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Sesquiterpene Production in Methyl Jasmonate-Induced *Persicaria minor* Cell Suspension Culture

(Penghasilan Seskuiterpena dalam Kultur Ampaian Sel *Persicaria minor* Diinduksi Metil Jasmonat)

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ABSTRACT

Sesquiterpenes are a three-isoprene unit compounds which belong to terpenoid family of secondary metabolites. These volatile compounds are one of the major constituents of essential oils in plants and plays major roles in plant signaling of defense mechanism. The effects of methyl jasmonate (MeJA) concentrations (100 and 200 μM) on the production of sesquiterpene compounds after incubation period for 1, 3, and 6 days were investigated in Persicaria minor *cell suspension culture. Headspace Solid-Phase Microextraction (HS-SPME) method was used to absorb volatile compounds from suspension cells and liquid medium. They were then analyzed through Gas Chromatography-Mass Spectrometry (GC-MS) to identify sesquiterpene compounds. Among the 15 sesquiterpene compounds identified, α- muurolene was found in significantly higher concentration in all MeJA treated cultures. The results showed that α-muurolene was detected in the suspension cells at the highest peak area of 14.17% at 100 μM MeJA treated cultures with 3-day incubation. Analysis of liquid medium of the treatments identified secretion of α-muurolene into the culture medium, with total peak area of 0.72%. These results showed that sesquiterpene production in MeJA induced* P. minor *suspension culture depended on the MeJA concentration and also culture incubation period.*

Keywords: α-muurolene; cell suspension culture; GC-MS; methyl jasmonate; sesquiterpene

ABSTRAK

Seskuiterpena adalah unit sebatian tiga isoprena yang tergolong dalam keluarga terpenoid metabolit sekunder. Sebatian meruap ini adalah juzuk utama dalam minyak pati daripada tumbuhan dan memainkan peranan penting sebagai pengisyaratan bagi mekanisme pertahanan tumbuhan. Kesan kepekatan metil jasmonat (MeJA) (100 dan 200 μM) ke atas penghasilan seskuiterpena dengan tempoh masa pengeraman 1, 3 dan 6 hari telah dikaji dalam kultur ampaian sel Persicaria minor. *Pengekstrakan-mikro ruang kepala fasa* (Headspace Solid-Phase Microextraction*) (HS-SPME) bersama kromatografi gas-spektrometri jisim (GC-MS) digunakan untuk mengenal pasti sebatian seskuiterpena daripada ekstrak ampaian sel. Daripada 15 sebatian seskuiterpena yang dikenal pasti, α-muurolin didapati hadir pada kepekatan yang signifikan bagi kesemua kultur yang diberi perlakuan MeJA. Keputusan kajian menunjukkan α-muurolin dikesan pada luas puncak tertinggi 14.71% dalam ampaian sel pada 100 μM MeJA dengan tiga hari pengeraman. Analisis ke atas medium cecair mendapati kehadiran rembesan α-muurolin dengan luas puncak keseluruhan 0.72%. Keputusan menunjukkan bahawa penghasilan seskuiterpena dalam ampaian sel terinduksi MeJA bagi* P. minor *adalah bergantung kepada kepekatan MeJA dan juga tempoh pengeraman.*

Kata kunci: α-muurolin; GC-MS; kultur ampaian sel; metil jasmonat; seskuiterpena

INTRODUCTION

Persicaria minor (Huds.) Opiz, synonymously known as *Polygonum minus* Huds. or 'kesum' by the locals is well recognized for its health benefits. Long used in traditional medicine, it treats digestive and dandruff problems. Being famous for its pharmacological and anti-inflammatory properties, this aromatic plant also lowers high cholesterol and blood pressure. Qader et al. (2012) reported that *P. minor* is a potential source of secondary metabolite production as it contains high levels of antioxidant properties. Their report was later supported by Ahmad et al. (2014) who showed antioxidant properties from volatile compounds of *P. minor* essential oil profiled from different tissues. In addition, this plant has been claimed to have other valuable pharmacological properties such as antibacterial, antifungal, antiulcer and antiviral (Vikram et al. 2014).

Early chemical analysis of essential oil by Yaacob (1987) showed that *P. minor* has high levels of aliphatic aldehydes containing of dodecanal (48.18%) and decanal (24.36%) as major components. Baharum et al. (2010) later identified 32 more compounds in *P. minor* essential oil through GC-MS and 48 compounds in total through two-dimensional gas chromatography-time of flight-mass spectrometry (GC×GC-TOF-MS) technique. Baharum et al. (2010) also reported that *P. minor* essential oil has terpene compounds as the major constituent, which strongly contributes to its flavour. Among the identified compounds,

eight sesquiterpene compounds were found through both GC-MS and GC×GC-TOF-MS analysis techniques, more than those reported by Yaacob (1987) where only β-caryophyllene was identified.

A study conducted by Ashraf et al. (2015), found β-caryophyllene produced as the main sesquiterpene and secreted into *P. minor* hairy root culture medium with a total peak area of 7.934%. This sesquiterpene compound has also been reported in jasmonic acid treated *P. minor* root extracts at 17.57% as the highest peak area along with other sesquiterpene compounds such as β-farnesena, trans-α-bergamotene, α-caryophyllene and α-panasinsen (Ismail et al. 2011). Studies by Ashraf et al. (2015) and Ismail et al. (2011) showed that *P. minor* provides a great platform for sesquiterpene production.

