RESEARCH PAPER

Development of Phytosomal Nanocarriers Loaded with *Phyllanthus Amarus* and *Ziziphus Mauritiana* Extract: Physicochemical Characterization and Antidiabetic Evaluation

Gaurav Kumar[1], Tripti Shukla[1]*

[1]*SAGE University, Indore, Kailod Kartal, Indore-Dewas By-Pass Road, Indore, Madhya Pradesh, 452020, India.

*Corresponding Author: Dr. Tripti Shukla, Professor, SAGE University, Indore, Madhya Pradesh, India. Email: drtriptishukla17@gmail.com

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ABSTRACT

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycaemia and associated with several long-term complications. Despite the availability of conventional antidiabetic therapies, their clinical utility is often limited by poor bioavailability, low therapeutic efficiency, and undesirable side effects. The present study was undertaken to develop and optimize phytosomal nanocarriers loaded with *Phyllanthus Amarus and Ziziphus Mauritiana* extract in order to enhance the bioavailability and therapeutic potential of its bioactive phytoconstituents. Hydroalcoholic extracts of *Phyllanthus Amarus and Ziziphus Mauritiana* were prepared and subjected to qualitative and quantitative phytochemical screening, which confirmed the presence of significant levels of phenolic and flavonoid compounds. Standardization of the extract was performed using high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), and mass spectrometry (MS), confirming the presence of major phytochemical markers including gallic acid, quercetin, and rutin. Phytosomal nanocarriers were formulated using phosphatidylcholine (lecithin) and cholesterol by the solvent evaporation and thin-film hydration techniques. The optimized phytosomal formulation (PPA2 and PZM2) exhibited a particle size of (109.7 nm and 131.2 nm) with a high entrapment efficiency of (91% and 90%), indicating successful incorporation of phytoconstituents within the phospholipid matrix. Physicochemical characterization demonstrated the formation of stable nanosized vesicles suitable for oral drug delivery. *In vitro* drug release studies showed sustained release with first-order kinetics and Fickian diffusion. Stability studies confirmed the optimized phytosomal formulation retained integrity over six months. Antidiabetic potential, assessed through enzyme inhibitory assays, revealed significant inhibition of α-amylase and α-glucosidase, indicating enhanced effects compared to the crude extract. The improved performance is linked to nanoscale size, high encapsulation efficiency, and increased solubility. The findings suggest that optimized phytosomal carriers of *Phyllanthus Amarus and Ziziphus Mauritiana* offer favorable characteristics, sustained release, stability, and promising antidiabetic activity, supporting further pharmacological research.

Key words: *Phyllanthus Amarus; Ziziphus Mauritiana; Phytosomal Nanocarriers; Phytosomes; Hydroalcoholic Extract; Phosphatidylcholine.*

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INTRODUCTION

1.1 Diabetes Mellitus

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death. Often symptoms are not severe, or may be absent, and consequently hyperglycaemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made. The long–term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease.

Several pathogenetic processes are involved in the development of diabetes. These include processes, which destroy the beta cells of the pancreas with

consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin [1]. Glucose is essential for providing energy for normal body functions. In diabetes, the blood glucose levels are increased due to relative or absolute deficiency of insulin. Insulin is a hormone. A Hormone is a chemical secreted by one of the glands in our body. This gland is situated in abdomen and is known as pancreas. Insulin acts as a gatekeeper that allows entry of glucose into the cell [2].

This is how blood glucose keeps on rising even if there is no food intake. There are two major types of diabetes as shown in Table 1. The first type *i.e.* insulin dependent diabetes. It can be treated only with insulin as the main drug. This is common in younger age group of patients. The second type is non-insulin dependent diabetes (Type II). This is more common in India. This usually affects people above 40 years of age. This group can be managed with diet, exercises and oral medications. Some patients in this group may require insulin sometime later in life [2].

Table 1: The comparison of two types of diabetes

Feature	Type I (IDDM)	Type II (NIDDM)
Age of onset	Younger age group	Above 30-35 years
Insulin level in blood	Negligible	Decreased or high
Bodyweight	Underweight or normal	Overweight or normal
Usual treatment	Insulin	Oral and Insulin
Inheritance	Less Significant	More Significant

Uncontrolled diabetes can lead to deleterious effects on every part of the body if not looked after well at the right time. Even if one may not, in actual sense suffer the consequences of the disease today, it needs to be adequately treated. Complacency and neglect is always dangerous. Poorly controlled diabetes can be a major aggravating factor for later diabetes complications. Some of these diabetes complications risks are shown in Table 2. This is only to make you aware how important it is to control diabetes and not to scare you about this disease [3].

Table 2: Complications of diabetes

Complication	Frequency, in comparison to non-diabetics
Retinopathy leading to blindness	25 times higher
Kidney disease	15-20 times higher
Foot problems	20-30 times higher
Paralysis	2 times higher
Heart disease	2-4 times higher

Estimation and interpretation of blood glucose is the only test for diagnosis of diabetes. World Health Organization (WHO) has laid down guidelines for diagnosis.

1.2. Role of Phytosomes as innovative delivery systems for the treatment of diabetes

The term “Phyto” refers to the plant, while “some” refers to cell-like [4]. Phytosomes are the vesicular drug delivery system enhancing the absorption and bioavailability of low-soluble drugs [4-5]. Phytosomes are complex of phospholipids and natural active phytochemicals, bound in their structures, obtained by the reaction between phosphatidylcholine (or any hydrophilic polar head groups) and plant extracts in an aprotic solvent as shown in Figure 1 [6-7]. These formulations exhibit improved pharmacological and pharmacokinetic properties as compared to prevalent preparations. The lipid-soluble phosphatidyl portion completely covers the hydrophilic phytoconstituent-choline complexes. Phytosomes have remarkable benefits such as high drug encapsulation, reveal a better stability profile (chemical bonds are formed among the polar head of the amphiphile molecule and phytoconstituent, and have a better bioavailability [8-9].

Only phytochemicals with an active hydrogen atom (-COOH, -OH, -NH2, -NH) can form phytosomes, allowing polyphenols abundant in plant foods-to bond with amphiphile molecules. Polyphenols may benefit various health issues, including cancer, inflammation, neurodegenerative and cardiovascular diseases, type 2 diabetes, and obesity [10]. Sou et al. created an intravenous curcumin phytosome for rats, showing high accumulation in bone marrow and spleen [11]. Phytosomes can merge with membranes and target normal cells, so their biological effects need assessment in animal models and clinical trials [12]. Studies confirm phytosome safety [13]. Additionally, pharmacokinetic and pharmacodynamic parameters must be evaluated to demonstrate benefits over pure phyto-constituents. Identifying the optimal dosage form is crucial for marketing [14].

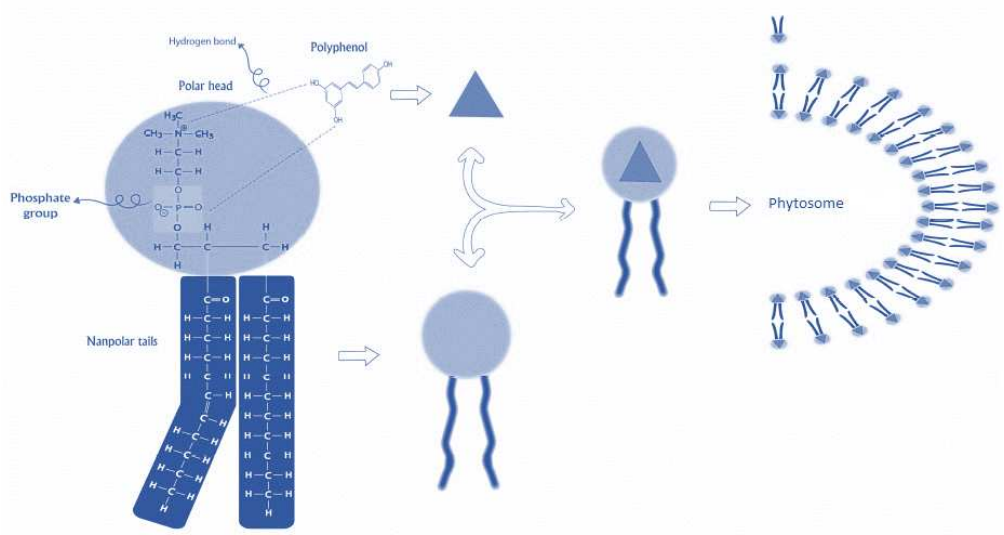


Figure 1: Principle for phytosome formation

1.3. ***Phyllanthus Amarus***

Phyllanthus Amarus is an important plant of Indian Ayurvedic system of medicine, well known for its medicinal properties and widely used worldwide. *Phyllanthus Amarus* is a plant of the family Euphorbiaceae and has about approximately 800 species, which are found in tropical and subtropical countries of the world [15-16]. The name '*Phyllanthus*' means "leaf and flower" as shown in Figure 2 & 3 and named so because of its appearance where flower, fruit and leaf appears fused [17].

Plant Profile (*Phyllanthus Amarus)*, Botanical Name: *Phyllanthus Amarus,* Family: Euphorbiaceae

Common name: Hindi: Bhuyiavla, Sanskrit: Bhumyaamalaki, Marathi: Bhuiavali, Tamil: Keelanelli, Bengali: Bhuiamla, Kannada: KiruNelli



Figure 2: Plant of *Phyllanthus Amarus*



Figure 3: Leaf of *Phyllanthus Amarus*

Phyllanthus Amarus is a branching annual glabrous herb that is 30-60 cm high and have slender, leaf-bearing branchlets, distichous leaves which are subsessile elliptic-oblong, obtuse, rounded base. Flowers are yellowish, whitish or greenish, auxillary, males flowers in groups of 1-3 whereas females are solitary. Fruits are depressed-globose like smooth capsules present underneath the branches and seeds are trigonous, pale brown with longitudinal parallel ribs on the back [18]. The plant has been found in Philippine, Cuba, Nigeria and among others. In India, *Phyllanthus Amarus is* widely distributed as a weed in cultivated and waste lands [19]. *Phyllanthus Amarus* herb has found its traditional usefulness in several health problems such as diarrhoea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders, scabies and wounds.

Phyllanthus Amarus has demonstrated hypotensive effects in animals and humans. People with a heart

condition and/or taking prescription of heart medications should consult their doctor before taking this plant. It may potentiate insulin and antidiabetic drugs. It contains a naturally occurring phytochemical geraniin. This chemical has been documented with negative chronotropic, negative inotropic, hypotensive and angiotensin-converting enzyme inhibitor effects in animal studies with frogs, mice and rats [20]. The antidiabetic potential of *Phyllanthus Amarus* investigated in an experiment model where fasted rats were made diabetic by single intraperitoneal injection of 120 mg/kg of alloxan monohydrates and then two doses of the aqueous and hydroalcoholic extract of *Phyllanthus Amarus* administered orally which were then compared with the normal control group that received distilled water only. After 15 days treatment the result demonstrates aqueous and hydroalcoholic extract of *Phyllanthus Amarus* decrease the blood glucose level significantly. Serum analysis of the treated experimental animals showed an increase in insulin and reduction in the malondialdehyde concentration, therefore demonstrated the potential antidiabetic property of aqueous and hydroalcoholic extract of *Phyllanthus Amarus* [21].

In another study the methanolic extract of *Phyllanthus Amarus* was found to inhibit lipid peroxidation & scavenge hydroxyl and superoxide radicals [22].

1.4. ***Ziziphus Mauritiana***

Ziziphus Mauritiana, commonly known as "Ber," is a medicinal plant traditionally used by indigenous communities for various biological applications, indicating its potential as a source of bioactive compounds for pharmaceutical and nutraceutical development.

It is also known as Indian Jujube; common jujube.

Botanical Name: *Ziziphus Mauritiana,* Family: Rhamnaceae

Common name: Hindi - Ber, Baer, Sanskrit – Badrah, Sanskrit - Ajapriya, kuvala, madhuraphala, karkandhu); Bengali - kool, ber, boroi, Marathi - Bar, bera, Gujarati, - Bor, bordi

Telgu – Reegu, Tamil - Elandai, yellandeelladu, Rajasthan - Bor, bordi

Ziziphus Mauritiana typically grows up to 512 meters (1639 feet) tall, with a spreading canopy. The leaves are glossy green, ovate to elliptic in shape, and arranged alternately along the branches and the flowers are small, yellow to greenish yellow, and fragrant. They are borne in clusters in leaf axils as shown in Figure 4. The fruit of *Ziziphus Mauritiana* is a small, round berrylike drupe, 23 cm in diameter, with a smooth skin that turns from green to yellow, and finally reddish brown or brownish black when ripe. The pulp is sweet and aromatic, containing a single seed. The bark is greyish brown and rough textured as shown in Figure 5.



Figure: 4 Leaf & Flower of *Ziziphus Mauritiana*



Figure: 5 Barks & Fruits of *Ziziphus Mauritiana*

In the present study, the leaves of *Ziziphus Mauritiana* were collected from Vindhya Herbal Garden, Bhopal, Madhya Pradesh, authenticated, shade-dried, pulverized, and extracted using a hydroalcoholic solvent system. *Ziziphus Mauritiana* is a highly nutritious and medicinally valuable fruit tree. It is rich in vitamins, minerals, phytochemicals, and bioactive compounds with antioxidant, anti-inflammatory, antimicrobial, and potential anticancer properties. Its nutritional benefits and diverse pharmacological activities make it an important candidate for functional foods, nutraceuticals, and phytomedicine [23]. Phytochemical screening of Ziziphus Mauritiana's hydroalcoholic extract identified various secondary metabolites, including alkaloids, carbohydrates, proteins, steroids, saponins, flavonoids, and phenolic compounds. The study also noted constituents like procyanidin B-2, B-3, granatin B, and ellagic acid derivatives. These findings highlight the diverse phytochemical profile of Ziziphus Mauritiana leaves, suggesting potential biological activity and value as a source of bioactive compounds [24].

