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JASMONIC AND SALICYLIC ACIDS ENHANCED PHYTOCHEMICAL PRODUCTION AND BIOLOGICAL ACTIVITIES IN CELL SUSPENSION CULTURES OF SPINE GOURD (*MOMORDICA DIOICA* ROXB)

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In vitro cell suspension culture was established for the production of commercially valuable phytochemicals in *Momordica dioica*. The influence of elicitors in jasmonic acid (JA) and salicylic acid (SA) increased their effect on phytochemical production and biomass accumulation in *M. dioica*. The results indicate that compared with non-elicited cultures, JA- and SA-elicited cell suspension cultures had significantly enhanced phenolic, flavonoid, and carotenoid production, as well as antioxidant, antimicrobial, and antiproliferative activities. Furthermore, elicited cultures produced 22 phenolic compounds, such as flavonols, hydroxycinnamic acids, and hydroxybenzoic acids. Greater biomass production, phytochemical accumulation, and biological activity occurred in JA- than in SA-elicited cell cultures. This study is the first to successfully establish *M. dioica* cell suspension cultures for the production of phenolic compounds and carotenoids, as well as for biomass accumulation.

Keywords: Carotenoids – cell suspension culture – jasmonic acid – phenolic compounds – salicylic acid

INTRODUCTION

Spine gourd (*Momordica dioica* Roxb. ex. Willd) is a highly nutritious cucurbit vegetable which is also used in traditional medicines from tropical regions [25]. It contains a considerable amount of triterpenoids, steroids, alkaloids, glycosides, saponins, polyphenols, carotenoids, vitamins, and other health promoting phytochemicals [22]. Polyphenols and carotenoids are used extensively in drugs due to their health benefits, such as antioxidant capacity, anti-aging and anti-carcinogenic effects, as well as protection from cardiovascular diseases [3, 18]. Plant-cell suspension cultures containing undifferentiated cells offer an attractive alternative for the production of bioactive secondary metabolites under a controlled environment [12]. Recently, successful biosynthesis of pharmaceutical compounds in cell suspension cultures have been reported in several plants [3, 10, 20]. Plant growth regulators are the most important factors in cell growth, differentiation, and metabolite formation [20], but very little

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information is available about influence of growth regulators on phenol and carotenoid production in an *in vitro* plant culture. Elicitation is one of the most efficient methods for improving secondary metabolite production in cell and organ cultures [9]. Jasmonic (JA) and salicylic acids (SA) are potent elicitors and plant defense hormones that play significant roles in regulating plant defense responses against numerous biotic and abiotic stresses. Jasmonic acid and SA have previously been used as elicitors in cell suspension cultures to enhance secondary metabolite production [12].

The main objective of this study was to evaluate whether JA and SA elicitors could effectively increase biomass, secondary metabolite (phenolic compounds and carotenoids) accumulation, as well as antioxidant, antimicrobial, and antiproliferative activities in *M. dioica* cell suspension cultures.

MATERIALS AND METHODS

Establishment of callus and cell suspension culture

Leaf explants of *M. dioica* were sterilized following procedures from our previous report [25] and aseptically grown on MS [13] medium containing sucrose (30 g/L w/v) and TDZ- (0.1, 0.5 and 1.0 mg/L) supplemented agar (8 g/L w/v), either alone or in combination with 1.0 mg/L naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), or 2,4-dichlorophenoxyacetic acid (2,4-D). Callus cultures were incubated for three weeks in a growth chamber at 25 ± 1 °C and a 16-h photoperiod (30 µmol m^{-2} s^{-1}; from 40-W white fluorescent lamps). Cell suspension cultures were initiated with friable callus placed in 250 mL Erlenmeyer flasks containing MS liquid medium (supplemented with 1.0 mg/L NAA and 0.5 mg/L TDZ). Cultures were kept under continuous agitation at 110 rpm in an orbital shaker and incubated at under the same temperature and light conditions as above.

