Berberine in the Medicinal Plant of Tali kuning (*Tinospora dissitiflora* Diels)

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Abstract

Two different approaches have been used to determine the bioactive compounds in the medicinal plant of Tali kuning (*Tinospora dissitiflora* Diels). Firstly, the conventional approach using column chromatography (CC), and preparative thin layer chromatography (PLC) eluted with benzene:ethyl acetate: formic acid (5:4:1, and 5:4:2, respectively), followed by CC eluted with benzene:methanol (3:2) were used to separate and isolate berberine from the chloroform fraction of Tali kuning. Structural elucidations of the isolated compounds were conducted using nuclear magnetic resonance (NMR) and mass spectrometry (MS). Secondly, a rapid and simple approach use the integrated intensities of proton signals for H-13 and H-8 of berberine on ^{1}H-NMR spectra, then the qualitative and quantitative determination of berberine in Tali kuning can be achieved directly from the crude extracts using ^{1}H-NMR. The proton signals for H-13 and H-8 of berberine on ^{1}H-NMR spectra, which appeared at empty regions as singlet, and without interference from the other signals, were available for qualitative determination of berberine. Whereas, the integrated intensity of proton signal for H-13 on ^{1}H-NMR spectrum was used for quantitative determination of berberine. Berberine content of Tali kuning was calculated manually based on the integrated intensity of proton H-13 from the authentic berberine chloride, which was 18.6 mg/g based on the weight of air-dried wood meal. This berberine content was comparable to that (22.78 mg/g) of Amur corktree (*Phellodendron amurense* Rupr), which is widely acknowledged for good producer of berberine.

Keywords: Tali kuning (*Tinospora dissitiflora* Diels), berberine, rapid quantitative and qualitative determination, ^{1}H-NMR.

Introduction

Tali kuning (*Tinospora dissitiflora* Diels), Menispermaceae, is widely used in West Papua, Indonesia for medicinal plants, mainly to alleviate a malarial symptom (Suebu 2002). Tali kuning refers to liana having yellow sap-stem. Decoction from the chopped stem of this plant is used for herbal medicine. However, our literature survey revealed that the bioactive compounds of this herbal plant are not clarified yet. When Tali kuning is used for further advanced utilization as herbal drugs, for example identification of chemical compounds in this plant should be determined with simple, rapid and repeatable methods. It is because in daily practices, the practitioners rarely use the pure compounds or single herbal drug to treat diseases, but they use a combination of several herbal drugs (Tang *et al*. 2009). Therefore, advanced and superior tools for rapid and simple determination of bioactive compounds of the medicinal herbs directly from the crude extracts are highly desirable and recommended.

Our preliminary study on the extractives from Tali kuning revealed that the main component was berberine and the bioactivity was mostly derived from this compound.

Conventional instruments such as column chromatography, preparative thin layer chromatography have been used to isolate bioactive protoberberine from the stem of *Mahonia japonica* (Hsieh *et al*. 2004), protoberberine alkaloids from *Fissistigma balanse* (Chia *et al*. 1997). However, these methods require several steps in extraction and separation, time consuming, and low sensitivity (Li *et al*. 2006). Rapid qualification and quantification of protoberberine in wood powder or its extract using ^{1}H-NMR have been employed successfully for *Cortex phellodendri* (Rutaceae) and have determined the traditional Chinese medicine prescriptions (Li *et al*. 2006). The similar ^{1}H-NMR was also used for rapid determination of protoberberine alkaloids in *Rhizoma coptidis* and quality control of commercial prescriptions (Li *et al*. 2009).

In this research, therefore, berberines from the chloroform fraction of Tali kuning using the conventional instrumental analyses were described. Rapid qualitative and quantitative determinations of berberine directly from the crude extracts of the stem wood powder from Tali kuning using ^{1}H-NMR were also reported. Furthermore, Amur corktree (*Phellodendron amurense* Rupr) was used for comparison of berberine content in Tali kuning because this plant has been reported for good producer of berberine (Tang *et al*.2009).

