



Influence of Climatic Conditions and Phenological Stages on Chemical Composition, Essential Oils and Anatomical Characteristics of *Ajuga iva* (L.) Schreb.

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ABSTRACT

Many species of *Ajuga* have medicinal values and widely used to treat many diseases. The medicinal values of *Ajuga iva* are associated with the containment of many pharmaceutical compounds as essential oils, diterpenoids, triterpenes and phenolic compounds. No information is currently available concerning the effect of different climatic conditions and phenological stages on chemical composition, essential oils or anatomical features of *Ajuga iva*. Therefore, this study aimed to investigate the effect of different climatic conditions and phenological stages on the contents of free sugars, phytohormone and essential oils, as well as anatomical characteristics of *Ajuga iva* (L.) Schreb. The leaves of *Ajuga iva*, showed the presence of high frequency of trichomes in upper and lower leaf surfaces which may limit light absorption, thereby reducing the risk of photoinhibition. The results indicated that the contents of chlorophyll a, total carbohydrates and most of the detected sugars were significantly increased during the flowering stage in dry season. HPLC analysis of *Ajuga* extracts identified 14 phenolic compounds most of which tended to increase in the flowering stage. Where the concentrations of pyrogallol increased 3.7-fold and gallic acids increased 4.6-fold, also the concentrations of catechol and vanillic acid increased intensely 8 and 7-fold, respectively. The results indicated that the yield of essential oils of the aerial parts of *Ajuga iva* have depended on the phenological stages and significantly increased in winter. The data of headspace GC-MS analysis of volatile oils indicated that the essential oils of *Ajuga iva* were dominated by volatile monoterpenes (C₁₀H₁₆) which have been shown to play important ecological roles in plant defence mechanisms. Sabinene was the main constituent in the essential oil of *Ajuga*, followed by α -thujene and α -pinene. The results indicated that the full flowering stage was considered as an ideal period for harvesting the high yield of plant hormones, sugars and phenolic compounds, as well as α -Thujene from *Ajuga iva*.

Keywords: *Ajuga iva*, anatomy, essential oils, phenolics, plant hormones

1. Introduction

Lamiaceae is well represented in the Mediterranean area and in Britain, comprises about 200 genera and 3300 species: aromatic, perennial, annual undershrubs or herbs. Most of the species belonging to the Lamiaceae are used widely in traditional medicine as a cure for many diseases (Carović-Stanko *et al.*, 2016).

Ajuga iva (L.) Scherb. is small woolly aromatic perennial flowering plant, the genus *Ajuga* belongs to family Lamiaceae, comprises 40 species. *Ajuga* is herbaceous plant that grows in rocky habitats in the western Mediterranean region and native to Asia, Europe, Northern Africa, and distributed across the temperate world (Yalcin and Kaya, 2006). The plant is rare and endangered (Batanouny *et al.*, 1999). The leaves and other aerial parts of the herbaceous plants have glandular hairs which secrete the volatile oil.

Ajuga has been used in Iran as a traditional medicinal herb to treat jaundice, joint pain and gout (Naghbi *et al.*, 2005), as well as anthelmintic (Bellakhdar *et al.*, 1991), antifungal and antimicrobial agents (Makni *et al.*, 2013). Generally, different species of *Ajuga* have medicinal values and widely used to treat many diseases such as hypertension, hyperglycemic, analgesia and fever (Pal and Pawar,

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2011), acute and chronic pharyngitis and pneumonia (Kirtikar and Basu, 1962; Agrawal and Tamrakar, 2005). These medicinal remedies are associated with the containment of *Ajuga* many pharmaceutical compounds as essential oils, diterpenoids, triterpenes, neo-clerodane-diterpenes, sterols, iridoid glycosides and flavonoids.

In fact, it is well documented that under different environmental and geographical conditions, the same species, can produce essential oils with different chemical profiles and biological effects (Maksimovi *et al.*, 2018; Mastinu *et al.*, 2021). No information is currently available concerning the effect of different climatic conditions and phenological stages on chemical composition, essential oils or anatomical features of *Ajuga iva*. Therefore, this study aimed to investigate the effect of different climatic conditions and phenological stages on the contents of free sugars, phytohormone and essential oils, as well as anatomical characteristics of *Ajuga iva* (L.) Schreb.

2. Materials and Methods

2.1. Plant materials

The aerial parts of *Ajuga iva* plant were collected in February during its vegetative stage and during flowering stage in July from Sidi Barani desert, Marsa Matrouh governorate. The samples of *Ajuga* plant were identified in the Herbarium of the Desert Research Center.

2.2. Ecological studies

2.2.1. The climate data

The climate data consist of average temperature and the rate of rainfall of the studied habitat, provided by Applied Agricultural Meteorological Laboratory.

2.2.2. Soil analysis

Soil samples were collected from the soil supporting the investigated plants at 3 random points at 0-30 cm. Soil texture (granulometric analysis) was determined according to Gee and Bauder (1986) using the international pipette. Soil EC and soil reaction (pH) in soil water suspension (1:2.5) was determined according to Page (1987), the content of Na and K were determined by using flame photometer (Jenway, PFP-7) and the concentration of Cl was determined according to Jackson (1967). The concentrations of magnesium, calcium, carbonate (CO₃) and bicarbonate ions (HCO₃) were determined according to the method of Rowell (1994).

