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Leaf and flavonoid production of perennial sow-thistle (*Sonchus arvensis* L.) at different growth stages

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Abstract

Perennial sow-thistle (*Sonchus arvensis* L.) is one of medicinal plants in Indonesia that is commonly used as diuretic, lithotripic and antiurolithic. This research studied the production and flavonoid content of perennial sow-thistle growth as wild plants, as basic information for *S. arvensis* cultivation. In situ observation on morphological characters then measurement of the plant biomass were conducted. The research was conducted with two sampling times i.e in May 2015 and September 2015. Wild plant samples of *S. arvensis* were taken from open field at Bogor Agricultural University (6033'23.0"SL, 106043'54.5"EL), Bogor, Indonesia. Plant materials were harvested at three phenological stages i.e. vegetative stage (bolting phase), the second stage was early generative stage (the flower buds begin to form) and the last one was maximum generative stage (full flowering). Five plants as replications were used for each stage of growth. Data were analyzed to compare the results between the phases of growth followed by the Pearson correlation tests to determine the relationship between variables. The results showed that maximum growth of basal leaves was found at vegetative stage, while maximum growth of stem leaves was at early generative stage. The highest flavonoid content of wild perennial sow-thistle was found at maximum generative stage.

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Introduction

Perennial sow-thistle (*Sonchus arvensis* L.) or called "tempuyung" in Indonesia is one of medicinal plants that is commonly used as diuretic, lithotripic and antiurolithic. *Sonchus arvensis* also has effects on human health through the detoxification and mobilization of blood circulation (Khan, 2012), valued as a delicious and nutritional herb and has been used for the treatment asthma, coughs, and other chest complaints and for calming the nerves, has insecticidal properties and anti-inflammatory activity (Xia and Liang, 2010), contains flavonoids and phenolic (Cinnamic acid) that play a role as antioxidants (Xu *et al.*, 2008). Extract of *S. arvensis* leaf can be used as a source of antioxidants that are effective, safe and commercially can be developed as medicines (Khan, 2012).

S. arvensis is originated from Europe and later spread to new areas such as the western part of Asia (Lemna and Messersmith, 1990). It is classified as an important species among the perennial weeds because of its unique vegetative reproductive system. It has a fast growing reproductive system which reproduces with roots and seed. It is deep-rooted with the whole plant filled with milky latex. It is a C3 plant and is adapted to various soil types but mostly the neutral or slightly alkaline soils meaning that it does not do well in highly acidic soils (Lemna and Messersmith, 1990). *S. arvensis* leaf are mainly found on the lower section of the stem and it develops rosettes at an early stage of growth, thereby increasing the photosynthetic area of the plant. The roots begin to thicken when *S. arvensis* plants have 5-7 leaves and at this juncture the plant is considered to have reached its compensation point i.e. the minimum level of root reserves (Vanhala, 2006). The plant will easily flower when develops under favourable conditions with respect to light and nutrition (Hakansson and Walgren, 1972).

S. arvensis generally grows wild as weeds and found in a wide range of habitats. It occurs in cultivated fields, in disturbed areas, along roadsides, and in ditches (Mc Williams, 2004).

Fulfilling the needs of simplicia, *S. arvensis* generally derived from wild plants. If the demand of raw material from wild plants continues, *S. arvensis* will be more difficult to find in the future. As a source of herbal medicine, plant cultivation is required to increase production of simplicia. Therefore, it is necessary to study the characteristics of *S. arvensis* growth as wild plants as a basic information for cultivation.

The aim of the study was to evaluate and compare leaf production and flavonoid content of *Sonchus arvensis* at different growth stages and to compare those variables at different observation times.

Methods

Experimental Site

The research was conducted from May to September 2015 with two sampling times, i.e. 10th May and 15th September 2015. Wild plant samples of *S. arvensis* were taken from the open field at Bogor Agricultural University, Darmaga, Bogor, Indonesia (6°33'23.0 "SA-106°43'54.5"EL). The plants grow wild on the roadside. Production and pigment analysis were conducted at Post-Harvest Laboratory, Department of Agronomy and Horticulture, Bogor Agricultural University while phytochemical analysis was conducted at Tropical Biopharmaca Research Center, Bogor Agricultural University, Indonesia.

