1.0 INTRODUCTION

Infectious diseases are caused by pathogens, which is clinically evident disease resulting from the presence of pathogenic microbial agents, including pathogenic viruses, pathogenic bacteria, fungi, protozoa, multicellular parasites, and uncharacteristic proteins known as prions. Infectious disease results from the interplay between those few pathogens and the defenses of the hosts they infect. The bacterial infections which contribute most to human disease are also those in which emerging and microbial resistance is most evident: diarrhoeal diseases, respiratory tract infections, meningitis, sexually transmitted infections, and hospital-acquired infections. Some important examples include penicillin-resistant *Streptococcus pneumoniae,* vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus,* multi-resistant salmonellae, and multi-resistant *Mycobacterium tuberculosis.* The development of resistance to drugs commonly used to treat malaria is of particular concern, as is the emerging resistance to anti-HIV drugs.

In recent years, an increase in the number of people in the world having health problems caused by various cancers, drug-resistant bacteria, parasitic protozoans, and fungi is a cause for alarm (Strobel and Gary, 2003). Antimicrobial agents are among the most valuable therapeutic agents in modern healthcare. Since their discovery during the 20th century, antimicrobial agents (antibiotics and related medicinal drugs) have substantially reduced the threat posed by infectious diseases. These gains are now seriously jeopardized by another recent development: the emergence and spread of microbes that are resistant to cheap and effective first-choice, or "first-line" drugs. Over the years, antimicrobials have saved the lives and eased the suffering of millions of people. By helping to bring many

serious infectious diseases under control, these drugs have also contributed to the major gains in life expectancy experienced during the latter part of the last century.

There are four common reactive oxygen species (ROS) in biological systems that are free radicals: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O2$). In addition, peroxynitrite ($ONOO^-$) and nitric oxide (NO) and other reactive nitrogen species (RNS) are called *free radical*s. Free radicals can be generated via a number of mechanisms, including normal physiological processes and processes resulting from external factors. For example, singlet oxygen is generated by photosensitization reactions wherein a molecule absorbs light of a given wavelength, exciting the molecule (Stanner et al., 2004; Willcox et al., 2004).

There are increasing evidences indicating that reactive oxygen species (ROS) and free radical-meditated reactions are involved in degenerative or pathological events such as cancer, cardiovascular disease, atherosclerosis, coronary heart ailment, Alzheimer's disease and diabetes. The oxidative damage to DNA, proteins and other cellular determinants seems inevitable when the concentration of ROS exceed tolerability of cells. Antioxidant could attenuate this oxidative damage of a tissue indirectly by increasing cells' natural defenses and or directly by scavenging the free radical species (Schinella *et al*., 2002).

Over the last decade, considerable experimental evidence has confirmed the importance for health of following a diet rich in antioxidants, which can protect the organism against the damage caused by these radicals. Some of these antioxidants are well known, such as vitamins (particularly vitamins E and C) and carotenoids, including β-

carotene. Furthermore, some polysaccharides have been disclosed to be potent antioxidants (Hou *et al*., 2005; Kim *et al*., 2003).

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Cancer begins when cells in a part of the body start to grow out of control. There are many kinds of cancer, but they all start because of out-of-control growth of abnormal cells (Jeanne and Carmen, 2009). Cancer is caused by both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis.

Cancer is not just one disease but many diseases. There are more than 100 different types of cancer. Most cancers are named for the organ or type of cell in which they start. For example, cancer that begins in the colon is called colon cancer and cancer that begins in basal cells of the skin is called basal cell carcinoma. Cancer types can be grouped into broader categories which include: Carcinoma as a cancer that begins in the skin or in tissues that line or cover internal organs; Sarcoma as a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue; and Leukemia as a cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood (Lim, 2001).

Cancer is treated with surgery, radiation, chemotherapy, hormone therapy, biological therapy, and targeted therapy. Choice of cancer treatment is influenced by

several factors, including the specific characteristics of cancer; overall condition; and whether the goal of treatment is to cure cancer, keep cancer from spreading, or to relieve the symptoms caused by cancer.

A growing number of fungi are the sources of novel and potentially life-saving bioactive secondary metabolites. Fungi produce an enormous array of secondary metabolites, some of which are important in industry. Fungi are well known for their vast diversity of secondary metabolites including antibiotics, amino acids, fuel (ethanol, biogas), single cell protein, biopesticeds, mycoherbicides, preservatives, vitamins, organic acid etc. Investigating the metabolites of fungi increased the chance of founding novel compounds.

In fungi, besides enzymes of biotechnological application and other products including biocontrol agents, the metabolites of pharmaceutical application are of great interest for counteracting common ailments. Bioactive fungi metabolites are believed to have capability to help in revitalization of immune system against a large number of pathogenic and non-pathogenic diseases. Investigating the metabolites of fungi increased the chance of founding novel compounds. Medicinal mushroom have widely used as tonics food and herb remedies since ancient times, are rich with medicinal properties and have been increasingly recognized through modern scientific research to have promising immunodulatory activities (Wasser, 2002). Mushrooms are considered to be a good source of proteins. Extracts from medicinal mushrooms have been used in traditional medication.

Penicilliopsis is a genus in the family *Trichocomaceae* phylum Ascomycota. It is teleomorphic fungus and classified under the Ascomycetes. The thallus (mycelium)

typically consists of a highly branched network of multinucleate, septate, and yellow in colour. It is easy to culture in the laboratory and has a fast growth within 48 hours.

To the best of our knowledge, the biological properties from *Penicilliopsis* sp. have not yet been reported. Therefore, the present study was carried out to evaluate the biological activities such as antioxidant, antimicrobial, cytotoxicity and anti human papillomavirus in *Penicilliopsis* sp. (KUM60280) collected from Malaysian forest.

The specific objectives of this study were:

1. To obtain mycelial biomass of *Penicilliopsis* sp. by liquid fermentation.
2. To extract bioactive compounds using methanol and dichloromethane.
3. To determine the antimicrobial activity of crude extracts of *Penicilliopsis* sp.
4. To evaluate the antioxidant property of *Penicilliopsis* sp. extracts based on its ability to scavenge free radicals and reducing power and to determine the total phenolic content of the extracts using the Folin-Ciocalteau method.
5. To analyze the cytotoxic potentials of methanol and dichloromethane crude exrtracts of *Penicilliopsis* sp. against cancer derived cell lines and normal cell lines.
6. To evaluate crude extracts of *Penicillliopsis* sp. for the the anti-human papillomavirus 16 E6 activity.

2.0 LITERATURE REVIEW

2.1 Characterization of Fungi

Fungi are unique group of organisms, different from all others in their behavior and cellular organization. Fungi have been traditionally used to produce a variety of important substances for the pharmaceutical and food industries (Marley *et al.*, 2004). Hence, primary and secondary metabolites, such as peptides, enzymes, organic acids and antibiotics produced by filamentous fungi are used for these purposes (Benette, 1998: Demain, 2000). Fungi are potential sources of secondary metabolites with biological activities. Fungi have been recognized for their utilization in developing and testing many kinds of hypotheses, as a model system in biology, as a creature of pure beauty. The fungi constitute a very large group of organisms found in virtually every ecological niche.

2.2 *Penicilliopsis* species

The genus *Penicilliopsis* was first described by Solms-Laubach (1886) on seeds of *Diospyros macrophylla* collected in the Botanical Garden of Bogor, Indonesia. It is the teleomorphic fungus, but was subsequently misapplied to various synnematous fungi. The detailed description clearly depicts an ascomycete with a phialidic anamorph. *Penicilliopsis* is a genus in the family *Trichocomaceae* phylum *Ascomycota*. The *Penicilliopsis* is classified under the Ascomycetes. Given conspicuous synnemata produced by these species and their common occurrence in tropical rain forests, it is surprisingly that the group has not been critically reexamined in the past fifty years. This fungus which

preferably lives on ground or rock is yellow in colour usually in branching form. However no investigation has been done to study the biological properties of this fungus. *Penicilliopsis* have been found fairly spread in the tropics especially from Brazil, Taiwan, Ivory Coast, Papua New Guinea, Indonesia, Singapore and Malaysia (Huei & Yu, 2001).

2.3 The Production of Secondary Metabolites by Fungi

Four groups of organisms are particularly good producers of secondary metabolites: plants, fungi, lichens, and actinomycetes, whereas yeasts, protozoa, and animals are less efficient producers (Frisvad *et al.,* 2007). Fungi are a major source of bioactive secondary metabolites which are of major importance in ecological chemical interactions (Camazine *et al.* 1983; Sterner, 1995). They can also play a major role in the combat of many human and animal diseases (Müller, 2001; Vicente *et al.* 2003; Peláez, 2005).

A fungal secondary metabolite is a chemical compound produced by a limited number of species in a genus, an order, or even a phylum and has a differentiation power. A profile of secondary metabolites compiled by the mycologyst is based on fungal extracts (Frisvad *et al.,* 2007). A classic example is penicillin produced commercially from a strain of *Penicillium chrysogenum* but originally discovered as a metabolite of *P. notatum.* Its discovery literally changed the course of medicine and has saved millions of lives. Other antibiotics produced by fungi are cephalosporins and griseofulvin (from fungus *P. griseofulvum*) to threat dermatophyte infections of humans, and fusidic acid (from various fungi) used to control *Staphylococci* than have become resistant to penicillin. Table 2.1 showed several fungal metabolites having practical applications in horticulture, medicine or research.

The pectic enzymes of fungi are used to clarify fruit juices. Whereas, fungal amylase is used to convert starch to maltose during bread-making, and fungal rennet is used to coagulate milk for cheese making. The wood-rotting fungus *Phanaerochaete chrysosporium* important in degrading lignin, has the potential to be developed for delignification of agricultural wastes and by-products of the wood-pulping industry so that the cellulose can be used as a cheap substrate for production of fuel alcohol by yeast (Deacon, 2000). Genetic engineering of fungi, particularly *Saccharomyces cerevisiae*, has been developed to the stage where the cells can be used as factories to produce pharmaceutical products, by the introduction of foreign (heterologous) genes.

Table 2.1 Fungal secondary metabolites produced commercially for pharmaceutical, agricultural and research uses (Deacon, 2000)

Usage	Product	Fungal source	Application
Medicine	Penicillin	*Penicillium chrysogenum*	Antibacterial
	Cephalosporin	*Chephalosporium acremonium*	Antibacterial
	Griseofulvin	*P. griseofulvum*	Antifungal
	Fusidin	*Fusidium coccineum*	Antibacterial
	Cyclosporin	*Trichoderma polysporum*	Immunosupressant
	Ergot alkaloids	*Claviceps purpurea*	Induces labour, migraine treatment
Agriculture	Zearalenone	*Gibberella zeae*	Growth promoter for cattle
		G. fujikoroi	Plant hormones

It is not unusual that different fungal species have one or more secondary metabolites in common. Cytochalasin D is one such metabolite. It is produced by several fungal species as different as *Coriolus vernicipes* (a basidiomycete), *Zygosporium masonii* (a zygomycete), and *Metarrhizium anisopliae*, *Engleromyces goetzii*, and *Hypoxylon terricola* (ascomycetes) (Cole & Schweikert, 2003). Many secondary metabolites are produced widely by different fungi (Cole & Schweikert 2003; Vicente *et al.* 2003). All species in *Penicillium* subgenus *Penicillium* series *Verrucosa* produce arabenoic acid (syn. verrucolone). All species in *Aspergillus* section *Circumdati* produce penicillic acid, except the phylogenetically outlying species *A. elegans* and *A. steynii* (Frisvad *et al.* 2004b). Whereas all species in *Aspergillus* section *Flavi*, except the phylogenetically outlying *A. avenaceus*, produce kojic acid (Frisvad *et al*., 2007). Emodin, an anthraquinone produced by fungi as different as *Cortinarius sanguineus* (basidiomycete) and *Penicillium islandicum* (ascomycete) (Turner, 1971), is also produce by rhubarbs (*Rheum rhaponticum*).

Technologies based on the degerative or synthetic activities of the fungi have become an integral part of the human society. Current commercial product of fungi include amino acids, antibiotics, fuel (ethanol, biogas), biopesticides, mycoherbicides, bread, cheese, fermented food, food(mushroom, etc), single cell protein, flavours, food colourants, preservatives, soy sauce, vitamins, organic acid and mycelial paper. Bioremediation, ensilage, biotransformation and many such processes involved the utilization of fungi. In the emerging 'age of biotechnology', the fungi are expected to provide a wider range of useful products and processes for human welfare under the banner of what is called 'fungal biotechnology '(Moss, 1990).

2.4 Antimicrobial Activity in Fungus

Antimicrobial is a general term given to substances including medicines that kill or slow the growth of microbes. Increasing use of antimicrobials in humans, animals, and agriculture has resulted in many microbes developing resistance to these powerful drugs. Many infectious diseases are increasingly difficult to treat because of antimicrobial-resistant organisms, including HIV infection, staphylococcal infection, tuberculosis, influenza, gonorrhea, candida infection, and malaria. Antimicrobial diseases are now more frequent than during the half of the century, being still difficult to diagnose clinically (Jian *et al.,* 2007). Several antimicrobials have been developed over the years to control these microorganisms. There is a need to search for new antimicrobial agents because infectious disease is still a global problem because of the development and spread of drug-resistant pathogens (Espinel *et al.,* 2001).

Research on biologically active compounds from fungus has always been of great interest for scientists looking for new sources of useful drugs against infectious diseases. Nowadays, bacterial resistance and its rapid increase is a major concern of global public health. Resistance development is an even bigger problem since the baacterial resistance is often not restricted to the specific antibiotic prescribed, but generally extends to other compounds of the same class. Alarmingly, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis*, vancomycin-resistant *enterococci* (VRE), amphicillin-resistant *Escherichia coli* and even vancomycin-resistant *Staphylococcus aureus* (VRSA) have emerged as common nosocomial infections (Gou *et al*., 2005). Although antimicrobial resistance was recognized soon

after the deployment of sulfanomides and penicillin, it now appears that the emergence of antibiotic-resistant bacteria is inevitable to most drugs. In the last two decades, the incidence of human fungal infections has increased dramatically, in parallel with the wide spread of incurable infectious diseases associated with antibiotic-resistant bacteria (Woong *et al*, 2006*).* As a consequences, new efforts to develop new antimicrobial agents are highly needed (Özlem *et al*., 2007).

To overcome the alarming problem of microbial resistance to antibiotics, the discovery of novel active compounds against new targets is a matter of urgency. Bacterial resistance to existing drugs is a constantly growing problem that, combined with a decline in the development of new antibiotics, presents a significant threat to human health (Christian *et al.,* 2007). Thus, microbes have the ability to release compounds that inhibit the growth of other microorganisms which depends on genetic factors and environmental condition. A number of 10–50 amino acid residues long, very often cationic peptides, have been identified from both vertebrate and invertebrate sources. They act in a variety of ways against many Gram-negative and Gram-positive organisms. The majority of experiments investigating their mode of action have focused primarily on the interaction of cationic peptides with model membrane systems.

In the global problem of bacterial resistance to the commonly used antibiotics these properties make cationic peptides valuable candidates as future therapeutic agents to combat bacterial, viral, and fungal infections (Karaman *et al*., 2003). Recently, the use of fungal products in medicinal treatment and in health foods has become very popular since

1990s and more so since the outbreak of the Severe Acute Respitory Syndrome (SARS) in China in 2003 (Dong and Yao, 2007).

In September 2001, World Health Organization (WHO) launched the first global strategy for combating the serious problems caused by the emergence and spread of antimicrobial resistance. Known as the WHO Global Strategy for Containment of Antimicrobial Resistance, the strategy recognizes that antimicrobial resistance is a global problem that must be addressed in all countries. No single nation, however effective it is at containing resistance within its borders, can protect itself from the importation of resistant pathogens through travel and trade. Poor prescribing practices in any country now threaten to undermine the potency of vital antimicrobials everywhere. Several antimicrobials have been developed over the years to control these microorganisms (Mencher and Wang, 2007).

A large number of fungal extracts and or extracellular products have been found to have antimicrobial activity, mainly the filamentous fungus *Penicillum* sp. Since the discovery of penicillin, the mycromycetes have been famous as producers of antibiotics and otheer "secondary metabolites" with biological activity (Ana *et al*., 2006). Kurobane *et al*. (1981) reported that *Penicillium brefeldianum* produce fulvic acid which possessed antiviral, antifungal, antioxidant and antibiotic activities. Maskey *et al.* (2003) isolated two active substances 8-O-methylaverufin and 1,8-Odimethylaverantin as new antifungal agents from *Penicillium chrysogenum*. Nam *et al.* (2000) found that compounds 8-Omethylsclererotiorinamine isolated from *Penicillium multicolor* showed antimicrobial activity.

2.4. 1 Antimicrobial Susceptibility Testing

Disc diffusion susceptibility is based on the inoculation of the surface of an agar plate with a suspension of the test organism followed by application of a paper disc containing a defined quantity of antimicrobial agent. Disc diffusion method is among the most widely adopted alternative approaches (Martin *et al.*, 2001). The antimicrobial agent diffuses from the paper disc into the surrounding medium and a gradient of antimicrobial concentration is established around the disc with the highest concentration immediately adjacent to the disc and progressively lower concentrations as distance from the disc increases. The interaction of the antimicrobial agents diffusing through the medium with the organism on the surface of the agar plate results in inhibition of bacterial growth for a variable distance around the paper disc. There is the relationship between the diameter zone of inhibition of bacterial growth around the disc and the MIC of the test organism for the particular antimicrobial agents. In general terms, the lower the MIC for a particular antimicrobial agent, the larger the diameter of the zone of inhibition growth.

A fundamental concept of *in-vitro* susceptibility testing is the measurement of minimum inhibitory concentration (MIC). The MIC is the lowest concentration of antimicrobial agent that presents visible growth of the microorganisms (Martin *et al*., 2001). The MIC is measured by exposing the organism, under conditions that are otherwise suitable for growth, to a range of concentrations of the antimicrobial agent (usually in dilution series). After a period of incubation that is at least sufficient to allow visible growth in the absence of antimicrobial agents, the culture medium is inspected to identify the lowest concentration of antimicrobial agents at which no growth is visible.

Determination of MIC can be costly and time consuming and as a result a number of alternative approaches to antimicrobial susceptibility testing have been developed.

2.5 Antioxidant Activity

Oxidation and reduction reactions must occur in pairs (i.e., when one atom or molecule is oxidized, another is reduced to defend against the potentially damaging effects of free radicals). Highly reactive molecules can oxidize molecules (i.e., remove electrons from molecules) that were previously stable and may cause them to become unstable species, such as *free radicals*. Free radicals are involved in the onset of many diseases such as cancer, rheumatoid arthritis, cirrcosis and arterioscelerosis as well as in degenerative process associated with ageing (Mariani *et al*. 2005).

Free radicals are produced in normal cell metabolism. A free radical is a chemical species with an unpaired electron that can be neutral, positively charged, or negatively charged. Although a few stable free radicals are known, most are very reactive (Paul *et. al.*, 2006). In free radical chain reactions, the radical product of one reaction becomes the starting material for another, propagating free radical damage. Metabolism is not the only source of free radicals. Halliwell and Gutteridge (2003) acknowledged that exogenous chemical and endogenous metabolic processess in the human body or in the food system might produce highly reactive free radicals, especially oxygen derived radicals which are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Most of organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT) or chemical compounds such as α-

tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione (Niki *et al.,* 1994).

Environmental pollutants are sources for free radicals including nitrogen dioxide, ozone, cigarette smoke, radiation, halogenated hydrocarbons, heavy metals, and certain pesticides (Katalinik *et al.,* 2006). Alcohol consumption can induce oxidative reactions in the liver. Certain chemotherapeutic agents including doxorubicin, cyclophosphamide, 5-fluorouracil, methotrexate, and vincristine can produce oxygen radicals at doses used in cancer patients. Increased physical activity can generate free radicals as the result of increased oxygen consumption during exercise.

There are three steps to the free radical chain reaction: initiation, propagation, and termination (Paul *et al.,* 2006). In the initiation step, free radicals are formed from molecules that readily give up electrons, such as hydrogen peroxide. In the propagation steps, the chain-carrying radicals are alternately consumed and produced. In the termination steps, radicals are destroyed. Thus, without termination by an agent such as an antioxidant, a single free radical can damage numerous molecules.

Antioxidants played an important role in lowering oxidative stresses caused by reactive oxygen species (ROS). ROS including superoxide anion radical, hydroxyl radical and hydrogen peroxide are generated under physiological and pathological stresses in human body (Nordberg and Arner, 2001). Some severe chronic diseases, such as arthritis, cancer, diabetes, cardiovascular diseases, inflammations and neurological disorders are related to the imbalance of ROS formation and their elimination (Halliwell, 1996).

Antioxidants protect against these complex diseases through scavenging free radicals and reducing hydrogen peroxide (Shahidi and Wanasundara, 1992). Despite the actions of antioxidant nutrients, oxidative damage occurs inevitably over time in most cells, and accumulation of this damage throughout life is believed to be a major contributing factor to aging and chronic diseases including cancer (Paul *et. al*, 2006). The balance between antioxidant and oxidation is believed to be critical in maintaining a healthy biological system (Judge *et al.*, 2005; Katalinik, *et al.*, 2006).

Recently, many reseachers have taken a great interest in fungi for their phenolic concentration and related total antioxidant potential (Mahfuz *et al*, 2007). A wide study variety of fungi have produced novel antioxidants (Yang *et al*., 2002). These include *Penicillium roqufortii, Aspergillus candidus, Mortierella sp., Emericella falconensis* and fungi of the genus *Acremonium.* Therefore, the identification and study of novel compounds characterized with antioxidant activity from fungal sources is an important strategy to improve human health condition and life quality.

Wild edible mushrooms are conventionally used in many Asian countries in both food and medicine (Isildak *et al*, 2004; Türkekul, *et al*, 2004, Manzi *et al,* 1999, Sanmee *et al.,* 2003**).** Mushrooms have also been reported as therapeutic food that is useful in preventing diseases such as hypertension, hypercholesterolemia and cancer. These functional characteristics are mainly due to their chemical composition (Manzi *et al*, 2001). In most countries, there is a well established consumer acceptance for cultivated mushrooms (*Agaricus bisporus, Pleorotus* spp., *Lentinus edodes, Vorveriella volvacea, Auricularia* spp.). Nevertheless, wild edible muhrooms are becoming more and more

important in our diet for their nutritional and pharmacological characteristics (Manzi *et al*., 2001).

