Fusaric Acid Induces DNA Damage and Post-Translational Modification Of p53 In Hepatocellular Carcinoma (HepG₂) Cells

By

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BSc. B. Med. Sc. (Hons) (UKZN)

Submitted in fulfilment of the requirements for the degree of

Master of Medical Science

in the Discipline of Medical Biochemistry and Chemical Pathology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

University of Kwa-Zulu Natal

Durban

2016

DECLARATION

This dissertation represents the original work by the author and has not been submitted in any form to another university. The work of others has been duly acknowledged in the text.

The research described in this study was carried out in the Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Science, College of Health Sciences, University of Kwa-Zulu Natal (Howard College Campus), Durban, under the supervision of Professor A.A. Chuturgoon, Dr S. Nagiah and Dr C. Tiloke.

30 November 2016

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ACKNOWLEDGEMENTS

PRESENTATIONS

Fusaric Acid Induces DNA Damage and Alters p53 in Liver Carcinoma (HepG$_2$) Cells

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College of Health Sciences Research Symposium (8th – 9th September 2016), University of Kwa-Zulu Natal, Durban, South Africa – 2nd Prize Poster Presentation, Masters Students Category

ABBREVIATIONS

a-K382-p53	Acetyl-lysine 382-p53
AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocator
Apaf-1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ARF-BP1	ADP-ribosylation factor binding protein 1
A-T	Adenine-Thymine
ATP	Adenosine tri-phosphate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad 3 related
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CA	California
CAT	Catalase
CAK	Cyclin-dependent kinase activating kinase
CCM	Complete culture media
chk1	Checkpoint kinase 1
chk2	Checkpoint kinase 2
CO_2	Carbon dioxide
CBP	CREB-binding protein
C_T	Cycle threshold

Cu^{2+}	Cupric ions
Cu^+	Cuprous ions
$CuSO_4$	Copper sulphate
DAS	Diacetoxyscirpenol
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DNA-PK	DNA-dependent protein kinase
DON	Deoxynivalenol
DR4/5	Death receptor 4/5
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's minimum essentials medium
FA	Fusaric acid
FADD	Fas associated death domain
FasL	Fas ligand
H_2O_2	Hydrogen peroxide
HAT	Histone acetyltransferase
HAUSP	Herpesvirus-Associated Ubiquitin-Specific Protease
HDAC	Histone deacetylase
HDAC1	Histone deacetylase 1
$HepG_2$ cells	Hepatocellular carcinoma cells
IC_{50}	50% Inhibition concentration
LMPA	Low melting point agarose

MDM2	Murine double minute 2
MgCl$_2$	Magnesium chloride
MO	Missouri
NaCl	Sodium chloride
NAD+	Nicotinamide adenine dinucleotide
NES	Nuclear export signal
NFDM	Non-fat dry milk
NLS	Nuclear localization signal
OH$^-$	Hydroxyl group
PBR	Peripheral benzodiazepine receptor
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
ppm	Parts per million
p-Ser15-p53	Phosphorylated-Serine 15-p53
p-Ser47-Sirt1	Phosphorylated-Serine47-Sirt1
PTP	Permeability transition pore
RBD	Relative band density
RT	Room temperature
SA	South Africa
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sirt1	Sirtuin 1
SOD	Superoxide dismutase

tBid	Truncated Bid
TBP	TATA-binding protein
TEMED	Tetramethylethylenediamine
TNF-α	Tumour necrosis factor alpha
TRADD	Tumour necrosis factor-receptor associated death domain
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloric acid
TTBS	Tris-buffered saline with Tween 20
USA	United States of America
VDAC	Voltage-dependent anion channel

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ABSTRACT

Fusaric acid (FA), a mycotoxin produced by the *Fusarium* species, is a common contaminant of maize and other agricultural products. FA is known to mediate toxicity in plants and animals; however, its mechanism of action is unknown. p53 is a tumour suppressor protein that is activated in response to cellular stress. Key functions of p53 include cell growth arrest, senescence and apoptosis. The function of p53 is regulated by a variety of post-translational modifications such as ubiquitination, phosphorylation and acetylation. p53 is ubiquitinated by the protein murine double minute 2 (MDM2) and degraded in the absence of cellular stress. The phosphorylation and acetylation of p53 occur during cellular stress and increase p53 stability and activity. The acetylation of p53 is regulated by histone acetyltransferases, CBP/p300, and histone deacetylases, HDAC1 and Sirt1. This study investigated the effect of FA on DNA integrity and the post-translational modifications of p53 in hepatocellular carcinoma (HepG$_2$) cells. Methods included: (i) culture and treatment of HepG$_2$ cells (IC$_{50}$: 104μg/ml FA, 24hrs); (ii) comet assay (DNA damage); (iii) western blot (protein expression of p53, phosphorylated-Ser15-p53 (p-Ser15-p53), acetylated-K382-p53 (a-K382-p53), acetyl-CBP (K1535)/p300 (K1499) (a-CBP (K1535)/p300 (K1499)), HDAC1, phosphorylated-Ser47-Sirt1 (p-Ser47-Sirt1) and MDM2); and (v) Hoechst 33342 assay (cell cycle and apoptosis analysis). FA caused DNA damage (89.98 ± 10.36μm vs. 33.99 ± 6.76μm, *p<0.0001*) in HepG$_2$ cells relative to the control. FA significantly increased the protein expression of p-Ser15-p53 (9.76-fold, *p=0.0002*), a-K382-p53 (1.75-fold, *p=0.0329*), p-Ser47-Sirt1 (1.24-fold, *p=0.0127*) and MDM2 (5.63-fold, *p=0.0094*) in HepG$_2$ cells. The expression of p53 (0.73-fold, *p=0.0034*), a-CBP (K1535)/p300 (K1499) (0.58-fold, *p=0.0043*) and HDAC1 (0.84-fold, *p=0.0006*) were significantly decreased in the FA treated cells. Hoechst analysis of HepG$_2$ cells showed that FA inhibited cell proliferation and induced apoptosis as evident by the absence of dividing cells and the presence of apoptotic bodies. FA is a genotoxic agent that increased p-Ser15-p53, decreased the interaction between MDM2 and p53 and increased the interaction between p53 and a-CBP/p300. This increased the acetylation of p53 (further achieved by the down-regulation of HDAC1 and inactivation of p-Ser47-Sirt1) leading to cell growth arrest and apoptosis of HepG$_2$ cells.

INTRODUCTION

Mycotoxin contamination of foods and feeds is a serious problem occurring worldwide (Milićević et al., 2010). Areas most often affected by mycotoxins include developing countries and poverty stricken areas where malnutrition is a major concern and the consumption of mycotoxin contaminated foods form a staple diet for many people (Bennett and Klich, 2003). The application of modern agricultural practices as well as regular government screening helps lower the risk of exposure to mycotoxins; however, it does not completely eliminate it. Hence, mycotoxin contamination is a recurring problem (Wild and Gong, 2010).

Mycotoxins are low molecular weight natural compounds produced as secondary metabolites of toxigenic moulds (Bennett and Klich, 2003, Milićević et al., 2010). Mycotoxins are common contaminants of a wide variety of foods and feeds and the consumption of mycotoxin contaminated foods may have a serious impact on human and animal health (Bennett and Klich, 2003, Milićević et al., 2010, Peraica et al., 1999, Zain, 2011). Several mycotoxins have been identified; many of which are carcinogenic, mutagenic, nephrotoxic, hepatotoxic and neurotoxic (Milićević et al., 2010, Peraica et al., 1999). Exposure to mycotoxins can occur via ingestion, inhalation and dermal routes leading to several diseases known as mycotoxicoses (Bennett and Klich, 2003, Milićević et al., 2010, Peraica et al., 1999, Zain, 2011).

The *Fusarium* species are a group of diverse and adaptable fungi that colonise plants causing diseases on agricultural crops with major economic losses (Bouarab, 2009). Fusaric acid (FA) also known as 5-butylpicolinic acid is a secondary metabolite and mycotoxin produced by the *Fusarium* species (Bacon et al., 1996, Fairchild et al., 2005). Fusaric acid is a fusariotoxin commonly found in maize and other cereal grains, which form an essential part of human food and animal feeds (Milićević et al., 2010). Therefore, the consumption of FA contaminated commodities may have adverse effects in humans and animals.

Fusaric acid is a Picolinic acid derivative and well-known metal chelating agent. The structure of FA is similar to that of Picolinic acid in that it contains the well conserved chelating structure of Picolinic acid as well as an additional fused aromatic ring structure or 5-butyl side chain (Bochner et al., 1980). Fusaric acid binds divalent cations and prevents these ions from functioning in biological processes (Swinburne, 2012).

Fusaric acid is known to affect both plants (D'Alton and Etherton, 1984, Diniz and Oliveira, 2009, Dong et al., 2012, Li et al., 2013) and animals (Abdul et al., 2016, Hidaka et al., 1969,

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Terasawa and Kameyama, 1971). However, little is known on the mechanism by which FA exerts its toxic effects. Previous studies have shown FA to cause changes in membrane permeability (D'Alton and Etherton, 1984), dysfunctions in mitochondrial activity (Abdul et al., 2016, Diniz and Oliveira, 2009), inhibition of respiration (D'Alton and Etherton, 1984, Pavlovkin et al., 2004, Telles-Pupulin et al., 1996) and ultimately cell death (Abdul et al., 2016, Stack et al., 2004). Fusaric acid is also known to have anti-hypertensive (Hidaka et al., 1969, Terasawa and Kameyama, 1971) and anti-tumour effects (Stack et al., 2004). Synergistic effects of FA with other mycotoxins produced by the *Fusarium* species such as Fumonisin B_1, deoxynivalenol (DON) and diacetoxyscirpenol (DAS) have also been reported (Bacon et al., 1995, Bungo et al., 1999, Fairchild et al., 2005, Ogunbo et al., 2007, Voss et al., 1999).

p53, often referred to as the guardian of the genome, is a tumour suppressor protein and transcription factor that is activated in response to cellular stress (Prives and Hall, 1999). Several stressors can activate p53 such as DNA damage, excessive oncogene activation, hypoxia and oxidative stress (Prives and Hall, 1999). Once activated, p53 mediates a plethora of functions including cell growth arrest, DNA repair, senescence and apoptosis (Barlev et al., 2001, Brooks and Gu, 2003, George, 2011, Reed and Quelle, 2014, Zhang et al., 2015). The function of p53 is regulated by a variety of post-translational modifications (van Leeuwen et al., 2013). These modifications include ubiquitination, phosphorylation and acetylation (Barlev et al., 2001, Brooks and Gu, 2003, Reed and Quelle, 2014, van Leeuwen et al., 2013, Zhang et al., 2015).

