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The Supercritical Fluid Extraction of Alkaloids from Papaya (*Carica papaya* L. var. Eksotika) Leaves

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ABSTRACT

Since the isolation procedures are easy, many of the earliest pure compounds isolated with biological activity were alkaloids. The nitrogen molecules in plant cells generally make the compound alkaline which make alkaloids exist in a salt form in plants. Thus, alkaloids are often extracted with water and/or acid solution system and then recovered as crystalline material by treatment using a base. The alkaloid carpaine was extracted from various part of *Carica papaya* L. var. Eksotika from field grown samples namely leaves, petiole and fruit peel, and from *in vitro* samples namely leaves, petiole, suspension cells and suspension liquid with only one artifact of impurity detected i.e. dehydrocarpaine II. Supercritical fluid extraction was analysed to obtain pure and high yield of carpaine compound as compared to conventional acid base extraction method. The ratio of ethanol/water/acetic acid used at 94.5:5:0.5 (v/v/v) was confirmed to be a better solvent system for carpaine extraction since only one pseudocarpaine i.e. dehydrocarpaine II was extracted together with carpaine. In this study, the application of single fluid of carbon dioxide in supercritical fluid extraction procedure generated pure and higher yield of carpaine compound. Additional centrifugation step should have contributed to a higher purity of the extracted carpaine.

Keywords: Alkaloids, *Carica papaya*, Eksotika, supercritical fluid extraction

INTRODUCTION

Papaya, *Carica papaya* L. (*C. papaya* L.) is one of the most widely grown crops in the tropical and sub-tropical region (Azarkan *et al*., 2003; Khuzhaev & Aripova, 2000) including Malaysia, where it is a smallholders' crop (Vilasini, 2000). This is because of its high demand as a multi-purpose fruit, not only as dessert fruits but also as a source for chemical compounds for medicinal use like papain, chymopapain and carpaine (Dawson, 1998; Litz, 1983). Eksotika is the main variety grown for export in Malaysia. This variety was developed and introduced by Malaysian Agricultural Research and Development Institute (MARDI) in 1987 to promote the development of papaya industry. The improved characteristics of this variety include fruit texture, taste, size, and also tolerant to Papaya Ring Spot Virus (PRSV) with good post-harvest quality to enable long transportation (Chan & Teo, 1994).

In herbal medical practice, 5000 alkaloids of all structural types are known and being explored. Alkaloids as secondary metabolites could be classified as true alkaloids, proto alkaloids, and pseudo alkaloids and were explored since 1819 (Rajnikant *et al*., 2005). Among the secondary metabolites classified as alkaloids in papaya plants, carpaine is the lead compound found in root, seed, bark, fruit peel, and leaves ranging from 1000 to 1500 ppm as reported by Burdick (1971). Studies on various aspect of carpaine have been carried out by many researchers since 1930's. Analysis of carpaine from *C. papaya* L. plants from various plant parts was carried out from separate studies; seed (Cheng & Tsai, 2004; Farias *et al*., 2007; Wilson *et al*., 2002); fruit (Azarkan *et al*., 2003; Knez *et al*., 2003; Nitsawang *et al*., 2006); root (Tang & Takenaka, 1983) and leaves (Coke & Rice, 1965; Govindachari &

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Narasimhan, 1953; Khuzhaev & Aripova, 2000; Tang, 1978).

However, in this study, carpaine was quantitatively determined from all plant parts of *in vivo* and *in vitro* origin and cell cultures. The chemical configuration of carpaine is a pyrolidine structure with a lactone moiety attached to the α-position (Govindachari, 2002) and consists of two identical substituted piperidine rings bridged by two ester groups (Burdick, 1971). The model suggested by Govindachari and Narasimhan (1953) indicated that the dimeric carpaine molecule is flexible and the two piperidine rings could assume the chair forms without restraint (Rajnikant *et al*., 2005) as shown in Figure 1.

Preceding studies have reported that the extraction of carpaine from papaya leaves inadvertently include some impurities such as ψcarpane, ψcarpamic acid, ψcarpaine (Govindachari, 2002), pseudocarpaine (Govindachari & Narasimhan, 1953; Khuzhaev & Aripova, 2000; Ogan, 1970), novel carpaine-derived macrocyclic ethers (Jacques *et al*., 1997), carpaine monoamides (Jacques *et al*., 1994) and dehydrocarpaine I and II (Tang, 1978). Hence, only a small amount of carpaine thrives to be extracted each time.

In this study, an improved conventional liquid-liquid acid base extraction method was developed to extract the right carpaine compound (Coke & Rice, 1968; Govindachari & Narasimhan, 1953; Khuzhaev & Aripova, 2000; Tang, 1978). However, following the supercritical fluid extraction (SFE) method was also developed for carpaine extraction by Knez *et al*. (2003). It is critical that the right carpaine compound is obtained, in order to qualitative analysed using an internal standard method of nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS).

MATERIALS & METHODS

General Remarks for Compound Extraction Experimental Procedure

Isolation of carpaine was achieved by using conventional acid base extraction technique as well as 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.

Plant Material

The matured leaves and petioles, and immature fruits of field grown plants *C. papaya* L. var. Eksotika [*C. papaya* (E)] were collected from the field plot of Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, while matured leaves and petioles of *in vitro* regenerant, and embryogenic suspension culture from the growth room of Plant Biotechnology Incubator Unit (PBIU), 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, ground and then powdered to 70 to 100 meshes.

