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Phytochemical constituents from the leaves of *Malva verticillata* L.

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Abstract
In the present study, a phytochemical investigation has been carried out for isolation and characterization of chemical constituents from the leaves of *Malva verticillata* L. The powder of dried leaves of *Malva verticillata* L. was extracted with petroleum ether and ethyl acetate. Attempt has been taken for isolation of compounds from petroleum ether and ethyl acetate extract of the leaves by chromatographic technique. Tetracontanyl palmitate has been isolated from petroleum ether extract and a ketone has been isolated from ethyl acetate extract. The structures of the compounds have been established by different spectroscopic techniques. Studies of antimicrobial activity of the isolated compounds were also done.

Keywords: *Malva verticillata* L., leaves, solvent extract, phytochemical, chromatography, spectroscopy

1. Introduction
World plant biodiversity is the biggest resource of herbal medicine and still now a major percentage around 60-80% of world population depend on plant-based medicines. Since ancient times, humans have used natural products, such as plants, marine organisms, in medicines to mitigate and treat all sort of physical illness. According to fossil records, the human use of plants as medicines may be traced back at least 60,000 years [1]. It is now clear that, the medicinal value of these plants lies in the bioactive phytochemical constituents that produce definite physiological effects on human body. These natural compounds formed the base of modern drugs as we use today. Natural products and related drugs are used to treat 87% of all categorized human diseases including bacterial infection, cancer and immunological disorders [2]. About 25% of prescribed drugs in the world originate from plants [3] and over 3000 species of plants have been reported to have anticancer properties [4]. About 80% of the population in developing countries relies on traditional plant based medicines for their primary health care needs [5]. Bangladesh has a rich and prestigious heritage of herbal medicines among the South Asian countries. More than 500 species of medicinal plants are estimated as growing in Bangladesh and about 250 species of them are used for the preparation of traditional medicines. However, the majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compound (s) [6].
Malva verticillata L. is one of the species of Malvaceae family. The leaves of *Malva verticillata* L. are commonly consumed as vegetables in the northern part of Bangladesh, locally known as Laffa or Napa Shak. Considering the importance of *Malva verticillata* L. much work has been done on its seed and different polysaccharides have been isolated previously such as MVS-IIA and MVS-IIG [7] as a neutral polysaccharides, MVS-V [8] as the major pectic peptidoglycan, MVS-I [9] also as a neutral polysaccharides, MVS-VI [10] as a novel acidic polysaccharides. On the other hand, in case of leaves antimicrobial activity and phytochemical analysis have been carried out for different solvent extracts [11]. However, according to literature survey, no report on phytochemical constituents of *Malva verticillata* L. leaves has been reported. Therefore, the present paper deals with petroleum ether and ethyl acetate extract of *Malva verticillata* L. leaves and the isolation and characterization of the biologically important chemical constituents from different extracts initially.

2. Experimental
2.1 Materials and Methods
All solvents and reagents used in this experiments were either of analytical or laboratory grade, purchased from Merck (Germany) and BDH (England) except petroleum ether which was commercial grade. Petroleum ether (PE) was obtained from commercial petrol after distilling in a glass distillation apparatus and collected the desire fraction.

Distilled solvent was used throughout the experiment. All fractions were monitored by analytical thin layer chromatography (TLC) using Merck pre-coated silica gel glass plates (0.25 mm) and spot detected under either I_2 chamber or UV-lamp. Fractions were dried at moderate conditions in Buchi Rotavapor, R-114 rotary evaporator. The column chromatography was carried out over silica gel 60 (230–400 mesh), purchased from Merck (Germany).

