ISOLATION STUDIES FROM STEM EXTRACT OF *PISTACIA INTEGERRIMA* STEW. EX BRAND

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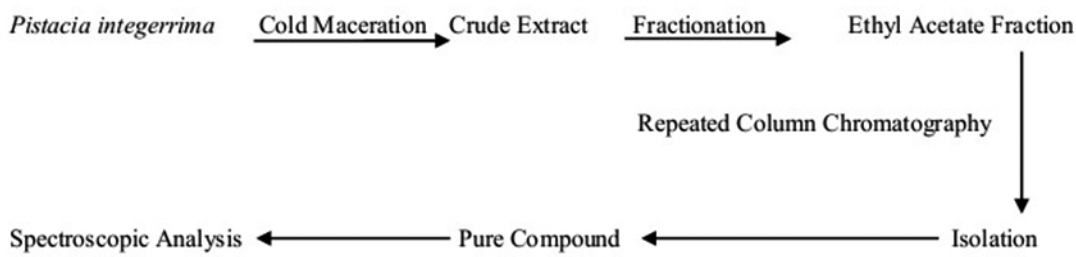
ABSTRACT

Plants have been source of fantastic natural compounds that range in complexity. Chemical studies on traditional medicinal plants have led to discovery of potent bioactive components**.** *Pistacia integerrima* belonging to family Anacardiaceae has been traditionally effective for rheumatic pain, analgesic and antipyretic effects and used in treatment of coughs, phthisis, asthma and dysentery. Natural products have served as basis for compounds to be utilized in drug discovery. Stem extract of *Pistacia integerrima* was investigated with the aim to find some novel components that can later be used as drugs or active pharmaceutical intermediates. Plant material was collected from Margalla Hills Islamabad, shade dried and subjected to cold maceration technique for extraction. Crude extract was partitioned into different fractions on polarity basis. Repeated normal and reverse phase column chromatography of ethyl acetate fraction supported by Thin Layer Chromatography have led to the isolation of pure single compound. Isolated compound was further analyzed towards structural elucidation using LC-MS, IR and NMR spectroscopy. Present finding will be a useful and novel addition in already known chemical components from *Pistacia integerrima.*

Keywords: *Pistacia integerrima*; Analgesic; Extract; Ethyl acetate fraction; Column chromatography; Nucleic magnetic resonance.



INTRODUCTION

Nature has gifted a great diversity of structures to living organisms including plants referred as natural products. Importance of natural products is understood historically and long synthetic routes, high costs and low yield of synthetic products further enhanced the value of natural products.[1] In addition as they have synthesized in living systems, natural products are more biological friendly than synthetic ones.[2] Plants act as a rich source of complex and highly varied structures (phytochemicals) unlikely to be synthesized in laboratories. Existence of plants in various ecological and geographical zones is further responsible for chemical diversity in them. These phytochemicals in turn serve as an indicator of complexity of plants.

Over hundred phytochemicals are being used as drugs and others served as basis of synthetic drugs. About 75% of these chemical substances were discovered during isolation studies on traditionally used medicinal plants.[3]

In Pakistan use of traditional medicines is very common and much influenced by wonderful biodiversity of medicinal plants nourish in its localities.[4] According to Federal Bureau of Statistics of Pakistan 80% of total 65.4% rural population of Pakistan rely on plant based traditional system of medicines for treatment of ailments.[5] One approach to increase utilization of plant based natural products in place of much costly artificial ones, is to chemically investigate those plants which are used in traditional system of medicines on prescription and practice of wise and elder men of community in Pakistan.[6] Different simple chromatographic techniques like Column Chromatography (CC) and Thin Layer Chromatography (TLC) can be employed to investigate the plants for potent chemical constituents. [7,8,9] Besides these, spectroscopic methods like Mass (MS), Infra Red (IR) and Nuclear Magnetic Resonance (NMR) spectroscopy provided further support to elucidate the structures of phytochemicals.[10,11,12]

