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In-Vitro Hepatoprotection Study, Cytotoxicity assay and Chromatographic Investigation of Phospholipids Fraction Isolated from *Cyperus esculentus* Tubers Growing in Egypt

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Abstract: The phospholipids content of *Cyperus esculentus* tubers were identified by both HPTLC and/or HPLC techniques. The identified phospholipid classes were Phosphatidic acids, Phosphatidylinositol, Phosphatidylethanolamines, Phosphatidylcholine, L-α Phosphatidyl DL-glycerol, Cardiolipin, and L-α-Lysophosphatidylcholine. The hydrolysis of total phospholipids showed that four fatty acids were attached to the phospholipid classes, and identified by GC/MS as Palmitic acid ($C_{16:0}$), Linoleic acid ($C_{18:2}$), Oleic acid ($C_{18:1}$) and Lignoceric acid ($C_{24:0}$). The major fatty acids were Palmitic and Oleic acids. Phosphatidylinositol ($C_{16:0, 18:1}$) was separated and identified by using HPTLC, HPLC and MALDI techniques. *In vitro* method of rat hepatocytes monolayer culture was used for the investigation of hepatoprotective and hepatotoxic effect of phospholipids fraction prepared from *C.esculentus* tubers, which were 25 and <1000 μgmL^{-1} respectively. In addition, the phospholipids fraction did not show any cytotoxic effect on both U937 and PBM cells untill a concentration of 30 μgmL^{-1}.

Key words *Cyperus esculentus*, HPLC, HPTLC, MALDI, Monolayer hepatocytes.

INTRODUCTION

Liver diseases are one of the major national health problems in Egypt. The need for the development of hepatoprotective agents has increased. Many years ago, tubers of *Cyperus* species were used for the treatment of several diseases such as hepatotoxicity (Mehta *et al.*, 1999) and as an anti-oxidative agent (Satoh *et al*., 2004). *C.esculentus* oily extract significantly lowered the serum levels of Alanine transaminase (ALT), Aspartate transaminase (AST) and Alkaline phosphatase (ALP) ($p \leq 0.005$) as compared to CCl_4- treated animals (Ameen *et al*., 1999). Phospholipids (PLs) are the major components of biological membranes. They consist of a polar headgroup, a phosphate group, a glycerol backbone, and two acyl chains of variable length and saturation. Phospholipids play various roles in biological systems. The most important is the formation of a bilayer membrane. This membrane provides structure for cells and organelles and maintains physiochemical properties (Camera *et al*., 2004). Phospholipids are precursors to arachidonic acid, which can be enzymatically broken down to leukotrienes, prostaglandins and thromboxanes that are all important modulators of inflammation. Phospholipids are also precursors to platelet activating factor and inositol triphosphate (Zemski Berry and Murphy 2004). Phospholipids constitute 60% of the lipid mass of a eukaryotic cell membrane (Han and Gross 2005). Therefore, minute changes in phospholipids can lead to changes in the membrane that have major consequences on cell function and viability.

Phospholipids from egg yolk are the major source used by the pharmaceutical nutrition industry. For nutritional supplements, egg phospholipids play no big role, because they have a relatively high cholesterol level and an unfavorable saturated fatty acid profile. Over ten years ago phosphatidylserine was a popular product in Europe, produced from bovine brain. The mad cow disease finally made this product unusable and challenged the researchers to find other ways to produce it from plants as soybeans and marine sources. The history of phospholipids does not end with soybeans. Our work aim to show other edible plants which could be used as a source of phospholipid. The study also shows the hepatoprotective, hepatotoxic and cytotoxic effect of the total phospholipids fraction of *C.esculentus* tubers.

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RESULTS AND DISCUSSION

2.1. Hepatotoxicity:

IC_{50} on monolayer of rat hepatocytes was determined for the total phospholipid extract of *C.esculentus* that showed no toxicity on hepatocytes until 1000 µg mL^{-1} concentration (Figure1).