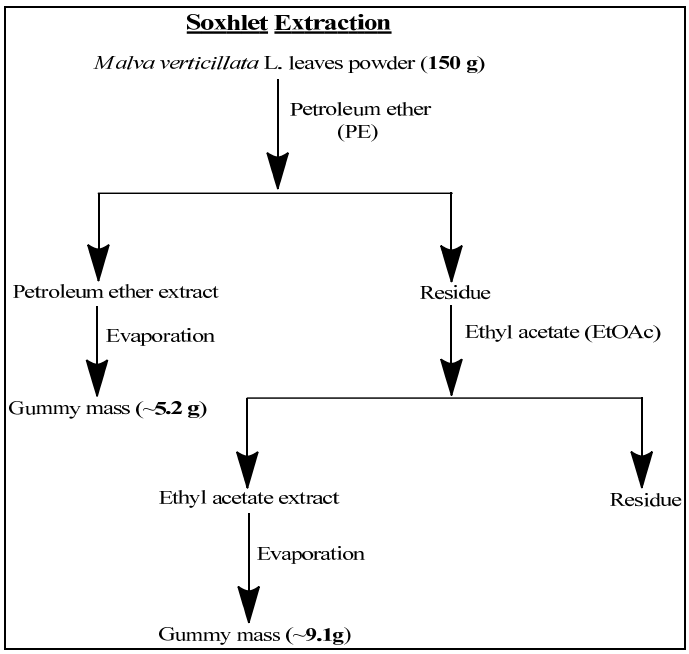
A UV-vis spectrum was recorded using the Shimadzu UV-1800 spectrometer, (Japan). A Shimadzu FTIR-84005 spectrometer (Japan) was used for recording infrared spectrum. Major bands (ν_{max}) were recorded as wave number (cm^{-1}) in KBr pellets. NMR spectra were recorded in $CDCl_3$ (Kanto Chemical Co. Inc.) at JNM-ECX-400, at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR as well. The internal standard for NMR was 0.03% tetramethylsilane (TMS). Chemical shifts (δ) are given in ppm with respect to TMS and coupling constants (*J*) are given in Hz. High-resolution mass spectrum (HRMS) was acquired on a MS spectrometer using EI (Electron Impact) techniques.

2.2 Sample preparation

Fresh *Malva verticillata* L. leaves (Napa Shak) were collected from the rural area of Dinajpur, northern district of Bangladesh during winter season. Fresh leaves were cut into small pieces and air-dried. The air-dried leaves were further dried in an oven at 40 °C, and then dried leaves were ground to powder (~200 mesh size). The powder was used throughout the investigation.

2.3 Extraction process

About 150 gm of powdered leaves of *Malva verticillata* L. was extracted by a set of soxhlet apparatus with petroleum ether (PE) and ethyl acetate (EtOAc) successively. The extracts were concentrated using a rotary evaporator (BUCHI Rotavapor, R-114, Japan) at a maximum temperature of 40 °C to give ~ 5.2 gm of petroleum ether extract and ~ 9.1 gm of ethyl acetate extract (Scheme 1).



Scheme 1: Extraction scheme of *Malva verticillata* L. leaves (Napa Shak)

Later, the crude petroleum ether extract was subjected to column chromatography on silica gel where n-hexane and ethyl acetate (EtOAc) were used at different ratio as mobile phase and a series of fractions were collected.

The fractions were monitored by thin layer chromatography (TLC) using different solvent systems and spots were trying to detect by UV-light and iodine vapor. The fractions were combined according to their R_f values. From the TLC pattern of all fractions, only SH1 showed one spot with tailing. Therefore, we target this fraction for our further purification process and try to figure out the actual chemical structure using different spectroscopic techniques. Rest of the fractions was not further analyzed due to small quantity. From that fraction SH1, a single white crystal like compound was isolated through consecutive chromatographic techniques (e.g. PTLC and column chromatography on silica gel). Eventually, the isolated compound was symbolized as SH1S1 (C1).

Later ethyl acetate extract (~9.0 gm) was fractionated by column chromatography into a series of fractions. All fractions were left undisturbed at refrigerator for several days, and after analyzing the fractions, a white crystalline solid compound was isolated along with other several compounds. The solid crystal was marked as Et-C1 and other compounds were marked as Et-C0 and Et-C2. Larger amount of extract is needed to reveal the characteristics of compounds. The isolated compounds SH1S1 (C1), and Et-C1 were characterized by different spectroscopic techniques (UV-vis, FT-IR, ^{1}H-NMR, ^{13}C-NMR and HRMS). The amount of compounds Et-C0 and Et-C2 was small and detail spectroscopic analysis was not completed yet.

