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GC-MS Analysis and *In-vitro* Apoptosis Induction and Anticancer Activity of Methanol Extract of *Aspergillus terreus* against Lung Cancer

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Abstract

The present study was focusing on qualitative and quantification of bioactive compounds present in *Aspergillus terreus* and evaluating its anticancer activity and apoptosis detection against lung cancer. Methods: *A. terreus* was sequentially extracted using the Soxhlet extraction technique with hexane, ethyl acetate, methanol and distilled water. Detection of bioactive compounds was done using Standard biochemical tests and GC-MS analysis was performed with NIST database to identify the bioactive compounds. The toxicity and anticancer activity of crude extract was investigated using MTT assay on L929 cells and lung cancer A549 cells whereas apoptosis study was conducted through Flowcytometry-based surface marker study on the A549 cancer cell line. Results: secondary metabolites analysis showed the presence of phenols and terpenoids as major constituents in the methanol extract whereas other solvent extracts have shown the absence of major bioactive compounds. Quantification studies showed that methanol extract has shown the phenolic content 179 µg/g of Gallic acid equivalent. The GC-MS analysis showed the presence of 1-Flurodecane, Methyl palmitate, Ethyl palmitate, 9, 12-Octadecanopic acid, 10-Octadecanoic acid, Methyl stearate, Octadecadeoinoate, Ethyl 9-hexadecanoate and 1-Monoarachidin as major bioactive compounds. Further, MTT based toxicity study on the L929 cell line revealed that methanol extract at lower concentrations like 50µg, 100µg and150µg shown more than 50% of cell viability and at higher concentration between 200µg-250 µg it was showing toxic nature with 47.89±0.01% viability. In case of anticancer activity against lung cancer A549 cell line the methanol extract have shown the dose dependent activity i.e the percentage of cell viability was decreased with increase in the concentration of methanol extract at 250µg the cell viability was found to be 35.12±0.005%. Flow cytometry based apoptosis study revealed that methanol extract has shown the inducing apoptosis in treated lung cancer A549 cells with percentage of 10.84. Conclusion: overall the present study shown that *A. terreus* possess different class of bioactive compounds and it has higher phenolic content. Toxicity study showed that methanol extract exhibited toxic nature at higher concentration on tested cell line and Anticancer and Apoptosis study revealed that methanol extract has shown the prominent with inhibiting the growth of lung cancer A549 cells through inducing apoptosis. Further, *A. terreus* would be a promising natural microorganism that has to be further researched in order to discover and isolate potent drug to treat cancer. Future studies will be on study of in-vivo animal studies and study of molecular mechanism of drug action on particular with anticancer study.

Keywords: *A. terreus*, Lung Cancer A549, MTT, Flow Cytometry, Apoptosis

INTRODUCTION

Cancer has become the most hazardous issue related to health problems in everyone, regardless of age, gender, ethnicity, wealth, or socioeconomic status. As a result, it is without a doubt the biggest challenge facing the medical system and the scientific community in the twenty-first century. About 10 million people die from cancer each year, making it the second biggest cause of death in the world, and this number is continually rising.[1] The main cancer treatment involves a number of therapeutic techniques, such as surgery, chemotherapy, and/or radiation.[2] Lung carcinoma, often known as lung cancer, affects both men and women. It is recognized as the leading cause of death in the globe. Some of the treatment options for breast cancer include surgery, radiation therapy, hormone therapy, chemotherapy, and targeted therapy. These medications do have some serious negative effects, though.[3] Traditional foundational elements of the healthcare system have been natural substances. Approximately 80% of the population has utilized alternative medicines for primary healthcare, according to research.[4] The chemotherapeutic drugs used today to treat cancer give patient's temporary relief and lengthen their lives, but they also have drawbacks like side effects, lack of selectivity, and high prices that not only lower patients' quality of life but are also out of reach for millions of patients in developing countries.[5] In the past, natural products have achieved tremendous advancements in the field of anti-cancer research;

more than 60% of anti-cancer medications used in clinical trials now come from natural sources, such as plants and microorganisms.[6]

Natural products produced by microorganisms offer a wide range of potential novel medicinal compounds. Microbial metabolites can be utilized as targets for the discovery and development of novel medications, primarily anticancer and antibiotics, according to pertinent reviews.[7,8] Some of the most amazing chemical factories currently known to science are found in filamentous fungi like Aspergillus, Penicillium, and Talaromyces. In light of this, a variety of bioactives, including mycotoxins, antifungal, and anticancer compounds, have been described in the literature over the past more than 100 years.[9] A variety of anticancer chemicals have been produced by marine fungi, which are a significant source of bioactive molecules.[10] The decomposition of substrates and animal remains, for example, is one way that marine fungus, which can be found in many habitats, contribute significantly to marine environments. Due to the evolution of fungal cell biology and feeding mechanisms, marine fungi are categorized as saprotrophs, parasites, or symbionts (epiphytic or endophytic).[11-13]

A typical fungus utilized extensively in the chemical and pharmaceutical sectors is *Aspergillus terreus*. It serves as the primary strain for making the significant chemical intermediary itaconic acid.[14] Mevinolin, a key cholesterol-lowering medication first discovered from *A. terreus*, is still manufactured commercially by *A. terreus* through submerged fermentation.[15] Additionally, it has been demonstrated that *A. terreus* from a particular eco-environment is a rich source of bioactive natural compounds.[16,17] There are very less experimental reports were available on the anticancer studies on the *A. terreus* hence with this background in the present study *A. terreus* was undertaken to study qualitative and quantitative analysis bioactive compounds present in it along with the anticancer and apoptosis study against lung cancer A549 cell line using in-vitro assays.

