Optimization of Processing Parameters for the Extraction and Microencapsulation of Soursop *(Annona muricata* L.*)* Leaf Extract

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I hereby declare that the data presented in this Dissertation report entitled, "Optimization of processing parameters for the extraction and microencapsulation of Soursop (*Annona muricata* L.) leaf extract" is based on the result of investigations carried out by me in the Botany discipline at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Aditi Venkatesh Naik and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given in the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Optimization of Processing parameters for the extraction and microencapsulation of Soursop *(Annona muricata* L.*)* leaf extract" is a bonafide work carried out by Ms. Priya Maruthi Velip under my supervision in partial fulfilment of the requirements for the award of degree of Master of Science in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

I am pleased to present this dissertation, which represents the culmination of my efforts during my Master's program in Botany at the Department of Botany, School of Biological Sciences and Biotechnology (SBSB), Goa University. This work was carried out under the guidance and support of my supervisor, Dr. Aditi Venkatesh Naik, Assistant Professor at the Botany Discipline, SBSB, Goa University. The focus of this dissertation is on the optimization of processing parameters for the production of encapsulated *A. muricata* leaf extract powder. *A. muricata*, commonly known as soursop, holds significant importance due to its medicinal properties and impact on human health. Through this research, we aimed to explore the most effective methods for extracting and encapsulating the beneficial compounds present in *A. muricata* leaves.

The dissertation is structured into four main chapters. Chapter 1 introduces the research topic, providing background information on *A. muricata* and the rationale behind selecting this research area. Chapter 2 reviews relevant literature, summarizing findings from previous studies on *A. muricata* conducted by various researchers. Chapter 3 outlines the materials and methods used in our research, detailing the experimental procedures and methodologies employed. Chapter 4 presents the results and discussion derived from our research findings. Through comprehensive experimentation, we discovered that shade drying is the optimal method for maintaining optimum moisture content and yield in *A. muricata* leaf extraction. Additionally, we found that methanol serves as the most effective solvent, yielding higher total phenols and acetogenin content, along with exhibiting superior DPPH radical scavenging activity. Moreover, our investigation revealed that a wall material combination of 2:1 Gum Arabic and sodium alginate provides enhanced structural and chemical stability to the encapsulated *A. muricata* leaf extract.

This dissertation represents not only the culmination of my academic journey but also a significant contribution to the field of botany and herbal medicine. It is my hope that the findings presented herein will serve as a valuable resource for future research endeavours and contribute to the broader understanding of the medicinal potential of *A. muricata*.

-Ms. Priya Velip

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Ms. PRIYA MARUTHI VELIP

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ABBREVIATIONS USED

Entity	Abbrevation
Absorbance of control	A_C
Absorbance of sample	A_S
Centimeter	cm
Degree Celsius	^oC
α-α-Diphenyl-β-picrylhydrazyl	DPPH
100% Distilled water	T1
Electron volt	eV
100% Ethanol	T4
80% Ethanol	T5
Freeze dry	FD
Grams	gm
Hours	hrs
Lethal concentration killing 50%	IC_{50}
Meter	m
100% Methanol	T2
80% Methanol	T3
Microgram	µg
Milligrams	mg
Milliliters	mL
Millimeter	mm
Nanometers	nm
Number	No.
Oven dry	OD
Percent	%
Reactive oxygen species	ROS
serial	Sr.
Shade dry	SD
(1:2) sodium alginate: Gum Arabic	S4
(1:2) sodium alginate: Gum Arabic: Distilled water extract	S5
(1:2) sodium alginate: Gum Arabic: Methanolic extract	S6
(1:2) sodium alginate: inulin	S1
(1:2) sodium alginate: inulin: Distilled water extract	S2
(1:2) sodium alginate: inulin: Methanolic extract	S3
Standard error	SE
Volume in volume	v/v
Weight in volume	w/v

ABSTRACT

Herbal and alternative medicines are increasingly recognized for their potential health benefits, with *Annona muricata* L., or Soursop (Annonaceae) standing out for its rich reservoir of bioactive compounds with antioxidant properties. This study aimed to optimize the extraction and microencapsulation processes of *A. muricata* leaf extract to enhance its antioxidant activity and bioavailability.

Phytochemical analysis revealed a diverse array of bioactive compounds in *A. muricata* leaves, including acetogenins, alkaloids, flavonoids, glycosides, saponins, tannins, and steroids, contributing to its extensive pharmacological profile. However, the challenge lies in preserving the bioactivity of these compounds during digestion, prompting the need for effective delivery systems. Through meticulous optimization of extraction parameters, such as shade drying, and microencapsulation techniques utilizing a blend of sodium alginate, inulin, and Gum Arabic, this study aimed to maximize the yield and stability of bioactive compounds. Emulsion stability, particle size distribution, and antioxidant activity were assessed to determine the efficacy of the microencapsulation process.

This study sheds light on the potential of *A. muricata* leaf extract as a natural antioxidant source with promising applications in functional foods, nutraceuticals, and pharmaceutical formulations. Furthermore, it accentuates the importance of optimizing extraction and encapsulation processes to harness the full therapeutic potential of medicinal plants like *A. muricata*.

CHAPTER 1: INTRODUCTION

1.1. MEDICINAL PLANTS

Sources derived from plants played an essential role in traditional medicines. Natural products have a major role in drug discovery and development of novel medicine (Cragg and Newman, 2013). In 1985 the World Health Organization (WHO) estimated that approximately 65% of the world's population primarily relied on plant-derived traditional medicines for primary health care (Farnsworth *et al*., 1985). The pharmacological effects of medicinal plants have been considered promising future drugs. In recent years, the revival of interest started in reclaiming medicinal plants as a source of potential drugs (Shakya, 2016).

1.1.1. Family: Annonaceae

Annona muricata belongs to family Annonaceae, also known as Custard-apple or Soursop family is one of the largest families in Magnoliidae. Annonaceae family occurs in tropical and subtropical regions of the world, consisting of 130 genera and 2,400 species. Annonaceae is comprised of mainly flowering plants. Members of this family are odorous due to the presence of essential oil. However, many of the species of this family are of great medicinal and pharmaceutical use (Rangel *et al*., 2024).

Annona is a genus of tropical fruit trees, belonging to the family Annonaceae. It ranks second largest genus in the family after *Guatteria* containing approximately 166 species. Many species are used in traditional medicines against numerous diseases (Badrie and Schauss, 2010).

1.1.2. *Annona muricata L.*

Annona muricata L. commonly known as Graviola, Soursop, Gaunabana, Pawpaw, and Sirsak is a member of the family Annonaceae. *Annona muricata* L. natively belongs to

warm tropical areas in South and North America and is distributed throughout tropical and subtropical parts of the world including India, Indonesia, Nigeria, and Malaysia (Pinto *et al*., 2005; Patel and Patel, 2015).

1.1.2.a. Scientific Classification

Kingdom: Plantae

Clade: Angiosperms

Order: Magnoliales

Family: Annonaceae

Genus: *Annona*

Species: *muricata*

Binomial name: *Annona muricata* L.

1.1.2.b. Botanical description

Annona muricata is a terrestrial evergreen, erect tree reaching 5–8 m in height consisting of an open, roundish canopy with alternately arranged, large, glossy, dark green leaves. The tree has large solitary yellow flowers on woody stalks (pedicels). Flowers are large and solitary, yellowish or greenish-yellow in color. Three outer petals are broadly ovate with heart-shaped bases and 3 inner petals are large, elliptical, and rounded. The edible fruits of the tree are large, oval, or heart-shaped and green in color, and frequently irregular lopsided composite. Fruit is derived from the fusion of many fruitlets, can weigh more than 4 kg, and the diameter varies between 15 and 20 cm (Pinto *et al*., 2005).

Fruit pulp consists of white fibrous juicy segments surrounding an elongated receptacle. In each fertile segment, there is a single oval, smooth hard, black seed {1/2}–

{3/4} in (1.25–2 cm) long. Fruit may contain as few as 5 or up to 200 or more seeds (Patel and Patel, 2015).

1.1.2.c. Medicinal potential of *Annona muricata*

Traditional medicinal uses of *Annona muricata* have been identified in tropical regions to cure diverse illnesses such as fever, pain, respiratory and skin illness, internal and external parasites, bacterial infections, hypertension, inflammation, diabetes, and cancer. Soursop is a good source of nutrition and its fruit contains annonacin, the most abundant annonacin. Acetogenins are a class of compounds that exhibit a wide range of biological activities (Badrie and Schauss, 2010).

Bark, leaves, and roots are considered antispasmodic, hypertensive, and sedative. Tea is made using different parts of the *Annona muricata* to cure various disorders such as inflammation of the mucus membranes, diabetes, and antispasmodic. Fruit (unripe) and leaf oil mixed with olive oil, and used externally to get rid of neuralgia, rheumatism, and arthritis. The fruit of the *Annona muricata* is known as cancer fruit and is edible with great health benefits (Coria-Téllez *et al*., 2018).

1.1.2.d. Annonaceous acetogenins

Acetogenins are long-chain fatty acid derivatives exclusively found in the members of Annonaceae (Lima *et al*., 2010). Acetogenins exhibit a wide range of biological activities such as anti-cancer, anti-parasitic, anti-inflammatory, anti-tumor properties and cytotoxicity against various types of cancer cell lines (Badrie and Schauss, 2010).

1.1.3. Extraction

Leaves of the soursop contain the largest diversity of phytochemical compounds such as alkaloids, terpenoids, flavonoids, steroids, fatty acids, phenolics, tannins, saponins

and acetogenins. Hence, its leaves have numerous benefits in many fields. These compounds are sensitive to higher temperatures and can be extracted using suitable techniques such as maceration and Soxhlet extraction using various solvent combinations (Alexander *et al*., 2024).

1.1.4. Encapsulation

Extraction and utilization of herbal extract derived from the foliage have been practiced from the past decade. Organic solvents are needed for herbal extraction which makes complicated formulation which is why human beings cannot take it directly. Bioactive compounds in herbal extracts are susceptible to physical and chemical environmental factors. To address this sensitivity, researchers have turned to encapsulation technology as a promising solution. This approach focuses on developing innovative procedures to preserve plant extracts and ensure their safe delivery, thereby enhancing the effectiveness of bioactive compound (Bomfim *et al*., 2016; Hani *et al*., 2017).

In recent years, there has been growing interest in exploring the bioactive constituents of foodstuffs, fueled by both scientific curiosity and the pharmaceutical industry's quest to incorporate health-beneficial compounds into diverse product formulations. However, a significant challenge arises concerning the availability of these ingredients within the body and their limited solubility in aqueous solutions (Nejatian *et al*., 2024).

Encapsulation emerges as a highly efficient and effective strategy for addressing challenges associated with bioactive compounds derived from herbal sources. The inherent vulnerability of these compounds to degradation, owing to factors like stomach pH and liver enzymatic activity, often limits their bioavailability in the bloodstream. Consequently, the insufficient delivery of bioactive compounds to targeted areas may fall below the minimum

effective level, diminishing their therapeutic efficacy. Encapsulation offers a solution by facilitating the delivery of plant extracts to their intended sites of action, circumventing barriers such as stomach acidity and liver metabolism. Moreover, encapsulated formulations, characterized by their reduced size, enhance the overall bioavailability of these compounds, ensuring optimal therapeutic outcomes (Yadav *et al*., 2020).

In modern times, there is a growing preference for alternative medicines and self-administered dietary supplements aimed at managing chronic conditions, rather than acute, life-threatening illnesses. The term "functional" pertains to the biologically active ingredients integrated into food products, as these bioactives are associated with health benefits linked to the prevention of various chronic diseases. On the other hand, "nutraceutical" primarily denotes the active components found in functional foods. Consistent consumption of functional foods has been linked to enhanced antioxidant activity, reduced inflammation, improved insulin sensitivity, and regulation of cholesterol levels (Alkhatib *et al*., 2017; George *et al*., 2021; Naskar *et al*., 2022).

1.1.5. Antioxidant activity

Antioxidants are compounds capable of impeding oxidation processes induced by atmospheric oxygen or reactive oxygen species (ROS). Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), such as free radicals, are notorious for triggering oxidative damage to various cellular components like proteins, enzymes, lipids, and nucleic acids. This oxidative stress can lead to cellular or tissue injury (Re *et al*., 1999; Mishra *et al*., 2012).

Superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), glutathione peroxidase (GPX), along with vitamins A, beta-carotene, C, and E, constitute a diverse array of enzymatic and non-enzymatic antioxidant defenses. These antioxidants operate in

concert, with their intracellular levels and actions intricately linked, collectively providing protection against oxygen toxicity and oxidative stress (Blois, 1958).

The DPPH assay, based on the stable free radical method, offers a straightforward, rapid, and sensitive approach to evaluating the antioxidant activity of plant extracts. When antioxidant molecules are present, the violet solution of DPPH in alcohol transitions to a colorless solution. This change occurs due to the delocalization of an electron pair across the entire molecule, rendering DPPH a stable free radical and preventing dimerization, unlike other free radicals. The delocalization of electrons imparts a deep violet hue, characterized by an absorption band centered around 517 nm in methanol solution (Rajan *et al*., 2011; Nirmaladevi *et al*., 2021).

1.2. AIMS AND OBJECTIVES

- To develop optimized protocols for drying and extraction of *Annona muricata* L. leaf extract.
- To employ TLC methods for the isolation of acetogenins within *A. muricata* leaf extract.
- To evaluate a range of wall materials for microencapsulation of *A. muricata* leaf extract.
- To investigate and quantify the enhanced antioxidant properties of *A. muricata* leaf extract post-encapsulation.

1.3. HYPOTHESES

Optimizing drying and extraction protocols for *Annona muricata* L. leaf extract is expected to significantly enhance efficiency and yield compared to conventional methods. Utilizing Thin Layer Chromatographic analysis, the isolation of Acetogenins within the leaf extract can be achieved, laying the groundwork for further investigation into their medicinal properties and potential drug development. Microencapsulation of the leaf extract using various combinations of wall materials is anticipated to identify the most effective

encapsulation agent for sustained release applications. Furthermore, encapsulation is projected to enhance the antioxidant properties of the leaf extract, thereby positioning it as a promising compound for incorporation into functional foods and pharmaceutical formulations.

1.4. SCOPE

The dissertation titled "Optimization of processing parameters for the extraction and microencapsulation of *Annona muricata* L. leaf extract" encompasses a comprehensive exploration of various methodologies to enhance the extraction and microencapsulation processes. It will evaluate different drying and extraction techniques, including shade drying, convection drying, and freeze drying, to identify the most effective protocol and solvent combination for extracting the *Annona muricata* leaf extract. Additionally, the study will assess a range of wall materials such as sodium alginate, Gum Arabic, and inulin for their suitability in microencapsulation. The resulting microcapsules will undergo characterization to analyze their size, stability, antioxidant capacity, and release profile, aiming to determine the optimal processing parameters.

Furthermore, the antioxidant activity of the encapsulated leaf extract will be scrutinized to evaluate its potential for integration into functional foods and pharmaceutical formulations. The scalability and standardization of the optimized processes will be considered to ensure practicality for future industrial applications.

A comparative analysis will be conducted to juxtapose various microencapsulation methods with traditional approaches, showcasing the enhancements achieved through optimization. Ultimately, the primary goal of this study is to refine processing parameters for the microencapsulation of *Annona muricata* L. leaf extract, with a focus on enhancing stability, bioavailability, and functionality.

CHAPTER 2: REVIEW OF LITERATURE

Annona muricata L. is known for its abundance of bioactive compounds, including acetogenins, alkaloids, flavonoids, and phenolic compounds, which contribute to its medicinal properties and health benefits. Additionally, *Annona muricata* L. is rich in volatile compounds, adding to its characteristic aroma and flavor profile. More than 200 compounds were isolated and identified from this plant.

2.1. MEDICINAL POTENTIAL OF *ANNONA MURICATA*

Phytochemical analysis conducted by Badrie and Schauss (2010) revealed that soursop, or *Annona muricata*, is a valuable nutritional source containing annonacin, the most prevalent acetogenin, found in various parts of the plant, particularly its fruit. The plant has been traditionally used for its antispasmodic, emetic, and sudorific properties. Additionally, *Annona muricata* leaves are used in decoction form to control head lice, while its fruits are known for their effectiveness against liver complaints and urethritis. The ethanolic extract of the bark or stem is also believed to reduce stress levels.

Further examination of the phytochemical contents, bioactivity, and toxicological aspects of *Annona muricata* leaf extracts by Coria-Téllez *et al*. (2018) confirmed its traditional medicinal applications across tropical regions. These applications encompass a wide range of illnesses, including fever, pain, skin ailments, internal and external parasites, bacterial infections, hypertension, inflammation, diabetes, and even cancer.

2.2. ANNONACEOUS ACETOGENINS

Meta-analysis studies of *A. muricata* have unveiled the presence of numerous active compounds, with a particular focus on a novel set of chemicals known as Annonaceous acetogenins, as reported by Lima *et al*. (2010).

In 1997, Purdue University published findings suggesting that Annonaceous acetogenins are not only effective in killing tumors resistant to anticancer agents but also exhibit a special affinity towards resistant cells. Recent research on acetogenins has further highlighted their ability to shut down intracellular pumps, thus killing multidrug-resistant cells by blocking the transfer of ATP. It is noteworthy that the leaves of *A. muricata* contain the highest amount of acetogenins, as noted by Naik and Sellappan (2021).

According to Campos *et al*. (2023), *A. muricata* is traditionally used extensively against inflammatory diseases and infections caused by fungi. The presence of acetogenins and alkaloids makes it a potential candidate for antifungal bioactivity. Particularly, the ethanolic extract of *A. muricata* has shown effectiveness against multi-drug-resistant Candida albicans.

2.3. DRYING AND EXTRACTION TECHNIQUES

De Souza *et al*. (2009) investigated the influence of various solvent extraction methods and their combinations on the metabolite yield of *A. muricata* leaves. They concluded that solvent mixtures outperformed pure solvents in extracting diverse fractions of metabolites. The mobile phase containing a mixture of methanol, acetonitrile, and water was deemed most suitable for basic fraction analysis, while an organic fraction analysis at 254 nm yielded favourable results.

Mahmud and Razak (2014) assessed the effects of different drying methods on the bioactivity of *Annona reticulata* leaf extract. Their results indicated that freeze drying was the most suitable method due to its operation at lower temperatures, preserving all compounds and secondary metabolites in the leaf extract.

Babu *et al*. (2018) evaluated various leaf drying procedures and their influencing mechanisms, suggesting that shade drying is the optimal method for extracting bioactive

compounds. They noted that parameters such as relative humidity, temperature, drying time, leaf size, shape, and age also impact the drying process.

Choi *et al*. (2020) explored the effects of different autoclave extraction times on the antioxidant, antidiabetic, and anti-inflammatory activities of phenolic compounds from *A. muricata* leaves. They found that water extracts exhibited high DPPH radical scavenging activity, ferric reducing antioxidant property, and α-glucosidase inhibitory activity stronger than that of acarbose, indicating their potential use as functional food ingredients in the food industry.

Tran *et al*. (2020) evaluated the impact of drying conditions on various parameters of soursop jelly. Their findings revealed that temperatures exceeding 50°C led to a decrease in total ascorbic acid content (TAA) and total phenolic content. Additionally, the highest protein content was observed at 40°C.

2.4. PHYTOCHEMICAL ANALYSIS AND BIOACTIVITY

Gavamukulya *et al*. (2014) investigated the phytochemical composition, antioxidant activity, and in vitro anticancer potential of ethanolic and water leaf extracts of *A. muricata*. Their study revealed the presence of various secondary metabolites such as alkaloids, phenols, steroids, flavonoids, anthraquinones, saponins, terpenoids, coumarins, tannins, cardiac glycosides, lactones, and phytosterols. The water leaf extract exhibited a higher total phenolic concentration, while the ethanolic leaf extract demonstrated *in vitro* cytotoxicity against tumor cell lines (EACC, MDA & SKBR3).

Naik and Sellappan (2019) employed chromatographic fingerprinting using HPTLC to analyze essential oils in different parts of *A. muricata*. They identified nine essential oil derivatives across various plant parts, with three restricted to seed extracts. Additionally, an essential oil with an Rf value of 0.17 was found in rind, seed, leaf, and bark extracts.

2.5. TLC ISOLATION OF ACETOGENINS

Luna *et al*. (2006) conducted the isolation of acetogenins from *Annona muricata* leaves through chromatographic methods using Kedde's reagent. Their findings confirmed the presence of annonacin (90%), isoannonacin (6%), and goniothalamicin (4%), and also demonstrated the anti-molluscicidal properties of *Annona muricata* leaves.

Vinothini and Growther (2016) performed Thin Layer Chromatographic (TLC) analysis of *A. muricata* leaf extract using different fractions of water and ethanol as extraction solvents. They utilized TLC sheets with silica gel 60 as the stationary phase and chloroform-methanol (9:1) as the mobile phase. Their study reported and confirmed the presence of acetogenins through the use of Kedde's reagent.

2.6. ANTIOXIDANT

Em and Ael (2016) conducted an analysis of fractions containing acetogenins derived from *Annona muricata* fruit pulp. They evaluated the total phenolic content and in vitro antioxidant activities using assays such as the DPPH assay and ABTS assay. Their findings revealed that the chloroform and aqueous leaf extracts exhibited the highest ABTS radical scavenging activity.

