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Research Article

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Formulation and evaluation of microsphere of Dalbergia sisso and its antimicrobial activity

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Abstract Microspheres play a very important role as particulate drug delivery system because of their small size and other efficient properties. These have been proved to be a suitable bridge to scale the distance over to formulate an effective dosage form, so simulate controlled drag release. These microspheres are used for drug delivery, wherein the drug can be encapsulated or in entrapped form Polymeric microspheres are ideal vehicles for many controlled delivery applications due to their ability to encapsulate a variety of drugs, biocompatibility, high bioavailability and sustained drug release characteristics Controlled release drug delivery employs drug-encapsulating devices from which therapeutic agents may be released at controlled rates for long periods of time, ranging from days to months. Such systems offer numerous advantages over traditional methods of drug delivery, including tailoring of drug release rates protection of fragile drugs and increased patient comfort and compliance.

The present research work is aimed at the formulation and evaluation of novel drug delivery system for Dalbergia sissoo extract.

Keywords Dalbergia sissoo, Microspheres, Biocompatibility, encapsulated, therapeutic agent.

1. Introduction

1.1 Nanotechnology

Nanotechnology is science of element and fabric that cope with particle length in nanometers. The word 'Nano' is derived from Latin word, which means dwarf. Nanomedicine offers with full-size monitoring, manage, repair, defense and improve human genetic device at molecular stage the usage of engineering nanostructures and nano devices. Nanotechnology has received loads of attention with by no means- visible-before enthusiasm due to its budding capacity. It has provided satisfactory lined determination and cognizance treatment of ailment at molecular degree. Pharmaceutical nanotechnology embraces utility of nanoscience to pharmacy as nano materials, and as gadgets like drug transport, investigative, imaging and biosensor materials. Pharmaceutical nanotechnology has furnished extra high-quality-tuned opinion and remedy of focused remedy of disorder at molecular degree. Nanomedicine for the shipping of lively antiviral molecules by way of nanocarriers, in particular, aims at obtaining higher efficiency and slight toxicity in affected person (Kingsley et al., 2006).

Pharmaceutical nanoparticles are defined as solid, submicron-sized (less than 100 nm in diameter) drug carrier that may or may not be biodegradable. The term nanoparticle is a combined name for both nanosphares and nanocapsules. Nanospheres are matrix system in which drug is uniformly dispersed, while nanocapsules are the system in which the drug is surrounded by a unique polymeric membrane. Nanostructures have the ability to protect drugs from the degradation in the gastrointestinal tract; the technology can allow target delivery of drugs to various areas of the body (Kingsley et al., 2006).

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The novel and potential applications of nanotechnology in pharmaceutics are; development of diagnostic tools, formulation of drug carrier systems and gene therapy. The advantages of nanotech drugs compared to conventional counterparts lie on the basis of particle size. Drugs/drug products with nano dimension can be used at a lower concentration and can leadto early onset of bioactivity. Nano drug delivery systems (nanopharmaceutics) are, but not limited to, nanocapsules, nanospheres, nanosponges, nanoemulsions, solid lipid nanoparticles, nanovesicular systems (liposomes, niosomes), molecular systems (inclusion complexes) and nanocrystals (Nikalje, A. P. 2015).

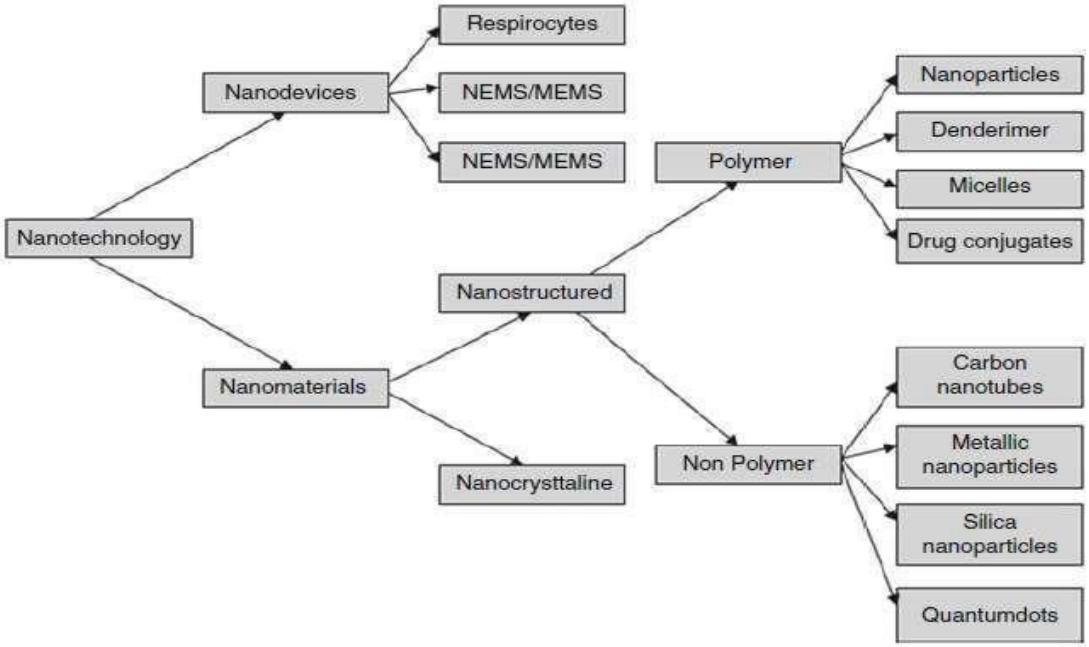

Figure 1: Various systems of Nanotechnology (Nikalje, A. P. 2015)

1.2 Novel drug delivery system (NDDS)

In the past few decades, considerable attention has been concentrated on the evolution of a novel drug delivery system (NDDS) for herbal drugs. Conventional dosage forms, including prolonged-release dosage forms, are unable to satisfy for both holding the drug component at a distinct rate as per directed by the requirements of the body, all through the period of treatment, as well as directing the phytoconstituents to their desired target site to obtain an utmost therapeutic response. In phytoformulation research, developing nano-sized dosage forms (polymeric nanoparticles and nanocapsules, liposomes, solid lipid nanoparticles, phytosomes, and nanoemulsion) has a number of advantages for herbal drugs, including enhancement of solubility and bioavailability, protection from toxicity, enhancement of pharmacological activity, enhancement of stability, improving tissue macrophage distribution, sustained delivery, and protection from physical and chemical degradation. Thus, the nano-sized NDDSs of herbal drugs have a potential future for enhancing the activity and overcoming problems associated with the plant medicines. Liposomes, which are biodegradable and essentially nontoxic vehicles, can encapsulate both hydrophilic and hydrophobic materials (Medina et al., 2004). With the progress in all domains of science and engineering, the dosage forms have developed from simple mixtures and pills to highly sophisticated technology, intensive drug delivery systems, which are known as NDDSs (Bhagwat et al., 2013).

1.3 Microspheres

Microspheres are polymeric micron range particles with sizes from 1 to 1000 µm .These microspheres are used for drug delivery, wherein the drug can be encapsulated or entrapped form. Based on the polymeric composition of microspheres, they can be classified into two types: natural and synthetic. Natural polymers include carbohydrates (e.g., chitosan, agarose, starch, alginate) and proteins (e.g., albumin, gelatin), while synthetic polymers include nonbiodegradable (e.g., polymethyl methacrylate, epoxy polymers) and biodegradable (polylactic acid/polyglycolic

lactic acid) (Prasad, Gupta, Devanna, & Jayasurya, 2014). Microspheres in drug delivery are used for targeted as well as prolonged drug release in the diseased area.

1.3.1 Types of Microspheres

1. Bioadhesive microspheres
2. Magnetic microspheres
3. Floating microspheres
4. Radioactive microspheres
5. Polymeric microspheres
   i) Biodegradable polymeric microspheres
   ii) Synthetic polymeric microspheres

1.3.2 Advantages of microspheres: (Mohan M *et al*., 2014)

- Particle size reduction for enhancing solubility of the poorly soluble drug.
- provide constant and prolonged therapeutic effect.
- provide constant drug concentration in blood thereby increasing patent compliance,
- Decrease dose and toxicity
- Protect the drug from enzymatic and photolytic cleavage hence found to be best for drug delivery of protein.
- Reduce the dosing frequency and thereby improve the patient compliance
- Better drug utilization will improve the bioavailability and reduce the incidence or intensity of adverse effects.
- Microsphere morphology allows a controllable variability in degradation and drug release.
- Convert liquid to solid form & to mask the bitter taste.
- Protects the GIT from irritant effects of the drug.
- Biodegradable microspheres have the advantage over large polymer implants in that they do not require surgical procedures for implantation and removal.
- Controlled release delivery biodegradable microspheres are used to control drug release rates thereby decreasing toxic side effects, and eliminating the inconvenience of repeated injections.

