid reagent]

Prepare a stock solution by dissolving 50 mg of vanillin in 2 mL of methanol, followed by the addition of 8 mL of sulfuric acid (98%). From this stock solution, prepare a 10% (v/v) dilution using methanol.

Chromatographic Conditions:

Table 4.5. Chromatographic Conditions of Gallic acid

Mode of Application	CAMAG Linomat 5 – Applicator
Filtration System	Whatman filter paper No. 1
Stationary Phase	MERCK – TLC / HPTLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from the base of the plate
Band length	8mm
Standard Volume for application	15 µL

Sample Volume for application	30 µL
Distance Between Tracks	20 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Saturation Time of Chamber	30 min
Mobile Phase	Toluene:Ethly Acetate:Formic Acid (5: 5: 1 v/v)
Visualization	254 nm
Quantification	254 nm

Table 4.6. Chromatographic Conditions of Quercetin

Mode of Application	CAMAG Linomat 5 – Applicator
Filtration System	Whatman filter paper No. 1
Stationary Phase	MERCK – TLC / HPTLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from the base of the plate
Band length	8mm
Standard Volume for application	10 µL
Sample Volume for application	30 µL
Distance Between Tracks	20 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Saturation Time of Chamber	30 min
Mobile Phase	Toluene:Ethly Acetate:Formic Acid (6: 4: 0.5v/v)
Visualization	@375 nm
Quantification	@375 nm

Table 4.7. Chromatographic Conditions of Arjunolic acid

Mode of Application	CAMAG Linomat 5 – Applicator
Filtration System	Whatman filter paper No. 1
Stationary Phase	MERCK – TLC / HPTLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10 mm

Development End Position	80 mm from the base of the plate
Band length	8mm
Standard Volume for application	5 µL
Sample Volume for application	30 µL
Distance Between Tracks	20 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Saturation Time of Chamber	30 min
Mobile Phase	Chloroform: Methanol (8.5: 1.5 v/v)
Visualization	@540 nm
Quantification	@540 nm

4.19 Animal study

4.19.1 Protocol details: IAEC approval no. for the study is PBRI/IAEC/30-09-2024/05.

4.19.1.1 Chemical requirement

Table 4.8. List of chemical used during the testing

Drug used	**Name of drug**	**Dose**
Name of test drug	**Sample-SNEEDS**	Treated with Dexamethasone (30 µg/kg body weight)
Name of std. drug	**Enalapril Maleate**	Dexamethasone + 5 mg/kg Enalapril Maleate
Drug used to induce disease	**Dexamethasone**	Dexamethasone + SNEDDS formulation
Identification code	**SNEEDS, EM, Dexa**	Treated with Dexamethasone (30 µg/kg body weight)

4.19.1.2 Test system

Table 4.9. Testing Protocol

Test System	Wistar Rats
Rationale	Well established animal model as per the published research work

Source	Animal House, PBRI, Bhopal
Number of animals/groups	Male: 18 Female: NIL
Age	10-12 weeks
Body weight range at acclimatization	Male: 150±30 g Female: NA
Acclimatization	7 Days
Room condition	22 ± 2°C
Diet	Regular Standard Diet (Keval sales Pvt. ltd.)
Water	*ad libitum*
Method of administration	Subcutaneous, Oral

4.19.1.3 Test condition details

Table4.10. Test condition details

Name	**Sample- Arjuna SNEEDS**	
Storage condition	**Temperature**	**Room temperature**
	Humidity	NA
	Container	Plastic
	Any other specification	NA
Safety precaution	Not specified. Handle as per the safety norms of the lab.	

4.19.2 Method: In-Vivo Anti-hypertension activity

4.19.2.1 Animals

Wistar albino rats, weighing between 150–200 g, were procured from the institutional animal facility at PBRI and housed under standardized laboratory conditions in sanitized, spacious polypropylene cages. The environment was maintained at a regulated temperature of 22 ± 2 °C with a 12-hour light/dark photoperiod. Throughout the experimental duration, the animals were granted unrestricted access to potable water and a standard pelletized diet (Normal Pellet Diet, NPD) sourced from Keval Sales Corporation, Vadodara.

4.19.2.2 Experiment

This study investigated the antihypertensive effects of a self-nanoemulsifying drug delivery system (SNEDDS) in male Wistar rats aged 8–12 weeks, with ethical clearance obtained

prior to experimentation. Hypertension was induced by daily subcutaneous administration of dexamethasone at 30 µg/kg body weight for 14 days. The animals were divided into three groups: a negative control group receiving only dexamethasone; a preventive group treated with the SNEDDS formulation both before and during dexamethasone exposure; and a curative group administered a standard antihypertensive drug orally from days 8 to 14 after hypertension was established.

Table 4.11. Design of experiment

S.No	Treatment Group	No of Animals	Dose
Group 1	Control	6/M	Treated with Dexamethasone (30 µg/kg body weight)
Group 2	Standard	6/M	Dexamethasone + 5 mg/kg Enalapril Maleate
Group 3	Test sample	6/M	Dexamethasone + SNEDDS formulation

4.19.2.3 Evaluation

4.19.2.3.1 Blood pressure

Blood pressure (systolic) was measured using a non-invasive tail-cuff plethysmography system. Blood pressure readings were taken on day 4, 7, 14, and 18, to evaluate the efficacy of the interventions.

CHAPTER 5

RESULT AND DISCUSSION

Chapter 5

Result and Discussion

5. Results and discussion:

5.1 Collection of samples:

T. arjuna bark was procured in the required amount from the local market of Jalandhar, Punjab.

5.2 Authentication:

The authenticity of *T. arjuna* bark was confirmed by the National Institute of Pharmaceutical Education and Research (NIPER), Mohali.

5.3 Organoleptic Characteristics of *T. arjuna*.

Table 5.1. Organoleptic Characteristics of *T. arjuna.*

S.No.	Characteristics	*T.arjuna*
1.	Colour	Greyish brown outer, reddish brown inner
2.	Odour	None
3.	Taste	Astringent
4.	Fracture	Short
5.	Surface	Rough

5.4 Quality control parameters of *T. arjuna.*

5.4.1. Common Name: *Arjuna*

5.4.2. Botanical name: *T. arjuna*

5.4.3. Part used: Bark

5.4.4. Physico-chemical Parameters: Dried sample of *Arjuna* bark was taken, and physicochemical parameters were performed.

Table 5.2. Results of Physico-chemical parameters

Parameters	Batches						Std.Div	Standard
	I	II	III	IV	V	VI		
Foreign Matter (%)	0	0	0	0	0	0	0±0	NMT 2%
Loss on Drying (105°C)	5.24	5.3	6.11	6.32	6.37	6.33	5.87±0.53	-
Total Ash (%	19.52	23.24	21.61	20.12	23.47	22.01	21.68±1.60	NMT25%

w/w)								
Acid insoluble Ash (%w/w)	1.04	1.007	1.03	0.99	0.94	0.97	0.99±0.03	NMT 1%
Alcohol Soluble extractive (%w/w)	39.05	39.64	40.66	42.01	40.71	42.33	40.73±1.28	NLT 20%
Water soluble extractive (%w/w)	37.94	34.84	40.21	42.62	42.95	43.31	40.31±3.37	NLT 20%

The physico-chemical parameters shows that foreign matter was not present in *T.arjuna.* Also, the result for other parameters were within the API standard limit i.e., LOD 5.87±0.53, Total ash 21.68±1.60, Acid-insoluble ash 0.99±0.03, Alcohol soluble extractive 40.73±1.28, water soluble extractive 40.31±3.37 respectively as shown in the table 5.2.

5.4.5. Phyto-chemical Analysis

Table 5.3. Result of Phyto-chemical Analysis (Water extract)

Compound	**Test Performed**	**Result (Batch)**					
		I	**II**	**III**	**IV**	**V**	**VI**
Alkaloids	**Dragendroff's reagent**	+	+	+	+	+	+
	Wagnor's reagent	+	+	+	+	+	+
	Mayer's reagent	+	+	+	+	+	+
Proteins	**Xanthoproteic test**	+	+	-	+	+	+
Flavonoids	**Lead acetate test**	+	+	+	+	+	+
	Shinoda test	+	+	+	+	+	+
Saponins	**Froth formation test**	+	+	+	-	+	+
Cardiac Glycosides	**Keller-killani test**	+	+	+	+	+	+
Steroids and triterpenoids	**Salkowski test (chloroform extract)**	-	-	-	-	-	-
Reducing sugar	**Fehling test**	+	+	+	+	+	+
Tannins and Phenolic compounds	**Ferric chloride test**	+	+	+	+	+	+

Table 5.4. Result of Phyto-chemical Analysis (Alcohol extract)

Compound	Test Performed	Result (Batch)					
		I	II	III	IV	V	VI
Alkaloids	Dragendroff's reagent	-	-	-	-	-	-
	Wagnor's reagent	+	+	+	+	+	+
	Mayer's reagent	+	+	+	+	+	+
Proteins	Xanthoproteic test	+	+	+	+	+	+
Flavonoids	Shinoda test	-	-	-	-	-	-
	Lead acetate test	+	+	+	+	+	+
Saponins	Froth formation test	-	-	-	-	-	-
Cardiac Glycosides	Keller-killani test	+	+	+	+	+	+
Steroids and triterpenoids	Salkowski test (chloroform extract)	+	+	+	+	+	+
Reducing sugar	Fehling test	+	+	+	+	+	+
Phenolic and Tannins ompounds	Ferric chloride test	+	+	+	+	+	+

Phytochemical analysis was done by preparing 2 extracts i.e., Water and alcohol, table no 5.3 shows the result for water extract while table no 5.4 shows the result for Alcohol extract, the test were repeated 6 time for both the extract and the analysis shows that all the 6 batches gives positive results for various phytochemicals as per the standards. And, all the required chemicals are present in the *T.arjuna* extract.

5.4.6. Heavy metal, Microbial limit test and Aflatoxin (by TLC)

Table 5.5. Result of Heavy metal analysis

S.No.	Test parameters	Results	Specifications
	Total		
1.	Lead (Pb)	Not detected	NMT 10.0ppm
2.	Cadmium (Cd)	Not detected	NMT 0.3ppm
3.	Mercury (Hg)	Not detected	NMT 1.0ppm
4.	Arsenic (As)	Not detected	NMT 3.0ppm

As presented in Table 5.5, heavy metal analysis of the *T. arjuna* sample revealed no detectable levels of heavy metals, indicating that the sample is free from harmful metallic contaminants.

Table 5.6. Result of Microbial Limit and pathogens test

S.No.	Test parameters	Results	Specifications
Microbial overload test			
1.	**Total microbial plate**	**170cfu/gm**	**$1x10^5$cfu/gm**
2.	**Total yeast and mould**	**Nil**	**$1x10^3$cfu/gm**
Pathogen Tests			
1.	***Salmonella***	**Absent**	**Should be absent**
2.	***Pseudomonas aeruginosa***	**Absent**	**Should be absent**
3.	***Staphylococcus aureus***	**Absent**	**Should be absent**
4.	***Escherichia coli***	**Absent**	**Should be absent**

Table 5.6 presents the results for microbial analysis, showing a total plate count of 170 cfu/g and an absence of yeast and molds, both conforming to the established regulatory limits.And, all pathogens including *Escherichia coli, Salmonellae, Pseudomanas aeruginosa and Staphylococcus aureus* were absent which shows that the collected *T. arjuna* sample is free from microbial contamination.

Table 5.7. Result of Aflatoxin

Test name	Test type	Result	Specification
Test for Aflatoxin (by TLC)	**B1 & B2**	**Absent**	B1 & G1-NMT 0.5 ppm
	G1 & G2	**Absent**	B2 & G2-NMT 0.1 ppm

As per the results depicted in table 5.7the aflatoxins analysis shows that no aflatoxins were detected in the *T.arjuna* sample.

5.4.7. Pesticide Residue:

Table 5.8. Observation of Pesticide Residue

Test Name	Test type	Result	Specification
Pesticide Residue	Alachor	Not detected	NMT 0.02 mg/kg
	Aladrin& Dieldrin	Not detected	NMT 0.05 mg/kg
	Chlordane	Not detected	NMT 0.05 mg/kg
	DDT	Not detected	NMT 1.00

			mg/kg
	Endosulfan	Not detected	NMT 3.00 mg/kg
	Malathion	Not detected	NMT 1.00 mg/kg

As shown in table 5.8, the pesticide residue analysis shows that no pesticide residuewas detected in *T.arjuna s*ample.

5.5 Preparation of extract:

Extract of *Arjuna* was prepared using the Soxhlet apparatus. Details of the extraction process are mentioned below.

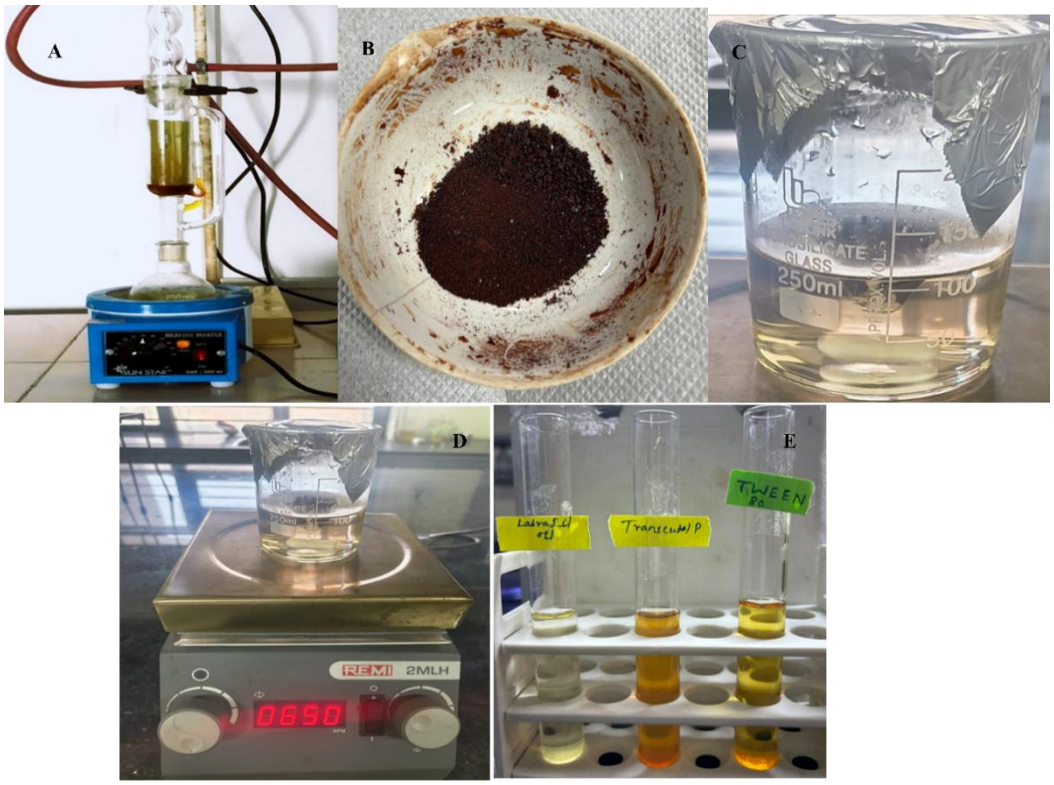


Fig.5.1. (A) Soxhlet apparatus (B) Dried *T. arjuna* extract (C) Addition of Drug, oil, surfactant and co-surfactant (D) Magnetic stirring at 650rpm

5.5.1 Extraction Batches

Table no. 5.9 shows the preparation of extract in 6 different batches with std. dev. The extraction process was conducted in six batches, each utilizing 100 g of dried coarse bark powder and 500 mL of methanol in a Soxhlet apparatus.

