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Salicylic Acid Spraying Affects Secondary Metabolites and Radical Scavenging Capacity of Drought-Stressed *Eriocephalus africanus* L.

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Abstract: Drought is among the most common abiotic stresses that significantly influence plants' growth and metabolic activities. In this study, *Eriocephalus africanus* L. (Asteraceae) was exposed to three levels of drought stress (irrigation with 75, 50, and 25% field capacity), together with foliar spraying of a plant hormone, salicylic acid (1, 2, and 3 mM SA), to observe the effect of drought stress and SA on its secondary metabolites. These growing conditions efficiently affected its total flavonoid and polyphenol contents (TFC and TPC, respectively). TFC and TPC increased by 53% and 35%, respectively, in stressed plants. Consequently, the radical scavenging activity improved by 140%. UPLC-ESI-MS/MS profiles of the extracts of control and stressed plants were assessed. Among identified polyphenols, 3,4-dicaffeoylquinic acid predominated in both samples, although it was detected in a greater percentage of stressed plants. Essential oils hydro-distilled from the plants showed a higher yield (1.05 ± 0.03% *v*/*w*) in stressed plants. Artemisia ketone prevailed in all oil samples' GC/MS chromatograms, with a higher yield (42%) recorded in stressed plants. In conclusion, drought stress and SA spraying triggered the production of phenolic and essential oil components and increased the radical scavenging activity of *E. africanus*. Thus, agricultural conditions are optimized to provide a continuous supply of plant materials with appropriate amounts of bioactive constituents for economic industrialization.

Keywords: elicitors; essential oil; polyphenols; industrial development; drug discovery; abiotic stress

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1. Introduction

Medicinal and aromatic plants are nowadays extensively incorporated in several industries for pharmaceutical, phytopharmaceutical, and health products manufacture, besides their widespread use in traditional and alternative medicine [1]. These industries need constant and reliable sources of standardized raw materials from cultivated rather than wild-harvested plants. However, reported data revealed that in certain cases, cultivated plants might not be as efficient as their wild versions [2]. Thus, agricultural conditions are optimized to provide a continuous supply of plant materials with appropriate amounts of bioactive constituents for economic industrialization.

Eriocephalus africanus L., also known as wild rosemary, is a native South African plant belonging to Asteraceae. The plant is tap-rooted and is extremely drought stress-tolerant [3,4]. It is used as a flavoring agent for culinary purposes and in folk medicine as an

anti-inflammatory, antimicrobial, expectorant, diuretic, and a diaphoretic [5]. Polyphenols and essential oil are the main two classes reported in *E. africanus* plant. Polyphenols are represented mainly by mono- and di-caffeoylquinic acids and flavonoids such as hesperetin and eriodictyol [3]. The essential oil exerts antioxidant, anti-inflammatory, and antimicrobial activities [6]. Additionally, aqueous extracts of the plant have demonstrated analgesic, antipyretic, and anti-nociceptive effects [7].

Environmental stresses affect both the quality and yield potential of plants. Water deficit, in particular, has a significant impact on the growth and metabolic activities of plants; besides, other environmental factors like improper temperature and soil salinity may also alter the plant's metabolic activity [8]. For medicinal and aromatic crops, drought stress may produce significant changes in the yield and composition of their metabolites [9]. Drought stress caused the accumulation of phenolics and flavonoids and improved the antioxidant capacity and lipid peroxidation in some Asteraceae plants like *Chrysanthemum morifolium*, *Cynara cardunculus*, and *Achillea* species [10].

Several commercially available chemicals are used as elicitors to modify and/or activate the accumulation of certain secondary metabolites in plants and subsequently affect their bioactivity [11]. The natural phytohormone salicylic acid (SA) occurs in plants at a very low concentration [12]. SA is a signal phytohormone compound responsible for inducing tolerance to certain biotic and abiotic stresses [13]. It is also thought to trigger the phenylpropanoid pathway, which leads to enhanced biosynthesis of some secondary metabolites [14,15]. Studies show that SA application caused the accumulation of polyphenolic compounds and essential oil in some plants like thyme, chickpea, and grape berries [16,17]. Nevertheless, reports concerning the influence of foliar spraying of SA on the quantity and quality of secondary metabolites in other medicinally important plants under field conditions are still limited.

This study aimed to investigate the effect of foliar spraying of the plant hormone salicylic acid on the secondary metabolites, as well as the radical scavenging capacity of drought-stressed *E. africanus* L., to set agricultural guidelines to optimize the growth and contents of the plant's secondary metabolites for industrial implementation and reduction of cultivation time and cost.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Seeds of *E. africanus* L. were purchased from the Ministry of Agriculture and Land Reclamation, Giza, Egypt, and planted in the medicinal farm of Mepaco. Medifood, Egypt. Mrs. Trease Labib, a consultant of plant taxonomy, Ministry of Agriculture, and the ex-director of Al-Orman botanical Garden, Giza, Egypt, kindly verified the plant's identity. Young plant cuttings were transplanted in plastic pots. A naturally illuminated greenhouse present in Future University, Cairo, Egypt, was used for plant growth (average temperature 25–30 °C, average relative % humidity, 20–40%). Plants were first transplanted in silty soil in small plastic pots (15 cm) until rooting (21 days). The rooted cuttings were transferred to larger pots (25 cm). Fertilizers (4 g of Ammonium sulfate (20.6% N) and 2 g of Potassium sulfate (48% K_2O)) were added after two weeks from transplantation. Plants were irrigated once per week.