For decades, cell suspension culture is believed to be a potential source for metabolite production. Cell suspension culture is an effective source of valuable metabolite compound production commercially. Fast cell proliferation due to cell growth initiation leading to higher metabolism rate compared to intact differentiated plants has been reported as the most important advantage of plant cell culture (Dörnenburg & Knorr 1995). Among commercially produced natural compounds through cell suspension cultures are vaccine against Newcastle disease virus (NDV) from tobacco (Liew & Hair-Bejo 2015), human glucocerebrosidase (GCD) enzyme from carrot (Shaaltiel et al. 2007) and human proteins from moss (*Physcomitrella patens*) (Ochoa-Villarreal et al. 2016).

Various approaches can be used for secondary metabolite production in cell suspension culture but elicitation is one of the most commonly used approaches for successful production of compounds. Elicitation is carried out by introducing a substance (elicitor) in small concentrations to a living system to initiate or improve the biosynthesis of specific compounds (Cai et al. 2011; Smetanska 2008). Few of the broadly studied elicitors are jamonic acid and its methylated derivative methyl jasmonate (MeJA), salicylic acid, yeast and fungal. A study conducted on *Mitragyna speciosa* suspension culture found that yeast extract has significantly enhanced alkaloid mitragynine content (Mohamad Zuldin et al. 2013), while chitosan produced highest 9-hydroxycanthin-6-one concentration in *Eurycoma longifolia* suspension culture (Keng et al. 2010). In *Euphorbia pekinensis* suspension culture, terpenoid biosynthesis (isoeuphpekinensin and euphol) was enhanced by 5.8 and 3.6-folds greater than control using *Fusarium* sp. E5 (Gao et al. 2011). Improved production of a sesquiterpene trilactone compound, bilobalide was reported by Kang et al. (2009) in *Ginkgo biloba* suspension culture through usage of a biotic elicitor, native *Candida albicans*.

Nevertheless, one of the most extensively studied elicitor MeJA has been widely reported to enhance metabolite compounds production in suspension cultures. For example, production of flavanoid compounds in *Hypericum perforatum* suspension culture (Sakhya et al. 2017) which produced 2.7-folds higher concentration than control cultures. MeJA treatment was also reported on *Ginkgo biloba* suspension culture which has increased bilobalide concentration by 6.25-folds in culture medium after 12-hours of exposure time (Kang et al. 2006). However, there is no reported study of MeJA effect in *P. minor* suspension culture. Previous study by Shukor et al. (2013) identified several compounds such as 2,2'-bioxirane, propanoic acid-2oxo-methyl ester, repandin A, 2-propanone,1,3-dihydroxy-, imidazolidine-2,4,5-trione and 2-acetyl-2-hydroxy-butyrolactone in *P. minor* suspension culture through jasmonic acid elicitation, but none were of sesquiterpene class compounds.

Extensive list of sesquiterpene compounds reported for *P. minor* through chemical analysis studies demonstrates this species as a prominent platform for sesquiterpene production. These valuable sesquiterpene compounds are believed to be beneficial for pharmaceutical industry, thus an efficient system for biosynthesis of these compounds is important. In this study, we have developed a suspension culture system for sesquiterpene production in *P. minor* through MeJA elicitation of different concentrations over different time points.

Materials and Methods

Callus Induction and Proliferation

Previously established *in vitro* grown *P. minor* cultures from Ulu Yam population were used as a source of explants. Semisolid medium using Murashige and Skoog (1962) (MS) salts supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 4 mg/L α-naphthalene acetic acid (NAA), optimized by Shukor et al. (2013) was used for callus induction. Sucrose (30 g/L) was added as a carbon source and 3.2 g/L gellan gum (Gelrite, Duchefa Biochemie) was used as a solidifying agent. The pH of the medium was adjusted to 5.7-5.8 with 1 N HCL and 1 N NaOH before autoclaving at 15 psi and 121°C for 20 min. *In vitro* grown leaves were excised at 1.0 × 1.5 cm each and transferred aseptically onto the callus induction medium. The cultures were incubated in a culture room of 16:8 (light:dark) photoperiod (irradiance of 22.26 μmol m^{-2} s^{-1}) and temperature 25 ± 1°C. Every two weeks, all explants were transferred onto fresh callus induction medium. Induced callus was then separated from the samples and subcultured onto fresh callus induction medium for proliferation. Incubation conditions and subculturing period remained the same.

Cell Suspension Culture Initiation

Liquid MS medium supplemented with 1 mg/L 2,4-D and 2 mg/L NAA was used for suspension culture initiation, based on Shukor et al. (2013) with some modification. Established culture line with good morphological characteristics (fragile and yellowish) and good proliferation rate was chosen for this study. Suspension culture was initiated by inoculating 1.0 g of callus into each flask with 50

mL suspension medium and incubated in a horizontal shaker rotating at 80 rpm, under light condition of 12:12 (light:dark) photoperiod at 25 ± 1°C. All samples were sub cultured into new medium every 2 weeks.

OBTAINING GROWTH CURVE

Growth curve was obtained by inoculating 0.5 g callus cells into each flask and incubated under 120 rpm agitation with same culture conditions, based on Srivastava et al. (2011). Suspension cells were drained from their liquid medium, air dried in laminar flow for 10 min and weighed for biomass. Biomass accumulation (fresh weight) was recorded until 45 days and analyzed for standard errors. Samples were then oven dried at 55°C for 24 h and weighed for dry biomass.

ELICITATION WITH METHYL JASMONATE (MEJA)

Elicitation of methyl jasmonate (MeJA) at 50, 100, 150, and 200 μM were tested in this study. MeJA was added into suspension culture at day 18 after culture initiation and incubated separately for 1, 3, 6, and 9 days. On sample harvesting day, suspension cells were drained and separated from liquid medium. They were then stored individually at -80°C for Headspace Solid-Phase Microextraction (HS-SPME) analysis.

