

Rapid Extraction and characterization of *E.coli* phospholipids and study of its potential application in liposomal drug delivery systems

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Abstract- Liposomes are the class of drug delivery systems which are considered as biodegradable, biocompatible, flexible, non-ionic and non-toxic and provides direct interaction of the drug with cell and can modulate the distribution of drug and also increase the therapeutic index of drugs. It can also be modified and used to deliver genes and protect some sensitive tissues to a particular kind of drugs. Manufacturing of liposomes requires use of expensive raw material (synthetic phospholipids) from specialized commercial suppliers makes the liposomal based drugs very expensive due to complex extraction procedure which time taking and products obtained in a very less amount. In this research work extraction of phospholipid of *E.coli* bacteria was done by solvent extraction procedure and characterized by paper chromatographic, spectroscopic techniques like UV- Visible, Photoluminescence, FTIR and dynamic light scattering techniques, particle size analyzer and zeta potential. It has been found that phospholipid so obtained has same properties as that of synthetic phospholipids and it can be an alternative to synthetic phospholipids to reduce the cost of the drug delivery systems. Such methods are ecofriendly, less time consuming and an abundant amount of product is obtained for the development of liposomes.

Keywords- liposome, drug delivery, phospholipids.

1. Introduction

Liposomes are the drug delivery systems used for the systemic administration of drugs. Liposome, a tiny vesicle made up of the same material as a cell membrane ¹. Its membranes are composed of natural phospholipids having a head group attracted to water and a tail group which is made up of a long hydrocarbon chain. The tail group is repelled by water and also may contain mixed lipid chains containing surfactant properties ². In the presence of aqueous medium, the heads are attracted to water and line up to form a surface facing the water and tails are repelled by water, and line up to form a surface away from the water same as in a cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer and the combined structure forms a bilayer ³. When phospholipid membranes are disrupted, they will reassemble themselves into tiny spheres which are smaller than a normal cell either as bilayers or monolayers ⁴. The bilayer structures are known as liposomes and the monolayer structures are known as micelles. As a drug delivery systems liposome played an important role in potent drug formulation to improve therapeutics⁵.

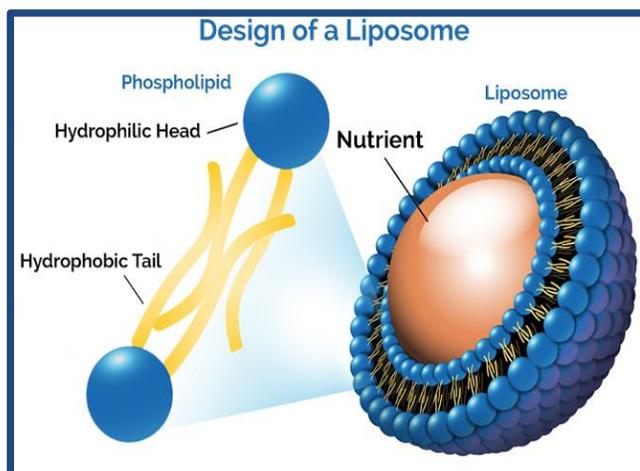
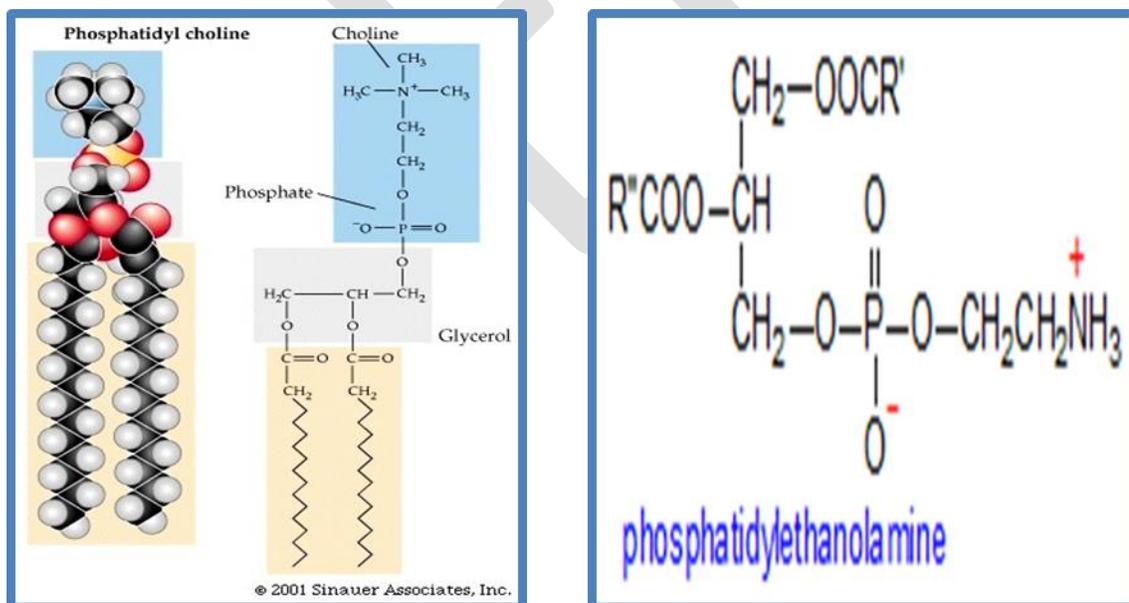


