NATURAL RED PIGMENT WITH ANTIBACTERIAL ACTIVITY FROM *Nigrospora sphaerica* CL-OP 30, AN ENDOPHYTIC FUNGUS ISOLATED FROM LEAF OF *Swietenia macrophylla* King

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by

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CZA	Czapek Dox agar
DMSO	Dimethyl sulfoxide
DPPH	Diphenyl-picrylhydrazyl
EC_{50}	50% maximal effective concentration
GCMS	Gas chromatography mass spectrometry
HMDS	Hexamethyldisilazine
INT	p-iodonitrotetrazolium violet salt
LC_{50}	50% lethal concentration
MBC	Minimal bactericidal concentration
MEA	Malt extract agar
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimal Inhibitory concentration
MRSA	Methicilin-resistant *Staphylococcus aureus*
OD	Optical density
PDA	Potato dextrose agar
R_f	Retention factor
SDA	Sabouraud dextrose agar
SEM	Scanning electron microscope
SmF	Submerged fermentation
TLC	Thin layer chromatography

TPC	Total phenolic content
UV	Ultra violet
YES	Yeast extract sucrose

PIGMEN MERAH SEMULA JADI DENGAN AKTIVITI ANTIBAKTERIA DARIPADA *Nigrospora sphaerica* CL-OP 30, KULAT ENDOFITIK YANG DIPENCILKAN DARIPADA DAUN *Swietenia macrophylla* KING

ABSTRAK

Keperluan untuk mendapatkan pigmen semula jadi daripada sumber mikrob telah berkembang dengan pesatnya memandangkan kebanyakam pigmen sintetik didapati merbahaya kepada manusia. Namun begitu, banyak pigmen semula jadi daripada sumber alamiah yang lain adalah tidak mencukupi untuk memenuhi keperluan industri. Dalam penyelidikan ini, enam pencilan kulat endofitik yang dipencilkan daripada daun *Swietenia macrophylla* telah dipilih sebagai subjek kajian tahap awal dan *Nigrospora sphaerica* CL – OP 30 telah dipilih sebagai sasaran tunggal kajian ini disebabkan oleh keupayaan kulat tersebut menghasilkan pigmen merah yang paling tinggi pada hari ke-14 dengan hasilan sebanyak 0.1471 OD/g berbanding dengan pencilan lain. Kulat tersebut didapati menghasilkan lebih banyak pigmen merah dalam keadaan pengkulturan bercahaya tanpa kehadiran ekstrak tumbuhan perumahnya dan saiz inoculum terbaik adalah dengan kehadiran dua plag kulat berdiameter 1 cm. Ekstrak etil asetat yang berwarna merah diekstrak daripada kaldu fermentasi mempamerkan aktiviti antibakteria yang ketara terhadap kesemua lapan bakteria Gram positif yang dikaji di mana zon perencatan berjulat daripada 16.0 ± 1.0 mm hingga 25.7 ± 3.1 mm, manakala kepekatan perencatan minimum pula adalah dalam julat 93.75 hingga 375.00 µg/mL. Kajian tentang corak masa pembunuhan pula menunjukkan aktiviti antimikrob ekstrak terhadap kedua-dua *Bacillus cereus* (pencilan klinikal) dan *Staphylococcus aureus* (pencilan bawaan makanan) adalah bergantung kepada kepekatan ekstrak yang digunakan. Keputusan daripada Mikroskop Elektron Imbasan memaparkan kesan ekstrak yang menyebabkan sel-sel *B. cereus* dan *S. aureus* mengecut, berkedut, berlekuk, berlubang atau pecah. EC_{50} daripada aktiviti penyingkiran radikal bebas DPPH adalah sebanyak 157.5725 µg/mL manakala purata kandungan fenolik adalah 82.77638 µg GAE/mg. Dalam kajian bioautografi, hanya satu tompok yang berwarna jingga dengan nilai R_f sebanyak 0.682 menunjukkan aktiviti antibakteria. Dengan menggunakan kromatografi turus, fraksi F2 dengan warna jingga terang telah dipencilkan dan didapati bahawa ia adalah satu-satunya fraksi aktif dengan aktiviti antibakteria dan mengakibatkan zon perencatan sebesar 19.7 ± 1.2 mm hingga 23.0 ± 1.0 mm, manakala nilai kepekatan perencatan minimum berjulat daripada 31.25 ke 125.00 µg/mL. Analisis GC-MS fraksi F2 tidak dapat mengesan pigmen semula jadi yang terdapat dalam fraksi F2. Kehadiran asid-asid lemak lain dan feniletanol dengan sifat antibakteria dapat dikesan. Ujian kesitotoksikan udang brin menunjukkan ekstrak mentah yang berwarna merah hanya bersifat kesitotoksikan kronik sahaja, sementara fraksi F2 pula bersifat kesitotoksikan akut dan kronik. Berdasarkan kepada kajian, fraksi F2 mempunyai aktiviti antibakteria yang lebih tinggi berbanding ekstrak mentah daripada kulat *N. sphaerica* CL-OP 30. Analisis LC-MS dicadangkan untuk pengajian pigmen semula jadi. Secara ringkasnya, sebatian pigmen daripada *N. sphaerica* CL-OP 30 berpotensi besar untuk dikaji secara mendalam sebagai agen pewarna dengan aktiviti antibakteria.