1.3.3 Disadvantages

- Drug discharge from measurements structure differs with an assortment of variables like inalienable and outward factors, food, and the pace of move through the gut
- The distinction in the delivery rate starting with one portion then onto the next
- Controlled discharge arrangement normally having a high amount of medications contains veracity and any vanishing of the measurements structure discharge trademark it might show to bring about the development of conceivable poisonousness and treatment disappointment
- Such measurement structures ought not be compacted or bitten Short drug stacking (limit of half) for the controlled delivery measurement structure
- Once managed it is difficult to eliminate the transporter totally from the body
- Parental conveyance of microsphere may act together or structure edifices with the blood segment (Kunchu et al ;2010)

1.3.4 Limitation (Kunchu *et al* ;2010)

Some of the disadvantages were found to be as follows

- The costs of the materials and processing of the controlled release preparation, are substantially higher than those of standard formulations.
- The fate of polymer matrix and its effect on the environment.
- The fate of polymer additives such as plasticizers, stabilizers, antioxidants and fillers.
- Reproducibility is less.
- Process conditions like change in temperature, pH, solvent addition, and evaporation/agitation may influence the stability of core particles to be encapsulated.
- The environmental impact of the degradation products of the polymer matrix produced in response to heat, hydrolysis, oxidation, solar radiation or biological agents. **(Mohan M *et al.*, 2014)**

1.3.5 Characteristics of microspheres: (Kalyan *et al* ;2010)

Table 1: Microsphere property

S. No.	Property	Consideration
1	Size Diameter	Uniformity/distribution
2	Composition	Density, Refractive Index, Hydrophobicity/hydrophilicity Nonspecific binding Autofluorescence
3	Surface Chemistry	Reactive groups Level offunctionalization Charge
4	Special Properties	Visible dye/fluorophoreSuperparamagnetic

1.4 Methods used in microsphere preparation

Choosing the method depends primarily on Character of a polymer been using, the drug, the factors equivocally determined by many formulations and technological factors as the size of the particles requirement, and the drug or protein should not be significantly impacted by the process, the reproducibility of the release profile and the method, there should be no stability Issue, in relation to the finished product. The various types of procedures used to prepare the microspheres using hydrophobic and hydrophilic polymers as matrix materials**.**

- The capacity to integrate medication doses which are relatively small.
- Stability of preparation after synthesis with a shelf spam which is clinically acceptable.
- Controlled particle size and dispensability for injection in the aqueous vehicles.
- Effective reagent release with strong control over a large time-scale.
- Biocompatibility of controllable biodegradability and chemical alteration response.

1.4.1Wax coating and hot melt

Wax used to encapsulate the main components, by dissolving or dispersing the product in melted wax. The waxy paste or mixture, such as frozen liquid paraffin, is released by high intensity blending with cold water. The water is heated up for at least an hour. The substance is stirred up for at least 1 hour. Then the external layer (liquid paraffin) is decanted and the microspheres are immersed in a non-miscible solvent and dry air is required to dry. For the surface ingredients, carnauba wax and beeswax can be used and both should be combined to obtain desirable characteristics.

1.4.2 Spray drying technique (Kunchu *et al* ;2010)

This was used to prepare polymer microsphere mixed charged with drug. This requires dispersing the raw substance into liquefied coating liquid, and then spraying the mixture into the air for surface solidification accompanied by rapid solvent evaporation. Organic solvent and polymer solution are formulated and sprayed in various weight ratios and drug in specific laboratory conditions producing microspheres filled with medications. This is fast but may lose crystalinity due to rapid drying.

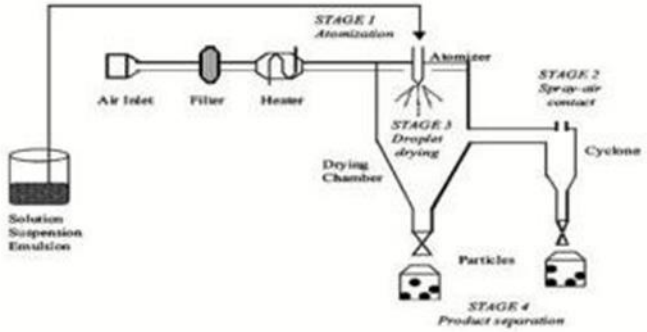

Figure 2: spray drying technique

1.4.3 Coacervation (Kunchu et al ;2010)

This method is a straight forward separation of macromolecular fluid into two immiscible types of material, a thick coacervate layer, comparatively condensed in macromolecules, and a distilled layer of equilibria. This method is referred to as basic coacervation, in the presence of just one macromolecule. If two or more opposite-charge macromolecules are involved, they are considered complex coacervation. The former is caused by specific factors

including temperature shift,Using non-solvent or micro-ions contributing to dehydration in macromolecules, since they facilitate interactions between polymer and polymer through polymer solvent interactions. This can be engineered to generate different properties on microsphere.

1.4.4Solvent evaporation (Kunchu et al ;2010)

The method of solvent evaporation has also been extensively used to preparation of PLA and PLGA microspheres which contain many various drugs. Several variables were identified that can significantly affect microspheric characteristics, such as solubility of drug, internal morphology, type of solvent, diffusion rate , temperature, polymer composition as well as viscosity, and drug loading. The efficacy of the solvent evaporation system to create microspheres relies on the effective entanglement of the active substance into the particles, and therefore this procedure is particularly efficient with drugs that are either insoluble or partially soluble in the liquid medium that constitutes the constant phase

1.4.5 Precipitation (Kunchu et al ;2010)

It is a modification of the form of evaporation. The emulsion is polar droplets scattered over a non-polar medium. The use of a co-solvent can extract solvent from the droplets.The subsequent rise in the concentration of polymers induces precipitation to create a microspheric suspension.

1.4.6 Freeze Drying (Kunchu et al ;2010)

Freeze-drying is effectively used in protein API microspheres praparation. The method is freezing, sublimation, main drying, and secondary drying. At the freezing step, account is taken of the eutectic point of the components. During the process, lyoprotectants or cryoprotectants will stabilise API molecules by removing water, creating a glass matrix, lowering intermolecular interaction by forming hydrogen bonds between the molecules or dipole - dipole interactions. It's a beneficial cycle for heat tolerant molecules, given its high expense. Freeze-drying produces solidification and then enables the reconstitution of particles in an aqueous media.

1.4.7 Single Emulsion Solvent Evaporation Technique (Kunchu et al ;2010)

This process requires polymer dissolution in an organic solvent accompanied by emulsification of an aqueous environment containing the emulsifying agent. The resulting emulsion is stirred for several hours in atmospheric conditions to allow the solvent to evaporate, which is then washed, rinsed and dried in desiccators. Designed and manufactured drugs microspheres with polymers by diffusion-evaporation method with emulsion solvent.

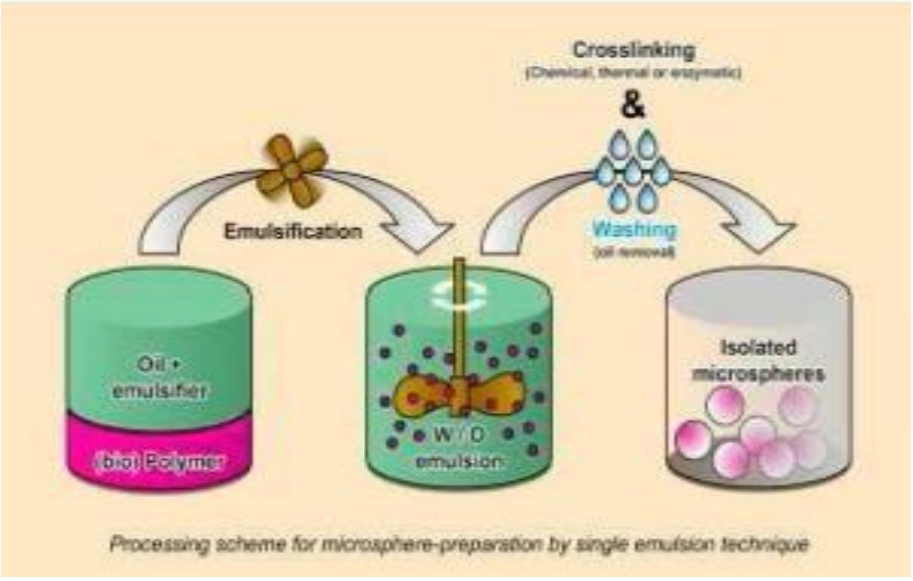

Figure 3: Single emulsion technique

1.4.8 Double emulsification method (Kunchu et al ;2010)

The Doppel-emulsion strategy requires mixing w / o / w or o / w / o processing the double emulsion. The aqueous solution of the product is distributed in a continuous lipophilic organic phase. The continuous step which consists of

a polymer solution eventually encapsulates medication Observed in the scattered aqueous layer to form primary emulsion. Prior to introduction to the aqueous solution of alcohol to form primary emulsion, the pre-formed emulsion is subjected to homogenisation or sonication. The microspheres filled with the drug prolonged the release of the medication 24 hours and were Observed to be diffusion and erosion regulated.

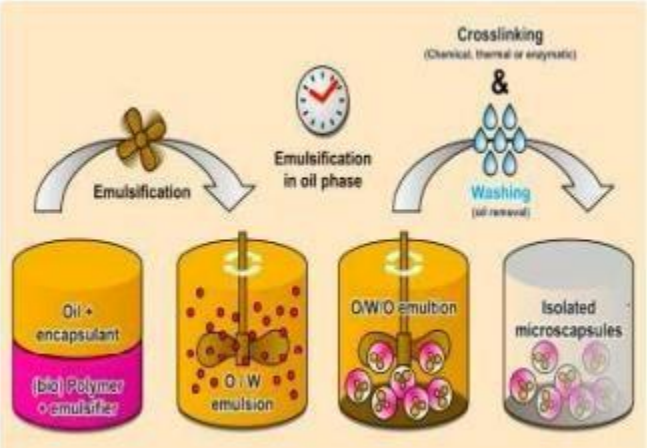

Figure 4: Double emulsion technique

1.4.9 Ionic gelation method (Kunchu et al ;2010)

Ionotropic gelation is depend on the tendency of polyelectrolytes to cross connect to develop hydrogel beads often called gelispheres in the existence of counter ions. Gelispheres are Circular cross linked polymeric hydrophilic agent capable of substantial gelation and thickening in model

biological fluids and drug release regulated by polymer relaxation via it. The hydrogel beads are formed by dumping a drug-laden polymeric solution into the polyvalent cations aqueous solution. The cations migrate through the drug-laden hydrophilic compounds, creating a three-dimensional lattice the moiety is ionically crosslinked. Biomolecules may also be placed into these gelispheres to maintain their three-dimensional form under moderate conditions.