STATISTICAL ANALYSIS

Experimental data were analyzed using Excel (Microsoft Inc.). Significant differences of compounds between different concentration of MeJA and α−muurolene compound between suspension cells and liquid medium were analyzed using student's T-test analysis ($p<0.05$) via excel. Data obtained were reported as mean ± standard error.

HEADSPACE SOLID-PHASE MICROEXTRACTION (HS-SPME)

Sesquiterpene analysis was carried out using HS-SPME following Ee et al. (2014). Samples stored at -80°C were defrosted at room temperature for 30 min. For suspension cell analysis, 1.5 g of suspension cells were measured and transferred into SPME vials, while for liquid medium analysis, 1.5 mL liquid medium was pipeted and transferred. Limonine (0.03 μL) as internal standard was then added into each of the samples and sealed using parafilm. The samples were vortexed and kept in ice to reduce any compound volatility. SPME fiber coated with poly-dimethylsiloxane (PDMS) was conditioned at 250°C for 30 min prior to inserting into the sealed vials exposing it to the headspace for 30 min at 60-70°C.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS

Analysis was carried out following Ee et al. (2014) on Agilent 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) coupled with 5975C inert MSD triple-axis detector (Agilent) with Electron Ionization (EI) as ion source. After sampling, the SPME fiber was retracted from the vials and immediately injected into GC inlet. Separation was achieved using 5% phenylmethylpolysiloxane column (AB-5MS; Abel Industries) with 30 m × 0.25 mm× 0.25 μm in size. Injection temperature was set at 50°C (held for 3 min) and temperature was increased 6°C at constant rate until 250°C. Helium was used as carrier gas at flow rate of 1.3 mL min^{-1} and mass range of 50-600 m/z. GC-MS was carried out on MeJA with incubation period 1, 3, and 6 days, while 9 days was left out because of the low detection starting at day 6.

MULTIVARIATE ANALYSIS

Sesquiterpene compound peaks were identified through mass spectrum library search using NIST, version 2. Sesquiterpene compounds identified in all replication samples were listed into a GC-MS peak schedule and analysed using SIMCA-P+ software version 12.0 (Umetrics, Sweden).

Results and Discussion

SUSPENSION CELL CULTURE ESTABLISHMENT

Growth curve for *P. minor* suspension culture was obtained for 45 days after suspension culture initiation (Figure 1). Lag phase was observed from cell culture initiation till the third day. Although the curve for exponential phase could not be seen clearly, it can be speculated to occur from third to sixth day of culture. Linear phase could be observed to have started at day 9. However, at day 21 suspension cells growth slowed down slightly until day 27 of culture initiation. Based on the observation on the steep curve of growth from day 30 to 36, maximum growth rate of *P. minor* suspension culture was achieved. Suspension cell biomass accumulation increased from 8.05 to 13.22 g (fresh weight) and from 0.79 to 1.03 g (dry weight) showing *P. minor* suspension cells were actively proliferating at an optimal state.

Towards the end of liner phase at day 39, 14.57 g suspension cells were accumulated. A difference on quantity of suspension cells proliferated from the day of initiation (0.5 g) to day 39 (14.57 g) of suspension culture initiation has been shown in Figure 2. Suspension cell proliferation rate was noticed to have slowed down from day 39 onwards and stagnant phase was observed from day 42. During this phase, slow suspension cell proliferation rate has been observed and this is believed to be due to the exhaustion of nutrients in culture medium to support continuous cell division and proliferation. Other possible reasons for this deceleration in growth are growth reduction due to cell signaling mechanism or cell death due to decrease in limited availability of nutrients, smaller physical area and air available for growth inside the vessel as well as gases exchange between cells and media inside the vessel (Bona et al. 2012; Pépin et al. 2004). Bona et al.

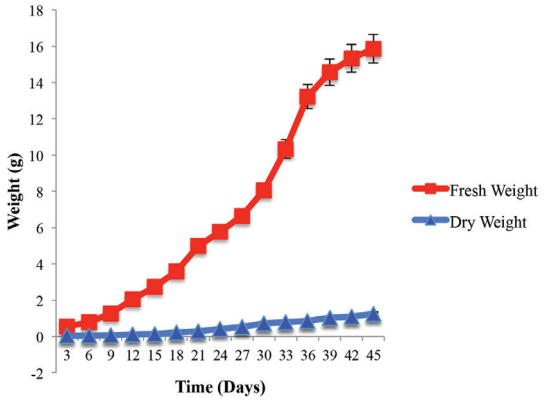

FIGURE 1. *Persicaria minor* suspension culture growth curve for fresh weight and dry weight (g) cultured in liquid MS medium supplemented with 1 mg/L 2,4-D and 2 mg/L NAA. Error bars represent the standard errors of three replicates

(2012) has stated that knowledge on cell suspension growth curve is important for logarithmic growth maintenance. Thus, based on the *P. minor* suspension growth curve obtained, day 18 was chosen for MeJA elicitation. Higher proliferation rate starting from day 18 probably helped in high and stable production of sesquiterpene compounds.

