

Anticancer and anti-inflammatory activity and untargeted metabolite profiling by UPLC-ESI-QTOF of crude extracts of mangrove endophytic fungus *Aspergillus stellatus* LM-03

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Abstract:

Mangrove ecosystem is diverse habitat for number of medicinally important microorganisms including fungi. Scientific research from last three decade emphasizes it potential in important secondary metabolites production, which have wide biological activities. The current study elaborates isolation of fungi from pneumatophore of mangrove plant *Avicennia marina* and investigation of ethyl acetate extract for antimicrobial, anti-inflammatory and anticancer activity. Ethyl acetate extract of the fungus displayed maximum anti-inflammatory activity with 76.39 % inhibition for by protein denaturation method and 22.52% inhibition for membrane stabilization method with respect to standard ibuprofen. Further, the anticancer activity of *Aspergillus stellatus* LM-03 exhibited against MCF-7 of breast cancer cell with an IC₅₀ value of 33.24 µg/ml. HPLC and UPLC-ESI-QTOF analysis displayed nine compound mainly alternariol monomethyl ether and methyl jasmonate, which are highly anticancer activity and moderate anti-inflammatory activity with other compounds as angelic anhydride, DL-3-(4-Hydroxyphenyl)lactic acid, 8,9-epoxyl-3-isobutyryloxy-10-(2-methylbutanoyl)thymol, 5-(6-methyl-7-oxooctyl) furan-2(5H)-one and phenyl-butyryl-glutamine with an unidentified compounds. The results of the current study indicate that it may be worthwhile to investigate *Aspergillus stellatus* LM-03 extract for promising bioactive metabolites with potential for medicinal use.

Keywords: Mangrove-pneumatophore-derived Fungus, Secondary Metabolites, Anti-inflammatory activity, anticancer activity, UPLC-ESI-QTOF.

1. Introduction

The mangrove ecosystem is a promising biodiversity hotspot, which is intensively studied in the last three decades in the search for unique metabolites of potential medicinal applications. The mangrove ecosystem occurs in around 112 countries mostly confined to latitudes between 30° S and 30° N. Differences in temperature in variations, high tidal range, high salinity, and strong winds with muddy anaerobic soil make this ecosystem unique resulting in special habitat for microorganisms (Tomlinson, P.B. 2001). This extreme environment stipulates diverse morphological and metabolic adaptations in microorganisms living in this ecosystem, which lead to unique secondary metabolites production to cope with extremities (Wu *et al.*, 2008). Around 1400-1500 secondary metabolites are isolated every year from marine organisms and microorganisms including phytoplankton, blue-red, and green algae, sponge, cnidarians, bryozoans, mollusks, tunicates, echinoderms, mangroves, and other intertidal plants and microorganisms including fungi and bacteria (Carroll *et al.*, 2023). Lin *et al.*, (2001) isolated xyloketal from the mangrove-derived fungus *Xylaria* sp., which drew special attention to mangrove microorganisms, especially fungi, and bacteria.

Aspergillus, including other fungal species *Penicillium*, *Fusarium*, *Trichoderma*, *phoma*, *Chaetomium* and *Acremonium* profoundly investigated for secondary metabolites production. Polyketides are the major secondary metabolites produced by these species followed by alkaloids, terpenoids, peptides and lipids and these compounds are with a broad range of biological activities (Kamat *et al.*, 2023). Mangrove-derived *Penicillium* (20%), *Aspergillus* (17%) and *Pestalotiopsis* (8%) give over 45% of secondary metabolites production from the year 1989 to 2020 (Chen *et al.*, 2022) and *Aspergillus* is predominant mangrove fungi which produce Polyketides and alkaloids. *Aspergillus* sp., as it is found ubiquitously in nature, gain the primary attention of researchers due to its extensive biosynthetic potential of secondary metabolites (SMs) with medicinal nutritional and agrochemical values (Yu *et al.*, 2021). According to the discovery of natural products (2023), around 3100 secondary metabolites were isolated from *Aspergillus* sp., and the major secondary metabolite from them is polyketides.

The present study summarizes isolation of *Aspergillus stellatus* LM-03 from the pneumatophore Mangrove plant *Avicennia marina*. Ethyl acetate extract of fungus investigated for its anti-anticancer and inflammatory, along with the HPLC and LCMS analysis of fractions studied for the prediction of the different compounds.

2. Results and Discussion

The samples were collected in the Lokhandawala Creek associated with Malad Creek, central west coast of Mumbai, Maharashtra, which is located inside the metropolitan city (Figure S1). The Malad

creek supports a good and widespread mangrove forest covering 8.41 sq. km. The LULC exhibited that the creek contains mudflats on the low tide that makes 0.69 sq. km in the present observation. Build-ups across the study area densely spread and mainly on the central part, that covers 25.60 sq. km. On the northwest part nearby the shore and inside the creek, there was a huge patch of barren land having 13.48 sq. km. Apart from this, there were some patches of dense vegetation (16.35 sq. km), sparse vegetation (1.44 sq. km), sandy beach (1.29 sq. km), rocky shore (0.62 sq. km), and agriculture (0.19 sq. km) along with about 31.91 sq. km water body. The Geographic features at the selected domain exhibit that the Lokhandawala zone is associated with mesotidal fluctuation as well as sewage and mangrove. The endophytic fungus was derived from the pneumatophore of the mangrove plant *Avicennia marina*. Based on the microscopic observation (figure S2A, S2B), taxonomical characteristics and sequence of the ITS ribosomal RNA gene, the fungus identified as *Aspergillus stellatus* LM-03. The figure S3, demonstrated the similarity of isolated *Aspergillus stellatus* LM-03 with its nearby family members. The main action of the anti-inflammatory agent is the inhibition of the cyclooxygenase system, which is responsible for the conversion of arachidonic acid to prostaglandins G2 to PGH2 along with peroxidation, which is associated with the formation of the long channels in the membranes. The opening of the channel occurs due to the release of chemical mediators. The extracellular activity of these enzymes due to acute and chronic inflammation (Sankari G. *et al.*, 2009). The anti-inflammatory activity of ethyl acetate extract of *Aspergillus stellatus* LM-03 performed by the protein denaturation method compared to standard ibuprofen and it found (Table S1) that the extract displayed 76.39 % inhibition compared to 83.22% of ibuprofen. The anti-inflammatory activity also performed by membrane stabilization method where ethyl acetate extract displayed 22.52% inhibition (Table S3). From these findings, we predict that *Aspergillus stellatus* LM-03 can produce secondary metabolites of having anti-inflammatory activity.

