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Enhancing the medicinal properties and phytochemical content of bitter melon (*Momordica charantia* L.) through elicitation with brassinosteroid, ethrel, and carrageenan

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Abstract

Bitter melon (*Momordica charantia* L.) is well-known for its high protein, steroid, alkaloid, mineral, lipid, triterpene, and phenolic compound content, as well as its medicinal properties, particularly its anti-diabetic effects.
To investigate the impact of elicitors on the morphology and phytochemical characteristics of bitter melon (Jounpouri cultivar) over two consecutive years (2018 and 2019), we conducted a field experiment. The study aimed to determine the effects of Ethrel, brassinosteroids (BRs), and k-carrageenan on yield and the production of anti-diabetic agents in *M. charantia* farm crops. The elicitors included ten levels, ranging from a control group to Ethrel (100, 300, and 600 mg l^{-1}), brassinosteroids (BRs) (0.1, 0.5, and 1 mg l^{-1}), and k-carrageenan (200, 400, and 600 mg l^{-1}). These characteristics included leaf area, leaf length, leaf width, fruit parameters, carbohydrate content, total phenols and flavonoid accumulation, antioxidant activity, total acid, ascorbic acid, momordicine, and charantin. Across both years, we observed the highest flavonoid accumulation and antioxidant activity in the Ethrel treatment group. Specifically, applying 0.5 mg l^{-1} BRs and 300 mg l^{-1} Ethrel led to an 18.8% and 14.8% increase in momordicine content, respectively. All elicitor treatments, particularly at 0.1 mg l^{-1} BRs, significantly increased leaf area, leaf length, and leaf width compared to the control group in both cropping years. Additionally, the application of all elicitors resulted in increased fruit weight, dimensions, and yield over the two consecutive years. Notably, in 2018, 600 mg l^{-1} Ethrel contributed to enhanced fruit weight and yield, while in 2019, 0.5 mg l^{-1} BRs exhibited the same effect. Metabolic and physiological changes in bitter squash induced by employed elicitors over two different years (2018–2019) are strongly dependent on a variety of environmental factors such as temperature and rainfall. In conclusion, using BRs as an elicitor has the potential to optimize the health benefits of bitter melon by increasing the content of two bioactive molecules, momordicine and charantin.

Keywords Antioxidant activity, Ascorbic acid, Bioactive molecules, Charantin, Momordicine

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Introduction

Bitter melon (*Momordica charantia* L.), a member of the Cucurbitaceae family, is a widely appreciated vegetable in Asia and Africa, and its cultivation has expanded to tropical and subtropical regions worldwide [1, 2]. In Iran, bitter melon was introduced three decades ago and is predominantly cultivated in the eastern and southern tropical regions. The plant has a significant place in traditional medicine, where it is used to treat diabetes in the form of fruit chips or as an infused tea. Additionally, grated immature fruits are mixed with yogurt to aid in blood glucose reduction after meals [1, 3]. Beyond its anti-diabetic properties, bitter melon is known for its diverse medicinal attributes, including anti-cancer, anti-microbial, anti-cholesterol, and anti-inflammatory effects [1, 3, 4]. Furthermore, bitter melon exhibits inhibitory activity against the human immunodeficiency virus (HIV) due to the presence of α- and β-momorcharin proteins [5]. Due to its rich contents of minerals (K, Ca, Zn, Mn, P, and Fe) and vitamins (A, C, E, B1, B2, B3, and B9), bitter melon surpasses other cucurbit plants in nutritional value [2, 6].

Among the many bioactive compounds in bitter melon, its enormous triterpenoids have garnered attention for their diverse biological effects, including antitumor, anti-diabetic, and anti-bacterial properties [4]. Notably, charantin, a cucurbitane-type triterpenoid comprising sitosteryl glucoside and stigmasteryl glucoside, is renowned for its potent anti-diabetic properties, effectively reducing blood sugar levels [4]. Similarly, momordicine, a bioactive saponin and cucurbitane-type triterpenoid extracted from bitter melon, demonstrates significant therapeutic potential in inhibiting head and neck cancer growth [7] and improving diabetic myocardial dysfunction [8].

The antioxidant properties of bitter melon have garnered attention in the treatment of various diseases, such as diabetes, cardiovascular disorders, brain diseases, arthritis, cancer, and immune system decline due to degenerative disorders [9, 10]. These antioxidant effects are attributed to the phenolic and flavonoid compounds present in the plant [2, 11].

Elicitation, achieved through the application of low amounts of abiotic or biotic molecules known as elicitors, represents a valuable approach to enhancing secondary metabolite accumulation in diverse culture systems [12]. Among these elicitors, carrageenans, categorized as abiotic elicitors, constitute a family of linear sulfated polysaccharides extracted from red edible seaweeds. Three primary types of carrageenan exist, varying in their degrees of sulfation: kappa-carrageenan with one sulfate group per disaccharide, iota-carrageenan with two sulfates per disaccharide, and lambda-carrageenan with three sulfates per disaccharide [13]. These compounds play a pivotal role in activating defense mechanisms in plants against pathogens and diseases [13, 14]. Additionally, carrageenans are known to regulate various morphological, physiological, and biochemical processes, including hormonal statuses such as auxin and gibberellin, ultimately leading to enhanced plant growth and yield [14]. For instance, carrageenan application has been shown to increase shoot length, leaf area, and leaf dimensions in basil [15].

Brassinosteroids (BRs), a class of polyhydroxysteroids recognized as endogenous phytohormones, play pivotal roles in plant growth and development. These roles encompass cell expansion, male fertility, pollen tube formation, vascular differentiation, and delayed senescence [16, 17]. BRs have been detected in all plant organs, including pollen, anthers, seeds, leaves, stems, roots, flowers, and grains. They have also been found in other interesting tissues, such as insect and crown galls, notably the galls of *Castanea crenata*, *Distylium racemosum*, and *Catharanthus roseus*. Pollen and immature seeds in particular tend to be especially rich sources of BRs, whereas their concentrations in vegetative tissue are very low compared to other plant hormones [17]. Brassinolides and castasterone were detected in rape pollen (*Brassica napus* L.) and seedlings of *Daucus carota*, *Phaseolus vulgaris*, and *Arabidopsis thaliana* by Liquid Chromatography–Mass Spectrometry (LC–MS) [17].

Furthermore, BRs have been reported to be involved in plant protection against both biotic and abiotic stresses [16, 18]. By influencing the uptake of essential inorganic ions, reducing toxic ion accumulation, and promoting ion homeostasis, BRs contribute to plant stress tolerance [19]. Notably, studies have shown that BRs elevate various physiological parameters, including growth, net photosynthetic rate, stomatal conductance, transpiration rate, total chlorophyll, carotenoids, polyphenols (flavonoid and anthocyanin) contents, in plants such as *Brassica juncea* L. under both stress and non-stress conditions [20]. Moreover, BRs treatment has been found to enhance fruit length and fruit weight in tomato plants [21].

Ethephon (2-chloroethyl phosphonic acid), a compound that releases ethylene, is widely utilized to stimulate various processes in plants, including germination, flower induction, fruit coloration, fruit ripening, fruit yield, and leaf, flower, or fruit abscission [22]. The impact of ethylene released from ethephon can be either beneficial or detrimental, as it regulates plant growth and development at different stages and concentrations [23]. Studies have demonstrated that lower concentrations of ethephon can enhance the leaf area of mustard, while higher concentrations inhibit it [24]. Ethephon significantly increased the number of female flowers, the sex ratio, and the yield in *Cucurbita pepo* L. at a 400 mg l^{-1}

concentration [25]. It is applied to plants in the form of a mist or spray. After application, ethephon penetrates through stomata and cuticles to the apoplast, where, at a pH of 5 and above, it decomposes to form ethylene, chloride, and phosphate. In recent years, Ethephon (2-chloroethylphosphonic acid), a liquid form of Ethrel that contains ethephon as its active component, has been used to release ethylene, making it the most widely used plant growth regulator. Spraying Ethephon on plants can promote fruit coloration, leaf, flower, or fruit abscission, fruit ripening, fruit yield, germination, and flower induction [26].

