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Natural alkaloid DNJ in mulberry and its application: An overview

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Abstract
The mulberry is mainly cultivated for its foliage which is the sole food for the silkworm *Bombyx mori* L. The polyhydroxylated piperidine alkaloid 1-DNJ is attracting attention due to its important biological function and it is the main active component in mulberry plant. However, the natural alkaloid DNJ content in mulberry attains importance because of its utility in various fields. Naturally it exists as a secondary metabolite product in plants, insects and in some microbial strains. But, the content of DNJ in plants and insects is relatively low. The present review summarizes the various sources of DNJ with special emphasis on mulberry, extraction, purification methods of DNJ and attempt is made to compile the available information regarding biosynthesis, candidate genes involved and factors influencing in its biosynthesis. 1-Deoxynojirimycin (DNJ) has its wide application as an anti-diabetic, antioxidant, anti-inflammatory, anti-aging and anti-obesity substance.

Keywords: Natural alkaloid DNJ mulberry is mainly cultivated for its foliage polyhydroxylated

Introduction
Morus, popularly known as mulberry belongs to the family Moraceae. It is a perennial shrub or tree representing an extremely important economic plant and its foliage is used in sericulture as the sole diet for the monophagous silkworm, *Bombyx mori* (Wang *et al.*, 2017) [52]. Three best known species of this group are *Morus alba*, *Morus rubra* and *Morus nigra*. This species is a monoecious - or dioecious plant of small- to medium-size, widely distributed in India, China, Japan, North Africa, Arabia, and Southern Europe as well as among other regions. Besides as food for silkworm, mulberry leaves are also been used in traditional Chinese medicine to treat some diseases since the *Morus species* is rich source of phenolic compounds, including flavonoids and anthocyanins, of great biological, pharmacological, and structural interest because of their antioxidant properties (Kumar and Chauhan, 2008) [31].
Several new functional food products that contain mulberry leaves have been manufactured in China, Japan, Korea and other countries (Asano *et al*., 2001) [5]. Korean scientists developed ice-cream containing mulberry leaf powder with functionality and palatability, and demonstrated that it could decrease blood glucose levels after consumption (Kim *et al*., 1999) [26]. Traditionally, the species are used for the prevention of liver and kidney diseases, joint damage, and anti-aging, due to their antioxidant properties (Mena *et al*., 2016) [39]. In addition, it has been shown to be an ally in the treatment of type 2 diabetes mellitus (DM2), due to its hypoglycaemic effects (Sanchez *et al.*, 2017) [45]. Further studies revealed that DNJ and its derivatives could inhibit hepatitis B (Mehta *et al*., 2013; Lazar *et al*., 2007) [38, 33] and hepatitis C (Durantel *et al*., 2001; Chapel *et al*., 2007) [15, 10], as well as glycosphingolipid storage disorders such as Gaucher disease (Cox *et al*., 2000) [12]. Iminosugars are monosaccharide analogues in which the ring oxygen has been replaced with an imino group. These iminosugars can inhibit α-glucosidase activity because of their structural resemblance to the sugar moiety of the natural substrate. As a result of this, many potential biological activities, such as anti-diabetic, antiviral, and anticancer effects, are associated with these naturally occurring iminosugars (Asano 2003) [6]. In the past few decades, different iminosugar compounds have been isolated and identified, including iminopyranoses, iminofuranoses, and tropane alkaloids.

Deoxy nojirimycin
The 1-Deoxynojirimycin or azasugar, a representative of iminopyranose alkaloid, was first reported in 1976 in the root bark of *Morus* species and named as moranoline (Yagi *et al.*, 1976) [58]. Till date more than 20 polyhydroxy alkaloids were identified from mulberry and also in silkworm.

DNJ and its derivatives were isolated from many plants and microbes including mulberry and found highest in mulberry compared to other plants (Asano *et al*., 2000: Qin-Xue *et al*., 2013) [4, 43]. 1- Deoxyiminosugars are chemically more stable than normal iminosugars because of absence of a hydroxyl group at the C_1 positions (Fig1). 1-Deoxynojirimycin belongs to the group of piperidine ring alkaloids derived from lysine (Robinson, 1917) [44] and are D-glucose analogues with an NH group substituted for the oxygen atom of the pyranose ring. Deoxynojirimycin is white in colour soluble in water and dimethyl sulfoxide, melts at 195 – 196°C with a Density 1.456 g/cm^3, Molar Mass is 163.173±0.06g/mol and Boiling point of 361.1±42.0 °C (Wang *et al*., 2017) [52].