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ABSTRACT

The demand for natural pigment from microbial sources are growing rapidly since most of the synthetic pigments are found to be hazardous to mankind, and most of the natural pigments obtained from other natural sources are insufficient for the industrial purposes. In this study, six endophytic fungal isolates which were isolated from *Swietenia macrophylla* were selected as the preliminary study subjects where *Nigrospora sphaerica* CL-OP 30 was eventually selected as the sole target for the study as the fungus produced highest yield of reddish pigment at day 14 with the 0.1471 OD/g of production compared to others. The higher pigmentation was favourable when the fungus was cultivated in light condition without the incorporation of host plant extract with the ideal inoculum size of the fungus, 2 fungal plugs with 1cm in diameter. The red-pigmented ethyl acetate extract from the fermentative broth exhibited significant antibacterial activity against all eight Gram positive bacteria tested where the inhibition zone ranged from 16.0 ± 1.0 – 25.7 ± 3.1 mm whilst the MIC ranged from 93.75 – 375.00 μg/mL. The time-kill assay showed that the antimicrobial activity of the extract against *Bacillus cereus* (clinical isolate) and *Staphylococcus aureus* (foodborne isolate) were concentration-dependent. The results from the Scanning Electron Microscopy study shows the extract made the cells of *B. cereus* and *S. aureus* shrunken, crumpled, dented, pitted, or even burst. The EC_{50} of DPPH free radical scavenging activity of the extract was determined to be 157.5725 μg/mL while the average of total phenolic content in the extract was 82.77638 μg GAE/mg. On the bioautography study, only one orange-coloured spot with R_f value at 0.682 showed antibacterial activity. Using open column chromatography, fraction F2 with bright orange colour was separated and found to be the only active fraction with antibacterial activity by exhibited inhibition zone ranged from 19.7 ± 1.2 mm to 23.0 ± 1.0 mm, while the MIC ranged from 31.25 – 125.00 μg/mL. The GC-MS analysis could not detect any natural pigment compounds. The presence of the other fatty acids and phenylethanol as the antibacterial compounds were detected. The brine shrimp cytotoxicity test indicated that the crude extract was chronically toxic whilst fraction F2 exhibited both acute and chronic toxicity. Based on the studies, the fraction F2 possessed greater antibacterial activity compared to the crude extract from *N. sphaerica* CL-OP 30. LC-MS is recommended in future study of natural pigment. the pigmented compounds from *N. sphaerica* CL-OP 30 have great potential to be explored as the colouring agent with antibacterial activity.

CHAPTER 1 INTRODUCTION

1.1. Problem statements

Nowadays, the synthetic pigments are widely applied in different industries, which involved in the colouration of textiles, cosmetics, inks, water colours, artist’s paints, leathers, papers and foods. The synthetic pigments also used in the agricultural research, light-harvesting arrays, photo electrochemical cells and also in hair colourings (Chidambaram *et al.*, 2013). Despite the importance and wide applications of synthetic pigments, various problems arise especially regarding to the ecology and human. Most of the synthetic pigments used in the product coloration are usually drained off together with the industrial waste into the environment which causes serious water and soil pollution. On the other hand, the synthetic pigments may also carcinogenic or toxic to human and cause various diseases including allergy reaction (Lang, 2009).

The use of natural products is a trend among the consumers as the health and safety awareness grows stronger day by day. People tend to choose natural products rather than synthetic goods to ensure the products are friendly to human body. Hence, the need to explore the world of natural pigments has emerged in order to find more eco-friendly and healthier colourants using green technology to fulfil the consumers’ desires.

However, current existing natural pigments are mostly known to be unstable against various factors including pH, temperature and light exposure. The hues provided are not as bright and as rich as provided by synthetic colourants. The productions of the natural pigment are various from batch to batch where no consistency of the pigments produced. Besides, it is costly to produce natural pigments as the production involves complex extraction from natural sources such as insects, flowering plants and invertebrates, which consequently increase the market prices of natural pigments (Ashfaq & Masud, 2002). Other than the disadvantages of current existing natural pigment stated above, the manufacturers also need to take extra precautions to prevent the contaminations or the pollutions of the natural sources which used to produce natural pigments (Wan *et al.*, 2012).

1.2. Rationale of the study

The search for natural metabolites from endophytes is an on-going trend for decades where people have their eyes on the natural product explorations from endophytes in pharmacological and agrochemical industries (Dreyfuss & Chapela, 1994).

Endophytes are organisms such as fungus or bacteria that live latently in any part of the plant without causing any apparent infection (Schulz *et al.*, 2002; Ramesh, 2006). The endophyte-host interaction may be mutualism, opportunistic parasitism, saprophytism or exploitation where the endophyte may lay dormant within the plant cells asymptomatically (Kusari *et al.*, 2012). A great number of endophytes have the potential to produce secondary metabolites that may be used directly or indirectly as the therapeutic agents against numerous diseases (Strobel *et al.*, 2004; Aly *et al.*, 2010; Kharwar *et al.*, 2011; Kusari *et al.*, 2012).

Endophytes may produce different secondary metabolites corresponding to the biotope due to the metabolic interactions between the fungi and the environment and the host as well (Schulz *et al.*, 2002). Many endophytes have the ability to produce wide spectrum of bioactive metabolites which are exclusive from a biochemical and molecular standpoint due to the ecological perspective and the host plant inhabited (Kusari *et al.*, 2012).

Some endophytic fungi were identified as the sources of anticancer, antidiabetic, insecticidal and immunosuppressive compounds. Other than those bioactive metabolites, endophytic fungi are also able to produce thermoprotective metabolites. The endophytes play role in making the plant to have the ability to adapt to stress condition by producing secondary metabolites with pharmaceutical importance (Ramesh, 2006). Besides, the endophytes especially endophytic fungi have been reported to be able to produce the same or similar bioactive metabolites as their host plants and also most bioactive compounds have been implicated in the protection against pathogens and herbivores (Radić & Štrukelj, 2012). In order to obtain the antibacterial compounds, the reasoning behind the host plant selection has mostly been investigating plants that are used in traditional medicine as the antibacterial agents. Endophytic microbes have been reported to have the ability to produce pigments such as melanin (Suryanarayanan *et al.*, 2004; Khanam & Chandra, 2015). Endophytic fungus *Bartalinia* sp. isolated from *Ixora coccinea* Linnaeus, Endophytic fungi producing orange pigment isolated from *Ginkgo Biloba* Linnaeus and endophytic bacteria from *Beta vulgaris* Linnaeus had been reported to produce red pigment (Liu *et al.*, 2008; Murugan & Mugesh, 2013; Khanam & Chandra, 2015).

The production of the bioactive metabolites may be optimized by altering the accessible culture and process parameters such as the media, aeration, temperature, pH and agitation. This could make the bioactive compounds production cost-effective, environment friendly, continuous and reproducible yield compliant to commercial scale-up. The production of the bioactive compounds using endophytes may independent of the variability influenced by the environmental conditions (Kusari *et al.*, 2012).

1.3 Objectives of study

The objectives of the research were as follows:

1. To screen five endophytes for pigment production.
2. To select the best fungal pigment producer.
3. To investigate the dependence of fungal pigment production to the presence of host plant extract and light.
4. To characterize the extracted fungal pigment.
5. To investigate the toxicity of the selected fungal extract and semi purified fraction.

