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Flavonoid compound of *Cucurbita moschata* at three different altitudes

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Abstract. *Suranto, Hidayati NR, Furqan M, Mahadjoeno E, Sajidan. 2023. Flavonoid compound of* Cucurbita moschata *at three different altitudes. Biodiversitas 24: 1853-1860.* The aims of this research were to examine the presence of flavonoids, both the total and the content of quercetin and kaempferol, in *Cucurbita moschata* (Duchesne) Duchesne ex Poir. leaf growing at different altitudes. First, Thin Layer Chromatography (TLC) was used to detect the presence of flavonoid substances, followed by the colorimetric $AlCl_3$ method to look at the Total Flavonoid Content (TFC). Finally, one-way ANOVA was employed to analyze quantitatively. Accordingly, High-Performance Liquid Chromatography (HPLC) method was used to test whether any member of flavonoid substances was found. The results showed that flavonoid substances of *C. moschata* leaf samples were detected, and the Rf value of every single altitude was not significantly different. However, the highest total flavonoid content was recorded from the middle altitude (1.369 mg QE/g); the lowest was found in the highest (0.244 QE/g). Furthermore, HPLC tests showed that retention times of quercetin at every single altitude from the lowest to the highest one were not much different. Meanwhile, kaempferol's retention time was recorded at 13.001 minutes for the lowest altitude and 12.955 and 12.965 minutes for the middle and highest altitudes, respectively. These preliminary results, especially the total flavonoid content in different altitudes, could detect various flavonoid members, such as quercetin and kaempferol, for the next project.

Keywords: Altitude, *Cucurbita moschata*, flavonoid, HPLC, TFC, TLC

INTRODUCTION

Pumpkin (*Cucurbita moschata* (Duchesne) Duchesne ex Poir), as one of the very important members of *Cucurbita* genus (Cucurbitaceae), has been recorded as the most popular fruit of the cultivated plants in the world (Hazra et al. 2007). This South Mexican plant (Whitaker 1981; Lim 2012) has been domesticated in other Mexican areas and South America. These very easily grown fruits of pumpkin have been noted to have many varieties and have been studied intensively for several decades. And this could be interpreted that this plant was easily growing and adapting to new environmental conditions, such as in tropical countries, e.g., Indonesia.

The multifunction of the pumpkin plant for human beings was recorded for the fruit and the leaves. Lim (2012) reported that the people of Kenya, Malawi, and Zimbabwe consumed pumpkin leaves. Besides, these leaves were also used as a medicinal plant for people in several tropical Asian countries and some parts of the Pacific regions. The very useful pumpkin leaves in curing intestinal and lung cancer cells within in vitro trial experiments (Kwak and Ju 2013). It is believed that the very use of these plants in human health was recorded not only due to the nutrition contained but also the bioactive substances within its leaves. A few bioactive compounds within pumpkin leaves were phenol, alkaloids, terpenoids, and flavonoids (Fidrianny et al. 2014; Suradkar et al. 2017). In addition, they noted that the highest concentration of flavonoids was recorded in these leaf plant organs compared to their fruits, rinds, and seeds (Kim et al. 2011). As a strong antioxidant, flavonoids have been recorded to have the capability to reduce the total number of free radicals. As an antioxidant, flavonoids could be used OH as an electron donor to reduce the total number of hydrogen peroxide (H_2O_2) with the help of the peroxide enzyme (Ferdinando et al. 2012).

The existence of bioactive substances within an individual plant is usually influenced by environmental conditions to adapt to new environmental changes. Many environmental conditions, such as altitudes, were also influenced by other environmental factors, such as temperature, relative humidity, and UV-B radiation (Körner 1999). Besides, oxygen level, soil condition, type of soil, and soil porosity were also considered to have influences in adapting pumpkin plants to the different altitudes (Yuliani et al. 2015). The contained flavonoid changes within plant organs could trigger the metabolism process change, which is usually catalyzed by several enzymatic reactions. This experiment used the TLC, TFC, and HPLC methods to examine any difference in their type of flavonoid substance when *C. moschata* plants grow at different altitudes.

MATERIAL AND METHODS

Sample location

Research locations for sampling purposes were determined at three different altitudes. The first location is

1-350 meters above sea level (masl), the second is 351-700 masl, and the third is 701-1050 masl. These locations were observed at three different times.

