Original Research Paper

Determination of 1-Deoxynojirimycin Content and Phytochemical Profiles from Young and Mature Mulberry Leaves of *Morus* Spp.

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Abstract: Mulberry leaves have been known to be rich in 1-Deoxynojirimycin (DNJ) which inhibits postprandial hyperglycemia by inhibiting α-glucosidase in the small intestine and thereby reducing the breakdown of starch and other complex sugar into glucose. Mulberry leaf extract also contains many other bioactive compounds and phytochemicals. They are very effective in supporting blood sugar levels and balancing cholesterol and triglycerides. The DNJ content in mulberry leaves depends on the leaf's variety and age. In this study, the phytochemical profile and DNJ content of young leaves and mature mulberry leaves in Indonesia were determined to further promote mulberry use using High Performance Liquid Chromatography (HPLC) and Pyrolisis Gass Chromatography-Mass Spectrometry (GC-MS). The phytochemicals test result for the four leaf samples showed that all four samples contain alkaloids, phenolics, flavonoids, tannins and terpenes. The mature leaves of *M. cathayana* contain the highest DNJ concentration (2.90 mg DNJ/g leaves) out of eight samples. DNJ has the potential to be used as a functional food for diabetic patients. Analysis using HPLC and Py-GC-MS confirmed that mulberry species contain many beneficial phytochemicals. This study presented that Indonesian mulberry leaves extracts to have high phytochemical compositions which very potential utilized in the medicinal field.

Keywords: 1-deoxynojirimycin, Mulberry, *Morus* spp., GC-MS, HPLC

Introduction

Mulberry is a plant from *Moraceae* family and *Morus* genus. Mulberry can grow in a wide variety of climate, from temperate to tropical, so this plant is cultivated in many regions, including Indonesia. Outside from its use as silkworm's feeds, mulberry leaves also used in traditional medicine because of its therapeutic effect (Ozgen *et al*., 2009) and pharmacology benefit (Song *et al*., 2009; Zafar *et al*., 2013).

In the medicinal field, many countries in Asia use mulberry leaves as a treatment for diabetes. The anti-diabetic activity of the mulberry leaves already verified through studies with animal experiments and human trials (Hunyadi *et al*., 2013). This activity comes from various bioactive constituents inside the mulberry leaves, with the one that contributes most for anti-diabetic effect is 1-deoxynojirimycin (DNJ) (Kimura *et al*., 2007).

DNJ is an alkaloid iminosugar, an analogue of glucose with -NH group substituting the oxygen atom on the pyranose ring. DNJ showed a high α-glucosidase inhibitory activity; therefore, it can be used to control the post-prandial blood glucose level on consumption (Hu *et al*., 2013; Liu *et al*., 2012). Beside the anti-diabetic effect, DNJ was also reported to have antioxidant, antimicrobial and anti-inflammatory activities (Yin *et al*., 2010).

Based on previous studies, it is found that DNJ content in mulberry leaves may vary in different species. The DNJ content also affected by other factors such as the age of the leaves and the growth environment (Hu *et al*., 2013; Yatsunami *et al*., 2008). Knowing this fact, this study aimed to further promote the use of mulberry leaves in Indonesia by determining DNJ content and the phytochemical profile of the mulberry leaves of *Morus alba* var. *kanva-2*, *M. alba* var. *multicaulis*, *M. bombycis* var. *lembang* and *M. cathayana* that was cultivated in Indonesia.

Materials and Methods

Plant Material

The mulberry leaf samples were collected from the University Farm of IPB University, Sukamantri, Bogor,

Indonesia. A total of eight samples from four mulberry varieties were selected. The samples were the young leaves and mature leaves of *M. alba* var. *kanva-2*, *M. alba* var. *multicaulis*, *M. bombycis* var. *lembang* and *M. cathayana*. The young and mature leaves were classified based on branch position. The first leaves and the fifth leaves from branch apex were classified as young leaves and mature leaves respectively. All leaf sample was lyophilized and ground to a powder before analysis.

