1.0 INTRODUCTION

Red rice is traditionally prepared by fermenting rice with a fungal starter from the genus *Monascus*, notably the *M. purpureus*, *M. pilosus*, or *M. ruber*. These fungi are chosen based on their pigments-producing ability and also various beneficial secondary metabolites. For thousands of years, the Chinese community has strong connection with red rice on the ground of its purported and renowned medicinal properties such as anti-hypertensive (Hsieh and Tai, 2003), anti-diabetic (Shi and Pan, 2010), and good in regulating blood circulation (Wu and Zhang, 1991). As such, the red rice has been a mainstay in the traditional Chinese medicine (TCM) ever since and has been included by the Chinese in their daily dietary intake.

However, as traditional as it is, red rice did not usually undergo formal safety and health inspection on its preparation and storage. Fungi belonging to the genus *Monascus* which are used as fermentation starters are widely known to produce citrinin during their metabolism (Blanc *et al.*, 1995a; 1995b; Li *et al.*, 2003).

Citrinin is a mycotoxin isolated from *Penicillium citrinum* (Hetherington and Raistrick, 1931). It is also produced by other species of *Penicillium* (Ei-Banna *et al.*, 1987) and *Aspergillus* (Kurata, 1990). On this basis, citrinin is also known as a naturally occurring contaminant in stored food commodities such as corn, wheat, barley and rice (CAST, 2003). Betina (1989) provided information on toxicity studies which showed that this mycotoxin is mainly nephrotoxic (toxic to kidney). Citrinin is also known to affect proximal tubules of the kidney in animals (Phillips *et al.*, 1980).

As a result of citrinin contamination, in recent years, there has been an increasing concern and much debate on whether or not the red rice is safe for human consumption. Questions raised revolve around its many and highly appraised beneficial values but with detrimental effects that may co-exist simultaneously. Here the argument

became profound on the ground that if the detrimental effects are not monitored, it will implicate health hazards to consumers.

Measures for quality control have always been overlooked when it came to traditional preparation. Nevertheless, extensive studies and numerous reports have attempted to highlight the detrimental effects of red rice. Although it has been commercialised in several countries (especially in Europe), but banned from several others (USA), the red rice continues to thrive within local and international Chinese communities. Poor and ill-supervised preparation and storage will only subject the red rice to chronic and deteriorated condition which in turn will render the red rice unsuitable for human consumption.

Like other foodstuffs, red rice upon storage has a high risk of contamination by detrimental microorganisms (especially the fungi) which might implicate hazard towards human consumption and subsequently, to human health. However, currently, there has been no work reported to assess its mycoflora status upon storage and the possible adverse effects they might bring upon.

The possibilities of detrimental effect suppressing the beneficial ones are common issues when dealing with traditional medicines such as the red rice. Without strong clinical studies, one can never be certain of when the beneficial or detrimental properties will outweigh each other.

In the western scientific community, however, this issue is taken rather seriously into consideration, with multiple agencies and industrial groups trying to commercialise the least detrimental and toxic red rice preparation resulted from years of studies and manipulation. Such the case, sadly, is not seen in Malaysia. With 26% Chinese, and strong if not great reliability on TCM, we still face the age-old complication which is lack of comprehensive surveys and monitoring programmes resulting in poor documentation on the health and clinical status of the red rice in question.

1.1 Objectives of Research

The present investigation was carried out with two-fold objectives;

a. to determine the occurrence of mycotoxigenic fungi in red rice
b. to evaluate the mycotoxins contaminating red rice marketed throughout the state of Selangor, Malaysia

2.0 LITERATURE REVIEW

2.1 Red Rice

Red rice is a bright reddish purple product of solid-state fermentation which acquires its colour from the metabolites of several fungi from the genus *Monascus*, most notably the *M. purpureus* (*purple* in Latin). It is consumed widely throughout the Asian countries (Ma *et al.*, 2000). In mainland China, it is included as a part of daily staple diet. Throughout the countries, it is known by many names; *Angkak* in Javanese, *Beni Koji* in Japanese, *Hong-Qu* in Chinese, *Hon-Chi* in Mandarin.

The red rice has a long and outstanding history where it has been used then (and still is) as a traditional Chinese food and medicine since as early as 800 A.D (Li *et al*., 1998). The use of red rice was found to be recorded in ancient Chinese books as early as Northern Sung dynasty (ca. 9^{th} century). During the Ming Dynasty (1368-1644), in Sung Ing-Shing's "*Tien Gung Kai Wu*", detailed procedures for production of red rice were recorded, and in Lee Shi-Jeng's "*Ben Cao Gang Mu - Dan Shi Bu Yi,*" (Compendium of Materia Medica, 1578 A.D.), it was stated that red yeast rice "functions in invigorating spleen and digestion, and promoting blood circulation and resolving blood stasis" (Fu *et al*., 2003).

Though it has long been within the Chinese and Asian community, recently there are a number of patents registered in Japan, the United States, France and Germany reporting the use of Monascal products (especially the red rice) as food colorant and additive (Silveira *et al.*, 2008). However, albeit being marketed as food supplement in USA, red rice usage as medicament has not been approved by the Food and Drug Administration (FDA) due to the presence of different concentrations of the mycotoxin citrinin (Kumari *et al.*, 2009).

By composition, red rice contains predominantly rice starches and sugars, yeast polyketides, fatty acids, pigments and condensed tannins (Martinkova *et al.*, 1999; Heber *et al.*, 2001).

2.2 Fermentation of Red Rice

Traditional production of red rice involves several steps. The knowledge of preparation was passed down from generation to generation. Modern and commercialised method adopted by factories is the modified way to suit large scale production and to meet the safety and health requirement set by the authorities. However, in certain districts, the production of red rice remains as small scale production, employing the traditional technique.

Traditionally, the rice used in the process is usually the normal, non-glutinous type to avoid clumping and to ease drying process. The rice is washed thoroughly before being steamed, and moistened by means of water spray. The moistened rice is then prepared on trays or in a rotary perforated bed. Inoculation with *Monascus* spp. starter occurred at this stage. The starter culture can be easily obtained at Chinese household shops. It can be either from a single species (*M. purpureus*, *M. pilosus*, or *M. ruber*) or a combination of it. Incubation of the inoculated rice at 34°C for two days takes place in a clean air-circulating chamber followed by a decrease to 25 - 30°C for the remaining five to ten days, or for some other producers up to two weeks, in which time fermentation occurs. During this period, fungi will grow and invade the rice grain while imparting its rich reddish-purplish colour, hence the name red rice. After this period, the fermented red rice is left on shelves for air-drying and subsequently put on the market in the forms of whole grain or as fine powder as shown in Figure 2.1 (Lee and Lee, 2002).

Figure 2.1 : Forms of red rice samples

2.3 Starters in Fermentation of Red Rice

The starter fungi in the fermentation of red rice belong to the genus *Monascus*. Taxonomically, these fungi belong to the Kingdom Fungi; Phylum Ascomycota; Class Eurotiomycetidae; Order Plectascales; Family Monascaceae; and Genus *Monascus*. The members of this genus constitute a group of homothallic ascomycetous fungi occurring worldwide (Kolotila and Volz, 1980).

The genus *Monascus* was studied by Dutch scientists in 1884 after the discovery of its use by villagers on a colonial island of Java (Van, 1884). A species isolated from red rice was named *M. purpureus* Went in 1895, recognising its purple coloration (Went, 1895). In 1983, Hawksworth and Pitt described in detail on the cultural and microscopical characteristics of *M. purpureus* Went, *M. pilosus* Sato, and *M. ruber*

Tiegh. These three representatives are among the most important members of the genus *Monascus* because of their use in the production of a range of oriental fermented food.

A larger list of *Monascus* species was tabulated in Table 2.1 below which is provided and maintained by www.indexfungorum.org. Index Fungorum is a community resource currently co-ordinated and supported by Commonwealth Agricultural Bureau International (CABI Bioscience); CBS Fungal Biodiversity Centre, Netherlands; and Landcare Research, New Zealand. It maintains the database of fungi (including yeasts, lichens, chromistan fungi, protozoan fungi and fossil forms) at species level and below.

Table 2.1 : Members of the genus *Monascus*

Species Name	Authority Names	Synonyme
Monascus albidulus	Zhong Q. Li & F. Guo	
Monascus albidus	K. Sato	*M. purpureus*
Monascus albidus var. *albidus*	K. Sato	
Monascus albidus var. *glaber*	K. Sato	
Monascus anka	K. Sato	*M. purpureus*
Monascus anka var. *anka*	K. Sato	
Monascus anka var. *rubellus*	K. Sato	
Monascus araneosus	K. Sato	*M. purpureus*
Monascus argentinensis	Stchigel & Guarro	
Monascus aurantiacus	Zhong Q. Li	
Monascus aurantiacus	Zhong Q. Li & F. Guo	
Monascus barkeri	P.A. Dang	
Monascus bisporus	L.R. Fraser	
Monascus eremophilus	A.D. Hocking & Pitt	
Monascus floridanus	P.F. Cannon & E.L. Barnard	
Monascus fuliginosus	K. Sato	
Monascus fumeus	Zhong Q. Li & F. Guo	
Monascus kaoliang	C.F. Lin & Iizuka	
Monascus lunisporas	Udagawa & H. Baba	

Table 2.1 cont.

Monascus major	K. Sato	*M. purpureus*
Monascus mucoroides	Tiegh	
Monascus pallens	P.F. Cannon, Abdullah & A. Abbas	
Monascus pilosus	K. Sato	
Monascus pubigerus	K. Sato	
Monascus purpureus	Went	
Monascus ruber	Tiegh	
Monascus rubiginosus	K. Sato	*M. purpureus*
Monascus rubropunctatus	K. Sato	
Monascus rutilus	Zhong Q. Li & F. Guo	
Monascus sanguineus	P.F. Cannon, Abdullah & A. Abbas	
Monascus serorubescens	K. Sato	
Monascus vini	Savul & Hulea	*M. purpureus*
Monascus vitreus	K. Sato	

In nature, members of the genus *Monascus* thrive in starch-rich substrates. Consequently, as starters for red rice fermentation, they are readily and commonly found on the rice or products derived from it such as Chinese red wine, fruit sauce, fermented bean curd, or fermented Sorghum (Hawksworth and Pitt, 1983).

2.4 Applications of Red Rice

Being associated with the Chinese community for over thousands of years, the importance and functions of red rice are more often than not reported in; (1) in food application, (2) in clinical/pharmacological application, and (3) in industrial application. These three applications have always been recorded and reported in interrelations.

2.4.1 Food Applications of Red Rice

In food application, red rice is used mainly as natural food colorant (Wong and Koehler, 1983; Francis, 1987; Fabre *et al*., 1993; Sheu *et al*., 2000). Red rice has been known to impart colour to several Chinese delicacies such as soybean cheese, sufu (red soybean curd), preserved dry fish, pickled vegetables, salted meats, roasted pork, roasted duck, and vegetable pork stew (Blanc *et al.*, 1998). Red rice not only imparts the rich purple-red colour to the foods, but also enhances the food flavour and provides natural aroma (Han *et al.*, 2001).

The rich red pigments of red rice are also of great advantage in substitution of the highly toxic nitrites [E 252 (potassium nitrate) and E 249 (nitrites)] that are used nowadays for the colouring and biological preservation of meat and sausage products (Blanc, 1998). A number of countries are gradually adopting this natural pigment to replace coal-tar dyes, as the latter have been implicated as carcinogens (Lee and Lee, 2001). And above all, it is vastly employed in the making of the Chinese red rice wine (*samsu* in Malay) much similar to its Japanese counterpart except for its colour (white rice wine; *sake*).

2.4.2 Medical Applications of Red Rice

From a medical standpoint, red rice exerts many clinical and health benefits, with the most notable attribute being anti-cholestrolemic. In the late 1970's, monacolins were first isolated from *Monascus* (Endo, 1979). This substance is somehow proven to be identical to the drug statin. Since then it has been isolated, purified, and commercialised based on its ability to inhibit cholesterol synthesis (and subsequently lower cholesterol levels) by blocking the action of 3-hydroxy-3-methylglutaryl

coenzyme A reductase (HMG-CoA reductase; rate-limiting enzyme in cholesterol biosynthesis). As a consequence, total cholesterol can circulate better and LDL-cholesterol will be lowered (Man *et al*., 2002; Journoud and Jones, 2004). Medicinal properties of red rice are tabulated in Table 2.2, and detrimental effects of red rice are tabulated in Table 2.3.

Table 2.2 : Medicinal properties of red rice

Medicinal Property	Reference
Anti-adipogenic	Jeon *et al.*, 2004
Anti-atherogenic	Endo *et al.*, 1985; Wei *et al.*, 2003
Anti-cancer / Anti-tumour	Yasukawa *et al.*, 1996; Yasukawa, 1998; Hong *et al.*, 2008
Anti-hypercholesterolemic	Endo, 1980; Endo *et al.*, 1985; Li *et al.*, 1998; Heber *et al.,* 1999; Bliznakov, 2000; Huang *et al.*, 2007; Jeun *et al.*, 2008
Anti-hyperlipidemic	Su *et al.*, 1995; Liu *et al.*, 1996; Shen *et al.*, 1996; Xie and Duan, 1996; Wang *et al.*, 1997; Liu *et al.*, 2003; Wei *et al.*, 2003; Journoud and Jones, 2004; Zhao *et al.*, 2004; Cicero *et al.*, 2005; Li *et al.*, 2005; Lin *et al.*, 2005; Mak *et al.*, 2005; Lee *et al.*, 2006; Sumioka *et al.*, 2006; Liao *et al.*, 2008
Anti-hypertensive	Kohama *et al.*, 1987; Tsuji *et al.*, 1992a; Tsuji *et al.*, 1992b; Tsuji *et al.*, 1992c; Kushiro *et al.*, 1996; Hsieh and Tai, 2003
Anti-hypertriglyceridemic	Wang *et al.*, 2000; Zhao *et al.*, 2003
Anti-inflammatory	Yasukawa, 1998; Zhao *et al.*, 2004
Anti-microbial	Wong and Bau, 1977; Wong and Koehler, 1981; Chen and Tseng, 1989; Wang *et al.*, 2002b; Kim *et al.*, 2006a; Kim *et al.*, 2006b
Anti-oxidant	Aniya *et al.*, 1999; Aniya *et al.*, 2000; Tseng *et al.*, 2003; Yang *et al.*, 2006; Pyo and Lee, 2007; Lee *et al.*, 2008
Anti-toxic	Aniya *et al.*, 1998

Table 2.3 : Detrimental effects of red rice

Side-effects	Reference
Anaphylaxis / Allergy	Wigger-Alberti *et al.*, 1999
Myopathy	Smith and Olive, 2003; Mueller, 2006; Polsani *et al.*, 2008
Rhabdomyolysis	Prasad *et al.*, 2002

2.4.3 Industrial Applications of Red Rice

In industries, the importance of red rice stands on the ground of diverse ability of its starter fungi to produce secondary metabolites of many functions and applications. These secondary metabolites are substances produced by *Monascus* spp. at a certain point in their life cycle. These substances are neither required nor vital to the fungal survival. But rather, they are just residue, waste or secretion as a result of the metabolism. Some of these substances might be harmless, and some might be harmful.

Through discovery and technology, fungi from the genus *Monascus* like other microorganisms, can be manipulated for the production of various metabolites and is useful as food additives and pharmaceuticals (Hsu *et al*., 2002). Among such metabolites is an antibacterial compound, 3-hydoxy-4-methoxy-benzoic acid (Wu and Wu, 2000), unsaturated fatty acids (Wang *et al.*, 1997) that may also help to reduce serum lipids, total cholesterol and triglyceride levels, and phytosterols such as beta-sitosterol and campesterol (Heber *et al.*, 1999), which are known to interfere with cholesterol absorption in the intestine (Moghadasian and Frohlich, 1999). Table 2.4 lists the secondary metabolites usually produced by fungi from the genus *Monascus* in red rice.

