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Research Article

# Analysis of Seasonal Variation in the Amounts of Phytochemicals in Kudzu (*Pueraria lobata*) Leaves Using HPLC with Evaporative Light Scattering Detection

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- Evaporative light scattering detector

**Abstract**

Kudzu (*Pueraria lobata*) is widely considered a weed in the USA. However, the roots and flowers of kudzu are commercially available as crude Oriental medicines. Since the leaves of kudzu contain similar phytochemicals, kudzu leaves seem to be a promising health food or herbal supplement. Therefore, we investigated the variation in amounts of phytochemicals in kudzu leaves during growth using a high performance liquid chromatographcoupled to an evaporative light scattering detector. The amount of total saponin obtained from collected kudzu leaves from May to January was fairly consistent (0.10%-0.19%). In contrast, the amount of total flavonoid varied from 0.78% (June 12) to 2.21% (October 23). The increase in content of flavonol glycosides was larger than that of isoflavone glycosides. Interestingly, the amount of quercetin glycosides gradually increased from May to November, whereas the amount of kaempferolglycosides did not change much. The total amount of phytochemicals increased until late autumn. Therefore, the most appropriate time to harvest the leaves would be late autumn, just before the leaves wither.

## ABBREVIATIONS

ELSD: Evaporative Light Scattering Detection

## INTRODUCTION

Kudzu (*Pueraria lobata*, Fabaceae) is widely spread over China and Japan. Puerariae Radix (the roots of *P. lobata*) is one of the most important Oriental crude drugs, used mainly as an antispasmodic agent [1]. The antispasmodic principles are attributed to isoflavones, represented as daidzein [2]. Further, the clinical application of Puerariae Radix for cardiovascular disease (hypertension, angina pectoris and myocardial infarction) is widespread in China [3]. We have described not only the structures of its saponins but also their hepatoprotective activities [4]. Puerariae Flos (the flowers of *P. lobata*) is a crude drug used to counteract the overconsumption of alcohol in traditional Japanese and Chinese therapeutic systems [4]. We found that the total saponin fraction was effective for treating alcohol intoxication [5]. We identified three saponins together with several isoflavones as active principles [6]. We also developed a method for simultaneous analysis of the total saponin and isoflavone fraction within the flowers using a high-performance liquid chromatography coupled to an evaporative light scattering detector [7].

In contrast, "the history of kudzu in the USA is short but telling" [8]. Imported into the USA from Japan, it first served as an ornamental in the southern USA in 1876. Its use in pasture became prevalent around 1910, while in the 1930s, it was recommended for erosion control. But due to its rapid growth, it

smothered trees and houses, and further use as a cover has been discouraged. In 1981, kudzu was about to be declared a pest [9].

On the other hand, kudzu leaves contain a lot of phytochemicals (Figure 1) [10]. We elucidated some of their structures as corresponding to kaikasaponin III (**10**), daidzin (**1**), genistin (**6**), and robinin (**2**) [6]. The biological activity of these phytochemicals is well known. For example, **10** have hepatoprotective [11-14] and 5α-reductase inhibitory [15] activity, **1** and **2** have estrogenic activity [16], **1** has antidipsotropic activity [17], and **2** has antioxidative activity [18]. This means that kudzu leaves are a promising health food or herbal supplement. These findings formed the basis of the experiments in this study, which focused on the efficient extraction of compounds in kudzu leaves.

Here we present our analysis of variation in the amounts of phytochemicals in kudzu leaves during growth using a high performance liquid chromatography coupled with an evaporative light scattering detector [7].

## MATERIALS AND METHODS

### Plant materials

The kudzu (*Pueraria lobata*) leaves used in this study were collected from the medicinal plant garden of Fukuoka University located in Fukuoka, Japan starting in May 2009 at intervals of about 2 weeks. After collection, the samples were dried immediately at 60°C for 2 hours, then crushed by a crusher and stored frozen at -20°C until analysis. Voucher specimens were deposited in the Laboratory of Pharmacognosy of Fukuoka University.

