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Research Article

Drought Stress and Recovery Modulate Physiological Traits and Metabolite Profiles in *Kaempferia galanga* L.

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ABSTRACT

Kaempferia galanga L. is a volatile compound-rich herb belonging to the ginger family and has been widely utilized in traditional medicine, pharmacology, and cosmetic applications. The aims of this study were to determine growth, physiological responses, and alterations in hexane extractable compounds, as well as antibacterial activity in plants subjected to water stress and recovery periods. The experiment was conducted using a randomized complete design with two experimental groups and four replications under well-irrigated water and drought conditions. The drought-affected plants, experiencing a 65.34% decrease in soil moisture, demonstrated only a 15.58% fall in leaf relative water content. Drought revealed nonsignificant effects on the plant biomass and pigments of the mature leaves. While physiological adaptation such as the elevation of chlorophyll *a+b* and carotenoids in the young leaves was observed, that may be related to photosynthesis during the recovery phase. The predominant groups of the phenylpropanoids, such as ethyl *p*-methoxycinnamate, and the aliphatics exhibited levels of 53.90% and 27.82%, respectively, in the control rhizomes. These contents remained at elevated levels during recovery, confirming that rhizomes had a unique characteristic that differed from leaves. Moreover, the hexane extracts derived from the drought-induced rhizomes showed a minimum inhibitory concentration of *Staphylococcus epidermidis* at 12,500 μg/mL, which was two times less than the control. The findings demonstrated that the native KG require an adequate water supply, and water deficit management influenced the proportions of plant metabolites. This study may establish a knowledge base for sustainable KG cultivation in future research.

Keywords: Rhizome propagation, Medicinal plant, Water stress, Hexane extract, Bioactivity

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Introduction

Kaempferia galanga (L.), also known as aromatic ginger or Pro-Hom, is an essential oil medicinal plant in the Kaempferia genus and the ginger family (Zingiberaceae). This genus comprises about 60 species found throughout Southeast Asia [1]. *K. galanga* (KG) is a small perennial herbaceous plant with an underground stem called a rhizome with pseudostems. KG is characterized by aromatic, fleshy, thick, pale-yellow rhizomes with several adventitious roots and single leaves that emerge from the rhizome. The leaves are relatively rounded in shape, typically two or more per plant, extending horizontally along the ground. The flowers that develop from the center of the leaves are white in color with a purple labellum. KG is generally grown and located in mixed deciduous forest habitats [2]. In terms of traditional medicinal uses, it has been included in Thailand's National List of Essential Medicines since 2013 due to its ethnobotanical importance. Additionally, the KG leaves are used as ornamental plants, auspicious symbols, and components in various religions [1, 2]. The leaves can be eaten as fresh vegetables and are also used in the production of value-added processed products in the form of seasoning powder. Various parts of plant, such as fresh or dried from rhizomes and leaves, as well as essential oils from rhizomes, are used for culinary and medicinal purposes, commonly associated with a variety of natural and synthetic chemical compounds [3].

Plants in the ginger family have a variety of many bioactive compounds that provide each plant with unique characteristics. A previous study on KG essential oils indicated extensive use in traditional and modern applications, for example, anti-allergic and anti-inflammatory properties [4], sleep-inducing effects [5], and potential for anti-cancer activity [6]. Similar studies revealed that the active compounds in KG such as phenylpropanoid esters, flavonoids, and benzoic acid derivatives may be associated with antibacterial, antioxidant, and anti-inflammatory activities [7, 8]. Moreover, ethyl *p*-methoxycinnamate of KG was related to analgesic activity, antimicrobial, neurodegenerative inhibitory, acetylcholinesterase, and antioxidant activities, as well as the inhibition of inflammatory cytokine synthesis [9]. And it was confirmed by genotoxicity analysis that the bioactive compound of KG exhibited a high level of safety at a concentration of 1 μg/mL, which can be applied in the cosmetics industry [10]. The phytochemical synthesis and accumulation of compounds in plants may depend on several factors, including plant genetics, geography, growth stage, and environment. Environmental influences can affect physiological and biochemical changes in plants, leading to differences between varieties, which are important for future applications [11]. Related studies demonstrated the impact of a water deficit on plant growth and the accumulation of important compounds. Reduction of the concentration of chlorophyll and total protein in lavender and lemon balm plants of the Lamiaceae family exposed to severe stress was observed [12]. Research in lavender plants grown under drought conditions revealed the enhancement of essential oil yields by means of the changes of compositions, such as the increasing of sesquiterpenes and the decreasing of monoterpenes [13]. In ginger (*Zingiber officinale*), the contents of phenolic compounds, 6-gingerol and 6-shogaol, were found increasing in plants grown in greenhouse conditions, although the rhizome weight decreased [14].

Consumers are increasingly prioritizing their health and seeking alternative healthcare products derived from natural sources, such as medicines, dietary supplements, and cosmetics, which is raising interest in herbs and spices. These plants are frequently harvested in their natural habitat and distributed as rhizomes for the pharmaceutical and medical sectors, for the production of essential oils, or for propagation. Studies indicated

that KG and *K. parviflora* are becoming rare and endangered in Bangladesh and India [8, 15]. Due to the increasing demand for these aromatic herbs in the family Zingiberaceae. The concerns have arisen that yields may be uncertain and insufficient to meet future growth. Furthermore, the knowledge background of metabolic evaluation of native aromatic ginger propagated off-season was limited. The aims of this research were to study the growth and the accumulation of leaf pigment contents and hexane-extractable compounds in leaves and rhizomes, as well as their antibacterial efficacy against the skin bacterium *Staphylococcus epidermidis* in KG cultivated under water-stress conditions and re-watering. The findings provided crucial data for future applications and are expected to support this species' potential usage as a sustainable source of natural antibacterial agents while improving our understanding of its phytochemical profile.

