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Research Article

Influence of Multiwalled Carbon Nanotubes and Biostimulators on Growth and Content of Bioactive Constituents of Karkade (*Hibiscus sabdariffa* L.)

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This work was carried out to evaluate the effect of different concentrations of multiwalled carbon nanotubes (MWCNT), 0, 500, and 1000 mg/L, and biostimulators (Delfan plus), 0 and 10 ml/L, and their interactions (sprayed on the plant shoot and soil until the complete wetness) on growth and production of bioactive constituents of karkade (*Hibiscus sabdariffa* L.) planted in plastic pots. After finishing experiment, growth parameters (length of shoot and root, number of leaves, branches, and fruits, chlorophyll content, and dry weight of calyces yield) were measured and also the active compounds of aqueous extracts calyces were measured by Gas Chromatography-Mass Spectrometry (GC-MS). The obtained results explained that the experimental treatment caused a significant increase in all measured parameters. GC-MS analysis shows quantitative and qualitative alterations of bioactive constituents of water extract from calyxes of karkade, where plant production of 60 active compounds at the combination of 1000 mg/L of MWCNT and 10 ml/L of Delfan plus was compared to untreated plants which produced 4 only.

1. Introduction

Medical plants researches have been expanded apparently during the last decade and international trend towards the use of natural plant remedies increased the need for more information about the properties and uses of medicinal plants. *Hibiscus sabdariffa* L., commonly known as roselle (English) and karkade (Arabic), belongs to Malvaceae and is an important annual crop grown successfully in subtropical and tropical climates [1]. The calyxes of karkade are rich in bioactive compounds including organic acids, phenolic compounds, vitamin A and vitamin C [2], flavonoid (anthocyanins), gossypetin, quercetin, pectin [3], saponins, glycosides, and alkaloids [4]. Additionally, Nnam and Onyeke [5] mentioned that calyxes contain 6.4% protein, 79.25% carbohydrates, 5.13% fat, 2.7% crude fiber, and 6.52% ash. Fresh or dried calyxes of karkade have been used traditionally as herbal drinks (cold and hot) because karkade has different therapeutic properties such as anticancer, antiviral, antifungal, antibacterial, antioxidant, antidiabetic, antihypertensive, antihyperlipidemia (anticholesterol), antiobesity, antispasmodic, and antitumor effects, decreasing the viscosity of the blood and stimulating intestinal peristalsis, and can be used to treat sore throats, coughs, and genital problems and it has also nephro/hepatoprotective and renal/diuretic effect and so forth [6].

In the recent years, there has been an increasing interest in nanoagriculture; the use of nanotechnology in agriculture and field application are highly important and are widely used in agricultural sciences, especially in the fields of biotechnology and tissue culture. The uptake of carbon nanomaterials by plants has shown a very recent field of nanoagriculture. Nanotubes are capable of interacting with biomolecules and creating functional nanosystems for transportation of other materials within cells, which leads to interaction of nanotubes and other compounds at morphological, cellular, and even molecular levels [7]. The application of biostimulators for different plants has positive effect on plant development due to containing high levels of free amino acids, total nitrogen, organic matter, and organic carbon [8].

Table 1: Characteristics of multiwalled carbon nanotubes (MWCNT).

Aspect	Powder
Colour	Black
Length	10–50 μm
Outer diameter	8–15 nm
Inside diameter	3–5 nm
Specific surface area	233 m^2/g
Purity	>95 wt%
True density	~2.1 g/cm^3
Bulk density	0.15 g/cm^3
Ash	<1.5 wt%
Electrical conductivity	>100 s/cm

Table 2: Characteristics of biostimulators (Delfan plus).

Physical-chemical characteristics		Chemical analysis	
Aspect	Liquid	Free amino acids	24.00%
Colour	Brown	Total nitrogen (N)	9.00%
Density	1.2 g/cc	Organic matter	37.00%
pH	7.2	Organic carbon	23.00%

Therefore, in the view of the medicinal importance of karkade calyxes, this work aimed to evaluate the effect of different concentrations of multiwalled carbon nanotubes and biostimulators (Delfan plus) on growth and quantitative and qualitative alterations of bioactive constituents measured by GC-MS.

2. Materials and Methods

A pot experiment was conducted in Biology Department, College of Science, University of Al-Qadisiyah, which included planting seeds of karkade plant in 18 plastic pots (5 seeds per pot) on 15/02/2016, and then the developing plants were reduced to one plant per pot. The experimental treatment was sprayed on the plant shoot and soil until the complete wetness; multiwalled carbon nanotubes (MWCNT) (Table 1) were added at three concentrations, 0, 500, and 1000 mg/L, on 10/05/2016. Biostimulators (Delfan plus) (Table 2) were added at two concentrations of 0 and 10 ml/L on 15/5/2016. The addition of two factors was repeated after one month, respectively.

After finishing the experiment (01/11/2016), growth parameters were measured, including length of shoot and root, number of leaves, branches, and fruits, dry weight of calyxes yield, chlorophyll content (measured by chlorophyll meter 502 SPAD), and the content of bioactive constituents per plant after preparation of aqueous extract of the calyxes of karkade harvested during periods of experience and then those bioactive constituents were measured by GC-MS technique (manufacturer: Shimadzu, GCMS-QP2010 Ultra system comprising a AOC-20i, Japan), according to the method of Srinivasan et al. [9].

2.1. Statistical Analysis. Data analysis was carried out using Randomized Complete Blocks Design (RCBD) in a factorial arrangement (3×2) with three replications per each treatment. Revised Least Significant Difference (RLSD) was used to compare treatment means at probability level of 0.05 when treatment effect was significant [10].