2.5.1 Antioxidant Assays

Several assays have been frequently used to estimate antioxidant capacities in fugus including 2,2- azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) / (ABTS), (Miller,1997), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams *et al*., 1995) ferric reducing antioxidant power (FRAP) (Benzie, 2003) and the oxygen radical absorption capacity (ORAC) (Ou *et al*., 2002).

2.5.1.1 The 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) Assay

The $DPPH^{\cdot}$ assay is one of the method in antioxidant testing. The $DPPH^{\cdot}$ is a stable free radical that can accept an electron or hydrogen radical converting into stable, diamagnetic molecule. The $DPPH^{\cdot}$ assay primarily evaluates proton radical scavenging activity. The 2,2-diphenyl-1-picrylhydrazyl, sometimes known as α,α-diphenyl-β-picrylhydrazyl ($DPPH^{\cdot}$) (molecular formula=$C_{18}H_{12}N_5O_6$; molar mass 384.33 g/mol), is a free radical compound usually tested to determine the free radical scavenging ability of antioxidant polyphenols in environment, including in plants, food and baverages. As this electron becomes paired off, the absorption decreases stoichiometrically with respect to the number of electron taken up. Such a change in absorbance produced in this reaction has been widely used to test the ability of several molecules to act as free radical scavengers (Dinis *et. al.*, 1994). Singh and Rajini (2003) reported that scavenging $DPPH^{\cdot}$ by

antioxidant due to their hrydrogen-donating ability. The hydrogen atoms or electrons donating ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple coloured methanol solution of $DPPH^{\cdot}$. The adequacy of the methodology was determined by a dose dependent curve and the absence of formulation interference in the detection of extracts antioxidant activity using $DPPH^{\cdot}$ assay.

According to Brand-Williams *et al.* (1995), the $DPPH^{\cdot}$ technique is performed in a polar medium such as methanol at ambient temperature without any additional oxygen. The radical is stable because of the spare electron delocalised over the whole molecule that is responsible to changes in colour from deep violet colour to light yellow due to picryl group (Mau *et al.*, 2001). The $DPPH^{\cdot}$ has a strong absorption band at 517 nm and paramagnetic because of its odd electron. However, it can accept an electron or hydrogen radical to become stable and diamagnetic.

Besides studing the sample extracts, a standard or positive control such as ascorbic acids or Butylated hydroxyanisole (BHA) should be included. $DPPH^{\cdot}$ and L-ascorbic acid should be prepared daily and kept in a flask wrapped with aluminium foil to protect from light, and stored at $4^{o}C$ in between measurement. L-ascorbic acid is an organic acid also known as Vitamin C that can be easily destroyed by light, heat and metal oxygen. It must be stored in the cold and dark and not in metal container.

The reaction was monitored until it has reached a plateau and the reaction kinetics was plotted. Determination of percentage inhibition at steady state was done from the plotted graphs (Sanchez-Moreno *et al.*, 1999). The values of percentage inhibition should

then be tranferred onto another graph for showing the percentage of inhibition as a function of antioxidant concentration. Mau *et al* (2001) stated that usage of the EC_{50} value has drawbacks in showing the results because the higher the antioxidant activity, the lower the value of EC_{50}. The disadvantage of presenting the results in bar graph is bars with low EC_{50} value are visually short in the graphs and mistakenly preceived as low efficiency.

2.5.1.2 The Reducing Power Assay

A simple, automated test measuring the ferric reducing ability of plasma, the FRAP assay, is presented as novel method for assessing ‘‘antioxidant power’’. Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. A biological antioxidant has been defined as ‘‘any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate’’ (Halliwell and Gutteridge, 1999). This definition is clear and covers every member of the antioxidant defense team. The method described measures the ferric reducing ability of plasma (FRAP). At low pH, when a ferric-tripyridyltriazine (Fe III -TPTZ) complex is reduced to ferrous (Fe II) form, an intense blue color with an absorption maximum at 593 nm develops (Benzie, 2003; Liu *et. al.*, 1982).

The reaction is nonspecific, and any half-reaction which has a less-positive redox potential, under reaction conditions, than the Fe III /Fe II -TPTZ half-reaction will drive Fe III -TPTZ reduction. Test conditions favor reduction of the complex and, thereby, color development, provided that a reductant (antioxidant) is present. Ferrozine is a compound closely related to TPTZ, has been widely used with excess ascorbic acid, to measure iron.

In the FRAP assay, excess Fe III is used and the rate limiting factor of Fe II -TPTZ, and color formation is the reducing ability of the sample.

2.5.2 Total Phenolic Contents in Fungal Metabolites

Phenolics are a source of natural antioxidants and closely associated with the aroma, acceptibility, taste, nutritional quality and stability of food by acting as colorants, flavourings and antioxidant. Phenolic acids are thought to play a positive role in prevention of human diseases and food industry. Phenolics are one of the major groups of non-essential dietary components that have been assiociated with the inhibition of atherosclerosis and cancer (Williams and Iatropoulus, 1997). The bioactivity phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavange free radicals (Decker, 1997).

The key role of phenolic compounds as scavangers of free radicals is emphasised in several reports (Komali *et al.,* 1999; Moller *et al*, 1999). The phenol are oxidized rapidly in solutions sufficiently alkaline to give appreciable concentration of the phenolate ions. Antiradical antioxidants act by donating hydrogren atoms to lipid radicals. Imeh and Kokhar (2002) reported that phenolics as antioxidants are important in food and biological systems because they are preferentially oxidized, thus sparing nutrients, cells and tissues.

Mushroom accumulates a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Phenolic compounds were found to have antioxidant activity in the inhibition of Low Density Lipid, LDL oxidation (Teissedre &

Landrault, 2000). Some edible mushrooms, which are widely consumed in Asian culture, have currently been found to posses antioxidant activity which is well correlated with their total phenolic content.

The Follin-Ciocalteau method is often used to measure total phenolic compound. The total phenolic contents of the extracts samples are expressed in miligrams per saving of gallic acid equivalent (GAE). Gallic acid (Formula Molecule = $C_7H_6O_5$; Formula Weight = 170.12) is preferable reference because of its stability, solubility, low price and purity. Singleton and Rossi (1965) stated that the active ingredient in the Follin-Ciocalteau reagent is yellow acidic solution with complex ion formed from phosphomolybdic and phosphotungstic acids. The colour formation is due to the reactions of monohydric phenols polyphenols, flavonoids, tannins and other readily oxidized subtances such as ascorbic acids. Such reagent oxidizes phenolates and the oxidation states of the acidsare reduced from +6 to a mixture of +6 and +5. A blue molybdenum tungsten omles was formed. Incubation is necessary for colour development because time is required for a complete reaction. Na_2CO_3 is added because phenol oxidizes rapidly in alkaline solutions. The carbonate should be added exactly three minutes after the Follin-Ciocalteau reagent was added. However, it is important to mix the sample and the Follin-Ciocalteu reagent under dilute conditions before addition of the carbonate. Na_2CO_3 (Formula Weight =105.99) is preferred over NaCN or NaOH because with NaCN, the blue colour fades instantly whereas NaOH provides a precipitation problem. The reference standard can be any convenience, known and reactive phenolic compound.

The limitation of the Folin-Ciocalteau method is the reaction is based on reduction–oxidation activities. Thus, the assay would not be specific to just phenolics but to any other substance that could be oxidised by the Follin reagent. Frankel *et al.(*1995) reported that the total phenolics determined in the Follin-Ciocalteau method do not give the abselute measurement of the amount of phenolics materials. Various phenolics compounds respond differently to this assay. Even though there is no specific extraction method for all phenolics present in a sample, extraction method, nature of the phenolic compound, presence of interfering substances, sample size, storage conditions and time and standard selections influence the analysis of the phenolics compounds. The extract usually contains a mixture of phenolic compounds that are soluble in the chosen solvent.

2.6 Cytotoxicity of Fungal Metabolites

Fungal growth occurs under favorable environmental conditions and it is associated with the production of secondary metabolites, many of which can be hazardous to vertebrates (animals and humans) (Kokkonen *et al*., 2005). Toxin of fungal origin is known as mycotoxins. The conditions which promote mycotoxin production are usually more restricted than those for mold growth. Mycotoxins are toxic secondary metabolites produced by about 200 identified filamentous fungi such as *Fusarium, Aspergillus* and *Penicillium* species, growing under a wide range of climatic conditions on agricultural commodities (grains, spices, fruits, coffee, nut) in the field and during storage (Krogh, 1987; Miller and Trenholm 1997). Their amount in food, beverages and feed has been recognised as potential threat to human and animal health, either caused by direct

contamination of plant materials or products thereof, or by "carry over" of mycotoxins and their metabolites into animal tissues, milk and eggs after intake of contaminated feed.

Evidence of mycotoxicoses can be traced back to ancient times and the middle ages (ergotism). However, it was not before 1960, that mycotoxins were identified as potential health hazard when 100 000 turkeys died from an acute necrosis of the liver after consuming groundnuts infected by *Aspergillus flavus* and contaminated by aflatoxins (X-disease) (Peter & Bernhard, 2006). Today, aflatoxins, trichothecenes, ochratoxin A (OTA), zearalenone (ZEN), fumonisins, moniliformin and patulin receive by far the greatest attention due to their frequent occurrence and their severe effects on animal and human health (Peter & Bernhard, 2006). Aflatoxins are secondary metabolites of the molds *Aspergillus flavus* and *Aspergillus parasiticus*. They are potent carcinogens, teratogens, genotoxics, nephrotoxic, neurotoxic, immunosuppressive and mutagens and pose severe hazards to animal and human health (Stavroula *et al*., 2006).

Less critical compounds and chronic intake of small amounts of mycotoxins may reduce feed intake and weight gain in animals and cause diarrhoea in humans (Peter & Bernhard, 2006). Several endemic diseases in Asia, Africa and Europe are now correlated to acute mycotoxin intoxication, such as Kwarshiorkor and Reye's syndrome (damage to liver and kidney caused by (aflatoxins) and Balkan Endemic Nephropathy (tumors of the upper urinary tract caused by ochratoxin A). Ochratoxin A (OTA) is a toxic metabolite produced by the molds *Aspergillus ochraceus* and *Penicillium verrucosum* and related species (Seudomore & Patel, 2000). The International Agency for Research on Cancer

(IARC) has classified OTA as a ‘‘possible human carcinogen’’ (Group 2B) (International Agency for Research on Cancer (IARC), 1995).

Factors contributing to their presence or production in food and feed include storage, environmental and ecological conditions, as temperature, relative humidity, the substrate and the use of fungicides, though the interrelations between all these factors are not yet well understood and toxin production cannot reasonably be predicted. Surveillance studies showed that mycotoxin contamination is a world-wide problem (Final report SCOOP: Placenta *et al*., 1999). It is estimated that 25% of the world's crop production and 20% of crop production within the European Union may be contaminated with mycotoxins (Peter and Bernhard, 2006). ZEN is non-steroidal estrogenic mycotoxin in corn, cereals and animal feeding (Placinta *et al.,* 1999; Muller *et al.,* 1998; Seudamore and Patel, 2000).

Medicinal fungi have been widely used as tonic foods and herbs remedies since ancient times and their medicinal properties have been increasingly recognized through modern scientific research (Jian *et al*., 2007). In the search of alternative medicines and natural therapeutics for cancer therapy, medicinal fungus is the most promising targets because of their notable immunomodulatory activities (Wasser, 2002). Because of the natural scarcity of most medicinal fungi, the cultivation of mycelia, fruiting bodies and the whole fungi by solid and submerged fermentation has become the main source of fungal supply (Smith *et al,* 2002; Chang and Miles, 2004). Novel anti-cancer drugs are also required due to the high worldwide mortality (Pisani, *et al.,* 1999).

2.6.1 *In vitro* Cytotoxicity Assay

In vitro assays are important and useful tools in toxicity assessment of various classes of environmental contaminants including fungal metabolites not only because they significantly reduce evaluation time, but also because they provide information about the mode of action of the toxicant (Francesca *et al*., 2004). In the search for new anticancer drugs, the most common screening methods employ cytotoxicity tests against panel of cancer cell lines. Depending on the design, *in vitro* cytotoxicity experiments could provide information of compounds with potential basal toxicity (intrinsic activity of chemicals that is common to all cell types), metabolism-mediated toxicity (chemicals require metabolic activation) and selective toxicity (chemicals interfere with cell-specific functions) (Robert *et al.,* 2007).

Anticancer drugs are designed to kill cells and such activity should be selective towards tumor cells. Therefore, it seems reasonable to utilize at the primary stage, *in vitro* assays to select the least toxic compounds among the most active ones (Joanna, *et al.*,, 2005). This information is crucial at the early stage of development of pharmaceutical drugs or crop protection chemicals.

In order to screen of large number of lead compounds in a relatively short period of time, cytotoxicity is routinely evaluated using permanent cells lines in 96-well plate format. *In vitro* tests are useful and necessary for screening purposes to define dose and time-dependent cytotoxicity, considered primarily as the potential of a compound to induce cell death, in different cell types (Eisbrand *et al*., 2002). In the exploration of anticancer

activity, the most common screening methods employed cytotoxicity tests against a panel of cancer cell lines to reveal compounds with the highest toxicity activity.

Cytotoxicity test is a screening method that typically uses permanent cell lines for ranking acute toxicities of parent compounds based on the basal cytotoxicity theory (i.e. chemical exert their acute toxic effects by interfering with basic cellular functions that are common to all mammalian cells (Barile and Cordona, 1998). It can also be used to assess the potential of bioactivation (generation of reactive and toxic metabolites) by using pairs of cells line and metabolic competent cell type, such as primary cultured hepatocytes (Gomez-Lechon *et al.,* 2001) or metabolic enzyme-transfected cells lines (Drobna *et al.,* 2005).

In past years, a number of methods have been developed to study cell viability and proliferation in cell culture. The most convenient, modern assays have been optimized for the use on microtiterplates (96-well format). This miniaturization allows many samples to be analyzed rapidly and simultaneously. Colorimetric and luminescene based assays allow samples to be measured directly in the plate using a microtiter plate reader or ELISA plate reader.

Cytotoxicity assays have been developed using different parameters associated with cell death and proliferation such as the integrity of the cell membrane, which can be measured by cytoplasmic enzyme activity released by damage cells. Another cytotoxicity assays was developed based on metabolic activity of viable cells using 3-(4, 5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) which can be reduced to a

blue colored formazan. Another parameter using neutral red, NR (3-amino-dimethylamino-2methyl-phenazine hydrochloride) has been used previously for the identification of vital cells in cultures (DeRenzis and Schechtman, 1973). This assay quantifies the number of viable, uninjured cells after their exposure to toxicants based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red over a range of concentration. Alteration of the cell surface or sensitive lysosomal membrane can lead to lysosomal fragility or other changes which are irrevisible.

Quantification of the dye extracted from the cells is done both by direct cell counts and by protein determinations of cell population (Borenfreund and Puerner, 1986). The amount of NR dye accumulated can be extracted from the lyososomes and quantitative spectophotometrically and compared to NR dye recovered from untreated control cell culture. Thus the assay can be applied to determine the cytotoxicity effects of a series of extracts (Riddle *el al,* 1986). The results are expressed as ED_{50} values which can be extrapolated from dose-response curves. The ED_{50} values refer to the effective dose (µg/ml or mol/L) required to inhibit the proliferation of cell culture by 50%. Linear regression was applied to correlate log-transformed *in-vitro* cytotoxicity data.

2.7 Human Papillomavirus

Human Papillomavirus (HPV) is an 8 kilobase naked DNA virus beloging to the family *Papillomaviridae.* It is a non-enveloped virus with double stranded DNA of about 55 nm in diameter. Human papillomaviruses (HPVs) infect epithelial cells of the skin and mucous membranes, and have been linked etiologically to cervical abnormalities including precancers, cervical cancer, warts, and recurrent respiratory papillomatosis (Burd, 2003).

HPVs are also associated with other malignancies such as squamous cell carcinomas of the anus, vulva, vagina, penis, and head and neck (Arends *et al.*, 1990). HPV infect basal cells in mucosa or skin, through micro lesion, and it is strongly dependent on differential status of the epithelium for its viral life cycle which is for the development of cervical cancer.

HPV are grouped by genotypes where they are characterized by degree of DNA sequence homology within specific viral genes. HPV types are considered distinct if their genomes have less than 90% homology in the DNA sequence of the L1 open reading frame (ORFs) (Bonnez and Reichman, 2005). HPV genomes contain about 10 designated ORFs . The ORFs are located on a single strand of the viral DNA.

The HPV genome is divided to early (E), late (L) and the long control regions (LCR). Figure 2.1 shows the genetic map of HPV. Region encodes viral proteins which function in viral DNA replication, transcriptional regulation and cellular tramsformation. LCR contain cis-regulatory elements such as important transcriptional enhancers and the origin of DNA replication (zur Hausen, 1996; Howley, 2001, Howley and Lowy, 2001).

The L region contains information for viral capsid proteins. The papillomavirus capsid (the protein shell that encloses the viral DNA) is composed of 360 copies of the L1 protein (Late 1: the major capsid protein) and approximately 12 copies of the L2 protein (Late 2: the minor capsid protein) (Bonnez, 1997).

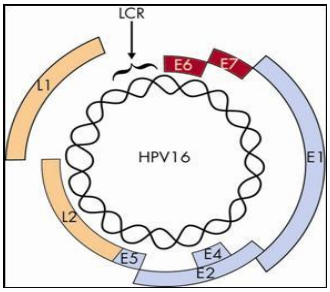


Figure 2.1: The genetic map of HPV (Howley and Lowy, 2001)

One copy of the double-stranded viral DNA is located inside the capsid shell. Five copies of the L1 protein combine to form a capsomer, and then 72 capsomers combine with one copy of viral DNA and 12 copies of the L2 protein at the center of each capsomer to form the infectious virus particle (Figure 2.2). The virus particle is an icosahedron which means that the capsomers are packed symmetrically in a regular arrangement, such that the virus particle can be rotated around several axes, but the appearance of the virus particle remains the same.

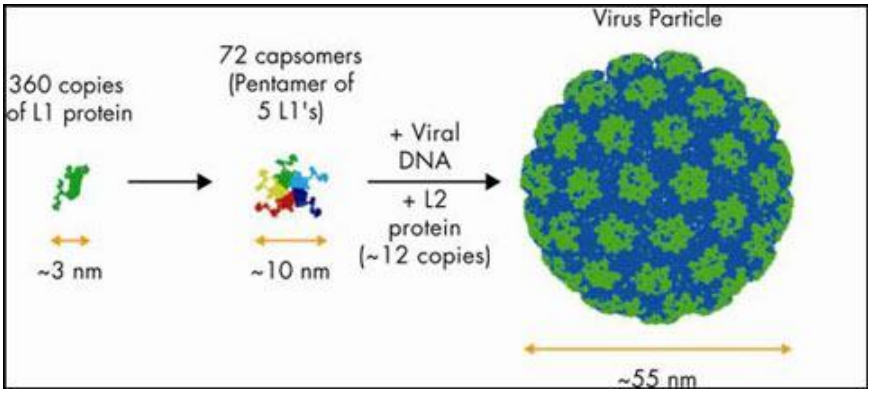


Figure 2.2: Organization of L1 and L2 proteins in the HPV particles (Bonnez 1997)

There are more than 100 distinct types of HPV that have been identified to date. Approximately 50 of these infect the epithelial membranes of the anogenital tract (Franco

et al., 2001 and Chan *et al*., 2002). Genital HPV strains are divided into two groups, based on their oncogenic potential and ability to induce viral associated tumors. The strains termed "high-risk strains" (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) are associated with intraepithelial neoplasia which more likely to progress to severe lesions and cancer (Franco & Ferenczy, 1999). Low-risk strains (HPV 6, 11, 42, 43, and 44) are associated with condylomata and low-grade cervical changes, such as mild dysplasia. Lesions due to low-risk HPV infection have a high likelihood of regression, little potential for progression, and are considered of no or low oncogenic risk (Helmerhorst & Meijer,2002).

Figure 2.3 shows the phylogenetic relationship based on DNA sequence of the gene for the L1 proteins between high-risk and low-risk HPVs (de Villiers *et al.,* 2004). There are 2 main groups of high-risk HPVs (species 5, 7, 6, and 9) and 2 main groups of low-risk HPVs (species 3, 13, 1, 8, and 10). For example, HPV-6 and HPV-11 (both low-risk typesare closely related to each other and are in the same species. HPV-6 and HPV-11 are the most common low-risk types of HPV, especially associated with warts.

Cervical cancer is one of the most common neoplastic diseases affecting women, with a combined worldwide incidence of almost half a million new cases annually, second only to breast cancer (Fronco *et al.*, 2003). HPV 16 is the most carcinogenic and most prevalent genotype detected in high grade squamous intraepithelial lesions high-grade cervical intraepithelial neoplasia and cancer of the uterine cervix (McFadden & Schumann, 2001).

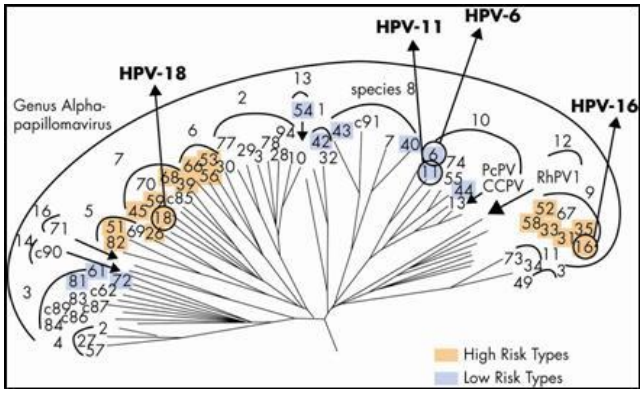


Figure 2.3: Phylogenetic Tree of the Sequences of High- and Low-risk Types of HPV

High-risk types of HPV are indicated with orange boxes, and low-risk types of HPV are indicated with blue boxes. (de Villiers, *et al.*,2004)

High-grade cervical intraepithelial lesions are most commonly associated with HPV 16, yet these strains are also frequently found to be the etiologic factor in minor lesions and mild dysplasia (McFadden and Schumann, 2001). HPV 16 is the predominant strain in almost all regions of the world, with the exception of Southeast Asia, where HPV 18 has the highest prevalence (Janicek & Averette, 2001). HPV 16 alone accounts for more than 50% of HPV infections (Jestreboff and Cymet, 2002). The targets of E6 proteins, which are expressed by HPV type 16 virus, are specific cellular transcription factors [p53, Rb family, cyclin-dependent kinase 2 (CDK 2), cyclins E and A] that regulate the cell cycle at the G1 checkpoint. The cell-cycle regulators, targeted by the oncogenes of tumor viruses play a major role in the control of G1–S progression in mammals.

