



Faculty of Resource Science and Technology

**Phenolic compounds, Antioxidant Capacity and Compound Profiles of
Extracts from Raw Sago Starch Fermentation with Endophytes**

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Extracts from Raw Sago Starch Fermentation with Endophytes

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Escalating prevalence of free radicals in the modern environment, driven by factors such as pollution and unhealthy lifestyles, poses a significant health concern. There is a need to explore the potential benefits and applications of antioxidants in mitigating the oxidative damage caused by free radicals and their role in promoting overall well-being. In the last decade, an increasing number of research findings and studies have addressed strategies to combat oxidative stress. This surge in interest stems from concerns regarding the potential health risks associated with synthetic drugs, which can contribute to silent yet perilous conditions like heart disease, hypertension, and cancer. As a result, there has been a growing exploration of natural-based materials as potential alternatives. Endophytic fungi, residing within the tissues of various plant species, have emerged as a promising alternative source of antioxidants that exhibit analogous properties to their host plants. This unique symbiotic relationship holds immense potential for the extraction of antioxidant compounds, offering new avenues for harnessing nature's own defences against oxidative stress. To implement this, one of the alternative ways is through value-added food. Antioxidant-enriched value-added food products achieved via innovative fermentation techniques have gained prominence as a sustainable approach to enhance both nutritional content and shelf-life, offering promising prospects for healthier dietary choices and reduced food waste. Despite an annual production of 14,000 tonnes of underutilized sago starch, this valuable resource remains largely untapped, presenting a pressing challenge for sustainable utilization and economic development, mainly in the region of Sarawak and in some small areas in Johor. This study aims to investigate innovative approaches to maximize the utilization of sago starch through fermentation, thereby unlocking its full potential for various applications in the food and industrial sectors. In this study, traditionally processed sago starch is being used

rather than industrially processed sago starch. The latter often loses crucial nutritional factors during its processing, whereas the former, known as raw sago starch (RSS) “*lemantak*” holds promise as a more nutritionally valuable option. Consequently, this research aims to explore the presence of potential phenolic compounds with antioxidant properties in fermented raw sago starch, offering an alternative avenue for extracting nutraceuticals from this resource. Additionally, employment of microbial fermentation with fungal endophytes to further enhance the production of phenolic compounds in traditionally processed RSS “*lemantak*”. Also, to fully maximise the potential of the fermented RSS “*lemantak*”, optimisation process was also carried out by manipulating several parameters such as effect of fungal endophytes consortia, effect of fermentation time, effect of extraction time and effect of solvent extraction. It is crucial that these parameters were manipulated to maximise the potential for phenolic compound production from fermented RSS “*lemantak*”. All selection was made based on the total phenolic and antioxidant profiles. It was found out that the best fungal consortia were consortium of *Aspergillus niger* and *Penicillium chermesinum* (AP) under solid-state fermentation condition. It was also found out the best fermentation period was on Day 7 while the best extraction time was 24 hrs of incubation under water bath extraction method at 50°C. Nevertheless, the best extraction solvent was methanol as compounds were more soluble in a more polar solvent. Extractives were also analysed through GC-MS method to identify the contributed bioactive compounds. Compounds such as Eicosane and 1-Eicosanol were found to be the most dominant in the extracts reported to have both antioxidant and antimicrobial activity. This opens the potential of fermented RSS into becoming new value-added food, harnessing the inherent nutritional benefits of sago starch and its newfound antioxidant capacity. As exploration in diverse applications of endophytic fungi continues in food science and nutrition, our findings

contribute to a deeper understanding of sustainable food processing and functional ingredient discovery.

Keywords: Fermentation, endophytes, raw sago starch, antioxidant, optimisation

***Penghasilan dan Pengekstrakan Sebatian Fenolik daripada Penapaian Pati Sagu
Mentah dengan Kulat Endofit***

ABSTRAK

Kemunculan radikal bebas yang semakin meningkat dalam persekitaran moden, yang dipacu oleh faktor-faktor seperti pencemaran dan gaya hidup yang tidak sihat, menjadi kebimbangan kesihatan yang signifikan. Terdapat keperluan untuk meneroka manfaat dan aplikasi potensi antioksidan dalam mengurangkan kerosakan oksidatif yang disebabkan oleh radikal bebas dan peranannya dalam meningkatkan kebahagiaan keseluruhan. Mutakhir ini, peningkatan penyelidikan dan kajian yang telah menganalisis pelbagai strategi untuk melawan tekanan oksidatif. Pertumbuhan minat ini timbul daripada kebimbangan mengenai risiko kesihatan yang mungkin berkaitan dengan antioksidan yang diperbuat secara sintetik, yang boleh menyumbang kepada keadaan berisiko seperti penyakit jantung, hipertensi, dan kanser. Lantaran daripada itu, kajian dan penyelidikan menggunakan bahan berasaskan semula jadi sebagai sumber alternatif yang berpotensi semakin meningkat atas isu keselamatan. Kulat endofit, yang menetap dalam tisu pelbagai spesies tumbuhan, muncul sebagai sumber alternatif yang menjanjikan sumber antioksidan yang mempunyai sifat analog dengan tumbuhan inang mereka. Hubungan simbiotik unik ini mempunyai potensi besar untuk penghasilan sebatian antioksidan, menawarkan jalan baru untuk memanfaatkan pertahanan semula jadi alam terhadap tekanan oksidatif. Untuk melaksanakan ini, salah satu cara alternatif adalah melalui makanan berharga tambah. Produk makanan berharga tambah yang diperkayakan dengan antioksidan melalui teknik fermentasi inovatif telah mendapat perhatian sebagai pendekatan mampan untuk meningkatkan kandungan pemakanan dan tempoh simpan, menawarkan prospek yang menjanjikan untuk pilihan pemakanan yang lebih sihat dan mengurangkan pembaziran makanan. Walaupun terdapat

pengeluaran tahunan sebanyak 14,000 tan, kanji sagu yang tidak dimanfaatkan menyebabkan sumber berharga ini masih belum digunakan sepenuhnya, lantas mengakibatkan cabaran yang mendesak dalam penggunaan yang mampan dan pembangunan ekonomi, terutamanya di Sarawak dan beberapa kawasan kecil di Johor. Kajian ini bertujuan untuk menyiasat pendekatan inovatif untuk memaksimumkan penggunaan kanji sagu melalui fermentasi, dengan itu membuka potensi penuhnya untuk pelbagai aplikasi dalam sektor makanan dan industri. Dalam kajian ini, kanji sagu yang diproses secara tradisional ditekankan sebagai subjek utama dan bukannya kanji sagu yang diproses secara industri. Hal ini demikian kerana, kanji sagu yang diproses secara industri sering kehilangan faktor pemakanan penting semasa pemprosesannya, manakala kanji sagu yang diproses secara tradisional yang juga dikenali sebagai 'kanji sago mentah (RSS) "lemantak",' menjanjikan sebagai pilihan yang lebih bernilai dari segi nutrisi pemakanan. Oleh itu, penyelidikan ini bertujuan untuk meneroka kehadiran sebatian fenolik berpotensi dengan sifat antioksidan dalam kanji sagu mentah yang diproses secara tradisional telah difermentasikan, menawarkan jalan alternatif untuk mengekstrak nutraseutikal daripada sumber ini. Selain itu, penggunaan fermentasi mikrob dengan kulat endofit untuk meningkatkan pengeluaran sebatian fenolik dalam RSS lemantak yang diproses secara tradisional. Selain itu, untuk memaksimumkan potensi sepenuhnya daripada RSS "lemantak" yang telah difermentasikan, proses pengoptimuman juga dilakukan dengan memanipulasi beberapa parameter seperti kesan konsortium kulat endofit, kesan tempoh fermentasi, kesan tempoh pengekstrakan, dan kesan pengekstrakan pelarut. Adalah penting bahawa parameter-parameter ini dimanipulasi untuk memaksimumkan potensi pengeluaran sebatian fenolik daripada RSS "lemantak" yang telah difermentasikan. Pemilihan kesemua ini dibuat berdasarkan profil fenolik keseluruhan dan profil antioksida. Didapati konsortium

kulat terbaik adalah konsortium Aspergillus niger dan Penicillium chermesinum (AP) di bawah keadaan fermentasi pepejal. Juga didapati tempoh fermentasi terbaik adalah pada Hari ke-7 manakala tempoh pengekstrakan terbaik adalah 24 jam di bawah kaedah pengekstrakan air panas pada suhu 50°C. Walau bagaimanapun, pelarut pengekstrakan terbaik adalah metanol kerana sebatian-sebatian lebih larut dalam pelarut yang lebih polar. Ekstrak juga dianalisis melalui kaedah GC-MS untuk mengenal pasti sebatian bioaktif yang menyumbang. Sebatian seperti Eicosane dan 1-Eicosanol didapati menjadi yang paling dominan dalam ekstrak yang dilaporkan mempunyai aktiviti antioksidan dan antimikrob. Ini membuka potensi RSS yang telah difermentasikan menjadi makanan berharga tambah baharu, memanfaatkan manfaat pemakanan asli kanji sago dan kapasitinya yang baru ditemui untuk antioksidan. Seiring dengan penjelajahan dalam pelbagai aplikasi kulat endofit dalam sains makanan dan pemakanan, penemuan kami menyumbang kepada pemahaman yang lebih mendalam tentang pemprosesan makanan yang mampan dan penemuan makanan berfungsi.

Kata kunci: Penapaian, kulat endofit, kanji sago mentah, antioksidan, pengoptimuman

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LIST OF ABBREVIATIONS

µl	Microlitres
BHA	Beta hydroxy acid
BHT	Butylated hydroxytoluene
°C	Degree Celsius
Consortia A	Fungal endophyte <i>Aspergillus niger</i>
Consortia AF	Fungal endophytes consortia of <i>Aspergillus niger</i> & <i>Fusarium equiseti</i>
Consortia AFP	Fungal endophytes consortia of <i>Aspergillus niger</i> , <i>Fusarium equiseti</i> & <i>Penicillium chermesinum</i>
Consortia AP	Fungal endophytes consortia of <i>Aspergillus niger</i> & <i>Penicillium chermesinum</i>
Consortia F	Fungal endophyte of <i>Fusarium equiseti</i>
Consortia P	Fungal endophyte of <i>Penicillium chermesinum</i>
Consortia PF	Fungal endophytes consortia of <i>Penicillium chermesinum</i> & <i>Fusarium equiseti</i>
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
g	Gram
g/L	Gram per litre
GAE	Gallic acid equivalent
GCMS	Gas chromatography mass spectrometry
GMM	Glucose minimal media
HPLC	High performance liquid chromatography
hrs	Hours

MEA	Malt extract agar
MEB	Malt extract broth
mg GAE/ g	Milligram of gallic acid equivalent per gram
min	Minutes
ml	Millilitres
mM	Millimolar
MS UV SPECTRO	Mass spectrometer ultraviolet spectrometer
nm	Nanometre
Na ₂ CO ₃	Sodium carbonate
OFAAT	One factor at a time
PFRAP	Potassium ferric reducing power
pH	Potential of hydrogen
RNA	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Rotation per minute
RSS	Raw sago starch
SMF	Submerged fermentation
SSF	Solid state fermentation
TBHQ	Tert-butylhydroquinone
TPC	Total phenolic content
UV-VIS Spectrometer	Ultraviolet-visible light spectrometer
w. b.	Wet basis
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Study Background

Natural products from plants have gathered significant attention due to the recent evidence suggesting their capacity to improve oxidative stress and other related diseases. Since ancient times, plants have been a reservoir of cures and treatments to many diseases and ailments. From different parts of plants, various kind of ailments were treated either applied externally or through consumption of the parts of the plants. Due to these values, plants were harvested for their benefits especially medicinal plants. However, when major harvesting of medicinal plants was carried out, over-harvesting should be considered and observed, as overharvesting will not only endanger the species of the medicinal plants and herbs, but this will also contaminate the gene pool of the species leading to a loss of genetic diversity. Although gene diversity can be restored through mutations, but it takes long time for it to recover. Moreover, habitat loss due to over-harvesting will also be occurred. Therefore, rather than fully utilising the medicinal plants, another organism which lives symbiotically, endophytic fungi, are being utilised instead.

This research utilised the endophytic fungi isolated from the host plant to help produce beneficial bioactive compounds as it was proven by studies that the bioactive compounds found in the endophytes and its host plant are within the same spectrum (Singh et al., 2016 & Kusari et al., 2012). Moreover, majority of endophytic fungi are non-pathogenic in nature making most of the derived metabolites are suitable for human usage due to the non-toxic property to mammalian cells (Ancheeva et al., 2020). It is well-known that the active compounds that have been isolated from fungal endophytes include

antibiotics, anticancer, antifungal, and antiviral. With the discovery of paclitaxel, an anticancer drug derived from the bark of *Taxomyces andreanae* from *Taxus brevifolia*, the surge of finding endophytic fungi with useful metabolites have been trending over the decades. These metabolites which was derived from the secondary metabolites produced in plants are biologically active compounds which comprises of phenolics, flavonoids and terpenoids classes, playing a major role in inhibiting the activity of the reactive oxygen species or free radicals which are harmful to the human body.

Oxidative stress can be associated to many human diseases such as inflammation leading to the pathogenesis of cancer (Maeda & Omata, 2008), cardiovascular diseases (Montetucco et al., 2011) and diabetes (Sell & Eckel, 2009). According to Gülçin (2020), oxidative stress can be defined as an imbalance between production reactive oxygen species and antioxidant defences.

1.2 Endophytic Fungi Produced Similar Bioactive Compounds to Host Plant

Years of research have studied the relationship between the isolated endophytic fungi and its host plant. Theoretically, the isolated endophytes may or may not produce similar compounds as its host plants. Despite of the numerous studies that claimed to produce compounds similar to its host plant, these isolated fungi required a certain stress or an activation of cluster of genes to induce the metabolite pathway via *in vitro* conditions to produce similar bioactive metabolites as their host plant (Sharma et al., 2021). Endophytes are microbes which colonize living, internal tissues of plants without causing any harm or symptoms of disease to their host plants as they live in symbiosis (Gouda et al., 2016). They are well known to produce secondary metabolites that are similar to the host which are beneficial to industries such as agriculture, cosmeceutical, and medical uses. Reports have

indicated that endophytes contribute substances that have various bioactivity reactions such as antimicrobial, antioxidant and enzyme production. As of now, only endophytic bacteria isolated from sago palm tissue culture were reported to possess antimicrobial activity (Labrador et al., 2014). No reports for endophytic fungi detected in sago palm were ever reported. Leveraging endophytic fungi-derived bioactive compounds in research is crucial for unlocking their untapped potential to combat drug-resistant pathogens, chronic diseases, and environmental sustainability, fostering innovation and progress in various scientific fields.

1.3 Problem Statement

The human body harbours defence mechanism against oxidative stress induced by free radicals. Apart from the innate defence system, synthetic drugs are also available to improve the capacity of the body defence mechanism against oxidative stress. But owing to harmful side effects of the synthetic drug, research on natural products has taken leap in recent years. From food technological view, antioxidants served as a preservative to prevent food from spoilage through oxidation, thus reducing the loss of nutrients, and maintaining the texture, colour pigment, taste, freshness, functionality, and aroma (Carocho & Fereira, 2013).

Sago starch is one of the staple foods derived from the underutilized crop *Metroxylon sagu* Rottb., the sago palm. The sago palms are commonly found in peat swamp areas widely distributed in Malaysia, Indonesia, and Papua New Guinea. In Malaysia, this palm is majorly found in the state of Sarawak and a few places in the state of Johor. Production of sago starch has long been utilised decades ago through different methods with one of it using the traditional way of processing it. Traditionally processed sago starch is commonly known as sago “*lemantak*” among locals. They are the most common native sago starch sold in the

wet markets of Sarawak and is an infamous local produce among the Sarawakian. The potential of sago starch is limitless. Studies reported that it contains high in amylopectin ranging from 69 to 78.8% making it as a potential bioplastic with high tensile properties (Arshad et al., 2019). Resistant starch is a selective starch that stimulates the growth and/or activity of one or more number of beneficial bacteria including *Lactobacilli* and *Bifidobacterial* and thus influencing positively of the host health which is known as the prebiotic effect (Arshad et al., 2019). Currently, there are no reports on the functional properties of sago “*lemantak*”, nor studies carried out to investigate the antioxidant ability of the sago starch “*lemantak*”. To further investigate on the functional properties of sago starch “*lemantak*,” this study also utilized fermentation techniques as a method to compare of the properties between unfermented sago starch and the fermented sago starch.

Fermentation is an old food processing technique since ancient times. This technique is known to make the food lasts longer by having a longer shelf life. Not only that, but it can also improve the texture, palatability and enhances the nutritional value of the food itself (Zhao et al., 2019). For instance, the *tempeh* and *tapai* production. Depending on the source of microorganism, fermentation can be classified as natural fermentation whereby the natural microflora in the substrates itself was used as the starter inoculum while seed fermentation is an artificially insemination of the microorganism upon the substrate. In general, the technique of fermentation is divided into solid phase fermentation and liquid phase fermentation which is also called submerged fermentation depending on the amount of water in the system. Studies have found out microorganisms used in fermentation feeds on carbohydrates to produce gases, acids, and enzymes which in turn modified the starch structure into a looser form which then generates delightful taste and tastes slightly sour (Yang & Tao, 2008). Therefore, this study focuses on the phytochemical properties,

specifically on the phenolic content and antioxidation capability of traditionally processed sago starch fermented with endophytic fungi.

In a way of preventing noncommunicable diseases from occurring, antioxidants help to balance out the oxidative stress formed by free radicals. Although synthetic drugs have been used to enhance the body's defense mechanism, but they always come with harmful side effects. Medicinal plants have always been known to be a treasure trove with beneficial metabolites. Instead of using a direct approach by harvesting medicinal plants, endophytic fungi which is known to possess similarly to their host plant is utilized and concurrently, reducing the risk of overharvesting medicinal plants which can lead to its extinction. Studying the fermentation process with endophytes could reveal novel bioactive compounds or metabolic pathways that could have broader applications in biotechnology or medicine. Without conducting the fermentation, these opportunities may be overlooked. By extracting metabolites from fermented sago starch with endophytes, this study focusses on assessing the phenolic content and antioxidant properties of traditionally processed sago starch fermented with endophytic fungi. Phenolic compounds are known for their antioxidant activity, which can help neutralize harmful free radicals in the body. If fermentation is not performed, the antioxidant capacity of the sago starch may remain limited, potentially missing out on the potential health benefits associated with increased antioxidant levels.

1.4 Hypothesis

Raw sago starch (RSS) "*lemantak*" will be able to provide both the growth support and production of novel antioxidant phenolic compounds through the fermentation of the raw sago starch "*lemantak*" by selected endophytic fungi. The fermentation of the raw sago

starch “*lemantak*” will also be able to increase the synthesis of phenolic compound products with increased antioxidant activity as compared to the unfermented sample.

1.5 Objectives

This study aimed to investigate potential phenolic compounds with antioxidant capacity from fermented raw sago starch with endophytes. This was achieved through the following set of objectives:

- i. To compare the solid-state fermentation and submerged fermentation type to produce phenolic compounds
- ii. To optimise the extraction parameters of the phenolic compounds from the fermented sample
- iii. To characterise the antioxidant and phenolic compounds produced

1.6 Chapter Summary

A general summary of the following chapters will be explained. In the chapter Introduction, a general view of the study is being represented. A brief background of the study was included to show the basis of the study is being conducted. The problem statement of the study was justified to work on the areas. Hypothesis served as framework and adjustment of the methodology as well as the objectives of the study.

Chapter 2 provides a detailed background study of the subject used in the study. The apprehension of utilising endophytic fungi and the raw sago starch in this study was explained in Chapter 2. Also, a brief overview of the main idea was also explained in the literature review to find a solution to solve the problem found from this study.

Chapter 3 shows a detailed explanation of the materials, source of sago starch and endophytic fungi as well as a detailed methodology of the study was laid out as a guide to carry out different tests. This chapter also serves as a framework for future research prospects.

Chapter 4 presents the findings of this study and a comparative reasonings based on previous starch-based studies. Problems occurred during the research was also stated and was overcome by finding suitable solutions.

Chapter 5 includes the conclusion and recommendations from this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This chapter describes the literature review for past research on sago starch and bioactive production through fermentation process. A detailed explanation to justify the was laid out. These will be explained further in the following subsections.

2.2 Free Radicals, Oxidative Stress, and its Mechanism of Destruction

Reactive oxygen species is a common term describing oxygen-containing free radicals and certain other radical compounds that can oxidize important biomacromolecules such as nucleic acids, proteins, and lipids. These molecules are the common target of these oxidative radicals. For instance, a lot of physiological and pathological phenomena are being associated with lipid peroxidation. Lipid peroxidation is an important marker in many human diseases associated with oxidative stress, especially arteriosclerosis and neurodegenerative diseases. Production of free radicals in living systems, drugs and food were produced primarily through oxidation process (Di Meo & Vendititi, 2020). Free radicals are reactive free radicals such as superoxide ($\text{-O}_2\bullet$), hydroxyl (-OH), peroxy ($\text{LOO}\bullet$), and hydroperoxyl ($\text{HOO}\bullet$) radicals and certain nonradical oxidizing agents such as hydrogen peroxide (H_2O_2), lipid hydroperoxide (LOOH), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1\text{O}_2$).

Major production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) derives from the powerhouse cell, the mitochondria. Produced as by-products in aerobic respiration by electron transport chain, the free radicals are also important in the

production of superoxide and nitric oxide by neutrophils and macrophages which helps in phagocytosis process (Adwas et al., 2019). According to Adwas et al. (2019), superoxide radicals in phagocytic cells can be considered as nonselective antibiotics, kills any infectious bacteria (and neutrophils) and possibly damaging surrounding tissue cells. Because these radicals contribute to the inflammatory response, the free radicals also promote cell proliferation (fibroblasts mitosis) which forms scar tissue and lymphocyte proliferation during clone production. On the other hand, other radicals such as the nitric oxide stimulates vasodilation of smooth muscles and thus improves blood flow to the site of inflammation (Hirst & Robson, 2011). Ultimately, it can be deduced that these by-products help minutely in different biological processes to protect the human body. However, exceeding a certain threshold of these free radicals, could cause emergence of non-communicable disease and thus jeopardizing our own body.

The production of free radicals such as ROS and RNS can be by endogenous or exogenous. Endogenous production of these free radicals is by cell-induced inflammatory mechanism and immunity cell activation, cancer, infections, and aging. Exogenous production is due to environmental pollution such as water and air pollution, alcohol consumption, heavy metals, and radiation exposure. Compounds from these factors are then degraded into ROS after entering the human body (Valko et al., 2007). The damage these free radical causes to the proteins, lipids and nucleic acids causes the development of diseases such as irritation, inflammation as well as diseases such as cancer, heart disease and diabetes (Haliwell, 2007).

Chan et al. (2015) stated that under normal circumstances, these free radicals are successfully eliminated by antioxidant defence mechanism through enzymes and non-enzymatic factors. Nevertheless, due to discrepancy of overproduction of ROS and failed to

buffer between the free radicals and antioxidant concentration eventually leads to cellular oxidative stress as shown in Figure 2.1. This state is known as oxidative stress.

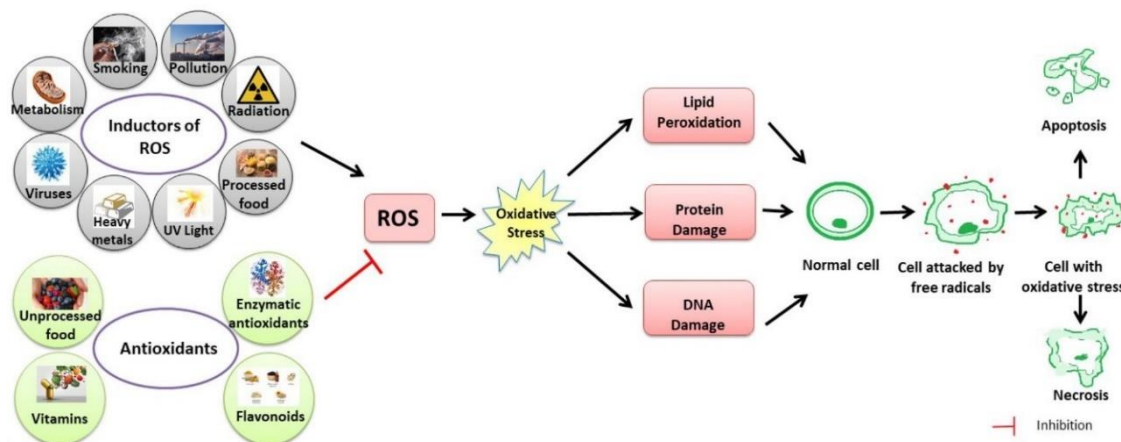


Figure 2.1: Schematic representation of the relationship between ROS, oxidative stress, and effects on the human body. Adapted from “Lifestyle, oxidative stress, and antioxidants: back and forth in the pathophysiology of chronic diseases,” by Shariffi-Rad et al. (2020).

The imbalance of this condition will mutilate biomacromolecules such as DNA, proteins and membrane lipids which then leads to the emergence of incurable diseases like atherosclerosis, stroke, cancer, and diabetes (Beckman & Ames, 1997). Not only that, the production of these free radicals also derived from lipid oxidation. In general, lipid oxidation produced lipid hydroperoxides and conjugated dienes or trienes. These by-products are unstable and will further breakdown to many secondary oxidation products, such as alcohols, aldehydes, hydrocarbons, and volatile compounds. As a result, this forms the bad odour from deterioration of food. This was further supported by the study conducted by Domínguez et al. (2019), which showed that the by-products of lipid oxidation could cause the formation of harmful compounds that induced several human pathologies, including atherosclerosis, cancer, inflammation, and ageing process. Oxidation does not only occur in human body, but also present in food processes when exposed to air (oxygen) and/or heat or light. These

oxidation reactions also reduce nutritional value due to loss of essential fatty acids and vitamins (Jacobsen et al., 2019). Thus, it is crucial that the mechanism of oxidation is to be understood to counter the negative impact of oxidative stress towards living beings as well as its impact in food industry.

2.3 Phenolic Compounds

Phenolic compounds are secondary metabolites available throughout the plant kingdom. They are compounds that contain a phenol moiety, involved in adaptation processes in plants during stress conditions like wounding or UV radiation exposure and as a defence mechanism against predators. Al Mamari (2021) described phenolic compounds as a structure of a phenol composed with a benzene ring that is substituted with a hydroxyl group. Systematically, this structure is known as a hydroxybenzene. These compounds can exist freely in the plants, although they are commonly bounded to sugars or proteins (Cosme et al., 2020).

Phenolic compounds are derived from several biosynthetic precursors including pyruvate, acetate, some amino acids such as phenylalanine and tyrosine, malonyl CoA, acetyl CoA through action of pentose phosphate, shikimate and phenylpropanoid metabolism pathways. Furukawa et al. (2004) stated that these group of metabolites have been broadly explored for their ability to combat oxidative stress, as chronic oxidative stress is often associated with a number of metabolic disorders and a variety of pathologies, such as obesity, diabetes and cardiovascular diseases.

Many terms were used to describe phenolic compounds such as polyphenols, bioactive compounds, and phytochemical depending on its structural and classes of phenolic compounds. Biological effects of phenolic compounds are mainly attributed to the ability to sequester or inhibit reactive oxygen and nitrogen species, transfer electrons to free radicals,

in addition to activating antioxidant enzymes, improving oxidative stress and inflammation (de Araújo et al., 2021). Plant derived constituents are bioactive and involved in potentiating the redox defence of body, prevention and counteracting oxidative stress and reducing free radical related cellular damage (Engwa, 2018). Biological properties of these compounds depend on several factors. Depending on the number and positions of the hydroxyl groups and its nature of substitutions on the aromatic rings, the antioxidant activity of the compounds is mainly sought which is related to the high tendency to chelate metals and stabilises the radicals (Roleira et al., 2018).

The classes of phenolics can be divided into 16 different classes depending on the length of its carbon chain (Cosme et al., 2020). The most studied phenolic compounds are the phenolic acids, tannins, and flavonoids. There are also classes such as simple phenols, polyphenols, stilbenes, lignans and lignin. These groups of phytochemicals have been extensively studied for many decades. Most of it derives from plant-based sources such as whole grains, legumes, fruits, and vegetables. They are ubiquitous in nature. Phenolic compounds display a wide range of biological activities such as antioxidant, antimicrobial, anti-inflammatory.

In addition, phenolic compounds can form different types of interactions, such as hydrogen bonds, ionic bonds, and hydrophobic interactions, with the components of the food matrix, therefore, it has been sought to optimize the production of phenolic compounds-enriched foods without altering its organoleptic and structural characteristics (Czajkowska–González et al., 2021).

Phenolic compounds can be analysed through different kind of techniques. Spectrophotometric techniques such as GCMS, HPLC, MS UV SPECTRO, UV-VIS SPECTRO represents some of the commonly used techniques in detecting this group.

Typical spectrophotometric technique in measuring the total phenolic content (TPC) in plants are usually detected by Folin-Denis and Folin-Ciocalteu methods. The latter procedure is commonly used as compared to the Folin-Denis as it is based on electron transfer which found to be preferable and thus frequently used in tests (Bunzel & Schendel, 2017).

2.4 Antioxidant Compounds

To counter the oxidative stress condition, antioxidant acts as a protective factor against the free radical radicals and helps to improve balance of reactive species and antioxidant concentration. The higher the concentration of reactive species in body, the more negatively it affects humans, animals, and plants (Olszowy, 2019). George and Abrahamse (2020) stated that antioxidants assumed the protector roles over these reactive species which consists of radical species, reactive oxygen, and nitrogen species by several mechanisms: preventing the formation of the reactive species, activation, and deactivation of enzymes, eliminating free radicals, formed chelate complexes with prooxidant metals, and repairing damages caused by the reactive species.

Similar to the free radical species, antioxidants can be originated from either endogenous or exogenous sources. Depending on their effect on intracellular free radicals, endogenous antioxidants often act prophylactically and often effectively repair damage caused by free radicals. Antioxidants which belonged to a subclass of phenolics are mostly sought after due to its prophylactic ability in preventing a disease from occurring and its consequences. Soobrattee et al. (2005) also mentioned that these compounds are also pursued because of its pluriparmacological effects owing to the many phenolic compounds found in a plant. Exogenous antioxidants, on the other hand, such as Vitamin C, was available upon consuming foods containing antioxidants such as fruits and vegetables. Other

antioxidants such as Resveratrol is a well known antioxidant blocking *in vitro* LDL oxidation, a biological phenomenon associated with the risk of coronary heart disease and myocardial infarction. Conti et al. (2016) described resveratrol to reduce cardiovascular risk factors, including blood lipids and VCAM-1, to improve cardiovascular function and physical capacity. Moreover, it was also shown that this antioxidant reduces inflammation in the vascular system in aged animals, leading to improved function blood vessel upon consumption in rodents as shown in a study by Gliemann et al. (2013).

In the field of medical and large-scale industry, there are two types of antioxidants available: natural-based and synthetic. Synthetic produced antioxidants are commonly used in food production as preservatives because they are more stable and has simpler extraction. On contrary, natural antioxidants are derived fully from natural products providing an alternative of bioactive properties and thus bringing additional value to the final products (Caleja et al., 2016). However, natural antioxidants are difficult to be extract due to its instability which leads to more expensive capital cost (Saad et al., 2007). Despite that, the harmful effects of synthetic derived antioxidant such BHA, BHT and TBHQ weighted more in the long run based on many toxicity tests conducted by researchers (Xu et al., 2021).

Some natural compounds are exogenous antioxidants which are hydrogen donors or quenchers of singlet oxygen anion. It can remove free radical intermediates or delaying oxidative reactions. Plant based foods containing vitamin C, D and E and various phyto ingredients are examples of natural antioxidants that function to promote cell cycle arrest and aiding in increasing immunity against viral infection (Khalid et al., 2022).

To determine the measurement of antioxidant potential in a substance, many chemical assays were designed to evaluate the antioxidant in either plant or food. Depending on the mechanism of the antioxidants, executing either through hydrogen atom transfer or

single electron transfer from potential antioxidants to free radicals and thus making the radicals to become more stable (Wright et al., 2001).

Several assays that are commonly used to test are: oxygen radical absorbance capacity (ORAC) assay, ABTS assay, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Trolox equivalent antioxidant assay (TEAC), and non-radical redox potential-based method such as Ferric reducing power (FRAP) assay and cupric reducing antioxidant power assay (CUPRAC) assay. The difference of each assay was further explained in Table 2.1 (Siddeeg et al., 2021).

Table 2.1: Comparison of commonly used antioxidant assay

Types	Dominating mechanism	Name of assay	Detection	Wavelength detection (nm)	Reference
Reactive oxygen species scavenging assay	Hydrogen atom transfer	ORAC	Fluoroscropy	Excitation wavelength (480)	Du & Xu (2014)
				Emission wavelength (520)	
	Electron transfer	DPPH	Spectrometry	515-528	Molyneux (2004)
	Electron transfer	TEAC	Spectrometry	734	Re et al. (1999)

Table 2.1 continued

Redox potential assay	Electron transfer	FRAP	Spectrometry	700	Oyaizu (1986)
	Electron transfer	CUPRAC	Spectrometry	450	Apak et al. (2004)

In this study, two antioxidant tests were selected which are, the DPPH assay and the FRAP assay. The two tests were shown to be reproducible in a study by Thaipong et al. (2006) upon tested with guava extracts. Moreover, it is also suggested by many studies under the assumption that using two assays improves the overall estimate of antioxidant capacity of the plant extracts. It was described that the possibility that each assay reflects a different aspect of the antioxidant behaviour of a plant extract (Qader et al., 2011). As both antioxidants are commonly used in detecting the ability of antioxidants, a comparison on its advantages and disadvantages of DPPH and PFRAP assay was carried out and tabulated in Table 2.2.

Table 2.2: The advantages and disadvantages of DPPH and PFRAP tests.

	Advantages	Disadvantages
2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay	• Rapid, simple, inexpensive and extensively used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods • This method may be utilized in aqueous and non-polar organic solvents and can be employed to investigate both hydrophobic and lipophilic antioxidants. (Prior et al. 2005). • Sensitive for radical reaction/HAT transfer or reduction of single e transfer (set)	• DPPH is not soluble in water. • Not physiological radical. Light, O₂, and pH have influence on DPPH absorbance. • Wide range of wavelength detection. • Small molecules react easier with DPPH than polymers. • The interaction of DPPH radical with other radicals results in a non-linear time response curve to steady state with varying antioxidant/DPPH ratios.

Table 2.2 continued

Potassium Ferric Reducing Power (PFRAP)	• Simple, cheap, robust • Unlike other ET-based methods, the FRAP assay is carried out under acidic pH conditions (pH 3.6) in order to maintain iron solubility and, more importantly, drive electron transfer which eventually increases redox potential, creating a shift in dominant reacting mechanism. (Hegerman et al., 1998).	• Power to reduce ferric³⁺ is not biologically relevant. • This test does not detect radical quencher (HAT) containing -SH groups like GSH and reaction time (kinetic) can vary strongly, especially polyphenols react slowly. Substances like biliverdin have a similar absorbance max. • Depends on analysis time which varies across substances
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2.5 Fermentation

Since ancient times, fermentation is a technique of food preservation, kept in a certain condition for a longer shelf-life. As such, the art of cheesemaking is believed to have been developed as early as 8000 years ago in the fertile crescent between Tigris and Euphrates in Iraq, where plants and animals have just been domesticated. Fermentation was then applied in bread and winemaking during the 4000 BC in Egypt. The technique then was further used in beer making, mouldy soybean as antibiotic in China and many others until the food in our own local kitchen. However, it was later discovered that with the utilization of microorganisms helps in extending longer shelf-life during fermentation and improve the

palatability. This then established an industry utilizing microbial fermentation to fit the demands of the expanding market back then (Ross et al., 2002).

Fermentation application in the food industry is a food processing technique which yields products that are not only shelf stable but better in sensorial qualities and health beneficial constituents (Bourdichon et al., 2012). Biotransformation of plant fermentation technology does not only apply to daily food consumed, but also towards waste products from different industries. This technique is advantageous over conventional chemically added procedure for acquiring natural active ingredients. According to Zhu et al. (2020), this process increases the physiological and biochemical activity of natural plants extracts due to their selective properties, environmental compatibility, mild conditions, and higher kinetics rate. Various enzymes for instance, lipase, protease, amylase, and or glucoamylase, generates or promotes the release or syntheses of bioactive compounds from substrate (Liu et al., 2020). Moreover, this fermentation technique has lower energy cost (Palacios-Bereche et al., 2014).

Microbial fermentation is a fermentation technique by using plants or plant-based materials, with the aid of microbial activity either bacteria or fungal to transform into value-added products and thus improve the product by its palatability and nutritional values in food and pharmaceutical industries. Fermentation process stimulates the production of corresponding metabolites from the plants via the help of microbial, for example, the *tempeh* and kimchi production (Lee et al., 2021).

In the book *Food, Fermentation, and Microorganisms*, the author quoted the definition of fermented foods as foods that have been exposed to the action of microorganisms or enzymes, with desirable biochemical changes cause major changes in the food (Bamforth & Cook, 2019). There are also several foods produced locally which also

uses fermentation technique to enhance the palatability and nutritional values such as the *tempeh*, *tapai*, *belacan* and *budu* production. Depending on the mode of fermentation technique, the suitability of the substrate and end-product varies depending on the supply and demand in the current market.

In such, the mode of fermentation used in the many industries will be further discussed. Moreover, a comparison of the two techniques will also be discussed.

2.5.1 Solid State Fermentation

Solid-state fermentation is a fermentation technique that occurred in little or near-absence of free water. Solid-state is advantageous over submerged or liquid fermentation as it is water-saving, energy-saving, and low-cost properties according to Chen (2013). According to the statement by Chen and Wang (2017), the substrate does not only serve as a physical structure for microbial growth but also offers as a carbon and nitrogen source and growth factors for the microbes used in the fermentation. Application of solid-state fermentation are diversely used to produce products such as enzymes. Bio pulping, a biomass pre-treatment which promotes in situ delignification of cellulose, commonly performed by fungi is another process that utilised solid state fermentation (Soccol et al., 2017). Martins et al. (2011) reviewed that SSF technique has been able to produce useful bioactive metabolites in shorter time as compared to SMF and higher yield without the need of aseptic conditions. This in turn decreases a significant amount of capital cost.

