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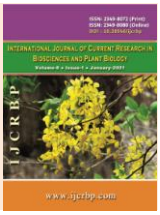

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Characterization and identification of compounds in neem (*Azadirachta indica*) seed oil

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Abstract

The seed oil of *Azadirachta indica* was obtained by Soxhlet extraction method. The maximum oil yield using petroleum ether was found 18.4% at a temperature of 70oC and a minimum yield of 13.5% at 30oC while at the same operating condition ethyl acetate and methanol resulted a maximum yield of 17.0% and 16.5% and a minimum of 12.0% and 13.0% respectively. So petroleum ether is a good solvent to afford high yield in this work. The oil was analyzed by GC-MS and result in the identification of the compounds (palmitoleic acid methyl ester, palmitic acid methyl ester, linoleic acid methyl ester, oleic acid methyl ester, stearic acid methyl ester, etc.) was obtained. In the present study various physical and chemical characteristics have been also studied. The results also indicated that the oil extracted from neem seed is the most acidic and non edible.

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Introduction

Plant oils are oils derived from plant sources, as opposed to animal fats or petroleum. Oils derived from plants have been used for thousands of years. Plant oils have been a healthy alternative to animal derived oils since their discovery. Plant oils are composed of compounds called triglycerides; which may exist in a highly unsaturated form or less (Ahmed et al., 1989).

Vegetable oils are substances derived from oil plants; they are composed of triglycerides which contain primarily polyunsaturated and mono-unsaturated fatty acids. Oil is extracted primarily from seeds. However, crude oil obtained needs to be refined in order to transform it into a range of useful products for industry and consumers. Oils improve flavor, lubricity, texture, and satiety to foods. They have also been found to have a major role in human nutrition. Oils and fats contain fatty acids essential for health that are not manufactured by the human body (O'Brien, 2009).

There are broadly two classes of plant oils: Essential Oils and Fixed Oils. Essential oils are volatile, and are usually derived from the non-seed parts of the plants. Most fixed oils are the so-called "fatty oils", and a majority of the fatty oils are derived from the

seeds – hence the term oil seeds, meaning oil-bearing seeds. Some of the fixed oils are derived from vegetables and nuts (Varghese and Naithani, 2002). The oil accumulation in *Azadirachta indica* is affected by a variety of factors such as seasonal and maturity, genetic and geographical variation (Terblanche, 2000; Marotti et al., 1994; Sanguanpong, 2010; Hussain et al., 2008). In this respect, there is no literature available on the chemical composition of neem seed oil from Bahir Dar, Ethiopia.

Materials and methods

Materials

Soxhlet extractor, dry oven (DAIHAN Scientific natural flow type oven, Japan), analytical balance (RADWAG; PS 360/C/1, China), sieve, heating mantle, thermometer, rotary evaporator (RE-2S-VD, German), pH meter (HI 99161, China), refractometer (HI 96841 Beer Refractometer, India), GC-MS (Agilent Technologies 7890B-5977A, China).

Chemicals (reagent)

N-hexane, ethanol, chloroform, petroleum ether, methanol, ethyl acetate, phenolphthalein, bromophenol, starch, DMSO, gentamicine.

Material preparation

Neem seed used in this study was obtained from Bahir Dar. Prior to use, the neem seeds was repeatedly washed to remove dirty and other impurities. Then, neem seeds were ground to different particle sizes.

Sample analysis

Determination of moisture content of the seeds

The cleaned sample was weighed and dried under shaded area until a constant weight was obtained. The percentage moisture in the kernel was calculated using the following:

$$Moisture\% = \frac{(W1-W2)*100}{W2} \ldots\ldots\ldots\ldots (1)$$

Where,
W1 = Original weight of the sample before drying;
W2 = Weight of the sample after drying.

Size reduction and sieve analysis of the seeds

The dried Neem seed was crushed by mechanical grinder (Retsch GmbH). The sample was sieved using set of sieves sizes arranged in descending order 1mm, 500µm and 200µm to obtain particular sizes of 500-1000 µm, 200-500 µm and ≤ 200 µm. This is because to investigate the effect of particle size on yield and quantity of oil.

