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IMPACT OF GEOGRAPHIC VARIATION ON PHYSICOCHEMICAL PROPERTIES OF NEEM (*AZADIRACHTA INDICA*) SEED OIL

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ABSTRACT: The applications of the seed oil are defined by its quality through many factors, including seed origin. The study aims to investigate the physicochemical characteristics to compare the oil quality of plant *Azadirachta indica* (Family: *Meliaceae*), grown in different geographical areas, Malaysia, and Sudan. Oils were extracted through the Soxhlet method via hexane for the duration of 6 h. The characteristics of the oils from Malaysia (OMY) and Sudan (OSD) are; lipid content: 37.03 and 39.96%; physical state: liquid for both; color: greenish-brown and brownish-yellow; odor: peanut with garlic and only peanut; density: 0.95 and 1.06 g/cm^3; refractive index: 70.90 and 69.80; pH: 4 and 5; moisture & volatile matter: 0.83 and 0.07%; acid value: 4.80 and 6.37 mg KOH/g; iodine value: 93.09 and 63.81 gI_2/100g; peroxide value: 8.49 and 1.67 meq O_2/kg; unsaponifiable matter: 1.84 and 0.91%; and free fatty acid: 4.75 and 3.21%. OMY and OSD recorded eight similar fatty acids: oleic- 20.46 and 52.02%, linoleic- 34.69 and 3.02%, stearic- 20.42 and 18.79%, palmitic- 18.66 and 20.79%, arachidic- 3.59 and 2.39%, behenic- 0.80 and 0.41%, lignoceric- 0.55 and 0.27%, and palmiticoleic acid 0.17 and 0.27%, respectively. Myristic- (0.05%) and lauric acid (0.02%) were found only in OSD. No significant differences observed in transmittance. These oils showed suitable characteristics for applications as non-drying oils, whereby the OSD has fewer tendencies towards oxidative rancidity. The overall study proves that the plant geographical variation influences the quality of the oil.

INTRODUCTION: Plant seeds are considered as one of the major sources for many of important phytochemicals, such as oils and its components. Since long ago, people worldwide have been using the seed oil for different purposes such as medicine, food, as well as fuel.

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Nowadays, the developing science and technology had also studied for various properties of the plant seed oil, in terms of replacing the existing petroleum with biodiesel, production of polyurethane coatings, as a modulator in rumen fermentation properties and the encapsulation of the oil for nano-emulsion [1, 2].

The worldwide known plant Neem, genus *Azadirachta* of *A. indica* (Family: *Meliaceae*) has been used for centuries since 2000-4000 BC. In terms of the Indian concept during the ancient times, the plant known to be, "the cure of all ailments" [3].

Previously, the uses of the various parts of the plant, such as the leaves, barks, roots, twig, seed kernel, and seed oil have been studied and reported. The seed oil of *A. indica* is one of the valuable parts of the plant, and its oil were extracted through pressing or solvent extraction methods. The oil had been used for Ayurvedic, pharmaceuticals, and pesticides for the treatment of acne, as repellents, for soap and toothpaste production and many other biological activities [4]. This plant now has a wide distribution over the world and could be found in many countries due to its essentiality in medicinal properties. Even though the species of the plant are the same, many other factors such as the geographical origin, genetic makeup, environmental condition, climate cultivation, and soil composition might result in differences of the characteristics of the plant [5]. Therefore, this study aimed to compare the quality of the seed oil, through their physicochemical properties of *A. indica* plant seed oil grown in two different regions Malaysia (Southeast Asia) and Sudan (North Africa).

MATERIALS AND METHODS:

Plant Materials: The seeds, were harvested directly from the trees in Malaysia and Sudan on May 2013, dried under the open air, and further dried in an oven at 40 °C. The entire study was conducted in Universiti Malaysia Pahang, Pahang, Malaysia. The samples were taken for initial moisture content *via* Moisture Analyzer (AND MS-70, Japan). The known weight of seeds was crushed by electric blender to reduce the particle size. All solvents and chemicals used in the study were of chromatography and analytical grades (Merck, Fisher, and Sigma from Germany, UK, and the US, respectively).