The experiment was performed in triplicate (196 pots); each replicate (64 pots) was divided into 4 groups, each irrigated with different Field Capacity, FC ($F_{1–4}$). Calculation of FC was achieved by weighing three large pots containing seven kg of soil (W1). Pots were fully immersed in a large water-filled container. After 24 h, pots were allowed to drain and then reweighed (W2). W2-W1 is considered 100% FC [18]. Plants were watered with 100/25%FC once per week and sprayed with distilled water/SA every 21 days for the control and stressed plants. After 105 days, plants were harvested early morning. Shade-drying in the open air (25–28 °C) was carried out for two weeks. Plants were then ground and kept in sealed containers in the refrigerator at 4 °C for further examination. Voucher

specimens are deposited in the Pharmacognosy research lab at the Future University in Egypt (E.1-16).

2.2. Chemicals and Solvents

Standard compounds (ascorbic acid, DPPH, gallic acid, rutin) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Salicylic acid, Folin–Ciocalteu reagent, and solvents were obtained from Merck, (Merck KGaA, Darmstadt, Germany).

2.3. Extraction of Plant Material

Dried aerial parts of the plants (250 g) were extracted using 70% ethanol by cold maceration (2 L × 3), at ambient temperature, for 4 h. Dry extracts were stored in sealed vials at 4 °C until analysis.

2.4. Measurement of Total Flavonoid and Total Polyphenol Contents (TFC and TPC)

TFC was measured in the plant extracts using the aluminum chloride colorimetric assay according to a published procedure [19] with slight modifications. Aliquots of tested samples (2 mL of methanol containing 10 mg dry extract of each plant) were mixed with 300 µL of (5%) sodium nitrite. After 6 min, 300 µL of (10%) aluminum chloride was added, and the reaction mixture was allowed to stand for another 6 min, then treated with 1 mL of (4%) sodium hydroxide, and the final volume was adjusted with deionized water to 6 mL. After a thorough mixing, the mixture was left at room temperature for 15 min, and the absorbance of the red color produced was measured at 510 nm. The TFC of each tested sample was calculated as a rutin equivalent (RE), as deduced from an established calibration curve (R^2 = 0.9962). The latter was prepared by adopting the same procedure using 0.2–0.6 mg/mL of rutin solutions in methanol. All determinations were performed in triplicates.

TPC of the plant extracts was determined as a gallic acid equivalent (GAE) according to the Folin–Ciocalteu colorimetric assay [20]. The calibration curve was made using gallic acid. Fifty µL aliquots of gallic acid (0.25–3 mg/mL) solutions in methanol were mixed with 50 µL Folin–Ciocalteu reagent (2 N) and 300 µL 20% anhydrous Na_2CO_3 with 3.5 mL deionized water (R^2 = 0.9978). The absorbance of the obtained color was measured at 728 nm after 30 min at 28 °C against a blank (prepared without adding Folin–Ciocalteu). TPC was estimated in the tested plant extracts on 50 µL samples of the methanol extracts (5 mg/mL) using the same procedure. TPC was expressed as a gallic acid equivalent, and triplicate experiments were carried out.

2.5. Evaluation of DPPH Radical Scavenging Activity

DPPH free radical (2,2-diphenyl-1-picrylhydrazyl) was used to assess the radical scavenging activity of the plant extracts according to a published procedure [21] with minor modifications. DPPH stock solution was prepared (8 mg/100 mL in methanol). A control prepared by mixing DPPH stock solution (10 mL) with methanol (10 mL) gave an absorbance at 517 nm (Ac) 0.91 ± 0.02 units. Plant extracts were prepared in different concentrations (20–400 µg/mL), and each was mixed with equal volumes of DPPH and left to stand for 30 min. in the dark. The absorbance of each mixture (As) was measured at the same absorbance. Standard ascorbic acid (20–100 µg/mL) was used to obtain the calibration curve. Percentage inhibition was calculated as follows: %I = [(Ac-As)/Ac] × 100. IC_{50} (median inhibitory concentration) values were determined using linear regression analysis of the %I vs. extracts concentration. The results were also expressed as ascorbic acid equivalent/mg dry weight, which was calculated as follows: IC_{50} ascorbic acid/IC_{50} sample = X mg ascorbic acid equivalent/mg DW.

2.6. Identification of Metabolites in Alcoholic Extracts via UPLC-ESI-MS/MS

The alcoholic extracts of the control treatment (F_1S_0) and the treatment with the highest TFC and TPC (F_4S_3) were analyzed using ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS). The analysis was carried out in negative ion acquisition mode on a Triple Quadrupole Mass Spectrometer (XEVO TQD, Waters Corporation, Milford, MA 01757, USA). Sample solutions (100 µg/mL in HPLC grade methanol) were filtered through a 0.2 µm PTFE membrane disc filter. Degassing was carried out by sonication. The injection volume was 10 µL. A reverse phase C18 column was used (ACQUITY UPLC-BEH C18, 1.7 µm particle size, 2.1 × 50 mm column). The mobile phase consisted of eluent A (0.1% formic acid in methanol) and eluent B (water acidified with 0.1% formic acid). Elution was a gradient with a flow rate of 0.2 mL/min as follows: 10% A (0–0.3 min), 10–90% A (0.3–18 min), 90% A (18–22 min), and 10% A (22–25 min). Negative ion ionization mode was used (mass spectra detected between m/z 100–900) with source temperature 150 °C; capillary voltage 3 kV; cone voltage 30 eV, de-cone gas flow, 50 mL/h, solvation temperature, 450 °C; and de-solvation gas flow, 900 L/h. Masslynx™ 4.1 software (Waters Inc., Millford, MA, USA) was used. Compounds were tentatively identified by their molecular weight, the fragmentation pattern of the mass spectrum, and comparison with previously published data.

