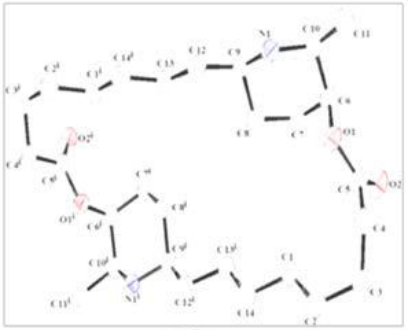
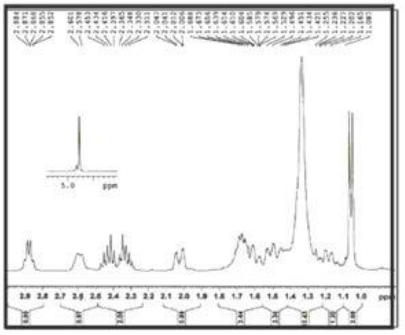
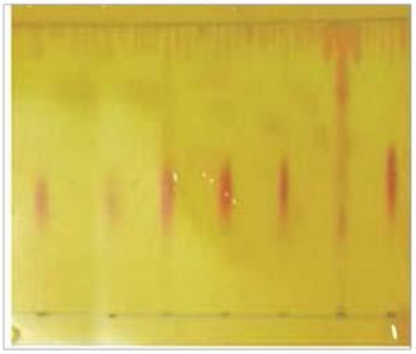




Chapter 4: Bioprospecting of Carpaine in *Carica papaya* Linn.



4. Bioprospecting of Carpaine in *Carica papaya* Linn.

4.1 Introduction

Alkaloids, produced as secondary metabolite for chemical defense against herbivorous, microorganisms, viruses and other plants, form one of the largest groups (about 20%) of known natural products [1], [2]. Alkaloids are more common in higher plants like Angiosperms, e.g. 60-70% of Apocynaceae and Solanaceae species accumulate alkaloids, however, few lower plants, like yew, a Gymnosperm and even fungi like Claviceps also produce alkaloids [3], [4]. The biosynthesis of alkaloids is not restricted to plants, these are produced in some animals such as marine sponges, worms, snails, insects, amphibia, fishes and birds as well [5].

The survey of literature reveals that Morphine was the first alkaloid, crystallized and identified by Sertoner in 1805 [6]. Similar intrinsically basic compounds isolated by W. Meissner and were named as 'alkaloids'. After isolating and studying a large number of alkaloids, Pelletier in 1983 summarized alkaloids as " alicyclic compound containing nitrogen in negative oxidation state with limited distribution among living organisms [7]."

Alkaloids can be classified on the basis of (a) bio synthetic path way and (b) chemical structure. From a structural point of view these are classified as

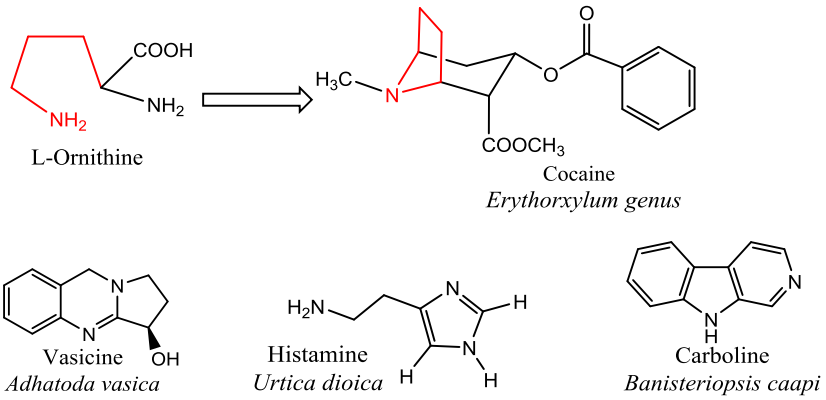


Figure 4.1: True alkaloids

True Alkaloid: Compounds having nitrogen as a heteroatom which are derived from different amino acids or biogenic amines by decarboxylation. They are usually found as ammonium salts in plant e.g. cocaine, histamine, carboline, peganine.

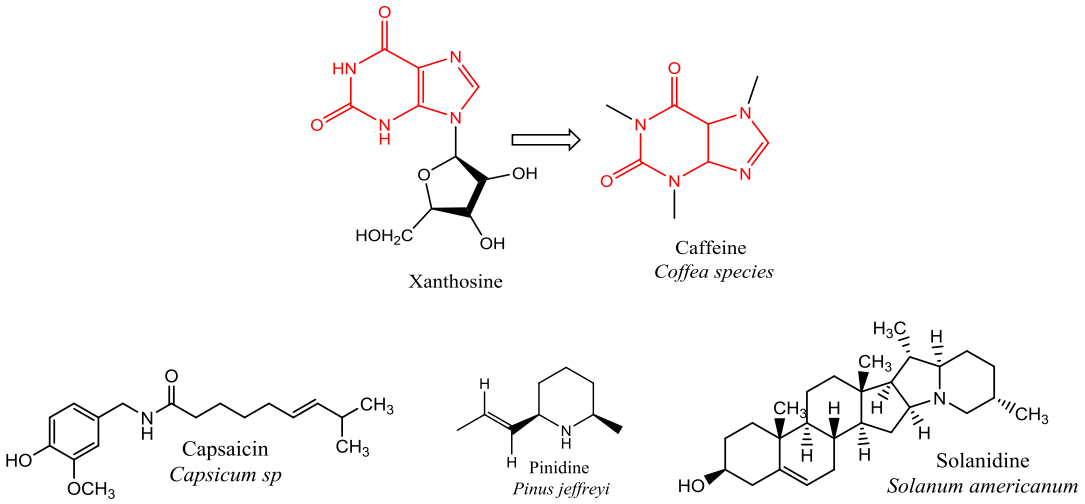


Figure 4.2: Pseudo alkaloids

Pseudo Alkaloid: Compounds having nitrogen as a heteroatom but not derived from amino acid, but rather they are derived from monoterpenes and derivative of acetate e.g. capsaicin, caffeine.