The ubiquitination of p53 occurs in the absence of cellular stress and is mediated by the protein murine double minute 2 (MDM2) (Dai and Gu, 2010, Brooks and Gu, 2003). MDM2 is the predominant negative regulator of p53 (Brooks and Gu, 2003). It adds ubiquitin chains to the C-terminal lysine residues targeting p53 for degradation by the 26S proteasome (Brooks and Gu, 2003, Dai and Gu, 2010, Reed and Quelle, 2014). In this way, MDM2 helps maintain low levels of p53 in the absence of cellular stress.

During DNA damage several protein kinases such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad 3 related (ATR) and DNA-dependent protein kinase (DNA-PK) are triggered (Barlev et al., 2001, Brooks and Gu, 2003, Reed and Quelle, 2014). These protein kinases phosphorylate p53 on several serine and threonine residues leading to the stabilization of p53 (Brooks and Gu, 2003, Dai and Gu, 2010).

p53 is also regulated by acetylation (Gu and Roeder, 1997). The acetylation of p53 occurs in response to DNA damage and increases the transcriptional activity of p53 preventing the

propagation of cells with damaged DNA (Dai and Gu, 2010, Brooks and Gu, 2003, Barlev et al., 2001, Reed and Quelle, 2014, Zhang et al., 2015). CREB-binding protein (CBP) and p300 are major histone acetyltransferases (HATs) involved in the acetylation of p53 whereas Sirt1 and HDAC1 are major deacetylases involved in the deacetylation of p53 (Zhang et al., 2015, Dai and Gu, 2010, Brooks and Gu, 2003). The acetylation of p53 is associated with enhanced p53 activity (Blattner, 2008) whereas the deacetylation of p53 is associated with the inactivation of p53 and cell survival (Lee et al., 2012).

This *in vitro* study investigated a possible mechanism of FA induced cytotoxicity in the hepatocellular carcinoma (HepG$_2$) cell line by determining the effect of FA on DNA integrity and the post-translational modifications of p53 over a period of 24 hrs. It was hypothesized that FA induced DNA damage and post-translationally modified the p53 protein in HepG$_2$ cells.

CHAPTER 1

LITERATURE REVIEW

1.1. Mycotoxins

Mycotoxins are secondary metabolites produced by moulds, mostly belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* (Yiannikouris and Jouany, 2002). These toxins are produced in cereal grains and animal feeds before, during and after harvests, in various environmental conditions (Yiannikouris and Jouany, 2002). Mycotoxins are capable of exerting harmful effects in humans, animals and crops and are responsible for causing illnesses and major economic losses (Peraica et al., 1999, Zain, 2011). The worldwide contamination of foods and feeds with mycotoxins is a significant problem (Zain, 2011). Aflatoxin, Fumonisins, Tricothecenes and Ochratoxins are the most common food contaminating mycotoxins. These mycotoxins are potent nephrotoxins, hepatotoxins and carcinogens that induce a wide variety of health problems in both humans and animals (Bennett and Klich, 2003, Peraica et al., 1999, Zain, 2011).

The interest in mycotoxins was first sparked in 1960 following the outbreak of Turkey X disease in which approximately 100 000 turkey poults died as a result of consuming aflatoxin *(Aspergillus flavus)* contaminated peanut meal (Peraica et al., 1999, Bennett and Klich, 2003). Since then, a large number of mycotoxins have been discovered and several outbreaks involving mycotoxins have occurred and still continue to occur globally (Yiannikouris and Jouany, 2002, Peraica et al., 1999).

Mycotoxins are low molecular weight natural products produced mainly by filamentous fungi (Bennett and Klich, 2003). Mycotoxins have no biochemical significance in the growth and development of the fungus and are often produced as an adaptive response to a change in the availability of nutrients and other environmental conditions (Zain, 2011). Mycotoxins are highly toxic compounds usually produced for purposes of self-defence or to dissolve cellular membranes as part of their fungal pathogenicity (Stack Jr et al., 2014). The chemical structures of mycotoxins vary considerably and account for their differences in biological effects (Yiannikouris and Jouany, 2002, Peraica et al., 1999). While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins. Their target and

concentration are both important to elicit a toxic effect (Bennett and Klich, 2003). For example, some fungal products such as penicillin are toxic to bacteria and are important prescribed antibiotics (Bennett and Klich, 2003).

Mycotoxins are common contaminants of a wide variety of food sources. Human exposure to mycotoxins may result from the consumption of contaminated foods as well as the carry-over of mycotoxins and toxic metabolites in animal products such as milk, meat and eggs (Yiannikouris and Jouany, 2002, Zain, 2011). However, skin-contact with mould infested substrates and inhalation of spore-borne toxins are also important sources of exposure (Bennett and Klich, 2003).

Exposure to mycotoxins lead to several, often unrecognized, diseases known as mycotoxicoses (Peraica et al., 1999, Zain, 2011). Mycotoxicoses are common in areas where there are poor methods of food handling and improper storage of food. It also frequently occurs in countries such as South Africa (SA) where malnutrition is a problem and where maize and cereal grains form a staple diet for many people (Bennett and Klich, 2003). The severity of mycotoxicoses vary among individuals and are dependent on the type of mycotoxin, the dose and length of exposure, route of exposure as well as the health and sex of the affected individual (Bennett and Klich, 2003, Peraica et al., 1999). The ability of mycotoxins to act synergistically with other chemicals to which the individual may have been exposed is also a major determinant of toxicity (Peraica et al., 1999). Mycotoxicoses, like all other toxicological syndromes, can be categorized as acute or chronic (Bennett and Klich, 2003). Acute or short-term toxicity generally have a rapid onset with an obvious toxic response whereas chronic or long-term toxicity usually occur at low doses and have irreversible effects (Bennett and Klich, 2003).

Mycotoxins are natural contaminants of food and therefore, their production is often inevitable. Several efforts to address mycotoxin exposure involve the removal of mycotoxin-contaminated commodities from the food supply by regular government screening. Despite efforts to control fungal contamination, extensive mycotoxin contamination is continuously reported in foods and feeds around the world (Pinotti et al., 2016).

1.2. Fusaric Acid: A *Fusarium* Mycotoxin

The genus *Fusarium* comprises of over 1 000 fungal species that cause diseases on agricultural crops with severe economic consequences worldwide (Bouarab, 2009). The *Fusarium* species are remarkably diverse and adaptable fungi found in soils globally (Bouarab, 2009). They are major plant pathogens and are capable of producing a wide variety of toxic metabolites. A large number of plants are symptomatically and asymptomatically infected by the *Fusarium* species causing immense damage and major economic losses (Bacon et al., 1996). These fungi have been identified on cereal crops in Western Europe and North America; wheat, cotton and barley in China; and rice in Japan, Taiwan and Thailand (Bouarab, 2009); where they have been implicated in causing head blights, ear rot and wilting (Bouarab, 2009).

Fusaric acid (FA ; 5-butylpicolinic acid or 5-n-butyl-pyridine-2-carboxylic acid) is a secondary metabolite and mycotoxin produced by several members of the *Fusarium* species (Fairchild et al., 2005). These include, among others, *Fusarium moniliforme*, *Fusarium oxysporum* and *Fusarium heterosporum* (Fairchild et al., 2005, Bacon et al., 1996). Fusaric acid is the most widely distributed mycotoxin produced by the *Fusarium* species and thus may serve as a presumptive indicator of *Fusarium* contamination in foods and feeds (Bacon et al., 1996).

Fusaric acid is a common contaminant of maize and other cereal grains such as barley, wheat millets and sorghum (Figure 1.1) (Bacon et al., 1996, Voss et al., 1999, Ogunbo et al., 2007). Fusaric acid is an often neglected mycotoxin and surveys to determine the concentration of FA in feeds and other agricultural products are limited (Voss et al., 1999). The concentration of FA naturally occurring in foods is approximately 643µg/kg (Streit et al., 2013). However, Bacon et al (1996) showed that the *Fusarium* species are capable of producing high levels of FA, ranging from 20 mg/ml to 1 080 mg/ml, in autoclaved maize (Bacon et al., 1996). Another study found that corn and swine feeds were contaminated with up to 36 ppm FA (Smith and Sousadias, 1993) and up to 12.4 ppm was found in toxic poultry and livestock feeds (Voss et al., 1999).