Additionally, ethephon treatment has been found to elevate anthocyanin content and the activities of superoxide dismutase and glutathione S-transferase in certain plants [27]. Moreover, the application of ethephon has been shown to increase flavonoid and antioxidant activity in watercress [28]. Ethephon-treated tomatoes were found to have higher ascorbic acid content, total acid (TA), and total soluble solids (TSS) [29]. Similarly, squash seedlings treated with Ethrel exhibited enhanced growth and fruit production [30].

Despite the increasing prevalence of diabetes and the growing interest in herbal medicines to minimize the side effects of chemical drugs, the impact of elicitors, particularly BRs and carrageenan, on the qualitative properties and secondary metabolites of bitter melon (*Momordica charantia* L.) is well-known for its high protein, steroid, alkaloid, mineral, lipid, triterpene, and phenolic compound content, as well as its medicinal properties. Hence, this study aims to evaluate the efficacy of different chemical stimulants, namely k-carrageenan, BRs, and ethephon, in modulating the content of total phenol and flavonoid, ascorbic acid, antioxidant activity, carbohydrates, momordicine, and charantin in bitter melon.

Materials and methods

Plant materials and growth conditions

The experiment was conducted at the research farm of the Faculty of Agriculture, University of Zanjan, Zanjan, Iran (latitude 36° 40′ 52″ N, longitude 48° 24′ 19″ E, and altitude 1593 m above sea level). The soil physicochemical properties were assessed before seed sowing, and the soil was found to have a loam clay sandy texture with pH 7.2, EC 1.12 dS m^{-1}, organic matter 1.11%, calcium carbonate 14.09%, nitrogen 0.08%, phosphorus 4.6 mg kg^{-1}, and potassium 154 mg kg^{-1}. The seedbed was prepared using a disk and leveler, and the field was divided into plots measuring 2 m wide and 10 m long. Bitter melon, *Momordica charanti*a L. (Jounpouri cultivar), seeds were soaked in water for 24 h before manual sowing with a spacing of 150×100 cm and a sowing depth of 2 cm. The first irrigation was applied immediately after sowing, followed by weekly drip irrigation in the spring and a 3-day interval during the summer. Weed control was done manually throughout the growing season. The ambrothermic graph of Zanjan City (Fig. 1) was used for reference during the two cropping years, 2018 and 2019.

Experimental design and treatments

The experiment followed a randomized complete block design with three replications for two consecutive years (2018 and 2019). Ten elicitor treatments (in aqueous solution form) were applied, including a control (distilled water), kappa-carrageenan (CAS Number: 11114-20-8, Sigma-Aldrich) (200, 400, and 600 mg l^{-1}), BRs (CAS Number: 280129-83-1, Sigma-Aldrich) (0.1, 0.5, and 1 mg l^{-1}), and Ethrel in Ethephon form (CAS Number: 16672-87-0, Sigma-Aldrich) (100, 300, and 600 mg l^{-1}). In order to better dissolve the elicitores, rinsed the main components with a few drops of methanol, then added distilled water to a volume of one liter. Bitter melon seeds were obtained from the Agricultural Research Center in Zabol, Iran. Elicitors were sprayed at the 4-8-leaf stage with a 4-day interval in the aqueous form. The control plants were sprayed with distilled water. Well-developed leaves at the full bloom stage were harvested for leaf parameter measurements, while fruit samples (100 g) were collected 16 days after fruit set to evaluate fruit characteristics.

Leaf characteristics

Leaf area was measured using a leaf area meter (VM-900 E/K) with three adult leaf samples taken randomly from nodes 8, 9, and 10. Leaf length and width were determined using Digimizer software (Digimizer v4.1.1.0).

Number of male flowers, number of female flowers, and sexual ratio

During the reproductive phase, three plants per experimental unit were selected, and daily counts of male and female flowers were recorded between 8 and 10 AM, after flower opening. The sexual ratio was calculated by dividing the number of male flowers by the number of female flowers.

Fruit characteristics and yield

Harvesting was carried out from the first fruit set every 15 days. Three fruits were randomly chosen from each experimental unit for assessment of fresh fruit weight using a digital balance (accuracy 0.001 g) immediately after harvest. The length and diameter of the fruits were measured using a digital caliper. Subsequently, the fruits were cut into rings, dried in an oven at 40 °C for 72 h, and weighed for dry weight measurement. Fruit yield per plant was calculated as the sum of fresh fruit weights from the three harvests.

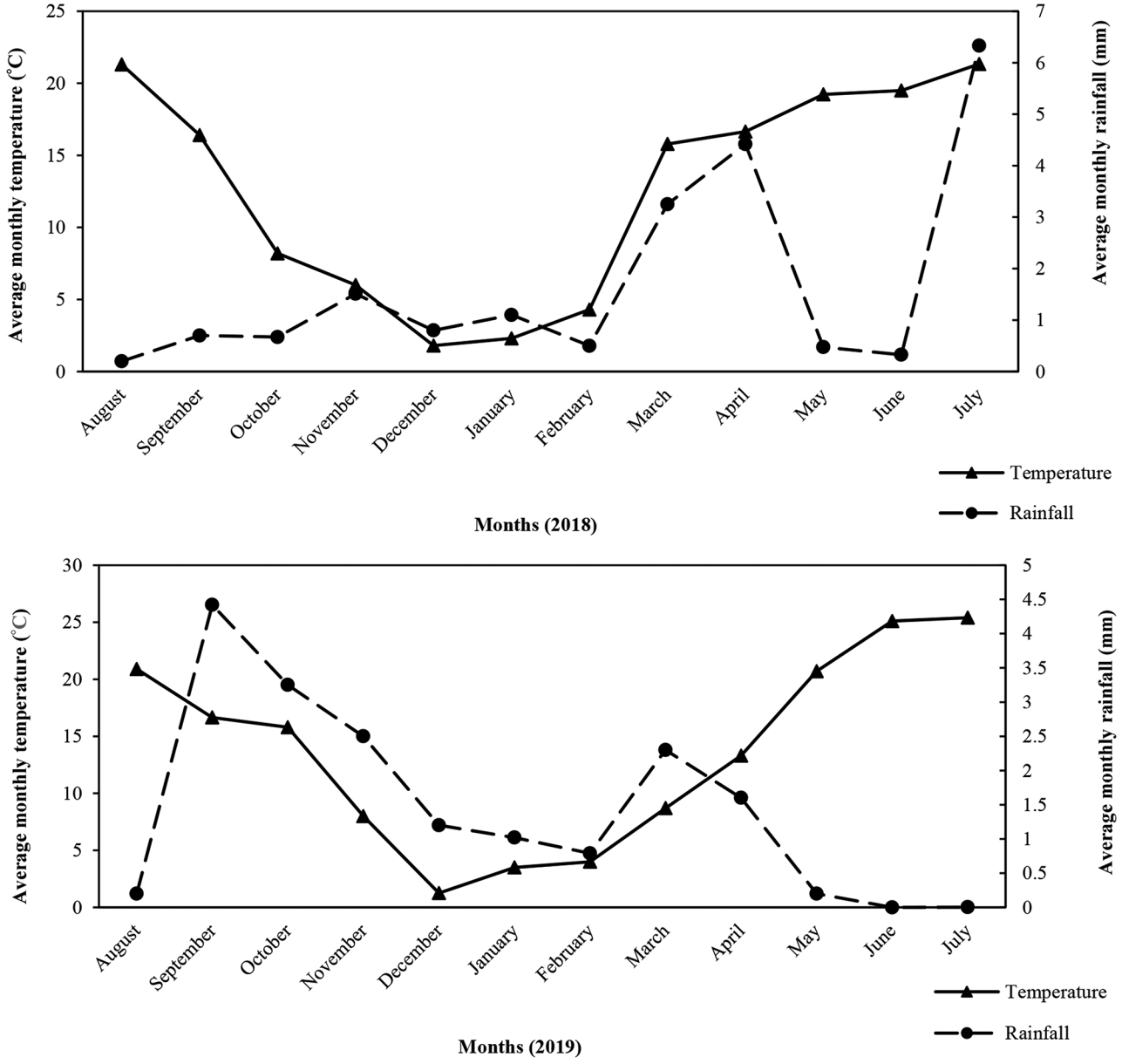

Fig. 1 Ambrothermic graph of Zanjan city for two consecutive cultivation year, 2018 and 2019