Table 2.4 : Metabolites from *Monascus* spp. in red rice

Metabolites	Reference
Acetylcholine	Chen and Liu, 2006
Acid-proteinase	Yasuda and Sakaguchi, 1998; Kuba *et al.*, 2005
Amine-oxidase	Frebort, 1997
Chitinase	Wang *et al.*, 2002a
Citrinin	Blanc *et al.*, 1995a; Blanc *et al.*, 1995b; Blanc *et al.*, 1998; Kycko *et al.*, 1998; Sabater-Vilar *et al.*, 1999; Shu and Lin, 2002; Hu and Chen, 2003; Li *et al.*, 2003; Wang *et al.*, 2003; Xu *et al.*, 2003a; Xu *et al.*, 2003b; Carvalho *et al.*, 2005; Chen and Hu, 2005; Liu *et al.*, 2005; Shimizu *et al.*, 2005; Wang *et al.*, 2005; Shimizu *et al.*, 2007; Pattanagul *et al.*, 2008
Ethanol	Chen and Johns, 1994
Fatty acid	Juzlova *et al.*, 1996b
Gamma amino-butyric acid	Tsuji *et al.*, 1992c; Su *et al.*, 2003; Wang *et al.*, 2003
Glucose-amylase	El-Sayed *et al.*, 2000
Lactone	Nozaki *et al.*, 1991
Lovastatin	Sayyad *et al.*, 2007
Mevinolin	Pattanagul *et al.*, 2008
Monakolin	Endo, 1979; Endo, 1980; Endo *et al.*, 1985; Negishi *et al.*, 1986; Hai, 1998; Xu *et al.*, 1998; Su *et al.*, 2003; Wang *et al.*, 2003; Hu and Chen, 2004; Chen and Hu, 2005
Peptidase	Liu *et al.*, 2004
Pigment	Lin, 1973; Manchand *et al.*, 1973; Hiroi *et al.*, 1975; Yoshimora *et al.*, 1975; Carels and Shepherd, 1977; Shepherd, 1977; Wong and Bau, 1977; Broder and Koehler, 1980; Nakawa *et al.*, 1980; Su and Huang, 1980; Wong and Koehler, 1981; Wong and Koehler, 1983; Evans and Wang, 1984; Mak *et al.*, 1990; Johns and Stuart, 1991; Lin and Demain, 1991; Chiu and Chan, 1992; Han and Mudgett, 1992; Lin *et al.*, 1992; Sato *et al.*, 1992; Bavavoda, 1993; Chen and Johns, 1993; Chiu and Poon, 1993; Blanc *et al.*, 1994;

Table 2.4 cont.

	Chen and Johns, 1994; Ju *et al.*, 1994; Juzlova *et al*, 1994; Lee *et al.*, 1994; Lin and Demain, 1994; Hamidi *et al.*, 1995; Lee *et al.*, 1995; Lin and Demain, 1995; Martinkova *et al.*, 1995; Hamidi *et al.*, 1996; Yasukawa *et al.*, 1996; Chen, 1997; Izawa *et al.*, 1997; Kujumdzieva *et al.*, 1997; Sato *et al.*, 1997; Watanabe *et al.*, 1997; de Vrije, 1998; Dussa and Kunz, 1998; Kujumdzieva, 1998; Matter and Luchese, 1998; Shin *et al.*, 1998; Slugen *et al.*, 1998; Ju et al., 1999; Martinkova *et al.*, 1999; Duliu *et al.*, 2000; Fenice *et al.*, 2000; Krairak *et al.*, 2000; Tseng *et al.*, 2000; Hsu *et al.*, 2002; Kim *et al.*, 2002; Carvalho *et al.*, 2003; Dominguez-Espinosa and Webb, 2003; Jung *et al.*, 2003; Carvalho *et al.*, 2005; Carvalho *et al.*, 2006; Kim *et al.*, 2006a; Kim *et al.*, 2006b; Babitha *et al.*, 2007; Kim *et al.*, 2007; Jeun *et al.*, 2008; Pattanagul *et al.*, 2008; Silveira *et al.*, 2008
Protease	Yasuda *et al.*, 1984; Tseng *et al.*, 2000; Liang *et al*., 2006

As shown in Table 2.4, many of the researches were directed to the production of pigments by *Monascus* spp. since the initial application of red rice was to produce food colorant. *Monascus* pigments are a group of secondary metabolites, called azaphilones, which share certain molecular structures and chemical properties (Blanc *et al.*, 1994; Juzlova *et al.*, 1996a).

It has also been shown that *Monascus* strains can produce at least six major pigments as depicted in Figure 2.2 (Juzlova *et al.*, 1996a). These pigments are divided into three groups: (1) orange pigments (rubropunctatin and monascorubrin), which have different side chains on the ozo-lactone ring; (2) red pigments (rubropunctamine and monascorubramine), which are nitrogen analogues of the orange pigments; and (3) yellow pigments (monascin and ankaflavin), which are the reduced forms of the two orange pigments.

Furthermore, it has been thoroughly discussed by several authors, reaching the same conclusion which is within a *Monascus* species the biosynthetic pathway of pigments and citrinin are related through the polyketide pathway (Barber and Staunton, 1979; Barber *et al*., 1981; Blanc *et al*., 1995).

Generally, citrinin is present in trace concentration (0.1 - 500 ppm) as compared to the *Monascus* pigments (10,000 - 100,000 ppm) (Zheng *et al*., 2009).

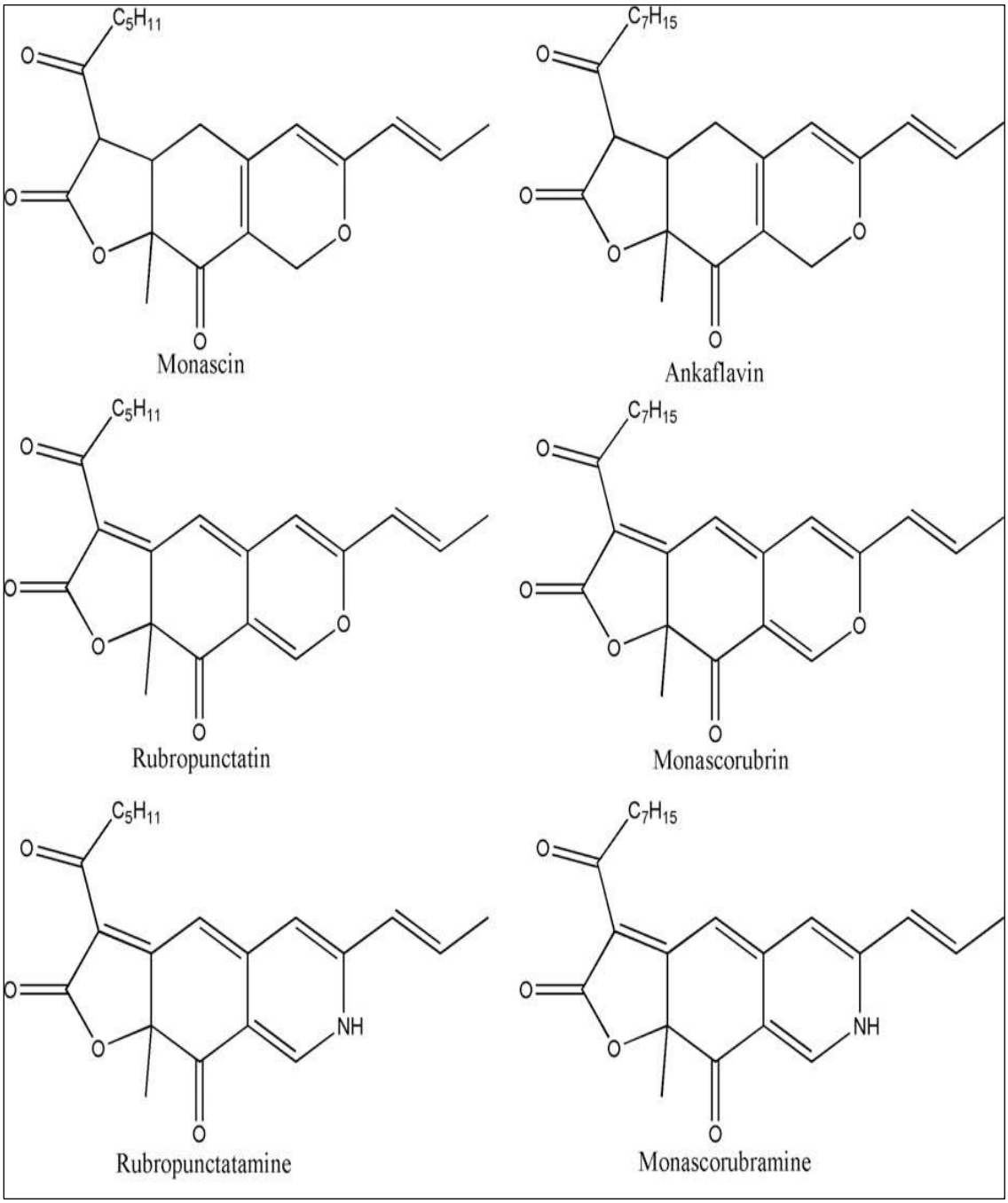

Figure 2.2 : Pigments of *Monascus* spp.

2.5 Consumption of Red Rice in Selangor

Selangor is one of the 13 states of Malaysia. Its geographical position in the centre of Peninsular Malaysia contributed to the state's rapid development and further contributed to its population. It is divided into nine administrative districts. The area covering districts and population from year 2008 to 2010 are tabulated in Appendix A.

By area, Selangor is approximately a small state within Malaysia (8,104 km^2 of 329,847 km^2). However, by population, Selangor is the most populated state with ethnic composition of 52.9% Malay, 27.8% Chinese, 13.3% Indian, and 6% other ethnic groups, and that constitutes about 18.6% of entire Malaysian population (Department of Statistics, Malaysia, 2009).

Most of the Chinese in Selangor still adhere to their customary and traditional belief including the application of TCM in daily life. TCM shops in Selangor are in good business with up to three to four shops operating within small Chinese establishment areas throughout the districts in Selangor. With this close proximity of the community and their belief, it is of critical importance to increase public awareness and knowledge on what they consume as health supplements.

Selangor Chinese community consumes red rice much in the same manner of their distant counterpart in Mainland China, Taiwan, or Korea. Among the most important and widely known application of red rice is in the making of the Chinese red rice wine (*samsu*; Malay). Traditionally, a small portion of red rice is added to a jar of water, usually ceramic or clay, and left for a certain duration where it undergoes liquid fermentation. The resultant red liquid is either consumed directly as *samsu*, or sprinkled on fried meat or chicken to impart red colouration, eliminate meaty odour, and give distinctive aroma and taste. To a certain extent, some might include red rice in the diet of a jaundice child (Leong, Ri Xin Ginseng Trading-person. commun.).

2.6 Mycotoxins in Red Rice

The worldwide contamination of foods and feedstuffs with mycotoxigenic fungi and subsequent mycotoxins contamination is a significant problem of great agro-economic importance. In fact, the economic impact of mycotoxins includes toxic effects on humans and animals with increased health care, veterinary care costs and reduced livestock production (Hussein and Brassel, 2001). At present, researches are being carried out to combat this complication from many perspectives (e.g. gene manipulation, enhanced protection, microbial inhibition) to avoid further damage and deterioration of world food and feedstuff sources.

As food of fungal origin, the red rice is also susceptible to mycotoxins contamination. Nevertheless, so far there has been no report on works concerning mycotoxins contamination of red rice except for citrinin. Following the early works of Blanc *et al.*, (1995a; 1995b; 1998), and since the fermentation of red rice was carried out by fungi from the genus *Monascus*, currently it is widely known that the most commonly associated mycotoxin with red rice is citrinin. In Malaysia however, no such work has ever been done to assess the safety and health status of red rice consumed here, although there has been works on its anti-bacterial properties (MARDI), and pigment production (UPM) (Mohd Noor, UPM-person. commun.).

2.6.1 Citrinin in Red Rice

The genus *Monascus* has been found to produce a toxic substance known as citrinin (Blanc *et al*., 1995) just as many other fungi in the order Eurotiales (Samuels, 1983). Figure 2.3 depicts the chemical structure of citrinin.

Citrinin's IUPAC name is (3R, 4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid with chemical formula of $C_{13}H_{14}O_5$. Citrinin is a teratogen (substance capable of interfering with foetal development) (Ciegler *et al.*, 1977), and hepato-nephrotoxin (toxic to liver and kidney) (Sabater-Vilar *et al.*, 1999), and is known to lead to serious health problems (Bennett and Klich, 2003) among which being implicated as a potential causative agent in human endemica Balkan nephropathy (IARC, 1986).

Citrinin was originally investigated for its antibacterial properties. But, this toxin has been reported to be a nephrotoxin and has been found to potentially be an immunostimulator rather than an immunosuppressor. Therefore, significant quantities of citrinin, while part of the diet, could lead to chronic and hard-to-diagnose kidney disease in susceptible individuals (CAST, 1989). Table 2.5 shows the levels of citrinin found in red rice and red-rice-derived products.

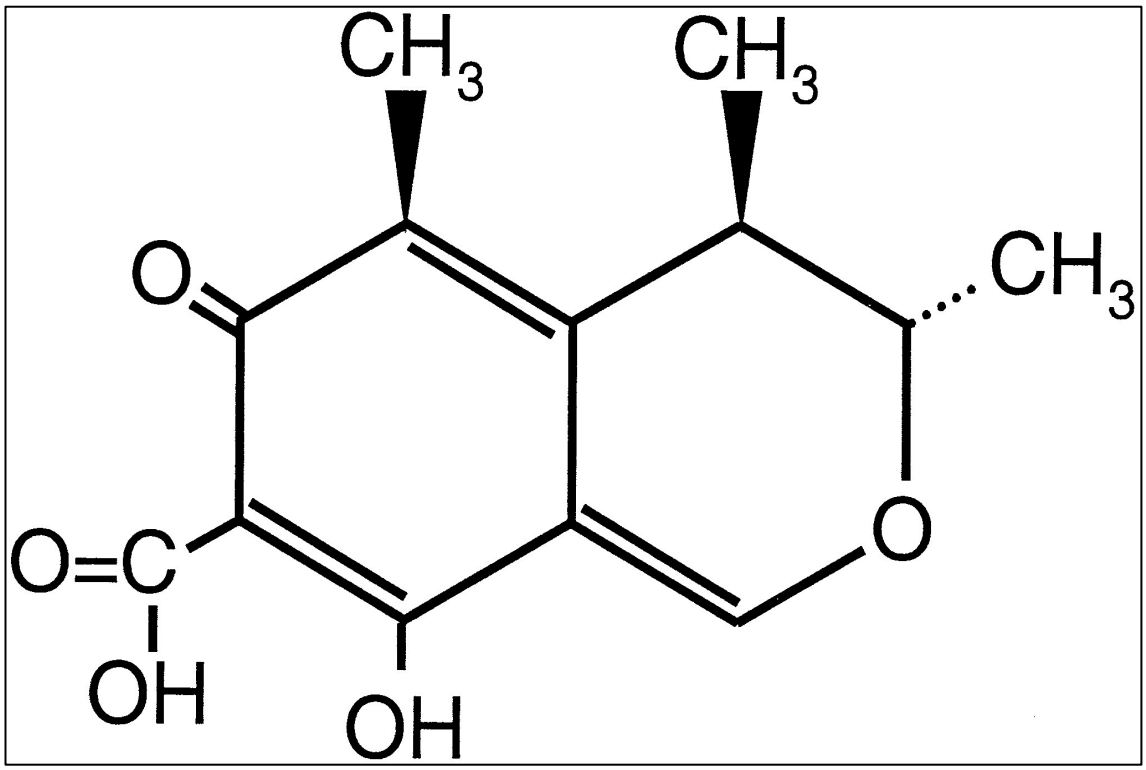

Figure 2.3 : Chemical structure of citrinin

Table 2.5 : Levels of citrinin in red rice and red rice products

Source	Citrinin Level	Reference
Red rice	14.3 mg / kg	Kumari *et al.*, 2009
Red rice	15.21 µg / g	Zheng *et al.*, 2009
Red rice	2,500 mg / kg	Eisenbrand, 2006
Liquid fermentation	56 mg / L	
Red rice	0.28 - 2,458.80 mg / kg	Liu *et al.*, 2005
Liquid fermentation	65 - 480 mg / L	Wang *et al.*, 2005
Red rice	4.2 - 25.1 mg / kg	Shu and Lin, 2002
Red rice	0.33 - 0.62 mg / kg	Ma *et al.*, 2000
Red rice	0.2 - 17.1 mg / kg	Sabater-Vilar *et al.*, 1999
Red rice	0.2 - 140 mg / kg	Xu *et al.*, 1999
Red rice	100 mg / kg	Blanc *et al.*, 1995b
Liquid fermentation	240 mg / L	

2.6.2 Toxicity Studies of Citrinin in Red Rice

In 1983, the toxicity of *Monascus* metabolites on foetal rat hepatocytes was demonstrated by Sako and co-workers (1983) who found that commercial preparations of *Monascus* pigments had an increasing effect on both A-glutamyl transpeptidase and glutathione-S-transferase (liver enzymes involved in detoxification processes). Earlier in 1979, an evident relationship between their induction and carcinogenesis has been reported (Boelsterli, 1979).

Similar hepatotoxic effects were observed on mice treated with citrinin (Bilgrami *et al*., 1988). *In vivo* studies have shown that citrinin adversely affects renal function and ultra-structure (Krejci *et al*., 1996). The citrinin action at cellular level is characterised by accumulation in the mitochondria and interference with the electron transport system (Ribeiro *et al*., 1997). This process, which depends on the pH and does

not affect the integrity of the cell membrane, leads to an inhibition of the synthesis of DNA and subsequently, of protein and RNA as well (Yoneyama and Sharma, 1987; Braunberg *et al*., 1992; Chagas *et al*., 1992a; Wasternack and Weisser, 1992). Furthermore, the mitochondrial dysfunction results in a decrease of liver glycogen and an inhibition of liver cholesterol and triglyceride synthesis (Chagas *et al*., 1992b).

In vivo exposure to citrinin caused renal clear-cell adenomes in Fischer 344 rats (Arai and Hibino, 1983). In addition, citrinin induced structural chromosomal aberrations after metabolic activation by rat and human microsome fractions (Thust and Kneist, 1979).