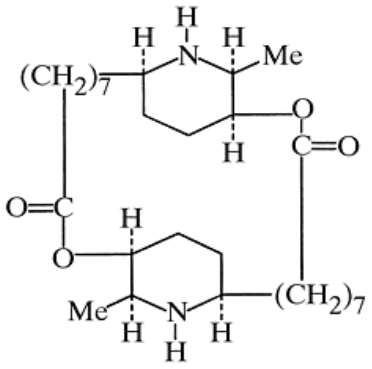


Figure 1. The chemical formula of carpaine, the alkaloid from *C. papaya* L. leaves according to Coke and Rice (1965).

Sample Extraction

Conventional Method

Field Grown Plant

The fresh leaves (500 g), petiole (150 g) and fruit peel (100 g) were collected in triplicate from MARDI. They were cleaned with distilled water and only healthy and disease-free samples were selected for further extraction. The samples were then air-dried at room temperature until dried. Later, the dried samples were ground to coarse powder and then used for alkaloid extraction.

In vitro Regenerant

The fresh leaves (25 g) and petioles (20 g) were collected in triplicate from PBIU. They were cleaned with distilled water and only healthy samples were selected for further extraction. The samples were then air-dried at room temperature until dried. Later, the dried samples were ground to coarse powder and then used for alkaloid extraction.

Embryogenic Suspension Culture

The fresh suspension culture (500 mL liquid and 100 g cells) were collected in triplicate from PBIU. Only the suspension cells were cleaned with distilled water and were then air-dried at room temperature until dryness. Later, the dried samples were ground to coarse powder and then used for alkaloid extraction. Suspension liquid was directly used for extraction without washing, dryness and grind.

Samples were soaked for 14 days in a mixture of absolute ethanol (EtOH, C_2H_5OH) (Merck), distilled water (H_2O) and glacial acetic acid (CH_3CO_2H) (Merck), in a ratio of 94.5:5:0.5, v/v/v as indicated in Table 1.

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 min. 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 H_2O: EtOH (49:1, v/v) and washed with diethyl ether [Et_2O, $(C_2H_5)_2O$] (50 mL) to remove the non-polar component. The aqueous layer was adjusted to pH 11 with potassium carbonate (K_2CO_3) solution (Merck) and then re-extracted with Et_2O (50 mL) (Rice & Coke, 1966). The ether extract was then washed with H_2O (50 mL) and 5% hydrochloric acid (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 with sodium sulfate (Na_2SO_4 anhydrous), (Merck) and Et_2O were 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 (±32ºC) until constant weight accepted.

Table 1. Solvent used for soaking.

No.	Types of Solvent	Combination Ratio
1.	Absolute ethanol : Distilled water : Glacial acetic acid	89 : 10 : 1 (Coke & Rice, 1965 and Tang, 1978)
2.	Absolute ethanol : Distilled water : Glacial acetic acid	94.5 : 5 : 0.5 (Modified)

Supercritical Fluid Extraction

Field Grown Plant

The fresh leaves (50 g), petiole (50 g) and fruit peel (50 g) were collected in triplicate from MARDI. They were cleaned with distilled water and only healthy and disease-free samples were selected for further extraction. The samples were then air-dried at room temperature until dryness. Later, the dried samples were ground to coarse powder and then used for alkaloid extraction.

In vitro Regenerant

The fresh leaves (5 g) and petioles (5 g) were collected in triplicate from PBIU. They were cleaned with distilled water and only healthy samples were selected for further extraction. The samples were then air-dried at room temperature until dryness. Later, the dried samples were ground to coarse powder and then used for alkaloid extraction.

Powdered *C. papaya* (E) ground samples were extracted for approximately 60 min in a dynamic loom, using an extraction fluid of pure carbon dioxide (CO_2) (supplied by Malaysian Oxygen, MOX) without other solvents, as indicated in Table 2. Extraction was carried out in triplicate.

Sequential modes of extractions were carried out with pure CO_2 but in a manner of varied temperature and pressure condiions as reported in Table 2 for each sample (three samples). All the extracts were collected into analytical grade EtOH (Merck) in a 250 mL volume of round bottle flask. The solvent was removed *in vacuo* at 40°C until constant weight of elucidated carpaine obtained (Knez *et al*., 2003). All of the extracts were dissolved in analytical grade of Et_2O (Merck) for storage prior to further analysis.

Characterization of Alkaloid Carpaine

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.

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; butanol (BuOH, C_4H_9OH), acetic acid (HOAc, C_3COOH), water (H_2O), methanol (MeOH, CH_4O), chloroform ($CHCl_3$), benzene (C_6H_6) and ethylene (C_2H_{12}) were used for TLC analysis as indicated in Table 3.

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.

Table 2. Solvent used in SFE.

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

Table 3. Solvent's combination used in TLC experiments.

No.	Types of Solvent	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} : H_2O	4 : 5 : 1

Nuclear Magnetic Resonance Spectrometry

The yellow needle-like crystals of carpaine obtained from acylation and SFE was dissolved in deuterated chloroform ($CDCl_3$) (Merck) and ^{1}H-NMR spectrum was taken on the fourier-transform nuclear magnetic resonance (JEOL 400 MHz FT-NMR) spectrometer (Manrique & Lajolo, 2002).

