BIOACTIVITY DETERMINATION OF *Cassia surattensis* SEED EXTRACT

by

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**Thesis submitted in fulfillment of the requirements
for the degree of
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LIST OF ABBREVIATIONS

DPPH	2,2-diphenyl-1-picrylhydrazyl
C. surattensis	*Cassia surattensis*
g	Gram
nm	nanometer
mm	milimetre
mg/ml	milligram/milliliter
µg/ml	microgram/milliliter
LD_{50}	median lethal dose
BHT	Butylated hydroxytoluene
BHA	Butylated hydroxyanisole
DNA	Deoxyribonucleic acid
NaOH	Natrium hydroxide
EDTA	Ethylenediaminetetraacetic acid
A.niger	*Aspergillus niger*
SEM	Scanning electron microscopy
MIC	Minimum inhibitory concentration
MFC	Minimum fungicidal concentration
STZ	Streptozotocin
HE	Hot water extract
EE	Cold ethanolic extract
CYP450	Cytochrome P450
DILI	Drug induced liver injury
Mrp	Multidrug resistance-associated protein
NAPQI	N-acetyl-p-benzoquinoneimine

GSH	Glutathione
ATP	Adenosine triphosphate
NAC	N-acetylcysteine
CCl_4	Carbon tetrachloride
SOD	Superoxide dismutase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
OBWI	Organ Body Weight Index
FTIR	Fourier transform infrared
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
PBS	Phosphate buffered saline
SNP	Sodium nitroprusside
SD	Standard deviation
TCA	Trichloroacetic acid
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
$O_2^{\bullet-}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical

PENENTUAN BIOAKTIVITI EKSTRAK BIJI *Cassia surattensis*

ABSTRAK

Cassia surattensis adalah tumbuhan berbunga yang telah digunakan secara tradisional sebagai makanan dan untuk tujuan perubatan di banyak negara. Kajian ini bertujuan untuk menentukan profil pencapjarian, aktiviti antioksidan, kesan hepatoprotektif, kesan sitotoksik ke atas sel Vero dan aktiviti genoprotektif menggunakan ujian komet. Keputusan kajian HPTLC menunjukkan biji *C. surattensis* mempunyai 10 puncak berbeza komponen kimia utama yang berbeza dan kajian logam berat menunjukkan biji tersebut bebas daripada tahap pencemaran logam berat yang tinggi. Ekstrak metanol biji *C. surattensis* menunjukkan aktiviti pemerangkapan radikal bebas DPPH dan radikal nitrik oksida yang baik dengan kuasa penurunan. Ekstrak biji *C. surattensis* mempunyai kandungan jumlah fenolik sebanyak 100.99 mg GAE/g berat kering dan terdapat korelasi positif antara kandungan jumlah fenolik dan aktiviti antioksidan ekstrak biji. Rawatan ekstrak biji *C. surattensis* secara signifikan menurunkan paras enzim hati dan berat relatif hati meningkat dalam hepatotoksisiti hati mencit yang diaruh dengan parasetamol. Kajian histopatologi membuktikan keberkesanan hepatoprotektif ekstrak metanol biji *C. surattensis* terhadap hepatotoksisiti hati diaruh oleh parasetamol. Kajian sitotoksisiti secara *in vitro* menunjukkan ekstrak biji *C. surattensis* tidak toksik terhadap sel Vero. Ekstrak biji *C. surattensis* juga menunjukkan keupayaan perencatan yang tinggi terhadap kerosakan oksidatif DNA yang diaruh oleh radikal bebas daripada H_2O_2 di dalam sel Vero dan menunjukkan bahawa ekstrak biji mempunyai kesan antigenotoksik yang efisien. Kesimpulannya, ekstrak biji *C. surattensis* menunjukkan ciri-ciri antioksidan, hepatoprotektif dan genoprotektif

yang baik berkemungkinan disebabkan oleh kehadiran kandungan fenolik dalam biji tumbuhan tersebut.

BIOACTIVITY DETERMINATION OF *Cassia surattensis* SEED EXTRACT

ABSTRACT

Cassia surattensis is a flowering plant that has been traditionally used in many countries for food and medicinal use. This study sought to determine the fingerprint profile, antioxidant activities, hepatoprotective effect, cytotoxicity effect on Vero cells and genoprotective activity using the Comet assay. The result of the HPTLC study revealed that the *C. surattensis* seed has 10 major different chemical component peaks and the heavy metal analysis showed that the seed was safe from high heavy metal contamination. The methanolic seed extract showed good antioxidant activities in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, nitric oxide radical scavenging activity and reducing power. *C. surattensis* seed extract contained total phenolic content of 100.99 mg GAE/g dry weight and there was a positive correlation between total phenolic content and the antioxidant activities of the seed extract. *C. surattensis* seed extract significantly reduced the elevated levels of serum liver enzymes and relative liver weight in paracetamol-induced liver hepatotoxicity mice. The histopathological examination verified the hepatoprotective effect of *C. surattensis* seed extract against the paracetamol-induced liver hepatotoxicity. *In vitro* cytotoxicity test demonstrated that *C. surattensis* seed extract was non cytotoxic against the Vero cells. *C. surattensis* seed extract also exhibited strong inhibitory effects against H_2O_2 - mediated DNA damage in Vero cells demonstrated that seed extract has an efficient antigenotoxic property. In conclusion, *C. surattensis* seed extract possessed antioxidant, hepatoprotective and genoprotective properties, which is probably due to the presence of phenolic content.