Sample extraction of *C. moschata*

Leaf samples of *C. moschata* were chosen as the material for examining the secondary metabolisms. A total of 500 grams of leaves were rinsed, selected, and air-dried for 21 days. Afterward, the dried leaves were mashed using a blender (Philip HR-2115). Finally, filtering was done to get the best leaf powder.

The secondary metabolism was extracted using the Bouzid et al. (2015) method with some modifications. The resulting extraction was then filtered using Whatman 1 filter paper and evaporated using a rotary evaporator (RE300) at 40°C. Evaporation was conducted using a water bath (607 WINA) and kept in the refrigerator at 4°C until used.

Thin Layer Chromatography (TLC) procedure

To detect the flavonoids, TLC was used using a mobile phase of chloroform: ethyl acetate (6:4 v/v), which was saturated in the static state of silica gel. As many as 10 mg of methanol extract were diluted in 1 milliliter (1 mL) of methanol. A total volume of 5 µL of the solution and 5 µL standard solution were spotted in the media, letting the solution dry. After that, the silica gel's bottom line was submerged at the bottom saturated mobile phase until the solution reached the top line. The dried silica plate was then detected using UV light at 254 nm after being steamed using ammonia (NH_3). The detected spots were then calculated for their movement based on their retardation factor (Rf), as used by Sambandam et al. 2016.

$$Rf = \frac{\text{the distance of movement solution from the first spot}}{\text{distance of solution movement}}$$

Total of Flavonoid Content (TFC)

The making of a quercetin standard solution

The quercetin standard solution was diluted 10 mg of quercetin in 10 mL of methanol solution. The 100 µL/mL quercetin concentration was made up by mixing 1 mL of the above quercetin solution in 10 mL methanol. The curve standard of quercetin was made from this solution by pipetting 2, 4, 6, 8, and 10 mL of the above solution (100 µg/mL) to be diluted to 10 mL methanol, respectively. This solution will produce a concentration of solutions that become 20 µg/mL; 40 µg/mL; 60 µg/mL; 80 µg/mL, and 100µg/mL, respectively. In addition, the above solutions were added 0.3 mL of 5% $NaNO_2$ (for 5 minutes), 0.3 mL of 10% $AlCl_3$ (6 minutes), and 2 mL IM NaOH. The standard solution for absorption will be visualized using UV-Vis Spectrophotometer using a wavelength of 415 nm (Shimadzu UV mini 1240 UV-Vis).

Calculate the total flavonoid content

The methods of colorimetric $AlCl_3$ were used to detect the total flavonoid content, as demonstrated by Ghasemzadeh et al. 2012 and Rebaya et al. 2014. The total flavonoid content will be calculated using UV-Vis Spectrophotometer (Shimadzu UV mini 1240 UV-vis) employing 415 nm wavelength. Absorbance values were then calculated by the formula listed below:

$$Y = ax + b$$

The first flavonoid concentration can be used to calculate the total flavonoid content as conducted by (Bag et al. 2015). The total flavonoids content could be calculated using the formula as follow:

$$F = \frac{c \times V \times Fp}{m}$$

Where:

YY : value of absorbance
xx : concentration of flavonoid (µg/mL)
a, ba, b : Constanta
F : total flavonoid content (mg quercetin/g dry weight)
C : early flavonoid concentration(µg/mL)
V : volume of used sampel (mL)
Fp : diluted factor
m : weight of the used sample (g)

The result of the calculation of total flavonoid content was measured in mg QE/g dried weight

Calculation of blank solution

A blank solution for every single solution resulted in a diluted quercetin standard. Therefore, each extract was made in the same procedure using a methanol solution to replace the extract and quercetin standard.

High-Performance Liquid Chromatography (HPLC)

Purification procedures of quercetin and kaempferol in each sample were conducted using gradient HPLC waters (2695), according to Jain et al. (2016), employing the mobile phase of H_2O: acetonitrile (45:55) containing 0.1% M-phosphoric acid. A total of 20 µL of each standard and sample were injected using a flow rate of 1.0 mL/min. The wavelength of 370 nm was chosen to detect the presence of quercetin and kaempferol substances.

