CHAPTER III
MATERIALS AND METHODS

3.1 Chemicals

Basal culture media used in this experiment, Murashige and Skoog's (MS) media (see Appendix) was purchased from GIBCOBRL. Plant growth regulators; 2,4-dichlorophenoxyacetic acid (2,4-D), 6-furfurylaminopurine (Kinetin, Kn), napthaleneacetic acid (NAA), 6-benzylaminopurine or N^6-benzyladenine (BA) were purchased from Fluka Laboratories, and thidiazuron (TDZ) was purchased from Riedel-de Haen (Germany). Elicitors; chitosan and methyl jasmonate were purchased from Sigma Chemical co. and Aldrich Chemical Co., respectively. (+)-Saccharose, L-ascorbic acid, and gelling agent (bacteriological agar) were purchased from Fisher chemicals, UNILAB, and GIBCOBRL, respectively. Both organic solvents used through out this experiment (dichloromethane and 95% ethanol) were analytical grade (LAB-SCAN). Water was deionised and distilled for 3 times prior to use.

3.2 Plant materials

Plant species studied through out this experiment were derived from various places as shown in Table 13.

Table 13 Plant species and sources

Plant species	Sources
Artemisia vulgaris var. *indica*	Plant garden, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
Cuminum cyminum	Plant garden, Faculty of Pharmacy, Rangsit University, Patumthani, Thailand
Fortunella japonica	Sireerukachart botanical garden, Mahidol University, Nakornpathom, Thailand
Pogostemon cablin	Plant garden, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

Both seeds of *Cuminum cyminum* and *Fortunella japonica* were grown and fully developed plant harvested.

All of these plant materials were identified by Assoc. Prof. Nijsiri Ruangrungsi, Ph.D., of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

3.3 Essential oil hydrodistillation

Essential oil was determined by the method described in the Association of Official Analytical Chemist (method 962.17, AOAC, 1990). Explants of each plant are shown in Table 14. Each explant was hydrodistillated in Clevenger type apparatus (Fig. 19). The exactly weight of explants was put into a 1000 ml round bottom flask, and distilled water was added into the flask to half-full. This flask was then connected to the apparatus for determination of essential oil. The content in this flask was distilled until two consecutive reading taken at one hour interval showed no change in oil content (approx four to six hours). After cooling, the oil volume was measured, calculated and expressed as millilitre of the oil per one hundred grams of sample. The essential oil was then collected and stored at 4 °C until being analysed for its chemical constituents by Gas Chromatography-Mass Spectrometry (GC-MS).

Table 14 Explants of individual plant species selected for essential oil hydrodistillation

Plant species	**Explants**
Artemisia vulgaris var. *indica*	Leaves
Cuminum cyminum	Fruits
Fortunella japonica	Leaves, peels
Pogostemon cablin	Leaves

Apparatus

The apparatus is constructed of resistant glass of low coefficient of expansion and has the following dimension:

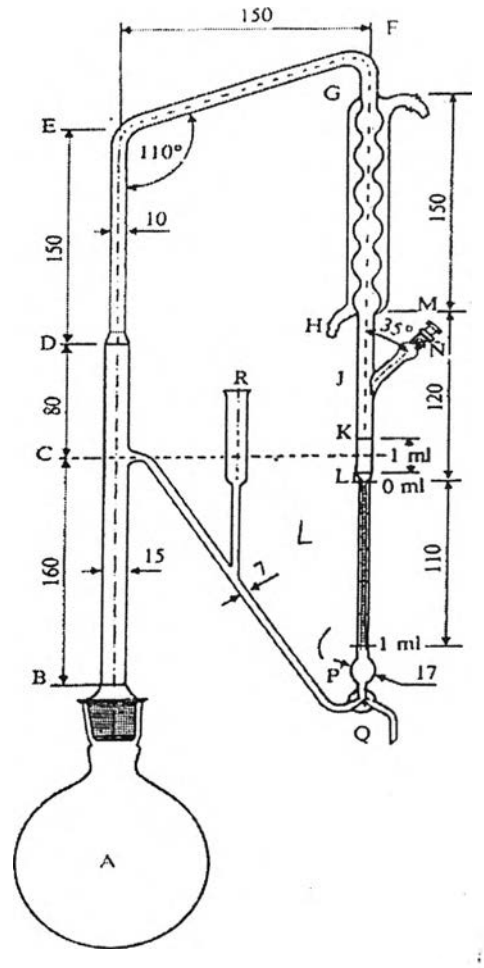
(a) a round-bottom flask (*A*) of suitable capacity with a short, ground-glass neck having an internal diameter of about 29 mm at the wide end;