Antimicrobial activity of the three isolated compounds Et-C0, Et-C1 and Et-C2 were performed [11]. Discs of three sample solutions e.g. Et-C0, Et-C1, Et-C2 were applied to the *Escherichia coli* bacteria solution. A positive control was made by placing antibiotic disc (Ciprofloxacin) on agar plate. Antibacterial activity was observed for Et-C0, Et-C1 and Et-C2 solutions with different inhibition zone.

3. Results and Discussion

The powder of dried *Malva verticillata* L. leaves was extracted with petroleum ether (PE) followed by ethyl acetate (EtOAc). The percentage of the extract was determined. The

ethyl acetate extract (6.07%) was higher than that of petroleum ether (PE) extract (3.46%).

The isolated compound SH1S1 (C1) from petroleum ether part was a white crystalline solid, which is highly soluble in $CHCl_3$, n-hexane and CH_2Cl_2. R_f value was obtained as 0.93 at 100% n-hexane, melting point 67-69 °C.

The FT-IR spectra of SH1S1 (C1) at KBr pellet showed (Fig. 1) different distinctive signals at 2910, 2810, 1731, 1463, 1171 and 719 cm^{-1} region. The absorption bands at 2910 cm^{-1} and 2810 cm^{-1} due to the presence of sp^3 C-H stretching (aliphatic) of either both $-CH_3$, $-CH_2-$ or >CH- group. The peak at 1731 cm^{-1} was for carbonyl group (>C=O) of an ester or aldehyde. The absorption band at 1171 cm^{-1} was due to –C-O- stretching. The absorption bands at 1463 cm^{-1} was the indication of $–CH_2-$ bending and an absorption band at 719 cm^{-1} was due to $–CH_2-$ bending and rocking of long aliphatic chain. The compound SH1S1 (C1) showed an UV absorption maximum at around 268 nm (very dilute solution in n-hexane) which was clearly due to π-π* electronic transition of ester (-COO-) functional group (Fig. 2).

The ^{1}H-NMR (400 MHz, $CDCl_3$, δ/ppm) spectra of the compound SH1S1 (C1) showed (Fig. 3) several peaks in particular regions. δ = 0.86-0.89 (aH & nH, t, *J*= 6.88 Hz), δ 1.25 (bH–eH & kH–mH, m), δ 1.55 (jH, m), δ 1.61 (fH, t, *J*=7.5 Hz), δ 2.28 (iH, t, *J*= 7.32 Hz), δ 4.03-4.06 (gH, t, *J*= 6.7 Hz). The observed ^{1}H NMR values were compared with the known compounds NMR spectra and our isolated compound showed a clear resemblance with dotriacontyl ester of docosanoic acid [12, 13]. The comparative NMR signals are shown in table 1.

Table 1: Comparative ^{1}H-NMR spectral data of isolated compound SH1S1 (C1)

No. of protons	Observed value (δ) ppm	Reported value[12,13] of triacontanyl palmitate (δ) ppm
2H (gH)	4.03-4.06	4.08
2H (iH)	2.28	2.1-2.3
2H (fH)	1.61	1.58
2H (jH)	1.55	1.54
(s+s*+6)H (bH–eH & kH–mH)	1.25	1.23
6H (aH & nH)	0.86-0.89	0.85-0.90

The ^{13}C-NMR (100 MHz, $CDCl_3$, δ/ppm) spectrum of the isolated compound SH1S1 (C1) showed (Fig. 4) carbon signals at different regions. δ 174.06 (>C=O), δ 64.40 ($-CH_2-O-$), δ 34.40 ($-CH_2-C=O$), δ 28.63 ($-CH_2-C-O-$), δ 25.93 ($-CH_2-CH_2-C-O-$), δ 25.02 ($-CH_2-CH_2-C=O$), δ 29.15 to 29.69 (long chain $-CH_2-$) δ 14.13 ($CH_3-(CH_2)s$*$-COO-(CH_2)s-CH_3$), δ 22.70 ($CH_3-CH_2-(CH_2)s$*$-COO-(CH_2)s-CH_2-CH_3$), δ 31.92 ($CH_3-CH_2-CH_2-(CH_2)s$*$-COO-(CH_2)s-CH_2-CH_2-CH_3$) [12, 13].