3. Results

The statistical analysis of Table 3 elucidates that increasing concentrations of both multiwalled carbon nanotubes (MWCNT) and biostimulators (Delfan plus) added to the plants alone or with each other lead to a significant increase in all studied parameters of karkade (length of shoot and root, number of leaves, branches, and fruits, chlorophyll content, dry weight of calyxes yield, and number of bioactive constituents) in comparison with those obtained from untreated plants; using the combination of 1000 mg/L MWCNT and 10 ml/L Delfan plus caused the highest increase which reached 30.47% length of shoot, 45.73% length of root length, 64.89% number of leaves, 62.08% number of branches, 60.98% number of fruits, chlorophyll content of 13.77%, and 48.30% dry weight of calyxes yield compared with untreated plants.

The GC-MS analysis of water extract from calyxes of karkade showed that untreated plants contain 4 bioactive constituents: 3,7,11,15-tetramethyl-2-hexadecen-1-ol; 1,1-bicyclohexyl, 2-(2-methylpropyl)-trans; 3-buten-2-one,4-(2-hydroxy-2,6,6-trimethylcyclohexyl); and 1,2-benzenedicarboxylic acid, diisooctyl ester (Table 4). 3-Buten-2-one, 4-(2-hydroxy-2,6,6-trimethylcyclohexyl) was identified as a major chemical constituent and gave the highest peak and relative area depending on the GC-MS chromatogram that showed 4 peaks (Figure 1).

Karkade treated without adding MWCNT and 10 ml/L Delfan plus produced 13 bioactive constituents (Table 5). Acetic acid was identified as a major chemical constituent depending on the GC-MS chromatogram that showed 13 peaks, while thujone gave the highest peak (Figure 2).

Result in Table 6 explained that karkade treated with 500 mg/L MWCNT and without adding Delfan plus produced 12 bioactive constituents. Depending on the GC-MS chromatogram in Figure 3, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester gave the highest relative area and peak compared to other compounds.

The treatment of karkade with a combination of 500 mg/L MWCNT and 10 ml/L Delfan plus showed 47 bioactive constituents (Table 7). Menthol, 1-(butyn-3-one-1-yl)-(1R,2S,5R) showed the highest relative area and peak compared to other compounds depending on the GC-MS chromatogram (Figure 4).

Karkade treated with 1000 mg/L MWCNT and without adding Delfan plus produced 41 bioactive constituents (Table 8). 1,2-Benzenedicarboxylic acid, diisooctyl ester gave the highest relative area and peak compared to other compounds depending on the GC-MS chromatogram (Figure 5). Finally, karkade treated with a combination of 100 mg/L MWCNT and 10 ml/L Delfan plus showed 60 bioactive constituents (Table 9). Depending on the GC-MS

Table 3: Significant effect of multiwalled carbon nanotubes and biostimulators (Delfan plus) in the studied characteristics of *Hibiscus sabdariffa* L.

Multiwalled carbon nanotubes concentration (mg/L)	Biostimulators (Delfan plus) concentration (ml/L)	Length of (cm)		Number of (per plant)			Chlorophyll content (SPAD)	DW of calyx yield (gm/fruit)	Number of bioactive constituents (per plant)
		Shoot	Root	Leaves	Branches	Fruits			
0	0	51.14	18.13	15.63	6.62	36.22	51.55	24.52	4
	10	57.44	22.43	23.33	11.42	44.13	53.42	29.72	13
500	0	52.13	23.54	25.31	8.32	57.81	54.82	31.61	12
	10	64.81	26.22	33.14	14.52	69.11	56.19	36.62	47
1000	0	59.73	28.73	29.46	10.74	77.54	57.22	40.41	41
	10	73.55	33.41	44.52	17.46	92.83	59.78	47.43	60
RLSD at 0.05		9.55	2.47	8.69	4.63	10.61	2.33	5.51	7.97

Table 4: Bioactive constituents of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment without multiwalled carbon nanotubes and biostimulators (Delfan plus).

Peak report TIC						
Peak	R. time	Area	Area%	Height	Height%	Name
1	12.62	93268	10.28	19921	24.88	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
2	13.56	70006	7.72	12624	15.77	1,1′-Bicyclohexyl, 2-(2-methylpropyl)-, trans-
3	18.57	689870	76.07	35869	44.80	3-Buten-2-one, 4-(2-hydroxy-2,6,6-trimethylcyclohexyl)-
4	20.42	53697	5.92	11659	14.56	1,2-Benzenedicarboxylic acid, diisooctyl ester
		906841	100.00	80073	100.00	

