Isolation and characterization of bioactive compounds from *Martynia annua* and its anticancerous activity against Huh-7 liver cancer cell lines

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Abstract

One of the fastest growing cancer types in the United States and the life-threatening illness is liver cancer. As a well-established model system for studying hepatocellular carcinoma (HCC), especially in relation to hepatitis C virus (HCV) infection, Huh7 liver cancer cell lines are regarded as critical in research because they enable scientists to examine the mechanisms of liver cancer development, drug efficacy and viral replication in a controlled laboratory setting. In essence, they serve as a vital tool for the development of treatments against liver cancer and diseases related to HCV. Compounds from medicinal plants are more preferred over synthetic antibiotics because they are less toxic, possess fewer side effects and are more easily absorbed by the body. Martynia annua is a medicinal plant found in the tropical and subtropical regions around the world, including Asia, Australia and Africa. In this study, a bioactive compound was isolated from Martynia annua and its anticancer activity was evaluated against the human liver cancer cell line Huh-7 using the MTT assay and DNA fragmentation assay. The crude extract of Martynia annua was subjected to fractionation using different organic solvents in increasing order of polarity.

The fractions that showed proper band formation and spots were then subjected to characterization techniques such as Gas chromatography-mass spectrometry (GC-MS), Fourier transform infrared spectroscopy (FTIR) and Nuclear magnetic resonance spectroscopy (NMR). The compound identified was oleic acid with molecular formula $C_{18}H_{34}O_2$ and molar mass 282. The compound was tested against an the Huh-7 cell line for cytotoxic activity and exhibited a significant decrease in cell numbers with IC_{50} value of 165.4µg/ml. Huh-7 cells treated with an IC50 concentration of Martynia annua extract for 24 hours. The current investigation demonstrated a decrease in cell survival due to the induction of DNA fragmentation.

Keywords: MTT assay, medicinal plant, liver cancer, *Martynia anuua.*

Introduction

With an anticipated 905,700 new cases and 8,30,200 deaths from liver cancer worldwide in 2020, it is a serious global health concern. Liver cancer ranks third globally in terms of cancer-related deaths and is the sixth most prevalent type of cancer diagnosed. Eastern Asia is home to more than half of the world's liver cancer cases and fatalities; 45.3% of cases and 47.1% of deaths occur in China alone[9,11,14]. The main risk factors for liver cancer include alcohol use, liver cirrhosis and hepatitis B and C infections. It is anticipated that between 2020 and 2040, the number of deaths from liver cancer will rise by 56.4%. One of the most widely used and thoroughly studied cell lines in liver cancer research is the Huh-7. The Huh-7 cells are well-differentiated and they possess stable growth patterns, making them suitable for long-term experiments.

Moreover, as they are tumorigenic, they help researchers to study the growth and formation of tumour. In hepatitis B and C research, they are used to study the pathogenesis and replication of hepatitis B and C viruses. In liver cancer biology, they play an important role to study metastasis, apoptosis and cell proliferation. In cancer therapy research, Huh-7 cells are utilized to determine the efficiency of targeted therapy and chemotherapy. Huh-7 cells are useful model for researching the biology of p53-related liver cancer since they carry a mutation in the p53 tumour suppressor gene. High telomerase activity, a characteristic of cancer cells, is seen in Huh-7 cells.

Among different medicinal plants, *Martynia annua* belongs to the plant group Martyniaceae and is commonly known as devil's claw or unicorn plants. This versatile plant previously came under the family Pedaliaceae, commonly known as the sesame family. It is indigenous to the New World and is found primarily in subtropical areas. Leaves and fruits are the most biologically active parts[22]. It is characterized by possessing a unilocular and bicarpellate ovary with parietal placentation[27]. The plant is a native of Mexico and is commonly known as Devil's claw in English, Kakanasika in Sanskrit, Vichchida in Gujarati and Bichu in Hindi[12,17].

Antioxidant qualities are possessed by this versatile *Martynia annua* leaves and roots contain fungicidal properties and are used to treat tuberculosis, sore throats and epilepsy. Fruits are also used as local sedatives. This plant in traditional medicine is used to cure tuberculosis, inflammation, wounds and sore throat. Nanoparticles have

also been synthesized from the leaves that possess significant antibacterial activities[20]. In gardens, *Martyniaannua* are grown as ornamental plants. It is actually a widely distributed weed and is noxious[26]. It was known to possess antitumour and proapoptotic effects on cancer cells and has antioxidant properties[13].

Hispidulin is the most abundant compound in flowers. It is considered to be a bioactive flavone and is a potent anti-cancer agent and also the strongest ligand of the GABA(A) receptor. The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood–brain barrier and exhibits anticonvulsive effects[18]. GC-MS studies revealed the presence of various compounds in seeds, leaves and fruits. The seeds contained stearic acid, palmitic acid, oleic acid, arachidonic acid, linoleic acid, malvalic acid and cyclopropenoid[7]. The leaves also possessed snapic acidc, p-hydroxy benzoic acid and chlorogenicacid and fatty acids such as stearic acid and palmitic acid. Pelargonidin-3 and cyaniding-3-galactoside are the compounds of flower[3].

Research has identified several phytocompounds present in *Martynia annua*. Iridoid glycosides are among the phytocompounds that have been found in *Martynia annua*. According to reports, these substances have antioxidant, antibacterial and anti-inflammatory properties. Triterpenoids that have been extracted from *Martynia annua*, including ursolic acid and oleanolic acid, have been demonstrated to possess anti-inflammatory, antibacterial and antioxidant properties[23]. Numerous research investigations have focused on the Huh-7 liver cancer cell line. Huh-7 cells have recently been employed to examine how various substances affect the development of liver cancer.

