CALLUS INDUCTION FROM EXPLANTS OF EURYCOMA LONGIFOLIA JACK (TONGKAT ALI) AND THE PRODUCTION OF 9-METHOXYCANTHIN-6-ONE FROM THE INDUCED CALLUS

ROSLI BIN NOORMI.

FBSB 2005 26
CALLUS INDUCTION FROM EXPLANTS OF
EURYCOMA LONGIFOLIA JACK
(TONGKAT ALI) AND THE PRODUCTION OF
9-METHOXYCANTHIN-6-ONE
FROM THE INDUCED CALLUS

ROS LI BIN NOORMI

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2005
CALLUS INDUCTION FROM EXPLANTS OF *EURYCOMA LONGIFOLIA* JACK (TONGKAT ALI) AND THE PRODUCTION OF 9-METHOXYCANTHIN-6-ONE FROM THE INDUCED CALLUS

ROSNI BIN NOORMI

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

June 2005
A study was carried out to determine 9-methoxycanthin-6-one distribution in intact plants and callus cultures of *Eurycorna longifolia* Jack. Qualitative analysis using TLC revealed that 9-methoxycanthin-6-one was present in leaf, petiole, stem, rachis, tap root, fibrous root, cotyledon and zygotic embryo of intact plant. The quantitative analysis using HPLC showed that the highest concentration of 9-methoxycanthin-6-one content was found in tap root. It was 4.10 mg/g DW tissues.

9-methoxycanthin-6-one was also present in callus tissues derived from different explants. The highest concentration was detected in fibrous root-derived callus (7.12 mg/g DW tissues). From the comparison between the data of callus tissues and intact plant parts, higher concentration of 9-methoxycanthin-6-one, more than 73.7 % was detected in callus tissues.
The ability of the callus to produce 9-methoxycanthin-6-one in different types of basal media (Murashige and Skoog, Gamborg, Schenk and Hildebrandt and White) were examined and identified. A basal MS medium exhibited the highest 9-methoxycanthin-6-one content (3.84 mg/g DW tissues). Hence, MS medium was selected for subsequent studies. The study on the effect of MS medium strength (quarter, half, full and double strength) on 9-methoxycanthin-6-one production showed that for full strength of MS, 4.97 mg of the compound per DW tissues could be obtained from callus cultured in quarter MS basal media.

The effects of five different carbon sources such as sucrose, glucose, fructose, sorbitol and mannitol [(0, 1.0, 2.0, 3.0, 4.0 and 5.0 % (w/v)] on 9-methoxycanthin-6-one production were studied separately. Two % (w/v) fructose promoted the production of 9-methoxycanthin-6-one (4.59 mg/g DW) and gained the highest yield compared to other carbon sources tested.

A series of studies was also carried out to examine the effects of various concentrations [(0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L in full strength MS medium supplemented with 3 % (w/v) sucrose)] of PGRs (plant growth regulators auxins) (2,4-D, picloram, dicamba, NAA and IAA) on callus growth and 9-methoxycanthin-6-one production. The addition of 3.0 mg/L dicamba increased the 9-methoxycanthin-6-one production (12.3 mg/g DW tissues).
The effects of different initial pH values (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) on growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* Jack callus cultures was observed. The highest 9-methoxycanthin-6-one production was obtained at pH 5.5 (1.53 mg/g DW tissues).

Feeding of each amino acids (DL-tryptophan, L-phenylalanine and L-tryrosine) at a series of concentrations (0, 1x10⁻⁴, 1x10⁻³, 1x10⁻², 1x10⁻¹, 1.0, 1x10¹, 1x10², 1x10³, 1x10⁴ μM) was observed to reduce the callus biomass growth as well as the 9-methoxycanthin-6-one production. The production of 9-methoxycanthin-6-one (2.34 mg/g DW tissues) in callus cultures also increased when the medium was supplemented with 1x10⁻¹ μM phenylalanine.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

