4.1 Induction and Development of Embryogenic Callus

Visual observations were made fortnightly for eight weeks on all immature embryo explants excised from *Carica papaya* L. variety Eksotika placed on CI medium supplemented with or without 250 mg/L carbenicillin. This concentration was chosen based on preliminary work done (data not shown).

During the first two weeks, explants excised showed different responses on the induction medium used. Initially friable callus was induced from the immature zygotic embryos after 2 weeks of culture on CI media. Subsequently upon sub-culture, embryogenic callus was observed after 3 weeks of culture on CI media. However, some of the explants produced masses of loose brown calli (Drew and Miller, 1989) which later became necrotic.

Explants gave different responses towards medium supplemented either with or without carbenicillin in terms of the production of callus and somatic embryos (***Figure 4.1***). The highest callus induction from immature embryos of *Carica papaya* L. variety Eksotika was 93.3 % ±11.8 and 80.7 % ±7.1 on the CI medium with and without 250 mg/L carbenicillin placed in the dark regime respectively. This result was significantly different from the means at the 5 % level of significance and p-value at 0.0538 (***Table 4.1***).

4.2 Formation of Somatic Embryos

In this study, somatic embryos were formed at the surface of the callus from most explants. It was shown that the best media for the induction of somatic embryos was 10 mg/L 2,4-D and 250 mg/L carbenicillin supplemented CI medium placed in the dark condition where 80.4 % [Total Number of Embryogenic Callus (Clumps) / Total Number of Explants (Clumps)] of the explants forming callus gave rise to somatic embryos (***Table 4.1***).

It was observed that embryogenic apical dome of the zygotic embryos were enlarged after 5 weeks and somatic embryos were first seen budding on the apical dome of the explants after 2 months of culture on the CI media. After 4 months, about 20 to 50 somatic embryos were produced from one immature zygotic embryo. The callus maintained high regenerative capacity after 5 months of culture on CI media (80.4 % ±13.6 and 71.2 % ±5.6 with and without 250 mg/L carbenicillin supplemented media respectively). This result was significantly different from the means at the 5 % level of significance and p-value at 0.0736 (***Table 4.1***).

Table 4.1: Response of immature zygotic embryo explants of *Carica papaya* L. var. Eksotika towards callus initiation and somatic embryo formation in culture media supplemented with and without 250 mg/L carbenicillin; ±s.d – standard deviation.

Treatment	Types of Culture	No. of Explant (Clumps)	No. of Embryogenic Callus (Clumps)	No. of Embryogenic Callus forming Somatic Embryo (Clumps)	Percentage of Embryogenic Callus forming Somatic Embryo (%±s.d)
A.	Callus Induction Media (CI) without 250 mg/L Carbenicillin	47	31	31	66.0±0.2
		32	21	21	65.6±8.6
		31	25	25	80.7±7.1
		11	8	8	72.7±6.6
	Average	30.3	21.3	21.3	71.2±5.6
	s.d	9.0	5.0	5.0	8.6
B.	Callus Induction Media (CI) with 250 mg/L Carbenicillin	30	28	28	93.3±11.8
		30	23	23	76.7±15.4
		24	15	15	62.5±13.8
		18	16	16	88.9±13.3
	Average	25.5	20.5	20.5	80.4±13.6
	s.d	5.7	6.1	6.1	0.1

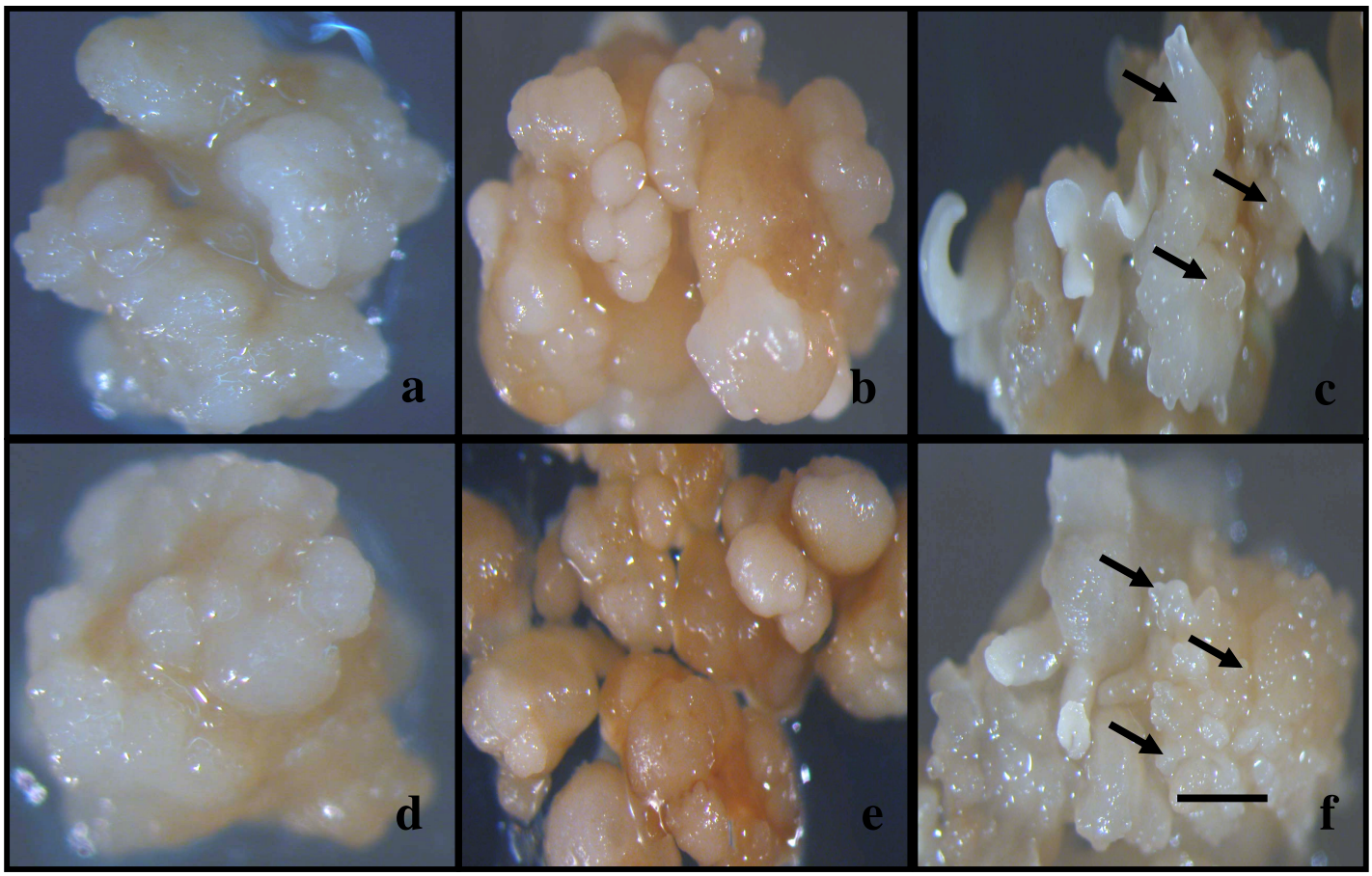


Figure 4.1: Comparison of the morphology and development somatic embryo induction in CI media with 250 mg/L carbenicillin (a, b and c) and without 250 mg/L carbenicillin (d, e and f) respectively. (a, b, d and e: callus proliferation of undifferentiated cells, c and f: direct formation of the somatic embryos). (10x) Bar at 0.1 mm.

4.3 Embryogenic Cell for Liquid Multiplication Culture

It was observed that callus placed on CI medium produced initial cells after 5 days in culture. The growth of the cells increased rapidly after the first sub-culture as indicated by the weight of the cells. Ten days after sub-culture, the embryos increased in size and regular sub-culture to fresh medium resulted in high production of somatic embryos (***Figure 4.2***).

It is important to ensure that the inoculum used to initiate and multiply the cell suspension must grow vigorously. Different plates with embryogenic callus were cultured and the callus growth was observed for about a month (***Table 4.2***). Callus from ***plate 4*** were selected as the best initial inoculum at 9.1 g ±0.9 compared to ***plate 1*** at 21.9 g ±0.3 (***Table 4.2***) of callus growth. For the cell suspension, it is crucial that the cells proliferate with ease in order to obtain a good inoculum in the liquid multiplication medium. In this study, one third of the callus was placed on CI medium while the remaining callus was placed in LM. Visual observation on the development of callus from various plates cultured on CI medium was compared (***Figure 4.3***). ***Figure 4.3.d*** showed the higher frequency of embryo formation from ***plate 4***.

Table 4.2: Response of immature zygotic embryos explant towards embryogenic callus induction in MS solid media with 250 mg/L carbenicillin supplemented media based; ±s.d – standard deviation.

Time of Incubation (Days)	0	5	10	15	20	25	**Sum**	**±s.d**
No. of Plate	**Callus Growth (g)**							
1	3.3	3.4	3.6	3.7	3.9	4.0	21.9	±0.3
2	3.1	3.2	3.4	3.6	3.8	4.1	21.2	±0.4
3	1.1	1.2	1.3	1.4	1.6	1.7	8.3	±0.2
4	0.2	0.8	1. 5	1.9	2.2	2. 6	9.1	±0.9
5	2.5	2.6	2.8	2.9	3.1	3.2	17.0	±0.3

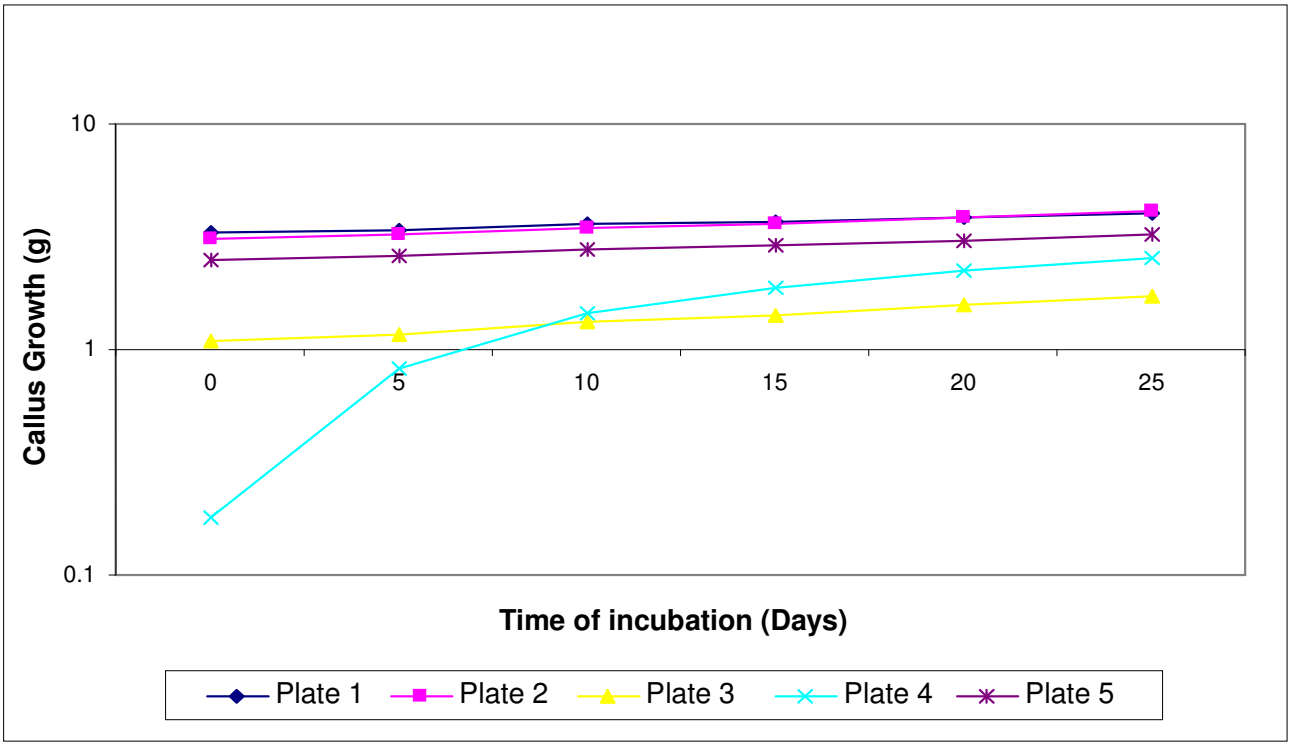


Figure 4.2: Time course of callus growth of *Carica papaya* L. var. Eksotika cultured on CI medium with 250 mg/L carbenicillin.

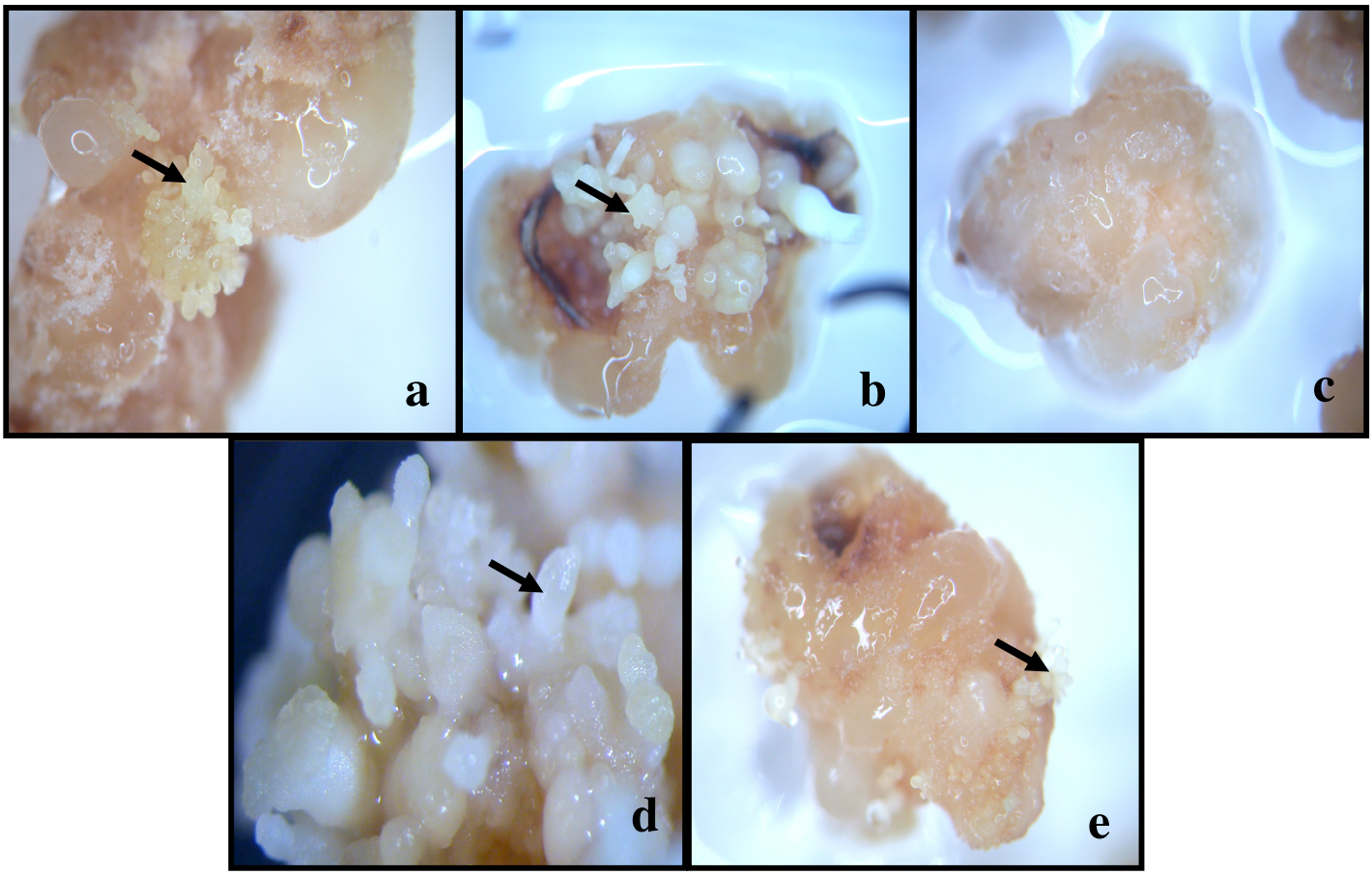


Figure 4.3: Different response of embryogenic callus from different plates towards the formation of somatic embryo. a. plate-1, b. plate-2, c. plate-3, d. plate-4 and e. plate-5. (10x) Bar at 0.1 mm.

4.4 Histochemical Test

The embryogenic and non-embryogenic nature of callus was determined by double staining method (Gupta and Holmstrom, 1978). Nuclei of cells stained with intense bright red indicated positive results of embryogenic callus and conversely, blue stained cells showed otherwise (***Figure 4.4***). Embryogenic callus were selected and used for multiplication in liquid media because of their totipotency and high regenerative capability rate.

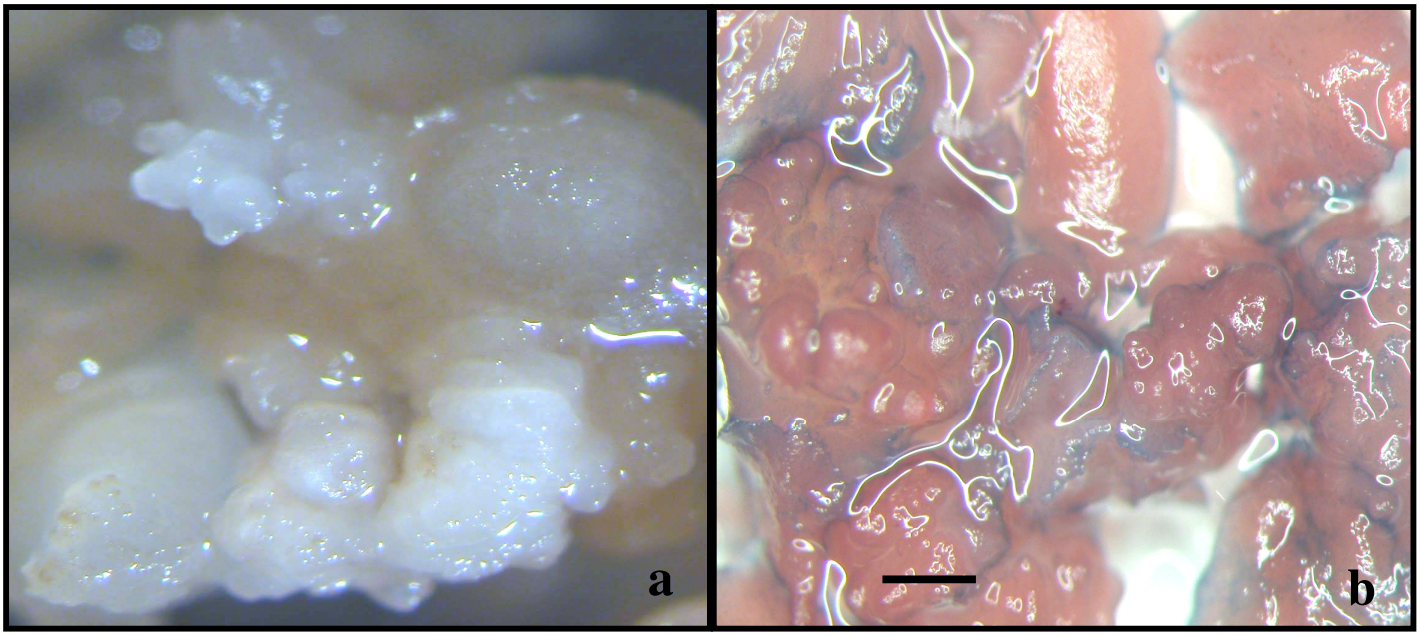


Figure 4.4: Globular and wet callus (a) double-stained red with acetocarmine (b). (10x) Bar at 0.1 mm.

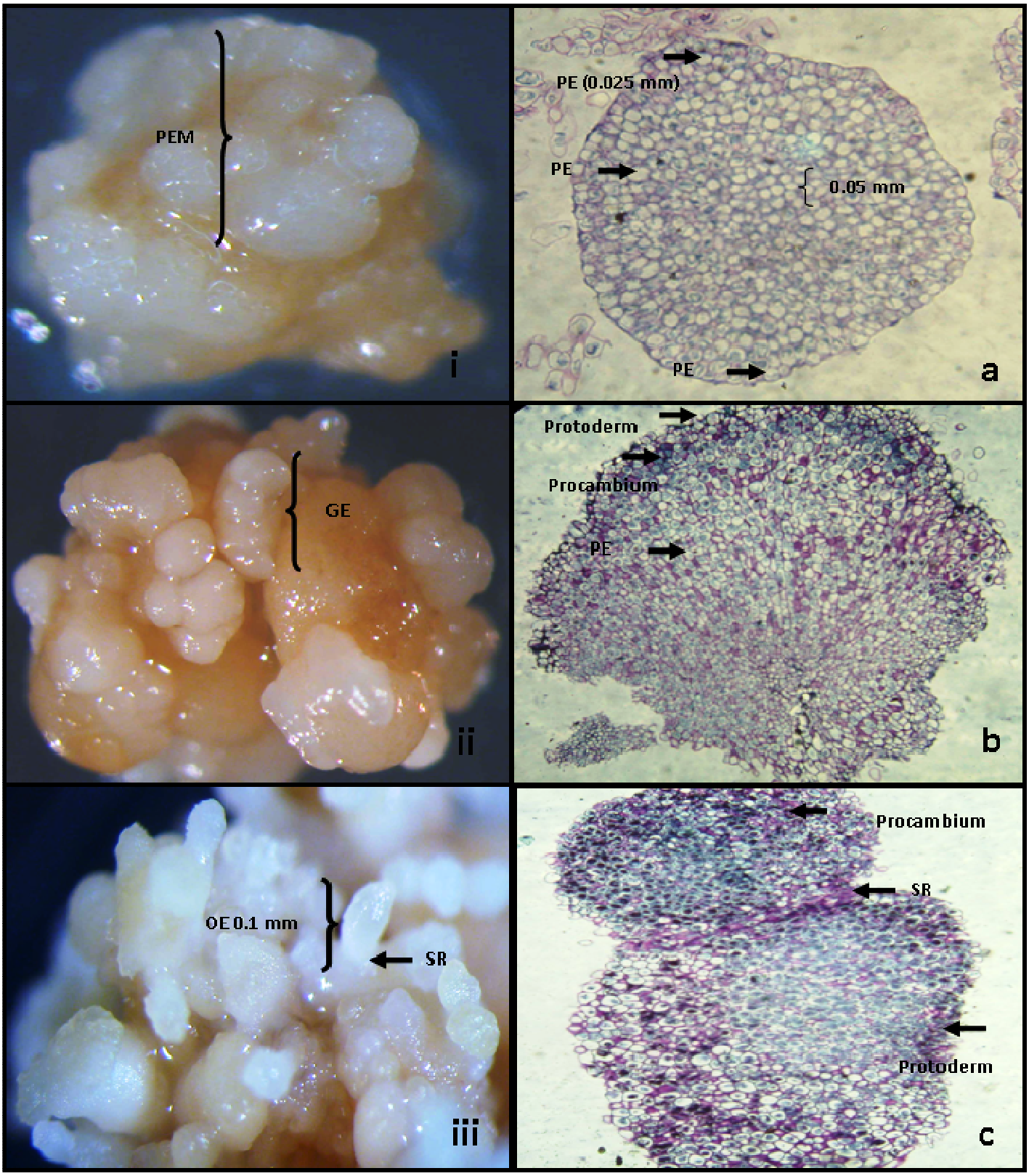
4.5 Histo-Anatomy Study

In the histo-anatomy studies, apparent observation showed groups of small meristematic cells in the whitish parenchymatic microcalli after one week of culture initiation. The cells located in the median zone of the endotesta dedifferentiated and subsequently divided to form callus (***Figure 4.5.i***). Subsequently, embryo develops within 14 days. Cells that accumulate starch (***Figure 4.5.ii***) acquired embryogenic characters and became isolated in a polysaccharide mucous like substances (***Figure 4.5.b***) and followed by visible protrusion of embryos (***Figure 4.5.iii***).