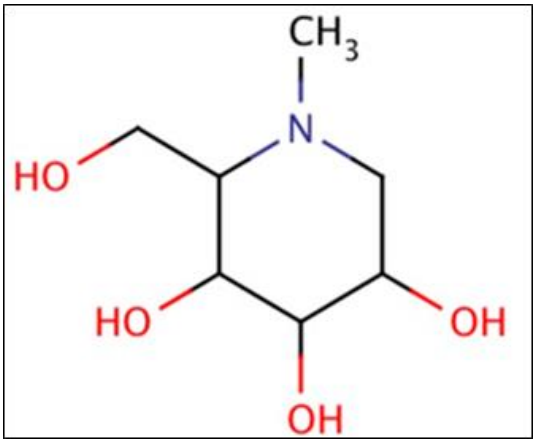


Fig 1: Structure and molecular formula of DNJ: $C_6H_{13}NO_4$

Sources of DNJ

In addition to mulberry, other plants also contain DNJ such as *Hyacinthus orientalis.* In this plant DNJ was extracted from the bud part by water: methanol (50:50) method and detected 31 mg of DNJ from 7.6 kg wet weight of bud. Kim *et al*. (1999) [26] obtained a pyrrolidine alkaloid and four other piperidine alkaloids from *Commelina communis* but its content was only 0.011%. In *Adenophora triphyllavar* var. japonica, DNJ as well as several other alkaloids were extracted (Fig. 2).

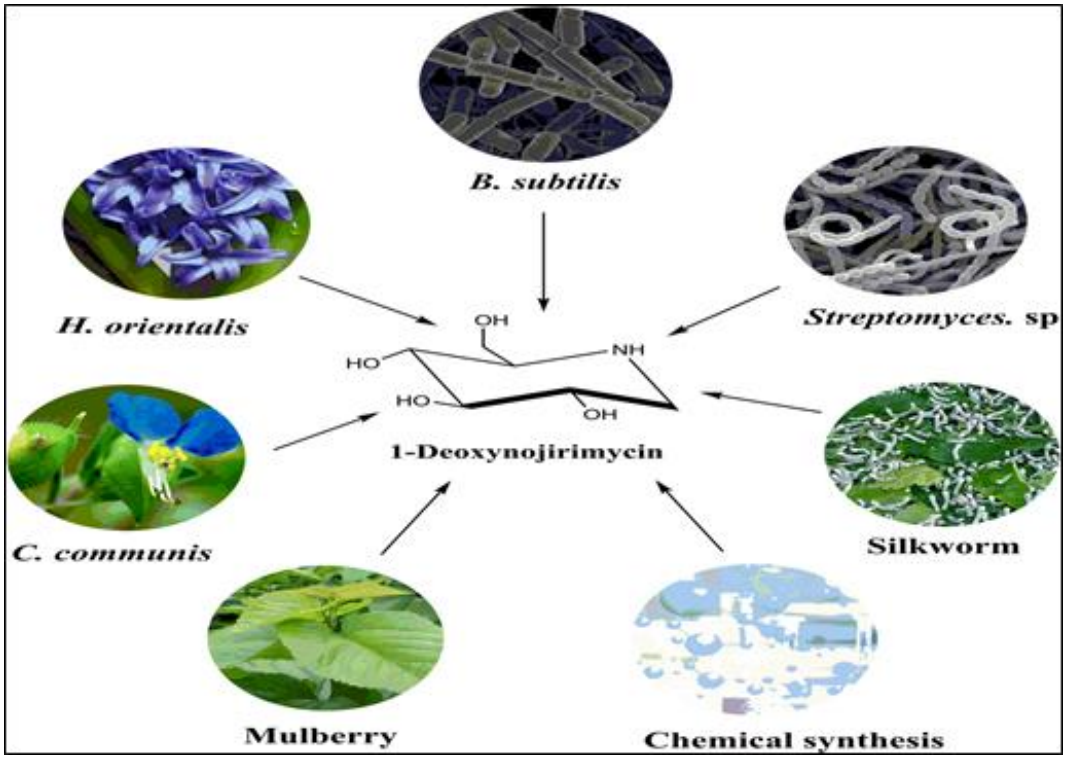


Fig. 2: Various sources of DNJ

Apart from plants the DNJ was also isolated from the lepidopteran insect, *Bombyx mori* and found in some strains of *Bacillus subtilis* (DSM704) and *Streptomyces species*. Similarly, Ezure *et al*. (1985) [16] isolated *Streptomyces lavendulae* (GC-148) from the soil and found about 4,200 mg/mL of DNJ in the culture filtrate. The findings clearly indicate that the natural source of DNJ was very low, making it too difficult to obtain a large quantity by traditional extraction methods. At present, DNJ and its derivatives were mainly obtained by chemical synthesis and the combination of chemical synthesis and microbial transformation (Song and Hollingsworth, 2000; Danieli and Murphy, 2001) [50, 13].

Biosynthesis of DNJ in mulberry

The two main biosynthesis pathways of lysine, involving more than 32 enzymes, were identified using the Kyoto

Encyclopedia of Genes and Genomes (KEGG) database. They are:

1) Diaminopimelic acid (DAP) pathway

This is utilized by most of the bacteria and green plants to produce lysine (Bhattacharjee, 1985) [8], and it begins with L-aspartate as the reaction substrate.

