Lipid Isolation Process and Study on Some Molecular Species of Polar Lipid Isolated from Seed of Madhuca ellitica

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Keywords: tandem high-resolution mass spectrometry, seeds, sulfoquinovosyldiacylglycerol, phospholipids, lipid molecular species, Madhuca ellitica

Abstract:

This study attempted the lipid extraction process from the seeds of Madhuca ellitica, a lipid-rich plant, and conducted a lipidomic analysis on molecular species of the obtained product. Total lipids of the crude seeds were found to contain 11.2% of polar lipids. The major fatty acids (FAs) of the polar lipids were palmitic (16:0), stearic (18:0), oleic (18:1n-9), and linoleic (18:2n-6) acids, which amounted to 28.5, 12.5, 44.8, and 13.2% of total FAs, respectively. The content and chemical structures of individual molecular species of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidic acid (PA), and sulfoquinovosyldiacylglycerol (SQDG) were determined by HPLC with a tandem high-resolution mass spectrometry (HRMS). The major molecular species were 18:1/18:2 PE, 16:0/18:1 PC, 18:1/18:2 PC, 16:0/18:2 PG, 16:0/18:1 PG, 16:1/18:1 PI, 16:0/18:1 PI, 18:0/18:2 PI, 16:0/18:1 PA, 18:1/18:2 PA, 16:0/18:1 SQDG, and 18:0/18:1 SQDG. The application of a tandem HRMS allows us to determine the content of each isomer in pairs of the monoisotopic molecular species, for example, 18:0/18:2 and 18:1/18:1. The evaluation of the seed polar lipid profile will be helpful for developing the potential of this tree for nutritive and industrial uses.

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Lipid Isolation Process and Study on Some Molecular Species of Polar Lipid Isolated from Seed of *Madhuca ellitica*

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Abstract: This study attempted the lipid extraction process from the seeds of *Madhuca ellitica*, a lipid-rich plant, and conducted a lipidomic analysis on molecular species of the obtained product. Total lipids of the crude seeds were found to contain 11.2% of polar lipids. The major fatty acids (FAs) of the polar lipids were palmitic (16:0), stearic (18:0), oleic (18:1n-9), and linoleic (18:2n-6) acids, which amounted to 28.5, 12.5, 44.8, and 13.2% of total FAs, respectively. The content and chemical structures of individual molecular species of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidic acid (PA), and sulfoquinovosyldiacylglycerol (SQDG) were determined by HPLC with a tandem high-resolution mass spectrometry (HRMS). The major molecular species were 18:1/18:2 PE, 16:0/18:1 PC, 18:1/18:2 PC, 16:0/18:2 PG, 16:0/18:1 PG, 16:1/18:1 PI, 16:0/18:1 PI, 18:0/18:2 PI, 16:0/18:1 PA, 18:1/18:2 PA, 16:0/18:1 SQDG, and 18:0/18:1 SQDG. The application of a tandem HRMS allows us to determine the content of each isomer in pairs of the monoisotopic molecular species, for example, 18:0/18:2 and 18:1/18:1. The evaluation of the seed polar lipid profile will be helpful for developing the potential of this tree for nutritive and industrial uses.

Keywords: *Madhuca ellitica*; lipid molecular species; phospholipids; sulfoquinovosyldiacylglycerol; seeds; tandem high-resolution mass spectrometry

1. Introduction

Buttercup tree or mahua, *Madhuca elliptica* (Pierre ex Dubard) H.J.Lam, *Madhuca longifolia* (Koenig) J.F. Macb (Synonyms, *Madhuca indica* Gmelin, *Madhuca latifolia* Macb., *Bassia latifolia* Roxb.;

Family, Sapotaceae) is a commercially important tree cultivated throughout the subtropical region of the Indo-Pak subcontinent and regarded highly for its flowers, fruits, timber, and seeds [1–3]. Previous reports have pointed out a myriad of valuable compounds, especially compounds of low molecular-weight including saponins, carbohydrates, triterpenoids, steroids, flavonoids, and glycosides, have been isolated from *Madhuca* [4–7]. Among these compounds, carotinoids, existing dominantly in the seeds of *Madhuca*, figure in certain medicinal applications for treatments of skin disease, rheumatism, headache, piles, and use as a laxative [8–10]. The *Madhuca* seed is also rich in fat (up to 58%) [11,12], suggesting potential use of the seed in the manufacture of laundry soaps, lubricants and biodiesel [13,14].

The base of the oil seeds of the plant is constituted of triacylglycerols (TG), which are neutral lipids. Also, natural seed fats contain a number of polar lipids, which are usually separated and lost during oil manufacturing. Among polar lipids, phospholipids hold great importance due to their nutritional value and bioactivities [15]. Each phospholipid class is a complex mixture of individual compounds, termed as "molecular species". Structures of phospholipids consists of the same polar group but different acyl chains of their molecules, which defines their nutritional value and bioactivities of phospholipid molecular species. Therefore, unambiguous identification of molecular species profile of the oilseed, through a lipidomic approach, may introduce possible application in the cosmetic, nutraceutical, and pharmaceutical industries [16,17]. Even though plant lipids and TG molecular species have been studied extensively in terms of compositional structure [18–20], information on the molecular species composition of phospholipids is scanty. For *M. elliptica*, previous studies have revealed its TG molecular species profile in seed fat and its solid and liquid fractions [21]. In addition, fatty acid (FA) composition of polar lipids and individual phospholipid class content of mahua butter from *M. elliptica* have been described [10]. The present work aimed to identify the chemical structure and quantitative composition of phospholipid molecular species from the *M. elliptica* seeds. The molecular species of sulfoquinovosyldiacylglycerols (SQDG), which contain a charged chemical group like phospholipid molecules, were also investigated. High performance liquid chromatography (HPLC), in tandem with high -resolution mass spectrometry (HRMS), was used as a lipidomic method [22]. Some important papers concerning molecular species of polar lipids in marine species, such as corals and red (hot) algae, have been recently published. For example, a previous study that investigated the distribution of tetracosapolyenoic acids (TPA) in molecular species of different phospholipid (PL) classes in the soft corals *Sinularia macropodia* and *Capnella sp.* from shallow waters of Vietnam showed some interesting results [23]. To be specific, Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) were found to be major PL classes of *S. macropodia* and *Capnella sp*. and more than 32 molecular species of these four PL classes were determined by high-resolution tandem mass spectrometry. The major molecular species of PL in both coral species were 18:1e/20:4 PE, 18:0e/20:4 PC, 18:0e/24:5 PS, and 18:0/24:5 PI. In two model red algae, *Polysiphonia* sp. and *Porphyridium sp.*, forms of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), the two most commonly found galactolipids in chloroplast membranes, were determined via positive-ion electrospray ionization/mass spectrometry (ESI/MS) and ESI/MS/MS [24]. In the study of Honda et al. (2019) the characteristics of glycerolipids, which are the substrates of eicosanoids production of *A. chilensis*, were investigated and compared to the reported values of *A. vermiculophyllum*. The results showed that monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylcholine (PC) were the major lipid classes in *A. chilensis* and accounted for 44.4% of the total lipid extract [25].

2. Materials and Methods

2.1. Materials

Ripe fruits of *M. ellitica* (1000 g) were collected from a plantation in Binh Duong province of Vietnam in February 2016 and were botanically identified by Dr. Nguyen Quoc Binh, Vietnam National

Museum of Nature—Vietnam Academy of Science and Technology. Seeds were cleaned, dried in cabinet dryer at 50 °C for 12 h, and stored at 4 °C after collection. Water content (%) in the *M. ellitica* seed was 10.27%. To prepare the sample for analysis, 100 g of sample seeds were finely ground using an electric grinder (KIKA Labortechnik M20), after which they were immediately subjected to lipid extraction. Neutral lipid standards, phospholipid standards (phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidic acid (PA)), and sulfoquinovosyldiacylglycerol (SQDG) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Extraction of Total Lipids (TL)

M. ellitica powder was then extracted for TL following a previously described method of Folch et al. (1957) [26] in which a Soxhlet extractor in combination with a solvent mixture of $CHCl_3$/MeOH (2:1, v/v) and aqueous solution of sodium chloride (0.75%) were used. After being separated overnight at 4 °C, the $CHCl_3$ layer was recovered and dehydrated with sodium sulfate. The extract was filtered and subjected to a rotary evaporator at 40 °C, and TL obtained were dissolved in $CHCl_3$ and stored at −20 °C. The content of TL was determined gravimetrically.

