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Biological investigation of *Lilium ledebourii* in Iran

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Abstract

Lilium ledebourii is a rare Asian species and is an endemic plant of Iran which grows in Gilan province in Damash highlands. In this study, the structural features of flower including the study of sections such as carpel, stamen, and tepals were performed. This plant had a full flower that consisted of six tepals (three petals and three petal-like sepals), six free stamens and a 3-lobed gynoecium. In the cross-section of tepals, the stomata as well as cells containing anthocyanins were observed. In stamens, anther (epidermis, endothecium, middle layers and an innermost tapetum), and filament were observed. In the gynoecium, ovary (ovary wall, locule, funiculus, anatropous ovule, different arrangements of vascular bundles, transmitting tissue and stomata), style (stylar canal and transmitting tissue), and dry stigma with elongated papillae tissue were observed. Besides, the presence of alkaloids in the bulb was tested using Wagner and Dragendorff reagents and the total alkaloid content in three types of methanol, ethanol, and butanol extraction was investigated. The ethanol extract had a significant difference with other extractions. The methanol extract was chosen for further investigation by gas chromatography-mass spectrometry (GC-MS) and two compounds of Pterin-6-carboxylic acid, and Pyrrolizin-1,7-dione-6-carboxylic acid, methyl (ester) were detected.

Keywords: Alkaloid, anatropous ovule, *Liliaceae*, papillae, petal-like sepal, tepal

بررسی زیست‌شناسی سوسن چلچراغ در ایران*

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خلاصه

سوسن چلچراغ (*Lilium ledebourii*) یک گونه نادر آسیایی از تیره سوسنیان است که بومی ایران بوده و در استان گیلان، در ارتفاعات منطقه داماش رشد می‌کند. در این مطالعه، ویژگی‌های ساختاری گل مشتمل بر مطالعه بخش‌هایی از قبیل برچه، پرچم و گلپارها انجام شد. این گیاه دارای یک گل کامل متشکل از شش گلپار (سه گلبرگ و سه کاسبرگ گلبرگ‌آسا)، شش پرچم آزاد و تخمدان سه برچه‌ای است. در سطح مقطع گلپارها، روزنه‌ها و همچنین سلول‌های حاوی آنتوسیانین‌ها مشاهده شدند. در پرچم‌ها، بساک (اپیدرم، لایه مکانیکی لایه‌های میانی و سلول‌های لایه پرستار میانی و میله مشاهده شد. در مادگی، تخمدان (دیواره، حفره، بند تخمک، تخمک آناتروپ، گروه‌های مختلف دسته‌های آوندی، بافت انتقال و روزنه‌ها)، خامه (کانال خامه و بافت انتقال دهنده) و کلاله خشک با بافت پاپیلای کشیده مشاهده شد. علاوه بر این، حضور آلکالوییدها در پیاز گل با استفاده از معرف‌های واگنر و دراژندورف مورد آزمایش قرار گرفت و محتوای کل آلکالوییدها در سه نوع عصاره متانولی، اتانولی و بوتانولی بررسی شد. عصاره اتانولی با سایر عصاره‌ها تفاوت معنی‌داری داشت. عصاره متانولی برای بررسی بیشتر با استفاده از کروماتوگرافی گازی-طیف‌سنجی جرمی (GC-MS) انتخاب شد و دو ترکیب Pterin-6-carboxylic acid و Pyrrolizin-1,7-dione-6-carboxylic acid, methyl (ester) شناسایی گردیدند.

واژه‌های کلیدی: آلکالویید، تخمک آناتروپ، پاپیله، سوسنیان، کاسبرگ شبیه گلبرگ، گلپار

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Introduction

Liliales is a group of Monocotyledon that consists of 10 families and is known by several features such as being a perennial, usually bulbous herb, lacks an onion-like odor, and superior ovary (Simpson 2010, Singh 2014). The family *Liliaceae* is subdivided into three tribes: *Lloydieae*, *Lilieae*, and *Tulipeae* (Kosenko 1999). The genus *Lilium* L. has about 100 species and dispersed in the northern hemisphere (Nishikawa *et al*. 1999, Hayashi & Kawano 2000). These plants have more expanded in temperate and cold regions (Hayashi & Kawano 2000) and many of them have been scattered in the eastern and western parts of Asia and the Himalayas (Kosenko 1999). The floral formula in the *Lilium* genus is: *p3+3, A3+3 G(3) (Ozen *et al*. 2012).

