RJC

RASĀYAN *J. Chem.*
Vol. 14 | No. 1 |343-350| January - March | 2021
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

PHYTOCHEMICALS CHARACTERIZATIONS OF NEEM (*Azadirachta indica* A. Juss) LEAVES ETHANOLIC EXTRACT: AN IMPORTANT MEDICINAL PLANT AS MALE CONTRACEPTIVE CANDIDATE

I. Seriana[1,2], M. Akmal[3,✉], Darusman[4], S. Wahyuni[5], K. Khairan[6,7,8] and Sugito[9]

[1]Graduate School of Mathematics and Applied Sciences, Universitas Syiah Kuala, Banda Aceh-23111, (Aceh) Indonesia
[2]Department of Midwifery, Polytechnic of Health Ministry of Health, Aceh Besar-23231, (Aceh) Indonesia
[3]Laboratory of Histology, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh-23111, (Aceh) Indonesia
[4]Faculty of Agriculture, Soil Science Department, Universitas Syiah Kuala, Banda Aceh-23111, (Aceh) Indonesia
[5]Laboratory of Anatomy, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh-23111, (Aceh) Indonesia
[6]Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Banda Aceh-23111, (Aceh) Indonesia
[7]Herbal Medicinal Research Centre, Universitas Syiah Kuala, Banda Aceh-23111, (Aceh) Indonesia
[8]Atsiri Research Centre, Universitas Syiah Kuala, Banda Aceh-23111, (Aceh) Indonesia.
[9]Laboratory of Pathology, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh-23111, (Aceh) Indonesia
✉Corresponding Author: akmal_kh@unsyiah.ac.id

ABSTRACT

Neem (*Azadirachta indica* A. Juss) is a tropical plant that belongs to the Meliaceae family. It has an antifertility effect that could be developed for a male contraceptive candidate. This study evaluated the phytochemicals characteristics of neem leaf ethanolic extracts from Kajhu and Limpok in Aceh Besar Regency, Aceh, Indonesia, through phytochemicals screening, FTIR, and GCMS analysis. The result showed the differences of active compounds of neem leaves ethanolic extract from Kajhu and Limpok. Neem leaves ethanolic extract from Kajhu have more active compounds than from Limpok area, which contains flavonoids, tannins, saponins, alkaloids, steroids. The main compound neem leaves ethanol extract in Kajhu is linolenic acid, while hexadecanoic acid is found in Limpok. Its active compounds serve as an antifertility agent that could disrupt gonadotropin hormone and spermatogenesis.

Keywords: Male Contraceptive Candidate, Phytochemicals Characteristics, *Azadirachta indica* A. Juss, Ethanol Extract

RASĀYAN *J. Chem.*, Vol. 14, No.1, 2021

INTRODUCTION

Neem (*Azadirachta indica* A. Juss) is a native plant from India, originating from the Meliaceae family that is widespread throughout the world and can be grown in tropical and subtropical countries. Neem has excellent medical benefits with various biological activities.[1] Neem has been known for centuries and is used in traditional Indian medicine since ancient times. Neem can be used to treat multiple diseases in almost all parts of the plant.[2] Several studies report that neem leaves have antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, immunomodulatory, anticancer, wound healing,nephroprotective,

http://dx.doi.org/10.31788/ RJC.2021.1415899

hepatoprotective, antimalarial, antifungal, antibacterial, neuroprotective,[1-3] antifertility and contraception.[4-7]