In recent years, development of antiviral products for certain highly pathogenic viruses with limited available treatments, such as viruses that may have biothreat potential,

is critically important and challenging. Antiviral drugs are a class of medication used specifically for treating viral infections. Like antibiotics, specific antivirals are used for specific viruses. Antiviral drugs are one class of antimicrobials, a larger group which also includes antibiotic, antifungal and antiparasitic drugs. They are relatively harmless to the host, and therefore can be used to treat infections. For example, *Metarhizium anisopliae* is an entomopathogenic fungus that secretes different types of destruxins (Kou *et al.*, 2005). Several studies demonstrated that destruxins have insecticidal (Roberst, 1981), phytotoxic, antiviral, nematocidal, and immunomodulating activities (Jegorov *et al.*, 1992). Destruxin B has suppressive effects on hepatitis B virus surface antigen gene expression in human hepatoma cells (Chen *et al.*, 1997).

2.7.2 Immunohistochemistry

Immunohistochemistry has emerged as a powerful investigative tool that can provide supplemental information to the routine morphological assessment of cells and tissues. The use of immunohistochemistry to study cellular markers that define specific phenotypes has provided important diagnostic, prognostic, and predictive information relative to diseases status and biology. There are numerous immunohistochemistry methods that may be used to localize antigens. The selection of a suitable method should be based on parameters such as the type of specimen under investigation and the degree of sensitivity required.

Immonohistochemistry is the localization of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent

dye, enzyme, radioactive element and collodal gold. The peroxidase –labeled antibody method, introduced in 1968, was the first practical application of antibodies to paraffin-embedded tissues and overcome some of the limitations of earlier fluorescent antibody method (Nakene, 1968). These pioneering studies using enzyme labels instead of fluorocent dyes opened the door to the development of modern methods of immunohistochemistry. The early immunohistochemistry approach used polyclonal antibody which was often in short supply and sometimes exerted significant variations. There are two ways to detect viral antigen; the direct method uses only one virus-specific antibody which is directly labelled wiith an indicator (fluorescein or alkaline phosphatase) and indirect method which uses two antibodies, one virus-specific antibody to bind the viral antigen and the second antispecies antibody to be labelled with the indicator (Lakeman, 1997).

In 1981, a new generation of immunohistochemical methods emerged with advent avidin-biotin methods, which remains widely used today (Hsu *et al.,* 1981). All avidin-biotin methods rely on strong affinity of avidin or strepavidin for the vitamin biotin. Strepavidin (from *Streptomyces avidinii*) and avidin (from chicken) both possess four binding sites for biotin. The biotin molecule is conjugated easily to antibodies and enzymes. In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex (Heras *et al.*, 1995).

In a similar method, the labelled strepavidin-biotin (LSAB) method also utilizes a biotinylated secondary antibody that links primary antibodies to a strepavidin-peroxidase conjugate. Since avidin is a glycoprotein and has an isoelectric point (pI) of 10, it has a

density to non-specifically bind to lectin-like and negatively charged tissue components at physiological pH. It has been largely replaced today by streptavidin. The test samples are incubated with primary specific antibody against the viral antigen. Then a secondary biotinylated antibody capable of binding to the primary antibody followed by Horse-raddish peroxidase (HRP) enzymes – conjugated with avidin or strepavidin to form a biotin –strepavidin HRP complex. Finally the test samples were exposed to a 3'-diaminobenzidine tetrahydrochloride (DAB) substrate. Upon reaction with HRP, the DAB subtrate will form a nondiffusing and insoluble dark brown precipitate at the site of the viral antigen (Forghani and Hagens, 1995). In both methods a single primary antibody subsequently is associated with multiple peroxidase molecules, and because of the large enzyme-to-antibody ratio, a considerable increase in sensitivity is achieved compared to direct peroxidase-comjugated method.

3.0 MATERIALS AND METHODS

3.1 *Penicilliopsis* sp. Culture and Maintenance

The mycelial cultures of *Penicilliopsis* sp. (KUM60280) was maintained on Malt Extract Agar, MEA (as described in Appendix A) slants and deposited at the Mycological Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya.

3.2 Production of Mycelial Biomass

Mycelial inoculum for liquid fermentation was prepared by inoculating centrally a plug taken from the outer rim of a mycelial colony onto Glucose-Yeast-Malt-Peptone agar, GYMP (Appendix A) and incubated for 4 days at 25^{o}C. Then, seven –mm plugs cut from outer rim of the mycelia colony using a sterile cork borer was aseptically inoculated into 50ml sterile GYMP in 250ml Erlenmeyer flasks. The inoculated mycelial were incubated at 25^{o}C for seven days on shaker incubator (Sanyo, Orbital Incubator) rotating at 200 rpm. After incubation, mycelia broth was harvested and freeze-dried.

3.3 Preparation of Extracts

Dried mycelia biomass was crushed into powder and extracted by soaking twice in methanol and dichloromethane (DCM) separately. Approximately 150ml methanol (Mallinckrodt Chemicals, USA) or dichloromethane was poured into 250ml Erlenmeyer flasks containing powdered mycelia biomass and shaken for 48 hours in rotary shaker rotating at 200 rpm at 25^{o}C. The mixtures were then filtered through (Whatman No. 1). The

methanolic or DCM filtrate was dried using a Büchi RotavaporR-114 (Switzerland) to yield the methanolic or DCM crude extract.

3.4 Determination for Antimicrobial Activities in *Penicilliopsis* sp.

3.4.1 Microorganisms Tested

Gram-positive pathogenenic bacteria comprising of *Bacillus subtilis*, *B. cereus* and *Staphylococcus aureus*, Gram positive oral bacteria, *Streptococcus sanguis, S. mutans and S. mitis*) and Gram-negative bacteria comprising of *Pseudomonas aeruginosa*, *P. vulgaris* and *Escherichia coli* were used as test organisms. Fungal species comprising of *Candida albicans*, *C. parapsilosis* and *Saccromyces pombe* were used to determine antifungal activities. The microorganisms tested were inoculated from Mycological Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya.

3.4.2 Preparation of Bacteria and Fungal Inoculum

Mueller-Hinton Agar nd Muller- Hinton Broth (MHB) (Appendix A) were used to grow Gram-positive bacteria. Sabouraud liquid medium (Appendix A) and Sabouraud dextrose agar (SDA) were used for growing of fungi. Inoculum was prepared by picking colonies from 24 hours old cultures. The density was adjusted by spectrophotometer to that of 0.5 McFarland standard at a wavelength of 530 nm to yield a stock suspension of 1×10^6

colony forming units per ml (cfu/ml) for bacterial suspensions and 2.5 x 10^3 cfu/ml intended for fungi.

3.4.3 Antimicrobial Activity of *Penicilliopsis* sp.

Antibacterial and antifungal activity of crude extracts was determined using disc diffusion assays. Modified agar diffusion method (Bauer *et al*., 1966) was used to determine antibacterial and antifungal activities. *Penicilliopsis* sp. extracts were dissolved in dimethylsulphoxide (DMSO) to obtain a concentration of 20mg/mL. DMSO 10% did not inhibit growth of bacteria and fungi. Briefly, a suspension of the test microorganisms (0.1 ml of 1 x 10^6 cfu/ml for bacteria and 0.1 ml of 1 x 10^5 cfu/ml for fungi and yeast) was lawned on appropriate agar in Petri plates (Muller-Hinton agar for bacteria and sabouraud dextrose agar for yeast). Sterile filter paper disc (6mm diameter) was impregnated with 20µl of the extract and then placed on the inoculated plates. Plates lawned with bacteria were incubated for 24 hours at 37^{o}C and 48 hours for fungi and yeast at 35^{o}C.

Commercial antibiotic namely Chloromphenicol (30µg/disc), Streptomycin (30µg/disc) and Kanamycin (30µg/disc) (Sigma, Madrid) were used as antibacterial standards while nystatin (100U Sigma, Madrid) was used as antifungal standard to determine the sensitivity for microbial species tested. The diameter of inhibition zones were measured in millimeters (ml). Inhibition zone diameters around each disc were measured and recorded at the end of the incubation time. The values measurements of inhibition zones were the average of three replicates.

3.5 Antioxidant Activity of *Penicilliopsis* sp.

The methanol and dichloromethane crude extracts were tested for antioxidant activity using 1-1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging assay and reducing power assay.

3.5.1 Scavenging Activity on 2-2-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) Radicals

DPPH radical scavenging activity was determined based on a procedure described by Brand-Williams *et al*. (1995) with a slight modification. One mM $DPPH^{\cdot}$ (Sigma-Aldrich) solution was prepared by dissolving 0.0039g of DPPH powder in 10ml methanol. The 1 mM solution was further diluted to 0.06 mM with methanol. Stock solutions of each dried extracts were prepared by dissolving 0.05g of dried extract in 1 ml methanol. An aliquout of 3.9ml of DPPH was added up to methanol and dichloromethane extracts to obtain 10-100mg/ml of solution.

The solution was mixed vigorously using a Shimadzu UV-1240-UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The decrease in absorbance was recorded at 0, 1, 2 minutes and every 15 minutes until the reaction reached a plateau. The steady state was determined by one-way analysis of variance (ANOVA) to compare decrease in absorbance values. Reaction reached a steady state after 1 hour at room temperature. in Antioxidant activity was compared with L-ascorbic acid (Sigma-Aldich) and Butylated hydroxyanisole, BHA (0.1mM-1.0mM) as a positive standard. All determinations were

performed in triplicates. The scavenging activity on DPPH was expressed as percentage radical scavenging calculated follows:

$$\% \text{ radical scavenging effect} = \frac{A_0 - A_S}{A_0} \times 100\ \%$$

A_0 refers to the absorbance of 0.06mM DPPH methanolic and DCM solution only whereas A_S the absorbance of the reaction mixture. The radical stock was prepared fresh daily.

3.5.2 Reducing Power Assay

The reducing power of *Penicilliopsis* sp. extracts were determined according to a modification method of Oyaizu (1986). Briefly, each extract in varying amounts of 5mg, 10mg, 15mg and 20mg was dissolved in one ml of methanol and subsequently 2.5 ml of 0.4M phosphate buffer (pH6.6) and 2.5ml 0f 1% (w/v) solution of potassium ferricyanide were added. The mixtures were incubated in water bath at 50^{o}C for 20 minutes. Following this 2.5ml of 10%, (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 650-x g for 10 minutes. Than, 2.5 ml aliquot of the upper layer was combined with 2.5 ml distilled water and 0.5 ml (500ul) of a 0.1% (w/v) solution of ferric chloride. Absorbance of reaction was read spectophotometrically at 700 nm. Increased absorbance of the reaction mixture indicates greater reducing power.

3.5.3 Statistical Analysis

Data were recorded as means ± standard deviations and analysed by SPSS (version 14 for Windows). One-way analysis of variance (ANOVA) was carried out to test for any significant differences between the means; the mean values of antioxidant activities between two extracts or two treatments were analyzed by independent-samples t-test. P-values less than 0.05 were considered to be statistically significant [*P <0.05; **P< 0.01;***P<0.001]. All analyses were performed in triplicates.

3.6 Total Phenolic Content of Extract

Total phenolic compound were determined as gallic acid equivalents (GAE) Follin-Ciocalteau's phenol reagent (2.0N), L-ascorbic acid, BHA and Gallic acid (3, 4, 5-trihydrobenzoic acid, Formula Molecule= $C_7H_6O_5$, Formula Weight = 170.12) were obtained from Sigma-Aldrich, whereas sodium carbonate (Na_2CO_3) was purchased from R & M Chemicals, United Kingdom. Solutions were prepared in distilled water at room temperature.

This method was a modification of that reported by Singleton and Rossi (1965). Two hundred and fifty microliters of 10% Follin-Cicocalteau's phenol reagent were added to 250µl of extracts (0.1 mg/ml) in 1.5 ml disposable plastic cuvettes and then mixed thoroughly. After three minutes, 500 µl of 10 % Na_2CO_3 was added to the mixture. After incubating for an hour in dark condition, the intensity of the blue colour was measured at 750 nm. A standard calibration curve was constructed using gallic acid with concentrations

1-5 µg/ml. Blank consisting of distilled water plus reagent without the extract samples and with L-ascorbic acid and BHA was prepared.

The absorbance values were converted to total phenolic content calculated based on the gallic acid calibration curve and expressed as gallic acid equivalents (GAE) in mg/g extracts. Three replicates were prepared for each concentration of extracts. The total of phenolic content was calculated using a calibration curve of gallic acid.

3.7 Evaluation of Cytotoxic Activity of *Penicilliopsis* sp. Extracts

3.7.1 Preparation of Stock Solution

Twenty milligrams of methanol and dichloromethane crude extract, respectively, were weight dissolved in 1 ml of Dimethyl Sulfoxide (DMSO, Sigma) in a 1.5 ml provial to provide a stock solution of 20 mg/ml and stored at -20^{o}C (Freezer, Acson) until use.

3.7.2 Cell Lines

In this study, six cancer cell lines and one normal cell line were used to analyse the cytotoxic activity of *Penicilliopsis* sp. (KUM60280). Human mouth epidermal carcinoma cell line (KB), Human epidermal carcinoma of cervix cell line (CaSki), Human colorectal cancer cell line (HT29), (HCT119), Human breast cancer cell line(MCF7), Human ovarian cancer cell line (SCOV3) and Human fibroblast cell line which is a normal cell line (MRC5) were purchased from American Tissue Culture Collection (ATCC, USA).

3.7.3 Maintenance of Cell

The cells were examined daily using an inverted microscope (Olympus) for signs of bacterial or fungal contamination. The 20% supplemented Rosewell Park Memorial Institute (RPMI) 1640 medium in the flask was replaced with fresh 10 % supplemented RPMI 1640 medium when colour change was observed. The cells were maintained in 10% supplemented RPMI 1640 medium in a 5% CO_2 incubator. Media change was performed at a twice-weekly interval or based on the colour changes from time to time. The cells were subcultured when the cell concentration exceeded 2 X 10^6 per ml.

3.7.4 Revival of Cells Lines

A provial containing cell line was removed from liquid nitrogen tank and plunged into a beaker of ice. It was then transferred to 37^{o}C water bath (Grand Instruments) for quick thawing using gentle hand agitation. The cells were transferred into a centrifuge tube (Falcon) containing 7 ml of 20% supplemented medium 199, DMEM, Mccoys, and RPMI 1640 medium and spun at 1000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 5 ml of 20% sumplemented medium and incubated in a 25 ml tissue culture flask at 37^{o}C in a 5% carbon dioxide (CO_2) water jacketed incubator (Jouan).

3.7.5 Subcultivation of Cells

Adherent cells attached and formed a single layer in a culture flask. The medium in the tissue culture flask was discarded. Confluent cells were washed using 10 ml of sterile phosphate buffered saline (PBS) pH 7.2. The cells were detached from the flask by incubating with 1 ml 0.25% accutase solution and 3 ml PBS solution for 5 to 10 minutes at 37^{o}C, and then sharply tapped to detach the cells from the flask. After the cells have detached, the cell suspension was transferred into a centrifuge tube and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and the cell pellet was resuspended in 3 ml of 10% supplemented medium. The uspension was split and transferred into different culture flasks each containing 7 ml culture media. The flasks were incubated at 37^{o}C in a 5% CO_2 incubator.

3.7.6 Cryopreservation of Cells

The cells with subconfluent or confluent monolayer were fed with fresh 10% supplemented RPMI 1640 medium 24 hours before freezing. The medium in the tissue culture flask was discarded and the cells were washed with 10 ml of sterile PBS pH 7.2. The cells were detached from the flask as described in section 3.7.5. After centrifugation, supernatant was discarded and the cell pellet was resuspended in 3 ml of freezing medium. The cell suspension was aliquoted in 1 ml amounts into 3 provials. The provials were left to stand in ice and kept in a polystyrene box. The box was kept in -70^{o}C freezer overnight after which, the provials were transferred and stored in a liquid nitrogen tank.

3.7.7 Serial Dilution of Extract Stock Solution

Aliquots of 10 µl from the extract stock solution (20 mg/ml) was diluted in 90 µl of sterile distilled water to produce a stock concentration of 2,000 µg/ml. It was then further diluted with 10% DMSO to provide final concentrations of 200 µg/ml and 20 µg/ml.

3.7.8 Incubation of Cell Lines with Extracts

The cells with confluent monolayer were detached from the flask as described before. Cells were detached from the flask with 0.25% accutase and washed in culture medium. The cell pellet was obtained by centrifugation at 1000 rpm for 5 minutes to remove fine dye crystals. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1 ml of 10% supplemented medium to produce a stock cell suspension. For cell enumeration, 100 µl of the stock cell suspension was transferred into a provial with 900 µl of 0.4% tryphan blue and mixed well. The haemacytometer (Scherf) was covered with a glass cover slip and 20 µl of the suspension with dye was loaded at two edges of the cover slip so that it could flow into the chambers by capillary action at the area. The haemacytometer was then examined under an inverted microscope and unstained viable cells were counted.

The cell suspension at a concentration of 3×10^4 cells/ml (concentration of 5×10^4 cells/ml was used for cell lines) was prepared according to the formula below:

$$P_1 \times 10^5 \times V_1 = P_2 \times V_2$$

P_1 : Average number of viable cells counted from the haemacytometer

10^5 : Counting chamber conversion factor & dilution factor dye

V_1 : Volume of stock cell suspension needed

P_2 : The desired cell concentration in the cell suspension

V_2 : Volume of 10% supplemented medium used for seeding

Cells were then plated in 96-well microtiter plate, at a concentration 30 000 cells/ml for each cell lines in a total volume of 190-200µl. A total of 200 µl of the cell suspension with known cell density was transferred into every well of a 96-wells plate (Nunc) and incubated overnight in a 5% CO_2 incubator 37^{o}C to allow the cells to adhere before addition of the extracts. The plate was observed under microscope and medium was discarded when the cells achieved 60-70% confluence. The cells were then incubated with fresh extracts solution serially diluted in 10% supplemented medium at varying concentrations (1, 10, 25, 50, 75 and 100 µg/ml) for 72 hours at 37^{o}C in a 5% CO_2 incubator. All the tests were performed in triplicates and untreated cells with just growth medium served as negative controls.

3.7.9 Neutral Red Cytotoxicity Assay

After the 72 hours incubation period, the medium with or without extracts was removed followed by the addition 200 µl of neutral red (NR) dye into respective wells. The plate was further incubated for 3 hours to allow maximum uptake of neutral red dye by the viable cells. The dye was discarded and the cells were rapidly washed with pre-warmed

washing solution. The neutral red dye was extracted from the viable cells by adding 200 µl of warm NR resorb solution into every well and was left to incubate at room temperature for 30 minutes in a bench top incubator (LT biomax 500). The plate was then gently agitated on a microplate shaker (LT Biomax 500) for 15 minutes and the optical density (OD) was determined spectophotometrically at 540 nm using an ELISA reader (Titertek Multiskan ® MCC/340).

The mean of measurements for each concentration was determined (*n*=3). The average data of the triplicates for each extracts concentration was expressed in terms of killing percentage relative to negative controls. The percentage of inhibition of each of the extract was calculated according to the following formula:

$$\% \text{ of inhibition} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

The ED_{50} is the concentration of extract that causes 50% inhibition or cell death. ED_{50} for each extract was extrapolated from the dose-response curves plotted. An extract that gave ED_{50} of 20µg/ml or less is considered active for the cytotoxic assay (Gerah *et al*.,1972). Linear regression analysis was used to compute the concentration of test agent needed to reduce the absorbency of NR by 50% (termed the midpoint cytotoxicity, or Effective Dose, ED_{50}).

3.8 Analysis for Anti-HPV 16 E6 Oncoprotein Activity of *Penicilliopsis* sp.

3.8.1 Cell Line

The cell line used in this study was the HPV16 containing cell line, CaSki.

3.8.2 Serial Dilution of *Penicilliopsis* sp. Stock Solution

Five different extract concentrations were used to study the anti-HPV 16 activity of *Penicilliopsis* sp. (KUM60280) extracts. Aliquotes of 180 µl from each extract stock solution (20 mg/ml) was diluted in 2.82 ml of sterile distilled water to produce a stock concentration of 200 µg/ml. Further dilution was carried out to provide final concentration of 600 µg/ml, 450 µg/ml, 300 µg/ml, 150 µg/ml and 75 µg/ml respectively.

3.8.3 Incubation of CaSki Cells with Extracts

One milliliter of serially diluted stocks were added into five tissue culture flasks containing cultured CaSki cells and 2 ml of fresh 10% supplemented RPMI 1640 medium to provide a final concentration of 200 µg/ml, 100 µg/ml, 50 µg/ml and 25 µg/ml, respectively. The cells were left to incubate with the extracts in a 5% CO_2 incubator at 37^{o}C for 72 hours. CaSki cells not treated with extracts of *Penicilliopsis* sp.(KUM60280) served as negative control.

3.8.4 Fixation of CaSki onto Slides

After 72 hours incubation period, the Caski cells were detached from the flask as described in 3.7.6. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1.2 ml of PBS pH 7.2. A total of 30µl of cell suspension was transferred into every well of three teflon coated slides (Immuno-cell Int.). The slides were left to dry overnight at room temperature. The slides were fixed with acetone (System) at -20^{o}C until use in immunohistochemical staining.

3.8.5 Detection of Human Papillomavirus (HPV) E6 protein Using 2-step Indirect Avidin-Biotin Immunoperoxidase Method

Immunohistochemical staining was carried out using the labeled Strepavidin Biotin Peroxidase kit (LSAB) and 3'-diaminobenzine tetrahydrochloride (DAB) Substrate system (Dako) according to specifications describe. All washing steps required constantshaking and incubationwith reagents were carried out in a humidified chamber.

The slides were immersed in decreasing concentrations of ethanol (Schlarlau) – 100 %, 95%, 90% and 80% for 2 minutes each and then washed in PBS pH7.6 for 10 minutes on the rocker platform (Bellco Biotechnology). The surrounding areas of each well were blotted dry using filter paper (Whatman) and 50 µl of 3 % hydrogen peroxide (BDH) was added onto every well to remove the endogenous peroxidase activity. The slides were

incubated for 10 minutes at 37^{o}C followed by washing in PBS pH 7.6 for 5 minutes on the rocker.