1.5 Characterization of Microsphere

Particle size analysis

The dried microsphere were determined by microscopic method using calibrated optical micrometer, the most commonly used techniques for microparticular visualisation are standard light microscopy (LM) (Caine et al., 2017).

Scanning electron microscopy (SEM) study

The Samples were analyzed through SEM and it was well qualified from a back scattered electron sensor for image analysis and conducting the x - Ray diffraction analysis (EDXA) for elemental structure determination where particular elements have been identified. In this method the sample was scanned in parallel lines using a centered electron beam. Microspheres were then placed on a sample holder for SEM characterization preceded by coating with a conductive metal like platinum or zirconium using a sputter coater. The sample was then scanned with a guided, fine electron beam. The surface properties of the sample were derived from the secondary electrons leaked from the sample surface (Caine et al., 2017).

Flow properties

The flow properties can analysed by determining the carr's compressibility index , Hausner ratio and resting angle of repose. A volumetric cylinder was used to assess bulk density and tapped density (Caine et al., 2017).

Thermal analysis

Thermal analysis techniques analyse these changes routinely by applying scheduled variations in temperature for heating and cooling, as well as applying defined Specimen atmospheres and pressures. The most widely observed properties include subtle variations in heat and enthalpy, weight loss or weight gain, Young's modulus, thermal expansion or shrinkage and evolution of gas (Caine et al., 2017).

Molecular size and shape

For ordinary representation of microspheres the Scanning Electron Microscopy (SEM) and Light Microscopy (LM) systems are most broadly utilized. Both can have the option to find the outer structure and state of microspheres. In a twofold walled microsphere, the light microscopy (LM) manages the cost of a covering boundary. It estimated infinitesimally the microsphere structures can be imagined when the covering. A microsphere surfaces assessment and cross-separated after particles can permit the scanning electron microscopy(SEM). For twofold walled frameworks assessment, Scanning Electron Microscopy can be utilized (Caine et al., 2017).

Density determination

Multi-volume pycnometer is utilized for the thickness of the microspheres isestimated. In a cup the example is precisely said something is set in the multi-volume pycnometer. At a consistent weight helium is start in the chamber and they permit extension. Results are diminished in weight inside the get together in this development. At the point when two progressive readings of weight decrease at various proportion at that point introductory weight are note. From two weight readings, the volume can decide the thickness of the microspheres transporter (Caine et al., 2017).

Angle of Contact

angle of contact is discovered the wetting property of a microparticle channel. The hydrophobicity or hydrophilicity term is inspecting microspheres' tendency. The interface of strong/air/water the point of contact should be estimated. The progressing and withdrawing point of contact are estimated by the addition of a bead in a roundabout cell mounted overthe goal of an improved magnifying instrument. 20°C inside a moment of affidavit of microspheres the contact points are estimated (Caine et al., 2017).

Electron spectroscopy for chemical analysis

The electron spectroscopy for substance investigation (ESCA) is pertinent for the surface science of the microsphere's assurance. A mean for the nuclear arrangement of the surface these stock the electron spectroscopy for the compound examination technique(ESCA). The assurance of surface corruption of the biodegradable microsphere is through the spectra. These spectra were gotten by utilizing ECSA (Caine et al., 2017).

Fourier transform infrared spectroscopy

Corruption of the polymeric lattice of the transporter framework is dictated by utilizing the FT- IR. Rotated complete reflectance's (ATR) are estimating the explored surface of the microspheres. From the ATR cell, the IR bar is passed and they reflected commonly through the example to supply IR spectra chiefly of surface material. The surface arrangement of the microspheres relying on assembling methodology and condition, this data gives the ATR-FTIR (Caine et al., 2017).

Determination of percentage yield

The percentage yield can be determined by calculating the measured amount of the product and the polymers used in the formulation of the microspheres and the Overall sum ofv microspheres produced (Caine et al., 2017).

Drug content

The mixture should be held aside to allow the particles to sediment and then wash. 1mL was moved into volumetric flask from the filtrate, and the volume was balanced with 0.1N NaOH. Drug was measured spectrophotometrically after the correct dilution (Caine et al., 2017).

Entrapment Efficiency

Lysate can decide the catch ability of the microspheres or the percent capture by permitting wash microspheres. According to monograph necessity, the lysate is then exposed to the assurance of dynamic constituents (Caine et al., 2017). The following equation is used for the calculation of encapsulation efficiency:

$$\% \text{ DEE} = \frac{Estimated\ drug\ content}{Theoritical\ percent\ drug\ content} \text{x } 100$$

1.6 Selection and collection of Plant-

Plant and plant was selected on the basis of ethno-botanical survey. Pharmacological investigations report and recent investigations were considered in respect of selected Plant. Observe that selected plant should possess the following properties: -

- Anti- ulcer activity
- Anti- oxidant activity
- Anti- inflammatory activity, anti- microbial activity etc.
- Having good quality and quantity of bio-constituents.

Fresh leaves of Dalbergia sissoo, free from disease were collected from local area. 340.12 gram of the powder prepared from shade-dried bulb was subjected to extraction by soxhlation method, for 24 hours using solvent (ex- Petroleum ether, Methanol) as nonpolar solvent at first.

1.7 Extraction

Soxhlet extraction method:

Maceration technique is an affordable way to get essential oils and bioactive compounds. Occasional shaking in maceration facilitate extraction by two ways; (a) increase diffusion, (b) remove concentrated solution from the sample surface for bringing new solvent to the menstruum for more extraction yield (Azmir et al.,2013). For experimental extraction in small scale, maceration generally consists of several steps.

- Firstly, plant samples were collected, washed, rinsed and dried properly.
- Secondly, grinding of plant materials into small particles or in powder form because it increases the surface area for proper mixing with solvent.
- Thirdly, in maceration process, specific solvent (according to sample & polarity) named as menstruum is added in a closed vessel & powder form of plants sample was subjected to extraction with different organic solvents petroleum ether and Methanol and allow standing for 4-5 days each.
- After that, the extract was filtered but the marc which is the solid residue of extraction is pressed to recover rest amount of solution (Azmir et al.,2013).
- Then, extract was transferred to beaker and evaporated; excessive moisture was removed and extract was collected in air tight container (Chandel et al, 2020) .

1.8 Determination of Percentage yield

The percentage yield of Dalbergia sissoo were determined as percentage of the weight of the extracts to the original weight of the dried sample used, using the formula (Duniya et al., 2018);

Formula:

$$\% \text{ Yield} = \frac{Weight\ of\ Extract}{Weight\ of\ plant\ material\ used} \text{x } 100$$

1.9 Solubility Determination

Solvent is a liquid that serves as the medium for reaction. It can serve two major purposes:

- Non-participatory to dissolve the reaction.
- Participatory a source of acid (protons), base (removing protons) or as a nucleophile (donating lone pair of electron).

Polarity is a separation of electric charge leading to a molecule or its chemical groups having an electric dipole moment, with a negatively charged end and positively charged end (Jain and Verma 2020).

1.10 Preparation of Reagents: (Indian Pharmacopoeia, 1996)

1. Molisch's Reagent- Dissolve 15gms of 1-napthnol in 100ml of alcohol or chloroform.
2. Fehling's solution (reagent for reducing sugar):
   - Fehling's A solution- Dissolved 34.66grms of copper sulphate in water and dilute it to 500ml.
   - Fehling's B solution- Dissolve 173grms of potassium sodium tartrate and 50grms of sodium hydroxide in water and dilute to 500ml.
3. Benedict's Reagent- With aid of heat, dissolve 173grms of sodium citrate and 100grms of sodium carbonate in 800ml of distilled water. Filter, if necessary & dilute to 850ml. Now dissolve 17.3grms of copper sulphate in 100ml of distilled water. Pour the copper sulphate solution to carbonate-citrate solution with constant stirring.

4. Barfoed's Reagent (reagent for reducing monosaccharide)- Dissolve 66grms of cupric acetate and 10ml of glacial acetic acid in distilled water and dilute to 1L.
5. Dragendorff's Reagent- Shake vigorously to dissolve 0.85grms of bismuth subnitrate in 10mL of glacial acetic acid and 40mL of water (solution A). Dissolve 8grms of potassium iodide in 20mL of water (solution B). Mix together in equal volumes of solutions A and B.
6. Mayer's Reagent- Dissolve 1.35grms of mercuric chloride in 60ml of distilled water and pour into a solution of 5grms of potassium iodide in 10ml of distilled water then, make up the volume till 100ml.
7. Hager's Reagent- Dissolve 1grms of picric acid in 100ml of distilled water.
8. Millon's Reagent- Dissolve 10grms of mercury in 10ml of concentrated nitric acid and dilute the solution with 20mI of distilled water.

1.11 Phytochemical investigation: -

Detailed phytochemical testing was performed to identify presence or absence of different phytoconstituents

1.12 Selection and collection of Plant: -

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1.17 Phytochemical investigation: -

Detailed phytochemical testing was performed to identify presence or absence of different phytoconstituents.

1.17.1 Qualitative estimation: (Yadav and Agarwala, 2011)

Table 2: Tests and procedure for qualitative estimation of extract:

S No	Qualitative Test	Procedure	Observation
1		**Test for Carbohydrates**	
1.1	Molisch's Test	Take 1ml of aqueous solution of extract & few drops of Molisch reagent(**α**-naphthol) & conc. Sulphuric acid was added along the wall of the wall of the test tube.	Formation of purple coloured ring at junction, indicate the presences of carbohydrates
1.2	Fehling's Test	Equal volume (1ml each) of Fehling's A & Fehling's B solution was taken in a test tube & 2ml of aqueous solution of extract was added followed byboiling for 5-10 minutes on water bath.	Formation of reddish brown coloured precipitate due to formation of cuprous oxide indicatedpresences of reducing sugar.