(b) a condenser assembly that closely fits the flask and consists of the following parts fused into one piece:

- a vertical tube (*BD*), 215 to 265 mm long and 14 to 16 mm in internal diameter,
- a bent tube (*DEF*) in which the distances *DE* and *EF* are each 145 to 155 mm long and 9 to 10 mm internal diameter
- a bulb-condenser (*GH*), 145 to 155 mm long and 9 to 10 mm in diameter at the restrictions,
- a vented stopper (*M*) and a tube (*N*) with an orifice of diameter about 1 mm that coincides with the vent. The wide end of the tube (*N*) is ground-glass, having an internal diameter of 10 mm,
- a tube (*HJ*), 75 to 85 mm long and 9 to 10 mm in internal diameter, making a 30 to 40 angle (*HJN*) with the tube (*JN*),
- a graduated tube (*LP*), graduated over 105 to 115 mm to give 1 ml subdivide in 0.01 ml. Above the graduation are two circular marks (*K* and *L*),
- a bulb-shaped swelling (*P*), about 2 ml in capacity,
- a three-way tap (*Q*), and
- a connecting tube (*CQ*), 7 to 8 mm internal diameter, fitted in the middle with a filling funnel (*R*). The junction (*C*) is at a level 20 mm higher than the uppermost graduation;

(c) a suitable heating device, allowing a fine control; and

(d) a vertical support with horizontal ring covered with insulation material.



Before use, clean the apparatus by successive washings with acetone, water and chromic acid cleansing mixture inverting several times, and rinse with water. Drain the apparatus and mount it in a place protects from a draught.

Figure 19 Clevenger type apparatus

3.4 Identification of essential oil obtained from hydrodistillation by using Gas Chromatography-Mass Spectrometry (GC-MS)

Each of the essential oil was diluted to 1:100 in methanol before being injected into GC-MS system. The condition of GC-MS was described as following. The spectrum was recorded and compared with the terpenes library (Adams, 1995).

One μl of the diluted essential oil was chromatographed by a Varian Saturn III, with the high performance fused silica capillary column; the film thickness was 0.25 μM, the length of column was 30 m and its internal diameter was 0.25 mm. The carrier gas used was helium (1 ml/min). The oven temperature programming was 60-240 °C at 3°C/min. The injector and detector temperature was 240°C and 280°C, respectively.

3.5 Media preparation for plant tissue culture experiments

3.5.1 Nutrition media

Murashige and Skoog's media (MS) was used as basal media through out this experiment. The compositions of this culture media were described in part B of Appendix and without any plant growth regulators.