Figure 1. Liposome (Ref: Integratedhealth)

As a drug delivery system liposomes have many advantages such as they provide controlled drug delivery and controlled hydration and also provide sustained release and can carry both water and lipid soluble drugs⁶. Liposomes are biodegradable, biocompatible, flexible and non-toxic and non-ionic. It provides direct interaction of the drug with cell and can modulate the distribution of drug and also increase the therapeutic index of drugs⁷.

1.1 Phospholipids : building block of liposome

Phospholipids are made up of two fatty acids (long chains of hydrogen and carbon molecules), which are attached to a glycerol 'head.' The glycerol molecule is also attached to a phosphate group, and this is the hydrophilic part of the molecule. The 'tail' ends of the fatty acid chains opposite the glycerol is the hydrophobic part of the molecule⁸.



A. Phosphatidylcholine

B. Phosphatidylethanolamine

Figure 2. Two main constituent of biological membrane

They are essential components of cell membranes and are found in small concentrations in other parts of the cell. It should be noted that all glycerophospholipid are members of the broader class of lipids known as phospholipids. The hydrophobic nature of lipid molecules allows membranes to act as effective barriers to more polar molecules. In this topic, we discuss the chemical and physical properties of the various classes of lipid molecules. The phosphate, together with such esterified entities, is referred to as a “head” group. Phosphatides with choline or ethanolamine are referred to as phosphatidylcholine (known commonly as lecithin) or Phosphatidylethanolamine (known commonly as cephalin), respectively. These phosphatides are two of the most common constituents of biological membrane⁹. Where phosphatidylcholine is the principal phospholipid in animals, PE is the principal one in bacteria. One of the primary roles for PE in bacterial membranes is to spread out the negative charge caused by anionic membrane phospholipids. In the bacterium E. coli, PE plays a role in supporting lactose permease's active transport of lactose into the cell, and may play a role in other transport systems as well. PE plays a role in the assembly of lactose permease and other membrane proteins. It acts as a 'chaperone' to help the membrane proteins correctly folds their tertiary structures so that they can function properly.^{9, 10}

1.2 Liposome in Global Healthcare Market

Present day liposomes are used as ideal drug delivery systems in pharmaceutical industry and this occupies a large are in global pharma market and drug delivery. Among the major areas Western Europe with 13% of the total market dominates followed by North America and South Asia with contribution of 12% and 11% respectively. The area like Oceania is also influenced by the liposomal preparations and contributed approximately 8% to the world market.



Figure 3. Global market of liposome (Ref: Asian J. Pharm. Tech)

In the present scenario USA is the leading producer and trader of liposomal based products. USA holds the top rank with 41% of the total world market. Taiwan and South Korea are among the good competitor for USA. Taiwan with 19% and Korea with 14% stood at

second and third spot. India too appeared on the frame and started the journey along with Hong Kong. Both the countries rank fifth with China holding the fourth spot with share of 10%. France and Spain are those countries which have strong pharmaceutical background and as far as the trading of liposomal products is concerned these countries stand at the sixth spot with individual share of six percent.¹¹ According to Cientifica, a nanotechnology research consultancy of European Space Agency, Nanocarriers will account for \$ 54.4 billion of \$ 136 billion market of Total Nanotechnology based drug delivery system by 2021. In which Liposome will account for \$ 15 billion by 2021 globally. Liposomes also have some disadvantages such as less stability, low solubility, short half-life, high production cost, and quick uptake by cells of reticulo-endothelial system.^{10, 11} But due to Biodegradability, Protective against leakage of Drugs to sensitive tissues, Non toxicity and easy modification, Liposome is most promising versatile drug carrier system used in drug delivery. Development of Liposomal gene delivery system (Lipoplex) has also proven the efficiency of Liposome in genetic engineering^{9, 11}.