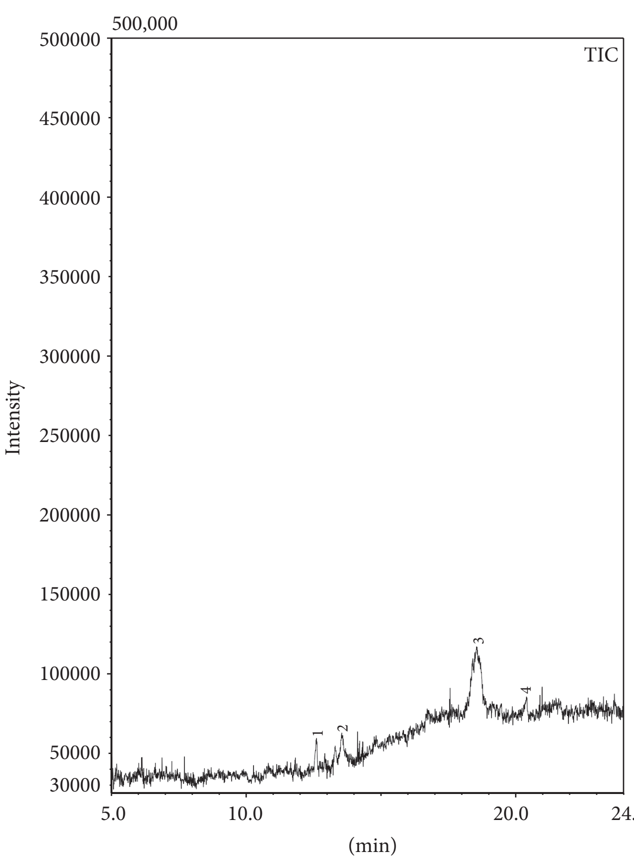

Figure 1: GC-MS chromatogram of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment without multiwalled carbon nanotubes and biostimulators (Delfan plus).

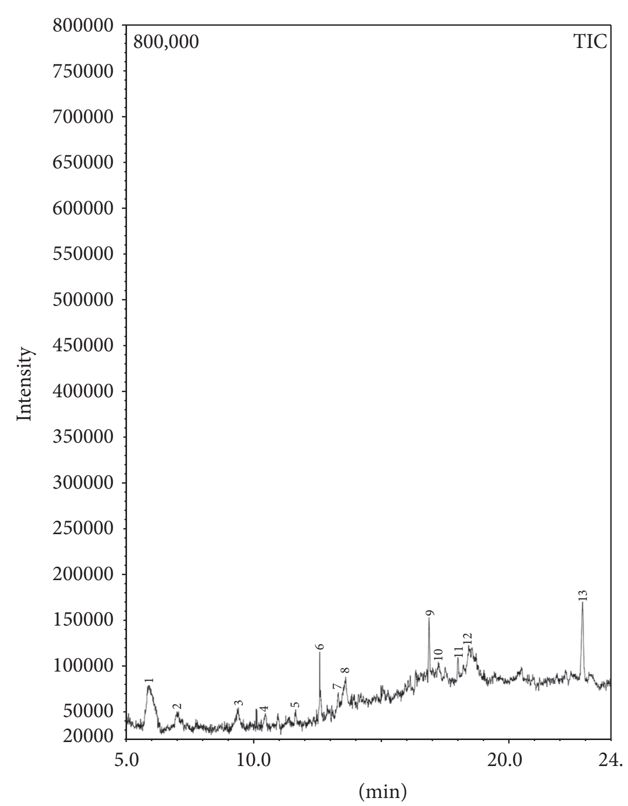

Figure 2: GC-MS chromatogram of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment without multiwalled carbon nanotubes and 10 ml/L biostimulators (Delfan plus).

Table 5: Bioactive constituents of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment without multiwalled carbon nanotubes and 10 ml/L biostimulators (Delfan plus).

Peak report TIC						
Peak	R. time	Area	Area%	Height	Height%	Name
1	5.87	924451	28.07	44787	9.86	Acetic acid
2	6.98	234129	7.11	18401	4.05	Camphor
3	9.39	264901	8.04	23849	5.25	Bicyclo[2.2.1]heptane, 2-methoxy-1,7,7-trimethyl-
4	10.45	66366	2.01	13395	2.95	2-Butenoic acid, 2-methyl-, (Z)-
5	11.63	58361	1.77	16685	3.67	1-Hexadecanol
6	12.58	237929	7.22	78034	17.18	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
7	13.31	45794	1.39	14885	3.28	1,3-Cyclopentadiene, 5,5-dimethyl-2-ethyl-
8	13.60	200938	6.10	28416	6.26	Diazoacetic acid, 2-isopropyl-5-methylcyclohexyl ester
9	16.87	187103	5.68	65937	14.52	3,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
10	17.22	96494	2.93	17373	3.82	3-Oxabicyclo[4.1.0]heptan-2-one, 4,4,7,7-tetramethyl-
11	18.00	72451	2.20	23296	5.13	12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-
12	18.42	487748	14.81	27530	6.06	3-Buten-2-one, 4-(2-hydroxy-2,6,6-trimethylcyclohexyl)-
13	22.88	416944	12.66	81663	17.98	Thujone
		3293609	100.00	454251	100.00	

Table 6: Bioactive constituents of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment of 500 mg/L multiwalled carbon nanotubes and without biostimulators (Delfan plus).

Peak report TIC						
Peak	R. time	Area	Area%	Height	Height%	Name
1	5.10	786921	11.31	30384	4.41	2-Pentanone, 4-hydroxy-4-methyl-
2	14.29	1861443	26.75	84961	12.34	2(3H)-Benzofuranone, hexahydro-3a,7a-dimethyl-, cis-
3	15.90	210700	3.03	35279	5.12	Tritetracontane
4	16.94	349173	5.02	37447	5.44	Docosyl pentafluoropropionate
5	17.63	11414	0.16	4964	0.72	Tetratriacontane
6	17.73	48075	0.69	15669	2.28	Tetratetracontane
7	17.81	41981	0.60	15121	2.20	Tritetracontane
8	19.17	820233	11.79	115136	16.72	Tetratriacontane
9	20.48	2197954	31.59	278588	40.46	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester
10	21.56	141100	2.03	16363	2.38	Eicosane, 2-cyclohexyl-
11	22.29	127819	1.84	23936	3.48	1-Heneicosanol
12	23.36	360897	5.19	30782	4.47	*cis*-13,16-Docasadienoic acid
		6957710	100.00	688630	100.00	

chromatogram in Figure 6, Naphthalenol,decahydro-1,4a dimethyl-7-(1-methylidene)-,[1R(1.alpha.,4a.beta.,8a.alpha.)] showed the highest relative area and peak compared to other compounds.

