ANTICANCER ACTIVITY OF MULBERRY SUSPENSION CULTURED CELLS INDUCED BY ELICITORS

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ABSTRACT Plant cell suspension culture combined with elicitor techniques has gained popularity in recent years. These techniques can increase phenolic content and attain consistent quality. The aim of this research was to study effect of 50 µM methyl jasmonate (MeJA) and jasmonic acid (JA) on inducing total phenolics content (TP), antioxidant activities; including 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and resveratrol content in a suspension culture of mulberry. It was found that dry cell weight (DW) of suspended mulberry cells was lower than the control when treated with MeJA and JA. However, the suspended mulberry cells treated with MeJA had the highest TP, antioxidant activities, and resveratrol content compared with JA treatment. TP evaluated by Folin–Ciocalteau method showed that suspended cells with 50 µM MeJA favored the phenolics production of 335.55±0.29 mg GAE/100 gDW after 6^{th} days of culture. Resveratrol contents of the cells cultured with 50 µM MeJA increased approximately 3-fold in 6^{th} days of culture. However, at day 6^{th}, mulberry suspension cells cultured with 50 µM MeJA had the highest antioxidant activity as indicated by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ where the values increased 2-fold when compare with the control. In addition, 0.516 g DW/mL mulberry cells cultured with 50 µM MeJA which had the highest TP, antioxidant activity and resveratrol content, resulting in decreased cancer cell lines; SW620, Chago-K1, KATO-III, Hep-G2, BT474 with their survival at 9.55±5.73%, 30.60±9.21%, 13.76±4.67%, 28.21±10.55% and 39.62±15.38% respectively.

1. INTRODUCTION

Mulberry can be grown in tropical and subtropical regions. It's a rich source of phenolic compounds including gallic acid, chlorogenic acid, sinapic acid, ferrulic acid (Radojković, et al., 2012) and resveratrol (Zhou, et al., 2013). Resveratrol, belonging to the stilbenoids family, possesses numerous important bioactivities, including anti-inflammatory, antioxidant (Shankar, et al. 2006) and anticancer such as liver cancer, colon cancer and stomach cancer (Tameda, et al., 2014). Resveratrol induced apoptosis in etoposide-resistant cancer cells is caused by activation of adenosine 5′ monophosphate (AMP)-activated protein kinase with a corresponding production of reactive oxygen species (ROS). ROS mediates the release of cytochrome C from mitochondria, which in turn leads to caspase activation and apoptosis (Hwang, et al., 2007). Phenolic compounds and resveratrol can be synthesized by plant cell suspension culture techniques (Vuong, et al., 2014). In addition, those techniques can be combined with an elicitor to increase compound content and achieve consistent quality. Methyl jasmonate (MeJA) and jasmonic acid (JA) are elicitors where a signal molecule in abiotic stress regulates the gene expression of defense-related protein (PR protein) as well as key enzymes in the phenylpropanoid pathway via stilbene synthase and chalcone synthase to produce phytochemicals in the plant. In this study, the effects of 50 µM MeJA and JA on cell growth, TP, antioxidant activities and resveratrol content in cultured cells of mulberry were investigated. Moreover, the usefulness of mulberry cell extract to inhibit the growth of cancer cell lines; 'Human colon adenocarcinoma (SW620)', 'lung cancer cell (Chago-K1)', 'Human liver hepatoblastoma (Hep-G2)', 'Human breast ductol carcinoma (BT474)' and 'Human gastric carcinoma (KATO-III)' were carried out.

2. EXPERIMENT

2.1 Callus formation

Callus cultures were initiated from mulberry leaves which they were subjected to surface sterilize with ethanol 70% (v/v) for 30 sec, then by NaOCl 7% (v/v)

for 15 min and NaOCl 5%(v/v) for 10 min, and finally rinsed 3 times with sterile distilled water for 5 min. The leaves were subjected onto a solid MS medium containing 2 mg/L 1-naphthaleneacetic acid (NAA), 1 mg/L 6-benzyladenine (BA), 0.2 mg/L kinetin, 20 g/L sucrose and 0.8% (w/v) agar, incubated for 4 weeks at 25ºC with 16 h light, and observed until initiation to callus around 45 days.

2.2 Cell suspension culture

The mulberry cell lines were grown in MS-medium at pH 5.6–5.8 which MS containing 2 mg/L NAA, 1 mg/L BA, 0.2 mg/L kinetin, without agar was used for suspension culture. About 125 mg callus tissues were transferred from solidified medium into 250 mL conical flasks containing 125 mL of liquid medium. The cultures were incubated on a rotary shaker operated at 110 rpm at 25±2°C.