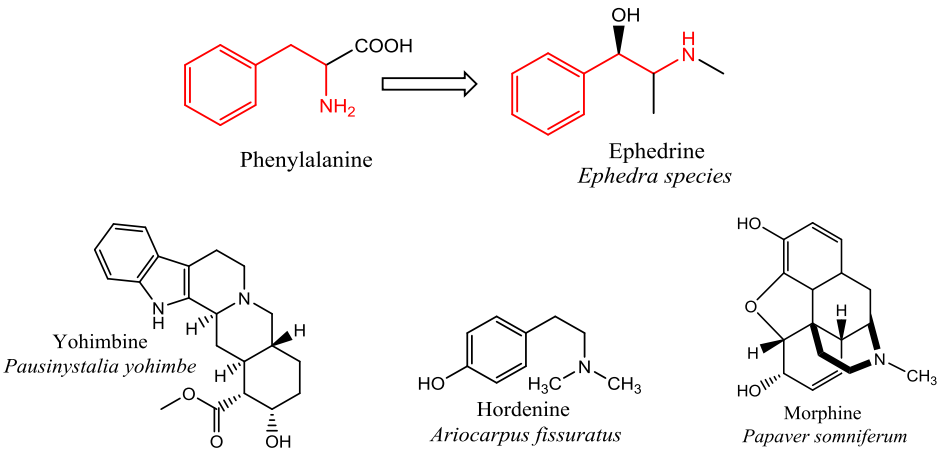


Figure 4.3: Proto alkaloids

Proto Alkaloid: Compounds which are derived from amino acid or biogenic amines but do not contain any heteroatom e.g. morphine, hordenine, yohimbine.

Usually alkaloids are present as salt in plants. Almost all alkaloid present in the plants are in solid state except few like nicotine which is brown liquid at room temperature [8]. Due to organic character of the salt of alkaloids, these are freely soluble in alcohol. Most of the alkaloids are intensely bitter in taste and often toxic in nature [1], [9].

Alkaloids exhibit a vast spectrum of bioactivity and many are used as medication, recreational drugs and entheogenic rituals [10].

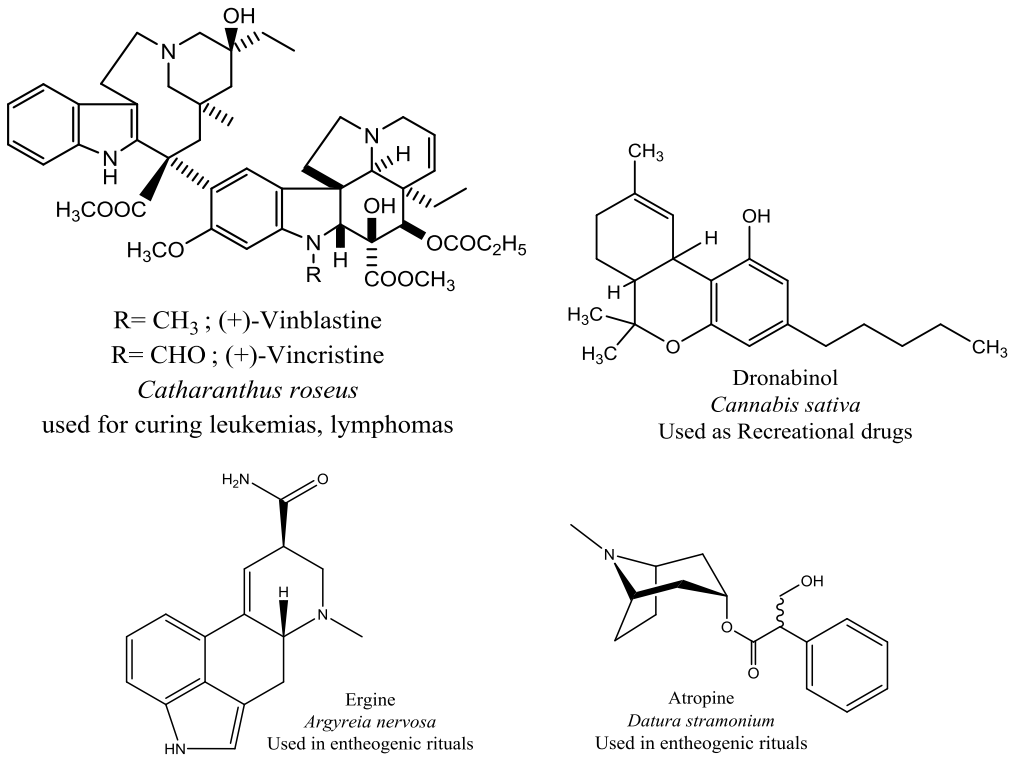


Figure 4.4: Few examples of alkaloids used as medication, recreational drugs and entheogenic rituals.

4.2 Alkaloids in *Carica papaya*

It is reported that papaya leaf contains many alkaloids, with carpaine as a lead component and pseudocarpaine, dehydrocarpaine I and II, choline, and methyl derivative of carpaine in minor quantities [11]. The alkaloid carpaine was first isolated by Greshoff in 1890 from *Carica papaya* leaves and was reported to be the

only alkaloid present, however, over a period of time many scientists discovered other components of the alkaloid groups present in *Carica papaya* leaves [12], [13]. The work done so far on isolation and characterization of the alkaloids present in *Carica papaya* leaves and different parts of the *Carica papaya* plant is summarized in Table 4.2.The most outstanding and a large volume of work on *Carica papaya* leaf alkaloids have been carried out by C. Govindachari and his group [14], [15].