4. Discussions

On the basis of previous results, the reasons for significant increasing growth indicators by adding factor are due to the fact that multiwalled carbon nanotubes (MWCNT) have sizes ranging from 8 to 15 nm (Table 1) and a large amount of surface area in relation to mass and thus have a high ability to penetrate the walls of plant cells and act as a smart transport system in the plant as well as multiple walls increasing the surface area of the mass; these results are consistent with Canas et al. [11] on the carrot, cabbage, lettuce, cucumber, and onion, Khodakovskaya et al. [12] on the tomato, and Heydari [13] on the anthurium.

Table 7: Bioactive constituents of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment of 500 mg/L multiwalled carbon nanotubes and 10 ml/L biostimulators (Delfan plus).

Peak report TIC						
Peak	R. time	Area	Area%	Height	Height%	Name
1	8.82	141226	0.94	14453	0.41	Borneol
2	9.46	162535	1.09	20610	0.58	1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-methylene-, (4aS-cis)-
3	9.93	248143	1.66	19212	0.54	2-Butenoic acid, 2-methyl-, (E)-
4	10.48	481302	3.22	80512	2.28	Dodecan-1-yl acetate
5	11.60	129429	0.87	20711	0.59	Cyclopropanecarboxylic acid, 1-hydroxy-, (2,6-di-t-butyl-4-methylphenyl) ester
6	12.35	108383	0.72	28348	0.80	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
7	12.64	269674	1.80	79065	2.24	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
8	13.00	244025	1.63	86620	2.45	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
9	13.33	293411	1.96	63445	1.80	(−)-Spathulenol
10	13.57	194491	1.30	30385	0.86	2-Pentadecanone, 6,10,14-trimethyl-
11	13.83	67118	0.45	19221	0.54	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahydro-α,α,4a,8-tetramethyl-, (2R-cis)-
12	14.66	45902	0.31	17959	0.51	Veridiflorol
13	14.76	93078	0.62	34590	0.98	2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octahydro-α,α,4a,8-tetramethyl-, [2R-(2.alpha.,4a.be)]
14	14.83	175845	1.18	81537	2.31	Hexadecanoic acid, ethyl ester
15	15.06	275082	1.84	28540	0.81	Hexadecanoic acid, ethyl ester
16	15.22	411108	2.75	109720	3.10	1-Hexadecen-3-ol, 3,5,11,15-tetramethyl-
17	15.68	400775	2.68	89433	2.53	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-
18	15.83	83819	0.56	31391	0.89	Limonen-6-ol, pivalate
19	16.04	141274	0.94	67137	1.90	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-
20	16.09	351866	2.35	191370	5.42	Longipinocarveol, trans-
21	16.36	116915	0.78	48041	1.36	Longipinocarveol, trans-
22	16.44	53494	0.36	20051	0.57	10-Methyltricyclo[4.3.1.1(2,5)]undecan-10-ol
23	16.78	159689	1.07	61966	1.75	Succinic acid, ethyl non-5-yn-3-yl ester
24	16.88	78982	0.53	33741	0.95	Bicyclo[2.2.1]heptan-2-ol, 4-chloro-1,7,7-trimethyl-, exo-
25	16.96	111111	0.74	40230	1.14	Dodecanoic acid
26	17.12	209656	1.40	76583	2.17	9,12-Octadecadienoic acid, ethyl ester
27	17.30	58891	0.39	24040	0.68	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-
28	17.58	67358	0.45	18877	0.53	Phytol
29	17.78	63878	0.43	21067	0.60	1,2-Benzenedicarboxylic acid, diisooctyl ester
30	18.00	309133	2.07	120882	3.42	4-(2,2-Dimethyl-6-methylenecyclohexyl)butanal
31	18.58	48232	0.32	21779	0.62	Hexadecanoic acid, 2-methylpropyl ester
32	18.66	149865	1.00	64467	1.82	Z,Z-8,10-Hexadecadien-1-ol
33	18.94	181596	1.21	51056	1.44	n-Hexadecanoic acid
34	19.37	244278	1.63	81298	2.30	Heptanoic acid
35	19.49	1286488	8.60	423334	11.98	Citronellyl tiglate
36	19.65	186885	1.25	53306	1.51	Bicyclo[2.2.1]heptan-2-ol, 2,3,3-trimethyl-
37	19.82	54252	0.36	18932	0.54	4-Methyldocosane
38	20.13	68098	0.46	24263	0.69	1-Eicosene
39	20.44	971615	6.50	98698	2.79	1-Cyclohexanone, 2-methyl-2-(3-methyl-2-oxobutyl)
40	20.62	122710	0.82	32654	0.92	3-Buten-2-ol, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-
41	20.82	177718	1.19	43055	1.22	(1S-(1Alpha,2alpha,4beta))-1-isopropenyl-4-methyl-1,2-cyclohexanediol
42	20.96	885998	5.92	264130	7.47	2H-Indeno[1,2-b]furan-2-one, 3,3a,4,5,6,7,8,8b-octahydro-8,8-dimethyl
43	21.54	753783	5.04	95420	2.70	1-(3-Methoxymethyl-2,5,6-trimethylphenyl)ethanone

Table 7: Continued.

