A DETAILED STUDY OF PHYTOSOMAL PREPARATION, CHARACTERIZATION AND HEPATOPROTECTIVE ACTIVITY OF HERBAL PLANTS ANDROGRAPHIS PANICULATA AND RHAPNUS SATIVUS.

Anjana Godiyal, Dr. Praveen Kumar, Spardha Trehan
Himalayan Institute of Pharmacy and Research, Rajawala, Dehradun

CHAPTER 1

1.INTRODUCTION

The liver plays an important role in maintaining and regulation of homeostasis of body. It is present in every biochemical reaction like growth, to cure diseases, provide nutrients, and energy to sustain healthy liver is crucial for wellbeing of an individual.

The liver filters blood that reaches stomach and intestine. The liver produces bile, which helps in breakdown of fats, elimination of contaminants, and maintenance of numerous micronutrients. The pancreas releases catalysts which help to break protein, fats and glucose.

The gall bladder collects bile which was produced by body. The hepatic region is separated into 4 parts: the bigger right and left lobes, and the smaller cortices and lobes. The left and right lobes are divided by "sickle-shaped" ligament which joins liver to abdomen. The hepatic lobe is divided into seven divisions, each with millions of fascicles (small lobes).

1.1 Hepatic damage

The toxic liver is damage caused by drugs and chemicals closely resembles a range of naturally occurring liver disorders. In fact, any patient without liver disease or unexplainable hepatitis is interviewed thoroughly about drug usage and chemical exposure. The universal form of untreatable illness in toxicity of liver is induce by drugs and chemicals.

1.1.1 Liver injury pattern

1.1.1.1 Hepatitis

The liver cell damages cause marked cell enlargement, with irregularly clumped cytoplasm and large clear spaces. Fat, iron, and retained biliary material are some of the substances which collects in liver. Necrosis All kinds of liver injury, mainly microbiological, toxic, circulatory, or traumatic, results in liver cell necrosis. The extent where liver lobule is involved in necrosis; changes.

1.1.1.2 Necrosis

All kinds of liver injury, mainly microbiological, toxic, circulatory, or traumatic, results in liver cell necrosis. The extent where liver lobule is involved in necrosis; changes. As a result, there are three types of acute liver cell necrosis: diffuse, zone and focal.

1.1.1.3 Apoptosis

Apoptosis is a distinct type of cell death which changes from cancer necrosis. It also includes condensation and nucleosomal DNA degradation.

1.1.1.4 Fibrosis

Liver disease, organ failure, and portal hypertension all are indication of liver cirrhosis, that causes liver transplantation. In initial stages, fibrosis develops with or around the central vein or fibrosis are deposited directly.

Table 1.1: Chemicals and pharmaceuticals cause liver damage.

S. No	Chemical	Effects
1.	Acetaminophen	Hepatic necrosis is induced by a toxic metabolite NAPQI generated by phosphorylation cytochrome p450.
2.	Amoxicillin	Anemia, liver dysfunction, and severe cytostatic encephalopathy all are signs of liver failure with a significant rise in SGOT and SGPT enzymes.
3.	Chlorpromazine	Transmittable inflammation of liver with features of jaundice.
4.	Ciprofloxacin	Cholestatic jaundice increases levels of SGPT, SGOT and alkaline phosphatase.
5.	Diclofenac	Raised ALT and experience intense, liver destruction, hepatitis, as well as other signs of liver failure acute cirrhosis all are signs of aseptic meningitis.
6.	Erythromycin	Elevated outstanding quality and AST levels hepatocellular cirrhosis with or without hepatitis.

1.2 Drug induced liver toxicity

The drugs are useful for management of several diseases which causes damage in liver cells as they are converted chemically to hydrophilic substances during metabolism. The method is known as biotransformation. The hydrophilic substances are excreted in urine or bile to get away of harmful metabolites. The conversion method contains two reactions namely phase I & phase II reactions. For phase I reaction, cytochrome P450 mechanism and its reaction contains metabolism of complex by corrosion or demethylation progression. The reaction produces a hydroxyl group which is captured by phase II reaction.

1.2.1 Paracetamol induced hepatotoxicity

Paracetamol is used for wellbeing to treat fever and pain management. It's an under-sleep aid which helps to treat minor headaches or muscle pain to serious discomfort. In general, paracetamol is used to reduce extreme pain in conjunction with other opioids and non-steroidal prodrugs (NSAIDs).

Although paracetamol, is healthy for single use in recommended doses, it causes severe liver failure. A normal dose of acetaminophen causes damage in ease caused by higher doses, based on climate and lifestyle.

1.2.2 Cholesterol-lowering drugs

To avoid myocardial infarction and strokes, statins are used by most commonly prescribed preventative medicines. Although statins are safe for long term use to lo were elevated LDL cholesterol, and are linked to

irregular liver failure. The levels of liver function in individuals on statins have been shown to raise by 5 per cent to 3percent of total in clinical studies.

1.2.3 Niacin

Vitamin-B3, also renowned as folate, is a form of drug which is used to decrease high blood cholesterol and triglyceride levels. High concentrations of niacin have just been linked to liver failure, likely to result in liver problems, reversibly raised AST and ALT levels, and in some instances, liver failure.

1.2.4 Antibiotics

1.2.4.1 Nitrofurantoin

Nitrofurantoin, an antibacterial drug, is being used to cure urinary tract infections (UTIs) caused by a variety of gram-negative and gram-positive bacteria. To cure UTI, 3 formulations of nitrofurantoin are presently available: a macrocrystalline form (Macrodantin), a crystallinity form (Furadantin), and a controlled release macrocrystalline form (Macrodantin) (Macrobid).

1.2.4.2 Augmentin

Augmentin is a penicillin and oxacillin amoxicillin which combines amoxicillin and clavulanic acid. It is effective against several pathogens, including H. influenzae, N. genital herpes, E. coli, Pneumococci, Streptococci.

1.3.2 Hepatoprotective Phyto extracts

1.3.2.1 Lady's thistle.

Lady's thistle (Silymarin), is derivative obtained from seeds of Silybum marianum L. (Family: Asteraceae or Compositae), has been used as a natural preparation for liver and biliary tract disorders. Silybin defends and rejuvenates liver cells in abundant liver disorders like cirrhosis, jaundice, and inflammation of liver.

1.3.2.2 Annona squamosa

Annona squamosa herb consists of alkaloid in the root. Other acetogenins have been isolated from the seeds, bark, and leaves. Raj et al examined the liver protective actions on Annona squamosa seed to alcohol acute liver failure. The liver toxicity was enhanced by giving 50% ethanol continuously for 7 days at a dose of 12.5 ml per kg. The extract of Annona squamosa seed (EEAS) was given in 200 mg and 400 mg /kg dose.

1.3.2.3 Silybum marianum

The plant phenolic constituents like flavonoids play a major role to treat liver disorders and they possess a potent hepatoprotective activity.

1.3.2.4 Chamomile recutita capitula

The impact of herbal tea (400 mg kg P.O.) on plasma and hepatocellular antioxidant, Na+ K+-ATP action, serum marker enzymes, total bilirubin, starch hepatocellular injury in rats was investigated to identify potential factors of connected in certain features.

1.3.2.5 Andrographis paniculata Andrographis paniculata is a widely used perennial herb found throughout the world. Andrographis is ethnobotanically used for the treatment of snake bites, bug bites, hyperglycaemia, diarrhoea, fever and malaria in Bangladesh, China, Hong Kong, India and Pakistan. GOT, GPT, reactive phosphate, glucose, bilirubin, cholesterol, and total protein have been used as evaluation markers.

1.4 The Phytosomes technology

The phytosomes is a Greek word which constitutes from two words Phyto means 'plant' and some alludes to a cell. The phytosome technology, developed by Indena S.p.A medicinal herbs possess liver protective action are lipophilic with melting point, which are freely soluble in non-polar solvents.

In liposomes, the active component is dissolved in medium of cavity or in layers of membrane. Unlike phytosomes; a liposome is arranged by adding hydrophilic molecules that surrounds the water. The phytosome is a special type of herbal extract preparation which possess greater efficacy than folk medicine. The low bioavailability and absorption of water- soluble phytochemicals, that can be overcome by phytosome technology as it distributes active phytochemicals in most efficient manner.

1.4.1 Hepatoprotective action of phytosomes

The phytoconstituents cause good therapeutic action to cure liver. Several studies indicated that phytosomes enhances bioavailability of Phyto constituents through oral and topical route of administration and hence bioavailability reduces the essential dose.

To rupture through cells and improves encapsulation efficiency of active constituents. This feature is used in drug carriers which are applied topically. As choline is necessary for normal functioning of liver, phosphatidylcholine is useful in phytosomes formulation which serves as a liver -protective and provides a synergistic action to protect liver.

The formation of a chemical bond between phenol and lipid improves the stability of bound constituents. The complex of phytoconstituents have a greater therapeutic benefit. The phytosomes are proven to be helpful in number of studies that increase phytoconstituents through dietary ingestion as well as the topical route of administration, drug release is enhanced, and required dose is decreased.