2.3. Physiological studies:

2.3.1. Anatomical examination

Fresh samples of *Ajuga iva*, collected from Sidi Barani desert in winter and summer seasons were kept in ethyl alcohol solution to fix and prepare them for anatomical studies. Four samples were sectioned by using microtome according to Paraffin Sectioning Method (Bani *et al.*, 2011; Mavi *et al.*, 2011). The staining slides were examined under Leica light microscope model DM-500, the images were acquired by using digital camera Leica ICC 50 HD with LAS E7 software version 2.1.0 2012.

2.3.2. Determination of photosynthetic pigments

The contents of photosynthetic pigments; chlorophyll-a (Chl a), chlorophyll-b (Chl b) and carotenoids were determined according to Sumanta *et al.* (2014). A known weight of fresh leaf sample (0.5g) was homogenized in 10 ml of 80% acetone, and the homogenate was centrifuged at 10,000 rpm for 15 min at 40 °C. The supernatant was filtrated, then 0.5ml of the filtrate was mixed with 4.5ml of solvent. The solution mixture was analyzed for chlorophyll a, chlorophyll b and carotenoids content and calculated according to the following equations:

$$\text{Chl a} = 12.25A_{663.2} - 279A_{646.8} \dots\dots\dots (1)$$

$$\text{Chl b} = 21.5A_{646.8} - 5.1A_{663.2} \dots\dots\dots (2)$$

$$C_x + c = (1000A_{470} - 1.82C_a - 85.02C_b) / 198 \dots\dots\dots (3)$$

Where: A = Absorbance, Chl a = chlorophyll a, Chl b = chlorophyll b, C x+c = carotenoids) and the results were expressed as (mg/100gFW).

2.4. Plant phytochemical studies

2.4.1. Preparation of samples

The aerial parts of *Ajuga iva* were dried in the oven at 60 °C and ground to fine powder and subjected to various analyses.

2.4.2. Determination of minerals content

Half g of dried sample was digested on a hot plate with 10ml concentrated sulphuric acid and (2-4ml) of perchloric acid was added. The clear solution was allowed to cool, then diluted to 100ml and used for analysis of minerals according to Baker and Smith (1974). The concentrations of K and Na were determined by a flame photometer (Jenway PFP7), calcium and magnesium contents were determined according to the method of Rowell (1994) by titration with ethylene diamine tetraacetic acid (EDTA). The concentrations of manganese (Mn), copper (Cu), zinc (Zn) and iron (Fe) were determined by using ICP emission spectroscopy (Jones, 1977). The dry matter of the plant was ashed and used to measure the concentrations of phosphorous according to Rowell (1994) and chloride according to (Jackson and Thomas, 1960). Nitrogen (N) content of sample was estimated by the method of Kjeldahl (1983) and crude protein was calculated as $N \times 6.25$ (James, 1995).

2.4.3. Determination of phenolic compounds

Phenolic compounds were determined by HPLC according to the method of Goupy *et al.* (1999) as follow: 5g of sample were mixed with methanol and centrifuged for 10 min at 1000rpm, the supernatant was filtered through a 0.2 µm Millipore membrane filter. In a vial ,1-3ml were injected into HPLC Hewllet Packard (series 1050) equipped with solvent degasser, auto sampling injector, ultraviolet detector set at 280 nm and quaternary HP pump series (1100). To separate phenolic compounds, packed column Hypesil BDS-C18, 4.0 x 250 mm was used at 35°C. Methanol and acetonitrile were used as mobile phases at flow rate of 1 ml/min. Phenolic acid standers were dissolved in mobile phase and injected into HPLC. Retention time and peak area were used to calculate phenolic compounds concentration by data analysis of Hewllet Packard software, Germany.

2.4.4. Determination of phytohormones

The plant hormones: gibberellic acid (GA), indole acidic acid (IAA), abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) were determined by reversed phase high performance liquid chromatography (HPLC).

2.4.4.1. Extraction of phytohormones

Two hundred milligrams (200 mg) of the tissue from leaves were weighed and suspended in 1 mL of ice-cold 50% Me-OH (-20°C) (Duportet *et al.*, 2012) at a ratio of 1: 5 (w/v) in 2-mL microcentrifuge tubes containing ceramic microbeads. The tubes were transferred to a microcentrifuge, and the extracts were centrifuged for 15 min at 4 °C and $13,000 \times g$. The supernatants were transferred into clean 2-mL microcentrifuge tubes while the pellets were resuspended with 500 µL of ice-cold 50% Me-OH and the extraction process repeated. The supernatants were combined and applied to 3-mL Strata-X-CSPE cartridges containing 30 mg of polymeric sorbent (33 µm, 85 Å particles, 0.9–1.2 milli-equivalents/g) (Phenomenex, Torrance, California, USA) for sample clean-up and concentration (Balcke *et al.*, 2012). The cartridges were conditioned with 1 mL of Me-OH and equilibrated with 1 mL of water before sample application onto the cartridge (1.5 mL). The SPE cartridges were eluted with 0.9 mL of 100% acetonitrile to release the phytohormone. All the solvents and samples applied to the cartridges were kept at ice-cold temperatures. The eluates from the cartridges were filtered through 0.22-µm filters, transferred into chromatography vials, and stored at -20°C for subsequent analyses.