2) a-aminoadipate (AAA) pathway

In this pathway, with α-ketoglutarate as the initiator, is preferentially used by fungi, euglena, and some bacteria (Kosuge and Hoshino, 1998) [30] and Mulberry. Only two enzymes have been identified in the biosynthesis of piperidine rings: lysine decarboxylase (LDC) (Gale and Epps, 1944) [17] and primary-amine oxidase (AOC) (Wilce *et al*., 1998) [56]. Lysine is first converted to cadaverine through the catalytic effect of LDC, followed by the AOC-catalysed formation of a piperidine ring structure and subsequent multi-step reactions to produce the corresponding products (Fig 3). The conversion of the piperidine ring to DNJ in the biosynthetic pathway is still unclear. DNJ has a piperidine ring structure with a hydroxymethyl group at position 2 and hydroxyl groups at positions 3, 4, and 5. Thus, DNJ is possibly formed through the methylation and hydroxylation of the 1-piperidine structure by methyltransferases and cytochrome P450 (CYP450) enzymes. The CYP450 enzymes exist in plant cells in both soluble and membrane-bound forms and are widely involved in plant secondary metabolic reactions, including hydroxylation, alkylation, and alkenyl epoxides, hydrocarbon oxidation, and dealkylation of nitrogen, sulphur, oxygen sites, and hydroxylation and oxidation of nitrogen sites (Wang *et al*., 2018) [53].

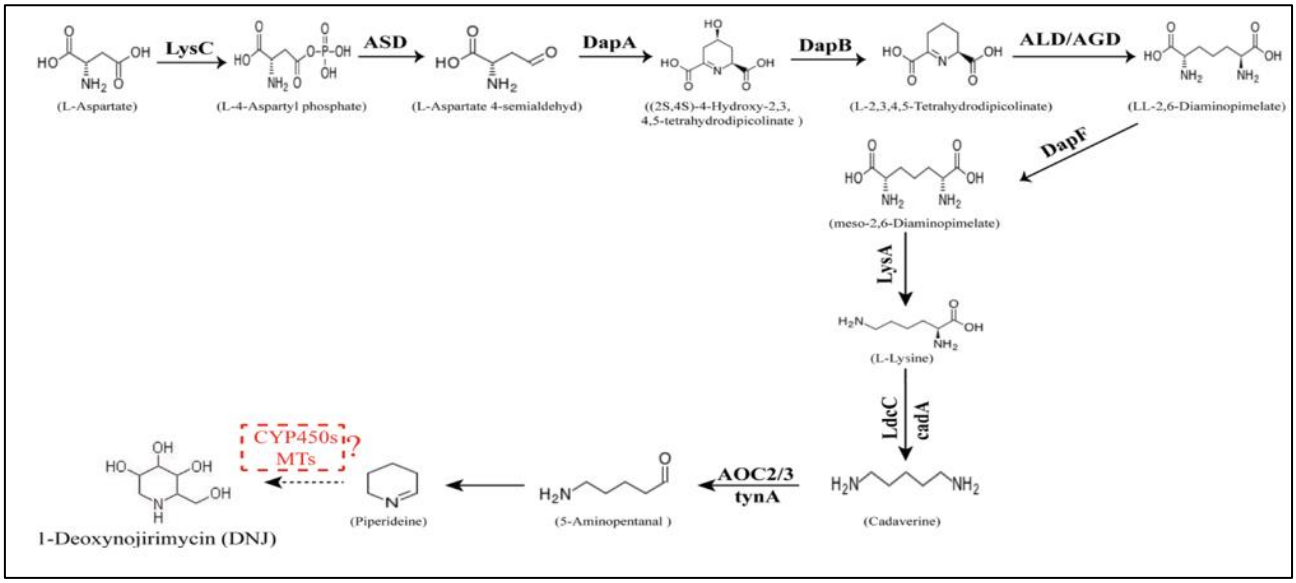


Fig 3: Pathway for the biosynthesis of DNJ alkaloids in mulberry (*Morus alba* L.).

LysC, aspartate kinase; ASD, aspartatesemialdehyde dehydrogenase; DapA, 4-hydroxy-tetrahydrodipicolinate synthase; DapB, 4-hydroxy-tetrahydrodipicolinate reductase; ALD/AGD, LL-diaminopimelate aminotransferase; DapF, diaminopimelate epimerase; LysA, diaminopimelate decarboxylase; LdcC/cadA, lysine decarboxylase; AOC2/3, primary-amine oxidase; CYP 450s, cytochrome P450s; MTs, methyltransferases.

Candidate genes involved DNJ biosynthesis in mulberry

A DNJ alkaloid biosynthetic pathway was outlined on the basis of differentially expressed transcripts and KEGG pathway assignments. In this pathway Lysine was generated using aspartic acid as a substrate, and then the lysine was used as a substrate to generate the DNJ alkaloid. Ten enzymes, encoded by 38 genes, were annotated in the Table 2. A total of 38 unique candidate genes were identified as being involved in DNJ alkaloid biosynthesis in mulberry and 9 unique genes had significantly differential expression. The expression level of 3 key genes encoding LDC and AOC were significantly correlated with DNJ content. 5CYP 450s, 2MTs, genes were significantly associated with DNJ content suggesting that 1- piperdine catalysed by CYP 450s enzyme and MTs to DNJ alkaloid (Wang *et al*., 2018) [53].