Cytotoxic activity of the identified fungal extract of *Aspergillus stellatus* LM-03 was determined against MCF-7 cell lines prepared at different concentrations (10, 40, and 100 µg/ml), after 24 hours of incubation activity determined by MTT assay. MTT cytotoxic assay is a quantitative colorimetric assay that measures the viability, proliferation, and induction of cells (Mosmann, 1983). After the addition of MTT, there was the formation of formazone crystal, where the yellowish MTT was reduced to dark-colored formazan by viable cells, which precipitates in the cytosol and dissolved after cell lysis whereas cells damaged due to toxic effect cannot transform MTT. The reaction mediated by the dehydrogenase enzymes present in the endoplasmic reticulum and mitochondria (Fotakis and Timbrell, 2006). From the MTT assay, it is found that extract displayed moderate anticancer activity against MCF-7 cell lines with an IC₅₀ value of 33.24 µg/ml. The Present study analyzed the ethyl acetate

extract of *Aspergillus stellatus* LM-03 for bioactive compounds via UPLC-ESI-QTOF analysis (figure S4 and S5). UPLC-ESI-QTOF analysis revealed the presence of 12 compounds, which were detected and identified, based on retention time and measured, masses (m/z). The UPLC-ESI-QTOF analysis of partially purified fractions (1, 2 and 3) of *Aspergillus stellatus* LM-03 revealed the presence of seven known compound and four unknown compound depending of highest retention time peaks and measured masses (m/z) (table S4 and figure S5). UPLC-ESI-QTOF chart in positive mode of fraction 1 suggesting occurrence of angelic anhydride while in negative mode displayed occurrence of DL-3-(4-Hydroxyphenyl)lactic acid, based on m/z values (figure S5). Chromatogram of fraction 2 in positive mode displayed presence of methyl jasmonate (Figure S5), 8,9-epoxyl-3-isobutyryloxy-10-(2-methylbutanoyl)thymol (figure S5) while in negative mode, alternariol monomethyl ether and two unknown compounds based on m/z values (figure S5). Chromatogram of fraction 3 in positive mode displayed occurrence of 5-(6-methyl-7-oxooctyl) furan-2(5H)-one (figure S5) and unknown compounds, while negative mode displayed phenyl-butyryl-glutamine and one unknown compound (figure S5) and unknown compound; [Table S4: retention time and measured masses (m/z)]. Since the 1940s, natural products and their derivatives have been employed extensively for human health, particularly for cancer and microbial infections (Newman *et al.*, 2016).

Numerous natural, synthetic, and semi-synthetic antibiotics have been employed since the discovery of penicillin. They are frequently separated from bacteria and fungus. Examples of fungi that produce enzymes and secondary metabolites with antibiotic activity include *Penicillium*, *Aspergillus*, *Fusarium*, *Cladosporium*, and yeasts. Penicillin, fusidic acid, and cephalosporin are just a few of the antibiotics made by fungi that have been successfully utilized to treat a variety of illnesses (Sethi *et al.*, 2013). Marine-derived *Aspergillus* and *Penicillium* spp. are both salt-tolerant, quickly growing species that may be found on a wide variety of surfaces, which accounts for the large number of compounds identified from these two genera. It is also known that several *Aspergillus* and *Penicillium* species produce extracts that have a variety of different actions (Buguni *et al.*, 2004). Unfortunately, the advent of infections that are drug-resistant and the proliferation of synthetic medications with adverse effects are driving up the demand for new bioactive molecules. Numerous secondary metabolites are produced by *Aspergillus* species, however only ochratoxin A is known to be a mycotoxin (Schuster *et al.*, 2002). Mangrove-associated fungi, the second-largest ecological group of marine fungi, are among the mangrove microbial community and not only play a crucial role in establishing and maintaining this biosphere, but they also represent a rich source of structurally distinctive and diverse bioactive secondary metabolites, drawing considerable interest from organic

chemists and pharmacologists (Chen et al., 2022). Due to the obvious mismatch between the declared diversity of farmed strains of fungi and the actual number of strains, fungi are nevertheless promising. In our study, the cultured fungal isolate was identified based on the culture morphology and spore molecular characteristic (figure S2). By using 18S rRNA gene sequencing, the chosen *Aspergillus* sp. LM-03 was recognized. The most promising method of molecular characterization was 18S rRNA gene sequencing, which was improved to identify amplified 18S rRNA and facilitate the phylogenetic variance among the fungal isolates (Awad et al., 2023). In order to identify closely related fungal species, the 501 base pair fungal sequence was nucleotide blasted (blast) in the NCBI database. According to the results, *Aspergillus stellatus* LM-03 (Accession number: OR888814), a mangrove-derived fungus, shares 98.39% and 98.21% of its sequencing with *Aspergillus stellatus* strain CLB43 and *Aspergillus stellatus* isolate EV36. Using the collected gene sequences, the MEGAX software's maximum likelihood and bootstrap consensus methods, a phylogenetic tree was created. The phylogenetic tree analysis, as shown in figure S3, showed that *Aspergillus stellatus* LM-03 belonged to the *Aspergillus* cluster and established a branch with *Aspergillus stellatus* strain CLB43 and *Aspergillus stellatus* isolate EV36. In this study, the selected fungal *Aspergillus stellatus* LM-03 isolate was grown in a PDB medium for 21 days. After incubation, the crude extracts (1.5 gm.) containing the metabolites were separated from the fermented medium with ethyl acetate. This was followed by screening for their anticancer and anti-inflammatory activity.

The chromatogram of the LCMS analysis of the partially purified fractions of *Aspergillus stellatus* LM-03 revealed the presence of bioactive compounds that might be involved in the above-described bioactivities. Among these compounds, angelic anhydride (figure S5) displayed the highest occurrence in the fungal extract. Till date no biological activity has been reported by angelic anhydride but Liang *et al.*, (2012) reported that using pure angelic anhydride and suitable base will find its wide use in the future in the preparation of angelate esters which often are moieties of natural products. DL-3-(4-Hydroxyphenyl) lactic acid (figure S5) obtained from the azalactone of 4-acetoxy- α -acetamidocannamic acid by two-stage hydrolase to the corresponding pyruvic acid followed by reduction (Saul and Trikojus *et al.*, 1948). This is the first report its occurrence in mangrove derived fungi and its acts as carcinogens and it was demonstrated to decrease considerably ascorbic acid concentration in the liver, adrenal glands and blood of mice. (Levchuk *et al.*, 1986). Methyl jasmonate (MeJA) (figure S5) has been discovered to be an essential cellular regulator that mediates a variety of developmental processes and defense responses to biotic and abiotic stimuli (Cheong et al., 2003) and key player in plant defense mechanism (Beal and ward 1998). Despite its role in plant defense, methyl jasmonate investigated for their anticancer and anti-inflammatory activity. Flesher E., (2005)

