3.1 Introduction

This chapter deals with the identification and characterization of bioactive compounds with medicinal significance by using Fourier transform infrared spectroscopy (FTIR), Nuclear magnetic resonance spectroscopy (NMR), 2D-NMR (COESY, NOSY, DEPT), Liquid chromatography mass spectrometry (LCMS), High resolution mass spectrometry (HRMS).

3.2 Methodology:

Plant extract was purified by column chromatography using silica gel (60-120 mesh) as a stationary phase and ethyl acetate: pet ether mixture as a mobile phase with gradual increase in polarity from hexane to 4% ethyl acetate: pet ether mixture. By increasing the polarity of solvents, fractions were collected and fractions having same R\textsubscript{f} value (by checking TLC) are combined and concentrated to get pure compound. The analysis of the sample was done using FTIR (recorded on JASCO FT/IR-5300- sample was prepared by dissolving 1 mg of compound in 10 micro liters of the dichloro methane (DCM), \textsuperscript{1}H, \textsuperscript{13}C NMR recorded on Bruker 400MHz NMR spectrometer (in CDCL3 with TMS as internal reference), DEPT, COSY and NOSY were recorded on Bruker 500 MHZ NMR spectrometer, LCMS were recorded on VG7070H mass spectrometer using EI technique or Shimadzu-LCMS-2010.

LC-MS data were obtained using electrospray ionization (positive mode) on a C-18 column at a flow rate 0.2 mL/min using MeOH/water (90:10) as eluent. Further, for accurate molecular weight identification high resolution mass spectrophotometer
Chemical Characterization

(HRMS) technique was used. For this, sample was dissolved in methanol and submitted to the analysis.

3.3 RESULTS:

The functional groups of the purified natural product were identified using standard FT-IR values. The reference values ~ 1697 and 1751 cm\(^{-1}\) corresponds to carbonyl groups and peak around 1637 cm\(^{-1}\) corresponds to alkene functionality. From this reference values, the purified compound is having keto, aldehyde and alkenic functional groups as shown in below figure 3.1.

![Fourier Transform Infrared spectra.](image)

**Figure. 3.1: Fourier Transform Infrared spectra.**
\(^1\)H-NMR spectrum of a more purified sample has displayed chemical shifts in the aliphatic region (0.5-2 ppm), a deshielded resonance at 2.8 ppm, and an olefinic resonance between 5-6 ppm. There were no aromatic resonances in the spectrum as there is no peak in the aromatic region (7-8 ppm). This spectrum clearly showing that there is a presence of aldehyde functionality by a peak at 10 ppm as shown in figure 3.2.

![Figure 3.2: \(^1\)H NMR spectra.](image)

The \(^{13}\)C spectrum contained resonances primarily in the aliphatic region from 10-40 ppm, but the spectrum also contained resonances indicative of carbons attached
to oxygen or otherwise deshielded by the electronegative environment (~80 ppm), olefinic carbons between 100-150 ppm, and two carbonyl carbons at 170 and 200 ppm. From this we can say that the purified compound is having one carbon attached to electronegative environment, two alkenic carbons and two carbonyl carbons as shown in figure 3.3. To confirm the structure of purified compound further we have carried out the 2D-NMR that is DEPT, COSY and NOESY.

Figure 3.3: $^{13}$C NMR spectra.

The $^{13}$C NMR and DEPT135 spectra revealed that there are two carbonyl carbons in the compound that was analyzed. The proton singlet at 2.8 ppm that integrates to one proton in a deshielded signal that is consistent with a proton near to
oxygen in the molecule. These data suggest that the *Saraca indica* compounds contains at least one ketone moiety. DEPT135 analysis confirmed the 50.1 ppm carbon to be a CH bearing carbon as shown in figure 3.4. Together, the NMR data suggested that the *Saraca indica* compound contains two olefinic carbons, a terminal bond as well as one involving a quaternary center and a ketone moiety, and a carbon bearing a single proton likely located next to the ketone moiety.

Figure 3.4: Distortion less Enhancement of Polarization Transfer spectrum.

