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Extraction and Characterization of Glycerides from Oil Seed of *Citrus maxima* Plant found in Manipur

Md. Abdul Halim Shah[1*], Bhaskarjyoti Bhattacharya[2], Dibakar Chandra Deka [2,3]

[1]Department of Chemistry, Dhanamanjuri University (D.M. College of Science) Imphal-795001, India.

[2]Department of Chemistry, Gauhati University, Guwahati, Assam, 781014, India.

[3]Mizoram University, Aizawl, Mizoram, 796004, India. Mail Id: mahalimshah@yahoo.com

KEYWORDS

Nobab, Pomelo, *Citrus maxima*, transesterification, non-edible vegetable oil, Athia, Biodiesel etc.

ABSTRACT:

Extraction of seed oil (Citrus maxima) was done by Solvent extraction technique on the crushed kernel using petroleum ether as the solvent. The oil was purified prior to further analysis by column chromatography over silica gel (60-120 mesh) using a mixture of petroleum ether and ethyl acetate (20:1) as the eluent. The purified oil was further trans esterified giving the final product known as biodiesel. The biodiesel is also known as the fatty acid methyl ester (FAME). Fatty acid methyl ester (FAME) composition of Citrus maxima seed oil was determined by NMR, IR and GC-MS analysis. The FAME of the oil of Citrus maxima was found to be 32.90 wt.% of methyl palmitate [C16:0], 29.70 wt.% of methyl linoleate [C18:2], 30.40 wt.% of methyl oleate [C18:1] and 7.00 wt.% of methyl stearate [C18:0]

1: INTRODUCTION

Most natural fats or oils are mixed triglycerides which may be saturated or unsaturated. Triglycerides are used as the storage form of energy and they are also important sources of energy for transport vehicles. Triglycerides are used in soap industry, in the manufacture of paints, varnishes, lacquers, synthetic detergents, glycerol, high molecular weight acids, vanaspati ghee, oil cloth, printing inks, hair oil, candles, polishes, medicine, drug for heart diseases and strokes, diabetes etc.

Manipur in North-East India is well known for its rich plant diversity. There are many plants which produce fruits with seeds highly rich in non-edible oils are growing in both plain and hill areas of Manipur (India). Most of these plants have no apparent economic value. Thus, such oils find very limited commercial uses and hence plants producing such oils are slowly disappearing because such plants are considered not important by farmers, government, any public and private sectors. As a result, plant diversity is dwindling. Biodiesel production from edible oils in large scale may cause Global imbalance to food supply and demand system. Hence the use of non-edible oils as the feedstock for biodiesel industries will spare edible oils for use in other industries of edible products [1]. The use of biodiesel now-a-days has become important for diesel engines and is getting worldwide attention because of its renewability, biodegradability, carbon neutrality and non-toxicity [2-5]. The developed countries like Brazil, Indonesia, Malaysia, USA, UK, Germany and Canada have already started using biodiesel blended petrol-diesel. Our country also desperately needed it as a substitute for Petro-diesel of self-reliance [6-7]. So, identification of fatty acid constituents in glycerides is essential. Biodiesel usually consists of methyl esters of long chain fatty acids and is made from nontoxic biological resources such as vegetable oils and animals fats by trans- esterification with methanol in presence of a catalyst [1-2, 9-10]. Catalysts may be acid, base and enzyme (lipase). Biodiesel can contribute in solving global warming and energy problems [1, 11-13]. Hence, non-edible vegetable oils and can be used as an alternative feedstock to produce bio-diesel [14-17].

