Chapter-5

Discussion

5.1 Fungal material collection

Fresh specimens of *S. stipitatum* were collected from various places of Palakkad district with the assistance provided by the tribal people during rainy seasons. However, it is available only in old termite nests under the soil in undisturbed abandoned areas and forests (Plate 1). Nowadays, it is not much seen in populated places since the soil is disturbed and polluted.

5.2 Molecular characterization

A molecular study carried out earlier on this taxon suspected that *S. stipitatum* is genetically similar to *Xylaria acuminatilongissima* (Latha *et al*, 2015). Recently collected fresh specimens of *S. stipitatum* and *X. hypoxylon* since *X. acuminatilongissima* is not available from tribal areas of Palakkad District, Kerala conducted molecular studies in fresh specimens immediately after collection. Have deposited *S. stipitatum* specimens at the National Fungal Culture Collection of India (NFCCI) - A National Facility (Ajrekar Mycological Herbarium - AMH) and identified and confirmed it as *S. stipitatum* based on morphological characters (Accession No. - AMH-10322). DNA barcode analysis using the ITS barcode region was performed to validate the species identification of the freshly obtained fungal specimens. ITS barcode sequences generated were submitted to GenBank, and the following accession numbers were obtained; *S. stipitatum* (384 bp) (MZ330799-MZ330800) and *X. hypoxylon* (363 bp) (MZ330801-MZ330802) collected from two different places of Malabar region. Similarity search in BLAST showed the highest homology (95.32%) with *X. acuminatilongissima* ITS sequence (Figure 2).

However, *X acuminatilongissima* has not yet been reported anywhere from India and reports of the taxa are available only from Taiwan (Ju and Hsieh, 2007; Cho *et al*., 2016). *S. stipitatum* is located only in undisturbed old abandoned closed termite nests

under the soil. Total lack of spores is another specialty of the species whereas, *the Xylaria* species belongs to Ascomycota. Moreover, this is the first ITS barcode sequence information of the *S stipitatum* available in the GenBank public domain and molecular studies on the species has not been reported earlier. Due to its restricted habitat, limited studies are available on this species. Even though *S. stipitatum* displayed 95.32% sequence similarity with *X. accuminatilongissima*, considering other factors like species morphology, reproductive stage and habitat preferences, it cannot be treated as any *Xylaria* species. Therefore, *S. stipitatum* has a unique separate species entity.

Multiple sequence alignment of ITS barcode sequences obtained in this study from *S. stipitatum* and *X. hypoxylon* also revealed several specific nucleotide changes, including indels, transitions or transversions at many positions (Figure 3). In addition, dendrogram based on ITS DNA barcode sequences of both genera also displayed two distinct clades, indicating the separate generic entities for *S. stipitatum* and *X. hypoxylon* (Figure 4).

Further, the hyphae mass of *S. stipitatum* dies very fast in contact with an open environment, and the dead specimen is often infested with other fungal species, including *Xylaria* (Ju *et al*., 2018). Thus, molecular analysis of a dead specimen may lead to the wrong categorization of the species status.

5.3 Preparation of fungal extracts

Fungal extracts were prepared after cleaning the specimen well in tap water and distilled water, then cut into small pieces and kept in a hot air oven for 48 hrs at 60˚ C. Then it was powdered, and the extraction using different solvents was done using a soxhlet apparatus. The solvents were then evaporated, and extracts were stored in a refrigerator for further studies.

5.3.1 Chemical screening

The extracts of *S. stipitatum* were prepared using the soxhlet apparatus. The solvents used were petroleum ether, chloroform, acetone, ethanol and distilled water. Petroleum ether contains phytosterols only, while chloroform extract contains phytosterols, triterpenoids and glycosides. Acetone extract shows the presence of tannins and lactones. Ethanol extract contains most number of secondary metabolites. They include alkaloids, flavonoids, phenols, aleurone grains and saponins. And aqueous extract contains naphthoquinones and phenols (Table 2). Since ethanol extract has a higher number of metabolites, it was chosen for further studies.