demonstrated its cytotoxic effects against cell lines representing a wide spectrum of malignancies, including prostate, breast and lung. It also induces death in leukemic cells isolated from the blood of chronic lymphocytic leukemia (CLL) patients and increased the survival of lymphoma bearing mice. Cesari and Carvalho (2014) retrieved its actions on cancer cell cycle, metabolism and apoptosis. It has multifaceted mode actions such as, arrests of cell cycle, its varied modes of operation. MJ Inhibits cell growth and proliferation by (1) stopping the cell cycle; (2) results in cell death through intrinsic/extrinsic pro-apoptotic, p53-independent apoptotic, and non-apoptotic (necrosis) pathways; (3) separates glycolytic and mitochondrial functions by removing hexokinase from the voltage-dependent anion channel; lowers mitochondrial membrane potential; favors cytochrome c release and ATP depletion. It also (4) causes reactive oxygen species-mediated reactions; (5) encourages MAPK stress signaling and redifferentiation in leukemia cells; (6) inhibits overexpressed proinflammatory enzymes in cancer cells, such as aldo-keto reductase 1 and 5-lipoxygenase; and (7) prevents cell migration and exhibits antiangiogenic and antimetastatic activities. It has anticancer effects by inhibiting growth in breast cancer cell MCF-7 (Yeruva et al., 2008). Dang *et al.*, (2008) and Sá-Nakanishi *et al.*, (2018) demonstrated that this natural product has potential anti-inflammatory activity. Umukoro *et al.*, (2017) also investigated methyl jasmonate, for its anti-inflammatory and membrane stabilizing activity. 8,9-epoxyl-3-isobutyryloxy-10-(2-methylbutanoyl)thymol (figure S5) is phenol esters demonstrated by spectroscopic data in positive mode. Alternariol monomethyl ether (figure S5) is the second most compound displayed with peak area of 35.44%. Raistrick *et al.*, (1953) firstly reported the production of alternariol monomethyl ether from the fungus *Alternaria tenuis*, compounds which strong cytotoxic and anticancer properties. It is leading mycotoxin produced by *Alternaria* fundal species (R. Thomas 1961; Pollock *et al.*, 1982; Torres *et al.*, 1998). AME (Alternariol monomethyl ether) has been investigated globally for its biological activities. Szliszka *et al.*, (2009) generated AME with others natural products from propolis (Bee glue) which is natural resinous products of honeybee, and AME displayed anti-inflammatory activity. Alternariol (AOH) and Alternariol monomethyl ether (AME) generated by *Alternaria alteranata* exhibited genotoxic and cytotoxic activity where AOH/AME-related immune suppression and morphological changes linked to the ability of these mycotoxins to cause cell cycle arrest at the G2/M phase (Grover and Lawrence 2017). Bensassi *et al.*, (2011) reported significant cytotoxic activity of AME against HCT116 cells by loss in cell viability mediated by activation of apoptotic process. It was found that AME activated the mitochondrial apoptotic pathway evidenced by the opening of the mitochondrial permeability transition pore (PTP), loss of the mitochondrial trans membrane potential ($\Delta\psi_m$) downstream generation of $O_2^{\bullet-}$, cytochrome c release and caspase 9 and 3 activation. This is the first report, depicting occurrence of AME in mangrove derived *Aspergillus* fungus. 5-(6-methyl-7-oxooctyl) furan-

2(5H)-one (figure S5) displayed antifungal activity but unable to exhibited other activities, isolated from marine derived bacterium *Streptomyces* (Cho KW *et al.*, 2001) and from our study it is found that it is firstly isolated from mangrove derived *Aspergillus* species. From the literature data, it observed that no significant biological activities (antimicrobial, anti-inflammatory and anticancer) reported from these two compounds. While negative mode displayed phenyl-butyryl-glutamine (figure S5).

In our study, ethyl acetate extract of *Aspergillus stellatus* LM-03 exhibited anti-inflammatory activity by protein denaturation and membrane stabilization method (Table S1 and S2). It was found that EtOAc extract exhibited 76.39% inhibition of protein denaturation while it displayed 22.52% inhibition by membrane stabilization method (Table 3). Ethyl acetate extract of *Aspergillus stellatus* LM-03 exhibited anticancer activity against MCF-7 cancer cell lines with an IC₅₀ of 33.24 µg/mL. As literature data above demonstrating anti-inflammatory and anticancer activity by certain compounds like methyl jasmonate and Alternariol monomethyl ether that were confirmed in fungal extract by LCMS analysis, we predict that the EtOAc extract of *Aspergillus stellatus* LM-03 exhibited moderated anti-inflammatory and strong anticancer activity. Further, the anti-inflammatory and anticancer activity of the EtOAc extract of *Aspergillus stellatus* LM-03 could be due to the cumulative action of all these compounds. These findings implies that bioactive substances from this specific fungal strain could be further purified and properly standardized for commercial use in the development of novel anti-inflammatory and anticancer medications.

4. Conclusions

The presents study confirms the biological activities of pneumatophore derived *Aspergillus stellatus* LM-03. The in vitro investigations of ethyl acetate extract of the *Aspergillus stellatus* LM-03 lead to its possible antinflammatory activity. Additionally, the cytotoxicity of the *Aspergillus stellatus* LM-03 ethyl acetate extract against the MCF-7 cancer cell line (human breast adenocarcinoma) demonstrated dose-dependent antiproliferative activity, inducing cell death. The study's findings suggest future research directions that aim to investigate in vivo and determine the mechanisms of action.

Based on the available spectral data of LCMS it was observed that partially purified fractions 1 and 2 of extract of *Aspergillus stellatus* LM-03 contain fatty acid derivatives, coumarins and isocoumarin derivatives, iridoid O—glycosides and butenolide compounds. Overall, the study finds that the isolated *Aspergillus stellatus* LM-03 can serve as a therapeutic model in the pharmaceutical and agricultural

industries and has tremendous promise for use in the discovery of novel anti-inflammatory and anticancer medicines.

Disclosure statement

The authors reported no potential conflict of interest.

Credit: authorship contribution statement

Avinash Awashank: Analysis and manuscript writing, **Supriya Tilvi:** Manuscript review and editing
Abhay B. Fulke: Conceptualization, Funding and manuscript writing, **G. Udhaba Dora:** Land Use Land Cover classification, manuscript writing, review and editing, **Karthi J:** Satellite image processing for Land Use land Cover classification.

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3. Experimental

3.1. Data and Methodology for the Land Use Land Cover (LULC).

The Sentinel 2A Level 2A processed to get the Land Use Land Cover (LULC) classes for the selected domain, Lokhandawala, Maharashtra (Fig. S1). The spatial resolution of this satellite image is 10 m, and the temporal resolution is 10 days (Copernicus Sentinel data, 2021). The raw images, 110 km x 110 km ortho-images in UTM/WGS84 projection processed using the Hexagon ERDAS Imagine 2022 software package. The satellite image extracted by cropping an area of 10 x 10 m² with keeping the sampling location at the center. At Lokhandawala (Maharashtra), the image sensed one day after the sampling time. The date and time of the image capture is 23 Mar 2021 11:06:41 in Indian Standard Time (IST), which is 05:30 hrs. ahead of Universal Time Coordinated (UTC).

