UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF FLAVONOIDS IN PEGAGA (CENTELLA ASIATICA L. URBAN) CELL SUSPENSION CULTURE

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

September 2010

DEDICATED TO:

FATHER, MOTHER, BROTHERS & JASON CHEE

WHO ALWAYS HAVE FAITH IN ME

AND

THEIR SUPPORTS HAVE GUIDED ME TO GONE THROUGH

ALL THE OBSTACLES IN LIFE

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PRODUCTION OF FLAVONOIDS IN PEGAGA (*CENTELLA ASIATICA* L. URBAN) CELL SUSPENSION CULTURE

By

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Septemeber 2010

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Centella asiatica L. Urban (Umbelliferea), locally known as 'Pegaga' was an herbal plant that had been used in traditional medicine in Asia for many centuries. Its medicinal values are attributed to the presence of flavonoid compounds. Since there was still no information available on the flavonoid production in cultured tissues, studied have been carried out in evaluating the distribution of the flavonoid content particularly kaempherol, quercertin, luteolin and apigenin in intact plants of the four accessions of *C. asiatica* collected several locations in Malaysia as well as in callus and cell suspension cultures. The total flavonoid content has been determined by using spectrophotometry methods. The flavonoid compounds present in the cell cultures have been analyzed with thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) techniques.

Results obtained from the studies revealed that the four accessions of *C. asiatica* which showed distinctive differentiation in morphological characteristic were

differed in the biochemical constituents especially in their flavonoid contents. The flavonoid constituents were detected in the range of 1.93 ± 0.094 to 8.99 ± 0.346 mg/g dry weight in the whole plant tissues. Flavonoids were also successfully detected in callus (0.98 ± 0.097 to 2.46 ± 0.021 mg/g dry weight) and cell suspension culture (0.67 ± 0.056 to 0.89 ± 0.044 mg/g dry weight). The flavonoid content found in cultured tissues were lower that that produced in the intact plant tissues. In the TLC analysis for the leaf tissues of intact plant, the possible of flavonoid compound might presented were kaempherol, naringerin, luteolin, (+)-catechin and rutin. Similar results were also been observed in callus, cell suspension and cell suspension media in the TLC analysis. Further investigation using HPLC showed that leaf tissues of intact plant accession UPM03 has rutin, luteolin, quercertin and kaempherol while others accession only have rutin or/and kaempherol. An additional flavonoid compound called apigenin was detected in callus and cell suspension. As a result, culture conditions such as different cell size aggregations, pH values, light, inoculum sizes as well as interaction between plant growth regulators have been assessed to optimize and enhance the selected flavonoid production in the chosen cell suspension cultures. Cell suspension UPM03 in aggregation size of 250-500 μm with the highest cell growth rate (0.69 g fresh weight/day) was suitable to produce flavonoid in 100 mL shake flask of *C. asiatica* suspension cells. The cells grow better and produce more flavonoid (3.66 ± 0.13 mg/g dry weight) under the initial pH of 5.7 with the presence of light when supplemented with 3 mg/L 2,4-D and 1 mg/L kinetin. In addition, the responses of cell to selected elicitors and precursors have also been investigated. The biosynthesis of flavonoid was elicited by the addition of 1mg/L of both yeast extract (7.32 ± 0.17 mg/g dry weight) and salicylic

UPM

acid (7.11 ± 0.16 mg/g dry weight). Elicition with 400 µM methyl jasmonate have increased flavonoid content to 7.82 ± 0.2 mg/g dry weight. However, the addition of 1mg/L casein hydrolysate (4.91 ± 0.15 mg/g dry weight) and chitosan (4.29 ± 0.13 mg/g dry weight) have no significant effect if compared to the control culture (5.08 ± 0.22 mg/g dry weight). The usage of 60 mg/L phenylalanine and 40 mg/L tyrosine have resulted in 14-fold and 3-fold increase in production of flavonoid, respectively. Further study on the synergistic effect of combination of precursor (60 mg/L phenylalnine) and elicitor (400 µM) showed that the cell suspension UPM03 not only produce the highest flavonoid content in cell (67.07 ± 6.47 mg/g DW) but also in the media (7.73 ± 0.12 mg/g). Thus, it was proven that the synergistic bioprocess was a very useful strategy to enhance the flavonoid production particularly luteolin in *in vitro* cultures of *C. asiatica*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN FLAVONOID MELALUI AMPAIAN SEL PEGAGA (*CENTELLA ASIATICA* L. URBAN)

Oleh

TAN SUAT HIAN

September 2010

Pengerusi: Professor Maziah Mahmood, PhD

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Centella asiatica L. Urban (Umbelliferea) yang dikenali sebagai pegaga oleh masyarakat tempatan merupakan sejenis pokok herba yang telah digunakan selama beberapa abad di negara Asia sebagai ubat tradisional. Nilai perubatannya adalah disebabkan kehadiran kompoun flavonoid. Oleh kerana ketiadaan maklumat dalam penghasilan flavonoid dalam tisu yang telah dikulturkan, maka kajian telah dijalankan untuk mengenalpasti taburan flavonoid terutamanya kaempferol, kuersetin, luteolin dan apigenin dalam 4 aksesi pokok induk *C. asiatica* yang telah dikumpulkan dari beberapa kawasan di Malaysia serta dalam kultur kalus and sel ampaian. Jumlah kandungan flavonoid telah ditentukan dengan penggunaan cara spectrofotometri. Komposisi flavonoid dalam kultur sel telah dianalisis dengan teknik TLC dan HPLC.