CHAPTER 2 LITERATURE REVIEW

2.1 Pigment

Colourants can be categorized as pigments or dyes. Pigments are coloured materials which are either inorganic or organic (Herbst & Hunger, 2004) that are essentially insoluble in the medium and usually used in the colouration of paints, printing inks, and plastics and they are also applied to paper, textiles, rubber, glass, ceramics cosmetics, crayons and building materials such as cement (Völz, 2001). The interaction of pigment with the medium involves the incorporation of pigment into the liquid medium (Christie, 2001). Some pigments maybe practically insoluble in some certain medium, yet they can still partially dissolve in another in particular conditions (Herbst & Hunger, 2004).

Pigments are the compounds that absorb light with the molecule-specific structure (chromophore) that capture the energy absorbed and is reflected and or refracted from a radiant source at the visible wavelength. Neural impulses are generated when the energy is captured by the eye, and then transmitted to the brain to be interpreted as a colour (Britton, 1983).

Unlike the dyes, pigments disperse instead of dissolve in the media they are incorporated with, and form a heterogeneous mixture (Lang, 2009). The pigments are distinctively different from dyes. Pigment is a coloured, black, white or fluorescent particulate organic or inorganic solid, which is usually insoluble and, essentially, physically and chemically stable against the vehicle or substrate which it is incorporated in. Therefore, the pigmentation phenomenon is by selective absorption and or by the scattering of light. Besides, a pigment will retain its crystalline or particulate structure. By contrast, dyes are soluble in the carrying medium and therefore lose the crystalline or particulate features in the solution when a dyestuff is used to impact colour to a material (Delgado-Vargas & Paredes-López, 2003).

The interaction of pigment with the medium involves the incorporation of pigment into the liquid medium. The pigmented medium is allowed to solidify by solvent evaporation, physical solidification or polymerization and the pigment is fixed in the solid polymeric matrix. Pigments have a weak affinity for their application medium and the pigment particles only attach to the surface where they had been applied with the medium (Christie, 2001).

Pigments can be classified according to their occurring in the nature whether they are synthesized chemically or they can be produced naturally by organisms (Hendry & Houghton, 1996). They are applied mostly in the colouration of the paints, inks, plastics, papers, textiles, rubber, cosmetics, cement and concrete, glass, and ceramics (Christie, 2001).

2.2 Classification of pigment

The pigments are classified based on their sources or their structures. Therefore, the pigments are either natural or synthetic and organic or inorganic in nature (Turner, 1988).

2.2.1 Natural pigments

Other than the synthetic pigments, there are some natural occurring pigments which can be derived from natural edible sources. The sources of the natural pigments are the plants including algae, vertebrates, invertebrates, fungi, lichens and bacteria (Hendry & Houghton, 1996). Natural pigments are generally defined as the materials extracted, isolated, or derived from plants, animals or microorganisms that show certain colour after applying on some substrate such as the textiles, papers and foods (Lauro & Francis, 2000). The curcumin extracted from turmeric, bixin from annatto seeds, and anthocyanins from red fruits are all the examples of the natural pigments used as the natural colourants in food manufacturing (Hendry & Houghton, 1996).

Some of the natural pigments are suggested to possess the ability to act as antioxidants and the presence of the natural pigments in the diet may reduce the risk of cardiovascular disease, cancer, and aging problems. Anthocyanins that present in fruit and vegetable products are anticarcinogenic, anti-inflammatory, antihepatotoxic antibacterial, antiallergic, antithrombotic and act as antioxidant. Carotenoids are synthesized by photosynthetic microorganisms and plants. Some of the carotenoids are vitamin A precursors and many show radical or single oxygen trapping activity and thus causing the antioxidant effects. Besides, carotenoids may reduce the risk of lung cancer, cervical dysplasia and cardiovascular diseases. Fruits and vegetables of green, orange, and red colour are the major sources of carotenoids for human. Curcuminoids show anti-inflammatory and anti-tumour-promoting effects (Lauro & Francis, 2000). Figure 2.1 shows several chemical structure of natural pigments.

A large number of pigment structures can be found abundantly in living organisms, such as anthocyanin group alone has more the 250 distinctive structures has been reported since the pigments have great structural complexity (Delgado-Vargas & Paredes-López, 2003).

The examples of the natural pigments are carotenoids, carmine, chlorophyll, xanthophyll, anthraquinone, indigoide (Otterstätter, 1999), betalains, monascus, gardenia yellow, paprika, annatto, lycopene, phycobilins, turmeric and anthocyanins (Lauro & Francis, 2000).

