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Hydnocarpus Pentandra Nanosuspension And In Vitro Anti Cancer Studies Against MCF7 Cell Line

Dr. P Premkumar[*1], Nikhila. M. Nair[2], Jency Abraham[3], Dr. Shajan Abraham[4]

[*1]Research Guide, Professor, Department of Pharmaceutics, SIMATS, Saveetha College of Pharmacy, Thandalam, Chennai-600077, Tamil nadu

[2,3]Research Scholars, SIMATS, Saveetha College of Pharmacy, Thandalam, Chennai-600077, Tamil nadu

[4]Research co-guide, Professor, HOD (Dept. of Pharmaceutics), Vice-Principal, Nazareth College of pharmacy, othera, Thiruvalla-689546, Kerala

***Corresponding Author:**

Dr. Premkumar

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ABSTRACT

Hydnocarpus pentandra, a medicinal plant widely known for its bioactive compounds, has shown significant therapeutic potential in various disease treatments. In recent years, nanotechnology-based drug delivery systems have gained attention for enhancing the efficacy of plant-derived bioactive compounds. This study focuses on the formulation and evaluation of Hydnocarpus pentandra nanosuspension and its in vitro anticancer activity against the MCF-7 breast cancer cell line. The nanosuspension was prepared using an optimized nano-precipitation technique, ensuring high drug loading efficiency, stability, and enhanced bioavailability. Characterization of the nanosuspension was carried out through particle size analysis, zeta potential measurement, polydispersity index (PDI), and scanning electron microscopy (SEM) to determine its morphology, stability, and dispersibility. The anticancer potential of the Hydnocarpus pentandra nanosuspension was evaluated through MTT assay, which measures cell viability in response to different concentrations of the formulation. The results demonstrated a dose-dependent cytotoxic effect on MCF-7 cells, indicating significant anticancer activity. The nanosuspension exhibited improved cellular uptake and enhanced cytotoxicity compared to the crude extract, suggesting that the nanoformulation plays a crucial role in increasing the bioavailability of the active compounds. Additionally, fluorescence microscopy and flow cytometry studies were performed to analyze apoptosis induction and cell cycle arrest mechanisms. The findings suggest that Hydnocarpus pentandra nanosuspension effectively inhibits the proliferation of MCF-7 cells through apoptosis-mediated pathways. The enhanced solubility, targeted drug delivery, and improved therapeutic efficacy of the nanosuspension make it a promising candidate for breast cancer treatment. Furthermore, the study highlights the potential of nanotechnology-based phytomedicines in cancer therapy, providing a novel approach for drug development. Future research should focus on in vivo studies and mechanistic evaluations to establish the clinical applicability of Hydnocarpus pentandra nanosuspension in oncology.

Keywords: *Hydnocarpus pentandra, nanosuspension, MCF-7 cell line, breast cancer, apoptosis, nanotechnology, in vitro anticancer activity*

1. INTRODUCTION

Nanotechnology is an emerging field in all areas of science, engineering and technology. Nano technology is seemed to have greatest impact in our daily life. It had improved and helped in many field of science. Nanotechnology is a novel interdisciplinary area of comprehensive research that combines medicine and other life sciences. It offers a potential for unique and novel approaches with broad spectrum of applications in cancer treatment including areas such as diagnostics, therapeutics and prognostics. Therefore nanotechnology opened a new vast exploiting area for cancer treatment. Most efforts to improve cancer treatment though nanotechnology are at the research or development stage. The development of drug delivery approach for the transportation of drug in a bioavailable and safe manner to the target site is now becoming an exceedingly important area of biopharmaceutical researches. The main advantage of the particles in the nanometric range is its improved physical and chemical properties. The major parameters in drug delivery include particle size, Surface area, hydrophobicity, crystallinity and surface charge[1].

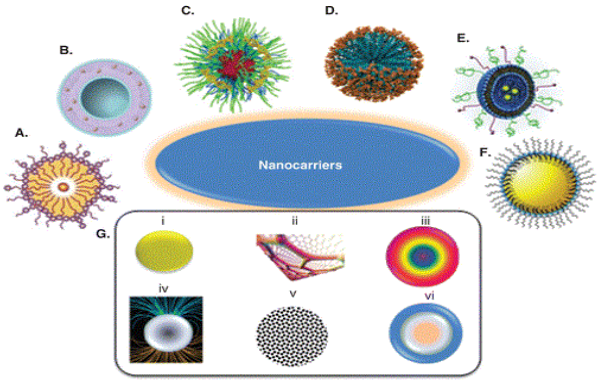


Figure 1: Nano carriers used in drug delivery system

Nanocarriers entrapping herbal drug will carry optimum amount of drug to their site of action bypassing all the barriers such as acidic Ph of stomach, liver metabolism and increase the prolonged circulation of the drug into the blood due to their small size[2].

A nanocarrier is nanomaterial mainly used as a transport module for another substance, such as a drug. Commonly used nanocarriers include polymers, dendrimers, solid lipid Nanoparticles, liposomes, micelles, carbon-based nanomaterials, quantum dots and other substances [3]. Nanocarriers are currently used in drug delivery and their unique characteristics demonstrate potential use in chemotherapy [4].

Polymeric Nanoparticles

Polymeric nanoparticles (PNP) are structures with a diameter ranging from 10 to 1000 nm. The PNPs are obtained from synthetic polymers, such as poly-∑- caprolactone {polyacrylamide and polyacrylate or natural polymers, Eg; albumin, DNA, Chitosan, gelatin. Based on in vivo behaviour, PNPs may be classified as biodegradable, i.e.,poly(L-lactide)(PLA),Poly-glycolide (PGA) and non-biodegradable, eg.polyurethane.PNPs are usually coated with non-ionic surfactants in order to reduce immunological interactions as well as intermolecular interactions between the surface chemical groups of PNPs [5]. They can be easily penetrated into the capillaries due to their small size and it is absorbed by cells, it will leads to the accumulation of drug at target sites [6].The applications of Nanoparticles include vascular endothelial dysfunction, oral delivery of insulin, brain drug targeting for neurodegenerative disorders like Alzheimer's disease [7].

Dendrimers

Dendrimers are nanometer-sized, highly branched and monodisperse macromolecules with symmetrical architecture. They consist of a central core, branching units and terminal functional groups [8]. The core together with the internal units, determine the environment of the nano cavities and consequently their solubilising properties, whereas the external groups, the solubility and chemical behaviour of these polymers. Different types of dendrimers, include polyamidoamine (PAMAM), polypropylene imine (PPI), Polylysine dendrimers have been used as host for both hydrophilic and hydrophobic drugs. An ideal dendritic drug-carrier must be non-toxic, non-immunogenic, preferably biodegradable; present an adequate biodistribution and allow tissue targeting [9].

Solid lipid Nanoparticles

Solid lipid nanoparticles are nanoparticles ranging from 50-1000nm that are made from lipids which remain in a solid state at room and body temperature.Lipids used include mono-,di-, or triglycerides, lipid acids and glyceride mixtures or waxes that are stabilized by the biocompatible surfactants [10]. There are several advantages of solid lipid Nanoparticles which include controlled drug release and drug targeting, protection of drug from chemical degradation, reduction of drug toxicity, enhancement of bioavailability, biodegradation, good tolerability, ability to incorporate both hydrophilic and lipophilic drugs, no problem with respect to large scale production and sterilization. The formulations incorporating herbal drugs in solid lipid nanopaticles include mouth washes (eg. Peppermint oil), gargles (eg Thymo and inhalations (eg Eucalyptus oil) [11].

Liposomes

Liposomes are a form of vesicles that consist either of many, few or just one phospholipid bilayers. The polar character of the liposomal core enables polar drug molecules to be encapsulated. Amphiphilic and lipophilic molecules are solubilised within the phospholipid bilayer according to their affinity towards the phospholipids. Liposomes have the distinct advantages of being both nontoxic and biodegradable because they are composed of naturally occurring substances[12]. Biologically active materials encapsulated within liposomes are protected to varying extent from immediate dilution or degradation, suggesting

drug carrier systems for the transport of drugs and other bioactive capsules to disease-affected organs. The unique ability of liposomes to entrap drugs both in an aqueous and a lipid phase make such drug delivery systems attractive for hydrophilic and hydrophobic drugs[13].

Micelles

These copolymers are composed of individual linear polymers that contain a hydrophobic and hydrophilic segment, which confers them the capacity to form micellar structures in aqueous media. These amphipathic polymers are arranged so that the hydrophobic part is located inside the structure, while the hydrophilic part is located outside, in contact with the aqueous medium[14]. Their advantages include high drug-loading capacity of the inner core and the possibility of their modulation to respond to various stimuli such as Ph and Temperature. Polymeric micelles are able to reach parts of the body that are poorly accessible to liposomes; accumulate more than free drugs in tumor tissues due to increased vascular permeability[15]. Thus polymeric micelles can be employed to administer chemotherapeutics in a controlled and targeted manner with high concentration in the tumor cells and reduced side effects[16].

Carbon Nano materials

Carbon nanomaterials include fullerenes and Nanotubes. Fullerenes are novel carbon allotrope with a polygonal structure made up exclusively of 60 carbon atoms[17]. These Nanoparticles are characterized by having numerous points of attachment whose surfaces also can be functionalized for tissue binding. Nanotubes have been one of the most sextensively used types of Nanoparticles because of their high electrical conductivity and excellent strength[18]. Carbon nanotubes can be structurally visualized as a single sheet of graphite rolled to form a seamless cylinder. There are two classes of carbon nanotubes: single walled (SWCNT) and multi-walled (MWCNT). Functionalized carbon nanotubes are emerging as novel components in nanoformulations for the delivery of therapeutic molecules[19].

Quantum Dots

Quantum dots are Nanoparticles made of semiconductor materials with fluorescent properties. It is crucial for biological applications[20]. Quantum dots must be covered with other materials, allowing dispersion and preventing leaking of the toxic heavy metals. Quantum dots glow very brightly when illuminated by ultraviolet light[21]. They can be coated with a material that makes the dots attach specifically to the molecule they want to track. Quantum dots bind themselves to proteins unique to cancer cells, literally bringing tumours to light[22].

Nanotechnology is the science that deals with the matter at the scale of one billionth of a meter (10^{-9}) = 1nm and it is the study of manipulating the matter at the atomic and molecular scale [23].

Nanoscience involves research to discover new behaviours and properties of matter which dimensions at the nanoscale.

Nanoscale which ranges roughly from 1 to 1000nm [24].

Pharmaceutical nanotechnology embraces applications of nanoscience to pharmacy as nanomaterial and as a device like drug delivery, diagnostic imaging and biosensor [25, 26] .

Nanomedicine is defined as submicron size molecules used for the treatment, diagnosis, monitoring and control of biological system.

Pharmaceutical nanotechnology has provided more fine –tuned diagnosis and focused treatment of disease at a molecular level. Pharmaceutical nanotechnology is most innovative and highly specialized field, which will revolutionize the pharmaceutical industry in near future [27]. Pharmaceutical nanotechnology presents revolutionary opportunities to fight against many diseases. It helps in detecting the antigen associated with diseases such as cancer, diabetes mellitus, neurodegenerative diseases, as well as detecting the microorganisms and viruses associated with infections[28, 29].