For plant growth regulators, auxins and cytokinins were used at various concentrations. These were;

Auxins:	α-Napthaleneacetic acid (NAA)
	2,4-Dichlorophenoxyacetic acid (2,4-D)
Cytokinins:	6-Benzylaminopurine or N^6-Benzyladenine (BA)
	6-Furfurylaminopurine (Kinetin, Kn)
	Thidiazuron (TDZ)

3.5.2 General preparation of semi-solid and liquid media

Approximately 800 ml of distilled water was measured and poured into a glass beaker. The basal media were then slowly added and gentle continuous stirred by using magnetic stirrer. After media completely dissolved, the requirement of sucrose, L-ascorbic acid, and plant growth regulator were added to the solution. The pH was adjusted to 5.7-5.8 with few drops of 1M sodium hydroxide and 1M hydrochloric acid. The solution was made up to 1 l with distilled water. In order to get the semi-solid medium, the gelling agent was then added at this time. Final concentrations of sucrose, L-ascorbic acid and gelling agent were 3% (w/v), 0.005% (w/v), and 10% (w/v), respectively. The solution was then heated and stirred to dissolving gelling agent completely. The media were poured into a suitable container. Semi-solid media were poured into 250-mL screw capped wide mouth bottles, and liquid media were poured into 250-mL Erlenmeyer flasks which were covered with aluminium foil. Both of them were then autoclaved at 121 °C (15 lb/inch^2 or 1 kg/cm^2) for 15 minutes. They were stored at room temperature prior to use.

3.6 Aseptic work

All the manipulations were carried out under aseptic conditions using a laminar flow cabinet with horizontal flow of sterilised air.

3.7 Germination of seedlings

Seedling of *Cuminum cyminum* and *Fortunella japonica* were germinated aseptically techniques as follows:

Fruits of *Cuminum cyminum* and seeds of *Fortunella japonica* were surface sterilised by dipping in 70% ethanol and shaking with 30% (w/v) hydrogen peroxide which containing Tween 80 as wetting agent, for 2-5 min, and then they were washed with sterilised distilled water for 3 times. Aseptic plant materials were aseptically transferred to pre-sterilised glass petri dishes, each one containing two pieces of Whatman No.1 filter paper and containing about 20 ml distilled water. Germination was carried out by incubation in the dark at 25 ± 2 °C. After they had germinated, they were put in 12 hour light/dark intervals to develop strong seedlings.

3.8 Preparation of leaf explants and surface sterilisation

Leaf explants of *Artemisia vulgaris* var. *indica* and *Pogostemon cablin* were surface sterilised by following methods prior to use in callus initiation.

Leaves of *Artemisia vulgaris* var. *indica* and *Pogostemon cablin* were surface sterilised by shaking with surface sterilising agent, various concentration of hydrogen peroxide or Clorox® containing Tween 80 as wetting agent (surfactant) for various times, depending on appearance and speciality of individual leaf explants. The compositions of surface sterilising agent were described in part C of Appendix. After that, they were washed three times with sterilised distilled water and then ready to used in callus initiation.

3.9 Initiation and maintenance of callus cultures

When individual seedlings were grown to over 1 cm, they were appropriated for use in callus initiation. Seedlings or surfaced sterilised leaf explants were cut into 1 x 1 cm^2 and aseptically transferred to the required semi-solid basal MS media containing L-ascorbic acid, sucrose and suitable plant growth regulators. They were gently pressed into the agar, and then incubated at the temperature of 25 ± 2 °C under dark or light conditions for various plants by using of cool white fluorescent tubes. The maintenance of callus cultures was carried out by incubating them at the same conditions for various plants, and subculturing old cultures to the new semi-solid basal MS media every 30-45 days, depending on the individual cell growth. When

the cultures grew to 3-4 cm^3 or media showed sigh of exhaustion and dehydration, they were selected healthy pieces, and aseptically transferred to 250-ml screw capped wide mouth bottles containing 50 ml of new media.

3.10 Initiation and maintenance of suspension cultures

After the fourth generation, 3-4 cm^3 of healthy callus cultures were aseptically transferred to Erlenmeyer flask containing 50 ml of liquid media and agitated with a magnetic stirrer (placed in the liquid media before autoclaving), to enable formation of suspension of single cells and small aggregates. Then these flasks were put on an orbital shaker, circulating at 120 rpm with the temperature of 25 ± 2 °C under dark or light conditions for various plants by using cool white fluorescent tubes. The suspension cultures were maintained by subsequently subculturing with a dilution of 1:4 to a new fresh liquid media at about 15-21 days, depending on individual cell growth.