Citrus maxima, Pomelo in English, locally called *Nobab* in Manipuri, is another important source of non-conventional oil. It belongs to *Rutaceae* family. The

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plant bears edible fruits spherical in shape (14-25 cm in diameter, weighing 0.78-2.50 kg) with a good number of small seeds inside (Figure 1). The seeds are rich in both kernel and oil. 35.80 g of dry seeds contain 19.40 g of kernel (54.19 wt.%) with (49.88 wt.%) oil after chromatographic purification. Fruits are popular but to be commercial exploited. Commercial exploitation of fruits would yield large quantities of seeds which can be used to produce oil. Pomelo is widely cultivated in China and South East Asia and is now widely available in the United States and in India including Manipur. The benefits of juice of Pomelo fruit are (i) prevention of urinary tract infections by its juice containing vitamin C killing bacteria which causes the infection, (ii) promotion of healing of skin by enzyme present in its juice, (iii) keeping of gums and teeth healthy because of vitamin C in its juice, (iv) keeping of hearth healthy due to a high content of potassium, which like vitamin C plays an important role in supporting the heart. Pomelo juice reduces cholesterol count in the body and promotes good cholesterol, (v) prevention of anaemia due to vitamin C in its juice that enhances the absorption of iron in body, (vi) wards off cold and flu by vitamin C in the juice by stimulating the action of antibodies and immune cells which guard the body against bacteria that cause cold, flu, asthma, bacterial infection, allergic and so on, and (vii) fights cancer. The skin of pomelo is very rich in bioflavonoids which fight cancer and help to reduce pancreatic, intestinal and breast cancer and the fibre, present in pomelo protects colon cancer, (viii) Pomelo can fight ageing as it contains spermidine which protect the cells from processes related to ageing and cell damage, (ix) The fibre is extremely significant for weight loss and the fruit has properties which help to burn the fat reducing the starch and sugar content in the body, (x) Prevention of osteoporosis: the pulp of pomelo rich in calcium and minerals encourages new bone development to sting bone health and decreases the potential risk of building brittle bones and (xi) aids digestion: the high vitamin C content in pomelo retains the elasticity of arteries and improves the digestive system. Pomelo is filled with dietary fibre which assists in preserving normal bowel motions and avoids haemorrhoids [19]. The Chinese believe that the pomelo tree is a sacred plant, so its leaves are spiritually cleaned. Pomelo fruit is the biggest in the citrus family. Apart from the uses noted above there are other numerous medicinal uses.

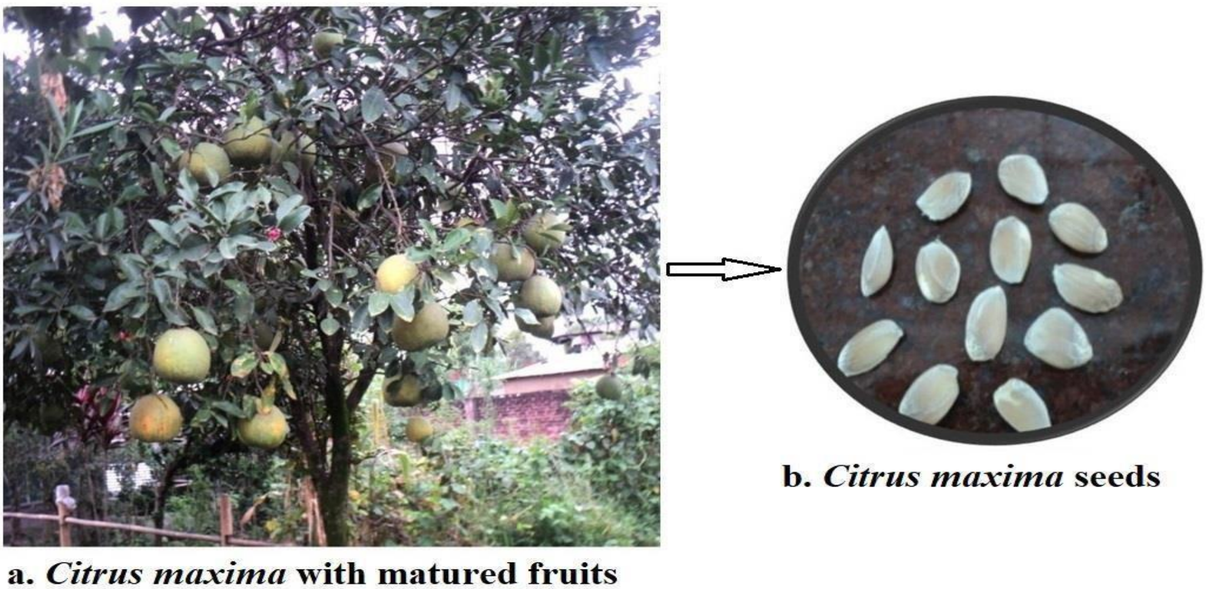

Figure 1: ***Citrus maxima*** **plant and seeds**

2: MATERIALS AND METHODS:

Citrus maxima seeds were collected from the Manipur University Campus, Canchipur, Imphal West (24.63

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94.02) (24°37'48.00" N 94°01'12.00" E), Manipur (India) during its availability of the season, November-April. The seeds were first cleaned and dried for 5/6 days in the sunlight, deshelled and the kernel crushed using a grinder prior to oil extraction. Methanol used was analytical grade (Mark Mumbai, India). All other solvents and chemicals used were analytical grade and they are procured from commercial sources and used as such without to further treatment. Oil was extracted from crushed and powdered kernel of *Citrus maxima* seeds in petroleum ether (bp 40-60°C) (10 ml/g) by stirring magnetically at room temperature using solvent extraction technique (27°C) for 4:30 hours. The solvent was removed at 45°C using a rotary vacuum evaporator (BUCHI Rotavapor R-200) to yield crude oil. This process was repeated 2-3 times with the seed cake using fresh solvent each time to extract most of the oil which was further dried by using vacuum pump. The oil was purified by column chromatography over silica gel (60-120 mesh) using a mixture of petroleum ether and ethyl acetate (20:1) as the eluent prior to transesterification is done. % oil content= (Weight of oil / Weight of powdered seeds) *100…. (1). The parameters of glycerides such as density, colour, refractive index, acid value, iodine value and saponification value were experimentally determined in accordance with the Association of Official Analytical Chemical Procedures [20] and these results are reported (Table 1).

Acid value (mg KOH / g) = $\frac{56.1 * V * N}{W}$ … (2). Here, V = titre value (mL), N = normality of KOH solution (determined by standardizing KOH solution with oxalic acid), W = weight of test sample taken in gram. Refractive indices of purified seed oils were determined by using the Abbe Refractometer (AW-24) at room of temperature, only two or three drops of oil was required. Densities of the purified oils were determined at room temperature (32^0C). For this, a clean and empty plastic centrifuge tube was taken and weighed. Accurately 1000 μL (= 1 mL) of the liquid sample was transferred into the tube with the help of a syringe and then weighed again. Then the density is determined based on mass per unit volume of oil. Iodine value = $\frac{12.69 * N * (V_B - V_S)}{W}$… (3) Here, VB = Volume of sodium thiosulphate solution used for the blank (mL), VS = Volume of sodium thiosulphate solution used for the oil sample (mL), N = Normality of sodium thiosulphate solution used, W = Weight of oil sample taken in gram. Saponification value = $\frac{56.1 * M * (V_B - V_S)}{W}$… (4). Here, VB = Volume of 0.5 M HCl solution used for the blank (mL), VS = Volume of 0.5 M HCl solution used for the oil sample (mL), M = Molarity of HCl used, W = Weight of oil sample taken in gram. % Moisture = $\{(W_1 - W_2)/W_1\}$ *100, ……………. (5). Here, W_1 = Initial weight of oil (in gram), W_2 = Final weight of oil (in gram)

Table 1. Physical parameters of *Citrus maxima* seed oil calculated using equation (1- 5)

Sl. No.	Parameters	Observed Values
1.	Colour	Pale yellow
2.	Oil content (wt. %)	49.88
3.	Density (g/cm^3)	0.9428
4.	Acid Value (mg KOH/g)	0.665
5.	Iodine value (gI_2/100g)	92.20
6.	Saponification value (mg KOH/g)	185.72
7.	Refractive Index	1.4612
8.	Moisture (%)	0.111

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The purified oil was trans esterified to fatty acid methyl esters (FAME) using a catalyst called Athia, a banana plants (ashes from the peels of banana fruits, variety used *Musa balbisiana*, (20 wt.% of the oil [18]. A mixture of the oil in methanol (10 ml/1g of the oil) and the catalyst (20 wt.% of the oil) was stirred vigorously magnetically at room temp (28°C) and the conversion completion of the reaction was monitored by thin layer chromatography (TLC).

