CHAPTER IV

5-LOX ASSAY-GUIDED ISOLATION AND IDENTIFICATION OF DAMMARANE TRITERPENOID 1 FROM *BORASSUS FLABELLIFER* SEED COAT

4.1. AIM AND OBJECTIVES

- To perform 5-LOX assay guided fractionation of *Borassus flabellifer* seed coat extract to isolate active principle (dammarane triterpenoid 1) using silica gel column chromatography since it exhibits significant 5-LOX inhibition.

- To elucidate the chemical structure of isolated active principle (dammarane triterpenoid 1) using various advanced spectroscopic techniques including, ^{1}H & ^{13}C NMR, MS and elemental analysis.

4.2. INTRODUCTION

Bioactive fractionation is a separation process based on bioactivity, in which a certain quantity of a mixture of compounds is divided during a phase transition, into individual compounds. In bioactivity-guided fractionation, extraction and separation of components are performed based on differences in their physicochemical properties as well as their biological activity. Fractions are collected using a technique called column chromatography based on differences in a specific property of the individual components.

Several bioactive compounds have been isolated using bioassay-guided fractionation. An acyphloroglucinol, a COX and 5-LOX dual inhibitor, was isolated from *Melicope ptelefolia* according to bio-activity guided fractionation (Shaari et al. 2011). Dammara-20,24-dien-3-one was isolated from the leaves of *Chisocheton polyandrous* using a bioassay-guided fractionation procedure (Chan et al. 2012).

Silica gel is the most commonly used as stationary phase for column chromatography and is a granular and porous form of silicon dioxide made from sodium silicate. Fractionation creates it possible to isolate components from a mixture. This property sets it apart from other separation techniques. Silica gel column chromatography has

wide applications including separation and purification of organic compounds, biomolecules and pharmaceutical components.

Thin Layer Chromatography, a chromatographic technique, is useful for determination of number of compounds in a mixture as well as for identification of compounds. High-performance liquid chromatography (HPLC), an analytical technique, is used to separate, quantify, and identify as well as assess the purity of each component in a mixture. Each component in the sample interacts differently with the adsorbent material (mostly silica), causing different flow rates for the different components for separation of the constituents as they flow out the column. HPLC is a technique commonly used for estimation of purity of component in the sample. Mass spectrometry (MS) is an analytical technique that used to determine the molecular weight and structural characterization of components. MS measures the mass-to-charge ratio of charged particles. LC-MS commonly used analytical technique for organic molecules that are thermally unstable. The separation of components in the sample is based on the difference in affinity of samples with stationary and mobile phases. It has wide applications in pharmaceutical and medical industry (Arpino and Patrick, 1992). It is commonly used in natural product research for identification and structural elucidation of compounds.

Nuclear magnetic resonance (NMR) spectroscopy is one of the versatile techniques for determination of structure of organic compounds. Spin transitions of only hydrogen nuclei are noticed in ^{1}H-NMR spectroscopy. δ value scale of ^{1}H-NMR ranges from 0-10 ppm with respect to Tetra methyl Silane (TMS) as internal standard. ^{13}C-NMR: 13C-NMR spectroscopy shows transitions of ^{13}C nuclei. DEPT is an advance technique to find out the number of hydrogens attached to a carbon atom (Neeraj et al., 2013). Among all available spectrometric methods, NMR is the analytical technique which provides a complete analysis and interpretation of the entire spectrum.

Elemental analysis is a process to analyze elemental composition of compounds. Elemental analysis can be qualitative (type of elements), as well as it can be quantitative (determining how much of an element is present). Molecular formula of compound can determine using elemental analysis. CHNX (C:Carbon; H:Hydrogen; N:Nitrogen and X:hetero elements) analysis is commonly used in structural elucidation of compounds. Melting point of a compound is the temperature at which it

transforms solid state to liquid at atmospheric pressure. It is useful for identification of compounds. According to American chemical society guidelines, ^{1}H & ^{13}C NMR, and mass spectra or elemental analysis data is minimum required for structural elucidation of organic compound.

