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PREPARATION AND CHARACTERIZATION OF PHYTOSOME OF NORBIXIN

Adarsh K. Mund, Abhinasa C. Guru, Rudra Pratap Singh*, Kishore K. Pradhani, Birendra Pangi and Manoj K. Pasayat

Department of Pharmaceutics Jeypore College of Pharmacy, Rondapalli - 764002, Odisha, India.

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***Corresponding Author**
Rudra Pratap Singh
Department of Pharmaceutics Jeypore College of Pharmacy, Rondapalli - 764002, Odisha, India.

ABSTRACT

Bixa orellana L. is reported to contain of several different classes of phyto-constituents including carotenoids, apo-carotenoids, sterols, etc. from all parts of this plant. Two types of compound of bixin (oil soluble) and norbixin, exhibit a wide range of pharmacological activities that can decrease the growth of bacteria, fungus and also proven antioxidant, anti-inflammatory acitivities. Norbixin is an alkaline water-soluble carotenoid originating from the hydrolytic removal of a methyl ester group of bixin. The key obstacle of norbixin, has low bioavailability, less soluble in water and it is rapidly eliminated from the body. The main objective of this study was to prepare the phytosome of Norbixin and evaluate it. The different molar ratio of 1:1, 1:2, 2:1 and 2:2 of phytosome of Norbixin containing norbixin and soya lecithin were prepared by the anti-solvent precipitation technique. The prepared phytosome was characterized for DSC, FT-IR, % EE, Particle size and zeta potential and SEM and evaluated by in vitro drug release study. FT-IR and DSC data showed the compatibility between norbixin and soya lecithin and also confirmed the no interaction between them. SEM study confirmed the crystalline structure, irregular shapes and smooth surface of optimized formulation. Thus, the prepared phytosome is, a carrier combining drug delivery system of soya lecithin and norbixin, is a more favorable option for topical application of norbixin.

KEYWORDS: Norbixin, Soya lecithin, Phytosome, *In vitro* drug release.

1. INTRODUCTION

Preparation of plants or their parts have been widely used in medicine since ancient times and till today use of phyto-medicines is widespread. Most of the biologically active constituents of plants are polar or water-soluble. However, water-soluble phyto-constituents like flavonoids, tannins, glycosidal aglycones etc. are poorly absorbed either due to their large molecular size, which cannot be absorbed by passive diffusion or due to their poor lipid solubility, thus severely limiting their ability to transport across lipid-rich biological membranes, resulting in their poor bioavailability. Bixa orellana L. commonly known as annatto belongs to the family Bixaceae. It is 3–6 m high bush native to Central and South America and is one of the oldest known natural dye yielding plants. Annatto extract, a natural carotenoid, has been employed in the food industry as an important colorant, mainly in dairy products such as cheese and butter.[1-2] It has been considered safe for human consumption, since it has been used for centuries in many parts of the world for the prevention and treatment of a number of heath disorders such as constipation, fevers, heartburn, asthma, scabies, ulcers, diarrhea, stomach upset, skin diseases, measles, anecdotal treatment of diabetes, allergy, leprosy, infectious diseases, burns, measles, gonorrhea, diarrhea, asthma, angina, tumors, skin problems, and urinary infections (oral and topic).[3-4] The pulp from seeds of this plant has long been used topically by indigenous people to enhance the beauty of lips which has led to the origin of B. orellana's nick name as lipstick tree.[5] Annatto has enormous number of applications in coloring and bleaching of dairy food products especially bakery products, cream deserts, butter milk deserts, rice flour, and corn starch.[6–8]