Lilium ledebourii (Baker) Boiss. is an endemic plant in Iran, which grows in Damash highlands (Gilan province, north of Iran). This plant grows at an altitude between 1750–2100 m (Azadi & Khosh-Khui 2007). It is a perennial herb having height between 50 and 150 cm, with thick stem, erect leaves, white flowers, capsule fruits, and anomocytic type of stomata on leaves (Farsam *et al*. 2003). The pollen grain is similar to *L. longiflorum* Thunb. and has the same characteristics as pollen type: monocolpate, pollen shape: a boat-like apertures: colpi and long with an end of more or less pointed, and pollen length: 107–110 µM (Kaviani *et al*. 2008). In *L. candidum*, *L. ciliatum*, *L. szovitsianum*, and *L. armenum*, all pollen grains are monosulcate; heteropolar, elliptical in polar view and oblate (Güven *et al*. 2014).

In the transverse section of *L. ledebourii* stem, an epidermal layer is observed that does not have anomocytic stomata. The stem has a thick cuticle, there is a multilayer collenchyma cell immediately after the epidermis, and thereafter there are 3–4 layers of sclerenchyma cells. The innermost layer of the cortex is encapsulated from parenchyma cells without intercellular space. Approximately 40–50 vascular bundles are dispersed without any particular qualities that surrounded by a sclerenchyma bundle sheath (Kaviani *et al*. 2008). The stamens are usually made up of the filament and anther that produce pollen. Anther is composed of four microsporangia that arise in two pairs (thecae) and are connected via a connective tissue (Rudall 2007).

Alkaloid is any active and heterocyclic chemical compound, containing nitrogen, which may be involved in some pharmacological or ecological activity (Roy *et al*. 2019). Over the years, a large number of alkaloids have been identified in wildlife, insects, marine organisms, microorganisms and plants. Alkaloids are now considered part of the chemical defense system in plants (Kruse *et al*. 2017). These valuable compounds, generally have pharmacological activity, especially for humans. Many of the pharmaceutical drugs used today are derived from alkaloids.

In the present study, the flower structure as well as alkaloids in the bulb of *L. ledebourii* plant were investigated. Since this plant is on the verge of extinction, therefore, attempt was made to investigate the first and most important step in identifying the structure of the reproductive organs (flowers). The genus *Lilium* processes one of the most important commercial flower of the world and success in this field, can provide a good economic opportunity. On the other hand, alkaloids are one of the most important groups of secondary metabolites in plants because of their uses as toxins to herbivores and medicines to humans. Unfortunately, no studies have been done on the flower structure and alkaloids in the bulb of this valuable plant. It seems that, before any preventive action to prevent the extinction of these rare species, the study of flower structure and secondary metabolites, especially in reproductive organs, is of great importance.

Materials and Methods

- Plant materials

After collecting the flowers from its natural habitat (Damash Village in the highlands of Gilan province, north of Iran), they were fixed in a FAA

solution (50% ethanol, 3.65% formaldehyde, and 5% glacial acetic acid) (Yamagishi & Akagi 2013), and transferred to the laboratory.

- Structural observations

In the laboratory, all types of sections (transverse and longitudinal) were taken manually. Besides, some samples were embedded in paraffin (Xing *et al*. 2010), and performed with precise sections by the microtome. Staining of the samples was done by Methylene blue, Alum carmine, Safranin, Hematoxylin, Eosin, and Toluidine blue (McLean & Ireland 1940). These sections and staining were performed in various organs of flower including carpel (ovary, style, and stigma), stamen (filament and anther), pedicel, and petal. The sections were observed with an Olympus light and stereo-microscope and then the images were recorded with a Sony digital camera. Petals, anthers, pollen grains, and stigma were investigated by scanning electron microscope (SEM) (Philips XL 30; Eindhoven, The Netherlands). The materials were vacuum-coated with a layer of gold (100 A thick) and photographed at a voltage of 15 kV.

- Alkaloids assay

- Alkaloid presence test

The presence of alkaloids was determined by Dragendorf and Wagner reagents. Some of the samples were dissolved in dilute HCl and then two Dragendorf reagent droplets were added. The presence of crystalline sediments indicated the presence of alkaloids in the sample. With Wagner's solution in the sample containing alkaloids, brick deposits are formed (Firdouse & Alam 2011).