1.4.2 Methods of phytosome formulation

1.4.5.1 Solvent evaporation precipitate

The solvent disappearance is an old & is commonly useful method for preparation of phosphatidylcholine complex. The drug & soya lecithin are placed in a 100 ml RBF and refluxed for 2 hours amid with 20 ml dichloromethane (DCM) at a tempt of higher than 50°C. 5-10 ml of the solution is strained. To get the precipitate, hexane was cautiously added by using magnetic stirrer to provide the concentrate which was sieved, dehydrated & placed in a desiccator.

1.4.5.2 Salting out method

The active constituent and phospholipid are dissolved in solvent like dioxane or acetone while the solution is being stirred overnight. The formed complex is isolated by precipitation on adding non-solvent like n-hexane.

1.4.5.3 Lyophilization technique

Both Phospholipid and phytoconstituent/plant extract are dissolved in suitable solvent and solution containing phytoconstituent were added to a solution containing phospholipid followed by stirring till complex formation takes place. The formed complex is isolated by lyophilization.

1.4.5.4 Mechanical dispersion method

Accurate amount of phospholipid was dissolved in diethyl ether in beaker kept into bath sonicator. Drug was dissolved in double distilled water and this solution was injected drop by drop into the beaker containing phospholipid while sonicating and then left for 15 min for further sonication. On evaporation of solvent milk white suspension forms as phytosomal complex.

1.4.6 Hepatoprotective action

The phosphatidylcholine act as a component which is added to the preparation of Phyto phospholipid complexes, and act as a liver-protective action. The choline acquires is necessary for the normal functioning of liver. In vitro study, have been shown that this phospholipid (PL) increases hepatic collagenase actions and helps to avoid the fibrous connective tissue and cirrhosis through reassuring collagen breakdown.

1.4.7 Advantages of phytosomes

- The phytosomes possess better pharmacokinetic and pharmacodynamics profile

. • The phytosomal vesicles have ability to give the drug via topical and oral route of administration.

- It enables that the medicament is given to the correct regions.
- Phytosomes administered the herbal drug, the security of the minerals in the leaf extracts should not be exaggerated.
- Improved greatly therapeutic efficacy.
- Entrapment efficiency is high and specified since medication is linked with lipid in the synthesis of nano particles.
- Drug entrapment of phytosomes is really not a concern.
- The creation of chemical bonds between phytocompounds and phosphatidyl molecules improves the stability of phytosomes.
- The formation of chemical bonds between phytoconstituents and phosphatidyl molecules increases the stability of liposomes.
- In skin care products, phytosomes are more advantageous than liposomes. Phytoconstituents have a greater clinical benefit.

1.4.8 Disadvantages

The phytochemicals from phytosomes are eliminated rapidly, which is a drawback.

1.4.9 Correlation among a Liposome and a Phytosome

A liposome differentiates from a specified item except that it is produced by mixing a water-soluble substance with phosphatidylcholine in a specific ratio under controlled settings. There really is no linkage between both the lecithin particles surrounding the soluble in liquid substance and thousands of phosphatidylcholine units surrounding the water-soluble ingredient.

1.4.10 Structural verification of Phyto phospholipid complexes

1.4.10.1 The partition coefficient and solubility

The active constituents, active component phenolic complex, and physiological mixture require evaluating soluble in water and unrefined solvents, and the n octanol/water solubility (P). The phenolic compounds, in average, have greater lipophilicity and solubility than energetic ingredient & thus characteristically have a greater solubility and biocompatibility as active ingredients.

1.4.10.2 Ultraviolet spectra (UV-spectra)

In UV wavelength range, with different absorption is useful to characterize the own structural property. The UV visible absorption features of constituents before and after complexation are found to change in majority of the studies.

1.4.10.3 Differential scanning calorimetry (DSC)

It is a kind of mass spectroscopy which requires a different technique (DSC). By compare the alteration in high temperature, the exterior of innovative peak, the evaporation of unique peaks, melting point and change in virtual peak region in DSC, interactions were observed. As comparison to a physiological mixture, Phyto phospholipid compounds possess radically different stretching vibration.

1.4.10.4 Fourier transform (FTIR)

FTIR is a way for morphological study that generates several efficient groups with separate band number, location, outline and concentration characteristics. By comparing the spectroscopy of lipid complex to that physical mixture, the formation of Phyto phospholipid compounds is demonstrated.

1.4.10.5 X ray diffraction (XRD)

X-ray diffraction technique that is used to identify microstructure and crystalline form of phyto-phospholipid complex formed.

1.4.10.6 Nuclear magnetic resonance imaging (NMR)

To identify the structure of compounds, 1H Magnetic and NMR spectra methods are being used commonly. Hydroxyl groups, rather than chemical bonds, are essential for both interaction between polyphenol and

phosphatidylserine. Angelico et al. showed that hydrogen atoms could develop between most of resveratrol between the polarity polyphenol metal ions and phosphatidylserine.

1.4.10.7 Phytosomal properties

A phytosome is a compound made up of a natural product and phospholipid. The organic phosphatidylcholine including such as soy phospholipids are created by mixing molar ratio amounts in an environment rich in phospholipids and phytoconstituents as appropriate solvent. The spectrographic evidence suggests the primary phospholipid substrate interacting of hydrogen bond formation.

1.5 Phytosomal commercial products

The different commercial products of phytosomes are mentioned in Table 1.2

Table 1.2: Plant product with source and indication.

S. No	Product	Plant Source (Chief constituent)	Indication
1.	Green Tea Phytosome	(Epigallocatechin 3-ogallate) *Camellia sinensis*	Oxidizing agent, antitumor, and cancer-prevention.
2.	Centella phytosome	*Centella asiatica* (Triterpine)	Cicatrizing, trophodermic
3.	Silymarin phytosome	*Silybum marianum*	Antioxidant protection to liver or skin.
4.	Bilberry mirto select phytosome	*Vaccinium myritillus* (triterpine)	(Anticinocide) potent antioxidant
5.	Rexatrol	(Resveratrol) Antioxidant and anti-aging	Antioxidant and anti-aging

CHAPTER 2

LITERATURE REVIEW

Manach C et al., (2004) stated that polyphenols are micronutrients present in our diet, and proof their role in decreasing the degenerative disorders like cancer and cardiovascular diseases. Studies on the identification of circulate metabolites, cellular uptake and intracellular metabolism with possible de-conjugation, biological activities of the conjugated metabolites are specific accumulation in several target tissues were evaluated.

Maiti K et al., (2005) conducted a study on quercetin (flavonoid) having free radical scavenging action. The free radical scavenging action of quercetin Phyto phospholipid complex is equivalent with quercetin at a dose of 10mg to 20 mg/kg body weight with plain quercetin.

Naik SR et al., (2006) studied that Ginkgo biloba possess neurocognitive enhancing effects. The mechanism of action of Ginkgo was related with antioxidant activities. In the present study, Ginkgo biloba phytosomes were administered to Wistar rats at a dose of 50 mg/kg and 100 mg/kg for 7 and 14 days. It was concluded that estimation of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase by Ginkgo biloba phytosome treatment.

Maiti K, et al., (2007) developed a new curcumin formulation along with the phospholipids was developed to overcome the limitation of absorption and to explore the protective activity of curcumin phospholipid complex on carbon tetrachloride induced acute liver damage in rats.

Verma and Vinayak et al., (2008) found that aqueous extracts of Andrographis paniculata significantly increased the actions of anti-oxidative defense enzymes like catalase, superoxide dismutase and glutathione and lowered glutamate content

Kamle et al., (2008) carried out study on formulation and development of polyherbal formulations and evaluated it for hepatoprotective activity against acute liver toxicity model of CCL4 and paracetamol induced liver damage in rats. The results indicated that the formulations F1 and F2 are effective both as prophylactic and therapeutic in experimental liver damage. The formulation F-2 showed better results for biochemical parameters as compared to F-1 formulation whereas the morphological, phenobarbitone sleeping time and histopathological studies were same for both the formulations.

Dandagi PM et al., (2008) studied that hepatoprotective efficacy of different extracts of Ferula asafoetida, Momordica charantia and N. jatamansi against liver toxicity.

Bhattacharya S et al., (2009) stated that lipophilic and molecular size are the two main restricted factors for molecules to pass the biological membrane which is absorbed followed by oral or by topical route. Phytosomes are produced by a patented method where the standardized plant extract or its constituents are bound to phospholipids, mainly phosphatidylcholine, produce a lipid compatible molecular complex.

Girish et al., (2009) carried out a study to evaluate six polyherbal liquid formulations (PLF) commercially available on carbon tetrachloride induced liver injury. The serum biochemical parameters like alanine

transaminase, aspartate transaminase and alkaline phosphatase were estimated. The CCl4 induced changes were significantly reversed.

Guruswamy et al., (2010) studied that the ethanolic extracts of three medicinal herbs (Asteracantha longifolia, Cyperus rotundus and Bryophyllum pinnatum) were prepared and evaluated for hepatoprotective activity in carbon tetrachloride induced liver damage in rats.

Gandhi A et al., (2012) evaluated that phytosomes are newly established herbal formulations which are better absorbed and as a result gives better bioavailability and actions than the conventional molecules or plant extracts.

Tatiya AU et al., (2012) performed a study to evaluate hepatoprotective activity of polyherbal formulation (PHF) having dried aqueous extracts of Andrographis paniculata, Phyllanthus niruri and Phyllanthus Emblica was studied. The results showed that the hepatoprotective action of formulation were useful for healthy liver due to combined action of all plant extracts along with constituents

Gupta A et al; (2013) carried out a study with the aim to isolate and identify the quercetin from polyherbal hepatoprotective formulation. Polyherbal formulations were developed by using fractionated extracts of Butea monospermic, Bauhinia variegate for the treatment of liver disorders and estimated for hepatoprotective action. The polyherbal tablets were formulated by wet granulation method using microcrystalline cellulose, aerosil and other excipients.

