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Identification and Exploration of Lecithin from Novel Sources and its Applications†

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Recent research on natural sources such as plants, microbes and other naturally derived compounds have grabbed much attention because of their reduced side effects in treating various ailments and biocompatibility. Technological advancements in the field of medicine have additionally triggered the researchers in developing novel systems for the treatment of many diseases among which cancer research has gained much importance in recent years. Lecithin, a naturally occurring compound present in egg yolk, soya bean oil and milk possess significant anticancer and antioxidant activity. It is composed of phospholipids, fatty acids, carbohydrates and a major constituent of phosphatidylcholine molecule, which is responsible for the normal functioning of nerve cells and cell membranes. The objective of the present work is to extract lecithin compound from Quail's egg using chemical extraction and its characterization using thin layer chromatography, gas chromatography-mass spectroscopy and nuclear magnetic resonance spectroscopy for the structural elucidation of various constituents present in the crude lecithin. Further, the extracted crude lecithin has been assayed for cytotoxic activity in normal cell line using MTT assay.

Keywords: Lecithin, Phosphatidylcholine, Cytotoxicity, MTT assay.

INTRODUCTION

Quail is a small size bird that belongs to pheasant family mainly feed on small insects and plants. There are nearly 45 varieties of Quail species present around the world, among which *Coturnix Japonica*[1] a brown coloured Quail variety is mainly bred in Indian farms for their meat and eggs. Quail's egg is smaller in size, with thin shells and brown spots over it having the weight in the range of 100-150 g (Fig. 1). It consists of 45 % of yellow yolk of its total content which is higher in proportion compared to hen's egg. They are considered to serve as a novel source of lecithin.

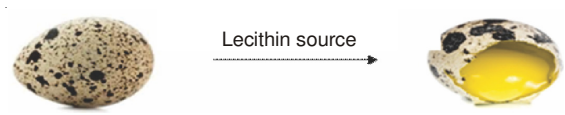

Fig. 1. Quail's egg

Lecithin is complex mixture of phospholipids (PL), fatty acids, carbohydrates with major constituent being phosphatidylcholine compound. Phospholipids grabs much attention because of their unique characteristic and simplicity in use. They are mainly used in pharmaceutical industries for the preparation of creams, gels and other formulations. They have wide applications in cosmetic and food industries too. Soya lecithin one of the major sources of phospholipids is obtained as by-product from oil refining industries which are highly impure in nature and so search of alternative sources for lecithin lead the researchers for the use of eggs. The main aim and objective of this work is to separate lecithin from Quail egg and to characterize the extracted crude lecithin qualitatively and quantitatively using TLC, GC-MS, NMR, FTIR and DSC analytical techniques and finding their potential uses in various therapeutic applications.

EXPERIMENTAL

Quail's eggs were purchased from Aishwarya quail farm, Pondicherry. Chemicals such as silica gel, acetone, chloroform and methanol used were of analytical grade.

Lecithin isolation from quail egg yolk: Modified singleton gray procedure was followed to isolate lecithin from quail egg yolk. Initially eggs were collected and needed yellow yolk was separated manually, followed by chemical extraction method for the isolation of lecithin[2]. To the yolk, solvent mixture of chloroform:methanol in the ratio of 2:1 was added and the mixture was stirred for 2 h at 200 rpm under room temperature. Then the mixture was transferred into separating flask and kept undisturbed for 1 h, later the clear solution settling down

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would contain the required lecithin compound which was collected separately. This solution was at last concentrated using ice cold acetone to make the required lecithin to get precipitated out[3,4]. Using vaccum filtration techniques precipitated lecithin was filtered and the isolated mass was stored in amber colour bottle and kept in deep freezer at (-20 ºC) until further use[5,6].