In this study, 5-LOX assay guided fractionation of *B. flabellifer* seed coat extract was performed using silica gel column chromatographic technique to isolate active compound. Further chemical structure of isolated active compound was determined using advanced spectroscopic techniques including NMR and mass spectra, and elemental analysis.

4.3. MATERIAL AND METHODS

4.3.1. Chemicals

Organic solvents (*n*-hexane, methanol, ethyl acetate) and silica gel **(**100-200 mesh) are purchased from Merck (India). All solvents and chemicals used in experiments were of analytical grade and purchased from local suppliers. All the chemicals and solvents used for chromatography were of HPLC grade.

4.3.2. Collection of plant material

Borassus flabellifer seed coat (tender) was collected from forests of north coastal Andhra Pradesh and identified by plant taxonomists, Department of Botany, Andhra University, Visakhapatnam, A.P., INDIA. A voucher specimen was deposited (deposition no. 22085) at Department of Botany, Andhra University, India.

4.3.3. Extraction

The dried powder of *B. flabellifer* seed coat was dissolved in methanol under reflux for 48 h and filtrated to remove the particulate matter. The solvent, methanol in seed coat extract was evaporated using rotary evaporator to get the residue (4% W/V).

4.3.4. Isolation and Identification of Bioactive Compound from *B. flabellifer* Seed Coat

This seed coat extract residue was fractionated with silica gel column chromatography using *n*-hexane and ethyl acetate solvent system (100:0, 99:1, 98:2, 97:3, 96:4, 96:4, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40). The collected fractions were analysed using thin layer chromatography (TLC) and same retardation factor (R_f) containing same spots were pooled as single fractions and evaluated for their 5-LOX inhibition activity. The bioactive fraction with good 5-LOX inhibition activity was then subjected to silica gel column chromatography with increasing polarity from *n*-hexane to ethyl acetate (100:0, 99:1, 98:2, 97:3, 96:4, 96:4, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35) to obtain further fractions. The collected fractions were analysed using TLC and same R_f value containing fractions were pooled as single fractions and again evaluated for their 5-LOX inhibition activity. Further bioactive fraction was run through HPLC to determine number and purity of the compounds (acetonitrile was mobile phase run with flow rate of 1.5 ml/min and detection at 220 nm). The structure of isolated bioactive compound was assigned based on melting point, ^{1}H & ^{13}C NMR data and elemental analysis (Teresa et al., 1985).

4.3.5. 5-LOX assay

5-LOX assay was performed according to modified UV-kinetic method (Reddanna et al., 1990). The complete method was described in Chapter 3.3.4.2.

4.3.6. Analytical techniques

4.3.6.1. Silica gel column chromatography

Silica gel used for column chromatography was 100-200 mesh size and activated at 110 ^{0}C for 5 hours before use.

4.3.6.2. Thin layer chromatography

Thin layer chromatography was done on commercially available alumina coated with silica gel. H_2SO_4:methanol (10:90) was used for spots development/dtection. UV cabinet (REMI) was used for spots observation.

4.3.6.3. Melting point

Melting point apparatus (Kumar) was used to determine the melting point of the compound

4.3.6.4. Liebermann-Burchard test for terpenoids:

Compound was mixed with 2 ml of acetic anhydride, boiled and cooled. Followed by concentrated H_2SO_4 was then added. Formation of brown ring at the junction of two layers as well as green coloration of the top layer and the formation of deep red color in the bottom layer indicate presence of triterpenoids.

4.3.6.5. HPLC :

HPLC (Shimazhu) analysis (acetonitrile was mobile phase run with flow rate of 1.5 ml/min and detection at 220 nm).

4.3.6.6. NMR :

JEOL –NMRspectrophotometer (^{1}H NMR: 200 MHz; ^{13}C NMR: 50 MHz) was used in the study. Deuterated solvent was used to dissolve the compound.

4.3.6.7. Mass spectrophotometer :

Finigan MAT90 Mass spectrophotometer instrument was used in the study.

4.3.6.8. Elemental analysis specifications :

Carlo Erba Model EA-108 elemental analyzers for the use in CHN elemental analysis.