Materials and Methods

Plant and growth conditions:

The propagation units, or rhizomes, of *Kaempferia galanga* L. received from Phetchabun, Northern Thailand. Rhizomes were prepared, immersed in 1% (w/v) Captane50 for 30 minutes (min), washed with water three times, and air-dried. Prior to planting, rhizomes underwent cold induction by being preserved at 4 °C for one week. Four rhizomes (per planting bags) were propagated in 8x12-inch bags with a substrate of soil, manure, and rice husk charcoal in a 2:1:1 ratio. The plants were maintained and watered every two days in an open greenhouse with a photoperiod of 10-12 hours (h) at a day/night temperature of 35 °C/25 °C. A randomized complete design featuring two treatments and four replications was performed, comprising an irrigated control and a drought treatment. Sixteen weeks after planting, a 30-day drought stress was applied, resulting in a severe water deficit as adapted from Kulak et al. [16] and Ahsan et al. [17], followed by a four-week recovery period. Samples were collected for analysis at two distinct periods including: post-drought and post-recovery conditions. On the 30th day of drought of 20-week-old plants, all fresh leaf samples were collected to determine leaf relative water content, leaf weight, leaf pigment contents, and hexane-extractable compounds. The underground rhizomes were continuously cultivated under 30 days recovery condition (D/R). The experiment was considered complete when the plants attained 24 weeks of post-planting age (24wk).

Leaf relative water content (RWC) and soil moisture (SM):

For each replication, three 2.5-centimeter leaf discs were extracted from three fully expanded leaves per plant to assess RWC. Following the measurement of fresh weight (FW), discs were submerged in distilled water in a petri dish for 12 h in darkness. Turgid weights (TW) were obtained after removal of excess water from their surfaces. Dry weights (DW) were recorded following oven-drying at 60 °C for 72 h. The RWC was calculated using the following equation: RWC (%) = (FW-DW)/(TW-DW)× 100 [16]. Soil sampling was performed for soil moisture (SM) analysis. Following the measurement of FW, soil was transferred to a petri dish, and DW was recorded after being oven-dried at 105 °C until a stable weight about 72 h was attained. SM was calculated using the equation: SM (%) = (FW-DW)/DW×100.

Determination of growth and pigment contents:

At recovery, materials including leaves, roots, and rhizomes were collected, and pigment content and hexane extract evaluations were performed. Growth parameters including number of shoots, fresh weight and dry weights of leaves, microrhizomes, mature rhizomes, and roots were quantified. For chlorophyll *a* +*b* and total carotenoid (Cx+c) quantification, 0.5 g of leaves were homogenized with 10 mL of 95% ethanol in 15 mL centrifuge tubes, incubated in the dark at 4 ◦C overnight following modifications from Sumanta et al. [18]. The homogenized mixture underwent centrifugation (Hettich universal 320R) at 9,000 rpm for 15 min at 4 ◦C. Optical density and absorbance were measured at 649, 664, and 470 nm using a spectrophotometer (Thermo Scientific Evolution 201 UV-Visible instruments). Pigment concentrations were calculated and expressed as mg per g of FW based on volume of extract (V) and tissue weight (wt).

Chl *a* = [13.36(OD664)−5.19(OD649)]×(V/1000×wt))

Chl *b* = [27.43(OD649)−8.12(OD664)]×(V/1000×wt))

Cx+c = [1000(OD470)−2.13(Chl *a*)−97.63(Chl *b*)]/209×(V/1000×wt))

Hexane extraction and Gas Chromatography-Mass Spectrometry (GC-MS) analysis:

Hexane extracts were obtained from the leaves and rhizomes of both control and drought-treated plants using solvent extraction. Briefly, 30 g of dried and powdered plant materials were extracted with 250 mL of 99% *n*-hexane (analytical reagent grade) at room temperature for 72 h. The solution was filtrated using Whatman No. 1 filter paper, followed by solvent evaporation via a rotary vacuum evaporator. The hexane extract was stored in a glass amber vial at 4 °C for subsequent examination, and the yield was determined using the following equation: Yield (%, w/w dry basis) = [weight of hexane extract (g)/weight of raw materials (g)] ×100

The chemical composition of the hexane extracts was analyzed using an Agilent GC-MS (Agilent 7890A GC-7000 Mass Triple Quad) as outlined by [19]. A DB-Wax fused silica capillary column (60 m× 0.25 mm i.d.× 0.25 μm film thickness) from J&W Scientific, Folsom, CA, was used for the injection. The column temperature was isothermally held at 30 °C for 5 min, subsequently programmed to increase at a rate of 5 °C/min to 240 °C, and then isothermally maintained at 240 °C for 20 min. The mass spectrometer operated in electron ionization mode, with the ion source temperature at 250 °C and the ionization energy at 70 eV. The scan mode was employed, with a scan range of 30 to 400 m/z. The data analysis was conducted using MassHunter software. Identification of chemical compounds was conducted by comparing mass spectra with the NIST mass spectral libraries (National Institute of Standards, 2011 edition). The content of the compounds was determined from the peak areas.

Determination of minimum inhibitory concentration (MIC) *and minimum bacterial concentration* (MBC) *values*:

The MIC of the hexane extracts against *Staphylococcus epidermidis* (DMST 15505) was determined using a twofold serial microdilution method with DMSO, in a concentration range of 50 to 0.0244 mg/mL. A bacterial suspension (10^8 cfu/mL) was diluted in Muller-Hinton Broth (MHB) at a 1:200 ratio, and 100 μL of this solution was combined with MHB and tested samples, with triplicate assays conducted. Resazurin was added as a colorimetric indicator, changing from blue to pink to indicate growth inhibition. MHB plus samples

served as the positive control, while MHB without samples used as the negative control. MIC values were the lowest concentrations in microplate wells showing residual blue after 24 h of incubation at 37 °C. The MBC was identified through subculturing, with no growth on sterile agar plates, marking the MBC value as the concentration demonstrating no visible growth [20].