Extraction of the Seed Oil: The oils of the *A. indica* seeds were extracted by solvent semi-continuous extraction method (Soxhlet) for 6 hours with n-hexane [6]. The extracted oils were filtered through filter paper (Whatman no. 2, 125 mm), and the solvent was evaporated using a rotary evaporator (model Buchi R-3, Switzerland) under reduced pressure and temperature and further dried under open air in a dark area.

The obtained oils were stored in hermetically closed dark bottles and kept on -4°C for further studies. The percentage of the lipids (w/w %) was calculated according to the following formula:

$$\% \text{ Lipid} = \text{Weight of oil (g)} \times 100\% / \text{Weight of sample (g)}$$

Physical State, Color, Odor, Freezing, Melting and Boiling Point Determination: Physical state at room temperature (25 °C) and color of the oils were determined visually, whereby odor was determined through the sensation of volatilized smell. For freezing point, the oil was filled in a clear glass vial, a thermometer was immersed into the oil, and the oil was solidified through the usage of ice blocks. The solidification temperature was recorded as freezing point.

The solidified oil was melted over a water bath at the temperature of 29 °C and the melting point was recorded. Again, around 10 mL of oil is filled in a clear glass vial and a thermometer was inserted. The vial was exposed to heat on a heating mantle and the oil was observed, whereby it starts circulating leading to boiling. The temperature at this point was recorded at boiling point [7].

Density, Refractive Index and UV-Vis Transmission Determinations: Density determination was done according to the following method, a small empty vial was weighed, and then filled with a known amount of oil up to the brim [8]. The vial was weighed again, and the density was calculated according to the following formula;

$$\text{Density } \rho = (\text{Weight of vial + oil}) - \text{Weight of vial} / \text{Volume of oil}$$

The refractive index of the oils was determined by using standard method [9]. This index was measured at 25 °C *via* pen Refractometer (Atago, Japan) with resolution and accuracy value of 0.1% and ± 0.2% in 10-60 °C. The measurement was repeated in triplicate, and the average value was reported. A dual-beam UV-Vis Spectrophotometer model Genesys 10S (Thermo Fischer Scientific, UK) was used to study the transmission characteristics of these oils.

The UV transparent quartz cell with the dimension of 10 × 1 mm, were loaded with 0.25 mL sample and scanned at the range of 190-800 nm. The transmission of the blank quartz cell characteristics was taken before the analysis of each sample.

Acid Value, Free Fatty Acid, Iodine Value, Peroxide Value, and Unsaponifiable Matter Analysis: The free fatty acids [10], iodine value [11], peroxide value [12] and unsaponifiable matter [13] were analyzed according to the standard methods. The acid value was determined by the following equation;

Acid value = % Fatty acid (as oleic) × 1.99

pH, Moisture, and Volatile Matter Analysis: pH was analyzed by using pH indicator strips (Merck, Germany). The universal indicator strip was dipped into the oil and was dried on a dry surface for 60 s. The reading was taken by comparing the color changes on the strips with the pH chart. Moisture and volatile matter were analyzed according to air-oven method [14] with some modification. About 5 g of oil, was weighed on a previously dried and tiered dish. The dish was covered with a loose lid and was heated in the oven at 105 ± 1 °C for one hour. The dish was removed from the oven, cooled in a desiccator and was weighed. The plate was re-heated for one hour. The cooling and weighing process was repeated until the weight change between two successive observations does not exceed one mg. The following formula is used to calculate the observations;

% of moisture and volatile matter = Weight loss of material on drying (g) × 100 / Weight of material (g)

Fatty Acid Composition and Percentage of Saturated and Unsaturated Fatty Acids: The crude oil was converted into fatty acid methyl ester, through transesterification reaction to determine the fatty acid composition. An amount of 100 mg of oil sample was dissolved in 10 mL of hexane in a test tube. An amount of 1 mL of 2 M KOH (Methanolic potassium hydroxide) was added into the same test tube and was a vortex. The hexane phase was collected and washed twice with 4 mL of water after 15 min and was further dried over anhydrous sodium sulfate. The fatty acid composition analysis was done by Gas Chromatography-Mass Spectrometry (GC-MS). The details of the chromatography equipment and settings are as shown in **Table 1**. The individual fatty acid composition was expressed as a percentage.