3.11 Measurement of growth parameter

Growth of callus and cell suspension cultures can be monitored by increasing in fresh or dry weight or cell number. For cell suspension cultures, increase in packed cell volume (PCV) or cell volume after sedimentation (CVS) is also a good indicator of growth (Franklin and Dixon, 1994).

3.11.1 Fresh and dry weight measurements

For callus culture, fresh weight was measured by transferring the entire callus (scrape off the medium) to a pre-weighed weighing boat (or container) and determines the fresh weight. For cell suspension cultures, collect the cells on a pre-weighed nylon membrane and determine the fresh weight (determine the weight of water retained by the membrane separately and subtract this amount from the measured fresh weight). Alternatively, transfer the entire contents of the cell culture flask to pre-weighed centrifuge tube. Spin the tube for 5-10 minutes at 200 g. Examine the supernatant for cells. If cells are present in the supernatant, repeat centrifugation until the supernatant is free from cells. Then carefully pipette out the entire medium without disturbing the pellet. Weigh the centrifuge tube with cells to determine the

fresh weight. After measuring the fresh weight, dry the samples in an oven at 60°C until no change in dry weight is observed.

Increase in fresh weight can also be measured without sacrificing the samples at the beginning of an experiment. Transfer callus or cells to pre-weighed Petri dishes or culture flask containing the medium. Weigh the Petri dish or the flask again and determine the weight of callus or cells added. At the end of an experiment, remove the entire callus or cells from a suspension culture and determine the final weight (Franklin and Dixon, 1994).

3.11.2 Packed cell volume (PCV) or cell volume after sedimentation (CVS)

The method which was used for measuring the growth of suspension cultures was to measure the height of the cell mass along the flask wall with a ruler fixed in an apparatus which kept the Erlenmeyer flask tilted in a fixed position (normally 60° for 250 ml flask). Finally the volume of the cells was defined as the cell volume after sedimentation (CVS) and expressed per flask suspension (Blom *et al.*, 1992). Fig. 20 shows the apparatus for measurement of the cell growth of *C. cyminum* suspension cultures.

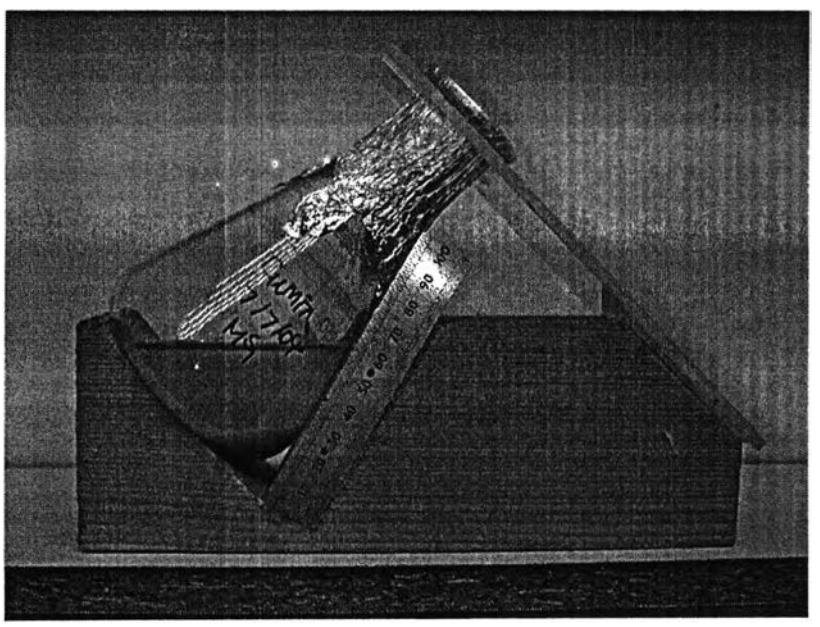


Figure 20 The apparatus for measuring cell growth of *Cuminum cyminum* cell suspension cultures