2.3. Lipid Fractionation

A column incorporated with 100 g of silica gel 60 (70–230 mesh, Sigma-Aldrich Co., St. Louis, MO, USA) was loaded with TL (3.4 g). Neutral lipids, glycolipids, and phospholipids were eluted with $CHCl_3$ (450 mL), acetone (230 mL), MeOH (60 mL), and MeOH/water (200 mL, 95:5, v/v). The MeOH-contained fractions were combined, taken to dryness, and polar lipids obtained were dissolved in $CHCl_3$ and stored at −20 °C.

2.4. Analysis of Lipid Class Compositions

One-dimensional thin layer chromatography (TLC) using precoated silica gel plates (6 cm × 6 cm) Sorbfil PTLC-AF-V (Sorbfil, Krasnodar, Russia) was employed to analyze TL composition. The development of plates commenced with *n*-hexan/Et_2O/AcOH (70:20:1, v/v/v) to their full length, followed with $CHCl_3$/MeOH/C_6H_6/NH_4OH (65:30:5:10, v/v/v) to 25% length. Following that, plates were subjected to a stream of air for drying, sprayed with 10% H_2SO_4 in MeOH and heated at 240 °C for 10 min. An image scanner (Epson Perfection 2400 PHOTO) operating in grayscale mode was used to record the chromatograms. An image analysis software (Sorbfil TLC Videodensitometer, Krasnodar, Russia) was used to determine percentages of lipid contents based on band intensity. Peak areas and lipid class percentages were calculated according to Hamoutene (2008) [27]. In the first and second direction of separation of polar lipids using two-dimensional silica gel TLC, $CHCl_3$/MeOH/C_6H_6/28% NH_4OH (65:30:10:6, v/v/v) and $CHCl_3$/MeOH/AcOH/CH_3COCH_3/C_6H_6/H_2O (70:30:4:5:10:1, v/v/v) were used, respectively. Identification of phospholipids on TLC plates was made using aforementioned authentic standards and the specific spray reagents [28]. The phospholipid content was evaluated with spectrophotometry following the digestion with perchloric acid [29].

2.5. Analysis of Lipid Class Compositions

To obtain fatty acid methyl esters (FAMEs), the lipids, contained in a screw cap vial, were treated with 2% H_2SO_4 in MeOH for 2 h at 80 °C under Ar. The purification was performed by TLC development in benzene. The preparation of 4,4-Dimethyloxazoline (DMOX) derivatives of FAs from FAMEs follows the study of Svetashev (2011) [30].

FAMEs were analyzed by gas chromatography (GC) employed on a Shimadzu GC-2010 chromatograph (Kyoto, Japan) at 210 °C. GC was performed in tandem with a flame ionization detector on a SUPELCOWAX 10 (Supelco, Bellefonte, PA, USA) capillary column (30 m × 0.25 mm× 0.25 μm). Temperatures of both injector and detector were maintained at 240 °C. Carrier gas was He at

30 cm/s. The identification of FAMEs was made by comparison with authentic standards (Supelco 37 Component FAME Mix; Supelco, Bellefonte, PA, USA) and a table of equivalent chain-lengths [31].

To identify structural composition of FAs, corresponding methyl esters and DMOX derivatives were analyzed by gas chromatography mass spectrometry (GC–MS) on a Shimadzu GCMS-2010 instrument (Kyoto, Japan) (electron impact at 70 eV) with a MDN-5s (Supelco, Bellefonte, PA, USA) capillary column (30 m × 0.25 mm ID). Carrier gas was He at 30 cm/s. The temperature parameter for analysis of FAMEs started at 160 °C, followed by an increase of 2 °C/min to 240 °C that was held for 20 min. Temperatures for both injector and detector temperatures were maintained at 250 °C. For analysis of GC–MS of DMOX derivatives, the temperature started at 210 °C, increased by 3 °C/min to 270 °C that was held for 40 min. The injector and detector temperatures were 250 °C for this analysis. The mass spectra of FAMEs were compared with the Mass Spectral Library: WILEY275.L and NIST 98 [32].

2.6. Analysis of Molecular Species of Polar Lipids

Determination of chemical structures and molecular species of PL was performed by high performance liquid chromatography, in conjunction with high-resolution mass spectrometry (HPLC−HRMS). In the HPLC separation of the polar lipids, content of Et_3N/AcOH (0.08:1, v/v) was fixed in the solvent system [33]. This permits an efficient electrospray ionization (ESI) and stabilizes ion signal by the simultaneous registration of positive and negative ions. A Shimadzu Prominence liquid chromatograph (Kyoto, Japan) was used to perform the HPLC−HRMS analysis of polar lipids. The instrument was equipped with two LC-20AD pump units, a high pressure gradient forming module, CTO-20A column oven, SIL-20A auto sampler, CBM-20A communications bus module, DGU-20A3 degasser, and a Shim-Pack diol column (50 mm × 4.6 mm ID, 5 µm particle size) (Shimadzu, Kyoto, Japan). Two solvent mixtures, A and B, formed the binary solvent gradient for HPLC separation. The mixture A consisted of *n*-hexane/2-propanol/AcOH/Et_3N (82:17:1:0.08, v/v/v/v) and mixture B consisted of 2-propanol/H_2O/AcOH Et_3N (85:14:1:0.08, v/v/v/v). The acceleration of gradient started at 5% of mixture B and reached 80% throughout the course of 25 min. After 1 min, the composition changed to 5% of mixture B over 10 min and maintained at 5% for another 4 min (the total run time was 40 min). The flow rate was 0.2 mL/min. Determination of lipids was performed by a high resolution tandem ion trap time of flight mass spectrometry with a Shimadzu LCMS-IT-TOF instrument (Kyoto, Japan). The instrument operates at both positive and negative ion mode during each analysis under ESI conditions. Temperature for ion source was set to 200 °C. The range of detection was m/z 100–1200. Negative and positive modes had their potential set at-3.5 and 4.5 kV, respectively. The drying gas (N_2) pressure was 200 kPa. The nebulizer gas (N_2) flow was 1.5 L/min. For identification of lipids, authentic standards were compared with the samples, which was performed using Shimadzu LCMS Solution control and processing software (v.3.60.361). Molecular species of each phospholipid class were determined by HRMS fragmentation pathways in comparison with standards [30]. Individual molecular species within each polar lipid class were quantified with respect to the peak areas for the individual extracted ion chromatograms [34].

3. Results

3.1. Total Lipid Composition

Total lipids (TL) constituted 34.0 ± 0.2% of the seed. The composition of TL is presented in Table 1 and Figure 1. The level of triacylglycerols (TG) was highest (63.2% of TL), followed by waxes (WX, 9.28% of TL), free fatty acids (FFA, 6.14% of TL), and diacylglycerols (DG, 5.17% of TL). Polar lipids constituted 11.2% of TL and contained glycolipids (GL, 6.78% of TL) and phospholipids (PL, 4.45% of TL). Five major classes were found in the total PL including Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA). These five classes amounted to 13.2, 13.1, 7.7, 4.9, and 2.9% of total PL, respectively.

Table 1. Lipid class composition (% of total lipid) and Phospholipid (PL) class (% of total Phospholipids) obtained from *Madhuca ellitica* seeds.

Lipid Class	Content (%)	Phospholipid (PL) Class	Content (%)
Waxes	5.28 ± 0.1	PC	30.7 ± 0.4
Triacylglycerols	63.2 ± 1.5	PE	29.6 ± 0.3
Diacylglycerols	5.17 ± 0.9	PI	17.2 ± 0.1
Free fatty acids	6.14 ± 0.6	PG	10.9 ± 0.2
Glycolipids	5.78 ± 0.2	PA	6.8 ± 0.1
Phospholipids	14.43 ± 1.3	LPE	4.8± 0.1

Results are given as the average of triplicate determinations ± standard deviation. PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PG: phosphatidylglycerol; PA: phosphatidic acid; LPE: Lyso-PE.