MATERIALS AND METHODS

2.1. Chemicals

Lecithin soya, 30%, HiMedia Laboratories Pvt. Ltd., Cholesterol, Kem Light Laboratories Pvt. Ltd. and Glibenclamide, Nicotinamide, and Streptozotocin were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and were stored at 2 - 4 °C for further use. All the other chemicals were of analytical grade and were purchased from standard commercial suppliers.

2.2. Collection and authentication of plant material

The plant *Phyllanthus Amarus & Ziziphus Mauritiana leaves* were collected from Vindhya herbal garden, Bhopal Madhya Pradesh in the year 2018 and authentication was done by Dr. Tayyab Saify, Professor and Head of the Department, Department of Botany, Gandhi PR College, Bhopal, Madhya Pradesh, India. The plant material were dried under shade, pulverised and kept in air tight container.

2.3. Extraction

A hydroalcoholic extract was prepared using 10:90 ratios by using soxhlet extraction. The solvent was removed under vacuum by using rotary evaporator. The obtained extract was dried in desiccator and stored in refrigerator for further use. The percentage yield was calculated.

2.4. Phytochemical screening

Qualitative phytochemical screening

Preliminary phytochemical screening of the extracts of *Phyllanthus Amarus & Ziziphus Mauritiana leaves* were carried out. The crude extracts were screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins and tannins by using standard procedures [25-27].

- **Test for Alkaloids:**

The chemical test were performed from neutral or slightly acidic solution of drug; following type of chemical test given by alkaloids are

- **Dragendorff's test**: Dragendorff's reagent (Potassium bismuth iodide) was added to the plant extract. Appearance of orange red coloured precipitate, confirmed the presence of alkaloids.
- **Mayer's test**: Adequate amount of plant extract was taken and few drops of Mayer's reagent (Potassium mercuric iodide) were added. Appearance of creamy-white coloured precipitate, confirmed the presence of alkaloids.
- **Hager's test**: Adequate amount of plant extract was taken and few drops of Hager's reagent (Saturated aq. solution of picric acid) were added.

Appearance of crystalline yellow coloured precipitate, confirmed the presence of alkaloids.

- **Wagner's test**: Few drops of Wagner's reagent (Dilute iodine solution) were added to the plant extract. Appearance of reddish-brown coloured precipitate, confirmed the presence of alkaloids.
- **Tannic acid test**: Few drops of tannic acid solution were added to the plant extract. Appearance of buff coloured precipitate, proved the presence of alkaloids.
- **Test for carbohydrates:**
- **Charring test**: The plant extract was taken in a test-tube and heating was done in presence of conc. H_2SO_4 produces charring with smell like burning sugar confirmed the presence of carbohydrate.
- **Molish test**: The plant extract was taken in a test tube and few drops of Molish reagent (α-naphthol) and conc. H_2SO_4 was added from sidewall of test tube. Appearance of purple coloured ring at junction, confirmed the presence of carbohydrates.
- **Iodine test**: To the plant extract few drops of iodine solution were added. Appearance of blue colour, which disappeared on heating and reappeared on cooling, revealed the presence of starch. This test is specifically for polysaccharides.
- **Barfoed test**: To the plant extract 2 ml of Barfoed reagent (Cupric acetate, acetic acid and water) was added and boiled. Appearance of brick red precipitate in 5 min, revealed the presence of monosaccharide while in 7 min, revealed the presence of disaccharide. It is used to make difference between monosaccharides and disaccharides.
- **Fehling solution test**: The test is used for reducing sugars and composed of two solutions, which are mixed in situ. Fehling solution A composed of 0.5% of copper sulphate whereas Fehling solution B composed of sodium potassium tartarate. Equal volumes of Fehling A and Fehling B solutions were mixed (1 ml each) and 2 ml of plant extract was added followed by boiling for 5-10 min on water bath. Appearance of reddish brown coloured precipitate due to formation of cuprous oxide, confirmed the presence of reducing sugar.
- **Benedict's test**: To the plant extract 1 ml of Benedict's solution (Copper sulphate and sodium hydroxide) was added and heated almost too boiling, appearance of green, yellow, orange, red or brown colour in order of increasing concentration of simple sugar in the test solution, due to formation of cuprous oxide, showed the presence of reducing sugars.
- **Test for protein and amino acids:**
- **Biuret test**: Few drops of Biuret reagent (Potassium hydroxide, copper sulphate and sodium potassium tartrate) were added to the plant extract. Appearance of violet or pink colour, showed the presence of proteins.
- **Millons test**: Few drops of Millons reagent (solution of mercuric and mercurous ions in nitric and nitrous acids) were added to the plant extract. White precipitate appears that turns red after heating on boiling water bath, showed the presence of proteins.
- **Ninhydrin test**: Few drops of 5% Ninhydrin solution were added to the plant extract and heated in boiling water bath for 10 min. Appearance of purple or bluish colour showed the presence of proteins.
- **Lead sulphide test**: 2 ml of 10% NaOH solution was added to the plant extract followed by few drops of lead acetate solution. The solution was shaken and boiled on water bath for few minutes. Appearance of black precipitate in presence of sulfur containing amino acids, showed the presence of proteins.
- **Xanthoproteic test**: Adequate amount of plant extract was taken and 2 ml of dil. $HNO_3$was added. Appearance of yellow precipitate, showed the presence of aromatic ring containing amino acid like tyrosine, tryptophan etc.
- **Test for steroids:**
- **Liebermann burchard test**: Plant extract was firstly extracted with chloroform and then few drops of acetic anhydride were added followed by conc. H_2SO_4 from side wall of test tube. A violet to blue coloured ring appeared at the junction of two liquid that showed the presence of steroid entity.
- **Salkovaski test**: Plant extract was firstly extracted with chloroform and then conc. $H_2SO_4$was added from sidewall of test tube. Appearance of yellow coloured ring at the junction of two liquid, which turns red after 2 min, confirmed the presence of steroid.
- **Trichloro acetic acid test**: Adequate amount of plant extract was taken and saturated solution of trichloro acetic acid was added. Colored precipitate appeared that proved the presence of steroid moiety.
- **Test for glycosides:**
- **Borntrager's test**: To the appropriate amount of plant extract dil. HCl was added, boiled and filtered. Equal volume of chloroform was added to the filtrate and shaken well. Chloroform layer was separated and ammonia was added. Appearance of

red colour in ammonical layer, showed the presence of anthraquinone.

- **Modified Borntrager's test**: An appropriate amount of plant extract was taken and dil. HCl was added followed by 5 ml $FeCl_3$. Boiling was done for 10 min on water bath, cooled and filtered, filtrate was extracted with CCl_4 or benzene and equal volume of ammonia solution was added. Appearance of pink to red colour, revealed the presence of anthraquinone moiety.
- **Keller Killiani test**: Plant extract was mixed with an equal volume of water and 0.5 ml of lead acetate solution, shaken, and filtered. The filtrate was extracted with chloroform, evaporated, and dissolved in 3 ml of glacial acetic acid with a few drops of FeCl3. When transferred to a test tube with 2 ml of concentrated H2SO4, a reddish brown layer appeared, turning bluish green after standing, indicating the presence of digitoxose.
- **Legal test**: An appropriate amount of plant extract was taken and equal volume of water and lead acetate solution was added, shaked and filtered. Filtrate was extracted with equal volume of chloroform and the chloroform extract was evaporated to dryness. The residue was dissolved in 2 ml of pyridine and 2 ml of sodium nitropruside was added followed by addition of NaOH solution to make alkaline. Appearance of pink colour, showed the presence of glycosides or aglycone moiety.
- **Baljeet test**: Sodium picrate solution was added to the plant extract. Yellow to orange colour appears that revealed the presence of aglycones or glycosides.
- **Test for saponins:**
- **Haemolysis test**: Few drops of plant extract were mixed with a drop of blood on slide. Appearance of ruptured RBC's, revealed the presence of saponins.
- **Foam test**: 10-20 ml of water was added to the plant extract and shaking was done for few minutes, Appearance of froth which persisted for 60-120 s, confirmed the presence of saponins.
- **Test for flavonoids:**
- **Ammonia test**: A piece of filter paper was dipped in the plant extract then exposed to ammonia vapor. Appearance of yellow spot on filter paper, showed the presence of flavonoids.
- **Shinoda test**: (a) Magnesium turning and dil. HClwere added to the plant extract. Appearance of red colour, showed the presence of flavonoids. (b) Zn turning and dil. HClwere added to plant extract. Deep red to magenta colour appeared that revealed the presence of dihydro flavonoids.
- **Vanillin-hydrochloric acid test**: Vanillin hydrochloric acid was added to plant extract. Appearance of pink colour, revealed the presence of flavonoids.
- **Test for phenolic compounds:**
- **5% Ferric chloride solution**: An appropriate amount of plant extract was taken and 5% $FeCl_3$ was added. Appearance of deep blue-black colour, confirmed the presence of phenolic compounds.
- **Lead acetate solution**: Lead acetate solution was added to plant extract and appearance of white coloured ppt., confirmed the presence of phenolic compounds.
- **Gelatin solution**: Gelatin solution was added to the plant extract. Appearance of white ppt., revealed the presence of phenolic compounds.
- **Potassium dichromate**: Appearance of red ppt., revealed the presence of phenolic compounds.
- **Dilute iodine solution**: Appearance of transient red colour, revealed the presence of phenolic compounds.
- **Dilute nitric acid**: Appearance of reddish to yellow colour, revealed the presence of phenolic compounds.
- **Acetic acid solution**: Appearance of red colour solution, revealed the presence of phenolic compounds.
- **Dil. potassium permanganate solution**: Appearance of de coloration, revealed the presence of phenolic compounds.
- **Test for fixed oils:**
- **Spot test**: Small quantity of plant extract was taken and pressed between two filter papers. Oil stains on the paper, showed the presence of fixed oil.
- **Saponification test**: Few drops of 0.5 N alc. KOH and phenolphthalein were added to the plant extract. Mixture was heated on a water bath for 1-2 h. Soap formation or partial neutralisation takes place that confirmed the presence of fixed oils.

Quantitative phytochemical screening

Determination of total phenolic content

Folin-Ciocalteu's method was used to determine total phenolic content. Folin-Ciocalteu's reagent is a mixture of phosphomolybdate and phosphotungstate and work by calculating amount of test substance required to inhibit the oxidation of the reagent. Folin-Ciocalteu's reagent was used as oxidising agent and Sodium carbonate was used as neutralizer. After 30 min absorbance was checked at 765 nm.

Method:

Preparation of test solution

1.5 ml of Folin-Ciocalteu's reagent and 1.2 ml of Na2CO3 were added to the hydroalcoholic plant extract. The reaction mixture was thoroughly mixed and was incubated in the dark for 30 min. After that absorbance of reaction mixture was taken at 765 nm.

Preparation of blank

Blank was prepared by adding 1.5 ml of Folin-Ciocalteu's reagent and 1.2 ml of Na2CO3 to hydroalcoholic mixture.

Preparation of calibration curve

Gallic acid served as the standard. A 100µg/ml stock solution was prepared in hydroalcohol. Aliquots (0.5 to 2.5 ml) were placed in volumetric flasks, then mixed with 10 ml hydroalcohol and 1.5 ml Folin-Ciocalteu's reagent. After 5 min, 4 ml of 20% sodium carbonate was added, and the volume was adjusted to 25 ml with distilled water. After 30 min, absorbance was measured at 765 nm, and a calibration curve was created. Results were reported as µg gallic acid equivalents (GAE) and percentage w/w [28-29].

Determination of total flavonoid content

Aluminium chloride colorimetric method was used to estimate total flavonoid content. Aluminium chloride forms acid stable complexes with the C4 keto group and either the C3 or C5 hydroxyl group of flavones and flavanols. In addition, aluminium chloride forms acid labile complexes with the ortho dihydroxyl groups in the A- or B- ring of flavonoids.

Preparation of calibration curve

10, 20, 40, 60, 80 and 100 µg/ml concentration of rutin was prepared. 1.5 ml of ethanol (95%), 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water were added to the diluted standard solution. Incubation was done at room temperature for 30 min and absorbance was recorded at 415 nm using UV spectrophotometer. A calibration curve of absorbance vs concentration was plotted.

Preparation of test solution

10mg/ml solution of hydroalcoholic extract of whole plant was prepared in ethanol. 0.5 ml of the above solution was added to 1.5 ml ethanol. 0.1 ml of 10 % aluminium chloride and 0.1 ml of 1M potassium acetate reagents were added to above solution. After that 2.8 ml of distilled water was added to solution and kept it for 30 min after that absorbance was taken at 415 nm.

Preparation of blank solution

2 ml of ethanol was added to 0.1 ml of 10% aluminium chloride and 0.1 ml of 1M potassium acetate reagents and then 2.8 ml distilled water was added. The amount of total flavonoids was calculated using the standard curve of rutin. Quantification was done on the basis of standard curve of rutin. Results were expressed as µg rutin equivalents and percentage w/w [30-31].

Standardization of extract

` *Phyllanthus Amarus & Ziziphus Mauritiana extract* was standardised by HPLC, HPTLC and MS analysis

HPLC

HPLC analysis was carried out using Empower 2 Software Build 2154 using C-18 column of 4.4×250mmn dimension was used as stationary phase and Isopropanol: formic acid (10:0.5) as mobile phase. Sample was prepared by suspending extract in HPLC graded methanol, 5 µl of the sample was injected and run for 30 min. Standardization was done by comparing the retention time (RT) of sample with the RT of standard [32-35].