- Extraction of alkaloids

To extract the alkaloids, 2 g of dry powder of the plant was first milled in 10 ml of methanol, ethanol, and butanol and then placed in an ultrasonic unit for one hour. The resulting solution was passed through Whatman No. 3 filter paper and then concentrated by the blower pump to a concentration of approximately 0.5 ml (Chebouat *et al*. 2013). In the next stage, it was completely dissolved in 10 ml of 5% sulfuric acid (Fileto-Pérez *et al*. 2015). The organic compounds such as tannins, fatty acids, etc. can be washed with an organic solvent such as diethyl ether (Meshram *et al*. 2015). In the next step, the soluble pH of ammonia or sodium hydroxide reached about 11. They were washed again with chloroform (an organic solvent) for three times. Finally, by evaporation of chloroform, the raw alkaloids were precipitated at the bottom of the dish (Erdemoglu *et al*. 2009).

- Reagent preparation

Green bromocruzol solution was obtained by dissolving 69.8 mg green bromocruzol in 3 ml 2N sodium hydroxide plus 5 ml distilled water by heat. The resulting solution was diluted in 1000 ml of distilled water. Two M phosphate buffer solution at pH = 4.7 was obtained by dissolving 71.6 g of sodium phosphate in one liter of distilled water. To achieve the desired pH in another container, 42.02 g of citric acid was dissolved in one liter of distilled water and gradually added to the first solution until it reached pH = 4.7 (Fazel *et al*. 2010, Tabasum *et al*. 2016).

- Standard curve

Pure atropine was prepared from the Tirakam Iranian Company to draw the standard curve as standard. For this purpose, 100 mg of it was dissolved in 10 ml of distilled water. For drawing the standard curve, different amounts of standard solution (0, 0.2, 0.4, 0.6, 0.8, 1, and 1.2 ml) were taken. Then 5 ml of phosphate buffer and 5 ml of green bromocruzol were added to each sample. It was washed with 5, 8, and 10 ml of chloroform with vigorous shaking, transferred to a 25 ml balloon of chloroform phase, and then reached volume by chloroform. The resulting solution was read three times at 415 nm. The read absorbance was then subtracted from the zero concentration and plotted on a standard diagram (Tabasum *et al*. 2016).

- Total alkaloids assay

Five mg of extracts (methanol, ethanol, and butanol) were dissolved in 10 ml of distilled water. Then,

one ml of each was harvested and 5 ml of phosphate buffer and 5 ml of green bromocruzol were added. The resulting solution was extracted with 5, 8, and 10 ml of chloroform. The chloroform phase solution was transferred to a 25 ml balloon and then reached volume by chloroform. Finally, each solution was read three times at 415 nm.

- GC-MS analysis

An Agilent GC-MS system was used to measure the alkaloid compounds in the samples at EI 70 eV. HP-5MS column (30 m × 0.25 mm × 0.25 µm) was used for this purpose. The temperature program of this experiment was as follows. They were stored at 120 ˚C for one min. Then, at a rate of 15 ˚C/min, the temperature rose from 120 ˚C to 210 ˚C. Thereafter, the temperature rose from 210 ˚C to 260 ˚C at 8 ˚C /min. Then the temperature reached 15 ˚C from 260 ˚C to 300 ˚C and the helium gas flow rate was set to 1 ml/min (Erdemoglu *et al.* 2009).

In the next stage, 2 mg of methanol extract was dissolved in 1 ml of methanol and injected into the GC-MS system. The methanol extraction was chosen because in previous experiments it was shown that, this extraction is suitable for analysis of alkaloid compounds (Zishan *et al*. 2017). The alkaloids and other compounds in the sample were identified by Mainlib database.

- Statistical analyses

Analyses of data were performed using SPSS 23 software for ANOVA and Duncan's multiple range test was used to compare the mean values for this experiment.

Results

- Structural observations

Lilium ledebourii had a complete and actinomorphic flower, which consisted of six tepals (three petal-like sepals, and three petals), six free stamens, and gynoecium with a superior ovary. The arrangement mod of sepals and petals in a floral bud is known as imbricate aestivation and the floral formula in this genus is: *p3+3, A3+3 G (3) (Fig. 1).

- Gynoecium

- Ovary

In the transverse section of *Lilium ledebourii* ovary (Fig. 2A), the axial placentation was determined. Epidermis that consisted of compressed square shape cells were covered by a thick cuticle except at the anomocytic stomata with 4–5 subsidiary cells.

The ovarian wall consisted of six vascular bundles with three locules. In this plant, locule was surrounded by transmitting tissue cells that very similar to the epidermis in shape (Fig. 2B). In fact, this tissue was continued from the same tissue in the style. In *L. ledebourii* each locule containing two anatropous ovules (ovule consisted of inner and outer integument and embryo sac), that connected to placenta via funiculus (Fig. 2C).