3.2. Sampling, Isolation, and Identification of Marine Fungus.

The samples collected in March 2021 in Lokhandawala Mangrove Mumbai India. Pneumatophore of mangrove plant collected and kept in sterile plastic bags carried, stored in microbiology laboratory until further use. *Aspergillus stellatus* LM-03 was isolated from the pneumatophore of the mangrove plant *Avicennia marina*. The fungus collected on PDA (potato dextrose agar) for 7 days. Identification carried out depending on morphological and conidial characters using the standard identification manual (Barnett *et al.*, 1972). The slide culture technique developed by Riddle R. W., (1950) used for the preparation of fungal mycelium on the cover slip.

3.3. Molecular characterization of fungal isolate.

The marine fungus was identified based on DNA sequences of the ITS ribosomal RNA gene. The fresh mycelium taken into a 2 ml micro-centrifuge tube. DNA identification carried out by using EZNA fungal DNA mini kit (OMEGA biotek. Inc). Genomic DNA was extracted and 18S rDNA region was amplified by using ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS2 (5'-TCCTCCGCTTATTGATATGC-3'). The amplification technique for the PCR reactions, which were carried out on a thermal cycler, included an initial denaturation step at 95 °C for 5 minutes, 34 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1.5 minutes, culminating in a final extension step at 72 °C for 10 minutes. Following ethidium bromide staining, the PCR results were analyzed using Agarose gel electrophoresis (1% agarose with 1× Tris-Borate-EDTA (TBE) buffer) and observed under UV illumination. The PCR products were sequenced using Sanger sequencing method (Sanger *et al.* 1977). The gene sequence was deposited in GenBank NCBI with the accession number OR888814.

3.4. Mass Cultivation of Fungal Isolates and Extraction.

Seed culture of *Aspergillus stellatus* LM-03 was prepared in 3 (flask) × 3(100 ml) in potato dextrose broth (consisting 200 g/L potato infusion, 20.0 g glucose and 1000 ml distilled water) and incubated at room temperature for 5-7 days. 50 ml of seed culture then transferred into already prepared 3.5 L × 3 conical flasks consisting of potato dextrose broth. The flasks then incubated at room temperature under static conditions for 21 days. After the incubation period, ethyl acetate solvent added to the flask and kept it for 7-8 hours. Then liquid broth+ solvent mixture and mycelium separated using a muslin cloth. The broth solvent mixture further followed by separation by vigorous vortexing to allow integration of secondary metabolites into ethyl acetate and allowed to stand constant. After separating the solvent from the broth, the solvent proceeded for rota evaporation to obtain crude extract. The ethyl acetate extract further chromatographed on silica gel column with CHCl₃: MeOH (9:1) to give three fractions (Fr. 1- Fr.3)

3.5. Biological evaluation of crude extract.

3.5.1. Antimicrobial activity of crude extract using disc diffusion assay.

Crude extracts of *Aspergillus stellatus* LM-03 tested for antibacterial activity against gram-positive and gram-negative bacteria with anti-fungal activity against fungi *candida albicans* (ATCC10231). The bacterial pathogens included were *Staphylococcus aureus* (ATCC6532), *Bacillus subtilis* (ATCC6633), and *Salmonella abony*. Muller Hinton Agar (MHA) for the antibacterial test and Sabouraud dextrose agar (SDA) used for the antifungal test. 20 microliter of 10 % crude extract pipetted on to 5mm disc until it is fully saturated. Then saturated disc placed onto Muller Hinton Agar (MHA) and Sabouraud dextrose agar. Tetracycline and fluconazole used as positive controls. The inhibition zone examined after incubation at room temperature for 24 hours (Balouiri *et al.*, 2016).

3.5.2. Anti-inflammatory activity of a crude extract.

3.5.2.1. In vitro Anti-inflammatory activity by Protein denaturation method.

In vitro, the anti-inflammatory activity carried out by the Protein denaturation method (Mounnissamy *et al.*, 2007). The reaction mixture (1 ml) consisted of 0.1 mL of egg albumin (from fresh hen's egg), 0.5 mL of Phosphate buffered saline (PBS, pH 6.4) and 0.4 mL of sample *Aspergillus stellatus* LM-03 at the concentration of 1mg/ml and a similar volume of double-distilled water served as control. Then the mixtures were incubated at (37 °C ± 2) in an incubator for 15 min and then heated at 70 °C for 5 min. After cooling, their absorbance measured at 660 nm by using a vehicle as blank. Ibuprofen at concentration 1 mg/ml) used as a reference drug and treated similarly for the determination of

absorbance. The percentage inhibition of protein denaturation calculated by using the following formula,

$$\text{Percentage inhibition} = \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100.$$

3.5.2.2. In vitro membrane stabilizing activity.

Membrane stabilizing activity used to assess the anti-inflammatory activity (Vallabh *et al.*, 2009). The standard drug used was IUB, AS. The reaction mixtures 4.5ml consists of 2ml hypotonic saline (0.25% NaCl) + 1ml 0.15M phosphate buffer (pH 7.4) + 100 μ l test solution (500 and 1000 μ g/ml)+ 0.5ml of 10% rat RBC in normal saline. The mixture incubated at 56°C for 30 minutes. The tubes were kept cooling under running tap water for 20 minutes. The mixture centrifuged for 3000 rpm for 10min and the absorbance of the supernatant measured at 560 nm.

3.5.3. Anti-cancer activity of crude extract.

The activity carried out by using the MTT assay method (Horiuchi *et al.*, 1988). Cells were incubated at a concentration of 1×10^4 cells/ml in culture medium for 24 h at 37°C and 5% CO₂. Cells were seeded at a concentration (70 μ l) 10^4 cells/well in 100 μ l culture medium and 100 μ l β -sitosterol and Stigma-sitosterol and its combination compounds (6.10, 12.5, 25, 50, 100 μ g/ml) into micro plates respectively (tissue culture grade, and 96 wells). Control wells incubated with DMSO (0.2% in PBS) and cell line. All samples incubated in triplicate. Controls maintained to determine the control cell survival and the percentage of live cells after culture. Cell cultures incubated for 24 h at 37 °C and 5% CO₂ in CO₂ incubator (Thermo scientific BB150). After incubation, the medium completely removed and Added 20 μ l of MTT reagent (5mg/min PBS). After addition of MTT, cells incubated for 4 hours at 37 °C in CO₂ incubator. Observed the wells for formazan crystal formation under microscope. The yellowish MTT reduced to dark colored formazan by viable cells only. After removing the medium completely. Added 200 μ l of DMSO (kept for 10 min) and incubate at 37 °C (wrapped with aluminum foil). Triplicate samples analyzed by measuring the absorbance of each sample by an Elisa microplate reader (Benesphera E21) at a wavelength of 570 nm (Sawant VJ *et al.*, 2016; Manikyam HK *et al.*, 2021).

3.6. Metabolite profiling of fractions of extract of *Aspergillus stellatus* LM-03 by analytical UPLC-ESI-QTOF-MS method.