HPTLC

The HPTLC fingerprint technique of a validated crude material act as a primary reference against which unknown material can be standardized [36]. HPTLC analysis of *Phyllanthus Amarus & Ziziphus Mauritiana* leaves extract was carried out with the help of CAMAG instrument. Sample was prepared in methanol and 100 µL capacity was applied using syringe. Toluene: ethyl acetate (7: 3) and anisaldehyde sulfuric acid was used as mobile phase and spraying agent respectively. Anisaldehyde sulfuric acid was used as spraying agent.

MS analysis

A hybrid quadrupole time of flight (Q-TofTM) mass spectrometer was used to analyse sample using 3 kV capillary voltage, 30V cone voltage and 800^0C temperature. Around 0.5 pmol of each example was infused into electrospray transport dissolvable by utilizing a microsyringe (50 µL) coupled to an infusion pump at 4µL/min flow, scanning was done upto 1500 m/zusing scan time of 5s [37]. MS analysis was performed to confirm the purity and quality of extract by using Mass spectrometer operating on hybrid quadrupole time of flight. Analysis was done by using standard conditions. A spectra was obtained by scanning from 100 to 1500 m/z [37-38].

1. **DEVELOPMENT OF PHYTOSOMES**

Accurately weighed quantity of lecithin 45 mg and cholesterol 15 mg was dissolved in 10 ml of chloroform in round bottom flask (RBF) and sonicated for 10 minutes using bath sonicator. Rotary evaporator (45-50 °C) does organic solvent removal. After complete removal of solvent thin layer of phospholipids mixture was formed. This film was

hydrated with hydroalcoholic extract of *Phyllanthus Amarus & Ziziphus Mauritiana rotary* evaporator (37-40°C for 1 hour). After hydration of mixture of lipid and plant extract was sonicated for 20 minutes by using Ultrasonic Probe Sonicator (Pci Analytics) in presence of ice bath for heat dissipation. Then prepared phytosomes were stored in freezer (2-8 °C) until used. Seven formulation batches of hydroalcoholic extract of *Phyllanthus Amarus* and *Ziziphus Mauritiana* leaves with the different ratio of lipid & lecithin are selected for formation of phytosomes as shown in Table 3 & 4 [39].

Table 3: Formulation composition of phytosome formulation of *Phyllanthus Amarus*

Formulation	Cholesterol	Lecithin	Chloroform	*Phyllanthus Amarus* hydroalcoholic extract	Cholesterol: lipid ratio
PPA1	15 mg	40 mg	10 ml	10 ml	1.5:4
PPA2	15 mg	45 mg	10 ml	10 ml	1.5:4.5
PPA3	15 mg	50 mg	10 ml	10 ml	1.5:5
PPA4	15 mg	55 mg	10 ml	10 ml	1.5:5.5
PPA5	15 mg	60 mg	10 ml	10 ml	1.5:6
PPA6	15 mg	65 mg	10 ml	10 ml	1.5:6.5
PPA7	15 mg	70 mg	10 ml	10 ml	1.5:7

Table 4: Formulation composition of phytosome formulation of *Ziziphus Mauritiana*

Formulation	Cholesterol	Lecithin	Chloroform	*Ziziphus Mauritiana* hydroalcoholic extract	Cholesterol: lipid ratio
PZM1	15 mg	40 mg	10 ml	10 ml	1.5:4
PZM2	15 mg	45 mg	10 ml	10 ml	1.5:4.5
PZM3	15 mg	50 mg	10 ml	10 ml	1.5:5
PZM4	15 mg	55 mg	10 ml	10 ml	1.5:5.5
PZM5	15 mg	60 mg	10 ml	10 ml	1.5:6
PZM6	15 mg	65 mg	10 ml	10 ml	1.5:6.5
PZM7	15 mg	70 mg	10 ml	10 ml	1.5:7

4. CHARACTERIZATION OF PHYTOSOMES

4.1. Quantification of entrapped and unentrapped drug

The both formulation (PPA & PZM) contains the hydroalcoholic extracts of *Phyllanthus Amarus & Ziziphus Mauritiana.* Standardization of extract was done by HPTLC method. In which quercetin taken as a marker to quantify the plant extracts. In HPTLC method used the silica gel 60F254 precoated TLC aluminium plates [E-Merck], Toluene: Ethyl acetate: Acetone: Formic acid (10:5:15:1) as solvent system and the plate was scanned at UV 366 nm and 254 nm using CAMAG TLC Scanner-3 and LINOMAT-V. Phytosomes (10 mg/ml) were centrifuged for 50 min at 41,000 × g and 40C using R-8C, REMI centrifuge machine (Lab Centrifuge, Ambala).The supernatant was collected and phytosomes pellets in the centrifuge tubes were redispersed in 10 ml distilled water to remove phytoconstituents (as for quercetin) adsorbed onto phytosomes. Centrifugation was repeated for 50 min and the supernatant was again collected and added to the first supernatant to comprise the unentrapped fraction of the phytoconstituents. The phytosomes pellets were solubilized using Triton X-100 (1%, w/v) and the released phytoconstituents was the phytosome-entrapped fraction. The entrapment efficiency (EE) of phytoconstituents (as for quercetin) in phytosomes was calculated after quantification of entrapped and unentrapped drug using HPTLC.

EE (%) = (amount of quercetin entrapped in phytosomes/overall amount of the quercetin in formulation) × 100 %

4.2. Morphology of phytosomes

The morphology of phytosome was observed by Transmission electron microscopy (TEM). The phytosome dispersions were deposited on carbon coated copper grids (400 mesh) and negatively stained with 1% w/v phosphotungstic acid. Phytosome morphology was viewed using a Philps CM 120 BioTwin transmission electron microscope (Philips Electron Optics BV, The Netherlands.

4.3. Size analysis of phytosomes

The size distribution of phytosomes was analyzed using Malvern mastersizer S laser diffraction size analyzer (Malvern Instruments Ltd., UK). A magnetically stirred cell dispersion unit (Malvern Instruments Ltd., UK) was employed with medium speed stirring in order to keep the phytosome dispersed during size measurement. The measurement position was 1.05 mm and polydisperse mode of analysis was chosen. These set up conditions permitted accurate measurement of particles having a size range between 0.1 and 10000 d.nm. The size and size distribution were measured by the instrument as ZAve [40].

4.4. Zeta potential

The measurement were made with a zetasizer 2000 DTS52013 (Malvern Instruments, UK) at 25°C. The

phytosomes were dilute with distilled water, loaded into capillary cell mounted on the apparatus and all experiment was performed at least in triplicate [41].

5. *IN VITRO* EVALUATION STUDIES OF PREPARED PHYTOSOMES

The *in vitro* releases of phytosomes were studied by using simple diffusion cell apparatus. The diffusion cell apparatus consists of glass tube with an inner diameter of 2.5 cm open at both ends, on end of the tube is tied with sigma dialysis membrane, which serves as a donor compartment. Phytosomes equivalent to 100 mg was taken in test tube and placed in 100 ml of phosphate buffer. The medium was stirrer and the temperature at 35-37°C. Periodically 10 ml of sample were withdrawn and after each withdrawn same volume of medium was replaced. Then the samples were assayed spectrophotometrically/HPTLC. The releases of all formulations were compared with pure hydroalcoholic extract [42].

5.1. Drug release kinetics

In the present study, the formulations (PPA2 & PZM2) were evaluated for phytoconstituents release kinetics. The drug release data were plotted using various kinetic equations (Zero order, first order, Higuchi's kinetics, Korsmeyer's equation, and Hixson-Crowell Cube root law) to evaluate the drug release mechanism and kinetics.

The data obtained from in vitro drug release studies were plotted as cumulative amount of drug released vs time, Zero order equation 1, log cumulative percentage of drug remaining vs time, first order equation 2 , cumulative percentage of drug released vs square root of time, Higuchi's model equation 3

$$C=K_0t \quad (1)$$

Where K_0 is the zero order rate constant expressed in units of concentration/time and t is the time in hours. A graph of concentration vs. time would yield a straight line with a slope equal to K_0 and intercept the origin of the axes.

$$\text{Log } C = \text{Log}C_0\text{-}kt/2.303 \quad (2)$$

Where C_0 is the initial concentration of drug, k is the first order constant, and t is the time.

$$Q = Kt^{1/2} \quad (3)$$

Where K is the constant reflecting the design variables of the system and t is the time in hours. Hence, drug release rate is proportional to the reciprocal of the square root time.

To evaluate the drug release with change in the surface area and the diameter of the particles, the data were also plotted using the Hixson-Crowell cube root law:

$$\sqrt[3]{Q_0} - \sqrt[3]{Q_t} = k_{HC}X_t \quad (4)$$

Where Q_t is the amount of drug released in time t, Q_0 is the initial amount of the drug in the microsphere, and K_{HC} is the rate constant for the Hixson-Crowell rate equation, as the cube root of the percentage of drug remaining in the matrix vs. time.

5.2. Mechanism of drug release

Koresmeyer*et al*'s equation (Equation 5). Log cumulative percentage of drug released vs log time was plotted to evaluate the mechanism of drug release from microspheres and the exponent n was calculated through the slop of the straight line [43-44].

$$M_t / M_\infty = Kt^n \quad (5)$$

Where M_t/M_∞ is the fractional solute release, t is the release time, K is a kinetic constant characteristic of the drug/polymer system, and n is an exponent that characterizes the mechanism of release of tracers. If the exponent n= 0.45, then the drug release mechanism is Fickian diffusion, and if $0.45 < n < 0.89$, then it is non-Fickian or anomalous diffusion.

Statistical analysis

The *in vitro* release results are expressed as the mean ± standard deviation. Multiple comparisons of means (Tukey Test) are used to substantiate statistical differences between groups, while student's t-test was used to comparison between formulation batches. Significance was tested at the 0.001 level of probability (p) [44].

Thermal analysis:

Thermo analytical measurement were carried out with Differential Scanning Calorimetry equipment (DSC-60, Shimadzu, Mumbai), 3.0 mg sample was placed into aluminium containers and heated at a constant rate of 10°C / min. between 40ºC to 120°C under nitrogen and argon and thermogram was recorded [45].

5.3. *In-vitro* antidiabetic activity

In-vitro antidiabetic activity of hydroalcoholic extracts was performed as per given models.

5.3.1. α-amylase inhibition assay

The α-amylase inhibitory activity was calculated according to the methods reported in previous literature [46-48]. Stock solution of hydroalcoholic extract was prepared in 20% DMSO. Hydroalcoholic extract (100 µg/ml), acarbose (100 µg/ml) and α-amylase (0.5 mg/ml) was prepared in 0.02M sodium phosphate buffer and preincubated at 25°C for 15 min. After this 100 µL of 1% starch solution in sodium phosphate buffer was added to the reacting mixture. The reaction mixture was incubated at 25°C for 30 min and DNSA (dinitrosalicylic acid) was added to stop the reaction, incubated in boiling water bath for

10 min, and then cooled to room temperature. The reaction mixture was then diluted using distilled water. Absorbance was measured using BIORAD iMark Microplate Reader at 595 nm. The reading was taken in triplicate and α-amylase inhibitory activity was calculated as percentage inhibition. The inhibitory activity was calculated as

Percentage inhibition= A_c- [A_s– A_o]/ A_c × 100

A_c=Absorbance of control; A_s= Absorbance of sample with enzyme; A_o= Absorbance of sample without enzyme

5.3.2. **α-glucosidase inhibition assay**

100 mM of phosphate buffer saline (pH 6.9) was used to prepare all the solutions. 95 µL of phosphate buffer saline was added to 96 wells microplate followed by the addition of 25 µL of α- glucosidase (0.5 U/ml). Then 30 µL of hydroalcoholic extract (100 µg/ml) or acarbose (100 µg/ml) was added to the mixture and incubated for 20 minutes in an incubator at 37 °C temperature. After incubation, 50 µL of p-nitrophenyl-α-Dglucopyranoside (5mM) was added and further incubated for 45 minutes at 37 °C. After incubation absorbance was taken at 415 nm in iMark Microplate Reader. Enzyme with substrate was positive control. The control represented 100% enzyme activity and did not contain any plant extract. The readings were taken in triplicate and results were expressed as mean ± SEM [49]. The percentage inhibition was calculated as

Percentage inhibition= A_c- [A_s– A_o]/ A_c × 100

A_c=Absorbance of control; A_s= Absorbance of sample with enzyme; A_o= Absorbance of sample without enzyme

5. STABILITY STUDY

Stability testing evaluates how drug substance or product quality changes over time under environmental factors like temperature and humidity, establishing retest periods or shelf lives. Test conditions are chosen based on climatic conditions categorized into four zones (I-IV). See Tables 5, 6, & 7 for storage conditions [50-53]. Stress testing is likely to be carried out on a single batch of the drug substance. The testing should include the effect of temperature (in 10° C increments [e.g., 50°C, 60°C] above that for accelerated testing), humidity (75% relative humidity [RH]) where appropriate, oxidation, and photolysis on the drug substance [54-55].

