MOLECULAR TOXICO-PATHOLOGY RESPONSES OF *EUDRILUS EUGENIAE* TO 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-b]PYRIDINE (PhIP) AND 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-f]QUINOXALINE (MeIQx)

by

BALOGUN WASIU GBOLAHAN

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

January 2019

ACKNOWLEDGEMENT

My sincere appreciation to the Almighty Allaah that made this project a reality and has kept me alive until this moment. I am grateful for all His mercies for me and my family. My sincere appreciation goes to my mentor and supervisor Associate Professor Md Azman Seeni Mohamed for giving me the opportunity to be involved in this work, fatherly support and guidance. I am also grateful to my co-supervisors Dr Hasnuri Mat Hassan and Dr Rabiatul Basria for helping at several times.

Special thanks to my parents for their enormous contribution; I am grateful for your support financially and spiritually. I also want to extend my appreciation to the lecturers at the Integrative Medicine Cluster and the entire staff of IPPT for helping me out, thank you for tolerating me. I must acknowledge the help of the staffs at the electron microscopy unit of School of Biological Sciences, Proteomics unit of Malaysian Genome Institute and animal research section of IPPT. My appreciation goes to Associate Professors Bakiah and Badrul Hisham for helping me during my tough period.

I am indebted to my friends and colleagues (Drs Ismail, Mogana Das, Nazilah, Hafiz, Mowaffaq, Annan, Moayad, Shuhaida, Egi, Yasser and others too numerous to mention) for their assistance during this programme. I wish to appreciate TWAS and USM for providing the scholarship which I used in sustaining myself. I also like to thank the grant awarding bodies who made it possible to carry out the work.

Lastly, I appreciate my wife Abdulwaheed Aishat and my kids Abdullah and Abdurrahman for their love and support at all time. You are my partner in progress and may Allaah keep us all in His care. I appreciate everybody for your contributions in all aspects of this endeavour.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CAT	Catalase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E. eugeniae	*Eudrilus eugeniae*
E. fetida	*Eisenia fetida*
GST	Glutathione-S-Transferase
H_2DCFDA	2-,7- dichlorodihydrofluorescein diacetate
HCAs	Heterocyclic Amines
IARC	International Agency for Research on Cancer
IPA	Ingenuity Pathway Analysis
IPKB	Ingenuity Pathway Knowledge Base
ISO	International Organization for Standardization
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
MeIQx	2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffered Solution
PhIP	2-Amino-1-methyl-6-phenylimiazo[4,5-b]pyridine
RIPA	Radioimmunoprecipitation Assay
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
SD	Standard Deviation

SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
TD_{50}	Median Toxic Dose
TEM	Transmission Electron Microscopy

LIST OF UNITS AND SYMBOLS

cm	Centimetre
°C	Degree Celsius
%	Percentage
mL	Millilitre
µl	Microlitre
mM	Millimolar
p	Statistical p-value
µg	Microgram
>	Greater than
≥	Greater or equals to
v/v	Volume per volume
w/v	Weight per volume
mg/mL	Milligram per millimetre

TINDAK BALAS TOKSIKOPATOLOGI MOLEKULAR OLEH *EUDRILUS EUGANIAE* TERHADAP 2-AMINO-1-METIL-6-FENILIMIDAZO[4,5-b]PYRIDINE (PhIP) DAN 2-AMINO-3,8-DIMETILIMIDAZO[4,5-f]QUINOSALIN (MeIQx)

ABSTRAK

Risiko karsinogenik pada makanan berprotin yang dimasak pada suhu tinggi adalah sangat berkait rapat dengan pembentukan heterosiklik amina (HCAs), bukti kehadirannya yang jelas dalam hidupan. Cacing tanah *Eudrilus eugeniae* (*E. eugeniae)* merupakan sejenis haiwan dalam tanah, terdedah dengan toksik daripada pencemaran alam sekitar, dan ianya satu model yang sesuai untuk memahami mekanisme agen toksik seperti HCAs. Oleh itu, kajian ini menjelaskan keupayaan tindakbalas *E. eugeniae* terhadap ketoksikan yang diinduksi oleh HCAs melalui kaedah OECD TG207 yang diubahsuai. Kumpulan *E. eugeniae* yang dibahagikan secara rawak masing-masing didedahkan kepada HCA (200, 400 and 600 mg/kg), MeIQx (300, 600 dan 900 mg/kg), pelarut DMSO dan air suling selama 28 hari berturut-turut. Di penghujung masa pendedahan, usus kecil dipotong, disimpan di dalam peti penyejuk beku bersuhu –80 □ bagi tujuan ujian molekul sementara itu yang lain masing-masing di simpan dalam cecair 10 % salin formal dan cecair Karnovsky bagi teknik mikroskop cahaya dan elektron. Pendedahan berterusan yang kronik terhadap PhIP dan MeIQx menyebabkan penghasilan spesis oksigen reaktif yang berlebihan, meningkatkan aktiviti glutation-S-transferase dan fosfatase alkali, dan pengurangan aktiviti katalase. Satu kajian fungsi toksiko-proteomik mendedahkan lebih lanjut bukti kerosakan oksidatif, gangguan dalam metabolisma tenaga dan kerosakan sel dalam usus kecil *E. eugeniae* yang terdedah kepada HCAs. Kemusnahan molekul berikutan pendedahan

HCAs adalah bergantung kepada kepekatan tahap keterukan toksisiti pada usus kecil, bukti kemusnahan pada kutikel, lapisan epitelium usus kecil, tisu kloragogenus, keleraian pada lapisan otot dan pembentukan ruang antara lapisan dalam dinding usus kecil. Kerosakan yang berterusan ini juga dipastikan dengan kerosakan pada setae, penyusutan kepadatan fiber otot, dan peralihan klorogosom kepada debris vesikel, pemerhatian melalui miroksop elektron imbasan dan transmisi. Hasil kajian ini mendedahkan secara meluas bahawa tahap keterukan toksisiti pada usus kecil berikutan pendedahan separa akut kepada HCAs adalah berpunca daripada kombinasi kerosakan oksidatif, tindakbalas keradangan, ketidakfungsian tenaga dan kerosakan seakan apoptosis pada tisu usus kecil *E. eugeniae* yang terdedah. Keunikan hasil kerja ini terkandung dalam penemuan berikut: kerosakan oksidatif dan ketidakaturan tenaga adalah pusat ketoksikan HCAs, kalmodulin dan kalretikulin adalah bio penanda tidak invasif yang penting dalam ketoksikan HCAs dan tahap keterukan ketoksikan HCAs adalah bergantung kepada tempoh pendedahan dan kepekatan. Akhir sekali, kajian ini telah membangunkan *E. eugeniae* sebagai satu piawai model *in vivo* bagi kajian ketoksikan HCAs.