Naik and Sellappan (2020) investigated antioxidant activity using an *in-situ* HPTLC-DPPH assay and assessed *in vitro* cytotoxicity on MCF-7 and SCC-40 cell lines. Their results showed higher cytotoxicity in leaves, suggesting potential bioactivity attributed to various phytochemicals present across different parts of Annona muricata.

2.7. WALL MATERIAL USED FOR ENCAPSULATION

2.7.1. Properties of wall material

Analysis conducted by Fang and Bhandari (2012) regarding spray drying, freeze-

drying, and related processes for food ingredient and nutraceutical encapsulation underscored the challenge of maintaining the stability of phytoactive compounds during food processing and storage. They emphasized the importance of encapsulation to enhance the stability of these substances against external factors.

Dragostin *et al*. (2017) emphasized specific characteristics required for effective encapsulation, including good rheological properties, scattering ability for bioactive compounds, emulsion stabilization, non-reactivity to active materials, complete release of core material, high solubility in nontoxic solvents, good cell viability, and low toxicity. These attributes are crucial for successful encapsulation and enhanced cellular functions.

Additionally, Bodbodak *et al*. (2022) suggested the incorporation of bioactives into different encapsulants as a method to improve the thermal stability of these compounds. They indicated the potential of encapsulation technology in enhancing the stability of natural bioactive ingredients by utilizing various gums, carbohydrates, and proteins. Their research highlighted that stronger interactions between the wall material and bioactive compounds lead to higher thermal resistance, contributing to the overall stability of encapsulated ingredients.

Ansari and Goomer (2022) investigated the potential of natural gums and carbohydrate-based polymers as encapsulants, emphasizing their superiority over lipid-based and protein-based carriers for encapsulation purposes. Their research revealed that these materials were more effective in preventing the degradation of bioactive compounds, indicating their promise in preserving the stability of these compounds during encapsulation processes.

2.7.2. Sodium alginate

Dantas *et al*. (2011) documented the wound-healing properties of sodium alginate

when integrated into chitosan-based films along with low-level laser therapy, suggesting its potential to enhance dermal burn healing.

Hecht and Srebnik (2016) provided an in-depth analysis of the structural characteristics of sodium alginate, highlighting it as a natural polysaccharide featuring a linear glycosidic bond of 1,4 linkages. They described it as a copolymer composed of β-D-mannuronic acid and α-L-guluronic acid, primarily extracted from brown algae. Additionally, they emphasized its remarkable water absorption and retention capacities, underscoring its diverse potential applications.

2.7.3 Inulin

Zabot *et al*. (2016) highlighted the significant thermal stability offered by inulin when used as a prebiotic encapsulant matrix for bioactive compounds, suggesting its potential as a wall material in encapsulation applications.

Du *et al*. (2023) provided a comprehensive description of inulin as a fructo-oligosaccharide consisting of fructose and glucose units linked with β 2-1 linkages at the ends of both chains. They noted its natural solubility as a dietary fiber and its classification as a partially digestible carbohydrate of fructan due to its unique glycosidic bond. Additionally, they emphasized its various bioactivities.

2.7.4 Gum Arabic

Ali *et al*. (2009) extracted Gum Arabic from dried exudates of *Acacia senegal* and *Acacia seyal*, highlighting its properties as a sticky, non-viscous soluble fiber. They emphasized its edibility and widespread use due to these favorable characteristics.

George *et al*. (2021) demonstrated the efficient encapsulation of bioactive-rich leaf extract of *Moringa oleifera* using maltodextrin, Gum Arabic, and a combination of both.

They suggested that the combination of maltodextrin and Gum Arabic provided superior stability compared to using maltodextrin or Gum Arabic alone. Additionally, they underscored the potential of these encapsulants as fortificants in processed food products.

2.8. ENCAPSULATION

Jordán-Suárez *et al*. (2018) delved into the microstructure of microcapsules containing *Annona muricata* leaf extract prepared via spray drying, using Gum Arabic and maltodextrin as encapsulants. They observed that higher concentrations of encapsulants improved the sphericity of the microcapsules.

Zulham *et al*. (2021) conducted a comprehensive review of various encapsulation methods and their stability in different plant extract samples. They found that Gum Arabic exhibited superior stability compared to other methods in the samples studied.

Jordán-Suárez *et al*. (2021) optimized processing parameters for the microencapsulation of *A. muricata* leaf extract to protect bioactive compounds from degradation post-separation. They proposed spray drying as a viable method for preserving these bioactive compounds effectively.

Nolasco-González *et al*. (2023) explored recent scientific advances in bioactive compounds from *Annona muricata*, *A. cherimola*, and *A. squamosa*, focusing on extraction, encapsulation technologies, and the potential biological activities of these compounds.

2.9. ENHANCED BIOACTIVITY POST ENCAPSULATION

Bomfim *et al*. (2016) delved into the chemical composition and antitumor potential of essential oil extracted from *Annona vepretorum* leaf complexed with β-cyclodextrin via microencapsulation, utilizing the Alamar blue assay. They also demonstrated the *in vivo* efficacy of the essential oil on mice inoculated with B16-F10 mouse melanoma. Their

findings highlighted an increase in *in vivo* tumor growth inhibition (62.66%) compared to essential oil alone after microencapsulation.

Zhang *et al*. (2020) conducted an analysis of the nanostructures of protein-polysaccharide complexes or conjugates for the encapsulation of bioactive compounds. They concluded that covalent and non-covalent interactions promote the formation of protein-polysaccharide conjugates and complexes, respectively. Various encapsulation approaches such as pH-driven encapsulation, liquid anti-solvent precipitation, heating and ionic gelation, and emulsion-based encapsulation were found to be broadly applicable for fabricating these bioactive compound-loaded protein-polysaccharide complexes or conjugates. This encapsulated bioactive compound exhibits promising nutrient enrichment to meet varying processing and consumption demands.

Ahmadzadeh *et al*. (2023) provided an analysis regarding the designing of future foods by employing 3D food printing technology to encapsulate bioactive compounds. Their research revealed that the incorporation of bioactive compounds into 3-dimensional matrices can improve protection during storage, enhance bioavailability, enable controlled release of bioactive compounds, and promote their incorporation into functional foods.

Sorasitthiyanukarn *et al*. (2024) conducted an examination of stability and biological activity enhancement of fucoxanthin through encapsulation in alginate/chitosan nanoparticles. Their research resulted in the development of a nanocarrier from alginate (ALG) and chitosan (CS) for the delivery of fucoxanthin (FX). Fucoxanthin nanoparticles were fabricated using oil-in-water emulsification and ionic gelation techniques. These nanoparticles exhibited enhanced stability under controlled environmental conditions and gradual fucoxanthin release in simulated gastrointestinal fluids. Furthermore, they demonstrated increased in vitro oral bioaccessibility, anti-inflammatory effects, and

gastrointestinal stability. The findings from Sorasitthiyanukarn and others highlight the potential of alginate/chitosan nanoparticles as an effective delivery system for fucoxanthin, offering improved stability and enhanced biological activity. This encapsulation strategy holds promise for the development of functional foods and nutraceuticals aimed at promoting health and preventing diseases associated with inflammation and oxidative stress.

In conclusion, the extensive research outlined in this review of literature underscores the paramount importance of optimizing processing parameters for the extraction and microencapsulation of *Annona muricata* L. leaf extract. Through meticulous investigation and experimentation, this study tries to shed light on the various methods and materials available for enhancing the stability, bioavailability, and functionality of the bioactive compounds present in *Annona muricata* L. leaf extract.

CHAPTER 3: MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Materials

This chapter deals with the materials and chemicals used and the methodology adopted for the present investigation titled "Optimization of processing parameters for the extraction and microencapsulation of Soursop *(Annona muricata* L.*)* leaf extract". The present investigation was carried out in Botany Discipline, School of Biological Sciences and Biotechnology, Goa University, Taleigao Plateau, Goa. The standard procedures along with modifications have been used for this study.

3.1.2. Chemicals

All the chemicals and reagents used in this study were of analytical grade and procured from Sigma Chemicals Co. (St. Louise, M. o., USA); HiMedia Laboratories Pvt. Ltd., Bombay; Sisco Research Laboratories Pvt. Ltd., Bombay.

3.2 METHODOLOGY

3.2.1. Collection of Plant Samples

Fresh leaf samples of *Annona muricata* L. were collected by making multiple visits to KOCL Research farm Kirbhatt, Nuvem, South Goa during October 2023 to March 2024. Matured and healthy leaves were collected in zip-lock polythene bags.

3.2.2. Preparation of Plant Samples for Drying

Collected leaf samples were washed thoroughly under the tap water to remove debris and other dust particles. Each leaf was wiped using laboratory-grade tissue paper to remove excess water and moisture. Further, the cleaned leaves were used for each type of drying procedure.

3.2.3. Drying Optimization

Controlled drying experiments were conducted using convection drying, freeze-drying, and shade-drying techniques to determine optimal conditions for *Annona muricata* leaf drying.

3.2.3.a. Shade Drying

Cleaned leaves of the *A. muricata* were shade-dried in a room with good ventilation at room temperature for 10 days until the desired moisture content was achieved (Babu *et al*., 2018).

3.2.3.b. Convection Drying

Cleaned leaves of *A. muricata* were dried in a hot air oven at 45ºC for 8-10 hours until the desired moisture content was obtained (Choi *et al*., 2020).

3.2.3.c. Freeze Drying

The cleaned leaves were freeze-dried at -107ºC by using a lyophilizer for 7-8 hours under the vacuum until consistent weight was achieved. The dried leaves of *A. muricata* were ground into a coarse powder using a mixer grinder and stored in an airtight container at 4ºC for further analysis and extraction (Babu *et al*., 2018; Mahmud and Razak, 2014).

3.2.4. Extraction Protocol

The sample for extraction was prepared by taking 10 g of leaf powder separately in a conical flask using 150 mL of various solvent combinations employing Maceration at 45ºC for 48 hours at 120 rpm and Soxhlet extraction at 35ºC for 12 hours, to extract bioactive compounds, for quantifying yields and analyzing metabolite fractions analysis (De Souza *et al*., 2009).

Leaf extracts were concentrated using a rotary vacuum evaporator at 35ºC for 90 minutes followed by lyophilization at -107ºC and stored at 4ºC until further use and analysis **(Table 3.1), (Fig. 3.1).**

3.3. ANALYTICAL STUDIES OF LEAF EXTRACTS

3.3.1. Determination of moisture content of *A. muricata* leaves

A total of 10 grams of fresh leaf samples from *A. muricata* were initially weighed. After each drying process, the weight was measured and divided by the initial fresh weight to determine the percentage of weight loss (Reeb *et al*., 1999).

3.3.2. Determination of pH of *A. muricata* leaf extracts

A 50 mL extract sample was placed in a beaker and equilibrated or tempered at 30°C in a water bath. The pH of the extract was measured directly by immersing the electrode into the sample using a digital pH meter. Prior to use, the pH meter was calibrated with standard buffer solutions of pH 4 and 9 at 30°C (Choi *et al*., 2020).

3.3.3. Determination of Total Extract Yield of *A. muricata* leaf

To ascertain the Total Extract Yield (TEY), 10 g of dried leaf powder underwent maceration and Soxhlet extraction using various solvent combinations as given in **Table 3.1** T1 (100% Distilled water), T2(100% Methanol), T3(80% Methanol: 20% Water), T4(100% Ethanol), T5(80% Ethanol: 20% water). Subsequently, the leaf extract was concentrated using a rotary evaporator, and the weight of the dried extract was recorded. Following assessment of the highest TEY, only one type of drying method was chosen for subsequent investigations (Zhang *et al*., 2007). To calculate TEY:

$$\text{Total Extract Yield (TEY)} = \frac{\text{Weight of dried leaf extract}}{\text{Weight of fresh leaf sample}} \times 100$$

Table 3.1 List of different solvent combinations used for extraction of *Annona muricata* leaf extract

Sample No.	Solvent combination
T1	100% Distilled water
T2	100% Methanol
T3	80% Methanol: 20% Water
T4	100% Ethanol
T5	80% Ethanol: 20% water

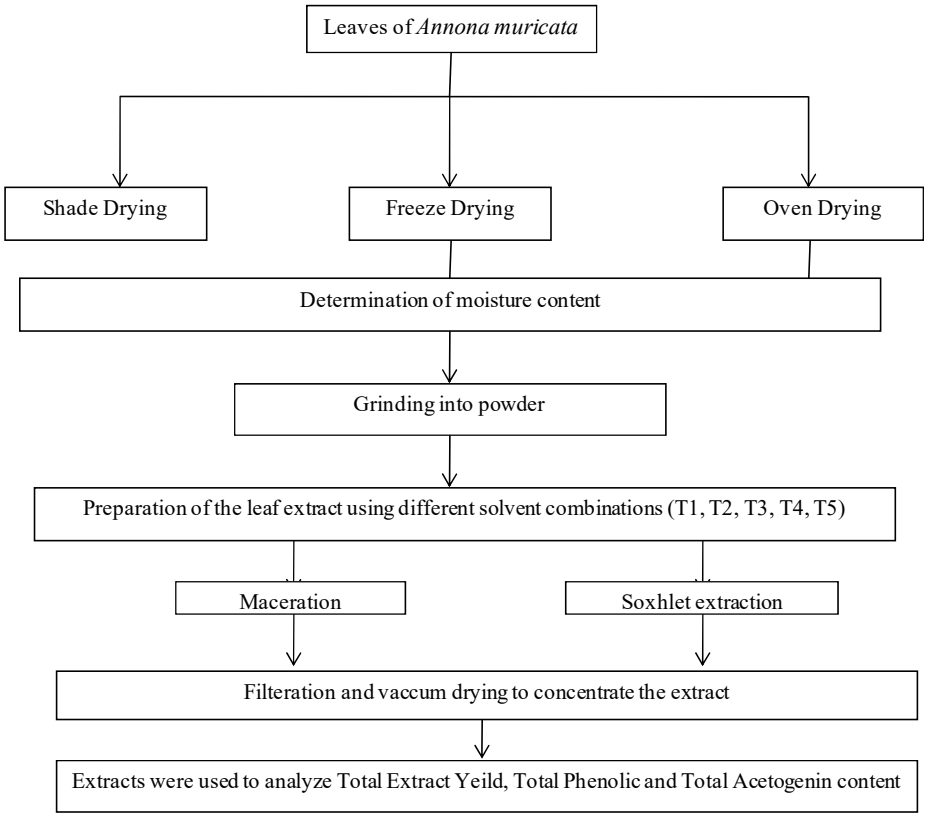


Fig. 3.1 Diagrammatic representation of sample preparation and extraction processes of *Annona muricata*

3.3.4. Thin layer chromatography (TLC) analysis of *Annona muricata* leaf extracts

To distinguish the bio-active compounds present in the crude leaf extracts, aluminum foil-baked silica gel 60 F_{254} plates; dimensions 10cm×10cm (Merck) were used. List of mobile phases, conditions, and derivatizing agents used for the detection of various secondary metabolites present in the leaf extract are given in **Table 3.2**.

Shade-dried leaf extracts with different solvent combinations (T1, T2, T3, T4, & T5) were loaded as a concentration band, 1.5 cm from the edge of their respective TLC plate, and allowed to dry. Where, T1-100% distilled water, T2-100% methanol, T3-80% Methanol: 20% distilled water, T4- 100% ethanol, and T5-80% ethanol and 20% distilled water were used as solvent combinations for extraction. In case of phenolic compounds, one extract spot of standard Gallic acid was loaded as T0-standard gallic acid.

The plates with dried extract were gently placed in the solvent chamber, closed, and left to run. The plates were removed from the solvent chamber when the solvent front had traveled ¾ (9 cm) of the plate's length (10 cm). The position of the solvent front was immediately marked with a soft pencil.

Separated bands were examined under Ultraviolet trans-illuminator visible, long UV (365 nm), and short UV (254 nm). The retention factor (Rf) value of different bands was calculated using the equation given by Wagner and Bladt (1996).

To calculate Rf value:

$$\text{Rf} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

Table 3.2 List of the mobile phase, conditions and derivatizing agents used for the detection of various secondary metabolites for TLC analysis

Class of compounds	Mobile phase	Derivatisation	Observation
Alkaloids	Toluene: Ethyl acetate: Methanol: Ammonia 25% (30:30:15:1) v/v/v/v	Dragendorff's reagent	Light orange bands spots visible light
Phenolic compounds	Tetrahydrofuran: toluene: formic acid: water (16:8:2:1) v/v/v/v	10% Ferric chloride solution	Grey or black spots under visible light
Flavonoids	Tetrahydrofuran: toluene: formic acid: water (16:8:2:1) v/v/v/v	10% Methanolic sulphuric acid	Fluorescent bands under long UV light.
Acetogenins	Chloroform: methanol (9:1) v/v	Vanillin sulphuric acid + heat100°C	Dark blue spots under visible light

3.4. PHYTOCHEMICAL ESTIMATION OF SECONDARY METABOLITES

3.4.1 Determination of Total Phenolic Contents

Total phenolic content was determined as Gallic acid equivalents (mg GAE/g) of dry extracts as per the Folin and Ciocalteu reagent method given by Singleton and Rossi (1965). Firstly, 1 mL of extract or Gallic acid (100-1000 µg/mL) was mixed with 5 mL 10% Folin-Ciocalteu Phenol reagent. After 15 minutes, 5 mL of 1 M sodium carbonate was added. The mixture was allowed to incubate in the dark for 30 min and absorbance was measured at 760 nm. The total phenol content was measured from a calibration curve where $y = 0.008x - 0.126$, $R^2 = 0.998$ was utilized for the determination of total phenolic content.

3.4.2. Determination of Acetogenin Contents

For the determination of total acetogenin content, Annonacin was used to derive a standard calibration curve. Standard solutions of Annonacin were prepared by serial dilutions using acetonitrile (10-100 µg/mL). 0.25 mL of 2% dinitrobenzoic acid and 0.25 mL of 5.7% alcoholic potassium hydroxide were added respectively in 1 mL of standard or leaf extract. The combination was mixed gently and absorbance was measured immediately where color reaction occurred within 1-2 minutes and disappeared quickly. The total acetogenin content was calculated based on a calibration plot ($y=0.0101x-0.055$, $R^2= 0.9923$) and expressed as mg annonacin equivalent (mg AE/g) of dried extract. All determinations were carried out in triplicate. Utilized UV-visible spectrophotometer for quantification of acetogenins measured absorbance at 505 nm (Aromdee *et al*., 2005).

3.5. ANTIOXIDANT ESTIMATION OF THE LEAF EXTRACTS

3.5.1. DPPH radical scavenging activity

The assessment of the antiradical activity against 2, 2-diphenyl-l-picrylhydrazyl (DPPH) was assessed by following the method given by Blois (1958) with slight modifications. Briefly, 1mL of 0.1 mM DPPH solution was added to 2 mL of leaf extract of varying concentrations (20, 40, 60, 80, 100 µg/mL). The mixture was shaken for 1 minute and left to stand in the dark at room temperature for 30 minutes. After that, the absorbance for the sample (A_{sample}) was measured at 517 nm against a methanol blank. A negative control ($A_{control}$) was taken after adding DPPH solution to 2 mL of the respective solvent. The percent of DPPH scavenging activity or % inhibition was calculated according to the equation:

$$\% \text{ Inhibition (I \%)} = \frac{\text{Acontrol} - \text{As}}{\text{Acontrol}} \times 100$$

Where, $A_{control}$ is the absorbance of the control, A_{sample} is the absorbance of the tested sample at the end of the reaction. The amount of sample necessary to decrease the absorbance of the DPPH by 50% (IC_{50}) was calculated graphically from the calibration curve using L- Ascorbic acid as standard.

A solvent combination with higher antioxidant activity, total phenolic content, and acetogenin content was further subjected to emulsion preparation of encapsulation.

3.6. ENCAPSULATION PROCESS USED FOR ENCAPSULATION OF LEAF EXTRACTS

3.6.1. Selection and preparation of the wall material

Encapsulation of optimized *Annona muricata* leaf extract was done by using a combination of different wall materials viz., sodium alginate, Inulin, and Gum Arabic. Lecithin was used as a surfactant and 2 sets were prepared one containing 1:2 sodium alginate and Inulin and the other one containing 2:1 Gum Arabic and sodium alginate of both methanolic and distilled water extract **(Table 3.3).**

3.6.1. a. Preparation of Sodium Alginate solution

10 g of Sodium Alginate (SA) was dissolved slowly in 80 mL of distilled water at 60^{o}C by continuously stirring it and after complete dispersion, the final volume was made up to 100 mL by adding distilled water. The prepared 10% Sodium Alginate solution was filtered using a muslin cloth to remove the foreign materials if any and used for the experiments (Shanmugasundaram, 2008).

3.6.1. b. Preparation of Gum Arabic solution

10 g of Gum Arabic (food grade) was dissolved slowly in 80 mL of distilled water at 60^{o}C and after complete dispersion; the final volume was made up to 100 mL by adding distilled water. It was kept under ambient conditions for 12 h to improve the film forming and emulsification properties. The prepared 10% Gum Arabic solution was filtered using muslin cloth to remove the foreign materials if any, and used for further experiments (Krishnan *et al*., 2005).