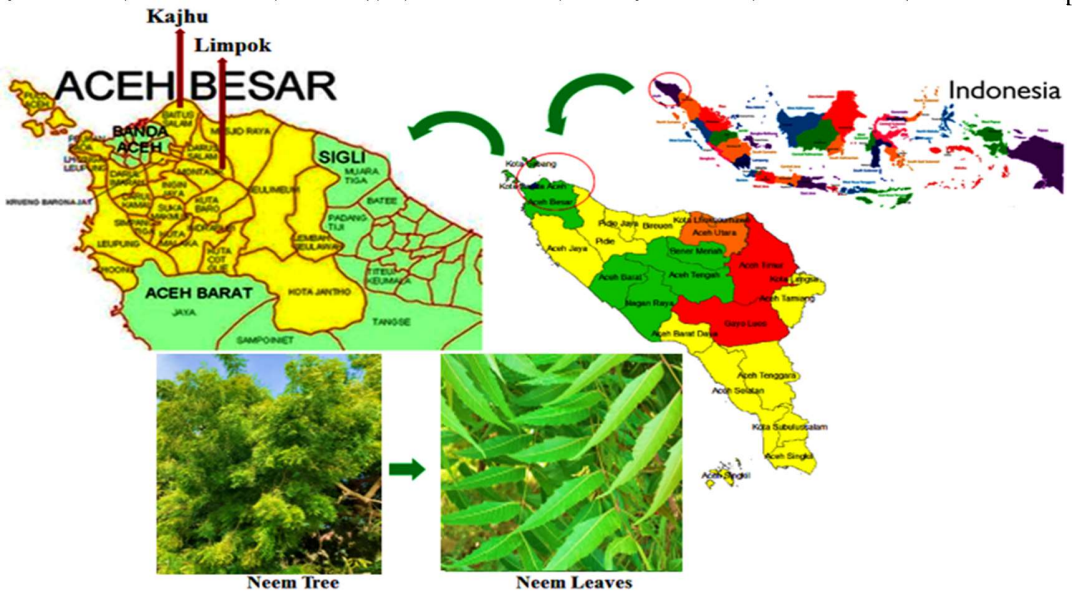

Fig.-1: The Location of Neem Plant (Kajhu and Limpok Aceh Besar Regency, Aceh, Indonesia)

Based on the phytochemical screening, neem leaves extract contains various active compounds such as steroids, alkaloids, flavonoids, saponins, terpenoids, glycosides, tannins, and phenolics.[4,5,8-10] Several active compounds have an antifertility effect.[5,11] *In vivo* studies have shown that neem leaf extract decreased sperm concentration, motility, viability, and increased sperm abnormalities.[12,13] Neem leaves extract caused changes in Follicle Stimulating Hormone (FSH) and testosterone that could disrupt spermatogenesis, which further affects sperm quality and male fertility.[12,14,15] Changes in FSH, testosterone, and sperm quality are standard criteria that serve as antifertility agents.[16] Thus, neem leaves ethanol extract has the potential to be developed as a male contraceptive candidate.

Until now, the phytochemicals constituent of neem leaf ethanolic extracts is varied. The local people in Aceh Besar Regency Aceh, Indonesia, have used neem leaf to treat diseases, including preventing pregnancy without knowing active compounds. To our knowledge, this is the first investigation of the antifertility potency of the neem leaves ethanolic extract from Kajhu and Limpok, Aceh, Indonesia. This study is aimed to analyze the phytochemical characteristics of neem leaves ethanolic extract through the phytochemicals screening, Fourier Transform Infrared (FTIR), and Gas Chromatography-Mass Spectroscopy (GCMS) analysis.

EXPERIMENTAL

Material

Ethanol 70%, hydrochloric acid 10%, NaCl, $FeCl_3$, aquadest, and neem leaves are collected from the Kajhu and Limpok, Aceh Indonesia. The neem leaves have been determined at the Herbarium Bogoriense, Biological Research Center, Indonesian Institute of Sciences (LIPI) Bogor, Indonesia (1454/IPH.1.01/If.07/VII/2019).

Preparation of Extract

Fresh neem leaves collected, air-dried, and made into powder. 500 gram (g) powdered were macerated with 5 L ethanol 70% for 7 days. The filtrate was evaporated with a rotary evaporator at 50ºC to give viscous extracts.

Soils Analysis

Soil analysis was carried out at the Soil Science Laboratory, Faculty of Agriculture, Universitas Syiah Kuala. The examination includes pH, C-organic, N-total, P-Bray, exchangeable K, cation exchange capacity (CEC), and electrical conductivity.