MATERIALS AND METHODS

Crude extraction

After the mass production the around 50 g dried biomass of *Aspergillus terreus* was subjected to the process of serial extraction using soxhlet apparatus for 6-12hrs by selecting different solvents system based on polarity i.e hexane, ethyl acetate, methanol and distilled water. After the extraction each extract was concentrated by rotary vacuum evaporator and evaporated to dryness.

Detection of secondary metabolites

In the present study the different solvent extracts of *A. terreus* were subjected for screening of secondary metabolites based on standard biochemical tests for tannins, flavonoids, steroids, anthocyanin, alkaloids, terpenoids, glycosides, quinones, cardiac glycosides, coumarins, phlobatannins, anthraquinone, or phenols.[18, 19]

Estimation of total phenolic content

Through the use of Spectrophotometry, the total phenolic content of the *Aspergillus terreus* methanol extract was determined.[20] The crude methanol extract was made by combining 0.5 ml (with stock concentration of 1mg/ml) of dissolved with 2.5 ml of distilled water that had been dissolved in 10% FCR and 2.5 ml of 7.5 percent Na_2CO_3. Further test sample was incubated for 45min in dark conditions at room temperature. After the incubation the absorbance was measured at 730nm with use of spectrometer. Concurrently, 0.5 ml of methanol was used to prepare blank in a similar manner, but without extract. Gallic acid's calibration curve was created in the 20–100mg/ml range. Finally, the amount of phenolics was reported as mg of gallic acid per gram of dry weight.

Table 1. Phytochemical analysis of different solvent extracts of *Aspergillus terreus*

Tests	Hexane Extract	Ethyl Acetate Extract	Methanol Extract	Water Extract
Alkaloids	-ve	-ve	-ve	-ve
Flavonoids	-ve	-ve	-ve	-ve
Glycosides	-ve	-ve	-ve	-ve
Phenols	-ve	-ve	+ve	-ve
Saponins	-ve	-ve	-ve	-ve
Tannins	-ve	-ve	-ve	-ve
Terpenoids	-ve	-ve	+ve	-ve
Steroids	-ve	-ve	-ve	-ve

+ve: Present; --ve: absent

In-vitro toxicity and anticancer activity of methanol extract of *A. terreus* against L929 cell line and lung cancer A549 cell line

In the present study toxicity as well as anticancer activity was performed using the MTT standard technique. In the current investigation, Methanol extract of *A. terreus* was tested on normal l929 cell line and lung cancer A549 cell line at different concentrations (50, 100, 150, 200, and 250 µg/ml). Untreated cells are taken as a negative control, whereas cisplatin-treated cells are used as a positive control. The selected cell lines were plated for 24 hours; the cells were maintained in serum-free media for 24 hours. After the growth the spent media was removed and replaced with different concentrations of methanol extract of *A. terreus* with media were added and incubated for 24 hours. After incubation the treated cells were subjected to MTT treatment for 3 hours later medium was taken out and 200 µl of DMSO was added. Further, the amount of formazan produced at 595 nm was measured using an Elisa reader after the. Based on optical density (O.D) readings, the percentage mortality for each exposure level and control groups was calculated. The following equation was used to calculate the IC50 value based on the percentage of cells that survived.[21]

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of test group}}{\text{Absorbance of control}} \times 100$$

Table 2. Cytotoxicity of methanol extract of *A. terreus* against non-cancerous L929 cell line

Treatment	Concen. in µg	Percentage of cell Viability
Methanol extract of *A. terreus*	50	91.70±0.00
	100	76.92±0.02
	150	65.99±0.00
	200	53.71±0.01
	250	47.89±0.01
Standard drug	15µg	72.46±0.01

*Results are taken in triplicates and expressed as Mean±SE

GC-MS profiling

The methanol extract of *Aspergillus terreus* was further subjected for the identification of probable compounds based on Gas Chromatography with Mass spectral analysis. In the present study GCMS of model GCMS-QP2010S was used for analysis. The methanol extract underwent for separation in fused silica type of closed column with helium gas as a mobile phase. The 1 µl aliquot of the extract was injected into the equipment with specifications of Linear Velocity, Pressure:65.0 kPa, Total Flow:24.0 mL/min, Column Flow:1.00 mL/min, Linear Velocity:36.8 cm/sec, Purge Flow:3.0 mL/min, and Final Temperature adjusted to 280°C and run for 6 min., the initial temperature of the column was set at 80°C, while the injector temperature was set at 260°C Based on a comparison of the components'

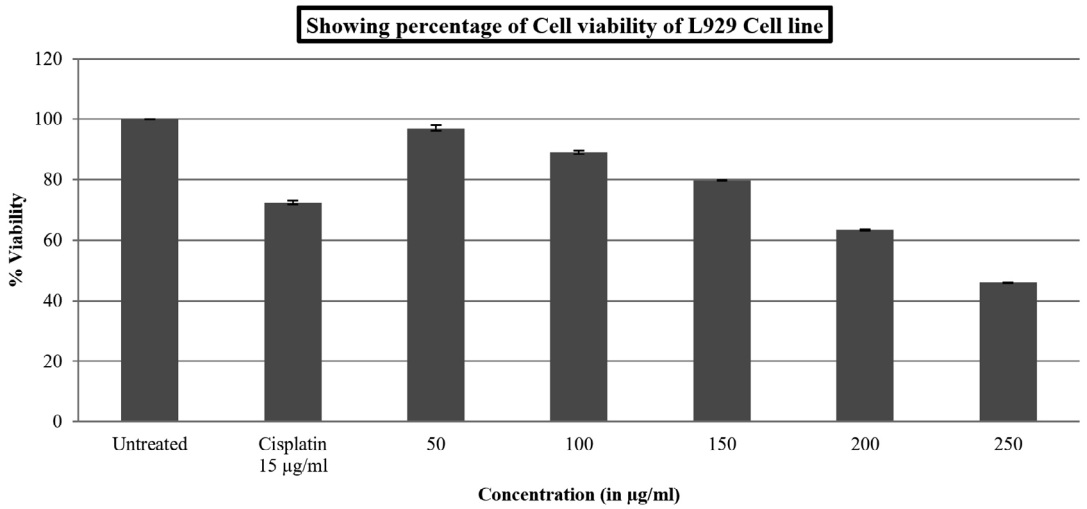

Figure 1. Effects of methanol extract of *Aspergillus terreus* on normal L929 cell line

mass spectra with those in the NIST mass spectral database, the components were identified.[22]