After completion of the reaction, the product mixture was extracted with petroleum ether (bp 40-60°C). The organic layer was washed with brine, dried over anhydrous Na_2SO_4 overnight and the solvent was removed under vacuum to yield the crude product which was further purified by column chromatography over silica gel using petroleum ether & ethyl acetate (20:1) as the eluent. The product was concentrated & evaporated to dryness on a rotary evaporator which was further dried using vacuum pump to remove the last traces of the solvents to yield pure biodiesel (FAME).

The composition of FAME mixture was estimated using Perkin Elmer Clarus 600 GC- MS. The column used was Elite 5 MS with initially held at 140°C for 5 min, increased to 240°C at 4°C/min, and then held for 5 min. The injector, transfer and source temperatures were 250°C and 150°C respectively. Carrier gas was helium and total scan time 35 min. El mode of ionization was applied and mass gas was from 20 to 400 Da. For identification of FAME library search was carried out using National Institute of Standards and Technology (NIST), National Bureau of Standards (NBS) and Wiley GC-MS library. Fatty acid profile of biodiesel from *Citrus maxima* seed oil is reported in Table 2. The ^1H and ^{13}C NMR spectra were recovered in Carbon Deuterium Trichloride ($CDCl_3$) at 300 MHz/5mm. NMR spectrometer and IR spectrum were recorded with a Perkin Elmer RXIFT-IR spectrometer as a thin film on KBr plate.

Fatty acid composition of the FAME prepared from *Citrus maxima* seed oil was determined by GC-MS analysis. Each peak of the gas chromatogram (Fig.2) was analysed and the fatty acid was identified using MS database. Each peak represents one fatty acid methyl ester. The three peaks in the gas chromatogram which means the presence of three different fatty acid methyl esters. The peak at the farthest distance on the right side in mass spectrum of any fatty acid methyl ester gives the molecular weight of the fatty acid. This peak is known as molecular ion peak. Retention time is the time taken when any peak develops. Based peak means the tallest peak in the mass spectrum due to the ion with the greatest relative abundance. The peak with the greatest m/z value is likely to be the molecular ion peak.

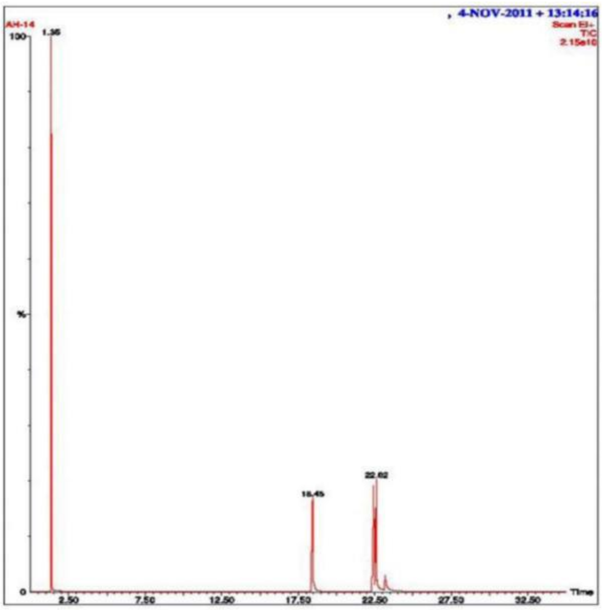

Figure 2: Gas Chromatogram of biodiesel from *Citrus maxima* Seed oil

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3: RESULTS AND DISCUSSION: The yield of the extracted and purified glycerides from *Citrus maxima* seed oil was found to be 49.88wt % at the room temperature (27°C) within 4:30 hours while the yield of trans esterified glyceride known as Fatty Acid Methyl Ester (FAME) was 57.9 wt. % at the room temperature (28°C) within 4:15 hours.