Extraction procedure

Neem seeds powder were weighed and put into the thimble of the Soxhlet extractor. 1:10 mL of the solvent (petroleum ether, ethyl acetate and methanol) was measured with a measuring cylinder and poured into the still pot of the Soxhlet extractor. The heat source was a heating mantle operating at a different temperature level. The solvent evaporated through the distillation path, thimble and the expansion adapter after which it condensed at the condenser unit of the Soxhlet extractor. At this point the condensed vapour returned to the thimble as liquid droplets and got in contact with the sample therein. When the solvent in the thimble rose to the level of the siphon top, the entire content of the thimble and siphon was emptied back into the still pot of the Soxhlet extractor. The process was repeated several refluxes till the extraction process were completed. Temperature was regulated using a thermometer.

Determination of the yield of neem oil extracted

The percentage of oil extracted was determined as:

$$\%yield = \frac{W1 - W2}{W1} * 100 \ldots\ldots\ldots\ldots (2)$$

Where,
W1- the sample powder placed in the thimble
W2- cake weight

Determination of the chemical properties of Neem oil

Determination of saponification value

This parameter is defined as the weight of potassium hydroxide, in milligrams, needed to saponify one gram of oil. The method for SV determination is based on the oil sample saponification by refluxing with a known excess of alcoholic potassium hydroxide solution. The

alkali required for saponification is determined by titration of the excess potassium hydroxide with standard hydrochloric acid; in the presence of phenolphthalein as indicator. A blank is also simultaneously prepared. 2 g of crude oil was added to 250 mL conical flask and 0.6 g of KOH was added to 30 mL of rectified spirit (absolute alcohol). Similarly, blank solution (all chemicals except oil) was prepared in another conical flask. Reflux condenser was attached to both sample and refluxed for25 min while stirring. After completion of refluxing, 5 drops of phenolphthalein indicator was added, while it was cool, titrated against 0.5 M HCl to the end point (Gohari et al., 2011). S.V was calculated using the following formula:

$$S.V = 56.1 * N * \frac{VO - V1}{W} \dots\dots\dots\dots (3)$$

Where,
N - Concentration of Hydrochloric acid
V_0 - Volume of HCl before titration
V_1 - Volume of HCl used after titration
W- Weight in gram of sample taken

Determination of acid value

Acid values are used as an indicator for edibility or otherwise of oils and suitability for use in industries. Oil (10 g) was added to 250 mL and 50 mL of neutral alcohol was added. Then the mixture was heated to 70°C for 3 minute. Phenolphthalein indicator (5 drops) was added to the prepared solution and titrated against 0.1 N NaOH to the end point, which is appearance of pink color. Similarly, blank solution was prepared and titrated against 0.1 N NaOH to the end point. Acid value was calculated using the following formula:

$$A.V = 56.1 * \frac{N(V1 - VO)}{W} \dots\dots\dots\dots (4)$$

Where,
N - Concentration of NaOH
V_0 - Volume NaOH used for the blank
V_1 - Volume of NaOH used for sample
W – Weight of sample taken

Determination of iodine value

The iodine value (IV) indicates the degree of unsaturation of the oil. It is defined as the number of grams of iodine absorbed by 100 grams of oil. The neem oil (0.3 g) with 10 mL carbon tetrachloride was dissolved in 250 mL. 20 mL of 0.2 N Wijs reagents, which is prepared by dissolving 8 g iodine trichloride in a mixture of 150 mL glacial acetic acid and 10 mL of carbon tetrachloride, were added and mixed after it was plugged with stopper. Then, the mixture was placed in dark at room temperature for 30 min and 15 mL of 10% potassium iodine solution and 100 ml distilled water was added. Four drops of 1% starch indicator was added and titrated against 0.1 M sodium thiosulfate solution till the disappearance of blue color. The color of samples with starch was dark-blue color and titrated till straw color appears (Sadoudi and Ali Ahmed, 2017).

$$I.V = \frac{12.69 * N * (VO - V1)}{W} \dots\dots\dots\dots (5)$$

Where,
N - Concentration of sodium thiosulfate
Vo - Volume of sodium thiosufate used for blank
V1 - Volume of sodium thiosufate used for sample
W - Mass of the sample