Table 5: General case for stability testing

Study	Storage conditions	Minimum time period covered by data at submission (months)
Long-term	25°C+/-2°C, 60% RH+/- 5% RH	12
Intermediate	30°C+/-2°C, 60% RH+/- 5% RH	6
Accelerated	40°C+/-2°C, 75% RH+/- 5% RH	6

RH= Relative Humidity

Table 6: Drug substance intended for storage in a refrigerator

Study	Storage conditions	Minimum time period covered by data at submission (months)
Long-term	5°C+/- 3°C	12
Accelerated	25°C+/-2°C, 60% RH+/- 5%RH	6

RH= Relative Humidity

Table 7: Drug substances intended for storage in a freezer

Study	Storage conditions	Minimum time period covered by data at submission (months)
Long-term	20°C+/- 5°C	12

6.1. Long-term and short-term stability studies

The short-term stability study was conducted to evaluate the liquid form of PPA2 & PZM2 at room temperature for up to 5 days. The long-term stability was conducted to monitor physical and chemical stabilities of liquid form of PPA2 & PZM2 at 2-8 °C. The stability parameters, such as pH, phyto-constituent's concentration, particle size distribution and entrapment efficiency were determined as function of storage time.

6.2. Accelerated conditions

At the accelerated storage conditions, a minimum of three time points, including the initial and final time points (0, 3 and 6 months) from a 6-month study are recommended. Where an expectation exists that the results from accelerated studies are likely to approach significant change criteria, increased testing should be conducted either by adding samples at the final time point or by including a fourth time point in the study design. When testing at the intermediate storage condition is called for as a result of significant change at the accelerated storage condition, a minimum of four time point, including the initial and final points (0, 6, 9, 12 months), from a 12-month study is recommended [56].

RESULTS AND DISCUSSION

7.1. Extraction

A brownish residue weighing 20.6 g and 20.02 g (yield of 4.12 % and 4.00 % w/w) was obtained from

hydroalcoholic extracts of *Ziziphus Mauritiana* & *Phyllanthus Amarus*. These were kept in airtight bottles in a refrigerator until used.

7.2. **Qualitative phytochemical screening**

7.2.1. **Phytochemical screening**

Phytoconstituents represent marker constituents present in plant drug which is responsible for its major therapeutic action. The preliminary phytochemical studies of hydroalcoholic extract of *Phyllanthus Amarus* reveals the presence of phenols, tannins and flavanoids as shown in Table 8. Many plants are now used for the traditional treatment of diseases, possibly due to the presence of these phytochemicals, which have been reported to possess high medicinal value. Phenols, flavanols, proanthocyanidins and flavonoids are very important plant bioactive component reported to possess strong antioxidant, antibacterial and numerous biological activities. Due to their molecules structures reported to contain a hydroxyl group or phenolics ring several studies have indicated that these compounds are very effective in scavenging free radicals due to their redox properties, thereby having the capacity to link with proteins and bacterial membranes to form complexes.

Table 8: Phytoconstituents of hydroalcoholic extracts of *Phyllanthus Amarus*

		Phyllanthus Amarus
Alkaloids	Dragendorff's test	-
	Mayer's test	-
	Hager's test	-
	Wagner's test	-
	Tannic acid test	-
Carbohydrates	Charring test	-
	Molish test	-
	Iodine test	-
	Barfoed test	-
	Fehling solution test	-
	Benedict's test	-
Protein and amino acids	Biuret test	+
	Millons test	+
	Ninhydrin test	+
	Lead sulphide test	+
	Xanthoproteic test	+
Steroids	Liebermann burchard test	-
	Salkovaski test	-
	Trichloro acetic acid test	-
	Borntrager's test	-
	Modified Borntrager's test	+
	Keller Killiani test	+
	Legal test	-
	Baljeet test	-
Saponins	Haemolysis test	-
	Foam test	-
	Ammonia test	+
	Shinoda test	+
Flavonoids	Vanillin-hydrochloric acid test	+
Phenolic Compounds	Lead acetate solution	+
	Gelatin solution	+
	Potassium dichromate	+
	Dilute iodine solution	+
	Dilute nitric acid	+
	Acetic acid solution	+
	Dil. potassium permanganate sol.	+
Fixed oils	Spot test	-
	Saponification test	-

(+) = Present, (-) = Absent

The results of qualitative phytochemical analysis of hydroalcoholic extracts of *Ziziphus Mauritiana* are shown in Table 9. In the hydroalcoholic extract of *Ziziphus Mauritiana* showed the positive results for almost all phytochemicals studied here except cysteine, glycosides, tannins and starch. In leaf extract analysis no proper demarcation could be revealed about most of the phytochemical constituents under study. Carbohydrates, reducing sugar and hexose sugar were found to be present only in hydroalcoholic extract of leaves. Presence of protein was qualitatively confirmed by hydroalcoholic extracts of leaves.

Table 9: Phytoconstituents of hydroalcoholic extracts of *Ziziphus Mauritiana*

		Ziziphus Mauritiana
Alkaloids	Dragendorff's test	+
	Mayer's test	+
	Hager's test	+
	Wagner's test	+
	Tannic acid test	+
Carbohydrates	Charring test	+
	Molish test	+
	Iodine test	+
	Barfoed test	+
	Fehling solution test	+
	Benedict's test	+
Protein and amino acids	Biuret test	+
	Millons test	+
	Ninhydrin test	+
	Lead sulphide test	+
	Xanthoproteic test	+
Steroids	Liebermann burchard test	+
	Salkovaski test	+
	Trichloro acetic acid test	-
Glycosides	Borntrager's test	-
	Modified Borntrager's test	-
	Keller Killiani test	-
	Legal test	-
	Baljeet test	-
Saponins	Haemolysis test	-

		Ziziphus Mauritiana
	Foam test	+
Flavonoids	Ammonia test	+
	Shinoda test	+
	Vanillin-hydrochloric acid test	+
Phenolic compounds	Lead acetate solution	+
	Gelatin solution	+
	Potassium dichromate	+
	Dilute iodine solution	+
	Dilute nitric acid	+
	Acetic acid solution	+
	Dil. potassium permanganate sol.	+
Fixed oils	Spot test	-
	Saponification test	-

(+) = Present, (-) = Absent

Solvents for extracting biomolecules from plants are selected based on the polarity of the target solute to ensure proper dissolution. Sequential use of multiple solvents can reduce similar compounds in the yield. The effectiveness of extracted phytochemicals from Phyllanthus Amarus is significantly influenced by the solvents and extraction methods employed [57]. Phyllanthin is present in almost all of the Phyllanthus species, and its supplementation is able to reduce mRNA expression of adipogenic genes and increase the expression of lipolytic genes in white adipose tissue. Moreover, phyllanthin increases the expression levels of mRNA of the insulin receptor and insulin receptor subtrate-1 in adipose and liver tissues, and hence provides protection against metabolic damage in tissues [58]. Obesity causes poor lifestyles by increasing health issues such as heart disease, type 2 diabetes, obstructive sleep apnea and cancers. The aqueous extracts of Phyllanthus play a vital role in the body and weight loss by significant improvement in serum glucose, liver enzymes, lipid profile, apolipoprotien A and kidney functions in afflicted obese rats [59].

The phytochemical profile of the leaves of *Ziziphus Mauritiana* previously reported are Procyanidin B-2, Procyanidin B-3, Granatin B, Neostrictinin, Ellagirannin isomer, kaempferol-3-O-□-D-6-(p-coumaroyl) glucopyranoside, ellagic acid glucoside, kaempferol-3-O-D-glucoside, ellagic acid deoxyhexose [60]. The knowledge of the chemical composition of *Ziziphus Mauritiana* seeds is in sharp contrast to the paucity of information regarding knowledge of the chemical constituents in *Ziziphus Mauritiana* leaves. In addition, *Ziziphus Mauritiana* leaves may contain constituents that possess hypoglycemic and anti-diabetic properties. It should be noted that the various *Ziziphus Mauritiana* leaf extracts appear to be very low in alkaloids, saponins and cardiac glycosides, which have higher toxicity potentials as compared to the larger amounts of terpenes, phenolics and flavonoids present which have low toxicity potentials [61].

7.3. **Quantitative phytochemical screening**

7.3.1. **Determination of total phenolic content**

Total phenols were expressed as mg/g of gallic acid equivalents. Figure 6 shows the standard graph of gallic acid and the equation is, $y = 0.8243x + 0.0135$, $R^2 = 0.99$, where y is absorbance at 765 nm and x is total phenolic content.

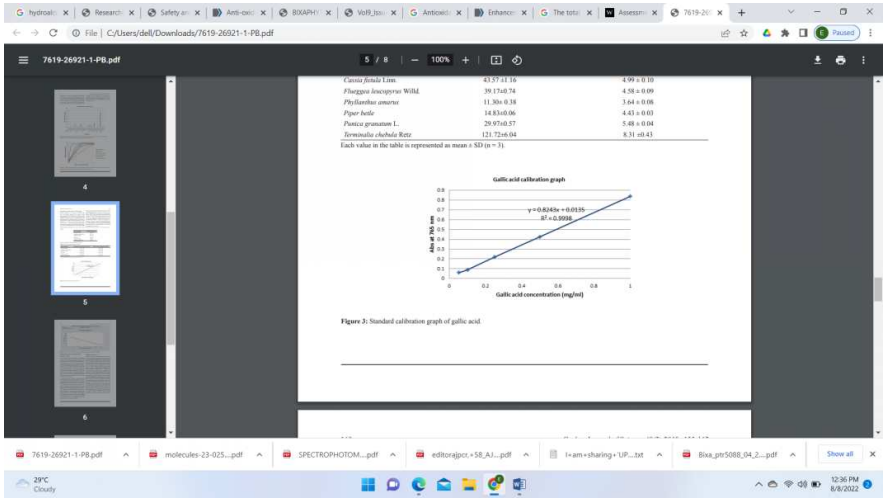


Figure 6: Standard calibration graph of gallic acid

Table 10: Results of total phenolic content of hydroalcoholic plant extracts

Extract	**Total phenolic content mg/GAE/g extract**
Phyllanthus Amarus	12.18 ± 0.64

Extract	Total phenolic content mg/GAE/g extract
Ziziphus Mauritiana	4.38 ± 1.58

Results of total phenolic content of hydroalcoholic extracts of *Phyllanthus Amarus and Ziziphus Mauritiana* are observed as 12.18 ± 0.64 and 4.38 ± 1.58, respectively as shown in Table 10. Different solvents have shown different effects in the extraction of phenolic compounds from the different parts of plants. This effect might depend on the polarity of the solvent as well as the specific part of the plant material. It has been reported that phenolic compounds are one of the major and diverse groups of phytochemical constituents in plants that have at least a single aromatic ring and one or more hydroxyl groups in their structures. Extracted phenolic compounds from plants have potential and different applications such as antitumor, antioxidant, antimicrobial, anti-inflammatory, antiviral, skin protection from UV radiation, analgesic, and antipyretic [62-64].

7.3.2. **Determination of total flavonoid content**

The content of flavonoids was expressed as mg/g of quercetin equivalents. Figure 7 shows the standard graph of quercetin and the equation is, y = 1.112x - 0.0164, R^2 = 0.9977, where y is absorbance at 430 nm and x is total flavonoid content.

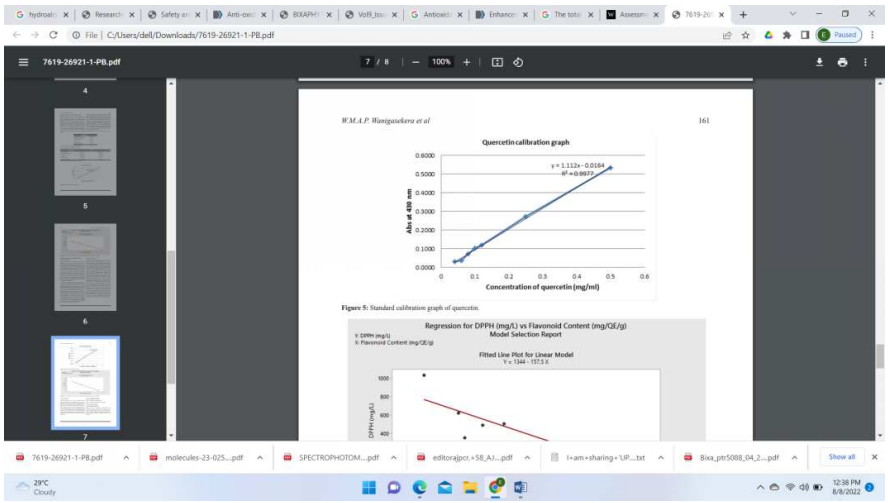


Figure 7: Standard calibration graph of quercetin

Table 11: Results of total flavonoid content of hydroalcoholic plant extract

Extract	Total flavonoid content mg/QE/g extract
Phyllanthus Amarus	8.45 ± 1.27
Ziziphus Mauritiana	11.47 ± 0.38

Results of total flavonoid content of hydroalcoholic extracts of *Phyllanthus Amarus and Ziziphus Mauritiana* are observed as 8.45 ± 1.27 and 11.47 ± 0.38, respectively as shown in Table 11. Flavonoid compounds are a major group of phenolic compounds that are responsible, along with carotenoids and chlorophylls, for colors in plants, as reported by [65]. Flavonoids contain in their chemical structure a variable number of phenolic hydroxyl groups and excellent properties of iron chelation and other transition metals, which give them a high antioxidant capacity; therefore, they play an essential role in the protection against oxidative damage and have therapeutic effects in a wide range of disease conditions [66-68].

7.4. **Standardization of extract**

Ziziphus Mauritiana & *Phyllanthus Amarus* leaves extract was standardized by HPLC, HPTLC and MS analysis.

7.4.1. **HPLC**

Standardization was done by comparing retention time of sample extract with the standard gallic acid, quercetin and rutin. Chromatogram of hydroalcoholic extracts of *Phyllanthus Amarus and Ziziphus Mauritiana* was shown in Figure 8 & 9.

