3.1 Plant Material

Seeds of immature papaya (*Carica papaya* L.) variety Eksotika (Subang x Sunrise Solo) were provided by Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor.

Immature hermaphrodite Eksotika fruits were randomly harvested from field grown trees as starting material. Fruits were picked between 90 to 100 days old after anthesis of female and hermaphrodite flowers. The desired seeds were three months old post anthesis, where the final sizes of the ovules were 3 to 4 mm long.

3.2 Preparation of Culture Medium

3.2.1 Embryogenic Callus Induction Medium

Embryogenic callus was initiated on CI medium which consisted of half strength Murashige and Skoog (1962) (MS) basal salts (***Appendix 1***) and enriched with myo-inositol (50 mg/L), full strength MS vitamins, adenine sulphate (45 mg/L), glutamine (100 mg/L), sucrose 6 % (w/v), 2,4-D (10 mg/L), solidified with 1.95 g/L phytagel (Vilasini *et al*., 2000) (***Appendix 2***). Two types of medium were prepared either with or without carbenicillin at 250 mg/L (Sigma Aldrich, USA).

pH of the media was adjusted to 5.8 prior to autoclaving as mentioned in 3.3. Embryogenic CI medium was dispensed into 9 cm Petri dishes.

3.2.2 Liquid Multiplication Medium

Embryogenic cell suspensions were multiplied in liquid multiplication medium, LM, with similar composition as CI (*3.2.1*), but reducing the concentration of 2,4-D to 2 mg/L (***Appendix 3***). For maintaining the culture, the concentration of 2,4-D was successively reduced 0.5 mg/L every 14 days. Cultures were kept without plant growth regulators until either four to five months or as required for regeneration. pH of the media was adjusted to 5.8 prior to autoclaving as mentioned in 3.3 (Wong *et al*., 2006).

3.2.3 Germination, Regeneration and Rooting Medium

Germination (G) and regeneration (R) medium consisted of MS basal salt supplemented with myo-inositol (108 mg/L), 3 % (w/v) sucrose, 0.2 mg/L BAP and NAA respectively, and solidified with phytagel (1.95 mg/L) (***Appendix 4***) until shoots appeared. Elongated shoots were transferred to medium consisting of full strength MS with 1 mg/L gibberellic acid (GA_3), 0.5 mg/L indole butyric acid (IBA), and 3.76 mg/L riboflavin (***Appendix 5***) for plant maturation. Finally, the plantlet were subjected to rooting medium consisting of full strength MS with 2.0 mg/L IBA (***Appendix 6***) for complete plant formation (Vilasini *et al*., 2000).

The pH of all media was adjusted to 5.8 with 1.0 M NaOH or 1.0 M HCl prior to autoclaving as mentioned in 3.3.

3.3 Sterilization Procedure

All culture containers, glassware and metal instruments used were sterilized to eradicate microorganisms present. Before drying in the oven at 50 ºC, containers, glasswares and

metal instruments were soaked in Teepol™, rinsed with tap water and distilled water for washing. Loosely capped culture tubes and jars, and also conical flasks covered with 2 layers of aluminum foils were used throughout the experiments. Subsequently, autoclaving was carried out at 121 ºC, 103.4 kPa for 20 minutes to ensure sterility.

Laminar airflow cabinet was sterilized with ultra violet (UV) light for 30 minutes before use. All working surface and items placed in the laminar flow were wiped with 70 % (v/v) ethanol.

3.4 Explant Culture

3.4.1 Preparation of Explants

Harvested fruits were washed thoroughly under running tap water and sprayed intermittently with 70 % (v/v) ethanol for surface sterilization before placing in the laminar flow cabinet. The immature seeds were taken from the immature fruit and were cut open to remove the immature zygotic embryos. The excised embryos were placed on solidified CI medium in Petri dishes. The cultures were placed in the dark at ± 25 ºC (Vilasini *et al*., 2000).

3.4.2 Callus Induction Medium

Observation was carried out every 14 days and the embryos were sub-cultured onto the same medium either every 2 weeks for 8 weeks or until the embryogenic callus developed. About 500 mg of the friable embryogenic callus (globular structure) was removed, and transferred to liquid multiplication medium with the same constituent as CI.

3.4.3 Establishment of Suspension Cultures

For embryogenic callus multiplication, 10 mL liquid multiplication medium (LM) was initially dispensed into 100 mL Erlenmeyer flasks with about 500 mg embryogenic callus as the inoculum. Subsequently, 10 mL of LM was added to the cultures after two weeks. Somatic embryos containing mostly the globular structures were sieved from the suspension cultures using a filter with 450 µm pore size.