Two types of compound Annatto extract, bixin (oil soluble) and norbixin (alkaline water soluble; hydrolyzed derivative of bixin), are available, and the latter is predominantly used as a food color in Japan.[9] Bixin is unique among the naturally occurring carotenoids because of its two carboxylic groups, one of which is a methyl ester. Norbixin is an alkaline water-soluble carotenoid originating from the hydrolytic removal of a methyl ester group of bixin, the major carotenoid found in the waxy surrounding material of Bixaorellana seeds. Almost no information is available on the biological properties of bixin and norbixin, possibly because they are not usually found in dietary fruits and vegetables. Nonetheless, both carotenoids (commercially known as annatto or E160b) are quite commonly found in a variety of industrialized food, as colorants.[10] The phospholipids soya lecithin used as a tool for heart health, coronary artery disease and improves circulation. Lecithin supplementation also helps to prevent blood clots**,** maintain the health of the liver through which excess fats

and energy-providing substances pass. Lecithin has an anionic charge that shown the positive effect in the repair of livers damaged by any number of conditions, including excess consumption of alcohol or other toxins.[11]

The key obstacle of Norbixin has the low bioavailability, less soluble in water and it is rapidly eliminated from the body. To overcome these issues and to make herbal therapy of norbixin more effectively, less costly, easily available and patient convenient, the nanoparticle based phytosome approach was selected. Phytosome is a newly introduced patented technology developed to incorporate the standardized plant extracts or water-soluble phyto-constituents into phospholipids to produce lipid compatible molecular complexes, which improves their absorption, solubility and bioavailability. Recent studies demonstrated the significance of phyto-phospholipid technology for standardized extracts of *Ocimum sanctum*, *Ginkgo biloba*, grape seed etc. Phytosomes of Silymarin, *Boswellic acid*, Naringenin, Lawsone, Curcumin and polyphenols improved the clinical efficacy without compromising the safety for various therapeutic activities. The main objective of this study was to prepare the phytosome of Norbixin to increase the permeability, bioavailability and stability.

2. MATERIALS AND METHODS

2.1. Materials

Standardized plant drug Norbixin was procured from Sigma Aldrich Pvt Ltd, Bangalore, India. Identity and purity of the drug was confirmed by HPLC analysis. Soya Lecithin (≥ 95% purity) was purchased from Hi-media Laboratories Pvt Ltd, Mumbai, India. All other chemicals and reagents were used of analytical grade.

2.2. Methods

2.2.1. Preparation of Phytosome of Norbixin

The different molar ratio of 1:1, 1:2, 2:1 and 2:2 of phytosome (NP1 - NP4) containing norbixin and soya lecithin were prepared by the anti-solvent precipitation technique as mentioned in **Table 1**. The specific weighed amount of norbixin and soya lecithin was taken into a 100 mL round bottom flask as per the above mentioned molar ratio of drug and phospholipid. It was refluxed at temperature not exceeding 60^{o}C for 2 h through adding of 20 mL of methanol. Then, the mixture was concentrated to 5-10 mL till getting of thin film. Hexane (10 mL) was added carefully with continuous stirring to get the precipitate, which was filtered and collected and stored in vacuum desiccators for overnight. The dried

precipitate was crushed in mortar and sieved through #100 meshes. The prepared phytosome was placed in amber colour glass bottle and stored at room temperature.[12-14]

2.2.2. Characterization and Optimization of Phytosome of Norbixin

The prepared phytosome of Norbixin was characterized for Physical compatibility studies, particle size and shape and evaluated by specific *in vitro* evaluation parameters.

2.2.2.1. Solubility Study

Saturation solubility of the phytosome of Norbixin and pure active phyto-constituent Norbixin was determined in buffer solutions and organic solvents media. Acidic pH 1.2 HCl, phosphate buffer pH 6.8 and pH 7.4, acetone, acetonitrile (ACN), dichloromethane (DCM), ethanol, methanol, dimethyl sulfoxide (DMSO) and distilled water (DW) were used to determine the concentration of solubility. In this respect, mechanical shaker was used to get the proper saturation of drug or formulation in a respective media. To accomplish this parameter, excess amount of sample was added to 5 mL volume of each media placed in the 10 mL volumetric flask and kept for shaking on mechanical shaker for 24 h. After the completion of shaking, 1 mL of aliquot was taken out from each sample and filtered through What man filter paper No 41 and absorbance was measured in the range of 200-400 nm on UV-Vis Spectrophotometer and calculations for solubility were done. Measurements were performed in triplicate.[15]

2.2.2.2. Physical compatibility study

To determine the drug-phospholipids interaction or compatibility, FT-IR spectroscopy and Differential scanning calorimetry (DSC) was used.