CHAPTER 1: INTRODUCTION

There is currently much interest in the therapeutic potential of medicinal plants as antioxidants in reducing free radical-induced tissue injury (Pourmorad *et al.,* 2006; Ashafa *et al.,* 2010). Reactive oxygen species (ROS) comprises both free radical and non-free radical oxygen intermediates such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^{\bullet}$). ROS are formed by the metabolism of oxygen in mitochondria, exogenous sources such as ionizing radiation and ultraviolet (UV) radiation. ROS can damage DNA by causing mutation and chromosomal damage, oxidize cellular thiols and initiate the peroxidation of membrane lipids by eliminating hydrogen atoms from unsaturated fatty acids (Halliwell and Gutteridge, 1985; Yin *et al.,* 2011).

There are frequent articles in newspapers or scientific literature citing the usefulness of the free-radical scavenging abilities of antioxidants and their general benefits to human health. There are a number of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). The use of synthetic antioxidants is being questioned because of their toxicity issues (Valentão *et al.,* 2002). Therefore, attention has been focused on the utilization of natural resources, like plants as natural antioxidants. The well known and traditionally used natural antioxidants from tea, wine, fruits, vegetables, spices and some natural antioxidants (example: rosemary and sage) are already exploited commercially either as antioxidant additives or as nutritional supplements (Schuler, 1990).

The liver is one of the vital organs of the body and the largest internal organ in the body. It is essential in keeping the body functioning properly. The liver has various functions, including the synthesis and breakdown of small and complex molecules that are important for normal vital functions.

Liver damage caused by a chemical agent or known as hepatotoxicity is a significant toxicological problem worldwide proportion and needs immediate attention. Hepatotoxicity is mainly caused by drugs, such as acetaminophen or commonly known as paracetamol. Paracetamol hepatotoxicity is caused by overdoses of the drug that may damage the liver. In the northern region of Malaysia, paracetamol was reported to be the main causative agent of poisoning cases during 3 years period from year 2000 to year 2002 (Fathelrahman *et al.*, 2006).

For hepatologists worldwide, there is no successful treatment to control the progression of the liver damage and the newly developed chemical/synthetic drugs often have side effects. The chemical hepatoprotective agent, such as silymarin and N-acetlycystine are available to protect or reduce liver damage and have side effects as well (Saller *et al.*, 2001; Schmidt and Dalhoff, 2001).

At present, there has been an increase in the interest and growth of traditional medicinal plant to promote liver health and overcome liver damage. There are considerable preclinical and clinical significant evidences of the natural hepatoprotective drugs from plants. Therefore, beneficial researches on suitable hepatoprotective drugs from natural resources are needed to replace chemical drugs.

Single cell gel electrophoresis or known as comet assay is a simple method used to measure DNA damage. This method is also used to evaluate the prevention and repairing ability of a sample on damaged DNA. Physiological processes and exposure to environmental stress can cause DNA damage and this eventually causes many human diseases, such as atherosclerosis, diabetes, neurodegenerative diseases and cancer (Marnett, 2000; Olinski *et al.*, 2002). Thus, there is a great interest to search for a potential genoprotective agent from natural sources.

Medicinal plants are a natural source of antioxidant, hepatoprotective and genoprotective agents. They are known as a good natural antioxidant because of its phytochemical properties such as vitamins (C, E), flavonoids (flavones, isoflavones, flavonones, anthocyanins) and polyphenols (ellagic acid, Tannis, Gallic acid) (Gupta and Sharma, 2006). The phytochemical properties of medicinal plants possess strong antioxidant activities and inhibit the free radical activities as these natural antioxidant molecules are electron rich. Antioxidant donates electrons to free radicals and neutralize them. This helps to protect the cells against the damaging effects of free radicals, which initiate various diseases (Gil *et al.,* 1999; Agbor and Ngogang, 2005). In addition, medicinal plants are an important source of natural antioxidant agents because of the less toxic nature and free from side effects compared to synthetic antioxidant (Latha *et al*., 1999; Agbor and Ngogang, 2005).

The plants are valuable in the treatment of a range of liver diseases and prevent the body from oxidative stress that could be caused by free radicals. The natural antioxidant agent from plants have potential genoprotective effects against DNA damage. Malaysia is rich in medicinal plant diversity in all three levels of biodiversity; species diversity, genetic diversity and habitat diversity (Yoga Latha *et al.,* 2005).

Cassia surattensis is one of the medicinal plants that is rich in medicinal values. The genus *Cassia* is well known for its diverse biological and pharmacological properties comprises about 600 species and is vastly distributed worldwide (Viegas Jr *et. al.,* 2004). The genus *Cassia* has been used as a potential medicinal plant since long ago (Chopra *et al*., 1956; Ayo *et al*., 2007). *C. surattensis* belongs to the family Fabaceae, distributed throughout Malaysia and are widely

grown as ornamental plants in tropical and subtropical areas. This plant species has been traditionally used in many countries as food products and for medicinal uses.

There were no known local uses of *C. surattensis*, the bark and leaves are said to be antiblenorrhagic (Perry, 1980). The decoction of the roots (Burkill, 1935) is commonly used to treat snake bites. The leaves are consumed for cough, sore throat and used for both internal and external cooling medicine. *C. surattensis* flowers and leaves have been studied extensively and the therapeutic properties such as antioxidant (Sangetha *et al.,* 2008), antimicrobial (Sumathy *et al.,* 2013) and antidiabetic (Ramesh Petchi *et al.,* 2012) have been reported. According to Deepak *et al.* (2013), *C. surattensis* seed showed good antioxidant, antifungal and antibacterial activities on bacterial and fungal cultures. A finding by El-Sawi and Sleem (2010), indicated the efficacy of *C. surattensis* leaf extract as hepatoprotective agent in CCl_4 - induced albino rats. However, more scientific studies on the beneficial activities of *C. surattensis* seed as compared to other parts of the plant and other frequently studied *Cassia* plants such as *Cassia fistula* is needed (Lavekar, 2009; Rahmani, 2015) to further verify the biological activities and acceptance of *C. surattensis* seed as a potential medicinal plant.