3.6.1. Instrumentation for UPLC-ESI-QTOF.

The Acquity H-class UPLC (Waters Corporation, Milford, MA, USA) was employed, which had an integrated vacuum degasser, automatic sample manager (Serial # C10UPA554M, Waters Corporation, Singapore), ultra-performance binary solvent manager (Serial # C10UPB081A, Waters Corporation,

Singapore), and injection volume range of up to 100 μ L with an optional extension loop. A C18 stationary phase (BEH C18, 50 x 1.0mm, 1.7 μ) was used for chromatographic separation. A photodiode array detector (DAD) was employed in conjunction with a Xevo G2-XS QToF (Serial # YFA1548, Waters Corporation, Wilmslow, UK) for mass spectrometric (MS) detection.

3.6.2. Method for UPLC-ESI-QTOF.

The mobile phase is made up of 0.1% formic acid in Water as an aqueous phase (A) and acetonitrile as an Organic modifier (B). Mobile phase delivered at a flow rate of 0.2 mL/min in the following gradient: initially %B is kept at 2% for 1 minute; 1 to 6 minutes (2 to 50% B), 6 to 12 minutes (50 to 95% B), held for 4 minutes, 16 to 17 minutes (95 to 2% B) and held at 2% for 3 minute. The samples (Fr. 1- Fr.3) were injected in a volume of 5 μ L each. The column temperature was kept at an optimal level throughout the chromatographic run (22 $^{\circ}$ C). For MS detection, a positive polarity electrospray combined ionization (ESCI) source used. The optimal instrument and acquisition parameters as follows: 50 L/hr. cone gas (nitrogen) flow; 750 L/hr. desolvation gas (nitrogen) flow; 45 $^{\circ}$ C probe temperature; 30 V sampling cone voltage; 150 $^{\circ}$ C source temperature; 80 V source offset voltage. The collision energy ramp varies from 6 eV-50eV (Argon, collision gas), and a mass range of 50 to 200 m/z. To acquire and process data, Waters Corporation's Mass Lynx software (V4.1, Milford, MA, USA) used.

Table S1: Anti-inflammatory activity of EtOAc extract of *Aspergillus stellatus* LM-03 by protein denaturation method

Compound/Standard	Concentration	OD	Mean	% Inhibition
Control	1000 μ g/ml	1.64	1.61	--
		1.59		
		1.62		
Standard	1000 μ g/ml	0.28	0.27	83.22
		0.30		
		0.25		
EtOAc extract of <i>Aspergillus stellatus</i> LM-03	1000 μ g/ml	0.41	0.38	76.39
		0.39		
		0.35		

Table S2: Anti-inflammatory activity of EtOAc extract of *Aspergillus stellatus* LM-03 by membrane stabilization method

Compound/Standard	Concentration	OD	Mean	% Inhibition
Control	1000 µg/ml	1.83	1.81	--
		1.80		
		1.85		
Standard	1000 µg/ml	0.70	0.70	61.53
		0.75		
		0.65		
EtOAc extract of <i>Aspergillus stellatus</i> LM-03	1000 µg/ml	1.40	1.41	22.52
		1.35		
		1.32		

Table S3: Anticancer activity of EtOAc extract of *Aspergillus stellatus* LM-03 against MCF-7 cells.

Compound/Standard	Conc. (µg/ml)	OD	Mean	% Inhibition	IC ₅₀ (µg/ml)
Control	10	1.238	1.345	--	
	40	1.477			
	100	1.320			
Standard drug: 5 FU	10	0.365	0.383	61.27	38.62
		0.387			
		0.398			
	40	0.303	0.318	67.84	
		0.351			
		0.301			
100	0.223	0.265	73.16		
	0.315				
	0.259				
EtOAc extract of <i>Aspergillus stellatus</i> LM-03	10	0.612	0.635	35.85	33.24
		0.606			
		0.687			
	40	0.690	0.618	37.57	
		0.517			
		0.647			
	100	0.521	0.612	38.14	
		0.675			
		0.641			

Table S4: Retention time (Rt) and respective measured masses (m/z) of fractions 1, 2 and 3 of extract of *Aspergillus stellatus* LM-03.

Fractions	Mode	RT	Neutral Mass (Da)	Observed m/z	Mass Error (mDa)	Formula	Proposed Molecule
fraction 1	Positive	6.912	182.216	183.0981	+0.8821	C ₁₀ H ₁₄ O ₃	Angelic Anhydride
fraction 1	Negative	6.92	181.16	181.0964	-0.0636	C ₉ H ₁₀ O ₄	DL-3-(4-Hydroxyphenyl) lactic acid
fraction 2	Positive	6.509	224.30	225.1487	+0.8487	C ₁₃ H ₂₀ O ₃	Methyl Jasmonate
fraction 2	Positive	9.944	334.41	335.1726	+0.7626	C ₁₉ H ₂₆ O ₅	8,9-epoxyl-3-isobutyryloxy-10-(2-methylbutanoyl)thymol
fraction 2	Negative	8.737	272.25	271.0358	-1.2142	C ₁₅ H ₁₂ O ₅	Alternariol monomethyl ether
fraction 2	Negative	9.444	--	339.0613	--	--	Unknown
fraction 2	Negative	9.950	--	333.1967	--	--	Unknown
fraction 3	Positive	6.510	224.296	225.1426	+0.8466	C ₁₃ H ₂₀ O ₃	5-(6-methyl-7-oxooctyl) furan-2(5H)-one
Fraction 3	Positive	7.621	--	397.1026	--	--	Unknown

fraction 3	Negative	7.675	292.33	291.1392	-1.19.8	C ₁₅ H ₂₀ N ₂ O ₄	Phenyl butyryl glutamine
fraction 3	Negative	9.950	--	333.1855	--	--	Unknown