2.2.2.2.1. Fourier Transfer Infrared spectrophotometer (FT-IR)

The physical compatibility study of pure active phyto-constituent Norbixin, physical mixture of Norbixin with soya lecithin and phytosome of Norbixin were performed by FT-IR (Perkin Elmer, Spectrum BX, USA) using KBr pellet method. Sample was crushed with KBr to get pellets at 600 kg/cm^2 pressure. Spectral data were taken in the range between 4000- 400 cm^{-1} to ascertain the structure and chemical interaction of drug, physical mixture and phytosome formulation.[16-17]

2.2.2.2.2. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry studies was conducted using DSC 60 (Shimadzu DSC60, Japan). Sample was weighed (2.00-10.00 ± 5 mg) and placed in the sealed aluminium crimp cell. The sample was scanned at 10^0C/ min up to 350^0C in the atmosphere of nitrogen. Peak transition onset temperatures were recorded.[16-17]

2.2.2.3. Determination of % yield

Determination of percentage yield of phytosome of Norbixin was calculated by the manual and established formula:[13,16]

$$(\%)\ \text{Yield} = \frac{\text{(Practical yield)}}{\text{(Theoretical yield)}} \times 100$$

Where,

Practical yield = The total weight of the formulation after completion of experiment.

Theoretical yield = The sum of the weight of the each ingredient which was used to get the formulation before proceeding the experiment.

2.2.2.4. Entrapment efficiency and Drug loading

To determine the percentage entrapment efficiency, 1-fold of phytosome of Norbixin was diluted with 10 mL of methanol using ultra-sonication. Then, it was centrifuged at 8,000 rpm for 15 min at -4^{o}C using cooling centrifuge machine. The supernatant was isolated and the amount of Norbixin and free Norbixin was determined by UV/Vis spectroscopy at 493 nm. Measurements were performed in triplicate.[16-17]

The Entrapment efficiency was calculated according to the following formula:

$$\text{EE}\ (\%) = \frac{\text{(Total amount of Norbixin)} - \text{(amount of free Norbixin)}}{\text{(Total amount of Norbixin)}} \times 100$$

The drug loading was calculated according to the following formula:

$$\text{DL}\ (\%) = \frac{\text{Total amount of Norbixin in the phytosome}}{\text{Total amount of phytosome of Norbixin}} \times 100$$

2.2.2.5. Particle size and Zeta Potential

The average diameter and surface charge property of phytosome of Norbixin was measured by laser diffraction using particle size analyser (Zetasizer 2000, Malvern Instruments Ltd., UK) at a fixed scattering angle of 90^o at 25^{o}C. Zeta potential is commonly used to

characterize the surface charge property and particle stability of phytosome which was expressed as the value of z-average size ± SD.[19]

2.2.2.6. Scanning electron microscopy (SEM)

Scanning electron microscopy was used to determine the particle size distribution and surface morphology of phytosome of Norbixin using JEOL JSM-6360 Scanning microscope (Japan). Dry samples was placed on an electron microscope brass stub and coated with gold in an ion sputter. Digital images were taken by random scanning of the stub at different magnifications.[16-17]

3. RESULT AND DISCUSSION

3.1. Preparation and Optimization of Phytosome of Norbixin

Since the norbixin was exhibiting good solubility in methanol, ethanol, phosphate buffer pH 7.4, dichloromethane and ethyl acetate, it was decided to blend with soya lecithin to form nanoparticle of phyto-phospholipid complex. The phytosome of norbixin (NP1-NP4) were prepared by anti-solvent precipitation technique and the qualitative results obtained from various characterized parameters such as percentage yield, entrapment efficiency, drug loading, particle size and zeta potential etc. were mentioned in **Table 1**.