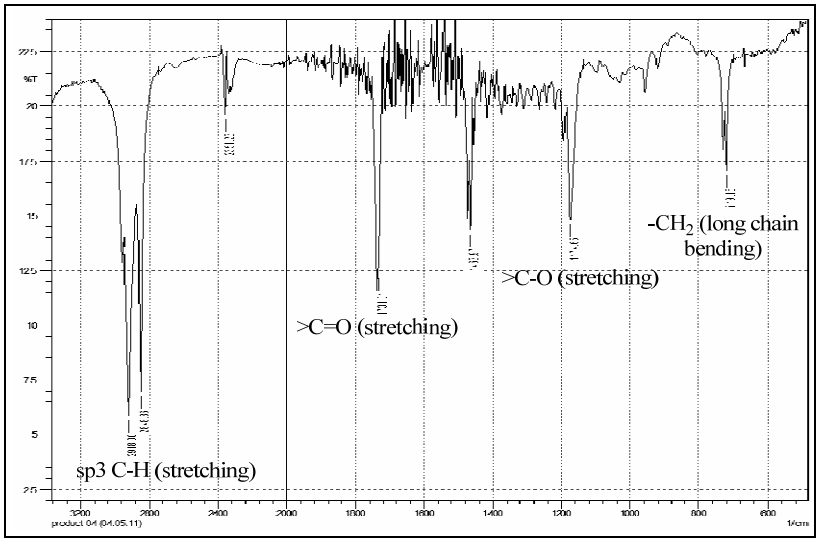


Fig 1: FT-IR spectra of SH1S1 (C1)

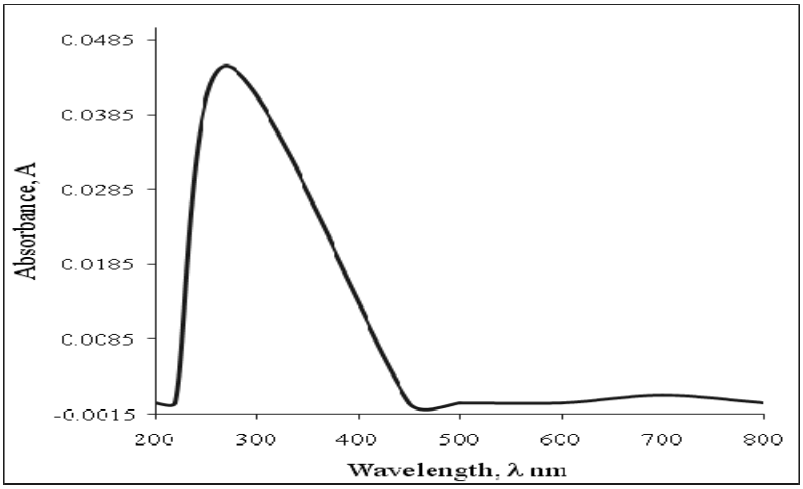


Fig 2: UV spectra of SH1S1(C-1)

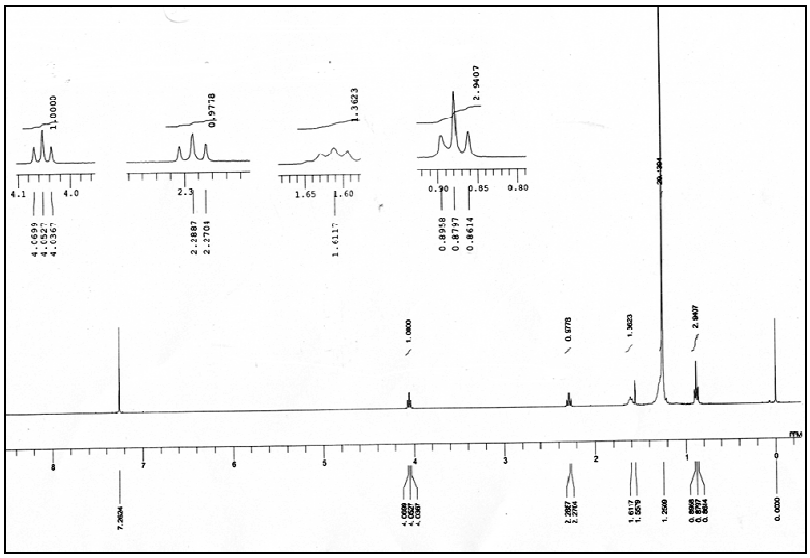


Fig 3: ^{1}H NMR spectra of SH1S1 (C1)

