2.1 Introduction

2.1.1 Origin of Papaya

The genus *Carica* L. is indigenous to tropical America and is believed to be native to Southern Mexico and neighbouring Central America. It is now present in every tropical and subtropical country and as far north and south as 32º latitude. Varieties of Batu Arang, Eksotika, Setiawan, Solo, Subang, Sunrise Solo, and Waimanalo papaya originated from natural hybridization (Chan and Teo, 1994).

2.1.2   Classification and Nomenclature

The family *Caricaceae* is comprised of four genera *Carica, Cyclimorpha, Jacaratia* and *Jarilla*. The genera *Cyclimorpha, Jacaratia* and *Jarilla* are small size trees with papain found in every part of the tree.

Most edible papaya is classified under the section *Carica*. Some examples are *Carica pentagona, Carica pubescens*, *Carica stipulata, Carica candamarcensis, Carica monoica, Carica erythrocarpa, Carica goudotiana* and *Carica quercifolia.* Only *Carica* genus has been planted for the fruit (Chan and Teo, 1994).

Carica papaya L. is classified under the Division *Magnoliophyta*; Class Magnoliopside; Subclass *Dilleniidae*; Order *Violales*; and Family *Caricaceae.*

There are several cultivars of papaya in Malaysia both local and introduced. The introduced cultivars are Cibinong, from *Indonesia*; Sunset and Sunrise Solo from *Hawaii*; Sunnybank from *Australia*; Honeydew from *India*; Maradol from *Cuba,* Cavite and Peradeniya from *Philippines* and Kokdum from *Thailand.* The agricultural

research in Malaysia has yielded several cultivars such as Eksotika, Batu Arang, Sitiawan, Subang and Taiping 2.

2.1.3 Synonyms and Common Names

There are almost 40 vernacular names of *Carica papaya* L. such as Arbre dè melon, Chamburo, Doeum lahong, Fan Kua, Gedang, Houng, Kepaya, Lapaya, Mapaza, Papaya, Thimbaw, and Wan Shou Kuo.

2.1.4 Etymology and Leave Characteristics

The foliage or leaves of *Carica papaya* L. emerge directly from the upper part of the stem in a spiral on horizontal petioles 30 to 90 cm long. The leaves are large, alternate, close together, palmately divided into 5 to 7 irregularly cut lobes, lobes glabrous, toothed, flat and are borne on leaf-stalks 30 or 60 cm in length, and which are peltately attached, and has prominent yellowish ribs and veins. Other characteristics comprised of long petiole, blade sub-orbicular, hollow, greenish or purplish-green to 80 cm long. The life span of a leaf is 4 to 6 months.

2.1.5 Plant Description

The papaya is a herbaceous and an indigenous plant with a soft stem which may grow as tall as 8 m height, in wild condition, as well as under cultivation. It is a short-lived, fast-growing, woody, large herb and varies in height and is about 5 or 7 cm to over 30 cm at the base in diameter. It normally flowers in 9 to 12 months producing whether male, female or hermaphrodite flowers.

2.1.6 Common Uses

Papaya is a multi-purpose fruit, not only as dessert but also as a source of chemical compounds use like papain, chymopapain and carpaine for medicinal cure (cancer,

heart disease, and anti-aging) (Craig, 1998). The International Tropical Fruits Network (TFNet) and Food and Agricultural Organization Statistic (FAOSTAT) reported that the world production of papayas has reached 5,951,000 metric tones (RM 100 million) in 2004 (Tropical Fruit Net, 2004).

2.1.7 Pharmacological Properties of Carica papaya L.

In vivo plants and plant cell cultures (*in vitro*) synthesize some of the unusual and complex chemicals for pharmaceuticals purposes (***Table 2.1***).

Carpaine is a major alkaloid and a lead compound that is present in papaya especially in in the leaves and traces of this alkaloid have also been found in its latex. It has been reported to possess varieties of pharmacological activities. It has been reported that carpaine at 2.0 mg/kg could reduced cardiac output, stroke volume, stroke work, and cardiac power (Hornick *et al*., 1978). Besides, carpaine also slows down the heart pulse rate and thus reduces blood pressure. However, higher doses can produce vasoconstriction. This alkaloid is also reported to be diuretic, antihelminthic, amoebicidal, and could inhibit growth of *Myobacterium tuberculosis* (Burdick, 1971).