2.5.2 Submerged Fermentation

Submerged or liquid fermentation are the most common type of fermentation used in industries. As its name, the liquid fermentation uses high amounts of liquid for the fermentation process. This type of fermentation can be classified into two categories: aerobic

and anaerobic fermentation. Antibiotics and enzymes are produced by aerobic fermentation. In aerobic fermentation, oxygen is incorporated into the liquid medium while the production of butanol is produced by fermentation under anaerobic mode where the addition of oxygen is disadvantageous. Facultative anaerobes are involved in certain fermentation processes, such as ethanol production. Submerged fermentation usually occurs in large bioreactors which constantly needs agitation and supply of nutrients to the microbial. This increases the overall costs of production and maintenance. However, despite the disadvantages of submerged fermentation, there are also reports conducted on the submerged fermentation for its beneficial production for instance, in pigment production as well as antibiotic production (Rafique et al., 2022; Ramesh et al., 2022).

2.5.3 Comparison between the Solid-State Fermentation and Liquid Fermentation

The most evident difference between the two fermentation types is the amount of liquid used in the process. Solid state as its name, is a fermentation technique that requires little to no water during the fermentation process. Conversely, submerged fermentation is liquid state fermentation that requires high amount of amount until the substrate is submerged throughout the process. However, as solid-state has low moisture content, the possibility of stunted growth of microorganisms may occur and thus affecting the production and/or extraction phenolic compounds. Unlike solid-state fermentation, submerged fermentation requires more maintenance as it constantly requires constant agitation and supply of nutrients for the growth of fungal. This in turn, proved to be easier monitoring the process parameters which could not be carried out under solid-state fermentation. A comparison between the type of fermentation is presented in Table 2.3 based on previous studies (Subramaniyam & Vimala, 2012; Hansen et al., 2015; Hermansyah et al., 2019).

Table 2.3: Comparison of solid-state fermentation and submerged fermentation

Solid-state fermentation	Factors	Submerged fermentation
Fermentation with microorganism grown on solid substrate in near absence of water	Process	Fermentation method commonly used in enzyme production using high water content
Can use composite and heterogenous products of agriculture, readily available	Substrates	Agriculture waste such as sludge and slurries can be used as substrates
May or may not be involved	Agitation	Agitation is essential
Nutrient concentration is not even as the microorganism only stays on the surface area	Nutrient distribution	Nutrients are evenly distributed due to the constant agitation
Simple fermentation equipment	Equipment	Complex equipment
Simplified and minimized downstream process and less waste	Extraction	Complex and expensive downstream process and difficulty in the waste disposal
Cheaper	Cost	Higher cost due to expensive media and expensive equipment

Higher yields have been proven when under solid state fermentation using *Clostridium thermocellum* for cellulase production under both submerged and solid-state fermentation whereby unit costs were calculated based on upscale production simulator (Zhuang et al., 2007). In terms of enzyme production, submerged fermentation is preferred in the industry due to better parameter control of the entire process. Kim et al. (2016) stated

that SSF is better for filamentous fungi which requires less moisture content while SMF is preferred for bacteria that requires higher moisture content. It was also stated that the fermentation modes determined on the secondary metabolite production as the different fermentation affects the difference of end products (Kim et al., 2016).

2.5.4 Application of SSF and SMF in production of phenolic compounds

Solid state and submerged fermentation are well known technique in phenolic production. Both methods play a crucial role in enhancing the yield and bioavailability of phenolic content, presenting versatile applications in various industries, including food, pharmaceuticals, and biotechnology. This brief overview highlights the diverse and valuable contributions of SSF and SMF in harnessing phenolic compounds for numerous applications. Barrios-Gonzales et al. (2005) stated that production of secondary metabolites begins when a threshold of one key nutrient such as carbon, nitrogen or phosphate source are depleted which then limits their growth.

A report by Bhanja et al. (2009) showed that in SSF method, the improvement of phenolic content and antioxidant properties of wheat grains by two different filamentous fungi (*Aspergillus oryzae* and *Aspergillus awamori*) were very impressive. It was also stated from the study that fermented wheat grains were considered to be antioxidant richer and healthier food supplement compared to non-fermented wheat grains. Several other studies have also shown that SSF process helps in phenolic production such as reported by da Costa Maia et al. (2020) in a study of phenolic production using five filamentous fungi in a brewer's spent grain fermentation. Upon fermentation, the amount of phenolic content detected has increased by seven times and in DPPH, it was detected five times more activity as compared to its non-fermented counterpart (da Costa Maia et al., 2020). Another study also has shown that the oats' total phenolic content (TPC) could be significantly increased

($p < 0.05$) through the utilization of an SSF system incorporating *M. anka* and *B. subtilis* (Chen et al., 2020). The findings suggested that the utilization of the SSF system incorporating *M. anka* and *B. subtilis* not only enhanced the phenolic contents but also altered the distribution of phenolic fractions in oats. Previous reports have highlighted that SSF has the potential to elevate the levels of free phenolic compounds in cereal products by breaking down cereal cell walls through the action of microbial enzymes (Abd Razak et al., 2015). This showed that SSF has an effect in increasing the production of phenolics.

Under SMF application, Jin et al. (2023) reported that through SMF application, the value of TPC increased to over 100 mg GAE/g within 6 days, whereas SSF did not change the TPC in chestnut inner shell which described that SMF also have potential in phenolic production. Another example of phenolic production in SMF application are also seen in other studies showed that mevastatin produced through SMF produced 490 mg/ml as compared to when it was produced through SSF method with *Penicillium citrinum* (0.0554 mg/ml) (Manzoni & Rollini, 2002; Chakravarti & Sahai 2002; Ahmed et al., 2006).

This study does not only implement the usual single microbial fermentation but utilising a co-culture microbial fermentation. A study conducted by Chen et al. (2021) showed that fermentation of co-culture with *M. anka*, *S. cerevisiae* and *B. subtilis* in corn kernels has shown higher total phenolic content compared to their unfermented corn kernel. It was speculated that the system works with microorganism to facilitate enzymatic hydrolysis which in turns destroys the cellular structure to regulate the phenolic mobilization in corn kernels. Another study by Khan et al. (2022) also showed impressive phenolic content detected in co-culture fermentation using lactic acid bacteria and yeast. It resulted in the highest amount of free, conjugated and bound phenolic detected. This shows that co-culture fermentation can bring rise to a different pathway to produce phenolic compounds.

There are many reasons that a co-culture fermentation has been on the rise as of recently. One of it being the intricate microbial interactions, whether symbiotic or synergistic, which give rise to the processes occurring in natural or controlled food fermentation (Adesulu-Dahunsi et al., 2020). Moreover, they exchange metabolites and molecular signals, leading each cell within the mixture to respond to the presence of others in the fermenting food matrix (Smid & Hugenholtz, 2010). Based on previous studies, this study is moving in the direction in finding an optimized culture of endophytes to gain higher activity of antioxidant and increased of phenolic content.

2.6 Sago Palm

The sago palm tree in Malaysia is commonly known as the *Metroxylon sagu Rottb.* It is widely found in the state of Sarawak and in some areas of southern West Malaysia in the state of Johor. The sago palm is known to be the underdog of the industry as the different parts of the palm can be fully utilized. Although there are many studies carried out using other parts of the sago palm, lesser research focused on the sago starch itself. Therefore, in this case, we will discuss further on the accomplishments of the current research on the use of sago starch.

2.7 Starch

Starch, in general is one of the main sources of carbohydrate in human diet. Starch has a wide range of application, from food industry to plastic industries. There are many types of starch available in the market. Starch derived from the potatoes, maize, sweet potato, and many others are used daily in cooking to making pastries. Starch from different plants differs in many aspects of its properties. For instance, the size of granule, breakdown viscosity, and pasting temperature. Depending on the source of starch, the uses also differ.

Suitability of flours and starches for specific requirements and uses requires the understanding of their functions and physiochemical properties (Wang et al., 2020).

Understanding these properties will certainly maximises the usage of the starch in different industries. However, current literatures reported on the physical properties but not on its physiochemical properties. It is suggested that investigating the full potential of different starches by determining both its physical, and phytochemical properties without altering their composition of nutritional, composition and health benefits will certainly boost many agriculture and industrial sectors.

2.7.1 Sago Starch

Sago starch is a type of starch that was extracted from the pith of sago palm. The starch is a food commodity among the locals of Sarawak and has abundant of uses in traditional food and glue cosmetics industries. The biggest plantations of the sago palm are in the areas of Mukah, Dalat and Oya in the state of Sarawak. The word “*lemantak*” is only used by the locals of Sarawak especially amongst Melanau ethnic group. Unlike the commercially produced sago starch, this sago starch “*lemantak*” is processed traditionally and is known to be a semi-wet sago starch due to its high-water content. In this study, raw sago starch (RSS) “*lemantak*” is used as substrate in the study as shown in Figure 2.2.



Figure 2.2: Sago starch bought from wet market in Kota Samarahan

Not only that, but the starch processing method also varies depending on its uses. In the market, the sago starch was known to have two different methods of extracting it. One, is through the modern mass production through factory, and the other method, is the traditionally processed of starch extraction. Although many reports have explained the differences in terms of physical characteristics of the two types of commercially sold sago starch, there are no reports in comparing their phenolic content. Based on a study by Kamal et al. (2017), there are major differences between the commercial sago starch and the traditional processed sago starch. The differences were seen from different aspects such as its granular size, starch composition and its FTIR composition. Raw sago starch “*lemantak*”, exhibits a threefold higher prevalence than sago flour. Despite being deemed of low quality in the global market due to its inefficient extraction and processing methods, “*lemantak*” is favoured locally for its elevated moisture content, particularly in the preparation of linut—a dish created by blending “*lemantak*” with hot boiling water, requiring a specific consistency. Microscopically, scanning electron microscopy revealed that “*lemantak*” possesses a

consistent granular shape and aggregation, with a diameter ranging from 6 to 60 micrometres. In contrast, commercial sago flour loses its granular appearance, displaying hollowness indicative of gelatinization during the drying process. The starch's colour, structure, and size in commercial flours vary due to differing drying techniques. Moreover, a research study carried out by Kamal et al. (2017) stated the traditionally processed sago starch has wider utilization and tastes better than the commercially produced ones.

2.7.2 Sago Starch Properties and its Current Uses in Industrial

Sago starch "*lemantak*" is a traditionally processed starch from the trunk of the sago palm. Differ from the commercial sago flour, sago starch "*lemantak*" has a unique viscosity when mixed with warm water which could not be obtained using the commercial sago flour. The commercialized flour has undergone additional modification processes to meet Malaysian's standard for food product. Currently, sago starch has been developed into food production of noodles, edible film, sago pearls and biscuits.

Sago starch "*lemantak*" has high moisture content up to 38.8%, which is higher than commercial standards. Industrial sago starch is 15% wet basis (w. b.) as stated in the Malaysia Standard of Requirements for Industrial Sago Starch (MS468: 1976) while maximum moisture content for edible sago starch is 13% w. b. as stated in the 'Requirements for Edible Sago Starch' (MS470:1992) (Kamal et al., 2019). Moisture content determines on the products to be made. This is because the quality of products is affected by higher moisture content as it does not hasten the oxidation of food but also affects the appearance of properties such as the gelatinization degree, crystallinity, amylose, and amylopectin degradation of starches (Liu et al., 2019).

In industrial processing, sago starch is widely used for pasting, gelatinisation and retrogradation process. These processes help in thickening and gelling of sauces and pie fillings, also in soups, porridges, and bakery products (Kamal et al., 2008).

There are many factors that affects the quality of sago starch. Processing time of the sago starch shows similar moisture content but differs in protein content, amylose-lipid content, and peak viscosity (Sim et al., 1991; Kamal et al., 2019).

There are also reports that fermented sago starch formed resistant starch whereby on being fed to gastrointestinal bacteria such as *Lactobacilli*, *Bifidobacterium*, *Escherichia coli* and *Campylobacter coli*, the growth of bacteria increases and among the selection of bacteria, *Lactobacilli* showed the highest prebiotic activity upon feeding on the retrograded starch (RS) formed by sago starch (Tan et al., 2015). Zhu (2019) concluded that depending on the type of RS and bacteria, sago starch-based RS has prebiotic function and thus has potential in prebiotic food.

Other than food security, the sago starch is also used to create composite films, ranging from incorporation in rubber latex composite films to creating edible starch-gelatin based films and nanofiber materials (Nouri & Nafchi, 2014; Suki et al., 2016; Yusof et al., 2018). The uses of sago starch in industrial are extensive. As compared to other starch sources, the sago starch production has lower cost with a high yield in return. Singhal et al. (2008) argued that sago palms are ecologically sustainable because some parts of the palms, such as fibrous roots, can remove contaminants and heavy metals. In sago palm plantations, Zhu (2019) found that sago forests are an excellent carbon sink for carbon sequestration.

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of Materials

3.1.1 Raw sago starch “*lemantak*” as Substrate

Fresh wet sago starch “*lemantak*” of 1 kg was purchased from a wet market in Bandar Riyal, Kota Samarahan. The sago was then brought back to the lab to be processed. The sago starch was first sieved and dried in oven at 50 °C for 18 hrs. This is to ensure that the fresh sago starch will last longer and does not easily contaminate by unwanted microorganisms. The weight of before drying and after drying were taken and calculated. Preparation of sago starch “*lemantak*” is to be used as the substrate in fermentation was carried out.

3.1.2 Total Phenolic Content and Antioxidant Activities

Folin-Ciocalteu’s reagent of 100 ml for total phenolic content assay was supplied by Merck (Germany) and 2,2-Diphenyl-picrylhydrazyl (DPPH) solution for scavenging activity by Sigma Aldrich (Germany). Gallic acid and ascorbic acid were used as standard for the phenolic test and antioxidant test. Both were supplied by Sigma Aldrich (Germany).

3.1.3 Standard Media

The isolates were cultured and maintained on malt extract agar and malt extract broth supplied by Himedia (India).

3.1.4 Fermentation Media

The media used in both solid-state fermentation and submerged fermentation was Glucose minimal media (GMM) were described in (g/L): 1.0 g of K_2HPO_4 , 0.01 g of $ZnSO_4 \cdot 7H_2O$, 0.05 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 3.0 g of $NaNO_3$ and 10 g of glucose (Ngieng et al., 2017).

3.1.5 Solvent for Extraction of Endophytic Fungi Secondary Metabolite

The extraction of endophytic fungi metabolite was done by using methanol, ethanol, and ethyl acetate supplied by Sigma. For GC-MS preparation, 100 ml of dimethyl sulfoxide (DMSO) were used and was supplied by Merck (Germany). All commercially available chemicals or reagents were used as received without further purification unless stated as shown in Appendix K. All solvents used were synthesis grade and used as received.

3.2 Methodology

3.2.1 Preparation of Selected Fungi as Inoculum

Three endophytic fungi were obtained from Molecular Genetic Laboratory 2. These fungi isolate namely *Aspergillus niger*, *Fusarium equiseti*, and *Penicillium chermesinum* were previously isolated from plants. *Aspergillus niger* was isolated from the sago bark shown in Figure 3.2 (a) while the latter two fungi, *Fusarium equiseti* and *Penicillium chermesinum* shown in Figure 3.2 (b) and Figure 3.2 (c) respectively, were isolated from the leaves of a medicinal plant, *Melastoma malachbrictum*, commonly known among locals as the “Senduduk” plant. Isolates were transferred onto malt extract agar (MEA) plate and grown for seven days. The fungi were maintained weekly for subsequent fermentation experiments. A number of three plugs from each of the pure isolates were then inoculated into malt extract broth (MEB) and left on a shaker at 140 rpm for seven days at room

temperature. The broth was removed, and 50 ml of Glucose minimal media (GMM) were added (Ngieng et al., 2017). The fungi-grown broth was then subjected to homogenization using Warring laboratory blender at medium speed to ensure that the broth is fully homogenised as shown in Figure 3.1. furthermore, to investigate higher phenolic production, this study also implements consortium of fungal cultures. The consortiums of fungal cultures are described in Table 3.1.

Table 3.1: Experimental setup of the different consortium that was investigated.

<i>Experimental setup</i>	<i>Aspergillus niger</i>	<i>Fusarium equiseti</i>	<i>Penicillium chermesinum</i>
<i>Control</i>	-	-	-
<i>Aspergillus niger</i>	+	-	-
<i>Fusarium equiseti</i>	-	+	-
<i>Penicillium chermesinum</i>	-	-	+
<i>Aspergillus niger + Fusarium equiseti</i>	+	+	-
<i>Aspergillus niger + Penicillium chermesinum</i>	+	-	+
<i>Fusarium equiseti + Penicillium chermesinum</i>	-	+	+
<i>Aspergillus niger + Fusarium equiseti + Penicillium chermesinum</i>	+	+	+

Foot note: “+” shows present fungal inoculum in fermentation system; “-” shows absence of fungal inoculum in fermentation system.



Figure 3.1: Blending of fungal mycelium grown with GMM in Warring Laboratory Blender



Figure 3.2 (a): *Aspergillus niger* growth of Day 5 on MEA



Figure 3.2 (b): *Penicillium chermesinum* growth of Day 7 on MEA



Figure 3.2 (c): *Fusarium equiseti* growth of Day 7 on MEA

3.2.2 Fermentation

In this subchapter, two types of fermentation method were tested which is known as the solid-state fermentation and the submerged fermentation. The two fermentations were compared, and the selected technique were further optimized with several other parameters. Selection of parameters were carried using one-factor-at-a-time (OFAAT) method to ensure the maximum production of phenolic compounds (Maharana et al., 2019). Manipulation of single factors at a time in each step of production devoid of supplied factors for comparative selection among the parameters.

3.2.2.1 Submerged Fermentation

In submerged fermentation, similar to the solid-state fermentation, a mass of 10 g of sago starch was added to the 250-ml Erlenmeyer flask and were then autoclaved. An aliquot of 50 ml of autoclaved glucose minimal media was added into the flask with 3 ml of respective fungal isolates mixed with GMM. The flasks were sterilised by flame before covered with aluminium foil and parafilm to decrease the possibility of contamination. The

flasks were then left in rotary shaker at 140 rpm in ambient temperature for seven days before proceeding for extraction of metabolites. All experimental were conducted in triplicates.

3.2.2.2 Solid State Fermentation

In solid-state fermentation, a mass of 10 g of sago starch were added into a 250 ml of Erlenmeyer flask with 4.5 ml of glucose minimal media and were autoclaved. The flasks were then left to be cooled to room temperature before adding 3 ml of fungal inoculum and were stirred thoroughly using a sterilized spatula. Moisture content of the solid-state system is required to be at a range of 75-78% (w/v) to ensure suitable growth condition for fungi. Exceeding or lesser than the range of moisture content will impede the growth of fungi used in this experiment. The opening of the flasks was sterilized over flame before covered with aluminium foil and parafilm to decrease the risk of contamination and left in static condition for seven days (Yin et al., 2019).

3.2.3 Extraction of Crude Metabolite

Metabolites were extracted by solvent extraction procedure using methanol as organic solvents. The fermented mass was taken and left to dry in oven for 24 hrs at a temperature of 50°C. Mass of before fermentation and after fermentation were taken as a record. The dried fermented mass was then grinded to powder-like form by using pestle and mortar. The samples were then added into 50 ml conical flask at a ratio of 1:10 (w/v) before adding the solvent. The flasks were left on rotary shaker for 24 hrs at 140 rpm at 50 °C in water bath. The extracts were filtered, and the crude extract were then left in fume hood. Crude extracts obtained was stored at 4 °C until further analysis (Bennour et al., 2020).

3.3 Optimization of Parameters

Optimization of several parameters were conducted. The parameters were divided into two phases: (1) the fermentation stage and (2) the extraction stage. In the fermentation stage, two parameters were observed which is the consortia of fungal isolates and the incubation period. While in extraction stage, the studied parameters were the extraction time, and the solvent extraction used.

Selection of best were carried out using Folin-Ciocalteu's method and DPPH radical scavenging assay. Each of the parameters were carried out using one-factor-at-a-time (OFAAT) method for selection of the best factors. Although single factor experiments are time consuming and data on factor interactions are lacking, they were used because they can provide data from fundamental effects of conditions or ranges of extraction factors (Bunmusik et al., 2023).

3.4 Phytochemical Analysis

Several phytochemical analyses were carried out to determine the total phenolic content by Folin-Ciocalteu method and DPPH radical scavenging activity of the fermented extracts. Moreover, a further antioxidant assay was also conducted by using the potassium ferric reducing power assay to investigate the reducing power ability of the extracts.

3.4.1 Total Phenolic Content (TPC)

Total phenolic content (TPC) of the crude extract from fermented mass were measured using Folin-Ciocalteu method as described by Chandra et al. (2014). Gallic acids were used as standard. 0.1 mg/ml stock solution of gallic acid was prepared by dissolving 10 mg of dry gallic acid in 1 ml of distilled water and then diluted up to 10 ml. The stock solution was stored at 4 °C. Working standards of between 0.1 to 0.02 mg/ml were prepared

by diluting the stock solution with distilled water. The extract was prepared at concentration of 2 mg/ml. An aliquot of 100 μ l of samples were added into a test tube of 750 μ l of Folin-Ciocalteu's reagent (diluted ten times with distilled water) and mixed. The mixture was left for 5 mins in the dark at room temperature. After that, an aliquot of 750 μ l of 7.5% NaCO₃ were added to the mixture and mixed. The extracts were then incubated for 90 mins in the dark at room temperature. The absorbance of samples was then read at 765 nm using Metertech sp 830 plus. The standard calibration of gallic acid was plotted. The calibration curve of the standard gallic acid used in calculation is exhibited in Appendix A. Several consortia of fungi were narrowed down for further analysis by choosing the top two consortia of endophytic fungi with the highest TPC value. The selected fungi were further analysed for their antioxidant capacity. Data was recorded and expressed in TPC mg/GAE g.

3.4.2 Antioxidant Assays

Antioxidant assays were conducted to test the antioxidant potential of the samples. Two tests were conducted namely, the 1-diphenyl-picrylhydrazyl commonly known as the DPPH radical scavenging assay and potassium ferric reducing power (PFRAP) assay.

3.4.2.1 DPPH Free Radical Scavenging Assay

The 1-diphenyl-2-picrylhydrazyl (α , α -diphenyl- β -picrylhydrazyl) (DPPH)) was from Sigma (Germany). The DPPH radical scavenging assay were carried out according to the method by Fitriansyah et al. (2018) with slight modification. Stock solution of DPPH was first prepared at a concentration of 0.2 mM using absolute methanol and was thoroughly mixed. The samples were firstly prepared at a starting concentration of 2 mg/ml and were then diluted by two-fold serial dilution. In a test tube, an aliquot of 200 μ l of different concentrations (1000 μ g/ml, 500 μ g/ml, 250 μ g/ml, 125 μ g/ml, 62.5 μ g/ml, 31.25 μ g/ml) of

test extracts in methanol with 800 µl of working solution 0.1 mM methanolic DPPH. The diluted extracts were mixed by pipetting up and down several times before incubating for 30 mins in the dark at room temperature. The sample were then transferred into cuvettes before read by spectrophotometer at 517 nm. The blank used was methanol and the negative sample was methanol and DPPH reagent. Ascorbic acid was used as standard. The calibration of ascorbic acid was conducted and shown in Appendix B. The scavenging effect on the free radicals were recorded and analysed by using the following formula (Formula 3.1):

$$\%Radical\ Scavenging\ Activity = \frac{(abs\ of\ negative\ control - abs\ of\ sample)}{(abs\ of\ negative\ control)} \times 100 \quad \text{Formula 3.1}$$

3.4.2.2 Potassium Ferric Reducing Power (PFRAP) Assay

Potassium ferric reducing power was carried out using the method by Abishek et al. (2019) with slight modification. The extracts were prepared in different concentrations (1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml) using two-fold serial dilutions. Briefly, 100 µl of test samples scaled up to 1 ml with methanol was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) followed by 2.5 ml of 1% potassium ferricyanide, further reaction mixture was incubated at 50 °C for 20 min. After incubation, 4.12.5 ml of 1% trichloroacetic acid was added and the content was centrifuged for 10 mins at 3500 rpm. Supernatant (2.5 ml) was taken, mixed with 2.5 ml of distilled water and 0.5 ml 1% FeCl₃ reagent and left to stand in room temperature for 10 mins. Absorbances of samples was recorded at 700 nm. Higher concentration of samples resulted in higher absorbance values and thus indicate higher reducing power.

3.5 Detection of Bioactive Compounds

3.5.1 Gas Chromatography-Mass Spectrometry (GCMS) Analysis

The positive bioactive compound was subjected to the instrument analysis GC-MS to identify the bioactive compound present in the fungal extracts. The purified fungal extract was prepared in the ethyl acetate. The sample is analysed in gas chromatograph equipped with detector. Firstly, the sample is introduced via an all-glass injector working in the split mode, with Helium gas as the carrier gas with linear velocity of 32 cm/s. The HP-5 fused silica capillary column (length – 30 m; Film thickness – 25 µm I.D – 0.2 mm) was used. The temperature program was follows: 80 – 240°C at 8 degree per min: 240 – 300°C at 12 degree per min and a 20 min hold at 300°C. The identification of bioactive compounds was done by matching the peak obtained with National Institute of Standard and Technology (NIST) library (Ibrahim et al., 2016).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Optimization of Phenolic and Antioxidant Production

4.1.1 Comparison between Different Types of Endophytes Consortia

Three endophytes were selected to be studied in this research. These endophytes were selected based on previous literature reviews on the potential phenolic produced by them. As such, the selected endophytes are *Aspergillus niger*, *Penicillium chermesinum* and *Fusarium equiseti*. These endophytes were previously isolated from plants and maintained in Molecular Genetic Laboratory, UNIMAS. Two of the fungal endophytes, *Aspergillus niger* and *Penicillium chermesinum* were isolated from a local medicinal plant, *Melastoma malachrichtum*, commonly known as “Senduduk”. Endophytes isolated from medicinal plants are highly desired due to the metabolites produced similar to their host by the means of symbiotic relationship with the host plant.

On the other hand, *Fusarium equiseti* was isolated from the bark of the sago tree. There are less literatures on the uses of sago bark in terms of bioactivity, however a study reported that the sago bark possesses a characteristic of antibacterial (Chusri et al., 2012). This shows that these endophytes are able of producing similar bioactive compounds to their host plants. The mutualism relationship between the endophytes with their respective host plants are due to the co-evolution in their survival as the host plant provides nutrients and home to the endophytes. In return, numerous bioactive compounds were generated by these endophytes as a remuneration to the host plant in combating pathogens, biotic and abiotic stresses.

4.1.1.1 Phenolic production

In this study, among the three isolates, *Aspergillus niger* (*A. niger*) shows the highest amount of phenolic content of 146.920 mg GAE/ g $\pm$ 24.75 and 203.65 mg GAE/ g $\pm$ 77.42 as shown in Figure 4.1 and Figure 4.2. In comparison to *Penicillium chermesinum* (*P. chermesinum*) and *Fusarium equiseti* (*F. equiseti*) which shows total phenolic content at 121.859 ± 19.46 mg GAE/ g and 129.038 ± 49.77 mg GAE/ g respectively under solid state fermentation. However, under submerged fermentation, *P. chermesinum* showed lower phenolic production of 14.231 ± 5.71 mg GAE/ g. There is also a slight increase of phenolic content for *F. equiseti* under submerged fermentation of 178.65 ± 51.99 mg GAE/ g. This shows that with fermentation process, the total phenolic content of the raw sago starch (RSS) “*lemantak*” with endophytic fungi can increase or decrease depending on the mode of fermentation and the genus of fungal endophytes used. An overview comparison of consortia under different fermentation is described in Figure 4.3.

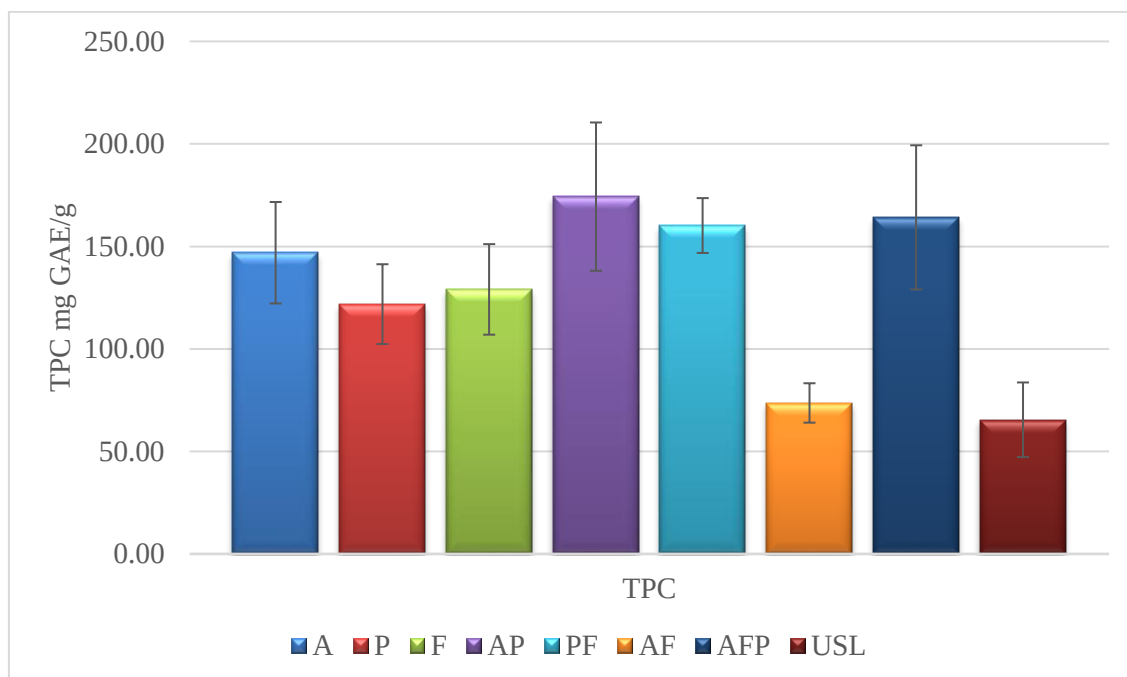


Figure 4.1: Total phenolic content under solid-state fermentation (SSF), (n=3)

Abbrev.: **A:** *A. niger*, **P:** *P. chermesinum*, **F:** *F. equiseti*, **AP:** *A. niger* & *P. chermesinum*, **AF:** *A. niger* & *F. equiseti*, **PF:** *P. chermesinum* & *F. equiseti*, **AFP:** *A. niger*, *F. equiseti* & *P. chermesinum*, **USL:** Uninoculated fermented sago “*lemantak*”

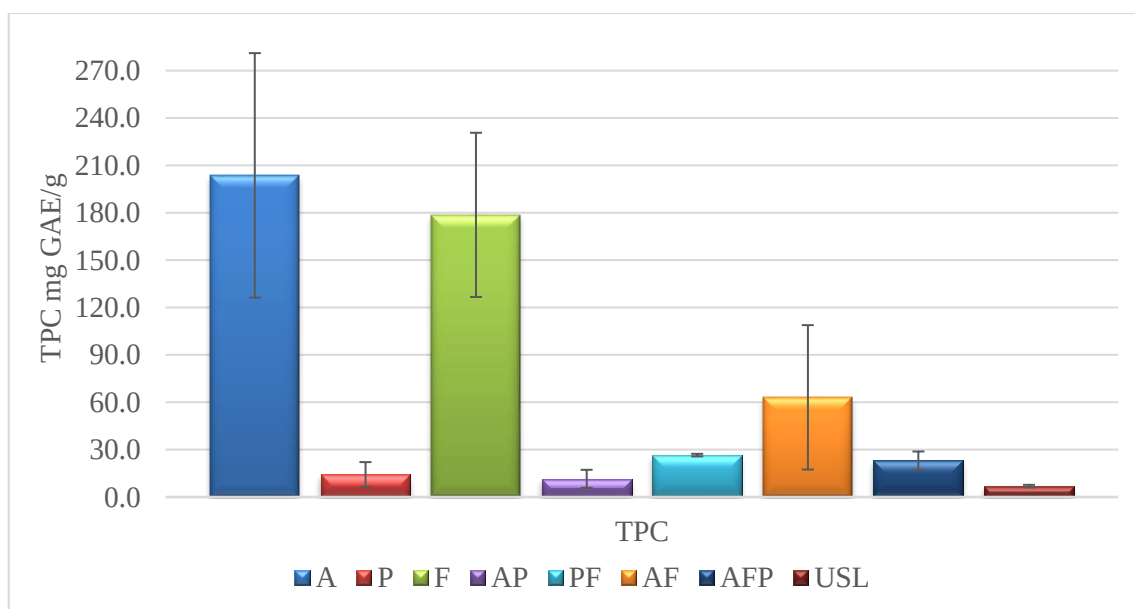


Figure 4.2: Total phenolic content under submerged fermentation (SMF), (n=3)

Abbrev.: **A:** *A. niger*, **P:** *P. chermesinum*, **F:** *F. equiseti*, **AP:** *A. niger* & *P. chermesinum*, **AF:** *A. niger* & *F. equiseti*, **PF:** *P. chermesinum* & *F. equiseti*, **AFP:** *A. niger*, *F. equiseti* & *P. chermesinum*, **USL:** Uninoculated fermented sago “*lemantak*”

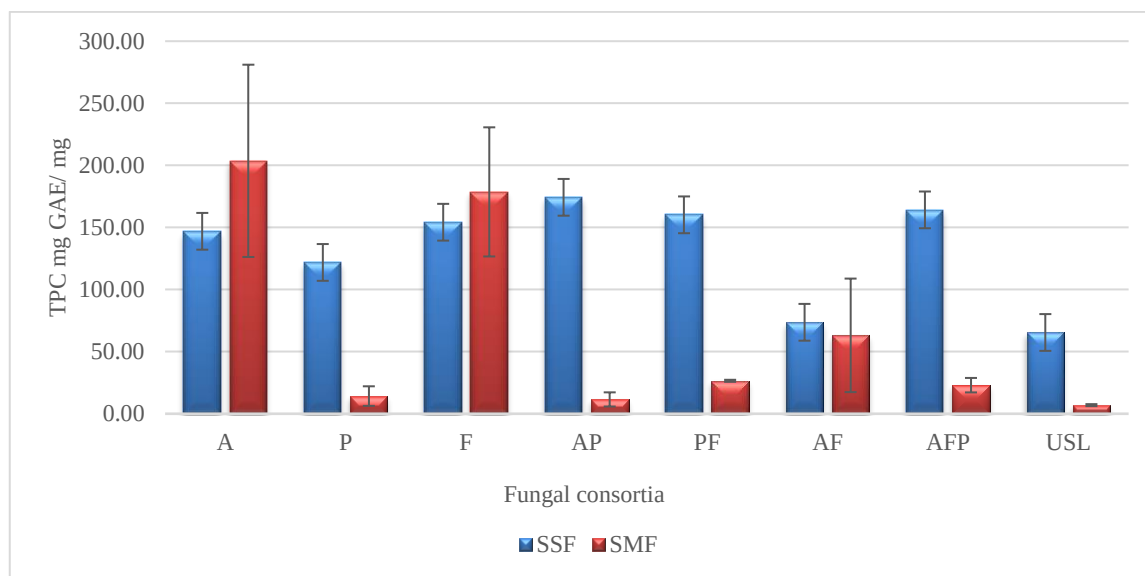


Figure 4.3: Comparison of total phenolic content in different consortia under different fermentation mode, (n=3)

Abbrev.: **A:** *A. niger*, **P:** *P. chermesinum*, **F:** *F. equiseti*, **AP:** *A. niger* & *P. chermesinum*, **AF:** *A. niger* & *F. equiseti*, **PF:** *P. chermesinum* & *F. equiseti*, **AFP:** *A. niger*, *F. equiseti* & *P. chermesinum*, **USL:** Uninoculated fermented sago “*lemantak*”

SSF7MEOH: solid state fermentation under seven days of methanolic extract

SMF7MEOH: submerged fermentation under seven days of methanolic extract

In this parameter test, the best endophyte to produce high phenolic content is *A. niger*. However, this study does not only focus merely on the difference of phenolic content produced by different genus of endophytes but also looking into consortia of these endophytes. For instance, the dual culture of the endophytes and a consortium of all the endophytes.

Interestingly, some of the dual cultures showed better total phenol content compared to their monoculture. The dual culture of *A. niger* and *P. chermesinum* (AP) showed slightly higher total phenol content of 174.295 ± 83.06 mg GAE/ g compared to their monoculture which strives at 146.920 ± 24.75 mg GAE/ g (*A. niger*) and 121.859 ± 19.46 mg GAE/ g (*P. chermesinum*). Besides that, the dual culture of *P. chermesinum* and *F. equiseti* (PF) also

showed higher total phenol content of 160.192 ± 13.37 mg GAE/ g compared to their monocultures.

However, under submerged fermentation, monoculture strives better as compared to the consortia. As shown in Figure 4.2, monoculture *A. niger* and monoculture *F. equiseti* showed higher total phenolic content as compared to other consortia. Overall, although the monoculture of *A. niger* and monoculture *F. equiseti* showed higher under submerged fermentation, the other consortia showed a lower in its phenolic production at more than 70% decrement. Interestingly, as for control, the uninoculated sago “*lemantak*” showed the lowest production of phenolic content of 6.94 ± 0.8 mg GAE/ g under submerged while under solid state fermentation, the sago “*lemantak*” showed total phenolic content of 65.45 ± 1.63 mg GAE/ g. The results obtained in this study for raw sago starch “*lemantak*” are also supported by a study by Duque et al. (2018). Upon investigating, it was found out the coarse fraction of the starch also plays a role in increasing phenolic content. As such, the highest exhibited in the study was at 42.76 ± 0.49 mg GAE/g sample. Reyes et al. (2017) claimed that fermentative ability affects the starchy matrices especially its physiochemical properties through the production of amylolytic enzymes.