Characterization of Extract
The characterization of the extract was determined based on methods developed by WHO[17] and the Ministry of Health of the Republic of Indonesia.[18] Extract analysis includes moisture and total ash content, water-soluble, and ethanol-soluble extract content.

Phytochemicals Analysis
Phytochemicals tests are based on the methods described by Harborne.[19] The phytochemical test includes alkaloids, flavonoids, saponins, tannins, terpenoids, and steroids.

Fourier Transform Infrared (FTIR) Spectroscopy Analysis
The FTIR spectrophotometer (Shimadzu Prestige-21) was used to record the spectra analysis. A sample of 10 mg was mashed with 100 mg KBr into a pellet with a size of 2 μm and was recorded in the spectra 400-4000 cm^{-1}.

Gas Chromatography-Mass Spectroscopy (GCMS) Analysis
A total of 5 μl of the filtered neem leaf ethanol extract was injected into GCMS (Agilent Technologies 7890A/5975A) using helium (He) as a carrier gas through a capillary column with constant pressure and a total rate of 1,2 ml/min and a split ratio of 8:1 psi. The injector temperature is 250ºC, the detector temperature is 230ºC, and the operating temperatures are 280ºC and 140ºC. The eluted component will be detected on a mass detector.

RESULTS AND DISCUSSION

Soil Analysis
The soil analysis showed that the soil pH of the Kajhu site was slightly alkaline, with deficient Carbon (C-organic), meager N total, medium P Bray, low exchangeable K, medium cation exchange capacity (CEC), and very low electrical conductivity (EC). The soil analysis from Limpok shows that the site had neutral pH, deficient Carbon (C-organic), meager N total, medium P Bray, low exchangeable K, low CEC, and low EC. Most of the soil analysis results from the two locations, i.e., Kajhu and Limpok, showed no differences, only the pH condition of Kajhu was slightly alkaline and Limpok had neutral pH.

Tabel-1: Soil Analysis of Neem Leaves from Kajhu and Limpok

No.	Characteristics of Soil	Kajhu		Limpok	
		Result	Description	Result	Description
1	pH	7.79	Slight alkaline	7.09	Neutral
2	C-organic (C)	0.05	Very low	0.09	Very low
3	N-total (N)	0.02	Very low	0.02	Very low
4	P-Bray (P)	9.60	Moderate	13.25	High
5	Exchangeable K	0.24	Low	0.17	Low
6	Cation exchange capacity (CEC)	16.80	Moderate	8.4	Moderate
7	Electrical conductivity (EC)	0.07	Very Low	0.13	Very Low

Characteristics of Extract
The characteristic of neem leaves ethanol extract analysis shows that the moisture content of neem leaf extracts from Kajhu and Limpok was 22.67% and 18.7%, respectively. The extract's moisture content should not exceed 30% to avoid the growth of fungi and microorganisms.[17,18] The total ash content of neem leaves extract of Kajhu and Limpok was 1.36% and 1.45%, respectively. The total ash content follows the standard determination of the total extract ash content. Ash content should have little value because this parameter indicates heavy metal contamination that can withstand high temperatures.[17,18] The soluble ethanol extract content of neem leaves extract from Kajhu and Limpok was 46,027% and 35,82%, respectively. The water-soluble extract content of neem leaves remove from Kajhu and Limpok was 17,97% and 12,33%, respectively. The ethanol-soluble extract content from Kajhu and Limpok was higher than the water-soluble extract content. Dissolved in ethanol are higher than in water. Ethanol is a suitable solvent, safe to consume, and can extract several bioactive compounds with various polarities.[20] The evaluation showed that the neem leaves ethanol extract of Kajhu and Limpok meet the standard drug parameters.