2.4.4.2. HPLC conditions

The extracts were analyzed on Shimadzu Class-VPV 5.03 (Kyoto, Japan) equipped with Shimadzu UV-VIS detector (SPD-10Avp) at 254 nm, LC-16ADVP binary pump, DCou-14 A degasser and phenomenex RP-18 (UK; 250 x 4.00 mm, 5 micron) column and heater set at 40 °C. Separation and

quantitation were carried out with a mobile phase of A (0.1% formic acid in Milli-Q water) and eluent B (0.1% formic acid in acetonitrile) at a constant flow rate of 0.4 mL/min. The conditions were: 85% of eluent A kept constant for 3 min, initiation of gradient by 70% of eluent A at 9 min, 50% of eluent A at 12 min, 5% of eluent A at 15 min kept constant for 2 min, and at 17 min brought back to 85% to flush the column.

2.4.5. Determination of free sugars

2.4.5.1. Sample preparation

Free sugars were determined using HPLC according to the method of (Zielinski *et al.*, 2014). One gm of plant sample was dissolved in 10ml of Milli-Q water (type 1). Then, filtered through filter membrane (0.22 µm) (Waters, Milford, MA, USA). An aliquot of 1.5 mL of these solutions was placed in vials for the analysis.

2.4.5.2. Equipment and operating conditions

The chromatographic system Agilent (series 1200) coupled to the refractive index detector was equipped with a quaternary pump, degasser and auto injector. The chromatographic data were obtained using the Agilent software. The samples were analyzed using an Aminex-carbohydrate HPX-87 column under isocratic condition with deionized water. The column temperature was maintained at 85 °C, the flow rate was 0.5 mL/min. and the detector at 50 °C. Sample detection was performed by comparing retention time's standards.

2.4.6. Determination of total carbohydrates

The total carbohydrates were determined using the phenol–sulfuric acid assay according to (Buyse and Merck, 1993). 0.3g of plant sample was dissolved in 10ml of 3%HCl and heated for 2-5 hours at 100 °C.

2.4.7. Determination of essential oil

2.4.7.1. Determination of the yield of volatile oils

About 200 g of plant powder of *Ajuga iva* were subjected to hydrodistillation method (Azmir *et al.*, 2013) for about 4 hours using Clevenger apparatus.

2.4.7.2. Analysis of volatile oils by Headspace Gas Chromatography–Mass Spectrometry (GC-MS).

Three grams of plant powder were placed into a 20 ml headspace vial. Then the vial was sealed with silicone rubber septa and aluminium caps. The sample was transferred to the headspace. The vial was heated for 20 min at 80°C while being agitated; and then introduced directly into the GC injector. The essential oils were analyzed by using Headspace method in combination with GC-MS. The GC-MS system (Agilent Technologies) was equipped with mass spectrometer detector (5977A) and gas chromatograph (7890B) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness). Headspace temperature program: oven temperature 80°C, needle temperature 85°C, transfer line temperature 90 °C and incubation time 20 min. Analyses were carried out using helium as the carrier gas at a flow rate of 1.0 ml/min at a split ratio of 1:30, injection volume of 1 µl and the following temperature program: 40 °C for 1 min; rising at 4 °C /min to 150 °C and held for 6 min; rising at 4 °C/min to 210 °C and held for 1 min. The injector and detector were held at 280 °C and 220 °C, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 50-550 and solvent delay 3 min. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

2.5. Statistical analysis

Physiological parameters and anatomical characters presented were assessed by numerical analysis performed by t test using SPSS for Microsoft Windows (Ver. 10.0, SPSS Inc., USA).

3. Results and Discussions

3.1. The Climatic conditions

The average high temperatures during the period of study in 2019 were 19.6, 17.9 and 18.1°C in December, January and February, respectively. Whereas the recorded average high temperatures during spring in April, May and June were 22.2, 24.1 and 27°C, respectively. The highest average high temperature (29°C) was recorded in August, while the lowest average high temperature (17.9°C) was recorded in January. The average rainfall rate in winter months; December, January and February were 33, 39 and 17mm, while in spring months, its rate was 5, 3 and 0 mm in April, May and June, respectively. The month with the highest rainfall rate ((39mm) was January. The dry period extended for three months from June to August. Meanwhile, the average duration of sunlight in December, January and February were 10.1, 10.4 and 11.1h, respectively. The month with the shortest days was December (Average sunlight: 10.1h), while the month with the longest days was June (Average sunlight: 14.2h).

3.2. The soil physicals and chemical properties

The soil chemical and physical analysis indicated that the soil of Sidi Barrani Desert is Loamy Sand in nature. The soil was rich with sand (62.41%) and silt (32.07 %). The percentages of clay, bicarbonate and calcium carbonate were 5.32%, 3.40% and 29.28%, respectively. The presence of silt and clay particles makes the carbonates very active, which may cause a reduction in the availability of phosphorous, zinc, manganese and copper (Moore *et al.*, 1990).

The results of the chemical analysis of soil solution, revealed that the soil pH was slightly alkaline (8.80) and soluble salts content, EC was 0.45 dS/m. The low contents of Ca²⁺(2.50 meql⁻¹), Mg²⁺(1.15 meql⁻¹), Na⁺ (0.61 meql⁻¹), K⁺ (0.40 meql⁻¹) and Cl⁻ (1.30 meql⁻¹) showed that the soil associated with *Ajuga* was lacking in nutrients and its fertility was depleted.