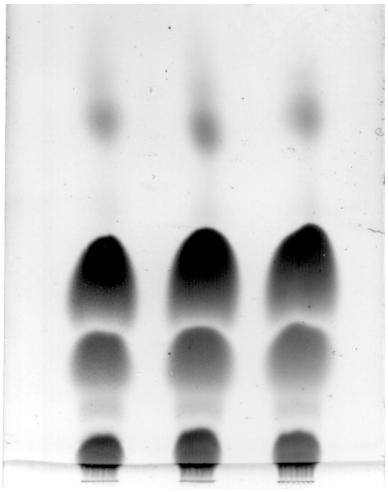

Figure 1. Lipid classes of the *M. ellitica* on Sorbfil thin layer chromatography (TLC).

3.2. Fatty Acid Composition of Total Lipids and Lipid Fractions

The FA composition of TL and their fractions, obtained from of *M. elliptica* seeds, is presented in Table 2. Oleic (18:1n-9), palmitic (16:0), stearic (18:0), and linoleic (18:2n-6) acids were the major FAs, collectively composing more than 98% of the total identified FAs. In general, the prevalence of unsaturated FAs is the major characteristic of the FA composition of TL. Oleic acid was the main FA (49.5%) followed by palmitic acid. The FA profiles of TL, neutral, and polar lipid fractions were similar. In addition, low levels of myristic (14:0), α-linolenic (18:3n-3), and arachidic (20:0) acids were detected.

Table 2. Fatty acid composition (% of total fatty acids) of total, neutral, and polar lipids obtained from *Madhuca ellitica* seeds.

Fatty Acids	Total Lipids	Neutral Lipids	Polar Lipids
14:0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
16:0	24.4 ± 1.2	25.7 ± 1.4	28.5 ± 1.7
16:1n-7	0.1 ± 0.1	0.1 ± 0.1	nd
18:0	11.5 ± 0.5	13.5 ± 2.3	12.5 ± 1.8
18:1n-9	49.5 ± 3.5	46.1 ± 2.8	44.8 ± 2.9
18:2n-6	13.6 ± 1.1	13.6 ± 0.9	13.2 ± 1.3
18:3n-3	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
20:0	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.1
Σ SFA	36.4 ± 0.4	39.8 ± 0.6	41.4 ± 0.5
Σ UFA	63.3 ± 1.7	60.0 ± 1.1	58.3 ± 1.3
SFA/UFA	0.58	0.66	0.71

nd: not detected or under 0.1% of fatty acid composition in each type phospholipid; SFA: saturated fatty acids. UFA: unsaturated fatty acids.

3.3. Chemical Structure of Molecular Species of Polar Lipids

The phospholipid classes of *M. ellitica* seeds were separated by HPLC–HRMS. The retention times of different molecular species of PE, PG, PA, PC, and PI were 5.90–6.87 min, 8.54–8.89 min, 9.18–9.37 min, 9.84–10.35 min, and 12.31–13.28 min, respectively. Among molecular species, four species including PE, PG, PA, and PI were revealed to be detectable at the MS^2 stage, whereas PC species were identified at the MS^3 stage [35]. HRMS spectra of PE molecular species are presented in detail as the examples of MS fragmentation (Figure 2A–D).

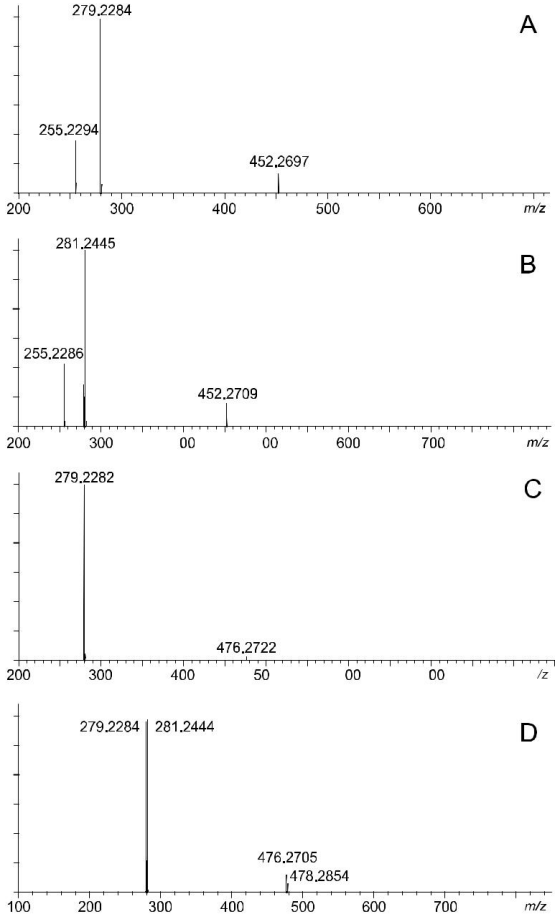

Figure 2. The high-resolution mass spectrometry (HRMS) spectra of (**A**) negative quasi-molecular ions $[M - H]^-$ and (**B**) positive cluster ions $[M + H + (C_2H_5)_3N]^+$ of phosphatidylethanolamine (PE) molecular species, MS^2 spectra of negative quasi-molecular ions $[M - H]^-$ at m/z 714.4988 (**A**), 716.5163 (**B**), 738.4975 (**C**), and 740.5140 (**D**) corresponded to 16:0/18:2 PE, 16:0/18:1 PE, 18:2/18:2 PE, and 18:1/18:2 PE molecular species of phosphatidylethanolamine MS^2 spectra of negative quasi-molecular ions $[M - H]^-$ at m/z 742.5296 (**A**) and 744.5436 (**C**) corresponded to a mixture of 18:1/18:1 PE + 18:0/18:2 PE and 18:0/18:1 PE molecular species of phosphatidylethanolamine from *Madhuca longifolia* seeds. The sub-pictures (**B**) and (**D**) show the spectra in a large scale.

Phosphatidylethanolamine (PE). Six signals of negative quasi-molecular ions $[M - H]^-$ at m/z 714.4988, 716.5163, 738.4975, 740.5140, 742.5296, and 744.5436, as well as six signals of positive cluster ions $[M + H + (C_2H_5)_3N]^+$ at m/z 817.6365, 819.6499, 841.6331, 843.6484, 845.6627, and 847.6749, were observed in the HRMS spectra of PE of *M. ellitica* seeds (Figure 1). Other less intensive peaks on Figure 1 corresponded to the signals of isotopic ions.

Six components were analyzed as described below. The MS^2 spectrum of the ions $[M - H]^-$ of component 1 (m/z 714.4988) (Table 3) contained the signal at m/z 452.2697 formed by the loss of neutral fragment of 262.2291 ($C_{18}H_{30}O$, calculated 262.2297) (Figure 2A). This neutral fragment

corresponded to dehydrated 18:2 acid. The MS^2 spectrum also contained the signals of carboxylate anions of 16:0 and 18:2 acids at m/z 255.2294 ($[C_{16}H_{31}O_2]^-$, calculated 255.2324) and m/z 279.2284 ($[C_{18}H_{31}O_2]^-$, calculated 279.2324), respectively. According to the elemental composition calculated and the value of monoisotopic molecular mass, component 1 was identified as palmitoyl linoleyl glycerophosphoethanolamine, 16:0/18:2 PE (Table 3). The MS^2 spectrum of the ions $[M - H]^-$ of component 2 (m/z 716.5163) contained the signal at m/z 452.2713 formed by the loss of neutral fragment of 264.2450 ($C_{18}H_{32}O$, calculated 264.2453) (Figure 2B). This neutral fragment corresponded to dehydrated 18:1 acid. The MS^2 spectrum also contained the signals of carboxylate anions of 16:0 and 18:1 acids at m/z 255.2294 ($[C_{16}H_{31}O_2]^-$, calculated 255.2324) and m/z 281.2443 ($[C_{18}H_{33}O_2]^-$, calculated 281.2480), respectively. Component 2 was identified as palmitoyl oleoyl glycerophosphoethanolamine, 16:0/18:1 PE (Table 1).

The MS^2 spectrum of the ions $[M - H]^-$ of component 3 (m/z 738.4975) contained the signal at m/z 476.2778 formed by the loss of dehydrated 18:2 acid (262.2197, $C_{18}H_{30}O$, calculated 262.2297) (Figure 2C). Only one signal of carboxylate anions at m/z 279.2283 ($[C_{18}H_{31}O_2]^-$) was found. This result showed that two acyl groups were identical. Component 3 was identified as dilinoleyl glycerophosphoethanolamine, 18:2/18:2 PE (Table 3).