Gas Chromatography / Mass Spectrometry

Mass spectrum was taken on the Hewlett-Packard (HP) 6890 Series, GC System spectrometer equipped with a Agilent DB-1ms (length: 30 m; ID: 0.25 mm; film thickness: 0.25 μm) with nitrogen (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 (5 ppm) into the spectrometer at the initial temperature at 70^{o}C for 2 min and increasing linearly at a rate of 10^{o}C per min to 250^{o}C and held for 10 min. The peak at retention time of 54.227 min in the gas chromatogram was identified as carpaine from the M^+ at 478.377 (Mahmood & Rahman, 1998).

RESULTS

Characterization of Alkaloid Carpaine

Conventional Method (Acid/Base Extraction)

Carpaine was isolated and crystallised from various parts of papaya plant from samples ranging from 20 to 500 g and 500 ml. Results of the amount isolated is shown in Table 4.

The purity of carpaine extracted (from acid/base extraction) from the field grown and *in vitro* samples of Eksotika was analysed using TLC and observed to be comparable to those reported earlier (Burdick, 1971; Coke & Rice, 1965). In addition, the melting point of the carpaine isolated corresponded to the reported melting point (Coke & Rice, 1968; Tang, 1978).

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 min (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.

Table 4. A distribution of carpaine extract from *C. papaya* (E) plant parts; Embryogenic cells suspension (ECS), not applicable (N.A) and standard deviation (±sd).

	Field Grown Plant			***In vitro* Regenerant and Embryogenic Cells Suspension**				
	Leaf	Petiole	Fruit Peel	Leaf	Petiole	Fruit Peel	ECS-Liquid	ECS-Cell
Quantity of Sample (g or ml)	500 500 500	150 150 150	100 100 100	25 25 25	20 20 20	N.A N.A N.A	500 500 500	100 100 100
Mass of Crystal (g)	0.213 0.270 0.285 ± 0.038	0.046 0.043 0.052 ± 0.004	0.027 0.027 0.022 ± 0.003	0.001 0.004 0.004 ± 0.002	0.003 0.001 0.003 ± 0.001	N.A N.A N.A N.A	0.024 0.023 0.020 ± 0.002	0.066 0.060 0.064 ± 0.003
Fraction of carpaine extract (%)	0.043 0.054 0.057 ± 0.007	0.031 0.029 0.035 ± 0.003	0.027 0.027 0.022 ± 0.003	0.004 0.016 0.016 ± 0.007	0.015 0.005 0.015 ± 0.006	N.A N.A N.A N.A	0.005 0.005 0.004 ± 0.001	0.066 0.060 0.064 ± 0.003

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 EtOH/H_2O/HOAc 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. 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 2 (*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 5).

The melting points (m.p.) for the crystalised carpaines isolated from different parts of the plant were observed to be between 110 to 121°C (literature m.p. 119 to 120°C) (Tang, 1979) (Table 6).