The complete procedure for discovering and developing a new therapeutic agent is highly expensive and tedious, often stretching over 10 to 15 years period (Nawaka and Ridley, 2003). The identification of the potential toxic compound from a plant is an imperative step in early drug development. The cytotoxicity assay provides information on the toxicity of the plant and to ensure the safety of the natural product for utilization. Previous study conducted by Sumathy *et al.* (2011) on the flower extract of *C. surattensis* using mice oral acute toxicity test showed the nature of the flower extract as nontoxic.

In the present study, the *in vitro* antioxidant activity and *in vivo* hepatoprotective activity of the seed extract of *C. surattensis* plant were carried out. The total phenolic content, fingerprint profiling, cytotoxicity and genoprotective and activities were also determined.

1.1 Objectives

The general objective of the study was to investigate the antioxidant and hepatoprotective activities of the seed of *C. surattensis* methanol extract. The selection of plant material was based on the known use of the plant species as food and herbal medicine. The specific objectives are as follows:

i. To profile methanol extract of the seed of *C. surattensis* by using spectroscopy (FTIR) and chromatography methods (HPTLC, HPLC).
ii. To evaluate *in vitro* antioxidant activity of the seed extract of *C. surattensis.*
iii. To evaluate the *in vivo* hepatoprotective activity of the seed extract of *C. surattensis*.
iv. To determine the cytotoxicity and the genoprotective activities of the seed extract of *C. surattensis*.

CHAPTER 2: LITERATURE REVIEW

2.1 *Cassia surattensis*

2.1.1 General characteristics

Cassia surattensis is a medium to large flowering tree (Figure 2.1). It is a native of India, Southeast Asia and Tropical part of Australia. *C. surattensis* is a fast growing tree with 12-15 feet tall, likes full sun and well-drained soil and bloom profusely during dry season. The leaves are even-pinnate, alternate, dark green, to about 7 inches long and usually have 6-9 opposite pairs of ovate to oblong leaflets. It has masses of bright yellow flower clusters and appears on almost every branch. The pea-like pod about 7 inches long, containing 3 to 5 seeds (Figure 2.2) and green in color initially, turning black once ripe (Hou *et al.*, 1996; Randell and Barlow, 1998; Wagner *et al.,* 2014). *C. surattensis* is commonly planted as a street or ornamental tree for its pretty yellow flowers (Hanelt *et al.*, 2001).

2.1.2 Taxonomy

C. surattensis is also known as *Senna surattensis*, *Cassia glauca* or *Senna glauca.* In Malay language, it is called kembang kuning or gelenggang. Table 2.1 shows the scientific classification of *C. surattensis* plant.

2.1.3 Medicinal Uses

In traditional medicine, medicinal plants are used for therapeutic purposes due to the presence of phytochemical with various medicinal properties. *C. surattensis* is a very popular herb amongst practitioners of traditional medicine, widely used as a decoction or infusion to treat various ailments. In Chinese traditions, leaves are used to treat constipation problem, sore throat and cough by consuming the infusion of boiled leaves orally (Hanelt *et al.,* 2001). *C. surattensis* is also used in folk medicine

as antihyperglycemic (Chopra *et al*., 1956; El-Sawi and Sleem, 2009). Bark and leaves of this plant are believed to be antihemorrhagic. It is also believed that Balinese rub the leaves of *C. surattensis* into both external and internal cooling medicines (Perry, 1980; Sangetha *et al.*, 2008b).

Table 2.1: Scientific classification of *C. surattensis* (Irwin and Barneby, 1982; Zhengyi and Raven, 2010)

CATEGORY	CLASSIFICATION
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Fabales
Family	Fabaceae / Leguminosae
Subfamily	Caesalpinioideae
Tribe	Cassieae
Subtribe	Cassiinae
Genus	*Cassia*
Species	*Cassia surattensis*
Common names	Golden Senna, Scrambled Egg Tree, Glossy Shower, Bush Senna, Scrambled egg tree bush and Scrambled egg tree

Figure 2.1: *Cassia surattensis* tree

Figure 2.2: The *C. surattensis* seeds. Magnification: 20X

2.1.4 Some identified chemical constituents of *C. surattensis*

The widespread use of *C. surattensis* in traditional medicine has stimulated more pharmacological studies over recent years. Phytochemical screening of *C. surattensis* flower has shown the presence of alkaloid, flavonoids, glycoside, tannins, saponins, phytosterols (Ramesh Petchi *et al.,* 2012). In previous studies, two anthraquinones (chrysophanol and physcion) were isolated from ethyl acetate extract of the bark (Tiwari and Misra, 1993). Phytochemical investigation of the pods leads to the isolation and structural elucidation of a new flavonol glycoside, 5,7- dihydroxy- 4'-methoxy-flavonol-3-*O*-β-D-galactopyranoside, along with chrysophanol physcion, kaempferide and quercetin (Rai *et al*., 1997). Luteolin-7-*O*-β-D-glucopyranosyl-(1-4)-*O*-α-L-arabinopyranoside (Salpekar and Khan, 1996), γ-sitosterolin and digitolutein were isolated from the seeds (Khare *et al*., 1994; Mazumder *et al.*, 2009). A new anthraquinone glycoside; 8-hydroxy-6-methoxy-3-methylanthraquinone-1-*O*-α-L-rhamnopyranosyl, 1-6-β-D-glucopyranoside (Rai and Roy, 1991; Gritsanapan and Nualkaew, 2002), as well as, chrysophanol, physcion, stearic acid, β-sitosterol and β-sitosterol- β -D-glucoside (Hemlata and Kalidhar, 1994) were isolated from the stem. A few compounds were isolated from the leaves of *C. surattensis* (El-Sawi, 2010) such as quercetin (Figure 2.3), rutin (Figure 2.4) and quercetin 3-*O*-glucoside 7-*O*-rhamnoside (Figure 2.5). This three compounds have been known for their antioxidant and hepatoprotective activities (Di Carlo *et al.,* 1999). Moreover, a water soluble nonionic biopolymer composed of D-galactose and D-mannose was isolated from the seeds of *C. surattensis* (Mishra *et al.,*1991).