Influence of auxins, cytokinins, sucrose, and media sources on biomass accumulation

The effects of auxins, cytokinins, sucrose, and media on cell growth and biomass production in cell suspension culture were determined. The experimental culture was 500 mg fresh mass (FM) of cells were grown in liquid MS supplemented with 40 g/L sucrose containing auxins (naphthalene acetic acid [NAA], indole-acetic acid [IAA], and 2,4-dichlorophenoxyacetic acid [2,4-D]) at differing concentrations (0, 0.5, 1.0, and 2.0 mg/L), combined with cytokinins (0, 0.1, 0.5, and 1.0 mg/L 6-benzylaminopurine [BAP] and thidiazuron [TDZ]). The control was MS devoid of growth regulators. The effects of using various MS media (B5 [11], NN [15], and N6 [5]) and sucrose (10, 20, 30, 40, and 50 g/L) were assessed. Cultures were harvested in duplicate at 7, 14, 21, 28, and 35 d post-cultivation, and then analyzed for

biomass accumulation and kinetics. Cultures were continuously agitated at 110 rpm and incubated in the conditions described under "Establishment of callus and cell suspension culture." After four weeks, the FM and dry mass (DM) of harvested cells were assessed.

Effects of jasmonic acid (JA) and salicylic acid (SA) elicitation on biomass accumulation and phytochemical production

Various JA or SA elicitor concentrations (0, 25, 50, 100 and 150 µM) were aseptically added on day 21 of cell suspension culture to the MS medium containing 40 g/L sucrose, supplemented with 1.0 mg/L NAA and 0.5 mg/L TDZ. The cultures were continuously agitated (110 rpm in an orbital shaker) under the conditions described in "Establishment of callus and cell suspension culture." After 28 d of culture, the FM and DM of harvested cells were evaluated. Cell suspensions were separated from the medium through filtering, rinsed with sterile water, and blotted before FM measurement. The DM was recorded after cell suspensions were oven-dried at 58 °C for 2 d.

Extraction and estimation of individual phenolic compounds using ultra-high performance liquid chromatography (UHPLC)

Elicited (JA and SA) and non-elicited cell suspension powder (1 g DM) were extracted following our published protocol [23, 24]. The presence of 22 phenolic compounds in the cell suspensions cultures was ascertained using UHPLC (Accela, USA) with a reverse phase column (C_{18}, 2.1 × 100 mm, 2.6 mm). The solvent, standard, and gradient procedures were as previously described [23, 24]. Phenolic compounds were identified in accordance with previously reported methods [25].

Estimation of total phenolic and flavonoid content (TPC and TFC)

Total phenolic content was quantified spectrophotometrically with the Folin–Ciocalteu assay [25]. Total flavonoid content was determined with the aluminum chloride spectrophotometric method [25].

Extraction and estimation of carotenoid content

Carotenoid extraction was conducted following previously described methods [18]. Elicited and non-elicited suspension cell samples (1 g) were pulverized in cold acetone (25 mL). The mixture was agitated for 10 min, followed by filtration using Whatman No. 1 filter paper. The filtrate was transferred into a separation funnel and partitioned with petroleum ether (20 mL). Acetone was removed via washing with

distilled water (100 mL) and discarding the lower phase, then repeating the process twice more. Next, the petroleum ether layer was filtrated using Whatman No. 1 filter paper covered with 5 g of anhydrous sodium sulfate to remove residual water. Petroleum ether extracts were pooled and volume-adjusted to 25 mL with petroleum ether. Extracts were spectrophotometrically analyzed (300–600 nm) using a UV-vis spectrophotometer and absorbance was measured at 450 nm to determine the total carotenoid content, calculated with the following formula:

$$\text{Total carotenoids (}\mu\text{g }\beta\text{-carotene g/L)} = \frac{A \times V\,(25\text{ mL}) \times 10^4}{E1\%_{1\text{cm}} \times P(1\text{ g})}$$

where A = Absorbance at 450 nm, V = Total extract volume, P = Sample weight, and $E1\%_{1\text{cm}}$ = Extinction coefficient of β-carotene in petroleum ether = 2592.