Pistacia integerrima is a deciduous tree belonging to family Anacardiaceae. Plant is widely distributed in East Afghanistan, Pakistan, and North West & West Himalaya to Kumaon. Traditionally plant has been effective for rheumatic pain, analgesic and antipyretic effects and used in treatment of coughs, phthisis, asthma and dysentery etc.[13] Galls of *Pistacia integerrima* are used to treat hepatitis and liver disorders in Pakistan.[14,15] They are also known for their use in diabetes, pain, fever and inflammatory conditions.[16] Chemical investigation of *Pistacia integerrima* leaf revealed that this interesting plant contains carotenoids, triterpenoids, catechins and flavonoids.[17,18] The polyphenolic contents in the leaves of *Pistacia integerrima* were also studied.[16] Based on the ethno-botanical importance and chemical composition of the plant it has been explored for many biological activities including anti-microbial, antioxidant, analgesic, cytotoxicity and phytotoxicity.[19] Based on the previous findings and medicinal importance of *Pistacia integrrima* aim of present investigation was to investigate the stem extract of *Pistacia integerrima* and elucidate the structure of pure isolated compounds using advance chromatographic and spectroscopic experiments with a hope to find some novel and interesting structure that will add the number of known natural compounds from this medicinally important plant species.

EXPERIMENTAL

Collection, Drying and Extraction:

Fresh *Pistacia integerrima* Stew. ex Brand plant material was collected from Margalla Hills of Pakistan and identified by Dr. Mir Ajab Khan, Department of Botany Quaid-i-Azam University Islamabad Pakistan. A voucher specimen was deposited in herbarium of Quaid-i-Azam University. Plant material was thoroughly washed under running tap water and dried under shade. Dried material was ground to fine powder. Cold maceration technique was used for extraction. Powdered plant material (2kg) was macerated in methanol (2000ml) in a closed glass container, stored at room temperature for one week, followed by vacuum filteration. This procedure was repeated many times. The combined methanol filtrates were concentrated after evaporating methanol under reduced pressure on a rotary evaporator (Buchi Rotavapor R-200) at 40 °C resulted in a greenish semi solid residue (400g).

Fractionation:

Fractionation of crude extract was carried out by suspending 400gm of extract in 200ml water and then partitioning with hexane, chloroform, ethyl acetate and methanol in order of increasing polarity by using separating funnel. All the five fractions including aqueous fraction were dried using rotary

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evaporator (Fig. 1). Fractions so obtained were hexane (15gm), chloroform (100gm), ethyl acetate (180gm), methanol (40gm) and aqueous (30gm). Scheme used in fractionation of crude methanol extract is summarized in Fig. 1.

Normal Phase Column Chromatography of Ethyl Acetate Fraction:

Ethyl acetate fraction was selected on basis of maximum quantity (180gm) and subjected to column chromatography in normal phase as well as reverse phase in different steps. Silica gel 60 (Sigma-Aldrich) with pore size 0.035-0.070 mm was used for normal phase column chromatography. Sample was loaded after adsorption on silica gel by making a uniform and even layer. Mobile phases starting from n-hexane (500ml) followed by chloroform (500ml), chloroform-ethyl acetate; 4:1(500ml), chloroform-ethyl acetate; 1:1 (500ml), chloroform-ethyl acetate; 1:4 (500ml), ethyl acetate (500ml), ethyl acetate-methanol; 4:1 (500ml), ethyl acetate-methanol; 1:1 (500ml) and methanol (500ml) were used. Elutions of 300ml were collected throughout. Organic solvents used were of analytical grade and were purchased from Sigma Alrdrich (Dorset UK). Each elution was analysed on TLC using silica plates (silica gel 60 F254 with fluorescent indicator. Eluted samples showing same Rf values, were combined resulted in nine (1-9) main groups (Fig. 2). Successive groups were dried at 45°C under vacuum using rotary evaporator.