Table 2.1: Pharmacological properties and significance of *Carica papaya* L. compounds

No.	Researchers	*Carica papaya* L. Compounds	Pharmacological Properties
1	Farias *et al*., 2007	α-amylase	Deleterious activity towards *Callosobruchus maculatus*
2	Nitsawang *et al*., 2006	Papain	Biocatalyst
3	Cheng and Tsai, 2004	Lipase	Enantioselective biocatalyst
4	Knez *et al*., 2003	Lipase	Biocatalyst
5	Azarkan *et al*., 2003	Chymopapain	Essential for the catalytic competence of the proteinase
6	Guillermo *et al*., 2002	Pectin	Pectinmethylesterase softening

			process
7	Wilberg *et al*., 1995	Carotenoid	Inhibition of cancer, immuno-enhancement, and prevention of cardiovascular disease
8	Jacques *et al*., 1994	Monoamides and diamide	Anticancer agents
9	Yamamoto and Tabata, 1989	Papain	Protease activity
10	Tang and Takenaka, 1983	Benzyl isothiocyanate	Resistance to the pathogen
11	Ogan, 1970	Choline	Stimulant action of acetylcholine on smooth muscle

2.1.8 Carica papaya L. var Eksotika

Eksotika or Eksotika I is a result of hybridization between varieties Sunrise Solo (Hawaii) and Subang (Malaysia) (Chan and Teo, 1994). The variety was produced after undergoing 15 years of breeding experiments carried out by the Malaysian Agricultural Research and Development Institute (MARDI) and this variety was launched to the nations on the 9th February 1987.

The variety Eksotika has great potential as an export and commercial crop because of the fruit cosmetics such as freckles and soft textures. Eksotika fruits weigh about 600 to 800 g with red flesh and high sugar content (Chan and Teo, 1994). Under environmental stress, such as in-stable climate effects the tree recovers well to produce fruit quickly. Comparatively, this variety is high yielding with red flesh and high sugar content (Chan and Teo, 1994) and amounting to about 75 tones per hectare. Eksotika is tolerant to Papaya Ring Spot Virus (PRSV) and have a good keeping quality or shelf life with delayed ripening characteristics (Vilasini *et al*., 2000).

Fruits are harvested after 9 months from planting. The infertility rate is between 22 to 47 %. Eksotika was developed from the Sunrise Solo with excellent fruit qualities but poor yield and small fruit and Subang 6 with large fruits which result in a hybrid that

reconstituted excellent fruit qualities and larger fruit (Chan, 2005). Eksotika papaya trees are of medium stature and start flowering after four months.

2.2   Economic Importance of Eksotika

Eksotika is the flagship fruits variety which has penetrated into previously untapped markets like Singapore, Hong Kong, China, Middle East countries and Europe. The advent of Eksotika had given the fruit industry a dramatic boost, particularly in generation of export earnings. In 1986, the year before Eksotika was released, the export revenue of papaya was a mere RM3 million. The export revenue has increased steadily every year, RM20 million in 1990, RM60 million in 2000, and has surpassed RM100 million in 2004.

To date the planting acreage and the export value of Eksotika has increased at a very rapid rate to become the most important export fruit in the country. Malaysia currently is the second most important exporter of papaya in the world, with an export value of USD22.5 million in 2004 (72,000 metric tone) after Mexico (MARDI, 2004).

2.3 Plant Tissue Culture

2.3.1 Introduction

Plant tissue culture is used as a term for protoplast, cell, tissues and organ culture under aseptic conditions. Tissue culture starts from various plant parts which have meristematic cells.

Tissue culture reveals the unique capacity of plant cells culture of its cellular totipotency to potentially give rise to whole plants. Through continuous cell division,

after a period of time, the plantlets are generated to produce the targeted number of plants (Bhattacharya, 2003).

A proper balance of particular nutrients and plant growth regulators in the culture medium are essential for the groeth and development of plant cells. Generally, high auxin seems to promote root initiation whereas high cytokinin tends to promote shoot initiation. The presence of plant growth regulators in the right balance has resulted in the production of both shoots and roots.

Special nutrient requirements and micro-environmental conditions must be established for each plant genotypes cultured. Dark condition generally produces the best callus growth but light seems to be necessary for differentiation of shoots.

2.3.2 Plant Regeneration

Plant regeneration through tissue culture can be accomplished via organogenesis and somatic embryogenesis.

2.3.2.1 Organogenesis

Organogenesis is more common and easier to control than the development of somatic embryos from cultured explants.

Plant production through organogenesis can be achieved through one of the three modes; production of adventitious organs from a callus derived from the explant, emergence of adventitious organs directly from the explant without an intervening callus phase, and production of plantlets from outgrowth of axillary buds (Bhattacharya, 2003).

By varying the growth regulator concentrations in the nutrient medium, shoots can arise directly from the explants itself or via an intermediate callus. Adventitious roots are produced either from the callus or regenerated shoots.