The sum percentage of saturated fatty acid was represented as total saturated fatty acids, whereas the sum of all unsaturated (mono- and polyunsaturated) was represented as total unsaturated fatty acids.

Statistical Analysis: Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS) software version 15.0, generating One-way ANOVA analysis. All values were expressed as a mean ± standard mean error. To determine whether there were any differences between the means, ANOVA and Duncan's new multiple range tests were applied to the result at 0.05 level of significance ($P < 0.05$). The Pearson correlation analysis was performed between variables.

TABLE 1: CHROMATOGRAPHIC SETTINGS FOR THE ANALYSIS OF *A. INDICA* OIL METHYL ESTER

Parameter	Setting
Chromatograph	Agilent Technologies7890A Gas Chromatography (GC) Systems coupled with Mass Spectrometry (MS) detector
Auto-sampler	GC autosampler
Column	Nonpolar capillary DB-1 of 100% dimethyl-polysiloxane with 30 m lengths, 0.25 mm diameter and 0.25 µm thickness
Carrier as	Helium at 1mL/min
Injector	Splitless mode with a n injector temperature of 250°C
Injection volume	1µL/L
Ion-source temperature	230 °C
Temperature program	60°C for 3 min, 240°C at the rate of 3°C/min and held for 10 min. Total runtime of 93 minutes
Lab data system	National Institute of Standards and Technology (NIST) Library Chem Station software

RESULTS: Table 2 and **3** shows various parameters on the physical and chemical properties of *A. indica* seed oils from Sudan (OSD) and Malaysia (OMY). The oil content for OMY and OSD were 37.03 and 39.96% respectively, and these values are represented in terms of lipid content **Table 2**. Both extracted oil present to be in a liquid state at the room temperature of 25 °C with

the color difference of brownish yellow and greenish-brown for OSD and OMY respectively. The OSD resembles like peanut, whereas the OMY smells with the combination of peanut and garlic. The boiling, freezing, and melting point was only studied for the OSD whereby the values attained are 230.0, 3.0 and 9.0 °C respectively. The density recorded for OSD is 1.06 g/cm^3 that are higher than OMY, 0.95 g/cm^3. The acid value, iodine value, peroxide value, pH and moisture and volatile matter that are obtained in this study for both OSD and OMY are 6.37 and 4.80 mg KOH/g, 63.81 and 93.09 gI_2/100g, 1.67 and 8.49 meq O_2/kg, 5.0 and 4.0, 0.07 and 0.83% respectively. The analysis of free fatty acid was represented in three forms of fatty acids; oleic-, lauric- and palmitic acid that achieves 3.21 and 4.75%, 1.24 and 3.36%, and 2.91 and 4.30% respectively for OSD and OMY. The transmittance values recorded for the different regions as plotted **Fig. 1** whereby the values are, visible: 83.36-87.43 and 78.64-77.79; UV-A: 83.36-40.84 and 78.64-37.49; UV-B: 40.84-0.11 and 37.49-0.10; and UV-C: 0.11-0.12 and 0.10-0.07 for the OSD and OMY respectively.

TABLE 2: PHYSICOCHEMICAL PROPERTIES OF A. INDICA SEED OIL FROM SUDAN AND MALAYSIA

Parameter		Unit	Experimental Value	
			OSD	OMY
Lipid content		%	39.96	37.03
Physical state at 25 °C		-	Liquid	Liquid
Color		-	Brownish-yellow	Greenish brown
Odor		-	Peanut	Combination of peanut and garlic
Boiling point		°C	230.0	ND
Freezing point		°C	3.0	ND
Melting point		°C	9.0	ND
Density at 25 °C		g/cm^3	1.06	0.95
Refractive index at 25 °C		-	69.80	70.90
Acid value (% free fatty acid as oleic)		mg KOH/g	6.37	4.80
Iodine value		gI_2/100g	63.81	93.09
Peroxide value		meq O_2/kg	1.67	8.49
Unsaponifiable matter		% by weight	0.91	1.84
pH		-	5.0	4.0
Moisture and volatile matter		% by weight	0.07	0.83
Free fatty acid value	Oleic	%	3.21	4.75
	Lauric	%	1.24	3.36
	Palmitic	%	2.91	4.30
Total saturated fatty acids		%	42.72	44.02
Total unsaturated fatty acids		%	55.31	55.32