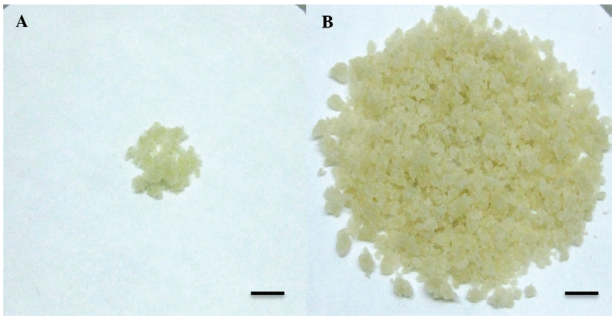

FIGURE 2. *Persicaria minor* suspension cell biomass accumulation on day 0 (0.5 g) (A) and day 39 (14.57 g) (B) in MS medium supplemented with 1 mg/L 2,4-D dan 2 mg/L NAA under light condition. Bar: 10 mm

EFFECTS OF MeJA ELICITATION ON SUSPENSION CELL FRESH WEIGHT ACCUMULATION

Suspension cultures were treated with four different concentrations of MeJA at day 18 of inoculation. Fresh weight at every period of incubation was observed (Figure 3). The 50 µM MeJA treated suspension culture showed an increase in cell fresh weight at day 3, followed with a gradual decrease from day 6 to day 9. Control treatment without any MeJA treatment was observed to increase in fresh weight accumulation throughout the 9 days of incubation. Addition of MeJA to the treated samples might have caused a stress to the suspension cells, subsequently reducing the growth rate, as shown in decreasing fresh weight accumulation after longer period or certain period of incubation.

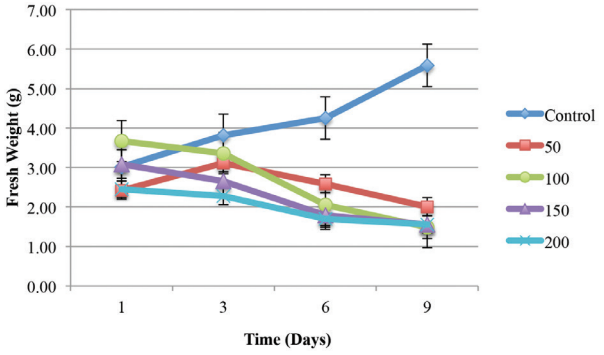

FIGURE 3. Fresh weight (g) callus accumulation in MeJA-treated *Persicaria minor* suspension culture following incubation. Bars represent standard errors of three replicates

EFFECTS OF MeJA ELICITATION ON CELL DRY WEIGHT ACCUMULATION

Dry weight of cells from 100, 150, and 200 µM MeJA treated suspension cultures were observed to have decreased with longer incubation period (Figure 4). Cultures treated with 50 µM MeJA were observed to have increased in dry weight accumulation from day 1 to day 3 of incubation but decreased in weight from day 6 to day 9. However, control treatment was observed to have a different pattern where there was a decrease in weight from day 1 to day 3 and an increase to day 6. At day 9, the cell weight decreased significantly. All treatments including the control showed a decrease in dry weight accumulation at day 9 of incubation.

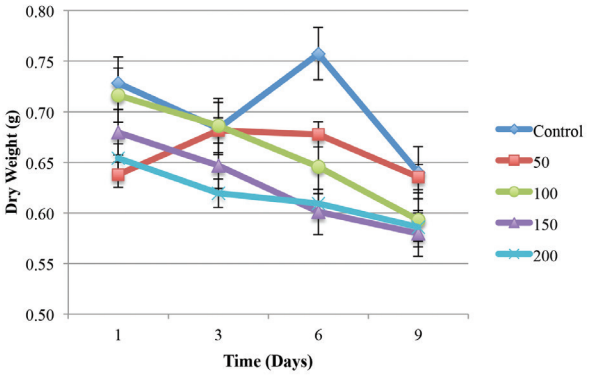

FIGURE 4. Dry weight (g) of callus for control treatment (without MeJA) and with 50, 100, 150, and 200 µM MeJA treated *Persicaria minor* suspension culture incubated for 1, 3, 6 and 9 days. Bars represent standard errors of three replicates

SESQUITERPENE ANALYSIS ON CELL SUSPENSION CULTURE AFTER MeJA ELICITATION

Cells from the suspension cultures treated with 100, and 200 µM MeJA and incubated for 1, 3, and 6 days were analysed for sesquiterpene compounds through SPME technique. These two treatment concentrations were chosen to be tested in order to obtain a fluctuation pattern of sesquiterpene compounds in suspension cells. Samples

without any MeJA treatment was also analysed as a control in this study. In this work, all analysis were conducted through GC-MS technique. Gas chromatography is one of the most widely used effective technique for separating and obtaining high-resolution components of volatile mixtures. As mass spectrometry is a powerful tool for structural elucidation of volatiles, combination of these techniques serves effective identification of volatile components in this study.

A total of 15 sesquiterpene compounds were identified in all the samples analysed and α-muurolene was identified with the highest peak area (14.17%) (Figure 5) in cultures treated with 100 µM MeJA with 3 days incubation period. The gas spectrum-mass chromatography for α-muurolene at same condition with retention time 21.2 min shown in Figure 6.

At day 1, this compound was identified at a very low concentration (0.02%) but showed an exponential increase at day 3 where it was identified with the highest peak area. However at day 6, this compound was only identified at 0.85%. Same pattern of fluctuation and deceleration was also observed in cultures treated with 200 µM MeJA with incubation period of 6 days. At day 3, α-muurolene was identified at 5.95% and decreased to 0.14% at day 6 of incubation.