MOLECULAR TOXICO-PATHOLOGY RESPONSES OF *EUDRILUS EUGENIAE* TO 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-b]PYRIDINE (PhIP) AND 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-f]QUINOXALINE (MeIQx)

ABSTRACT

The carcinogenic risks of highly heated proteinaceous foods are at large associated with the formation of heterocyclic amines (HCAs), evidence in their substantial presence in the biota. The earthworm *Eudrilus eugeniae* (*E. eugeniae*) is a terrestrial ubiquitous animal, susceptible to toxicity from environmental contamination, and a suitable model for understanding the mechanism of toxic agents such as HCAs. Thus, this study elucidated the vulnerability of *E. eugeniae* to HCAs induced toxicity using a modified OECD TG207 approach. Randomised groups of *E. eugeniae* were exposed to PhIP (200, 400 and 600 mg/kg), MeIQx (300, 600 and 900 mg/kg), solvent DMSO and distilled water respectively for 28 consecutive days. At the end of the exposure, the intestines were excised, stored in –80 °C freezer for molecular examination while others were prefixed in 10% formal saline and Kanovsky fluid for light and electron microscopy techniques respectively. Sub-acute exposure to either of PhIP and MeIQx resulted in overproduction of reactive oxygen species, increased glutathione-S-transferase and alkaline phosphatase activities, and a depletion in the catalase activity. A functional toxico-proteomics study further revealed evidence of oxidative damage, disturbance in energy metabolism and cellular damages in the intestine of the *E. eugeniae* exposed to HCAs. Complementing the molecular disruptions following HCAs exposures is the dose-dependent severity intestinal toxicity, evidenced in the destruction of the cuticle, intestinal epithelium, chloragogenous tissues, disintegration of the muscular layer and the interlayer space

formation in the intestinal wall. These progressive damages were also confirmed with the damage to the setae, loss of compactness of the muscle fibres and transition of the chloragosomes to debritic vesicles, observed with the scanning and transmission electron microscopy respectively. The results from this study extensively show that the severity of the intestinal toxicity following sub-acute exposure to HCAs is progressive due to the combined oxidative damages, inflammatory responses, energy dysfunctions and apoptotic-like damages to the intestinal tissues of the exposed *E. eugeniae.* The novelties of this work include in the concluding that the oxidative damage and energy dysregulation are central to HCAs toxicity, calmodulin and calreticulin are vital non-invasive biomarkers in HCAs toxicity and that the severity of HCAs toxicity depends on the exposure period and concentration. This study has established *E. eugeniae* as a standard *in vivo* model for studying HCAs toxicity.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Heterocyclic amines are a group of toxic compounds found mostly in fried meats and fish (Sugimura *et al.,* 2004; Fede *et al.,* 2009). They were initially thought to be limited only to fried foods, but further efforts have shown their occurrence in other sources such as indoor and outdoor air, diesel exhaust particles, cigarette smoke, cooking fumes, rain water, incineration-ash and soil (Liu *et al.,* 2013). They have also been detected in biological samples such as urine, plasma, bile and faeces (Teunissen *et al.,* 2010). Heterocyclic amines usually find their way into the environment especially in the soil through domestic waste, industrial waste and rainwater (Warnert, 2013). Popular member of this group include 2-Amino-3methylimidazo[4,5-f]quinoline (IQ), 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), and 2-Amino-1-methyl-6-phenylimiazo[4,5-b]pyridine (PhIP) (Gibis, 2016). PhIP was detected in an incineration-ash collected from garbage-burning plants in a metropolitan city. The toxic effects of heterocyclic amines can affect several organs of the body. Using rodent as a model, organs such as liver, oral cavity, mammary gland, small and large intestines, Zymbal gland, clitoral gland and skin have been reported as target organs for heterocyclic amines toxicity (Dumont *et al.,* 2010).

Invertebrate animals such as *enchytraeidae* and earthworms are the major residents of the soil; they represent 60 – 80 % of the total soil biomass (Blouin et al., 2013). They are either saprovores (ingesting mineral particles from organic debris) or microbivores (decaying bacteria and fungi) (Blouin et al., 2013). Earthworms

significant presence in the soil expose them to many substances released from industrial and domestic wastes which could be carcinogenic, hence, their suitability for testing the effects of chemicals on the soil (Sanchez-Hernandez, 2006). The biodegradability of earthworm varies, it can completely degrade compounds such as polycyclic aromatic hydrocarbons but cannot degrade heavy metals rather it bioaccumulates them (Natal□da□Luz et al., 2012). According to the Organisation for Economic Co-operation and Development (OECD) TG 207 (1984) and International Organization for Standardization (ISO) Standard Number 11268-1 (2008), *Eisenia fetida andrei* are the most common species of earthworm used for acute and chronic toxicity assays of industrial chemicals and for the determination of effects of pollutants on reproduction. In Malaysia and neighbouring countries, the earthworm species *Eudrilus eugeniae* are the most common species found in the pasture (Blakemore, 2015). They are used for vermicomposting in the agrarian communities. Several studies have also used the earthworms to study the toxicological effects of chemicals on the soil (Sharma and Satyanarayan, 2011; Vijaya *et al.,* 2012; Fernando *et al.,* 2015).

Despite empirical evidence showing the ability of earthworms to survive toxic assaults in soil from toxic agents, there is little information on the way and manner the earthworms survive the toxic assault. In specific, there is limited information on the effects of PhIP and MeIQx potent food carcinogens on the earthworm, an organism with a high biodegradability. In the study, *Eudrilus eugeniae* was used as an *in vivo* model to study the toxic effect of the MeIQx and PhIP on the living component of the soil using different concentrations and understanding the molecular mechanism behind its toxicity.

1.2 Objectives of the Study

The principal aim of the study was to evaluate and analyse the toxico-pathological responses of the intestine of the earthworms following exposure to the food carcinogens (PhIP and MeIQx) and to elucidate the mechanisms of the pathological responses using the light and electron microscopy, biochemical and molecular techniques.

