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Isolation of Two Isolates from *Abrus precatorius* Methanolic Extract Using Spectrometry

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Abstract

Combination of column chromatography, thin layer chromatography and neutron magnetic resonance spectroscopy can be used for isolation and characterization of phytochemical principles. The study was carried out to further re-assess the credibility and the problems associated with the methods using methanolic extract of *Abrus precatorius* leaf. Column and thin layer chromatography led to separation of two n-hexane isolates, H_1 (yellow with R_f=0.121) suspected to be alkaloid. But the spectra may be flavonoid, quinone, flavanquinone and glycoside and H_2 (bluish-green with R_f=0.608) suspected to be mixtures of aliphatic and aromatic compounds in addition to methanol-d4. Neither of the isolates contained a single compound in sufficient purity for further assessment.

Keywords: Impurity; Spectroscopy; *Abrus precatorius*; Isolate

Introduction

Chromatography is a technique for separating the components of a mixture by selective adsorption or absorption [1]. Various regulatory authorities are emphasizing on the purity and the identification of impurities in active pharmaceutical ingredients. Spectroscopy is one of the methods used to isolate and characterize pharmaceutical principles and impurities [2]. Whereas impurity is any component of the drug product that is not the chemical entity defined as the drug substance or an excipient in the drug product [3]. Sources of impurities are organic, which include decarboxylation and hydrolysis photolytic cleavage, which include enantiomeric inorganic, reagents, ligands, catalysis and heavy metals. Others are in-process production related impurities, which include stereochemistry, solvent, synthetic intermediate, by-products, storage, leachable and extractable related impurities [2,4-11]. Since the large number of compounds under investigation in drug discovery presents a significant analytical challenge for the detection, quantitation, and characterization of the compounds alone, spectroscopy of isolates from methanolic extract of *Abrus precatorius* leaf was carried out with a view to identifying and characterizing the isolates and the likely problems associated with isolation [12].

Materials

Collection of the plant materials

The plant materials (leaves) used for the study were obtained from Ajule in Ofu Local Government Area of Kogi State, Nigeria. The plant was collected between July and August and identified by a botanist in the Department of Biological Science, Usmanu Danfodiyo University, Sokoto where a voucher specimen with voucher number 2005029 has been deposited.

Preparation of methanolic extract

The plant materials (leaves) collected were air dried to a constant weight under an open shade and pulverized with the help of a mortar and pestle to fine powder. Five hundred grammes (500 g) of *Abrus precatorius* leaf powder was dissolved in 1200 ml of methanol, stirred for one hour and filtered with Whatman filter paper no. 1 into measuring cylinder. The filtrate was therefore concentrated at 60oC in a desiccator [13].

Column chromatographic separation of the methanolic extract using different organic solvents

The crude methanolic extract was fractionated with methanol, ethylacetate, chloroform and n-hexane solvents using column and thin layer chromatography. The two isolates (H_1 and H_2) obtained from n-hexane fraction were identified using 1H proton nuclear magnetic resonance spectroscopy [14,15]. Ten gram (10.0 g) of dried crude methanolic extract of *Abrus precatorius* leaf was separated using column chromatographic method. About 200.0 g slurry of activated silica gel (70-230 mesh) was packed to two-third the length of the glass column (150 × 1.5 cm, 1D). The methanol extract was dissolved in 60 ml of methanol-water mixture (1:2) v/v translating to 20 ml of methanol and 40 ml of water and the mixture was introduced into the column. The column was eluted with 1.2 L of n-hexane, 1.0 L of chloroform, 1.2 L of ethylacetate and finally washed with 1.0 L of methanol using the flow rate of 5 cm^3/s. This afforded four solvent fractions: methanol, ethyl acetate, chloroform and n=hexane.

Purification of the n-hexane fraction

Five gram (5.0 g) of the n-hexane fraction was separated using silica gel column chromatograph. The column was eluted with mixtures of n-hexane and chloroform, starting with n-hexane: chloroform (1:2) and finishing with n-hexane: Chloroform (0:5). Thereafter the column was

eluted with chloroform and ethylacetate mixture, starting with chloroform: Ethylacetate (1:3) and finishing with chloroform: ethylacetate (0:7). The column was finally washed with methanol. Fractions of 20 ml each were collected amounting to a total of 60 fractions. Fractions 1-11 were mainly fatty matter and therefore discarded. Fractions 12-22 and fractions 23-30 were pooled together for thin layer chromatographic separation. Fractions 30-60 had very negligible mass and were discarded also.