The pale-yellow colour of the *Citrus maxima* seed oil was due to the presence of natural pigments like tocopherols, carotenoids and their derivatives. The yield of the oil was moderate. Density and iodine value of *Citrus maxima* seed oil were found to be 0.9428 g/cm^3 and 92.20 gI2/100 respectively which are comparable to those of soya bean oil and sunflower oil. The acid value of this oil was found to be 0.665 mg KOH/g which is within the limit for industrially useful oil. Saponification value was 185.72 mg KOH/g whose value is suitable for soap making and cosmetic industries. Refractive Index of this oil was 1.4612 which is not very much different from those recorded for conventional seed oils such as palm oils (1.445-1.451), cotton seed oil (1.468-1.472), safflower oil (1.473-1.476) and soya bean oil (1.4728) at 25’C. Moisture was found to be 0.111% (low value) which is suitable good quality and contamination does not take place easily due to its low value of moisture. Low moisture content is an essential criterion for commercial oil.

3.1: Analysis of FAME of Citrus maxima

The mass spectra of methyl palmitate, methyl linoleate, methyl oleate and methyl stearate are shown in Figure 3a-3d. The molecular ion peak and base peak are also presented in Table 3.

^{1}HNMR (300 MHz, CD Cl_3): 5.28 -5.42 ppm, 3.66 ppm, 2.79 ppm, 2.29 ppm,1.99 -2.01 ppm, 1.24 – 1.29 ppm, 0.89 ppm. ^{13}C NMR (75MHz, $CDCl_3$): 174.36 ppm, 129.74 -130.20 ppm, 51.46 ppm, 14.09 - 34.09 ppm.

FTIR (thin film): 1728.22 cm^{-1}, 1608.63 cm^{-1}, 2912.51 cm^{-1}, 2735.06 cm^{-1}, 3082.25 cm^{-1}, 1209.37 cm^{-1}, 1056.99 cm^{-1}, 781.17 cm^{-1}. Relative percentages of fatty acid esters were calculated from the total ion chromatography by computerized integrator and results are presented (Table 2). Fatty Acid Methyl Ester (FAME) from Citrus maxima consists of 15.35 wt.% of methyl palmitate (C16:0), 47.70 wt.% of methyl linoleate (C18:2), 30.54 wt.% of methyl oleate (C18:1) and 6.41 wt.% of methyl stearate (C20:0).

Table 2: Composition of biodiesel from *Citrus maxima* seed oil

Entry	Retention time (mm)	FAME	wt.%
1	18.54	Methyl palmitate	15.35
2	22.77	Methyl linoleate	47.70
3	22.93	Methyl oleate	30.54
4	23.30	Methyl stearate	6.41

Table 3: Molecular ion and base peaks of FAME from *Citrus maxima* seed oil

Entry	FAME	Molecular ion peak (m/z)	Base peak (m/z)
1	Methyl palmitate	270	74
2	Methyl linoleate	294	67
3	Methyl oleate	296	55
4	Methyl stearate	298	74

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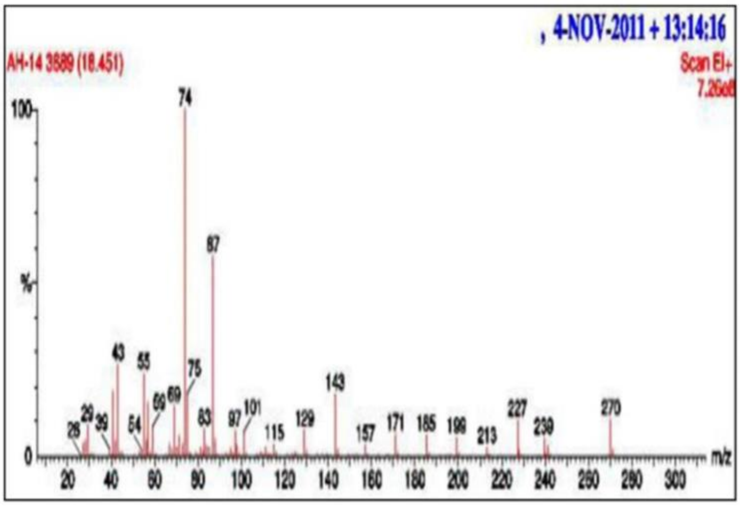

