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Quantitative Analysis of Neem Extract Complex using Phosphatidyl choline obtained from Soya Lecithin oil

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ABSTRACT

The neem tree has been known for its unique properties both in improving human health and against insects. Azadirachtin is a tri terpenoid limonoid obtained from various parts of the neem. Phosphatidyl choline is the most important phospholipid which helps in crossing the lipid barrier of the cell membrane and thus helps in easy absorption of the compound/drug. In the present work we are combining the isolated Azadirachtin and PC to form a complex, which can enhance the bio availability and easy absorption of the compound/drug. First of all, Azadirachtin is extracted from the dried neem kernel powder in a Soxhlet extractor using Di-Chloro methane as a solvent. Phosphatidyl choline is extracted and isolated from soya lecithin oil through reflux, using Methanol + Sodium hydroxide as solvent. The TLC, UV, HPLC, FTIR and DSC reports indicate the separation of the compounds from their crude forms. Azadirachtin and Phosphatidyl choline separated are refluxed together for 2 hours and dried in a Rota evaporator to form a 'Complex'. The absorbance's of Azadirachtin, Phosphatidyl choline and the Complex are measured at 220, 230 and 217nm. The qualitative analysis of Azadirachtin, Phosphatidyl choline and the Complex (1:1) were carried out by HPLC, on a C-18 column. Aceto nitryl: Methanol: 1% Triethyl amine p^H 4 (60:40:1) was used as mobile phase at a flow rate of 1ml/min, at 210nm.The isolation of Phosphatidyl choline from soya lecithin oil used is a new method and the formation of Complex is an innovative work.

Keywords: Phosphatidyl choline, Azadirachtin, Complex, UV spectrophotometer, HPLC, FTIR, DSC.

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INTRODUCTION

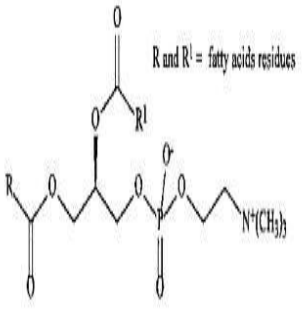
Phytochemistry is the study of the 'chemistry of plants'. Like animals, plants produce a wide variety of chemical compounds, called metabolites, as part of their normal life processes. The phytochemical investigation of a plant involves the following steps: extraction of the plant material; separation and isolation of the constituent of interest; characterization of the isolated compounds; investigation of bio synthetic pathways to particular compounds and quantitative evaluations.[1]

The scientific name of neem is 'Azadirachta indica' and belongs to family 'Meliaceae'. Neem compounds belong to a general class of natural products called ‘limonoid’. Azadirachtin is the most important and active component. Other components are Nimbin, Nimbinin, Nimbidin, oleic stearic and Palmitic acids, Quercitin and other limnoids. Its melting point is 160ºC and molecular weight of is 720.Azadirachtin is currently considered as neem’s main agent for controlling insects. ‘It appears to cause 90% of the effect on most pests. Research over the past years has shown that unlike chemical insecticides, neem compounds work on the insect’s hormonal system, not on the digestive or nervous system and therefore do not lead to development of resistance in future generations.[2,3]

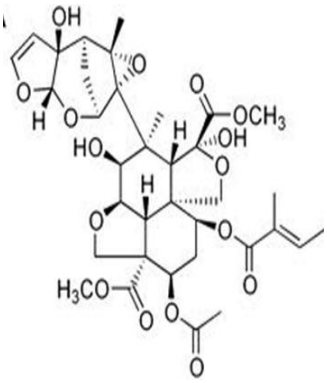
The scientific name of soya beans is 'Glycine max.' which belongs to the family 'Fabaceae.' Soya beans have an important phospholipid “Phosphatidyl choline” (PC) in it. Phosphatidyl choline, which is composed of fatty acid, choline, glyceryl and phosphoryl group. Its melting point is 229.5ºC and molecular weight is 790. It provides phosphorus for body growth and is the main source of choline and essential fatty acid, it has good emulsification properties.[4]

The newly created “Complex/phytosome” structures contain the active ingredients of the herb bounded to phospholipids. The phospholipid molecular structure includes a water-soluble head and two fat-soluble tails. Because of this dual solubility, the phospholipid acts as an effective emulsifier. By combining these properties the bioavailability for lipid soluble drugs is enhanced.[5,6]

