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Physio-chemical assessment of beauty leaf (*Calophyllum inophyllum*) as second-generation biodiesel feedstock

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ABSTRACT

Recently, second-generation (non-vegetable oil) feedstocks for biodiesel production are receiving significant attention due to the cost and social effects connected with utilising food products for the production of energy products. The Beauty leaf tree (*Calophyllum inophyllum*) is a potential source of non-edible oil for producing second-generation biodiesel because of its suitability for production in an extensive variety of atmospheric condition, easy cultivation, high fruit production rate, and the high oil content in the seed. In this study, oil was extracted from Beauty leaf tree seeds through three different oil extraction methods. The important physical and chemical properties of these extracted Beauty leaf oils were experimentally analysed and compared with other commercially available vegetable oils. Biodiesel was produced using a two-stage esterification process combining of an acid catalysed pre-esterification process and an alkali catalysed transesterification process. Fatty acid methyl ester (FAME) profiles and important physicochemical properties were experimentally measured and estimated using equations based on the FAME analysis. The quality of Beauty leaf biodiesels was assessed and compared with commercially available biodiesels through multivariate data analysis using PROMETHEE-GAIA software. The results show that mechanical extraction using a screw press produces oil at a low cost, however, results in low oil yields compared with chemical oil extraction. High pressure and temperature in the extraction process increase oil extraction performance. On the contrary, this process increases the free fatty acid content in the oil. A clear difference was found in the physical properties of Beauty leaf oils, which eventually affected the oil to biodiesel conversion process.

However, Beauty leaf oils methyl esters (biodiesel) were very consistent physicochemical properties and able to meet almost all indicators of biodiesel standards. Overall this study found that Beauty leaf is a suitable feedstock for producing second-generation biodiesel in commercial scale.

Therefore, the findings of this study are expected to serve as the basis for further development of Beauty leaf as a feedstock for industrial scale second-generation biodiesel production.

1. Introduction

Rapid growth in population, urbanisation and energy demand, together with the reduction of conventional petroleum-based oil reserves and degradation in air quality are continuously motivating researchers to find more sustainable and cleaner energy sources.

As a consequence, biodiesels produced from vegetable oil feedstocks are receiving significant attention as an option to fossil-based diesel. The first recorded production of biodiesel occurred in 1937 via transesterification and using palm oil as feedstock. Biodiesel research continued from this time, but its potential was not fully realised until the 1970s energy crisis when interest in alternative fuels was renewed (Jayed et al., 2009). Since this time, a multitude of feedstocks for biodiesel has been assessed for industrial scale production. In general, biofuels offers numerous profits over fossil-based fuels including ability to produce from regionally available biomass sources, lower greenhouse gas emissions, enhanced biodegradability, and enhanced sustainability characteristics (Reijnders, 2006; Ellabban et al., 2014). Biodiesel typically contains 10%–45% of O_2 by weight while fossil-based diesel has virtually do not contain any O_2. This higher O_2 content in biodiesel helps for better and complete combustion compared with petroleum diesel. Moreover, biodiesels typically contain less sulphur and nitrogen that improves air quality from fuel combustion (Hoekman et al., 2012). At the same time, the rise in production and consumption of biodiesels has focused attention on biodiesel quality standards (Behçet, 2011).

A large number of potential biodiesel feedstocks have been examined in last few decades (Goodrum and Geller, 2005; Holser and Harry-O'Kuru, 2006; Rahman et al., 2014; Raadnui and Meenak, 2003; Lin and Li, 2009; Marchetti et al., 2008; Leung and Guo, 2006). However, only a few feedstocks including rapeseed, soybean, sunflower, tallow, waste cooking oil, etc. are being utilised for the commercial produced of biodiesel at industrial scale (Jahirul et al., 2013). These commercial biodiesels are made using edible oil feedstocks and are typically referred to as first-generation biodiesels (Rashid and Anwar, 2008). The prime criticism of first-generation biodiesels is that it is using edible oil and high-quality agricultural land for biodiesel production. Farmers have the option to sell the vegetable oil to the food market or the biodiesel production market. If the biodiesel production market is offering a higher price, farmers will choose this option more often than not to make a living. This is of specific concern in poorer nations where yields utilised for biodiesel generation dislodge the creation of nourishment harvests, hence bringing about a lack. Supply and interest direct that a deficiency will result in a value climb, which nations, for example, Malaysia are as of now encountering. This issue brought on worldwide open deliberation because of the 2007–2008 world nourishment value emergencies. Distinctive contentions exist in regards to the reason for this emergency. However, there has been the hypothesis that the expanded utilisation of biodiesel brought about a nourishment deficiency and resulting cost increment (Kingsbury, 2007). Thus, an option must be considered which wipes out the hindrances of conventional first-generation biodiesels that do not compete with food production.

In a recent study (Ashwath, 2010), a substantial number of non-eatable oil seed plants were been identified which have the potential to be used as biodiesel feedstocks. Those feedstocks are commonly referred as second-generation biodiesel that have the ability to grow on previously cleared or degraded land. Among those, *Beauty leaf* was recognised as one of the most potential feedstock biodiesel production as a result of the high oil productivity of the seeds. *Beauty leaf* is a moderately sized (8–20 m high) plant, grows in mixed cultures with minimal cultivation (Mohibbe Azam et al., 2005). The tree naturally grows in the sub-tropical and tropical atmosphere (with in the temperature between 18 and 33 °C) and free draining soils close to shorelines. It is frequently found in clay soils within Australia, India, Sri Lanka and throughout central and southern Asia including Indonesia (Jahirul et al., 0000). Moreover, the *Beauty leaf* tree has the potential for the production of 16,000 kg of dry oil bearing seeds in a year utilising one hector of the land area (Mohibbe Azam et al., 2005; Okano, 2006).

However, the potential of *Beauty leaf* oil as a source of second-generation biodiesel is yet to be utilised commercially because of the absence of knowledge on the production process and biodiesel quality. Therefore, this study aims to access different oil extraction methods for *Beauty leaf* oil seed and to evaluate the quality of the oil and biodiesel produced.

2. Methods

2.1. Beauty leaf oil seed preparation

Seed preparation is critical in optimising the oil extraction process from plant to oil seed. This is because the physical conditions such as size, hardness and dryness of seeds and kernels varies significantly from one species to another. Several steps are involved including seed collection, kernel extraction and drying. Fig. 1 shows *Beauty leaf* seed preparation steps and brief descriptions of these steps are given in following sections.

Dry *Beauty leaf* seeds were mostly collected from the coastal locations of northern Queensland, Australia though local seed supplier. The seeds were than cracked open manually to expose and obtain the oil bearing kernels. To reduce kernel damage and oil loss, seed-cracking was done with care using two tools that are stompers and mallets. About 51 kg of useable wet kernels was produced from cracking of 140 kg of *Beauty leaf* seeds resulting in a kernel yield of 36%. Assuming a seed productivity of 16,000 kg of dry seeds per year per hectare, it is likely that the *Beauty leaf* plant can produce ~5800 kg of wet kernel per year in a hectare of land area.

The kernels of *Beauty leaf* seeds naturally contain high moisture that needs to be removed for effective oil extraction. Drying was conducted using Kernels were put in the foil trays; by 2 kg per tray to guarantee the kernels was spread enough for uniform drying. A Clayson Electric oven with temperature controller was used for this purpose. The trays were weighed before placed in the drying oven for three days at 40 °C. After that the temperature of the oven was increased to 70 °C and the drying progress was monitored by measuring the weight of a few times in a day. Because a fan-forced oven was used, the tray positions in the oven seemed to impact on its drying, especially those trays nearest to the oven walls. To reduce this effect, the trays were rotated in the oven to ensure uniform drying rates. The seed was dried until it was observed that the weight was remaining constant for one day. The moisture content of the kernels was approximately 32%. Therefore, it is expected that about 3960 kg of the dry kernel can be produced from *Beauty leaf* plant per hectare per year.