One study discovered that sofosbuvir inhibits DENV1 genome replication in human hepatic Huh-7 cells, indicating that it might have antiviral properties. The function of particular genes in Huh-7 cells has been investigated in other investigations. The impact of various treatments on liver cancer cells has also been studied using Huh-7 cells. Researchers discovered that in hepatocellular carcinoma, curcumin mediated resistance to lenvatinib through the EGFR signaling pathway. Additionally, a study demonstrated that senescence is induced by preventing methionine catabolism[8].

Given the traditional use of *Martynia annua* in medicine and its rich phytochemical composition, there is a strong rationale for conducting research on its anticancer potential. The aim of the study is to specifically identify the bioactive compounds by isolating and characterizing phytochemicals present in *Martynia annua*, which may lead to the discovery of novel anticancer agents. Assessment of the anticancer activity of *Martynia annua* extracts and isolated compounds using *in vitro* (cell-based) methods may provide valuable insights into their therapeutic potential. Elucidation of the molecular mechanisms underlying the anticancer effects of *Martynia annua* compounds by performing assays may facilitate the development of targeted therapies.

The whole plant was chosen for the extraction and purification of bioactive chemicals to prove their efficacy in the treatment of cancer. Using several solvents, the current work aims to extract the physiologically active substance from *Martynia annua* leaves in order to study, separate and purify the chemical components responsible for anticancer activity.

Material and Methods

Collection and preparation of plant material: The entire *Martynia annua* plant was collected from Padappai in the Kancheepuram district of Tamil Nadu. The species was identified at the National Institute of Siddha, Ministry of AYUSH (Govt of India), Chennai and the voucher/specimen number is NISMB7182024 dated 03.12.2024. After three weeks of shade drying, the plant material was ground into a fine powder with an electronic blender. The plant was pulverized and 25 g sample was subjected to extraction using Soxhlet in the following increasing polarity order: hexane, methanol and water. In addition, the extract was concentrated using a rotary evaporator and kept for later use at 4˚C[1].

Fractionation of the extract by column and thin layer chromatography: Using the ideal solvent ratios, the crude extract of *Martynia annua* was subjected to silica gel (100–200) mesh size normal phase column chromatography. The 100g of silica gel and the non-polar solvent hexane were combined to make a slurry, which was then agitated with a glass rod to get rid of glass bubbles. After that, the column was filled with the homogenous suspension. In order to facilitate the sample's simple distribution within the prepackaged silica gel column, 2g of silica gel and crude extract were combined to create a fine green powder prior to column loading. The ground material was layered over a silica column and to maximize the elution and isolation of different organic compounds from the plant-based extract, gradient solvent systems were used.

The solvent system used to elute the column was hexane:chloroform, then chloroform:ethylacetate and lastly ethylacetate:methanol in the ratio of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100. Oleic acid, a mono-unsaturated omega- 9 fatty acid, can be extracted using hexane, a nonpolar solvent, often used as an initial solvent for column equilibration and sample loading. A moderately polar solvent, ethyl acetate was used as a gradient solvent to elute oleic acid from the column. This stepwise gradient elution approach effectively separated compounds based on their polarity from non-polar to polar.

The 30 fractions in total were taken from each solvent system. Proper band formations or spots were observed only in the fractions 16- 24 and they showed the same Rf values

of 0.92. These fractions were carried out for further purification. TLC plates were run again with the selected fractions, re-extracted using various solvents and the band areas were scraped off from the plates and the substance was eluted from the silica with ethanol or methanol and was given for characterization studies to identify the compound[1].

Gas Chromatography-Mass Spectroscopy Analysis: With a glass column SGE BPX5 and a capillary dimension of 30 m x 0.25 mm x 0.25 µ, the GC-MS instrument (GCMS-QP-2010) was used to analyze the extracts of *Martynia annua* and its fractions. The temperature inside the oven was programmed to 80–260°C. The temperatures at the interface and the inlet were 200°C and 250°C respectively. Helium was used as the carrier gas, flowing at 1.0 ml/min. A temperature of 200°C was maintained for the ion source and spectra were recorded at the SRM Institute of Science and Technology, Tamil nadu, India[24,25].

FTIR Spectroscopic Analysis: The liquid compound was analyzed using attenuated total reflectance (ATR) and it is the most common method of FTIR spectroscopy for analysing liquid plant samples. The liquid sample is ensured to be free from contaminants and impurities. If required, remove particulate debris from the sample by filtering it with an appropriate filter. A gentle cloth and a light detergent were used to thoroughly clean the ATR crystal. To get rid of any remaining detergent, the ATR crystal was rinsed with purified water. To avoid water stains, gently wipe the ATR crystal dry. A tiny drop (about 1-2 µl) of the prepared sample should be carefully placed onto the ATR crystal's center. Using a pipette tip or a soft cloth, the sample was uniformly distributed across the ATR crystal.

For the best spectral quality, make sure the sample and the ATR crystal are in good contact. As necessary, the instrument's settings were modified (such as resolution and scan number). To extract useful information, the spectrum was analyzed using software techniques (such as spectral subtraction and peak assignment). The peak value in the infrared radiation portion of the FTIR spectrum was used to determine the functional group of the active chemical component [5].

^{1}H NMR and ^{13}C NMR Spectra: 1H NMR and 13C NMR techniques were used to identify bioactive compounds in extracts. Spectra were collected using Bruker spectrometers at 10 MHz for 1H NMR and 200MHz for 13C NMR, using DMSO and D_2O (methanol) solvents. Chemical shifts were measured in ppm (δ) and coupling constants were expressed in Hertz[16].