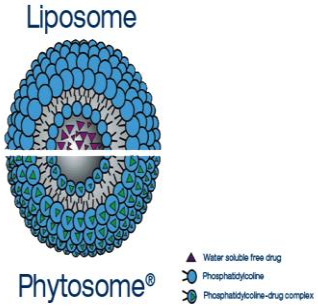
Here we are discussing about neem and its properties; the emulsifying property of Phosphatidyl choline from soya lecithin and conversion of Azadirachtin and Phosphatidyl choline to form a Complex. Because of the 'complex' valuable components of the herbal extract are protected from destruction by digestive secretions and gut bacteria, thus it enhances the utilization of the key components of the plant extract.[6]



Structure of Phosphatidyl choline



Structure of Azadirachtin



Picture 1 Picture showing difference between liposome and a phytosome/complex

MATERIALS AND METHODS

Materials:

The materials used in the work are: Soya lecithin oil was obtained from Raja Lakshmi Stores, Hyderabad. Neem seeds were obtained from the nearest vegetable market, Hyderabad.

Chemicals and Solvents used:

Hexane, Acetone, Sodium hydroxide, Dichloro methane and Chloroform of LR grade were obtained from Akshaya chemicals of SD fine chemicals, Mumbai.Aceto nitryl, Methanol, Water of HPLC grade were obtained from Akshaya chemicals, Mumbai. The Tri ethylamine of AR grade obtained from G.R. Merck.Silica 60 GF_{254} - TLC Plates pre coated aluminium sheets, Merck.

Instruments:

Reflux set up, Soxhlet extraction set up, Shimadzu UV-visible Spectrophotometer (model UV-1800), Schimadzu HPLC, Bruker Alpha-T FTIR, Horizon WC-56-KBr Press, DSC instrument.[7,8]

Methods for collection of fruits, isolation of kernels and extraction of the compounds: [9-11]

Neem consists of bitterness in varied content, but a huge quantity of bitter compounds like Azadirachtin and nimbin are present in the kernels of the neem seed. Riped neem fruits are collected and depulped then the seeds are washed with clean water. The seeds are dried and the

outer shell is removed to obtain kernels. These kernels are ground coarsely such that no oil comes out of them.[2]

Picture 2 Dried neem seeds

Picture3 Dried neem kernels

Extraction of Azadirachtin:

Weigh about 500g of coarse neem kernel powder and fill it up in a thimble, then place in a Soxhlet extractor and extract it with 600-700ml Dichloro methane. Keep it on a heating mantle and heat to reflux for about 12 hours.[15] When the kernel powder is extracted with solvents like Dichloro methane liminoids and other constituents get dissolved in it, leaving the seed cake without any actives. The solvent can be recovered by distillation.

The distilled or concentrated solution is kept for cooling. Hexane is added to the concentrate which produces precipitate. It is then filtered using Vaccum pump and the residue is dried which gives a pale greenish colored powder. This powder consists of Azadirachtin and a very little quantity of Nimbin. 500g of neem kernel powder on extraction with Dichloro methane gives about 1g of Azadirachtin.

Picture4 Isolated Azadirachtin powder

TLC confirmation:

A small amount of Azadirachtin is dissolved in methanol. Spot the sample on a TLC plate and place it in a beaker consisting of mobile phase Iso-Propanol: Hexane(2.5:17.5). Run till 3/4th of the plate and place it in an Iodine chamber. It gives two pale yellow colored spots. The upper

spot indicates Azadirachtin and the below one indicates nimbin. The literature review shows that there is no much work done on extraction of Azadirachtin from kernels of neem seeds using Di-Chloro methane as a solvent and HPLC analysis at 210nm.[12-15]

Extraction of Phosphatidyl choline from lecithin oil:

Soya lecithin oil is a brown colored thick material. Weigh about 50g of oil and dissolve it in a solution of 10g NaOH+250ml Me OH and stir it. Keep this solution for reflux for about 2 hours. Cool it and separate the MeOH soluble part, remove MeOH insoluble part. Add ice water to the methanol soluble part, the insoluble separates. Filter this and dry the residue obtained. The methanol insoluble residue is washed with acetone. To the filtrate add hexane and separate using a separating funnel. Collect the above hexane layer from the separating funnel and repeat this extraction twice. As Phosphatidyl choline is soluble in hexane, it gets dissolved in the hexane solution. The hexane solution obtained above is distilled. Acetone is added to the distilled residue and then filtered. On drying the residue a pale yellow colored powder is obtained. Each batch of 50g of oil gives about 1g of Phosphatidyl choline. From TLC of the obtained powder and on checking the melting point we can confirm Phosphatidyl choline.