The specific objectives of the research work are:

1. To assess the toxicity potentials of PhIP and MeIQx to *Eudrilus eugeniae* using the contact and soil exposure tests
2. To evaluate the toxico-histopathological effects of different concentrations of PhIP and MeIQx on the intestine and body wall of *Eudrilus eugeniae*
3. To evaluate the toxic effects of the PhIP and MeIQx on the biochemical markers of oxidative stress and alkaline phosphatase
4. To evaluate the ultrastructural changes induced by PhIP and MeIQx in the digestive system of *Eudrilus eugeniae*
5. To evaluate the apoptosis response of the intestine of the *Eudrilus eugeniae* after exposure to PhIP and MeIQx
6. To identify potential biomarkers for investigating the toxic effects of PhIP on the intestine of *Eudrilus eugeniae* using a functional proteomic approach

1.3 Significance of the Study

The main significance of the study was to add to the scientific understanding of the effects of toxic chemicals (especially food carcinogens) on organisms using earthworms as a model. The study would also help in the profiling of the proteins that

can be used as potential biomarkers for diagnosing heterocyclic amine toxicity in human.

1.4 Problem Statement and Hypothesis

There is a growing concern about the levels of toxic agents and the risks associated with them. Consequently, understanding the mechanism of toxicity and identification of potential biomarkers for the measurement of the effects has become an important tool for the assessment. However, there is no information on the response of the earthworm to heterocyclic amines, and the mechanism of toxicity of heterocyclic amines on the earthworms.

Hence, to answer the questions raised from the objectives of this thesis, several experiments were conducted using behavioural, histological, electron microscopy (scanning and transmission), biochemical and molecular techniques.

Finally, the study hypothesized that heterocyclic amines are toxic to the intestine of the earthworms and cause damages to the intestine of the earthworms.

1.5 Flowchart

The flowchart of the study is shown in figure 1.1.

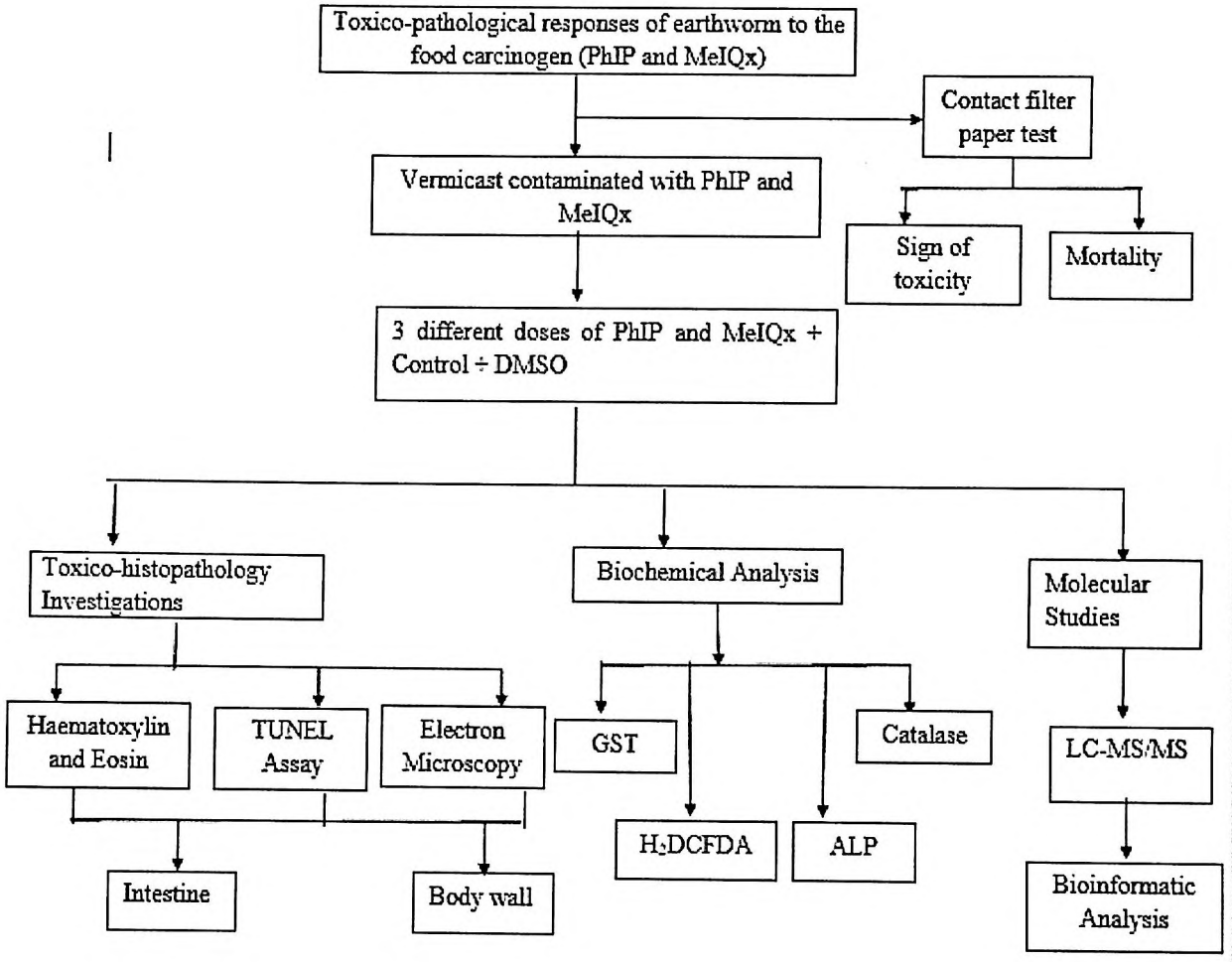


Figure 1.1: Schematic illustration for the design of experiments

CHAPTER 2

LITERATURE REVIEW

2.1 Molecular Toxicology of Carcinogens

Toxic agents are agents that causes adverse effects on the biological system. Exposure to these agents can cause genotoxicity, immunotoxicity, cytotoxicity, carcinogenicity, tissue damage, reduced growth and development, through different cellular pathways (Carpentar and Bushkin-Bedient 2013). These agents could be in chemical, biological or physical in form (IARC, 2017). These agents are identified through epidemiological studies and experimental studies using primate and non-primate animals (Takayama *et al.,* 2008). The agent exposed to, the dosage, the duration and the organs affected, and other factors, all contribute to determining the pathway of cellular responses leading to toxic effects in the cells and ultimately to carcinogenesis (Carpentar and Bushkin-Bedient, 2013). The International Agency for Research on Cancer (IARC) which published authoritative reports on carcinogenic agents based on studies by authorities in the field of cancer biology have identified 1012 toxic agents that can cause cancer in human. Table 2.1 showed the toxic agents as classified by the IARC monographs. The group 1 includes Aflatoxins and benzene, group 2A include IQ and DDT, and group 2B include PhIP and MeIQx while the group 3 include Reserpine and Actinomycin D. Only the organic compound, Caprolactam was listed as a member of group 4. IARC reported that there is limited evidence that group 2A and 2B which are the group of most heterocyclic amines are carcinogenic (IARC, 2017).