The characterization results of phytosome of Norbixin are shown in **Table 1**. NP2 contains the blending of Norbixin and soya lecithin (1:2), has shown the efficient 72.92±0.06% yield as compared to the other phytosomes. The entrapment efficiency and drug loading of NP varies from 59.04±0.07 to 88.03±0.24% and 02.37±0.06 to 13.07±0.04%. It was found that NP2 showed maximum 88.03±0.24% entrapment efficiency and 06.15±0.93% drug loading, respectively due to the proper bounding of Norbixin with the polar head of soya lecithin as compare to the others. The particle size of NP varies from 23.36±0.10 nm to 197.05±0.00 nm. It was found that NP2 showed lowest 23.36±0.10 nm (**Fig. 1**) particle size, due to the availability of numbers of Norbixin molecule and combined interaction with soya lecithin. The zeta potential of NP2 was found to be 6.92 ±0.06 mV with 0.20 ±0.1 PDI as shown in **Fig. 2**. The results of NP2 suggested that the selected ratio of Norbixin and soya lecithin (1:2) favoured the formation of complex, resulting in the formation of uniformly distributed nano sized phyto-phospholipid vesicular cells. Based on optimum results, NP2 was selected as an optimized phytosome which was further characterized and evaluated by different evaluation parameters.

3.2. Characterization of optimized Phytosome of Norbixin

To characterize the functional groups and the formation of phytosome of norbixin, FT-IR spectroscopy was used. In the FT-IR spectra (**Fig. 3**), the peak at 3439 cm^{-1} attributed to phenol O-H stretching group, 1620 cm^{-1} due to alkenyl C=C stretching while peaks at 682 cm^{-1} corresponds to aromatic C-H bending group of norbixin, respectively. The strong peaks at 1733 cm^{-1}, 2925 cm^{-1} & 2854 cm^{-1} in soya lecithin could be due to C=O absorption and stretching & deformation of methyl group. The peak at 1457 cm^{-1} observed in soya lecithin due to bending vibration of CH_2 group. These peaks were found and not shifted after the analysing of physical mixture of norbixin and soya lecithin which suggested that there was no interaction between norbixin and soya lecithin. A shift from 3439 cm^{-1} to 3328 cm^{-1} and 1620 cm^{-1} to 1602 cm^{-1} compared to norbixin and a shift from 1733 cm^{-1} to 1654 cm^{-1} compared with soya lecithin were exhibited by optimized phytosome of NP2. These changes confirmed the formation of phytosome by the hydrogen bonding between the –OH group of phenolic ring of norbixin and P=O group of soya lecithin.

Furthermore, DSC compatibility study was performed to characterize the phytosome of norbixin. As shown in the DSC thermogram (**Fig. 4**), a sharp endothermic peak of norbixin at 203.51°C and a broad peak of soya lecithin were found at 157.16°C, respectively. The same two melting peaks were also observed in the physical mixture of norbixin & soya lecithin at 216.66°C and 154.80°C, respectively. One peak was shifted little more and another peak was shifted slightly lower which may be due to the hydrophobic interaction between norbixin and soya lecithin. The thermogram of NP2 exhibited two peaks at 153.74 and 193.82°C and the characteristic peaks of norbixin and soya lecithin were not observed. These observations suggested the absence of norbixin and soya lecithin interactions.

The solubility study of optimized NP2 was observed in different organic solvents and buffers and also compared with active phyto-constituent Norbixin. From the solubility comparison results, it was concluded that the optimized phytosome NP2 was showing freely soluble to sparingly solubility profile (**Fig. 5**) in all solvents as compared to Norbixin due to the wettability and dispersion properties of soya lecithin. The surface morphology, shape and structure of the optimized NP2 at various magnifications are shown by scanning electron microscope (SEM; **Fig. 6**). It was observed that the crystalline particles of Norbixin were associated with soya lecithin that was forming colloidal phytosome of NP2 with irregular

particle shapes and crystalline structure with smooth surface. These results confirm the vesicle formation of Norbixin using soya lecithin as amphiphilic lipid.

