

# The isolation and identification of antimalarial agents of the n-hexane fraction of the neem leaves (*Azadirachta indica* A. Juss)

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**Abstract.** Following the study which confirmed the antimalarial activity of the n-hexane fraction of the neem leaves (*Azadirachta indica* A.Juss), a research that aimed to reveal the active compounds act as antimalarial agents had been conducted. In this study, the n-hexane fraction of neem leaves were analyzed using the phytochemical, spectroscopic and chromatographic methods. The chemical compounds, suspected to have an antimalarial activity, were isolated using the thin-layer chromatographic method with several solvents which different in their polarity. The visualizing reagents were used to characterize the compounds. The result showed that the n-hexane fraction of neem leaves contained of steroids, terpenoids and fenols. Analysis by gas chromatography-mass spectroscopy showed that fenol derivatives existed in the largest amount. The compounds were identified as benzyl benzoat (25.63%), tetracosane (17.51%) and hexadecanal (14.61%).

**Key words:** n-hexane, neem leaves, steroid, terpenoids, fenols and benzyl benzoat

## Introduction

Although the therapeutic potency of herbal medicines has been confirmed by many studies, the effects and the safety has been questioned since plants contains various compounds including therapeutic and toxic agents. This fact causes different responses shown by patients when the medicine is administered into their bodies. A compound can reduce, increase or even abolish the pharmacologic activity of the other compound (Katzung, 2001; Siswandono and Sukardjo, 1995; Ganiswarna, 2009).

To prevent the negative effects of using natural medicines, the active agents should be obtained as a pure compound. Isolation followed by purification could be carried out using extraction methods in which several solvents with different polarity are used. The amount, type and concentration of compounds in each fraction are identified using phytochemical, spectroscopic and chromatographic methods (Harbone, 1987; Stahl, 1985).

There were several studies investigated the effect of neem leaves extract (*A.indica* A.Juss) on malarial parasites, cancer, diabetes and hypertension. Analysis of the active compound that exist in the other part of neem tree also have been conducted. However, the identification of compounds in n-hexane fraction of neem leaves that showed potent activity as in vivo antimalarial agent have not been undertaken yet (Maryatun et al., 2008).

## Materials and Methods

### **The extraction of active compounds from neem leaves (*A. indica* A.Juss)**

The neem leaves extraction was carried out using maceration. The finely cut neem leaves (1000 g) was soaked in 10L metanol. The mixture was allowed to react for 5 days while stirred frequently. Then the liquid phase was separated by filtration. This procedure was repeated three times using fresh solvent (metanol). The total liquid phase was then saturated followed by hexane fractionation. The collected hexane fraction was saturated by vacuum rotary evaporator at 40°C and then dried by freeze dryer. The amount of required solvent, how many times the process was repeated and the amount of obtained fractions were recorded. The fraction was sealed in dark-colour bottles and stored in refrigerator.

### **Isolation of analyte using Thin-layer chromatography**

N-hexane fraction was diluted to get solution 0.1%. Two to ten (2-10) µL of the solution was spotted on silica plate (10x10cm) using 10 µL-micropipette. The next spotting was undertaken after the previous one dry. The spotted plate was introduced into chromatography system in which selected solvents are used as mobile phases. The spots were eluted by suitable solvents. The plate was then removed and allowed to dry in open space. To identify the compounds, the plate was sprayed with visualizing reagents and

heated at 90-110°C. The result was copied onto transparant paper and milimeter paper to know Rf value. The Rf was the ratio of the distance travelled by component to the distance travelled by solvents

#### **Analysis of flavonoid**

The n-hexane fraction of neem leaves (100 mg) was extracted using etanol 80%. The mixture was heated at 60°C and then was sentrifuged. Five (5) µL of solution was spotted on cellulose (stationary phase). The sample were eluted in ethyl acetate-formic acid-acetic acid-water: 100-11-11-27 (Santos et al., 1978). The chromatogram was analysed under UV light (254 nm) and then reacted with ammonia followed by identification by UV light at 365 nm. The result was compared to rutin (Wagner, 1984).

#### **Analysis of steroid**

Into 100 mg of n-hexane fraction of neem leaves was added 2 mL of n-hexane. The mixture was shaken in vortex. The sample (5 µL) was spotted onto silica gel plate and eluted in benzene-ethyl acetate (65:35) to the final line. The chromatogram was allowed to dry and then reacted with a visualizing reagent, KOH etanolic 10%. Identification by UV light was undertaken at 254 and 365 nm. The result was compared to beta-sitosterol.