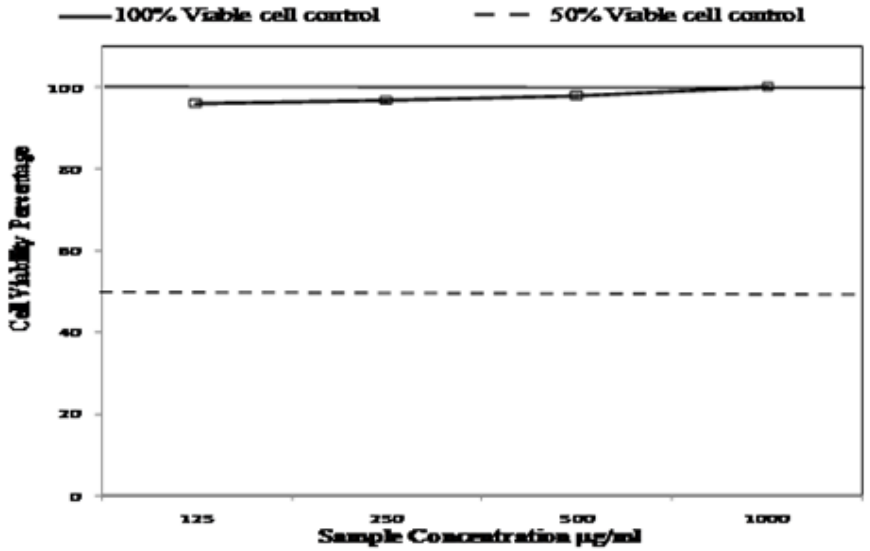

Fig. 1: Viability of Monolayer of Rat Hepatocytes after 2 hrs Treatment with Different Concentrations of the Extracts Using MTT Colourimetric Assay. Each Point Represents the Mean (n=3).

2.2. Evaluation of Hepatoprotective Activity:

The total phospholipids fraction had a hepatoprotective activity at a concentration of 25 µg mL^{-1} (Figure 2).

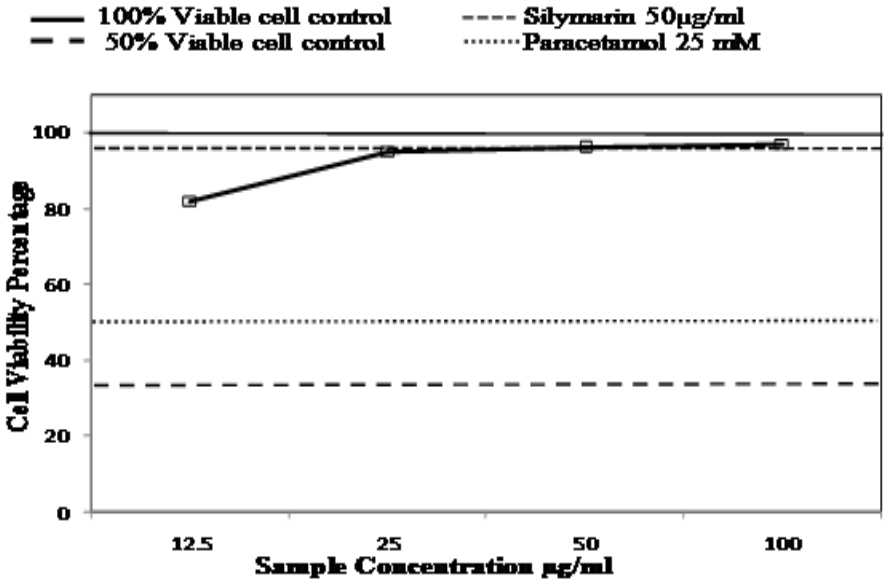

Fig. 2: Viability of Monolayer of Rat Hepatocyte after 2 hrs Treatment with Different Concentrations of the Extracts Followed by Treatment with 25 mM paracetamol for 1hr. in comparison with 50 µg Silymarin as Control Using MTT Colourimetric Assay. Each Point Represents the Mean (n=3).

2.3. Cytotoxicity Evaluation:

The IC_{50} of the phospholipids fraction did not show hepatotoxicity until a concentration of 30µgmL^{-1} for both U937 and PBM cells (Figure 3).

