Changes in the content of bioactive pharmaceutical-industrial compounds of *Euphorbia trigona* Mill. treated with hormones

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Abstract

The effects of auxin and gibberellin were investigated on *Euphorbia trigona* plants grown in a complete randomized block design under greenhouse conditions. One-minute pretreatments including auxin regulators NAA, IBA, and IAA, (0, or 500 mg/l), followed by treatments containing IAA, IBA and NAA (250 mg/l), and also GA treatment (250 mg/l) without any pretreatments were applied on two-month old cuttings. After 4 months, fresh and dry weights of shoots and roots, and shoot total terpenoid contents were measured. In some samples, shoot extract was analyzed using GC-MS. Fresh and dry weights of shoots did not show any response to the treatments and pretreatments. NAA treatment had the most positive affected root fresh and dry weights. The highest significant increase in terpenoid content was observed in NAA treatment and NAA and IBA pretreatments. GC-MS analysis showed that the chemical compounds in the extract mainly included sesquiterpenes, diterpenes, fatty acids, esters, and steroids. Bis-2-ethylhexyl phthalate contents under NAA treattment and control were 49.31% and 9.59%, respectively; Hexadecanoic acid contents in NAA and control were 2.73% and 2.12%, respectively; 6,10,14-trimethyl-2-pentadecanone content in NAA treatment and control were 3.16% and 1.42%, respectively, and Neophytadiene content under control and gibberellin treatment were 0.94% and 1.64%, respectively. Applying hormones is suggested to result in a positive effect on terpenoids and fatty acids anabolic pathways in *Euphorbia trigona*.

Keywords: Indole acetic acid, Indole butyric acid, GC-MS, growth regulator, Naphthalene acetic acid, Terpenoid

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Introduction

Herbs are one of the most important sources of medicinal compounds, and most modern drugs are derived from herbal products (Ekta et al., 2012). Herbal products are also commonly used in traditional medicine, because they are readily available, inexpensive and effective (Saad and Said, 2011).

A medicinal plant is a plant whose whole or parts are used fresh, dried, or processed to diagnose, treat, prevent, or assist in the physiological functions and maintaining the health of the human, animals, and other plants (Jalilvand et al., 2011). Essential oils and plant extracts have

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compounds with different biological effects, including antimicrobial properties, and as a result herbal medicines with less side effects can be used to treat bacterial infections (Singh et al., 2012).

Secondary metabolites determine important aspects of human food quality (color, taste, and favor). Some of them, e.g. plant pigments, are important for the diversity of flowers and ornamental plants. A number of plant secondary metabolites are used in the production of drugs, insecticides, food seasonings, dyes, and fragrances (Vaishnav and Demain, 2011). Some herbs are also very effective in treating skin diseases by having secondary metabolites as a plant defense mechanism against microbes (Lourith and Kanlayavattanakul, 2013).

Some relatively common effective metabolites found in different species of euphorbia genus plants contain metabolites with several medicinal or even industrial applications. Studies on different species of Euphorbia indicate the beneficial therapeutic properties of these plants for the treatment of various diseases such as cancer, rheumatism, asthma, bacterial infections, and nerve pain (Yang et al., 2021). Euphorbiaceae family contain diterpene and triterpene esters, lectins, and alkaloid compounds with important medicinal properties (Cavalcante et al., 2020). Many species of Euphorbia are used to treat skin injuries such as warts, wounds, boils, dermatitis, psoriasis, eczema, sunburn, and also hair loss. Their latex is also used to heal wounds (Ernst et al., 2015). Furthermore, in some African countries, the leachate and extracts of plants belonging to this family are commonly used to repel plant pests, especially insect larvae and mollusks in flooded farms, and also to control bacterial, fungal, and viral diseases of crops (Mwine et al., 2013). Fais and coworkers' (Fais et al., 2021) reported the presence of terpenoid and sterol compounds in some species of the genus Euphorbia. Isolated compounds of the genus Euphorbia, contain flavonoids, triterpenoids, alkanes, amino acids and alkaloids. The anti-inflammatory, antioxidant and antitumor effects of some flavonoid compounds originating from Euphorbiaceae, are also described in previous research (Aslanturk and Celik, 2013). Kebede and coworkers (Kebede et al., 2021)showed that some species of Euphorbia have antimicrobial, and antiviral properties, and are potential drug precursor sources. Phytochemical study of the *Euphorbia fischeriana* by Meng et al.(Meng et al., 2021) also showed the cytotoxic and anti-cancer properties of some novel diterpenoids.