Materials and Methods

Plant Material

The stem (12 cm of diameter) of Tali kuning (*T. dissitiflora* Diels) was collected from Manokwari, West Papua. Mr. Djitmau from the Herbarium Manokwarienses, State University of Papua, identified the voucher speciments of this plant, which were in agreement with New Guinea Forest Department (NGF) collection No.12272 and Indonesian Biodiversity Information System (IBIS 2011). The bark of Amur corktree was obtained from our collection at Monobe Campus, Kochi University, Kochi, Japan.

Extraction, Separation and Isolation

One kilogram of wood powder (having moisture

content of 15%) from Tali kuning was extracted with MeOH (1L) at room temperature for 48 h and 1 L of methanol extract was obtained by filtration using glass funnel. These extraction procedures were repeated five times and 5 L of methanol extracts were collected, and evaporated with vacuum evaporator to give 9.91 g of dried methanol extract. This dried methanol extract was dissolved in 10 ml of MeOH and partitioned with hexane (100 ml) to give 0.25 g of hexane fraction. The residue was partitioned with 100 ml of chloroform to result 7.02 g of chloroform fraction. The remaining residue was re-partitioned with acetone (100 ml) and 0.36 g of acetone fraction was obtained. MeOH fraction of 1.76 g was obtained after the remaining dried residue was partitioned with MeOH (100 ml). The chloroform fraction, as major fraction (1.92 g) was chromatographed over column chromatography (CC) eluted with benzene: ethyl acetate : formic acid (5:4:1*)* to give 40 fractions. Fractions 9~15 were combined and evaporated to give dried residue of 1.06 g, and permeated to preparative thin layer chromatography (PLC) developed using similar eluent with increasing polarity (5:4:2). The yellow band on PLC was scratched out and dissolved in methanol. The methanol solution was filtered with filter papers. Condensation of this solution gave a brown crystal of compound**1** (60 mg). The mother solution was evaporated and further re-chromatographed over CC using benzene:methanol (3:2) and yellow powder of compound **2** (15.9 mg) was collected. The previous procedures (PLC and CC, respectively) were also employed to the fractions 16~19 (0.20 g) to afford a yellow powder of compound **3** (190 mg), while from the fractions 20~28 (0.080 g) gave yellow powder of compound **4** (44 mg).

Compounds (1, 2, 3, and 4) had UV λ_{max} spectra at 222, 225, 236, 264, and 347 nm. EI-MS m/z recorded from all the isolated compounds (1~ 4) showed base peak at 336 (100) indicating the molecular ion ($C_{20}H_{18}NO_4$). The NMR (^1H and ^{13}C) spectra of the isolated compounds were summarized as follows: ^1H-NMR(CD_3OD) : 3.26(2H,t,J=6.0Hz, H-5), 4.92(2H,t,J=6.0Hz, H-6), 4.11(3H, s, OCH_3), 4.20(3H, s,OCH_3), 6.11(2H, s, OCH_2O), 6.96(1H,s,H-4), 7.65(1H,s,H-1), 8.00(2H,d,J=8.8Hz,H-12), 8.11(2H,d,J=8.8Hz,H-11), 8.69(1H,s,H-13), 9.79 (1H,s,H-8). ^{13}C-NMR (CD_3OD): 28.19(C-5), 57.19(C-6), 57.67(C-10-OCH_3), 62.54(C-9-OCH_3), 103.66(O-CH_2-O-), 106.55(C-1), 109.38(C-4), 121.49(C-14a), 121.87(C-13), 123.33(C-8a), 124.51(C-12), 128.10(C-11), 131.90(C-4a), 135.18(C-12a), 139.68(C-14), 145.78(C-8), 146.40 (C-9), 149.92(C-2), 152.02(C-10), 152.17(C-3).