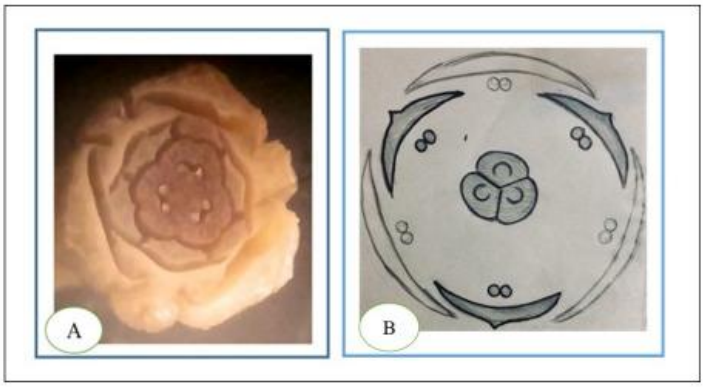


Fig. 1. Imbricate aestivation arrangement of tepals in *Lilium ledebourii*: A. Binocular stereo-microscope, B. Schematic diagram.

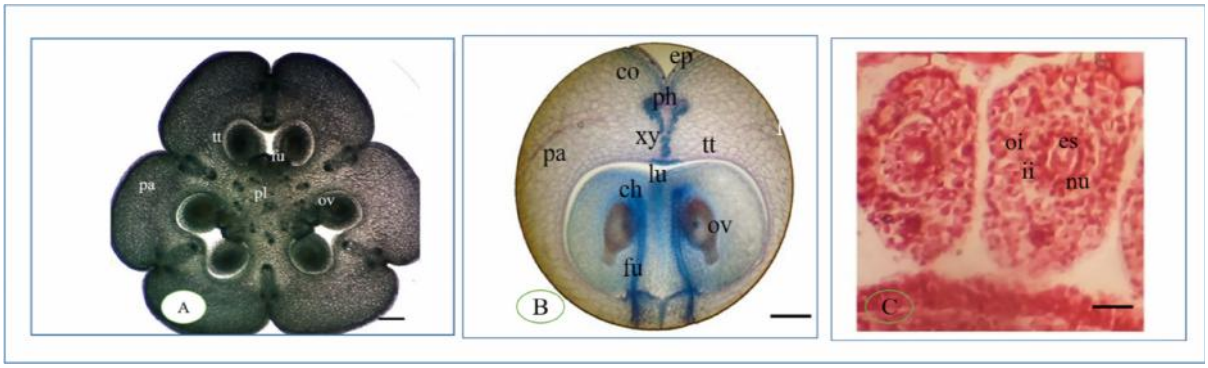


Fig. 2. Gynoecium in *Lilium ledebourii*: A. Cross section of ovary (4x), B. Close up of locule (10x), C. Ovules in the longitudinal section. ch: chalaza, pa: parenchyma, pl: placenta, ov: ovule, fu: funiculus, tt: transmitting tissue, ep: epidermis, ph: phloem, xy: xylem, lu: locule, es: embryo sac oi: outer integument, ii: inner integument, nu: nucellus (Bar = 100 µm).

- Style

Style in the *Lilium ledebourii* was triangular-shaped and about 3.5 cm in height. The number of vascular bundles in the style was an indicator of the number of carpels (Fig. 3). In the transverse section, it was revealed that, the style is covered by cuticle. Epidermis in style was compact and consisted of square shape cells. Under the epidermis, there were parenchymal cells surrounding the vascular bundles. Central stylar canal provides a pathway for the movement of the pollen tube toward the ovary. Stylar canal was surrounded by transmitting tissue, which consisted of square-shape cells that compressed together. These results showed that, the styles in *L. ledebourii* are hollow.

- Stigma

The stigma is a special secretion tissue to receive pollen grains. In the transverse section, it was observed that, the epidermis cells had a variety of elongated papillae that were surrounded by the primary cell wall, had a waxy cuticle, and surround the parenchyma tissue (Fig. 4A).

The image of the SEM microscope revealed that, there was a specific adhesion and interaction between the grain and papillae of the stigma (Fig. 4B).