Liposomal based drugs are still very expensive due to high cost of production. Liposomes are made up of phospholipids (synthetic lipid) which are very much expensive. Hence to reduce the cost there must be some alternatives for the building blocks of liposome-phospholipids. Phospholipids are generally found in natural living system like plant, animals and microbes¹². The natural phospholipids are described in pharmacopeias and relevant regulatory guidance documentation of the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Natural phospholipids are in general well known to regulatory authorities. In addition, their track record as excipient with very high tolerability and biocompatibility is outstanding. So Extraction and Characterization of phospholipid from natural resources is done and furthermore, Synthesis of Liposome from such natural phospholipid can also be done. Synthetic phospholipids play compared to natural phospholipid (including hydrogenated phospholipids), as derived from the number of drug products containing synthetic phospholipids, a minor role. Natural phospholipids are used in oral, dermal, and parenteral products including liposomes¹³.

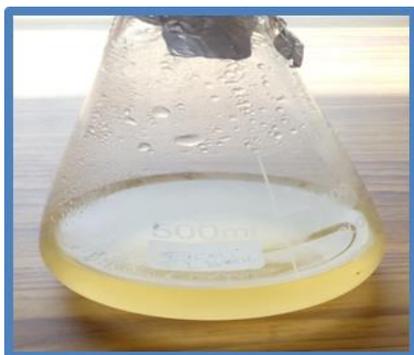
Natural phospholipids instead of synthetic phospholipids should be selected as phospholipid excipients for formulation development, whenever possible, because natural phospholipids are derived from renewable sources and produced with more ecologically friendly processes and are available in larger scale at relatively low costs compared to synthetic phospholipids. Synthetic phospholipids are obtained by a series of complex and time taking process by using harmful and toxic solvents. Such methods are hazards for human health and environment. Also, the cost of extraction is very high and the production is low. Hence, liposomes made from synthetic phospholipids are very much expensive and not accessible to common people. Whereas natural phospholipids are easy to extract, purified and isolate and can be obtained on a large scale with reproducible quality and are less expensive. Phospholipids are well established excipients for pharmaceutical applications. Extraction of such natural phospholipids is crucial to exploits its potential use. Bacterial cell membrane (E.coli) contains abundant Phosphatidylethanolamine which are the main building blocks of Liposome. So,

such natural phospholipid can be used for the synthesis of liposome instead of using synthetic and expensive phospholipid procured from lipid manufacturers. Natural phospholipids are purified from, e.g., soybeans or egg yolk using nontoxic solvent extraction and chromatographic procedures with low consumption of energy and minimum possible waste. Here in this research work, we have extracted the E.coli phospholipids and characterization was done to study its potential application in the development of liposomes and also we report that the bacterial cell can be economical source of phospholipids.

2. Material and Methods

2.1 Extraction of bacterial phospholipids

Bacterial Species E.coli strain MTCC 1687 was procured from CSIR-Institute of Microbial Technology, Chandigarh, India. Bacteria were cultured in 250ml Luria-Bertani (LB) Broth and left of growth in incubator at 37°C at 200rpm for 24 hr. Turbidity was observed after 24 hrs, which shows full growth of bacteria in the culture medium and an optical density was measured at 600nm. Modified Bligh and Dayer Method (Bligh & Dayer, 1959)¹⁴ was used with in which Chloroform and methanol was added in the ratio 1:2 to the bacterial culture. 100 ml bacterial culture solution was taken in flask and 125 ml Chloroform and 250 ml Methanol solution was added to it. The solution was stirred with magnetic stirrer for 10 minutes.