The prevalence of mycotoxins in foods and feeds is a major focus of several studies (Darwish et al., 2014, Hawkins, 2013, Hawkins, 2014). A recent study determined the prevalence of mycotoxins in corn and other cereal grains (Hawkins, 2014). In particular, approximately 30% of wheat and 79% of corn were contaminated with FA (Figure 1.2) (Hawkins, 2014). This is of great concern as maize and cereal grains form an integral part of people's diets and such a high degree of contamination increases the possibility of animal and human exposure to FA, posing a major health hazard when consumed.
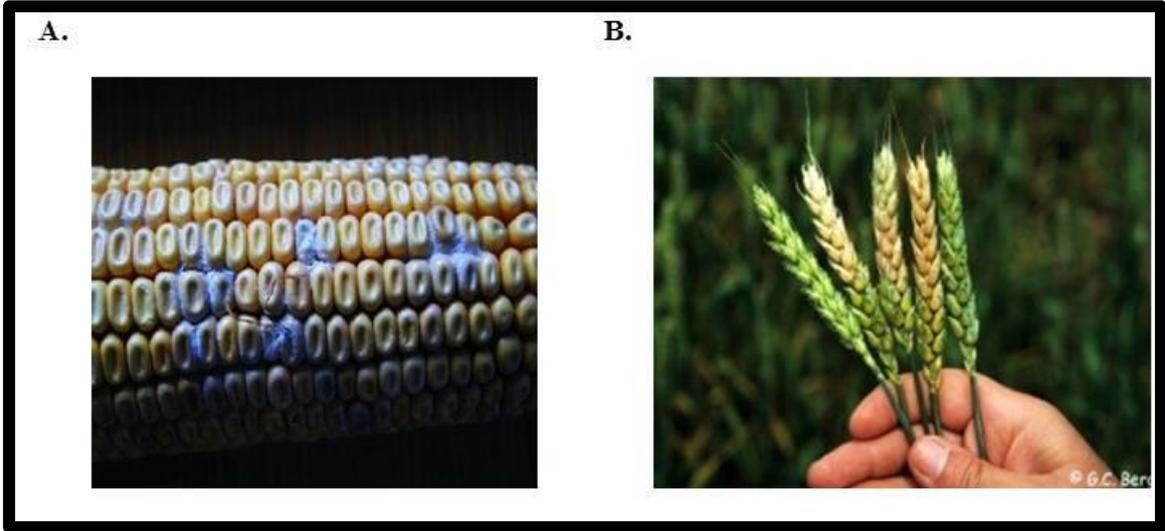
Figure 1.1: *Fusarium* contaminated maize (A) and wheat (B) (Griessler, 2008, Bergstrom, 2014)
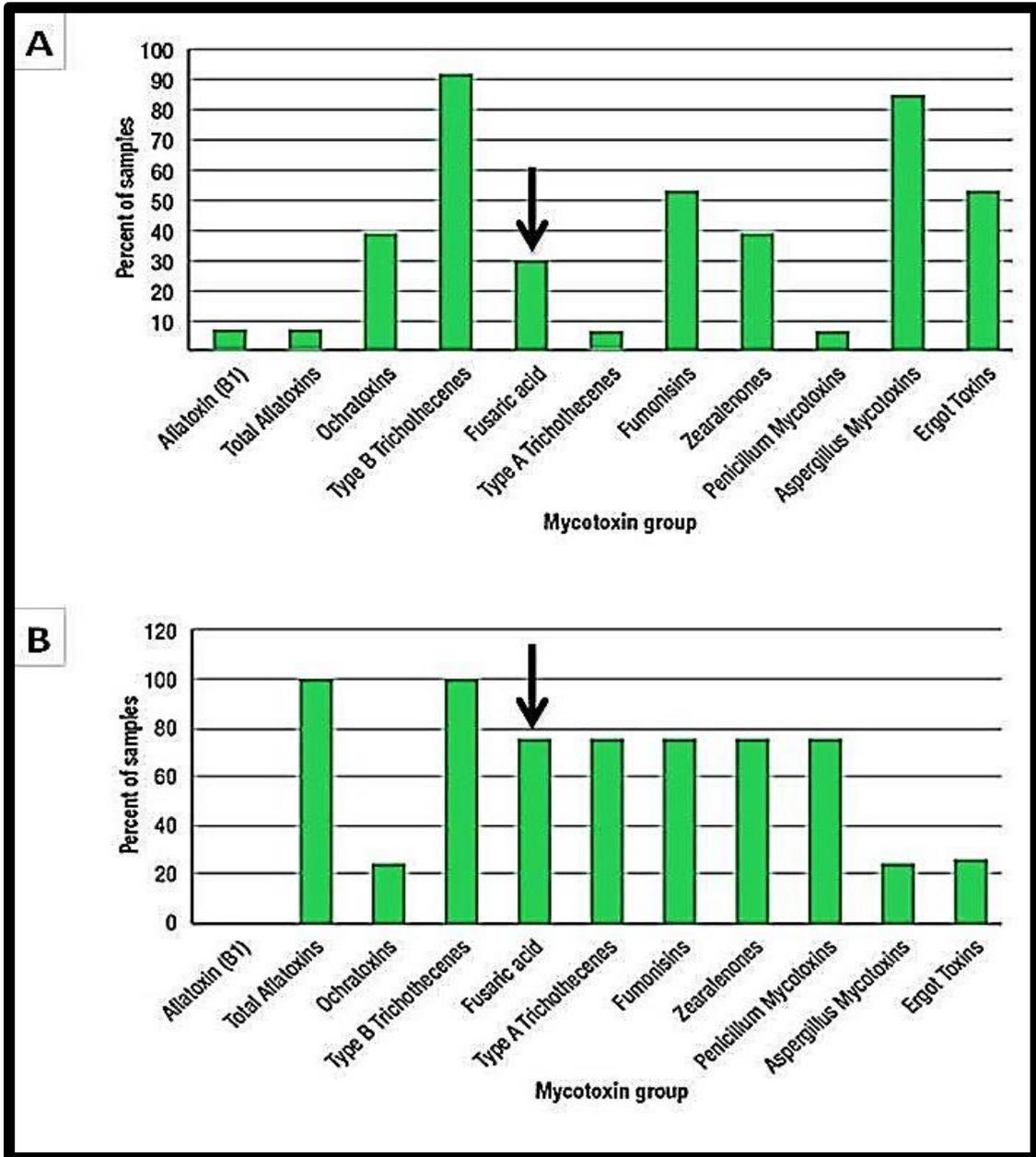
Figure 1.2: Percentage of wheat (A) and corn (B) contaminated with FA (Hawkins, 2014)

1.2.1. Structure of Fusaric Acid

Fusaric acid is a product of *Fusarium* fungal fermentation and a physiologic metabolite of tryptophan and Picolinic acid (Stack et al., 2004). Picolinic acid also known as 2-picolinic acid and 2-pyridine carboxylic acid is a six membered ring structure and isomer of nicotinic acid that consists of the chemical formula, $C_6H_5O_2N$ (Grant et al., 2009). The ability to act as a metal chelating agent and induce an anti-proliferative effect is one of the most widely studied characteristics of Picolinic acid (Fernandez-Pol et al., 1977, Grant et al., 2009). Picolinic acid is a bidentate metal chelating agent known to efficiently chelate copper, iron, zinc and cadmium (Fernandez-Pol et al., 1977, Grant et al., 2009). These metal ions are essential for the growth of cells and their chelation by Picolinic acid may provide a mechanism by which this compound exerts its anti-proliferative effect (Grant et al., 2009). A previous study conducted by Fernandez-Pol (1977), showed that Picolinic acid inhibits the growth of normal rat kidney (NRK) cells in a dose-dependent manner. This may occur by interfering with the specific metal requirements for cell growth and by altering NAD+ metabolism (Fernandez-Pol et al., 1977). Picolinic acid is a pyridine derivative that has the capacity to undergo exchange reactions with the nicotinamide moiety of NAD+ and thus forms NAD+ analogues (Fernandez-Pol et al., 1977, Bochner et al., 1980).

Fusaric acid is a Picolinic acid derivative and well-known metal chelating agent consisting of the chemical formula, $C_{10}H_{13}O_2N$ (ŠroBároVá et al., 2009). It contains the well conserved chelating structure of Picolinic acid and an additional fused aromatic ring structure or 5-butyl side chain (Figure 1.3) (Bochner et al., 1980). The butyl side chain is an important component in the structure of FA as it increases the lipophilicity of FA, enabling it to penetrate cell membranes which are mostly composed of lipids (Bochner et al., 1980). The structure of FA also contains a hydroxyl (OH^-) group that acts as a proton donor and is responsible for most of the weak acid properties of FA.

Fusaric acid is a pyridine derivative and chelator of divalent cations (Li et al., 2013). Like Picolinic acid, it is capable of forming conjugates with zinc, iron, copper and manganese thereby, forming chelates that make these ions inaccessible (Li et al., 2013). This occurs via the N-atom of the pyridine ring of FA which binds with the carboxyl group of the metal thereby, preventing these ions from functioning in various biological processes (Swinburne, 2012).
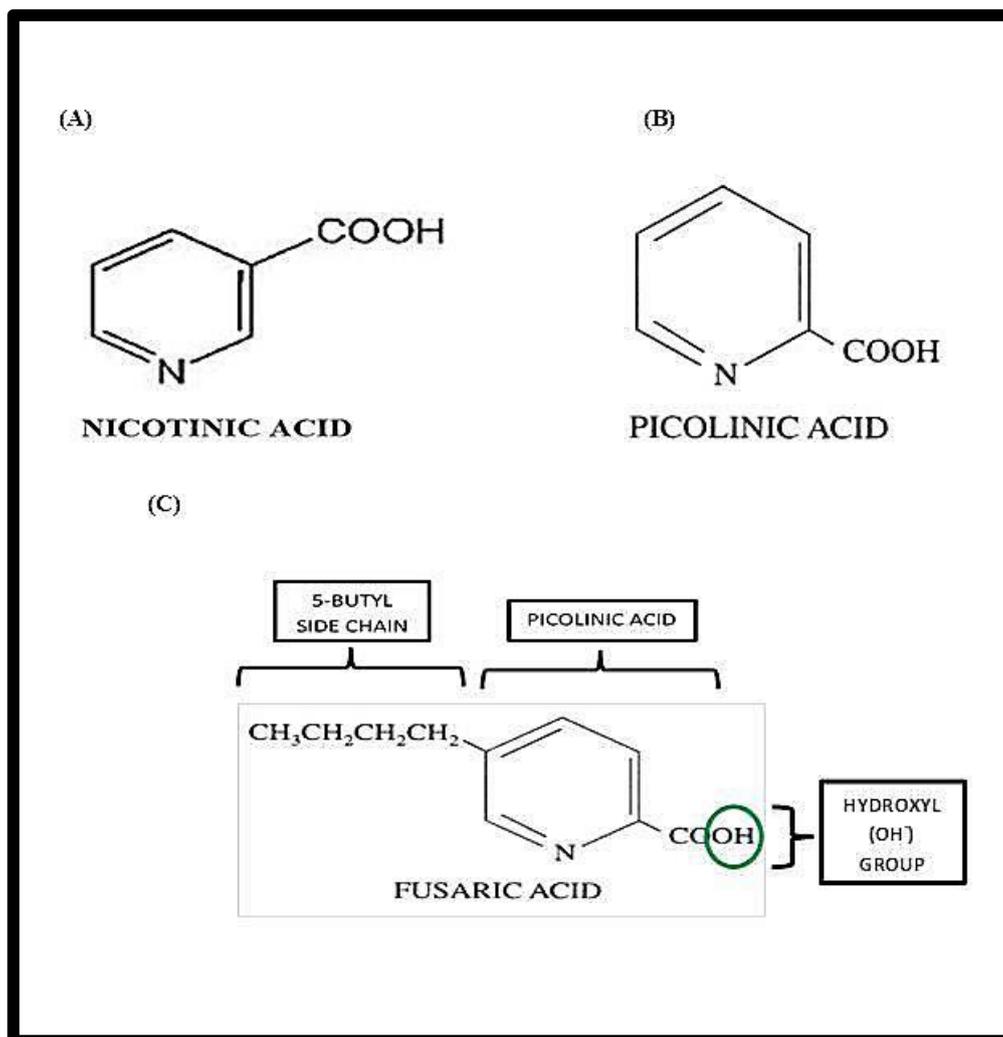
Figure 1.3: Structural comparison between nicotinic acid (A), Picolinic acid (B) and Fusaric acid (C) (May et al., 2000)