The surrounding areas of wells were blotted dry and 10 µl of diluted HPV16 E6 antibody (Chemicon), were diluted in 1:50 with sterile distilled water (10 µl/ml + 490 µl/ml sterile distilled water) added to four wells on the one end of the slide. 30µl of sterile distilled water was added to four wells on the other end of slide. These cells, which were untreated with the anti-HPV 16-18E6 antibody, served as negative controls. The slides were incubated again in a bench top incubator (LT Biomax 500) at 30^{o}C for 1 hour. Later, slides were washed twice in PBS pH 7.6 for 15 minutes each. Afterwards 30µl of secondary antibody were added to each well followed by incubation at 37^{o}C for 30 minutes then added with 30µl biotinylated anti-mouse and anti-rabbit immunoglobulin (Dako) streptavidin – horse radish peroxidase (Dako).

Subsequently slides were washed in PBS pH 7.6 for 5 minutes, after which, the wells were filled with 30µl of liquid DAB substrate – cromogen (Dako) which is mixture of 40µl of chromogen and 2ml of DAB subtrate and incubated for 20 minutes at 37^{o}C until the desired colour intensity developed. The slides were rinsed in distilled water and then counterstained with Mayer's hematoxylin solution (Fluka) for two minutes. The slides were rinsed again with distilled water and then immersed into 30% ammonia solution (Systerm) for 10 seconds. After the last rinse with distilled water, the slides were mounted with pre-warmed glycergel (53^{o}C, Dako), covered with covered slip and left to dry overnight in the dark. The stained slides were visualized under the light microscope (Olympus).

4.0 RESULT

4.1 Crude Extract and Mycelia Biomass from *Penicilliopsis* sp.

Penicilliopsis sp. (KUM60280) sporulated after 4 days grown on Glucose-Yeast-Malt-Peptone (GYMP) agar at 25^{o}C. Colony diameter on GYMP reached 5 cm diameter, velvety, with crenate, subsurface margins, amber to honey, with synnemata abundantly produced branched with pure yellow pigments diffused into media, while the underslide turn yellowish in colour. Plate 4.1 showed sporulation of synemata region on surface of colonies. Figure 4.1 (a and b) illustrates the morphology of *Penicilliopsis* sp. showed oval shaped conidia located in an ascus.

Plate 4.1 *Penicilliopsis* sp. (KUM 60280) mycelia growing on Glucose-Yeast-Malt-Peptone agar

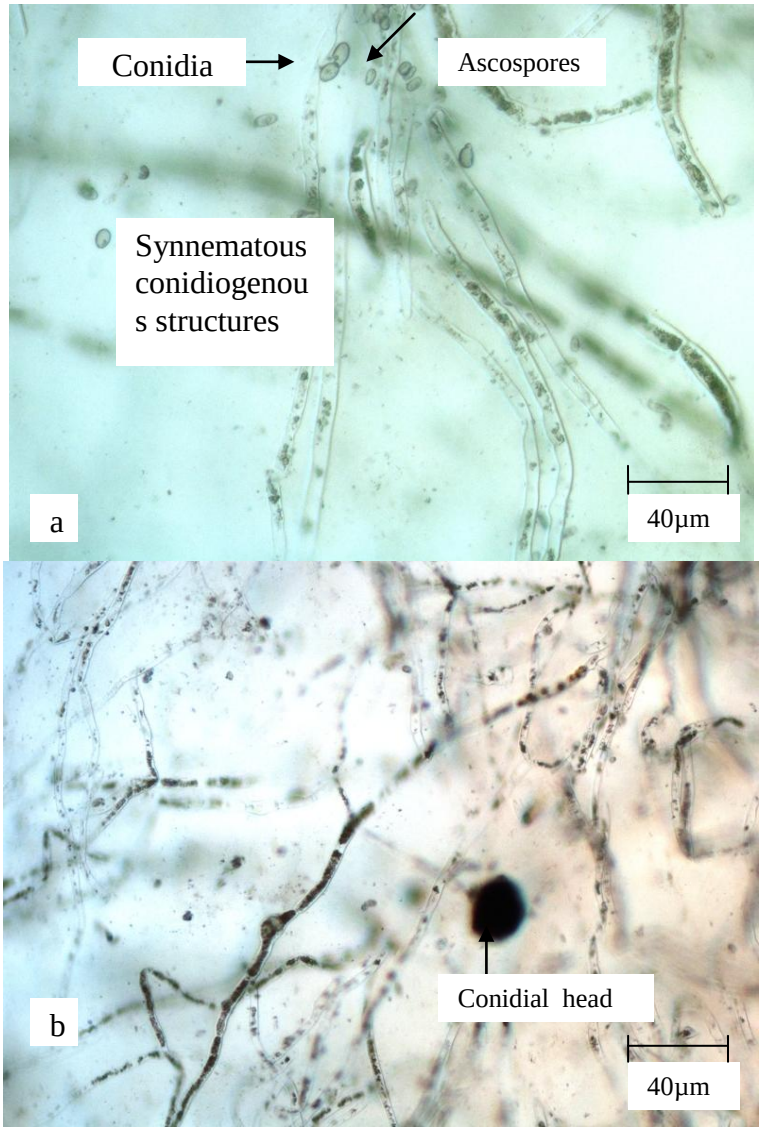


Figure 4.1 (a and b) Morphology of *Penicilliopsis* sp. using light microscope of 400 X magnification.

Table 4.1 shows the yield of mycelial biomass and the yield of crude extract in methanol and dichloromethane from culture grown in Glucose-Yeast-Malt-Peptone (GYMP) media. The amount of mycelial biomass for methanol extract was 31.19 gram/gram whereas mycelial biomass for DCM was 19.93 gram/gram. Extract yield in methanol showed the higher percentage then DCM. This might indicate that most of the solutes in methanol extract were polar components.

Table 4.1 Percentage yield crude extracts of *Penicilliopsis* sp. (KUM60280)

Type of Extract	Mycelial biomass (g/ml)	Crude extract dry weight (g) / (g)
Methanol	350.28	31.19
Dichloromethane	218.63	19.93

4.2 Antimicrobial Activity of *Penicilliopsis* sp.

Antibacterial and antifungal activities of crude extracts were determined using modified disc diffusion method (Bauer *et al*., 1966). A total 12 microorganisms consisting of Gram positive and Gram negative bacteria and yeast were tested. The *in-vitro* antimicrobial activity was assessed by the diameter size of inhibition zones.

Standard antibiotics (streptomycin, kanamycin, chloramphenicol and nystatin) were used as a positive control. The dimethyl sulfoxide (DMSO) used to dissolvethe extract was a negative control and did not show any inhibiton against all microorganisms. Table 4.2 presents the zones of inhibition produced by *Penicilliopsis* sp.extracts.

Methanol extract did not show any antimicrobial activity against all test microorganisms. Dichloromethane extract of *Penicilliopsis* sp. displayed low inhibition activity against all test bacteria and fungi ranging from 7-9.7mm diameter. Inhibition zones against all microorganisms were detected in halo, indicating a weak inhibition. The most

susceptible species was *Bacillus cereus*, followed by *Escherichia coli*, while the highest antifungal activity was agains *S. pombe*.

4.3 Antioxidant Activity of *Penicilliopsis* sp.

Extracts of *Penicilliopsis* sp. were subjected to screening for their possible antioxidant activity. Antioxidant capacity was evaluated using $DPPH^{\cdot}$ free radical-scavenging and reducing power.

4.3.1 $DPPH^{\cdot}$ Scavenging Effect

The $DPPH^{\cdot}$ scavenging method, utilizes free radical with a characteristic absorption at 517 nm which measures a violet solution. Antioxidant activity was defined as the amount of antioxidant necessary to decrease the initial $DPPH^{\cdot}$ concentration by 50%, which represent the effective dose (EC_{50}). Figure 4.2 illustrates the free radical scavenging of extracts tested at various concentrations.

Free radical scavenging of ascorbic acid and butylated hydroxylanisole (BHA) were also determined and acts as a positive control (Figure 4.3). Ascorbic acid and BHA were observed to react quickly with the $DPPH^{\cdot}$ radical.

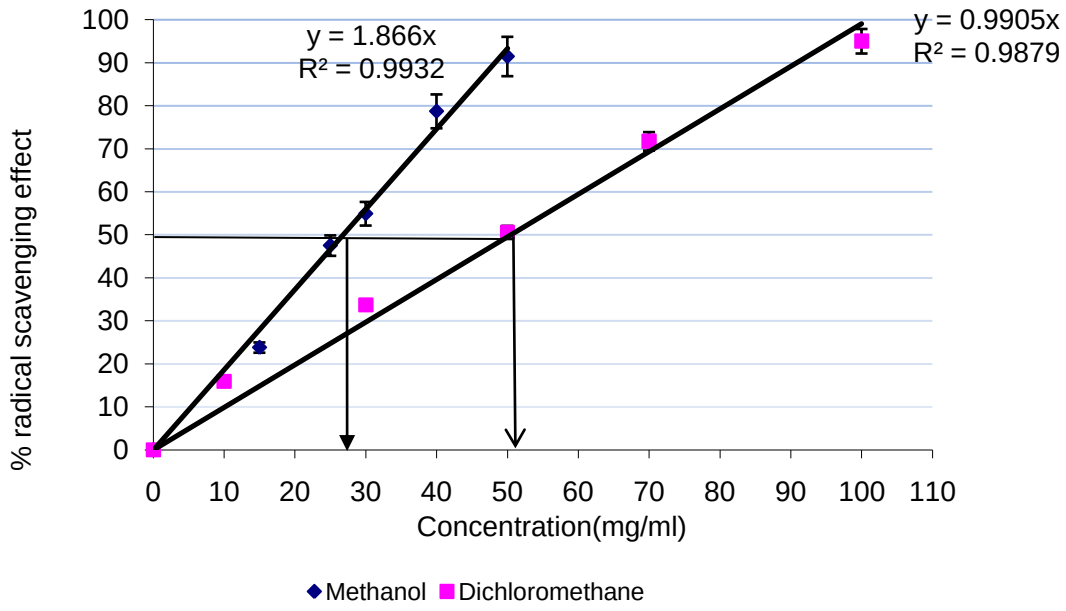


Figure 4.2 DPPH scavenging activity of *Penicilliopsis* sp. mycelia extracts.

The EC_{50} value of positive control which is ascorbic acid is 0.1 mM or 100 µM equivalent to 0.078 mg/ml while EC_{50} for BHA is 0.2 mM or 200 µM which is equivalent to 0.11 mg/ml. Free radical scavenging activity of the extracts exhibit dose dependent response. The EC_{50} was determined after 60 minutes i.e. the time taken for maximum DPPH radical being scavenged (Appendix).

Table 4.2 Antimicrobial activity of the extract of *Penicilliopsis* sp. (KUM60280) (20 µg/disc) against test microorganisms using disc diffusion method

	Diameter of inhibition zone (mm)				
Microorganisms/ Extract	**Dichloromethane**	**Kanamycin**	**Streptomycin**	**Chloromphenicol**	**Nystatin**
Gram positve bacteria					
Bacillus cereus	9.67 ± 1.70	29.3 ± 1.40	30.0 ± 0.40	33.7 ± 1.20	NT
Bacillus subtilis	8.33 ± 0.5	2.20 ± 0.80	2.30 ± 1.40	3.30 ± 3.60	NT
Satphylococcus aureus	8.33 ± 0.5	2.57 ± 1.70	1.93 ± 1.40	23.0 ± 4.90	NT
Oral bacteria (Gram +ve)					
Streptococcus mitis	7.30 ± 1.20	23.7 ± 1.20	18.0 ± 0.4	18.0 ± 1.40	NT
Streptococcus mutans	7.70 ± 0.40	15.30± 1.70	18.7 ± 1.70	16.7 ± 1.90	NT
Streptococcus sanguis	8.30 ± 0.50	17.7 ± 0.05	18.7 ± 2.60	17.7 ± 1.20	NT
Gram negative bacteria					
Escherichia coli	8.70 ± 1.20	26.0 ±1.20	20.3 ± 1.40	24.7 ± 2.10	NT
Pseudomonas aeruginosa	8.33 ± 0.4	29.0 ± 0.8	24.7 ± 1.90	33.7 ± 1.90	NT
Pseudomonas vulgaris	7.67 ± 0.4	31.0 ± 2.90	22.3 ± 0.50	33.0 ± 1.40	NT
Antifungal Activity					
Candida albican	7.00 ± 0.8	NT	NT	NT	26.3 ± 0.8
Candida parapsilosis	7.67 ± 0.5	NT	NT	NT	2.78 ± 0.9
Saccromyces pombe	8.00± 0.5	NT	NT	NT	2.20 ± 1.60

Values are means ± SD (mm) of three separate experiments; '-'no inhibition zone; NT: not tested

*No inhibition were shown by the methanol extract

The concentrations of methanol extract were 15, 25, 30, 40 and 50 mg/ml, and dichloromethane extracts were 10, 30, 50, 70 and 100 mg/ml. The effective concentrations of each fraction and reference compound required to scavenge 50% of the DPPH radicals. The methanol extracts showed better DPPH radical scavenging activity rather than dichloromethane extract. The polar methanol extract was able to reduce the stable free radical 2,2-diphenyl-1-picrylhydrazine (DPPH) to yellow-colored DPPH with an EC_{50} 26.8 mg/ml whereas EC_{50} of dichloromethane extract was 50.5 mg/ml.

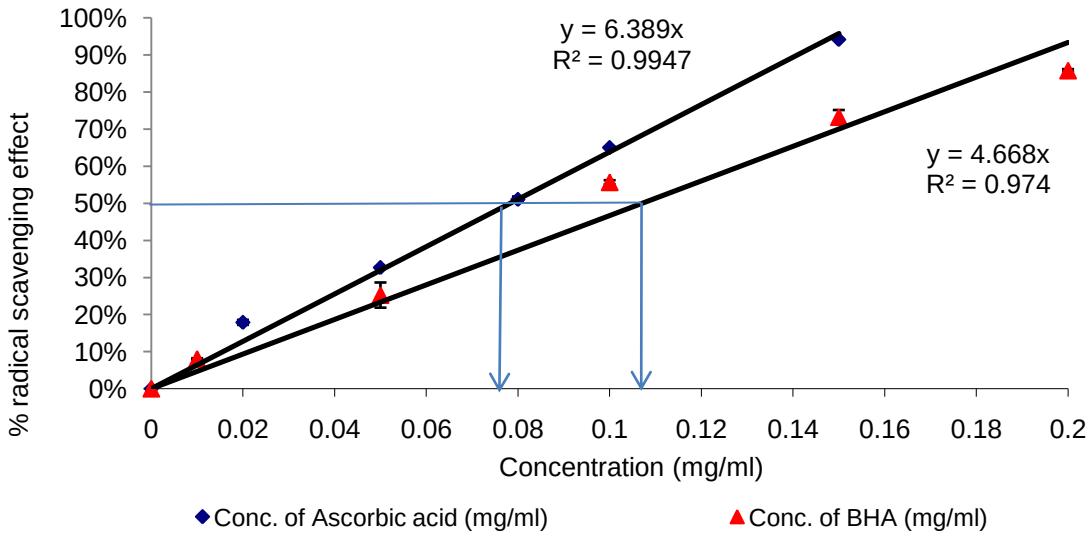


Figure 4.2 DPPH scavenging activity of ascorbic acid and butylated hydroxylanisole (BHA).

The synthetic antioxidant agent, ascorbic acid and BHA still have shown better antioxidant activity because the EC_{50} values of ascorbic acid and BHA were 0.078 mg/ml and 0.11 mg/ml respectively. The effective concentration of extracts and positive control required to scavenge 50% of the DPPH radical, (the EC_{50} values) are presented in Table

4.3. Methanolic extract showed higher scavenging activity with EC_{50} value of 26.8 mg/ml while dichloromethane extract showed EC_{50} value of 50.5 mg/ml.

Table 4.3 Scavenging effects of fractions of *Penicilliopsis* sp. extracts on $DPPH^{\cdot}$ radical

Sample	EC_{50} (mg/ml)
Dichloromethane	50.5
Methanol	26.8
Ascorbic Acid	0.078
BHA	0.11

4.3.2 The Reducing Power Assay

Total reducing power was determined as described by Oyaizu, (1986) with modification. An increased in absorbance of the reaction mixture indicated increased reducing power. Figure 4.4 kshows the reductive capabilities of *Penicilliopsis* sp. extracts compared to ascorbic acid and butylated hydroxylanisole (BHA) as a positive control.

The reducing power of both crude extracts from *Penicilliopsis* sp. increased with the concentration. The reducing powers of methanolic extract and dichloromethane extracts from mycelia were 1.161 and 0.431 mg/ml, respectively at 5 mg/ml. However, the reducing power of ascorbic acid and BHA showed excellent reducing powers of 1.933 and 2.539, respectively which are significantly higher than crude extracts from *Penicilliopsis* sp. (KUM60280) at the same concentration.

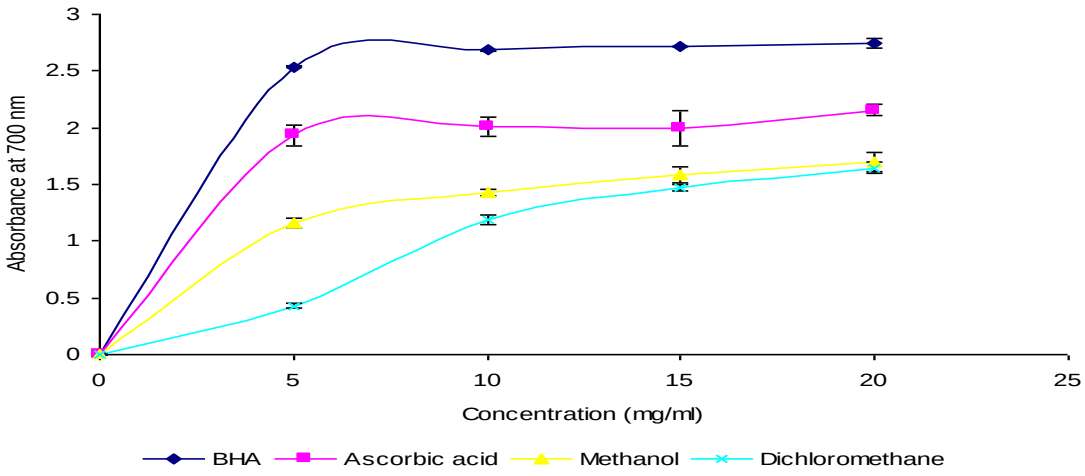


Figure 4.4 Reducing power of *Penicilliopsis* sp. extracts, ascorbic acid and butylated hydroxylanisole (BHA). Average of three replicates.

4.4 Total Phenolic Content assay

The Follin-Ciocalteau was used to measure the total phenolics content in the *Penicilliopsis* sp. (KUM60280) extracts. Results were expressed as gallic acid equivalents (mg/100ml). The antioxidant activity has been found to be correlated to the total phenolic content due to their property of scavenging free radicals. Therefore, the correlation of total phenolic content of the *Penicilliopsis* sp. extracts.

Table 4.4 Amounts of total phenolics content equivalent gallic acid (GAE) in g GAE/100 g extracts

Samples	g/(GAE)/100 g extracts
Methanol extract	107.87
Dichloromethane extract	58.43
BHA	9723.25
Ascorbic acid	932.9

The total phenolic content of Penicilliopsis sp. (KUM60280) extracts expressed as a gallic acid equivalent (GAE) per 100 gram extracts are presented in table 4.4. The total phenolics content in methanol extract was significantly higher than dicloromethane, respectively, found to be 107.87g GAE/100 g methanol and 58.43g GAE/100 g extract, respectively. The result indicated that the higher the antioxidant activity obtained for the methanol fraction, the higher is the total phenolic content.

4.5 Cytotoxicity Activity of *Penicilliopsis* sp.

In the present study, methanol and dichloromethane extracts from *Penicilliopsis* sp. were evaluated for their cytotoxic activities against human mouth epidermal carcinoma cell line (KB), human epidermal carcinoma of cervix cell line (CaSki), human colorectal cancer cell line (HT29), (HCT119), human breast cancer cell line (MCF7) human ovarian cancer cell line (SCOV3) and normal human fibroblast cell line. The cells were grown using different medium such as RPMI 1640, DMEM, Mc COYs, MEM, DMEM and 199 medium. The cell lines were incubated with methanol and dichloromethane extract of *Penicilliopsis* sp. at varying concentrations of 1, 10, 25, 50, 75, and 100 μg/ml for 72 hours in a 5 % carbon dioxide (CO_2) at 37^{o}C. The negative controls consisted of cells lines not treated with *Penicilliopsis* sp. extracts.

The cells were exposed to the extracts at various concentrations. Generally, based on NR assay, methanol and dichloromethane extracts of *Penicilliopsis* sp. were considered not cytotoxic against the cell lines tested. Figures 4.6 to 4.12 were plotted, to show different cytotoxic profiles when treated using different crude extracts from *Penicilliopsis* sp. The

cytotoxic effect of crude extracts of *Penicilliopsis* sp. against cell lines increased with increasing concentrations of extracts, showing killing percentage. The present study shows that the pattern of killing percentages shown by methanol extract ranged from 20-30 % at 1 µg/ml, 30—40% at 10 µg/ml, 40-55 % at 50 µg/ml, 55-60% at 75 µg/ml and 70-80 µg/ml at 100 µg/ml. While dichloromethane extract ranged from 1-10% at 1 µg/ml, 20-30% at 10 µg/ml, 30-40% at 25 µg/ml, 40-50% at 50 µg/ml, 50-60% at 75 µg/ml and 60-70% at 100 µg.

The cytotoxic data obtained were expressed as a killing percentage relative to negative controls. ED_{50} value is the concentration of extract which results in 50% killing of cells. The ED_{50} values were extrapolated from the dose-response curves plotted cell employed more than 50% cytotoxicity inhibition. Results exerted that the ED_{50} of MCF7 cells line against crude methanol extract and dichloromethane extract was 56.221µg/ml and 48.912 µg/ml, respectively. The ED_{50} of methanol extract in MCF7 appeared is more potent compared their ability to retard more than 50% of cell growth even at low concentration as 1 µg/ml. It was followed by CaSki cell; results show that the extracts marked cytotoxicity effect was 40.183 µg/ml for methanol and 41.451 µg/ml for dichloromethane. KB cells lines exerted less cytotoxicity effects by given lower ED_{50} were 40.882 µg/ml for methanol and 30.063 µg/ml dichloromethane respectively. Table 4.5 stated ED_{50} values of *Penicilliopsis* sp. (KUM60280) extracts against various cell lines. Graph plotted (Figure 4.6 to 4.12) shows that ED_{50} values were all more than 20 µg/ml indicating that they are not strongly cytotoxic.