4A. Turbidity observed in culture medium



4B. Stirring solution on magnetic stirrer

Figure 4

After this 125 ml methanol was added and stirred for 1 minute then 125 ml dH₂O was added. Then the sample was centrifuged at 1000 rpm for 10 minutes. Biphasic system was observed. The bottom phase contains phospholipid and upper phase has methanol and water. Pasteur pipette was used to separate the bottom phase from upper phase. About 100ml of sample was obtained. Solvent was evaporated using rotary evaporator. The extracted sample solution contains phospholipid. The solution was filtered and stored in brown vials and labeled. Various analytical tools were used to characterize the sample.

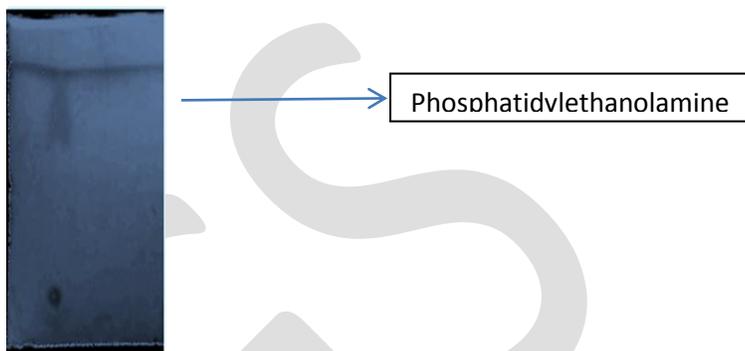
2.2 Characterization of phospholipids

2.2.1 Thin Layer Chromatograph Analysis

Thin Layer Chromatography Analysis of sample was done to detect the presence of phospholipid. Chloroform/Methanol/Ammonia is mixed in the ratio 65:35:4 (v/v/v) and a solvent system were prepared. TLC silica gel plate was dipped into the solution and after ten minutes plate was removed from the solution, dried and watched under UV Light.



5A. TLC Plate



5B. TLC analysis under UV Light

Figure 5

Spotted thick line was observed under UV Light hence, presence of Phosphatidylethanolamine phospholipid was confirmed by Thin Layer Chromatography agreements with previous work.^{15, 16}

2.2.2 UV-Visible Spectra Analysis

UV Visible spectroscopy is an important analytical tool which involves the promotion of electrons from ground state to the higher energy or excited state. UV Spectroscopy is type of absorption spectroscopy in which light of ultra-violet region (200-400nm) is absorbed by the molecule. Absorption of the ultra violet radiations results in the excitation of the electrons from ground state to higher energy state. The energy of UV radiation that is absorbed is equal to the energy difference between the ground state and higher energy state. Absorption of incident radiation by bonding/nonbonding electrons represents a high energy (~100 kCal/mole) transition. This corresponds to a high frequency, i.e. low wavelength, absorption band which is observed at 200 ~ 800 nm in the UV and visible range of detection. In Solution, electronic absorption spectra are found with broad, generally unresolved bands.^{17, 18} UV-Visible spectroscopy of the sample was done on SHIMADZU UV-3600 Plus and data were taken and studied. It is generally observed that phospholipid UV absorption occurs between 200 nm to 400 nm.^{19, 20}

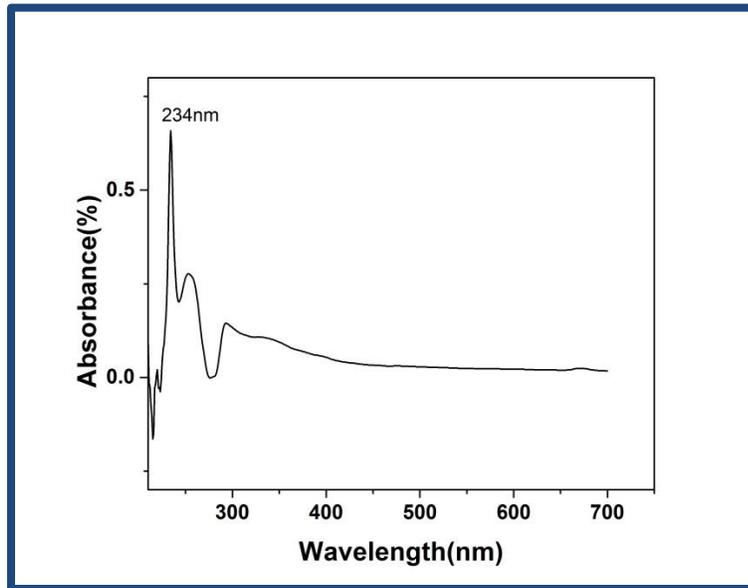


Figure 6. UV-Visible spectra of Phosphatidylethanolamine (PE)

Since, Phospholipid has no chromophore groups so UV detection can be carried out between 200-250 nm. From graph it is observed that a sharp peak is obtained at 234 nm and it is coincident with other experiments²⁰.