The spectral data obtained from the isolated compounds were analyzed and compared systematically to those reported in the published literatures (Chia *et al*. 1997; Hsieh *et al*. 2004; Min *et al*. 2006; 2007).

Chemicals and Instrumental Analysis

All chemicals used were analytical grade (Wako Ltd, Osaka, Japan). Glass column (30 cm in length and 3.5 cm in diameter, Vidrex P) and silica gel 60 (70~230 mesh) (Nacalai tesque Inc, Kyoto, Japan) were run for CC. Thin layer chromatography (TLC) and PLC were done by using precoated plates with K5F silica gel 150 A (5 x 10 cm, 200 μm in thickness, silica gel 150 A (Whatman Ltd, Maidstone, England)), and precoated plates with silica gel 60 F_{254} (20 x 20 cm, 2 mm in thickness of precoated silica gel 60 F_{254}, (Merck, Damstadt, Germany), respectively. Berberine chloride hydrate (Tokyo Chemical Industry Co Ltd, Tokyo, Japan) was used as standard. UV spectra were recorded on a Shimadzu UV-VIS 1200 spectrometer. Mass spectra (MS) were measured on a Shimadzu 2010 LC-MS (Shimadzu Co., Kyoto, Japan) using a packed column COSMOSIL 5C18-AR-II (4.6 x 250 mm, Nacalai Tesque), eluent (MeOH) and UV detector.^1H-NMR and^{13}C-NMR spectra were detected by a JEOL JNM-EX400 (JEOL Ltd., Tokyo, Japan) at 400 MHz for ^1H and 100 MHz for ^{13}C, respectively.

Rapid Qualitative and Quantitative Determination of Berberine using^1H-NMR

A rapid qualitative and quantitative determination of berberine from the crude extracts of Tali kuning was conducted using the method employed by Li *et al*. (2006, 2009) with some modification. Ten milligrams of stem and bark powders (moisture contents of 11.15 and 7.54% for Tali kuning and Amur corktree, respectively) were extracted with MeOH (0.5 ml) using sonication at room temperature for 30 min and MeOH extracts were obtained by filtration with filter papers. These extraction procedures were repeated for three times. The MeOH extracts were combined and dried up with vacuum evaporator. The dried MeOH extracts were dissolved in methanol-d_4 (0.5 mL containing 84.4 μg anthracene) and transferred to the NMR test tube for ^1H-NMR measurement. Three replicates were employed. Authentic berberine chloride of 500 μg dissolved in methanol-d_4 (0.5 mL containing 84.4 μg anthracene) was used for determining berberine quantity in the crude extract using^1H-NMR.

^1H-NMR spectra were recorded in methanol-d4 (99.9%) using JEOL JNM-ECX 500(JEOL Ltd., Tokyo, Japan). For each sample, 100 scans were recorded with the following parameter: 0.187 Hz/point; spectral width, 14400Hz; pulse width, 4.0 μs; relaxation delay, 2s. For quantitative analysis, peak area was used and the start and end points of the integration of each peak were selected manually.

Results and Discussion

Spectral Features of Berberine

UV absorptions recorded from the compounds (1~4) were dominantly detected at 222, 225, 236, 264 and 347 nm, indicating those of berberine and in well agreement with those reported by Min *et al*. (2006) and Grycova *et al*. (2007). MS base peak at m/z 336 (100) was appeared in all

compounds, suggesting the molecular ion of berberine ($C_{20}H_{18}NO_4$), and having similarity with those reported in the references (Hsieh *et al*. 2004; Min *et al*. 2006, 2007). Compounds (1, 2, 3, and 4) isolated from Tali kuning have yellow to brownish color, which was similar to the color of berberine. Usually berberine has bright color (yellow to orange) and typically yellow (Huang *et al*. 2005). Berberine is one of the protoberberine and quaternary alkaloids contributing to the color of the plant tissue (Huang *et al*. 2005) found in the plant families of Berberidaceae, Papaveraceae, Rutaceae, Rubiaceae, Rhamnaceae, Runumculaceae, Magnoliaceae, and Annonaceae, including Menispermaceae (Vennerstrom and Klayman 1988).