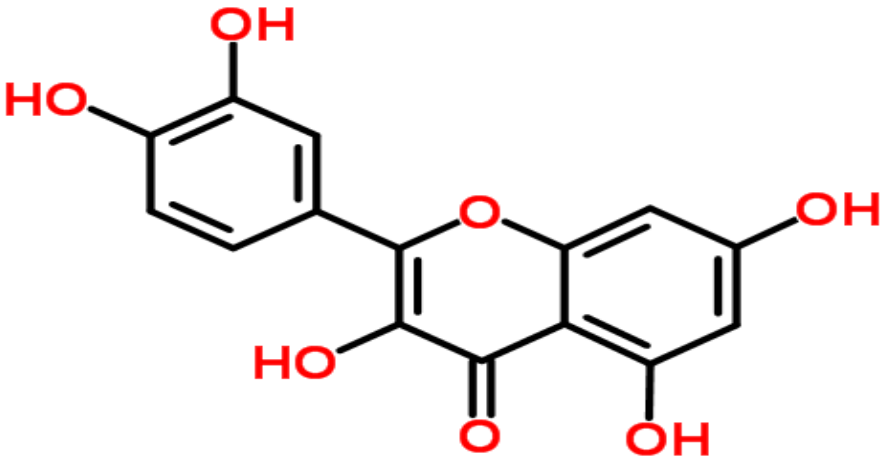

Figure 2.3: Quercetin
Source: *Chemspider*

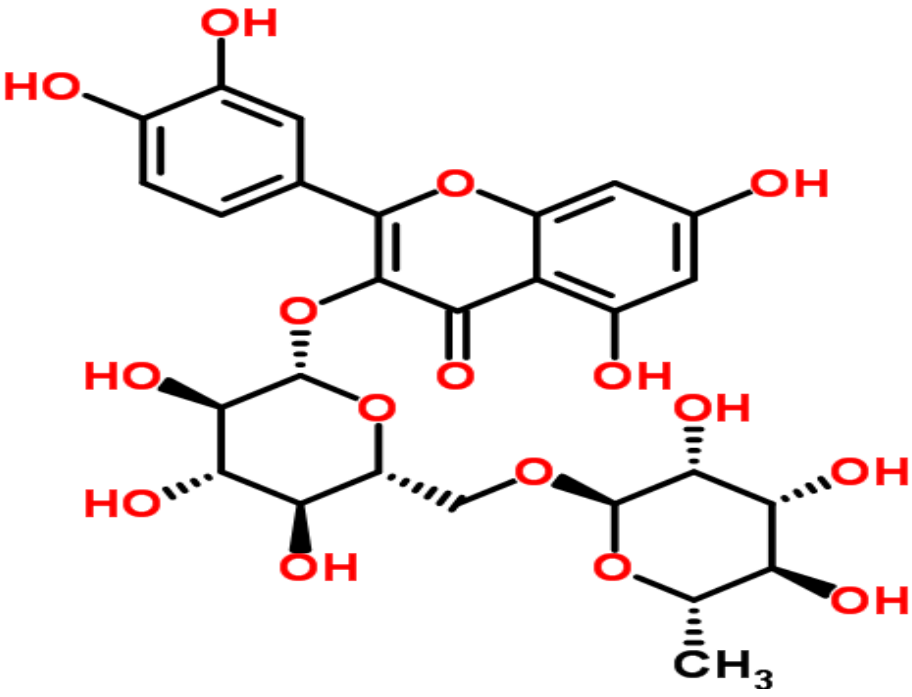

Figure 2.4: Rutin
Source: *Chemspider*

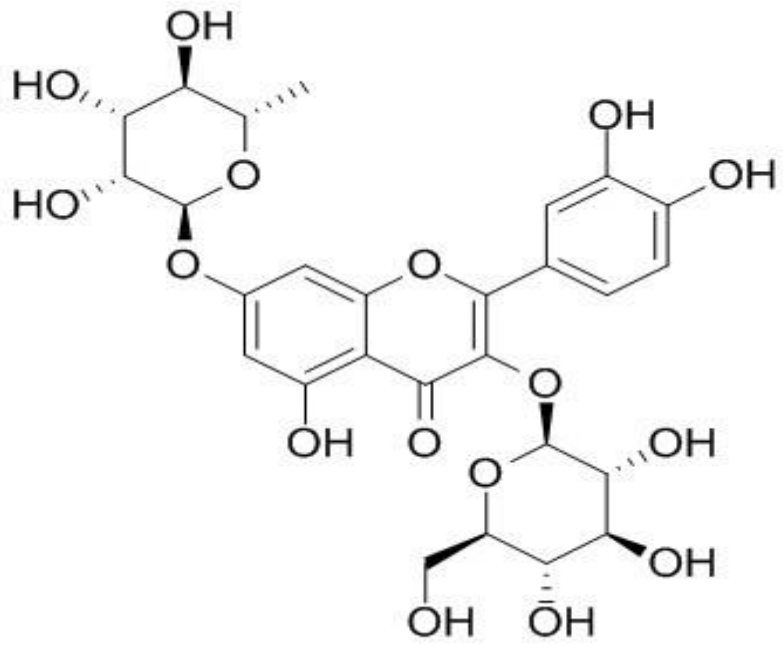

Figure 2.5: Quercetin 3-*O*-glucoside 7-*O*-rhamnoside
Source: *ChemFaces*

2.1.5 Biological activities of *C. surattensis*

2.1.5.1 Antifungal

The methanolic flower extract of *C. surattensis* has been evaluated for their antifungal activity against human pathogenic microorganism *Aspergillus niger* by using the agar disc diffusion method, broth dilution method, the percentage of hyphal growth inhibition and scanning electron microscopy (SEM) observation (Sumathy *et al.,* 2013). The extract exhibited good antifungal activity with zone of inhibition 15 mm and minimum inhibitory concentration (MIC) 6.25 mg/ml. The flower extract of *C. surattensis* exhibited considerable antifungal activity against *A. niger* with an IC_{50} of 2.49 mg/ml on the hyphal growth. Percentage of hyphal growth inhibition of this fungus was determined at concentrations 0.195, 0.39, 0.78,1.562, 3.125, 6.25, 12.5, 25, 50 and 100 mg/ml. For minimum fungicidal concentration (MFC) the flower extract showed only moderate reduction (42%) at 1.562 mg/ml of hyphal growth, while inhibition was substantial at 12.5 mg/ml with 100%. The percentage reduced from 62% at 6.25 mg/ml to 0 at 0.195 mg/ml. In SEM squashed, collapsed, empty and deformation of hyphae were the major changes observed, while shrunken conidiophores were the obvious alteration. Morphological alterations observed on *A. niger* caused by the flower extract could be the contribution of chemical compounds present in the *C. surattensis* flower.

The seed extracts showed antifungal activity against all fungal cultures. The acetone *C. surattensis* seed extract showed inhibition zone 22 mm against *Aspergillus niger,* 27 mm against *Penicillum chrysogenum*, 25 mm against *Sacchomyces cerevesi* and 27 mm against *Candida albicans*. While the methanolic seed extract showed inhibition zone 24 mm against *Aspergillus niger*, 22 mm against

Sacchomyces cerevesi and 23 mm against *Penicillium chrysogenum* and 21 mm against *Candida albicans* (Deepak *et al.,* 2013).

2.1.5.2 Antibacterial