3.3. Anatomical features of the leaves of *Ajuga iva* during different Phenological stages

The morphological and anatomical features of *Ajuga* leaves during vegetative and flowering stages (Figs. 1 & 2), indicated that the leaves of *Ajuga* are characterized by the presence of non-glandular and glandular hairs (Fig. 1(c)), like the leaves of other species of Lamiaceae (Werker *et al.*, 1985). The non-glandular trichomes are well distributed on the epidermis and surround the stoma to shield the stoma and oil glands from intensive heat during hot and dry seasons. The flowers are bisexual, zygomorphic, grow in dense leafy spikes and are yellow in colour. The corolla is bilabiate with an elongated lower limb consisting of 2 small lateral lobes and long central bifid lobe, a minute reduced upper limb and the stamens didynamous (Evans, 2009). The average stem length is 15-20 cm, covered by dense hairs with crowded linear revolute-margined leaves (Täckholm, 1974).

Mediterranean-type ecosystems are characterized by certain temperature and rainfall regimes that disturb the growth of plants in both the summer and winter seasons (Mitrakos 1980; Larcher 2000). *Ajuga iva* is a seasonally dimorphic plant, which presents entirely different appearances in winter and summer. The morphology and length of leaf were significantly affected by seasons and significantly different between the vegetative and flowering stages as shown in Fig (1). Average leaf length was 3.73±0.05 during vegetative stage and 1.56±0.05 during flowering stage.

As shown in Figure (2) and Table (1), there was a significant increase in the thickness of mesophyll layer and elongation of parenchymatic palisade during vegetative stage, which reflects the high photosynthetic capacity during this period as reported by Von Caemmerer and Evans (1991) and Oguchi *et al.* (2005). The thickness of mesophyll with additional layers of palisade cells helps to promote using light efficiently and reduce the light damage under low temperatures in winter (Huner *et al.* 1998; Terashima *et al.* 2001). Also, a high frequency of trichomes in *Ajuga iva* (Fig.1) may limit light absorption, thereby reducing the risk of photoinhibition. Glandular trichomes are epidermal appendages and considered the primary secretory organ in Lamiaceae. The essential oils are often found in glandular hairs that present on the leaf surface, trichomes, in a bulbous, sub-cuticular chamber, in droplets of fluids located under the surfaces of leaves or in the secretory canals of plant-cell walls plant (Venkatachalam *et al.*, 1984; Abdelmajeed *et al.*, 2013). The glandular trichomes include peltate and capitate types. The peltate trichomes are embedded in the epidermis, each trichome consists of one basal epidermal cell and a short cylindrical stalk cell with a large round secretory head, when the trichomes

are touched, the secretion product is released to the outside. Whereas capitate trichomes consisted of a one-celled glandular head, subtended by a stalk of one cell, after the production of trichomes secretory materials, these materials are extruded to the outside immediately as reported by Werker (1993).



Fig. 1: Morphology of *Ajuga iva* (L.) Schreb during vegetative stage(a) and flowering stage(b), glandular trichomes on the leaf upper and lower surfaces (c), *Ajuga* leaf and cross section during vegetative (d (1-2))and during flowering stages (d (3-4)).

Previous studies showed that the peltate trichomes produce most of the essential oils in the Lamiaceae (Werker, 1993; Clark *et al.* 1997; Huang *et al.*, 2008). Trichomes may thus have multiple functions and trichome density may evolve in response to variations in several environmental factors. According to Ehrlinger (1984) and Løe *et al.* (2007) the plants with high density of leaf trichomes such as *Ajuga* in this study can indicate that the plant inhabits in environments that are dry or cold, where UV-radiation is intense, and in areas where the risk of being damaged by herbivorous insects is high.