Table 2.1: Toxic Agents as Classified by the IARC Monographs

Group	Characteristics	Number of agents
Group 1	Carcinogenic to humans	120
Group 2A	Probably carcinogenic to humans	82
Group 2B	Possibly carcinogenic to humans	311
Group 3	Not classifiable as to its carcinogenicity to humans	499
Group 4	Probably not carcinogenic to humans	1

Source: (IARC, 2018)

Most toxic agent cause their adverse effects by DNA damage (Helleday *et al.,* 2014). The damage to DNA which carries genetic information could be from internal or external sources (Helleday *et al.,* 2014). The internal sources come from reactions and actions going on inside the cells. They include enzymatic conversions, errors during replication, the release of reactive oxygen species, deamination and bile acids (Helleday *et al.,* 2014). The external sources come from outside the body especially the environment. They include the non-ionizing ultraviolet radiation, tobacco smoke, genotoxic chemical compounds, infections and drugs (Cunningham *et al.,* 2011; Kanavy and Gerstenblith, 2011; Helleday *et al.,* 2014).

Many models have been used to study molecular toxicology. This is necessary because the initiation, progression and the side effects of toxic agents are difficult to study in human. Primates and other animals that have similar genetic makeup have been used to study toxicology. Rodents are used in basic research because of their mature transgenic abilities, clear genetic settings, relatively small size and quick

breeding (Xia and Chen, 2011). Primates such as chimpanzee are also used because of their shared genetic evolution, system biology and metabolism with human (Xia and Chen, 2011). Other animals that have added a clue to the knowledge of toxicology include fishes, cats, dogs and *Drosophila* (Miles *et al.,* 2011; Cekanova and Rathore, 2014). Organisms such as yeast have added to scientific knowledge on the role of cell cycle and maintaining cell polarity in cancer (Guaragnella *et al.,* 2014), the egg extracts of *Xenopus laevis* have also added to the current understanding of the biochemical pathways and modifications of the proteins important for normal cellular function (Gillespie *et al.,* 2012). Finally, the nematode *Caenorhabditis elegans* was important in the understanding of the genetic pathways that regulate apoptosis (Arvanitis *et al.,* 2013) while fruit flies (*Drosophila melanogaster*) have helped in identifying the pathways that are dysregulated during the formation of cancer (Rudrapatna *et al.,* 2012).

While animal studies have provided some headway about toxicity research, the use of cell lines for *in vitro* assays has also allowed scientists to conduct research mimicking cancerous conditions (Birbrair *et al.,* 2014). However, there is plausibility in *in vitro* testing because of advances in high throughput screening (Raies and Bajic, 2016). This has led to the use of computational methods for predicting toxicity. This is termed "in silico toxicology". There are now bioinformatic tools such as Toxmatch, ToxTree and ChemIDplus have helped in toxicity prediction (Raies and Bajic, 2016).

2.1.1 Role of Oxidative Stress in Toxicity

Under normal physiological states, there is a balance in the generation of free radicals and the complex antioxidant defence system (Ravi Kiran and Aruna, 2010). This balance helps the organism to prevent the overproduction and accumulation of

reactive oxygen species (ROS) which can lead to oxidative stress (Ravi Kiran and Aruna, 2010). Toxic agents induce their toxic effects by continuously producing ROS coupled with a faulty antioxidant defence system and DNA repair mechanisms (Klaunig *et al.,* 2010). The overproduction of ROS stimulates the signal transduction pathways leading to the activation of key transcription factors such as AP1, Nrf2 and NF-κB (Klaunig *et al.,* 2010). The activation of these key transcription factors altered the patterns of gene expression which ultimately leads to carcinogenesis (Klaunig *et al.,* 2010). The level of ROS production *in vivo* or *in vitro* is usually measured using colourimetric, spectrophotometric and enzymatic methods.

Sources of ROS production can be endogenous or exogenous. Endogenous sources of ROS include oxidative phosphorylation, Cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Klaunig and Kamendulis 2004; Klaunig *et al.,* 2010). Immune cells such as neutrophils, eosinophils, and macrophages can also be additional endogenous sources of ROS production and are major contributors to the cellular reactive oxygen species (Klaunig and Kamendulis 2004; Klaunig *et al.,* 2010). Exogenous sources of ROS include redox cycling compounds, Haber-Weiss and Fenton reactions from reduced metal ions and radiation (Klaunig and Kamendulis 2004; Klaunig *et al.,* 2010). The role of ROS appears to be an important and nearly universally common step in toxic agent-induced cellular responses. The role of ROS in toxicity development is summarised in figure 2.1.

