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ORIGINAL ARTICLE

Acremonium strictum Isolated Oleic Acid Induces Apoptotic DNA Fragmentation in Mcf-7 Cell Line.

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ABSTRACT
Fungal endophytes are the microorganisms that colonize the inner tissue of plants and create a symbiotic relationship with each other. These endophytes are the rich producers of secondary metabolites that can be used for medicinal, industrial or agriculture purpose. The current study was undertaken to explore the secondary metabolome especially the fatty acid profile of Acremonium strictum, a fungal endophyte of various plants. A. strictum was inoculated on potato dextrose broth and checked for fatty acid production. Fatty acids were extracted using chloroform, methanol and water solvents. Fatty acid methyl esters were made from respective fatty acids and run on Gas Chromatography. The oleic acid fraction was extracted and further purified by High-Performance Liquid Chromatography. On average 9.12g/100ml of biomass was obtained from culture filtrates. Fatty acid profiling revealed 22.99 and 19.27mg/100g of oleic acid and pentadecenoic acid respectively were produced by A. strictum. The biopotency of oleic acid was checked on MCF-7 cells via apoptotic DNA fragmentation and DPPH assay. The DPPH assay showed at 200µg/ml concentration the scavenging activity of oleic acid reached 51.2%, which was comparable to that of reference standard butylated hydroxytoluene. Isolated DNA from oleic acid-treated cells showed DNA smearing at IC50 of 1.25 mg/ml, which was optimum enough to induce fragmentation in MCF-7 cell. Altogether the current study showed the biopotency of endophyte produced oleic acid on MCF-7 cells. This study opens new avenues to treat fatty acids as anti-cancer agents and hence more like studies are warranted to replicate the results.
Keywords: *Acremonium strictum*, Endophyte, Oleic acid, DNA fragmentation, Gas Chromatography

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INTRODUCTION

Fungal endophytes are extremely diverse microorganisms that live within plant tissues but usually remain asymptomatic [1]. They have been considered plant mutualists, benign commensals or latent pathogens. Being ubiquitously invasive in plant tissue many of them have been isolated from a range of host plants. They are considered good producers of bioactive compounds or secondary metabolites, that can be isolated from the host plant directly or from the endophytic fungi. The plethora of novel metabolites comprising various agrochemicals, industrial and chemotherapeutic substances possess the major advantage of low toxicity, minor environmental impacts and bioavailability [2].

Endophyte *Acremonium strictum* comprises a diverse group of specialized fungi that can be found in different host plants. They are included in the group of endophytes being disseminated by spores. *A. strictum* acts as plant-herbivore mediators with negative effects on larval growth rate and mortality; act as entomopathogen and show antagonistic effects against the pathogens; serves as a source of biological control agents of pests [3]. Certain *Acremonium* endophytes also help in enhancing plant growth and improved persistence even under conditions of drought and higher temperatures. Additionally, it has been previously shown that their produce metabolic products like pyrrocidines, polyketide aminoacid derived antibiotics that help in host defence mechanisms against the microbial pathogens [4]. Along with primary metabolites, a wide range of pharmaceutically significant compounds belonging to all structural classes were found to be produced by *A. strictum*. For example range of alkaloids, steroids, terpenoids,

polyketones, flavonoids, quinols and phenols as well as some chlorinated compounds were reported from this fungus [5].

Fatty acids are well-known antioxidants that help in preventing cancer in therapeutics. Although they are obtained from various living sources, endophytes remain unexplored as a rich source of fatty acids. However previous studies indicate extraction of fatty acids from filamentous fungi of various other ecologies such as mycorrhizal fungi, soil saprophytes, marine fungi etc [6]. Several unsaturated fatty acids and a few saturated branched-chain fatty acids exhibit antiproliferative activity against various cancer cells both *in vitro* and *in vivo* like breast cancer, liver cancer, colon cancer, etc. The anticancer mechanisms of free polyunsaturated fatty acids (linoleic acid, α - linolenic acid, γ - Linolenic acid), monounsaturated fatty acid and novel branched-chain derivatives like methyl oleate etc. have been proposed from various viewpoints based upon several studies [7-8]. Tested on tumour cells, the cytotoxicity of these fatty acids was correlated with extensive lipid peroxidation, cryptic glycolysis, membrane lipid modification and blood vessel remodelling [8]. I*n vitro* cytotoxic activities against cancer cell lines are determined using MTT (3, (4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) assay technique. In metabolically active cells the yellow water-soluble tetrazolium salt MTT is converted to purple, water-insoluble formazan by dehydrogenase enzymes of the mitochondria. The resulting intracellular formazan is directly proportional to the number of metabolically active cells. This colourimetric reaction can be measured spectrophotometrically. Antioxidant studies of free fatty have been analyzed via 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay methods showing potent free radical scavenging activity. Hence, the present study was carried to exploit the anticancer and antioxidant free radical scavenging activity of oleic acid in MCF-7 cell line via apoptotic DNA fragmentation assay.