Analysis of data

Therefore, to analyze the presence and absence of flavonoid substances were conducted qualitatively and quantitatively. Meanwhile, flavonoid content was analyzed quantitatively using an Analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). The presence of quercetin and kaempferol was analyzed by comparing the retention times of the sample and the standards used.

RESULTS AND DISCUSSION

Thin layer chromatography

Figure 1 shows the yellow spot formed for the leaf extract of *C. moschata* and the quercetin standard. These yellow spots were detected when the mobile phase elution process was conducted. Table 1 shows only little variations between Rf values of quercetin at three different altitudes resulting from every leaf extract of *C. moshata*. These Rf values ranged between 0.81 and 0.85; the quercetin standard was 0.78. It was interesting to note that the highest Rf value (0.85) was recorded at the middle altitude.

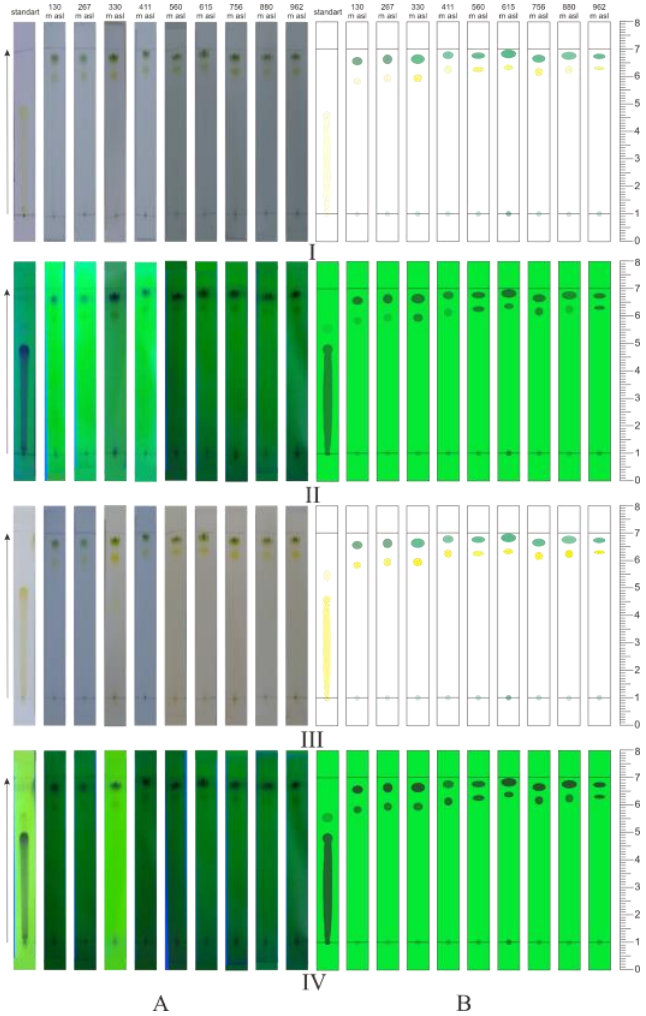

Figure 1. Thin Layer Chromatography leaf extract of *Cucurbita moschata* from different altitudes before and after being treated with NH_3. A. Original spots of TLC, B. Redrawing spots of TLC. Note: I: Visible light before NH3 steaming; II: UV 254 nm before NH3 steaming; III: Visible light after NH3 steaming; IV: UV 254 nm after NH3 steaming

Total of Flavonoid Content (TFC)

Leaf extracts of methanol of *C. moschata* from different altitudes and quercetin standards were observed in their absorbance at a wavelength of 415 nm. The quercetin standard absorbance was calculated to get the standard quercetin curve. This standard curve is shown in Figure 2.

Figure 2 shows the increasing quercetin standard absorbance and the increasing quercetin standard from 20 µg/mL to 100 µg/mL. The Y=0.0308x+0.0115 with R2=0.9686 of quercetin standard curved was formed based on that standard. This regression equation Y=0.0308x+0.0115 was used to calculate the flavonoid content of each leaf extract of *C. moschata* at every altitude. The total flavonoid content of flavonoid samples at three different altitudes is presented in Figure 3.