4.4. RESULTS AND DISCUSSION

4.4.1. 5-LOX Assay Based Isolation of Dammarane Triterpenoid 1 From *B. flabellifer* Seed Coat Extract

As significant 5-LOX inhibitory activity of seed coat extract, first separation of seed coat extract using silica gel column chromatography using *n*-hexane to ethyl acetate solvent systems (100:0, 99:1, 98:2, 97:3, 96:4, 96:4, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40), yielded seven major fractions (F1 (inactive), F2 (IC_{50} > 100 µg/ml), F3 (IC_{50} = 91.21±9.32 µg/ml), F4 (IC_{50} = 7.21±0.75µg/ml), F5(IC_{50} > 100

µg/m), F6 (IC_{50} > 100 µg/ml) and F7(IC_{50} > 100 µg/ml)) (Figure 4.1). F4 fraction showed significant 5-LOX inhibition thus further F4 was fractionated using silica gel column chromatography with increasing polarity from hexane to ethyl acetate (100:0, 99:1, 98:2, 97:3, 96:4, 96:4, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35), to yield 3 fractions, $F4_{(1)}$ (IC_{50} = 63.12 µg/ml), $F4_{(2)}$ (IC_{50} = 4.31±0.51 µg/ml) an $F4_{(3)}$ (IC_{50} > 100 µg/ml). Among three fractions, $F4_{(2)}$ showed predominant 5-LOX inhibition with IC_{50} of 4.31±0.51 µg/ml which is comparable or more that of the positive control NDGA (IC_{50} = 4.5±0.12 µg/ml). Further studies demonstrated that $F4_{(2)}$ also inhibited COX-1&-2 but not inhibited PLA2 activity.

Further bioactive fraction $F4_{(2)}$ was run through HPLC to determine number and purity of the compounds (acetonitrile was mobile phase run with flow rate of 1.5 ml/min and detection at 220 nm) and found single peak with almost 97% purity at 12 min (retention time). HPLC analysis demonstrated that single compound is present in $F4_{(2)}$ (Figure 4.2).