The average yield of the extract was 29.06±1.7. The maximum yield was 29.14 gm and lowest was 26.21 gm.

Table 5.9. Detail of extraction

Sr.No.	Wt. of dried bark (gm)	Solvent System	Temp (°C)	Duration (hrs)	Wt. of extract obtained (gm)	Mean ± % dev
1.	100	Methanol (500ml)	60	18	27.88	29.06±1.7
2.					29.14	
3.					31.75	
4.					29.47	
5.					29.95	
6.					26.21	

5.5.2 UV absorbance calibration curve

Table 5.10. UV absorbance

Sr.No.	Conc (µg/ml)	Absorbance
1.	10	0.246
2.	20	0.367
3.	30	0.478
4.	40	0.562
5.	50	0.687

A 25 mg/mL concentration of the extract was analyzed using a UV spectrophotometer (UV-1800, Shimadzu, Japan) across the 200–800 nm wavelength range to identify the maximum absorbance (λmax). The findings, illustrated in Figure 5.2 and detailed in Table 5.10, were subsequently used to evaluate the linearity of the extract.Different concentrations of extract were analyzed, and absorbance were recorded atλmax 207 nm. The graph was plotted between the different concentrations and absorbances. The linear regression analysis yielded the equation Y = 0.0108x + 0.1449, accompanied by an R^2 value of 0.9967. The proximity of the R^2 value to 1 confirms the sample's adherence to Beer-Lambert's law, demonstrating excellent linearity. These findings are depicted in Figure 5.2.

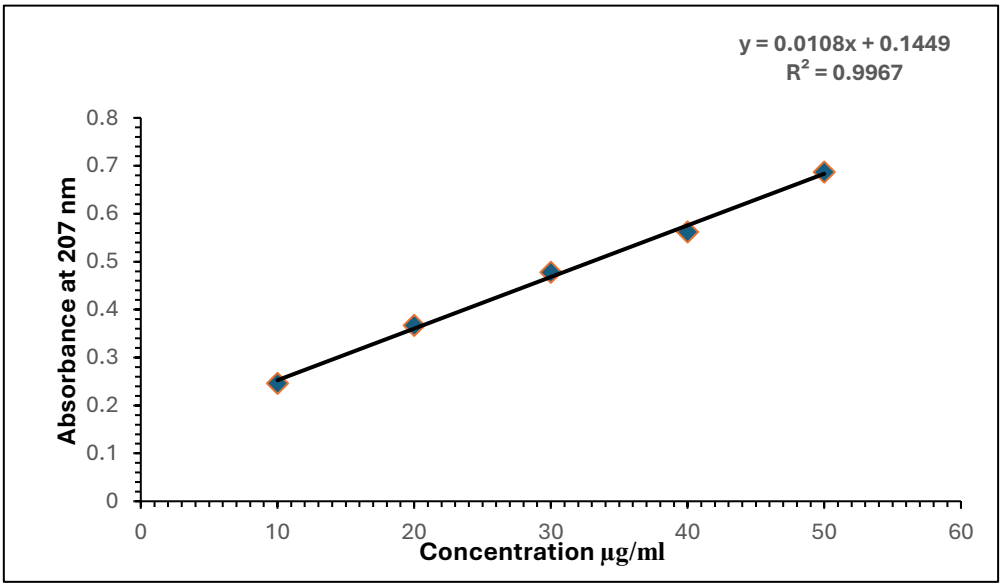


Fig. 5.2. UV calibration curve

5.5.3 Thin Layer Chromatography (TLC)

Table 5.11. Rf values of *T. arjuna* extract

Sample	**Solvent system**	**Ratio**	**Rf value (After vaporizing in iodine chamber)**
T. arjuna	Chloroform: Methanol	8:2	0.92,0.97

Several Rf values were calculated during the TLC of above extract using the suitable solvent system and 0.92 is compared with the standard giving the violet color and validate the presence of cardiac glycosides in the extract of *T. arjuna*.

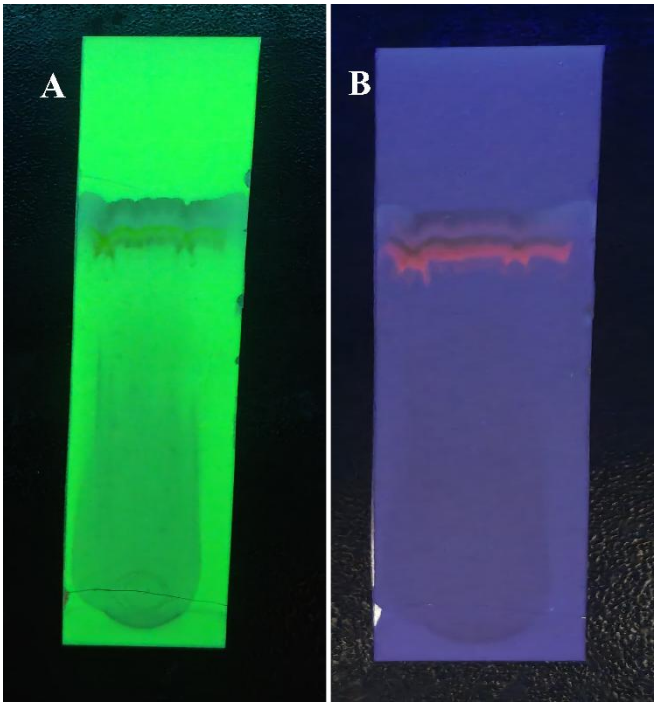


Fig.5.3 TLC plate A at 210 nm and B at 254 nm

5.6 Preparation of SNEEDS

5.6.1 Solubility of Vehicle

Table 5.12. Solubility study of *T. arjuna* extract

Vehicle	Solubility in batches			St. Dev of Solubility
	I	II	III	
Castor oil	0.145	0.149	0.147	0.147±0.002
Coconut oil	0.339	0.335	0.337	0.337±0.002
Sunflower oil	0.191	0.189	0.188	0.189±0.0015
Sesame oil	0.98	0.97	0.96	0.97±0.01
Labrafil M 1994 CS	15.200	15.199	15.198	15.199±0.001
Labrafac CC	0.330	0.335	0.332	0.332±0.0025
Labrasol	0.398	0.397	0.396	0.397±0.001
Tween 60	0.776	0.779	0.800	0.785±0.013
Tween 20	0.885	0.887	0.888	0.886±0.0012
Tween 80	8.9	8.7	9.0	8.866±0.152
Transcutol P	8.800	8.779	8.778	8.785±0.012
PEG 400	7.071	7.075	7.073	7.073±0.002

The solubility of *T. arjuna* extract was assessed across different vehicles. Consequently, Labrafil M 1994 CS, Tween 80, and Transcutol P were identified as the most suitable excipients for the development of the *T. arjuna* SNEDDS formulation.

Fig.5.4. Solubility of nanoemulsion vehicles

5.6.2 Emulsification

Table 5.13. result of different ratios of oil, surfactant, co-surfactant

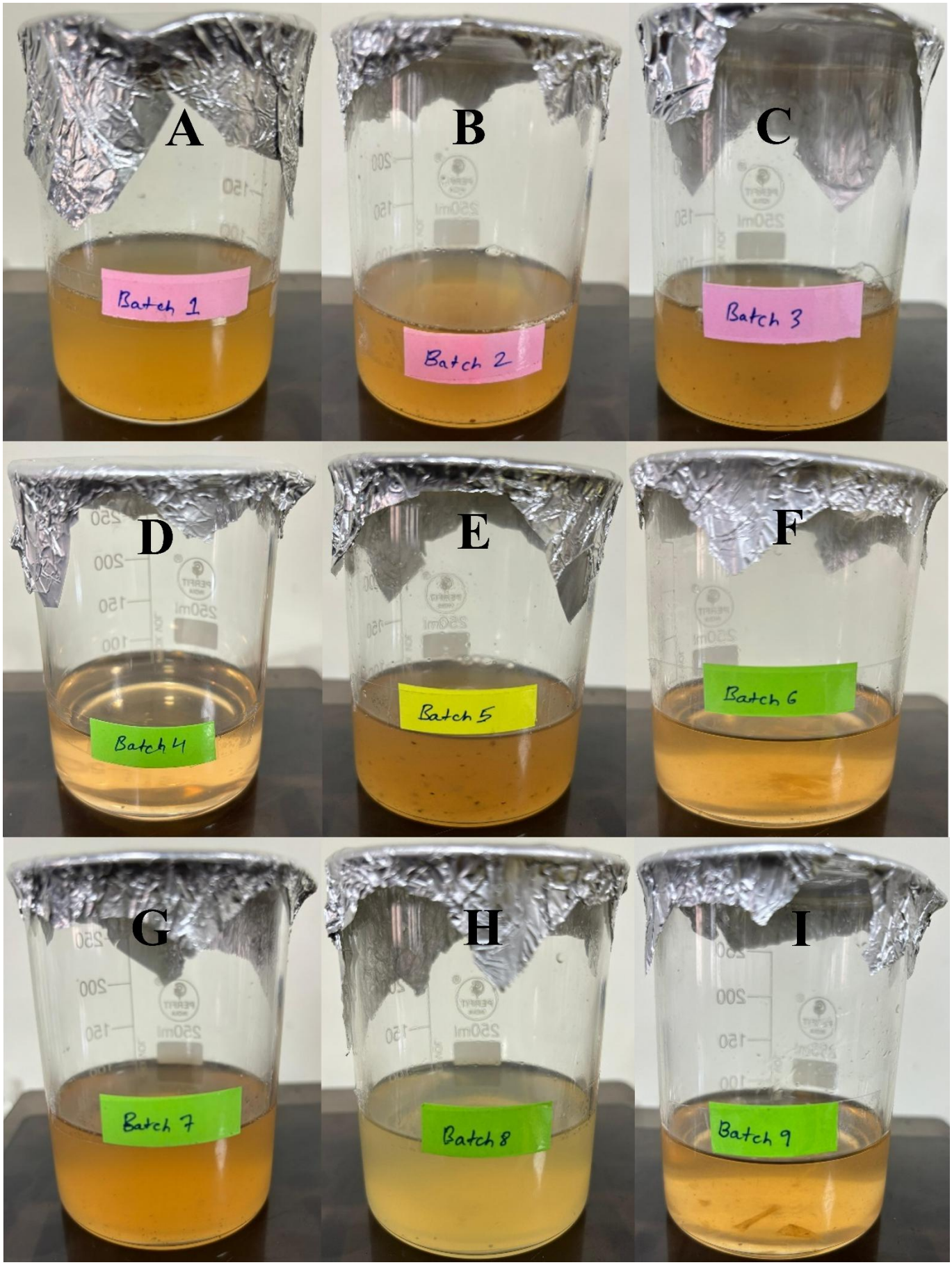
Batch No.	Oil (µl)	Surfactant (µl)	Co-surfactant (µl)	Sample (µl)	Distilled water (ml)	Result
1.	0.1	0.5	0.4	150	100	Creaming
2.	0.2	0.1	0.8	150	100	Creaming
3.	0.3	0.35	0.35	150	100	Cracking
4.	0.2	0.2	0.6	150	100	Clear
5.	0.6	0.2	0.2	150	100	Cracking
6.	0.2	0.4	0.4	150	100	Turbid
7.	0.3	0.3	0.4	150	100	Creaming

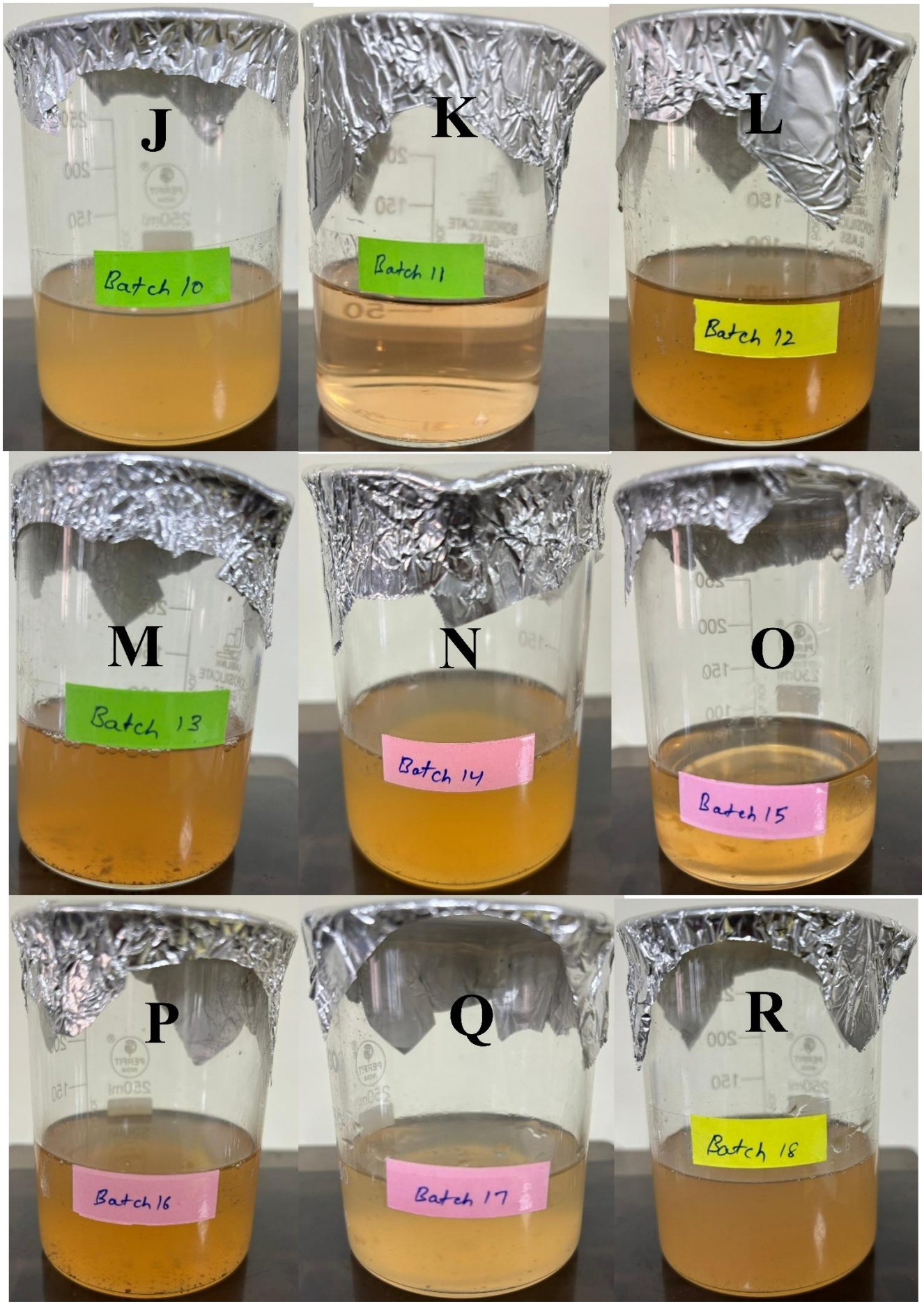
8.	0.2	0.7	0.1	150	100	Creaming
9.	0.5	0.1	0.4	150	100	Turbid
10.	0.2	0.53	0.27	150	100	Creaming
11	0.1	0.1	0.8	150	100	Clear
12.	0.3	0.23	0.47	150	100	Cracking
13.	0.4	0.27	0.33	150	100	Cracking
14.	0.2	0.5	0.3	150	100	Creaming
15.	0.1	0.4	0.5	150	100	Turbid
16.	0.5	0.2	0.3	150	100	Cracking
17.	0.3	0.6	0.1	150	100	Creaming
18.	0.4	0.3	0.3	150	100	Creaming
19.	0.1	0.6	0.3	150	100	Cracking
20.	0.4	0.2	0.4	150	100	Very turbid
21.	0.3	0.47	0.23	150	100	Creaming
22.	0.4	0.40	0.2	150	100	Very turbid
23.	0.1	0.3	0.6	150	100	Clear
24.	0.4	0.5	0.1	150	100	Very turbid
25.	0.3	0.5	0.2	150	100	Cracking
26.	0.3	0.2	0.5	150	100	Very turbid and creaming
27.	0.9	0.37	0.53	150	100	Turbid

Twenty-seven SNEDDS formulations incorporating *T. arjuna* bark extract were prepared by systematically varying the ratios of oil, surfactant, and co-surfactant to optimize the delivery system.