Unlike solid-state fermentation where factors such as moisture content and the oxygen are negligible, submerged fermentation requires these factors to transport the amylolytic enzymes to the substrate (Valencienne et al., 2020). These enzymes facilitated in breaking down long chains of carbohydrate to simpler chains in a metabolite action. In return, by-products such as secondary metabolites, lactic and citric acid was formed. This may be one of the factors contributing to the low phenolic production detected in submerged fermentation compared to the solid-state fermentation. This is implied that under solid-state

fermentation, more phenolic content was produced with different consortia of fungal endophytes. This was also demonstrated that the genus *Aspergillus* and *Fusarium* produced higher phenolic content regardless of the fermentation mode as shown in Figure 4.1 and Figure 4.2.

A statistical analysis was carried out to observe whether the isolates used has significant differences in terms of phenolic content production. It was observed that there are no significant differences in phenolic content among the isolates ($P > 0.05$) under solid state fermentation as shown in Appendix P. However, under submerged fermentation, it was observed that there is significant difference among the isolates ($P > 0.05$) (Appendix O). This showed that under submerged fermentation, the choice of fungal endophytes and its consortia does affect the production of phenolic content. In solid state fermentation, regardless of the isolates used, the phenolic content produced is not affected by the fungal endophytes and its consortia. The results shown in this study is similar in a study by Amaya-Chantaca et al. (2022) whereby grape pomace in solid state fermentation showed higher phenolic content (2.262 g L⁻¹ of hydrolysable tannins) compared to under submerged fermentation (0.182 g L⁻¹ of hydrolysable tannins). It is deduced that there might be other factors that influences the production of phenolic content in solid state fermentation. This would be recommended for future works to further optimised the production of phenolic content.

Adeleke and Babalola (2022) stated that 80% of population in most developing countries uses medicinal plants as their primary healthcare. However, the growth of medicinal plants encounters numerous obstacles stemming from issues such as excessive harvesting without proper replanting, ecological imbalances, human-induced activities in

nature, and habitat destruction due to pest infestations (Palanichamy et al., 2018). These factors collectively lead to a decline in plant populations and limit their exploration for medicinal purposes. Hence, the endophytes derived from medicinal plants which possess similar metabolites as their host plant are selected. Other than that, the biodiversity of active compounds is also one of the reasons endophytes are being selected in this study. Although utilising endophytic fungi from medicinal plants seems promising as it could help in a more sustainable drug discovery, it is an ongoing area of research, and the translation of findings into practical therapeutics may take time. Nevertheless, the exploration of these natural sources holds great potential for the development of new medicines that could contribute to decreasing various ailments.

In previous studies, the genus of *Aspergillus* is widely used in many industrial applications such as food industries and pharmaceuticals. Usage of *Aspergillus niger* in the production of citric acid within the industry is immeasurable (Ramesh & Kalaiselvam, 2011). Recent findings have found out that *Aspergillus oryzae* has the ability to alleviate inflammation in *Mycoplasma pneumoniae* pneumonia showing a pharmaceutical potential within the *Aspergillus* species (Lee et al., 2023). *Aspergillus* species can contribute to the synthesis of various phenolic acids, including protocatechuic acid, p-hydroxybenzoic acid, and others, depending on the specific strain and fermentation conditions (Valencienne et al., 2020).

On the other hand, it was reported that *Fusarium equiseti* isolated from mangrove filament possesses antioxidant activity (Prathyusha et al., 2018). Although the genus *Fusarium* are known to be producing mycotoxins such as beauvericin (BEA), a study showed that the mycotoxin has antibacterial effect against several strains of bacteria, with

highest damage on *C. perfringens* (Meca et al., 2010). Moreover, an investigation conducted by Liu et al. (2019) has shown that *F. equiseti* isolated from a Chinese medicinal plant to possess significant antibacterial activity against several bacteria such as *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Shigella castellani*, and *Escherichia coli*. This shows that *F. equiseti* do possess antimicrobial activity. It is suggested that in-depth research using this genus can eventually produce a product with antibacterial properties.

There are several reasons that might contribute to the production of phenolic compounds in submerged fermentation state. Submerged fermentation requires high amount of liquid, constant stirring, and continuous supply of nutrients. These fungi may be producing higher amount of phenolic under lesser liquid condition while under submerged condition, they might be producing other bioactive compounds such as enzymes and vitamins. Moreover, the relationship of the fungi consortia under submerged fermentation could also contribute to the production of phenolic compound. The symbiosis between the fungi could also be the reason that the amount of phenolic are produced lower due to various reasons. Yu et al. (2021) stated that the consortium formed in mixed cultures of microorganisms differs from the pure culture because of its cellular interaction, evolution, and survival. This also may complicate in terms of food diverse flavours, metabolites produced and others.

The three endophytic fungi used in this study are well known to produce certain bioactive compounds. For instance, the genus *Penicillium* produced Penicillin, the infamous first antibiotic (Gaynes, 2017). Not only that, *Aspergillus niger* is also known to be widely used in food application and produced bioactive compounds such as 5-hydroxymethyl-2-furancarboxylic acid which can be used as anaesthetic in medical treatment (Wei et al.,

2022). The extract of *A. niger* from petroleum ether also shows antibacterial activity against several bacteria strains such as *Agrobacterium tumefaciens*, *Erwinia carotovora*, and *Ralstonia solanacearum* (Wei et al., 2022). The genus *Fusarium* was known to be producing harmful mycotoxin. However, Elavarasi et al. (2017) stated that the *Fusarium spp.* isolated from a mangrove plant, shows cytotoxicity more than anticancer compound, Rubrofusarin, an anticancer compound. *Fusarium equiseti* from marine derived showed antibacterial and phytotoxic ability upon growing on rice medium (Li et al., 2020). This tallies with the results of the study whereby upon growing on solid-state medium, the total phenolic content of RSS “*lemantak*” is known upon fermentation with different fungal consortia with the best total phenolic content exhibited by the genus *A. niger* and consortia of *P. chermesinum* and *F. equiseti* at seven days of fermentation.

4.1.1.2 Antioxidant production

To investigate the antioxidant capacity of these extracts, antioxidant tests were conducted to test the ability of the extracts in stabilises free radicals. There are several ways to investigate the antioxidant potential of a certain subject. For example, the DPPH scavenging method whereby free radicals were scavenged by DPPH electrons, transferring electrons to the unstable radicals turning it into stable form. In such, this study uses the DPPH radical scavenging method as a preliminary method to select the best endophyte consortia.

Based on Figure 4.4 and Figure 4.5 which exhibits the results of DPPH scavenging activity of endophytes consortium, certain endophytes and their consortia showed relatively high inhibition activity. The higher the inhibition activity, the greater the potential for it to be an antioxidant. This is because the inhibition activity shows that the free radicals underwent delocalization process to become stable and thus reducing the number of free

radicals as free radicals could bring many harmful effects to the living organisms. The formation of stable phenoxy radical lead by the ability of electron donating substituents or presence of hydrogen with the ability of reducing free radicals to delocalize the unpaired electron in a substance (Gülçin, 2012). Gülçin (2020) also stated that the ability of polyphenolic compounds depends on the redox properties of their phenolic hydroxyl groups and the potential for electron delocalization across chemical structures.

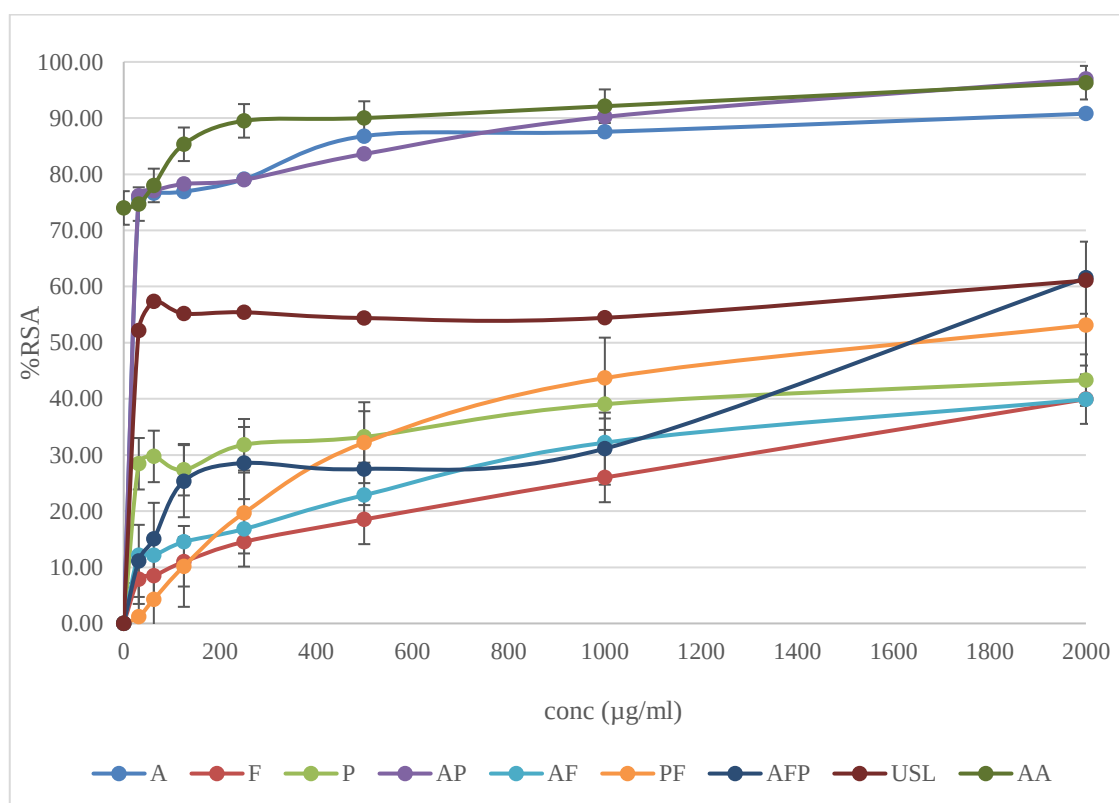


Figure 4.4: DPPH radical scavenging activity solid state fermentation, (n=3)

Abbrev.: **A:** *A. niger*, **P:** *P. chermesinum*, **F:** *F. equiseti*, **AP:** *A. niger* & *P. chermesinum*, **AF:** *A. niger* & *F. equiseti*, **PF:** *P. chermesinum* & *F. equiseti*, **AFP:** *A. niger*, *F. equiseti* & *P. chermesinum*, **USL:** Uninoculated fermented sago “*lemantak*”, **AA:** Ascorbic acid

In Figure 4.4, it is shown that the best consortia that exert high inhibition activity was the monoculture *A. niger* (A). Followed by AP > USL > PF > P > AFP > AF > F. Interestingly, the abiotic control, RSS “*lemantak*” showed high scavenging activity against free radicals as compared to when it is fermented with endophytes and its consortia. In 2018,

a study conducted by Duque et al. showed lesser phenolic content of raw sago starch at different coarse fraction. In his study, the highest exhibited phenolic content was by sago starch of coarse fraction C60 at $IC_{50} 2.63 \pm 0.19$. This shows that the RSS “*lemantak*” may already possessed or produced antioxidant compounds without additional fermentation process nor upon fermentation with endophytes. However, there are no means to compare the current study with other researchers conducted on sago starch of its phenolic content as there are almost little to no studies conducted on the raw sago starch. When fermented with fungal endophytes, the scavenging activity increases and thus increasing its potential as an antioxidant producer and its potential as a value-added product. Besides that, the consortia of *A. niger* and *P. chermesinum* (AP) also showed the higher activities among other consortia at a range of 96.94 - 76.17% of inhibition activity upon fermentation with RSS “*lemantak*”. This shows that under fermentation with RSS “*lemantak*”, antioxidant potential of the endophytes increases and thus increasing its capability as a value-added product.

In Figure 4.5, it is observed that the best scavenging activity was also exhibited by monoculture *A. niger* under submerged fermentation. Followed by PF > monoculture *F. equiseti* > AF > AP > AFP > monoculture *P. chermesinum* > control. However, in terms of the scavenging activity as the concentration decreases, it showed that consortia PF has better scavenging activity as compared to monoculture *A. niger*.

Surprisingly, under submerged fermentation, the scavenging activity of the abiotic control (RSS “*lemantak*”) showed a decrement of almost 40% in activity. The decline in which may have altered the antioxidant compounds’ activity (Shah et al., 2022).

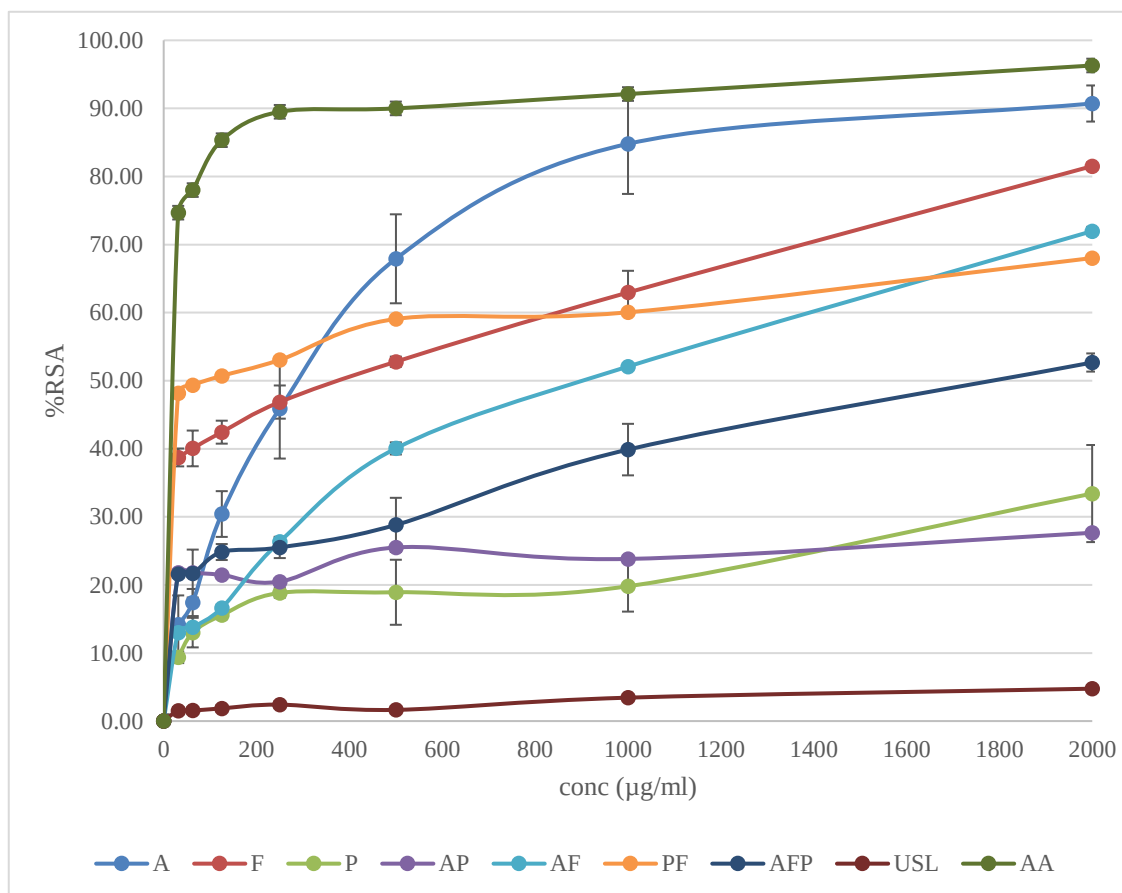


Figure 4.5: DPPH radical scavenging activity submerged fermentation, (n=3)

Abbrev.: **A:** *A. niger*, **P:** *P. chermesinum*, **F:** *F. equiseti*, **AP:** *A. niger* & *P. chermesinum*, **AF:** *A. niger* & *F. equiseti*, **PF:** *P. chermesinum* & *F. equiseti*, **AFP:** *A. niger*, *F. equiseti* & *P. chermesinum*, **USL:** Uninoculated fermented sago “*lemantak*”, **AA:** Ascorbic Acid

Inappropriate levels of antioxidants may be toxic and act as an oxidation stimulator instead as an antioxidant (Yousefian et al., 2014). This supports the study to select consortia AP and PF based on their phenolic content and antioxidant ability. Whether the difference of fermentation method affects the production of phenolic and its antioxidant capacity will be further investigated and explained in the subsequent subchapter.

Moreover, it could be highlighted here that the radical scavenging activity of consortia AP and monoculture *A. niger* under submerged fermentation mode shown in the DPPH test could rivalled to that of the standard, ascorbic acid which unknowingly is the best scavenger compound. There are also reports regarding the ascorbic acid having a negative side as an antioxidant whereby ascorbic acid is deemed as a double-faced prooxidant material. Ascorbic acid contain ascorbate whereby apart from reducing oxidising sources, it also reduces metal ions such as Fe^{3+} and Cu^{3+} . In return of this process, a Fenton reaction occurs in which ascorbate produces high reactive free radicals in presence of transition metal ions (Naidu, 2003). The formation of these free hydroxyl was reported to be inducing damage to DNA by causing cleavage of the phosphodiester backbone and modification of DNA bases (Crott, 1999). Therefore, there is a need to search for a suitable standard which does not causes any side effects that are detrimental to the human health. It is proposed that the extracts of monoculture *A. niger* and consortia AP may have the potential of establishing a natural-based product perhaps in pharmaceutical and food industry.

In the preliminary testing of this study, different consortia were carried out under fermentation. The extracts were collected and tested for total phenolic content and antioxidant ability. It is seen that the consortia of fungal *A. niger* and *P. chermesinum* (AP) produced the highest total phenolic content and higher antioxidant capacity under solid-state fermentation. In contrary, there are two candidates for submerged fermentation which are the combination of *P. chermesinum* and *F. equiseti* (PF) and monoculture *A. niger* based on its total phenolic content (TPC) and DPPH scavenging activity.

It is observed that there are three potential consortium candidates to be considered which are monoculture *A. niger*, *P. chermesinum* and monoculture *A. niger*. However, only

two are selected which are consortia *A. niger* and *P. chermesinum* (AP) and consortia *P. chermesinum* and *F. equiseti* (PF). The rationale for selection is justified for a number of reasons based on its phenolic content and its antioxidant capacity as a free radical scavenger.

Consortia AP showed higher phenolic content compared to its monoculture and also was selected due to its ability as a radical scavenger by demonstrating high activity in antioxidant activity as observed in the results obtained. Similarly, as its total phenolic content, this consortium also has potential in scavenging activity rivalled that of its monoculture *A. niger* and monoculture *P. chermesinum* respectively.

On contrary, consortium *P. chermesinum* and *F. equiseti* showed lower phenolic content in both solid state and submerged fermentation. For consortium *P. chermesinum* and *F. equiseti*, scavenging activity showed higher at lower concentration. Total phenolic content is lower than that of produced by consortium AF, consortium AF showed lower scavenging ability in antioxidant. This showed that AF possess lower number of phenolic compounds that contributes to its antioxidant capacity. Moreover, PF showed higher phenolic content when incubated under solid state fermentation as compared to monoculture *A. niger*. As this study strives to seek potential parameters with higher antioxidant activity, consortia PF was selected based on the criteria in submerged. Consortia PF has at least 50% of inhibition activity even at the lowest concentration. It is interesting to note that consortia PF may possess better antioxidant potential than monoculture *A. niger*. Due to these reasons, the selected consortia are consortium AP and consortium PF.

4.1.2 Comparison between Fermentation Types

A study on the fermentation method was also carried out to see whether this parameter affects the production of total phenolic content and antioxidant capacity. The

fermentation method that was carried out were solid-state fermentation and submerged fermentation. As the name, a solid-state fermentation was carried out in a little to near absence of water while the substrate in submerged fermentation is fully immersed in liquid. As the fermentation method varies from one another, it is suggested that the phenolic production also varies depending on the fermentation method.

From this study, it was found out that the solid-state fermentation showed higher total phenolic content compared to submerged fermentation as shown in Figure 4.5. Comparing the fungal endophytes consortia AP in two fermentation mode, it is shown that the under solid-state fermentation, the amount of phenolic content is higher than the production under submerged fermentation. A similar trend was also observed on consortia PF and in the uninoculated sago "*lemantak*".

Based on the results obtained from the study, it is shown that production of phenolic content is higher under the solid-state fermentation (SSF) compared to submerged fermentation (SMF) as shown in Figure 4.6.

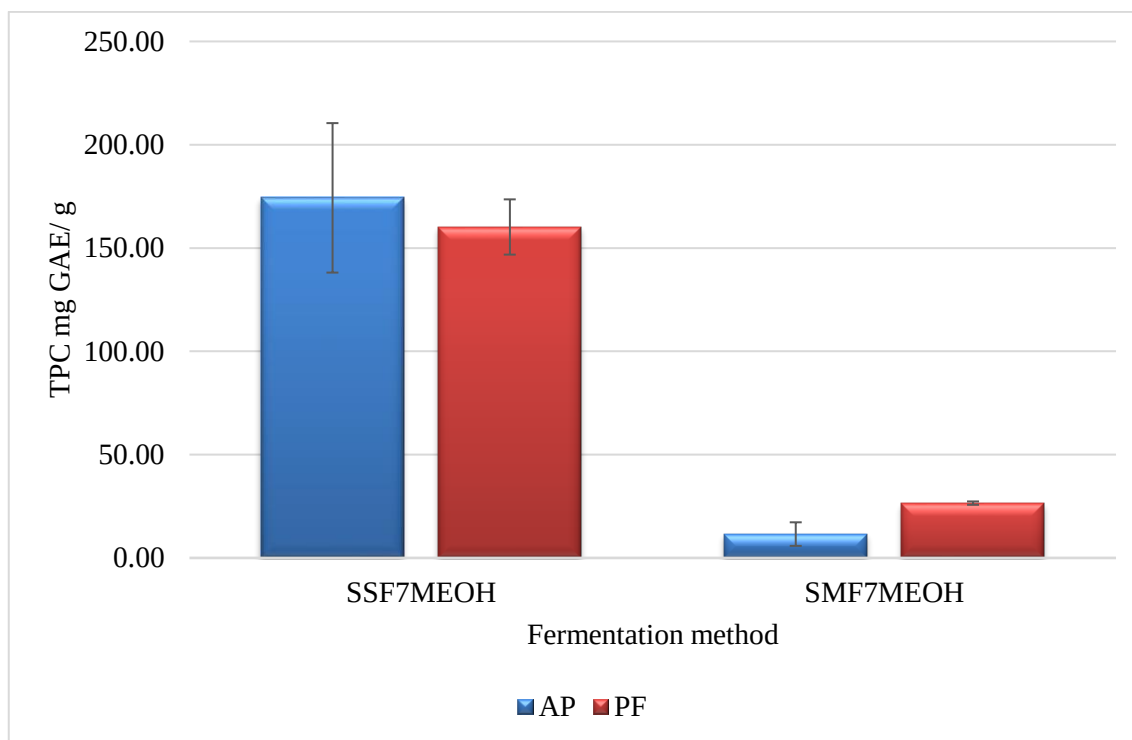


Figure 4.6: Comparison of total phenolic content of methanolic extract of fermented sago starch by different fungal endophytes consortia under different fermentation method, (n=3)

Abbrev: **AP:** *A. niger* & *P. chermesinum*, **PF:** *P. chermesinum* & *F. equiseti*

SSF7MEOH: solid state fermentation under seven days of methanolic extracts

SMF7MEOH: submerged fermentation under seven days of methanolic extracts

Figure 4.7 showed the DPPH scavenging activity of Day 7 extracts in consortia of endophytes AP and PF as well as USL in different fermentation mode. It is observed that SSF-AP prevails in scavenging free radicals compared to SSF-PF and SS7-USL under solid state fermentation. This showed that consortia AP showed good scavenging activity under solid state fermentation (96.940 – 76.143%) at different concentrations. While under submerged fermentation, SM7-PF extract showed better scavenging activity compared to other extracts of SM7-AP and SM7-USL. The results obtained in this parameter showed similarities between the total phenolic content and radical scavenging activity between the extracts whereby both SS7-AP and SM7-PF showed highest in total phenolic content and

scavenging activity. There is a possibility that there are phenolic compounds that may be contributing to the radical scavenging activity.

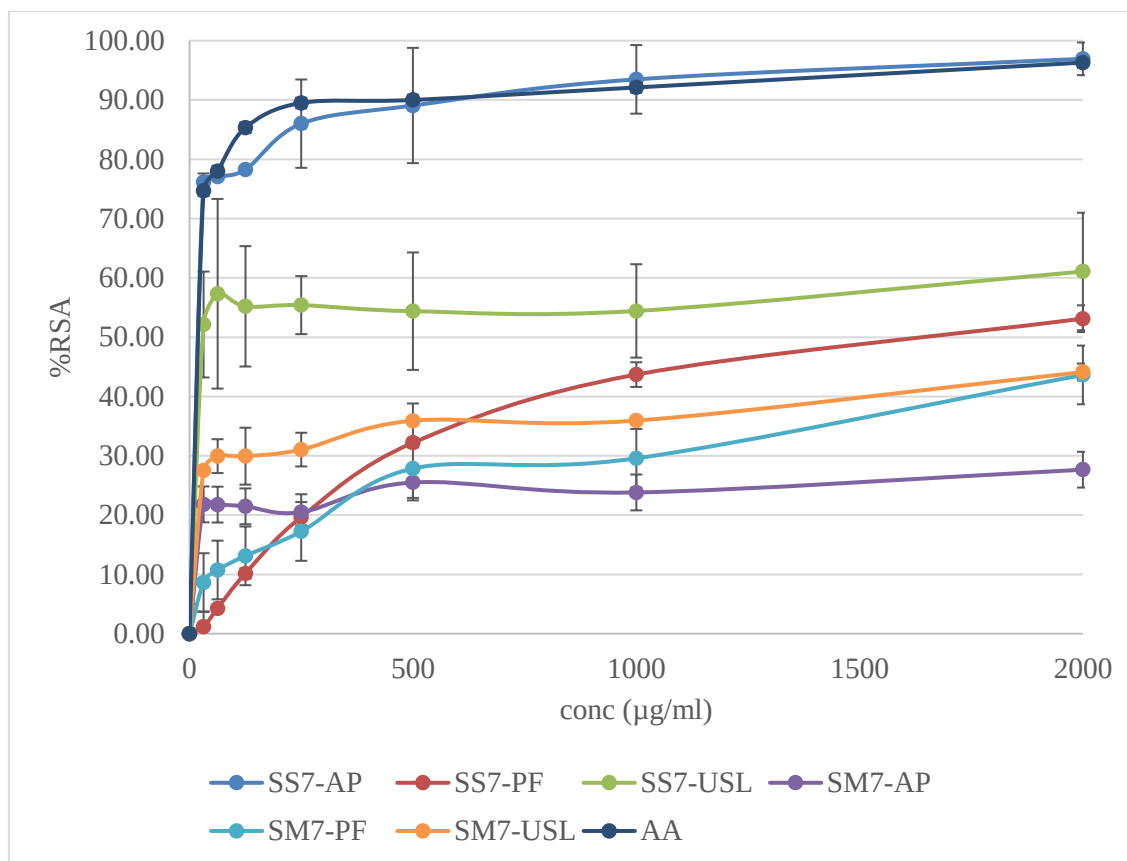


Figure 4.7: DPPH scavenging activity between fermentation methods in different consortia, (n=3)

Abbrev: **SSF7-USL**: solid state fermentation extract under seven days of uninoculated fermented RSS, **SSF7-AP**: solid state fermentation extract under seven days with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SSF7-PF**: solid state fermentation extract under seven days with fungal endophytes consortia *P. chermesinum* and *F. equiseti*, **SM7-USL**: submerged fermentation extract under seven days of uninoculated fermented RSS, **SMF7-AP**: submerged fermentation extract under seven days with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SMF7-PF**: submerged fermentation extract under seven days with fungal endophytes consortia *P. chermesinum* and *F. equiseti*, **AA**: ascorbic acid

It can be implied that solid state fermentation method showed better production of phenolic content as compared to submerged. The condition of SSF which requires lesser liquid is more suitable for the growth of endophytes used in this study. Moreover, selection

of SSF as the most suitable fermentation technique to produce bioactive compound from fermented RSS will not only be cost effective for production of value-added products based on fermented RSS as SSF uses lower capital cost and thus more economical in a larger scale. In contrary, SMF requires continuous maintenance such as constant agitation, supply of nutrients for microbial growth. However, depending on the end-product or demand of the market, antioxidant metabolites through submerged fermentation can be useful especially in probiotic food industry as mentioned in a study by de Oliveira et al. (2020). Furthermore, it can be proven that microbial fermentation helps in converting products with many potentials whereby in this study, the production of phenolic compounds from fermented RSS “*lemantak*” and the antioxidant capacity of RSS “*lemantak*”. Therefore, from this study, we can conclude that SSF is better than SMF in maximising production of phenolic, particularly on the antioxidant potential from fermented RSS “*lemantak*”.

Solid-state fermentation supports controlled release of nutrients as the same substrate can be used over a long fermentation period as stated in a study by Subramaniam and Vimala (2012). Contrarily, submerged fermentation utilized substrates rapidly which requires constant supply of nutrients. However, the benefit of submerged fermentation is that the purification process is easier especially for extraction of secondary metabolites as the metabolites are diffused to the liquid during fermentation. Solid-state fermentation (SSF) and submerged fermentation (SMF) has major differences especially during the downstream processing. In comparison to solid-state fermentation, submerged fermentation requires filtration and purifying process upon gaining the products.

Aspergillus niger produces much higher titers and have higher productivity in SSF system than in SMF system as shown in a study by Venegas et al. (2013). In fact, the enzyme

pectinase produced higher in SMF than in SSF, showing the absence of catabolite repression to produce pectinases by SSF (El Enshasy et al., 2018). However, a study by Viniegra-González et al. (2003) demonstrated that it is possible to obtain higher enzyme productivity in SSF, as compared to SMF, even when growth is restricted by physical hindrances (excessive packing density) if the mould happens to have a higher enzyme yield factor and a lower rate of decay.

In summary, SSF cultures appear to function naturally as fed-batch cultures with fast oxygenation but slow sugar supply. This process has the added advantage of being a static process that does not use mechanical energy. In contrast, SMF cultures act as a homogeneous system. It takes a lot of energy to supply oxygen fast enough to meet the high oxygen demand. Such methods require automated fed-batch delivery of substrates to avoid catabolite repression (Viniegra-Gonzalez et al., 2003). In a study by Amaya-Chantaca and colleagues (2022), a comparative study of fermentation method on grape pomace showed that higher number of polyphenols were produced through solid state fermentation than in submerged fermentation. The findings from this study were in line with reports from Amaya-Chantaca et al. (2022) and several other studies.

In an overall overview, the use of fermentation helps in increasing the production of phenolic compounds as shown in the study. The outcome of each fermentation varies for each substrate hence it is crucial to select suitable mode of fermentation depending on the end target. Therefore, from this study, it can be concluded that SSF is better than SMF in maximising the production of phenolic and antioxidant by utilizing RSS "*lemantak*" as substrate.

4.1.3 Comparison between Incubation Periods

Incubation period in fermentation is the time that the microorganisms were exposed to the substrate to maximize the productivity of the organism whereby the product of interest may be a bioactive metabolite, an enzyme, a vitamin, or recombinant protein.

Initially, a preliminary test was conducted with a maximum duration of 21 days with a seven-day interval (Appendix C). However, the initial test showed that increased production of phenolics with antioxidant ability was detected within the range of 7 to 14 days as shown in Appendix C. Therefore, a further investigation was conducted by investigating the range of 14 days. A second set of incubation period was further determined with an interval of two days which are shown in this study. In this study, a range of fifteen days were setup with two-day intervals. The selected endophytic fungi from previous trial were used in this parameter according to the one-factor-at-a-time (OFAAT) method. Implementation of solid-state fermentation was carried out for this parameter as the chosen fermentation mode was deemed to be the highest in the production of phenolic contents and its antioxidant ability based on previous criteria trial.

As demonstrated in Figure 4.8, the production of phenolic varies at different incubation period. A trend of increase can be observed in the phenolic production under consortium AP. The highest production was observed on Day 7, with drastic decrease on Day 9 and a steady increase was observed onwards until Day 15. Therefore, the highest phenolic production is on Day 7 followed by Day 15 > Day 13 > Day 11 > Day 9 for consortium AP. Contrarily, for consortium PF, the highest production can be observed on Day 15. Followed by Day 11 > Day 13 > Day 7 > Day 9. It is important to note that the

overall production of phenolic content on dual culture PF is higher than in dual culture AP excluding Day 7 for AP which is much higher compared to Day 15 PF.

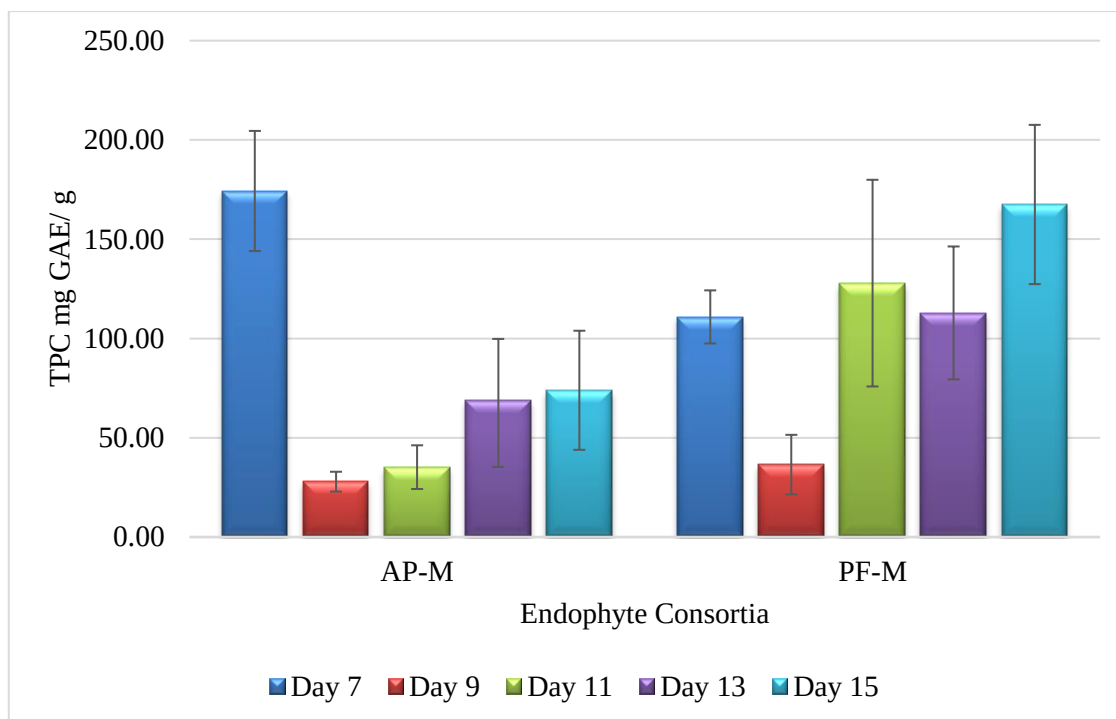


Figure 4.8: Total phenolic content of methanolic extract of fermented sago starch by selected fungal endophytes consortia at different incubation period, (n=3)

Abbrev: **AP-M**: fungal endophytes consortia *A. niger* & *P. chermesinum* in methanol extract; **PF-M**: fungal endophytes consortia *P. chermesinum* & *F. equiseti* in methanol extract

In general, the production of phenolic content for dual culture PF in average is more than 100 mg GAE/ g as compared to dual culture AP. This shows that fungal endophytes consortium PF could be producing more phenolic content as the period of incubation increases. Therefore, it can be assumed that this dual culture is suitable for longer period of incubation and thus can reduce the cost of production. It is evident that mixed cultures permit for better efficient usage of substrate based on the results from this study. It can be seen in Figure 4.9, the scavenging ability of consortium AP tallies with the phenolic content results in Figure 4.8. For consortium AP, the antioxidant activity on Day 7 shows the highest. This

is followed by Day 15 > Day 13 > Day 11 > Day 9. This also suggested that among the phenolic compound that was produced, majority compounds produced are with antioxidant ability. A further investigation was carried out to validate this finding.

In contrast, the DPPH scavenging activity for consortium PF is lesser on Day 15 as compared to the results shown in Figure 4.9. The highest antioxidant activity of consortium PF observed on Day 13. Followed by Day 7 > Day 11 > Day 15 > Day 9 as shown in Figure 4.10. Although the extract of consortia PF showed higher total phenolic content on Day 15, in contrast, the antioxidant activity of the mentioned extract has the lowest inhibition activity. A possible reason to this occurrence that the phenolic compounds contributing to the antioxidant is lesser than the ones in extracts of consortia AP. As illustrated, S7-AP showed the highest antioxidant activity and could rivalled to the standard. A further investigation on the characteristic of S7-AP is needed. The activity shown in antioxidant activity of S7-AP is in line with the results of total phenolic content.