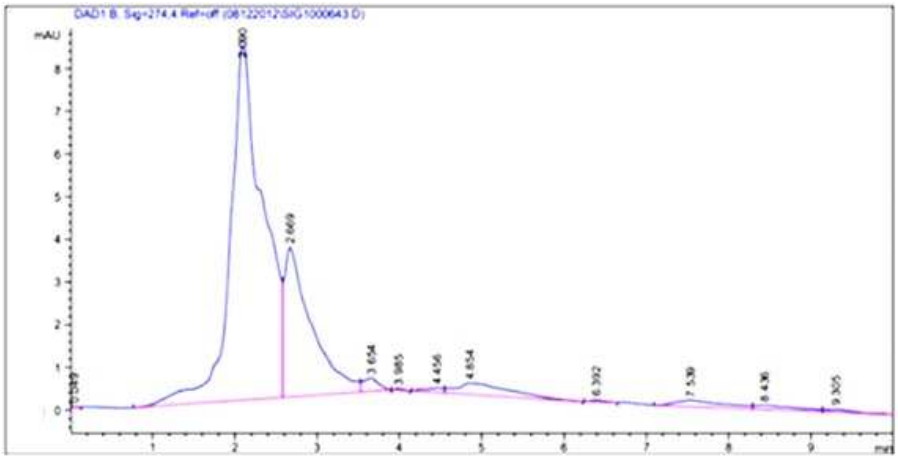


Figure 8: Chromatogram of hydroalcoholic extract of *Phyllanthus Amarus*

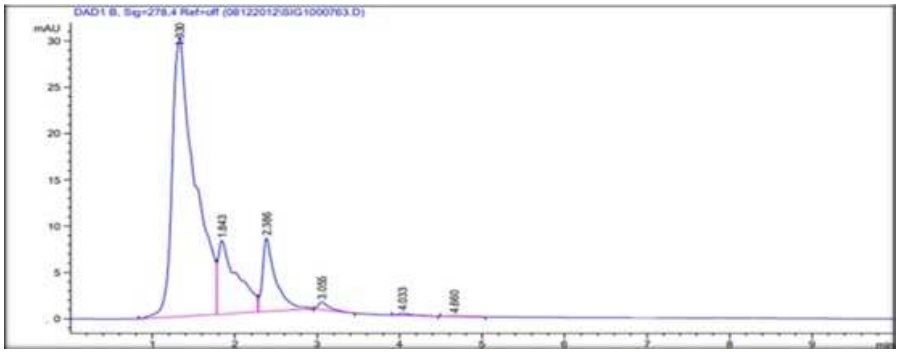


Figure 9: Chromatogram of hydroalcoholic extract of *Ziziphus Mauritiana*

The HPLC chromatogram of the reference standard of gallic acid is shown in Figure 10 and detection was done at 274 nm. The retention time of gallic acid standard was found to be 1.30 min. The HPLC chromatogram of the reference standard of quercetin is shown in Figure 11 and detection was done at 278 nm. The retention time of quercetin standard was found to be 1.32 min. The retention time of quercetin in the hydroalcoholic extract of *Ziziphus Mauritiana* was found to be 1.330 min. The HPLC chromatogram of the reference standard of rutin is shown in Figure 12 and detection was done at 255 nm. The retention time of rutin standard was found to be 2.024 min. The retention time of rutin in the hydroalcoholic extract of *Phyllanthus Amarus* was found to be 2.0 min.

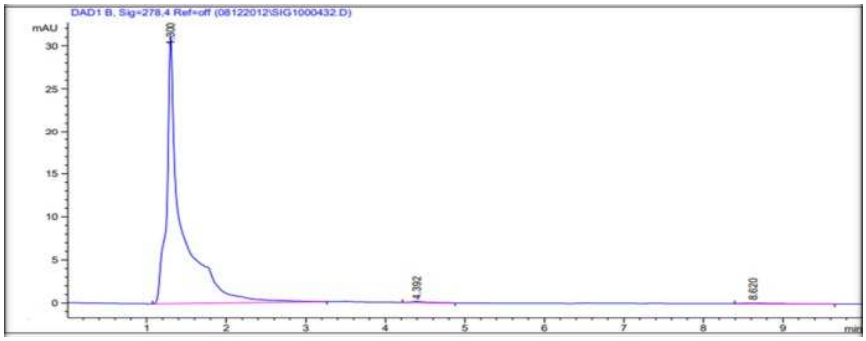


Figure 10: HPLC chromatogram of the reference standard of gallic acid (at 274 nm)

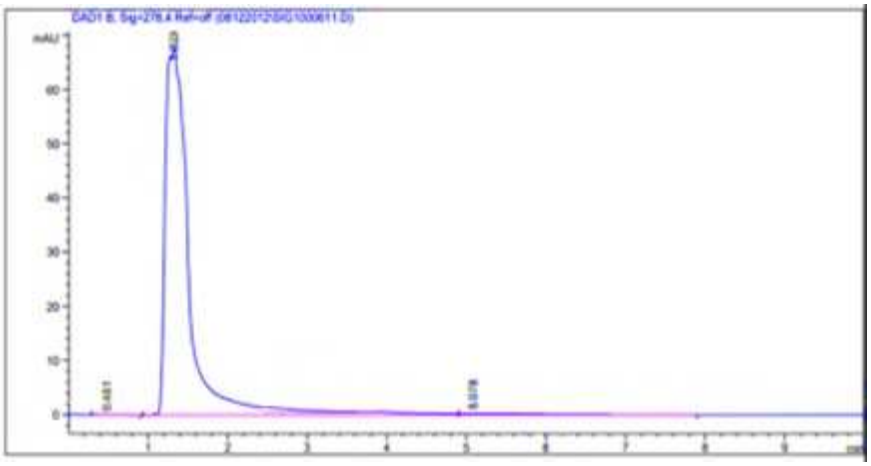


Figure 11: HPLC chromatogram of the reference standard of quercetin (at 278 nm)

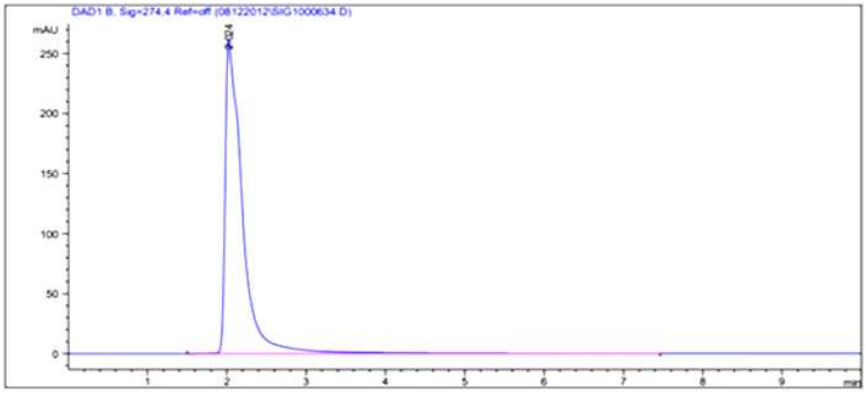
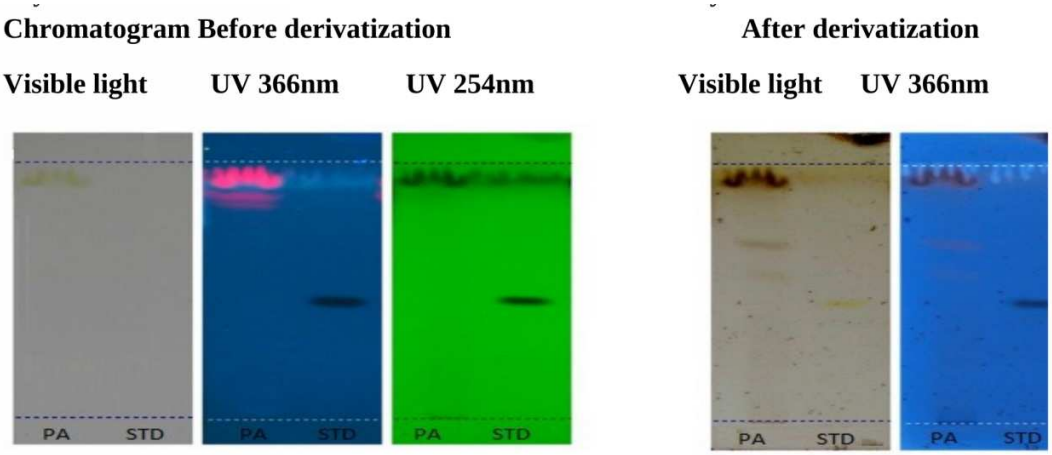


Figure 12: HPLC chromatogram of the reference standard of rutin (at 255 nm)

7.4.2. **HPTLC**

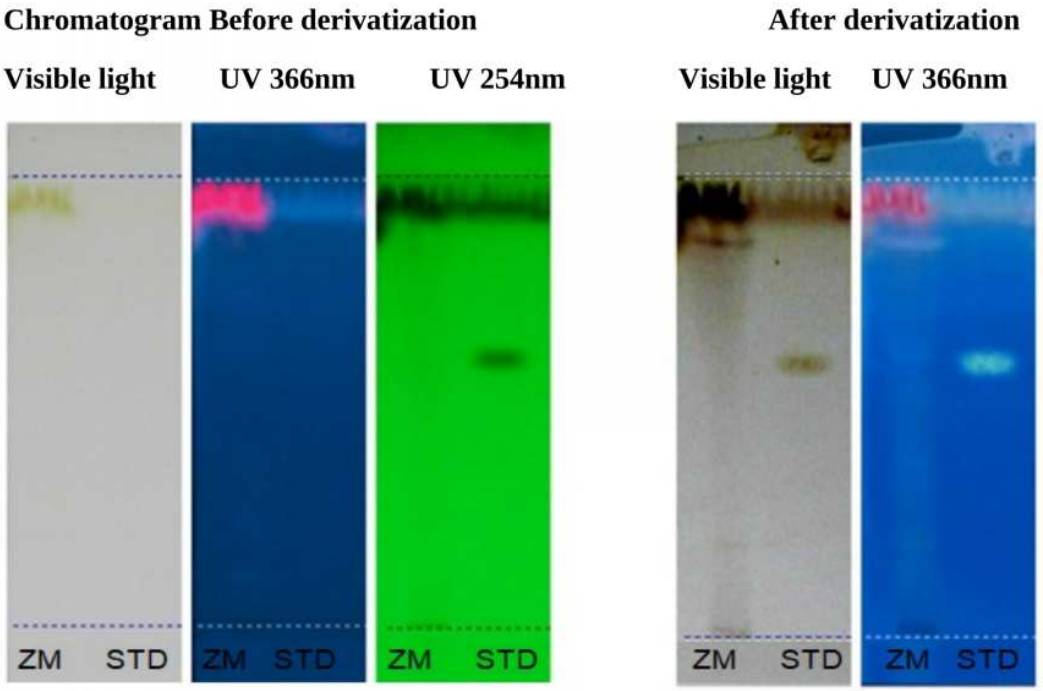
The evaluation of crude extract is an integral part of correct identity. HPTLC is useful as a phytochemical marker and more effective in the field of plant taxonomy also for the identification of plants through secondary metabolites. HPTLC fingerprinting is proved to be a linear, precise, and accurate method for herbal identification. Such finger printing is useful in quality control of herbal products and checking for the adulterants. Therefore, it can be useful for the evaluation of different marketed pharmaceutical preparations [69-71]. HPTLC fingerprint profiles of secondary metabolites in hydroalcoholic extract unveils the occurrence of the secondary metabolites flavonoids.



PA: *Phyllanthus Amarus*, STD: Standard

Figure 13: HPTLC chromatogram of hydroalcoholic extract of *Phyllanthus Amarus*

From the sample of *Phyllanthus Amarus* leaf extract compounds with R_f values of 0.02, 0.08, 0.15, 0.18, 0.25 0.33, 0.36, 0.47, 0.55, 0.68. 0.85 and for standard 0.49 were detected as shown in Figure 13.



ZM: *Ziziphus Mauritiana*, STD: Standard

Figure 14: HPTLC chromatogram of hydroalcoholic extract of *Ziziphus Mauritiana*

From the sample of *Ziziphus Mauritiana* leaf extract compounds with R_f values of 0.02, 0.07, 0.13, 0.16, 0.21, 0.25 0.31, 0.46, 0.58, 0.70, 0.76, 0.76, 0.86, 0.92 and for standard 0.67 were detected as shown in Figure 14. Phytoconstituents were identified based on their R_f values. Identified phytoconstituents were confirmed by the visualization at different wavelengths of light and by densitometric analysis of the plate. The reported data based on the HPTLC fingerprint approach can also be proposed as a quick and reliable analytic model for the Pharmacognostic study of plant raw materials used in commercial products.

7.4.3. **MS analysis**

To obtain a mass spectrum, the sample must first be ionized. The instruments in the Mass Spectrometry Laboratory are capable of all modern ionization techniques. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation,

and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is then produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound. In addition, it provides information on the positive ions formed in the ionization process, which is linked to the chemical structure of the molecule and the nature of the bonds. This technique is widely used for analyzing compounds from natural products.