2.2.3 Photoluminescence Spectral Analysis

Photoluminescence (PL) spectroscopy is a useful technique for the study and characterization of materials and dynamical processes occurring in materials, specifically the optical properties of the materials. Photoluminescence, which occurs by virtue of electromagnetic radiation falling on matter, may range from visible light through ultraviolet, X-ray, and gamma radiation. It has been shown that, in luminescence caused by light, the wavelength of emitted light generally is equal to or longer than that of the exciting light (i.e., of equal or less energy). As explained below, this difference in wavelength is caused by a transformation of the exciting light, to a greater or lesser extent, to non-radiating vibrational energy of the atoms.²¹

The Characterization of the liquid sample was done on the HORIBA Fluoromax-4 Photoluminescence spectrophotometer taking 234 nm as here it is observed that the emitted radiation has higher wavelength than the excited radiation as it was 234 nm in the case of UV-Visible Spectrophotometer. Here, the wavelength of emitted radiation is 407 nm which is found to be coincident with the previous research papers.^{21, 22}

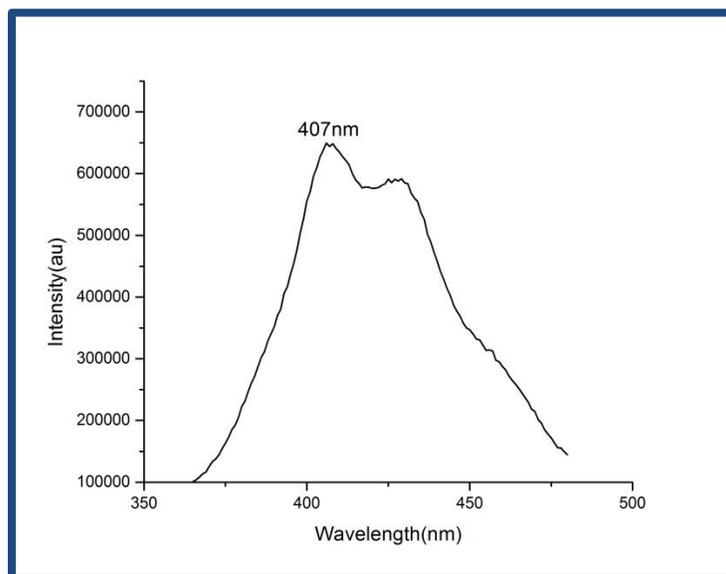


Figure 7. Photoluminescence Spectra of Phospholipid

2.2.4 FTIR Spectra Analysis

Fourier Transform Infra-Red Spectroscopy is one of the most powerful analytical tools for determining the presence of various organic or inorganic functional groups in the sample. Each molecule has specific vibrational frequency on account of the absorption of the IR radiation. FTIR of the sample was done using Parkin Elmer® Spectrum-2 FTIR Spectrophotometer in KBr at a Relative Humidity (RH) of 20 % in the ambient. The resolution was 1cm^{-1} . A Phosphatidylethanolamine molecule has one amine group, phosphate group, a carbonyl group, alcohol and alkyl groups. In FTIR Characterization of the sample these functional groups were detected with their respective vibrational frequencies. These functional groups have vibrational frequency are in coincidence with the FTIR analysis of previous research and experimental work^{23, 24, 25, 26}. The stretching frequency of various functional groups in the sample is given below:

Sl.No	Vibrational Frequencies (In cm^{-1})	Functional Group Present
1.	1261	Phosphate (PO_2^-)
2.	3397	Alcohol (OH^-)
3.	2924	Amine (NH_3^+)
4.	1667	Carbonyl ($\text{C}=\text{O}$)
5.	2853	Hydrocarbon ($\text{C}-\text{H}$)

Table 1 -Vibrational frequencies of functional groups present in the sample.