Apoptosis study by Flowcytometry

The pulmonary lung cancer A549 cells were plated in a 6-well flat bottom microplate with cover slips and incubated in CO_2 incubator at 37°C for 24 hours. After the attaining cells confluence the used media was replaced with fresh media with known concentration (204.16µg) of methanol extract of *A. terreus* and incubated for 24 hours. Cells were given two PBS washes after the incubation. 500 x g centrifuged for 5 minutes at 4°C. Re-suspend the cell pellets at 1 x 10^6 per mL in ice-cold 1X Binding Buffer after discarding the supernatant. Maintain tubes on ice. Then carefully mix in 2 L of PI and 5 L of AbFlour 488 Annexin V. Incubate tubes for 15 minutes in the dark while keeping them on ice. Gently stir in 400 L of ice-cold 1X binding buffer. Analyze cell preparations using flow cytometry within 30 minutes. FlowJo X 10.0.7 software was then used for analysis.[23]

Statistical analysis

In the current study, the experiments executed in triplicates (n=3) and after the performance and analysis the results were quoted as the mean ± standard deviation or standard error.

RESULTS AND DISCUSSION

In 2020, there are projected to be 18.1 million cancer cases worldwide, based on the report of Global Cancer Statistics it showed that in case of men and women approximate 9.3 million and 8.8 million respectively cancer incidents were identified.[24] In 2020, 12.5 percent and 12.2 percent of all new cases of cancer were diagnosed as breast and lung cancer, respectively. With 1.9 million new cases, colorectal cancer was the third most prevalent cancer in 2020, making up 10.7% of all new cases.[25] In 2020, 15.4% of all new cases of malignancy in males will be lung cancer, making it the most incident cancer type in men worldwide. Resistance to chemotherapy and reoccurring illnesses are also significant hindrances. To improve the effectiveness of treatment, new therapeutic approaches or anticancer drugs may be considered. To create anticancer drugs, a variety of plants, marine life, and microbes are used as sources. It is well recognized that bacterial and fungal metabolites are important

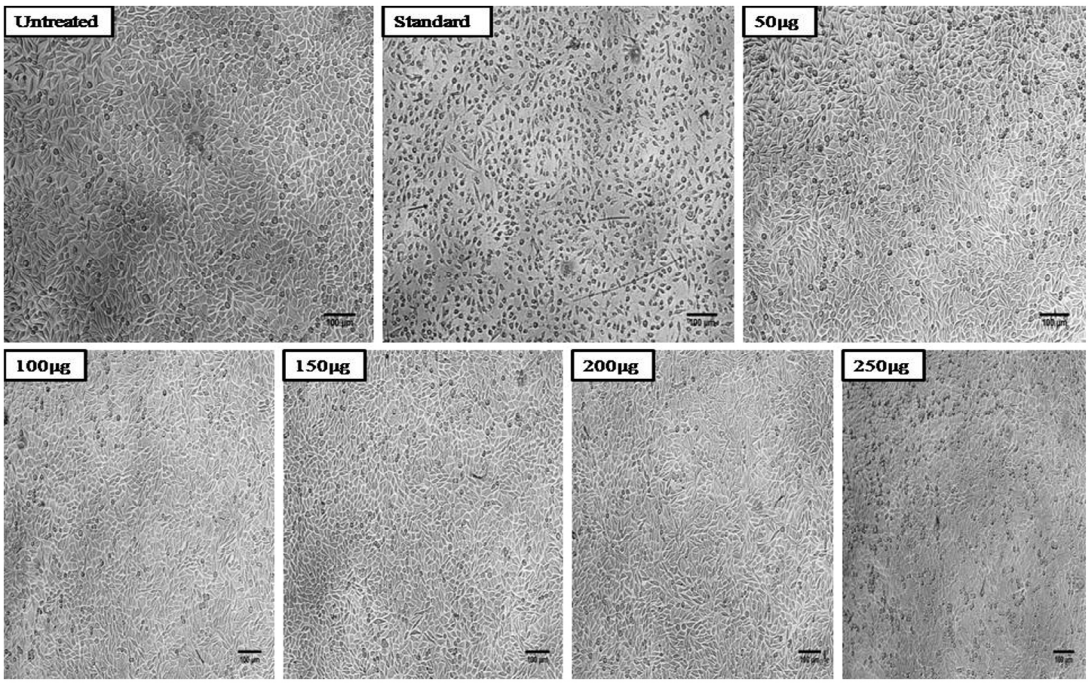

Figure 2. Showing morphological effects of methanol extract of *A. terreus* on L929 cell line

sources of bioactive substances in microorganisms. However, there are currently not many anticancer medications made from microbial metabolites.