2.2. Oil extraction

Oil was extracted from the kernel by three different methods that are: mechanical oil extraction, chemical oil extraction at the atmospheric condition and accelerated solvent extraction under high pressure and temperature condition. Each of the extraction methods has its advantages and limitations. A brief description of the oil extraction methods conducted in this study is given in the following sections.

2.3. Mechanical oil extraction using oil press (OP)

A Mini 40 electric motor powered screw press shown in Fig. 2 was used for the mechanical oil extraction. Beauty leaf kernel was found to be very hard to process utilising the screw press due its physical properties, and several cycles were required to extract the oil. It was also difficult to control the soft kernel paste after one pass and to keep the process clean. Two operators were obliged to go continually to the screw press, and the rate of oil generation
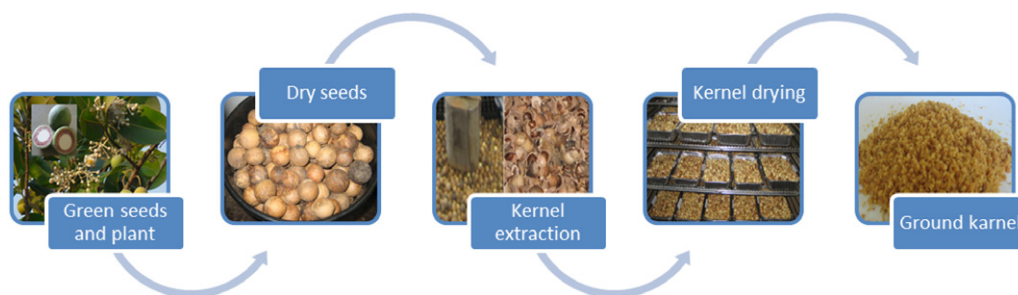
Fig. 1. Flow chart of *Beauty leaf* seeds preparation.

Fig. 2. *Beauty leaf* oil extraction using a screw press.

was very low, taking over an hour to process just about 200 g of sample. Mixing of rice husks with kernels significantly accelerated the rate of oil production. It was also observed that temperature (both ambient and barrel/product) have a significant impact on the oil yield. This was evident when attempting to expel oil at low ambient temperatures (e.g. cold mornings) which took longer.

2.4. Chemical oil extraction using n-Hexane (nHX)

In this process, oil was extracted using *n*-hexane as an oil solvent at ambient conditions. The ground kernels and *n*-hexane were mixed at 2:1 weight ratio and put into conical flasks. To ensure that kernels were wetted uniformly, an initial stir was given on it and then mixture were left on the orbital mixture for eight hours for absorbing oil by *h*-hexane. The samples (*n*-hexane and oil mixture) were then filtered and left open for 8–20 h under the fume hood (Fig. 3) for the natural evacuation of *n*-hexane. At this time, the samples were weighted in every 2 h to determine the evaporation rate of *n*-hexane. After complete evaporation of solvent, the oil was collected for analysis. It was observed that the *n*-hexane oil extraction method resulted in a much greater oil yield than the mechanical oil extraction process.

2.5. Accelerated solvent extraction (ASE)

The accelerated solvent extraction (Dionex^{TM} ASE 350$^{®}$) machine is shown in Fig. 4(a) was used to extract *Beauty leaf* oil at high pressure and temperature using accelerated solvent extraction method. The oil extractor comes with an automated extraction control system that uses elevated temperatures and pressures to achieve extractions in a short period. Measured samples were inserted into metal sample cells, and the desired operating conditions were set using the control interface. Although the machine allows for the use of up to 3 different types of solvents

only *n*-hexane was used as the solvent for lipid extraction. The electric oven maintained the cell contents at the selected operating temperature throughout the extraction process and was set to 150 °C. *N*-hexane was pumped into the cell with pressurised nitrogen gas to achieve a pressure of 1600 psi. After the extraction process was completed, all the extracted oil samples were collected into the vessels in the collection tray (Fig. 4(a)). The solvent was separated from the extracted sample using the Dionex^{TM} SE$^{®}$ 400 solvent evaporator system as shown in Fig. 4(b).

2.6. Oil Yield

The *Beauty leaf* oil yield from the three extraction techniques are shown in Fig. 5. All the results are averages of three replicates for each extraction method. Overall the highest oil yield was obtained using the ASE oil extraction method that produced 39.5 g of oil per 100 g of dry kernels. The static *n*-hexane extraction methods produced about 35 g of oil from 100 g of dry kernels. These results indicated a 4%–5% oil yield increase for the higher pressure and temperature conditions. This result is likely to be due to the improvement in solvation power of *n*-hexane under higher temperatures. With increases in temperature, the thermal energy of the solvent increases, which help to overcome cohesive and adhesive interactions. Moreover, higher temperatures increase the molecular motion of molecules and decrease hydrogen bond interactions. Higher pressure facilitates more interactions between the solvent and oil especially oil that is trapped in pores and would normally not be contacted by solvents under ambient conditions. These results indicate that about 1.56 tons of oil per hectare per year can be produced from *Beauty leaf* plant using chemical extraction methods with high pressure and temperature. The results also indicate that solvent oil extraction methods are more repeatable, and, given the relative ease of preparation, it is considered to be more reproducible.

Fig. 3. Chemical oil extraction.
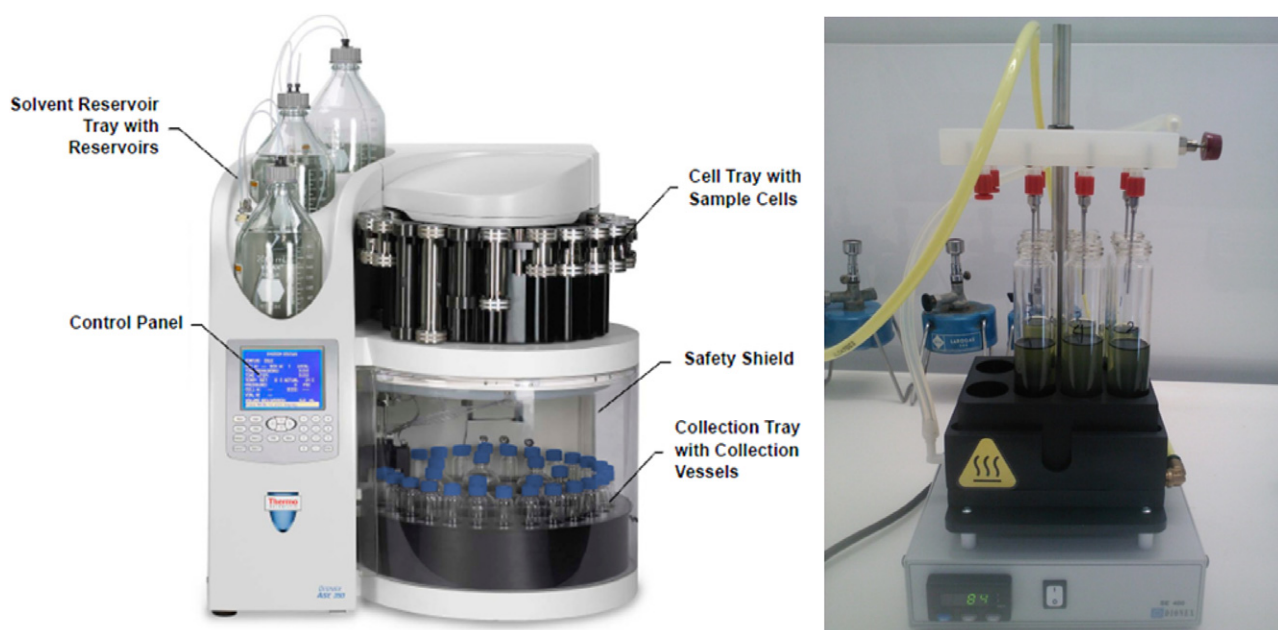
Fig. 4. ASE oil extraction (a) Dionex™ ASE 350®. (b) Solvent removal with the flow of nitrogen.
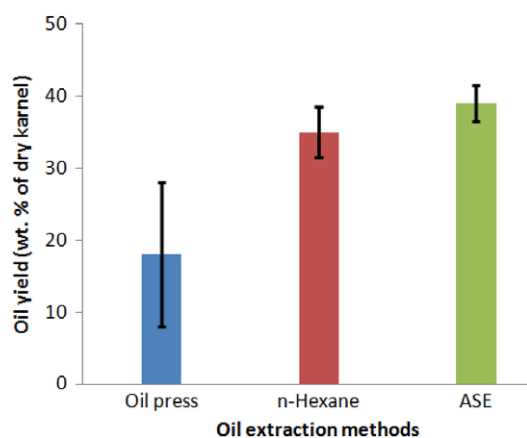
Fig. 5. Beauty leaf oil yield from three different extraction methods.