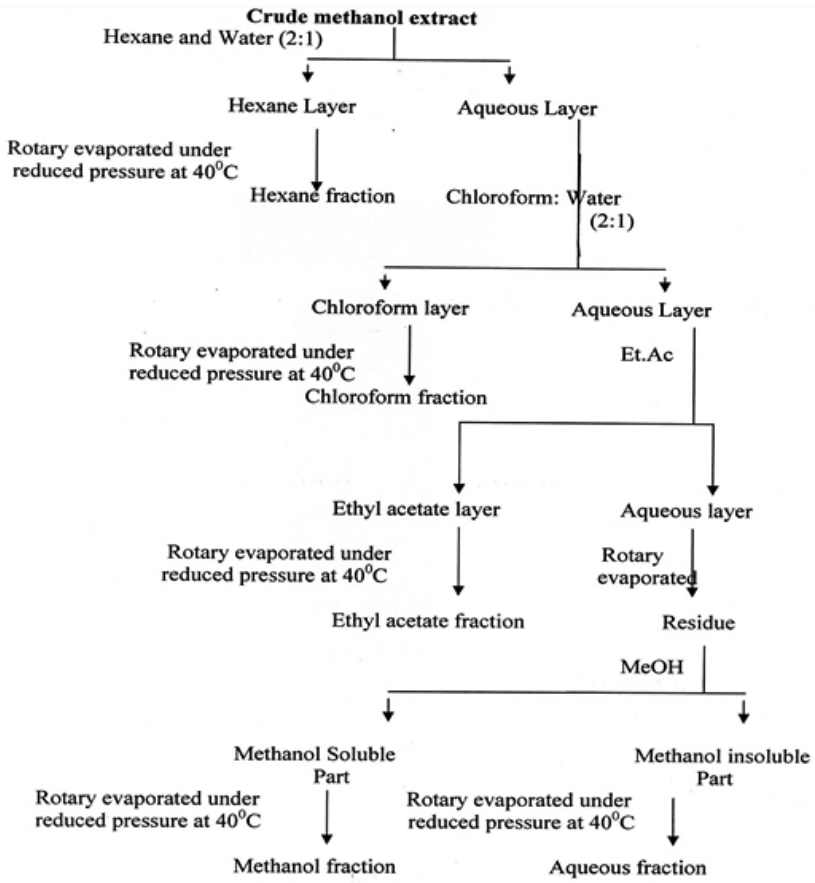

Fig. 1: Summary of scheme used for fractionation process.

Column Chromatography of Group 2:

Group 2 was further purified by column chromatography after loading on silica gel. Starting from n-hexane (300ml), hexane-chloroform; 1:1 (300ml), hexane-chloroform; 1:4 (300ml), chloroform (300ml), chloroform-ethyl acetate; 9:1(300ml), chloroform-ethyl acetate; 8:2 (300ml), chloroform-ethyl acetate; 7:3 (300ml), chloroform-ethyl acetate; 5:5(300ml), chloroform-ethyl acetate; 2:8 (300ml) and finally ethyl acetate (500 ml), elutions of 50ml were collected and subjected to TLC. Seven groups (A-G) were obtained on the basis of Rf values (Fig. 2). Successive groups were dried under vacuum using rotary evaporator at 45°C.

Column Chromatography of Group F:

Group F was further selected for analysis using column chromatography on silica gel. Elutions (25ml) starting from n-hexane (200ml), hexane-chloroform; 8:2 (200ml), hexane-chloroform; 7:3(200ml), hexane-chloroform; 5:5(200ml), hexane-chloroform; 2:8 (200ml), chloroform (200ml), chloroform-ethyl acetate; 8:2 (200ml), chloroform-ethyl acetate; 7:3 (200ml), chloroform-ethyl acetate; 5:5 (200ml), chloroform-ethyl acetate; 3:7 (200ml), chloroform-ethyl acetate; 2:8 (200ml) and ethyl acetate (200ml) were collected and subsequently subjected to TLC and were further pooled in to three sub groups (a-c) on the basis of Rf values (Fig. 2). Successive subgroups were dried under vacuum using rotary evaporator at 45 °C.

Reversed Phase Column Chromatography of Group b:

Reversed phase chromatography was utilized at this step to remove polar impurities from the sub-group b. Starting from water: methanol, 1:1(100ml), water: methanol, 4:6 (100 ml), water: methanol, 2:8(100ml), methanol (100ml), ethyl acetate (100ml) and chloroform (100ml), elutions of 15ml were collected and grouped into seven groups (b1-b7) on the basis of Rf values (Fig. 2).

Column Chromatography of b6 in Reverse Phase:

Group b6 was subjected to reverse phase column chromatography. Stepwise elutions (10 ml) were collected in different mobile phases starting from water-methanol; 1:1 (80ml), methanol (80ml), methanol-ethyl acetate; 1:1 (80ml), methanol-ethyl acetate; 2:8 (80ml), ethyl acetate (80ml), ethyl acetate-chloroform; 1:1 (80ml) and chloroform (80ml). Three groups (1-3) were obtained (Fig. 2) and group 2 indicated a single compound which was further purified by repeated crystallization under vacuum.

Physical Characteristics of Isolated Compound:

Appearance of compound was noted based on visual observation and its melting point was determined on GallenKamp (Sanyo) instrument.