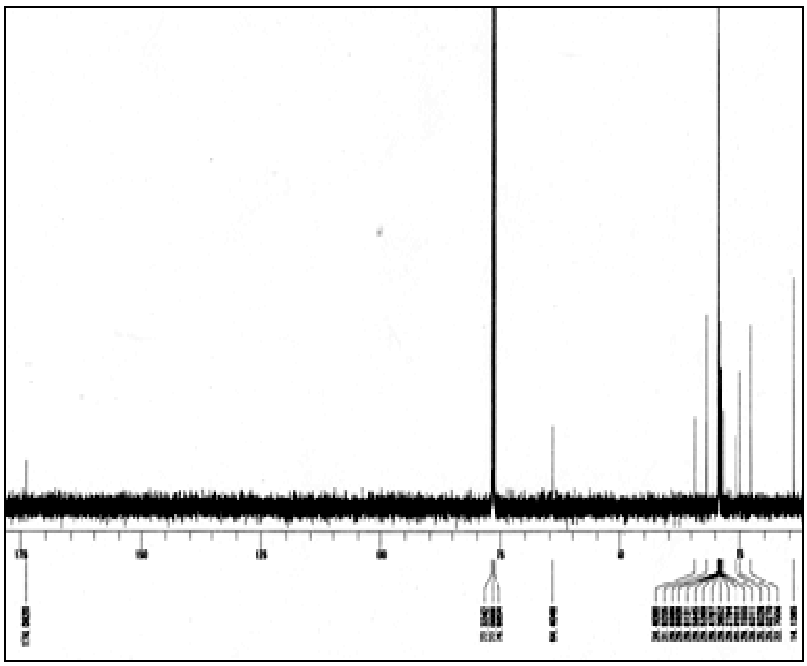


Fig 4: ^{13}C NMR spectra of SH1S1 (C1)

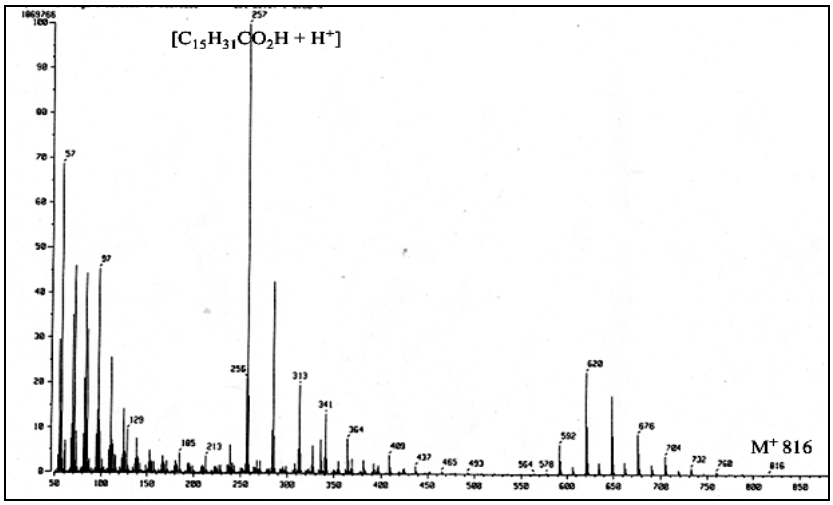


Fig 5: HRMS spectrum of SH1S1 (C1)

From the above-mentioned physical characterization and spectral data analysis (e.g. UV-vis, FT-IR, ^{1}H-NMR and ^{13}C-NMR) of the compound SH1S1 (C1), it was observed that, the isolated compound might be "long chain ester" having molecular formula of $C_nH_{2n}O_2$. A tentative molecular structure was shown in fig. 6.