2.7. Comparison of oil extraction methods

During *Beauty leaf* oil extraction using the three different oil extraction methods; it was observed that all the methods have techno-economic merits and demerits compared with each other. For example, although the ASE method had higher oil yields over the other two techniques, it requires high investment, sophisticated equipment and skilled operators. Table 1 presents a summary of *t* the merits and demerits of oil extraction techniques investigated in this study.

2.8. Oil analysis

Experiments were led to focus on the quality of the oil extracted in terms of density, kinematic viscosity, higher heating value, acid value and surface tension. Kinematic viscosity was tested utilising a Brookfield DV-III Rheometer according to the ASTM D445 test standard. Oil surface tension and density were measured by ASTM D971-12 and ASTM D1298 standard test methods in a KSV Sigma 702 Tensiometer. The higher heating value of biodiesel was measured following the ASTM D240-09 test standard using a Parr 6200 oxygenated bomb calorimeter. The acid value of oils was measured using D5555-95 (2011) test standard. All experiments were undertaken in triplicate, and average results were used. The experimental results are shown in Table 2 along with similar parameters of other vegetable oil results obtained from the

Table 1
Merits and demerits of the extraction methods.

Methods	Merits	Demerits
Oil press	● No contamination of solvent ● Less processing cost ● Less consumables required ● Low preparation is required ● Whole seeds or kernels can be processed	● Low oil yields ● Skilled operators require ● Performance highly depends on kernel physical condition ● Relatively dirty process ● Filtration or degumming process of oil is required ● Low and inconsistent oil production ● High oil loss ● Labour and time intensive
n-hexane	● Consistence in oil generation performance ● Less oil loss ● Relatively simple process ● Suitable for bulk oil extraction ● Low capital investment ● No especial equipment required	● High potential for solvent contamination ● Using *n*-hexane required high safety concern ● Relatively costly ● High hexane requirement ● Only kernel can be processed
ASE	● Automatic technique ● Condition can be optimised ● More efficient ● Clean process ● Relatively less solvent consumption ● Less time and labour incentives ● High oil yield	● Very high initial cost ● High preparation required ● Special equipment and skill required ● Potential for solvent contamination ● Only kernel can be processed

Table 2
Physical properties of *Beauty leaf* oil.

Vegetable oil		Acid value (mgKOH/g)	Density (kg/l)	Surface tension	Higher heating value (MJ/kg)	Kinematic viscosity (40 °C, cSt)
Beauty leaf[a]	*Oil press*	*36.26*	*0.964*	*30.85*	*38.10*	*56.74*
	n-Hexane	*24.00*	*0.936*	*26.60*	*39.52*	*42.24*
	ASE	*39.22*	*0.945*	*27.39*	*39.34*	*44.05*
Rapeseed[b]		0.39	0.907	23.24	40.05	38.25
Canola[b]		0.16	0.912	33.00	39.74	33.34
Soybean[b]		0.82	0.914	25.13	39.62	32.85
Sunflower[b]		0.20	0.916	23.77	39.49	31.63
Cottonseed[b]		0.30	0.914	34.10	39.40	33.70
Palm[b]		0.90	0.916	31.00	40.14	39.65

[a] Experimental.
[b] Literature (Altın et al., 2001; Chouaibi et al., 2012; Lang et al., 2001; Demirbas, 2003; Anand et al., 2010; Singh and Singh, 2010; Keng et al., 2009; Yusaf et al., 2011; Halpern, 1949; Doll et al., 2008; Atabani et al., 2013a).

literature. *Beauty leaf* oil obtained from the screw press showed higher density, surface tension, kinematic viscosity and lower heating value compared to oil obtained from ASE and ambient *n*-hexane methods. This might be due to the presence of suspended small particles remaining in the oil from mechanical extraction although large particles were removed via centrifugation. The acid values of oil from the screw press (36.26 mgKOH/g) and ASE oil (39.22 mgKOH/g) were much higher than the acid value of oil obtained from ambient *n*-hexane extraction (24 mgKOH/g). The high pressure and temperature involved in ASE and press oil might handle creating high free fatty acid in the oil. When compared with conventional vegetable oils, all of the *Beauty leaf* oil samples showed much higher acid values in Table 2. These results confirm that raw beauty-leaf oil is not suitable directly as a fuel for diesel engine application because of having high acid value and kinematic viscosity and conversion to fatty acid methyl esters is required prior to use as a fuel.

2.9. Biodiesel production

Like other conventional vegetable oils shown in Table 2, the kinematic viscosity of *Beauty leaf* oils (42.24–56.74 cSt) are much higher than that of petroleum diesel (2–3 cSt). Higher kinematic viscosities of the fuel increases the drag forces in the fuel system and injection pump, engine deposition and wear in the fuel pump materials and injectors. This can adversely influence fuel spray, fuel–air mixture formation and the combustion process that eventually affects engine performance and emissions (Jahirul et al., 2013). Therefore, Raw *Beauty leaf* oils, as well as other vegetable oils, are not suitable for the direct use a diesel engine fuel. To overcome this difficulty, raw vegetable oils are needed to go through a chemical reaction called transesterification. In this process, vegetable oils (tri-glycerides) react with alcohols (e.g., methanol, ethanol) in the presence of acid or alkali catalysed, producing fatty acid alkyl esters and glycerol. The physical properties of fatty acid alkyl esters (commonly referred as biodiesel) are close to conventional diesel which normally suitable for direct use in a diesel engine. This vegetable oil to biodiesel conversion technique is very popular in the commercial biodiesel production due to its high conversion efficiency, simplicity, low conversion cost and the fuel qualities of the product (Lin et al., 2011; Gerpen, 2005; Issariyakul et al., 2007). After the biodiesel conversion is complete, glycerol is removed as a by-product and the esters are purified to produce clean biodiesel (Fernando et al., 2007). One of the problems with transesterification reaction is that the reaction of free fatty acid (FFA) and alkali catalyst can produce shop (Fig. 6) which eventually reduce biodiesel yield. Therefore, for reducing FFA acid, a pre-esterification process is usually used with oil containing a high level of FFA. A typical pre-esterification process uses homogeneous acid catalysts, like sulphuric acid, or heterogenous 'solid-acid' catalysts, to pre-esterify the free fatty acids (Zhang and Jiang, 2008; Haas, 2005; Samios et al., 2009) as indicated in Fig. 7.