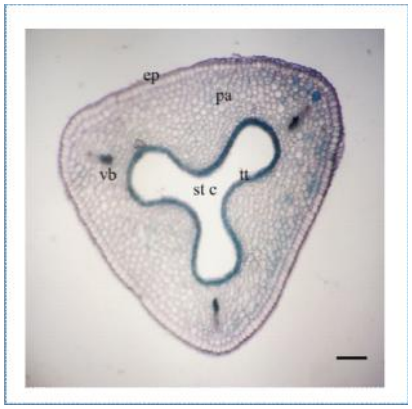


Fig. 3. Transverse section of style in *Lilium ledebourii*. ep: epidermis, pa: parenchyma, tt: transmitting tissue, vb: vascular bundle, stc: stylar canal (Bar = 100 µm).

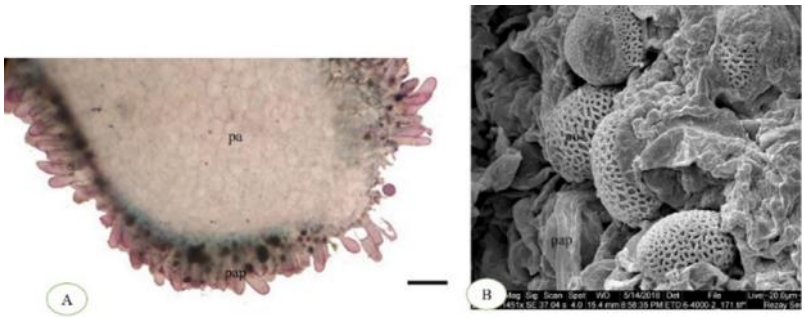


Fig. 4. Transverse section of stigma in *Lilium ledebourii*: A. Light microscope (x4), showing papillae with primary cell wall, B. SEM, showing pollen on the stigma surface within elongated papillae. pa: parenchyma, pap: papillae, po: pollen (Bar = 100 µm).

- Stamen
- Anther

Anther in *L. ledebourii*, which consisted of four microsporangia was separated into two pairs (thecae) linked by a connective. Each theca possesses two sporangia or anther locules divided by a septum (Figs 5A & B). The anther wall consisted of several layers of cells. In the transverse section, four distinct layers were observed: epidermis, endothecium, middle layers, and an innermost tapetum. The epidermis was an outermost layer of anther that has a protective function in the mature anther; epidermal cells were very smooth. The endothelium was the hypodermal layer of the anther wall that usually consisted of a single layer. The endothecium cells were radially elongated, had reached the maximum development and endothecial cells typically develop fibrous thickenings, which contributed to the anther dehiscence mechanism. Middle layers consisted of single layers that developed secondary thickenings similar to that of endothecium cells. Innermost layer of the anther wall was a single layer tapetum that completely surrounds the sporogenous tissue (Figs 5C & D).

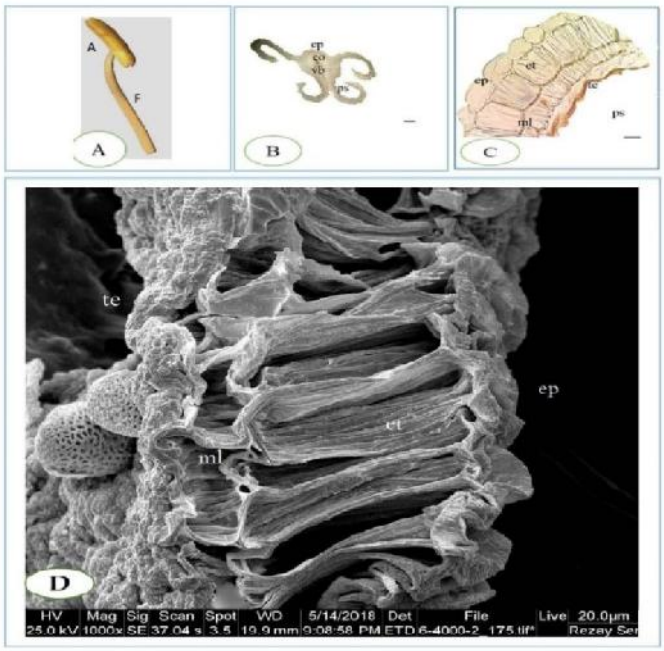


Fig. 5. Androecium in *Lilium ledebourii*: A. Stamen consists of anther and filament, B. Transverse section of anther, C. Hand section of pollen sac (x10), D. SEM micrograph of pollen sac layers. a: anther, f: filament, ep: epidermis, co: connective, vb: vascular bundle, et: endothecium, ml: middle layer, te: tapetum, ps: pollen sac (Bar = 100 µm).