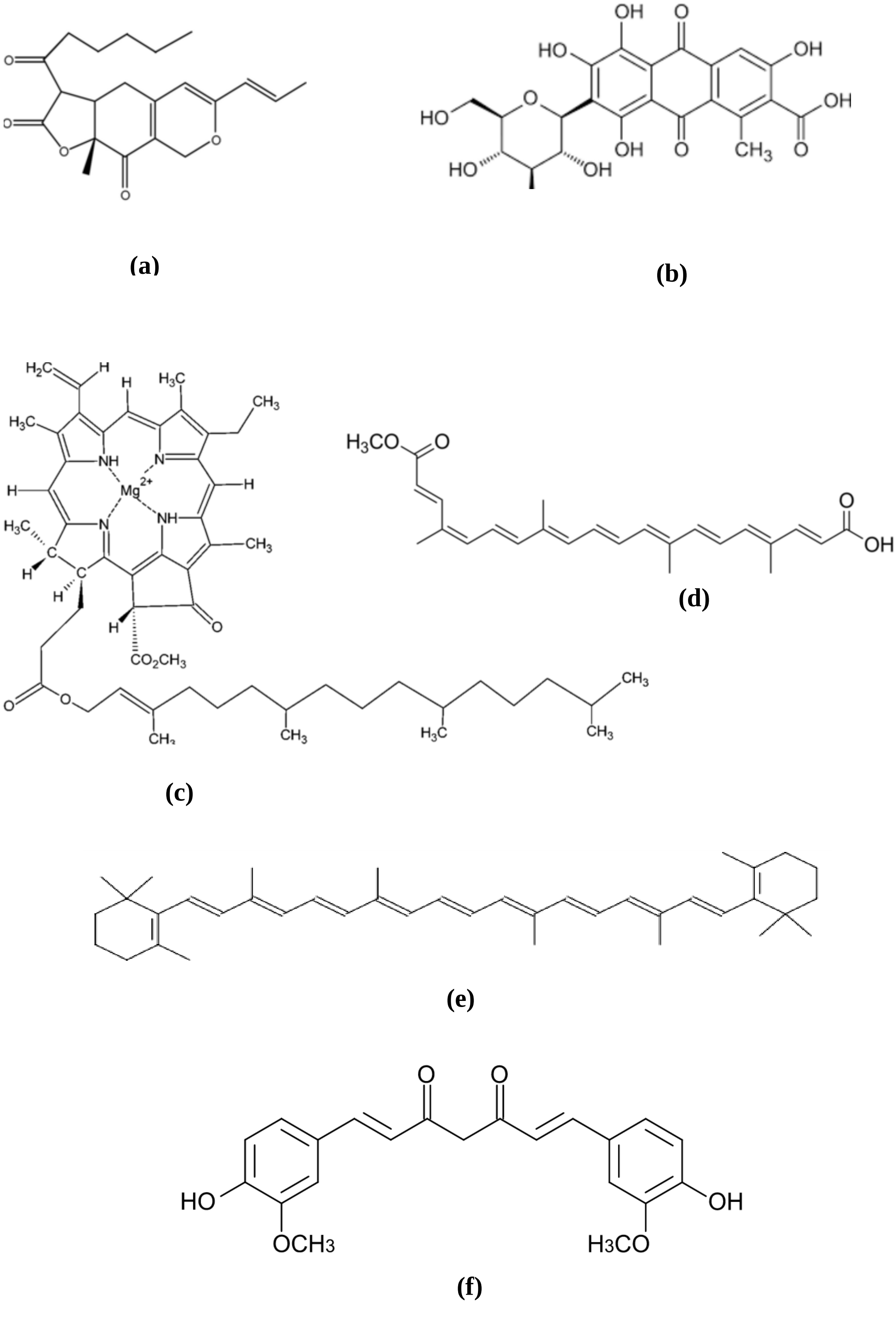
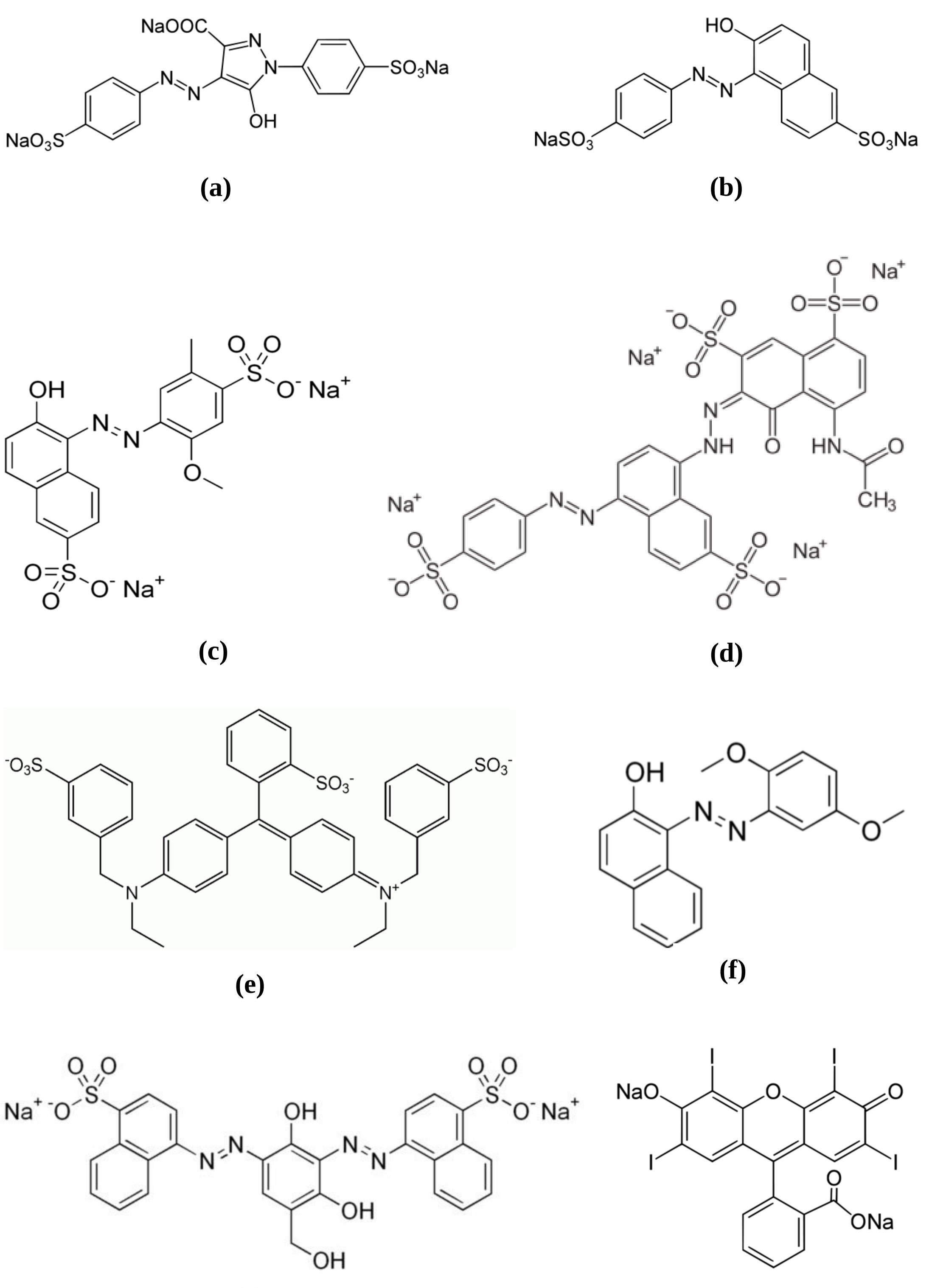


Figure 2.1: Chemical structures of several natural pigments; (a) Monascin, b) Carminic acid, (c) Chlorophyll a, (d) Bixin, (e) β-carotene, (f) Curcumin (Hendry & Houghton, 1996).

2.2.2 Synthetic pigments

Synthetic pigments are those pigments that do not occur naturally in nature and are produced by chemical synthesis. Some of the examples of the synthetic pigments are the sunset yellow, carmoisine and tartrazine. Besides that, there are also some natural-identical colours that are chemically synthesized so as to be identical chemically to the natural pigments, for example β-carotene, riboflavin and canthaxanthin (Hendry & Houghton, 1996). Synthetic pigments are applied in a wide range of coloured materials such as paints, plastics, printing inks, photographs, ceramics and cosmetics (Christie, 2001). The discovery and the introduction of synthetic pigments never stop and today, more than 700 synthetic colourants have been manufactured (Delgado-Vargas & Paredes-López, 2003). The most recognized examples of synthetic pigments are azo colourants, triaryl methane, xanthen colourant, quinophtalone, and phtalocyanin (Otterstätter, 1999). Some chemical structures of synthetic pigments were shown in Figure 2.2.