A schematic of a two-step process of biodiesel production from high FFA contained *Beauty leaf* oil is shown in Fig. 8. A triple neck
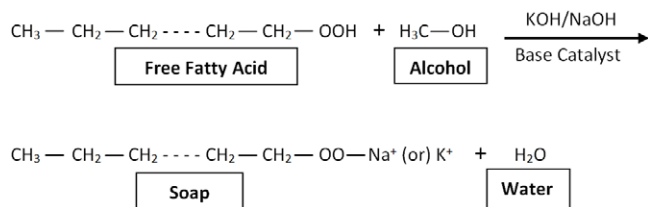
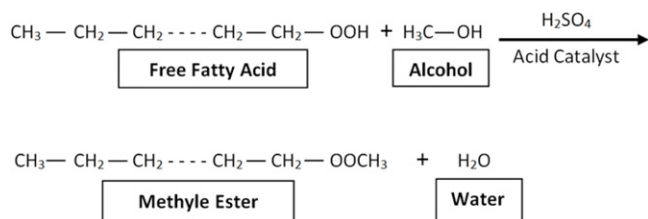**Fig. 6.** Soap formation in oils contains high FFA (Jahirul et al., 2013).

Fig. 7. Acid pre-esterification (Jahirul et al., 2013).

Fig. 8. Two step bio-diesel production process from *Beauty leaf oil*.

bottom flask reactor, shown in Fig. 9(a) was used for both acid-catalysed pre-esterification and base-catalysed transesterification. For each experiment, oil was carefully transferred into the reaction flask and preheated in an oil bath to the reaction temperature. In acid-catalysed esterification, sulphuric acid (H_2SO_4) was used as catalyst, sodium methoxide ($NaOCH_3$) was used for base-catalysed transesterification. The reaction parameters were set according to the findings of Jahirul et al. (2014) where they have conducted a study to determine the optimum reaction condition for converting biodiesel from *Beauty leaf* oil. At the completion of acid-catalysed pre-esterification, the mixture was centrifuged in a self-standing tube for 7 min to separate the methanol–water and esterified oil phases as shown in Fig. 9(b). The majority of the excess methanol, sulphuric acid and impurities were separated into the top phase. The bottom phase containing the oil was collected for base-catalysed transesterification. The procedures were undertaken in triplicate, and average values were taken. It was found that after acid-catalysed pre-esterification, the acid value of *Beauty leaf* biodiesel reduced to 5.14, 3.66, and 6.30 respectively for screw press, ambient *n*-Hexane and ASE extracted oils. Fig. 9(c) shows the product phase after base-catalysed transesterification, where the top layer containing crude *Beauty leaf* biodiesel was collected and washed to remove the soap, unreacted methanol and another contaminant. The average methyl ester conversion for the screw press, *n*-Hexane and ASE extracted beauty leaf oils were 75.47%, 90.76% and 83.76%, respectively. The results clearly indicated the dependency of the biodiesel conversion process the on the presence of free fatty acid in the base oil. Therefore after analysing these results, it is clear that methyl ester production efficiency not only depends on feedstock but also the oil extraction methods.

3. Result and discussion

3.1. Biodiesel analysis

The chemical composition of biodiesels is very important for determining their suitability for automobile engine application. Chemically, all biodiesels are mono alkyl esters of fatty acids, commonly referred as fatty acid methyl or ethyl esters. Depending on the feedstock and production process, the fatty acids are different about the chain length, the degree of unsaturation or presence of other chemical functions. Fatty acids are commonly designated by two numbers: the first number represents the total number of carbon atoms in the fatty aci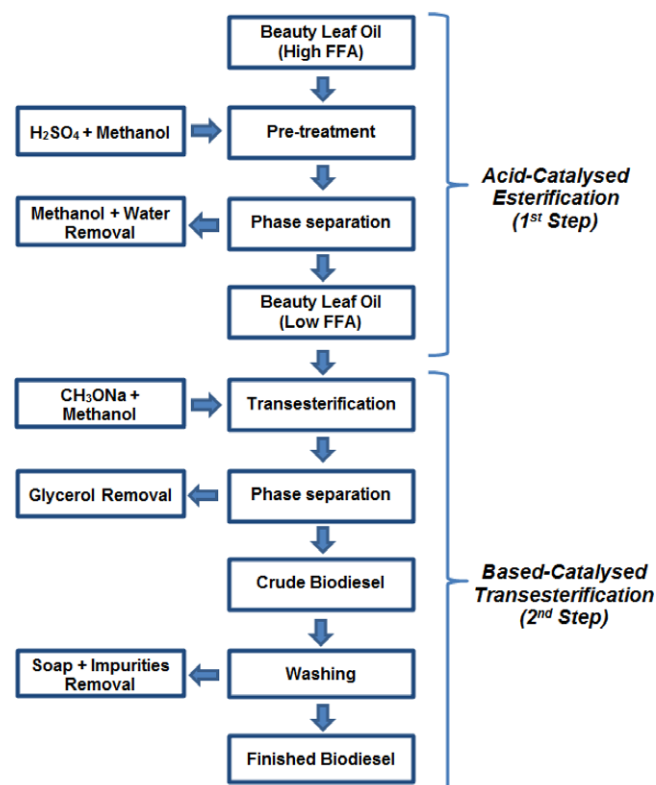d and the second is the number of double bonds. For example, 18:2 designates linolenic acid that contains 18 carbon atoms and two double bonds. Table 3 shows the fatty acid methyl ester composition of Beauty leaf oil (BLOME) produced through three different oil extraction methods along with traditional biodiesel obtained from soybean (SOME), canola (COME), palm (POME), rapeseed (ROME) and sunflower (SOME) oil feedstocks. The FAME composition of *Beauty leaf*, soybean and canola biodiesels were analysed by gas chromatography and flame ionisation detection (GC-FID) by EN 14103 standards. The gas chromatograph (GC) was a Hewlett–Packard 6890 System fitted with Varian Select™ 30 m × 0.32 mm × 0.25 μm column. FAME compositions of other biodiesel were collected from literature (Halpern, 1949). The prominent fatty acids found in chemical composition of biodiesels were Palmitic (Hexadecanoic, C16:0), Stearic (Octadecanoic, C18:0), Oleic (9-Octadecenoic, C18:1) and Linolenic (9, 12-Octadecadienoic, C18:2) acids. Three main types of fatty acids were found in the biodiesel samples: saturated (Cn:0), monounsaturated (Cn:1) and polyunsaturated with two or three double bonds (Cn:2, 3). The percentage of these compounds for each vegetable oil is given in Table 3. Based on this composition, average chain length (ACL) and an average number of the double bond (ANDB) were estimated using Eqs. (1) and (2).

$$ACL = \sum n \cdot (Cn:0, 1, 2, 3, wt\%) \tag{1}$$

$$\begin{aligned} ANBD = [1 \cdot (Cn:1, wt\%) + 2 \cdot (Cn:2, wt\%) \\ + 3 \cdot (Cn:3, wt\%)] / 100 \end{aligned} \tag{2}$$

where, *n* is the number of carbon atom in the fatty acid chain.