Cell Culture: Huh-7 (Human hepatocellular carcinoma) was procured from the National Centre for Cell Sciences (NCCS) located in Pune, India. Cells were kept in the phase of logarithm and developed in Dulbecco's modified Eagle Medium (DMEM) enhanced with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100µg/mL streptomycin. They were kept in an incubator with 95% air that was humidified at 37°C and 5% CO_2.

Anticancer activity: The samples anticancer activity was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay) on the Huh-7 cell line[4]. The cells were seeded onto 96-well microplates (1 x 10^6 cells/well) and they were allowed to develop to 70–80% confluence in an incubator with 5% CO2 for 48 hours at 37°C. After that, the medium was changed and the cells were exposed to various sample concentrations before being incubated for a full day. After 24 hours, the morphological differences between the treated and untreated (control) cells were examined under a digital inverted microscope (20X magnification) and captured on camera. After that, the cells were cleaned with phosphate-buffer saline (PBS, pH 7.4) and each well received 20 µL of the (MTT) solution (5 mg/mL in PBS).

After that, the plates were left in the dark at 37ºC for two hours. After dissolving the formazan crystals in 100 µL DMSO, the absorbance at 570 nm was measured using spectrometry. To determine the percentage of viable cells, the following formula was used:

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100

DNA fragmentation assay: One of the distinctive biochemical features of apoptosis is the cleavage of DNA by a specific nuclease called caspase-activated DNase (CAD). During apoptosis, the DNA strand is cleaved by CAD and generates a number of DNA fragmentations of 180-200 base pairs known as DNA ladders. These DNA fragments can be extracted from the cells and visualized using agarose gel electrophoresis. Agarose gel electrophoresis and DNA extraction were carried out using the protocol[20]. In short, DMEM medium containing 10% FBS was used to plate 1 × 10^6 cancer cells/ml per well in 6 well plates. After that, the cells were cultured for 24 hours under 37°C with 5% CO_2.

The medium was then taken out, cleaned with PBS and replaced with fresh serum-free medium. It was then maintained for one hour at 37°C in a CO_2 incubator. The cells were treated with IC_{50} concentration of the isolated compound for 24 hours. From the cell lysate, the DNA was extracted as follows after incubation. The cells were transferred into microfuge tube after washing the cells with PBS and addition of 0.5 ml of lysis buffer. Mixture was incubated at 37 °C for one hour, followed by the addition of 4 µl of proteinase K and it was further incubated for three hours at 50 °C.

0.5ml of phenol, chloroform and isoamyl alcohol (25:24:1) were added, mixed and centrifuged for 10 minutes at 4 °C at 10,000 rpm. Following centrifugation, 2 volumes of ice-cold absolute ethanol and 1/10 volume of 3 M sodium acetate were added to the supernatant and the mixture was then

allowed to sit on ice for 30 minutes in order to precipitate DNA. Centrifugation was carried out to pellet DNA at at 4 °C, 13,000 rpm for 10 minutes. After aspirating the supernatant, 1.0 milliliter of 70% ethanol was added to wash the pellet. Until all traces were eliminated, the centrifugation process was repeated and the the pellet was dried for about half an hour at room temperature and then reconstituted in 50 microliters of TE buffer. DNA samples containing 10 µg/ml were electrophoresed in a 1% agarose gel containing ethidium bromide for one hour under 90 V in a gel tank filled with TBE buffer. Using a UV transilluminator, the gels were examined [21].

Results and Discussion

Characterization of bioactive compounds of *Martynia annua*: The crude extract of *Martynia annua* was subjected to silica gel (100–200) mesh size normal phase column chromatography using the ideal solvent ratios. 2g of silica gel and crude extract were combined to create a fine green powder prior to column loading. The ground material was layered over a silica column and to maximize the elution and isolation of different organic compounds from the plant-based extract, gradient solvent systems were used. The stepwise gradient elution approach of column chrmomatography effectively separated compounds based on their polarity from non-polar to polar. 30 fractions in total were taken from each solvent system.

Proper band formations or spots were observed only in the fractions 16- 24 in the solvent ethyl acetate and they showed the same Rf values of 0.92. These fractions were carried out for further purification. TLC plates were run again with the selected fractions, re-extracted using various solvents and the bands areas were scraped off from the plates and the substance was eluted from the silica with ethanol or methanol as it helps to desorb or wash off the compounds from the silica gel due to its ability to disrupt hydrogen bonding and polar interations. Then the compound was identified.

Gas chromatography mass spectroscopy method for the identification of bioactive compounds: The present study on methanolic extract of *Martynia annua* leaf revealed the presence of six major bioactive compounds by the GC–MS. These compounds are dodecanoic acid, hexadecanoic acid, octadecanoic acid ethyl ester and cis-13-octadecenoic acid (Fig.1). Monosaturated fatty acid that is widely found in both plants and mammals, is oleic acid. It has a characteristic lack of color and smell. This particular kind of omega-9 fat promotes membrane fluidity and guards against oxidative stress from free radicals. Oleic acid (OA) has a strong ability to induce apoptosis in tumor cells through the activation of caspase-3[19].

FTIR Spectroscopic Analysis for Identification of Functional Groups in Methanolic Extract: Based on the peak value in the infrared spectrum, the FTIR spectrum determined the functional group of the active chemical components present in the extract. The peaks represent the stretching and bending vibrations that occur between sample molecules. Based on its ratio, the functional groups of the constituents were separated when the extract was put through the FTIR.