- Filament

Filaments in *Lilium ledebourii* were 3.3 cm high and had a diameter of approximately 1.7 mm. They were typically cylindrical and slender. In the transverse section, the filament possessed a parenchymatous ground tissue surrounding the vascular bundle, which consisted of a single vascular bundle (Fig. 6).

- Pollen

According to our observations, all pollen grains (monosulcate and heteropolar) in polar views were elliptical and their surface ornamentation was reticulum cristatum. Polar axis was 100.980 µm and equatorial axis was 132.411 µm. The P/E ratio was 0.762. In this study, the sulcus length (Slg) was 111.279 µm, the sulcus width (Slt) was 27.490 µm, and the Slg/Slt rate was 4.047. The sulcus was narrow, it was deep all over, and was almost equal to the equatorial axis (Fig. 7).

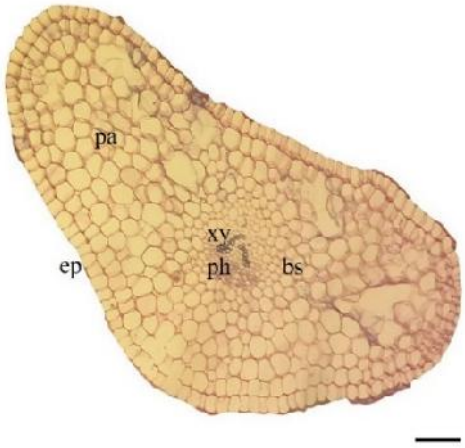


Fig. 6. Filaments in *Lilium ledebourii*, parenchymatous ground tissue surrounding the vascular bundle (x10). pa: parenchyma, ep: epidermis, ph: phloem, xy: xylem (Bar = 100 µm).

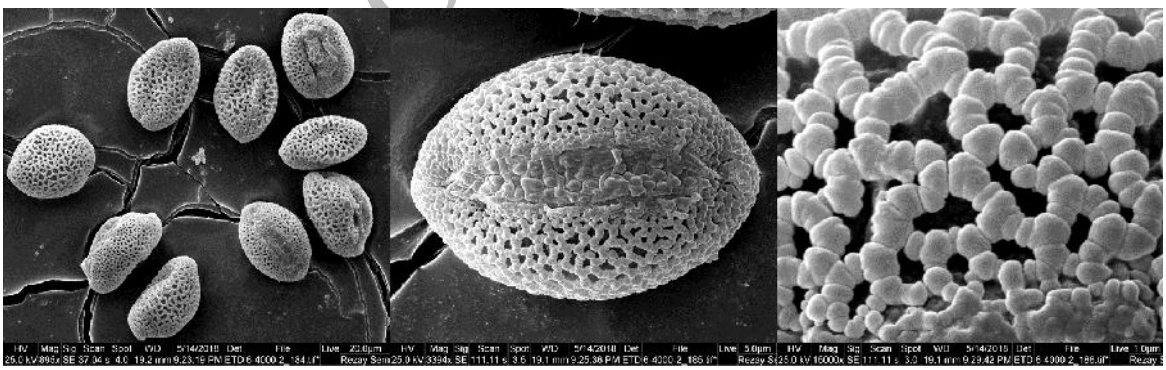


Fig. 7. SEM micrographs of pollen grain in *Lilium ledebourii.*

- Tepals

In *Lilium ledebourii*, the outer two types of floral organs were modified leaf-like structures that had six tepals, of which there were three sepals and three petals (Figs 8A & D).

Sepal in *Lilium ledebourii* was very similar to petals in color, size, and shape (Table 1). Both petals and sepal had purple spots on their adaxial surface; however, the number of spots in the petals was much more than the sepals.

The observation of petal surfaces in *L. ledebourii* revealed that petal spots were arranged around the vascular bundles in the parenchymal cells. Another difference between petals and sepals was the absence of a stomatal at both surfaces (abaxial and adaxial) in the petals, while both surfaces of the sepals had stomata (Figs 8B, C, E & F).

The SEM observation on the petal surface showed that, spots and nectaries were developed from epidermal cells that occur on adaxial surface of petals and more or less on sepal surface (Figs 8G, J & K). In the transverse sections of petals and sepals, it was observed that they consisted of abaxial and adaxial epidermis and parenchymatous ground tissue that surrounding the vascular bundle. Parenchymal and epidermal cells overlying vascular bundles in adaxial surface of petal can provide a place for the accumulation of anthocyanin pigments (Figs 8H & I).