According to research, phytochemicals have anticancer properties, and many of them are being employed to treat this well-known illness.[26-28] Recently several research findings revealed that phytochemicals isolated from plant origin have been used as potent drugs to cure many disorders but at the same time it is associated with several practical difficulties working with plants such as slow growth, abnormal habitat with less percentage of reproducibility of targeted desired phytochemcials.[29,30] Natural substances are a well-known significant source of potent anticancer medicines that help us fight cancer. Plants, microorganisms, and marine species make up the majority of the principal sources. A variety of substances that are successfully employed as chemotherapy drugs, including daunorubicin, doxorubicin, actinomycin D, bleomycin, and mitomycin C, are mostly derived from bacteria.[31] Numerous fungal metabolites are still being developed and tested in human therapeutic trials. For instance, the anti-angiogenesis agent fumagillin made from *A. fumigates*.[32] It was discovered that phenylahostin, isolated from *A. ustus*, is cytotoxic to a number of cancer cell lines, including those from the breast, colon, lung, ovary, and leukaemia.[33] It has been demonstrated that the mycotoxin anguidine, a member of the trichothecenes family and generated by a number of Fusarium species, prevents leukaemia cells from proliferating.[34]

Due to the production of primary metabolites that have beneficial products like enzymes, such as -amylase, amyloglucosidase, hemicellulase, and glucose, as well as citric acid, Aspergillus species are important economically.[35-38] Itaconic acid, a co-polymer used in the manufacturing of paint, is produced by *A. terreus*. *A. terreus* has the ability to produce secondary metabolites that can be exploited to produce therapeutically useful products, such as statins, a medication used to decrease cholesterol.[34,37]

According to the accepted practice for biochemical testing for secondary metabolites, the phytochemical analysis for *A. terreus* in the current study was carried out. The results of the phytochemical study indicate that, among the various solvent extracts, the *Aspergillus terreus* methanol extract has demonstrated the presence of phenols and terpenoids as key phytochemical constituents. The outcomes are displayed in Table 1. Phenols in general have drawn a lot of attention due to their crucial physiological functions (such as hormones, aliphatic membrane anchors, maintaining membrane structure), ecological

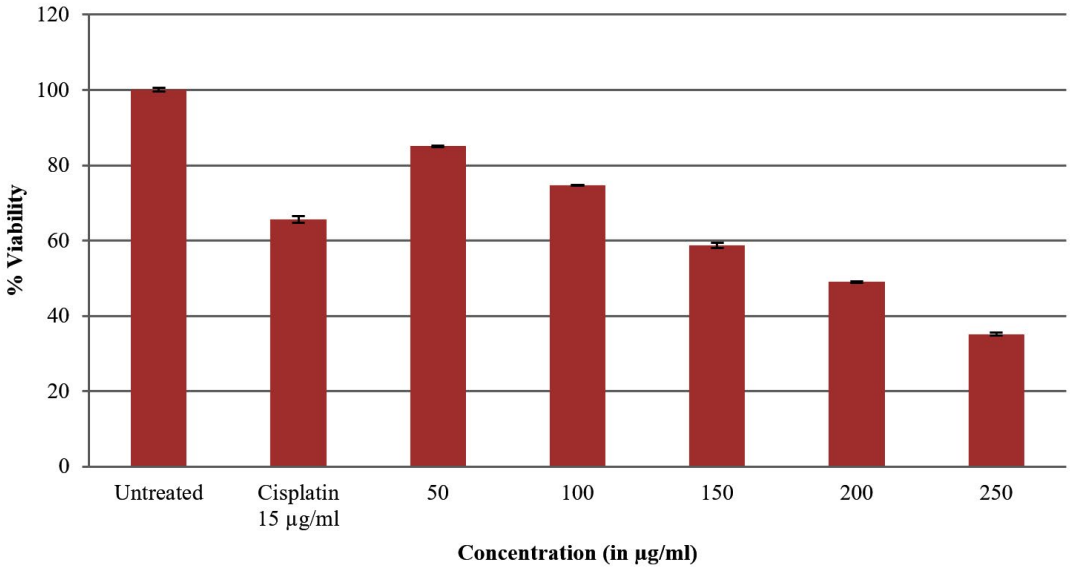

Figure 3. Percentage of cell viability of lung cancer A549 cells treated by methanol extract of *Aspergillus terreus*

functions (such as defence compounds, insect/animal attractants), and their numerous uses in pharmaceutical and industrial applications, ranging from flavors and fragrances to disinfectants and lubricants.[38,39] Triterpenoids, which are these substances' primary bioactive components, have analgesic and anticancer effects.[40]

Based on phytochemical findings, the total phenolic content of the methanol extract has been quantified using standard Gallic acid