Similarly to other biodiesels shown in Table 3, *Beauty leaf* oil biodiesels were also high in Palmitic (C16:0), Stearic (C18:0), Oleic (C18:1) and Linolenic (C18:2) acids esters. Mono-unsaturated stearic (C18:1) acid methyl ester is the most prominent consisting of 38.6%–40.29% by weight followed by poly-unsaturated Linolenic (22.81%–27%), saturated Stearic (16.59%–18.64%) and Palmitic (14.48%–14.73%). *Beauty leaf* oil biodiesels have higher saturated
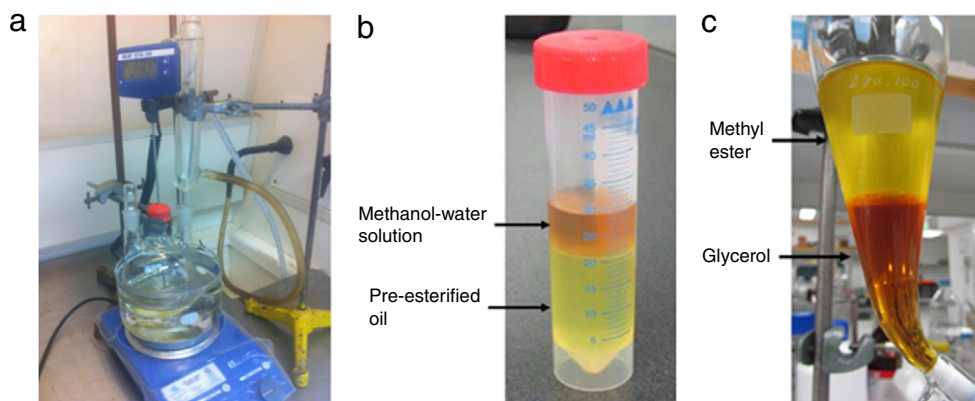
Fig. 9. (a) Esterification reactor (b) acid-catalysed pre-esterification product; (c) base-catalysedtransesterification product.

Table 3
The fatty acid distributions of *Beauty leaf* and commercial biodiesels.

FAME	Formula	BLOME[a]			SOME[a]	COME[a]	POME[b]	ROME[b]	SFOME[b]
		OP	ASE	nHX					
Lauric	C12:0	0	0	0	0	0.4	0.1	0	0
Myristic	C14:0	0	0	0	0	0.53	0.7	0	0
Palmitic	C16:0	14.73	14.48	14.68	13.04	13.19	36.7	4.9	6.2
Palmitoilic	C16:1	0.19	0.22	0.24	0.28	5.6	0.01	0	0.1
Stearic	C16:1	0	0.04	0	0	0	0	0	0
Oleic	C18:0	16.59	18.64	18.25	6.32	3.04	6.6	1.6	3.7
Linoleic	C18:1	39.3	40.29	40.18	26.59	47.1	46.1	33	25.2
Linolenic	C18:2	27	22.81	23.23	45.34	27.2	8.6	20.4	63.1
Gondonic	C18:3	0.28	0.17	0.19	6.9	5.23	0.3	7.9	0.2
Erucic	C20:0	0.95	1.04	1.02	0.44	0.55	0.4	0	0.3
Lauric	C20:1	0.29	0.24	0.23	0.3	0.95	0.2	9.3	0.2
Myristic	C22:1	0	0	0	0	0	0	23.0	0.1
Saturated (wt%)		**32.7**	**34.7**	**34.4**	**20.4**	**18.8**	**44.6**	**6.5**	**10.4**
Mono-unsaturation (wt%)		**39.86**	**40.88**	**40.51**	**27.28**	**53.97**	**46.31**	**65.30**	**25.30**
Poly-unsaturation (wt%)		**27.28**	**22.98**	**23.42**	**52.24**	**32.43**	**8.90**	**28.30**	**64.00**
Average chain length (ACL)		**17.74**	**17.74**	**17.74**	**17.76**	**17.67**	**17.28**	**19.01**	**17.94**
Average number of double bond (ANBD)		**0.95**	**0.87**	**0.87**	**1.32**	**1.19**	**0.64**	**1.22**	**1.53**

[a] Experiment.
[b] Literature (Hoekman et al., 2012; Singh and Singh, 2010; Jahirul et al., 2014).

esters consisting of 32.7%–34% which after palm oil biodiesel that is 44.6%. However, *Beauty leaf* biodiesel showed higher long chain saturation factor (10.72–11.81) over palm oil biodiesel (7.37). This is because palm oil biodiesel is richer in short chain saturated Palmitic acid esters compared with *Beauty leaf* biodiesel. Overall the chemical compositions of *Beauty leaf* biodiesels are closer to palm oil biodiesel than any other biodiesels shown in Table 3.

3.2. Fuel properties

Biodiesel properties from any types of feedstock need to meet the relevant quality standard before being accepted as an acceptable automobile fuel. However, the properties biodiesels are different from one feedstock to another due to differences in the compositional profiles describe above. In some cases, properties also vary in similar feedstocks from different origins and production processes. Therefore, biodiesels produced from any feedstocks are needed to meet the recognised quality standards for the commercial use as diesel engine fuel. The most internationally recognised biodiesel standards are EN14214 (in Europe) and ASTM D-6751 (in the USA). Numerous other countries have defined their standard, which in many cases derive from either EN14214 or ASTM D-6751. With the increasing production of biodiesel within Australia and as a part of the *Fuel Quality Standards Act 2000*, the Australian government has released a biodiesel fuel standard, "Fuel Standard (Biodiesel) Determination 2003'. This

standard is an adaptation of the above US and EU standards and fuel standards differ only slightly to conform to Australian climate related requirements. A summary of the important fuel quality parameters of *Beauty leaf* oil biodiesels and conventional biodiesels across all three standards are shown in Table 3. Among the fuel properties listed in Table 4, kinematic viscosity, density, higher heating value and acid value were obtained from the experiment for beauty leaf biodiesels, soybean and canola biodiesel. A similar experimental procedure was followed for these four parameters were described in the previous section. For comparison purposes, experimental data for above-mentioned parameters were obtained from literature (Ramos et al., 2009) for palm, rapeseed and sunflower oil biodiesel. The other fuel property parameters were estimated using the empirical equations based on chemical composition published in the literature. The equations used in this study to estimate the biodiesel properties were carefully selected from the published research papers.

3.3. Kinematic viscosity

One of the most significant fuel properties of biodiesel is Kinematic viscosity (KV). It plays a dominant role in the fuel atomisation, fuel–air mixture formation and combustion process particularly at the cold weather when an increase in viscosity affects the fluidity of fuel. The higher the KV, the higher is the pressure loss in the fuel line and injection pump, therefore, resulting in increases

Table 4
Fuel properties of *Beauty leaf* oil biodiesel and commercial biodiesels.