Thin layer chromatographic separation of the isolates

Trial thin layer chromatography was done using different solvents to ascertain the best solvent combination for the separation, chloroform: methanol (2:1) was discovered to be the best binary solvent. The pooled fractions were re-dissolved in the eluting solvent and were spotted on a pre-coated aluminum TLC plate (20 × 16 cm). The plates were placed in a solvent tank containing the binary solvent and allowed to stand for some times. Two bands were obtained after the separation and their R_f values calculated. Band 1 (yellowish, R_f=0.121) was scraped and re-dissolved in methanol. The mixture was filtered and the filtrate concentrated in-vacuo to obtain isolate 1 (H_1). Also, band 2 (bluish-green, R_f=0.608) was scraped and re-dissolved in the mobile phase. The mixture was filtered and concentrated in-vacuo to obtain isolate 2 (H_2).

Spectroscopic analysis of the isolate

The method of Azogu [14] was adopted to determine both the full and expanded spectra of isolates H_1 and H_2.

Results and Discussion

The yield of crude methanolic extract of *A. precatorius* leaf was 14.0%.

Percentage (%) yield=Weight of extract yielded/ Weight of powder × 100

% yield=70 × 100/500 g

% yield=14.0%

The yield of methanolic extract was 14.0%.

Column chromatographic separations yielded two isolates. The H_1 isolate with R_f=0.121 is yellow in colour and the H_2 isolate with R_f=0.608 is blush-green in colour (Figure 1).

Proton nuclear magnetic resonance spectroscopy of the isolates H_1 and H_2 revealed full spectra as well as expansions of the region containing signals, with somewhat increased intensity for each sample H_1 and H_2 (Figures 2-5). Neither of the samples contains a single compound in sufficient purity for further assessment. The spectra are full of signals apart from larger ones corresponding to the solvent (methanol-d4). The remaining signals correspond to aliphatic and aromatic compounds (Figures 2 and 4). There appears to be a little bit of all the classes of phytochemicals (Figures 2-5). Hence, it would not be possible to solve any structures from these spectra. More thorough purification of the putative compounds is needful.

The retention factor (R_f) of a compound, is a ratio of the distance a compound travels up the TLC plate to the distance the solvent travel. More strongly adsorbed compounds don't travel far up the TLC plate and thus have lower R_f as seen in H_1 isolate (R_f=0.121). Although, more polar solvents for example (methanol: water) can compete with the polar silica so that the components stay dissolved in a more polar solvent than in the less polar solvents. In that case, all the components travel further up the TLC and have higher R_f as seen in H_2 isolate with R_f (0.608). The structure of a molecule (analyte or solvent) is responsible for its intermolecular forces with other molecules, which in turn controls the compound's physical and chemical characteristics. In chromatography, the intermolecular forces between an analyte and the solvent and the analyte and the stationary phase control the analytes migration [16].

For two closely migrating components, optimum resolutions are usually obtained when R_f's of both compounds are between 0.2 and 0.6, for column chromatography, it is best if resolution is optimized where the R_f's of the two closely migrating components are 0.2 and 0.4. One of the most effective methods to improve resolution is to alter the composition of the solvent system so that the components affinities for the mobile phase versus the solid phase are differently changed. Changing the chemical nature of the solvent system, such as changing a hydrogen bonding solvent to a solvent, which cannot hydrogen bond to the analyte, is often the most effective. Another effective way to improve resolution is to decrease the diameter of the analyte spots. This can be achieved by applying smaller spots [17].