Plant Material

S. arvensis plants were harvested at three growth stages i.e. vegetative stage (stem begins to growth), with a mean height of plant 4.5 cm. The second stage was early generative stage (the flower buds begin to form), with a mean height of plant 92 cm and the last stage was maximum generative stage (full flowering stage), with a mean height of plant 125.8 cm (Fig. 1). Five plants as replication were harvested from every growth stage then transferred to the laboratory for further analysis. Fresh plant samples were separated into leaves, stems, flowers and roots.

The following measurements were fresh weight. The leaves were oven dried (60°C) for 3x24 hours and measured as dry weight.

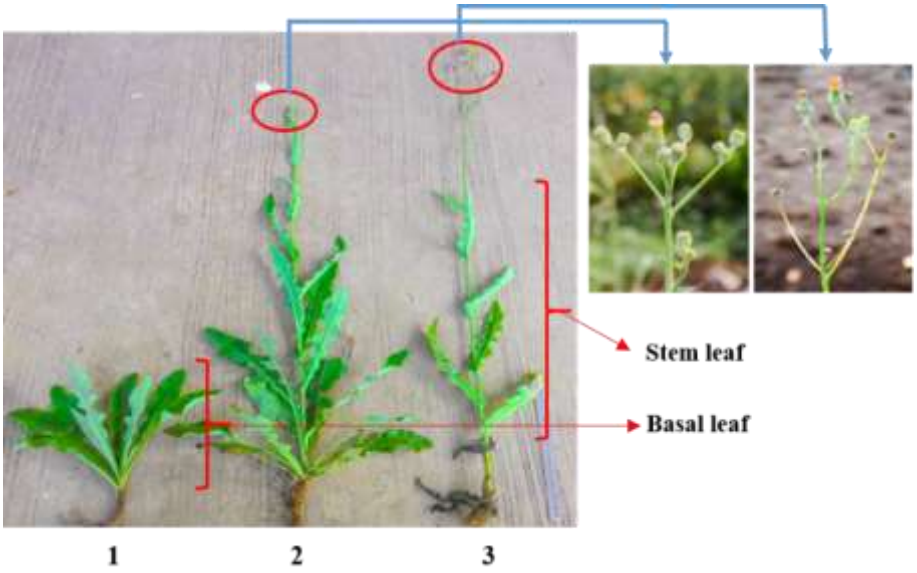

Fig. 1. Morphology of perennial sow-thistle at different growth stages. Note: (1) The vegetative stage; (2) The early generative stage and (3) The maximum generative stage

Equipment and Chemical

Materials used for chemical analysis were analytical grades of quercetin and luteolin standard, tert-buthylhydroquinone (TBHQ) (Sigma-Aldrich; St. Louis, MO). Methanol, ethanol, KH_2PO_4, HCl, CH_3COOK, $AlCl_3$, acetonitrile, acetone (E-Merck; Darmstadt, Germany). Laboratory equipment for analysis includes a Shimadzu UV-1800 Spectrophotometer (Japan) with 2.34 probes UV, Centrifuge Heraeus labofuge-400R, oven Sanyo MOV-212F, Vortex Thermo Scientific maximix™, Ultrasound Assisted Reaction (UAR) Branson™ 8510, Hitachi HPLC System UV-VIS Detector L-2420 with zorbax SB C-18 column.

Pigment Analysis

Fresh leaves from each growth stage were taken as samples for measuring the content of total chlorophyll, anthocyanin and carotenoid. The pigment analysis follows Sims and Gamon, (2002) with modification. About 0.02g fresh leaf was placed in a mortar and grinded into powder. The powder was then added with 2mL of the extraction solvent 85% acetone and 15% Tris stock buffer (1%w/v Tris final concentration; adjusted to pH 8 with HCl).

The extract was centrifuged at 12000rpm for 3min. A defined quantity of supernatant (1mL) was removed and diluted to 3mL then vortex.

Absorbance was measured at 537, 663, 647 and 470nm by using spectrophotometer UV-Vis. The pigment content was then calculated according to equation (1–4). [1] Anthocyanin = $(0.08173 * A_{537}) - (0.00697 * A_{647}) - (0.002228 * A_{663})$; [2] Chl a = $0.01373 * A_{663} - 0.000897 * A_{537}) (0.003046 * A_{647})$; [3] Chl b = $(0.02405 * A_{647}) - (0.004305 * A_{537}) - (0.005507\ A_{663})$; [4] Carotenoids = $(A_{470}$ $(17.1*(CHl_a+CHl_b)- 9.479*$ *Anthocyanin*$))/119.26$