The MS^2 spectrum of the ions $[M - H]^-$ of component 4 (m/z 740.5140) contained the signals at m/z 478.2854 and 476.2705 formed by the loss of neutral fragments of dehydrated 18:2 (262.2286, $C_{18}H_{30}O$, calculated 262.2297) and 18:1 acids (264.2435, $C_{18}H_{32}O$, calculated 264.2453) (Figure 2D). The other two signals at m/z 281.2444 and 279.2284 were attributed to the anions of 18:1 and 18:2 acids, respectively. Component 4 was identified as oleoyl linoleyl glycerophosphoethanolamine, 18:1/18:2 PE (Table 3).

The MS^2 spectrum of component 5 $[M - H]^-$, m/z 742.5296) contained six signals formed two compact groups (Figure 2A). Three major signals at m/z 283.2545 ($[C_{18}H_{35}O_2]^-$), 281.2443 ($[C_{18}H_{33}O_2]^-$), and 279.2285 ($[C_{18}H_{31}O_2]^-$) (Figure 2B) corresponded to the anions of 18:0, 18:1, and 18:2 acids, respectively. Three weak signals at m/z 478.2866, 476.2707, and 460.2808 were formed by the loss of neutral fragments of 264.2430 ($C_{18}H_{32}O$, calculated 264.2453), 266.2589 ($C_{18}H_{34}O$, calculated 266.261), and 282.2488 ($C_{18}H_{34}O_2$, calculated 282.2559), respectively.

These neutral fragments also originated from 18:1 and 18:2 acids. Component 5 was identified as a mixture of isomeric dioleoyl glycerophosphoethanolamine (18:1/18:1 PE) and stearoyl linoleyl glycerophosphoethanolamine, 18:0/18:2 PE (Table 3). The ratio between 18:1/18:1 PE and 18:0/18:2 PE was 4.4/1 according to the intensity of the corresponding ion peaks. The MS^2 spectrum of the ions $[M - H]^-$ of component 6 (m/z 744.5436) showed the signals at m/z 480.3046, 283.2577, and 281.2441 (Figure 2C,D). Similar to components 1 and 2, component 6 was identified as stearoyl oleoyl glycerophosphoethanolamine, 18:0/18:1 PE (Table 3).

Thus, seven PE molecular species, such as 16:0/18:2 PE, 16:0/18:1 PE, 18:2/18:2 PE, 18:1/18:2 PE, 18:1/18:1 PE, 18:0/18:2 PE, and 18:0/18:1 PE, were identified.

Phosphatidylcholine (PC). Six signals of negative acetylated molecular ions $[M + CH_3COO]^-$ at m/z 816.5760, 818.5889, 820.5943, 840.5712, 842.5888, and 844.6059, as well as six corresponded signals of positive quasi-molecular ions $[M+ H]^+$, were observed in the HRMS spectra of PC of *M. ellitica* seeds (Table 4). The ions $[M + CH_3COO]^-$ of each component lost methyl acetate (CH_3COOCH_3) at the MS^2 state. The subsequent fragmentation formed the anions characterized acyl groups of most components at the MS^3 stage. In MS^3 spectra of ions $[M - H - CH_3COOCH_3]^-$, the signals at m/z 255.2320, 283.2602, 281.2474, and 279.2332 indicated the presence of acyl groups of 16:0, 18:0, 18:1, and 18:2 acids, respectively. According to the MS^3 data, the elemental composition calculated, and the value of mono-isotopic molecular mass, we identified components 1, 2, and 5 as 16:0/18:2 PC, 16:0/18:1 PC, and 18:1/18:2 PC, respectively (Table 4). Component 6 were identified as a mixture of 18:1/18:1 PC and 18:0/18:2 PC with the ratio 1.58:1. We could not observe MS^3 fragmentation of components 3 and 4 because of their low concentration. We suggest that these components are 16:0/18:0 PC and 18:2/18:2 PC on the base of their elemental composition and mono-isotopic molecular mass (Table 4).

Table 3. Molecular species of phosphatidylethanolamine (PE) from *Madhuca ellitica* seeds.

No.	Molecular Species	ESI-MS		Monoisotopic Molecular Mass		Molecular Formula	MS^2		
							Fragment Ion $[M - H - X]^-$ *		X
		$[M + H + Et_3N]^+$ m/z	$[M - H]^-$ m/z	Measured	Calculated		m/z	Composition	
1	16:0/18:2 PE	817.6365	714.4988	715.5061	715.5152	$C_{39}H_{74}NO_8P$	452.2697	$C_{21}H_{44}NO_7P$	$C_{18}H_{30}O$
							279.2284	$C_{18}H_{31}O_2$	$C_{21}H_{43}NO_6P$
							255.2294	$C_{16}H_{31}O_2$	$C_{23}H_{43}NO_6P$
2	16:0/18:1 PE	819.6499	716.5163	717.5236	717.5309	$C_{39}H_{76}NO_8P$	452.2713	$C_{21}H_{44}NO_7P$	$C_{18}H_{32}O$
							281.2443	$C_{18}H_{33}O_2$	$C_{21}H_{43}NO_6P$
							255.2294	$C_{16}H_{31}O_2$	$C_{23}H_{45}NO_6P$
3	18:2/18:2 PE	841.6331	738.4975	739.5048	739.5152	$C_{41}H_{74}NO_8P$	476.2778	$C_{23}H_{44}NO_7P$	$C_{18}H_{30}O$
							279.2283	$C_{18}H_{31}O_2$	$C_{23}H_{43}NO_6P$
4	18:1/18:2 PE	843.6484	740.5140	741.5213	741.5309	$C_{41}H_{76}NO_8P$	478.2854	$C_{23}H_{46}NO_7P$	$C_{18}H_{30}O$
							476.2705	$C_{23}H_{44}NO_7P$	$C_{18}H_{32}O$
							281.2444	$C_{18}H_{33}O_2$	$C_{23}H_{43}NO_6P$
							279.2284	$C_{18}H_{31}O_2$	$C_{23}H_{45}NO_6P$
5	18:1/18:1 PE (4.4) 18:0/18:2 PE (1)	845.6627	742.5296	743.5369	743.5465	$C_{41}H_{78}NO_8P$	478.2866	$C_{23}H_{46}NO_7P$	$C_{18}H_{32}O$
							476.2707	$C_{23}H_{44}NO_7P$	$C_{18}H_{34}O$
							460.2808	$C_{23}H_{44}NO_6P$	$C_{18}H_{34}O_2$
							283.2545	$C_{18}H_{35}O_2$	$C_{23}H_{43}NO_6P$
							281.2443	$C_{18}H_{33}O_2$	$C_{23}H_{45}NO_6P$
							279.2285	$C_{18}H_{31}O_2$	$C_{23}H_{47}NO_6P$
6	18:0/18:1 PE	847.6749	744.5436	745.5509	745.5622	$C_{41}H_{80}NO_8P$	480.3046	$C_{23}H_{48}NO_7P$	$C_{18}H_{32}O$
							283.2577	$C_{18}H_{35}O_2$	$C_{23}H_{45}NO_6P$
							281.2441	$C_{18}H_{33}O_2$	$C_{23}H_{47}NO_6P$

ESI-MS: electrospray ionization-mass spectrometry. * Precursor ion $[M - H]^-$.

Table 4. Molecular species of phosphatidylcholine (PC) from *Madhuca ellitica* seeds.