Table 1: Candidate genes related to DNJ alkaloid biosynthesis in mulberry

Gene	***Enzyme***	***No. All[a]***	***No. Up[b]***	***No. Down[c]***
LysC	aspartate kinase	10	0	2
ASD	aspartate-semialdehyde dehydrogenase	2	0	0
DapA	4-hydroxy-tetrahydrodipicolinate Synthase	4	0	1
DapB	4-hydroxy-tetrahydrodipicolinate Reductase	1	0	0
ALD/AGD	LL-diaminopimelate aminotransferase	3	0	1
DapF	diaminopimelate epimerase	3	0	0
Lys A	diaminopimelate decarboxylase	2	0	0
LdcC	Lysine decarboxylase	1	0	1
AOC2/3	primary-amine oxidase	12	1	3
Total number of transcripts		**38**	**1**	**8**

Note: (**a**)The total number of transcripts related to DNJ biosynthesis. (**b**)The number of transcripts with up regulated expression significantly in mulberry samples M7 and M11**. (c)**The number of transcripts with down regulated expression significantly in mulberry samples M7 and M11.

Factors influencing the biosynthesis of 1-deoxynojirimycin in mulberry

DNJ biosynthesis is highly influenced by weather conditions and other properties of the growing areas of the host plants *viz*, Mulberry varieties, geographic locations, soil properties, meteorological factors and harvesting time (Lou *et al.*, 2011) [36].

Varieties

Bajpai and Baskar Rao (2014) [7] screened four mulberry varieties of south India to quantify the 1-deoxynojirmycin in different varieties namely K-2, S-13, S-34, and V-1(Fig 3). The DNJ content in different mulberry varieties ranged from 0.68-2.78 mg/gm. The order of 1-deoxynojirimycin concentration in varieties was K-2 > S-13 > S-34 > V-1 (Fig.4). Though V-1 variety was developed by breeding methods (interspecific hybridization of S30 x Ber C776). It contains less DNJ when compared to the K-2 variety which exhibited highest content of 1-deoxynojirimycin, and was developed by natural selection (OPH). Study also coincide with values obtained by other researchers from different varieties and in different countries (Kimura *et al.*, 2007; Yatsunami *et al*., 2008; Wei, *et al.*, 2009; Kefei *et al.*, 2011 and Qin-Xue *et al.*, 2013) [28, 61, 55, 25, 43]. The DNJ content observed that the different Chinese mulberry leaves ranged from 1.57 to 3.48 mg/gm (Wei, *et al.*, 2009) [55]. In general, the results revealed that DNJ content was less in Indian varieties as compared to the Chinese varieties.

Chaluntorn *et al*. (2012) [11] observed leaf position in plant is also a factor responsible for DNJ concentration, and the DNJ content was highest in shoots followed by young leaves and mature leaves. Wei-qi (2006) [54] studied variation in mulberry plant organs and the sequence of concentration was observed as Branch Phloem> Leaves > Branch Xylem and the DNJ concentration was ranging from 0.40-4.9 mg/gm when recorded with the help of HPLC-PDA.