NMR Detection

^{1}H-NMR signal patterns of compounds 1, 2, 3, and 4 were fundamentally similar to those of berberine reported by Chia *et al*. (1997), Hsieh *et al*. (2004), and Min *et al*. (2006, 2007), as illustrated in Figure 1. ^{13}C-NMR spectra of the compounds 1, 2, 3, and 4 also have similarity to that of the berberine reported in the published literatures (Chia *et al*. 1997; Hsieh *et al*. 2004; Min et al. 2006; 2007). Two methoxy signals on D-ring were appeared at δ 57.68 ppm for C-10 and at δ 62.54 ppm for C-9. Carbon signals of C-2 and C-3 connected with dioxymethylene were detected at δ 149.93 and δ 152.21 ppm, respectively. Figure 2 showed the elucidated structure of the isolated compound, berberine.

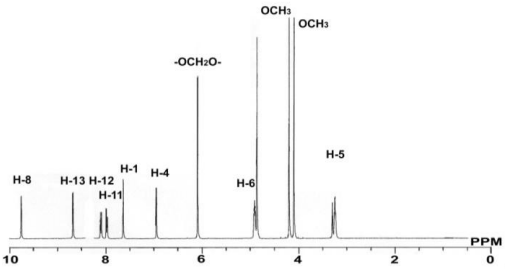

Figure 1. ^{1}H-NMR spectrum of berberine isolated from the chloroform fraction of Tali kuning (*T. dissitiflora* Diels).

^{1}H-NMR spectra have valuable information used for qualitative and quantitative determination, because ^{1}H-NMR spectral pattern of berberine was very typical (Figure 1). This figure showed that the proton signals of H-13 and H-8 of berberine isolated from the chloroform fraction of Tali kuning on ^{1}H-NMR spectrum appeared at δ 8.69 ppm and δ 9.79 ppm, respectively similar to that reported by Li *et al*. (2006). Because these two proton signals are very typical for berberine and they could be used for berberine qualitative determination (Li *et al*. 2006, 2009). For this purpose, internal standard was required and anthracene was selected for it because its integrated proton intensity was stable for 24 hours and appeared at δ 8.44 ppm, very closed to spectrum regions of targeted proton of H-13 and H-8 (Li *et al*. 2006).

Rapid Determination of Berberine from the Methanol Extract of Two Medicinal Plants using ^{1}H-NMR

Figure 3 (A) demonstrated a ^{1}H-NMR spectrum of the authentic berberine chloride. Whereas the ^{1}H-NMR spectra of berberine recorded for the crude extracts of stem wood powders from Tali kuning and bark powders from Amur corktree were shown in Figure 3 (C), and Figure 3 (B), respectively. Figure 3 showed that typical berberine protons of H-13 and H-8 on ^{1}H-NMR spectra were appeared on the downfield regions at δ 8.69 (1H,s) and δ 9.79 (1H,s) ppm for H-13 and H-8, respectively. As illustrated in Figure 3 (C) and 3 (B) for berberine from Tali kuning and Amur corktree, respectively, these two typical protons (H-13 and H-8) of berberine were also resonanced at the same chemical shifts, where H-13 was detected at δ 8.69 (1H,s) ppm and H-8 was at δ 9.79 (1H,s) ppm. Both proton signals appeared as singlet, and well separated from the other proton signals. More importantly, these two proton signals were detected at the same chemical shifts consistently as illustrated in Figures 3 (A), 3 (B), and 3 (C), respectively. These results were similar to that in the ^{1}H-NMR of berberine from *Cortex phellodendri* reported by Li *et al*. (2006).