At the end of the first subculture, high frequency embryogenesis was expressed by cleavage divisions within some of cells. This was characterized by the multiplication of embryogenic cells and of young, globular embryos (***Figure 4.5.ii*****),** developed into true somatic embryos during the third and fourth sub-culture (***Figure 4.5.iii***) and also by the production of friable somatic embryos (***Figure 4.5.iv*****).**

Shoot initials at an early stage were dome shaped from the apical region of embryo axis with densely stained cells (***Figure 4.5.v***) and subsequently developed into a typical apical meristem covered with leaf primordial.

Corresponding histological sections of the embryogenic developmental stages of the cultured explants, immature zygotic embryo, removed 90 to 100 days old after anthesis of female and hermaphrodite flowers are shown in ***Figures 4.5*** (***a, b, c, d*** and ***e***)


PEM
i
PE (0.025 mm)
PE
0.05 mm
PE
a
GE
ii
Protoderm
Procambium
PE
b
OE 0.1 mm
SR
iii
Procambium
SR
Protoderm
c

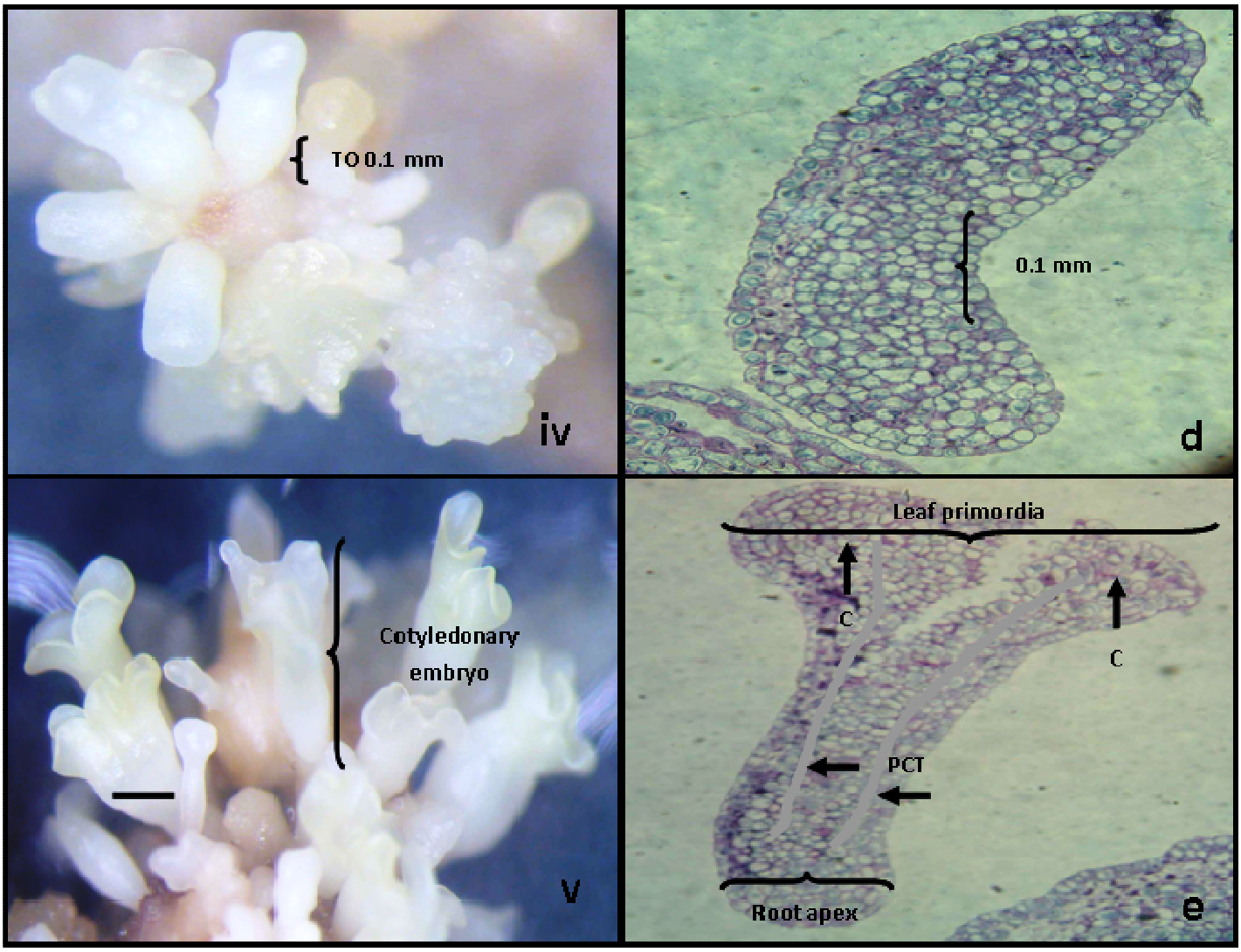


Figure 4.5: Longitudinal sections of the embryogenic cells after days 14, 21, 40, and 60 days of inoculations in MS medium containing 10 mg/L 2,4-D (i: pro-embryo, ii: globular embryo, iii: oblong and heart shape embryo, iv: torpedo shape embryo and v: cotyledon embryo) (10x), and (a, b, c, d and e) showing the phases of the embryogenesis (10x). C: Cotyledon, GE: Globular embryo, OE: Oblong embryo, PCT: Pro-cambial traces, PE: Pro-embryo, PEM: Pro-embryo mass, SR: Suspensor region, TO: Torpedo embryo.

4.6 Somatic Embryo Multiplication in Liquid Culture

It was clearly observed that the multiplication and growth parameter of cells (somatic embryo) in suspension cultures were multiplied in LM medium with 250 mg/L carbenicillin. Batch cultures technique was used for initiating single cell cultures. Cell suspensions were grown in 100 to 250 mL flasks each containing 20 to 50 mL of culture media.

Upon transfer to the agitated LM medium, large masses of cells were produced Suitable embryogenic callus for initiation of cell suspension were easily dispersed and released into the liquid medium in a few seconds due to the mechanical effects of cell clusters breaking in the liquid media.

Proliferation of globular somatic embryos from embryogenic callus became apparent after 1 to 2 month(s) of culture. The pro-embryos increased in number rapidly and produced suspension of highly uniform embryos. By regular sub-culturing, the cells in fresh medium containing 2 to 5 mg/L 2,4-D continued to proliferate for 12 months without apparent loss of regenerative potential.

However, the regenerative potential of the suspensions was reduced to 4 to 5 months when cultured in liquid media without 2,4-D. The embryos became mature if prolonged culture on this media for a subsequent 5 weeks period.

In this work, 2,4-D was found to be the major plant growth regulator affecting the competence of the single cells in liquid culture. Cell divisions in suspension cultures

were encouraged by the agitation of orbital shaker at 100x g served to aerate the culture and thus a higher growth rate at the exponential phase than those of callus in solid medium.

Three cell lines were established from the cell suspension growing in LM medium with carbenicilin and the growth responses were compared by determining the weight and volume of the suspension cells. It was observed that for ***line 1*** the weight and volume of the cell after 30 days was the highest compared to the other cell lines. However, in ***line 2*** although the weight of the cell was higher than ***line 3***, the volume was observed to be vice versa (***Table 4.3***). The development of the FW during the entire growth cycle from each individual batches could be used to select batches with desired growth characteristics. The growth of the cells was determined by collecting cells on pre-weighed (in wet condition) circular nylon filter. Cell volume after sedimentation, was determined by aliquoting 15 mL cells in centrifuge tubes and centrifuged at 1000g where the volume of cells was determined.

Table 4.3: Relationship between cell volume after sedimentation (CVS) and fresh weight of cells (FW) from three different cell lines of batch suspension cultures of *Carica papaya* L. var. Eksotika. From each cell line CVS and FW were measured during the entire growth cycle.

Items / **Batch of Cell Suspension**	**Flask**	**Incubation Period (Days)**		
		10	**20**	**30**
50 mL LM in 250 mL Erlenmeyer flask		**Weight of Callus (g)**		
	1	0.7	1.9	7.8
	2	*1.9*	*3.0*	*2.8*
	3	*2.0*	*2.1*	*2.0*
		Volume of Cells (mL)		
	1	1.4	1.9	1.9
	2	*1.1*	*0.2*	*0.3*
	3	*1.6*	*0.9*	*0.8*

The multiplication rate of the suspension cultures was higher on LM medium with 250 mg/L carbenicillin in the light regime than the ones on medium without carbenicillin in the same cultural condition with multiplication frequency at 1.7 % and 2.6 % respectively (***Table 4.4***). This was shown by taking the fresh weight of the embryogenic cell suspensions.

Table 4.4: Response of embryogenic cell multiplication in LM media with and without 250 mg/L carbenicillin; ±s.d – standard deviation.

No.	Types of Culture	Incubation Period (Days)				Average	±s.d
		0	10	20	30		
		Fresh Weight of Embryogenic Cell Suspension (ECS) (g)					
1.	Liquid Multiplication Media (LM) without 250mg/L Carbenicillin	0	1.1	1.2	1.7	1.3	±0.3
2.	Liquid Multiplication Media (LM) with 250mg/L Carbenicillin	0	1.3	0.7	2.6	1.5	±1.0

A non-invasive method for routine-estimation of suspension cultures which performed reproducible growth cycles were done by taking fresh weight of two different cell lines treated with carbenicillin and control (without carbenicillin). Fresh weight (FW) was expressed as gram per 150 mL of suspension cells and the time course of FW was measured every 10 days (***Figure 4.6***). The data in ***Figure 4.6*** showed that the cells in media without carbenicilin were actively growing from day 0 to 10 and with carbenicilin from day 20 to 30 (***Table 4.4***). After 30 days, the FW of ECS was higher in media with carbenicillin than control.

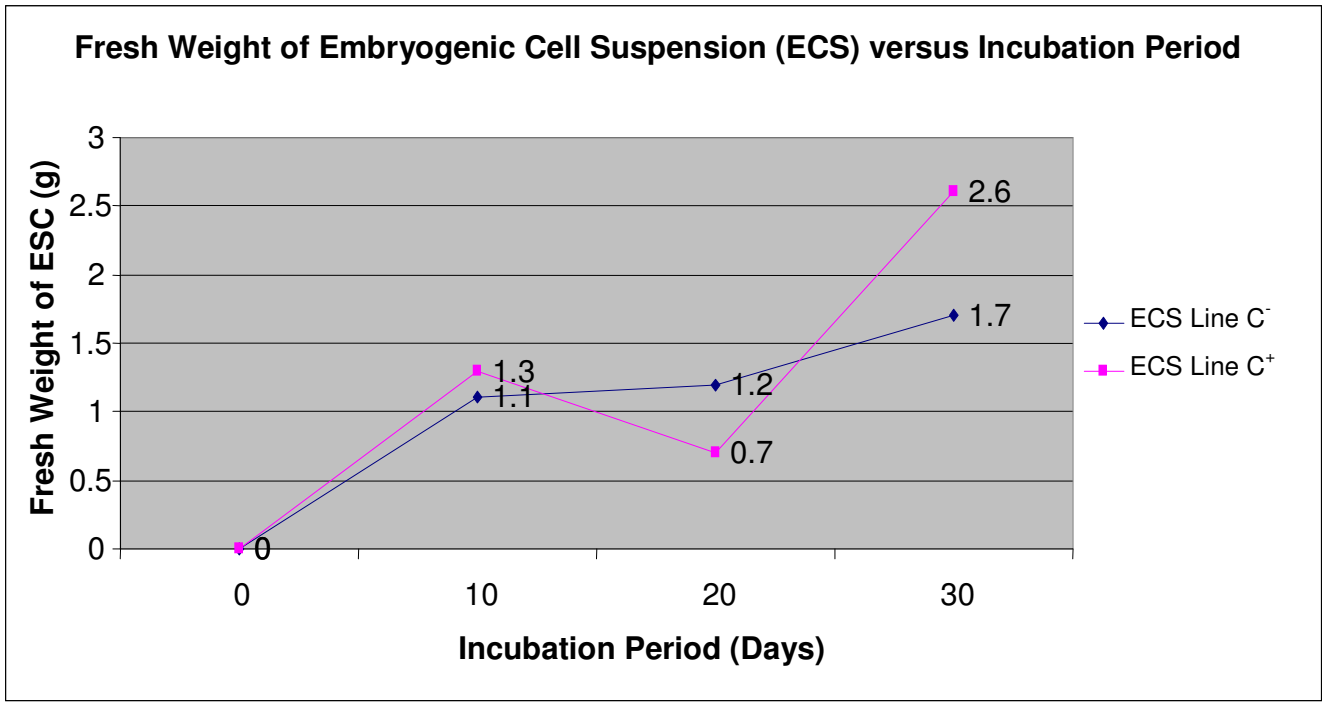


Figure 4.6: Comparison of time course of fresh weight (FW) of cell suspension grown in batches of 150 mL in Erlenmeyer flasks between LM medium with (C^+) and without (C^-) 250 mg/L carbenicillin. ECS: embryogenic cell suspension.

4.7 Cell Viability Assessment

After 2 months of continuous agitation, suspended cells (***Figure 4.7.i***) developed into embryos. High variation in cell characters were observed producing rounded, elongated and oval shaped cells with varying sizes and types (cytoplasmic and vacuolated). Assessment of cell viability using FDA solution showed that only cells with dense cytoplasm fluoresced (***Figure 4.7.iv***). Large numbers of suspension cells showed 65 % viability with dense cytoplasm fluoresced under ultraviolet (UV) light. These cells were small, rounded and with distinct nucleus.

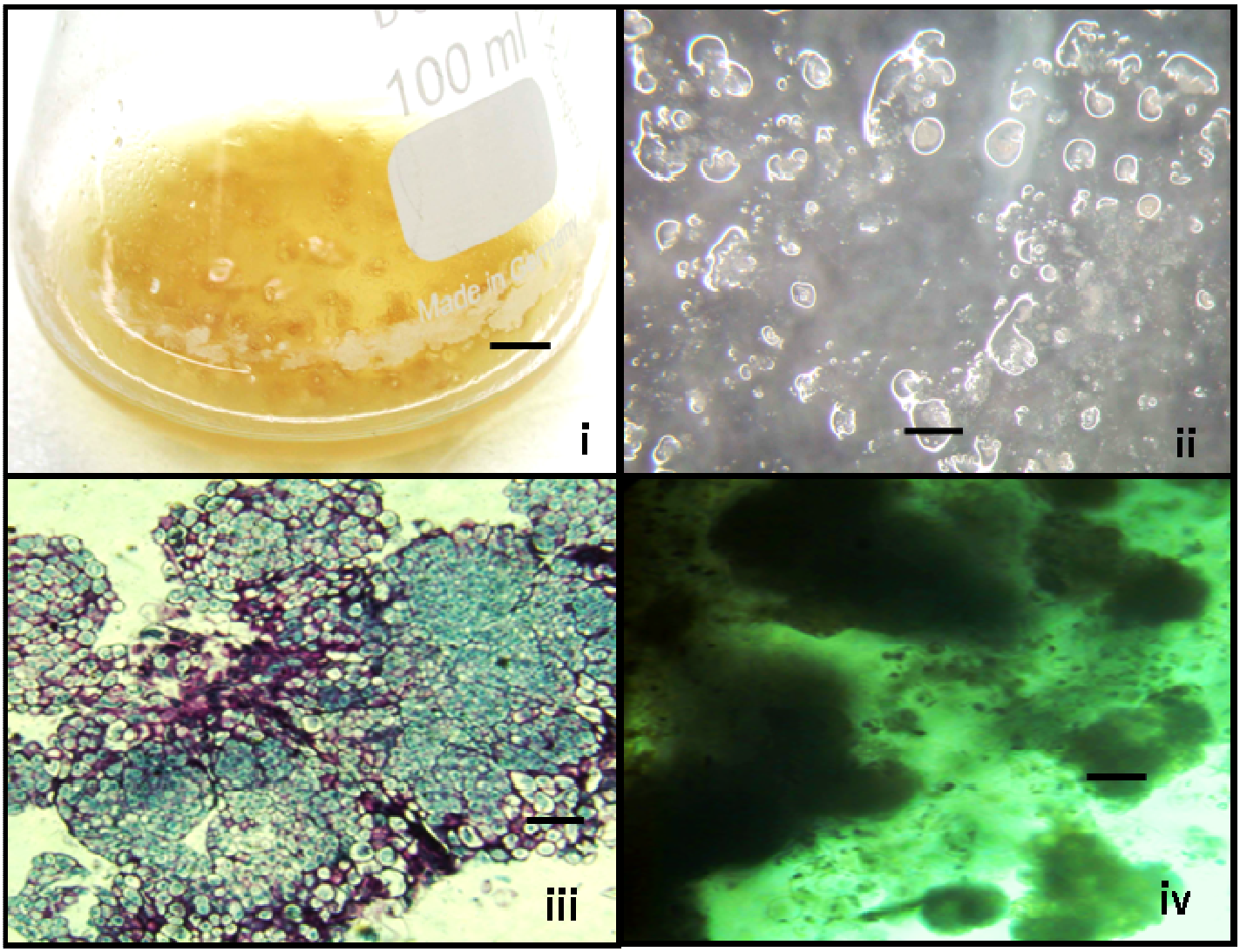


Figure 4.7: Suspended cells (i), Developed somatic embryos (ii), Cytoplasmic and non cytoplasmic cell (iii) and Fluorescing cells with dense cytoplasm (iv). (10x) Bar at 1.0 cm (i) and 0.05 mm (ii, iii and iv).

4.8 Somatic Embryo Germination

An effective and optimal culture medium for somatic embryo germination was germination (G) media supplemented with 0.2 mg/L BAP and NAA cultured in the light regime where 100 % germination was observed (***Table 4.5***). The viability of somatic embryos to form plantlet was influenced by the use of suitable G medium (***Table 4.5***).

In this study, plant germination from somatic embryo was obtained from embryogenic callus treated with CI and 250 mg/L carbenicilin. Observations were made twice a week for 4 months. The plants form shoots within the 3 months of culture period.

When normal somatic embryo germinated, hypocotyls raised cotyledons and shoot apex appeared directly on the medium and elongation of epicotyl led shoot apex away from cotyledons (***Figure 4.8.iii***). When the abnormal somatic embryos germinated, only aberrant cotyledons were obtained, and shoot apex became stunted.

Germination of cells from embryogenic suspension culture showed that the somatic embryos germinated in a highly synchronous manner; where the hypocotyl became swollen followed by rapid shoot multiplication. Concurrently, chlorophyll development becomes obvious in the cotyledons and these little shoots undergo plant regeneration stage (***Figure 4.8.c*** and ***d***).

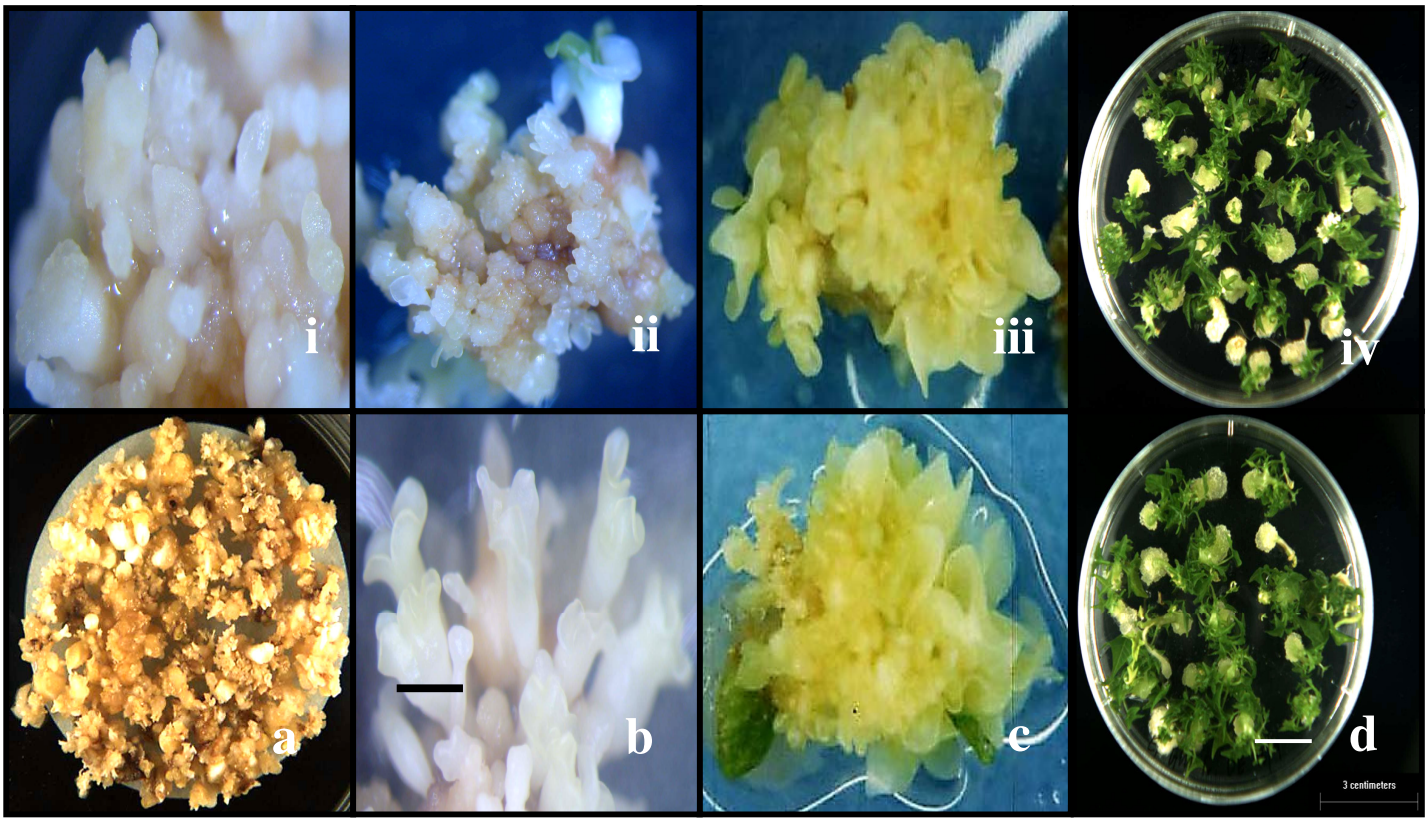


Figure 4.8: The developmental stage of somatic embryo. Proliferating embryoid from solid medium (i, ii and iv) and liquid multiplication medium (a, b and d); A fully developed embryoid (ii and b); Shoots with tri-lobed leaf and hypocotyl (iii and c); and Matured shoots (iv and d). (10x) Bar at 0.5 mm (i, ii, a and b) and 3.0 cm (iii, iv, c and d).

Table 4.5: The effects of MS supplemented with 250 mg/L carbenicillin on callus growth, somatic embryo, and 0.2 mg/L BAP and NAA in solid medium measured by the number of explants which produced callus and shoots.

Items / Types of Media	Plate	No. of Immature Zygotic Embryo	No. of Embryogenic Callus	Percentage of Lifely Embryogenic Callus
Callus Induction Media (CI) with 250 mg/L Carbenicillin	1	10	10 clumps	100
	2	10	10 clumps	
	3	10	10 clumps	
	4	10	10 clumps	
	5	10	10 clumps	

	Plate	No. of Somatic Embryos	No. of Shooted Embryos	Percentage of Regenerated Shoots
Germination Media (G) with 0.2 mg/L BAP and NAA	1	7 clumps	7 clumps	100
	2	8 clumps	8 clumps	
	3	9 clumps	9 clumps	
	4	7 clumps	7 clumps	
	5	10 clumps	10 clumps	

4.9 Regeneration of Plantlet

On G medium, average of 88.4 % matured single embryos formed distinct structural and developmental features with organized embryo cells (shoot) before reaching the stage of mature plantlets. Data is shown in ***Table 4.6***.