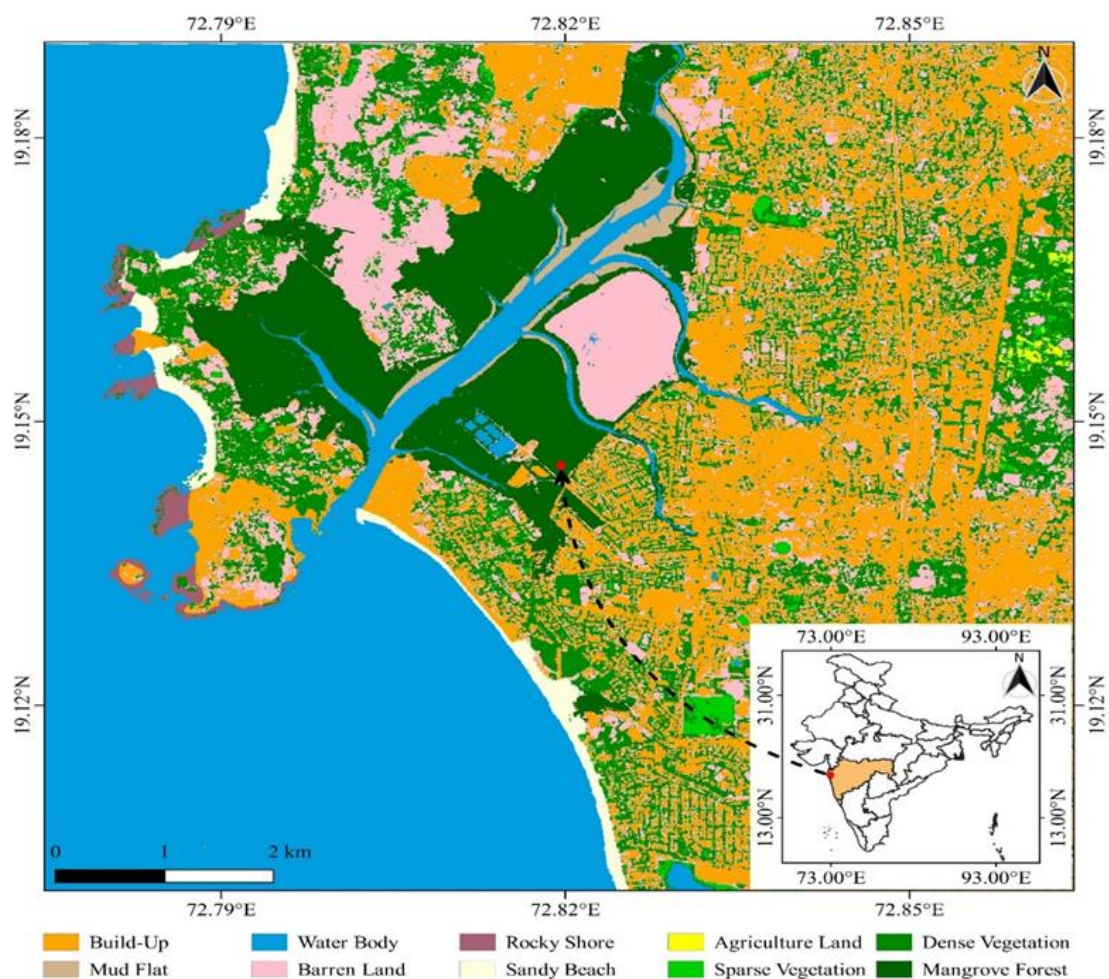


Figure S1: The sampling location and geographic features of the study area, Lokhandawala, Maharashtra

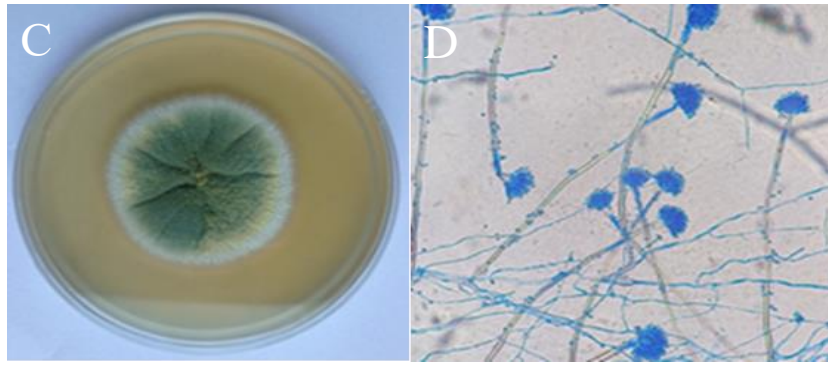


Figure S2: Pure culture of *Aspergillus stellatus* LM-03 on PDA (A) and Microscopy of pure culture of *Aspergillus stellatus* LM-03 (B).

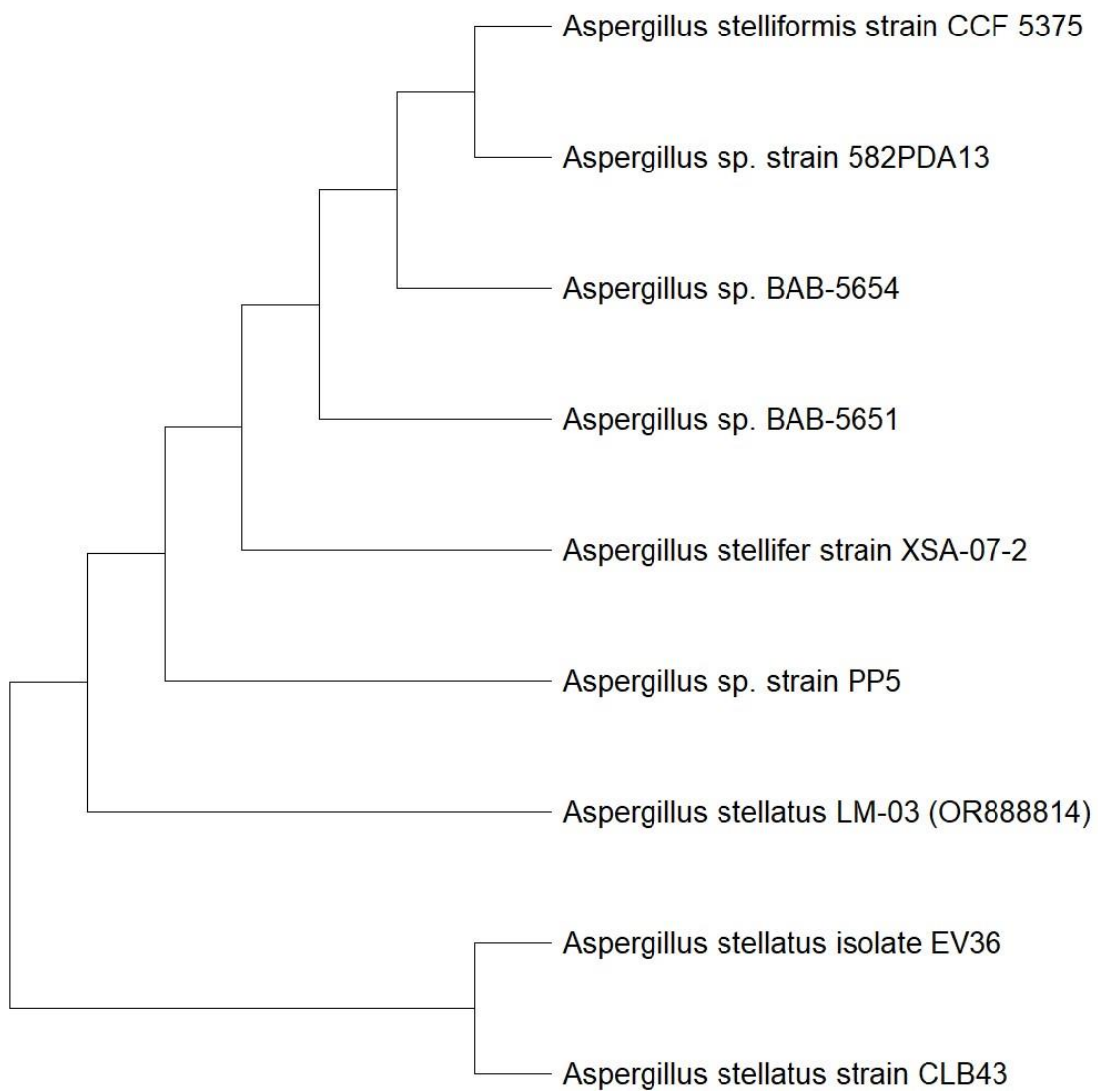
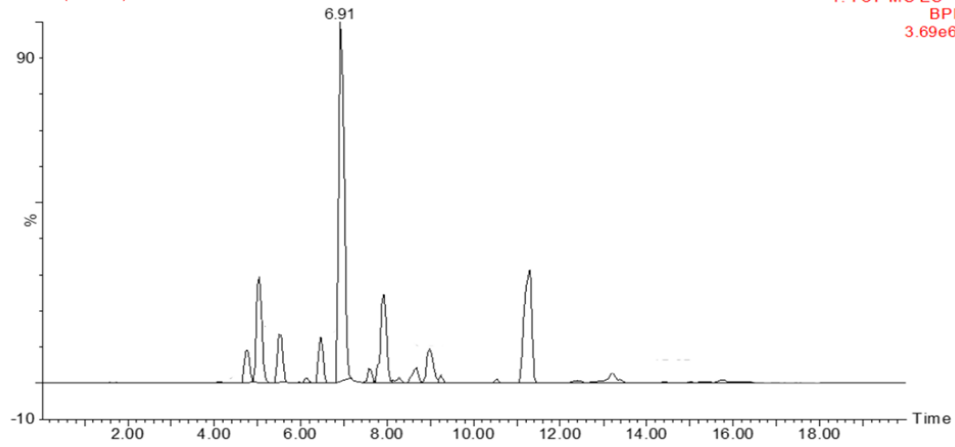