Table Captions

Table 1: Formulation of Phyto-phospholipid complex of Norbixin.

Table 2: Preparation of antioxidant standard curve; various concentrations of Trolox (0-100 µM) were prepared by diluting 0.2 mM stock of standard Trolox with 0.075 M phosphate buffer.

Figure Captions

Figure 1: Particle size of Optimized Norbixin phyto-phospholipid complex (NP2).

Figure 2: Zeta potential of Optimized Norbixin phyto-phospholipid complex (NP2).

Figure 3: IR spectrum of (I) Norbixin (II) Soya lecithin, (III) Physical mixture of Norbixin and soya lecithin and (IV) Norbixin phyto-phospholipid complex (NP2).

Figure 4: DSC thermogram of (i) Norbixin (ii) Soya lecithin (iii) Physical mixture of Norbixin and soya lecithin and (iv) Norbixin phyto-phospholipid complex (NP2).

Figure 5: Solubility comparison of Norbixin with NP2.

Figure 6: Surface morphology of optimized NP2 at 6000x and 12000x magnifications.

Table 1: Characterization and Optimization of Phytosome of Norbixin.

Formulations		Evaluation Parameters					
Phytosomes	**N:S**	**Yield (%)**	**Entrapment efficiency (%)**	**Drug loading (%)**	**Particle size (nm)**	**Zeta Potential (mV)**	**PDI**
NP0	0:1	21.07±0.05	00.00±0.00	00.00±0.00	98.03±0.18	-13.05 ±0.03	0.04 ±0.0
NP1	1:1	39.01±0.19	59.04±0.07	02.37±0.06	51.21±0.04	-7.10 ±0.00	0.51±0.2
NP2	1:2	72.92±0.06	88.03±0.24	06.15±0.93	23.36±0.10	6.92 ±0.06	0.20 ±0.1
NP3	2:1	69.02±0.03	80.06±0.31	05.03±0.27	158.04±0.01	16.28 ±0.01	0.68±0.6
NP4	2:2	81.22±0.11	68.09±0.05	13.07±0.04	197.05±0.00	24.09 ±0.09	0.71±0.2