Qualitative analysis of lecithin by TLC: Thin layer chromatography technique was performed using laboratory procedures. Glass slides were thoroughly cleaned with distilled water followed by ethanol and were dried. Silica gel slurry was prepared by mixing appropriate amount of silica with distilled water and made into paste using mortar and pestle. Prepared slurry was uniformly poured on the glass slides to get a uniform thickness of the TLC plate. Solvent system of chloroform:methanol:water of 65:25:4 ratio was prepared and the TLC chamber was saturated with solvent mixture before starting the experiment for the detection of lecithin[7]. Both the isolated lecithin and soya lecithin (reference standard for crude) were loaded as small spot on the prepared plate and was kept in the saturated solvent chamber for run[8]. The solvent was allowed to move through till three-fourth of the plate. Then they were removed, air dried and placed in iodine chamber for the detection of the sample.

$$R_f \text{ value} = \frac{\text{Distance travelled by the substance}}{\text{distance moved up by the solvent}}$$

Gas chromatography-mass spectroscopy (GC-MS): Gas chromatography mass spectroscopy was done to detect the compounds present in the crude lecithin sample. GC-MS analysis was performed by injecting 1 µL of sample into the oven, which was dissolved in suitable chloroform:methanol (2:1) solvent with helium as a carrier gas maintained at constant flow rate. GC-MS oven was programmed from 70-260 ºC which increases at the rate of 10 ºC/min. Mass spectroscopic analysis was done with an electron ionization at a voltage of 70 eV and the whole process was carried out for 40 min for complete analysis. Finally spectral results of crude sample were obtained with respect to retention time and peak area.

Nuclear magnetic resonance spectroscopy (NMR): NMR spectroscopy is based on the phenomena of nuclear magnetic resonance which gives elaborate information about structural and chemical properties of various compounds present in the crude sample. NMR of both carbon (^{13}C) and proton (^{1}H) were done in order to find carbon and hydrogen atoms containing functional groups of compounds and their pattern of arrangement in them which can provide a clear idea of the crude sample. NMR spectroscopy was performed by dissolving extracted crude lecithin in chloroform (deuterium as isotope of hydrogen) solvent for analysis. External magnetic field was applied under reduced pressure in order to get chemical shift of the compounds[9]. Based on the nuclear magnetic resonance frequencies, molecules in the compound experiences different spins of hybridization and undergoes different stretches which gives the complete structural elucidation of the crude sample.

Fourier transform infrared spectroscopy (FT-IR): Fourier transform infrared spectroscopy was done to find interactions and compatibility between molecules in the sample. Analysis was performed using ATR techniques, which was based on the aspect of measuring the intensity of internally reflected beams from the samples. The procedure consists of dispersing the sample into KBr and it was compressed to thin film using high pressure. Later these films were placed in between the cells of two IR transparent windows for total internal reflections[11,12]. Lecithin sample was made into a thin film and it was placed in between the cells, it was scanned in the wave number range of 4000-400 cm^{-1} and the output spectra obtained were recorded.

Differential scanning calorimetry (DSC-TGA): DSC was performed to find the exothermic and endothermic changes taking place in sample with respect to rise in temperature. It gives information like glass transition temperature, recrystallization and melting point of the sample. TGA was performed to find the weight loss of the sample with respect to time and temperature increase. They also help in determining the thermal stability and degradation nature of the sample as a function of heat. The process was carried out by placing 3-8 mg of crude lecithin in alumina pan under nitrogen (N_2) atmosphere as carrier gas with flow rate of 30 mL/min at a scanning rate of 10-500 ºC/min. Thermograms of sample were obtained with distant peaks with respect to temperature[11,13].