Total phenolic content

Dried fruit powder (0.5 g) was mixed with 10 ml of 80% methanol and shaken for 24 h at 120 rpm min^{-1} in darkness at room temperature. The extract was then filtered using Whatman paper No. 1 and stored at 4 °C until further analysis. The total phenolic content was determined using a modified Folin-Ciocalteu method. Specifically, 200 µl of methanolic extract was mixed with 400 µl of Folin-Ciocalteu (Sigma Alderich, Germani, Cas No.1.09001.0100) reagent (diluted with distilled water at a 1:10 ratio) and 400 µl of 7% sodium carbonate solution. The solution was incubated at room temperature for 30 min, and the absorbance was measured at 765 nm using a UV-visible spectrophotometer (PerkinElmer - Lambda 25-USA). The total phenolic content was calculated as mg of gallic acid equivalent using an equation obtained from the calibration curve.

Total flavonoid content

The total flavonoid content was measured using a colorimetric method. 500 µl of methanolic extract was mixed with 1.5 ml of 80% methanol, 100 µl of 10% aluminum chloride (Merck, Germani, NO. 801081) solution, 100 µl of 1 M potassium acetate (CH_3CO_2K, Merck, Germani, NO. 104820), and 2.8 ml of distilled water. The samples were incubated at room temperature for 40 min, and the absorbance was measured at 415 nm using a UV-visible spectrophotometer. Quercetin (Sigma-Aldrich, USA,

$C_{15}H_{10}O_7$, NO. Q4951) was used as the standard, and the data were expressed as mg of quercetin equivalent per 100 g of fresh weight.

Antioxidant activity

Antioxidant activity was evaluated using a free radical scavenging assay with DPPH as the free radical. Methanolic extract (250 µl) was mixed with 750 µl of DPPH ($C_{18}H_{12}N_5O_6$, Merck, Germany, NO. 300267) solution (2 mg of DPPH dissolved in 50 ml of pure methanol). The mixture was incubated in the dark at room temperature for 30 min, and the absorbance was recorded at 517 nm using a UV-visible spectrophotometer. The percentage of DPPH free radical scavenging was calculated using the formula:

Percentage of DPPH free radical scavenging = (Ac-As) / Ac × 100.

Where Ac and As are the absorbances of the control and the sample, respectively.

Total soluble solids (TSS)

The total soluble solid content of a solution is determined by the index of refraction. This is measured using a refractometer and is referred to as the degree. In our study, fresh fruit (100 g) was divided into smaller pieces, and the juice was extracted using a juicer machine. Total soluble solids were determined using a digital refractometer (RHB32, Italy) [21].

Total acid (TA)

To prepare the fruit extract, the first two fruits were selected from each replicate. Then the juices were extracted by an electric juicer. Moreover, the fruit juice was passed through Whatman filter paper No. 1. The resulting extract was used to measure total organic acid. Five milliliters of fruit juice were diluted with 45 ml of distilled water and titrated with 0.1 N sodium hydroxide until the pH stabilized at 8.1–8.2. The total acid was calculated and expressed as % protocatechuic acid [21].

Ascorbic acid determination using the iodometric titration method

The ascorbic acid content of the fruit juice was determined using an iodometric titration method. A starch indicator solution (1%) was prepared and added to 10 ml of juice. The mixture was titrated with an iodine solution until a stable, light gray color was obtained. The amount of ascorbic acid was calculated using the formula: A = (S × N × F × 88.1×100) / 10, where A is the content of L-ascorbic acid in the sample (mg l^{-1}), S is the amount of iodine solution consumed (0.05 M) at the end point of titration (ml), N is the normality of the iodine solution in mol l^{-1} (0.01), and F is the consumed iodine solution factor (0.885).

Carbohydrate determination

The carbohydrate content was determined using the phenol-sulfuric acid method. Ten milliliters of 95% ethanol were added to 0.2 g of samples, and the mixture was placed in a water bath at 80 °C for 1 h. Then, 1 ml of 0.5% phenol and 5 ml of 98% sulfuric acid were added to 1 ml of the extract, and the mixture was allowed to cool. The absorbance was measured at 483 nm using a UV-visible spectrophotometer, and the standard curve was plotted using D (+)-glucose.

Momordicine measurement

Momordicine was extracted using cold maceration. Fruit powder (0.5 g) was homogenized with 10 ml of 80% ethanol on a shaker for 3 h, followed by centrifugation at 2000 rpm for 15 min. Then, 1 ml of dimethyl sulfoxide (DMSO) was mixed with 9 ml of pure methanol of HPLC grade. Afterward, 100 µl of ethanolic extract was combined with 900 µl of the DMSO-methanol solution, filtered with a 0.45 µm syringe filter, and subjected to sonication at 20 °C for 15 min before HPLC analysis. HPLC analysis was performed using a C-18 column with a mobile phase of 70:30 (v/v) methanol and deionized water at a flow rate of 1 ml min^{-1}. The detector was a UV diode array [31].

Charantin measurement

Charantin was extracted using cold maceration. Fruit powder (0.5 g) was homogenized with 10 ml of 80% ethanol on a shaker for 3 h, followed by centrifugation at 2000 rpm for 15 min. The extract was filtered with a 0.45 µm syringe filter and purified by sonication at 20 °C for 20 min before HPLC analysis. HPLC analysis was performed using a C-18 column with a mobile phase of 98:2 (v/v) methanol / deionized water at a flow rate of 1 ml min^{-1}. The detector was a UV diode array [32].

Statistical analysis

Data from the randomized complete block design were subjected to a combined analysis of variance for the two years. The data were tested for normality before statistical analysis and found to be normal for all traits. The general linear model (GLM) procedure of SAS statistical software, version 9.2 (SAS, Institute Inc., 2009), was used for data analysis. Differences between means were evaluated for significance using the least significant difference (LSD) test ($p \leq 0.05$).