Albrecht Kossel was the first to coin the term secondary metabolites in 1891. Fungi-derived secondary metabolites are also promising compounds used in different industries (Aly *et al*., 2010). Numerous bioactive metabolites have recently been discovered with a wide range of biological activities, including antibiotics, antioxidants, antitumor, and anti-inflammatory properties (Devi, 2020). So, secondary metabolites are utilized in the treatment of a variety of illnesses like cancer, infections, inflammations etc. as bioactive compounds. Secondary metabolites have a wide range of uses in food, pharmaceuticals, medicine, and agriculture, since they contain antifungal, antibacterial, anticancer, and anti-inflammatory properties and act as a biocontrol agent against a variety of insects, pests, and diseases. These compounds are presently one of the major findings of scientific research. According to study, secondary metabolites screened from a vast fungal population should be employed in various sectors with no negative consequences (Singh and Sharma, 2020).

Alkaloids are a large group of organic compounds that contain a nitrogen atom or atoms in their structure. Alkalinity is caused by the nitrogen atoms in these

substances. They have a diverse range of activities such as analgesic, antitumor, antimalarial, antihypertensive etc. (Al-Snafi, 2021). Secondary metabolites like alkaloids, flavonoids, tannins, saponins, coumarins etc., act as the sources of anti-inflammatory agents (Mohammed *et al*., 2014). Phenols include one or more phenol groups as a common feature, and they range in complexity from basic molecules with one aromatic ring to very complex polymeric compounds. They possess anti-inflammatory, antioxidant, antiseptic, analgesic phytoestrogenic properties etc.. Flavonoids are the most common sort of phenol found in nature. Flavonoids have a chroman ring with an aromatic ring in positions 2, 3 or 4 in their structural skeleton. Flavonoids have antioxidant, anticancer, anti-inflammatory, antiviral, and antibacterial effects and a cytoprotective impact on the coronary and circulatory systems and the pancreas and liver. These qualities make them one of the most appealing natural compounds for supplementing existing therapeutic choices (Cazarolli *et al*., 2008). Saponins are carbohydrate-based molecules having a polycyclic aglycone moiety linked to a steroid (steroidal saponins) or triterpenoid (triterpenoidal saponins) moiety. Saponins have a large number of pharmacological effects. Antitumor, anti-inflammatory, expectorant, sedative, and analgesic properties are all found in saponins (Builders and Philip, 2019).

5.3.2 GC-MS Analysis

The GC-MS exhibits 10 peaks at given retention times 4.16, 9.18, 9.80, 11.30, 13.05, 13.97, 14.73, 21.20, 25.90, and 30.41. The compounds identified were 2-Propanone, 1-(dimethylamino)-; 2-Pyrrolidinone; N-Trimethylsilyl-2-pyrrolidinone; 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester; Hexanediamide, N,N’-di-benzoyloxy-; 2,5-Methylene-d,l-rhamnitol; 2,5-Methylene-d,l-rhamnitol; 2,5-Methylene-d,l-rhamnitol; Ribitol; 1,2-Benzenecarboxylic acid, diisooctyl ester

respectively (Figure 4). The structure of obtained compounds is also elucidated (Figure 6).

The GC-MS analysis shows the presence of some bioactive compounds in the extract. 2-Pyrrolidinone has significant antioxidant and anticancer activity (Thangam *et al*., 2013). Many derivatives of 2-Pyrrolidinone have proved to possess anti-inflammatory activity. In addition, the template 2-Pyrrolidinone also contributes to the anti-inflammatory activity of new compounds (Moutevelis-Minakakis *et al*., 2011). The most prevalent chemicals found in the extract are ribitol and 2,5-Methylene-d,l-rhamnitol. The primary bioactive compound ribitol has been shown to possess excellent anti-inflammatory and anticancer activities. (Ashour & Wink, 2011).