2.3.2.2 Somatic Embryogenesis

Somatic (or asexual) embryogenesis is the production of embryo-like structures from somatic cells. A somatic embryo is an independent bipolar structure and is not physically attached to the tissue of origin. Somatic embryos can develop and germinate to form plants in a manner analogous to germination of zygotic embryos.

Production of somatic embryos from cell, tissue or organ cultures may proceed either directly (without an intervening callus phase) or indirectly via callus culture. Direct embryogenesis usually occurs from explants maintained on solid culture medium and can be utilized for micro-propagation for a more limited range of species.

However, indirect embryogenesis from liquid cell suspension is particularly attractive for micro-propagation, as long as genetic stability can be maintained (Litz and Conover, 1983).

2.3.2.2.1 Germination of Somatic Embryo

For germination, generally somatic embryos must be exposed to a low temperature to enable them to germinate. Somatic embryos can be germinated readily on solid culture medium without hormones and plantlets develop to a size suitable for transfer to soil or vermiculite (Bhattacharya, 2003). However, some plants require hormones for germination (Stasolla and Yeung, 2003, Sharma and Millam, 2004 and Jones *et al*., 2006).

During the growth of cell suspension cultures, cells and embryos are evenly exposed to nutrients and hormones. The media components can be controlled precisely to induce development of somatic embryos in a uniform manner. In liquid culture, the pro-embryo cell clusters and somatic embryos usually develop as separate structures suspended in the medium. Thus the cells can easily be sieved, centrifuged, sub-cultured and manipulated as required.

2.3.4 Single Cell Culture

Single cell cultures have been produced from tissue cultures which have been subjected to continuous shaking. Under these conditions, small cell clumps or individual cells particularly if the callus is soft and friable-slough-off into the culture medium.

Plant cell suspension cultures provide a relatively homogenous population of cells, readily accessible to exogenously applied chemicals and grow under defined, aseptic conditions. Suspension cell cultures are initiated by transferring preferably friable callus inoculums to agitated liquid nutrient medium of the same compositions as that used for callus growth.

Relatively large initial inoculums is advantageous, as this will ensure that sufficient single and/or small clumps are released into the medium to provide a suitable high cell density for subsequent growth (Dixon and Gonzales, 1994). Agitation rates on an orbital shaker between 70 to 150 rpm with an orbital motion stroke of 2 to 4 cm are often used.

Agitation serves both to aerate the culture and to disperse the cells which, either as a result of cell division at the callus surface or mechanical effect breaks away from the

inoculum tissue (Litz and Conover, 1983). Basically there are two types of cultures, batch cultures and continuous cultures.

Batch cultures are used for initiating single cell cultures. Cell suspensions are grown in flasks containing liquid culture medium. The cultures are continuously propagated by routinely taking a small aliquot of the suspension and transferring it to a fresh medium.

Continuous cultures are culture vessels that have been designed to grow large-scale cultures under steady state for a long period by adding fresh medium and draining out the used medium (Blom, 1992). Continuous cultures may be of the close or open types. In the former, the addition of fresh medium is balanced by outflow of the old medium. The cells from the out-flowing medium are separated mechanically and added back to the culture. In close continuous cultures, cell biomass continues to increase as the growth proceeds. In contrast to the open continues cultures, the inflow of the medium is accompanied by a balancing harvest of an equal volume of the culture (medium and cells) (Blom, 1992 and de Almeida *et al*., 2000).

2.4 Tissue Culture of Papaya

There are many published reports on the tissue culture of papaya. ***Table 2.2*** shows the use of various plant growth regulators (PGRs) on the formulation of papaya tissue culture for plant regeneration.

Table 2.2: Formulation of papaya tissue culture

Items No.	Author (s)	Basal Medium	Plant Growth Regulator (s) and Supplements
1	Tsay and Su (1985)	Murashige and Skoog (MS)	NAA and BA
2	Jordan(1986)	Nitsch and Nitsch (N)	NAA and BA
3	Chen *et al*. (1987)	Murashige and Skoog (MS)	GA_3, and NAA
4	Mondal *et al*. (1994)	Murashige and Skoog (MS)	IBA and BAP
5	Monmarson *et al*. (1995)	Murashige and Skoog (MS)	ABA and kinetin
6	Jordan and Velozo (1996)	Nitsch and Nitsch (N)	BA, IAA, NAA
7	Sutanto *et al*. (1999)	Murashige and Skoog (MS)	BAP
8	Yu *et al*. (2001)	Murashige and Skoog (MS)	BA
9	Renukdas *et al*. (2003)	Murashige and Skoog (MS)	B_5 vitamins, boric acid and, picloram
10	Bhattacharya *et al*. (2003)	Murashige and Skoog (MS)	BAP and NAA
11	Rimberia *et al*. (2005)	Murashige and Skoog (MS)	BA and NAA

2.4.1 Plant Growth Regulators

They are various classes of plant growth regulators - auxins, cytokinins, gibberellins, abscisic acid, steroid, ethylene and brasino steroids. They are either naturally occurring in plants or their analogs are synthetically made.