Mean ± SD, n = 3 (N: Norbixin, S: Soya lecithin), PDI = Polydispersity Index

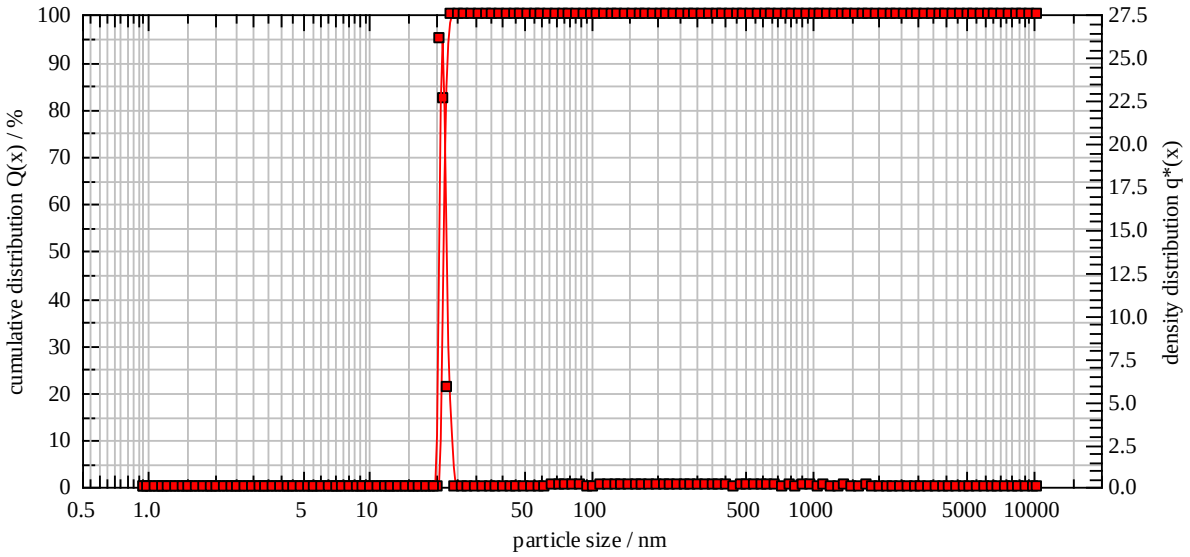

Figure 1: Particle size of Optimized Norbixin phyto-phospholipid complex (NP2).

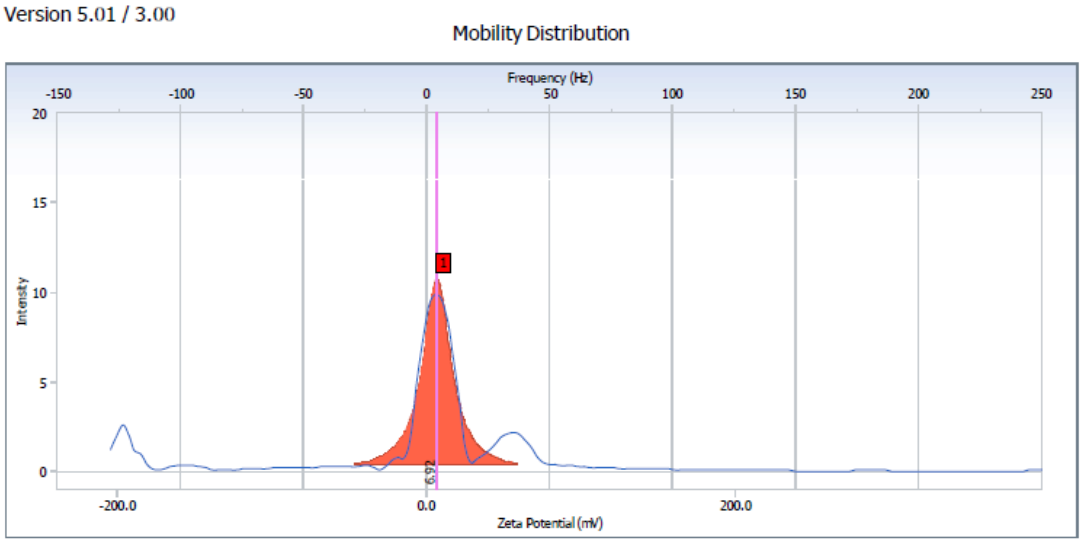

Figure 2: Zeta potential of Optimized Norbixin phyto-phospholipid complex (NP2).