Statistical analysis:

Statistical analyses, including the Independent-Samples T-Test and Duncan Multiple Range Test, were conducted using the IBM SPSS Statistics software program, version 22. Differences at the 0.05 threshold were considered significant.

Results and Discussion

Plant growth, leaf RWC, and photosynthetic pigment contents

This study investigated the growth and physiological responses of KG cultivated during the off-season over a duration of 24 weeks. Four months after planting, the plants experienced the 30th day of drought (D) (Figures 1A & 1B), after which all leaves were taken for data analysis. The rhizomes were subsequently retained in the soil and subjected to a recovery condition to promote the plant's acclimatization until the experiment's conclusion (D/R). Upon the completion of the water deficit phase, soil moisture (SM) and leaf relative water content (RWC) were determined. It was shown that the plants were cultivated under severe drought stress with 12.08% SM (about 28% field capacity) and normal irrigation with 34.86% SM (80% field capacity) [16, 17]. The stressed-treated plants, grown under a 65.34% decrease in SM, exhibited only a 15.58% fall in leaf RWC, both statistically significant in comparison to the well-irrigated control (Figure 1C). The drought-leaves demonstrated a markedly reduced fresh weight (FW) (186.16±7.54 g) compared to the control group (299.75±45.17 g); however, no significant change in dry weight (DW) was observed (Table 1; Figure 1A & 1B). Following recovery, both the above- and below-ground parts of the 24-week-old plant were harvested for further analysis, and the increasing trends in the biomass of leaves were observed. Leaf FW increased from 29.66±1.37 g to 38.77±7.47 g, while the DW rose from 1.52±0.09 g to 2.23±0.72 g in control and drought-treated plants, respectively. There were no significant differences in the number of shoots, fresh and dry biomass of leaves, or new young rhizomes (microrhizomes) between the control and recovery groups. However, the reduction in the FW of mature rhizomes and roots was significantly observed (Table 1; Figures 1D & 1E).

The extraction of photosynthetic pigments from fresh leaves using 95% ethanol, with concentration evaluated by spectrophotometric methods, was determined. The contents of chlorophyll *a*+*b* (Chl *a*+*b*) and total carotenoids (Cx+c) were slightly greater but non-significant in the well-irrigated control than in the drought-treated leaves (Figure 2A). During recovery of mature leaves, the comparable contents of the pigments of the stressed leaves and the control were observed (Figures 2B & 2C). However, an upward trend in pigment concentrations in the young leaves was noted; the levels of chl *a*+*b* in the control, compared to the recovered leaves, rose from 3.9869±0.6761 to 4.8911±0.8729 mg/gFW, and Cx+c increased from 0.9611±0.1128 to 1.0837±0.1743 mg/gFW, respectively (Figures 2B & 2C).

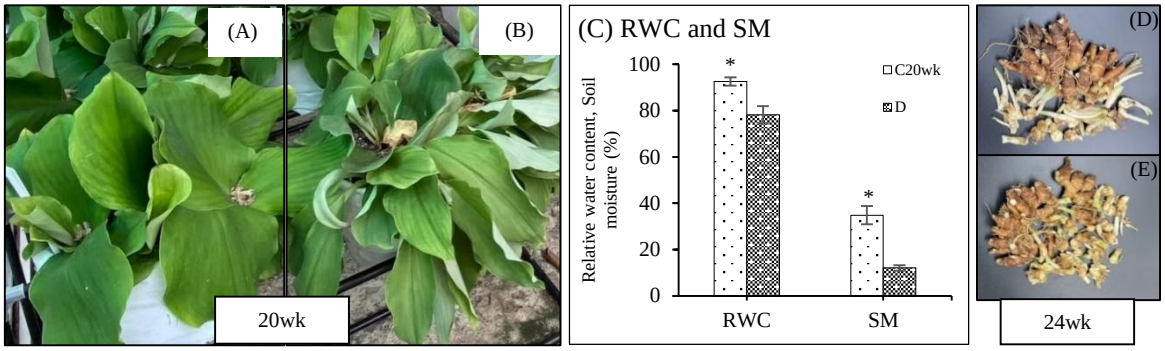

Figure 1 Comparison of *K. galanga* grown under (A) well-irrigated control and (B) drought, (C) leaf relative water content (RWC) and soil moisture (SM) (%) of 20-week-old (20wk) plants, and the rhizomes of (D) control and (E) recovery of 24-week-old (24wk) plants. n=4; D–30 days after drought treatment; R–re-watering; *Statistically significant at P-value < 0.05 at the 95% confidence range.

Table 1 The t-test evaluated *K. galanga* growth subjected to drought treatment and recovery.

Growth Parameter (unit per replicate)		**Treatment**		**P-value**
		C20wk	**D**	
Leaves (post drought stress)	FW	299.75±45.17	186.16±7.54	**0.048***
	DW	18.28±2.14	16.31±0.82	0.422ns
		C24wk	**Recovery; D/R**	
Shoot numbers		41.75±0.75	35.75±4.05	0.195ns
Leaves (post re-watering)	FW	29.66±1.37	38.77±7.47	0.275ns
	DW	1.52±0.09	2.23±0.72	0.103ns
Microrhizomes	FW	77.32±8.68	57.26±11.91	0.245ns
	DW	5.65±0.55	5.45±1.17	0.885ns
Mature rhizomes	FW	119.43±2.69	107.42±3.23	**0.046***
	DW	10.83±0.67	10.70±0.40	0.883ns
Roots	FW	12.97±1.59	6.88±1.19	**0.022***
	DW	1.20±0.20	0.89±0.14	0.261ns

*Statistically significant at P-value < 0.05; n=4; ns, P-value > 0.05, indicating an absence of significant difference at the 95% confidence range; C24wk–24-week-old control plant; D –30 days after drought treatment; R–re-watering; FW–Fresh weight; DW–dry weight.