Table 1. Value of retardation factor (Rf) of quercetin standard and methanol leaf extract of *Cucurbita moschata* using TLC test at three different altitudes

	Altitude range	Altitude (m asl.)	Rf value
Quercetin (standard)	-	-	0.78
1	Lower (I)	130	0.81
2		267	0.81
3		330	0.81
4	Middle (II)	411	0.85
5		560	0.85
6		615	0.85
7	Upper (III)	756	0.83
8		880	0.83
9		962	0.83

Note: I (1-350 m asl), II (351-700 m asl), III (701-1050 m asl); no. 1 to 9 were the number of samples used

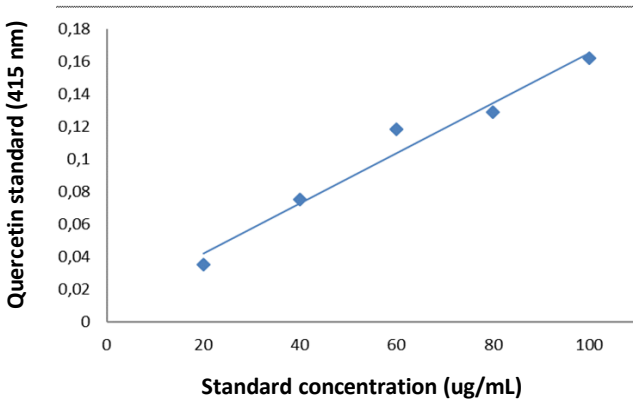

Figure 2. The curve of the quercetin standard is the base calculation of total flavonoid content in this experiment

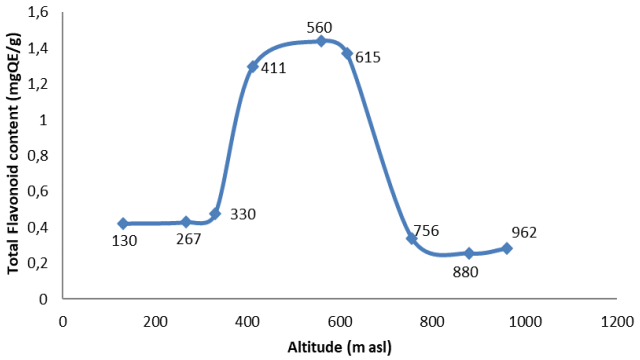

Figure 3. Total flavonoid content of *Cucurbita moschata* leaves extracted methanol sampled from every single altitude (mg QE/g)

It was recorded that total flavonoid content would increase along the altitudes; these could be seen on the altitudes of 130, 267, 330, and 411 masl, respectively; the optimum result was recorded at 560 masl. On the other hand, the decreasing amount of total flavonoids started from 615 masl up to below 880 masl. Based on total flavonoid content at every altitude, they were classified into three groups depending on their altitude ranges. As seen in Figure 4, there were significant differences in their total flavonoid content between the three altitudes group. The highest total flavonoid content (1.369 mgQE/g) was found at the middle altitudes ranging between 351 and 700 masl. This result differed significantly from low altitudes areas of 1-350 masl (0.442 mgQE/g) and higher altitudes areas of 701-1050 masl (0.244 mgQE/g). These results showed that the optimum production of quercetin was recorded in the middle area rather than the lower or upper locations.

High Performance of Liquid Chromatography (HPLC)

Quercetin and kaempferol as the member of flavonoids substance on the leaf extract methanol of *C. moschata* have been detected by comparing the retention times standard of quercetin and kaempferol with retention times samples at different altitudes. Figures 5 and Figure 6 show 7.827 min for quercetin and 12.878 min for kaempferol, respectively. Every single sample showed its peak of flavonoid substance. The two flavonoid derivatives, quercetin, and kaempferol, showed slightly different retention times. The time for quercetin found at lower altitudes was 7.792 min; meanwhile, 13.011 min was recorded for the kaempferol (Figure 7). At the middle altitude, the peak of quercetin was observed at 7.786 and 12.955 for kaempferol (Figure 8). In addition, this substance of kaempferol at the highest altitude was detected at 12.965 min and 7.775 for quercetin, respectively (see Figure 9).