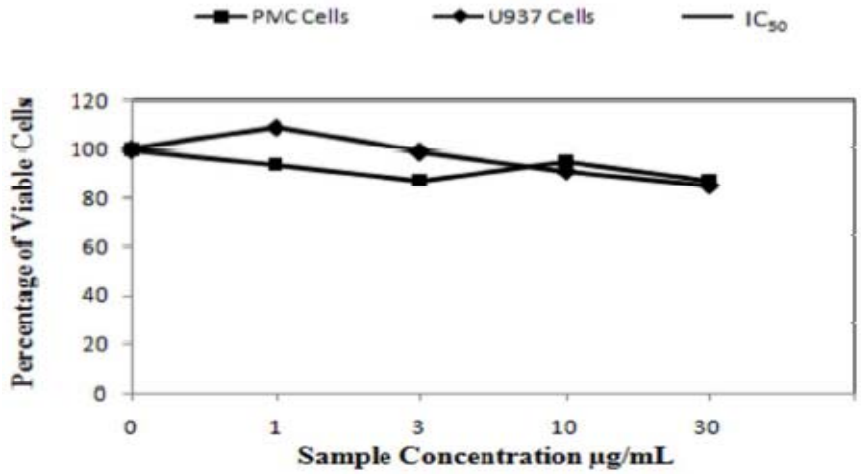

Fig. 3: Viability of U937 Cells after 72 hrs Treatment with Different Concentrations of the Extracts Using XTT Colourimetric Assay. Each Point Represents the Mean ± S.D (n=3).

2.4. HPTLC:

When comparing between methanol fraction and the ten PL references used, it appeared that the methanol fraction contains L-α-Phosphatidyl-DL-glycerol, Cardiolipin, L-α- Phosphatidylethanolamine, L-α-Lysophosphatidylcholine, L-α-Phosphatidylinositol, L-α- Phosphatidylcholine and L-α-Phosphatidic acid. In addition, there are neutral lipids and triglycerides in the fraction. (Figure 4).

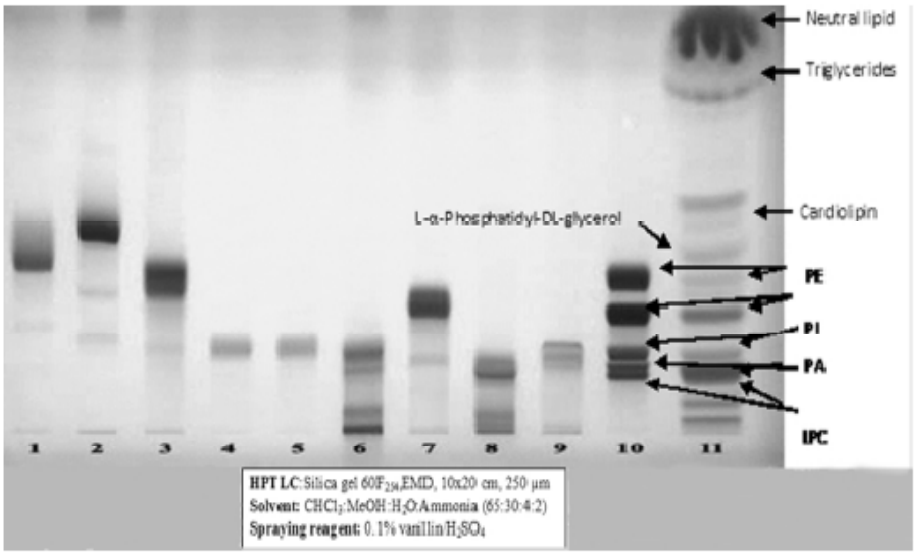

Fig. 4: Track 1: L-α-Phosphatidyl-DL-glycerol (10mg/5ml), Track 2: Cardiolipin (10mg/5ml), Track 3: L-α-Phosphatidylethanolamine (PE) (10mg/5ml), Track 4: Sphingomyelin (10mg/5ml), Track 5: L-α-Lysophosphatidylcholine (LPC) (10mg/5ml), Track 6: L-α-Phosphatidylinositol (PI) (10mg/5ml), Track 7: L-α-Phosphatidylcholine (PC) (10mg/5ml), Track 8: L-α-Phosphatidic Acid (PA) (10mg/5ml), Track 9: L-α-Phosphatidyl-L-serine (10mg/5ml), Track 10: Avandi mixture of five phospholipids, Track 11: Methanol fraction (Total PL classes).

2.5. HPLC:

By comparing the methanol fraction with the standards, four classes of phospholipids were identified. The classes are Phosphatidic acid, Phospatidylinositol, Phosphatidylethanolamine and Phosphatidylcholine.