Visual assessment was conducted to evaluate the clarity and turbidity of the prepared emulsions. Samples 4,11 and 23 exhibited clarities across all batches of SNEDDS. Conversely, turbidity was observed in samples 6, 9, 15, 20, 22, 24, 26 and 27. Creaming occurred in samples 1, 2, 7, 8, 10, 14, 17, 18 and 21 while phase separation was observed in

samples 3, 5, 12, 13, 19 and 25. Based on the experimental findings, a ternary phase diagram was developed to identify the optimal region for SNEDDS formulation. Comprehensive observations for each formulation batch are provided in Table 5.13.


A
Batch 1
B
Batch 2
C
Batch 3
D
Batch 4
E
Batch 5
F
Batch 6
G
Batch 7
H
Batch 8
I
Batch 9


J
Batch 10
K
Batch 11
L
Batch 12
M
Batch 13
N
Batch 14
O
Batch 15
P
Batch 16
Q
Batch 17
R
Batch 18

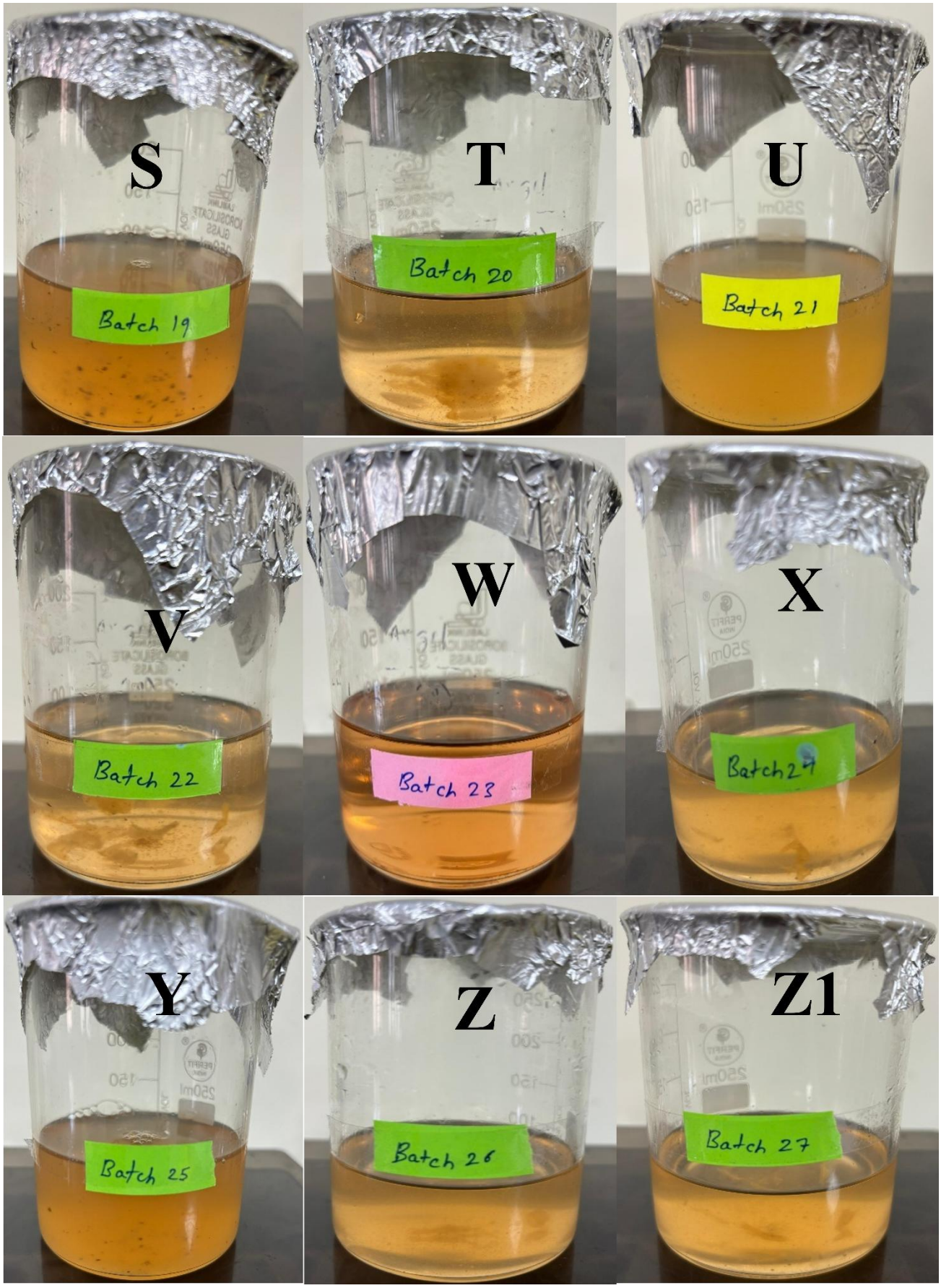


Fig. 5.5. Pictures of Different batches of SNEEDS

5.6.3 Ternary Phase Diagram

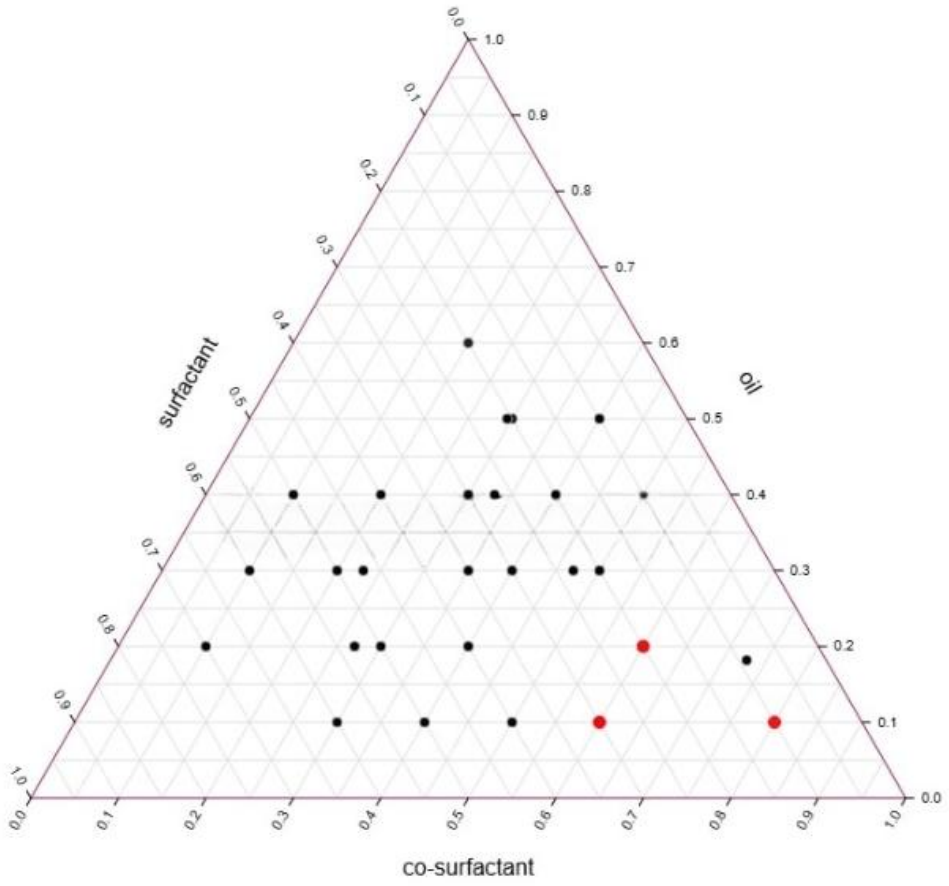


Fig.5.6. Ternary phase diagram showing oil phase, surfactant and co-surfactant

5.6.4 Design of experiment (DOE)

5.6.4.1. QbD-based formulation optimization studies

Various SNEDDS formulations were developed by adjusting the ratios of oil, surfactant, and co-surfactant in accordance with the Design of Experiments (DoE) framework. The resulting data were effectively represented by a quadratic model, reflecting the influence of the independent variables on the formulation responses. Detailed results are provided in Table 5.14.

Table 5.14. Response of CQAs of SNEEDS

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Run	A:Oil	B:Surfactant	C:Cosurfactant	Globule Size	Zeta Potential	PDI
				nm	mV	

1	0.1	0.3	0.6	329.5	-29.7	0.233
2	0.15	0.03	0.7	717	-33.2	0.367
3	0.15	0.2	0.86	789.5	-47.8	0.814
4	0.2	0.1	0.8	668.4	-30.2	0.528
5	0.15	0.2	0.7	702.8	-31.9	0.497
6	0.15	0.2	0.7	702.8	-31.9	0.497
7	0.1	0.3	0.8	924.8	-58.2	0.866
8	0.06	0.2	0.7	461.3	-37.5	0.621
9	0.15	0.2	0.7	702.8	-31.9	0.497
10	0.1	0.1	0.6	311.2	-33.9	0.288
11	0.23	0.2	0.7	843.7	-53.9	0.746
12	0.15	0.2	0.53	427.7	-30.9	0.52
13	0.15	0.2	0.7	702.8	-31.9	0.497
14	0.15	0.2	0.7	702.8	-31.9	0.497
15	0.2	0.1	0.6	712.9	-39.9	0.574
16	0.15	0.2	0.7	702.8	-31.9	0.497
17	0.2	0.3	0.8	576.4	-37.4	0.675
18	0.2	0.3	0.6	655	-34.7	0.726
19	0.15	0.36	0.7	717	-33.3	0.548
20	0.1	0.1	0.8	338.5	-30.4	0.225

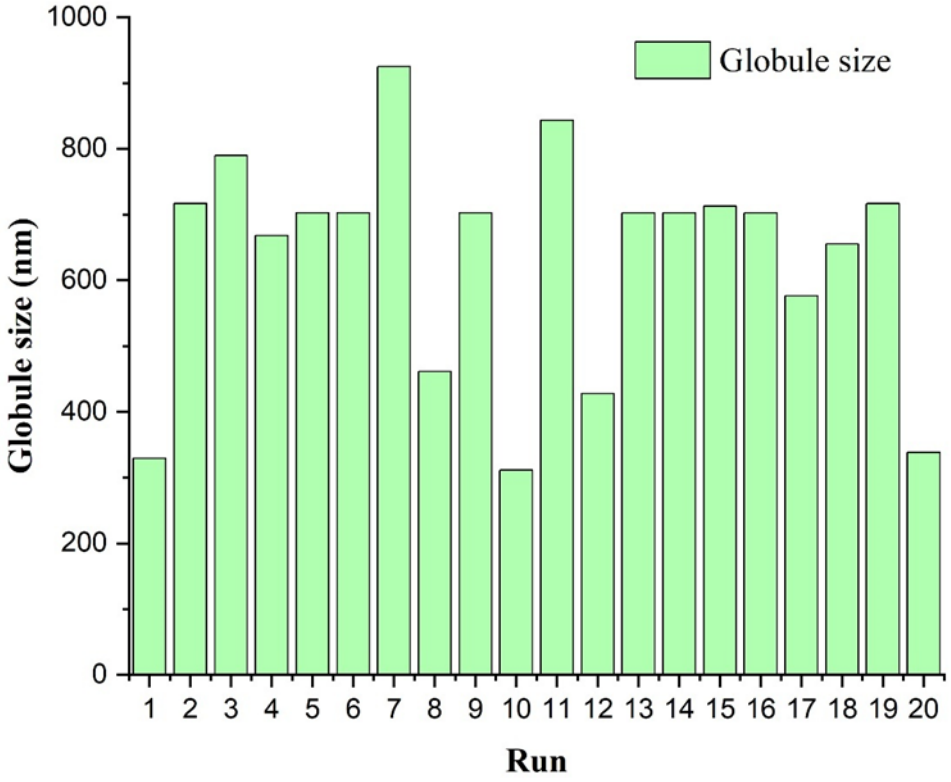


Fig.5.7. Bar graph of globule size vs various formulations

As depicted in Figure 5.7, the globule sizes among the various formulations ranged from a minimum of 311.2 nm in run 10 to a maximum of 924.8 nm in run 7, indicating considerable

variation in particle size across the tested batches. It was observed that lower range of all oil, surfactant and cosurfactant was affected by the globule size of lower size range, oil, surfactant and co-surfactant keeping 0.1 to 0.15, 0.1 to 0.2 and cosurfactant 0.6 to 0.65. A strong mutual interaction of all three factors also exist which tend to cause decrease in the globule size.