The filtrate was transferred to 10 mL of the conditioned and fresh medium respectively. For the following sub-culture (two weeks), all medium was removed and replaced with 20 mL of fresh medium. Subsequently, the cultures were sub-cultured every two weeks by adding 10 mL of LM medium until the volume reached 50 mL. Cultures were maintained at ± 25 ºC, with a 16 hr photoperiod at 2000 lux under continuous agitation on a rotary shaker at 100rpm (Wong *et al*., 2006).

Cells of embryogenic suspension (5 mL) were transferred onto CI medium following the protocols in 3.2 prior to culture in G medium for germination and regeneration. Plantlets regenerated from the embryos were sub-cultured every two weeks for a period of 3 to 4 months until mature.

3.5 Verification of Embryogenic Callus

3.5.1 Histochemical Test

Desired embryogenic callus was initially selected with double staining technique. A sample of the cells to be examined was placed onto a slide and a few drops of 1 % (v/v) acetone carmine were added. The samples were heated for a few seconds followed by

rinsing with distilled water 2 to 3 times. A few drops of 0.1 % (v/v) Evan's Blue was added to the sample and were incubated for 2 to 3 minutes and rinsed again with distilled water 2 to 3 times (Gupta and Ibaraki, 2006). The excess water was discarded and finally examined under a light microscope (Carl ZEISS, DSM940A).

3.5.2 Histological Studies

Samples of calli and somatic embryos at various stages of embryogenesis were fixed in a ratio of formalin: glacial acetic acid: ethanol (5: 5: 90) for 48 hrs. The mixtures were then dehydrated through graded series of tertiary butyl alcohol followed by embedding in paraffin wax. Finally, the rotator microtome sections (10 μm thickness) was used and the sections were stained with 1 % v/v toluidine blue O (Fernando *et al*., 2001).

3.6 Viability Test

Fluorescein diacetate stain (FDA) was used as viability test for the suspension cells. A drop of FDA stock solution 0.1 % (w/v) in acetone, stored in the dark at 4 °C) was added to 10 mL of the liquid multiplication medium. A sample of the cells to be examined was placed onto a slide and a few drops of the diluted FDA solution were added (Jalil *et al*., 2008). The sample was incubated for a few minutes before the cover slip being placed over the sample, and finally examined under an inverted microscope (400x) (Carl ZEISS, DSM940A).

3.7 Observation and Growth Measurements

3.7.1 Callus Cultures

Cultures were observed every 2 weeks for 8 weeks. The percentages of immature zygotic embryos (IZE) forming embryogenic callus (EC) was recorded as follows:

$$\frac{\text{IZE forming EC}}{\text{Total number of IZE}} \times 100$$

Observations of embryogenic callus that developed into somatic embryos were also carried out. The percentages of embryogenic callus (EC) that developed into somatic embryos (SE) in the same medium composition and condition were scored as follows:

$$\frac{\text{Number of EC clumps forming SE}}{\text{Total number of EC clumps}} \times 100$$

The regeneration of shoots from the somatic embryos on regeneration medium was monitored. The percentage of shoots regeneration from somatic embryos (SE) was recorded as follows:

$$\frac{\text{Number of SE forming shoots}}{\text{Total number of SE}} \times 100$$

3.7.2 Growth Curve of Suspension Cultures

Stable suspension cultures were used to determine the growth curve. Suspension cultures were considered stable once single clumps of callus (cytoplasmic cells) were formed. To determine the packed cell volume (PCV), a known volume of uniformly dispersed suspension was transferred to a 15 mL polypropylene tube. The tubes were spinned in a

bench centrifuge for 5 minutes at 200x g. PCV is the volume of the pellet as a function of the volume of culture, and is usually expressed as ml pellet per litre culture or a percentage (Blom *et al*., 1992).

3.7.3 Histological Analysis

Various stages of calluses and somatic embryo was viewed and examined under a binocular microscope.

3.7.4 Viability of Cell Suspension

To determine the percentages of cell suspension viability, the number of cells in a field of view under bright field and the number of fluorescent cells in the same field under the UV illumination was determined as follows:

$$\frac{\text{Number of Fluorescent Cells (under UV illumination)}}{\text{Total Number of Cells (under bright field)}} \times 100$$

3.7.5 Experimental Replicate

Throughout the experiments in 3.2, 3.4, 3.5, 3.6, and 3.7, five replicates with 10 explant/samples were used in each experiment. Experiment was repeated at least twice.

3.8 Student ‘t’ Distribution Test

In this study, Student ‘t’ test was used to determine the significant differences between the treated mean samples and the control mean values.