3.6.1.c. Preparation of Inulin solution

20 g of Inulin (food grade) was dissolved slowly in 80 mL of distilled water at 60ºC and after complete dispersion; the final volume was made up to 100 mL by adding distilled water. It was kept under ambient conditions for 12 h to improve the film-forming and emulsification properties. The prepared 20% Inulin solution was filtered using muslin cloth to remove the foreign materials if any, and used for further experiments (Krishnan *et al*., 2005).

3.6.1.d. Emulsification

10 mL of leaf extract solution (10 mg/mL) was added to the 100 mL wall material combination solution to obtain core material. Plant extract was added to each wall material combination except control. This immiscible mixture was emulsified in a high-speed emulsifier for 5 min at 3000 rpm until the leaf extract was dispersed completely in 1 mL of lecithin which was added to aid the emulsification process (Choudhary *et al*., 2020; Shaikh *et al*., 2006) **(Table 3.4).**

3.6.2. Preparation of microencapsulated powders using Lyophilizer

The resultant emulsion after emulsification was kept at (4 ± 2ºC), and later placed in the Lyophilizer for freeze drying under high vacuum pressure at -107°C for 8-9 hours until complete removal of the moisture. The microencapsulated dried emulsion was collected and ground into a fine powder using a motor and pastel. Homogenized powder was stored in an air-tight container at 4°C for further analysis.

Table 3.3 List of combinations of wall materials (T1= 100% Distilled water leaf extract, T2= 100% Methanolic leaf extract, T3= 80% Methanol + 20% Water leaf extract, T4= 100% Ethanol leaf extract, T5= 80% Ethanol + 20% Water leaf extract).

The ratio of wall materials	Wall materials
1:2 w/w	Sodium alginate: inulin: T1
2:1 w/w	Gum Arabic: Sodium alginate: T1
1:2 w/w	Sodium alginate: inulin: T2
2:1 w/w	Gum Arabic: Sodium alginate: T2

Table 3.4 Wall material combinations and preparation of emulsion using *Annona muricata* leaf extract (SA= Sodium Alginate, I=Inulin, D.W. = 100% Distilled Water, M= 100% Methanol, G.A. = Gum Arabic).

Sample ID	Wall material ratio	Wall material combination
S1	1:2	SA: I
S2	1:2	SA: I: D.W extract
S3	1:2	SA: I: M extract
S4	2:1	GA: SA
S5	2:1	GA: SA: D.W. extract
S6	2:1	GA: SA: M extract

3.7. CHARACTERIZATION OF EMULSION

3.7.1. Particle size and zeta potential

The particle size and Zeta potential of the prepared particles present in the emulsions was measured by using a Zeta/Nanoparticle analyzer, with a dynamic light scattering system. The particle size and Zeta potential was analyzed separately until three constant readings were obtained wherein 1.5 mL of each emulsion was initially dispersed in 5 mL of distilled water (Choudhary *et al.*, 2020).

3.7.2. Emulsion Stability Index (ESI)

For the emulsion stability estimation, 10 mL of each emulsion sample was separately taken in the graduated cylinders and kept at 40ºC and unseparated phase was measured and the stability of the emulsion was measured in terms of stability index percentage:

$$\text{ESI (\%)} = \frac{H1}{H0} \times 100$$

Where, H0 stands for the initial emulsion volume and H1 stands for the unseparated phase volume of the emulsion (Binsi *et al.*, 2017).

3.7. ANALYSIS OF SPRAY-DRIED ENCAPSULATED POWDER

3.8.1. Bulk density

The analysis of bulk density was carried out by using the method of Nadeem *et al*. (2013) with slight modifications. Briefly 5 grams of encapsulated Freeze-dried *Annona muricata* leaf extract was taken into a 10 mL graduated cylinder (kept at uniform level) and the volume of the encapsulated *A. muricata* leaf extract was recorded. The ratio of the mass of the encapsulated *Annona muricata* leaf extract to its volume was taken as the value of bulk density. To calculate Bulk density = $\frac{\text{Mass of encapsulated leaf extract}}{\text{Volume of encapsulated leaf extract}t} \times 100$

3.8.2. Tapped Density

The Tapped density of the encapsulated freeze-dried *Annona muricata* leaf extract was evaluated by applying the method of Ozdikicierler *et al*. (2014) with slight modifications. 5 grams of encapsulated powder was taken in a 10 mL graduated cylinder and the volume was observed when the sample was tapped 20 times from a height of 15 cm on the pinnacle of a rubber mat. Tapped density is calculated according to the equation:

$$\text{Tapped density} = \frac{\text{Mass of encapsulated leaf extract}}{\text{Tapped volume}} \times 100$$

3.8.3. Flowability

The Flowability of the encapsulated freeze-dried *A. muricata* leaf extract was analyzed by determining the value of the Carr index (CI) and Hausner ratio (HR) (Jinapong *et al*., 2008). Bulk and tapped densities were used to determine the value of CI and HR as follows:

$$\text{Carr Index (CI)} = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100$$

$$\text{Hausner ratio (HR)} = \frac{\text{Tapped density}}{\text{Bulk density}}$$

The values of the Carr index and Hausner ratio in terms of the flow property were analyzed which indicated the quality of the powder (Lebrun *et al*., 2012) **(Table 3.5).**

Table 3.5 Specification for Carr index and Hausner ratio for comparison of the flowability of the encapsulated powder of *Annona muricata* leaf extract.

Flowability	***Car Index (CI) (%)***	***Hausner ratio (HR) (%)***
Excellent	0-10	1.00-1.11
Good	11-15	1.12-1.18
Fair	16-20	1.19-1.25
Passable	21-25	1.26-1.34
Poor	26-31	1.35-1.45
Very poor	32-37	1.46-1.59
Very, very poor	>38	>1.60

3.8.4. Antioxidant stability of encapsulated freeze-dried *A. muricata* leaf extracts powder

To evaluate the stability of the entrapped antioxidants, the encapsulated powder was dissolved in sodium citrate solution and the antioxidant activity was analyzed by the DPPH radical scavenging assay (see section 3.5.1). Wherein, 20 mg of encapsulated leaf extracts were dissolved in 5 mL of Sodium citrate solution till complete dissolution at room temperature and filtered through Whatman No. 1 filter paper. Further, DPPH procedure according to (section 3.5.1) was employed, where the absorbance was analyzed at 517 nm (Deladino *et al*., 2008).

3.8.5. Scanning Electron Microscopy (SEM) and Energy Dispersive X- Ray Analysis of encapsulated powder.

The morphological structure of the encapsulated powder was analyzed under optimal conditions using Scanning Electron Microscopy (SEM). All the samples of encapsulated powder were coated using spurr by vacuum evaporator and analyzed using SEM at 20 kV with a working distance of 9.5 mm. The scanned images were taken digitally using XT Microscope software. In addition to imaging the surface morphological structure, elemental analysis was also determined by EDX (Energy Dispersive X-ray) detector. EDX spectrum of the samples was analyzed, by Quanta FEG 250 (Carneiro *et al*., 2013).

CHAPTER 4. RESULTS AND DISCUSSION

This chapter presents the research findings of the present study alongside pertinent discussions. The results are organized in alignment with the study objectives and are illustrated using section-wise Figures and Tables. Each section delves into specific aspects of the study, offering insights and interpretations that contribute to a comprehensive understanding of the research outcomes. These findings underscore the significance of *Annona muricata* as a valuable source of natural compounds with diverse health-promoting properties. While all parts of the *Annona muricata* tree are recognized for their curative benefits, our study specifically focused on the leaves of *Annona muricata*. This deliberate choice was made to explore the bioactive potential and extraction methods tailored to this plant component, aiming to harness its therapeutic properties effectively. By narrowing our focus to the leaves, we aimed to deepen our understanding of their phytochemical composition and optimize extraction techniques for enhanced utilization in pharmaceutical and nutraceutical applications (**Plate 4.1**).

4.1. ANALYTICAL STUDIES OF LEAF EXTRACTS

4.1.1. Determination of moisture content of *Annona muricata* leaves.

There was minimal variation observed in the moisture content among shade-dried, freeze-dried, and oven-dried leaves, with the highest and lowest moisture content recorded at 69.59 ± 0.166 and 66.88 ± 0.162, respectively. Freeze-dried leaves exhibited slightly higher moisture content compared to oven-dried leaves, while shade-dried leaves maintained an optimal moisture content of 67.92 ± 0.065.

During the drying process, factors such as pressure, temperature, light, and heat influenced the moisture content. Freeze-drying involved drying at -107°C under high vacuum pressure, resulting in moisture removal. Conversely, oven-drying at 45°C led to

Plate 4.1: ***Annona muricata*** **(a) Habit, (b) Leaves, (c) Bud, (d) Flower, (e) Fruit**

moisture loss due to exposure to heat. In contrast, shade-drying at room temperature without heat or pressure preserved an optimal moisture level.

These findings suggest that the shade-drying method is preferable for maintaining the ideal moisture content of leaves. This conclusion aligns with the results of previous studies, such as that conducted by Babu *et al*. (2018), which also advocated for shade drying as the optimal procedure for preserving bioactive compounds during leaf extraction **(Table 4.1) (Fig. 4.1).**

4.1.2. Determination of pH of *A. muricata* leaf extracts

There was a notable difference in pH values among all five solvent combinations of shade-dried, freeze-dried, and oven-dried leaf extracts, with the highest and lowest pH values recorded at 7.05 ± 0.005 and 5.21 ± 0.005 in T1 and T4 macerated leaf extracts of shade-dried and oven-dried samples, respectively.

Throughout the extraction process, physical variables such as increased temperatures, applied force, and the choice of solvent combinations influenced the pH values of the leaf extracts. Elevated temperatures tend to increase acidity, leading to a gradual decrease in pH values, resulting in the oven-dried extract exhibiting the lowest pH values compared to shade-dried and freeze-dried extracts.

These findings mirror those of a similar evaluation study conducted by Mphahlele *et al*. (2016), which explored the effects of extraction methods and elevated temperatures on pH levels. Their study also observed significant pH variation in pomegranate juice extracts, highlighting the impact of extraction conditions on pH values **(Table 4.2) (Fig. 4.2).**

4.1.3. Determination of Total Extract Yield of *A. muricata* leaf extracts

A total of five solvent combinations (T1, T2, T3, T4, & T5) and three types of drying methods were utilized to determine the total extract yield of the leaf extract. In this study, all the Soxhlet extracts exhibited higher total extract yield compared to the macerated extracts.

The Total Extract Yield (TEY) of macerated leaf samples in different individual solvents and solvent combinations were obtained in the following order: 100% Methanol (T2) > 100% Ethanol (T4) > 80% Methanol (T3) > 80% Ethanol (T5) > 100% Distilled water (T1). Across all drying methods, i.e. shade dry, freeze dry, and oven-dry, 100% Methanol (T2) yielded the highest TEY, with values of 7.01 ± 0.251, 6.59 ± 0.404, and 6.27 ± 0.404, respectively. Whereas 100% Distilled water (T1), 80% Methanol (T3) & 80% Ethanol (T5) yielded lowest in all drying methods with 4.42 ± 0.152, 4.57 ± 0.2 and, 5.19 ± 0.251 in oven drying. 4.49 ± 0.3, 5.66 ± 0.404 and 5.55 ± 0.360 in freeze drying.

On the other hand, 100% Distilled water (T1), 80% Methanol (T3), and 80% Ethanol (T5) yielded the lowest TEY in all drying methods, with values of 4.42 ± 0.152, 4.57 ± 0.2, and 5.19 ± 0.251 in oven drying, and 4.49 ± 0.3, 5.66 ± 0.404, and 5.55 ± 0.360 in freeze drying. The high total extract yield with 100% Methanol (T2) can be attributed to the presence of more methanol-soluble compounds compared to other solvents. This finding aligns with previous research where methanol demonstrated the maximum yield of extract for all plant parts of *Annona muricata* compared to other solvents. It is suggested that the phytochemicals extracted by methanol contain polar properties, whereas other solvents may extract compounds with semi-polar properties (Naik and Sellappan, 2019) **(Table 4.3) (Fig 4.3) and (Plate 4.2).**

Table 4.1 Determination of the moisture content of *Annona muricata* leaves under shade drying, Freeze drying and Oven drying. Data represented mean values ± Standard deviation (n=3).

Sr. No.	*Type of drying*	*Moisture content*
1.	Shade drying	67.92 ± 0.065
2.	Freeze drying	69.59 ± 0.166
3.	Oven drying	66.88 ± 0.162

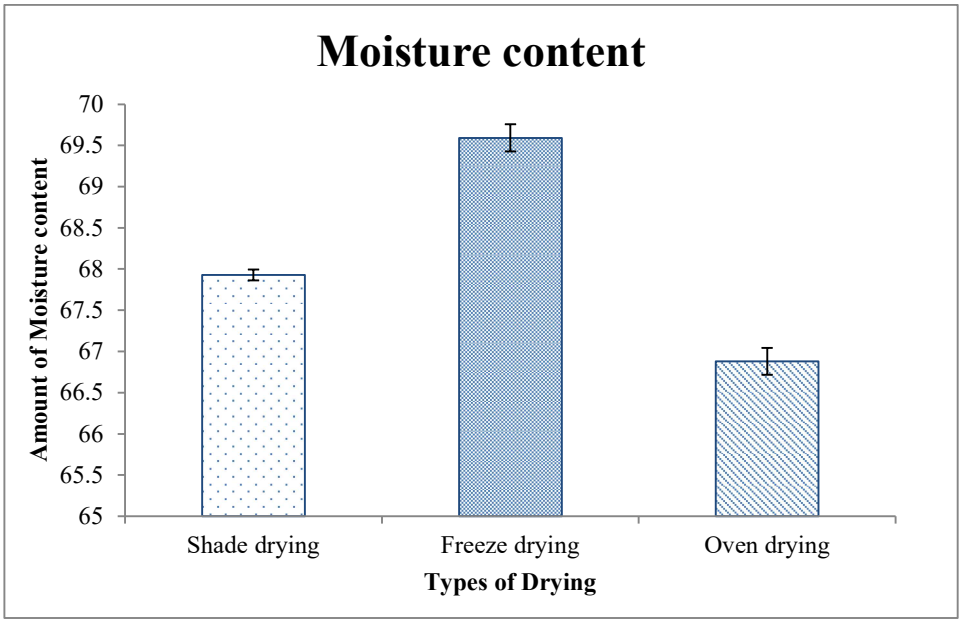


Fig. 4.1 Determination of the moisture content of *Annona muricata* leaves using shade drying, freeze drying and oven drying methods

Table 4.2 Determination of pH of *Annona muricata* leaf extracts in different solvents (T1= 100% Distilled water leaf extract, T2= 100% Methanolic leaf extract, T3= 80% Methanol+20% Water leaf extract, T4=100% Ethanol leaf extract, T5=80% Ethanol + 20% Water leaf extract).

	pH in different solvent combinations (Note: Data represented mean values ± standard deviation (n=3))				
Type of drying	**T1**	**T2**	**T3**	**T4**	**T5**
Shade drying	7.05 ± 0.005	5.84 ± 0.005	6.02 ± 0.005	5.29 ± 0.005	5.85 ± 0.005
Freeze drying	7.03 ± 0.005	5.86 ± 0.005	6.17 ± 0.005	5.30 ± 0.011	5.73 ± 0.005
Oven drying	7.02 ± 0.005	5.59 ± 0.005	5.97 ± 0.005	5.21 ± 0.005	5.59 ± 0.005

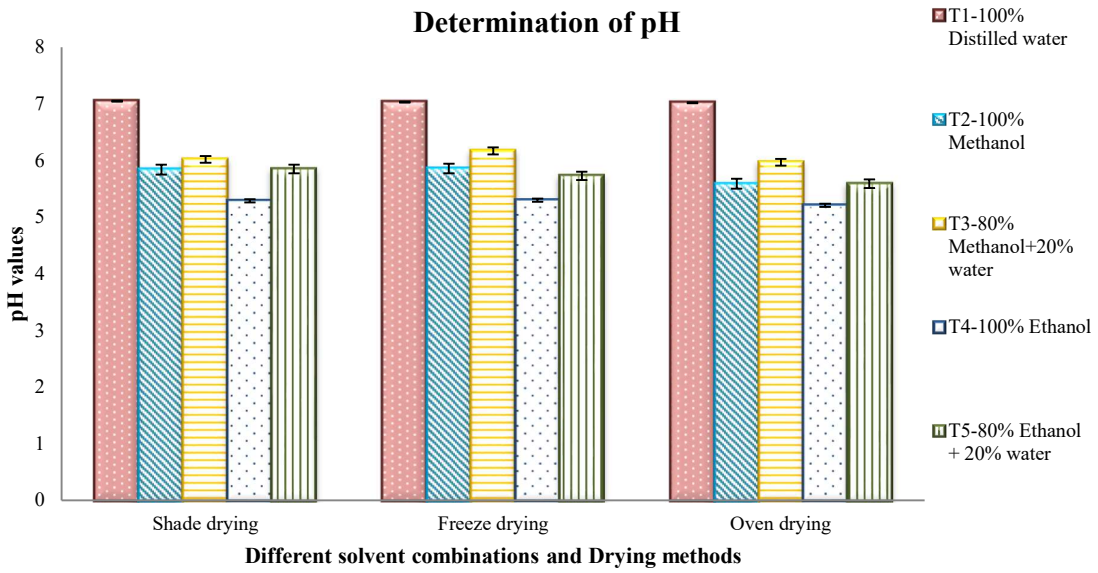


Fig.4.2 Determination of pH of *Annona muricata* leaf extracts using various solvent combinations and drying methods.

Table 4.3 Determination of Total Extract Yield (TEY) of *A. muricata* leaf extract using various solvent combinations a (T1= 100% Distilled water extract, T2= 100% Methanolic extract, T3= 80% Methanol+20% Water leaf extract, T4=100% Ethanol extract, T5=80% Ethanol + 20% Water extract).

Drying methods	***TEY % in different solvent combinations (Note: data represented mean values ± standard deviation (n=3))***.				
	T1	**T2**	**T3**	**T4**	**T5**
Shade dry	5.63 ± 0.351	7.01 ± 0.251	5.79 ± 0.208	6.74 ± 0.264	5.77 ± 0.251
Freeze Dry	4.49 ± 0.3	6.59 ± 0.404	5.66 ± 0.404	6.27 ± 0.251	5.55 ± 0.360
Oven Dry	4.42 ± 0.152	6.27 ± 0.404	4.57 ± 0.2	6.01± 0.4	5.19 ± 0.251

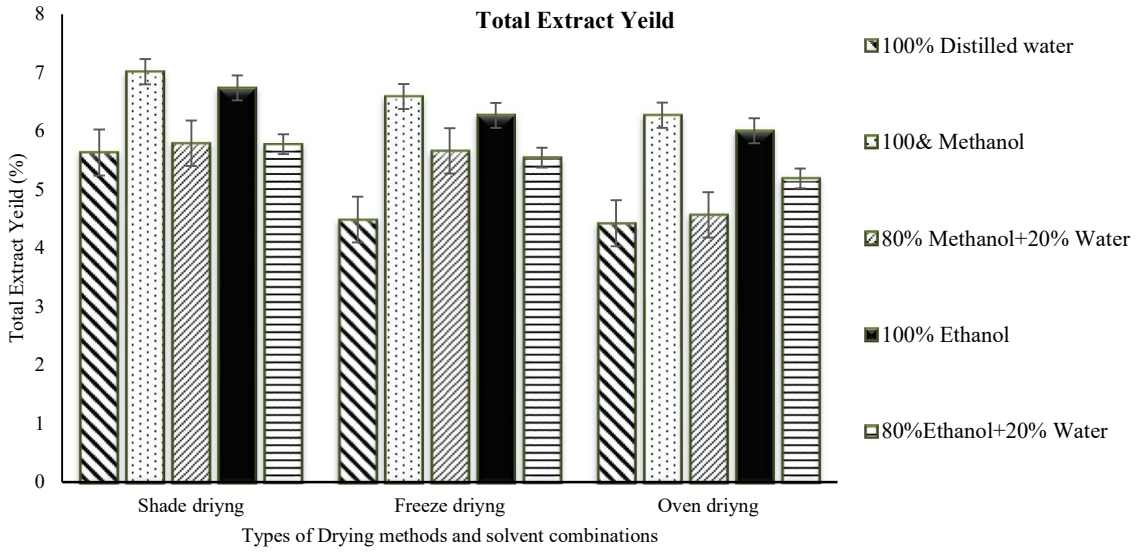


Fig. 4.3 Determination of Total Extract Yield (TEY) % of *Annona muricata* leaf extract using various solvent combinations and drying methods