Table 4.7 showed that the average percentage of normal plantlets formed was 88 % whereas 12 % showed abnormality. It could be concluded that the regenerative potential from each callus clumps differed ranging from 67 to 100 %.

Table 4.6: Germination percentage of embryogenic cells from suspension culture.

Items / Medium	Clumps of 1 ml Embryogenic Cells	No. of Germinated Somatic Embryo per Clumps of Embryogenic Cells	Percentage of Germinated Somatic Embryo
Germination Media (G)	16 clumps	13/16	81.3
	20 clumps	20/20	100
	21 clumps	19/21	90.5
	24 clumps	21/24	87.5
	26 clumps	22/26	84.6
	28 clumps	25/28	89.3
	35 clumps	30/35	85.7
	Average of Percentage		88.4

Table 4.7: The plantlet development via various stages of incubation medium measured by percentage (%) of somatic embryos from liquid culture which produced shoots.

Plate	No. of Somatic Embryos Clumps	No. of Somatic Embryos Produced Shoots	No. of Normal Plantlet	No. of Abnormal Plantlet	Percentage of Normal Plantlet	Percentage of Abnormal Plantlet
1	7	7	5	2	71.4	28.6
2	7	7	6	1	85.7	14.3
3	6	6	6	0	100	0
4	7	7	5	2	71.4	28.6
5	5	5	5	0	100	0
6	8	8	8	0	100	0
7	9	9	9	0	100	0
8	7	7	7	0	100	0
9	6	6	5	1	83.3	16.7
10	7	7	7	0	100	0
11	6	6	4	2	66.7	33.3
12	10	10	7	3	70	30
13	7	7	7	0	100	0
14	7	7	5	2	71.4	28.6
15	7	7	7	0	100	0
Total					88	12

In this work, maturation of somatic embryo was achieved on solidified G medium but it was also reported that vigorous embryos could also be obtained in liquid medium (Gupta and Ibaraki, 2006). It was observed that for the completion of maturation process, each embryo must achieve both morphological (***Figure 4.9***) and physiological maturity.

The complete process of plant regeneration encompasses a series of defined developmental stages from cell aggregate to heart shaped to cotyledonary for 2 and 4 weeks respectively. Several sub-cultures on the G medium supplemented with BAP and NAA (0.2 mg/L) followed by culturing on regeneration (R) medium consisted of GA_3 (1 mg/L), IBA (0.5 mg/L) and riboflavin (0.37 mg/L) (***Appendix 5***) produced 96.4 % of plantlets (***Table 4.8***).

Observation for rooting development was monitored after 3 weeks of culture. The best rooting medium for the rhizogenesis of the new *in vitro* derived shoots was induced under *in vitro* conditions in A2 media, where the minimum percentage achieved was 50 % (***Table 4.9***). A2 medium consisted IBA (2.0 mg/L) (***Appendix 6***). The formation of profuse roots and normal plantlets (***Figure 4.9***) in this study required a specific auxin type and concentration to suit the initial needs of the developmental tissue. In this work, 2.0 mg/L IBA was used to induce the outgrowth of axillary roots.

Acclimatization is the final stage where plantlets will be transferred from jars into polybags (***Figure 4.9.iv***) for 2 months for the purpose of hardening the vegetative structures and later to the field in an open area where the conditioning of regenerants were essential for adaptation.

For acclimatization processes, when the first set of matured tri-lobed leaves appeared (***Figure 4.9.iii***), the plantlets were transferred to polybags with commercial soil (Jaya Tanah Baja™) for 1 to 2 months in the nursery. After reaching a desirable size (10 to 15

cm), plants were then transplanted to the field. This will enable plant physiological stabilization in order to adapt with external atmosphere.

Table 4.8: Regeneration percentage of somatic embryos cultured on G medium when transferred to R medium.

Items / Medium	No. of Clumps of Somatic Embryo	No. of Germinated Somatic Embryo per Clumps of Somatic Embryo	No. of Regenerated Somatic Embryo	Percentage of Regeneration
Regeneration Media (R)	15 clumps	14/15	14/14	100
	25 clumps	25/25	22/25	88
	25 clumps	25/25	24/25	96
	25 clumps	23/25	23/23	100
	25 clumps	19/25	18/19	94.7
	25 clumps	25/25	24/25	96
	25 clumps	25/25	25/25	100
	Average of Percentage			96.4

Table 4.9: Assessment of roots formation in various rooting media. All media are described in Appendix 6.

No.	Types of Media	Types of Explants		No. of Cultures	No. of Rooted Plants	Percentage of Rooted Plants (%)
		Direct Embryogenesis	Indirect Embryogenesis			
1	A1 *(Control)*	-	√	12	3	25
2	A2		√	12	7	58.3
3	A3	√ (4)	√ (7)	11	3 / 3	75 / 42.9
4	A4	√	-	14	4	28.6
5	A5	√	-	5	0	0
6	B	√	-	12	0	0
7	C	√	-	15	0	0

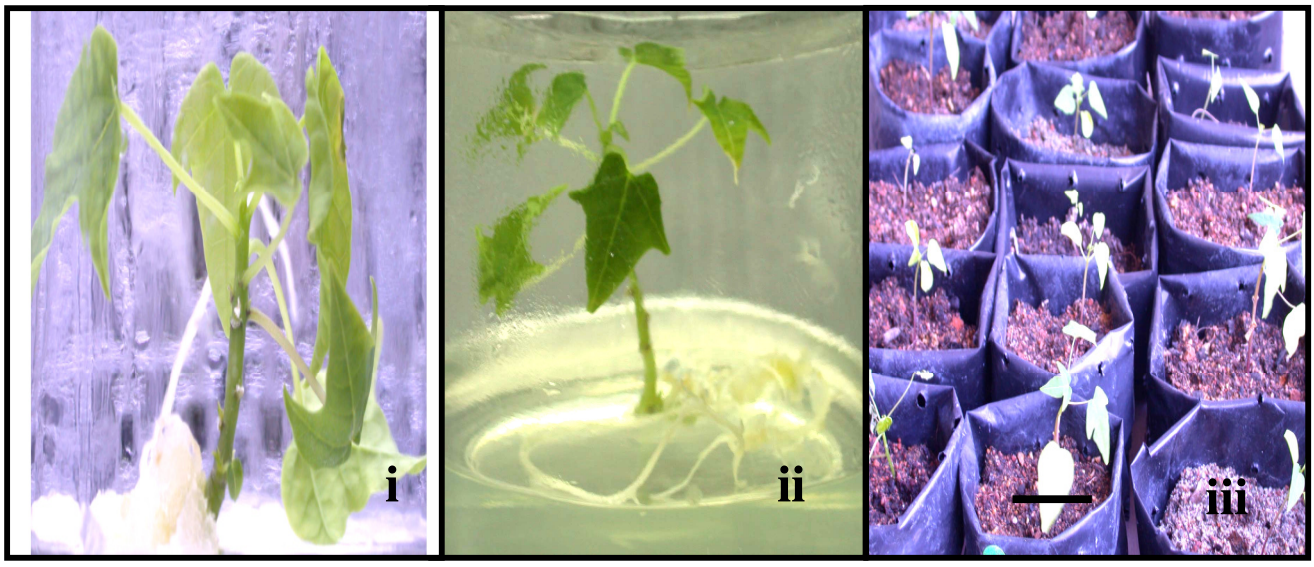


Figure 4.9: The formation of profuse roots and normal plantlets. A shoot with tri-lobed leaf and hypocotyl (i); A plant with well developed roots (ii); and A plant grown in soil (iii). (10x) Bar at 3.5 cm.

4.10 Characterization of Alkaloid Carpaine

4.10.1 Conventional Method (Acid/Base Extraction)

4.10.1.1 Field Grown Plant, In vitro Regenerant and Embryogenic Cells Suspension

Carpaine was isolated and crystallised from various parts of papaya plant from samples ranging from 20 to 500 g. Results of the amount isolated is shown in ***Table 4.10***.

Table 4.10: A distribution of carpaine extract from *Carica papaya* L. var. Eksotika plant parts; ECS: Embryogenic Cells Suspension; -: Not Applicable; ±s.d – standard deviation

Types of Sample									
Batches		***In vivo***			***In vitro***				
		Plant Parts							
		Leaf	Petiole	Fruit Peel	Leaf	Petiole	Fruit Peel	ECS-Liquid	ECS-Cell
Amount (g or ml) of Sample	Pre-Extraction	500	150	100	25	20	-	500	100
		500	150	100	25	20	-	500	100
		500	150	100	25	20	-	500	100
Weight (g) of Crystal	Post-Extraction	0.213	0.046	0.027	0.001	0.003	-	0.024	0.066
		0.270	0.043	0.027	0.004	0.001	-	0.023	0.060
		0.285	0.052	0.022	0.004	0.003	-	0.020	0.064
		±	±	±	±	±	-	±	±
		0.038	0.004	0.003	0.002	0.001	-	0.002	0.003
Percentage of carpaine extract (%)		0.043	0.031	0.027	0.004	0.015	-	0.005	0.066
		0.054	0.029	0.027	0.016	0.005	-	0.005	0.060
		0.057	0.035	0.022	0.016	0.015	-	0.004	0.064
		±	±	±	±	±	-	±	±
		0.007	0.003	0.003	0.007	0.006	-	0.001	0.003

The purity of carpaine extracted (from acid/base extraction) from the leaves of field grown plant and *in vitro* regenerant of Eksotika was analysed using TLC and observed to be comparable to those reported earlier (Coke and Rice, 1965; Burdick, 1971; and Tang, 1979).

In addition, the melting point of the carpaine isolated corresponded to the reported melting point (Tang, 1978, Burdick, 1971, Ogan, 1970 and Coke and Rice, 1968).

In this study one of the pseudo-carpaine, i.e. the dehydrocarpaine II, was detected only in a very minor quantity in the gas chromatogram with the retention time of 54.87 minutes (i.e., approximately 10 % as compared to carpaine as observed in the gas chromatogram). This is confirmed by mass spectrum with a peak observed at *m/z* 476. This compound was, however, not isolated.

Both the pseudo-carpaine is not easily separated from carpaine through the conventional purification techniques such as re-crystallization. Coke and Rice (1965) and Tang (1979) used ethanol/water/acetic acid in a ratio of 89:10:1 (v/v/v) to soak the samples for the carpaine extract. In our method, the same solvent mixture was used. However, in our method, a gradient ratio of 94.5:5:0.5 (v/v/v) of the solvents was used resulting in better separation of the carpaine from the dehydocarpaine II which is approximately 10 % the amount of carpaine as observed in the gas chromatogram (***Figure 4.14*** and ***4.15***). Presumably, the more non-polar medium encouraged better extraction of carpaine which is non-polar. In addition, the extracts were subjected to centrifugation which removed all solid particles, thus providing a cleaner sample for crystallization.

From conventional (acid/base) extraction experiments conducted, carpaine was obtained as dull yellow needle-like crystals as shown in ***Figure 4.10*** (*Crystal system/space group*: orthorhombic/$P2_12_12_1$) (Rajnikant *et al*., 2005). The R_f value (BuOH-HOAc-H_2O, 4:1:5 v/v/v) on TLC was 0.43±0.11 (***Table 4.11***)

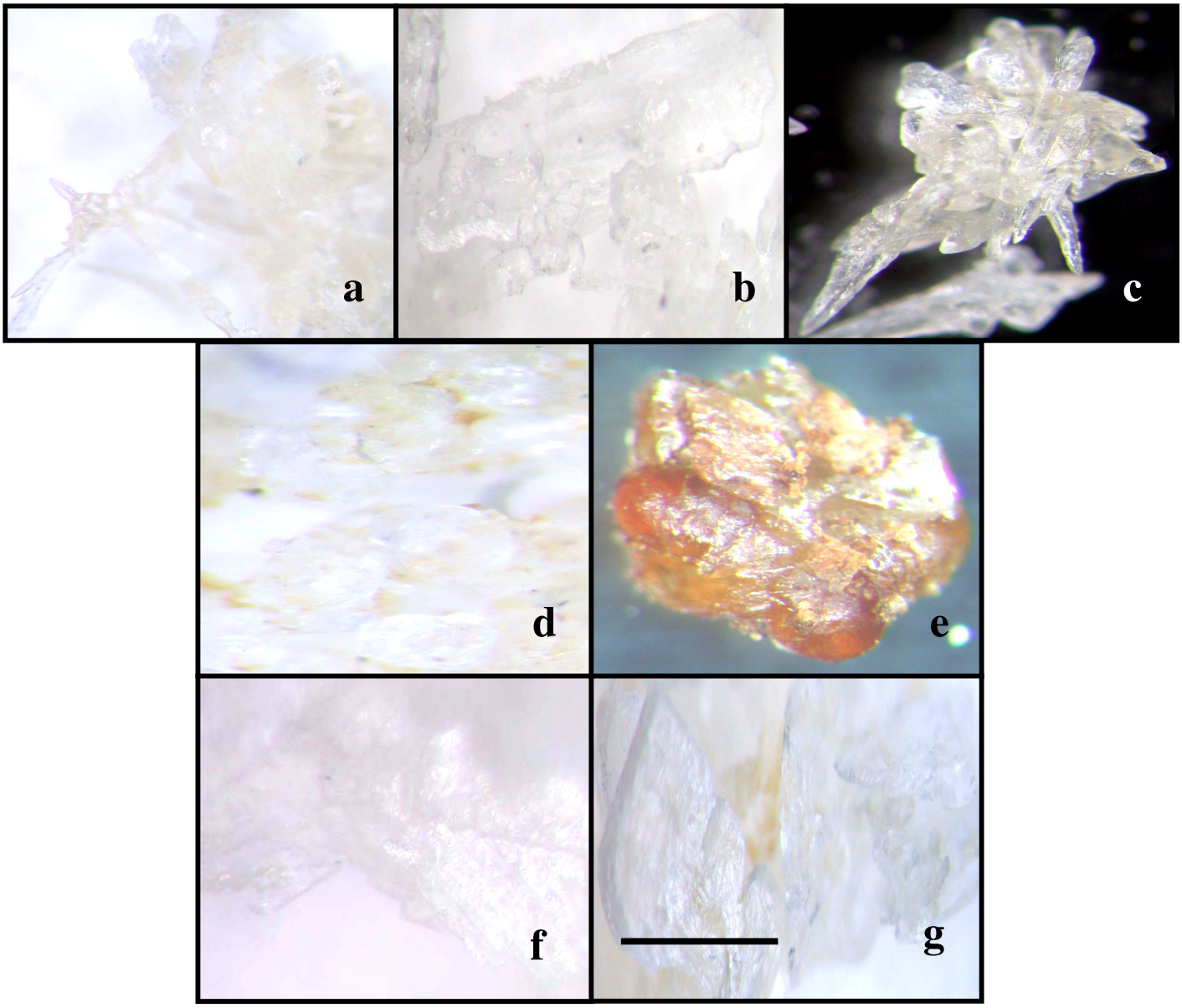


Figure 4.10: Pictures of carpaine isolated from the leaf of the field grown plants (a), petiole (b), and fruit peel (c); *in vitro* plantlets leaf (d) and petiole (e); and embryogenic cells suspension (f) and liquid (g) through conventional acid/base extraction. (10x) Bar at 2.0 mm.

Table 4.11: The pooled fractions subjected to thin layer chromatography; FGP: Field Grown Plants; IvP: *In vitro* Plantlets; ECS: Embryogenic Cells Suspension; Not Applicable: -; Ave: Average; and ±s.d: standard deviation

Types of Sample	Leaf				Petiole				Fruit Peel				ECS Liquid Medium				ECS Cells			
	Replicate																			
	1	2	3	Ave	1	2	3	Ave	1	2	3	Ave	1	2	3	Ave	1	2	3	Ave
FGP	0.13	0.36	0.38	0.29 ± 0.14	0.43	0.43	0.43	0.43 ± 0.00	0.53	0.60	0.54	0.56 ± 0.04	-	-	-	-	-	-	-	-
***Iv*P**	0.30	0.38	0.38	0.35 ± 0.05	0.34	0.36	0.36	0.35 ± 0.01	-	-	-	-	-	-	-	-	-	-	-	-
ECS	-	-	-	-	-	-	-	-	-	-	-	-	0.51	0.53	0.48	0.51 ± 0.03	0.54	0.57	0.50	0.54 ± 0.04

The melting points (m.p.) for the crystalised carpaines isolated from different parts of the plant were observed to be between 110 to 121 ^{o}C (literature m.p. 119 to 120 ^{o}C) (Tang, 1979) (***Table 4.12***).

Table 4.12: Observed melting points of crystals isolated from various parts of plants.

No.	Sample	Temperature (^{o}C)	Time (sec.)
1.	Crystalline of *In vivo* Plants Leaf	119	±10
2.	Crystalline *In vivo* Plants Petiole	110	±10
3.	Crystalline *In vivo* Plants Fruit Peel	121	±10
4.	Crystalline *In vitro* Leaf	120	±10
5.	Crystalline *In vitro* Petiole	110	±10
6.	Crystalline Cells Suspension	118	±10
7.	Crystalline Suspension Liquid	121	±10

The ^{1}H NMR and mass spectra for the sample extracted from the leave are shown in ***Figures 4.12 (a)*** and ***(d)*** while the spectra for all other samples extracted from the different parts of the plant are also attached in ***Figure 4.12***.

The ^{1}H NMR spectrums revealed the following signals: δ ^{1}H (400 MHz, $CDCl_3$): 1.01 (6 H, d, *J* = 7 Hz, 2 C<u>H</u>CH_3), 1.3 - 1.7 (28 H, m, 2 $(CH_2)_7$), 1.9 - 2.6 (10 H, m, cyclic-H), 2.85 (2 H, q, *J* = 7 Hz, 2 C<u>H</u>CH_3) and 4.7 (1 H, bs, 2 H, 2 NH). This spectrum is identical to that reported by Sato and co-workers (Sato *et al*., 2003). Mass spectrometry found a peak at *m/z* 478.377 in the spectrum which corresponded to ***Figure 4.14 (a)*** and ***(d)*** $C_{14}H_{25}N_2O_4$, as reported by Tang (Tang, 1979).

4.10.2 Supercritical Fluid Extraction

4.10.2.1 Field Grown Plant and In vitro Regenerant

In this study, the weight of papaya samples used ranged from 20 to 500 g. Supercritical fluid extraction (SFE) of these samples furnished crystalline carpaine as displayed in ***Table 4.13*** with the average volume of solvent used to be about 3033.2 g in one hour showed in ***Table 4.14.***

Table 4.13: A distribution of carpaine extract from *Carica papaya* L. var. Eksotika plant parts; ECS: Embryogenic Cells Suspension; -: Not Applicable; ±s.d – standard deviation

Types of Sample									
Batches		***In vivo***			***In vitro***				
		Plant Parts							
		Leaf	Petiole	Fruit Peel	Leaf	Petiole	Fruit Peel	ECS-Liquid	ECS-Cell
Amount (g or ml) of Sample	Pre-Extraction	50	50	50	5	5	-	-	-
		50	50	50	5	5	-	-	-
		50	50	50	5	5	-	-	-
Weight (g) of Crystal	Post-Extraction	1.988	0.231	0.454	0.018	0.003	-	-	-
		1.8	0.22	0.444	0.015	0.002	-	-	-
		1.98	0.216	0.44	0.015	0.003	-	-	-
		±	±	±	±	±	-	-	-
		0.106	0.008	0.007	0.002	0.001	-	-	-
Percentage of carpaine extract (%)		0.994	0.116	0.227	0.36	0.06	-	-	-
		0.900	0.110	0.222	0.3	0.04	-	-	-
		0.990	0.108	0.220	0.3	0.06	-	-	-
		±	±	±	±	±	--	-	-
		0.053	0.004	0.004	0.035	0.012	-	-	-

Table 4.14: Volume of CO_2 used during the extraction procedures

No.	Initial volume of CO_2 (g)	Final volume of CO_2 (g)	Total of CO_2 used (g)	Sample
1	285066	281424	3642	*In vivo* leaf
2	285723	281424	4299	*In vivo* petiole
3	288536	285723	2813	*In vivo* fruit peel
4	291877	288536	3341	*In vitro* leaf
5	292948	291877	1071	*In vitro* petiole

Carpaine obtained from SFE gave dull yellow cubic-like crystals as shown in ***Figure 4.11*** (*Crystal system/space group*: orthorhombic/$P2_12_12_1$) (Rajnikant *et al*., 2005), The R_f value (BuOH-HOAc-H_2O, 4:1:5 v/v/v) for the compound was observed to be 0.45 ±0.08 (***Table 4.15***)

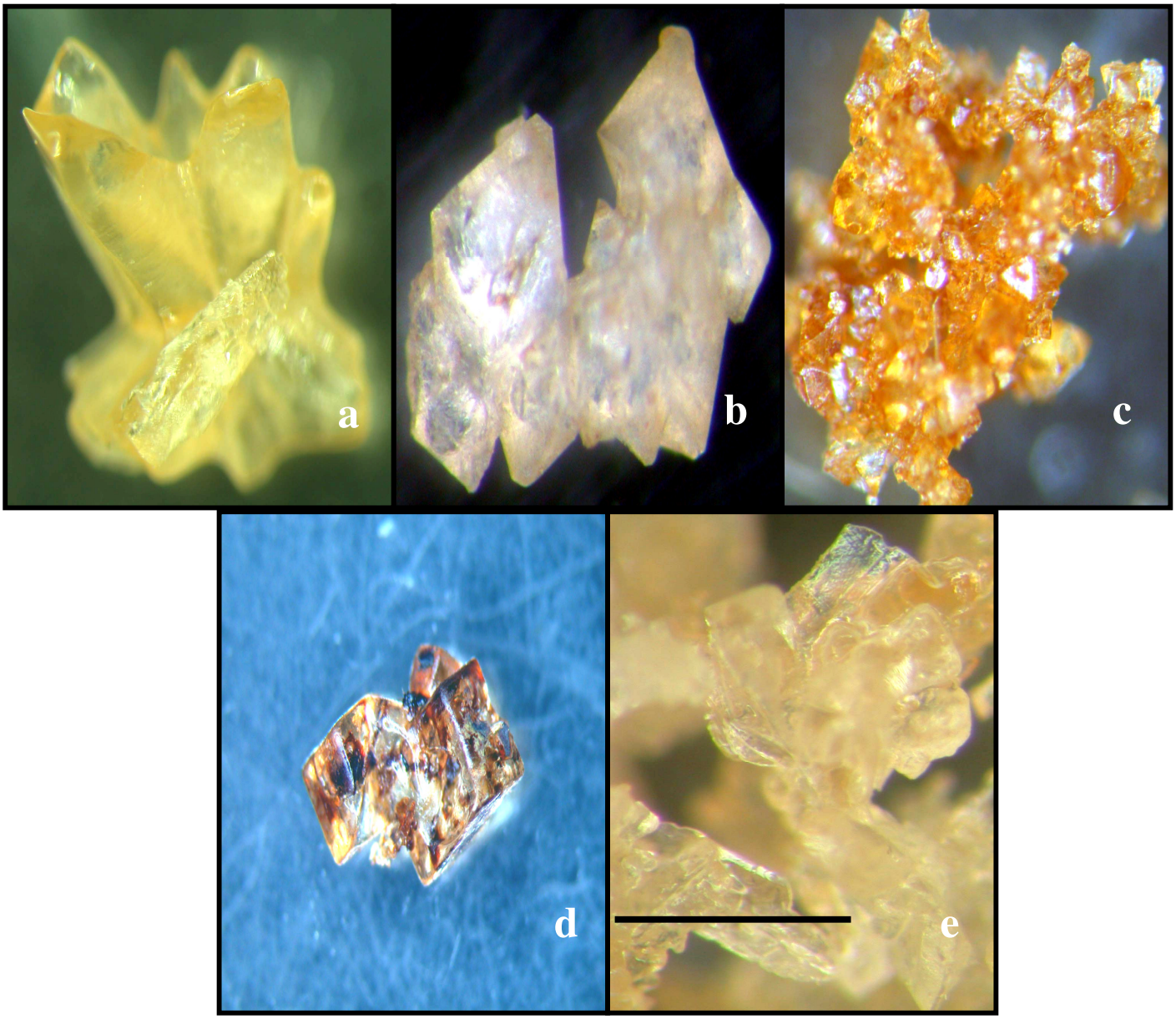


Figure 4.11: Pictures of carpaine isolated from the leaf of field grown plants (a), petiole (b), and fruit peel (c); and *in vitro* plantlets leaf (d) and petiole (e) through supercritical fluid extraction. (10x) Bar at 0.025 cm.

