Lipid Classification and Characterization of *Terminalia Belerica* Seed Oil From Tripura

B Anjaneyulu¹, T Ravinder¹, Sudhan Debnath², Sanjit Kanjilal¹ and P P Chakrabarti*¹

¹Centre for Lipid Research, CSIR-Indian Institute of Chemical Technology Hyderabad
²Department of Chemistry, Maharaja Bir Bikram College, Agartala, Tripura

**ABSTRACT**

*Terminalia belerica* fruit is known for its medicinal properties and is currently exploited by manufacturers of Indian traditional ayurvedic medicines. Apart from its abundance in some states of India, organized farming of this tree has been started in some places. The seed of this fruit was found to be a good source of oil (38%) and is not commercially exploited. In this study, attempts were made to extract oil from the *Terminalia belerica* seeds and analyze the oil for the physico-chemical characteristics. *Terminalia belerica* fruits were collected from the forests of Tripura and the seeds were dried and powdered after the separation of pulps. The soxhlet extraction of seeds yielded 38.0% of lipids. Various physico-chemical properties like acid value (AV), iodine value (IV), saponification value (SV), unsaponifiable matter (USM) and phosphorous content etc were determined following standard methods and was found to have AV of 0.9, IV of 71.2, SV of 185.8 USM of 1.7% and 181.6 ppm of phosphorus. The total lipids were separated into neutral lipid (NL), glycolipid (GL) and phospholipids (PL) fractions by using column chromatography and found to contain 98.9%NL, 0.4%GL and 0.7%PL respectively. Among the phospholipids PE was obtained as the major phospholipid along with PC and PI. The triacylglycerol (TAG) composition was also determined. The molecular species with effective carbon number (ECN) C48 and C46 were found to be the major ones. Fatty acid composition of the oil extracted from the seed was determined. The oil was found to contain oleic acid (43.1%), palmitic acid (28.3%), linoleic acid (17.0) and stearic acid (10.6%) as major fatty acids. Unsaponifiable fraction of seed oil was analyzed for sterol composition and found to have β-Sitosterol as the major sterol. The total tocopherol content was found to be about 660 ppm. The fruit of *Terminalia belerica* is generally regarded as safe for human consumption.

The lipid composition of seed oil also did not show any unusual fatty acids. These data obtained may help for commercial exploitation of the *Terminalia belerica* seed oil.

**KEYWORDS**: *Terminalia belerica*, physico- chemical characteristics, fatty acid composition, molecular species, phospholipid composition.

**INTRODUCTION**

*Terminalia belerica*, known as vibhitaki in traditional literature in India, is one of the most common herbs for medicinal uses¹. This is used for controlling cough and nourishment of lungs, throat, voice, hair and eyes. Another important use of the fruit is in treatment of liver disorders²,³. The fruit also has purgative properties ⁴ and it was used as cardiac depressant⁵. The fruit contains hosts of antioxidants and nuteraceutiacals like gallic acid, ellagic acid, ethyl gallate, beta-sitosterols, rhamnose etc.⁶. The uses of fruit as anthelmentic, antiseptic, astringent and expectorant are also known in traditional Indian medicine ⁷. In recent years, researchers have focussed their interest and are thoroughly studying the medicinal aspects of this particular fruit. Jadon et al. has studied the protective effect of this fruit extract against the carbon tetrachloride induced damage in albino rats ⁸ and concluded that the damage caused by carbon tetrachloride exposure could be revised to normal condition by *Terminalia belerica* fruit extract in combination with gallic acid. The ayuvaredic medicine in India has also experienced paradigm shift in recent past and lots of scientific studies being done in various laboratories worldwide to understand the working mechanism of these medicines and on isolation of bio-active molecules from these medicinal plants. Organized forming of these plants and herbs have started in a big way as the sales of ayurvedic medicines are having a steady rise. This tree is available in huge quantities in Uttar Pradesh, Madhya Pradesh, Chhattisgarh, Punjab, Maharashtra and almost all the North-eastern states. However, no data is available on the total production of these
herbs in India. Though the fruits are being studied thoroughly and many literature reports are available on its uses, no significant data is available in literature on the seed and oil obtained from this seed. The seeds are not exploited for any commercial benefits. The biodiesel industry in India is facing short comings of raw materials and recently some researchers have utilized the oil extracted from the 'Terminalia belerica' seeds as raw materials for biodiesel preparation. However, detailed studies on the physico-chemical properties of the oil was not performed. In this present study, the oil was collected from a forest of Tripura, a north-eastern state. The fatty acid compositions of the oil and various lipid fractions obtained from the oil were determined. The oil obtained was totally analyzed for its physico-chemical properties. The molecular species present in the triglycerides were estimated. The oil was also analyzed for phosphorous contents and the phospholipids compositions also determined. These data may be useful for various types of commercial exploitation other than biodiesel preparation.