Table 4.1: Extraction of carpaine

Scientists	**Amount of plant material**	**Yields (%)**	**Remarks**
Barger *et al* [16]	100 kg	0.018	C_2H_5OH:CH_3COOH:H_2O (80:0.5:19.5) as weak acid, Base is not specified
Tang *et al* [17]	5 kg	0.040	C_2H_5OH:CH_3COOH:H_2O (89:1:10) as weak acid & K_2CO_3 as a weak base
Head *et al* [18]	500 g	0.080	C_2H_5OH:HCl:H_2O (80:1:19) as weak acid & K_2CO_3 as a weak base
Govindachari *et al* [11]	14 kg	0.110	C_2H_5OH:CH_3COOH:H_2O (90:1:9) as weak acid & aq. NH_3 as a weak base
Ogan *et al* [19]	2.5 kg	0.007	C_2H_5OH:CH_3COOH:H_2O (90:1:9) as weak acid & aq. NH_3 as a weak base + use of Reinecke's reagent with Ag_2SO_4
Rapoport *et al* [20]	150 pound	0.040	C_2H_5OH:CH_3COOH (95:5) as weak acid & K_2CO_3 as a weak base
Julianti *et al* [21]	1g	0.011-3.7	Pressurized liquid extraction
Bukhori *et al* [22]	5-50g	0.961	Supercritical fluid extraction

The structure of carpaine was a mystery, which was solved by Rice *et al* through painstakingly difficult chemical and spectral analysis [23]. The final confirmation of its existence as a dimer came from X-ray crystallographic analysis by Kabaleeswaran *et al* [24].

Present chapter is about isolation, characterization and quantification of carpaine in different parts of *Carica papaya i.e.* bioprospecting.

4.3 Isolation and characterization of carpaine

Many methods have been used for isolation of carpaine including conventional, pressurized liquid extraction and super critical extraction. Among conventional methods, Govindachari *et al* have reported the highest yields. The application of novel techniques utilizing pressurized liquid and super critical fluids for extractions are reported to be much more efficient, yielding better amounts of carpaine [21], [22]. However these techniques require principally expensive set up. Due to unavailability of these facilities, the present work of extraction was carried out by modifying conventional method used by Govindachari *et al* [11].

4.3.1 Materials and Methods

4.3.1.1 Chemicals

All chemicals used were of analytical reagent grade (AR). Acetic acid, ethanol, chloroform, cyclohexane, petroleum ether, liquor ammonia, ethyl acetate, n-hexane, acetone were purchased from the Merck (India). Conductivity water was used in all steps of the extraction.

4.3.1.2 Plant material

The plant material was collected as described in PART I section 2.2.1.1.

4.3.1.3 Extraction

The dried, powdered leaves (500 g) of *Carica papaya* refluxed for 12 h in ethanol and acetic acid (90:1). The extract was filtered and the process was repeatedly twice. The combined alcoholic extracts were concentrated to a small volume at ordinary pressure

initially and later under reduced pressure. The residue was treated with water (500 mL), acidified with acetic acid and kept overnight. Celite filtration was done to remove sticky resinous material from aqueous extract. The acidic extracts were concentrated under *vacuum*. The clear solution was repeatedly extracted with ether to remove chlorophyll and other highly non-polar phytochemicals. The aqueous fraction was treated with acetone to remove highly polar compounds. The whole solution was centrifuged, decanted, distilled and concentrated under *vacuum*. The concentrated portion was diluted with 500 mL distilled water. The aqueous solution was basified with liquor ammonia and kept overnight. The liberated base was extracted with diethyl ether, dried over Na_2SO_4 bed, and concentrated under *vacuum*. Crude alkaloids were obtained as sticky light brown solid. The crude alkaloids were dissolved in the minimum amount of n-hexane and cooled at 0 $^{\circ}$C overnight. The crystals of carpaine were collected and recrystallized from n-hexane.

Following parameters as given in table 4.2 were varied to optimize the extraction process.

Table 4.2: Method optimization of carpaine extraction

S. No	Acetic acid (%)	pH using Base	Temp (°C)	Base	Time (days)	Yield (mg)
1	0.5	8	60*	NH_3	18	10.1
2	0.5	8	100	NH_3	10	10.1
3	0.5	10	60*	NH_3	18	11.9
4	0.5	10	100	NH_3	10	12.5
5	1	8	60*	NH_3	18	25.3
6	1	8	100	NH_3	10	29.4
7	1	10	60*	NH_3	18	32.8
8	**1**	**10**	**100**	**NH_3**	**10**	**40.1**
9	1.5	8	60*	NH_3	18	10.1
10	1.5	8	100	NH_3	10	13.5
11	1.5	10	60*	NH_3	18	17.5
12	1.5	10	100	NH_3	10	18.8
15	1	10	100	K_2CO_3	10	10.7
16	1	10	100	Na_2CO_3	10	1.9

* stands for vacuum used

The method was optimized for *Carica papaya* leaves and the same procedure was used for extraction of carpaine from other parts i.e. seeds, root, stem, fruits and petioles of the plant.

The carpaine extracted from each part was subjected to identification and quatitation.

4.3.2 Identification and characterization of the carpaine

TLC plate (7x5cm) spotted with crude alkaloid extracted from different parts of *Carica papaya* Linn. plant at 1cm apart from each other and run with EtOAc: MeOH: Formic acid (5:1:1) solvent. The plate was finally stained with dragendroff reagent. The melting temperatures were determined by capillary method. FTIR spectra of carpaine collected from each part of plant were recorded as KBr pellet on Perkin

Elmer RX1 model in the range of 400 - 4000 cm^{-1}. Optical rotation was recorded on Jasco P2000 polarimeter. The ^{1}H NMR, ^{13}C NMR, DEPT -135 and 2D NMR spectra in deuterated chloroform ($CDCl_3$) were recorded on Bruker (400 MHz) NMR spectrometer. Crystallographic data were collected on Xcalibur, Eos, Gemini diffractometer with a graphite monochromator using CuKα radiation. All the parameters were in good agreement with the data which were produced in CCDC 138024 [24].