The standard normal distribution of the means of a random sample, $t = (x-\mu) / Sx$ is applicable if reliable estimate of S sample size is small. As Sx is estimated from a small sample, it is subject to sampling error and will depart more or less widely from the true x. The accuracy of the estimate increases as the number of degrees of freedom on which it is base increases' (Murdoch and Barnes, 1998, Dawson *et al*., 1986 and Gomez and Gomez, 1984).

3.9 General Remarks for Compound Extraction Experimental Procedure

Isolation of carpaine was achieved by using conventional acid base extraction technique as well as supercritical fluid extraction (SFE), Thar Technologies Process Suite. The esterification of carpaine was monitored on the silica gel 60 F_{254} aluminium base thin layer chromatography (TLC) (MERCK, Darmstadt, German).

The purity of the compound extracted was examined by its physical properties such as melting point (m.p) (MP-ID Fargo Model 3500 Bishop Graphics Inc. with 10x Deluxe), proton nuclear magnetic resonance (^{1}H NMR) spectrum (JEOL JNM-LA400 FT-NMR System) and GC [Hewlett-Packard (HP) 6890 Series] chromatogram.

3.10 Plant Material

The matured leaves of mother plants *Carica papaya* L. var. Eksotika I were collected from the field plot of Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, while matured leaves of *in vitro* regenerant, and cell suspension from the growth room of Plant Biotechnology Incubator Unit, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur. The samples were air dried at room temperatures (±32 °C) for about 14 days, grounded and then powdered to 70 to 100 meshes.

3.11 Sample Extraction

3.11.1 Conventional Method

3.11.1.1 Mother Plant

Batches of 500 g in triplicate of ground leaves were used.

3.11.1.2 In vitro Regenerant

Batches of 50 g in triplicate of ground leaves were used.

3.11.1.3 Embryogenic Suspension Cells

Batches of 500 mL (liquid) and 50 g (cells) in triplicate of suspension cells were used.

Samples were soaked for 14 days in a mixture of absolute ethanol (C_2H_5OH) (MERCK), distilled water and glacial acetic acid (CH_3COOH) (MERCK), in a ratio of 95.5:5:0.5, v/v/v.

Table 3.1: Solvent used for soaking.

No.	Types of Solvent	Combination Ratio
1.	Absolute ethanol : Distilled water : Glacial acetic acid	89 : 10 : 1 (Coke and Rice, 1965 and Tang, 1978)
2.	Absolute ethanol : Distilled water : Glacial acetic acid	95.5 : 5 : 0.5 (Modified)

The mixture was filtered through a Whatman filter paper (Qualitative Circles 150 mm diameter) and the filtrate was subjected to Hettich Zantrifugen, EBA 12 R centrifugation

at 5000 rpm for 15 minutes. The resulting pellet was discarded and the supernatant was collected.

The combined supernatant was evaporated *in vacuo* at 60 °C (BÜCHI Rotavapor, R-114 and BÜCHI Waterbath, B-480) to obtain a thick dark brown slurry product. The dark brown syrup was then added into a mixture of distilled H_2O: CH_3COOH (49:1, v/v) and washed with diethyl ether (50 mL) to remove the non-polar component. The aqueous layer was adjusted to pH 11 with K_2CO_3 solution (MERCK) and then re-extracted with Et_2O (50 mL) (Rice and Coke, 1966).

The ether extract was then washed with distilled H_2O (50 ml) and 5 % HCl solution (50 ml) (MERCK) was added. The acidic aqueous layer was separated from the ether layer and the pH was again adjusted to 11. This procedure was repeated twice and the final ether extract was dried (Na_2SO_4 anhydrous), (MERCK) and Et_2O was removed *in vacuo* at ±30 °C (Tang, 1978).

The brown slurry obtained yielded a precipitate of dull yellow needle-like carpaine crystals upon chilling at -20 °C in a Sanyo Biomedical Freezer, MDF-US37D.

The yield of each extraction was determined after drying at room temperature (±32 ºC) until constant weight accepted.

3.11.2 Supercritical Fluid Extraction

3.11.2.1 Mother Plant

Batches of 5 g in triplicate of ground leaves were used.

3.11.2.2 In vitro Regenerant

Batches of 5 g in triplicate of ground leaves were used.

3.11.2.3 Embryogenic Suspension Cells

Batches of 5 g (cells) in triplicate of suspension cells were used.

Powdered *Carica papaya* L. var. Eksotika I ground samples (5 g each) were extracted for approximately 60 minutes in a dynamic loom, using an extraction fluids of pure carbon dioxide (CO_2) (supplied by Malaysian Oxygen, MOX) without othert solvents, as indicated in ***Table 3.2***. Extraction was carried out in triplicate.