Extract preparation

Elicited and non-elicited cell suspension powder (1 g DM) were subjected to extraction with 50 mL of methanol (95%) and kept at room temperature for 24 h with repeated shaking. Subsequently, the solution was passed through Whatman No. 1 filter paper and concentrated until dry. Dried methanolic extract was then dissolved in the minimum amount of methanol necessary and stored at 4 °C until needed for subsequent analyses on biological activities.

Antioxidant activities

Previously published protocols [23, 24, 25] were used to measure antioxidant activities via DPPH free-radical scavenging, reducing power, the phosphomolybdenum method, and the metal ion-chelating assay.

Antibacterial and antifungal activities

Staphylococcus aureus (KACC 10778), *Bacillus subtilis* (KACC 10111), *Pseudomonas aeruginosa* (KACC 11085), *Escherichia coli* (KACC 10495), *Candida albicans* (KACC 30062), *Aspergillus niger* (KACC 41687), and *Fusarium oxysporum* (KACC 40053) were used to test for antibacterial and antifungal activity. Tests were performed using the NCCLS disc diffusion method [23, 24, 25].

Antiproliferative activity

Two human cancer cell lines (colon HT-29 and estrogen-dependent breast MCF-7) were used for cytotoxicity screening of elicited and non-elicited cell culture extracts in *M. dioica.* Briefly, human cells were added to 96-well plates (5×10^3 cells well^{-1}) and treated for 48 h with *M. dioica* culture extracts (12.5, 25, 50, 100, and 200 µg/mL per elicited and non-elicited group). Cell viability was assayed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric method [8].

Experimental design and data analysis

All experiments were performed in triplicate and the data are presented as mean ± standard deviation (SD). One-way ANOVA analysis followed by a Duncan's test was used to determine significant differences ($P \leq 0.05$) in the statistical software package.

RESULTS AND DISCUSSION

Establishment of callus culture

The highest callus frequency (89.0%; with yellowish friable features) occurred after three weeks of culturing *M. dioica* leaf explants with 1.0 mg/L NAA and 0.5 mg/L TDZ. Used alone, 0.5 mg/L TDZ resulted in a maximum callus frequency of 59.0%, with green and friable features. However, calli were green or white and compact in response to TDZ combined with IAA or 2,4-D, both of which induced less callogenesis than the NAA plus TDZ combination. As a potent bioregulator of *in vitro* morphogenesis, TDZ mimics effects of both auxin and cytokinin on growth and differentiation in cultured explants [19]. Furthermore, TDZ was more efficient for callus formation when combined with NAA than with the other two auxins. This outcome was corroborated by a previous study in *Rhodiola crenulata* cell suspension cultures, showing friable callus induction by a TDA and NAA mixture [19].

Effects of growth regulators, sucrose, media, and growth kinetics on biomass accumulation in cell suspension culture

Different concentrations of three auxins (NAA, 2,4-D, and IAA) were tested for their effects on *M. dioica* cell suspension cultures. Among them, 1.0 mg/L NAA in MS led to more biomass accumulation than 2,4-D and IAA (Fig. 1A, B, C). When combined with 0.5 mg/L, 1.0 mg/L NAA induced maximum biomass accumulation (Fig. 1D). This combination also produced a high phenolic content in callus cultures of *Artemisia absinthium* [3] and *Lallemantia iberica* [16]. Next, we investigated the effects of sucrose (10–50 g/L in MS) on biomass accumulation (Fig. 1E). We found

that 40 g/L sucrose was suitable for biomass accumulation. Our results are corroborated by another study that also revealed higher biomass accumulation and secondary metabolite production after treatment with 40 g/L of sucrose [14, 20]. We then demonstrated that of the different media tested (MS, NN, B5, and N6), MS was superior and induced maximum biomass accumulation (Fig. 1F). Several previous studies have also found MS suitable for biomass accumulation and secondary metabolite production in cell suspension cultures [3, 20]. Tracking biomass (FM and DM) accu-