A) Plant hormones are organic compounds other than nutrients produced by plants which, in low concentrations, regulate plant growth and development. They usually move within the plant from a site of production to a site of action.

B) Plant growth regulators are either synthetic compounds or plant hormones that effect plant physiological processes. They regulate growth by mimicking hormones, by influencing hormone synthesis, degenerate, or translocation, or possibly by modifying hormonal action sites.

2.4.1.1 Auxins

In nature, hormones of this group are involved with elongation of stems and internodes, tropism, apical dominance, abscission, and rooting. The auxins commonly used in tissue culture are naphthaleneacetic acid (NAA) and dichlorophenoxyacetic acid (2,4-D). Of these, NAA is widely used for cell division, cell elongation and rooting and, in interaction with a cytokinin, for shoot proliferation. 2,4-D is effective for the induction and growth of callus.

Studies of the physiology of auxin action showed that it was involved in such varied plant activities as stem growth, root formation, lateral bud inhibition, abscission of leaves and fruits, activation of cambial cells, and others. Indole-3-acetic acid (IAA) was identified as a naturally occurring compound having considerable auxin activity and was soon found to promote adventitious root formation (Gamborg, 2002).

It is now well accepted and has been subsequently confirmed that in plant tissue culture, auxin in general either natural or artificially applied, is a requirement for the initiation of adventitious roots on stem and it has been shown that the division of the first root initial cells are dependent upon either applied or endogenous auxin.

Formation of preformed root initials in stems apparently is dependent upon the native auxins in the plant plus an auxin synergist; together these lead to synthesis of ribonucleic acid (RNA) which is involved in the initiation of the root primordial (Gamborg, 2002).

2.4.1.2 Cytokinins

These PGRs are concerned with cell division and shoot proliferation. In tissue culture media, cytokinins are incorporated mainly for cell division and differentiation of

adventitious shoots from callus and organs. These compounds are also used for shoot proliferation. An example of cytokinin is 6-benzylaminopurine (BAP).

Various natural and synthetic compounds such as zeatin, kinetin, and 6-benzyladenine have cytokinin activity. Generally, applied synthetic cytokinins have not stimulated or prevented root initiation. At relatively low concentrations, when cytokinins are applied to the medium at an early developmental stage, roots are initiated while at higher concentrations an inhibitory response is exhibited (Gamborg, 2002).

However, at a later stage of development, root initiation did not occur. The influence of cytokinins in root initiation may thus depend upon the particular stage of initiation and the concentration of cytokinin.

2.4.1.3 Interaction between Auxin and Cytokinin

Cytokinin is related to auxins in controlling the organ differentiation. In a study, cytokinin at relatively high concentrations promoted bud formation and inhibited root formation. Auxins, at high concentrations, gave the opposite effect.

These were interacting relationships, however, between auxins and cytokinins. At low concentrations, IAA promoted bud formation, enhancing the cytokinin influence. Also, at low concentrations, kinetin stimulated the effect of IAA on root promotion (Gamborg, 2002).

Temperature is an influencing factor in these relationships. High temperature, 81 ° F (27 ° C), itself inhibited bud formation and opposed the stimulatory effects of cytokinin on this process as well as the suppressing effect of cytokinin on root formation. On the other hand, the auxin effects were stimulated under long days at

this temperature as compared to lower temperatures [60 $^\circ$ F (15 $^\circ$ C)] (Gamborg, 2002).

Under low light intensities, however, cytokinin neither nor auxin level and regeneration ability was affected by temperature. It would appear that the regenerative ability of the explant is due to complex interaction of temperature, photoperiod, and light intensity, controlling the levels of endogenous auxins and other growth regulators.

2.4.1.4 Gibberellins

The gibberellins are a group of closely related, naturally occurring compounds, and known principally for their effects in promoting stem elongation. At relatively high concentrations (up to 10^{-3} M) they have consistently inhibited adventitious root formation (Gamborg, 2002).

There is evidence that this inhibition is a direct local effect which prevents the early cell divisions involved in transformation of mature stem tissues to a meristematic condition. Gibberellins have a function in regulating nucleic acid and protein synthesis and may be suppressing root initiation by interfering with these processes. At lower concentrations, however, (10^{-11} to 10^{-7} M) gibberellin has promoted root initiation (Gamborg, 2002).