1.2.2. Effects of Fusaric Acid

Fusaric acid is a non-specific fungal toxin known to have numerous effects in both plants and animals (ŠroBároVá et al., 2009). However, the exact mechanism of action is yet to be elucidated. Fusaric acid is a well-known phytotoxin that causes wilt disease symptoms (necrotic spots on leaf blades, shrivelling and drying of leaves and shrinking of the stems and petioles) in a variety of plants (Rani et al., 2009, Pirayesh et al., 2015). Other effects of FA on plants include alterations in membrane permeability (Pavlovkin et al., 2004, D'Alton and Etherton, 1984), increased electrolyte leakage (D'Alton and Etherton, 1984) and inhibition of respiration (D'Alton and Etherton, 1984, Pavlovkin et al., 2004).

Fusaric acid is a cell membrane permeating weak acid and is therefore, potentially toxic as a proton conductor (Bochner et al., 1980). It alters the mitochondrial membrane potential of cells and decreases ATP production (Diniz and Oliveira, 2009, D'Alton and Etherton, 1984, Pavlovkin et al., 2004). This may occur by inhibiting cytochrome c oxidase and the ATPase/ATP synthase pump (Telles-Pupulin et al., 1996). Fusaric acid impairs mitochondrial function and biogenesis in HepG$_2$ cells; and induces apoptosis by increasing the activity of caspases-3/7 (Abdul et al., 2016). Fusaric acid also elevates the oxidative stress biomarker lipid peroxidation and increases the activity of anti-oxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (Sapko et al., 2011).

Fusaric acid is toxic to mice (intra peritoneal LD$_{50}$ 80mg/kg and intravenous LD$_{50}$ 100mg/kg) and death caused by the lethal dose has been attributed to its hypotensive effect (Hidaka et al., 1969, Pirayesh et al., 2015). The ability of FA to cause a significant decrease in blood pressure has also been observed in cats, dogs, rabbits and rats as a result of the inhibition of dopamine-β-hydroxylase (Pirayesh et al., 2015, Hidaka et al., 1969), a key enzyme in the synthesis of the neurotransmitter norepinephrine (Fairchild et al., 2005). Fusaric acid is a potent inhibitor of dopamine-β-hydroxylase and a decrease in the endogenous levels of norepinephrine has been observed in the heart, spleen, brain and adrenal glands of rats (Terasawa and Kameyama, 1971, Hidaka et al., 1969).

The removal of essential metal ions by FA may serve as a mechanism by which this compound exerts its toxicity (Stack et al., 2004). Studies on several human cancers (mainly epidermoid cancers such as adenocarcinoma and hepatocellular carcinoma) revealed that FA has anti-tumour activity (Rani et al., 2009, Stack Jr et al., 2014, Stack et al., 2004). Fusaric acid has anti-tumour activity against head and neck squamous cell carcinoma (HNSCC) by increasing DNA damage and preventing its synthesis and repair (Stack et al., 2004, Stack Jr et al., 2014). This may occur due to the chelation of divalent cations from catalytic DNA associated metalloproteins (Stack et al., 2004) and inactivation of zinc finger proteins (Rani et al., 2009). Two HNSCC cell lines (UMSCC-1 and Cal-27 cells) and a mouse model showed FA to have both tumouristatic and tumouricidal effects respectively (Stack et al., 2004). The removal of metal ions by FA inhibits metal-containing oxidative enzymes (Rani et al., 2009) and alters the conformation of proteins that require metal ions in order to maintain structural stability. Fusaric acid also increases cytokine production in Hep-2 (human cervical carcinoma cells) and Hep-DOC (docetaxel-resistant Hep-2 cells) cells and this may be responsible for the cytostatic and cytocidal effects of FA (Ye et al., 2013). Fusaric acid also inhibits the proliferation of WI-38

fibroblasts and is a potent inhibitor of DNA synthesis in MDA-MB-468 cells and WI-38 fibroblasts (Fernandez-Pol et al., 1993).

Another study showed FA to be toxic to mice by chelating calcium causing a delay in bone ossification and affecting the growth of foetuses (Reddy et al., 1996). Fusaric acid was also shown to be toxic to zebrafish by chelating copper and inhibiting the enzyme lysyl oxidase resulting in notochord malformation (Yin et al., 2015).

Fusaric acid is known to enhance the toxicity of other mycotoxins in both plants and animals (Bacon et al., 1996). Fusaric acid is not the only mycotoxin produced by the *Fusarium* species. Other mycotoxins such as zearalenones, trichothecenes and fumonisins are also produced by the *Fusarium* species (Bennett and Klich, 2003, Peraica et al., 1999). These mycotoxins are common contaminants of various food commodities and are often produced in combination with FA. Therefore, the toxicity of FA may be ascribed to its ability to act synergistically with other mycotoxins (Bacon et al., 1996).

A study conducted on pigs indicated that FA increased the toxicity of deoxynivalenol (a β-trichothecene) by competing with tryptophan for binding to blood albumin and therefore, increased the concentration of free tryptophan in the blood. This leads to an increased uptake of tryptophan in the brain and increased serotonin synthesis (Smith et al., 1997).

Exposure to FA enhanced vomiting, feed refusal and brain metabolism in pigs given trichothecenes (Smith and MacDonald, 1991). However, Ogunbo et al. (2007), showed no toxic synergy between FA and T2-toxin in broiler chicks and young turkey poults. This is in keeping with the findings of Fairchild et al. (2005) in which FA had no significant effect on body weight and body weight gains in turkey poults when fed up to 300 ppm of FA. Fusaric acid also dose-dependently increased food intake in chicks (Bungo et al., 1999).

Synergism between 4, 15-diacetoxyscirpenol (DAS) (a type A trichothecene) and FA has been demonstrated in insects (Dowd, 1988). Fusaric acid enhanced the toxicity of DAS in insects as it increased mortality from 5% with DAS alone to over 20% with DAS and FA combined (Dowd, 1988). However, FA antagonized the negative effects caused by DAS alone (Dowd, 1988). Similar results were obtained by Fairchild et al. (2005) in which turkey poults fed a combination of DAS and FA showed a recovery in body weight and body weight gains as opposed to a diet with FA and DAS alone.

Fumonisin B_1 is another mycotoxin produced by the *Fusarium* species (Bennett and Klich, 2003, Milićević et al., 2010, Peraica et al., 1999, Zain, 2011). A study into the effect of FA and

Fumonisin B_1 on chicken embryos revealed that the treatment of chicken embryos with FA and Fumonisin B_1 alone did not cause a toxic response. However, when combined a toxic response was observed (Bacon et al., 1995). This suggests that FA may have a synergistic effect with Fumonisin B_1 (Bacon et al., 1995). Although, no synergistic effects were observed with FA and Fumonisin B_1 in rats (Voss et al., 1999).

1.3. Apoptosis: Programmed Cell Death

Apoptosis (programmed cell death) is responsible for the deletion of cells in normal tissues as well as in some pathological states (Kerr et al., 1994). It is a sequential process involving cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and the formation of apoptotic bodies. The apoptotic bodies are phagocytosed and digested by nearby resident cells (macrophages and other phagocytes), preventing the spillage of intracellular contents and avoiding inflammatory changes (Kerr et al., 1994). Apoptotic bodies not subjected to phagocytosis are released into the adjacent lumen where they display progressive dilation and degradation of cytoplasmic organelles in a process known as necrosis. Necrosis differs from apoptosis in which the cell swells and the plasma membrane ruptures releasing cytosolic contents into the extracellular space where they produce an inflammatory response (Woodle and Kulkarni, 1998).

1.3.1. Caspases

Caspases also known as death proteases are a group of cysteine proteases responsible for the initiation and execution of apoptosis (Hengartner, 2000). Caspases consist of an active-site cysteine and are known to cleave substrates after aspartic acid residues (Asp-Xxx) (Hengartner, 2000). In order to control the apoptotic process, caspases are initially synthesized as inactive zymogens. These zymogens consist of three domains – an N-terminal pro-domain, a p20 domain and a p10 domain. Proteolytic cleavage of these zymogens between the p20 and p10 domains as well as between the pro-domain and p20 domain leads to the activation of caspases (Hengartner, 2000). Caspases act by inactivating or activating apoptotic-regulatory proteins and are responsible for most of the morphological changes observed during apoptosis (Hengartner, 2000).

Initiator caspases such as caspase-2, -8 and -9 are the apical caspases in apoptosis and their activation is required for the cleavage and activation of the downstream executioner caspases-3, -6 and -7. The activation of executioner caspases usually ensures that apoptosis occurs (Hengartner, 2000, Hongmei, 2012).

1.3.2. Pathways of Apoptosis

Apoptosis is triggered by a variety of physiological death signals as well as pathological cellular insults (Hongmei, 2012). It occurs via several pathways of which the two most common pathways involve caspase activation and are termed the intrinsic/mitochondrial pathway and the extrinsic/death receptor pathway.