Daleya AB. et al, (2014) studied the hepatoprotective actions of ethanolic leaf extract of Andrographis paniculata on thioacetamide-induced hepatotoxicity in rodents and it was found no acute toxicity was observed.

Allam A N et al., (2015) studied that the curcumin phytosomes were prepared by solvent evaporation method and formulated as free flowing powder and semisolid formulation to increase curcumin content in soft gels. Phytosomal powder was characterised in terms of drug content and zeta potential.

Vakillodin S et al., (2015) studied that the aim of the present study was to explore the hepatoprotective and antioxidant activity of Citrullus colocynthis fruit. The hepatoprotective profile of methanolic extract of Citrullus colocynthis (MECCF) fruit was investigated on rodents, which were made hepatotoxic using paracetamol.

Shukla et al., (2016) reported the significant choleretic actions of andrographolide in rodents. The findings suggested the multiple doses pretreatment against ethanol enhanced liver protective action.

Bui Thanh tung et al., (2017) developed a phytosomal curcumin complex and evaluated the hepatoprotective activity of the complex on paracetamol induced liver toxicity in mice. Results showed that the phytosome has stronger hepatoprotective effect as compared to plain curcumin extract.

Fatima T et al., (2017) carried out a study to evaluate the hepatoprotective action of polyherbal formulation (Andrographis paniculata, Boerhaavia difusa, Eclipta alba, Picrrorrhiza kurroa in wistar albino rodents using carbon tetrachloride induced hepatic damage experimental animals.

Bhumika et al; (2017) carried out a study to investigate the hepatoprotective action of aqueous methanolic extract of polyherbal formulation andindividual extracts of Calotropis procera, Gymnema sylvestre and Lawsonia inermis leaves.

Ali M et al; (2018) discussed several herbal medicines with hepatoprotective properties. Several animal models were used to study hepatotoxicity along with their mechanism of action

Ravi G S et al; (2018) carried out a study on amphiphilic drug lipid nano

complex of rutin egg phosphatidylcholine (EPC) to enhance its bioavailability

and investigated the impact of the complex on hepatoprotetion and antioxidant

action.

Anitha V et al., (2019) focused on method of preparation, properties, advantages characterisation and applications of phytosomes as drug carrier system.

Cheemalapati, VN et al; (2019) focused on review of medicinal herbs and compounds separated from them with antiulcer and hepatoprotective activity such as Andrographis paniculata, Eclipta alba, Picrorrhiza kurroa and Silybum marianum.

Fristiohady A et al., (2020) investigated that the phytocomponents of Wualae fruit extract and its cell-defensive action on CCl4-induced hepatotoxicity. The phytochemical screening was done for the assessment of alkaloids, flavonoids, saponins, tannins and terpenoids. The extract was performed on Wistar male rats to assess the lipid profile test on Wualae fruit extract at a dose of 200, 300, and 400 mg/kg body weight for seven days and induced with CCl4 the next day. The findings revealed that extract consists of alkaloids, flavonoids, tannins, and terpenoids and it was found that values of various serum biochemical parameters were decreased.

Sudhir K et al., (2020) studied that Phytophospholipid complex method is used as a feature which is important in all-purpose addition of plant extracts. The method has effectively determined the irrational uncertainty of plant-based drugs.

Kamra P et al., (2021) evaluated the hepatoprotective activity of methanolic whole plant extract of Persicaria hydropiper on CCL4 induced hepatotoxicity model. In the study, the hepatotoxicity was induced by intra-peritoneal injection of carbon tetrachloride in rats for seven days. After that the extract was given at two different doses of 200 mg/kg and 400 mg/kg body weight for seven days. Reference standard used was silymarin. The results indicated that the dose dependent hepatoprotective action of plant extract.

Sudhir K et al., (2020) studied that Phyto phospholipid complex method is used as a feature which is important in all-purpose addition of plant extracts. The method has effectively determined the irrational un Aims and objectives certainty of plant-based drugs.

CHAPTER 3

AIMS

A Detailed Study of Phytosomal Preparation, Characterization and Hepatoprotective Activity on Herbal Plants Andrographis Paniculata and Rhapnus Sativus.

OBJECTIVES

The main problem of Andrographis paniculata and other plant extracts and phytochemicals taken for investigation of hepatoprotective activity is less clearance rate and low elimination half-life, when give as such in oral solid or liquid dosage forms (Aurvedical or natural products).

So, the primary objective was to solved the issue by conversion of Andrographis paniculata into phytosomes with other extracts and phytoconstituent combinations, i.e. Raphanus sativus, by taking their bio markers from market along with adding another dimension, that of making novel synergistic combinations, as their combinations have not been earlier so formulated, and the possibility that the formulation would possess good synergistic effect.

The studies include:

1. Preformulation characterisation.
2. Design & development of phytosomal formulation with different markers and phytochemical composition.
3. In-vitro characterization of prepared phytosomal formulation
4. Efficacy comparisons of prepared phytosomal formulation with traditional formulation.
5. Selection of optimized formulation for investigation of hepatoprotective action.
6. Evaluation of hepatoprotective activity by appropriate comparative estimation of hepatic biochemical biomarkers, antioxidant potential.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

Materials sourced from Hi-Media laboratories Pvt. Ltd., and Loba chemicals, including Soya-lecithin, CCl_4, bismuth carbonate, $CaCl_2$, copper sulphate, $FeCl_3$, Hcl, I_2, ninhydrin, nitric acid, phloroglucinol, KI, Sodium potassium tartarate, ruthenium red, safranin dye, sodium acetate, NaI, NaOH, nitroprusside sodium, sudan red II, sulphuric acid, tannic acid and vanillin sulphuric acid were used for extraction, phytochemical investigation and formulation. The solvents and equipments used for extraction, phytochemical investigation and formulations are listed in table no. 4.1 and 4.2.

Table 4.1: Chemicals list.

S. No	Chemicals used	Grade	Manufacturers
1.	Dichloromethane	LR	Loba chemie pvt. Ltd Mumbai
2.	Alpha napthol	LR	Loba chemie pvt. Ltd Mumbai
3.	H_2SO_4acid	LR	Loba chemie pvt. Ltd Mumbai
4.	Phloroglucinol	AR	Evonik Lab, Mumbai
5.	Conc. Hcl	LR	Loba chemie pvt Ltd
6.	Dil. Hcl	LR	Loba chemie pvt Ltd
7.	Picric acid	LR	Loba chemie pvt Ltd
8.	Dragendorff's reagent	LR	Loba chemie pvt Ltd
9.	Hager's reagent	LR.	Loba chemie pvt. Ltd
10.	NaOH	LR.	Loba chemie pvt. Ltd
11.	Methanol	LR.	Loba chemie Pvt Ltd
12.	Hexane	LR	Loba chemie Pvt Ltd

Table 4.2: List of equipment's.

S. No	Equipments used	Manufacturers
1.	Electronic balance	Citizen, Mumbai India
2.	Digital PH meter	Mahavir Scientific chemicals and instruments, Chandigarh, India.
3.	Magnetic stirrer	Scientific chemicals and instruments Chandigarh.
4.	UV visible laboratories Pvt. Ltd.	Systronics, Mumbai India.
5.	Hot air oven	Mahavir scientific, Chandigarh.
6.	Melting point apparatus	Perfit, India.
7.	Rotary evaporator	Perfit, India
8.	Orbital shaking incubator	Remi India
9.	Dissolution apparatus	Lab India,
10.	Differential scanning colorimeter	Mattler Toledo
11.	Zeta analyzer	Malvern instrument ltd

4.2 Methods

The phytoconstituent markers of Andrographis paniculata and Rhapanus sativus were taken from Simpsons pharma Mumbai.

4.2.3.1 Preliminary photochemical screening

The phytochemical tests were done for screening of phytoconstituent marker for phytochemical constituents. The phytochemical screening of Andrographis paniculata, and Rhapanus sativus for the estimation of carbohydrates, proteins, amino acids, steroids, glycosides, anthracene glycosides, saponin glycosides, flavonoids, alkaloids and tannins were carried out. The hydroalcoholic extract was analysed to carry out the phytochemical screening

A) Alkaloids test: A small portion of dissolvable ether, chloroform ethanol and water extracts with drops of dilute hydrochloric acid and filtered. The filtrates were tried with different alkaloidal reagents as shown below:

i) Mayer's reagent: To the filtrate add few drops of mayer's reagent cream color precipitate were observed.

ii) Hager's test: To 2 ml of the filtrate when 2 drops of the picric acid were added from the test tube, it gives yellow color.

iii) Wagner's test: It is used for the estimation of alkaloids in natural products. Mostly alkaloids contain of potassium mercuric iodide solution to give red colored precipitate.

B) Anthracene glycosides

i) Borntrager's test: The test solution when heated on a water bath and filtered, add 1 ml of organic solvent (CCL4) and equal amount of alkali i.e. NaOH was added to the filtrate and shaken for 10 minutes. The appearance of pink to red color indicates the presence of anthraquinone moiety.