In certain study, gibberellic acid was noted to inhibit both adventitious bud and root formation, probably by blocking the organized cell divisions which initiate formation of bud and root primordial. Lowering the natural levels of gibberellin in the tissues stimulate adventitious root formation in cuttings.

2.5 Alkaloids

Plant produces a complex array of secondary metabolites which may not be directly involved in the organism's growth and development. Although many of these metabolites can be synthesized in the laboratory, their synthesis is usually complex and tedious with low yields.

Alkaloids are often classified according to the nature of the nitrogen-containing structure, for examples piperidine, pyrolidine, and indole, though the structural complexity of some examples rapidly expands the number of subdivision. They are organic nitrogenous bases heterocyclic which occur mainly in plants as their salts of common carboxylic acids such as citric, lactic, oxalic, acetic, malic and tartaric acids as well as fumaric, benzoic, aconitic and veratric acids. Their amine character produces alkaline solutions in water and hence, the origin of their name, alkaloids. The biological activities of many alkaloids are often dependent upon their amine function being transformed into a quarternary system by protonation at physiological pHs (Govindachari, 2002).

The nitrogen atoms in alkaloids originate from an amino acid, and, in general, the carbon skeleton of the particular amino acid precursor which forms the alkaloid is also largely retained in its structure, though the carboxylic acid carbon is often lost through decarboxylation. Accordingly, alkaloids are further subdivided into groups which are based on their amino acid precursors (Bennet *et al*., 2004). The classification of alkaloids has been stated into three main kinds such as true alkaloids, proto alkaloids and pseudo alkaloids. True and proto alkaloids are derived from amino acids, have heterocyclic ring with nitrogen and does not have heterocyclic ring with

nitrogen respectively. Whereas, pseudo alkaloids is not derived from amino acids and having heterocyclic ring with nitrogen. Other than that, alkaloids are also subdivided into groups based on the chemical classification, and numerous classes of alkaloids are possible such as ornithine, tyrosine, tryptophane, pyridine and lysine (Rajnikant *et al*., 2005).

There is a variety of structural types of alkaloid such as monocyclic, dicyclic, tricyclic, and tetracyclic, as well as cage structures.

Alkaloids are also renowned for their potent pharmacological activities. It has a wide range of important clinical use such as hypertension, analgesics, and treatment of anti-spasmodic (Newman *et al*., 2003). For example, vincristine is one of the most potent antileukemic drugs and quinine with the principal antimalarial compound. In another example, carpaine, an alkaloid from extracts of *Carica papaya* has been shown to possess anti-hypertension activities (Hornick *et al*., 1978).

2.6 Carpaine

Carpaine description shown in ***Table 2.3*** can be found in *Carica papaya* L. and *Vasconcellosia hasta Carnel*, *Caricaceae* which can be isolated largely from the leaves of the plant. It has been reported to cause bradycardia and central nervous system depression (Hornick *et al*., 1978 and Burdick, 1971). In natural product chemistry, carpaine has been classified under the group of alkaloids and it has a macrocyclic dilactone structure, a cyclic hydrocarbons that contain multiple rings and share one or more atoms (Govindachari, 2002).

Carpaine has been documented as monoclinic prisms from acetone which sublimees at 120° C under 0.05 mm pressure $[\alpha]^{12}_D + 24.7°$ (c = 1.07 in ethanol). It is slightly soluble in water and soluble in most organic solvents except petroleum ether (Govindachari and Narasimhan, 1954). Table 2.3 describes some of the physical and chemical properties of carpaine.

Table 2.3: Properties of carpaine compounds (Barger *et al*., 1937)

Items	Details
Name	Carpaine
CAS type 1 name	13, 26-dimethyl-2, 15-dioxa-12, 25-diazatricyclotriacontane-3, 16-dione
Chemical name	12, 25-Diaza-13, 26-dimethyl-2, 15-dioxatricyclo [22.2.2.2 <11, 14>] triacontane-3, 16-dione.
Formula	$C_{28}H_{50}N_2O_4$
Molecular mass	478.70 g/mol
Melting point	121 °C
Solubility	30 mg/ml in water at 80 °C, less than 0.5 mg/ml at 25 °C
pH in aqueous medium	4.6
Crystal structure	The molecule is flexible and the changes in the conformations are brought about by the hydrogen bonding of the protonated-N atoms with the two Br atoms and the water molecule
Chemical drawing	

2.7 Compound Extraction

Compound extraction is the method of separating the constituents of a mixture utilizing preferential solubility of one or more components in a second phase. Commonly, this added second phase is a liquid, while the mixture to be separated may be either solid or liquid. If the starting mixture is a liquid, then the added solvent must be immiscible or only partially miscible with the original and of such a nature that the components to be separated have different relative solubility in the two liquid phases. There are various techniques of extractions such as solid/liquid extraction, liquid/gas extraction and acid/base extraction will be discussed and utilized.