Results

Leaf characteristics

The leaf area, leaf length, and leaf width were significantly influenced by both the elicitors and the effect of the cultivation year × elicitors (Table 1). In both cropping years, the application of elicitors resulted in a significant

Table 1 Analysis of variance of different elicitors on the studied traits of *Momordica charantia* L. in two cultivation year

		Mean of squares									
Source of variation	df	Leaf area	Leaf length	Leaf width	Number of male flowers	Number of female flowers	Sexual ratio	Fruit fresh weight	Fruit dry weight	Fruit width	Fruit length
cultivation year (Y)	1	1.74^{ns}	4.159^{ns}	0.1794^{ns}	15.001^{ns}	3.75^{ns}	78.585^{ns}	1315628.6^{**}	7134.71^{**}	50.288^{ns}	1958.42^{ns}
Y(R)	4	214.879	1.24	4.061	115.333	0.6	14.285	2733.897	11.725	31.64	911.821
Elicitor (E)	9	3551.09^{**}	22.603^{**}	18.118^{**}	524.141^{**}	5.794^{**}	41.437^{ns}	106473.79^{**}	321.369^{**}	355.105^{**}	4512.92^{**}
Y × E	9	619.31^{*}	6.037^{**}	5.856^{**}	254.333^{ns}	14.861^{**}	172.948^{**}	92238.77^{**}	157.662^{**}	123.774^{**}	3048.32^{**}
Error	36	235.122	1.551	0.7831	124.593	0.767	20.471	3048.16	21.644	30.03	412.036
Coefficient of variation (%)		22.2	10.6	8.4	29.3	27.8	29.8	21.4	24.3	13.8	13.7
		Mean of squares									
Source of variation	**df**	**Fruit yield**	**Total phenolic content**	**Total flavonoid content**	**Antioxidant activity**	**TSS**	**TA**	**Vitamin C**	**Carbohydrate**	**Momordicine**	**Charantin**
cultivation year (Y)	1	1562771.7^{ns}	526556.5^{**}	2.483^{**}	249.86^{ns}	11.441^{*}	0.0043^{ns}	6.37^{ns}	12.476^{**}	812.544^{**}	1606.84^{ns}
Y(R)	4	938350.2	222.675	0.069	53.389	1.496	0.0404	1.047	0.1242	4.347	596.97
Elicitor (E)	9	6743693.7^{**}	9648.407^{**}	0.115^{*}	266.204^{**}	0.494^{ns}	1.247^{**}	1.811^{**}	8.106^{**}	696.382^{**}	94467.77^{**}
Y × E	9	7488783.1^{**}	7378.6^{**}	0.0865^{ns}	83.678^{ns}	0.432^{ns}	1.366^{**}	0.0632^{ns}	5.915^{**}	8.748ns	104.9856
Error	36	183002.4	436.187	0.0476	59.92	0.259	0.0344	0.3097	0.1523	40.39	334.99
Coefficient of variation (%)		19.5	16.1	14	11.2	10.2	10.7	24.8	13.3	13.4	11.2

*, $p \leq 0.05$; **, $p \leq 0.01$; ns, not significant

TSS, Total soluble solids; TA, total acid

increase in the leaf area, leaf length, and leaf width of bitter melon compared to the control. Additionally, control plants exhibited the lowest values for leaf area, leaf length, and leaf width, while plants treated with 0.1 mg l^{-1} BRs showed the highest values (Table 2).

Number of male flowers, number of female flowers, and sexual ratio

The number of male flowers was significantly affected by the application of elicitors (Table 1). The results indicated that the highest number of male flowers was observed in plants treated with 600 mg l^{-1} Ethrel, followed by plants treated with 1 mg l^{-1} BRs, showing an increase of 66.7% and 48.2%, respectively, compared to the control (Fig. 2). Conversely, the number of male flowers decreased by up to 27.5% with the application of 200 mg l^{-1} k-carrageenan compared to the control (Fig. 2).

The number of female flowers was significantly influenced by the effect of the cultivation year × elicitors (Table 1). In the first year (2018), the number of female flowers increased with the application of all three levels of Ethrel (100, 300, and 600 mg l^{-1}) and 0.1 mg l^{-1} BRs compared to the control. The treatment with 600 mg L^{-1} Ethrel resulted in the highest number of female flowers in the first year (Table 2). On the other hand, in the first year, the other treatments showed a decreasing effect on the number of female flowers, and they did not differ significantly from the control (Table 2). In the second year (2019), all elicitors led to an increase in the number of female flowers compared to the control, with the highest number observed with the application of 400 mg l^{-1} k-carrageenan, followed by 1 mg l^{-1} BRs (Table 2).

The sexual ratio was significantly affected by the effect of the cultivation year × elicitors (Table 1). In the first year (2018), the sexual ratio was significantly increased by the application of 400 mg L^{-1} K-carrageenan, followed by 1 mg l^{-1} BRs, compared to the control, while it was significantly decreased by the application of Ethrel (300 and 600 mg l^{-1}) compared to the control (Table 2). In the second year (2019), all elicitors resulted in a decrease in the sexual ratio compared to the control, with the lowest sexual ratio of 79.5% reduction observed with 400 mg l^{-1} K-carrageenan (Table 2).

Fruit characteristics and yield

The fruit fresh weight and fruit dry weight were influenced by the effects of the year, elicitors, and the cultivation year × elicitors (Table 1). Similarly, fruit width, fruit length, and fruit yield were influenced by the effects of elicitors and the effect of the cultivation year× elicitors (Table 1). In both cropping years, the application of elicitors resulted in a significant increase in the fruit fresh weight, fruit dry weight, fruit width, fruit length, and fruit yield of bitter melon compared to the control. In the first year (2018), the treatment with 600 mg l^{-1} Ethrel led to the highest fruit fresh weight, fruit dry weight, fruit width, and fruit length, while the highest fruit yield was obtained in plants treated with 200 mg l^{-1} K-carrageenan, followed by 600 mg l^{-1} Ethrel, compared to the control (Table 2). In the second year (2019), the fruit fresh weight, fruit dry weight, and fruit yield of bitter melon were significantly increased by the application of 0.5 mg l^{-1} BRs, while fruit width and fruit length were significantly elevated by the application of 0.1 mg l^{-1} BRs and 100 mg l^{-1} Ethrel, respectively (Table 2).

Total phenolic content

The total phenolic content was found to be significant ($p<0.01$) due to the effects of year, elicitors, and cultivation year× elicitors (Table 1). In both cropping years, the application of elicitors led to an increase in the total phenolic content compared to the control. In the first year (2018), the treatment with 0.5 mg l^{-1} BRs and 300 mg l^{-1} Ethrel resulted in the highest amount of phenolic compound (Table 2). In the second year (2019), the highest amount of phenolic compound was obtained with the application of 300 mg l^{-1} ethrel (Table 2).

Total flavonoid content

The total flavonoid content was affected by the effect of the cultivation year and elicitors (Table 1). The results indicated that the content of flavonoid was higher in the first year (2018) compared to the second year (2019), which could be attributed to the difference in environmental factors between the two consecutive cropping years (Table 3). Additionally, all elicitors led to an increase in the total flavonoid content compared to the control, with the highest content observed with the application of 1 mg l^{-1} BRs and 600 mg l^{-1} Ethrel, resulting in an increase of 41.7% and 37%, respectively, compared to the control (Fig. 3).

Antioxidant activity

Antioxidant activity was only found to be significant ($p<0.01$) due to the effects of elicitors (Table 1). The comparison of different elicitors on antioxidant activity revealed that the lowest level of antioxidant activity was observed in the control plants. All elicitors had a positive effect on antioxidant activity compared to the control, with the highest level of antioxidant activity (increase of 43.3%) observed with the treatment of 100 mg l^{-1} Ethrel (Fig. 4).