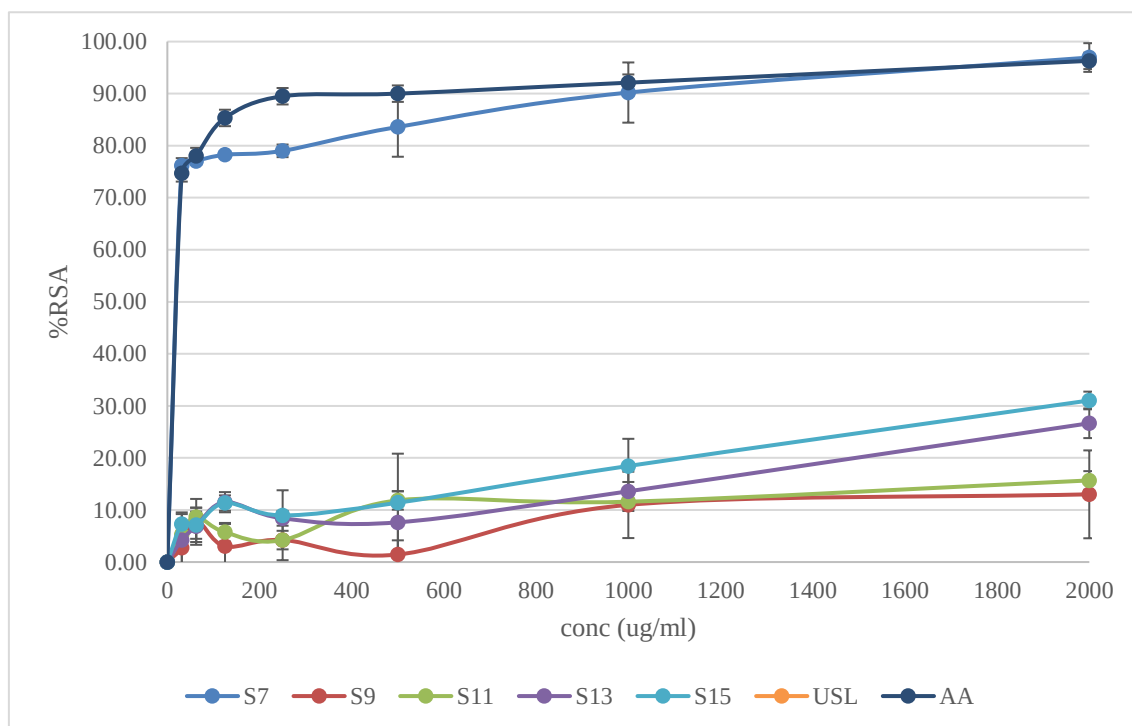


Figure 4.9: DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes of consortia AP under solid state fermentation (SSF), (n=3)

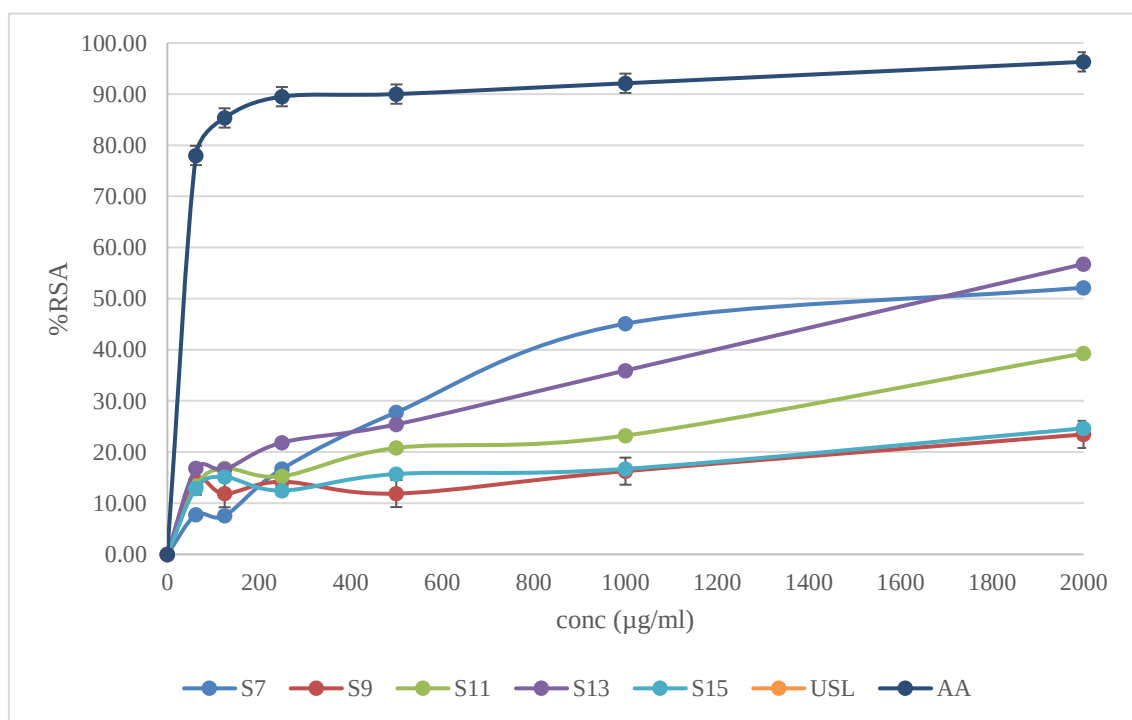


Figure 4.10: DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia PF under solid state fermentation (SSF), (n=3)

A study by Krisnaningsih et al. (2019) discovered that the increased fermentation period in yoghurt with taro starch as stabilizer increases total solid caused by lactic acid bacteria (LAB) cell proliferation. Whereby this condition increases the nutritional content of the produced yoghurt. Moreover, another study also showed that at a certain period, the production of a substance could increase and decrease after the optimised period (Nadeem et al., 2015). This study showed in tandem as previous research that the production of phenolic content decreases after a certain period and subsequently increases. Other factors may contribute to the results, and thus require more investigation.

From this experimental parameter, it is shown that incubation period is crucial to determine the optimum production of phenolic compounds as well as the antioxidant ability to scavenge free radicals. It is also shown that the consortia AP shows highest phenolic content and scavenging ability on Day 7. On the other hand, consortia PF showed better phenolic content and scavenging activity at Day 13 under methanolic extracts.

4.1.4 Comparison between Extraction Time

In this parameter, the extraction time was investigated to determine whether the parameter affects the extraction of phenolic content. Some studies researched that the extraction time could be influencing the polyphenols extracted from plant as the components are exposed to a certain duration under a certain temperature range (Che Sulaiman et al., 2017). Delgado et al. (2019) had stated that increased polyphenol was found out after extracting at an increased temperature which enhances their solubility, promoting their diffusion rate and thus maximises the polyphenols transfer. The study also observed degradation of compounds, vaporization, and loss of solvents once the temperature exceeded a certain value. This study used a temperature of 50°C which was deemed to be optimal for

polyphenol extraction. Therefore, this study also investigated the possibility of phenolic production influenced by extraction time.

4.1.4.1 Total Phenolic Content

As for the other parameters, best selection from previous parameters were carried forward to be further selected. As of this point, the selected parameters were solid state fermentation (SSF), seven days period of incubation (D7) and fungal endophytes consortia AP. Under this experiment, the extraction time parameter was investigated at different extraction time which are at 4 hrs, 8 hrs, and 16 hrs extraction. However, it is best to highlight here that at this parameter, all the previous selected conditions were also maintained to observe and select the best parameter that optimizes the production of phenolic compounds with antioxidant activity and the results are discussed in the following subchapter. Moreover, results from submerged fermentation extracts are also displayed in Appendix E for a comparison of activity between the fermentation type. The fermented RSS was conducted under the same condition and mass except for extraction time.

Based on Figure 4.11 - 4.14, it is shown that the total phenolic content of fermented sago starch with different fungal endophytes and its consortia differs as longer extraction time was imposed. This is also shown throughout extended fermentation periods. In Figure 4.11, fungal endophytes consortia AP of Day 7 showed the highest TPC of 174.30 mg GAE/g. Out of all the 15 extracts, only consortia AP of Day 7, consortia PF of Day 7 and consortia AP of Day 11 showed high amount of TPC. For uninoculated fermented sago starch "*lemantak*" (USL), lowest phenolic content was shown on Day 15 and highest amount of phenolic content was observed on Day 7. The remaining samples at 4 hrs showed TPC less than 50 mg GAE/g. Nevertheless, at 4 hrs of extraction with solvents, the presence of phenolic content from the fermented sago starch can be seen.

Figure 4.12 showed the total phenolic content of fermented sago starch extracts after 8 hrs of extraction. In general, extract from consortia AP of Day 15 showed the highest phenolic content at 120 mg GAE/g. The trend of phenolic content of consortia AP over period of fermentation is as follows: D15 > D13 > D7 > D11 > D9. In comparison to 4 hrs of extraction, the phenolic content varies after 8 hrs of extraction as some of the extracts showed a decrease in phenolic content while some of the extracts had an increase over time. This could be due to the longer exposure to heat and promotes the release of phenolic compounds from the extracts. A trend of increasing phenolic content after 8 hrs was seen in consortia PF with a highest peak at Day 13 and a slight decrease in phenolic content after Day 15 of fermented period in contrast to the 4 hrs of extraction whereby Day 7 has the highest phenolic content. For USL, total phenolic content of Day 7 prevails after 8 hrs. Total phenolic content in this extract was seen to be increasing until Day 9 and decreases after Day 11.

At 16 hrs of extraction, consortia AP, consortia PF and the extract of USS showed the highest phenolic content at Day 7 as seen in Figure 4.13. In consortia AP and USS, both showed the lowest phenolic content at Day 15. For extract of consortia PF, phenolic content was observed the lowest on Day 9. However, it is to note here that the amount of extractable phenolic content of all extracts is lower than the extracts of any other period of extraction at 4, 8 and 24 hrs. Nevertheless, extract of consortia AP under Day 7 exhibits the highest phenolic content after 16 hrs of extraction of 154.73 mg GAE/g. Only the extracts of Day 7 and Day 13 fungal endophytes consortia AP extract showed the phenolic content of more than 50 mg GAE/g.

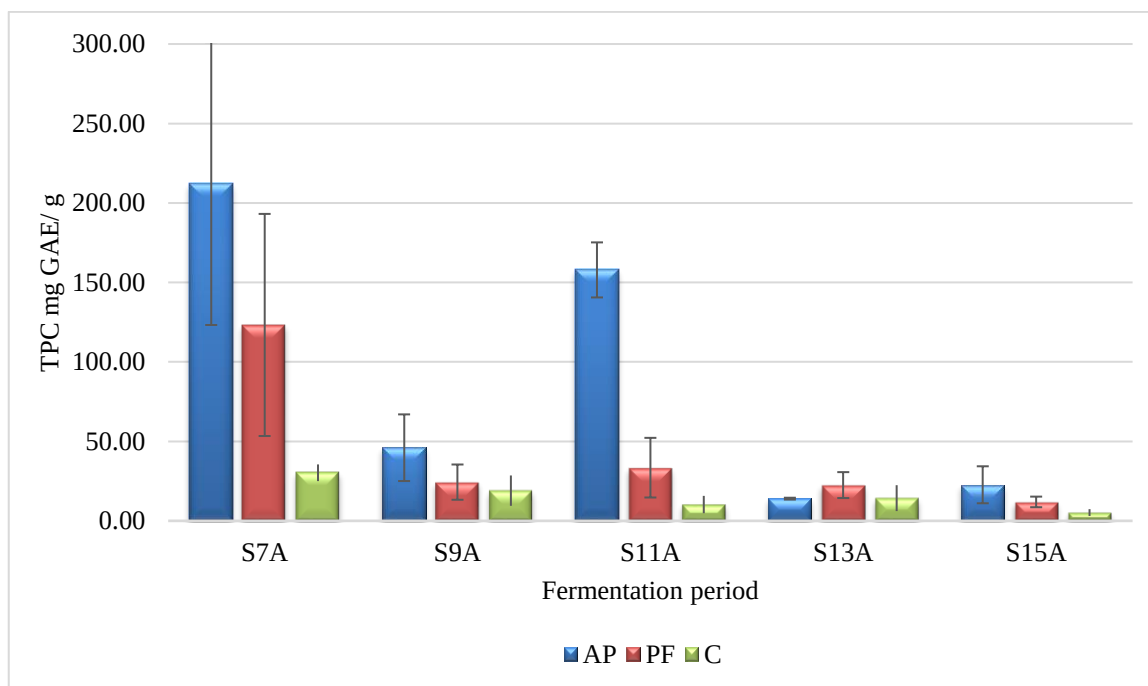


Figure 4.11: Total phenolic content of methanolic extracts of fermented sago starch by different fungal endophytes consortia under solid state fermentation (SSF) after 4 hrs of extraction at different fermentation period, (n=3)

Abbrev.: **AP**: endophyte consortia *A. niger* & *P. chermesinum*, **PF**: endophytes consortia *P. chermesinum* & *F. equiseti*, **C**: uninoculated fermented RSS

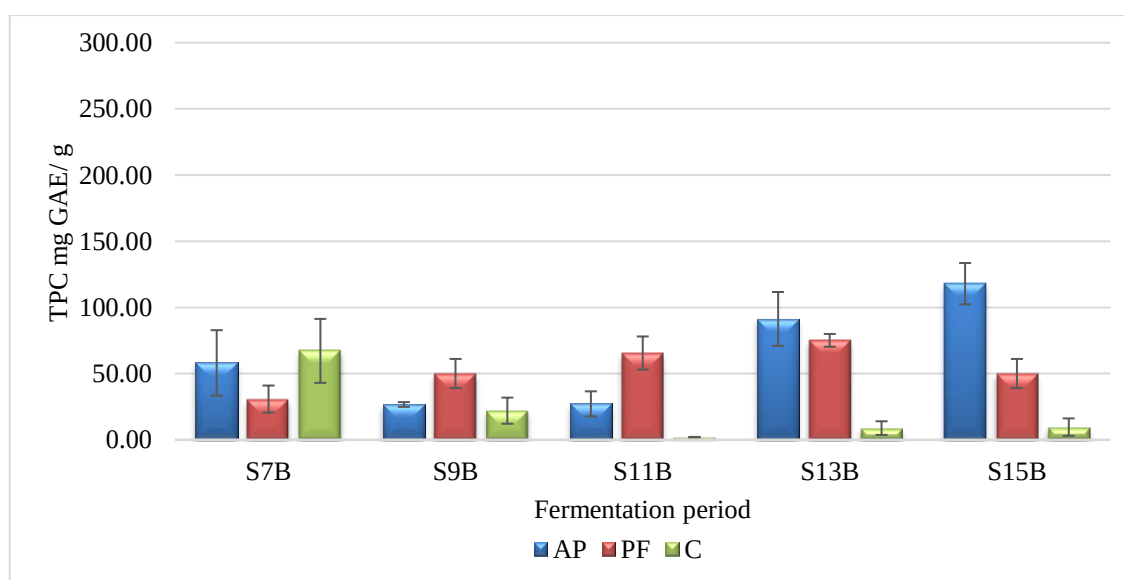


Figure 4.12: Total phenolic content of methanolic extracts of fermented sago starch by different fungal endophytes consortia under solid state fermentation (SSF) after 8 hrs of extraction at different fermentation period, (n=3)

Abbrev.: **AP**: endophyte consortia *A. niger* & *P. chermesinum*, **PF**: endophytes consortia *P. chermesinum* & *F. equiseti*, **C**: uninoculated fermented RSS

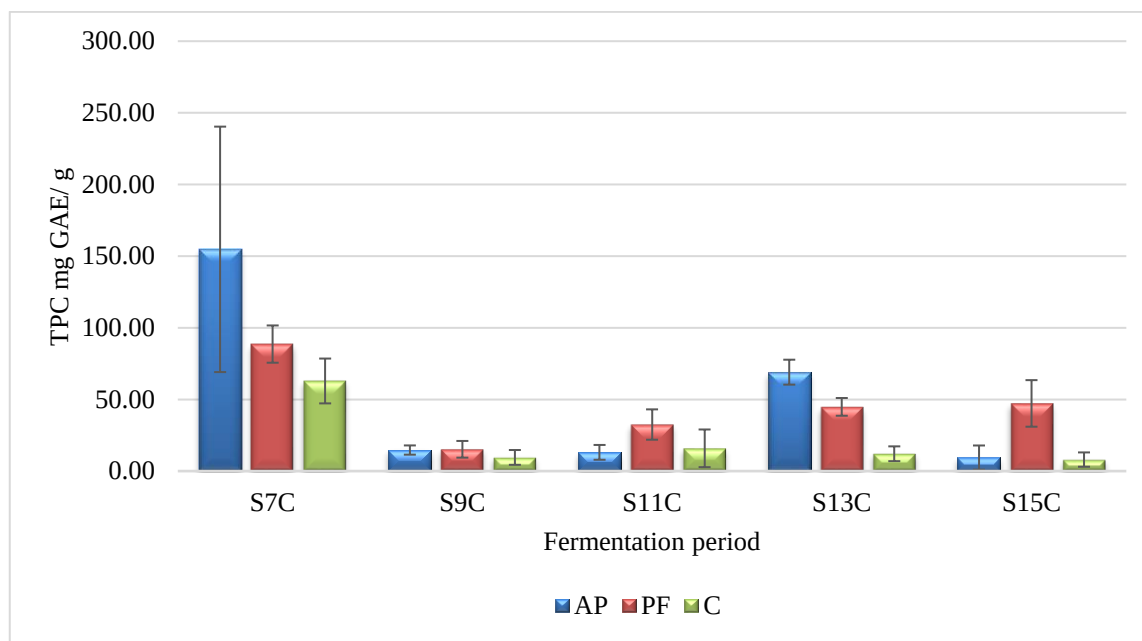


Figure 4.13: Total phenolic content of methanolic extracts of fermented sago starch by different fungal endophytes consortia under solid-state fermentation (SSF) after 16 hrs of extraction at different fermentation period, (n=3)

Abbrev.: **AP**: endophyte consortia *A. niger* & *P. chermesinum*, **PF**: endophytes consortia *P. chermesinum* & *F. equiseti*, **C**: uninoculated fermented RSS

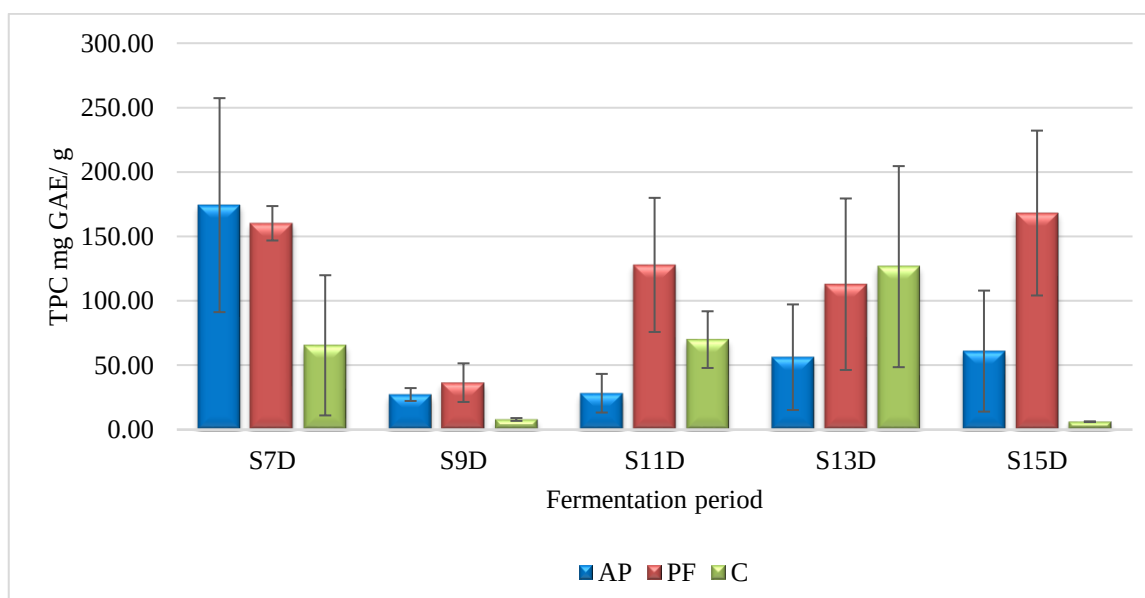


Figure 4.14: Total phenolic content of methanolic extracts of fermented sago starch by different fungal endophytes consortia under solid state fermentation (SSF) after 24 hrs of extraction at different fermentation period, (n=3)

Abbrev.: **AP**: endophyte consortia *A. niger* & *P. chermesinum*, **PF**: endophytes consortia *P. chermesinum* & *F. equiseti*, **C**: uninoculated fermented RSS

The three extracts of different fermentation period were subjected to different extraction time of 4, 8, 16 and 24 hrs. The timeframe chosen was based on preliminary test whereby an initial of 24 hrs period were imposed on the extracts. In this sub parameter, a minimum of 4 hrs were selected based on the previous preliminary tests. Arrangement of the results are displayed in breakdowns at different fermentation periods to carefully select the periods or timeframe that gives the optimum range of phenolic content and high scavenging ability.

4.1.4.2 Antioxidant activity at Day 7

Based on Figure 4.15 (a) which showed the DPPH activity of methanolic extract of fermented sago starch after 4 hrs of extraction after Day 7 under SSF. It is also observed that the scavenging activity of the three extracts subjected to SSF increased as the extraction time increases (8 hrs and 16 hrs) which can be observed in Figure 4.15 (b) and Figure 4.15 (c). This shows that the longer the fermented RSS were exposed to the solvent, more soluble phenolic compounds are able to be extracted.

In Figure 4.15 (a), fungal consortia PF showed better scavenging activity followed by fungal endophytes consortia AP (S7AP) and control (S7C). Whilst in Figure 4.15 (b), consortia AP showed better scavenging activity as compared to the extract of the same consortia at 4 hrs after extraction.

It is also observed that the extract of 16 hrs extraction of fungal endophytes consortium AP showed the highest scavenging activity of more than 76.19% of inhibition as compared to fungal endophytes consortium PF and the control, USL (consortia PF: 21.61% at low concentration and 83.40% of inhibition at high concentration; USL: 11.20% at low concentration and 74.30% at high concentration) at 16 hrs of extraction.

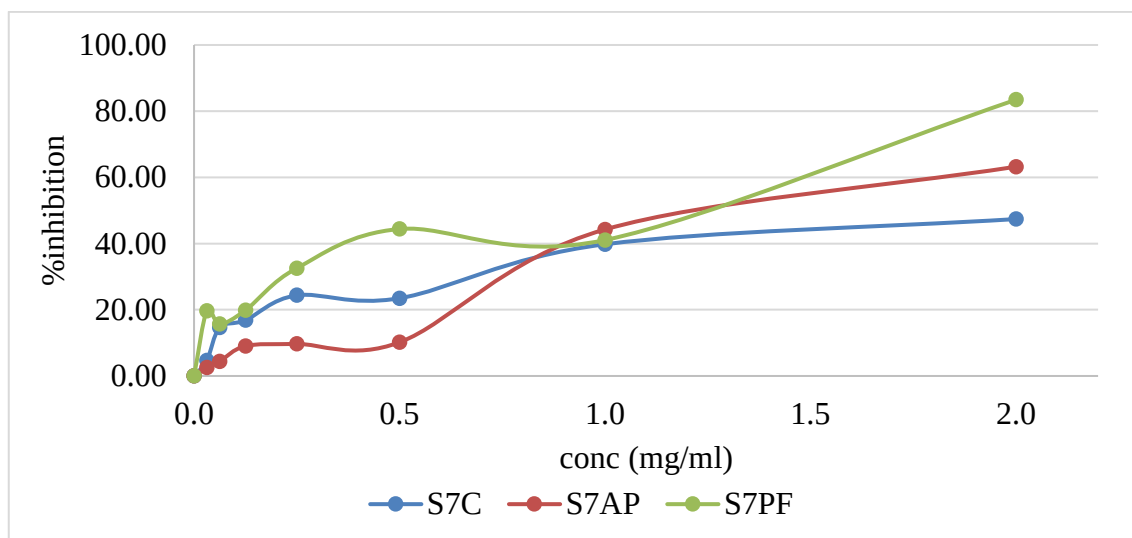


Figure 4.15 (a): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under solid state fermentation (SSF) after 4 hrs of extraction time at Day 7 of incubation period, (n=3)

Abbrev.: **S7-C:** solid state fermentation extract under seven days of incubation with uninoculated RSS, **S7-AP:** solid state fermentation extract under seven days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S7-PF:** solid state fermentation extract under seven days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

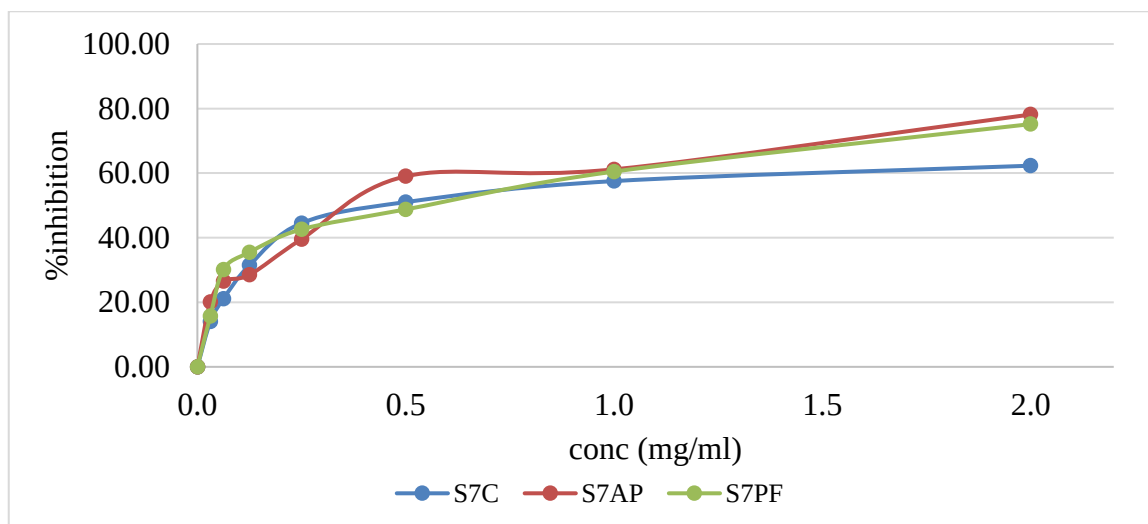


Figure 4.15 (b): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under solid state fermentation (SSF) after 8 hrs of extraction time at Day 7 of incubation period, (n=3)

Abbrev.: **S7-C:** solid state fermentation extract under seven days of incubation with uninoculated RSS, **S7-AP:** solid state fermentation extract under seven days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S7-PF:** solid state

fermentation extract under seven days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*.

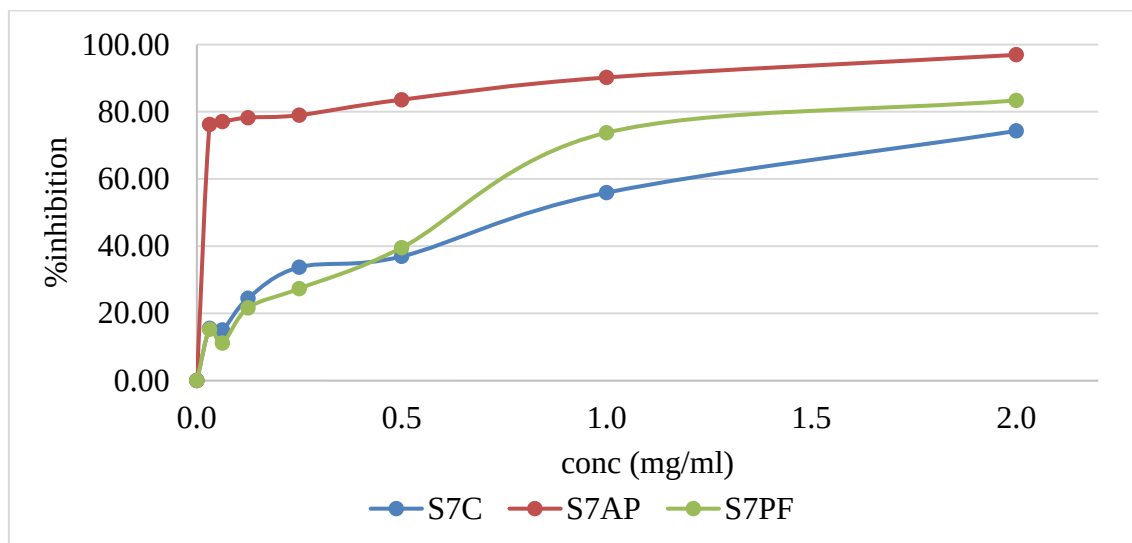


Figure 4.15 (c): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under solid state fermentation (SSF) after 16 hrs of extraction time at Day 7 of incubation period, (n=3)

Abbrev.: S7-C: solid state fermentation extract under seven days of incubation with uninoculated RSS, S7-AP: solid state fermentation extract under seven days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, S7-PF: solid state fermentation extract under seven days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

4.1.4.3 Antioxidant activity at Day 9

In Day 9 extracts shown in Figure 4.16 (a) - (c), solid state fermentation (SSF) extract Day 9 of fungal endophytes consortia AP (S9AP) showed a decrease in trend as the time increases. At 4 hrs of extraction, extract S9AP had already exhibit 60.48% of scavenging activity. However, after 8 hrs, the amount decreases slightly as shown in Figure 4.16 (b) which further decreases as time increases up to 16 hrs. Unlike the SSF extract Day 9 of fungal endophytes consortia PF (S9PF) which showed a slight increase in activity as the time increases. S9PF showed scavenging activity of 36.17% after four hrs of extraction and a slight decrease was observed after 8 hrs. Although at 16 hrs of extraction the activity demonstrates high scavenging activity of 71.73%. This shows that this extract showed better antioxidant activity at longer time of extraction, contrarily to S9AP whereby as time

increases, the antioxidant activity decreases. On the other hand, SSF extract of uninoculated fermented sago starch (control) (S9C) showed no changes in the first 8 hrs. But it can be observed that after 16 hrs of extraction, the scavenging activity of S9C showed a twofold increase from 21.55% to 48.26%. Similar to S9PF, extract S9C showed better antioxidant activity as time increases.

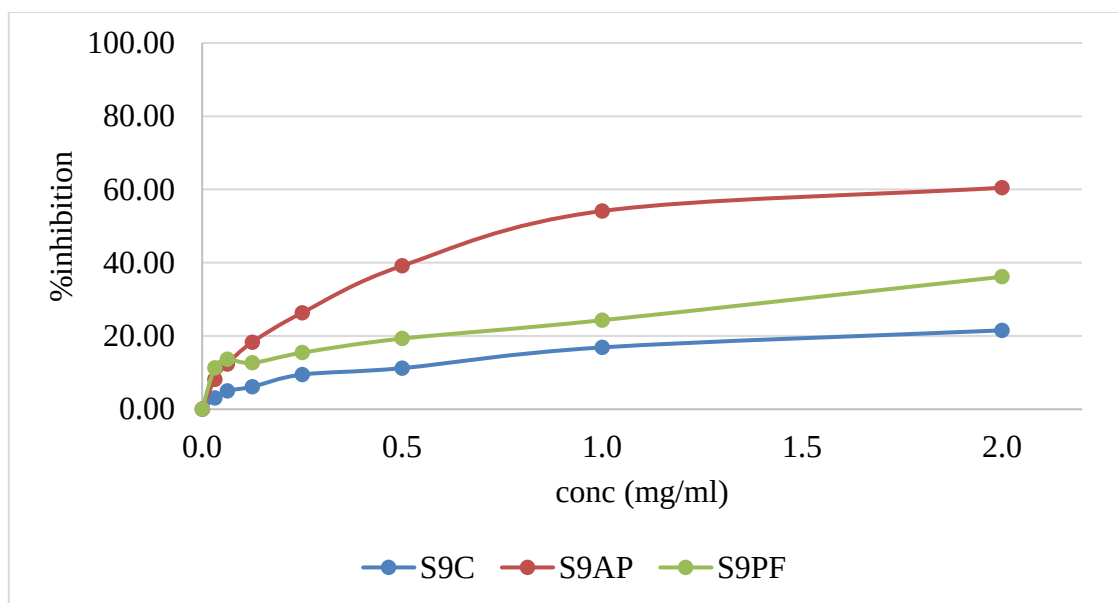


Figure 4.16 (a): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 4 hours of extraction time at Day 9 of incubation period, (n=3)

Abbrev.: **S7-C:** solid state fermentation extract under seven days of incubation with uninoculated RSS, **S7-AP:** solid state fermentation extract under seven days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S7-PF:** solid state fermentation extract under seven days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

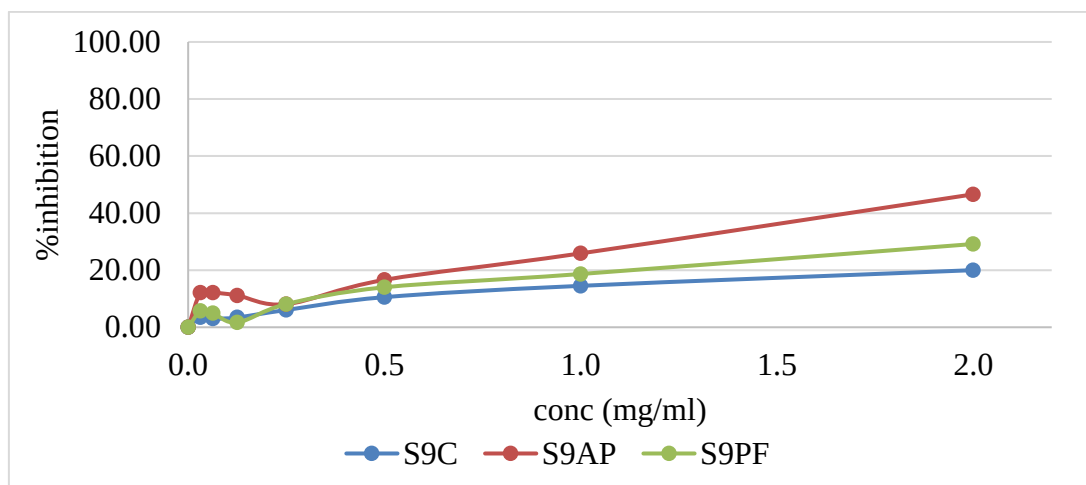


Figure 4.16 (b): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 8 hours of extraction time at Day 9 of incubation period, (n=3)

Abbrev.: **S9-C:** solid state fermentation extract under nine days of incubation with uninoculated RSS, **S9-AP:** solid state fermentation extract under nine days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S9-PF:** solid state fermentation extract under nine days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

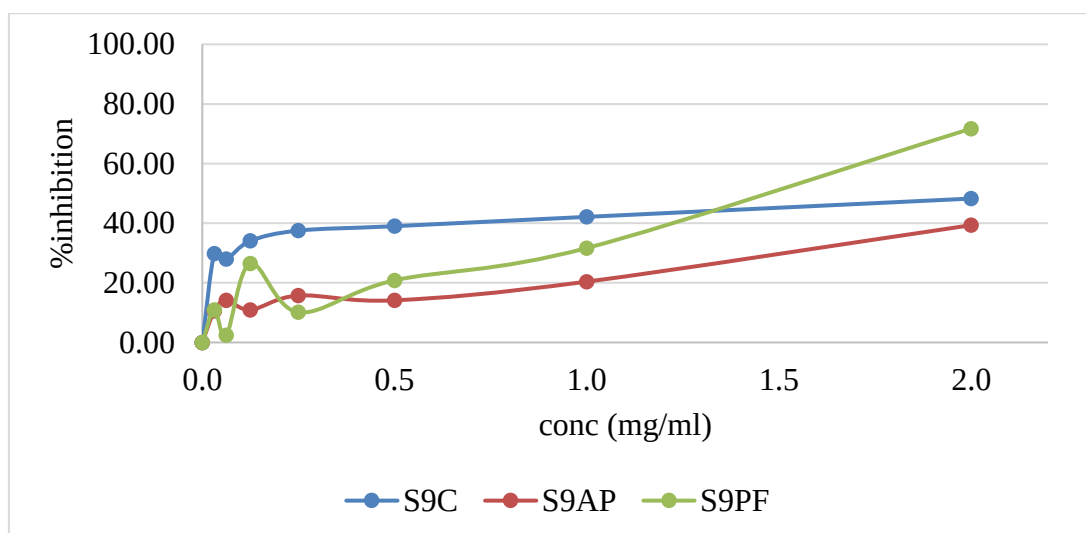


Figure 4.16 (c): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 16 hours of extraction time at Day 9 of incubation period, (n=3)

Abbrev.: **S9-C:** solid state fermentation extract under nine days of incubation with uninoculated RSS, **S9-AP:** solid state fermentation extract under nine days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S9-PF:** solid state

fermentation extract under nine days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

4.1.4.4 Antioxidant activity at Day 11

On Day 11 as shown in Figure 4.17 (a) - (c), all SSF extracts showed a decrease as the time increases. This could be assumed that the antioxidant constituents might be sensitive to heat and thus has lower activity as the time increases. As seen in Figure 4.16 (a), solid state fermentation (SSF) extract of Day 11 with fungal endophytes consortia AP (S11AP) showed decrease up to 16 hrs of extraction. Similarly, extract of Day 11 with fungal endophytes consortia PF (S11PF) also showed major decrease as time increases. However, in uninoculated fermented sago starch (S11C) extract, the scavenging activity showed similar results as time increases. This can be assumed that the sago starch does not produce high inhibition activity after 11 days by itself. With the help of microbial cultures, the enhancement of scavenging activity was observed. Although the scavenging activity of S9C is not as high as compared to other extracts, this showed that the fermented sago starch possessed inhibition activity of free radicals. This result is in concurrent with a study using fermented okra seeds whereby the antioxidant and phenolic content decreases over increment of fermentation period (Adetuyi & Ibrahim, 2014). The author suggested that it may be resulted from the action of an enzyme polyphenol oxidase affecting diffusion of phenolics in cell liquids and oxidation of diffused phenolics.

According to Azmir et al. (2013), temperature also affects the extraction efficiency of bioactive compounds. In their study, it is shown that the antioxidant activity decreases as the temperature increases. This is because at higher extraction temperature, there may be loss of volatile compounds due to their sensitivity towards temperature (Azmir et al., 2013). Other than temperature, time of extraction influences the extraction efficiency which is shown in this study. Although of a different extraction system, it is shown that increase of

extraction time promotes plant cell wall to break. But with a prolonged extraction time may causes the temperature in the system to increase and thus obliterating the heat-sensitive bioactive compounds (Bunmusik et al., 2023).