4.3.3 Results and Discussion

Optimized condition for quantitative extraction of carpaine from *Carica papaya* leaves was achieved by varying concentration of acetic acid (%), pH, extraction temperature and different bases.

Use of different concentrations of acetic acid (0.5, 1.0, 1.5% v/v) revealed that 1% v/v is the best for maximum extractions whereas with 0.5% of acetic acid concentration carpaine extraction is incomplete and concentration above 1.0% results into sticky mass with lower yields. Extraction under two different alkaline conditions i.e. pH 8 and pH 10 indicated that yields were better at *p*H 10. The conventional method required *Carica papaya* leaves infusion for 3 days, to shorten this time, in the present method, the *Carica papaya* leaves were refluxed at 100°C or at 60°C (under vacuum) for a day. The results showed that refluxing at 100°C yielded better quantities. Comparison of extraction with different bases was done using strong (NaOH, KOH) to weak bases (NH_3, Na_2CO_3, K_2CO_3) indicated that use of ammonia during significantly increased the extraction yield.

Once the method was optimized for extraction of *Carica papaya* leaves, the same procedure was used for the extraction of carpaine from different parts of the plant, stem, root, fruits, seeds and petioles.

4.3.3.1 Identification of Carpaine

Carpaine was obtained as white crystalline powder from all the parts of plant. The TLC results [Figure 4.5] of the isolated carpaine indicated relatively good purity of the extracted compound. The Rf values for the extracts from different parts of the plant matched with that for the standard carpaine.

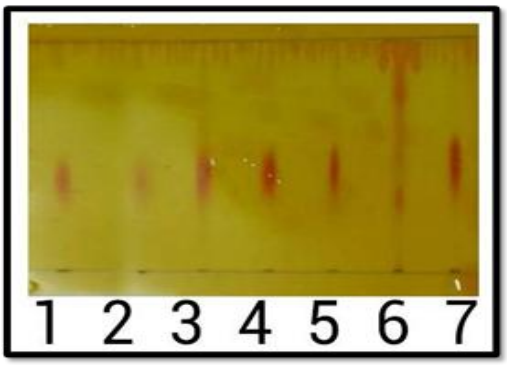


Figure 4.5: Comparative TLC (1-Standard, 2-Seeds, 3-root, 4-stem, 5-leaves, 6-fruit, 7- petioles)

Table 4.3: Rf values of different plant parts of *Carica papaya*

Sl. No.	Plant parts	Rf Values
1	Standard	0.45
2	Seeds	0.45
3	Root	0.45
4	Stem	0.45
5	Leaves	0.47
6	Fruit	0.35
7	Petioles	0.50

The melting temperatures of carpaine obtained from various parts are tabulated below in Table 4.3 having melting point 117-120 $^{\circ}$C.

Table 4.4: Melting points of various parts of plants

Sl. No.	Plant parts	Temperature (◦C)
1	Seeds	119
2	Root	118
3	Stem	120
4	Leaves	119
5	Fruit	117
6	Petioles	119

IR spectrum of carpaine isolated from *Carica papaya* leaves [Figure 4.6] exhibited absorptions for secondary nitrogen at 3323 cm^{-1} (m, NH), 2925cm^{-1} (s, CH), 2850 cm^{-1} (s, CH) and ester carbonyl at 1712 cm^{-1} (w, CO), 1465 cm^{-1} (s, CH_2), 1375 cm^{-1} (s, CH_3), 1100 cm^{-1} (w, C-O).

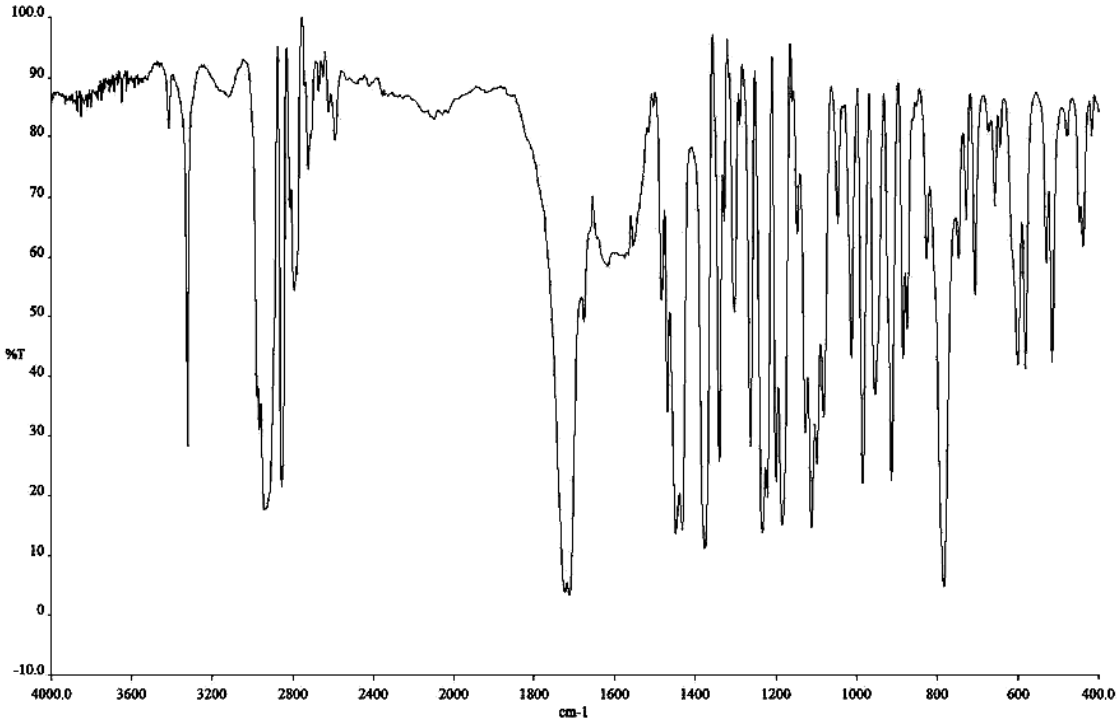


Figure 4.6: IR spectra of carpaine extracted from *Carica papaya* leaves.