Total soluble solids, total acid, and ascorbic acid

The total soluble solid was only affected by the effect of the year (Table 1). The results indicated that the total soluble solids were higher in the first year (2018) compared to the second year (2019), which could be attributed to

Table 2 Mean comparison of the effect of the cultivation year and elicitors on the leaf area, leaf length, leaf width, number of female flowers, sexual ratio, fruit fresh weight, fruit dry weight, fruit width, fruit length, fruit yield, total phenolic content, total acid, and carbohydrate content in *Momordica charantia* L

Year	Elicitor	Leaf area (cm^2)	Leaf length (cm)	Leaf width (cm)	Number of female flowers	Sexual ratio	Fruit fresh weight (g)	Fruit dry weight (g)	Fruit width (mm)	Fruit length (mm)	Fruit yield (g $plant^{-1}$)	Total phenolic content (mg GAE/ 100 g FW)	TA (%)	Carbohydrate (mg Glucose/g DW)
2018	Control	42.12^{ef}	9.7^{fg}	8.89^{gh}	2.33^{efg}	16^{b-e}	33.27^{k}	2.39^{k}	27.3^{j}	70.28^{i}	326.6^{h}	84.92^{f}	2.4^{bc}	1.79^{gh}
	Ethrel 100 mg L^{-1}	48.37^{ef}	10.59^{ef}	9.49^{gh}	3^{c-f}	17.69^{bcd}	68.47^{ijk}	7.46^{h-k}	42.03^{b-e}	136.1^{efg}	672.5^{gh}	181.83^{e}	1.7^{ef}	3.98^{bc}
	Ethrel 300 mg L^{-1}	63.28^{de}	$10.87d^{ef}$	10.1^{efg}	4.33^{bc}	9.68^{e-h}	103.93^{h-k}	6.26^{ijk}	32.79^{f-j}	147.6^{def}	684.2^{gh}	315.75^{b}	1.07^{ij}	5.78^{a}
	Ethrel 600 mg L^{-1}	47.79^{ef}	10.11^{ef}	9.07^{gh}	7.67^{a}	7.29^{fgh}	233.21^{ef}	15.72^{fg}	65.3^{a}	205.86^{a}	3369.4^{c}	260.58^{c}	1.5^{fg}	1.81^{gh}
	Carrageenan 200 mg L^{-1}	82.74^{cd}	14.33^{ab}	12.11^{cd}	2^{fg}	11.33^{d-h}	46.42^{k}	4.35^{jk}	29.05^{hij}	135.86^{efg}	6495.5^{a}	190^{de}	2.1^{cd}	1.36^{h}
	Carrageenan 400 mg L^{-1}	81.71^{cd}	12.01^{cde}	11^{def}	1.33^{g}	28.17^{a}	106.09^{h-k}	8.3^{g-k}	43.44^{bcd}	167.13^{b-e}	3140.1^{c}	197.33^{de}	1.83^{de}	1.93^{gh}
	Carrageenan 600 mg L^{-1}	79.63^{cd}	13.2^{bc}	11.62^{cd}	2^{fg}	16^{b-e}	154.36^{f-i}	14.75^{fgh}	48.6^{b}	184.14^{abc}	2416.4^{d}	205.83^{de}	2.3^{bc}	0.61^{i}
	Brassinosteroid 0.1 mg L^{-1}	101.42^{abc}	14.42^{ab}	12.76^{bc}	2.67^{d-g}	13.42^{d-g}	164.85^{fgh}	7.39^{h-k}	41.8^{b-f}	146.53^{d-f}	2341.1^{d}	224.5^{d}	1.57^{ef}	4.59^{b}
	Brassinosteroid 0.5 mg L^{-1}	80.32^{cd}	13.72^{abc}	11.41^{cde}	1.67^{fg}	18.36^{bcd}	144.47^{f-i}	11.89^{g-j}	46.14^{bc}	193.65^{ab}	2007.8^{de}	352.08^{a}	1.6^{ef}	1.5^{h}
	Brassinosteroid 1 mg L^{-1}	64.25^{de}	10.94^{def}	8.87^{gh}	2^{fg}	22.39^{ab}	47.76^{jk}	3.66^{k}	30.66^{g-j}	153.94^{c-f}	2096.1^{d}	219.42^{d}	1.43^{fgh}	1.48^{h}
2019	Control	24.78^{f}	7.64^{g}	7.19^{l}	1.33^{g}	22.33^{ab}	137.85^{g-j}	11.4^{g-j}	27.99^{ij}	88.31^{hi}	689.3^{gh}	11.5^{h}	1.2^{ghi}	0.53^{i}
	Ethrel 100 mg L^{-1}	48.28^{ef}	9.91^{f}	9.03^{gh}	2.33^{efg}	12.94^{d-g}	219.02^{efg}	13.71^{ghi}	42.99^{bcd}	207.12^{a}	$1095.1f^{g}$	49.42^{g}	3.13^{a}	2.33^{fg}
	Ethrel 300 mg L^{-1}	46.61^{ef}	9.98^{ef}	8.1^{hi}	2.67^{d-g}	18.33^{bcd}	357.48^{d}	34.77^{cd}	33.81^{e-j}	136.22^{efg}	1787.4^{def}	52.17^{fg}	1.23^{ghi}	5.24^{a}
	Ethrel 600 mg L^{-1}	51.96^{e}	10.17^{ef}	9.61^{fg}	2.33^{efg}	21^{abc}	203.04^{efg}	28.5^{de}	43.38^{bcd}	121.51^{fgh}	1015.2^{gh}	48.08^{g}	3^{a}	2.63^{ef}
	Carrageenan 200 mg L^{-1}	94.05^{bc}	13.38^{bc}	11.55^{cde}	4^{bcd}	6.83^{gh}	211.37^{efg}	21.75^{ef}	39.29^{c-g}	137.27^{d-g}	1056.8^{g}	31.25^{gh}	0.83^{j}	3.53^{cd}
	Carrageenan 400 mg L^{-1}	108.5^{ab}	14.63^{ab}	13.59^{ab}	7.67^{a}	4.57^{h}	466.04^{c}	43.37^{ab}	36.81^{d-i}	156.53^{cde}	2330.2^{d}	43.73^{gh}	1.1^{ij}	4.33^{b}
	Carrageenan 600 mg L^{-1}	49.34^{ef}	9.93^{f}	9.09^{gh}	3.67^{b-e}	15.14^{b-e}	701.53^{b}	38.76^{bc}	44.36^{bcd}	113.13^{gh}	3507.6^{c}	35^{gh}	2.5^{b}	4.31^{b}
	Brassinosteroid 0.1 mg L^{-1}	125.61^{a}	15.67^{a}	14.93^{a}	1.67^{fg}	14.67^{c-f}	264.41^{e}	29.39^{de}	44.46^{bcd}	142.18^{d-g}	1322.1^{efg}	39.83^{gh}	1.6^{ef}	3.25^{de}
	Brassinosteroid 0.5 mg L^{-1}	58.12^{de}	10.59^{ef}	9.68^{fg}	3.67^{b-e}	9.92^{e-h}	853.66^{a}	46.94^{a}	37.81^{c-h}	154.36^{c-f}	4268.3^{b}	25.08^{gh}	1.13^{hij}	4.59^{b}
	Brassinosteroid 1 mg L^{-1}	80.98^{cd}	12.74^{bcd}	11.46^{cde}	4.67^{b}	11.71^{d-h}	649.99^{b}	31.67^{cd}	37.8^{c-h}	170.2^{bcd}	3250^{c}	22.58^{gh}	1.6^{ef}	3.22^{de}

For each parameter, data with the same letter are not significantly different (LSD test $p \leq 0.05$)