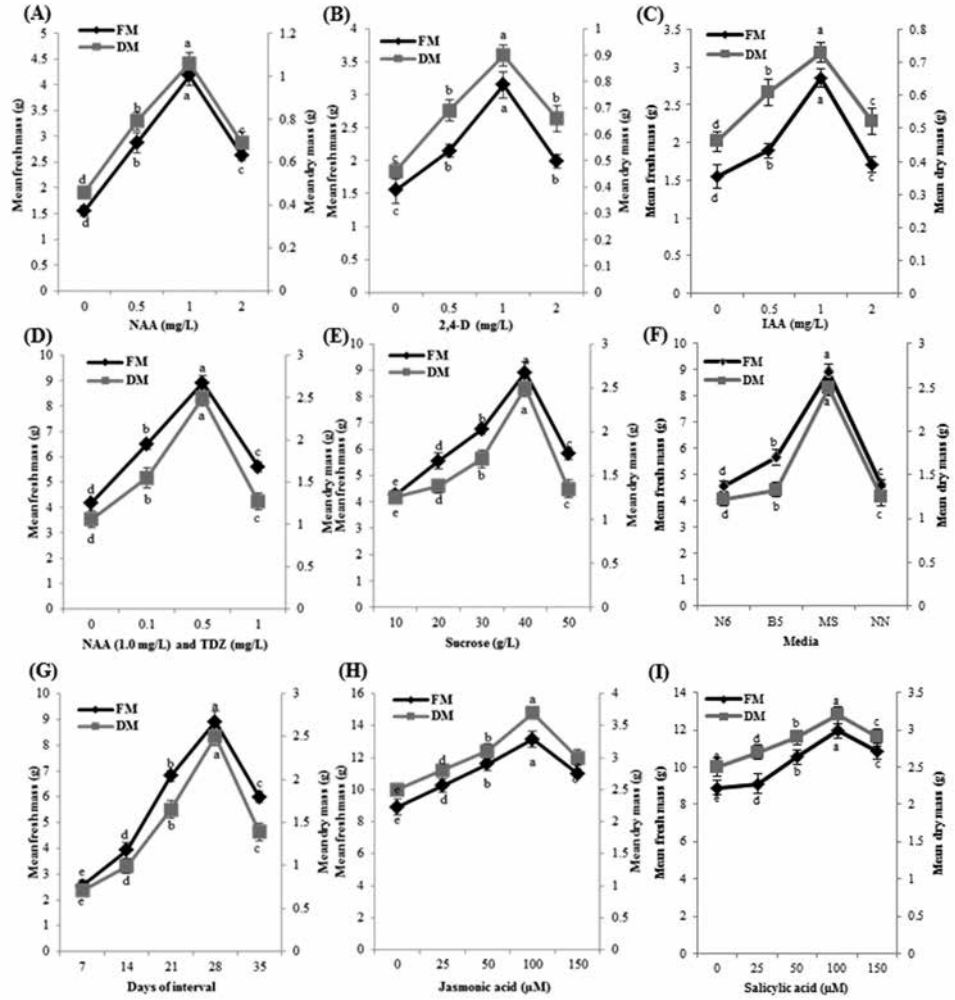

Fig. 1. Effects of plant growth regulators, sucrose, media, elicitors, and growth kinetics on biomass accumulation in *M. dioica* cell suspension cultures. **A**. naphthalene acetic acid (NAA); **B**. 2,4-dichlorophenoxyacetic acid (2,4-D); **C.** indole-3-acetic acid (IAA); **D**. NAA 1.0 mg/L with thidiazuron (TDZ); **E**. sucrose; **F**. media; **G**. growth kinetics; **H**. jasmonic acid (JA); **I**. salicylic acid (SA). Means ± SD of triplicates followed by the same letters are not significantly different according to Duncan's test at $P \leq 0.05$

mulation patterns across the course of the experiment showed that levels were at maximum on day 28 (Fig. 1G). In accordance with our study, 28-d-old cell suspension culture of *W. somnifera* [14] and *A. absinthium* [3] exhibited high metabolite production and biomass accumulation.