Fig.5.8. Bar graph of Zeta potential vs various formulation

The zeta potential measurements varied between -29.7 mV (observed in run 1) and a peak value of -58.7 mV, as depicted in Figure 5.9. Notably, lower zeta potential values were associated with reduced oil content (0.1 to 0.12), while higher zeta potentials corresponded to increased concentrations of surfactant and cosurfactant, ranging from 0.25 to 0.3 and 0.75 to 0.8, respectively. These observations are clearly illustrated in the contour plots shown in

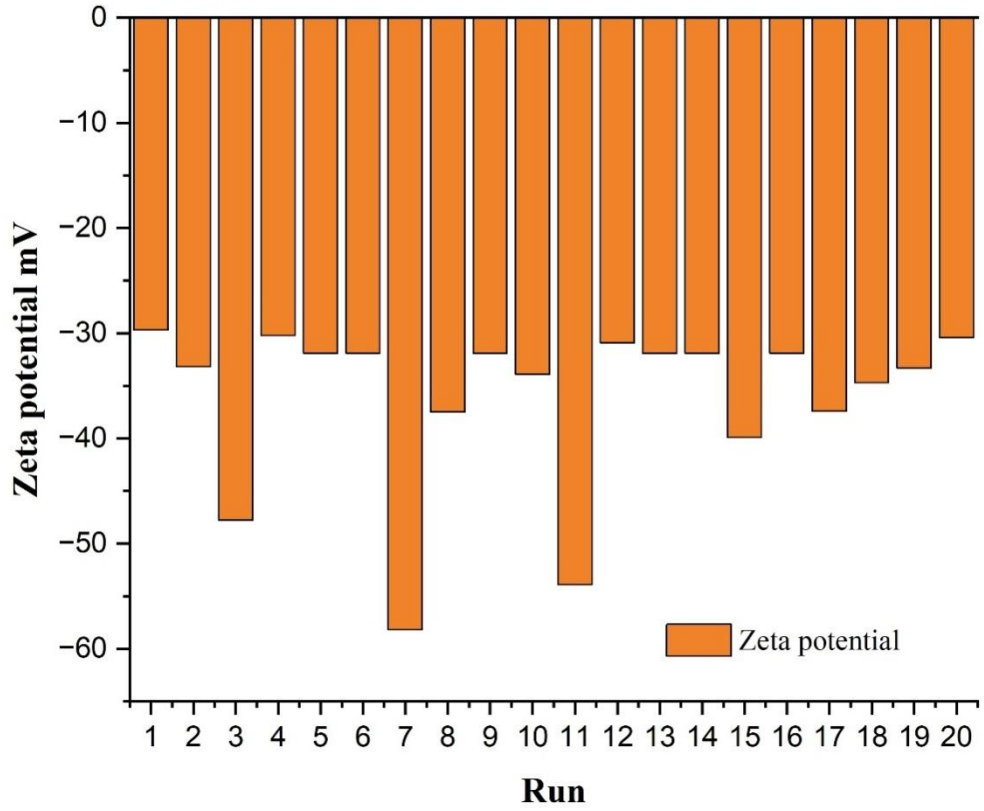
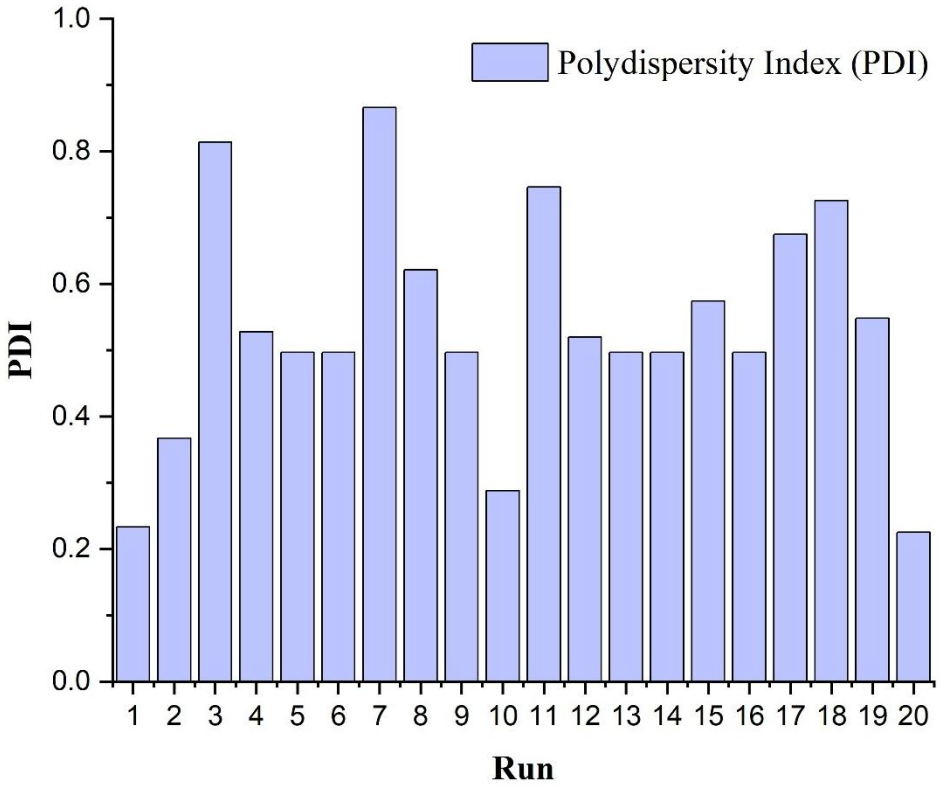


Figure 5.8.

The minimum PDI was observed as 0.16 (run 10) and maximum was found to be 0.51 (run 13) (Fig 5.9). PDI give the description about distribution pattern of the particles. As in case of run 13, where wider PDI was observed along with larger average particle size is observed, faster sedimentation is expected owing to instability. Whereas in the case of run 10, PDI shows proximity of average particle diameter to its median diameter. Hence, it indicates better chances of stability.

Fig.5.9 Bar graph of PDI vs various formulations

Statistical evaluation of the model was conducted using Design of Experiments (DoE) tools, including fit summary, sequential model sum of squares, lack-of-fit tests, and model summary statistics, as presented in Table 5.15. The selection of quadratic models for all responses was supported by a highly significant prob > F value ($P < 0.0001$), elevated R-squared values, low standard deviations, and reduced predicted residual error sum of squares (PRESS). Analysis of variance (ANOVA) was performed to assess the significance and



effect size of the main variables and their interactions, as detailed in Table 5.16. The F-values for the responses Y1, Y2, and Y3 were 5.69, 4.09, and 4.21, respectively, with corresponding P-values below 0.05, confirming the statistical significance of the models.

The adequacy of the model was confirmed by adequate precision values of 8.905, 7.174, and 7.404 for the respective responses, all surpassing the threshold value of 4. This indicates a strong S/N ratio suitable for exploring the design space. Additionally, the difference between adjusted R^2 and predicted R^2 remained below 2 across all responses, demonstrating model consistency. The model's statistical significance was further validated by a p-value less than 0.05, supporting the appropriateness of the quadratic model.

Table 5.15 Fit summary and Anova for globule size (Y_1), Zeta potential (Y_2), PDI (Y_3)

Fit summary			
Source	Sequential p-value	Adjusted R^2	Predicted R^2

	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
Linear	0.0280	0.3322	0.0205	0.3165	0.0346	0.3444	-0.0379	-0.4403	-0.0116
2FI	0.0143	0.0531	0.0833	0.6167	0.3284	0.5082	-0.4442	-0.4077	-0.4182
Quadratic	0.1759	0.0460	0.1725	0.6894	0.5939	0.6032	-0.3660	-0.6840	-0.7500

Sequential Model Sum of Squares [Type I]									
Source	Sum of Squares			df			Mean Square		
	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
Mean vs Total	8.051E+06	26093.09	5.74	1	1	1	8.051E+06	26093.09	5.74
Linear vs Mean	2.388E+05	229.05	0.2609	3	3	3	79612.43	76.35	0.0870
2FI vs Linear	1.763E+05	432.74	0.1256	3	3	3	58766.16	144.25	0.0419
Quadratic vs 2FI	55576.79	300.94	0.0744	3	3	3	18525.60	100.31	0.0248
Cubic vs Quadratic	64856.25	231.18	0.0930	4	4	4	16214.06	57.79	0.0233
Residual	27116.73	30.50	0.0286	6	6	6	4519.45	5.08	0.0048
Total	8.614E+06	27317.50	6.32	20	20	20	4.307E+05	1365.88	0.3160

Sequential Model Sum of Squares [Type I]						
Source	F-value			p-value		
	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Mean vs Total						
Linear vs Mean	3.93	1.23	4.33	0.0280	0.3322	0.0205
2FI vs Linear	5.18	3.33	2.78	0.0143	0.0531	0.0833
Quadratic vs 2FI	2.01	3.83	2.04	0.1759	0.0460	0.1725
Cubic vs Quadratic	3.59	11.37	4.87	0.0798	0.0058	0.0430
Residual						
Total						

Lack of Fit Tests									
Source	Sum of Squares			df			Mean Square		
	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Linear	3.238E+05	995.36	0.3216	11	11	11	29440.75	90.49	0.0292
2FI	1.475E+05	562.62	0.1960	8	8	8	18443.72	70.33	0.0245
Quadratic	91972.98	261.68	0.1217	5	5	5	18394.60	52.34	0.0243
Cubic	27116.73	30.50	0.0286	1	1	1	27116.73	30.50	0.0286
Pure Error	0.0000	0.0000	0.0000	5	5	5	0.0000	0.0000	0.0000

Model Summary Statistics									
Source	Std. Dev.			R^2			Adjusted R^2		
	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3

Linear	142.27	7.89	0.1418	0.4245	0.1871	0.4479	0.3165	0.0346	0.3444
2FI	106.54	6.58	0.1228	0.7378	0.5405	0.6635	0.6167	0.3284	0.5082
Quadratic	95.90	5.12	0.1103	0.8365	0.7863	0.7912	0.6894	0.5939	0.6032
Cubic	67.23	2.25	0.0691	0.9518	0.9751	0.9508	0.8474	0.9211	0.8443

Model Summary Statistics						
Source	Predicted R^2			PRESS		
	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Linear	-0.0379	-0.4403	-0.0116	5.840E+05	1763.56	0.5893
2FI	-0.4442	-0.4077	-0.4182	8.126E+05	1723.55	0.8262
Quadratic	-0.3660	-0.6840	-0.7500	7.686E+05	2061.94	1.02
Cubic	-9.6231	-4.4910	-9.8381	5.977E+06	6723.28	6.31

Table 5.16. Summary of ANOVA of CCD for optimized formulation

Responses	**Regression Parameters**		**Observed P-value***
	R^2	F_{cal}	
Globule Size	0.8365	5.69	0.0060
Zeta Potential	0.7863	4.09	0.0193
PDI	0.7912	4.21	0.0175

*Indicates statistical significance at $P<0.05$.

Using DOE software, the finalized mathematical models for responses Y1 through Y3 were developed based on coded variables, as detailed below. Within these models, positive coefficients represent synergistic interactions, whereas negative coefficients indicate antagonistic interactions.

Y_1 (Globule Size) = +705.78 + 98.98 A +33.29 B + 81.13 C -94.31 AB -93.21 AC

+66.74 BC -37.23 A^2 -14.42 B^2 -52.75 C^2

The positive coefficient of all factors individually indicates the increased influence of their amount on globule size. Whereas the negative coefficient with a high value indicates the mutual interaction of oil surfactant and oil and cosurfactant. This is also represented in the perturbation plot.

Y2 (Zeta potential) = -32.00 -1.29 A -1.89 B -3.40 C +2.70 AB +4.00 AC -5.55 BC -4.23 A^2 +0.1748 B^2 -1.98 C^2

The observed negative effect of increasing oil, surfactant, and cosurfactant concentrations indicates that reduced proportions of these components are associated with lower zeta potential values.

The magnitude of the effect can be further predicted through the value of the coefficient of cosurfactant, which indicates its prominent impact compared to the other two factors. This is represented as such in the perturbation plot. A strong self-interaction and a mutual interaction of oil with cosurfactant were predicted. The possibility of alteration of zeta potential seems primarily controlled by cosurfactants since their function is to impart stability to the globules by working at the globule interface.

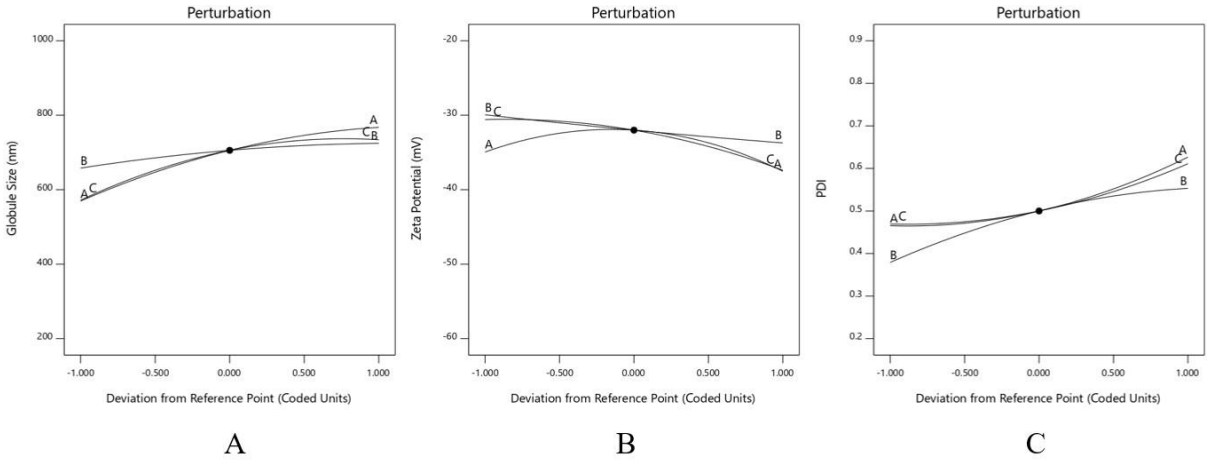
Y_3 (PDI) = =+0.5001 +0.0806 A +0.0871 B +0.0708 C-0.0359 AB -0.0834 AC +0.0864 BC +0.0460 A^2 -0.0339B^2 +0.0401 C^2

Similar mutual interaction of oil-surfactant and oil-cosurfactant was observed in PDI. Increase in all factors indicated rise in PDI. Self interactions of weak magnitude were observed with all the factors.

The perturbation plots were also plotted to understand the effect of factors on responses. The flat lines indicate no/minimal effect of a factor on responses, whereas deep curvature indicates a significant effect. As depicted in Fig 5.10 (perturbation plot), all three factors individually marginally impact all three responses. whereas a strong mutual interaction was seen between oil-surfactant and oil-cosurfactant ratios that alter globule size, zeta potential and PDI.

The polynomial equations obtained were employed to generate 2D contour and 3D response surface plots, revealing outcomes that aligned well with the model's predictions.

Fig 5.10 Perturbation plot for Globule size (A), zeta potential (B), PDI (C)



A B C

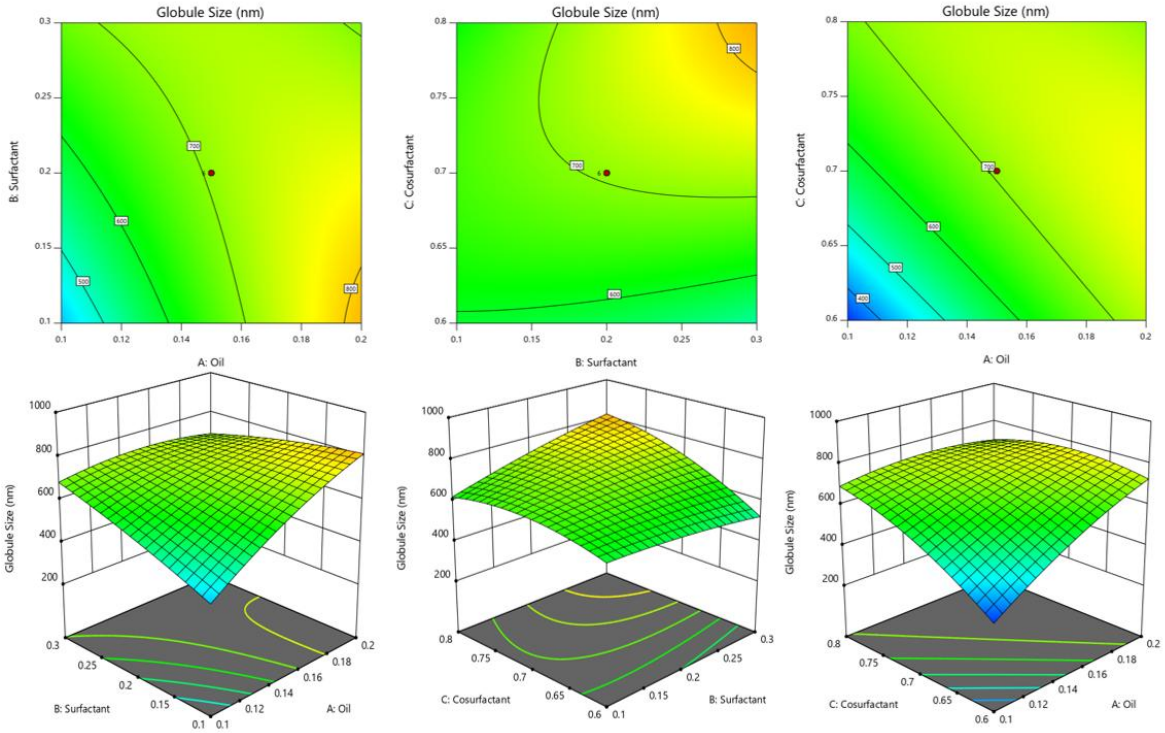


Fig. 5.11 2D contour plot (A) and 3D response surface plots (B) for globule size

The globule size is primarily influenced by the phospholipid content rather than the SDC, as illustrated in Fig. 5.11. At concentrations above 85 % of phospholipids, globule size is expected to decrease i.e. at higher phospholipids and at lower amount of edge activator. Interaction plot indicates both factors play their role in globules size determination with low

interaction of each other. Whereas, at soy lecithin concentration below 85 %, both factors show strong interplay of interactions to have impact on globule size.