Figures 4.5 (a,b, and c) show negative control consist of untreated CaSki, KB and HT 29 cells respectively. Negative controls exhibited normal proliferation rate and showed no sign of abnormal death after 72 hours incubation.

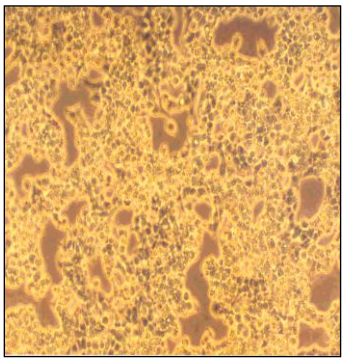


Figure 4.5(a)

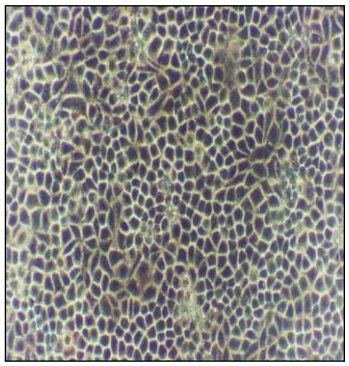


Figure 4.5(b)

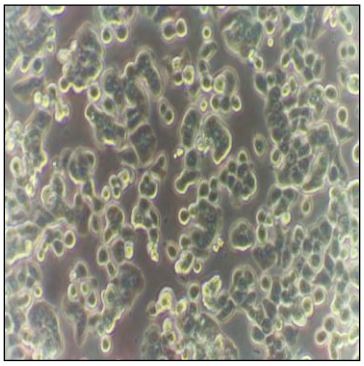


Figure 4.5(c)

Figure 4.5: Photomicrograph of: (a): KB cells incubated in Medium 199 (b): CaSki cells incubated in RPMI 1640 medium (c): HT29 cells incubated in RPMI 1640 medium

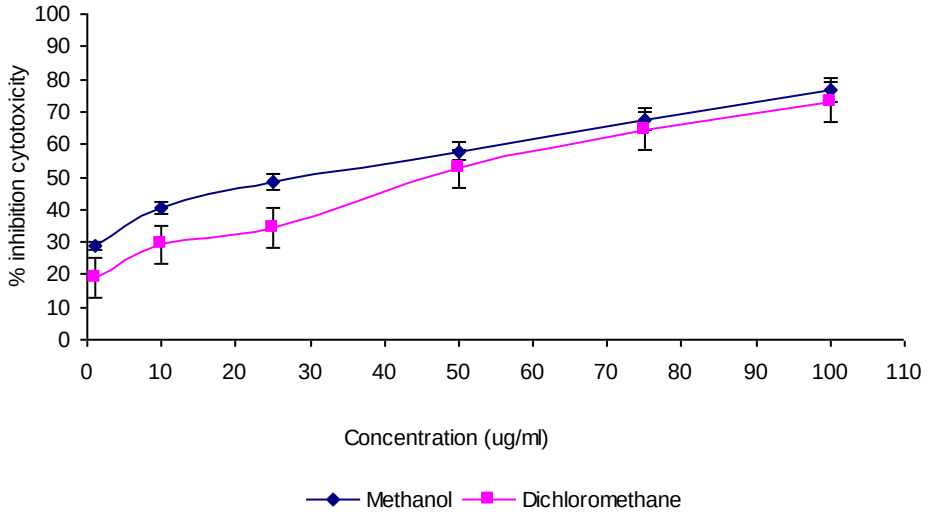


Figure 4.6 The *in vitro* growth inhibitions of MCF7 cells by crude extract of *Penicilliopsis* sp. determined by using the Neutral Red Cytotoxic assay.

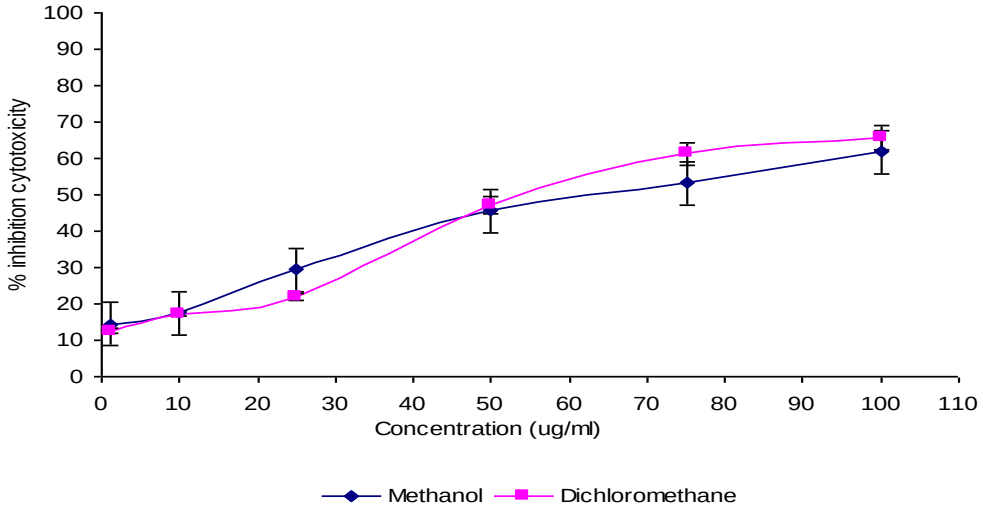


Figure 4.7 The *in vitro* growth of CaSki cells by crude extract of *Penicilliopsis* sp. determine by the Neutral Cytotoxic assay.

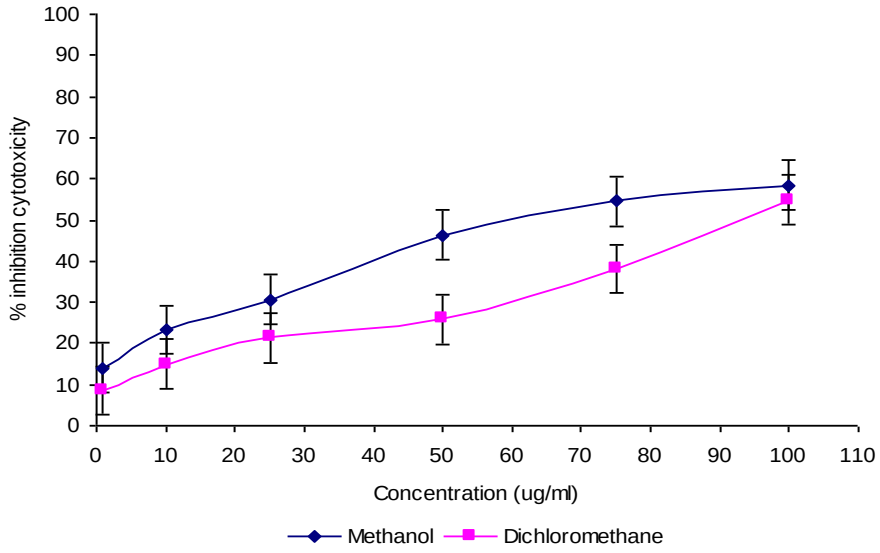


Figure 4.8 The *in vitro* growth of KB cells by crude extract of *Penilcilliopsis* sp. determine by the Neutral Red Cytotoxic assay.

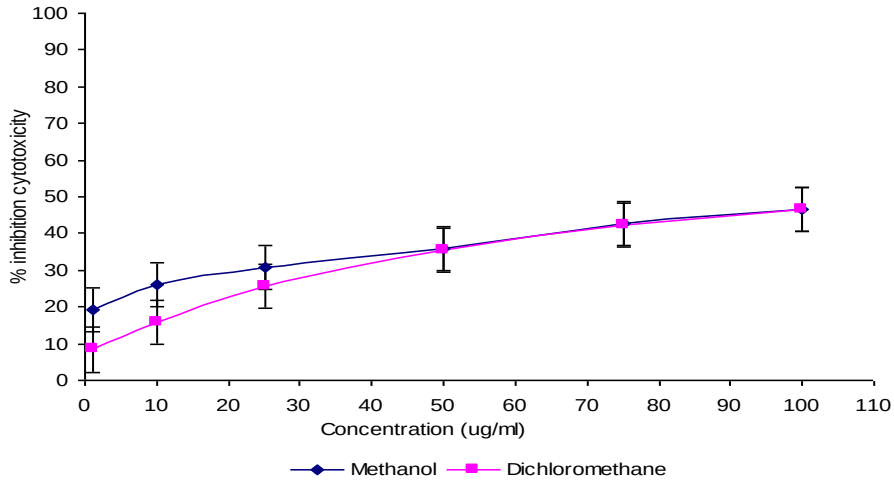


Figure 4.9 The *in vitro* growth inhibitions of HT29 cells by crude extract of *Penicilliopsis* sp. determine by the Neutral Red Cytotoxic assay.

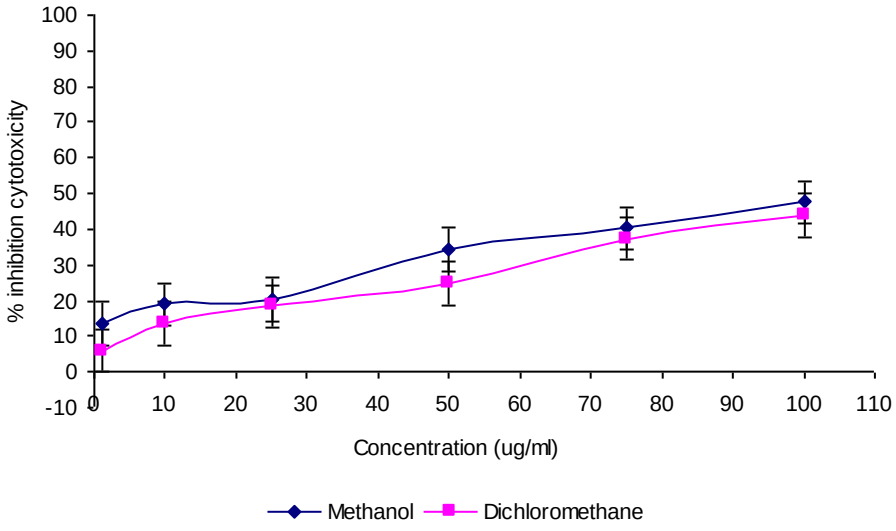


Figure 4.10 The *in vitro* growth of SCOV3 cells by crude extract of *Penicilliopsis* sp. determine by the Neutral Red Cytotoxic assay.

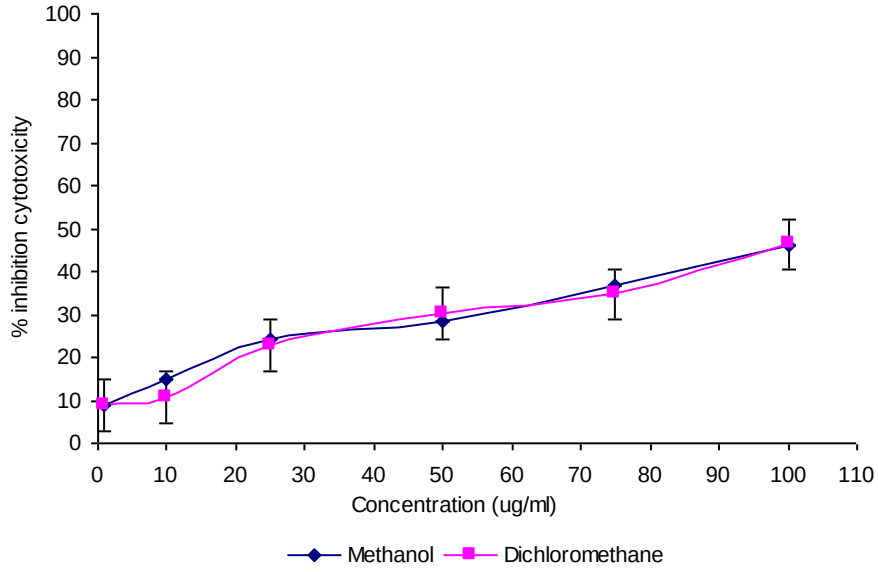


Figure 4.11 The *in vitro* growth of HCT119 cells by crude extract of *Penicilliopsis* sp. determine by the Neutral Red Cytotoxic assay.

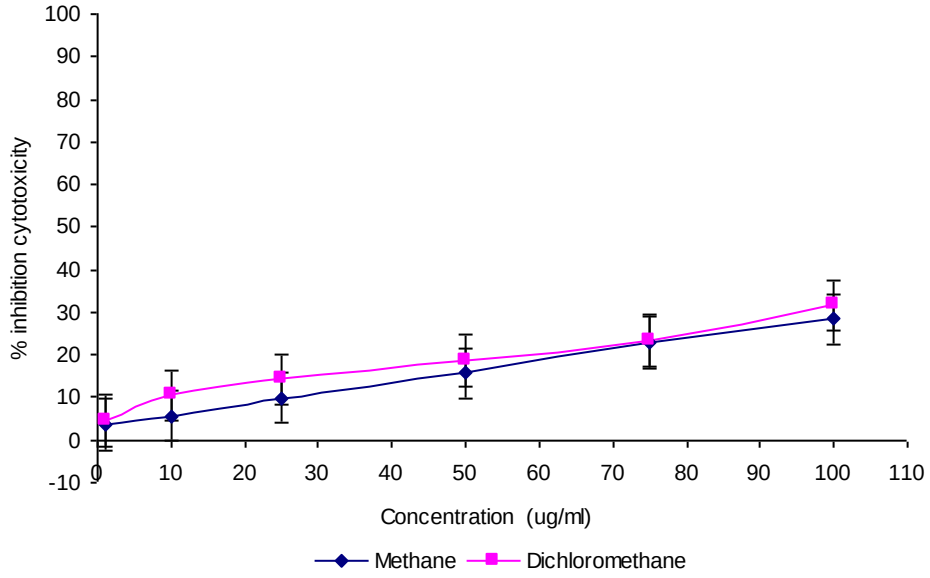


Figure 4.12 The *in vitro* growth of MRC5 cells by crude extract of *Penicilliosis* determine by the Neutral Red Cytotoxic assay.

Table 4.5 ED_{50} values of *Penicilliopsis* sp. (KUM60280) extracts against various cancer cell lines.

	Penicilliopsis KUM60280 ED_{50} (µg/ml)	
	Methanol	Dichloromethane
MCF7	56.221	48.912
CaSki	40.183	41.451
KB	40.882	30.063

Human mouth epidermal carcinoma cell line (KB); Human epidermal carcinoma of cervix cell line (CaSki); Human breast cancer cell line (MCF7)

4.6 Anti-HPV 16 E6 Oncoprotein Activity in *Penicilliopsis* sp.

Methanol and dichloromethane extracts of *Penicilliopsis* sp. were evaluated for potential anti humanpapilloma virus, HPV16 E6 activity. CaSki cells were treated with the extracts at 4 different concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml) for 72 hours at 37^{o}C.

The 3 steps indirect HRP – DAB immunoperoxidase technique using anti HPV-16 E6 monoclonal antibody were successfully applied in this study to analyze the expression of E6 oncoprotein still present in treated and untreated Caski cells. The staining color was categorized as: no stain (-), weak (+), moderate (2+), strong (3+) and very strong (4+). The suppressing effect of the testing extract is considered as weak for (4+), moderate for (3+), moderate for (2+) and high for (+). The presence of E6 oncoprotein was noted based on the appearance of reddish brown stain either in nuclear or cytoplasmic regions of Caski cells.

Negative controls used in this study were the CaSki cells not treated with the extract and incubated with anti- HPV 16, E6 monoclonal antibody (Negative control A) as well as CaSki cells not treated with the extracts and not incubated with monoclonal antibody (Negative Control B). The results of staining of both CaSki cells incubated with or without HPV monoclonal antibody are shown in Plate 3 (a and b). All CaSki cells incubated with monoclonal antibody show strong reddish brown (4+) stains and were morphologically intact. This is due to high expression of the HPV16 E6 oncoprotein. Negative control A was found to exert strong reddish-brown stain indicated high expression of E6 oncoprotein.

While the CaSki cells which incubated without HPV monoclonal antibody appears to be unstained (-).

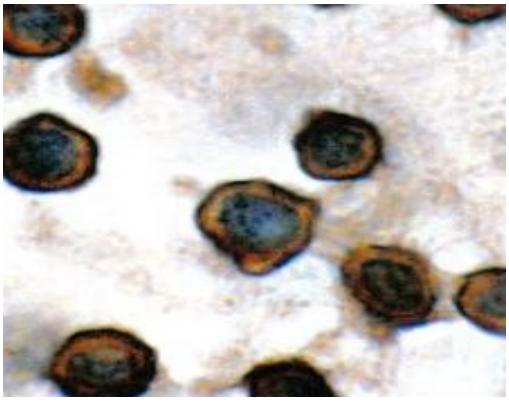


Plate3(a)

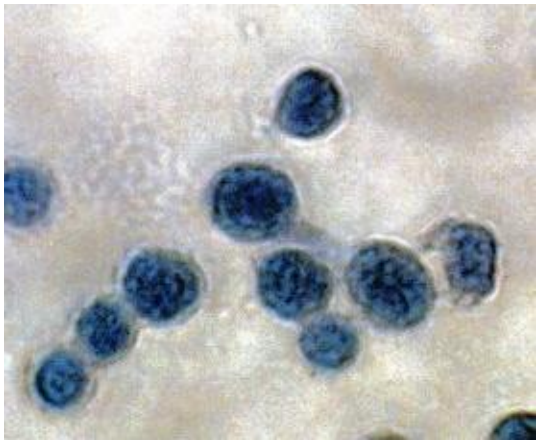
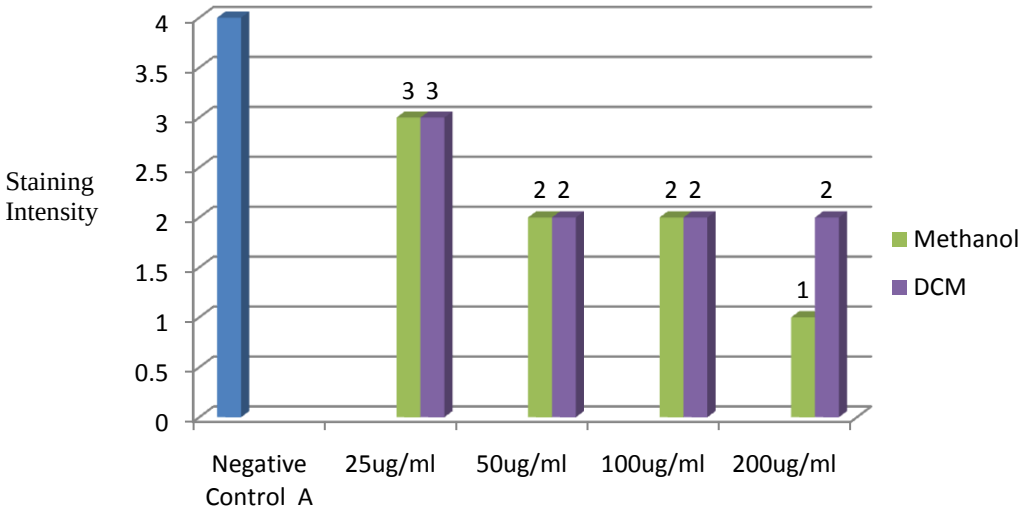


Plate 3(b)

Plate 3 (a) CaSki cells not treated with *Penicilliopsis* KUM60280 extracts but incubated with anti-HPV-16 E6 monoclonal antibody. Cells showed very strong staining (4+) in the nuclear and cytoplasmic regions (400x) (b) CaSki cells not treated with *Penicilliopsis* sp. (KUM60280) extracts without anti-HPV-16 E6 monoclonal antibody. Cell showed no staining (-) was observed under 400x magnification.

The different intensities of staining reflected the expression of HPV-16 E6 oncoprotein still presenting of CaSki cells treated with different extracts at various concentration. The results were compared between crude extracts of *Penicilliopsis* sp. (KUM60280) and negative control (Figure 4.13). Table 4.6 and table 4.17 summarized and the morphology the expression of HPV-16, E6 oncoprotein analyzed extra treated CaSki cells.



(*Negative control A for CaSki not treated with *Penicilliopsis* sp.)

Figure 4.13: Staining intensities of CaSki cells treated with *Penicilliopsis* sp.

Classification for the staining intensity as: no stain (0), weak (1+), moderate (2+), strong (3+) and very strong (4+)

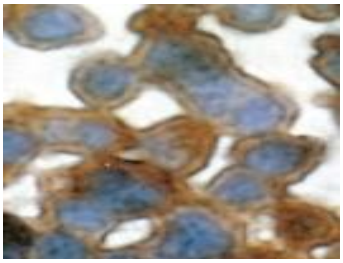
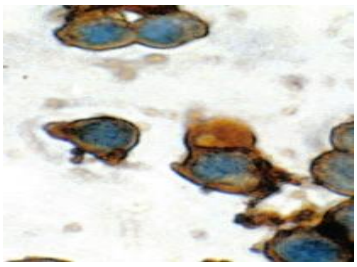
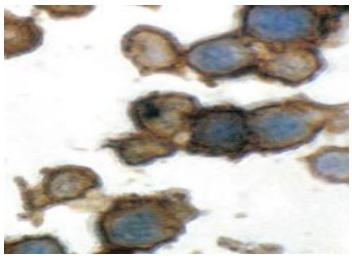
Stanining intensity of the reddish-brown stains in CaSki cells decreased from 4+ to 2+ when treated with 50µg/ml and 100µg/ml of methanol extract. Than futher decreased to + was observed at higher concentration of 200µg/ml. This suggest that methanol extract of

Penicilliopsis sp. (KUM60280) was successful in producing some inhibition on the expression of HPV16 E6 oncoprotein on a dose dependent manner where higher extract concentrations produce stronger suppression on the oncoprotein.