Property	Unit	Biodiesel standard			BLOME[a]			SOME[a]	COME[a]	POME[b]	ROME[b]	SFOME[b]
		Australian	ASTM D6751	EN 14214	OP	ASE	nHX					
Kinematic viscosity @40 °C	mm^2/s	3.5–5	1.9–6	3.5–5	4.46	4.34	4.38	3.86	5.45	4.5	4.4	4.2
Density	kg/m^3	0.860–900	n/a	860–900	0.894	0.892	0.893	0.863	0.871	0.874	0.877	0.880
HHV	MJ/kg	n/a	n/a	n/a	40.85	40.52	40.46	40.78	41.59	41.24	41.55	41.26
Acid number	mg KOH/g	0.8, max	0.5, max	0.5, max	0.88	1.00	0.76	0.34	0.91	0.12	0.16	0.15
Oxidation stability	h	n/a	3, min	6, min	4.14	4.44	4.42	2.71	3.21	5.31	3.09	1.88
Iodine value	g iod/100 g	n/a	n/a	120, max	81.44	74.81	75.24	119.47	107.07	57	109	132
Cetane number	–	51 min	47, min	51 min	56.53	58.42	58.39	47.89	49.16	61.80	52.02	44.90
Linolenic acid content	% (m/m)	n/a	n/a	12, max	0.28	0.17	0.19	6.9	5.23	0.3	7.9	0.2
Flash point	°C	120, min	93, min	120, min	145.64	143.06	143.65	160.87	162.00	176	170	177
CFPP	°C	Report	Report	Report	2.45	4.11	3.92	−5.76	−2.94	10	−10	−3

[a] Experimental.
[b] Literature (Hoekman et al., 2012; Singh and Singh, 2010; Ramos et al., 2009; Atabani et al., 2014, 2013b).

in engine deposits and, shoot formation, requiring more energy to pump the fuel and increasing wear on fuel pump elements and injectors. In contrast, low fuel KV is not desirable because it will not provide sufficient lubrication for the precision fit of fuel injection pumps, resulting in leakage or increased wear (Jahirul et al., 2013). Therefore, the upper and lower limit of biodiesel KV is defined in all biodiesel standards shown in Table 4. The KV of produced *Beauty leaf* biodiesels was 4.38–4.46 mm^2/s that are at the acceptable limit according to all biodiesel standards. Table 3 also shows that the KV of *Beauty leaf*, palm, rapeseed and sunflower oil biodiesels were quite similar at around 4.4 mm^2/s whereas soybean oil biodiesel has the lowest (3.86 mm^2/s) and cottonseed oil biodiesel has the highest kinematic (5.45 mm^2/s). The *Beauty leaf* biodiesel made from oil through oil press showed slightly higher KV compared with chemical oil extraction that may be due to the higher viscosity of the same feedstock. Overall there is only minor variation in KV was found between three different *Beauty leaf* biodiesels.

3.4. Density

Density is defined as mass per unit volume of the liquid fuel commonly expressed in units of kg/m^3. Density is an important property for automobile fuel because it influences the amount of fuel injected in the engine cylinder. Changes in fuel density will influence engine output power due to a different mass of fuel injected which directly affects engine performance. Comparing crude vegetable oil (Table 2) and vegetable oil methyl ester (Table 4), it can be seen that esterification process reduces the density by 7%–8%. *Beauty leaf* biodiesel produced from oil obtained through mechanical extraction showed slighter higher density compared with that of other *Beauty leaf* biodiesels. However, the densities of all biodiesels shown in Table 3 were in the acceptable range specified by Australian and European biodiesel standards.

3.5. Higher heating value

The higher heating value indicates energy content in the fuel per unit mass. Therefore, the conventional unit of higher HHV is kJ/g or MJ/kg. The HHV of *Beauty leaf* biodiesels was found to vary from 40.85 to 40.96 MJ/kg with very little fluctuation among the *Beauty leaf* oil source. These results indicate that the HHV of vegetable oil methyl ester is about 4% higher than the crude vegetable oil as show in Table 2. Table 4 shows that the biodiesel produced from non-edible *Beauty leaf* oil produced has an HHV close to that of commercial biodiesel produced from edible vegetable oil feedstocks.

3.6. Acid number

Acid number (AN) indicates the amount of carboxylic acid present, such as in fatty acids. It is expressed as the amount of KOH (mg) required for neutralising 1 g of fatty acid methyl ester or biodiesel. Fuel with a high acid number can cause a higher level of lubricant degradation and severe corrosion in engine fuel systems (Haseeb et al., 2011). AN is set to a maximum of 0.5 KOH/g in both European (EN14214) and American (ASTM D6751) biodiesel standards whereas the Australian standard allow slightly higher AN, setting the maximum value at 0.8 KOH/g. Naturally, the AN of crude *Beauty leaf* oils were very high compared with the traditional edible vegetable oil shown in Table 2. Table 4 shows that a significant reduction of this acid value occurred in the two-stage biodiesel production process utilised in this study. However, the acid number of *Beauty leaf* oil biodiesels remained high when compared with other commercial biodiesels. Table 4 indicates that oils obtained through oil press, *n*-Hexane and ASE produced biodiesel were 0.88, 0.76 and 1.00 KOH/g respectively. Although only biodiesel from *n*-Hexane oil met the Australian biodiesel standard (Table 4), the other biodiesels were only slightly higher than the standard. It is likely that with further optimisation, biodiesel from *Beauty leaf* oil should be able to meet EN standards.

3.7. Oxidation stability

Oxidation stability (OS) is the indication resistance of the degradation of fuel due to oxidation during long-term storage. Biodiesels show less oxidative stability compared with petroleum diesel due to their different chemical composition, and this is one of the major issues that limits the wide spread use biodiesel as a fuel in automobile engines. In this study, OS of beauty leaf biodiesels and conventional biodiesels are estimated from the fatty acid composition of biodiesels using Eq. (3) proposed by Wang et al. (2012).

$$OS = -0.0384 \times DU + 7.770. \tag{3}$$

All of the biodiesels listed in Table 4 failed to meet the ASTM standard in terms of oxidation stability, which is 3 h minimum. Only *Beauty leaf* and palm biodiesel were in the range of European standard of oxidation stability. *Beauty leaf* biodiesels showed oxidation stability from 4.12 to 4.42 h that was much higher than all conventional biodiesel except palm oil biodiesel. This is because the presence of double bonds in the chains influences the rate of oxidation. The feedstocks with a higher concentration of polyunsaturated fatty acids are much more prone to oxidation than the feedstocks contains saturated or monounsaturated fatty

acids. For the same reason, *Beauty leaf* biodiesel obtained from oil press showed less oxidation stability then other types. An overall oxidation estimation result confirms that *Beauty leaf* biodiesel is a better fuel in terms of OS then most of the commercial biodiesel.

3.8. Iodine value

Iodine value (IV) is a critical parameter concerning to biodiesel quality on the ground that higher IV biodiesel prompts a higher rate of polymerisation of glyceride that increases fuel thickness, resulting the formation deposits on engine fuel system and adversely affecting fuel injector spray patterns. This property is set to a maximum value of 120 g I_2/100 g according to EN14214 standard. IV of beauty leaf biodiesel and conventional biodiesels were determined from the chemical composition of biodiesel using Eq. (4) developed by Kalayasiri et al. (1996).

$$IV = \sum_i \left(\frac{254 \times N_i \times D_i}{M_i} \right) \qquad (4)$$

where N_i, D_i and M_i are the percentages, a number of double bonds and molecular weight of the i_{th} fatty acid methyl ester.

The IV results tabulated in Table 4 indicate that all biodiesels meet the EN14214 standard except the sunflower oil methyl ester. The IV of *Beauty leaf* oil biodiesels (74.81–81.44 I_2/100 g) were well below the allowable limit and also below most of the commercial biodiesel. Only palm oil biodiesel showed better result than *Beauty leaf* biodiesel in IV which was estimated 57 I_2/100 g. Oil press *Beauty leaf* biodiesel showed slightly higher IV due to having a higher degree of unsaturation compared to other types.