2.7. Isolation of Essential Oils

E. africanus powdered aerial parts (25 g from each treatment) were hydro-distilled using Clevenger-type apparatus for 3 h. Essential oils were dried over anhydrous sodium sulfate and saved in sealed amber vials, at 4 °C, until use.

2.8. Analysis of Essential Oil Composition via GC-MS

An Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA, equipped with a capillary column (RTX-5MS, 30 m × 0.32 mm, film thickness 0.25 µm), was used for GC-MS analysis of *E. africanus* oils. An Agilent 5975C mass selective detector was coupled to the column. The initial oven temperature was 40 °C for 2 min, then raised to 210 °C (at a rate of 5 °C/min); injector and detector temperatures were set at 290 and 300 °C, respectively. Helium was used as a carrier with a 2 mL/min flow rate. Manual sample injection (0.1 µL) was performed in split mode. EI mode (35–500 m/z range) was used for recording mass spectra. The ionization voltage was 70 eV. The ion source temperature was set at 230 °C. RI (retention indices) were calculated using a homologous series of C5–C24 n-alkanes. Compounds were identified by spectra comparison with MS libraries (Wiley) and by comparing RI with those previously reported in the literature [22]. For quantitation of relative percentages of individual components, peak areas were measured.

2.9. Statistical Analyses

Analysis was performed in triplicate. Values are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) via GraphPad Prism® v.5 software (GraphPad Software Inc., La Jolla, CA, USA) was used to analyze the results. Significant differences among means of different treatments were separated using Bonferroni posttests at $p \leq 0.05$.

3. Results

3.1. Effects of Drought Stress and SA Spraying on Fresh Weight, Dry Weight, TFC, TPC, Radical Scavenging Activity, and Oil Yield (Table 1)

Drought stress negatively affected both the plant fresh and dry weights, however, SA reversed this impact up to a concentration of 2 mM (at 3 mM fresh and dry weights of plants started to decrease).

Both drought stress and SA spraying led to a significant rise in TFC in the investigated plant. TFC increased from 109.3 ± 0.4 mg RE/g dry weight in the control treatment F_1S_0 to 167.7 ± 0.2 mg RE/g dry weight in the highest stressed treatment F_4S_3, meaning that the applied conditions resulted in a 53% increase in TFC.

Similarly, the TPC was increased from 181 ± 0.7 mg GAE/gm dry weight in the control treatment F_1S_0 to 243 ± 0.7 mg GAE/g dry weight the highest stressed treatment F_4S_3, thus increasing by 35% due to the applied water stress and SA spraying.

The radical scavenging activity increased significantly in stressed plants. IC_{50} value decreased from 28.9 ± 0.4 µg/mL in F_1S_0 to 12.1 ± 0.4 µg/mL in F_4S_3 (140% increase in antioxidant capacity).

Essential oils of *E. africanus* samples were obtained as a pale-yellow liquid with a characteristic rosemary-like odor. Oil yield in F_1S_0 (control) was 0.86 ± 0.12% *v*/*w*, while F_4S_3 1.05 ± 0.03% *v*/*w*. Generally, it was observed that there is an increase in the oil yield of about 31% due to drought stress and SA spraying.

Table 1. Effects of drought stress and SA spraying on fresh weight, dry weight, TFC, TPC, and radical scavenging activity.