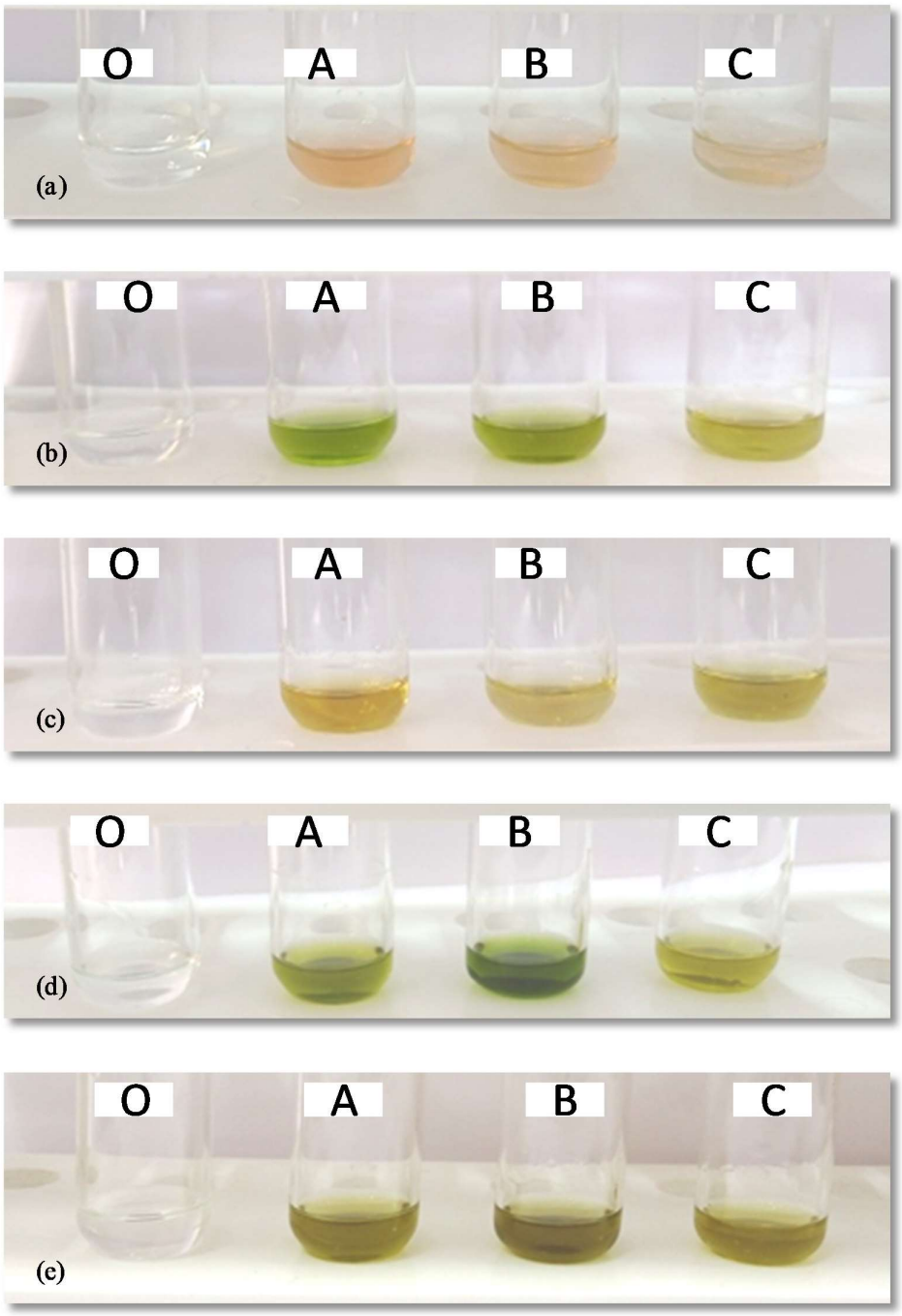


Plate 4.2: Leaf extracts of *Annona muricata* using solvent combinations and (A) shade drying, (B) Freeze drying, (c) Oven drying methods. (a) 100 %distilled water, (b) 100 % Methanol, (c) 80 % Methanol, (d) 100 % Ethanol (e) 80 % Ethanol. (O) is the control pure water without leaf extract.

4.1.4. Thin layer chromatography (TLC) analysis of *A. muricata* leaf extracts

4.1.4.a. TLC isolation of Alkaloids

The TLC plate was prepared and run using a specific solvent system **(Table 3.2)**, resulting in a total of 28 distinct bands. Among these bands, 7 were observed in extracts T2, T3, T4, and T5, while no bands were observed in T1. Notably, extracts T2 (100% methanol) and T4 (100% ethanol) exhibited more intense coloured bands compared to T3 (80% methanol) and T5 (80% ethanol).

The identification of bands was conducted by dipping them into Dragendorff's reagent, which revealed orange-coloured bands indicative of alkaloid compounds. Specifically, the band with an Rf value of 0.5 consistently showed the presence of alkaloids, exhibiting an orange colour under visible light in all solvent combinations (T2, T3, T4, and T5). These findings align with previous Thin Layer Chromatographic analyses conducted by Wagner and Bladt (1996), which confirmed that alkaloids typically exhibit an Rf value of 0.5 **(Table 4.4) (Plate 4.3).**

4.1.4.b. TLC isolation of phenols

The TLC plate was prepared and run using a specific solvent system **(Table 3.2)**, resulting in a total of 28 distinct bands. Notably, 1 band was observed in T0 (standard Gallic acid), while the maximum of 7 bands were observed in T3 and T5. Conversely, the least number of bands was observed in T1. T3 (100% methanol) and T5 (100% ethanol) exhibited more intense coloured bands compared to T3 (80% methanol) and T5 (80% ethanol).

Identification of the bands was performed by dipping them into a 10% Ferric chloride solution, revealing grey or black spots indicative of phenols under visible light. The standard gallic acid band, with an Rf value of 0.88, appeared dark blue in visible light, confirming

that corresponding bands with the same Rf value were of phenolic compounds across all solvents **(Table 4.5), (Plate 4.4).**

4.1.4.c. TLC isolation of Flavonoids

The TLC plate was prepared and run using a specific solvent system **(Table 3.2)**, resulting in a total of 30 distinct bands. The highest number of bands was observed in T2 (100% methanol), while only 1 band was observed in T1 (100% distilled water). An equal number of bands were observed in T3, T4, and T5. T2 (100% methanol) and T4 (100% ethanol) exhibited more intense coloured bands compared to T3 (80% methanol) and T5 (80% ethanol).

Identification of the bands was conducted by dipping them into a 10% Methanolic sulphuric acid reagent, revealing fluorescent bands of flavonoids under long UV light. The band with an Rf value of 0.8 appeared fluorescent red under long UV, confirming that the band consisted of flavonoids in all the solvents. These results were compared to Thin Layer Chromatographic analysis conducted by Wagner and Bladt (1996), which confirmed that the Rf value of flavonoids can indeed be 0.8 **(Table 4.6) (Plate 4.5).**

4.1.4.d TLC isolation of Acetogenins

The TLC plate was prepared and run using a specific solvent system **(Table 3.2)**, resulting in a total of 13 distinct bands. Three bands were observed in each of the solvent combinations T2, T3, T4, and T5, respectively, while only one band was observed in T1. Notably, T2 (100% methanol) and T4 (100% ethanol) exhibited more intense coloured bands compared to T3 (80% methanol) and T5 (80% ethanol).

Identification of the bands was performed by dipping them into a Vanillin sulphuric acid reagent, followed by heating the plate at 100°C for 5 minutes, which revealed dark blue

spots under visible light. The bands with Rf values of 0.7, 0.8, and 0.9 appeared dark blue under visible light after reagent dipping, confirming the presence of acetogenins.

These findings were compared with similar studies on bioactive compounds of *Annona muricata* leaf extracts using chromatographic techniques by Prayitno *et al*. (2016), which also detected acetogenins using vanillin sulphuric acid. They reported that *Annona muricata* leaves extracts contain acetogenins with functional groups like terpenoids and lactones, as well as tetrahydrofurane, which appear violet in color under visible light after dipping into the vanillin sulphuric acid **(Table 4.7) (Plate 4.6).**

Table 4.4 TLC isolation of Alkaloids

Solvent system	*Sample*	*Number of distinct bands*	*Rf value*	*Colour of band after reagent dipping under visible light*	*Possible compound*
Toluene: Ethyl acetate: Methanol: Ammonia 25% (30:30:15:1) v/v/v/v	T1(100%Distilled water extract)	0	0	0	0
	T2(100% methanolic extract)	7	0.89	Dark green	Unknown
			0.72	Light blue	Unknown
			0.68	Green	Unknown
			0.66	Light pink	Unknown
			0.59	Orange	Alkaloids
			0.47	Brown	Unknown
			0.40	Grey	Unknown
	T3 (80% methanol+20% water extract)	7	0.89	Dark green	Unknown
			0.72	Light blue	Unknown
			0.71	Green	Unknown
			0.66	Light pink	Unknown
			0.58	Orange	Alkaloids
			0.47	Brown	Unknown
			0.40	Grey	Unknown
	T4 (100% Ethanol extract)	7	0.89	Dark green	Unknown
			0.79	Light blue	Unknown
			0.74	Green	Unknown
			0.64	Light pink	Unknown
			0.5	Orange	Alkaloids
			0.47	Brown	Unknown
			0.39	Grey	Unknown
	T5 (80%ethanol + 20%water extract)	7	0.89	Dark green	Unknown
			0.78	Light blue	Unknown
			0.75	Green	Unknown
			0.66	Light pink	Unknown
			0.51	Orange	Alkaloids
			0.47	Brown	Unknown
			0.40	Grey	Unknown

Table 4.5 TLC isolation of Phenols

Solvent system	***Sample***	***Number of distinct bands***	***Rf value***	***Colour of band after reagent dipping under visible light***	***Possible compound***
	T0 (Gallic Acid)	1	0.88	Dark Blue	Phenols
Tetrahydrofuran: toluene: formic acid: water (16:8:2:1) v/v/v/v	T1(100%Distilled water extract)	1	0.78	Black Spot	Phenols
	T2(100% methanolic extract)	6	0.88	Blue	Phenols
			0.85	Yellow	Unknown
			0.78	Dark Blue	Phenols
			0.61	Blue	Phenols
			0.30	Brown	Unknown
			0.23	Grey	Unknown
	T3 (80% methanol+20% water extract)	7	0.96	Blackish Blue	Phenols
			0.88	Blue	Phenols
			0.85	Yellow	Unknown
			0.78	Dark Blue	Phenols
			0.61	Blue	Phenols
			0.30	Brown	Unknown
			0.23	Grey	Unknown
	T4 (100% Ethanol extract)	4	0.88	Blue	Phenols
			0.85	Yellow	Unknown
			0.78	Dark Blue	Phenols
			0.61	Blue	Phenols
	T5 (80%ethanol + 20%water extract)	7	0.96	Blackish Blue	Phenols
			0.88	Blue	Phenols
			0.85	Yellow	Unknown
			0.78	Dark Blue	Phenols
			0.61	Blue	Phenols
			0.30	Brown	Unknown
			0.23	Grey	Unknown

Table 4.6 TLC isolation of Flavonoids

Solvent system	***Sample***	***Number of distinct bands***	***Rf value***	***Colour of band after reagent dipping under long UV***	***Possible compound***
Tetrahydrofuran: toluene: formic acid: water (16:8:2:1) v/v/v/v	T1(100%Distilled water extract)	1	0.90	Light Blue	Unknown
	T2(100% methanolic extract)	8	0.84	Fluorescent Red	Flavonoids
			0.82	Black	Unknown
			0.80	Blue	Unknown
			0.77	Blue	Unknown
			0.71	Light Blue	Alkaloids
			0.61	Dark Blue	Unknown
			0.51	Navy Blue	
			0.39	Fluorescent Green	Flavonoids
	T3 (80% methanol+20% water extract)	7	0.90	Fluorescent Red	Flavonoids
			0.82	Black	Unknown
			0.78	Blue	Unknown
			0.72	Light Blue	Unknown
			0.64	Dark Blue	Unknown
			0.53	Navy Blue	Unknown
			0.42	Fluorescent Green	Flavonoids
	T4 (100% Ethanol extract)	7	0.88	Fluorescent Red	Flavonoids
			0.84	Black	Unknown
			0.78	Blue	Unknown
			0.72	Light Blue	Unknown
			0.69	Dark Blue	Unknown
			0.65	Navy Blue	Unknown
			0.52	Fluorescent Green	Flavonoids
	T5 (80%ethanol + 20%water extract)	7	0.89	Fluorescent Red	Flavonoids
			0.82	Black	Unknown
			0.78	Blue	Unknown
			0.71	Light Blue	Unknown
			0.63	Dark Blue	Unknown
			0.51	Navy Blue	Unknown
			0.38	Fluorescent	Flavonoids

Table 4.7 TLC isolation of Acetogenins

Solvent system	***Sample***	***Number of distinct bands***	***Rf value***	***Colour of band after reagent dipping under visible light***	***Possible compound***
Chloroform: methanol (9:1) v/v	T1(100%Distilled water extract)	1	0.95	Dark Blue	Acetogenin
	T2(100% methanolic extract)	3	0.78	Dark Blue	Acetogenin
			0.52	Dark Blue	Acetogenin
			0.28	Pink	Unknown
	T3 (80% methanol+20% water extract)	3	0.78	Dark Blue	Acetogenin
			0.52	Dark Blue	Acetogenin
			0.28	Pink	Unknown
	T4 (100% Ethanol extract)	3	0.76	Dark Blue	Acetogenin
			0.49	Dark Blue	Acetogenin
			0.29	Pink	Unknown
	T5 (80%ethanol + 20%water extract)	3	0.80	Dark Blue	Acetogenin
			0.56	Dark Blue	Acetogenin
			0.29	Pink	Unknown

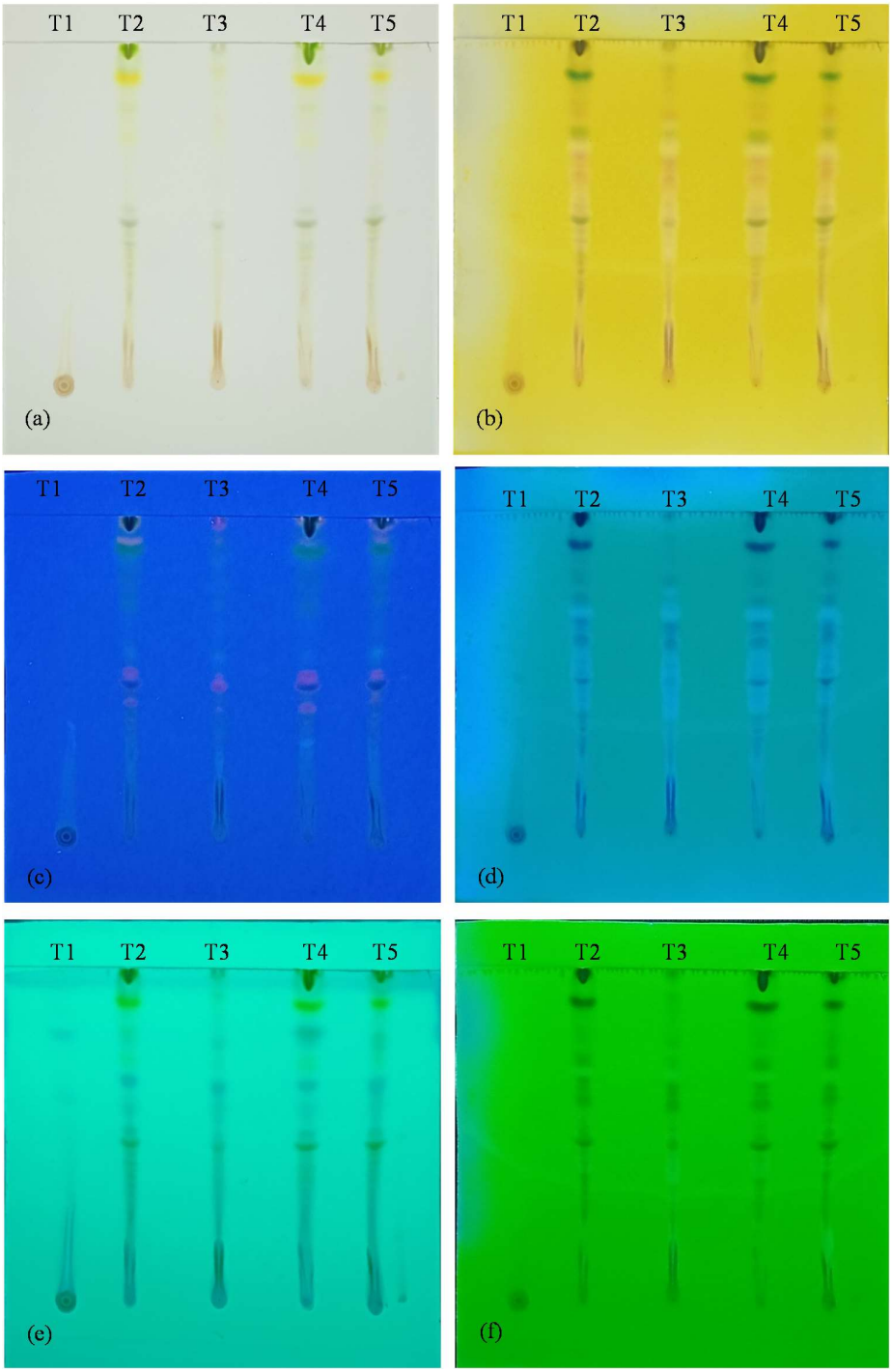


Plate 4.3: TLC isolation of alkaloids before and after derivatization a) under visible light before, b) under visible light after, c) under long UV before, d) under long UV after, e) short UV before & f) short UV after (T1= 100% Distilled water leaf extract, T2= 100% Methanolic leaf extract, T3= 80% Methanol+20% Water leaf extract, T4=100% Ethanol leaf extract, T5=80% Ethanol + 20% Water leaf extract).

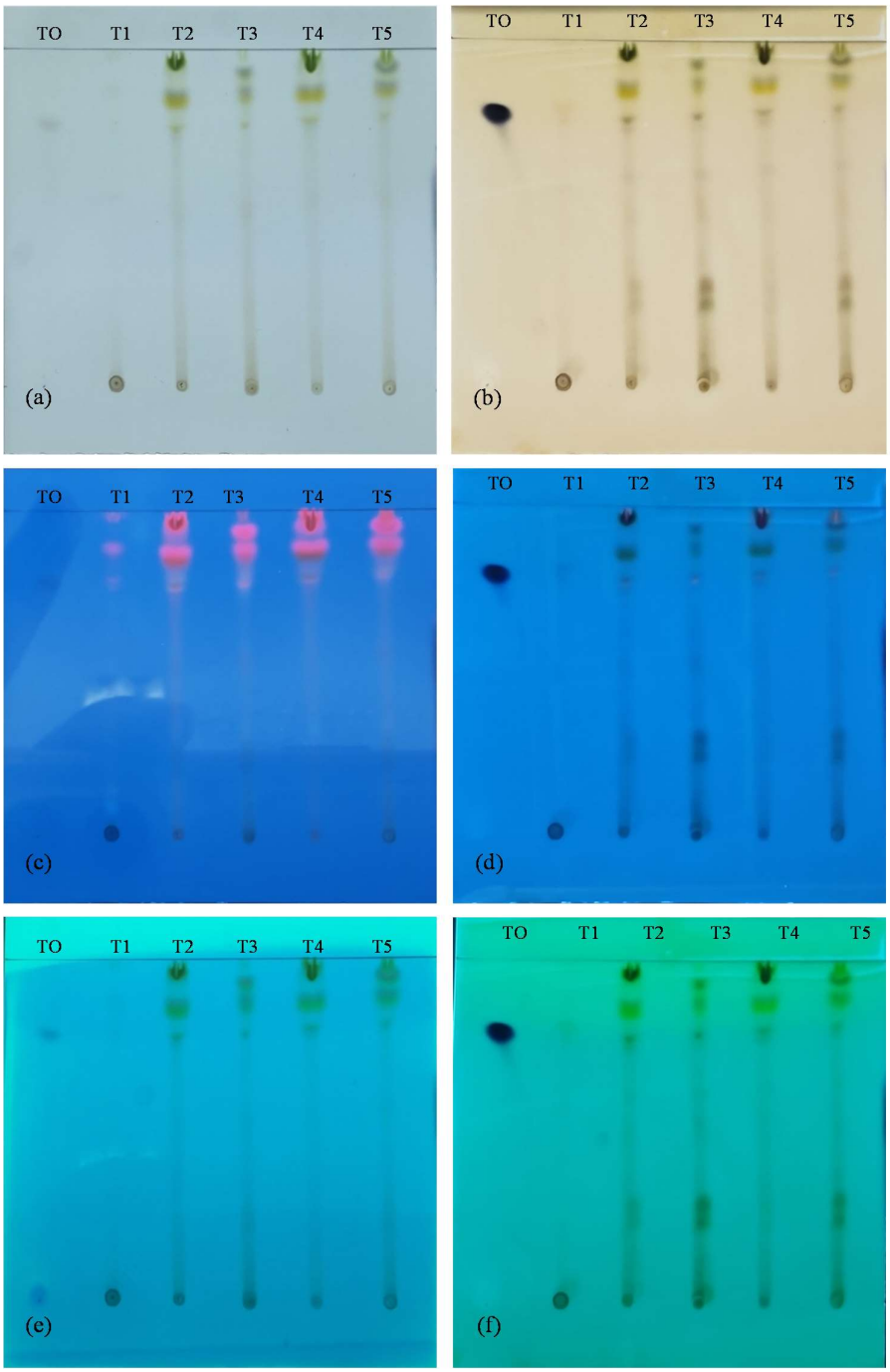


Plate 4.4: TLC isolation of phenolics before and after derivatization a) under visible light before, b) under visible light after, c) under long UV before, d) under long UV after, e) short UV before & f) short UV after (T1= 100% Distilled water leaf extract, T2= 100% Methanolic leaf extract, T3= 80% Methanol+20% Water leaf extract, T4=100% Ethanol leaf extract, T5=80% Ethanol + 20% Water leaf extract).