TA, total acid

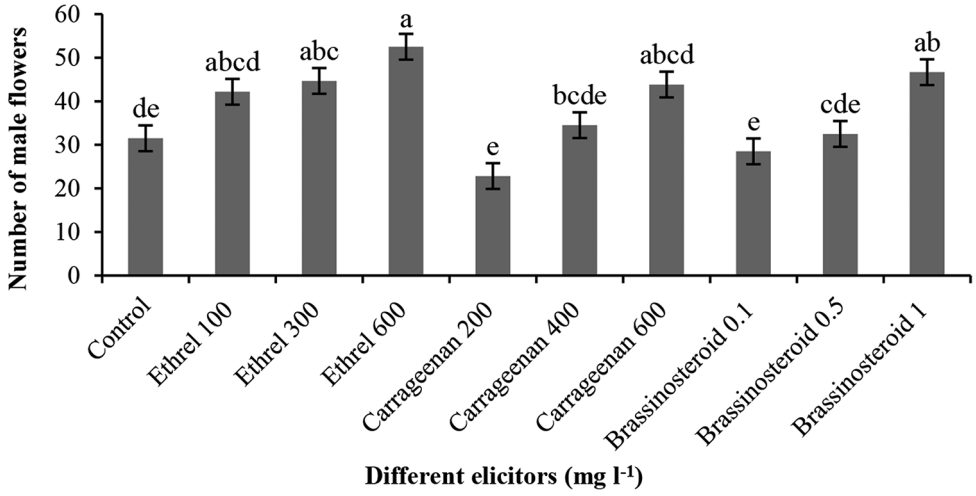

Fig. 2 Mean comparison of different elicitors on the number of male flowers in *Momordica charantia* L. Columns with the same letters do not differ significantly (LSD test $p \leq 0.05$)

Table 3 The mean comparison of the simple effect of the cultivation year on the total flavonoid content, total soluble solids, and momordicine content in *Momordica charantia* L

Year	Total flavonoid content (µg/g DW)	Total soluble solids (°Brix)	Momordicine (µg/g DW)
2018	1.77[a]	5.41[a]	50.99[a]
2019	1.36[b]	4.54[b]	43.64[b]

For each parameter, data with the same letter are not significantly different (LSD test $p \leq 0.05$)

the difference in environmental factors between the two cropping years (Table 3).

Total acid was found to be significant ($p<0.01$) due to the effects of elicitors and the effect of the cultivation year × elicitors (Table 1). In the first year (2018), the total acid decreased with the application of all elicitors compared to the control, with the lowest total acid (decrease of 55.4%) observed with the treatment of 300 mg l^{-1} Ethrel (Table 2). In the second year (2019), the highest amount of total acid was observed with the application of 100 mg l^{-1} Ethrel, while the lowest amount (a decrease of 30.8%) was obtained with the treatment of 200 mg l^{-1} K-carrageenan compared to the control (Table 2).

Ascorbic acid content was only affected ($p<0.01$) by the effect of elicitors (Table 1). The comparison of different elicitors on ascorbic acid content indicated that the highest amount of ascorbic acid (increase of 44.5%) was recorded with the treatment of 400 mg l^{-1} K-carrageenan (Fig. 5).

Carbohydrate content

The carbohydrate content was found to be significant ($p<0.01$) due to the effects of year, elicitors, and

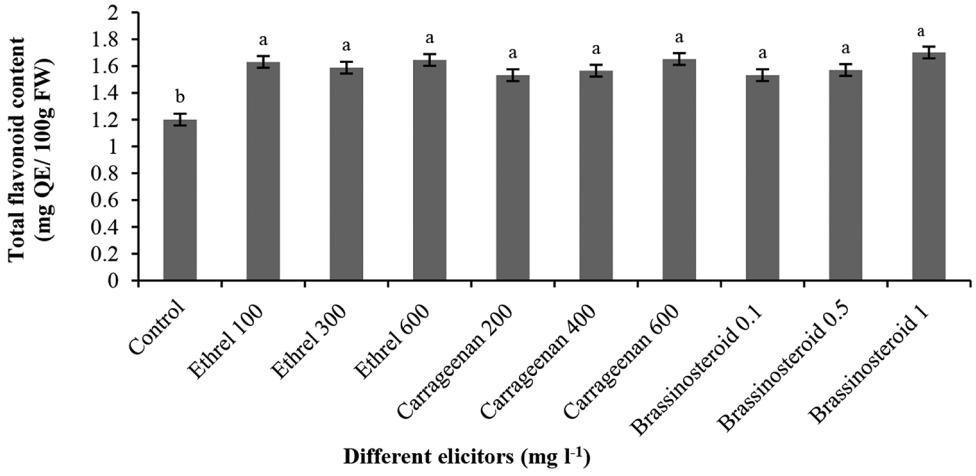

Fig. 3 Mean comparison of different elicitors on the total flavonoid content in *Momordica charantia* L. Columns with the same letters do not differ significantly (LSD test $p \leq 0.05$)

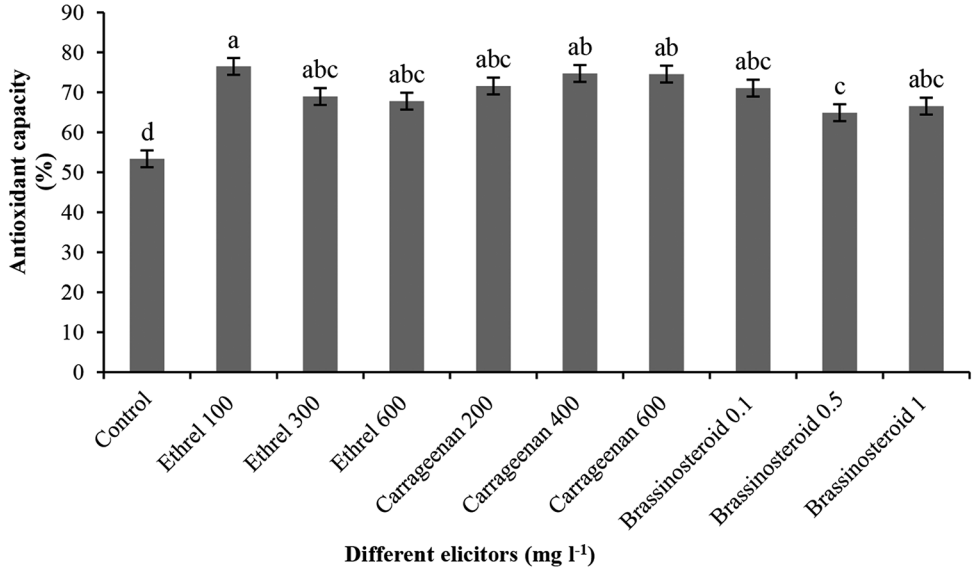

Fig. 4 Mean comparison of different elicitors on the antioxidant activity in *Momordica charantia* L. Columns with the same letters do not differ significantly (LSD test $p \leq 0.05$)

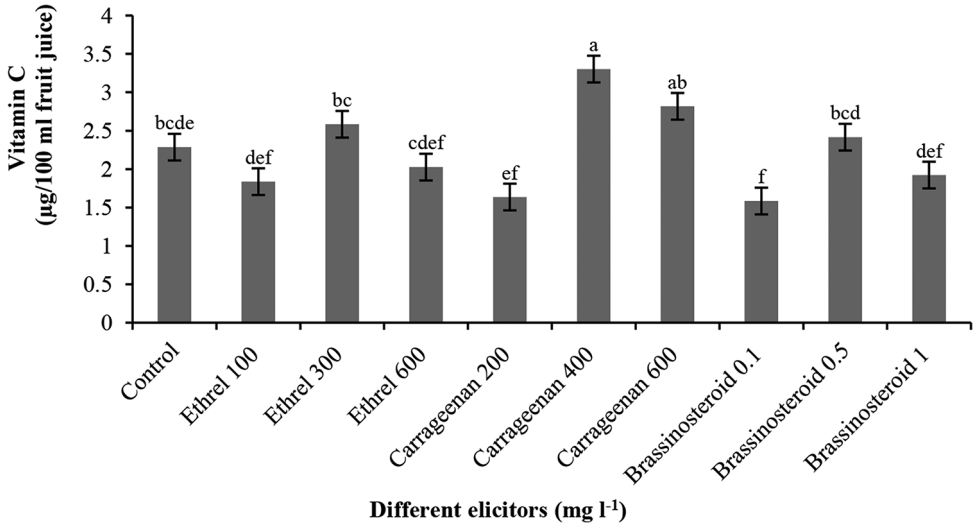

Fig. 5 Mean comparison of different elicitors on the vitamin C content in the fruit of *Momordica charantia* L. Columns with the same letters do not differ significantly (LSD test $p \leq 0.05$)

the effect of the cultivation year× elicitors (Table 1). In the first year (2018), the carbohydrate content was significantly increased with the application of 300 mg l^{-1} Ethrel, followed by 0.1 mg l^{-1} BRs, compared to the control (Table 2). In the second year (2019), all elicitors had a positive effect on the carbohydrate content compared to the control, with the highest content of carbohydrates observed with the treatment of 300 mg l^{-1} Ethrel (Table 2).