The total value of quercetin peak area shown at the chromatogram on the lower altitude was 202.99 unit volume area); accordingly, 32330.09 and 1616.49 unit volume areas were recorded. These pictures were smaller than the quercetin standard (2937857.89 unit volume area). This picture was also shown by kaempferol: 738.02, 1302.05, and 241.71 unit volume areas for the lower, middle, and highest altitudes, respectively, compared to the standard of 872371.28 unit volume area.

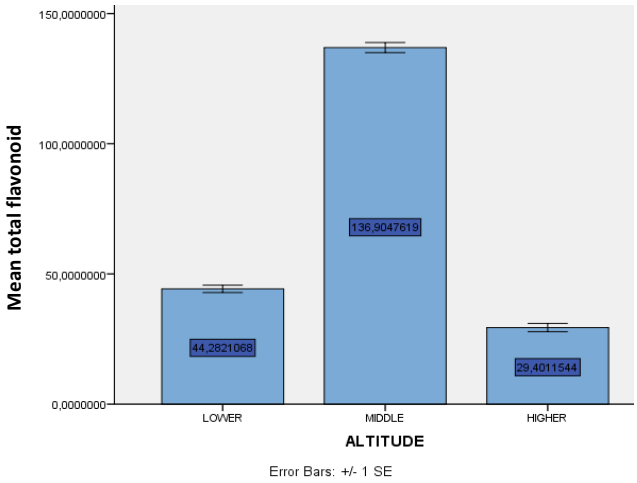

Figure 4. The average total flavonoid content in methanolic extract of *Cucurbita moschata* leaves at every single altitude (mgQE/g); Error bars +/- 1 SE