Nanosuspension are colloidal dispersions of nanosized drug particles stabilized by surfactants [30].They can also defined as a biphasic system consisting of pure drug particles dispersed in an aqueous vehicle the diameter of the suspended particle is less than 1µm in size [31, 32]. Reduction of drug particles to nanometer range leads to an enhanced dissolution rate due to increased surface area and saturation solubility [33] .The nanosuspension can also be lyophilized or spray dried and the Nanoparticles of nanosuspension can also be incorporated in a solid matrix [34, 35]. Nano is a Greek word which means 'dwarf' .Nano means it is the factor of 109 or one billionth. Some comparisons of nanoscale are given below,

0.1 nm - Diameter of one Hydrogen atom

2.5 nm - Width of a DNA molecule

1 micron – 1000nm

1nm = 10^{-9}m = 10^{-7}cm = 10^{-6}mms

Micron - 10^{-6}m = 10^{-4}cm = 10^{-3}mm [36, 37].

Figure 2: Types of Nanosuspension

Advantages:

1. Can be applied for poorly water soluble drugs.
2. Can be given by any route.
3. Physically more stable than liposomes.
4. Most cost effective.
5. Reduction in tissue irritation.
6. Rapid dissolution and tissue targeting can be achieved by IV route of administration.
7. Improvement in biological performance due to high dissolution rate & saturation
8. Solubility of the drug.
9. Provide ease of manufacture and scale up for large scale production.
10. Possibility of surface-modification of Nanosuspension for site specific delivery.
11. Improved dose proportionality [38, 39].

Table 1: Formulation Consideration of nanosuspension

Excipient	Function	Example
Stabilizers	Wet the drug particles thoroughly, prevent Ostwald's ripening and agglomeration of Nanosuspension, providing steric or ionic barrier	Lecithins, poloxamers, polysorbate, cellulosics, povidones
Co surfactants	Influence phase behaviour when micro emulsions are used to formulate nanosuspensions	Bile salts, dipotassium glycerrhizinate, transcutol, glycofurol, Ethanol, isopropanol
Organic solvent	Pharmaceutically acceptable less hazardous solvent for formulation of nanosuspension	Methanol, ethanol, chloroform, isopropanol, ethyl acetate, ethyl formate, butyl lactate, triacetin, propylene carbonate, benzyl alcohol
Other Additives	According to the requirement of the route of Administration or the properties of the drug moiety	Buffers, salts, polyols, osmogens, cryoprotectants etc.

Techniques of Preparation of Nanosuspension:

Mainly there are two methods for preparation of nanosuspensions. The conventional methods of precipitation (Hydrosols) are called 'Bottom up technology'. In Bottom up technology the drug is dissolved in a solvent, which is then added to non-solvent to precipitate the Crystals. This technology is that during the precipitation procedure the growing of the drug crystals needs to be controlled by addition of surfactant to avoid formation of micro particles. The 'Top Down Technologies' are the disintegration methods and are preferred over the precipitation methods [40, 41].

The 'Top Down Technologies' include Media Milling(Nanocrystals), High pressure homogenization in water(Dissocubes), High pressure homogenization in non-aqueous media(Nanopure) and combination of precipitation and High pressure homogenization(Nanoedge).

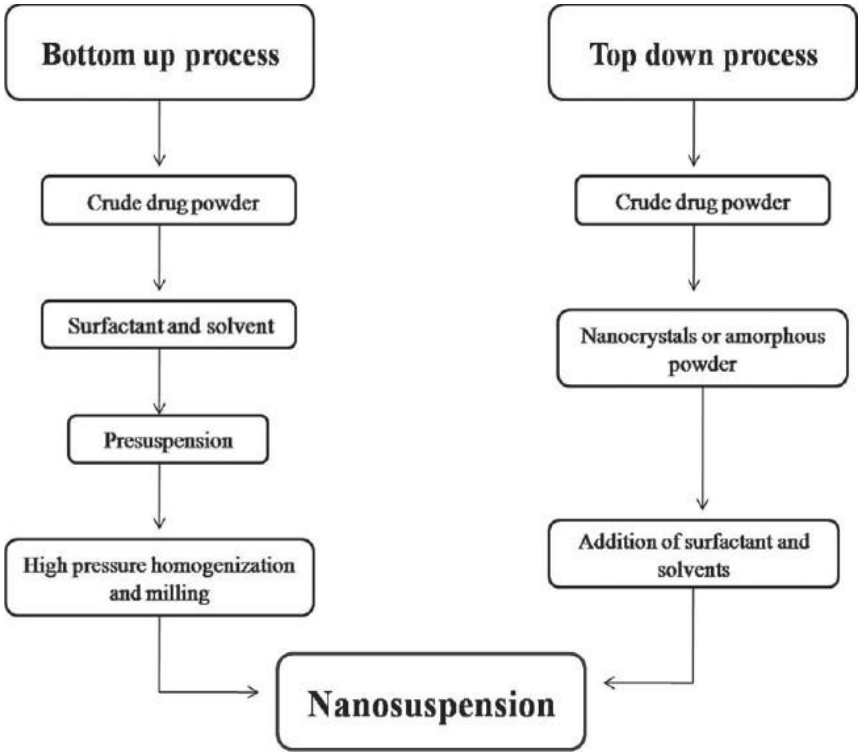


Figure 3: Formulation Techniques

1. **Media milling (nanocrystals or nanosystems)**

The method is first developed and reported by Liversidge (1992). The nanosuspensions are prepared by using high-shear media mills [42]. The milling chamber charged with milling media, water, drug and stabilizer is rotated at a very high shear rate under controlled temperatures for several days(at least2-7 days). The milling medium is composed of glass, Zirconium oxide or highly cross-linked polystyrene resin. The high energy shear forces are generated as a result of the impaction of the milling media with the drug resulting into breaking of microparticulate drug to nanosized particles[43].

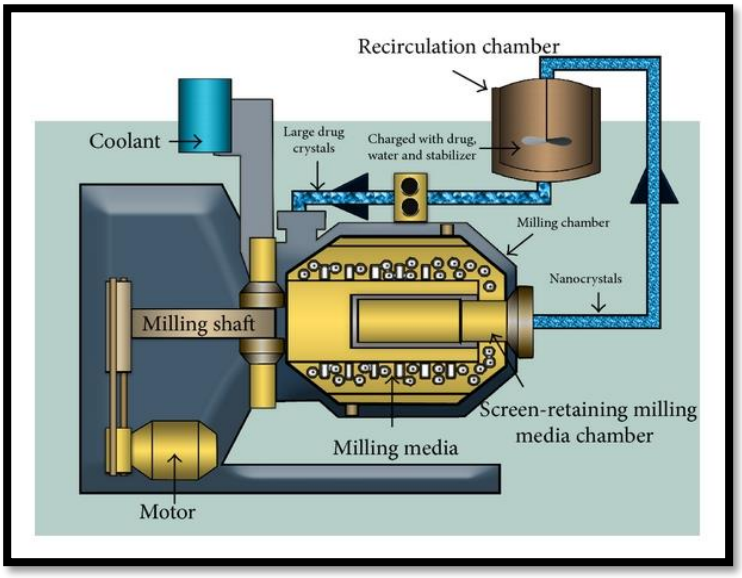


Figure 4: Media milling Process

Advantages

1. Very dilute as well as highly concentrated nanosuspensions can be prepared by handling 1mg/ml to 400mg/ml drug quantity.
2. Nano sized distribution of final Nano sized product.

Disadvantages

1. The media milling technique is time consuming.
2. Some fractions of particles are in the micrometer range.
3. Scale up is not easy due to mill size and weight [44].

2. Homogenization

a) High pressure homogenization (Dissocubes)

It is the most widely used method for the preparation of nanosuspensions of many poorly water soluble drugs. Disoccubes are engineered using piston-gap-type high pressure homogenizers. High pressure homogenizers consist of a high pressure plunger pump with a subsequent relief valve. The task of the plunger pump is to provide the energy level required for the relief. The relief valve consists of a fixed valve seat and an adjustable valve [45, 46].

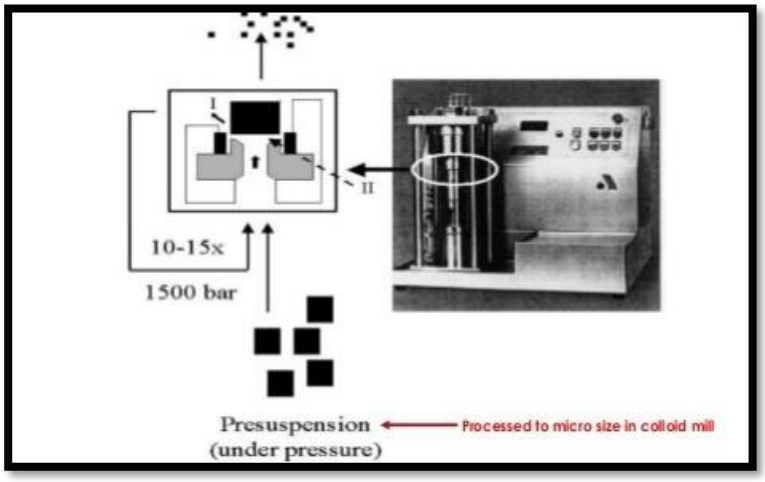


Figure 5: High pressure homogenization process

Principle

In this technique suspension is forced by a pressure plunger pump through a narrow valve under high pressure. When the suspension is allowed to as through the orifice the static pressure will be reduced below the boiling pressure of water which results in the boiling of water and formation of gas bubbles. When it leaves the orifice pressure will be normal and bubbles will implode. So surrounding particles will rush into the surface which causes the size reduction [47, 48].

Advantages

- It does not cause the erosion of processed materials.
- Very dilute as well as highly concentrated nanosuspensions can be prepared by handling 1mg/ml to 400mg/ml drug quantity.
- It is applicable to the drugs that are poorly soluble in both aqueous and organic media.
- Disadvantages
- Pre processing like micronization of drug is required.
- High cost instruments are required that increases the cost of dosage form [49].

b) **Homogenization in non aqueous media (Nano pure)**

Nano pure is the water free media or water mixture. In Nano pure technology the drug suspension in the non aqueous

media when homogenized at 0◦c or even the freezing point and hence called as deep freeze homogenization .

c) **Nano edge**

The precipitated drug Nanoparticles have tendency to continue crystal growth to the size of microcrystal. They need to be processed with high energy forces (Homogenization). They are in completely amorphous, partially amorphous or completely crystalline which create problems in long term stability as well as in bioavailability, so the precipitated particle suspension is subsequently homogenized which preserve the particle size obtained after the precipitation step [50].

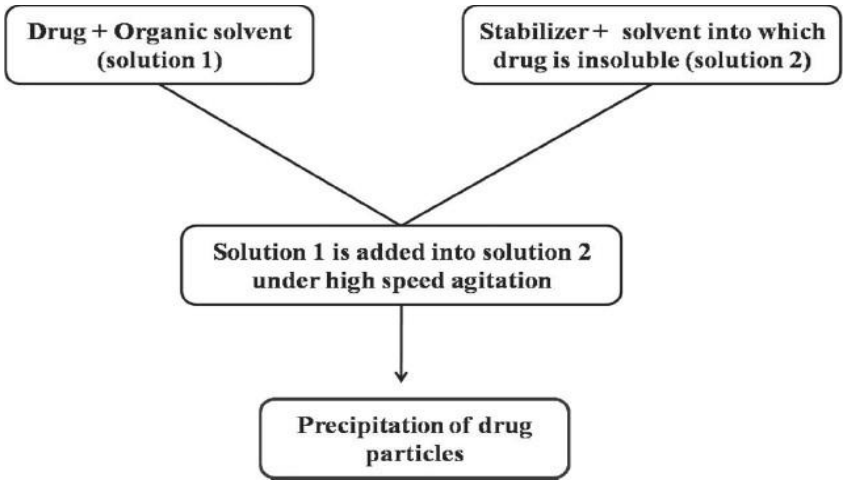


Figure 6: Nano edge process

3. Precipitation

The most common method of precipitation used is anti solvent addition method in which the drug is dissolved in an organic solvent and this solution is mixed with a miscible anti solvent. Mixing processes vary considerably [51]. Precipitation has also been coupled with high shear pharmaceutically acceptable and less hazardous water –miscible solvents, such as ethanol and isopropanol, and partially water miscible solvents, such as ethyl acetate, ethyl formate, butyl lactate, triacetin, propylene carbonate and benzyl alcohol [52].