Total Flavonoid

Fresh harvested leaves were washed with water, drained, and oven dried at 60°C for 3 x 24h then were crushed into powder. About 0.1g of dry powder was placed in microtube and extracted by shaking with 1 mL of 95% aquaeous ethanol, then stored at freezer (-2°C) and allowed to stand for 24 hours. Plant extract was then heated for 60 min at 60°C and centrifuged at 12000 rpm for 5 minutes. The extract was dilluted by taking 0.5mL and dissolved in 0.5mL of ethanol then centrifuge at 12000rpm for 5 minutes. Aliquots of supernatat were reserved for the total flavonoid content measurement. The $AlCl_3$ method was used to determine total flavonoid content of *Sonchus arvensis* extracts as described by Chang *et al.* (2002) with modification. Plant liquid extract of 0.1mL was added with ethanol (1.5mL), 10% aluminium chloride (0.1mL), 1M potassium acetate (0.1mL),

distilled water (3.2mL) separately then mixtured using a vortex. Absorbance at 415nm was measured following incubation for 30min at 27°C. Total flavonoid were quantified based on standar curves of 100mg.L^{-1} quercetin y= 0.0294x + 0.0881 (R^2=0.9995).

Flavonoid Analysis

The flavonols quercetin and flavones luteolin were quantified in *S. arvensis* extracted according to the method by Hertog *et al.* (1992) and Andarwulan *et al.* (2010). Dry powder (1g) were extracted with 40 mL of 62.5% methanol containing 2g.L^{-1} TBHQ and then added with 10mL of 6M HCl. After refluxing at 90°C for 2 h with occasional swirling, the extract was allowed to cool then filtered using whatman no. 2 and was subsequently made up to 100mL with methanol and sonicated for 5min. Approximately 2mL was filtered through a 0.45µm whatman filter for organic solvent and 20µL was injected onto an HPLC Hitachi UV-VIS with zorbax SB C-18 column (Japan), and eluted with isocratic 25% acetonitrile and 0.025M KH_2PO_4 at a flow rate of 0.9mL/min. Flavonoids were quantified on the basis of comparison standard at 370nm. Flavonoid content was calculated by comparing a standard area and sample area. HPLC detection limit is 0.004mg.kg^{-1} .

Data Analysis

Data were analyzed using Microsoft Excel 2010 and Statistical Analysis Software (SAS) 9.2. The letters following number that showed the differences among treatment were based on the margin of error (α=0.05). The data were also analyzed with Student t-test and Pearson correlation test. Production of bioactive compunds were calculated using the following formula:

Production of bioactive compounds = Dry weight of leaf (g per plant) x total flavonoid content (%)

Result

Leaf Morphological Character

The growth of *S. arvensis* can be devided into three stages. The first stage is vegetative stage i.e. basal leaves in rosette grow fully then the stem starts to grow.

Following stem elongation, the number of leaf at the base (rosette) decreases. The second growth stage when stem leaves grow until initial flowering (early generative). The last stage was maximum generative stage when there was no basal leaf found and followed by full flowering and produce seeds.

Number of Leaf at Different Growth Stages

There was a difference in the number of leaf at three different growth stages (Fig. 2), but similar pattern of leaf number was found in both time of observation. The number of basal leaf was higher during vegetative stage, then declined up to 50% when *S. arvensis* has entered budding stage. It might be replaced by fast growth of stem leaves at early generative stage. However at the last stage i.e. maximum generative stage, the number of stem leaves also decreased.

These pattern of leaf number following growth stage occured in both times of observation. These result also suggest that the vegetative stage could be characterized by the maximum growth of basal leaf. After this stage, the plant must reduce its growth (basal leaf) and prepare it self to the budding and flowering stages. There was no difference of leaf number between both times of observation in each growth stage based on Student t-test (P>0.05).

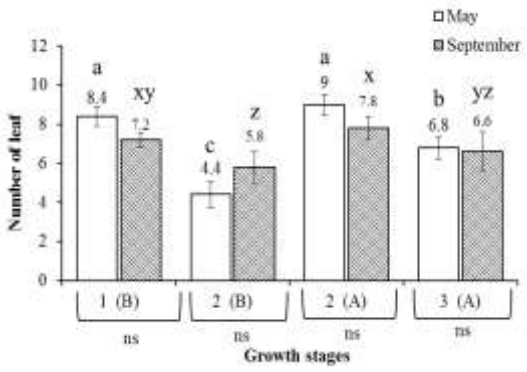

Fig. 2. Number of leaf at different growth stages. Note: (1) Vegetative stage, (2) early generative stage, (3) maximum generative stage. B: Basal leaf, A: Stem leaf. Numbers followed by the same letters (a,b,c) were not significantly different at May observation. Meanwhile, numbers followed by the same letters (x,y,z) were not significantly different at September observation. ns : not significant based on Student t-test with α= 0.05.