Figure S3: Molecular phylogenetic analysis of *Aspergillus stellatus* LM-03 by maximum likelihood method.

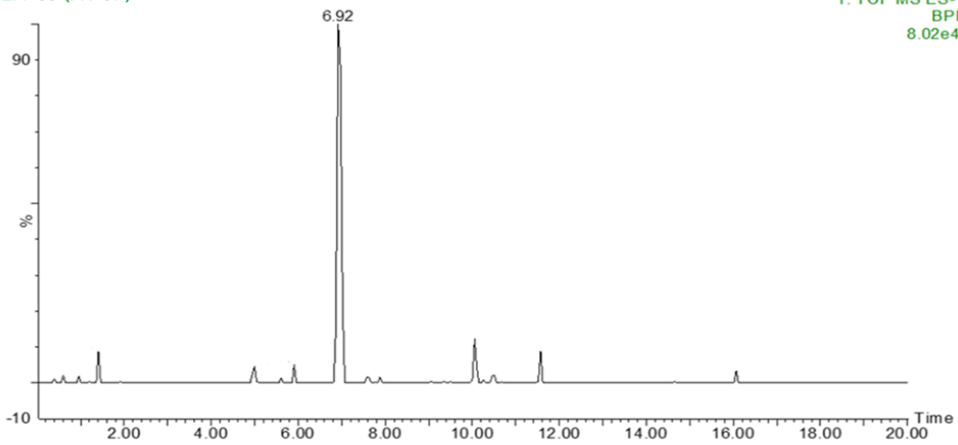
LM-03 (Fr.-01)

1: TOF MS ES+
BPI
3.69e6



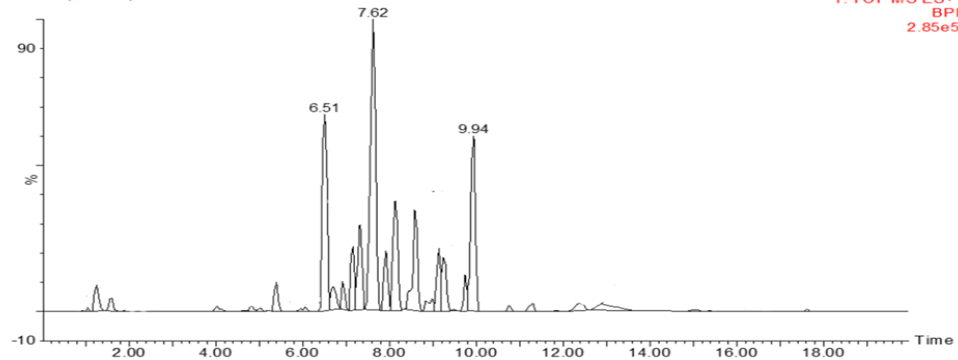
LM-03 (Fr.-01)

1: TOF MS ES-
BPI
8.02e4



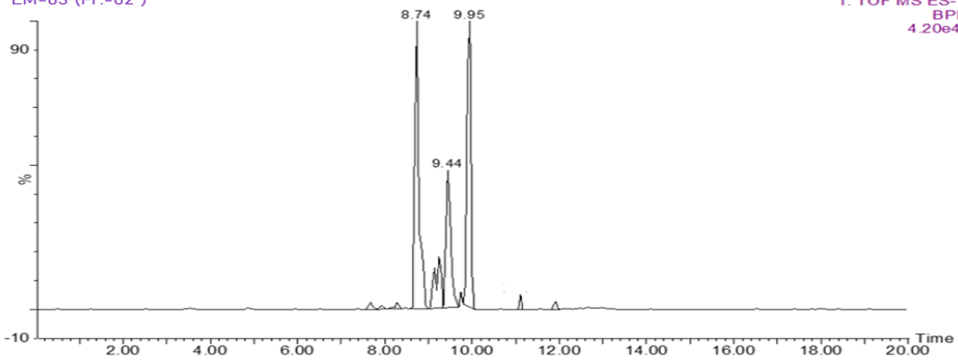
LM-03 (Fr.-02)

1: TOF MS ES+
BPI
2.85e5



LM-03 (Fr.-02)