3.12 Study on the effect of plant growth regulators on callus formation and appearance

Various types and concentrations of plant growth regulators in MS media were varied to study their effects on callus formation of the investigated plants. Two auxins (NAA and 2,4-D) and two cytokinins (BA and Kn) were used at the concentration of 0.1-1 mg/l. The effect of various plant growth regulators on callus formation was measured by investigating the individual growth and determination of the fresh weight (FW) and dry weight (DW).

3.13 Study on the effect of light on callus formation and appearance

Callus cultures of various plants were incubated at temperature of 25 ± 2 °C under different light conditions; 24-h light, 12-h light/12-h dark, and 24-h dark. The effect of various light conditions on callus formation was measured by investigating the individual growth and determination of the fresh weight (FW) and dry weight (DW).

3.14 Study on chemical constituents of essential oil produced by plant cell cultures

3.14.1 Extraction of chemical constituents of essential oil produced by plant cell cultures

Extraction of callus cultures for their volatile constituents involved initial separation of callus and media, these were prefrozen, and then freeze dried for over 24 h using freeze dryer. The dried materials were ground with a pestle and mortar into fine powders. A known amount of dried materials was extracted with sufficient redistilled dichloromethane to produce a suspension of the powder. This suspension was then filtered and evaporated under nitrogen and concentrate to 100 µl.

Cell suspension cultures were extracted either directly or after separation of the cells and liquid media. After suspension were filtered, extraction of cells was carried out using method mention above, and media were extract directly using a certain amount of redistilled dichloromethane. Alternatively, whole suspension cultures were extracted with redistilled dichloromethane by stirring with a magnetic stirrer on an orbital shaker for 3 h. Following the phase separation, the volume of redistilled dichloromethane was reduced with nitrogen flow to 100 µl.

3.14.2 Analysis of essential oil constituents by Gas Chromatography (GC)

1 µl of the concentrated extract prepared above was chromatographed by gas chromatography. A Hewlett Packard 5890 Gas Chromatography, fitted with a flame ionization detector (FID), was used for analysis of essential oil constituents. The column used was high performance fused silica linked methyl silicon capillary column; the film thickness was 0.25 µM, the length of column was 30 m and its internal diameter was 0.22 mm. The carrier gas was a mix ratio of hydrogen : oxygen : nitrogen (25 : 20 : 45). The results were recorded on a Hewlett Packard 3392A integrator. Normally, the oven temperature programming was 50-250°C at 7°C / min, and isothermal for 3 min. The injector and detector temperature was 250°C and 280°C, respectively.

3.14.3 Identification of essential oil constituents produced by plant cell culture by Gas Chromatography-Mass Spectrometry (GC-MS)

Determination of mass spectra was carried out using Kratos Concept 25 spectrometer operated at 70 eV, equipped with a Sun Mash 3 computer data output. The operating conditions of gas chromatography were similar to those used in GC analysis, but with helium as carrier gas. Mass spectrometer conditions were as follow; ionisation current, 1 A; ionisation potential, 70 eV; source temperature, 150 °C; resolution, 1000; scan speed, 1 sec/decade. Identification was based on sample retention time data, and electron impact mass spectra (EI-MS). Comparison was made with those held on Masslynx software, relevant references and literature reviews (Jenning and Shibamoto, 1980, Adam, 1995, Silverstein and Webster, 1998).

3.15 Shoot regeneration (Organogenesis)

Various types and concentrations of plant growth regulators in MS media were varied to study their effect on organ formation. Two auxins (NAA and 2,4-D) and three cytokinins (BA, Kn, and TDZ) were used at the concentration of 0.5, 1, 2, 3, and 5 mg/l. The effect of various plant growth regulators on shoot formation was measured by investigation of individual growth of various plants, fresh weight determination, and dry weight determination.