Nowadays, people tend to use natural pigments instead of synthetic pigments to gain healthier lives. Due to the reduction of the use of synthetic pigments, the market for synthetic pigments has been shrunken but it is still there because of some of their important properties. The synthetic pigments have a variety of hues that can be adjusted to the desired shades, the use of synthetic pigments also ensures the uniformity in the product colourations and they are rather stable against pH (Delgado-Vargas & Paredes-López, 2003).



(a) (b)

(c) (d)

(e) (f)

(g) (h)

Figure 2.2: Chemical structures of several synthetic pigments; (a) Tartrazine, (b) Sunset yellow FCF, (c) Allura red AC, (d) Brilliant black BN, (e) Brilliant blue FCF, (f) Citrus red 2, (g) Chocolate brown HT, (h) Erythrosine (Hendry & Houghton, 1996).

2.2.3 Organic pigments

The market for organic pigments grows larger at the end of the 19th century since high demand of organic pigments as the result of the development of textile dye industry at that time.

At first, organic pigments were the secondary product of the dyestuff industry. The salts of anionic and cationic dyes, also known as “lakes” dissolve in the water and react with other substrates to produce organic pigments (Delgado-Vargas & Paredes-López, 2003). The first synthetic organic pigment in the world was available in the 1930s, which is the copper phthalocyanine that can dissolve in the water and act as dyes. Later on in 1958 and 1984, pigments based on quinacridones and diketo-pyrrolo-pyrroles were commercialized respectively (Zollinger, 2003).

Organic pigments have the following properties such as bright and intense colour, light and weather fastness, low cost, heat resistant, non-toxic, and high colour strength. The properties make organic pigments non-biodegradable and they are not easily dissolve in water and most of the solvents and thus non-toxic to the environment (Hikmet & Mustafa, 2014). Some chemical structures of organic pigments as displayed in Figure 2.3.

Organic pigments such as azo pigments provide the basic colour of yellow, red, orange organic pigments which are still in use nowadays. Organic pigments offer intense bright colour and perform moderately in fastness of the pigments. Despite the fastness moderate fastness property, the production of organic pigments is rather expensive due to its high performance of in retaining their superior colour properties by providing good colour strength, intensity and brightness of the pigments. In contrast to high reflective index of inorganic pigments, organic pigments have low reflective index, which means most of the organic pigments are transparent and have poor opacity. Some of the high performance organic pigments are quinacridones, isoindolines, perylenes, diketopyrrolopyrroles, dioxazines, and perinones (Christie, 2001).

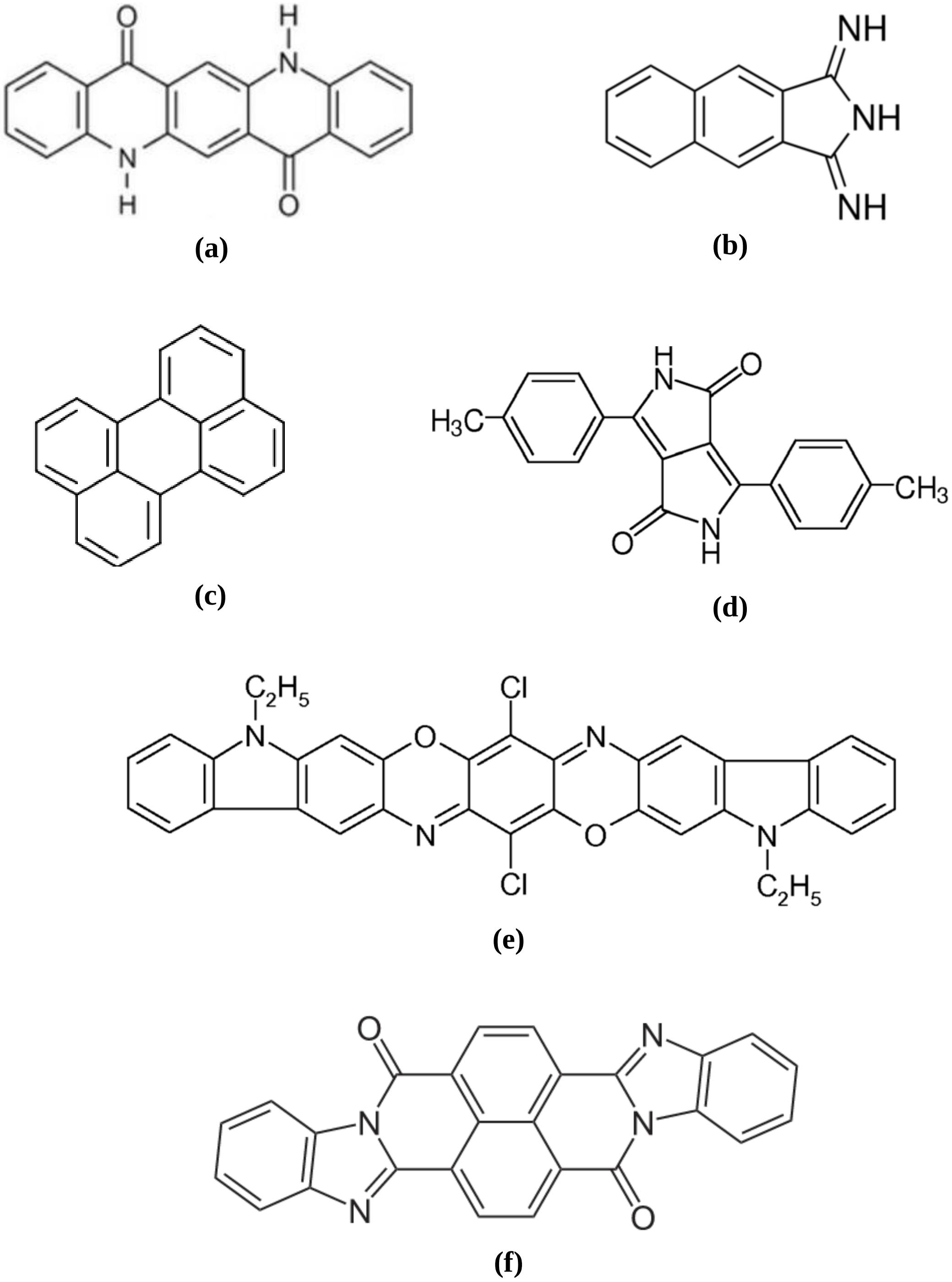


Figure 2.3: Chemical structures of several Organic pigments; (a) quinacridone, (b) isoindoline, (c) perylene, (d) diketopyrrolopyrrole, (e) dioxazine, and (f) perinone (Christie, 2001)