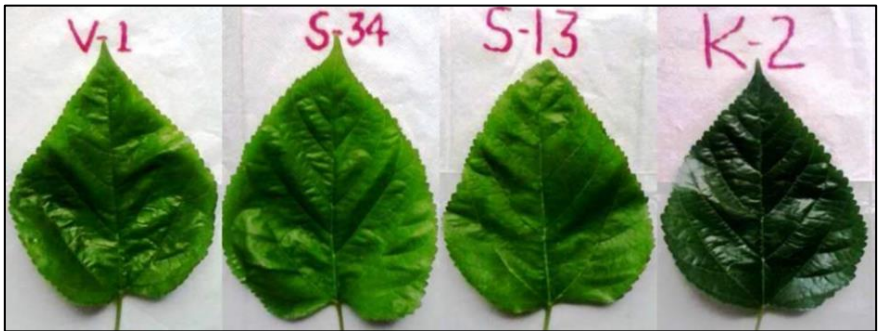


Fig 3: Leaves of Mulberry Varieties V-1, S-34, S-13 and K-2

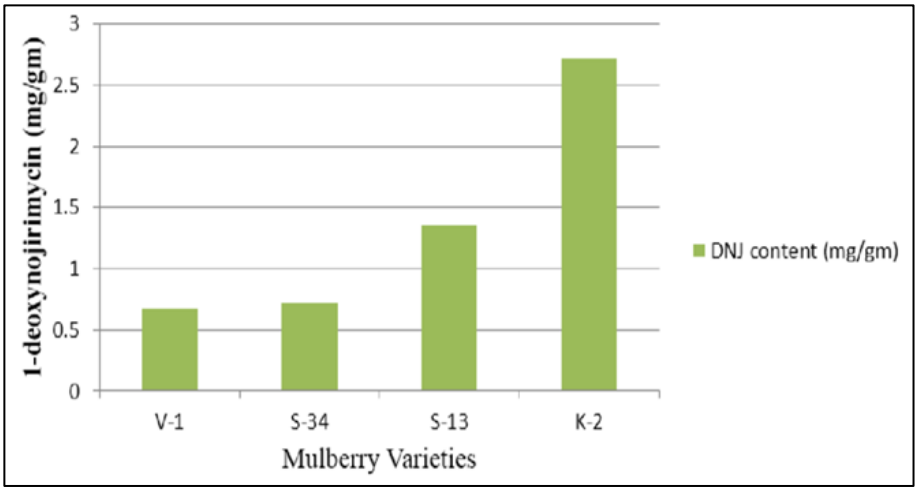


Fig 4: Deoxynojirimycin content of mulberry leaves in four varieties

Geographical location and Characteristics of soil

Lou *et al*. (2011) [36] observed a positive correlation with latitude and mulberry DNJ content. The higher latitude matches a higher content of DNJ (Table 2). The pH value and mineral element content of the soil from the same mulberry fields when analysed revealed that the alkaline soil was more suitable to the biosynthesis of DNJ. The DNJ content and total nitrogen content in the soil is negatively correlated. There is no obvious relationship between DNJ content and the content of other mineral elements in soil (Table 2).

Table 2: The relationship between DNJ content, geographic location and soil characteristics.

Geographical location	Latitude	Total N (%)	Available P (%)	Available K (%)	As (µg/g)	Cd (µg/g)	Cr (µg/g)	Hg (µg/g)	Pb (µg/g)	pH	DNJ content (%)
Yinchuan (W)	38.5	0.13	0.058	0.24	23.0	0.22	29	6.9	15	8.49	0.2076
Xuzhou (E)	34.3	0.32	0.180	0.27	13.0	0.11	26	6.9	19	8.74	0.1857
Tongxiang (E)	30.6	0.26	0.200	0.24	8.9	0.15	35	9.4	32	6.90	0.1488
Zhenjiang (E)	32.1	0.25	0.079	0.25	7.4	0.13	27	7.9	25	6.88	0.1375
Hefei (C)	31.8	0.22	0.055	0.23	2.9	ND	34	9.4	28	6.99	0.0088
Mengzi (S)	23.5	0.25	0.160	0.23	44.0	0.92	73	19.0	45	7.92	0.0643

Weather and harvest time

Mulberry young leaves from same position of different trees were collected in an interval of 2 h in a day from the same variety, Husang 32 growing in the same field to elucidate the effect of weather and harvest time in a day on the mulberry DNJ content. Lou *et al*., 2011 [36], observed that two active periods of DNJ biosynthesis are 06:00 to 10:00 a.m. and 6:00 p.m. (Fig. 5A& B) in both sunshine and cloudy day.

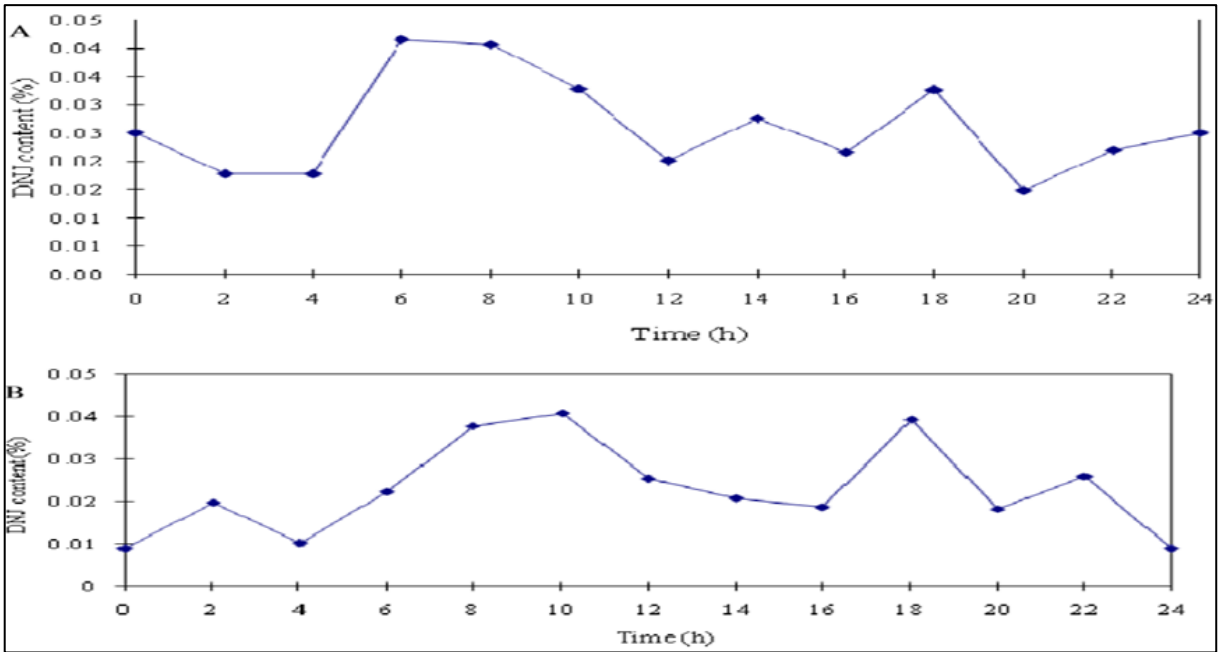


Fig 5: DNJ content at various harvest time of mulberry variety Husang 32 during sunshine day and cloudy day.