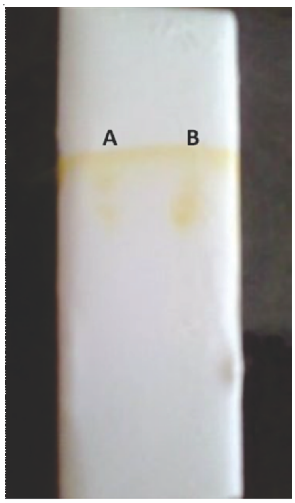
Cytotoxicity efficacy of lecithin: MTT assay is a colourimetric assay done to determine the viability of cells *in vitro* condition[14]. MTT assay was done for crude lecithin to check the cytotoxicity level using NIH3T3 normal cell line at various concentrations. NIH3T3 cell lines were seeded in 96 well plates and incubated for 24 h. Lecithin was loaded in the plate and incubated for 1 h, later plates were removed from incubator and 10 µL of MTT was added and incubated for 4 h. Formazan crystals were formed in the plate and to that 100 µL of isopropyl alcohol was added and kept in shaker till it gets dissolved. The solution was separated and analyzed for absorbance at 570 nm.

$$\text{Inhibition } (\%) = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

RESULTS AND DISCUSSION

Qualitative analysis: Brownish yellow colour spots in the TLC plate confirmed the compounds of quail egg. Lecithin detected is equivalent to soya lecithin which was used as reference standard. R_f value was calculated for both the detected egg lecithin and standard soya lecithin and it was found to be similar in the range of 0.8 (Fig. 2). Isolated compound from quails egg was proved to be lecithin with reference 2.

Molecular mass determination: The output spectrum from GC-MS was analyzed and the identified compounds were given in Table-1. Compounds in the crude sample were identified based on the comparison of spectral data with that of standard compounds and by matching with that to NIST library software preloaded. Spectral peak area gave an idea of probable compounds being present in the sample. It was seen that derivatives of palmatic acid and Stearic acid were found to be in the percentage range of 61.67 and 43.3 which were the group of fatty acids and triglycerides confirming that the isolated mass contains the compounds of lecithin. Structures of these compounds are shown in Fig. 3a,b and the GS-MS spectra of crude lecithin is given in Fig. 4.



A - Qualis egg lecithin (Test)

B - Soya lecithin (Standard)

Fig. 2. TLC profile of crude lecithin with soya lecithin as standard

TABLE-1
COMPOUNDS DETECTED IN CRUDE LECITHIN USING GC-MS ANALYSIS

Compound name	m.w.	m.f.	Probability
4,6-Dimethoxy-7-(5-methyl-1-pyrrolin-2-yl)-2,3-diphenylindole	410	$C_{27}H_{26}N_2O_2$	81.68
Hexadecanamide, N-(2-hydroxyethyl)	299	$C_{18}H_{37}NO_2$	79.56
Isopropyl dodecanoate	242	$C_{15}H_{30}O_2$	79.19
Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$	61.67
Tetradecane	198	$C_{14}H_{30}$	55.53
Pentadecane	212	$C_{15}H_{32}$	54.51
Dotriacontane	450	$C_{32}H_{66}$	52.52
Ether, methyl 1-octadecenyl	282	$C_{19}H_{38}O$	43.3
Heneicosane	296	$C_{21}H_{44}$	35.84
Dodecanamide, N-(2-hydroxyethyl)	243	$C_{14}H_{29}NO_2$	35.71
Isochiapin	350	$C_{19}H_{26}O_6$	25.50
Octacosane	394	$C_{28}H_{58}$	21.96

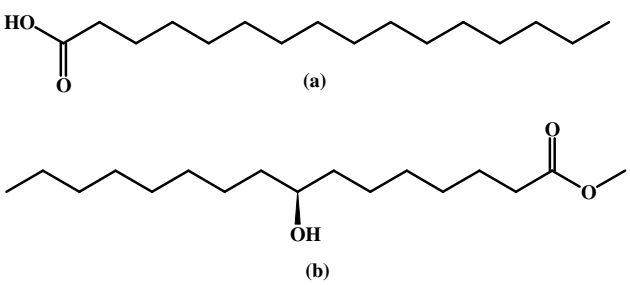


Fig. 3. (a) Structure of palmitic acid. (b) Structure of stearic acid

Nuclear magnetic resonance spectroscopy (NMR): Peaks obtained from the carbon ^{13}C and proton ^{1}H NMR (Fig. 5) revealed the presence of carbon and hydrogen atom containing compounds in them. From ^{13}C NMR spectra chemical shifts in the range of 14.24-54.39 ppm confirmed the presence of non polar alkyl and ester compounds and shift at 76.74-77.59 ppm indicated the presence of carbohydrates groups in them. In proton ^{1}H NMR spectra the chemical shift from 0.837-7.261

