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Isoflavonoid Components of Leaves of *Cajanus cajan*

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Abstract We studied to identify useful components in the leaves of *Cajanus cajan*. In the petroleum ether and ethyl acetate fractions, TLC was done, substances of which R_f values were 0.95, 0.90, 0.87, 0.72, 0.61 and 0.55 respectively were identified, their spectra were tested by UV, IR, MS, ^{1}H NMR and isoflavonoids, namely genistin, genistein, daidzin, daidzein, ononin and formononetin were identified.

Key words pigeon pea (*Cajanus cajan*), genistin, genistein, daidzin, daidzein, ononin, formononetin, isoflavonoid

Introduction

The great leader Comrade **Kim Il Sung** said.

"If we manufacture Koryo medicines in quantity and use them, we can prevent diseases and better protect the people's health."("**KIM IL SUNG** WORKS" Vol. 39 P. 196～197)

Pigeon pea (*Cajanus cajan* (L.) Mill) is a perennial shrub belonging to Fabaceae which is derived in India [16].

Protein content in its seeds is different according to varieties, but contains 18.5～26.3% of dry mass. The protein consists of 16 kinds of amino acids, so pigeon pea becomes ideal supplementary food in countries where rice is the staple food [1].

Leaves contain 19% of protein [16], organic acids, abundant vitamin A and B [8].

Phenolic components in leaves of pigeon pea have been actively studied since 1970s [4－7, 12, 18].

From stem and bark of pigeon pea, a researcher separated and identified 5 isoflavonoids of which 3 compounds were antifungal isoflavones, one was isoflavanone and the other was cajanol [12].

Another scholar separated and identified cajanin, cajanol, cajaflavanone and 2 isoflavones from root and seeds of pigeon pea [20].

In India, genistin was separated and identified from root, and isoflavon glycoside, genistein, 2′-O-methylcajanon, cajaisoflavone from root bark [4－7].

In a certain country, stilben was separated from leaves of pigeon pea and its structure was clarified by MS and NMR (^{1}H, ^{13}C) [13].

17 kinds of flavonoids have been separated and identified from root, stem, seeds and leaves of pigeon pea [4－8, 12, 18].

Many researchers studied the effect of protein fraction of pigeon pea seeds on cholesterol content and neutral lipid content in rats and revealed that feeding 18% protein fraction added to feedstuff

lowered the contents significantly [9, 14, 17].

Many researchers reported that isoflavonoids in pigeon pea leaves expanded the coronary arteries and brain blood vessels and decreased oxygen consumption, so they were highly effective in treatment and had no toxicity when they were used in hyperpiesia, dizziness, stenocardia, cerebral arteriosclerosis, hyperlipemia and so on [2, 3].

Especially, it was clarified that isoflavonoids such as genistein in pigeon pea leaves inhibited angiogenesis when several kinds of cancer cells in human bodies were increasing [2, 3, 11, 15].

Because genistein has specific apoptosis inducing function on several kinds of human cancer cells, its anticancer effect is very high [10, 11]. And genistein is a phytoestrogen and it acts like estrogens [19, 21].

Until now, many researchers have analyzed components in several organs such as seeds, stem and root of pigeon pea, but there are no data, which deal with isoflavonoids of leaves.

We separated and identified isoflavonoid components in leaves of pigeon pea cultivated in ecological conditions of Korea in order to identify components which are used effectively for prevention and treatment of hyperlipemia and arteriosclerosis.

1. Materials and Method

1.1. Materials

Pigeon pea (*Cajanus cajan*) was planted on May 10, 2009 and harvested on Oct. 25 in Pyongyang area. Then its leaves were used as material for analysis after drying in shade until the water content became 10%.

1.2. TLC analysis

Absorbent Silica gel G_{60} F_{254} (glass plate), Silica gel H (for column)

Development solvent ethyl acetate–methanol (10 : 1) for the 1st development, benzol–ethyl acetate (7 : 3) for the 2nd development

Developer iodine vapor, 5% solution of ferric chloride

If one spot appeared by dropping 10～30 μg by 1st and 2nd dimension TLC, it was considered as single substance and tested.