Table 3.2: Solvent used in supercritical fluid extraction (SFE).

No.	SFE Fluid	Pressure (bar)	Temperature (ºC)
1.	CO_2	400	40
2.	CO_2	450	45
3.	CO_2	500	50

Sequential modes of extractions were carried out with pure CO_2 but in a manner of varied temperature and pressure conditions as reported in ***Table 3.2*** for each sample (three samples).

All the extracts were collected into analytical grade C_2H_5OH (Merck) in a 250 ml volume of round bottle flask (Knez *et al.*, 2003). The solvent was removed *in vacuo* at 40 °C until constant weight of elucidated carpaine obtained. All of the extracts were dissolved in analytical grade of Et_2O (MERCK) for storage prior to further analysis.

3.12 Characterization of Alkaloid Carpaine

3.12.1 Melting Point

The yellow needle-like crystals of carpaine obtained from acylation and SFE in triplicate were subjected to melting point analysis to assess its degree of purity.

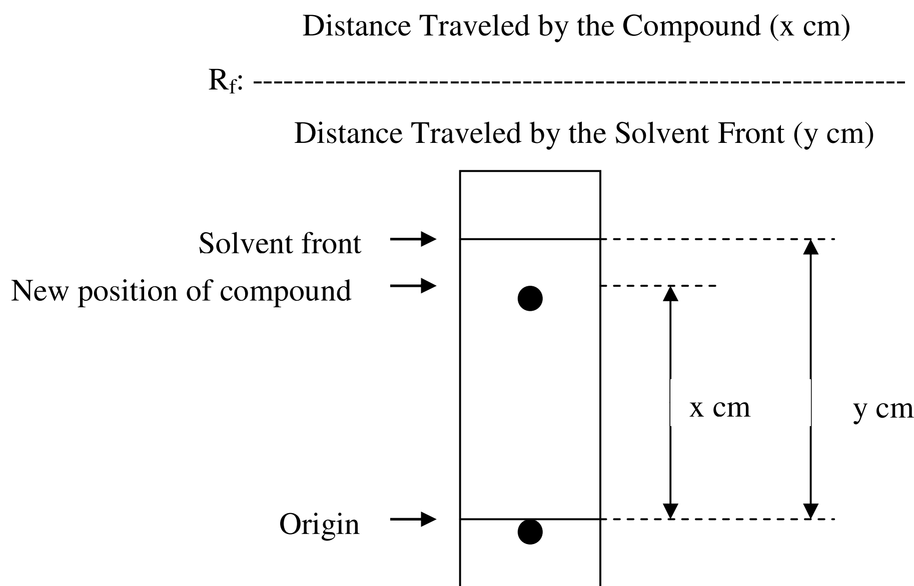
3.12.2 Thin Layer Chromatography

The yellow needle-like crystals of carpaine obtained from acylation and SFE was dissolved in dry Et_2O (MERCK). Various combinations of solvents were used for TLC analysis as indicated in ***Table 3.3***.

Table 3.3: Solvent's combination used in TLC experiments

No.	Solvent's Combination	Ratio
1	BuOH : HOAc: H_2O	4 : 1 : 5
2	MeOH : $CHCl_3$	3 : 7
3	$CHCl_3$: C_6H_6	1 : 9
4	$CHCl_3$: C_2H_{12}	4 : 5 : 1

TLC is used to support the identity of a compound in a mixture when the retention factor, R_f of a compound is compared with the R_f of a known compound was counted following:



3.12.3 Magnetic Resonance Spectrometry

The yellow needle-like crystals of carpaine obtained from acylation and SFE was dissolved in $CDCl_3$ (MERCK) and ^1H NMR spectrum was taken on the FT-NMR (JEOL 400 MHz FT-NMR) spectrometer (Manrique and Lajolo, 2002).

3.12.4 Gas Chromatography / Mass Spectrometry

Mass spectrum was taken on the HP (Hewlett-Packard) 6890 Series, GC System spectrometer with nitrogen, MOX (N_2), as the carrier gas. The sample was first silylated with N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (SUPELCO), dissolved in diethyl ether and injected into the spectrometer at the initial temperature at 70 ^oC for 2 minutes and increasing linearly at a rate of 10 ^oC per minutes to 250 ^oC and held for 10 minutes. The peak at retention time of 54.227 minutes

in the gas chromatogram was identified as carpaine from the M^+ at 478.377 (Mahmood and Rahman, 1998).