Changes in the medicinal and antimicrobial compounds, methyl palmitate, methyl stearate, and bis (2-ethylhexyl) phthalate in American agave (*Agave Americana* L. cv Marginata) under urea treatment

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Abstract

In the present study, the effect of urea fertilizer was investigated on growth rate, leaf terpenoids, and bioactive compounds detectable by GC-MS in American agave leaf extract under pot and greenhouse cultivation. Two concentrations of urea 46% fertilizer (5 and 10 g l^{-1}) was used with irrigation, in 8-leaf stage, and their effects were compared with plants irrigated with no fertilizer as control in a randomized complete block design. Applying urea fertilizer at both concentrations increased the fresh and dry weights of leaves and roots. Urea fertilizer had no significant effect on leaf terpenoid content, but leaf extract analysis by GC-MS showed a positive effect on increasing the content of methyl palmitate and methyl stearate, which are valuable medicinal compounds with antimicrobial, anti-inflammatory, anti-cancer, and antioxidant properties. Utilization of urea fertilizer, doubled the percentage of these compounds in leaf extract. However, urea fertilizer reduced the content of bis (2-ethylhexyl) phthalate, which has also medicinal properties comparable to the other two mentioned compounds. The positive effect of urea fertilizer in terms of a significant increase in octadecane and methyl palmitate (about twice) with usability as a biofuel is another important finding of the present work.

Keywords: GC-MS, methyl ester, nitrogen fertilizer, octadecane, succulent, terpenoid

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Introduction

American agave is native to Mexico and the United States in New Mexico, Arizona, and Texas, but is now cultivated worldwide as an ornamental plant (Anderson, 1999). This plant is adapted to many areas, including parts of South America, the Southern Mediterranean zones, Africa, India, China, Thailand, and Australia (Oudhia, 2007).

The five major parts of the agave plant, namely flowers, leaves, stems, seeds, and buds, are edible. Edible leaves are boiled and the buds, stems and branches are roasted as food. In Asia, agave flower stalks are also dried or roasted to consume. Its grain flour is used for making bread or as a bread ingredient (Cruz-Rubio et al., 2018).

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Agave syrup is a healthy alternative for many purposes. Low-fat agave syrup is used in low-calorie diets. Its leaf tea is also used to control blood sugar and treatment of digestive problems (Oudhia, 2007).

Ethno pharmacologically, agave species have been used to treat bacterial diseases such as ulcers and gastrointestinal infections, urological disorders, and dysentery, as well as diseases related to oxidative stress such as cancer and diabetes (Madrigal-Santillán et al., 2022). On the other hand, anti-inflammatory properties (Hernández-Delgado et al., 2021), anti-hypertensive (Álvarez-Chávez et al., 2021), immunomodulatory (El-Hawary et al., 2020), antiparasitic (López-Romero et al., 2018), and antifungal (Esquivel-Chávez et al., 2021) properties have been reported for agave species. According to a 2015 study by Thakur et al., American agave leaf extract possesses anti-Leishmania antiparasitic activity. The sap is also antiseptic, diaphoretic, diuretic, and antifungal. Furthermore, the extract of its fallen leaves is laxative and its leaf extract is used to treat bruises. Agave juice is also used to treat diarrhea and dysentery (Thakur et al., 2015).

Pharmacologically, the most important secondary metabolites of American agave are flavonoids, terpenoids, saponins and hecogenins. Hecogenin is used in pharmacy to make cortisone and sex hormones (Ghoghari and Rajani, 2006). Studies by some researches (El-Hawary et al., 2020) showed that aqueous extracts and steroidal sapogenins of American agave have good anti-inflammatory activity. Also, methanolic extract of American agave has strong cytotoxic properties.

In general, the use of nitrogen fertilizers at different stages of plant growth in addition to their effects on growth and development, can change the content of primary and secondary metabolites of the plant (Rahmani et al., 2021).