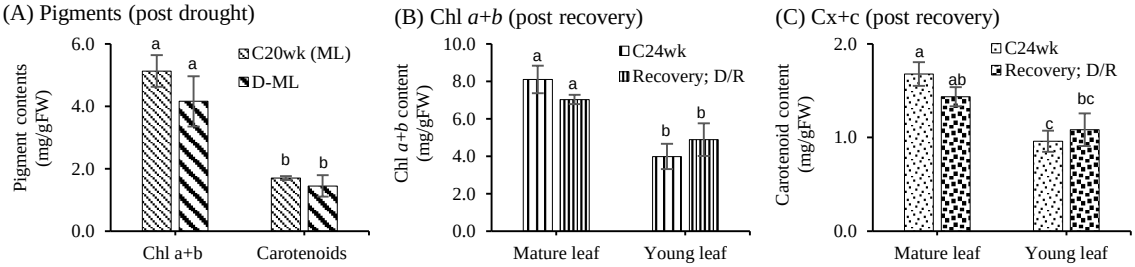

Figure 2 Photosynthetic pigment contents of *K. galanga* subjected to (A) drought and (B and C) recovery. n=4; C20wk, C24wk–20, 24-week-old control plant; D–30 days after drought treatment; R–re-watering; ML–Mature leaf. *Statistically significant DMRT ($p < 0.05$) are indicated by different lowercase letters within each graph.

There were no differences between recovery and the control plants in some growth parameters, such as the dry mass of rhizomes, leaves, roots, and shoot numbers, as well as leaf pigment contents. It indicated that KG exhibited effective physiological adaptation under this experimental condition of 30-day severe water deficit (28% field capacity) followed by recovery. Results showed comparable contents of total chlorophyll and carotenoids in both the control and stressed leaves, whereas the enhancement of pigment accumulation was observed in the young leaves of stressed plants. The leaf RWC revealed a substantial degree of water shortages; even with limited soil moisture, the plants maintained a significant water content in their leaves, exceeding 75%. It suggested that leaves may be essential for generating organic compounds through photosynthesis and distributing those compounds to support other plant structures in response to environmental stress. It was clearly shown that the KG plant experienced the actual drought stress on the plants without causing plant death. A distinctive morphological structure with thick leaves and a fleshy rhizome might serve to store water and various other compounds, enabling it to tolerate arid conditions [1, 2]. Typically, KG indigenous to mixed deciduous forest environments are known to endure dry conditions in their natural habitat [2]. Study on drought stress responses of C3 (kale) and C4 (barnyard grass), concluded that both C3 and C4 plants experienced a significant reduction in leaf chl *a+b* content, rubisco (a crucial CO_2-fixing enzyme) activity, and photosynthetic rate [21]. These responses and parameters related to oxidative stress were more pronounced in kale, classified as a C3 plant, similar to the KG plant. A similar study, Indian ginseng of the Solanaceae family (*Withania somnifera*) grown under drought stress, exhibited the reduction of photosynthetic parameters such as stomatal conductance, chlorophyll content and leaf RWC. However, chl *a*, chl *b*, and chl *a+b* pigments, and genes associated with detoxification and osmoregulation were recovered after re-watering [22]. The findings showed that some adaptations to tolerate drought, therefore maintaining their internal water balance, were enhanced. The mature rhizomes may further function as water and nutrient storage that could be utilized by other parts of the plant, such as young rhizomes and shoot-bearing leaves, to ensure the plant's survival.

Chemical composition of K. galanga hexane extracts

The hexane extracts obtained from dried KG samples, including leaves and rhizomes, exhibited distinct physical characteristics. The leaf extracts appeared as clear brownish-yellow liquids, whereas the rhizome extracts showed a darker brownish-yellow coloration with observable crystalline structures. Both extracts emitted a characteristic camphoraceous aroma. The extraction yields (%) varied depending on plant part, developmental

stage, and cultivation conditions. Higher yields were obtained from rhizomes compared to leaves, and from mature leaves compared to young leaves. In addition, plants grown under well-irrigated (control) conditions exhibited greater extraction yields than those subjected to drought stress (Table 2).

Table 2 The extraction yields (%) on a dry weight basis of *K. galanga* subjected to drought and recovery.

Sample	**Hexane extract yield (%)**			**color**
	C20wk	**D**		
Leaf	mature	mature	young	
	4.92	4.69	2.53	Clear brownish yellow
	C24wk	**Recovery; D/R**		
Mature leaf	5.70	4.58		Clear brownish yellow
Mature rhizome	14.89	13.28		Dark brownish yellow

Note: The extracts were obtained from samples combined from all four replicates of each treatment. C20wk, C24wk–20-week-old and 24-week-old control plants; D–30 days after drought treatment; R–re-watering.

The extraction from rhizomes of the control plant (C24wk) yielded 14.89%, greatly above the 13.28% yield obtained from drought-treated rhizomes on a dry weight basis. GC-MS analysis of the plant extracts was identified by about fifty potential chemicals. The GC-MS chromatograms illustrated the predominant chemical constituents from the dried rhizomes (Figure 3), and their relative abundance (%) in the hexane extracts was shown in Table 3.