Peak report TIC						
Peak	R. time	Area	Area%	Height	Height%	Name
44	22.20	370247	2.48	48444	1.37	Fumaric acid, ethyl 2-methylallyl ester
45	22.88	304991	2.04	43927	1.24	7-Hexadecenal, (Z)
46	23.13	252921	1.69	27372	0.77	Cyclopropanemethanol,. alpha.,2-dimethyl-2-(4-methyl-3-pentenyl)-, [1.alpha.(R*),2.alpha.]-
47	23.72	3349386	22.39	561901	15.90	Menthol, 1′-(butyn-3-one-1-yl)-, (1R,2S,5R)-
		14956656	100.00	3533773	100.00	

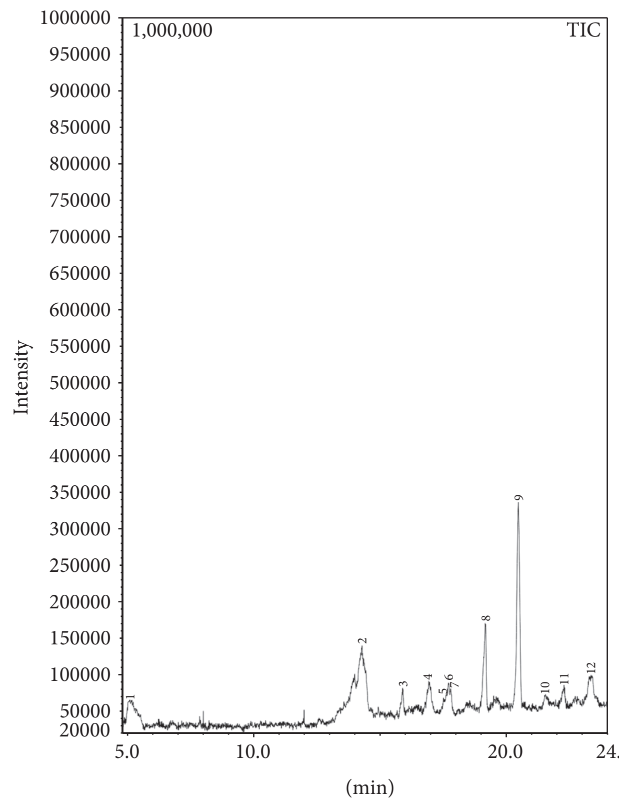

Figure 3: GC-MS chromatogram of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment of 500 mg/L multiwalled carbon nanotubes and without biostimulators (Delfan plus).

Extensive research has shown that the penetration of MWCNT into the plant cells can bring about changes in metabolic activity, leading to an increase in biomass. The tubular nanoparticle is easy to transport inside the plant. MWCNT increased water uptake through changes in the lipid formation, rigidity, and permeability of cell membrane and also enhanced net assimilation of CO_2. MWCNT might act as regulators for growth or could regulate the marker genes to enhance cell culture growth by increasing cell wall formation, cell divisions, and water transport [14, 15]. MWCNT can penetrate cell wall of leaves and induce growth of plants and development (enhancement of shoot length and root length). They also could organize nutrients absorption in the plant such as increased nitrogen, potassium, calcium, and phosphorus while decreasing sodium percent and as a result increasing total chlorophyll [16].

Additionally, MWCNT can be introduced in the plant cell by their attachment to carrier proteins, through aquaporins, using ionic channels, by endocytosis, and through their merging with organic compounds. In the particular case of MWCNT, their entrance is associated mainly with the creation of new pores in the cells [17]. The characteristics are unique of MWCNT (nanoparticle size, ability to penetrate cells, high biochemical reactivity, and rapid distribution inside plant system) and make them an attractive tool for crop management techniques. In this respect, specific types of nanoparticles in low doses have not been found to be harmful to plants but instead are capable of activating specific physiological processes, promoting photosynthesis and nitrogen metabolism, and therefore improving the growth of the plants [18]. They also play a key role in cell wall reinforcement during plant development, cell elongation,

Table 8: Bioactive constituents of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment of 1000 mg/L multiwalled carbon nanotubes and without biostimulators (Delfan plus).