TLC confirmation:

Dissolve some Phosphatidyl choline in chloroform. Spot it on a TLC plate and place it in a beaker containing mobile phase $CHCl_3$: Me OH: liquid NH_3 (6:4:1). Run 3/4th TLC plate the dip it in a reagent (ammonium molybdate + conc. sulphuric acid + water) and place under tap water. Dry it on a hot plate purple-blue colored spot is obtained which confirms the presence of Phosphatidyl choline.

The literature review shows that there is no work done to isolate Phosphatidyl choline from soya lecithin oil using methanol and sodium hydroxide as the mobile phase.[16-21]

FORMULATION OF A COMPLEX:

1:1 complex:

Complex or a Phytosome is an innovative transport system that show better pharmacokinetic and therapeutic profile than other non complexed herbal extracts. The Complex helps to reduce polarity of active substances hence making them easily absorbable. Here we have formulated a Complex so that Azadirachtin can be easily absorbable. Take about 100mg of obtained Phosphatidyl choline and add 100mg of Azadirachtin to it. Then add $CHCl_3$ and Me OH in the ratio of 1:1 to it, keep it for reflux for 2 hours. Cool it and concentrate the solution in a Rota evaporator. Finally a Complex is thus formulated.[22]

STUDIES ON CHARACTERIZATION OF COMPOUNDS AND COMPLEX:[7,8]

UV spectroscopy:

Different concentrations of Azardiracthin, Phosphatidyl choline and Complex were prepared, scanned in the UV region from which the wavelengths 220nm & 230nm were selected for Azadirachtin and Phosphatidyl choline. While 217nm is selected as wavelength for the Complex, where they show maximum absorbance. As low concentration (below 10mcg) does not show any absorbance, high concentrations (above 100mcg) were taken for calibration. The calibration curves were linear over the concentration ranges of 30-70µg/ml for Azadirachtin,500-900µg/ml for Phosphatidyl choline and 200-600µg/ml for the Complex. Absorbance's were plotted versus respective concentrations. The calibration curves were shown in Figure 1,2 and 3.

High Performance Liquid Chromatography:

Optimization of separating conditions:

Solvent selectivity (solvent type), solvent strength (percentage of organic solvent in mobile phase), strength and p^H of buffer, wavelength etc., were varied to determine the chromatographic conditions that gave the best separation. When Aceto nitryl: Water: Triethyl amine (60:40:1) are used as mobile phase the peak of Phosphatidyl choline was absent shown in Figure.4. Hence Aceto nitryl: methanol: Triethyl amine is used as mobile phase which gave good sharp peaks.

Optimized chromatographic conditions:

The final optimized conditions are given below:

Stationary phase	Reverse phase phenomenex® luna 5µ, c 18(2) 100A (250 x 4.6 mm i.d)
Mobile phase	Aceto nitryl : methanol :1% triethyl amine adjusted to p^H 4.
Solvent ratio	(60 : 40 : 1) v/v
Detection wavelength	210 nm
Flow rate	1ml/min
Operating pressure	146 kgf
Temperature	Room temperature
Mode	Isocratic
Retention times	Azadirachtin - 3.8 min, Phosphatidyl choline - 7.8 min Complex (1 : 1) - 3.8 and 7 min

Preparation of sample solutions:

Weigh about 10mg of the compounds Azadirachtin powder, Phosphatidyl choline, Complex and transfer into 10ml volumetric flasks. They are extracted with methanol.The volumetric flask was sonicated for 2-3 minutes to affect the complete dissolution of the compounds and the solution was made upto the volume with methanol and filtered. Suitable aliquots of compound and its solution were prepared and injected to HPLC to obtain concentration in the linearity range.

Recording of chromatograms:

A steady baseline was recorded with optimized chromatographic conditions and the compound solutions were injected and chromatograms were recorded. Retention times of Azadirachtin, Phosphatidylcholine and Complex were found to be 3.8m, 7.8m, 3m and 7m shown in Figure 5,6 and 7 Peaks areas of the sample or individual compounds chromatograms were compared with that of the complexes to confirm the formation of the complex.

