Quantitative HPLC analysis of flavonoid in three different solvent extracts in leaves of *Gynura procumbens*

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**Abstract.** In Malaysia, traditional folks call *Gynura procumbens* as Sambung nyawa or translated as longevity life. The name given might be because of the claim made by them that the leaves extracts seems to be valuable and possess high theraphatic potential treatment of various diseases. Extraction of the leaves have been conducted by many researchers to identify the properties for antidiabetic/antihyperglycemic, antihypertensive, antimicrobial, antioxidant, antiinflammatory, antiulcer, anticancer, ant herpes and immunomodulatory. Extracts have been obtained using different solvent. Four alcoholic extracts and one water extract were studied separately. Soxhlet extraction was conducted in the following manner: plant material (3.0 g) was placed in the Soxhlet apparatus. Extraction process was carried out for three hours using 99.9% ethanol, methanol and water as a solvent (250 mL) until the solvent discoloration (3 hours). Yield of extraction techniques was determined by evaporating the solvent under vacuum using rotary evaporator. Previous research on solvent extraction of *Gynura procumbens* were also compared to whereby the methanol extract obtained by other researcher was 12.2%, ethanol extract with 14.73% and 27.83%. There is also one study extracting *Gynura procumbens* extracts via water, however the yield is not mentioned. High performance liquid chromatography (HPLC) coupled with ultra violet (UV) detector has been used for the separation and quantification of kaempferol from the *Gynura procumbens* leaves extract. Reversed phase high performance liquid chromatography separation was performed, using 0.1% phosphoric acid:acetonitrile as eluent in mobile phase. Our results confirm previous reports concerning the presence of several flavonoids.

1. **Introduction**

Kaempferol was one of the valuable bioactive compounds that can be found in *Gynura procumbens* leaves extract. Kaempferol is a sub grouped under flavonols in flavonoid compounds. Kaempferol can also be found highly in kale, endive, spinach and berries. Kaempferol is soluble in water, ethanol, methanol, diethyl ether and n-hexane (1). In 2016, an overview on the biological activities of *Gynura procumbens* was prepared by a group of researchers from Malaysia (2). According to this overview, the most solvent used for the extraction of *Gynura procumbens* leaves was ethanol.
A wide variety of extraction technique to determine kaempferol, quercetin and myricetin has been developed. According to Kaewseejan et al., (2015) kaempferol from *Gynura procumbens* extract were detected at 370 nm with UV-diode array detector and were separated using gradient elution of 1% acetic acid and acetonitrile at 38ºC (3). They extract the compounds via stirring with 95% ethanol three times. Jain and Shaikh (2016) able to extract kaempferol from crude drug extracts using methanol. The compound were detected at 370 nm with water:acetonitrile (45:55) mobile phase containing 0.1% O-phosphoric acid (4). There is also an extraction using methanol-25% HCl (4:1) by a group of researchers in China of *Sedum* crude medicines. The mobile phase used was methanol-0.4% phosphoric acid (47:53) and kaempferol was detected at 360 nm (5).

Besides kaempferol, quercetin and myricetin can also be found *Gynura procumbens* extract (6). Table 1 and figure 1 shows the structure, formula and molecular weight of kaempferol, quercetin and myricetin.

![Figure 1](image_url)

**Figure 1.** Structure of kaempferol, quercetin and myricetin showing the functional group attached.

| Common name | Formula | Molecular weight (g/mol) |
|-------------|---------|-------------------------|
| Kaempferol  | C_{15}H_{10}O_{6} | 286.239 |
| Quercetin   | C_{15}H_{10}O_{7} | 302.238 |
| Myricetin   | C_{15}H_{10}O_{8} | 318.237 |

Solvent extraction via *Soxhlet* has widely being applied to extract valuable components from plants. This technique not only can extract components from leaves (7), it can also extract compounds from stem, seeds (8), flower and roots (9) of plants. Besides solvent extraction, other technique also can be applied to extract desired components from plants such as microwave assisted extraction (10), maceration (11), supercritical fluid extraction (12) and ultrasound assisted extraction (13). Each technique has its own advantages and selected technique were usually based on constraints such as time, cost and complexity of equipment set up. Solvent extraction is chosen for this study to meet the time constraints, the availability of the equipment in the lab and low cost needed running the experiments.

The objectives of this study were to compare the yield of *Gynura procumbens* leaves extract using methanol, ethanol, water, ethanol/water and methanol water. Then, the extract will further be analyzed using HPLC to determine flavonoid compounds.
2. Material and methods

2.1. Plant material
The raw materials of the Gynura procumbens leaves were supplied by local company, Herbagus Sdn Bhd based in Kepala Batas, Penang was already ground and were below 10% humidity for the moisture content. It is then stored in a dry place until further use. The particle size distribution received by the supplier was determined by sieving. Table 2 shows the particle size distribution and the range in mm.

| Particle size range (mm) | Particle size distribution (%) |
|--------------------------|-----------------------------|
| x < 0.3                  | 4.2289                      |
| 0.5 > x > 0.3            | 8.5639                      |
| 1.18 > x > 0.5           | 54.9425                     |
| 2 > x > 1.18             | 26.0990                     |
| x > 2                    | 6.1658                      |

2.2. Standards and reagents
Kaempferol, quercetin and myricetin standard analytical grade (purity > 98%) of brand ChemFaces were purchased from Scienfield Expertise PLT. All solvents used for extraction and chromatography were of analytical grade (R&M Chemicals and QReC). Methanol HPLC grade was used for extract dissolve for analysis process.