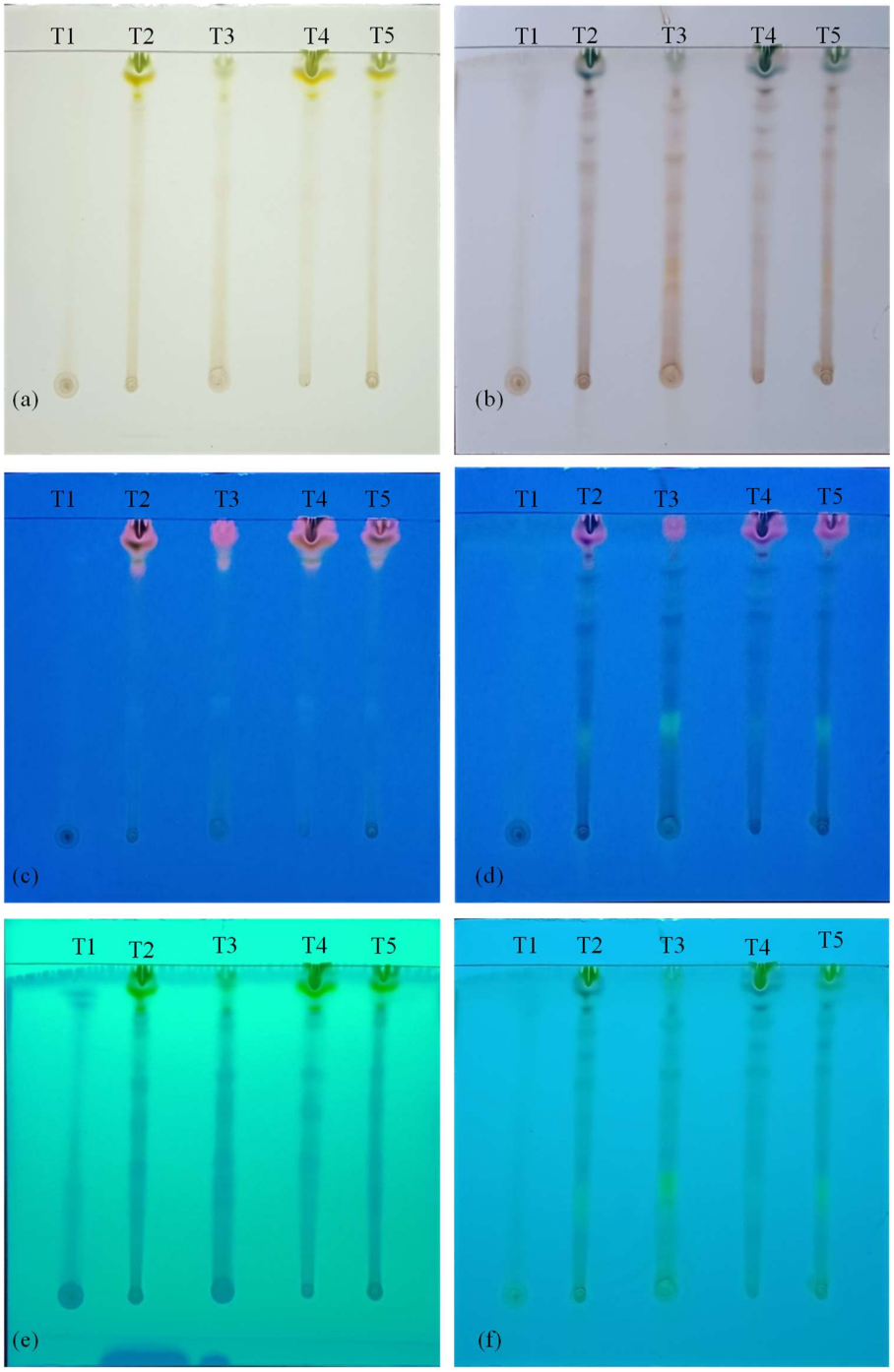


Plate 4.5: TLC isolation of flavonoids before and after derivatization a) under visible light before, b) under visible light after, c) under long UV before, d) under long UV after, e) UV before & f) short UV after (T1= 100% Distilled water leaf extract, T2= 100% Methanolic leaf extract, T3= 80% Methanol+20% Water leaf extract, T4=100% Ethanol leaf extract, T5=80% Ethanol + 20% Water leaf extract).

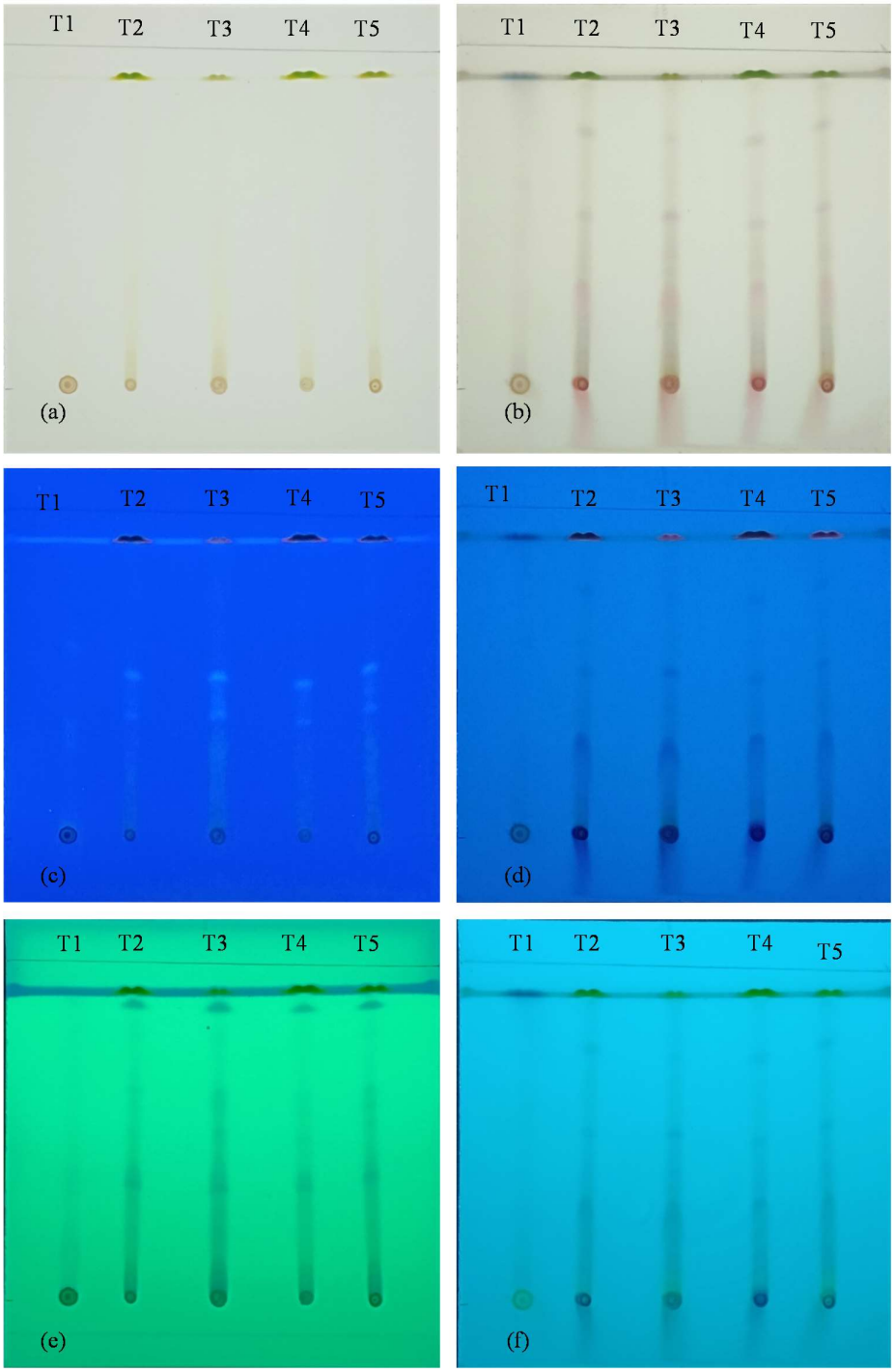


Plate 4.6: TLC isolation of Acetogenins before and after derivatization before and after derivatization a) under visible light before, b) under visible light after, c) under long UV before, d) under long UV after, e) short UV before & f) short UV after (T1= 100% Distilled water leaf extract, T2= 100% Methanolic leaf extract, T3= 80% Methanol, T4=100% Ethanol leaf extract, T5=80% Ethanol + 20% Water leaf extract).

4.2. PHYTOCHEMICAL ESTIMATION OF SECONDARY METABOLITES

4.2.1. Determination of Total Phenolic Contents of extracts

The total phenol content (TPC) of leaf extract with different solvent combinations was measured using Folin Ciocalteu's method. Phenol compounds in plants are associated with antioxidant activities because phenolic compounds contain redox properties hence, they behave like singlet oxygen quencher, hydrogen donor, and reducing agent (Choi *et al*., 2020). T2 (100% Methanol) extract had the highest amount of total phenolic contents in shade drying (1.10 ±0.007), Freeze drying (0.89 ± 0.002), and Oven drying (0.72 ± 0.002) per mg of GAE respectively. Whereas minimum total phenolic concentration was found in T1 (100% distilled water). The extractability of the phenolic compounds is dependent upon the polarity of the phenolic compound. The above results are compared with the evaluations done by Naik and Sellappan (2019) on physicochemical and phytochemical analysis of different plant parts of *Annona muricata* using different solvents and concluded that methanolic extract contains the highest yield of extract and polyphenols. Another researcher Babbar *et al*. (2014) also determined a correlation between polyphenols and *in vitro* antioxidant activity.

Based on the above data shade of drying showed the presence of the highest amount of total phenolic compound, extract yield, optimum moisture content, and pH. The data suggest that shade drying is the best type of drying to maintain the optimum phytochemical and physiochemical properties of *Annona muricata* leaf extracts. Therefore, shade drying was further subjected to the determination of acetogenin content and antioxidant activity. The findings of the present study were compared with a similar study done by Babu *et al*. (2018) which suggested that shade drying is the best method for the extraction of bioactive compounds **(Table 4.8) (Fig. 4.4, 4.5).**

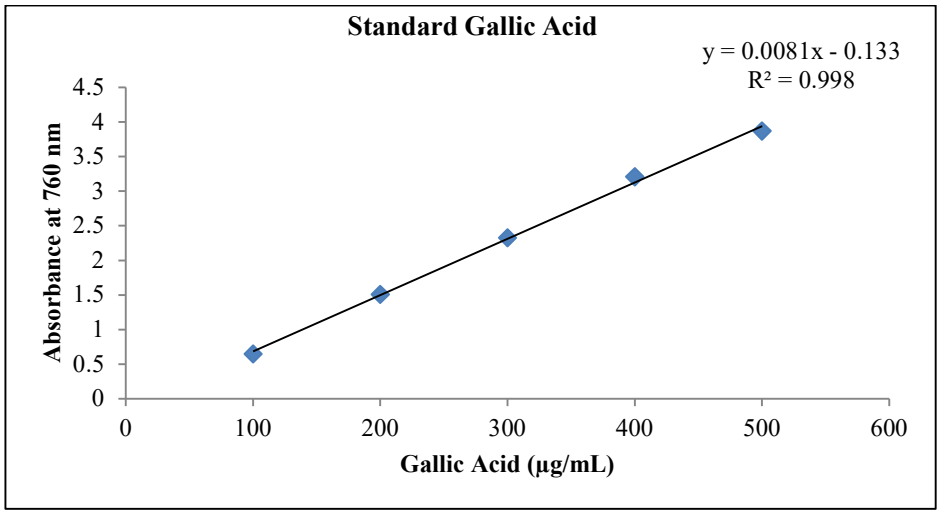


Fig. 4.4 Calibration curve for Gallic acid for determination of Total Phenolic Content (mg of Gallic Acid equivalents (GAE)/g of extracts) at varying concentration.

Table 4.8 Total Phenolic Content (TPC) in T1 (100% distilled water), T2 (100% methanol), T3 (80% methanol), T4 (100% ethanol) and T5 (80% ethanol) leaf extract of *Annona muricata* by different drying methods. Data represented mean values ± Standard deviation (n=3).

Drying	***Concentration of TPC mg of Gallic Acid equivalents (GAE)***				
	T1	**T2**	**T3**	**T4**	**T5**
Shade Drying	17.27 ± 0.005	17.73 ± 0.007	17.65 ± 0.01	17.4 ± 0.001	17.6 ± 0.007
Freeze Drying	17.15 ± 0.007	17.51 ± 0.002	17.41 ± 0.001	17.27 ± 0.020	17.41 ± 0.004
Oven Drying	17.09 ± 0.02	17.35 ± 0.002	17.30 ± 0.006	17.07 ± 0.011	17.28 ± 0.003

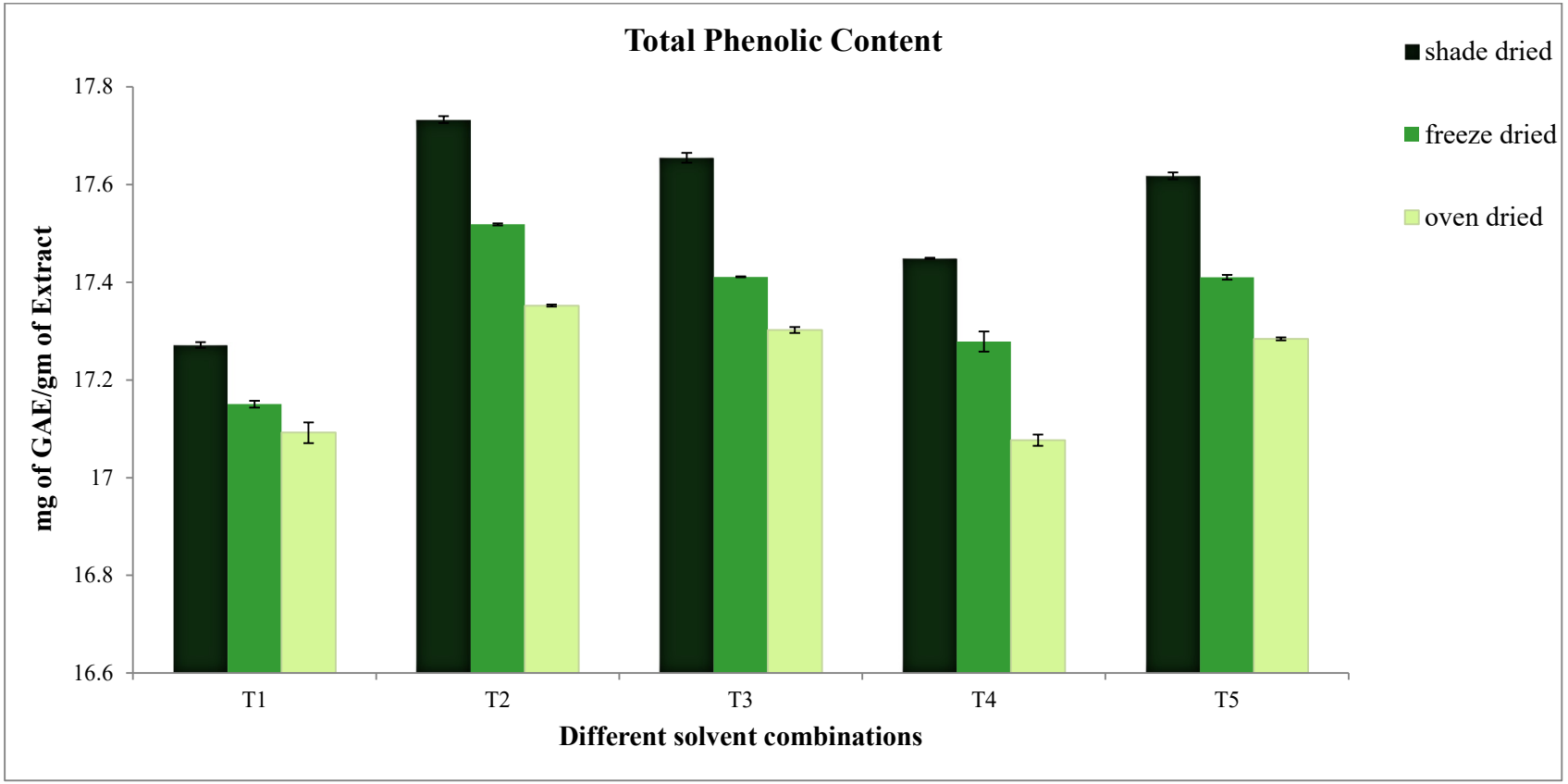


Fig. 4.5 Determination of Total Phenolic Content (TPC) in mg of Gallic Acid equivalent (GAE)/g of *Annona muricata* leaf extract in different solvent combinations and drying methods. (Note: T1 (100% distilled water), T2 (100% methanol), T3 (80% methanol + 20% Water), T4 (100% ethanol) and T5 (80% ethanol + 20% Water) leaf extract of *Annona muricata* by different drying methods. Data represented mean values ± Standard deviation (n=3)).

4.2.2. Determination of Acetogenin Contents

The acetogenin content present in the leaf extract with different solvent combinations was determined using Annonacin standard by spectrophotometric analysis. Among the solvent combinations tested, T2 (100% Methanol) exhibited the highest concentration of acetogenins, followed by T4 (100% Ethanol), while the lowest amount was detected in T1 (distilled water).

These findings align with a study conducted by López-Romero *et al*. (2022), which indicated that seeds of *Annona muricata* contain a higher amount of acetogenins, with solubility in the order of methanol > ethanol > water. Additionally, the study established a correlation between the acetogenin content and the bioactivity of the extracts, suggesting that higher acetogenin content leads to increased bioactivity **(Table 4.9) (Fig. 4.6, 4.7).**

4.2.3. DPPH radical scavenging activity

DPPH is a stable, purple-coloured compound with an absorbance at 517 nm, which is reduced upon receiving an electron or proton from an antioxidant, resulting in the formation of 2,2-diphenyl-1-picrylhydrazine, a yellow or colourless compound. Antioxidants, crucial in disease prevention, function as free radical scavengers, prooxidant metal complexes, singlet oxygen quenchers, and reducing agents (Mishra *et al*., 2012). The study demonstrated an inverse relationship between antioxidant potential and the IC_{50} value, as absorbance values decreased with increasing extract concentration, leading to a rise in percentage inhibition.

Using the leaf extract of shade-dried *Annona muricata* with different solvent combinations, the inhibitory action against the DPPH radical was examined. The radical scavenging activity of the extracts, ranked from highest to lowest, was observed as follows: T2 (100% Methanol) > T4 (100% Ethanol) > T5 (80% Ethanol + 20% Water) > T3 (80%

Methanol + 20% Water) > T1 (100% Distilled water). T2 (100% Methanol) exhibited the lowest IC_{50} value of 116 µg/mL, while T1 (100% Distilled water) showed the highest IC_{50} value of 439 µg/mL.

These findings are consistent with conclusions drawn by Romero *et al*. (2022), indicating that the methanolic seed extract of *Annona muricata* displays superior reducing power compared to the distilled water seed extract, with a lower IC50 value for in vitro antioxidant activity.

Phenolic compounds, abundant in plants, exhibit potent antioxidant and free radical scavenging properties, contributing to defence mechanisms. These compounds function through various radical quenching mechanisms, including metal scavenging and electron transfer. Higher molecular weight phenolic compounds possess greater radical neutralization capacity. The observed results in the DPPH assay may be attributed to the presence of polyphenols and other phytoconstituents, which can mitigate oxidative damage associated with diseases such as AIDS, cancer, cataracts, and neurological ailments (Nirmaladevi et al., 2021).