2.3 Elicitor treatment procedure

Elicitors were added when cells began their log phase of sigmoid growth curve. To investigate the effects of 50 µM MeJA and JA, cells were cultured in MS-based liquid. The flasks (in triplicate) were incubated on an incubator shaker at 110 rpm in a light, temperature-controlled room at 25±1°C. Samples in were taken on 3, 6, 9, 12 and 15 days after the addition of elicitors. Then cell cultures were harvested from the culture medium on 3, 6, 9, 12 and 15 days after treated with MeJA and JA. The cell dry weight (gDW) was recorded.

2.4 Effect of elicitor on secondary metabolite and antioxidant activity

2.4.1 Determination of total phenolic content

Briefly, an aliquot (1 mL) of extracted solution was mixed with 5 mL of a 10-fold diluted Folin–Ciocalteu reagent with thorough shaking. After 5 min, 4 mL of Na_2CO_3 (7.5%, w/v) was added and the mixture was allowed to stand for 60 min in the dark. Absorbance was measured at 765 nm.

2.4.2 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) assay

In brief, one milliliters of DPPH (1 mM DPPH radical solution in 95% ethanol) solution was mixed with 1 mL of extract, vortexed and then incubated for 30 min at room temperature adapted by Brand-William, et al., 1995. The reduction in the absorbance at 517 nm was recorded using a UV–vis spectrophotometer and results were expressed in mM Trolox equivalents (TE) per 100 g dry weight of the sample.

2.4.3 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) assay

ABTS$^{\bullet+}$ assay was followed by Cai, et al., 2004. The 7.0 mM ABTS and 2.45 mM potassium persulfate were mixed for the production of ABTS cation (ABTS$^{\bullet+}$) and kept in the dark (12 h, 22±1°C). ABTS solution was diluted with 80% (v/v) ethanol until an absorbance of 0.70 (±0.05) was obtained at 734 nm. For sample analysis, 4 mL of diluted ABTS$^{\bullet+}$ solution was added to 1 mL of methanolic extract and mixed thoroughly. The reaction mixture was allowed to stand (25±2°C, 6 min, dark) and then the absorbance was recorded in a UV–vis spectrophotometer at 734 nm. All data were expressed in mM Trolox equivalents (TE) per 100 g dry weight (mM TE/100 g FCW) of the sample.

2.4.4 Determination of resveratrol content

The trans-resveratrol content was determined by high pressure liquid chromatography (Agilent 1200 Series, Agilent Technologies), equipped with DAD detector at 306 nm and autosampler. Separation was achieved using a ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm id, 5 µm packing); the column temperature was maintained at 40 °C. The HPLC conditions were described previously which column was eluted with solvent A (glacial acetic acid in water) and solvent B (20% phase A and 80% acetonitrile) gradient at a flow rate of 1.0 mL/min. The extract was identified and compared with a trans-resveratrol standard.

2.5 *In vitro* cytotoxicity assay

Cytotoxicity assay was performed using MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide. Briefly, Cancer cell line (BT474, Hep-G2, SW-620, Chago-K1 and KATO-III) were seeded onto well plate at a density of $5x10^3$ cells/well. Then 2 µl of mulberry cell extract (0.516 g DW/mL) were added to each well and incubated at 5% CO_2 for 72 h, doxorubicin used as a positive control. MTT was added to cell supernatant at 10 µL/well. Cells were incubated for 4 h, then, 10 mL of DMSO was added to dissolve crystals in the well. The formation of formazan was measured by microplate reader at 540 nm.

3. ANALYSIS

3.1 Effect of elicitor on cell growth

The cell growth subjected with 50 µM MeJA and JA was shown in Table 1. It was found that the dry biomass of mulberry suspension cells cultured with MeJA and JA was lower than the control cells. As compare between two elicitors, the highest cell growth was found in cells treated with 50 µM JA in 9th days which it was 7.15 g DW/L. Cell growth of cells treated with MeJA was lower than cells treated with JA.