Determination of peroxide value

The PV of an oil or fat is used as a measurement of the extent to which rancidity reactions have occurred during storage. *A. indica* oil weighing 5 g was added to 250 mL conical flask and 30 mL of solvent (acetic acid – chloroform) was added. Then 0.5 mL of KI, 30 mL of distilled water was added to the mixtures. A few drops of starch were added to insure the presence of free iodine by the appearance of blue color. The mixture was titrated by 0.1N sodium-thiosulfate until the blue color just vanishes. Similarly, blank solution (all chemicals except oil) was prepared in another conical flask (Sadoudi and Ali Ahmed, 2017). P.V was calculated using the following formula:

$$PV\left({}^{meq}/_{kg}\right) = \frac{N * (Vs - Vb) * 1000}{W} \dots\dots\dots\dots (6)$$

Where,
N - Concentration of sodium thiosulfate
Vo - Volume of sodium thiosulfate used for blank
V1 - Volume of sodium thiosulfate used for sample
W - Mass of the sample

Gas Chromatography-Mass Spectroscopy

Analysis of the Neem oil was done using Gas Chromatography with Mass spectrometer to know the

composition of oil. Gas Chromatography-Mass Spectrometry (GC-MS) is a method that combines the features of liquid chromatography and mass spectrometry to identify different substances within a test sample.

Results and discussion

Percent yield of extract in Soxhlet extractor

The percentage yield of neem seed oil extracts showed variation with reference to particle size, temperature and solvent employed (Tables 1-5, Fig. 1). The particle size of the neem seed used for the extraction of oil, *viz.*, ≤200, 200-500 and 500-1000 µm showed that the smaller particle size yielded higher when compared with the larger particle size in the solvent system used (petroleum ether, ethyl acetate and methanol).

The percentage extract yield of 21.5, 16.00 and 12.5 was recorded in petroleum ether, ethyl acetate and methanol, respectively on 6 h of extraction time followed by the extraction time of 4 h and 2 h.

Table 1. Total %yield in Soxhlet extraction of different particle size of need seed and time using petroleum ether as a solvent.

Particle size (µm)	Cake wt, and yield (%)	Extraction time (hours)		
		2	4	6
≤200	Cake wt (g)	6.9	8.0	8.6
	Yield (%)	17.5	20.0	21.5
200-500	Cake wt (g)	6.3	7.3	8.4
	Yield (%)	15.75	18.25	21.0
500-1000	Cake wt (g)	5.0	5.9	7.2
	Yield (%)	12.5	14.75	18.0

Table 2. Total %yield in Soxhlet extraction of different particle size of need seed and time using ethyl acetate as a solvent.

Particle size (mm)	Cake wt, and yield (%)	Extraction time (hours)		
		2	4	6
≤200	Cake wt (g)	4.9	5.7	6.4
	Yield (%)	12.25	14.25	16.00
200-500	Cake wt (g)	4.3	5.3	5.9
	Yield (%)	10.75	13.25	14.75
500-1000	Cake wt (g)	3.0	3.9	4.2
	Yield (%)	7.50	9.75	10.5

Table 3. Total %yield in Soxhlet extraction of different particle size of need seed and time using methanol as a solvent.

Particle size (µm)	Cake wt, and yield (%)	Extraction time (hours)		
		2	4	6
≤200	Cake wt (g)	4.2	4.6	5.0
	Yield (%)	10.5	11.5	12.5
200-500	Cake wt (g)	3.7	4.2	4.9
	Yield (%)	9.25	10.5	12.25
500-1000	Cake wt (g)	2.0	2.7	3.2
	Yield (%)	5.0	6.7	8