2.6. Hydrolysis and GC/Mass:

From table (1), it is clear that palmitic acid (saturated fatty acid) and oleic acid (unsaturated fatty acid) are the major fatty acids attached to all phospholipid zones. According to Cevec (1993)**,** the saturated fatty acid group is linked to phospholipid at carbon one of the glycerol backbone and linked to the unsaturated fatty acid at carbon two of the glycerol group. From those results, we concluded that palmitic acid is attached to carbon one of the glycerol group and oleic acid is attached to carbon two of the glycerol group.

Table 1: Gas Liquid Chromatography analysis of the hydrolyzed fatty acid groups from the phospholipid zones.

Compound Name	RR_t (min)	Area Percentage				
		Zone 1	Zone 2	Zone 3	Zone 4	Zone 5
Palmitic acid ($C_{16:0}$)	1	28.2	23.53	31.94	46.47	37.38
Linoleic acid ($C_{18:2}$)	1.16	-	11.72	4.81	7.57	10.88
Oleic acid ($C_{18:1}$)	1.17	71.8	52.10	27.21	45.96	51.74
Unknown	1.46	-	-	36.05	-	-
Lignoceric acid ($C_{24:0}$)	1.76	-	12.65	-	-	-

RR_t = Relative to Palmitic acid retention time = 21.033 min.

2.7. Matrix-Assistant Desorption/Ionization (MALDI):

The molecular weight of zone 1 is 218 m/z. The base peak is 760 m/z and the main peaks were at 734,786,798,808 and 824 m/z.

On considering the R_f value, TLC comparison with phospholipid standards, HPLC and the hydrolysis data of zone 1 showed that it is phosphatidylinositol with palmitic acid connected to Carbone 1 of the glycerol backbone and oleic acid attached to carbon 2 of the glycerol backbone.

3. Experimental:

3.1. Plant Material:

Tubers of *Cyperus esculentus* L. (Family Cyperaceae) were collected from Rashid area (Borg Rashid), Egypt; October 2008. The plant was kindly brought by Prof. Dr. Salah Zarad; *Water Relation and Field Irrigation Department-National Research Center, Cairo, Egypt* and identified by Prof. Dr. Kamal Zaid, Botany Department, Faculty of Science, Cairo University. A voucher specimen by the number of 222, is deposited at the Faculty of Science's herbarium, Cairo University, The plant was washed with tap water, shade dried and finely crushed. The crushed tubers were kept in the refrigerator at 4 °C.

3.2. Isolation and Preparation of Rat Hepatocytes Monolayer Culture:

A primary culture of rat hepatocytes was prepared according to (Seglen, 1976) method, which was modified by (Kiso *et al*., 1983) using a waster male rat (250-300 gm) The rat was obtained from the animal house of the NRC (National Research Center, Cairo). Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Guide, 1985).

3.3. IC_{50} Determination on Rat Hepatocytes Monolayer Culture:

After 22-24 hours, the rat hepatocyte monolayer was washed twice with Phosphate Buffer Saline (PBS). In order to determine IC_{50}, different concentrations were prepared for each sample (100 – 1000 µg mL^{-1}). After two hours of cells incubation with the extract, cell viability was determined using the MTT assay. The assay was performed according to the method of (Mosmann, 1983) modified by Carmichael *et al*., (1987). Absorbance of formasan crystals produced by viable cells was read at 540 and 630 nm dual wave-length using the Automatic Kinetic Microplate Reader (Labsystems Multiskan RC reader). Each experiment was repeated three times and the mean absorption of each concentration was calculated. A graph plotted with x-axis showing the different concentrations of the extract used and the y-axis showing the absorbance percentage of viable cells. The IC_{50} was graphically determined from the concentration that yielded an absorption coinciding with the 50% of cells that received no extract.

3.4. Evaluation of Hepatoprotective Activity:

The primary rat hepatocyte monolayer was prepared as in section 3.2. Different concentrations were prepared from phospholipids fraction (12.5-100 µg mL^{-1}) using serial dilutions technique by dissolving in DMSO (1% maximum concentration). For each concentration, three replicates were carried out; in addition to positive control that was 50 µg mL^{-1} Silymarin. The plate was incubated for 2 hrs at 37 ºC and 5% CO_2, then washed twice with PBS. A 200 µL of 25 mM paracetamol was added to each well. After one hour of cells incubation with the paracetamol, cell viability was determined using the MTT assay as in section 3.3. The concentration of the extract that was able to protect the cells from the hepatotoxic effect of paracetamol by hundred percent was considered hepatoprotective.

