5. Chapter 5: Extraction and isolation of Anacardic Acid

5.1 Extraction of Cashew Nut Shell Liquid (CNSL), and group isolation of Anacardic Acid

The CNSL was extracted from the raw cashew nut shells using soxhlet extraction, as the CNSL contains three major components viz. Cardol, Cardanol, and Anacardic Acid, therefore the compound of our interest i.e Anacardic Acid was further isolated from the mixture using an acid-base reaction technique involving several steps. Furthermore, $Ana_{C15:3}$ and $Ana_{C15:0}$ were isolated among all Anacardic Acid subtypes using column chromatography. The various steps involved in the extraction and isolation process are described in this section.

5.1.1 Extraction of CNSL

CNSL was extracted from cashew nuts shells of *Anacardium occidentale L*. by solvent extraction method as described by J.H.P. Tyman et al. 1989 (Tyman, Johnson et al. 1989). Briefly, intact cashew nut shells were frozen for 72 h and hammered to have the final size in the range of 2 to 4 mm. These were loaded into a thimble of soxhlet extractor, solvent extraction preceded with light petroleum ether (boiling range 40-60°C). Extraction was allowed for 24 h, followed by concentration of the CNSL by vacuum evaporation.

5.1.2 Isolation of Anacardic Acid from CNSL

Group isolation was performed by acid-base reaction principle using calcium hydroxide as optimized and reported by Paramashivappa et al. 2001 (Paramashivappa, Kumar et al. 2001) followed by identification and quantification using HPLC method. Briefly;

1. 100 g CNSL was dissolved in 600 mL of 5% aqueous methanol solution.
2. $Ca(OH)_2$ (50 g) was added slowly while stirring followed by incubation for 3 h at 50°C under gentle stirring. Calcium anacardate was precipitated in this step.
3. Calcium anacardate precipitate was filtered followed by washing with 200 mL

methanol and dried under vacuum at 45-50°C for 2 h.

4. Calcium anacardate was further suspended in 440 mL water followed by the addition of 60 mL of concentrated HCl under stirring.
5. Twice extraction of the resultant solution with ethyl acetate (150 mL each time) yielded Anacardic acid in the upper organic phase.
6. The organic layer was further washed with distilled water followed by drying over anhydrous sodium sulfate and concentration under reduced pressure.
7. Identification and quantification by using HPLC.

5.1.3 Isolation of C15:3 and C15:0 among the Anacardic Acid subtypes

Activated RP silica C18 was used for column packing. The silica was activated overnight at 100°C and packed in a column (height to diameter ratio; 8:1). The column was filled with methanol and passed through the column bed to clean before use. The sample was placed on the top end of the column and methanol was passed through the bed. Ten fractions were collected and tested using TLC analysis. Further, fractions containing more than one component were subjected to further column chromatography. The process was replicated until the pure compounds were obtained. The purity of the isolated compounds was further confirmed by using HPLC while the identity was assured using FT-IR and ^{1}H NMR (Yuliana, Nguyen-Thi et al. 2014).

5.2 HPLC analysis

The HPLC analysis was performed by using HPLC (Waters, Singapore) equipped with 515 HPLC Pump connected with 2998 Variable Wavelength detector (Photodiode Array Detector). The data acquisition was performed by Empower software. The chromatographic separation was performed by using a spherisorb C 18 column (250 mm x 4.6 mm, packed with 5 microns), considering as stationary phase. The mobile phase consisted of Acetonitrile: Water: Trifluoroacetic acid (90:10:0.1), with a flow rate of 1

mL/min. and the eluent was monitored at a wavelength of 312 nm. The column temperature was maintained at 25°C temperature and injection volume 10 µL was used while the total run time was 20 min. The mobile phase was filtered through 0.45 µm nylon membrane (Millipore) prior to use (Oiram Filho, Alcântra et al. 2017).

5.3 Identification of the compounds using FT-IR and ^{1}H NMR

5.3.1 FT-IR

The FT-IR spectra of the isolated components ($C_{15:3}$ and $C_{15:0}$) were obtained using FT-IR, Bruker Alpha II FTIR Spectrometer (Bruker Corporation, Germany). The samples were scanned at a spectral range of 500–4000 cm^{-1} with a resolution of 2 cm^{-1} and 128 scans for each run. The samples were first diluted with KBr powder and compressed to form pellets for performing the measurements (Hamad and Mubofu 2015).