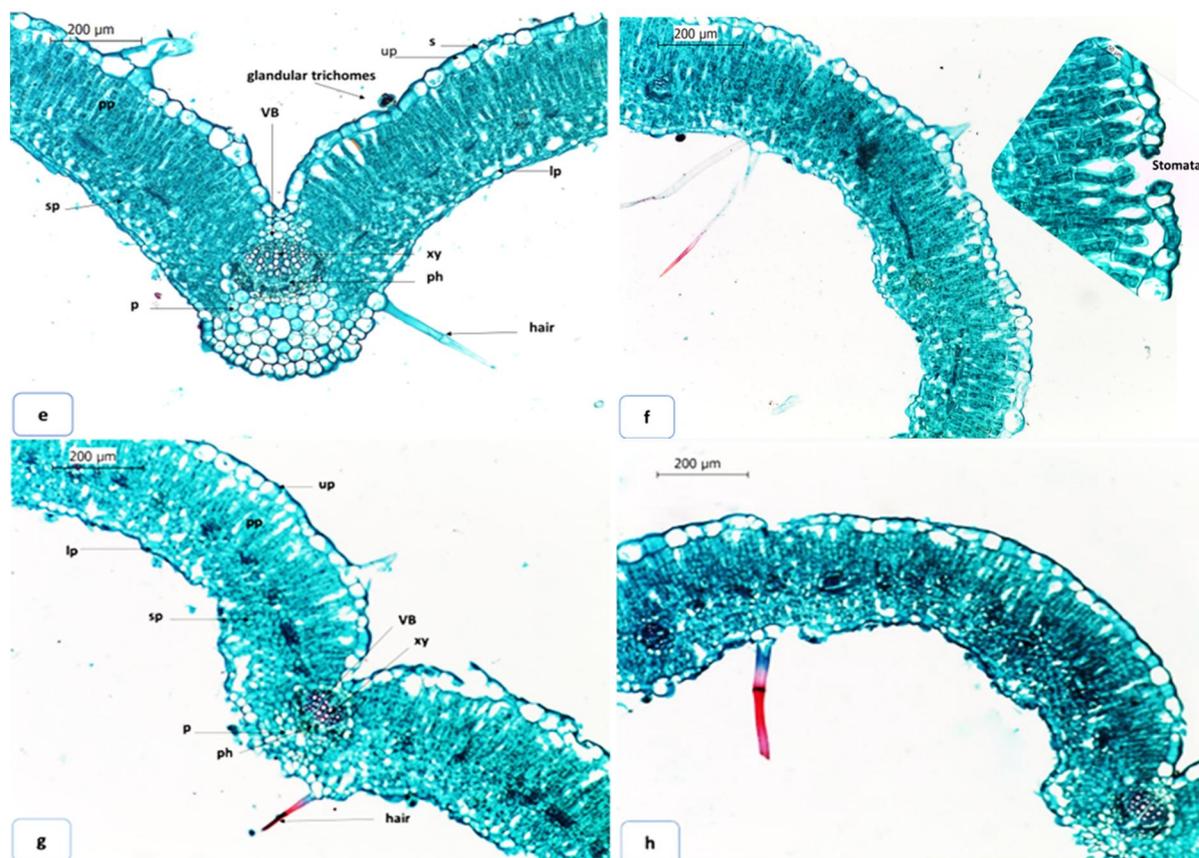


Fig. 2: Transverse sections of the lamina of *Ajuga iva* in winter (e-f) and summer (g-h); up: upper epidermis, lp: lower epidermis, pp: palisade parenchyma, sp: spongy parenchyma, p: parenchyma, ph: phloem, VB: vascular bundle, xy: xylem, s: stomata (Scale bar 200µm).

Table 1: The anatomical characters of the leaf of *Ajuga iva* during vegetative and flowering stages

Characters	Vegetative stage	Flowering stage
Average length of upper epidermis (µm)	28.90±3.52 ^a	33.64±1.80 ^a
Average length of lower epidermis(µm)	12.58±2.42 ^a	11.37±0.73 ^a
Average thickness of palisade parenchyma layer(µm)	134.92±2.57 ^a	101.75±7.70 ^b
Average length of palisade parenchyma(µm)	35.82±3.42 ^a	21.47±2.32 ^b
Average Length of leaf vascular bundle (VB) (µm)	134.8±1.77 ^a	74.42±4.05 ^b
Average width of leaf VB (µm)	183.56±1.70 ^a	83.59±3.07 ^b
Average thickness of the leaf midrib (µm)	390.67±0.80 ^a	182.23±1.02 ^b
Average Length of parenchymatic tissues layer below VB (µm)	141.87±4.49 ^a	81.86±1.65 ^b
Average area of parenchyma cells (µm ²)	1578±139.7 ^a	349±101.9 ^b
Average thickness of spongy parenchymatic tissues layer(µm)	55.00±0.81 ^b	70.52±0.91 ^a
Average length of leaf (cm)	3.73±0.05 ^a	1.56±0.05 ^b

Values are expressed as mean ± SD (n=3), in each row values followed different letters are significantly different at p<0.05

The images of leaf cross sections of *Ajuga iva* showed that the leaf lamina rolls transversally to the midrib. Leaf-rolling may be related to the water potential in the leaf and resulted from a top–bottom differential elastic shrinkage in the leaf cross-section (Mouliya, 2000) and may be related to the accumulation of phytohormones (Krishna, 2003; Talaat and Shawky, 2012). Moreover, leaf rolling is a good indicator of drought tolerance (Amelework *et al.*, 2015) and results in decreasing leaf temperature and rate of transpiration (Richards *et al.*, 2002), decreasing stomata closure and represents an important drought-avoidance mechanism under drought stress (O’Toole *et al.*, 1979).

3.4. The content of photosynthetic pigments in *Ajuga* during different phenological stages

As shown in Table 2, the content of primary photosynthetic pigment, chlorophyll a (Chl a) was significantly affected by phenological stage, as its value was 2.16±0.16 mg/100gFW in vegetative stage and significantly decreased to 1.10±0.12 mg/100g FW in flowering stage in dry season. The reduction in the content of chlorophyll was reported in some crop species, especially in tolerant genotypes more than in sensitive genotypes (Sairam *et al.*, 1997). Whereas the concentration of Chl b was significantly increased to 15.8 ± 0.07 mg/100g FW in flowering stage in dry season.

The obtained data indicated that there was a significant reduction in the ratio of Chl a to Chl b from 2.977±0.435 during vegetative stage in winter to 0.070±0.008 during flowering stage in summer. The reduction of the ratio of chlorophyll a to b was previously reported in resistant species of tomato under stress conditions, this indicated that photosystem II protects the plant against low water stress (Ghorbanli *et al.*, 2013).

Table 2: The content of photosynthetic pigments in *Ajuga* during different phenological stages

Stages	Chlorophyll a (mg/100g FW)	Chlorophyll b (mg/100g FW)	Carotenoids (mg/100g FW)	Chl a/Chl b
Vegetative	2.16±0.16 ^a	0.73±0.05 ^b	1.45±0.08 ^a	2.97±0.43 ^a
Flowering	1.10±0.12 ^b	15.8±0.07 ^a	1.60±0.07 ^a	0.07±0.01 ^b

Values are expressed as mean ± SD (n=3), in each column values followed different letters are significantly different at p<0.05.