C) Glycosides test

i) Keller Killiani test: To the 1ml of glacial acetic acid contains containing small portion of Ferric chloride and 1 ml of concentrated sulphuric acid was included to the concentrate and appearance of shading showed the presence of glycosides.

D) Saponins test

Foam test: 1 ml of the test solution of extract was diluted with distilled water with 10mland stirred in a graduated cylinder for15 minutes. The development of foam shows the presence of saponins.

E) Flavonoids test

Shinoda test: Few pieces of magnesium ribbon and concentrated hydrochloric acid were added to the methanolic extract. Appearance of pink to red color indicates the flavonoids presence.

F) Tannins test Ferric chloride test: To the powdered sample, add 2 drops of 5% of ferric chloride solution. Blue or green color gives the indication of tannins.

G) Carbohydrates test Molisch Test: It is used to give test for carbohydrates. In this test, conc. sulphuric acid converts into furfural which reacts with alpha naphthol solution to give purple coloured compound.

Fehling's test: It is used to identify the reducing sugars. Fehling A & Fehling B gives deep blue color.

H) Amino acids & proteins test Ninhydrin test: The ninhydrin test is useful for the estimation of amino acids. It gives blue color to the samples containing amino acids.

Biuret test: The Biuret test is used for the estimation of proteins it gives purple color. **Millon's test:** It is useful for the estimation of soluble proteins. It gives reddish brown color to the compound.

Xanthoproteic acid test: It is useful for the estimation of proteins. It gives yellow color to the compounds.

4.2.4 Preparation of phytosomes

The phytosomes were prepared by reacting with natural phospholipid (soy PC) with the phytoconstituents biomarkers Rhapanus sativus and Andrographis paniculata in a 1:1:1, 1:2:1, 2:1:1, 1:1:2 with a molar ratio in a reflux condenser. The reaction was completed by refluxing with 20 ml of dichloromethane in a 100 ml round bottom flask for 2 hrs at a temperature not more than 40°C. The resulting clear solution was evaporated, and 10 ml of n-hexane was added to it with continuous mixing. The precipitate was sieve and dried under vacuum to eliminate traces of solvents, which causes in formation of a thin film. The thin film was separated and stored in an amber-colored glass bottle had been flushed with nitrogen and kept at a room temperature. The dried precipitate crushed in a pestle mortar and then sieved through 100 meshes.

4.2.5 Characterisation of phytosomes

4.2.5.1 Fourier transform infrared spectroscopy

FTIR studies were done in AKUMS PVT LTD. HARIDWAR.FTIR spectra of pure drug andrographolide and formulated phytosomes were obtained by predictable potassium bromide pellet/disc method by FTIR. The scanning range was 400 4000 cm−1.

4.2.5.2 Determination of size of particles

The particle size of phytosomes were done in AKUMS PVT LTD. HARIDWAR using Beckmann Coulter particle size analyser. Photon Correlation Dispersion (NANO ZS Malvern equipment) has been used to estimate the size of the particles of the phytosomes, and zeta potential was measured via surface charge in an electric field.

4.2.5.3 Percentage yield

The prepared phytosomes were dried properly and weighed accurately. The weight was divided by calculation of total weight of drugs with non-volatile recipients. % Yield = Weight of phytosomes formed / Weight of drugs and nonvolatile solvent multiplied by 100.

4.2.5.4 Drug entrapment efficiency

100 mg of formulated phytosomes were dried out accurately and isolated in 50 ml distill water. The content was stimulated for 2 hour and cleaned via what man filter paper of aperture range about 45 micrometres. It was suitably thinned and analysed spectrophotometrically at λ max of 266 nm. The quantity of drug entrapped was intended from standard calibration curve

Entrapment efficiency= Amount of drug in phytosomal formulation/ Theoretical amount of drug in phytosomal preparation X 100

4.2.5.5 Differential scanning calorimetry

DSC studies were done from AKUMS PVT LTD. HARIDWAR Differential scanning calorimeter (Mettler-Toledo; STAR SW9.20, USA) has been used to generate heat curves of phytosomes and physiological mixture of phosphotidylcholine extracts. Over a maximum temperature of 25–200°C, each sample was examined at one rate of 10°C/min. The coolant flow rate was fixed steady at 5 millilitre per minute.

4.2.5.6 Scanning electron microscopy

SEM studies of prepared formulations were done in AKUMS PVT LTD. HARIDWAR Scanning electron microscope (SEM)-quanta 200 has been used to assess morphology characteristics. The phytosomes were spread on a round aluminium tube precoated using silvery adhesive and put in the device's monitoring area. It was then inspected under the SEM at different magnification, and micrographs were produced. SEM has been used to analyze the material at low vacuum. The pressure was kept at a constant of 5.99–6.02 torr. It's a secondary electron detector which has been utilized

4.2.5.7 Transmission electron microscopy

TEM studies of prepared formulations were done from AKUMS PVT LTD. HARIDWAR morphological features were done using transmission electron microscopy (TEM-100S Microscope; JOEL Ltd., Tokyo, Japan). The samples were diluted with methanol in ratio of 1:10 and sonicated for 10 min. A drop of phytosome was fixed onto a carbon glazed grid, left to form a thin film and the phytosome images were seen with the help of TEM.

4.2.5.8 In-vitro drug release

In vitro release characteristics of phytosomal suspension was studied using dissolution test apparatus. 5 ml of phytosomal capsule was taken placed in USP basket type II suspended in 900 ml of phosphate buffer at pH 7.4 as dissolution test medium maintained at temperature of 37±0.5ºC with 100 rpm. 5ml of dissolution sample was withdrawn at several time intervals for the period of 24 hours to analyze the amount of phytoconstituent released at 223 nm in UV spectrophotometer.

4.3 Statistical analysis

The statistical analysis was carried out using one-way analysis of variance (ANOVA).

The results are presented as mean ± standard deviation. A statistically significant difference was considered at $P < 0.05$.

CHAPTER 5

RESULTS & DISCUSSION

5.1 Phytochemical screening

Table 5.1: Preliminary Phytochemical Screening (Chemical Tests).

S.NO	Chemical Test	Andrographis paniculata	Raphanus sativus
1.	Dragendorff's reagent	+ve	+ve
2.	Carbohydrates	+ve	+ve
3.	Molisch reagent	+ve	+ve
4.	Glycosides	+ve	+ve
5.	Saponins	+ve	+ve
6.	Tannins	+ve	¯ve
7.	Proteins and amino acids	+ve	+ve
8.	Flavonoids	+ve	¯ve

+ Present, - Absent

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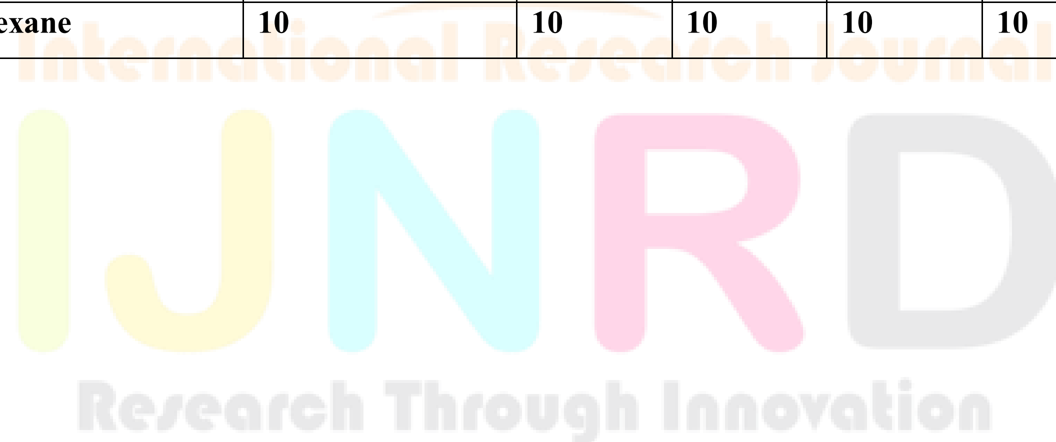
Table 5.2: Phytosomal formulations.

S.NO	API/EXCIPIENTS	FORMULATION CODE				
		F1(QTY)	F2(QTY)	F3(QTY)	F4(QTY)	F5(QTY)
1.	Andrographis paniculata (BIO MARKER)	5 ML	7 ML	9 ML	11 ML	13 ML
2.	Rhapanus sativus (BIO MARKER)	5 ML	7 ML	9 ML	11 ML	13 ML
3.	Dichloromethane	10	9	8	7	6
4.	Alpha napthol	12	11	10	9	8
5.	H2SO4acid	8	7	7	7	7
6.	Phloroglucinol	5	4	4	4	4
7.	Conc. Hcl	8	8	8	8	8
8.	Dil. Hcl	10	10	9	9	7
9.	Picric acid	7	7	6	6	4
10.	Dragendorff's reagent	2	2	2	2	2
11.	Hager's reagent	2	2	2	2	2
12.	NaOH	6	6	6	6	6
13.	Methanol	10	10	10	10	10
14.	Hexane	10	10	10	10	10



5.2 Standard plot of andrographolide

The graph indicates the standard plot of absorbance vs conc. The equation Y= 0.058x-0.024 and R2 = 0.996 shows linear relation between conc vs absorbance.