Keputusan telah menunjukkan bahawa 4 aksesi *C. asiatica* mempunyai pembezaan dari segi ciri-ciri morfologi yang menonjol dan berbeza dalam komposisi biokimia

terutamanya dalam kandungan flavonoid. Kandungan flavonoid dalam pokok induk dikesan dalam lingkungan 1.93 ± 0.094 ke 8.99 ± 0.346 mg/g berat kering. Kandungan flavonoid juga berjaya dikesan dalam kalus (0.98 ± 0.097 ke 2.46 ± 0.021 mg/g berat kering) dan sel ampaian (0.67 ± 0.056 ke 0.89 ± 0.044 mg/g berat kering). Kandungan flavonoid yang dikesan dalam kultur tisu adalah lebih rendah daripada pokok induk. Dalam analisi TLC untuk daun anak pokok, flavonoid kompound yang mungkin hadir ialah kaempferol, naringerin, luteolin, (+)-katekin dan rutin. Keputusan yang sama juga dikesan dalam kalus, sel ampaian dan media sel ampaian dalam TLC analisis. Kajian seterusnya dengan HPLC menunjukkan tisu daun anak pokok aksesi UPM03 mempunyai rutin, luteolin, kuersetin dan kaempferol manakala aksesi lain hanya mempunyai rutin atau/dan kaempherol. Oleh itu, keadaan kultur seperti saiz gumpalan yang berbeza, nilai pH, kehadiran cahaya, saiz inokula serta interaksi hormon telah ditaksir untuk mengoptimumkan dan menambah penghasilan flavonoid yang terpilih dalam kultur sel ampaian yang dipilih. Sel ampaian dengan saiz gumpalan 250-500 µm dengan kadar penumbuhan yang paling tinggi (0.69 g berat basah/hari) sesuai digunakan untuk penghasilan menggunakan 100 mL kalalang goncang. Sel–sel ampaian ini tumbuh dengan lebih baik dan menghasilkan lebih banyak flavonoid (3.66 ± 0.13 mg/g berat kering) dalam keadaan pH 5.7 diikuti dengan kehadiran cahaya apabila ditambahkan dengan 3 mg/L 2,4-D dan 1 mg/L kinetin. Tambahan pula, kajian telah dijalankan pada tindakbalas sel terhadap penggunaan elisitor dan pembekalan prekursor tertentu. Penghasilan flavonoid telah digalakkan dengan penambahan 1 mg/L kedua-duanya ekstrak ragi (7.32 ± 0.16 mg/g berat kering) dan asid salisailik (7.11 ± 0.16 mg/g berat kering). Penggunaan 400 µM metil jasmonat telah menambahkan kandungan

flavonoid ke 7.82 ± 0.2 mg/g berat kering. Walau bagaimanapun, penambahan 1 mg/L kasin hydrolisat (4.91 ± 0.15 mg/g berat kering) dan kitosan (4.29 ± 0.13 mg/g berat kering) tidak mempunyai kesan yang ketara jika dibandingkan dengan sampel kawalan (5.08 ± 0.22 mg/g berat kering). Penggunaan 60 g/L fenilalanina dan 40 mg/L tirosina telah meningkatkan penghasilan flavonoid masing-masing sebanyak 14-kali ganda dan 3-kali ganda. Kajian tentang kesan sinergistik dalam kombinasi prekursor dan elisitor menunjukan ia adalah strategi yang amat berguna dalam meningkatkan penghasilan flavonoid terutamanya luteolin dalam kultur *in vitro C. asiatica*. Kajian seterusnya dalam kesan kombinasi precursor (60 mg/L phenylalanina) dan elisitor (400 µM tyrosina) menunjukkan sel ampaian UPM03 bukan sahaja menghasilkan kandungan flavonoid yang terbanyak dalam sel (67.07 ± 6.47 mg/g berat kering) malahan dalam media (7.73 ± 0.12 mg/g). Kesimpulan, proses penggabungan penggunaan ialah satu strategi untuk meningkatkan penghasilan flavonoid terutamanya luteolin dalam kultur *C. asiatica.*

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I certify that an Examination Committee met on 7th September 2010 to conduct the final examination of Tan Suat Hian on her Doctor of Philosophy thesis entitled "Production of Flavonoids in *Centella asiatica* L. urban (Pegaga) Cell Suspension Culture" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations that have been duly acknowledged. I also declare that it has not been previously and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

TAN SUAT HIAN

Date: 6/9/10

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