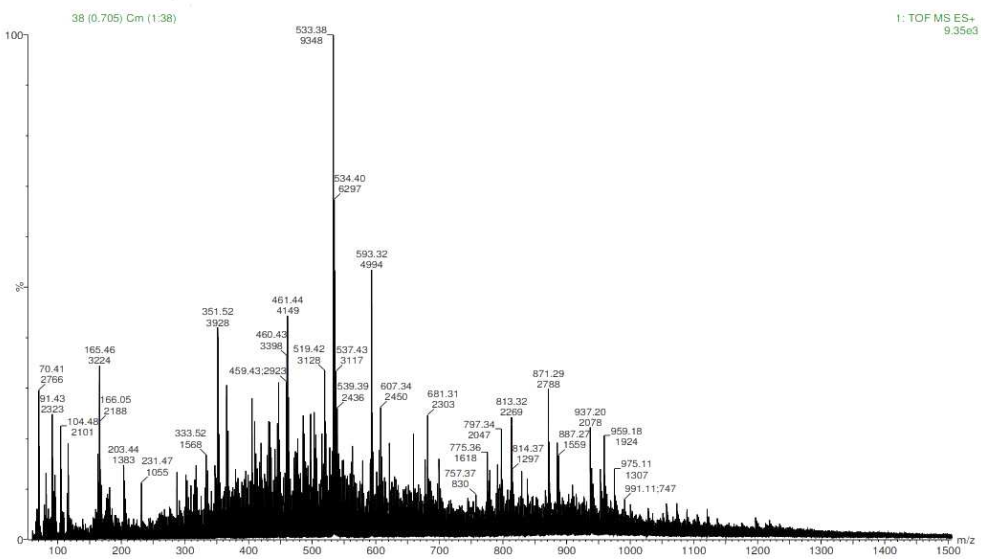


Figure 15: Mass spectra of hydroalcoholic extract of *Phyllanthus Amarus*

The doubly charged molecular ion of the sample was subjected to Q-Tof-MS/MS analysis and the results are detailed in given spectra (Figure 15 & Figure 16). From the spectra following m/z values i.e. 165.46, 231.47, 333.52 351.52 and 607.34 obtained that corresponds to gallic acid, kaempferol, quercetin, chlorgenic acid and rutin.

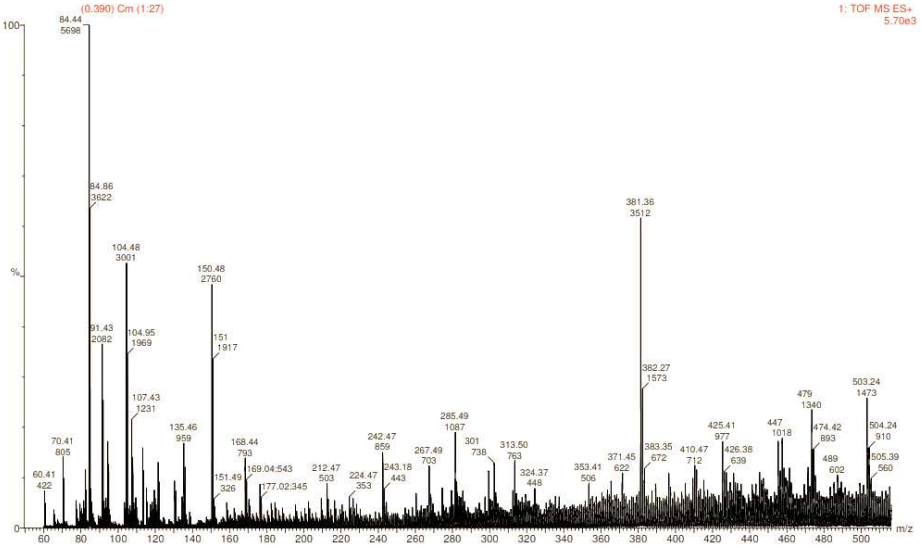


Figure 16: Mass spectra of hydroalcoholic extract of *Ziziphus Mauritiana*

A molecular ion peak of 479 m/z for quercitin-3-glucoside and 301 m/z for quercetin is obtained. A peak at 504.24 m/z corresponds to quercetin-3-acetylhexoside. A peak at 489 m/z corresponding to kaempferol-acetylglucosideis obtained that gives a fragment ion peak at 285 m/z. An another peak at 301 m/z is obtained that gives fragment ion peak of 151 m/z value. All peaks are in accordance with the

previously reported literature and suggesting the quality of extract.

7.5. **Phytosomes characterization**

For quantification of PPA & PZM phyto constituents (flavonoids), HPTLC method was used and quercetin taken as the marker compound. The standard curve of quercetin was prepared by HPTLC method & UV spectrophotometer method. In HPTLC method obtained the Y = 532.67x + 539.83 and R2 = 0.9929 and in UV spectroscopy method Y=0.0907x + 0.0655 and R2 = 0.9989. These above equations used in quantification of quercetin, which represent the quantity of phyto constituents present in the phytosomes. The entrapment efficiency of *Phyllanthus Amarus* and *Ziziphus Mauritiana* phytosomes were 91 & 90 % respectively as shown in Figure 17.

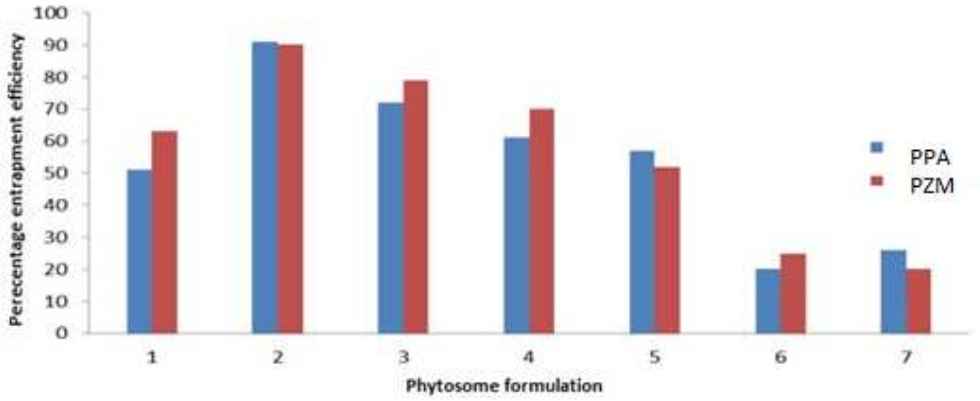


Figure 17: Encapsulation efficiency of PPA (Phytosomes of *Phyllanthus Amarus*) & PZM (Phytosomes of *Ziziphus Mauritiana*)

The design of experiments is a structured, organized method used to determine the relationship between the factors affecting a process and the output of that process [72]. In the development of any pharmaceutical product an important issue is to design a formulation with optimized quality in a short time period and minimum number of trials and cost-effective compared to conventional methods of formulating dosage forms [73]. Solvent evaporation method was selected for the preparation of phytosomes containing plant extracts. Seven formulation batches of hydroalcoholic extract of *Phyllanthus Amarus* and *Ziziphus Mauritiana* leaves with the different ratio of lipid & lecithin and selected on the basis of formation of phytosomes, encapsulation efficiency and desired particle size. On the basis of morphology (digital photographs & TEM), particle size and encapsulation efficiency, PPA2 & PZM2 batches were selected as the optimized formulation as shown in Table 12 and then evaluated further.

Table 12: Optimized formulation composition of phytosome

Formulation	Cholesterol	Lecithin	Chloroform	Hydroalcoholic extract	Cholesterol: lipid ratio
PPA2	15 mg	45 mg	10 ml	10 ml	1.5:4.5
PZM2	15 mg	45 mg	10 ml	10 ml	1.5:4.5

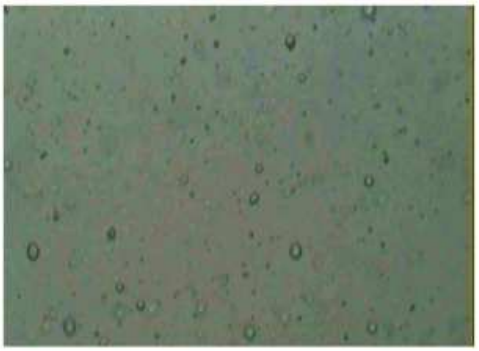


Figure 18 (A): Digital microscopy of PPA2 PZM2 (Phytosomes of *Phyllanthus Amarus*).

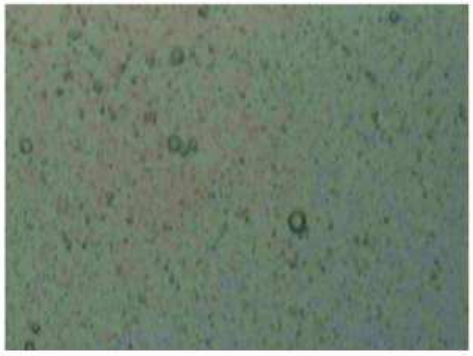


Figure 18 (B): Digital microscopy of (Phytosomes of *Ziziphus Mauritiana*).

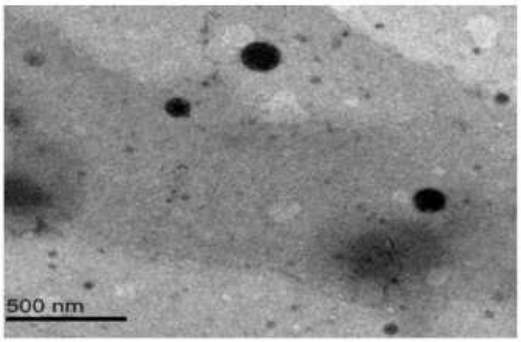


Figure 19 (A): TEM of phytosomes of *Phyllanthus Amarus*

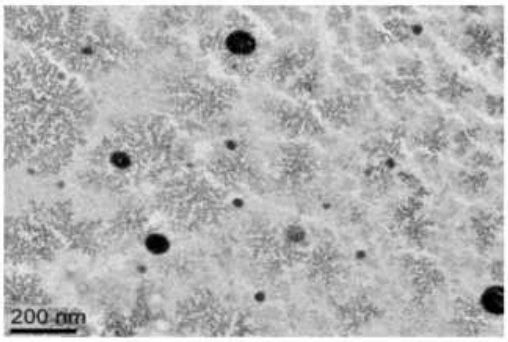


Figure 19 (B): TEM of phytosomes of *Ziziphus Mauritiana*

Uniform, regular and rigid vesicles were observed in digital microscopic view. TEM provided the evidence of vesicle formation and their morphology evaluation analysis showed small, spherical, and unilamellar vesicles. Vesicles between size range of 100nm-500nm were observed in scanning electron microscopic view as shown in Figure 18 & 19.

7.5.1. **Size analysis of phytosomes:**

The size distribution of phytosomes was analyzed using Malvern mastersizer S laser diffraction size analyzer (Malvern Instruments Ltd., UK).

Table 13: Physiochemical characteristics of the *Phyllanthus Amarus* and *Ziziphus Mauritiana* phytosome formulation

Formulation	z-Average diameter (d.nm)	Zeta potential (mV)
PPA2	109.7	-21.0
PZM2	131.2	-23.5

The average size of the *Phyllanthus Amarus* and *Ziziphus Mauritiana* phytosomes were 109.7 & 131.2 nm as shown in Figure 20 & 21.

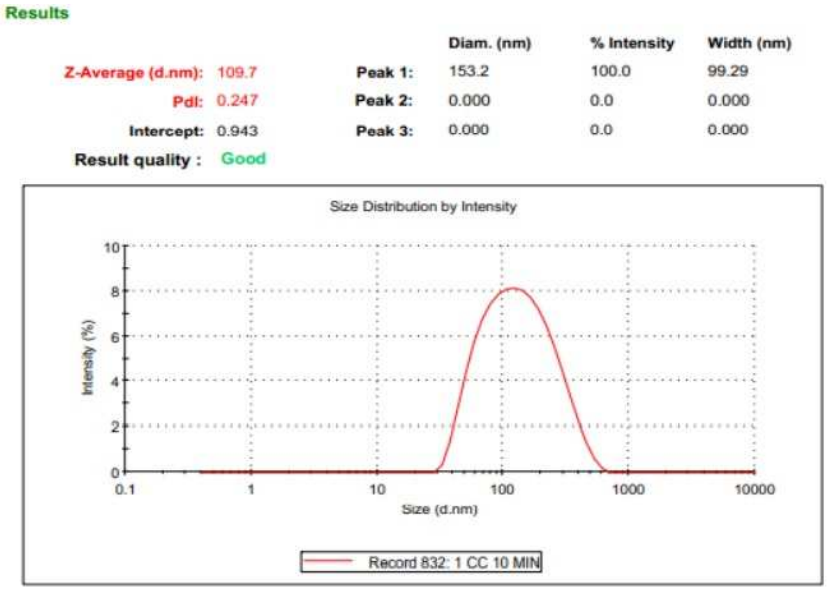


Figure 20: Size distribution by intensity of *Phyllanthus Amarus* phytosome formulation (PPA2)

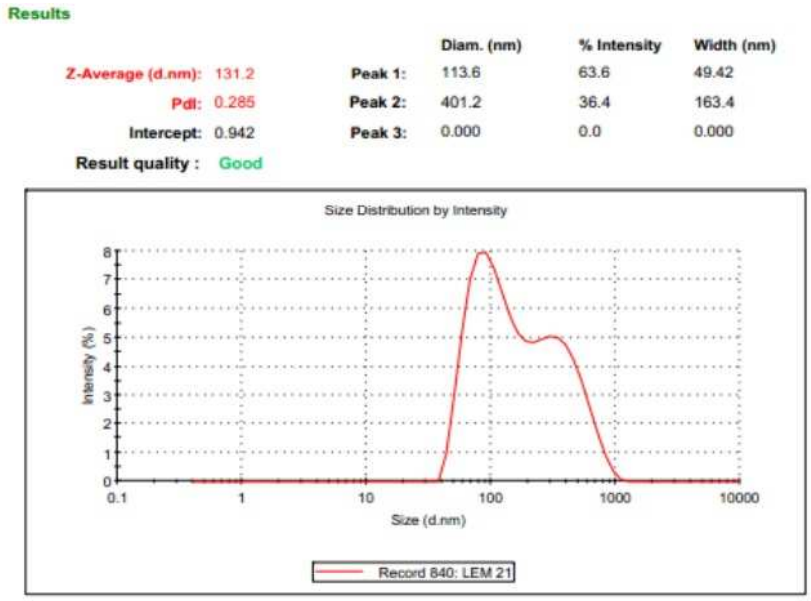


Figure 21: Size distribution by intensity of *Ziziphus Mauritiana* phytosome formulation (PZM2)

7.5.2. **Zeta potential**

The membrane surface potential plays an important role in the rate of aggregation and fusion of vesicles and hence in the physical stability of phytosomes. As far as zeta potentials are concerned, all types of phytosomes had negative electrophoretic mobilities as shown in Figure 22 & Figure 23.