3.5. The content of minerals in *Ajuga* during different phenological stages

The obtained results (Table 3) showed that the contents of minerals, protein and consequently nutrition value of *Ajuga iva* were significantly affected by phenological stages. The contents of nitrogen, sodium, potassium, phosphorus, chloride and iron were significantly decreased in flowering stage, whereas the contents of calcium, zinc and copper were significantly increased in flowering stage.

Table 3: The content of minerals and total protein in *Ajuga iva* during vegetative and flowering stages

Minerals	Phenological stages	
	Vegetative stage	Flowering stage
Nitrogen g /100g	2.13±0.015 ^a	1.55±0.200 ^b
Sodium g /100g	0.87±0.020 ^a	0.39±0.005 ^b
Potassium g /100g	1.78±0.025 ^a	1.55±0.115 ^b
Calcium g /100g	0.86±0.015 ^b	1.74±0.021 ^a
Magnesium g /100g	0.51±0.015 ^a	0.51±0.011 ^a
Phosphorus g /100g	0.22±0.020 ^a	0.09±0.020 ^b
Chloride g /100g	0.61±0.020 ^a	0.31±0.015 ^b
Iron mg /100g	59.9±1.543 ^a	40.92±0.161 ^b
Manganese mg / 100g	2.71±0.030 ^a	2.22±0.025 ^b
Zinc mg /100g	1.72±0.020 ^b	2.55±0.025 ^b
Copper mg/100gm	1.27±0.017 ^b	1.91±0.0251 ^a
Total protein (g/100g)	13.33±0.091 ^a	9.68±0.125 ^b

Values are expressed as mean ± SD (n=3), in each row values followed different letters are significantly different at p<0.05

3.6. The content of phenolic compounds in *Ajuga* during different phenological stages

HPLC analysis of *Ajuga* extracts identified 14 phenolic compounds (Table 4), most phenolic compounds in *Ajuga* tended to increase in the flowering stage. Where the concentrations of pyrogallol increased 3.7-fold and gallic acids increased 4.6-fold in flowering stage, also the concentrations of catechol and vanillic increased intensely 8 and 7-fold, respectively. The content of catechin increased from 47.30 in wet season during vegetative stage to 152.57 µg/g during flowering stage in dry season. Similarly, the contents of catechol and ferulic increased from 7.80 and 29.54 µg/g during vegetative stage to 63.80 and 45.43 µg/g during flowering stage, respectively. The contents of 4-Aminobenzoic and coumarin increased from 1.37 and 12.81 during vegetative stage to 7.76 and 21.36 µg/g during flowering stage, respectively.

The results of HPLC analysis indicated that chlorogenic acid was detected only in the sample that collected during flowering stage. In agreement with the obtained results, several researchers have reported that the biosynthesis of phenolic was stimulated in the flowering stage among different species of Lamiaceae such as *Mentha pulegium* (Salem *et al.*, 2018), *Melissa officinalis* (Saeb *et al.*, 2011), *Origanum majorana L.* (Hamrouni *et al.*, 2009) and mint species mainly during the time of ultraviolet radiation (Shahmohamadi *et al.*, 2014; Tomson and Kruma, 2017). The increase in phenolic content during flowering in the dry season is an adaptive response to stress conditions which provides protection of leaf tissue against UV-B penetration thus acting as antioxidant, also it has several functions in plant as antibiotics, natural pesticides, attractant for pollinators (Heldt, 1997). Therefore, the presence of high levels of this compound in *Ajuga iva* may explain the high antioxidant capacity of ethanolic extracts of *Ajuga*, collected in summer season during flowering stage as reported by El-lamey (2020). So, the full flowering stage was considered as an ideal period for harvesting the high yield of phenolic compounds from *Ajuga iva*.

Table 4: The content of phenolic compounds in *Ajuga iva* during vegetative and flowering stages

Phenolic compounds (µg/g)	Phenological stages	
	Vegetative stage	Flowering stage
Pyrogallol	79.36	294.81
Gallic	1.97	9.17
3-OH Tyrosol	3.63	8.55
Catechol	7.80	63.80
4-Aminobenzoic	1.37	7.76
Catechin	47.30	152.57
Chlorogenic	-	82.18
p-OH-benzoic	9.05	14.17
Caffeic	41.00	27.54
Vanillic	10.99	75.95
Caffeine	112.59	127.44
Ferulic	29.54	45.43
Ellagic	75.60	238.85
Coumarin	12.81	21.36

3.7. The content of phytohormones in *Ajuga* during different phenological stages

As shown in Table (5), the content of phytohormones increased in flowering stage. The of content of Indole acetic acid (IAA) increased progressively from 0.010 µg/g in vegetative stage to 0.874 µg/g in flowering stage, similarly, the contents of abscisic acid (ABA) and gibberellic (GA₃) increased from 0.004 and 0.183 in vegetative stage to 1.240 and 0.302 µg/g in flowering stage, respectively. No remarkable change in the contents of salicylic and jasmonic acids. According to Blázquez and Weigel (1999), the plant hormones that significantly affect flower bud differentiation are gibberellic acid, zeatin riboside (ZR), abscisic acid and indole acetic acid.