Table-2: The Characteristics of Neem Leaves Ethanolic Extract from Kajhu and Limpok

No.	Characteristics of extract	Extract (%)	
		Kajhu	Limpok
1	Moisture content	22.67	18.70
2	Total ash content	1.36	1.45
3	Ethanol soluble extract content	46.03	35.82
4	Water-soluble extract content	17.97	12.33

Yield of Extract

The percentage yield of neem leaves ethanolic extract from Kajhu and Limpok was 2,64 and 2,12%, respectively. These results indicate that various plantation locations of neem leaves will produce a different percentage of yield extract. The yield value indicates the amount of active compound in an extract. Geographically, Kajhu is located in the coastal area at 05°35'41" North and 95°22'20" East with an elevation of 1 m above sea level (asl) while Limpok is located at 05°33'39" North and 95°22'12" East with a height of 3 m above sea level (asl).

Table-3: Location and Percentage Yield of Neem Leaves Ethanolic Extract

No.	Sample location	Height	Weight of sample	Weight of extract	Yield
1	Kajhu	1 m asl	500 g	13,2 g	2,64 %
2	Limpok	3 m asl	500 g	10,6 g	2,12 %

Phytochemicals Analysis

The neem leaves ethanolic extract from Kajhu contains alkaloids, flavonoids, tannins, saponins, and steroids. Meanwhile, the ethanolic extract of neem leaves from Limpok contains flavonoids, tannins, saponins, and steroids. Terpenoids are not found in Kajhu and Limpok, alkaloids are only found in the ethanolic extract of neem leaves from Kajhu. The active compound difference is influenced by several factors, such as geographical location, climate, genetic variation, agronomy, storage of neem plants[21], and other various environmental factors such as light, temperature, groundwater, fertility, and soil salinity.[22] Synthesis and accumulation of active compounds depend on the environmental conditions of the plant.[23]

A study conducted by Al-Hashemi and Hussain[24] in Oman found that the ethanol extract of neem leaves contains alkaloids, flavonoids, tannins, and saponins. Another study from Indonesia found that the ethanol extract of neem leaf contains flavonoids, terpenoids, alkaloids, tannins, and saponins.[25] The study in Nigeria found that ethanol extracts of neem leaf had tannins, saponins, alkaloids, phenolics, flavonoids, glycosides, and terpenoids.[26] The results of prior studies indicate differences in active compound profiles of neem leaf ethanol extract according to the neem plant's original location. The active compound of neem leaves can vary between regions, countries, genotypes, and neem plants themselves.[21]

Kajhu is an area in Aceh Besar regency, Aceh, Indonesia, located on the coast. Kajhu has slightly alkaline soil that affects the active compound of neem plants. Meanwhile, Limpok is an area located far from the coast with neutral soil conditions. According to Gahukar[21], neem plants that grow in sandy soils (alluvial soils) have high active compounds. Moreover, soil salinity conditions can increase oxidative stress due to the increased production of reactive oxygen species. They can change plant metabolism. Thus plants produce a large number of active compounds to clean or detoxify reactive oxygen species.[22] This condition is responsible for the differences in the secondary metabolites of neem plants from Kajhu and Limpok.

Table-4: Phytochemicals Constituent of Neem Leaves Ethanolic Extract from Kajhu and Limpok

Sample location	Phytoconstituent					
	Alkaloids	Flavonoids	Tannins	Saponins	Terpenoids	Steroids
Kajhu	+	+	+	+	-	+
Limpok	-	+	+	+	-	+

Note: + = positive containing secondary metabolites; - = negative containing active compound