LC-MS Analysis:

LC-MS analysis was done on WATER's LCT micromass with TOF (Time of Flight) mass spectrophotometer using Electron Spray Ionisation connected to Alliance auto sampler injection system.

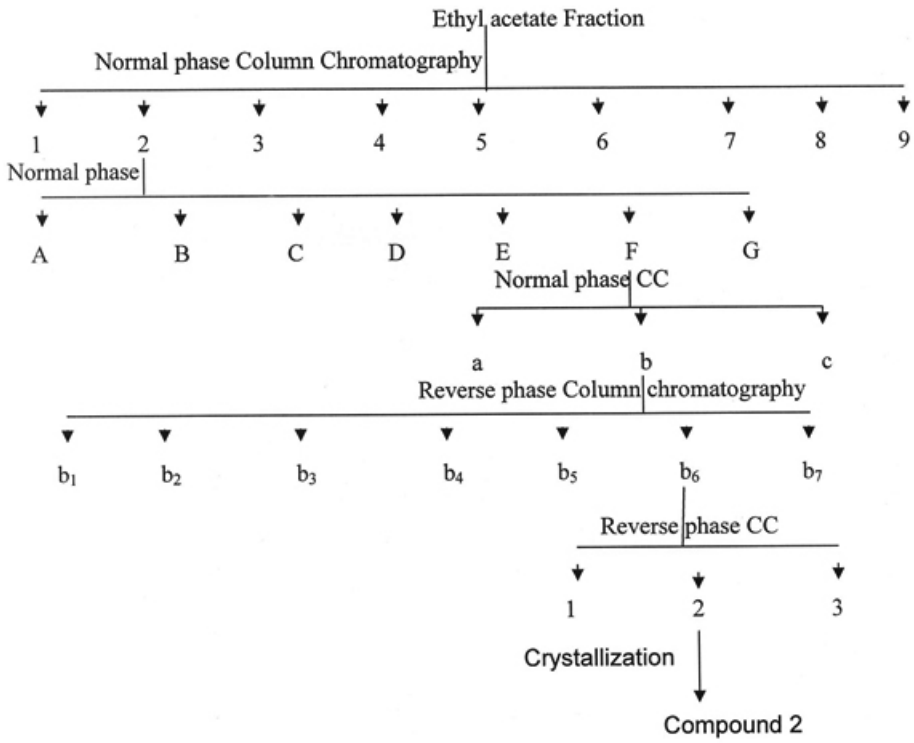

Fig. 2: Summary of scheme used in isolation of compound.

IR Analysis:

IR analysis was done by using Thermo Nicolet 380 FT-IR spectrophotometer with OMINIC version 7.3 control and processing software from Thermo Electron Corporation, using Sodium chloride discs. Absorption bands were quoted in wave number (cm-1).

NMR Analysis:

NMR spectra were recorded using a JEOL Eclipse 400 NMR spectrometer with Jeol DELTA version 7.2 control and processing software was used to perform different NMR experiments, where peak positions were quoted on the scale relative to an internal standard. Compound was dissolved in deutrated methanol (CD_3OD) and solution was pipetted in clean NMR tubes for analysis.

RESULTS AND DISCUSSION

The paper reports on isolation of a single compound from ethyl acetate fraction of *Pistacia integerrima*. Compound appeared as white needle like crystals. Spectroscopic data is as under:

250mg; m.p 204-207°C; UV (CH_3-COO-CH_2-CH_3: $CHCl_3$) 254nm; IR(KBr) cm^{-1} : 831 (C-Cl), 1599 (C_6H_6), 1613 (NH_2), 1736 (CO), 3346 (OH); 1H NMR(CD_3OD) δ / ppm : 6.61 (d, 1H, *j* 8.6Hz, H-8), 6.51 (d, 1H, *j*. 8.6, H-10), 6.36 (d, 1H, *j*. 2.38, H-14), 6.29 (d, 1H, *j*. 2.38, H-13), 6.235 (d, 1H, *j* . 2.56, H-5), 4.4876 (s, 3H, H-16), 2.63 (d, 1H, *j*. 12.45, H-7); ^{13}C NMR (CD_3OD) δ / ppm: 117.48 (C,C-1), 151.18 (C,C-2), 157.65 (C,C-3), 156.96 (C,C-4), 45.40 (CH,C-5), 129.24 (C,C-6), 102.80 (CH,C-7), 115.64 (CH,C-8), 128.72 (CH,C-10), 111.44 (CH,C-13), 130.14 (CH,C-14), 169.28 (C,C-15), 43.91 (CH_3,C-16); MS *m* / *z* : 508 (M^+) and 365(M^+ - 342).