4. Emulsification-solvent evaporation technique

This technique involves preparing the solution of drug by it emulsification in another liquid that is non-solvent for the drug. Evaporation of the solvent leads to precipitation of the drug. Crystal growth and particle aggregation can be controlled by creating high shear force using a high speed stirrer [53].

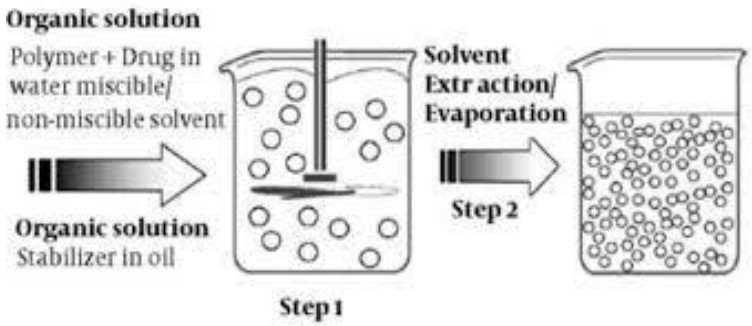


Figure 7: Emulsification-solvent evaporation technique

4. Supercritical fluid process

Novel nano sizing and solubilisation technology whose application has increased particle size reduction via supercritical fluid (SCF) process..A supercritical fluid (SF) can be defined as a dense non condensable fluid. Supercritical fluids are fluids whose temperature and pressure are greater than its critical temperature (CT) and critical pressure (CP) [54]. A SCF process allows micronization of drug particles within narrow range of particle size, often to sub-micron levels. Current SCF processes

have demonstrated the ability to create nano particulate suspensions of particles 5 to 2000 nm in diameter. The low solubility of poorly water soluble drugs and surfactants in supercritical Co2 and high pressure required for these processes restrict the utility of this technology in the pharmaceutical industry[55].

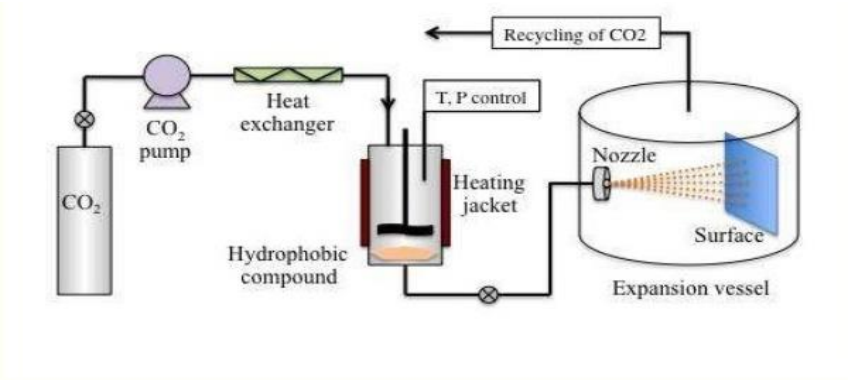


Figure 8: Supercritical Fluid Process

Characterization of Nanosuspension

Nanosuspension are evaluated as same as conventional suspensions such as appearance, colour, odour, assay, related impurities etc. Along with that particle size, zeta potential, morphology, dissolution study, in-vivo studies are also performed.

Particle size

Particle size and particle size distribution are two important parameters since it will affect the saturation solubility, dissolution rate, stability, and in-vivo behaviour of nanosuspensions. Any change in the particle size will leads to the change in the solubility and dissolution. Particle size determines the physiochemical behaviour of the drug. Particle size can be determined by SEM or TEM analysis [56]. Particle size distribution can be determined by photon correlation spectroscopy (PCS) or laser diffraction (Id). Particle size distribution will be expressed in Polydispersity index (pi).pi value of 0.1-0.25 indicates fairly Nano size distribution where as its value greater than 0.5 indicates a very broad distribution [57].

Surface charge (Zeta potential)

Zeta potential will be determine the stability of nanosuspension. A minimum zeta potential of 30mv is required where as in case of combined electrostatic or steric stabilizer, a zeta potential of 20mv would be sufficient [58].

Crystalline state and particle morphology

When the drug undergoes Nano sizing the crystalline nature and particle morphology will change. This can be detected by this method. X-ray diffraction analysis is mainly used for the determination of the solid state of the particle and is supplemented by scanning electron microscopy [59].

Saturation solubility and dissolution velocity

Nanosuspension will increase the solution solubility and dissolution velocity. It also help for the in vitro behaviour of the formulation. When the particle size reduced to Nano metric range, dissolution velocity and dissolution pressure will increase which leads to the solution solubility due to the changes in the surface tension [60].

Applications

Nanosuspension has wide range of applications especially in case of low solubility and low bioavailability drugs. They are mentioned below.

1. Intravenous administration

The Parenteral route of administration provides a quick onset action , rapid targeting and reduced dosage of the drug. It is the preferred route for drugs undergoing first-pass metabolism and those that are not absorbed in the GIT or degraded in the GIT [61]. One of the important applications of nanosuspension technology is the formulation of intravenously administered products [62]. Intravenous administration results in several advantages, such as administration of poorly soluble drugs without using a higher concentration of toxic co-solvents, improving the therapeutic effect of the drug available as conventional oral formulations and targeting the drug to macrophages and the pathogenic microorganisms residing in the macrophages [63].

2. Bioavailability enhancement

The poor oral bioavailability of the drug may be due to poor solubility, poor permeability or poor stability or poor stability

in the gastrointestinal tract (GIT) [64, 65]. Nanosuspensions resolve the problem of poor bioavailability by solving the twin problems of poor solubility and poor permeability across the membrane [66, 67]. Bioavailability of poorly soluble oleanic acid, a hepatoprotective agent, was improved using a nanosuspension formulation. The therapeutic effect was significantly enhanced, which indicated higher bioavailability [68]. This was due to the faster dissolution (90% in 20 min) of the lyophilized nanosuspension powder when compared with the dissolution from a coarse powder (15% in 20 min) [69].

3. Pulmonary administration

Aqueous nanosuspension can be nebulised using mechanical or ultrasonic nebulizers for lung delivery. Because of their small size, it is likely that in each aerosol droplet at least one drug particle is contained, leading to a more uniform distribution of the drug in lungs [70]. They also increase adhesiveness and thus cause a prolonged residence time. Budenoside drug Nanoparticles were successfully nebulised using an ultrasonic nebulizer [71].

4. Ocular administration

Ocular delivery of the drugs as nanosuspensions is to provide a sustained release of drug. Certain drugs have poor solubility in lachrymal fluid. If it is formulated as Nanoparticles its saturation solubility and bioavailability will increase. It is mainly applied for hydrophobic drugs. It increases the residence time include sac. The best example of nanosuspension is ibuprofen. The anti-inflammatory activity of ibuprofen increased compared with the aqueous formulation [72].

5. Oral Drug Delivery

Because of the numerous advantages oral route is the most preferable route for many of the drugs especially in the case of oral administering antibiotics such as atovaquone and bupravaquone. By making it in Nano size, its solubility and bioavailability will increase. The oral administration of naproxen Nanoparticles leads to an area under the curve (AUC) (0-24 h) of 97.5 mg-h/l compared with naproxen nanosuspension and naproxen tablets. In the case of diazole nanosuspension has absolute bioavailability of 82.3 and the conventional dispersion only 5.2% [73].

6. Drug targeting

Nanosuspensions can also be used for targeting as their surface properties and changing of the stabilizer can easily alter the in vivo behaviour[74]. The drug will be taken up by the mononuclear phagocytic system to allow regional-specific delivery. This can be used for targeting anti mycobacterial, fungal or leishmanial drugs to the macrophages if the infectious pathogen is persisting intracellularly[75].

7. Mucoadhesion of the Nanoparticles

Nanoparticles orally administered in the form of a suspension diffuse into the liquid media and rapidly encounter the mucosal surface. The particles are immobilized at the intestinal surface by an adhesion mechanism referred to as ‘’bio adhesion’’. From this moment on, the concentrated suspension acts as a reservoir of particles and an adsorption process takes place very rapidly. The direct contact of the particles with the intestinal cells through a bioadhesive phase is the first step towards particle absorption [76]. The adhesiveness of the nanosuspensions not only helps to improve bioavailability but also improves targeting of the parasites persisting in the GIT [77].

2. CANCER/TUMOR

Figure 9: Cancer Cells

Cancer is a group of diseases which is caused due to uncontrolled growth of the cells and forms from the extra mass tissue known as tumour. The loss of apoptotic nature by the cells in their metabolic pathway leads to cancer. Cigarette smoking, tobacco intake, alcohol intake, poor diet and exposure to UV rays lead to cancer. Different organs can be effect by cancer cells like lungs, kidney, eyes, heart, brain etc. Cancer cells also spread in blood stream and causes blood cancer [78]. The person who works in the chemical factories, nuclear reactors, drainage system and mining are most prone to cancer. Treatments such as surgery, chemotherapy and radiation therapy, bone marrow transplantation are used to treat cancer in different stages [79].

Tumours are found in all kinds of tissue;

It can be of two types;

a) Benign

b) Malignant

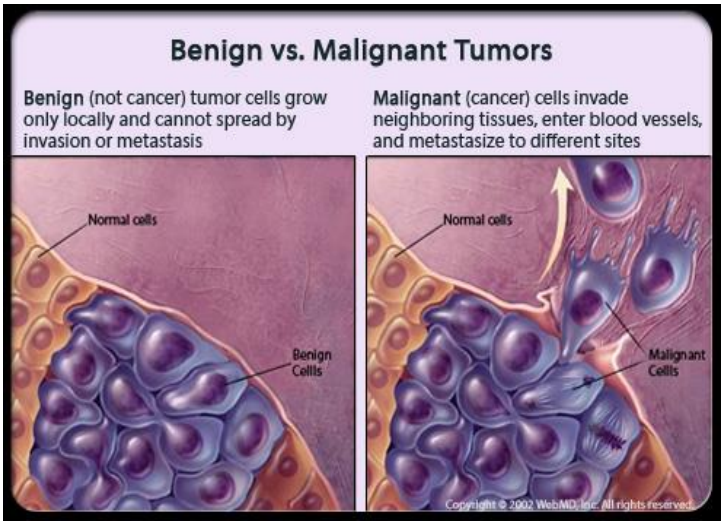


Figure 10: Benign and Malignant tumour

Benign

Benign tumours are not cancer. They usually can be removed and, in most cases, they do not come back. Most important, cells from benign tumours do not spread to other parts of the body. Cells from benign tumours stay together and often they are surrounded by a containing membrane. Benign tumours are not usually a threat to life.