The NMR spectrum of an organic compound recorded as a solution (usually 5-10%) sometimes exhibits small peaks arising from the solvent. The solvents normally chosen do not contain hydrogen. Usually, the deuterated solvents contain a small proportion of isotopically isomeric molecules containing hydrogen instead of deuterium, and this will give rise to an additional peak(s) in the proton spectrum [18]. The shift range in Figures 3 and 5 is 1.0-9.0 ppm whereas the shift range in Figures 2 and 5 is between 1.0 and 5.5 ppm. Hydrogen-bonding and proton exchange are concentration dependent. The chemical shifts of hydrogen-bonded protons e.g. O-H or N-H in alcohols or amines can vary greatly (4-5 ppm) depending on concentration. Also chemically labile protons can exchange with each other if the solvent contains a labile deuteron then, because the solvent is usually in excess, it can exchange with the labile protons of the sample, effectively removing them with the sample. The concentration of the sample should be sufficient and 1-10% are common and a volume of about 0.5 ml is required [19]. Despite purification of the samples (H_1 and H_2), the isolates contained oil. The spectra of fixed oils are characterized by four sets of signals the olefinic protons, the four glyceride methylene protons, methylene protons adjacent to the double bonds, and proton of the remaining saturated carbon atoms [20]. Chemical shifts are expressed in units downfield from Tetramethylsilane (TMS), usually at 6 MHz. However, shift values up field of TMS are rarely encountered [15]. TMS is dissolved to the extent of 0.5% in a solution of the sample. The H_1 isolate which was yellow with R_f (0.121) could be flavonoid, glycoside and saponin whereas the H_2 isolate which was bluish-green with R_f (0.608) could be alkaloid. Interpretation of various chromatograms showed that blue colouration revealed phenolic acids and yellow orange revealed flavonoid, yellow colour revealed flavonoid, glycoside and saponin whereas blue colour revealed alkaloid [21,22]. Wide range of resonances for protons is 1-10 ppm and Peaks close to TMS are usually methyl groups [14].