*Values were recorded as mean average; ND: Not determined

TABLE 3: FATTY ACID COMPOSITION (%) OF *A. INDICA* SEED OIL FROM SUDAN AND MALAYSIA

Fatty Acid	Formula	Systematic Name	Structure	Weight (%)	
				OSD	OMY
Oleic acid	$C_{16}H_{34}O_2$	9-octadecenoic acid	C18:1	52.02	20.46
Linoleic acid	$C_{18}H_{32}O_2$	9,12-octadecadienoic acid	C18:2	3.02	34.69
Stearic acid	$C_{16}H_{36}O_2$	Octadecanoic acid	C18:0	18.79	20.42
Palmitic acid	$C_{16}H_{32}O_2$	Hexadecanoic acid	C16:0	20.79	18.66
Arachidic acid	$C_{20}H_{40}O_2$	Eicosanoic acid	C20:0	2.39	3.59
Behenic acid	$C_{22}H_{44}O_2$	Docosanoic acid	C22:0	0.41	0.80
Lignoceric acid	$C_{24}H_{48}O_2$	Tetracosanoic acid	C24:0	0.27	0.55
Palmiticoleic acid	$C_{16}H_{30}O_2$	9- hexadecenoic acid	C16:1	0.27	0.17
Myristic acid	$C_{12}H_{28}O_2$	Tetradecanoic acid	C14:0	0.05	-
Lauric acid	$C_{24}H_{24}O_2$	Dodecanoic acid	C12:0	0.02	-

*The obtained results in terms of fatty acid methyl esters from GC-MS library data system was reviewed, and the final results were listed out in the form of fatty acid chains.

The determination of free fatty acids through the transesterification reaction of OMY and OSD detected eight similar fatty acids: oleic- 20.46, 52.02%, linoleic- 34.69, 3.02%, stearic- 20.42, 18.79%, palmitic- 18.66, 20.79%, arachidic- 3.59, 2.39%, behenic- 0.80, 0.41%, lignoceric- 0.55,

0.27%, and palmiticoleic acid 0.17, 0.27%, respectively. The OSD present to have extra two extra fatty acid, myristic- (0.05%) and lauric acid (0.02%).

DISCUSSION: The obtained yields are agreeing with literature stating that *A. indica* seed contains 25-45% oil on a dry matter basis [15]. The difference in the color intensity of the oils that are brownish-yellow and greenish-brown for Sudanese and Malaysian oil respectively might be attributed due to the presence of various pigments, such as the chlorophyll content. The green color of the immature seeds disappears upon maturation resulting in chlorophyll retention [16].

Besides, there is also a report stated that the presence of moisture contents at greater levels impacts the color of the oil, whereby the moisture rises the chlorophyll content and thus contribute in an increment of color intensity [17]. The normal and thermal oxidation process of oil contributes towards the deterioration of lipids, and thus it might also influence the color changes of the oil compared to the initial color of the oil upon [16, 18].

For the past several years, many compounds had been isolated and identified from *A. indica* seed oil and one of the compounds, tignic acid (5-methyl-2-butanic acid) had been identified to be responsible in the typical odor of the oil [19, 20].

The boiling, freezing and melting point of OSD is almost in, similar to the reported results whereby 258.0, 7.0 and 10.0 °C for boiling, freezing and melting point respectively [8]. Literature had reported a density value of 102.00 kg/m^3 (1.02 g/cm^3), that is closer to the value of the Sudan oil [21]. The density varies as the concentration of the wall material varies at which more heavy material fits into spaces between the particles, the higher is the density [22].