The methanol and acetone extracts of *C. surattensis* seed showed antibacterial activity against several bacterial cultures and the acetone seed extract showed highest antibacterial activity with inhibition zone of 13 mm against *Escherichia coli,* 26 mm against *Klebsiella pneumoniae*, 13 mm against *Bacillus subtilis,* 12 mm against *Salmonella typhi* and 18 mm against *Staphylococcus aureus* (Deepak *et al.,* 2013). On the other hand, methanolic extract of *C. surattensis* leaves showed high activity against three bacterial strains; *Bacillus subitlis, Escherichia coli* and *Staphylococcus aureus* with inhibition zone of 20, 17, 20 mm, respectively and MIC = 8-16 mg/ml (Mortada *et al.,* 2011). In a study by Voon *et al*. (2012), reported that methanol extract of *C. surattensis* flower exhibited good antibacterial and antifungal activities against *Proteus mirabilis, Staphylococcus aureus, Escherichia coli, Salmonella typhi, Micrococcus spp., Enterobacter aerogenes, Bacillus subtilis, S.sonnei, A. lipoferum, Klebsiella pneumoniae, Pseudomona aeruginosa , Candida albicans* and *Aspergillus niger*.

2.1.5.3 Antidiabetic

The antidiabetic activity of *C. surattensis* flower was evaluated using normoglycaemic and streptozotocin (STZ) - induced diabetic rats using oral administration of hot water extract (HE) and cold ethanolic extract (EE). In normoglycaemic rats, both HE and EE significantly lowered the blood glucose level in a dose-dependent manner. In glucose tolerance test, both extracts of *C. surattensis* flowers gave significant effect by lowering the blood glucose level at the end of

60 minutes after glucose loaded and even lower level at the end of 120 minutes and 180 minutes. For HE extract, at the dose of 400 mg/kg body weight (bw), the glucose concentration at 60 minutes was 136.5 ± 3.2 mg/dl, 126.2 ± 4.2 mg/dl at 120 minutes and 118.2 ± 3.4 mg/dl at 180 minutes. For EE extract (dose 400 mg/kg bw), the glucose concentration at 60 minutes was 139.4 ± 3.4 mg/dl, 128.9 ± 2.3 mg/dl at 120 minutes and 119.2 ± 3.3 mg/dl at 180 minutes. The HE and EE of *C. surattensis* flower enhanced glucose utilization, so the blood glucose level was significantly decreased in glucose loaded rats. The efficacy of the antidiabetic activity of both HE and EE extracts was almost comparable to standard drug, Glybenclamide (Ramesh Petchi *et al.,* 2012).