Although the positive effects of nitrogen fertilizer on American agave has already been reported in studies on various aspects of agave species in different contexts, e.g. on its alkaloid contents (Jokar et al., 2021), there is little research in Iran. This research avenue can be important because of the increasing cultivation of this non-native medicinal plant as an ornamental plant in Iran. Consequently, the present study has focused on the effects of urea fertilizer on growth and especially on the content of some bioactive and valuable metabolites of *Agave Americana* cv. Marginata.

Materials and Methods

American agave cv. Marginata plants at nearly similar developmental stage, including 2 to 3 leaves in each plant, were prepared from the greenhouse of Islamic Azad University, Gorgan and were transferred to pots containing perlite, coco peat, and peat moss with equal proportions. Irrigation was conducted regularly for 6 months with ordinary water. After 6- to 8-leaf stage, foliar application of treatment solutions containing urea fertilizer 46% at two concentrations of 5 or 10 g L^{-1} was started and continued to 6 stages with 14-day intervals for treated pots. The control pots were irrigated with water and no fertilizer.

Growth parameters

Fresh and dry weight of roots and shoots were measured as growth parameters. To determine dry weight, plant samples, including shoots and roots, were first dried at 90 ℃ for 24 hours in an electric oven and then weighed by a digital scale.

Total terpenoid assay

Following the method described in Ghorai, et al. (Ghorai et al., 2012), 7 ml of 95% methanol was added to 1 g of fresh shoots and homogenized with a mortar and pestle. The mortar was then placed in an ice bath, and the plant sample was ground to obtain a homogeneous solution, which was then transferred to test tubes. After centrifugation in 4000 rpm at room temperature for 15 minutes, the supernatant was collected in another test tube. Then 3 ml of chloroform was added to the collected supernatant, and after vortex, 500 µl of the mixture was left for 3 minutes; then, 200 µl of concentrated sulfuric acid was added to each of the test tubes and the test tubes were cooled in an ice bath for a maximum of 15 minutes and placed in the dark at room temperature for 1.5 to 2 hours. The supernatant was then drained very slowly and 3 ml of methanol

(95%) was added to the reddish-brown precipitate. When completely dissolved, the ODs were read at 538 nm against blank (methanol). For making standard samples, linalool was dissolved in methanol, prepared with different concentrations, and treated as above mentioned procedure for plant methanol extracts. The content of terpenoids was then calculated with reference to the standard equation in terms of micro equivalents of linalool per gram of fresh weight.

GC-MS analysis

To isolate and detect various organic compounds of plant extracts, first the plant sample was weighed and then dried in the shade. The plant powder was then suited to extraction. To do this, Soxhlet equipment and N-hexane solvent were used. In the next step, the obtained solution was concentrated by means of a rotary apparatus, and the solvent was separated from the sample.

Then the different compounds in the samples were separated from each other by column chromatography (column chromatography 18, white silica gel with 60 particle size and 350 mesh, Merck) with the help of different solvents. The first stage solvent contained 15% water, 25% ethanol and 60% propanol. The second stage contained 15% methanol, 50% propanol, and 35% butanol, and the third stage solvent contained 30% propanol and 70% butanol.

After collecting the samples in large plates, the samples were dried in the shade for 48 hours at room temperature. In this assay, 6 fractions were obtained. After drying, the dried extracts were collected, weighed, and dissolved in the last solvent before they were used for injection into a GC-MS device (Shimadzo TQ-8050).

Statistical Analysis

The treatments were applied in a complete randomized block design, with four replications for each treatment and measurement. One replication was considered for GC-MS analysis obtained from a mixture of shoot powder of five plants. Significance of treatment effects was assessed using one-way ANOVA and differences

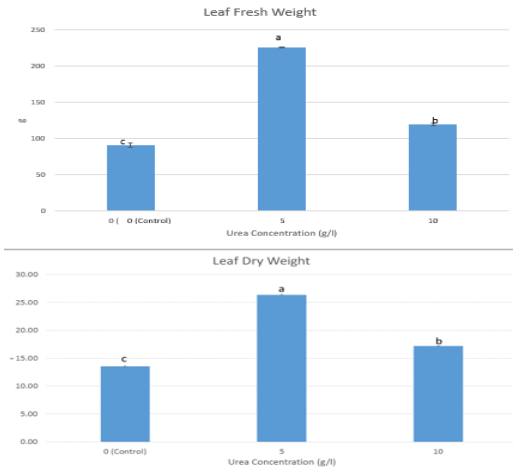


Fig. I. Comparison of the effect of different concentrations of urea fertilizer on fresh and dry weight of leaves; columns and bars indicate means and standard error. The same letters indicate the absence of a significant difference (P≤0.05) between the compared groups.