The ^{1}H-NMR spectrum indicated clearly the presence of plane of symmetry which shows two monomer of carpamic acid combined together forming cyclic structure by ester linkage.

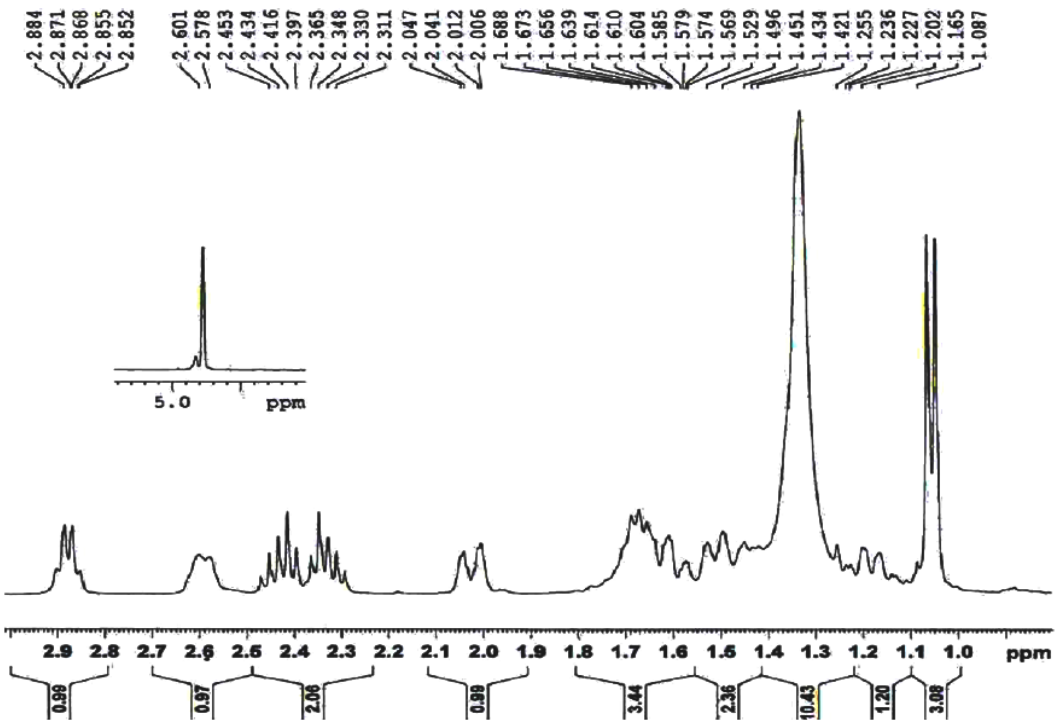


Figure 4.7: ^{1}H NMR spectra of carpaine

1.4–1.34 (9H, m, 5-, 7-, 8-, 9-, 10-, 11-H), 1.24-1.70 (2H, m, 7-, 8-H), 1.17–1.07 (3H, m, 7-, 8-, 15- H), 1.57-1.45 (2H, m, 5-,7-, 8-, 9- H), 1.71-1.61 (3H, m, 4-, 12- H), 2.05–2.01 (1H, dd, 4-H, J= 16Hz), 2.37-2.30 (1H, m, 13- H), 2.48-2.4 (1H, m, 6- H), 2.60-2.59 (1H, br, d, 2-H, J= 4Hz), 2.90-2.87 (1H,dd, 3-H, J= 12Hz),4.78 (1H, s, 1-H).

The^{13}C NMR and DEPT spectra Figure 4.8 and Figure 4.9 displayed signals for 14 carbons.