Examples of Benign Tumours

Papilloma	A projecting mass on the skin (for example, a wart)
Adenoma	A tumour that grows in and around the glands
Lymphoma	A tumour in fatty tissue
Osteoma	A tumour originating in the bones
Myoma	A tumour of muscle tissue
Angioma	A tumour usually composed of small blood or lymph vessels (for Example, a birth mark)
Nevus	A small skin tumour of one variety of tissues (for example, a mole)

Malignant

Malignant tumours are cancer. Cancer cells can invade and damage tissues and organs near the tumour [80]. Cancer cells also can break away from a malignant tumour and enter the lymphatic system or the blood stream, which is how cancer can spread to other parts of the body. The characteristics feature of the cancer is the cell's ability to grow rapidly, uncontrollably and independently from the tissue where it started. The spread of cancer to other sites or organs in the body through the blood stream or lymphatic system is called metastasis.

Malignant tumour generally can be classified in two categories.

Carcinomas: These cancers originate in the epithelium. The epithelium is the lining cells of an organ. Carcinomas are the most common type of cancer. Common sites of carcinomas are the skin, mouth, lung, breast, stomach, colon and uterus.

Sarcomas: Sarcomas are cancers of connective and supportive tissue of all kinds. Sarcomas can be found anywhere in the body, and they often form secondary growths in the lungs [81].

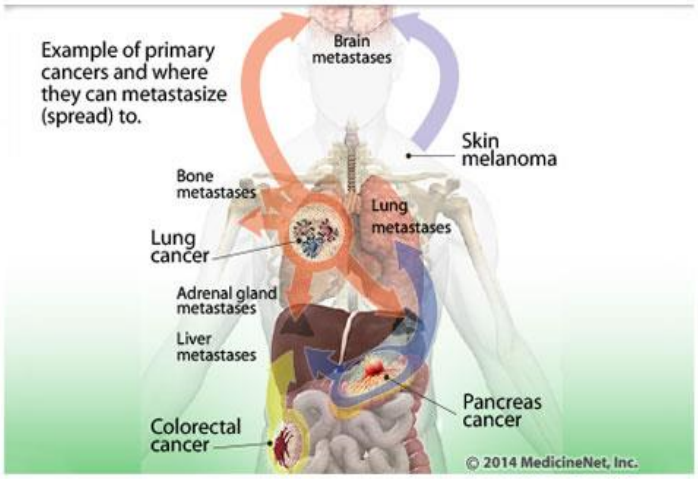


Figure 11: Metastasis of cancer

Metastasis, is the spread of a cancer or disease from one organ or part to another not directly connected with it. The new occurrences of disease thus generated are referred to as metastases. In metastasis, cancer cells break away from where they first formed (primary cancer), travel to other areas of the body through the blood stream or the lymph system. If the cells travel through the lymph system, they may end up in nearby lymph nodes or they may spread to other organs. More often, cancer cells that break off from the main tumour travel through the blood stream. Once in the blood, they can go to any part of the body. Many of these cells die, but some may settle in a new area, benign to grow, and form new tumours. The spread of cancer to a new part of the body is called metastasis [82].

Cancer cells have to go through several steps to spread to new parts of the body:

1. They have to be able to break away from the original tumour and enter the blood stream or lymph system, which can carry them to another part of the body.
2. They need to attach to the wall of a blood or lymph vessel and move through it into a new organ.
3. They need to be able to grow and thrive in their new location.
4. They need to be able to avoid attacks from the body's immune system.

Going through all these steps means the cells that start new tumours may no longer be exactly the same as the ones in the tumour they started in. This may make them harder to treat [83].

3. CHARACTERISTICS OF THE CANCER

Proto-oncogenes: genes that encourage the growth of a cell

A mutation can turn the normal genes into a cancerous oncogenes that force extreme cell division. Oncogenes can encode signalling molecules such as growth factors, or components of the signalling cascades that regulate the mediate the cellular responses to such signalling molecules.

Tumour suppressor genes: genes that stop excessive growth of the cell.

If a cell starts to divide excessively, its neighbour sends inhibiting factors to quieten it down. Such factors either act directly or trigger inhibitory factors in the rogue cell. A key phase in the development of a cancer cell comes when it develops one or more mutations is called tumour suppressor genes- which enable it to ignore its neighbours. Mutations can knock out a cell-surface receptor for inhibiting factors, or a critical component of the cascades inside the cell that receive and process the signal. Other mutations can disable proteins such as p53, which trigger the cell to commit suicide if its DNA becomes damaged, or its signalling cascades go out of control.

Angiogenic genes: genes that control a cell's blood supply

Angiogenesis is the proliferation of a network of blood vessels that penetrate into cancerous growths, supplying nutrients and oxygen and removing waste products. Tumour angiogenesis actually starts with cancerous tumour cells releasing molecules that send signals to surrounding normal host tissue. This signalling activates certain genes in the host tissue that, in turn, make proteins to encourage growth of new blood vessels.

Metastasis genes: controlling the spread of cancer

Metastatis is to be called as the spreading nature of the cancer from one organ or part to another non-adjacent organ or part throughout the body [84].

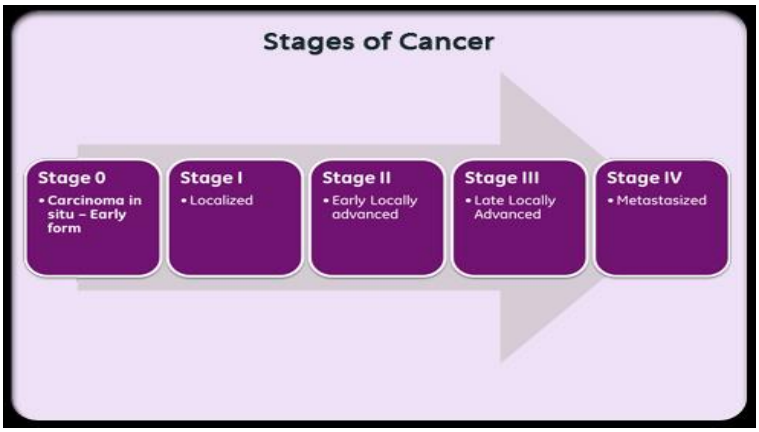


Figure 12: Stages of cancer

Cell cycle

The cell cycle is the series of events that takes place in a cell leading to its division and replication that produces two daughter cells. In prokaryotes which lack a call nucleus, the cell cycle occurs via binary fission. In cells with a nucleus, as in eukaryotes, the cell cycle can be divided into three periods : interphase, the mitotic phase, and cytokinesis. During interphase the cell grows, accumulating nutrients needed for mitosis preparing it for cell division and duplicating its DNA. During the mitotic phase the cell splits itself into two distinct daughter cells. During the final stage, cytokinensis, the new cells is completely divided. To ensure the proper division of the cell there are control mechanisms known as cell cycle check points [85].

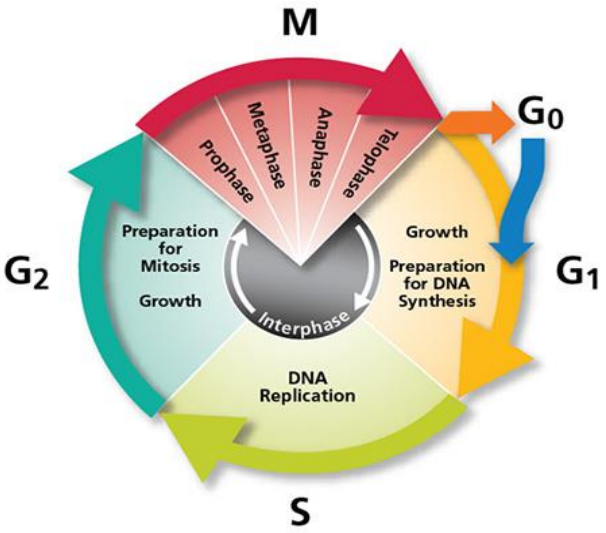


Figure 13: Cell cycle

Symptoms of cancer

Everyone should be familiar with certain signs that may indicate early cancer. It is important to report immediately, before the condition spreads. It is unfortunate that early stages of cancer are typically painless; because they are painless, diagnosis and treatment are often delayed.

Early symptoms can include

- Unaccountable weight loss
- Unusual bleeding or discharge
- Persistent indigestion
- The presence of white patches inside the mouth or white spots on the tongue [86].

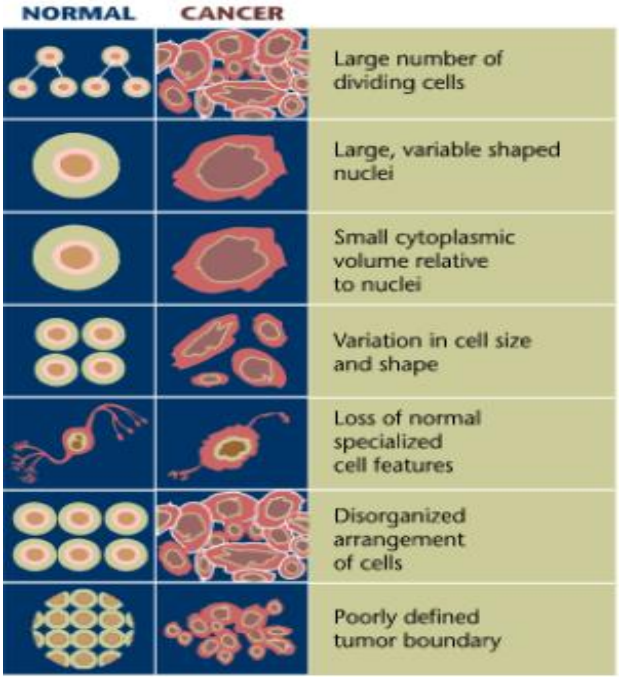


Figure 14: Differences of normal and cancer cells

Detection of cancer

Early detection and prompt treatment are directly responsible for increased survival rates.

Tools for cancer detection include

- Self-exams
- Biopsy(the removal of living tissue for the purpose of microscopic examination of cells)
- Ultrasound(the use of reflected high-frequency sound waves to differentiate various kinds of tissue)
- Computed tomography(CT)(the use of x-rays to produce a cross-sectional picture of the body parts)
- Magnetic resonance imaging(MRI)(the use magnetic fields and radio waves to show changes in soft tissues without the use of x-rays) [87].

Risk factors for cancer

Because cancer is not a single disease, it does not have a single cause. Many causes or risk factors can contribute to a person's chance of getting cancer. Risk factors are different with each type of cancer. Risk factors can include such things as genetic factors, life style factors which include Tobacco, Diet, Infectious agents, Occupational Exposure, Reproductive Factors, Sedentary life style, Alcohol/Drugs, pollution etc.

Genetics play a large role for many cancers, such as breast and colon cancer. This means that a family's history can be a risk factor for some types of cancers [88].

Lifestyle factors

Personal choices we make about the way we live our lives can increase our chance of developing cancer. These choices are called lifestyle factors, and they include smoking, heavy drinking and eating foods that have excess calories, high fat, and low fiber. Other factors that increase risk are related to sexual contact and sunlight exposure.

Tobacco

Thirty percent of all cancers are attributed to smoking or chewing tobacco. Cigarette smoking is also associated with cancers of the mouth, pharynx, larynx, oesophagus, pancreas, kidney and bladder.

Diet

Researchers found that different types of food you eat affect your risk of developing cancer. Approximately 30% of cancers are related to diet.

Infectious agents

Some viruses have the ability to transform cells into cancer. Examples include(a) human papilloma virus(HPV) and cervical cancer, and (b) Epstein-Barr virus and lymphoma[89].