Table 1. Tepals size in *Lilium ledebourii*

Tepal	Length (cm)	Width (cm)
Sepal	6.7	1
Petal	6.6	1.2

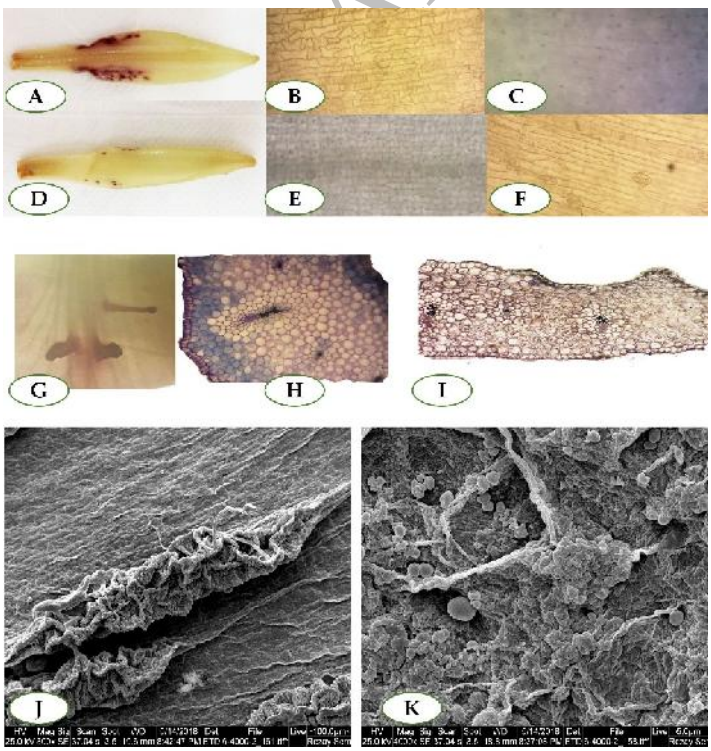


Fig. 8. Tepals in *Lilium ledebourii*: A. Petal, B-C. Abaxial and adaxial surface of petals that lack the stomata, D. Sepal, E-F. Abaxial and adaxial surface of petals that containing the stomata, G, J. Epidermal outgrowths that occur on adaxial surface of petals, K. Nectaries, H-I. Transverse sections of petals and sepals.

- Alkaloids assay

The presence of alkaloids in the bulb of *Lilium ledebourii* was confirmed using Wagner and Dragendorff reagents. The total alkaloid contents in methanol, ethanol, and butanol extracts of *L. ledebourii* bulb were 0.066, 0.081, and 0.059 µg/mg, respectively (Fig. 9). There was a significant difference in total alkaloid content between ethanol extract and other extracts (methanol and butanol). However, there was no significant difference between methanol and butanol extracts.

The methanol extraction was analyzed by GC-MS and the compounds in samples were identified using Mainlib and Replib databases. As presented in table 2; 10 compounds were detected. Among the compounds obtained from the Alkaloids Dictionary, Pterin-6-carboxylic acid compound was an alkaloid. Also, Pyrrolizin-1, 7-dione-6-carboxylic acid compound was very similar to the pyrrolizidine alkaloids family that, it is likely to be part of the alkaloids in this family.

Discussion

Lilium ledebourii flower such as other *Liliaceae* family, had six tepals (three petal-like sepals, and three petals), six free stamens and three-lobed gynoecium (Glimn-Lacy & Kaufman 2006), and its flora formula is similar to other *Lilium* that reported previously (Ozen *et al*. 2012). Gynoecium consisted of an ovary, style(s) and stigma(s). Ovary had axial placentation that consisted of locules for formation ovule. In each theme, two ovules appear that, linked to the placenta by funiculus that already reported (Rudall 2007). Transmitting tissue cells that observed in this plant were already reported by Singh & Walles (1992). Anomocytic stomata on ovary were similar to the leaves that already reported (Farsam *et al*. 2003). Style and stigma were 3-lobed in *L. ledebourii* that are similar to other *Lilium* sp. (*Liliaceae*) (Rudall 2007).

Anther wall in *Lilium ledebourii* consisted of several layers in order from outside to inside, epidermis, endothecium, one to three middle layers and an innermost Tapetum that is similar to *Lilium polyphyllum* that already reported (Dhyani *et al*. 2009). *L. ledebourii* anthers had two lobes with four pollen sac that already reported (Kaviani *et al*. 2008).