Chemical composition of the *A. indica* (neem) oils

The chemical composition of neem seed oil extracted with petroleum ether and ethyl acetate are shown in Tables 6 and 7. The chemical composition of neem seed oil extracted with petroleum ether as revealed by GC-MS were 9,12-Octadecadienoic acid, (Z,Z)-methyl ester, 9-Octadecenoic acid, methyl ester, (E)-Hexadecanoic acid, Methyl stearate, 11-Octadecenoic acid-methyl ester, β-Sitosterol, Eicosanoic acid, cis-11-Eicosenoic acid, methyl ester, phenol, 2,2'-methylene bis[6-(1,1-dimethylethyl)-4-methyl-7,10-Octadecadi-enoic acid, Docosanoic acid methyl ester, Diisooctyl phthalate, Heptadecanoic acid and Methyl tetradecanoate (Table 6). Whereas, the ethyl acetate extract of neem seed oil showed the chemical compounds such as Benzene acetic acid, Undecanoic acid, 10-methyl- methyl ester, 7-Hexadecene, (Z)-, Methyl tetradecanoate, (Z)-Methyl hexadec-11-enoateHexadecanoic acid, Hexadecanoic acid,

Cyclopropaneoctanoic acid, 2-hexyl- methyl ester, Heptadecanoic acid, 9,12-Octadecadienoic acid, (Z,Z)-methyl ester, 9-Octadecenoic acid (Z)- methyl ester, 9-Octadecenoic acid, (E)-Methyl stearate, Linoleic acid ethyl ester and Ethyl Oleate in different peak areas (Table 7).

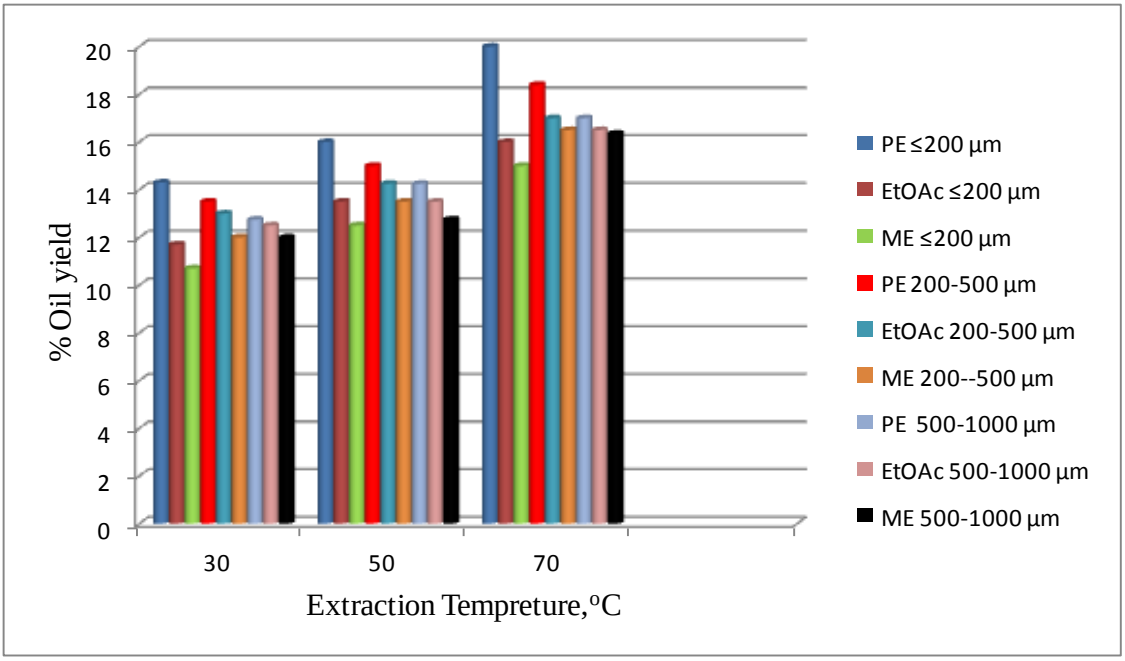

Fig. 1: The effect of temperature on percentage yield of neem seed oil.

Factors that determine the composition and yield of the essential oil obtained are numerous. In some instances it is difficult to segregate these factors from each other, since many are interdependent and influence one another (Terblanche, 2000). These variables may include seasonal and maturity variation, geographical origin, genetic variation, growth stages, part of plant utilized and postharvest drying and storage (Marotti et al., 1994). Seasonal and maturity variation factors are interlinked with each other, because the specific ontogenic growth stage will differ as the season progresses. There are variations in the chemical profile of essential oils from various plants collected during different seasons. The essential oils yields varied considerably from season to season and was also influenced by the micro-environment (sun or shade) in which the plant was growing. Results obtained by Badi (Sanguanpong, 2010) also indicated that timing of harvest is critical to both yield and oil composition.