3.9. Cetane number

Cetane number (CN) is a utilised used diesel fuel quality parameter and is an estimation of the ignition quality of diesel fuels. It is identified with the ignition delay (ID) time, that is, the time that passes between injection of the fuel into the cylinder of a diesel engine and the onset of burning (Knothe, 2005). Higher CN is desirable for ensuring good cold start properties and reducing the development of white smoke. Oppositely, lower CN may bring about diesel knocking and enhancing the exhaust emissions. Australian and European biodiesel standard limit the CN to a minimum value of 51 whereas ASTM standard limit it a minimum value of 47 as shown in Table 4. In this study, CN of biodiesel was calculated using Eq. (5) proposed by Kalayasiri et al. (1996).

$$CN = 46.3 + \left(\frac{5458}{SV} \right) - (0.225 \times IV) \qquad (5)$$

where *SV* is the saponification value estimated using Eq. (6)

$$SV = \sum_i \left(\frac{560 \times N_i}{M_i} \right). \qquad (6)$$

Table 4 indicates excellent ignition quality of biodiesel produced from *Beauty leaf* oil biodiesels. The CN of *Beauty leaf* biodiesels was 56.53–58.39; much higher than the minimum recommended value of 51. Moreover, the CN of beauty leaf biodiesels are far better than most of the commercial biodiesel produced from edible oil. The CN of palm oil biodiesel was found to be 61.80, slightly higher than *Beauty leaf* oil biodiesel. This is because palm oil contains a higher percentage of saturated methyl ester. Oil press biodiesel showed slightly lower CN then the other beauty-leaf oil biodiesels that may be due to the higher linoleic acid content, which increases the degree of unsaturation and hence reduces the CN.

3.10. Flash point temperature

The flash point (FP) is the lowest temperature at which the fuel will begin to vaporise to form an ignitable mixture when it comes in interacts with the air. Australian and European biodiesel specification required flash point temperature at least 120 °C, whereas in the US the minimum requirement level is 93 °C. FP temperature in °C was calculated from the linear recreation Eq. (7) developed by Agarwal et al. (2010).

$$\begin{aligned} FP = {} & 205.226 + 0.083 \times C16{:}0 - 1.723 \times C18{:}0 \\ & - 0.5717 \times C18{:}1 - 0.3557 \times C18{:}2 - 0.467 \times C18{:}3 \\ & - 0.2287 \times C22. \end{aligned} \qquad (7)$$

Table 3 shows that the FP temperature of *Beauty leaf* biodiesels was between 143.06 and 145.64 °C that is higher the minimum requirement specified in the biodiesel standards. While comparing with commercial biodiesel, *Beauty Leaf* biodiesel showed lower flash point temperature. It is noted that very high flash point temperature of automobile fuel is not desirable because it can cause cold engine start-up problems, misfiring and ignition delay, which increases carbon deposition in the combustion chamber (Szybist et al., 2007). No significant variation in FP temperature was noted among the different *Beauty leaf* biodiesel results shown in Table 4.

3.11. Cold filter plug point (CFPP)

One of the real issues connected with the utilisation problems associated with the use of biodiesel in countries with a frosty atmosphere is their poor cold flow properties when compared with petroleum diesel fuels. The parameter determines the cold flow property biodiesel be called cold-filter plugging point (CFPP). It is the least temperature at which a fuel portion will go through a standardised filtering device in a determined time (Jahirul et al., 2013). Cold filter plugging point (CFPP) in °C were calculated from Eq. (8) proposed by Ramos et al. (2009).

$$CFPP = (3.1417 \times LCSF) - 16.477 \qquad (8)$$

where, long chain saturation factor estimated using Eq. (9)

$$\begin{aligned} LCSF = {} & 0.1 \cdot (C16{:}0, \text{wt\%}) + 0.5 \cdot (C18{:}0 \text{ wt\%}) \\ & + 1 \cdot (C20{:}0 \text{ wt\%}) + 1.5 \cdot (C22{:}0 \text{ wt\%}) \\ & + 2.0 \cdot (C24{:}0 \text{ wt\%}). \end{aligned} \qquad (9)$$

The cold temperature properties of biodiesel should be reported according to the Australian, European and US although the limits are not specified. However it is commonly understood that biodiesels with low CFPP, CP and PP are better options for diesel engine fuels operating in cold weather condition. Table 4 shows that all cold temperature properties of *Beauty leaf* biodiesels were much higher than that from most commercial biodiesel. Among the biodiesel showed in Table 4, palm oil biodiesel showed highest CFPP temperature followed by *Beauty leaf* oil biodiesel. The rapeseed oil biodiesel followed by soybean oil biodiesel showed the lowest cold temperature properties. The average CFPP of *Beauty leaf* biodiesel was found 3.5, 12.6 and −2.9 °C, respectively. Beauty leaf oil produced through oil press showed slightly better cold weather properties due to having a higher linolenic acid methyl ester content.

3.12. Validation of beauty leaf biodiesel

To become a successful alternative of diesel fuel, *Beauty leaf* biodiesel should have the suitable chemical composition to ensure compliance with standard biodiesel properties. The fuel properties of beauty leaf biodiesel from three different extraction
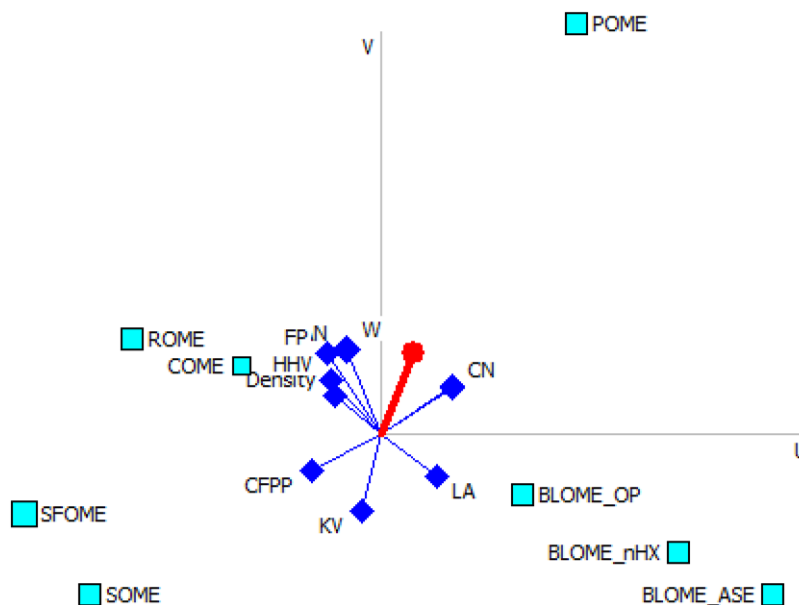
Fig. 10. GAIA plot for eight biodiesel showing ten criteria and decision vector. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Variables and preference used in PROMETHEE-GAIA analysis.

Variables	Preference for PROMETHEE-GAIA
Kinematic viscosity (KV)	Min
Density	Min
Higher heating value (HHV)	Max
Acid number (AN)	Min
Oxidation stability (OX)	Max
Iodine value (IV)	Min
Cetane number (CN)	Max
Linolenic acid (LA)	Min
Flash point (FP)	Max
Cold filter plug point (CFPP)	Min

Table 6
Corresponding ranking and Phi value of biodiesels.

Rank	Biodiesel	Phi
1	POME	0.16
2	ROME	0.05
3	BLOME_OP	0.02
4	BLOME_nHX	0.01
5	BLOME_ASE	−0.01
6	COME	−0.06
7	SFOME	−0.08
8	SOME	−0.10

methods were analysed and compared with five other commercially available biodiesels. To determine the suitability of *Beauty leaf* biodiesels compared to other biodiesels based on 14 criteria (fuel properties): CN, IV, OX, AN, HHV, KV, Density, FP, CFPP, Linolenic acid, ACL, MUFA and PUFA a multi-criteria decision method (MCDM) software was used. In this study, the PROMETHEE-GAIA were used because of their rational decision vector that stretches towards the preferred solution compared to other MCDM (Islam et al., 2015) (see Table 5).