Extraction process of DNJ in mulberry

The method of DNJ extraction in mulberry leaves, using cellulase produced by microorganisms such as *Trichoderma reesei* ATCC 26921, *Aspergillus niger* DSMZ821, *Trichoderma viride* CICC 40502 and *T. koningii* CICC 13012 was developed and the amount of DNJ extracted after 12 hours of pretreatment with *Trichoderma reesei* fermentation fluid was maximum (1.995 mg/g of leaf powder) which was 18 times higher than that of the distilled water-soaked group (Gu *et al*., 2013) [18] and the microorganism *Ganoderma lucidum* was screened for fermenting mulberry leaves in order to improve the extraction efficiency of DNJ. The extracting efficiency determined by reverse phase HPLC equipped with a fluorescence detector (HPLC-FD) was up to 0.55 per cent (Jiang *et al.*, 2014) [23].

DNJ Purification

Ion exchange resin chromatography, which takes advantage of electric charges on target products in the sample, is a finer separation method than macroporous resin chromatography. During the operations, however, it may produce harmful waste such as ammonium hydroxide (Luo and Wu. 2012) [37]. Another purification method is through solvent extraction, which is based on the different solubility of the target compounds in the sample. DNJ was extracted from mulberry branch leachate with diethyl ether, ethyl acetate and 1-butanol separately and HPLC was used to examine each solvent extraction (Liu *et al.*, 2014) [35].

DNJ Detection methods

DNJ has no chromophore group in its molecules, and this causes difficulties in quantification and direct analysis. Therefore, the molecule needs derivatization in order to be detected. There are many detection methods like high-performance liquid chromatography (HPLC), fluorescence detection (Kim *et al*., 2003) [27], HPLC- evaporative light scattering detection (Kimura *et al*. 2004), hydrophilic interaction chromatography (HILIC)-mass spectrometry (MS) (Nakagawa *et al*., 2007) [40], HPLC-MS/MS (Nuengchamnong *et al*. 2007) [42], high-performance anion-exchange chromatography with pulsed amperometric detection (Yoshihashi *et al.*, 2010) [62], which are usually considered as complicated, costly and time-inefficient, and may cause environmental pollution. A method of Direct analysis in real time mass spectrometry (DART-MS), for qualitative and quantitative analysis of DNJ in mulberry leaves was found better compared with the HPLC method that uses a fluorescence detector (Xu *et al*., 2015) [57]. DART-MS can process 8 samples in 6 minutes, while only three samples were handled by HPLC in a 45-minute period. Additionally, the DART-MS method is more environmentally friendly because it does not consume toxic reagents or produce toxic compounds like other methods.

Applications of DNJ

Diabetes

Diabetes mellitus is the consequence of complex metabolic disorder characterized by high blood glucose levels with defects in insulin secretion and is associated with high risks of cardiovascular disease (American Diabetes Association, 2007; Jiao *et al.*, 2017) [1, 22]. Mulberry leaf extracts had authenticated proofs of anti-diabetic effects in experimental animals (Andallu *et al*., 2001; Kuriyama *et al*., 2008 and Naowaboot *et al.*, 2009) [2, 32, 41], Mulberry leaves are considered as an herbal medicine for the treatment of diabetes for decades (Ji *et al*., 2016) [24]. In DNJ administered humans, DNJ blocks this α-glucosidase activity which affects conversion of disaccharides to monosaccharides, therefore disaccharides cannot be digested as well as absorbed and are passed into the intestine and eventually excreted. Thus, it reduces glucose absorption and lowers blood sugar levels (Fig.7) (Kiran Thakur *et al.*, 2019) [29].