5.3.2 ^{1}H NMR

Proton (^{1}H) NMR spectra of the separated Anacardic Acid components ($C_{15:3}$ and $C_{15:0}$) were recorded using AVH D 500 AVANCE III HD 500 MHz one bay NMR Spectrometer (Bruker BioSpin International AG, Germany). The test samples were dissolved in dimethyl sulfoxide (DMSO) at 298 ± 0.1 K, and chemical shifts (in ppm) were recorded using tetramethylsilane (TMS) as the internal standard (Philip, Da Cruz Francisco et al. 2008).

5.4 Results and Discussion

Anacardic Acid was isolated from CNSL using soxhlet extraction while Anacardic Acid components ($C_{15:3}$ and $C_{15:0}$) were further isolated employing the acid-base reaction principle using calcium hydroxide. The HPLC analysis was performed for confirmation of the isolated compounds while the identity of the Anacardic Acid components ($C_{15:3}$ and $C_{15:0}$) were confirmed using FT-IR and ^{1}H NMR.

5.4.1 HPLC analysis

HPLC chromatogram of Anacardic Acid and Anacardic Acid components ($C_{15:3}$ and $C_{15:0}$) is depicted in Figure 5.1, which clearly indicates four different peaks of Anacardic Acid. The retention time of the peaks were observed at 2.665, 3.257, 4.353, and 5.342 min while the HPLC chromatograms of Anacardic Acid components, $C_{15:3}$ and $C_{15:0}$ indicated peaks at 2.615 and 5.327 respectively, indicating corresponding RT values similar to the Anacardic Acid. In a similar study by Philip et al. 2008, Anacardic Acid was isolated using supercritical carbon dioxide and the HPLC data indicated HPLC peaks in line with our findings. Four different peaks were reported at RT values 2.83, 3.93, 5.49, 6.75, and 9.33 min (Oiram Filho, Alcântra et al. 2018).

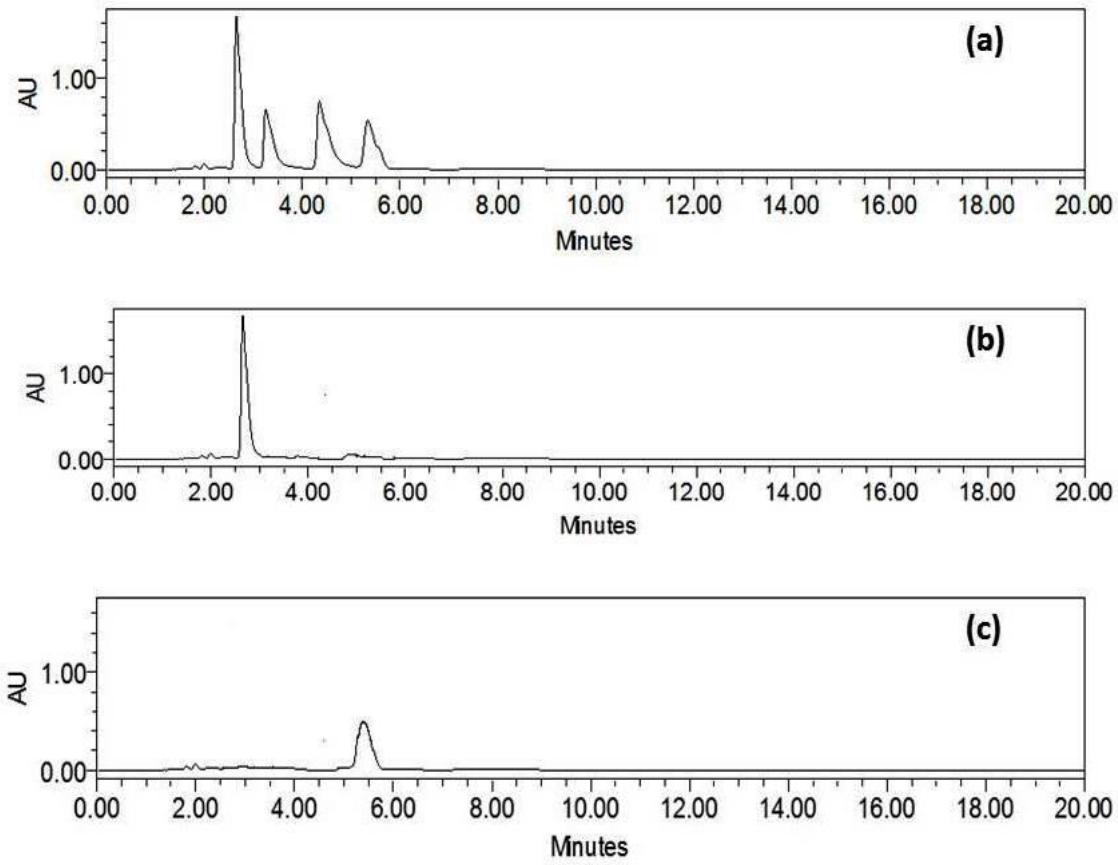

Figure 5.1: Representative HPLC profiles of Anacardic Acid (a), $Ana_{C15:3}$ (b), and $Ana_{C15:0}$ (c).