MATERIALS AND METHODS

Materials

Terminalia belerica (TB) fruits were collected from the forests of Tripura and the seeds were dried after the separation of pulp. Standards of Fatty acid mixtures and triglycerides were purchased from M/s Sigma Chemicals, St Louis, USA. Standard tocopherol and tocotrienols were procured from M/s Sigma Chemicals, St Louis, USA. Silica gel (60–120 mesh) for column chromatography was purchased from Acme Synthetic Chemicals, Mumbai, India. Pre-coated thin layer chromatography (TLC) plates (silica gel 60 F) were procured from Merck, Darmstadt, Germany. Reference samples of phosphatidylethanolamine (PE), phosphatidylcholine (PC), Phosphatidylinositol (PI), cardiolipin (CL) were purchased from M/s Sigma Chemicals, St Louis, USA. HPLC grade solvents were procured from M/s Merck, Mumbai, India. All other analytical grade reagents and solvents were purchased from M/s S.d.fine Chemicals, Mumbai, India.

Methods

Extraction of oil from Terminalia belerica Seed: The dried Terminalia belerica seeds were crushed to get powder and the oil was extracted using soxhlet extractor with hexane as solvent. After 6-8 hr of extraction, the lipid extract was concentrated using a rotary evaporator and dried completely under reduced pressure to obtain the seed oil.

Physico-Chemical Characteristics of oil: Different physico chemical characteristics like acid value, iodine value, peroxide value, moisture & volatiles, saponification value, Unsaponifiable matter, refractive index, tocopherol content were determined according to AOCS Official methods and phosphorous content was determined by IUPAC method. The density of the oil was determined by using Automatic Density Meter (Anton Parr, DMA 4500) according to the method prescribed by ASTM D4052.

Separation of lipid classes: Total lipids of Terminalia belerica seed oil (10 g) was separated in to neutral, glyco- and phospholipids by silica gel column chromatography with the elution of chloroform, acetone and methanol respectively. The results of class of lipids were taken in average of triplicate column results. Glycolipids and phospholipids were qualitatively identified by developing TLC using solvent system chloroform / methanol / water (65:25:4 v/v/v) and spraying with α-naphthol and ammonium molybdate as spray reagents respectively.

HPLC analysis of phospholipids: The phospholipid fraction obtained from column chromatography was qualitatively analyzed by normal phase HPLC equipped with a quaternary pump and an evaporative light scattering detector (ELSD 2000, Alltech, Deer field, IL, USA). The drift tube temperature of the ELSD was set at 50°C and the nebulizing gas (nitrogen) flow rate was 1.5 lit/min, with impactor ‘on’ mode. The concentration of the sample 1 mg/ml was injected to a SunFire Prep Silica column (5um, 4.6 x 250 mm; Sun Fire columns, Waters, Ireland) at a mobile phase flow rate of 0.5 ml/min. A binary gradient solvent system composed of eluent A [chloroform/ methanol/ ammonium hydroxide (80:19.5:0.5, v/v/v)] and eluant B [chloroform/methanol/ammonium hydroxide/water (60:34:0.5:5.5, v/v/v/v)] was used for elution following the method described by Avalli and co-workers. The Identification of phospholipid fractions was carried out by comparing the retention times of the respective commercial standards.

Preparation of fatty acid methyl esters: Fatty acid methyl esters (FAME) of total, neutral, glycolipid and phospholipids fractions of the oil were prepared according to the methods described by Christie. The oil (10-20 mg) was taken in 15 ml of 2% H2SO4 in methanol reagent and refluxed for about 4 hours. After complete conversion as monitored by TLC, solvent was partially removed and the remaining mixture was extracted with ethyl acetate and the combined ethyl acetate layers were washed with water until neutral. Then the ethyl acetate extract was dried over anhydrous sodium sulphate and evaporated under reduced pressure on a rotary evaporator to obtain FAME.