Influence of elicitors (JA and SA) on biomass accumulation, carotenoid production, and phenolic compound production in cell suspension culture

The effect of elicitors on biomass accumulation, as well as the production of phenolic compound and carotenoids, was tested in *M. dioica* cell suspension cultures. Elicitors significantly increased biomass (FM and DM) accumulation and phytochemical (phenols and carotenoids) production, suggesting a synergistic effect on cell proliferation and phytochemical biosynthesis (Figs 1H, I and 2A–C). Biomass accumulation in 100 μM JA- or SA-cultured cells was significantly higher than accumulation in non-elicited cell suspension cultures (Fig. 1H, I). Additionally, carotenoid, total phenolic, and flavonoid content was higher in 100 μM elicited cell cultures than in non-elicited ones (Fig. 2A–C). Our results were consistent with previous reports on JA and SA elicitation increasing total phenolic and flavonoid content in cell suspension cultures of *Panax ginseng* [2] and *Artemisia absinthium* [3, 4]. Another study also demonstrated that the highest pigment production (a sixfold increase in carotenoid yield from that of non-elicited cells) was achieved on cultures treated with methyl jasmonate (MeJA), supporting our results [18].

Qualitative and quantitative evaluations were performed with Ultra-HPLC on phenolic compounds from non-elicited cell suspension cultures (MS containing 40 g/L sucrose supplemented with 1.0 mg/L NAA and 0.5 mg/L TDZ) and elicited cell extracts (Table 1). Phenolic compounds were identified through retention-time comparisons, and calibration curves were then calculated from UV spectra of authentic standards and the quantitative data. Both elicited and non-elicited cell culture extracts contained flavonols, hydroxybenzoic acid, and hydroxycinnamic acid, but the former contained more of these compounds (Table 1). This action of JA and SA is consistent; they enhance phenolic acid composition in *Vitis vinifera* suspension culture [17], as well as increasing *p*-hydroxybenzoic and syringic acid content in *Schisandra chinensis* calli [21]. We also found that gentisic, gallic, *p*-hydroxybenzoic, syringic, caffeic, and ferulic acids were higher in JA- and SA-elicited cell cultures than in non-elicited cell cultures. Similar to our study, gallic acid content was higher in elicited cell suspension cultures of *Acer ginnala* [7] and *A. absinthium* [3], while caffeic acid content was higher in SA-elicited cell cultures of *Salvia miltiorrhiza* [6]. Furthermore, we showed that quercetin, rutin, myricetin, kaempferol, and naringenin content was higher in JA- and SA-elicited cell cultures than in non-elicited cell cultures. Veratric acid, hesperidin, and vanillin levels were also higher in elicited cell cultures than in non-elicited cell cultures (Table 1). Corroborating our results, MeJA and SA led to flavonoid content being higher by 2.1- and 1.5-fold, respectively, in *Hypericum*

Table 1
Ultra-high performance liquid chromatography analysis of phenolic compounds in jasmonic acid (JA)- or salicylic acid (SA)-elicited and non-elicited *M. dioica* cell suspension cultures