1.3	Benedict's Test	Equal volume of Benedict's reagent and extract were taken ina test tube and heat the solution in water bath for 5-10 minutes.	Solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution which indicated the presenceof reducing sugar.
1.4	Barfoed's Test	Take 1 ml of extract and Barfoed's reagent in a test tube and heated on water bath for 2 minutes.	Red colour due to formation of cupric oxide indicates the presence of monosaccharide.
2.		**Test for Alkaloids**	
2.1	Dragendroff's Test	Take 1ml of extract dissolved in alcohol & few drops of aceticacid and Dragendroff's reagentwas added in a test tube.	Formation of orange red precipitate indicated the presences of alkaloids.
2.2	Mayer's Test	Take 2-3 ml of extract solution in a test tube & few drops of Mayer's reagent were added along sides of tube.	Formation of white or creamy precipitate indicates the presence of alkaloids.
2.3	Hager's Test	Take 1 ml of extract solution ina test tube, & few drops of Hager's reagent were added.	Formation of yellow colour precipitateindicates the presence of alkaloids.
2.4	Wagner's Test	Take 1-2 ml of extract solution, & few drops of Wagner's reagent were added in a test tube.	Formation of reddish brown precipitateindicates the presence of alkaloids.
3.		**Test for Saponins**	
3.1	Froth Test	The extract was diluted with distilled water and shaken in graduated cylinder for 15 minutes.	The formation oflayer of foam indicates the presence of saponins.
4.		**Test for Triterpenoids & Steroids**	
4.1	Salkowski's Test	The extract was dissolved in chloroform & equal volume of Conc. Sulphuric acid was added.	Formation of bluish red to cherry red colour in chloroformlayer & green fluorescence in acid layer indicate the presence of steroids.
4.2	Libermann-Burchard's Test	The extract was treated with chloroform & few drops of acetic anhydride were added in above solution, boiled and cooled. Then, Conc. Sulphuric acid was added through the sidesof the test tube.	Formation of brown ring at the junction of two layers, if upper layer turned green, indicate presence of steroids and formation of deep red colour indicate presence of triterpenoids
5.		**Test for Tannin & Phenolic Compounds**	
5.1	Ferric ChlorideTest	Take small amount of extract & dissolved in distilled water. Then, 2 ml of 5% ferric chloride solution was added to above solution.	Formation of darkblue, precipitate indicates presence of phenolic compounds.
5.2	Lead Acetate Test	Small amount of extract was dissolved in distilled water. To above solution few drops of lead acetate solution was added.	Formation of white precipitate indicates presence of phenolic compounds.
5.3	Gelatin Test	Some quantity of extract was dissolved in distilled water. To above solution 2 ml of 1% gelatin solution containing 10% sodium chloride was added.	Development of white precipitate indicates presence of phenolic compounds.

6.		**Test for Flavonoids**	
6.1	Shinoda's Test	Take 1ml of extract in alcoholic solution, few magnesiumturnings & few drops of conc. HCL were added and heated on a water bath.	Formation of red pink colour indicated the presence of flavonoid.
7.		**Test for Glycosides**	
7.1	Borntrager's Test	Take 3 ml of extract solution,dilute sulphuric acid was added, & boiled for 5 minutes and filtered. In cold filtrate, equal volume of benzene orchloroform was added and shake it well. The organic solvent layer was separated and ammonia was added to it.	Formation of pink tored colour in ammonical layerindicates presence ofanthraquinone glycosides.
7.2	Keller-Killiani Test	Take 2 ml of extract solution, 3 ml of glacial acetic acid and 1 drop of 5% ferric chloride were added in a test tube. Then, 0.5 ml of concentrated sulphuric acid was added carefully by the side of the test tube.	Formation of blue colour in the acetic acid layer indicates the presence of Cardiac glycosides.
8.		**Test for Protein & Amino acids**	
8.1	Biuret's Test	The extract was treated with 1 ml of 10% sodium hydroxide solution in a test tube and heated. A drop of 0.7% copper sulphate solution was added to the above mixture.	The formation of violet or pink colour indicates the presence of proteins.
8.2	Ninhydrin Test	Take 1-2ml of extract solution ina test tube & 3 drops of 5% ninhydrin was added and heated for 10 minutes in boiling water bath.	Development ofpurple or bluish colour indicates presences of amino acid.
8.3	Millon's Test	Take 3ml of extract solution in atest tube & 5ml of Millon's reagent was added.	White precipitate formed which onheating turned tobrick red, indicate the presence of protein.

1.17.2 Quantitative Tests

A. Spectrophotometric Quantification of Total Phenolic Content:

Procedure–Folin-Ciocalteu Assay was used for the determination of the total phenolic content in plant extract. The extracts (0.1 mL and 1mg/ml) were mixed with 2.5 mL of Folin-Ciocalteu Reagent and 2mL of 7.5% sodium carbonate and then the resulting solutions were allowed to stand for 30 minutes at room temperature before the absorbance was read spectrophotometrically. Subsequently, they were diluted to 5 mL and the absorbance was read instantly at 760 nm. Calibration curves were composed using standard solutions of Gallic Acid Equivalent (GAE) mg/gm. Concentration of 20, 40, 60, 80, and 100 mg/mL of Gallic aid was prepared. A blank solution was also prepared for the same situation and reagents for the preparation of the standard and sample solutions. The Folin-ciocalteu reagent is sensitive to reducing compounds including polyphenols. They produce a blue colour upon reaction. This blue colour was measured spectrophotometrically (Tangco et al., 2015).

B. Spectrophotometric Quantification of Total Flavonoid Content: -

Procedure-Aluminium chloride colorimetric method (Chang et al., 2002) was used for the determination of flavonoid content. 1 ml of each extract solution was mixed with 2.5ml of distilled water. Then, 75µl of sodium nitrite was added and mixed. After this stand for3 minutes before adding 0.15ml Aluminium chloride (100g/L) was added and allowed to stand for 5 minutes. Then, 0.5ml of 1 M sodium hydroxide was added. The mixture was shaken and mixed thoroughly. Absorbance of mixture was estimated at 510 nm using UV spectrophotometer. The calibration curve was calculated using Rutin as the standard. Total flavonoid content was determined from the calibration curve and results were indicated as mg Rutin equivalent per gram dry extract weight (Parthasarathy et al., 2009).

1.18 Formulation of microspheres by Solvent Evaporation method

Microspheres containing extract (Dalbergia sissoo) as a core material were prepared by Solvent Evaporation method. Extract (Dalbergia sissoo), HPMC and EC were dissolved in a mixture of ethanol and dichloromethane (1:1) at room temperature (As in table 3). This was poured into 250 mL water containing 0.01% Tween-80 maintained at a temperature of 30–40 °C and subsequently stirred at 300 rpm agitation speed for 45 minutes to allow the volatile solvent to evaporate. The microspheres formed were filtered, washed with water and dried in oven at 37°C. (Fartyal et al., 2011).

Table 3: Composition of microsphere formulation

Formulations (Code)	Polymer HPMC (mg)	Polymer Ethyl cellulose (mg)	Extract (mg)	Temperature °C	Solvent ratio (1:1) ethanol/DCM
F1	300	50	100	30-40°C	5ml:5ml
F2	250	100	100	30-40°C	5ml:5ml
F3	200	150	100	30-40°C	5ml:5ml
F4	150	200	100	30-40°C	5ml:5ml
F5	100	250	100	30-40°C	5ml:5ml

1.19 Evaluation parameter of extract loaded microsphere

Particle size

The particle size is one of the most important parameter for the characterization of microspheres. The size of microspheres was measured using Malvern Zeta sizer (Malvern Instruments). The dispersions were diluted with Millipore filtered water to an appropriate scattering intensity at 25°C and sample was placed in disposable sizing cuvette. The size data is documented in Table 17. (Singh and Vingkar 2008).

Zeta potential

The zeta potential was measured for the determination of the movement velocity of the particles in an electric field and the particle charge. In the present work, the microspheres was diluted 10 times with distilled water and analyzed by Zetasizer Malvern instruments. All samples were sonicated for 5-15 minutes before zeta potential measurements. The zeta potential data is documented in Table 18. (Đorđević et al., 2015).

Scanning Electron Microscopic (SEM)

The electron beam from a scanning electron microscope was used to attain the morphological features of the extract loaded microspheres were coated with a thin layer (2–20 nm) of metal(s) such as gold, palladium, or platinum using a sputter coater under vacuum. The pretreated specimen was then bombarded with an electron beam and the interaction resulted in the formation of secondary electrons called auger electrons. From this interaction between the electron beam and the specimen's atoms, only the electrons scattered at 90° were selected and further processed based on Rutherford and Kramer's Law for acquiring the images of surface topography (Anwer et al., 2019).

1.20 Antibacterial activity of Microsphere by Well diffusion assay

Preparation of Nutrient Agar Media

28 g of Nutrient Media was dissolved in 1 litre of distilled water. pH of media was checked before sterilization. Media was sterilized in autoclave at 121oC at 15 lbs pressure for 15 minutes. Nutrient media was poured into plates and placed in the laminar air flow until the agar was get solidified.

Well Diffusion Assay

The bacterial suspension of E. coli was standardized to 108 CFU/ml of bacteria and kept into the shaker. Then, 100µl of the inoculums from the broth (containing 108 CFU/ml) was taken with a micropipette and then transferred to fresh and sterile solidified Agar Media Plate (Mohammadi-Sichani et al., 2012). The agar plate was inoculated by spreading the inoculums with a sterile spreader, over the entire sterile agar surface. Three wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer. The wells were then formed for the inoculation of the microsphere, Microsphere and extract (1mg/ml) solution. 100 µl of the sample was loaded. It was allowed to diffuse

for about 30 minutes at room temperature and incubated for 18-24 hours at 37o C. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of tested compounds. The zone of inhibition (ZOI) was observed and measured in mm. Zones were measured to a nearest millimeter using a ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, non-reflecting background. The diameters of the zone of complete inhibition (as judge by unaided eye) were measured, including the diameter of the well (Manandhar et al., 2019).