MATERIAL AND METHODS

Extract of Oleic acid

Extraction of the Oleic acid was carried out employing the procedures of Strobel & Daisy 2003 [10]. Briefly, at the end of the incubation period inoculated flasks were collected and the culture fluid was passed through four layers of cheesecloth to remove solids and extracted with chloroform, methanol and water solvents. Both mycelia and filtrate were separately subjected to solvent extraction. The fresh mycelium from each flask was washed three times with sterile distilled water to remove adherent filtrate. The collected mycelium was then dried in a hot air oven at 60°C, crushed in a mortar, extracted with the solvent to obtain intracellular metabolites. Both crushing and extraction were repeated three times, left in a separating funnel for 15 min to precipitate. The crude extract was collected.

Total extraction of cellular lipids was done by disrupting the cells using a solvent mixture of chloroform, methanol and water to perform the extraction and the solvent ratios were balanced such that a single phase is formed with the sample water [11]. The lipids were suspended in the chloroform phase. The fat content was determined in an aliquot of the chloroform phase by weighing the lipids after evaporation of the solvent. Modification of this method was made by using hexane and acetone instead of chloroform and methanol. 30 mg dried biomass was powdered using mortar and pestle to disrupt the cells, extracted with 5 ml of acetone followed by 5 ml of hexane and the fractions were collected. The tube containing the fraction was centrifuged and the supernatant was collected. The remaining biomass was re-extracted twice by the addition of solvents that a total volume of 20 ml was then concentrated and diluted with the same solvent mixture for further analysis.

Gas Chromatography (GC)

Oleic acid along with other fatty acids was converted to Fatty acid methyl esters (FAMEs) before processing for GC. FAMEs were analyzed using GC equipped with Flame Ionization Detection (GC-FID). The FAME standards were prepared in a hexane solution at concentrations of 0.2 mg/ml. GC was performed on Agilent 6890 series Gas Chromatograph equipped with ALS 7673 auto-injector, FID and the capillary column VF- 23MS (30 m x 0.25 mm x 0.25 μ). Injector and detector temperatures were maintained at 230 and 250°C respectively. The oven was programmed for 2 min at 160°C then increased to 180°C at 6°C/min, maintained for 2 min at 180°C, increased further to 230°C at 4°C/min and finally maintained for 10 min at 230°C. The carrier gas nitrogen was used at a flow rate of 1.5 ml/min. The injection volume was 1 ml with a split ratio of 50:1.

Purification of Oleic acid

The extracted fatty acid methyl esters were then subjected to High-Performance Liquid Chromatography (HPLC). The analytical conditions were as follows: pump, LC- 5A (Shimadzu); column, Inertsil ODS-2 (4.6 × 250 mm; GL Science, Tokyo, Japan); detector, SPD-2A (Shimadzu); wavelength, 205 nm; mobile phase, acetonitrile/water (95:5, vol/vol); flow rate, 1 ml/min; and column temperature, 30°C. The fraction

containing oleic acid ester was eluted, concentrated and subjected for GC-FID analysis under the same conditions described above comparing with the standard.