Occupational Exposure

Occupational exposure includes high-risk occupations such as uranium miners, asbestos factory workers, certain chemical plant workers, and workers in nuclear power plants.

Reproductive factors

The reproductive factors category refers mostly to women's risk factors. For example, the risk of breast cancer goes up if a woman does not have children before the age of 30. Sexually transmitted disease also increases the risk of cervical cancer.

Sedentary lifestyle

Not moving around much during the day may increase the risk of cancer. The body's own defences work better when you exercise and maintain an ideal weight. Moderate exercise such as walking or climbing a flight of stairs can help.

Alcohol/Drugs

Alcohol contributes to the risk of developing cancer. People who drink too much or abuse drugs may not eat well or take care of themselves, which will increase their overall risk of cancer.

Pollution

Although people think environmental pollutions is a major of cancer, in fact few cancers have been found to be caused by pollution, but research is still ongoing. Many of the cancers is not known. Other actors that interact to increase the risk of cancer are age, hormonal balance, response to stress and status of the immune system [90].

Figure 15: Hydnocarpus pentandra

Hydnocarpus pentandra is an Indo-Malayan genus belonging to the family Flacourtiaceae [91]. Five species of Hydnocarpus are reported to occur in India viz, *H.alpina, H. Kurzii, H macrocarpa, H.pentandra* and *H.pendulus* [92,93]. These various species of Hydnocarpus are also used in traditional medicine in china, Thailand, Malaysia and Myanmar for several skin disorders [94]. Out of the five species, *H.pentandra* is the most widely distributed species.It is primarily used for treating leprosy and skin disorders [95]. The extracts and compounds isolated from this plant show a wide spectrum of biological properties, including anti-bacterial, anti-leprotic, anti-tubercular, anti-psoriatic, anti-rheumatic, hypolipidemic, anti diabetic, anti cancer, anti-inflammatory, anti-oxidant activities[96, 97].

Morphology

This is a tree up to 10 m (33 ft) tall. The tree is deciduous and as well as evergreen too. Bark is brownish, fissured; blaze pinkish. Branch lets are round, minutely velvet-hairy. Leaves are simple, alternate, carried on 0.7-2.2 cm (0.28-0.87 in) long stalks. Leaves are 8-23 x 3.5-10 cm (3.1-9.1 x 1.4-3.9 in), usually oblong to elliptic- oblong, tip long-pointed, often falling off, base narrow, margin toothed, papery, hairless [98]. Midrib is raised above, secondary nerves 5-7 pairs. Flowers are borne in short cymes or solitary, in leaf axils. Petals are white. Berry is woody, round, 6-10 cm (2.4-3.9 in) across usually brown tomentose, black when young; seeds numerous. The flowering takes place from January to april. Flowers are greenish white in colour and grow solitary or recemes [99].

Trees of the species that yield chaulmoogra oil grow to a height of 12-15 m (39-49 ft) and in India trees bear fruits in August and September. The fruits are ovoid some 10 cm (3.9 in) in diameter with a thick woody rind. Internally they contain 10-16 black seeds embedded in the fruit pulp [100]. The seeds account for some 20% of the fruit weight. A typical tree produces 20 kg (44 Ib) of seed/ annum. The kernels make up 60-70% of the seed weight and contain 63% of pale yellow oil (mukherjee). The oil is unusual in not being made up of straight chain fatty acids but acids with a cyclic group at the end of the chain. Seeds are ovoid, irregular and angular, 1 to 1 ¼ inches long, 1 inch wide, skin smooth, grey, brittle; kernel oily and dark brown. Fatty oil is obtained by expression, known officially as Gynocardia oil in Britain, as oleum chaulmoograe in the U.S.A [101].

4. AIM & OBJECTIVES

Cancer is one of the major concerns, as it is one of the leading cause of death worldwide. There are over 100 various types of cancer; each is categorized by the type of the cell that affected. Several million of deaths were caused due to cancer and this was dramatically increased recently. Depending upon the type and stage of cancer, treatments include combina tion of surgery, radiation therapy, chemotherapy, immune therapy. Chemotherapy is the major therapeutic approach for cancer treatment.. However, conventional chemotherapy has several limitations like lack of aqueous solubility, lack of selectivity and multi drug resistance. In short, chemotherapy will shorten the survival of patients' overtime. Therefore, we need to develop anti-cancer drugs with novel nanotechnology for the effective treatment of cancer.

Hydnocarpus pentandra is an Indo-Malayan genus belonging to the family Flacourtiaceae. It has showed potent anti-cancer activity against cancer cell growth. The extracts and compounds isolated from this plant show a wide spectrum of biological properties, including anti-bacterial, anti-leprotic, anti-tubercular, anti-psoriatic, anti-rheumatic, hypolipidemic, anti diabetic, anti-cancer, anti-inflammatory, antioxidant activities. However in pharmacetical field its use is still limited due to its poor solubility. The formulation of poorly water soluble drugs has always been a challenging problem faced by the pharmaceutical scientists.

Large proportions of newly discovered drugs are water insoluble, and therefore poorly bioavailable, contributing to deserted development effort. .Novel approaches are rapidly progressing aimed to solve the problems associated with these newly discovered drugs. Nano suspension technologies have emerged as promising strategy for the efficient delivery of poorly soluble drugs. Among different methods Nano precipitation technique is the simplest method and has been successfully employed in the formulation of Nanosuspension. It has several advantages such as higher drug loading capacity, dose concentration in infected tissues, lower incidence of side effects of excipients..Therefore, we need to develop effective drug Nano suspension formulation against cancer treatment.

This inspired us to develop a nanosuspension formulation of *Hydnocarpus pentandra* leaf residue and investigation of its Anti-cancer studies in an *in vitro* model against MCF 7 cell line.

Objectives of the study

1. Formulation of nanosuspension of *Hydnocarpus pentandra* by nano precipitation method
2. Characterization of nanosuspension of *Hydnocarpus pentandra*
    - Zeta potential analysis of *Hydnocarpus pentandra* nanosuspension using photon correlation Spectroscopy
    - Scanning Electron Microscope (SEM) analysis of *Hydnocarpus pentandra* nanosuspension
3. Solubility study of *Hydnocarpus pentandra* nanosuspension
4. Stability study of *Hydnocarpus pentandra* nanosuspension
5. *In vitro* anti-oxidant activity of *Hydnocarpus pentandra* nanosuspension
6. *In vitro* cytotoxicity study of *Hydnocarpus pentandra* nanosuspension against MCF 7 cell line

5. REVIEW OF LITERATURE

Kalpesh S. Wagh et.al; (2011) studied Nanosuspension: a new approach of bioavailability enhancement. It emphasized that

solubility is essential factor for drug effectiveness, independent of the route of administration..The formulation of poorly water soluble drugs has always been a challenging problem. The study focused on the various methods of preparation of nanosuspension with their advantages and disadvantages, formulation In vivo-in vitro evaluation method of nanosuspension and their application in drug delivery system. It concluded that the transformation of any drug to drug Nanoparticles leading to an increase in saturation solubility, dissolution velocity and providing the general feature of an increased adhesiveness to surfaces is one of the most important achievements [102].

Bala Krishna et.al; (2011) studied on nanosuspension in drug delivery. The study described about the preparation methods, characterization and applications of the nanosuspension. Nanotechnology has emerged as an tremendous field in the medicine. Nanosuspensions are part of Nanotechnology. Many of the drug candidates are exhibiting poor aqueous solubility. The use of drug nanosuspension is an universal formulation approach to increase the therapeutic performance of these drugs in any route of administration [103].

Vishal R. Patel et.al; (2011) reviewed Nanosuspension: An approach to enhance solubility of drugs. This review article describes the preparation methods, characterization and applications of the nanosuspension. For large scale production of nanosuspensions, media milling and high-pressure homogenization technology have been successfully used. Striking characteristics, like improvement of dissolution velocity, increased saturation solubility, improved bioadhesivity, versatility in surface modification, and ease of postproduction processing have widened the applications of nanosuspensions for various routes of administration. The applications of nanosuspensions in oral and parental routes have been very well established in this article [104].

Mitesh Patel et.al; (2011) reviewed on Nano suspension: A Novel Approach for drug delivery system.This study described the methods of nanosuspension production, formulation, evaluation and applications in pharmaceutical drug delivery as well as the marketed products. Drugs with poor solubility and low bioavailability are called ‘brick dust’ candidates once abandoned from formulation development work can be rescued with nanosuspension technology. A nanosuspension not only solve the problems of poor solubility and bioavailability but also alters the pharmacokinetics of drug and thus improves drug safety and efficacy. Nanosuspension technology can be combined with traditional dosage forms such as tablets, capsules, pellets, and can be used for Parenteral products [105].

Geeta vikram yadev et.al; (2012) studied on Nanosuspension: A promising drug delivery system. This study deals with the special features of nanosuspension, the preparation methods, advantages of such methods, characterization of nanosuspensions, patents, marketed products and their applications for hoping to make easy, the future research in this area. Attractive features such as increased dissolution velocity, increased saturation solubility, improved bioadhesivity, versatility in surface modification and ease of post-production processing, have widened the applications of nanosuspensions for various routes. The applications of nanosuspensions in Parenteral and oral routes have been very well investigated and applications in pulmonary and ocular delivery have been realized [106].

Paun J.S. et.al;(2012) studied on Nanosuspension: An emerging trend for bioavailability enhancement of poorly soluble drugs. This study takes account of introduction, advantages, properties, formulation consideration, preparation, characterization and application of the nanosuspensions. Improved bio-adhesiveness, versatility in surface modification and ease of post-production processing have widened the applications of nanosuspensions for various routes. Nanosuspension technology can be combined with traditional dosage forms: tablets, capsules, pellets and also can be used for Parenteral products. The advances in production methodologies using emulsions or micro emulsions as templates and precipitation method have provided still simpler approaches for production but with limitations [107].

Sarita et.al (2012); studied on Eudragit –Based Nanosuspension of poorly Water-soluble Drug Formulation and In vitro-In Vivo Evaluation. The study was performed to investigate potential of Eudragit RPLO-based nanosuspension of glimepiride, for the improvement of its solubility and overall therapeutic efficacy, suitable for peroral administration. Nanoprecipitation method being simple and less sophisticated was optimized for the preparation of nanosuspension. Physicochemical characteristics of nanosuspension in terms of size, Zeta potential, Polydispersity index, entrapment efficiency (% EE) and in vitro drug release were found within their acceptable ranges.Stability study revealed the nanosuspension was more stable at refrigerated condition with no significant changes in particle size distribution. In vivo studies showed that nanosuspensions exhibited better pharmacokinetic profile, efficiently reduced blood glucose level and maintained it to desirable level as compared to GLM nanosuspension. Therefore, GLM nanosuspension can be expected to gain considerable attention for improved therapeutic activity for the treatment of diabetes mellitus [108].

Masilamani et.al; (2012) studied on the effect of formulation and process variables on drug content and entrapment efficiency of aceclofenac nanosuspension.This study described the effect of polymer and surfactant concentration (formulation variables) and sonication time/agitation speed (process variables) on drug content and entrapment efficiency of nanosuspension of a water insoluble drug aceclofenac.The prepared nanosuspension of aceclofenac were analyzed for the drug content, entrapment efficiency and other evaluation parameters. These studies concluded that polymer and surfactant concentration with desired sonication time and agitation speed have significant effect on drug content and entrapment efficiency [109].