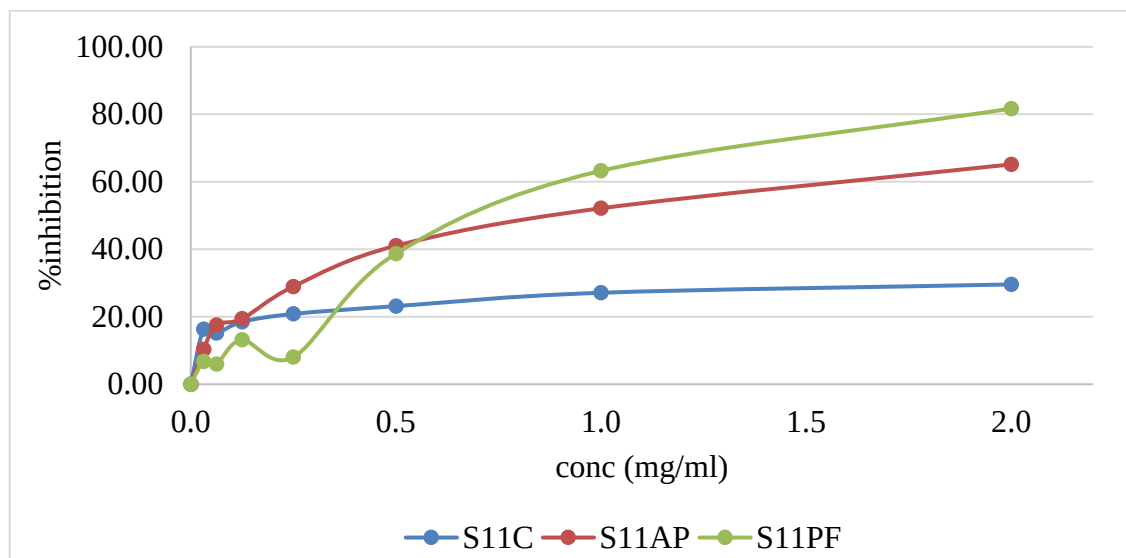


Figure 4.17 (a): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 4 hours of extraction time at Day 11 of incubation period, (n=3)

Abbrev.: **S11-C:** solid state fermentation extract under 11 days of incubation with uninoculated RSS, **S11-AP:** solid state fermentation extract under 11 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S11-PF:** solid state fermentation extract under 11 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

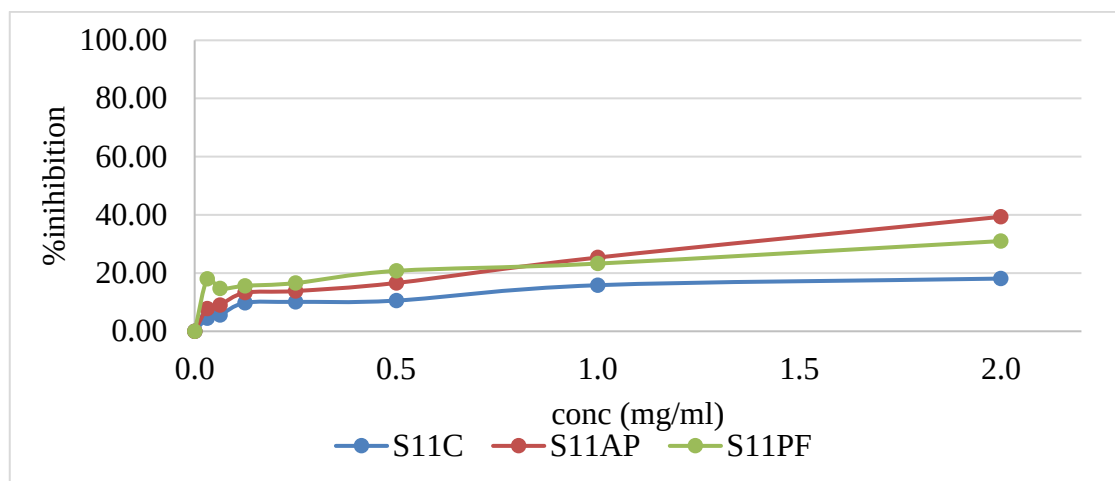


Figure 4.17 (b): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 8 hours of extraction time at Day 11 of incubation period, (n=3)

Abbrev.: **S11-C:** solid state fermentation extract under 11 days of incubation with uninoculated RSS, **S11-AP:** solid state fermentation extract under 11 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S11-PF:** solid state fermentation extract under 11 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

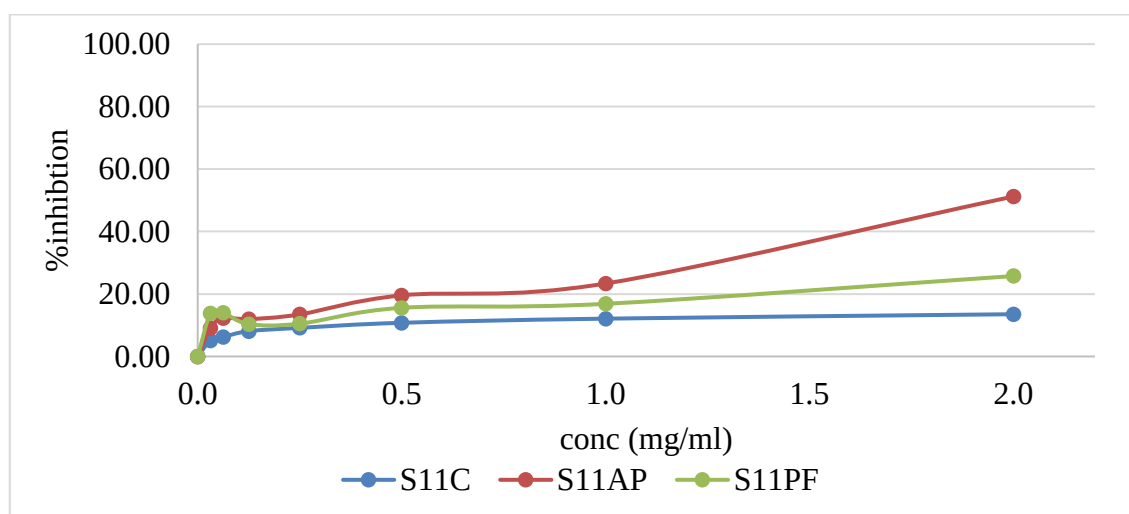


Figure 4.17 (c): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 16 hours of extraction time at Day 11 of incubation period, (n=3)

Abbrev.: **S11-C:** solid state fermentation extract under 11 days of incubation with uninoculated RSS, **S11-AP:** solid state fermentation extract under 11 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S11-PF:** solid state fermentation extract under 11 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

4.1.4.5 Antioxidant activity at Day 13

Figure 4.18 (a) - (c) showed the scavenging activity of Day 13 extracts at different extraction time under solid state fermentation. The scavenging activity of Day 13 with fungal endophytes consortia AP under solid-state decreases with longer extraction time. However, an increasing trend of scavenging activity was exhibited by Day 13 with fungal endophytes consortia PF under solid-state fermentation. This can be assumed that Day 13 with fungal endophytes consortia PF showed more compounds contributing to the antioxidant profile are more extractable as time increases. This highlighted that the antioxidant-contributing compounds can be extracted only after being exposed to a longer period of extraction.

It can be demonstrated that Day 13 of fermented sago starch without fungal endophytes (S13C) showed antioxidant activity no more than 45% in average which shows that the antioxidant capacity at Day 13 of fermentation decreases. Moreover, it can also be observed that a similar trend of scavenging activity of Day 13 without fungal endophytes was shown on both solid-state fermentation and submerged fermentation (Appendix F). This also shows that there is no discrepancy between both fermentation techniques on Day 13 of unfermented RSS "*lemantak*".

Day 13 with fungal endophytes consortia AP, on the other hand, shows a high antioxidant activity after 4 hrs of extraction and a decrease in antioxidant ability as the time increases which can be seen in Figure 4.18 (a) - (c) under solid-state fermentation. This shows that Day 13 with fungal endophytes consortia AP extract is sufficient to exhibit antioxidant ability after 4 hrs of extraction as the inhibition activity showed at 71.81% at average. As shorter time of extraction is more favourable in industrial as it also reduces the cost.

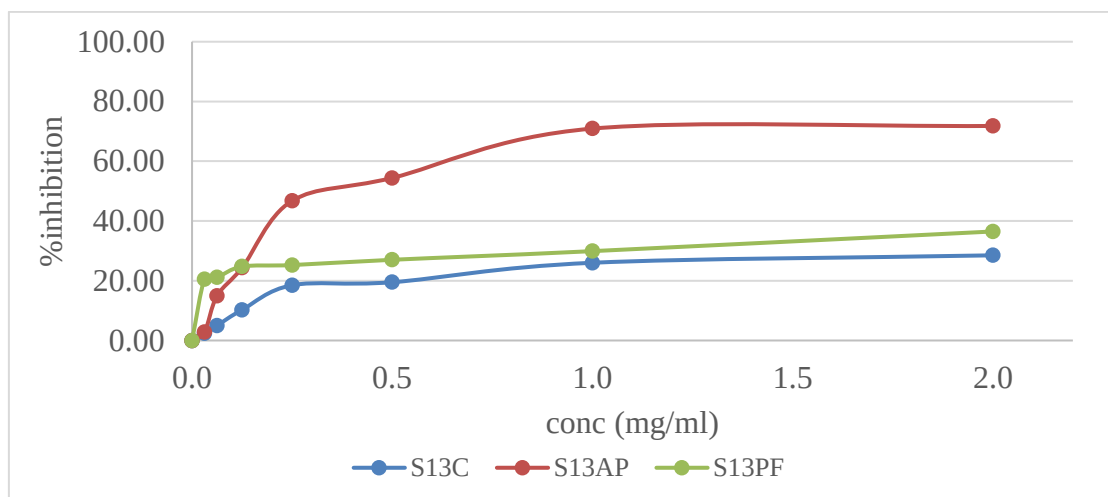


Figure 4.18 (a): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 4 hours of extraction time at Day 13 of incubation period, (n=3)

Abbrev.: **S13-C:** solid state fermentation extract under 13 days of incubation with uninoculated RSS, **S13-AP:** solid state fermentation extract under 13 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S13-PF:** solid state fermentation extract under 13 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

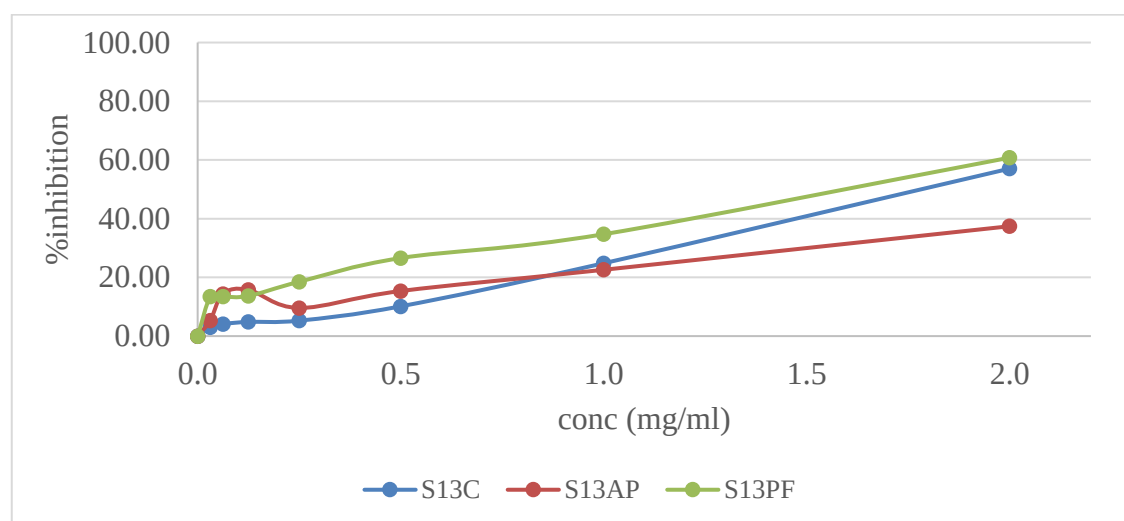


Figure 4.18 (b): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 8 hrs of extraction time at Day 13 of incubation period, (n=3)

Abbrev.: **S13-C:** solid state fermentation extract under 13 days of incubation with uninoculated RSS, **S13-AP:** solid state fermentation extract under 13 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S13-PF:** solid state fermentation extract under 13 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

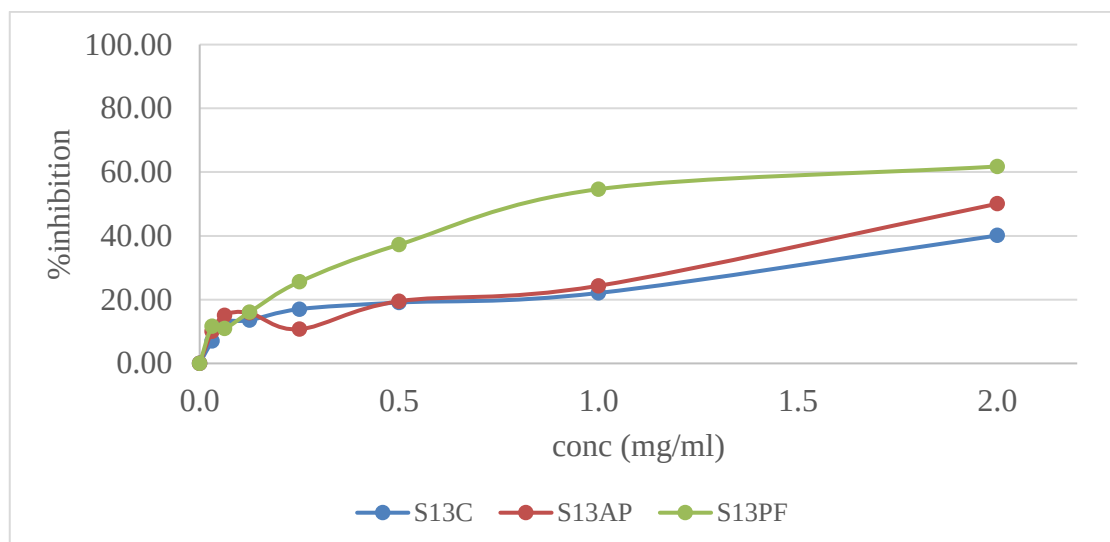


Figure 4.18 (c): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 16 hours of extraction time at Day 13 of incubation period, (n=3)

Abbrev.: **S13-C:** solid state fermentation extract under 13 days of incubation with uninoculated RSS, **S13-AP:** solid state fermentation extract under 13 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S13-PF:** solid state fermentation extract under 13 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

4.1.4.6 Antioxidant activity at Day 15

In Figure 4.19 (a) - (c) displayed the radical scavenging activity of Day 15 extracts in solid state fermentation. In general, the extracts under SSF showed a low scavenging activity after 4 hrs of extraction which then increases by double after 8 hrs. However, the ability to scavenge free radicals decreases at 16 hrs of extraction. It can be assumed that the best extraction for extracts under SSF for Day 15, is at 8 hrs of extraction.

Under solid-state fermentation, Day 15 with fungal endophytes consortia AP (S15AP) shows a low scavenging activity of an average less than 22% at 4 hrs of extraction. However, the activity increases by almost quadruple fold more at 8 hrs of extraction and decreases drastically after 16 hrs. Next, Day 15 with fungal endophytes consortia PF (S15PF) also showed a low scavenging activity at 4 hrs. This activity then was observed to increase

slightly at 8 hrs of extraction which then also decreases after 16 hrs. Nevertheless, Day 15 without fungal endophytes (S15C) showed no difference as the time of extraction increases. This could mean that there are lesser amounts of antioxidant compound-contributing that can be extracted for Day 15 fermented sago starch “*lemantak*”.

This can be assumed that 8 hrs of extraction are suitable for phenolic extraction of Day 15 under solid state fermentation.

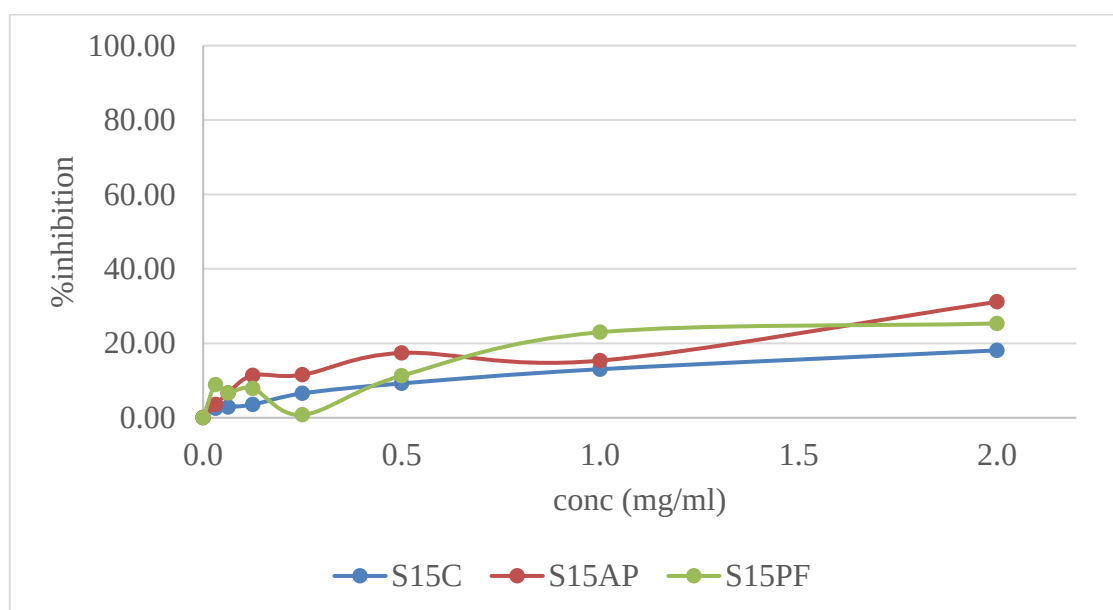


Figure 4.19 (a): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 4 hours of extraction time at Day 15 of incubation period, (n=3)

Abbrev.: **S15-C:** solid state fermentation extract under 15 days of incubation with uninoculated RSS, **S15-AP:** solid state fermentation extract under 15 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S15-PF:** solid state fermentation extract under 15 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

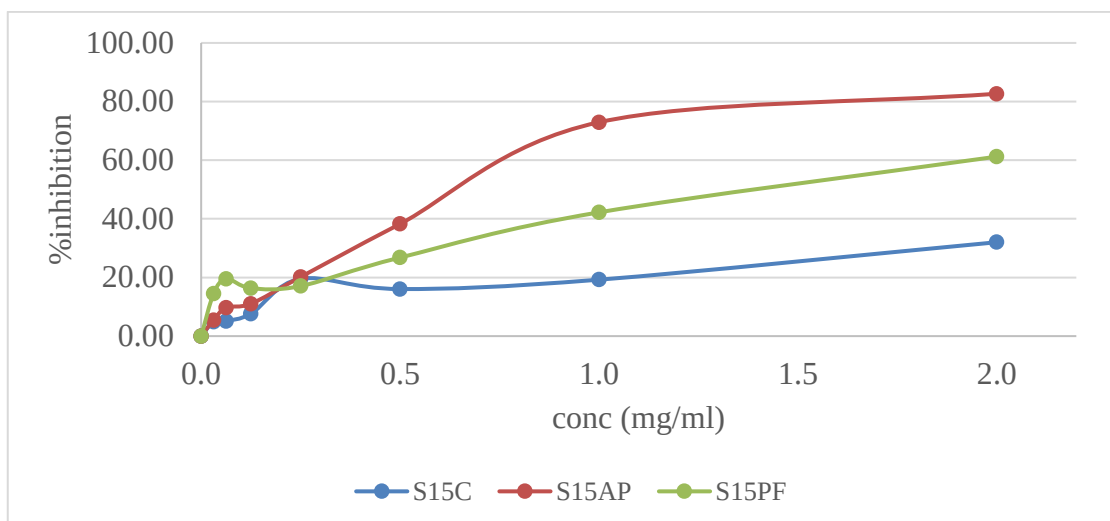


Figure 4.19 (b): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 8 hours of extraction time at Day 15 of incubation period, (n=3)

Abbrev.: **S15-C:** solid state fermentation extract under 15 days of incubation with uninoculated RSS, **S15-AP:** solid state fermentation extract under 15 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S15-PF:** solid state fermentation extract under 15 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

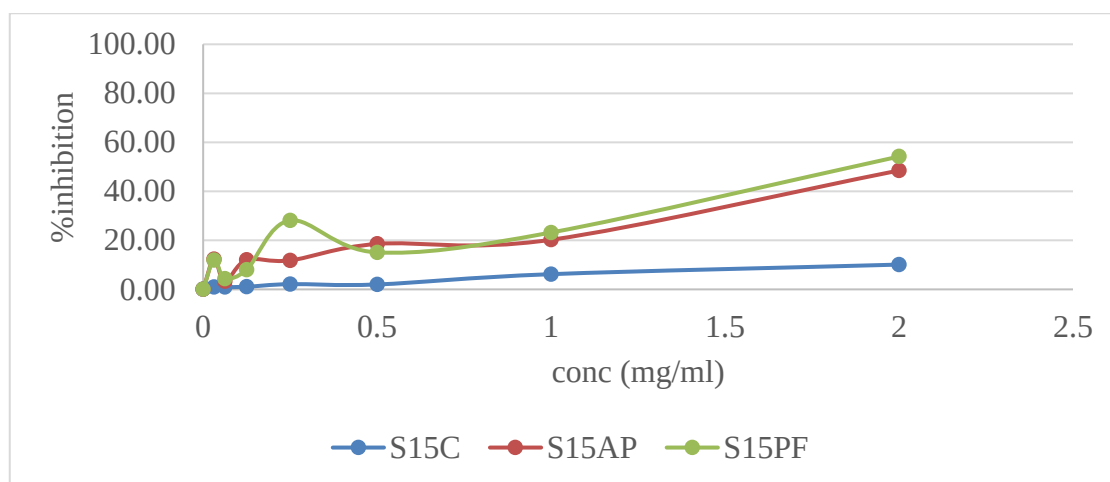


Figure 4.19 (c): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes consortia under solid state fermentation (SSF) after 16 hours of extraction time at Day 15 of incubation period, (n=3)

Abbrev.: **S15-C:** solid state fermentation extract under 15 days of incubation with uninoculated RSS, **S15-AP:** solid state fermentation extract under 15 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **S15-PF:** solid state fermentation extract under 15 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

As explained by Hammoudi et al. (2017), many studies have investigated the kinetics of polyphenol extraction from plant material. In their study, it was summarized that the process can be divided into two phases, (1): a rapid phase and (2) a slow phase. This first phase can be interpreted as the presence of solutes or compounds at surface sites of the plant material while the second stage, the slow phase shows a molecular diffusion of solutes from internal sites through the pores (Herodez et al., 2003; Spigno et al., 2007).

In this study, the two-stage extraction can be observed, the first increase of polyphenol concentration was demonstrated at 8 hrs, and some shown at 4 hrs of extraction while the slow extraction (after 16 hrs) was characterized by improved polyphenol content with the development of low extraction. It is also possible that due to over longer period, the compounds have lower solubility. It was also reported that longer extraction period may affect in increase of phenolic content however decreases the overall antioxidant activity (Alu'datt et al., 2010). Other than that, longer exposure to solvent under a certain temperature, can disrupts the nature of the compound as unstable compounds can disintegrate after long exposure of temperature.

From this parameter, it can be concluded that the most suitable extraction period is at 8 hrs long for longer fermentation period such as more than thirteen days of incubation. However, for shorter fermentation period such as seven days of incubation, 16-24 hrs are the most suitable time based on the phenolic content and DPPH radical scavenging activity.

4.1.5 Comparison between Extraction Solvents

Solvent extraction is widely used to extract active ingredients from plants. In general, extraction of bioactive compounds goes through several stages: (1) the solvent diffuses into the solid matrix; (2) the solute then dissolves in the solvents; (3) the solvent is diffused out of the solid matrix and last, (4) the collection of extracted solutes. Factors boosting the

diffusion and solubility of the above steps will assist the extraction. According to Zhang et al. (2018), the extraction efficiency can be affected by factors such as properties of the solvent, the solvent-to-solid ratio, the particle size of the raw materials, extraction temperature and extraction duration (Hernandez et al., 2009). Other than that, the yield of extraction is also affected by polarity of solvent, pH, temperature, extraction time and composition of the samples (Do et al., 2014). Hence, the screening process is important to identify the best solvent for a specific extraction procedure and thus complete the optimal antioxidant task for a certain sample.

This study uses different solvents to investigate the solvent that maximises the extraction of polyphenols. The solvent used in this study are methanol, ethanol, and ethyl acetate.

4.1.5.1 Total Phenolic Content in different solvents

As illustrated in Figure 4.20, the total phenolic content in different solvent was demonstrated and varies in different solvents. From the Figure 4.20, it is observed that methanolic fungal endophytes consortia AP and fungal endophytes consortia PF shows higher total phenolic content as compared to ethanol and ethyl acetate extract of both consortia. The trend of total phenolic content from different solvent is as follows: Methanol > Ethanol > Ethyl acetate. As the incubation period increases, the extraction of polyphenols from different solvent also varies.

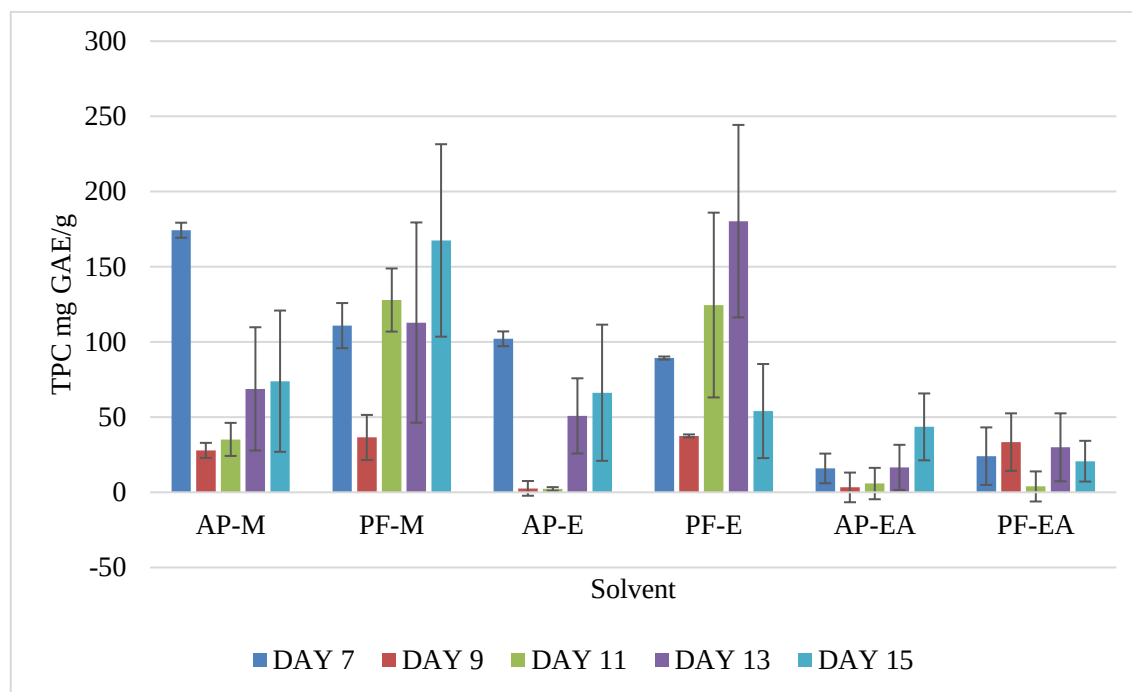


Figure 4.20: Total phenolic content of fermented sago starch by different fungal endophytes consortia in different solvents, (n=3)

Abbrev.: **AP-M**: fungal endophytes consortia AP under methanol extract, **PF-M**: fungal endophytes consortia PF under methanol extract, **AP-E**: fungal endophytes consortia AP under ethanol extract, **PF-E**: fungal endophytes consortia PF under ethanol extract, **AP-EA**: fungal endophytes consortia AP under ethyl acetate extract, **PF-EA**: fungal endophytes consortia PF under ethyl acetate extract

For fungal endophytes consortia AP under methanol extraction, Day 7 shows the highest phenolic content extracted at 174.29 ± 30.24 mg GAE/g while Day 9 showed the lowest at 27.88 ± 5.0 mg GAE/g. For ethanolic AP, Day 7 also shows the highest phenolic content at 102.11 ± 13.07 mg GAE/g. For ethyl acetate extracts, on the other hand, although the overall extracted phenolic compounds were not as high as those of methanolic and ethanolic extracts, Ethyl acetate extract of AP shows high phenolic content on Day 15 at 43.53 ± 12.33 mg GAE/g.

Next, for fungal endophytes consortia PF, methanol extraction also shows better extraction based on the total phenolic content compared to ethanolic and ethyl acetate extracts of consortia PF. Methanol extraction works the best on Day 15 (167.50 ± 15 mg

GAE/ g) while ethanol extracts the highest on Day 13 (180.32 ± 31.23 mg GAE/ g). Although total phenolic content of ethyl acetate extracts by consortia PF are incomparable with methanol and ethanol extracts, it is noted that the Day 9 of Ethyl acetate extracts (33.40 ± 10 mg GAE/ g) exhibits higher phenolic content among other incubation period of ethyl acetate extracts. Interestingly, the TPC shown by fungal endophytes consortia PF showed better under different incubation period of methanolic which is on Day 15. This could be due to that the phenols produced on Day 15 are more soluble with methanol while ethanolic PF extracted more phenol compounds on Day 13.

Statistically, the type of solvents does affects extraction of the total phenolic content significantly. However, there is no relationship between incubation period and type of solvent used significantly affects the total phenolic content. Therefore, it can be deduced that the phenolic content is affected by type of solvent used in the study.

Some of the phenolic compounds are more soluble in methanol while some are soluble in ethanol which can be seen from this study. This is also reflected on the phenolic content under ethyl acetate. It can also be caused by the complex formation of some phenolic compounds in extracts soluble in methanol, ethyl acetate, and ethanol. Furthermore, the solubility and release properties of polyphenols are affected by their structural differences. For example, a compound's structure has a large impact on its level of polarity, binding, and interaction with the sample matrix (Alara et al., 2021). Due to their structural composition, high molecular weight phenols are often insoluble in terms of solubility. This is mainly because of the extractability and solubility of different plant extracts/ residues in different solvents as all of them are not extracted in a particular solvent which can be seen from this study as shown in the phenolic content that varies among the type of solvents used.

Furthermore, the stability of phenolic compounds varies due to their heterogeneous distribution within the plant. For example, some phenolic compounds are stable, while others are oxidative, thermolabile, and volatile. Recovering polyphenols from sources is a difficult task, as most foods and plants have high levels of enzymatic activity (Bohlin, 1998). These phenolic compounds can have more phenolic groups or higher molecular weight than phenol ethyl acetate. Based on the TPC results, the best extraction solvent was methanol in both consortia. Methanol showed a significant difference in total phenolic content as compared to ethanol and ethyl acetate as methanolic extracts showed higher content as compared to the latter solvents.

To investigate the antioxidant ability of fermented samples extracted by different solvent, an antioxidant DPPH radical scavenging activity assay was carried out. Selection of the best solvent was determined by both total phenolic content (TPC) and DPPH radical scavenging activity.

4.1.5.2 Antioxidant activity under different solvents

In the obtained results as shown in Figure 4.21 - 4.23, methanolic extracts shown to be the best extraction solvent in an overview. Under methanol extraction, fungal endophytes AP shows higher scavenging activity as compared to PF with the highest activity on Day 7 as shown in Figure 4.21. The trend shows a drastic decrease on Day 9 and gradually increases from Day 11 to Day 15. It is to be highlighted here that although consortia AP showed better scavenging activity in general, consortia PF showed higher scavenging activity on Day 13 as compared to consortia AP. The amount of scavenging activity by Day 13 with fungal endophytes consortia PF is almost by 50% more than the activity shown by Day 13 with fungal endophytes consortia AP. This could be assumed that certain components contributing to its antioxidant ability are being produced on Day 13 by this consortium.

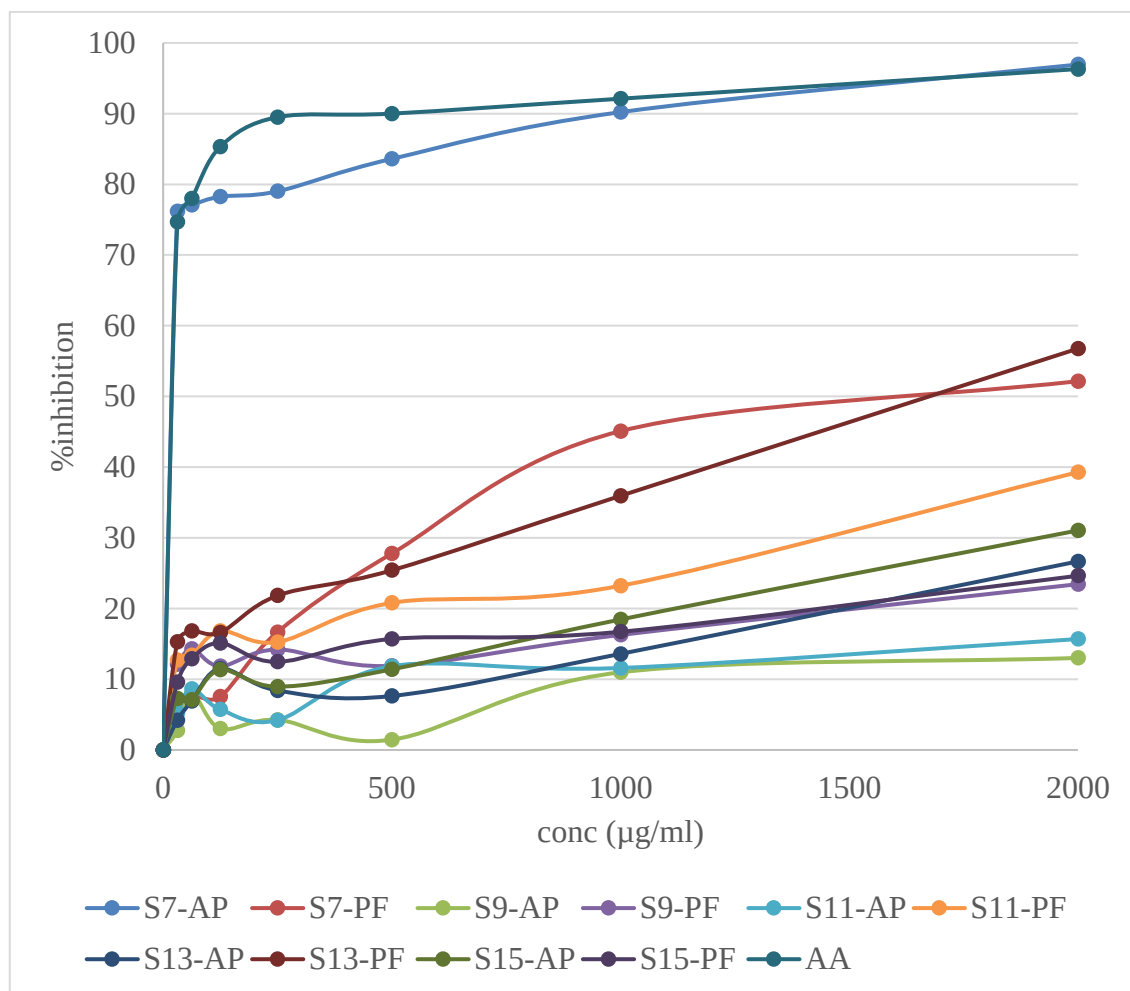


Figure 4.21: DPPH radical scavenging activity for methanolic extracts in different incubation period of both fungal endophytes' consortia AP and PF, (n=3)

Abbrev: **S7-AP:** solid state fermentation of seven days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S7-PF:** solid state fermentation of seven days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S9-AP:** solid state fermentation of nine days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S9-PF:** solid state fermentation of nine days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S11-AP:** solid state fermentation of 11 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S11-PF:** solid state fermentation of 11 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S13-AP:** solid state fermentation of 13 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S13-PF:** solid state fermentation of 13 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S15-AP:** solid state fermentation of 15 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S15-PF:** solid state fermentation of 15 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **AA:** Ascorbic acid

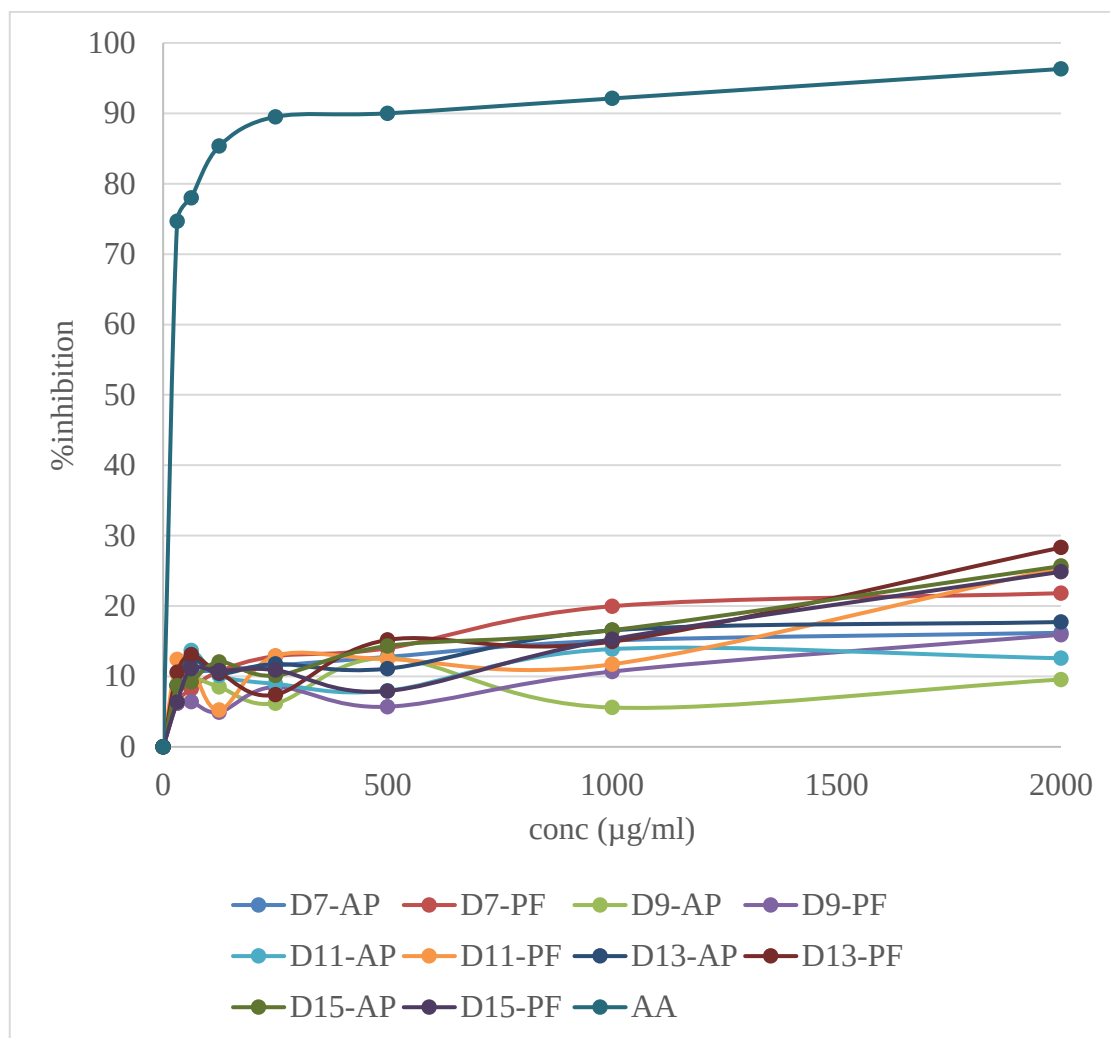


Figure 4.22: DPPH radical scavenging activity for ethanolic extracts in different incubation period of both fungal endophytes' consortia AP and PF, (n=3)