In 1996, Krejci and co-workers reported citrinin at 20 mg / kg showed acute nephrotoxicty in dogs which was characterised by significant changes in renal function which included proteinuria, glucosuria and reductions in inorganic phosphorus excretion rate.

Later in 2005, there has been a report of citrinin content at 0.28 - 2458.80 mg / kg in a commercial red rice, of which lipid extracts range from 1.8 - 4.7 mg / mL caused 50% cell death to human embryonic kidney cell (HEK293) (Liu *et al*., 2005). In 2006, Golli and co-workers described cytotoxicity of citrinin in Vero cells (Green Monkey kidney cells) showing IC_{50} at 32.5 µg / g. In the same year, citrinin was found to be nephrotoxic and teratogenic in chronic toxicity studies in rats at a dosage of 50 mg / kg body weight / day after 60 weeks in the test animals (Eisenbrand, 2006). In 2007, it was found that citrinin at 3.75 µg / g promoted mouse embryo apoptosis and developmental injury of mouse blastocysts *in vitro* and *in vivo* (Chan, 2007; Chan and Shiao, 2007).

Reports on the mutagenicity and genotoxicity of citrinin are rather alarming. In short-term mutagenicity tests, citrinin was negative in the Ames test in *Salmonella thyphimurium* up to 400 µg / plate (Kuzchuk *et al.*, 1978; Wuergler *et al.*, 1991), whereas it causes single- and double-strand breaks in the DNA of intact *Escherichia*

coli cells and induces DNA repair synthesis in permeabilised *E. coli* cells (Martin *et al.*, 1986). In addition, an increased frequency of base pair substitutions was found in a mutant reversion test in *E. coli* (Brakhage *et al.*, 1988). Finally, chromosomal abnormalities such as chromosome/chromatids breaks, gaps and acentric fragments were found in bone marrow cells of mice, treated orally with citrinin (Jeswal, 1996).

On the whole, these results indicate that it might no longer be considered safe to consume red pigments of *Monascus* as food additives without verifying the absence of citrinin. Although there are not enough evidence linking Monascal products to nephropathy or other adverse medical effects in the consumers, precaution should be taken. Effort should be undertaken to identify non-citrinin producing strains, possibly by disruption of the citrinin synthesis route. Furthermore, contamination of *Monascus* products with citrinin should be avoided as citrinin can exert health implications as outlined here.

3.0 MATERIALS AND METHODS

3.1 Sampling of Red Rice

Fifty (50) samples of red rice (100 g each in sterile sample bottles) were obtained from traditional Chinese medicine stores throughout nine (9) administrative districts in Selangor, Malaysia. Storage conditions (cold or room temperatures) of rice samples from respective stores were recorded. Cold condition in which samples were kept was within the range of 4 - 10°C (refrigeration temperature). Rice samples were examined promptly, or refrigerated at 4°C for not more than 36 hours before analysis.

3.2 Isolation of Mycotoxigenic Fungi

Isolation of mycotoxigenic fungi was performed on two solid media namely DRBC (Dichloran Rose Bengal Chloramphenicol) and CD (Czapek Dox), using both dilution and direct-plating techniques. DRBC (Merck, Germany) and CD (Oxoid, England) agar were obtained commercially and prepared according to Appendices B1, and B2. Both media were autoclaved (Tomy, Japan) at 121°C for 15 min at 15 psi. Autoclaved media were poured on plates and left overnight to solidify.

For dilution plating, 10 g of rice samples were aseptically weighed and blended with 90 mL sterile 0.1% peptone water (1 g peptone in 1 L distilled water) using a surface-disinfected waring blender (Torrington, USA) for five minutes. This gave a 1:10 (10^{-1}) dilution. 10^{-2}, 10^{-3}, and 10^{-4} dilutions were achieved by three serial 10-fold dilutions with sterile 0.1% peptone water. These four dilutions served as inocula for mycotoxigenic fungal isolation and were transferred separately with different sterile

pipettes onto DRBC and CD by spread-plate method using a sterile bent glass rod. Inoculation of rice samples were done in triplicates for each dilution on DRBC and CD.

For direct plating, 10 g of rice samples were directly sprinkled over the surface of DRBC and CD agar plates.

All inoculated plates were incubated at room temperature (25°C) for seven days (inverted after first day of incubation).

3.3 Enumeration of Mycotoxigenic Fungi

Enumeration of mycotoxigenic fungal isolates was performed only on DRBC agar. Dichloran contained in DRBC will restrict fungal hyphal growth and ease enumeration. Dilution plating technique as described earlier was employed on DRBC. DRBC cultures were incubated in the dark because photo-degradation of rose bengal yields toxic substances to fungi. Plates with 10 - 100 colony-forming units (CFU) were used for counting and the results were expressed as CFU per gram (CFU/g). CFU (of all culturable fungi) and isolation frequency (of each genus) were calculated according to Rosa *et al.*, (2008):

CFU : n x d.f. x 10 ----------> colony forming unit

n : number of colonies appeared on plate (best in the range of 10-100)

d.f : dilution factor of samples

10 : multiplication of 0.1 mL of aliquot plated to reach 1 mL

F : (ns / N) x 100 -----------> isolation frequency

ns : number of samples from which a certain genus occurred

N : total number of samples

3.4 Identification of Mycotoxigenic Fungi

Identification of fungal isolates was carried out by means of morphology (macro and micro). After seven days of incubation, all colonies grown on the agar surface was aseptically transferred onto fresh CD agar to obtain pure culture. All subcultures were incubated at room temperature (25°C) for another seven days (inverted after first day of incubation).

3.4.1 Macroscopic Observation

Macroscopic observation was carried out using an upright/educational microscope (Olympus, Japan) by directly observing the fungal culture in agar plates. During macroscopic observation, fungal isolates showing similar characteristics (colour, conidial structure) were grouped and labelled. One representative for each labelled groups were subjected to microscopic observation.

3.4.2 Microscopic Observation

Microscopic observation was carried out using a microscope attached to a computer and digital camera (Nikon, Japan) by means of wet-mound slide technique. Steps to prepare wet-mound slides are shown in Appendix C. During microscopic observation, smaller characteristics (shape, texture, and ornamentation of conidiospores, seriation) with measurements were taken into consideration. Data obtained were then analysed, compared and referred to published manual of identification. In this study, special attention was attributed to certain mycotoxigenic food fungi of the genera *Aspergillus* and *Penicillium*. Thus, the manuals used were Raper and Fennel, (1977) for

Aspergillus, Ramirez (1982) for *Penicillium*, and Hawksworth and Pitt, (1983) for red rice starter fungi (genus *Monascus*).

3.5 Analysis of Mycotoxins

In this study, mycotoxins were evaluated in two continuous steps; enzyme-linked immunosorbence assay (ELISA) test, and immunoaffinity column (IAC) clean-up. RIDASCREEN® ELISA kits and RIDA® IAC columns were obtained from R-Biopharm AG (Darmstadt, Germany). Methods pertaining to both procedures were followed as manufacturer's instructions. R-Biopharm AG (Darmstadt, Germany) holds the patented methodology for mycotoxins quantification and food samples clean-up through their in-house developed procedures (Michelle, R-Biopharm (M)-person. commun.).

Before the samples were subjected to mycotoxins evaluation by RIDASCREEN® ELISA test, the samples underwent clean-up process by reason of intense red colouration that might interfere during the ELISA procedure. For clean-up, red rice samples were ground to fine powder form and sieved to obtain standard size. Samples that have undergone clean-up process were stored in a cool place and protected against light until ready for ELISA test. Clean-up procedure was done by means of RIDA® immunoaffinity column (IAC). The basis for IAC is antigen-antibody reaction. The RIDA® column contains a gel suspension to which monoclonal antibodies are attached covalently. The antibodies are specific for only one type of antigen (either the aflatoxin or ochratoxin-A). Toxin present will be bound by the monoclonal antibodies and all other substances will not be retained by the column thus eliminating the possible interference of high pigmentation of the samples. A hundred per cent methanol causes a

denaturation of the antibodies. Therefore, the antigen (aflatoxin or ochratoxin-A) will be set free during the elution process.

3.5.1 Extraction and Clean-up for Citrinin (RIDA® general column)

For citrinin, there was no immunoaffinity column developed at present for samples clean-up. RIDA® general column (alumina powder) usually used to clean high-pigmented food samples was applied instead. Since it was a general column, recovery rate for citrinin was tested. For clean-up, 5 g of ground sample was weighed and added to a suitable container with 12.5 mL 70% methanol (v/v). The mixture was homogenised vigorously for three minutes with shaker at 150 rpm. The homogenised mixture was then filtered through Whatman No. 1 filter paper. One millilitre filtrate was added to 9 mL distilled or deionised water. The entire diluted solution was passed through the clean-up column. The solution was pushed at 1 - 2 drops per second. Clean-up filtrate was used in the ELISA test (50 µL per well). The recovery was tested by passing a known amount of citrinin solution down the column and was subjected to the same ELISA procedure to evaluate the recovered amount.

3.5.2 Extraction and Clean-up for Aflatoxin (RIDA® immunoaffinity column)

Five grams of ground sample was weighed and added to a suitable container with 25 mL 70% methanol (v/v). The mixture was homogenised vigorously for 10 minutes with shaker at 150 rpm. The homogenised mixture was then filtered through Whatman No. 1 filter paper. Five millilitres of the filtrate was added to 15 mL of distilled or deionised water. The entire 20 mL diluted sample solution was passed through the column. Before starting the procedure, RIDA® columns were brought to

room temperature. Caps plugged on top and tip of the columns were removed prior to use. The storage buffer above the gel was rinsed off within the first washing step. Second washing step involved passing 2 mL distilled or deionised water through the column. The column was then filled up with prepared sample solution slowly and continuously at a flow rate of approximately one drop per second. The eluted solution was discarded. Third washing step was done by rinsing the column with 10 mL distilled or deionised water. Air was pressed through the column for approximately 10 seconds to ensure complete removal of residual fluids. A clean and closable vial was placed directly below the column. Elution of trapped aflatoxin to antibodies inside the column was done with 0.5 mL 100% methanol passing slowly through the column at a flow rate of approximately one drop per second to ensure complete elution of the aflatoxin. Eluate residue was collected by pressing air thoroughly through the column for 30 seconds. Eluate containing aflatoxin was diluted at 1:10 with sample dilution buffer (provided by manufacturer). Samples were then measured for aflatoxin by RIDASCREEN® Aflatoxin ELISA test.

3.5.3 Extraction and Clean-up for Ochratoxin (RIDA® immunoaffinity column)

Ten grams of ground sample was weighed and added to a suitable container with 20 mL 60% acetonitrile (v/v). The mixture was homogenised vigorously for 20 minutes with shaker at 150 rpm. The homogenised mixture was then filtered through Whatman No. 1 filter paper. The entire filtrate was passed through the RIDA® column. Before starting the procedure, columns were brought to room temperature. Caps plugged on top and tip of the columns were removed prior to use. The storage buffer above the gel was rinsed off within the first washing step. Second washing step involved passing 2 mL 90% PBS / Methanol (v/v) through the column (Appendix D1). The column was then

filled up with prepared sample solution slowly and continuously at a flow rate of approximately one drop per second. The eluted solution was discarded. Third washing step was done by rinsing the column with 10 mL distilled or deionised water. Air was pressed through the column for approximately 10 seconds to ensure complete removal of residual fluids. A clean and closable vial was placed directly below the column. Elution of trapped ochratoxin-A to antibodies inside the column was done with 1 mL 100% methanol passing slowly through the column at a flow rate of approximately one drop per second to ensure complete elution of the aflatoxin. Eluate residue was collected by pressing air thoroughly through the column for 30 seconds. Eluate containing ochratoxin-A was added to 1 mL 0.13 M sodium hydrogen carbonate buffer (Appendix D2) and was mixed thoroughly. Samples were now ready for ochratoxin-A evaluation by RIDASCREEN® Ochratoxin-A ELISA test.

3.6 Quantification of Mycotoxins

Three mycotoxins were quantitated based on the predominant occurrence of their producer fungi in red rice samples; citrinin (*Monascus* spp., *Penicillium chrysogenum*), aflatoxin (*Aspergillus flavus*), and ochratoxin-A (*Aspergillus niger*). Evaluation of mycotoxins was carried out by using RIDASCREEN® ELISA test. The basis of the test is also the antigen-antibody reaction. The wells in the microtiter strips are coated with capture antibodies directed against the respective antigens (citrinin, aflatoxin, ochratoxin-A). Addition of RIDASCREEN® enzyme conjugate, RIDASCREEN® enzyme substrate (urea peroxide) and RIDASCREEN® chromogen (tetramethylbenzidine) completed the ELISA procedure. The addition of the RIDASCREEN® stop solution changed the solution from blue to yellow that can be

measured photometrically at 450 nm. All toxins samples were covered from direct light during incubation.

3.6.1 Quantification of Citrinin

RIDASCREEN® Citrinin ELISA kit was used to quantify citrinin. Sufficient numbers of wells were inserted into the microwell holder for all standards and samples to be run. Not more than three strips (24 wells) were run if single pipette was used thus time lapse between well to well and overall pipetting duration did not interfere with the stipulated incubation time. RIDASCREEN® Standard and sample positions were recorded. Fifty microliters standards and extracted samples were pipetted into separate wells (duplicate wells for each); using new pipette tips for each standard or sample. Fifty microliters RIDASCREEN® anti-citrinin antibody was then added to each well. The plate was shaken gently and manually and incubated for 10 minutes at room temperature. After incubation, the microwells were subjected to washing procedure. The washing procedure started by draining the liquid out of the wells into a sink. The microwell holder was tapped upside down onto a clean filter towel (three times in a row) to remove all remaining liquid from the wells. By using a wash bottle, the wells were filled with distilled or deionised water (250 µL per well). The washing procedure stopped by emptying the microwells and drying process. Washing procedures were repeated for two more times to ensure complete removal of liquid. One hundred microliters RIDASCREEN® enzyme conjugate solution was added to the well, mixed, and incubated again for 10 minutes at room temperature. For the second time, washing procedures were repeated. One hundred microliters RIDASCREEN® substrate/chromogen was added to each well, mixed, and incubated for another 5 minutes at room temperature. Finally, 100 µL RIDASCREEN® stop solution was added

to each well and mixed. Absorbance was measured at 450 nm with an ELISA reader (Tecan, Austria) within 10 minutes after the addition of stop solution.

3.6.2 Quantification of Aflatoxin

RIDASCREEN® Aflatoxin ELISA kit was used to quantify aflatoxin. Microtiter wells were prepared and positions of standards and samples were recorded. Fifty microliters RIDASCREEN® standards and samples were added to separate duplicate wells. Fifty microliters RIDASCREEN® enzyme conjugate and 50 µL RIDASCREEN® antibody solution were then added to each well and mixed gently by shaking the plate manually and incubated for 30 minutes at room temperature. Washing procedure was done thrice. After that, 50 µL RIDASCREEN® substrate and 50 µL RIDASCREEN® chromogen were added to each well, mixed, and incubated for another 30 minutes at room temperature. One hundred microliters RIDASCREEN® stop solution was added to each well, and mixed. Absorbance at 450 nm was read within 30 minutes after the addition of stop solution.

3.6.3 Quantification of Ochratoxin

RIDASCREEN® Ochratoxin ELISA kit was used to quantify ochratoxin. Microtiter wells were prepared and positions of standards and samples were recorded. Fifty microliters RIDASCREEN® standards and samples were added to separate duplicate wells. Fifty microliters RIDASCREEN® enzyme conjugate and 50 µL RIDASCREEN® antibody solution were then added to each well and mixed gently by shaking the plate manually and incubated for 30 minutes at room temperature. Washing procedure was done three times by using the washing buffer (Appendix D3). After that,

100 µL RIDASCREEN® substrate/chromogen were added to each well, mixed, and incubated for another 15 minutes at room temperature. One hundred microliters RIDASCREEN® stop solution was added to each well, and mixed. Absorbance at 450 nm was read within 30 minutes after the addition of stop solution.

3.7 Data and Statistical Analyses

Independent sample t-test, correlation and regression analyses of parameters (mycotoxigenic fungal load, mycotoxins, individual producer fungi, storage conditions) were done statistically by using Statistical Package for Social Sciences (SPSS Version 16.0; New York, USA). Data, means of data, and standard deviation of data were provided in respective tables under Appendices G1-5. A good standard deviation of data should be below 0.05 ($p \leq 0.05$).

For RIDASCREEN® ELISA assays, a software (RIDA®SOFT Win) was used to automatically calculate the absorbance readings without constructing standard curves. Particular care was taken on dilution factors in the preparation for each toxin before computing the data into the software. The results for mycotoxins content were standardised to ppb (part per billion; equals to µg/kg) since regulation concerning mycotoxins are usually reported in that unit.

4.0 RESULTS

4.1 Sampling Sites and Number of Samples

The state of Selangor is divided into nine administrative districts, namely: Gombak, Hulu Langat, Hulu Selangor, Klang, Kuala Langat, Kuala Selangor, Petaling, Sabak Bernam, and Sepang. In Figure 4.1, name and number in boxes denote the districts with the number of samples obtained.