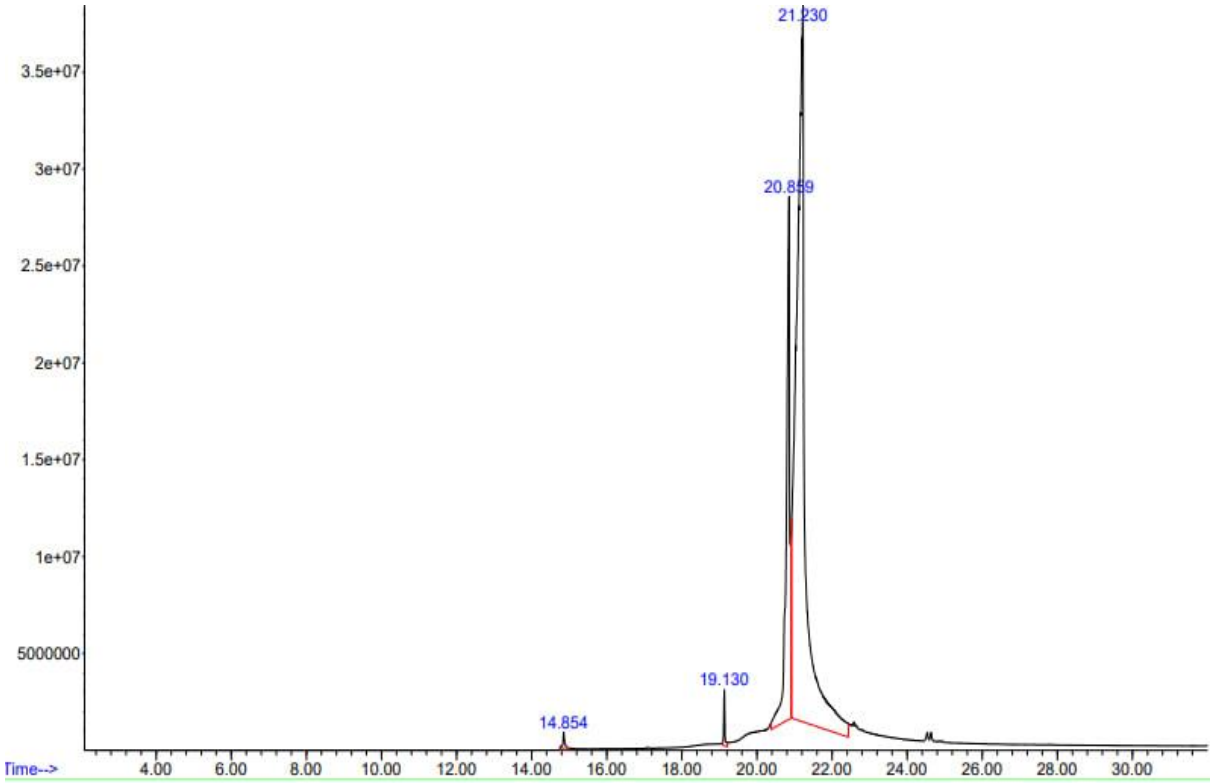
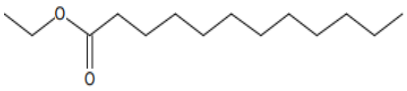
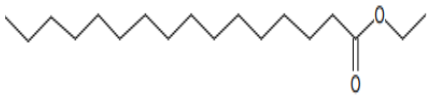
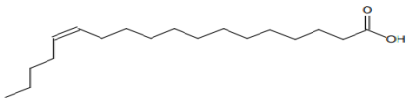


Figure 1: Chromatogram of *Martynia annua* isolated compounds

Table 1
Identified compounds in Chromatogram of *Martynia annua*

Name	Formula	MW	RT	Area %
Dodecanoic acid	$C_{14}H_{28}O_2$	228	14.854	0.436%
Hexadecanoic acid	$C_{18}H3_6O_2$	284	19.130	0.747%
9-Octadecenoic acid ethyl ester	$C_{20}H_{38}O_2$	310	20.859	21.150%
cis-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282	21.230	77.666%

Table 2
Identified compounds with their chemical formula and biological activities

S.N.	Compounds	Chemical Structures	Biological Activities
1.	Dodecanoic acid		Antiviral, antifungal, anti-inflammatory, wound healing, immune modulation, Antimicrobial
2.	Hexadecanoic acid		Antioxidant, anti-inflammatory, hepatoprotective, cytotoxic, antimicrobial
3.	9-Octadecenoic acid ethyl ester		Skin conditioning, low toxicity anti-inflammatory
4.	cis-13-Octadecenoic acid		Anti-inflammatory, lipid metabolism modulation, anti-cancer and anti-obesity effects