Abbrev: **S7-AP**: solid state fermentation of seven days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S7-PF**: solid state fermentation of seven days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S9-AP**: solid state fermentation of nine days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S9-PF**: solid state fermentation of nine days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S11-AP**: solid state fermentation of 11 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S11-PF**: solid state fermentation of 11 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S13-AP**: solid state fermentation of 13 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S13-PF**: solid state fermentation of 13 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S15-AP**: solid state fermentation of 15 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S15-PF**: solid state fermentation of 15 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **AA**: Ascorbic acid

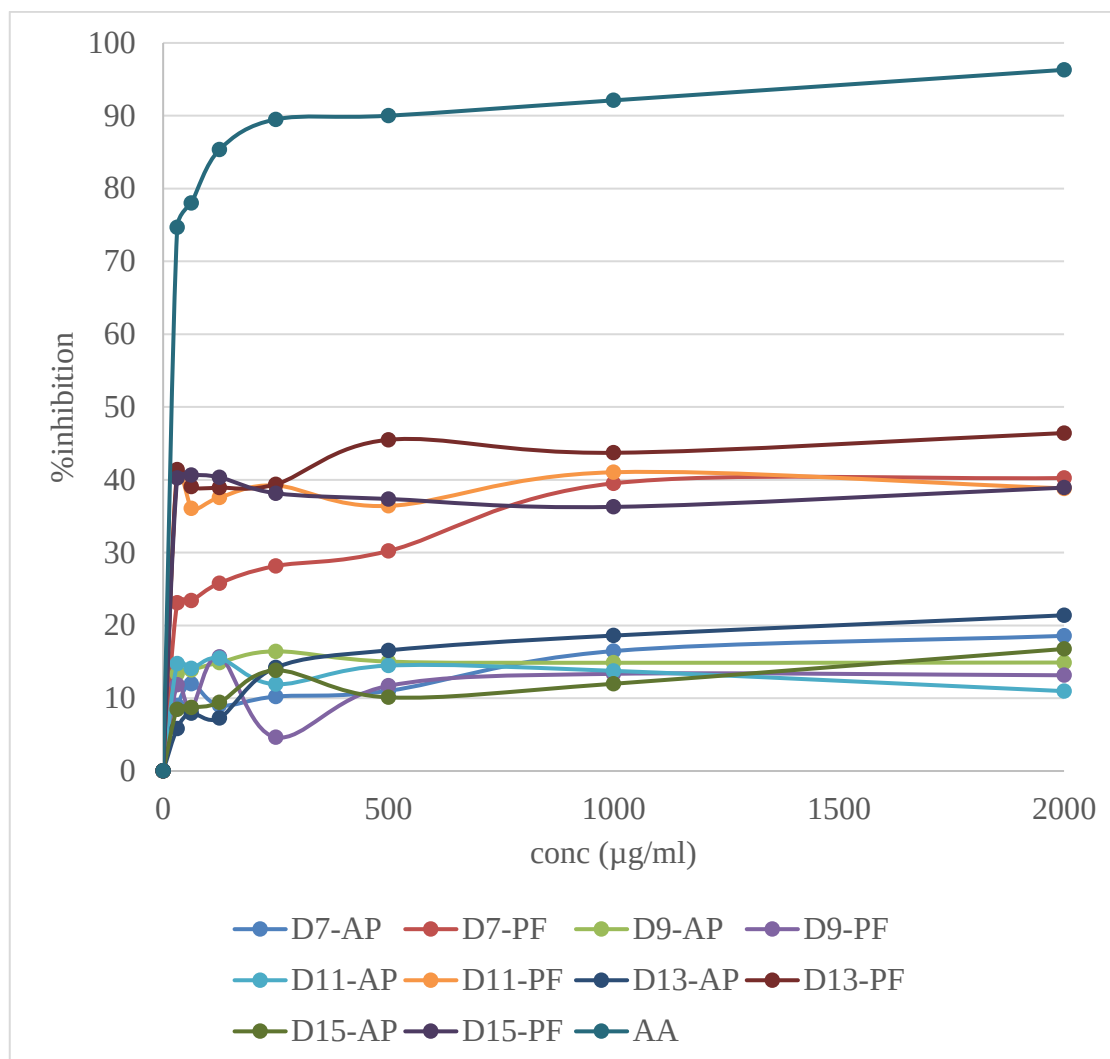


Figure 4.23: DPPH radical scavenging activity for ethyl acetate extracts in different incubation period of both fungal endophytes' consortia AP and PF, (n=3)

Abbrev: **S7-AP**: solid state fermentation of seven days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S7-PF**: solid state fermentation of seven days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S9-AP**: solid state fermentation of nine days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S9-PF**: solid state fermentation of nine days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S11-AP**: solid state fermentation of 11 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S11-PF**: solid state fermentation of 11 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S13-AP**: solid state fermentation of 13 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S13-PF**: solid state fermentation of 13 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **S15-AP**: solid state fermentation of 15 days extract with fungal endophytes consortia *A. niger* & *P. chermesinum*, **S15-PF**: solid state fermentation of 15 days extract with fungal endophytes consortia *P. chermesinum* & *F. equiseti*, **AA**: Ascorbic acid

In Figure 4.22, the scavenging activity shown by ethanolic extraction are lower than those methanolic extracts. From the Figure 4.22, it is shown that the overall activity is less than 30% of inhibition activity. The highest activity is shown by the Day 13 with fungal endophytes consortia PF extract which is at 28.31% at the highest concentration of 2000 µg/ml while the lowest activity is exhibited by Day 9 with fungal endophytes consortia AP extract. The scavenging activity of ethyl acetate extracts are shown in Figure 4.23. In an overview, the activity shown by ethyl acetate extracts showed an overall of less than 50% of scavenging activity. However, it is to note here that the DPPH scavenging activity of ethyl acetate showed better as compared to the ethanolic extracts. The extracts that showed the highest activity is also exhibited by Day 13 with fungal endophytes consortia PF at 46.42% of inhibition activity under ethyl acetate extraction while the lowest activity is being illustrated by Day 9 with fungal endophytes consortia PF. This shows that extract Day 13 with fungal endophytes consortia PF has interesting antioxidant potential under both ethanol and ethyl acetate extraction. A further test such as TLC chromatography is recommended to investigate the compounds in the extracts that gives its antioxidant ability. It is also to note that aqueous extraction had been carried out previously. However, due to the limitations of the study, extraction using aqueous was eliminated as the extracts formed a viscous texture which then impede the progress and organic solvents were then used.

To obtain desired phytochemicals from plant material, it is a common procedure to use organic solvents for extraction. This is because it is efficient, easy to use, fast, and widely applicable technique. Depending on many factors, the extraction yield varies as the most important factors depends on the type and polarity of the solvent applied (Dai & Mumper, (2010 as cited by Pintać et al., 2018). Nevertheless, there is no unique solvent or optimal extraction procedure to fully acquire the polyphenols from plant material such as the sago

starch. As of now, there are scarce information on the extraction of polyphenols from fermented sago starch “*lemantak*.”

A study stated that the different polarity of solvent had significant effect on the phenolics compound and antioxidant activity in higher content in more polar solvents (Turkmen et al. 2006 as cited in Zuhair et al., 2013). Moreover, it is proposed that phenolic compounds are often affiliated with other biomolecules such as proteins, terpenes, chlorophylls, inorganic compounds, and solvent must be found suitable for the extraction (Rajbhar et al., 2015). As starch is the highest content in sago starch “*lemantak*”, the amount of phenolic content shown in this study are due to its fermentation action by the endophytes. The sago starch “*lemantak*” also has a maximum of protein content 0.25% (Ahmad et al., 1999) which could also mean that, by its own, the sago starch produced lesser phenolic compounds. The amount of protein content also plays a part in high phenolic content as shown in the phenolic profile of chia seeds (Alcântara et al., 2019). Depending on the substrate or plant material, several others showed different efficacy of solvent. For instance, a study carried out by Musa et al. (2011) mentioned that extraction of total phenols from grape seeds (*Vitis vinifera*) has low effectiveness using acetone, methanol, and water (Zuhair et al., 2013). Mokrani and Madani (2016) showed that 60% acetone was the most efficient solvent to extract high total phenolic content from peach fruits.

The solubility of antioxidant compounds in solvent also play an important role as it is affecting the recovery of compounds at the time of extraction. Therefore, indirectly it has an impact in extraction process as it can raise the solubility of antioxidant compounds (Allothman et al., 2009). Solvent selectivity specifies towards a specific class of compounds. In a study by Pavlić et al. (2021), extractions by organic solvent shows lowest selectivity towards terpenoids however was found highest during hexane extraction. It was also stated

that polyphenols are moderately polar compounds, but the difference in structures could significantly influences their polarity (Pavlić et al., 2021).

Other than that, the polarity of the solvent also affects the ability to dissolve a selected group of antioxidant compounds. López et al. (2011) discussed that it is quite complex to compare between yield, total phenolic content, and antioxidant capacity. This is because each extract may possess different classes of phenolics of which holds diverse antioxidant potentials. This statement supports the results from the study as different solvent exhibits varying antioxidant activities and phenolic content.

From the results, it can be assumed that methanol is the best solvent while ethyl acetate is the weakest solvent to extract phenolic compounds from fermented sago starch “*lemantak*.” This is because ethyl acetate has a low recovery of polar phenolics as ethyl acetate extracts is non-polar while methanol is a polar solvent and thus is able to extract more polar phenols. Usage of ethyl acetate as a solvent was to determine the range of phenol compounds that can be extracted. This is because ethyl acetate is known to be a lesser polar solvent. A study suggested that a lesser polar solvent has better antioxidant and antimicrobial upon extracting gallotannins from *Galla chinensis* (GAC) (Tian et al., 2009). However, in this study, methanol showed as a better solvent in extracting compounds that attribute to high antioxidant activity. On the other hand, ethyl acetate extracts showed lower antioxidant activity. It can be assumed that the phenolics contained in the fermented RSS “*lemantak*” could be more soluble in a polar solvent. Other than that, extraction power of solvent depends on the nature of compound and solvent, the matrix or its substrate has considerable influence.

Preliminary extraction via aqueous extraction was also carried out but due to certain circumstances, the aqueous extraction proved to be a challenge. This is because upon eliminating water from the extract, the extracts turn out very viscous-like and it becomes

difficult even after immersing it back in pure water. For future recommendation, perhaps it would be worth to look into the aspects of aqueous extraction or a greener extraction utilising a mix of both organic solvents and water.

For industries that require high extraction efficacy of polyphenols, methanol would present the best option. However, the downside of methanol utilization in extracting phenolics is due to its toxic characteristics which brings to a serious problem for application of food and pharmaceutical applications (Rosa et al., 2017). To overcome this, many studies had investigated combinations of different solvent to reduce the toxicity of products during production. A movement towards a greener extraction has been on the move for the past few years by combining solvents and/or with water for higher efficiency of extraction. Therefore, as both methanol and ethanol have better efficiency in phenolic extraction, a further test on the antioxidant potential was carried out using Potassium Ferric Reducing Power (PFRAP).

To summarize the parameters that have been conducted, it was perceived that the suitable parameters to maximize the production of phenolic content are fermentation mode of solid-state fermentation (SSF), with fungal endophytes consortia of *A. niger* and *P. chermesinum* (AP), at a fermentation period of seven days (D7), extracted at 24 hrs under 50 °C in water bath incubator in methanol.

An additional antioxidant assay was added to support the antioxidant test carried out.

4.1.6 Potassium Ferric Reducing Power (PFRAP) Assay

From all the parameters that was carried out, it was found out that SSF extracts with a duration of 24 hrs extraction showed better phenolic content as well as its ability in scavenging free radicals using DPPH method. To further investigate its antioxidant potential, another antioxidant test was carried out. Potassium Ferric Reducing Power (PFRAP) assay

was an alternative method to investigate the antioxidant ability of the extracts. This is because PFRAP uses a different mechanism of testing the antioxidant potential as the method of PFRAP transfers electron from Fe^{3+} , reducing it to form Fe^{2+} electrons. The reducing potential of a sample is determined by its ability to convert Fe^{3+} to Fe^{2+} (Siddeeg et al., 2020). In this method, the yellow colour of the test solution changes to various shades of green and blue depending on the level of reducing agents (antioxidants) in the sample. These reducing agents reduce the Fe^{3+} /ferricyanide complex to the iron form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Mokrani & Madani, 2016). In general, as an ET-based non-radical method, the FRAP assay has little to do with the radical quenching process (HAT mechanism) occurring in lipid systems and is claimed to have poor correlation with other measures of antioxidant activity. Therefore, it is suggested that this assay can be used in combination with other methods to distinguish the dominant mechanisms of different antioxidants (Prior et al., 2015 as cited by Zhong and Shahidi, 2015).

Figure 4.24 demonstrated the current results from fermented RSS "*lemantak*" using the alternative method of PFRAP. It is to note that the FRAP assay using Benzie and Strain (1996) method was also carried out previously, however the results showed up negative using the method. Results from the FRAP assay are attached in Appendix J. This could be due to the extraction method or sample preparation that could had affected the results of FRAP. Moreover, the sample suitability with the method was also considered. During the troubleshooting of the conventional FRAP method, results remained negative and thus an alternative of FRAP assay using potassium ferricyanide as radical generator was employed.

As shown in Figure 4.24, it is observed that different extracts from different fermentation period and fungal endophytes consortia possess different reducing power

ability. It was exhibited that the highest reducing power was shown by the fungal endophytes consortia PF from Day 7 under solid state fermentation extract (PF7) at 0.912 absorbance value. Followed by extracts of AP13 > AP11 > AP7 > PF13 > AP9 > AP15 > PF11 > PF9 > PF15.

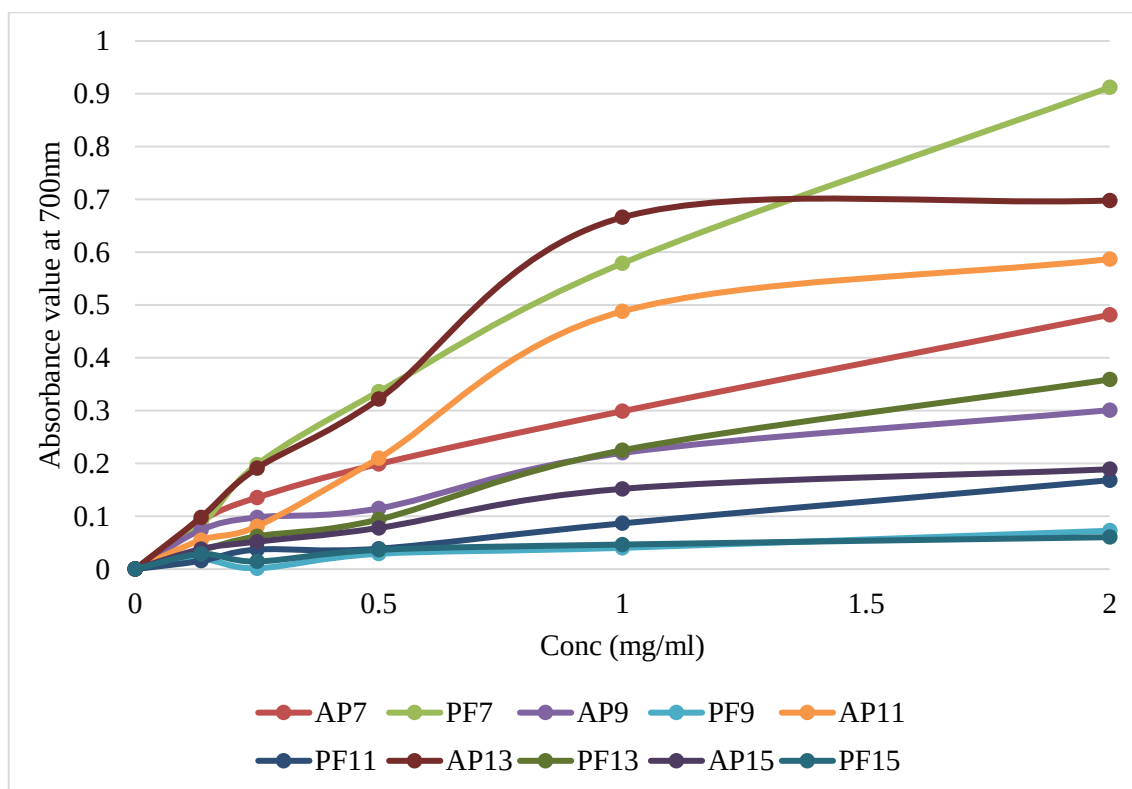


Figure 4.24: Methanolic PFRAP at different incubation period in solid state fermentation (SSF) with both fungal endophytes' consortia AP and PF, (n=3)

Abbrev.: **AP7**: Fungal endophyte consortia *A. niger* & *P. chermesinum* under seven days of fermentation extract, **PF7**: Fungal endophyte consortia *P. chermesinum* & *F. equiseti* under seven days of fermentation extract, **AP9**: Fungal endophyte consortia *A. niger* & *P. chermesinum* under nine days of fermentation extract, **PF9**: Fungal endophyte consortia *P. chermesinum* & *F. equiseti* under nine days of fermentation extract, **AP11**: Fungal endophyte consortia *A. niger* & *P. chermesinum* under 11 days of fermentation extract, **PF11**: Fungal endophyte consortia *P. chermesinum* & *F. equiseti* under 11 days of fermentation extract, **AP13**: Fungal endophyte consortia *A. niger* & *P. chermesinum* under 13 days of fermentation extract, **PF13**: Fungal endophyte consortia *P. chermesinum* & *F. equiseti* under 13 days of fermentation extract, **AP15**: Fungal endophyte consortia *A. niger* & *P. chermesinum* under 15 days of fermentation extract, **PF15**: Fungal endophyte consortia *P. chermesinum* & *F. equiseti* under 15 days of fermentation extract

For fungal endophytes consortia AP, the highest reducing power was displayed best by extract of Day 13 at 0.698 absorbance value, followed with Day 11, Day 7, Day 9, and Day 15. Contrarily, the result shown by extract of fungal endophyte consortia AP differs with its DPPH radical scavenging activity results. This may be attributed to the mechanism of the PFRAP assay. Antioxidant can counteract on two different methods either through hydrogen atom transfer (HAT) or single electron transfer (SET) (Ioannou et al., 2015). Although these two mechanisms can occur in parallel, Prior et al. (2005) suggested that factors such as the structure of antioxidant and its properties, solubility, partition coefficient and solvent system can determine its predominant mechanism and thus could determine whether the mechanism is based on HAT or SET system.

In scavenging activity, extract of fungal endophytes consortia of *A. niger* and *P. chermesinum* (AP7) of Day 7 showed the most dominant of radical scavenging activity of an almost 96.94% at 2 mg/ml but in PFRAP assay, Day 7 is the third highest of reducing power at an absorbance of 0.482. This shows that the antioxidant compound in these extracts react differently due to the predominant mechanism of the antioxidant compound containing in the extract itself. A similar pattern was also exhibited by Day 13 of fungal endophytes AP (AP13) in its DPPH radical scavenging activity (26.67%) and its potassium ferric reducing power assay (0.698 absorbance value) whereby it is more dominant in reducing activity relative to its scavenging of free radical ability. This also suggests that AP13 may contain more antioxidant that counteract radicals in SET-based mechanisms.

For fungal endophytes PF, Day 7 of fungal endophytes *P. chermesinum* and *F. equiseti* (PF7) showed best reducing power of 0.912 absorbance in 2 mg/ml in relative to extracts of other fermentation periods. The reducing power ability can be seen in a trend of PF7 as the most prevailing extract followed by PF13 > PF11 > PF15 > PF9.

A similar trend in scavenging activity was also observed by the extracts of fungal endophytes consortia PF whereby a slight discrepancy between the extract of Day 7 and Day 13. The trend also followed by the extract of Day 11 > Day 15 > Day 9. The results can be explained that the predominant mechanism occurred simultaneously to both mechanisms as stated by Ioannou et al. (2015).

Huang et al. (2005) also mentioned that in addition to the differences in mechanism from the HAT reactions which occurred normally between antioxidants and peroxy radicals, DPPH is a durable nitrogen radical bearing no resemblance to the highly reactivity and transient peroxy radical involved in lipid peroxidation. It has been proposed that due to this fact, many antioxidants that act quickly with peroxy radicals react slowly or are even immune to DPPH.

The FRAP test is a typical SET-based method measuring reduction of complex ferric ion ligand to the intensely blue ferrous complex (Fe^{2+}). The condition for this test to be conducted is to be in acidic condition of pH 3.6 to ensure iron stability. Reaction at low pH decreases the ionisation potential that drives electron transfer and increases redox potential causing as shift in the dominant reaction mechanism (Munteanu & Apetrei, 2021). However, the downside of this PFRAP analysis is that the Prussian blue tends to precipitate and stains the measurement cuvette which further disrupts inaccurate reading. Therefore, it is highly recommended that the timing of adding the ferric ions must be considered to avoid error during measurement. It is also highly recommended that this test was to be coupled with another antioxidant test of a different dominant mechanism for detection of different types of antioxidants. The use of FRAP assay has been globally used throughout much research on beverages, plant materials, lipid substances and dietary substance due to its well validated

and used to investigate various effects on human lifestyle, diet, stages of growth medical treatment, and alternative therapies (Munteanu & Apetrei, 2021).

Nevertheless, a report by Duque et al. (2011) showed that FRAP values obtained from coarse and fine unfermented sago flour is greater than those reported in berries (Pellegrini et al., 2003) which supports the results from this study whereby antioxidant capacity can be observed from raw sago starch "*lemantak*". Moreover, the sago starch mentioned by Duque et al. (2011) also showed positive results upon using sago starch as substrate for fermentation with lactic acid production showing another potential of the fermented sago starch as prebiotic food. Nevertheless, it is to highlight that the sago starch "*lemantak*" used in this study underwent fermentation process which affects the total phenolic content and antioxidant capacity of the substrate itself. This can also be seen from the results of this study whereby an increase of phenolic content and antioxidant ability in fermented samples were demonstrated.

To summarise, results shown from this parameter exhibited that fermented sago starch with fungal endophytes consortia AP showed better antioxidant activity at Day 13 while fermented sago starch with fungal endophytes consortia PF showed better activity at Day 7. However, under DPPH radical scavenging activity, fermented sago starch with consortia AP strives better in scavenging free radical due to existence of antioxidant compounds that are more dominant in SET. While fungal endophytes consortia PF of Day 7 showed better capacity in reducing Fe^{3+} to Fe^{2+} which may be due to the presence of active compounds that contribute to the reducing power which was exhibited in this parameter. Interesting results also showed in submerged fermentation extracts which are exhibited in Appendix J. This study also screened and identifies compounds presented in the extracts

from the study through gas chromatography mass spectrometry method and will be further discussed in the next section.

4.1.7 Gas Chromatography-Mass Spectrometry

In this study, screening of constituents through gas chromatography method was conducted. To note, there is no specific compounds that is being targeted throughout this study as this study focuses on the optimization of phenolic compounds production by several parameters particularly on the production process. This is also the first report on identification of compounds produced through RSS "*lemantak*" fermentation. Although there are other methods in detecting bioactive compounds such as liquid chromatography-mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC), this study utilises gas spectrometry-mass spectrometry (GC-MS) to detect available bioactive compounds as a method to screen without targeting any specific bioactive compounds. Unlike LC-MS which uses a liquid phase column and ionization to separate based on their mass-to-ratio (m/z), GC-MS uses heating via gas carrier (mobile phase) and further categorizes the compounds into its m/z . Moreover, GC-MS provides better chromatographic separation as compared to LC-MS (Yuan, 2004). In terms of sample form, GC-MS required samples to remain in gaseous form and is more suitable for volatile or semi-volatile substances that are readily to vaporize. On the other hand, HPLC can only analyse in liquid form but could detect larger, less table and non-volatile substances as no exposure to high temperature during detection (GenTech Scientific, 2021).

The bioactive compounds presented in methanol extracts were identified using Gas Chromatography Mass Spectrometry (GC-MS) analysis of the fermented RSS "*lemantak*" with fungal endophytes consortia. Through this analysis, the name, area (%), retention time, molecular formula and biological properties of the detected bioactive compounds were

perceived. By comparing the mass spectra data from the National Institute of Standards and Technology (NIST 2017, USA) database, the bioactive compounds presented were identified. In this study, A detailed record of bioactive compounds presented in each extract and the selected GC-MS chromatogram were exhibited in Appendix K.

Based on the results of evaluation obtained, there are in total of 290 of bioactive compounds where 144 compounds were found in solid state fermentation extracts while 146 compounds were discovered from submerged fermentation extracts across different fungal consortia. In this study, it was found out that the compounds detected differs from one another depending on each parameter such as fermentation type, incubation period, endophytic fungi, and solvent that were used in the process. This is shown in Table 4.1 - 4.2 whereby different compounds were extracted from the fermented RSS "*lemantak*" in different fermentation technique.

Besides that, it is also observed that although similar compounds were being produced in the extracts by different fungi, the amount of the compound detected also differs from one another. In total, there are at least 192 bioactive compounds found under fermented RSS with single fungal isolates and at most of 195 constituents under mixed consortia in both fermentation method. Table 4.1 and Table 4.2 shows the highest compound found in methanolic extracts under different mode of fermentation in different consortia on Day 7. Further analysis and discussions on the identified bioactive compounds and their biological properties were focused on the extract D7AP and D13PF based on their antioxidant capacity in DPPH radical scavenging assay and reducing power capacity. The chromatogram of extract D7AP and D13PF are exhibited in Figure 4.25 and 4.26. The biological properties of

the three highest area% of each extract from Day 7 of fermentation were also discussed further.

Table 4.1: The GC-MS identified compounds from SSF mode of different fungal consortia. The table shows the three highest area% of compounds found in different extracts.

Sample	Compounds	Area%
S7-A	Phenol, 2,4,6-tris(1-phenylethyl)-	23.13
	1H-Benzotriazole	12.92
	1-Docosene	10.94
S7-P	1H-Benzotriazole	10.73
	1-Docosene	10.49
	n-Hexadecanoic acid	9.36
S7-F	Tetrapentacontane	15.46
	1H-Benzotriazole	11.3
	Octacosanol	9.41
S7-C	9-Octadecenoic acid, methyl ester, (E)-	17.58
	Hexadecanoic acid, methyl ester	16.99
	1-Docosene	7.36
S7-AP	Disiloxane, 1,3-bis(1,1-dimethylethyl)-1,1,3,3-tetramethyl	14.23
	1-Eicosanol	10.95
	1H-Benzotriazole	9.19
S7-AF	4H-Pyran-4-one, 5-hydroxy-2-(hydroxymethyl)-	17.46
	1H-Benzotriazole	10.19
	Octacosanol	7.67
S7-PF	n-Hexadecanoic acid	11.27
	1-Eicosanol	9.23
	N-(tert-butyl dimethylsilyl) trifluoroacetamide	7.95

Table 4.1 continued

S7-AFP	Disiloxane, 1,3-bis(1,1-dimethylethyl)- 1,1,3,3-tetramethyl	14.6
	Phenol, 2,4-bis(1-phenylethyl)-	7.14
	Octacosanol	6.39

Footnotes: **A:** *A. niger*, **P:** *P. chermesinum*, **F:** *F. equiseti*, **AP:** *A. niger* & *P. chermesinum*, **AF:** *A. niger* & *F. equiseti*, **PF:** *P. chermesinum* & *F. equiseti*, **AFP:** *A. niger*, *F. equiseti* & *P. chermesinum*, **USL:** Uninoculated fermented sago “*lemantak*”

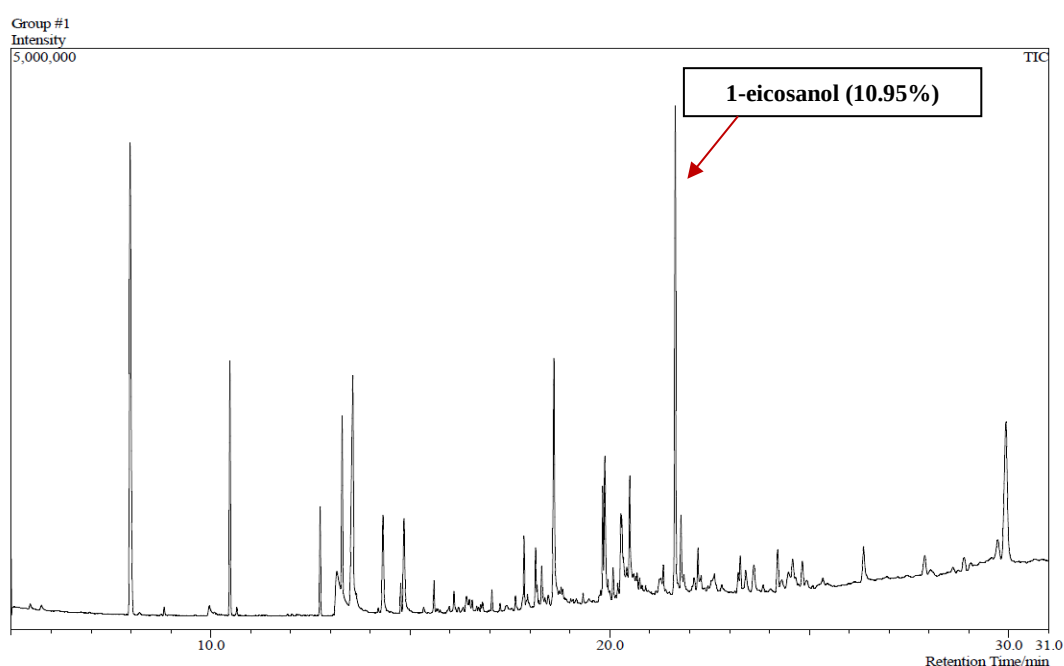


Figure 4.25: Chromatogram of Day 7 of fungal endophytes consortia *A. niger* & *P. chermesinum* (AP) under solid state fermentation

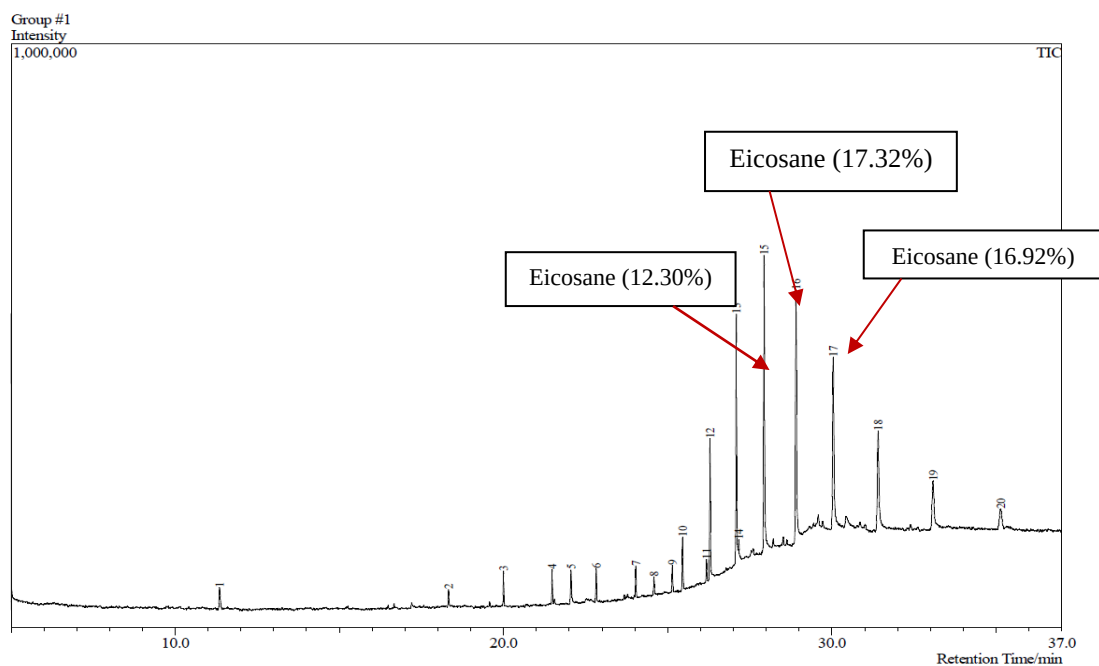


Figure 4.26: Chromatogram of Day 13 of fungal endophytes consortia *P. chermesinum* & *F. equiseti* (PF) under solid state fermentation

In total, there are 41 compounds detected in D7-AP methanol extract under solid state fermentation while it was found out that D13-PF only contained 10 bioactive compounds in methanol extraction under solid state fermentation. Appendix J showed the biological activities of the identified compounds in both D7-AP and D13-PF. The extract of D7-AP contained highest area% of bioactive compounds of 1-Eicosanol (10.95%), followed by: 1H-Benzotriazole (9.19%), Octabenzene (7.71%) and n-hexadecanoic acid (6.63%). It is also to note that, the total area% detected by compound Disiloxane, 1,3-bis(1,1-dimethylethyl)-1,1,3,3-tetramethyl cumulatively at 14.23%. In methanolic D13-PF extract, it was found out that the bioactive compound Eicosane exhibits the highest cumulatively at 46.54%, followed by dotriacontane (27.88%), Heneicosane (10.15%) and Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15 (6.51%).

Compounds screened in both extracts exhibited significant biological activity, as reported in previous references. The compounds exhibited have been reported to display

antioxidant activity. These bioactive compounds have been found to exhibit a wide range of biological properties, as reported in previous references. It can be observed that most of compounds may contribute to the antioxidant capacity of both extracts. It is observed that compounds with highest area (%) in extract D7-AP such as 1-Eicosanol (10.95%), Octabenzene (7.81%) and n-Hexadecanoic acid (6.63%) are infamous compounds in attributed to antioxidant activity. Not only that, but these compounds also have potential as antimicrobial.

On the other hand, compounds with highest area (%) from D13-PF extracts has shown its ability in antioxidant. Compounds such as eicosane (46.54%) cumulatively, dotriacontane (27.88%) and heneicosane (10.15%) also was proven to possess ability in antimicrobial activity against *Streptococcus pneumoniae* and *Aspergillus fumigatus* (Vanitha et al., 2020).

Therefore, it can be inferred that the bioactive compounds identified in methanolic extract from D7-AP and D13-PF were most likely to play the most significant role attributing to the potent antioxidant ability in respective of each assays conducted.

Table 4.1 showed different compounds extracted from fermented sago starch with different fungal endophytes and its dual cultures. From Table 4.1, it is seen that several compounds are detected higher in different extracts such as Phenol, 2,4,6-tris(1-phenylethyl)-, detected the highest in S7-A, 1H-Benzotriazole showed the highest in S7-P, Tetrapentacontane exhibits the highest in S7-F and other compounds such as Disiloxane, 1,3-bis(1,1-dimethylethyl)-1,1,3,3-tetramethyl, 4H-Pyran-4-one, 5-hydroxy-2-(hydroxymethyl)-, n-Hexadecanoic acid which was detected the highest in other fungal consortia shown in Table 4.1.

Recently, a study reported that the compound detected from *Ulva reticulata*, phenol, 2,4,6-tris(1-phenylethyl)- is a compound known with antibacterial and antioxidant properties (Sivaranjani et al., 2021). This compound is detected in S7-A and S7-AFP which showed at 23.13% and 7.14%, respectively. In S7-A, the compound was seen to be higher than in extract by consortia AFP. This shows that this compound might be generated by *Aspergillus niger*.

According to Ferdosi et al. (2022), the flower *Cassia fistula L.* also exhibits 1-Docosene which shows antibacterial activity. The compound, 1-docosene belongs to unsaturated fatty acid group which serves as oxygen receptors and are strong radical scavengers. It is also reported that this compound also shows anticarcinoma activity in other studies (More et al., 2022).

Disiloxane, 1,3-bis(1,1-dimethylethyl)-1,1,3,3-tetramethyl is an organosilicon compound, which is also found in GC-MS results of ethanolic prickly pear pulp (El-Beltagi et al., 2019). However, there are no established bioactivity on this compound.

4H-Pyran-4-one, 5-hydroxy-2-(hydroxymethyl)- found in S7-AP is also known as the kojic acid. This compound is known have antioxidant activity (Nuraini et al., 2019). Nuraini et al. (2019) has found this compound produced by an endophytic fungus from an endemic plant in South Kalimantan. Results from their study showed that this compound was produced by the fungal genera *Aspergillus*. Therefore, it can also be suggested that the compound found in this study is contributed by the *Aspergillus niger* as this compound was also found in the extract produced by S7-A (Table 4.2).

n-hexadecanoic acid is compound shown to possess anti-inflammatory, antioxidant, and hypocholesterolemic activity (Grace & Kolawole, 2020). It is observed that this

compound was detected the highest in extract S7-PF. In its monoculture, *F. equiseti* does not produced this compound with fermented RSS “*lemantak*”. However, *P. chermesinum* produced this compound but detects in a smaller amount (Table 4.1). This shows that with consortium, the amount of n-hexadecanoic acid can be increased which also explains the DPPH results whereby fungal endophytes consortia PF does exhibits higher antioxidant ability as compared to the fermented RSS with monocultures.