Sutradhar et.al; (2013) reviewed increasing possibilities of nanosuspension.This review describes the methods of pharmaceutical nanosuspension production including advantages and disadvantages, potential benefits, characterization tests and pharmaceutical applications in drug delivery. In this case, nanosuspension formulations can be considered as a promising candidate. Various techniques described in this review al10one or in combination can be successfully used to solve the poor bioavailability problem of hydrophobic drugs and drugs which are poorly soluble in aqueous and organic solutions. By emphasizing this technology, our society will be benefited financially also. Thus, nanosuspension technology is able enough to bring enormous immediate benefits and will revolutionize the research and practice of medicine in the field of pharmacy[110].

Arunkumar et.al; (2013) reviewed nanosuspension technology and its applications in drug delivery. This article reviews the current methods used to prepare nanosuspensions and their application in drug delivery..Nanosuspensions of pure drug offer a method to formulate poorly soluble drug and enhances the bioavailability of several drugs. Nanosuspensions can be formulated for various routes of administration such as oral, Parenteral, ocular, topical and pulmonary routes. This technology is gaining significance as the number of molecules with solubility and bioavailability related problems are increasing day by day. Thus, nanotechnology can play a vital role in drug discovery programs to increase aqueous solubility as well as bioavailability of poorly soluble drugs [111].

Priyank et.al; (2013) reviewed nanosuspension trends and technologies .Poor water solubility has become a major challenge for the formulation of the compound. Nanosuspension has the potential to overcome this problem. Change of material into the nanodimension change the properties. This review outline various advantages of nanodimensional particles [112].

Rupali L. Shid et.al; (2013) reviewed nanosuspension: A review. Nanosuspension solved poor bioavailability problem of hydrophobic drugs and drugs which are poorly soluble in aqueous and organic solutions. Production techniques such as media milling and high pressure homogenizer are used for large scale production of nanosuspensions. Nanosuspension can be administered through oral, Parenteral, pulmonary, ocular and topical routes. Since nanotechnique is simple, less requirements of excipients, increased dissolution velocity and saturation solubility many poor bioavailability drugs are formulated in Nanosuspension form [113].

Shanti Bhushan Mishra et.al; (2013) reviewed Nanosuspension of phyllanthus amarus extract for improving oral bioavailability and prevention of paracetamol induced hepatotoxicity in Sprague- Dawley rats. This study evaluated and compared the hepatoprotective effects of the ethanolic extract of P.amarus(PAE) and its Nanoparticles(PAN) on paracetamol induced acute liver toxicity in Sprague-Dawley rats. In conclusion they found that an oral dose of Phyllanthus amarus Nanoparticles that is five times less than the oral dose of Phyllanthus amarus extract could exhibit a similar hepatoprotective effect. This study could serve as a useful reference to allow the future exploitation of nanoparticulate system as a novel preventive and therapeutic measure for the treatment of hepatic and other various physiological disorders [114].

Amudha et.al; (2014) studied on Formulation of Nanosuspension drug delivery system containing coriander sativum extracts. Nanosuspension containing *coriander sativum* was prepared by solvent evaporation method followed by homogenization and they are evaluated for following parameters like particle size, zeta potential, PDI, drug content and in vitro drug release. The results showed that the solvent evaporation method followed by homogenization was an optimized technique for the preparation of Nanosuspension, which lead to better results like high efficiency, high drug content and sodium lauryl sulphate was a better choice of surfactant to reduce the particle size and leads to uniform distribution of the particles in Nanosuspension . While considering the zeta potential, particle size and PDI, it concludes that N2 is the best formulation among all the four formulations. Thus nanosuspension was a best alternative dosage form for natural herbal extracts [115].

Steffi et.al; (2014) studied on preparation, characterization and stabilization of curcumin Nanosuspension. Nanosuspension was prepared by bottom up method using acetone and water as solvent system in which curcumin was dissolved in acetone and added to this solution drop wise into a beaker containing water with constant stirring. Different amount of SDS was used as stabilizer and and the stability was optimized. Nanosuspension was characterized using particle size analyzer, zeta potential analyzer and SEM. The result showed the method adopted for the preparation of nanosuspension was found to be good. Then the addition of stabilizer SDS may be the better choice for the stabilization of curcumin nanosuspension when the suspension is required to be kept for a period of about a month [116].

Abirami et.al; (2014) studied on Herbal nanoparticles for anticancer potential- a review . This study described about various nano particulate technologies that have been studied for the delivery of herbal medicines and which are gaining more attention for improved therapeutic response and bioavailability. However, several problems such as poor solubility, poor bioavailability, low oral absorption, instability and unpredictable toxicity of herbal medicines limit their use. In order to overcome such problems, Nanoparticles can play vital role. Hence, different nanoparticles including polymeric nanoparticles, liposomes, pro liposomes, solid lipid nanoparticles and micro emulsions utilization show potential to deliver herbal medicines with better therapy [117].

Roya Yadollahi et.al; (2014) studied on Nanosuspension technologies for delivery of poorly soluble drugs. This review described preparation methods for nanosuspensions , typical characterization techniques, several applications for drug

delivery design and different administration routes such as Parenteral , pulmonary, oral and ocular. It also described the recent progress in therapeutic nanosuspensions produced by various techniques such as high pressure homogenization, media milling and emulsification. Attractive characteristics of nanosuspensions such as uniform nanosized particles, improved solubility in biological media and adhesiveness, increased drug concentrations, and residence time at the absorption sites enable the innovative design of a new class of drug delivery systems. Such nanosized drug formulations have a number of benefits for drug therapies including high surface area, controllable nanosize dimensions and tailored surface chemistry[118].

Samar A. Afifi et.al; (2015) studied on Nanosuspension: An emerging trend for bioavailability enhancement of Etodolac. Etodolac nanosuspension were prepared by Ph shift or antisolvent method in presence of different stabilizers. The dissolution of nano sized ET was significantly enhanced compared with the crude pure drug. The results showed that the particle size minimization produced by Ph shift method was not the major determining factor in the dissolution improvement. Rather, the type of stabilizer used in the formulations was of greater importance. The results also demonstrated that nano precipitation can thus be a simple and effective approach to produce submicron particles of poorly water-soluble drugs..Nanosnization of Etodolac had the potential to overcome absorption limitations of the poorly soluble drug [119].

Zhiping Wang et.al; (2015) studied on Berberine Nanosuspension enhances hypoglycaemic efficacy on streptozotocin induced diabetic C57BL/6 Mice.In this stsudy, the low solubility and poor membrane permeability of Ber were enhanced by NS technology. This study demonstrated that Ber-NS possessed excellent antidiabetic activity in diabetic mice model. Moreover, Ber-NS produced a superior hypoglycaemic and TC and body weight reduction and less adverse effects compared with bulk Ber and Met. Therefore, Ber-NS may be explored as a novel potential antidiabetic agent for the functional food and pharmaceutical purpose. This study also provides evidences to support the therapeutic effects of compound NS for treatment of diabetes in china [120].

Kiran Thadkala et.al; (2015); studied on formulation, optimization and evaluation of oral nanosuspension tablets of nebivolol hydrochloride for enhancement of dissolution rate.This study was performed to improve the solubility and dissolution rate of poorly soluble drug,nebivolol hydrochloride by nanosuspension tablet prepared using microcrystalline cellulose povidone and croscarmellose sodium.The physicochemical compatibility of the drug and excipients were studied by Infrared spectroscopy and differential scanning calorimetry.This study shows that nanosuspension tablets changes the properties of poorly soluble drugs like nebivolol hydrochloride and increases the wetting property and surface area of the drug particle and indirectly increases the dissolution and oral bioavailability of drugs.Thus study shows that nanosuspension tablets are promising alternative technique for improving dissolution rate and bioavailability of drugs[121] .

Nazish Jahan et.al; (2015) studied on Formulation and characterization of nanosuspension of herbal extracts for enhanced antiradical potential. This review presents the nanosuspension approach for increasing the aqueous solubility and thereby bioactivity of important herbal extracts. Nanosuspensions of the seeds of three plants extract (Silybum marianum, Elettaria cardamomum and coriandrum sativum) were prepared by using polyvinyl alcohol(1.5%w/v) as a stabilizer. Prepared Nanoparticles were characterized by scanning electron microscope. Activity of nanosuspension formulation was assessed by using four in vitro antioxidant assays. These synthesized Nanoparticles were found to be more effective against quenching free radical than their crude extracts and standards. This study shows that nanosuspension of herbal medicines potentiates the antioxidant potential [122].

6. MATERIALS AND METHOD

Materials

- Petroleum ether
- Ethanol
- Acetone
- Polyvinyl alcohol
- Curcumin
- Methanol
- DPPH (22, diphenyl-1-picryl hydrazyl)

Instruments

- Magnetic balance (Unibloc Shimadzu)
- Magnetic stirrer (Rotek magnetic stirrer, 230 V, 50 cpc, 10 AC, 40 Watts)
- UV-1700 Pharmaspec-Shimadzu, (Ge Nei-Bangalore)
- Orbital shaker incubator (GeNei, Bangalore)

- Millipore water purifier (Millipore SAS, France)
- Lyophiliser
- Refrigerated centrifuge

Preparation of *Hydnocarpus pentandra* leaf residue

From *Hydnocarpus pentandra* leaves:

Fresh leaves of *Hydnocarpus pentandra*, (figure 15) were identified and collected from Chalakudy, Thrissur, Kerala and was authenticated by Dr. Jalaja S Menon, Kerala Agricultural University, Thrissur. The collected leaves were washed several times with water to remove the dust particles and then sun dried to remove the residual moisture and grinded to form powder.

The powdered leaves (5g) were macerated with petroleum ether to remove fatty substances; the marc was further exhaustively extracted with 95% ethanol for 3 d (3x3 I) by cold percolation method and centrifugation at 10,000 rev min^{-1}. The extract was separated by filtration and concentrated on rotavapour (Buchi, USA) and thus 1 gm of solid residue was obtained.

Formulation of nanosuspension of *Hydnocarpus pentandra* by nano precipitation technique

Nano precipitation technique was applied to prepare *Hydnocarpus pentandra* with slight modification. Briefly, 5 g of *Hydnocarpus pentandra* residue was dissolved in 15 ml of acetone and ethanol (3:1) by sonication at 20 W for 30 s. The resulting solution was then slowly injected (1 ml min^{-1}) with a syringe connected to a thin Teflon tube, into 25 ml water containing polyvinyl alcohol (PVA) 1.5% w/v with continuous magnetic stirring at 1000 rpm. The resulting emulsion obtained was then diluted in 25 ml PVA solution (0.2% w/v in water) in order to minimize coalescence and the mixture was continuously stirred (500 rpm) for 6 h at room temperature to allow solvent evaporation and nano particle formation. The resulting nanosuspension was subsequently cooled down to $^{-}$18◦c and Lyophilized [123].

2. **Characterization of *Hydnocarpus pentandra* nanosuspension**

Zeta potential analysis of *Hydnocarpus pentandra* nanosuspension using photon correlation spectroscopy

The electrophoretic mobility (Zeta potential) measurements were made using the Malven Zetasizer (Nano ZS90, Malvern Instruments) at 25◦c. The zeta potential is caused by the net electric charge contained within the region bounded by the slipping plane, and also depends on the location of that plane. The determination of the zeta potential of a nanosuspension is essential as it gives an idea about the physical stability of the nanosuspension. The zeta potential of a nanosuspension is governed by both the stabilizer and the drug itself.