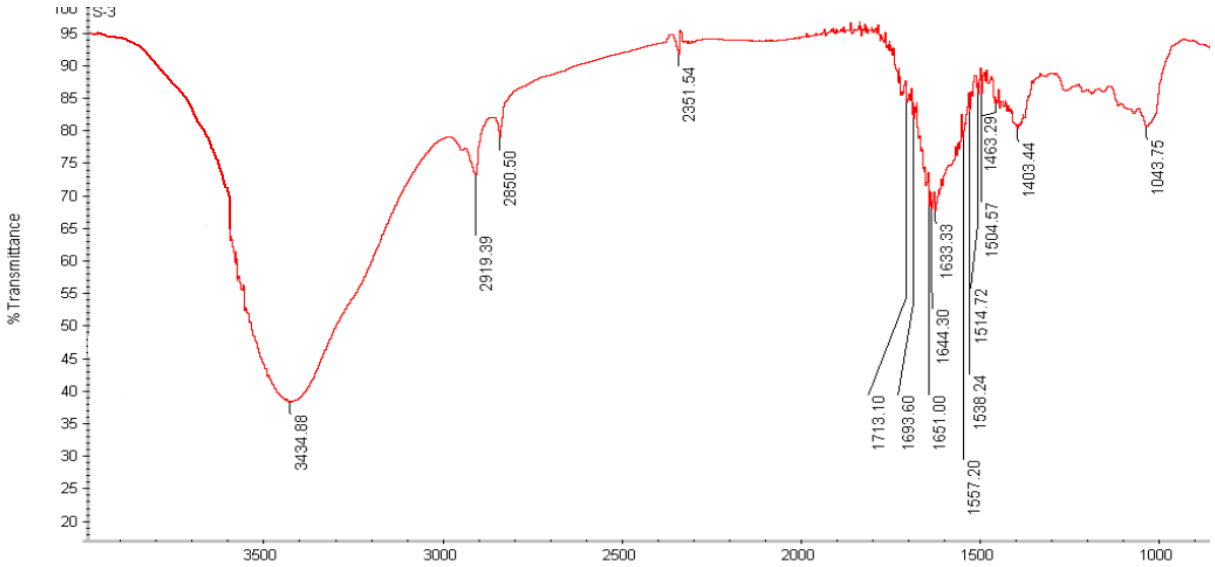


Figure 2: FTIR peak values of *Martynia annua* methanolic extract

In fig. 2, the peak values indicating functional groups were shown. The FTIR spectroscopy exhibited a broad band at 3434.88cm^{-1} indicating the presence of a hydroxyl group, 2919.39cm^{-1} for C-H group, 1713.10cm^{-1} showing the presence of a carbonyl group, 1633cm^{-1} for the C=C group, 1463cm^{-1}for the C-H bonding frequency and 1043cm^{-1} for the C-O group indicating the presence of the compoundoctadecanoic acid[2]. Absorption peaks at 1713.10cm^{-1} indicated the presence of functional groups C=O and peaks at 2850 indicated C-H stretching and peaks at 1043 indicatedC-C stretching for ethyl ester. Peaks at around 1500cm^{-1} due to the presence of -CH_2- groups in its longer chain, indicated the presence of hexadecanoic acid. Characteristic peaks at 2919cm^{-1} and 2850.50cm^{-1} due to C-H stretching vibrations and a prominent peak at around 1713.10cm^{-1} for the carbonyl (C=O) group indicate the presence of dodecanoic acid**.** Thereby the functional groups were confirmed with the FTIR.

Nuclear Magnetic resonance spectroscopy for the Identification of the Structure of the Active Constituents
^{13}C-NMR Spectroscopy: ^{13}C-NMR spectra showed two signals in the unsaturated carbon region at δ130.22 and δ114.06. The signal at δ139.27 might be a carbonyl carbon atom. The signal at δ14.10 might be due to methyl carbons and all other signals at δ22.56, δ22.68, δ26.73, δ27.19,

δ28.95, δ29.15, δ29.35, δ31.43, δ31.52, δ31.92 were due to methyl carbons in a long chain.

^{1}H-NMR Spectroscopy: The ^{1}H-NMR spectrum of the compound exhibited signals at δ0.83 as a pair of triplets overlapping each other, indicating the presence of methyl groups at δ1.32 for a long chain of methylene proton, at δ1.62 for methylene groups to C=C group,and at δ2,12 for methylene group to carbonyl group. δ multiple signals appeared at 3.71 which may be attributed to the oxymethylene group and at 5.43 for the unsaturated proton.

The 13C NMR, 1H proton and MS fragmentation were used to predict and clarify the structure. The chemical formula $C_{18}H_{34}O_2$ was found for the stable compound and the structure of the compound was suggested to be fatty acid (oleic acid) (Fig. 5). We anticipate the presence of oleic acid based on the spectral research mentioned above.

The predominant compound is cis-13-octadecenoic acid is an omega-7 monounsaturated fatty acid (MUFA) that exerts distinct metabolic and physiological effects. A key biological function is its conversion into conjugated linoleic acid (CLA**)**, a bioactive lipid associated with anti-inflammatory, anti-obesity and potential anti-cancer effects. Research indicates that oleic acid may positively influence lipid profiles by balancing LDL and HDL cholesterol levels while lowering triglycerides, though outcomes depend on overall dietary intake.

Additionally, it exhibits modest antibacterial properties, particularly against Gram-positive pathogens and could play a role in shaping gut microbiota. Its presence in human breast milk suggests possible importance in early development. Though structurally classified as a *trans* fat, oleic acid's natural origin and metabolic fate distinguish it from industrially produced harmful variants. Ongoing studies investigate its therapeutic applications for metabolic syndromes and inflammatory conditions.

Anti-cancer activity: The effect of botanical extracts at different concentrations, such as 50,100,150,200, 250 µg/ml was tested on Huh-7 liver cancer cell line by MTT assay at incubation period of 24 hours[10]. The standard drug used was doxorubicin. The MTT test is a precise and straightforward technique that yields valuable quantitative information about the antiproliferative capacity of natural extracts. Cell analysis designated in figure 7 shows that the *Martynia annua* extract caused development inhibition of Huh-7 liver cancer cell lines in dose dependent manner. The botanical extract at IC_{50} value of 165.4µg/ml showed cytotoxicity against liver cancer cell lines. The cells reduced in size with obvious cell shrinkage and the number of cells diminished by chromatin clumping and destruction of monolayer.

DNA fragmentation: One of the hallmarks of apoptosis is DNA breakage. Two indicators that indicate the induction of apoptosis include DNA fragmentation and an irregular reduction in cell size, wherein the cells shrink and contract. A crucial aspect of apoptosis is the fragmentation of DNA into oligonucleosomal size pieces. Huh-7 cells treated with an IC50 concentration of *Martynia annua* extract for 24 hours in the current investigation demonstrated a decrease in cell survival due to the induction of DNA fragmentation. The apoptotic induction in the interspersed smears in the lanes is depicted in figure 9.