5.3.3 LC-MS Analysis

LC-MS performed in dual ion mode showed the presence of many bio-active compounds (Table 3,4 & Figure 7,8). More compounds were obtained through the positive ion mode but high abundance compounds are less. The most abundant compounds present in the extract are amino acid combination Arg Phe Arg, eflornithine, monobenzone, C16 sphinganine etc.. The compound eflornithine is commonly used to retard facial hair growth in women. It was previously developed and approved as a trypanosomiasis antiprotozoan medication. Recently it has been found that eflornithine is very effective in treating malignant gliomas. As a single drug, eflornithine shows an effect against recurrent gliomas. In anaplastic gliomas, eflornithine in conjunction with procarbazine, lomustine, and vincristine chemotherapy improves progression-free survival and overall survival by around 2.5 years compared to PCV treatment (Levin *et al*., 2018). Monobenzone is a depigmenting agent. And it has been widely used in the treatment of vitiligo for past

years (Rordam *et al*., 2012). KDM1A gene is widely expressed in cancer tissues and promotes cancer initiation and progression through various cellular signalling pathways. Monobenzone is an effective inhibitor of KDM1A and can potentially inhibit the spreading of stomach cancer (Ma *et al*., 2021). Other compounds also exhibit many medicinal uses. (23R)-1alpha,23,25-trihydroxy-24-oxo vitamin D3/ (23R)-1alpha, 23, 25-trihydroxy-24-oxo cholecalciferol, (24R,25S)- 25, 26-epoxy-1alpha, 24- dihydroxy-27 norvitamin D3 / (24R,25S)- 25, 26-epoxy1alpha, 24-dihydro are derivatives of vitamin D3. Clobetasol Propionate- a corticosteroid is used to treat various skin diseases like psoriasis, eczema, dermatitis etc. (Feldman & Yentzer, 2009). Tetrahydrodeoxycorticosterone has the potential anxiolytic property (Kunovac and Stahl, 1995). Finasteride is used to treat symptomatic benign prostatic hyperplasia (BPH) in men who have an enlarged prostate, as well as to treat male pattern hair loss (androgenetic alopecia) (Kaufman *et al*., 1998).

Thus, the present findings enumerates that the secondary metabolites and the major bioactive compounds present in the sample obtained through GC-MS and LC-MS may have contributed the antioxidant, antitumor, and anti-inflammatory potential.

5.4 In vitro cytotoxicity assay

New antitumor agents obtained from natural sources were primarily evaluated on their cytotoxic efficacy against cancer cell lines or in vivo antitumor models. The majority of existing cytotoxicity tests are based on changes in plasma membrane permeability or the absorption of dyes that are usually expelled by live cells. In the current study, the cytotoxicity of the fungal extract was estimated by the trypan blue dye exclusion method, in which the dead cells uptake the dye and appear blue under the microscope. Two cell lines were used for the in vitro cytotoxicity assay such as, DLA and EAC.

In both cell lines the percentage cytotoxicity increases in a dose dependent manner. In DLA cells it exhibits 12.5% cytotoxicity in 10 µg/mL, 15.8% in 20 µg/mL, 26.7% in 50 µg/mL, 34.2% in 100 µg/mL and at highest concentration 200 µg/mL it exhibits 51.6% cytotoxicity. Then in EAC cells 10% in 10 µg/mL, 14.2% in 20 µg/mL, 20% in 50 µg/mL, 28.3% in 100 µg/mL and at 200 µg/mL exhibits 35.8% cytotoxicity. IC_{50} value of cytotoxicity in DLA cell is 191.09 µg/mL. So, the extract shows more cytotoxicity in DLA cells than that of the EAC cells (Table 6,7 and Figure 13).

5.5 Animal experiments

All the animal experiments were carried out with prior approval from Institutional Animal Ethical Committee with approval number- ACRC/IAEC/18(2) P-2, following the internationally accepted laboratory animal use and care guidelines and rules of CPCSEA (Approval no. of institution – 149/PO/Rc/S/99/ CPCSEA).