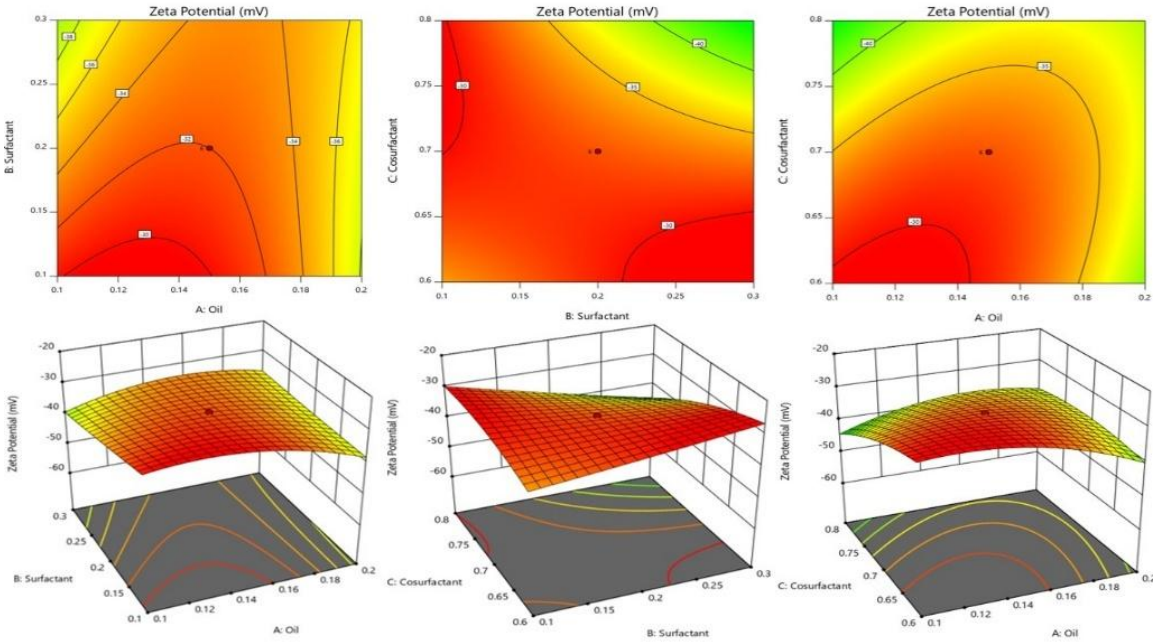


Fig. 5.12. 2D contour plot and 3D response surface plots for Zeta potential

For zeta potential, both factors play the role to impart stability to the globules. Therefore, both the factors are important to impart stability to dispersion Fig 5.12.

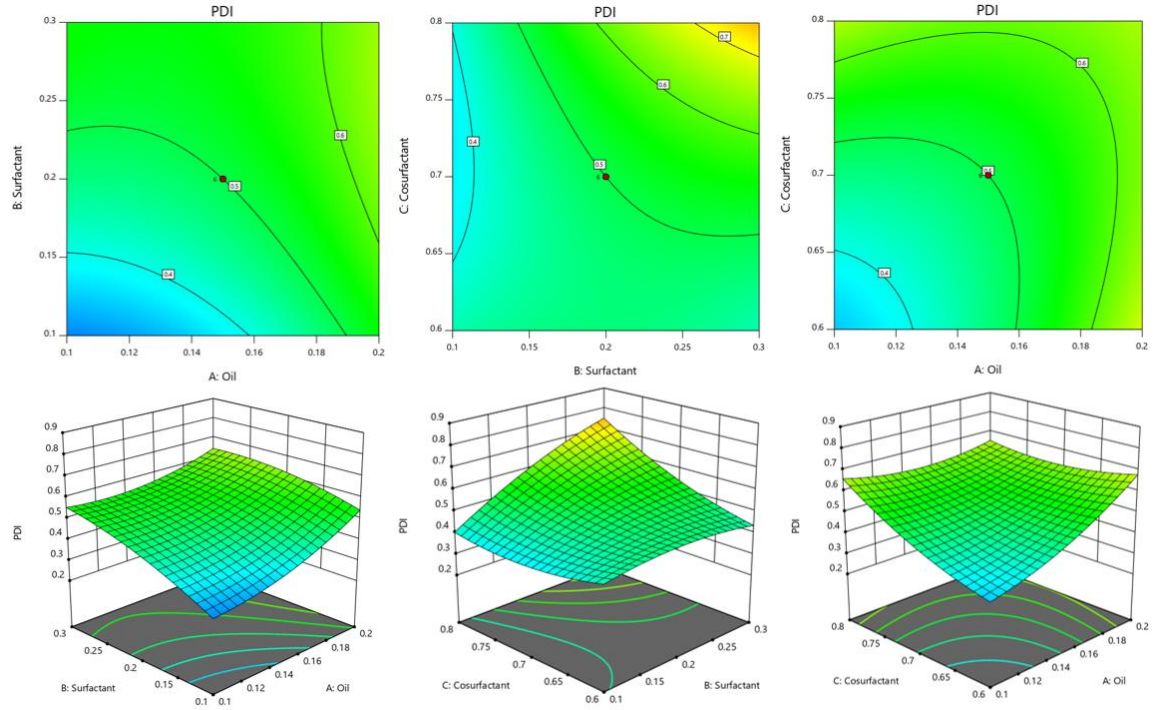


Fig.5.13 2D contour plot and 3D response surface plots for PDI

Like globule size, the impact of both the factors is seen on PDI Fig 5.13. The effect is more

significant when the amount of phospholipids and edge activator is increased beyond 85 mg and 15 mg respectively. Although the impact is less significant, it is similar when the concentration of both factors decreases linearly.

5.6.4.2. Optimized formulation and validation studies

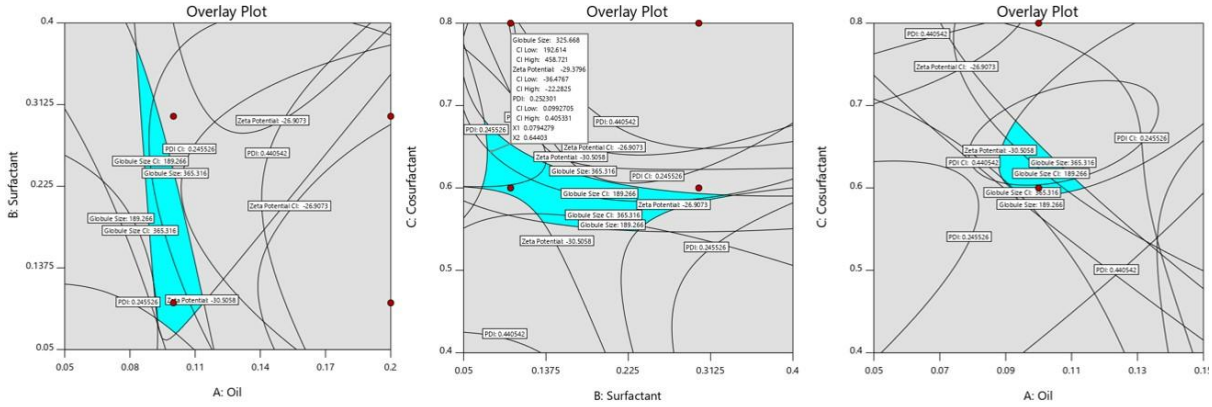


Fig. 5.14. Overlay plot indicating the location of optimized flexi-liposomal formulation

The optimized overlay plot was identified using DoE software Fig 5.14. The CCD model predicted particle size as 325.668 nm, zeta potential -29.37 mV, and PDI as 0.252. Applying a graphical optimization model, the best fit optimum formulation was selected, containing oil: surfactant: cosurfactant in the ratio of 0.1:0.1:0.8. The optimized formulation is depicted within the defined optimal region of the overlay plot. A detailed comparison of the experimental and predicted responses, along with their percentage errors, is provided in Table 5.17. The percentage errors ranged from % to %, all remaining within the acceptable limit of ±10%. This close agreement between predicted and experimental results underscores the high predictive accuracy of the experimental design used for SNEDDS optimization.

5.6.4.3 Evaluation of globule size, zeta potential and PDI

Table 5.17. Validation of experimental results with predicted values and percentage error

Response	Predicted value	Average Experimental value	% Error
Globule size (nm)	325.668	329.5	1.17%
Zeta potential (mV)	-29.3796	-29.7	1.12%
PDI	0.252301	0.233	-7.64%

5.7 Drug release

Table 5.18. (%) of drug release of SNEEDS and Extract

Time	% Drug release of SNEDDS	% Drug release of Extract
0	0	0
5	31.16	13.82
15	39.29	19.08
30	51.55	26.17
60	57.01	34.05
90	65.58	40.45
120	78.58	45.48
150	85.08	49.37
180	90.25	58.4
210	96.89	62.28

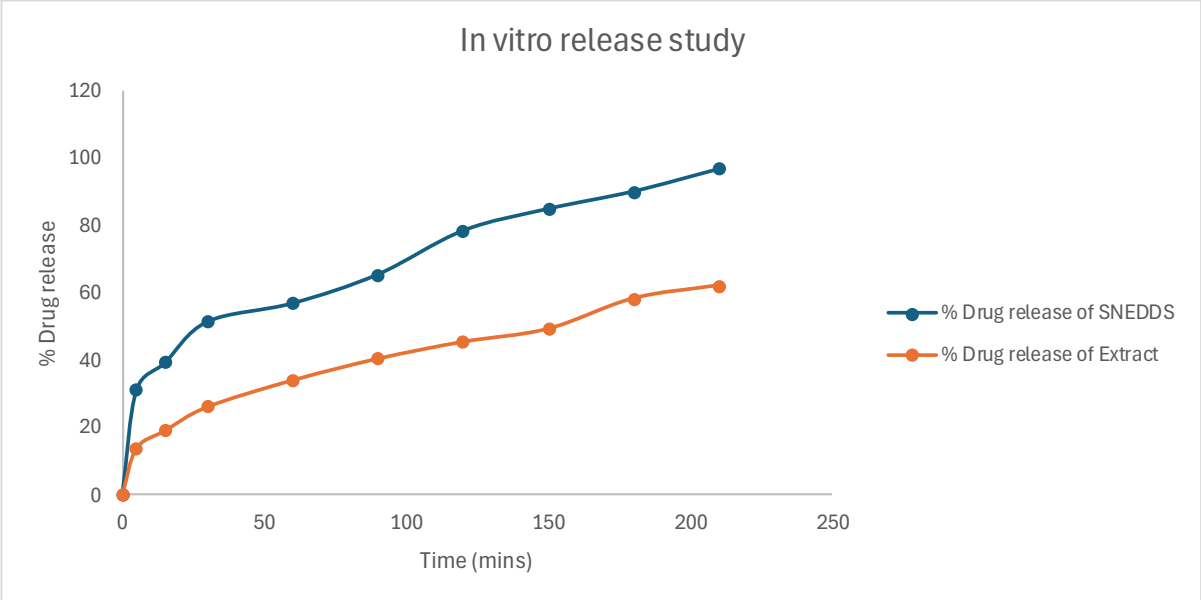


Fig. 5.15 Maximum amount of drug release

The collected samples were filtered and analyzed for drug concentration at 207 nm using a UV-visible spectrophotometer, with appropriate blanks used for comparison. Maximum amount of drug release is calculated as 96.89% for SNEDDS and 62.28% for Extract.

5.8 Characterization of SNEEDS

Various characterization techniques were employed to evaluate the SNEDDS, such as organoleptic assessment, determination of entrapment efficiency, particle size analysis, zeta potential measurement, and scanning electron microscopy (SEM). The outcomes of these

tests are detailed below.

5.8.1 Entrapment efficiency

The absorbance of supernatant liquid after centrifugation of SNEDDS formulation was observed 0.277. The unentrapped drug concentration was calculated i.e.24.31%, using standard calibration curve or with the help of linear equation (Y= 0.0108x + 0.01449). Finally, percentage Entrapment of *T. arjuna* SNEDDS was found to be 60.66% using the following formula.

$$EE = \frac{Amountofentrapped\ drug}{Amount\ of\ total\ drug} x100$$

5.8.2 PDI & Globule size Analysis

Within Self-Nanoemulsifying Drug Delivery Systems (SNEDDS), the polydispersity index (PDI) serves as an indicator of the homogeneity and consistency of particle size distribution in the nanoemulsion. PDI values range from 0 to 1, where a lower value (typically below 0.3) indicates a more homogeneous droplet size distribution, which is desirable for stability and consistency in drug delivery. In SNEDDS, a low polydispersity index (PDI) signifies a homogenous droplet size distribution, which contributes to improved stability by reducing the risk of droplet aggregation and phase separation. In contrast, a high PDI denotes greater heterogeneity in droplet sizes, which can negatively impact the formulation's stability and therapeutic performance.

Table 5.19. Results of PDI & Globole size analysis

Results			**Size (d. nm)**	**% intensity**	**St Dev (d. nm)**
Globule size (d. nm)	329.5	Peak 1	350.7	96.5	138.1
PdI	0.233	Peak 2	5030	3.3	592.4
Intercept	0.941	Peak 3	74.25	0.2	5.746

Globule size analysis of the *T. arjuna* self-nanoemulsifying drug delivery system (SNEDDS) was conducted using a Particle Size Analyzer. The results indicated an average globule diameter of 329.5 nm with a polydispersity index (PDI) of 0.233. Since the PDI value falls within the acceptable range of 0.1 to 0.5, it confirms the formulation's homogeneity and uniform droplet size distribution. These results are illustrated in Fig. 5.16.