FTIR:[7]

After the synthesis of organic or natural compound in the laboratory, we use infrared spectroscopy (IR) to analyze the product and verify the compounds identity. It is done by KBr press method.

- The sample is ground in a mortar & pestle and mixed with approximately 100 times as much KBr in the same grinding apparatus.
- Prepare a pellet on a KBr press, such that the sample should form a translucent disc at the end of compression process.
- Load into the IR spectroscopy and scan it.
- Interpret the spectrum with an interpretation chart. These peaks represent functional groups of the molecule analyzed and show how each covalent bond bends or stretches in response to infrared radiation moving through the sample.
- Usually % transmittance is on the y-axis and wave number is on the x-axis.
- Look for peaks in the characteristic regions of the graph/spectra.
- Compare the peaks marked with list of common functional groups and their absorptions.
- Thus by matching the peaks we can identify the compounds and any changes made in the functional groups.
- Change in the functional groups and bond formation indicate the formation of a Complex when compared to individual samples.
- The IR spectra of Azadirachtin, Phosphatidyl choline and Complex are shown in Figure 8,9and10

Differential Scanning Calorimetry: [8]

The samples of Azadirachtin, Phosphatidyl choline and Complex were analyzed by DSC. The basic principle underlying this technique is that when the sample undergoes a physical transformation such as phase transitions, more or less heat will need to flow to it than the reference to maintain both at the same temperature. Whether less or more heat must flow to the

sample depends on whether the process is exothermic or endothermic. DSC is a linear heating method, which results in a cyclic heating of the sample. Generally when samples are exposed to heat, they emit energy/heat (exothermic).But in case of complex formation the sample absorbs energy /heat (endothermic) which can be seen as a dip in the peak spectra. Thus we can confirm the formation of a Complex from the spectra. The data/graphs of DSC for the samples are shown in Figure 11,12and 13

RESULTS AND DISCUSSION

TLC studies:

The TLC of Phosphatidylcholine gave blue – purple coloured spot which indicated presence of Phosphatidyl choline. Azadirachtin gave pale yellow colored spot with mobile phase Isopropanol: Hexane in the ratio 2.5:17.5 when placed in an Iodine chamber.

UV studies:

Phosphatidyl choline showed UV absorbance at 230nm, Azadirachtin showed absorbance at 220nm and the Complex showed absorbance at 217 nm. The calibration data and the calibration curves were shown in Table 1,2,3 and Figure 1,2,3

Table 1 Calibration data of Azadirachtin

Concentration	Absorbance
10mg	0.122
20mg	0.202
30mg	0.268
40mg	0.343
50mg	0.446
60mg	0.538
70mg	0.632
80mg	0.147
90mg	0.813
100mg	1.198

Table 2 Calibration data of Phosphatidyl choline

Concentration	Absorbance
100mg	0.002
200mg	0.003
300mg	0.009
400mg	0.016
500mg	0.019
600mg	0.024
700mg	0.030
800mg	0.035
900mg	0.041
1000mg	1.10

Table 3 Calibration data of the Complex

Concentration	Absorbance
100mg	0.146
200mg	0.183
300mg	0.247
400mg	0.314
500mg	0.382
600mg	0.450
700mg	0.536
800mg	0.612
900mg	0.612