5.5.1 Toxicity study

To understand the drug's toxicity, a toxicity study was conducted before going on to in vivo animal studies. Six Swiss albino mice were considered in this research. Three males and three females. Acute toxicity testing was carried out. In other words, a single dose with a greater concentration of the medication was administered. The concentration used was 2 g/kg b.wt. By dissolving ethanol extract in water, the drug was prepared. The death rate of the mice was assessed after the medication was given to them orally. The dosage was calculated using the results of an acute toxicity study conducted in accordance with OECD standards 423, which showed that the ethanol extract did not cause mortality at dosages up to 2 gm/kg b. wt. Furthermore, during the research period, no toxicity symptoms were seen in the dosage group up to 2 g/kg b.wt.

The drug concentrations used in the animal studies were 200 mg/kg b.wt. and 50 mg/kg b.wt. A larger dose of 1/10th of the amount used in the acute toxicity trial was chosen, as was a lower dose.

5.5.2 In vivo antitumor study

5.5.2.1 DLA-induced solid tumor model

Dalton's Lymphoma Ascites is a weakly differentiated malignant tumor that may be transplanted and grows in both solid and ascitic forms. It is seen in mice as lymphocytes (Kleinsmith, 2006). Lymphoma is a disease that affects lymphocytes and the lymphatic system, including the spleen, thymus, liver, and other lymphatic organs (Kalaiselvi *et al*., 2012). Swiss albino mice with DLA-induced solid tumors were treated with ethanol extract of *S. stipitatum* and compared to mice treated with the standard drug cyclophosphamide.

Ethanol extract of *S. stipitatum* exhibited a significant reduction in tumor volume when compared to the control group, which was left untreated after the induction of DLA cells. The tumor volume was measured for 30 days after the induction of DLA cells and drug administration for 10 days. Initially, the tumor volume gradually increased in all groups. But the rate of increase is less when compared to the control group. In the control group, after the 6^{th} day, it shows a tremendous increase in the tumor volume until the 30^{th} day. And on the 30^{th} day, tumor volume was 3.174 ±0.113 cm^3. But in other groups, even though the tumor volume increased, it was much less than the control group. In the standard group (diclofenac treated), the tumor volume on the 30^{th} day was only 0.738 ±0.077 cm^3. In the *S. stipitatum* ethanol extract treated ones in higher dose (200 mg/kg), tumor volume was 0.941 ±0.121 cm^3 and in lower dose 1.389 ±0.09 cm^3. Thus, it shows a dose-dependent decrease in the tumor volume, which shows the dose-dependent

increase in the antitumor activity of the extract. The result obtained for the ethanol extract is almost comparable with that of the standard group, and it reveals that the ethanol extract of *S. stipitatum* has significant antitumor properties (Table 10,11; Figure 14 & Plate 2).

5.5.2.2 EAC-induced ascites tumor model

Ehrlich Ascites Cancer is aggressive, quickly growing, undifferentiated carcinoma (Segura *et al*., 2000). It is hyperdiploid from the outset, with an excellent transplantable capacity, a limited lifespan, 100% malignancy, and lack tumor-specific transplantation antigens (Kaleoğlu and İşli, 1977). It grows in all strains of mice and may be found in both ascites and solid forms. EAC are the most susceptible to chemotherapy because they are undifferentiated and have a fast development rate, and they mimic human tumors (Ozaslan *et al*., 2007). EAC induced ascites tumor model of Swiss albino mice was treated with 2 doses of ethanol extract of *S. stipitatum* and compared with the mice treated with standard drug cyclophosphamide. In addition, the increase in lifespan of each group of mice was evaluated to identify the potential of the administered drug.

The ethanol extract of *S. stipitatum* at a higher dose (200 mg/kg) exhibited excellent antitumor activity by increasing the life span of EAC-induced mice. In the untreated control group, the average lifespan observed was 19.8 days. In standard, it increases to 28.6 days, in higher dose extract-treated group lifespan was 30.7 days and in lower dose extract-treated group average lifespan was 26.6 days. The percentage increase in the life span of drug-treated groups was calculated by comparing it with the control group. In the standard group, the percentage increase in lifespan was 44.4%. In the 200 mg/kg ethanol extract-treated group, the percentage increase in life span was 55%, and in the lower dose treated group, it was 34.3%.