Apoptotic DNA Fragmentation Assay

MCF-7 and Vero cell lines were purchased from National Centre for Cell Sciences Pune. The cells were thawed in a water bath at 37°C for approximately one to two minutes and added to 10 ml of pre-warmed (37°C) antibiotic-free RPMI 1640 (Sigma) growth medium containing 10% foetal calf serum (FCS) (Sigma). The cells were harvested and centrifuged (500 × g, 5 min, 25°C) to obtain a cell pellet and the supernatant was discarded. The pellet was resuspended in a 10 ml pre-warmed total growth medium and transferred to a cell culture dish. The cells were incubated in a humidified CO_2 incubator (5% CO_2) at 37°C until the monolayer was sub-confluent. The cells were treated with different concentrations of oleic acid for 16 hours. After treatment 0.5 ml of cell suspension was suspended and centrifuged at 200 x g at 4°C for 10 min. 0.5 ml of TTE (10 mM Tris (pH 7.4), 5 mM EDTA, 0.2% Triton solution was added to the pellet and vortexed vigorously. To separate fragmented DNA from intact chromatin, tubes were centrifuged at 20,000 × g for 10 min at 4°C. Carefully supernatant was transferred in new tubes and 0.5 ml volume of ice-cold 5 M NaCl was added and vortexed again vigorously. 0.7 ml of ice-cold isopropanol was added again and vortexed vigorously. Precipitation was allowed to proceed overnight at -20°C. After precipitation DNA was recovered by pelleting for 10 min at 20,000 × g at 4°C. The supernatant was discarded by aspiration or by rapidly inverting tubes and drops of fluid were carefully removed which remains adherent to the wall of the tubes with a paper towel corner. Pellets were rinsed by adding 0.5-0.7 ml ice-cold ethanol and centrifuged at 20,000 × g for 10 min at 4°C. Again supernatant was discarded by aspiration or by rapidly inverting tubes. The tubes were air-dried in an upright position for at least 3 h before proceeding. DNA was dissolved by adding to each tube 20-50 µl of TE (10 mM Tris–HCl (pH – 7.5) and 1 mM EDTA) solution. Samples of DNA were mixed with loading buffer by adding 10X loading buffer to a final concentration of 1X. Electrophoresis was run in standard 1X TBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA). DNA bands were visualized by placing the gel on a UV Transilluminator.

RESULTS AND DISCUSSION

The fungal isolate procured from IMTECH was evaluated by morphological typing and staining after sub-culturing. *A. strictum* colonies were white to pale pink slow-growing often compact and moist, became powdery, suede-like with age. Hyphae were fine and hyaline and produced mostly simple awl-shaped erect phialides of 20–40 µm. Conidia were usually one-celled (macroconidia) hyaline and pigmented, globose to cylindrical, and mostly aggregated in slimy heads at the apex of each phialide (Figure 1).

Figure 1: The morphotype of *A. strictum* and its microscopic visualization

The biomass of the dried cultures of *A. strictum* was weighed and the results were obtained. Higher yield of nearly 9.87, 5.85 and 11.65 g of biomass per 100 ml of *A. strictum* were obtained from the triplicates with the cultures grown in PDB under conditions of pH 6 and room temperature with 12 h cycle of light and dark conditions. The average biomass of *A. strictum* was 9.12g/100ml. Employing the fatty extraction method a total volume of 1 ml extract was obtained from 9g of the dried biomass. The final concentration of the extract was 13.7mg/ml. 1 ml of this extract was run on the TLC. The TLC profile revealed 4 different spots with Rf values of 0.54, 0.41, 0.32 and 0.7 indicating the presence of palmitic acid, stearic acid, oleic acid and triglycerides compared with the palm oil as standard.

The FAME profile of *A. strictum* revealed the presence of 7 fatty acids with a high percentage of oleic acid of 22.99mg/100g. Comparing the results of fatty acid profiling with the control strain, revealed the production of 6 fatty acids in common, yielding a higher percentage of oleic acid of 22.99mg/100 g followed by pentadecenoic acid of 19.27mg/100g. Further the overall percentage of total monounsaturated fat reveals an 80% (87.86mg/100g) increase and polyunsaturated fat reveals a 12% (12.14mg/100g) increase of fatty acid profile from the parent strain. Comparative qualitative analysis shows the difference in the production of the following six fatty acids: myristoleic acid, pentadecenoic acid, heptadecenoic acid, palmitoleic acid, oleic acid and linoleic acid (Table 1).

Table 1: Fatty acid profile of *A. strictum* as obtained by GC

S.No.	Fatty acid	Yield (mg)/100 g
1	Caprylic acid	0
2	Lauric acid	0
3	Tridecanoic acid	0
4	Myristoleic acid	14.15
5	Pentadecanoic acid	19.27
6	Palmitic acid	0
7	Palmitoleic acid	17.94
8	Heptadecenoic acid	13.51
9	Elaidic acid	0
10	Oleic acid	22.99
11	Linoelaidic acid	0
12	Linoleic acid	12.14
13	Tricosanoic acid	0
14	Lignoceric acid	0
15	Saturated fat	0
16	Poly unsaturated fat	12.14
17	Transfat	0

The purification of the oleic acid was carried by HPLC. Fractions were collected via HPLC and the methyl ester of oleic acid was eluted at 13.7 min (Figure 2). The fraction was further concentrated. Following comparisons with the standard via GC-FID, the purified oleic acid was checked further for its biological activity.