Plant organ growth is enhanced by the association of auxin with other hormones such as gibberellin, and the balance of these hormones and the ratio between them is important in achieving the best results (Muraro et al., 2011). Studies on growth regulators have also shown that they can alter the metabolism of some secondary metabolites in plants (Rui et al., 2020). The use of hormonal pretreatments to accelerate the rooting of succulent plant cuttings is a common breeding method in greenhouses. Studies have also been conducted in Iran on the effect of fertilizers on succulent and ornamental plants ((Jokar et al., 2022); (Rahmani et al., 2021)). However, in Iran and in the world, little scientific research has been done focusing on the response of succulent plants to exogenous hormones. The present study investigated these responses to auxin pretreatment and hormonal treatments of auxin and gibberellin in *Euphorbia triguna* with an emphasizes on changes in the growth and content of some bioactive compounds.

Materials and Methods

Preparation of cuttings and culture medium

This research was carried out in the Farhikhtegan Greenhouse, Gorgan Azad University in the form of a complete randomized block design with four replications. In the beginning of March 2020, leaf bearing shoot cuttings, with a length of 13-15 cm and a diameter of 7-10 cm were prepared from two-year-old *Euphorbia trigona* plants. The selected cuttings were kept in the shade for two weeks to dry the end of the cuttings.

Pre-treatments and treatments

To prepare hormonal solutions, the appropriate values were dissolved in ethanol 96% and distilled water, with suitable volumes. Cuttings were pretreated with each of the auxin hormones including indole acetic acid (IAA), indole butyric acid (IBA), and naphthalene acetic acid (NAA) at a

Table 1
Introduction of treatments and pretreatments used in the present study

Treatment abbreviation	Pretreatment IBA500 mg/l	Pretreatment IAA500 mg/l	Pretreatment NAA500mg/l	Treatment IBA250mg/l	Treatment IAA250mg/l	Treatment NAA250mg/l	Treatment GA250mg/l
C (Control)	-	-	-	-	-	-	-
GA	-	-	-	-	-	-	+
P-IBA	+	-	-	-	-	-	-
IBA	+	-	-	+	-	-	-
P-IAA	-	+	-	-	-	-	-
IAA	-	+	-	-	+	-	-
P-NAA	-	-	+	-	-	-	-
NAA	-	-	+	-	-	+	-

concentration of 500 mg/l. For this purpose, 2-3 cm from the bottom of the cuttings were placed in hormonal solutions for one minute and then planted in soil-filled pots. Coco peat and perlite in the ratio of 3 to 8 were used as suitable soil for cultivating of the cuttings. After one week, the first fungicide irrigation was performed. In the beginning of spring, irrigation was done once every two weeks, and during the summer it was done weekly. Hormonal treatments and fertilizer utilization (N20P20K20 fertilizer, 3 g/l) were done every 14 days at 8 stages by foliar application. The control plants, without pre-treatment or hormonal treatment, only underwent irrigation with fungicides and fertilizers like the other plants. Table 1 presents treatment groupings and their related abbreviations. As is shown in this table, GA treatments did not undergo any pretreatments.

Growth parameters

In the beginning of September 2020, all cuttings were slowly removed from the planting bed. Then, the roots were separated from the cuttings and the fresh weights of roots and shoots were recorded. Then, to measure the dry weights of stems and roots, the plants were placed in an oven and dried at 90 ℃ for 24 hours before weighing.