1: TOF MS ES-
BPI
4.20e4



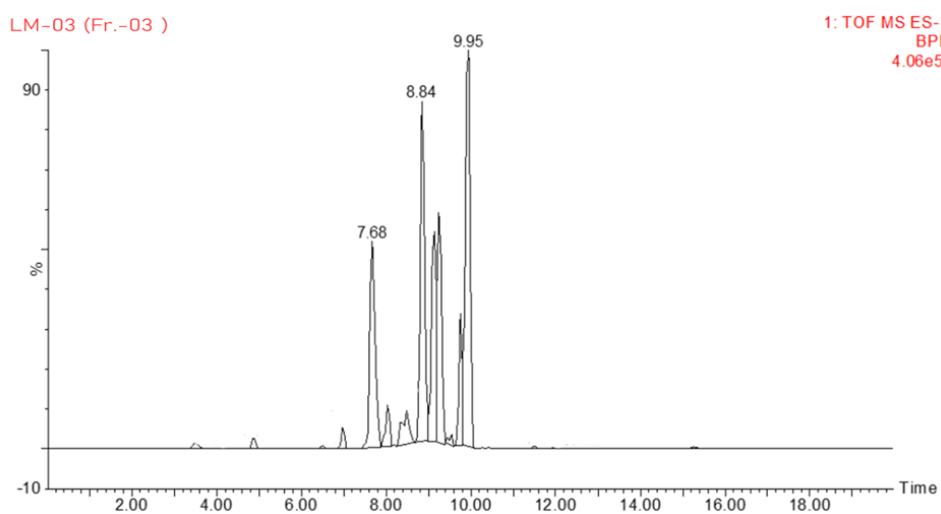
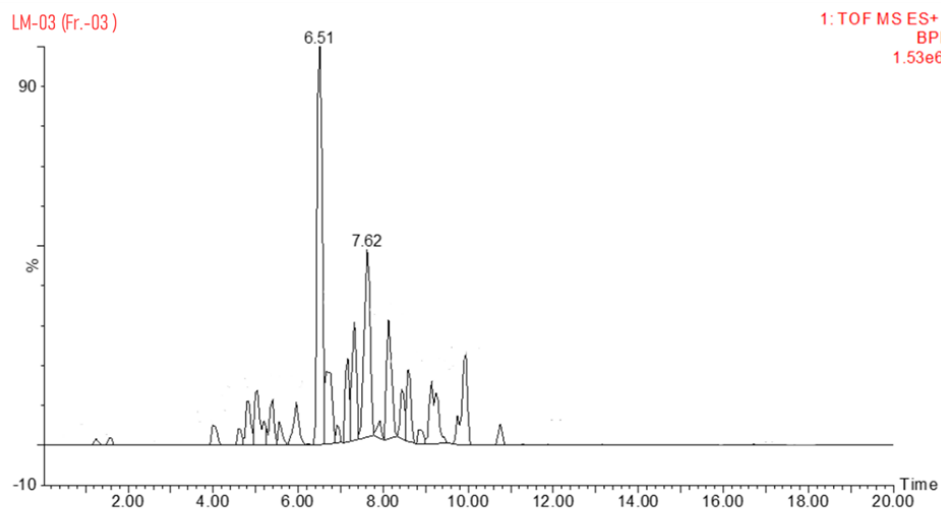


Figure S4: Base peak chromatograms of fractions 1, 2 and 3 of *Aspergillus stellatus* LM-03 in positive and negative mode.

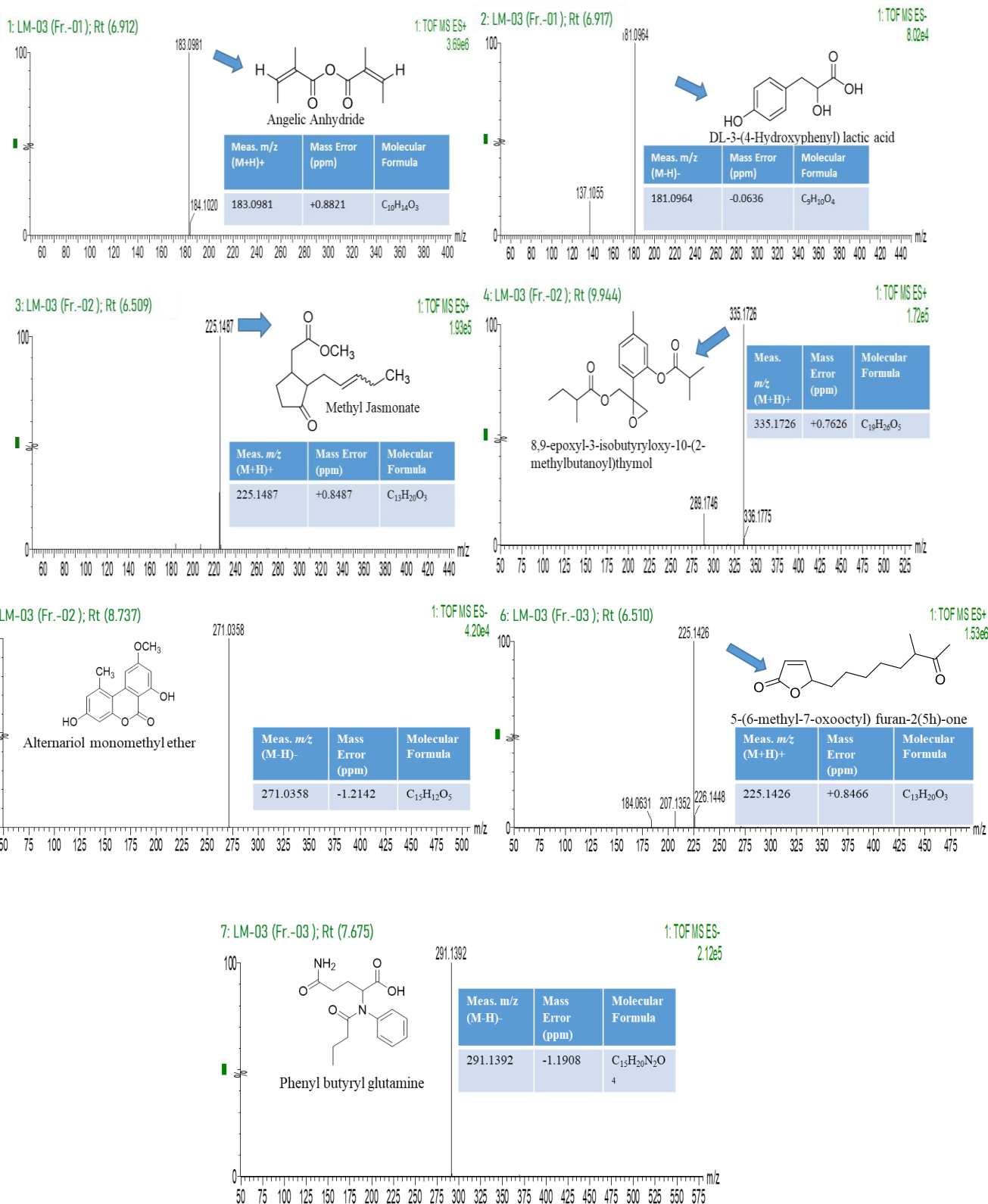


Figure S5. MS/MS spectrum of compounds identified from ethanolic extract (EtOAc) of *Aspergillus stellatus* LM-03 by UPLC-MS/MS analysis. Angelic anhydride (1), DL-3-(4-Hydroxyphenyl) lactic acid (2), Methyl jasmonate (3), 8,9-epoxyl-3-isobutyryloxy-10-(2-methylbutanoyl)thymol (4), Alternariol monomethyl ether (5), 5-(6-methyl-7-oxooctyl) furan-2(5H)-one (6), phenyl-butryryl-glutamine (7).