Scanning electron microscope (SEM) analysis of nanosuspension

The surface morphology of the prepared nanosuspension was determined for by using scanning electron microscopy (JEOL model JSM-6390LV). A portion of sample was placed on a carbon film coated copper grid for SEM. Studies were performed at 80kv using JEOL model JSM-6390 LV, Japan equipped with selected area electron diffraction pattern (SAED). The copper grid was fixed in to sample holder and placed in a vacuum chamber of the scanning electron microscope and observed under low vacuum and SEM images were recorded [I24, 125].

Solubility studies

Saturation solubility is defined as the maximum quantity of a compound (solute) that can be dissolved in a certain quantity of a specific solvent at a specified temperature. However it is well known that the saturation solubility also depends upon the modification of polymorphic compounds and the particle size, if the particle size is less than 1µm are present. Solubility studies were performed using a shaker (orbital shaker incubator, GeNei, Bangalore). Excess of drug residue and Lyophilized Nanosuspension were separately added in 20 ml distilled water and stored at 37±0.5◦C. After 24 hours of shaking, suspensions were filtered and analyzed using UV spectrophotometer at 370 nm (UV-1700 Pharmaspec, Shimadzu). Experiments were carried out in triplicate, and solubility data were averaged [I26, I27].

Stability studies

Stability is an important factor in case of nanosuspension. In case of suspensions the possibility of sedimentation and cake formation are more. The particles will settle down and it would not redisperse uniformly after shaking. So the dose will be changed, bioavailability will be less which leads to the decreased pharmacological output. When we are formulating the drug in to nanometer range the particle size will be less than 1µm, so it would not sediment rapidly it will suspend in the solvent more time and redispersion will be fast. The stability was measured at different temperature and find out at which temperature it would be more stable. The prepared Lyophilized Nanosuspension was kept at room temperature (RH-85%) and 4◦C (RH-20%) for six months at specific time period. A portion of the sample were taken and subjected to SEM analysis for the determination of stability [128].

***In vitro* antioxidant activity**

Anti oxidant activity can be evaluated by scavenging of the stable DPPH radical. This model is extensively used as it is less

time consuming than the other methods. DPPH can be accepting an electron and hydrogen radical and can be converted into a stable diamagnetic molecule. DPPH contain an odd electron, and so it has a strong absorption at517nm. When this electron becomes pairs off, the absorption decreases stochiometrically with respect to the electron taken up. Such change in the absorbance produced in this reaction has been widely applied to assess the capacity of numerous molecules to act as free radical scavengers.

Free radical scavenging activity of all the concentrations of lyophilized Nanosuspension of Hydnocarpus pentandra were determined by DPPH assay method and compound with curcumin used as standard.

Chemicals used

2.2-diphenyl-1-picryl hydrazyl (DPPH)

Methanol

Curcumin

Preparation of solutions

Preparation of 1000µg/ml stock solution of *Hydnocarpus pentandra* nanosuspension

10mg of Nanosuspension was taken and dissolved in methanol. The volume was made up to 10mL with methanol.

Preparation of 0.1%v/v, 1%v/v and 10%v/v formulated nanosuspension

From the above solution 1.0mL aliquot was transferred to a 10mL standard flask and the volume was made up to 10mL with methanol for 10%v/v solution. From the stock solution 1.0mL and 0.1mL aliquots were transferred to a 100mL standard flask and the volume was made up to 100mL with methanol for 1%v/v and 0.1%v/v solution.

Preparation of 0.2mM DPPH solution

0.00789g of DPPH was taken in a 100mL standard flask and dissolved in 100mL of methanol. The final volume was made up to 100ml with methanol.

Preparation of 0.1%v/v, 1%v/v and 10%v/v standard solution:

10mg of curcumin was taken in a 10mL standard flask and dissolved in methanol. The volume was made up to 10mL with methanol. From this solution 1.0mL aliquot was transferred to a 10mL standard flask and the volume was made up to 10mL with methanol for 10%v/v solution. From the stock solution 1.0mL and 0.1mL aliquots were transferred to a 100mL standard flask and the volume was made up to 100mL with methanol for 1%v/v and 0.1%v/v solution.

Procedure for the evaluation of antioxidant activity:

1.5ml of 2.0mM of DPPH solution was added to 1.5mL of different concentration of the formulated Nanosuspension. Another series of solution were prepared by taking 1.5mL of methanol and 1.5ml of different concentrations of formulated Nanosuspension. The above solutions were allowed to react at room temperature for 30min. after 30 min the absorbance values were measured at 517nm. Curcumin was used as the reference standard. Percentage of scavenging activity was calculated by using the following formula.

$$\% \text{ scavenging} = \frac{(Ab+As)-Am}{Ab} \times 100$$

Ab = absorbance of 1.5mL of DPPH +1.5mL of methanol at 517nm.

Am= absorbance of 1.5mL of DPPH +1.5mL of Nanosuspension at 517nm.

As= absorbance of 1.5mL of metanol+1.5mL of Nanosuspension at 517nm [129].

***In vitro* cytotoxicity activity of *Hydnocarpus pentandra* nanosuspension**

The 3-(4,5 – dimethylthiazol-2,5-diphenyltetrazolium bromide) dye reduction assay was conducted to diagnose the cytotoxic activity of the formulated nanosuspension. MCF7 cells were plated onto 48 wells plates, 18 hours before the commencement of the test. Growth medium used was DMEM with 10% Fetal Bovine Serum. The plates were incubated in an animal cell culture incubator, maintained at 37^0C with 5% carbon dioxide. The wells achieved 70% confluency at the time of testing. The original growth medium in the 48 well plate was removed and the samples prepared above were added to the wells. The plates were returned to the incubator for 96 hours. At the end of 96 hours, the media in the wells were carefully removed and fresh complete growth media was added. To each well MTT solution (5 mg/ml of MTT dissolved in PBS) was added and replaced in the incubator for 3 hours. After 3 hours, the medium was carefully removed from the wells, and DMSO was added to each well and kept on a rocking platform for efficient mixing and extraction of formazan dye from the cells by DMSO. After 30 minutes, the absorbance of the DMSO was measured at 570 nm, in a multi-well spectrophotometer.

The average absorbance of the “control” wells was taken as 100% and all other absorbance values were calculated based on

this and plotted on the graph [130].

7. RESULTS & DISCUSSIONS

Hydnocarpus pentandra nanosuspension

Nanosuspension of *Hydnocarpus pentandra* was prepared by Nano precipitation method. This method involves the precipitation of a drug from an organic solution and the diffusion of the organic solvent in the aqueous medium in the presence or absence of a surfactant. Organic medium containing saturated drug solution is injected into a stirred aqueous solution containing a stabilizer as a surfactant. Therefore, this study indicated that Nano precipitation method is a simple, more facile, less complex, comparatively cheaper, less energy consuming and easy for large scale production.

Characterization of *Hydnocarpus pentandra* nanosuspension

Zeta Potential Analysis of nanosuspension of *Hydnocarpus pentandra*

The Zeta potential value of formulated lyophilized nanosuspension was obtained as -8.24 mv. In order to obtain a nanosuspension exhibiting good stability, for a nanosuspension a minimum zeta potential of ±30mv is required. Therefore this study indicated that the formulated lyophilized nanosuspension was sufficiently stable.

The zeta potential is caused by the net electric charge contained within the region bounded by the slipping plane, and also depends on the location of that plane. The determination of the Zeta potential of a nanosuspension is essential as it gives an idea about the physical stability of the nanosuspension. The zeta potential of a nanosuspension is governed by both the stabilizer and the drug itself.

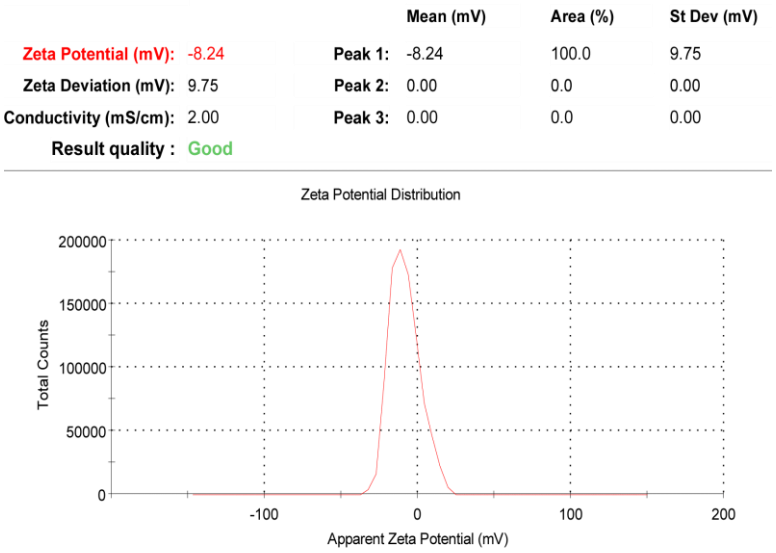


Figure 16: Zeta potential analysis of *Hydnocarpus pentandra* nanosuspension

SEM analysis of *Hydnocarpus pentandra* nanosuspension

The particle size of the formulated nanosuspension was found to be within the range of ~ 320 nm-480 nm. Therefore the formulation is in nanoscale. Nano grinding is a critical process which used to obtained appropriate particle size reduction and stability of nanosuspension. The mean particle size and width of the particle size distribution are important characterization parameters as they govern the saturation solubility, dissolution velocity and physical stability.

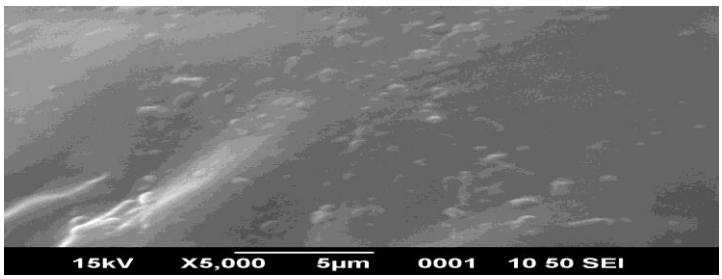


Figure 17: SEM analysis of *Hydnocarpus pentandra* nanosuspension

Solubility studies of *Hydnocarpus pentandra* nanosuspension

Solubility of *Hydnocarpus pentandra* after 24 hr shaking was found to be 5.3 ± 0.32.mg/ml. Solubility of *Hydnocarpus pentandra* lyophilized nanosuspension after 24 hr shaking was found to be 10.5 ± 0.52 mg/ml.

The study indicated that nanosuspension increase the saturation solubility as well as dissolution velocity. From the solubility of *Hydnocarpus pentandra* was extremely low being only 5.3 mg/ml. There was the significant enhancement in the solubility of the Lyophilized nanosuspension produced by Nano precipitation method. This is because the saturation solubility increases with decreasing particle size below 1000 nm.