Table1 Effect of elicitors on cell growth (g DW/L) of the mulberry suspension cultured cells.

treatment	cell growth (g DW/L)				
	3 day	6 day	9 day	12 day	15 day
control	2.75±0.01^a	6.36±0.01^a	7.19±0.01^a	7.26±0.01^a	6.71±0.01^a
MeJA 50 µM	2.67±0.07^a	5.65±0.05^c	6.95±0.05^b	6.60±0.40^b	5.70±0.40^b
JA 50 µM	2.75±0.05^a	6.05±0.05^b	7.15±0.15^a	6.60±0.10^b	6.65±0.05^a
F-test	ns	**	ns	ns	ns
C.V. (%)	1.99	0.74	1.31	3.73	3.88
LSD	0.11	0.09	0.19	0.53	0.51

3.2 Effect of elicitors on total phenolic content and antioxidant activity

In this study, stress responses from 50 µM MeJA and JA on TP were monitored (Fig.1). It was found that TP in mulberry cell treated with MeJA and JA was higher than the control. However, the greatest TP was found in the mulberry cell suspension cultures treated with 50 µM MeJA in 6th days (335.55 mg GAE/100 g DW). The elicitor suspension cultured cells showed darker color than the control (Fig 2). The freely suspended cells as well as medium were yellow to orange in color. It might be due to the intracellular and extracellular production of phenolics as Schripsema et al. (1999) demonstrated that this color has been attributed to phenolic formation.

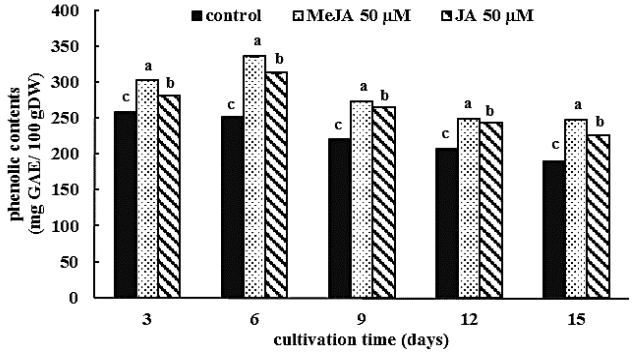

Fig. 1 Effect of MeJA and JA on total phenolic content of mulberry suspension cultured cells.

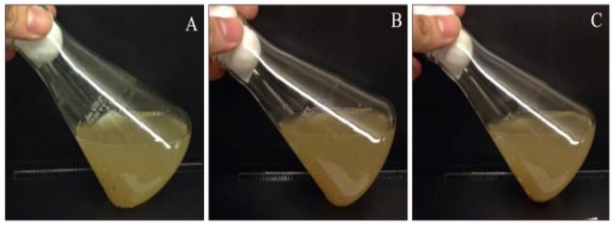

Fig. 2 Mulberry suspension cultured cells treated with MeJA and JA; control (A) 50 µM MeJA (B) and 50 µM JA (C).

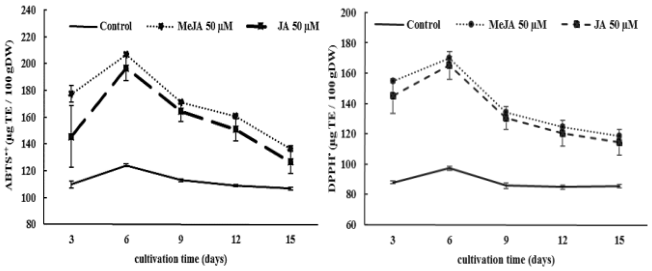

Fig. 3 Effect of MeJA and JA on $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ of mulberry suspension cultured cells.

The effect of 50 µM MeJA and JA on antioxidant activity as assessed by the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ in the mulberry suspension cultured cells were shown in Fig. 3. The highest $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (169.90 µgTE/100g DW and 206.52 µg TE/100g DW) was observed in mulberry cell suspension cultured with MeJA on 6 days. After 6 days, they decreased drastically at day 9th. This finding was consistent with the results of the TP which MeJA and JA could up-regulate the gene expression of defense-related protein (PR protein) (Belhadj, et al., 2008), as well as phenylalanine ammonium lyase (PAL) (the key enzymes in the phenylpropanoid pathway via with stilbene synthase and chalcone synthase and produce phenolic) (Bauer, et al., 2011). In addition, this finding was similar to Ali, et al., 2014 in suspension culture of *Artemisia absinthium* L. which its antioxidant activity ($DPPH^{\bullet}$) increased when treated with MeJA and JA.