1.3.2.1. The Intrinsic/Mitochondrial Apoptotic Pathway

The intrinsic apoptotic pathway is initiated in response to radiation, viral infection, irreparable DNA damage and oxidative stress (Hongmei, 2012). The mitochondria are central components of the intrinsic apoptotic pathway as it is the site where most of the pro- and anti-apoptotic molecules are located (Gross et al., 1999). Anti-apoptotic molecules such as BCL-2 are found on mitochondrial membranes whereas pro-apoptotic molecules such as BAX and cytochrome c are found in the cytosol and within the mitochondria respectively (Gross et al., 1999).

BAX translocates from the cytosol to the mitochondria, following a death signal, where it undergoes a conformational change causing it to become an integral membrane protein (Gross et al., 1999). BAX interacts with members of the permeability transition pore (PTP; adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC) and peripheral benzodiazepine receptor (PBR)) on the mitochondrial membrane resulting in the opening of the PTP and the release of cytochrome c and apoptosis inducing factor (AIF) from the mitochondria (Gross et al., 1999, Hongmei, 2012). The release of cytochrome c enables binding with the apoptotic protease activating factor-1 (Apaf-1), pro-caspase-9 and ATP to form an apoptosome. Apoptosome formation activates caspase-9, consequently activating executioner caspases-3/7 resulting in apoptotic cell death (Figure 1.4) (Hengartner, 2000, Fulda, 2010).

BCL-2 is an anti-apoptotic molecule that plays an important role in maintaining the integrity of the mitochondrial membrane. BCL-2 inhibits cellular free radical formation, cytochrome c

release and the activation of caspases thus preventing apoptosis (Rego et al., 2001). Following apoptotic stimuli, the BH3-domain-only molecule (BIM) translocates to the mitochondria where it interacts with BCL-2 to antagonize its anti-apoptotic activity and promote apoptosis (Gross et al., 1999).

1.3.2.2. The Extrinsic/Death Receptor Pathway

The binding of death inducing ligands or soluble factors to death receptors on cell membranes leads to the activation of the extrinsic apoptotic pathway. In this pathway, death ligands such as tumour necrosis factor alpha (TNF-α), tumour necrosis factor related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL) bind to their death receptors on the cells surface, type 1 TNF receptor (TNFR1), death receptor 4/5 (DR4/5) and Fas respectively (Okada and Mak, 2004, Hongmei, 2012). These death receptors have an intracellular death domain that recruits adaptor proteins such as TNF-receptor associated death domain (TRADD), Fas associated death domain (FADD) and cysteine proteases such as caspase-8. The binding of an adaptor protein to the death ligand-death receptor complex results in the formation of a death inducing signalling complex (DISC). DISC is responsible for the assembly and activation of pro-caspase-8 to caspase-8. Caspase-8 activates caspase-3/7 which leads to apoptosis (Figure 1.4) (Gross et al., 1999, Hongmei, 2012, Fulda, 2010).

The activation of the extrinsic apoptotic pathway can lead to the activation of the intrinsic apoptotic pathway via the BH3-domain-only molecule, Bid (Gross et al., 1999). The activation of caspase-8 (via the extrinsic apoptotic pathway) results in the cleavage of cytosolic p22 Bid at the amino terminus leading to the generation of a p15 carboxy-terminal fragment of Bid, truncated p15 Bid (tBid). tBid translocates to the mitochondria and directly activates pro-apoptotic proteins to induce mitochondrial outer membrane permeabilization (Chipuk and Green, 2006). This leads to the release of cytochrome c and activation of the intrinsic apoptotic cascade (Figure 1.4) (Gross et al., 1999).
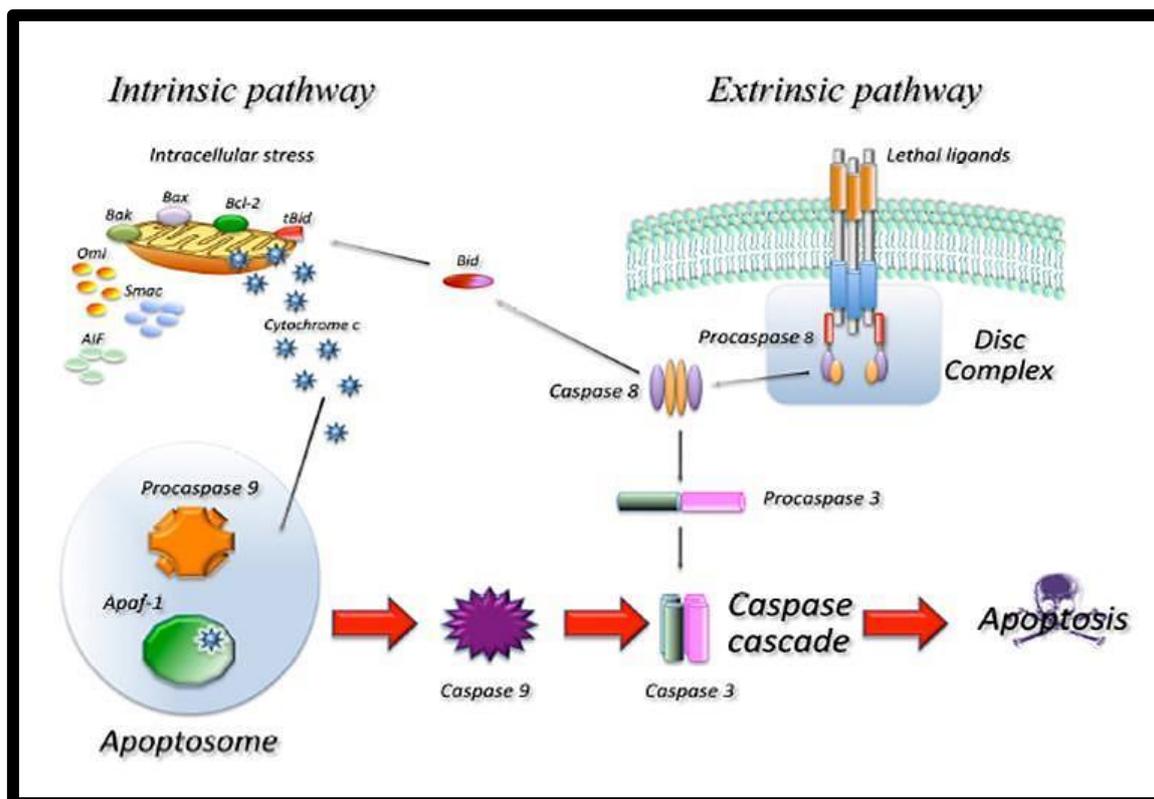
Figure 1.4: Extrinsic and Intrinsic Pathways of Apoptosis (Favaloro et al., 2012)

1.4. DNA Damage

Irreparable DNA damage is the major initiating event in apoptosis. DNA damage is caused by several endogenous (oxidative stress, replication errors) and exogenous (UV radiation, chemicals, toxins) agents. Common types of DNA damage include covalent modifications of bases (oxidation, hydrolysis, alkylation), breaks in the DNA backbone occurring in one (single-strand) or both (double-strand) DNA strands and crosslinks between bases on the same or opposite DNA strands and between DNA and protein molecules (Figure 1.5) (Sancar et al., 2004). Cells have evolved numerous mechanisms of halting cell division and repairing DNA damage (Sancar et al., 2004). However, sometimes DNA damage is so extensive it cannot be repaired and the affected cell is removed via apoptosis (Hongmei, 2012). In the event that apoptosis does not occur, normal functioning of the cell is disrupted and base lesions accumulate increasing the possibility of mutations and cancer development.
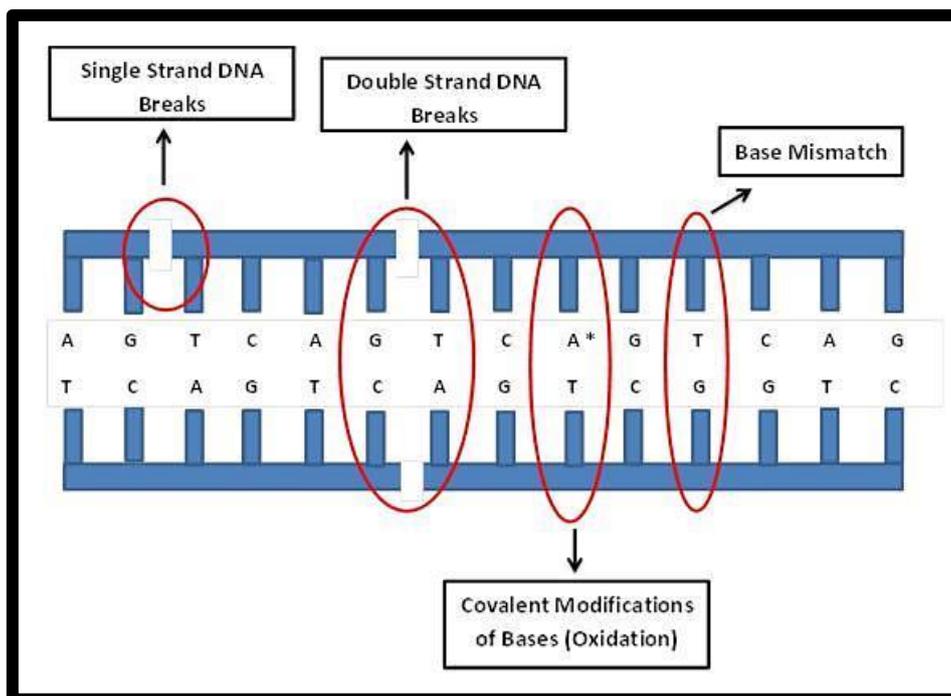
Figure 1.5: Common types of DNA damage (Prepared by author)

1.5. The p53 Tumour Suppressor Protein

DNA damage is the most common cause of p53 activation. p53 is a tumour suppressor protein and transcription factor involved in regulating the expression of genes critical for cell cycle arrest and apoptosis (Brooks and Gu, 2003, George, 2011, Reed and Quelle, 2014). It is encoded by one of the most frequently mutated genes in human cancers and more than 50% of cancers have been reported to contain mutated or inactive p53 (Barlev et al., 2001, George, 2011, O'Brate and Giannakakou, 2003, Zhang et al., 2015).