Assessing terpenoid contents

Following Ghorai et al. method (Ghorai et al., 2012), 7 ml of 95% methanol was added to 1 g of fresh plant sample in a mortar. The mortar was then placed in an ice bucket, and the plant was ground to obtain a homogeneous solution, which was then transferred into test tubes. Test tubes were centrifuged at 4000 rpm under room temperature condition for 15 minutes, and the supernatant was collected. Then, 3 ml of chloroform was poured into a test tube, to which 500 µl of the collected supernatant was added. After stirring, the test tubes were left for 3 minutes and then 200 µl concentrated sulfuric acid was added to each tube. The test tubes were then cooled in an ice bucket 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 95% methanol was added to the reddish-brown precipitate. When completely dissolved, the light absorptions were determined at 538 nm against blank (95% methanol). To prepare a standard sample, 200 µl of linalool solution in methanol of known concentration was added to 1.5 ml of chloroform. The concentration of total terpenoids was calculated in terms of microequivalent linalool per gram fresh weight using the standard equation.

Phytochemical assay

The plants collected for the experiment were dried under normal conditions and away from sunlight and then were ground by an electric mill. After setting the Soxhlet apparatus, extraction was performed for 9 hours using four grams of plant powder and 250 ml of 96% ethanol as solvent. The

Table 2
GC-MS detectable compounds in shoot extract of *Euphorbia trigona*: control samples

Number	Chemical Name	%
1	2-Pentadecanone, 6,10,14-trimeth.	1.42
2	Hexadecanoic acid	2.12
3	Bis(2-ethylhexyl) phthalate	9.56
4	1-Heptacosanol	1.02
5	3,4-Secolupa-4(23),20(29)-dien-3-oic acid, methyl ester	1.48
6	Stigmasterol	1.46
7	(23S)-ethylcholest-5-en-3.beta.-ol	9.56
8	Lup-20(29)-en-3-ol, (3.beta.)	2.12
9	benzo[b]cyclopropa[1m]fluorenone	2.12
10	alpha.-Amyrin	2.57
11	Lupeol	2.57
12	3-.beta.-Fluoro-androsta-5,16-dien	1.71
13	7-Methoxymethyl-1-(trimethylsilyl)-1-[1,1-bis(trimethylsilyl)ethyl]-2-oxa-1-sila	2.20
14	9,19-Cyclolanost-25-en-3-ol, 24-methyl-, (3.beta.,24S)-	4.95
15	HOP-22(29)-EN-3BETA-OL	1.09
16	Epifriedelinol	8.37
17	4-epi-FRIEDELIN	2.47

solvent flask was placed in liquid paraffin. After the extraction process was completed, the sample extracts were collected and purified by a rotary apparatus (rotary evaporation under suction) for 24 hours. The remaining solvent was evaporated in the air and the extract was dried and carefully weighed, dissolved in methanol, and injected into GC Mass apparatus, (Shimadzo TQ-8050) to identify the organic compounds in the sample.

Statistical Analysis

The experiments were conducted in a complete randomized blocks design with 4 replications for each treatment and each measurement, except for GC-MS analysis with a single replication, obtained from a mixture of five shoots' powder. Significance of treatment effects was assessed using one-way ANOVA and differences between treatments were investigated using Tukey test at p≤0.05. Statistical measurements were performed using SPSS program (Version 23), and the relevant graphs were drawn using Excel software (Microsoft Office, 2016).

Results

As shown in Fig. (I), the effect of the treatments used in this study on the fresh weight and dry weight of the stem was not significant. However, these effects on root fresh weight were significant (Fig. II). The minimum and maximum root fresh weights were related to control plants and NAA treatment, respectively. Control plants did not show a significant difference from other treatments at p≤0.05. Also, except for the IAA treatment and the IBA pretreatment, the differences between the other treatments and the NAA treatment were significant.