Figure 3 (a): Mass spectrum of methyl palmitate

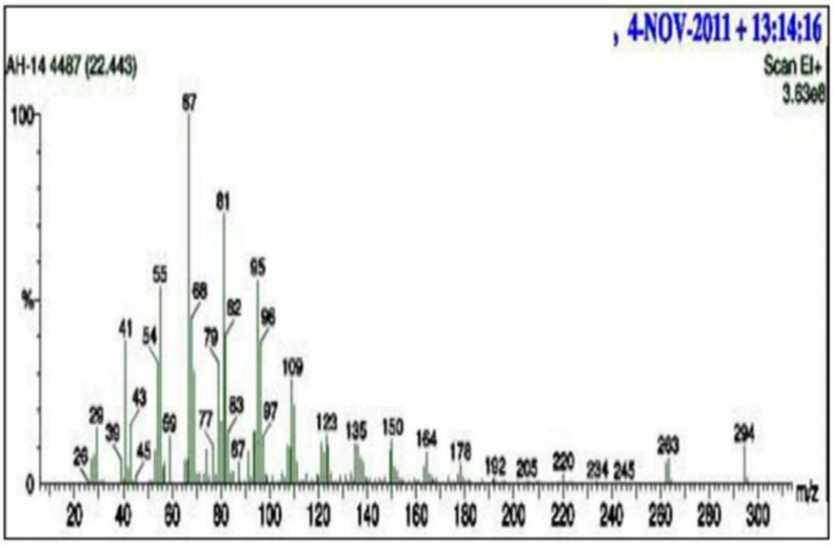

Figure 3 (b) Mass spectrum of methyl linoleate

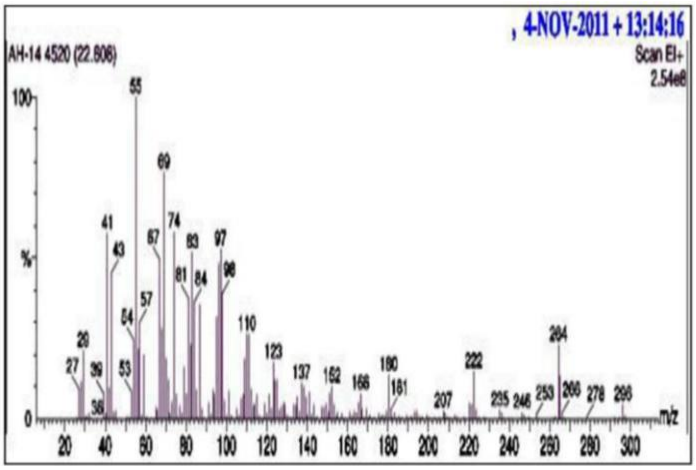

Figure 3 (c): Mass spectrum of methyl oleate

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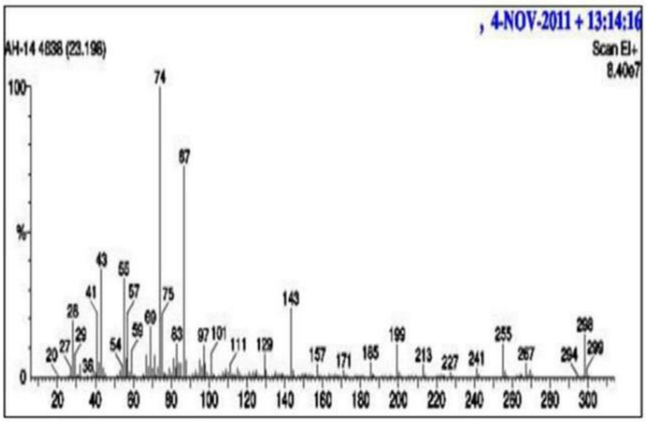

