Isolation and Structure Modification of Biologically Active Compound Nimbolide from *Azadiracthta indica* A. Juss. Var. *siamensis* Valeton

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Abstract

Malaria is found throughout the tropical and sub-tropical regions of the world and causes more than 300 million acute illnesses and at least one million deaths annually. Moreover the increase of drug resistance of *Plasmodium falciparum* remains to be serious problems. In order to continue antimalarial activity evaluation, nimbolide was isolated from the leaves of *Azadiracthta indica* A. Juss. Var. *siamensis* Valeton and its structure was modified. Experimentally, the ground-dried leave was extracted with acetone. Acetone extract was washed with hot hexane and crystallized with methanol. Recrystallization with dichloromethane : hexane mixture (1:1) provided nimbolide with 0.204% yield. The pure nimbolide reacted with 10% methanolic-KOH at 0°C to yield a reaction product of 17.02%. Structures of nimbolide and final product were illustrated by spectroscopy methods, i.e., infrared spectrophotometry, ultraviolet-visible spectrophotometry, nuclear magnetic resonance spectrometry and gas chromatography-mass spectrometry. It can be concluded that the effectiveness and convenient methods for nimbolide isolation and structure modification were developed.
Keywords: Anti-malarial, Nimbolide, *Azadiracthta indica* A. Juss. Var. *siamensis* Valeton

Introduction

Malaria remains to be one of the serious problems in tropical countries because of the increase in *Plasmodium falciparum* strains resistant to conventional antimalarials (WHO, 1998). Thailand is a great resource of medicinal plants and many of them have claimed to be used as antimalarials. Hence, utilization of these plants has been considered a possible alternative to solve this problem. Clinical trials of about 30 kinds of medicinal plants were carried out during the second World War due to lacking of antimalarial drugs and some of them showed antimalarial activity (Ketusinh, 1948). In additon, *in vitro* antimalarial activity of various Thai medicinal plants were investigated (Suppakun, 1983).

Azadiracthta indica Meliaceae is widely discovered in South, Southeast Asia and West Africa. In Thailand, leaves of *A. indica* A. Juss.Var. *siamensis* Valeton (locally called Sadao tree) are extensively used as vegetable, and the leaves and other parts of the plant are traditionally used for a variety of ailments. Aqueous extract of leaves in particular is used as remedy for malaria, similar to the practice in Nigeria (Okpanyi and Ezeukwu, 1981). The terpenoid lactone nimbolide (Figure 1)

from ethanol extraction of promising *A. indica* was identified (Ekong, 1967) and found to inhibit *P. falciparum* in culture with a moderate potency (EC_{50} 0.95 mg/ml) (Rochanakij *et al*., 1985). Therefore, it is promising to use nimbolide as a starting material for preparing structure-related compounds for antimalarial activity. In this report, we described the isolation process and attempted to enhance the antimalarial activity of nimbolide via chemical modification.

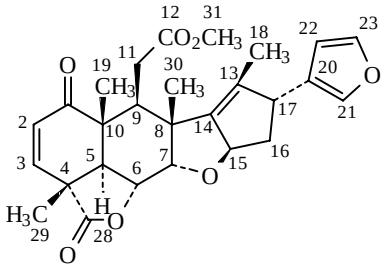

Figure 1. Chemical structure of nimbolide

Materials and Methods

Plant materials

Leaves of *Azadiracthta indica* A. Juss. Var. *siamensis* Valeton were collected in Phitsanulok, Thailand in August 2000.

Chemicals

Acetone, hexane and methanol were commercial grade and obtained from Rattana Trading Co. (Thailand). Analytical grade hexane, hydrochloric acid, methanol and potassium hydroxide were purchased from Merck (Damstadt, Germany). Chloroform and dichloromethane were analytical grade and obtained from Lab Scan Co. (Thailand). Thin layer chromatography (TLC) aluminium sheets 20x20 cm silica gel 60 F_{254} was obtained from Merck (Damstadt, Germany).

Apparatus

Melting points were determined with an electrothermal melting point apparatus (Buchi 535, Japan). Infrared (IR) spectra were determined by KBr pellet technique with Spectra 2000 (Perkin Elmer, Germany). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of nimbolide was obtained by a Bruker Avance300-Av300 and nimbolide derivatives spectra were obtained by a Jeol JMN-A500 spectrometer. The chemical shifts are reported in δ values in parts per million (ppm) downfield from tetramethylsilane

(TMS) as the internal standard. The samples were determined by gas chromatography (GC, Varian Star 3400cx, California, USA) using packed column (Porapak N 80/100 2m x 1/8″s.s., Varian, USA). EI mass spectra were measured with a gas chromatography-mass spectrometer (GC-MS, Varian Star 3400cx-Varian Saturn 3, California, USA) instrument using a capillary column (DB-5MS, 30m x 0.250 mm, J&W Scientific, USA). The electron energy was 70 eV.

Extraction and isolation of nimbolide from leaves of *A. indica* var. *siamensis*

Fresh leaves of *A. indica* var. *siamensis* were dried in a hot-air oven at 60°C for 2 hours and milled to fine powder. The dried powder (1 kg) was macerated in acetone at room temperature for 3 days and then filtered. The dark green filtrate was evaporated under reduced pressure until dryness. The black gum residue was digested in hot hexane (250 ml) and the hexane solution was decanted. The process was repeated until the hexane washing appeared colorless (8-10 times). Methanol (200 ml) was added to the dark green residue and the mixture was kept in a refrigerator (4°C) overnight. Then the newly form crystal was filtered and washed with cold methanol resulting in white crystal. The white crystal was crystallized twice from dichloromethane : hexane (1:1). The colorless plate crystal of nimbolide (2.04 g, 0.204%) was obtained and identified by melting point measurement, IR spectrophotometry, ^{1}H-NMR spectrophotometry, gas chromatography and GC-MS spectrometry.