1.3. Extraction and separation of total isoflavonoids and individual substances

Total isoflavonoid was extracted and separated with methanol, petroleum ether and ethyl acetate and individual substances were separated by column chromatography and recrystallization.

1.4. Measuring instruments and devices used for identification of individual substances

In experiment are used infrared water measuring instrument “Kett F-1A”, trace melting point measuring instrument “Yanaco”, polarimeter “Cartzeless-OT”, ultraviolet spectrophotometer “Shimadzu UV-265”, infrared spectrophotometer “Shimadzu IR-420”, mass spectrometer “JMS-DX300”, ^{1}H-nuclear magnetic resonance spectrometer “Brüker WM-250” (measuring condition: inner standard substance TMS, frequency 100MHz)

2. Results and Discussion

2.1. Composition of total isoflavonoids

By TLC of total isoflavonoids of pigeon pea leaves, 10 spots appeared of which R_f values are 0.95, 0.9, 0.87, 0.72, 0.61 and 0.55 and the main spot's R_f was 0.72.

2.2. Extraction and separation of total isoflavonoids and individual substances

500g of dry leaves of pigeon pea was extracted 3 times for 24 hours in 25℃ with 10 times of methanol and the extract was condensed by reducing pressure to 100g of methanol extract (water content 30%). This was dissolved in 200mL of hot water, put for 2 hours, filtered and the sediments were thrown away, and the filtered solution was extracted 3 times with 60mL of petroleum ether respectively, the extracts were added, condensed to 6g of petroleum ether fraction (mother liquor).

The mother liquor of petroleum ether extraction was extracted 5 times with 60mL of ether respectively, added, dehydrated with anhydrous sodium sulphate, decompressed and condensed to 9g of ether extract. This was dissolved in 40mL of alcohol and analyzed by column chromatography. Substance 2 (R_f 0.95, 60mg) was separated from fractions No. 29－38, substance 4 (R_f 0.9, 100mg) from No. 19－25 and substance 6 (R_f 0.87, 120mg) from No. 4－16 respectively.

The residue of petroleum ether extraction was extracted with 60mL of ethyl acetate respectively, added, dehydrated with anhydrous sodium sulphate, decompressed and condensed to 14g of ethyl acetate fraction. This was dissolved in 95% and the sediments were separated, dissolved in 50% solution of warm ethanol and put for 12h, and formed crude crystal was recrystallized with hot water resulting in pale yellow needle-shaped crystal (R_f 0.72, 1 000mg, substance Ⅰ).

Then, pale yellow crude crystal powder taken after evaporating alcohol from 95% alcohol extract was dissolved in hot distilled water, sucking filtered and condensed to 20mL of filtrate, and put for 12h in 4℃. The formed crystal was recrystallized with hot alcohol to colorless pillar-shaped crystal (R_f 0.61, 560mg, substance Ⅲ).

Insoluble sediment (2.4g) was dissolved in alcohol, mixed with absorbent (10g), dried, passed through 0.1mm sieve, filled carefully in column (5cm×30cm) which comprised silica gel (100g) and separated with ethyl acetate: methanol (10∶1). (separated fraction 3mL, eluting rate 1mL/min) Every separated fraction was identified by TLC. Fractions (No. 13－20) were evaporated to be needle-shaped crystal. This was recrystallized with mixed solution of alcohol and ether (1∶1) to be needle-shaped crystal (R_f 0.55, 400mg, substance Ⅴ).

2.3. Identification of individual substances

2.3.1. Identification of individual substances separated from ethyl acetate fractions

Identification of substance Ⅰ This is pale yellow needle-shaped crystal, dissolved in hot methanol and ethyl acetate, and almost insoluble in chloroform and ether.

Melting point: 253～255℃

Specific rotatory power: $[\alpha]_D^{25}$ -29.7^{o} (0.02mol/L NaOH)

Molecular weight: 270 (genin, EI-MS)

Color reaction: dark green in the reaction with ferric chloride solution, yellow in the reaction with undiluted sulfuric acid and sodium hydroxide, and pale yellow in the reaction with hydrochloric acid-magnesium.