Figure 3 (d): Mass spectrum of methyl stearate

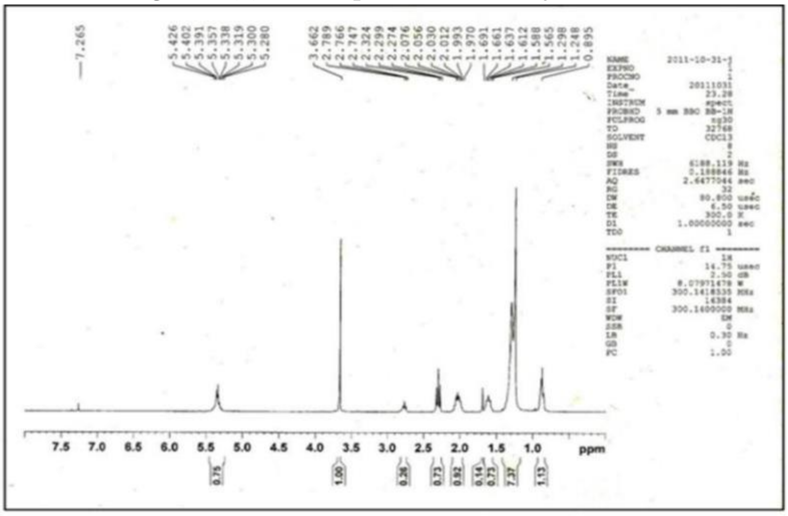

Figure 4: ^{1}H NMR spectrum of biodiesel from *Citrus maxima* seed oil

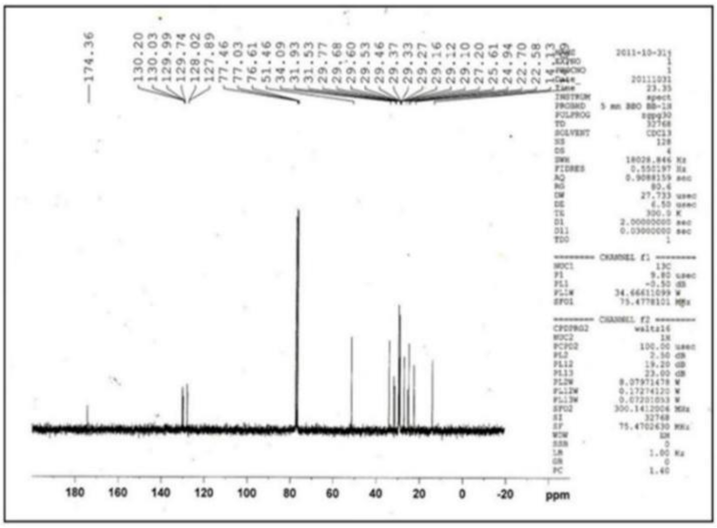

Figure 5: ^{13}C NMR spectrum of biodiesel from *Citrus maxima* seed oil

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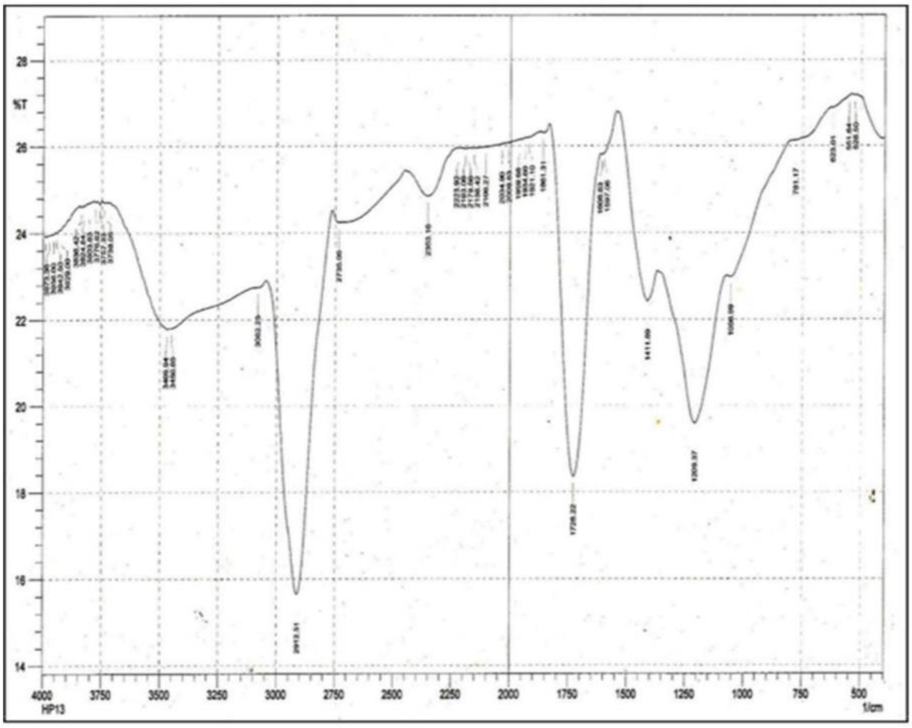