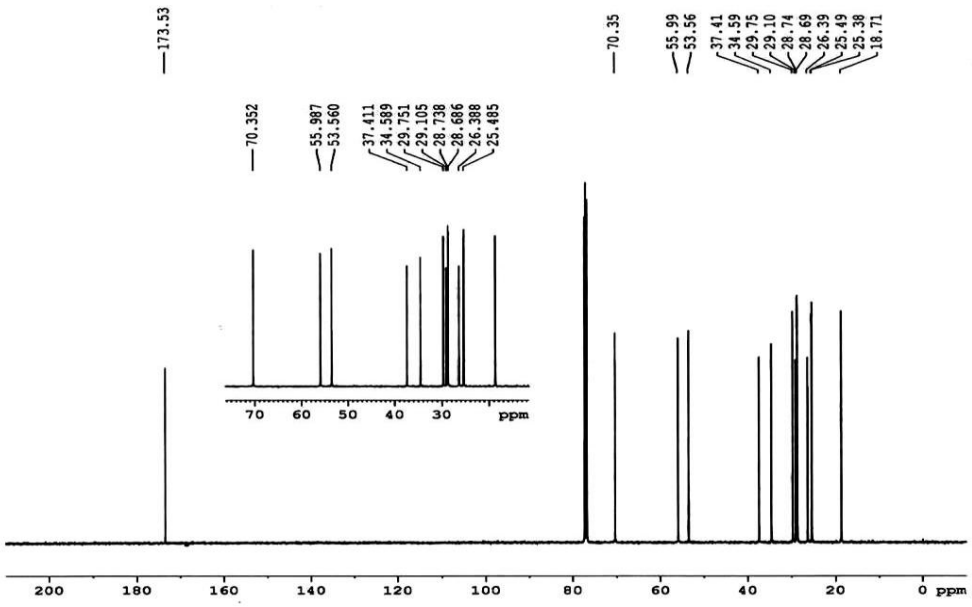


Figure 4.8: ^{13}C NMR spectra of carpaine

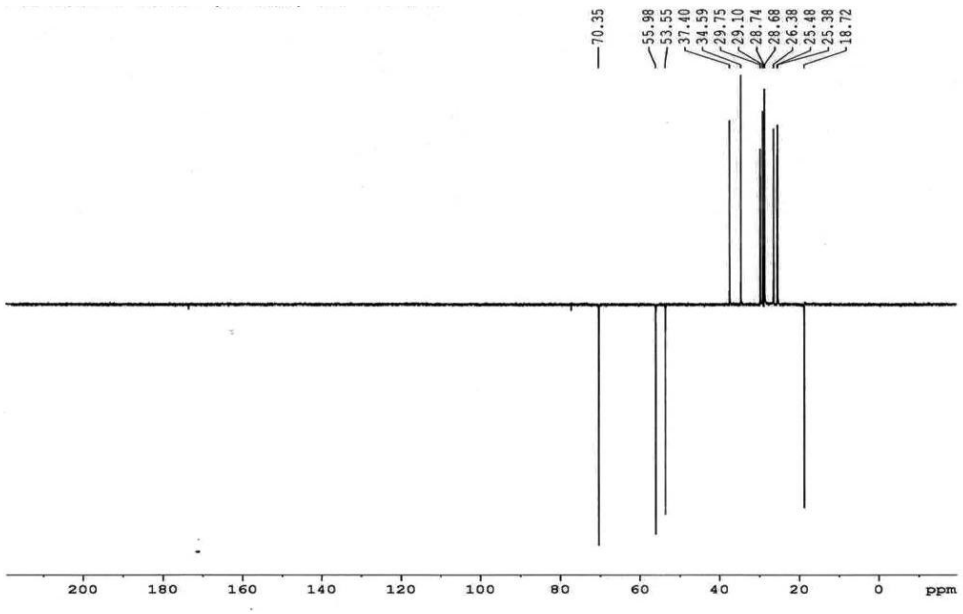


Figure 4.9: DEPT NMR spectra of carpaine

Due to plane of symmetry all 28 carbons were divided into 14 pairs of carbons, one pair of methyl groups (δC 18.71 ppm), 9 pairs of methylenes (δC 37.40, 34.59, 29.75, 29.10, 28.74, 28.68, 26.38, 25.48, 25.38 ppm), three pair of methines (δC 70.35, 55.99, 53.56 ppm), one pair of carbonyl group (δC 174.53ppm).

Crystals suitable for X-ray crystallographic analysis were obtained from slow evaporation of hexane solution of carpaine isolated from *Carica papaya* leaves. The final X-ray crystallographic diffraction analysis of revealed Crystal data: $C_{28}H_{50}N_2O_4$, M=478.70 gm, of dimensions 0.25×0.18×0.13 mm with orthorhombic system. Its structure and relative stereochemistry, directly supported the structural assignment of carpaine space group P212121, a=5.4712(2)Å, b=14.4801(4)Å, c=18.6963(7)Å, V=1481.19(9)Å3, Z=2, d=1.073 g/cm^3. The total number of independent reflections measured were 2416, of which 2137 observed [$|F|^2 \geq 8\sigma([|F|^2)$]. The crystal structure was solved by the direct method SHELX-2013 [25].

Carpaine structure is reported (Sato et al) and all data are matching with reported one [26].

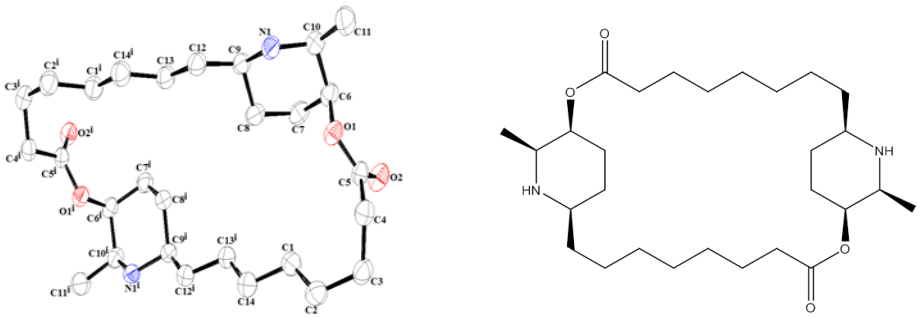


Figure 4.10: ORTEP of carpaine

Table 4.5: Crystal data of carpaine

Parameters	**Carpaine**
Empirical formula	$C_{28}\ H_{50}\ N_2\ O_4$
Formula weight	478.70
Temperature (K)	293(2)
Wavelength (Å)	1.54184
Crystal system	Orthorhombic
Space group	P 21 21 21
a (Å)	5.4712(2)
b (Å)	14.4801(4)
c (Å)	18.6963(7)
α (°)	90.00
β (°)	90.00
γ (°)	90.00
Volume ($Å^3$)	1481.19(9)
Z	2
Density (Mg/m^3)	1.073
Absorption coefficient (μ/mm^{-1})	0.555
F(000)	528
Crystal size (mm)	0.25 x 0.18 x 0.13
Theta range for data collection (°)	3.861 to 72.095
Index ranges	$-5 \leq h \leq 6$, $-17 \leq k \leq 14$, $-22 \leq l \leq 19$
Reflections collected	3607
Independent reflections	2416 [R(int) = 0.0190]
Data / restraints / parameters	2416 / 0 / 163
Goodness-of-fit on F^2	1.075
Final R indices [I>2sigma(I)]	R1 = 0.0487, wR2 = 0.1260
R indices (all data)	R1 = 0.0552, wR2 = 0.1375
Largest diff. peak and hole (eA^{-3})	0.154 and -0.143