GC analysis: The fatty acid composition was determined with a Agilent 6890N series Gas Chromatograph
equipped with a flame ionization detector (FID) on a split injector. A fused silica capillary column (DB-225MS, 30 X 0.25mm i.d., J & W Scientific, USA) was used for separation. The oven temperature was programmed at 160°C for 2 min, increased to 230°C at 5°C/minute and hold for 20 minutes at 230°C. The injector and detector temperatures were maintained at 230 and 250°C respectively. The nitrogen gas used as carrier at the flow rate of 1 ml/minute. Identification of fatty acids was carried out by comparing them with the retention time of respective commercial standards.

**Molecular species determination by HPLC:** Triglyceride molecular species were estimated by High performance liquid chromatography (HPLC) equipped with a quaternary pump and an evaporative light scattering detector (ELSD 2000, Alltech, Deerfield, IL, USA). The drift tube temperature of the ELSD was set at 50°C and the nebulizing gas (nitrogen) flow rate was set at 1.5 L/min. The concentration of sample in acetone solution was 1 mg/ml and was injected on a reversed phase silica column (X Bridge™ C18, 5μm, 4.6 x 250 mm). The molecular species were eluted with mobile phase acetone (100%) at a flow rate of 1 ml/min. The molecular species of *Terminalia belerica* seed oil was tentatively identified by their equivalent carbon numbers (ECN).

**Regiospecific analysis of Terminalia belerica seed oil:** Positional distribution of fatty acids was carried by porcine pancreatic lipase mediated hydrolysis as described by Christie. 20 mg of the oil was mixed with tris buffer (pH 8.0, 4 ml), calcium chloride solution (0.4 ml, 2.2%) and bile salt solution (1 ml, 0.05%). To this 10 mg of pancreatic lipase was added, the mixture was incubated for 3 min at 40°C. The reaction was then stopped by adding ethanol followed by addition of 1.5 ml of 6 N HCl. The hydrolysis product was extracted with diethyl ether and washed with water until neutral and dried completely under reduced pressure. The hydrolyzed product was separated on thin layer chromatography (TLC) with a mobile phase of hexane/ethyl acetate/acetic acid (70/30/1, v/v/v). The bands corresponding to 2-monglyceride and free fatty acids were separated. The fractions were converted in to fatty acid methyl esters using 2% sulfuric acid in methanol reagent. The mean composition of each fatty acid in 1 and 3 positions is calculated from the intact triacylglycerols and in sn-2 position by following the equation:

\[
\text{Position 1 and 3} = \frac{[(3x \ TAG)-SN-2]}{2}
\]

**RESULTS AND DISCUSSION**

Oil was extracted from the *Terminalia belerica* seed collected from Tripura using hexane and found to contain 38.0% of lipids. Physico chemical properties of the oil were determined and are shown in Table 1. The analytical data was comparable with the data reported earlier.  

**TABLE 1**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
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<tbody>
<tr>
<td>FFA (%)</td>
<td>0.45</td>
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<tr>
<td>Iodine value (g/100 g)</td>
<td>71.2</td>
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<tr>
<td>Peroxide value(ppm)</td>
<td>0.5</td>
</tr>
<tr>
<td>Moisture &amp; volatiles (%)</td>
<td>0.12</td>
</tr>
<tr>
<td>Saponification value</td>
<td>185.8</td>
</tr>
<tr>
<td>Density at 40°C (g/cm³ )</td>
<td>0.89410</td>
</tr>
<tr>
<td>Phosphorous content (ppm)</td>
<td>181.6</td>
</tr>
<tr>
<td>Unsaponifiable matter (%)</td>
<td>1.7</td>
</tr>
<tr>
<td>Refractive index at 30°C</td>
<td>1.3081</td>
</tr>
<tr>
<td>Viscosity at 40°C (cst)</td>
<td>35.6</td>
</tr>
<tr>
<td>Triglycerides (%)</td>
<td>97.0</td>
</tr>
<tr>
<td>Diglycerides (%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Monoglycerides (%)</td>
<td>0.20</td>
</tr>
<tr>
<td>Tocopherol (ppm)</td>
<td>660</td>
</tr>
<tr>
<td>Oxidation stability at 110°C ( hr)</td>
<td>23.8</td>
</tr>
</tbody>
</table>

The *Terminalia belerica* seed oil after extraction appeared as light yellow in colour with liquid form at room temperature. The analytical data revealed that, the oil is having free fatty acids content about 0.45%, which is low compared to earlier reported data. The iodine value observed 71.2, which tells about the unsaturation of the T B seed oil. From the iodine value, the oil can be considered as non-drying oil and also having the combination of saturated and unsaturated fatty acids. The peroxide value of the oil was found to be 0.5 ppm. This clearly indicates the quality of the seed oil after extraction was good low level of deterioration.