Based on total phenolic contents, total acetogenin content, and DPPH radical scavenging activity, the leaf extracts exhibiting the most promising results, namely T1 (100% Distilled water) and T2 (100% Methanol), were selected for further encapsulation via emulsion preparation **(Table 4.10) (Fig. 4.8, 4.9 & 4.10).**

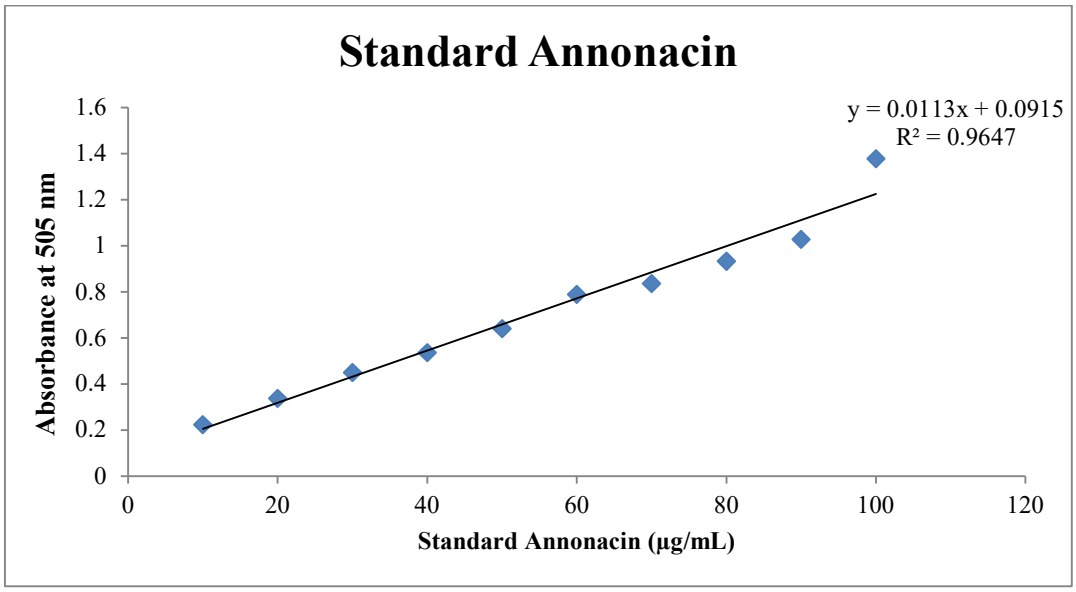


Fig 4.6 Calibration curve for Annonacin Standard for determination of total Acetogenin content (mg of Annonacin equivalents g of extracts) at varying concentration.

Table 4.9 Total amount of Acetogenin content in (T1) 100% Distilled water extract, (T2) 100% Methanolic extract, (T3) 80% Methanol+20% Water leaf extract, (T4) 100% Ethanol extract, (T5) 80% Ethanol + 20% Water extract leaf extract of *Annona muricata* by shade drying. Data represented mean values ± Standard deviation (n=3).

Solvent combination	*Concentration(µg/ml)*	*Total acetogenin content (mg of Annonacin/g of extract)*
T1	1000	37.81 ± 0.013
T2	1000	108.9 ± 0.062
T3	1000	72.09 ± 0.011
T4	1000	97.12 ± 0.065
T5	1000	61.03 ± 0.013

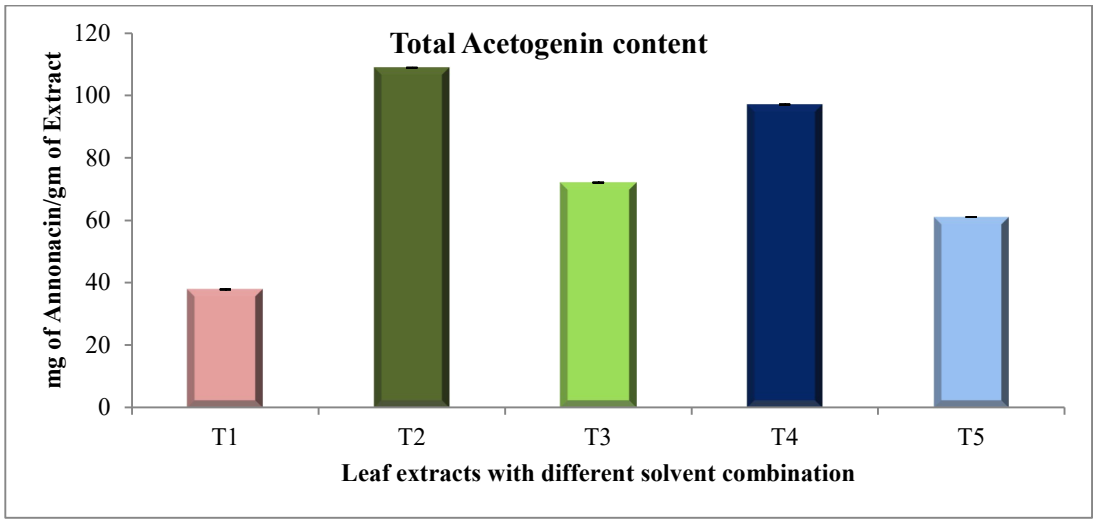


Fig. 4.7 Determination of Total Acetogenin in mg of Annonacin of *A. muricata* leaf extract in different solvent combinations and drying methods. (T1-100% Distilled water, T2-100% Methanol, T3-80% Methanol, T4-100% Ethanol and T5-80% Ethanol).

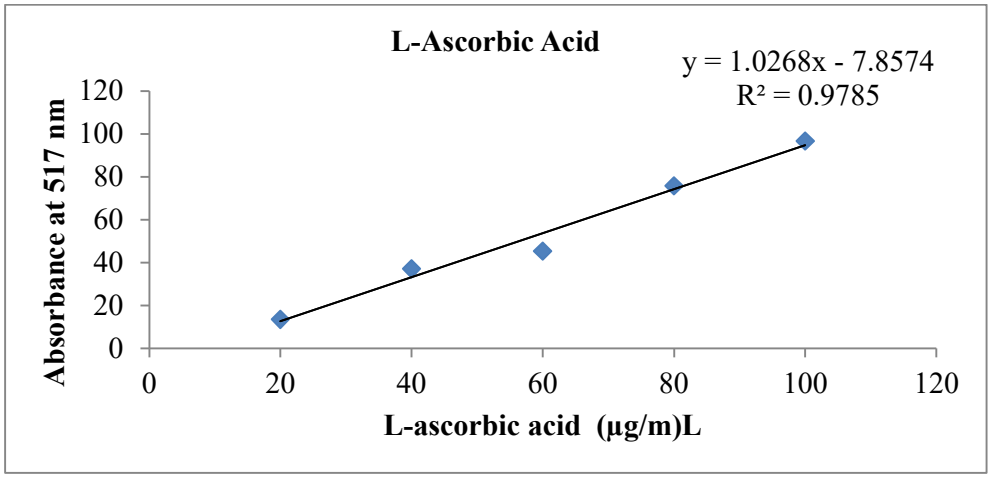


Fig 4.8 DPPH radical scavenging activity of L-Ascorbic Acid at varying concentrations.

Table 4.10 DPPH free radical scavenging assay % scavenging activity of DPPH by L-Ascorbic acid and leaf extracts of *Annona muricata* with different solvent combinations. Data represented mean values ± Standard deviation (n=3).

Sr. No.	*Concentration (µg/mL)*	*L-Ascorbic Acid*	*Leaf extracts with different solvent combinations*				
			T1	**T2**	**T3**	**T4**	**T5**
1	20	13.51 ± 0.003	28.88 ± 0.001	28.61 ± 0.002	26.57 ± 0.003	26.38 ± 0.002	27.59 ± 0.002
2	40	37.12 ± 0.001	30 ± 0.002	31.94 ± 0.002	29.16 ± 0.002	28.98 ± 0.002	28.79 ± 0.001
3	60	45.37 ± 0.002	31.48 ± 0.001	36.01 ± 0.003	29.53 ± 0.003	31.85 ± 0.002	32.68 ± 0.001
4	80	75.92 ± 0.002	32.12 ± 0.001	40.92 ± 0.002	31.85 ± 0.002	35.92 ± 0.002	34.44 ± 0.002
5	100	96.79 ± 0.015	32.87 ± 0.001	47.5 ± 0.003	33.51 ± 0.002	36.94 ± 0.002	37.5 ± 0.003

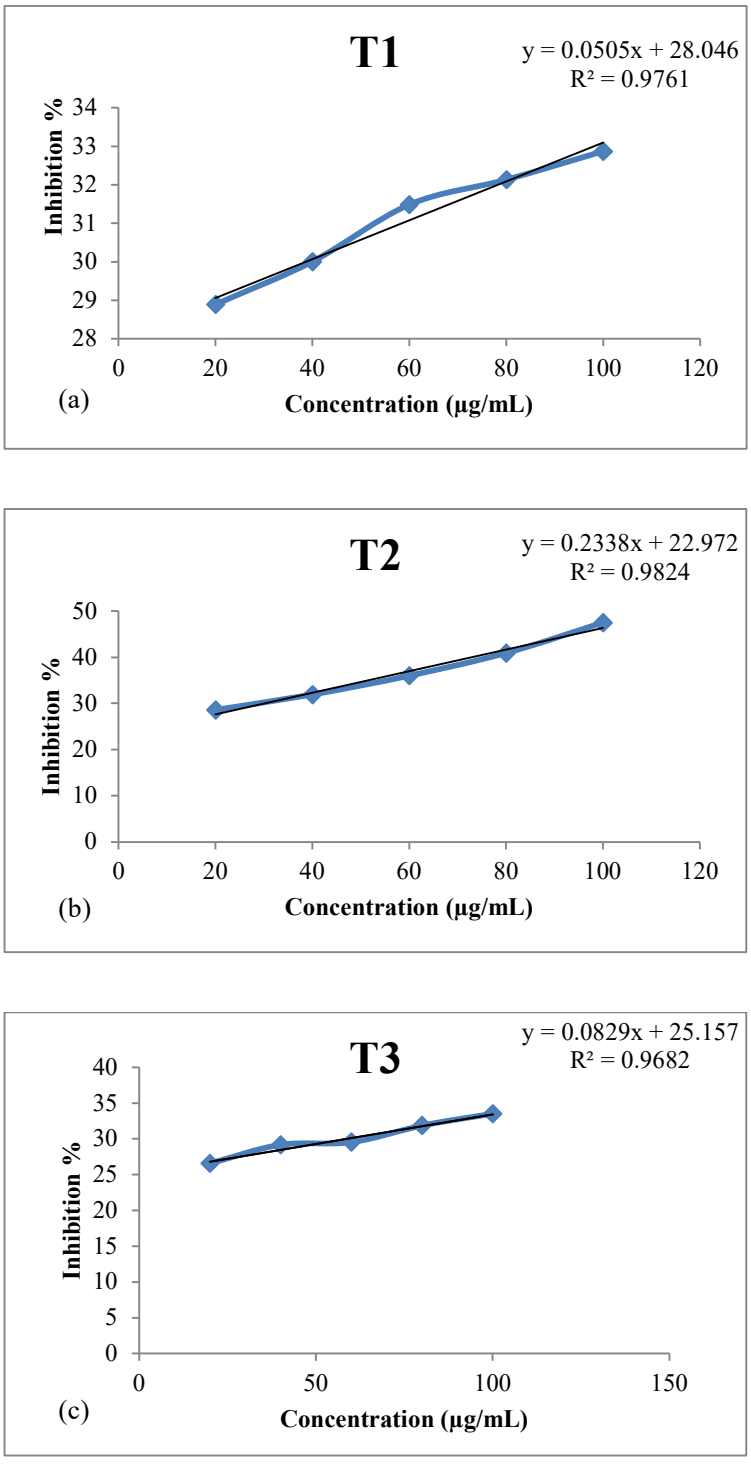


Fig 4.9. DPPH radical scavenging activity of (a) T1-100% distilled water, (b) T2-100% methanol, (c) T3- 80 % methanol of shade dried leaf extract of *Annona muricata*.

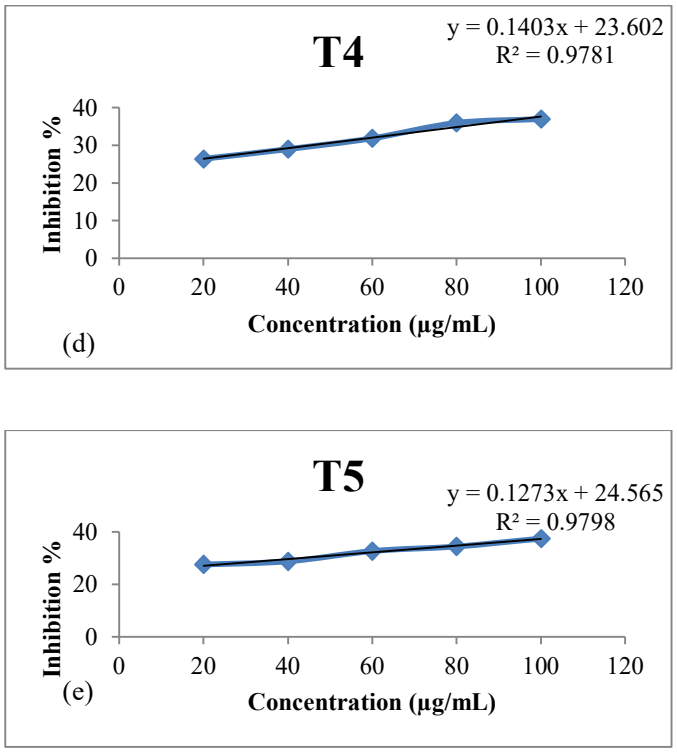


Fig 4.9 DPPH radical scavenging activity of (d) T4-100% Ethanol, (e) T5- 80% ethanol of shade dried leaf extract of *Annona muricata*.

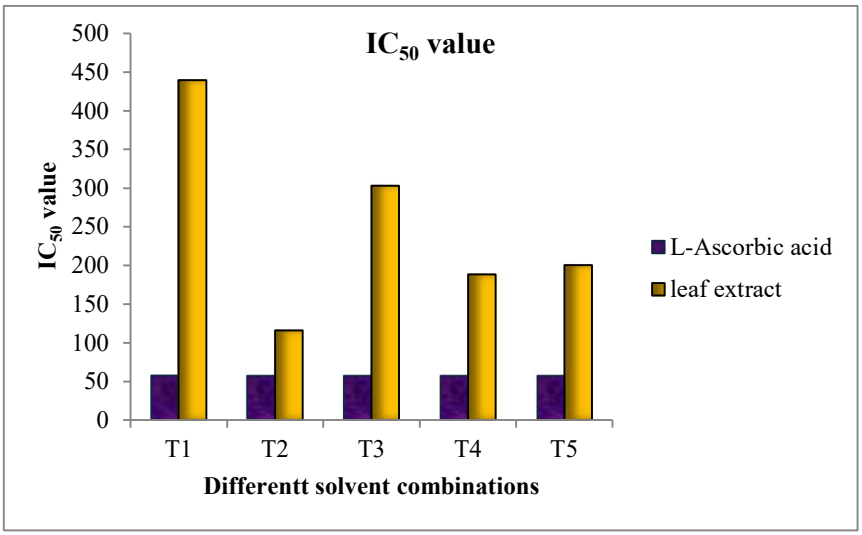


Fig 4.10 Comparison between IC_{50} value of L-ascorbic Acid and shade dried leaf extract of *Annona muricata* in different solvent combination (T1, T2, T3, and T4 & T5).

4.3. CHARACTERIZATION OF EMULSION

4.3.1. Particle size assessment of emulsion

The particle size of all the emulsion samples with *Annona muricata* leaf extracts and 1% lecithin was done by Dynamic Light Scattering. The analyzed particle size of all the emulsion samples ranged between 6943.47 ± 0.003 to 250 ± 0.005 nm in diameter. The highest particle size was observed in S1 control for 1:2 sodium alginate and Gum Arabic. Whereas the lowest particle size was seen in S4, control for 2: 1 Gum Arabic and sodium alginate. Both encapsulated samples containing extract showed lower particle size in 1:2 sodium alginate and inulin compared to that of control, contrasting to that both the encapsulated sample containing 2:1 Gum Arabic: sodium alginate showed higher particle size than the control **(Fig.4.11), (Plate 4.7) and (Plate 4.8**).

The size of the particle is affected by several factors such as drying temperatures, moisture content, and pressure hence there is a huge variation in size of the encapsulates. Present study shows similarity with the study reported by Bashir *et al*. (2024) suggesting that freeze drying causes an increase in particle size compared to spray drying and Gum Arabic is best to maintain the particle size and better encapsulation. Another researcher Jordán-Suárez *et al*. (2018) conducted the investigations on microstructure *Annona muricata* leaf extract microcapsules, reported that the microstructure of the microcapsules was closely linked to the concentration of the encapsulants which in turn determine the physical and chemical characteristics of the encapsulated powder.

4.3.2. Emulsion Stability Index (ESI)

The emulsion stability index was analyzed after the time of homogenization to check the stability of encapsulated *Annona muricata* leaf extract samples at intervals of 24 hours, 72 hours, and 7 days. The stability index of the emulsion samples was analyzed at the

interval of 24 hours and showed very little precipitation in the S1(1:2 sodium alginate: inulin), S2(1:2 sodium alginate: inulin: distilled water leaf extract), S4 (2:1 Gum Arabic: sodium alginate), and S6 (2:1 Gum Arabic: sodium alginate: methanolic leaf extract). Whereas there was no precipitation in the S5 (2:1 Gum Arabic: sodium alginate: distilled water leaf extract) sample with ESI 99.33 ± 0.1. The maximum amount of precipitation observed in the S3 (1:2 sodium alginate: inulin: 100 % methanolic leaf extract) sample with ESI is 99.33 ± 0.1. The emulsion stability index of all the extracts decreased with an increase in time interval. Out of all the emulsion samples S2 and S3 containing distilled water extract showed higher ESI % and sample S3 showed lowest ESI %. Sample S1 and S4 without leaf extract showed less ESI % compared to the distilled water extract. The observation depicted that the leaf extract of *Annona muricata* plays a vital role in the stability of extracts **(Table 4.11) (Fig.4.11)**.

The stability was affected by the ratios of wall material and plant extracts. The results were compared with the study done by Jarzębski *et al*. (2019) which concluded that the emulsion with the hemp oil with pea protein stabilizer has high stability. The encapsulating material also affects the stability of the emulsions. Hence the emusions containing the methanolic leaf extract in both the wall material combinations showed lower emulsion stability compared to the distilled water extract containing emulsions.

Table 4.11 Emulsion Stability Index (%) of emulsion with leaf extracts at the interval of 24 hours, 4 days, & 7 days after the time of homogenization

Sample name	*ESI (24 h) ± SD %*	*ESI (Day 4) ± SD %*	*ESI (day 7) ± SD %*
S1	96 ± 0.5	92.33 ± 0.1	82 ± 0.2
S2	96 ± 0.2	94.66 ± 0.1	94 ± 0.2
S3	75.66 ± 0.2	64.33 ± 0.1	55.33 ± 0.2
S4	98 ± 0.2	96.33 ± 0.1	82.66 ± 0.2
S5	99.33 ± 0.1	96.66 ± 0.1	94 ± 0.2
S6	94 ± 0.1	90.66 ± 0.1	84.66 ± 0.3

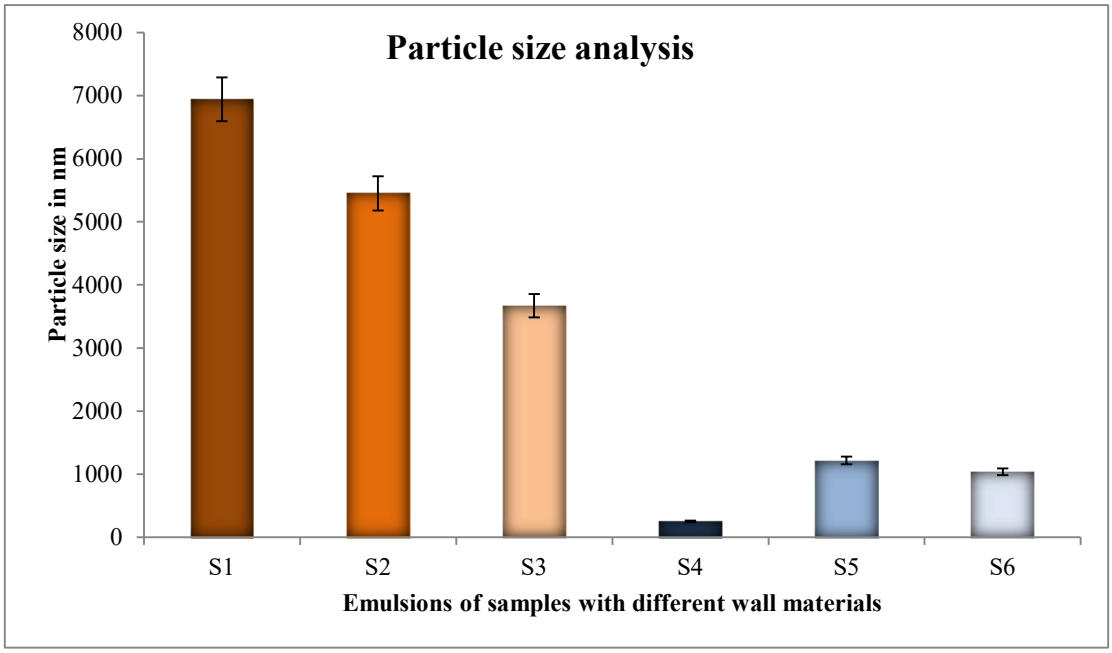


Fig. 4.11 Particle size analysis of the optimized emulsion samples encapsulated with *Annona muricata* leaf extracts. (S1 (1:2 sodium alginate: inulin), S2 (1:2 sodium alginate: inulin: distilled water leaf extract), S3 (1:2 sodium alginate: inulin: 100 % methanolic leaf extract), S4 (2:1 Gum Arabic: sodium alginate), S5 (2:1 Gum Arabic: sodium alginate: distilled water leaf extract), S6 (2:1 Gum Arabic: sodium alginate: methanolic leaf extract).