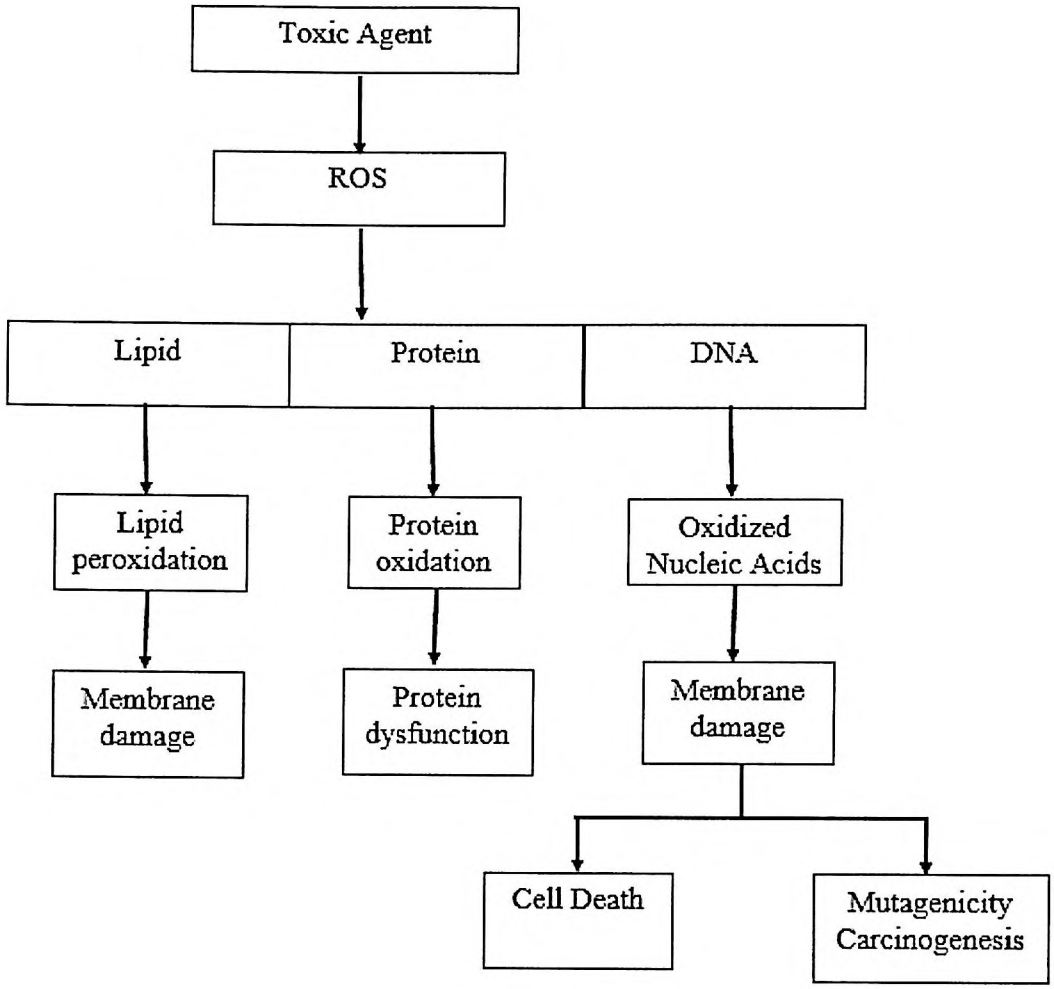


Figure 2.1: Consequences of toxic-induced oxidative stress. The figure illustrates the potential outcomes of reactive oxidative species when not counterbalanced by antioxidant defences of the cell.

Source: Lourenço, 2012

Cellular antioxidants are complex webs of system produced by the cell to maintain cellular redox balancing. The antioxidant can be divided into enzymatic and non-enzymatic. Prominent enzymatic antioxidant includes superoxide, catalase, thioredoxin, glutaredoxin and glutathione peroxidase; while common examples of the non-enzymatic antioxidant include glutathione, catechins, vitamins A, C, and E (Klaunig *et al.,* 2010). Polymorphism in this enzymatic antioxidant causes DNA

damage which can increase the risk of an individual getting cancer (Khan *et al.,* 2010). Although one-third of cancers are related to food, yet they are also a good source of antioxidants. Foods with a high amount of antioxidants include small red bean, wild blueberry, red kidney bean and cranberry (Selby-Pham *et al.,* 2017). These foods are good antioxidants because they are rich sources of polyphenols and flavonoids which have antioxidant properties (Selby-Pham *et al.,* 2017). Spices such as clove, turmeric, basil, curry powder, ginger, pepper, paprika, garlic, coriander, onion and cardamom are also rich in polyphenols, therefore, are good sources of antioxidant (Paur *et al.,* 2011). Popular herbs such as thyme, marjoram, tarragon, oregano, savoury and dill weed are also good sources of antioxidants (Paur *et al.,* 2011).

2.1.2 Genes and Proteins Implicated in Toxicity

Understanding the genes and proteins implicated in toxicity is important in understanding the progression of toxicants and how to manage it. Tumour suppressor genes which are the genes encoding proteins that inhibit cell transformation and whose inactivation, is advantageous for tumour cell growth and survival have been reported to be implicated in toxicity. The role of tumour suppressor genes in the progression of toxicity differs depending on the stages and type of toxicant exposed to (Vlahopoulos *et al.,* 2008).

The tumour suppressor genes are involved in several important biological process which are highly conserved. These functions include cell cycle regulation, cell differentiation, signal transduction, programmed cell death, cell adhesion and repair of DNA errors arising from exposure to toxic agents (Weinberg, 2014).

Identifying proteins encoded in toxicity is usually done using immunohistochemical techniques which allow for visualising the distribution and

relative presence of these proteins expressed in various tissue samples (Duraiyan *et al.,* 2012). Some group of proteins are differentially expressed between the various forms of toxic samples. Additionally, differential expression of proteins is used to differentiate between the toxicant-exposed tissues and normal tissues (Duraiyan *et al.,* 2012). Other proteomic methods used for identifying protein implicated in toxicity testing include two-dimensional gel electrophoresis, immunoblotting and mass spectrometry. Transcriptional changes measured using the nucleic acids may not reflect accurately the changes at the protein level, hereby, preventing mechanistic interpretations (Wang *et al.,* 2010a). There are usually inconsistencies in the data generated by nucleic acid research, which means it must be correlated with a number of proteins synthesized (Wang *et al.,* 2010a). Therefore, proteomic studies are important to provide indications at a molecular level of the effects of the exposure to toxicants, but most importantly, to the development of more sensitive and specific biomarkers that may be employed as early warning signals for the exposure to toxicants.

2.2 Environmental Pollution

Environmental pollution is the contamination of the environment by pollutants that cause a threat to the environment and its inhabitants. The pollutants could be from natural sources such as environmental chemicals or anthropogenic sources such as oil spillage and car exhaust. It is quite difficult to classify pollutants but there are about 330 substances or compounds that have been listed as pollutants or potential pollutants because they have adverse effects on the environment and its inhabitant. Among the substances that have been listed as environmental pollutants are heterocyclic amines, pesticides, heavy metals, radioactive wastes and polycyclic aromatic hydrocarbons (Duffus, 2002; David, 2005; Choi *et al.,* 2010; Teunissen *et al.,* 2010).

Heterocyclic amines were initially thought to be only restricted to proteinaceous foods as they have been isolated and identified from fish and meat, but recent studies have found these compounds in environmental media (Teunissen *et al.*, 2010; Warnert, 2013). Heterocyclic amines have been detected in outdoor air, indoor air, diesel exhaust particles, incineration ash from garbage burning plants, cigarette smoke, cooking fumes, soil and rainwater (Teunissen *et al.*, 2010; Liu *et al.*, 2013). Heterocyclic amines have also been detected in urine, saliva, plasma, faeces and bile which are biological specimens (Fede *et al.*, 2009; Teunissen *et al.*, 2010; Gu *et al.*, 2010). Heterocyclic amines find their way into the environment through combustion of various materials which are later released into the water bodies and soil through domestic and industrial waste. Prominent members that have been found in environment samples include Trp-P-1, Trp-P-2, MeIQx and PhIP (NTP, 2014). Examples of environmental media where heterocyclic amines have been found are shown in figure 2.2.