### Extraction

Analysis was conducted referring to a published protocol [7]. Powdered kudzu leaves (1g) were extracted with 9 mL EtOH-$H_2O$ (6:4) solution for 2 hours at 60°C. The slurry was centrifuged at 20,000g for 5 min. The supernatant (1 mL) was diluted fourfold with $H_2O$. The entire sample was applied to an Oasis HLB cartridge (Waters, Milford, MA) and then washed with MeOH-$H_2O$ (9:1) solution, followed by MeOH. The MeOH fraction was evaporated to dryness to give the final extract.

### HPLC apparatus and analytical conditions

The analytical apparatus consisted of a CCPM-II pump (Tosoh, Tokyo, Japan) and a Model 300s evaporative light scattering detector (HPLC-ELSD system) (SofTA, Westminster, CO, USA). HPLC-ELSD conditions were as follows: column, $C_8$ reversed-phase column (Shiseido, Capcell Pak C8 DD 250 mm × 4.6 mm i.d., Shiseido, Japan); solvent A, $H_2O$ with 0.05% trifluoroacetic acid (TFA); solvent B, $H_2O$:$CH_3CN$ (4:6) with 0.05% TFA; column temperature, 40°C; flow rate, 1.0 mL/min; spray chamber temperature, 40°C; drift tube temperature, 60°C. Elution was performed as follows at the given time points: solvent A/solvent B (84:16, 0 min) → solvent A/solvent B (70:30, 15 min) → solvent A/solvent B (42:58, 22 min) → solvent A/solvent B (34:66, 30 min) → solvent A/solvent B (34:66, 35 min) → solvent A/solvent B (0:100, 45 min) → solvent A/solvent B (0:100, 50 min). Retention times (in min) of flavonoids were 16.2 (daidzin, **1**) [19], 16.5 (robinin, **2**) [6], 19.2 (quercetin 3-*O*-robinobioside, **3**) [20], 20.1 (rutin, **4**) [6], 21.3 (kaempferol 3-*O*-robinobioside, **5**) [21], 22.1 (genistin, **6**) [19], 22.2 (nicotiflorine, **7**) [19], and 25.0 (6″-*O*-malonylgenistin, **8**) [22]. Retention times (min) of saponins were 37.4 (soyasaponin I, **9**) [23], and 40.0 (kaikasaponin III, **10**) [6]. Peaks were identified based on comparison of retention times with those of constituents separated from *P. lobata* leaves.

### Preparation of sample solutions

Extracts were dissolved in 100% MeOH to obtain a final volume of 0.5 mL. About 0.5 mL of the solution was centrifuged at 20,000g for 5 min, and then 20.0 μL of this solution was injected into the HPLC-ELSD system for analysis. Samples were prepared in duplicate.

### Quantification

Four standard solutions containing the same concentrations of 10 analytes (0.063-0.5 mg/mL) were injected in triplicate. Calibration curves were plotted logarithmically using peak area (y-axis) versus concentration (x-axis) for each analyte and fitting appropriate calibration equations to the results. The concentration of each analyte ($C_{hplc}$) in HPLC sample solution was quantified by calculation from the peak area of each analyte using the resulting calibration curves. The calibration curves of