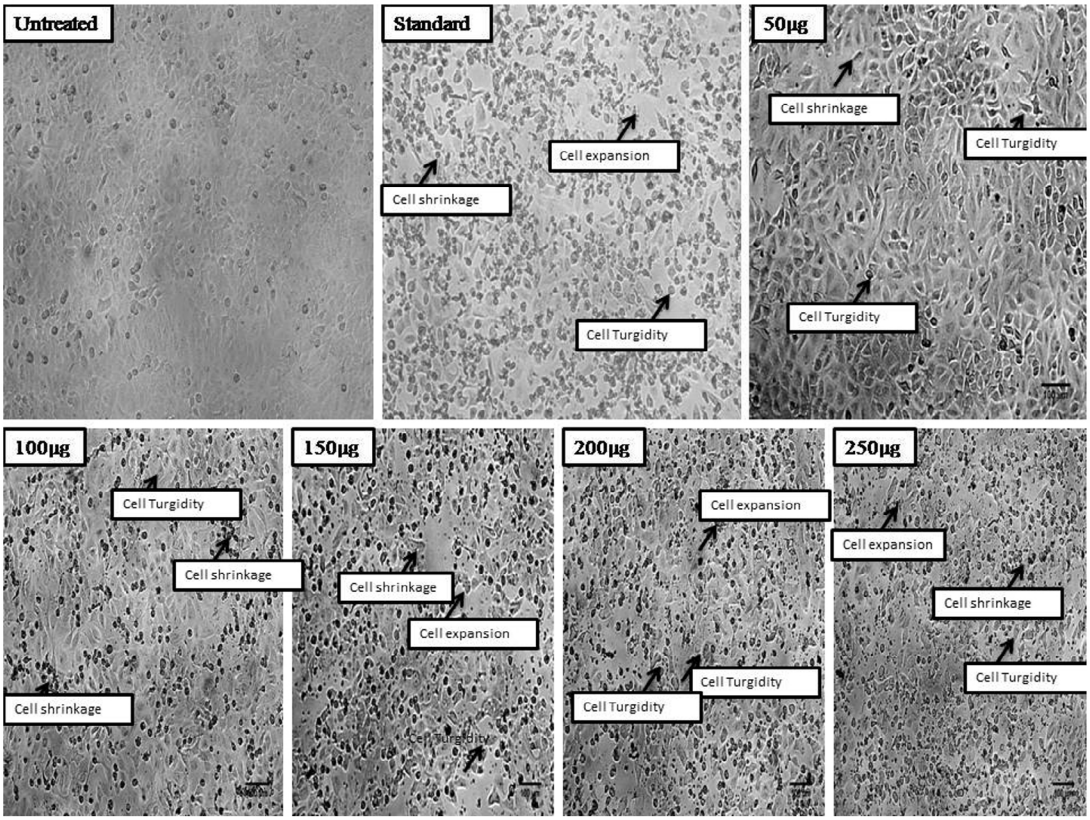

Figure 4. Morphological effects of methanol extract of *A. terreus* on lung cancer A549 cells

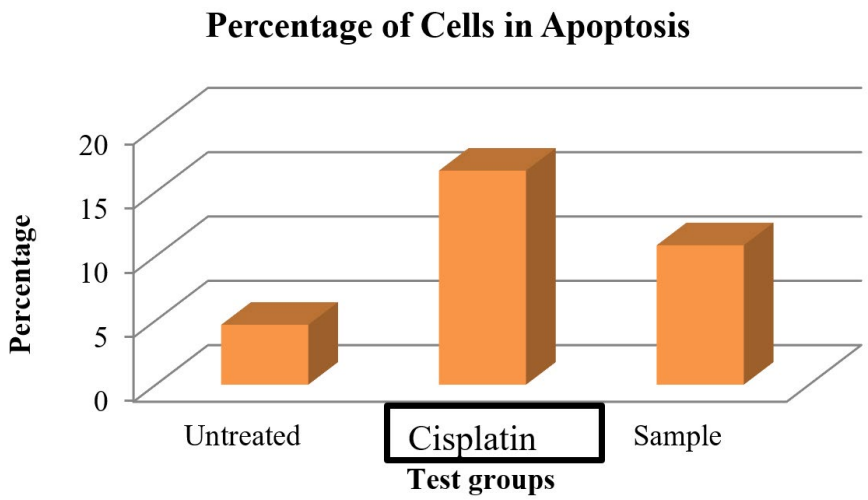

Figure 5. Percentage of Apoptosis of lung cancer cells

calibration curve (Y=0.001X+0.113) and it was found to be 176 µg/g. Phenols have the nature of antioxidant and possess several applications such as fight against ageing, cancer, and other diseases of the heart.[41] Applications of phenolic to enhance the nutritional content and quality of food are proliferating quickly in the food business.[42]

The methanol extract of *A. terreus* was tested for toxicity on the non-cancerous transformed fibroblast cell line L929 using the conventional MTT cell viability assay method before being examined for anticancer activities. In this test, the viability of the L929 cell line was assessed after exposure to methanol extract at various doses (50, 100, 150, 200, and 250µg/ml). The percentage of cell viability in the methanol extract-treated dropped as extract concentrations rose. Nearly higher concentrations of the extract have demonstrated its toxicity, with more than 50% of the cells being inhibited. Table 2 and Figure 1 present the findings. Figure 2 depicts the morphological impact of *A. terreus* methanol extract on the L929 cell line.

Despite considerable advancements in medical technology for its diagnosis and treatment, cancer is arguably the most deadly

Table 3. Percentage of cell viability in lung cancer A549 cells treated by methanol extract of *Aspergillus terreus*

Sample	Concentrations in µg	% of Cell viability	IC50 value in µg
Methanol extract of *Aspergillus terreus*	50	85.06±0.0025	204.16
	100	74.68±0.0015	
	150	58.70±0.011	
	200	48.98±0.0025	
	250	35.12±0.0055	
Standard drug Cisplatin	15	65.60±0.014	11.43