Treatment	Fresh Weight * (g)	Dry Weight * (g)	TFC ** (mg RE/g Dry Weight)	TPC ** (mg GAE/g Dry Weight)	Radical Scavenging Activity ** (IC_{50} µg/mL)	Essential Oil Yield ** (% *v*/*w*)
F_1S_0 ***	22.5 ± 1.6 [a]	9.1 ± 1.0 [a]	109.3 ± 0.4 [a]	181.4 ± 0.7 [a]	28.9 ± 0.4 [a]	0.86 ± 0.12 [a]
F_1S_1	23.8 ± 1.8 [a]	9.6 ± 1.0 [a]	111.3 ± 0.3 [a]	184.6 ± 0.6 [a]	25.2 ± 0.9 [b]	0.96 ± 0.07 [b]
F_1S_2	27.8 ± 0.9 [b]	9.9 ± 1.1 [a]	114.8 ± 0.1 [b]	188.0 ± 0.9 [a]	24.7 ± 0.5 [b]	0.99 ± 0.02 [b]
F_1S_3	26.2 ± 0.8 [b]	8.8 ± 0.7 [b]	117.8 ± 0.2 [b]	191.4 ± 0.9 [b]	24.1 ± 0.5 [b]	0.97 ± 0.01 [b]
F_2S_0	21.5 ± 0.9 [a]	8.5 ± 0.8 [b]	126.2 ± 0.3 [b]	194.9 ± 0.3 [b]	23.8 ± 1.0 [b]	0.91 ± 0.07 [b]
F_2S_1	22.8 ± 0.8 [a]	9.2 ± 0.7 [a]	130.8 ± 0.3 [b]	198.1 ± 0.7 [b]	22.2 ± 0.9 [b]	1.00 ± 0.07 [b]
F_2S_2	26.6 ± 1.0 [b]	9.3 ± 0.9 [a]	139.7 ± 0.3 [b]	202.9 ± 0.3 [b]	20.4 ± 0.6 [b]	1.02 ± 0.10 [b]
F_2S_3	26.0 ± 0.8 [b]	8.3 ± 0.8 [b]	142.1 ± 0.1 [b]	206.9 ± 0.7 [b]	18.6 ± 0.4 [b]	1.00 ± 0.01 [b]
F_3S_0	19.8 ± 1.5 [b]	7.9 ± 0.9 [b]	149.9 ± 0.1 [b]	214.8 ± 0.9 [b]	17.8 ± 0.2 [b]	0.91 ± 0.02 [b]
F_3S_1	21.0 ± 1.0 [a]	8.7 ± 0.4 [b]	151.4 ± 0.3 [b]	217.0 ± 0.7 [b]	16.2 ± 0.8 [b]	1.02 ± 0.20 [b]
F_3S_2	23.5 ± 1.1 [b]	8.9 ± 0.9 [b]	155.8 ± 0.2 [b]	222.4 ± 0.9 [b]	15.6 ± 0.7 [b]	1.04 ± 0.02 [b]
F_3S_3	22.1 ± 1.0 [a]	8.0 ± 0.6 [b]	159.4 ± 0.2 [b]	225.9 ± 0.7 [b]	15.1 ± 0.4 [b]	1.00 ± 0.04 [b]
F_4S_0	16.7 ± 1.6 [b]	6.8 ± 0.7 [b]	160.3 ± 0.2 [b]	230.3 ± 0.3 [b]	14.6 ± 0.6 [b]	0.95 ± 0.02 [b]
F_4S_1	18.3 ± 0.8 [b]	7.5 ± 0.8 [b]	162.3 ± 0.1 [b]	234.5 ± 1.2 [b]	14.5 ± 0.7 [b]	1.03 ± 0.05 [b]
F_4S_2	19.6 ± 0.8 [b]	8.0 ± 0.7 [b]	163.7 ± 0.3 [b]	238.9 ± 0.6 [b]	13.9 ± 0.4 [b]	1.04 ± 0.02 [b]
F_4S_3	17.0 ± 0.7 [b]	7.0 ± 0.6 [b]	167.7 ± 0.3 [b]	243.4 ± 0.7 [b]	12.1 ± 0.4 [b]	1.05 ± 0.03 [b]

$F_{1,2,3,4}$: 100, 75, 50, 25% FC respectively. $S_{1,2,3,4}$: 0, 1, 2, 3 mM SA, respectively. Significant differences among means of different treatments were separated using Bonferroni posttests at $p \leq 0.05$ (*: n = 9, **: n = 3) with all treatments compared to the control plant I_1/SA_0 (irrigated at 100% FC and sprayed with distilled water. ***: control treatment irrigated with 100% FC and sprayed with distilled water only. Means followed by different letters in same row denote significant difference at $p < 0.05$.

3.2. UPLC-ESI-MS/MS Metabolic Profile of Alcoholic Extracts

The alcoholic extracts of the control treatment (F_1S_0) and the treatment with highest TFC and TPC (F_4S_3) were analyzed using UPLC-ESI-MS/MS. Thirty-two compounds were identified in the extracts of both treatments, although with qualitative variability. The major detected polyphenol in the two samples was 3,4-dicaffeoylquinic acid. Five compounds disappeared from the control sample, viz. the polyphenols caffeic, ferulic, and 1-caffeoylquinic acids, and the polyacetylenes dehydrofalcarinone and dehydrofalcarinol. Meanwhile, eight compounds appeared in the chromatogram of F_4S_3 extract: the sesquiterpene lactone ivangustin, the polyphenol 1,4-caffeoylquinic acid, two flavones pectolinarigenin and apigenin, the flavonol kaempferol, and the flavone glycoside apigenin-7-glucuronide (Table 2, Figure 1).

Table 2. UPLC-ESI-MS/MS in negative ion ionization mode of F_1S_0 and F_4S_3 ethanol extracts.