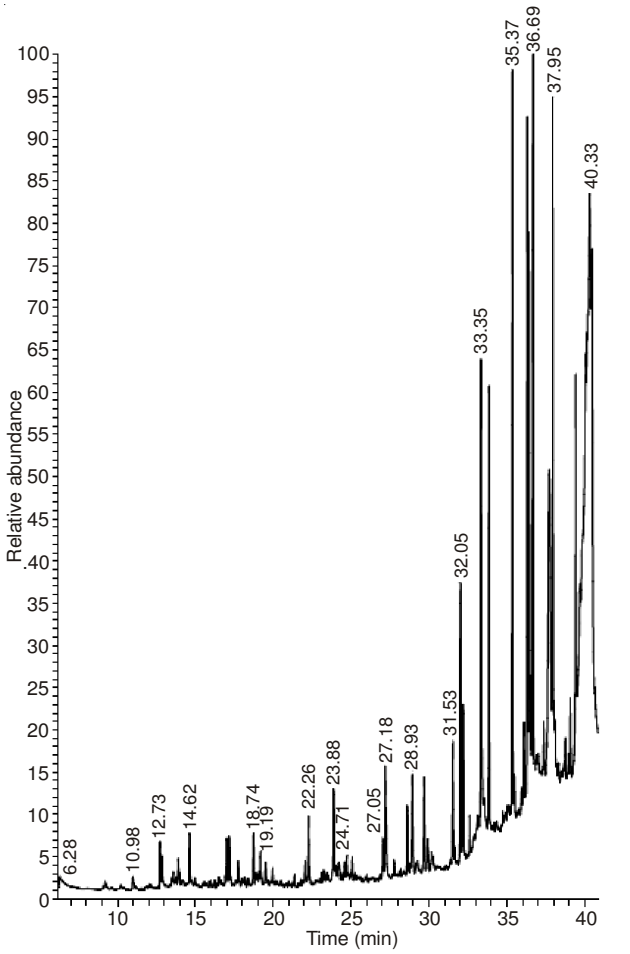


Fig. 4. GC-MS Spectra of crude lecithin

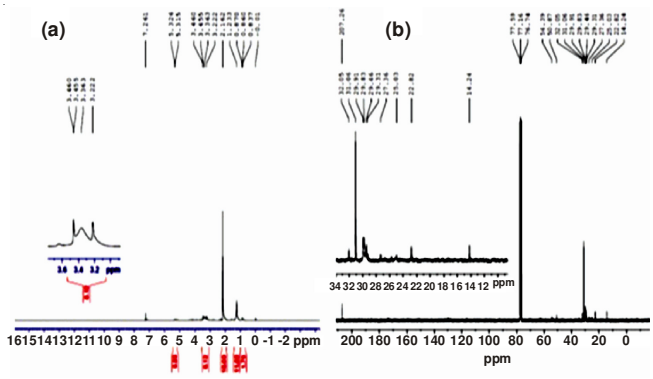


Fig. 5. NMR spectra's of crude lecithin (a) (^{1}H NMR) (b) (^{13}C NMR)

ppm revealed the presence of inorganic orthophosphate and monoester phosphate groups present in the sample[9,12]. It also elaborated various structural elucidations of compounds present in them.