Leaf Fresh and Dry Weight at Different Growth Stages

Different pattern shown between the first (May) and second (September) observation (Fig. 3). The first observations shows higher fresh and dry weight of basal leaf (B) at vegetative stage than at early generative stage. A consistent of 50% decrease in the fresh and dry weight of leaf was observed during the early generative stage and the full flowering stages. The second observation did not show significant different in fresh and dry weight of leaf between vegetative stage, budding stage and full flowering growth stages. The changes of fresh and dry weight of leaf in every growth stage, could become the beginning of knowledge in the development of perennial sow-thistle cultivation standard.

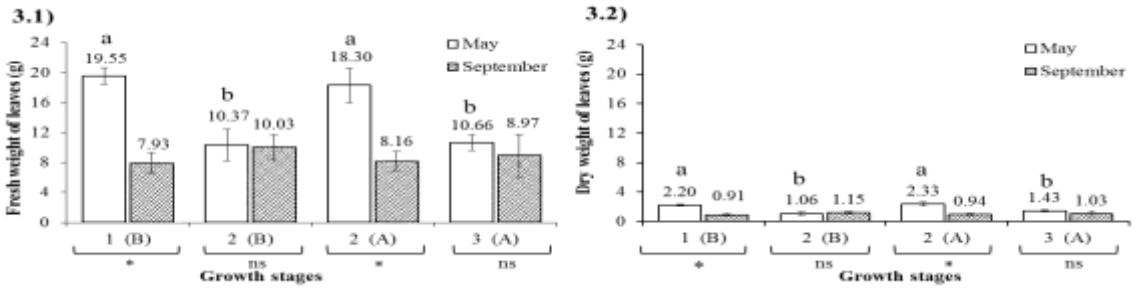

Fig. 3. Fresh weight (3.1) and dry weight (3.2) of leaves at different growth stages. Note: (1) Vegetative stage; (2) The vegetative stage; (3) The maximum generative stage; B: Basal leaf; A: Stem leaf. Numbers followed by the same letters (a,b,c) were not significantly different at May observation. ns : not significant; *: significant ($P<0.05$) based on Student t-test with $\alpha= 0.05$.

Total chlorophyll and carotenoid contents were found differences among the growth stages at September observation (Table 1). Total chlorophyll and carotenoid contents of basal leaf were found highest at vegetative stage. Furthermore total chlorophyll content at stem leaf were higher at maximum generative stage compared to early generative stage.

Differences in the growth stages affects the anthocyanin content in both time of observation. Anthocyanins content of basal leaf were found higher at the early generative stage than the vegetative stage then anthocyanins content of stem leaf were found higher at maximum generative stage compared to the early generative stage (Table 1).

Flavonoid Content at Different Growth Stages

Luteolin concentration was found higher than quercetin in all growth stages (Table 2). The highest concentration of luteolin are shown by stem leaf at maximum generative stage at May (First observation) i.e. 15.41 mg/100g DW. Quercetin concentration at vegetative stage was undetected by HPLC system (<0.0045 mg.kg^{-1}).

Table 1. Total chlorophyll, Content and anthocyanin concentration of perennial sow-thistle at different growth stage.

Growth stages	May			September		
	Total chlorophyll (mg.g^{-1}FW)	Carotenoid (mg.g^{-1}FW)	Anthocyanin (mg.100g^{-1}FW)	Total chlorophyll (mg.g^{-1}FW)	Carotenoid (mg.g^{-1}FW)	Anthocyanin (mg.100g^{-1}FW)
1 (Basal)	1.21	0.26	0.049 b	2.95 a	0.60 a	0.050 b
2 (Basal)	1.16	0.22	0.068 a	1.66 c	0.36 c	0.069 ab
2 (Stem)	1.28	0.27	0.051 b	2.18 bc	0.46 bc	0.060 b
3 (Stem)	1.25	0.24	0.062 a	2.50 b	0.53 ab	0.082 a

Note: (1) Vegetative stage; (2) early generative stage; (3) maximum generative stage; Number followed by the different letters in the same row were significantly different ($\alpha=5\%$); FW= Fresh weight.