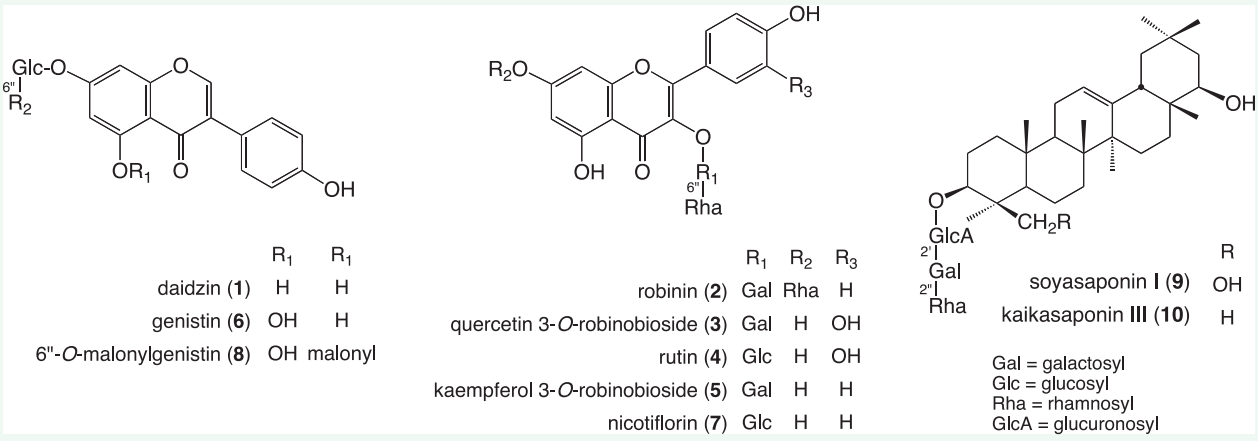


**Figure 1** Phytochemicals in kudzu (*Pueraria lobata*) leaves.

**1**–**10** were respectively Y = 0.483X – 3.610, Y = 0.510X – 4.091, Y = 0.478X – 3.226, Y = 0.539X – 4.596, Y = 0.580X – 5.000, Y = 0.553X – 4.946, Y = 0.578X – 4.952, Y = 0.525X – 4.030, Y = 0.568X – 4.654, Y = 0.534X – 4.423, where Y = log A, with A = peak area, and X = log $C_{hplc}$, with $C_{hplc}$ = concentration (mg/mL). The correlation coefficients (r values) of **1**–**10** were respectively 0.9935, 0.9973, 0.9718, 0.9993, 0.9968, 0.9777, 0.9959, 0.9508, 0.9978 and 0.9998.

The yield (C) of each analyte in each sample was calculated according to Euation 1:

$$C = \frac{C_{hplc} \times V1 \times V3}{V2 \times M1} \quad (1)$$

where C is the amount of analyte in the sample (mg/g), $C_{hplc}$ the analyte concentration of the HPLC solution (mg/mL), V1 the HPLC sample volume (0.5 mL), V2 the volume of extracted solution loaded onto the cartridge (1 mL), V3 the volume of extracted solution (9 mL), and M1 the weight of the sample (1 g).

## RESULTS AND DISCUSSION

An HPLC system with a UV absorbance detector is typically used for analyzing flavonoids. However, simultaneous analysis of saponins and flavonoids has been difficult due to their difference in absorption coefficient. An HPLC system coupled to an evaporative light scattering detector is the only technique affording sufficient accuracy and sensitivity. In principle, area percent calculations from evaporative light scattering detection (ELSD) chromatograms can be used to determine the weight percent of each component in a mixture [24]. Therefore, HPLC coupled to ELSD is a good technique for the identification and simultaneous analysis of saponins and flavonoids in kudzu leaves.

Figure 2 shows representative HPLC profiles of kudzu leaves collected from the medicinal plant garden of Fukuoka University. Characteristic twin peaks corresponding to robinin (**2**) and rutin (**4**) appeared. Four minor peaks corresponding to other flavonol glycosides (**3**, **5** and **7**) and a malonylated isoflavone glucoside (**8**) were also observed. On the other hand, the peaks corresponding to two isoflavone glucosides (**1** and **6**) and two saponins (**9** and **10**) were relatively small.