According to Dr Duke’s phytochemical and ethnobotanical databases, the compound octacosanol, is another fatty alcohol class substance which showed antiparkinsonian, antiviral (Khan et al., 1999), and hypocholesterolemic activity (Pedersen, 1987). This compound is also found in other plants such as *Ophiopogon intermedius*, *Prosopis glandulosa*, and other organisms (LOTUS).

On the other hand, 1-Eicosanol is a natural product that is available in *Lonicera japonica* and *Artemisia baldshuanica*. This compound is also known as the arachidyl alcohol possessing bioactivities such as cancer-preventive according to Dr. Duke’s phytochemical and ethnobotanical database (n.d). Another study suggested that this compound has antibacterial effect (Chatterjee et al., (2018) as cited by Ebin (2021)). Another study also showed that 1-eicosanol found from *Streptomyces spp.* isolated from soil sample showed that it has significant larvicidal activity against mosquitos’ larvae from *Culex* and *Aedes* species (Nandhini, 2020).

Other than that, tetrapentacontane is an alkane class compound is known to have anti-inflammatory and antioxidant properties (Fairuz et al., 2019). This compound was also found in hexane leaf extract of *Vernonia amygdalia* which is also known in possess several other therapeutic agents.

Unfortunately, there is no available bioactivity in the compound 1H-Benzotriazole.

Table 4.2: The GC-MS identified compounds. Highlighted area% shows the highest area% of compounds found in different extracts.

Sample	Compounds	Area%
SM7-A	Oleic acid	29.61
	n-Hexadecanoic acid	11.01
	Phenol, 2, 4-bis(1-phenylethyl)-	8.56
SM7-P	Oleic acid	32.4
	n-Hexadecanoic acid	14.38
	Octadecanoic acid	5.63
SM7-F	Oleic acid	18.42
	Phenol,2,4-bis(1-phenylethyl)-	7.69
	n-Hexadecanoic acid	5.41
SM7-C	Oleic acid	36.03
	n-Hexadecanoic acid	14.21
	octadecanoic acid	7.31
SM7-AP	Oleic Acid	31.57
	n-Hexadecanoic acid	11.8
	Octadecanoic acid	5.54
SM7-AF	Oleic Acid	34.44
	n-Hexadecanoic acid	14.07
	Octadecanoic acid	6.13

Table 4.2 continued

SM7-PF	9,12-Octadecadienoic acid (Z, Z)- 9-	12.06
	Octadecenoic acid (Z)-, methyl ester	8.98
	Methyl 10-trans,12-cis-octadecadienoate	8.04
Oleic Acid		26.74
SM7AFP	n-Hexadecanoic acid	12.19
	Octadecanoic acid	7.93

Footnotes: A: *A. niger*, P: *P. chermesinum*, F: *F. equiseti*, AP: *A. niger* & *P. chermesinum*, AF: *A. niger* & *F. equiseti*, PF: *P. chermesinum* & *F. equiseti*, AFP: *A. niger*, *F. equiseti* & *P. chermesinum*, USL: Uninoculated fermented sago “*lemantak*”

The GC-MS results from the submerged fermented extracts are found to be more than 30 compounds per consortia extracts.

From the results, most compounds detected are from fatty acid class. As such, Table 4.3 showed that the dominating compound found is oleic acid from all extracts apart from extract SM7-PF. The extract SM7-PF, however, showed to be producing compound 9,12-Octadecadienoic acid (Z, Z)- (12.06%), Octadecenoic acid (Z)-, methyl ester (8.98%) and Methyl 10-trans,12-cis-octadecadienoate (8.04%). All three compounds belonged to fatty acid class, specifically, these compounds are derivatives of linoleic acid. Linoleic acid is an essential fatty acid compound that humans and other animals requires for various biological processes upon ingesting.

Other compounds that were detected were oleic acid, hexadecenoic acid, and n-hexadecanoic acid. Most extracts have these three compounds. For extract SM7-A and SM7-F, another compound, Phenol,2,4-bis(1-phenylethyl)- was detected in these two extracts with an area% of 8.56% and 7.69% respectively.

Oleic acid, hexadecenoic acid and n-hexadecanoic acid belonged to fatty acid class. Research stated that oleic acid possessed anti-inflammatory effect by influencing arachidonic acid metabolism (Carrillo Pérez et al., 2012a). Several other studies by Martínez et al. (2005), Girao et al. (1986), and Carrillo Pérez et al. (2012b) showed that this compound also can cause inhibition of cell proliferation on different cell tumour lines. Hexadecenoic acid possesses antioxidant and antimicrobial effect as stated in a study by Huda et al. (2015), and Abubakar and Majinda (2016) as cited by Sikandar et al. (2020). Ravi and Krishnan (2017) also mentioned that n-hexadecanoic acid shows characteristics of cytotoxicity against HCT-611 cell lines in MTT assay.

Therefore, it can be suggested that SMF RSS extracts showed useful constituents with anticancer and hypercholesterolaemic activities.

To note, several other compounds such as 1-Nonadecene, and several others was also detected that possesses notable bioactivities.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

It was found out that the fermentation process helps in increasing phenolic content in raw sago starch. It is also shown that solid-state fermentation helps to enhance more production of phenolic content and antioxidant capacity of the sago “*lemantak*” compared to submerged fermentation. This shows that fermentation method under less moisture condition is preferable to produce phenolic content with high antioxidant activity with the selected endophytes. Moreover, several parameters were also optimized to establish a benchmark to enhance the phenolic content and its antioxidant. The method carried out for the parameters were investigated in one factor at a time (OFAAT) method whereby one factor was investigated at one time through trials and errors. Parameters that were investigated in this study were the fermentation incubation period, effect of fungal consortia, the effect of solvent extraction and the effect of extraction time. Although single factor experiments are time-consuming and lack of data on factor interactions, they provide fundamental data on conditions or ranges for factors of extraction that has a significant statistical.

Parameters that were manipulated are in two separate phases: the fermentation phase and the extraction phase. From the results that were discussed, it is observed that the strains or fungal consortia effects the production of phenolic and antioxidant activity in submerged fermentation but under solid state fermentation, the strains of endophytes do not influence the fermentation type, and it was concluded that the consortia of *Aspergillus niger* and *Penicillium chermesinum* were the best strains to be use in fermentation with raw sago starch

“*lemantak*”. It was also discovered that during the seventh day of fermentation was the best for this fungal consortium in enhancing both phenolic and antioxidant activity of fermented RSS “*lemantak*”. This shows that the optimum maturity of the fungi also affects the production of beneficial phenolic compounds. The extract of consortia AP performed best at seventh day of fermentation which then further extracted in methanol solvent after 24 hrs of extraction time. This is most likely due to the matrix formed by combination of fermented sago starch and the fungal endophytes which requires longer time to release the phenolic compounds.

On the other hand, fermented sago starch by consortium *Penicillium chermesinum* and *Fusarium equiseti* showed the best on Day 13 which consumes a longer fermentation period as compared to fermented sago starch by consortia AP. However, it is to be highlighted that the antioxidant activity of PF is higher than that of AP at this point of fermentation. This was due to the compound Eicosane that was discovered based on the GC-MS results. Therefore, it is known that Day 13 with fungal endophytes consortia PF extract has high potential in antioxidant capacity. It is also determined that methanolic extracts of both consortia also showed the best solvent extractor as compared to ethanol and ethyl acetate which is less polar than methanol. It was also further determined that the best extraction time was in the range of 4-8 hrs in water bath extraction for longer fermentation period while 16-24 hrs are recommended for extracts of longer fermentation period.

From the GC-MS results that was conducted, most of the dominant compounds are shown contributed to the antioxidant ability. The present findings also found out that there are antimicrobials compound showed. Hence, there is a need to isolate and purify the phytochemicals or molecules from the crude extracts that possessed significant antimicrobial

activities, including AP and PF of submerged fermentation to serve as potential antimicrobial against the microbes causing diseases.

5.2 Recommendations

The availability of current study consumes a long time therefore it is highly recommended to implement new techniques using response surface methodology (RSM) as it will ease and shorten the period of several processes especially in optimizing the parameters for maximum phenolic production. As extracts from fermented sago starch by consortia AP and PF showed high antioxidant activity, it is also recommended to be further characterized in the downstream processes and to be purified to obtain the compounds responsible for the antioxidant. Moreover, another test should also be screened for its toxicity through *in vivo* method on live cells and animal modelling before developing if it were to be implemented as a nutraceutical product for human consumption and usage. It is also recommended that spray drying and freeze drying to be implemented on the fermented sago starch extracts to observe the stability conditions for longer shelf life.

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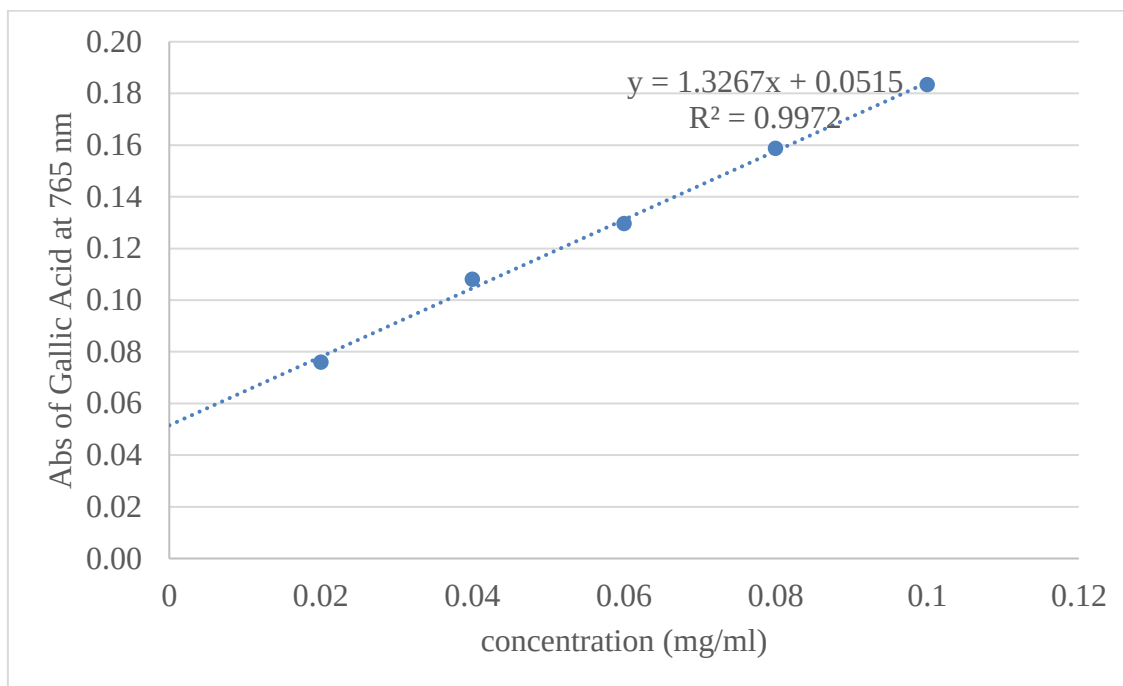
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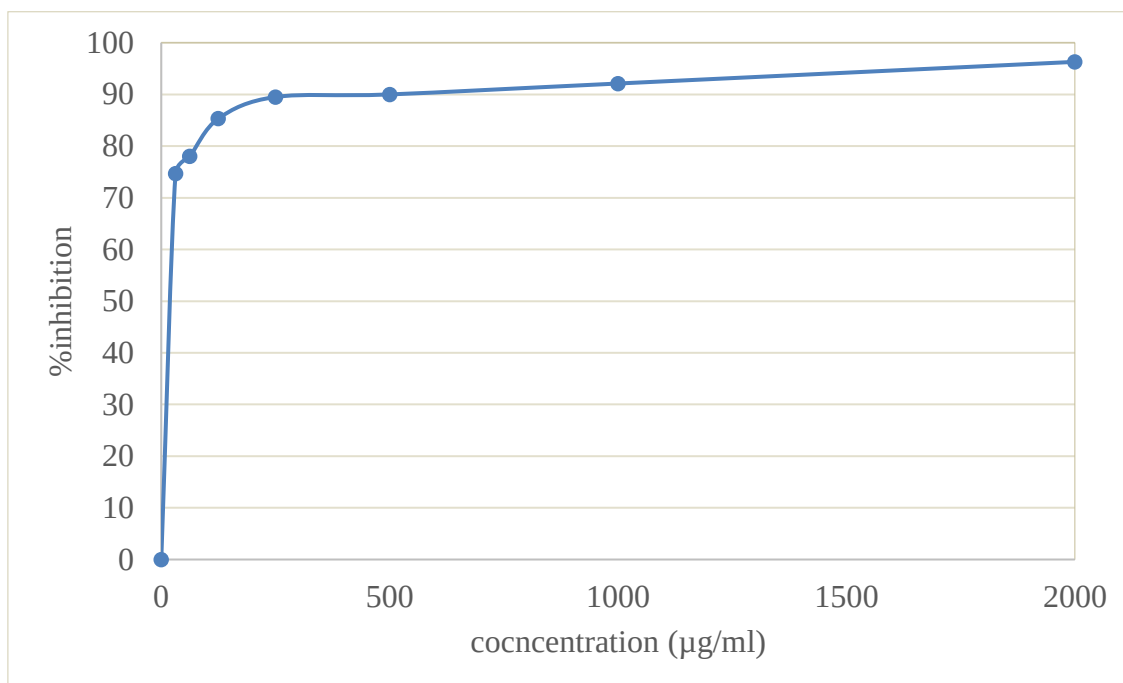
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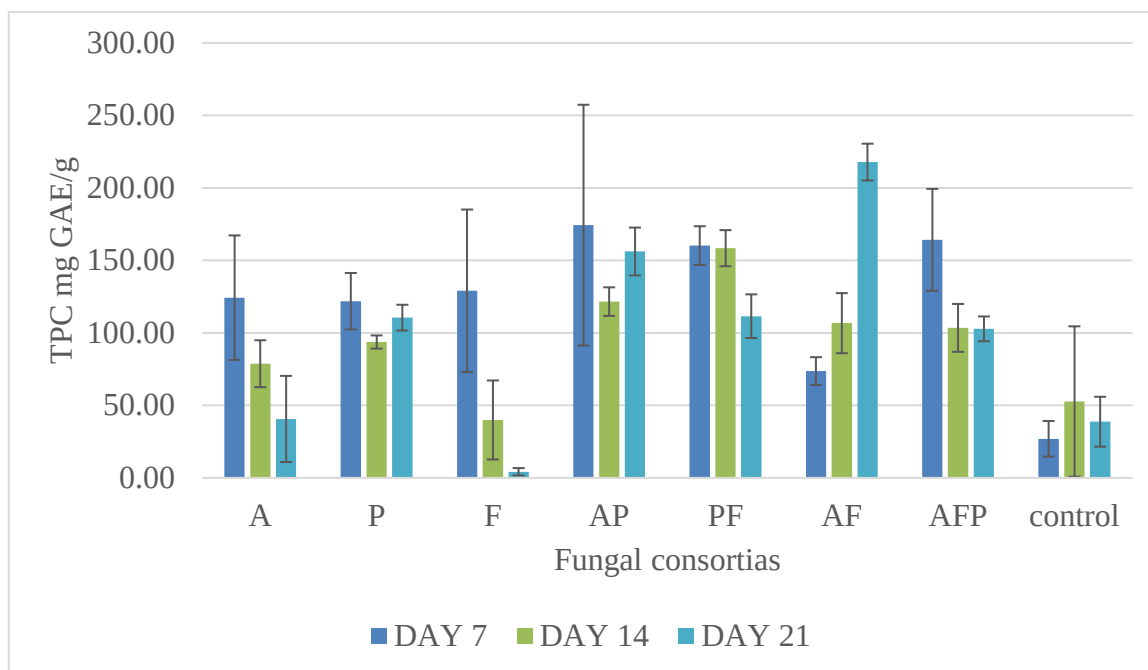
APPENDICES



Appendix A. Standard curve for Gallic acid

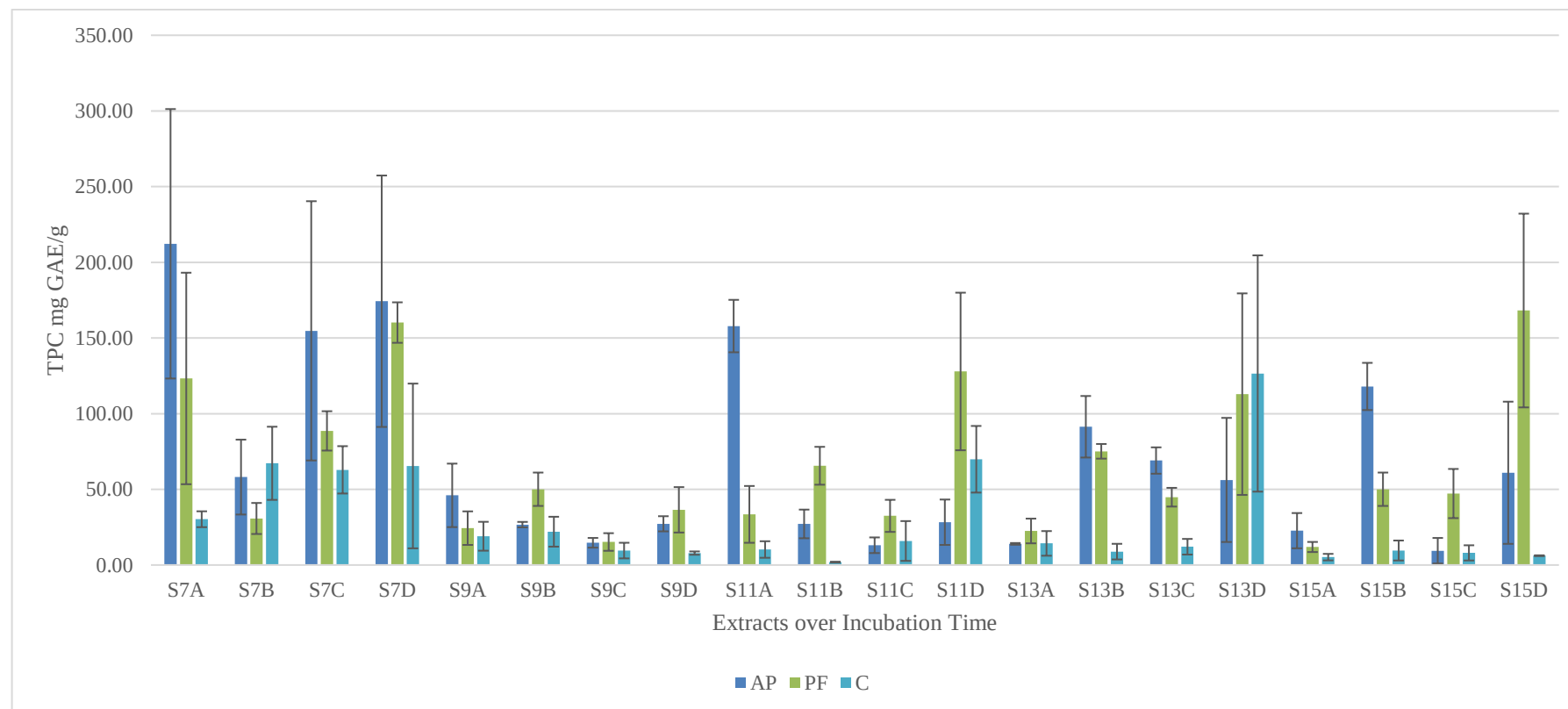


Appendix B. Standard curve for Ascorbic acid



Appendix C. Total phenolic content of different fungal endophytes consortia over 21 fermentation days in solid state fermentation

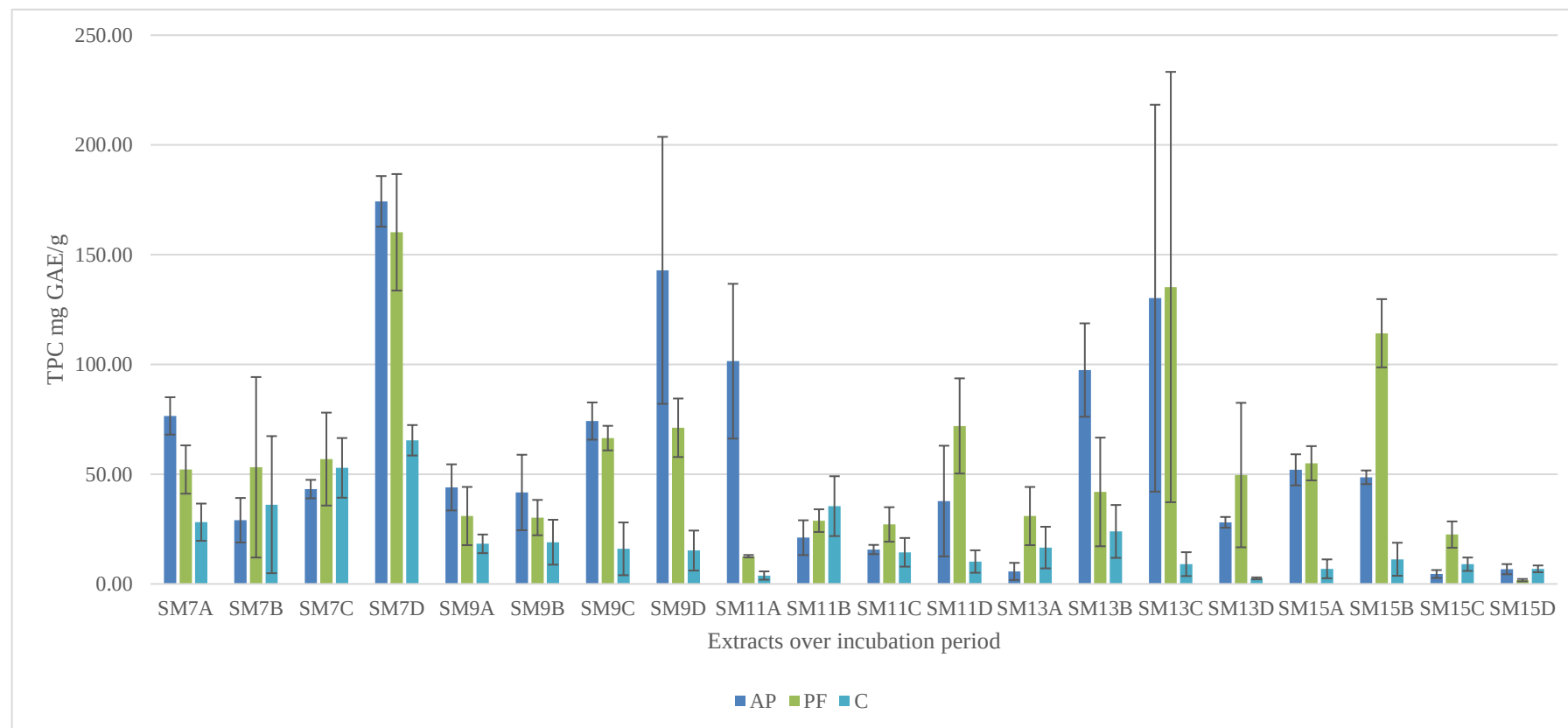
A: *A. niger*, **P:** *P. chermesinum*, **F:** *F. equiseti*, **AP:** *A. niger* & *P. chermesinum*, **AF:** *A. niger* & *F. equiseti*, **PF:** *P. chermesinum* & *F. equiseti*, **AFP:** *A. niger*, *F. equiseti* & *P. chermesinum*



Appendix D: Total phenolic content of fungal endophytes extracts consortia AP, fungal endophytes consortia PF and controlled extracts without fungal inoculum over 4, 8, 16 and 24 hrs of extraction period under solid state fermentation.

Abbrev.: **AP**: endophyte consortia *A. niger* & *P. chermesinum*, **PF**: endophytes consortia *P. chermesinum* & *F. equiseti*, **C**: uninoculated fermented RSS.

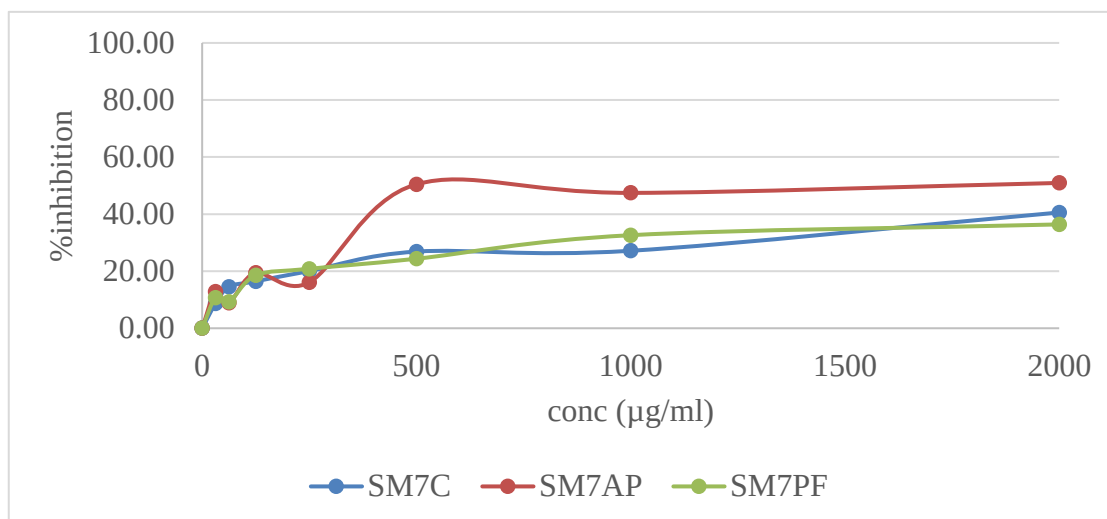
A: extracted under 4 hrs in water bath at 50 °C; **B**: extracted under 8 hrs in water bath 50 °C; **C**: extracted under 16 hrs in water bath 50 °C; **D**: extracted under 24 hrs in water bath 50 °C



Appendix E: Total phenolic content of fungal endophytes extracts consortia AP, fungal endophytes consortia PF and controlled extracts without fungal inoculum over 4, 8, 16 and 24 hrs of extraction period under submerged fermentation.

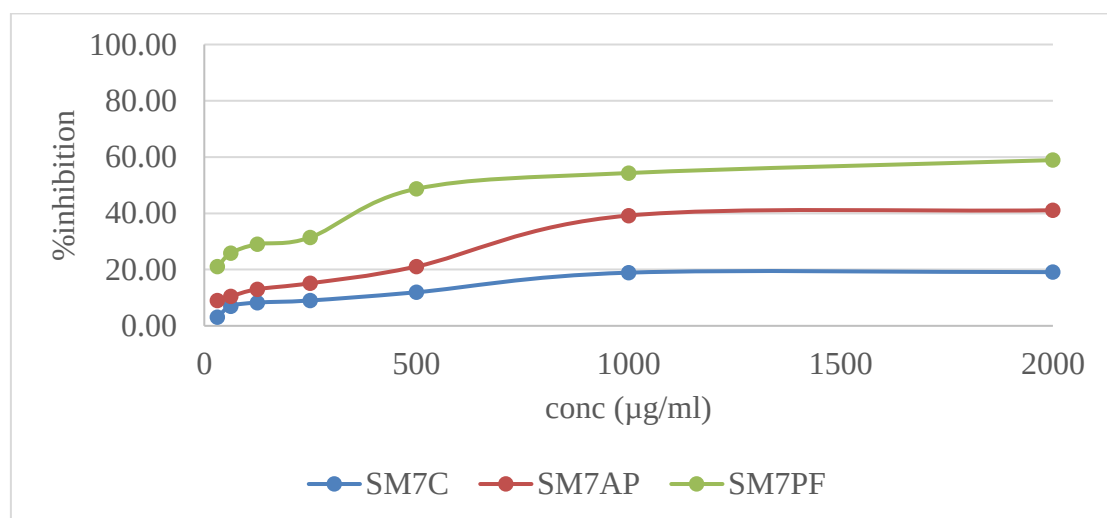
Abbrev.: **AP**: endophyte consortia *A. niger* & *P. chermesinum*, **PF**: endophytes consortia *P. chermesinum* & *F. equiseti*, **C**: uninoculated fermented RSS

A: extracted under 4 hrs in water bath at 50 °C; **B**: extracted under 8 hrs in water bath 50 °C; **C**: extracted under 16 hrs in water bath 50 °C; **D**: extracted under 24 hrs in water bath 50 °C



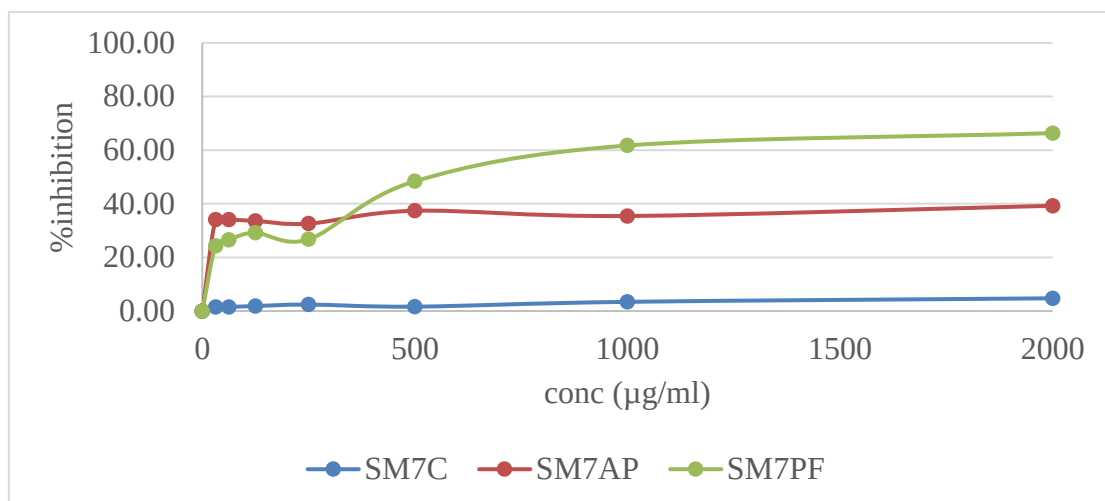
Appendix F (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 4 hrs of extraction time at Day 7 of incubation period.

Abbrev.: **SM7-C:** submerged fermentation extract under seven days of incubation with uninoculated RSS, **SM7-AP:** submerged fermentation extract under seven days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM7-PF:** submerged fermentation extract under seven days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



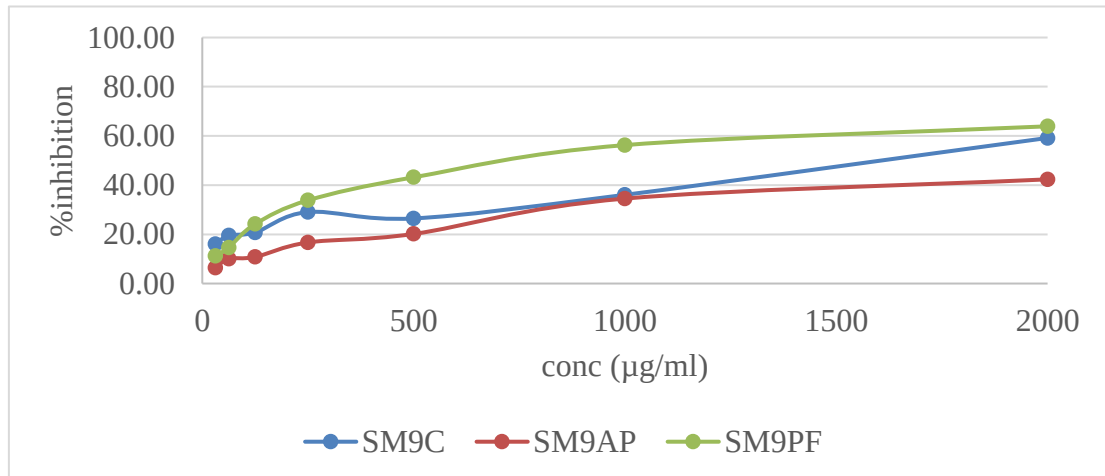
Appendix F (2): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 8 hrs of extraction time at Day 7 of incubation period.

Abbrev.: **SM7-C:** submerged fermentation extract under seven days of incubation with uninoculated RSS, **SM7-AP:** submerged fermentation extract under seven days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM7-PF:** submerged fermentation extract under seven days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



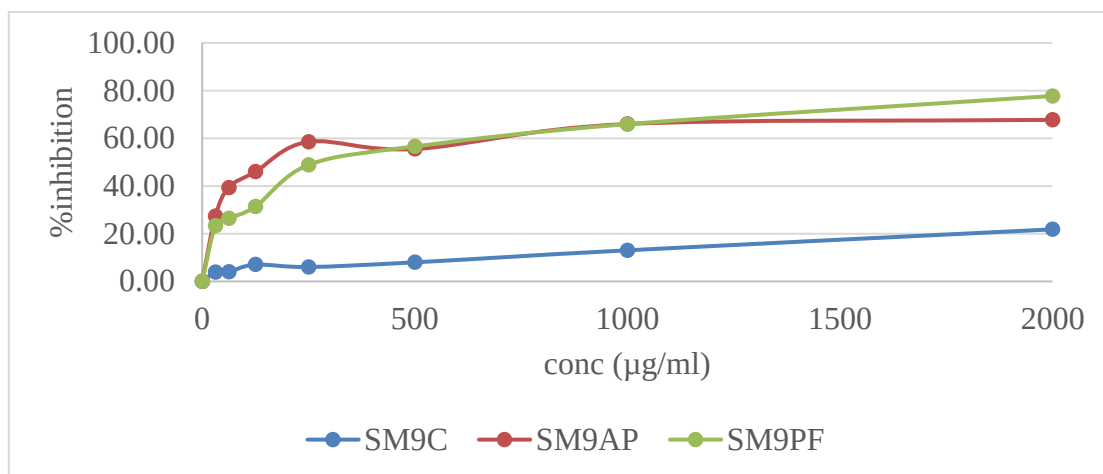
Appendix F (3): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 16 hrs of extraction time at Day 7 of incubation period.

Abbrev.: **SM7-C:** submerged fermentation extract under seven days of incubation with uninoculated RSS, **SM7-AP:** submerged fermentation extract under seven days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM7-PF:** submerged fermentation extract under seven days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



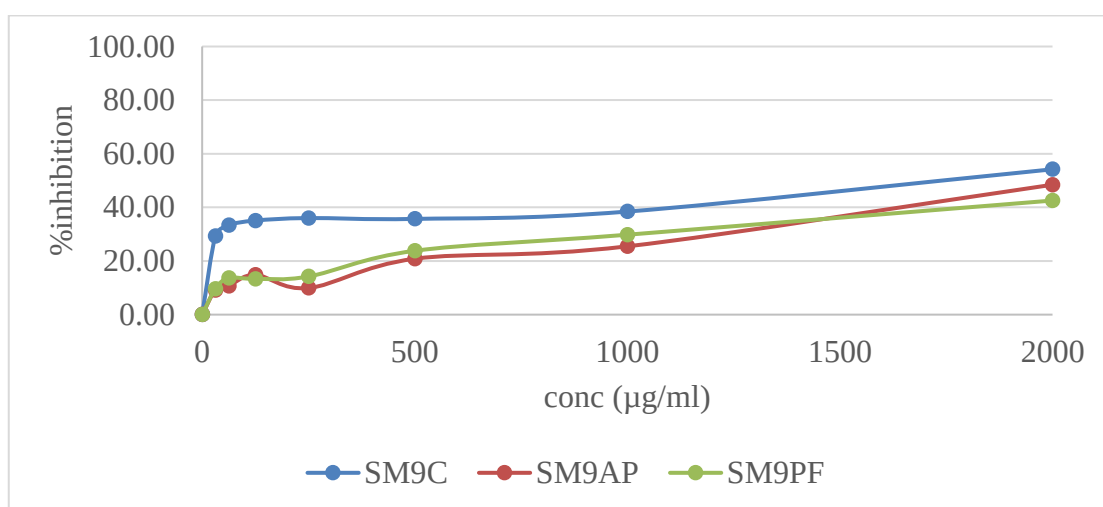
Appendix G (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 4 hrs of extraction time at Day 9 of incubation period.

Abbrev.: **SM9-C:** submerged fermentation extract under nine days of incubation with uninoculated RSS, **SM9-AP:** submerged fermentation extract under nine days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM9-PF:** submerged fermentation extract under nine days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



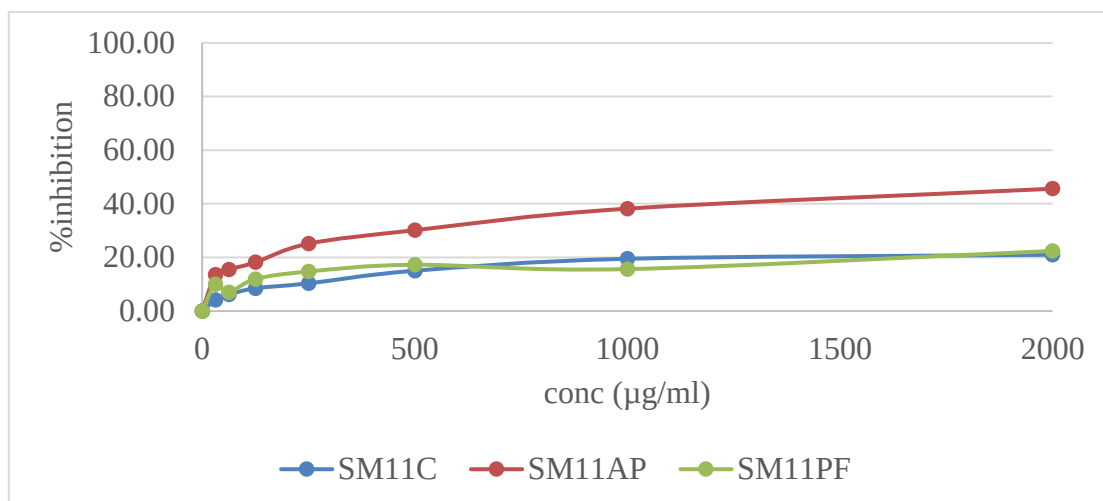
Appendix G (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 8 hrs of extraction time at Day 9 of incubation period.