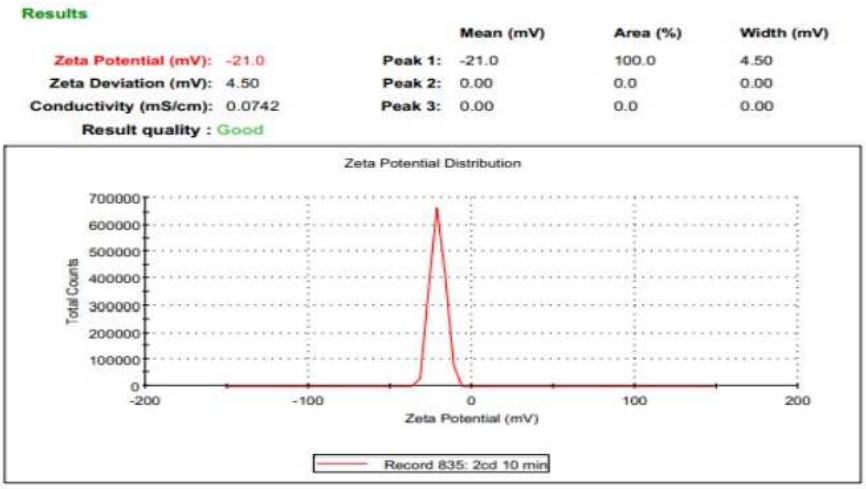


Figure 22: Zeta Potential distribution of *Phyllanthus Amarus* phytosome formulation (PPA2)

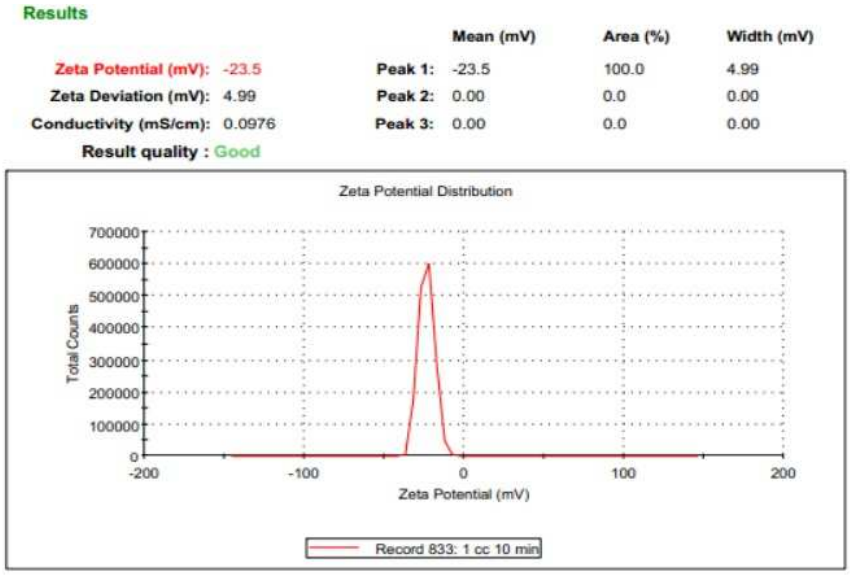


Figure 23: Zeta Potential distribution of *Ziziphus Mauritiana* phytosome formulation (PZM2)

A net negative charge in phosphatidylcholine increases zeta potentials, while kinetic stability relates to membrane charge and electrostatic repulsion overcoming London dispersion forces. Vesicles repel when their double layers overlap, creating an entropic force. This surface charge forms an energy barrier that, when surpassing thermal energy, ensures kinetic stability. Physiochemical characteristics of the *Phyllanthus Amarus* and *Ziziphus Mauritiana* phytosomes formulation are summarized in Table 13.

7.6. ***In-vitro*** **release studies**

The *In-vitro* releases of *Phyllanthus Amarus* and *Ziziphus Mauritiana* phytosomes were studied by using simple diffusion cell apparatus. Phytosomes equivalent to 100 mg of *Phyllanthus Amarus and Ziziphus Mauritiana* phytosomes were taken in test tube and placed in 100 ml of phosphate buffer. The medium was stirrer and the temperature at 35-37°C. The PPA2 & PZM2 batches showed the 89.73 & 87.09 % cumulative release as shown in Figure 24.

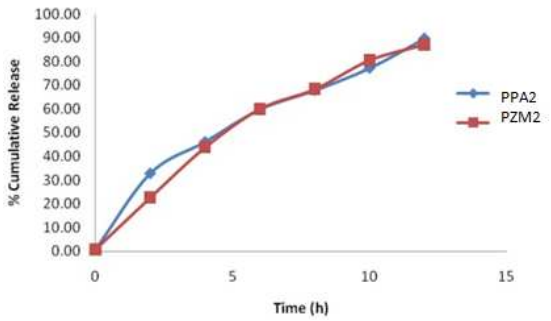


Figure 24: *In vitro* release of PPA2 (Phytosomes of *Phyllanthus Amarus)* & PZM2 (Phytosomes of *Ziziphus Mauritiana)*

7.7. **Drug release kinetics**

The kinetic treatment reflected that release data of PPA2 & PZM2 showed R2 value of 0.9938 & 0.9904 for first order or 0.9446 & 0.9601 for zero order equation respectively, indicating that release of plant phytoconstituents follows first order kinetic further in kosrsemeyerpeppas & higuchi equations n value were 0.259 & 0.266 showed the Fickian diffusion (Refer Figure 25 & Figure 26).

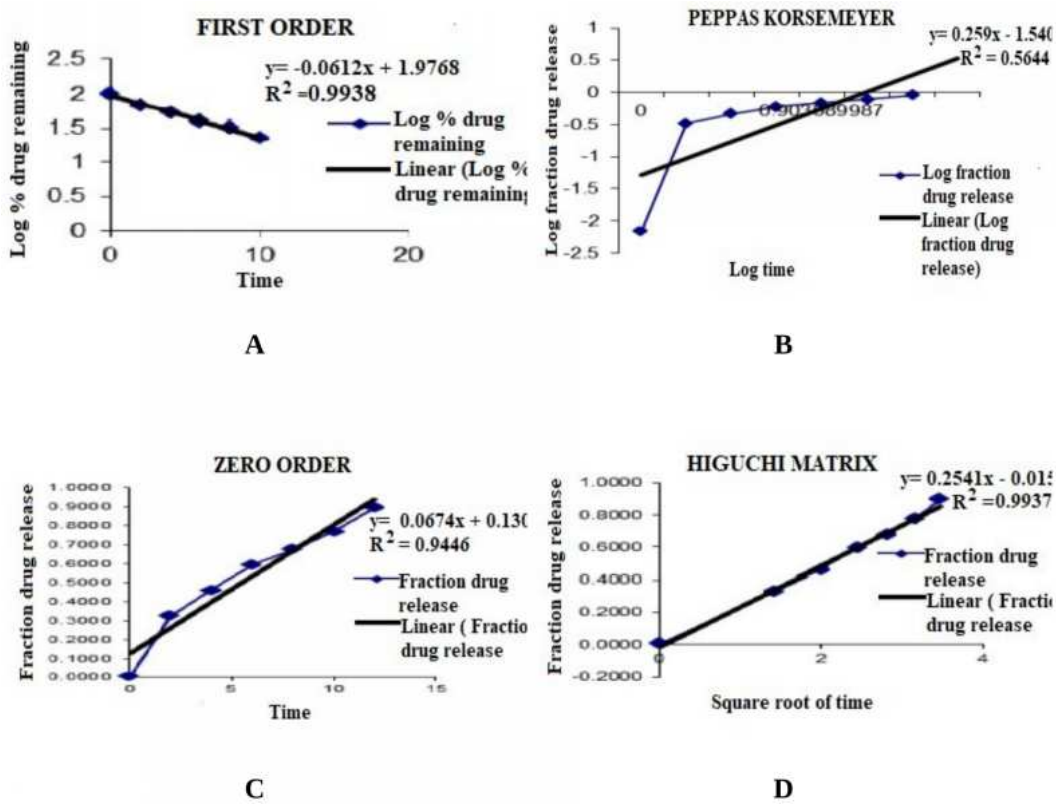


Figure 25: Release kinetics of PPA2, (A) First order plot, (B) Korsemeyerpeppas plot, (C) Zero order plot, (D) Higuchi matrix plot.

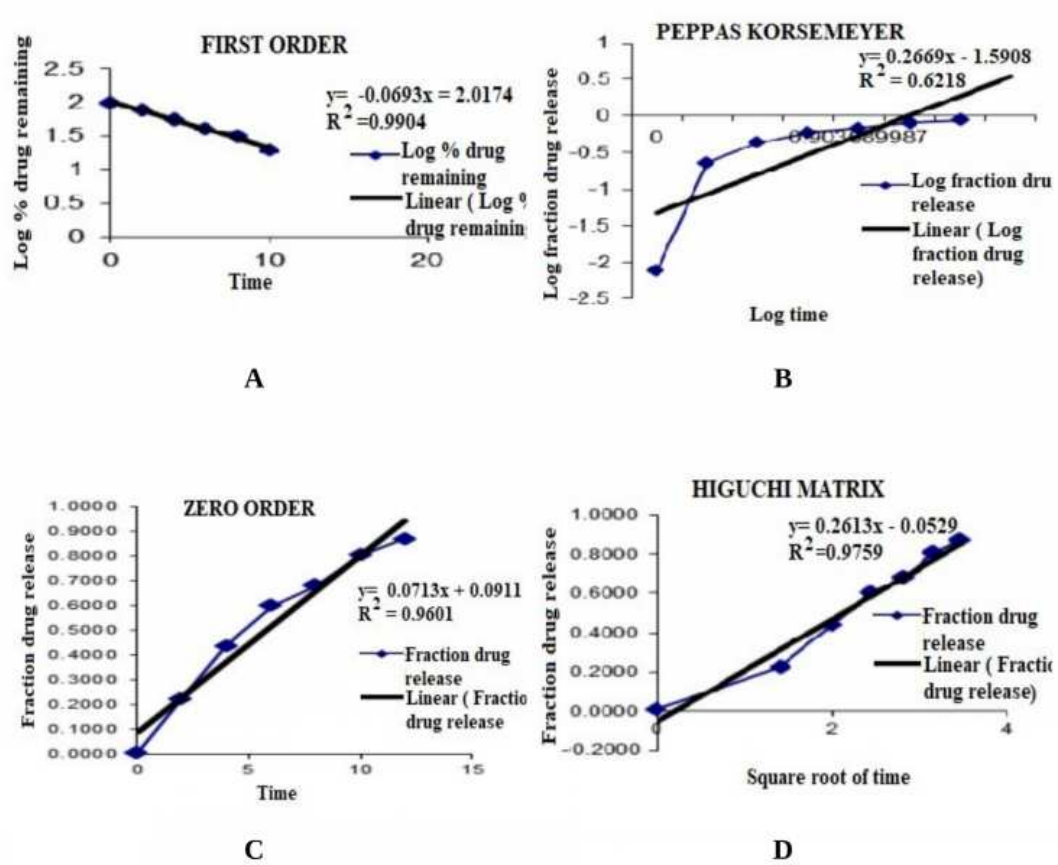


Figure 26: Release kinetics of PZM2,(A) First order plot, (B) Korsemeyerpeppas plot, (C) Zero order plot, (D) Higuchi matrix plot.

7.8. **Stability testing**

Table 14: Stability data of PPA2 & PZM2 stored at room temperature

Time/ Days	pH		Marker concentration (%)		Particle size (nm)		EE (%)	
PPA2/ PZM2	PPA2	PZM2	PPA2	PZM2	PPA2	PZM2	PPA2	PZM2
1 month	7.58	7.57	101	100	105	110	91	90
2 month	7.58	7.57	100	100	101	110	91.2	89.7
3 month	7.58	7.55	100	100	105	109	90.3	89.3
4 month	7.56	7.57	100	100	105	110	90.1	87.6
5 month	7.58	7.57	100	99.8	104	108	90	88.7

The short-term stability showed that the PPA2 & PZM2 were physically and chemically stable for up to 4 days at room temperature (Table 14). There was no appreciable change in phytosome size, pH, plant extract entrapment efficiency, and drug contents. The long-term stability showed that PPA2 & PZM2 were physically and chemically stable at 2-8 ^{0}C up to 6 months (Table 15). The drug contents were decrease within in the specifications (≥ 95% 0f initial) set for the product for up to 6 months at storage conditions. No significant changes were observed in mean particle size, pH and entrapment efficiency during stability studies

Table 15: Stability data of PPA2 & PZM2 stored 2-8 ^{0}C

Time/ Days	Ph		Marker concentration (%)		Particle size (nm)		EE (%)	
PPA2/ PZM2	PPA2	PZM2	PPA2	PZM2	PPA2	PZM2	PPA2	PZM2
1 month	7.58	7.57	100	100	105	110	91	90
2 month	7.56	7.58	99.9	99.6	105	109	90.7	89.5
3 month	7.54	7.56	98.4	99.1	104	107	90.5	88.9
4 month	7.52	7.54	97.2	98.5	105	109	90.9	89.9
5 month	7.55	7.52	96.3	96.8	103	108	90.2	87.8

7.9. **Thermal analysis of phytosomes**

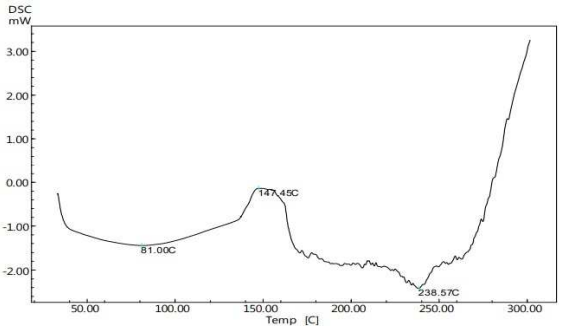


Figure 27: DSC of PPA2 (Phytosomes of *Phyllanthus Amarus*).