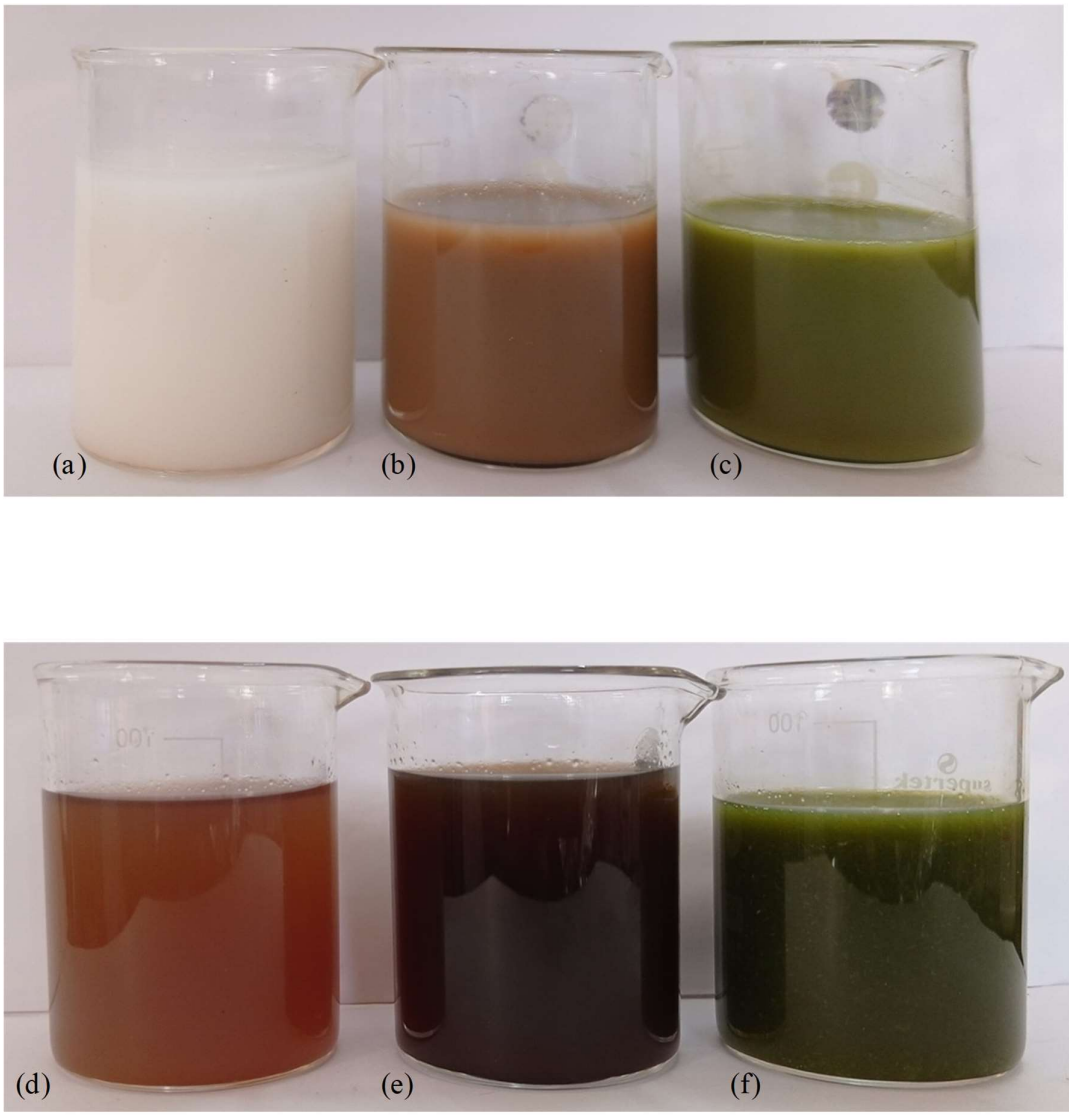


Plate 4.7: Emulsion preparation using different wall materials and leaf extract of *Annona muricata* (a) sample S1 (1:2 sodium alginate: inulin) (b) Sample S2 (1:2 sodium alginate: inulin: distilled water leaf extract), (c) Sample S3 (1:2 sodium alginate: inulin: 100 % methanolic leaf extract), (d) Sample S4 (2:1 Gum Arabic: sodium alginate), (e) Sample S5 (2:1 Gum Arabic: sodium alginate: distilled water leaf extract), (f) Sample S6 (2:1 Gum Arabic: sodium alginate: methanolic leaf extract).

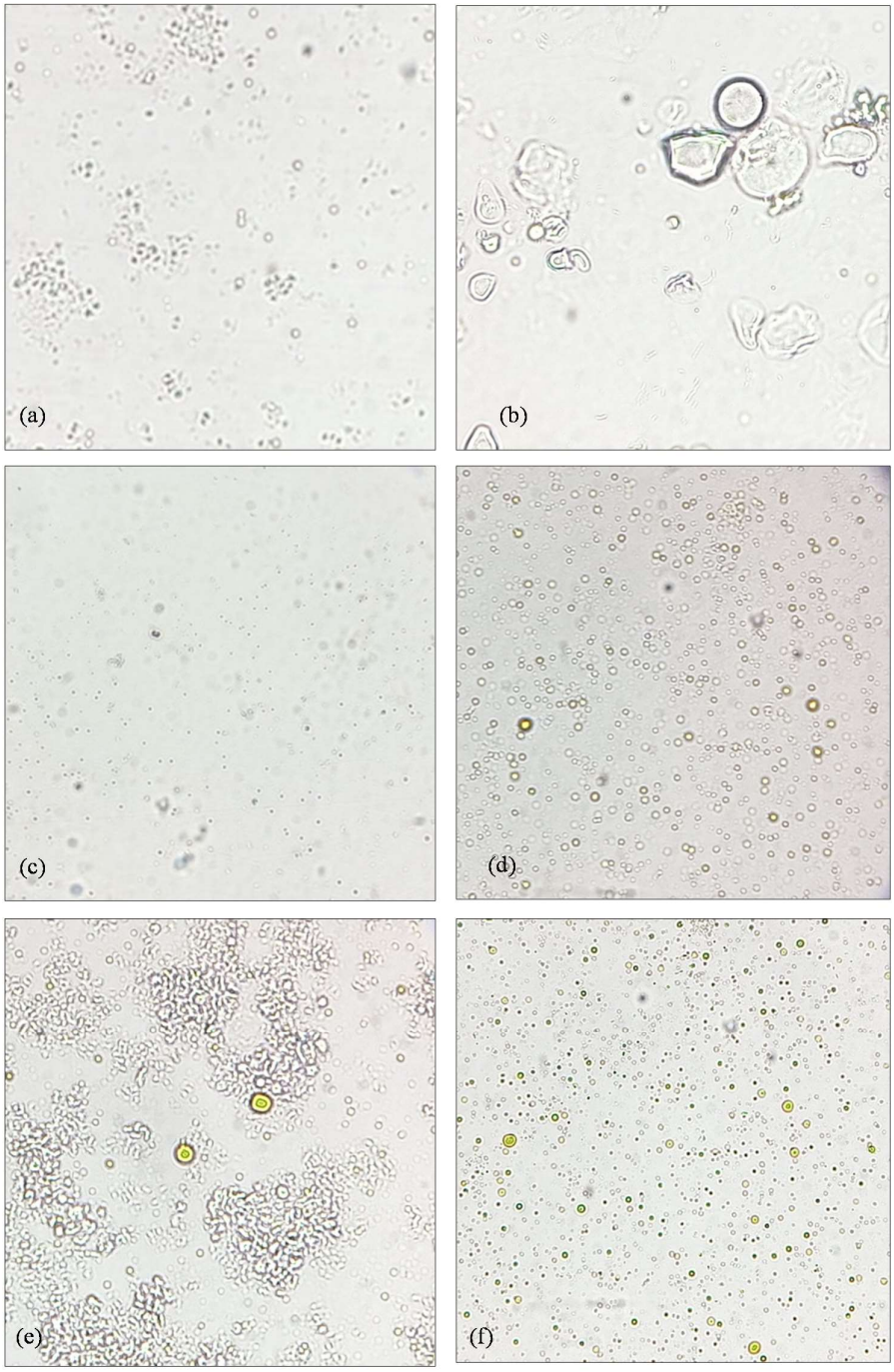


Plate 4.8: Microscopic analysis of: emulsion of encapsulated leaf extract of *Annona muricata* (a) Sample S1 (1:2 sodium alginate: inulin) (b) Sample S2 (1:2 sodium alginate: inulin: distilled water leaf extract), (c) Sample S3 (1:2 sodium alginate: inulin: 100 % methanolic leaf extract), (d) Sample S4 (2:1 Gum Arabic: sodium alginate), (e) Sample S5 (2:1 Gum Arabic: sodium alginate: distilled water leaf extract), (f) Sample S6 (2:1 Gum Arabic: sodium alginate: methanolic leaf extract).

4.4. ANALYSIS OF SPRAY-DRIED ENCAPSULATED POWDER

4.4.1. Bulk density, Tapped density, and Flowability.

Bulk density, tapped density, and flowability play critical roles in packaging and transportation as they directly impact the amount of product that can be accommodated within a container. Samples containing a 2:1 ratio of Gum Arabic and sodium alginate (S4, S5, and S6) exhibited higher bulk density and tapped density but lower flowability. Conversely, samples containing a 1:2 ratio of sodium alginate and inulin showed lower bulk density and tapped density but higher flowability. The presence of air in samples with low bulk and tapped density may enhance oxidation, potentially reducing the storage stability of freeze-dried powder, as observed in samples S1, S2, and S3 compared to S4, S5, and S6 (**Table 4.12), (Fig. 4.12), and (Plate 9).**

The bulk density and tapped density of the samples were influenced by several factors, including the ratio of wall material, particle size, and temperature. These factors collectively impact the flowability of the encapsulated samples. The analysis conducted aligns with the findings presented by Abdullah and Geldart (1999), indicating that powder flowability tends to improve with larger particle sizes and lower bulk and tapped densities.

4.4.2. Antioxidant activity of encapsulated freeze-dried *A. muricata* leaf extract powder

To assess the antioxidant activity in the encapsulated freeze-dried leaf extract, a DPPH radical scavenging assay was conducted following the dissolution of encapsulated particles in a sodium citrate solution, as the wall materials are not soluble in alcoholic solvents. The results indicate that the encapsulated extract exhibits higher antioxidant activity compared to that of the non-encapsulated leaf extracts. Specifically, S3 and S6 demonstrate greater antioxidant activity due to the incorporation of methanolic leaf extract, as opposed to S2 and S5, which contain distilled water leaf extracts. This outcome aligns

with findings from the analysis conducted by Sorasitthiyanukarn et al. (2024), which indicated an increase in in vitro oral bioaccessibility, anti-inflammatory effect, gastrointestinal stability, and antioxidant activity of Fucoxanthin after encapsulation into a matrix compared to non-encapsulated Fucoxanthin.

Thus, the data suggests that DPPH radical scavenging activity increases upon encapsulation, indicating that bioactive compounds may act more effectively post-encapsulation, likely due to the safe release of the encapsulated leaf extract.

Dragostin *et al*. (2017) conducted investigations highlighting the importance of polymers in the encapsulation process. They reported that encapsulation provides several advantages, including the protection of bioactive compounds from the harmful effects of various physicochemical agents, extension of the viability of compounds, and safe drug delivery to the targeted site. These findings suggest that the enhanced bioactivity observed post-encapsulation may be attributed to these factors.

Table 4.12 Bulk density, tapped density and flowability of the freeze-dried encapsulated *A. muricata* leaf extracts.

Sample	***Bulk density***	***Tapped density***	***Car Index (CI) (%)***	***Hausner's ratio (HR) (%)***	***Flowability***
S1	40.95 ± 0.5	50.05 ± 0.5	18.18 ± 0.5	1.22 ± 0.5	Fair
S2	41.42 ± 0.5	55.23 ± 0.5	25.00 ± 0.5	1.33 ± 0.5	Passable
S3	40.54 ± 0.2	54.67 ± 0.2	25.84 ± 0.2	1.34 ± 0.2	Passable
S4	47.16 ± 0.1	61.98 ± 0.1	23.91 ± 0.1	1.31 ± 0.1	Passable
S5	47.57 ± 0.3	65.1 ± 0.3	26.92 ± 0.3	1.36 ± 0.3	Poor
S6	49.47 ± 0.1	69.26 ± 0.1	28.57 ± 0.1	1.40 ± 0.1	Poor

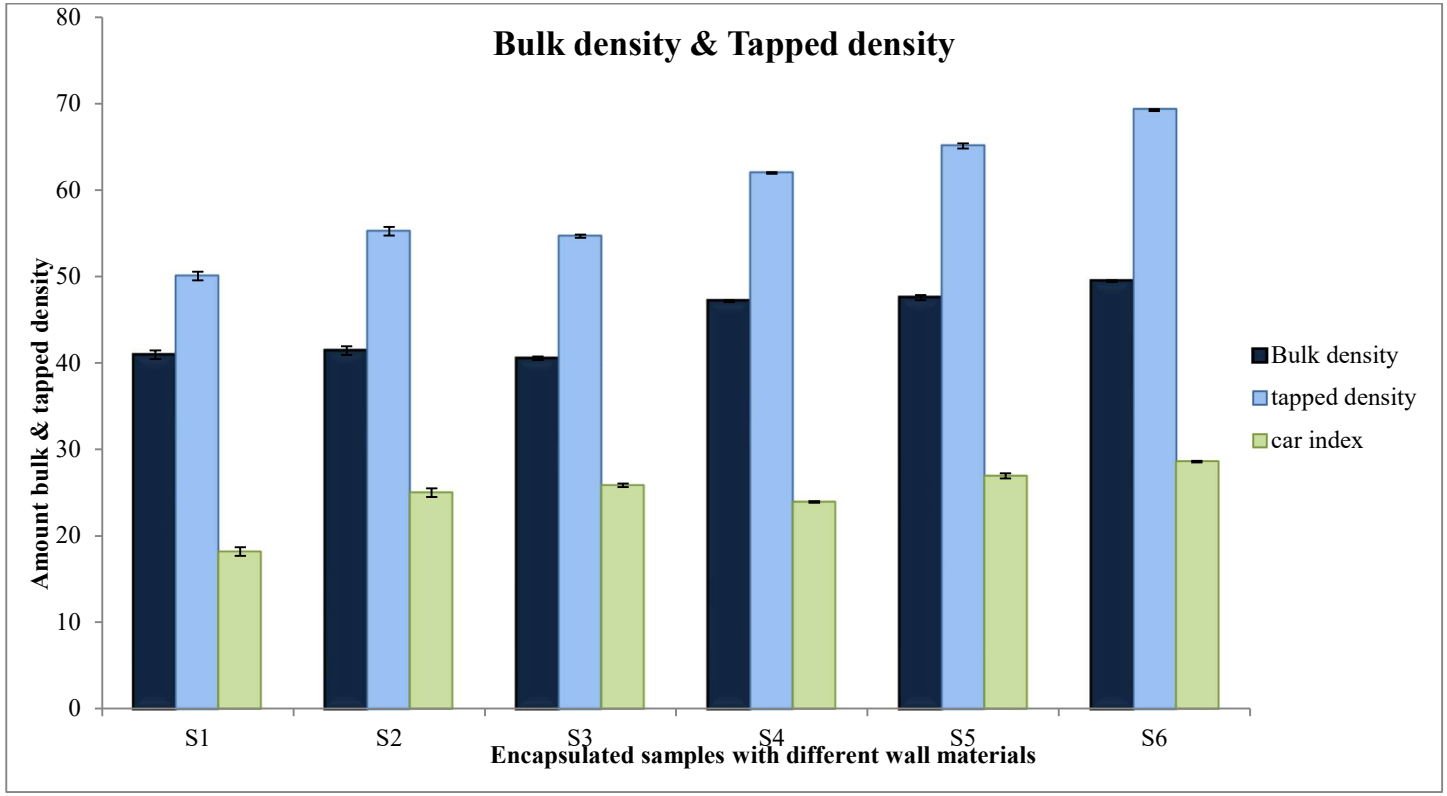


Fig.4.12 Bulk and tapped density analysis of the optimized encapsulated samples with *Annona muricata* leaf extract

Table 4.13 DPPH free radical scavenging assay % scavenging activity of DPPH by L-Ascorbic acid and encapsulated leaf extracts of *Annona muricata* with different solvent combinations. Data represented mean values ± Standard deviation (n=3).

Concentration (µg/mL)	***L-Ascorbic Acid***	***Encapsulated powder of Leaf extracts with different wall material combinations***					
		S1	**S2**	**S3**	**S4**	**S5**	**S6**
20	13.51 ± 0.003	1.56 ± 0.002	1.74 ± 0.002	4.69 ± 0.003	2.48 ± 0.002	2.76 ± 0.002	5.15 ± 0.004
40	37.12 ± 0.001	32.57 ± 0.002	3.95 ± 0.002	8.10 ± 0.003	3.95 ± 0.002	3.13 ± 0.003	10.86 ± 0.003
60	45.37 ± 0.002	3.22 ± 0.001	8.01 ± 0.001	9.94 ± 0.002	5.61 ± 0.001	5.34 ± 0.002	12.70 ± 0.002
80	75.92 ± 0.002	4.60 ± 0.002	10.31 ± 0.003	42.54 ± 0.002	6.53 ± 0.003	10.03 ± 0.004	42.54 ± 0.002
100	96.79 ± 0.015	6.19 ± 0.001	18.04 ± 0.02	48.98 ± 0.006	7.36 ± 0.007	11.23 ± 0.003	48.98 ± 0.006

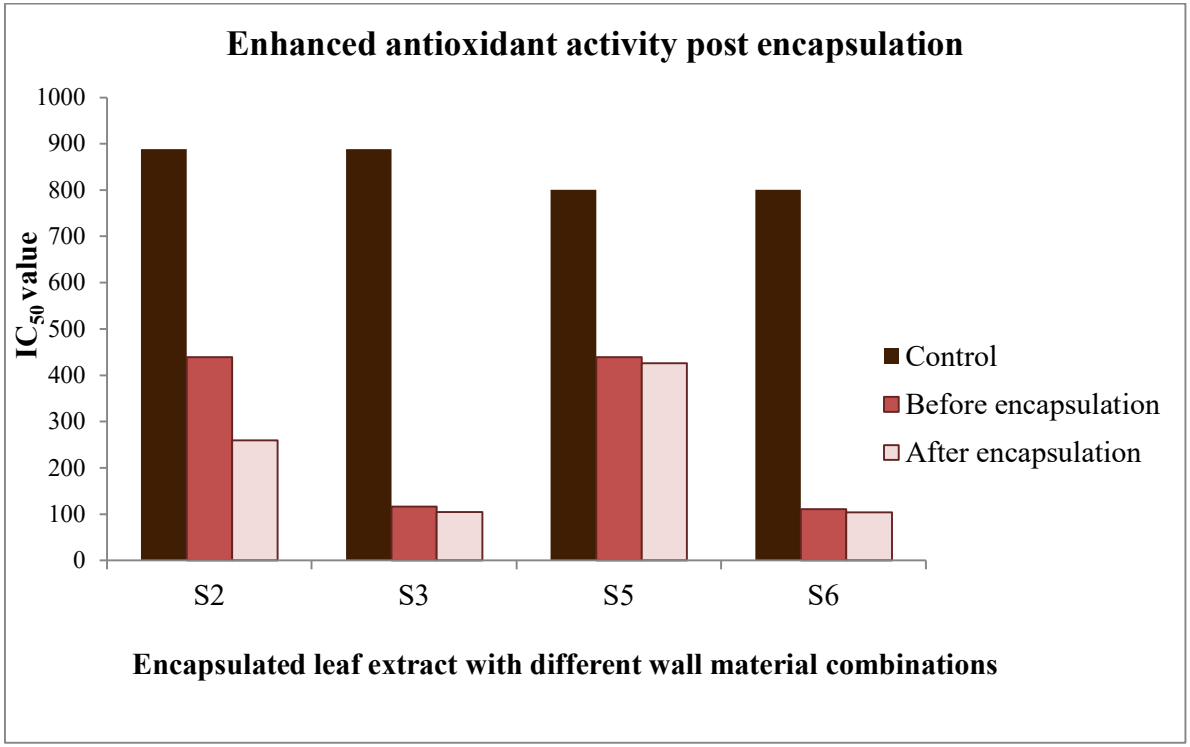


Fig 4.13 Comparison between IC_{50} value before and after encapsulation of shade dried leaf extract of *Annona muricata* in different wall material (S2, S3, S5 & S6) with control.