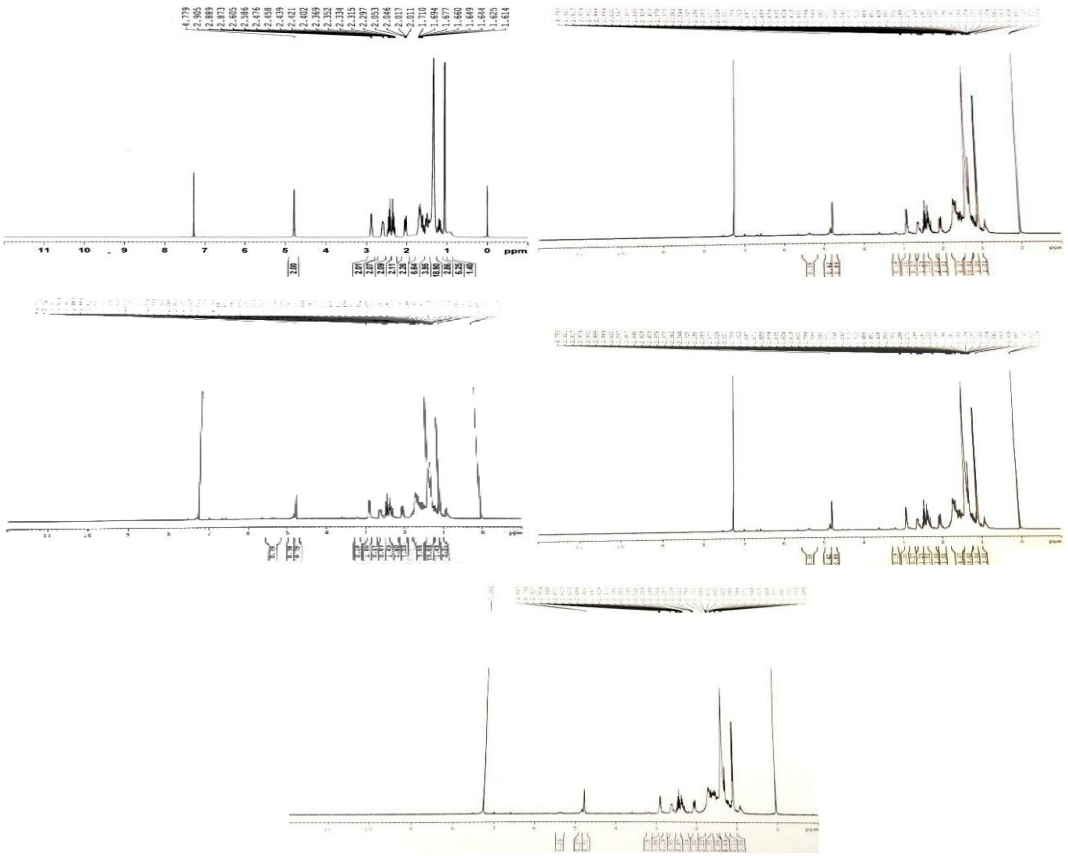


Figure 4.11 [A]: 1H NMR of Standard (Carpaine) **[B]:** 1H NMR of root sample **[C]:** 1H NMR of stem sample **[D]:** 1H NMR of leaves sample **[E]:** 1H NMR of petiole sample

4.4 Quantitation of carpaine in different parts of *Carica papaya*

A method for quantitative determination of carpaine was developed and validated while studying bio guided assay of the various sub fractions of alkaloids in section 3.12. However due to some unavoidable circumstances it was not available for the quantification of carpaine in the various parts of *Carica papaya*. To overcome this difficulty, LCMS-MS method on a different instrument with different internal standard and conditions was developed and validated.

4.4.1 Development and validation of LCMS-MS method for carpaine

4.4.1.1 LCMS/MS protocol

The chromatographic system consisted of Waters Acquity UPLC^{TM} system (Waters, Milford, MA, USA) connected to a hybrid linear ion trap triple-quadrupole mass spectrometer (API 4000 QTRAP™ MS/MS system from AB Sciex, Concord, ON, Canada) via electrospray (Turbo V) ion source. The Waters Acquity UPLCTM system was equipped with a binary solvent manager, sample manager, column oven and photodiode array detector (PAD). The control of LC-MS/MS system, data acquisition and processing was done by Analyst software (version 1.5.1, AB Sciex). LC separation was performed on CSH C18 column (100 mm × 2.1 mm id, 1.7µm) maintained at 25 ˚C. The mobile phase consisted of 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B). Gradient elution was performed as follows: 0-0.8 min, 25-30% B; 0.8-1.0 min, 25-30% B; 1.0-2.0 min, 30-90% B, 2.0-2.4 min, 90-90% B; 2.4-2.7 min, 90-20% B and finally, the initial conditions was held for 0.3 min for re-equilibration. The flow rate was kept at 0.25 mL/min throughout the analysis. The sample injection volume was 1 µL.

The MS instrument was operated in positive electrospray ionization mode with MRM acquisition at the unit resolution for Q1 and Q3. The optimized conditions for the electrospray source were as follows: ion spray voltage, 5500 V; curtain (CUR) gas, 20 psi; nebulizer gas (GS1), 50 psi; heater gas (GS2), 50 psi; ion source temperature, 550°C; collision activated dissociation (CAD) gas and the interface heater was on. Multiple reactions monitoring (MRM) mode was utilized to detect the compounds of interest.