2.2.4 Inorganic pigments

The use of inorganic pigments can be traced back to prehistoric times and some of them are still in use nowadays. 20th century was the golden age for the inorganic pigments. During 20th century, inorganic pigments such as the black pigment, titanium dioxide and other chemically synthesized coloured pigments, for examples, oxides, cadmium sulphides, leads chromates and Prussian blue were produced (Christie, 2001).

Inorganic pigments are commercialized in the form of fine powders and used as the colourants to many ceramic materials such as porcelain enamels and ceramic bodies. They are acting as the decorative and protective coatings on the ceramic materials. The pigments used in ceramic colourations must possess the stabilities at high temperature and chemically inactive to other substances in any circumstances. Inorganic pigments are the only pigments containing metallic chromophores as the elements of the first transition for the colouration of ceramic colourations (Lang, 2009).

Todays, some of the inorganic pigments have been used as the food additives to act as certain roles such as anticaking agent, emulsifier, acidity regulator, and stabilizer and most importantly, provide colour to the foods, such as calcium carbonate, magnesium hydroxide, and magnesium chloride (Delgado-Vargas & Paredes-López, 2003).

Generally, inorganic pigments are heat resistant and stable against light, weathering, chemicals and solvents. Besides, inorganic pigments can be produced at lower cost compared to organic pigments. An additional benefit of using inorganic pigments is they tend to have higher opacity compared to organic pigments as their reflective indexes is higher than that of the organic pigments. However, inorganic pigments show poor colour intensity and brightness compared to organic pigments (Christie, 2001).

Inorganic pigments have dull shades, except Chrome Yellow, Molybdate Red and cadmium-based pigments. The inorganic pigments have limited spectral range, and that results in restricted option of hues. Besides, inorganic pigments often have limitation in the applications. As for example, paint containing Prussian Blue can only be used as the interior house paint instead of exterior paint since Prussian Blue must nor exposed to alkalis to protect the paint's colour and other properties. In addition, the inorganic pigments have poor tinctorial strength and lack in brightness. Therefore, inorganic pigments are restricted to be applied in printing ink (Herbst & Hunger, 2004).

2.3 Classification and molecular affinities of natural pigments

There are not more than six groups of biological pigment structures in the world, which are tetrapyrroles, isoprenoids, quinones, benzopyrans, N- heterocyclic compounds, and metalloproteins.

There are around 28 cyclic and 6 linear tetrapyrroles. Isoprenoids consist of more than 600 carotenoids, and over 4100 flavonoids including 250 anthocyanins in the group benzopyrans. For the group of quinones, the members are widely distributed, such as the wide distributed anthraquinones and naphthaquinones. Further, the compound of N-heterocyclic group, indigoid, one of the oldest known colourants, betalains, purines, pterins, flavins, phenazines and phenoxazines are the major compounds of the N- heterocyclic group. The group metalloproteins is a group consists of a large number of proteins which can be found abundantly and essentially in living organisms. The members of the group include haemoglobin, myoglobin, ceruloplasmin, and haemovanadin (Delgado-Vargas & Paredes-López, 2003).

2.3.1 Tetrapyrroles

Basically, tetrapyrroles can be found easily in living organisms as a lot of organisms have the ability to produce them. Tetrapyrrole derivatives generally have two types of structures, which are linear and cyclic arrangements that have pyrrole ring in both kinds of molecules.

Plant bilins such as phytochrome, phycocyanin and phycoerythrin are one of the most common pigments found in the world. Phytochrome, a linear tetrapyrrole, is an orange-red to far red pigment which was found in the year of 1959. Phytochrome presents in two different stable and inconvertible types, Pr and Pfr. Phycobilins are the deeply coloured pigment-protein complexes which can dissolve in water and some appear to be fluorescent. Phycobilins can be found in red algae, blue-green algae and cryptomonad. Phycocyanin is blue with red fluorescence and phycoerythrin is yellow-orange molecules with bright orange fluorescence.

The most important tetrapyrroles among the tetrapyrrole derivatives are the members of the chlorophyll subgroup. Chlorophylls are common in the chloroplasts of higher plants and most algae. Only chlorophyll a and b can be found in superior plants, ferns, green algae, mosses and prokaryotic organisms, while the other types of chlorophyll can be isolated and detected from other groups such as other algae and bacteria (Delgado-Vargas & Paredes-López, 2003).

2.3.2 Isoprenoids

Isoprenoids are also being recognized as terpenoids which are ubiquitous in nature as they play a lot of important roles and functions as hormones, pigments and phytoalexins. The isoprenoid derivatives can be grouped into three different subgroups which are quinones, carotenoids and iridoids. As not all of the quinones are isoprenoid derivatives, therefore quinones are usually not discussed as the members of isoprenoid. Iridoids are the biosynthetic precursors of alkaloids and they have been only found in the more developed dicotyledoneous plants, and that property makes the iridoids become the taxonomic marker.

Carotenoids are the most important pigments in the group of isoprenoid. Carotenoids are lipids and the members of carotenoids are the most widely distributed compared to all the other classes of natural pigments. Carotenoids are categorized as terpenoids and are produced by the isoprenoid pathway (Delgado-Vargas & Paredes-López, 2003). They can be found throughout plants, algae and sometimes occurred as animal and microbial pigments.

Carotenoids can be extracted from the natural tissues using polar solvents and isolated carotenoids are very sensitive to heat and light. The light-absorption properties of carotenoids enable them to play an important role in photosynthesis, photoprotection, phototropism and photoreception. Other than those roles, carotenoids are commonly used in the food industry as colourants due to their naturally occurrence in many foods. Carotenoids are also needed for the human to prevent or heal the health problems due to the vitamin A deficiency and act as protective agents against certain skin diseases caused by light (Yong, 2012).