No.	Phenolic compounds	Concentration (µg/g dry mass)		
		Non-elicited	SA-elicited	JA-elicited
Hydroxybenzoic acid				
1	*p*-Hydroxybenzoic acid	121.12±1.0^{g}	135.25±1.5^{g}	149.15±1.0^{i}
2	Gallic acid	352.50±1.5^{d}	374.12±2.0^{d}	395.21±2.0^{e}
3	Protocatechuic acid	36.21±1.0^{k}	28.10±1.0^{j}	31.00±1.0^{n}
4	Syringic acid	51.50±1.0^{j}	74.25±1.0^{j}	98.52±1.5^{k}
5	Gentisic acid	428.71±2.0^{b}	445.65±2.5^{b}	498.15±2.0^{b}
	Total	990.04^{c}	1057.37^{b}	1172.03^{a}
Hydroxycinnamic acid				
6	Caffeic acid	424.15±2.5^{b}	425.00±2.0^{c}	434.25±2.0^{d}
7	*p*-Coumaric acid	84.42±1.5^{i}	71.23±1.0^{j}	79.50±1.0^{l}
8	Ferulic acid	115.12±1.5^{g}	137.15±1.5^{g}	158.20±1.5^{h}
9	Chlorogenic acid	18.00±1.0^{m}	21.15±1.0^{j}	29.31±1.0^{n}
10	*o*-Coumaric acid	22.10±1.0^{m}	25.10±1.0^{j}	25.00±1.0^{o}
11	*t*-Cinnamic acid	11.21±0.5^{n}	12.10±0.5^{k}	18.00±0.8^{p}
	Total	675.00^{c}	691.73^{b}	744.26^{a}
Flavonols				
12	Myricetin	244.50±2.0^{f}	292.30±2.0^{f}	315.21±1.5^{g}
13	Quercetin	440.15±1.0^{a}	471.12±2.0^{a}	495.50±2.0^{a}
14	Kaempferol	325.40±2.0^{e}	351.00±1.5^{e}	369.00±2.0^{f}
15	Catechin	380.12±1.0^{c}	344.15±3.0^{e}	372.45±2.5^{f}
16	Rutin	420.00±1.5^{b}	442.00±2.0^{b}	465.50±2.0^{c}
17	Naringenin	105.11±1.0^{h}	114.00±1.0^{h}	125.12±1.0^{j}
18	Biochanin A	19.22±0.5^{m}	21.15±1.0^{j}	25.00±1.0^{o}
	Total	1934.50^{c}	2035.72^{b}	2167.78^{a}
Others				
19	Vanillin	20.10±1.0^{m}	25.00±1.0^{j}	27.15±1.0^{o}
20	Veratric acid	110.15±1.0^{h}	95.00±1.0^{i}	100.11±1.0^{k}
21	Homogentisic acid	31.20±0.5^{l}	35.15±0.5^{i}	42.00±0.5^{m}
22	Hesperidin	41.14±1.0^{k}	45.00±1.0^{h}	44.00±0.7^{m}
	Total	202.59^{b}	200.15^{c}	213.26^{a}

Mean±SD within a row followed by the same letters are not significantly different according to Duncan's Test at $P \leq 0.05$.

perforatum cell suspension cultures than in non-elicited cells [26]. Together, these data suggest that elicitation with JA or SA is a promising alternative method for increasing phytochemical (phenols, flavonoids, and carotenoids) production and cell growth in *M. dioica* cell suspension cultures.

Effects of elicitors (JA and SA) on antioxidant activity in cell suspension culture

The antioxidant capacity of elicited and non-elicited cell cultures was evaluated using free radical scavenging, reducing potential, phosphomolybdenum assays, and metal chelating activity. Jasmonic acid- and SA-elicited cells increased antioxidant activity in *Panax ginseng* [2] and *A. absinthium* [3]. Similarly, higher antioxidant activity was exhibited in elicited cells than in non-elicited cells (Fig. 2D). Elicited cells also had more antioxidant potential than non-elicited cell cultures, as seen in the former's reducing capacity (Fig. 2E). Using the phosphomolybdenum method, we found that the antioxidant capacity of elicited cell culture extracts was higher than that of non-elicited cell culture extracts (Fig. 2F). Finally, elicited cells had a greater percentage of metal scavenging capacity than non-elicited cells (Fig. 2G). The higher phenolic, flavonoid, and carotenoid levels in the JA- and SA-cultured cells directly influenced their antioxidant potential.