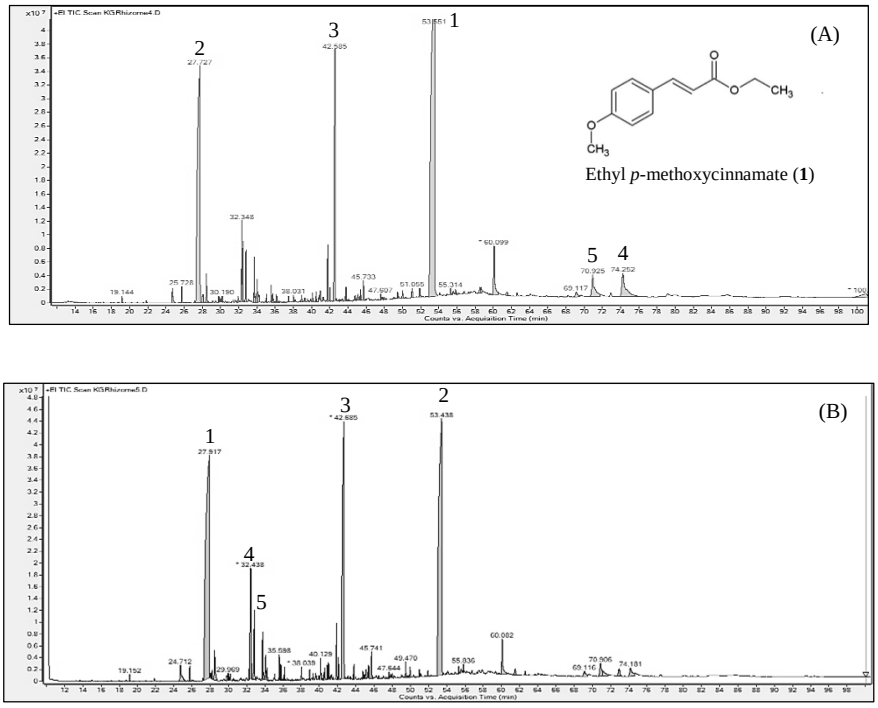

Figure 3 GC–MS chromatogram of hexane extract obtained from *K. galanga* rhizomes of 24-week-old plants at the end of the experiment of (A) control showing structure of the ethyl *p*-methoxycinnamate, and (B) recovery. The numbers 1-5 represent the five predominant chemical constituents with the highest relative abundances in descending order, as presented in Table 3; D–30 days post-drought treatment; R–re-watering.

Table 3 Relative abundance (%) of the predominant chemical compositions in the hexane extract of KG plants subjected to drought and recovery compared to the control.

RT (min)	Possible compound	Chemical groups	Relative peak area (%)*						
			Leaf					Rhizome	
			C20wk	D-YL	D-ML	C24wk	D/R-ML	C24wk	D/R-Rz
24.695	Tetradecane	4	ND	ND	ND	ND	ND	0.51	0.94
27.300	**Pentadecane**	4	1.76	1.63	1.89	0.59	0.69	**22.47**$^{(2)}$	**31.62**$^{(1)}$
28.455	α-Gurjunene	2	ND	ND	0.10	ND	ND	0.71	0.94
32.348	**Heptadecane**	4	0.28	0.12	0.24	ND	0.10	1.81	**2.93**$^{(4)}$
32.467	(-)-Borneol	2	ND	ND	ND	ND	ND	1.06	ND
32.483	endo-Borneol	2	0.53	0.12	0.47	ND	0.13	ND	1.16
32.785	**8-Heptadecene**	4	0.46	0.17	0.43	0.16	0.18	1.14	**1.72**$^{(5)}$
33.718	6,9-Heptadecadiene	4	ND	ND	ND	ND	ND	0.76	0.85
34.045	γ-Cadinene	2	0.23	0.11	0.23	0.09	0.10	0.53	0.81
37.511	Phytol, acetate	2	1.57	0.43	1.49	0.77	0.16	ND	ND
37.523	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	2	0.34	0.08	0.32	0.20	0.63	0.13	ND
39.545	Caryophyllene oxide	2	0.65	0.14	0.47	0.34	0.28	ND	ND
41.746	(*Z*)6,(*Z*)9-Pentadecadien-1-ol	4	2.11	0.96	1.92	0.90	1.16	1.13	1.25
42.481	**Cinnamic acid, ethyl ester**	1	0.74	1.58	0.75	0.33	0.42	**9.38**$^{(3)}$	**15.43**$^{(3)}$
45.741	Epi-13-Manool	2	ND	ND	ND	ND	ND	0.37	0.61
49.523	*cis*-Ethyl *p*-methoxycinnamate	1	6.25	1.21	5.93$^{(4)}$	3.54	4.17	0.13	0.30
52.454	Phytol	2	4.22	1.95	4.67	9.80	9.97$^{(5)}$	ND	ND
53.173	***trans*-Ethyl *p*-methoxycinnamate**	1	24.88$^{(1)}$	16.44$^{(3)}$	22.71$^{(1)}$	14.56$^{(2)}$	19.46$^{(1)}$	**44.39**$^{(1)}$	**28.91**$^{(2)}$
60.172	*n*-Hexadecanoic acid	3	14.52$^{(2)}$	16.79$^{(2)}$	14.48	13.82$^{(3)}$	11.66$^{(3)}$	2.20	1.62
63.001	2H-Pyran-2-one, tetrahydro-6-nonyl-	5	0.71	ND	ND	ND	ND	ND	ND
63.012	Palmitoleic acid	3	ND	ND	ND	ND	0.73	ND	ND
63.055	9-Hexadecenoic acid	3	ND	ND	ND	0.85	ND	ND	ND
68.432	Octacosane	4	0.77	ND	ND	ND	0.38	ND	ND
68.492	Tetracosane	4	ND	ND	1.29	ND	ND	ND	ND
69.171	Octadecanoic acid	3	0.89	1.75	0.99	1.27	1.19	0.42	0.42

Table 3 Relative abundance (%) of the predominant chemical compositions in the hexane extract of KG plants subjected to drought and recovery compared to the control. *(cont.)*