						Peak report TIC
Peak	R. time	Area	Area%	Height	Height%	Name
1	7.02	765601	2.17	56572	0.86	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-
2	8.74	82130	0.23	15029	0.23	Pentanoic acid, 4-methyl-
3	8.87	81976	0.23	10541	0.16	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-
4	9.38	520061	1.47	49940	0.76	Isoborneol
5	9.87	32654	0.09	10191	0.16	Caryophyllene
6	9.91	9809	0.03	5883	0.09	Geranyl ethyl ether 2
7	10.44	1145521	3.24	132334	2.01	2-Butenoic acid, 2-methyl-, (E)-
8	11.01	145689	0.41	24839	0.38	Dodecane, 2,6,11-trimethyl-
9	11.29	78677	0.22	17466	0.27	Hexanoic acid
10	11.66	64866	0.18	19188	0.29	1-Heneicosanol
11	12.35	39374	0.11	14212	0.22	Heptadecane, 2,6,10,15-tetramethyl-
12	12.69	106924	0.30	11102	0.17	2-Butanone, 4-(2,2,6-trimethylcyclohexyl)-
13	13.30	356944	1.01	73595	1.12	Caryophyllene oxide
14	13.56	91558	0.26	33046	0.50	Octadecane, 1-chloro-
15	13.82	291034	0.82	89776	1.37	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-
16	14.66	168921	0.48	78432	1.19	Heneicosane
17	14.75	79761	0.23	32959	0.50	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]
18	14.83	408734	1.16	176706	2.69	2-Pentadecanone, 6,10,14-trimethyl-
19	15.09	467380	1.32	68319	1.04	1-Heneicosanol
20	15.26	319379	0.90	83584	1.27	Caprolactam
21	15.67	32473	0.09	18890	0.29	2-Bromotetradecane
22	16.05	318485	0.90	143648	2.19	Selina-6-en-4-ol
23	16.09	300889	0.85	133271	2.03	Hexadecanoic acid, ethyl ester
24	16.44	281577	0.80	65032	0.99	1H-3a,7-Methanoazulene-6-methanol, 2,3,4,7,8,8a-hexahydro-3,8,8-trimethyl-, [3R-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]
25	16.60	473246	1.34	223525	3.40	Heneicosane
26	16.79	283275	0.80	117213	1.78	8-Hydroxy-2,2,8-trimethyldeca-5,9-dien-3-one
27	17.49	92528	0.26	39301	0.60	Hexadecane, 2,6,11,15-tetramethyl-
28	18.00	293141	0.83	105445	1.60	Eicosanoic acid
29	18.19	136422	0.39	55398	0.84	2-Decene, 2,4-dimethyl-
30	18.31	447480	1.27	149416	2.27	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-
31	18.51	2937254	8.31	1043306	15.88	Eicosane, 10-methyl-
32	18.80	151566	0.43	54379	0.83	2,6-Octadienal, 2,6-dimethyl-8-(tetrahydro-2H-2-pyranyloxy)
33	19.57	5322093	15.06	604327	9.20	Hentriacontane
34	20.75	11971493	33.88	1173046	17.85	1,2-Benzenedicarboxylic acid, diisooctyl ester
35	20.89	1963448	5.56	620603	9.45	Heneicosane
36	20.97	441729	1.25	151709	2.31	Oxalic acid, 2-ethylhexyl pentadecyl ester
37	21.66	798268	2.26	78439	1.19	cis-13,16-Docasadienoic acid
38	22.25	84015	0.24	25211	0.38	(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-methanol
39	22.34	448534	1.27	81446	1.24	Tritetracontane
40	22.90	2376704	6.73	483543	7.36	Cyclopropanemethanol,. alpha.,2-dimethyl-2-(4-methyl-3-pentenyl)-, [1.alpha.(R*),2.alpha.]-
41	23.70	925582	2.62	199546	3.04	Pentadecanoic acid
		35337195	100.00	6570408	100.00	

Table 9: Bioactive constituents of water extract of calyxes of *Hibiscus sabdariffa* L. from combination treatment of 1000 mg/L multiwalled carbon nanotubes and 10 ml/L biostimulators (Delfan plus).

Peak Report TIC						
Peak	R. time	Area	Area%	Height	Height%	Name
1	5.98	179688	0.89	14717	0.29	1-[t-Butyl]-3-[2-hydroxyethyl]-2-thiourea
2	6.53	206765	1.03	16890	0.33	1,3,3-Trimethylcyclohex-1-ene-4-carboxaldehyde, (+,−)-
3	6.99	477865	2.38	39661	0.77	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-
4	7.75	17402	0.09	8436	0.16	2-Octenoic acid, 7-hydroxy-, ethyl ester
5	7.99	277810	1.38	25340	0.49	2-Cyclohexen-1-one, 3-methyl-
6	8.72	58849	0.29	11308	0.22	Decanoic acid, 3-methyl-
7	8.89	61164	0.30	11322	0.22	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (Z)-
8	9.38	478340	2.38	65463	1.27	Borneol
9	9.84	13698	0.07	7413	0.14	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, [1aR-(1a.alpha.,4a.alpha.,7.alpha.,7a.beta.,7b. alpha.)]
10	9.88	11189	0.06	8044	0.16	Spiro[cyclopropane-1,8′(1H′)[3a.6]methano[3ah]cyclopentacycloocten]-10′-one, octahydro-, (3'as,6'R,9'ar)
11	10.48	861378	4.28	116784	2.27	2-Butenoic acid, 2-methyl-, (E)-
12	10.99	54684	0.27	15680	0.31	Decane, 2-methyl-
13	11.29	23403	0.12	13933	0.27	Heptanoic acid
14	11.40	96098	0.48	29717	0.58	2-Hexanol, 3,3,5-trimethyl-2-(3-methylphenyl)-
15	11.62	96495	0.48	30081	0.59	n-Nonadecanol-1
16	12.34	43727	0.22	14425	0.28	Decane, 2-methyl-
17	12.58	72201	0.36	17495	0.34	E-6-Octadecen-1-ol acetate
18	12.96	95214	0.47	31350	0.61	1-Dodecanol
19	13.30	413748	2.06	114634	2.23	Tricyclo[3.1.0.0(2,4)]hexane, 3,6-diethyl-3,6-dimethyl-, trans-
20	13.56	58576	0.29	18742	0.37	2-Cyclohexene-1-methanol, 2,6,6-trimethyl-
21	13.81	333618	1.66	122979	2.40	Nerolidyl acetate
22	13.94	257018	1.28	76005	1.48	2,3-Dipropyl-cyclopropanecarboxylic acid, ethyl ester
23	14.00	27371	0.14	18033	0.35	Tetradecanoic acid, ethyl ester
24	14.15	101642	0.51	25064	0.49	10s,11s-Himachala-3(12),4-diene
25	14.24	103868	0.52	28501	0.56	2H-Pyran-2-one, 4-hydroxy-6-methyl-
26	14.36	65809	0.33	24124	0.47	Terpin Hydrate
27	14.66	219672	1.09	107246	2.09	2-Bromotetradecane
28	14.74	136624	0.68	55418	1.08	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]
29	14.83	943433	4.69	399599	7.78	2-Pentadecanone, 6,10,14-trimethyl-
30	15.02	72288	0.36	29358	0.57	2-Piperidinone, 1-(3,4,5,6-tetrahydro-2-pyridinyl)-
31	15.11	221117	1.10	85066	1.66	7-Oxabicyclo[4.1.0]heptane, 2,2,6-trimethyl-1-(3-methyl-1,3-butadienyl)-5-methylene-
32	15.22	334667	1.66	83563	1.63	2H-Cyclopentacyclooctene, 4,5,6,7,8,9-hexahydro-1,2,2,3-tetramethyl-
33	15.34	21275	0.11	13398	0.26	Heptanoic acid, anhydride
34	15.82	179493	0.89	59327	1.16	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-
35	16.04	2866546	14.25	855621	16.66	1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene)-, [1R-(1.alpha.,4a.beta.,8a.alpha.)]-
36	16.41	159687	0.79	39983	0.78	9-Isopropyl-1-methyl-2-methylene-5-oxatricyclo[5.4.0.0(3,8)]undecane
37	16.59	474369	2.36	220498	4.29	Tricosane
38	16.78	432738	2.15	180735	3.52	Limonen-6-ol, pivalate
39	16.96	250742	1.25	76006	1.48	(−)-Spathulenol
40	17.12	144462	0.72	59742	1.16	Longipinocarveol, trans-
41	17.23	116967	0.58	29430	0.57	1-Cyclohexyl-1-(2-methylenecyclohexyl)ethanol
42	17.39	149266	0.74	44782	0.87	Cyclohexane, butyl-
43	17.57	118813	0.59	37221	0.72	(1-Ethylbuta-1,3-dienyl)benzene

