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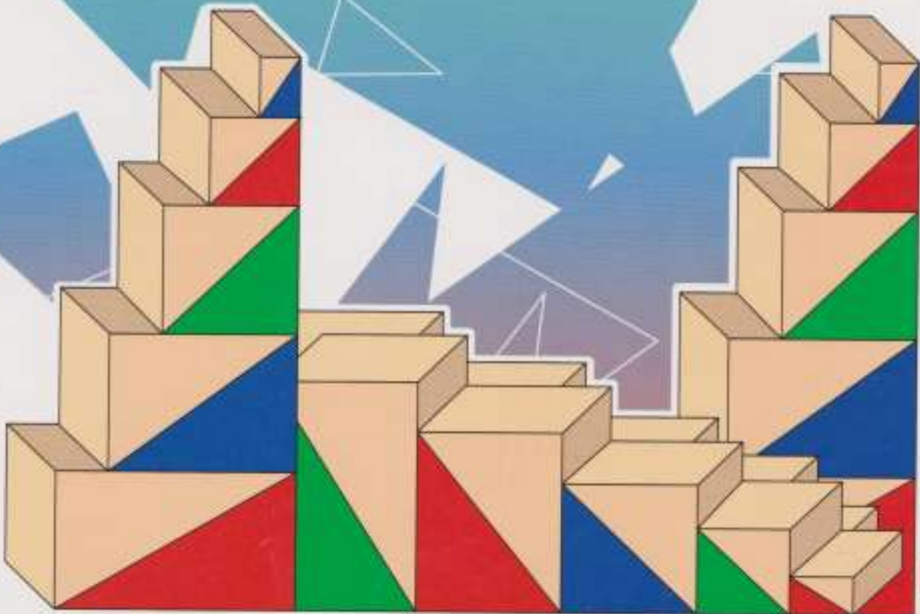

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Strong antifeedancy of berberine in Tali kuning (*Tinospora dissitiflora* Diels) against Japanese subterranean termite *Reticulitermes speratus* Kolbe

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ABSTRACT Berberine ($C_{20}H_{18}NO_4$) was isolated from chloroform fraction of Tali kuning (*Tinospora dissitiflora* Diels), eluted with column chromatography (CC) and preparative thin layer chromatography (PLC) using solvent systems of benzene:ethyl acetate:formic acid 5:4:1 and 5:4:2, respectively, as well as CC with benzene:methanol (2:3). Antifeedancy of the isolated compound against Japanese subterranean termite *Reticulitermes speratus* Kolbe was evaluated, compared to that of chloroform fraction and berberine chloride standard. Three replicates and levels of concentration 12.5, 15, and 50 mg/ml were employed. The results indicated that by day-7 at 50 mg/ml the termite survivors were 60, 69, and 61 % recorded from berberine, chloroform fraction, and berberine chloride, respectively. However, there was no surviving termite by the 14th day at the similar concentration from three samples tested. Interestingly, all experimental variables gave a similar class IV for coefficient of antifeedancy (CA) value of 75≤CA≤100, indicating a strong antifeedancy.

Key words: Tali kuning, *Reticulitermes speratus* Kolbe, berberine alkaloid, strong antifeedancy

Ｔａｌｉ　ｋｕｎｉｎｇ中のベルベリンのヤマトシロアリに対する強摂食阻害

ワヒュディ[*1*2]、大谷慶人[*1]、市浦英明[*1]