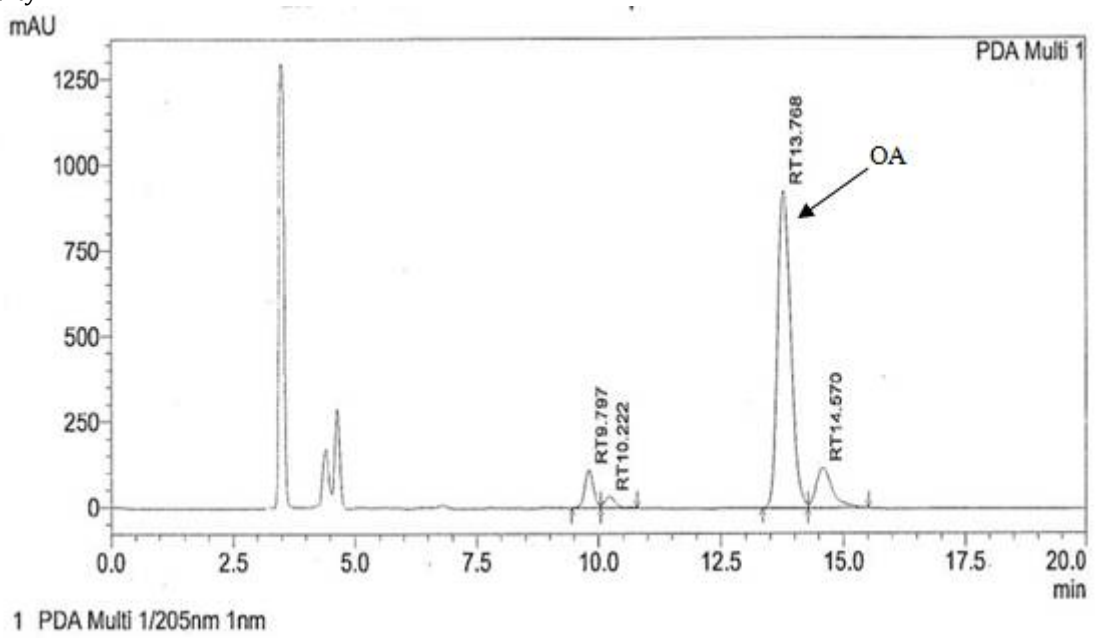

Figure 2: HPLC chromatogram for the purification of Oleic Acid

The radical scavenging activity of oleic acid fraction was determined using the α,α-diphenyl-β-picrylhydrazyl (DPPH) assay. The free radical scavenging capacity of the oleic acid was noted to be increased in a concentration-dependent manner. The sample was able to reduce the stable, purple-coloured radical DPPH into yellow-coloured DPPH-H. At a concentration of 200µg/ml, the scavenging activity of oleic acid reached 51.2%, which was comparable to that of reference standard butylated hydroxytoluene (Figure 3). The cell death by DNA fragmentation was examined by conducting agarose gel electrophoresis of DNA samples isolated from MCF-7 cell lines treated with the oleic acid sample. Healthy control cells maintained in culture medium alone did not show DNA fragmentation. However, the DNA isolated from oleic acid-treated cells revealed DNA ladders consisting of fragments when stained with ethidium bromide (Figure 4). Consistent with the DPPH assay the cell lines treated with higher concentrations of oleic acid showed DNA smearing (slight DNA degradation) and the IC50 concentration of oleic acid - 1.25 mg/ml was optimum enough to induce fragmentation in MCF-7 cell.