1.21 Stability studies

The Extract loaded Microsphere formulation was packed and were placed in the stability test chamber and subjected to stability studies at accelerated testing (250C±20C and 60 ± 5% RH) and (400C±20C and 70 ±5% RH) for 3 months. The formulation was checked for evaluation parameter particle size and Zeta potential studies at the interval of 30, 45, 60, 90 days (3 month) months. The formulation was tested for stability under accelerated storage condition for 3 months in accordance to International Conference on Harmonization (ICH) guidelines. Formulation was analyzed for the change in evaluation parameter particle size and zeta potential studies. All Results were compared against final formulation of 0 days as the reference.

3. RESULTS AND DISCUSSION

3.1 Percentage Yield

In phytochemical extraction the percentage yield is very crucial in order to determine the standard efficiency of extraction for a specific plant, various sections of the same plant or different solvents used. The yield of extracts received from the Dalbergia sissoo is shown in Table: 4

Table 4: Percentage Yield of crude extracts of Dalbergia sissoo extract

S.No	Solvent	Theoretical weight	Yield(gm)	% yield
1	Pet ether	300	1.35	0.47%
2	Methanol	284.25	6.58	2.33%

3.2 Preliminary Phytochemical study

Table 5: Phytochemical testing of extract

S. No.	Experiment	Pet. Ether extract	Methanolic extract
1.	**Test for Alkaloids**		
1.1	Dragendroff's test	Absent	Present
1.2	Mayer's reagent test	Absent	Present
1.3	Wagner's reagent test	Absent	Present
1.3	Hager's reagent test	Absent	Present
2.	**Test for Glycoside**		
2.1	Borntrager test	Absent	Present
2.2	Legal's test	Absent	Present
2.3	Killer-Killiani test	Absent	Present
3.	**Test for Carbohydrates**		
3.1	Molish's test	Absent	Absent
3.2	Fehling's test	Absent	Absent
3.3	Benedict's test	Absent	Absent
3.4	Barfoed's test	Absent	Absent
4.	**Test for Proteins and Amino Acids**		
4.1	Biuret test	Absent	Absent
5.	**Test for Flavonoids**		
5.1	Alkaline reagent test	Absent	Present
5.2	Lead Acetate test	Absent	Present
6.	**Test for Tannin and Phenolic Compounds**		

6.1	Ferric Chloride test	Absent	Present
7.	**Test for Saponins**		
7.1	Foam test	Present	Absent
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	Present	Present
8.2	Libbermann-Burchard's test	Present	Present

3.3 Quantitative Analysis

Preliminary phytochemical testing of crude extracts confirmed the presence of phenolics and flavonoids in plant material. To estimate their amount total phenolic (TPC) and total flavonoid content (TFC) assays were performed.

3.3.1 Total Phenolic content (TPC) estimation

Table 6 Standard table for Gallic acid

S. No.	Concentration (µg/ml)	Absorbance
1.	20	0.139
2.	40	0.169
3.	60	0.195
4.	80	0.229
5.	100	0.263

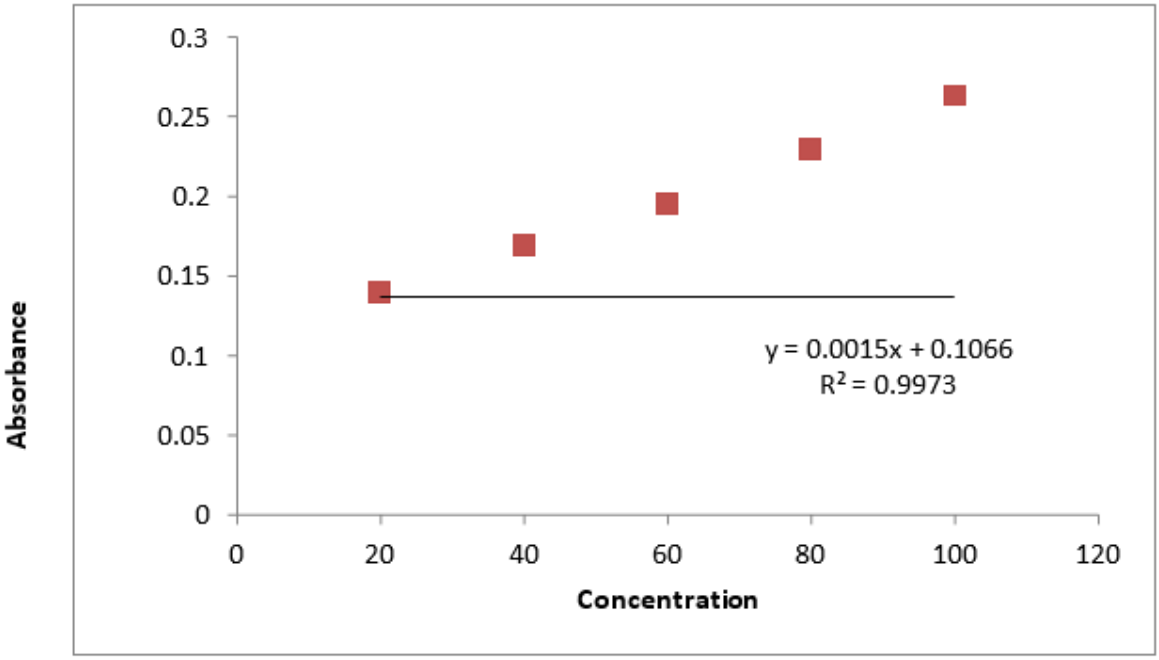

Figure 5: Graph represent standard curve of Gallic acid

3.3.2 Total Phenolic Content in extract

Table 7: Total Phenolic Content

S. No	Absorbance	TPC in mg/gm equivalent of Gallic Acid
1	0.145	
2	0.169	63.05 mg/gm
3	0.182	

Table 8-Total Phenolic Content of extract Dalbergia sissoo

Extracts	Total Phenolic content (mg/gm equivalent of Gallic acid)
Methanol	63.05

3.3.3 Total Flavonoids content (TFC) estimation

Table 9 Standard table for Rutin

S. No.	Concentration (µg/ml)	Absorbance
1.	20	0.167
2.	40	0.196
3.	60	0.252
4.	80	0.291
5.	100	0.315

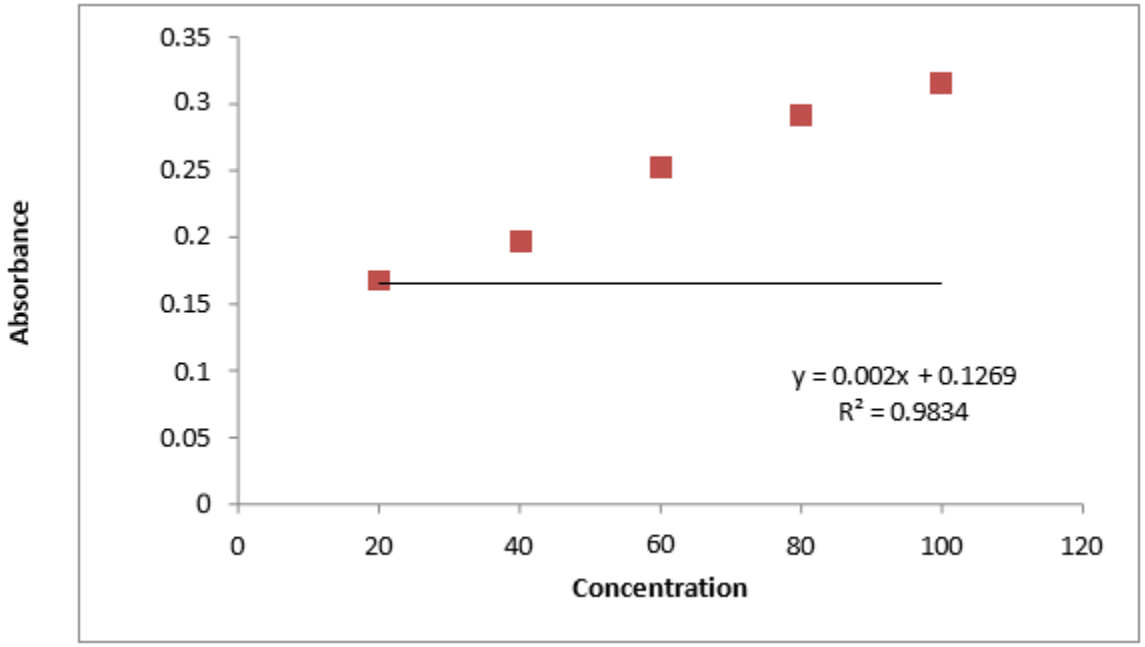

Figure 6: Graph represent standard curve of Rutin

3.3.4 Total Flavonoid Content in extract

Table 10: Total Flavonoid Content

S .No	Absorbance	TFC in mg/gm equivalent of Rutin
1	0.132	
2	0.153	15.23 mg/gm
3	0.176	

Table 11: Total Flavonoid Content of extract Dalbergia sissoo

Extracts	Total Flavonoid content (mg/gm equivalentof rutin)
Methanol	15.23

3.5 Organoleptic properties

Table 12: Organoleptic properties of Plant extract

Organoleptic properties	Observation

Colour	Reddish
Odour	Characteristic
State	Solid

An evaluation of the plant extract organoleptic qualities, including color, odor, and state, was conducted. Plant extract was discovered to have a Dark reddish colour to it when tested. Extract has a Characteristic odor and has a solid state form, according to research conducted on it. Extract exhibited the same color, odor and state as the requirements for these characteristics. Result show in Table 12.