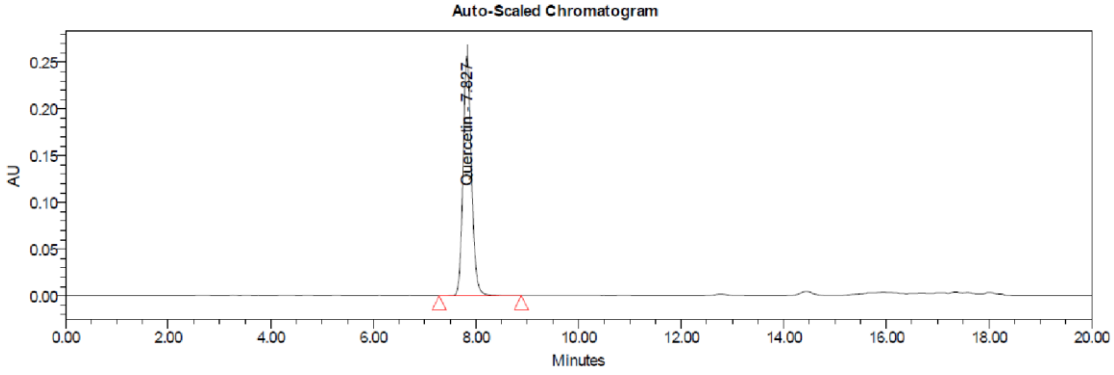

Figure 5. Chromatogram HPLC of quercetin standard

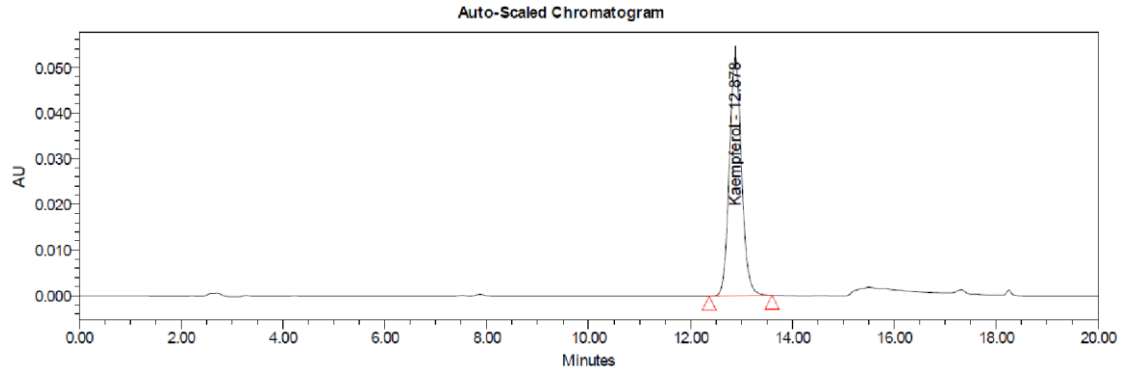

Figure 6. Chromatogram HPLC of kaempferol standard

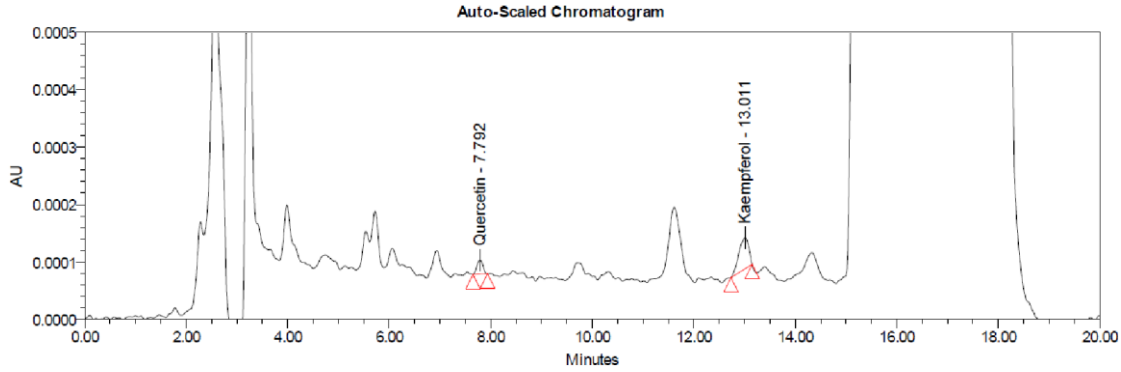

Figure 7. Chromatogram HPLC of *Cucurbita moschata* leaf extract at a lower altitude

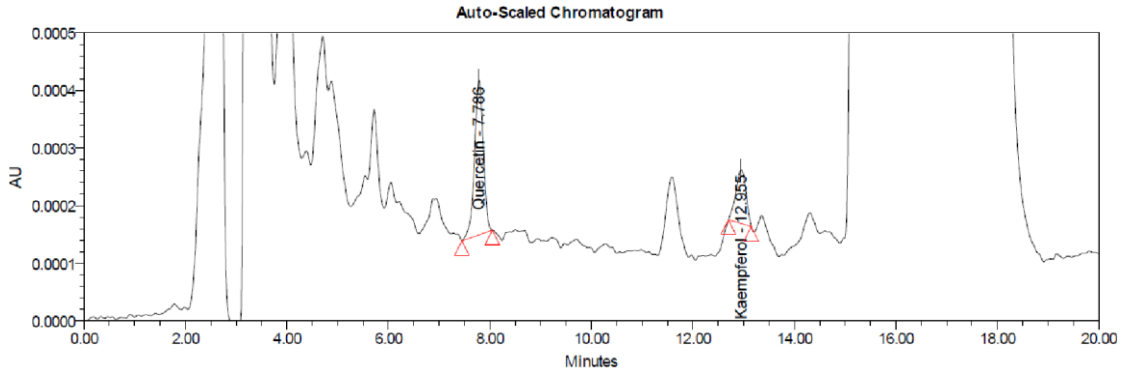

Figure 8. Chromatogram HPLC of *Cucurbita moschata* leaf extract at middle altitude