Table 5: The content of phytohormones in *Ajuga iva* during vegetative and flowering stages

Phenological stages	Salicylic acid (µg/g)	Indole acetic acid (µg/g)	Abscisic acid (µg/g)	Gibberellic acid (µg/g)	Jasmonic acid (µg/g)	ABA/GA ₃	ABA/IAA
Vegetative	0.095	0.010	0.004	0.183	0.001	0.022	0.400
Flowering	0.114	0.874	1.240	0.302	0.003	4.106	1.418

The effects of phytohormones on plants during flowers formation differ between plants. For example, in biennial and long-day (LD) plants, gibberellic acid can facilitate flower formation, while in apple and *Arabidopsis thaliana*, the formation of the flower is inhibited by it. According to Domagalska *et al.* (2010) and Gazzarrini and McCourt (2003), the flower bud differentiation during the formation of the flower is influenced by the hormones content and the ratio between them through their effect on the use of nutrients. In this study, the high ratio of ABA: GA₃ (4.106) and ABA: IAA (1.418) may contribute to flower bud differentiation during the flowering stage in *Ajuga iva* as reported by Feng *et al.* (2006) in roses plants. Also, the participation of IAA in the differentiation and development of each flower organ has been reported by Zhang *et al.* (2014).

3.8. The contents of free sugars and total carbohydrates in *Ajuga* during different phenological stages

The contents of free sugars, glucose, inulin and galactose increased in summer season during the formation of flower, while the contents of sucrose and sorbitol increased in winter season during vegetative stage (Table 6).. The results showed no remarkable change in the contents of fructose, mannitol and stachyose and a slight increase in glucuronic acid during flowering stage. Sugars act as osmolytes that can assist plants to maintain water within cells and protect cellular component from dehydration (Wood and Goldsbrough, 1997) and have a protective role for chloroplast from damage in conditions of water deficit during flowering stage (Santarius,1973).

The concentration of total carbohydrates increased significantly during the formation of flower; their value increased from 4.35±0.03g/100g during vegetative stage to 4.72±0.03g/100g during flowering stage.

Table 6: The contents of free sugars and total carbohydrates in the Arial parts of *Ajuga* during vegetative and flowering stages

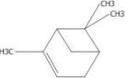
Free sugars g/100g	Phenological Stages	
	Vegetative Stage	Flowering Stage
Glucose	0.6074	0.9749
Xylose	0.0489	0.0180
Fructose	1.7426	1.6823
Sucrose	3.3749	1.0121
Ribose	0.1189	0.0664
Stachyose	0.3798	0.3472
Inulin	0.2507	4.0510
Maltose	-	0.0469
Raffinose	0.1178	0.0783
Galactose	0.1461	0.4305
Rhamnose	-	0.1219
Glucuronic acid	0.0602	0.0995
Arabinose	-	0.3011
Mannitol	0.0433	0.0415
Sorbitol	0.0942	0.0356
Total carbohydrates (g/100g)	4.35±0.03^b	4.72±0.03^a

3.9. The main constituents of the essential oils of *Ajuga* during different Phenological stages

The data of headspace GC-MS analysis of volatile oils (Table 7), (Figs. 3 & 4) indicated that the essential oils of *Ajuga iva* were dominated by volatile monoterpenes (C₁₀H₁₆) which have been shown to play important ecological roles in plant defense mechanisms through indirect chemical and physical defences (Phillips and Croteau, 1999). When the plant is subjected to mechanical damage resulting from attacking by microbes or herbivores, volatile monoterpenes are directly emitted to the atmosphere which helps in the recruitment of herbivore predators and pheromone attraction/repelling of herbivores (Erbilgin and Raffa, 2001; Reddy and Guerrero, 2004).

The Phenological stages showed differently the levels of major compounds. The percentages of alpha -thujene, rimantadine and nonacosane increased during flowering stage under extreme environmental condition, while the percentage of Sabinene, alpha -Pinene and (2S, 3S)-2,3-Epoxy-1-hexanol increased during vegetative stage. According to (Gali-Muhtasib, 2006), higher content of β-thujone has been reported during the flowering period. Sabinene was the main constituent in plant samples, collected during vegetative (68.14%) and flowering stages (53.45%). Accumulation of phytohormones during flowering stage may lead to increased leaf-rolling as reported by Krishna (2003) and Talaat and Shawky (2012). The effects of different environmental conditions such as the soil pH, growth at different altitudes, geographic location, and /or season, as well as plant age on the quality and the main constituents of volatile oils have been reported by others (Ravid *et al.*, 1992; Saleh *et al.*, 1987).

Table 7: The main constituents of the essential oils in *Ajuga iva* during vegetative and flowering stages

Peak	RT	Component	Molecular Formula	Stereo structure	Vegetative Area Sum %	Flowering Area Sum %
1	11.382	α-Thujene	C ₁₀ H ₁₆		5.39	17.83
2	11.62	α -Pinene, (-)-	C ₁₀ H ₁₆		16.18	14.01
3	13.13	Sabinene	C ₁₀ H ₁₆		68.14	53.45
4	15.00	Rimantadine	C ₁₂ H ₂₁ N		2.92	7.11
5	30.90	(2S,3S)-2,3-Epoxy-1-hexanol	C ₆ H ₁₂ O ₂		3.95	3.43
6	35.45	Nonacosane	C ₂₉ H ₆₀		3.41	4.17
Total (%)					99.9	100

RT, Retention time (as min), all contents are relative, comparing according to total amount of essential oil, which is found in the plant.