Thermal analysis by DSC-TGA: Analysis of crude lecithin using DSC-TGA techniques elaborates the thermal properties of the sample (Fig. 6). DSC spectral peaks at 75.24, 274.85 and 302.53 ºC show the glass transition temperature, recristallization point and melting point of the crude lecithin sample, respectively. The recorded thermogravimetric spectra showed two distinct degradation points, which confirmed coexistence of more than one degradation process. At lowest temperature of around 80 ºC it showed the breakage of water

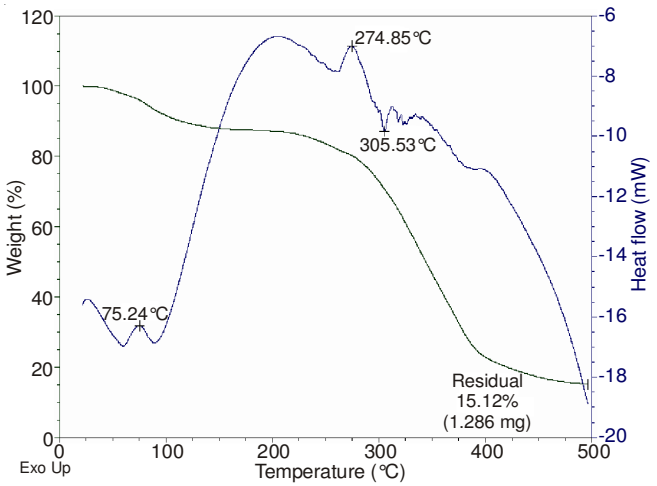


Fig. 6. DSC-TGA curve of crude lecithin

linkages and at higher temperature of 400 ºC showed the complete degradation of the crude lecithin compound[11-13].

Fourier transform infrared spectroscopy: FTIR spectroscopy using ATR technique was done and the spectral curve was obtained for crude lecithin sample. Fig. 7 presents various bands of spectra obtained in between the range of 4000-400 cm^{-1} which were due to different chemical bond stretching and bending of molecules in the sample. Fig. 7 shows the FTIR spectra of crude lecithin and their characteristic functional groups stretching were elaborated in Table-2.

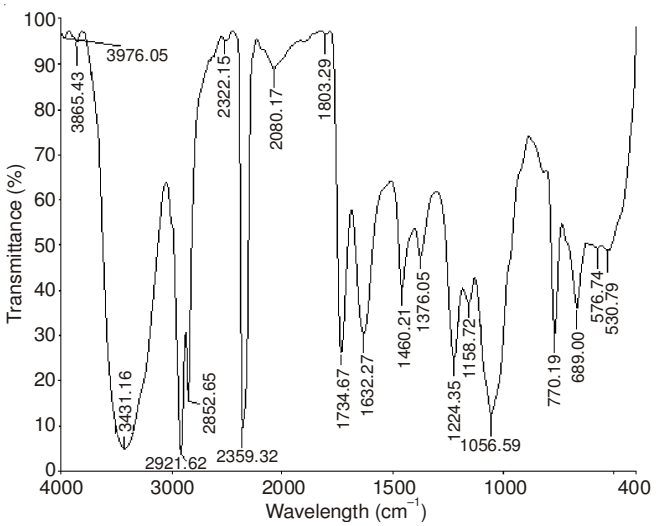


Fig. 7. FTIR spectra of quail egg lecithin crude

TABLE-2
FUNCTIONAL GROUPS PRESENT IN CRUDE LECITHIN AND THEIR MOLECULAR STRETCHING

Functional groups	Molecules	Wave number (cm^{-1})
Alkyl	C-H Stretch	2322.15
Aldehydes	=C-H Stretch	2852.65
	C=O Stretch	1734.67
Esters	C- O Stretch	1056.59
		1124.35
Carbonyl	C=O Stretch	1803.29
Alkenes	C=C Stretch	1632.27
	=C-H Bending	770.19
Alkanes	-C-H Stretch	2852.65
		1376.05
Acid	-O-H Stretch	2921.62
	C-O Stretch	1224.35
Amide	N-H Stretch	3431.16
Aromatic	C=C Stretch	1460.21

Cytotoxicity study of lecithin: Extracted crude lecithin from quail's egg was evaluated for cytotoxic potentials in normal cell line (NIH3T3) using MTT assay techniques (Figs. 8 and 9). *In vitro* studies were carried out using various concentrations such as 12.5, 25, 50, 100 and 200 µg/mL and the percentage of cell viability observed was in the range of 98.44, 97.80, 92.30, 85.07 and 79.12, respectively. From the data obtained it is concluded that lecithin at a higher concentration of 200 µg/mL showed less cytotoxicity in normal cell line. Lecithin as a biocompatible material can be utilized in cosmet ics and in other pharmaceutical preparations and in developing non toxic human friendly products.