INDUKSI KALUS DARIPADA EKSPLAN
_Eurycoma longifolia_ Jack (Tongkat Ali) DAN
PENGHASILAN 9-METOKSIKANTIN-6-ON
DARIPADA KALUS YANG TELAH DIINDUKSI

Oleh

**ROSNI BIN NOORMI**

_Jun 2005_

Pengerusi : Profesor Maziah Mahmood, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Kajian telah dijalankan untuk menentukan taburan 9-metoksikantin-6-on di dalam pokok induk dan kultur kalus _Eurycoma longifolia_ Jack. Analisa secara kualitatif menggunakan TLC telah menunjukkan bahawa 9-metoksikantin-6-on hadir di dalam daun, petiol, batang, rakis, akar tunjang, akar serabut, kotiledon, dan zigot embrio daripada pokok induk. Analisa secara kuantitatif menggunakan HPLC pula telah menunjukkan bahawa kepekatan tertinggi sebatian 9-metoksikantin-6-on (4.10 mg/g berat kering) telah hadir di dalam pokok induk iaitu di dalam akar tunjang. 9-metoksikantin-6-one juga telah di kesan hadir dalam tisu kalus daripada eksplan yang berbeza. Kepekatan tertinggi (7.12 mg/g berat kering) telah dikesan di dalam kalus akar serabut. Keputusan menunjukkan bahawa kepekatan 9-metoksikantin-6-on telah di dapati lebih tinggi (73.66%) di dalam tisu kalus berbanding daripada pokok induk.
Keupayaan kalus untuk menghasilkan 9-metoksikantin-6-one di dalam jenis media asas MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), WH (White, 1963) dan B5 (Gamborg et al., 1968)) yang digunakan telah diperiksa. Didapati bahawa media asas MS mempamerkan kandungan 9-metoksikantin-6-one tertinggi (3.84 mg/g berat kering). Media MS telah digunakan untuk kajian selanjutnya. Sementara itu, kajian terhadap kesan media MS ke atas kekuatan media (suku, separuh, penuh dan dua kali ganda kekuatan) telah menunjukkan bahawa penghasilan 9-metoksikantin-6-one tertinggi (4.97 mg/g berat kering) telah diperolehi daripada kultur kalus di dalam suku kekuatan media asas MS.

Kesan lima sumber karbon yang berbeza seperti sukrosa, glukosa, fruktosa, sorbitol dan manitol [0, 1, 2, 3, 4, dan 5 % (w/v)] ke atas penghasilan 9-metoksikantin-6-one telah dikaji satu persatu. Pemberian fruktosa pada 2% (berat/ispadu) telah mempamerkan penghasilan 9-metoksikantin-6-one (4.59 mg/g berat kering) dan merupakan penghasilan tertinggi berbanding dengan sumber-sumber karbon yang dikaji.

Kajian juga telah dijalankan untuk memeriksa kesan pelbagai kepekatan [(0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) dalam media asas MS penuh dibekalkan dengan 3 % (berat/ispadu) sukrosa] pengatur pertumbuhan tumbuhan (PGRs) (2,4-D, pikloram, dikamba, NAA and IAA) pada pertumbuhan kalus dan penghasilan 9-metoksikan-6-on. Penambahan 3.0 mg/L dikamba telah meningkatkan penghasilan 9-metoksikan-6-on (12.3 mg/g berat kering).
Kesan perbezaan pH awal (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) pada pertumbuhan kultur kalus dan penghasilan 9-metoksikan-6-on daripada Eurycoma longifolia Jack telah diperhatikan. Penghasilan 9-metoksikan-6-on tertinggi telah berupaya diperolehi pada pH 5.5 (1.53 mg/g berat kering).

Pembekalan setiap asid amino (DL-triptofan, L-fenilalanina and L-tirosina) pada kepekatan (0, 1x10^{-4}, 1x10^{-3}, 1x10^{-2}, 1x10^{-1}, 1.0, 1x10^{1}, 1x10^{2}, 1x10^{3}, 1x10^{4} \mu M) telah diperhatikan untuk meningkatkan pertumbuhan kalus juga penghasilan 9-metoksikan-6-on. Penghasilan 9-metoksikan-6-on (2.34 mg/g berat/ispadu) juga telah meningkat apabila media dibekalkan dengan 1x10^{-1} \mu M fenilalanina.
ACKNOWLEDGEMENTS

In the name of Allah, Most Gracious, Most Merciful. Due to His willingness, the completion of his study was made possible.