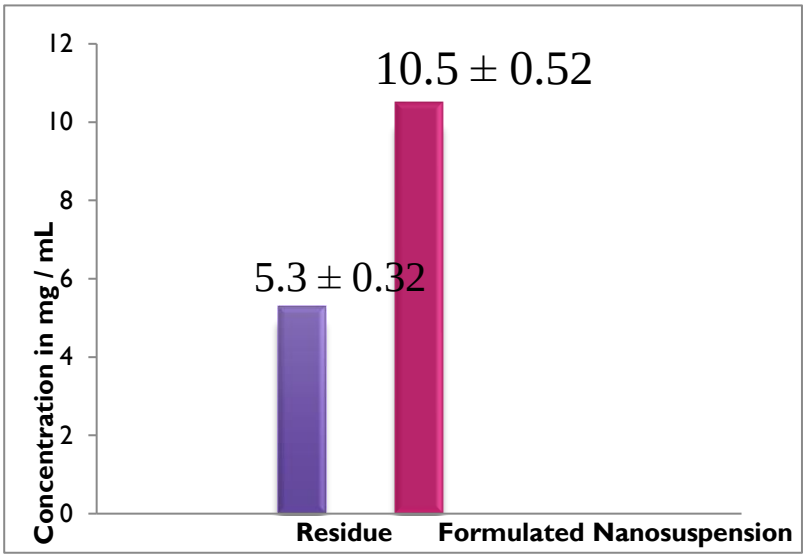


Figure 18: Solubility study of *Hydnocarpus pentandra* nanosuspension

Stability studies of *Hydnocarpus pentandra* nanosuspension

Lyophilized nanosuspensions were stored at 4˚C and at room temperature respectively. Particle size was monitored over six months. Stored at the lower temperature and at the room temperature, the particle size showed no significant change over six months. Then the particle size of the nanosuspension was found to be within the range of ~ 320 nm to 480 nm. Therefore solubility study showed that the formulated nanosuspension was stable in room temperature 25 °c (RH 85%) as well as cold temperature 4 °c (RH 20%).

Table 2: Stability study of *Hydnocarpus pentandra* nanosuspension

Duration	**Particle size (nm)**	
	4 °c **RH 20%**	RT, 25°C **RH 85%**
0	305 nm	320 nm
3	320 nm	360 nm
6	380 nm	390 nm

***In vitro* antioxidant study of *Hydnocarpus pentandra* nanosuspension**

Antioxidant activity can be evaluated by scavenging of the stable DPPH radical. This model is extensively used as it is less time consuming than the other methods. DPPH can accept an electron and hydrogen radical and can get converted into a stable diamagnetic molecule. DPPH contain an odd electron, and so it has a strong absorption at 517nm. When this electron becomes pairs off, the absorption decreases stochiometrically with respect to the electron taken up. Such change in the absorbance produced in this reaction has been widely applied to assess the capacity of numerous molecules to act as free radical scavengers.

Table 3: Antioxidant activity of curcumin by DPPH method

Extract	Concentration (% V/V)	Absorbance of Curcumin + DPPH (517nm)	Absorbance of Curcumin+ methanol (517nm)	% Scavenging
Curcumin	0.1	0.280	0.013	73.08
	1	0.138	0.015	87.06
	10	0.073	0.019	94.55

Table 4: Antioxidant activity of *Hydnocarpus pentandra* nanosuspension

	Concentration (%V/V)	Absorbance of NS + DPPH (517nm)	Absorbance of NS + Methanol (517nm)	% Scavenging
Formulated NS	0.1	0.269	0.013	74.19
	1	0.120	0.034	91.33
	10	0.069	.035	96.57

The formulated nanosuspension have hydrogen donating ability or can scavenge free radicals and at 10% v/v showed significant antioxidant activity.*Nanosuspension of Hydnocarpus pentandra exhibited significant antioxidant activity using DPPH method at dose dependent manner*.

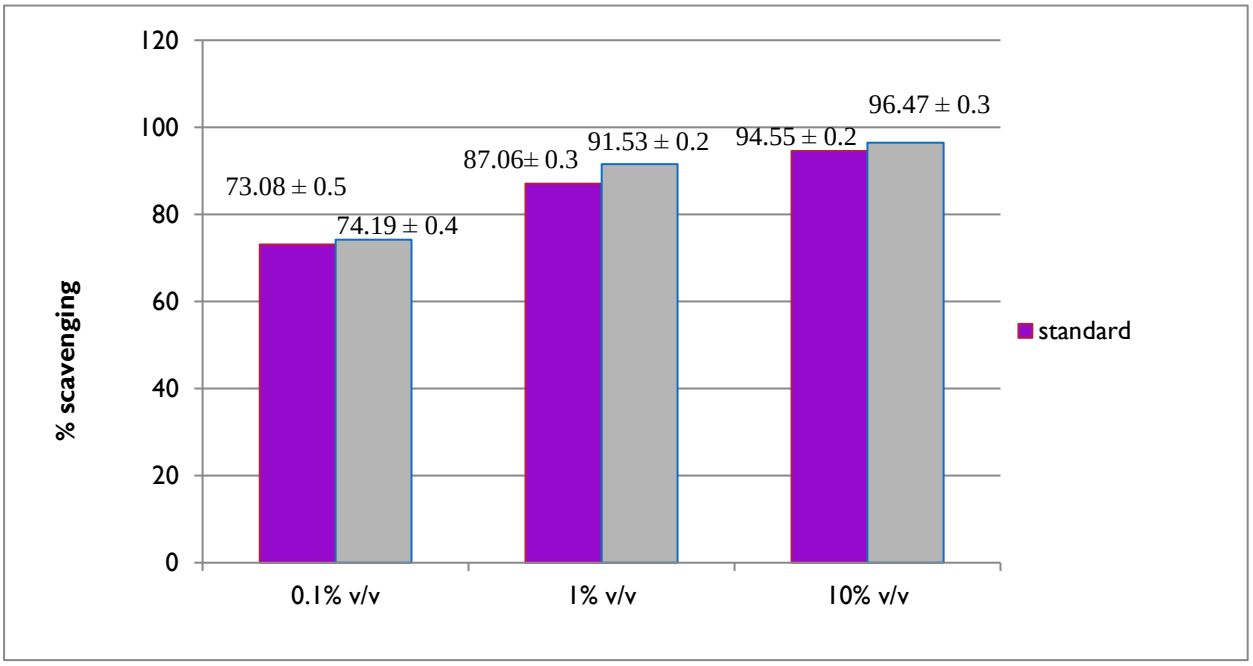


Figure 19: Antioxidant activity of *Hydnocarpus pentandra nanosuspension* compared with curcumin

In vitro* cytotoxicity study of nanosuspension of *Hydnocarpus pentandra

In vitro cytotoxicity activity against human breast cancer MCF 7 cell line was evaluated at different concentration (0.1 %v/v, 1%v/v, 10%v/v) by MTT assay. The in vitro screening of the *Hydnocarpus pentandra* nanosuspension showed significant cyto toxicity activity at against the human breast cancer cell line. The results obtained are shown in Table 5 & Figure 20. Our cytotoxicity analysis of the sample shows a direct dose-response relationship; cyto toxicity increased at higher concentration.

Table 5: % Cytotoxicity of *Hydnocarpus pentandra* nanosuspension

Sl. No.	Concentration (%V/V)	Percentage Cytotoxicity
1	0.1	92.62
2	1	85.53
3	10	57.17

The average absorbance of the "control" wells was taken as 100% and all other absorbance values were calculated based on this and plotted on the graph. The Formulated lyophilized nanosuspension at 10% v/v showed significant cytotoxicity activity. *Nanosuspension of Hydnocarpus pentandra exhibited significant anticancer* ***activity*** *against MCF 7 cell line at dose dependent manner*.

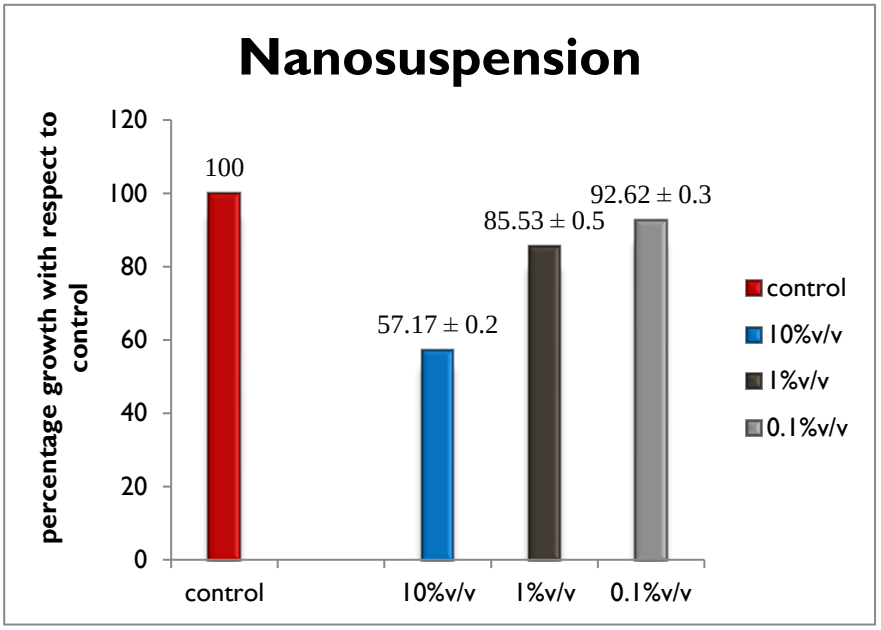
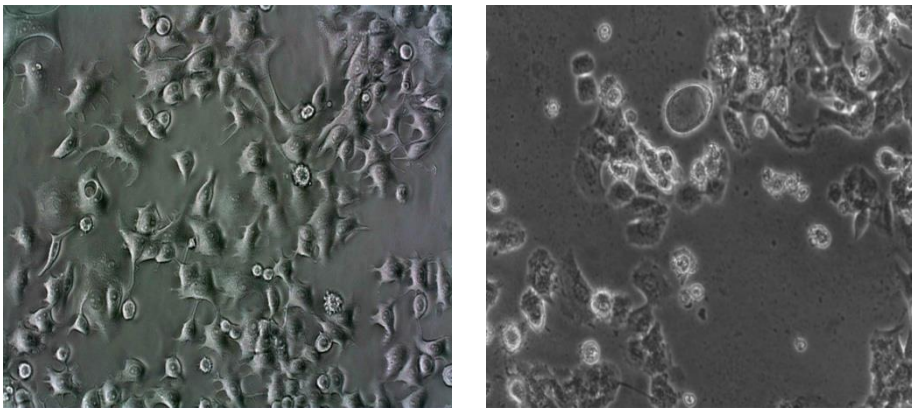


Figure 20: % Cytotoxicity of *Hydnocarpus pentandra* nanosuspension



Control MCF-7 Cell line

MCF-7 Cells treated with *Hydnocarpus pentandra* Nanosuspension

Figure 21: *In vitro* cytotoxicity study of *Hydnocarpus pentandra nanosuspension*

8. SUMMARY & CONCLUSION

Medicinal plants are mainly used for traditional Indian medicine and act as dietary agents for the treatment of various diseases. The poorly water soluble drugs have always been a challenging problem faced by pharmaceutical scientists. In this case nanosuspension formulation can be considered as promising candidate. Nanosuspension formulation have been largely solved the solubility as well as dissolution problems to improve drug absorption. It has therapeutic advantages, such as simple method of preparation, applied for poorly water soluble drugs/ extract most cost effective, suitable for large scale production.

Nanosuspension of *Hydnocarpus pentandra* was prepared by nano precipitation method. Particle size of formulated lyophilized nanosuspension showed range from ~ 320 to 480 nm. Zeta potential value of formulated nanosuspension was obtained as -8.24 mv. Solubility study indicated that formulated nanosuspension enhanced the solubility of the *Hydnocarpus pentandra.* Stability study showed nanosuspension was stable in RT and cold temperature. Nanosuspension of *Hydnocarpus pentandra* exhibited significant anti oxidant activity using DPPH method and anti cancer activity against MCF 7 Cell line at dose dependent manner.

The study concluded that nanosuspension of *Hydnocarpus pentandra* exhibited anti oxidant activity as well as anticancer activity.

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