Table 4.15: The pooled fractions subjected to thin layer chromatography; FGP: Field Grown Plants; *Iv*P: *In vitro* Plantlets; Not Applicable: -; Ave: Average; and ±s.d: Standard Deviation

Types of Sample	**Leaf**				**Petiole**				**Fruit Peel**			
	Replicate											
	1	2	3	Ave	1	2	3	Ave	1	2	3	Ave
FGP	0.40	0.55	0.41	0.45 ± 0.08	0.43	0.53	0.42	0.46 ± 0.06	0.53	0.57	0.50	0.53 ± 0.04
***Iv*P**	0.40	0.40	0.38	0.39 ± 0.01	0.41	0.40	0.41	0.41 ± 0.006	-	-	-	-

The melting point of the carpaine extracted from SFE corresponded to the reported melting point (Tang, 1978, Burdick, 1971, Ogan, 1970 and Coke and Rice, 1968), indicating the carpaine to be pure as indicated in ***Table 4.16***.

In addition, one of the pseudo-carpaine, i.e. the dehydrocarpaine II, was detected only in a very minor quantity (10 %) in the gas chromatogram at the retention time of 54.87 minutes.

The mass spectrum of the peak observed at *m/z* 476 confirmed the presence of the dehydrocarpaine II. This compound was, however, not isolated.

The melting points (m.p.) of the isolated carpaines were found to be between 98 to 120 ^{o}C (literature m.p. 119 to 120 ^{o}C) (Tang, 1979).

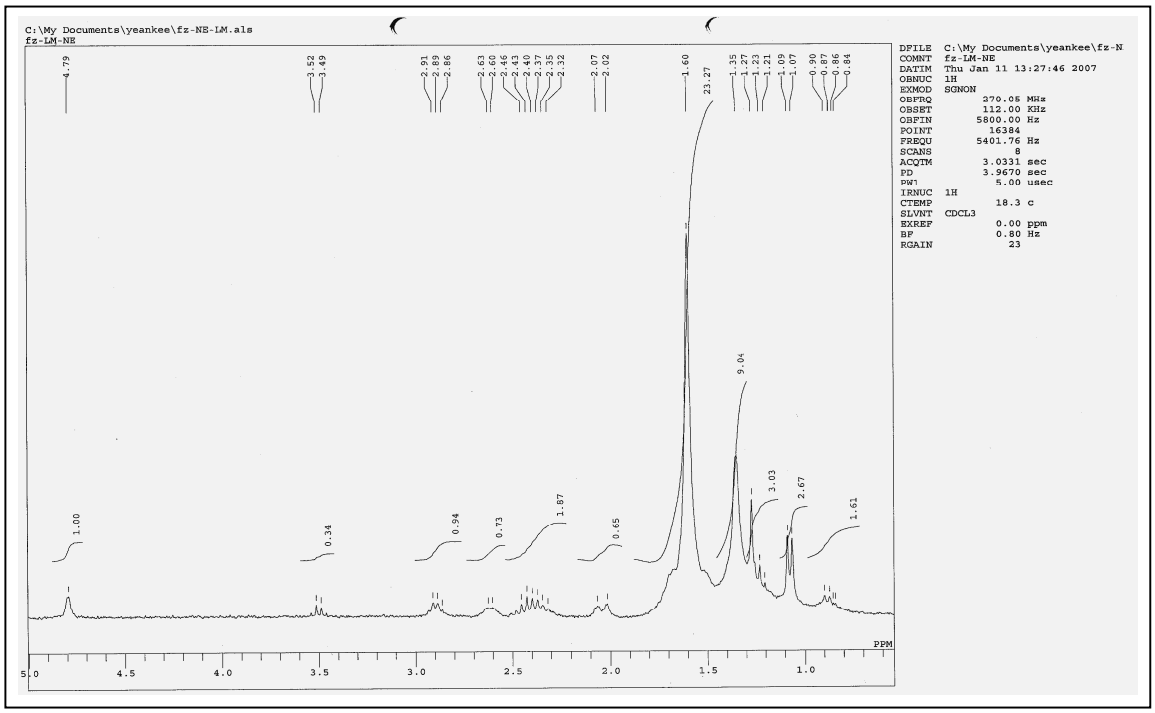
Table 4.16: Observed melting points of crystals isolated from various parts of plants.

No	Sample	Temperature (ºC)	Time (sec.)
1.	Crystalline of *In vivo* Plants Leaf	119	±10
2.	Crystalline *In vivo* Plants Petiole	98	±10
3.	Crystalline *In vivo* Plants Fruit Peel	108	±10
4.	Crystalline *In vitro* Leaf	120	±10
5.	Crystalline *In vitro* Petiole	106	±10

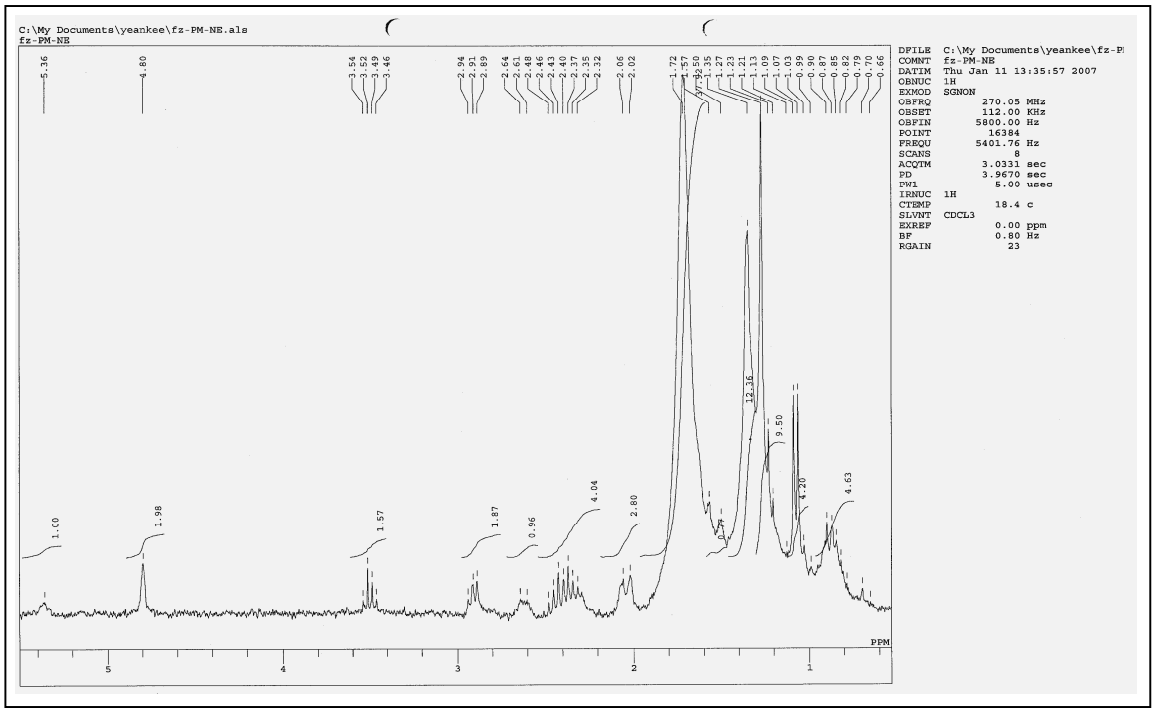
The ^{1}H NMR and mass spectra for the ample extracted from the leaves are shown in ***Figure 4.13 (a)*** and ***(d)*** while the spectra for all other samples extracted from the different parts of the plant are also attached in ***Figure 4.13***.

The ^{1}H NMR spectrums revealed the following peaks for carpaine: δ ^{1}H (400 MHz, $CDCl_3$): 1.01 (6 H, d, *J* = 7 Hz, 2 C<u>H</u>CH_3), 1.3 - 1.7 (28 H, m, 2 $(CH_2)_7$), 1.9 - 2.6 (10 H, m, cyclic-H), 2.85 (2 H, q, *J* = 7 Hz, 2 C<u>H</u>$CH_{3)}$ and 4.7 (1 H, bs, 2 H, 2 NH). ***Figure 4.13 (a)*** and ***(d)***. This spectrum is identical to that reported by Sato and co-workers (Sato *et al*., 2003). Mass spectrometry found a peak at *m/z* 478.377 in the spectrum which corresponded to (***Figure 4.15.a*** and ***d***) $C_{14}H_{25}N_2O_4$, as reported by Tang (Tang, 1979).

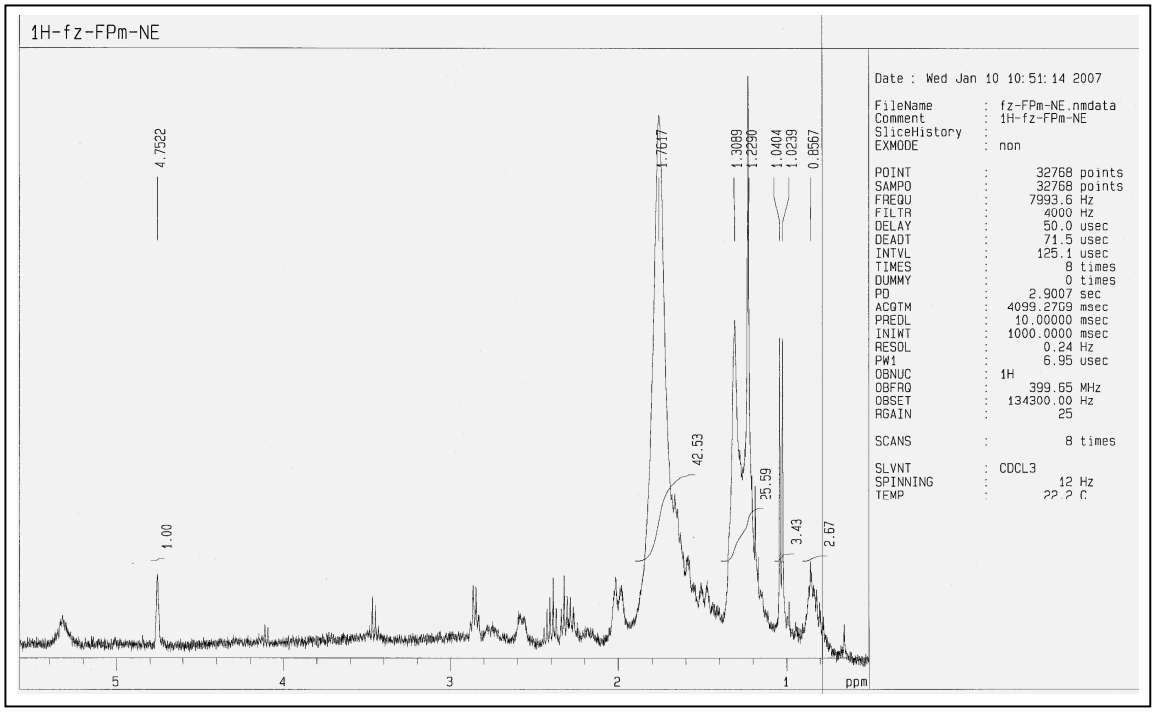
Figure 4.12: Magnetic Resonance Spectrometry of samples from acid/base extraction



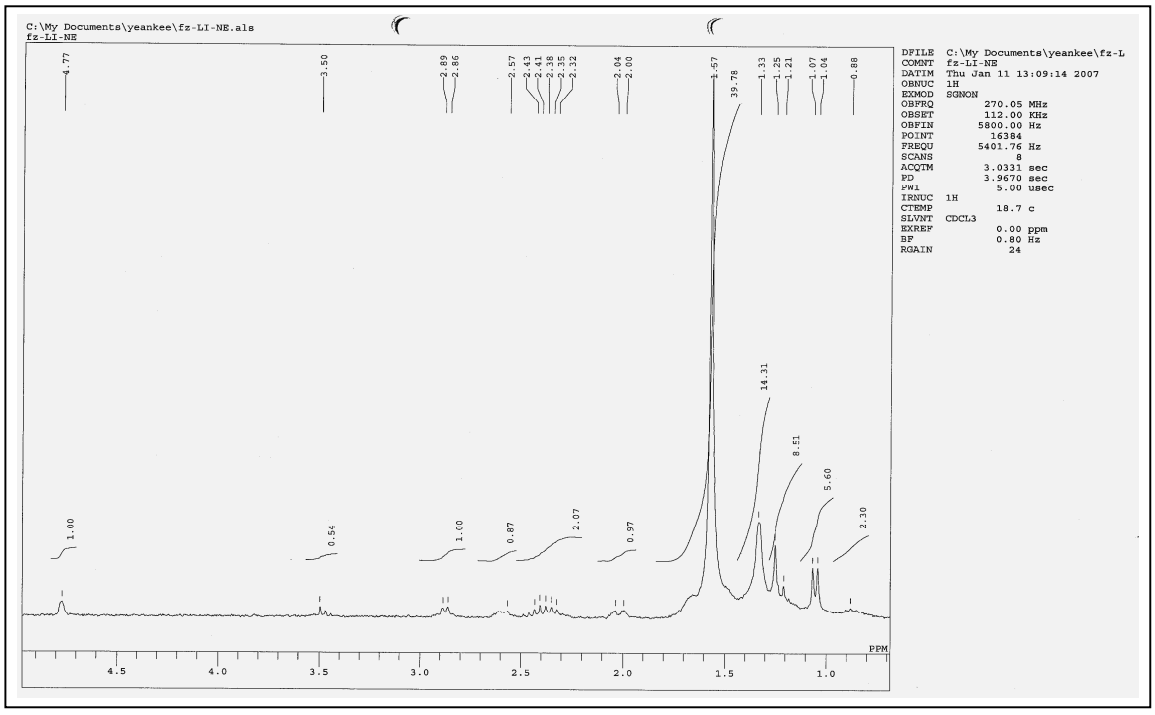
a) ^{1}H NMR of carpaine from *in vivo* leaf



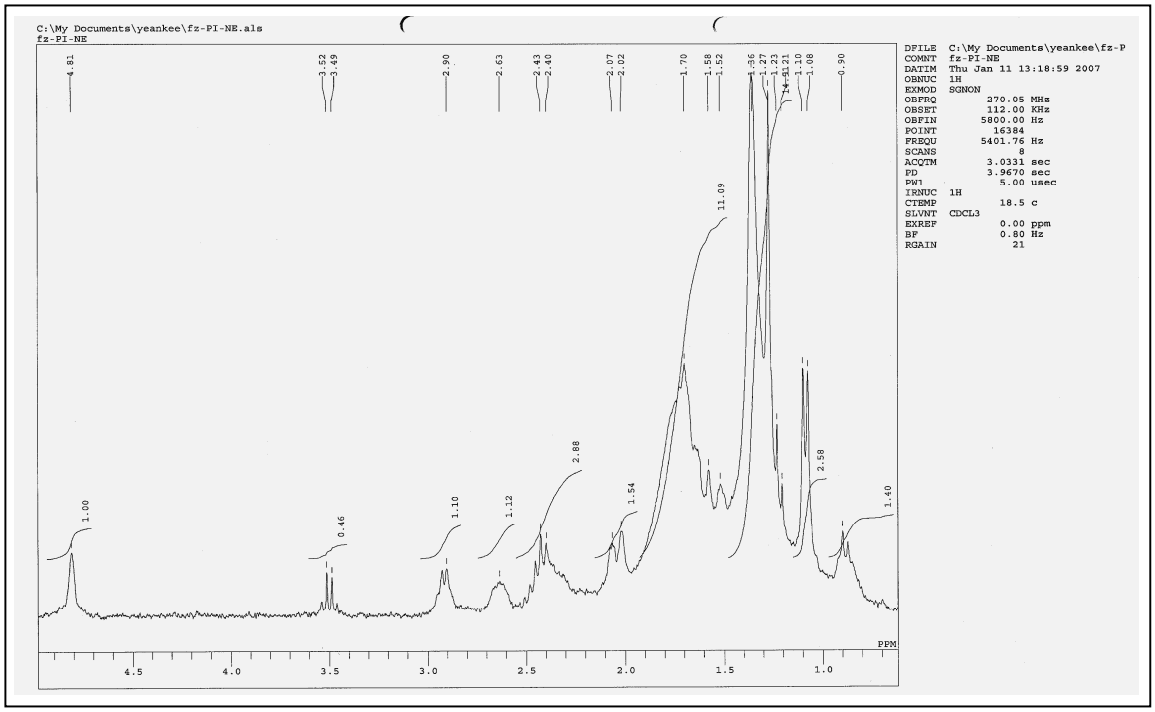
b) ^{1}H NMR of carpaine from *in vivo* petiole



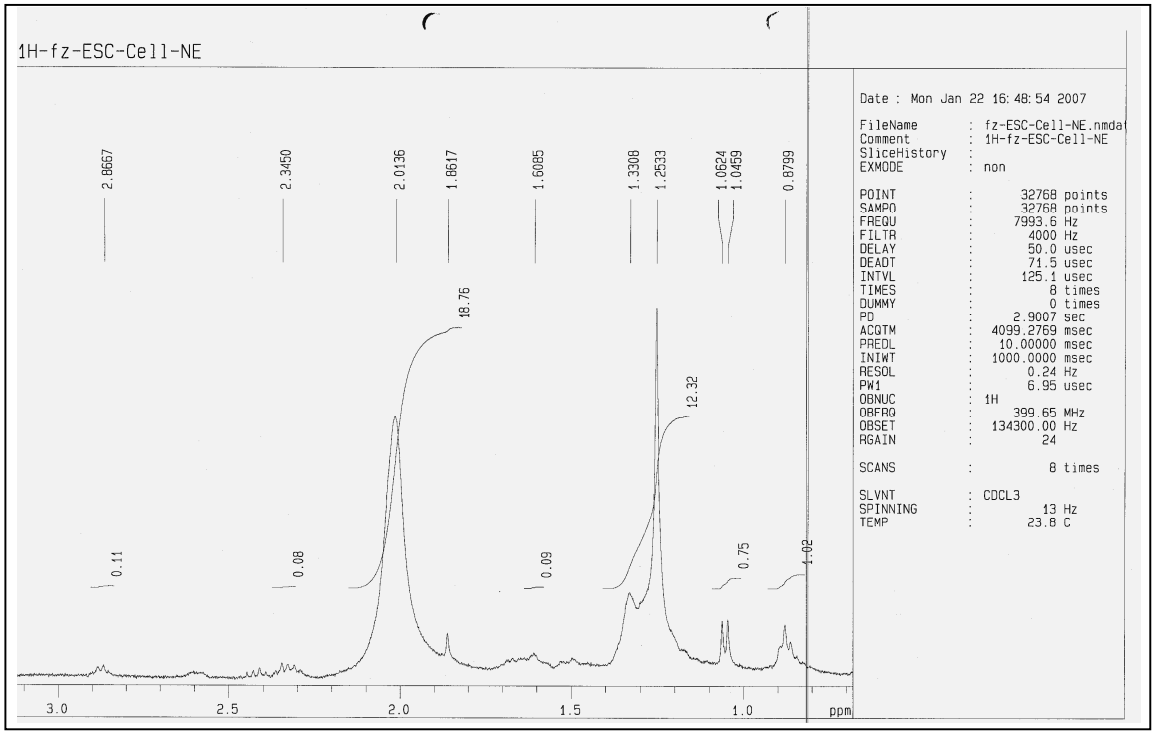
c) ^{1}H NMR of carpaine from *in vivo* fruit peel



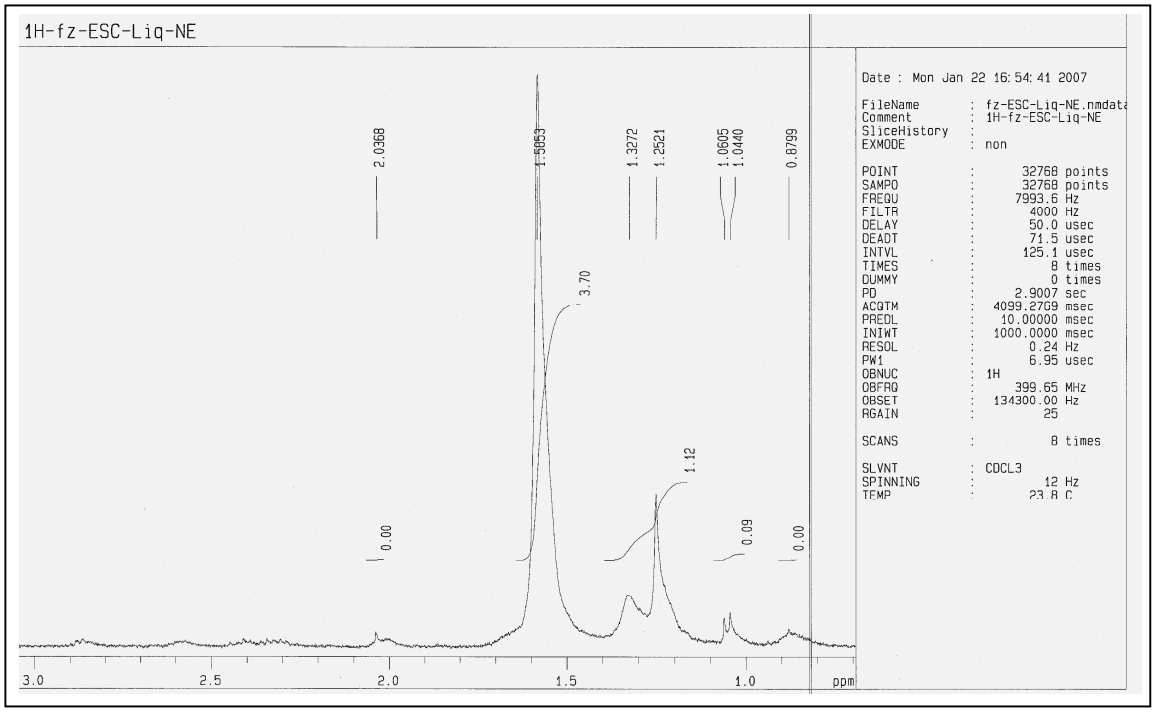
d) ^{1}H NMR of carpaine from *in vitro* leaf