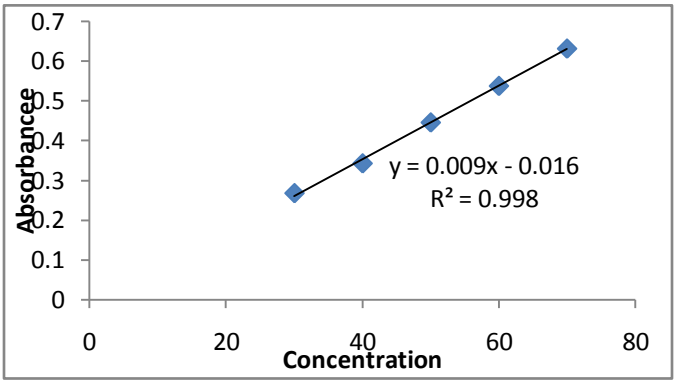


Figure 1: Calibration curve of Azadirachtin

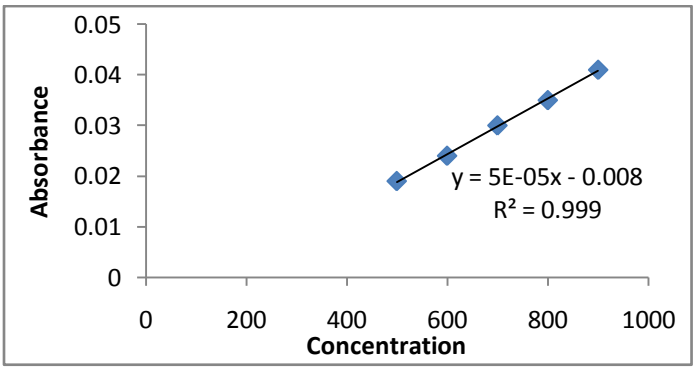


Figure 2: Calibration curve of Phosphatidyl choline

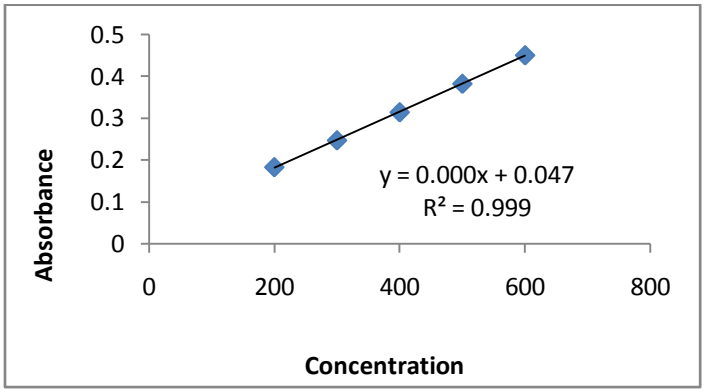


Figure 3: Calibration curve of Complex

HPLC studies:

The HPLC analysis of the samples showed chromatograms with different peak area, % area and retention times. Depending on the peaks and retention time, the compounds were confirmed. Azadirachtin showed peak at 3.8 min, Phosphatidyl choline showed peak at 7.8 min. Complex showed 2 peaks exactly at 3.8min and 7min, which shows the formation of complex. The chromatograms of Azadirachtin, Phosphatidyl choline and Complex are shown in Figure 5, 6, 7.

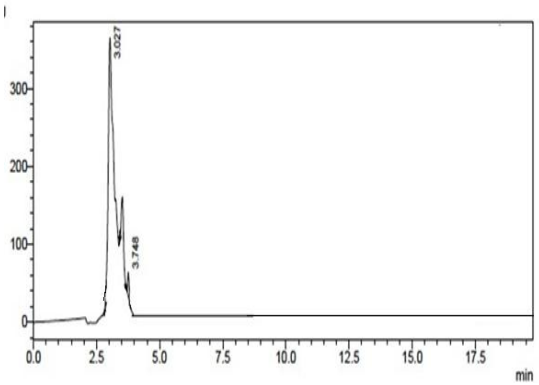


Figure 4: Chromatogram of Phosphatidyl choline in ACN: Water : TEA (60:40:1)

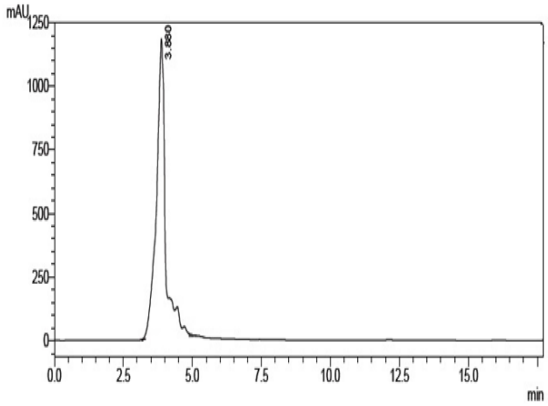


Figure 5: Chromatogram of Azadirachtin in Aceto nitryl: methanol: tri ethyl amine (60:40:1) at 210nm