Peak N°	Rt	Compound Name	[M-H]-	Formula	MS/MS	Rel.% *		References
						F_1S_0 **	F_4S_3 ***	
1	0.79	Quinic acid	191	$C_7H_{12}O_6$	MS^2[191]: 173	6.63	4.74	[23]
2	0.88	1-Caffeoylquinic acid	353	$C_{16}H_{18}O_9$	MS^2[353]: 191, 173, 109	1.53	0.00	[4]
3	5.12	3-Caffeoylquinic acid	353	$C_{16}H_{18}O_9$	MS^2 [353]: 191, 179, 135, 173	9.85	10.07	[24]
4	5.77	5-Caffeoylquinic acid	353	$C_{16}H_{18}O_9$	MS^2[353]: 191, 179	0.26	0.48	[4]
5	6.76	Ivangustin	247	$C_{15}H_{20}O_3$	MS^2[247]: 231	0.00	0.35	[25,26]
6	6.97	Chlorogenic acid methyl ester	367	$C_{17}H_{20}O_9$	MS^2[367]: 191, 17, 135	0.00	0.24	[4]
7	7.80	Quercetin	301	$C_{15}H_{10}O_7$	MS^2[301]: 273, 179, 151	1.60	1.60	[24]
8	8.26	Eriodictyol 7-glucuronide	463	$C_{21}H_{20}O_{12}$	MS^2[463]: 287, 107	6.30	8.85	[4]
9	8.83	Catechin	289	$C_{15}H_{14}O_6$	MS^2[289]: 166, 124, 115, 76	2.16	4.00	[24]
10	9.12	3,4-Dicaffeoylquinic acid	515	$C_{25}H_{24}O_{12}$	MS^2[515]: 353, 173, 179, 191, 135	19.29	23.37	[4]
11	9.82	Hesperetin	301	$C_{16}H_{14}O_6$	MS^2[609]: 301, 286, 213	2.27	1.98	[4]
12	10.61	3,5-Dicaffeoylquinic acid	515	$C_{25}H_{24}O_{12}$	MS^2[515]: 353, 191,173, 127	10.90	12.06	[4]
13	10.79	Eriodictyol	287	$C_{15}H_{12}O_6$	MS^2[287]: 151	6.13	4.75	[4]
14	11.39	1,4-Dicaffeoylquinic acid	515	$C_{25}H_{24}O_{12}$	MS^2[515]: 353, 203, 299, 255, 173, 179	0.00	0.52	[4]
15	11.72	4,5-Dicaffeoylquinic acid	515	$C_{25}H_{24}O_{12}$	MS^2 [515]: 471, 337, 163	2.92	4.48	[4]
16	12.24	Isorhamnetin	315	$C_{16}H_{12}O_7$	MS^2[315]: 164, 151	3.09	3.77	[23]
17	13.33	Jaceosidin	329	$C_{17}H_{14}O_7$	MS^2[330]: 315	3.01	3.47	[23]
18	14.29	Eupatilin	343	$C_{18}H_{16}O_7$	MS^2[343]: 330, 168	2.15	1.34	[23]
19	15.75	Gallic acid	169	$C_7H_6O_5$	MS^2[169]: 125	0.47	0.21	[24]
20	19.72	Caftaric acid	311	$C_{13}H_{12}O_9$	MS^2[311]: 179, 149	0.25	0.15	[23]
21	20.09	Kaempferol	285	$C_{15}H_{10}O_6$	MS^2[285]: 239, 187, 143	0.00	1.51	[23,24]
22	20.36	Dehydrofalcarinone	239	$C_{17}H_{20}O$	MS^2[239]: 55, 41	0.26	0.00	[25]
23	20.47	Dehydrofalcarinol	241	$C_{17}H_{22}O$	MS^2[241]: 140, 139, 95	0.78	0.00	[25]
24	20.51	Caffeic acid	179	$C_9H_8O_4$	MS^2[179]: 163, 145, 135	0.85	0.60	[24]
25	21.04	Apigenin-7-glucuronide	446	$C_{21}H_{18}O_{11}$	MS^2[445]: 296, 175	0.00	0.32	[23]
26	21.45	Ferulic acid	193	$C_{10}H_{10}O_4$	MS^2[193]: 173, 133	0.59	0.00	[4]
27	22.20	Apigenin	269	$C_{15}H_{10}O_5$	MS^2[269]: 151, 107	0.00	0.30	[23]
28	22.39	14-Hydroxydexoxyivangustin	279	$C_{15}H_{20}O_5$	MS^2[279]: 162, 262, 246, 167	0.52	0.74	[20,21]
29	22.63	Luteolin	285	$C_{15}H_{10}O_6$	MS^2[285]: 151, 133	0.96	0.82	[23]
30	23.00	Pectolinarigenin	313	$C_{17}H_{14}O_6$	MS^2[313]: 299, 271	0.00	1.27	[23]
31	23.53	11-Hydroxy-4,5-secoeudesmane-4,5-dione	253	$C_{15}H_{26}O_3$	MS^2[253]: 196, 170, 52, 111, 82	2.54	1.63	[25,26]
32	24.02	Naringenin	271	$C_{15}H_{12}O_5$	MS^2[271]: 151, 119, 107, 93	0.39	1.80	[23]
		Total% of identified constituents				85.92	97.56	

* For quantitation of relative percentages of individual components, peak areas were measured (single analysis). ** F_1S_0: Control plants normally irrigated (100% FC) without spraying of SA, *** F_4S_3: plants irrigated with 25% FC and sprayed using 3 mM SA.

Peak (10) is identified as 3,4-Dicaffeoylquinic acid, the major polyphenol detected in *E. africanus* L. ethanol extracts. The applied drought stress (25%FC) and sprayed SA (3 mM) caused a 23% increase in its concentration.

3.3. Effects of Drought Stress and SA Spraying on Essential Oil Composition

GC-MS analysis of the essential oils of *E. africanus* could detect 29 constituents, among which 27 were common in all samples, although with quantitative variability. Oxygenated mono- and sesquiterpenes dominated the overall composition of the oils, followed by sesquiterpene hydrocarbons. Meanwhile, monoterpene hydrocarbons appeared to be rare, and were only represented by α-pinene. The principal identified components were artemisia ketone, Juniper camphor, epi-γ-eudesmol, β-eudesmol, yomogi alcohol, α-cedrol, and aromadendrene epoxide. In most samples, artemisia ketone was predominant (Table 3).