The results are represented as mean± standard error

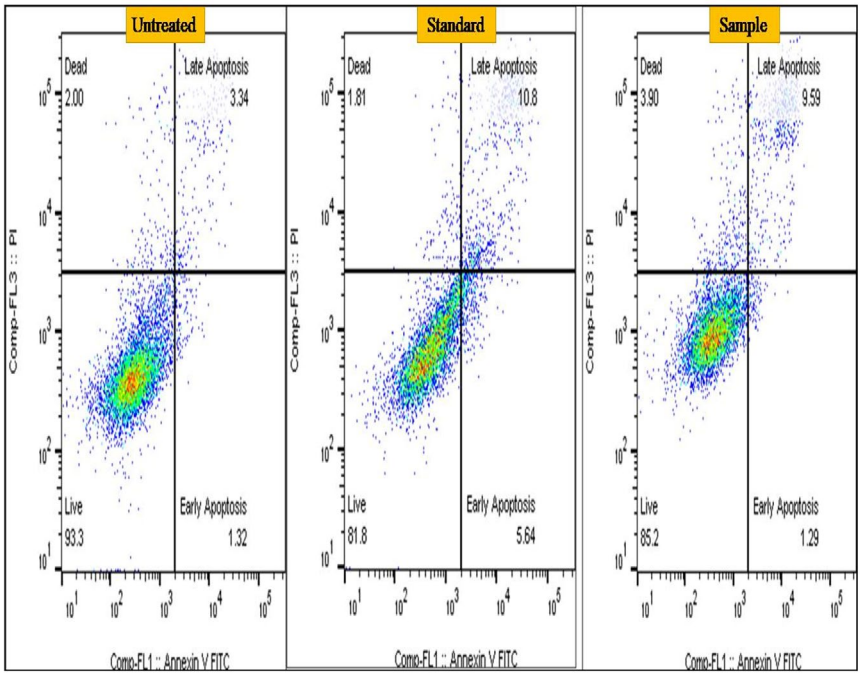

Figure 6. Detection of early and late apoptosis in lung cancer A549 cells treated by methanol extract of *A. terreus*

Table 4. GC-MS identified compounds present in Methanol extract of *Aspergillus terreus*

Peak No.	Compound name	Retention time	Base m/z	Nature	Uses
1.	1-Flurodecane	21.183	57.05	Aliphatic hydrocarbon	Used in the organic synthesis
2.	Methyl palmitate	28.368	74.05	Fatty acid methyl ester	It was used in the soaps and detergents industry and also acts as anti-inflammatory and anti-fibrotic agent
3.	Ethyl palmitate	29.681	88.05	Ethyl ester	It is used to produce soaps, cosmetics, and industrial mold release agents.
4.	9, 12-Octadecanopic acid		31.595	67.05	Fatty acid It is used for the treatment or prevention of cardiac arrhythmias.
5.	10-Octadecanoic acid		31.694	55.05	Fatty acid It is used in hardening soaps, softening plastics and in making cosmetics, candles and plastics.
6.	Methyl stearate	32.154	74.05	Fatty acid Methyl ester	It is used as solubilizing agents and unfolding of proteins.
7.	Octadecadeoinoate	32.797	67.05	Fatty acid	Cosmetics
8.	Ethyl 9-hexadecanoate		32.884	55.05	Ethyl ester It is used to produce soaps, cosmetics
9.	1-Monoarachidin	38.665	57.10	Natural prenylated resveratrol	Anticancer and Antioxidant

and progressing disease, posing a mortality danger to the entire planet. All cells are subject to oxidative stress; hence oxidation and free radicals generation leads to the incident of various tumor locations' carcinogenesis.[43] Both men and women can develop lung cancer, commonly known as lung carcinoma. It is regarded as the leading global cause of death. Lung cancer can be treated in a variety of ways, including surgery, radiation therapy, hormone therapy, chemotherapy, and targeted therapy, but each of these approaches has potentially severe adverse effects. Natural remedies have long been a cornerstone of medical care. Nearly 80% of the population,

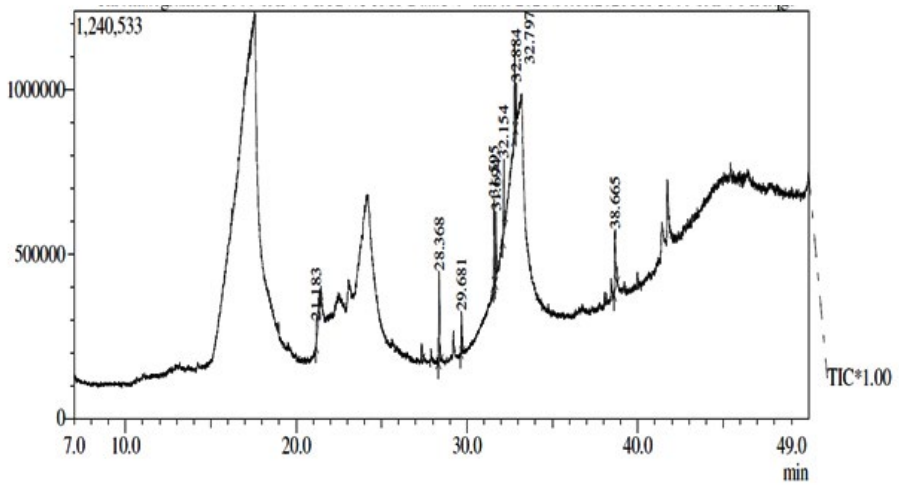

Figure 7. GCMS analysis of methanol extract of *A. terreus* (Continued...)