3.16 Methods for improving chemical constituents of essential oil produced by plant cell cultures

3.16.1 Feeding precursor and biotransformation

3.16.1.1 Substrate feeding and extraction of biotransformation products from suspension cultures

Standard substrates were obtained from commercial sources, with the purity being over 98%. They were mixed with 70% ethanol to get clear solutions, and added to the suspension cultures by passing through preautoclaved Mobile Phase Filters (0.45 μm pore size, Whatman). The final concentrations of the substrates were 100 ppm. The control readings were made without substrate added to cultures, and media added substrates without cell cultures. The cultures were incubated in the conditions mentioned above in 3.10.

After cultures were harvested, suspension cultures were analysed using the method in 3.14, and the yields of biotransformation products were calculated by comparing the peak heights of products with total peak height from integration of GC chromatogram.

3.16.1.2 Time course study

For time course study, 5 ml samples were taken using sterile 5 ml pipette in sterile conditions at regular time intervals. They were placed into 15 ml screw stopped test tubes containing 1 ml of n-pentadecane (76.9 ppm) as internal standard, shaken for 3 h with 5 ml of dichloromethane, and then centrifuged at 3,000 rpm for 10 min. The organic layer was passed through a Whatman No.1 filter paper, then evaporated under nitrogen flow to concentrate to 100 μl, and subjected to analysis using GC. The concentrations of substrate and biotransformation products were calculated by using normalisation method.

3.16.1.3 Feeding precursors of each major chemical constituents in individual cell suspension cultures

In attempt to increase level of major chemical constituents, the precursor of each compound was fed into cell suspension cultures in the early of stationary phase. Each precursor has been shown below in Table 15.

Table 15 Precursors fed into individual cell suspension culture attempt to increase level of major constituent

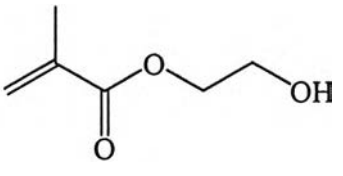
Plant species	Major chemical constituents	Precursors
Artemisia vulgaris var. *indica*	(+)-davanone	geraniol
Fortunella japonica	d-limonene	geraniol, nerol, linalool
Cuminum cyminum	cuminaldehyde	gerniol, cuminol
Pogostemon cablin	patchouli alcohol	farnesol

3.16.1.4 Use of HEMA co-polymers for biotransformation

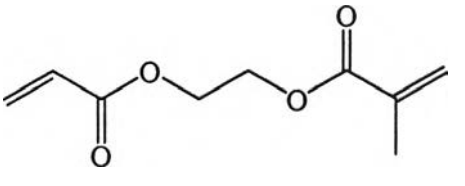
3.16.1.4.1 Preparation of p-HEMA discs

A certain amount of 2-hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) were mixed with redistilled water in test tube, and nitrogen passed through the tube for 30 minutes, then the polymer cylinder was produced by radiation (1 M Rad dose ^{60}Co, 18 hr). The resulting cylinder was cut into thin discs (1.4 cm diameter and 1 mm thickness). Fig. 21 shows the structures of the monomer and crosslinking agent. Substrates were loaded by mixing with the monomers before the polymerisation. Concentrations of substrate and products in the polymer discs were examined as follows:

Discs were left in redistilled dichloromethane for several days until no volatile compounds were released into organic phase. Then the dichloromethane layer was concentrated to 100 μl under nitrogen prior to GC analysis.