FTIR Analysis

The FTIR analysis of the ethanolic extract of neem leaves from Kajhu and Limpok shows the strong absorption at a wavenumber of 2927 and 2858 cm^{-1}. It is strengthened by the absorption of wavenumbers

of 1450 and 1381 cm^{-1}, indicating the C-H functional group. This wavenumber is thought to be originated from steroid and saponin compounds. Uptake at wave number 1743 cm^{-1} indicates C=O, which is assumed to be derived from tannins. The absorption band at a wavenumber of 1033 and 1154 cm^{-1} represents C-O ether group presence, while the absorption at a wavenumber of 1450 cm^{-1} with low intensity indicates O-H groups' presence, which is thought to be originated from flavonoid compounds. O-H functional groups, C-H aliphatic, C=C aromatic, and C-O indicate the presence of flavonoid compounds. The existence of vibrations in the wavenumber of 1033 cm^{-1} is the absorption of C-N. This is strengthened by the absorption period at a wavenumber of 1743 cm^{-1} with C═O carboxylic group and wavenumber of 1033 cm^{-1} with a stretching vibration of C-O alcohol. This shows the existence of alkaloids. The data shows that the alkaloid compounds contain O-H, N-H, C-N, C=C, C-O alcohols, and C=O carboxylate group. The FTIR analysis showed that FTIR spectrum of neem leaves ethanolic extract from Kajhu and Limpok had no significant difference due to the same type of plant. Even so, there is a slight change in the wavenumber between the two FTIR spectra but it shows insignificant and does not affect the change in the composition of the compound functional groups contained in the sample.

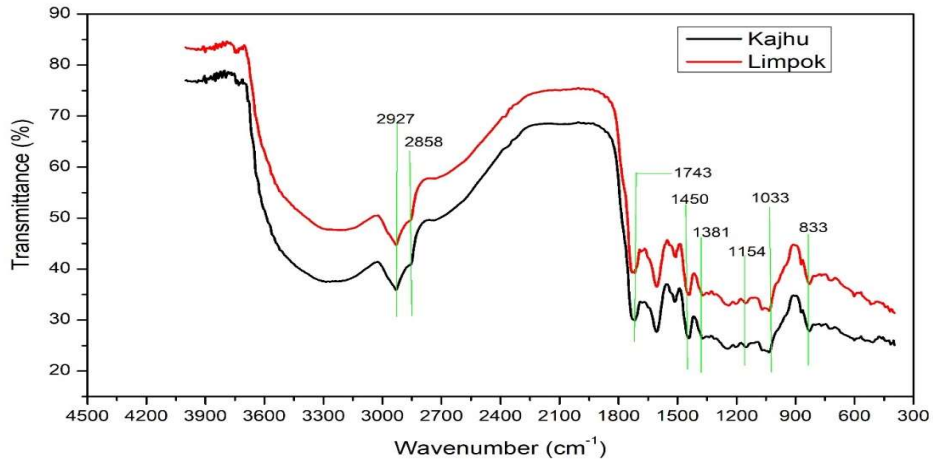

Fig.-2: FTIR Spectrum of Neem Leaves Ethanolic Extract from Kajhu and Limpok

GCMS Analysis

The GCMS analysis shows that ethanol extracts of neem leaves from Kajhu contain 20 active compounds while Limpok contains 17 active compounds. The main compound of ethanol extract of neem leaves from Kajhu was linolenic acid (23,0%) and Limpok was n-hexadecanoic acid (28,74%). Interestingly, the ethanol extract of neem leaves from Kajhu contains higher linolenic acid than from Limpok with 23,0% and 18,0%, respectively. Differences in the bioactive compounds are closely related to the origin of the neem plant. The composition of active compounds of plant species can vary according to the geographical location, which corresponds to soil type, precipitation level, light intensity, and humidity.[21] The geographical location and various environmental factors such as temperature, light, groundwater, fertility, and soil salinity greatly affect the chemical composition of a plant.[22]

Table-4: GCMS of Neem Leaves Ethanolic Extract from Kajhu and Limpok

No.	Neem leaves from Kajhu	% Area	No	Neem leaves from Limpok	% Area
1	2-isopropenyl-1-methyl-4(1-methylethlidne)-vinylcyclohexane)	1.05	1	gamma.-elemena	2.30
2	neophytadiene	7.65	2	germacrene b	1.49
3	neophytadiene	1.93	3	7-ethyl-9,9-dimethylspiro (4.5) dec-7ene-1,4 dione	1.45
4	neophytadiene	2.91	4	neophytadiene	5.50
5	1,2-benzenediccarboxylic acid, dibutyl ester	1.13	5	neophytadiene	1.05
6	hexadecanoic acid, ethyl ester	1.76	6	1,2-benzenedicarboxylic acid, dibutyl ester	3.78
7	n-hexadecanoic acid	11.91	7	1,2-benzenedicarboxylic acid, dibutyl ester	3.20