On the other hand, in CaSki cells treated with dichloromethane extract of *Penicilliopsis* sp. the staining intensity of the reddish brown stains, decreased only slightly from 4+ to 2+ when treated with 50µg/ml. No futher decrease was observed at 100µg/ml and 200µg/ml. These suggest that dichloromethane extract only slightly suppressed the expression of HPV16 E6 oncoprotein.

In this present study, CaSki cells treated with methanol and dichloromethane of *Penicilliopsis* sp. remained morphologically intact at lower concentrations but some lyses was observed at 100µg/ml and 200µg/ml (Figure4.6 and 4.7). Generally, the HPV 16 E6 oncoprotein was not suppressed in CaSki cells by the crude extracts of *Penicilliopsis* sp.

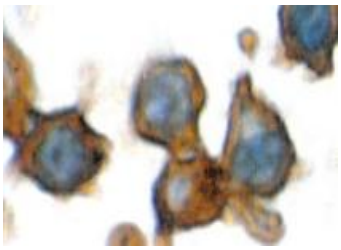
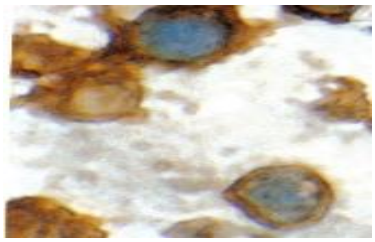
Table 4.6 Anti HPV effect methanol extract of *Penicilliopsis* sp. on CaSki cell

	Concentration of extract (µg/ml)	Intensity of reddish-brown stain	Morphology of cells
	25	3+	No intact and no lysis
	50	2+	No intact and no lysis
	100	2+	No intact and some lysis
	200	+	No intact and majority of cell was lysis

CaSki cells treated with methanol extract of *Penicilliopsis* sp. (KUM60280) (400x magnification)

Note: Classification for the intensity of staining as no stain (-), weak (+), moderate (2+), strong (3+) and very strong (4+)

Table 4.7 Anti HPV effect of dichloromethane extract of *Penicilliopsis* sp. on CaSki cell

	Concentration of extract (µg/ml)	Intensity of reddish-brown stain	Morphology of cells
	25	3+	No intact and no lysis
	50	2+	Intaact and no lysis
	100	2+	Some intact and some lysis
	200	2+	Some intact and some lysis

CaSki cells treated with dichloromethane extract of *Penicilliopsis* sp. (KUM60280) (400x) Note: Classification for the intensity of staining as no stain (-), weak (+), moderate (2+), strong (3+) and very strong (4+)

5.0 DISCUSSIONS

5.1 Yield of Mycelial Biomass and Crude Extract of *Penicilliopsis* sp.

The process efficiency is qualitatively related to extraction yield. Incubation of *Penicilliopsis* sp. at 25^{o}C for 48 hours under shaking condition gives optimum growth of mycelial biomass. *Penicilliopsis* sp. sporulated immedieatly on day seven because medium was incubated under shaking condition where nutrient uptake is more efficient, giving more rapid growth and that growth become homogenous (Tuner, 1971).

In the present study, the yield crude extracts of *Penicilliopsis* sp. gives optimum growth of mycelium. It was noted, the difference yield of crude methanol extract which was notably higher propagation than dichloromethane as listed in Table 4.1. This might indicate that most of the solutes in methanol crude extract were polar components compared to dichloromethane, an indication of the presence components in the *Penicilliopsis* sp. Besides the evaluation of the quantitative efficiency of extracting process, the yield values are not directly related to their qualitative efficiency. Peterson and Bridge (1994) reported known that culture medium can affect the presence or absence of secondary metabolites and/or their level of production by fungi.

In has been reported, fungal culture has potential advantages for higher mycelial and polysaccharide production in a more-compact space over a shorter incubation time and availability of convenient control with less chance of contamination (Kim *et al*., 2003; Lee *et al*., 2004). Many investigators have attempted to obtain optimal submerged cultures for mycelial polysaccharides production from several fungi (Dong & Yao, 2007). To the best

of our knowledge, the nutritional requirements for submerged culture of *P. baumii* Pilát have not been demonstrated. To achieve higher yield of mycelial polysaccharide in a culture, it is a prerequisite to design an optimal production medium and a set of optimal process operating conditions.

5.2 Antimicrobial Activity of *Penicilliopsis* sp.

This study was carried out using agar diffusion assay and modified slightly by Bauer *et al*. (1966). The antimicrobial effect of crude extracts of *Penicilliopsis* sp. were tested against six Gram positive bacteria including three oral bacteria, three species of Gram negative bacteria and three species of fungal as summarized in Table 4.2. The antimicrobial activity of methanol and dichloromethane extracts of *Penicilliopsis* sp. was shown by the size of inhibition zone (mm). However, both of crude extracts of *Penicilliopsis sp*. failed to show antibacterial and antifungal activity. From the results, methanolic extracts of *Penicilliopsis* sp. did not exhibit antibacterial and antifungal activity (Table 4.2) whereas the dichloromethane extracts exhibited weak inhibition zone less than 10 mm. Based on the results, it was possible to conclude that the crude dichloromethane extract had a mild activity and broader spectrum than those of methanol extract.

Cintia *et al.* (2007) reported that the tested bacterial and fungi grew inside the halo, indicating a weak inhibition power. The maximal inhibition zones of *Penicilliopsis* sp. extracts were *Bacillus cereus* followed by *Escherichia coli*. As reported by Cosentino *et al.* (1999) and Karaman *et al.* (2003), Gram positive bacteria are more sensitive to crude extract than Gram negative bacteria. Previous studies have suggested that the differences in

cell wall structure between Gram positive and Gram negative bacteria might be the reason. The Gram negative bacteria have an outer membrane acting as a barrier to many environmental substances, including antibiotics (Palombo and Sample, 2001). The difference in sensitivity between Gram-positive and Gram-negative bacteria might be attributed to the differences in morphological constitutions between these microorganisms. Gram-negative bacteria have an outer phospholipidic membrane containing lipopolysaccharide components. On the other hand, the Gram-positive bacteria only have an outer peptidoglycan layer which is not as an effective permeability barrier as the former.

As reported by Aziz *et al*. (2007), the antibacterial properties of *Laetiporus sulphureus* were not as effective as the commercial drugs. But, microorganisms become resistant to antibiotics overtime. Previously become it has been demonstrated that mushrooms show antimicrobial effects (Hur *et al*., 2004; Ishikawa *et al*., 2001; Sheena *et al*., 2003).

Developing new antimicrobial agents that are cell selective is a major challenge, especially when the target is the cell membrane (Gregory *et al.,* 2006). The results of *in-vitro* antifungal activities showed that all target compounds had no potent *in-vitro* antifungal activities. A variety of studies have been conducted in an attempt to isolate natural antifungal substances with potential pharmaceutical utilization, and to develop and design new synthetic or semi-synthetic drugs (Lopez-Garcia *et al.,* 2005; Viejo-Diaz *et al,* 2005; Alcouloumre *et al.,* 1993). The mycelium contains compounds with wide-ranging antimicrobial activity (Aziz *et al*, 2002). Sung *et al.* (1999) reported the antifungal activity of peptide against various pathogenic fungi isolated from patient and fluconazole-resistant

fungi, its synergism with other antifungal drugs, the rate of fungicidal activity, and its cytotoxicity against mammalian cell.

5.3 Antioxidant Activity of *Penicilliopsis* sp.

Recently, numerous reports have described antioxidants and compounds with radical-scavenging activity present in fruits, vegetables, herbs, and cereal extracts (Gray *et. al.*, 2002; Hou *et. al.*, 2005 and Nutiila, 2003). A wide variety of fungi produced novel antioxidant compound (Ishikawa, 1992; Yang *et al.*, 2002). These include *Penicillium roquefortii, Aspergillus, Candidus, Mortirella* sp., *Emericella falconensis* and fungi of the genus *Acremonium*. In the present study, *Penicilliopsis* sp. demonstrated antioxidant properties of methanolic and dichloromethane from mycelia biomass when analysed using a range of testing system *in vitro;* DPPH assay and the reducing power assay.

The results suggest that *Penicilliopsis* sp. extracts have potent antioxidant activities. Single electron transfer reactions (DPPH and FRAP) can be relatively slow and measure relative percent decrease in product rather than kinetics or total antioxidant capacity. The inhibition of accumulation of colored reagents (DPPH) in the presence of antioxidants is not always linearly correlated to antioxidant concentrations. Due to the complexity of the oxidation-antioxidation processes, it is obvious that no single method is capable of providing a comprehensive picture of the antioxidant profile of studied sample.

5.3.1 DPPH· Free Radical Scavenging Activity

Free DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of extracts. As antioxidants donate protons to these radicals, the absorbance decreases. The decrease in absorbance is taken as a measure of the extent of radical scavenging. Radical-scavenging capacities of the extracts, measured by DPPH assay, are shown in Figure 4.2. It was observed, that in line with the increase seen in the amount of methanol and dichloromethane extracts of *Penicilliopsis* sp., an increase in DPPH free radical-scavenging occurred. The inhibition value increases with concentration. Vitamin C oxidation was compared with those of *Penicilliopsis* sp. extracts, Vitamin C and BHA.

It was found that inhibition values of both *Penicilliopsis* sp. crude extracts and the standards increased with concentration. For example; at 20mg/ml concentration, methanolic and dichloromethane crude extracts of *Penicilliopsis* sp., showed 35%, 25% of scavenging effect, whereas 20 mg/ml concentrations of BHA and ascorbic acid showed 19% and 12%, respectively. While at 50 mg/ml concentrations, 91% for methanol, 51 % for dicholoromethane and 56% and 66% respectively for ascorbic acid and BHA. According to this, it is possible that the high inhibition value of the *Penicilliop* sp.extracts were due to the high concentration of phenolic compounds (see Table 4.3). Previously in a study performed on *Laetiporus sulphureus* (Bull.) Murrill. extracts, inhibition values both of *L. sulphureus* ethanol and the standards increased parallel with the elevation of concentration in the linoleic acid system (Aziz *et al*., 2006).

In this study, the methanol extracts showed higher antioxidant activities than dichloromethane extracts. Polar compounds extracts exhibited stronger activity than non

polar compounds in accordance with free radical scavenging activity results. This result could be explained by the 'polar paradox' (Frankel *et al.,* 1994). According to this theory, polar antioxidants would be more effective in non-polar media, and non-polar antioxidants more active in polar media. As a result, methanol extracts have excellent capacity in both $DPPH^{\cdot}$ and FRAP.

The antioxidant properties assayed herein were summarized in Figure 4.3 and results are normalized and expressed as EC_{50} values (mg various extracts per ml) for comparison among the crude extracts with positive control as illustrated in Figure 4.2. Scavenging effects of *Penicilliopsis* sp. extracts on $DPPH^{\cdot}$ radicals increased concentrations. The decrease in the concentration of $DPPH^{\cdot}$ radical due to the scavenging ability of extracts from *Penicilliopsis* sp. and standard is very significant ($P<0.01$). With regard to EC_{50} values of antioxidant activities by $DPPH^{\cdot}$ radical scavenging activity, effectiveness was in a descending order: BHA>ascorbic acid>methanol> dichloromethane. The EC_{50} scavenging activities were 0.078 > 0.107 > 26.80 > 50.48 mg/ml, respectively. The results indicated that crude extracts of *Penicilliopsis* sp. (KUM60280) have a noticeable effect of scavenging free radicals. However, the scavenging effect of BHA and ascorbic acid are higher than crude extracts of *Penicilliopsis* sp.

This study showed crude extracts from *Penicilliopsis* sp. have some components within the fragments significantly strong radical-scavenging component and have a potency to become one of antioxidant medicine. Various extracts might react with free radicals, particularly the peroxy particle which is the major propagators of the autooxidation chain of fat, thereby terminating the chain reaction (Frankel, 1991; Gordon, 1990; Shahidi and Wanasundra, 1992). Methanolic extracts from edible mushroom species might react with

free radicals, which are the major initiator of the autoxidation chain of fat, thereby terminating the chain reaction (Frankel, 1991; Gordon 1990). Activity of natural antioxidants has been shown to be involved in termination of free radicals reaction (Shimada *et al*., 1992). Furthermore Herraiz *et al*. (2003) found that an essential amino acid L-trytophan could react with phenolic aldehydes in food to form phenolic tetrahydroβ-carboline alkaloids that scavenged 2,2-azinobis/93-ethyl-benzothiazoline)-6-sulfonic acid (ABTS) effectively.

Antioxidants are believed to play a significant role in the body defense system against free radical. Free radicals are harmful by-products generated during normal cellular metabolisms, which could initiative oxidative damage to body (Abidi and Ali, 1999; Halliwell & Gutteridge, 1999). Besides playing an important role in physiological systems, antioxidants have been used in food industry to prolong the shelf life of foods, especially those in rich in polyunsaturated fats. These components in the food are readily oxidized by molecular oxygen and are major cause of oxidative deterioration, nutritional losses, off flavour development and discolorization. The additional of synthetic antioxidant such as butylated hydroxylanisole (BHA), butylated hydroxyltoluent (BHT), propyl gallate etc has been widely used industrially to control lipid oxidation in food (Gurdip *et al.*, 2007).

5.3.2 The Reducing Power

The reducing capacity of a compound may served as a significant indicator of its potential antioxidant activity. For the measurement of the reductive ability, this study has

investigate the Fe^{3+} and Fe^{2+} transformation in the presence of the crude extracts of *Penicilliopsis* sp. using the method of Oyaizu (1986). The reducing power of both *Penicilliopsis* sp. extracts increased with the concentrations. The increase in absorbance of the reaction mixture was interpreted as increase in reducing activity of the *Penicilliopsis* sp. and the result was compared to gallic acid (positive control). Figure 4.4 indicates the reductive capabilities of crude methanolic and dichloromethane. Among the crude extracts methanolic showed better reducing power than dichloromethane, i.e 1.161 and 0.431 mg/ml respectively.

According to the present study, it is suggested that methanol fraction has remarkable potency to donate electron to reactive free radicals, converting them into more stable nonreactive species and terminating the free-radical chain reaction. However, BHA and ascorbic acid showed higher activities than methanol and dichloromethane. The reducing power of crude extracts and standard compounds followed the order BHA>ascorbic acid>methanol>dichloromethane.

Previous studies observed a direct correlation between activities and reducing power of certain fungus extracts (Pin, 1998; Pin *et. al.*, 1999). Pin (1998) stated the reducing properties are generally associated with the presence of reductones which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reducing power assays often used to evaluate the ability of natural antioxidant to donate electron (Dorman *et al.*, 2003, Yildirim *et al.*, 2000). The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition

of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997).

5.4 Total Phenolics Contents in Extract of *Penicilliopsis* sp.

The $DPPH^{\cdot}$ assay reflects the ability of phenolics present in crude extracts of *Penicilliopsis* sp. to inhibit radical formation. All the results were expressed as gallic acid equivalents (g /(GAE) / 100g). Total phenol were the major naturally occurring antioxidants components founds and their contents were in descending order BHA (9723.25 g/(GAE)/100g) > ascorbic acid (932.9 g/(GAE)/100g) > Methanol (107.87 g/(GAE)/100g) > Dichloromethane (58.43 g/(GAE)/100 g) (Table 4.4).

Phenol is important plants constituent because of their scavenging ability due to their hydroxyl groups (Hatano *et al.,* 1989). It is important, therefore to determine the total phenol content in order to evaluate the possible synergistic or antagonistic effect on their contribution to the total phenol to the total of antioxidant capacity. The phenolics compounds may contribute directly to the antioxidative action (Duh *et al.,* 1999). Vitamin C is highly bioavailable and is therefore the most important water-soluble antioxidant in cells and efficient scavenger of reactive oxygen species (Halliwell, 1996). Phenol such as BHT and gallate were known to be effective antioxidants (Madhavi *et al.,* 1996).

Due to their scavenging abilities on free radicals and chelating abilities on ferrous ions (Latilo and Fraga, 1998), phenols contain good antioxidant properties, antimutagenic properties and anticancer properties (Ahmad & Mukhtar, 1999). Yen *et al.,* (1993) found

that the antioxidant activity of the methanolic extract from peanut hulls correlated with it's content of totals phenols. This activity is increased with presence of polyphenols in particular (Behera *et al*., 2005). Therefore the higher contents of total phenols in the crude extracts were responsible for their better antioxidant properties. .

Phytochemicals, such as phenolic compounds are considered beneficial for human health, decreasing the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation (Silva *et al*., 2004; Pulido *et al*, 2000 and Tseng *et al*, 1997). Phenolic constituents, such as flavanoids, phenolic acids, and tannin are well known for their high antioxidant activity (Rice-Evants *et al.*, 1996). Many studies indicate linear relationship between total phenolics and antioxidant activivty (Djeridane *et al*, 2006; Kim *et al.*, 2004 and Kim, *et al*, 2006).

In this study it was found that phenolic compounds are major contributors to antioxidant activity, since total phenolics and antioxidant activity showed a good correlation coefficient of R^2=0.9972 (Appendix B). A similar result was previously observed by Kim *et al.*, (2003). Ana *et al.*, 2007 reported that it is important to determine the total phenolic content in order to evaluate the possible synergistic or antagonistic effect on their contribution to the total antioxidant capacity. Phenolics measured by the Folin-Ciocalteau assay are essentially simple soluble phenolics. The release of hydrophobic phenolic antioxidants and ellagic acid enrichment is thought to occur through the molecular rearrangement of simple phenolics and hydrolysis of complex polyphenols such as tannins by fungal enzyme systems (Vettem and Shetty, 2002).

Previously, lignin or polymeric phenolic degradation activities were linked to phenolic antioxidant mobilization from soybean substrate during solid-state bioprocessing by dietary fungi such as *Rhizopus oligosporus* or *Lentinus edodes* (McCue *et al*., 2004). Recently, fungus (mushroom) are considered to be a good source of protein and total phenolic content such as variegatic acid and diboviquinone, which have been found in mushroom (Cheung, *et al.*, 2003).

Correlation of antimicrobial activities to phenolic content, DPPH radical inhibition and ellagic acid provides insights into potentially different mechanisms of action of these antimicrobials. Soluble phenolics are thought to exert their antimicrobial effect by causing hyperacidification at the plasma membrane interface of the microorganism, which potentially results in disruption of the H+-ATPase required for ATP synthesis. Different sensitivities of the microorganisms against the phenolic content in the extracts may be because of the difference in their membrane structure and associated cell wall differences between gram positive and gram negative bacterial species (Vettnem *et al*, 2004).

Tannins and hydrolyzed tannins are known to exhibit inhibitory effects on microorganisms (Acamovic and Stewart, 1992). Tannins and their hydrolyzed products have been shown to inhibit the growth of microorganism by sequestering metal ions critical for the microbial growth and metabolism (Kainja *et al.*, 1998; McDonald, *et al.*, 1996) or by inhibiting critical functions of the bacterial membrane such as ion channels and proteolytic activity (Muhammed, 1997).

5.5 Cytotoxicity Activity of *Penicilliopsis* sp. Using *in vitro* Neutral Red

Cytotoxic anticancer drug efficacy is contingent upon differences in drug sensitivity between normal and tumor tissue. These drugs often small, with little difference between the doses required for optimal antitumor activity (effective dose) and that which produces unacceptable toxicities in normal cells (Chatelu *et al.,* 2003). The cytotoxicity of the studied extrcts requires special attention in view of the major damage they cause to cell functions, which result in the ability of cell to proliferate. *In vitro* cytotoxicity assays are useful to define basal cytotoxicity, for example the intrinsic ability of a compound to cause cell death as a consequence of damage to several cellular functions. The cytotoxic effect may be considered an earlier indication of cellular damage, with possible biological consequences. The ideal anticancer agents would exert minimal adverse effects on normal cell with maximal capacity to kill tumor cells and/or inhibit tumor growth. Inhibition of proliferation of tumor cells without inducing cell death could potentially reduce cytotoxicity to normal, non-tumor host cells, particularly if those normal cell are low- or non- proliferating (Ruen, 2006).

Cytotoxicity test using human cell lines are appropriate for screening of the potential hazardous effects of a large number of chemicals on human health. It have been reported in past years, a numerous of methods number have been developed to study cell viability and proliferation in cell culture. In the search for new drug for anticancer the most common screening methods employ cytotoxicity tests against cell lines. Therefore, it seems reasonable to utilize, at the primary screening stage, *in vitro* toxicity assays to select the least toxic compounds among the most active ones (Joanna *et al,* 2005). There are many *in vitro* methods, which have been developed to test toxicity towards particular cells. One or

more of them can be chosen if specific type of toxicity is expected. One of the tests used for evaluating toxicity of new anticancer agents *in vitro*, is colony forming unit assay using cell from human. In the drug discovery process, it is desired to find not only the most active, but also the least toxic substances. It is important that such selection could be done at the very beginning of developmental process, at the stage of *in vitro* studies.

This study was carried out to evaluate the cytotoxic potential of methanol and dichloromethane extracts of *Penicilliopsis* sp. on human mouth epidermal carcinoma cell line (KB), human epidermal carcinoma of cervix cell line (CaSki), human colorectal cancer cell line (HT29), (HCT119), human breast cancer cell line (MCF7), human ovarian cancer cell line (SCOV3) and normal human fibroblast (MRC5). The method used was *in vitro* neutral red (NR) cytotoxicity assay. This technique is simple and rapid based on the lysomal incorporation of NR dye by viable cells assay which can be conveniently carried out in 96-well microtiter plates (Borenfreund and Puerner, 1986). The cells cultured were observed under micsroscope from time to time to monitor the growth of CaSki cells. Only living cells are able to manage the active uptake of neutral red. Visual inspection or staining of cells followed by cell counting used in this studies to determine toxic effects of toxicty The cells were than incubated with fresh serial diluted extracts 1, 10, 25, 50, 75 and 100µg/ml when 60-80% confluence in growth was achieved. Based on NR assay, methanol and dichloromethane extracts of *Penicilliopsis* sp. (KUM60280) were considered not cytotoxic against the cells line tested.