4.2 Fungal Count by Storage Conditions

Distribution of fungal count (CFU/g) by storage condition (cold and room temperatures) is shown in Figure 4.2. Exact figures for both conditions are tabulated in Appendix E. In general, fungal counts for all samples were above the permissible limit set by Malaysian and international standards, with the highest count at 2.1×10^6 CFU/g, lowest count at 1.4×10^4 CFU/g, and mean at 2.2×10^5 CFU/g. However, there was no significant difference ($p = 0.069$; > 0.05) between fungal counts on red rice samples stored under cold and room temperatures (Independent Samples t-Test; Appendix G1).

For isolation of fungi, there was no significant difference between DRBC and CD agar. Although both agar contain slightly different growth nutrients, both agar contain the same amount of antibiotic chloramphenicol (premixed in DRBC, supplemented in CD). Fungi isolated from one sample exhibited almost similar occurrence on both agar.

For enumeration of fungi, fungal isolates obtained from DRBC agar were counted. Dichloran consisted in the DRBC agar restricted hyphal growth and eased enumeration of fungal colonies.

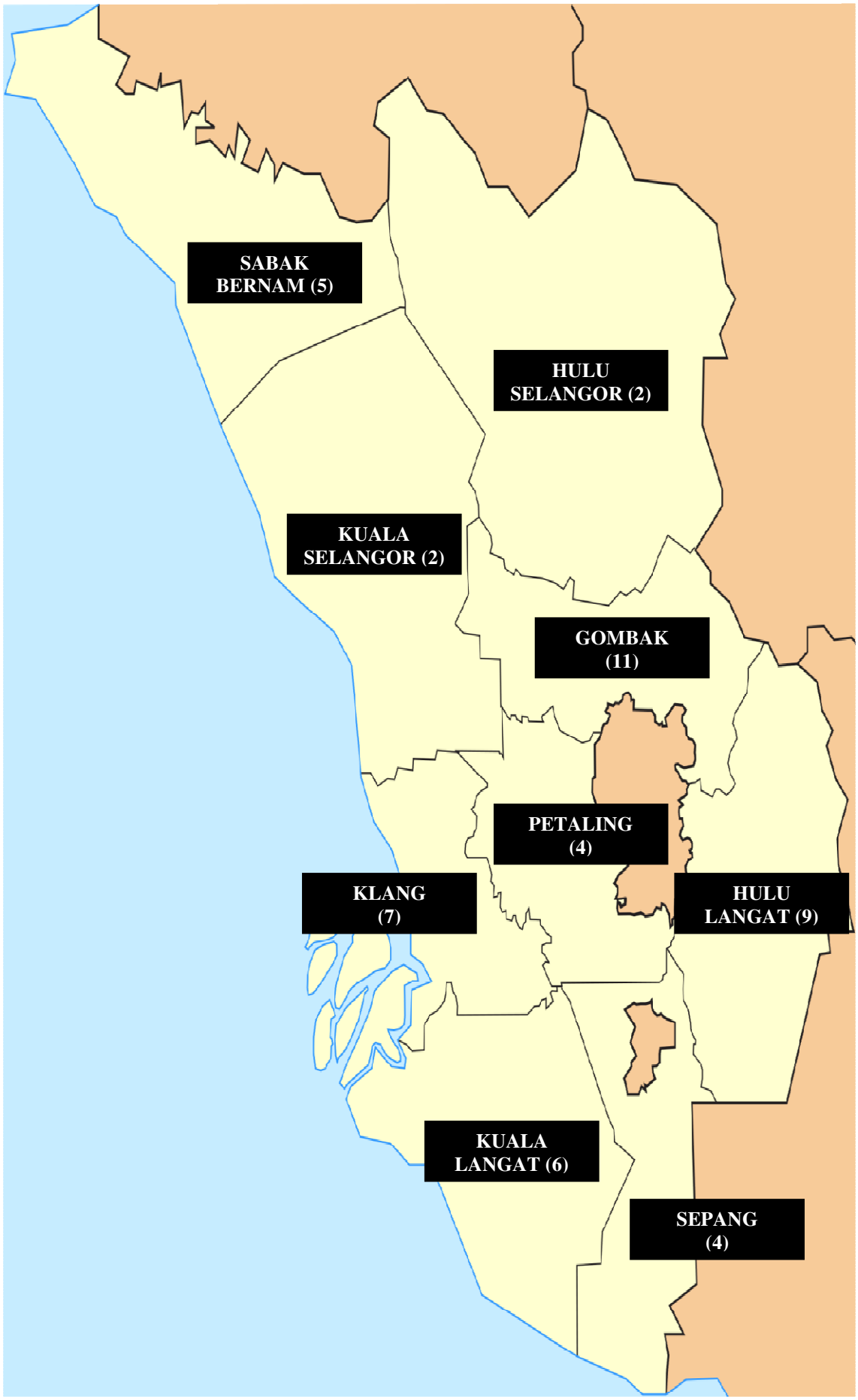

Figure 4.1 : Map of Selangor by districts and number of samples

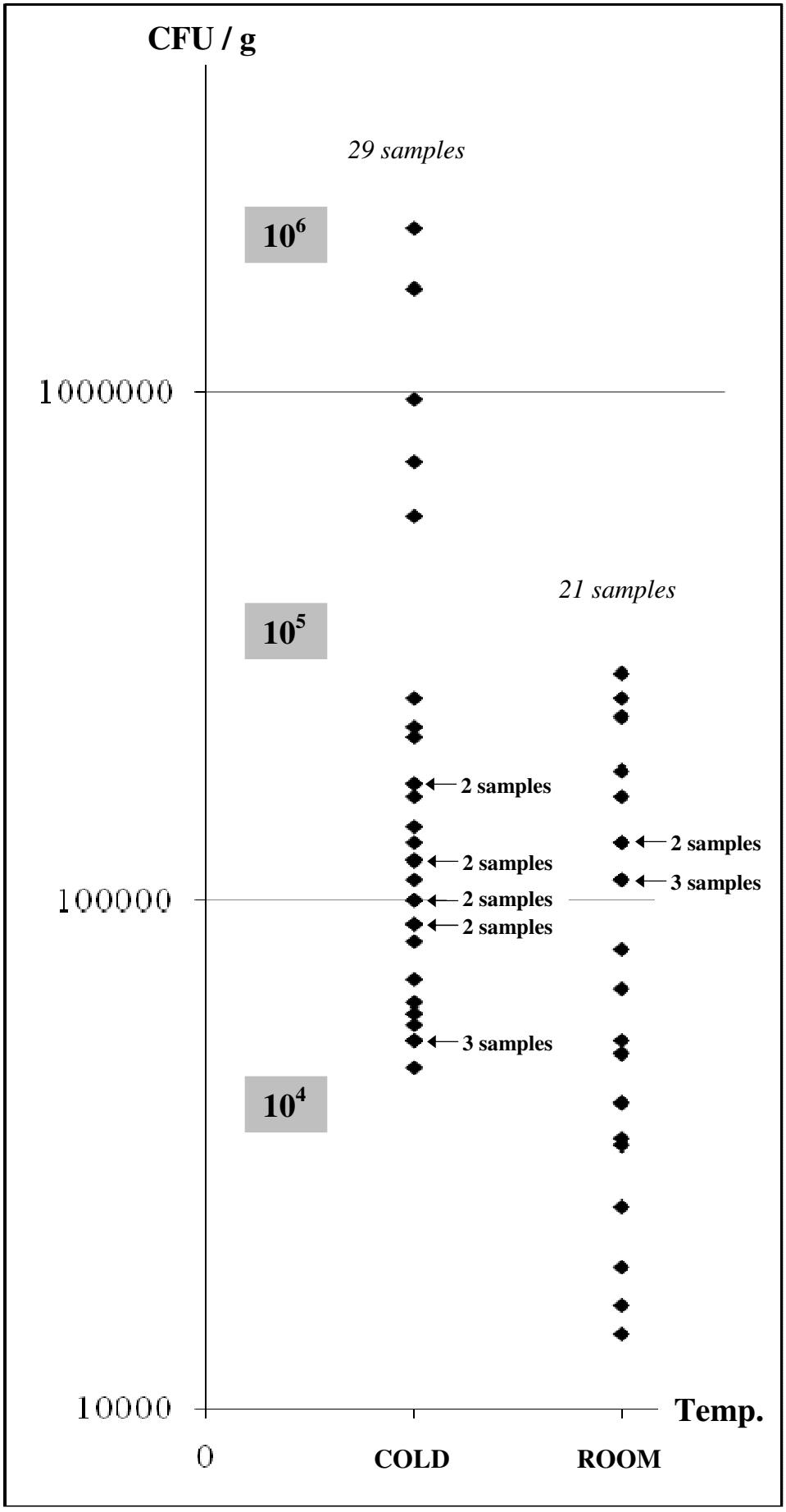

Figure 4.2 : Fungal count by storage conditions

4.3 Morphological Identification of Dominant Fungi

Although the *Monascus* spp. were not included in any of the mycotoxigenic fungi on foods and feedstuffs, it is imperative however to also identify the starter fungi for red rice production. Reported below are identification images (macro- and micromorphology) for *Monascus purpureus*, *Monascus pilosus*, *Aspergillus flavus*, *Aspergillus niger*, and *Penicillium chrysogenum* isolated from red rice samples.

4.3.1 Morphology of *Monascus purpureus*

Macroscopically on Czapek Dox agar, *M. purpureus* colonies were small in diameter (8-22 mm), plane (*flat*), sparse (*thinly scattered*), with little aerial growth, white or reddish mycelium initially, and became strongly pigmented as colonies mature, in orange or red shades, usually reddish orange, and soluble pigments (diffused into agar) in shades of orange or pink usually produced (Figure 4.3a).

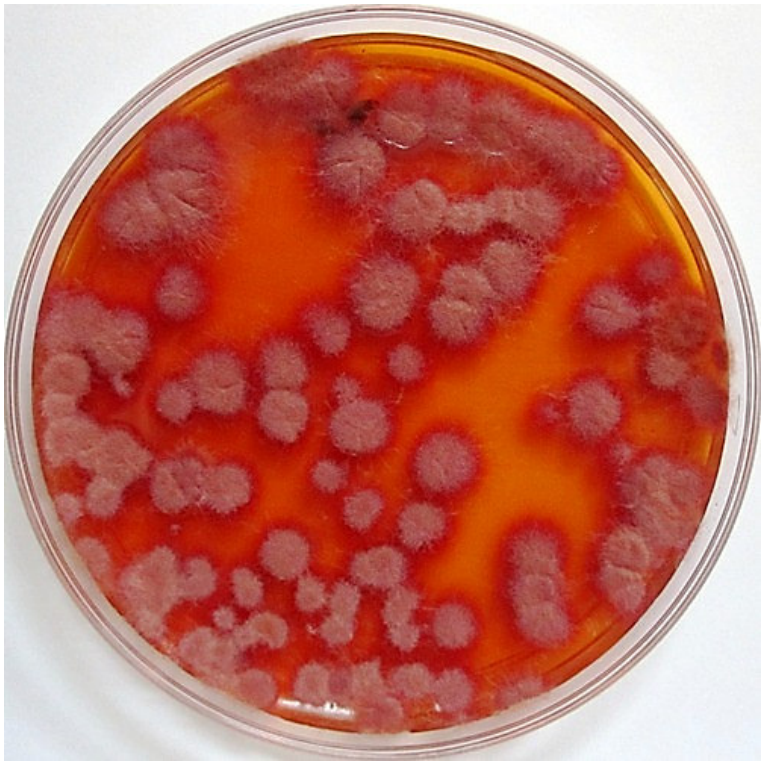

Figure 4.3a : Cultural characteristic (macroscopy) of *Monascus purpureus*

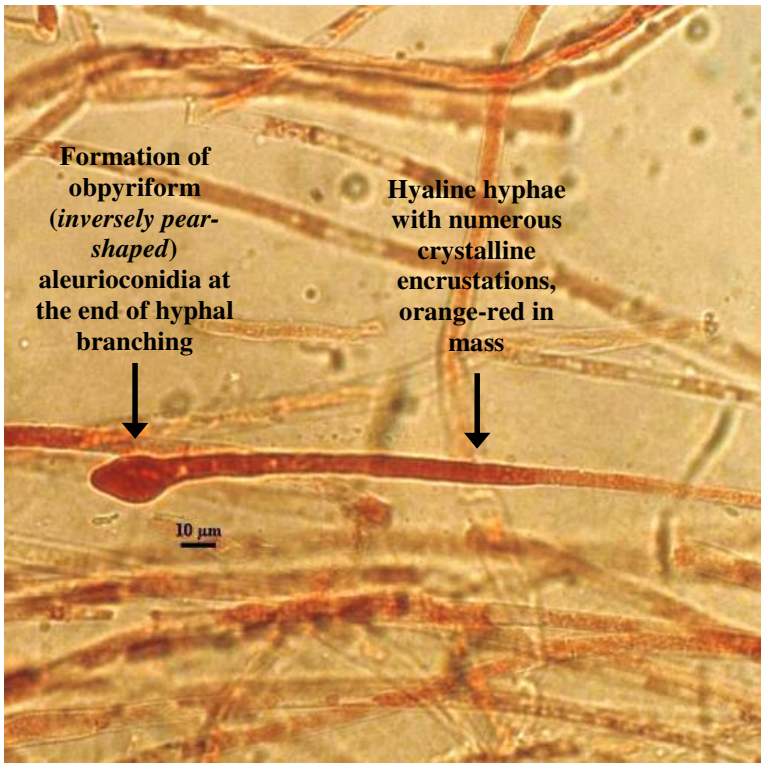

Figure 4.3b : Hyphae and formation of aleurioconidia in *Monascus purpureus*

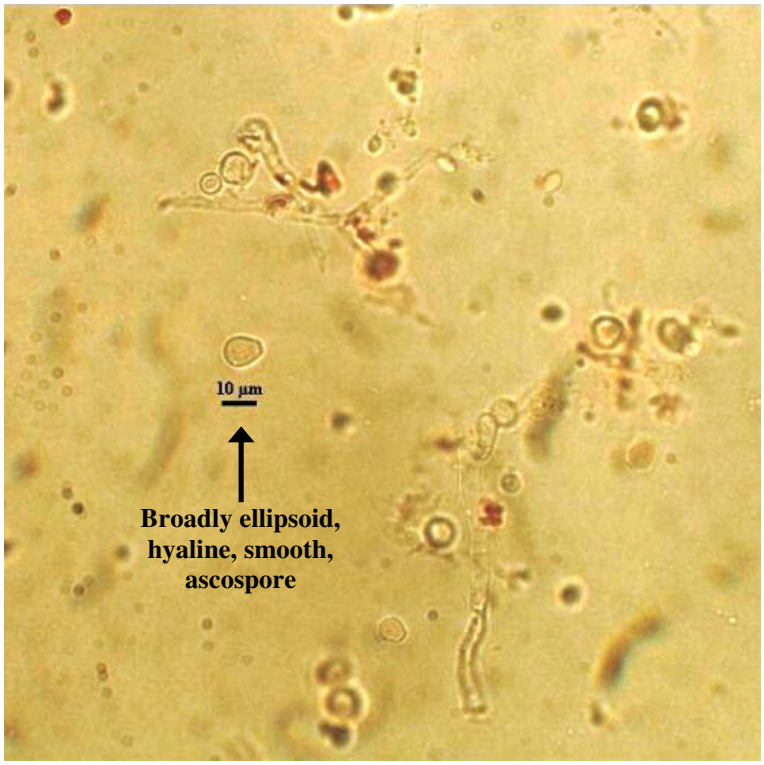

Figure 4.3c : Ascospore of *Monascus purpureus*

4.3.2 Morphology of *Monascus pilosus*

As opposed to cultural characteristics of *M. purpureus* depicted in Figure 4.3a, on Czapek Dox agar, colonies of *M. pilosus* were relatively larger in diameter (10-40 mm), fimbriate (*with edges*), floccose (*cottony*), with pastel red margins, and deep red centres (Figure 4.4a).

Figure 4.4a : Cultural characteristic (macroscopy) of *Monascus pilosus*

Since *M. purpureus* and *M. pilosus* are of the same genus, they have several common characteristics. However, they were readily distinguished by growth rates and thus the size of colonies on day seven of incubation (*M. purpureus* slower and smaller), and the size of ascospores (*M. purpureus* broader). Microscopic characteristics comparison of *M. purpureus* and *M. pilosus* are tabulated in Table 4.1. Characterisation key in identifying species from the genus *Monascus* are tabulated in Appendix F1.