Sample Extraction

Procedures of sample extraction followed the description by Hu *et al.* (2013) and Vichasilp *et al.*, (2009). Ninety milligrams of each sample was mixed with 2.5 mL 0.05 M HCl, followed by sonication using ultrasonicator at 180 W and 40.000 Hz for 10 min. The solutions then centrifuged at 17.000 g for 15 minutes. Initial supernatants were collected and the residues were re-extracted as described above. Both supernatants then pooled and diluted with distilled water to a final volume of 6 mL.

Preparation of DNJ Stock Solution

Five milligrams of standard DNJ (Sigma Aldrich, USA) was mixed with 500 µL distilled water to obtain the DNJ solution of 10 g/L. Seventy-five microliters of this solution was then added to 5 mL 0.05 M HCl and diluted with distilled water to a final volume of 10 mL. DNJ concentration of the stock solution was 75 mg/L. The stock solution was stored at 4°C until further use.

Derivatization of DNJ

Procedures for DNJ derivatization conducted by Kim *et al.* (2003). Ten microliters of extract sample of DNJ standard solution was mixed with 10 µL 0.4 M potassium borate buffer (pH 8). Twenty microliters of 9-Fluoroenylmethyl Chloroformate (FMOC-Cl) 5 mM in acetonitrile was added, the solution then vortexed for 30 seconds and incubated at 25°C for 20 min. After that, 10 µL 0.1 M glycine was added to stop the reaction. The solution was diluted with 950 µL 0.1% acetic acid to stabilize DNJ-FMOC and filtered using nylon syringe filter (0.45 µm). Ten microliters of the filtrate was injected to HPLC to determine the DNJ concentration.

Preparation of Standard Solution

DNJ stock solution was derivatized according to the method described in DNJ derivatization. The derivatized stock solution was then diluted with distilled water to make standard solutions with a concentration of 2.28, 4.56, 9.13, 18.25, 37.5 and 75 mg/L. The standard solutions were analyzed by HPLC and the relationship between peak area on chromatogram with DNJ concentration was used to construct a standard curve (Hu *et al.*, 2011).

Qualitative Phytochemical Tests

The phytochemicals tested for its presence were alkaloids, phenolics, flavonoids, tannins and terpenes. For those tests, four drops of each sample were added with reagents corresponding to each tests on the spot plate.

HPLC Analysis

The derivatized extracts of all eight samples and standard solutions were analyzed with HPLC to determine their DNJ content. HPLC analysis was done using acetonitrile-acetic acid 0.1% (v/v) as the mobile phase with 1:1 ratio at 1.0 mL/minutes elution rate for 14 minutes, silica C18 column at ambient temperature as stationary phase and VWD detector set at 254 nm. After each analysis, the column was washed using methanol for 16 minutes to remove impurities on the column and equilibrated with mobile phase for 10 min (Kim *et al.*, 2003). All the chemicals used in the HPLC analysis were of HPLC grade and the water used was double distilled. The HPLC analysis of each sample was done in triplicate. The data presented were the mean ± Standard Deviation (SD).

Py-GC-MS Analysis

Ground leaf samples were analyzed using Py-GC-MS instrument QP2010 (Shimadzu, Duisburg). The stationary phase was capillary column (Phase Rtx-5MS) 60 m ×0.25 mm ID and the mobile phase was helium gas. Pyrolysis temperature was set at 400°C (Nandagopal *et al.*, 2014).

Results and Discussion

Qualitative Phytochemical Tests

The result of the qualitative phytochemical tests of eight mulberry leaf samples is presented in Table 1. This result revealed that alkaloids, phenolics, flavonoids, tannins and terpenes are present in all eight leaf samples.

Phytochemicals are bioactive compounds that mainly found in plants. Phytochemicals can be divided into a few major groups, which are alkaloids, phenolics and terpenes (Niratker *et al.*, 2014). The result of preliminary qualitative tests in this study shows that all mulberry leaf samples contain all major groups of phytochemicals, which are alkaloids, phenolics, flavonoids, tannins and terpenes. The phytochemicals in the mulberry leaves are responsible for its therapeutic effect in the medicinal field.