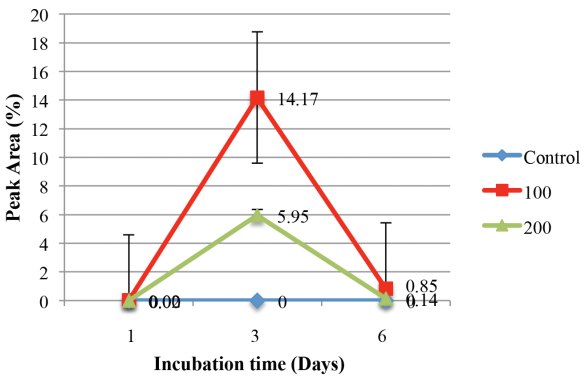

FIGURE 5. The α-muurolene peak area (%) for control (without MeJA), 100, and 200 µM MeJA treated *Persicaria minor* suspension culture incubated for 1, 3 and 6 days. Bars represent standard errors of three replicates

EFFECTS OF MEJA CONCENTRATION AND INCUBATION PERIOD ON SESQUITERPENE COMPOUNDS

Among the 15 sesquiterpene compounds identified, five compounds (α-santalene, sesquiphellandrene, γ-gurjunene, α-bisabolene and γ-cadinene) were not identified at 3 days incubation period under 100 µM MeJA treated suspension culture (Table 1). However, these compounds

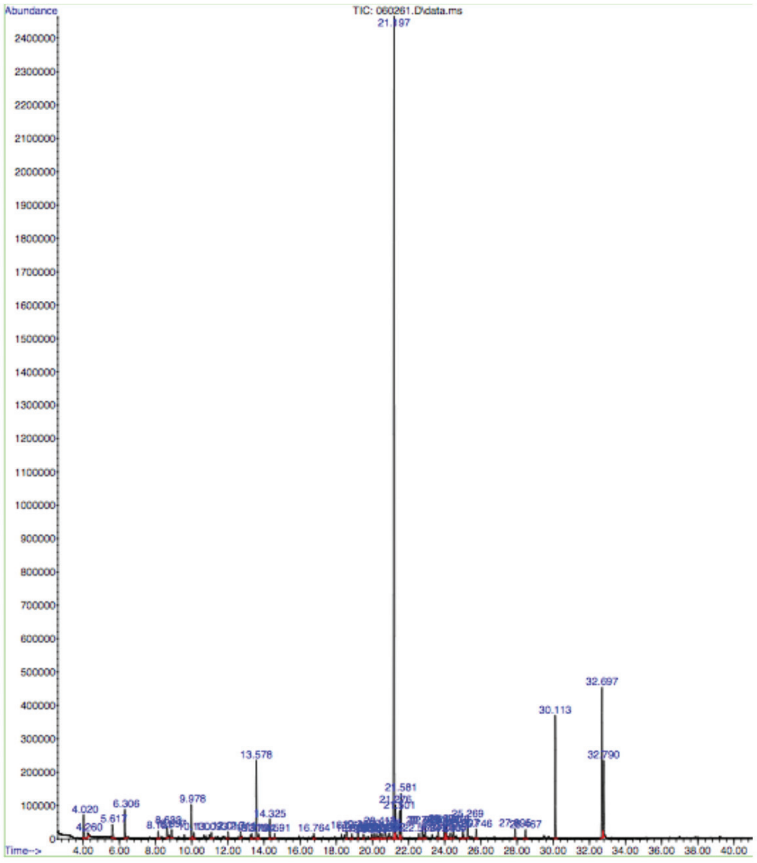

FIGURE 6. Gas spectrum-mass chromatography of α-muurolene compound's peak at retention time 21.197 min for 100 µM MeJA treated *Persicaria minor* suspension culture incubated for 3 days

TABLE 1. Peak area (%) of compounds from 100 and 200 µM MeJA treated *Persicaria minor* suspension cultures incubated for 1, 3 and 6 days

Retention time	Sesquiterpene Compounds	Molecular Formula	Molecular Weight (MW)	Ion Fragmentation (*m/z*)	Peak area (%)					
					1-day		3-day		6-day	
					100 µM MeJA[a]	200 µM MeJA[b]	100 µM MeJA[a]	200 µM MeJA[b]	100 µM MeJA[a]	200 µM MeJA[b]
18.5744	α-cubebene	$C_{15}H_{24}$	204.357	161, 119, 105	-	-	0.15	0.09	0.04	-
19.4971	α-santalene	$C_{15}H_{24}$	204.357	94, 93, 41	-	-	-	0.25	-	-
20.2160	sesquiphellandrene	$C_{15}H_{24}$	204.357	93, 69, 41	-	-	-	0.09	-	-
20.4130	alloaromadendrene	$C_{15}H_{24}$	204.357	105, 91, 41	-	-	0.28	0.07	0.09	-
20.7057	germacrene D	$C_{15}H_{24}$	204.357	161, 159, 105	-	-	0.25	0.17	-	-
21.1956	α-muurolene	$C_{15}H_{24}$	204.357	161, 105, 91	0.02	-	14.17	5.95	0.85	0.14
21.5009	α-amorphene	$C_{15}H_{24}$	204.357	161, 119, 105	-	-	0.45	0.22	0.10	-
21.5836	δ-cadinene	$C_{15}H_{24}$	204.357	161, 134, 119	-	-	0.77	0.19	-	-
22.7670	γ-muurolene	$C_{15}H_{24}$	204.357	161, 105, 81	-	-	0.25	0.13	0.08	-
22.9898	γ-gurjunene	$C_{15}H_{24}$	204.357	161, 107, 81	-	-	-	0.08	-	-
23.6386	α-bisabolene	$C_{15}H_{24}$	204.357	93, 91, 41	-	-	-	0.27	0.05	-
24.0459	selina-3,7(11)-diene	$C_{15}H_{24}$	204.357	161, 137, 84	-	-	0.14	-	-	-
24.0903	valencene	$C_{15}H_{24}$	204.357	161, 121, 95	-	-	0.32	-	-	-
24.3066	1,4-Methano-1H-indene, octahydro-1,7a-dimethyl-4-(1-methylethenyl)-,[1S-(1.**α**.,3a.β.,4.**α**.,7a.β.)]-	$C_{15}H_{24}$	204.357	108, 93, 41	-	-	0.14	-	-	-
24.3129	γ-cadinene	$C_{15}H_{24}$	204.357	105, 119, 133	-	-	-	0.30	-	-

were identified in cultures treated with 200 µM MeJA with the same incubation period of 3 days (Table 1). A total of 12 compunds were identified in 200 µM MeJA treatment cultures except selina-3,7(11)-diene, valencene and 1,4-Methano-1H-indene,octahydro-1,7a-dimethyl-4-(1-methylethenyl)-,[1S-(1.α.,3a.β.,4. α.,7a.β.)] at 3 days incubation period (Table 1).