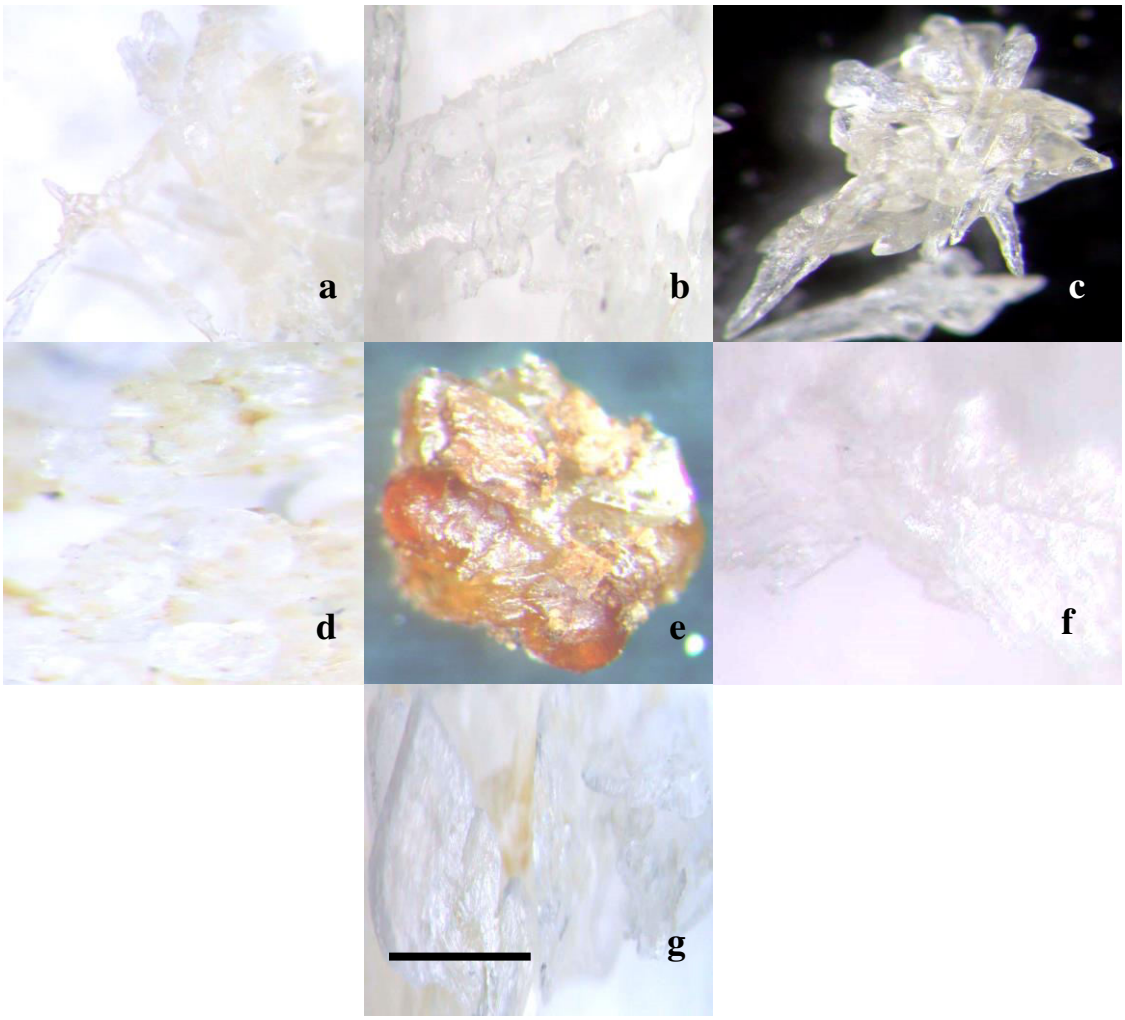


Figure 2. The texture of carpaine isolated from the field grown plants (a to c), *in vitro* regenerant (d and e) and embryogenic cells suspension (f and g) samples. Leaf (a), petiole (b) and fruit peel (c); leaf (d) and petiole (e); and suspension cells (f) and liquid (g) through conventional acid/base extraction. (10x) Bar at 2.0 mm.

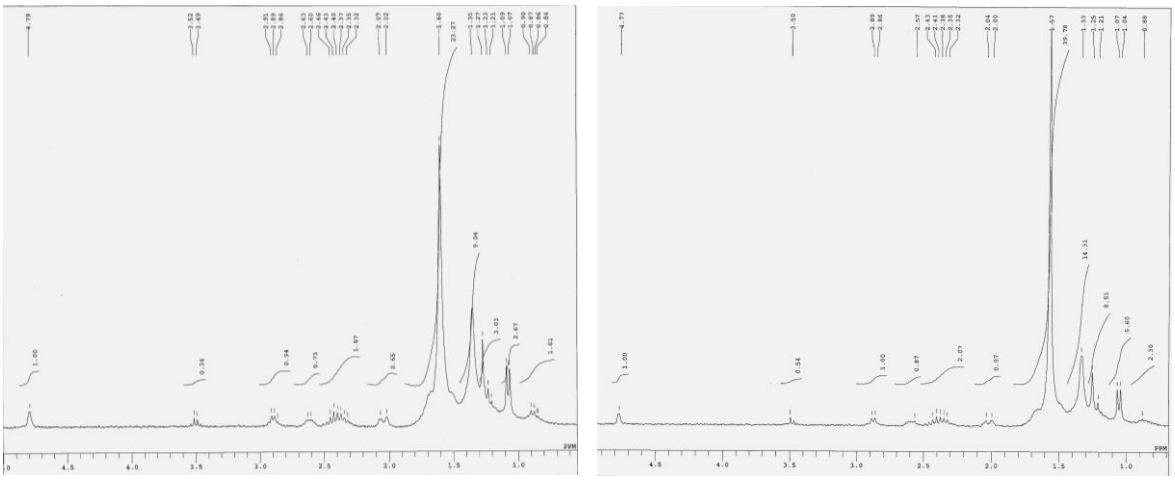
Table 5. The pooled fractions subjected to TLC. Field grown plants (FGP), *in vitro* regenerant (IvR), embryogenic cells suspension (ECS), not applicable (N.A), average (Ave) and standard deviation (±sd).

Types of Sample	Leaf	Petiole	Fruit Peel	ECS Liquid	ECS Cells
	Ave	Ave	Ave	Ave	Ave
FGP	0.29±0.14	0.43	0.56±0.04	N.A	N.A
IvR	0.35±0.05	0.35±0.01	N.A	N.A	N.A
ECS	N.A	N.A	N.A	0.51±0.03	0.54±0.04

Table 6. Observed melting points of crystals isolated from various parts of plants.