5.4.2 FT-IR

The FT-IR spectra of Anacardic Acid $C_{15:3}$ ($Ana_{C15:3}$) and Anacardic Acid $C_{15:0}$ ($Ana_{C15:0}$) is demonstrated in Figure 5.2. Both the spectra revealed most of the common peaks at 3420 and 1304 cm^{-1} (Ar =OH), 3400–2400, 1645 cm^{-1} and 1304 cm^{-1}(-COOH), 3009 cm^{-1} (Ar =H and vinyl-H), 1607 cm^{-1} (aliphatic C-C), and 1446 cm^{-1} (aromatic C-C) while Anacardic Acid $C_{15:3}$ showed additional peaks at 2924 and 2849 cm^{-1} (aliphatic C=H). In a study by Philip et al 2008, the isolated Anacardic Acid revealed FT-IR peaks similar to our results (Philip, Da Cruz Francisco et al. 2008).

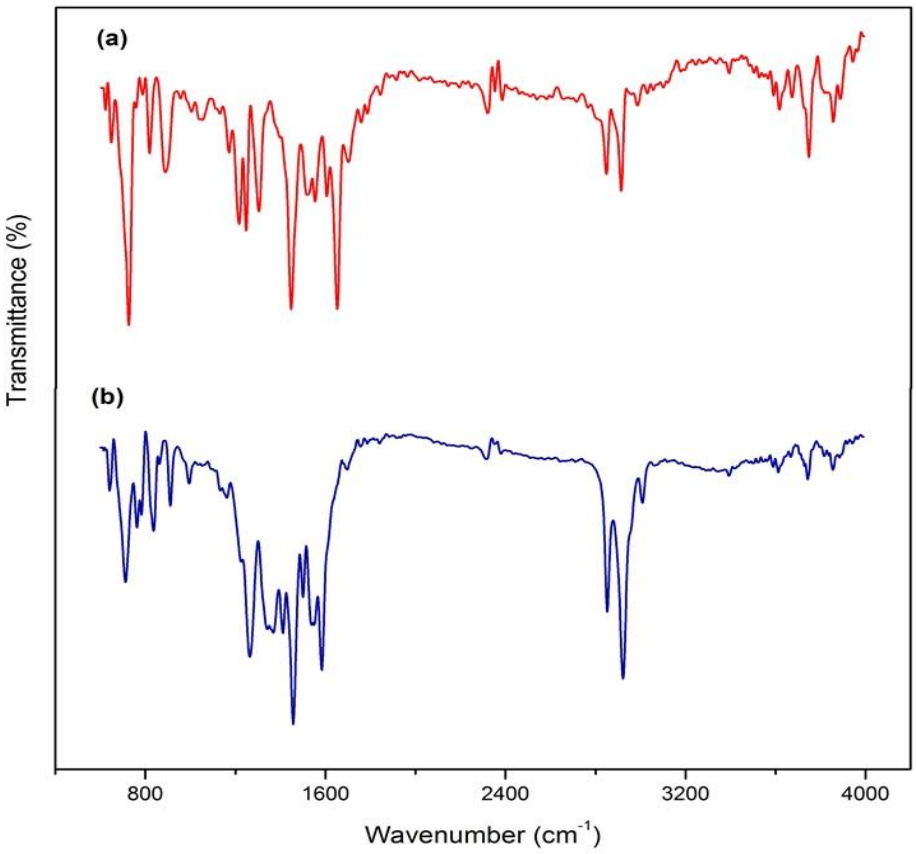

Figure 5.2: Comparative FT-IR spectra of $Ana_{C15:3}$ (a) and $Ana_{C15:0}$ (b).

5.4.3 ^{1}H NMR

The ^{1}H NMR spectra of $Ana_{C15:3}$ depicts aromatic moiety with absorption bands at ~7.012 ppm, ~6.503 ppm, and ~6.428 ppm due to H-4 (t), H-5 (d), and H-3 (d), respectively.