3.6 Solubility study

Table 13: Solubility study of Extract

Solvents	Observation/Inference
Methanol	Freely Soluble
Ethanol	Freely Soluble
DMSO	Freely Soluble
Water	Soluble

4 . Discussion

The solubility of Extract was determined in various non-volatile or volatile liquid vehicles such as Dimethyl sulfoxide, methanol, ethanol, and water shown in Table 13. From the results, it was observed that the extract is freely soluble in Ethanol, Methanol and DMSO and soluble in water.

4.1 Evaluation parameter of microsphere formulations

Particle size determination

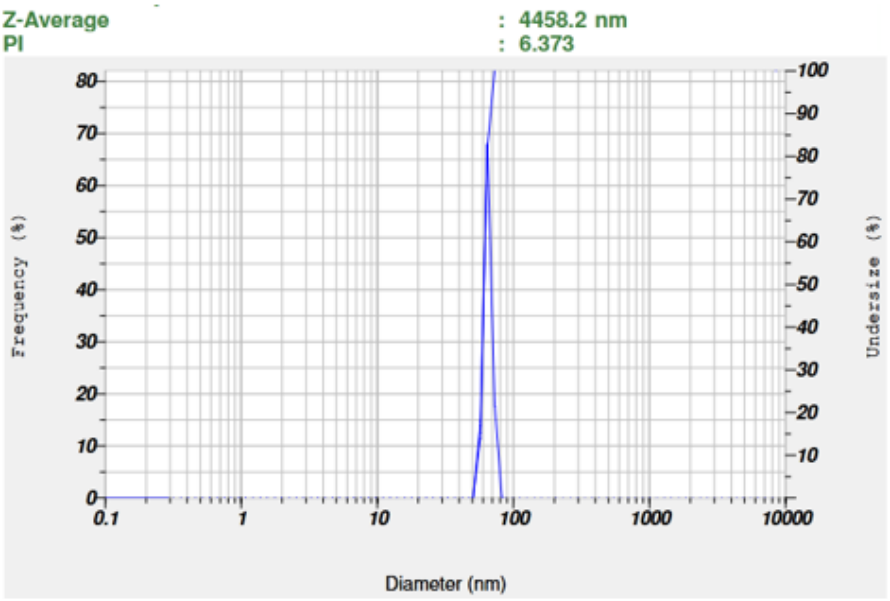

Figure 7: Particle size (F1)

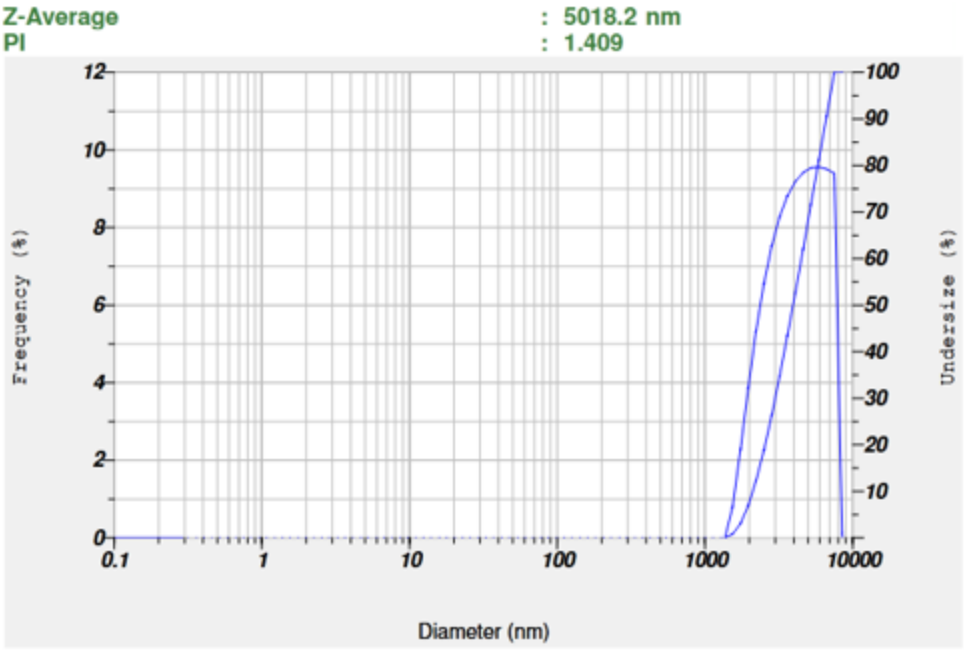

Figure 8: Particle size (F2)

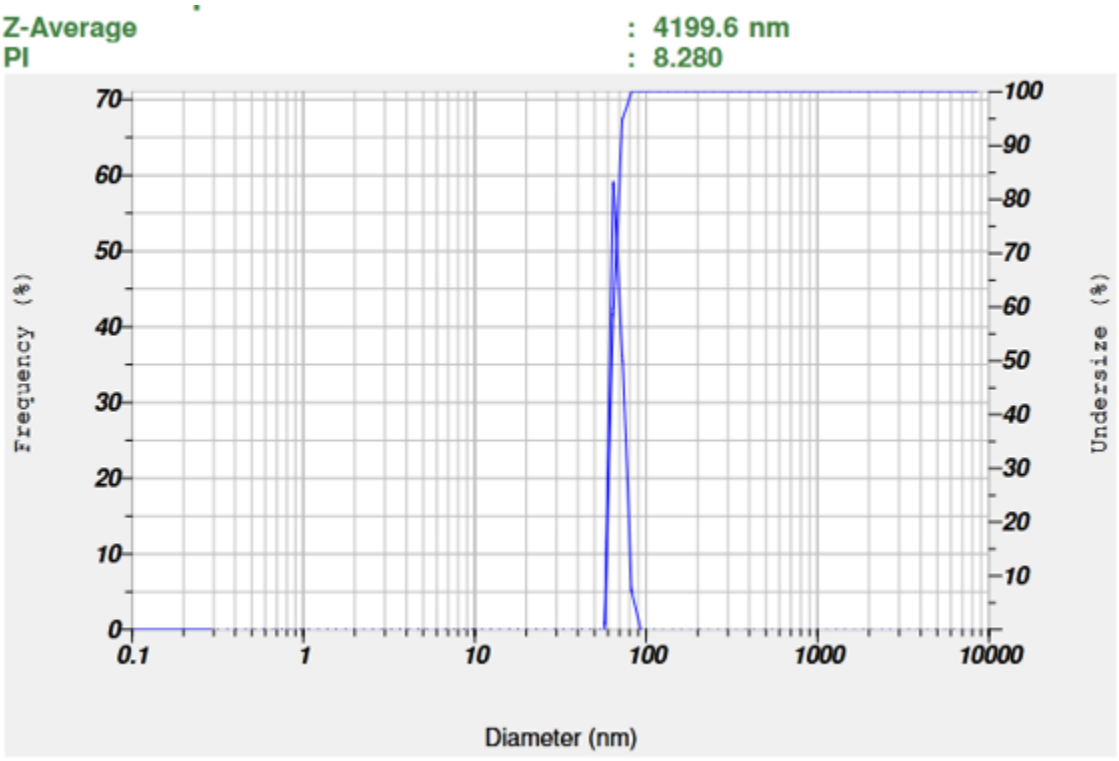

Figure 9: Particle size Figure (F3)

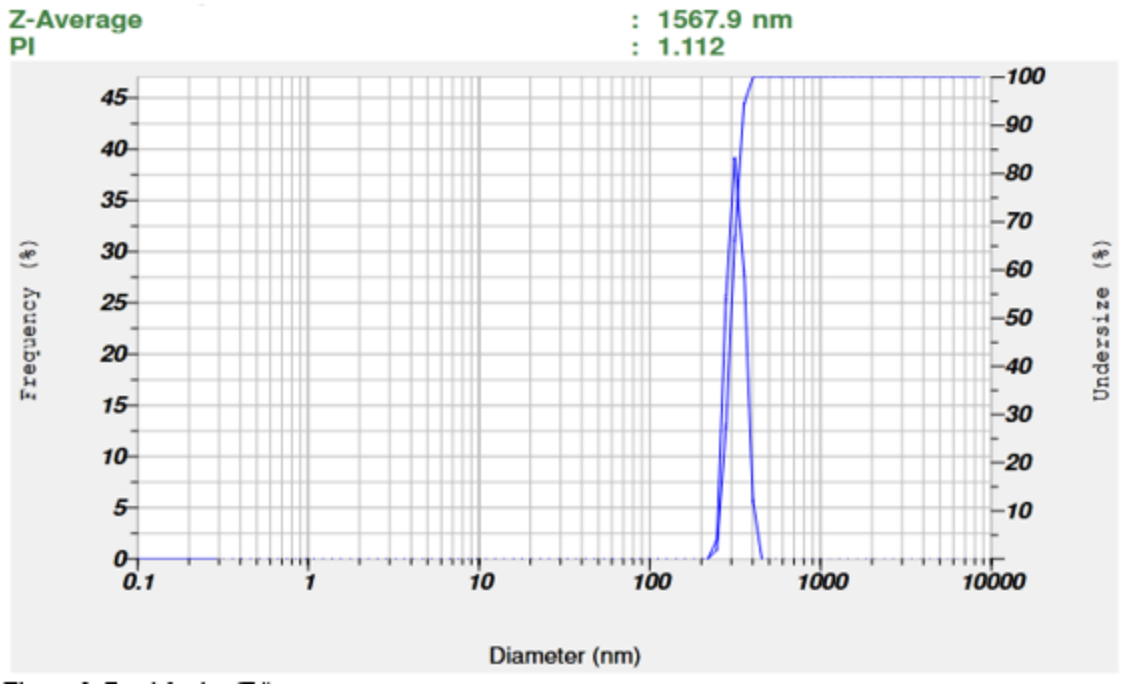

Figure 10: Particle size (F4)