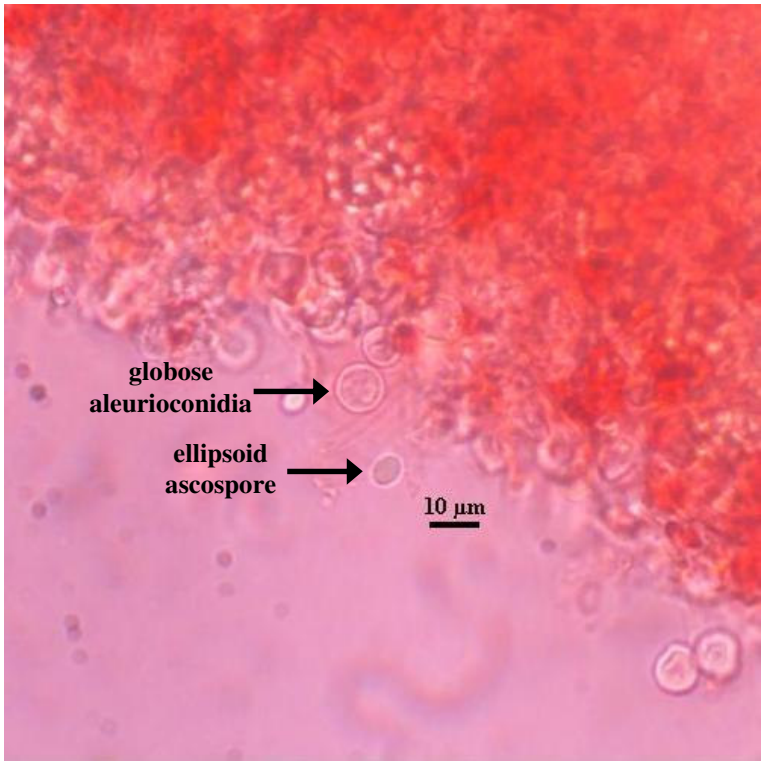

Figure 4.4b : Aleurioconidium and ascospore of *Monascus pilosus*

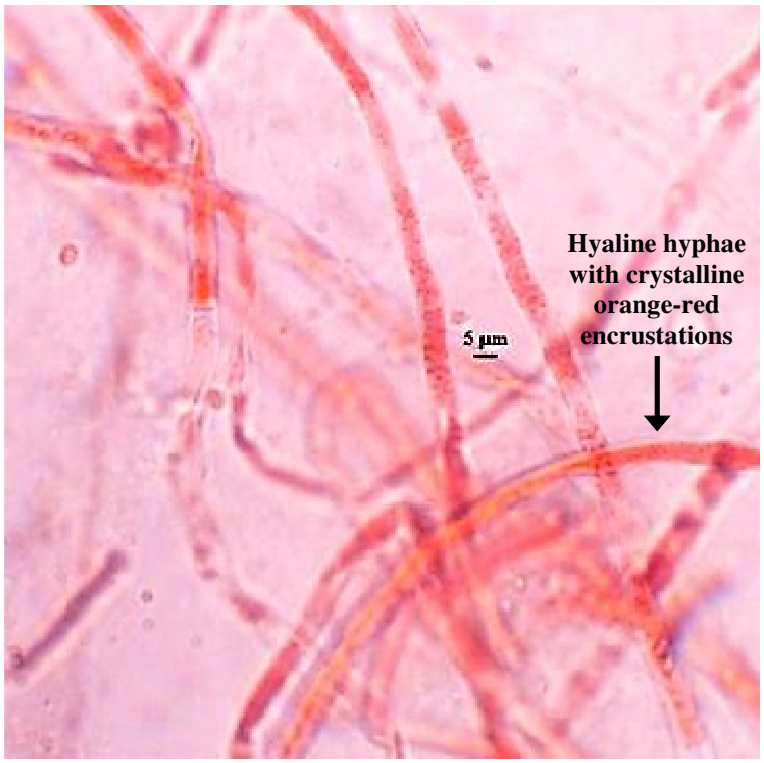

Figure 4.4c : Hyphae of *Monascus pilosus*

Table 4.1 : Comparison of microscopic characteristics between *Monascus purpureus* and *Monascus pilosus*

Characteristics	*Monascus purpureus*	*Monascus pilosus*
Mycelia	Sparse to abundant	Sparse
Hypha	Irregularly branched, hyaline, guttulate (*having one or more oil-like drops*), constant in thickness (3-5µm), walls with numerous crystalline encrustations, orange-red in mass. **Figure 4.3b**	Irregularly branched, hyaline, guttulate, uneven in thickness, smooth, walls with occasionally crystalline encrustations, orange-red in mass. **Figure 4.4c**
Aleurioconidia (*conidia developed from blown-out of hyphal branch*)	Singly or in short chains, obpyriform (*inversely pear-shaped*) to globose, hyaline, truncate (*horizontal*) at base, guttulate, with thick, smooth walls. 8-11 by 8-10 µm. **Figure 4.3b**	Singly or in short chains, obpyriform to globose, hyaline, truncate at base, guttulate, with thick, smooth walls. 5-14 by 5-9 µm. **Figure 4.4b**
Cleistothecia (*closed fruitbody without opening, with asci not regularly arranged*)	Globose, arising singly from hyphae, 25-70 µm (normal; 45-60 µm), peridium (*wall*) hyaline with 2-5 µm thickness	Globose, arising singly from hyphae, 25-55 µm, peridium hyaline with 1.5-4 µm thickness
Asci	Filled with compact mass of ascospores	Filled with compact mass of ascospores
Ascospores	Broadly ellipsoid, hyaline, smooth, 6-7 by 4.5-5 µm **Figure 4.3c**	Ellipsoid, hyaline, smooth, 5-7 by 3-3.5 µm. **Figure 4.4b**

4.3.3 Morphology of *Aspergillus flavus*

On Czapek Dox agar, *A. flavus* colonies were in yellow-green shades with normal growth rate attaining 5 cm diameter in 10 days (6 - 7 cm fast; 3 - 4 cm slow) at room temperature (24 - 26°C) with thin but close-textured mycelium. The colonies were plane, producing abundant conidial structures. Young conidial heads were in yellow shades and becoming greener in age and did not shift colour to brown during the maturation process. Reverse was uncoloured and, exudate and odour were absent. Binocular / dissecting microscope (Olympus, Japan) was used to observe the conidial heads of this isolate (image not provided). It was observed that this isolate produced radiate (round; spreading from a centre) conidial heads. Characterisation key in identifying members from the group *A. flavus* are tabulated in Appendix F2.

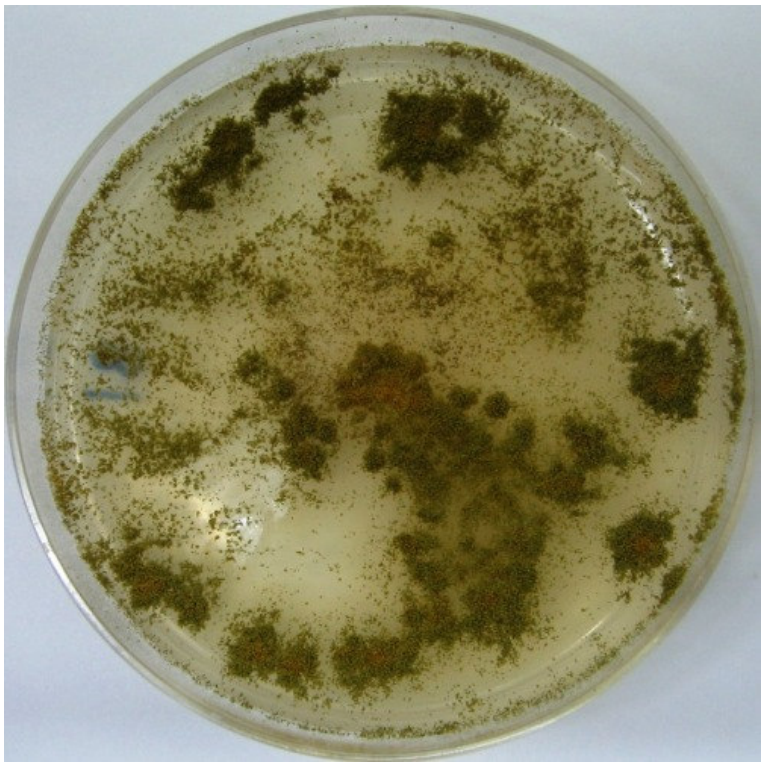

Figure 4.5a : Cultural characteristic (macroscopy) of *Aspergillus flavus*

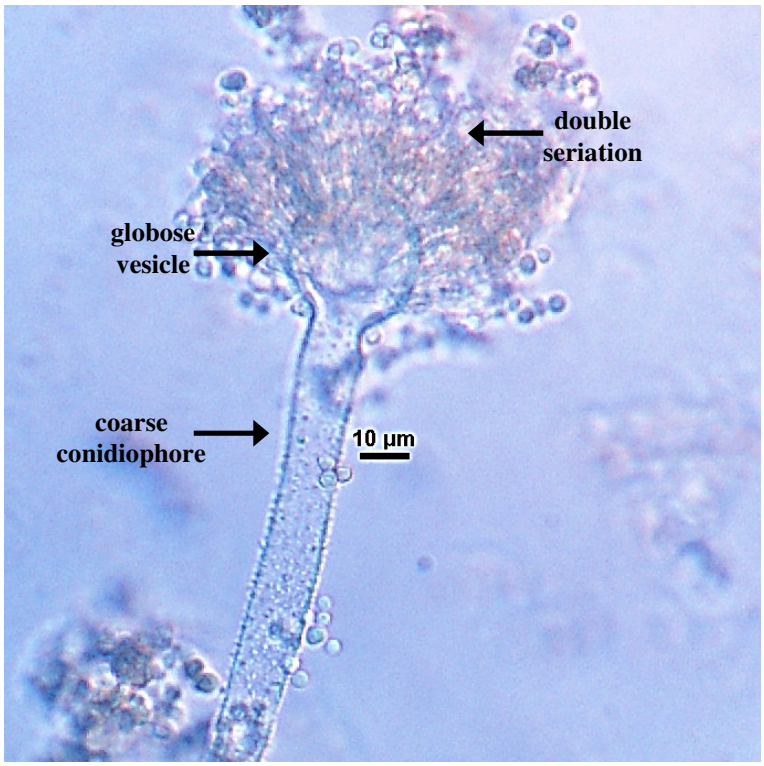

Figure 4.5b : Microscopic structures of *Aspergillus flavus*

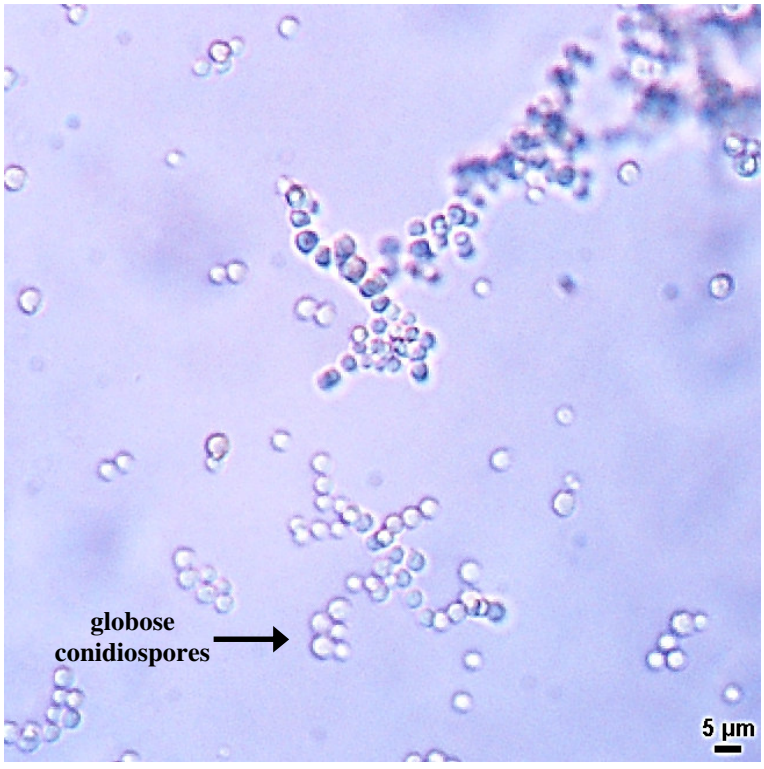

Figure 4.5c : Conidiospores of *Aspergillus flavus*

Depicted in Figure 4.5b are microscopic structures of matured *A. flavus*. It has globose vesicle (normally seen in aged cultures) with the diameter of 25 - 45 µm, and double seriation sterigmata (sterigmata are either single or double but never co-existed). The conidiophores are heavy-walled, uncoloured and coarsely roughened, with stalk (a part of the whole length of a conidiophore located immediately below the vesicle) measured at 11.3 µm which fits within the range as described by Raper and Fennel, (1977) at 10 - 20 µm. In Figure 4.5c, conidia appeared globose, with the diameter of 3.5 - 4.5 µm.

4.3.4 Morphology of *Aspergillus niger*

On Czapek Dox agar, *A. niger* colonies consisted of compact to fairly loose basal mycelium, in white to faintly yellow shades, bearing abundant and crowded conidial structures which are typically carbonaceous black to naked eyes. Like the basal mycelium colour, reverse is usually white, colourless, and pale yellow (colony centre).

Microscopically, as depicted in Figure 4.6b, conidial heads are usually large, black (or deep brownish) and globose, with lengthy conidiophores (1.5 - 3.0 mm by 15 - 20 µm). Conidiophores are colourless, with smooth, thick walls (2.0 - 2.5 µm).

Figure 4.6c depicts one conidial head of *A. niger*. Double seriation was observed at very close arrangement with brownish shades. Primary sterigmata are usually (20 - 30 µm by 5.0 - 6.0 µm). Secondary sterigmata are usually shorter (7 - 10 µm by 3.0 - 3.5 µm).

Figure 4.6d depicts several conidiospores of *A. niger*. They are typically globose at maturity mostly in 4.0 - 5.0 m diameter, heavy-walled, irregularly roughened, and echinulated. Characterisation key in identifying members from the group *A. niger* are tabulated in Appendix F3.