Full scan product ion spectra of carpaine and piperidine as internal standard (IS) were recorded with MRM mode through the transition from precursor ion m/z 479.0 to product ion m/z 240.0.

Linearity was determined by the analysis of standard solutions in the range of 10 to 5000 ng/mL in methanol containing 100µL of 10µL/L piperidine (IS). Calibration curves were constructed by plotting peak area ratio (y) of carpaine to the internal standard, versus their corresponding concentration (x). Linearity was accessed by weighted (1/x), linear regression of calibration curves generated in triplicate on three consecutive days using analyst-internal standard peak ratio. Parameters obtained from the calibration curve were used for calculating carpaine concentration in different parts of the *Carica papaya*.

4.4.1.2 Validation

The method was validated according to the guidelines of International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use [27] and to recommendations of the *United States Pharmacopeia* (2009).
The LODs and LOQs were calculated based on S/N (signal to noise ratio) of the compound at which S/Ns were detected as 3:1 and 10:1, respectively.

4.4.1.3 Statistical analysis

Values are expressed as mean ± SEM and data were analyzed using one-way ANOVA followed by the Dunnett's Multiple Comparison Test using GraphPad Prism (version 5; Graph- Pad Software Inc., San Diego, CA, USA). The significance level was set at $p < 0.05$.

4.4.2 Results and Discussion

For quantitation studies, two mass transitions for carpaine were used (i.e. qualifier and quantifier) and one for IS resulted in well-shaped peaks at 0.60 min and 0.80 min with good sensitivity (Figure 4.12).

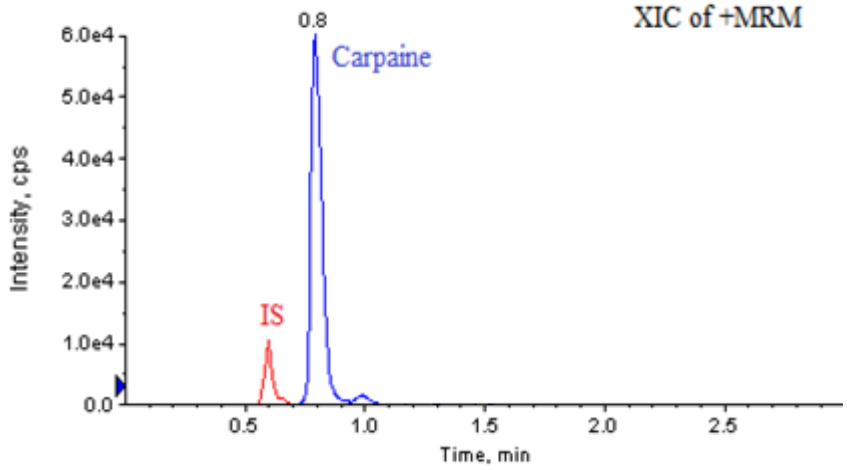


Figure 4.12: XIC of Carpaine along with piperidine as an internal standard (IS)

The calibration curve was plotted for 479 m/z value corresponding to the parent ion.

LOD and LOQ were determined based on the MS response of a serial dilution of standard solution. LOD and LOQ were 0.09 ng/ mL (S/N ≥ 3) and 0.25 ng/mL (S/N ≥ 10), respectively (Table 4.6). Good linearity was observed over the concentration range 10 to 5000 ng/mL for carpaine.

Table 4.6: Regression data, LOD, LOQ for carpaine

m/z	component	Regression	r^2	LOD(ng/mL)	LOQ(ng/mL)
479	Carpaine	y=22700x-23.8	0.9991	0.09	0.25

The intraday (n=5) and inter day (n=9) analysis precision RSD% for intraday was 1.35 and precision RSD% for interday was 2.09. The accuracy (recovery) (n=3) was 89.1. All validation parameters reflect the good precision and accuracy of the method for quantification of carpaine.

The quantitation results showed the presence of carpaine in all the parts of papaya plant and among them, leaves contained highest amount of carpaine followed by fruit. Comparison of carpaine content between different age group of papaya leaves was also done i.e. tendril leaves (≤ 1 week), 2-3 weeks leaves and matures leaves (> 3

week). Mature leaves contained highest amount of carpaine as compared to other age group of leaves.

Table 4.7: Quantity of carpaine present in different parts of papaya plant

Papaya parts	**Content (mg/g)**
Leaves (mature)	9.30
Fruit pulp	4.90
Fruit Peel	1.99
Roots	1.86
Stem	1.04
Petioles	0.72
Seeds	0.65
Tendril leaves	0.76
2-3 weeks older leaves	0.57

Previous reports suggested the presence of carpaine in leaves, petioles and fruits only whereas from LCMS-MS data it is found that almost all parts of papaya plant contains carpaine [11], [13], [16], [17], [19], [21], [22]. Julianti et al collected older and younger papaya leaves samples from different parts of western Java, Indonesia to study the carpaine composition and tried to correlate amount with age of the leaves using LCMS technique [21]. However, they couldn't observe an apparent relationship between age and alkaloid content. Similarly in our case, there is no clear change in carpaine content was observed as the age of leaves varies from tendril to mature one.

4.5 Conclusion

Modified method gives good yield in short time as compared to other traditional methods.

A new LCMS-MS method was developed and validated for the detection and quantification of carpaine from all parts of *Carica papaya* using piperidine as internal standard. This method was found to be fast, simple and sensitive with good precision and accuracy which can be reproducible with controlled parameters [28].

Qualitative and quantitative analysis of carpaine in different parts of *Carica papaya* was done by TLC followed by derivatization, ^{1}H-NMR and LC-MS-MS method respectively. The respective analysis confirmed that all the parts of papaya plant contains alkaloids i.e. carpaine.

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