The refractive index value is significant since the amount of unsaturated fatty acids and long-chain hydrocarbon. The refractive index of the OSD (69.80) is slightly lower compared to the OMY (70.90). This is attributed by the low amount of unsaturated fatty acid, length of the hydrocarbon chain, molecular weight and degree of unsaturation as well as conjugation [23]. The acid value is the relative measure of rancidity as free fatty acids, which are formed during decomposition or hydrolysis of oil glycerides, due to the action of moisture, temperature and/or lypolytic enzyme lipase. The acid values obtained are agreeable with past studies. Oxidation and hydrolysis processes is also a factor that led towards increment in acid value as the percentage of unsaturated fatty acids increase [17].

Among various factors of oil classification, the drying quality of the oil is also being considered, whereby it could be drying, semi-drying, or non-drying oil through the analysis of the iodine value [24]. The iodine value for OMY and OSD suggests that it is a non-drying oil and it is comparable to the standard iodine value of less than 100 gI_2/100g [25] by its physical state of being liquid at room temperature of 25°C, underexpose air condition. The low iodine value represents the fewer amounts of unsaturated bonds, and thus the oil has fewer tendencies to go through oxidative rancidity [17].

On the other hand, the oil from Malaysia had also undergone some chemical decomposition process, whereby the peroxide value is high compared to OSD. The peroxide value indicates the rancidity process whereby the higher peroxide value, proves higher oxidation level and the deterioration of lipids [26]. Theoretically, oil that shows a high amount of peroxide value is more prone to undergo rancidity that affects the total quality of the oil [23].

The moisture and volatile matter analysis prove that both oils contain a small amount of moisture and volatile matters. Thus, the presence of water or moisture contributes towards hydrolysis that results in breaking up of triglycerides into glycerols and free fatty acids [17]. This process was accelerated due to the presence of the action of lipase enzymes as the catalyst.

Therefore, these reactions, both oxidation, and hydrolysis, reduce the amount of unsaturated free fatty acids and thus contribute towards the reduction of the iodine value and average molecular weight and increases the acid value [17].

Unsaponifiable matter consists of constituents such as sterols, higher molecular weight alcohols, pigments, waxes, and hydrocarbon which do not react with bases during the formation of soap [27]. Extracted *A. indica* oil through mechanical pressing

and the unsaponifiable matter analysis resulted in 0.8% that is closer to the obtained results in this study [28]. Due to the small value of the unsaponifiable matter, *A. indica* had been studied in the application of biodiesel production [29].

Based on the results of the fatty acid composition as in **Table 3**, the oils differ in terms of the major and minor fatty acid chains. The total percentage of fatty acid chains were 98.03% and 99.34% for OSD and OMY respectively. The values are represented as the relative percentage area from the sum of all identified peaks. The preponderance chain detected in the OSD was the monounsaturated oleic acid (52.02%), whereby the poly-unsaturated linoleic acid present to be dominant in the OMY (34.69%). The analysis of OSD showed two extra fatty acids, namely; myristic and lauric acid at a very low percentage of 0.05 and 0.02 % respectively.

In contrast, analysis of volatiles in the seed and cake of the same plant reports the presence of myristic and lauric acid at the value of 0.127 and 0.191% [30].

Overall, an author had stated that the various fatty acid composition of the same plant from different areas is varied due to its genetic makeup. The sequence arrangement, according to the increasing percentage of fatty acid shows that the arachidic, behenic, lignoceric, and palmiticoleic acid were present to be at the same sequence level of fifth to eight places. It means that the main four detected fatty acid chains, linoleic, oleic, stearic, and palmitic acid differ in sequence from the first to the fourth place.

The overall results of this analysis show that the unsaturated fatty acid makes about half of the composition, whereby the mono-unsaturated are 20.63 and 52.29%; polyunsaturated are 34.69 and 3.02%; and the saturated fatty acids were recorded to be the balance at the level of 44.02 and 42.72% respectively for both OMY and OSD. The obtained results in a current study of fatty acid compositions of OSD agree with many past studies reported that the oleic acid is predominant for *A. indica* seed oil and its percentage lie between 25-61.9% [30-32].