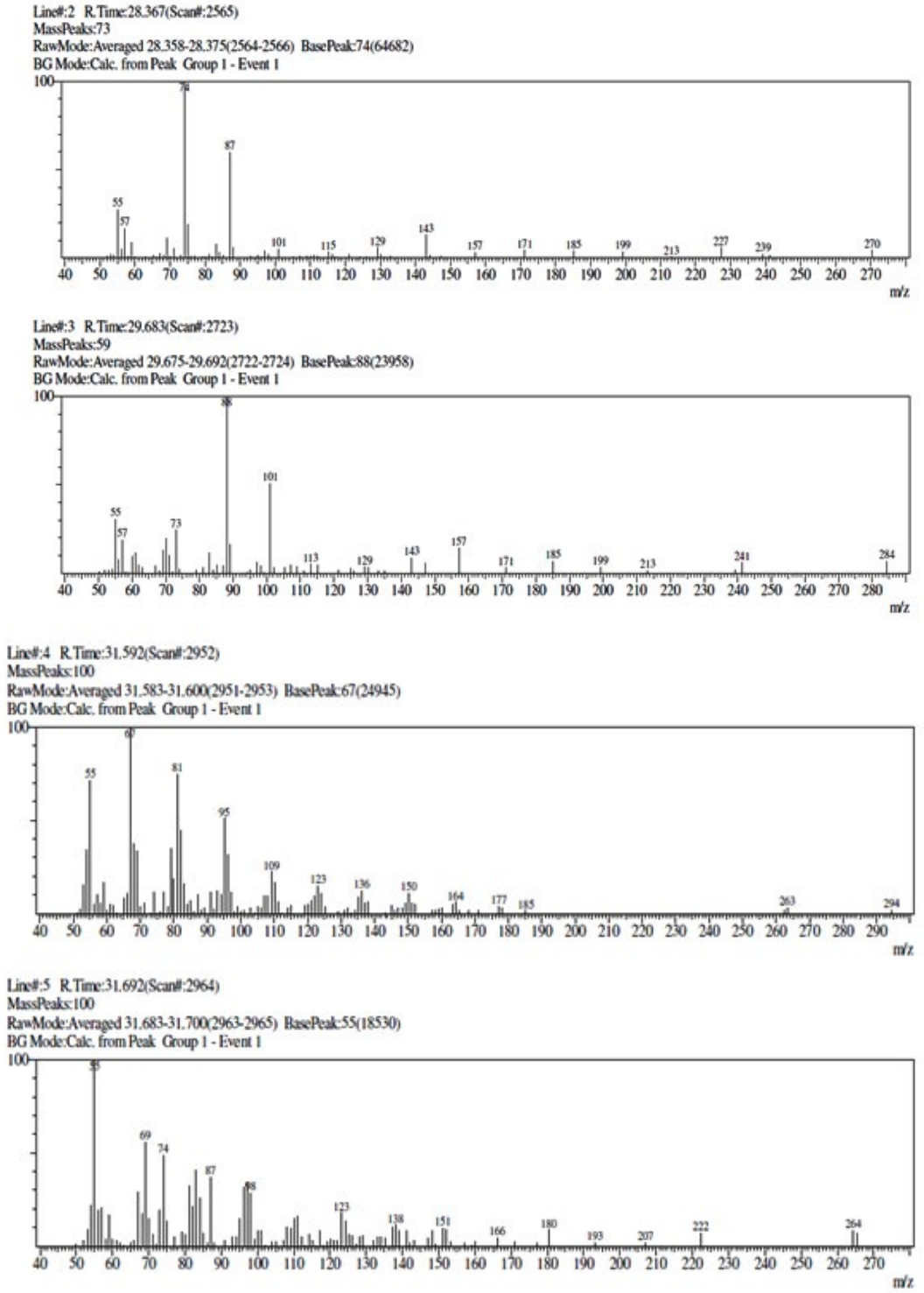

Figure 7. GCMS analysis of methanol extract of *A. terreus* (Continued...)