In the plant, terpenoid compound used to attract insect (Wadood *et al.*, 2013). From recent studies, it was discovered that this compound mostly responsible for this effect is some iminosugars and phenolics compounds (Hunyadi *et al.*, 2013; Liu *et al.*, 2012). These various phytochemicals contain in the mulberry leaves can be further studied to determine its physiological actions on the human and can be seen as a potential source of useful drugs.

Table 1: Phytochemical test results of leaf samples

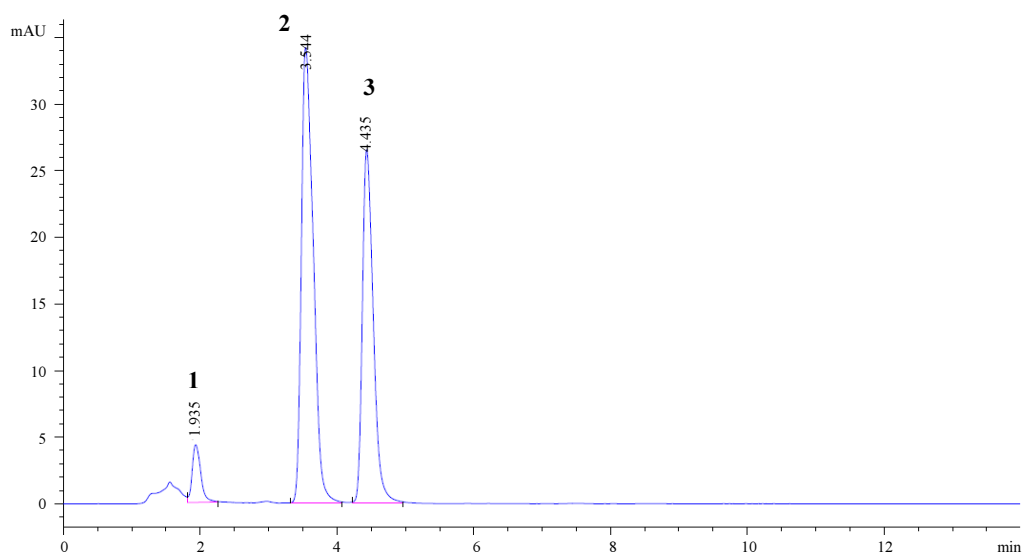| Phytochemicals	*M. alba* var. *kanva-2*		*M. alba* var. *multicaulis*		*M. bombycis* var. *lembang*		*M. cathayana*	
	Young	Mature	Young	Mature	Young	Mature	Young	Mature
A. Alkaloids								
Wagner's test	+	+	+	+	+	+	+	+
Mayer's test	+	+	+	+	+	+	+	+
B. Phenolics								
FeCl$_3$	+	+	+	+	+	+	+	+
C. Flavonoids								
NaOH test	+	+	+	+	+	+	+	+
D. Tannins								
Pb-acetate test	+	+	+	+	+	+	+	+
E. Terpenes								
Liebermann-burchard's test	+	+	+	+	+	+	+	+

Fig. 1: Chromatogram standard curve. Number 1 represents FMOC-OH; 2) FMOC-DNJ; 3) FMOC-Gly

DNJ is one of the iminosugars found in the mulberry leaves. Many of the iminosugars show inhibitory activity against glucosidases, so they have been receiving great attention in the pharmaceutical industry. DNJ is a potent α-glucosidase inhibitor while also having antioxidant, antimicrobial and anti-inflammatory activity (Hu *et al.*, 2013). It has an excellent α-glucosidase inhibitory activity in vitro and is suitable to use as a functional food to treat diabetic patients (Kim *et al.*, 2003). Thus, it is necessary to find a good source of DNJ since DNJ has high potential to overcome diabetic.

HPLC Analysis

The standard solutions were analyzed by HPLC and the results obtained were used to construct a standard curve (Fig. 1). The equation from the standard curve was then used to calculate the actual DNJ concentration in the leaf samples.

The chromatogram from HPLC analysis of leaf samples is shown on Fig. 2. The DNJ retention time is around 3.5 min.

The result of DNJ determination by using HPLC is presented in Table 2. The result showed that the highest DNJ content is in mature *M. cathayana* leaves. The result also showed that the mature leaves of all samples contain higher DNJ concentration.