Abbrev.: **SM9-C:** submerged fermentation extract under nine days of incubation with uninoculated RSS, **SM9-AP:** submerged fermentation extract under nine days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM9-PF:** submerged fermentation extract under nine days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



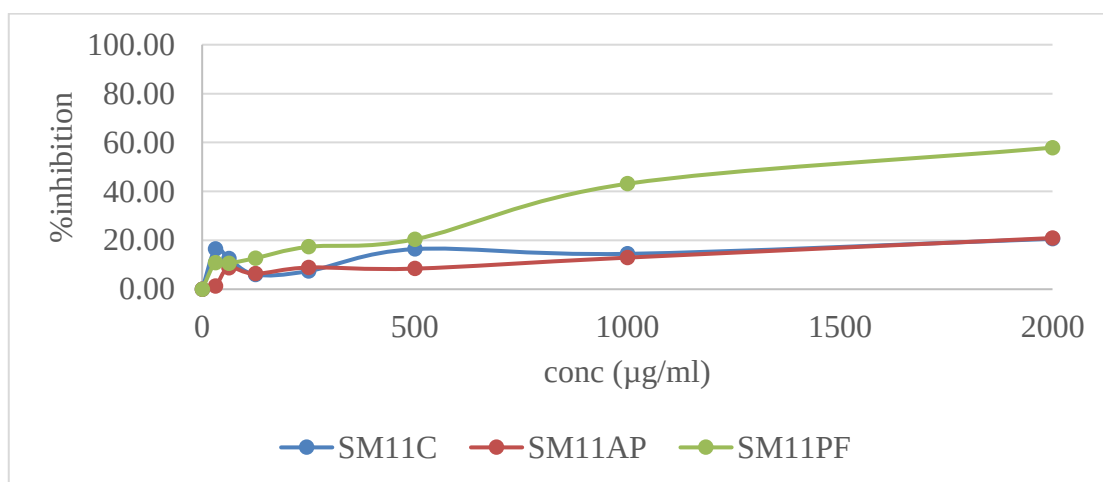
Appendix G (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 16 hrs of extraction time at Day 9 of incubation period.

Abbrev.: **SM9-C:** submerged fermentation extract under nine days of incubation with uninoculated RSS, **SM9-AP:** submerged fermentation extract under nine days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM9-PF:** submerged fermentation extract under nine days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



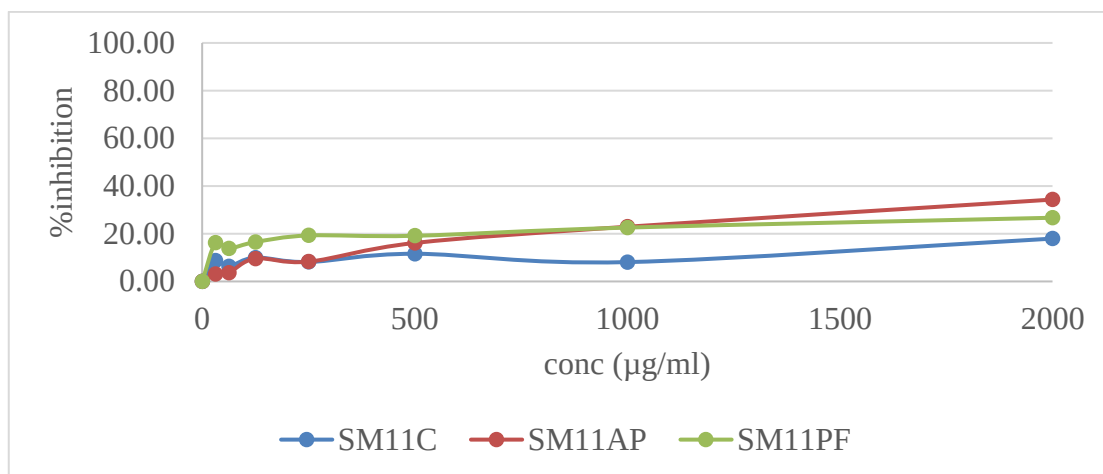
Appendix G (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 4 hrs of extraction time at Day 11 of incubation period.

Abbrev.: **SM11-C:** submerged fermentation extract under 11 days of incubation with uninoculated RSS, **SM11-AP:** submerged fermentation extract under 11 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM11-PF:** submerged fermentation extract under 11 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



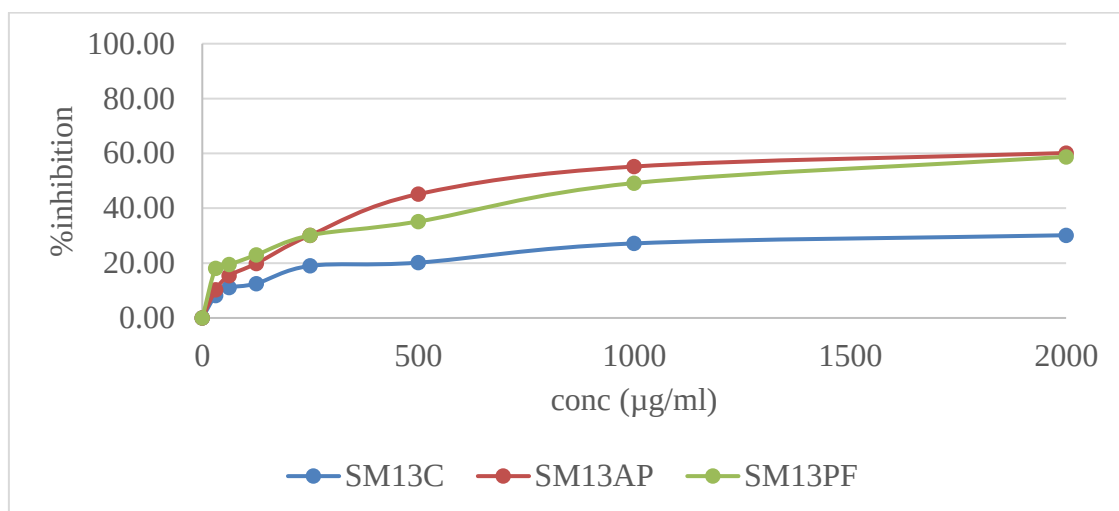
Appendix G (2): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 8 hrs of extraction time at Day 11 of incubation period.

Abbrev.: **SM11-C:** submerged fermentation extract under 11 days of incubation with uninoculated RSS, **SM11-AP:** submerged fermentation extract under 11 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM11-PF:** submerged fermentation extract under 11 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



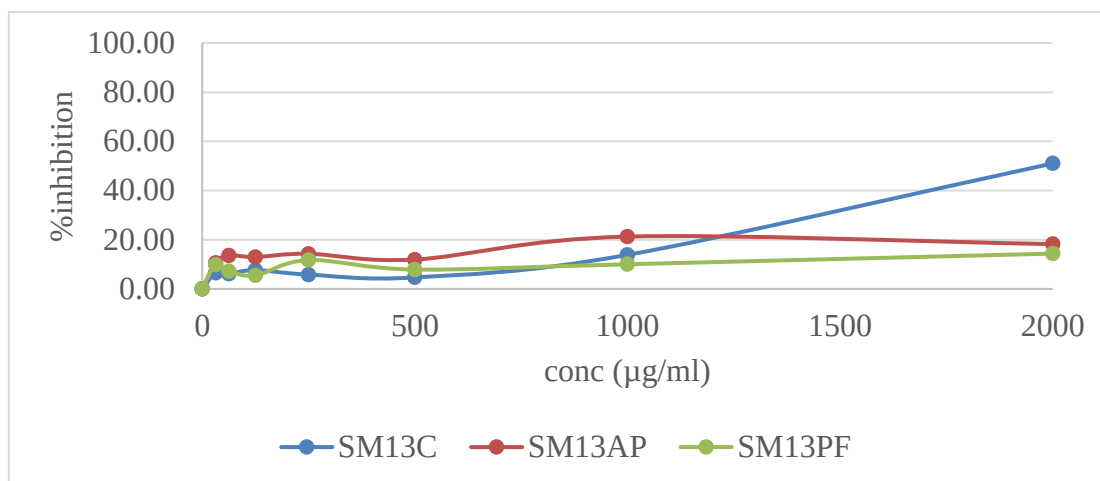
Appendix G (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 16 hrs of extraction time at Day 11 of incubation period.

Abbrev.: **SM11-C:** submerged fermentation extract under 11 days of incubation with uninoculated RSS, **SM11-AP:** submerged fermentation extract under 11 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM11-PF:** submerged fermentation extract under 11 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



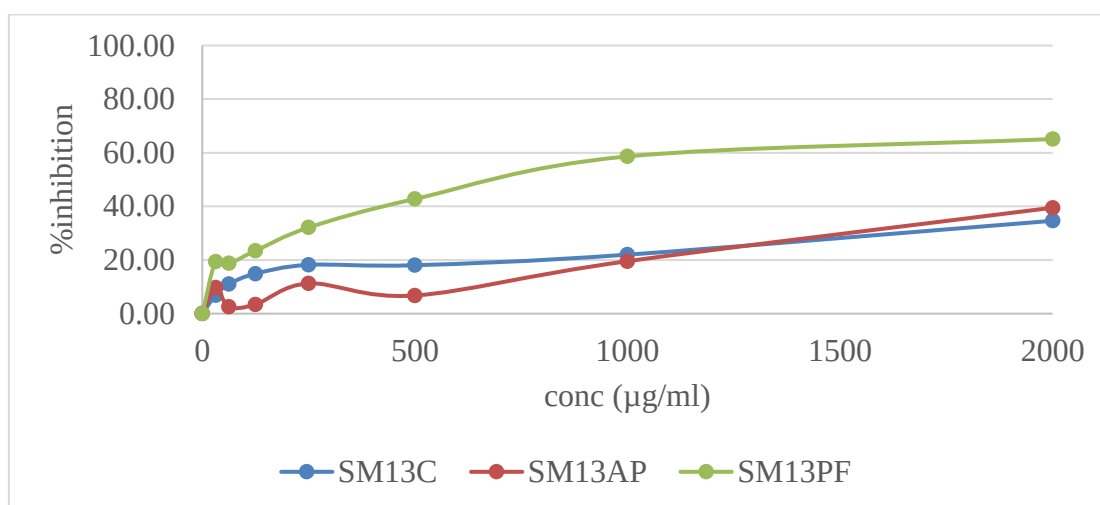
Appendix H (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 4 hrs of extraction time at Day 13 of incubation period.

Abbrev.: **SM13-C:** submerged fermentation extract under 13 days of incubation with uninoculated RSS, **SM13-AP:** submerged fermentation extract under 13 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM13-PF:** submerged fermentation extract under 13 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



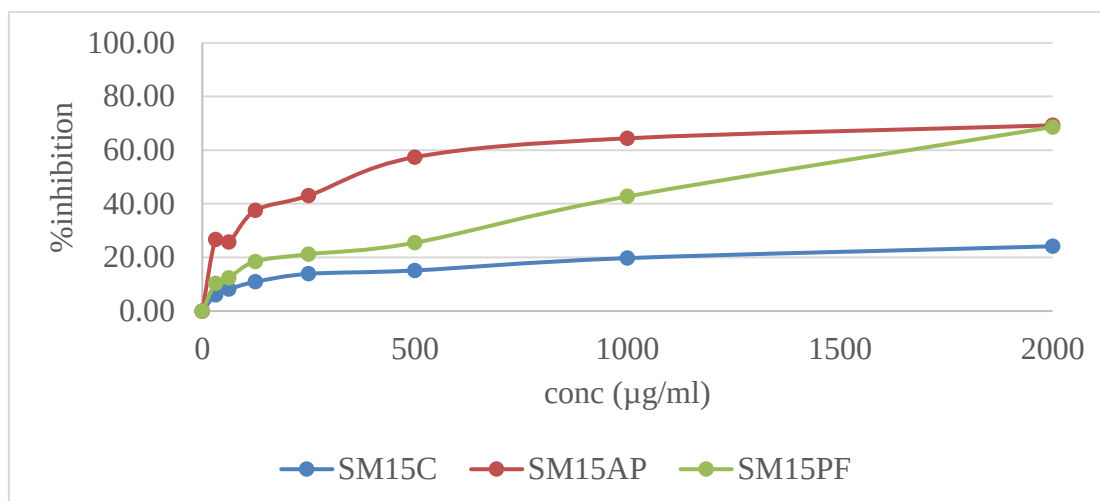
Appendix H (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 8 hrs of extraction time at Day 13 of incubation period.

Abbrev.: **SM13-C:** submerged fermentation extract under 13 days of incubation with uninoculated RSS, **SM13-AP:** submerged fermentation extract under 13 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM13-PF:** submerged fermentation extract under 13 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



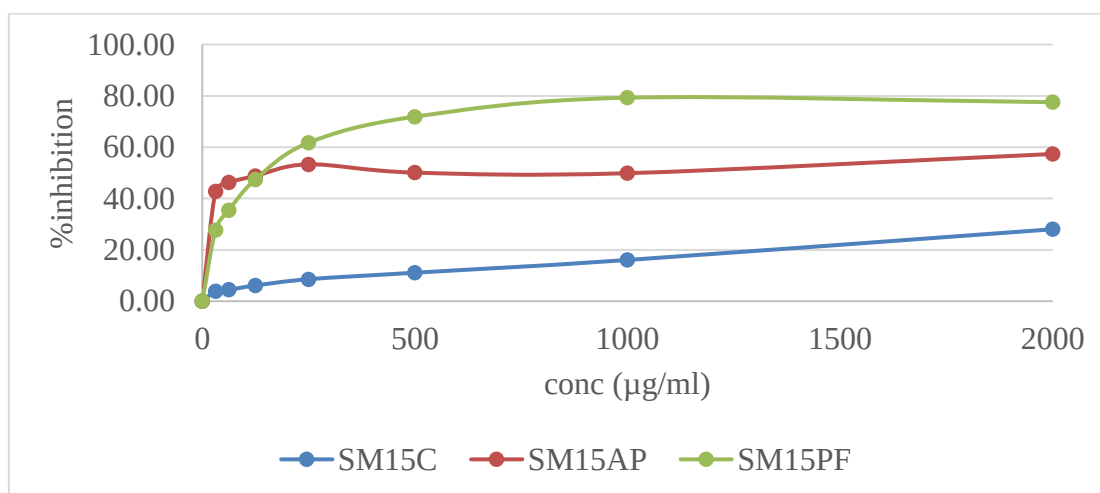
Appendix G (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 16 hrs of extraction time at Day 13 of incubation period.

Abbrev.: **SM13-C:** submerged fermentation extract under 13 days of incubation with uninoculated RSS, **SM13-AP:** submerged fermentation extract under 13 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM13-PF:** submerged fermentation extract under 13 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



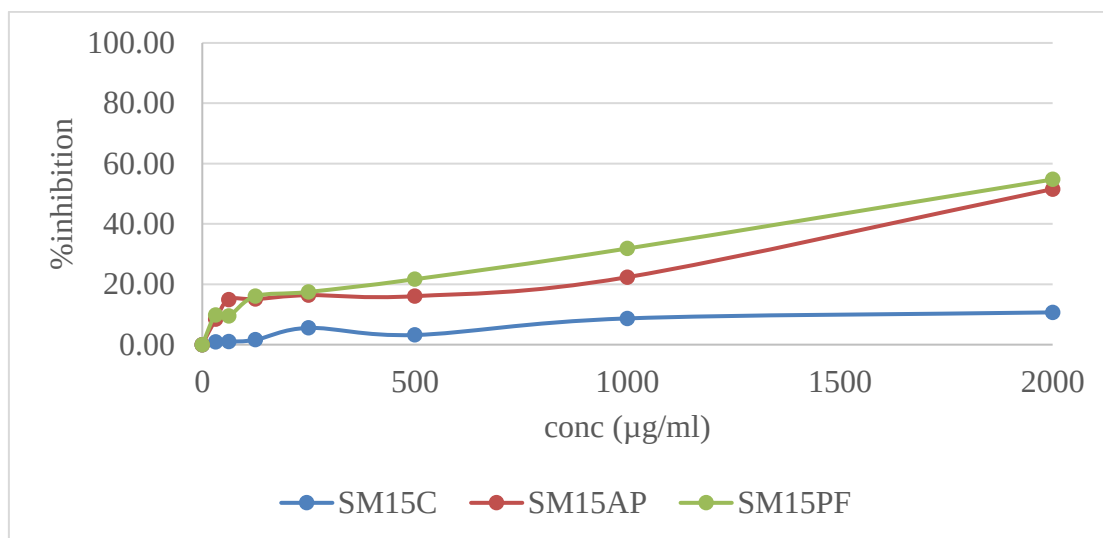
Appendix I (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 4 hrs of extraction time at Day 15 of incubation period.

Abbrev.: **SM15-C:** submerged fermentation extract under 15 days of incubation with uninoculated RSS, **SM15-AP:** submerged fermentation extract under 15 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM15-PF:** submerged fermentation extract under 15 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



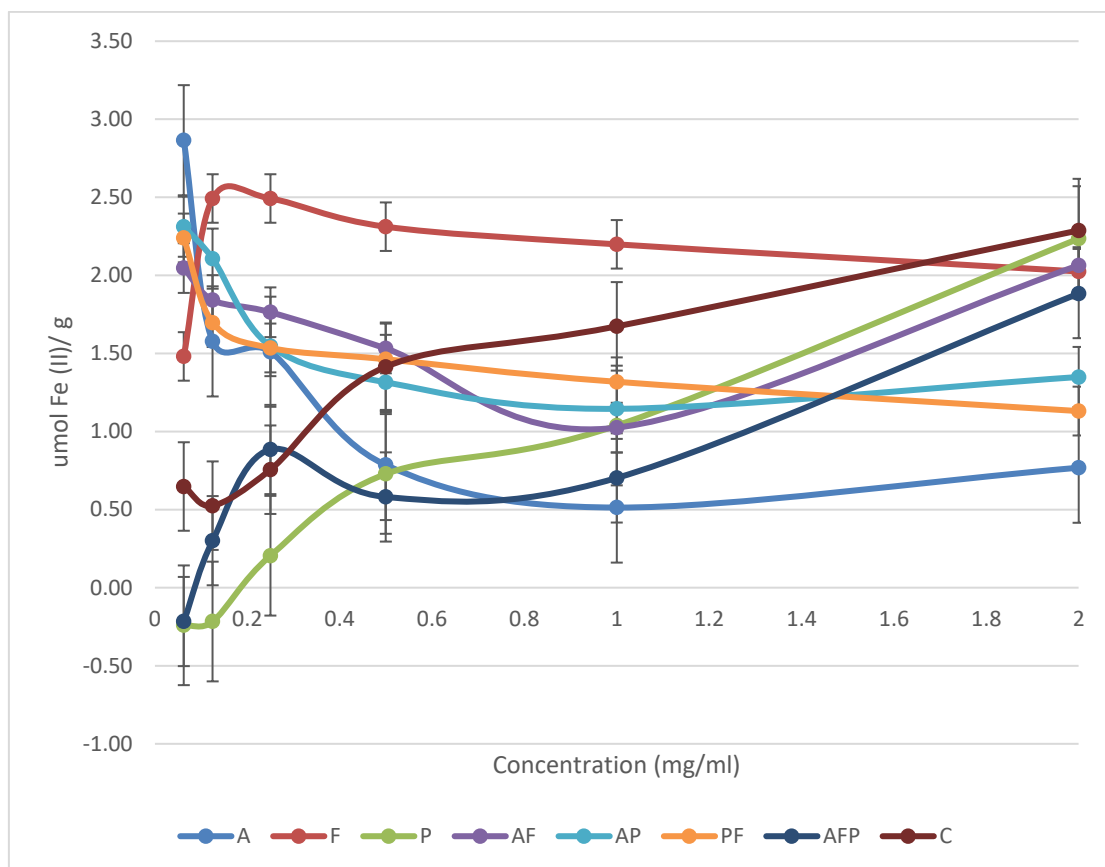
Appendix I (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 8 hrs of extraction time at Day 15 of incubation period.

Abbrev.: **SM15-C:** submerged fermentation extract under 15 days of incubation with uninoculated RSS, **SM15-AP:** submerged fermentation extract under 15 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM15-PF:** submerged fermentation extract under 15 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*

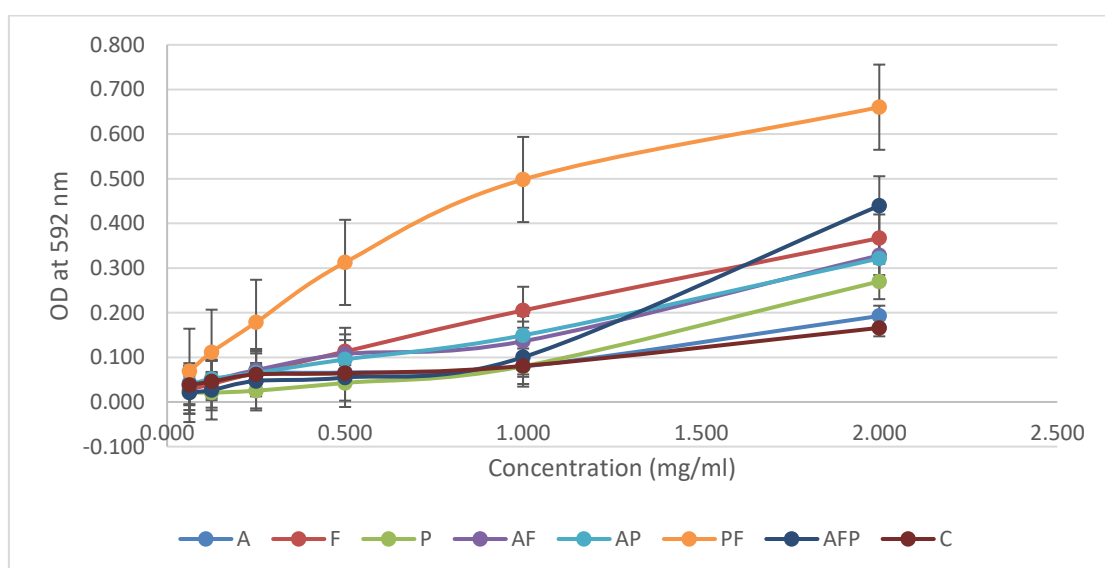


Appendix I (1): DPPH scavenging activity of methanolic extract of fermented sago starch by fungal endophytes under submerged fermentation (SMF) after 16 hrs of extraction time at Day 15 of incubation period.

Abbrev.: **SM15-C:** submerged fermentation extract under 15 days of incubation with uninoculated RSS, **SM15-AP:** submerged fermentation extract under 15 days of incubation with fungal endophytes consortia *A. niger* and *P. chermesinum*, **SM15-PF:** submerged fermentation extract under 15 days of incubation with fungal endophytes consortia *P. chermesinum* and *F. equiseti*



Appendix J (i). Average FRAP activity of different fungal consortia extract under solid state fermentation at seven days of fermentation in methanol.



Appendix J (ii). Average FRAP activity of different fungal consortia extract under solid state fermentation at seven days of fermentation in methanol based on its absorbance at 592 nm.

Appendix K: The list of chemicals used for making Glucose Minimal Media

Chemicals	Amount used (g/L)
K₂HPO₄	1.0
ZnSO₄.7H₂O	0.01
CuSO₄.5H₂O	0.05
MgSO₄.7H₂O	0.5
FeSO₄.7H₂O	0.01
NaNO₃	3.0
Glucose	10.0

Appendix L: Two-way ANOVA for Total Phenolic Content between fermentation type and fungal isolates

ANOVA: Two-Factor with Replication

SUMMARY	SSF	SMF	Total
<i>C1</i>			
Count	3	3	6
Sum	196346.2	-14807.7	181538.5
Average	65448.72	-4935.9	30256.41
Variance	2.96E+09	16321499	2.68E+09
<i>A1</i>			
Count	3	3	6
Sum	372884.6	25961.54	398846.2
Average	124294.9	8653.846	66474.36
Variance	1.84E+09	1.24E+08	4.8E+09
<i>F1</i>			
Count	3	3	6
Sum	387115.4	357307.7	744423.1
Average	129038.5	119102.6	124070.5
Variance	3.14E+09	1.63E+10	7.79E+09
<i>P1</i>			
Count	3	3	6
Sum	365576.9	610961.5	976538.5
Average	121859	203653.8	162756.4
Variance	3.79E+08	5.99E+09	4.56E+09

<i>AF1</i>			
Count	3	3	6
Sum	220961.5	189423.1	410384.6
Average	73653.85	63141.03	68397.44
Variance	92307692	2.09E+09	9.05E+08

<i>AP1</i>			
Count	3	3	6
Sum	522884.6	22884.62	545769.2
Average	174294.9	7628.205	90961.54
Variance	6.9E+09	62179487	1.11E+10

<i>PF1</i>			
Count	3	3	6
Sum	480576.9	58269.23	538846.2
Average	160192.3	19423.08	89807.69
Variance	1.79E+08	1.52E+08	6.08E+09

<i>AFP1</i>			
Count	3	3	6
Sum	492500	69038.46	561538.5
Average	164166.7	23012.82	93589.74
Variance	1.24E+09	34072978	6.49E+09

<i>Total</i>			
Count	24	24	
Sum	3038846	1319038	
Average	126618.6	54959.94	
Variance	2.94E+09	6.92E+09	

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample (fungal isolate)	6.63E+10	7	9.47E+09	3.655458	0.005218	2.312741
Columns (Fermentation technique)	6.16E+10	1	6.16E+10	23.77625	2.85E-05	4.149097
Interaction	7.75E+10	7	1.11E+10	4.271831	0.00197	2.312741
Within	8.29E+10	32	2.59E+09			
Total	2.88E+11	47				

- (P > 0.05)
- There is a significant difference among the type of isolates used
- There is a significant difference between fermentation technique
- There is a significant difference in the interaction between isolate used and fermentation technique

Appendix M: One-way ANOVA for incubation period in consortia AP

ONE WAY ANOVA: Single Factor (incubation period for consortia AP)

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
7	3	522.8846	174.2949	6898.57
9	3	81.73077	27.24359	1478.748
11	3	84.80769	28.26923	1487.13
13	3	168.6538	56.21795	4721.055
15	3	182.8846	60.96154	1557.84

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	44150.43	4	11037.61	3.418625	0.052279	3.47805
Within Groups	32286.69	10	3228.669			
Total	76437.12	14				

No significant difference between incubation period of AP

Appendix N: One way ANOVA for incubation period of consortia PF

ANOVA incubation period of Consortia PF

ANOVA: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
7	3	330.318	110.106	111.9344
9	3	109.4231	36.47436	3542.357
11	3	383.6538	127.8846	2709.911
13	3	338.6538	112.8846	4433.136
15	3	504.4231	168.141	78570.61

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	27325.68	4	6831.421	0.382208	0.816541	3.47805
Within Groups	178735.9	10	17873.59			
Total	206061.6	14				

No significant incubation period of PF

Appendix O: One way ANOVA among isolates under submerged fermentation

ANOVA: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
USL	3	-14.8077	-4.9359	16.3215
A	3	610.9615	203.6538	5993.491
F	3	357.3077	119.1026	16264.07
P	3	25.96154	8.653846	124.4083
AF	3	189.4231	63.14103	2088.363
AP	3	22.88462	7.628205	62.17949
PF	3	58.26923	19.42308	152.2189
AFP	3	69.03846	23.01282	34.07298

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	109639.9	7	15662.84	5.065782	0.003466	2.657197
Within Groups	49470.24	16	3091.89			
Total	159110.1	23				

There is a significance difference in submerged fermentation. Consortia of fungi does affects phenolic content in submerged fermentation.

Appendix P: One way ANOVA among isolates under solid state fermentations

ANOVA: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
USL	3	196.3462	65.44872	2961.144
A	3	372.8846	124.2949	1842.357
F	3	387.1154	129.0385	3142.456
P	3	365.5769	121.859	378.7475
AF	3	220.9615	73.65385	92.30769
AP	3	522.8846	174.2949	6898.57
PF	3	480.5769	160.1923	178.8462
AFP	3	492.5	164.1667	1236.736

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	34173.02	7	4881.86	2.33426	0.075988	2.657197
Within Groups	33462.33	16	2091.395			
Total	67635.35	23				

There are no significance differences between isolates in SSF. Under SSF, consortia of fungi do affects phenolic content.

Appendix Q

ANOVA: Two-Factor with Replication

SUMMARY	4	8	16	Total
<i>AP</i>				
Count	3	3	3	9
Sum	675.059	116.1908	522.8846	1314.134
Average	225.0197	38.73027	174.2949	146.0149
Variance	678.3672	1431.024	6898.57	9208.804

<i>PF</i>				
Count	3	3	3	9
Sum	381.951	62.62746	480.5769	925.1554
Average	127.317	20.87582	160.1923	102.795
Variance	716.777	327.1706	178.8462	4283.148

<i>C</i>				
Count	3	3	3	9
Sum	84.428	120.338	196.3462	401.1122
Average	28.14267	40.11267	65.44872	44.56802
Variance	8.552476	1207.883	2961.144	1316.512

<i>Total</i>				
Count	9	9	9	
Sum	1141.438	299.1563	1199.808	
Average	126.8264	33.23958	133.312	
Variance	7618.663	827.863	5137.479	

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	46649.47	2	23324.73	14.56953	0.000173	3.554557
Columns	56445.13	2	28222.57	17.6289	5.75E-05	3.554557
Interaction	33205.91	4	8301.477	5.185422	0.00587	2.927744
Within	28816.67	18	1600.926			
Total	165117.2	26				

At day 7, there is significant difference among samples as extraction increases.

Appendix R

ANOVA: Two-Factor with Replication

SUMMARY	4	8	16	Total
<i>AP</i>				
Count	3	3	3	9
Sum	138.0587	509.4149	29.89954	677.3732
Average	46.01958	169.805	9.966513	75.26369
Variance	439.762	32163.51	74.56706	13440.83
<i>PF</i>				
Count	3	3	3	9
Sum	73.08624	127.8838	47.786	248.7561
Average	24.36208	42.62795	15.92867	27.63956
Variance	122.8856	226.7688	14.32359	230.6965
<i>C</i>				
Count	3	3	3	9
Sum	45.139	45.137	16.459	106.735
Average	15.04633	15.04567	5.486333	11.85944
Variance	26.88577	63.17611	19.66579	50.27872
<i>Total</i>				
Count	9	9	9	
Sum	256.284	682.4358	94.14454	
Average	28.476	75.8262	10.4605	
Variance	336.7798	13224.02	47.72181	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	19611.5	2	9805.752	2.662071	0.097096	3.554557
Columns	20517.82	2	10258.91	2.785095	0.088346	3.554557
Interaction	22953.56	4	5738.389	1.557861	0.228272	2.927744
Within	66303.08	18	3683.505			
Total	129386	26				

Day 9, there is no significant difference among samples as extraction period increases. There is no significant difference in extraction period.

Appendix S
ANOVA: Two-Factor with Replication

SUMMARY	4	8	16	Total
<i>AP</i>				
Count	3	3	3	9
Sum	473.6538	103.8895	230.5769	808.1202
Average	157.8846	34.62982	76.85897	89.79114
Variance	300	1513.766	3160.108	4185.994
<i>PF</i>				
Count	3	3	3	9
Sum	87.11538	617.1154	35.98562	740.2164
Average	29.03846	205.7051	11.99521	82.24626
Variance	234.7633	58986	71.27315	23451.15
<i>C</i>				
Count	3	3	3	9
Sum	7.692308	3.846154	41.73077	53.26923
Average	2.564103	1.282051	13.91026	5.918803
Variance	6.743097	1.269724	22.09073	43.75719
<i>Total</i>				
Count	9	9	9	
Sum	568.4615	724.851	308.2933	
Average	63.16239	80.539	34.25481	
Variance	5313.712	24146.21	1835.058	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	38752.12	2	19376.06	2.712214	0.093418	3.554557
Columns	9839.463	2	4919.731	0.688652	0.515006	3.554557
Interaction	83015.71	4	20753.93	2.905084	0.051205	2.927744
Within	128592	18	7144.001			
Total	260199.3	26				

Day 11

No significant diff between samples at different extraction time

Extraction time does not influence phenolic content extraction

However, phenolic content of samples depends on the extraction time as there is significant difference.

Appendix T
ANOVA: Two-Factor with Replication

SUMMARY	4	8	16	Total
<i>AP</i>				
Count	3	3	3	9
Sum	419.0385	274.0385	273.2692	966.3462
Average	139.6795	91.34615	91.08974	107.3718
Variance	1350.641	413.1657	1496.647	1402.256
<i>PF</i>				
Count	3	3	3	9
Sum	526.7308	172.3846	943.8462	1642.962
Average	175.5769	57.46154	314.6154	182.5513
Variance	70330.92	948.7304	122891.3	60969.12
<i>C</i>				
Count	3	3	3	9
Sum	285.7692	532.1154	13.09431	830.9789
Average	95.25641	177.3718	4.364769	92.33099
Variance	19854.01	85216.03	16.12402	31888.5
<i>Total</i>				
Count	9	9	9	
Sum	1231.538	978.5385	1230.21	
Average	136.8376	108.7265	136.69	
Variance	24098.07	24510.36	50318.57	

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	42053.65	2	21026.82	0.625555	0.546199	3.554557
Columns	4716.636	2	2358.318	0.070161	0.932498	3.554557
Interaction	144327.2	4	36081.8	1.073446	0.398567	2.927744
Within	605035.1	18	33613.06			
Total	796132.6	26				

Day 13

- No significant difference among samples as extraction time increases
- No significant difference among extraction time
- No significant difference among the two factors at this period of incubation

Appendix U
ANOVA: Two-Factor with Replication

SUMMARY	4	8	16	Total
<i>AP</i>				
Count	3	3	3	9
Sum	68.08936	23.5957	18.87804	110.5631
Average	22.69645	7.865232	6.292679	12.28479
Variance	161.5978	47.61256	65.27753	130.0622
<i>PF</i>				
Count	3	3	3	9
Sum	35.73416	37.36233	125.0743	198.1708
Average	11.91139	12.45411	41.69143	22.01898
Variance	11.14856	121.9285	81.31581	271.3441
<i>C</i>				
Count	3	3	3	9
Sum	14.4304	19.756	18.472	52.6584
Average	4.810133	6.585333	6.157333	5.850933
Variance	5.277485	13.66044	11.82645	8.334778
<i>Total</i>				
Count	9	9	9	
Sum	118.2539	80.71402	162.4243	
Average	13.13932	8.968225	18.04715	
Variance	105.3392	52.94269	354.0752	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	1192.664	2	596.3318	10.32818	0.001029	3.554557
Columns	371.735	2	185.8675	3.219134	0.0638	3.554557
Interaction	1866.903	4	466.7258	8.083463	0.000649	2.927744
Within	1039.29	18	57.73835			
Total	4470.592	26				

On Day 15, there is significant difference among samples as extraction time increases
However, there is no significant difference in extraction time
There is a significant difference of samples depending on extraction time.

Only Day 7 and Day 15 showed significant difference as extraction time increases. Day 11 showed significant difference on the interaction between extraction time and samples (fungal consortia).

Appendix V: SOLVENT USED

Consortia AP

ANOVA: Two-Factor with Replication

SUMMARY	7	9	11	13	15	Total
<i>M</i>						
Count	3	3	3	3	3	15
Sum	522.8846	81.73077	84.80769	168.6538	182.8846	1040.962
Average	174.2949	27.24359	28.26923	56.21795	60.96154	69.39744
Variance	6898.57	1478.748	1487.13	4721.055	1557.84	5459.794
<i>E</i>						
Count	3	3	3	3	3	15
Sum	71.289	7.884615	348.2692	152.5	198.6538	778.5967
Average	23.763	2.628205	116.0897	50.83333	66.21795	51.90645
Variance	26.28827	24.01381	3214.546	5745.759	2050.937	3197.192
<i>EA</i>						
Count	3	3	3	3	3	15
Sum	5.002	-2.11538	-17.5	49.80769	130.5769	165.7712
Average	1.667333	-0.70513	-5.83333	16.60256	43.52564	11.05142
Variance	2.335	108.0375	109.073	1191.913	6045.759	1407.466
<i>Total</i>						
Count	9	9	9	9	9	
Sum	599.1756	87.5	415.5769	370.9615	512.1154	
Average	66.57507	9.722222	46.17521	41.21795	56.90171	
Variance	8350.34	577.4696	4170.27	3260.947	2519.457	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	26896.63	2	13448.32	5.819765	0.007315	3.31583
Columns	16771.1	4	4192.774	1.814425	0.152083	2.689628
Interaction	54807.22	8	6850.903	2.964731	0.014358	2.266163
Within	69324.01	30	2310.8			
Total	167799	44				

Under AP, there is significant diff between solvent used.

However, there is no significant diff among incubation periods. Phenolic content produced at different incubation period are influenced by the solvent used

Appendix X

Consortia PF

ANOVA: Two-Factor with Replication

SUMMARY	7	9	11	13	15	Total
<i>M</i>						
Count	3	3	3	3	3	15
Sum	330.318	109.4231	383.6538	338.6538	504.4231	1666.472
Average	110.106	36.47436	127.8846	112.8846	168.141	111.0981
Variance	111.9344	3542.357	2709.911	4433.136	78570.61	14718.68
<i>E</i>						
Count	3	3	3	3	3	15
Sum	219.887	112.5	540.9615	540.9615	108.0769	1522.387
Average	73.29567	37.5	180.3205	180.3205	36.02564	101.4925
Variance	182.1376	1.035503	34800.94	34800.94	3473.385	15094.86
<i>EA</i>						
Count	3	3	3	3	3	15
Sum	76.202	100.1923	41.34615	41.34615	62.11538	321.202
Average	25.40067	33.39744	13.78205	13.78205	20.70513	21.41347
Variance	17.56316	366.0256	510.2564	510.2564	183.6292	286.067
<i>Total</i>						
Count	9	9	9	9	9	
Sum	626.407	322.1154	965.9615	920.9615	674.6154	
Average	69.60078	35.7906	107.3291	102.3291	74.95726	
Variance	1430.899	980.7733	14943.27	15199.08	25485.22	

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	72741.26	2	36370.63	3.322245	0.049738	3.31583
Columns	29821.82	4	7455.455	0.681012	0.610568	2.689628
Interaction	63144.51	8	7893.063	0.720985	0.671629	2.266163
Within	328428.2	30	10947.61			
Total	494135.8	44				

There is a significant difference on solvent used

No significant difference in incubation period

Total phenolic content does influence by solvent used but not on its incubation period