3.5. Cytotoxicity Assay on Leukaemia Cell Lines:

Human leukaemia cell lines which named as histiocytic lymphoma cells U937 (ATCC CRL 1593) were maintained in RPMI 1640 medium (Sigma Chemical) supplemented with 1% (v/v) heat-inactivated FCS, 100 units/mL penicillin G, 100 µgmL^{-1} streptomycin and 0.025 µgmL amphotericin B and was incubated at 37 ˚C and 5% CO_2. Leukemia cells (U937) were seeded with a density of 0.25×10^5 cells mL^{-1} in a 96-well plate. For each well 1 µL of different concentrations (1, 3, 10 & 30 µgmL^{-1}) of the plant extract were added in triplicates. After 72 hours of the incubation in 5% CO_2 at 37˚C, a mixture containing 50 µL XTT test solution (2 mL XTT + 40 µL electron-coupling reagent) was added to each well. After 2 hours of incubation at 37˚C in the 5% CO_2 incubator, the absorbance of the samples was measured at dual wavelength of 450 and 650 nm using an ELISA reader (Multiskan EX Labsystem, Helsinki, Finland). The cytotoxicity of the extracts was determined by comparing the response of cell lines to the extracts with the response of cells to DMSO controls. The 50% inhibition concentration (IC_{50}) was the concentration of crude extracts or pure constituents that inhibited the growth of leukaemia cell lines by 50%.

3.6. Cytotoxicity assay on Normal Cells:

Fifty milliliters of peripheral blood were withdrawn from the forearm vein of human volunteers (Brought from the Red Cross). Peripheral blood mononuclear cells (PBMC) were isolated from the puffy coat. The PBMC were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 100 units/mL penicillin G, 100 µgmL^{-1} streptomycin and 0.025 µgmL^{-1} amphotericin B. The assay was conducted on 2.5×10^6 cells mL^{-1} PBMC (200µL/well) in a 96-well microplate, with 1 µL of various concentrations of samples (1, 3, 10 & 30 µgmL^{-1}), in triplicate. Culture plates were incubated at 37˚C for 72 hours in a 5% CO_2 incubator. At the end of incubation time, the cytotoxicity of extract against PBMC was assayed with XTT method. The data calculated according to the method described above. The concentration of 50% inhibition (IC_{50}) was the concentration that achieved 50% cytotoxicity against PBMC.

3.7. Reagents:

Pure analytical- reagents or HPLC- grade solvents were used. Such as chloroform, methanol, acetonitrile, hexane, acetone, ammonia and phosphoric acid, which were purchased from Merck or Fluca. Phospholipid standards, L-α-Phosphatidylcholine (PC), L-α-Phosphatidylethanolamine (PE), L-α-Phosphatidyl-L-serine, L-α-Phosphatidylinositol (PI), L-α-Phosphatidic Acid (PA), L- α-Phosphatidyl-DL-glycerol, L-α-Lysophosphatidylcholine (LPC), Sphingomyelin and Cardiolipin from soybean, were purchased from Sigma (St. Louis, MO, USA). Phospholipid standard for the HPLC analysis, PC, PE, PI and LPC from soybean, were purchased from Avanti,Polar lipid, Inc. Molybdenum blue, spraying reagent for phospholipid detection was purchased from Sigma (St. Louis, MO, USA) and 0.1%Vanillin-H_2SO_4 spraying reagent was prepared according to Reich and Schibli (2006).

3.8. Sample Preparation:

Total lipids were extracted according to Iverson *et al*., (2001), from air dried crushed tubers (50 gm) by sonication (Chua *et al*., 2009) with $CHCl_3$: MeOH (2:1) (200 mL x 2). The extract was dried at 40°C under vacuum. The dried extract (8.65 gm) was dissolved in acetone (Hoevet *et al*., 1968, Rhodes and Lee 1957), filtered and left overnight at 4°C. The residue was separated using cooling centrifuge at 1°C for 10 minutes at 4500 rpm. The acetone soluble and insoluble parts were dried at 40°C under vacuum; giving a yield of (0.85 and 0.56 gm respectively).