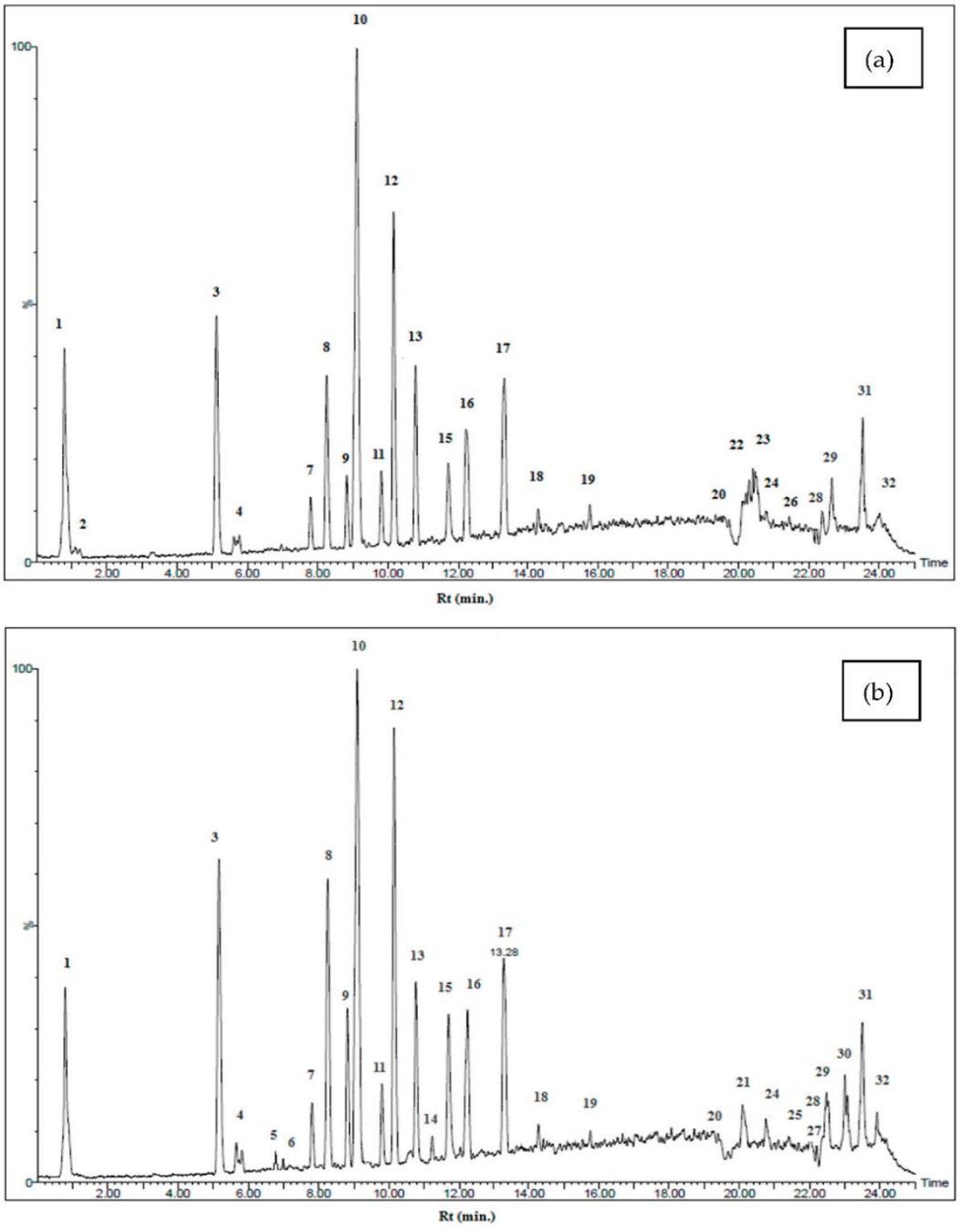

Figure 1. UPLC-ESI-MS/MS chromatograms in negative ion ionization mode of the ethanol extracts of *E. africanus*. (**a**) F_1S_0: Control plants normally irrigated (100% FC). (**b**) F_4S_3: plants irrigated with 25% FC and sprayed using 3 mM SA. Numbers in the chromatograms denote peak numbers on Table 2.

Table 3. Effects of drought stress and foliar spraying of salicylic acid (SA) on the chemical composition of the essential oils of the aerial parts of *E. africanus* treatments.