ベンゼン・酢酸エチル・ギ酸（5:4:1，5:4:2）を展開溶剤としたカラムクロマトグラフィーと分取薄層クロマトグラフィー、更にベンゼン・メタノール（3:2）を用いたカラムクロマトグラフィーにより、Tali Kuning (*Tinospora dissitiflora* Diels) の抽出物のクロロホルムフラクションからベルベリンを単離した。単離化合物のヤマトシロアリに対する生物活性を評価し、クロロホルムフラクション、塩化ベルベリン標品と比較した。全て３回繰り返し試験を行い、試料の濃度は 12.5、15、50 mg/ml を用いた。その結果、50mg/ml の濃度で、７日目までにベルベリン、クロロホルムフラクション、塩化ベルベリン標品ではそれぞれ、60、69、61 %のシロアリ生存率を示した。しかし、14 日後では全ての試料において生き残ったシロアリはなかった。全ての試料の摂食阻害係数（CA）は 75≤CA≤100 の範囲にあり、強い摂食阻害を意味するクラスⅣに分類された。

キーワード：Tali kuning，ヤマトシロアリ，ベルベリン，強摂食阻害

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1. Introduction

Tali kuning (*Tinospora dissitiflora* Diels) is a climbing plant or liana, having yellow inner stem, and being widely used as medicinal plant for antimalarial purposes in West Papua, Indonesia.[1] However, our literature survey revealed that the bioactive constituents of this plant are not determined yet and scientifically need to be clarified. Many phytochemical constituents are widely known in possessing antitermitic activity. Antitermitic activity of essential oils taken from eucalypt in Thailand against subterranean termite *Coptotermes formosanus* Shiraki have been reported.[2] Continuing to search an alternative eco-friendly antitermiticidal insecticide from naturally occurring substances, evaluating and testing different plant origin constituents in Tali kuning are our next targets. Japanese subterranean termite *Reticulitermes speratus* Kolbe,[3] one of two subterranean termite species the most economically important species in Japan[4] and worldwide[5], and a serious insect pest for papers and wood,[6] was used in this research. Therefore, this research is designed to isolate and elucidate bioactive compounds in Tali kuning and evaluate antifeedancy against the Japanese subterranean termite *R. speratus* Kolbe.

2. Materials and Methods

2.1. Materials

A mature stem of Tali kuning (12 cm diameter) was collected from Manokwari, West Papua. The air-dried stem was powdered and passed through 40-mesh sieve. The subterranean termites *Reticulitermes speratus* Kolbe were collected from the field of Kochi prefecture, Japan on November 2010.

2.2. Chemicals and analytical instruments

All chemicals used were analytical grade (Wako Ltd, Osaka, Japan). Column chromatography (CC) was conducted by using a glass column (30 cm in length and 3.5 cm in diameter, Vidrex P) of silica gel 60 (70-230 mesh) (Nacalai tesque Inc., Kyoto, Japan). Thin layer chromatography (TLC) and preparative thin layer chromatography (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. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-VIS 1200 spectrometer, Mass spectra (MS) were measured on a Shimadzu GC-MS QP 5050 (Shimadzu Co., Kyoto, Japan), and ^{1}H-NMR and ^{13}C-NMR spectra were detected by a JEOL JNM-EX400 (JEOL Ltd., Tokyo, Japan) at 400 MHz and 100 MHz, respectively.

2.3. Isolation and spectrometry

One kilogram of wood powder (15 % of

moisture content) was extracted with MeOH (1 ℓ) at room temperature for 48 hours, filtered with glass funnel (25G-3), and MeOH extract (1 ℓ) was collected. The residue was extracted again with pure MeOH (1 ℓ) for 48 hours and filtered using the same glass funnel. This procedure was repeated three times and gave 3 ℓ of MeOH extract. The combined extract (5 ℓ) was evaporated to give dried extract (9.91 g). This dried extract was dissolved with 10 ml MeOH, and then fractionated with 100 ml hexane to earn hexane fraction (0.25 g). The residue was further fractionated with 100 ml chloroform to give chloroform fraction (7.02 g). The similar procedure was employed to earn acetone (0.36 g) and methanol (1.76 g) fractions. The major fraction, chloroform fraction (1.92 g) was eluted with column chromatography (CC) and preparative TLC using mixture of three solvents, benzene : ethyl acetate : formic acid (5:4:1) and (5:4:2), respectively, followed by CC with benzene : MeOH (2:3), which leaded the isolation of berberine ($C_{20}H_{18}NO_4$). The NMR (^{1}H and ^{13}C) spectra were summarized as follows:

^{1}H-NMR:(CD_3OD) : 3.17(2H, t, J=6.4Hz, H-5), 4.84(2H, t, J=6.8Hz, H-6), 4.00(3H, s, OCH_3), 4.10(3H, s, OCH_3), 6.00(2H, s, OCH_2O), 6.86(1H, s, H-4), 7.55(1H, s, H-1), 7.90(2H, d, J=9.2Hz, H-12), 8.01(2H, d, J=9.2Hz, H-11), 8.59(1H, s, H-13), 9.67 (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).

2.4. Antifeedancy test

A contact method using filter paper (Whatman No. 3, 3 cm in diameter) was used for antifeedancy test. Antifeedancy of the isolated berberine against Japanese subterranean termite *R. speratus* Kolbe was evaluated, where chloroform fraction and berberine chloride standard were also used as comparison. Three replicates and levels of concentration 12.5, 25, and 50 mg/ml were employed. Quartz sand (2 g) was layered on the Petri plate (6 cm in diameter and 1 cm height) bottom, and distilled water was added to the quartz sand for maintaining relative humidity. Fifty workers, which are older than 3rd instar termites, were used. Termites were allowed to feed and dead termites were monitored daily for two weeks. Three replicates were used and filter papers treated with MeOH only were designed for control. Termite survivor and coefficient of antifeedancy (CA) were used to evaluate antifeedant activity. Termite survivor was expressed as a percentage of the number of surviving termites counted daily to the number of initial termites. CA was calculated using formula developed by Ohmura et al.,[8] with some modification and can be described as follows: | KK-EE/KK+EE | x 100 %, where KK and EE are the weight loss of the control and treated filter papers, respectively. Complete antifeedancy reaches when CA value reaches a maximum of 100 %. Moreover, the CAs are grouped into five classes as follows[8]: feeding preference, class I ($0 \leq CA < 25$), class II ($25 \leq CA < 50$), class III ($50 \leq CA < 75$), class IV ($75 \leq CA < 100$).

3. Results and discussion

3.1. Berberine from Tali kuning

Berberine isolated from Tali kuning was yellow-brownish crystal. The spectrometric data (UV, EI-MS, ^{1}H and ^{13}C-NMR) of the compounds isolated from Tali kuning were compared systematically with those data reported in the literatures.[9-12] Both spectroscopic data were consistent with the cited literatures and had similar signal patterns. Maximum absorptions of UV spectra were detected at 222, 225, 236, 264 and 347 nm, and EI-MS showed a base peak at *m/z* 336 (100) suggesting molecular ion peak of berberine ($C_{20}H_{18}NO_4$). ^{1}H-NMR shows singlet signals at δ 7.55 and δ 6.86 were assigned for H-1 and H-4 on A-ring, triplet signals at δ 3.17 (2H, t, J=6.4Hz) and δ 4.84 (2H, t, J=6.4Hz) for H-5 and H-6 on the isoquinoline B-ring. Other singlets detected at δ 9.67 and δ 8.59 for H-8 and H-13 on C-ring were also noticeable with the doublets at δ 8.01 (1H, d, J=8.8 Hz) for C-11 and δ 7.90 (1H, d, J=8.8 Hz) for C-12 on D-ring. Singlet signals at δ 4.00 (3H) and δ 4.10 (3H) were assigned for two methoxy (OCH_3) and singlet at δ 6.00 (2H) was attributable to the protons of methylenedioxy (OCH_2O). For ^{13}C-NMR signals, two methoxy signals on D-ring were shown at δ 57.67 for C-10 and at δ 62.54 for C-9. Methylenedioxy connected at C-2 and C-3 on the A-ring was detected at δ 103.66, and the C-2 and C-3 at δ 149.92 and at δ 152.17, respectively.