On the other hand, in the case of oil from Malaysia, another study supported the current results that the major content of *A. indica* seed oil was linoleic acid at 38.26% and followed by oleic acid at 34.09% [33]. It has also previously been reported that the compositions of *A. indica* seed oil were; palmitic-, stearic-, oleic-, arachidic- and behenic acid lies between 17.30-34.30, 6.60-24.0, 25.40-57.90, 1.24-1.30, and 0.23-1.73% respectively [30].

The fatty acid profile could significantly change due to the storage and climatic conditions whether it could increase with a period of storage, air, heat, traces of metal, peroxides, light, or double bonds present in the oil [34] and thus leads towards the deterioration of the quality [2].

This plant seed oil had been reported to be one of the most suitable feedstocks for biodiesel production according to the fatty acid methyl ester profile that becomes one of the key factor [1].

The Ultraviolet (UV) light is classified into three different categories of UV-A (400-315 nm), UV-B (315-280 nm), and UV-C (280-200 nm) according to its wavelength 35. The UV-Visible transmission profiles of these oils were illustrated in **Fig. 1**. The overall results on the UV-Visible analysis show that the OMY absorbs heavily, as indicated by the lower transmittance value. The transmittance of both oils decreases as the wavelength decrease from UV-A, UV-B, UV-C. The UV-C ray is classified as the short wavelength radiation and was reported to be the most dangerous. But, this radiation could not reach the earth's surface as it could not penetrate through the atmosphere.

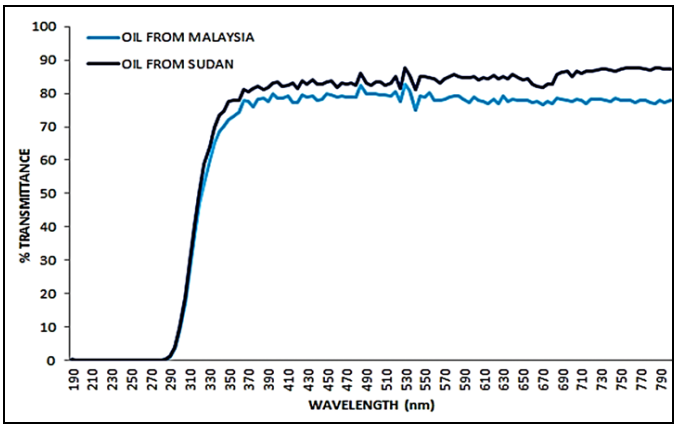

FIG. 1: UV-VISIBLE TRANSMITTANCE SPECTRA OF *A. INDICA* SEED OILS

Whereas UV-A and UV-B rays, long and medium wavelength radiation could not be completely filtered and thus they do contribute towards consequent health effects up to cancer [35].

There is no significant difference observed between the transmittance values of both oils. Therefore, a medium is required to absorb a specific amount of these radiations, and both of these oils have the capability in absorbing radiation up to 99.889 and 99.094%. It has also previously been reported that *A. indica* seed oil from India does show transmittance below 60% at UV-B region and these oils are used as applicant mostly in the southeastern part of India [36]. In terms of the overall quality of oil, it is said that the quality decrease as the storage life is longer and the factors that influences are the decrease in the iodine value and refractive index and also the increase in the acid number.

Even though both seeds are obtained from the same family of Meliaceae; there is a slight difference in the physicochemical properties as few factors might have influenced such as geographical origin and environmental condition of the plant, climate cultivation, soil composition, time of fruit harvesting and maturity and the drying process [5].

CONCLUSION: This study provides an insight into the physicochemical characteristics of Neem seed oils from Malaysia and Sudan. *A. indica* seed oil demonstrates promising properties that could be a potential source for unlimited applications.

UV-Visible transmittance of the oils suggests these oils for such applications to solve current skin problem that is attributed to the penetration of UV light. The obtained results showed some variations that might be attributed due to several factors. Moreover, the geographical variation of the plant source has impacts on several properties that would influence the quality of seed oil.

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CONFLICT OF INTEREST: Nil

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