Figure 4.6a : Cultural characteristic (macroscopy) of *Aspergillus niger*

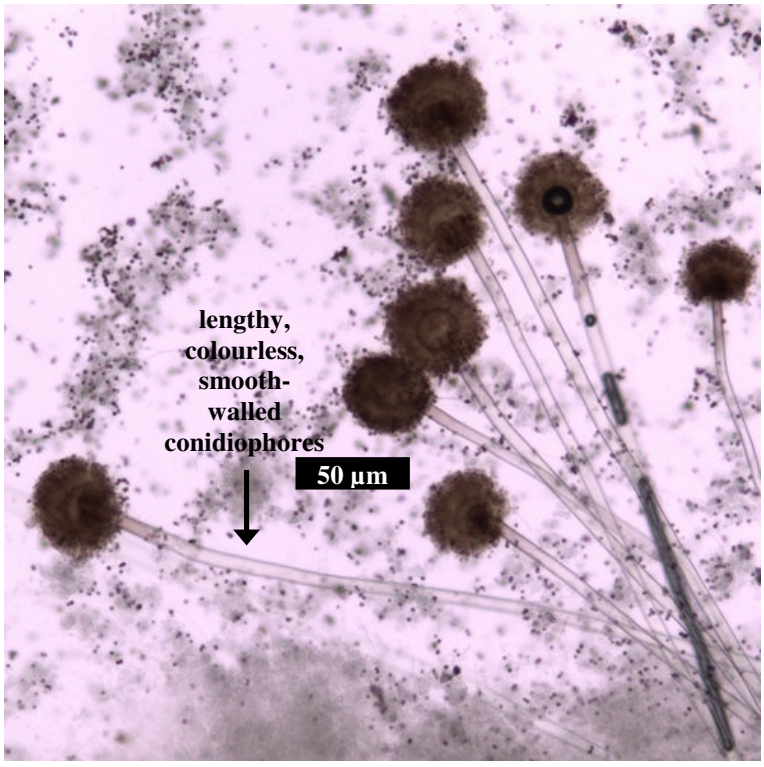

Figure 4.6b : Microscopic structures of *Aspergillus niger*

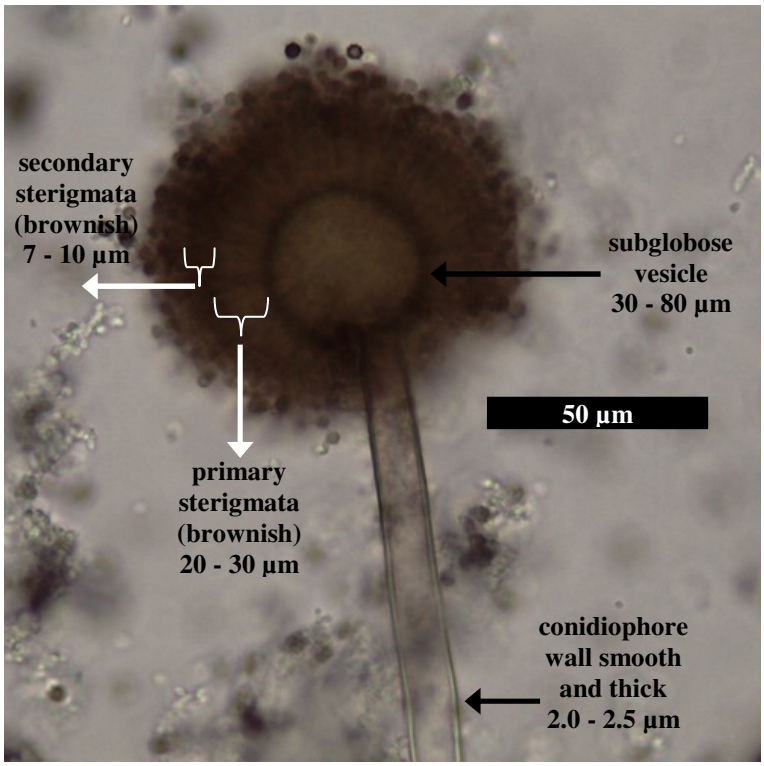

Figure 4.6c : Conidial head of *Aspergillus niger*

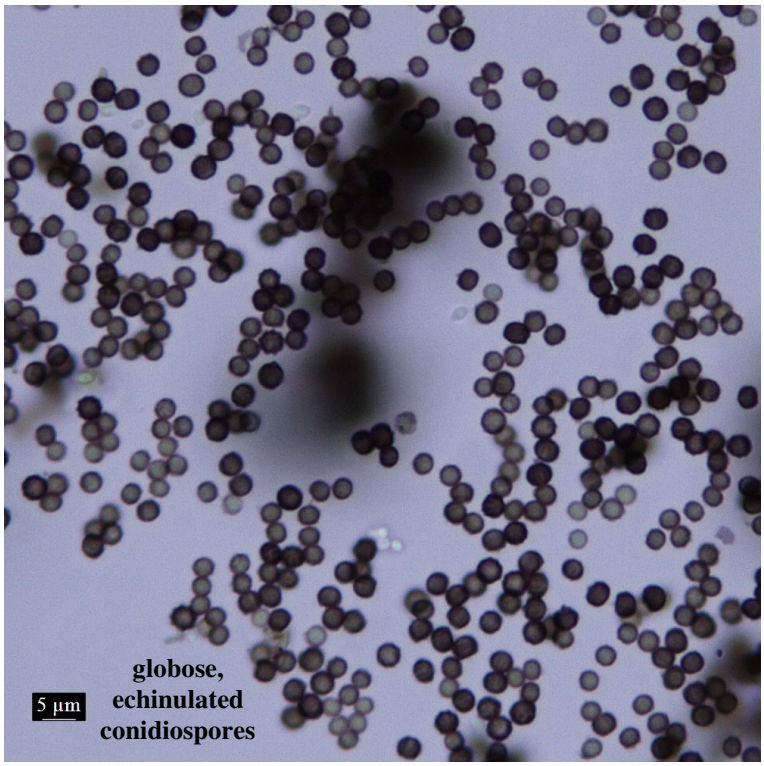

Figure 4.6d : Conidiospores of *Aspergillus niger*

4.3.5 Morphology of *Penicillium chrysogenum*

On Czapek Dox agar, *Penicillium chrysogenum* colonies were typically velvety, close textured and thin. Vegetative hyphae are usually creamish, or yellowish, and becoming deep green or bluish green with the ripening of conidia (Figure 4.7a).

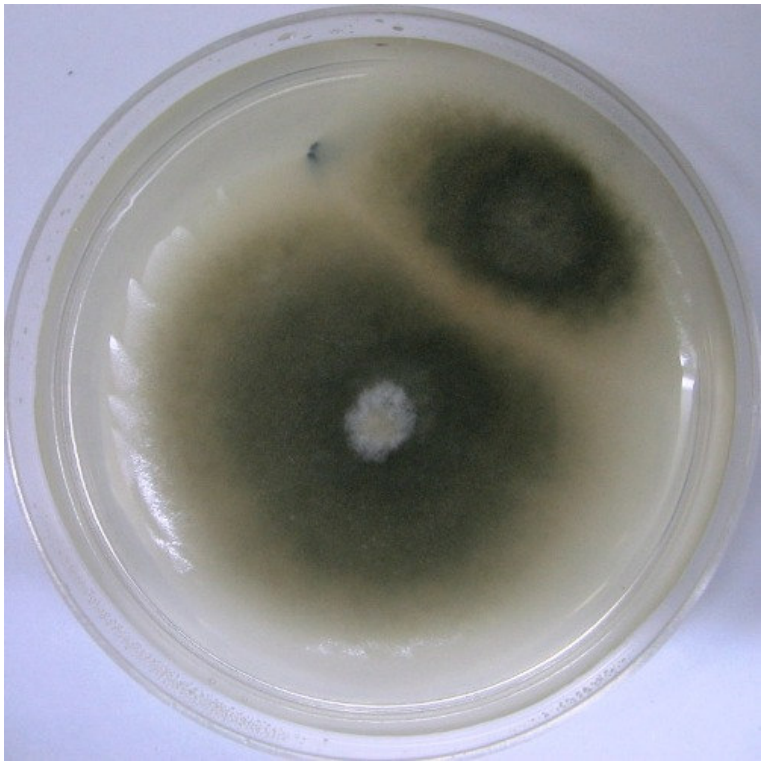

Figure 4.7a : Cultural characteristic (macroscopy) of *Penicillium chrysogenum*

Characterisation key in identifying members from the group *Penicillium chrysogenum* are tabulated in Appendix F4. As shown in Figure 4.7b, the conidiophore is usually smooth-walled and variable in length (150 - 400 µm), commonly branched, with branches (15 - 25 µm) usually bearing 2 - 4 metulae (10 - 13 µm) which support phialides which in turn support the chains of conidiospores or phialidospores. Figure 4.7c depicts conidiospores or phialidospores of *P. chrysogenum*. They are typically globose or subglobose, mostly 3.0 - 4.0 µm by 2.8 - 3.5 µm, and smooth-walled.

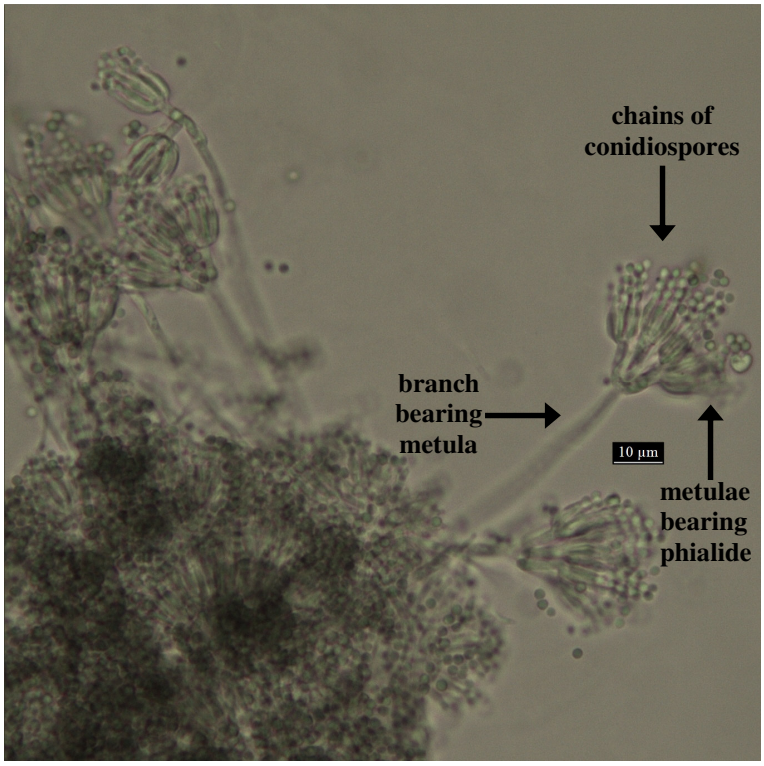

Figure 4.7b : Microscopic structures of *Penicillium chrysogenum*

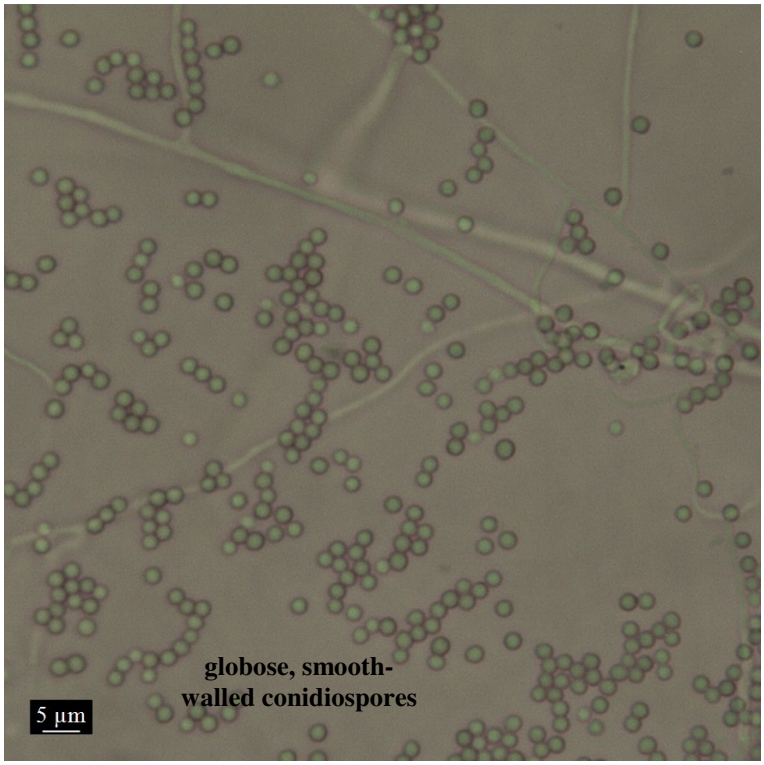

Figure 4.7c : Conidiospores of *Penicillium chrysogenum*

4.4 Isolation Frequency of Mycotoxigenic Fungi

Isolation frequency of mycotoxigenic fungi from 50 red rice samples are shown in Table 4.2. The starter fungi (*Monascus* spp.) were present in all samples (100%), followed by *Penicillium chrysogenum* (62%), *Aspergillus niger* (54%), and *A. flavus* (44%). Other fungi were also isolated but at very low frequency such as *Penicillium* sp. B (4%), *Penicillium* sp. P (4%), *Penicillium* sp. W (6%), *Penicillium* sp. Y (6%), *Aspergillus clavatus* (4%), and *A. versicolor* (2%). Non-mycotoxigenic fungi such as zygomycetous fungi (34%), *Cladosporium* sp. (12%), and *Chrysosporium* sp. (2%) were also isolated. Of the 50 samples, eight samples did not give any isolates except for the starter fungi. For the starter fungi (*Monascus* spp.), 29 samples were identified as *Monascus purpureus*, and 21 samples as *M. pilosus*.

Table 4.2 : Isolation frequency of mycotoxigenic fungi from red rice

Sample	Fungal count (CFU / g)	*Monascus purpureus*	*Monascus pilosus*	*Aspergillus flavus*	*Aspergillus niger*	*Penicillium chrysogenum*
01	**1.6×10^6**	√	**-**	√	√	√
02	**5.7×10^5**	√	**-**	**-**	√	**-**
03	**1.1×10^5**	-	√	√	√	√
04	**2.5×10^4**	-	√	**-**	√	√
05	**8.0×10^4**	√	**-**	√	**-**	√
06	**1.6×10^5**	√	**-**	**-**	√	√
07	**2.1×10^6**	-	√	√	√	√
08	**1.6×10^5**	√	**-**	√	√	√
09	**1.0×10^5**	√	**-**	√	√	√
10	**5.7×10^4**	-	√	**-**	**-**	√
11	**5.3×10^4**	-	√	√	√	√
12	**1.1×10^5**	√	**-**	√	**-**	√
13	**2.5×10^5**	-	√	**-**	**-**	**-**
14	**1.4×10^4**	-	√	√	√	**-**

Table 4.2 cont.

15	**4.0×10^4**	-	√	**-**	**-**	**-**
16	**2.3×10^5**	-	√	**-**	**-**	**-**
17	**1.1×10^5**	√	**-**	**-**	√	√
18	**3.4×10^4**	√	**-**	√	**-**	√
19	**2.5×10^5**	√	**-**	**-**	√	√
20	**2.2×10^5**	-	√	**-**	**-**	**-**
21	**6.0×10^4**	√	**-**	√	√	√
22	**1.9×10^4**	√	**-**	**-**	**-**	√
23	**7.3×10^5**	√	**-**	**-**	√	√
24	**6.7×10^4**	√	**-**	√	**-**	√
25	**1.7×10^5**	-	√	**-**	√	**-**
26	**1.3×10^5**	√	**-**	√	√	**-**
27	**1.0×10^5**	√	**-**	√	√	**-**
28	**1.6×10^4**	-	√	**-**	**-**	**-**
29	**2.8×10^5**	-	√	**-**	**-**	**-**
30	**7.0×10^4**	√	**-**	√	√	√
31	**5.3×10^4**	-	√	**-**	**-**	**-**
32	**5.0×10^4**	-	√	**-**	**-**	√
33	**1.3×10^5**	-	√	**-**	**-**	√
34	**1.4×10^5**	√	**-**	**-**	**-**	**-**
35	**4.7×10^4**	√	**-**	√	**-**	**-**
36	**1.3×10^5**	√	**-**	**-**	**-**	√
37	**8.3×10^4**	√	**-**	**-**	√	√
38	**9.0×10^4**	√	**-**	√	**-**	√
39	**1.2×10^5**	-	√	**-**	√	**-**
40	**6.3×10^4**	√	**-**	**-**	√	**-**
41	**1.2×10^5**	√	**-**	√	√	√
42	**9.0×10^4**	√	**-**	√	**-**	**-**
43	**5.3×10^4**	√	**-**	**-**	**-**	√
44	**3.3×10^4**	-	√	**-**	√	√
45	**2.1×10^5**	√	**-**	√	√	√
46	**1.8×10^5**	-	√	**-**	**-**	√

Table 4.2 cont.

47	**1.7 x 10^5**	√	**-**	√	√	**-**
48	**1.1 x 10^5**	√	**-**	**-**	**-**	√
49	**9.7 x 10^5**	-	√	√	√	√
50	**5.3 x 10^4**	-	√	**-**	√	**-**
Isolation Frequency		**58 %**	**42 %**	**44 %**	**54 %**	**62 %**

4.5 Mycotoxins in Red Rice

Three mycotoxins determined in this study were citrinin, aflatoxin, and ochratoxin-A, and results are tabulated in Tables 4.3 - 4.5. Evaluation was done by principles of immunology (immunoafinity for clean-up column, ELISA test for mycotoxins evaluation).

4.5.1 Citrinin in Red Rice

Tabulated in Table 4.3 are the amounts of citrinin in 50 samples of red rice. Citrinin was found in all 50 samples (100%) with all samples giving readings above the permissible limits by Malaysian (Table 5.2) and also European standards (Table 5.3). Highest amount of citrinin was at 20.65 ppm, and lowest at 0.23 ppm.

Table 4.3 : Citrinin in red rice

Sample	*R^2	Absorbance (450nm)		Mean	**St. Dev.	***Dil. Fac.	ppm / $mgkg^{-1}$
01	**R^2 : 0.9776**	0.658	0.638	0.648	0.014	25	**2.77**
02		1.076	1.086	1.081	0.007	25	0.96
03		1.179	1.373	1.276	0.137	25	0.62
04		1.311	1.072	1.192	0.169	25	0.75

Table 4.3 cont.

05		0.621	0.851	0.736	0.163	25	**2.19**
06		0.846	0.938	0.892	0.065	25	**1.49**
07		1.739	1.721	1.730	0.013	25	0.45
08		1.259	1.231	1.245	0.020	25	**1.25**
09		1.105	0.884	0.995	0.156	25	**1.97**
10	**R^2 : 0.9485**	0.790	0.824	0.807	0.024	25	**2.80**
11		0.794	0.585	0.690	0.148	25	**3.55**
12		0.252	0.368	0.310	0.082	25	**9.85**
13		1.780	1.573	1.677	0.146	25	0.74
14		1.561	1.476	1.519	0.060	25	**1.02**
15	**R^2 : 0.9670**	0.735	0.457	0.596	0.197	25	**6.15**
16		1.401	1.327	1.364	0.052	25	**1.37**
17		1.280	1.220	1.250	0.042	25	**1.68**
18		1.007	0.929	0.968	0.055	25	**2.82**
19		1.188	1.148	1.168	0.028	25	**2.17**
20		1.564	1.543	1.554	0.015	25	**1.23**
21	**R^2 : 0.9773**	1.314	1.090	1.202	0.158	25	**2.07**
22		1.305	1.026	1.166	0.197	25	**2.18**
23		1.123	1.168	1.146	0.032	25	**2.25**
24		0.628	0.891	0.760	0.186	25	**4.03**
25		1.732	1.739	1.736	0.005	25	0.37
26		1.123	1.235	1.179	0.079	25	**1.24**
27	**R^2 : 0.9970**	0.759	0.918	0.839	0.112	25	**2.53**
28		0.986	0.941	0.964	0.032	25	**1.93**
29		0.747	0.557	0.652	0.134	25	**3.92**
30		0.500	0.495	0.498	0.004	25	**6.01**
31		0.809	0.772	0.791	0.026	25	**2.91**
32		0.745	0.611	0.678	0.095	25	**3.63**
33	**R^2 : 0.9912**	0.700	0.678	0.689	0.016	25	**3.55**
34		0.185	0.207	0.196	0.016	25	**16.45**
35		0.234	0.229	0.232	0.004	25	**13.62**
36		0.192	0.224	0.208	0.023	25	**15.40**

Table 4.3 cont.