3.3 Effect of elicitor on resveratrol

The resveratrol contents in mulberry cell suspension cultures treated with MeJA and JA are presented in Fig. 4. The resveratrol content in the treated cells rapidly increased and reached the maximum on 6th days. The accumulation of resveratrol contents by 50 µM MeJA treated cells was greater than the JA and control. The highest amount of resveratrol produced by mulberry cells cultured in suspension containing 50 µM MeJA on day 6 was 12069.14 µg/100g DW. MeJA and JA could up-regulate the activity of PAL, the key enzymes in the phenylpropanoid pathway via with stilbene synthase and chalcone synthase (Shen, et al., 2012), and stilbene synthase controlled the biosynthesis of resveratrol.

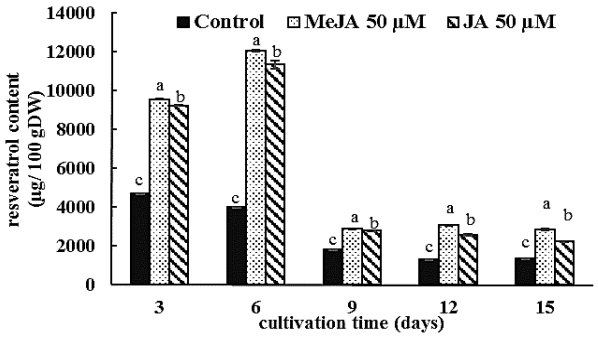

Fig. 4 Effect of MeJA and JA on resveratrol content of mulberry suspension cultured cells.

3.4 Effect of mulberry cell extract on cancer cell line

Microscopic analysis of cancer cell lines treated with mulberry cell extract revealed a reduction in cell number and altered structural features of cells apparent changes as shown in Fig. 5. These observations correlated with the results of the MTT assay indicated that mulberry cell extract contained compounds which had anticancer properties. Mulberry cell extract showed a significant effect on the survival of the all cancer cell lines (Fig. 6). All cancer cells also began to lose viability at this cell density when they were exposed to a 0.516 g DW/mL *Morus alba* cells cultured with 50 µM MeJA. Mulberry cell extract could decrease cancer cell lines; SW620, Chago-K1, KATO-III, Hep-G2, BT474 with their survival at 9.55%, 30.60%, 13.76%, 28.21% and 39.62% respectively. However, the doxorubicin (control) 10 ug/mL showed a statistically significant reduction in the

viability of the all cancer cell lines which were similarly to the mulberry cell extracts of 0.516 g DW/ mL.

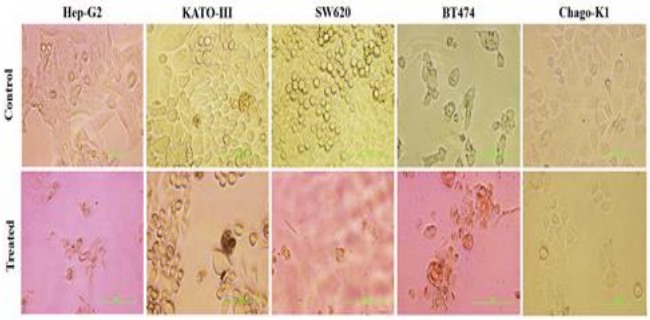

Fig. 5 Microscopic analysis of cancer cell lines treated with mulberry cell extracts of 0.516 gDW/mL.

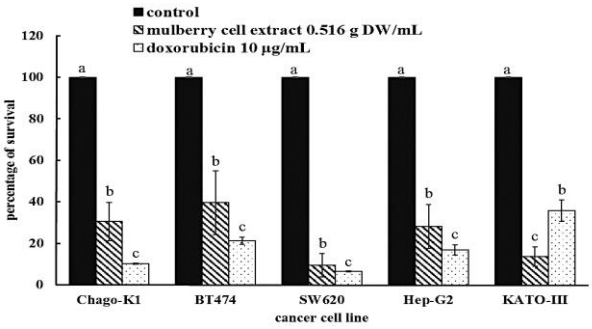

Fig. 6 Effect of mulberry cell extracts and doxorubicin on percentage cell survival.

CONCLUSION

Although methyl jasmonate (MeJA) and jasmonic acid (JA) could inhibit cell growth, they could enhance total phenolics, antioxidant activities, and resveratrol contents in cultured cells of mulberry. The best condition to enhance the total phenolic, antioxidant activity and resveratrol content was 50 µM MeJA after 6th days of mulberry cell suspension cultures. Mulberry cell extract treated with 50 µM MeJA could have high potent anticancer cell activities.

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