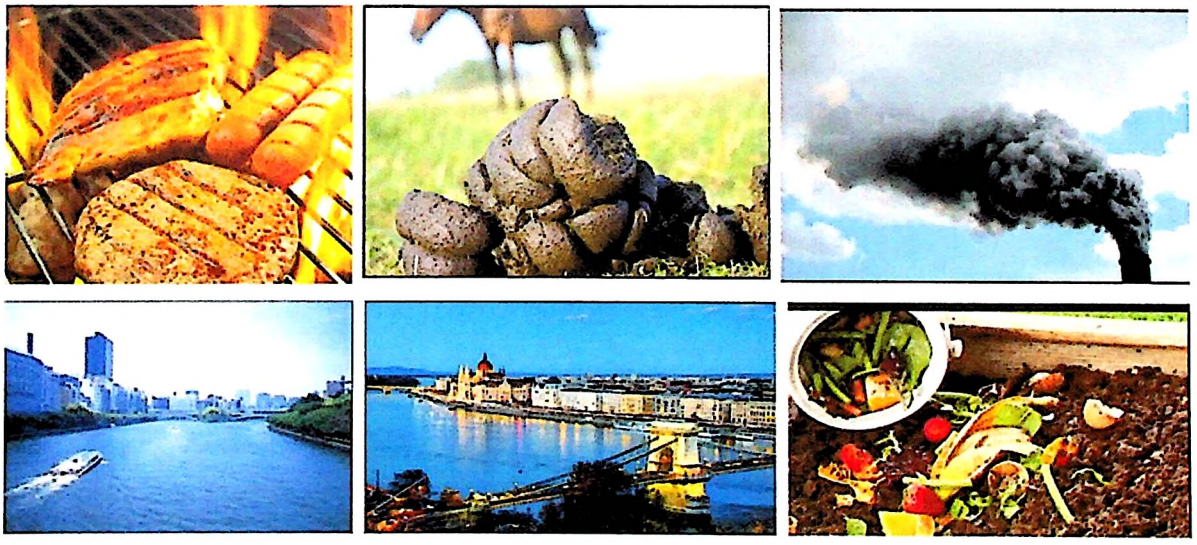


Figure 2.2: Examples of environmental media where heterocyclic amines have been detected. Heterocyclic amines have been detected in river water, faeces and outdoor air

Polycyclic aromatic hydrocarbons (PAHs) are carcinogenic substances that are released into the environment when there is burning of oil, gas, wood and other organic substances (Choi *et al.,* 2010). They are also found in cooked meat and fish (Choi *et al.,* 2010). They are about 100 in number and are ubiquitous in the environment but the United States Environmental Protection Agency (USEPA) has regulated on 16 of them which are considered as high priority pollutant; they include benzo[a]pyrene, dibenz(a,h)anthracene, benz(a)anthracene and benzo(b)fluoranthene (Choi *et al.,* 2010). They can occur naturally in the environment such as those released during volcanoes and creosote (Ravindra *et al.,* 2008). They can also occur following anthropogenic activities such as dredging, mining, bush burning and fossil fuel extraction (Ravindra *et al.,* 2008). Results from both human and animal studies have shown that PAHs could affect various systems and induce developmental defects (Ramesh *et al.,* 2011).

Heavy metals such as chromium, cadmium and lead are naturally residential in the environment and its media but in a minimal amount (Duffus, 2002). The presence of an uncontrolled amount of these metals in the environment can affect the environment and its biotic components. When heavy metals are released into the environment usually from industrial wastes, paints, mining and vehicle emissions (Stankovic and Stankovic, 2012); they degrade the quality of the water, air and soil and cause health issues in animals and plants that get exposed to them (Baird and Cann, 2012). Places that have experienced heavy metals contamination include the lead poisoning from mining in Zamfara state, Nigeria and mercury poisoning in Minamata, Japan (Amasawa *et al.,* 2016; Tirima *et al.,* 2016).

Pesticides are important agricultural tools, they are needed to wade off the attack of pests on farmland. Despite their benefits, pesticides have toxic effects on the

environment and biotic components. According to the Stockholm Convention on the use of persistent organic pollutants, 9 of the 12 known and dangerous organic chemicals are used as organochlorine pesticides (Gilden *et al.,* 2010). The effects of pesticides are widely felt in both developing and developed nations. In the United States alone, the environmental and human effect of pesticides is valued at about $9.6 billion (David, 2005). The public health and groundwater contamination by pesticides are valued at $1.1 billion and $2 billion respectively (David, 2005).

Radioactive wastes are wastes that contain radioactive materials. These wastes are highly dangerous and need to be kept away from the unprotected individuals. They cause death such as those seen in Adamawa state in Nigeria from uranium exposure. Several factors influence the effects of radioactive waste on the environment and its inhabitant; they include the type of ionising radiation, chemical properties and energy of the radioactive elements (Lourenço et al., 2012; Carpentar and Bushkin-Bedient 2013).

Other prominent pollutants that have been reported include persistent organic pollutants (POP) such as aldrin, chlordane, endrin and toxaphene (El-Shahawi *et al.,* 2010) and environmental persistent pharmaceutical pollutants (EPPP) such as estradiol, cardiovascular medicines, antibiotics and any drugs that can pollute the environment (Kümmerer, 2010).

2.2.1 Rationale for Selecting Heterocyclic Amines

Among the common carcinogens known and found in the environment such as heavy metals, polycyclic aromatic hydrocarbons and heterocyclic amines, the least studied are the heterocyclic amines. Much is not known about the effects of this group on the inhabitants of the soil. To the best of my knowledge, this is the first work

elaborating on the effects of heterocyclic amines on an inhabitant of the terrestrial environment.

Heterocyclic amines (HCAs) were used in this experiment considering that Malaysia and neighbouring countries experience environmental conditions that can cause the release of HCAs into the environment. In Malaysia and other countries of South-Eastern Asia, haze is experienced annually. It has been reported that HCAs are found in rainwater collected from Singapore during haze (Wu et al., 1995). Another experience that causes the release of HCAs is bush burning; in Malaysia and its neighbouring countries, farmers burn their farmland at the end of a planting season in anticipation of the next planting season, HCAs have been reported in the environment after the burning of grasses and incineration ash (Wu et al., 1995).

Several factors were considered before selecting PhIP and MeIQx as the representative of the HCAs to study the toxicological response of earthworm in the study. Some of the factors considered include occurrence in the environment, exposure from food and toxicity.

The most popular member of HCAs that have been reported to occur in the environment includes PhIP, MeIQx, Trp-P-1, Trp-P-2 and 4,8-DiMeIQx (NTP, 2014). PhIP and MeIQx are among the most ubiquitous member of the HCAs that has been found in environmental media. They have been found in the popular Yodo River in Japan. They have also been detected in rainwater collected during haze in South East Asia (Wu et al., 1995).