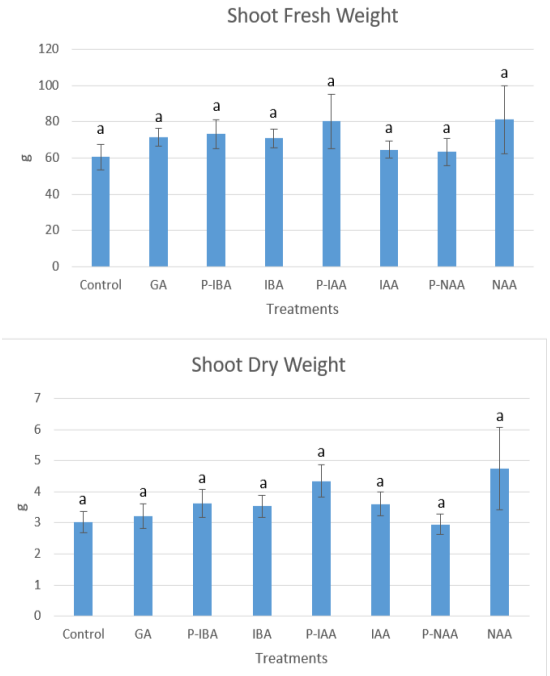

Fig. I. Effects of auxin and gibberellin on shoot fresh and dry weights of *Euphorbia trigona*; columns and error bars represent average and standard errors, respectively. Similar letters indicate no significant difference at p≤0.05.

Table 3
GC-MS detectable compounds in shoot extract of *Euphorbia trigona*: butyric acid pretreatment

No	Name	%
1	Neophytadiene	1.29
2	2-Pentadecanone, 6,10,14-trimeth	1.73
3	n-Hexadecanoic acid	1.67
4	Bis(2-ethylhexyl) phthalate	33.10
5	Decanedioic acid, bis(2-ethylhexyl) ester	1.55
6	9,14-Dioxo-3a,3d,6a,7,8,9,14,15,16,16a-decahydro-1H,6H-dicyclopenta[2,1-a:1,2-k] ,	1.23
7	Stigmasterol	1.90
8	D:A-Friedooleanan-2-one	1.29
9	Stigmast-5-en-3-ol, (3.beta)	11.48
10	(23S)-ethylcholest-5-en-3.beta.-ol	11.48
11	benzo[b]cyclopropa[1m]fluorenone	2.15
12	beta.-Amyrin	2.63
13	7,8-Dihydroxy-.alpha.-dunnione	1.54
14	4H-Pyrano[3,2-h][1]benzoxepin-4-one, 6,9-dihydro-5-hydroxy-2-(hydroxymethyl)-8-m	1.54
15	(E)-2-[1-(2,5-Dimethyl-3-furyl)propyldene]-3-isopropylidenesuccinic	1.54
16	Lanosterol	1.53

Fig. (II) also shows the significant effects of treatments on root dry weight. Control and pretreatment with NAA had the lowest values and treatment with NAA had the highest values. The difference between these two treatments and other treatments and also the differences between NAA and other treatments were not significant at p≤0.05.

The effects of treatments on total terpenoid content were significant (Fig. III) with the highest values related to IBA and NAA pretreatments and NAA treatment, which showed no significant difference. The control and other treatments showed the lowest values and were statistically in the same group while their differences from the three mentioned treatments were significant at p≤0.05.

Analysis of the control plants' shoot extract with GC-MS, showed 17 compounds with percentages above 1% (Table 2). Major compounds were Bis (2-ethylhexyl) phthalate,9.56%, (23S)-ethylcholest-5-en-3.beta.-ol,9.56%, Epifriedelinol,8.37%, and 9,19-Cyclolanost-25-en-3-ol, 24-methyl-, (3.beta.,24S), 4.95%. Each of the other compounds' contribution was less than three percent of the extract.