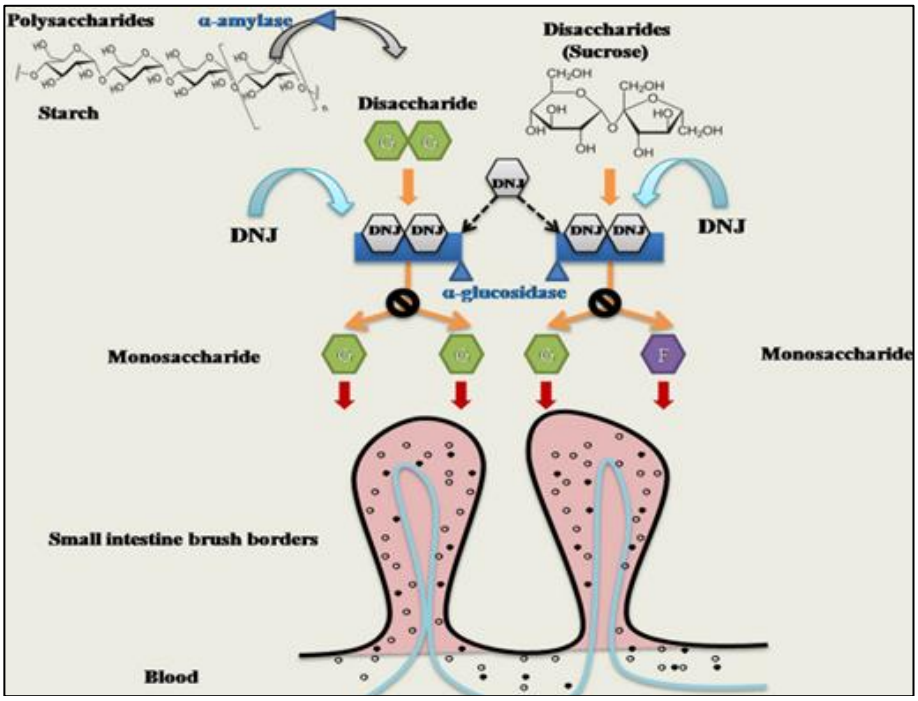


Fig 7: Possible mechanism of DNJ as α-glucosidase inhibitor in the human digestive tract

Cancer

Cancer is the foremost reason of worldwide health threat with cancer-related fatality. The DNJ can directly act on cancer cells and the higher DNJ concentration was observed in colorectal cancer tissues due to the similarity of DNJ structure to that of glucose (Shuang *et al*., 2017) [48]. In many countries, the dietary treatment, especially caloric restriction is adopted for cancer prevention but this also leads to considerable stress in a human body which proves its drawback for cancer prevention (Tsuduki *et al*., 2013) [51]. The DNJ does not have any direct effect in cancer prevention but it can reduce D-glucose analogue and helps in reduction of glucose bioavailability with a caloric restriction effect because glucose requirement in cancer cells is higher than normal cells and hence, DNJ could inhibit the growth of these cells (Seyfried *et al*., 2011) [47].

DNJ in aging

Aging is linked to deterioration of memory and an increased prevalence of neural disorders (Driscoll *et al*., 2003) [14]. Mulberry derived DNJ can delay cellular senescence (the process of deterioration with age) which is promoted under metabolic changes, imbalance in insulin, glucose homeostasis, and decrease in neural factors in the brain (Shuang *et al*., 2014) [49].

Anti-obesity activity of DNJ

Obesity is characterized by an increase in adipose tissue which alters the secretion of various adipokine proteins (Hu *et al*., 1996) [20]. Among them, adiponectin has positive correlation with insulin secretion, glucose metabolism, and fatty acid oxidation (Yamauchi *et al*., 2001) [60]. Administration of DNJ can prevent diet-induced obesity by increasing levels of adiponectin. Tsuduki *et al*. (2013) [51] studied the anti-obesity effect of 1-deoxynojirimycin (DNJ) in the diet-induced obese mouse model. A decrease in adipocyte size was observed in mice treated with DNJ compared with the control (+) group. The mean adipocyte sizes in the DNJ1 and DNJ5 groups were 80 and 70 per cent of that in the control (+) group, respectively (Fig 8). Hence, DNJ inhibited adipocyte hypertrophy in a dose-dependent manner, therefore DNJ exhibited an anti-obesity effect.

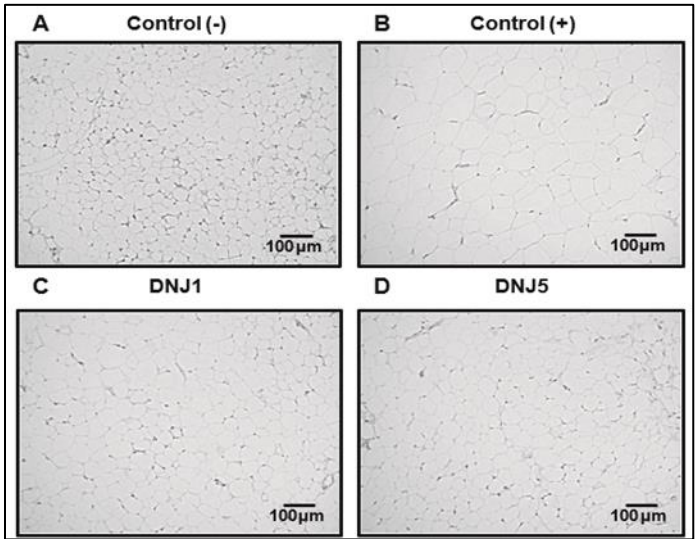


Fig. 8: Effects of DNJ on the mesenteric adipose tissue of mice. A) control (-): control diet B control (+): The high-fat diet to mice

DNJ for Coronary heart disease (CHD)