e) ^{1}H NMR of carpaine from *in vitro* petiole

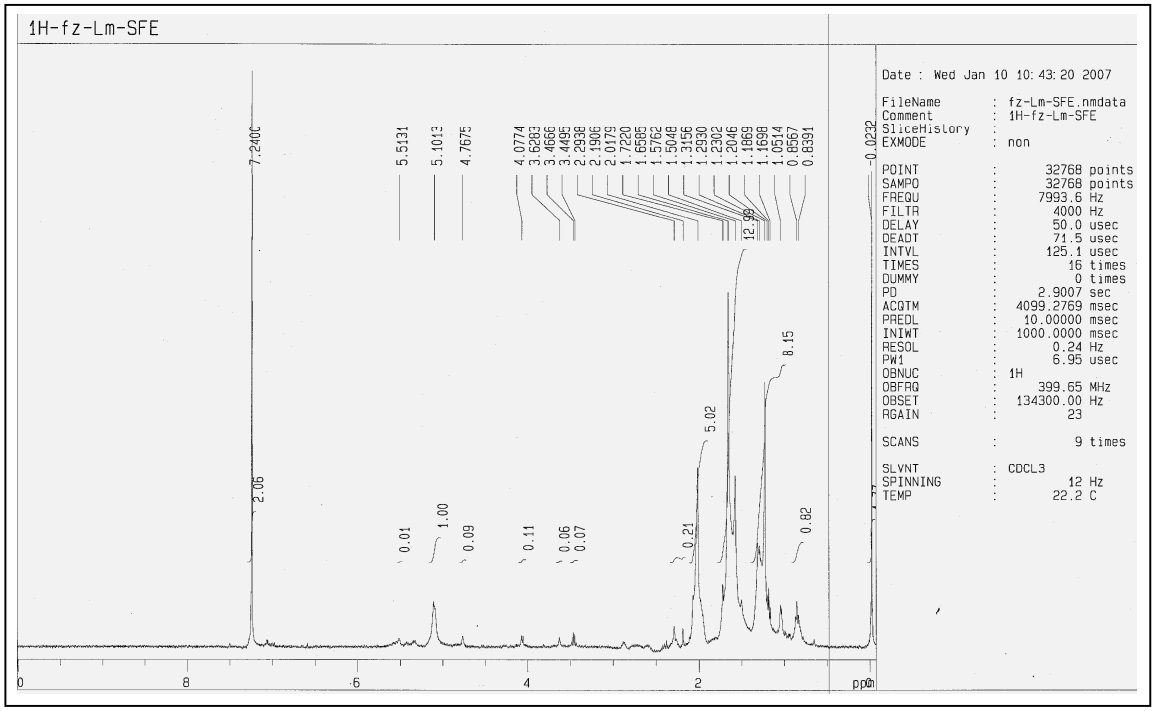


f) ^{1}H NMR of carpaine from suspension cell

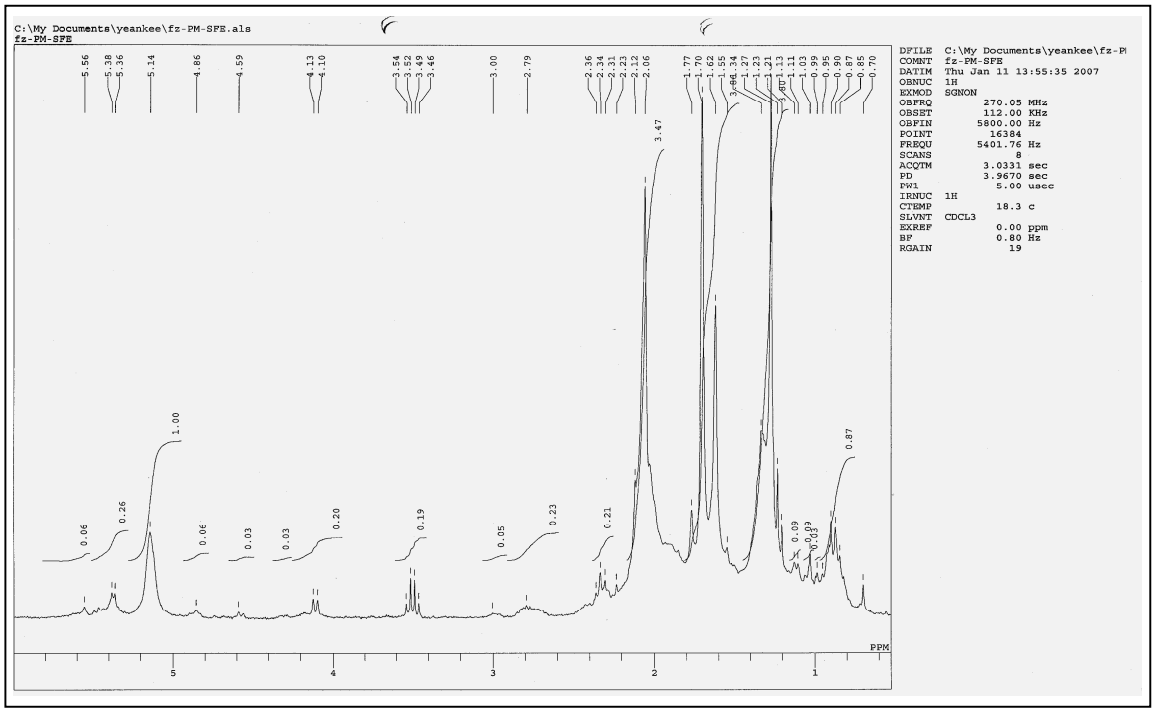


g) ^{1}H NMR of carpaine from suspension liquid

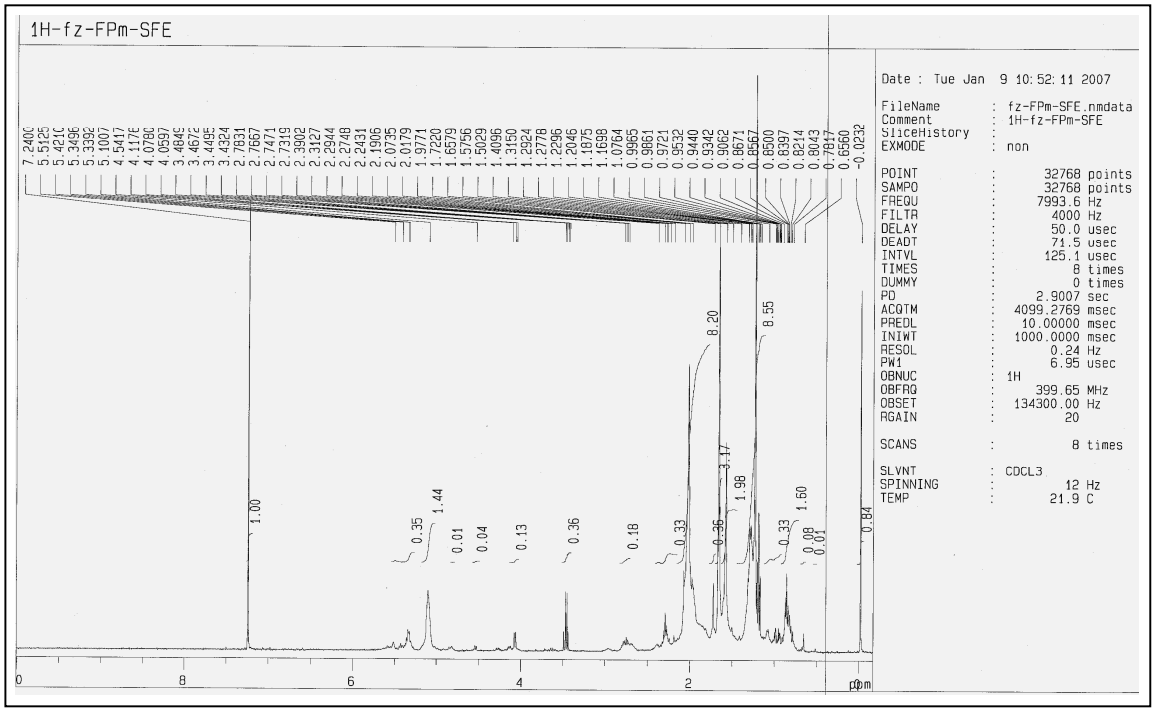
Figure 4.13: Magnetic Resonance Spectrometry of sample from SFE



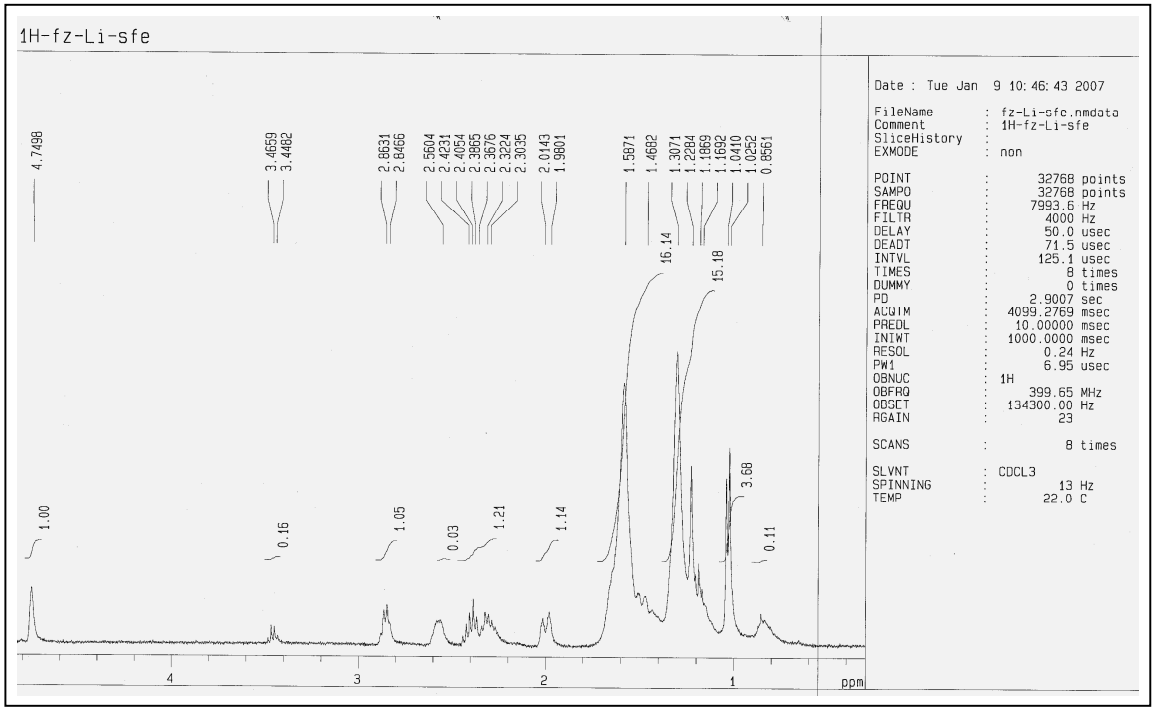
a) ^{1}H NMR of carpaine from *in vivo* leaf



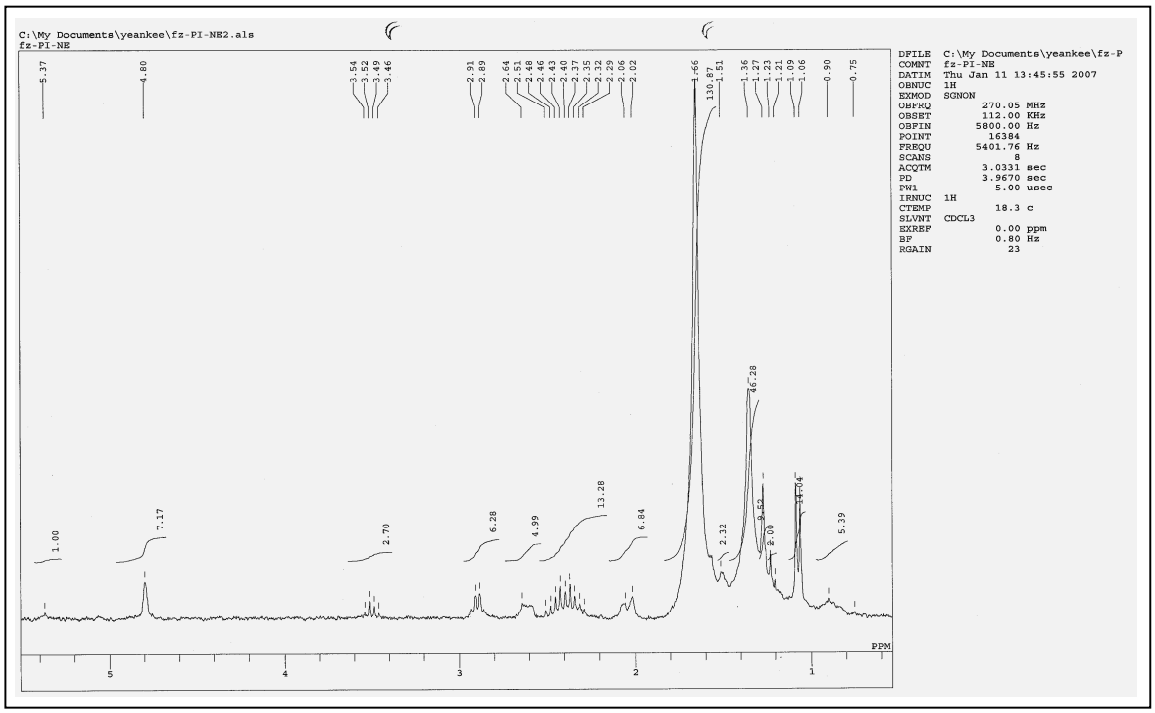
b) ^{1}H NMR of carpaine from *in vivo* petiole



c) ^{1}H NMR of carpaine from *in vivo* fruit peel

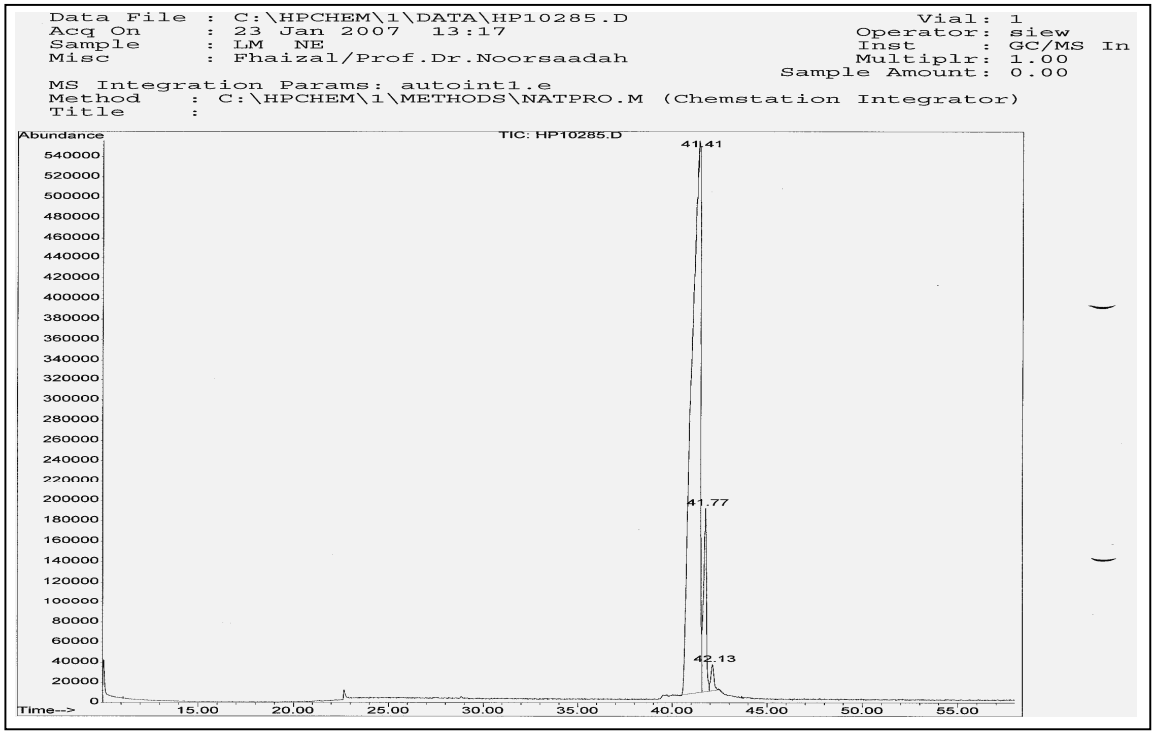


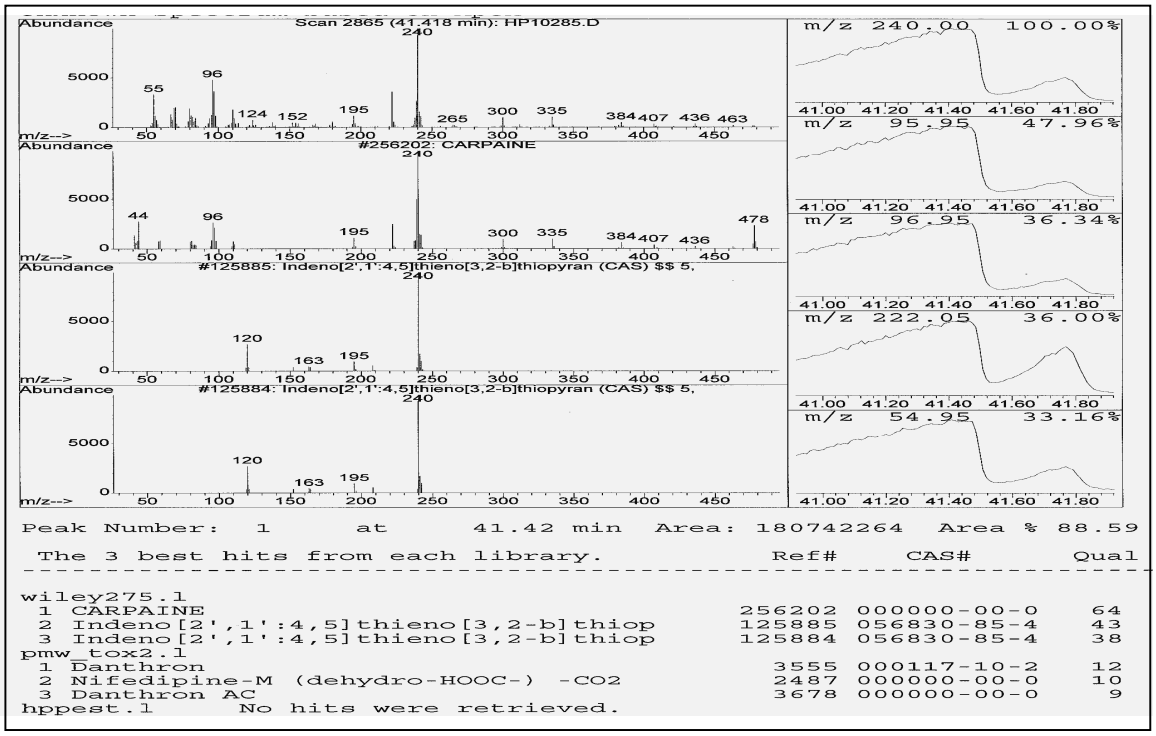
d) ^{1}H NMR of carpaine from *in vitro* leaf



e) ^{1}H NMR of carpaine from *in vitro* petiole

Figure 4.14: GC-MS of samples from acid/base extraction

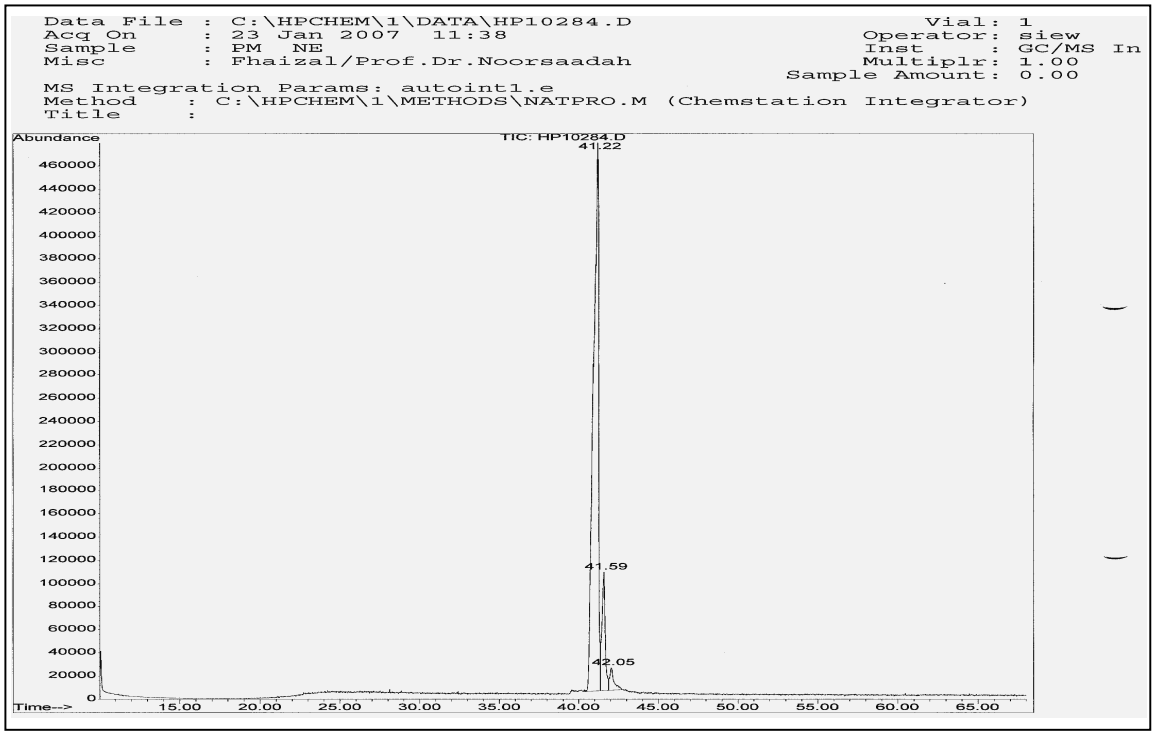


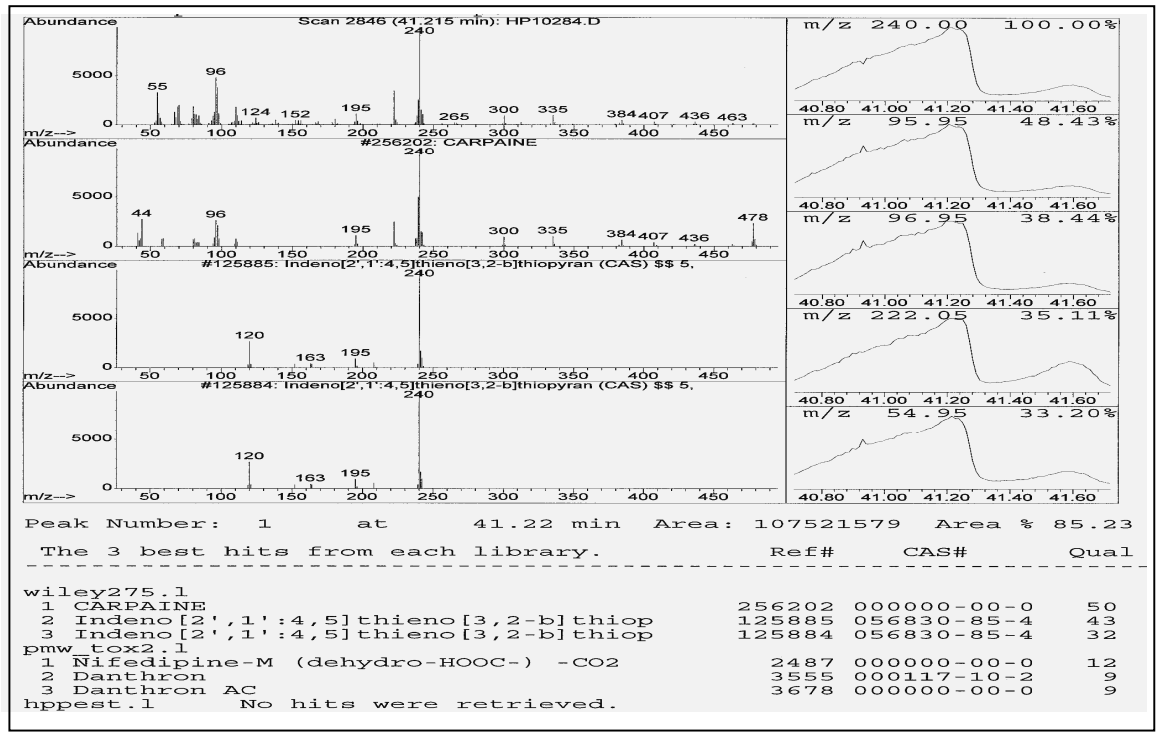


Peak Number: 1 at 41.42 min Area: 180742264 Area % 88.59

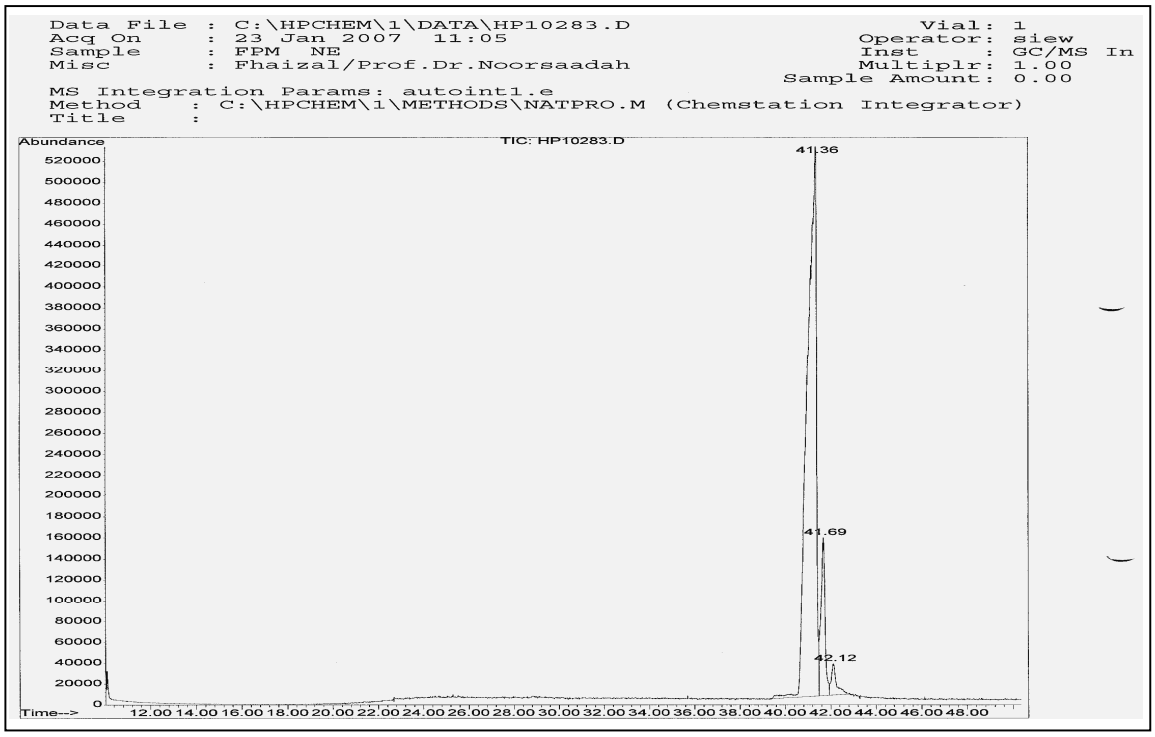
The 3 best hits from each library.	Ref#	CAS#	Qual
wiley275.l			
1 CARPAINE	256202	000000-00-0	64
2 Indeno[2',1':4,5]thieno[3,2-b]thiop	125885	056830-85-4	43
3 Indeno[2',1':4,5]thieno[3,2-b]thiop	125884	056830-85-4	38
pmw_tox2.l			
1 Danthron	3555	000117-10-2	12
2 Nifedipine-M (dehydro-HOOC-) -CO2	2487	000000-00-0	10
3 Danthron AC	3678	000000-00-0	9
hppest.l No hits were retrieved.			