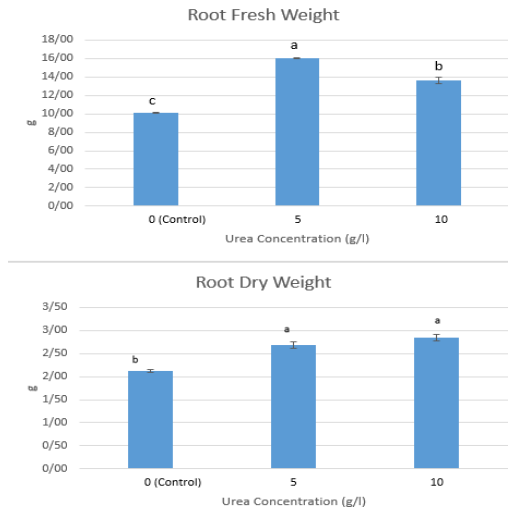


Fig. II. Comparison of the effect of different concentrations of urea fertilizer on fresh and dry weights of roots; columns and bars indicate means and standard error. The same letters indicate the absence of a significant difference (P≤0.05) between the compared groups.

between treatments were investigated using Tukey test (P≤0.05) through SPSS statistical software (version 23). Graphs were drawn using Excel software (Microsoft Office, 2016).

Results

Growth

Urea fertilizer at lower concentrations had a more increasing effect on fresh and dry weight of leaves and fresh weight of roots (Figs. I and II). The

positive effects of urea fertilizer with higher concentration was also significant but less than those of lower concentrations. However, the results of root dry weight measurement showed that the most positive and significant effect was related to the higher concentration, with no significant difference from lower concentration.

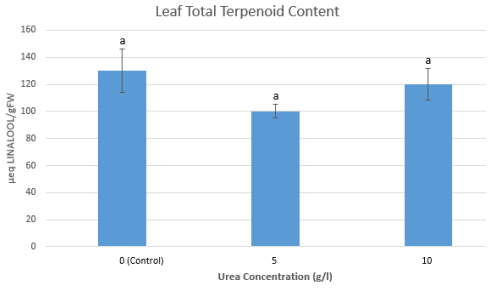


Fig. III. Effect of different concentrations of urea on leaf terpenoid contents; columns indicate means and standard error. The same letter indicates no significant difference (P≤0.05) between the compared groups.

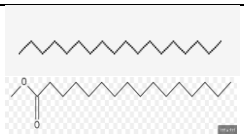
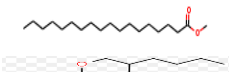
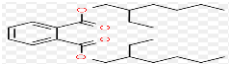
Comparison of leaf terpenoid content

The effect of different concentrations of urea fertilizer on the total content of leaf terpenoids was not significant (Fig. III).

GC-MS analysis of leaf extract

Gas chromatography-mass spectrometry (GC-MS) analysis profile and the structure of the major components of The American Agave extracts are presented in Table 1 and Fig. (IV). The comparison of major compounds above 2% identified by GC-MS in American agave leaf extract (total of 0.16 g)

Table 1
Introduction of chemical coordinates of the major compounds identified by GC-MS in American agave leaf extract

Chemical name	Formula	Molar mass (g)	Chemical structure
Octadecane	$C_{18}H_{38}$	254.297	
Hexadecanoic acid,methyl ester	$C_{17}H_{32}O_2$	270.256	
Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298.287	
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.564	