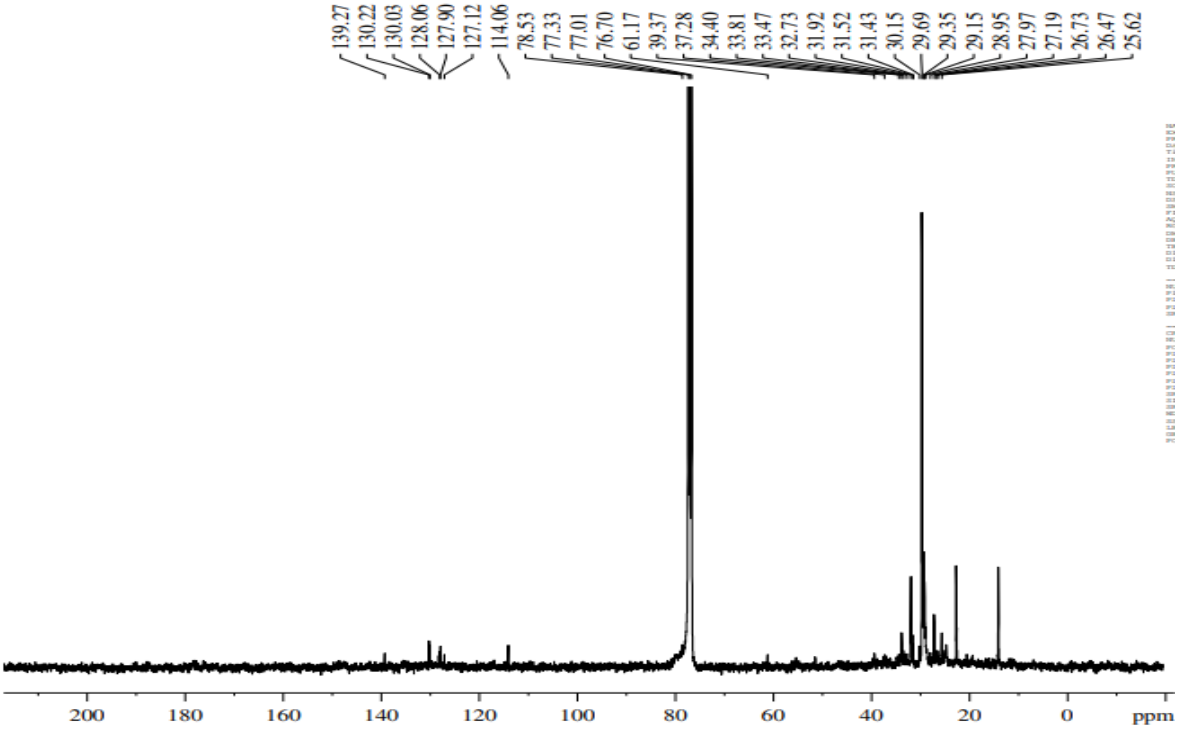


Figure 3: ^{13}C-NMR spectra of isolated compound from *Martynia annua* extract

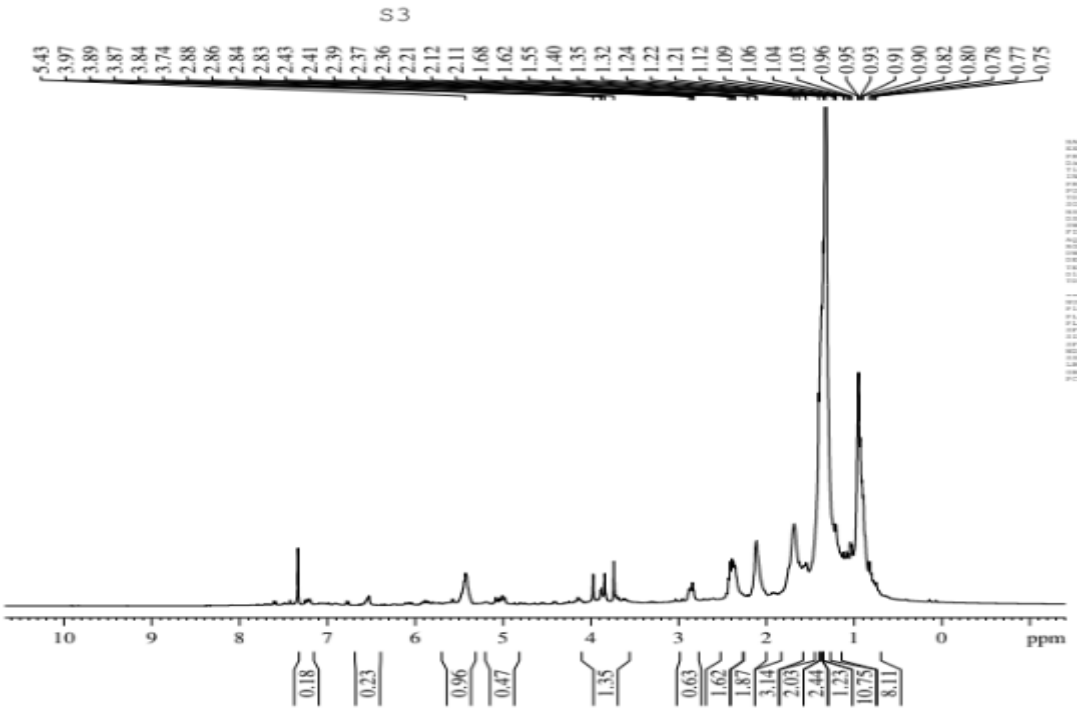


Figure 4: ^{1}H-NMR spectra of isolated compound from *Martynia annua* extract

Figure 5: the structure of secondary metabolites isolated fatty acid (OLEIC ACID).
Mol wt = 280.0, Mol formula = $C_{18}H_{34}O_2$