UV, $\lambda_{max}^{ethanol}$(nm): 261.2, 332.1

IR, $\nu_{max}^{KBr}(cm^{-1})$: specific peaks appeared in 3 445 (ν_{OH}), 1 653 ($\nu_{C=O}$ in γ-pyrone ring), 1 616, 1 576, 1 514 ($\nu_{C=C}$ in aromatic ring), 1 265, 1 179, 1 094, 1 040 (ν_{C-O}), 902 (β-saccharide), 824 (benzol substitute). This showed that the molecular structure of substance Ⅰ comprises hydroxyl group, γ-pyrone ring, benzol ring, β-glucoside bond, therefore it is a glycoside in which isoflavone and β-saccharide are bound.

MS, m/e (%): 270 (M^+), 152 (A^+), 118 (B^+), 134, 125, 103, 88, 76, 68, 55, 40. The specific peak of which m/e value is 270 would be genin molecular ion peak which appeared when genin without saccharide part lost one electron and ionized.

^{1}H NMR, δ(ppm): 6.48(1H, d, J=1.86 C_6—H) and 6.73(1H, d, J=1.86 C_8—H) are divided into coupling constant 1.86 because 2 protons act in *m*-position. 6.84(2H, d, J=8.61 $C'_3-C'_5-H$) and 7.42(2H, d, J=8.61 $C'_2-C'_6-H$) are divided into coupling constant J=8.61 because 2 protons act respectively in *o*- and *m*-position. 8.42 (1H, S, C_2—H), 9.65 (1H, S, C_4—OH), 12.94 (1H, S, C_5—OH) and 5.07 (1H, d, J=6.06) show that proton acts in 1 position, the constant J=6.06 becomes supplementary evidence of β-glucoside bond.

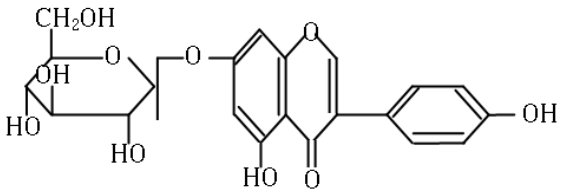


Fig. 1. Genistin (genistein-7-glucoside)

In order to identify substance Ⅰ more concretely, it was hydrolyzed, so it was clarified that its binding saccharide is glucose and genin is genistein. The physical properties and structure analysis data of substance Ⅰ were consistent with those of glycoside genistin, so it was identified as genistin (Fig. 1).

Identification of substance Ⅲ This is colorless column-shaped crystal, dissolved in hot methanol and ethyl acetate and acetone, and insoluble in chloroform and ether.

Melting point: 214～217℃

Specific rotatory power: $[\alpha]_D^{25}$ -32.1^o (0.02mol/L KOH)

Molecular weight: 254 (genin, EI-MS)

Color reaction: colorless in the reaction with ferric chloride solution, yellow in the reaction with undiluted sulfuric acid and sodium hydroxide, and colorless in the reaction with hydrochloric acid-magnesium.

UV, $\lambda_{max}^{ethanol}$(nm): 248.6, 301.4

IR, $\nu_{max}^{KBr}(cm^{-1})$: specific peaks appeared in 3 410 (ν_{OH}), 1 620 ($\nu_{C=O}$ in γ-pyrone ring), 1 563, 1 510 ($\nu_{C=C}$ in aromatic ring), 1 250, 1 174, 1 092, 1 042 (ν_{C-O}), 900 (β-saccharide), 880, 851 (benzol substitute). This showed that the molecular structure of substance Ⅲ comprises hydroxyl group, γ-pyrone ring, benzol ring, β-glucoside bond, therefore it is a glycoside.

MS, m/e (%): 254 (M^+), 136 (A^+), 118 (B^+), 89, 63, 44, 28. The weak specific peak of which m/e value is 254 appeared and it would be genin molecular ion peak which appeared when genin without saccharide part lost one electron and ionized.