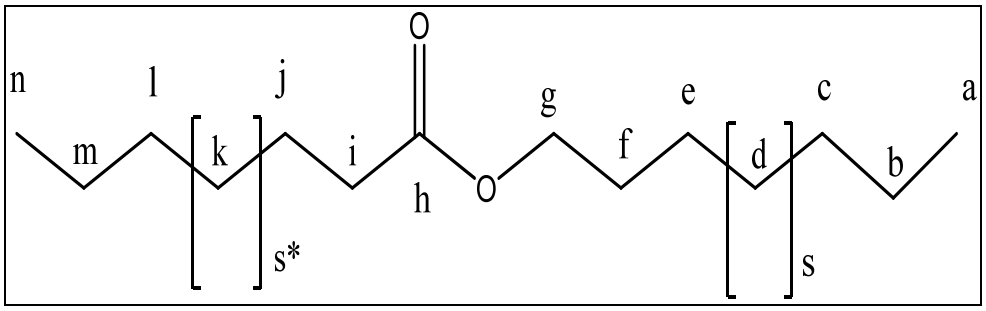


Fig 6: Tentative structure model of SH1S1 (C1)

In the Mass spectrum (HRMS) of the compound SH1S1(C1) peaks were observed (Fig. 5) at various regions including EI-MS (m/z) at M^+ 816, 760, 732, 704, 676, 620, 592, 578, 564, 493, 465, 437, 409, 364, 341, 313, 257, 256, 213, 185, 129, 97, 57 etc. The molecular mass of the compound was found at 816 and the base peak was at 257. In the low-*m*/*z* range of the spectrum, it presents a series of peaks regularly spaced by 14 mass units, indicated the presence of alkane structure. The region of high values above 500, showed a specific pattern of peaks regularly spaced every 28 units, from 592 to 732. This mass spectrum is perfectly compatible with the molecular long-chain wax esters [14]. The peaks at 409, 437, 465, and 493 respectively corresponding to the ion fragments $[C_{24}H_{49}CO_2]^+$, $[C_{26}H_{53}CO_2]^+$, $[C_{28}H_{57}CO_2]^+$, $[C_{30}H_{61}CO_2]^+$, and $[C_{32}H_{65}CO_2]^+$ [14]. The series of peaks in the mass range 592-732 correspond to the molecular ions of the different long-chain esters present in our isolated plant ester SH1S1 (C1).

According to the mass spectral data analyses, the molecular formula of the compound was confirmed as $C_{56}H_{112}O_2$. Molecular mass at 816 belongs to the saturated mono wax ester $[C_{56}H_{112}O_2]^+$. After analyzing all spectroscopic data, it is clear that the isolated compound might be tetracontanyl palmitate ($C_{56}H_{112}O_2$ = 816) or dotriacontyl tetracosanoate ($C_{56}H_{112}O_2$ = 816) because both have the same UV, IR, ^{1}H NMR and ^{13}C NMR spectra (Fig. 7).