In contrast, only α-muurolene compound was identified at 100 µM MeJA treated cultures at 1 day incubation period (Table 1). This compound was also solely identified at 200 µM MeJA treated cultures at 6 days incubation period (Table 1). All other compounds were not identified at 1 day incubation period in both 100 µM and 200 µM MeJA treatments. Effect of MeJA on sesquiterpene production was also reported by Ito et al. (2005) on *Aquilaria sinenesis* cell suspension culture. Three sesquiterpene compounds (α-guaiene, γ-guaiene and α-humulene) were identified in cells treated with MeJA (0.1mM) after 7 days and extracted through SPME coupled with GCMS. Following research by Kumeta and Ito (2010) to quantify these three sesquiterpene compounds showed that the production of all three sesquiterpene (α-guaiene, γ-guaiene and α-humulene) increased after 12 h of incubation with MeJA, compared to 0 and 6 h. This shows the effect of optimized MeJa incubation period towards production sesquiterpene compounds.

MULTIVARIATE ANALYSIS

Corelation between MeJA concentration and incubation time in sesquiterpene compounds production was analysed through multivariate analysis. Through this analysis approach, variables contributing to sesquiterpene production were plotted. In principal component analysis (PCA) score plot, three groups of samples were clearly separated (Figure 7). The three groups are treatments of 3 days incubation period at 100 µM MeJA, 6 days incubation period at 100 µM MeJA and 3 days incubation period at 200 µM MeJA. For the identification of the sesquiterpene compound responsible for the separation between these groups of treatments, loading scatter plot was analysed. In the loading plot, almost all the compounds were scattered close to each other, except for α-muurolene which was analysed to be scattered far from the y-axis (Figure 8). Thus, this α-muurolene compound was found to be responsible for the seperation of the treatments in PCA score plot. The α-muurolene was also reported in *Ptychopetalum olacoides*, a very popular tropical medicinal herb, to have vast pharmaceutical properties, such as memory enhancement (da Silva et al. 2004), antinociceptive and neuroprotective effects (Siqueira et al. 2003), antinociceptive effects (Vaz et al. 1998) and erectile dysfunction or vasorelaxant effects (Antunes et al. 2001). This compound has also been identified in juniper oil (Bailon et al. 2017) which showed antimicrobiotic properties and inhibition to *Escherichia coli* ATTC 25922 strain. Dursun et al. (2017) also claimed that olive leaf from the variety 'Halhalı' accumulated with high level of α-muurolene at 4.05% to have an important and beneficial effects on human health.

EXUDATION OF A-MUUROLENE INTO LIQUID MEDIUM

In this study, liquid medium analysis was carried out on 100 µM MeJA treated samples with 3 days incubation period. This treatment was chosen due to the highest α-muurolene peak area identified in its suspension cells (14.17%). Liquid medium sample of the treatment was identified with 0.72% of α-muurolene peak area. Comparison of α-muurolene concentration between suspension cells (14.17%) and liquid medium (0.72%) of the same treatment showed that they were significantly different through T-test analysis ($p< 0.05$) (Figure not shown). Although the concentration of the compound is not high in liquid medium, its identification has showed that there has been a certain degree of this compound being released from suspension cells to extracellular culture environment (medium). This process, which commonly known as two-phase culture system is an important approach that has been studied to strategically increase secondary metabolite production in suspension culture. This system promotes continuous extraction of

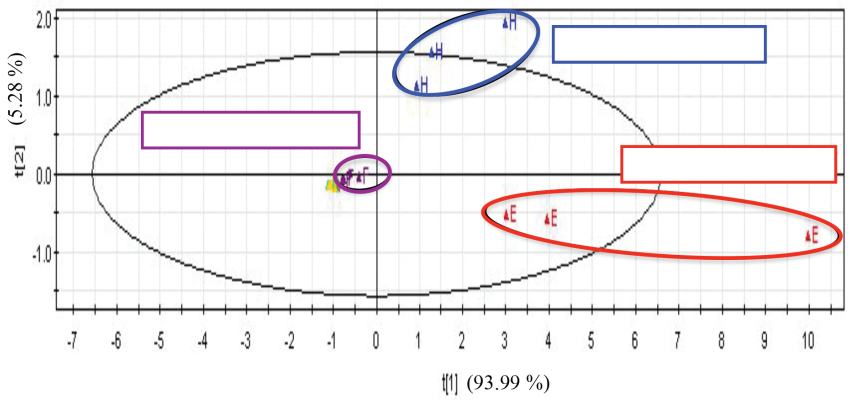

FIGURE 7. PCA score plot for control treatment (without MeJA) and with 50, 100, 150, and 200 µM MeJA treated *Persicaria minor* suspension culture incubated for 1, 3 and 6 days