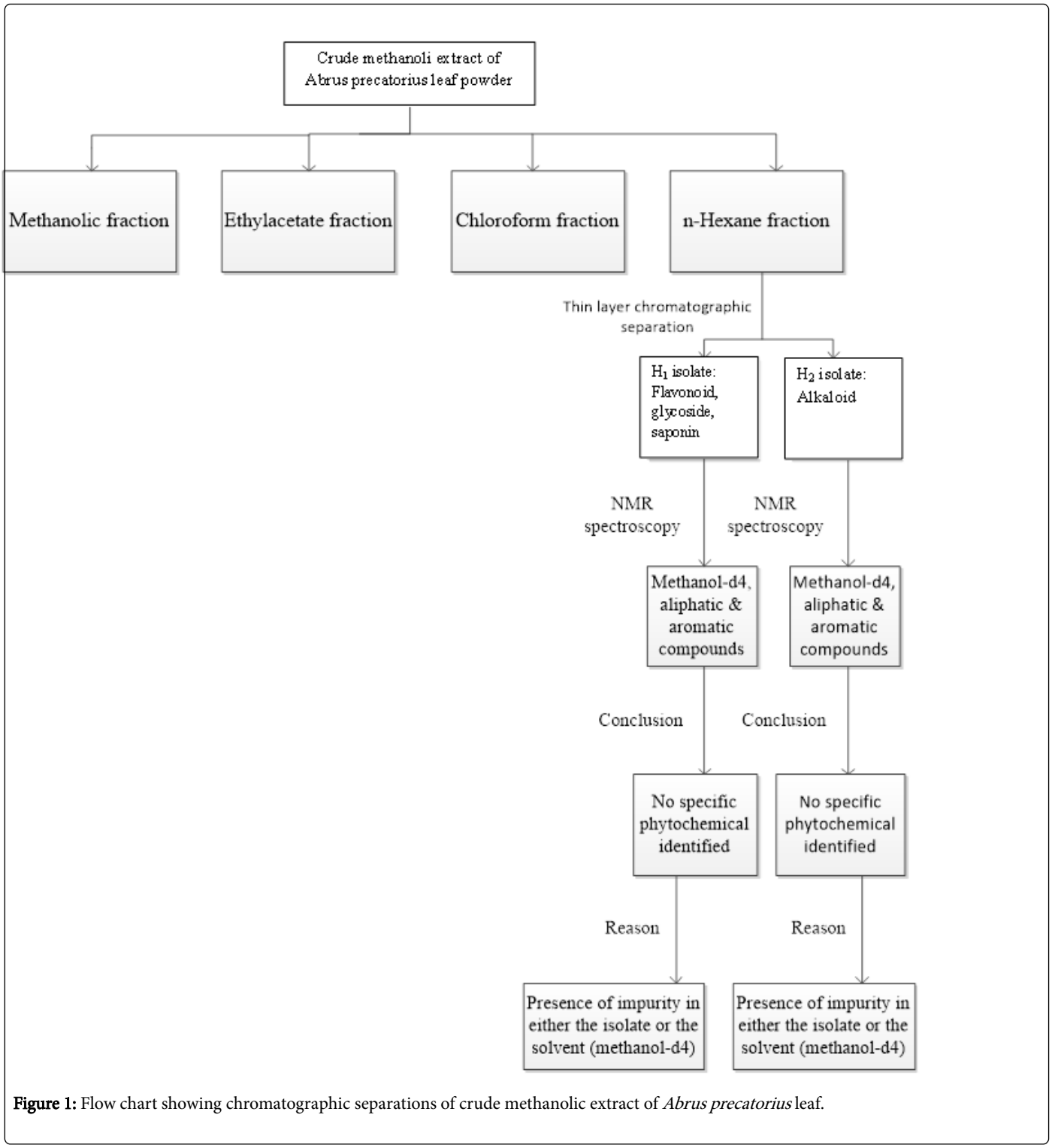

Figure 1: Flow chart showing chromatographic separations of crude methanolic extract of *Abrus precatorius* leaf.

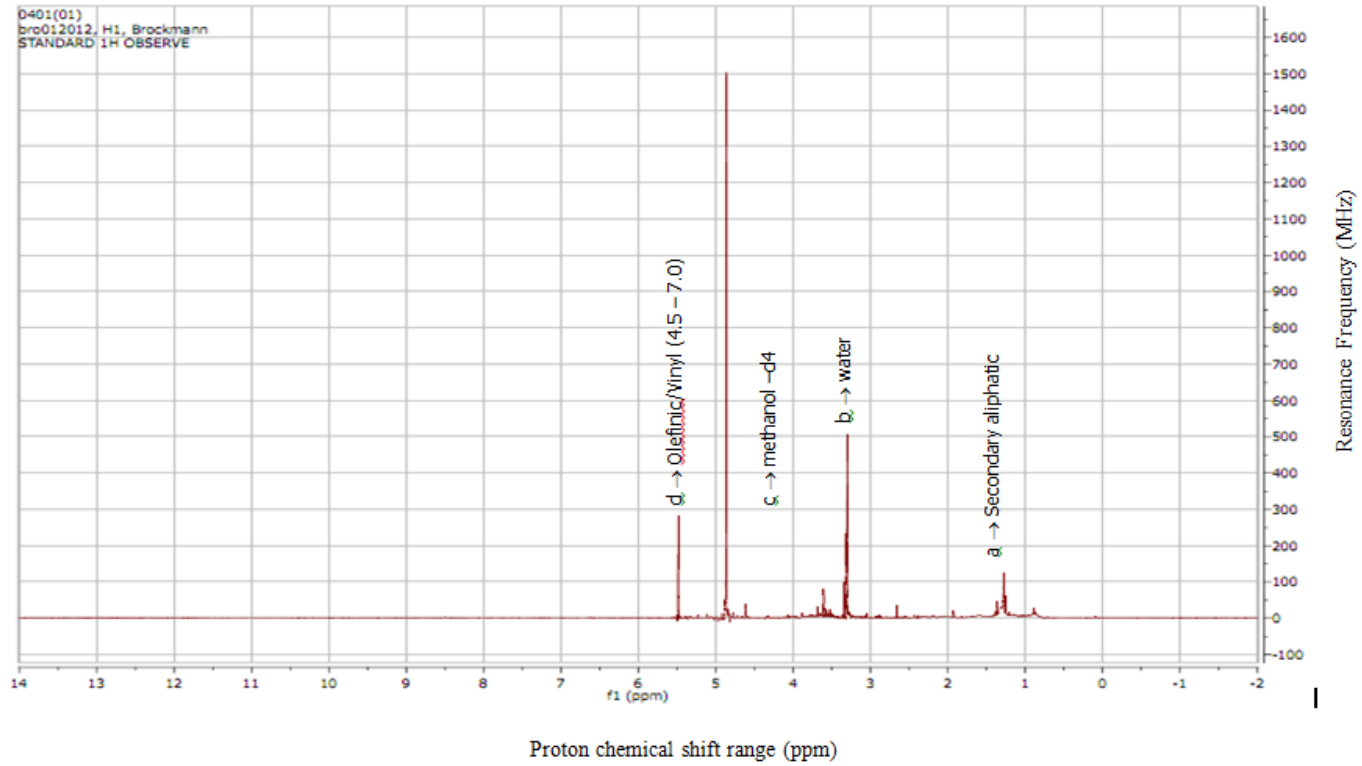

Figure 2: The full spectrum of H_1 isolate.