p53 plays a major role in several cell signalling pathways and is activated in response to DNA damage, hypoxia, excessive oncogene activation and other stressors (Figure 1.6) (Prives and Hall, 1999). Once activated, p53 acts as a critical regulator of cell proliferation by functioning as a checkpoint protein to monitor DNA damage, arrest the cell cycle and initiate DNA repair prior to cell division (Barlev et al., 2001). p53 also mediates apoptosis in cells with irreparable DNA damage. In this way, p53 functions to maintain genomic stability and is often referred to as the "guardian of the genome" (Brooks and Gu, 2003, George, 2011, Reed and Quelle, 2014).

p53 also activates the transcription of certain genes such as MDM2, p21 and BAX which are involved in regulating cell proliferation and apoptosis. It serves as a negative transcription factor to down-regulate the gene expression of BCL-2 (Kang et al., 2015) and can bind directly to anti-apoptotic BCL-2 proteins and activate pro-apoptotic BCL-2 proteins (eg. BAX). This enables p53 to regulate mitochondrial outer membrane potential and apoptosis (Chipuk and Green, 2006).
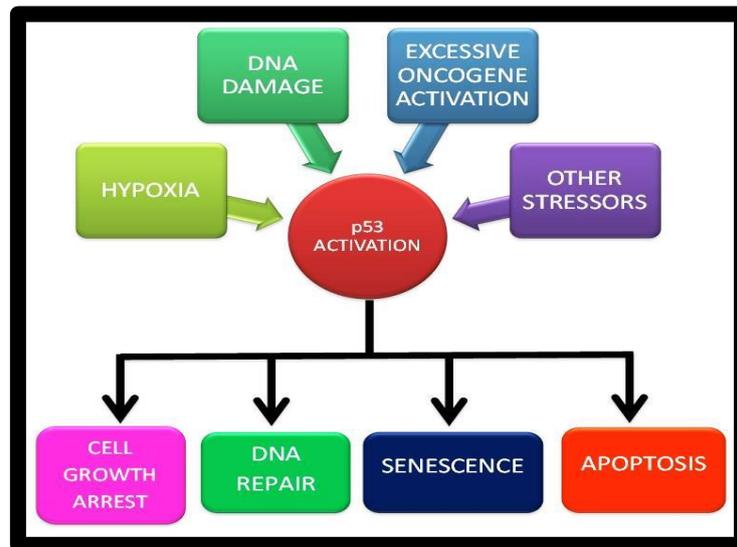
Figure 1.6: Activation and function of p53 (Prepared by author)

1.5.1. The Structure of p53

The p53 gene, *TP53*, is located on the short arm of human chromosome 17 (17p13.1) and encodes a 53kDa nuclear phosphoprotein upon translation (George, 2011). The p53 protein consists of 393 amino acids and is made up of three main regions – an acidic N-terminal region, a central core region and a basic C-terminal region (Figure 1.7). The N-terminal region includes a transactivation domain and a proline rich domain. The transactivation domain consists of amino acids 1 to 42 and is involved in the activation of transcription factors (George, 2011, Lambert et al., 1998). It interacts with several proteins such as MDM2, TATA-binding protein (TBP) and protein kinases that regulate the stability and activity of p53 (Chao et al., 2003, Lambert et al., 1998, Thakur et al., 2012). The proline rich domain consists of amino acids 63 to 97 and is required for p53-mediated apoptosis.

The central core region comprises of a DNA-binding domain (amino acids 98 to 292) that interacts with DNA in a sequence-specific manner. The DNA binding domain consists of two copies of the 10bp consensus sequence, 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' (Pu=A/G, Py=T/C), separated by approximately 0 to 13bp (Benchimol, 2001) and it is the site where the majority of cancer associated mutations are detected (Prives and Hall, 1999). These mutations are usually missense mutations that alter the conformation of the DNA-binding domain and prevent the binding of p53 to DNA (Dai and Gu, 2010, Prives and Hall, 1999). This domain is stabilized by the tetrahedral coordination of a zinc ion with cysteine 176, histidine 179, cysteine 238 and cysteine 242 (Joerger and Fersht, 2010).

The C-terminal region comprises of a tetramerization domain and a regulatory domain. The tetramerization domain (amino acids 324 to 355) is involved in the oligomerization and activation of p53. The regulatory domain (amino acids 363 to 393) functions as a transcriptional regulator and is involved in recognizing DNA damage. The C-terminal region also contains three nuclear localization signals (NLS) and two nuclear export signals (NES) that are responsible for the import and export of p53 to and from the nucleus (O'Brate and Giannakakou, 2003).
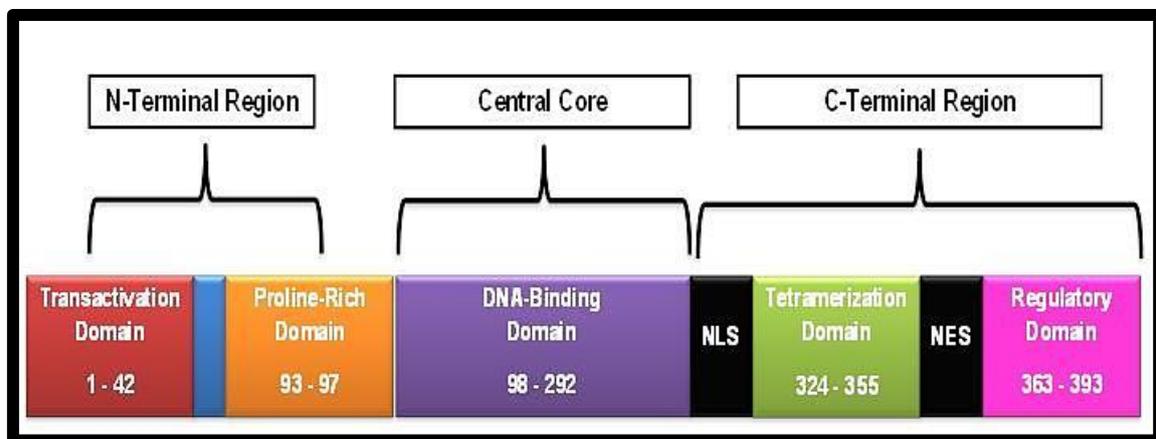
Figure 1.7: Structure of p53 (Prepared by author)

1.5.2. Post-translational Modifications of p53

p53 is synthesised in the cytoplasm where it remains latent in the absence of cellular stress (George, 2011, O'Brate and Giannakakou, 2003). Exposure to stress such as DNA damage, hypoxia and excessive oncogene activation causes p53 to translocate to the nucleus where it

exerts its transcriptional effects (O'Brate and Giannakakou, 2003). p53 can also function in a transcription-independent manner in the cytoplasm to regulate processes such as autophagy and apoptosis (Dai and Gu, 2010, Green and Kroemer, 2009).

The tight regulation of p53 function is essential for maintaining normal cell growth and preventing tumourigenesis (Brooks and Gu, 2003). p53 is subjected to a variety of post-translational modifications that regulate its function as a tumour suppressor protein (van Leeuwen et al., 2013). These modifications occur mainly in the N- and C-terminal regions of the protein and include ubiquitination, phosphorylation and acetylation (Barlev et al., 2001, Reed and Quelle, 2014, van Leeuwen et al., 2013). Other post-translational modifications of p53 include neddylation, sumoylation and methylation (Dai and Gu, 2010).

1.5.2.1. Ubiquitination of p53

In normal healthy cells, p53 is a short-lived protein (half-life of approximately 10 min) that is maintained at very low levels due to the continuous ubiquitination by the protein MDM2 (Thakur et al., 2012). MDM2 is synthesised in the cytoplasm and must translocate to the nucleus where it functions to ubiquitinate p53. The phosphorylation of MDM2 on Serine 166 and 168 by phosphoinositol 3 kinase (PI3K) and protein kinase B (PKB) result in the translocation of MDM2 from the cytoplasm to the nucleus (O'Brate and Giannakakou, 2003).

Ubiquitination refers to the covalent conjugation of one or more ubiquitin molecules to a protein substrate. This process plays a key role in regulating p53 stability and localization. MDM2 is a major E3 ubiquitin ligase and negative regulator of p53 (Dai and Gu, 2010, Brooks and Gu, 2003). It ubiquitinates p53 at lysine (K) residues (K370, K372, K373, K381, K382 and K386) within the C-terminal regulatory domain (Dai and Gu, 2010). p53 can be mono-ubiquitinated or poly-ubiquitinated depending on the level of MDM2. High levels of MDM2 poly-ubiquitinates p53 whereas low levels of MDM2 mono-ubiquitinates p53 (Dai and Gu, 2010).

The ubiquitination of p53 occurs in the nucleus and depending on the level of ubiquitination; p53 can undergo one of two fates. Poly-ubiquitinated p53 is generally degraded in the nucleus by the 26S proteasome whereas mono-ubiquitinated p53 inhibits DNA binding and is exported from the nucleus to the cytoplasm (Dai and Gu, 2010, Green and Kroemer, 2009, Reed and Quelle, 2014). In the cytoplasm, mono-ubiquitinated p53 can become further ubiquitinated by ARF-BP1 and Pirh2 and then degraded by the 26S proteasome (Dai and Gu, 2010, Green and Kroemer, 2009). Mono-ubiquitinated p53 in the cytoplasm was also shown to travel to the

mitochondria where it becomes de-ubiquitinated by the mitochondrial enzyme, Herpesvirus-Associated Ubiquitin-Specific Protease (HAUSP). This leads to the generation of the apoptotically active non-ubiquitinated p53 which induces mitochondrial outer membrane permeabilization thus, triggering the release of pro-apoptotic factors and causing apoptosis in a transcription-independent manner (Figure 1.8) (Green and Kroemer, 2009). HAUSP is a direct antagonist of MDM2 activity and functions by specifically removing ubiquitin molecules from p53 when stimulated by DNA damage. Therefore, HAUSP protects p53 from MDM2-mediated degradation and allows the rapid accumulation of p53 upon DNA damage (Brooks and Gu, 2003).