I would like to express my deeply appreciation and gratitude to the chairman of my supervisory committee, Prof Hjh. Dr. Maziah Mahmood, for her help, guidance and constant support in making the completion of this thesis a success. I am also grateful to Professor Dr. Chan Kit Lam from School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang for his supports, advice and generous assistance. The help rendered by my supervisory committee, Assoc. Prof. Dr. Nor‘aini Mohd. Fadzillah and Dr. Janna Ong Abdullah are greatly appreciated.

I greatly appreciate my parents, Norsiah Bt Maggon and Noormi Bin Hussain for their encouragement. To all of my lab-mates namely Sobri H, Anna, Ramani P, Tee CS, CY, Deswina, Chew, Chia Yee, Wai Sum Y, Sree, Suzita S, Nurkhanani, Sinarwati, Naila, Ali, Wai Sun, Hatta, and Uncle Thong, I thank for theirs strongly support.

Last but not least, my appreciation goes to the members from Dr. Radzali’s lab; Ms. Loo Wai Sum and Kak Aishah their support during the whole study period and also others from Dr. Yunus’s lab; Arif Khalid for his valuable guidance throughout HPLC analysis.
I certify that an Examination Committee met on 22nd June 2005 to conduct the final examination of Rosli bin Noormi on his Master of Science thesis entitled “Callus Induction from Explants of Eurycoma longifolia Jack (Tongkat Ali) and the Production of 9-methoxycantine-6-one from the Induced Callus” 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:

Mohd Arif Syed, PhD  
Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Chairman)

Radzali Muse, PhD  
Associate Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

Mohd Puad Abdullah, PhD  
Lecturer  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

Sompop Prathanturarug, PhD  
Professor  
Faculty of Pharmacy  
Mahidol University, Thailand  
(External Examiner)

GULAM RUSUL RAHMAT ALI, PhD  
Professor/Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia  

Date: 22 Jul 2005
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

**Maziah Mahmood, PhD**  
Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Chairman)

**Chan Kit Lam, PhD**  
Professor  
School of Pharmaceutical  
Universiti Sains Malaysia  
(Member)

**Nor’aini Mohd. Fadzillah, PhD**  
Associate Professor  
Faculty of Science  
Universiti Putra Malaysia  
(Member)

**Janna Ong Abdullah, PhD**  
Lecturer  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Member)

---

**AINI IDERIS, PhD**  
Professor/Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 11 AUG 2005
DECLARATION

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

ROSNI BIN NOORMI

Date: 18 JULY 2005
TABLE OF CONTENTS

ABSTRACT          ii
ABSTRAK           v
ACKNOWLEDGEMENTS  viii
APPROVAL          ix
DECLARATION       xi
LIST OF TABLES    xiv
LIST OF FIGURES   xvi
LIST OF ABBREVIATIONS  xx

CHAPTER

1  INTRODUCTION  1

2  LITERATURE REVIEW   5
   2.1  Eurycoma longifolia Jack (Tongkat Ali)  5
   2.2  Major Chemical Compounds in Tongkat Ali  6
      2.2.1  Quassinoids  8
      2.2.2  Alkaloids  10
      2.2.3  Squalenes  12
      2.2.4  Others  12
   2.3  Plant Secondary Metabolites Production in Cell Cultures  13
      2.3.1  Callus cultures  14
      2.3.2  Cell suspension cultures  17
      2.3.3  Hairy roots cultures  21
   2.4  Factors Influencing the Production of Secondary Metabolite in Cell Cultures  23
      2.4.1  Manipulation of Basal Media  24
      2.4.2  Carbon Sources  25
      2.4.3  Phytohormones  25
      2.4.4  pH  27
      2.4.5  Precursors  28
      2.4.6  Elicitors  29