In GAIA plane, the criteria that lie near to (±45°) are corresponded, while those lying in reverse bearing (135°–225°) are against related, and those in a generally orthogonal course have no or less impact (Espinasse et al., 1997). The preference function criteria (fuel property) were modelled as a minimum (i.e. lower values are preferred for good biodiesel) or maximum (higher values are preferred for good biodiesel). The selection of preference function also influences the direction of criteria. For example, IV and CN were inversely related but still showed in the same direction within ±45°. This is because the Cetane number were preferred to maximum, but iodine number was preferred to a minimum, as shown in Table 4, which has was suggested by Islam et al. (2013). Therefore, criteria that are in the same preference (min/max) and lie close to ±45° are correlated. The direction and length of criteria are indicative to their influence on decision vector (marked as red line in Fig. 10) (Islam et al., 2013), such that the very short length of some criteria, in particular, 'Density' and 'HHV', indicate the little effect on the decision vector.

The choice vector demonstrates the best samples, (*i.e.*, those that adjust to the course of this vector) and the furthest criteria towards the choice vector are the most ideal (Figueira et al., 2005). In this study, equally weighted criteria showed (Fig. 10) that POME was most aligned with the decision vector and its farthest position from the centre gave it the highest ranking.

Table 6 shows the overall ranking of the different biodiesel and the three biodiesel from *Beauty leaf*, BLOME_OP, BLOME_nHX and BLOME_ASE, were placed third, fourth and fifth, respectively, in the middle of the overall rankings. The Phi value is the net flow score that could be negative or positive depending upon the angular distance from the decision vector and the distance from the centre. Biodiesel from soybean oil was at the bottom of the ranking compared with other biodiesel. It can be seen from Fig. 10 that the quality of *Beauty leaf* biodiesel in terms of fuel properties does not depend on oil extraction methods. The results of this analysis indicate the ability of *Beauty Leaf* biodiesel to compare with commercially available first-generation biodiesels.

The quality ranking analyses of biodiesel shown in the previous section was conducted with a similar weighting of all parameters. However, the significance of some fuel properties depends on the environment where it will be stored and utilised. In tropical/subtropical location, CFPP was not through to be of significant here. High temperatures of this area are, however, likely to influence the oxidative stability of the biodiesel. On the other hand, in the winter climate condition CFPP are more important than oxidation stability. Therefore, ranking sensitivity analysis was conducted for the fuel properties CFPP and OS by increasing the weighting from 1 (equal to other parameters) to 10, and the results are shown in Table 7. A significant change in ranking was found for both

Table 7
Comparative rank shift with different OS and CFPP weighting.

	OS					CFPP						
Weighting	1–3	4–6		6–10		1–2	3–4		5–6		7–10	
POME	1	1	–	1	–	1	5	↓	7	↓	8	↓
ROME	2	5	↓	6	↓	2	1	↑	1	–	1	–
BLOME_OP	3	2	↑	2	↑	3	6	↓	6	–	6	–
BLOME_nHX	4	3	↑	3	↑	4	7	↓	8	↓	7	↑
BLOME_ASE	5	4	↑	4	↑	5	8	↓	5	↑	5	–
COME	6	6	–	5	↑	6	3	↑	3	–	3	–
SFOME	7	7	–	7	–	7	4	↑	4	–	4	–
SOME	8	8	–	8	–	8	2	↑	2	–	2	–

Black arrows upward: rank increase; Black arrows downward reduce rank; Hyphen: no ranking change.

OS and CFPP. POME always ranked 1 with the increasing of OS weighting. At the same time, the rank of *Beauty leaf* biodiesels were improved and was ranked just after POME. In contrast, the rank of ROME dropped dramatically with the weighting increase of OS. On the other hand, an opposite trend was observed when the weighting was increased for CFPP. Both POME and *Beauty leaf* biodiesels dropped in rank and were placed at the bottom in the ranking table. Therefore, as for palm oil biodiesel, the Beauty leaf oil biodiesels are unlikely to be suitable for cold climate conditions, especially in winter. These results indicate that beauty biodiesels are a better choice for tropical/sub-tropical regions than and colder climate conditions.

4. Conclusion

Second-generation biodiesel is gaining more interest in the market as a sustainable alternative of diesel fuel. However, to produce biodiesel from new sources and continue to develop these in the market, various aspects must be examined. In this study, the potential of Beauty leaf plant was evaluated as a source of second-generation biodiesel. Oil was extracted from dry seed kernels using three different oil extraction methods, and oil properties have been analysed. Oil has been esterified to produce biodiesel using a two-step esterification technique, and the physicochemical properties were assessed. From the results obtained in this study the following conclusion can be made.

The performance of *Beauty leaf* oil extraction using an oil press resulted in a low oil yield. This drawback was overcome using chemical oil extraction using *n*-hexane as oil solvent. Furthermore, the oil yield further increased by 3%–4% with high pressure and temperature extraction. The highest oil yield was found on average 51.5% of dry kernels in ASE extraction method, which suggested that *Beauty leaf* plant can produce about 1.56 tons of oil per year per hectare. When comparing quality with edible vegetable oils, conventionally used as biodiesel feedstock, in terms of acid value, density, kinematic viscosity, surface tension and higher heating value, *Beauty leaf* oil showed much higher acid values resulting from high free fatty acid contents. Chemical oil extraction under atmospheric conditions produced oil containing relatively low levels of free fatty acids. However, those results have illustrated that raw *Beauty leaf* oil may not be suitable for direct use in diesel engines. Another drawback of Beauty leaf oil is that conventional base-catalysed trans-esterification cannot be used directly for biodiesel production. Therefore, a two-step esterification process, involving acid-catalysed pre-esterification and base-catalysed trans-esterification, was used in this study. During the first stage of this process, the acid value was significantly reduced to the acceptable limit for base-catalysed trans-esterification. The highest biodiesel conversion efficiency was found to be 93.05% for the oil produced by chemical oil extraction in atmospheric condition, whereas oil obtained from screw press and ASE methods showed 75.74% and 83.76%, respectively, under similar reaction conditions, which is due to variations in the acid value of the respective oils.

Beauty leaf oil biodiesels mostly comprise esters of saturated Hexadecanoic (C16:0) and Octadecanoic (C18:0) acid, mono-unsaturated 9-Octadecenoic acid (C18:1) and poly-unsaturated 9, 12-Octadecadienoic (C18:2). This biodiesel is rich in saturated methyl esters compared with commercial biodiesels, except biodiesel from palm oil and is also rich in long chain saturation factors. Like palm oil, this makes Beauty leaf oil biodiesel better in terms of most of fuel properties, including kinematic viscosity, density, higher heating value, oxidation stability, iodine value, cetane number, flash point, linoleic acid content. On the other hand, Beauty leaf biodiesels are performed worse in terms of cold temperature properties and free fatty acid content. However, *Beauty leaf* biodiesel can meet the American, European and Australian biodiesel standards. The multivariate data analysis using PROMETHEE-GAIA software indicated that biodiesel from *Beauty leaf* oil could be a better option for automobile engine application compared with much other commercial biodiesel, including biodiesel from cotton seed, sunflower and soybean oil, especially in tropical/sub-tropical regions.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

MI has made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data and manuscript drafting.

RB has given to conception, design and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved and final approval of the version to be published.

WS has given to conception, design and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved and final approval of the version to be published.

AN has involved providing experimental facilities and data accusation and participated in the sequence alignment.

RM has been involved manuscript revising critically for important intellectual content.

RM has been carried out in data analysis.

FM has been involved in drafting the manuscript and has been carried out in the data analysis.

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