Synthesis of nimbolide derivative

Nimbolide (235 mg) was treated with 10% methanolic-KOH (75 ml) at 0°C for 3.5 hrs. The solution was acidified with 6 *N* HCl to pH 5 and kept at room temperature to complete precipitation. A methanolic solution was collected and evaporated under reduced pressure to give viscous residue. The residue was crystallized with 50% methanol to provide a yield reaction product of 17.20%. The structure of reaction product was illustrated by spectroscopy methods, IR spectrophotometry and ^{1}H-NMR spectrometry.

Results and discussion

The isolation of nimbolide from *A. indica* var. *siamensis* leaves by hexane extraction was reported by Nair *et al*. (1997) with more complicated processes. In this report we describe simpler solvent extraction and crystallization techniques without further column chromatographic method and obtained higher yield. Extraction of 1 kg-dried powder leaves of *A. indica* var. *siamensis* resulted in nimbolide (2.04g, 0.204%), $C_{27}H_{30}O_{7}$, m.p. 242-246°C, lit. m.p. 245-247°C (Ekong, 1967). The purity of nimbolide

(99%) was obtained from GC chromatogram. The GC-MS revealed a molecular ion peak (M^+) and also base peak at *m/e* 466 that are identical to molecular weight of nimbolide. The IR spectra exhibited three strong bands of different characteristic carbonyl groups. The bands at 1,671, 1,728 and 1,776 cm^{-1} were assigned to be C=O stretching vibration of conjugated carbonyl, ester and lactone, respectively.

The ^{1}H-NMR spectrum of the solution of nimbolide in deuterated dimethyl sulfoxide (DMSO) was in accordance with published previously by Kigodi (1989). The signal at 1.20 (3H, s), 1.34 (3H, s), 1.44 (3H, s) and 1.67 (3H, s) were due to methyl proton on C_{19}, C_{30}, C_{29} and C_{18}, respectively. The methoxy proton gave a signal at 3.50 (3H, s). The signal at 2.10 (1H, m) and 2.18 (1H, m) were due to protons on C_{16}. The signal at 2.34 (1H, dd) and 3.22 (1H, dd) were obviously due to methylene protons of side chain. The signal at 5.88 (1H, d) and 7.24 (1H, d) were due to proton on vinylic C_2 and C_3, respectively. Three protons on furan ring gave rise to a signal at higher field (d6.22, 7.18 and 7.28).

The hydrolysis reaction of nimbolide in the present of 10% methanolic-KOH provided a yield nimbolide derivative of 17.20% and its structure is identified in Figure 2.

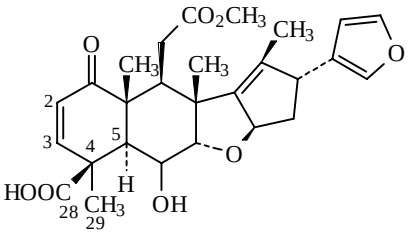

Figure 2. Chemical structure of nimbolide derivative

The IR spectra showed three strong bands of different characteristic carbonyl groups. The bands at 1,676, 1,721 and 1,747 cm^{-1} were assigned to be C=O stretching vibration of conjugated carbonyl, ester and carboxylate carbonyl, respectively. The band at 3348 cm^{-1} was obviously due to hydroxyl group.

The ^{1}H-NMR spectrum of the solution of nimbolide derivative in deuterated dimethyl sulfoxide (DMSO) was in accordance with published previously by Kigodi (1989). The signal at 1.26 (3H, s), 1.34 (3H, s), 1.58 (3H, s) and 1.73 (3H, s) were due to methyl proton on C_{19}, C_{30}, C_{29} and C_{18}, respectively. The methoxy proton gave a signal at 3.68 (3H, s). The signal at 2.05 (1H, m) and 2.11 (1H, m) were due to protons on C_{16}. The signal at 2.28 (1H, dd) and 3.34 (1H, dd) were obviously due to methylene protons of side chain. The signal at 5.83 (1H, d) and 6.70 (1H, d) were due to proton on vinylic C_2 and C_3, respectively. Three protons on furan ring gave rise to a signal at higher field (δ 6.33, 7.28 and 7.33) and the signal at 2.90 (1H, d) was obviously due to hydroxy proton.

The chemical structure of nimbolide has two labile functional group, i.e., ester and lactone ring. A comparison between these two functional groups, the 5-memberred lactone ring has more strain than aliphatic ester. Therefore, lactone ring is easier to hydrolyze by methanolic-KOH to give carboxylic acid and hydroxyl groups, which are convenient for further chemical reaction such as introducing alkyl amino side chain or sugar moiety into nimbolide derivative.

Conclusion

Nimbolide was isolated from leaves of of *A. indica* var. *siamensis*. The hydrolysis reaction of nimbolide involving the cleavage on the lactone ring is carried out by 10% methanolic-KOH solution to yield nimbolide derivative. In further study, these compounds will be evaluated for antimalarial activity.

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