3  MATERIALS AND METHOD  31
   3.1  Plant Materials  31
   3.2  Sterilization of explants  31
   3.3  Callus Induction  32
   3.4  Multiplication and Maintenance  32
3.5 Growth Curves
3.6 Effect of different media composition on callus growth and 9-methoxycanthin-6-one production
  3.6.1 The influence of different basal media
  3.6.2 Carbon sources
  3.6.3 Plant growth regulators (auxins)
  3.6.4 pH
  3.6.5 Amino acids
3.7 Analysis of 9-methoxycanthin-6-one
  3.7.1 Preparation of 9-methoxycanthin-6-one standard
  3.7.2 Extraction of 9-methoxycanthin-6-one from callus and intact plant part
  3.7.3 Analysis of 9-methoxycanthin-6-one using thin layer chromatography (TLC)
  3.7.4 Analysis of 9-methoxycanthin-6-one using high performance liquid chromatography (HPLC)
  3.7.5 Statistical analysis

4 RESULTS AND DISCUSSION
  4.1 Induction of *Eurycoma longifolia* Jack Callus Cultures from different explants
  4.2 Growth of *Eurycoma longifolia* Jack callus cultures
  4.3 Study on distribution of 9-methoxycanthin-6-one in intact plant part and callus culture of *Eurycoma longifolia* Jack by using TLC and HPLC method
  4.4 Effect of different medium composition on growth and 9-methoxycanthin-6-one content
  4.5 Effect of in different MS nutrient strength composition on growth and 9-methoxycanthin-6-one content
  4.6 Effects of Different Plant Growth Regulators (auxins) and 9-methoxycanthin-6-one content
  4.7 Effects of different carbon sources on growth and 9-methoxycanthin-6-one content
  4.8 Effects of different initial pH on growth and 9-methoxycanthin-6-one
  4.9 Effects of different amino acids on growth and 9-methoxycanthin-6-one content