8	n-hexadecanoic acid	6.45	8	n-hexadecanoic acid	28.74
9	n-hexadecanoic acid	4.88	9	cycloprone, 1,1,2,2-tetramethyl-3-(2-phenylethenylideno)-	1.01
10	azulene, 7-ethyl-1,4-dimethyl-	1.23	10	phytol	9.32
11	(2e)-3,7,11,15-tetramethyl-2-hexadecen-1-ol	6.74	11	linolenic acid	18.00
12	phytol	3.58	12	octadecanoic acid	2.15
13	1,4,6,8-tetramethyl-(1,3a-(13c)-azulene	3.73	13	trioctylphodlhine	1.74
14	linolenic acid	23.00	14	pentacosane	8.24
15	linolenic acid	4.63	15	4-methoxy-2-butyn-1-ol	3.66
16	2',5'-dimethoxy-2-biphenylcarboxylic acid	3.22	16	stigmastan-3, 5-diene	1.29
17	nanocosane	5.04	17	vitamin e	1.10
18	stigmastan-3,5-diene	1.29			
19	octacosane	3.35			
20	stigmastan-3,5-diene	1.76			

The active compound of the neem leaves ethanol extract from Kajhu and Limpok has an antifertility effect. Previous studies showed a change in FSH and testosterone concentrations in male rats that were given neem leaf extract.[14,15,27,28] Flavonoids, steroids, alkaloids, tannins, and saponins disrupt the hypothalamus-pituitary-testis axis characterized by changes in the concentration of FSH and testosterone. Flavonoid and steroid compounds can influence FSH and testosterone production, flavonoids and steroid which have a structure similar to cholesterol that can affect those hormones.[29,30] Another report found that alkaloid compounds can suppress the release of gonadotropin hormones which cause reduced FSH and testosterone in target organs, resulting in the decreased testicular function that inhibits spermatogenesis.[31] Likewise, saponin compounds which are derivatives of terpenoids and steroids, can affect the function of reproductive hormones.[32] Meanwhile, tannins, which are the same group of polyphenolic compounds like flavonoids, can cause changes in gonadotropin hormones that change in testicular function and spermatogenesis.[33]
The GCMS analysis showed that linolenic acid was the most abundant compound from the Kajhu neem leaf ethanol extract. Linolenic acid is an unsaturated fatty acid belonging to the omega-3 group. These fatty acids cause a decrease in FSH concentration and an increase in testosterone concentrations.[34,35] Another fact has found that these unsaturated fatty acids can affect sperm quality through the fatty acid metabolism pathway. It affects hormones and testicular function.[36] The ethanol neem leaves extract from Limpok contains many hexadecanoic acids, which is saturated fatty acid. This fatty acid also causes an imbalance of gonadotropin hormones which causes impaired testicular function and spermatogenesis.[37] Changes in the concentration of FSH and testosterone will provide negative feedback to the hypothalamus and anterior pituitary to inhibit the secretion of gonadotropin hormones, i.e., FSH and LH. The inhibition of FSH and testosterone secretion disrupts spermatogenesis.[38]

CONCLUSION

The ethanolic extract of neem leaves has a different active compound due to the geographical location and environment where the neem plant grows. The neem plants from Kajhu have a higher number of active compounds with an antifertility effect compared to the neem leaves from Limpok. Phytochemical characteristics neem leaves ethanol extract from Kajhu and Limpok meet the standard drug parameters for male contraception candidates.

ACKNOWLEDGMENT

The author would like to express gratitude to the Ministry of Health of the Republic of Indonesia for the fellowship program.

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[RJC-5899/2020]