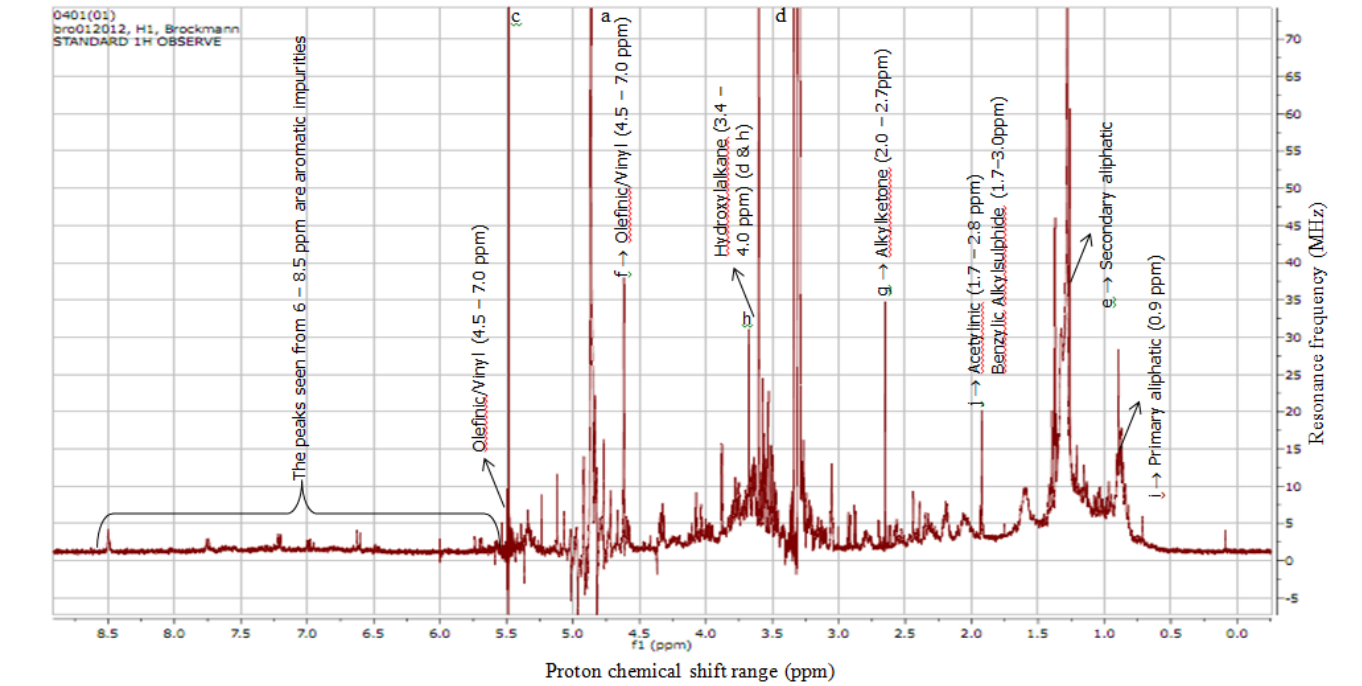

Figure 3: The expanded spectrum of H_1 isolate.