2.1.5.4 Antioxidant

The evaluation of antioxidant activity through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity by Deepak *et al.* (2013), showed that acetone extract of *C. surattensis* seed exhibited maximum antioxidant activity with 76.11% inhibition than other extracts in comparison to standard drug ascorbic acid. A study on antioxidant assessment on *C. surattensis* flowers (Chew *et al.,* 2009) and leaves (Thilagam *et al.,* 2012) proved that they have good antioxidant activities. In another study by Sangetha *et al.* (2008), the antioxidant properties of *C. surattensis* (flowers, leaves, stem and pod) extracts were evaluated through DPPH radical scavenging activity and xanthine oxidase inhibition assay. *C.surattensis* flowers revealed the best antioxidant property, presenting much lower IC_{50} values (423.32 µg/ml for DPPH assay and 11.1 µg/ml for xanthine oxidase assay). Furthermore, the highest antioxidant contents (polyphenols) were found in these extracts (657.2392 + 2.0321 mg GAEs/g extract). The scavenging effects of flowers, stem and leaves extracts on DPPH radicals were excellent ($P < 0.05$), especially in the case of *C. surattensis*

flower (93.54% at 1.0 mg/ml). The inhibition values were also remarkably good for stem (81.97% at 1.0 mg/ml) and leaves (66.28% at 1.0 mg/ml), but *C. surattensis* pod (45.72% at 1.0 mg/ml) revealed a low value of antioxidant activity compared with BHT (66.23% at 1.0 mg/ml). Mortada *et al.* (2011) revealed that among the methanol extracts of the tested *Cassia* species, the total antioxidant activity of *C. surattensis* was the most active (556.65 ± 2.45 mg equivalent to ascorbic acid/g extract). In a previous study, there appeared to be a positive correlation between the total antioxidant capacity of the methanolic extracts of the tested *Cassia* species and their total phenolic contents (El-Hashasa, 2010). It is also reported that the phenolic compounds are responsible for antimicrobial properties and antioxidant activity (Vasudevan, 2009; Panda, 2010).

2.1.6 Toxicological assessment

The cytotoxic activity of methanolic extract of *C. surattensis* flower was evaluated based on primary brine shrimp assay and *in vivo* toxicity study. The extract was found not toxic in brine shrimp assay with lethal concentration 50 (LC_{50}) value of 3.32 mg/ml. In the oral acute toxicity study, mice were administrated orally with a single dose of 5000 mg/ml extract and observed for 14 days for any toxicity sign. Based on the body weight and histopathological examination, the *C. surattensis* flower extract was found to be nontoxic as there were no significant differences in the body weight of the mice. Histopathology analysis on subjected organs did not reveal any pathological condition in the treatment group. Doses of *C. surattensis* flower up to 5000 mg/kg bw appear to be safe in a mouse model. In short, the methanolic flower extract of *C. surattensis* did not show any toxicity signs based on the brine shrimp assay and oral acute toxicity findings (Sumathy *et al.*, 2011). In another study, the toxicity of *C. surattensis* flower was tested using chronic

administration of hot water extract (HE) and cold ethanolic extract (EE). Both extracts was found to be non-toxic and well tolerated, as there are no overt signs of toxicity, hepatotoxicity or renotoxicity following chronic oral administration (Ramesh Petchi *et al.,* 2012). The leaf extract of *C. surattensis* seems to have no toxicity since the value of LD_{50} (8.6 gm/kg bw) was up to 80 times of effective dose (El-Sawi and Sleem, 2009).

2.2 Free radicals

Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons in its outer shell and can be formed when oxygen interacts with certain molecules. They are highly reactive molecules capable of independent existence (Halliwell and Gutteridge, 2007). Free radicals are derived either from normal essential metabolic processes in the body or from external sources such as exposure to X-rays, cigarette smoking, air pollutants and industrial chemicals.

Bi-radical nature of oxygen readily accepts unpaired electrons to form a series of partially reduced species collectively known as ROS including $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, peroxy (ROO), alkoxy (RO) and nitric oxide (NO). Generation of ROS is an integral feature of normal cellular function like mitochondrial respiratory chain, phagocytosis, ovulation and fertilization (Valko *et al.,* 2004; Genestra, 2007). Their production, however multiplies several folds during pathological conditions. The superoxide radicals generated by the mitochondrial electron transport chain participate in several reactions yielding various ROS such as H_2O_2 and $OH^{\bullet}$ (Valko *et al.,* 2007). These ROS are capable of damaging biologically relevant molecules in cells and tissues such as DNA, proteins, carbohydrates and lipids that lead to cell damage and homeostatic disruption (Young and Woodside, 2001). They induce undesirable oxidation, causing membrane damage, protein modification, DNA

damage, and cell death induced by DNA fragmentation and lipid peroxidation (Frei, 1997; Willcox *et al.,* 2004; Pacher *et al.,* 2007).

This oxidative damage, associated with ROS is believed to be involved not only in the toxicity of xenobiotics but also in the pathophysiological role in the aging of skin and several diseases like heart disease (atherosclerosis), cataract, cognitive dysfunction, cancer, critical illness such as sepsis and acute respiratory distress syndrome, chronic inflammatory diseases of the gastrointestinal tract, organ dysfunction and disseminated intravascular coagulation.

2.3 Antioxidant

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants scavenge free radicals which delay or inhibit cellular damage (Halliwell, 1995; Kensler, 2007). These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction, to protect the cells against free radicals toxic effects (Jeong, 2006).

2.3.1 Mechanism of antioxidant action

Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor and metal-chelating agents (Krinsky, 1992). The antioxidant can function in one of the two principles mechanisms of action (Rice-Evans and Diplock, 1993). The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS initiators by inhibiting chain-initiating catalyst. For the first mechanism, when a radical release or steals an electron, a second radical is formed. The last one exerts the same action on

another molecule and continues until either the free radical formed is stabilized by a chain-breaking antioxidant such as vitamin C and vitamin E, or it simply disintegrates into an inoffensive product. For the second mechanism, an antioxidant enzyme like superoxide dismutase and catalase can prevent oxidation by reducing the rate of chain initiation, for example by scavenging initiating free radicals (Young and Woodside, 2001).