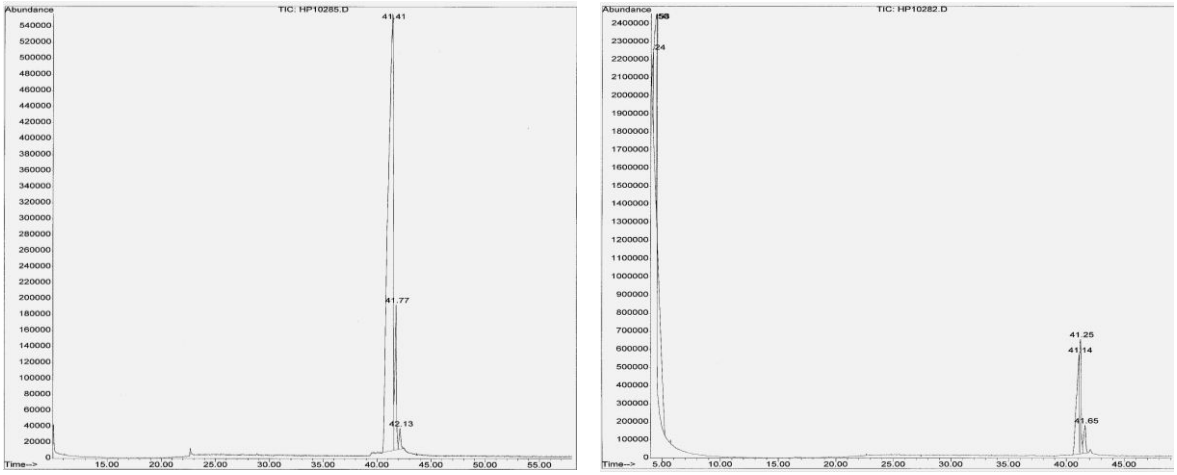
No.	Sample	Temperature (ºC)	Time (sec.)
1.	Crystalline of FGP leaf	119	±10
2.	Crystalline of FGP petiole	110	±10
3.	Crystalline of FGP fruit peel	121	±10
4.	Crystalline of IvR leaf	120	±10
5.	Crystalline of IvR petiole	110	±10
6.	Crystalline of ECS cells	118	±10
7.	Crystalline of ECS liquid	121	±10

The ^{1}H-NMR and mass spectra for the sample extracted from the leave are shown in figures 3(a) and (b) while the spectra for all other samples extracted from the different parts of the plant are also attached in Figure 3. 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 *et al*. (2003). Mass spectrometry found a peak at *m/z* 478.377 in the spectrum which corresponded to Figures 4(a) and (b) $C_{14}H_{25}N_2O_4$, as reported by Tang (1979).



(a) (b)

Figure 3. NMR spectrum of samples from acid/base extraction. ^{1}H-NMR of carpaine from FGP leaf (a) and IvR leaf (b).



(a) (b)

Figure 4. GC-MS of samples from acid/base extraction. Mass spectrum of carpaine from FGP leaf (a) and IvR leaf (b).

Supercritical Fluid Extractions

In this study, the quantity of papaya samples used ranged from five to 50 g. Supercritical fluid extraction of these samples furnished crystalline carpaine as displayed in Table 7 with the average volume of solvent used to be about 3033.2 g in one hour showed in Table 8.

Carpaine obtained from SFE gave dull yellow cubic-like crystals as shown in Figure 5 (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 and 0.39 ±0.01 for FGP and IvR leaf; 0.46 ±0.06 and 0.41 ±0.006 for FGP and IvR petiole; and 0.53 ±0.04 for FGP fruit peel respectively (Table 9).

Table 7. A distribution of carpaine extract from *C. papaya* (E) plant parts; Embryogenic cells suspension (ECS), not applicable (N.A) and standard deviation (±sd).

	Types of Sample (Plant Parts)							
	Field Grown Plant			***In vitro* Regenerant and Embryogenic Cells Suspension**				
	Leaf	Petiole	Fruit Peel	Leaf	Petiole	Fruit Peel	ECS-Liquid	ECS-Cell
Quantity of Sample (g or ml)	50	50	50	5	5	N.A	N.A	N.A
	50	50	50	5	5	N.A	N.A	N.A
	50	50	50	5	5	N.A	N.A	N.A
Mass of Crystal (g)	1.988	0.231	0.454	0.018	0.003	N.A	N.A	N.A
	1.8	0.22	0.444	0.015	0.002	N.A	N.A	N.A
	1.98	0.216	0.44	0.015	0.003	N.A	N.A	N.A
	±	±	±	±	±			
	0.106	0.008	0.007	0.002	0.001	N.A	N.A	N.A
Fraction of carpaine extract (%)	0.994	0.116	0.227	0.36	0.06	N.A	N.A	N.A
	0.900	0.110	0.222	0.3	0.04	N.A	N.A	N.A
	0.990	0.108	0.220	0.3	0.06	N.A	N.A	N.A
	±	±	±	±	±			
	0.053	0.004	0.004	0.035	0.012	N.A	N.A	N.A

Table 8. 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	FGP leaf
2.	285723	281424	4299	FGP petiole
3.	288536	285723	2813	FGP fruit peel
4.	291877	288536	3341	IvR leaf
5.	292948	291877	1071	IvR petiole

Table 9. The pooled fractions subjected to TLC. Field grown plants (FGP), *in vitro* regenerant (IvR), not applicable (N.A), average (Ave) and standard deviation (±sd).

Types of Sample	Leaf	Petiole	Fruit Peel
	Ave	Ave	Ave
FGP	0.45±0.08	0.46±0.06	0.53±0.04
IvR	0.39±0.01	0.41±0.006	N.A