The activity of the ethanol extract is in a dose-dependent manner. Activity increases in the higher dose treated group. That reveals that the ethanol extract of *S. stipitatum* has promising antitumor activity even better than the standard treated group (Table 12 and 13; Figure 15, and Plate 3). This discloses the significance of further studies in this fungus to obtain the exact compounds behind the activity and formulate the precise drug combination to use in chemotherapy.

5.5.3 In vivo antioxidant study

In the in vivo antioxidant study with ethanol extract of *S. stipitatum*, when the untreated control group animals exposed to sodium fluoride (NaF) showed a weaker profile of antioxidant in both blood and liver tissues, it was noticed that pre-treatment with *S. stipitatum* ethanol extract remarkably enabled them to overcome the stress. This is observed from the elevated levels of catalase, SOD, reduced glutathione, GPx and GR in the liver, and catalase and SOD in blood, which was considerably enhanced with reference to control. Lipid peroxidation, which is the oxidative degeneration of lipids caused by the free radicals, is also reduced in the groups which were pre-treated with drugs (Table 8 and 9). Cascade processes that produce free radicals and glutathione-depleting agents promote oxidation in cells, resulting in cellular damage. Peroxyl radicals, superoxide radicals, singlet oxygen, and hydroxyl radicals are all known to cause DNA damage. There are some synthetic antidotes for fluoride poisoning, such as DMSA (meso-2, 3-dimercaptosuccinic acid) and BAL (2, 3-dimercapto-1-propanol), when administered alone, they might produce adverse effects. Recent research has found that polyphenols and flavonoids produced from natural sources can reduce fluoride-induced damage in cells. Phenols and flavonoids present in fungi are already proved to possess antioxidant activity (Cazarolli *et al*.,

2008). Its presence might have reduced oxidative stress, which would not have been accomplished otherwise if pre-treatment had not been used.

5.5.4 In vivo anti-inflammatory study

5.5.4.1 Acute carrageenan induced model

Carrageenan-induced inflammation is the most suitable procedure for screening acute anti-inflammatory agents (Greenwald, 1991). The current investigation on ethanol extract of *S. stipitatum* reveals its ability to remarkably reduce paw edema in a dose-dependent manner. In carrageenan, the inhibitory effect of extract at high dose (200 mg/kg) was 83.33%, the low dose (50 mg/kg) was 66%, and that of standard diclofenac was 88.88% (Table 14). In all groups, there is an initial increase in the paw thickness for the first 2 hrs after the induction of carrageenan. But then it starts to reduce, and the drug-treated groups show a drastic reduction compared to the control. The decrease in the paw edema in the control group is significantly slighter, and it will not reduce beyond a specific limit. In the standard group, it almost gets reduced to the normal level. And the drug-treated ones also show a good result (Figure 16).

5.5.4.2 Chronic formalin-induced model

The formalin-induced paw edema model is the best method for screening chronic anti-inflammatory agents closely related to human arthritis (Greenwald, 1991). In the formalin-induced model, the inhibitory effect of extract at high dose (200 mg/kg) was 94%, the low dose was 80%, and standard diclofenac was 98% (Table 15). Here also, all groups show an increase in paw edema initially, but it is comparatively less in drug-treated groups. Then from the 3rd day, onwards edema starts to decrease, and ethanol extract treated ones and standard treated ones almost

come to the normal paw thickness. Thus, the *S. stipitatum* ethanol extract at high and low doses gives a promising result comparable to the standard drug (Figure 17).