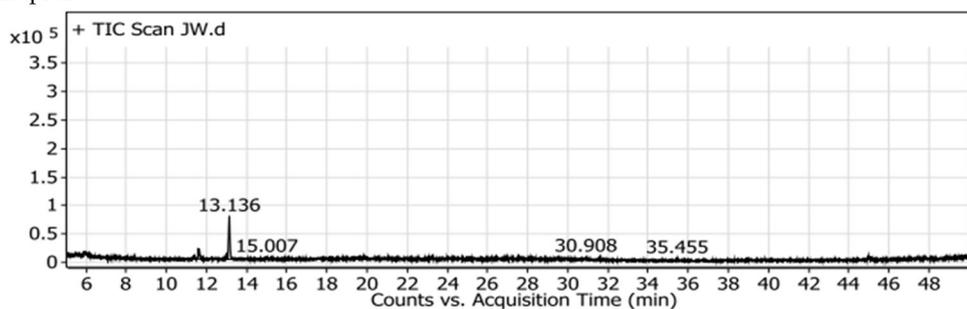


Fig. 3: Chromatogram of essential oil of *Ajuga iva* during vegetative stage.

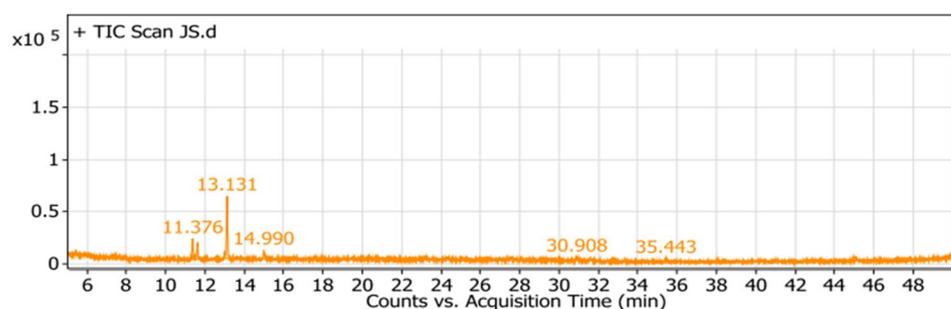


Fig. 4: Chromatogram of essential oil of *Ajuga iva* during flowering stage

The high content of essential oils can be used as an indicator of the plant capacity of tolerance to environmental stress. Because they assist the plants to adapt the environmental stress conditions as, drought, high temperature and intense radiation and pollution with heavy metals (Abu-Darwish and Abu-Dieyeh, 2009; Abu-Darwish, 2009). Therefore, natural selection favours the survival of population with the high content of essential oils that has a higher adaptive value and a high chance of survival as reported by Stevović, *et al.* (2011).

The results indicated that the yield of essential oils of the aerial parts of *Ajuga iva* has depended on the phenological stages; where the yield of oils was 0.35% in vegetative stage and significantly decreased to 0.15% in flowering stage. This increase may be attributed to the increase in photosynthetic rate and the production of photosynthetic carbon which resulted from the increase of photosynthetic pigments concentration in winter season that invests largely in the biosynthesis of secondary metabolites, including essential oils Padda and Picha (2008). Similarly, Jamali *et al.* (2013) reported that the decrease in essential oils content during the fruiting stage as a response to the leaf senescence and due to the activation of a sequence degradative chemical reaction during catabolic pathways. Furthermore, the reduction of the surface area of the leaf of *Ajuga* during the formation of flower can affect the photosynthetic capacity, decrease the nutrient availability (Candolfi-Vasconcelos and Koblet, 1990) and consequently decrease the biosynthesis of essential oils.

4. Conclusion

The results indicated that the content of photosynthetic pigments in the leaves of *Ajuga iva* was significantly different between vegetative and flowering stages, which may be resulted from the variations in the size of leaf and difference in anatomical features, especially the thickness of mesophyll tissues and elongation of parenchymatic plastid, which considered as adaptive responses to the difference in climatic conditions. The leaves of *Ajuga iva* showed the presence of high frequency of trichomes, which may limit light absorption, thereby reducing the risk of photoinhibition. The images of leaf anatomy of *Ajuga iva* showed that the leaf lamina rolls transversally to the mid-rib. Leaf-rolling may be related to the accumulation of phytohormones and results in decreasing leaf temperature and rate of transpiration, decreasing stomata closure and represents an important drought-avoidance mechanism under drought stress during flowering stage. Total carbohydrates and most of the detected sugars were significantly increased during the flowering stage in dry season. HPLC analysis of *Ajuga* extracts identified 14 phenolic compounds most of which tended to increase in the flowering stage. Where the concentrations of pyrogallol increased 3.7-fold and gallic acids increased 4.6-fold, also the concentrations of catechol and vanillic acid increased intensely 8 and 7-fold, respectively. The results indicated that the yield of essential oils of the aerial parts of *Ajuga iva* have depended on the phenological stages and significantly increased in winter. The data of headspace GC-MS analysis of volatile oils indicated that the essential oils of *Ajuga iva* were dominated by volatile monoterpenes ($C_{10}H_{16}$) which have been shown to play important ecological roles in plant defence mechanisms. Sabinene was the main constituent in the essential oils of *Ajuga*, followed by α -thujene and α -pinene. The results indicated that the full flowering stage was considered as an ideal period for harvesting the high yield of plant hormones, sugars and phenolic compounds, as well as α -Thujene from *Ajuga iva*.

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