No.	Molecular Species	ESI-MS	Monoisotopic Molecular Mass		Molecular Formula	MS^2	MS^3	
		$[M + CH_3COO]^-$ m/z	Measured	Calculated		Fragment Ion * $[M + CH_3COO - C_3H_6O_2]^-$ m/z	Fragmentation **	
							m/z	Composition
1	16:0/18:2 PC	816.5760	757.5621	757.5622	$C_{42}H_{80}NO_8P$	742.5389	279.2274	$C_{18}H_{31}O_2$
2	16:0/18:1 PC	818.5889	759.5750	759.5778	$C_{42}H_{82}NO_8P$	744.5532	281.2462 255.2320	$C_{18}H_{33}O_2$ $C_{16}H_{31}O_2$
3	16:0/18:0 PC	820.5943	761.5804	761.5935	$C_{42}H_{84}NO_8P$	746.5599	ND	-
4	18:2/18:2 PC	840.5712	781.5573	781.5622	$C_{44}H_{80}NO_8P$	766.5309	ND	-
5	18:1/18:2 PC	842.5888	783.5749	783.5778	$C_{44}H_{82}NO_8P$	768.5511	281.2432 279.2352	$C_{18}H_{33}O_2$ $C_{18}H_{31}O_2$
6	18:1/18:1 PC (1.58) 18:0/18:2 PC (1)	844.6059	785.5920	785.5935	$C_{44}H_{84}NO_8P$	770.5674	283.2602 281.2474 279.2332	$C_{18}H_{35}O_2$ $C_{18}H_{33}O_2$ $C_{18}H_{31}O_2$

ND: not detected. * Precursor ion $[M+CH_3COO]^-$. ** Precursor ion $[M+CH_3COO–C_3H_6O_2]^-$.

Phosphatidylinositol (PI). The main molecular species of PI from *M. ellitica* seeds produced five negative quasi-molecular ions $[M - H]^-$ at m/z 833.5188 ($[C_{43}H_{78}O_{13}P]^-$), 835.5328 ($[C_{43}H_{80}O_{13}P]^-$), 857.5155 ($[C_{45}H_{78}O_{13}P]^-$), 861.5463 ($[C_{45}H_{80}O_{13}P]^-$), 863.5635 ($[C_{45}H_{82}O_{13}P]^-$) (Table 5). These five components constituted more than 96% of total PI. Positive quasi-molecular ions also formed.

MS^2 fragmentation of the ions $[M - H]^-$ of PI were more complex than that of PE or PC and gave more ions. Generally, the MS^2 spectra the ions $[M - H]^-$ of PI contained the signals of FA carboxylate anions and several fragments, which arise from the loss of each FA, each dehydrated FA, two FAs, two dehydrated FAs, and different combinations of inositol and acyl fragments.

As an example, MS^2 fragmentation of the ions $[M - H]^-$ of component 1 (m/z 833.5188) is explained in detail (Table 5). The ions at m/z 281.2474 $[C_{18}H_{33}O_2]^-$, 279.2307 $[C_{18}H_{31}O_2]^-$, and 255.2346 $[C_{16}H_{31}O_2]^-$ corresponded to carboxylate anions of 18:1, 18:2, and 16:0, respectively.

The loss of neutral acids 16:1, 16:0, and 18:2 gave ions at m/z 579.2901 ($[M - H - C_{16}H_{32}O_2]^-$), 577.2784 ($[[M - H - C_{16}H_{30}O_2]^-$), and 553.2813 ($[M - H - C_{18}H_{32}O_2]^-$, calculated 553.2783), respectively. The loss of dehydrated 18:2 and 16:0 led to the formation of ions at m/z 571.2904 ($[M - H - C_{18}H_{30}O]^-$) and 595.2769 ($[M - H - C_{18}H_{30}O]^-$), respectively. The appearance of ions at m/z 297.0388 (calculated 297.0381) was caused by the simultaneous loss of 16:0 and 18:2 (or 16:1 and 18:1). The ions at m/z 315.0464 (calculated 315.0487) were formed by the simultaneous loss of dehydrated 16:0 and 18:2 (or 16:1 and 18:1). The loss of inositol and acyl fragments, namely ($[C_6H_{10}O_5 + C_{16}H_{30}O_2]$, ($[C_6H_{10}O_5 + C_{16}H_{32}O_2]$, and ($[C_6H_{10}O_5 + C_{18}H_{32}O_2]$, gave the ions at m/z 417.2418, 415.2268, and 391.2253, respectively, characteristic for PI. Thus, compound 1 was identified as a mixture of 16:1/18:1 PI and 16:0/18:2 PI. Similar to our approach described above, compounds 2, 3, and 5 were identified as individual 16:0/18:1 PI, 18:2/18:2 PI, and 18:0/18:1 PI, respectively, whereas compound 4 contained a mixture of 18:0/18:2 PI and 18:1/18:1 PI (Table 5).

Phosphatidylglycerol (PG). Molecular species of PG of *M. ellitica* seeds were identified according to the monoisotopic molecular mass of negative quasi-molecular ions $[M - H]^-$, their elemental compositions, and MS^2 fragmentation indicated acyl groups of PG molecules. Six major signals of the negative quasi-molecular ions $[M - H]^-$ at m/z 745.4944, 747.5092, 749.5212, 771.5092, 773.5253, and 775.5380 were observed in the HRMS spectra (Table 6). MS^2 spectra of the ions $[M - H]^-$ contained the signals of carboxylate anions of 16:0 ($[C_{16}H_{31}O_2]^-$), 18:0 ($[C_{18}H_{35}O_2]^-$), 18:1($[C_{18}H_{33}O_2]^-$), and 18:2 ($[C_{18}H_{31}O_2]^-$) acids. The signals of the ions, which lost neutral FAs, or dehydrated FAs, or glycerol fragment ($C_3H_6O_2$) were also observed in some MS^2 spectra of the ions $[M - H]^-$ of PI molecular species. According to MS/MS data, the molecular species 16:0/18:0 PG, 16:0/18:1 PG, 16:0/18:2 PG, 18:0/18:1 PG, 18:0/18:2 PG, 18:1/18:1 PG, and 18:1/18:2 PG were identified (Table 6). These seven molecular species constituted about 99% of total PG.

Phosphatidic acid (PA). Six signals of the negative quasi-molecular ions $[M - H]^-$ at m/z 671.4643, 673.4798, 695.4660, 697.4803, 699.4949, and 701.5098 were detected in the HRMS spectra of molecular species of PA from *M. ellitica* seeds (Table 7). Similar to other phospholipid classes, MS^2 spectra of the ions $[M - H]^-$ of PA contained the signals of carboxylate anions of 16:0 ($[C_{16}H_{31}O_2]^-$), 18:0 ($[C_{18}H_{35}O_2]^-$), 18:1($[C_{18}H_{33}O_2]^-$), and 18:2 ($[C_{18}H_{31}O_2]^-$) acids. The signals of the ions $[M - H]^-$, which lost two neutral FAs or two dehydrated FAs, were also observed in these MS^2 spectra. The components, which produced ions related to three FAs, were considered as a mixture of two isotopes. For example, compound 5 (m/z 699.4949, $[M - H]^-$) was identified as a mixture of 18:1/18:1 PA and 18:0/18:2 PA. On the whole, the molecular species 16:0/18:1 PA, 16:0/18:2 PA, 18:0/18:1 PA, 18:0/18:2 PA, 18:1/18:1 PA, 18:1/18:2 PA, and 18:2/18:2 PA were identified (Table 7).

Table 5. Molecular species of phosphatidylinositol (PI) from *Madhuca ellitica* seeds.

No.	Molecular Species	ESI-MS	Monoisotopic Molecular Mass		Molecular Formula	MS^2	
		$[M-H]^-$ m/z	Measured	Calculated		Fragment Ion * $[M-H-X]^-$ m/z	X
1	16:1/18:1 PI 16:0/18:2 PI	833.5188	834.5256	834.5258	$C_{43}H_{79}O_{13}P$	595.2769 579.2901 577.2784 571.2904 553.2813 417.2418 415.2268 409.2352 391.2253 297.0388 281.2474 279.2307 255.2346	$C_{16}H_{30}O$ $C_{16}H_{30}O_2$ $C_{16}H_{32}O_2$ $C_{18}H_{30}O$ $C_{18}H_{30}O_2$ $C_{22}H_{40}O_7$ ($C_6H_{10}O_5 + C_{16}H_{30}O_2$) $C_{22}H_{42}O_7$ ($C_6H_{10}O_5 + C_{16}H_{32}O_2$) $C_{24}H_{40}O_6$ ($C_6H_{10}O_5 + C_{18}H_{30}O$) $C_{24}H_{42}O_7$ ($C_6H_{10}O_5 + C_{18}H_{32}O_2$) $C_{34}H_{64}O_4$ ($C_{16}H_{32}O_2 + C_{18}H_{32}O_2$) $C_{25}H_{45}O_{11}P$ $C_{25}H_{47}O_{11}P$ $C_{27}H_{47}O_{11}P$
2	16:0/18:1 PI	835.5328	836.5401	836.5415	$C_{43}H_{81}O_{13}P$	597.3036 579.2939 571.2876 553.2805 435.2438 417.2389 409.2351 391.2250 297.0373 281.2484 255.2337	$C_{16}H_{30}O$ $C_{16}H_{32}O_2$ $C_{18}H_{32}O$ $C_{18}H_{34}O_2$ $C_{22}H_{40}O_6$ ($C_6H_{10}O_5 + C_{16}H_{30}O$) $C_{22}H_{42}O_7$ ($C_6H_{10}O_5 + C_{16}H_{32}O_2$) $C_{24}H_{42}O_6$ ($C_6H_{10}O_5 + C_{18}H_{32}O$) $C_{24}H_{44}O_7$ ($C_6H_{10}O_5 + C_{18}H_{34}O_2$) $C_{34}H_{64}O_4$ ($C_{16}H_{32}O_2 + C_{18}H_{34}O_2$) $C_{25}H_{47}O_{11}P$ $C_{27}H_{49}O_{11}P$
3	18:2/18:2 PI	857.5155	858.5228	858.5258	$C_{45}H_{79}O_{13}P$	577.2805 415.2264 279.2337	$C_{18}H_{32}O_2$ $C_{24}H_{42}O_7$ ($C_6H_{10}O_5 + C_{18}H_{32}O_2$) $C_{27}H_{47}O_{11}P$