The mulberry leaves are known to have high DNJ content. From the eight leaf samples analyzed, the DNJ content greatly varies between leaf samples. This is because the mulberry varieties and the age of mulberry leaves could affect DNJ concentration (Hu *et al.*, 2013; 2011). In this study, *M. cathayana* leaves have the highest DNJ content over all sample with a concentration of DNJ in the mature leaves of *M. cathayana* contain DNJ as high as 2.90 mg DNJ/g leaves. For comparison, the DNJ content of the mulberry leaves also has been determined in other studies.

Table 2: DNJ determination of leaf samplesA

Leaf samples	DNJ Concentration (mg DNJ/g leaves) ± SD
Morus alba var. *kanva-2*	
Young leaves	0.07±0.03
Mature leaves	0.27±0.08
M. alba var. *multicaulis*	
Young leaves	0.077±0.018
Mature leaves	0.157±0.058
*M. bombyci*s var. *lembang*	
Young leaves	0.136±0.039
Mature leaves	1.413±0.023
Morus cathayana	
Young leaves	0.94±0.11
Mature leaves	2.90±0.57

The studies on China mulberry varieties got the DNJ content ranging from 0.10-1.60 mg DNJ/g leaves (Hu *et al.*, 2013), while studies on Thailand mulberry varieties were found DNJ content ranging from 0.30-3.20 mg DNJ/g leaves (Vichasilp *et al.*, 2009). This makes the DNJ content of mature *M. cathayana* is quite high compared to the DNJ content of another mulberry varieties. All of the mature leaves also have higher DNJ content young leaves, which differ with another study that found that DNJ content is higher in young leaves than in mature leaves (Hu *et al.*, 2011). This is possible because of the difference in variety and growth environment of the mulberry. However, the definite biosynthesis pathway of DNJ is still not known (Yatsunami *et al.*, 2008).

HPLC chromatograms showed a different pattern for the young and mature leaves from all species in this study. This means that the nitrogen-containing compound from the young and mature leaves of those species are different since the FMOC derivatizes the primary and secondary amine containing a compound in the samples (Kim *et al.*, 2003). Interestingly, some other peaks arose in particular both *M. bombycis* var. *lembang* young and mature leaves on Fig. 2E and 2F. These peaks might represent the other constituents such as limonene, benzoic acids, flavonoid derivatives, anthocyanins and polyphenols. Mulberry leaves are believed can be used as potential food materials due to their nutritional and functional components (Yu *et al.*, 2018). Mulberry is also known to have fatty acid (linolenic acid, palmitic acid, oleic acid) content. Moreover, many volatile constituents also found in mulberry, such as acetic acid, ethyl butyrate, 3-hydroxyl-2-butanone, ethyl acetate, 3-methyl butanal, 2-methyl butanal, heptanal, hexanal, methional, trans-2-hexanal, 2-octenone, hexanoic acid, benzaldehyde, methyl hexanoate, 2-ethylhexanal, octanal, ethyl hexanoate, limonene and ethyl decanoate (Zhang *et al.*, 2018). All HPLC chromatogram showed the highest peak on retention time at 4.4 minutes, where this peak was presumed to belong l-limonene.

These constituent compositions may vary according to different ecological conditions (Yu *et al.*, 2018).

Py-GC-MS Analysis

The analysis of leaf samples using Py-GC-MS gives a pyrogram which detect many organic compounds. From all the organic compounds detected, the phytochemicals that were found are listed in Table 3. All the phytochemicals were in terpenes group. Mature leaves of all samples give a wider variety and higher amount of terpenes with this analysis.