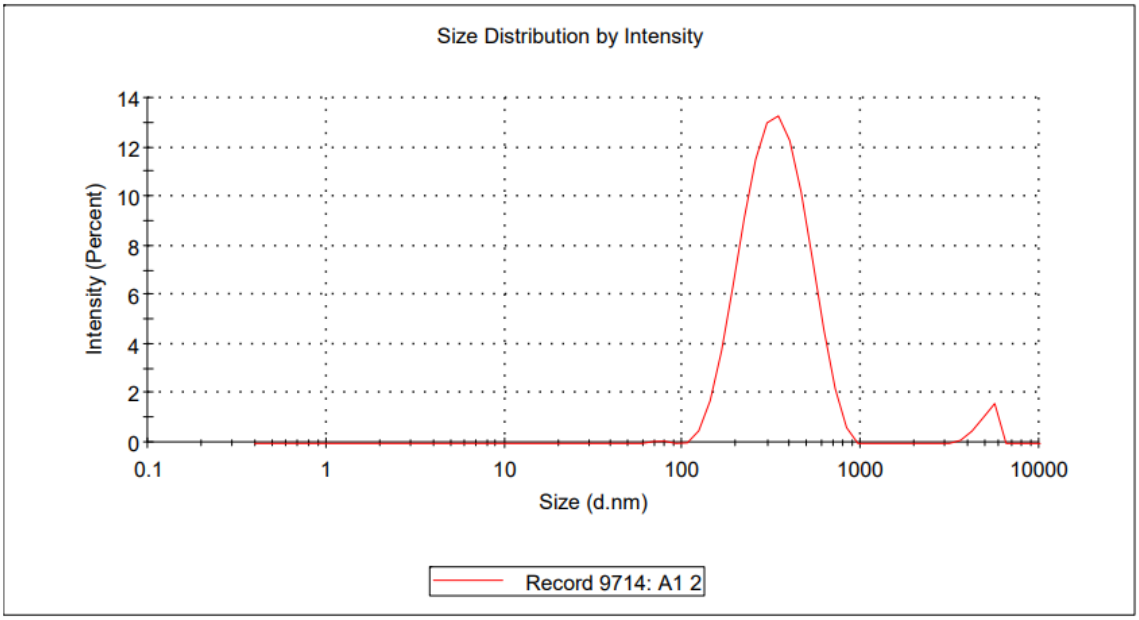


Fig.5.16. PDI & Globule size graph

5.8.3 Zeta Potential Analysis

In Self-Nanoemulsifying Drug Delivery Systems (SNEDDS), zeta potential serves as a key indicator of nano-emulsion stability. It represents the surface charge of the oil droplets within the formulation, influencing their electrostatic repulsion and thereby preventing droplet aggregation. A high absolute zeta potential value (either positive or negative) usually signifies a stable SNEDDS, where the droplets remain dispersed, ensuring efficient drug delivery. Conversely, a low zeta potential may result in droplet coalescence, diminishing the stability of the nano-emulsion and potentially impairing the efficacy of the drug delivery system. Zeta potential analysis was performed to determine the stability of the SNEDDS formulation, as adequate surface charge helps prevent particle aggregation and contributes to long-term colloidal stability. [100].

Table 5.20. Results of Zeta potential analysis

Results			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV)	-29.7	Peak 1	-29.7	100	8.2442
Zeta Deviation (mV)	8.24	Peak 2	0.00	0.0	0.00

Conductivity (mS/cm)	0.0722	Peak 3	0.00	0.0	0.00

The zeta potential of the *T. arjuna* self-nanoemulsifying drug delivery system (SNEDDS) was measured using a Particle Size Analyzer and found to be -29.7 mV. This value reflects the steric stabilization imparted by the surfactant and co-surfactant components within the formulation. The results are presented in Fig. 5.17.

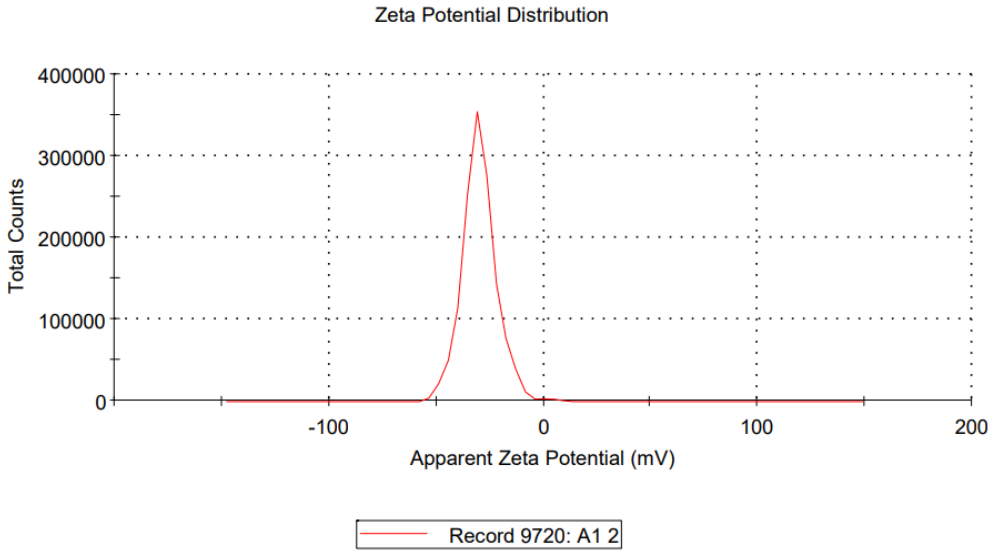


Fig.5.17. Showing zeta potential distribution of *T.arjuna* SNEEDS

5.9 Scanning electron microscopy (SEM)

Field Emission Scanning Electron Microscopy (FESEM) serves as an essential analytical tool in the evaluation of SNEDDS, offering high-resolution imaging to assess the surface morphology and structural features of nanoemulsion droplets. This technique enables precise observation of droplet shape, size, and distribution at the nanoscale, thereby confirming the homogeneity and uniform dispersion of the formulation. SEM analysis demonstrated a smooth and uniform surface morphology without visible drug crystals. This indicated successful adsorption and molecular dispersion of the formulation within the carrier matrix. [101]. This detailed analysis helps ensure that the SNEDDS has the desired nanoscale features for effective drug delivery, as droplet size and morphology can directly impact the formulation's stability, bioavailability, and therapeutic efficiency. The Scanning electron microscopic (SEM) scanning of the prepared SNEDDS of *T. arjuna* was done at Lovely Professional University, CIF, Phagwara. The following scanned images were obtained. Scanning electron microscopy Fig. 5.18 of SNEEDS loaded with extract of *T.arjuna* at 200000x. the size of the particle was measured in the range of 81.1-164nm and the average size of the particles were 117.57nm

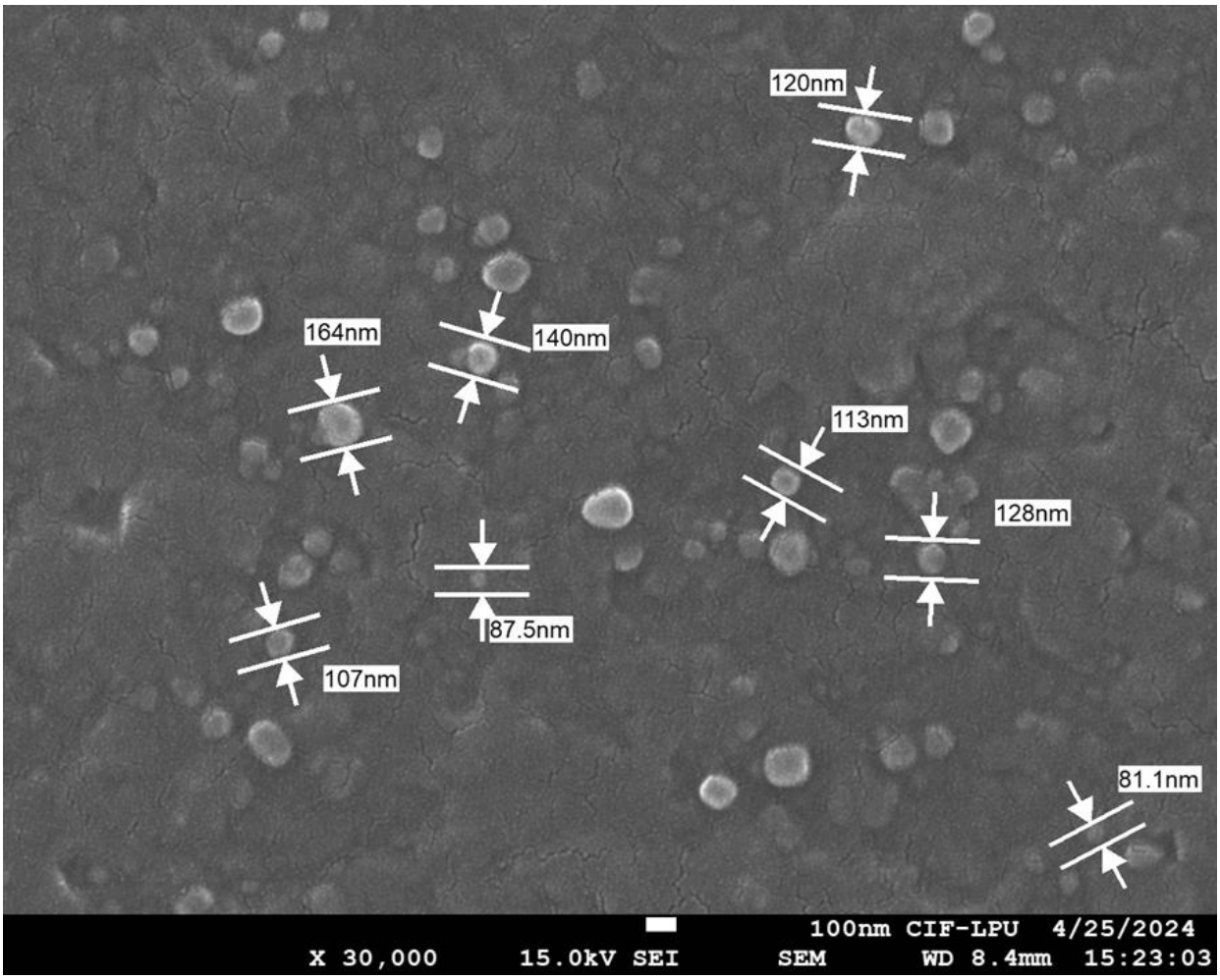


Fig.5.18. Scanning Electron microscopy showing particle size of SNEEDS

5.10 Accelerated stability study:

Table 5.21. Results of Accelerated stability study

Accelerated Stability Study (temp:40± 2^{O}c,75 ±5% RH)				
Sr. No.	**Parameters**	**0 Month**	**3rd Month**	**6th Month**
Organoleptic analysis				
1	Description	Brown coloured Transparent liquid	Brown coloured Transparent liquid	Brown coloured Transparent liquid
2	Odour	Characteristic	Characteristic	Characteristic
Physico-chemical analysis				
1	Specific Gravity	1.000	0.990	0.990
2	pH (Direct)	9.04	8.95	8.71
3	Viscosity by	0.79cP	0.75 cP	0.71cP

	Ostwald			
Instrumental analysis				
1	%Quercetin by HPTLC	0.00007%	Not Applicable	0.00005%
2	%Gallic acid by HPTLC	0.0013%		0.0012%
3	%Arjunolic acid by HPTLC	0.00024%		0.00020%
Microbiological analysis				
1	Total Microbial PlateCount	290 cfu/g	Not Applicable	137 cfu/g
2	Total Yeast &MouldCount	Absent		Absent
3	*Staphylococcus aureus*	Absent		Absent
4	*Salmonella sp.*	Absent		Absent
5	*Pseudomonas aeruginosa*	Absent		Absent
6	*Escherichia coli*	Absent		Absent
Kew-word: API–Ayurvedic Pharmacopoeia of India; %- Percentage w/w, ppm-Parts per millions, NA–Not applicable, ND-Not detected, cfu/g– Colony forming unit per gram				

Table 5.22 Globule size, Zeta potential, and Entrapment efficiency.

Parameter	**0 Months**	**6 Months**	**p value**
Globule size (nm)	330.0 ± 2.15	332 ± 3.08	0.4086
PDI	0.233 ± 1.69	0.255 ± 2.46	0.9904
Zeta Potential (mV)	-29.7 ± 1.03	-26.0 ± 2.91	0.1065

5.11 HPTLC study

5.11.1 Gallic acid

Table 5.22. Chromatographic Conditions of gallic acid

Mode of Application	CAMAG Linomat 5 – Applicator
Filtration System	Whatman filter paper No. 1
Stationary Phase	MERCK – TLC / HPTLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from the base of the plate
Band length	8mm
Standard Volume for application	15 µL
Sample Volume for application	30 µL
Distance Between Tracks	20 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Saturation Time of Chamber	30 min
Mobile Phase	Toluene: Ethly Acetate: Formic Acid (5: 5: 1 v/v)
Visualization	254 nm
Quantification	254 nm

Table 5.23. % of Gallic acid present in samples

Parameters	**Gallic acid**	**SNEDDS**	***T. arjuna* Bark extract**
Weight	**2.2 mg**	1118 mg	104.5 mg
Rt	**0.50**	**0.50**	**0.50**
AUC	**43178**	236.0	252.0
%Gallic acid	**-**	0.013%	0.090%

Table 5.24. R_f Value of Gallic acid

Track 1	**Track 2**	**Track 3**
--	**--**	**0.27**
0.50	**0.50**	**0.50**

--	--	0.62
72	72	--
--	77	0.76
79	--	--
--	--	0.88

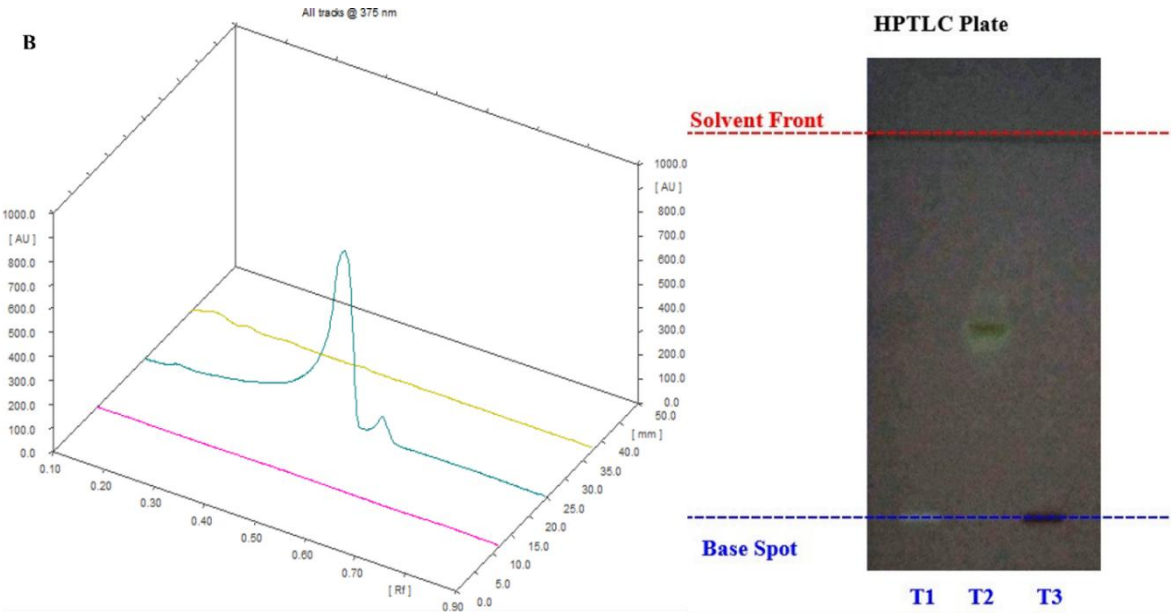
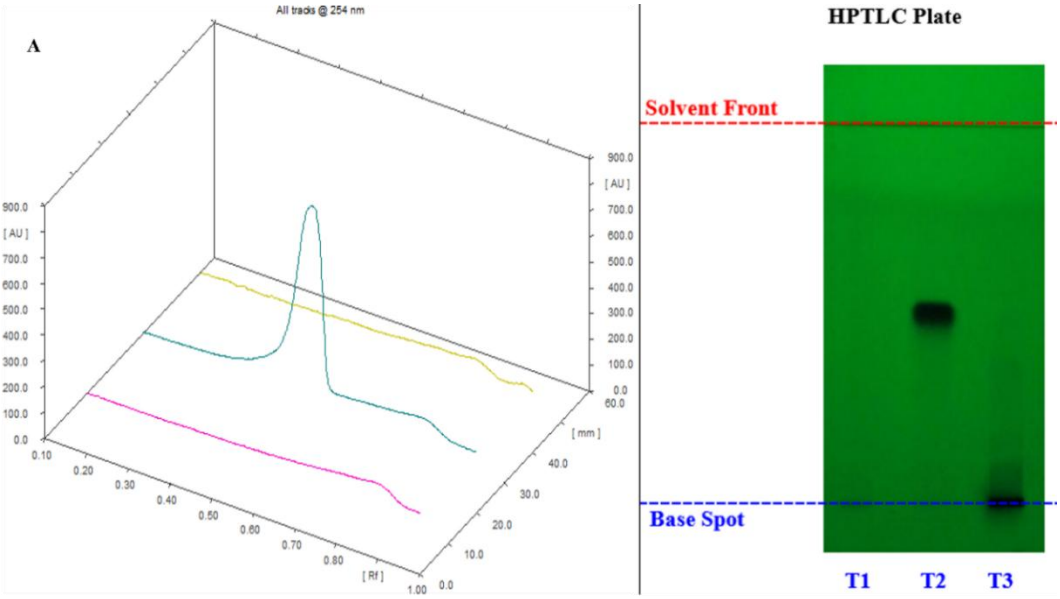