Table 3, shows 16 compounds with above 1% contribution to the shoot extract of butyric acid-pretreated plants. The main constituents were Bis (2-ethylhexyl) phthalate, 33.10%, Stigmast-5-en-3-

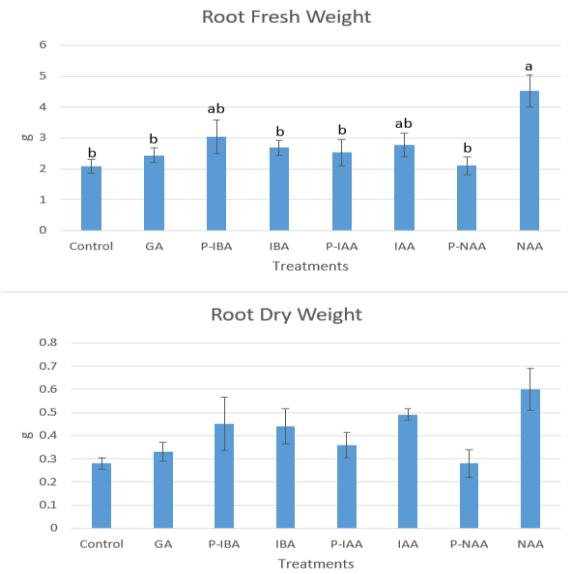

Fig. II. Effects of auxin and gibberellin on root fresh and dry weights of *Euphorbia trigona*; columns and error bars represent average and standard errors, respectively. Similar letters indicate no significant difference at p≤0.05.

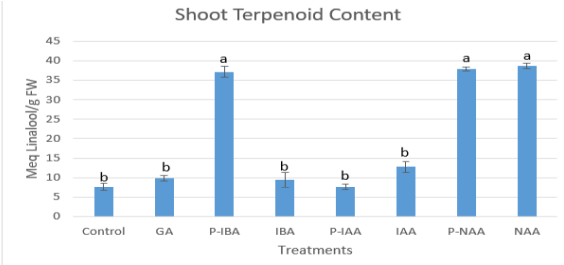

Fig. III. The effects of auxin and gibberellin on the terpenoid content of shoots in *Euphorbia trigona;* columns and error bars represent average and standard errors, respectively. The same letters indicate no significant difference at p≤0.05.

Table 4
GC-MS detectable compounds in shoot extract of *Euphorbia trigona*: Naphthalene acetic acid treatment

No	Name	%
1	Loliolide	1.21
2	2-Pentadecanone, 6,10,14-trimethyl-	3.16
3	Hexadecanoic acid, methyl ester	1.16
4	n-Hexadecanoic acid	2.73
5	Bis(2-ethylhexyl) phthalate	49.31

Table 5
GC-MS detectable compounds in shoot extract of *Euphorbia trigona*: Gibberellin treatment

Number	Chemical Name	%
1	Neophytadiene	1.64
2	n-Hexadecanoic acid	1.11
3	Phytol	1.36
4	Bis(2-ethylhexyl) phthalate	11.27
5	3-Phenyl-5,10-secocholesta-1(10),2-dien-5-one	1.03
6	Stigmasterol	4.51
7	Lanosta-8,24-dien-3-ol, (3.beta)	2.13
8	23S)-ethylcholest-5-en-3.beta.-o	8.14
9	Alnulin	1.89
10	beta.-Amyrin	2.10
11	Lanosterol	1.80
12	2-(2',3',4',5',6'-Pentamethylphenyl)azulene	1.84
13	12-Hydroxy-13-methoxy-8,11,13-podocarpatriene	1.84
14	3-.beta.-Fluoro-androsta-5,16-diene	1.84
15	7-Methoxymethyl-1-(trimethylsilyl)-1-[1,1-bis(trimethylsilyl)ethyl]-2-oxa-1-sila	1.83
16	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)	3.51
17	14-Hydroxy-13-methoxy-8,11,13-podocarpatriene	9.06
18	Epifriedelinol	11.33
19	D:A-Friedooleanan-3-one friedelin	3.20
20	4-epi-FRIEDELIN	3.20

ol, (3.beta), 11.48%, and (23S)-ethylcholest-5-en-3.beta.-ol, 11.48%. Other compounds composed less than 3% of the extract.