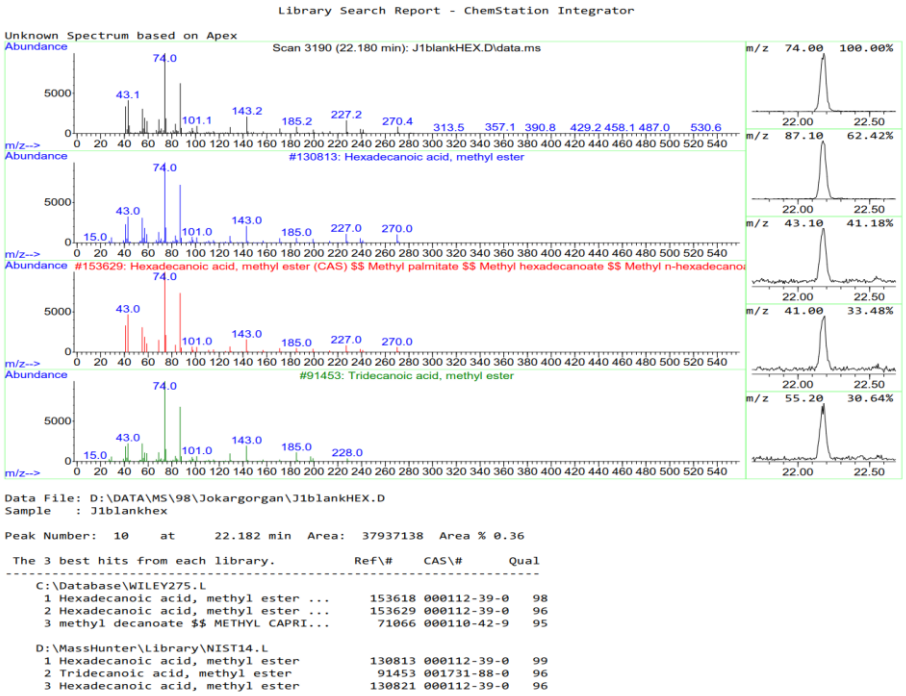


Fig. IV. Gas chromatography-mass spectrometry (GC-MS) analysis profile

Table 2
Comparison of the presence of major compounds identified by GC-MS in American agave leaf extract in control and urea fertilizer (10 g / l) treatment

Treatment	Similar Possible Names in GC-MS Library	Highest confidence percent	CAS Number
Control	Octadecane (CAS) $$ n-Octadecane $$ Octadecan	96	000593-45-3
Urea Fertilizer	Octadecane (CAS) $$ n-Octadecane $$ Octadecan	93	000593-45-3
Control	Hexadecanoic acid, methyl ester (CAS) $$ Methyl palmitate $$ Methyl hexadecanoate $$ Methyl n-hexadecanoate $$ Uniphat A60 $$ Metholene 2216 $$ Palmitic acid methyl ester $$ Palmitic acid, methyl ester $$ n-Hexadecanoic acid methyl ester $	99	000112-39-0
Urea Fertilizer	Hexadecanoic acid, methyl ester (CAS) $$ Methyl palmitate $$ Methyl hexadecanoate $$ Methyl n-hexadecanoate $$ Uniphat A60 $$ Metholene 2216 $$ Palmitic acid methyl ester $$ Palmitic acid, methyl ester $$ n-Hexadecanoic acid methyl ester $	98	000112-39-0
Control	Octadecanoic acid, methyl ester (CAS) $$ Methyl stearate $$ Methyl octadecanoate $$ Methyl n-octadecanoate $$ Stearic acid methyl ester $$ Kemester 9718 $$ Stearic acid, methyl ester $$ n-Octadecanoic acid methyl ester $$ Methyl-octadecanoate $$	97	000112-61-8
Urea Fertilizer	Octadecanoic acid, methyl ester (CAS) $$ Methyl stearate $$ Methyl octadecanoate $$ Methyl n-octadecanoate $$ Stearic acid methyl ester $$ Kemester 9718 $$ Stearic acid, methyl ester $$ n-Octadecanoic acid methyl ester $$ Methyl-octadecanoate $$	95	000112-61-8
Control	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (CAS) $$ Bis(2-ethylhexyl) phthalate $$ DOP $$ DEHP $$ DOF $$ DNOP $$ Octoil $$ Fleximel $$ Sicol 150 $$ Eviplast 81 $$ Staflex DOP $$ Eviplast 80 $$ VestinolAH $$ Truflex DOP $$ Bisoflex81 $$ Witcizer Di-(2-ethylhexyl)phthalate	91	000117-81-7