3.9. HPLC Analysis:

HPLC technique was performed using Dionex Summit IV, consisting of P680 pump and a fixed wave-length UV 200 nm filter. The flow-rate of the mobile phase was fixed at 2mL min^{-1}.The mobile phase was acetonitril: methanol: phosphoric acid (260:10:3). The stationary phase; is normal phase silica column, 5µm (25cm x 4.6 mm), Supelco with column temperature of 25 °C.

3.10. Mass Analysis:

The MALDI instrument used was; Applied Biosystems Voyager-DE STR (University of Huston, Huston, Texas, USA). Accelerating voltage was 20 kV. The extraction mode used was delayed, with an extraction delay time of 100 nsec and number of laser shots were 50 per spectrum.

3.11. ***Thin Layer Chgromatography:***

The insoluble acetone part is rich in phospholipids (PL). For the purification of PL from neutral lipids and triglycerides, 2gm of total acetone insoluble part was fractionated over 85gm silica gel 60 (Flucka, , particle size 0.2-0.5 mm). The column was eluted with chloroform, acetone and methanol; respectively. PL classes eluted with methanol (60 mg) were identified by comparing their R_f with standards using high performance thin layer

silica plates (silica gel readymade glass plates $60F_{254}$, EMD, 20x20 cm, 250 µm) (Figure 4). Ten mg of methanol fraction were dissolved in 3 mL $CHCl_3$ for preparative TLC (silica gel 60 F_{254}, pre-coated plate for preparative layer chromatography, glass, layer thickness 2mm, 20x20 cm, EMD) and developed by solvent system $CHCl_3$:MeOH:H_2O:Ammonia (65:30:4:2). Five zones of phospholipids where marked, scratched off and eluted with methanol. The eluted 5 zones were dried at 40 °C under vacuum and spotted on TLC. The TLC showed that zones 1,2 and 4 were single spots. HPLC was used to compare the retention time of those three zones with the standard under the same condition.

3.12. GC/ MS Chromatography:

Each phospholipid zone isolated in section 3.11, was hydrolyzed to identify the fatty acid methyl ester groups attached to the glycerol part of the phospholipids using GC/MS. (Table 1).

GC/MS was carried out using an HP5890 Series II Gas Chromatography, HP 5972 Mass Selective Detector and Agilent 6890 Series Autosampler. A Supelco MDN-5S 30 m by 0.25mm capillary column with a 0.5 µm film thickness was used with helium as the carrier gas at a flow rate of 1.0 ml per min. The GC oven temperature was programmed at an initial temperature of 130˚C for 1 minute, then heated up to 300˚C at 5˚C/min and held at 300˚C for 5 minutes. Injector and detector temperatures were set at 250˚C. Mass spectrometry runs in the electron impact (EI) at 70eV. The identification of the chemical constituents was determined by their GC retention times and the interpretation of their mass spectra. The results were confirmed by using the mass spectral library search of the National Institute of Standards and Technology (NIST) database.

4. ***Conclusion:***

The phospholipids content of *C.esculentus* tuber showed a hepatoprotective effect at 25 µg mL^{-1} and did not show any hepatotoxicity or cytotoxicity untill a concentration of (1000 and 100 µg mL^{-1}, respectively). The chemical composition of *C.esculentus* tubers phospholipids were identified with both HPTLC and HPLC technique. From HPTLC, seven classes of phospholipid were identified namely L-α-Phosphatidyl-DL-glycerol, Cardiolipin, L-α- Phosphatidylethanolamine, L-α-Lysophosphatidylcholine, L-α-Phosphatidylinositol, L-α-Phosphatidylcholine and L-α-Phosphatidic acid. At the same time, four classes were identified by HPLC technique namely Phosphatidic acid, Phospatidylinositol, Phosphatidylethanolamine and Phosphatidylcholine.

The four fatty acid group attached to the phospholipid classes was identified by the GC/MS technique after hydrolysis of the phospholipid classes. The identified fatty acids were Palmitic acid ($C_{16:0}$), Linoleic acid ($C_{18:2}$), Oleic acid ($C_{18:1}$) and Lignoceric acid ($C_{24:0}$). The major fatty acids were Palmitic and Oleic acid.

For the first time Phosphatidylinositol ($C_{16:0,\ 18:1}$), was isolated and identified from *C.esculentus* tubers growing in Egypt.

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