Table 9: Continued.

Peak	R. time	Area	Area%	Height	Height%	Name
44	17.91	52764	0.26	16286	0.32	Benzeneethanol,. alpha.-ethyl-
45	18.00	278732	1.39	107308	2.09	Dodecanoic acid
46	18.18	203495	1.01	68303	1.33	But-2-enoic acid, amide, 3-methyl-N-methallyl-
47	18.46	799323	3.97	240431	4.68	Heptacosane
48	18.65	168921	0.84	74823	1.46	9,12-Octadecadienoic acid, ethyl ester
49	19.46	224542	1.12	39309	0.77	Phytol
50	19.92	156623	0.78	28959	0.56	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester
51	20.21	144167	0.72	40315	0.79	Oxacycloheptadec-8-en-2-one
52	20.31	219523	1.09	59841	1.17	Tetradecanoic acid
53	20.50	49486	0.25	17568	0.34	1,3-Dioxolane, 4,4,5-trimethyl-2-pentadecyl-
54	20.60	195477	0.97	54141	1.05	Fumaric acid, ethyl 2-methylallyl ester
55	20.80	253010	1.26	76165	1.48	Tetratetracontane
56	20.94	441843	2.20	129566	2.52	Oxalic acid, 2-ethylhexyl pentadecyl ester
57	21.05	123783	0.62	38025	0.74	Cyclohexane, 1,5-dimethyl-2,3-divinyl-
58	21.83	1095808	5.45	63024	1.23	1-Octacosanol
59	22.88	1803739	8.97	328164	6.39	Cyclohexan-1-ethanol, 1-hydroxymethyl-
60	23.70	2578313	12.82	469027	9.14	n-Hexadecanoic acid
		20119328	100.00	5134389	100.00	

Peak Report TIC

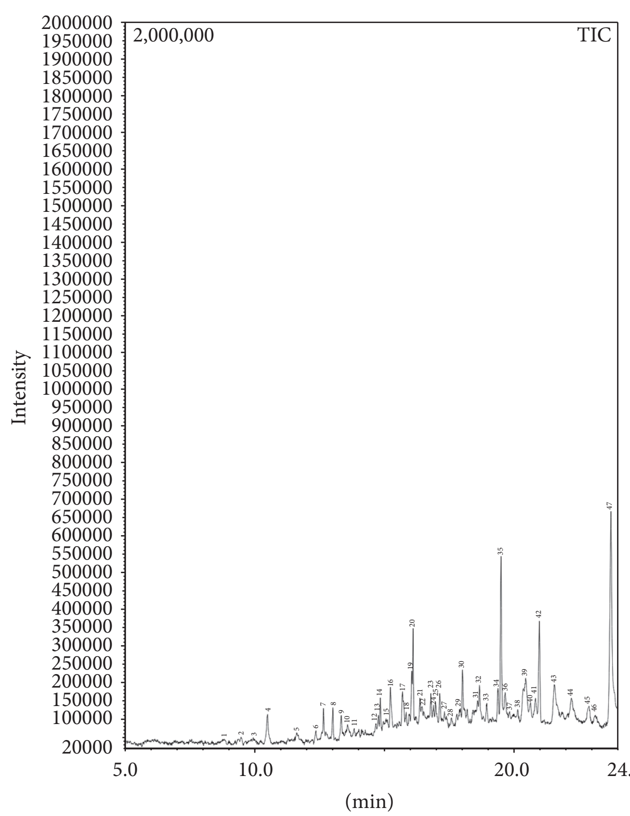

Figure 4: GC-MS chromatogram of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment of 500 mg/L multiwalled carbon nanotubes and 10 ml/L biostimulators (Delfan plus).