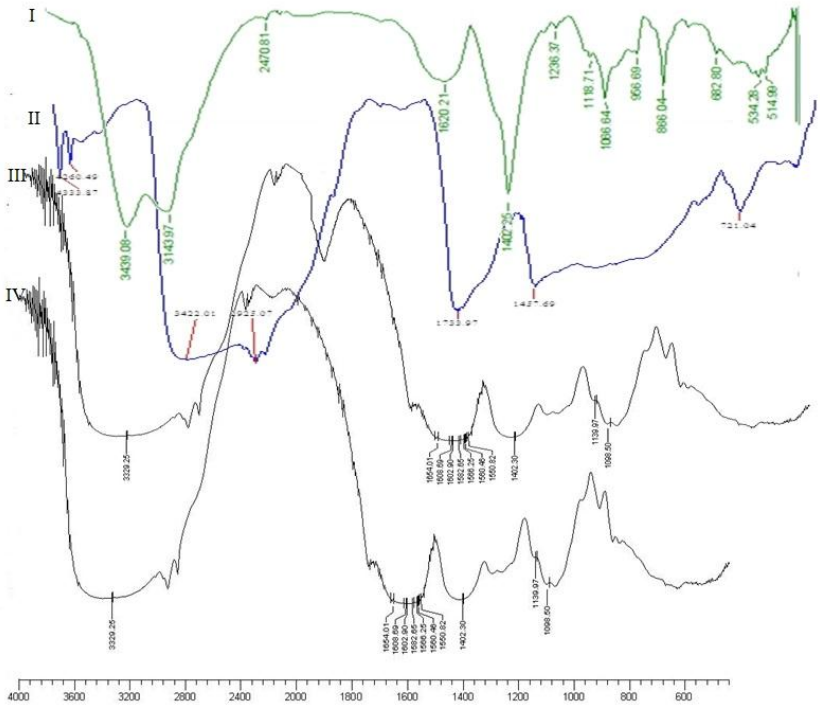

Figure 3: IR spectrum of (I) Norbixin (II) Soya lecithin, (III) Physical mixture of Norbixin and soya lecithin and (IV) Norbixin phyto-phospholipid complex (NP2).

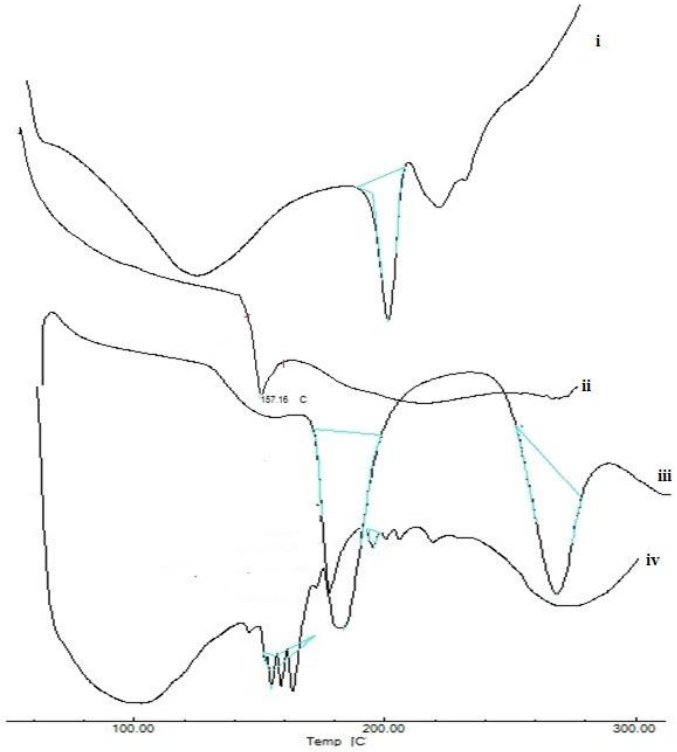

Figure 4: DSC thermogram of (i) Norbixin (ii) Soya lecithin (iii) Physical mixture of Norbixin and soya lecithin and (iv) Norbixin phyto-phospholipid complex (NP2).