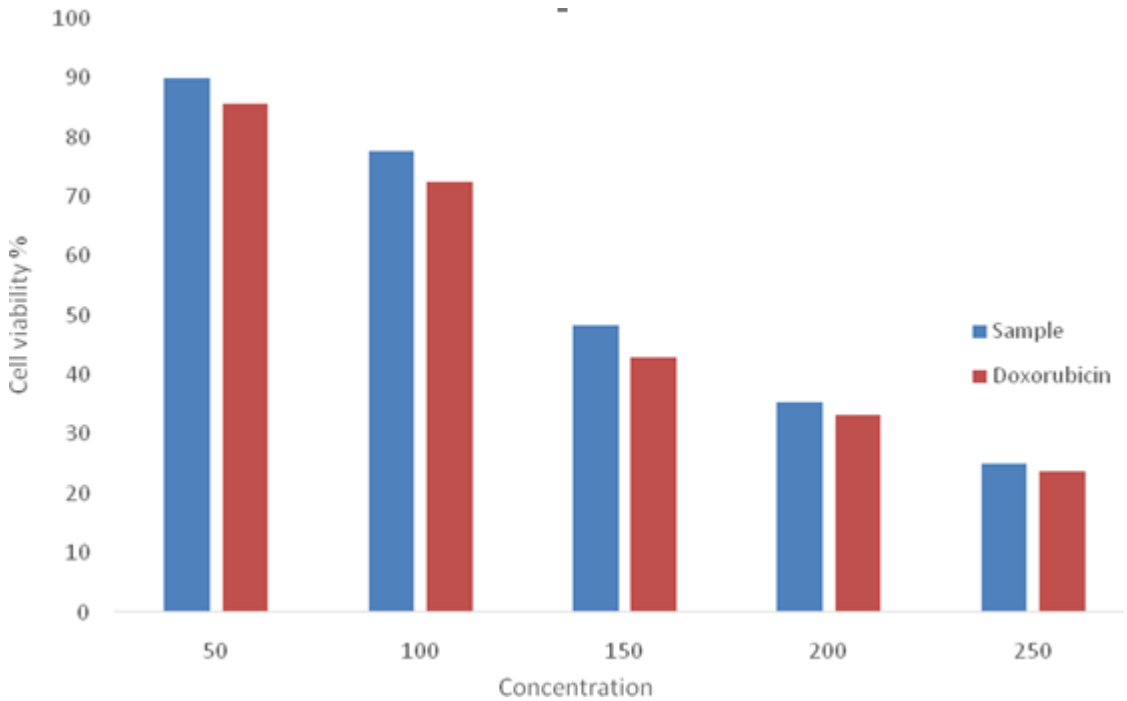
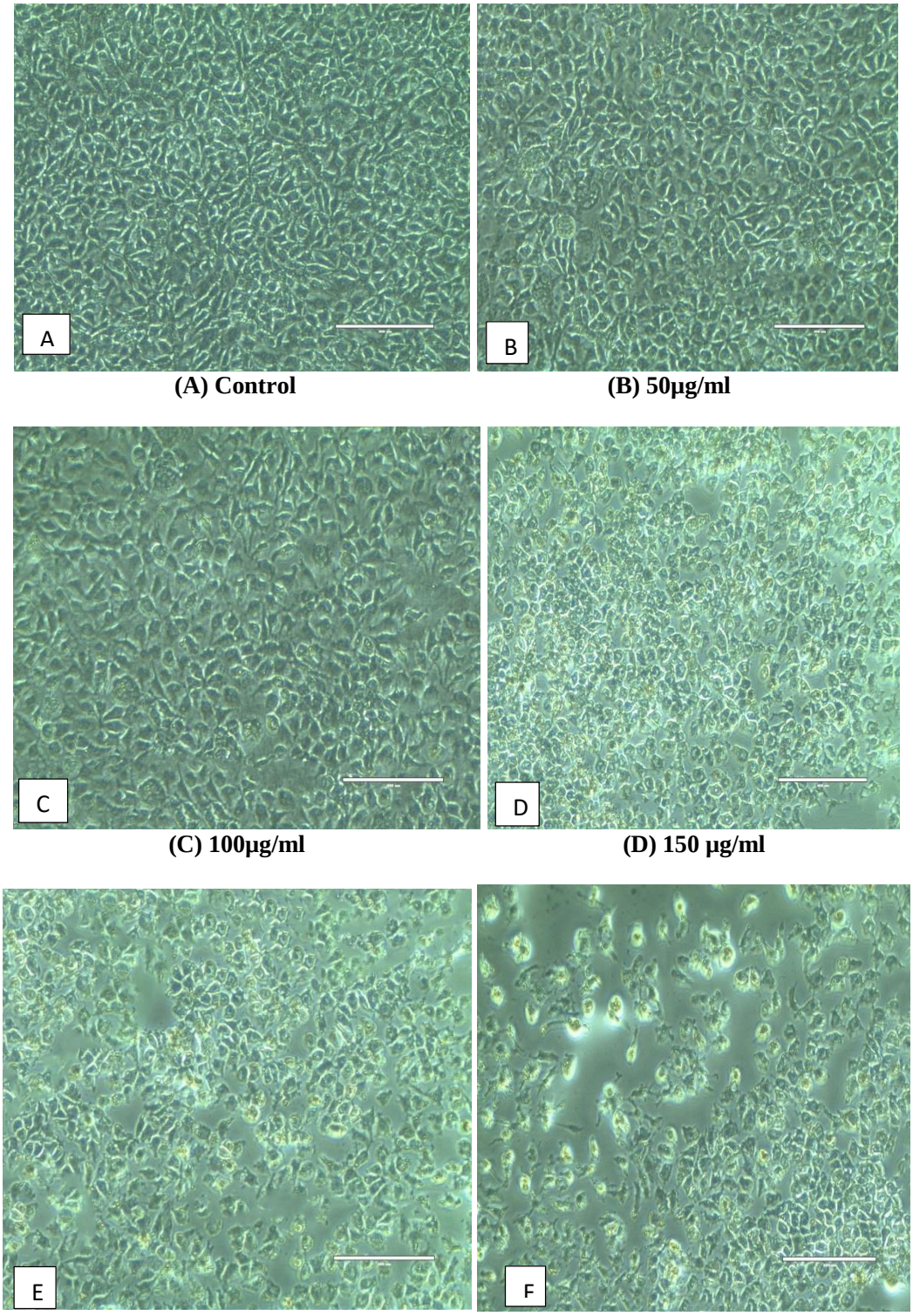