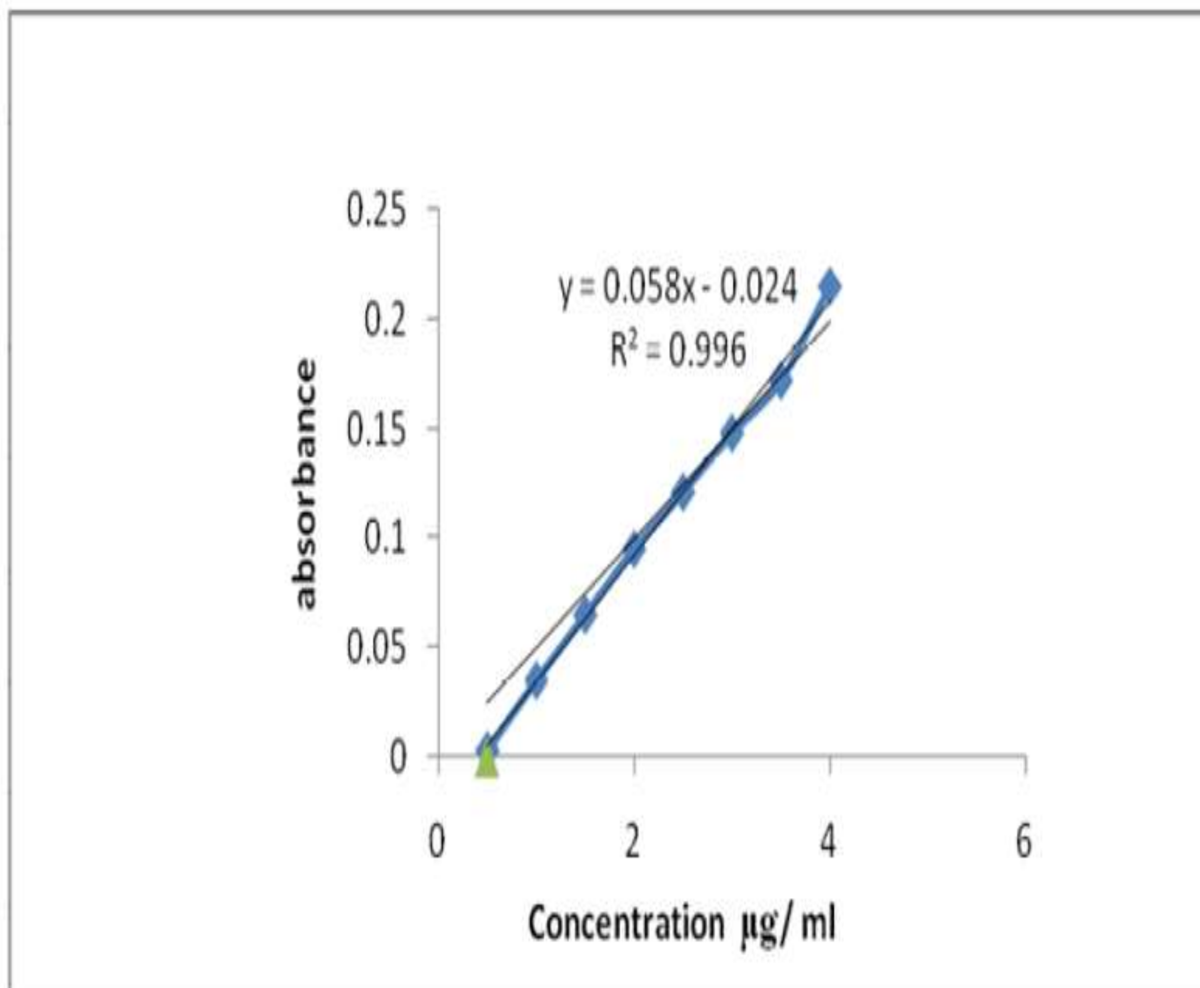


Figure 5.1: Conc. vs absorbance of andrographolide.

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5.3 Standard plot of Quercetin

The graph indicates the standard plot of absorbance vs conc. Equation Y= 0.065 x - 0.041 and R2 = 0.983 shows linear relation between concentration vs absorbance.

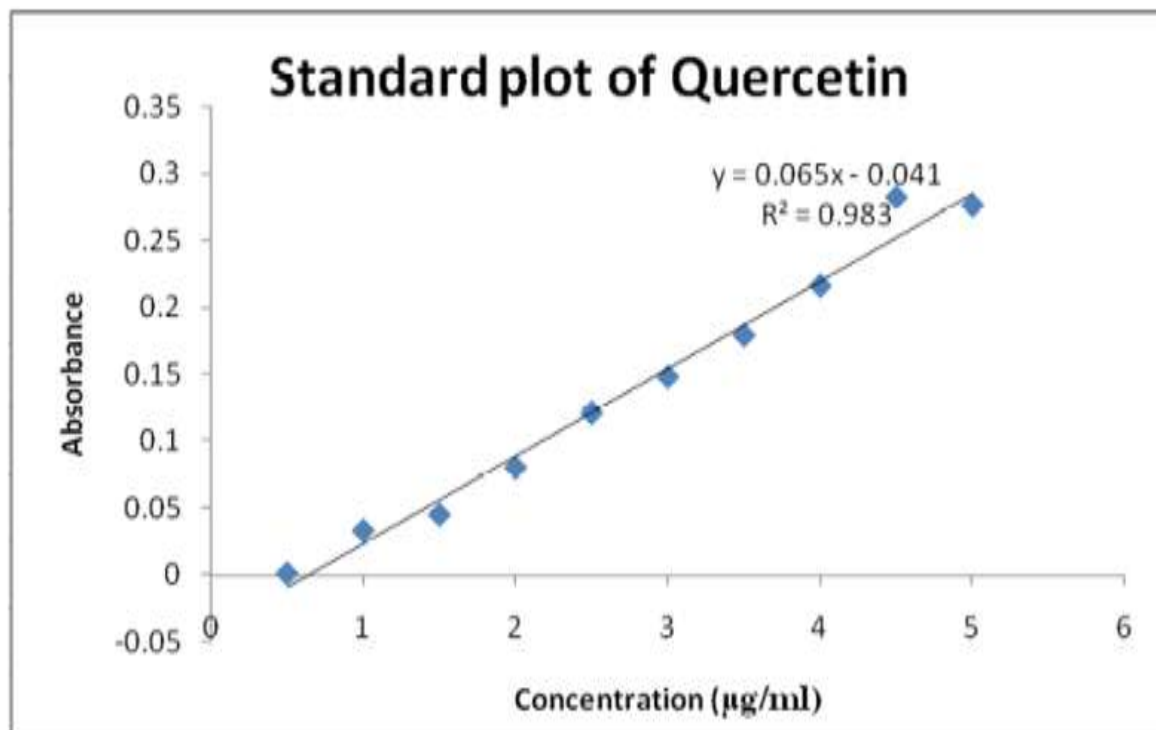

Figure 5.2: Conc vs absorbance of Quercetin.

5.4 Optical microscopy

Formulated Phyto phospholipid complexes were viewed under the microscope, a drop of suspension formed was taken on a glass slide and excess of suspension was drained off with filter paper and was allowed to dry. The sample formed was then viewed under optical microscope to visualize the vesicle formation.

5.5 Particle size and size distribution

Particle size and size distribution of formed Phytophospholipid complex was assessed using photon correlated spectroscopy with dynamic light scattering on Zeta-sizer. For determination of size the samples were dispersed in isopropyl alcohol by stirring on magnetic stirrer for the period of 10 minutes. The dispersion so obtained was analysed using Zeta sizer.

The particle size of formulation of F1-is 153.2 nm, F2= 158.5 nm, F3= 611.14 + 08 nm, F4= 631.24 nm and F5= 620.34 nm. Polydispersity index of formulation F1= 0.484, F2= 0.216, F3= 0.343, F4=631.24 nm and F5=0.443.

5.6 Percentage yield

Percentage yield of Phyto-phospholipid complex as give in Equation 1 was estimated by taking into consideration the initial amount of powder extract and phospholipid taken and amount of phyto-phospholipid complex formed finally.

Percentage yield = Weight of phytosomes formed X 100 Weight of phytoconstituent and phospholipids

Table 5.3: Percentage yield of prepared formulations.

S.NO	FORMULATION CODE	% YIELD
1.	F1	80.4
2.	F2	80.5
3.	F3	77.3
4.	F4	81.8
5.	F5	89.5

5.7 Entrapment efficiency

Entrapment efficiency was estimated as per the following equation to calculate the amount phytoconstituent entrapped within the vesicular structure. For this Phyto phospholipid complex equivalent to 100mg of andrographolide was weighed and dispersed in water by stirring at room temperature for 2 hours followed by centrifugation for 20 minutes at 1000 rpm. The content was then filtered using what man filter paper and analysed by measuring absorbance 321 nm using double beam UV Spectrophotometer.

Entrapment efficiency = Amount of phytoconstituent entrapped Actual amount of phytoconstituent X 100

Table 5.4: Entrapment Effeciency of prepared formulations.

S.NO	FORMULATION CODE	% EE
1.	F1	81.6
2.	F2	84.5
3.	F3	87.3
4.	F4	89.8
5.	F5	92.5

5.8 Scanning electron microscopy (SEM)

Morphological characteristics of formed phytosomal complexes were observed by taking SEM images. The sample to be studied was spread on aluminium stub pre coated with silver glue and viewed under SEM at varying magnifications to record micrograph. The phospholipids complex was made up of phospholipids and drugs and appeared column shape.