Table 1 summarizes the amounts of flavonoids (**1**–**8**) and saponins (**9** and **10**) in kudzu leaves collected from May to January at intervals of about 2 weeks. The amount of total saponin obtained from the kudzu leaves collected in each period was fairly consistent (0.10%–0.19%). In contrast, the amount of total flavonoids varied from 0.78% (June 12) to 2.21% (Oct. 23). Variation in the total amounts of flavonoids and saponin is illustrated in (Figure 3). The amount of flavonoids gradually increased from May to December. Kudzu leaves exposed to bright sunlight grew quickly, and it is likely that the flavonoid content gradually increased due to storage. (Figure 4) shows details of the variation in total flavonoids, i.e., a comparison of flavonol glycosides and isoflavone glycosides. The amounts of both phytochemicals gradually increased from May to December. The increase in flavonol glycosides was larger than for isoflavone

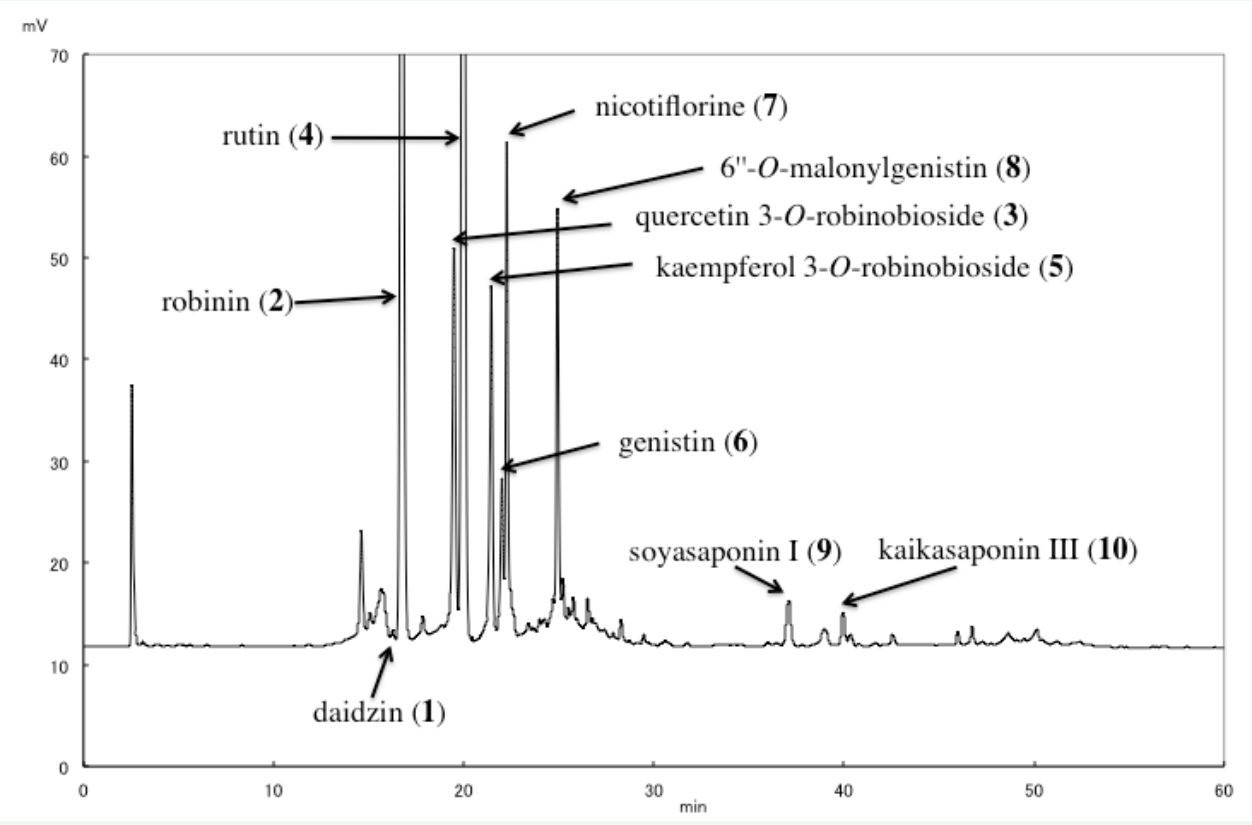


**Figure 2** HPLC profiles of kudzu (*Pueraria lobata*) leaves.

**Table 1**: Percentage of flavonoids (**1**-**8**) and saponins (**9**, **10**) in kudzu (*Pueraria lobata*) leaves.

| Collection Date | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Total flavonoid | 9 | 10 | Total saponin | Total contents |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| May 22 | ND | 0.44 | 0.08 | 0.13 | 0.10 | 0.04 | 0.04 | 0.09 | 0.91 | 0.07 | 0.07 | 0.14 | 1.05 |