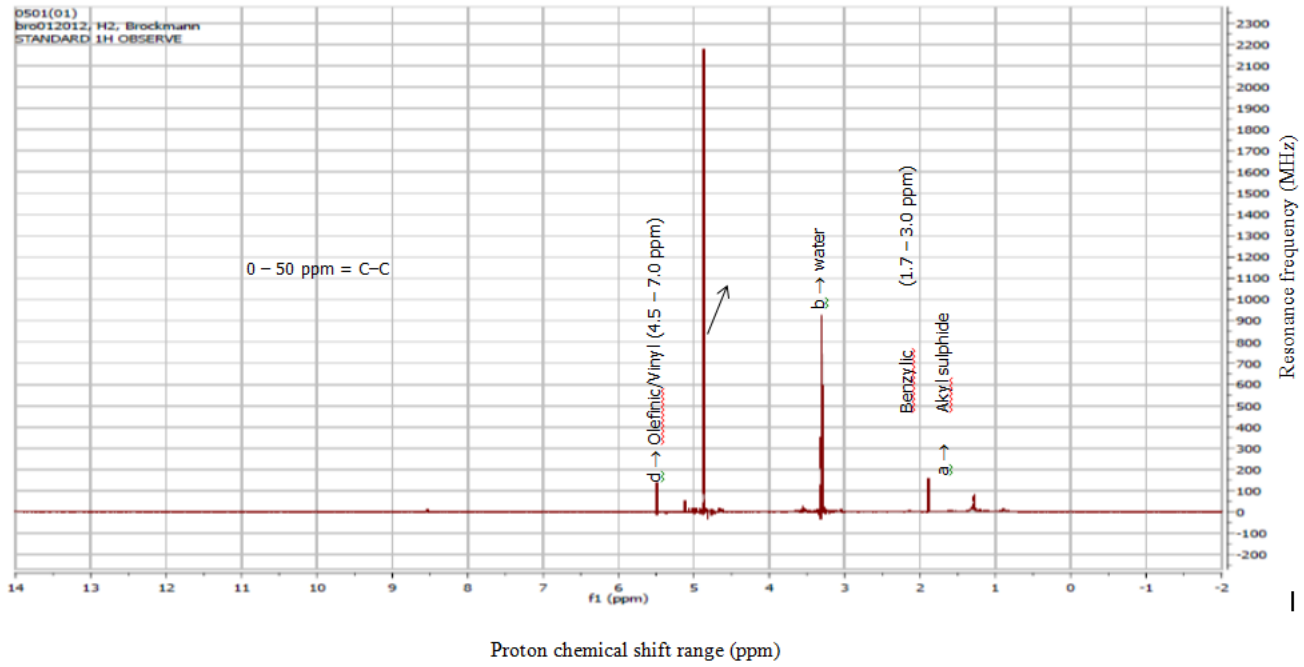

Figure 4: The full spectrum of H_2 isolate.

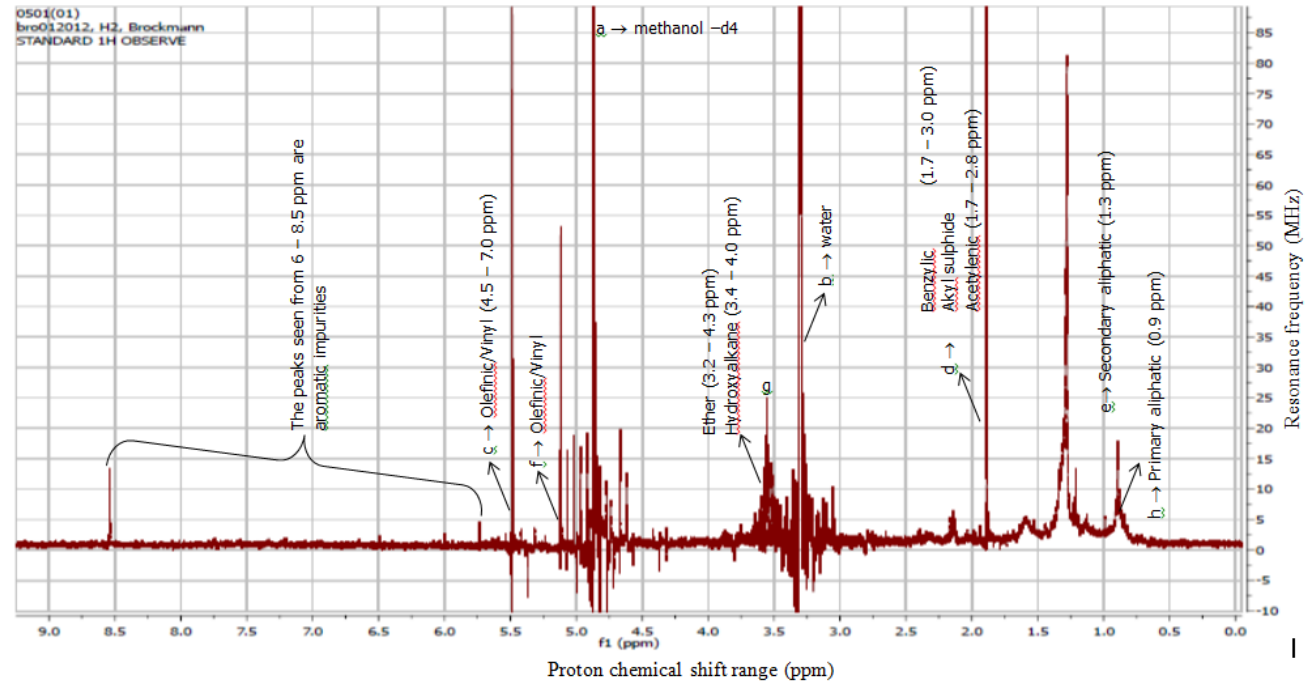

Figure 5: The expanded spectrum of H_2 isolate.