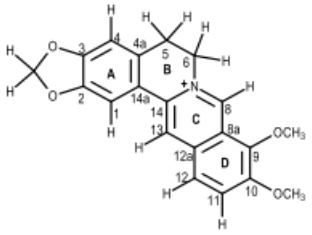

Figure 2. Elucidated structure of berberine isolated from the chloroform fraction of Tali kuning.

^{1}H-NMR of authentic berberine chloride (Figure 3 (A)) showed that proton signal of internal standard (anthracene) was detected at δ 8.44 ppm, and its integrated intensity was used as 1, which was equal to 84.4 μg of anthracene. The integrated intensites of anthracene in Figure 3 (B) and Figure 3 (C) also used as 1 for calculation of berberine contents of the crude extracts from stem wood and bark powders of Tali kuning and Amur corktree, respectively.

Figure 3 (A) demonstrated that the proton H-13 of authentic berberine chloride was detected at δ 8.69 ppm, similar to those illustrated in Figures 3 (B) and 3 (C), respectively. The proton H-13 of authentic berberine chloride had integrated intensity of 1.91, which is equal to 500 μg of berberine. On the other hand, the integrated intensities of protons H-13 of berberines from the crude extracts of bark and stem wood powders of Amur corktree (Figure 3 (B)) and Tali kuning (Figure 3 (C)) were 0.84 and 0.69, respectively.

From the aforementioned data, the berberine contents in the crude extracts from the bark and stem wood powders of Amur corktree and Tali kuning can be calculated.

Berberine contents in the two medicinal plants determined by integrated intensities of proton H-13 of berberine on ^{1}H-NMR spectra are summarized in Table 1.

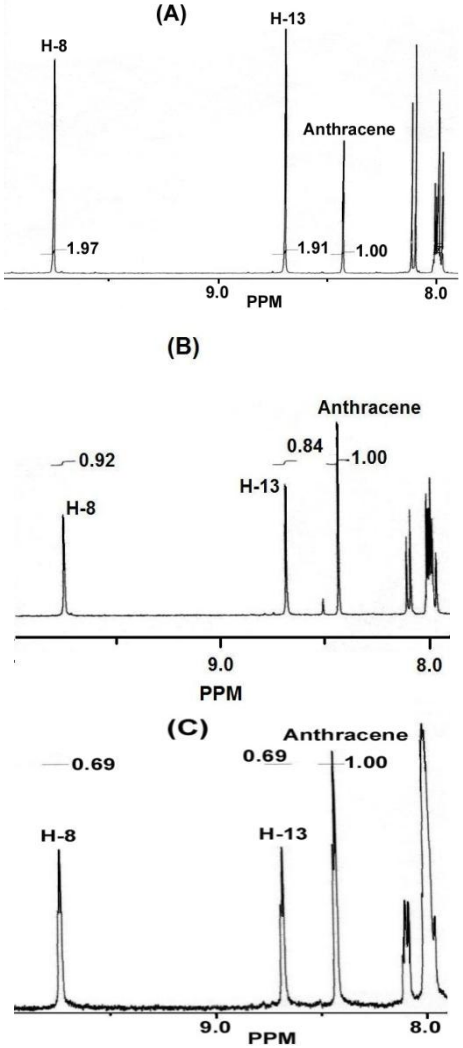

Figure 3. ^{1}H-NMR of the samples with internal standard (anthracene) in methanol-*d*4. (A):Berberine chloride, (B):Extract from Amur corktree, (C):Extract from Tali kuning.

Table 1 indicated that berberine content of the crude extracts from the bark powders of Amur corktree was 22.78 mg/g of air-dried powders. This quantity was higher than that (18.06 mg/g) of the crude extracts from the stem wood powders of Tali kuning. However, the percentage of berberine content in Tali kuning based on the weight of dried extract was higher (12.04%) than that (8.34%) in Amur corktree (Table 1). It is because Tali kuning wood powder had higher moisture content (11.15%) and less extract content (1.50 mg) than the Amur corktree, 7.5% and 2.73 mg, respectively.