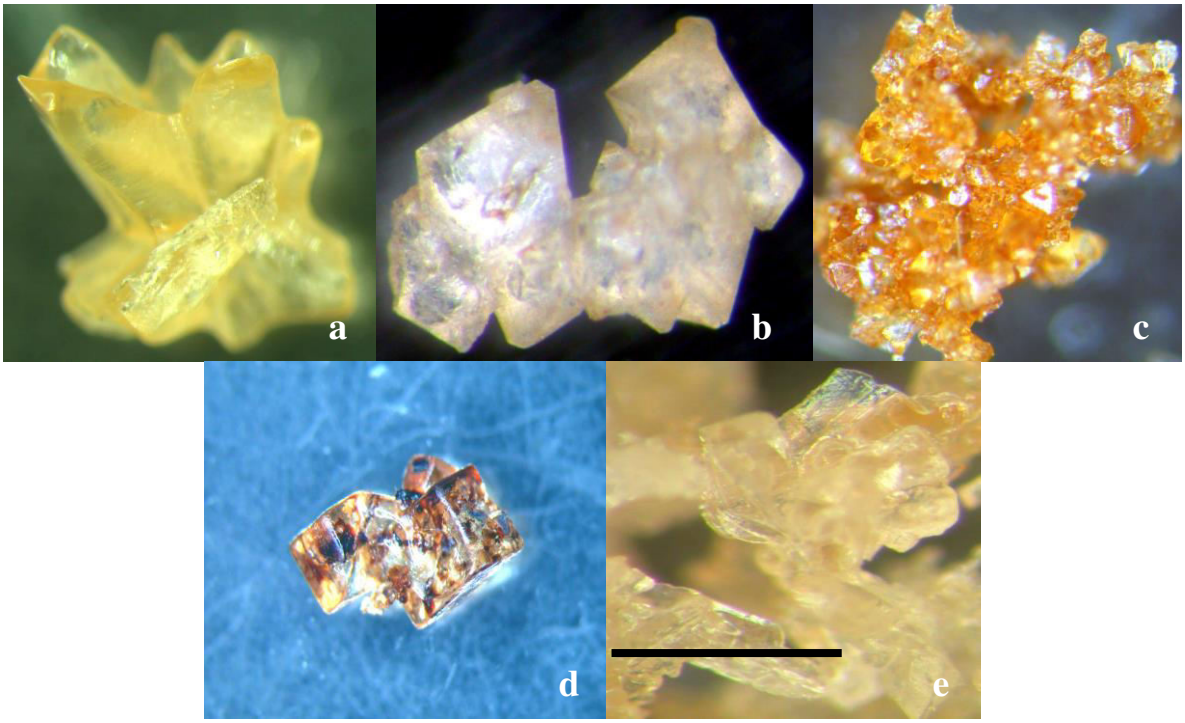


Figure 5. The texture of carpaine isolated from the field grown plants (a to c) and *in vitro* regenerant (d and e) samples. Leaf (a), petiole (b) and fruit peel (c); leaf (d) and petiole (e) through SFE. (10x) Bar at 0.025 cm.

The melting point of the carpaine extracted from SFE corresponded to the reported melting point (Coke & Rice, 1968; Tang, 1978), indicating the carpaine to be pure as indicated in Table 10.

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 min. The mass spectrum of the peak observed at *m/z* 476 confirmed the presence of the dehydrocarpaine II. This compound was however, not isolated (Figures 4 and 7).

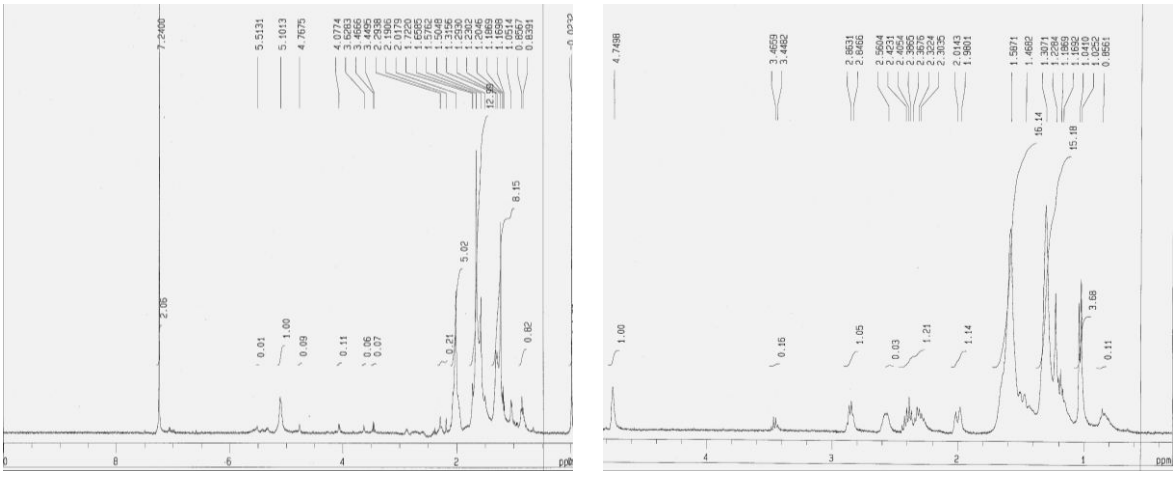
The melting points (m.p.) of the isolated carpaines were found to be between 98 to 120°C (literature m.p. 119 to 120°C) (Tang, 1979).

Table 10. Observed melting points of crystals isolated from various parts of plants.