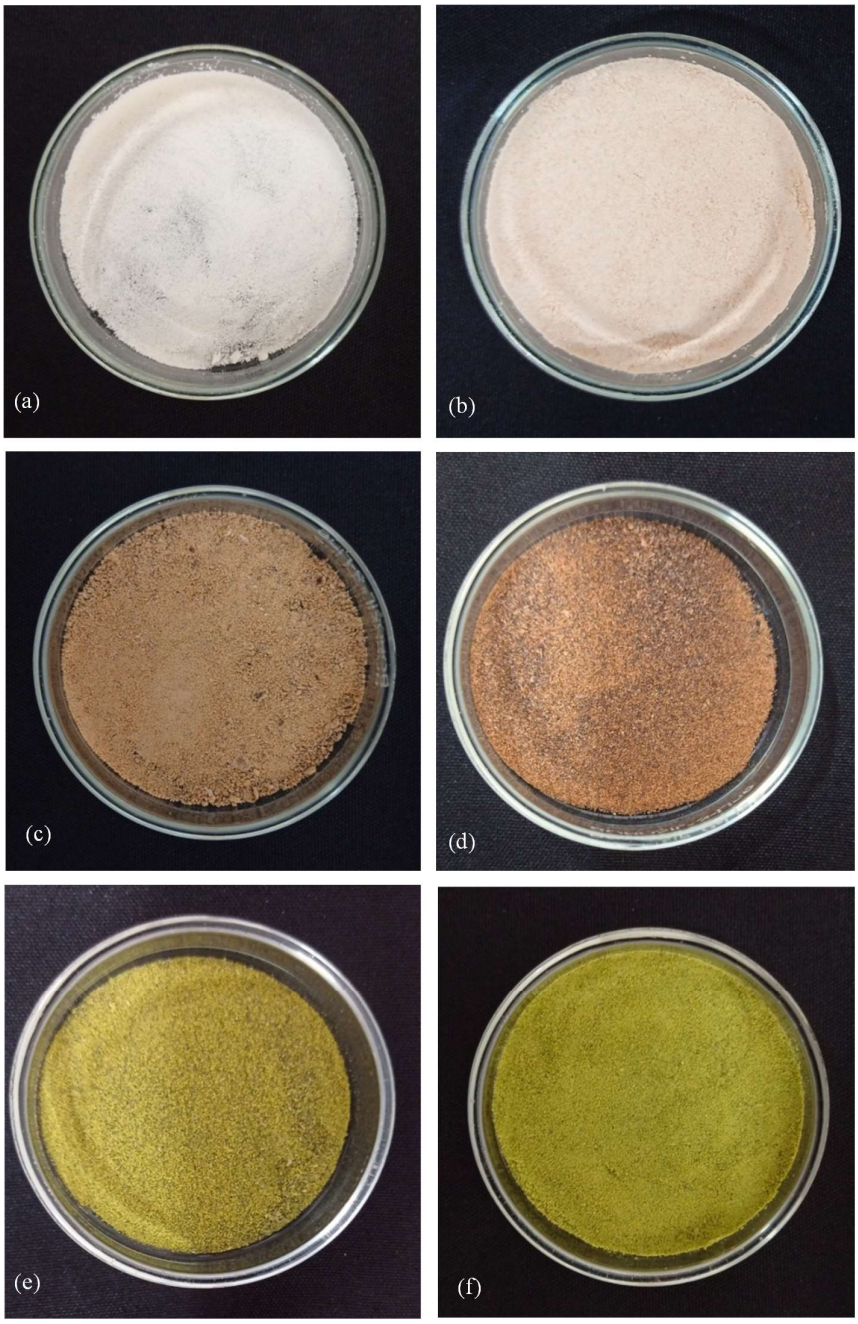
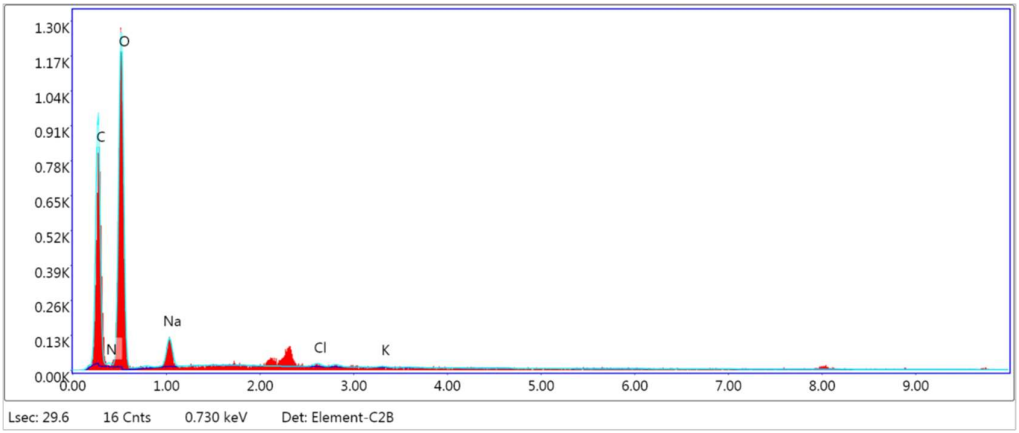


Plate 4.9: Freeze dried powder of encapsulated leaf extract of *Annona muricata* (a) Sample S1 (1:2 sodium alginate: inulin) (b) Sample S2 (1:2 sodium alginate: inulin: distilled water leaf extract), (c) Sample S3 (1:2 sodium alginate: inulin: 100 % methanolic leaf extract), (d) Sample S4 (2:1 Gum Arabic: sodium alginate), (e) Sample S5 (2:1 Gum Arabic: sodium alginate: distilled water leaf extract), (f) Sample S6 (2:1 Gum Arabic: sodium alginate: methanolic leaf extract).

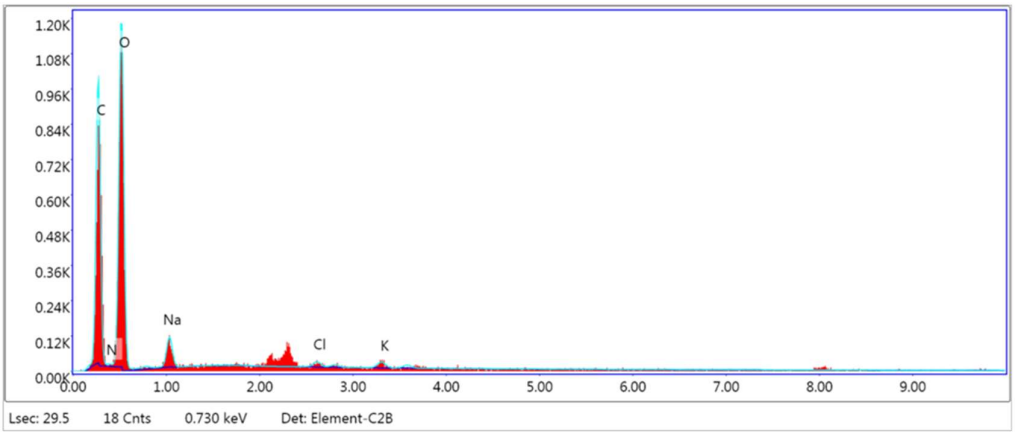
4.4.3. Scanning Electron Microscopy (SEM) and EDX analysis of encapsulated leaf sample

The Scanning Electron Microscopy of the encapsulated leaf extract was conducted to determine the surface morphological characteristics. Both encapsulated samples (S3 & S6) containing methanolic leaf extract exhibited dented surface characteristics. The wall material combination containing a 2:1 ratio of Gum Arabic and sodium alginate showed better morphological characteristics compared to that of a 1:2 ratio of sodium alginate and inulin. Freeze drying resulted in the entrapment of the microcapsules of extract in the whole emulsion. Additionally, the extent of powder preparation by grinding of freeze-dried encapsulation also affected the morphological characteristics. This analysis aligns with similar work conducted by Hani *et al*. (2016), which reported that the methanolic extract exhibited dentate behaviour, while the deionized water extract of *Moringa oleifera* displayed smooth surface morphology under Scanning Electron Microscope examination **(Plate 4.9 and 4.10).**

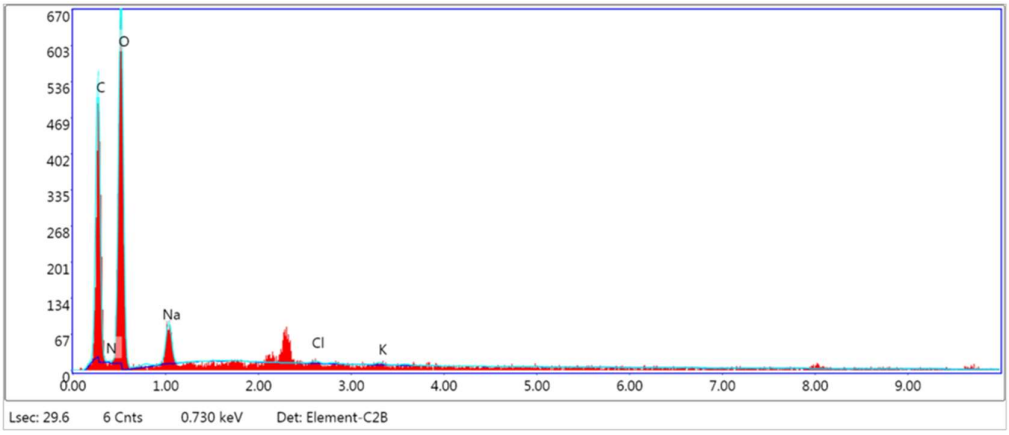
The EDX (Energy Dispersive X-ray) analysis is a technique used to determine the elemental composition and estimate their relative abundance. The data generated by EDX consists of spectra showing peaks corresponding to the elements making up the true composition of the sample being analysed. In the EDX spectra of all the encapsulated samples, peaks corresponding to the elements were observed in the following order of abundance: oxygen > carbon > sodium > nitrogen > chlorine > potassium, respectively. Both the control samples showed similar EDX spectra, with one peak indicating impurity. This peak was observed in all the encapsulated samples as well, with its intensity increasing in samples S2, S3, S5, and S6, respectively. Notably, sample S6 exhibited the highest element composition among all the samples analysed **(Fig. 4.14).**



(a)

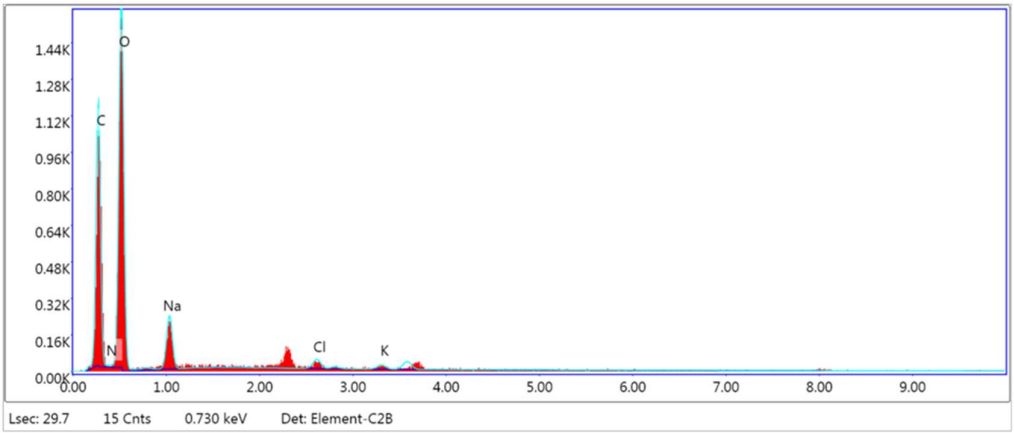


(b)

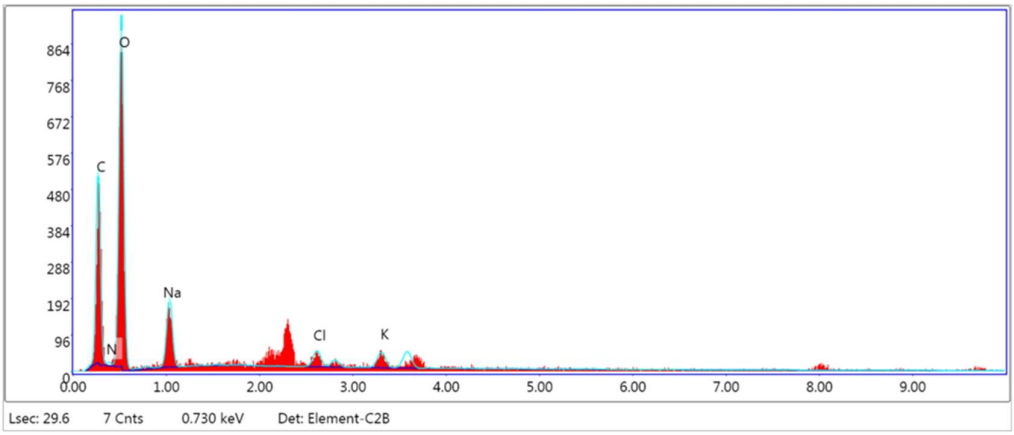


(c)

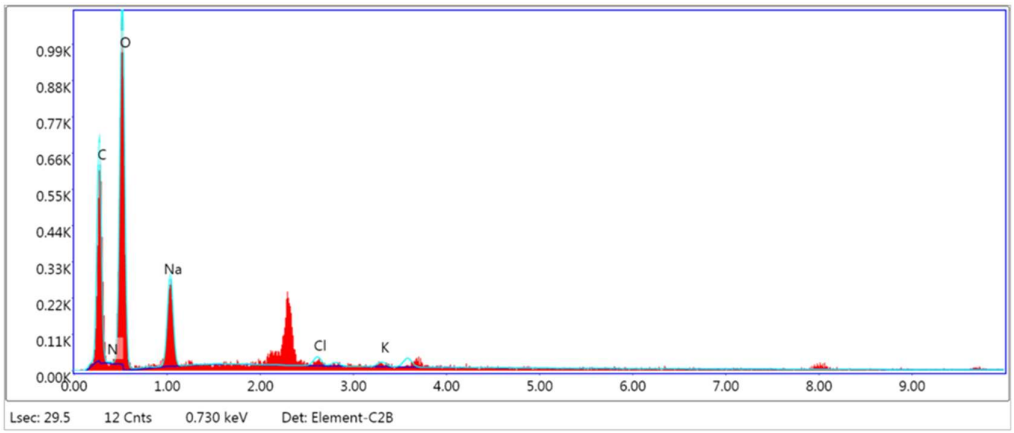
Fig 4.14 Energy Dispersive X-Ray Analysis of the encapsulated freeze-dried *Annona muricata* leaf extract powder of (a) Sample S1, (b) Sample S2, (c) Sample S3.



(d)



(e)



(f)

Fig 4.14 Energy Dispersive X-Ray Analysis of the encapsulated freeze-dried *Annona muricata* leaf extract powder of (d) Sample S4, (e) Sample S5, (f) Sample S6.

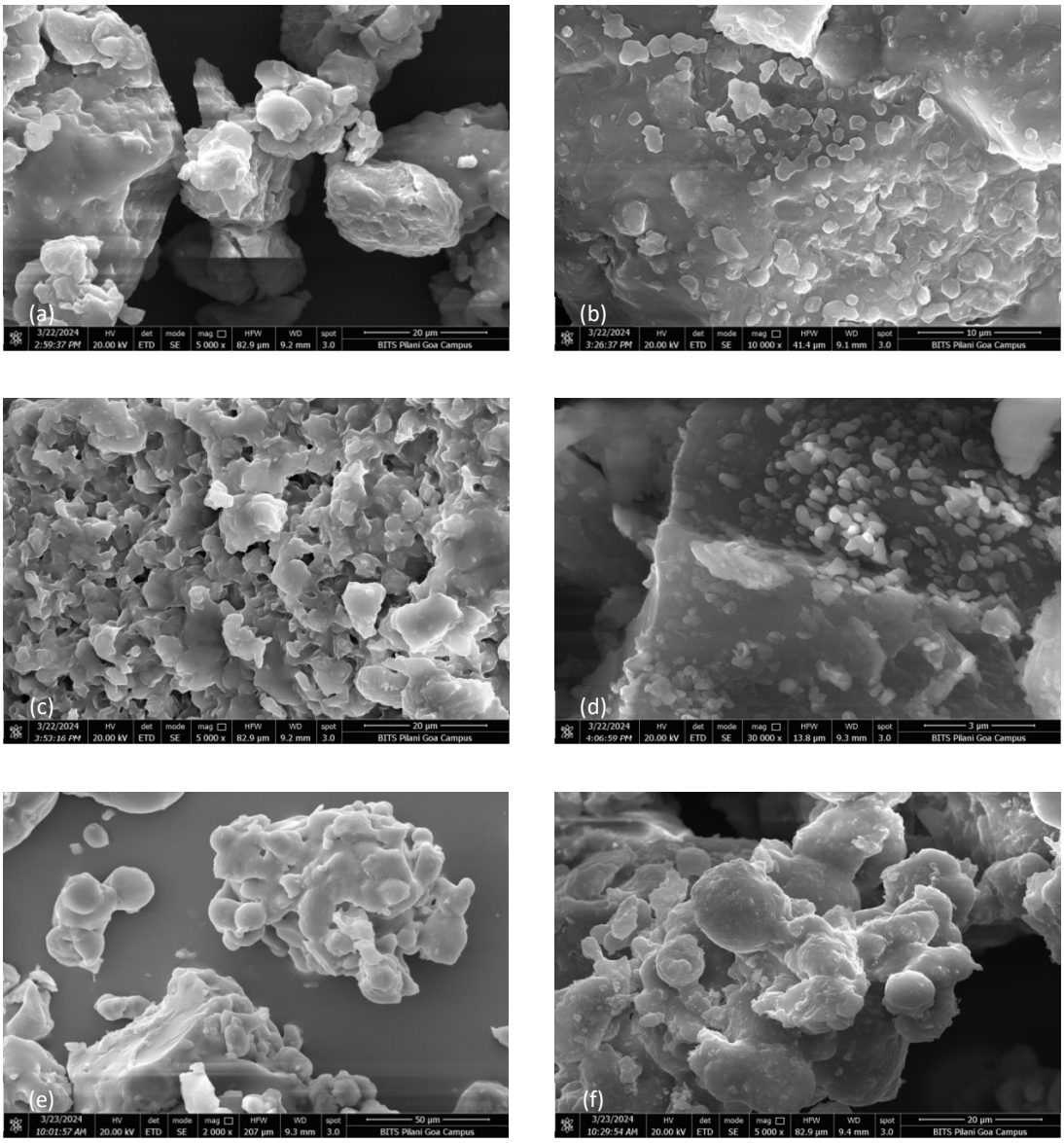


Plate 4.10: SEM analysis of Freeze dried powder of encapsulated leaf extract of *Annona muricata* (a) Smple S1 (1:2 sodium alginate: inulin) (b) Sample S2 (1:2 sodium alginate: inulin: distilled water leaf extract), (c) Sample S3 (1:2 sodium alginate: inulin: 100 % methanolic leaf extract), (d) Sample S4 (2:1 Gum Arabic: sodium alginate), (e) Sample S5 (2:1 Gum Arabic: sodium alginate: distilled water leaf extract), (f) Sample S6 (2:1 Gum Arabic: sodium alginate: methanolic leaf extract).

CHAPTER 5: CONCLUSION

In this comprehensive study on *Annona muricata* leaf extract encapsulation, various methodologies were employed to optimize drying, extraction, and encapsulation protocols. Analytical studies revealed shade drying as the optimal method for maintaining moisture content and total extract yield. Thin layer chromatographic analysis confirmed the presence of alkaloids, phenols, flavonoids, and acetogenins in the extract. Phytochemical estimations highlighted the highest content of total phenols and acetogenins in the 100% methanol extract. DPPH radical scavenging activity indicated superior antioxidant potential in the methanolic extract compared to other solvent combinations.

Further, emulsion preparation was conducted using different solvent combinations, with six wall material combinations produced. Particle size analysis and emulsion stability assessment revealed the superior performance of the sample containing a 2:1 ratio of Gum Arabic to sodium alginate.

Post-encapsulation, antioxidant activity was significantly enhanced, suggesting effective delivery and release of bioactive compounds. This promising outcome underscores the potential application of encapsulated *Annona muricata* leaf extract in functional foods. The study contributes valuable insights into the development of encapsulation strategies for enhancing the stability and bioactivity of medicinal plant extracts.

The results demonstrated that the optimized processing parameters significantly enhanced the antioxidant activity and stability of the *A. muricata* leaf extract. Microencapsulation not only improved the bioavailability of bioactive compounds but also enhanced their shelf life and potential therapeutic efficacy.

CHAPTER 6: SUMMARY

In this study, we aimed to develop freeze-dried encapsulated powder of *Annona muricata* leaf extract, leveraging encapsulation strategies to enhance stability for potential therapeutic applications. We addressed the absence of standardized industrial production methods by focusing on optimizing protocols for drying, extraction, and powder production. Four key objectives guided our methodologies, with analytical studies revealing shade drying as the optimal method for maintaining moisture content and total extract yield.

Thin layer chromatographic (TLC) analysis confirmed the presence of alkaloids, phenols, flavonoids, and acetogenins in the leaf extracts. Phytochemical estimation highlighted the highest content of total phenols and acetogenins in the 100% methanol (T2) sample. DPPH radical scavenging activity indicated superior antioxidant potential in the 100% methanol extract compared to other solvent combinations.

Further, emulsion preparation was undertaken using various solvent combinations, resulting in six distinct wall material combinations. Particle size characterization and emulsion stability assessment demonstrated the superior performance of the 2:1 Gum Arabic to sodium alginate combination.

Post-encapsulation, antioxidant activity was significantly enhanced, indicating effective delivery and release of bioactive compounds. This suggests the potential for future application of encapsulated *Annona muricata* leaf extract in functional foods. Overall, our study contributes valuable insights into the development of encapsulation strategies to enhance the stability and bioactivity of medicinal plant extracts for therapeutic purposes.

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