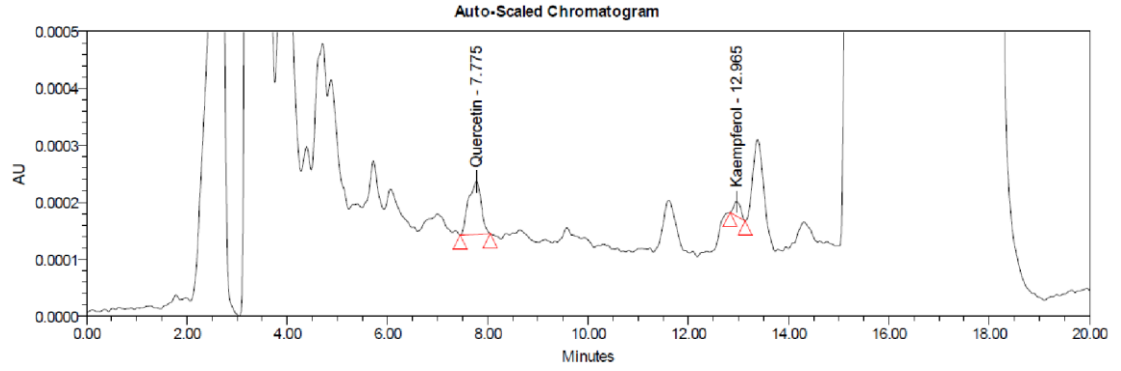

Figure 9. Chromatogram HPLC of *Cucurbita moschata* leaf extract at upper altitude

Discussion

Quercetin has been recorded as one of the very crucial substances of antidepressants. This is due to its antioxidant content, anti-implation, and also neuroprotective effects. Therefore it would be a potential substance in treating and preventing depression (Silvestro et al. 2021). In addition, quercetin and kaempferol have been noted as anticancer by blocking the Nuclear receptor 4A1(NR4A1)-regulated responses in Rhabdomyosarcoma (RMS). These substances are also believed to function in hypersensitive and cardioprotective substances in the human body. Meanwhile, they could influence seedling and pollen development, photosynthesis, and plant induction within the plant life cycle. Moreover, they could grow well by regulating the ROS signal, which could modulate the auxin and ABA hormones (Singh et al. 2021; Jan et al. 2022).

Quercetin is noted as flavonol and classified as a group of glycan flavonoids and is usually easily eluted using 60:40 chloroform and ethyl acetate (Wagner and Bladt 2001). The typical yellow spot would be noted after being treated with ammonia (NH_3) gas on the 254 F of silica plat. This ammonia gas has been reported to be one of the best flavonoid reactants in producing yellow color in visible light (Dwiatmaka 2010). These pictures confirmed that the leaf extract of *C. moschata* contained a flavonoid substance with quercetin as standard using a wavelength of 254 nm; the GF 254 silica plat would be fluorescence due to chromophore groups that eventually produce dark spots.

Recent studies in extracting the leaf samples were shown by Sonam et al. (2017). They recorded that flavonoids substance extracted from leaves of *Reinwardtia indica* Dumort. produce Rf values of 0.80-0.92. Accordingly, Janakiraman and Jeyaprakash (2015) reported a similar Rf value (0.84) of extracted methanol of the flavonoid substance of *Vitex negundo* L., which is indicated as quercetin, was found. These results followed the result of *C. moschata* leaf (0.81, 0.83, 0.85), in which the lower altitude (0.81) was slightly lower compared to the middle and higher altitudes (0.83 and 0.85). And these results were very close to Janakiraman and Jeyaprakash's findings. Interestingly, more total flavonoids (1.369 mg QE/g) in this experiment were found at the middle altitude when this result was compared to Kwak and Ju (2013), in which they only got 0.736 mg QE/g of dried weight. However, this result was still higher, while it was compared to other altitudes.

The difference in plant metabolic content has been believed to be influenced by environmental conditions, especially in producing secondary metabolisms. This follows Jan et al.'s (2022) statement that flavonoid concentrations would depend on growth condition development phases, type of organs, and environmental conditions. It is recorded the highest total flavonoid content in *Matricaria chamomilla* L. cv. Bona was evident at the highest altitude compared to other lower altitudes. This phenomenon may be due to more UV-B radiation which eventually is triggered by more flavonoids produced (Ganzera et al. 2008). However, this statement was not the same as this finding. This condition may correlate with high rainfall conditions at that altitudes. The highest rainfall at higher altitudes could be influenced by the limitation of plants in absorbing sunlight during overcast. Meanwhile, normal light intensity and good microclimate quality would potentially increase plants' total secondary metabolism (Magagnini et al. 2018). The normal light intensity would benefit the plants' optimal photosynthesis, which could produce optimal total carbons for photosynthetic purposes of secondary metabolism. And those have been supported by Wang et al. (2022) opinion that light intensity was very potentially inducted to the accumulation of flavonoids and their enzyme system, which trigger the production of flavonoids such as Phenylalanine Ammonia Lyase (PAL) and 4-coumaric acid coenzyme A ligase (4CL). The result, especially the total flavonoid content in the middle altitude, followed what Krishnamurthy and Sarala (2013) did. They recorded that the highest flavonoid concentration, *Artocarpus gomezianus* Wall. ex Trécul, was found at middle altitude; meanwhile, the lowest concentration was reported from the highest and lowest altitudes.