Carotenoids usually distributed in plants, animals and microbes. They act as the photosynthetic tissues to assist in the photosynthesis process in higher plants and algae （Teh Faridah, 2012）. The other non-photosynthetic related carotenoids may also found in flowers or fruits that are yellow, orange or red in colour. As for examples, violaxanthin in yellow flowers such as dandelion and daffodil, fruits like tomatoes and apricots contain *β*- carotene and lycopene. In kingdom Animalia, carotenoids such as *β*- carotene, *ϒ*- carotene, canthaxanthin and astaxanthin can be found in vertebrates, birds, fishes, amphibians, reptiles, and invertebrates for the colouration of the outer appearances. In microbes such as fungi (*Blakeslea trispora*, *Cantharellus cibarius* and *Rhodotorula* spp.) and bacteria (*Flavobacterium* sp., *Sarcina* sp. and *Corynebacterium* sp.) contain carotenoids such as *β*- carotene, *ϒ*-carotene or canthaxanthin (Britton, 1983).

2.3.3 Quinones

Quinones are the largest group in number and structural variation of pigments. The pigments range from pale yellow, orange, red, purple and brown to almost black. They can be categorized as benzoquinones, naphthaquinones, anthraquinones, and extended quinones where more complex polymeric quinones are grouped (Delgado-Vargas & Paredes-López, 2003). Almost all quinones are solid and crystallize readily.

The members of quinones can be found readily in higher plants, fungi, bacteria and in animals such as arthropods and echinoderms. Quinones usually occur in higher plants such as bark, heartwood, and roots, but they rarely contribute to the external colour of higher plant tissues. Different quinones have different functions. Some quinone pigments are used as the dyes (Britton, 1983). More and more of the naturally occurring quinones have been reported to possess the antimicrobial activity (Lenta *et al.*, 2007; Mohanlall *et al.*, 2011; Kremer *et al.*, 2012; Kosalec *et al.*, 2013; Wu *et al.*, 2014).

2.3.4 Benzopyrans

The most widespread benzopyrans are flavonoids that can be found abundantly in each part such as flowers, leaves, stems and fruits of the vascular plants. Flavonoids are water-soluble and they are phenolic compounds that are consisted by two aromatic rings and one central pyran ring.

Basically, most of the flavonoids can only be seen under ultraviolet light but some of the members show pale yellow colour and contribute yellow colour to some flowers. The only truly pigment compounds in this group are the anthocyanins which provide wide spectrum of colours, orange to blue colour in flower petals, fruits, leaves and roots. In most of the visible pigmented compounds, the anthocyanidins such as cyanidin (blue-red), delphinidin (Purple-blue) and malvidin (purple) bound to one or more sugars to dictate the colour of the anthocyanidins (Hendry & Houghton, 1996).

In nature, anthocyanins are brightly pigmented to attract other organisms such as insects to help in pollination, seed dispersal, and antifeedant. Anthocyanins have the ability to act as the antioxidant agents. Other than the antioxidative property, anthocyanins are suggested to possess the abilities to help in photosynthesis and provide photoprotection to the host plants. Anthocyanins such as grape extracts are used as the food colouring agents (Delgado-Vargas & Paredes-López, 2003).

2.3.5 N- Heterocyclic compounds

Purines can be detected in all living organisms as they are the essential structural elements of the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Purines only contribute limited range of colours such as white, semi-transparent cream colours and silvery colours in fishes.

Pterins contribute white, cream, yellow, and red colours in insects such as butterflies and moths. Other than the insects, they also exist in vertebrate eyes, human urine, and bacteria such as Lactobacillus casei. Pterins are the cofactors in the synthesis of other pigments, ommochromes. Xanthommatin is the product of the synthesis from tryptophan through kynurenine and it contributes red or brown colour to insect eyes.

Flavins can be found abundantly in microorganisms and plants. The most widespread member of this group is the riboflavin which appears to give yellow colour to the marine invertebrates. Riboflavin is highly water-soluble, imparts bright yellow colour in concentrated solution and the absorbance spectrum is similar to carotenoid (Delgado-Vargas & Paredes-López, 2003).

Phenazines are pigmented compounds that can be detected in bacteria, more specifically, common in certain species of *Pseudomonas* and *Streptomyces*. Phenazines appear in bright yellow, but exceptions include pyocyanins and iodinin which are deep-blue and violet-blue colours respectively. Occasionally, phenazines can be isolated from the infected wounds of mammalians due to the bacterial invasion. Phenazins are relatively stable and have the ability to dissolve in the water.

Phenoxazines are structurally similar to phenazines and they can be found in fungi and insects by imparting some particular colours such as yellow, golden yellow and dark brown. Pink-red phenoxazine, also known as actinomycin, which shows antibiotic activities, can be isolated from *Streptomyces* spp. (Hendry & Houghton, 1996).

2.3.6 Metalloproteins

Metalloproteins are the metal-binding proteins. Generally, the trace metals such as zinc, cobalt, iron, manganese, chromium, nickel and copper can be found in living organisms as the natural constituents of proteins where the metal ions are used in wide variety of biological functions (Wilson *et al.*, 2004).

Many metalloproteins rarely successfully isolated from the living organisms as the quantities may not be sufficient for purification or the compounds do not remain intact during the isolation or purification process, for examples, cytochrome c is bright scarlet in reduced form and Fe-S ferredoxin is yellow in colour before they are isolated and becomes colourless compound.

The more known metalloproteins are metalloporphyrins where porphyrins contacted other metal ions such as iron. The example for metalloporphyrins is iron-binding porphyrins which gives red to purple colour to the compounds, forming haems like red-coloured haemoglobin and myoglobin. Copper metalloproteins usually found in blue to blue-green colour with the exception of bronze-purple-coloured turacin. Vanadium metalloporphyrins is green apple in colour while zinc metalloporphyrins give pink to brown colour to the shell of the birds' eggs (Hendry & Houghton, 1996).

2.4 The need in exploring natural pigment

Synthetic pigments are not accepted by some consumers due to the health safety and that results in the more interests in the exploration of more natural pigments which are more reliable than the synthetic pigments (Sikorski, 2007).

The pigments used in tattoo industry were reported to cause some allergic reactions to human especially red pigments (Zwad *et al.,* 2007). The tattoo artists tend to mix the organic pigments with insoluble metallic elements such as cadmium, mercury, silicon, titanium, aluminium, and iron to get the desired colours. Granulomatous and lichenoid hypersensitivity reactions were caused by the mercury or cadmium incorporated in red tattoo ink and aluminium in permanent eyeliner tattoo. Pseudolymphomatous reaction was only reported to occur at the red coloured tattoo (Ravneet *et al.*, 2009).