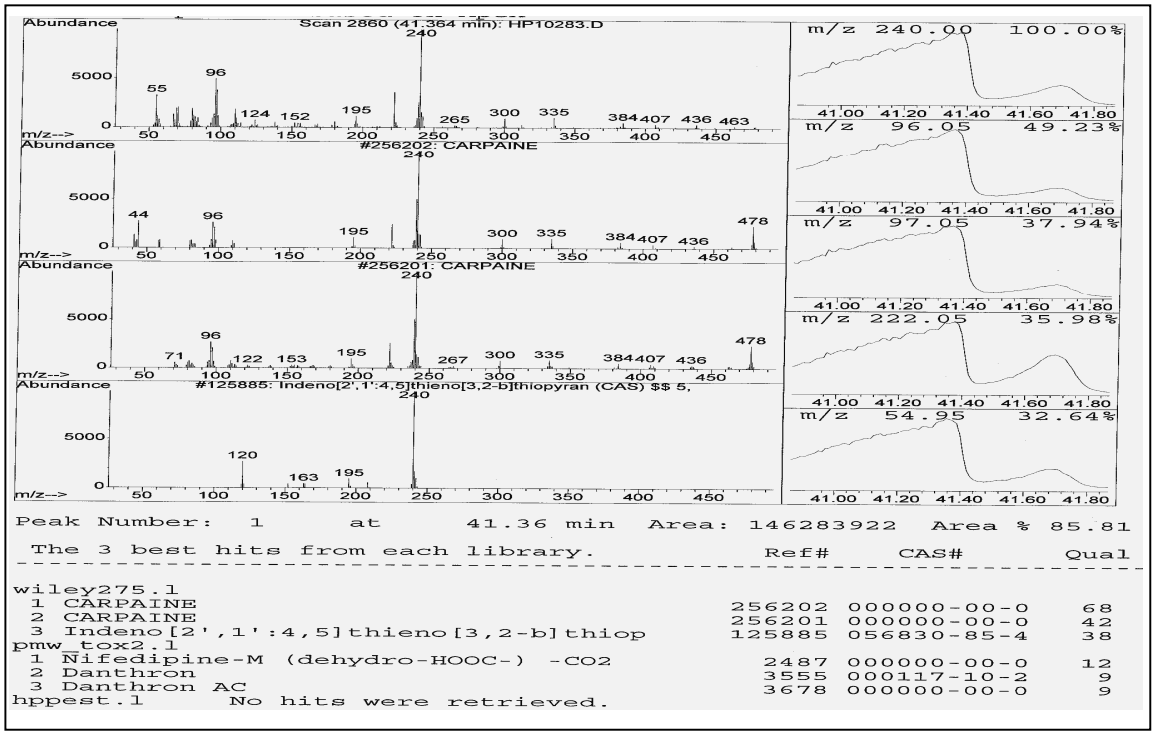
a) Mass spectrum of carpaine from *in vivo* leaf





b) Mass spectrum of carpaine from *in vivo* petiole



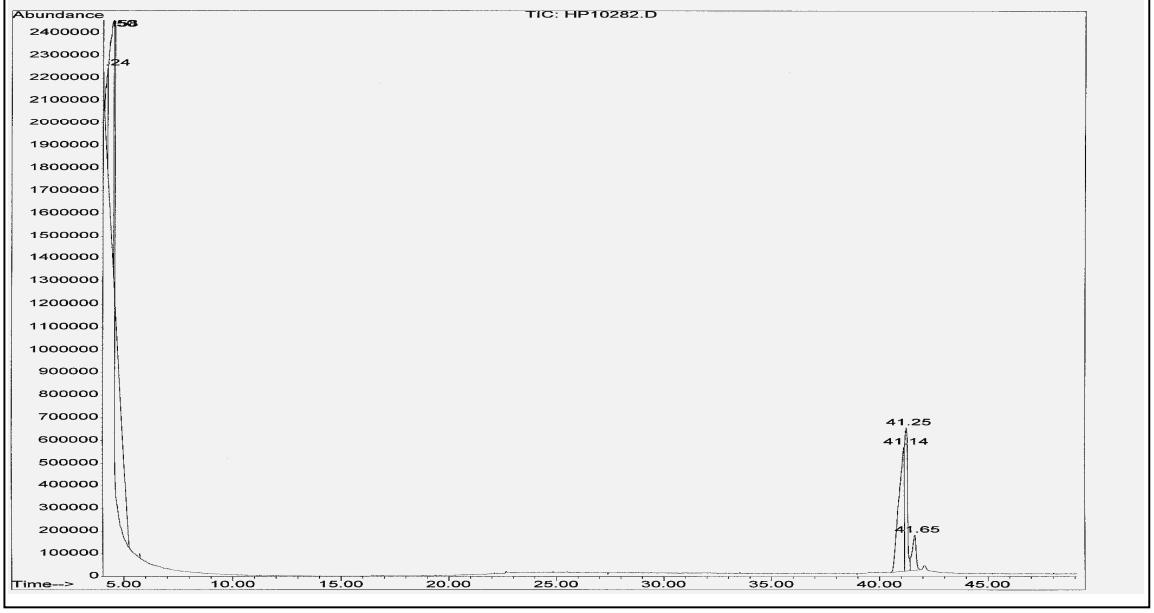


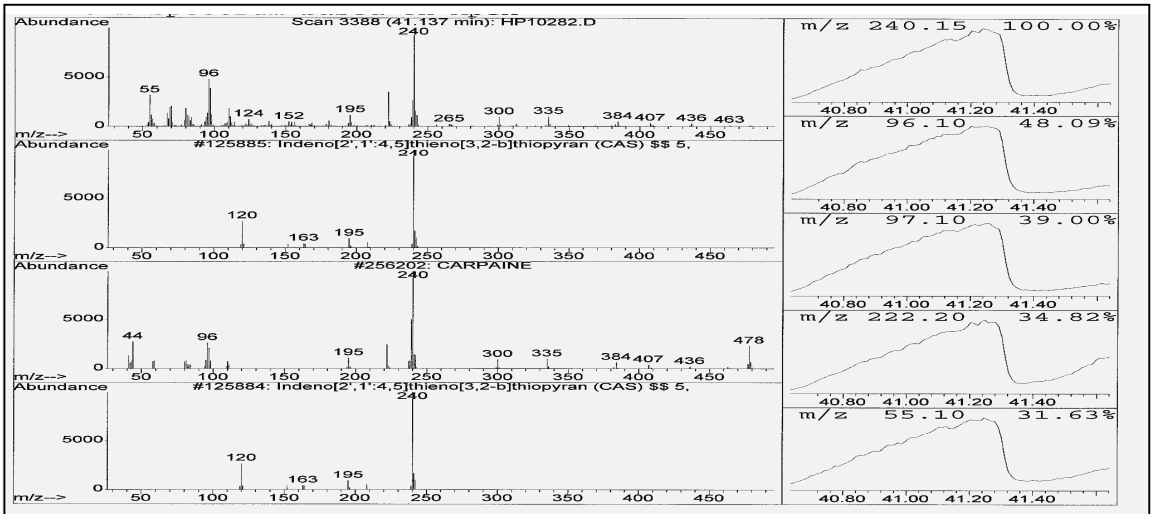
Peak Number: 1 at 41.36 min Area: 146283922 Area % 85.81

The 3 best hits from each library.	Ref#	CAS#	Qual
wiley275.l			
1 CARPAINE	256202	000000-00-0	68
2 CARPAINE	256201	000000-00-0	42
3 Indeno[2',1':4,5]thieno[3,2-b]thiop	125885	056830-85-4	38
pmw_tox2.l			
1 Nifedipine-M (dehydro-HOOC-) -CO2	2487	000000-00-0	12
2 Danthron	3555	000117-10-2	9
3 Danthron AC	3678	000000-00-0	9
hppest.l No hits were retrieved.			

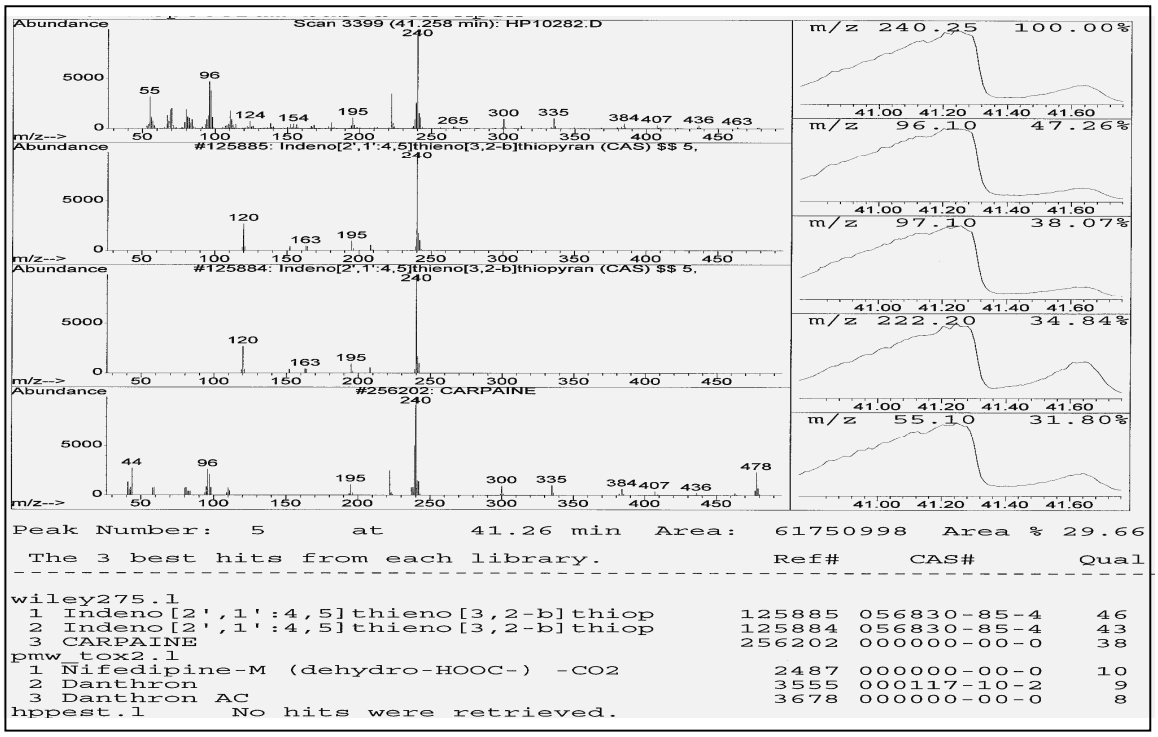
c) Mass spectrum of carpaine from *in vivo* fruit peel

```
Data File  : C:\HPCHEM\1\DATA\HP10282.D                    Vial: 1
Acq On     : 23 Jan 2007   10:02                          Operator: siew
Sample     : LI   NE                                      Inst    : GC/MS
Misc       : Fhaizal/Prof.Dr.Noorsaadah                   Multiplr: 1.00
                                                          Sample Amount: 0.00
MS Integration Params: autoint1.e
Method     : C:\HPCHEM\1\METHODS\NATPRO.M  (Chemstation Integrator)
Title      :
```

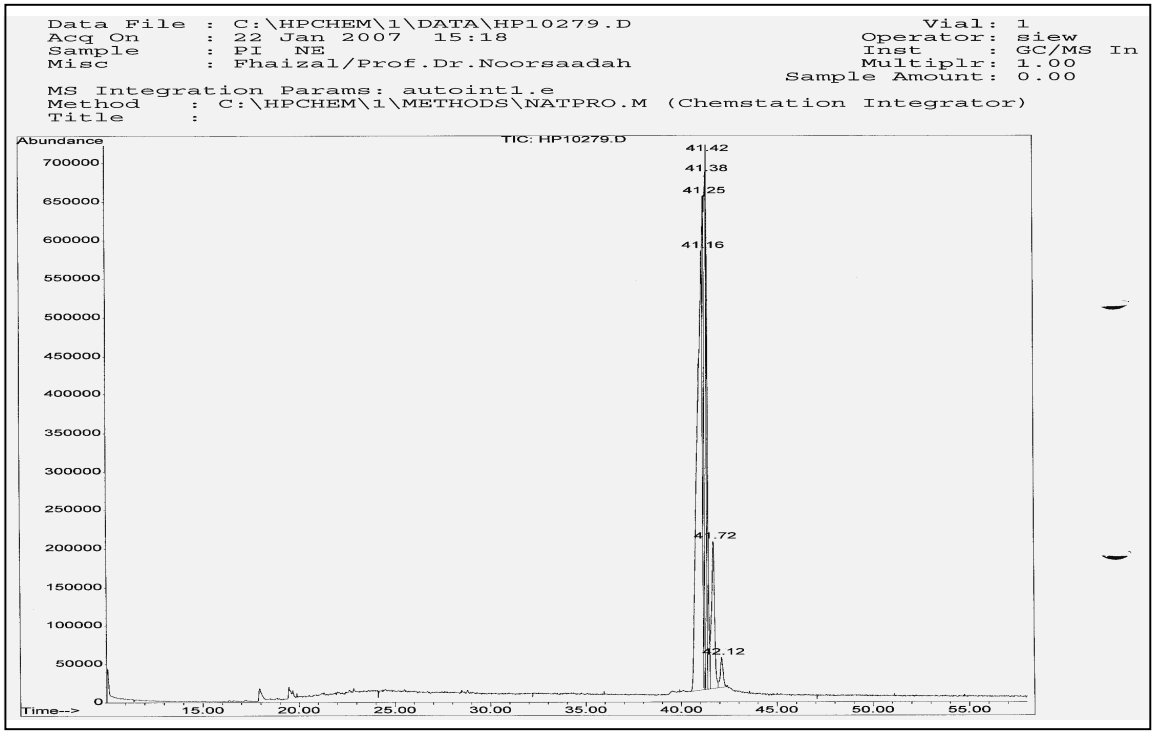


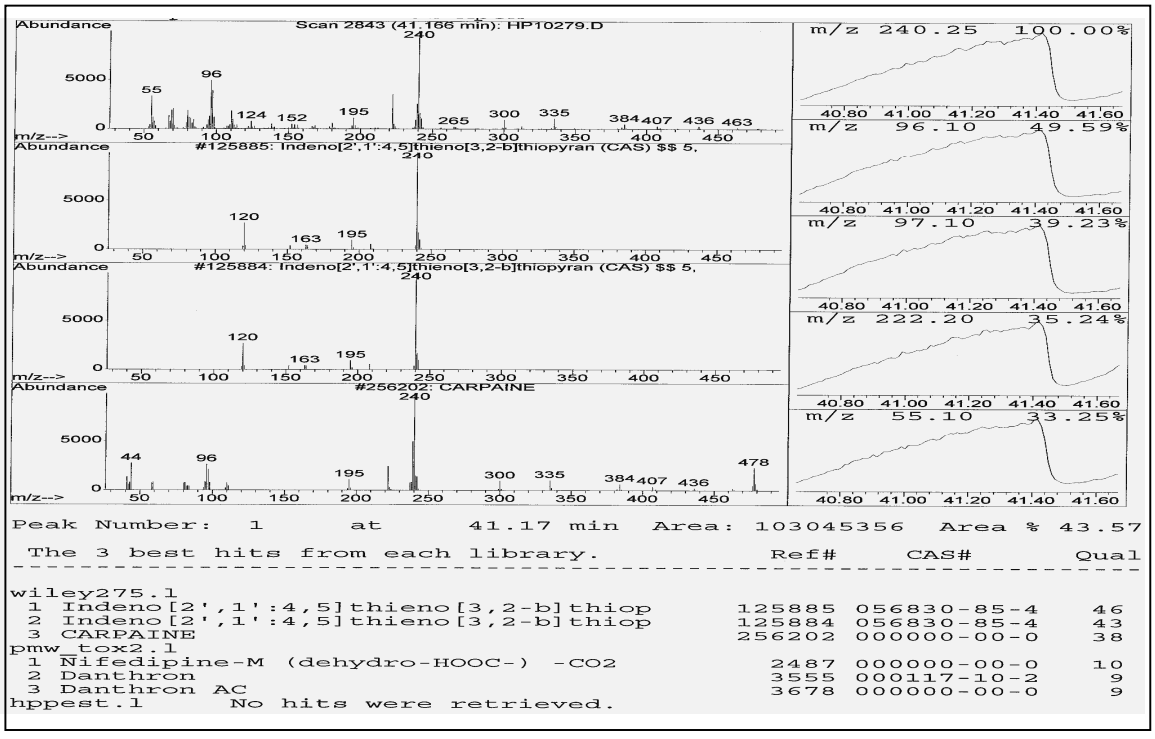


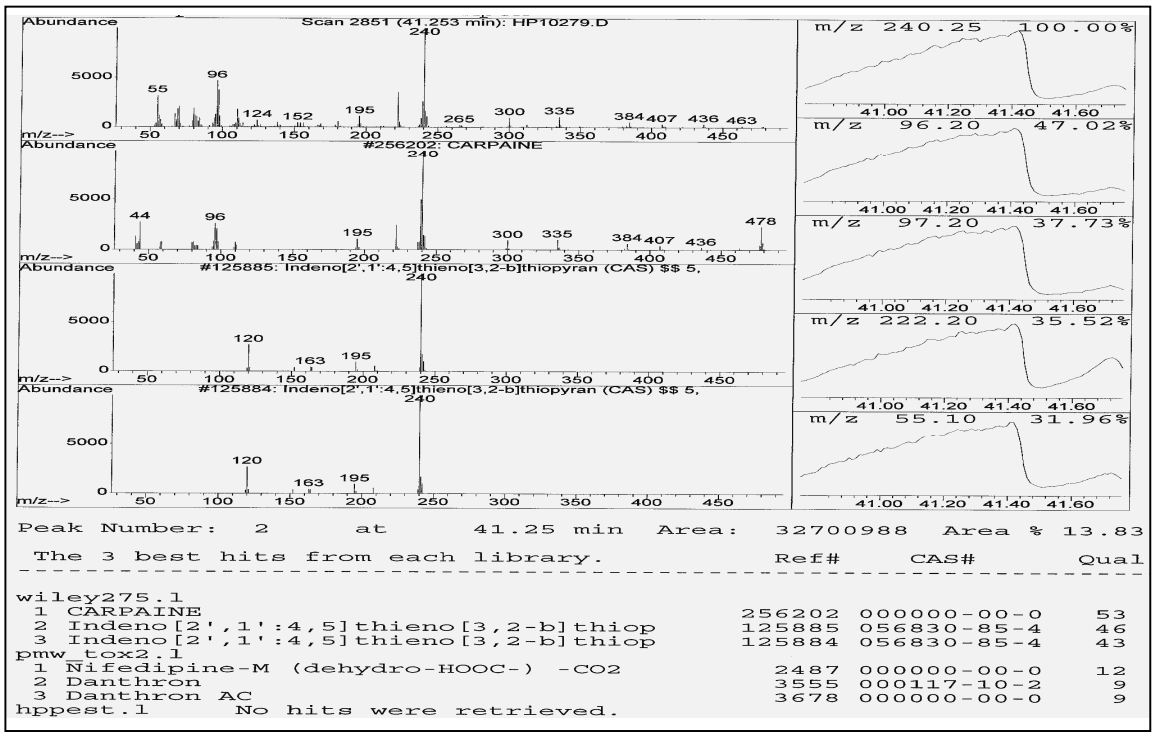

```
Peak Number:  4      at      41.14 min   Area:   94624929   Area % 45.45

 The 3 best hits from each library.                Ref#      CAS#         Qual
-------------------------------------------------------------------------------

wiley275.l
  1 Indeno[2',1':4,5]thieno[3,2-b]thiop          125885  056830-85-4     46
  2 CARPAINE                                     256202  000000-00-0     43
  3 Indeno[2',1':4,5]thieno[3,2-b]thiop          125884  056830-85-4     43
pmw_tox2.l
  1 Nifedipine-M (dehydro-HOOC-) -CO2              2487  000000-00-0     12
  2 Danthron                                       3555  000117-10-2      9
  3 Danthron AC                                    3678  000000-00-0      9
hppest.l      No hits were retrieved.
```