5 CONCLUSION
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>List of plant secondary metabolites isolated from <em>Eurycoma longifolia</em> Jack.</td>
<td>7</td>
</tr>
<tr>
<td>2.2</td>
<td>List of selected plant secondary metabolites derived from cell cultures.</td>
<td>15</td>
</tr>
<tr>
<td>4.1</td>
<td>Percentage of <em>Eurycoma longifolia</em> Jack callus induced from leaves explants in the induction media (MS) supplemented with different auxins after 30 days of cultures in the basal MS medium supplemented with 3% (w/v) of sucrose and incubated at pH 5.7, 25 ± 2 °C in the dark.</td>
<td>40</td>
</tr>
<tr>
<td>4.2</td>
<td>Percentage of <em>Eurycoma longifolia</em> Jack callus induced from petiole explants in the induction media (MS) supplemented with different auxins after 30 days of cultures in the basal MS medium supplemented with 3% (w/v) of sucrose and incubated at pH 5.7, 25 ± 2 °C in the dark.</td>
<td>43</td>
</tr>
<tr>
<td>4.3</td>
<td>Percentage of <em>Eurycoma longifolia</em> Jack callus induced from rachis explants in the induction media (MS) supplemented with different auxins after 30 days of cultures in the basal MS medium supplemented with 3% (w/v) of sucrose and incubated at pH 5.7, 25 ± 2 °C in the dark.</td>
<td>44</td>
</tr>
<tr>
<td>4.4</td>
<td>Percentage of <em>Eurycoma longifolia</em> Jack callus induced from stem explants in the induction media (MS) supplemented with different auxins after 30 days of cultures in the basal MS medium supplemented with 3% (w/v) of sucrose and incubated at pH 5.7, 25 ± 2 °C in the dark.</td>
<td>45</td>
</tr>
<tr>
<td>4.5</td>
<td>Percentage of <em>Eurycoma longifolia</em> Jack callus induced from tap root explants in the induction media (MS) supplemented with different auxins after 30 days of cultures in the basal MS medium supplemented with 3% (w/v) of sucrose and incubated at pH 5.7, 25 ± 2 °C in the dark.</td>
<td>46</td>
</tr>
<tr>
<td>4.6</td>
<td>Percentage of <em>Eurycoma longifolia</em> Jack callus induced from fibrous root explants in the induction media (MS) supplemented with different auxins after 30 days of cultures in the basal MS medium supplemented with 3% (w/v) of sucrose and incubated at pH 5.7, 25 ± 2 °C in the dark.</td>
<td>47</td>
</tr>
<tr>
<td>4.7</td>
<td>Percentage of <em>Eurycoma longifolia</em> Jack callus induced from cotyledon explants in the induction media (MS) supplemented with different auxins after 30 days of cultures in the basal MS medium supplemented with 3% (w/v) of sucrose and incubated at pH 5.7, 25 ± 2 °C in the dark.</td>
<td>48</td>
</tr>
</tbody>
</table>
Percentage of *Eurycoma longifolia* Jack callus induced from zygotic embryo explants in the induction media (MS) supplemented with different auxins after 30 days of cultures in the basal MS medium supplemented with 3% (w/v) of sucrose and incubated at pH 5.7, 25 ± 2 °C in the dark.
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Eurycoma longifolia</em> Jack (Tongkat Ali) Plant.</td>
<td>3</td>
</tr>
<tr>
<td>2.1</td>
<td>Chemical Structures of the quassinoids isolated from leaves of <em>Eurycoma longifolia</em> Jack.</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>The chemical structure of canthin-6-one compound isolated from the roots of <em>Eurycoma longifolia</em> Jack.</td>
<td>11</td>
</tr>
<tr>
<td>2.3</td>
<td>The chemical structure of 9-methoxycanthin-6-one compound</td>
<td>11</td>
</tr>
<tr>
<td>4.1</td>
<td>Induction of <em>Eurycoma longifolia</em> Jack callus cultures derived from leaf explants in MS basal medium + B5 vitamins, 3% (w/v) sucrose, 0.25% (w/v) gelrite agar supplemented with different hormones concentration (0, 1, 2, 3, 4, 5 mg/L) after 30 days of cultures.</td>
<td>52</td>
</tr>
<tr>
<td>4.2</td>
<td>Induction of <em>Eurycoma longifolia</em> Jack callus cultures derived from petiole explants in MS basal medium + B5 vitamins, 3% (w/v) sucrose, 0.25% (w/v) gelrite agar supplemented with different hormones concentration (0, 1, 2, 3, 4, 5 mg/L) after 30 days of culture.</td>
<td>53</td>
</tr>
<tr>
<td>4.3</td>
<td>Induction of <em>Eurycoma longifolia</em> Jack callus cultures derived from rachis explants in MS basal medium + B5 vitamins, 3% (w/v) sucrose, 0.25% (w/v) gelrite agar supplemented with different hormones concentration (0, 1, 2, 3, 4, 5 mg/L) after 30 days of culture.</td>
<td>54</td>
</tr>
<tr>
<td>4.4</td>
<td>Induction of <em>Eurycoma longifolia</em> Jack callus cultures derived from stem explants in MS basal medium + B5 vitamins, 3% (w/v) sucrose, 0.25% (w/v) gelrite agar supplemented with different hormones concentration (0, 1, 2, 3, 4, 5 mg/L) after 30 days of culture.</td>
<td>55</td>
</tr>
<tr>
<td>4.5</td>
<td>Induction of <em>Eurycoma longifolia</em> Jack callus cultures derived from tap root explants in MS basal medium + B5 vitamins, 3% (w/v) sucrose, 0.25% (w/v) gelrite agar supplemented with different hormones concentration (0, 1, 2, 3, 4, 5 mg/L) after 30 days of culture.</td>
<td>56</td>
</tr>
<tr>
<td>4.6</td>
<td>Induction of <em>Eurycoma longifolia</em> Jack callus cultures derived from fibrous root explants in MS basal medium + B5 vitamins, 3% (w/v) sucrose, 0.25% (w/v) gelrite agar supplemented with different hormones concentration (0, 1, 2, 3, 4, 5 mg/L) after 30 days of culture.</td>
<td>57</td>
</tr>
<tr>
<td>4.7</td>
<td>Induction of <em>Eurycoma longifolia</em> Jack callus cultures derived from cotyledon explants in MS basal medium + B5 vitamins, 3% (w/v)</td>
<td>59</td>
</tr>
</tbody>
</table>
sucrose, 0.25% (w/v) gelrite agar supplemented with different hormones concentration (0, 1, 2, 3, 4, 5 mg/L) after 30 days of culture.