Yellowish to brownish alkaloids from the chloroform fraction of the medicinal plant of Tali kuning have been isolated and their structures were also elucidated, which was berberine ($C_{20}H_{18}NO_4$), using conventional methods of CC and PLC. Traditional methods such as crystallization, CC, and acidic extraction are frequently used to obtain the individual alkaloids from the plant extracts (Grycova *et al*. 2007).

Using integrated signal intensity of proton H-13 and H-8 on ^{1}H-NMR spectra, a rapid qualification and quantification of berberine in the crude extracts of the stem wood powder from Tali kuning was successfully achieved. Berberine quantity (18.06 mg/g of air-dried wood powders) was comparable to that (22.78 mg/g of air-dried bark powders from Amur corktree). Amur corktree was used for comparison of berberine content, because this medicinal plant has been widely known for good producer of berberine (Tang *et al*. 2009).

The quantities of berberine in the crude extracts from the two medicinal plants, Tali kuning and Amur corktree, respectively, are comparable to that (14.07 mg/g of air-dried wood meal) in *P. amurense* Rupr collected from Taiwan determined using the similar method of ^{1}H-NMR (Li *et al*. 2006). It should be considered that the whole stem of Tali kuning was used for determination of berberine content, while only the bark of Amur corktree was used for it. It means that abundance of berberine in Tali kuning is totally considerable. Quantitative differences in berberine contents in the plants are affected by several factors (climate, environment, and soil composition), and the occurrence of berberine in some plants is restricted to a specific part of the plant body, but in the other plants is dispersed through the whole body with varying rates in different plant tissues (Grycova *et al*. 2007).

It is generally claimed that the diverse geographical origin of the plant make the content of alkaloids quite different one to another (Li *et al*. 2006, 2009). Therefore, a rapid and simple determination of bioactive compounds in the plants using ^{1}H-NMR is necessary to estimate the medicinal values of it. These findings support the arguments that ^{1}H-NMR is an effective tool superior to HPLC in detecting and determining the chemical constituents in herbal medicine, and has been utilized for quality control of commercial traditional Chinese medicine (Li *et al*. 2006, 2009). Furthermore, it is applicable for determining berberine contents in the crude extracts from the stem wood powder of Tali kuning.

Berberine is gaining international popularity due to its great varieties of biological and pharmacological activities (Grycova *et al*. 2007). This includes the inhibition of DNA synthesis, protein biosynthesis, the inhibition of membrane permeability, and the uncoupling of oxidative phosphorylation (Schmeller *et al*. 1997). Other pharmacological activities of berberine have been reported for anti-inflammatory activity (Ivanovska and Philipov 1996), and antimalarial activity (Vennerstrom and Klayman 1998).

Table 1. Quantitative determination of berberine in the methanol extractives from bark and stem wood powders of Amur corktree and Tali kuning using integrated intensities of H-13 signals on ^{1}H-NMR spectra.

Medicinal Plants	Moisture Content (%)	Weight of dried extracts (mg) ± SD from 10 mg of air-dried wood meal	Berberine	
			Quantity (μg) ± SD in 10 mg of air-dried wood meal	Percentage based on the weight of oven-dried extract (%) ± SD
Amur corktree	7.54	2.73±0.24	227.8 ± 5.7	8.34± 0.96
Tali kuning	11.15	1.50 ± 0.22	180.6 ± 6.4	12.04 ± 1.90

Each value represents the value from three replicates; the following letters are standard deviation (SD).

Conclusions

It is highly advised that Tali kuning (*T. dissitiflora* Diels) can be recommended for new producer of berberine, because the berberine quantity in this plant is comparable to that of the Amur corktree (*P. amurense* Rupr), which has been known as good producer of berberine. Importantly,^{1}H-NMR can be employed to detect the bioactive compounds in the medicinal herbs, determine the medical values of it, and maintain the quality control of medicinal plants, as they are rarely sold in single herbal medicine but are sold in mixed ones.

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