3. Solvent extraction
Soxhlet extraction was conducted in the following manner: plant material (3.0 g) was placed in the Soxhlet apparatus. Extraction process was carried out for three hours using ethanol, methanol ethanol/water, methanol/water and water as a solvent (250 mL) until the solvent discoloration (3 hours). Yield of extraction techniques was determined by evaporating the solvent under vacuum using rotary evaporator. The dried extracts were kept in the fridge until analysis. Yield (Y) was expressed as grams of dry extract per 3 g of dry plant material (g/3 g DP).

4. High Pressure Liquid Chromatography (HPLC) analysis

4.1. Preparation of Working Standards and Sample Solutions
Entire HPLC method development was done using kaempferol, quercetin and myricetin. Preparation of standard solution and sample solution was done using methanol of HPLC grade solvents.

4.1.1 Working standard of kaempferol, quercetin and myricetin. 2mg of standard dissolved in methanol and made up to 100mL volume to yield a concentration of 0.02mg/mL or 20 µg/ml stock solution. Then from the 20 µg/ml stock solution further dilution with methanol into 10, 5, 3, 2 and 1 µg/ml is prepared and filtered through 0.45 µm NY membrane filter. This was then used for HPLC analysis.

4.1.2 Sample solutions. For analysis, alcoholic dried extract of Gynura procumbens was dissolved in methanol at concentrations of 1-5 mg/ml. All of these solutions were sonicated at 60°C for 30 min to ensure all solids were completely dissolved before analysis by HPLC. Then it is filtered through 0.45µm NY membrane filter and ready to be injected. The same processes were followed for the preparation of sample extract in water.
Quantitative data were calculated from the calibration curves. Regression equation and coefficient ($R^2$) were also determined. Content of flavonoid compound were expressed as milligrams per gram of extract (mg/g).

4.2. HPLC procedure
The HPLC analysis was conducted at Analysis laboratory in Faculty of Engineering Built and Environment, UKM, Malaysia. It was performed with an Agilent LC 1100 Series HPLC. The analysis process were carried out with a C-18 Phenomenex XD 250 mm column, volume injection of 20µl and a flow rate of 1.0 ml/min. All analyses were performed at 35°C, with a mobile phase of Phosphoric acid (0.1%):Acetonitrile of 65:35 ratio, and was pumped into the column. The UV absorbance of the eluent was measured at 220nm.

5. Results
5.1. Yield of extraction
Water gives the highest yield with 22.48% compared to other alcoholic extracts. Both ethanol extracts (pure and with water) give higher yield than methanol. The colour of the extracts was mostly medium to dark green for alcoholic extracts, and brown tea-like for water extract. Different solvent gives different form of residue extract (Table 3).

| Extracting solvent  | Yield (%) | Colour of extracts  | Residue form  |
|---------------------|-----------|---------------------|---------------|
| Methanol            | 11.55     | Dark green          | Sticky paste form |
| Ethanol             | 16.19     | Medium dark green   | Sticky paste form |
| Water               | 22.48     | Brown tea-like      | Flakes        |
| Methanol/water      | 13.15     | Medium dark green   | Sticky gel form |
| Ethanol/water       | 15.23     | Medium dark green   | Sticky gel form |

Previous research on solvent extraction of *Gynura procumbens* were also compared to whereby the methanol extract obtained by other researcher was 12.2% (14), ethanol extract with 14.73% (15) and 27.83% (3). There is also one study extracting *Gynura procumbens* extracts via water, however the yield is not mentioned (16).

Both extract from pure ethanol and methanol dissolve easily in methanol for further flavonoid compound analysis. Because of that, these two samples were proceeded to next process for HPLC analysis. This is supported by Singh & Bharati (2014). They described that, kaempferol, quercetin and myricetin easily dissolve in alcohol and not in water (Table 4).

| Flavonoids    | Water       | Alcohol          | Acetic acid |
|---------------|-------------|------------------|-------------|
| Kaempferol    | Slightly soluble | Boiling: Readily soluble | n.a         |
| Quercetin     | Hot water: Slightly soluble | Boiling: Soluble | Glacial: Soluble |
|               | Cold water: Insoluble |                   |             |
| Myricetin     | Boiling water: Sparingly soluble | Soluble | Insoluble |

5.2. HPLC analysis
The total run time was found to be less than 9 min, while the retention times of kaempferol, quercetin and myricetin were observed at 8.40, 5.46 and 3.88 min, respectively. This indicates that the present HPLC method is rapid, easy, and convenient. The presence of flavonoids in the plant extract was confirmed by comparison of their retention times and overlaying of ultraviolet (UV) spectra with those
of standard compounds. Regression equation for all compound able to get value of $R^2 = 0.99$ with the range concentration of the calibration curve of 1 to 20 $\mu$g/ml.

Ethanol extract shows higher amount of kaempferol and quercetin than from methanol extract. Myricetin however shows slightly higher amount in methanol extract than in ethanol extract (Table 5). A study by a group of researchers in Thailand claimed that the crude ethanolic extract contain 464.53, 135.87 and 251.10 $\mu$g/g of kaempferol, quercetin and myricetin respectively (3).

Table 5. Flavonoid compound content of alcoholic Gynura procumbens leaves extracts.

| Common name | Methanol extract (mg/g) | Ethanol extract (mg/g) |
|-------------|-------------------------|------------------------|
| Kaempferol   | 0.59                    | 0.70                   |
| Quercetin    | 0.05                    | 0.37                   |
| Myricetin    | 0.14                    | 0.12                   |

6. Conclusion
Gynura procumbens has successfully extracted using solvent extraction method. Five different solvents were used as an extracting solvent. As reported in table 3 and table 5, higher yield does not proportionally mean that it will give a higher amount of flavonoids. The flavonoids were separated based on the solubility principle of the solvent used during the solvent extraction process. Further study on analyzing compounds extracted using water and alcohol/water solvent will be conducted using other dissolving solvents.

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