The results was obtained from NR assay were expressed as *effective dose, ED_{50}* values which refer to the effective extracts concentration that reduce the proliferation of cultures by 50% inhibition. Results indicated that the percentage of killing increased with

the concentration of extracts tested. The results from ED_{50} value was extrapolated from the-dose response curves. Ridell *el al.*, (1986) reported that the dose-response is considered more reliable than value based]. Figure 4.6 till 4.12 illustrated cytotoxic activity of crude extracts against cell lines. From the graph plotted, ED_{50} for methanol and dicloromethane extracts of *Penicilliopsis* sp. were all more than 20mg/ml. It shows that crude extracts of *Penicilliopsis* sp. was not actively cytotoxic against cells lines tested. Its might be explained because of the cell line's origin was huge difference, which implies that the sensitivity for exposure with different chemicals might vary as well. The cytotoxic effect was elicited by certain metabolite that may be enhanced or reduced by interacting metabolites to a different degree for different cells lines. The cell lines were used in this study has been used for a long time which cells have changes in their ability. Previous study on the anti-tumor activities of *Cordyceps* species have mainly focused on their water or alcohol extracts, which contain mostly polar components (Zhang *et al*, 2004).

The neutral red (NR) assay was used in this present study was performed after 72 hours treatment of *Penicilliopsis* sp. against seven cancer cell lines and one normal cell. This technique based on the incorporation of the supravital dye neutral red, into the lysosomes of viable cells (Borenfreund, *et al.,* 1995; Babich and Tipton, 2002). Based on Riddell *et al.*, (1986), suggested 48 to 72 hours incubation period for cell lines to react with extracts using the NR method as compared to 24 hours because the latter may give an unacceptable number of false negative indications of cytotoxicity. Short incubation period will lead to failure to discriminate between chemicals which have genuinely different cytotoxicity. Tryphan blue dye was used as for counting cancer and normal cell lines because they are frequently used for toxicological purpose, low cost and quick response.

Tryphan blue exclusion is used generally to quantify the reduction in percentage of viable cells and the cytotoxic effect.

The literature reports several studies on cytotoxicity of fungal metabolites in mammalian cell lines (Dambach *et al,* 2005 ; Gomez *et al,* 2001). It has been discovered that an endophytic fungus (*Taxomyces andreanae*) also produced the anticancer drug paclitaxel (Taxol®) derived from Pacific yew (Stierle *et al.,* 1995). Francesca *et al.,* (2004) found the toxicity of sixteen fungal metabolites produced by some entomopathogenic fungi or biological control fungi agents was evaluated on lepidoptera *Spodoptera frugiperda* (SF-9). This mycotoxin is extremely toxic to leucocytes and other rapidly dividing cells resulting *in vivo* and *in vitro* immunosuppressing effects. Bioactivity-guided fractionation of this extract resulted in the isolation of a novel dihydroxanthenone (type of anticancer) which is named as globosuxanthone (Kithsiri *et al.,* 2006). Dominique *et al.* (2002), evaluated a new cytotoxic tatralone derivative from *Humicola grisea*, filamentous fungi from wood. *Inonotus obliquus* (Chaga mushroom), one of the well-known medicinal mushroom, has been used as remedies for gastric disorders and cancer and there are many reports published on anticancer and anti-inflammatory activities (Wasser and Weis, 1999; Hwang *et al*., 2003; Kim *et al*., 2006; Chen *et al*., 2007; Youn *et al*., 2008).

5.6 Anti- HPV 16 E6 Oncoprotein Activity of *Penicilliopsis* sp.

Cervical cancer is one of the most common neoplastic diseases affecting women, with a combined worldwide incidence of almost half a million new cases annually, second

only to breast cancer. Basic and epidemiologic research conducted during the past 15–20 years have provided overwhelming evidence for an etiologic role for infection with certain types of sexually-transmitted human papillomavirus (HPV) as the primary cause of cervical cancer. Persistent infection with carcinogenic human papillomavirus (HPV) types is widely considered a necessary cause in the development of cervical cancer (Wright and Shiffman, 2003). The relative risks of cervical cancer following HPV infection as ascertained in case-control and cohort studies are among the highest in cancer epidemiology (Francoc *et al*., 2001).

The E6 oncogene of the HPV-16 is almost regularly expressed in the cervical tumours and in the derived cell lines. Oncogene with alteration properties sepecially interacts with the p53 protein and has a direct degradation through the ubiquity pathway (Schwarz, 1990; Herrington, 1994). Constant expression of E6 oncoprotein will interfere with host cell regulatory proteins and lead the host cell into a proliferative state required for malignant transformation (Arends *et al*, 1998). Therefore, screening for natural compounds with HPV-16 E6 oncoprotein suppressing effect is important.

In the present study, the CaSki cells were treated with methanol and dichloromethane extracts of *Penicilliopsis* sp. extracts at varying concentration (25 □g/ml, 50 □g/ml, 100 □g/ml and 200 □g/ml). The presence of E6 oncoprotein was noted based on the emergence of reddish brown stain either in nuclear or cytoplasmic regions of Caski cells. Results were interpreted by the intensity of colour of the reddish brown stain. The staining color was categorized as: no stain (-), weak (+), moderate (2+), strong (3+) and very strong (4+). The appearance of reddish-brown stain indicates the presence of HPV-16

E6 oncoprotein in CaSki cells. Generally, all CaSki cells incubated with monoclonal antibody was found strong reddish brown (4+) stains and were morphologically intact. This is due to high expression of the HPV16 E6 oncoprotein. Negative control A was found to exert strong reddish-brown stain indicated high expression of E6 oncoprotein.

In the present study, methanol extract of *Penicilliopsis* sp. showed stanining intensity of the reddish-brown stains in CaSki cells decreased from 4+ to 3+ when treated with 25µg/ml and 50µg/ml. Than further decreased to 2+ and + at higher concentration of 100ug/ml and 200µg/ml, respectively. This shows that methanol extract of *Penicilliopsis* sp. (KUM60280) was able to produce some inhibition on the expression of HPV16 E6 oncoprotein on a dose dependent manner where higher extract concentrations produce stronger suppression on the oncoprotein. The staining intensity of reddish brown in CaSki cells treated with dichloromethane extract of *Penicilliopsis* sp. (KUM60280), decreased only from 4+ to 2+ when treated with 50µg/ml. There was no further decreased observed at 100µg/ml and 200µg/ml. It showed that dichloromethane extract of *Penicilliopsis* sp. only slightly suppressed the expression of HPV16 E6 oncoprotein.

According to Ghasaq *et al*.(1999), the relatively low level of detection by immunohistochemistry can be attributed not only to the lower sensitivity of the method but also to factors related to the nature of the antibodies and the nature of the antigenic target. First, in malignant lesions, not all of the E6 transcripts are translated into full-length E6 protein (Nilson, *et al.*, 1996). Second, it is conceivable that fixation and processing of the tissues may have adverse effects on the epitope of the E6 protein and their binding efficiency has only been tested *in vitro*. Third, the E6 protein (like E7, the other HPV gene

expressed early in the replicative cycle of HPV) has a short half-life thus rapid degradation might reduce its detection by immunohistochemistry in our samples.

The technique used in this study to evaluate the presence of HPV 16 E6 oncoprotein was the two-step indirect avidin-biotin immunoperoxidase immunocytochemistry method. Avidin, is a large glycoprotein, can be labeled with peroxidase and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies. The technique involves three layers which is the first layer is unlabed. The reagents are readily manufactured in market and no extraordinary equipment is required to carry out the staining protocol. Sometimes false-negative staining occur which can attribute the HPV 16 E6 activity by over fixation or overlapping of cells. False-positive may be resulted from incomplete elimination of endogenous enzyme activity. Endogenous enzyme activity can be suppressed or avoided by pretreatment with H_2O_2 prior to incubation with monoclonal antibody (Forghani and Hagens, 1995). False-positive results may be seen due to non-immunological binding of protein or substrate reaction products. They may also caused by endogenous enzymes such as myeloperoxidase, leucocyte alkaline phosphatase and hemoglobin pseudoperoxidase, primarily in frozen cells and depending on the type of enzyme label used to visualize the reaction (Elias *et al,* 1989).

The negative controls were untreated CaSki cells (were not incubated with *Penicilliopsis* sp. (KUM60280) extracts). Some of these cells were incubated with anti-HPV 16 E6 monoclonal antibody (Negative control A) whereas the others were not (Negative control B). The cells incubated with anti-HPV monoclonal antibody exhibited very dark reddish-brown stain in the nuclear and or cytoplasm. This indicated that the

HPV-16 E6 oncoprotein was intense and highly expressed in untreated CaSki cells. The control not incubated with anti-HPV 16 E6 monoclonal antibody did not exhibit reddish-brown stain. This correlates well with the absence of specific anti-HPV 16 E6 primary antibodies needed to detect the E6 oncoprotein.

In this present study, fresh solvent was used for each run because expected time duration influenced the functional stability of biological substance, including immunohistochemistry (IHC). Incubation times or temperatures other than those specified may give erroneous results. In this study, the temperature of incubation period during addition of antibody was decreased from 45^{o}C to 30^{o}C. Generally, incubation times can be extended if little or no background was detected. In IHC, antibody titer and dilution as well as incubation time and temperature are tightly interwoven in their effect on staining quality. Optimum antibody titer is defined as the highest dilution of an antiserum (or monoclonal antibody) that result in maximum specific staining with the least amount of background under specific test conditions. This highest dilution is determined primarily by the absolute amount of specific antibodies present. The stability of a diluted antibody can vary over time depending upon the dilution. Correct dilutions will contribute to the quality of staining if they are prepared accurately and consistently. Antibodies should be stored according to the conditions detailed on the specification sheet. If frozen antibody are utilized, than repeated freezing and thawing should be avoided. Frozen antibodies must be stored in small aliquots until periodic assay verification for detect unacceptable changes in reactivity. In addition, phosphate buffered saline (PBS), should not be used as a diluents unless recommended by the manufacturer.

6.0 CONCLUSION

Fungus is known to produce unusual classes of compounds with a wide variety of biological activities (including antimicrobial, antiviral, and antitumor properties). Therefore, fungus is very attractive as a potential source of new therapeutic agents. No work has been done to investigate the bioactivity of extracts from *Penicilliopsis* sp. mycelia.

In this present study, that extracts of *Penicilliopsis* sp. (KUM60280) only exhibited good antioxidant activity evaluated by DPPH scavenging activity and total reducing power. However crude extracts of *Penicilliopsis* sp. did not exhibit antimicrobial activity. The extracts of *Penicilliopsis* sp. are not actively cytotoxic against human mouth epidermal carcinoma cell line (KB), human epidermal carcinoma of cervix cell line (CaSki), human colorectal cancer cell line (HT29), (HCT119), human breast cancer cell line (MCF7), human ovarian cancer cell line (SCOV3) and normal human fibroblast (MRC5). Further evaluation using immunocytochemistry technique showed that the extracts did not suppress the expression of HPV 16 E6 oncoprotein in CaSki cells. Although the results from the present study are not sufficient to predict the final biological effectiveness of *Penicilliopsis* sp. It nevertheless served as a practical preliminary screening tool to evaluate biological activities of *Penicilliopsis* sp.

Real success in this area will probably only be achieved through an interdisciplinary approach, whereby the knowledge is combined with the technology and structural tool for mycologists. It should be noted however that not all experiments lead to the discovery of a

totally new chemical compound; occasionally the structural probing reveals a known bioactive compounds, although its source might be novel.

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Appendix A

Preparation of solution, chemical and media

Malt Extract Agar (MEA)

Malt Extract Agar (MEA) agar weighing 13.44 g was dissolved in 400 ml of distilled water in an Erlenmeyer flask using a magnetic stirrer. The solution was poured into universal bottles until half full, than autoclaved at 121^{o}C for 20 minutes. To make agar slants, the universal bottles were tilted are certain angle to give a reasonable slope. The agar in the bottles was left to harden at room temperature.

Potato Dextrose Agar (PDA)

Potato Dextrose Agar (PDA) agar weighing 24 g was dissolved in 400 ml of distilled water in an Erlenmeyer flask using a magnetic stirrer. The solution was poured into universal bottles until half full, than autoclaved at 121^{o}C for 20 minutes. To make agar slants, the universal bottles were tilted are certain angle to give a reasonable slope. The agar in the bottles was left to harden at room temperature.

Muller-Hinton Agar (MHA)

Muller-Hinton Agar (MHA) was agar weighing 38 g was dissolved in 400 ml of distilled water in an Erlenmeyer flask using a magnetic stirrer. The solution was poured into universal bottles until half full, than autoclaved at 121^{o}C for 20 minutes. To make agar slants, the universal bottles were tilted are certain angle to give a reasonable slope. The agar in the bottles was left to harden at room temperature.

Muller-Hinton Broth (MHB)

Muller-Hinton Broth (MHB) was agar weighing 38 g was dissolved in 400 ml of distilled water in an Erlenmeyer flask using a magnetic stirrer. The solution was poured into universal bottles until half full, than autoclaved at 121^{o}C for 20 minutes. To make agar slants, the universal bottles were tilted are certain angle to give a reasonable slope. The agar in the bottles was left to harden at room temperature.

Yeast Peptone Dextrose Agar (YPDA)

Yeast Peptone Dextrose Agar (YPDA) agar weighing 13.44 g was dissolved in 400 ml of distilled water in an Erlenmeyer flask using a magnetic stirrer. The solution was poured into universal bottles until half full, than autoclaved at 121^{o}C for 20 minutes. To make agar slants, the universal bottles were tilted are certain angle to give a reasonable slope. The agar in the bottles was left to harden at room temperature.

Sabouraud dextrose agar (SDA)

Sabouraud dextrose agar (SDA) agar weighing 65 g was dissolved in 400 ml of distilled water in an Erlenmeyer flask using a magnetic stirrer. The solution was poured into universal bottles until half full, than autoclaved at 121^{o}C for 20 minutes. To make agar slants, the universal bottles were tilted are certain angle to give a reasonable slope. The agar in the bottles was left to harden at room temperature.

Preparation of Glucose-Yeast-Malt-Peptone (GYMP) agar

A 500 ml Erlenmeyer flask was filled with 0.4 g $MgSO_4.7H_2O$, 0.4g NH_4Cl g KH_2PO_4, 0.4 g K_2HPO_4, 6.0 g glucose3.2 g peptone, 3.2 g malt extract and 3.2 g yeast extract and 70.0 g bacteriological agar. The contents were dissolved in 400 ml of distilled water using a

magnetic stirrer. The flask was covered with non-absorbent cotton plugs and aluminium foil, then autoclaved at 121^{o}C for 20 minutes. The sterilized medium was left to cool, then poured into Petri dishes aseptically and allowed them to harden.

Cell Culture

Basic Medium 199

One sachet of medium 199 (Flow lab, Australia) that containing Earle's salt with L-Glutamine and HEPES (N-2-Hydroxylethyl-Piperazine-N-2-Ethane-sulfonoc Acid, Sigma, USA) without sodium bicarbonate (Flow Lab). Was made up to 1 litre with distilled water. Two grams of sodium bicarbonate ($NaHCO_3$, Merck, Germany) was added to the medium. The medium was filter sterilized using a 0.22 ?m filter membrane. (Schleicher & Schuell) and stored at 4^{o}C for up to 4 months.

RPMI 1640 Medium

One bottle of RPMI 1640 powder with Earle's salts and L-glutamine (Flow lab) was made up to one litre with sterile distilled water. Two grammes of sodium bicarbonate (Merck) and 0.5206 g of N – 2 – hydroxyrthyl – piperazine – N' – 2 – ethane – sulfonic acid (HEPES, Sigma) were added. The medium was stirred and pH was adjusted to pH 7.4. The medium was filter sterilized using 0.2 µm cellulose filter membrane (Schleicher & Schuell) and stored at 4^{o}C up to a shelf life of 2 months.

10% Supllemented RPMI medium and 199 medium

100 ml of 10% supplemented RPMI 1640 medium and medium 199 was prepared by mixing 90 ml of basic medium, 10 ml of heat inactivated foetal bovine serum (FBS was heated at 56^{o}C for 30 minutes, Flowlab), 2 ml of penicillin/streptomycin (PAA Laboratory

HmbG) and 1 ml of amphostat B (Flowlab) in a beaker. The pH was adjusted according to the colour of the medium by adding hyrochloric acid (Systerm) or sodium hydroxide (Systerm). The desired ph 7.2 – 7.4 where the medium should appear as reddish orange. The medium was filter sterilized using 0.2 µm cellulose filter membrane and stored at 4^{o}C up to two weeks.

20% Supplemented RPMI 1640 Medium and Medium 199

50 ml of 20% supplemented RPMI 1640 medium and medium 199 was prepared by mixing 45 ml of 10% supplemented medium and 5 ml of heat inactivated foetal bovine serum in a beaker. The medium was filter sterilized using 0.2 µm cellulose filter membrane and stored at 4^{o}C up to 2 weeks. This 20% supplemented medium was used as revived cell.

Freezing Medium

The freezing medium consisted of 50% inactivated foetal bovine serum, 40% RPMI 1640 medium and 10% dimethyl sulfoxide (DMSO, Sigma). The solution was mixed well by using a 20 ml disposable syringe (Terumo) and filters membrane and kept 4^{o}C. Freshly prepared freezing medium was used for cryopreservation of cells.

Phosphate Buffer Saline (PBS) ph 7.2

The phosphate buffer saline was prepared using 1.52 g of sodium phosphate anhydrous (Na_2HPO_4, Merck) and 8.5 g of sodium chloride (NaCl, BDH Lab Supplies), which were dissolved in 1 litre of sterile distilled water. The solution was stirred and the pH was adjusted to pH 7.2. the solution was then filtered into a sterile bottle using filter paper (Whatman 541). The solution was autoclaved (P-Selecta) and kept at room temperature.

Phosphate Buffer Saline (PBS) pH 7.6

The phosphate buffer saline was prepared using 1.5 g of potassium phosphate anhydrous (K_2HPO_4, BDH Lab Supplies, 0.2 g of potassium dihyrogen orthophosphate (KH_2PO_4, Merck) and 7.75 g of sodium chloride (NaCl, BDH Lab Supplies), which were dissolved in 1 litre of sterile distilled water. The solution was stirred and pH was adjusted to pH 7.6. The solution was then filtered using filter filter paper and kept at rom temperature.

10% Dimethyl Sulfoxide (DMSO)

The 10% DMSO solution was prepared by mixing DMSO (Sigma) with sterile distilled water in a ration of 1:9. Freshly prepared 10% DMSO was used for the neutral red cytotoxic assay.

0.4% Tryphan Blue

0.4% Tryphan blue was prepared by dissolving 0.2g powdered tryphan blue (Sigma) in 50 ml of sterile distilled water. The solution was kept at room temperature.

Neutral Red Stock Solution

Neutral red stock solution was prepared by dissolving 0.04 g of neutral red (ICN Biomedicals Inc.) in 10 ml of sterile distilled water to provide a final concentration of 4 mg/ml. The stock solution was wrapped with aluminum foil and stored at 4^oC.

Neutral Red – Medium

The neutral red stock solution was further diluted with 10% RPMI 1640 medium to provide a final concentration of 50 g/ml in a centrifuge tube. The solution was wrapped with aluminum foil and kept at room temperature. The solution was centrifuged twice at 1, 500 rpm for 10 min to removed any precipitate of dye crystals.

Washing Solution

The washing solution consisted of 1 ml of formaldehyde (Sigma) and 1 g of calcium chloride ($CaCl_2$, Sigma), which were dissolved in 100 ml distilled water and stored at 4^oC.

Resorb Solution

The resorb solution was prepared by adding 1 ml of glacial acetic (BDH Lab Supplies) with 9 ml absolute ethanol (HmbG chemicals) and 50 ml absolute ethanol (HmbG chemicals) and 50 ml distilled water. The solution was store 4^oC.

3% Hydrogen Peroxide

The 3% hydrogen peroxide was prepared by mixing 1 ml of 30% hydrogen peroxide (H_2O_2, BDH Lab Supplies) with 9 ml of distilled water. The solution was wrapped with aluminum foil and kept at room temperature.

Antioxidant Activity Assay

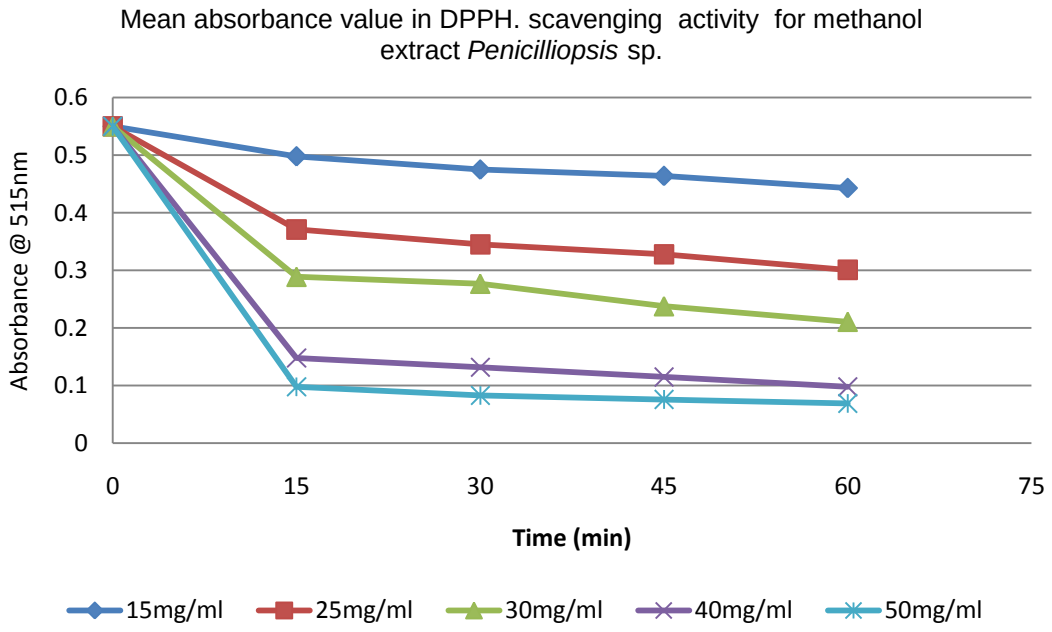
Preparation of stock solution

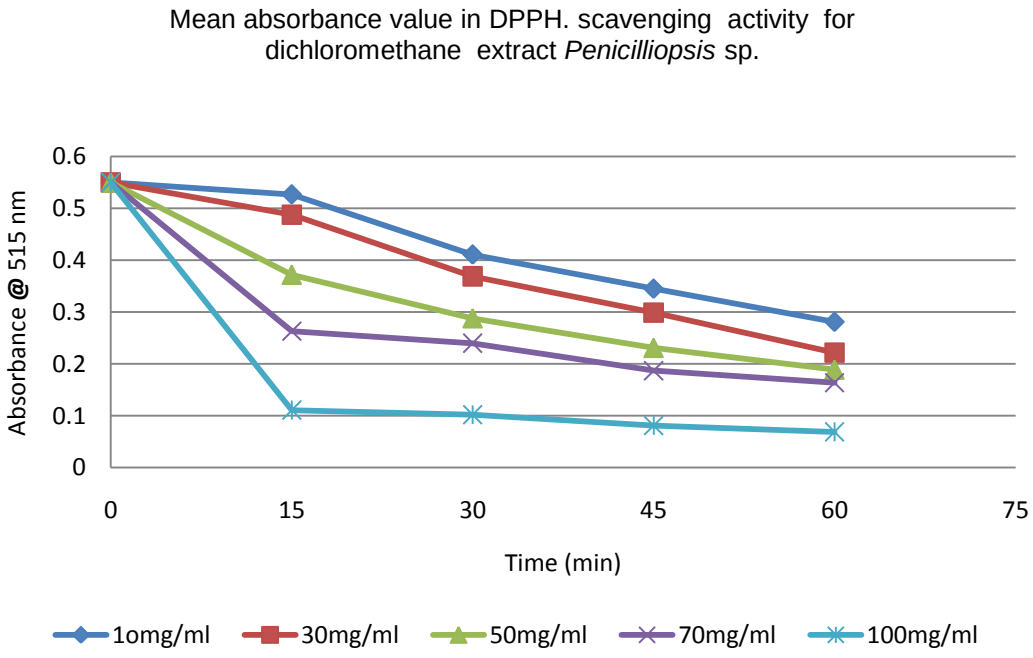
A stock solution of 20 mg/ml of each extract was prepared and wrapped in aluminium foil. The crude extracts were dissolved in methanol (Systerm).