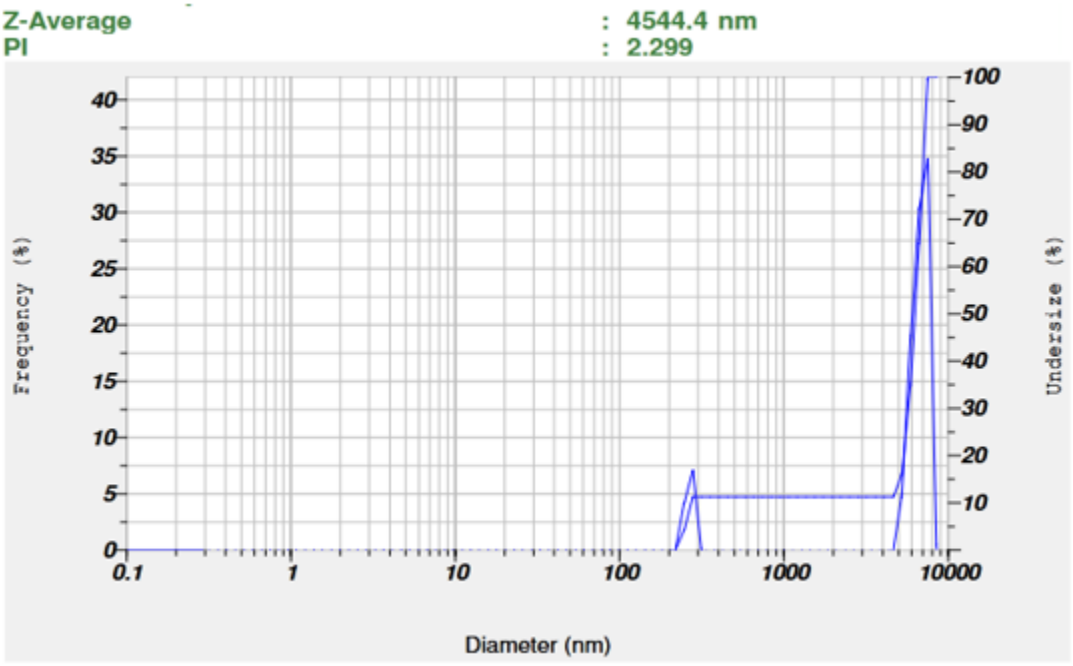

Figure 11: Particle size (F5)

Table 14: Result of Particle

S.No.	Formulations	Particle size (nm)	PI Value
1.	F1	4458.2 nm	6.373
2.	F2	5018.2 nm	1.409
3.	F3	4199.6 nm	8.280
4.	F4	1567.9 nm	1.112
5.	F5	4544.4 nm	2.299

4.1.1 Size of all formulations

The particle size is one of the most important parameter for the characterization of microsphere. The average particle sizes of the prepared microsphere formulation were measured using Malvern zeta sizer. Particle size analysis showed that the average particle size of microsperes was found to be range between 1567.9 to 5018.2 nm. These particle size values indicate that the all formulated microsphere is under the range of microsphere and F4 is the lowest particle size of all formulation shown in above table 14.

4.1.2 Zeta potential determination

Zeta Potential (Mean) : 8.4 mV
Electrophoretic Mobility Mean : 0.000065 cm^2/Vs

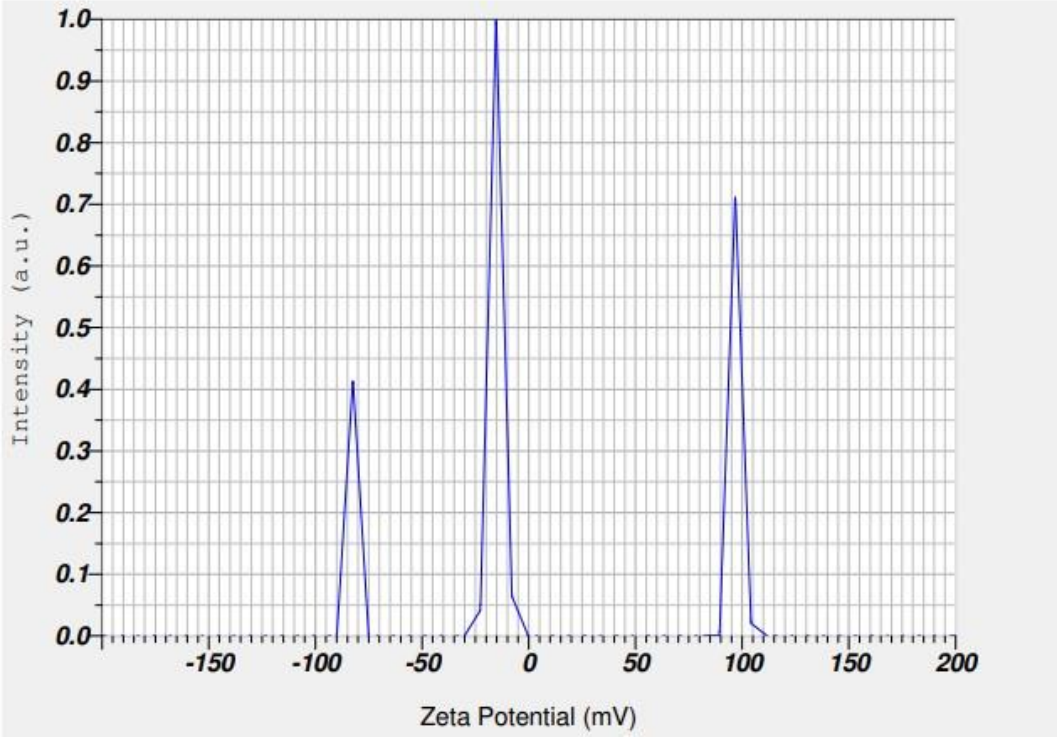

Figure 12: Zeta potential (F1)

Zeta Potential (Mean) : 19.4 mV
Electrophoretic Mobility Mean : 0.000150 cm²/Vs

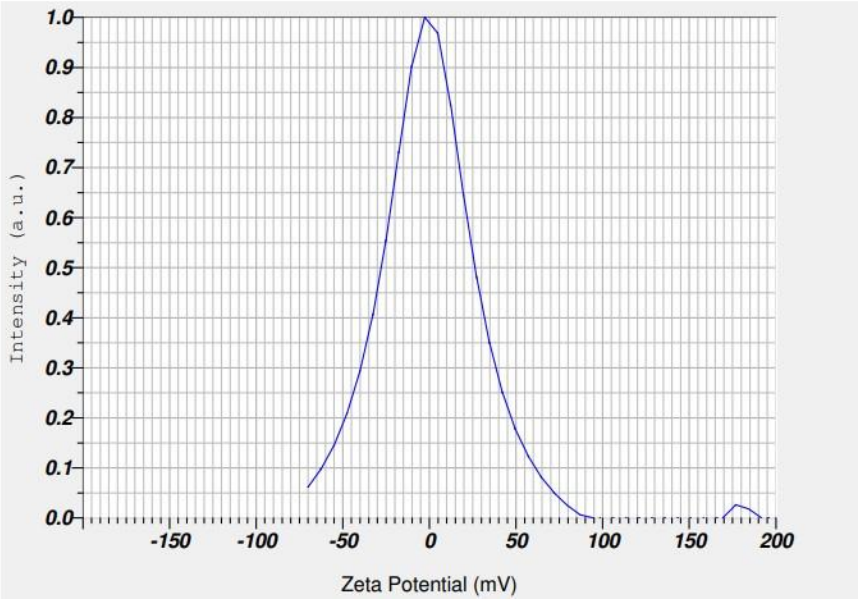

Figure 13: Zeta potential (F2)

Zeta Potential (Mean) : -1.8 mV
Electrophoretic Mobility Mean : -0.000014 cm²/Vs

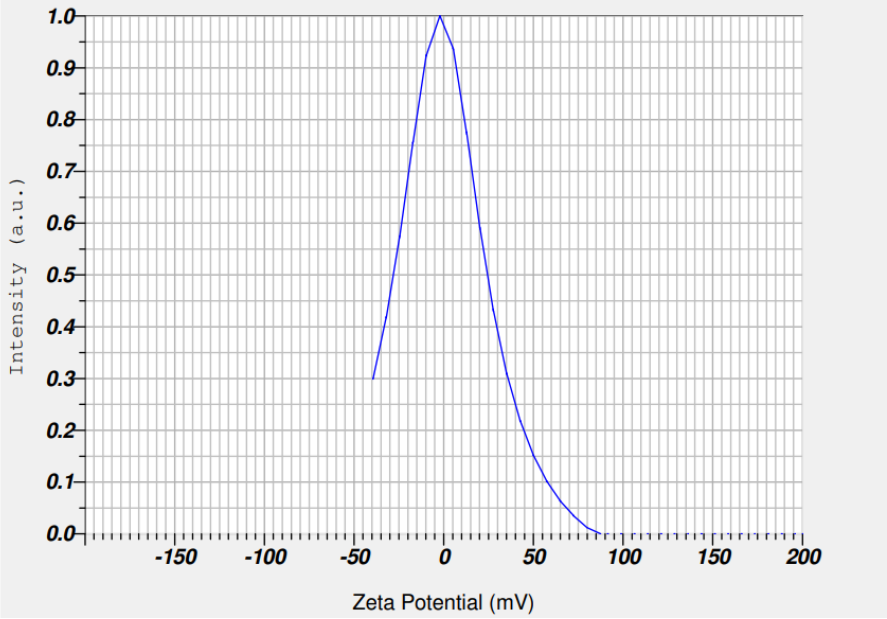

Figure 14: Zeta potential (F3)