MDM2 can also inhibit p53 transcriptional activity by binding directly to the N-terminal region of p53 and blocking the access of transcription factors to the transactivation domain of p53 (George, 2011, Brooks and Gu, 2003). The MDM2 gene is an important transcriptional target of p53 and the stress-induced increase in p53 levels can induce the expression of MDM2, which in turn downregulates p53, creating a negative feedback loop (Dai and Gu, 2010, Gu et al., 2008). However, during apoptosis MDM2 is cleaved by caspase-3 generating a 60kDa fragment (molecular weight of MDM2 is normally 90kDa) that is capable of binding to p53 but lacks the characteristic E3 ubiquitin ligase activity (Pochampally et al., 1998). In this way, p53 is not targeted for degradation and hence it accumulates within cells.

MDM2 has been established as an oncogene as its overexpression has been observed in several human cancers such as breast cancer, oesophageal cancer and ovarian cancer (Momand et al., 1998). Overexpression of MDM2 conveys tumourigenic potential by diminishing the ability of p53 to induce cell cycle arrest and apoptosis in response to cellular stress (Pochampally et al., 1998). The importance of MDM2 in regulating p53 activity was further determined by the observation that *MDM2* null mice die as a result of p53-dependent apoptosis (Pochampally et al., 1998).

1.5.2.2. Phosphorylation of p53

p53 stabilization and transcriptional activation are crucial early events in a cell's battle against genotoxic stress (Brooks and Gu, 2003). The p53 protein harbors an array of serine and threonine residues in the N-terminal transactivation domain and the C-terminal regulatory domain that are phosphorylated or dephosphorylated in response to genotoxic stress (Brooks and Gu, 2003, Dai and Gu, 2010).

Following DNA damage, p53 is imported from the cytoplasm to the nucleus where it becomes phosphorylated by several protein kinases such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad 3 related (ATR), DNA-dependent protein kinase (DNA-PK), checkpoint kinases 1 and 2 (chk1 and chk2) and cyclin-dependent kinase activating kinase (CAK) (Figure 1.8) (Barlev et al., 2001, Brooks and Gu, 2003, Dai and Gu, 2010, Reed and Quelle, 2014). These kinases are activated by various forms of DNA damage and directly phosphorylate p53 on serine (Ser-15, Ser-20, Ser-33, Ser-37 and Ser-46) and threonine (Thr-18) residues (Reed and Quelle, 2014). Chk1 and chk2 can also phosphorylate C-terminal residues (Ser-313, Ser-314, Ser-315, Thr-377 and Ser-378) in response to DNA damage (George, 2011, Reed and Quelle, 2014). The de-phosphorylation of p53 at Ser-15 and Ser-37 negatively regulates p53-dependent cell death and promotes cell survival (Li et al., 2006). Several studies show that DNA damage induced phosphorylation on Ser-15 and Ser-37 of p53 alleviates its inhibition by MDM2 and is essential for the stabilization of p53 during cellular stress (Shieh et al., 1997). However, others argue that the phosphorylation of p53 is necessary for its acetylation by increasing its interaction with transcriptional coactivators and histone acetyltransferases (Barlev et al., 2001, Kang et al., 2015, Lambert et al., 1998, Tang et al., 2008).

1.5.2.3. Acetylation of p53

The role of histone acetylation and its involvement in the regulation of transcription has long been a topic of interest. Histone acetylation, catalysed by histone acetyltransferases (HATs), refers to the covalent linkage of an acetyl group from acetyl coenzyme A to the epsilon (ε) amino group of lysine residues (Delcuve et al., 2012, Tang et al., 2008). Histone deacetylation is catalysed by histone deacetylases (HDACs) and involves the removal of acetyl groups from the lysine residues of histones (Delcuve et al., 2012). There are four classes of histone deacetylases – class I (HDACs 1, 2, 3 and 8), class II (HDACs 4, 5, 6, 7, 9 and 10), class III (Sirtuins 1-7) and class IV (HDAC 11). Histone acetylation and deacetylation modify chromatin structure and are known to affect many DNA based events such as replication and transcription (Delcuve et al., 2012).

p53 was the first non-histone protein shown to be regulated by acetylation and deacetylation (Gu and Roeder, 1997). Several studies have shown that p53 acetylation is a crucial event that occurs in response to genotoxic stress and is essential for increasing p53 stability and stimulating its transcriptional activity (Barlev et al., 2001, Zhang et al., 2015, Solomon et al., 2006, Kang et al., 2015). Acetylation of p53 was also shown to promote its ability to bind to

DNA in a sequence-specific manner (Sakaguchi et al., 1998, Luo et al., 2004, Gu and Roeder, 1997) and recruit transcriptional co-activators to specific p53 response elements (Barlev et al., 2001).

Histone acetyltransferases such as CREB-binding protein (CBP)/p300 and p300/CBP-associated factor (PCAF) mediates acetylation of the C-terminal lysine residues of p53 through interaction with its N-terminal region. Eight acetylation sites (K120, K320, K370, K372, K373, K381, K382 and K386) have been identified for p53, six of which were found to occur in the C-terminal regulatory domain of p53 (K370, K372, K373, K381, K382 and K386) (Barlev et al., 2001, Dai and Gu, 2010, Reed and Quelle, 2014). K382 is the major lysine residue acetylated in response to DNA damage and an increase in the acetylation of K382 has been shown to correlate with an increase in p53 phosphorylation (Reed and Quelle, 2014). K320 in the tetramerization domain of p53 is acetylated by PCAF, which favours cell survival by promoting p53 mediated activation of cell cycle arrest target genes (Dai and Gu, 2010). The acetylation of K120 in the DNA-binding domain of p53 was shown to be essential for p53-dependent apoptosis but has no effect on the induction of p21 and cell growth arrest (Tang et al., 2008). Recently it was observed that another lysine residue, K164, in the DNA-binding domain of p53 is acetylated by CBP/p300 in response to DNA damage (Tang et al., 2008) and the acetylation of this lysine residue is involved in regulating a vast amount of p53 targets (Dai and Gu, 2010).

Histone deacetylation plays a major role in inhibiting p53-dependent transcriptional activity, cell growth arrest and apoptosis (Reed and Quelle, 2014, Zhang et al., 2015). The major deacetylases involved in deacetylating p53 are HDAC1 and sirtuin (Sirt) 1 (Brooks and Gu, 2003). HDAC1 is a zinc-dependent enzyme closely related to the yeast counterpart, *RPD3* (Delcuve et al., 2012). HDAC1 is found mainly in the nucleus and is responsible for deacetylating p53 at C-terminal lysine residues (K320, K373 and K382) thereby, inhibiting p53-dependent transcription (Dai and Gu, 2010, Reed and Quelle, 2014, Zhang et al., 2015).

Sirt1 is a class III NAD^+-dependent deacetylase closely related to the yeast counterpart, *Sir2* (silent mating type information regulation 2). Sirt1 is predominantly localized in the nucleus where it catalyses the deacetylation of acetyl-lysine residues in a reaction that cleaves NAD^+ and generates O-acetyl ADP-ribose (Solomon et al., 2006). Sirt1 has been shown to deacetylate p53 specifically on K382, which is important for inducing cellular senescence in cells with minimal DNA damage (Wang et al., 2008). In addition, Sirt1 causes p53 hypo-acetylation and inhibits p53-mediated apoptosis. This is in accordance with a study by Cheng et al. (2003) in which p53 hyper-acetylation and apoptosis were observed in *Sirt1*-deficient mice. The

deacetylation of p53 by Sirt1 has also been shown to promote p53 nuclear export resulting in its accumulation within the cytoplasm. Increased cytoplasmic p53 may enhance its passage to the mitochondria where it induces apoptosis. Hence, Sirt1 may have a role in inducing apoptosis in a transcription-independent manner (Yi and Luo, 2010). Deacetylation of p53 by Sirt1 can also promote its ubiquitination and subsequent degradation (Barlev et al., 2001, Tang et al., 2008).

Recently, it has been shown that p53 can be deacetylated in the cytoplasm and the deacetylase responsible for catalysing this reaction is the cytoplasmic Sirt2 (van Leeuwen et al., 2013). Sirt7 was also found to interact with and deacetylate p53 *in vitro* (Vakhrusheva et al., 2008). This is in accordance with a study in which *Sirt7*-deficient cells were found to contain hyper-acetylated p53 (Vakhrusheva et al., 2008).