No.	Sample	Temperature (ºC)	Time (sec.)
1.	Crystalline of FGP leaf	119	±10
2.	Crystalline of FGP petiole	98	±10
3.	Crystalline of FGP fruit peel	108	±10
4.	Crystalline of IvR leaf	120	±10
5.	Crystalline of IvR petiole	106	±10

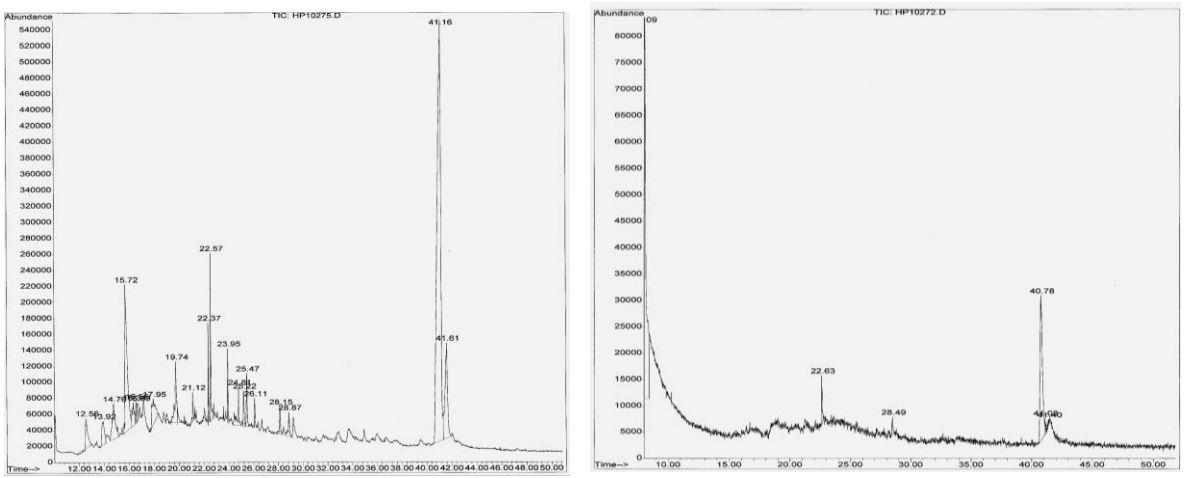
The ^{1}H-NMR and mass spectra for the sample extracted from the leaves are shown in Figures 6(a) and (b) while the spectra for all other samples extracted from the different parts of the plant are also attached in Figure 6.

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), Figures 6(a) and (b). This spectrum is identical to that reported by Sato *et al*. (2003). Mass spectrometry found a peak at *m/z* 478.377 in the spectrum which corresponded to Figures 7(a) and (b) $C_{14}H_{25}N_2O_4$, as reported by Tang (1979).



(a) (b)

Figure 6. NMR spectrum of samples from SFE. ^{1}H-NMR of carpaine from FGP leaf (a) and IvR leaf (b).



(a) (b)

Figure 7. GC-MS of samples from SFE. Mass spectrum of carpaine from FGP leaf (a) and IvR leaf (b).

DISCUSSION

Other researchers have isolated the alkaloid carpaine from the leaves of the *C. papaya* L. var. Uzbekistan, Nigerian and Solo (Govindachari, 2002; Khuzhaev & Aripova, 2000). However, the amount of carpaine they extracted from the Uzbekistan, Nigerian and Solo variety was quite low, ranging from 0.025 to 0.4% and rather impure since it contains other compounds such as dehydrocarpaine I and dehydrocarpaine II.

In this work, the major alkaloid isolated from the leaves of *C. papaya* (E) is carpaine (Govindachari *et al*., 1954) and another nitrogenous base compound, i.e., pseudocarpaine (Khuzhaev & Aripova, 2000) which is a stereoisomer of carpaine. This stereoisomer however, is only isolated in very small quantity (10%). Previous studies for carpaine isolation gave low quantity and poor quality. Therefore, it seemed worthwhile to investigate whether the low yield observed in the carpaine isolated were due to some factors in the regulation of secondary metabolism at all levels such as genes, enzymes, transport and compartmentalization or due to the extraction and refinement procedures.

Our work in isolating carpaine from *C. papaya* (E) leaves using the methods of Govindachari *et al*. (1954); Coke & Rice (1968); Ogan (1970); Tang (1978) and Khuzhaev & Aripova (2000) yielded an impure carpaine samples with lower yield. In an attempt to harvest the carpaine in a purer and higher yields, modification to conventional acid/base extraction method were carried out. In addition, extraction was also carried out using the SFE method to determine which of the two methods is more reliable and efficient in giving carpaine in a purer and higher yield. The amount of carpaine isolated from the various sources of plant parts using the two different techniques is shown in Table 11.

The yield of carpaine isolated (with acid/base extraction) from FGP and IvR leaves was 0.256 g or 0.051% and 0.003 g or 0.012% respectively. Meanwhile, the yield of carpaine isolated (with SFE) from FGP and IvR leaves was 1.923 g or 0.961% and 0.016 g or 0.32% respectively.

Our results showed improvement in the isolated yield compared to those reported for Nigerian and Solo plants 0.175 (0.012%) (Khuzhaev & Aripova, 2000; Tang, 1978) as when the yielded carpaine was up to ±1:5 and 1:50 from the respective acid/base and SFE methods. In addition, the use of younger leaves compared to the previous reports where mature leaves were used seem to enhance the quantity of carpaine produced. The evidence took place with the explanation of the nature of secondary metabolite production reported by Satoh and Flores (1990). In nature (FGP), the roots of plants produce a complex array of secondary metabolites, possibly because they live in an environment of soil, which is permeated with pathogens and predators.