Methylenedioxy and methoxy are typical substituents of berberine, and structure of berberine is illustrated in Figure 1.

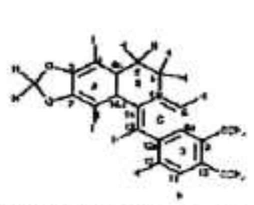
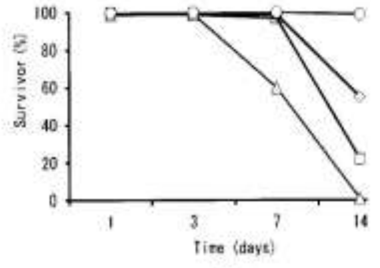


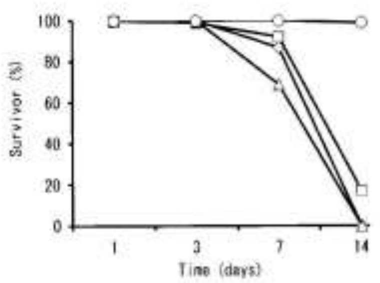
Figure 1 Structural formula of berberine in Tali kuning.

3.2. Termite survivors

Cumulative termite survivors were counted at the 14[th] day and expressed in percentage. One-way analysis of variance (ANOVA) indicates that termite survivors recorded from three samples were statistically significant ($p=0.03$), where berberine (7.4 %) is different to berberine chloride (12 %), but chloroform fraction (26.7 %) is different with the two previous compounds. Termite survivors counted from three concentration levels were also highly significant (p=0.003). The lowest survival rate of 1.4 % was recorded from the highest concentration (50 mg/ml), followed by 18.1 and 26.7 % from concentrations of 12.5 and 25 mg/ml, respectively. In addition, those termite survivors were less than that recorded from the control treatment which was of 99%. Termite survivors counted from this experiment were lower than those of the antifeedancy activity of berberine chloride from *Phellodendron amurense* against *R. speratus* Kolbe[6], the same subterranean termite as this study. These workers[6] reported that termite survivors against berberine chloride at dosages of 100 μg applied on the paper disc (8 mm in diameter) counted at 10, 20 and 30 days were 90.0, 63.3 and 30.0 %, while 300 μg dosage gave survivors of 80.0, 56.7 and 16.7 % for 10, 20 and 30 days, respectively. This is probably due to higher concentration employed in this experiment. Another possibility is that as described on the experimental section, a 3rd instar *R. speratus* Kolbe were used here, while termite nymphs were used by the previous workers.[6] Various wood extracts from Brazilian hardwoods were also active against *Nasutitermes corniger*, where alkaloidal extract from heartwood of *Bowdichia virgilioides* Kunt (Papilionoideae) at 100 mg/ml killed all termite in 4 days, and no termite were survived after day 7 at 25, 50 and 100 mg/ml exposing to ethyl acetate extract from wood of *Anadenanthera colubrina* (Vell.) Brenan Var Cebil (Griseb) Von Reist Alt (Mimosidaeae).[7]



(A)



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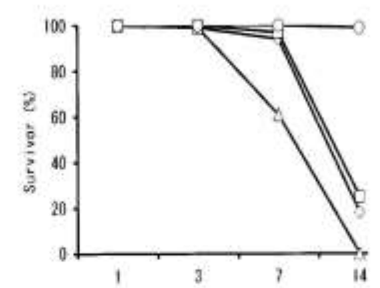

(C)

Figure 2 Survival rate for *Reticulitermes speratus* Kolbe against berberine (A), chloroform fraction (B) and berberine chloride (C). Concentrations were (◇) 12.5 mg/ml, (□) 25 mg/ml, and (△) 50 mg/ml. MeOH was used as control (○). Each point represents the mean of three replications.

In this experiment, chloroform fraction from Tali kuning was less active, where 50 mg/ml gave termite survivor of 69 and 0% at 7 and 14 day, respectively, compared to those collected from Brazilian wood extract.[6] This is probably due to lower level of the sample concentration and termite species used.