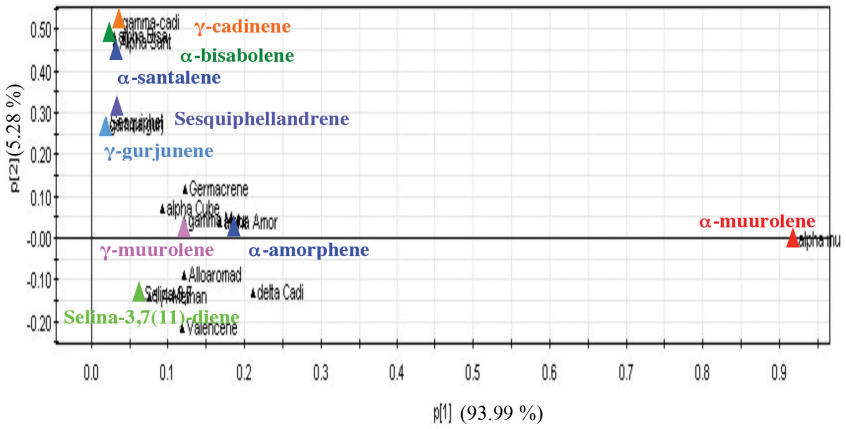

FIGURE 8. PCA loading scatter plot for control treatment (without MeJA) and with 50, 100, 150, and 200 µM MeJA treated *Persicaria minor* suspension culture incubated for 1, 3 and 6 days

compounds of interest through their enhanced release into extracellular medium.

Cai et al. (2012) reported that compounds released into extracellular culture medium are prone to degradation of enzymes (phosphatase acid, DNAse, α-mannosidase, ß-glucosidase, and lipase) which are also secreted into the medium due to lack of storage tissue (vacuole) in undifferentiated cells of suspension cultures. This could probably be one of the explanations for low α-muurolene concentration achieved in liquid medium of this study.

CONCLUSION

Cell suspension culture appears to be a promising technique for *in vitro* production of valuable secondary metabolites. In this study, we achieved sesquiterpene production through MeJA elicitation. Different MeJA concentration treatments with different incubation times affected sesquiterpene production pattern differently. MeJA at 100 µM concentration with short incubation period (1 day) did not trigger production of many sesquiterpene compounds found in longer incubation period (3 days and 6 days) treatments. In general, among 1, 3, and 6 days of incubation periods investigated, 3 days gave higher peak area of sesquiterpene compounds in comparison to 1 and 6 days. Elicitation with 200 mM MeJA incubated for three days were found to have the highest quantity of sesquiterpenes identified with a total number of 12 compounds. However, the compound of interest in this study, α−muurolene was identified with the highest peak area (14.17%) in 100 µM MeJA elicitated treatment incubated for three days. We believe this study will serve as a platform for establishing continuous production of sesquiterpene compounds through elicitation.

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REFERENCES

Ahmad, R., Baharum, S.N., Bunawan, H., Lee, M., Noor, N.M., Rohani, E.R., Ilias, N. & Zin N.M. 2014. Volatile profiling of aromatic medicinal plant, *Polygonum minus* in different tissues and its biological activities. *Molecules* 19(11): 19220-19242.

Antunes, E., Gordo, W.M., de Oliveira, J.F., Teixeira, C.E., Hyslop, S. & De Nucci, G. 2001. The relaxation of isolated rabbit *Corpus cavernosum* by the herbal medicine Catuama and its constituents. *Phytotherapy Research* 15(5): 416-421.

Ashraf, M.F., Che Mohd Zain, C.R., Zainal, Z., Ismail, I., Noor, N.M., Anuar, N., Markom, M. & Ismail, I. 2015. Establishment of *Persicaria minor* hairy roots and analysis of secreted β-caryophyllene in medium broth. *Plant Cell, Tissue and Organ Culture* 121(1): 11-20.

Baharum, S.N., Bunawan, H., Ghani, M.A., Mustapha, W.A.W. & Noor, N.M. 2010. Analysis of the chemical composition of the essential oil of *Polygonum minus* Huds. using two-dimensional gas chromatography-time-of-flight mass spectrometry (GC-TOF MS). *Molecules* 15: 7006-7015.

Bona, C.M., Santos, G.D. & Biasi, L.A. 2012. *Lavandula* calli induction, growth curve and cell suspension formation. *Revista Brasileira de Ciências Agrárias* 7(1): 17-23.

Cai, Z., Kastell, A., Knorr, D. & Smetanska, I. 2012. Exudation: An expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. *Plant Cell Reports* 31(3): 461-477.

Cai, Z., Kastell, A., Mewis, I., Knorr, D. & Smetanska, I. 2011. Polysaccharide elicitors enhance anthocyanin and phenolic acid accumulation in cell suspension cultures of *Vitis vinifera*. *Plant Cell, Tissue and Organ Culture* 108(3): 401-409.

Da Silva, A.L., Piato, A.L.S., Bardini, S., Netto, C.A., Nunes, D.S. & Elisabetsky, E. 2004. Memory retrieval improvement by *Ptychopetalum olacoides* in young and aging mice. *Journal of Ethnopharmacology* 95(2-3): 199-203.

Dörnenburg, H. & Knorr, D. 1995. Strategies for the improvement of secondary metabolome production in plant cell cultures. *Enzyme and Microbial Technology* 17: 674-684.

Dursun, A., Güler, Z., Özkan, D. & Bozdoğan-Konuşkan, D. 2017. Identification of volatile compounds (VCs) in the leaves collected from 'Gemlik', 'Halhalı' and 'Sarı Hasebi' olive tree varieties. *International Journal of Secondary Metabolite* 4(3): 195-204.

Ee, S.F., Mohamed-Hussein, Z.A., Othman, R., Shaharuddin, N.A., Ismail, I. & Zainal, Z. 2014. Functional characterization of sesquiterpene synthase from *Polygonum minus*. *The Scientific World Journal* 2014: 840592.