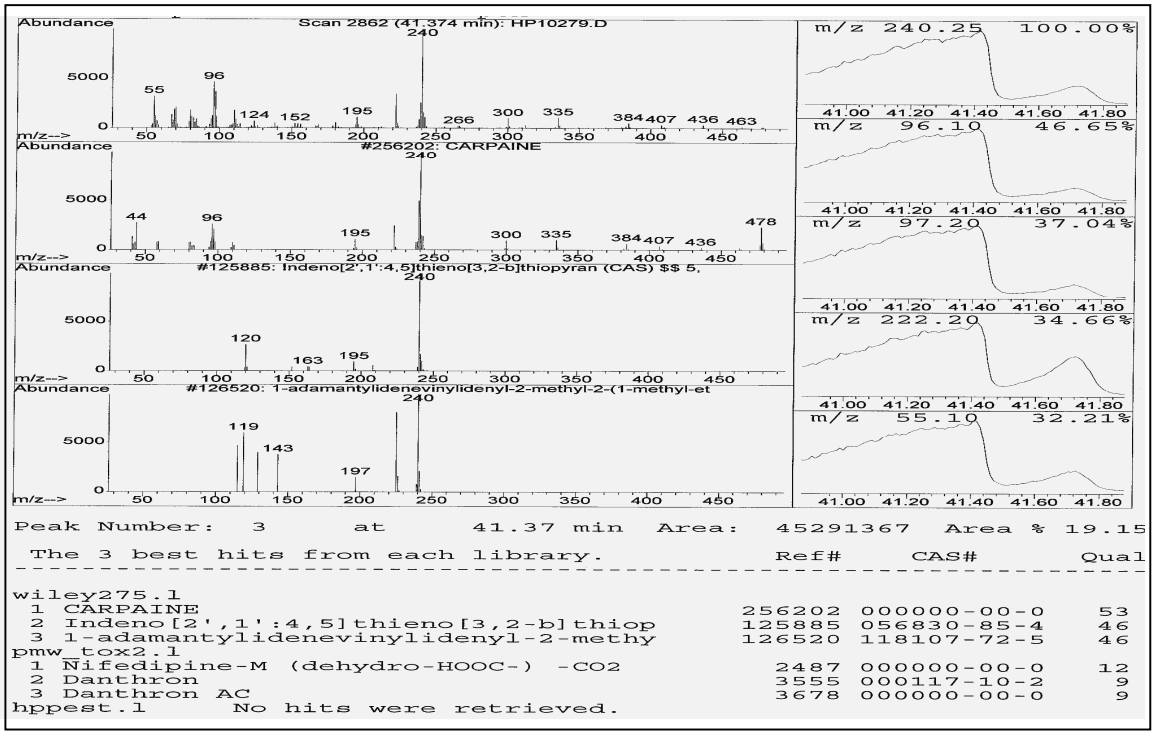


d) Mass spectrum of carpaine from *in vitro* leaf

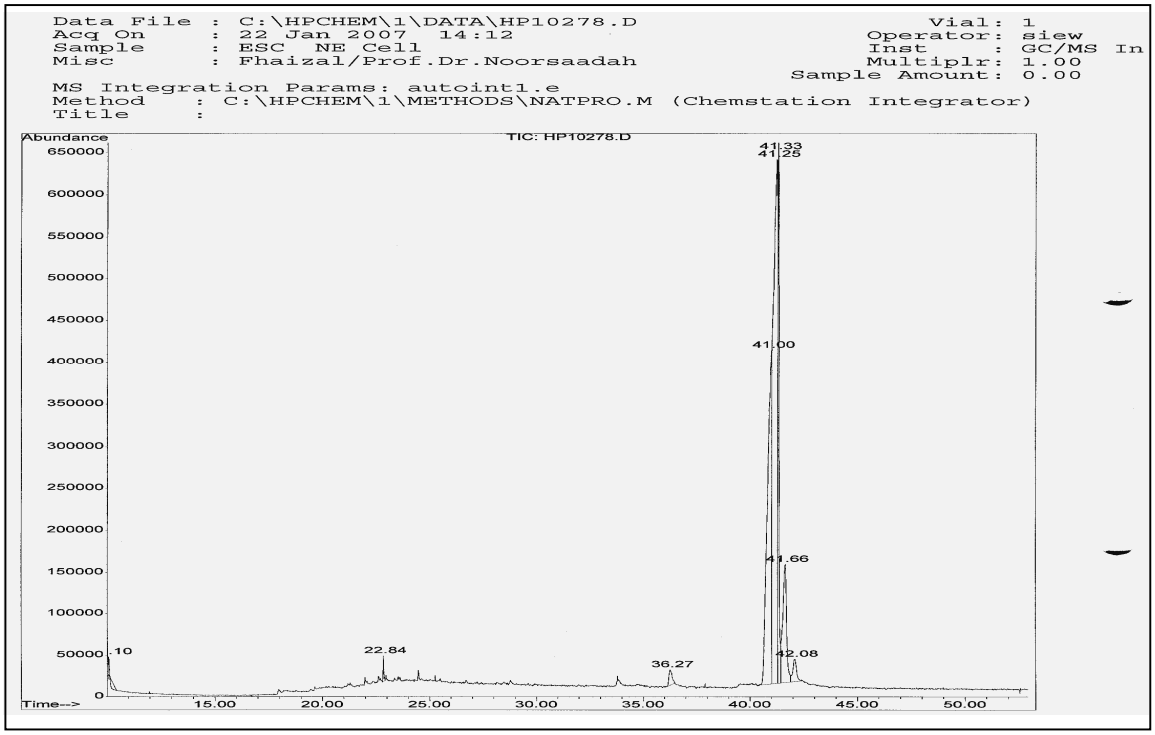

Data File : C:\HPCHEM\1\DATA\HP10279.D
Acq On : 22 Jan 2007 15:18
Sample : PI NE
Misc : Fhaizal/Prof.Dr.Noorsaadah
Vial: 1
Operator: siew
Inst : GC/MS In
Multiplr: 1.00
Sample Amount: 0.00
MS Integration Params: autoint1.e
Method : C:\HPCHEM\1\METHODS\NATPRO.M (Chemstation Integrator)
Title :
Abundance
TIC: HP10279.D
41.42
41.38
41.25
41.16
41.72
42.12
Time-->

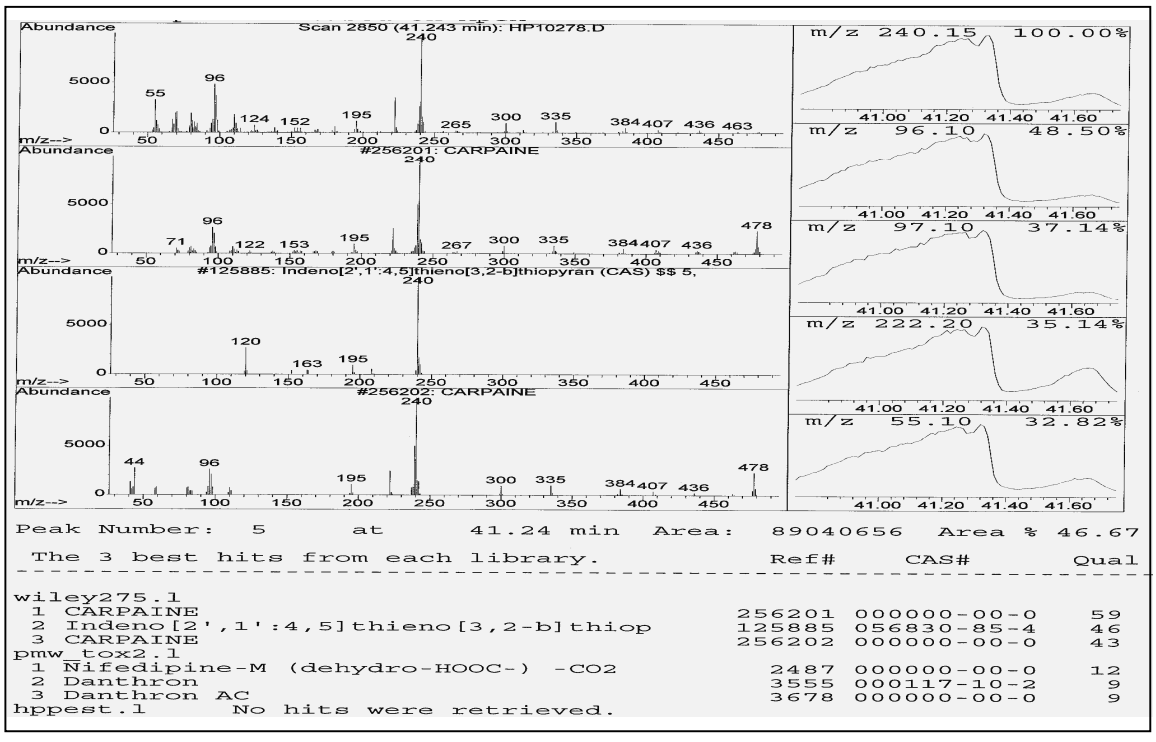

Abundance
Scan 2843 (41.166 min): HP10279.D
#125885: Indeno[2',1':4,5]thieno[3,2-b]thiopyran (CAS) $$ 5,
#125884: Indeno[2',1':4,5]thieno[3,2-b]thiopyran (CAS) $$ 5,
#256202: CARPAINE
m/z-->
m/z 240.25 100.00%
m/z 96.10 49.59%
m/z 97.10 39.23%
m/z 222.20 35.24%
m/z 55.10 33.25%
Peak Number: 1 at 41.17 min Area: 103045356 Area % 43.57
The 3 best hits from each library. Ref# CAS# Qual
wiley275.l
1 Indeno[2',1':4,5]thieno[3,2-b]thiop 125885 056830-85-4 46
2 Indeno[2',1':4,5]thieno[3,2-b]thiop 125884 056830-85-4 43
3 CARPAINE 256202 000000-00-0 38
pmw_tox2.l
1 Nifedipine-M (dehydro-HOOC-) -CO2 2487 000000-00-0 10
2 Danthron 3555 000117-10-2 9
3 Danthron AC 3678 000000-00-0 9
hppest.l No hits were retrieved.





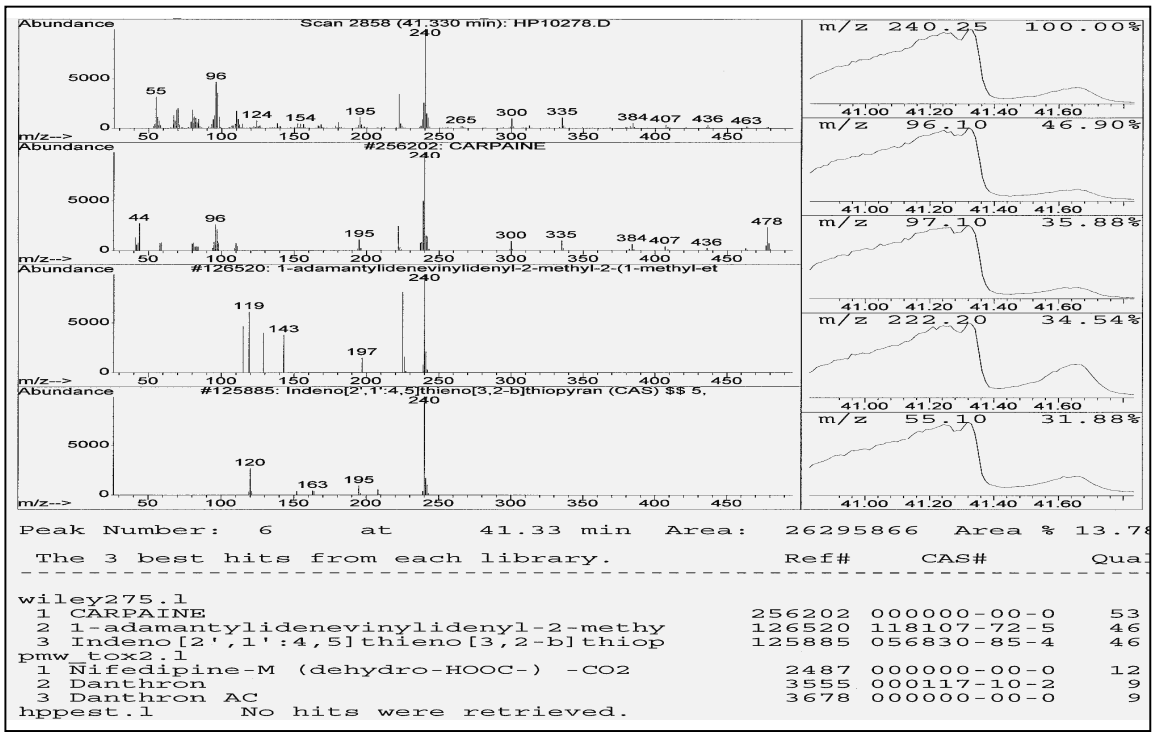
e) Mass spectrum of carpaine from *in vitro* petiole



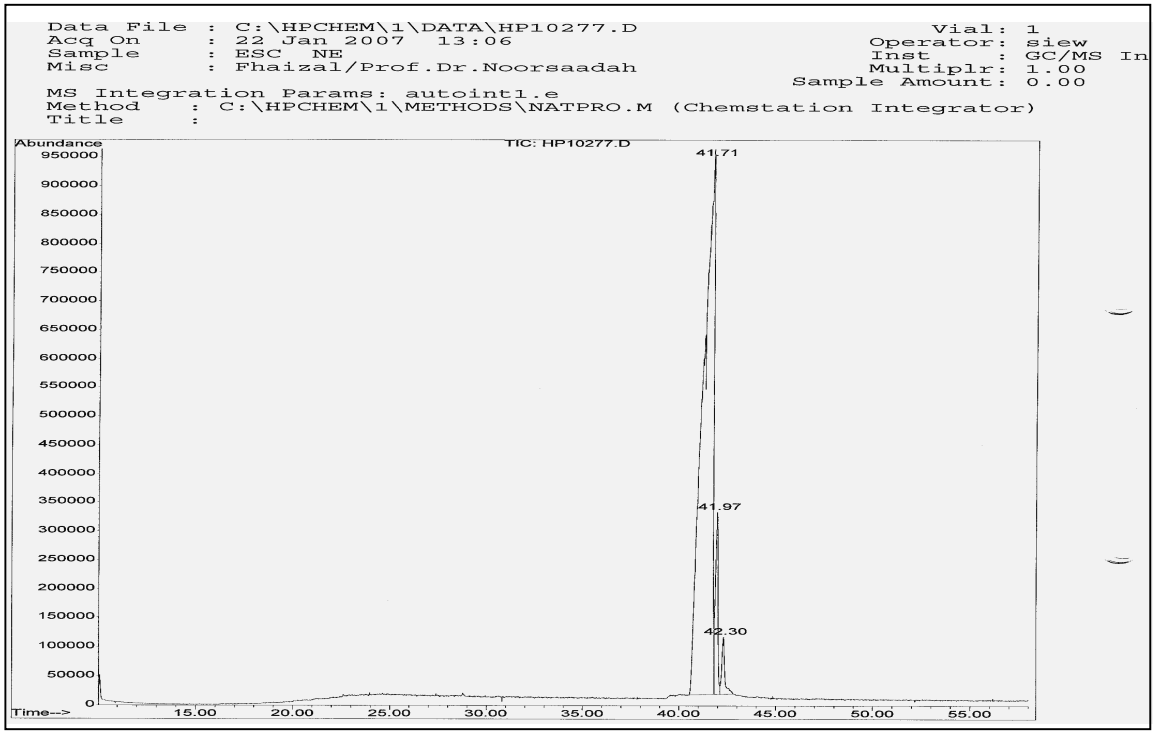


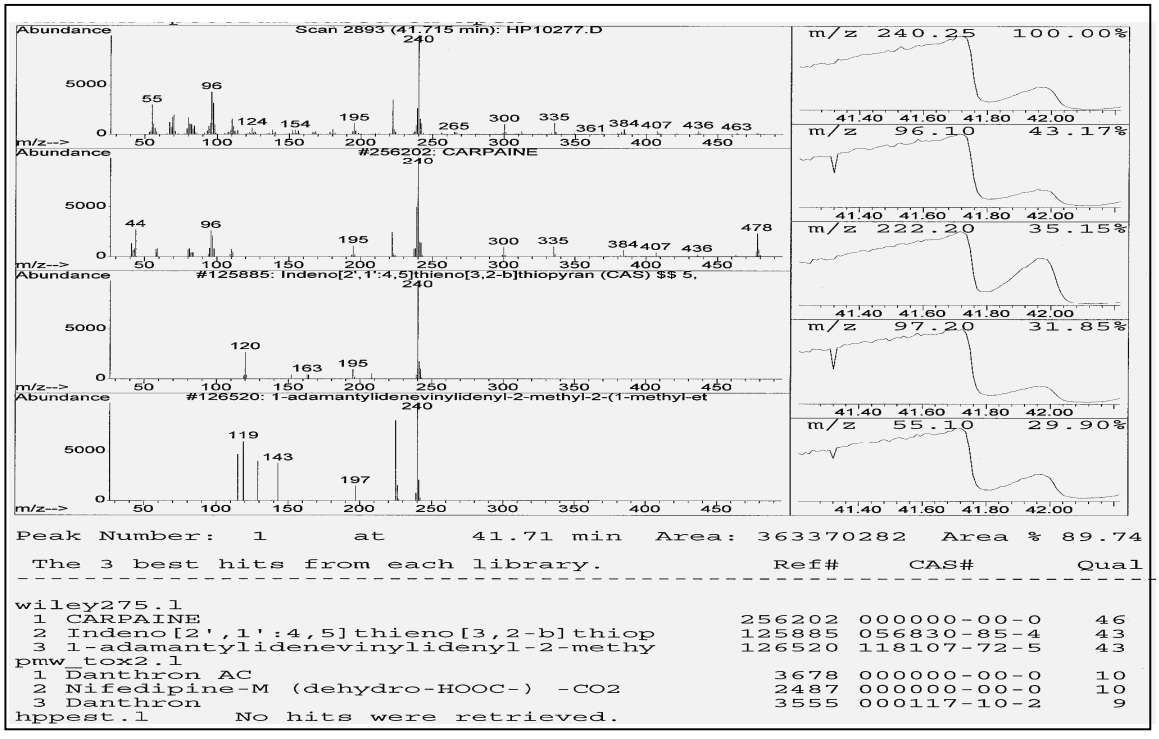
Peak Number: 5 at 41.24 min Area: 89040656 Area % 46.67

The 3 best hits from each library.	Ref#	CAS#	Qual
wiley275.l			
1 CARPAINE	256201	000000-00-0	59
2 Indeno[2',1':4,5]thieno[3,2-b]thiop	125885	056830-85-4	46
3 CARPAINE	256202	000000-00-0	43
pmw_tox2.l			
1 Nifedipine-M (dehydro-HOOC-) -CO2	2487	000000-00-0	12
2 Danthron	3555	000117-10-2	9
3 Danthron AC	3678	000000-00-0	9
hppest.l No hits were retrieved.			



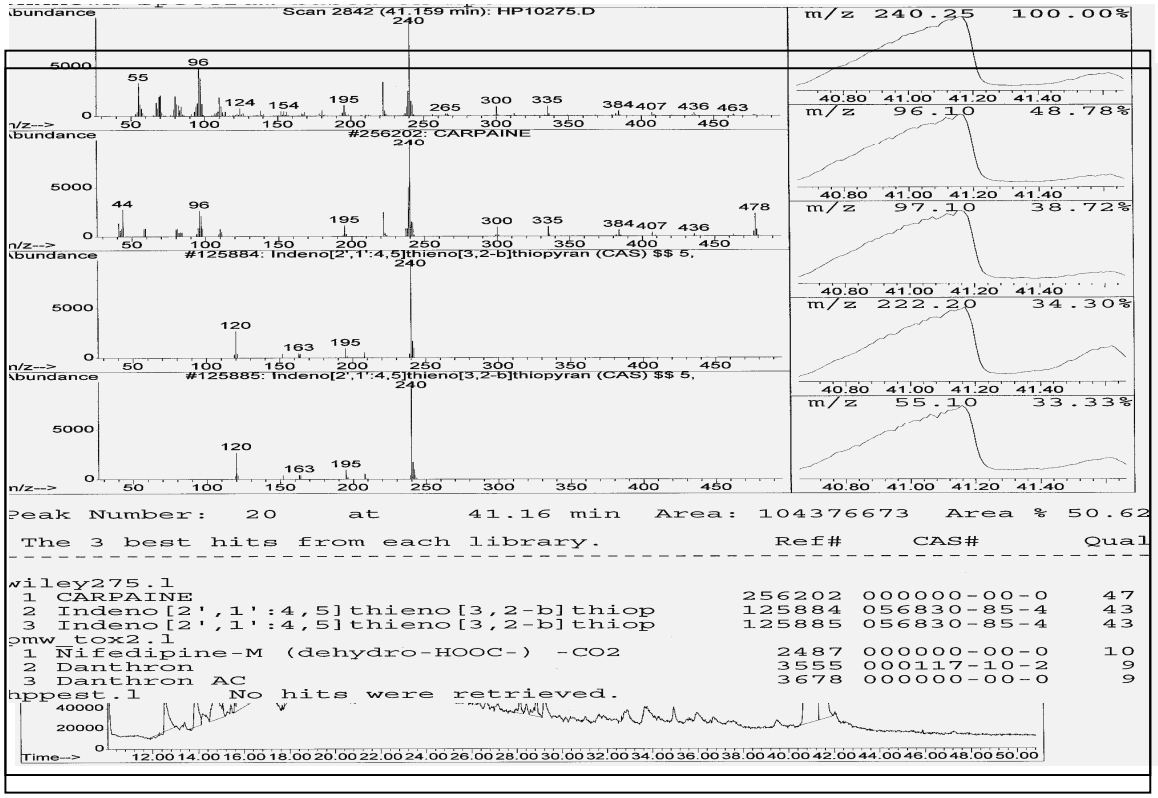
f) Mass spectrum of carpaine from suspension cell



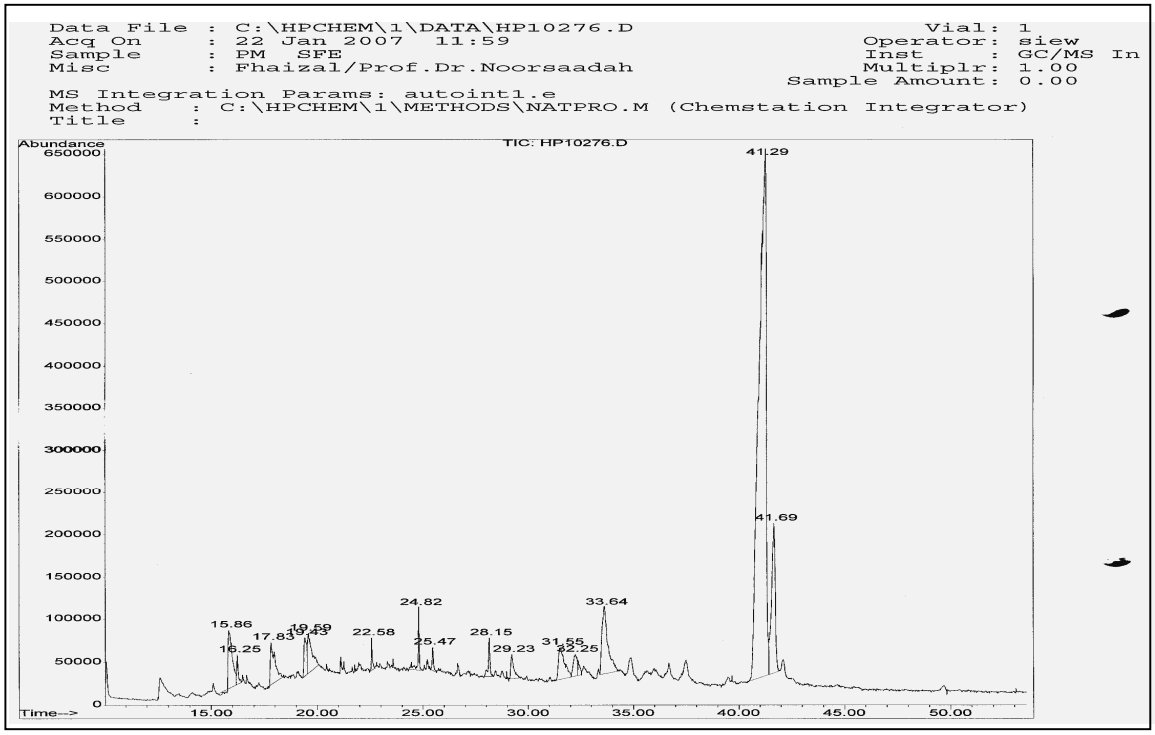


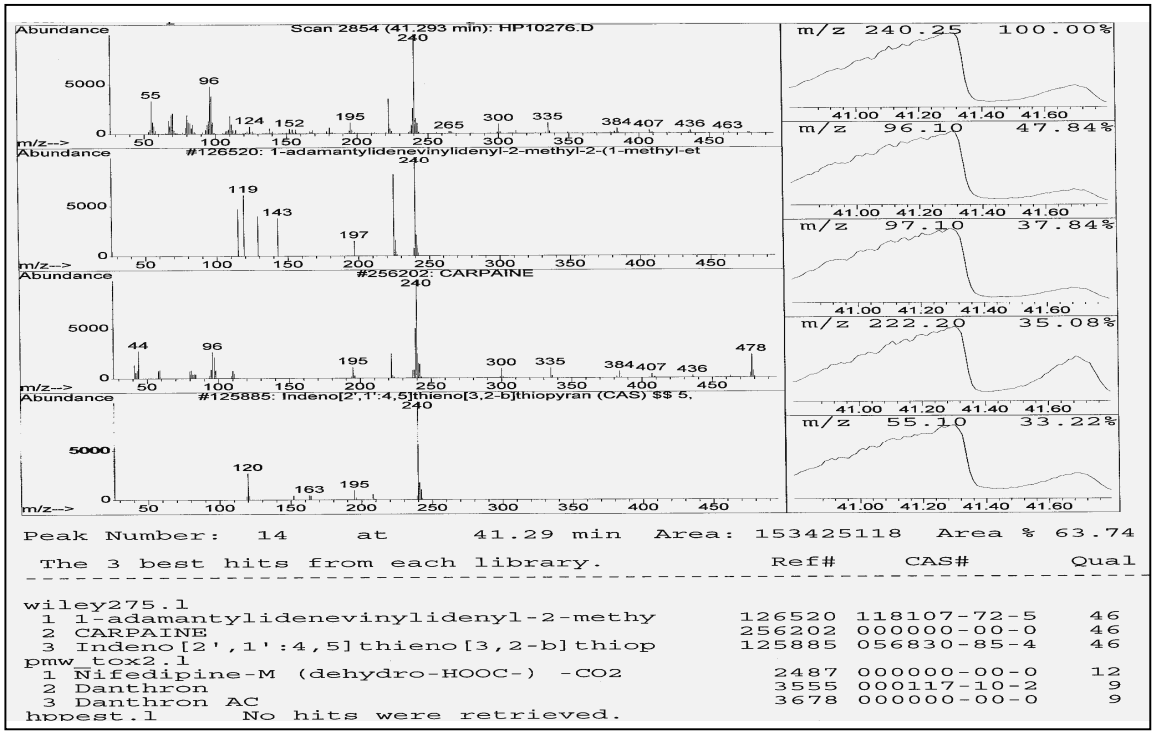
g) Mass spectrum of carpaine from suspension liquid