Exposure to HCAs is primarily from consumption of cooked food infected with heterocyclic amines; however, they have also been detected in biological samples, beer, wine, cigarette smoke, and diesel exhaust. The concentration of HCAs exposed

to from food ranges from 1 ng/kg to 17 ng/kg bw per day (Joshi *et al.,* 2015). In a review by Keating *et al.,* (1999), it was shown that the dietary intake of HCAs in a day ranges from 160 to 1800 ng per person. The authors showed that PhIP followed by MeIQx followed by IQ and lastly MeIQ represent the highest dietary intake of HCAs in human beings. The study showed clearly that based on exposure from food, both PhIP and MeIQx represent the most common HCAs found in cooked food (Keating *et al.,* 1999).

While results from human exposure to HCAs have been quite confusing, data from animal studies have shown that HCAs are highly toxic substances. According to Sugimura *et al.,* (2004), the toxic dose of popular HCAs in rodents (rats and mice) shows them as highly toxic compounds. The median toxic dose (TD_{50}) in rats and mice are outlined in Table 2.2.

Table 2.2: TD_{50} of major HCAs in mice and rats

Heterocyclic Amines	Rats (mg/kg/day)	Mice (mg/kg/day)
PhIP	2.2	64.6
MeIQx	0.7	11
IQ	0.7	14.7
MeIQ	0.1	8.4
AαC	–	15.8
MeAαC	6.4	5.8

Adapted from Sugimura *et al.,* (2004)

2.3 Chemistry of Heterocyclic Amines

The history of HCAs goes back to 1977 when it was discovered that smoke particles formed from cooking proteinaceous foodstuffs contained a significant amount of mutagens by trapping with glass-fibre filters (Gibis, 2016). HCAs are the product of reaction when sugar, creatinine (creatine) and amino acids combine at high temperatures (Teunissen *et al.,* 2010). Using various purification techniques, about 30

HCAs have been isolated and their chemo-structural analysis has been known (Gibis, 2016). The formation of HCAs in a cooked meat or fish increases with increase in temperature and duration of cooking, this also depends on the cooking method and the type of meat cooked (Teunissen *et al.,* 2010).

HCAs can be divided into 2 based on how they are formed. The first is produced by heating mixtures of monosaccharides, amino acids and creatine at high temperature, this group includes IQ, MeIQ, MeIQx, and PhIP while the second is produced by decomposing amino acids and proteins at high temperature through radical reaction, this group includes Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC and MeAαC (Gibis, 2016). The first group cannot be inactivated by converting an exocyclic amino group to a hydroxyl group by diluting with nitrite, unlike the second group which can be inactivated (Gibis, 2016).

Reports have shown that the toxic effects of HCAs could be suppressed by flavonoids, antioxidants and chlorophyll derivatives in animal studies (Jamali *et al.,* 2016; Joshi *et al.,* 2015). The formation of HCAs in food can also be reduced with the use of a microwave oven and avoiding direct exposure of meat/fish to flames (Teunissen *et al.,* 2010).

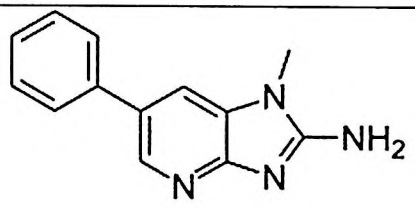
2.3.1 Chemistry of PhIP

2-Amino-1-methyl-6-phenylimiazo[4,5-b]pyridine popularly known as PhIP with a chemical formula of $C_{13}H_{12}N_4$ is an IARC Group 2B carcinogen (IARC, 2017). The National Toxicology Program (NTP) also listed PhIP as reasonably anticipated to be a human carcinogen (NTP, 2014). It has a CAS registry number of 105650-23-5. The chemical is soluble in organic solvent and usually diluted in DMSO and methanol

in the laboratory (O'Neil, 2006). PhIP has been described as the most abundant and most studied member of the HCA (NTP, 2014). It has peculiar properties used in differentiating it from other HCAs. These are highlighted in table 2.3.

PhIP is found naturally in cooked fish and meat but they have also been synthesised artificially which has made experiments to assess their carcinogenicity easy. PhIP can be synthesised from 3-amino-2-phenylpropenal and creatinine heated in the presence of N,O-bis(trimethylsilyl)acetamide at 120 °C for 2 hours (Lindström, 1995). This approach yielded a 26 % PhIP from the experiment. PhIP can be decomposed by heating, thereby emitting toxic fumes of nitrogen oxides (Lewis, 2004).

Table 2.3: Physical and chemical properties of PhIP

Property	Value/Description
CAS Number	105650-23-5
Chemical formula	$C_{13}H_{12}N_4$
Chemical name	2-Amino-1-methyl-6-phenylimiazo[4,5-b]pyridine
Molecular weight	224.26
PubChem Identifier	1530
Chemical structure	
Physical appearance	off-white solid
Melting point	327 to 328 °C
Boiling point	468.9 °C
Solubility in water	407.1 mg/L
Density (at 20 °C)	1.3 gcm^{-3}
Vapour pressure (at 25 °C)	8.09×10^{-9} mmHg

Log partition coefficient	2.23
Extinction coefficient	19,400 at 316 nm
Refractive index	1.699
Flash point	237.4 °C

Source: Lewis, (2004); O'Neil, (2006); USEPA, (2009)

Exposure to PhIP is harmful and can lead to various side effects such as mutagenicity, cytotoxicity, genotoxicity but this depends on other factors. Some of these factors include the amount of PhIP exposed to, the duration of exposure and route of exposure (Alaejos and Afonso, 2011). Other factors that influence the harmfulness of PhIP include what other chemicals was the individual exposed to, age of the individual, sex, family history, lifestyle details, and state of wellness (Alaejos and Afonso, 2011).

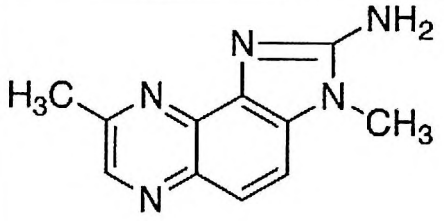
2.3.2 Chemistry of MeIQx

2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline popularly known as MeIQx with a chemical formula of $C_{11}H_{11}N_5$ belong to the Group 2B carcinogen according to the IARC monograph (IARC, 2017). NTP has also named MeIQx as reasonably anticipated to be a human carcinogen (NTP, 2014). MeIQx can be decomposed by heating, thereby emitting toxic fumes of nitrogen oxides (Lewis, 2004). It has a CAS registry number of 77500-04-0. The chemical is soluble in organic solvent and usually diluted in DMSO and methanol in the laboratory (O'Neil, 2006).