The oil was also found to contain the phospholipids 181.6 ppm, which are most important in forming emulsions. The result indicated that, the oil contained 1.7% of unsaponifiable matter, which contains mostly tocopherols and sterols apart from hydrocarbons. Among all the sterols β-sitosterol was found to be the major and campsterol, stigma sterols were observed in low level. The total tocopherol content as considered as vitamine E was around 660 ppm in the seed oil. γ-tocopherol (584.7 ppm) is the major tocopherol and α-tocopherol (42.0 ppm), δ-tocopherol (33.2 ppm) were observed in minor quantities. Almost the similar composition of tocopherol...
content was observed compared to sunflower oil\textsuperscript{27}. The saponification value of the oil was estimated as 185.8 mg/KOH and that comparable with other vegetable oils. The seed oil contained triglycerides almost 97.0\%, diglycerides 0.25\% and monoglycerides 0.2\%. The oxidative stability of the seed oil was obtained as 23.8 hr, which was higher in compared with earlier reported data\textsuperscript{10}. The seed oil contains the tocopherols and phospholipids; this could be the reason for the T B seed oil oxidatively stable. According to Kotake-Nara et al, the synergetic effect between tocopherols and phospholipids influence the stability of the oils\textsuperscript{28}. The viscosity (35.6 cst) and density (0.894 g/cm\textsuperscript{3}) of the oil was found to be in lower ranges compared to earlier report\textsuperscript{10}.

The fatty acid composition of the seed oil was determined by using gas chromatography after conversion in to fatty acid methyl esters and the results shown in Table 2. The positional distribution of fatty acids of \textit{Terminalia belerica} seed oil was also given in Table 2.

The total oil was separated in to neutral, glyco- and phospholipids fractions with the elution of chloroform, acetone and methanol respectively. Further, the phospholipid fraction was analyzed for individual class of lipids by using HPLC. The results observed that, phosphatidylethanolamine (80.1\%) were present as the major phospholipid followed by phosphatidylcholine (16.7\%) and phosphatidylinositol (3.2\%).

<table>
<thead>
<tr>
<th>ECN</th>
<th>% of Triacylglycerol composition</th>
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<tbody>
<tr>
<td>C42</td>
<td>LLL</td>
</tr>
<tr>
<td>C44</td>
<td>LLO</td>
</tr>
<tr>
<td>C46</td>
<td>OOL/POL</td>
</tr>
<tr>
<td>C48</td>
<td>OOO/POO/POP/OSL</td>
</tr>
<tr>
<td>C50</td>
<td>OOS/SOP</td>
</tr>
</tbody>
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L- Linoleic, O- Oleic, P- Palmtic, S- Stearic acid.

The triacylglycerols compositions as molecular species were determined by using reversed phase HPLC and the results obtained were shown in Table 3. The molecular species are reported as effective carbon number (ECN) of triglyceride. The results revealed that, the molecular species of the \textit{Terminalia belerica} Seed oil obtained primarily in 5 types which were in the range of triglyceride having ECN C42-C50. The species of triglyceride of the seed oil having C48 is the major component, followed by C46, C50 and others.

### CONCLUSIONS

The oil obtained (38\%) from \textit{Terminalia belerica} seeds collected from forest of Tripura was thoroughly analysed for its physico-chemical characteristics. No unusual fatty acid was identified and this oil was found to have oleic acid as the major fatty acid (43.1\%) followed by palmitic (28.3\%), linoleic acid (17\%) and stearic acid (10.6\%). The oil was found to have 660 ppm of tocopherol. The major phospholipid present in the oil was phosphatidyl ethanol amine. The fruit of this tree is regarded as safe and has many medicinal uses. The data generated about the oil obtained from the \textit{Terminalia belerica} seed may help in further commercial exploitation of the herb.
ACKNOWLEDGEMENT

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REFERENCES


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