FTIR data are obtained are in coincident with the data obtained by Pohle et al ²⁷. The results for the phospholipids studied here clearly demonstrate that the conformational properties critically depend on their actual lipid structure, sample composition and sample temperature.

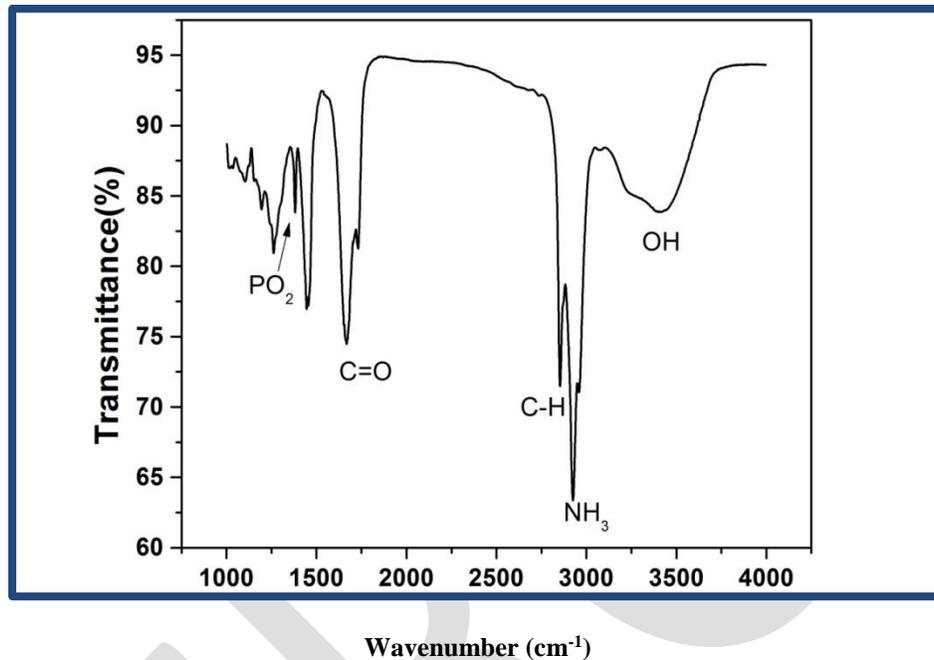


Figure 7. FTIR Spectra of Phospholipid

2.2.5 Particle Size Analysis

For particle size analysis was done on Malvern Zetasizer and a detailed study was done and an average particle size was found to be 965.5 nm by series dilution of the sample. The important colloidal characteristics of an emulsion system include its particle size. Typically, there may be particle size variation in the molecules chosen for making drug delivery systems such as Phosphatidylethanolamine. It is important to opt best size analysis methods for study smaller particle emulsion system. Simple calculations suggested that a micro emulsion was formed only when sufficient water was present to satisfy the hydration of both the phospholipid head groups and the hydroxyl groups of the co-surfactant associated with the droplet. Micro or nano emulsion cannot be considered as inert as drug molecule can alter their properties. Emulsions are generally inherently unstable system thermodynamically and therefore, one would expect them undergo change in their particle size with time ^{28, 29, 30}.