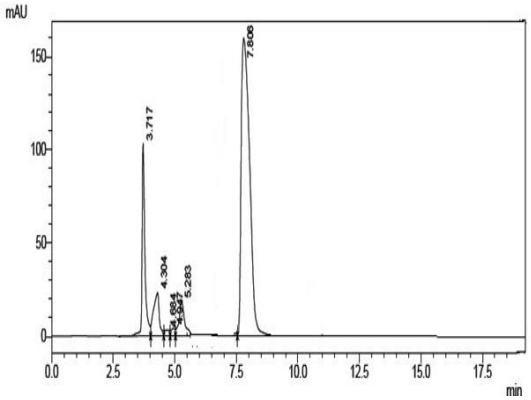


Figure 6: Chromatogram of Phosphatidyl choline in Aceto nitryl: methanol: tri ethyl amine (60:40:1) at 210nm

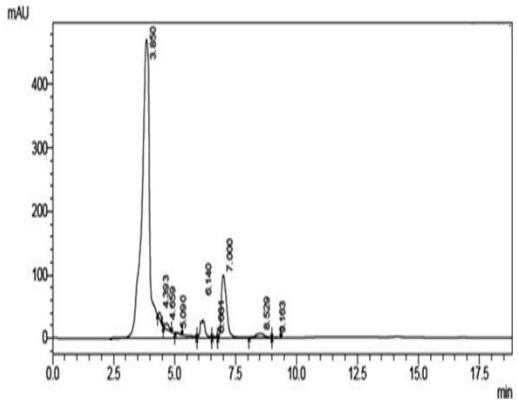


Figure 7: Chromatogram of Complex in Aceto nitryl: methanol: tri ethyl amine (60:40:1) at 210nm

IR studies:

The IR spectra of azadirachtin extract has shown a characteristic band at 1742cm^{-1} The IR spectra of Phosphatidyl choline has shown a band at 1540cm^{-1} The band of Azadirachtin is not observed in the Complex , but a band at 1540cm^{-1} indicates the presence of Phosphatidyl choline and indicates the complex formation through the functional group responsive for the band at 1744cm^{-1} The IR spectra's of Azadirachtin, Phosphatidyl choline and complex are shown in Figure 8,9and 10

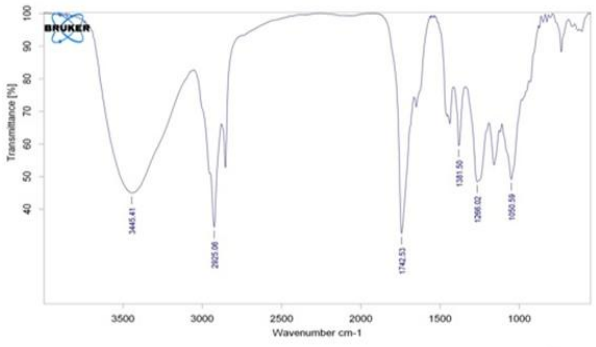


Figure 8: IR spectra of Azadirachtin

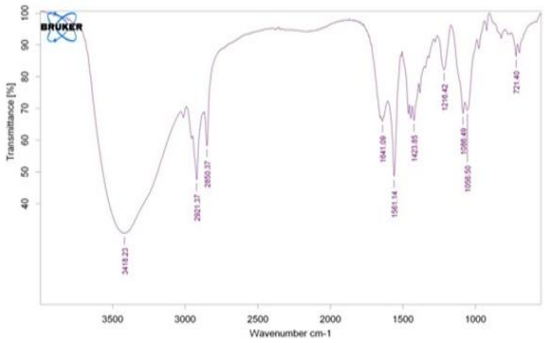


Figure 9: IR spectra of Phosphatidyl choline

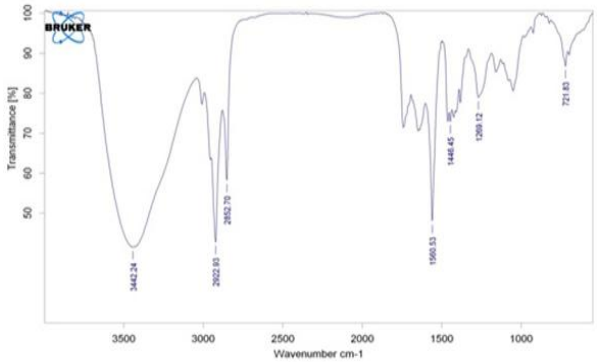


Figure 10: IR spectra of Complex

DSC studies:

Azadirachtin showed a peak on heating at 135.6^{0}C, where as Phosphatidyl choline showed a peak at 125.6^{0}C both are slightly endothermic which corresponds to its melting point.The Complex gave a huge dip in the peak as it absorbed heat in the process of formation of the Complex. The dip in the peak of Complex corresponds to its melting point indicates the Complex formation. The spectra of Azadirachtin, Phosphatidyl choline and complex are shown in

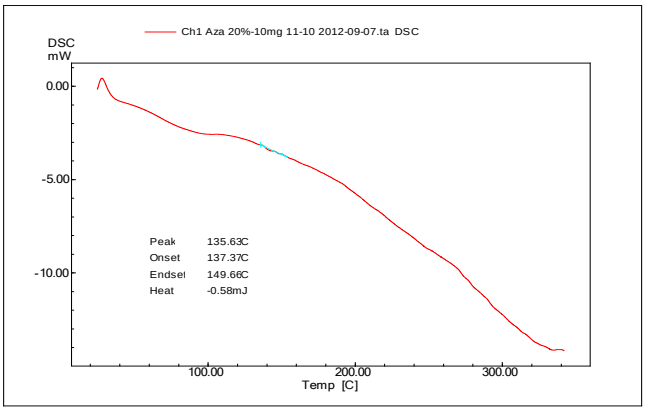


Figure 11: DSC image of Azadirachtin

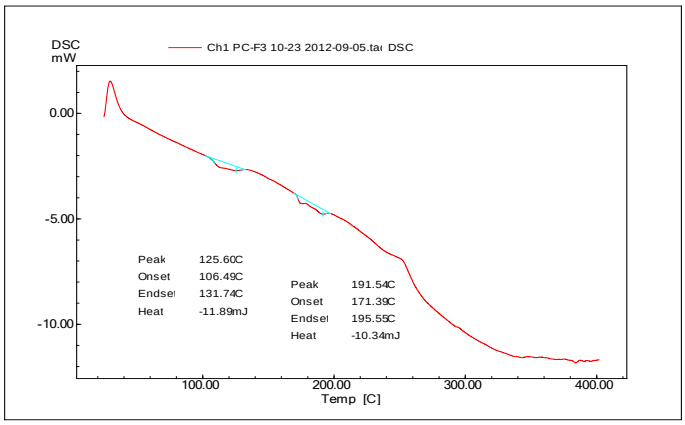


Figure 12: DSC image of Phosphatidyl choline

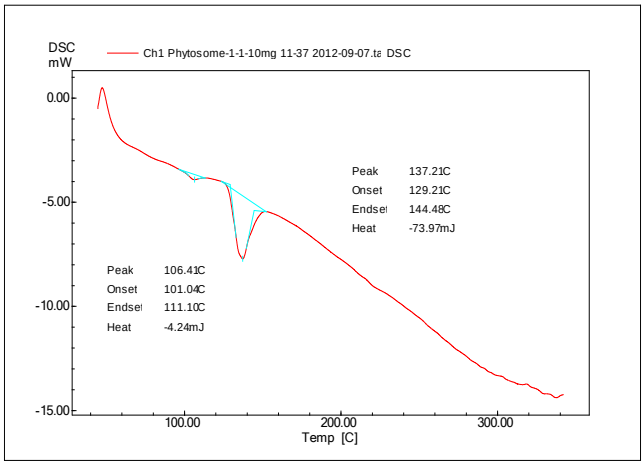


Figure 13: DSC image of Complex

CONCLUSION

The process adopted in this work to isolate, separate and purify the Phosphatidyl choline from soya lecithin oil is feasible and innovative. The oil residues were removed from impurities firstly and then separated by extraction.The process adopted for isolation and separation of Azadirachtin from dried neem kernel powder is simple and feasible. The product thus obtained is 40% pure and is reproducible. The method used for the formation of Complex using Phosphatidyl choline and Azadirachtin is innovative and has reproducibility in the formation of the Complex. From the UV, IR, DSC and HPLC it is confirmed that the methods adopted have resulted in the purification of Phosphatidyl choline from soya lecithin oil, standard and reproductive Azadirachtin from neem kernel, formation of Azadirachtin-Phosphatidyl choline complex in the ratio of 1:1

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