Fig.5.19 3D Chromatogram and HPTLC plate of (A) Gallic acid (B)Quercetin



5.11.2 Quercetin

Table 5.25. Chromatographic Conditions of gallic acid

Mode of Application	CAMAG Linomat 5 – Applicator
Filtration System	Whatman filter paper No. 1
Stationary Phase	MERCK – TLC / HPTLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from the base of the plate
Band length	8mm
Standard Volume for application	10 µL
Sample Volume for application	30 µL
Distance Between Tracks	20 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Saturation Time of Chamber	30 min
Mobile Phase	Toluene: Ethly Acetate: Formic Acid (6: 4: 0.5 v/v)

Visualization	@375 nm
Quantification	@375 nm

Table 5.26. % of quercetin

Parameters	Quercetin	SNEDDS	*T. arjuna* Bark extract
Weight	2.1 mg	1118 mg	104.5 mg
Rt	0.50	0.50	0.50
AUC	26.0	236.0	252.0
%Quercetin	-	0.0007%	0.042%

Table 5.27. R_f Value of Quercetin

Track 1	Track 2	Track 3
--	**--**	**0.27**
0.50	**0.50**	**0.50**
--	**--**	**0.62**
72	**72**	**--**
--	**77**	**0.76**
79	**--**	**--**
--	**--**	**0.88**

5.11.3 Arjunolic acid

Table 5.28. Chromatographic Conditions of Arjunolic acid

Mode of Application	CAMAG Linomat 5 – Applicator
Filtration System	Whatman filter paper No. 1
Stationary Phase	MERCK – TLC / HPTLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from the base of the plate
Band length	8mm
Standard Volume for application	5 µL

Sample Volume for application	30 µL
Distance Between Tracks	20 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Saturation Time of Chamber	30 min
Mobile Phase	Chloroform: Methanol (8.5: 1.5 v/v)
Visualization	@540 nm
Quantification	@540 nm

Table 5.29. % of Arjunolic acid present in samples

Parameters	**Arjunolic acid**	**SNEDDS**	***T. arjuna* Bark extract**
Weight	1.0 mg	1118 mg	104.5 mg
Rt	0.55	0.55	0.55
AUC	7316.2	26.6	454.7
%Arjunolic acid	-	0.0024%	0.268%

Table 5.30. R_f Value of Arjunolic acid

Track 1	**Track 2**	**Track 3**
--	**--**	**0.27**
0.50	**0.50**	**0.50**
--	**--**	**0.62**
72	**72**	**--**
--	**77**	**0.76**
79	**--**	**--**
--	**--**	**0.88**

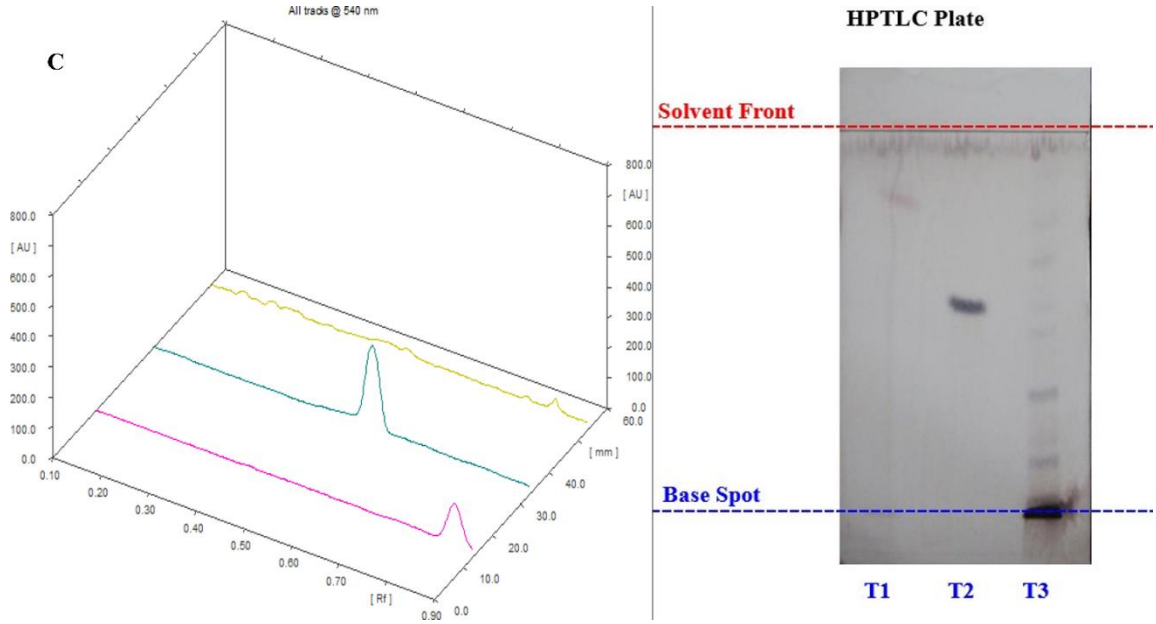


Fig.5.20. Chromatogram and HPTLC plate of Arjunolic acid

5.12 Animal Study

Physical parameters: The evaluation of body weight and blood pressure yielded the following results, which are systematically presented below for comprehensive analysis.

5.12.1 Body weight

The statistical data represented in the graph shows that no group shows the significant effect of treatment on rats. Throughout the treatment period, from Day 0 to Day 18, the body weight of all animals remained relatively stable. No significant changes were observed, indicating that the treatment did not adversely affect the animals. A slight increase in body weight suggests normal growth and overall good health, thereby confirming the absence of any toxic effects from the administered formulation.

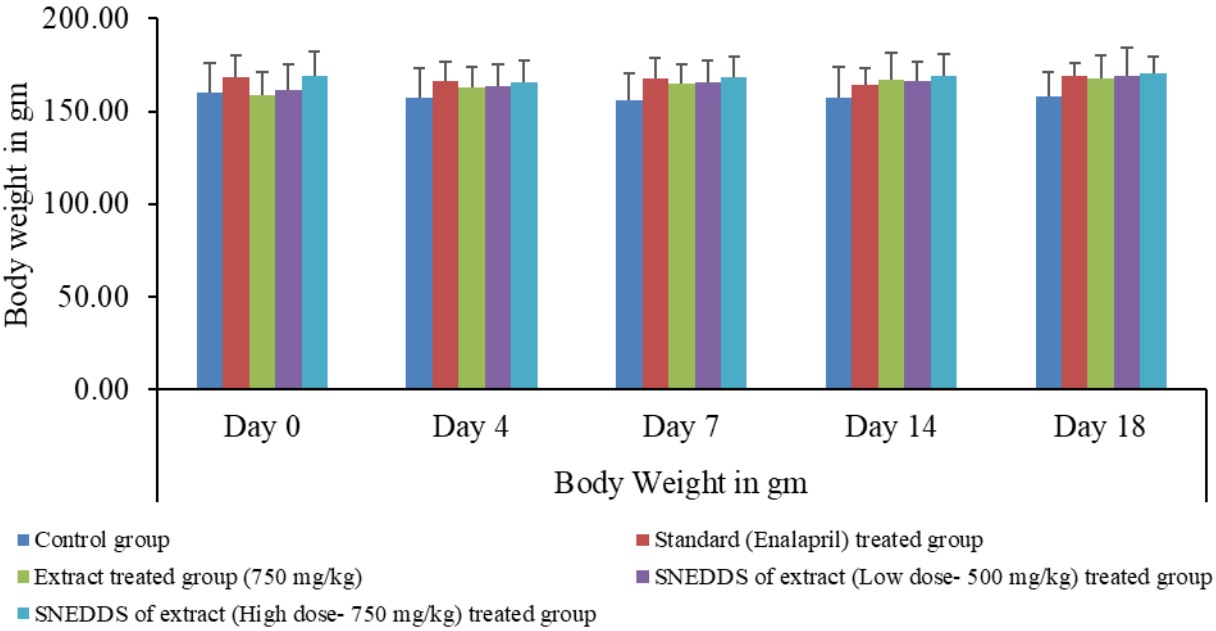


Fig.5.21. Effect of different treatments on body weight of rats: Data represented by means ±SEM.

5.12.2 Systolic blood pressure Assessment:

*,**,*** denotes p<0.05, p<0.01, p<0.001 statistically significant difference as compared to control group. On day 14th statistically significant difference was observed in standard (Enalapril) treated group and SNEDDS of extract (Hight dose- 750 mg/kg) treated group and no change in other 2 groups. Then on 18th day significant difference in all the groups were observed as compared to control group, standard (Enalapril) treated group and SNEDDS of extract (Hight dose- 750 mg/kg) treated group shows more significant results and extract treated group and SNEDDS of extract (low dose- 500 mg/kg) treated group shows slightly less effect compared to other two groups. The result shows that even on high dose the test drug does not show any harmful effects, hence the test drug is safe and effective.

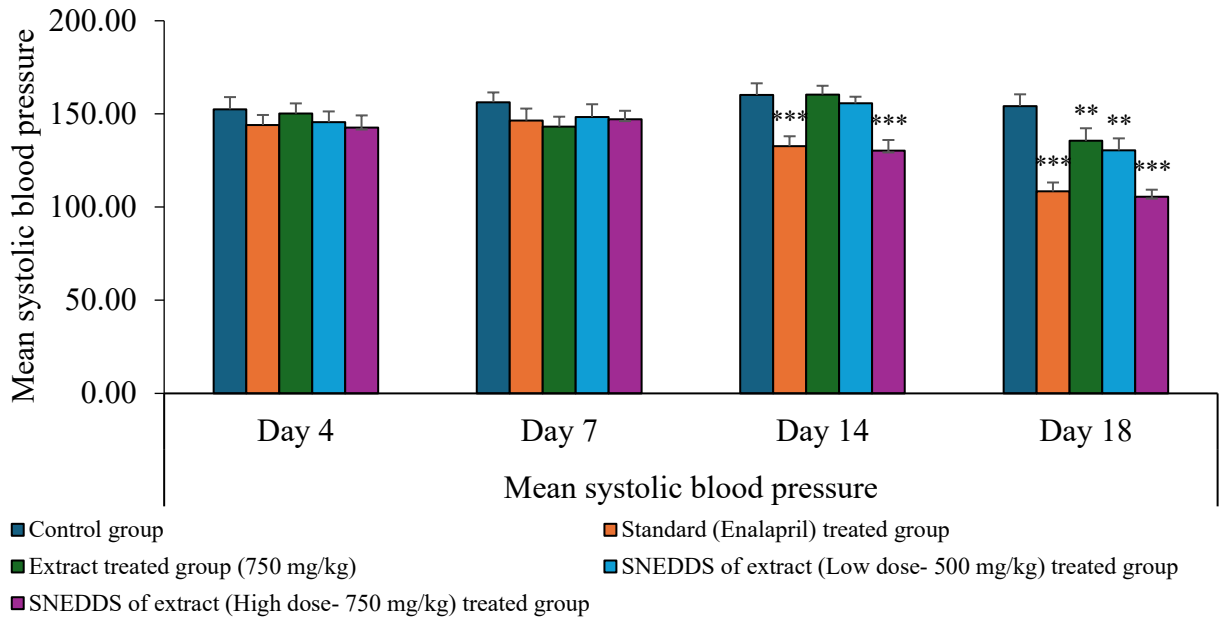


Fig.5.22. Effect of different treatments on Systolic Blood Pressure of Wistar rats after the induction of hypertension: Data represented by means ± SEM. Statistical significance level was considered as p<0.05(*), p< 0.01(**) and p<0.001 (***)

CHAPTER 6
SUMMARY
&
CONCLUSION

Chapter 6: Summary and conclusion

The development and assessment of a Self-Nanoemulsifying Drug Delivery System (SNEDDS) containing the stem bark extract of *T. arjuna* demonstrated significant potential for improving the therapeutic efficacy of this well-established cardioprotective agent. The formulation process commenced with the standardization of the extract using Thin Layer Chromatography (TLC), which verified the presence of key bioactive phytoconstituents and ensured the consistency and quality of the extract employed.

The formulation of SNEDDS was systematically optimized and evaluated based on key physicochemical attributes, including an average globule size of 329.5 nm, a polydispersity index (PDI) of 0.233, and a zeta potential of -29.7 mV. These parameters collectively confirm the development of a stable and homogenous nanoemulsion with efficient dispersion behavior. Furthermore, in vitro drug release studies demonstrated the formulation's ability to achieve controlled and sustained release, which is expected to enhance the bioavailability of the therapeutic phytoconstituents present in the *T. arjuna* extract.

The accelerated stability studies revealed that the formulation preserved its physical and chemical stability when subjected to stress conditions for six months, suggesting a robust shelf-life potential for the SNEDDS. The samples were checked for globule size, zeta potential, and entrapment efficiency. Since the p-values for both samples, i.e., at 0 months and 6 months, are greater than 0.05. This shows that the two formulations are statistically similar when stored under stability conditions for 6 months. Furthermore, High-Performance Thin-Layer Chromatography (HPTLC) analysis confirmed the sustained presence of essential phytoconstituents post-formulation, thereby validating the chemical integrity of the *T. arjuna* extract throughout the preparation process.

In vivo studies carried out on animal models provided strong evidence of the formulation’s antihypertensive efficacy. Marked reductions in blood pressure were observed in the treated groups, particularly at higher dose levels, demonstrating efficacy comparable to standard antihypertensive agents. Importantly, the body weight of treated animals remained stable throughout the study, indicating that the formulation did not produce any harmful or systemic adverse effects even during prolonged administration.

The study results indicate that all groups showed a normal and comparable increase in

body weight (about 8–12%) over 18 days, suggesting no signs of toxicity. In contrast, a significant reduction in systolic blood pressure was observed, particularly in the SNEDDS high-dose group (approx. 26%) and the standard Enalapril group (approx. 25%), indicating strong antihypertensive activity. The extract and SNEDDS low-dose groups showed moderate reductions (approx. 19% and 16%, respectively), while the control group showed minimal change (approx. 6%). These findings demonstrate that the SNEDDS formulation exhibits a dose-dependent antihypertensive effect comparable to the standard drug.