4.8 Induction of *Eurycoma longifolia* Jack callus cultures derived from embryo explants in MS basal medium + B5 vitamins, 3% (w/v) sucrose, 0.25% (w/v) gelrite agar supplemented with different hormones concentration (0, 1, 2, 3, 4, 5 mg/L) after 30 days of culture.

4.9 Three weeks old *Eurycoma longifolia* Jack callus after four times of subculture.

4.10 Growth of *Eurycoma longifolia* Jack leaf-derived callus (dry weight) cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.11 Growth of *Eurycoma longifolia* Jack petiole-derived callus (dry weight) cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.12 Growth of *Eurycoma longifolia* Jack rachis-derived callus (dry weight) cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.13 Growth of *Eurycoma longifolia* Jack stem-derived callus (dry weight) cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.14 Growth of *Eurycoma longifolia* Jack tap root-derived callus (dry weight) cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.15 Growth of *Eurycoma longifolia* Jack fibrous root-derived callus (dry weight) cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.16 Growth of *Eurycoma longifolia* Jack cotyledon-derived callus (dry weight) cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.17 Growth of *Eurycoma longifolia* Jack zygotic embryo-derived callus
(dry weight) cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.18 Morphology of three weeks old Callus cultures of *Eurycoma longifolia* Jack derived from different explants types cultured in a basal MS medium supplemented with B5 vitamins, different auxins incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.19 TLC spot of 9-methoxycanthin-6-one on aluminium sheets coated with Silica gel 60 *F*254 and developed with 25 DC-Alufolien Kiesel gel 60 *F*254 using chloroform and methanol solvent (Intact plant part).

4.20 TLC spot of 9-methoxycanthin-6-one on aluminium sheets coated with Silica gel 60 *F*254 and developed with 25 DC-Alufolien Kiesel gel 60 *F*254 using chloroform and methanol solvent (Callus cultures).

4.21 Distribution of 9-methoxycanthin-6-one in different plant parts and callus cultures of *Eurycoma longifolia* Jack.

4.22 Growth and 9-methoxycanthin-6-one productions of *Eurycoma longifolia* Jack callus cultures in different basal medium supplemented with B5 vitamins and incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.23 Growth and 9-methoxycanthin-6-one productions of *Eurycoma longifolia* Jack callus cultures in different MS nutrient strength medium supplemented with B5 vitamins and incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.24 Effect of different PGRs (auxins) concentrations on Growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* Jack callus cultures in different basal medium supplemented with B5 vitamins and incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.25 Growth and 9-methoxycanthin-6-one productions of *Eurycoma longifolia* Jack callus cultures in different carbon sources supplemented with B5 vitamins and incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.

4.26 Changing of initial pH profile in medium culture supplemented with B5 vitamins and incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light with respect to growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* Jack callus cultures.