There are many reports in the literature showing the variation in the yield and chemical composition of the essential oil with respect to geographical regions (Uribe-Hernandez et al., 1992). Variations in the yield and chemical profile of essential oils, collected from different geographical locations, respectively. Such differences could be linked to the varied soil textures and possible adaption response of different populations, resulting in different chemical products being formed, without morphological differences being observed in the plants (Hussain et al., 2008). Genotype is typically defined as "the genetic make-up of an organism, as characterized by its physical appearance or phenotype", while chemotype is generally defined as "a group of organisms that produce the same chemical profile for a particular class of secondary metabolites". Genetic makeup of the plant is one of the most important contributors to their essential oil composition (Hussain et al., 2009). Other factors which affect the growing plants thus leading to variations in oil yield and composition, include part of plant used; post-harvest drying; length of exposure to sunlight; availability of water, height above sea level, plant density, time of sowing and the presence of fungal diseases and insects. The oil composition and yield may also change as a result of the harvesting methods used, the isolation techniques employed, the moisture content of the plants at the time of harvest and the prevailing extraction conditions (Hussain et al., 2009).

Table 4. Physical property determination, experimental result of the characterization of extracted neem seed oil by petroleum ether.

Physical property	Unit	Experimental value	Standard value
Refractive index at 20°C	-	1.47381	1.45-1.4705
Density	kg/m^3	0.9506	0.900-0.9850
Odour	-	Garlic	Garlic-peanut
Colour	-	Yellow	Yellow
Specific gravity	-	0.9538	0.948 - 0.964
pH	-	3.4	3.4-5.5
Brix at 20°C	%	73.385	ND

ND –not determined before.

Table 5. Physical property determination, experimental result of the characterization of extracted neem seed oil by ethyl acetate.

Physical property	Unit	Experimental Value	Standard value
Refractive index at 20°C	-	1.43746	1.45-1.4705
Density	kg/m^3	0.9862	0.900-0.985
Odour	-	Garlic	Garlic-peanut
Colour	-	Yellow	Yellow
Specific gravity	-	0.9479	0.948 - 0.964
pH	-	3.3	3.4-5.5
Brix at 20°C	%	58.014	ND

ND –not determined before.

Table 6. Chemical composition of neem seed oil extracted by petroleum ether.

Peak	RT	Area (%)	Height (%)	Chemical name	Molecular formula
8	36.858	100	100	9,12-Octadecadienoic acid (*Z*,*Z*)-, methyl ester	$C_{19}H_{34}O_2$
9	36.934	28.22	70.46	9-Octadecenoic acid, methyl ester, (*E*)-	$C_{19}H_{36}O_2$
5	33.561	17.64	40.36	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$
11	37.316	6.32	16.97	Methyl stearate	$C_{19}H_{38}O_2$
10	36.978	1.41	5.25	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$
23	53.871	1.16	0.28	β-Sitosterol	$C_{29}H_{50}O$
15	40.741	0.62	1.57	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$
14	40.299	0.49	1.31	cis-11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$
16	42.375	0.44	0.98	Phenol, 2,2'-methylene bis[6-(1,1-dimethylethyl)-4-methyl-	$C_{23}H_{32}O_2$
12	39.187	0.32	0.6	7,10-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$
18	44.905	0.29	0.43	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$
19	45.37	0.28	0.38	Diisooctyl phthalate	$C_{24}H_{38}O_4$
7	35.448	0.27	0.72	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$
3	29.405	0.26	0.77	Methyl tetradecanoate	$C_{15}H_{30}O_2$