These results highlight the dose-responsive nature of the antihypertensive effect and reinforce the safety and tolerability of the SNEDDS formulation. Altogether, the findings support the effectiveness of *T. arjuna* SNEDDS in improving the therapeutic profile of the extract while maintaining a high safety margin. Incorporation of a polymeric amorphous system improved the physical stability of SNEDDS and reduced drug precipitation. The formulation maintained sustained dissolution characteristics during storage conditions. [102].

In summary, the developed SNEDDS based on *T. arjuna* stem bark extract offers a promising approach for antihypertensive therapy. It enhances solubility, ensures controlled release, provides long-term stability, and demonstrates notable in vivo efficacy and safety. The optimized *T. arjun*a SNEDDS developed using a QbD approach exhibited improved dissolution characteristics and enhanced oral bioavailability. The study demonstrated that systematic optimization of formulation variables significantly influenced SNEDDS performance. SNEDDS significantly enhanced the solubilization and oral absorption of *T. arjuna* through nano-sized droplet formation. The optimized formulation showed approximately a two-fold increase in bioavailability compared with standard drug. [103]. These encouraging outcomes lay the foundation for future clinical studies and the potential development of a novel, plant-based nanoformulation for cardiovascular care.

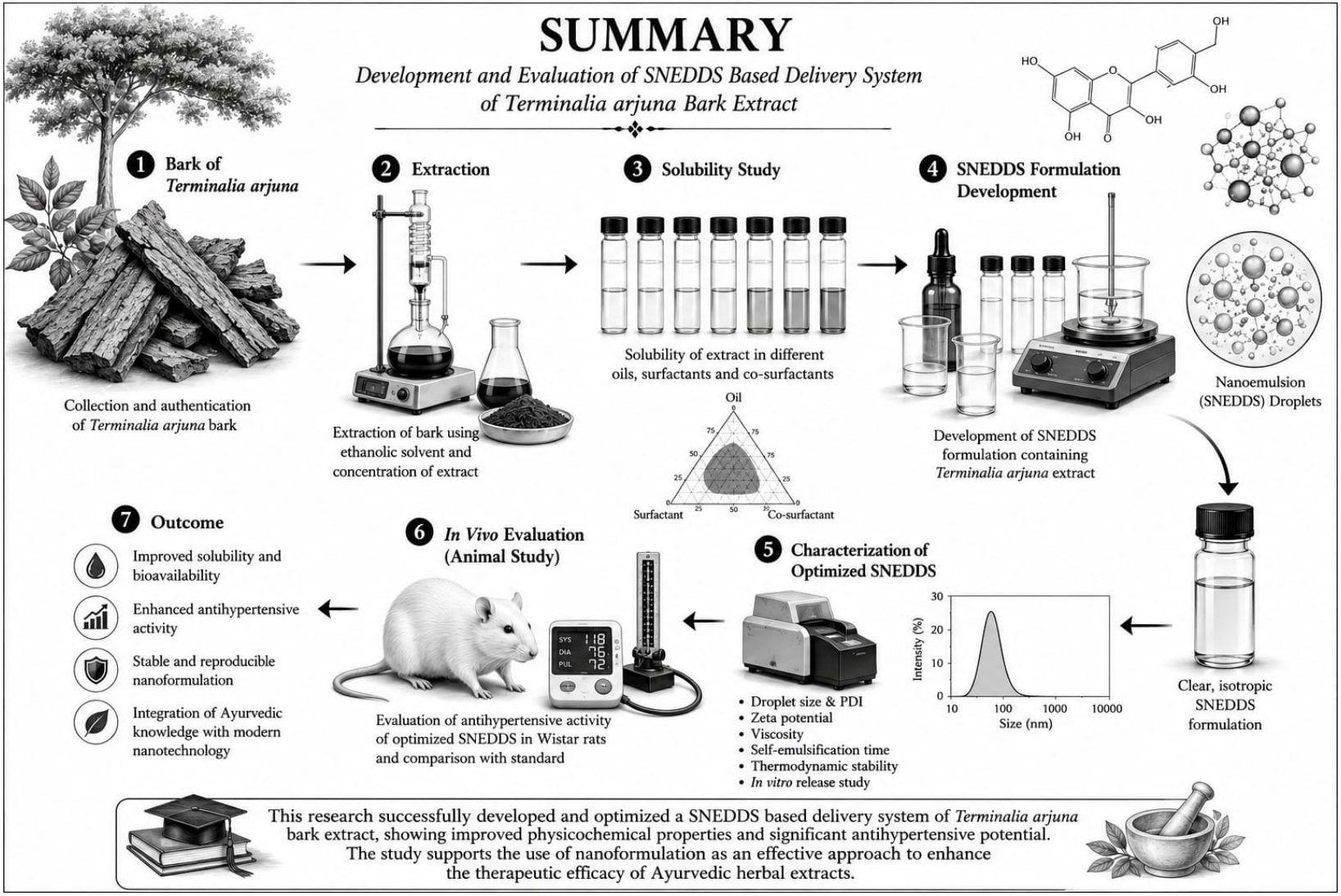


Fig: 6.1 Schematic Representation of the Overall Research Workflow

CHAPTER 7

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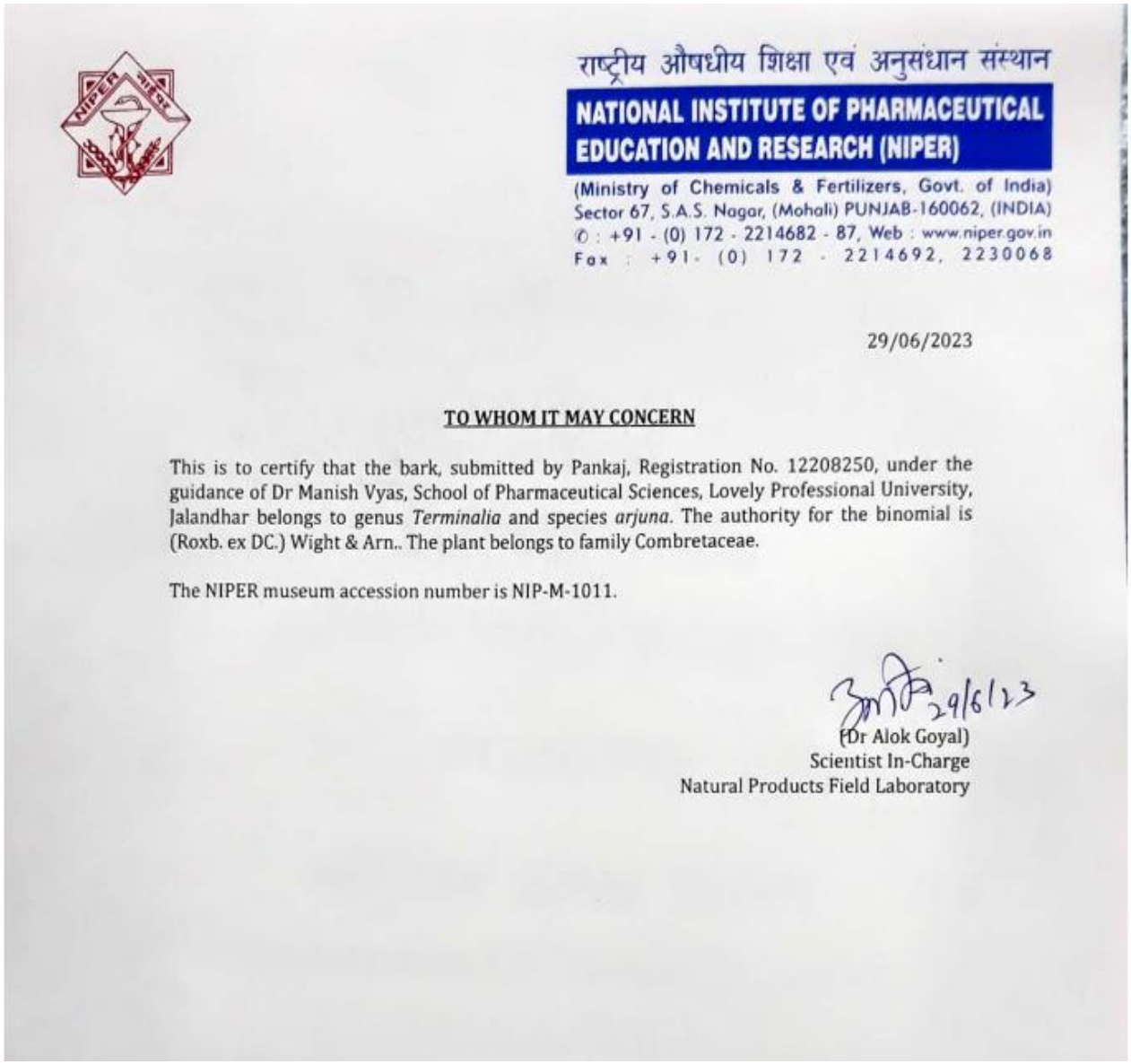
Appendix 1



राष्ट्रीय औषधीय शिक्षा एवं अनुसंधान संस्थान

NATIONAL INSTITUTE OF PHARMACEUTICAL EDUCATION AND RESEARCH (NIPER)

(Ministry of Chemicals & Fertilizers, Govt. of India)
Sector 67, S.A.S. Nagar, (Mohali) PUNJAB-160062, (INDIA)
✆ : +91 - (0) 172 - 2214682 - 87, Web : www.niper.gov.in
Fax : +91- (0) 172 - 2214692, 2230068

29/06/2023

TO WHOM IT MAY CONCERN

This is to certify that the bark, submitted by Pankaj, Registration No. 12208250, under the guidance of Dr Manish Vyas, School of Pharmaceutical Sciences, Lovely Professional University, Jalandhar belongs to genus *Terminalia* and species *arjuna*. The authority for the binomial is (Roxb. ex DC.) Wight & Arn.. The plant belongs to family Combretaceae.

The NIPER museum accession number is NIP-M-1011.

29/6/23

(Dr Alok Goyal)
Scientist In-Charge
Natural Products Field Laboratory

Appendix 2

HERBHEAL
CARE BY NATURE

CIN No.- U24233PB2008PTC032243

ISO 9001

Herbal Health Research Consortium Pvt. Ltd.

(A GOVT. OF INDIA SPONSORED & PB. GOVT. ASSISTED AYUSH CLUSTER PROJECT)

Works at : Village Khyala Khurd, Ram Tirth Road, Amritsar
Ph.: 01858-262025, Fax.: 01858- 262026 E-mail: herbheal@ymail.com
Corrs. Add: 277, East Mohan Nagar, Amritsar - 143006

Government Approved Testing Laboratory
Lic no. 1/ASU/TESTING LAB./PB./2014 **Dated:10/07/2023**

CERTIFICATE OF ANALYSIS

(The Drugs and Cosmetics Act 1940 and the Rules Thereunder)

Sample	**Extract Samples**	**A.R. No.**	06/2023/PR/001
Supplied by	Pankaj (12208250) L.P.U	**Sample Quantity**	-
Mfd. by	-	**Ref date**	30/06/2023
Batch No.	-	**D/M**	-
Date of receipt	16/06/2023	**D/E**	-

Sr.No.	TESTS	RESULTS	SPECIFICATIONS
1.	**Microbial test**		
	a) Total Microbial Plate Count	170cfu/gm	1×10^5 cfu/gm
	b) Total Yeast and Moulds	Nil	1×10^3 cfu/gm
2.	**Pathogen Tests**		
	a) Escherichia coli	Absent	Should be Absent
	b) Staphylococcus aureus	Absent	Should be Absent
	c) Pseudomonas aeruginosa	Absent	Should be Absent
	d) Salmonella	Absent	Should be Absent
3.	**Test For Aflatoxins (by TLC)** (B1, B2 and G1,G2)	Absent Absent	B1 & G1- NMT 0.5 ppm B2 & G2- NMT 0.1 ppm
4.	**Heavy Metals**		
	a) Lead (as Pb)	Not Detected	NMT 10.0 ppm
	b) Cadmium (as Cd)	Not Detected	NMT 0.3 ppm
	c) Mercury (as Hg)	Not Detected	NMT 1.0 ppm
	d) Arsenic (as As)	Not Detected	NMT 3.0 ppm
5.	**Pesticide Residue**		
	a) **Alachor**	Not Detected	NMT 0.02 mg/kg
	b) **Aldrin&Dieldrin**	Not Detected	NMT 0.05 mg/kg
	c) **Chlordane**	Not Detected	NMT 0.05 mg/kg
	d) **DDT**	Not Detected	NMT 1.00 mg/kg
	e) **Endosulfan**	Not Detected	NMT 3.00 mg/kg
	f) **Malathion**	Not Detected	NMT 1.00 mg/kg

Remarks: The Product complies with prescribed standards of In-house Specifications

Prepared By 10/07/2023

Checked By 10/07/2023

Approved By 10/07/2023

"End of report"

Note:
1. Sample(s) not drawn by us unless otherwise stated.
2. Total liability of this laboratory is limited to the invoiced amount.
3. Test certificate in full or parts shall not be used for promotional or publicity purpose.

Appendix 3

VASU Research Centre

Ministry of AYUSH Approved
Ayurvedic Testing Laboratory
(License No.: GATL/08)

SNEDDS	**AR No.**	VARS/RS/25/03/011
	Report Date	06/10/2025
Name of Scholar	Mr. Pankaj Verma Lovely Professional University, Panjab.	

ACCELERATED STABILITY STUDY (TEMP: 40 ± 2 °C, 75 ± 5 % RH)

Sr. No.	Parameters	0 Month	3rd Month	6th Month
ORGANOLEPTIC ANALYSIS				
1	Description	Brown coloured transparent liquid	Brown coloured transparent liquid	Brown coloured transparent liquid
2	Odour	Characteristic	Characteristic	Characteristic
PHYSICO-CHEMICAL ANALYSIS				
1	Specific Gravity	1.000	0.990	0.990
2	pH (Direct)	9.04	8.95	8.71
3	Viscosity by Ostwald	0.79 cP	0.76 cP	0.75 cP
INSTRUMENTAL ANALYSIS				
1	% Quercetin by HPTLC	0.00007 %	Not Applicable	0.00005 %
2	% Gallic acid by HPTLC	0.0013 %		0.0012 %
3	% Arjunolic acid by HPTLC	0.00024 %		0.00020 %
MICROBIOLOGICAL ANALYSIS				
1	Total Microbial Plate Count	290 cfu/g	Not Applicable	137 cfu/g
2	Total Yeast & Mould Count	Absent		Absent
3	*Staphylococcus aureus*	Absent		Absent
4	*Salmonella sp.*	Absent		Absent
5	*Pseudomonas aeruginosa*	Absent		Absent
6	*Escherichia coli*	Absent		Absent

Kew-Word: API – Ayurvedic Pharmacopoeia of India; % - Percentage w/w, ppm - Parts per millions, NA – Not applicable, ND - Not detected, cfu/g – Colony forming unit per gram.

Appendix 4

Ref ...PBRI/IAEC/2024105 Date ..22-11-24

CERTIFICATE

This is to certify that the following project has been approved by the Institutional Animal Ethical Committee, Pinnacle Biomedical Research Institute (PBRI), Bhopal (Reg. No. 1824/PO/RcBi/S/15/CPCSEA).

Project Title	Development and characterization of SNEDDS of *Terminalia arjuna* Linn. and its Anti-hypertensive activity in experimental animals
Protocol Approval Number	PBRI/IAEC/30-09-24/015
Investigator Name	Pankaj
Approval Date	30-09-2024

IAEC
Pinnacle Biomedical Research Institute
22/11/24

Dr. Mohd. Azaz Khan
Chairperson, IAEC
PBRI