Py-GC-MS is a powerful technique to analyze the component of the samples. It only needs a minute amount of sample and the sample does not need to be derivatized before analysis. With the pyrolysis, the sample component will either flash vaporized if the molecule is volatile and thermally stable or degraded into lighter molecules if the molecule is large and hard to volatilize (Montalbani, 2012). The pyrolysis of the eight leaf samples were done to see whether the phytochemicals in the samples can be detected by this method. The results showed that only a few simple terpenes on each sample were successfully analyzed. The simple terpene is easier to volatilize because it is lack of polar group, while the other phytochemicals like alkaloids and phenolics mostly contain amide and hydroxyl group respectively, making them more polar and harder to volatilize (Kusch, 2012). The other phytochemicals present in the sample are degraded and detected as a simpler organic molecule in the pyrogram, so it is not possible to analyze the whole phytochemicals content of the mulberry leaf samples using this technique. The terpenes found on the leaf sample pyrograms cannot be treated as total terpenes contents since it is unknown if all the terpenes are successfully volatilized or not. The volatile terpenes detected in the mulberry leaves like chamigrene and farnesene may play a role in the leaves defense mechanism against the pathogen (Courtois *et al.*, 2012). But, some of the terpene fractions found in the sample have a beneficial effect. The phytol is known to have antimicrobial properties as phytomedicine (Rajab *et al.*, 1998) and the limonene is known to have anticancer properties (Zheng *et al.*, 1992).
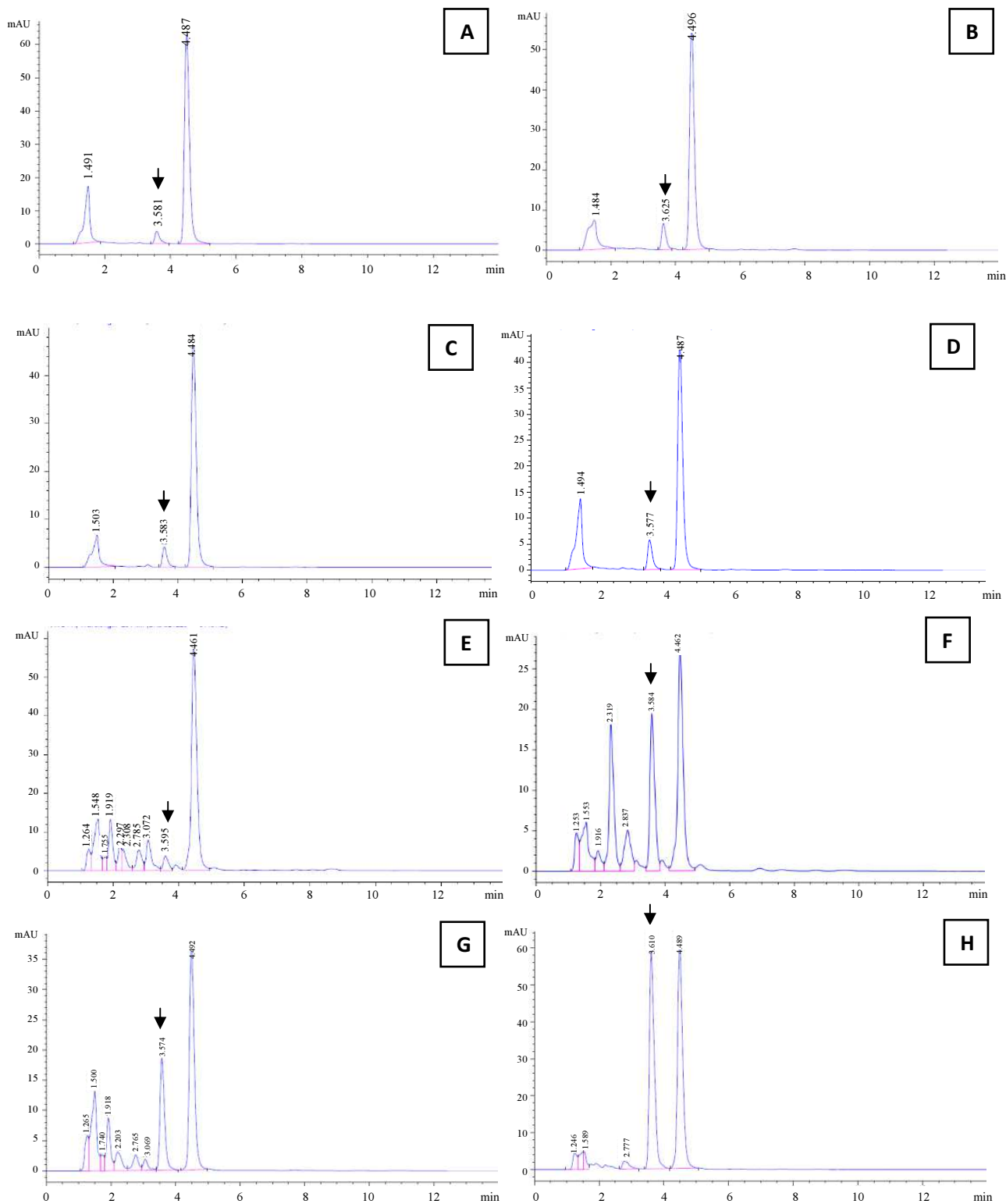
Fig. 2: Chromatogram of leaf samples: (A) *M. alba* var. *kanva-2* young leaves (B) *M. alba* var. *kanva-2* mature leaves (C) *M. alba* var. *multicaulis* young leaves (D) *M. alba* var. *multicaulis* mature leaves (E) *M. bombycis* var. *lembang* young leaves (F) *M. bombycis* var. *lembang* mature leaves (G) *M. cathayana* young leaves (H) *M. cathayana* mature leaves. DNJ 's peaks showed by the arrow