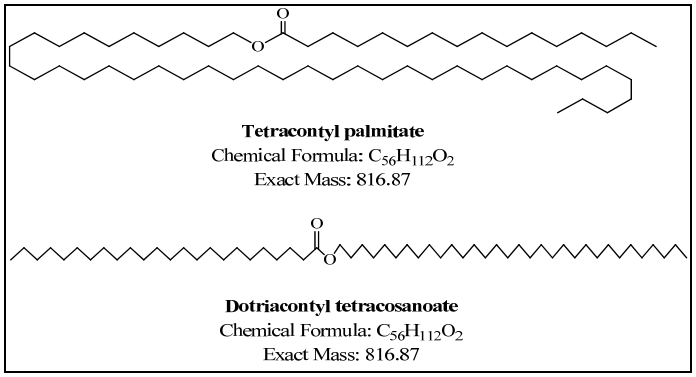
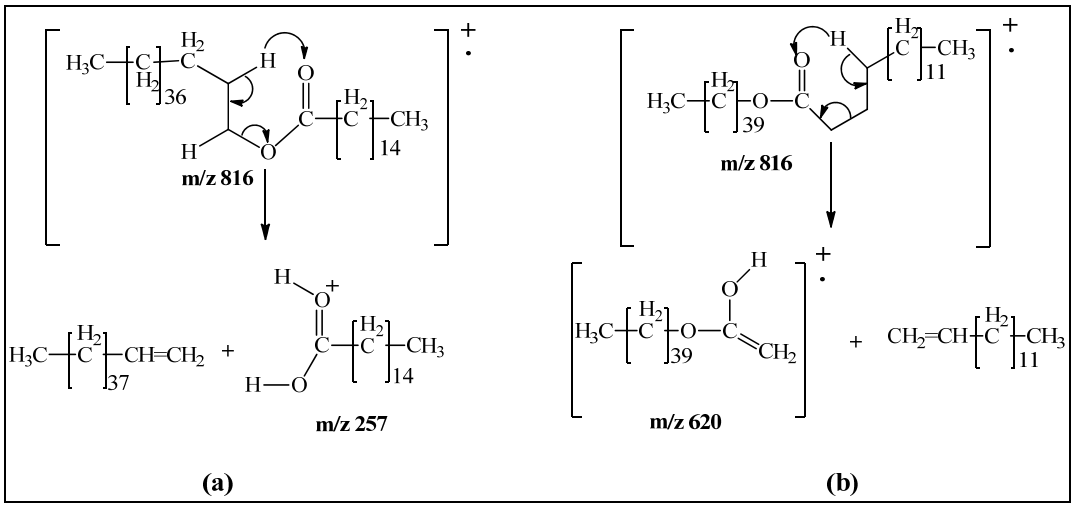


Fig 7: Probable molecular structure of SH1S1 (C1)



Scheme 2: Selective fragmentation pattern of SH1S1 (C1)

But, the mass of m/z = 257 $[M-559]^+$ or $[CH_3(CH_2)_{14}CO_2H_2]^+$ to a fragment ion of ester (palmitic acid ion) and m/z = 620 $[M-196]^+$ or $[CH_3(CH_2)_{40}CO_2H]^{-+}$ provide the clear indication that our isolated compound is tetracontanyl palmitate not dotriacontyl tetracosanoate (Scheme 2).

Again, the ethyl acetate extract was fractionated into different fractions by silica gel column chromatography. A white crystalline compound was observed along with some other yellowish gummy compounds. The crystalline compound was marked as Et-C1 and the gummy compounds were marked Et-C0 and Et-C2 as well. Analyzing of rest of the fractions is under progress.

The compound Et-C1 was a white crystalline solid. It was soluble in n-hexane, dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$) and ethyl acetate (EtOAc). The IR spectrum of the compound Et-C1 showed absorption bands at 2917, 2848, 1705, 1471, 1463 and 718 cm^{-1}. The peak at 1705 cm^{-1} was for carbonyl group (>C=O) of carboxylic acid or ketone, but the absence of an absorption band for O-H confirms that the carbonyl group was from ketone. From physical characteristics and spectral analysis of FT-IR, ^{1}H-NMR, ^{13}C-NMR of the compound Et-C1, some similarities was found with the previous compound SH1S1 (C1). Now in the IR spectrum, the absence of absorption bands for O-H and C-O led to the compound as ketone having long chain. In the ^{1}H-NMR spectrum (Fig. 8), a triplet around δ 2.3 ppm indicates the presence of methylene protons next to the carbonyl carbon ($-CH_2-CO-$), at the same time the absence of oxygenated methylene protons ($-CO-O-CH_2-$) which led to the compound as a ketone. The compound Et-C1, as ketone having long chain, also supported by the presence of a peak at about δ 178 ppm for carbonyl carbon (-**C**O-) and the absence of oxygenated methylene carbon (-CO-O-**C**H_2-) at about δ 60-65 ppm in ^{13}C-NMR.