Effects of elicitors (JA and SA) on antimicrobial activity in cell suspension culture

Elicited cell suspension cultures from various plants had increased antibacterial and antifungal activity [1]. Disc diffusion analysis indicated that elicited and non-elicited cell culture extracts both exhibited (Gram-positive and Gram-negative) antibacterial and antifungal activities. However, antibacterial and antifungal activities were higher in JA and SA cell culture extracts than in non-elicited cells (Fig. 2H). These results were compared to the antibacterial and antifungal effects, respectively, of chloramphenicol and thymol as positive controls, thereby demonstrating that suspension cell culture extracts of *M. dioica* could be used for treating bacterial and fungal diseases.

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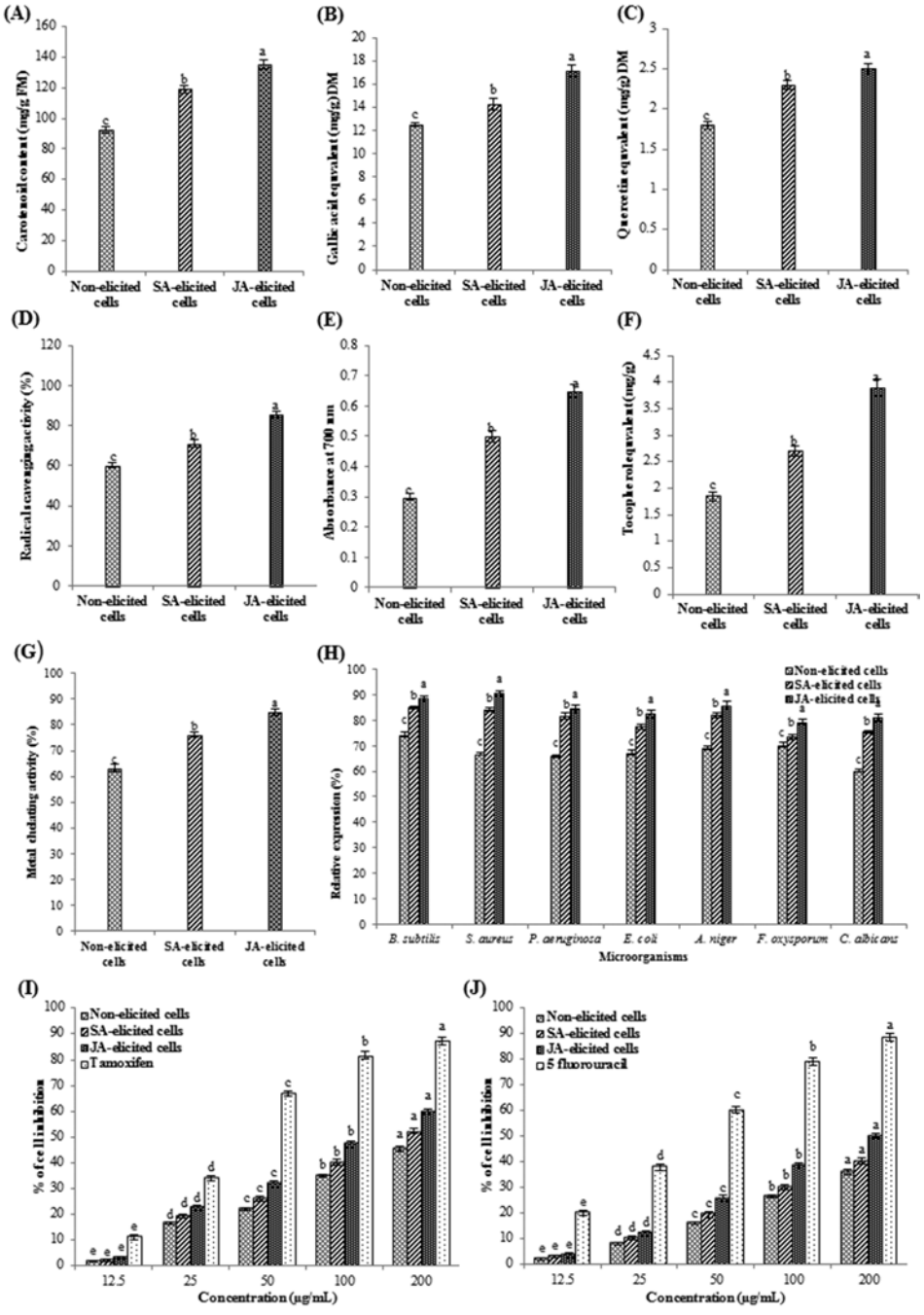
Fig. 2. Effects of JA and SA on phytochemical production, as well as antioxidant, antimicrobial, and antiproliferative activities in *M. dioica* cell suspension cultures. **A**. Carotenoid content; **B**. total flavonoid content; **C.** total phenolic content; **D**. free radical-scavenging activity (using DPPH); **E**. reducing power; **F**. results from the phosphomolybdenum method; **G**. metal chelating activity; **H**. antimicrobial activity assayed with the disc diffusion method; **I**. MCF-7 cell line inhibition; **J**. HT-29 cell line inhibition. Means±SD of triplicates followed by the same letters are not significantly different according to Duncan's test at $P \leq 0.05$