Solid/liquid extraction is the most common technique in isolating compounds from natural products since it allows soluble components to be removed from solids using solvent. In solid/liquid extraction, a solvent is first added to a solid. Any insoluble material is then separated from the solution by gravity or vacuum filtration, and soluble material is 'extracted' into the solvent. In natural product extraction, often the raw materials such as the leaves or bark of plants are soaked with an appropriate solvent for several days before filtering (Azarkan *et al*., 2003).

Liquid/liquid extraction is a method to separate compounds based on their relative solubility in two different immiscible liquids, using a separatory funnel. In this method, the substance to be extracted is first dissolved in a solvent. This substance is then extracted from one liquid phase into another liquid phase. By this process a soluble compound is usually separated from an insoluble compound. Some examples of where this extraction technique is used are in the production of fine organic compounds and the processing of perfumes.

Acid/base extraction is a sequential liquid/liquid extractions process to purify acids and bases from mixtures based on their chemical properties. It is routinely performed for the isolation of compounds from crude extracts of natural products like alkaloids.

The fundamental theory behind this technique is that addition of an acid to a mixture of an organic acid and base will result in the acid remaining uncharged, while the base will be protonated and conversely, the addition of a base to a mixture of an organic acid and base will result in the base remaining uncharged, while the acid is deprotonated to give the corresponding salt. These salts are ionic and would be water soluble while the neutral natural products are not. In this technique, a mixture is dissolved in a suitable organic solvent such as dichloromethane or diethyl ether. An aqueous solution of the acid or base is added to the mixture, and the pH of the aqueous phase is adjusted to bring the compound of interest into its required form. After shaking and allowing for phase separation, the phase containing the compound of interest is collected. A flowchart of the process involved in this technique is shown in *Figure 2.1*.
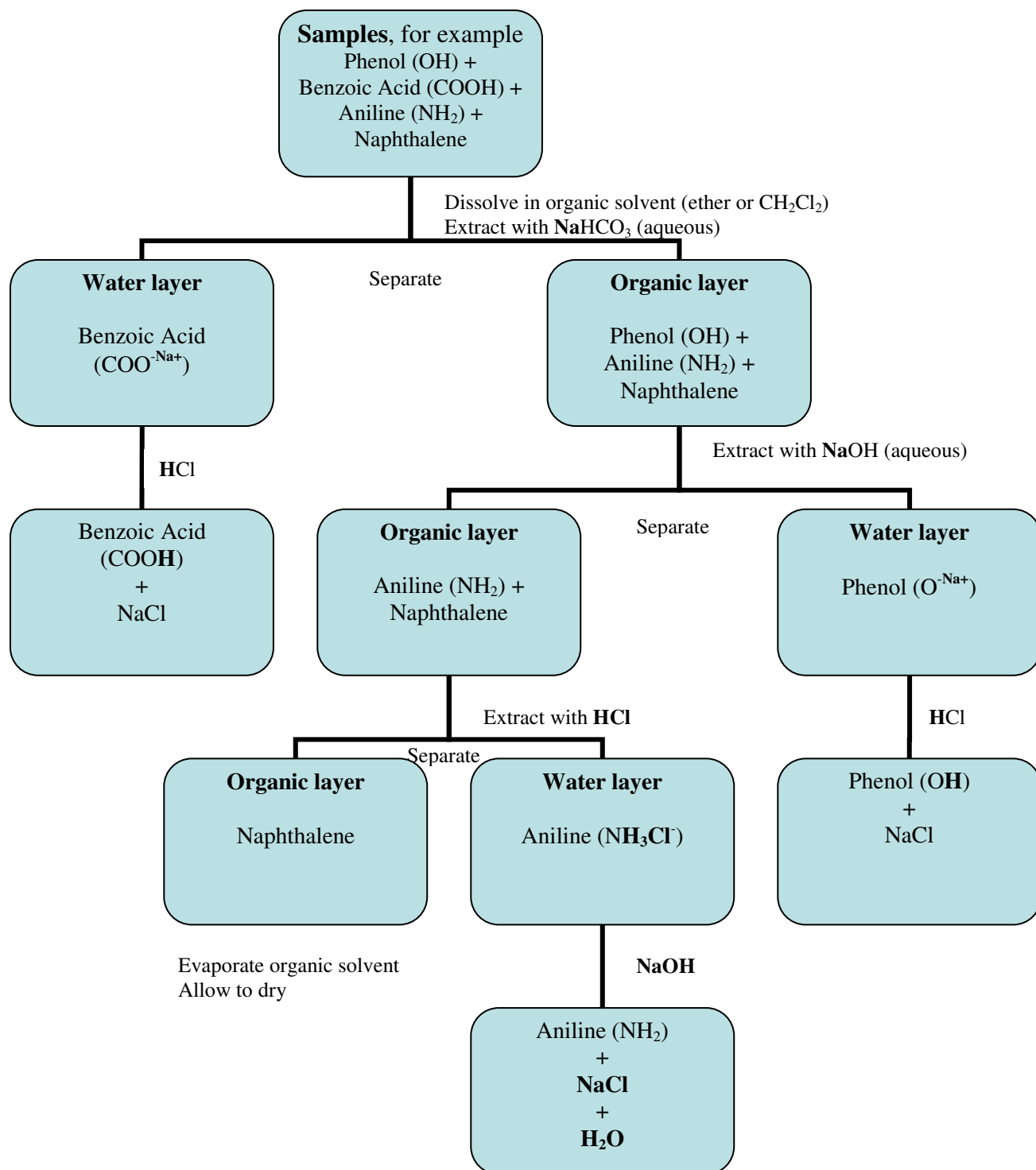
Figure 2.1: Flow chart of the acid/base extraction process