Table 5. *Cont.*

No.	Molecular Species	ESI-MS	Monoisotopic Molecular Mass		Molecular Formula	MS^2	
		$[M-H]^-$ m/z	Measured	Calculated		Fragment Ion * $[M-H-X]^-$ m/z	X
4	18:0/18:2 PI 18:1/18:1 PI	861.5463	862.5536	862.5571	$C_{45}H_{81}O_{13}P$	599.3202	$C_{18}H_{30}O$
						597.3037	$C_{18}H_{32}O$
						595.2894	$C_{18}H_{34}O$
						581.3095	$C_{18}H_{32}O_2$
						579.2945	$C_{18}H_{34}O_2$
						577.2775	$C_{18}H_{36}O_2$
						437.2665	$C_{24}H_{40}O_6$ ($C_6H_{10}O_5 + C_{18}H_{30}O$)
						435.2511	$C_{24}H_{42}O_6$ ($C_6H_{10}O_5 + C_{18}H_{32}O$)
						433.2306	$C_{24}H_{44}O_6$ ($C_6H_{10}O_5 + C_{18}H_{34}O$)
						419.2571	$C_{24}H_{42}O_7$ ($C_6H_{10}O_5 + C_{18}H_{32}O_2$)
						417.2394	$C_{24}H_{44}O_7$ ($C_6H_{10}O_5 + C_{18}H_{34}O_2$)
						415.2241	$C_{24}H_{46}O_7$ ($C_6H_{10}O_5 + C_{18}H_{36}O_2$)
						297.0370	$C_{36}H_{68}O_4$
						283.2622	$C_{27}H_{45}O_{11}P$
						281.2484	$C_{27}H_{47}O_{11}P$
						279.2328	$C_{27}H_{49}O_{11}P$
5	18:0/18:1 PI	863.5635	864.5708	864.5728	$C_{45}H_{83}O_{13}P$	701.5027	$C_6H_{10}O_5$
						599.3203	$C_{18}H_{32}O$
						597.3032	$C_{18}H_{34}O$
						581.3097	$C_{18}H_{34}O_2$
						579.2943	$C_{18}H_{36}O_2$
						419.2573	$C_{24}H_{44}O_7$ ($C_6H_{10}O_5 + C_{18}H_{34}O_2$)
						417.2393	$C_{24}H_{46}O_7$ ($C_6H_{10}O_5 + C_{18}H_{36}O_2$)
						297.0376	$C_{36}H_{70}O_4$
						283.2627	$C_{27}H_{47}O_{11}P$
						281.2485	$C_{27}H_{49}O_{11}P$

* Precursor ion $[M-H]^-$.

Table 6. Molecular species of phosphatidylglycerol (PG) from *Madhuca ellitica* seeds.

No.	Molecular Species	ESI-MS	Monoisotopic Molecular Mass		Molecular Formula	MS^2	
		$[M-H]^-$ m/z	Measured	Calculated		Fragment Ion * $[M-H-X]^-$ m/z	X
1	16:0/18:2 PG	745.4944	746.5017	746.5098	$C_{40}H_{75}O_{10}P$	391.2224 279.2303 255.2336	$C_{21}H_{38}O_4$ ($C_3H_6O_2 + C_{18}H_{32}O_2$) $C_{22}H_{43}O_8P$ $C_{24}H_{43}O_8P$
2	16:0/18:1 PG	747.5092	748.5164	748.5254	$C_{40}H_{77}O_{10}P$	281.2482 255.2323	$C_{22}H_{43}O_8P$ $C_{24}H_{45}O_8P$
3	16:0/18:0 PG	749.5212	750.5285	750.5411	$C_{40}H_{79}O_{10}P$	283.2598 255.2321	$C_{22}H_{43}O_8P$ $C_{24}H_{47}O_8P$
4	18:1/18:2 PG	771.5092	772.5164	772.5254	$C_{42}H_{77}O_{10}P$	491.2788 281.2477 279.2325	$C_{18}H_{32}O_2$ $C_{24}H_{43}O_8P$ $C_{24}H_{45}O_8P$
5	18:1/18:1 PG 18:0/18:2 PG	773.5253	774.5326	774.5411	$C_{42}H_{79}O_{10}P$	283.2598 281.2478 279.2328	$C_{24}H_{43}O_8P$ $C_{24}H_{45}O_8P$ $C_{24}H_{47}O_8P$
6	18:0/18:1 PG	775.5380	776.5524	776.5567	$C_{42}H_{81}O_{10}P$	493.2940 491.2773 419.2554 417.2406 283.2608 281.2478	$C_{18}H_{34}O_2$ $C_{18}H_{36}O_2$ $C_{21}H_{40}O_4$ ($C_3H_6O_2 + C_{18}H_{34}O_2$) $C_{21}H_{42}O_4$ ($C_3H_6O_2 + C_{18}H_{36}O_2$) $C_{24}H_{45}O_8P$ $C_{24}H_{47}O_8P$

* Precursor ion $[M-H]^-$.

Table 7. Molecular species of phosphatidic acid (PA) from *Madhuca ellitica* seeds.

No.	Molecular Species	ESI-MS	Monoisotopic Molecular Mass		Molecular Formula	MS^2	
		$[M-H]^-$ m/z	Measured	Calculated		Fragment ion * $[M-H-X]^-$ m/z	X
1	16:0/18:2 PA	671.4643	672.4716	672.4730	$C_{37}H_{69}O_8P$	409.2375 391.2271 279.2274 255.2313	$C_{18}H_{30}O$ $C_{18}H_{32}O_2$ $C_{19}H_{37}O_6P$ $C_{21}H_{37}O_6P$
2	16:0/18:1 PA	673.4798	674.4871	674.4887	$C_{37}H_{71}O_8P$	417.2394 409.2332 391.2242 281.2469 255.2318	$C_{16}H_{32}O_2$ $C_{18}H_{32}O$ $C_{18}H_{34}O_2$ $C_{19}H_{37}O_6P$ $C_{21}H_{39}O_6P$
3	18:2/18:2 PA	695.4660	696.4727	696.4730	$C_{39}H_{69}O_8P$	433.2324 415.2228 279.2319	$C_{18}H_{30}O$ $C_{18}H_{32}O_2$ $C_{21}H_{37}O_6P$
4	18:1/18:2 PA	697.4803	698.4876	698.4887	$C_{39}H_{71}O_8P$	435.2511 433.2351 417.2405 415.2241 281.2458 279.2316	$C_{18}H_{30}O$ $C_{18}H_{32}O$ $C_{18}H_{32}O_2$ $C_{18}H_{34}O_2$ $C_{21}H_{37}O_6P$ $C_{21}H_{39}O_6P$
5	18:0/18:2 PA 18:1/18:1 PA	699.4949	700.5022	700.5043	$C_{39}H_{73}O_8P$	437.2641 435.2511 419.256 417.2382 415.2223 283.2608 281.2479 279.2322	$C_{18}H_{30}O$ $C_{18}H_{32}O$ $C_{18}H_{32}O_2$ $C_{18}H_{34}O_2$ $C_{18}H_{36}O_2$ $C_{21}H_{37}O_6P$ $C_{21}H_{39}O_6P$ $C_{21}H_{41}O_6P$
6	18:0/18:1 PA	701.5098	702.5171	702.5200	$C_{39}H_{75}O_8P$	437.2633 435.2503 419.2546 417.2385 283.2621 281.247	$C_{18}H_{32}O$ $C_{18}H_{34}O$ $C_{18}H_{34}O_2$ $C_{18}H_{36}O_2$ $C_{21}H_{39}O_6P$ $C_{21}H_{41}O_6P$

* Precursor ion $[M-H]^-$.