(A)
Carotenoid content (mg/g FM)
Non-elicited cells
SA-elicited cells
JA-elicited cells
(B)
Gallic acid equivalent (mg/g) DM
(C)
Quercetin equivalent (mg/g) DM
(D)
Radical scavenging activity (%)
(E)
Absorbance at 700 nm
(F)
Tocopherol equivalent (mg/g)
(G)
Metal chelating activity (%)
(H)
Relative expression (%)
Non-elicited cells
SA-elicited cells
JA-elicited cells
B. subtilis
S. aureus
P. aeruginosa
E. coli
A. niger
F. oxysporum
C. albicans
Microorganisms
(I)
% of cell inhibition
Non-elicited cells
SA-elicited cells
JA-elicited cells
Tamoxifen
Concentration (µg/mL)
(J)
5 fluorouracil

Effects of elicitors (JA and SA) on antiproliferative activity in cell suspension culture

Antiproliferative activities against cancer cell lines (MCF-7 and HT-29) in elicited and non-elicited cell extracts were evaluated. The inhibitory abilities of these extracts were compared with those of standard tamoxifen and 5-fluorouracil for the MCF-7 and HT-29 cell lines, respectively (Fig. 2I, J). Elicited cell extracts (200 µg/mL) showed the strongest antiproliferative activity against MCF-7 and HT-29 cells, whereas non-elicited cell extracts displayed weak inhibition. We also observed that MCF-7 cells experience more inhibition than HT-29 cells (Fig. 2I, J). In line with our results, a previous study also reported that phytochemical constituents inhibited MCF-7 and HT-29 cell proliferation [27]. In summary, the MTT assay results indicated *M. dioica* cell suspension cultures can inhibit the growth of breast and colon cancer cell lines.

CONCLUSIONS

Cell suspension cultures of *M. dioica* have the potential for commercial-scale studies by the pharmaceutical industry. Phenolic groups (e.g. flavonols, hydroxybenzoic acid, hydroxycinnamic acid, and derivatives) were higher in JA- and SA-elicited culture cells than in non-elicited cell cultures. Bioactive compounds (carotenoids, phenolic compounds, and flavonoids) and biological activities (antioxidant, antibacterial, antifungal, and anticancer) were also higher in JA- and SA-elicited cell cultures than in non-elicited cultures. This established elicitor system could be useful for biochemical and bioprocess engineering aimed at efficiently producing bioactive compounds in *M. dioica* cell suspension cultures.

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