2.8 Supercritical Fluid Extraction

Super-critical fluid extraction (SFE) is a technique that uses a supercritical fluid as a solvent in an extraction. When a fluid is taken above its critical temperature (Tc) and critical pressure (Pc), it exists in a condition called "the super critical fluid state" (*Figure 2.2*). The density of the gas can be altered by varying its pressure, resulting in the super critical fluid having a density ranging between those of a gas and a liquid. By manipulating the pressure and temperature of the supercritical fluid, a pure substance can be selectively extracted. Carbon dioxide and water are the most commonly used supercritical fluids in SFE.
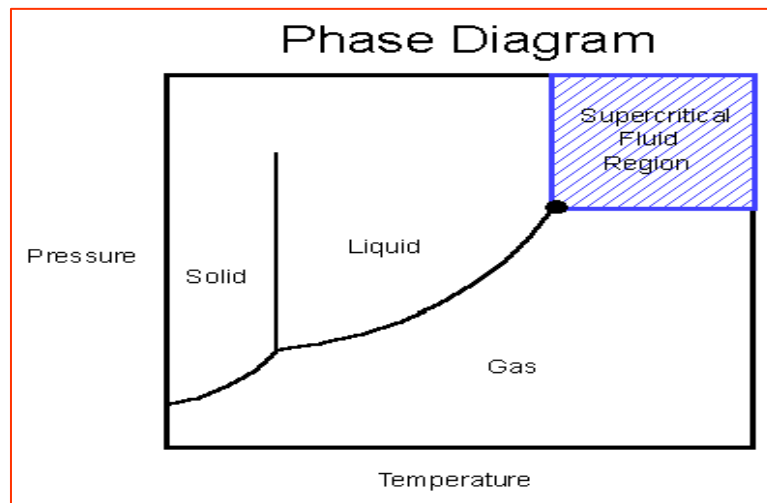

Figure 2.2: Carbon dioxide pressure-temperature phase diagram

SFE has some advantages over the more conventional extraction processes. For example, unlike the more traditional methods of extraction, SFE uses no additional solvents in the process. This results in substantial cost savings due to reduction in

post-processing steps and clean-up procedures. In addition, it has been reported that the resulting extracted product is of the purest quality (Knez *et al*., 2003).

2.9 Chromatography

Chromatography is a method for separating complex mixtures (two or more) which relies on the differential affinities of the substances to be separated for a gas or liquid mobile medium or for a stationary adsorbing medium (such as paper or silica gel) through which they are passed. Several types of chromatography are routinely used in organic chemical extraction and separation namely thin layer chromatography (TLC), gas chromatography (GC) and liquid chromatography (LC).

2.9.1 Thin Layer Chromatography

Thin layer chromatography (TLC) is a simple, quick, and inexpensive method for separating and extracting components from a mixture. It is often performed on a sheet of glass, plastic or aluminum foil which is coated with a thin layer of adsorbent material such as silica gel, aluminium oxide, or cellulose. This layer of adsorbent is known as the stationary phase. Sample comprising the different analytes is then applied onto the plate and a solvent or solvent mixture known as the mobile phase is drawn up the plate via capillary action.

Since different analytes ascend the TLC plate at different rates, separation could be achieved due to the differences in their attraction to the stationary phase and the difference in their solubility in the solvent. By changing the solvent, or perhaps using a mixture of solvents, the separation of components can be adjusted. The distance that each compound travels up the TLC plate is measured by the retention factor (R_f)

value. Comparison of the Rf value of the compound with a standard can help in the identification of the compound in the mixture.