Momordicine and charantin contents

The momordicine content was affected ($p<0.01$) by the effect of the cultivation year and elicitors (Table 1). The results indicated that the content of momordicine was higher in the first year (2018) compared to the second year (2019), which may be attributed to the variation in environmental factors between the two consecutive cropping years (Table 3). Furthermore, the comparison of different elicitors on momordicine content showed that the highest content of momordicine (increase of 18.8% and 14.8%) was obtained with the treatment of 0.5 mg l^{-1} BRs and 300 mg l^{-1} Ethrel, respectively, compared to the control (Fig. 6).

Charantin content was only significant ($p<0.01$) due to the effects of elicitors (Table 1). The comparison of different elicitors on charantin content indicated that all

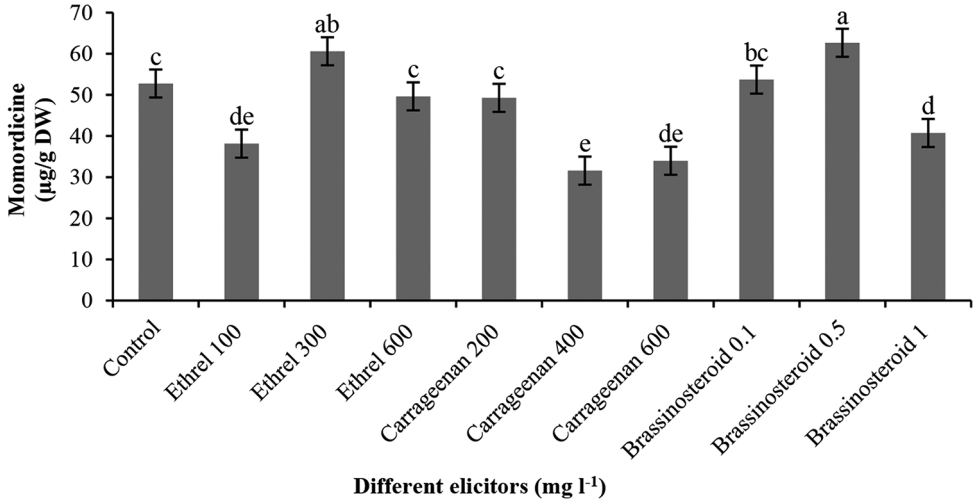

Fig. 6 Mean comparison of different elicitors on the momordicine content in the fruit of *Momordica charantia* L. Columns with the same letters do not differ significantly (LSD test $p \leq 0.05$)

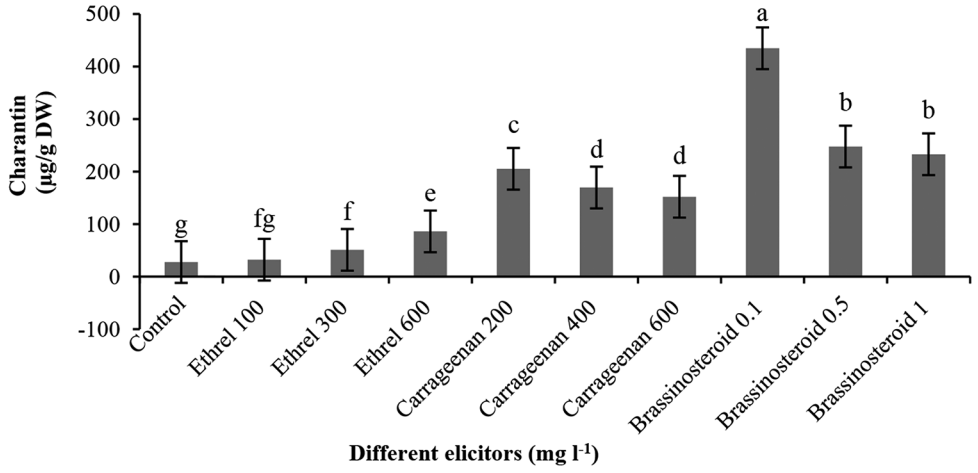

Fig. 7 Mean comparison of different elicitors on the charantin content in the fruit of *Momordica charantia* L. Columns with the same letters do not differ significantly (LSD test $p \leq 0.05$)

elicitors had a positive effect on charantin content compared to the control, with the highest content observed with the treatment of 0.1 mg l^{-1} BRs (Fig. 7).

Discussion

In this study, we investigated the effects of carrageenan, BRs, and ethylene on the growth, antioxidant activity, and secondary metabolites of bitter melon over two consecutive years. Our findings revealed significant differences in all traits of bitter melon between the consecutive years, confirming the influence of weather on fruit quality and the plant's responses to various elicitors.

These observations align with the earlier study, which also highlighted the variability in the chemical composition of tomato fruits across consecutive years and its correlation with weather conditions and the application of ethephon [33]. The differences in yield and phytochemical contents between 2018 and 2019 can be attributed to variations in weather conditions such as temperature and rainfall. For instance, higher temperatures and lower rainfall in 2019 may have contributed to increased stress on the plants, leading to higher production of certain secondary metabolites [34]. The climate has a significant impact on plant physiology, structures, and functions. Factors such as temperature, global climatic changes, and seasonal variation affect processes like photosynthesis, nutrient cycling, and transpiration, as well as the production of primary and secondary metabolites [35]. Plants experience a range of temperature levels and are affected by the changing seasons, as well as by the overall trend towards warmer and drier climates. These factors influence the chemical compositions of plants, with volatile chemicals being particularly affected.

Brassinosteroid, a growth stimulator known for promoting cell division and elongation, exhibited positive effects on bitter melon growth, including increased plant height, leaf number, and leaf area. These findings are consistent with Swamy and Rao research and further validate our study's results [33]. Likewise, we observed that K-carrageenan treatment positively influenced leaf area, which is in agreement with Ahmadi Mousavi et al.'s findings [15], indicating elevated growth parameters in basil treated with K-carrageenan. Ethrel treatment resulted in the highest number of leaves and leaf area in squash fruit, corroborating the work of Shafeek et al. [25]. The stimulatory effects of these elicitors on cell division and elongation contributed to enhanced plant growth [19, 25].

We also investigated the effects of BRs and Ethrel on flower number and sexual ratio in bitter melon. The results were consistent with previous studies [25, 35–37], suggesting that these elicitors promote root growth, leading to stronger shoot growth and an increased "female to male flowers" ratio. Papadopoulou and Grumet reported that the application of BRs and ethephon increased total female flowers in cucumber and expressed that increased femaleness by the BRs treatment may be attributed to the increment of ethylene production due to BRs [36]. Application of 400 mg l^{-1} Ethrel in the seedlings of cucumber increased the number of female flowers per plant and sex ratio compared to control [37]. Shafeek et al. reported that foliar application of Ethrel at a high level (150 mg l^{-1}) produced a higher number of female flowers in *Cucurbita pepo* L. compared to all treatments and control [25]. Ezzo et al. reported that squash seedlings treated with 250 ppm Ethrel increased plant growth and female flowers which after that improved fruit production and its goodness [30]. It has been reported that BRs play a regulatory role in the early fruit development of cucumber plants [38].