Another absorption band was observed at 15.93, which was due to the proton present in the -COOH group. The hydrogens present in double bonds of the alkyl chain showed absorption at different values of δ. Alkenyl protons showed absorption at 5.829–4.953, while methylene (-CH_2) absorptions were observed at ~3.052, ~2.804, ~2.015, and ~1.484. Aliphatic -CH_2 showed absorption at δ = 1.252. The absence of alkenyl protons at 5.829–4.953 was observed in Figure 5.3, indicating a saturated alkyl side chain corresponding to $Ana_{C15:0}$. Morais et al 2017 isolated Anacardic Acid and reported the ^1H NMR shifts of isolated copound which were found to be in accordance with our findings (Morais, Silva et al. 2017).

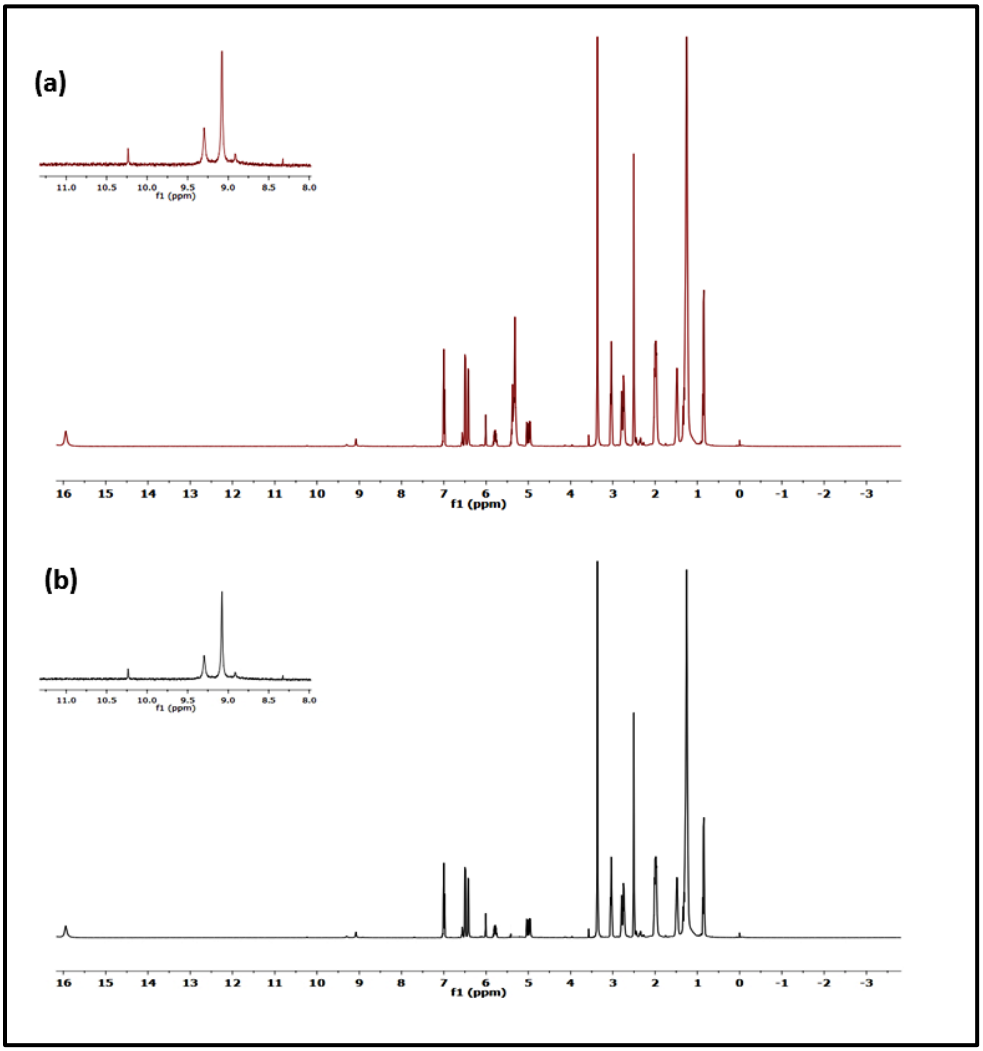

Figure 5.3: ^1H NMR spectra of $Ana_{C15:3}$ (a) and $Ana_{C15:0}$ (b).