#### **Analysis of terpenoid**

Into 100 mg of n-hexane fraction of neem leaves was added n-hexane. The sample (5 µL) was spotted onto silica gel plate and eluted in toluene-ethyl acetate (95:5) to the final line. The product was analysed with UV light (254 nm). The visualizing reagent, vanilin-asam sulfat was sprayed on the plate which then was reanalysed with UV light at 365 nm. The result was compared to carvone.

#### **Analysis of alkaloid**

A 120 mg of n-hexane fraction of neem leaves was reacted with ammonia 10% and extracted by chloroform. The chloroform layer was separated and then evaporated. Into evaporated product was added chloroform to make 1 mL solution. Ten (10)µL of solution was spotted silica gel plate and eluted in metanol-ammonia (100:1,5) to the final line. The product was analysed with UV light (254 nm). The visualizing reagent, Dragendorf was sprayed on the plate which then was reanalysed with UV light at 365 nm. The result was compared to quinin.

#### **Analysis of fenols**

Into 100 mg of n-hexane fraction of neem leaves was added 2 mL of n-hexane. The mixture was shaken in vortex. The sample (5 µL) was spotted onto silica gel plate and eluted in methanol-formic acid 10% (95:5) to the final line. The plate was allowed to dry. The product was reacted with a visualizing reagent, ferri chloride. Identification by UV light was undertaken at 254 and 365 nm (Santos et al., 1978).

#### **Gas chromatography-mass spectroscopy analysis**

The instrument was GC-MS Schimadzu QP-2010S with operational specification as follows: ionisation type: Electron impact (EI), coloumn: capillary coloumn HP-5MS, lenght:30 cm, internal diameter: 0.25 mm, temperature: 100°C. The mobile gas was helium, pressure 16.5 Kpa, with split injector at 300°C, the injection speed of sample was 36.8 ml/minute and the flow rate in coloumn was 0,6 mL/minute. The coloumn was packed with stationary phase which sticked on celite or chromosorb A as support material.

The sample was dissolved in n-hexane to get 50 ml solution which then was injected into injection port through. The gas (helium, 16.5 Kpa) flow was set to be constant to carry the sample to the coloumn so that the separation could occur effectively. The detector produced the signals that transmitted to the recorder which then showed the result as peaks that were different in retention time. The temperature of injector and deteector was kept stabile at 300°C, while the temperature of capillary coloumn was increasingly change started from100°C.

#### **Results and Discussion**

Isolation of antimalarial compounds contained in neem leaves by metanol extraction followed by hexane fractionation was effective with 100 gram yield. The n-hexane fraction eluted in thin layer chromatography showed several compounds detected under UV 254 nm and 365 nm (after reacted with specific visualizing reagents). the result shown in Table 1.

Table 1. Thin layer chromatography and phytochemistry analysis

n-HDN	Rf value	Visualizing reagents	Colours	Standard	Result
Flavonoid	-	Ammonia vapour	-	Rutin	Negative
Steroid	0.84	Lieberman-Burchard	Violet	Beta-sitosterol	Positive
Terpenoid	0.21; 0.24; 0.33; 0.81 and 0.96	Vanilin-sulphuric acid	Red brown-violet	Carvone	Positive
Alkaloid	-	Dragendorf	-	Quinin	Negative
Fenol	0.75	Ferri chloride	Dark green	Fenol	Positive

Detection of flavonoid under UV short wavelength showed no compound observed to have same Rf value or same colour to Rutin, the standard that contained flavonoid. The same result was shown after the plate was sprayed with ammonia vapour and then analysed under UV light 365 nm. This indicated that there was no flavonoid extracted in hexane. One spot with Rf value was same to beta-sitosterol, was observed in steroid analysis. The result showed that unidentified steroid presented in n-hexane fraction. The chromatography separated 5 terpenoid compounds which could be seen under UV light 365 nm as red brown-violet spots after reacted with vanilin-sulphuric acid. In this analysis, the standard was carvone. Alkaloid analysis found no compound classified to alkaloid detected under UV light 254 nm, while the result after reacting with Dragendorf reagent showed one spot with different Rf value and different colour compared to quinin, the standard. One substance, shown as a dark green spot, was detected on TLC plate in fenol analysis after been sprayed with ferri chloride.