Inflammation is mediated by the activation of prostaglandins, Platelet Activating Factor (PAF), and other mediators of inflammation like TNF-α, interleukin, NO etc. (Hwang *et al*., 1986). And it is also attributed to the release of histamines, kinins, serotonins, etc. (Larsen and Henson, 1983). Here the anti-inflammatory activity of ethanol extract in both models is more or less comparable with that of the diclofenac, the conventional anti-inflammatory drug. And the anti-edematous effect maybe because of the inhibition of histamine release or the inhibition of cyclooxygenase enzymes responsible for the formation of prostaglandins. Cyclooxygenase inhibition proves to be more effective for the inhibition of carrageenan-induced inflammation. Thus, it might be downregulating the prostaglandin synthesis and the Coxygenase-2, which promotes the prostaglandin synthesis (Giuliano and Warner, 2002). Then the proliferative phase of inflammation is represented by the formalin-induced paw edema (Ahmed and Ramabhimalah, 2012). So, the drug also seems to act by inhibiting the proliferative phase of inflammation.

5.6 Mycosynthesis of silver nanoparticles

Silver nanoparticles have been used as antibacterial agents, in industrial, household, and healthcare-related products, optical sensors, medical device coatings, and cosmetics, in pharmaceutical and food industries, in orthopedics, diagnostics, drug delivery, and as anticancer agents, and have ultimately improved the tumor-killing effect (Chernousova and Epple, 2013). The use of AgNPs in cancer is grouped into two categories: diagnostic and therapeutic. Several research groups have reported using AgNPs as nanocarriers for enhanced targeted delivery,

chemotherapeutic drugs, radiation effect and photodynamic therapy (Zhang *et al*., 2016).

The fungus *S. stipitatum* was utilized to produce stable silver nanoparticles, which were visually examined by converting the fungal aqueous filtrate incubated with $AgNO_3$ solution into a dark brown color due to AgNP deposition caused by surface plasma resonance. (Figure 9). In the control group, there was no color change (Krishnaraj *et al*., 2010). The color change in the solution showed the production of AgNPs. By altering the color intensity of the aqueous filtrate with $AgNO_3$, the AgNPs were well distinct in the solution after 1 day of incubation, with no aggregation. AgNPs generate a brown solution in water due to the surface Plasmon resonances (SPR) action and reduction of $AgNO_3$ (Bansal *et al*., 2010).

5.6.1 UV- Visible spectra analysis of silver nanoparticles

UV-Visible spectroscopy was used to characterize the nanoparticles and to examine their optical properties. AgNPs are formed when the color changes from cloudy white to brown. The absorption spectra result corresponded adequately with this phenotypic change. The production of AgNPs, which are typical of silver nanoparticles, was studied using UV-Visible spectroscopy in the 350-600 nm range, with the highest absorption owing to the excitation of surface Plasmon vibrations occurring at 440 nm regions indicates the presence of silver nanoparticles. These findings agree with the study of silver nanoparticles conducted by Verma *et al*. (2010).

5.6.2 SEM Analysis of silver nanoparticles

The scanning electron microscopy (SEM) gives significant details on the shape and dimension of the nanoparticles. The biosynthesized silver nanoparticles spherical in form, indicating that they were surface deposited silver nanoparticles. The size of

the nanoparticles shown in the result are larger due to the aggregation of nanoparticles and capping agents. The monodispersed, spherical and particle size were strongly reliant on AgNPs' properties. These findings were in accordance with the results of Pal *et al*. (2007).

5.6.3 TEM Analysis of silver nanoparticles

Transmission electron microscopy (TEM) provided further knowledge of the morphology and size of AgNPs. The figure 12. shows a typical TEM images of silver nanoparticles. Several researchers had previously studied the biosynthesized silver nanoparticles using TEM (Balaji *et al*., 2009). The average size of the nanoparticles ranges from 12-28 nm, according to data acquired from transmission electron micrographs.

5.6.4 XRD analysis of silver nanoparticles

The crystalline structure of silver nanoparticles was revealed by X-ray diffraction patterns (Figure 13). Peaks at 2θ values of 38.148, 43.885, 64.488, and 77.398 correspond to crystal planes (1 1 1), (2 0 0), (2 2 0), and (3 1 1), respectively. The results match those published by the Joint Committee on Powder Diffraction Standards (JCPDS) File No. 04-0783 for silver (face-centric cubic). XRD has been used to characterize silver nanoparticles by several researchers. The obtained results are as per the standard diffraction of past scientific reports (Qian *et al*., 2013).