37	**R^2 : 0.9971**	1.660	1.576	1.618	0.059	25	0.23
38		0.486	0.571	0.529	0.060	25	**4.21**
39		1.003	1.086	1.045	0.059	25	**1.12**
40		0.313	0.308	0.311	0.004	25	**9.37**
41		0.182	0.270	0.226	0.062	25	**14.49**
42		0.160	0.187	0.174	0.019	25	**20.65**
43	**R^2 : 0.9895**	1.661	1.500	1.581	0.114	25	0.39
44		0.784	0.919	0.852	0.096	25	**2.13**
45		1.122	1.125	1.124	0.002	25	**1.18**
46		0.505	0.457	0.481	0.034	25	**5.39**
47		0.496	0.519	0.508	0.016	25	**5.00**
48		0.890	1.001	0.946	0.079	25	**1.74**
49		0.518	0.478	0.498	0.028	25	**5.13**
50		0.878	0.743	0.811	0.096	25	**2.34**

**** : Regression Co-efficient***
***** : Standard Deviation***
****** : Dilution Factor***

4.5.2 Aflatoxin in Red Rice

Tabulated in Table 4.4 are the amounts of aflatoxin in 50 samples of red rice. Aflatoxin was found in 46 samples (92%) with 35 samples giving readings above the permissible limits by Malaysian (Table 5.2) and also European standards (Table 5.3). Overall, aflatoxin readings were lower than that of citrinin. Highest amount of aflatoxin was at 77.33 ppb, and lowest at 0.61 ppb.

Table 4.4 : Aflatoxin in red rice

Sample	*R^2	Absorbance (450nm)		Mean	**St. Dev.	***Dil. Fac.	*A. flavus*	ppb / μgkg^{-1}
01	**R^2 : 0.9368**	0.781	0.476	0.629	0.216	50	√	**29.89**
02		0.702	0.722	0.712	0.014	50	**-**	**23.08**

Table 4.4 cont.

03		0.645	0.622	0.634	0.016	50	√	**29.53**
04		1.296	1.110	1.203	0.132	50	-	not detected
05		0.320	0.337	0.329	0.012	50	√	**77.33**
06		1.070	1.196	1.133	0.089	50	-	not detected
07	**R^2 : 0.9868**	0.794	0.854	0.824	0.042	50	√	**5.20**
08		0.927	0.917	0.922	0.007	50	√	< 5
09		1.025	1.005	1.015	0.014	50	√	< 5
10		0.875	0.867	0.871	0.006	50	-	< 5
11		0.949	0.973	0.961	0.017	50	√	< 5
12		0.764	0.848	0.806	0.059	50	√	**5.82**
13		0.680	0.677	0.679	0.002	50	-	**11.56**
14		0.708	0.636	0.672	0.051	50	√	**11.96**
15	**R^2 : 0.9482**	1.000	1.107	1.054	0.076	50	-	**5.54**
16		1.010	1.034	1.022	0.017	50	-	**7.22**
17		1.151	1.163	1.157	0.009	50	-	not detected
18		0.556	0.558	0.557	0.001	50	√	**40.47**
19		0.897	0.781	0.839	0.082	50	-	**17.43**
20		1.081	1.033	1.057	0.034	50	-	**5.38**
21		1.250	1.090	1.170	0.113	50	√	not detected
22		0.820	0.819	0.820	0.001	50	-	**18.68**
23		1.056	1.005	1.031	0.036	50	-	**6.80**
24	**R^2 : 0.9889**	0.685	0.673	0.679	0.009	50	√	**29.37**
25		1.055	0.973	1.014	0.058	50	-	**10.32**
26		1.423	1.458	1.441	0.025	50	√	< 5
27		1.300	1.281	1.291	0.013	50	√	< 5
28		1.308	1.351	1.330	0.030	50	-	< 5
29		1.179	1.188	1.184	0.006	50	-	**5.52**
30		0.786	0.735	0.761	0.036	50	√	**22.91**
31		1.119	1.101	1.110	0.013	50	-	**7.36**
32		1.100	0.904	1.002	0.139	50	-	**10.74**
33	**R^2 : 0.9992**	1.149	1.061	1.105	0.062	50	-	< 5
34		0.850	0.899	0.875	0.035	50	-	**12.96**

Table 4.4 cont.

35		0.786	0.768	0.777	0.013	50	√	**18.23**
36		0.977	0.960	0.969	0.012	50	-	**9.08**
37		0.883	0.807	0.845	0.054	50	-	**14.43**
38		0.975	0.888	0.932	0.062	50	√	**10.51**
39		0.378	0.393	0.386	0.011	50	-	**70.72**
40		0.695	0.653	0.674	0.030	50	-	**25.67**
41		0.962	0.882	0.922	0.057	50	√	**10.88**
42	**R^2 : 0.9942**	1.091	1.078	1.085	0.009	50	√	**8.94**
43		1.177	1.119	1.148	0.041	50	-	**6.76**
44		1.321	1.237	1.279	0.059	50	-	< 5
45		1.217	1.202	1.210	0.011	50	√	< 5
46		1.157	1.188	1.173	0.022	50	-	**5.99**
47		1.188	1.304	1.246	0.082	50	√	< 5
48		1.081	1.130	1.106	0.035	50	-	**8.20**
49		0.858	0.818	0.838	0.028	50	√	**20.18**
50		0.618	0.559	0.589	0.042	50	-	**40.64**

**** : Regression Co-efficient***
***** : Standard Deviation***
****** : Dilution Factor***

4.5.3 Ochratoxin-A in red rice

Tabulated in Table 4.5 are the amounts of ochratoxin-A in 50 samples of red rice. Ochratoxin-A was found in all 50 samples (100%) of red rice, but none of the samples (0%) gave readings above the permissible limits by Malaysian (Table 5.2) and also European standards (Table 5.3). Ochratoxin-A readings were much lower than that of citrinin, and aflatoxin. Highest amount of ochratoxin-A was at 2.48 ppb, and lowest at 0.23 ppb.

Table 4.5 : Ochratoxin-A in red rice

Sample	*R^2	Absorbance (450nm)		Mean	**St. Dev	***Dil. Fac.	*A. niger*	ppb / μgkg^{-1}
01	**R^2 : 0.9905**	0.864	0.825	0.845	0.028	2	√	< 1
02		0.749	0.641	0.695	0.076	2	√	**1.16**
03		0.652	0.603	0.628	0.035	2	√	**1.32**
04		1.048	1.013	1.031	0.025	2	√	< 1
05		0.730	0.725	0.728	0.004	2	-	**1.09**
06		0.427	0.395	0.411	0.023	2	√	**2.19**
07		1.673	1.647	1.660	0.018	2	√	< 1
08		1.461	1.399	1.430	0.044	2	√	< 1
09		0.979	0.930	0.955	0.035`	2	√	< 1
10	**R^2 : 0.9919**	0.524	0.545	0.535	0.015	2	-	**1.54**
11		1.480	1.402	1.441	0.055	2	√	< 1
12		1.217	1.238	1.228	0.015	2	-	< 1
13		1.263	1.273	1.268	0.007	2	-	< 1
14		1.221	1.186	1.204	0.025	2	√	< 1
15		0.999	0.931	0.965	0.048	2	-	< 1
16		0.366	0.352	0.359	0.010	2	-	**2.48**
17		1.362	1.318	1.340	0.031	2	√	< 1
18		0.791	0.781	0.786	0.007	2	-	< 1
19	**R^2 : 0.9911**	0.593	0.607	0.600	0.010	2	√	**1.50**
20		0.474	0.495	0.485	0.015	2	-	**1.94**
21		1.182	1.215	1.199	0.023	2	√	< 1
22		1.386	1.351	1.369	0.025	2	-	< 1
23		0.522	0.517	0.520	0.004	2	√	**1.78**
24		1.023	1.029	1.026	0.004	2	-	< 1
25		1.396	1.536	1.466	0.099	2	√	< 1
26		0.994	1.004	0.999	0.007	2	√	< 1
27		1.204	1.090	1.147	0.081	2	√	< 1
28	**R^2 : 0.9860**	1.735	1.651	1.693	0.059	2	-	< 1
29		0.945	0.851	0.898	0.067	2	-	< 1
30		1.614	1.563	1.589	0.036	2	√	< 1

Table 4.5 cont.

31		0.927	0.893	0.910	0.024	2	**-**	< 1
32		0.968	0.961	0.965	0.005	2	**-**	< 1
33		1.364	1.327	1.346	0.026	2	**-**	< 1
34		0.771	0.711	0.741	0.042	2	**-**	**1.15**
35		0.587	0.569	0.578	0.013	2	**-**	**1.58**
36		1.120	1.050	1.085	0.050	2	**-**	< 1
37		1.196	1.195	1.196	0.001	2	√	< 1
38		0.844	0.778	0.811	0.047	2	**-**	< 1
39		1.087	1.036	1.062	0.036	2	√	< 1
40		1.112	1.094	1.103	0.013	2	√	< 1
41		0.481	0.499	0.490	0.013	2	√	**1.73**
42		0.422	0.399	0.411	0.016	2	**-**	**2.15**
43		1.340	1.292	1.316	0.034	2	**-**	< 1
44	**R^2 : 0.9941**	0.671	0.677	0.674	0.004	2	√	**1.13**
45		0.716	0.700	0.708	0.011	2	√	**1.05**
46		1.618	1.441	1.530	0.125	2	**-**	< 1
47		0.777	0.771	0.774	0.004	2	√	< 1
48		0.538	0.506	0.522	0.023	2	**-**	**1.59**
49		0.978	1.010	0.994	0.023	2	√	< 1
50		0.612	0.611	0.612	0.001	2	√	**1.29**

**** : Regression Co-efficient***
***** : Standard Deviation***
****** : Dilution Factor***

5.0 DISCUSSION

5.1 Sampling of Red Rice in Selangor

The sampling region in this study is the state of Selangor, Malaysia. As of 2010, Selangor has the largest population in Malaysia at 5,411,324. The ethnic composition of Selangor consists of Malay at 52.9%, Chinese at 27.8%, Indian at 13.3%, and other ethnic groups at 6% (Preliminary Count Report, 2010).

The aim of sampling was to obtain red rice samples which are normally sold by traditional Chinese medicine shops. During the course of sampling which was carried out within the period of January to December 2009, it was found that these shops thrive successfully and closely to the Chinese community with three to ten shops operating even within a small municipal district.

Using over-the-counter approach with shop owners, it was learnt that the red rice is included in daily intakes by the Chinese (to sprinkle on fried meat, chicken or pork; to use as fermentation starter of Chinese red wine; or just as colorant in soups or vegetables). Many shops visited were out of red rice supply at time of sampling indicating high demand among the Chinese community.

On the average, these shops sold about 15 to 30 kg of red rice per month depending on the area (large districts with few shops sold more) with customers buying 0.2 to 0.5 kg of red rice depending on the application and method of cooking (Chee, Fui Onn Enterprise-person. commun.). Compared to normal white rice (RM3 - 6 / kg), red rice is more expensive at RM25 - 35 / kg. It was also learnt that majority of shoppers obtained their supply of red rice from abroad (China, Taiwan, Thailand). Restricted by time and man-power, the number of red rice samples were limited to 50 (n = 50).

During the visits to these traditional Chinese medicines stores, variations in storage condition were noted. Some shops have the red rice stored in bare condition without packaging and was placed inside wooden drawer alongside many other herbs. Some have the red rice stored in packaging. Some shops stored their red rice in the refrigerator (cold temperature), and many other at room temperature (especially those occupying wooden drawer stack case). Some shops were equipped with air-conditioning system, while other shops operated within an open-air surrounding. These variations of storage condition might give impacts on microbial contamination of red rice samples. Indication on high demand and daily consumption of red rice within the Chinese community presents a potent foundation in alarming the public with possible health consequence of red rice.

5.2 Enumeration of Fungi on Red Rice

Fungal counts for 50 red rice samples stored under cold (n = 29) and room temperatures (n = 21) were carried out. For all 50 samples, fungal growth detected in the enumeration of fungal load consisted of 100% of the red rice starters, *Monascus* spp. with relatively high addition of fungi from other genera (*Aspergillus*, *Penicillium*).

By being a fungal starter, *Monascus* spp. were present on the red rice as early as the fermentation started and remained viable upon storage regardless of what the storage condition is. Unlike freezing temperature (-20°C) which inhibits all normal types of microbial growth, cold temperature (4°C) does not. The latter merely slows down the growth rate. As such, there should be no difference between fungal loads of both temperatures as confirmed by Independent Samples t-Test conducted which gave no significant difference (p = 0.069; > 0.05) between fungal counts on red rice samples stored at cold and room temperatures. In other words, storage temperatures (cold or

room) did not affect the viability of *Monascus* spp. This finding in turn might suggest that traditional post-processing technique of red rice (outdoor- or indoor-drying) did not eliminate resistant *Monascus* ascospores.

One common characteristic of genus *Monascus* is the production of ascospores capable of surviving heat treatments and subsequently growing under reduced oxygen levels resulting in food spoilage. The ascospores can tolerate hot air treatments for short periods at 96°C (Domsch *et al.*, 1980). At this point, viability of *Monascus* ascospores is of health concern. Increased viability of the ascospores tends to spoil the red rice if the rice is subjected to improper storage condition and rendering the rice as unfit for human consumption. Post-processing technique should consider the elimination or sterilisation of *Monascus* ascospores.

Even though the red rice is consumed daily or weekly by the Chinese community, the quantity taken might vary depending on type of cooking (fermentation of *samsu*, sprinkle on fried meat, red rice porridge). So far, there have been no reports on direct food poisoning alleged to red rice consumption. However, red rice usage as alternative medication has not been approved by the Food and Drug Administration (FDA) mainly due to the presence of different concentrations of the mycotoxin citrinin (Kumari *et al.*, 2009).

Although no significant difference was detected between cold and room temperatures, high readings of fungal counts (1.4×10^4 to 2.1×10^6 CFU/g) were noted from all 50 samples. This occurrence might be attributed to poor manufacturing practice (open-air surroundings, lack in use of packaging). In 2006, Fuat and co-workers conducted a mycoflora test on several poly-herbal products from Malaysia. They found that the fungal counts are safely within the permissible limits because of the use of air-tight packaging. This finding would be true in the case of red rice samples obtained in this study, where most samples were stored in bare (unpacked) condition.

In general, fungal counts for all red rice samples were above the permissible limit set by Malaysian Ministry of Health of not more than 5.0 x 10^2 CFU/g (Newsletter of the Drug Control Authority Malaysia, 1999), and International Commission for Microbiological Specification of Foods of 10^2 to 10^5 CFU/g (Elliot, 1980). These limits apply to all types of yeasts and moulds.

5.3 Isolation Frequency of Mycotoxigenic Fungi

Mycotoxigenic fungi were isolated and identified from the 50 red rice samples. The widely known food-borne fungal genera; *Aspergillus*, *Penicillium* (Filternborg *et al.*, 1996) were represented by *Penicillium chrysogenum* (62%), *Aspergillus niger* (54%), and *A. flavus* (44%). The starter fungi present in all samples were divided into *Monascus purpureus* (58%), and *Monascus pilosus* (42%). From the list of mycotoxigenic fungi isolated, possible mycotoxins produced can be identified. Table 5.1 listed the mycotoxigenic fungi with potential mycotoxins (Cole and Cox, 1981).

Table 5.1 : Mycotoxigenic fungi with mycotoxins

Aspergillus flavus	***Aspergillus niger***	***Penicillium chrysogenum***
Aflatoxin Aflatrem Aspergillic acid Cyclopiazonic acid Kojic acid Nitropropionic acid Sterigmatocystin	Malformin Nigragillin Ochratoxin	Citrinin Penicillic acid

Presence of food-borne fungal genera (*Aspergillus*, *Penicillium*) at high frequencies in red rice samples evaluated here was attributed to the fact that these fungi are mostly air-borne, and readily contaminate foods in storage. Open-air surroundings

of shops selling red rice, and lack of use of proper packaging only served to increase the risk of air-borne fungal contamination. Similar works with high frequencies of *Aspergillus* and *Penicillium* and subsequent mycotoxins contamination were also reported in stored cocoa beans (Sanchez-Hervas *et al.*, 2008), maize grains (Janardhana *et al.*, 1999), and rice grains (Reddy *et al.*, 2009).

From all 50 samples, eight samples did not exhibit any growth except for the starter fungi (one sample with *M. purpureus* and seven samples with *M. pilosus*). However, from these eight samples, aflatoxin and ochratoxin-A were identified. Absence of *A. flavus* and *A. niger* albeit the presence of aflatoxin and ochratoxin-A might be explained according to Larry and Beuchat, (2003) who stated that the presence of mycotoxigenic fungus in a food may not definitely point out that mycotoxin is present. On the other hand, the absence of mycotoxigenic fungus may not necessarily guarantee that mycotoxin is not present in the food, since growth followed by death of mycotoxigenic fungi may occur at any point before the food is analysed. Therefore, further quantification on mycotoxins was carried out to verify their presence in red rice samples.

5.4 Mycotoxigenic Fungi and Mycotoxins in Red Rice

Mycotoxins contamination of food and feedstuffs, and subsequent loss and damage to the world food supplies has been a serious complication. Various laws and legislation have been adopted by countries around the world to combat and curb further deterioration of food commodities.