Rt (min.)	Compound	RI *	0 mM SA				1 mM SA				2 mM SA				3 mM SA			
			F_1	F_2	F_3	F_4	F_1	F_2	F_3	F_4	F_1	F_2	F_3	F_4	F_1	F_2	F_3	F_4
			Relative abundance%															
Monoterpene hydrocarbons																		
9.18	α-Pinene	931	0.55	0.54	0.45	0.44	0.56	0.54	0.46	0.45	0.56	0.50	0.41	0.40	0.61	0.75	0.55	0.46
Oxygenated monoterpenes																		
11.34	Yomogi alcohol	991	2.35	2.29	2.20	2.19	2.45	2.39	2.39	2.25	2.49	2.43	2.42	2.30	2.79	2.78	2.60	2.33
12.79	1,8-Cineol	1026	1.82	1.79	1.79	1.66	1.85	1.80	1.80	1.76	1.96	1.93	1.89	1.88	2.71	2.64	2.30	1.99
13.34	Artemisia ketone	1058	39.00	38.39	38.35	38.01	41.89	41.81	41.63	41.25	42.71	42.65	42.18	42.00	32.55	31.68	31.46	31.45
14.02	Artemisia alcohol	1075	0.69	0.68	0.68	0.66	0.77	0.78	0.77	0.76	0.83	0.79	0.76	0.76	0.98	0.96	0.89	0.80
16.55	Pinocarvone	1159	0.35	0.43	0.44	0.50	0.42	0.44	0.45	0.56	0.46	0.42	0.48	0.49	0.49	0.58	0.58	0.59
17.59	Myrtenol	1192	0.15	0.59	0.16	0.45	0.19	0.17	0.20	0.24	0.06	0.17	0.19	0.19	0.21	0.24	0.29	0.40
% *Oxygenated monoterpenes*			**44.36**	**44.17**	**43.62**	**43.47**	**47.57**	**47.39**	**47.24**	**46.82**	**48.51**	**48.39**	**47.92**	**47.62**	**39.73**	**38.88**	**38.12**	**37.56**
Sesquiterpene hydrocarbons																		
22.98	α-Copaene	1301	0.97	0.92	0.88	0.81	0.98	0.93	0.92	0.85	1.05	1.04	0.96	0.94	0.93	0.99	0.96	0.72
23.96	β-Caryophyllene	1308	1.76	1.70	1.65	1.65	1.78	1.79	1.79	1.72	1.82	1.83	1.80	1.74	2.21	2.21	2.10	1.82
25.63	α-Humulene	1450	0.65	0.59	0.59	0.58	0.76	0.69	0.60	0.60	0.76	0.70	0.63	0.62	0.93	0.92	0.90	0.71
25.91	Aromadendrene	1458	0.55	0.54	0.54	0.52	0.56	0.54	0.55	0.55	0.67	0.63	0.61	0.61	0.83	0.79	0.78	0.66
25.97	Bicyclogermacrene	1494	1.17	0.99	0.93	0.84	1.19	1.12	0.96	0.91	1.44	1.43	1.40	0.96	1.30	1.24	1.16	0.96
27.55	α-Selinene	1516	—	—	—	1.77	0.55	—	0.83	1.79	0.10	0.49	0.59	0.49	0.61	2.24	2.06	2.74
29.71	δ-Cadinene	1403	0.67	0.63	0.71	—	0.58	0.47	0.60	0.49	0.10	0.58	0.78	0.41	0.44	0.69	0.17	0.99
% *Sesquiterpene hydrocarbons*			**5.77**	**5.37**	**5.30**	**6.17**	**6.40**	**5.54**	**6.25**	**6.91**	**5.94**	**6.70**	**6.77**	**5.77**	**7.25**	**9.08**	**8.13**	**8.60**
Oxygenated sesquiterpenes																		
26.89	α-Cedrol	1486	2.57	2.55	2.54	2.36	2.59	2.56	2.54	2.45	2.66	2.58	2.53	2.18	2.84	2.69	2.66	2.62
27.39	β-Caryophyllene epoxide	1526	0.53	1.89	0.53	0.52	0.53	0.43	0.53	0.54	0.43	0.56	0.55	0.59	0.57	0.64	0.62	0.54
28.02	Spathulenol	1475	1.48	1.19	1.13	1.09	1.93	1.29	1.21	1.16	1.95	1.40	1.40	1.11	1.22	1.20	1.14	1.10
28.18	Caryophyllene oxide	1450	1.35	1.27	1.27	1.17	1.45	1.43	1.42	1.46	1.78	1.56	1.51	1.49	2.59	2.04	1.93	1.53
28.51	Gauiol	1595	0.59	0.87	0.94	0.94	0.49	0.44	0.84	0.91	0.49	0.51	0.73	0.54	0.32	0.95	0.65	1.71
28.71	Epi-γ-eudesmol	1619	6.19	5.46	4.92	4.74	6.24	5.41	4.99	4.81	6.33	5.47	5.12	4.88	7.24	6.36	6.86	5.68
29.28	γ-Eudesmol	1630	0.95	0.93	0.91	0.60	0.99	0.95	0.75	0.61	1.02	1.10	0.83	0.75	1.01	0.91	0.67	0.71
29.45	Aromadendrene epoxide	1639	2.45	2.44	2.39	2.34	2.49	2.45	2.40	2.33	2.67	2.51	2.50	2.41	2.54	2.49	2.43	2.16
29.37	β-Eudesmol	1644	3.37	3.33	2.83	2.74	3.42	3.35	2.99	2.81	3.67	3.47	3.12	2.89	3.22	3.48	2.99	2.54
29.85	α-Eudesmol	1650	0.95	0.88	0.84	0.79	0.98	0.91	0.88	0.78	1.17	0.96	0.91	0.91	1.37	1.24	1.24	1.02
29.89	α-Cadinol	1652	0.94	0.92	0.85	0.77	0.45	0.41	0.89	0.89	0.44	0.41	0.90	0.48	0.49	0.96	1.03	1.89
30.03	Junipor camphor	1661	17.88	17.02	16.86	16.40	18.89	17.50	17.14	16.56	19.48	17.61	17.02	16.99	18.66	17.00	16.77	15.56
30.21	β-Bisabolol	1668	0.72	0.74	0.63	0.69	0.65	0.43	0.70	0.63	0.24	0.45	0.80	0.51	0.44	0.78	0.79	1.22
32.70	α-Bisabolol	1681	0.65	0.90	0.79	0.69	0.40	0.76	0.63	0.53	0.28	0.25	0.55	1.03	0.85	0.78	0.43	1.16
34.64	Isobicyclogermacre-nal	1730	0.64	0.73	0.77	0.68	0.22	0.76	0.66	0.54	0.06	0.24	0.55	0.79	0.84	0.74	0.45	1.28
% *Oxygenated Sesquiterpenes*			**41.26**	**41.12**	**38.20**	**36.52**	**41.72**	**39.08**	**38.57**	**37.01**	**42.67**	**39.08**	**39.02**	**37.55**	**44.20**	**42.26**	**40.66**	**40.72**
% Total identified constituents			**91.94**	**91.20**	**87.57**	**86.60**	**96.25**	**92.55**	**92.52**	**91.19**	**97.68**	**94.67**	**94.12**	**91.34**	**91.79**	**90.97**	**87.46**	**87.34**