^{1}H NMR, δ (ppm): 8.07 (1H, d, J=8.60 C_5－H) appeared as double line because the corresponding proton acted with proton of *o*-position (C_6－H) 7.42 (2H, d, J=8.60C_2, C_6－H) and 6.83 (2H, d, J=8.60 C_3, C_6－H) appeared as double lines when corresponding protons acted symmetrically each other. 8.40 (1H, S, C_2－H) appeared by the action of proton of C_2 position, protons of C_6 and C_8 and positions appeared as multiple lines between 7.09～7.25ppm. Namely the proton of C_6 position was divided into coupling constant J=8.60 by interaction with proton of C_5 position, then reacted with C_8 proton of meta-position, so C_6 proton was divided into 4 lines. Then C_8 proton reacted with C_6 proton of meta-position to be divided into coupling constant J=1.80 (double line), so C_6 and C_8 protons appeared in multiple lines (6 lines). C_1 proton of substance Ⅲ appeared as double lines in 5.11ppm (1H, d, J=7.5), which shows that substance Ⅲ has β－glucoside bond.

In order to identify substance Ⅲ more concretely, it was hydrolyzed, so it was clarified that its binding saccharide is glucose and genin is daidzein. The physical properties and structure analysis data of substance Ⅲ were consistent with those of glycoside daidzin (Fig. 2).

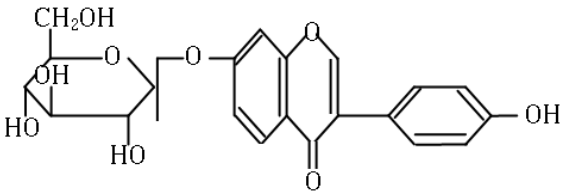


Fig. 2. Daidzin (4′-hydroxyisoflavone-7-glucoside)

Identification of substance Ⅴ This is colorless needle-shaped crystal, dissolved in methanol and ethanol, and soluble in water and ether to a slight degree.

Melting point: 210～213℃

Specific rotatory power: $[\alpha]_D^{25}$ -23.5^{o} (pyridine)

Molecular weight: 268 (genin, EI-MS)

Color reaction: Colorless in the reaction with ferric chloride solution, yellow in the reaction with undiluted sulfuric acid and sodium hydroxide, and pale yellow in the reaction with hydrochloric acid-magnesium.

UV, $\lambda_{max}^{ethanol}$(nm) : 249, 299

IR, $\nu_{max}^{KBr}(cm^{-1})$: Specific peaks appeared in 3 390 (ν_{OH}), 1 637 ($\nu_{C=O}$ in γ－pyrone ring), 1 577, 1 509 ($\nu_{C=C}$ in aromatic ring) and 897 (β－glucoside bond). This showed that the molecular structure of substance Ⅴ comprises hydroxyl group, γ－pyrone ring, benzol ring and β－glucoside bond, therefore it is a glycoside.

MS, m/e(%): 268 (M^+, genin), 136 (A^+), 132 (B^+), 90, 58, 46, 28. The very weak specific peak of which m/e value is 268 would be genin molecular ion peak which appeared when genin without saccharide part lost one electron and ionized.

^{1}H NMR, δ (ppm): 3.82 (3H, S, －OCH_3), 8.22 (1H, S, C_2－H) and 8.17 (1H, d, J=9.36, C_5－H) appeared as double lines because the protons of corresponding positions reacted with *o*-position (C_6－H). 7.51 (2H, d, J=8.98 $C_2'-C_6'-H$) and 6.97 (2H, d, J=8.98 $C_3'-C_5'-H$) also appeared

as double lines due to the reactions with the proton of corresponding position. And multiple lines appeared at 7.05～7.29ppm by protons of C_6 and C_8. On the other hand, 5.05 (1H, d, J=6.68) showed that proton reacts at 1 position in saccharide molecule. The value of coupling constant of C_1 proton showed that substance Ⅴ comprises glycoside bond.

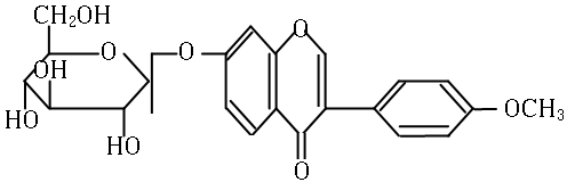


Fig. 3. Ononin (formononetin-7-glucoside)

In order to identify substance Ⅴ more concretely, it was hydrolyzed, so it was clarified that its binding saccharide is glucose and genin is formononetin. The physical properties and structure analysis data of substance Ⅴ were consistent with those of glycoside ononin, so it was identified as ononin (Fig. 3).