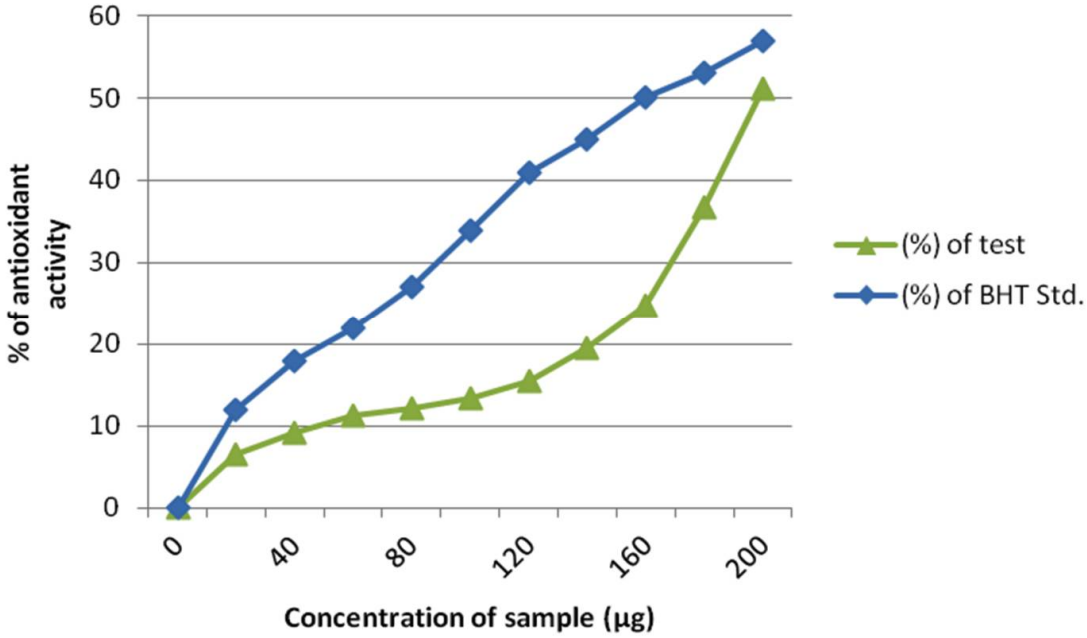

Figure 3: Antioxidant activity of Oleic acid using DPPH assay

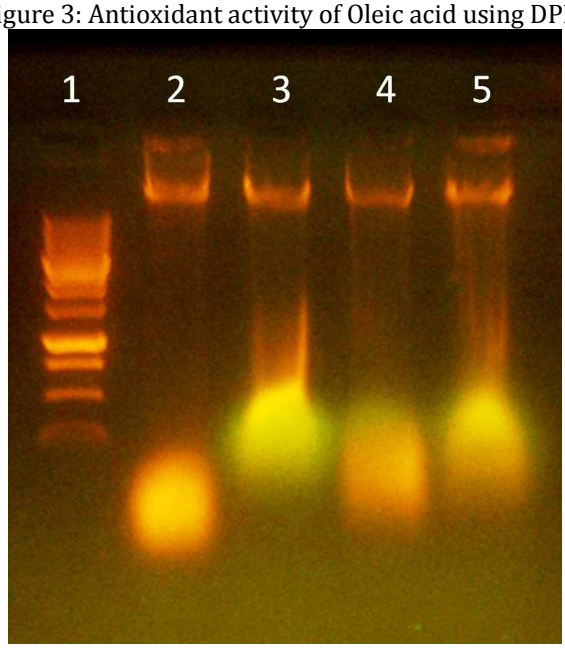

Figure 5: Apoptotic DNA fragmentation assay in MCF-7 cell lines and. The lanes 1 to 5 designate control ladder, 10, 1.25, 0.625, 0.312 mg/ml of oleic acid

The fungal metabolome consists of secondary metabolites of endo- and exo origin. Their production can be enhanced by biotic and abiotic environmental stress [12]. These secondary metabolites are extracted by

organic solvents and separated whose profiling has been used quite extensively for taxonomic purposes. The secondary metabolites along with fatty acids are extracted from a mycelial mat of various fungi. In our study, the culture filtrate of *A. strictum* varied the quality of chemical compounds like alkaloids, flavonoids fatty acids etc. Similarly, secondary and primary metabolites have been extracted from various endophytic fungi like *Alternaria* sp., *Chaetomium* sp., *Curvularia* sp., etc. using various solvents like methanol, hexane, dichloromethane, ethyl acetate and butanol and later tested for potency via antimicrobial, antileishmanial, anticancer and antioxidant studies [13]. Similarly, in our results, the solvent used in our study proved a good agent for the extraction of various metabolites. Fatty acid production from the fungal mycelial mats is almost directly proportional to biomass production. We have observed the fungal biomass have a profound effect on the magnitude of metabolites obtained from them. this is already proved by other studies. Summner JL et al (1969) observed that in *Penicillium atrovenetum* there were changes in the lipid constituents during the growth and development. This led to total fatty acids increase during the log phase and the major fatty acids observed were Palmitic, Stearic, Oleic and Linoleic [14]. The results inferred in the present study, optimization of the media conditions in standardizing for higher biomass yields have proven the fact that the higher the biomass, the higher was the percentage of free fatty acids and hence the acid value. Higher yield of biomass per 100 ml of culture media was obtained with the cultures grown in PDB under conditions of pH 6 and room temperature, with 12 h cycle of light and dark conditions yielding 36.3, 27.6 and 15.2 percentages of free fatty acids with an acid number of 72.23, 54.9 and 30.24 compared to control. The increase in Oleic acid production due to salt elicitors in the media implies the role of metal ions to act as cofactors for the enzymes involved in the fatty acid synthesis. The production of oleic acid from palmitic or stearic acid is catalyzed by the enzyme complex δ-9-desaturases.