In the present study, mycotoxins quantitated were compared to the permissible limits outlined in the Malaysian Food Regulation (Food Regulations, 1985), and by extension, to the limits set by the European Union (F.A.O, 2003).

Tables 5.2 and 5.3 show the limits of various mycotoxins in foods by Malaysian government, and European Union respectively.

Table 5.2 : Malaysian limits of mycotoxin in foods

Food	Mycotoxins	Maximum permitted proportions (µg/kg)
Groundnuts	Aflatoxin	15
Milk	Aflatoxin	0.5
Others	**All types of mycotoxins**	**5**

Adapted from : Malaysian Food Regulation, 1985

Table 5.3 : European limits of mycotoxin in foods[1]

Food	Mycotoxins	Limit (µg/kg)[2]
Groundnuts, nuts, dried fruit, processed products (for direct human consumption)	Aflatoxin B_1	2
	Aflatoxin Total[3]	4
Groundnuts (subjected to sorting, before human consumption)	Aflatoxin B_1	8
	Aflatoxin Total	15
Nuts, dried fruits (subjected to sorting, before human consumption)	Aflatoxin B_1	5
	Aflatoxin Total	10
Cereals, processed products (for direct human consumption)	Aflatoxin B_1	2
	Aflatoxin Total	**4**
Cereals (subjected to sorting, before human consumption)	Aflatoxin B_1	2
	Aflatoxin Total	4
Maize (subjected to sorting, before human consumption)	Aflatoxin B_1	5
	Aflatoxin Total	10
Spices	Aflatoxin B_1	5
	Aflatoxin Total	10
Raw cereals	**Ochratoxin-A**	5
Cereals, processed products (for direct human consumption)		**3**
Dried vine fruits		10
Fruit juices and fruit nectar	Patulin	50
Concentrated fruit juices		50

Table 5.3 cont.

Spirit drinks, fermented drinks		50
Solid apple products (for direct human consumption)		25
Apple juice and solid apple products, (for infants)		10
Other infant food		10
Cereals products	Deoxynivalenol	500
Flour		750
Milk	Aflatoxin M_1	0.05

Adapted from : European Union, 2003
1 : *Section for feedstuffs excluded*
2 : *µg / kg = ppm = part per million*
3 : *Total = Aflatoxin B_1, B_2, G_1, G_2,*

From Tables 5.2 and 5.3 above, it is imperative to note that certain mycotoxins (citrinin, T-2, zearalenone, fumonisin) were not in the outlined list. This might be due to different legislations adopted by different countries. As such, citrinin quantitated in this study will fall under *'other mycotoxin'* section in the Malaysian Food Regulation, (1985). Red rice falls under the *'cereals and processed products intended for direct human consumption'* section in the European Union, (2003), and *'other foods'* in Malaysian Food Regulation, (1985). In Japan, the maximum allowance level of citrinin in red rice is much higher at 200 ppb (ng / g), but its authorised limit is under consideration in China and the European Economic Community (Mandt, 1998).

5.4.1 *Monascus* spp., *Penicillium chrysogenum*, and Citrinin in Red Rice

Citrinin was present in all 50 samples of red rice (100 %). Citrinin content quantitated from all samples was found to exceed the permissible limits by Malaysian legislation (Food Regulations, 1985) at 5.00 ppb (Table 5.2). However, the Malaysian

limit was set for all mycotoxins other than aflatoxins. European Union did not list this mycotoxin in their legislation (Table 5.3).

Highest amount of citrinin was at 20648.75 ppb (~21 ppm), and lowest at 229.50 ppb (~0.2 ppm). This result is far higher than the actual citrinin standard (0.2 ppm ; Japanese standard). This result is also in accordance with the levels of citrinin found in red rice and red rice-derived products reported by several researchers at 14.3 ppm (Kumari *et al.*, 2009), 15.21 ppm (Zheng *et al.*, 2009), 4.2 - 25.1 ppm (Shu and Lin, 2002), and 0.2 - 17.1 ppm (Sabater-Vilar *et al.*, 1999), although some authors reported higher readings (Table 2.4).

Presence of citrinin in red rice samples is an almost certain occurrence since *Monascus* spp. that were used as starters in the fermentation have been found to produce citrinin at some point during metabolism (Blanc *et al.*, 1995a). Presence of *Penicillium chrysogenum* as an air-borne contaminant will also add to the risk of citrinin contamination since it is also a known citrinin producer (Ei-Banna *et al.*, 1987).

Relationship between fungal count (CFU / g) on red rice samples and citrinin level was analysed by regression method (Linear Regression; Appendix G2). It was found that the relationship between fungal count and citrinin was significantly (p = 0.036; < 0.05) not linear (R^2 = 0.017) [regression scale; 0 - 0.09 = none, 0.1 - 0.3 = small, 0.31 - 0.5 = medium, 0.51 - 1.00 = large]. This finding suggested that citrinin production is independent on the fungal load and that it may have been produced during fermentation of the red rice.

Production of citrinin on foods depends on various factors. In a review by Xu *et al.*, (2006) it was described that citrinin production was influenced by nutritional factors (oxygen supply, carbon and nitrogen sources, fatty acids), and environmental factors (water activity, temperature, preservation techniques, circulation and storage techniques of commodity).

Earlier in section 5.2, it was proven that storage conditions (room and cold temperatures) had no significant affect (p = 0.069; > 0.05) on fungal load from red rice samples obtained in this study, and by extension, to citrinin production itself (R^2 = 0.017; none linear). Assuming that the rice used by traditional red rice producers was the normal rice, like usual practice, and not the glutinous rice, the content of carbon, nitrogen, and fatty acids should not differ significantly. The fermented rice will undergo air-drying. As such, oxygen supply, and water activity should also not differ significantly. In-door or out-door drying of the fermented rice during traditional preparation technique will only concern the risks of air-borne microbial contamination.

Citrinin is produced as a secondary metabolite during metabolism, and *Monascus* spp. being the fermentation starters will also have a lagging period during the life-cycle in which time they undergo metabolism and produce secondary metabolite. So, no matter how long the rice is left for fermentation, the citrinin is only produced during that certain period of time. That being said, length of fermentation should not affect the production of citrinin.

In addition to that, in this regard, variables in citrinin production might come from the two species of *Monascus* spp. isolated from red rice samples *M. purpureus* (29 samples) and *M. pilosus* (21 samples). Independent t-Test conducted (Appendix G3) showed a significant difference (p = 0.037; < 0.05) between the levels of citrinin produced by *M. purpureus* (means = 5,179.1 ppb) and *M. pilosus* (means = 2,432.9 ppb). This result suggested that *M. purpureus* is a higher producer for citrinin in red rice samples obtained in this study. This finding almost resembles that of Wang *et al.*, (2005) who tested variability in citrinin production by 23 samples of *Monascus* spp. and reported that *M. purpureus* (9 samples) and *M. pilosus* (4 samples) yielded citrinin at 183.44 ± 84.14 ppm, and 152.50 ± 19.07 ppm, respectively.

Citrinin is a potent nephrotoxic. However, up to date, there has been no report indicating that citrinin is the main cause for kidney cancer on human. Nevertheless, several works on non-human patients have been reported with varying susceptibility depending on species. At 20 ppm citrinin induced acute nephrotoxicty in dogs (Krejci *et al.*, 1996). At 1.8 to 4.7 ppm citrinin caused 50% cell death to human embryonic kidney cell (HEK293) (Liu, *et al*., 2005). At 32.5 ppm citrinin caused 50% cell death in Vero cells (Green Monkey kidney cells) (Golli *et al.*, 2006). At 3.75 ppm citrinin promoted mouse embryo apoptosis and developmental injury of mouse blastocysts *in vitro* and *in vivo* (Chan, 2007; Chan and Shiao, 2007).

Based on the information obtained over the counter, a customer might purchase 0.2 to 0.5 kg of red rice for weekly consumption on average. From the study, citrinin was detected in all 50 samples at 0.2 to 20 ppm. Assuming the customer consumes 0.2 kg of red rice, there is a chance that he might ingest approximately 4 ppm of citrinin. Even though this level is lower than 20 ppm which can cause acute nephrotoxicity in dogs (Krejci *et al.*, 1996), it is still far higher than the approved standard for citrinin in food at 0.2 ppm (Mandt, 1998).

So far, there have been no reports on nephrotoxicity caused by long-term exposure of citrinin at low amount. However, based on the data collected by Malaysian Ministry of Health on the number of kidney cancer patients in Malaysia from 2002 to 2006, there is a significant difference among the major races in Malaysia whereby the Chinese remained highest in number of kidney cancer patients during the surveyed period from 2002 to 2006 (National Cancer Registry, 2006).

Kidney cancer might be caused by many factors. Since there is no work on citrinin toxicity done on human subject, one cannot simply implicate citrinin as the major factor. However, several works by other authors discussed earlier might pave way for the implication and validation of nephrotoxicity of citrinin.

5.4.2 *Aspergillus flavus* and Aflatoxin in Red Rice

Aflatoxin was present in 46 samples of red rice (92%). Out of that, 35 samples (70%) were quantitated with aflatoxin content exceeding the permissible limits by Malaysian legislation (Food Regulations, 1985) at 5.00 ppb for foods other than groundnuts (15 ppb), and milk (0.5pbb) (Table 5.2), and European Union at 4.00 pbb (Table 5.3). Overall, aflatoxin readings were lower than that of citrinin. Highest amount of aflatoxin was at 77.33 ppb, and lowest at 0.61 ppb.

From 50 samples of red rice, *A. flavus* was present in 22 samples (44 %). However, aflatoxin was present in 46 samples (92%). This might due to the death of *A. flavus* at any point of red rice processing, or the fungus was not able to be resuscitated on agar medium on account of too little spores, but has released the aflatoxin on the rice nevertheless.

Another possibility of low percentage of occurrence of *A. flavus* as compared to high percentage of aflatoxin was that *A. flavus* was inhibited by the citrinin released by the starter fungi, *M. purpureus* and *M. pilosus*. As one of mycotoxins, citrinin possesses antibiotic, bacteriostatic, antifungal and antiprotozoal properties (Hanika *et al.*, 1983; Bilgrami *et al.*, 1988; Berndt, 1990). It may also be possible that *A. flavus* is among the species susceptible to the antifungal effect of citrinin.

Appendix G4 shows the percentage of (possible) inhibition of *A. flavus* against increasing concentration of citrinin from 50 red rice samples. However, no linear pattern of inhibition can be deducted (R^2 = 0.0011). One possible explanation for failure to obtain linear pattern of inhibition might due to the irregular distribution of citrinin concentration where 38 out of 50 samples gave citrinin reading within the range of 0 to 4.99 ppm. Nevertheless, the highest range of citrinin concentration (15.00 - 20.65ppm) gave the highest percentage of inhibition *A. flavus* (66.67%).

As an air-borne fungus, ascospores of *A. flavus* might have contaminated the red rice during storage. So far, there has been no work reporting on the contamination of *A. flavus* and aflatoxin on red rice, although many works suggested that this fungus is major storage contaminant (Patel *et al.*, 1996; Sales and Yoshizawa, 2005; Schatzmayr *et al.*, 2006).

Aflatoxins are a type of naturally occurring mycotoxins mainly produced by various strains of *Aspergillus*, such as *A. flavus*, *A. parasiticus*, *A. tamarii*, and *A. nomius*. Aflatoxins exist in many classes (B_1, B_2, G_1, G_2, M_1, M_2) and are known to exert immunotoxic, mutagenic, and carcinogenic effects (Moss, 1998). Aflatoxins are also a potent hepatotoxic (toxic to liver) and were classified as Group I Carcinogen by the International Agency for Research on Cancer (IARC, 1993). High presence of aflatoxin (70% of all samples in this study) by account of poor storage condition of red rice warrants consideration since red rice is consumed daily by the Chinese community.

5.4.3 *Aspergillus niger* and Ochratoxin-A in Red Rice

Ochratoxin-A was present in all 50 samples of red rice (100%). However, all the readings were within the permissible limits set by Malaysian legislation (Food Regulations, 1985) at 5.00 ppb for foods other than groundnuts (15 ppb) (Table 5.2), and milk (0.5pbb), and European Union at 3.00 pbb (Table 5.3). Highest amount of ochratoxin-A was at 2.48 ppb and lowest at 0.23 ppb. Out of 50 samples of red rice, 27 samples (54%) were contaminated by *A. niger*. However, ochratoxin-A was found in all samples. Similar possibility of inhibition effect of *A. flavus* by citrinin from *Monascus* spp. as discussed earlier might also occur on *A. niger* and thus explains the presence of lower *A. niger* albeit high contamination percentage of ochratoxin-A.

Appendix G5 shows the percentage of (possible) inhibition of *A. niger* against increasing concentration of citrinin from 50 red rice samples. Almost linear pattern of inhibition can be deducted ($R^2 = 0.7556$). Highest range of citrinin concentration (15.00 - 20.65ppm) gave the highest percentage of inhibition *A. niger* (66.67%).

It is also noteworthy that although all red rice samples were contaminated by ochratoxin-A, none of the readings exceeded the permissible limits set either by Malaysian regulation, or European Union. The low levels of ochratoxin-A quantitated from red rice samples may be due to the fact that *A. niger* is not the major or potent producer of ochratoxin-A.

Ochratoxin-A is originally produced by *A. ochraceus*, hence the name (Meri *et al.*, 2005). If accumulated in the body at a concentration high enough, ochratoxin-A can be nephrotoxic (toxic to kidney), immunosuppressive, and also carcinogenic (Kuiper-Goodman and Scott, 1989). Eventhough *A. niger* also produces ochratoxin-A, it is still considered as GRAS (generally regarded as safe, USFDA, 1998) due to the low level of ochratoxin-A production.

However, it is still worrying as *A. niger* is also known to produce oxalic acid (LD_{50} 150 ppm in rat) that can cause gastroenteritis, kidney problems and skin ulceration; kojic acid (LD_{50} 250 ppm in rat) that can increase skin sensitivity by reducing melanin in skin; malformin A (LD_{50} 3.1 ppm in rat), and malformin C (LD_{50} 0.9 ppm in rat) that can cause abdominal pain and vertigo (USEPA, 1997).

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The present study was aimed to (1) determine the occurrence of mycotoxigenic fungi, and (2) evaluate the mycotoxins contaminating red rice marketed throughout the state of Selangor, Malaysia.

Overall fungal loads (CFU/g) for all 50 red rice samples examined in this study were 1.4 x 10^4 CFU/g (lowest), 2.1 x 10^6 CFU/g (highest) with average at 1.8 x 10^5 CFU/g. These readings were highly above the permissible limit for fungal contamination of herbal preparation set by Malaysian Ministry of Health of not more than 10^2 CFU/g. These readings also exceeded the normal microbiological profile of cereal grains and cereal products drafted by the International Commission for Microbiological Specification of Foods of 10^2 to 10^5 CFU/g for dry cereal mixes.

Based on analyses and observation, three predominant mycotoxigenic fungi were isolated from 50 red rice samples obtained in this study namely *Penicillium chrysogenum* (62%), *Aspergillus niger* (54%), and *Aspergillus flavus* (44%). Starter fungi in the fermentation of red rice were also isolated namely *Monascus purpureus* (58%) and *Monascus pilosus* (42%).

Immunological assay employed in this study confirmed the presence of three mycotoxins from all red rice samples namely citrinin, aflatoxin, and ochratoxin-A. Citrinin was present in 50 samples (100%) at 0.23 to 20.65 ppm. Aflatoxin was present in 46 samples (92%) at 0.61 to 77.33 ppb. Ochratoxin-A was present in 50 samples (100%) at 0.23 to 2.48 ppb. Citrinin (100%), and aflatoxin (76.09%) were present critically above the permissible limit set by Malaysian Ministry of Health of not more than 5 ppb of all mycotoxins in food, and European Union at 4 ppb for aflatoxin.

Meanwhile, none of the samples contained ochratoxin-A above the permissible limit (not more than 5 ppb for Malaysian limit; not more than 3 ppb for European limit).

6.2 Recommendation

High number of fungal load above microbial limit, presence of mycotoxigenic fungi, and subsequent presence of mycotoxins above toxin limit in traditionally-prepared red rice samples examined in this study might collectively pose significant health hazard towards human consumption.

Presence of fungal contaminants only served as an initial indicator of possibility for mycotoxins contamination. This might be due to improper production, handling, transportation, and storage practice of red rice as usually is observed in common traditional preparation. Presence of mycotoxins above the permissible limit on the other hand, is evidence that the traditional food in question is no longer safe for human consumption. It is henceforth suggested that traditional production of red rice should adhere to scientific inspection and clinical regulation, and that toxicity studies on the nephrotoxic effect of citrinin produced by the starter fungi in red rice should be carried out to confirm the safety and health status of red rice. If the citrinin from *Monascus* spp. used in the fermentation of red rice is proven to have direct effect on consumer's health especially the functions of kidney, the public and industry should immediately be alerted, health regulation governing the consumption of red rice should be promulgated, and the search for scientific solution should begin, possibly on molecular technology by modifying the genes controlling citrinin synthesis pathway.