Table 2. Luteolin, quercetin and total flavonoid contents of perennial sow-thistle at different growth stages.

Growth stages	May			September		
	Luteolin (mg.100g^{-1}DW)	Quercetin (mg.100g^{-1}DW)	Total Flavonoid (mg SK.100g^{-1}DW)	Luteolin (mg.100g^{-1}DW)	Quercetin (mg.100 g^{-1}DW)	Total Flavonoid (mgSK.100g^{-1}DW)
1 (Basal)	3.71	0.00	81.85	8.11	0.00	92.52
2 (Basal)	5.16	0.13	87.59	13.52	0.43	108.72
2 (Stem)	11.16	0.59	94.50	5.47	0.11	104.02
3 (Stem)	15.41	0.44	101.27	12.45	1.15	121.96

Note: (1) Vegetative stage; (2) early generative stage; (3) maximum generative stage; DW: Dry weight; Basal leaf; Stem leaf.

The highest content of total flavonoid was found in stem leaf (A) at maximum generative stage. This findings showed that there was positive correlation between flavonoid concentration and the growth stage at each position leaf. Total flavonoid concentration of basal leaf have increases when entering the early generative stage. The increase of flavonoid concentration was also shown by stem leaf in following stage i.e. when maximum generative stage.

Production of Total Flavonoid

The production of bioactive compounds was determined based on the dry weight and total flavonoid content. The highest production of total flavonoid was from stem leaves at early generative stage (13.78 mg per plant), at May (first observation) (Fig. 4).

The first observation showed a decrease in the production of bioactive compound of basal leaves and stem leaves when entering the next stage.

Different trend was found in the second observation (September) where an increase in the production of bioactive at basal leaves and stem leaves when entering the next stage.

Production of bioactive compunds differed between observations indicated that environmental factors also affected the production and metabolism of plants.

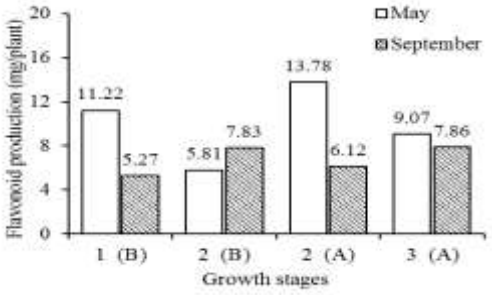

Fig. 4. Production of total flavonoid content at different growth stages. Note: (1) Vegetative stage; (2) early generative stage; (3) maximum generative stage; B: Basal leaf; A: Stem leaf.

Correlation Between Variables of Observation

There were positive correlations among the bioactive compounds (Table 3). Total flavonoid content were positively correlated to anthocyanin ($P<0.05$), quercetin ($P<0.05$) and luteolin content ($P>0.05$). Furthermore, there were negative correlation between leaf dry weight with anthocyanin, luteolin, quercetin and total flavonoid content ($P>0.05$).

Table 3. Correlation analysis between variables.

Variable	Anthocyanin	Luteolin	Quercetin	Total flavonoid	DW leaf
Anthocyanin	1.00	0.32	0.62	0.72*	-0.56
Luteolin		1.00	0.67	0.63	-0.05
Quercetin			1.00	0.79*	-0.01
Total flavonoid				1.00	-0.48
DW leaf					1.00

Note: *= significant base on Pearson correlation test ($P<0.05$). DW= Dry weight.

Discussion

The current study showed that one life cycle of *S. arvensis* can be devided into three growth stages with different morphological characteristics.

The first stage is vegetative stage i.e. basal leaves in rosette grow fully then the stem starts to grow. Following stem elongation, the number of leaf at the base (rosette) decreases.

The second growth stage when stem leaves grow until initial flowering (early generative). The last stage is full flowering stage when there was no basal leaf found, and followed by maximum generative and produce seeds. This is accordance with (Lemna and Messersmith, 1990) who explains that the initially growth of *S. arvensis* leaf was by forming a rosette close to the ground surface, and then developed at elongated stems.