2-Hydroxyethyl methacrylate
(HEMA)



Ethylene glycol dimethacrylate
(EGDMA)

Figure 21 Chemical structure of 2-hydroxyethyl methacrylate (HEMA); a monomer, and ethyleneglycol dimethacrylate (EGDMA); a cross linking agent

3.16.1.4.2 Substrate feeding using p-HEMA discs

Discs containing substrates were sterilized with 30% (w/v) hydrogen peroxide for 1 minute, they were then washed with sterilized distilled water, and placed in cell suspensions cultures, 7 days after subculture. The control experiments were carried out by addition of a disc containing substrate to the media without cell cultures, and addition of a disc without substrate to cell cultures, under the same conditions as cell suspension cultures throughout the experiment.

The concentrations of products and substrate were monitored by removing 5 ml aliquots from the suspensions at fixed time intervals, and extracting and analyzing substrates and biotransformation products according to the method outlined in 3.14.

3.16.2 Plant growth regulators

Various types and concentrations of plant growth regulators in MS media were varied to study their effect on callus formation and essential oil production of the investigated plants. Two auxins (NAA and 2,4-D) and three cytokinins (BA, Kn, and TDZ) were used at the concentration 0.1-1 mg/l. The effect of various plant growth regulators on essential oil production was measured by method described in 3.14

3.16.3 Light

Callus cultures were incubated at the temperature of 25 ± 2 °C under different light conditions; 24-h light, 12-h light/12-h dark, and 24-h dark. After forth generation, callus cultures were harvested and essential oil constituents were obtained and measured by method described in 3.14

3.16.4 Elicitation

Chitosan and methyl jasmonate (MEJA) were used as elicitors in this experiment.

3.16.4.1 Elicitation with chitosan

One gram of chitosan (Sigma Chemical Co.) was purified by dissolving in 90 ml of 0.1 M acetic acid, and centrifuged for 30 min, and then the insoluble fractions were discarded. This procedure was performed four times. After centrifugation, the supernatant was precipitated by adjusting its pH to 8.0. The precipitate was washed with distilled water and dried at 60°C. The dried solid was ground to obtain a purified chitosan powder. Chitosan solution was obtained by dissolving purified chitosan powder in 0.1 M acetic acid (1 g chitosan/90 mL acetic acid) and adjusting the pH to 5.0. Chitosan concentration was varied from 50 to 400 ppm for determination of optimum concentration. Suspension cultures of *Cuminum cyminum* and *Fortunella japonica* were used over for 21 days under the conditions as described above, except that chitosan was added to the cell cultures unless otherwise stated. The control experiment was carried out by adding each concentration of chitosan to media without cells under the same conditions as the cultures. The essential oil constituents were obtained and measured by the method described in 3.14, after 7 days, 14 days, and 21 days.

3.16.4.2 Elicitation with methyl jasmonate (MEJA)

Suspension cultures of *Cuminum cyminum* and *Fortunella japonica* were subcultured to new media and then 10 ppm, 50 ppm, and 100 ppm MEJA (in 70% ethanol solution) were added to cultures through a preautoclaved Mobile Phase Filter (0.45 μM pore size, Whatman) individually. The control experiment was carried out by adding each concentration of MEJA to media without cells under the same conditions as the cultures. The essential oil constituents were obtained and measured by method described in 3.14, after 7 days, 14 days, and 21 days.

3.16.5 Permeabilisation

Tween-20 was used as permeabilising agent in this experiment. When these suspension cultures were harvested, they were subcultured to new media containing with various concentrations of Tween-20 (0.5% w/v, 1% w/v, 1.5% w/v, and 2% w/v). Then essential oil constituents were obtained and measured by method described in 3.14, after 7 days, 14 days, and 21 days.

3.16.6 *In situ* product removal (two-phase system)

n-Hexadecane was used as the second phase for accumulating essential oil constituents released in culture media. 10 mL of *n*-hexadecane was added into new media and autoclaved by method described in 3.5.2 prior to use. Each of suspension culture was subcultured into new media containing *n*-hexadecane and essential oil constituents were obtained and measured by method described in 3.14, after 7 days, 14 days, and 21 days.