Figure 4.15: GC-MS of samples from SFE

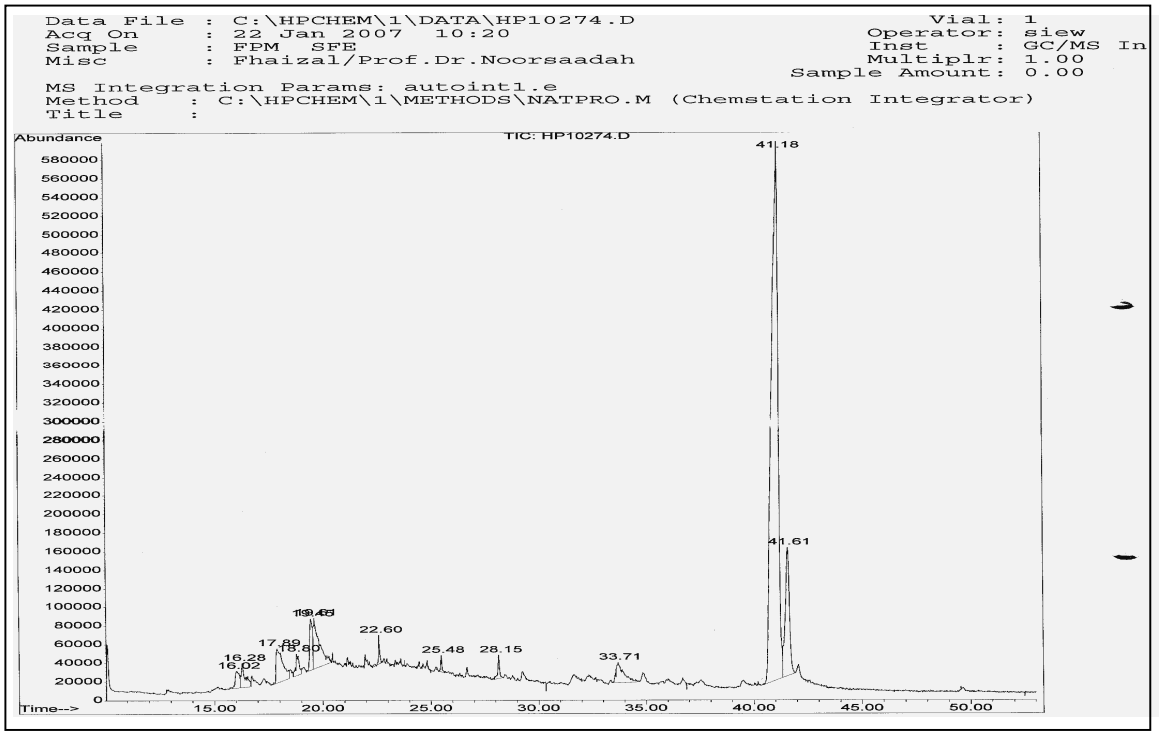


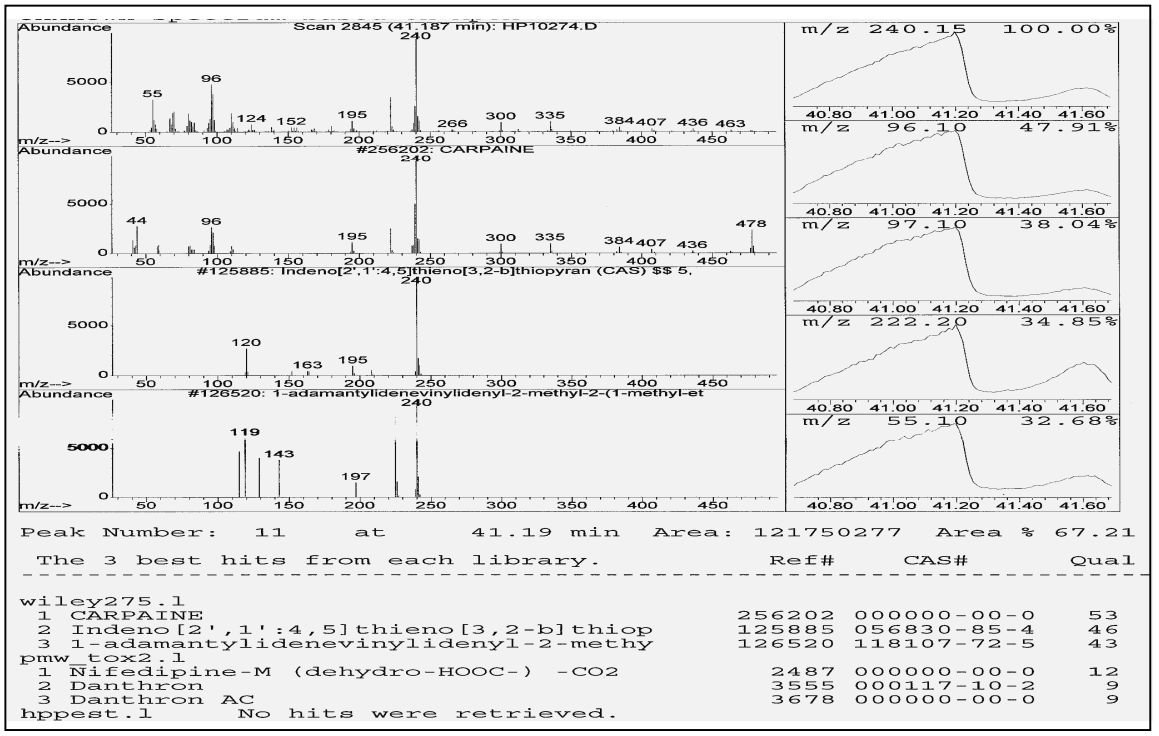
a) Mass spectrum of carpaine from *in vivo* leaf



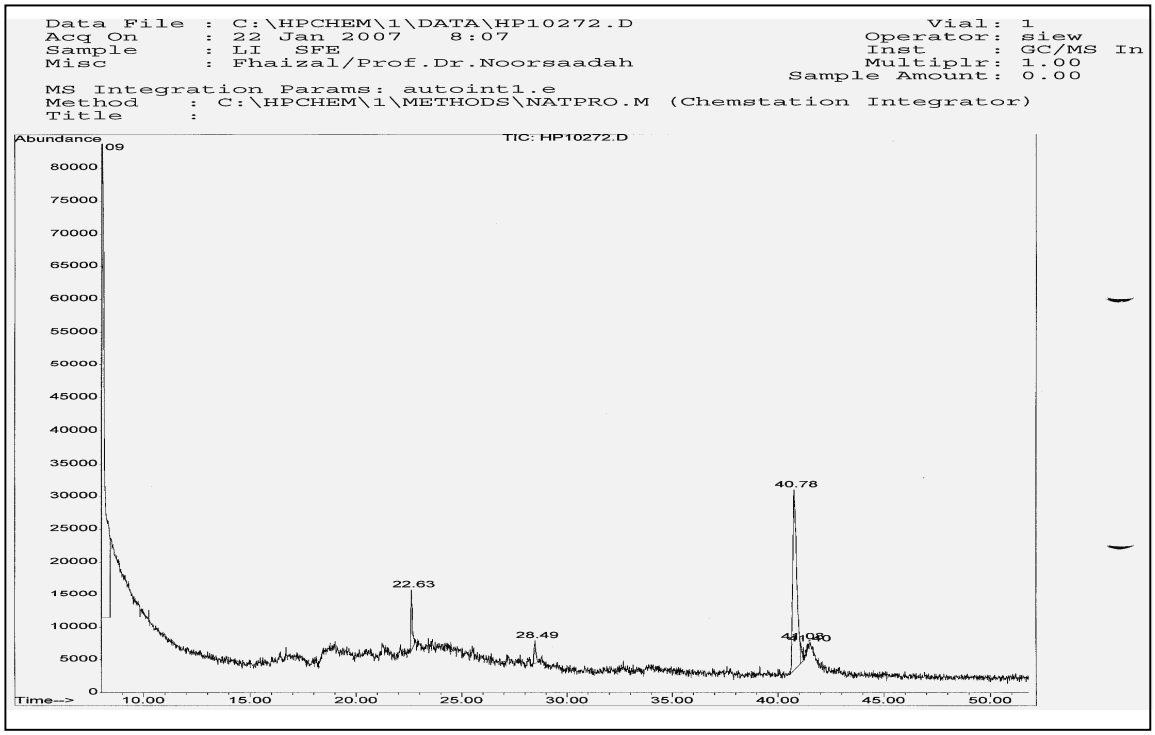


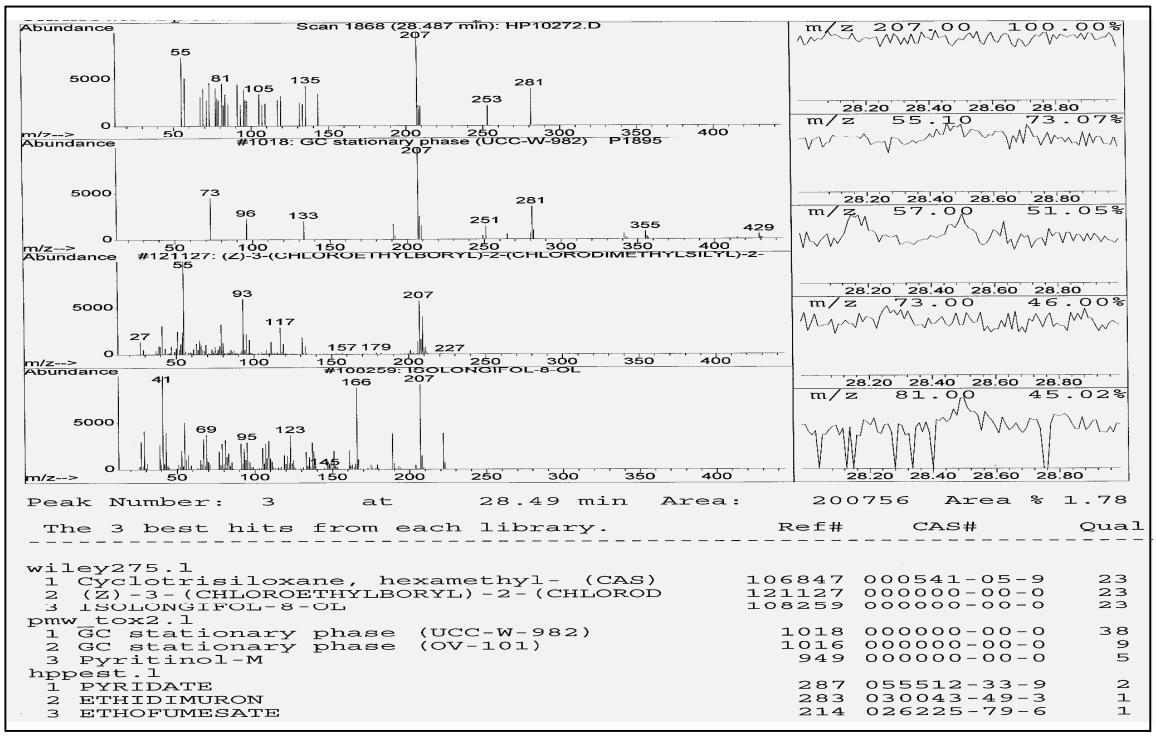
b) Mass spectrum of carpaine from *in vivo* petiole



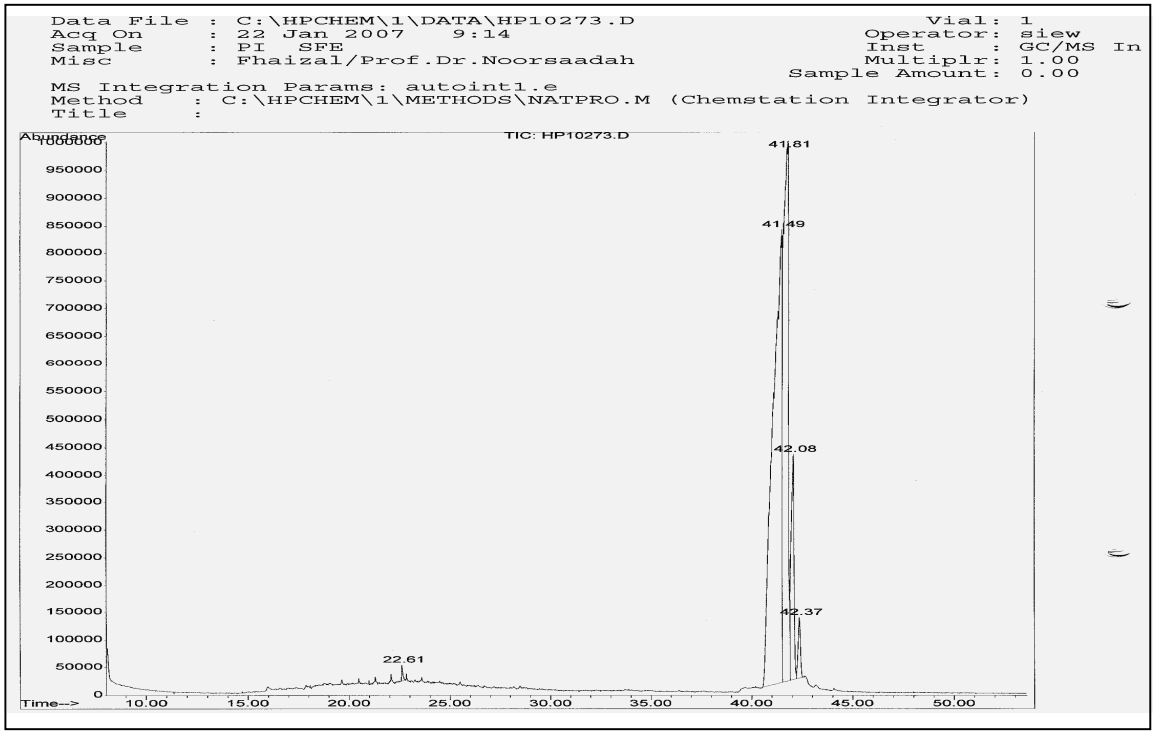


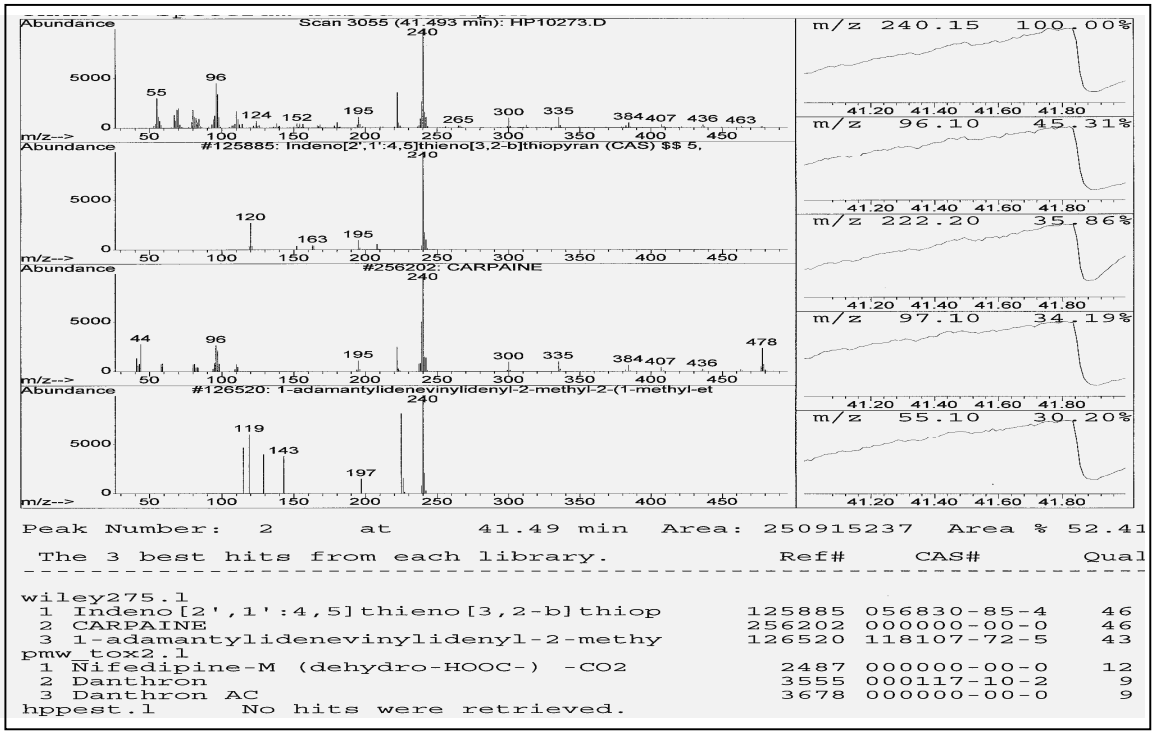
c) Mass spectrum of carpaine from *in vivo* fruit peel

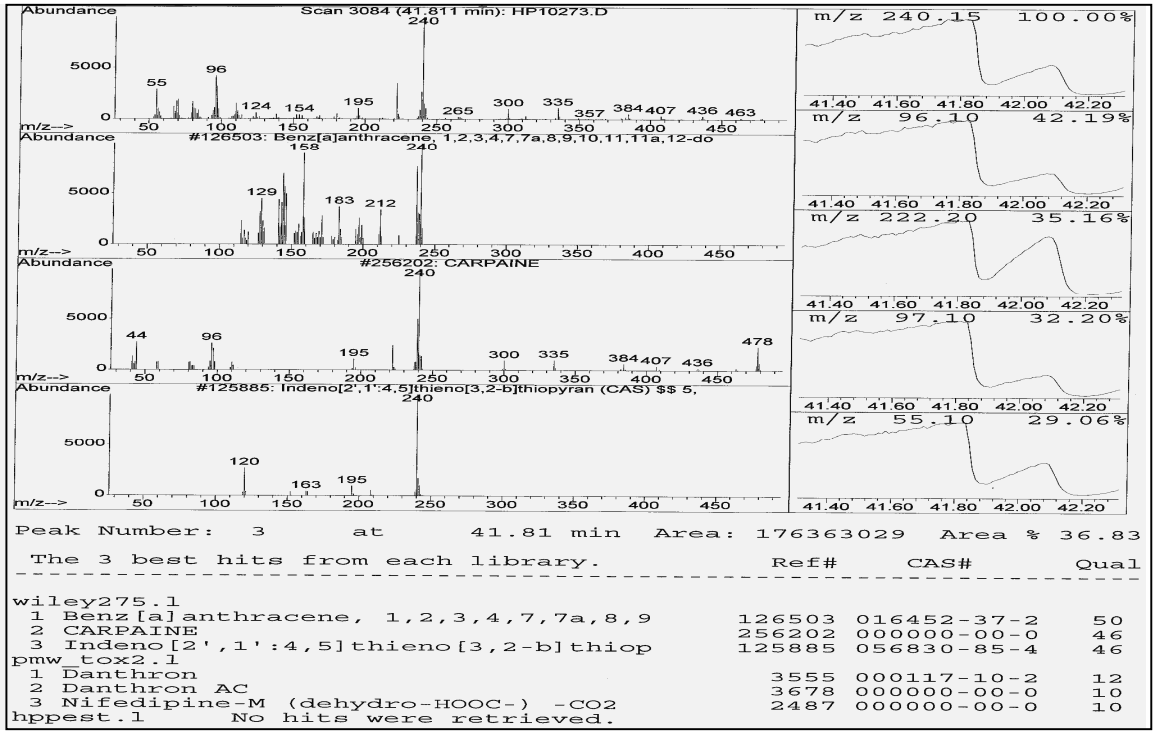




d) Mass spectrum of carpaine from *in vitro* leaf


Data File : C:\HPCHEM\1\DATA\HP10273.D
Acq On : 22 Jan 2007 9:14
Sample : PI SFE
Misc : Fhaizal/Prof.Dr.Noorsaadah
Vial: 1
Operator: siew
Inst : GC/MS In
Multiplr: 1.00
Sample Amount: 0.00
MS Integration Params: autoint1.e
Method : C:\HPCHEM\1\METHODS\NATPRO.M (Chemstation Integrator)
Title :
Abundance
TIC: HP10273.D
41.81
41.49
42.08
42.37
22.61
Time-->


Abundance
Scan 3055 (41.493 min): HP10273.D
#125885: Indeno[2',1':4,5]thieno[3,2-b]thiopyran (CAS) $$ 5,
#256202: CARPAINE
#126520: 1-adamantylidenevinylidenyl-2-methyl-2-(1-methyl-et
m/z-->
m/z 240.15 100.00%
m/z 96.10 45.31%
m/z 222.20 35.86%
m/z 97.10 34.19%
m/z 55.10 30.20%
Peak Number: 2 at 41.49 min Area: 250915237 Area % 52.41
The 3 best hits from each library. Ref# CAS# Qual
wiley275.l
1 Indeno[2',1':4,5]thieno[3,2-b]thiop 125885 056830-85-4 46
2 CARPAINE 256202 000000-00-0 46
3 1-adamantylidenevinylidenyl-2-methy 126520 118107-72-5 43
pmw_tox2.l
1 Nifedipine-M (dehydro-HOOC-) -CO2 2487 000000-00-0 12
2 Danthron 3555 000117-10-2 9
3 Danthron AC 3678 000000-00-0 9
hppest.l No hits were retrieved.



e) Mass spectrum of carpaine from *in vitro* petiole