Various methods have been developed to measure total antioxidant capacities such as the ORAC or the DPPH assay [15]. The free radical scavenging capacity of the oleic acid was noted to be increased in a concentration-dependent manner at a concentration of 200µg/ml. The scavenging activity of oleic acid reached 51.2%, which was comparable to that of reference standard BHT used. Certain studies conducted by Cho *et al,* (2010) showed functional oil containing monoacylglycerol-oleic acid had the strongest radical scavenging and antioxidant activities against copper-mediated low-density lipoprotein (LDL) oxidation and the strongest inhibitory activity against LDL-associated phospholipase and exhibited potent activation of paraoxonase activity which contributes to the maintenance of antioxidant activity. Fatty acid micelles scavenged superoxides in an unsaturation-dependent manner that is evident through few studies where supplementation of Human Aortic Endothelial Cells with unsaturated fatty acids resulted in the lower formation of ROS**.**

Several mono- and polyunsaturated fatty acids have shown to inhibit the growth of malignant cells *in vitro*. The effect of oleic acid on the viability of the MCF-7 cell line evaluated the dose-dependent inhibitory effect against the cell line tested. The IC50 values of oleic acid were found to be 1.25 mg/ml against MCF-7. Previously the discovery of oleic acid as the major component of olive oil in a healthy Mediterranean diet to protect against breast cancer [17] was evaluated. Additionally, oleic acid and its two branched-chain derivatives were tested *in vitro* for their anticancer activities against two cancer cell lines MCF-7 and HT-29 exhibited highly significant antitumor activity [18].

Anticancer assays are often sustained with DNA fragmentation studies to check for cell apoptosis. The induction of toxicity can be investigated by changes in cell size, granularity, membrane integrity and DNA fragmentation using flow cytometry and electrophoretic studies. Present studies have proven to induce fragmentation that IC50 concentration of oleic acid - 1.25mg/ml and 62.5µg/ml was optimum enough in MCF-7 cell lines to cause DNA smearing or slight DNA degradation. This is in concord with other previously published studies where few fatty acids have evoked a DNA smearing in malignant tissues**.** For example, assays conducted for palmitic, stearic, oleic, linoleic, arachidonic, docosahexaenoic and eicosapentaenoic acids on a macrophage cell line was investigated have shown the same results [19]. Several studies have also reported the induction of DNA fragmentation and loss of membrane integrity in different cell types after treatment with fatty acids [20]. Muralidhar *et al.* (2004) showed that oleic acid and palmitic acid promote apoptosis of human monocyte-derived macrophages [21]. The mechanisms of fatty acids to induce cell death involved changes in mitochondrial transmembrane potential and intracellular neutral lipid accumulation. It involves reactive oxygen species-mediated DNA fragmentation that is being enhanced by unsaturated fatty acids leading to necrosis [22, 23]. Further studies indicate that oleic acid-rich olive oil suppresses Her-2/***neu*** over expression, which in turn interacts synergistically with anti-Her-2/***neu*** immunotherapy by promoting apoptotic cell death of breast cancer cells with Her-2/***neu*** oncogene amplification [24].

CONCLUSION

The study implicated the endophytic fungi *A. strictum* as a good source of fatty acids especially oleic acid. Hence can be utilized as an alternative source of oleic acid production. The oleic acid fraction has evoked a good response as a therapeutic agent for the breast cancer cell line. However more parallel studies on other malignant cells are warranted to replicate the results. Altogether, the study reveals a novel method for the utilization of oleic acid produced from *A. strictum* in clinical therapeutics.

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COMPETING INTERESTS

We declare no competing interests

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