For quantitation of relative percentages of individual components, peak areas were measured. * RI: retention indices determined on RTX-5MS capillary column. F_1–F_4 are 100, 75, 50, 25% field capacity of irrigation water, respectively. Italics indicate the major identified classes while bold/italics indicates the sum of the percentages.

4. Discussion

In the current study, the effect of foliar spraying of SA on drought-stressed *E. africanus* L. was investigated, aiming to optimize growth conditions to produce bioactive metabolites. Drought, as well as several other stress factors, have been shown to elicit the production of certain metabolites [27]. In the present study, the accumulation of valuable antioxidant compounds (flavonoids and polyphenols) was obtained in drought-stressed plants sprayed with 3 mM SA (F_4S_3). This treatment showed an increase in the total flavonoid and polyphenols contents and the radical scavenging activity of the plant (Table 1). These findings agree with data reported on SA, which has been reported to play a key role in plant growth, development, and responses to abiotic stresses such as salinity and drought stress [23]. It also could trigger the phenylpropanoid pathway, which leads to increased biosynthesis of some secondary metabolites [28,29]. SA affects the transcriptional activities of the receptors to promote defense gene expression and thus promote plant immunity, which helps it face different physiological stresses [30]. Exposure to prolonged drought stress increases excitation energy and levels of ROS (reactive oxygen species) [31]; this noxious effect is reversed by exogenous SA application (in small concentrations) that can improve the antioxidant system in stressed plants [32]. Moreover, SA triggers the phenylpropanoid pathway, leading to increased biosynthesis of some secondary metabolites, especially terpenoids and flavonoids, with a defense-related function [33]. Several studies showed that hormone signaling in specific cells and cellular domains can facilitate improved plant responses to drought [34]. Earlier investigations on other plants like *Salvia miltiorrhiza*, cabbage, caraway, cucumber, calendula, and basil revealed that the application of SA in low concentration stimulated the production of phenolic compounds [35,36]. Additionally, considerable differences were observed in phenolic, flavonoid, and anthocyanin contents between control and water-stressed *Chrysanthemum morifoilum* [37]. SA induced the accumulation of mRNA of phenylalanine, leading to enhanced production of phenylalanine and, consequently, the accumulation of phenylpropanoids such as phenolic acids [15]. The radical scavenging capacity of the plant extracts was also significantly improved in F_4S3 treatment (Table 1). A positive correlation is usually found between the phenolic compounds and the antioxidant effect. This activity is mainly attributed to the phenolic components' redox properties and chemical structures that can neutralize the reactive oxygen species [38]. Previous studies also showed that the combination of SA with other elicitors as propolis in small concentrations may enhance the antioxidant capacity of some crops like tomato [39]. The major identified phenolic in the UPLC-ESI-MS/MS profiles, 3,4-dicaffeoylquinic acid (Table 2, Figure 1), was reported to have strong antioxidant activity [40,41]. Other Asteracea plants like *Achillea*, *Silybum marianum*, and globe artichoke showed increased antioxidant activity due to exposure to drought stress [10,42].

Drought stress and SA spraying were able to increase the essential oil yield (Table 1). SA improved the essential oil yield in drought-stressed plants like *Carum copticum*, *Lippia citriodora*, and *Rosmarinus officinalis* [43,44]. This activity may be due to the antioxidant capacity of essential oils, which could help the plant face stress [45]. Drought stress negatively affected the production of certain essential oil components (Table 3); however, SA application was found to reverse this effect, as observed for artemisia ketone, Juniper camphor, epi-γ-eudesmol, β-eudesmol, yomogi alcohol, α-cedrol, and aromadendrene epoxide.

5. Conclusions

In conclusion, foliar spraying of salicylic acid significantly improved the production of the targeted bioactive secondary metabolites (phenolic compounds and essential oil components) and radical scavenging capacity in the drought-stressed *E. africanus* L. plants as compared to control plants (non-stressed sprayed with SA-free distilled water). Concerning the influence on secondary metabolites of treated *E. africanus* L. samples, a remarkable increase was observed from initial values recorded in control plants in its total flavonoid, phenolic, and essential oil contents (53%, 35%, and 31% increase, respectively). Additionally, the major identified component in alcoholic extracts of the treatment (3,4-

dicaffeoylquinic acid) increased by about 23%, and artemisia ketone content of its volatile oil increased by about 10%. The radical scavenging activity increased by about 140% due to the applied conditions.

Collectively, a quarter of the amount of irrigation water was used to obtain higher percentages of the needed metabolites with the spraying of a commercially available cheap elicitor, SA. Therefore, optimizing the plant growth conditions suitable for each species is of economic importance for reducing cultivation cost and time and providing valuable raw material for implementation in the food and pharmaceutical industry. Future studies on the different genotypes of *E. africanus* are recommended to assess how the different genotypes would respond to drought and salicylic acid spraying.

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