Figure 6: IR spectrum of biodiesel from *Citrus maxima* seed oil

The ^{1}H NMR spectrum of biodiesel from *Citrus maxima* seed oil is presented in (Figure 4). The multiplet 5.28-5.42 ppm represents the olefinic protons(–CH=CH–). A singlet signal at 3.66 ppm is representing methoxy protons of the ester functionality of the biodiesel. A signal is seen at 2.79 ppm which represents bis-allylic protons (–C=C– CH2–C=C–) of the unsaturated fatty acid chain. The triplet at 2.29 ppm (t, ^3J = 7.5 Hz) may be due to the ∝-methylene protons to ester (–CH2– CO2Me). The ∝-methylene protons to double bond (– CH2–C=C–) is seen as a multiplet at 1.99-2.01 ppm. The - methylene protons to ester (CH2–C–CO2Me) also appear as a multiplet at 1.24-1.29 ppm indicates the protons of backbone methylene of the long fatty acid chain. The terminal methyl protons (C–CH3) at 0.89 ppm appear as a multiplet.

The ^{13}C NMR spectrum of biodiesel from *Citrus maxima* seed oil is shown in the (Figure 5). The signal at 174.36 ppm represents the carbonyl carbon of the ester molecular and the olefinic carbons appear at 129.74 and 130.20 ppm. The signal at 51.46 ppm suggests methoxy carbons of esters. The methylene and methyl carbons of fatty acid moiety appear in the range from 14.09 to 34.09 ppm.

In IR spectrum of biodiesel from *Citrus maxima* seed oil (Figure 6) a sharp signal at 1728.22 cm^{-1} is indicative of strong absorption by ester carbonyl stretching frequency. The weak signal at 1608.63 cm^{-1} is indicative of strong absorption by ester carbonyl stretching frequency. Strong and sharp signals at 2912.51 and 2735.06 cm^{-1} are due to C– H stretching frequencies. The absorbance at 3082.25 cm^{-1} indicates the =C–H stretching frequency. The bands at 1209.37 and 1056.99 cm^{-1} are expected for C-O-C stretching vibrations. The observation of an absorption peak at 781.17 cm^{-1} suggests the CH2 rocking.

4. CONCLUSION

The yield of the extracted and purified glycerides from *Citrus maxima* seed oil was found to be 49.88 wt.% at the room temperature (27°C) within 4:30 hours while the yield of trans esterified glyceride known as Fatty Acid Methyl Ester (FAME) was 57.9 wt.% at the room temperature (28°C) within 4:15 hours. The colour, density, acid value, iodine value, saponification number, refractive index and moisture of the *Citrus maxima* seed oil were found to be pale yellow, 0.9428 g/cm^3, 0.665 mg KOH/g, 92.20 gI2/100 g, 185.72 mg KOH/g, 1.4612 and 0.111 % respectively. The biodiesel from *Citrus* *maxima* seed oil, after extraction and purification by column chromatography, was prepared by heterogeneous transesterification process and analysed for its fatty acid methyl esters composition using IR, NMR and GC-MS. This study found that FAME from *Citrus maxima* seed oil consists of 15.35 wt. % of methyl palmitate (C16:0), 47.70 wt. % of methyl linoleate (C18:2), 30.54 wt.% of methyl oleate (C18:1), and 6.41 wt. % of methyl stearate (C20:0). The molecular ion peak of methyl palmitate, methyl linoleate, methyl oleate and methyl stearate, were observed at 270, 294, 296, and 298 respectively as was expected.

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