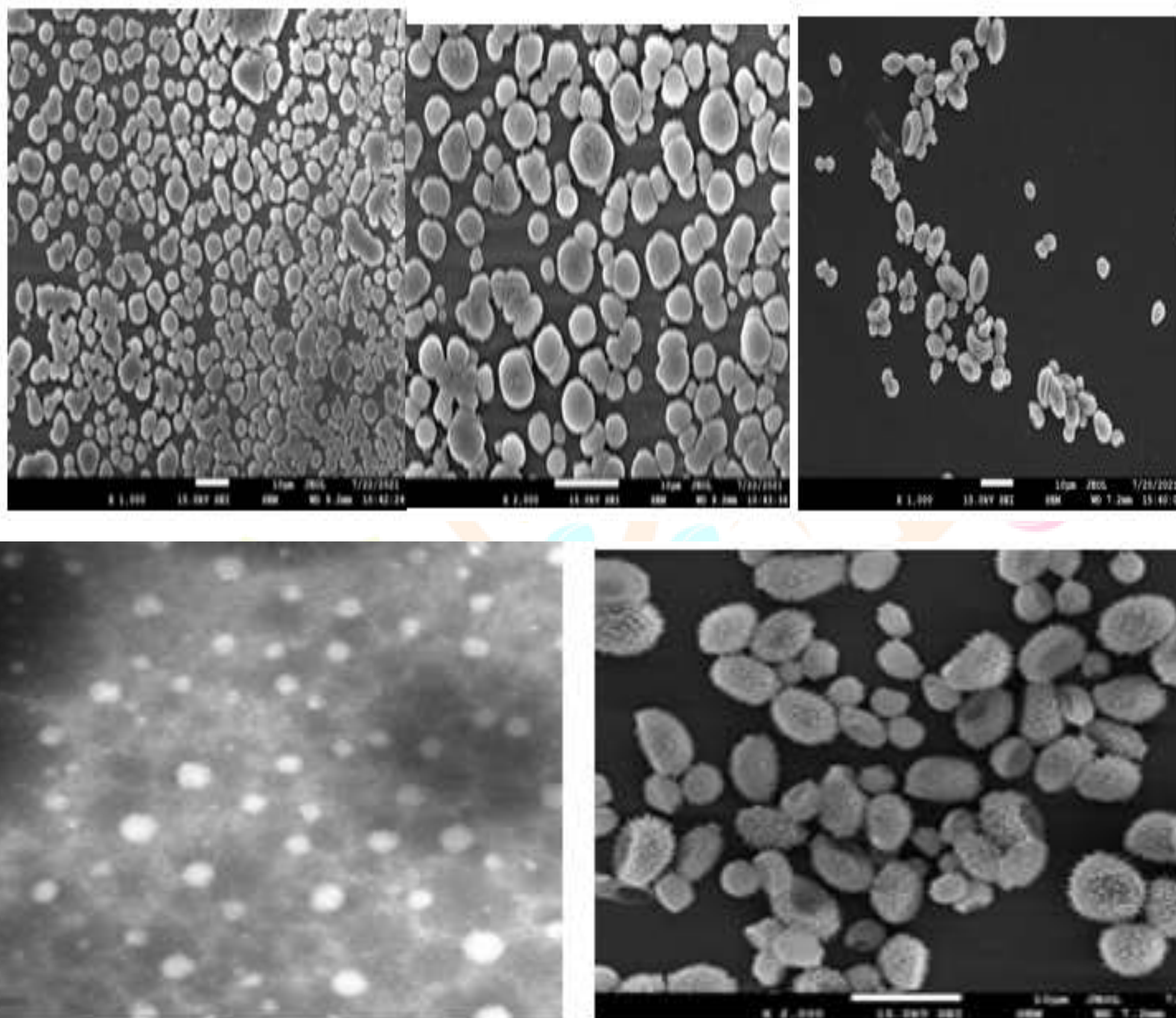

Figure 5.3: SEM images of prepared Phytosomal formulations(F1-F5).

5.9 Transmission electron microscope:

The TEM analysis revealed phytosomes were sphere-shaped and smooth particles. The internal diameter of prepared phytosomes was 2.17 nm, which corresponds with size attained to SEM images. The particle size distributions of the phytosomes were of uniform size indicates the non-appearance of aggregation of phospholipids.

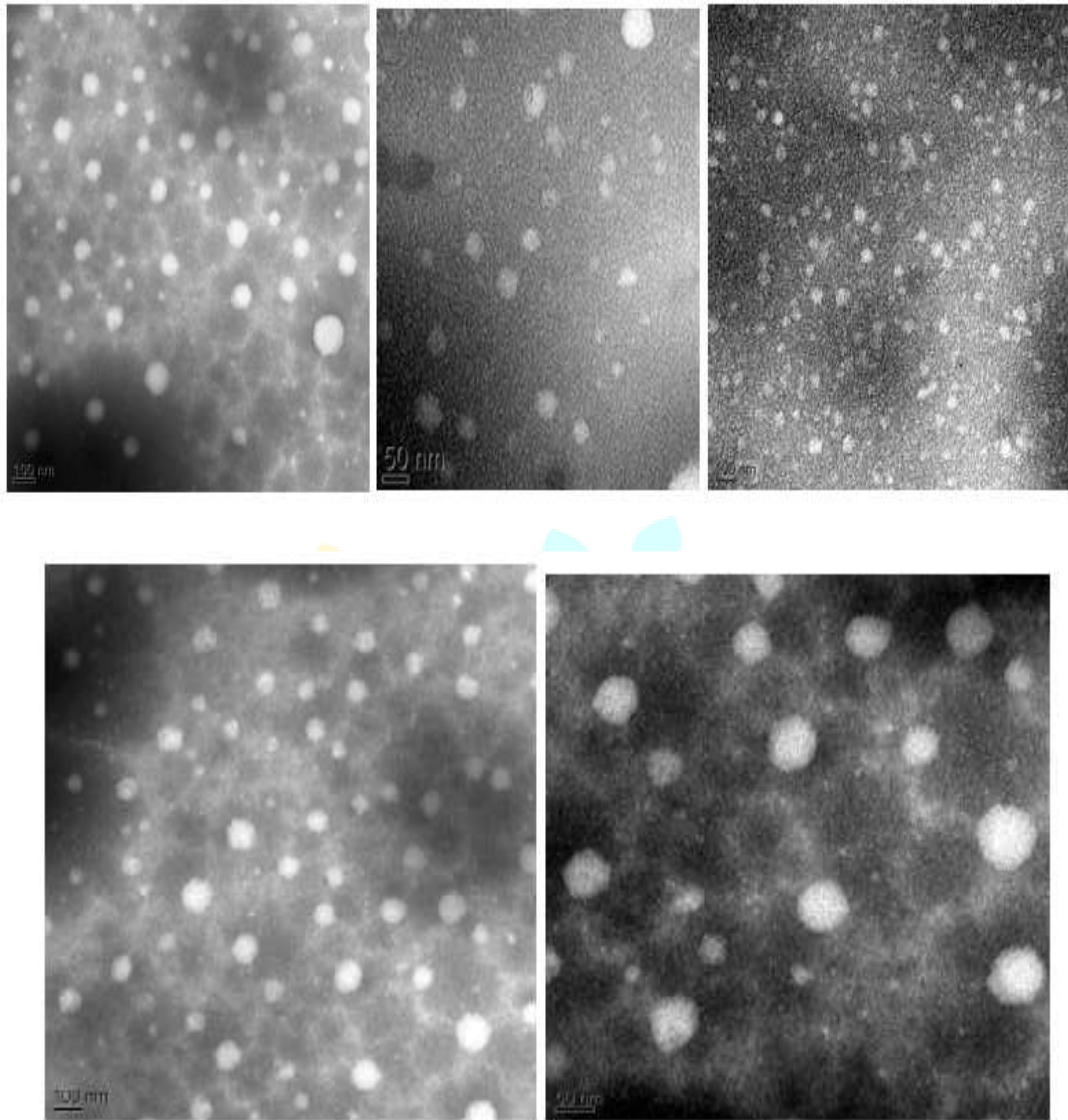

Figure 5.4: TEM images of prepared Phytosomal formulations(F1-F5).

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5.10 FTIR Studies

FTIR studies of (Andrographis paniculata, Raphanus sativus), phosphatidylcholine and prepared phytosome shown below. It explored much information about formulations stability and drug availability patterns. The medium infrared range consists of 4000–400 cm−1.