2.9.2 Gas Chromatography

Gas-liquid chromatography (GLC), or often simply called gas chromatography (GC), is a common type of chromatography used for separating and analyzing compounds that can be vaporized without decomposition. In GC, the mobile phase is a carrier gas, usually an inert or unreactive gas such as helium or nitrogen while the stationary phase is a column consisting of either a microscopic layer of liquid or polymer on inert solid support in a glass or metal tubing.

The gaseous compounds being analyzed will interact with the walls of the column, which is coated with different stationary phases which causes each compound to elute at a different time, known as the retention time of the compound. The comparison of retention times is what gives GC its analytical usefulness.

In a GC, the process of separating the compounds in a mixture occurs with the gas phase passing through a column which is located in an oven where the temperature of the gas can be controlled. Here, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas. In this aspect, GC is similar to fractional distillation, since both processes separate the components of a mixture primarily based on boiling point (or vapor pressure) differences. However, the advantage of a GC is that it can be used on a much smaller scale (i.e. microscale) while fractional distillation is typically used to separate components of a mixture on a large scale.

2.10 Characterization of Extract

The most commonly used technique for characterization of compounds is the spectroscopic technique, in particular the nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS). NMR and MS have two advantages, i.e. they are rapid, and they are most effective for a fraction of milligram quantities of samples.

2.10.1 Mass Spectrometry

Mass spectrometry is a useful technique for determining the molecular mass as well as the structural information of a molecule. It uses the difference in the mass-to-charge ratio (*m/z*) of ionized atoms or molecules which have distinctive fragmentation patterns that provide structural information to identify their structural components which is recorded onto a mass spectrum.

A mass spectrum is a pattern representing the distribution of components (atoms or molecules) by mass which is given in a plot of intensity vs. mass-to-charge ratio graph of the molecule. In this plot, the *x*-axis is the *m/z* value which represents a relationship between the mass of a given ion and the number of elementary charges that it carries. For example, for the ion $C_2H_5^+$, a peak is observed at 29 *m/z* and that of the $C_7H_7^+$ (tropylium ion), a peak is observed at 91 *m/z*. The *y*-axis of a mass spectrum represents the signal intensity of the ions, often measured in counts per second (cps). There are many factors that can also affect signal intensity in a non-proportional way. For example, the size of the ion will affect the velocity of impact which in turn will affect the signal output. Alternatively, in systems such as Fourier

transform ion cyclotron resonance (FTICR) the signal intensity is related to the amplitude of the free induction decay signal.

2.10.2 Magnetic Resonance Spectrometry

NMR spectrometry is a form of absorption spectrometry in which a sample is subjected to electromagnetic radiation in the radio-frequency region at frequencies governed by the characteristics of the sample. NMR spectrometer must be tuned to a specific nucleus such as ^1H and ^{13}C. When placed in a magnetic field, the active nuclei (such as ^1H or ^{13}C) will absorb at a frequency characteristic of its isotope. The resonance frequency, energy of the absorption and the intensity of the signal are proportional to the strength of the magnetic field. For example, in a 21 tesla magnetic field, protons resonate at 900 MHz. It is common to refer to a 21 T magnet as a 900 MHz magnet, although different nuclei resonate at a different frequency at this field strength. Since both this frequency shift and the fundamental resonant frequency are directly proportional to the strength of the magnetic field, the shift is converted into a field-independent dimensionless value known as the chemical shift. The chemical shift is reported as a relative measure from some reference resonance frequency. Many types of information can be obtained from an NMR spectrum. For example, the chemical shift tells us the chemical environment of the nuclei which can be used to obtain some structural information about the molecule in a sample. Different protons in a molecule resonate at slightly different frequencies, indicating the different environment to which they are exposed. For example, protons on an aromatic ring will have a chemical shift value of about 7 ppm (relative to tetramethysilane at 0 ppm). An NMR spectrum is often represented by a plot of the frequencies of the absorption versus intensities constitutes (Tang, 1978 and Mahmood and Abd. Rahman, 1998).

2.11 Objective of Study

In this work, micropropagation via somatic embryogenesis will be carried out to better understand the process of embryogenic callus induction, cell suspension multiplication, and the regeneration of plantlets, and to establish a source of *in vitro* culture material.

This study aims to improve the method for extraction of carpaine from *Carica papaya* L. var. Eksotika. Characterization of carpaine will be carried out using the NMR and GC-MS spectroscopic techniques. Comparison for the accumulation of carpaine in different parts of the field grown plant as well as *in vitro* regenerant via acid base extraction versus supercritical fluid extraction was carried out. The information gathered will be used in establishing ways to enhance the accumulation of carpaine in *Carica papaya* L. var. Eksotika.