Stable angina pectoris (SAP) in patients with coronary heart disease (CHD) and blood stasis syndrome (BSS) is a potentially serious threat to public health.1-Deoxynojirimycin (DNJ),which is a unique polyhydroxy alkaloid, is the main active component in mulberry (*Morus indica* L.) leaves and may exhibit protective properties in the prevention of SAP in patients with CHD.The improvement in SAP score, associated symptoms, and blood stasis syndrome (BSS) was seen after DNJ treatment. (Yan ma *et al*., 2019) [59].

a) Anti-inflammatory activity of DNJ

Before the treatment, the statistical difference for the serum levels of CRP, IL-6, and TNF-a was insignificant between the two groups. After the 4-week treatment, the serum levels of

inflammatory factors CRP, IL-6, and TNF- a in EG were reduced compared with those in CG (Table 3). The results suggest that DNJ treatment increased the anti-inflammatory features of the patients.

Table 3: The comparison of serum levels of inflammatory cytokines before and after treatment

Cytokines		*Control group*	*Treatment group*
CRP(µg/ml)	Before	4.65	4.79
	After	3.96	3.57
IL-6 (pg/ml)	Before	150.12	150.68
	After	146.95	139.65
TNF (pg/ml)	Before	66.98	66.29
	After	65.34	62.11

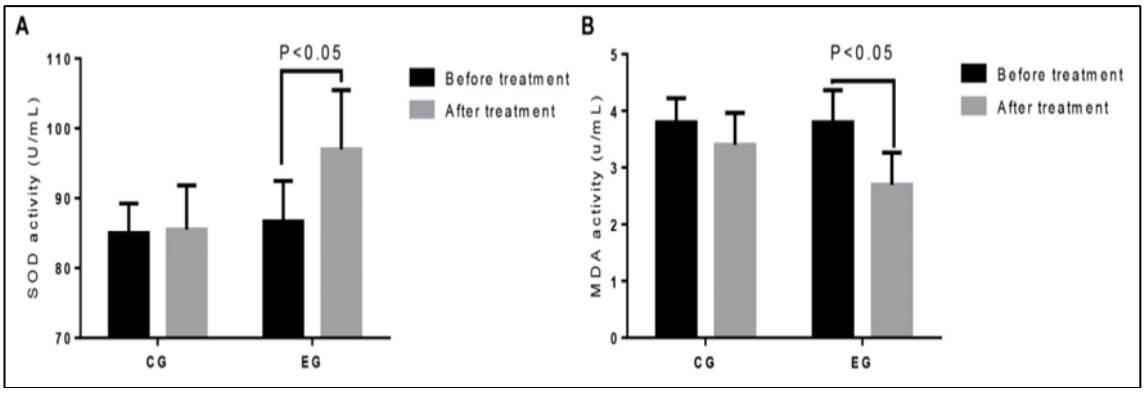
a. C-reactive protein (CRP) b. Interleukin-6(IL-6) c. Tumour necrosis factor-α(TNF-α) Control group - conventional treatment and treatment group DNJ treatment

b) Antioxidant activity of DNJ

Superoxide dismutase (SOD): Body's most crucial antioxidant as it is responsible for disarming the most dangerous free radicals of all the highly reactive superoxide radicals. Superoxide dismutase enzyme that helps break down potential harmful oxygen molecules in cells, might prevent damage to tissues.

Malondialdehyde (MDA): Marker for oxidative stress results from lipid peroxidation of poly unsaturated fatty acids.

Serum SOD levels were increased, whereas the MAD levels in EG were reduced compared with those in CG (Fig 9). The results suggest that DNJ treatment increased the antioxidant capacities of the patients.



(A) SOD activity (B) MAD activity

Fig 9: The comparison of antioxidant activities between two groups

b) Anxiety and depression level:

The Self-rating Anxiety Scale (SAS) and Hamilton Depression Scale (HAMD) scores in DNJ treated group were reduced relatively compared to those in conventional treatment group. The results suggest that DNJ treatment improved the anxiety and depression of patients with stable angina pectoris.

Conclusion

Mulberry foliage is used in sericulture as the sole diet for the monophagous insect silkworm, *Bombyx mori.* Apart from food for silkworm, mulberry leaves are used as traditional medicine to treat disease as it contains a rich source of phenolic compounds. DNJ as biologically active molecule with promising health effects can be the hope for many health-related issues. Its sources include mulberry, dayflower, and several bacterial strains. Identification of key genes involved in DNJ alkaloid biosynthesis will provide a basis for the further analysis of its biosynthetic pathway and ultimately for the realization of synthetic biological production. Currently, the key genes involved in DNJ biosynthesis in mulberry leaves have not been identified, as most studies were geared towards DNJ bioactivities, total content, and effective separation and purification methods. The low amount of DNJ in mulberry leaves and inconsistent yields demands complex procedures for extraction and purification.1-Deoxynojirimycin possesses antihyperglycemic, anti-obesity and antiviral features, also regulate the Glycometabolism, lipid and fat metabolism. There is a need to explore the possibilities of identifying DNJ based cost effective products for better use in future.

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