Gao, F., Yong, Y. & Dai, C. 2011. Effects of endophytic fungal elicitor on two kinds of terpenoids production and physiological indexes in *Euphorbia pekinensis* suspension cells. *Journal of Medicinal Plants Research* 5(18): 4418-4425.

Ismail, I., Gor, M.C., Mohammed-Hussein, Z.A. & Noor, M.N. 2011. Alteration of abiotic stress responsive genes in *Polygonum minus* roots by jasmonic acid elicitation. In *Plants and Environment,* edited by Vasanthaiah Kambiranda, D. Rijeka, Croatia: InTech-Open Access Company. pp. 49-88.

Ito, M., Okimoto, K., Yagura, T., Honda, G., Kiuchi, F. & Shimada, Y. 2005. Induction of sesquiterpenoid production by methyl jasmonate in *Aquilaria sinensis* cell suspension culture. *Journal of Essential Oil Research* 17(2): 175-180.

Kang, S.M., Min, J.Y. & Kim, Y.D. 2006. Effects of methyl jasmonate and salicylic acid on the production of bilobalide and ginkgolides in cell cultures of Ginkgo biloba. *In Vitro Cellular & Developmental Biology-Plant* 42: 44-49.

Kang, S.M., Min, J.Y., Kim, Y.D., Karigar, C.S., Kim, S.W. & Goo, G.H. 2009. Effect of biotic elicitors on the accumulation of bilobalide and ginkgolides in *Ginkgo biloba* cell cultures. *Journal of Biotechnology* 139(1): 84-88.

Keng, C.L., Wei, A.S. & Bhatt, A. 2010. Elicitation effect on cell biomass and production of alkaloids in cell suspension culture of the tropical tree *Eurycoma longifolia*. *Research Journal of the Costa Rican Distance Education University* 2(2): 239-244.

Kumeta, Y. & Ito, M. 2010. Characterization of δ-guaiene synthases from cultured cells of *Aquilaria*, responsible for the formation of the sesquiterpenes in Agarwood. *Plant Physiology* 154(4): 1998-2007.

Liew, P.S. & Hair-Bejo, M. 2015. Farming of plant-based veterinary vaccines and their applications for disease prevention in animals. *Advances in Virology* 209434: 1-12.

Mohamad-Zuldin, N.N., Said, I.M., Noor, N.M., Zainal, Z., Chew, J.K. & Ismail, I. 2013. Induction and analysis of the alkaloid mitragynine content of a *Mitragyna speciosa* suspension culture system upon elicitation and precursor feeding. *The Scientific World Journal* 209434: 1-11.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassay with tabacco cultures. *Physiology of Plant* 15: 473-497.

Ochoa-Villarreal, M., Howat, S., Hong, S., Jang, M.O., Jin, Y.W., Lee, E.K., & Loake, G.J. 2016. Plant cell culture strategies for the production of natural products. *BMB Reports* 49(3): 149-158.

Pépin, M., Archambault, J., Chavarie, C. & Cormier, F. 2004. Growth kinetics of *Vitis vinifera* cell suspension cultures: I. Shake flask cultures. *Biotechnology and Bioengineering* 47(2): 131-138.

Qader, S.W., Abdulla, M.A., Chua, L.S. & Hamdan, S. 2012. Potential bioactive property of *Polygonum minus* Huds (kesum) review. *Scientific Research and Essays* 7(2): 90-93.

Sakhya, P., Marslin, G., Siram, K., Beerhues, L. & Franklin, G. 2017. Elicitation as a tool to improve the profiles of high-value secondary metabolites and pharmacological properties of *Hypericum perforatum*. *Journal of Pharmacy and Pharmacology* 71(1): 70-82.

Shaaltiel, Y., Bartfeld, D., Hashmueli, S., Baum, G., Brill-Almon, E., Galili, G., Dym, O., Boldin-Adamsky, S.A., Silman, I., Sussman, J.L., Futerman, A.H. & Aviezer, D. 2007. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement theraphy of Gaucher's disease using a plant cell system. *Plant Biotechnology Journal* 5(5): 579-590.

Shukor, M.F.A., Ismail, I., Zainal, Z. & Noor, N.M. 2013. Development of a *Polygonum minus* cell suspension culture system and analysis of secondary metabolites enhanced by elicitation. *Acta Physiology Plant* 35(5): 1675-1689.

Siqueira, I.R., Fochesatto, C., da Silva, A.L., Nunes, D.S., Battastini, A.M., Netto, C.A. & Elisabetsky, E. 2003. *Ptychopetalum olacoides*, a traditional Amazonian 'nerve tonic', possesses anticholinesterase activity. *Pharmacology Biochemistry and Behavior* 75(3): 645-650.

Smetanska, I. 2008. Production of secondary metabolites using plant cell cultures. *Food Biotechnology* 111: 187-228.

Srivastava, P., Sisodia, V. & Chaturvedi, R. 2011. Effect of culture conditions on synthesis of triterpenoids in suspension cultures of *Lantana camara* L. *Bioprocess and Biosystems Engineering* 34(1): 75-80.

Vaz, Z.R., Mata, L.V. & Calixto, J.B. 1998. Analgesic effect of the herbal medicine Catuama in thermal and chemical models of nociception in mice. *Phytotherapy Research* 11(2): 101-106.

Vikram, P., Chiruvella, K.K., Ripain, I.H.A. & Arifullah, M. 2014. A recent review on phytochemical constituents and medicinal properties of kesum (*Polygonum minus* Huds.). *Asian Pacific Journal of Tropical Biomedicine* 4(6): 430-435.

Yaacob, K.B. 1987. Kesom oil: A natural source of aliphatic aldehydes. *Perfumer Flavorist* 12: 27-30.

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