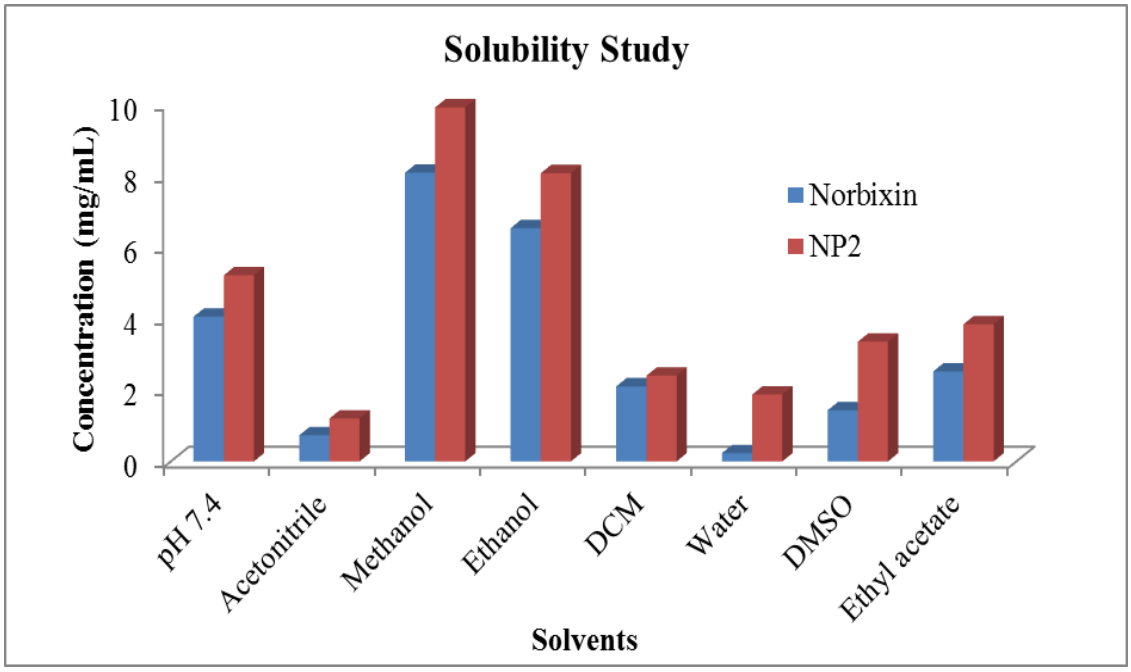

Figure 5: Solubility comparison of Norbixin with NP2.

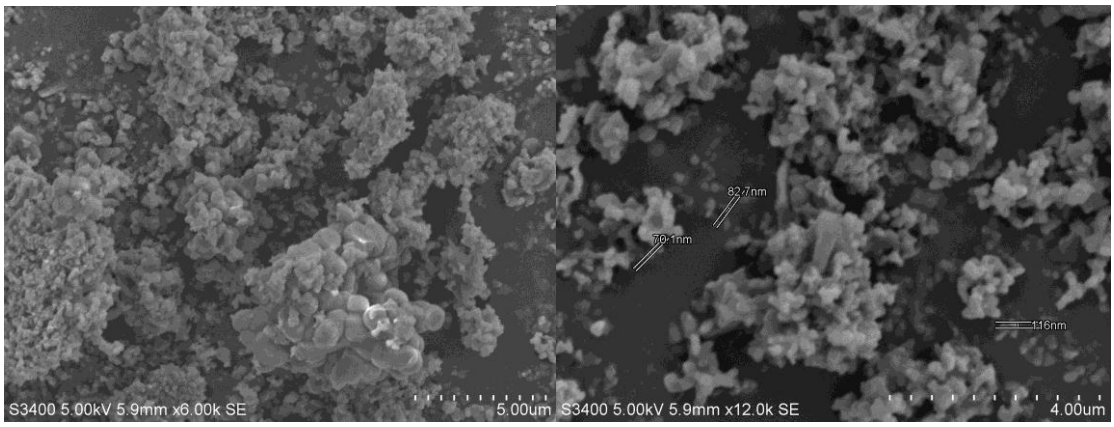

Figure 6: Surface morphology of optimized NP2 at 6000x and 12000x magnifications.

4. CONCLUSION

In this study, we prepared the phytosome of norbixin by anti-solvent precipitation technique. This method was less costly as well as more efficient to produce the formulation in less time. The different molar ratios of phytosome were prepared and characterized for FT-IR spectroscopy, DSC thermograms, percentage yield, entrapment efficiency, drug loading, particle size and shape confirms the formulation. Thus, the prepared phytosome is, a carrier combining drug delivery system of soya lecithin and norbixin, is a more favorable option for oral application of norbixin and also may be helpful in overcoming the limitations of existing drug delivery systems like other water soluble constituents.

Conflict of Interest

The authors declared that there are no conflicts of interest.

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