More structural detail of the *Saraca indica* metabolite was mined from the aliphatic region of the spectroscopic data. The $^1$H NMR spectrum contains two singlet resonances at 1.1 ppm and 1.2 ppm that integrated to 3 H and 7 H respectively. These
resonances suggested the presence of three methyl group on the compound. The COSY and DEPT135 spectra confirmed that the 1.1 ppm singlet was linked to a CH/CH3 carbon at 24.6 ppm. The 7 H singlet at 1.2 ppm was found to be linked to two CH/CH3 carbons at 22.5 and 32.8 ppm. Because these three methyl groups were all singlets and not split by any other proton signals so, they all appeared to be attached to quaternary centers. Furthermore, the deshielded aliphatic carbon resonance at 32.8 ppm linked to a non-deshielded resonance at 1.2 ppm is characteristic. These resonances are consistent with Vicinal-dimethyl groups attached to a quaternary center.

The 1H NMR spectrum also showed a number of signals with CH2 splitting patterns between 1-2 ppm. Analyzing the DEPT and COSY spectrum, it revealed that in addition to the three methyl carbons on the major component of Saraca indica, there were six additional carbons bearing hydrogens. All of these carbons (18.1, 23.0, 26.9, 29.6, and 40.5 ppm) showed multiple proton interaction suggesting these carbons had at least two attached protons. The DEPT135 confirmed that these six carbon signals were CH2 signals for the Saraca indica compound. The rigid and sharp shapes of the CH2 peaks suggested that they were involved in a ring system.

The many structural details for the Saraca indica compound obtained suggested that the metabolite was likely to be a steroid as shown in figure 3.5. This reasoning was based on the aliphatic nature of the compound, the non-aromatic ring system, the olefinic bonds, methyl groups as well as the carbonyl and hydroxyl/keto moieties that are all common components of steroid compounds.
Further analysis of the COSY data revealed that another olefinic proton at 6.8 ppm is not coupled with another olefinic proton suggesting this olefinic bond must contain a quaternary carbon as shown in figure 3.5. An olefinic bond containing a quaternary center is further supported by the presence of a quaternary carbon in the olefinic region of 100-150 ppm in the DEPT135 (Pretsch et al., 2000).

Identification of individual Steroid components was made based on the mass spectral data obtained for our compound, and by comparison with literature information. The major components of the Saraca Indica (purified compound)
Chemical Characterization

retained the common Steroid core. Fragment peak at m/z 329 corresponding to the [M-1] as shown in figure 3.6.

Figure 3.6: Liquid chromatography mass spectrometry spectrum.

High resolution mass spectroscopy spectrum revealing the molecular weight of the purified natural product is 328.0040 as displayed in figure 3.7.
**Figure 3.7: High Resolution Mass Spectroscopy spectrum**
The isolated natural product was identified and its Chemical composition given below

![Chemical Structure]

Chemical Formula: C\textsubscript{21}H\textsubscript{28}O\textsubscript{3}

Exact Mass: 328.2

Molecular Weight: 328.004

\textit{m/z}: 328.20 (100.0%), 329.21 (23.1%), 330.21 (3.2%)

Elemental Analysis: C, 76.79; H, 8.59; O, 14.61

3.4 Discussion:

Based on the above biochemical experiments, it suggests the molecular structure of extracted ketosterol from the bark of \textit{Saraca indica} as elucidated by $^1$H, $^{13}$C and 2D NMR having Steroid ring, two carbonyl groups (carbonyl stretching around 1697 and 1751 cm\textsuperscript{-1} in IR and aldehyde peak at 10 ppm), two alkenic moieties (alkenic stretching around 1637 cm\textsuperscript{-1} and alkenic protons around 5-6 ppm) groups, one carbon attached oxygen (around 3 ppm) and three methyl groups (around 1-2 ppm) similar to steroid moiety shown above. This is further confirmed by the LC-MS and ESI-MS as molecular weight 328 is matching with structure of ketosterol with a molecular formula C\textsubscript{21}H\textsubscript{28}O\textsubscript{3} as proposed from our observations.