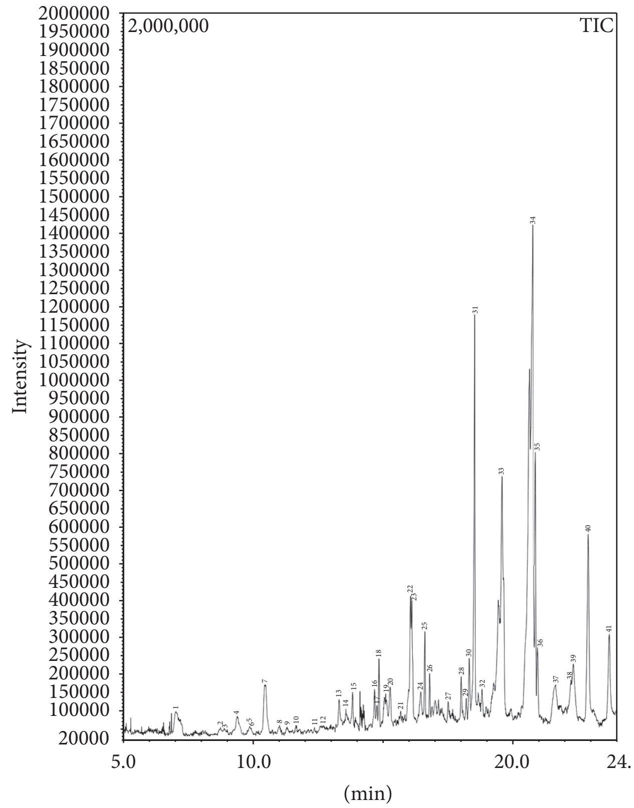

Figure 5: GC-MS chromatogram of water extract of calyxes of karkade (*Hibiscus sabdariffa* L.) from combination treatment of 1000 mg/L multiwalled carbon nanotubes and without biostimulators (Delfan plus).

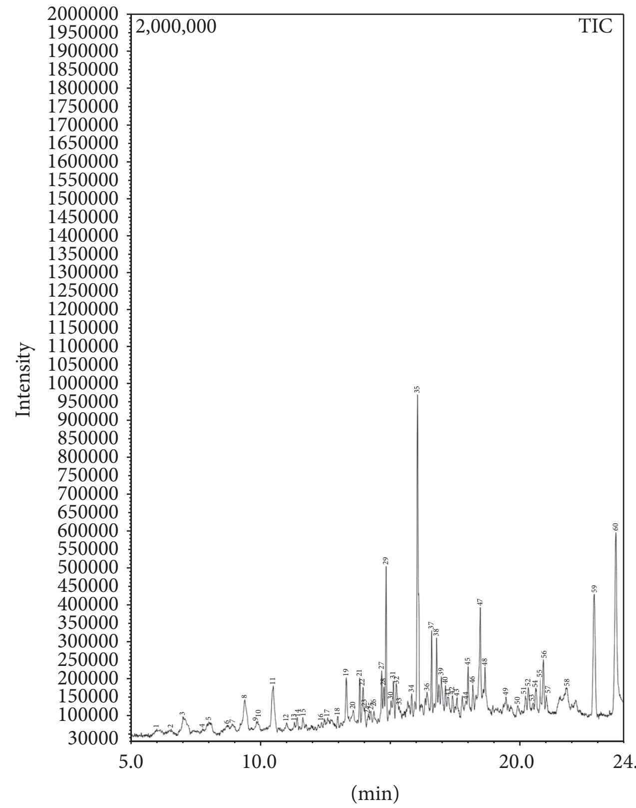

Figure 6: GC-MS chromatogram of water extract of calyxes of *Hibiscus sabdariffa* L. from combination treatment of 1000 mg/L multiwalled carbon nanotubes and 10 ml/L biostimulators (Delfan plus).

reproduction, and photosynthesis and in response to external signals regulate water channels (aquaporins). There are many reports indicating that aquaporins are central components in plant water relations and are crucial for root water uptake and are a limiting factor for plant growth [19].

Meanwhile, biostimulators (Delfan plus) also caused a significant increase in all studied parameters because they contain free amino acids, total nitrogen, organic matter, and organic carbon (Table 2). These results were in agreement with Saeed et al. [20] on soybean, El-Zohiri and Asfour [21] on potato, and Abo Sedera et al. [22] on strawberry.

Biostimulators act as source of plant growth hormones like gibberellins that could directly or indirectly impact the physiological activities of plant growth and development. The division in the plant with addition of biostimulators leads to increasing the rate of photosynthesis of production of secondary metabolic compounds of plants, particularly in the case of abiotic stress.

Application of exogenous amino acid improves plant growth by increasing the concentration of photosynthetic pigments (ALA is a common precursor to tetrapyrrole) [23, 24], as well as antioxidative enzyme activities [25]. And it affects processes involved in plant nitrogen metabolism (Iwai et al. [26]). Also, amino acids in biostimulant have positive effects on plant growth and yield and significantly relieve the injuries caused by abiotic stresses [8]; the addition of high concentration of multiwalled carbon nanotubes to plant probably causes stress to the plant.

5. Conclusions

Data from results suggest that experimental factors have an important role in causing quantitative and qualitative variations of their medically active ingredients compared with untreated plants. Combination treatment of 1000 mg/L multiwalled carbon nanotubes and 10 ml/L biostimulators (Delfan plus) caused an increase in all studied parameters of karkade.

Conflicts of Interest

The author declares that they have no conflicts of interest.

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