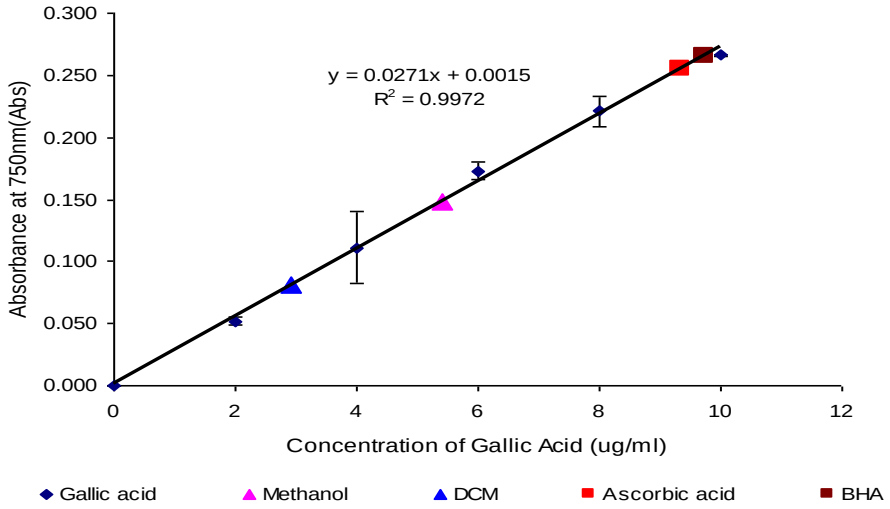
APPENDIX B (EXPERIMENTAL DATA)

Results

Reaction kinetics of scavenging effect on 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) radical



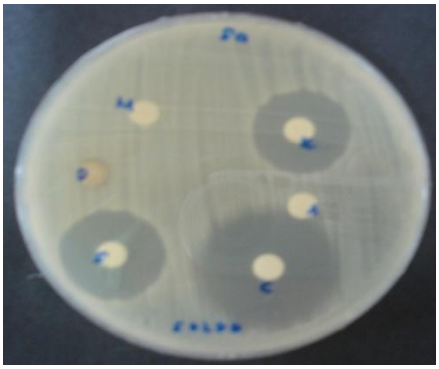




Standard calibration curve for total phenolics contain obtained from gallic acid using Follin-Ciocalteau method.

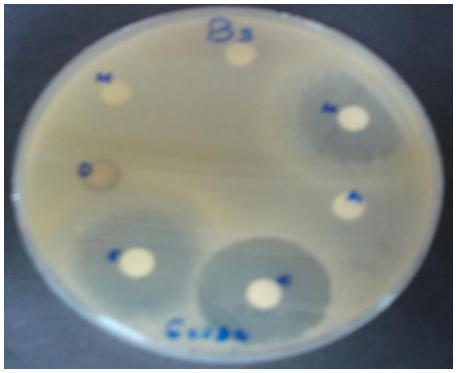
Antimicrobial

Gram positive bacteria



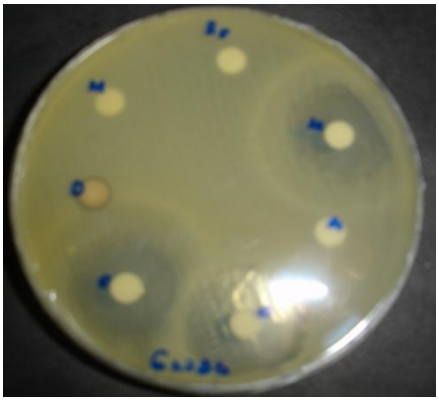
Penicilliopsis sp. (KUM60280) against *Staphylococcus aureus*, Sa

M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin



Penicilliopsis sp. (KUM60280) against *Bacillus subtilis*, Bs

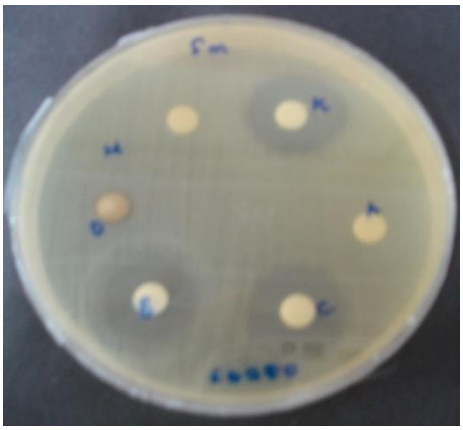
M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin



Penicilliopsis sp. (KUM60280) against *Bacillus cereus*, Bc

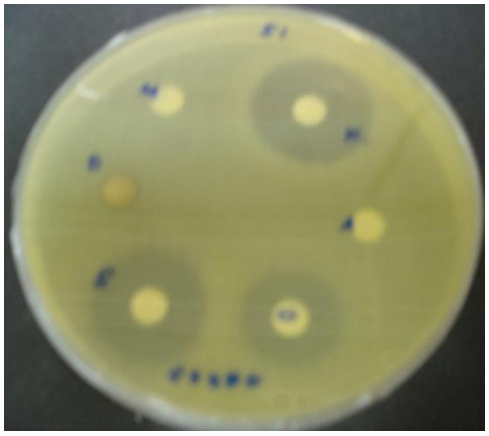
M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin

Oral Bacteria



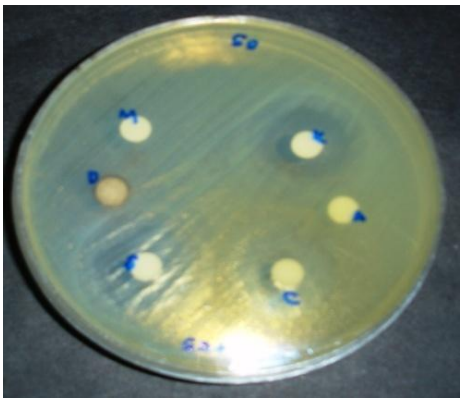
Penicilliopsis sp. (KUM60280) against *Streptococcus mutan*, Sm

M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin



Penicilliopsis sp. (KUM60280) against *Streptococcus mitis*, Si

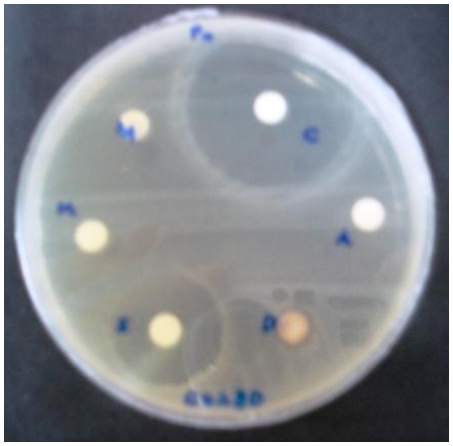
M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin



Penicilliopsis sp. (KUM60280) against *Streptococcus sanguis*, Ss

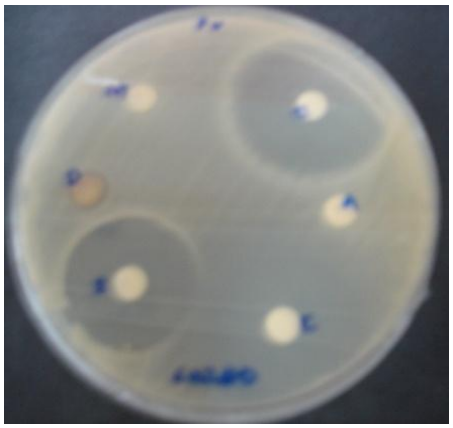
M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin

Gram negative bacteria



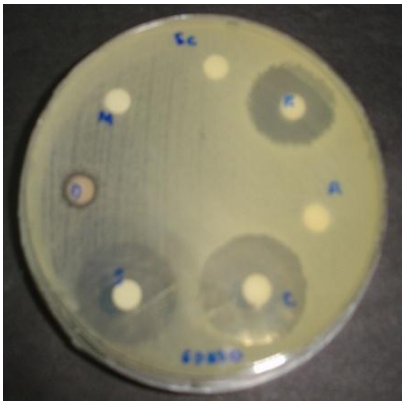
Penicilliopsis sp. (KUM60280) against *Pseudomonas aeruginosa*, Pa

M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin



Penicilliopsis sp. (KUM60280) against *Pseudomonas vulgaria*, Pv

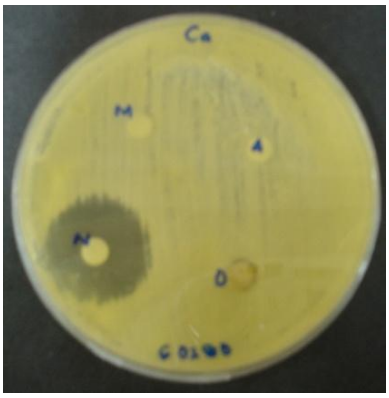
M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin



Penicilliopsis sp. (KUM60280) against *Escherichia coli,* Ec

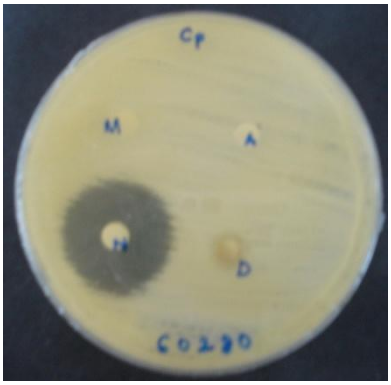
M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin

Anti-fungal



Penicilliopsis sp. (KUM60280) against *Candida albican,* Ca

M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K: Kanamycin; N:Nystatin



Penicilliopsis sp. (KUM60280) against *Candida parasilopsis*, Cp

M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K:Kanamycin; N:Nystatin

Penicilliopsis sp. (KUM60280) against *Saccromyces pombe,* Sp

M: Methanol; D; Dichloromethane; S; Streptomycin; C: Chloromphenicol; K : Kanamycin; N:Nystatin

Appendix C: Statistic

One-way ANOVA to compare decrease in absorbance values to determine time taken to reach steady state during scavenging of DPPH·

Methanol
25mg/ml

TIME	Count	Mean	Homogeneous Groups
60	3	0.157667	X
45	3	0.183667	XX
30	3	0.211333	X
15	3	0.264667	X
2	3	0.368	X
1	3	0.383667	XX
0	3	0.408	X

Contrast	Difference	+/- Limits
0 - 1	0.0243333	0.0277497
0 - 2	*0.04	0.0277497
0 - 15	*0.143333	0.0277497
0 - 30	*0.196667	0.0277497
0 - 45	*0.224333	0.0277497
0 - 60	*0.250333	0.0277497
1 - 2	0.0156667	0.0277497
1 - 15	*0.119	0.0277497
1 - 30	*0.172333	0.0277497
1 - 45	*0.2	0.0277497
1 - 60	*0.226	0.0277497
2 - 15	*0.103333	0.0277497
2 - 30	*0.156667	0.0277497
2 - 45	*0.184333	0.0277497
2 - 60	*0.210333	0.0277497
15 - 30	*0.0533333	0.0277497
15 - 45	*0.081	0.0277497
15 - 60	*0.107	0.0277497
30 - 45	0.0276667	0.0277497
30 - 60	*0.0536667	0.0277497
45 - 60	0.026	0.0277497

30mg/ml

TIME	Count	Mean	Homogeneous Groups
60	3	0.133333	X
45	3	0.159	X
30	3	0.184333	X
15	3	0.237333	X
2	3	0.352	X
1	3	0.371667	X
0	3	0.407	X

Contrast	Difference	+/- Limits
0 - 1	*0.0353333	0.0191531
0 - 2	*0.055	0.0191531
0 - 15	*0.169667	0.0191531
0 - 30	*0.222667	0.0191531
0 - 45	*0.248	0.0191531
0 - 60	*0.273667	0.0191531
1 - 2	*0.0196667	0.0191531
1 - 15	*0.134333	0.0191531
1 - 30	*0.187333	0.0191531
1 - 45	*0.212667	0.0191531
1 - 60	*0.238333	0.0191531
2 - 15	*0.114667	0.0191531
2 - 30	*0.167667	0.0191531
2 - 45	*0.193	0.0191531
2 - 60	*0.218667	0.0191531
15 - 30	*0.053	0.0191531
15 - 45	*0.0783333	0.0191531
15 - 60	*0.104	0.0191531
30 - 45	*0.0253333	0.0191531
30 - 60	*0.051	0.0191531
45 - 60	*0.0256667	0.0191531

40mg/ml

TIME	Count	Mean	Homogeneous Groups
60	3	0.0606667	X
45	3	0.0703333	X
30	3	0.121	X
15	3	0.159333	X
2	3	0.287	X
1	3	0.308	X
0	3	0.354	X

Contrast	Difference	+/- Limits
0 - 1	*0.046	0.0236622
0 - 2	*0.067	0.0236622
0 - 15	*0.194667	0.0236622
0 - 30	*0.233	0.0236622
0 - 45	*0.283667	0.0236622
0 - 60	*0.293333	0.0236622
1 - 2	0.021	0.0236622
1 - 15	*0.148667	0.0236622
1 - 30	*0.187	0.0236622
1 - 45	*0.237667	0.0236622
1 - 60	*0.247333	0.0236622
2 - 15	*0.127667	0.0236622
2 - 30	*0.166	0.0236622
2 - 45	*0.216667	0.0236622
2 - 60	*0.226333	0.0236622
15 - 30	*0.0383333	0.0236622
15 - 45	*0.089	0.0236622
15 - 60	*0.0986667	0.0236622
30 - 45	*0.0506667	0.0236622
30 - 60	*0.0603333	0.0236622
45 - 60	0.00966667	0.0236622

50mg/ml

TIME	Count	Mean	Homogeneous Groups
60	3	0.0626667	X
45	3	0.0653333	X
30	3	0.0663333	X
15	3	0.0726667	X
2	3	0.207	X
1	3	0.232	X
0	3	0.286333	X

Contrast	Difference	+/- Limits
0 - 1	*0.0543333	0.0112067
0 - 2	*0.0793333	0.0112067
0 - 15	*0.213667	0.0112067
0 - 30	*0.22	0.0112067
0 - 45	*0.221	0.0112067
0 - 60	*0.223667	0.0112067
1 - 2	*0.025	0.0112067
1 - 15	*0.159333	0.0112067
1 - 30	*0.165667	0.0112067
1 - 45	*0.166667	0.0112067
1 - 60	*0.169333	0.0112067
2 - 15	*0.134333	0.0112067
2 - 30	*0.140667	0.0112067
2 - 45	*0.141667	0.0112067
2 - 60	*0.144333	0.0112067
15 - 30	0.00633333	0.0112067
15 - 45	0.00733333	0.0112067
15 - 60	0.01	0.0112067
30 - 45	0.001	0.0112067
30 - 60	0.00366667	0.0112067
45 - 60	0.00266667	0.0112067

DCM

10 mg/ml

Time	Count	Mean	Homogeneous Groups
60	3	0.228	X
45	3	0.341667	X
30	3	0.371667	X
15	3	0.393667	X
2	3	0.436333	X
1	3	0.455333	XX
0	3	0.465333	X

Contrast	Difference	+/- Limits
0 - 1	0.01	0.0233547
0 - 2	*0.029	0.0233547
0 - 15	*0.0716667	0.0233547
0 - 30	*0.0936667	0.0233547
0 - 45	*0.123667	0.0233547
0 - 60	*0.237333	0.0233547
1 - 2	0.019	0.0233547
1 - 15	*0.0616667	0.0233547
1 - 30	*0.0836667	0.0233547
1 - 45	*0.113667	0.0233547
1 - 60	*0.227333	0.0233547
2 - 15	*0.0426667	0.0233547
2 - 30	*0.0646667	0.0233547
2 - 45	*0.0946667	0.0233547
2 - 60	*0.208333	0.0233547
15 - 30	0.022	0.0233547
15 - 45	*0.052	0.0233547
15 - 60	*0.165667	0.0233547
30 - 45	*0.03	0.0233547
30 - 60	*0.143667	0.0233547
45 - 60	*0.113667	0.0233547

30 mg/ml

Time	Count	Mean	Homogeneous Groups
60	3	0.228	X
45	3	0.341667	X
30	3	0.371667	X
15	3	0.393667	X
2	3	0.436333	X
1	3	0.455333	XX
0	3	0.465333	X

Contrast	Difference	+/- Limits
0 - 1	0.01	0.0233547
0 - 2	*0.029	0.0233547
0 - 15	*0.0716667	0.0233547
0 - 30	*0.0936667	0.0233547
0 - 45	*0.123667	0.0233547
0 - 60	*0.237333	0.0233547
1 - 2	0.019	0.0233547
1 - 15	*0.0616667	0.0233547
1 - 30	*0.0836667	0.0233547
1 - 45	*0.113667	0.0233547
1 - 60	*0.227333	0.0233547
2 - 15	*0.0426667	0.0233547
2 - 30	*0.0646667	0.0233547
2 - 45	*0.0946667	0.0233547
2 - 60	*0.208333	0.0233547
15 - 30	0.022	0.0233547
15 - 45	*0.052	0.0233547
15 - 60	*0.165667	0.0233547
30 - 45	*0.03	0.0233547
30 - 60	*0.143667	0.0233547
45 - 60	*0.113667	0.0233547

50 mg/ml

Time	Count	Mean	Homogeneous Groups
60	3	0.219667	X
45	3	0.270333	X
30	3	0.316667	X
15	3	0.356	X
2	3	0.376333	X
1	3	0.398	X
0	3	0.416667	X

Contrast	Difference	+/- Limits
0 - 1	0.0186667	0.0200727
0 - 2	*0.0403333	0.0200727
0 - 15	*0.0606667	0.0200727
0 - 30	*0.1	0.0200727
0 - 45	*0.146333	0.0200727
0 - 60	*0.197	0.0200727
1 - 2	*0.0216667	0.0200727
1 - 15	*0.042	0.0200727
1 - 30	*0.0813333	0.0200727
1 - 45	*0.127667	0.0200727
1 - 60	*0.178333	0.0200727
2 - 15	*0.0203333	0.0200727
2 - 30	*0.0596667	0.0200727
2 - 45	*0.106	0.0200727
2 - 60	*0.156667	0.0200727
15 - 30	*0.0393333	0.0200727
15 - 45	*0.0856667	0.0200727
15 - 60	*0.136333	0.0200727
30 - 45	*0.0463333	0.0200727
30 - 60	*0.097	0.0200727
45 - 60	*0.0506667	0.0200727

70 mg/ml

Time	Count	Mean	Homogeneous Groups
60	3	0.225	X
45	3	0.289667	X
30	3	0.320667	X
15	3	0.353333	X
2	3	0.374333	X
1	3	0.383	X
0	3	0.394667	X

Contrast	Difference	+/- Limits
0 - 1	0.0116667	0.0204618
0 - 2	0.0203333	0.0204618
0 - 15	*0.0413333	0.0204618
0 - 30	*0.074	0.0204618
0 - 45	*0.105	0.0204618
0 - 60	*0.169667	0.0204618
1 - 2	0.00866667	0.0204618
1 - 15	*0.0296667	0.0204618
1 - 30	*0.0623333	0.0204618
1 - 45	*0.0933333	0.0204618
1 - 60	*0.158	0.0204618
2 - 15	*0.021	0.0204618
2 - 30	*0.0536667	0.0204618
2 - 45	*0.0846667	0.0204618
2 - 60	*0.149333	0.0204618
15 - 30	*0.0326667	0.0204618
15 - 45	*0.0636667	0.0204618
15 - 60	*0.128333	0.0204618
30 - 45	*0.031	0.0204618
30 - 60	*0.0956667	0.0204618
45 - 60	*0.0646667	0.0204618

100 mg/ml

Method: 95.0 percent LSD

Time	Count	Mean	Homogeneous Groups
60	3	0.175667	X
45	3	0.213333	X
30	3	0.238333	X
15	3	0.287	X
2	3	0.321	X
1	3	0.331333	XX
0	3	0.345	X

Contrast	Difference	+/- Limits
0 - 1	0.0136667	0.01869
0 - 2	*0.024	0.01869
0 - 15	*0.058	0.01869
0 - 30	*0.106667	0.01869
0 - 45	*0.131667	0.01869
0 - 60	*0.169333	0.01869
1 - 2	0.0103333	0.01869
1 - 15	*0.0443333	0.01869
1 - 30	*0.093	0.01869
1 - 45	*0.118	0.01869
1 - 60	*0.155667	0.01869
2 - 15	*0.034	0.01869
2 - 30	*0.0826667	0.01869
2 - 45	*0.107667	0.01869
2 - 60	*0.145333	0.01869
15 - 30	*0.0486667	0.01869
15 - 45	*0.0736667	0.01869
15 - 60	*0.111333	0.01869
30 - 45	*0.025	0.01869
30 - 60	*0.0626667	0.01869
45 - 60	*0.0376667	0.01869