RT (min)	Possible compound	Chemical groups	Relative peak area (%)*						
			Leaf					Rhizome	
			C20wk	D-YL	D-ML	C24wk	D/R-ML	C24wk	D/R-Rz
71.189	9-Octadecenoic acid, (*E*)-	3	ND	15.63$^{(4)}$	ND	ND	ND	ND	0.50
72.439	**Oleic Acid**	3	10.42$^{(4)}$	0.80	9.98$^{(3)}$	9.92$^{(5)}$	9.64	**2.32$^{(5)}$**	0.90
74.354	**9,12-Octadecadienoic acid (*Z*,*Z*)-**	3	14.25$^{(3)}$	21.68$^{(1)}$	13.08$^{(2)}$	17.91$^{(1)}$	16.47$^{(2)}$	**3.38$^{(4)}$**	0.82
79.307	9,12,15-Octadecatrienoic acid, (*Z*,*Z*,*Z*)-	3	6.26$^{(5)}$	9.89$^{(5)}$	4.83$^{(5)}$	12.67$^{(4)}$	10.59$^{(4)}$	ND	ND
91.130	Vitamin E	5	ND	1.08	ND	2.74	0.77	ND	ND
91.865	1-Heptatriacotanol	4	ND	1.24	2.40	0.70	1.75	ND	ND
99.962	Phytol, acetate	2	ND	0.35	1.82	1.35	2.98	0.99	ND
102.067	Stigmasterol	2	ND	0.71	ND	0.80	ND	ND	ND
	sum		91.84	94.86	90.49	93.31	93.61	93.83	91.73
	Other compounds		8.16	5.14	9.51	6.69	6.39	6.17	8.27
	Total		100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total EPMC (*cis*- & *trans*-)			**31.13**	**17.65**	**28.64**	**18.10**	**23.63**	**44.52**	**29.21**
The total possible compounds			50	50	50	50	53	50	53

Note: *Peak numbers (1-5 in parentheses) in rhizome column correspond to those specified in Figure 3A-3B. 5 chemical groups are shown: 1-phenylpropanoids, 2-terpenoids, 3- fatty acids and lipids, 4-aliphatics, 5-others. RT−retention time; C20wk, C24wk−20-week-old and 24-week-old control plants; EPMC−Ethyl *p*-methoxycinnamate; D−30 days post drought; R−re-watering; YL−young leaf; ML−mature leaf; ND−not detected.

The four levels of a semi-quantitative analysis: very high [VH], high [H], medium [M], and low [L], based on a comparative study of the relative peak areas of the compounds from different samples was obtained (Table 4). The compounds were further classified into five major chemical groups based on their biosynthetic origins and chemical structures including phenylpropanoids, terpenoids, fatty acids/lipids, aliphatics and others (Table 5). Results showed that the hexane extracts contained both primary metabolites or primary-associated physiological adaptation such as phytol and stigmasterol, a major component of chlorophyll and membrane lipid bilayer, respectively, and vitamin E (tocopherol) function as an antioxidant protecting chloroplast from oxidative stress. And secondary metabolites or specialized metabolites such as monoterpenoids, sesquiterpenoids, diterpenoids, phenylpropanoids/aromatic esters, aliphatic hydrocarbons, long-chain alcohols, and lactones were involved in plant defense and stress adaptation [22-24].

Table 4 The cumulative relative peak area (%) and semi-quantitative assessment of five major chemical groups across seven samples of leaf and rhizome.

Chemical groups	Semi-quantitative levels*						
	Leaf					Rhizome	
	C20wk	D-YL	D-ML	C24wk	D/R-ML	C24wk	D/R-Rz
1 Phenylpropanoids	**31.87[H]**	19.23[M]	**29.39[H]**	18.43[M]	**24.05[H]**	**53.90[VH]**	**44.64[VH]**
2 Terpenoids	7.54[M]	3.89[L]	9.57[M]	13.35[M]	14.25[M]	3.79[L]	3.52[L]
3 Fatty acids/Lipids	**46.34[VH]**	**66.54[VH]**	**43.36[VH]**	**56.44[VH]**	**50.28[VH]**	8.32[M]	4.26[L]
4 Aliphatics	5.38[M]	4.12[L]	8.17[M]	2.35[L]	4.26[L]	**27.82[H]**	**39.31[H]**
5 Others	0.71[L]	1.08[L]	ND	2.74[L]	0.77[L]	ND	ND

Notes: *Values represent the cumulative relative peak area (%) of chemicals classified into primary groups, as detailed in Table 3, with semi-quantitative levels indicated in brackets: very high [VH] ≥40%, high [H] = 20 to <40%, moderate [M] = 5 to <20%, low [L] <5%. C20wk, C24wk–20-week-old and 24-week-old control plants; D–30 days post drought; R–re-watering; YL–young leaf; ML–mature leaf; ND–not detected.

Table 5 The major chemical groups classified into primary, secondary and specialized metabolites.

Chemical groups	Sub- groups	Metabolite types	Examples of compounds
1 Phenylpropanoids	Cinnamate derivatives	Secondary	Cinnamic acid, ethyl ester Ethyl *p*-methoxycinnamate
2 Terpenoids	Monoterpenoids	Secondary	(-)-Borneol; endo-Borneol
	Sesquiterpenoids	Secondary	α-Gurjunene; γ-Cadinene; Caryophyllene oxide
2 Terpenoids	Diterpenoids	Primary	Phytol
		Secondary/Specialized	Phytol, acetate; 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; Epi-13-Manool
	Phytosterols	Primary	Stigmasterol
3 Fatty acids/Lipids	Saturated	Primary	*n*-Hexadecanoic acid; Octadecanoic acid
	Monounsaturated	Primary	Palmitoleic acid; 9-Hexadecenoic acid; 9-Octadecenoic acid, (*E*)-; Oleic Acid
	Polyunsaturated	Primary	9,12-Octadecadienoic acid (*Z*,*Z*)-; 9,12,15-Octadecatrienoic acid, (*Z*,*Z*,*Z*)-
4 Aliphatics	Alkanes / Alkenes	Secondary/Specialized	Tetradecane; Pentadecane; Heptadecane; 8-Heptadecene; 6,9-Heptadecadiene; Octacosane; Tetracosane
	Long-chain Alcohols	Secondary/Specialized	(*Z*)6,(*Z*)9-Pentadecadien-1-ol; 1-Heptatriacotanol
5 Others	Tocopherols	Primary	Vitamin E
	Lactones	Secondary	2H-Pyran-2-one, tetrahydro-6-nonyl-

The modification of hexane extractables in plants under water stress had both positive and negative effects. Increased levels of phenylpropanoids and aliphatics in drought-stressed rhizomes enhanced the chemical defenses and antibacterial activities of the plant, suggesting potential advantages for bioactive functions. However, this alteration caused lower extraction yields, reduced fatty acids and terpenoids contents, and decreased the fresh weight of the rhizomes, indicating that these conditions could have affected growth and biomass. Consequently, while water stress enhanced certain compounds, it involved compromises that influenced plant productivity and general condition, factors that warranted consideration in cultivation methods.