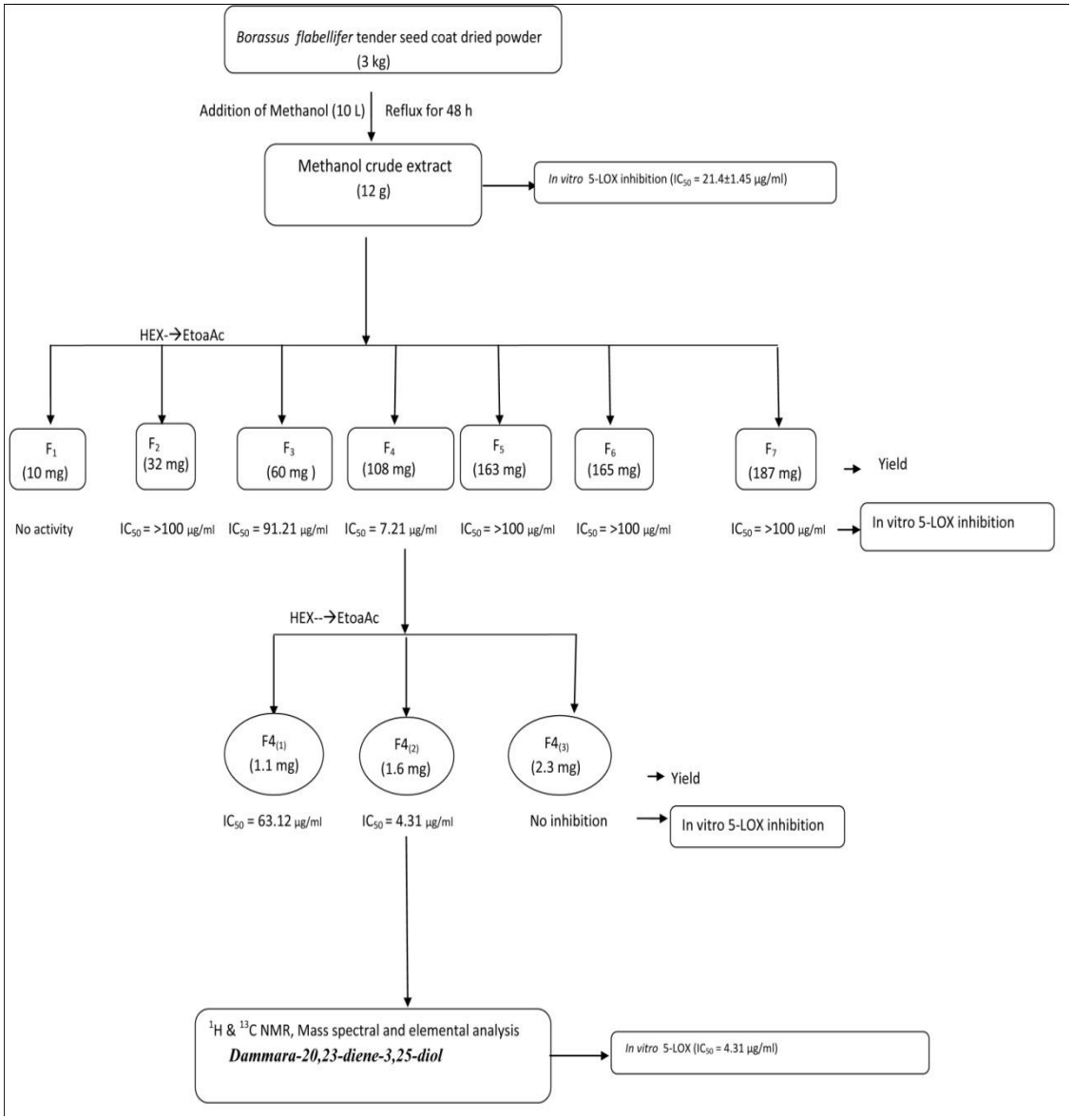

Figure 4.1. 5-LOX assay-guided isolation of dammarane triterpenoid 1 from *Borassus flabellifer* seed coat