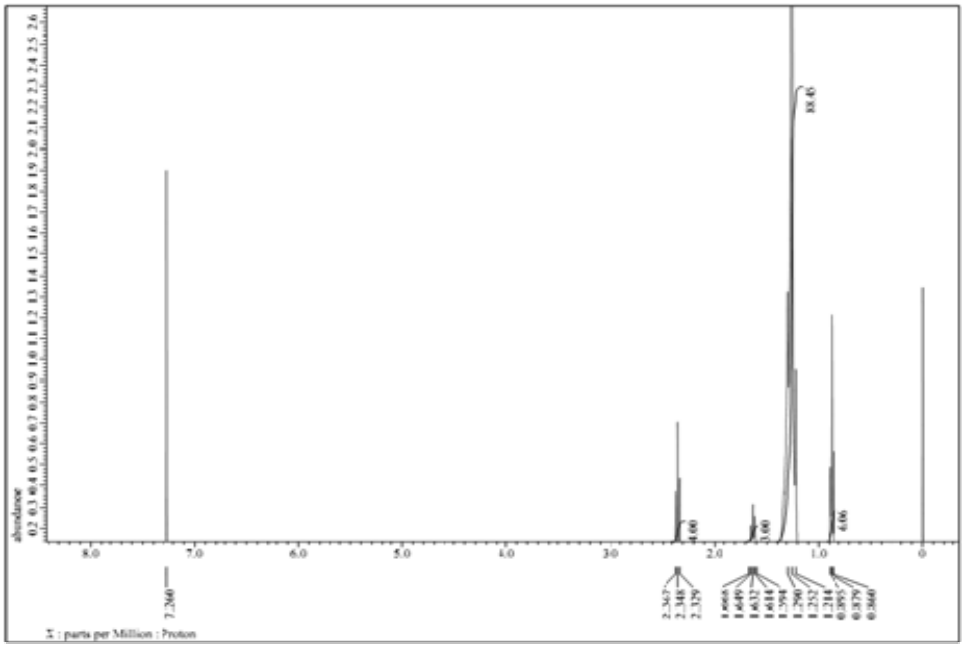


Fig 8: ^{1}H NMR spectra of Et-C1

From the above-mentioned analytical discussion and comparison with SH1S1 (C1), the compound Et-C1 was found to be "long chain ketone" having molecular formula of $C_nH_{2n}O$. So, the structure of the compound Et-C1 was tentatively proposed below (Fig. 9):

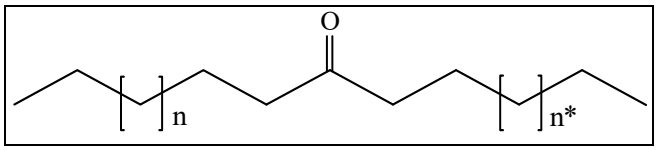


Fig 9: Proposed structure of compound Et-C1

Further spectroscopic study is going on to establish the final structure of this ketone.

We have also checkout the antibacterial activity of the isolated compounds. Analyzing the result it was clear that, the compounds Et-C0, Et-C1 and Et-C2 were active against gram-negative *Escherichia coli* bacteria. The activity was increased with increasing the polarity of the compounds. The highest inhibition zone, 7.00 mm, was observed for the compound Et-C2 (Table 2).

Table 2: Data of antibacterial activity test against *Escherichia coli* of the isolated compounds

Entry[a]	Compound	Activity	Zone of inhibition (mm)
1	Et-C0	Active	2.50
2	Et-C1	Active	4.00
3	Et-C2	Active	7.00
Positive control	Ciprofloxacin	Active	13.00

[a] Screening was performed in an incubation at 35-37 °C for 24-48 h.

4. Conclusion

In this report, we have successfully isolated and characterized a new wax ester tetracontanyl palmitate ($C_{56}H_{112}O_2$) and a ketone from *Malva verticillata* L. leaves (Napa shak). Wax esters have diverse biological functions in bacteria, insects, mammals, and terrestrial plants and are also important substrates for a variety of industrial applications [15]. Further purification of other fractions and methanol extract is our ongoing research. We are also looking the antibacterial activity of the methanol extract against some gram-positive and gram-negative bacteria.

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