| Jun. 12 | ND | 0.41 | 0.08 | 0.14 | 0.07 | 0.02 | 0.03 | 0.04 | 0.78 | 0.06 | 0.05 | 0.11 | 0.89 |
| Jun. 29 | ND | 0.42 | 0.11 | 0.17 | 0.08 | 0.02 | 0.04 | 0.05 | 0.88 | 0.09 | 0.10 | 0.19 | 1.07 |
| Jul. 17 | ND | 0.41 | 0.22 | 0.26 | 0.14 | 0.02 | 0.07 | 0.12 | 1.24 | 0.07 | 0.10 | 0.17 | 1.41 |
| Aug. 4 | ND | 0.40 | 0.21 | 0.25 | 0.10 | 0.02 | 0.06 | 0.13 | 1.17 | 0.08 | 0.10 | 0.18 | 1.35 |
| Aug. 17 | ND | 0.41 | 0.34 | 0.29 | 0.14 | ND | 0.09 | 0.15 | 1.42 | 0.06 | 0.11 | 0.17 | 1.59 |
| Aug. 28 | ND | 0.39 | 0.50 | 0.31 | 0.15 | 0.03 | 0.11 | 0.17 | 1.66 | 0.05 | 0.12 | 0.17 | 1.83 |
| Sep. 11 | ND | 0.37 | 0.50 | 0.32 | 0.16 | ND | 0.12 | 0.22 | 1.69 | 0.05 | 0.10 | 0.15 | 1.84 |
| Sep. 24 | ND | 0.39 | 0.60 | 0.33 | 0.18 | 0.03 | 0.12 | 0.21 | 1.86 | 0.04 | 0.08 | 0.12 | 1.99 |
| Oct. 9 | ND | 0.38 | 0.54 | 0.33 | 0.15 | 0.04 | 0.12 | 0.22 | 1.77 | 0.03 | 0.07 | 0.10 | 1.87 |
| Oct. 23 | 0.06 | 0.38 | 0.67 | 0.36 | 0.23 | 0.05 | 0.17 | 0.29 | 2.21 | 0.04 | 0.06 | 0.10 | 2.31 |
| Nov. 6 | ND | 0.42 | 0.42 | 0.33 | 0.18 | 0.06 | 0.13 | 0.23 | 1.76 | 0.06 | 0.06 | 0.12 | 1.88 |
| Nov. 20 | 0.05 | 0.40 | 0.59 | 0.36 | 0.20 | 0.06 | 0.14 | 0.27 | 2.06 | 0.05 | 0.08 | 0.13 | 2.18 |
| Dec. 4 | 0.04 | 0.38 | 0.53 | 0.36 | 0.16 | 0.06 | 0.13 | 0.28 | 1.95 | 0.06 | 0.07 | 0.12 | 2.07 |
| Dec. 17 | 0.05 | 0.39 | 0.57 | 0.34 | 0.16 | 0.06 | 0.15 | 0.34 | 2.06 | 0.06 | 0.08 | 0.14 | 2.20 |
| Jan. 18 | 0.07 | 0.41 | 0.48 | 0.34 | 0.19 | 0.09 | 0.17 | 0.27 | 2.02 | 0.07 | 0.06 | 0.13 | 2.15 |