Table 3: Secondary metabolite constituents from Py-GC-MS analysis

Leaf samples	Secondary metabolites	Class	Concentration
Young *M. alba* var. *kanva-2*	Limonene	Monoterpenes	1.12%
	Phytol	Diterpenes	0.69%
Mature *M. alba* var. *kanva-2*	Phytol	Diterpenes	8.16%
	Limonene	Monoterpenes	6.33%
	Citronella	Monoterpenes	1.87%
Young *M. alba* var. *multicaulis*	Neophytadiene	Sesquiterpenes	4.52%
	Limonene	Monoterpenes	13.53%
Mature *M. alba* var. *multicaulis*	Dihidrocarveol	Monoterpenes	0.65%
Young *M. bombycis* var. *lembang*	N/A*		
Mature *M. bombycis* var. *lembang*	Limonene	Monoterpenes	5.58%
	Cycloundecene	Sesquiterpenes	0.44%
	Neophytadiene	Sesquiterpenes	3.81%
Young *M. cathayana*	N/A*		
Mature *M. cathayana*	Limonene	Monoterpenes	14.24%
	Phytol	Diterpenes	3.24%
	Chamigrene	Sesquiterpenes	0.81%
	Farnesene	Sesquiterpenes	0.73%

*Not Available

However, the root rot may threat the mulberry plant. It has been reported that *Macrophomina phaseolina* (Tassi) *Goid* causes charcoal rot which has the predominant pathogen to which all productive mulberry cultivars. Thus, controlling the quality of mulberry plant may maintain the phytochemical constituents in mulberry (Pinto *et al.*, 2018). Limonene also detected in *M. bombycis* var. *lembang* mature leaves, 1-limonene and cyclododecane are secondary metabolites are known to have antimicrobe, antiviral, immunosuppressant, antiallergic, skincare and trigger hair growth (Nahbar *et al.*, 2012). Previous studies showed that mulberry plants have potential pharmacological properties as antidiabetes by naturally inhibiting α-glucosidase activity. Liu *et al.* (2015) demonstrated that mulberry leave extracts presented Ki value was 56.53 mg/mol as a competitive inhibition mode against α-glucosidase. However, the other parts of mulberry are also have potential antidiabetic effect as well. Thus, it suggested that mulberry plant is a good nutritional source as natural antidiabetic agent (Liu *et al.*, 2015).

Conclusion

In the course of the research, all samples contain various phytochemicals, including alkaloids, phenolics, flavonoids, tannins and terpenes that can be beneficial to our health, which DNJ as one of them.

DNJ is known to have an antidiabetic effect. The mature leaves of *M. cathayana* contain the highest DNJ of all the eight samples used (2.90 mg DNJ/g leaves) and have the potential to be used as functional foods for diabetic patients. The mulberry leaves also still contain many beneficial phytochemicals to be further discovered.

Author's Contributions

All authors participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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