Thermal analysis has become an important tool for characterization dry extracts and plant products. The DSC photo visual showed the degradation process of samples. The DSC shown the characteristic peaks of lecithin, cholesterol and phytoconstituents of hydroalcoholic extract *Phyllanthus Amarus* leaves (81.90^0 , 147.45^0& 238.57^0C) and hydroalcoholic extract *Ziziphus Mauritiana* leaves (137.45^0& 231.37^0C) as shown in Figure 27 & 28, respectively.

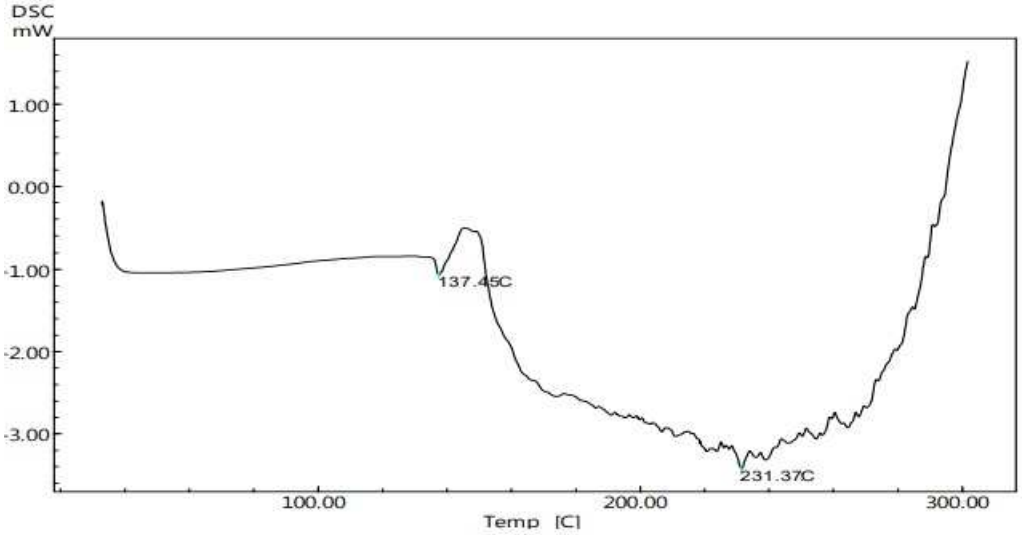


Figure 28: DSC of PZM2 (Phytosomes of *Ziziphus Mauritiana)*.

The phytosomal drug delivery technique was found suitable for use in the delivery of *Phyllanthus Amarus* and *Ziziphus Mauritiana* phytoconstituents. This technique is capable of modifying the phytoconstituent release profile so as to allay the challenges associated with immediate release profiles. Finally, there is need to conduct in vivo studies on the absorption of the *Phyllanthus Amarus* and *Ziziphus Mauritiana* phytosomal complex to verify if the intestinal permeability and bioavailability does improve as a result of the increased lipophilicity of the phytoconstituents.

7.10. **FTIR study**

The IR spectrum Figure 29 (A) of the empty phytosomes showed characteristic absorption. FTIR results show bands at 3373.52 cm-1 for hydroxyl (-OH), 2924.43 and 2854.18 cm-1 for C–H stretching of fatty acids, 1734.02 cm-1 for carbonyl stretching, 1466.34 cm-1 for C-H bending, 1238.67 cm-1 for P=O stretching, 1087.51 cm-1 for P–O–C stretching, and 969.49 cm-1 for choline N-(CH3)3 stretching. These findings align closely with Zhang et al. (2016), which reported similar peaks. The FT-IR spectrum of the phytosomes of *Phyllanthus Amarus* extract exhibited a characteristic band at 3293.83 cm-1 for a hydroxyl (-OH) group and at 2926.43 cm-1 (for C-H stretching) and 1627.82 cm-1. This indicates the presence of a carbonyl (C=O) group and 1346.36 cm-1 for (C-H bending) and 991.68 for (C-O group) as shown in Figure 29 (B).

The FT-IR spectrum of the phytosomes of *Ziziphus Mauritiana* showed significant changes. The absorption peak of hydroxyl (O–H) stretching of empty phytosomes shifted to lower wave number at 3352.62 cm-1 in complex. This could indicate the interaction of plant extract to polar end of the PC through formation of strong hydrogen bonding between hydroxyl groups of phospholipid and extract

phytoconstituents in phytosomes as shown in Figure 29 (C). [75-76]. Shifting of the P=O absorption band of PC molecule to lower frequency (1235.92 cm-1) further indicates the sensitivity of these vibrations to the formation of H-bonds [77].

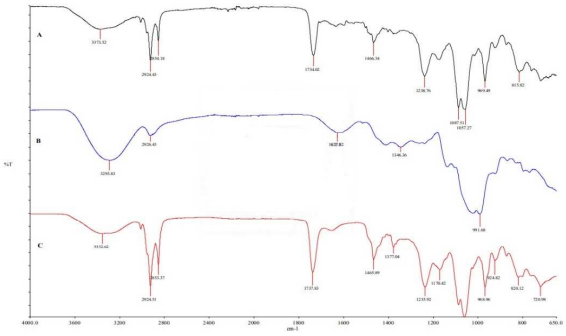


Figure 29: FTIR spectra of (A) empty phytosomes, (B) PPA2 (Phytosomes of *Phyllanthus Amarus*) and (C) PZM2 (Phytosomes of *Ziziphus Mauritiana*)

7.11. ***In-vitro*** **anti diabetic activity**

In-vitro antidiabetic activity of hydroalcoholic extracts of *Phyllanthus Amarus* and *Ziziphus Mauritiana* leaves was performed as per given models.

7.11.1. **α-amylase inhibition assay**

The % inhibition of α-amylase activity of hydroalcoholic extracts of *Phyllanthus Amarus* and *Ziziphus Mauritiana* at the concentrations of 20, 40, 60, 80 and 100 µg/ml as shown in Table 16 was studied in comparison with standard acarbose. The standard acarbose showed 70.83±1.48% (at concentration 100 µg/ml) inhibitory activity of α-amylase with IC50 value 24.85 µg/ml. *Phyllanthus Amarus* (at concentration 100µg/ml) exhibited 61.35 ±1.10 % of α-amylase inhibitory activity with an IC50 value of 26.97 µg/ml. *Ziziphus Mauritiana* (at concentration 100 µg/ml) exhibited 63.05±0.55 % of α-amylase inhibitory activity with IC50 values 28.53 µg/ml respectively. Results obtained from the above investigation showed that *Ziziphus Mauritiana* showed more potent inhibitory activity of α-amylase when compared with *Phyllanthus Amarus*.

Table 16: % inhibition of α-amylase with IC50 values

Concentrations (µg/mL)	*Phyllanthus Amarus*			*Ziziphus Mauritiana*			Acarbose		
	% inhibition	SD	IC_{50} values (µg /mL)	% inhibition	SD	IC_{50} values (µg /mL)	% inhibition	SD	IC_{50} values (µg /mL)
20	12.99	±0.36		17.21	±0.65		22.50	±0.90	
40	21.61	±0.98		30.48	±0.96		36.25	±0.69	
60	38.56	±1.32	26.97	35.34	±1.14	28.53	46.63	±0.79	24.85
80	50.54	±0.69		52.59	±1.19		62.47	±0.69	
100	61.35	±1.10		63.05	±0.55		70.83	±1.48	

Data are mean ± SD or % ± SD for triplicate measurements.

7.11.2. **α-glucosidase inhibition assay**

The % inhibition of α-glucosidase activity of hydroalcoholic extracts of *Phyllanthus Amarus* and *Ziziphus Mauritiana* at the concentrations of 20, 40, 60, 80 and 100 µg/ml as shown in Table 17 was studied in comparison with the standard drug i.e. acarbose.

Table 17: % inhibition of α-glucosidase with IC50 values

Concentrations (µg/mL)	*Phyllanthus Amarus*			*Ziziphus Mauritiana*			Acarbose		
	% inhibition	SD	IC_{50} values (µg/ mL)	% inhibition	SD	IC_{50} values (µg/ mL)	% inhibition	SD	IC_{50} values (µg /mL)
20	40.39	±1.73		41.01	±1.85		50.57	±0.94	
40	55.01	±1.91		50.91	±1.01		60.70	±0.86	
60	63.83	±0.33	35.83	62.80	±2.93	38.23	77.13	±1.71	32.48
80	81.92	±1.26		71.62	±1.46		92.31	±2.66	
100	92.71	±2.92		82.17	±2.26		97.67	±0.89	

Data are mean ± SD or % ± SD for triplicate measurements.

The standard acarbose showed 97.67 ± 0.89 % (at concentration 100 µg/ml) inhibitory activity of α-glucosidase with IC50 value 32.48 µg/ml. *Phyllanthus Amarus* (at concentration 100 µg/ml) exhibited 92.71 ± 2.92 % of α-glucosidase inhibitory activity with an IC50 value of 35.83 µg/ml. Percentage inhibition for *Ziziphus Mauritiana* (at concentration 100 µg/ml)

exhibited 82.17 ± ±2.26 % of α-glucosidase inhibition with an IC50 value of 38.23 µg/ml. Results obtained from the above investigation showed that *Ziziphus Mauritiana* showed more potent inhibitory activity of α-glucosidase when compared with *Phyllanthus Amarus.*

CONCLUSION

The present study successfully developed and optimized phytosomal nanocarriers containing Phyllanthus amarus and Ziziphus mauritiana extracts to enhance their physicochemical properties, stability, and in vitro antidiabetic potential. The extracts exhibited total phenolic contents of 12.18 ± 0.64 and 4.38 ± 1.58 mg GAE/g, and total flavonoid contents of 8.45 ± 1.27 and 11.47 ± 0.38 mg QE/g, respectively. The optimized formulations, PPA2 and PZM2, demonstrated particle sizes of 109.7 and 131.2 nm, with entrapment efficiencies of 91% and 90%, respectively. The formulations exhibited sustained release and maintained physicochemical stability for up to six months under refrigerated storage. In antidiabetic assays, P. amarus and Z. mauritiana extracts showed α-amylase inhibition of 61.35 ± 1.10% and 63.05 ± 0.55% at 100 µg/mL, with IC_{50} values of 26.97 and 28.53 µg/mL, respectively. Their α-glucosidase inhibition reached 92.71 ± 2.92% and 82.17 ± 2.26%, with IC_{50} values of 35.83 and 38.23 µg/mL, respectively. Overall, these findings highlight the potential of phytosomal nanocarriers to improve the delivery and stability of plant-derived bioactive compounds while supporting their promising in vitro antidiabetic activity. Further in vivo and clinical studies are required to validate their therapeutic efficacy and safety in diabetes management.

LIST OF ABBREVIATIONS

Abbreviation	Full Form
ANOVA	Analysis of Variance
ATR	Attenuated Total Reflectance
DLS	Dynamic Light Scattering
DSC	Differential Scanning Calorimetry
EE	Entrapment Efficiency
FTIR	Fourier Transform Infrared Spectroscopy
GAE	Gallic Acid Equivalent
HPLC	High-Performance Liquid Chromatography
HPTLC	High-Performance Thin-Layer Chromatography
IC_{50}	Half Maximal Inhibitory Concentration
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
LC–MS	Liquid Chromatography–Mass Spectrometry
m/z	Mass-to-Charge Ratio
MS	Mass Spectrometry
Nm	Nanometre
PDI	Polydispersity Index
PC	Phosphatidylcholine
PPA	*Phyllanthus Amarus* Hydroalcoholic Extract
PPA2	Optimized *Phyllanthus Amarus* Phytosomal Formulation
QE	Quercetin Equivalent
Q-ToF-MS	Quadrupole Time-of-Flight Mass Spectrometry
Rf	Retention Factor
Rpm	Revolutions Per Minute
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
w/w	Weight per Weight
°C	Degree Celsius
Mg	Microgram
µg/mL	Microgram per Millilitre
Mm	Micrometre

ETHICAL APPROVAL

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

HUMAN AND ANIMAL ETHICAL RIGHT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, and no funding was required to conduct these review data.

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AVAILABILITY OF DATA AND MATERIALS

The data supporting this study's findings will be available in the cited references.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

The manuscript was written through the contributions of all authors. The all contribution individually as per; "**GK;** Writing the paper and Original Draft Preparation, **TS:** Guide, Investigation & writing-Reviewing, Editing and supervision". All authors have approved the final version of the manuscript.

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