The hexane extracts showed that ethyl *p*-methoxycinnamate (EPMC) was the primary marker compound, displaying the highest concentrations in both leaves and rhizomes, with a significant majority in the rhizome, thus rendering it suitable as a chemical marker in the KG plant. The predominant group of phenylpropanoids and aliphatics exhibited very high levels of 53.90% [VH] and high levels of 27.82% [H], respectively, in the control rhizomes (C24wk) (Table 4). This value remained at elevated levels in rhizomes during recovery (D/R-Rz), confirming that KG rhizomes have a unique characteristic that differs from those of the leaves. Hexane was used for the extraction of primary and secondary metabolites, serving as a non-polar solvent. The hexane-extractable metabolites were subsequently evaluated using GC-MS according to a typical procedure. A report on Zingiberaceae; *Curcuma caesia* Roxb. and *K. parviflora*, indicated that hexane extraction effectively isolated lipid-soluble chemicals, such as terpenoids, alkanes, steroids, and specific flavonoids [25]. However, its use often requires complementary extraction with more polar solvents to achieve a comprehensive metabolite profile of a plant sample such as phenolics, flavonoids, glycosides, amino acids, sugars and organic acids [6, 25]. In addition, research on the major compound, EPMC, in *K. galanga* dried rhizome showed that the hydrodistillation process, GC-FID, and GC-MS analysis exhibited 52.54% EPMC. While, the organic solvent extraction following the HPLC method, 1.85% EPMC was obtained by the nonpolar hexane extraction, which was less effective than other solvents, such as moderately polar acetone (2.24%) or low polar chloroform (1.99%) [6].

In KG leaves, fatty acids and lipids were the predominant metabolites, exceeding about 43-67% [VH] in all samples (Table 4), representing the primary indicator between leaves and rhizomes. The mature leaves impacted by the drought (D-ML) had values comparable to the 20wk-control leaves. The most significant alterations were observed in the drought young leaves (D-YL) with a fatty acid content of 66.54% [VH]; EPMC decreased, and oleic acid was markedly low; however, the values of trans-octadecenoic acid, vitamin E, and stigmasterol were enhanced. It revealed that young leaves reacted to drought conditions. The changes in primary-related metabolites indicated an effort to preserve membrane integrity, and vitamin E could function similar to tocopherols in preventing lipid peroxidation within chloroplasts [23]. The adaptation of mature leaves (D/R-ML) was found during the recovery phase, which had elevated levels of phytol and diterpenoids, along with a restoration of total EPMC (23.63%, Table 3).

The results of the principal compounds of KG plants provided some interest in antibacterial activities. KG essential oil has been studied for its utilization in traditional herbal preparations in various sectors such as herbal compress balls, hair care, cosmetics, and the production of food and beverages [6, 10, 26]. Here, *Staphylococcus epidermidis* (DMST 15505), a common skin bacterium, was examined with KG extract to study its preliminary potential against acne-causing bacteria. The minimum concentration of the examined chemical required to inhibit and destroy *S. epidermidis* through color transition of resazurin, with blue indicating

inhibition and pink representing normal bacterial growth, was investigated [20]. A blue color at a minimum inhibitory concentration (MIC) of 12,500 μg/mL was observed from the recovered rhizomes, which was two-times less than the control (Table 6). Further analysis showed that the equivalent minimal bactericidal concentration (MBC) of 50,000 μg/mL was observed from both the control and recovered rhizomes, with MBC/MIC ratios of 2.00 and 4.00, respectively. In contrast, the positive control of erythromycin, a naturally derived antibiotic, showed extremely low MIC of 6.10 μg/mL and MBC of 12.20 μg/mL values. The cinnamic acid derivatives (4-isopropylbenzyl cinnamide) exhibited effective antimicrobial activity with an MIC value of 128 μg/mL against both *S. aureus* (ATCC-35903) and *S. epidermidis* (ATCC-12228) and were classified as bactericidal types [27]. Previous studies on the antibacterial properties of *K. galanga*, employing the agar diffusion technique, revealed that *Staphylococcus aureus*, a gram-positive bacterium like *S. epidermidis*, is commonly utilized as a model to evaluate the efficacy of secondary plant metabolites. The application of ethyl *p*-methoxycinnamate rich KG hexane extract (66.39%) at different concentrations ranging from 50 to 500 μg/mL showed that a concentration of 500 μg/mL produced the largest inhibitory zone [10]. Similarly, the MIC values for essential oil derived from the KG rhizome against *S. aureus* at concentrations of 1.2% and 2.4% (about 12,000 - 24,000 μg/mL) were reported [28]. Furthermore, the MBC/MIC ratio of less than or equal to 4 was identified as an indicator correlated with antibiotic activity [10, 27]. Table 6 indicated a ratio of ≤4 across all samples, signifying that the KG hexane extract required high concentrations in order to demonstrate a mild antimicrobial effect against *S. epidermidis* in comparison to the antibiotic erythromycin.

Table 6 Minimal inhibitory concentration of hexane extract from rhizomes for inhibiting and destroying *Staphylococcus epidermidis* (DMST 15505)

Examined substances (μg/mL)	***S. epidermidis***		**MBC/MIC ratio**
	MIC	**MBC**	
1. C24wk	25,000.00	50,000.00	2.00
2. D/R rhizome	12,500.00	50,000.00	4.00
3. Erythromycin (positive control)	6.10	12.20	2.00

Notes: MIC–Minimal Inhibitory Concentration; MBC–Minimal Bactericidal Concentration; 3 measurements/ sample; C24wk–24-week-old control plant; D–30 days post drought; R–re-watering.