2.3.2. Identification of individual substances separated from ether fractions

Identification of substance Ⅱ This is pale yellow needle-shaped crystal, dissolved in methanol, ethanol, ethyl acetate and acetone, and insoluble in water and benzol.

Melting point: 292～295℃

Molecular weight: 270 (MS)

Color reaction: green in the reaction with ferric chloride solution, yellow in the reaction with undiluted sulfuric acid and sodium hydroxide, and pale yellow in the reaction with hydrochloric acid-magnesium.

UV, $\lambda_{max}^{ethanol}$(nm) : 261, 332

IR, $\nu_{max}^{KBr}(cm^{-1})$: Specific peaks appeared in 3 410 (ν_{OH}), 1 643 ($\nu_{C=O}$ in γ－pyrone ring), 1 612, 1 561, 1 514 ($\nu_{C=C}$ in aromatic ring), 1 201, 1 142, 1 040(ν_{C-O}), 908, 882, 847 (benzol substitute). This substance should be genin type because it has not adsorption by β－glucoside bond.

MS, m/e (%) : 270 (M^+ 100), 150 (A^+), 118 (B^+), 241, 135, 89, 78, 69, 51. The specific peak of which m/e value was 270 showed that the molecular weight is about 270. And the specific peak of which m/e value is 152 is divided ion peak of A-ring type which has 2 hydroxyl group, which showed that these two divided ions are the main compounds of substance Ⅱ. Namely substance Ⅱ would be isoflavone compound in which A ring is combined with B ring.

In order to identify substance Ⅱ more concretely, TLC analysis and mixed melting test were done with control of genistein sample. The results showed that substance Ⅱ was consistent with the physical properties and structure analysis data of genistein (Fig. 4).

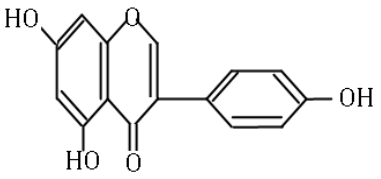


Fig. 4. Genistein (4′, 5, 7-tri-hydroxyisoflavone)

Identification of substance Ⅳ This is pale yellowish white needle-shaped crystal, dissolved in hot methanol, ethanol, acetone and ethyl acetate, soluble in ether to a slight degree and insoluble in water, benzol and chloroform.

Melting point: 315～320℃ (methanol)

Molecular weight: 254 (MS)

Color reaction: Colorless in the reaction with ferric chloride solution, yellow in the reaction with undiluted sulfuric acid and sodium hydroxide, and light red in the reaction with hydrochloric acid-magnesium.

UV, $\lambda_{max}^{ethanol}$(nm): 248.4, 301.6

IR, $\nu_{max}^{KBr}(cm^{-1})$: Specific peaks appeared in 3 220 (ν_{OH}), 1 620 ($\nu_{C=O}$ in γ-pyrone ring), 1 592, 1 513 ($\nu_{C=C}$ in aromatic ring), 1 237, 1 189, 1 042 (ν_{C-O}), 885, 840, 787 (benzol substitute).

MS, m/e (%): 254 (M^+ 100), 255, 226, 141, 139, 136 (A^+), 119, 118 (B^+), 89, 80, 63. The strong specific peak of which m/e value is 254 would be genin molecular ion peak which appeared when genin lost one electron and ionized.

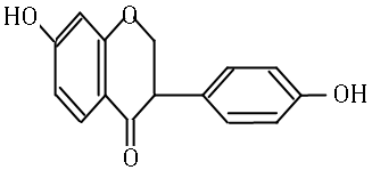


Fig. 5. Daidzein (4′, 7-dihydroxyisoflavone)

The above experimental results showed that substance Ⅳ was consistent with the physical properties of daidzein (Fig. 5).