Figure 6: Effects of isolated compounds on Huh-7 liver cancer cell line



(A) Control **(B) 50µg/ml**

(C) 100µg/ml **(D) 150 µg/ml**

(E) 200 µg/ml **(F) 250 µg/ml**

Figure 7: Effects of different concentrations of *Martynia annua* extracts on Huh-7 liver cancer cell lines.

One possible cause of the smearing could be necrosis of post-apoptotic cells. By contrast, the untreated control cells did not show any sign of fragmentation or cell death[15].

Conclusion

The research demonstrated that a bioactive compound, oleic acid extracted from the *Martynia annua* plant exhibited notably strong cytotoxic effects on the live cancer cell line and had not been isolated in the plant before. Although there are few reports of compounds from this family being isolated, some of these compounds may be present in other members of the Martyniaceae family. Therefore, the plants primary anticancer components were fatty acids with excellent selectivity between malignant and healthy cells. It offers a fresh approach to anticancer drug development, with its highly selective index allowing for minimal toxicity to normal or healthy tissue in the body.

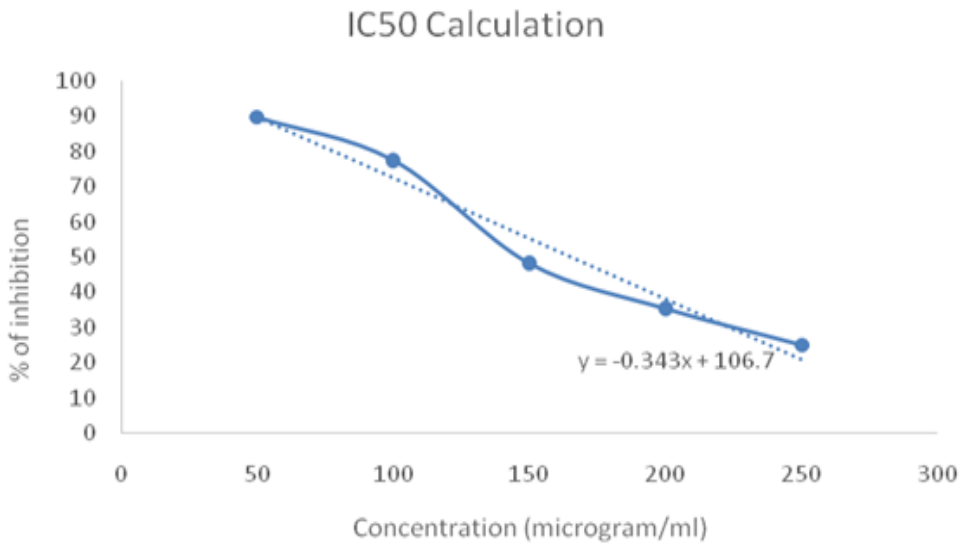
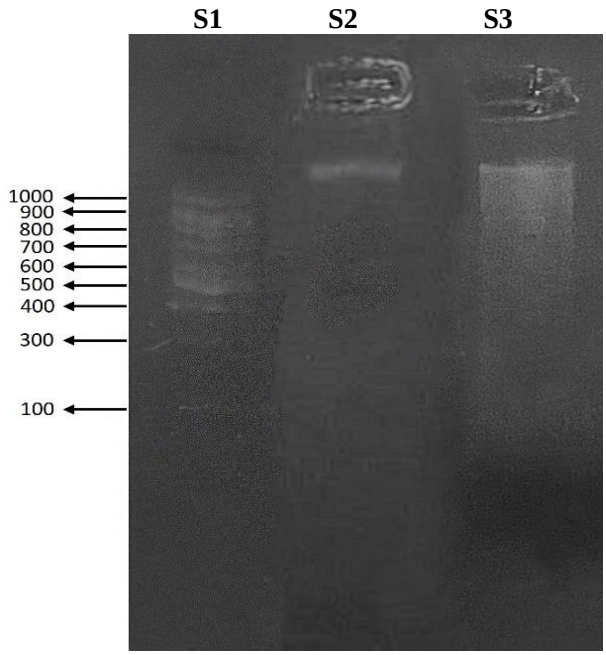


Figure 8: IC_{50} Calculation of oleic acid



S1 – Ladder
S2 – Control untreated Huh-7 cells
S3 – Sample 1 treated Huh-7 cells
Figure 9: DNA fragmentation of Huh-7 cells treated with the isolated compound.

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