The presence of alkaloids in the bulb of *L. ledebourii* was confirmed using the Dragendorf and Wagner and Meyer tests, which is in accordance with the results of the study on *Lilium candidum* (Devi *et al*. 2016). There are several reports of the presence of types of alkaloids in the bulb of the *Lilium* genus (Buckingham *et al*. 2010, Mimaki & Sashida 1990). The total alkaloid content observed in the bulb of *L. ledebourii* was very close to the reported value (Meng *et al*. 2015).

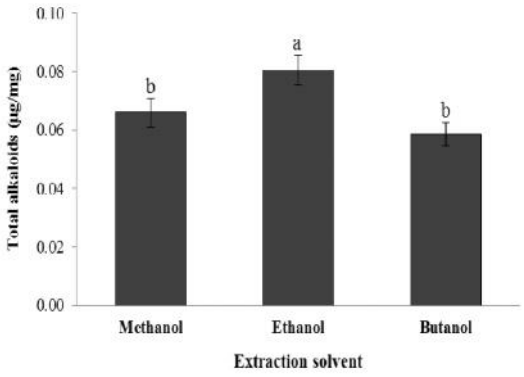
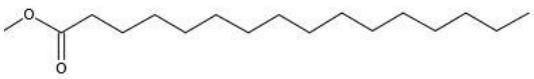
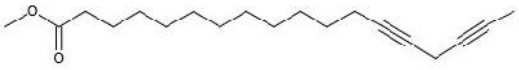
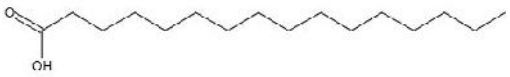
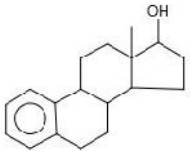
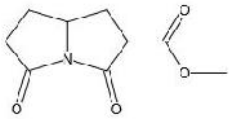
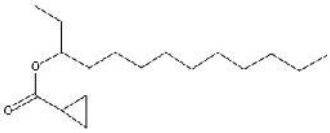
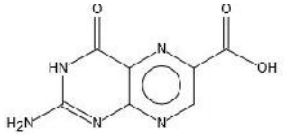
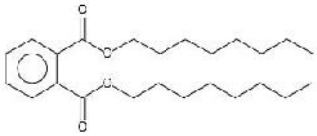
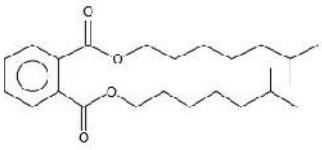


Fig. 9. The content of alkaloid compounds in methanol, ethanol, and butanol extractions of *Lilium ledebourii* bulb.

Table 2. Compounds identified by GC-MS from methanol extraction of *Lilium ledebourii*

Compound name	Chemical formula	Chemical structure
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	
13,16-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	
Estra-1,3,5(10)-trien-17β-ol	$C_{18}H_{24}O$	
Pyrrolizin-1,7-dione-6-carboxylic acid, methyl (ester)	$C_9H_{11}NO_4$	
3-Cyclopropylcarbonyloxytridecane	$C_{17}H_{32}O_2$	
Pterin-6-carboxylic acid	$C_7H_5N_5O_3$	
3-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	
Di-n-octyl phthalate	$C_{24}H_{38}O_4$	
1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	

The composition of Pyrrolizin-1,7-dione-6-carboxylic acid, methyl (ester) has previously observed in plants such as *Adiantum capillus-veneris* Linn (Vadi *et al*. 2017) and *Frankenia pulverulenta* L. (Altameme 2017). This compound has antitumor and antiviral properties (Hussein *et al*. 2016). Pterin-6-carboxylic acid belongs to the Pteridine family of alkaloids and exhibits unique properties such as fluorescence. This family has a significant role in metabolism. These compounds can be found in plants such as *Adiantum capillus-veneris* Linn (Vadi *et al*. 2017) and *Artemisia judaica* (Shiboob 2018). Its mass is 207.148, which is exactly the same as that of extracted in *L. ledebourii* (Buckingham *et al*. 2010). Pterin-6-carboxylic acid has reported to possess many biological activities such as antitumor and antioxidant activities (Hussein *et al*. 2016).

In this study, the reproductive organs of *L. ledebourii* were studied and compared with other species of *Lilium*. Besides, the presence and content of alkaloids in the bulb of this plant were investigated. These findings can be effective in reducing the danger of extinction and increasing the economic value of this valuable plant. However, further biochemical and genetic investigations are needed to identify the unknown factors that affect the plant survival and distribution.

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