TLC mobile phase optimized was ethyl acetate:chloroform 6:4 and Rf value calculated was 0.6. Compound was found UV active at 254nm. Compound was unable to elute from the GC-MS column, so, LC-MS technique was utilized which showed a molecular ion peak at 508m/z (Fig. 3). The peak represented an adduct of sodium which was confirmed by running a sample (Sucrose) of known molecular weight (342), the adduct of which gave a molecular ion peak of 365m/z (Fig. 4). Adduct formation particularly by alkali metals in LC-MS analysis has been reported and discussed in literature.[20,21,22,23,24]

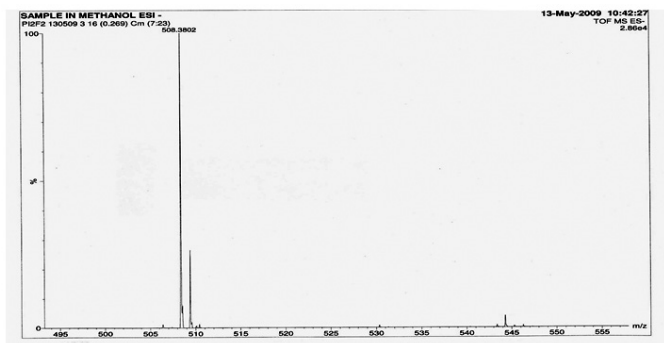

Fig. 3: LC-MS spectrum of compound isolated from ethyl acetate fraction. Values on X-axis represent m/z of isolated compound and Y-axis represents its % age in sample.

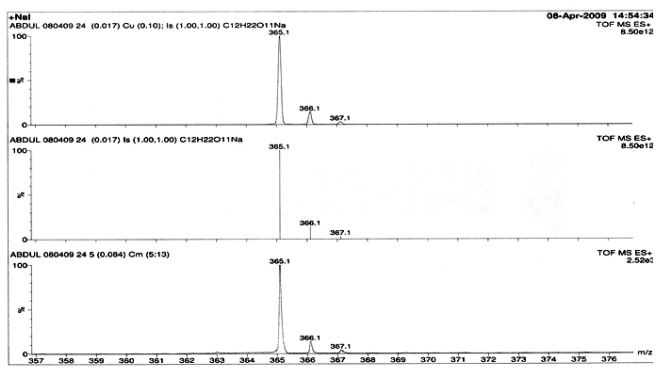

Fig. 4: LC-MS spectrum of standard sample of sucrose used as reference. Peak at 365 represents a Na adduct of sucrose. The actual molecular weight of which is 342.

Looking at the IR spectroscopic data, which act as a big source for natural products analysis, an absorption peak at 1736cm^{-1} represents a ketonic group in the molecule while the peak at 3346 cm^{-1} is due to OH group in molecule (Fig. 5). Peak at 1599cm^{-1} represents aromatic absorption. UV absorption around 254nm is also an indication of double bond in structure.[25]A sharp band at 1513cm^{-1} in spectrum is characteristic of triazine compounds.[23] Absorption peak at 831cm^{-1} represents C-Cl stretch and at 1613cm^{-1} represents NH_2 group in structure (Fig. 5).

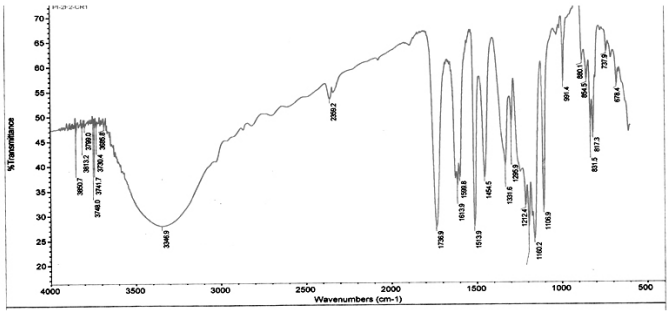

Fig. 5: IR spectrum of compound isolated from ethyl acetate fraction. Absorption bands quoted in wave number (cm^{-1}) represent functional groups in compound.

The peak positions shown by the ^{13}C NMR along with combine Carbon and DEPT spectrum are shown in Fig. 6 & 7 while the peak positions shown by the ^{1}H NMR in (Fig. 8, 8a & 8b).