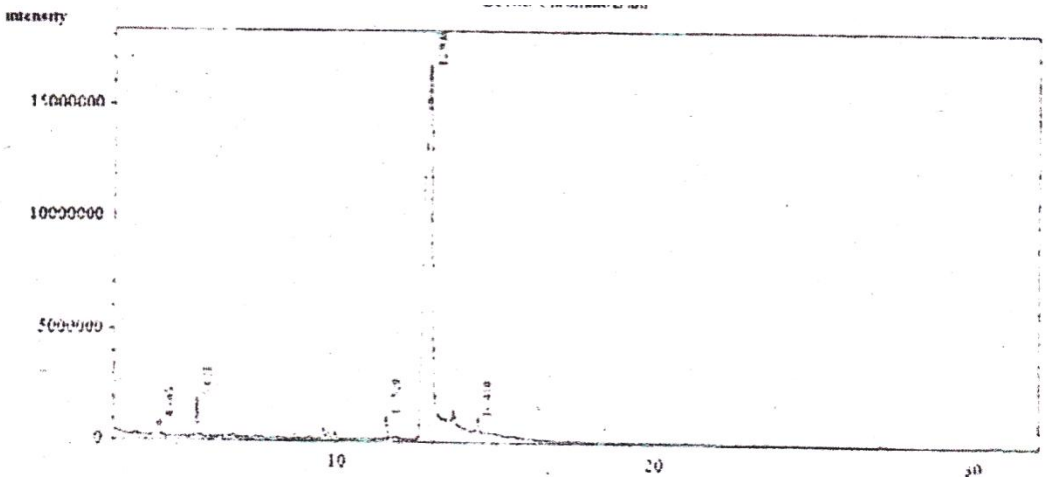

Figure 4.2. HPLC chromatogram of Fraction $4_{(2)}$

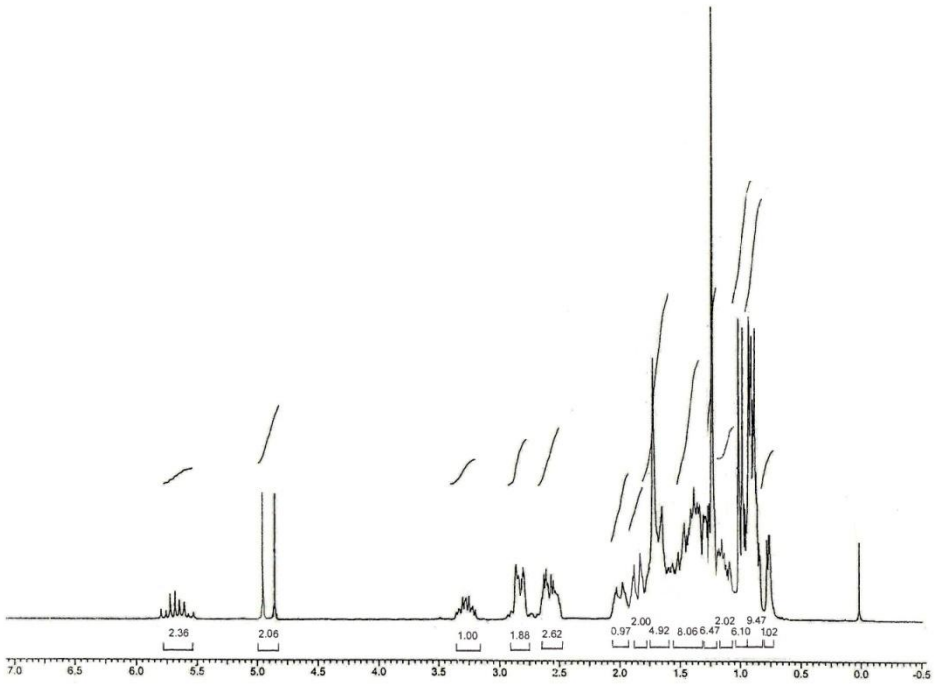

Figure 4.3. ^{1}H NMR spectrum of dammarane triterpenoid 1

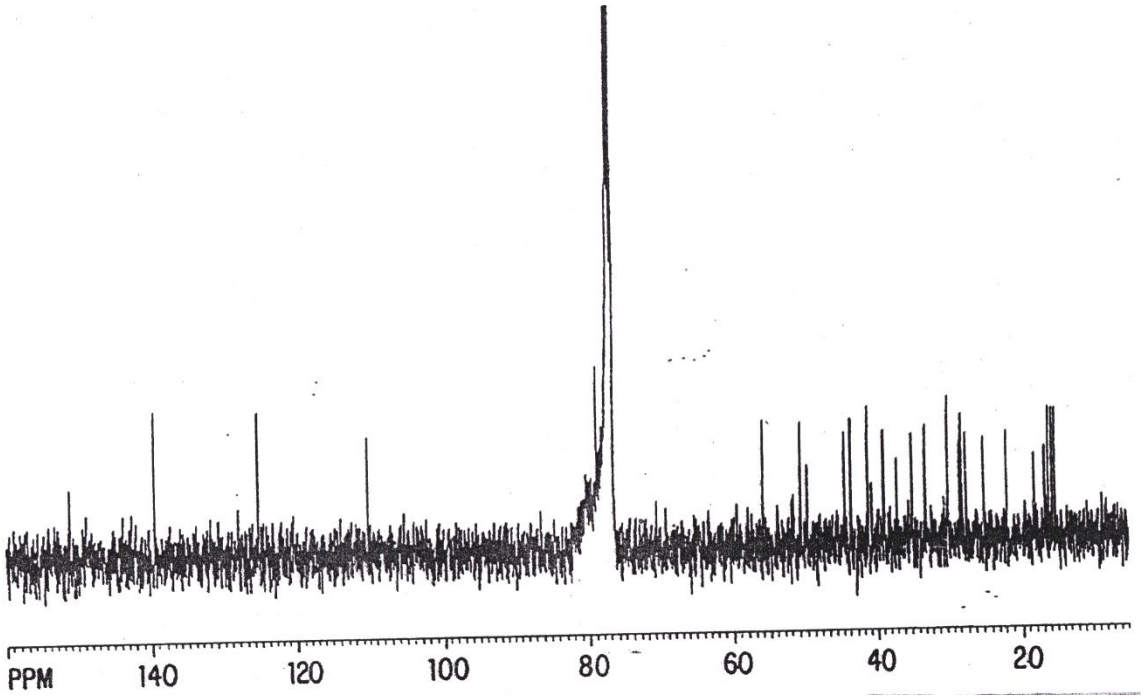

Figure 4.4. ^{13}C NMR spectrum of dammarane triterpenoid 1

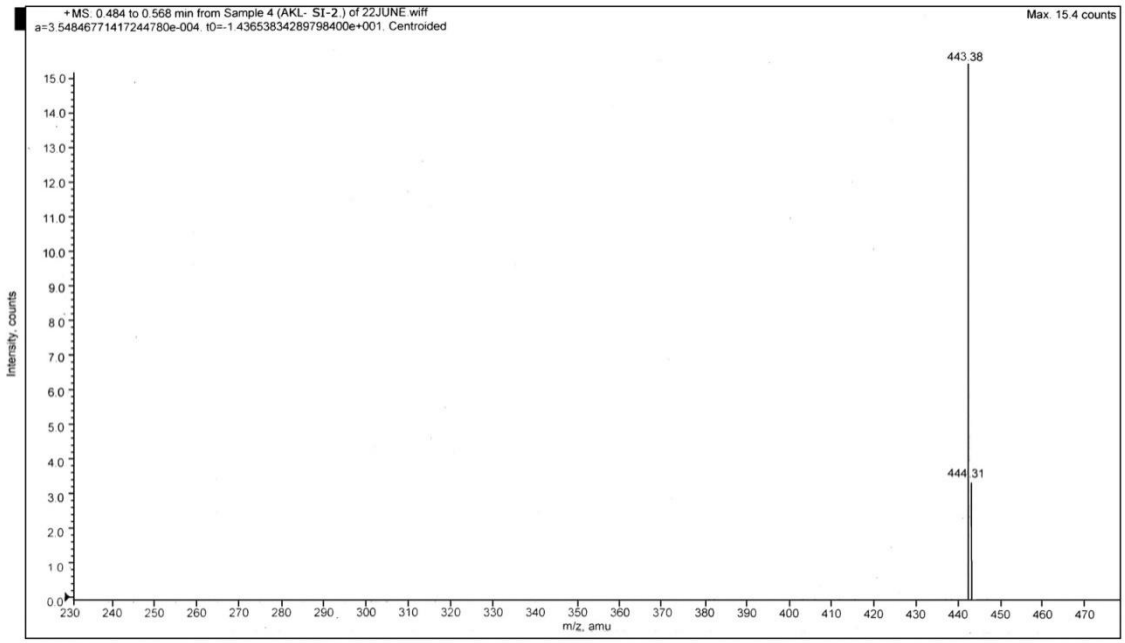

Figure 4.5. Mass spectrum of dammanrane triterpenoid 1

4.4.2. Structural elucidation of dammarane triterpenoid 1 using advance spectroscopic techniques

Dammarane triterpenoid 1 (Dammara-20,23-diene-3,25-diol) (Figure 4.6**)** was the compound present in $F4_{(2)}$, determined based on melting point (92.4 ^{0}C), ^{1}H and ^{13}C NMR data (Table 4.1), and elemental analysis (Anal. Calcd. for $C_{30}H_{50}O_2$: C, 81.39%; H, 11.38%. Found: C, 81.31%, H, 11.42%). Liebermann-Burchard test is also positive. ^{13}C spectra five signals (15.8, 16.2, 16.21, 28.03 & 15.37) indicated to five CH_3-C carbons and 151.3, 110.0 & 125.5, 139.5 signals correspond to alkene carbons. Finally, 29.82, 29.83 signals indicated to CH_3-C carbons adjacent to the OH group and 70.0 signal due to OH group flanked to the quaternary-C atom is evident the dammarane triterpenoid 1 structure (Figure 4.4). Side chain (–C(=CH_2)-CH_2-CH=CH-C(OH)Me_2) of dammarane triterpenoid 1 is identified based as 4.85 (1H, s) and 4.92 (1H, s) protons indicated to CH_2 protons of terminal alkene. 5.62 (bd, 1H) & 5.65 (dt, 1H) protons identified as alkenes protons. 2.68 indicated to methylene (CH_2) protons. Finally, 1.32 six protons belongs to two methyl groups (Figure 4.3). 444 (M+1) mass of dammarane triterpenoid 1 is also evidence for this molecule (Figure 4.5).