Synthetic azo dyes can be created abundantly in low cost. However, the azo dyes are mutagenic and carcinogenic to mammals as some of the toxic aromatic amines produced by the azo compounds during the bioprocess have the ability to react with the DNA-binding intermediates to cause the mutation. Sudan dyes I, II, III, IV, and Para red are azo dyes that have been classified as potential carcinogens and mutagens to mammals (Xu *et al.*, 2010).

Synthetic azo colourants such as Sudan dyes have been reported to be category three of carcinogens which are harmful to human health. The dyes are banned in most countries and their use as food additives are restricted (Pan *et al.*, 2012). However, the dyes are still illegally used in some products such as waxes, fuels, foods, and drinks since they are cheaper and have great intensify power in creating red-orange colours (Chang *et al*., 2011; Sun *et al.*, 2014).

Sunset yellow is a permitted synthetic food colouring at certain dose. However, the study using Sunset yellow on splenocytes suggested that even at the non-cytotoxic dose, Sunset yellow still has the ability to suppress the splenocytes, caused immune suppression on T-cells, B-cells, and some cytokines, thus, expose human body to greater harm (Ashish *et al.*, 2013).

Eigenmann and Haenggeli (2004) suggested that the artificial food colourants have great potential to induce attention-deficit hyperactivity disorder (ADHD) among children. A study regarding the use of artificial colourings among preschool children proved that there was a significant difference in their behaviour. The children showed hyperactive behaviour after artificial colourings intakes, and after the withdrawal of artificial food colourants, there was a great reduction in hyperactivity among the children (Bateman *et al.*, 2004).

The introduction of the synthetic colourant, tartrazine among a group of children who aged from 2 – 14 years old proposed that the intake of tartrazine caused some of the children to experience sleep disturbance, impatient and dysphoric reactions (Rowe & Rowe, 1994). Allura red AC is a dark red, synthetic azo colourant which is water soluble and used in food colourings such as drinks and candies (Wu *et al.*, 2014). As suggested by Mccann *et al.* (2007), Allura red AC caused significant increase in hyperactivity behaviour among two different categories of children.

2.5 Natural distribution of pigments

Generally, natural pigments act as some important roles in particular metabolic or physiological processes. The most well-known examples are the role of chlorophyll in photosynthesis to provide the whole ecosystem with oxygen and harmoglobin as the oxygen carrier in human body. The pigments might provide the colouration to the organism itself as well. Since the prehistoric times, human already collect the pigmented items and crush them to get the colour desired. The sources of the natural pigments are generally from plants, animals, insects and microorganisms (Britton, 1983). Different sources of natural pigments will be further discussed in the following subtopics.

2.5.1 Fungal pigments

More than 1000 pigments have been identified in fungi (Delgado-Vargas & Paredes-López, 2003). The fruiting bodies of the fungi are often brightly pigmented and some of the pigmentations are due to the exposure of the fungal sap to air often causes the pigmented oxidation production. So far, over 1, 000 types of pigment have been described and reported from all classes of fungi including slime moulds (Hendry & Houghton, 1996).

The chlorophyll which is abundant in plants and algae is completely absent in fungi while carotenoids can only be found in four orders of the Phragmobasidiomycetidae and one order of Ascomycetes. Some pigments can be found in only trace amounts such as riboflavin. Riboflavin (vitamin B2) is very important in the electron transport in most organisms and is colourless. However, in the fungal genera Russula and Lyophyllum, riboflavin and its close derivatives are found to be deep yellow colour (Hendry & Houghton, 1996).

The betalains, melanins, and a relatively small number of carotenoids and certain anthraquinones are the only pigments which are common in fungi and plants or animals. Most of the pigments found in fungi have not been found in other organisms. Fungi, especially the more simple single-celled organisms can be cultivated in large scale, and this characteristic offers the great potential for exploration in the field of natural pigments (Hendry & Houghton, 1996).

Terphenylquinone compounds show blue colour n dyeing processes. They can be obtained from *Sarcodon*, *Phellodon*, and *Hydnellum* as well as from *Telephora* spp. Besides, Grevillines together with terphenylquinones are important colourants from *Boletales* sp. Grevillines produce yellowish to reddish hues. The richly coloured appearance of the fruit bodies of *Cortinarius* sp appears in brown, red, olive green, and even violet colour. The main pigment from *Cortinarius* sp is anthraquinone which is one of the most important classes of dyestuffs among commercial colourants (Bechtold & Mussak, 2009).

Anka red pigment is produced by the *Monascus* fungus which known as the *koji* fungus in Japan, “ang-kak” or “red rice mold” in China since 1884 (Attokaran, 2011). The genus *Monascus* produces several natural pigments which range from red, yellow and orange. *Monascus purpureus* is the major red pigment producer. *Monascus purpureus* are involves in the fermentation of red rice to produce red rice wine where the colour of the product is consistent, stable and soluble in water (Lauro & Francis, 2000).

Monascus sp. survives on the carbohydrates that can be found in the substrates the fungus is growing on. In the pigment production, the regulation of oxygen and carbon dioxide results in higher yield of the pigment production (Delgado-Vargas & Paredes-López, 2003). The anka red pigment is currently used as the food colourant in meat processing, food productions, sauces, and ice cream in Asia (Attokaran, 2011).

Other than *Monascus* sp., there are more fungi have been reported to have the ability to produce various colours. As reported by Cho *et al.,* in the year of 2002, *Paecilomyces sinclairii*, a type of edible and high pigment-producing fungus, produced a different colours of water soluble pigment at different pH such as red, violet and pink using submerged fermentation system. The pigmentation of *Penicillium* sp. such as *Penicillium herquei*, *Penicillium purpurogenum*, *Penicillium aculeatum, Penicillium oxalicum, Penicillium pinophilum* and *Penicillium persicinum*, have been reported and studied intensively (Robinson *et al.*, 1992; Gautschi *et al.*, 2004; Mapari *et al.*, 2005; Mapari *et al.*, 2008).

Besides than the fungi, lichens also have the ability to produce pigments. The pigments from lichens have been used as dyes, particularly as textile dyes since ancient times. These fungal-algal and fungal-cyanobacterial associations are usually