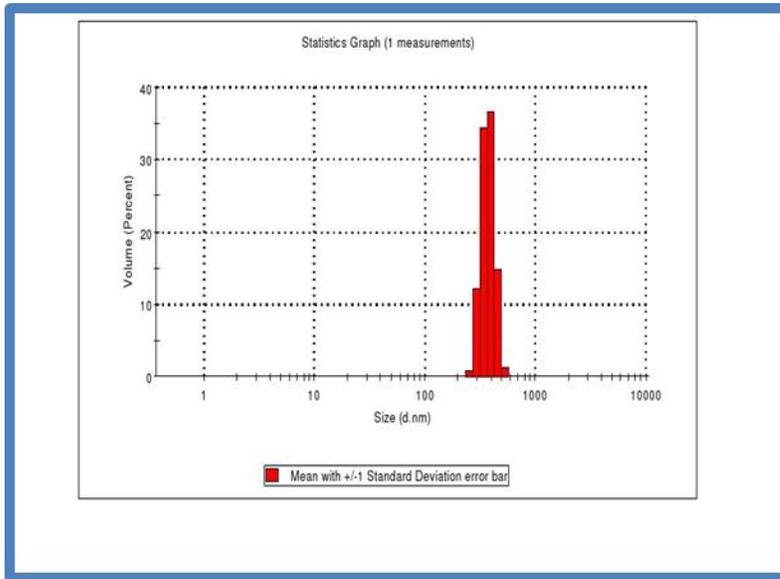


Figure 8 Static Graph of Particle Size

2.2.6 Zeta Potential Analysis

The zeta potential is a key indicator of the stability of colloidal dispersions. The magnitude of the zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is small, attractive forces may exceed this repulsion and the dispersion may break and flocculate. Zeta potential is related to the charge on the surface of the particle, and so influences a wide range of properties of colloidal materials, such as their stability, interaction with electrolytes, and suspension rheology. The Zeta Potential of the sample was done on the Malvern Zetasizer and the potential found to be -7.5mV which is due to higher probability of stability of phospholipid in the emulsion system.^{31, 32, 33, 34}

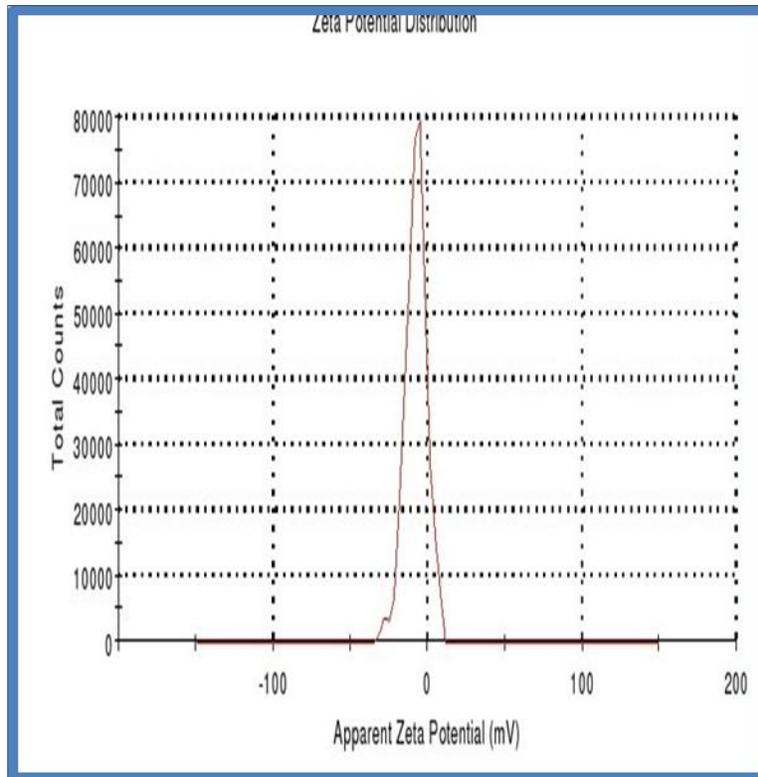


Figure 9. Zeta Potential of Phospholipid molecule (in mV)

3. Result and Discussions:

3.1 Bacterial Culture

It is essential to harvest healthy cells so as to obtain abundant amount of phospholipids. This was indicated by absorbance peak at A_{600} nm that doesn't count for dead cells in the culture. Also, a good turbidity has shown good bacterial colony growth.

3.2 Characterization of phospholipids

Characterization of phospholipids was done by chromatographic method and various spectroscopic techniques. It has shown characteristic peak absorption at 234 nm in UV region and luminescence peak at 407 nm. While the in FTIR spectra analysis the presence of phosphate, alcohol, amine, carbonyl functional groups were shown at a vibrational frequency of 1261, 3397, 2924, 1667, 2853 per centimeter respectively. The particle size and zeta potential of phospholipid molecule were found to be 965.5 nm and -7.5 mV respectively with agreement with previous research works.

4. Conclusion

In this research work we have successfully demonstrated the rapid and easy extraction of phospholipid from bacterial cells. It is important to conclude that such economical way of extraction as compared to expensive synthetic lipids commercially available

from limited manufacturers and suppliers can open the door for innovative research work for liposomes as model drug delivery system. Further work in this area can promote inexpensive but effective investigations of several water soluble drug that can be encapsulated and delivered to the cells. The drug delivery systems prepared from natural resources is quite feasible, economic and having low production cost which also reduced the price of drugs and easily available to common people.

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