**Abbreviations:** ND: Not Detected

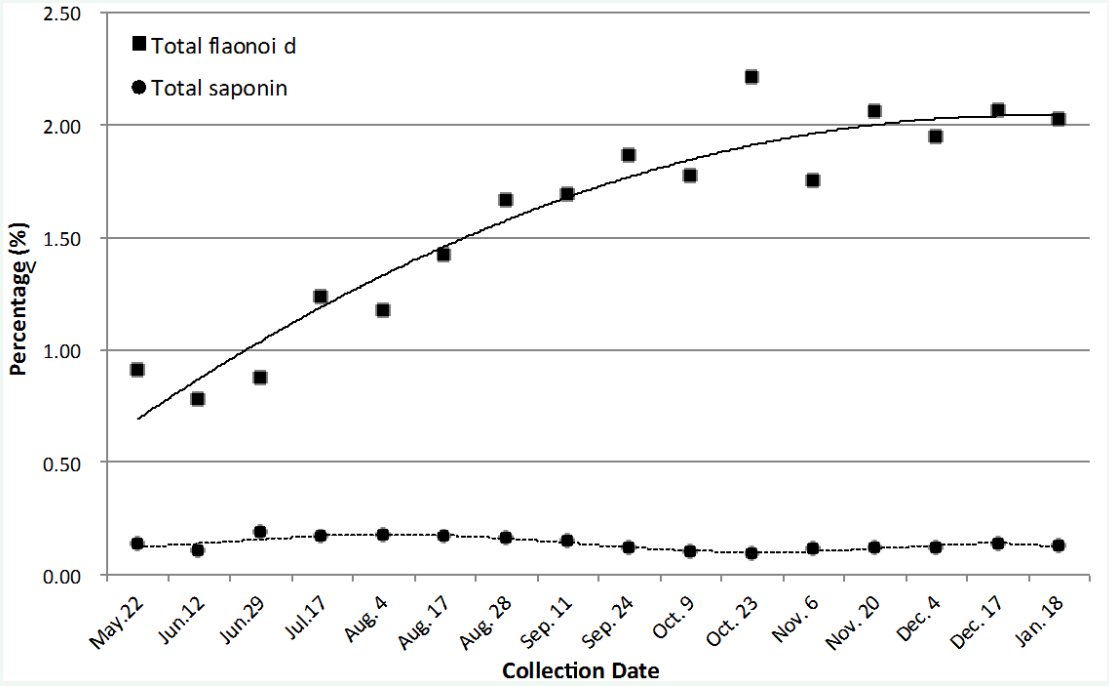


**Figure 3** Variation in percentage of flavonoids and saponins from May to January.

glycosides. Interestingly, of the flavonol glycosides, the amount of quercet in glycosides gradually increased from May to November, whereas the amount of kaempferol glycosides did not change much (Figure 5). The total amount of phytochemicals increased until late autumn (Table 1). Since kudzu leaves wither when it snows, the most appropriate time to harvest the leaves would be late autumn, just before the first snowfall.

## CONCLUSION

Since the leaves of kudzu contain many phytochemicals having various biological activities, kudzu leaves are a promising health food or herbal supplement. We investigated the variation in amounts of phytochemicals in kudzu leaves during growth using an HPLC system coupled to an evaporative light scattering detector. The amount of total saponin obtained from collected