H and ^{13}C NMR provided sufficient information about molecule structure. The DEPT (Distortionless Enhancement by Polarization Transfer) experiment (Fig. 7) indicated absence of CH_2 units in compound. HETCOR (Hetronuclear Chemical Shift Correlation) spectrum (Fig. 9) provided information on carbon proton connectivity as well as quaternary carbons which do not show any proton linked to them. These experiments explained quaternary carbons showing comparatively weaker signals at δ117, 129, 151, 156, 157 and 169ppm as reported in literature. Carbon at δ169-170ppm appeared to be carbonyl as far as reference ranges for carbon chemical shifts are concerned. Similarly chemical shifts at δ117, 129, 151, 156 and 157ppm also predict aromaticity of structure in reference to ranges for carbon chemical shifts reported in literature.

^{1}H NMR indicates presence of 3 singlets at δ 4.4 - 4.5ppm. These three singlets appear to be linked with carbon at δ 45ppm in ^{13}C spectrum further proved by DEPT and HETCOR experiments. Duplets appear at δ 6.2 and 6.3ppm showed coupling effect. Similarly duplets at δ 6.5 and 6.6ppm showing same *j* value of 8.6 appear to be coupled. Protons in the range of δ 6.2-6.6ppm represent CH further supported by DEPT and HETCOR spectra (Fig. 7 & 9).

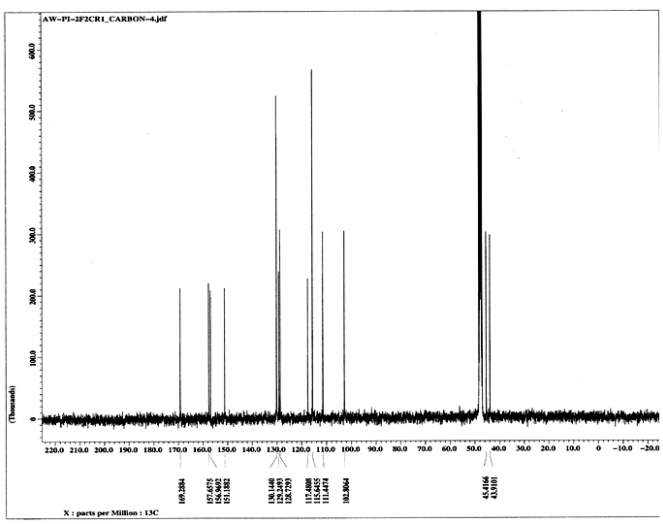

Fig. 6: Carbon NMR spectrum of compound isolated from ethyl acetate fraction. Values at base represent chemical shifts in ppm.

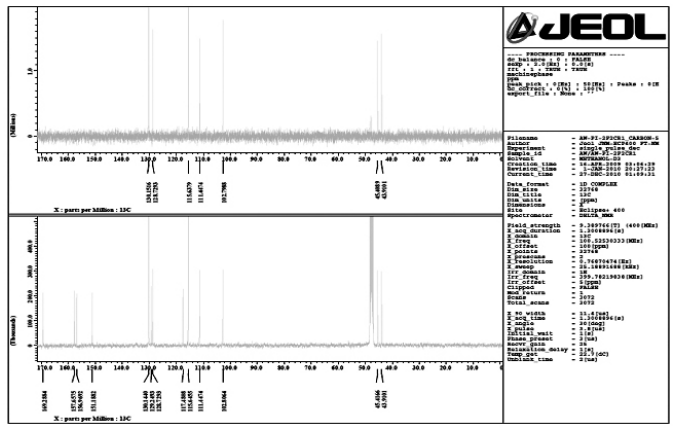

Fig. 7: Combine carbon and DEPT spectra of compound isolated from ethyl acetate fraction. It represents a comparison of carbon spectrum with DEPT spectrum with all upright peaks showing presence of only CH and CH_3 groups. Absence of inverted peaks represents absence of CH_2 groups in compound.

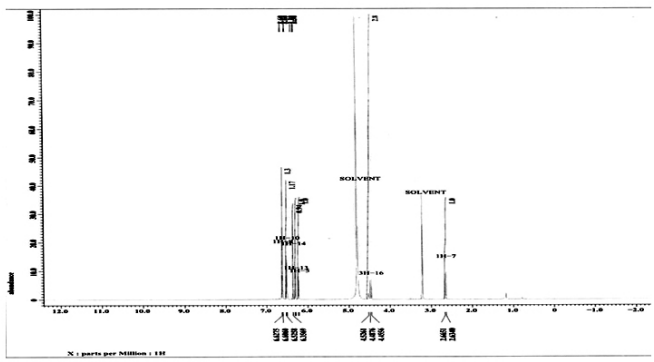

Fig. 8: Proton NMR Spectrum of compound isolated from ethyl acetate fraction. Values at the base represent chemical shifts in ppm.