Zeta Potential (Mean) : -8.7 mV
Electrophoretic Mobility Mean : -0.000068 cm²/Vs

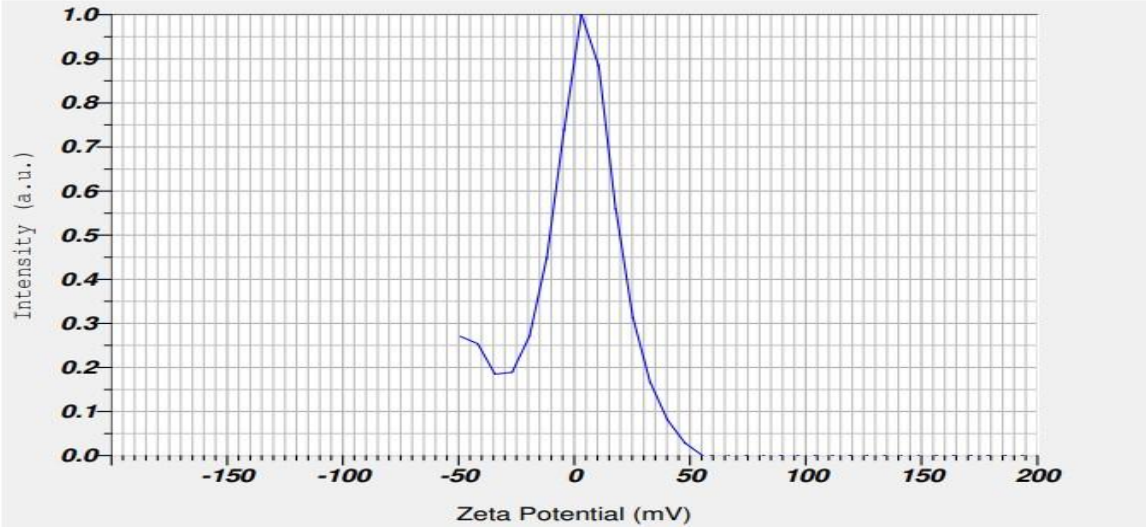

Figure 15: Zeta potential (F4)

Zeta Potential (Mean) : 10.6 mV
Electrophoretic Mobility Mean : 0.000082 cm²/Vs

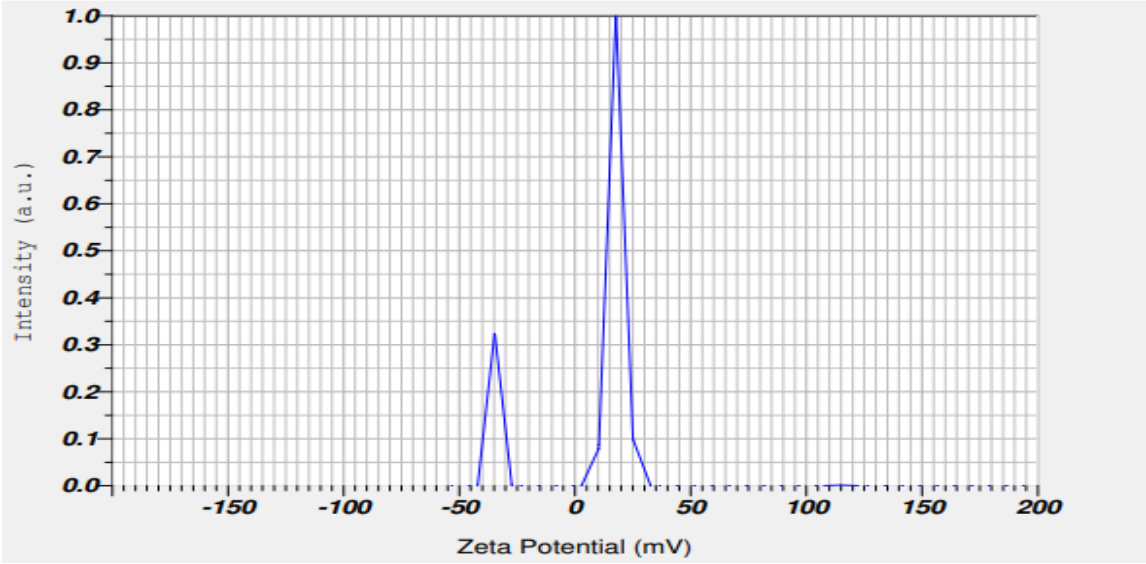

Figure 16: Zeta potential (F5)

Table 15: Result of Zeta potential of all formulations

S.No	Formulation	Zeta potential
1	Microsphere (F1)	8.4 mV
2	Microsphere (F2)	19.4 mV

3	Microsphere (F3)	-1.8 mV
4	Microsphere (F4)	-8.7 mV
5	Microsphere (F5)	10.6 mV

Zeta potential analysis is carried out to find the surface charge of the particles. The magnitude of zeta potential is predictive of the colloidal stability. Zeta potential was found to be all formulation range -1.8 to 10.6 mV with peak area of 100% intensity. These values indicate that the all formulated microsphere is stable. Results show in above table 15.

4.1.3 Scanning electron microscopy characterization of F4 formulation

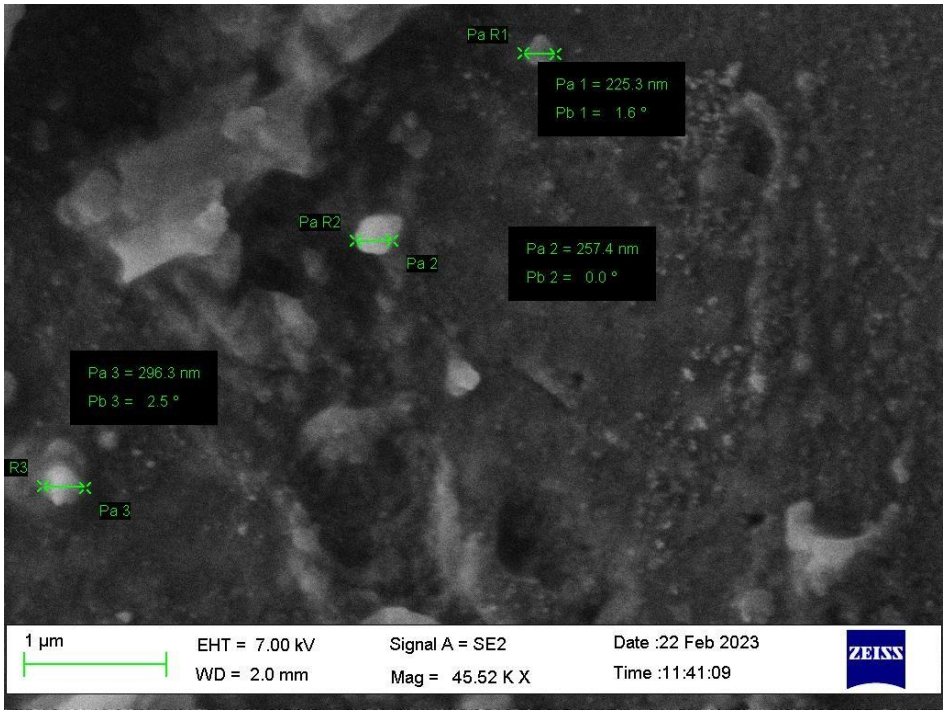

Figure 17: SEM

SEM analysis was performed to determine their microscopic characters (shape & morphology) of prepared microsphere. Drug loaded microsphere were prepared and dried well to remove the moisture content and images were taken using scanning electron microscopy. Scanning electron micrograph of the prepared microsphere at 45.52 kx magnification showed that the microsphere were smooth surface morphology and spherical shape. The smooth surface morphology an spherical shape of microsphere was clearly observed in the SEM images. Show in above figure 17

4.2 Results of antimicrobial activity of microsphere F4 formulation

4.2.1 Antimicrobial activity of Formulation against E.coli

Table 16: Antimicrobial activity of Formulation, placebo and plant extract against E.coli

S.No.	Sample Name	Zone of Inhibition (mm)
1.	Placebo	7 mm
2.	Plant extract	14 mm
3.	Formulation	18mm

Table 17: Stability Study of Microsphere (F4) formulation

S. No	Time (Days)	$25^0C±2\ ^0C$ and 60 ± 5% RH			$40^0C±2\ ^0C$ and 70 ±5% RH		
		Appearance	Particle Size nm	Zeta potential V	Appearance	Particle Size nm	Zeta potential mV
1.	0	Solid Powder	1567.9 nm	-8.7 mV	Solid Powder	1567.9 nm	-8.7 mV
2.	30	Solid Powder	1567.7 nm	-8.6 mV	Solid Powder	1567.2 nm	-8.6 mV
3.	45	Solid Powder	1568.0 nm	-8.3 mV	Solid Powder	1568.2 nm	-8.8 mV
3.	60	Solid Powder	1567.1 nm	-8.9 mV	Solid Powder	1568.1 nm	-8.7 mV
4.	90	Solid Powder	1567.4 nm	-8.9 mV	Solid Powder	1567.8 nm	-8.9 mV

Formulation were found to be stable, both physically and chemically, for a period of 3 months at accelerated stability conditions (250C±2 0C and 60 ± 5% RH) and (400C±2 0C and 70 ±5% RH). Physicochemical parameters, including appearance, Zeta potential and particle size were not altered significantly. Results of assay and other evaluation criteria at periodic time points of stability studies are summarized in Table 17.

CONCLUSION

The formulation and characterization of extract loaded microspheres was performed in the present research work. SEM photographs confirmed the shape and formation of the microspheres. The results revealed that experimental conditions allowed a uniform distribution of the extract in microspheres having no significant effect on drug-polymer interaction. Finally, there results of this investigation elucidate that the process and formulation variables could be effectively altered to achieve the desired characteristics of the microspheres for novel delivery of the extract. These microspheres are used for drug delivery, wherein the drug can be encapsulated or entrapped form. In future it can also be formulated in various dosage forms. Extract: Polymer ratio and extract had significant effect on zeta potential and particle size. From the scanning electron microscopy (SEM) study observed that microspheres were spherical and fairly smooth surface.

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