The further termite survival rates counted three times at intervals of 3,7, and 14 days, were illustrated in Figure 2A, 2B, and 2C.

As can be seen from Figure 2A-2C, three chemical substances at 50 mg/ml indicated that termite survivors decreased dramatically after 7 days. The order of termite survivors after 7 days was 60, 69, and 61 % for berberine, chloroform fraction, and berberine chloride, respectively. Finally at this concentration, no termites survived after 14 days.

3.3. Coefficient of Antifeedancy

One-way ANOVA revealed that CAs recorded from three chemical substances tested were significantly ($p=0.02$) regardless of concentration levels used, where berberine chloride (92.13 %) is different to berberine (87.34 %), but chloroform fraction (87.84 %) is not different to the other two chemical compounds. On the other hand, CA counted from three concentration levels were not significant ($p=0.6$), 88.2, 88.92, and 90.18 % for 12.5, 25, and 50 mg/ml, respectively. Further analysis using post-hoc test was conducted and the results are summarized in Table 1.

Table 1. Coefficient of antifeedancy recorded from three substances at three concentration levels against Japanese subterranean termites *Reticulitermes speratus* Kolbe

Termite species	Concentration. (mg/ml)	Coefficient of antifeedancy (%)$^{a, ab}$		
		Berberine	Chloroform fraction	Berberine chloride
Reticulitermes speratus Kolbe	12.5	86 ± 1.3^{a} (IV)	85 ± 0.5^{a} (IV)	94 ± 1.7^{ab} (IV)
	25	88 ± 4.8^{a} (IV)	89 ± 0.6^{a} (IV)	89 ± 3.7^{a} (IV)
	50	88 ± 1.6^{a} (IV)	90 ± 0.8^{a} (IV)	93 ± 1.1^{ab} (IV)

^{a}Mean (n=3) using 50 termites per replicate. Values followed by different letter (a-b) are highly significant following the Fisher's least significant difference (LSD) ($p<0.01$). Brackets represent the class of CA values.

Table 1 shows that means for CA recorded from naturally occurring constituents in Tali kuning (berberine and chloroform fraction) gave similar mean values, following Fisher LSD post-hoc test (a), compared to the berberine chloride (a and ab). The CA values recorded from this experiment (Table 1) and those reported in the published paper[8] have the similar grade of class IV, indicating strong antifeedancy. It is because the CA values for strong antifeedancy fall into $75 \leq CA \leq 100$. They reported that three flavonoids at 16 μl permeated into the paper disc (13 mm in diameter) had antifeedant activity against subterranean termite *C. formosanus* Shiraki, and gave the CA values of 81, 77.1, and 76.8 % for quercetin, taxifolin and naringenin, respectively.[8] The CA values reported in this study were higher (87, 88, and 92 % for berberine, chloroform fraction and berberine chloride, respectively) than those in the paper.[8]

Naturally occurring substances are rich in bioactive compounds that offer, either jointly or independently, toxicity, feeding deterrence or repellence,[13] even though the exact mechanism by which wood extractives kill, deter or repel termite is still unclear.[7] Various naturally occurring constituents, such as alkaloids,[6,13] flavonoids,[8] essential oils,[2-3] and wood extractives,[7] have been reported to be active against subterranean termites *R. speratus* Kolbe, *C. formosanus* Shiraki and *N. corniger*, respectively. They could be useful alternatives for antitermitic pesticides, because they are renewable, environmentally safe, and less toxic to the other creatures.

4. Conclusion

A strong antifeedancy compound in Tali kuning (*T. dissitiflora* Diels) was isolated, and structural analyses elucidated it was berberine. Naturally occurring constituents, berberine and the chloroform fraction from Tali kuning showed strong antifeedancy against Japanese subterranean termite *R. speratus* Kolbe, and these constituents could be used as alternative substances for termite control.

5. References

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