Additionally, BRs and Ethrel treatment positively impacted fruit development, leading to increased fruit weight and dimensions. In tomato plants, BRs was found to be better with maximum fruit length, fruit breadth, and fruit weight than other concentrations and treatments [21]. Increased fruit weight and dimensions by BRs and Ethephon can be due to the increased leaf area, in which leaf area increases carbon metabolism and the production of complex compounds, such as carbohydrates, that are transmitted to the fruits, leading to an increase in fruit weight and dimensions and finally fruit yield [39, 40]. Furthermore, BRs enhance essential nutrient uptake, net photosynthetic rate, stomatal conductance, and total chlorophyll content, all of which contribute to higher fruit yield [19, 20], aligning with our study's results. Carrageenan can increase fruit yield by regulating morphological, physiological, and biochemical processes and hormonal statuses [41].

Regarding fruit quality, the application of BRs at various concentrations improved ascorbic acid content and TSS [21, 42]. This enhancement can be attributed to the regulation of ethylene production by BRs, as ethylene plays a significant role in fruit ripening and quality [21]. Moreover, altering endogenous BR levels has been linked to improved fruit quality [32]. We also observed increased ascorbic acid and TA in K-carrageenan and Ethrel-elicited plants compared to the control in the second year, which is consistent with Moniruzzaman et al.'s findings regarding the application of ethephon in tomato [29]. Additionally, Shafeek et al. reported increased ascorbic acid and TSS in squash fruit with a foliar spray of Ethrel [25]. The timing of maximum ascorbic acid content, occurring 10 days after fruit set, aligns with Sung Goo et al., observations [43]. The higher accumulation of ascorbic acid may be attributed to decreased activity of the ascorbic acid oxidase enzyme [44]. These results collectively support the role of elicitors in improving fruit quality in bitter melon.

Elicitors also play a crucial role in promoting plant defense responses and the production of secondary metabolites, such as phenol and flavonoid [20, 29, 45]. We observed an increase in total phenol and total flavonoid contents in late-stage fruit maturity with all elicitors, particularly with 0.5 mg l^{-1} BRs and 300 mg l^{-1} Ethrel treatment. This increase can be attributed to the antioxidant activity of phenolic compounds, which act as defense mechanisms in response to elicitation [46]. Our findings are in agreement with Klimek-Szczykutowicz et al. (2022), who reported elevated flavonoid levels and antioxidant activity with ethephon treatment in watercress. Similarly, Barba-Espín et al. found increased total phenolic content with ethephon treatment in carrot [47]. BRs have been shown to enhance flavonoid, total phenolic, and anthocyanin contents in *Brassica juncea* L. under both stress and non-stress conditions [20], supporting our observations of increased flavonoid, total phenolic content, and antioxidant activity with Ethrel and BRs treatment. These effects may be attributed to heightened phenylalanine ammonia-lyase (PAL) enzyme activity [47], which increases as a result of external elicitor application and affects the expression of genes involved in phenolic compound biosynthesis [48–50].

Additionally, BRs treatment improved PAL activity in summer tea [50], and K-carrageenan treatment increased PAL activity in basil plants [15]. Bi et al. also reported that chickpea is elicited by K-carrageenan at a concentration of 100 µg GAE ml^{-1} of accumulated isoflavones and related pterocarpans (phenolics) and using the polysaccharides for plants [51]. Catechin and epicatechin belong to the subgroup of flavanol in bitter melon [52]. Other researchers [28, 53] had claimed that different elicitors can have various effects on the production of phenolic

compounds, flavonoids, and antioxidant activity, which was also confirmed in our study. In this study, the antioxidant activity of the bitter melon fruit was increased synchronously with phenol and flavonoid accumulation, especially in the treatment of BRs and Ethrel. Carbohydrates are basic compounds required to produce secondary metabolites, such as phenolic compounds, through the shikimic acid pathway [54].

Moreover, elicitors, including BRs and Ethrel, influenced carbohydrate content in our study. The exogenous application of BRs has been shown to promote carbohydrate metabolism in several plants, increasing the activity of INVs and SuSyn enzymes as well as sugar content [55–57]. The application of BRs to tomato fruit effectively induced ripening, elevating soluble sugars and ascorbic acid [58]. Similarly, we found that the carbohydrate content was highest with the Ethrel and BRs treatments, consistent with the study of Moreira et al., where the Ethrel treatment retained appropriate carbohydrate amounts to improve the yield and quality of 'Ponkan' mandarin fruits [59].

Furthermore, the application of elicitors, especially BRs and Ethrel, optimized the production of anti-diabetic agents (momordicine and charantin) in bitter melon. Similarly, other researchers have described that the accumulation of the secondary metabolites can be enhanced by the exogenous application of BRs, such as essential oils in peppermint [60] and catechin and theanine in tea [50], and ethylene has been linked to the increase of rutin, quercetin, and cyanidin 3-O-glucoside in tartary buckwheat and trigonelline in fenugreek [55, 61]. Moreover, salicylic acid, jasmonic acid, and gibberellin have been found to incrementally impact the content of secondary metabolites [31, 62–65]. The exogenous application of BRs and other plant growth regulators has been shown to elevate the activity of secondary metabolism-related enzymes, leading to increased production of secondary metabolites [50].

Conclusions

In conclusion, the application of different elicitors significantly influenced the phytochemical traits of bitter melon. Specifically, treatments with 200 mg l^{-1} K-carrageenan, 0.1 mg l^{-1} BRs, and 0.5 mg l^{-1} BRs demonstrated the most favorable results. These elicitors effectively enhanced leaf and fruit characteristics, with 0.1 mg l^{-1} BRs showing particular efficacy for leaf traits and 200 mg l^{-1}K-carrageenan, 0.5 mg l^{-1} BRs, and 600 mg l^{-1} Ethrel being optimal for fruit yield. The study also highlighted the important roles of total phenolic, total flavonoid, ascorbic acid, and antioxidant activity in free radical scavenging. Moreover, significant increases in momordicine and charantin, essential anti-diabetic agents, were observed in plants elicited with the tested compounds, especially BRs. This study represents the first report on the production of total phenol, total flavonoid, ascorbic acid, carbohydrates, momordicine, and charantin in bitter melon plants elicited with Ethrel, K-carrageenan, and BRs. The developed protocol holds promise for sustainable and efficient production of valuable phytochemicals in medicinal plants, with potential implications for biochemical and bioprocess engineering.

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Author contributions
A.K and M.S: Conceptualization, Methodology, Software, Validation, Supervision, Visualization, Writing- Original Draft. Z.M: Formal Analysis, Investigation, Resources, Data curation, Project administration, Writing - Original Draft. M.T, F.R and M.G: Software, Writing - Review and preparation of final version. All authors have read and agreed to the published version of the manuscript.

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Data availability
All the data generated or analyzed during the current study were included in the manuscript. The raw data is available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
We all declare that manuscript reporting studies do not involve any human participants, human data, or human tissue. So, it is not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Statement of compliance
The authors confirm that all the experimental research and field studies on bitter melon plants, including the collection of plant material, complied with relevant institutional, national, and international guidelines and legislation.

Statement on experimental research and field studies on plants
We confirmed that all methods were performed in accordance with the relevant guidelines, regulations and legislation of Iran.

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