3.4. Composition of the Molecular Species of the Phospholipids

The percentages of the individual molecular species described above within each phospholipid class (PE, PC, PI, PG, and PA) are combined in Table 8. The composition of total phospholipids (Table 1) was used to determine the percentages of the individual molecular species within total phospholipids of *M. ellitica* seeds (Table 8). The weight content of each molecular species was calculated with regard to the content of phospholipids in total seed lipids (44.5 g/kg). Five molecular species (16:0/18:1, 16:0/18:2, 18:1/18:1, 18:1/18:2, and 18:2/18:2) amounted about 91% of total PE. The major molecular species of PC were 16:0/18:1, 18:0/18:2, and 18:1/18:2. It was found that 16:0/18:1 was the main molecular species of both PI and PG.

Table 8. Content of molecular species of phospholipids obtained from *Madhuca ellitica* seeds.

Phospholipid Class	Molecular Species	Concentration		
		% of Each Phospholipid Class	% of Total Phospholipids	mg/kg of Total Lipids
PE	16:0/18:1 PE	19.21	5.77	2567
	16:0/18:2 PE	16.13	4.85	2158
	18:0/18:1 PE	4.64	1.39	620
	18:0/18:2 PE	3.96	1.19	529
	18:1/18:1 PE	17.43	5.23	2329
	18:1/18:2 PE	27.18	8.16	3632
	18:2/18:2 PE	11.44	3.44	1529
PC	16:0/18:0 PC	3.96	1.19	530
	16:0/18:1 PC	30.11	9.05	4026
	16:0/18:2 PC	13.27	3.99	1774
	18:0/18:2 PC	17.10	5.14	2287
	18:1/18:1 PC	10.79	3.24	1443
	18:1/18:2 PC	19.78	5.94	2645
	18:2/18:2 PC	4.98	1.50	666
PI	16:0/18:1 PI	44.18	7.70	3427
	16:0/18:2 PI	1.01	0.18	78
	16:1/18:1 PI	20.92	3.65	1623
	18:0/18:2 PI	23.08	4.02	1790
	18:1/18:1 PI	5.25	0.92	407
	18:2/18:2 PI	2.02	0.35	157
	Other PI	3.54	0.62	275
PG	16:0/18:0 PG	9.42	1.06	469
	16:0/18:1 PG	43.47	4.87	2167
	16:0/18:2 PG	22.05	2.47	1099
	18:0/18:1 PG	7.93	0.89	395
	18:0/18:2 PG	2.77	0.31	138
	18:1/18:1 PG	8.32	0.93	415
	18:1/18:2 PG	5.14	0.58	256
	Other PG	0.90	0.10	45
PA	16:0/18:1 PA	29.01	1.91	852
	16:0/18:2 PA	15.98	1.05	469
	18:0/18:1 PA	7.05	0.47	207
	18:0/18:2 PA	4.74	0.31	139
	18:1/18:1 PA	18.19	1.20	534
	18:1/18:2 PA	19.62	1.29	576
	18:2/18:2 PA	5.41	0.36	159

Results are given as the average of triplicate determinations. PE: phosphatidylethanolamine; PC: phosphatidylcholine; PI: phosphatidylinositol; PG: phosphatidylglycerol; PA: phosphatidic acid.

About 82% of PA were comprised of four molecular species (16:0/18:1, 16:0/18:2, 18:1/18:1, and 18:1/18:2). Overall, three molecular species, namely 16:0/18:1 PC (9.05%), 18:1/18:2 PE (8.16%), and 16:0/18:1 PI (7.7%), were mostly abundant in total phospholipids. Total phospholipids contained 32.05, 38.68, 15.97, and 5.65% of the unsaturated molecular species with one, two, three, and four double bonds in their acyl groups, respectively. The content of saturated molecular species was low (2.25% of total phospholipids). Molecular species with 18:3 were not identified, probably, because of their low concentrations.

3.5. Sulfoquinovosyldiacylglycerol (SQDG)

A pure SQDG fraction was isolated from polar lipids by preparative TLC and analyzed by HPLC–HRMS. Ten major signals of the negative quasi-molecular ions $[M - H]^-$.at m/z 765.4858, 793.5157, 815.4939, 817.5106, 819.5253, 839.4893, 841.5061, 843.5215, 845.5368, and 847.5527 were detected in the HRMS spectra of the SQDG fraction (Table 9). The elemental composition and the value of monoisotopic molecular mass confirmed the presence of SQDG in total lipids of *M. ellitica* seeds. The signals of carboxylate anions in MS^2 spectra of the ions $[M - H]^-$ of SQDG allowed us to identify fourteen molecular species which constituted about 99% of total SQDG. There were 14:0/16:0, 16:0/16:0, 16:0/18:1, 16:0/18:2, 16:0/18:3, 16:1/18:1, 18:0/18:1, 18:0/18:2, 18:0/18:3, 18:1/18:1, 18:1/18:2, 18:1/18:3, 18:2/18:2, and 18:2/18:3 SQDG (Table 9).

The main molecular species was 16:0/18:1 SQDG (28.09%), followed by 18:0/18:1 SQDG (17.97%), and 16:0/18:2 SQDG (12.65%). Unsaturated components predominated in the SQDG fraction, whereas the content of saturated molecular species was low (7.51% of total SQDG). Several molecular species of SQDG contained one 18:3 acyl group, but a component with two 18:3 acyl groups was not found.

Table 9. Molecular species of sulfoquinovosyldiacylglycerol (SQDG) from *Madhuca ellitica* seeds.

No.	Molecular Species	Content, mol. %	ESI–MS	Monoisotopic Molecular Mass		Molecular Formula	MS^2	
				Measured	Calculated		Fragment Ion [a] $[M - H - X]^-$ m/z	X
1	14:0/16:0	0.36	765.4858	766.4901	672.4730	$C_{39}H_{74}O_{12}S$	537.2731	$C_{14}H_{28}O_2$
							509.2461	$C_{16}H_{32}O_2$
2	16:0/16:0	7.15	793.5157	794.5198	794.5214	$C_{41}H_{78}O_{12}S$	537.2723	$C_{16}H_{32}O_2$
3	16:0/18:3	2.04	815.4939	816.5011	816.5057	$C_{43}H_{76}O_{12}S$	559.2593	$C_{16}H_{32}O_2$
4	16:0/18:2 +	12.65	817.5106	818.5179	818.5214	$C_{43}H_{78}O_{12}S$	563.2875	$C_{16}H_{30}O_2$
	16:1/18:1	4.72					561.2734	$C_{16}H_{32}O_2$
							537.2730	$C_{18}H_{32}O_2$
5	16:0/18:1	28.09	819.5253	820.5325	820.537	C43H80O12S	563.2892	$C_{16}H_{32}O_2$
							537.2747	$C_{18}H_{34}O_2$
6	18:2/18:3	0.77	839.4893	840.4965	840.5057	$C_{45}H_{76}O_{12}S$	561.2694	$C_{18}H_{30}O_2$
							559.2581	$C_{18}H_{32}O_2$
7	18:2/18:2 +	2.44	841.5061	842.5134	842.5214	$C_{45}H_{78}O_{12}S$	563.2841	$C_{18}H_{30}O_2$
	18:1/18:3	0.75					561.2733	$C_{18}H_{32}O_2$
							559.2638	$C_{18}H_{34}O_2$
8	18:0/18:3 +	0.99	843.5215	844.5287	844.537	$C_{45}H_{80}O_{12}S$	565.3002	$C_{18}H_{30}O_2$
	18:1/18:2	5.76					563.2883	$C_{18}H_{32}O_2$
							561.2728	$C_{18}H_{34}O_2$
							559.2573	$C_{18}H_{36}O_2$
9	18:0/18:2 +	7.12	845.5368	846.5441	846.5527	$C_{45}H_{82}O_{12}S$	565.3005	$C_{18}H_{32}O_2$
	18:1/18:1	8.75					563.2894	$C_{18}H_{34}O_2$
							561.2738	$C_{18}H_{36}O_2$
10	18:0/18:1	17.97	847.5527	848.5599	848.5683	$C_{45}H_{84}O_{12}S$	565.3009	$C_{18}H_{34}O_2$
							563.2903	$C_{18}H_{36}O_2$

[a] Precursor ion $[M - H]^-$.