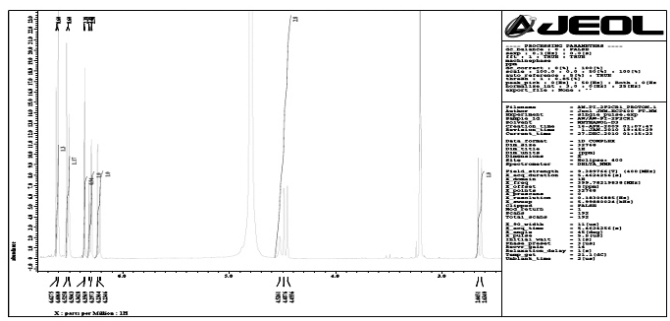

Fig. 8a: Magnified form of Proton NMR spectrum of compound isolated from ethyl acetate fraction. Integration of peaks showing number of protons coming under a peak. Values at the top of peaks represent coupling constant (j value) for protons.

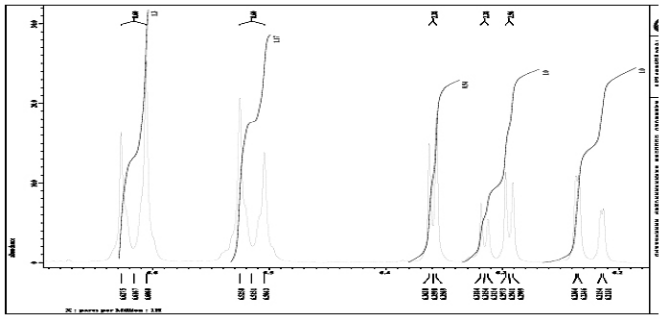

Fig. 8b: Magnified form of individual peaks in Proton NMR spectrum of compound isolated from ethyl acetate fraction representing their splitting, integration and coupling constants.

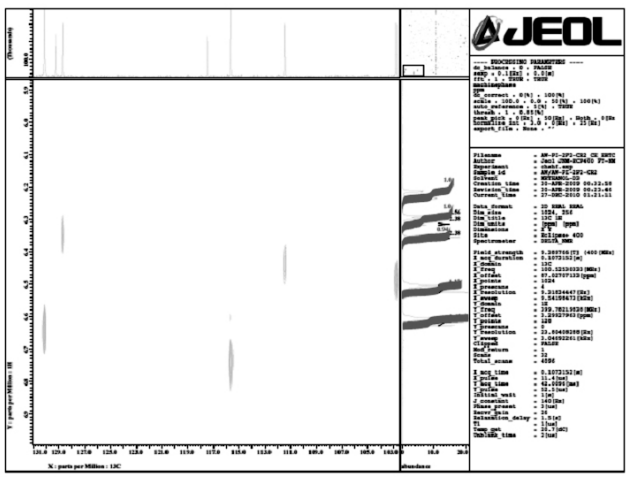

Fig. 9: HETCOR spectrum of compound isolated from ethyl acetate fraction. Spectrum represents Carbon- Proton connectivity along with quaternary carbons with no proton linked to them.

Results of spectroscopy indicates the isolation of chlorinated natural compound from *Pistacia integrrima*. Natural chlorinated compounds are not common in plants, although they are found frequently in marine algae and fungi.[28,29] more than 130 compounds has been isolated from higher plants with chlorine. Members of family Asteraceae are known to have diversity of such compounds.[30] The compound recently isolated from Pistacia integrrima may be tested for its biological activities as cholrinated compounds isolated from Pea plant are known to most active biological compounds.[31] Author is towards more experimentation to overall finalize the structure of this novel compound from *Pistacia integerrima*.

CONCLUSION

In conclusion chromatographic techniques like column chromatography and TLC in combination with spectroscopic analysis like MS, IR and NMR have proved as an effective tool for isolation and structural elucidation of natural products. Exploring local flora of Pakistan like in this study will help in defining the secretes of nature in medicinal plants into chemically defined novel structures.

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