Identification of substance Ⅵ This is white needle-shaped crystal, dissolved in ethanol and acetone, and soluble in ether to a slight degree, and insoluble in water, benzol and chloroform.

Melting point: 262～264℃

Molecular weight: 268 (MS)

Color reaction: Colorless in the reaction with ferric chloride solution, yellow in the reaction with undiluted sulfuric acid and sodium hydroxide, and light red in the reaction with hydrochloric acid-magnesium.

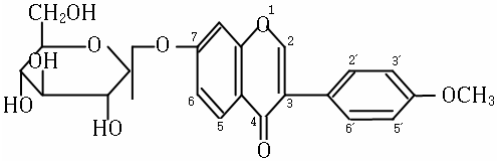
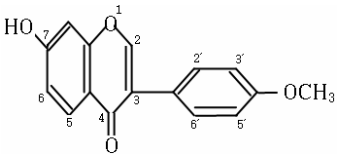
UV, $\lambda_{max}^{ethanol}$(nm): 248.8, 298.8

IR, $\nu_{max}^{KBr}(cm^{-1})$: Specific peaks appeared in 3 145 (ν_{OH}), 1 634 ($\nu_{C=O}$ in γ-pyrone ring), 1 594, 1 567, 1 507 ($\nu_{C=C}$ in aromatic ring), 1 248, 1 191, 1 022 (ν_{C-O}).

MS, m/e (%): 268 (M^+ 100), 136 (A^+), 132 (B^+), 108, 89, 77, 63, 51, 44. The specific peak of which m/e value was 268 showed that the molecular weight of this substance is about 268, that of 136 was a divided ion peak of A-ring which has one hydroxyl group, and that of 108 was a divided ion peak of B-ring which has one methoxy group, which showed that those were the main compound of this substance.

^{1}H NMR, δ (ppm): 3.82 (3H, S, OCH_3), 7.48 (2H, d, J=8.98 $C'_2-C'_6-H$), 6.96 (2H, d, J=8.98 $C'_3-C'_5-H$), 8.13 (1H, S, C_2-H), 8.07 (1H, d, J=8.80 $C^+|-H$), 6.95 (1H, d, J=8.80 C_6-H), 6.87 (1H, d, J=2.02 C_8-H). As the result of ^{1}H NMR, the chemical variation values of all protons were slight higher than substance Ⅴ (ononin) because in ononin glucose was bound in 7 position, so the electrons moved a bit in the whole molecule. The results of ^{1}H NMR of substances Ⅴ and Ⅵ were compared in table.

Table. Comparison of the chemical variation values of substance Ⅴ (ononin) and substance Ⅵ

Position of groups and hydrogen	Substance Ⅴ	Substance Ⅵ
		
$-OCH_3$	3.82(3.8216)	3.82(3.8179)
C_2-H	8.22(single line)	8.13(single line)
C_5-H	8.17(double lines J=9.36)	8.07(double lines J=8.80)
C'_2, C'_6-H	7.51(double lines J=8.98)	7.48(double lines J=8.98)
C'_3, C'_5-H	6.97(double lines J=8.99)	6.96(double lines J=8.98)
C'_6, C'_8-H	7.05～7.29(multiple lines)	6.85～6.99(multiple lines)

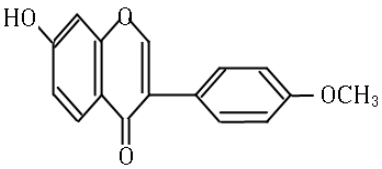


Fig. 6. Formononetin (7-hydroxy-4′-methoxyisoflavone)

As shown in table1, the ^1H NMR data of substance Ⅵ was very similar to those of substance Ⅴ. Namely the chemical variation values in 3.82, 7.48, 8.13, 8.07 and 6.87ppm were higher a bit than those of ononin. But there was no signal by binding saccharide. Therefore, substance Ⅵ should be formononetin which was formed by removing glucose molecular from ononin (Fig. 6).

Conclusion

Glycosides such as genistin, daidzin and ononin and aglycons such as genistein, daidzein and formononetin were separated and identified as isoflavonoids in leaves of *Cajanus cajan*.

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