For this reason, they evolved capacities for synthesizing all manner of defence chemicals that may accumulate in the roots, be secreted into the rhizosphere, and later transported to the shoot. Meanwhile, in IvR plants the secondary metabolites are produced by the roots at low levels until the roots are confronted with a contamination caused by pathogen, at which time they may increase the synthesis and secretion of the metabolite to try to ward off the contamination. Due to this reason, they exhibited the responses of defense mechanism towards latent bacterial contaminants which certain internal chemicals may produce and accumulated in the roots and later being transported to the shoot.

The relative amount of carpaine isolated from different plant parts showed that in the IvR samples, highest carpaine content is in the petiole and the lowest content to be in the leaves. However, in the FGP samples, the highest carpaine content was found in the leaves and the lowest content in the fruit peel. Secondary metabolites such as carpaine are produced in the organelles (Nishioka & Funatsu, 1999; Yazaki, 2005). Organelles function as transported plant cell systems (Jørgensen *et al*., 2005; Yazaki, 2005). The extracts in our study had been subjected to centrifugation to remove all solid particles. This presumably resulted in an increase amount of carpaine isolated since the organelles are plasmolysed during the centrifugation, releasing more carpaines into the media (Burkart, 2002).

Table 11. Quantity and fraction of carpaine extract from FGP and IvR leaves of *C. papaya* (E).

Samples	Types of Extraction	
	Acid/Base	Supercritical Fluid
FGP leaves	0.256 g (0.051 %)	1.923 g (0.961%)
IvR leaves	0.003 g (0.012 %)	0.016 g (0.32%)

In this study, the isolation procedure employed ethanol/water/acetic acid (v/v/v) as extraction solvents in the conventional extraction method, while carbon dioxide (gram per hour) was used in SFE. More carpaine was extracted through SFE than from acid/base extraction (Table 11). In SFE, extraction is carried out at lower temperatures. Perhaps this helps to lessen any possible deterioration in the extracts. In addition, since CO_2 was used as the mobile phase, there would be no oxygen in the extraction process. This would possibly reduce significantly the chances of oxidation of the extracts during the extraction process. The more prominent advantage in using SFE is that the mobile phase is CO_2, which is gaseous. Thus, in SFE there is no residual solvent in either the extract or in the raffinate. This translates into reduction in the loss of compounds extracted during post-processing and clean-up procedures.

The purity of carpaine extracted from the leaves of Eksotika was determined by TLC (R_f value of 0.43±0.11 and 0.45±0.08 from both mode of extraction) and found to be comparable to those reported earlier (R_f value of 0.4 for carpaine and 0.15 for dehydrocarpaine II) (Burdick, 1971 & Tang, 1978). In addition, the proton NMR and mass spectrum of the carpaine isolated which is observed to be pure and identical to those reported by Tang (1978) and by Sato *et al*. (2003).

In our work, only one pseudo-carpaine compounds were isolated, namely dehydrocarpaine II (white needle-like crystal, m.p. 65 to 66°C), whilst Govindachari *et al*. (1954); Coke and Rice (1968); Tang (1978) and Khuzhaev and Aripova (2000) obtained both dehydrocarpaine I and II. However, dehydrocarpaine I and dehydrocarpaine II are not easily separable from carpaine through the conventional acid/base purification techniques such as re-crystallization. Tang (1978) and Coke and Rice (1968) used ethanol/water/acetic acid in a ratio of 89:10:1 (v/v/v) to soak the leaves of *C. papaya* L. var. Solo for carpaine extract. However, they obtained a mixture of carpaine, pseudocarpaine and dehydrocarpaine I and II at ratio 31.5 (or 0.0115% of carpaine) to 68.5 (or 0.025% of dehydrocarpaine I and II).

They obtained a mixture of carpaine, dehydrocarpaine I and dehydrocarpaine II with higher concentration of dehydrocarpaine I and dehydrocarpaine II than that of carpaine (Khuzhaev & Aripova, 2000; Tang, 1978). The carpaine crystals obtained by the previous researchers are usually contaminated with two pseudocarpaines, and its purity is dependent upon the techniques used in separating them. In our work, modification of two-phase aqueous organic extraction and two-step precipitation methods proposed by Coke and Rice (1968) and Tang (1978) were used for purification of carpaine from the clarified papaya crude alkaloid. Although the same solvent mixture was used, the ratio used in this study was 94.5:5:0.5 (v/v/v). This solvent system is more non-polar which presumably encourages better extraction of the non-polar carpaine. With all these modification, we were able to separate the carpaine from the dehydrocarpaine II.

CONCLUSION

The alkaloid carpaine, was extracted from various parts of *C. papaya* (E) field grown plants namely the leaves, petiole and fruit peel, and cell cultures namely leaves, petiole, suspension cells and suspension liquid with one impurity compound detected namely dehydrocarpaine II. In IvR samples, petiole gave the highest carpaine content and the

lowest content found in the leaves. Meanwhile, in FGP samples, leaves gave the highest carpaine content and the lowest content detected in the fruit peel. Supercritical fluid extraction was identified as a more efficient method for getting pure and high yield of carpaine compared to conventional acid/base extraction method.

The ratio of ethanol/water/acetic acid used at 94.5:5:0.5 (v/v/v) was confirmed to be a better solvent system for carpaine extraction compared to the system ethanol/water/acetic acid used at 89:10:1 (v/v/v) used by Coke and Rice in 1968. However, carbon dioxide used as supercritical fluid (SF) in SFE compared to water seemed to furnish pure and higher yield of carpaine. Additionally, centrifugation may also contributed to the greater yields of the carpaine extracted observed.

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