MeIQx is formed during the cooking of muscle-derived foods including fish and meat. It can also be synthesized in the laboratory for research purposes. Grivas and Olsson (1985) synthesized MeIQx from 4-fluoro-o-phenylenediamine with a yield of 21 %. The cyclisation with [^{14}]C-cyanogen bromide at the last step gave a [^{14}]C

label. The authors reported that an attempt to synthesized MeIQx from p-fluoroaniline gave a lesser yield although avoided the separation of isomers (Grivas and Olsson, 1985). Like other HCAs, MeIQx have its own properties which can be used in identifying it. These are highlighted in table 2.4.

Table 2.4: Physical and chemical properties of MeIQx

Property	Value/Description
CAS Number	77500-04-0
Chemical formula	$C_{11}H_{11}N_5$
Chemical name	2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline
Molecular weight	213.24
PubChem identifier	62275
Chemical structure	
Physical appearance	Pale orange to brown crystalline solid
Melting point	295 to 300 °C
Boiling point	458.4 °C
Solubility in water	398 mg/L
Density (at 20 °C)	1.47 gcm^{-3}
Vapour pressure (at 25 °C)	1.7×10^{-8} mmHg
Log partition coefficient	1.01
Extinction coefficient	41,000 at 273 nm
Refractive index	231°C
Flash point	1.776

Source: Lewis, (2004); O'Neil, (2006); USEPA, (2009)

Exposure to MeIQx like other HCAs is harmful and could lead to various side effects such as mutagenicity, cytotoxicity, genotoxicity although this depends on other factors such as the amount of MeIQx exposed to, lifestyle details, the duration of exposure, family history and route of exposure (Alaejos and Afonso, 2011).

2.4 Toxicokinetics of Heterocyclic Amine

The ways and methods by which HCAs are absorbed, distributed, metabolised and excreted from the body are similar. HCAs enter the body orally when they are eaten in food or inhaled during smoking. They are usually distributed into major organs especially the organs of the digestive and respiratory systems. The liver is the most metabolically active tissue in the biotransformation of HCAs in both rodents and humans (Gu et al., 2010). However, interspecies differences have been reported in both catalytic activity and regioselectivity of liver enzymes involved in the metabolism of HCAs (Gu et al., 2010), potentially affecting their biological activities and making questionable the use of animal models for estimating risks of HCAs for human health. The metabolism of HCAs released metabolites which form DNA adducts, these adducts are genotoxic or potential genotoxic compounds. The HCAs and their metabolites can be excreted in urine and faeces. They can be found also in bile and hair. Urinary excretion level of a member of the heterocyclic amines can serve as an approximate measure of another member in estimating exposure to these compounds in humans that are exposed to them (Konorev et al., 2015). Several factors influence the degree of agreement between the urinary excretion levels of heterocyclic amines and they include uneven contents of heterocyclic aromatic amines in food, enzymatic and interindividual metabolic differences, and methods of analysis (Konorev et al., 2015).

The pathway of toxicity of HCAs in human and animals followed a similar pattern. There is Cytochrome P450-mediated ring oxidation which is followed by conjugation to sulphate or glucuronic acid which is followed by direct phase II conjugation to the exocyclic amine groups (Dumont *et al.,* 2010). The enzymatic activation is initiated by P450-mediated N-oxidation of the exocyclic amine groups forming the mutagenic N-hydroxy intermediates which possibly will react directly with DNA (Dumont *et al.,* 2010).

Comparing the toxicokinetics of PhIP and MeIQx, Stillwell *et al.,* (1997) reported that the metabolism and deposition of MeIQx are more strongly influenced by the enzymatic activity of CYP1A2 than those of PhIP. However, there is no relationship between the activity of NAT2 and the unmetabolized levels of MeIQx and PhIP excreted via urine (Stillwell *et al.,* 1997). The parent HCAs including PhIP and MeIQx together with their primary oxidative metabolites are further converted into a variety of phase II metabolites which are known genotoxic species; they include glucuronides, sulphate esters, and acetylated products (Stillwell *et al.,* 1997).

2.4.1 Toxicokinetics of PhIP

Research to understand the toxicokinetics of PhIP have been carried out in both human and experimental animals. Conditions such as bile ligation can affect the bioavailability of PhIP; when PhIP was orally administered to rats with bile ligation, there was increased bioavailability of PhIP by 2.5-fold in rats with bile duct ligation when compared with the sham-operated rats (Ferguson and Flora, 2005).

In a pharmacokinetic study in mice, the authors showed that when 1 mg/kg of [^{14}C]PhIP was administered to the mice, the area under the curve for the orally

administered PhIP was elevated in *Bcrp* by 2.9 fold in mutant mice when compared to the wild-type mice (van Herwaarden *et al.,* 2003). Using *in vivo* and *in vitro* technique, the *Bcrp* gene effectively affects the exposure of mice to PhIP by reducing the uptake of PhIP from the lumen of the alimentary tract and mediating elimination by the liver, biliary system and intestine (van Herwaarden *et al.,* 2003).

The metabolism of PhIP follows a sequence according to Fede et al., (2009). The Cytochrome P450 enzymes catalyse the oxidation of the exocyclic amine group of PhIP to form 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (HONH-PhIP). HONH-PhIP can undergo conjugation by sulfotransferases (SULTs) or N-acetyltransferases (NATs), to produce highly reactive esters that bind to DNA or undergo solvolysis to produce 2-amino-1-methyl-6-(5-hydroxy)phenylimidazo[4,5-b]pyridine (5-HO-PhIP) (Fede *et al.,* 2009). The Cytochrome P450s further catalyse oxidation at the 4′ position of PhIP to form 2-amino-1-methyl-6-(4′-hydroxy)-phenylimidazo[4,5-b]pyridine (4′-HO-PhIP), a principal detoxication product of PhIP in experimental animals which occurs at considerably lower levels (Fede *et al.,* 2009). Human P450s primarily catalyse the formation of HONH-PhIP as the major oxidation product (Fede *et al.,* 2009). PhIP and its metabolite HNOH-PhIP undergo conjugation by uridine diphosphate glucuronosyltransferases 1A1 (UGT1A1) isoforms to produce N2- and N3-glucuronide conjugates (Fede *et al.,* 2009). The glucuronide conjugates of HONH-PhIP are detoxication products in the metabolism of PhIP although bacterial glucuronidases can hydrolyse HON-PhIP-N3-Gl to liberate HONH-PhIP for further metabolism and formation of DNA adduct (Fede *et al.,* 2009). The metabolism pathway of PhIP is described in figure 2.3.