2.3.2 Antioxidant classification

The major antioxidant enzymes directly involved in direct elimination of ROS are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Pacher *et al.*, 2007; Valko *et al.*, 2007). The non-enzymatic antioxidants that belong to endogenous antioxidants such as glutathione, coenzyme Q10, melatonin, uric acid and bilirubin are produced during normal metabolism in the body (Droge, 2002; Willcox *et al.*, 2004). Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E, vitamin C and B-carotene (Levine *et al.*, 1991). The body cannot produce these micronutrients and must be supplied in the diet or supplements. Phytonutrients are being increasingly appreciated for their antioxidant activity, one example is phenolic compounds (Gale, 2001).

2.3.3 Phenolic compounds

Phenolic compounds are a large and diverse group of secondary plant metabolites that are widespread in the plant kingdom (Strube *et al.*, 1993). Phenolic compounds are synthesized in plants partly as a response to ecological and physiological pressures such as pathogen attack, UV radiation and wounding (Zulak *et al.*, 2006; Diaz *et al.*, 2010; Kennedy and Wightman, 2011). The basic structural feature of

phenolic compounds is an aromatic ring bearing one or more hydroxyl groups (Chirinos *et al.,* 2009). This compound has a wide range of structures such as flavonoids, tannins and phenolic acids (Figure 2.6). Flavonoids are most common phenolics and often responsible alongside the carotenoids and chlorophylls for their blue, purple, yellow, orange and red colors. The flavonoid family includes flavones, flavonols, iso-flavonols, anthocyanins, anthocyanidins and catechins (Rong, 2010; Ferreira and Pinho, 2012).

"Tannins" is a general term used for phenolic substances and can be divided into proanthocyanidins, galloyl and hexahydroxydiphenoyl esters and their derivatives, gallotannins and ellagitannins (Haslam, 1998). While phenolic acids are one of the main phenolic classes and occurs in the form of esters, glycosides or amides. Phenolic acids have two essential groups hydroxycinnamic and hydroxybenzoic acid. Hydroxycinnamic acid derivatives include ferulic acid, caffeic acid and *p*-coumaric acids, while hydroxybenzoic acid derivatives consist of gallic acid, vanillic acid and syringic acid. Phenolics have strong *in vitro* and *in vivo* antioxidant activities, which are associated with their ability to scavenge free radicals, break radical chain reactions, and chelate metals (Niki, 2010; Yang *et al.,* 2010; Chiang *et al.,* 2013). This property enables this compound to prevent heart disease (Hoye, *et al.,* 2008; Jin and Mumper, 2010) reduces inflammation (Zhang *et al.,* 2011; Mohanlal *et al.,* 2012) and cancer (Ramos, 2008; Sawadogo *et al.,* 2012)

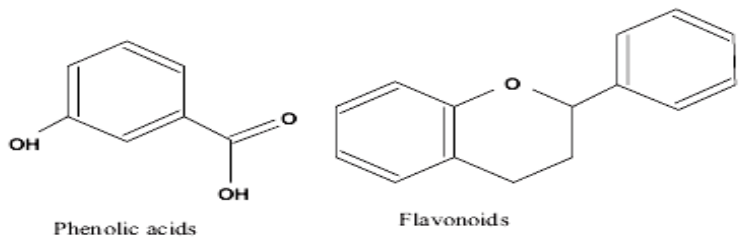

Figure 2.6: Basic structures of phenolic acids and flavonoids.
Source: InTech (2014)

2.3.4 Evaluation of antioxidant activity

There are many *in vitro* methods that have been developed to evaluate the antioxidant capacities of natural antioxidants either as pure compounds or as plant extracts. The *in vitro* methods are divided into two main groups based on the reactions; i) Hydrogen atom transfer reactions like Oxygen Radical Absorbance Capacity (ORAC), Total radical trapping antioxidant potential (TRAP) and β carotene bleaching; and ii) Electron transfer reactions like DPPH radical scavenging assay, superoxide anion radical scavenging assay, hydroxyl radical scavenging assay, nitric oxide radical scavenging assay and total phenolic content assay (Niki, 1993; Rice-Evans and Diplock,1993). These methods are popular due to their high speed and sensitivity. However, it is important to use more than one method to evaluate the antioxidant capacity of plant materials because of the complex nature of phytochemicals (Chanda and Dave, 2009).

2.4 Liver

2.4.1 Functions of the Liver

The liver is the largest metabolic organ of the body and is positioned beneath the diaphragm in the right hypochondrium of the abdominal cavity (Ellis, 2011). The liver has a wide range of functions in the body, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions. It is the major drug-metabolizing and drug-detoxifying organ of the body. It is a unique organ where it can regenerate the significant loss of liver cells due to drug toxicity or other damages (Mehendale, 2005). The liver helps in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction. In addition, it aids metabolism of

carbohydrate, protein and fat, secretion of bile and storage of vitamins (Ahsan *et al.*, 2009). The liver performs a variety of important host defense and metabolic functions that include synthesis of acute-phase proteins, gluconeogenesis, detoxification, and clearance of endogenous mediators, as well as secretion of pro-inflammatory cytokines (Pastor *et al.*, 1995).

2.4.2 Drug Metabolism in liver

The liver is the primary site for metabolism of almost all drugs because it is relatively rich in a large variety of metabolizing enzymes. Drug metabolism is a process where drug molecules are chemically altered, usually to more polar metabolites that exhibit increased water solubility to allow excretion in urine or bile. Drug metabolism is likely to be a byproduct of metabolic pathways that metabolize endogenously synthesized compounds (endobiotics) such as steroids, sterols, bile acids and eicosanoids (Nebert and Russell, 2002).