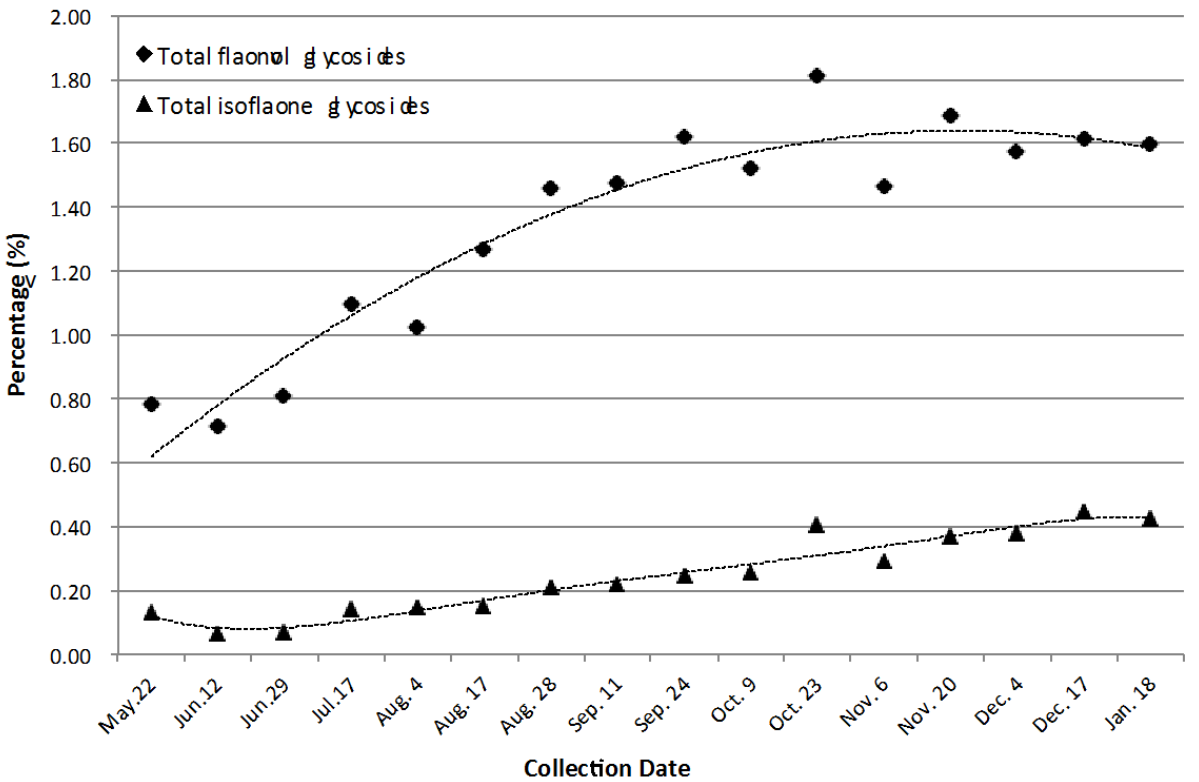


**Figure 4** Variation in percentage of flavonol glycosides and isoflavone glycosides from May to January.

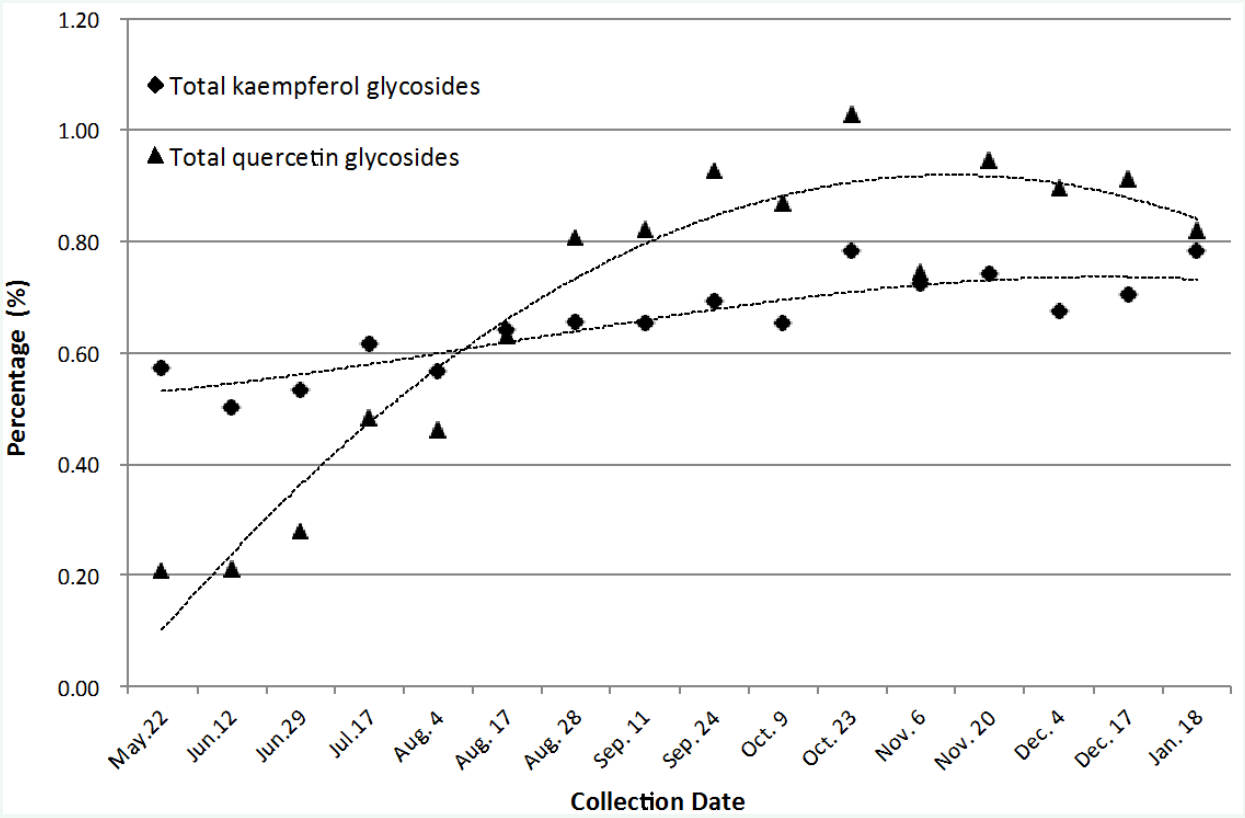


**Figure 5** Variation in percentage of quercetin glycosides and kaempferol glycosides from May to January.

**Table 2:** Percentage of phytochemicals in kudzu (*Pueraria lobata*) leaves.

| | | Aglycones | Compounds | Average Content |
|---|---|---|---|---|
| Flavonoid (1.59 %) | Isoflavone (0.25%) | Daidzein | Daidzin (**1**) | 0.02 ± 0.03 % |
| | | Genistein | Genistin (**6**) | 0.04 ± 0.02 % |
| | | | 6''-*O*-malonylgenistin (**8**) | 0.19 ± 0.09 % |
| | Flavonol (1.34 %) | Kaempferol | Robinin (**2**) | 0.40 ± 0.02 % |
| | | | Kaempferol 3-*O*-robinobioside (**5**) | 0.15 ± 0.04 % |
| | | | Nicotiflorine (**7**) | 0.10 ± 0.04 % |
| | | Quercetin | Quercetin 3-*O*-robinobioside (**3**) | 0.40 ± 0.19 % |
| | | | Rutin (**4**) | 0.29 ± 0.08 % |
| Saponin (0.14 %) | | Soyasapogenol B | Soyasaponin I (**9**) | 0.06 ± 0.01 % |
| | | Sophoradiol | Kaikasaponin Iii (**10**) | 0.08 ± 0.02 % |

kudzu leaves from May to January was fairly consistent. In contrast, the amount of total flavonoid increased gradually. The increase in content of flavonol glycosides was larger than that of isoflavone glycosides. The total amount of phytochemicals increased until late autumn. Therefore, the most appropriate time to harvest the leaves would be late autumn, just before the leaves wither.

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