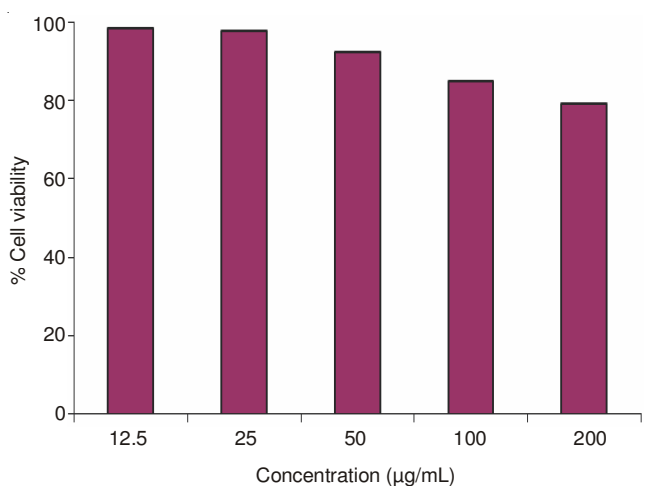


Fig. 8. Percentage of cell viability against lecithin at different concentrations

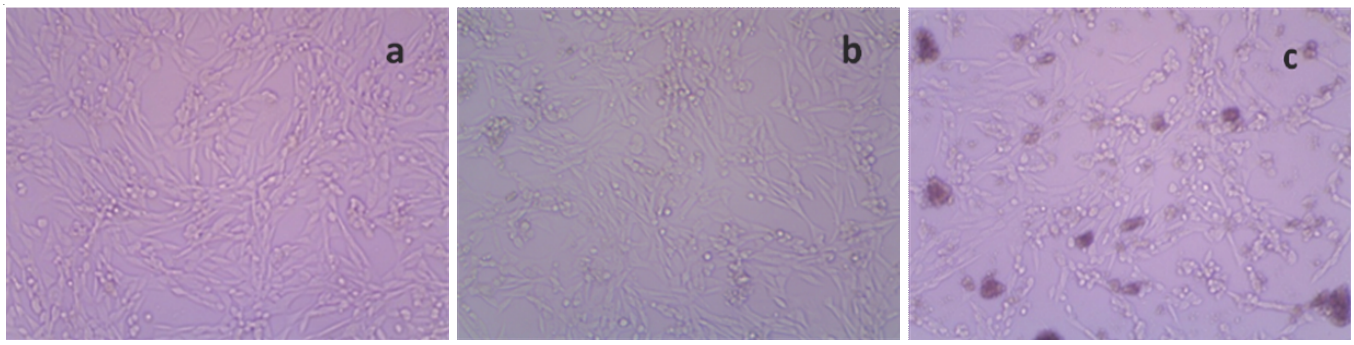


Fig. 9. NIH3T3 cell line treated with Quails egg lecithin. (a) control (b) 12.5 µL/mL (c) 200 µL/mL

Conclusion

Lecithin was successfully isolated from quail egg and analyzed both quantitatively and qualitatively by well established techniques like TLC, GC-MS, NMR, DSC-TGA, FTIR and also evaluated for cytotoxic potential. The data of the results were encouraging and depicted that the isolated compound contains fatty acids and esters in them. NMR and FTIR also confirmed the presence of these components. Present study inferred that it can be used as biocompatible material for various applications in pharmaceutical industry and in cosmetic industries for developing creams and gels.

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