between the control and urea treatment samples in the control samples are shown in Fig. (V) and Table 2. These compounds, which accounted for 47.19% of the GC-MS detectable extract, included bis (2-ethylhexyl) phthalate (15.03%), Methyl hexadecanoate (or methyl palmitate (14.96%), methyl octadecanoate or methyl stearate (9.5%), and octadecane (7.7%).

In the samples obtained from the treatment of 10 gL^{-1} urea fertilizer (total of 0.26 g), the major compounds identified by GC-MS in the extract were methyl palmitate (29.65%), methyl stearate (19.22%), and octadecane (18.29%), which accounted for 67.16% of the extract. The sum of their percentage increased to 69.14% with the addition of bis (2-ethylhexyl) phthalate (1.98%). Fig. (IV) presents a profile of gas chromatography-mass spectrometry (GC-MS) analysis.

Discussion

The results of this study showed that urea fertilizer at both concentrations had a significant positive effect on the fresh and dry weights of leaves, which is in agreement with the research reported by Liu et al. (Liu et al., 2020)on the use of nitrogen fertilizers.

Also, studies by some researchers (Iqbal et al., 2020)showed that nitrogen fertilizers led to the

development of the root system, which is in agreement with the results of the present work.

Terpenoids play an important role in the development of medicinal properties of agave(López-Romero et al., 2018). Based on the results of the present work, and despite the positive effects of urea fertilizer on the growth of American agave, the overall content of terpenoids did not change significantly. On the other hand, the results of leaf extract analysis by GC-MS also indicated the predominance of non-terpenoid bioactive compounds from the group of alkanes, methyl esters, and phthalates. This in turn shows that the main biosynthetic pathways toward alkanes, fatty acids, and fatty alcohols are of considerable importance in American agave as a tropical plant with prominent cuticle(Salgado, 2021).

On the other hand, considering the known anti-inflammatory, anti-hypertensive, immunomodulatory, anti-parasitic, and antimicrobial properties of agave species and in terms of the contents of Table 3, it can be concluded that non-terpenoid compounds play a significant role in these medicinal properties. Also, urea fertilizer by improving the content of methyl esters on the one hand and reducing the content of some important phthalates, specifically, bis (2-ethylhexyl) phthalate, can play both positive and negative roles in promoting medicinal and antimicrobial properties of American agave.

In comparison with control, the treatment with 10 gL^{-1} urea fertilizer could increase the total amount of extraction (0.26 g as compared to 0.16 g). Also, as urea fertilizer could increase the percentage of octadecane and methyl palmitate about 2 times, the positive role of this fertilizer in promoting anabolic pathways toward alkanes, fatty acids, and fatty alcohols, and the potential of this plant as a source of biofuels should not be overlooked.

Conclusion

Urea fertilizer increased the growth of ornamental-medicinal succulent plant, American agave cultivar Marginata. This fertilizer did not have a significant effect on the total content of terpenoids, but had a positive effect on increasing the content of methyl palmitate and methyl stearate, which are valuable medicinal compounds with antimicrobial, anti-inflammatory, anti-cancer, and antioxidant properties. However, urea fertilizer reduced the content of bis-(2-ethylhexyl) phthalate, which has some valuable properties comparable to above mentioned compounds.

The positive effect of urea fertilizer on the significant increase in octadecane and methyl palmitate levels, which have a potential to be used as a biofuel, is another important result of the present work.

The results of this study showed that urea fertilizer with both concentrations had a significant positive effect on the fresh and dry weight of leaves, which is in agreement with the research of Liu et al. (Liu et al., 2020) on the use of nitrogen fertilizers.

Also, studies and Asif et al. (2020), showed that nitrogen fertilizers lead to the development of the root system, which is in agreement with the results of the present work.

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