4. Discussion

The seeds of *Madhuca* are the base of numerous food products including a variety of lipid substances. Some of which are commonly referred to as "fat", "oil" or "butter" [13]. Technologies of their manufacturing are adopted to obtain the substances with target properties. Different methods are used for lipid analysis of these seed products and fresh seeds. Therefore, the comparable information on the lipid composition of *M. ellitica* seeds is limited.

The seeds of *M. ellitica* were found to be rich in lipids. The seed contains a considerable amount of crude non-polar compounds (up to 61% of dw), which can be extracted by organic solvents. However, the lipid percentage does not seem to exceed 50% and depends on the extraction method [10,11,13].

Classic Folch's method yielded 34% of total lipids from fresh seeds and indicated that the lipid contents in *M. ellitica* seeds and other oilseed crops are similar [23]. Quality and utility of seed oils are mainly determined by their FA composition. The previous studies of the FA composition of total lipids

have shown that 16:0 (11.7–25.9%), 18:0 (19.1–32.2%), 18:1n-9 (32.9–48.6%), and 18:2n -6 (9.4–15.4%) were the major FAs found in Indian *M. longifolia* seeds [8,13,21].

The FAs of total lipids of our samples (Table 2) contained 18:0 at low content (11.5%) and 18:1n-9 at high content (49.5%). The oils with the high content of 18:1n-9 and the very low level of 18:3n-3 (0.1%) are suitable for some cosmetic and pharmaceutical preparations. Some polyunsaturated FAs (PUFAs), such as 20:5n-3 and 22:6n-3, were earlier detected in mahua butter from the Indian buttercup [10] but were not found in the present study (Table 2). To our knowledge, these n-3 PUFAs are common for marine plants and animals. The enrichment of the polar lipid fraction with PUFAs was reported for animal lipids, but not for plant lipids (Table 2). The FA profiles of total, neutral, and polar lipids from *M. ellitica* seeds were quite similar (Table 2). Unusual FAs were not found in neutral lipids from *M. ellitica* seeds, in spite of the fact that seed TG often contain rare FAs [32]. The elevated content of 16:0 in the polar lipids (Table 2) has been previously observed in *M. ellitica* seeds [10]. In total lipids (TL) of oil seeds, the level of neutral lipids is highest, followed by glycolipids (GL) and phospholipids (PL). Neutral lipid classes, first of all, triacylglycerols (TG), prevailed in TL from *M. ellitica* seeds (Table 1). Extraction with hexane produced TL containing 91.2 and 0.2% of TG and PL, respectively [10]. The use of Folch's method [23] showed that *M. ellitica* seed TL contained 4.5% of PL (Table 1). According to Ramadan and others (2006), the predominant PL subclasses were PE (57.7%) followed by PC (30.6%), while PI and PS were isolated in smaller quantities [10]. Our results confirmed that PE, PC and PI were the major PL subclasses, while PG was detected instead of PS. Additionally, PA was observed in the lipids of *M. ellitica* seeds. It is undoubted that the lipids of *M. elliptica* contained PG because its' chemical structures were confirmed by mass-spectrometry (Table 6). Generally, both PG and PS are known to be in seed lipids [19]. The discrepancy in PL class composition of *M. ellitica* may be explained by different extraction methods applied for TL preparation.

Phospholipids (PL) are recognized to play multiple roles in cell processes. PL form a bilayer of cell membranes and, therefore, are involved in important functions of the cell, such as energy transduction, signal transduction, trans-membrane transport and cell-cell recognition. The wide range of these biochemical processes explains the need for high diversity in phospholipid structure [36]. At the same time, only a few molecular species within each PL class demonstrate high biological activities. A potential pharmaceutical importance of an individual molecular species mainly depends on the chemical structure of its acyl groups [37–39]. Thus, common chemical characteristics, such as lipid class composition and total FA composition of each lipid class, are not enough for the detailed description of polar components of seed lipids. A lipidomic study of lipid molecular species is necessary for the use of these seeds effectively [40].

Fast determination of the profile of lipid molecular species became available as a result of the development of chromatography–mass-spectrometry [41–43]. The amounts of neutral lipids in seed oils are known to be the highest, followed by glycolipids and phospholipids [44]. Correspondingly, analysis of molecular species of neutral lipids and glycolipids are performed on a regular basis [19,45], but data on PL molecular species are scanty [46]. For *M. ellitica* seeds, molecular species profiles of TG of different fat products were earlier described [21], but data on PL molecular species were absent. In the present study, the chemical structure and the content of PL molecular species of *M. ellitica* seeds were determined for the first time by a high-resolution tandem mass-spectrometry. This MS method allows both the detection of lipid molecular species in a presence of other low-molecular weight compounds and the determination of acyl group chemical structures of each lipid molecular species [22]. Thirty-four molecular species belonging to five PL classes were identified (Table 3). It is likely that using the molecular species profile as a description of the PL class may be preferable to using the FA profile. Indeed, total FAs of PL contained 58.3% of unsaturated FAs (Table 2), while unsaturated PL molecular species amounted to 97.7% of total PL (Table 3). In animal PL, polyunsaturated components are concentrated in position sn-2 with saturated FAs most abundant in position sn-1. In plants, the differences between the two positions are relatively minor. We did not determine the positional distributions of FAs in PL from the seeds of *M. longifolia*, but we showed

that half of the molecular species contain two unsaturated acyl groups (Table 8). This distribution is explained by some differences between lipid metabolism in plants and other organisms [43]. The results will be important to determine nutraceutical and economical utility of *M. ellitica* seeds. Similar to PL, a charged group presents in molecules of sulfoquinovosyldiacylglycerol (SQDG), which is one of the important glycolipid (GL) classes in plants [47]. Membranes of chloroplasts and other plastids are enriched in GL. Thylakoid membranes of the chloroplasts are the site of the light reactions of photosynthesis. According to the important role of GL, these classes have been found in all seed oils. However, Kadri recently observed a lack of SQDG in polar lipids of *Pinus halepensis* seeds [19]. SQDG has been previously detected in the seeds of *M. longifolia* by Ramadan and others [10]. To confirm the presence and the structure of SQDG, this lipid class was obtained during the isolation of the polar lipid fraction from the seeds of *M. ellitica*. A profile of molecular species of SQDG was investigated similarly to that of PL. Among fourteen molecular species, three major species (16:0/18:1, 18:0/18:1, and 16:0/18:2) amounted to 58.7% of total SQDG (Table 9). The molecular species of SQDG containing two unsaturated acyl groups (for example, 18:1/18:1, 18:1/18:2, 18:2/18:2, 18:2/18:3) were also found. The compositions of the SQDG molecular species have been described in different phyla, for example, a soil bacterium [48], cyanobacteria [49,50], microalgae, seaweeds [51], *Arabidopsis* leaves [45], and sea urchin [52]. These molecular species were characterized by the high degree of saturation of FAs and contained one or two saturated acyl groups. Antibacterial, antitumor, and antiviral activities were reported for SQDG. Since the biological activities depend on a FA saturation degree, we suppose that unsaturated molecular species of SQDG from the seeds of *M. ellitica* enhance the pharmacological potential of these compounds.

5. Conclusions

The seed oil from the buttercup tree *M. ellitica* is widely used in India and Indochina. However, a detailed analysis of its polar lipid fractions has not been performed. A lipidomic approach showed that the seeds of *M. ellitica* contain a variety of polar lipid compounds with both biotechnological potential and pharmaceutical interest. Our research enhances the industrial potential of *M. ellitica* and shows that their seeds may be a good source of polar lipids, which contain about 95% of unsaturated components. Further studies are needed to extend knowledge concerning the distribution of SQDG molecular species between oil seeds.

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