Recent evidence for the highest flavonoid content, particularly in the middle altitude, was also shown by Yuliani et al. (2019) when they worked with *Elephantopus scaber* L. and *Aeratum conyzoides* L. They argued that the highest secondary metabolism was usually related to the soil condition. The quercetin was only detected in the cultivated plants but not in the uncultivated area. In addition, the cultivation method of plants and the environmental factor were noted to influence the presence of secondary metabolism besides the environmental condition. As Yuan (2020) reported in *Dendrodium officinale* Kimura & Migo, further factors such as temperature and intra-cellular CO_2 concentration were also recorded to be crucial factors in forming the biochemical trait of *Camellia sinensis* (L.) Kuntze leaves (Hazra et al. 2021). A further finding was reported by Zhang et al. (2021) in which improvement in CO_2 concentration could develop the plant protection system, which grows on the surface of cadmium-contaminated soil by synthesizing the flavonoids.

Based on the HPLC test on derivative flavonoid substances on the leaf of *C. moschata*, two substances were detected (quercetin and kaempferol), which are parts of the strong antioxidant substances (Vellosa 2011; Ozgen et al. 2018). This finding followed Kuponiyi et al. (2013) in which they recorded that quercetin, kaempferol, and 5,7-diethyl-3,4-dimethoxy-2', 6'dimethyl flavan were also detected in leaf samples of *C. moschata*. Meanwhile, kaempferol, a flavonoid substance, was detected in the seed of *C. moschata* (Sakshi et al. 2018). Furthermore, Cao et al. (2010) and Kulczynski and Gramza-Michalowska (2019) reported quercetin, rutin, isoquercetin, astragalin, and myricetin were detected within this fruit.

The wide use of the HPLC-DAD in plotting the flavonoids based on plant part and geographical origin was reported by (Khuluk et al. 2021). Those flavonoids e.g., orientin, hyperoside, rutin, myricetin, luteolin, quercetin, kaempferol, and apigenin. Therefore, HPLC was employed to purify the existence of quercetin and kaempferol substances within the *C. moschata* sample. The

chromatogram showed that small differences in retention time at each altitude were evident. However, the widest area of quercetin and kaempferol substance was found at the middle altitude compared to other altitudes. It is well understood that environmental condition usually influences bioactive substances within the plant. This was recorded by Senica et al. (2016), the existence of metabolite on secondary metabolism in *Sambucus nigra* L. leaf, flower, and fruit was influenced by altitude. In addition, Su et al. (2017) noted that both altitude and precipitation have been recorded to influence their quercetin and kaempferol on the leaves of *Hippophae rhamnoides* L. subsp. intensify. Besides, different seasonal effects have also been considered to influence the metabolism of secondary metabolites of plants growing at different altitudes (Oruc et al. 2017).

Another total flavonoid content recorded with the highest quercetin was found in plants growing in the middle altitude (Sulastri 2018). The condition of growing plant areas, particularly in the middle altitude, generally determines the highest secondary metabolism, such as quercetin. It is recorded that abiotic stress usually could cause a dropping the production of drug plants. Davoudi et al. (2022) recorded that Heat Shock Protein 70_s at *C. moschata* (CMoHSP70_s) would be useful in fading plants under environmental stress. During growth and development, plants interact with different high and ultraviolet radiation, temperature stress, drought stress, salinity stress, as well as another factor such as soil conditions and even chemical stress, which could give effect decreasing plant physiological properties (Li et al. 2020; Taratima et al. 2022). One of the above conditions may result in the quercetin concentration being different.

In conclusion, plants growing at different altitudes have made it possible to be detected in their secondary metabolisms, such as flavonoids and antioxidant substances. The highest total flavonoid contents found at the middle altitude show that this plant habitat may contribute to their secondary metabolism products. The detected quercetin and kaempferol have become good preliminary results for further exploring the potential of *C. moschata* to be presented as an antioxidant material in medical drug substances, particularly for Indonesian natural plants in the near research project.

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