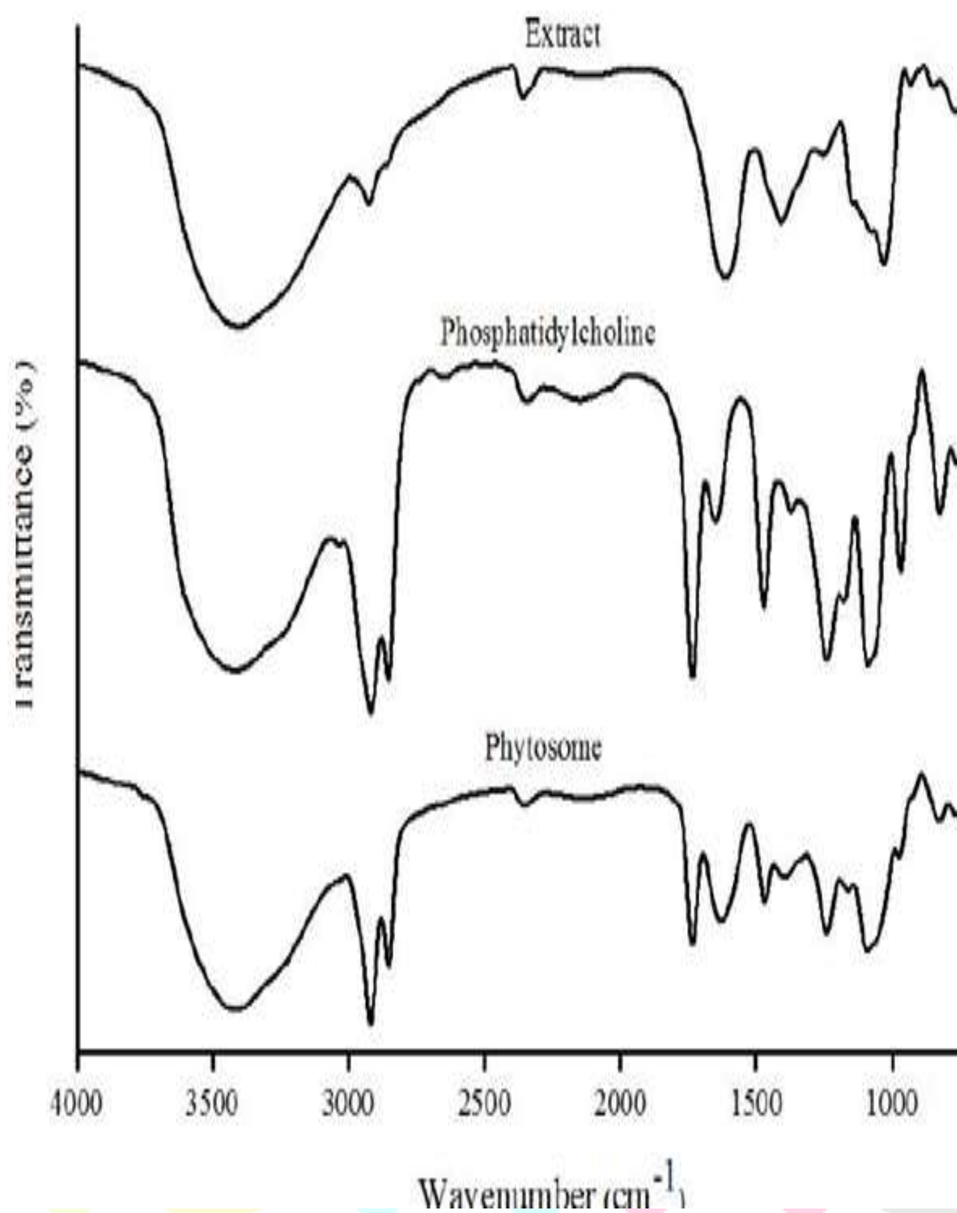

Figure 5.5: FTIR images of prepared Phytosomal formulations.

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Table 5.5: Functional group peaks by FTIR.

S.No.	Functional Group	Frequency (cm^{-1})	Interpretation
1.	O-H	3400-3500	Presence of medium intensity hydroxyl group (extract) and phytosomes
2.	H-O	2928-2900	Presence of very low intensity carboxyl O-H (extract), and sharp in phytosomes
3.	-C=O	1700-1710	Presence of ketone group of medium intensity in (extract) but low in

5.11 DSC thermograms

DSC thermograms of andrographolide were done to investigate the drug nature whether it is crystalline or amorphous form for a prepared formulation and find out the possibilities of interactions with additional ingredients.

The DSC thermograms of semi-purified of combination of all extracts, SPC and optimized phytosomal preparations. DSC scan of large amount of soya phosphatidylcholine explored two broad endothermic peaks which gives the important features of amorphous substances at melting point of 226.77 °C, and 213.51 °C showed the lipid movements in hot condition changes powder to crystalline state.

The prepared phytosomal formulation system consisted of SPC lipid, while semi-purified extracts was present in the lipid bi-layer. In the DSC, thermogram a sharp peak of a maximum at 226°C appeared in the semi-purified 213.51°C but no other endothermic or exothermic peaks were observed. In formulation, a depression of the melting point of plant extracts containing and SPC from 226.89 °C and three new peaks with maxima at 213°C respectively appeared in phytosomal formulation.

At the same time, the melting enthalpy values of all extracts showed drastic depression from -65.36 J/g and -57.70 J/g to -4.00 to 18.52 j/g, respectively. The overall interactions of SPC and semi-purified extracts and phytosomal formulation were concluded by elimination of endothermic peak (s), appearance of new peak (s), change of peak shapes and its onset, peak temperature/melting point and relative peak area or melting and enthalpy are shown below:

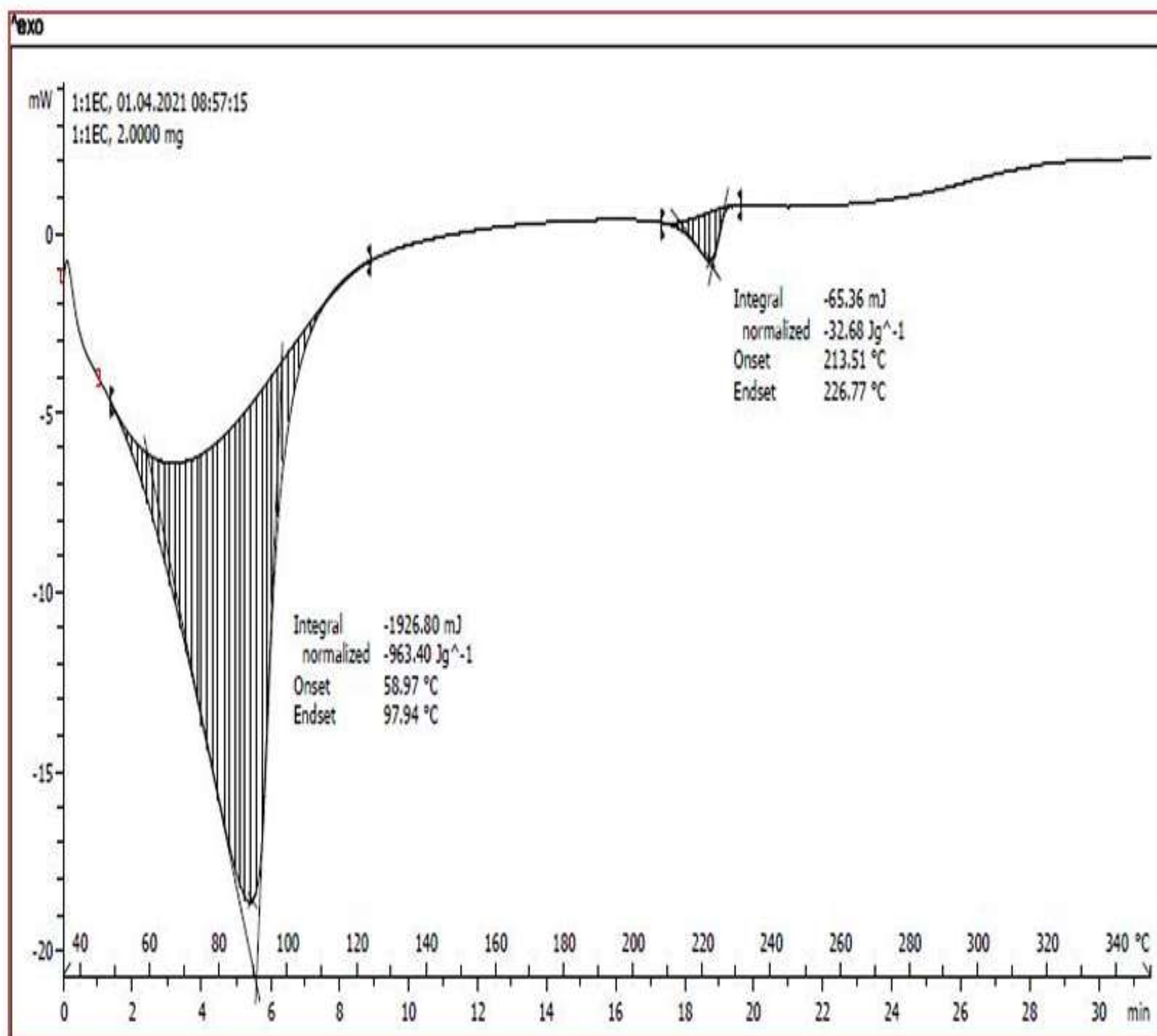

Figure 5.6: DSC images of prepared Phytosomal formulation.

5.11 In-vitro drug release

In vitro release characteristics of phytosomal suspension was studied using dissolution test apparatus. 5 ml of phytosomal capsule was taken placed in USP basket type II suspended in 900 ml of phosphate buffer at pH 7.4 as dissolution test medium maintained at temperature of 37±0.5ºC with 100 rpm. 5ml of dissolution sample was withdrawn at several time intervals for the period of 24 hours to analyze the amount of phytoconstituents released at a 223 nm in UV spectrophotometer. The formulations F5 possessed higher in vitro release studies i.e.…92.5% when compared with other formulations, F1, F2, F3 and F4 The percentage cumulative release graph is mentioned.