It is known that the underground part of KG, or the rhizome, is the major part for phytochemical synthesis and contains numerous cells that accumulate essential oil. The yields of hexane extract were related to these specific characteristics, which rhizomes produced a higher percentage than the leaves. Physiological responses under water stress showed that the biomass of leaves was consistent with the contents of photosynthetic pigments. The young leaves exhibited elevated concentrations of chlorophyll *a+b* and total carotenoids during recovery when compared to the control, signifying a restoration of photosynthesis alongside membrane repair status [22]. Previous studies have suggested that the plant's tolerant characteristics under abiotic stresses might be related to the ability of the antioxidant system, including the ability to reduce oxidative damage in cells [23, 24]. Sun et al. [29] studied that the volatile organic compound isoprene of hybrid *Populus* clones may be essential for maintaining chloroplast membrane integrity during plant recovery from dehydration

combined with heat stress. Water stress, especially recovery conditions, not only induced changes in growth and physiology but also led to variations in both the content and composition of hexane extract compared to the control. It suggested that different plant parts, leaf and rhizome, served distinct and significant functions; leaves were primarily essential for drought responses and photosynthetic recovery. Meanwhile, rhizomes served as the primary plant tissues for synthesis and storage of key phytochemicals involving defensive mechanisms [10, 30]. GC-MS results clarified an elevation in the accumulation of key compounds under severe drought conditions, but a decrease in the relative abundance of some compounds was detected. These specific compounds may contribute to plant adaptation and play a crucial role in plant defense mechanisms. This was revealed that KG hexane extract contained ethyl *p*-methoxycinnamate as its dominant compound, which was found in the highest concentration. Data showed that phenylpropanoid esters and other predominant chemicals upregulated in drought-stimulated rhizomes may also be involved in the inhibition of *S. epidermidis* (DMST 15505) growth. This study showed that the recovered rhizomes demonstrated more inhibiting effect of *S. epidermidis* than the control, although the total EPMC were lower (29.21% vs. 44.52%, Table 4). This inhibitory effect may be related to a change in the balance of chemical components rather than mainly the key compound. The recovered rhizomes exhibited a higher percentage of aliphatics (39.31% vs. 27.82%), which may be related to phenylpropanoid esters to disrupt bacterial membranes even at lower EPMC. This was consistent with previous findings indicating that combining cinnamic acid derivatives with lipophilic substances could improve membrane permeability. Additionally, the ratios of unknown substances that also contributed to bioactivity may change due to metabolic changes that occurred during the recovery phase. These results implied that the overall metabolite composition of the KG hexane extract had a greater impact on its antibacterial activity than any individual compound, underscoring the need for further fractionation and bioassay-guided investigations to identify the precise active constituents.

Various reports have shown that the compound was important for a wide range of applications, including the activities of anti-angiogenic, anti-inflammatory, anti-cancer [4, 6, 9], and antibacterial [7, 8]. For antibacterial purposes, inhibition of bacterial growth is due to several important natural substances found in plants or newly formed synthetic compounds. An analysis of antibacterial efficacy revealed that the certain synthetic cinnamides and cinnamates were directly involved in the inhibition of enzymes crucial in fatty acid synthesis, a fundamental component of bacteria membranes [27]. Furthermore, a report revealed that KG essential oil was the abundant source of ethyl *p*-methoxycinnamate, which increased membrane solubility for allowing active compounds to penetrate bacterial cell membranes and disrupt intracellular structures [10]. Stress resistance appeared to depend on the level of stress as well as species or varieties of the plants. Studies have also been undertaken regarding the effects of water scarcity on yield and essential oil composition in other members of the ginger family. The varying degrees of dehydration influenced the volatile oil content and main chemical accumulation in cassumunar ginger (*Z. montanum*). The severe 120-day water deficit resulted in elevated essential oil and sabinene levels, with no impact on antioxidant activity, whereas the greatest terpinen-4-ol content was detected at a moderate water deficit [30]. In addition, the cultivation of ginger (*Z. officinale*) under drought conditions affected rhizome fresh weight and essential oil yields and enhanced the levels of phenolic compounds, α-zingiberene, α-farnesene, and geranyl acetate, while decreasing levels of β-sesquiphellandrene and β-bisabolene [14]. The synergistic interactions of multiple compound groups, such as cinnamic acid derivatives

combined with antibiotics, may enhance the efficacy of essential oils in various aspects [27]. This study demonstrated that although stressed plants had lower phenylpropanoid levels, the balanced ratio of hydrocarbons and terpenoids may have a synergistic effect, allowing the main active compounds to work more efficiently than in the control plants. The present study examined the antibacterial efficacy of KG hexane extracts but did not assess their toxicity in mammalian or skin cell types. The extracts demonstrated potential for cosmetic and topical antibacterial purposes, cytotoxicity and skin irritation must be assessed prior to practical utilization. Thus, evaluating the safety profile and broader capabilities of KG extracts using cell viability and skin irritation assays was recommended in future studies.

Conclusions

This research studied the growth, physiological responses, alterations in hexane extractable compounds, and their bioactivity of *K. galanga* cultivated under water deficit condition and recovery. Water stress affected fresh weight and altered the relative content of major compounds in the hexane extract. There were no impacts on the plant biomass and pigment of the mature leaf under drought conditions. Physiological adaptation such as the elevation of photosynthetic pigments in the young leaves was observed during recovery. The modification of chemical profiles, especially ethyl *p*-methoxycinnamate, resulting in their internal ability, including antimicrobial activity. The hexane extract derived from recovered rhizomes showed greater *S. epidermidis* growth inhibition than the control. The results suggested that efficient plant propagation and adequate water supply should be considered for each plant species. This experiment may provide a framework for KG cultivation in future studies and sustainable applications.

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