Drug metabolism is often divided into two phases of biochemical reaction - phase 1 and phase 2. Some drugs may undergo only phase 1 or only phase 2 metabolism, but more often, the drug will undergo phase 1 and then phase 2 sequentially. In phase 1 reaction, the enzymes carry out oxidation, reduction, or hydrolytic reactions. This prepares the drug for phase 2 in which enzymes form a conjugate of the substrate (the phase 1 product). These processes tend to increase water solubility of the drug and this facilitates excretion as well as decreasing pharmacological activity. Most of phase 2 reactions occur in the hepatocyte cytoplasm and involve conjugation with endogenous substance (example glucuronic acid, sulfate, glycine) via transferase enzymes. Chemically active products from phase 1 are made relatively inert and suitable for elimination by the phase 2 step

(Liston *et al.*, 2001). The most important family of metabolizing enzymes in the liver is cytochrome P450.

2.4.2.1 Role of cytochrome P450 enzymes

The predominant catalysts of phase I metabolism in the liver is cytochrome P450 (CYP450), a microsomal superfamily of isoenzymes that catalyzes the oxidation of many drugs. Cytochrome P450 isoenzymes are a group of heme-containing enzymes primarily located in the lipid bilayer of the smooth endoplasmic reticulum (ER) of hepatocytes ranging from bacteria to humans (Nelson *et al.*, 1996; Denisov *et al.*, 2012).

CYP450 plays an important role in oxidative metabolism of many exogenous compounds from a variety of drug classes (example, thiazolidinediones and meglitinides), environmental chemicals and pollutants, and natural plant products. CYP450 acts as a catalyst in oxidative reactions in organs such as the liver, intestine, kidney, lung, and brain (Jefferson, 1998; Schoch *et al.*, 2008). They develop gradually to allow organisms to metabolize foreign chemicals (Nemeroff *et al.*, 1996; Jefferson, 1998). A major function of CYP450 is to metabolize lipid soluble chemicals into water-soluble compounds for excretion in bile or urine. Cytochrome P450 enzymes are essential for the production of cholesterol, bile-acid biosynthesis, steroids, vitamin D3 synthesis and metabolism, retinoic acid hydroxylation prostacyclins, and thromboxane A_2 (Nebert and Russell, 2002; Lynch and Price, 2007). There are more than 50 CYP450 enzymes, but these enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 metabolize 90 percent of drugs (Slaughter, 1995; Wilkinson, 2005). Cytochrome P450 enzymes can be inhibited or induced by drugs, causing drug-drug interactions. This can lead to unexpected adverse reactions or therapeutic failures. Cytochrome P450 enzymes

often interact with warfarin, antidepressants and antiepileptic drugs (Lynch and Price, 2007)

2.5 Hepatotoxicity

The liver is an important target organ of the toxic drugs, xenobiotics and oxidative stress because of its unique metabolism and relationship to gastrointestinal tract. Hepatotoxicity is defined as hepatic toxicity or damage to the liver cells caused by exposure to a drug or other chemical agents called hepatotoxins. This results the liver to function irregularly (Vichitra *et al.,* 2013). Drug induced liver injury (DILI) is one of the most frequent cause of liver injury that poses a major clinical problem and challenge to drug regulators (Russman *et al.,* 2009). Drug induced liver injury makes up a total of 5% of all hospital admissions and 50% of all acute liver failures (McNally, 2006). The idiosyncratic drug reactions with more than 75 percent of cases cause liver transplantation or death (Ostapowicz *et al.,* 2002). One of the most frequent reason a drug withdrawn from the market because of its hepatic adverse effect and it is estimated more than 900 drugs cause the liver damage (Rajamani *et al.,* 2010).

2.6 Mechanism of liver injury

There are a number of mechanisms that initiate hepatic injury or aggravate the ongoing injury processes. Many chemicals damage the prominent intracellular organelle mitochondria. The impaired organelle releases excessive amounts of ROS that leads to hepatic cell injury. Liver injury also significantly aggravated by the accumulation of inflammatory cells such as neutrophils and activation of sinusoidal lining cells, especially Kupffer cells (Jaeschke, 2000). Activated Kupffer cells release ROS, cytokines and chemokines, which promote oxidative stress in injuries

caused by toxicants. The activation of some enzymes in the cytochrome P-450 system such as CYP2E1 involves production of injurious metabolites that promote oxidative stress (Jaeschke *et al.,* 2002). Injury to hepatocyte and bile duct cells lead to accumulation of bile acid in the liver and this promotes further liver damage or injury (Patel *et al.,* 1998; Davit-Spraul *et al.,* 2009).

2.7 Types of Hepatotoxicity

2.7.1 Acetaminophen - induced hepatotoxicity

Acetaminophen, also known as paracetamol or N-acetyl-p-aminophenol (APAP). This drug is most commonly used as analgesics to reduce fever and mild to moderate pain. Acetaminophen induced hepatotoxicity has been linked with a number of cirrhosis, hepatitis and suicide attempts cases. Acetaminophen if taken in overdose, can cause severe hepatotoxicity that leads to liver failure and nephrotoxicity depletion (Masubuchi *et al.,* 2005). In therapeutic dose, acetaminophen is converted by drug metabolizing enzymes to water-soluble metabolites and eliminated in the urine (Ramachandran and Kakar, 2009; Hinson *et al.,* 2010)

Paracetamol induced liver injury is a classic case of drug - induced liver injury (DILI-1). This case can be tested in more than one strain of rodents (Mehendale, 2005) and show a significant dose-dependency in both animals and humans. Oxidative stress plays a significant role in the hepatotoxicity caused by paracetamol and research have been done on antioxidants as an alternative treatment against paracetamol toxicity (Avila *et al.,* 2011)**.**

Figure 2.7 shows that acetaminophen is directly conjugated with glucuronic acid or sulfate by glucuronyl transferases or sulfonyltransferases. The conjugated compound is eliminated into the bile by multidrug resistance-associated protein (Mrp2) or into the blood by Mrp3 (Chen *et al.,* 2003). The remaining unconjugated