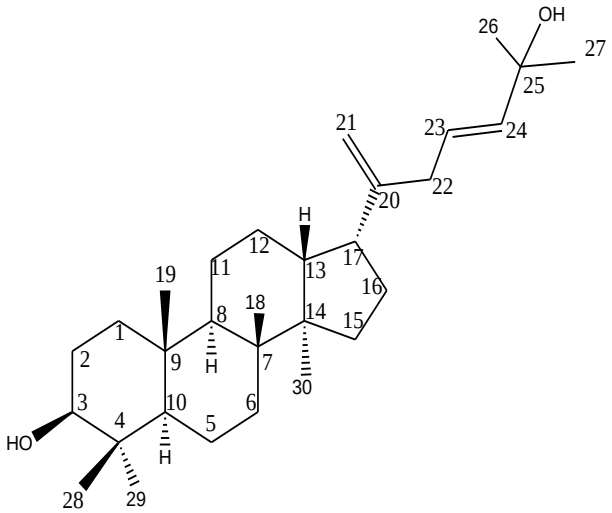

Fig. 4.6. Chemical structure of Dammarane triterpenoid 1 (*Dammara-20,23 diene-3,25-diol*)

Table 4.1. ^{1}H and ^{13}C NMR spectral data of Dammarane triterpenoid 1.

	NMR spectral analyses of dammarane triterpenoid 1	
C/H Position	^{1}H	^{13}C
1	1.25-1.60 (m)	39.1
2	1.25-1.60 (m)	27.4
3	3.20-3.35 (m)	79.0
4	--	38.9
5	0.73-0.76 (bd)	55.8
6	1.25-1.60 (m)	18.3
7	1.25-1.60 (m)	35.0
8	--	40.8
9	1.25-1.60 (m)	51.0
10	--	37.2
11	1.25-1.60 (m)	21.30
12	1.25-1.60 (m)	24.93
13	1.95-2.05(m)	47.61
14	--	49.44
15	1.25-1.60 (m)	31.38
16	1.25-1.60 (m)	28.2
17	2.50-2.58 (m)	43.8
18	0.97 (s)	15.8
19	0.82 (s)	16.2
20	--	151.3
21	4.85,4.95 (s)	110.0
22	2.60-2.65 (bd), 2.78-2.85 (bs)	41.2
23	5.62 (dt)	125.5
24	5.66 (d)	139.5
25	--	70.0
26	1.46 (s)	29.82
27	1.46 (s)	29.83
28	0.79 (s)	16.21
29	0.99 (s)	28.03
30	0.89 (s)	15.37

4.5. CONCLUSIONS

As significant 5-LOX inhibition activity, fractionation of *Borassus flabellifer* seed coat extract was done using silica gel chromatography based 5-LOX inhibition activity. In first separation of seed coat extract using silica gel column chromatography with increasing polarity from hexane to ethyl acetate, yielded seven major fractions (F1-F7). Among obtained fractions, F4 fraction showed significant 5-LOX inhibition thus further fractionated using silica gel column chromatography, to yield 3 fractions ($F4_{(1)}$ - $F4_{(3)}$). Among three fractions, $F4_{(2)}$ showed predominant 5-LOX inhibition. $F4_{(2)}$ also inhibited COX (1 & 2) but not inhibited PLA2 activity. Chemical structure of dammarane triterpenoid 1 (Dammara-20,23-diene-3,25-diol) was the compound present in $F4_{(2)}$, determined based on ^{1}H & ^{13}C NMR spectral and elemental analyses.