TLC is used to follow the separation and to check for purity. R_f versus elution strength and differences in solvent intermolecular forces (selectively) as well as optimization of resolution (Rs) are important in TLC. Having R_f of 0.121 and 0.608 show that the two isolates are less polar and polar compounds respectively. If TLC shows that the analyte is contaminated, either re-extraction or re-crystallization will be necessary before spectroscopic analysis. TLC in phytochemistry is the first source devoted to supplying state-of-the art information on TLC

as it applies to the separation, identification, quantification and isolation of medicinal plant components [22]. The choice of the appropriate solvent is one of the most important factors for obtaining extracts with a high concentration of bioactive compounds. In general, the highly hydroxylated aglycones forms of phenolic compounds are soluble in alcohols such as ethanol and methanol [23]. Less polar solvents such as ethylacetate, n-hexane, acetone and chloroform are used for the lesser polar and the highly methoxylated aglycones form [24]. The most polar phytochemical compounds can be extracted using water [25]. The position of an absorption peak relative to that of the reference compound is known as the chemical shift [18]. Chemical shift changes induced by solvents are relatively small. Paramagnetic metal complexes can cause broadening of the NMR signal [15] as seen in the two expanded sepctra of H_1 and H_2 samples (Figures 3 and 5). However, the peak seen in Figure 2 at 1.4 ppm (-CH3), another at 3.4 ppm (-OD), at 4.8 ppm (CD3) and at 5.5 ppm (-C=C-H) may suggest aliphatic to aromatic compounds. However, the Figure 3 showed the first peak at 1.8 ppm (-C=C-H), 3.4 ppm (-OD), 4-8 ppm (CD3) and 5.5 ppm (-C=C-H). When interpreting NMR spectra, begin from left to right, and note the number of signal sets (singlets, doublets). Establish the number of peaks which in the case of 1H NMR spectra will indicate the number of sets of equivalent hydrogens in the compound. However, meaningful structural assignment would generally require a combination of information from elemental analysis and from chemical and physical characteristics of the compound and its spectral data [14]. The position of an absorption in the NMR spectrum is therefore denoted, not only by the absolute frequency value, but also, by its relationship to the absorption frequency of a reference compound. The normal reference compound for 1H spectra is tetramethylsilane [(CH3) Si, TMS]. All the protons in TMS are equivalent, and the proton nuclei in the vast majority of organic compounds absorb down field from the single TMS signal. Tetramethylsilane is not soluble in water. The δ value is obtained by dividing the position in hertz by the instrument frequency in Megahertz and is expressed in parts per million. The δ value may relate to any reference compound and therefore the particular reference used (e.g. TMS) must be quoted. Earlier literature used the similar T scale, this related solely to TMS, which was given a value of 10. The two scales can be readily interconverted since $\gamma\ \tau=10-\delta$ [18]. The singlet peak at 4.8 ppm is methanol-d4 a standard used in this study. A shift of 4.8 ppm had been reported for methanol-d4 in a separation study [26].