Figure 1 presents the chromatogram of Gas chromatography-Mass spectroscopy (GC-MS) obtained for n-hexane fraction of neem leaves. It shows the percentage of each compound which transformed to vapour phase when it was placed in the instrument at the set temperature and pressure. Based on the instrument work mechanism, it could be assumed that there might be some compounds that did not vaporize. The compounds might be those that were classified to steroid or terpenoid that had been identified in phytochemical analysis.

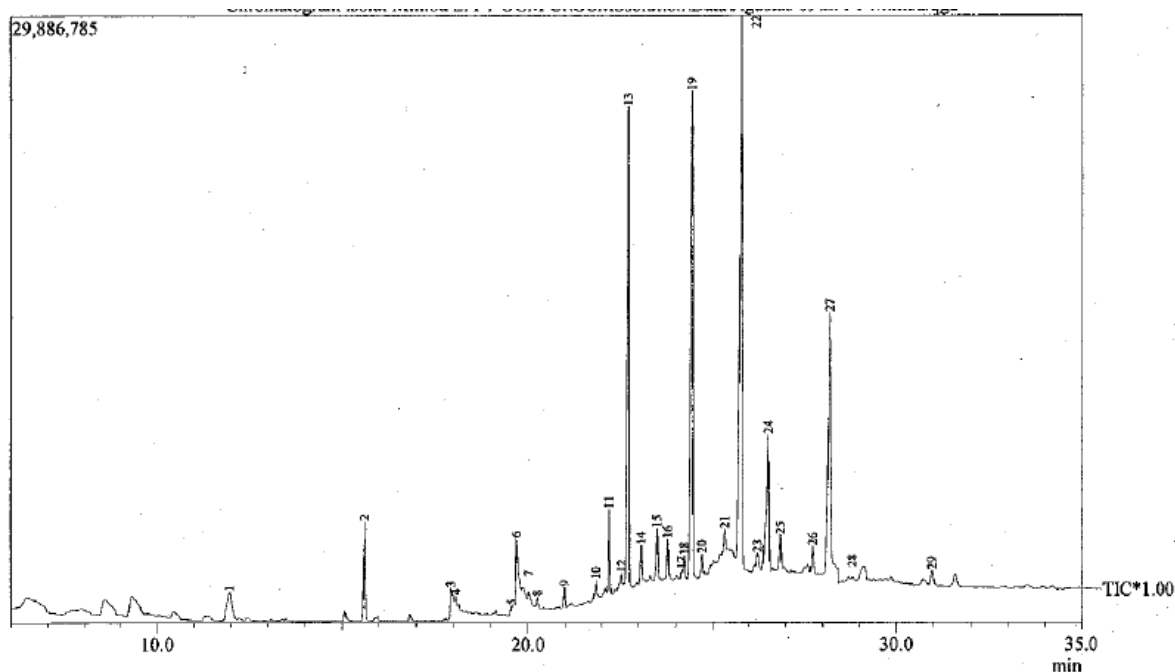


Figure 1. The chromatogram of n-hexane fraction of neem leaves obtained from Gas chromatography-Mass spectroscopy (GC-MS)

The substances analysed by GC-MS was identified as phenols which included compound 22 identified as benzyl benzoat (25,63%), compound 19 identified as tetracosane (17.51%) and compound 13 identified as hexadecanal (14.61%). This result was not compared to others since no publication related to this available yet. These fenols which presented in the n-hexane fraction are suspected to have antifungal activity for dermatitis tretments, can be used in oral gargle antiseptic, anti acne and preservatives (Biswas et al., 2002).

The steroids and terpenoids extracted from neem leaves needs to be investigated further to know the specific compounds in detailed. The possible active agents with antimalarial activity are terpenoid derivatives.

### Conclusions

The n-hexane fraction of neem leaves (*Azadirachta indica* A. Juss) contained steroid, terpenoid and fenol compounds. The fenols were identified as benzyl benzoat (25.63%), tetracosane (17.51%) and hexadecanal (14.61%). The steroid and terpenoid derivatives presents in the fraction are remained on the investigation to reveal the potential antimalarial agents which are suspected being classified to terpenoid.

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