Table 5.6: Formulations F5 showing % DR & % CDR.

S.NO	TIME	ABSORBANCE	CONCENTERATION	% DRUG RELEASE	%CUMULATIVE DRUG RELEASE
1.	8	0.002	0.15	0.015	0.015
2.	16	0.004	0.24	2.98	2.995
3.	24	0.005	0.42	8.56	11.55
4.	32	0.007	0.61	11.98	23.53
5.	40	0.008	0.83	15.04	38.57
6.	48	0.01	1.78	17.12	55.69
7.	56	0.04	3.76	18.15	73.84
8.	64	0.05	4.25	19.71	93.55

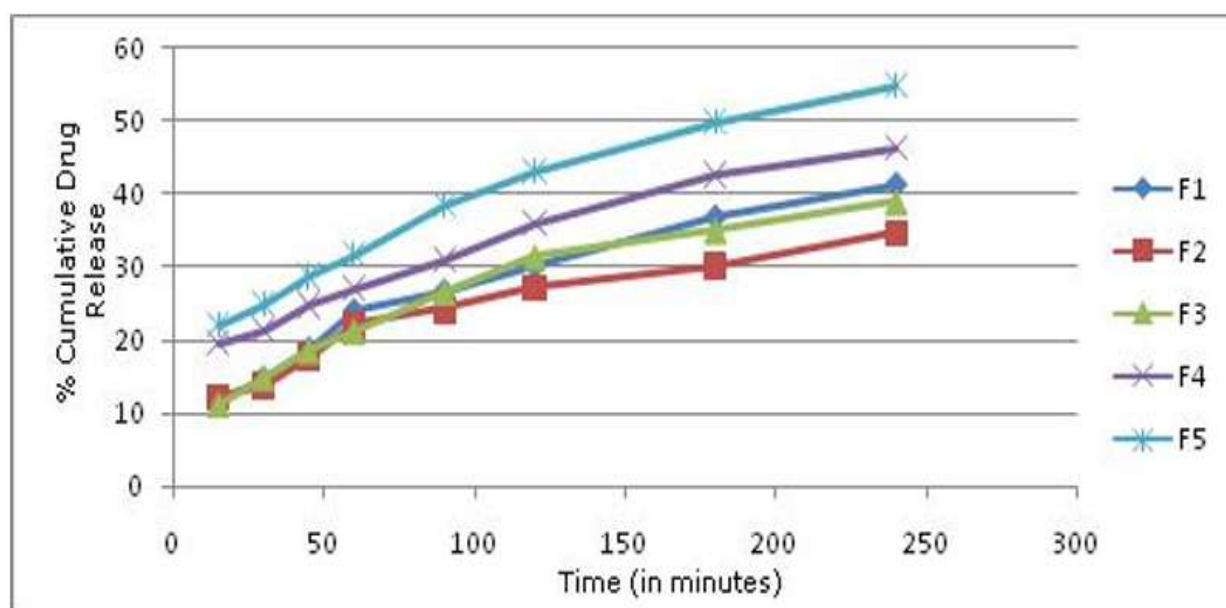

Figure 5.7 %: Cumulative drug release along with time for all formulations.

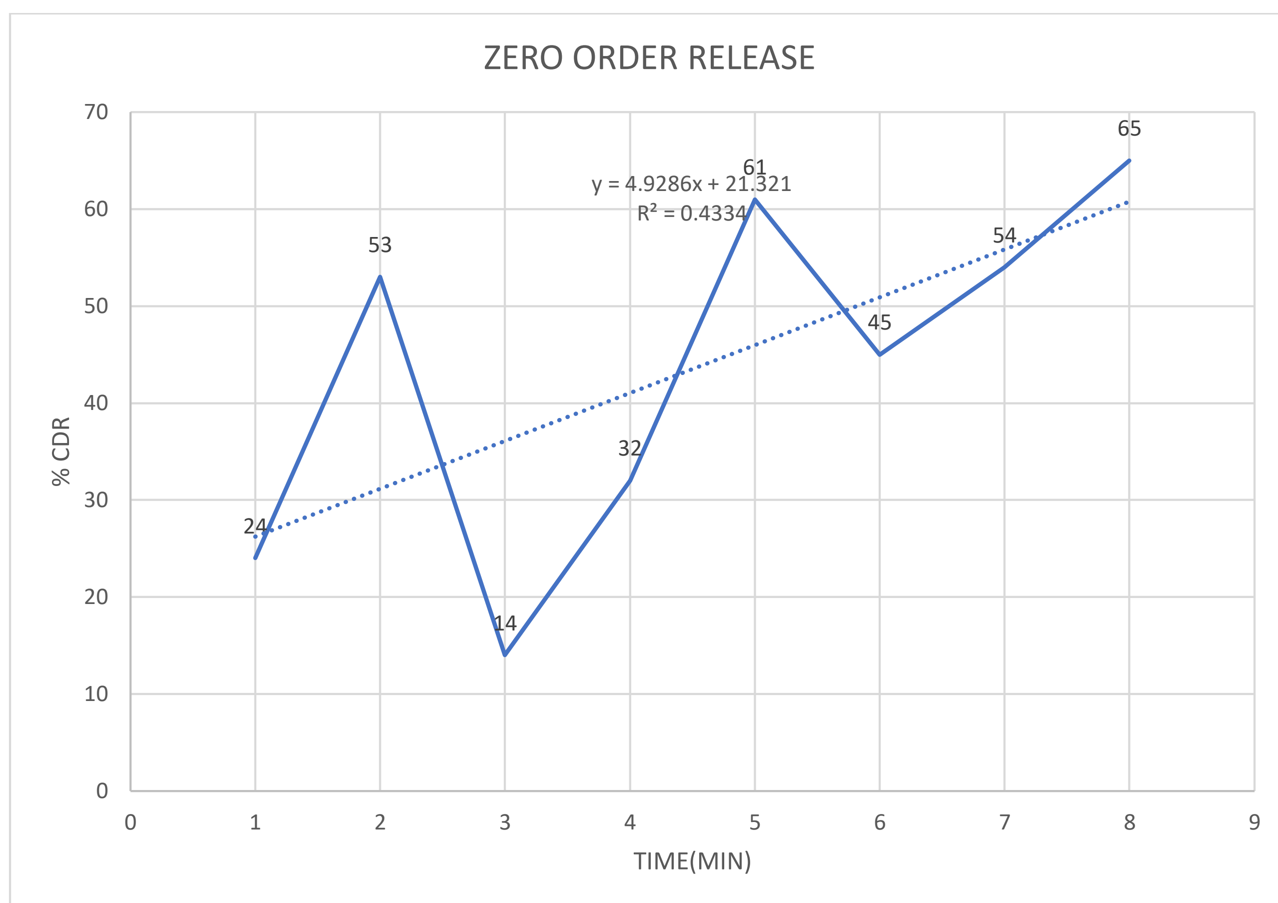

Figure 5.8: Standard curve of F5 showing zero order release.

CHAPTER 6

6.1 SUMMARY & CONCLUSIONS

Andrographis paniculata (AP) is a plant used in traditional medicines worldwide for its medicinal properties, and for liver disorders. Despite numerous studies on its efficacy, the precise mode of action responsible for its hepatoprotective activity.

Extensive research has been conducted on AP to explore its phytochemical constituents and antioxidant mechanisms, and its potential in treating liver disorders such as hepatitis, cirrhosis, and jaundice has been highlighted.

This plant contains various diterpenoids (neo andrographolide, andrographolide (AGL), and dehydroandrographolide), phenolic compounds (caffeic acid, chlorogenic acid, and protocatechuic acid), xanthones (1,8-dihydroxy-3,7-dimethoxyxanthone1,8-dihydroxy-3,7-dimethoxyxanthone) and flavonoids (apigenin, luteolin, and 7-O-methylwogonin), but their composition and concentration may vary based on geographical, season, plant parts, and phenological growth stage

Andrographolide, a bitter, colourless ent-labdane diterpene lactone is the main active compound in AP, present in all plant parts, but highest in concentration in the leaves). Its pharmacological properties include anti-allergic, antibacterial, anticancer, antidiabetic, anti-dyslipidemic, anti-inflammatory, anti-leishmanial, antiviral, antipyretic, analgesic, hepatoprotective,

All the test perfomed shows positive result and prove its action in favour of hepatoprotective activity.

The phenolic compounds of Rhamnus sativus, quercetin and catechins, decreased ROS levels, alanine transaminase (ALT) and aspartate aminotransferase (AST) levels in APAP-induced HepG2 cells, thus the hepatoprotective effect of the Brassicaceae's functional ingredients was confirmed and phytochemical screening test also confirmd.

The various five formulations were prepared, out of which F5 shows best result like in-vitro drug release of 93.55%, DSC data of prepared phytosomal prepared formulations, FTIR Studies, TEM images (with diameter of 2.17 nm), SEM images, Entrapment efficiency F5(92.5%), percentage yield (89.5%), particle size of formulation of F5= 630.34 nm. Polydispersity index of formulation of F5=0.443.

CHAPTER 7

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