Results of GC-MS analysis of the extract, obtained from naphthalene acetic acid-treated plants are presented in Table 4. Five compounds were identified with percentages above 1%. The most important compound was Bis-2-ethylhexyl phthalate that contributed for 49.31% of GC-MS detectable compounds. Each of the other compounds composed not more than 3% of the extract.

GC-MS analysis also showed that 20 compounds with a higher percentage than 1% were detectable in the extracts of plants treated with gibberellin (Table 5). The major compounds were bis-2-ethylhexyl phthalate (11.27), Epifridlinol (11.33), 14-hydroxy-13-methoxy 8,11,13, -podocarpetrin (9.06), 23- Ethyl cyst-5-N-3-beta (8.14), stigmasterol (4.51), cycloanest-25-N-3-1, 24-methyl (3-beta) (3.51), D: A -Apifridolnan- 3- It (3.20), 4- Epi-Friedlin (3.20) Other compounds contributed to less than 3% of the extract.

Comparison of the results obtained from the identification of compounds from extracts of control plants and other treatments showed that 4 compounds including bis-2-ethylhexyl phthalate, hexadecanoic acid, 6,10,14-trimethyl pentadcanone (hexahydropharencil acetone), and neofitadine were common among them. Also, comparison of the percentage of all 4 compounds in the shoot extracts showed higher values for treatments than the control samples.

Discussion

Based on the results of the present study, roots showed more sensitivity to hormonal effects than shoots. Fresh and dry weights of shoots did not

show significant responses to the hormonal treatments and pretreatments applied in the study. NAA treatment had the most positive effect on root fresh and dry weights. Li et al. (2020) found that the use of IBA and NAA hormones leads to better root formation and the amount of root formation depends on the type and concentration of auxin and the method of hormone application, which ultimately improves or fails in rooting. These findings are consistent with the results of the present study. Other researchers have also shown that the use of plant growth regulators such as auxin and especially NAA, is suitable for improving rooting characteristics (Ma et al., 2014). However, the results of the present work do not agree with the results of some other research in that the growth of the aerial parts was not sensitive to auxins. For example, auxin has been reported to induce shoot phototropism (Fankhauser and Christie, 2015), or shape shoot architecture (Gallavotti, 2013), and this clearly indicates the sensitivity of plant shoots to auxin regulators.

The results of the present work suggest that gibberellin had no significant effect on the total content of terpenoids while the effect of auxin was significant. The highest significant increase in terpenoid content was observed in NAA treatment and NAA and IBA pretreatments.

Gas chromatography-mass spectrometry identified many compounds in Euphorbia trigona extract with therapeutic or industrial properties. One of the most important compounds identified was bis-2-ethylhexyl phthalate. With the chemical formula C24H38O, the molecular weight of this compound is 390 g/mol and its melting point and evaporation temperatures are 50 ℃ and 385 ℃, respectively. Bis-2_ethylhexyl phthalate reduces water absorption due to its lipophilicity and increases the adhesion time of the adhesive compounds in manufacturing compressed products. In the kneading process, it will delay the baking and increase the chemicals in the lignification process. In papermaking, due to the reduction of bonding between the fibers in general, it can reduce the strength properties of the paper (Sampson and De Korte, 2011). Bis-2-ethylhexyl phthalate has been identified as an anti-leukemic, anti-mutagenic, antimicrobial and anti-cytotoxic activity (Lee et al., 2000) and its presence in euphorbia plants has been reported in some previous studies (Abd-Elhakim et al., 2019).

Potential of plants' use in the treatment of antileukemia, is especially due to the high content of bis (2-ethylhexyl) phthalates (Lee et al., 2000). Hexadecanoic acid that has also been observed in acacia wood, eucalyptus bark, night wood, inner woolid, outer wood and juniper root, is also an important industrial compound ((Tajik et al., 2014); Hosseini et al., 2011). The main application of this compound is in the production of PVC resins and vinyl chloride, which is added to plastic for flexibility (Hossini Hashemi, 2011). Hexadecanoic acid (palmitic acid) found in the extracts of control and treatments samples in this study, is a predominant fatty acid also found in many plants and seeds including *Euphorbia tirucalli* (de Souza et al., 2019) and *Euphorbia gaillardotii*, and *Euphorbia macroclada* (Ertas et al., 2015). Murakami et al. (Murakami et al., 2020) reported that palmitic acid modulates the immune system by acting on immune T cells.