4.27 Growth and 9-methoxycanthin-6-one productions of *Eurycoma longifolia* Jack callus cultures.
Jack callus cultures by addition of phenilalanine, tryptophan and tyrosine supplemented with B5 vitamins and incubated at pH 5.7, 25 ± 2 °C with a photoperiod of 16 hours fluorescent light.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>Dicamba</td>
<td>3,6-Dichloro-o-aniscic acid</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
</tr>
<tr>
<td>Fwt</td>
<td>Fresh weight</td>
</tr>
<tr>
<td>G</td>
<td>Gram</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>NAA</td>
<td>α-Naphthaleneacetic acid</td>
</tr>
<tr>
<td>PGR(s)</td>
<td>Plant growth regulator(s)</td>
</tr>
<tr>
<td>Picloram</td>
<td>4-Amino-3,5,6-trichloropicolinic acid</td>
</tr>
<tr>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>Distance of the substance over distance of the solvent movement</td>
</tr>
<tr>
<td>R&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Retention time (min)</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight for volume</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>Dicamba</td>
<td>3,6-Dichloro-o-anisic acid</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
</tr>
<tr>
<td>Fwt</td>
<td>Fresh weight</td>
</tr>
<tr>
<td>G</td>
<td>Gram</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>NAA</td>
<td>α-Naphthaleneacetic acid</td>
</tr>
<tr>
<td>PGR(s)</td>
<td>Plant growth regulator(s)</td>
</tr>
<tr>
<td>Picloram</td>
<td>4-Amino-3,5,6-trichloropicolinic acid</td>
</tr>
<tr>
<td>Rₜ</td>
<td>Distance of the substance over distance of the solvent movement</td>
</tr>
<tr>
<td>Rᵣ</td>
<td>Retention time (min)</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight for volume</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

It is well known that plants produce a variety of economically important secondary metabolites, with approximately 4,000 new discoveries every year, total up to over 100,000 known compounds (Wei et al., 2002). Some of the plant biochemicals are used in the healthcare, food, flavour and cosmetics industries (Frank and Masanaru, 1995; Pascal and Johan, 2002). Meanwhile, others are used for the production of agrochemicals, fragrances, colours and biopesticides (Ramachandra and Ravishankar, 2002; Verpoorte and Memelink, 2003). Examples of plant secondary metabolites used for the production of pharmaceuticals are dopamine, morphine, codeine, reserpine, and the anticancer drugs such as vincristine, vinblastine and taxol (Frank and Masanaru, 1995).

It was estimated that only 10 - 15 % of the known higher plant species had been investigated for their important bioactive compounds (Za’rate et al., 2001). Natural drug production from plants frequently involves extraction from living plants. This method is often tedious, costly and low yields. Furthermore, the target compounds may only be available seasonally (Kutney, 1998).

Thus, plant cell culture methodologies have the potential to overcome these problems (Gao et al., 2000). Plant tissue culture offers an alternative approach for the production and manufacturing of natural and additional plant secondary products (Oksman -
Caldentey et al., 1994). This technology also offers an attractive source for the production of high-value secondary metabolites (Alfermann and Petersen, 1995; Stockigt et al., 1995). This technology has the advantages over the conventional agriculture productions as it is independent on geographical seasonal variations, the continuous supply of products has uniform quality and yield is ensured. In addition, it is possible to produce novel compounds and increase the production efficiency by applying cell culture technology (Ramachandra and Ravishankar, 2002).

Moreover, in vitro culture enables the possibility to harvest the desired natural products without contamination of pesticides, herbicides or insecticides, and also to overcomes the natural heterogeneity in plant materials and variations in product contents (Tatischek et al., 1991). In vitro techniques can be an important approach to produce useful secondary products (Aziz et al., 2002). There have been a number of reports on using plant and organ tissues to produce a wide range of different secondary compounds (Rhodes et al., 1990; 1997).

The plant material used in this study is Eurycoma longifolia Jack (Figure 1), which is also known locally as ‘Tongkat Ali’ and ‘Pasakbumi’, in Indonesia. This plant is reputed to increase male virility and sexual prowess and has gained notoriety as a male aphrodisiac (Kuo et al., 2003). Pharmacological evaluations on the various compounds isolated from Eurycoma longifolia Jack showed that it also possessed anti-malaria (Kardono et al., 1991); anti-ulcer (Tada et al., 1991); cytotoxic (Morita et al., 1990; Kardono et al., 1991; Itokawa et al., 1992; Morita et al., 1993); antimalarial