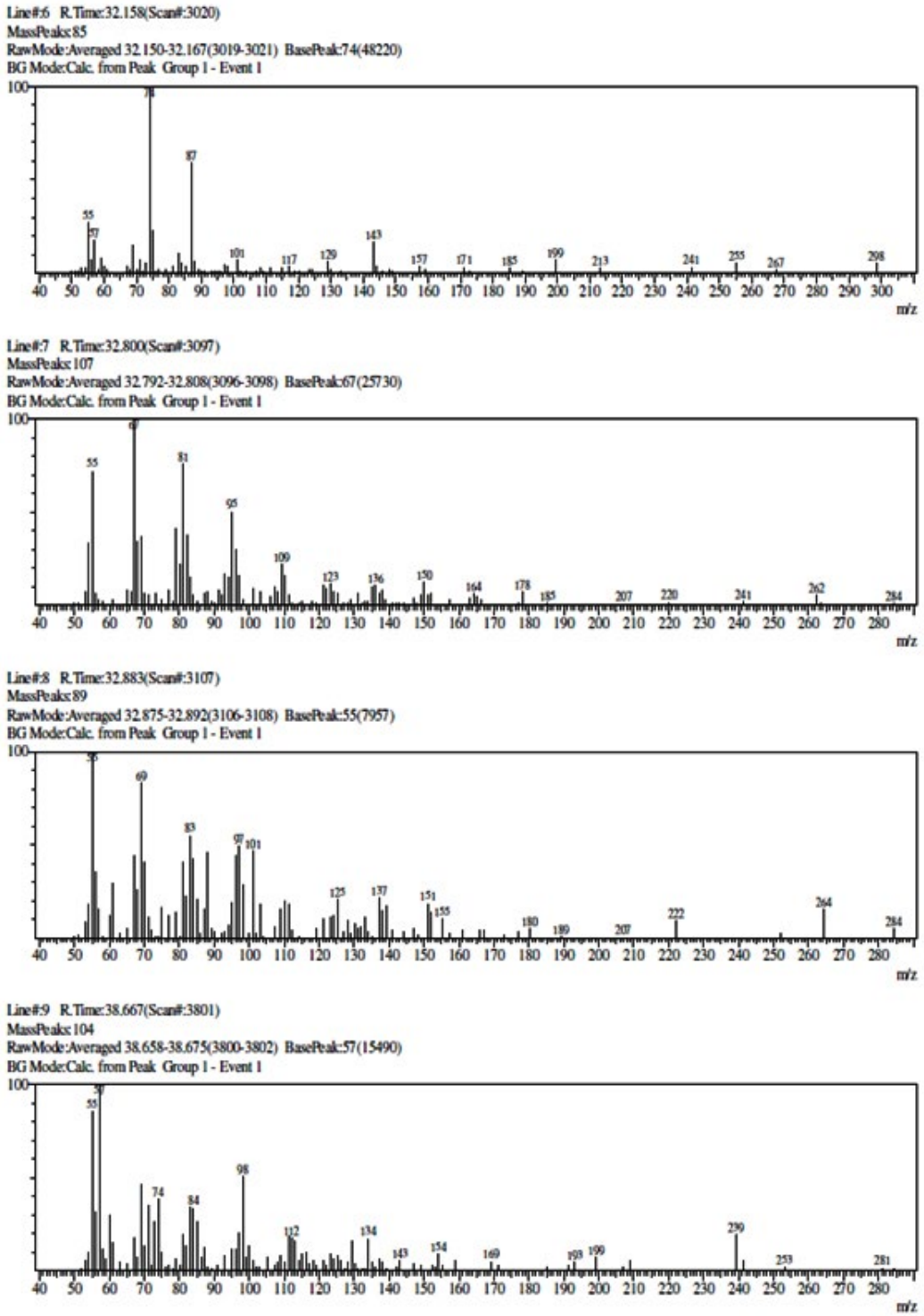

Figure 7. GCMS analysis of methanol extract of *A. terreus*

on average, uses natural medicine systems for primary healthcare, according to observations3. In the present study different concentrations of the methanol extract of *A. terreus* (50µg, 100µg, 150µg, 200µg and 250µg) were treated to the lung cancer A549 cell line. The cell viability results shown that as the concentration of test sample was increased there is gradual decrease in the percentage of cell viability. In the methanol extract treated A549 cells the percentage of viable cells in lung cancer was found to be 85.06% at 50µg concentration and at higher concentration i.e. 250µg it was found to be 35.12 and for standard drug cisplatin it was about 65.60 (Table 3 and Figure 3). It was evident from looking at the cell viability tested methanol extract shown appreciable results and demonstrated to be more efficient against lung cancer A549 cell lines. These findings even correlate with the morphological results and the MTT outcomes are correlated. Compared to untreated cells, the test samples of treated A549 have different cellular shape like elongated, ruptured and uneven shapes whereas untreated cells had a distinct spherical shape without any cellular spaces. Contrarily, in extract treated cells it was found dose dependant manner with formation of intra cellular spaces between the cells were seen (Figure 4) with several apoptotic characteristics such as cell expansion, cell shrinkage, membrane blabbing and cells turgidity. These were all characteristics of cells going through apoptosis. The test samples may be causing the lung cancer A549 cell line to undergo apoptosis, according to MTT and microscopic examination findings.

Apoptosis (planned cell death) is characterized by a number of different features such as release of protein phosphatidylserine through the plasma membrane, internucleosomal DNA breaking, membrane blabbing, chromatin condensation and fragmentation, and membrane blabbing.[44] As a result, one of the best cancer treatment methods is triggering apoptosis.[45] Phosphatidylserine (P.S.) on the plasma membranes outer layer functions as a marker site in causing the early stages of apoptosis.[46,47] The membrane's outer layer contains phosphatidyl-serine (P.S.) is specifically binds with the targeted protein like Annexin V.[48] The process of phosphatidyl-serine translocation is ongoing. In the current work, A549 lung cancer cells that have undergone treatment were used to determine early and late apoptosis using flow cytometry. The findings of current work showed that untreated cells experienced a small percentage of apoptosis that may have been caused by natural mechanisms, whereas methanol extract treated A549 cells shown around 10.84% of cells with apoptosis and whereas for standard drug cisplatin it was found to be 16.64% (Figure 5 and Figure 6).

Gas chromatography (GC), one of the most used techniques in chromatography procedures, has emerged as one of the most crucial instruments for the separation of phytocompounds. Recently, GC-MS has solidified its position as a potent method for identifying secondary metabolites in both plant and non-plant species.[49] In the current investigation, GC-MS analysis was used to determine the potential chemicals present in the methanol extract. The gas chromatogram has nine strong peaks, according to the GC-MS findings. To identify the substances, each peak will be put through a similarity search utilizing the NIST library database. 1-Flurodecane, Methyl palmitate, Ethyl palmitate, 9, 12-Octadecanopic acid, 10-Octadecanoic acid, Methyl stearate, Octadecadeoinoate, Ethyl 9-hexadecanoate, and 1-Monoarachidin have all been detected in a methanol extract of the *A. terreus* (Table 4 and Figure 7). These reported compounds are known to have a variety of uses, including use in the cosmetics sector, detergents, flavouring agents, and the pharmaceutical industry. According to Sermakkani and Thangapandian,[50] 9, 12-Octadecandionoic acid has anti-inflammatory, antimicrobial, hypocholesterolemic, and hepatoprotective effects.

CONCLUSION

Aspergillus terreus biomass was used in the current study, and it was extracted using various polar solvents to produce different extracts, which were then tested for the presence of secondary metabolites. Methanol extract contains phenols and terpenoids among the various solvent extracts. The presence of a

greater phenolic content in the methanol extract is also confirmed by a quantification analysis. In-vitro toxicity research findings shown that methanol extract was proven to be less toxic at lower concentrations but at the range of the concentration of 200-250ug/ml it was showing toxicity on tested normal cell line L929. Anti-cancer research A549 lung cancer cell line MTT assay demonstrates that methanol extracts exhibits dose-dependent activity as the concentration is raised. Flow cytometry study clearly shows that methanol extract was inducing apoptosis in selected lung cancer A549 cell line. The GC-MS analysis showed the presence of probable bioactive compounds present in the methanol extract of *A. terreus*. Overall study concludes that in the future the *A. terreus* can be taken as natural microorganism for the isolating potent anticancer drug. Further, in future there is a need for additional research on the separation, purification, and structural elucidation of chemicals needed for a specific activity with a specific mechanism.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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