Since fractions 1-11 were mainly fatty matter and were therefore not collected, the larger signals suggest methanol-d4 (solvent) and the smaller signals suggest aliphatic compounds. Hence the spectra contain methanol-d4 (alcohol) and aliphatic compounds suggesting the need for more thorough purification of the isolates H_1 and H_2 to guarantee identification of putative compounds. The band 1, which was yellowish in colour and dissolved in methanol, may be flavonol, quinone or flavanquinone. Quinones are generally yellow crystalline solids usually insoluble in water but soluble in ether and sublime on heating. In the nuclear magnetic resonance spectrum, the absorption by the proton in the hydroxyl group gives rise to a broad peak, the chemical shift of which is rather variable; the peak disappears on deuteration [18]. Nuclear magnetic resonance spectroscopy (NMR) measures the absorption of electromagnetic radiation in the radio-frequency region of roughly $4MH_2$ to 750 MH_2, which corresponds to a wavelength of about 75 m to 0.4 m. Nuclei of atoms rather than outer electron are involved in the absorption process [19]. Of the about 100 different kinds of nuclei that possess an intrinsic angular momentum perhaps proton is the one of most general interest at the present time [27]. The precise frequency from which energy is absorbed gives an indication of how an atom is bond to, or located spatially with respect to other atoms. Thus NMR offers an excellent physical means of investigating molecular structure and molecular interactions. NMR spectra may also be used for compound identification, by a finger print technique, and sometimes as a specific method of assay for the individual components of a mixture. Paramagnetic impurities in either sample or solvent cause resonance line broadening and small amount of oxygen dissolved in solution may cause considerable loss of spectrum resolution (line broadening) [15] as seen in our study. The rate of magnetic field sweep is important; too slow a sweep leads to saturation effects, whereas a fast sweep results in ringing. Provided ringing is not excessive and does not distort the resonance signal, it is indicative of good field homogeneity. The resolution of spectrum is its clarity of division into distinct bands corresponding to atomic or electronic transition. Resonance line width is governed, in part, by magnetic field homogeneity, and therefore in assessing any spectrum, an index of resolution is desirable. The resolution of a resonance band is conveniently expressed by its width in Hz at half height. If a proton is bonded to a carbon atom that is directly bonded to an electronegative element, electron density around the proton will be inductively pulled away, resulting in a de-shielding of the proton. Consequently, the resonance position of the proton will be shifted downfield from TMS. A greater down shift will occur if the proton is directly bonded to the electronegative element, and the higher the electronegativity, the greater the down shift. Conversely, the resonance position will shift to higher field (shielded) with decreasing electronegativity. Thus, the relative positions of resonance peaks in the NMR spectrum are a measure of the extent to which the protons are shielded or deshielded [14]. The NMR solvent used to acquire the spectra contain a maximum of 0.05% and 1.0% TMS (v/v) respectively since deuterium has a spin of 1. Triplets arising from coupling to deuterium have the intensity ratio of 1:1:1. NMR spectra of neat deuterated solvent always exhibit a peak due to H_2O in addition to the residual solvent peak (Figures 2 and 4). When the exchange rate between and HDO is slow on the NMR timescale, the water peak appears as two peaks a singlet corresponding to H_2O and 1:1:1 triplet corresponding to HDO [27].

Aliphatic C-H protons appear between 5-O ppm. Aliphatic fragments adjacent to heteroatoms or electron withdrawing groups fall into the 2-5 ppm region. Saturated C-H fall into the 2-0 ppm region. If you attach 2 electron withdrawing groups to the carbon, the proton may appear in 5-6 ppm region were alkenes normally showed up. Alpha and beta unsaturated aliphatic aldehydes possess growth inhibitory and bactericidal activity. This property depends on the length of the aliphatic carbon chain and the species of microorganisms [28]. This may suggest *in vivo* and *in vitro* antimicrobial activity of *Abrus precatorious* leaf extract against *Plasmodium berghei*, Plasmodium falciparum, *Trypanosoma brucei* rhudesiense, *Trypanosoma cruzi*, *Leishmania donovoni* and rat skeletal myoblasts [13,29]. Other susceptible microorganisms are *Streptococcus pyogenes*, *Streptococcus pneumonia*, *Salmonella typhimurniro*, *Escherichia coli*, *Klebsiella pneumonia* [30-33]. To analyse the effect of solvent type on the extraction of flavonoids, the crude methanolic extract can be partitioned to give non-polar (n-hexane and dichloromethane) and polar (ethylacetate, acetone and water fractions). Although aqueous phase is the most effective phase for the isolation of flavonoid aglycones from plant material [34]. The n-hexane solvent can be used to extract esters from medicinal plants [35]. Terpenoids and furanoterpenes are responsible for *in vitro* activity against *P. falciparum* [36]. Aerucyclamides A-D is heterocyclic peptides and

nostocarboline, an alkaloid respectively produced by Microcystis aeruginosa and Nostoc displayed activity against *P. falciparum* with a pronounced selectivity towards rat myoblasts [37]. Antiprotozoal activities are significantly correlated with cytotoxicity and the major determinants for activities are α and β-unsaturated and structural elements, also known to be essential for other biological activities of sesquiterpene lactones. However, certain compounds are more toxic against protozoa than against mammalian cells while others are more cytotoxic than active against the protozoa.

Conclusion

The inability to identify and characterize the two isolates (H_1) with R_f (0.121) and H_2 with R_f (0.608) using neutron magnetic resonance spectroscopy may be attributed to contamination of the isolates at the level of chromatographic separations and spectroscopy. However, H_1 was suspected to be alkaloid whereas and H_2 was suspected to be flavonoid, quinone, flavanquinone and glycoside, respectively.

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