The current observation indicated loss of leaf number (basal leaf) during the development of plants until flowering and seed production. Tavaviza (2012) reported that the dry weight of the below ground structures of *S. arvensis* decreased gradually during the early phenological stages, reached a minimum, and then increased again during further phenological development. Lemna and Messersmith (1990) explained that in perennial sow-thistle growth, the initial loss in weight of the below ground structures can be attributed to the allocation of stored food reserves to above ground plant parts and also to respiration of the old root and development of fine roots. Declerck (1995) also explained that differences in the growth stage and the position of leaf affects the structure of the plant, number of leaf and density of stomata.

The study showed that the total flavonoid content of basal and stem leaves were differed between growth stages (Table 2). Infact, many previous works reported significant changes of flavonoid content with physiological stage (Sellami *et al.,* 2009; Karray *et al.,* 2010; Moraes *et al.,* 2013). They reported that flavonoid content was higher when plants enters generative stage compared to that in the vegetative stage. Males *et al.* (2003) also indicated that the aerial parts of *Crithmum maritimum* collected before flowering and at the beginning of flowering were the richest in total flavonoid and total phenol content.

The result of this current study shows that *S. arvensis* contains luteolin at all growth stages with higher level than quercetin. Similar studies have also reported on the comparison of the content of luteolin and quercetin that are different in some types of medicinal plants (Hertog *et al.,* 1992; Andarwulan *et al.,* 2011).

Quercetin is the most commonly studied compounds among the compounds of flavonoid group (Lakhanpal and Rai, 2007), but many recent studies provide information that there are other flavonoid compounds that also acts as an antioxidant, anti-carcinogenic mechanism and one of them is luteolin (Seelinger *et al.,* 2008).

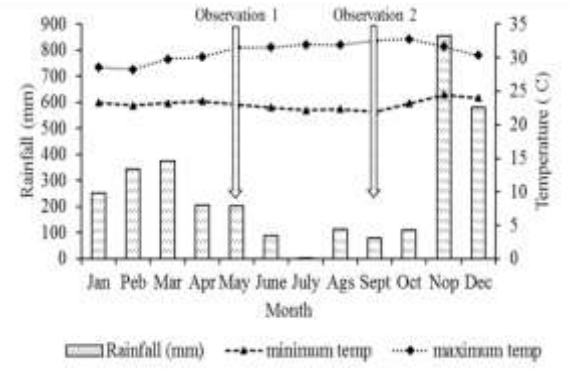

Fig. 5. Rainfall (mm) and average of daily temperature (°C) at Darmaga, Bogor 2015. The Arrows indicate the sampling times.

Leaf number, leaf fresh and dry weight at May (first) observation generally were higher than those at September (second) observation. These was possibly related to the differences in rainfall distribution during the observation periods. If we calculate the rainfall at three months before the observation, then we get that during February-April, the total rainfall was 926 mm whereas during June-august the total rainfall was 204.2mm (Fig. 5).

Low rainfall is expected to affect the decline in leaf production. Hakansson and Wallgren. (1972) explained that growth of *S. arvensis* will not bloom when environmental conditions are not favourable and is capable of flowering quickly when water, light and nutrients are available. Zollinger and Kells (1991) also reported that high temperature was expected to affect the decline in leaf production of *S.arvensis*.

Variation of flavonoid concentration during the growth of *S.arvensis* confirmed the influence of both phenological stages and climate factors on production and release of these metabolites.

In the other hand, the opposite peak of accumulation of flavonoid during the late vegetative stage and the early generative stage might be due to the fact that during this stage, the plant protection is mainly secured by phenolics which are highly accumulated during this stage (Sellami *et al.,* 2009). The accumulation of total flavonoid during the maximum generative stage could be related to ecological roles such as intensifying antifungal defences and attracting pollinators (Langenheim, 1994).

Regarding these variations in the accumulation of secondary metabolites in *S. arvensis* plants, it could be concluded that the physiological stage of the plant affects the choice of the best harvesting time. Our results showed that perennial sow-thistle (*Sonchus arvensis*) is a good source of flavonoid particularly during the maximum generative stage. When considering biomass production, harvesting at early generative stage would be favoured.

Conclusion

Based on three growth stages of *Sonchus arvensis* i.e. vegetative stage, early generative stage and the maximum generative stage, it is known that early generative stage produces the highest total of leaf number and total of leaf dry weight. Maximum growth of basal leaves was found at vegetative stage, while maximum growth of stem leaves was at early generative stage. The highest total flavonoid content of *Sonchus arvensis* was found at maximum generative stage.

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