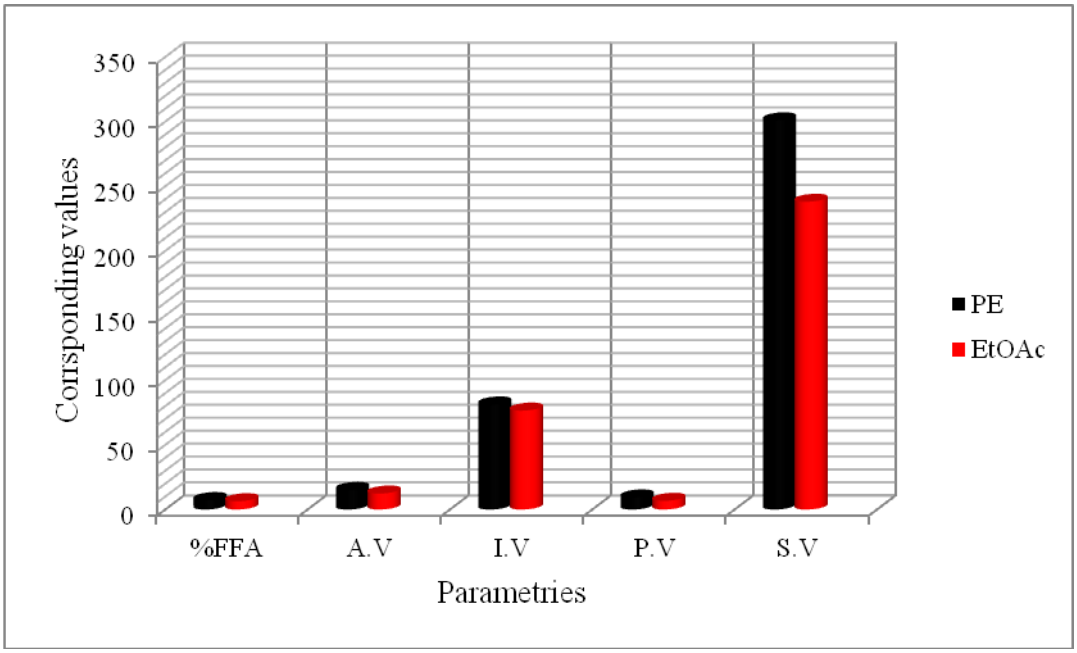

Fig. 2: Chemical characteristics of Neem oil with reference to different parametries.

Table 7. Chemical composition of neem seed extracted by ethyl acetate.

Peak	RT	Area	Height	Chemical Name	Molecular Formula
1	15.705	407351.41	129215.95	Benzene acetic acid, methyl ester	$C_9H_{10}O_2$
2	24.86	277276.55	94242.71	Undecanoic acid, 10-methyl-, methyl ester	$C_{13}H_{26}O_2$
3	26.442	279148.04	104321.61	7-Hexadecene, (*Z*)-	$C_{16}H_{32}$
4	29.401	447024.83	159424.44	Methyl tetradecanoate	$C_{15}H_{30}O_2$
5	33.095	258057.05	75362.09	(*Z*)-Methyl hexadec-11-enoate	$C_{17}H_{32}O_2$
6	33.553	30952280.97	9498069.61	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$
7	34.842	1278854.68	430551.61	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$
8	34.978	275859.62	67589.95	Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester	$C_{18}H_{34}O2$
9	35.448	460130.74	157519.26	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$
10	36.83	176380248.8	26284257.53	9,12-Octadecadienoic acid, (*Z*,*Z*)-, methyl ester	$C_{19}H_{34}O_2$
11	36.906	52247248.12	17346907.35	9-Octadecenoic acid (Z)-,methyl ester	$C_{19}H_{36}O_2$
12	36.958	2257340.67	995940.84	9-Octadecenoic acid, methyl ester, (*E*)-	$C_{19}H_{36}O_2$
13	37.303	11963927.04	4078428.14	Methyl stearate	$C_{19}H_{38}O_2$
14	37.91	9793271.76	3463530.45	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$
15	38.01	3021336.5	936795.04	Ethyl Oleate	$C_{20}H_{38}O_2$

Conclusions

The present study forms the baseline data for the chemical composition of compounds present in oil from neem (*A. indica*) seeds collected from the geographical location Bahir Dar, Ethiopia. It can be used for comparing variations in chemical compounds present in neem seed oil of different geographical locations with respect to extraction method and the type of solvent used. The particle size of the neem seed used for extraction of oil is playing important role in the extract yield as the smaller size yielded higher percentage of yield in all the three kinds of extracts used in the present study.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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