The main components identified in *Euphorbia hirta* Linn include hexadecanoic acid, phytol, hexaecane, hexadecane-1-1, 1 and 2 benzene d-carboxylic acid (Gopi et al., 2015). Palmitic acid was also detected in *Tetracera scanden* methanolic extract. Methanolic extract of this plant was reported to have strong antioxidant properties (Soleha et al., 2020).

Another chemical compound that was common in controls and treatments is 6,10,14-trimethyl 2-pentadcanone. In the ethanolic extract of *Phyllanthus niruri* from the Euphorbiaceae family, the combination of 2-pentacanone, 6, 10, 14, trimethyl with other compounds has been reported (Kaur et al., 2017). Essential compounds obtained from flowering aerial parts of *Lamium album* L. were also analyzed and 6,10,14-trimethyl-2-pentadcanone and 4-hydroxy-4-methyl-2-pentananone were identified as the main constituents of essential oil (Morteza-Semnani et al., 2016).

In their, study Salehi et al. (Salehi et al., 2021) showed that phytol and pentacanone were

common components of the oils found in ficus species. In another study, the essential oils of *Herniaria incana* were identified by GC-MS. The main constituents were 6,10,14-trimethyl 2-pentadcanone (37.6%) and palmitic acid (4.0%) (Lazari et al., 2000).

Eclipta alba L. extract is traditionally used to treat Campbellobacter diarrhea. Trimethyl-2-pentadcanone in this extract can inactivate the enzyme arginine decarboxylase and disrupt the metabolic cycle of bacteria (Lazari et al., 2000).

Neofitadine was another common compound identified in the control and treatment samples. Neofitadine is an alkene which has been reported to have antioxidant properties and can have anti-inflammatory potential (Swamy et al., 2017). In *Euphorbia hirta* latex, neofitadine as a natural derivative of unsaturated hydrocarbons, was reported` by GC-MS analysis. The latex of this plant has allochemical properties and has shown antifungal activity (Menon et al., 2020).

The data obtained from this study showed that 4 compounds, namely 23-ethylcest-5-n-3-beta, stigmasterol, phytol, and beta-amirin were present in the extracts of control plants and plants pretreated with gibberellin and butyric acid but not in the plant extracts treated with naphthalene acetic acid. The percentage of stigmasterol, phytol, and beta-amirin in the extracts of plants treated with gibberellin had a higher percentage than the control. Therefore, to obtain a higher percentage of stigmasterol, phytol, and beta-amirin, better results can be achieved with gibberellin treatment.

Conclusion

According to the results of the present study, the roots of Euphorbia trigona was more sensitive to hormonal treatment than its shoots. Also, NAA treatment had the most positive effect on root growth. A sharp and significant increase in terpenoid content was observed in NAA treatment and NAA pretreatment as well as in IBA treatment, which calls for future studies on Euphorbia trigona as a biological source of medicinal terpenoids.

Mass spectrometric analysis using GC-MS indicates that there is a high contribution of terpenoid compounds including diterpenes, triterpenes, and sterols in the plant extracts. The effects of the hormone type and its mechanism of action on the metabolic pathways related to bioactive compounds, including terpenoids, may be different.

Practically, the positive role of some hormonal treatments in the significant increase of some compounds with medicinal-industrial uses in the extract of this plant is important. From this perspective, the most significant finding of the present study is the role of NAA treatment in increasing the commercial composition of bis-ethylhexyl phthalate by five times with pharmaceutical-industrial application in plant extracts so that the share of this compound in the GC-MS detectable compounds of shoot extract is about50%.

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