1.5.3. The Role of MDM2 in Regulating p53 Acetylation

The role of MDM2 in regulating the acetylation of p53 has received much interest over recent years. Some studies show that MDM2 can decrease the acetylation of p53 by ubiquitinating CBP/p300 and mediating its export from the nucleus to the cytoplasm, where it acts as an E4 ubiquitin ligase to further ubiquitinate p53 and enable its degradation (Dai and Gu, 2010, Reed and Quelle, 2014, Zhang et al., 2015). Another study showed that p53 is ubiquitinated and acetylated on the same C-terminal lysine residues and hence, MDM2 and CBP/p300 may compete for the same binding sites (Brooks and Gu, 2003, Reed and Quelle, 2014, Zhang et al., 2015). Acetylation of p53 inhibits MDM2-mediated ubiquitination by blocking the recruitment of MDM2 to p53 responsive promoters and *vice versa* (Zhang et al., 2015). MDM2 also promotes p53 deacetylation by recruiting HDAC1 to acetylated p53 complexes (Ito et al., 2002). The unmodified lysine residues then become substrates for MDM2-mediated ubiquitination resulting in p53 degradation (Ito et al., 2002).
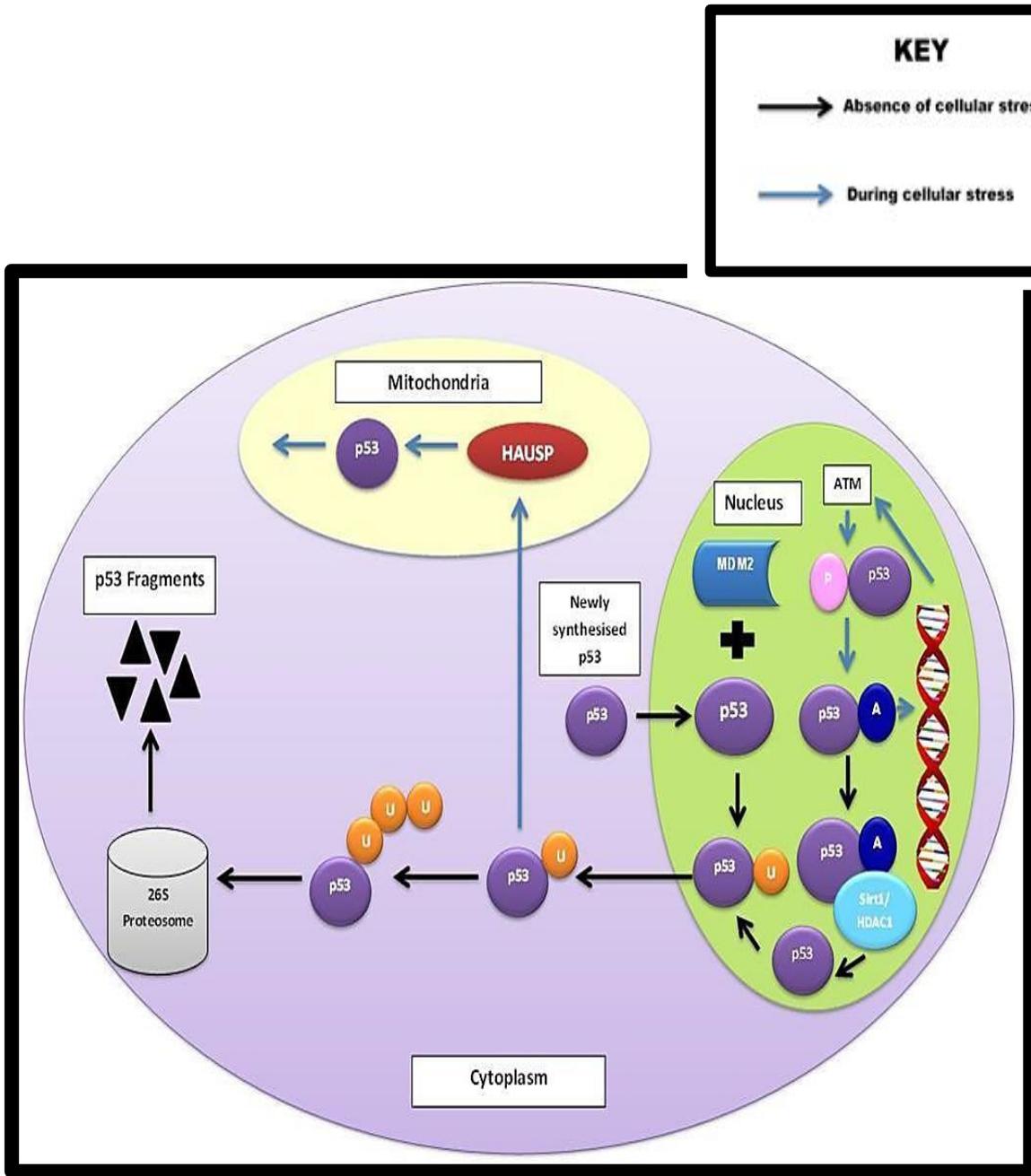
Figure 1.8: Regulation of p53 (Prepared by author)

1.6. Problem Statement, Aims and Objectives

Problem Statement

Mycotoxin contamination of foods and feeds is a serious problem occurring worldwide (Milićević et al., 2010). South Africa is the major producer of maize and other cereal grains such as barley, wheat millets and sorghum. It is responsible for producing large quantities of agricultural crops each year. However, the lack of proper food storage methods accompanied with the hot and humid weather of SA provide ideal conditions for the growth of pathogenic fungi and the production of mycotoxins. Fusaric acid is an often neglected mycotoxin that contaminates a wide variety of food sources especially, maize which forms part of the staple diet. Several studies have reported FA to be a toxic agent in both plants (D'Alton and Etherton, 1984, Diniz and Oliveira, 2009, Dong et al., 2012, Li et al., 2013, Sapko et al., 2011, Telles-Pupulin et al., 1996) and animals (Terasawa and Kameyama, 1971, Smith and Sousadias, 1993, Ogunbo et al., 2007, Hidaka et al., 1969, Dowd, 1988, Bungo et al., 1999, Fairchild et al., 2005, Voss et al., 1999). However, the exact mechanism by which this mycotoxin exerts its toxicity is still unknown. p53 is a major stress response protein that regulates a variety of cellular processes and therefore, its activation may provide a possible mechanism by which FA induces its toxicity. The ability of FA to induce DNA damage and activate p53 has not been previously reported.

Aim

To determine the effect of FA on DNA integrity and the post-translational modifications (phosphorylation and acetylation) of p53 in hepatocellular carcinoma (HepG$_2$) cells.

Null Hypothesis

Fusaric acid does not cause DNA damage and post-translationally modify p53 in HepG$_2$ cells.

Objectives

- ✓ To determine the effect of FA on DNA integrity in HepG$_2$ cells at 24 hrs using the single cell gel electrophoresis (SCGE)/comet assay.

- ✓ To determine the effect of FA on the protein expression of p53, phosphorylated-Ser15-p53, acetylated-K382-p53, MDM2, acetylated-CBP (K1535)/p300 (K1499), HDAC1 and phosphorylated-Ser47-Sirt1 in HepG$_2$ cells at 24 hrs using western blot.

- ✓ To determine the effect of FA on the cell cycle and apoptosis in HepG$_2$ cells at 24 hrs using the Hoechst 33342 assay.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Fusaric acid (FA), isolated from the fungus *Gibberella fujikuroi* (F6513), was purchased from Sigma-Aldrich (St. Louis, MO, USA). The human hepatocellular carcinoma (HepG$_2$) cell line was purchased from Highveld Biologicals (Johannesburg, SA). Cell culture equipment (cell culture flasks etc.) and reagents (Eagle's Minimum Essentials Medium (EMEM), trypsin, trypan blue etc.) were purchased from Lonza Biotechnology (Basel, Switzerland). Western Blot equipment and reagents were purchased from Bio-Rad (Hercules, CA, USA). All other reagents were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

2.2. Cell Culture

2.2.1. Introduction

The HepG$_2$ cell line, epithelial in morphology and consisting of 55 chromosome pairs, was derived from the liver tissue of a 15-year old Caucasian male possessing a well differentiated hepatocellular carcinoma. They are adherent cells that grow as monolayers or small aggregates in cell culture. Although often referred to as a liver cancer cell line, the HepG$_2$ cells are a common and reliable model for liver toxicity testing. These cells contain many liver-specific genes, which produce plasma proteins, retain cell surface receptors, and can synthesise, assemble and secrete many low and high density lipoproteins similar to those of normal human hepatocytes (Dehn et al., 2004). HepG$_2$ cells are also rich in Phase I (cytochrome P450 mono-oxygenases) and Phase II (glucuronic and sulphate conjugation) biotransformation enzymes that play a vital role in normal biotransformation reactions and are essential for the detoxification of various compounds (Dehn et al., 2004, Soldatow et al., 2013, Wilkening et al., 2003).

2.2.2. Cell Culture Conditions

HepG$_2$ cells were allowed to grow (37°C, 5% CO$_2$) in 25cm^3 sterile cell culture flasks containing complete culture media (CCM; Eagle's Minimum Essentials Medium (EMEM) supplemented with 10% foetal calf serum, 1% penicillin-streptomycin-fungizone and 1% L-glutamine). Cells were rinsed every two days in 0.1M phosphate buffered saline (PBS) and reconstituted with CCM (5ml). Once approximately 90% confluent, the cells were rinsed in 0.1M PBS and incubated (37°C, 5% CO$_2$) with the relevant treatment for 24 hrs. Thereafter, the cells were detached by incubation in 1ml trypsin (37°C, 5% CO$_2$, 5 min) and used for the relevant assays. Cells were counted using the trypan blue cell exclusion method.

2.3. Preparation of Fusaric Acid Treatment

A 1mg/ml FA stock solution was prepared by dissolving 1mg of FA in 1ml 0.1M PBS. An inhibitory concentration of 50% (IC$_{50}$: 104μg/ml) was obtained from literature (Abdul et al., 2016) (Appendix A) and used in all subsequent assays. Treatment of HepG$_2$ cells were conducted over 24 hrs (37°C, 5% CO$_2$) with 5ml of 104μg/ml FA (treatment). An untreated control (CCM only) was also prepared.

2.4. Comet Assay

2.4.1. Introduction

The comet assay also known as the single cell gel electrophoresis (SCGE) is a simple and rapid technique used to detect DNA strand breaks in individual cells (Collins, 2004, Fairbairn et al., 1995, Tice et al., 2000). It was first introduced by Ostling and Johansen in 1984 and was later modified by Singh et al. (1988) to improve sensitivity.

Cells are embedded in agarose gel on a microscope slide. The cells are lysed with detergents and high molarity sodium chloride to remove cellular proteins and the DNA is allowed to unwind in an alkaline medium. The cells are then electrophoresed at high pH resulting in structures that resemble comets, with a distinct head comprising of intact, undamaged DNA and a tail consisting of fragmented, damaged DNA, when viewed using fluorescent microscopy

(Collins, 2004, Tice et al., 2000). The length of the comet tail is directly proportional to the amount of DNA damage.

The comet assay is based on the principle that the structure of DNA is a highly organised supercoil tightly packed with matrix proteins and chromatin in the nucleus. However, when DNA becomes damaged this highly organised structure is disrupted causing the individual DNA strands to relax. During electrophoresis, the applied electric field causes the negatively charged DNA (due to the phosphate backbone) to migrate towards the positively charged anode. Undamaged DNA is too large and does not migrate and hence do not produce comet-like images. However, the small fragments of damaged DNA migrates faster producing comet-like images when viewed using fluorescent microscopy (Collins, 2004, Fairbairn et al., 1995, Tice et al., 2000).

2.4.2. Protocol

The comet assay was used to determine the degree of DNA damage in $HepG_2$ cells. Following incubation of $HepG_2$ cells with FA for 24 hrs, the supernatants were removed, the cells were rinsed three times in 0.1M PBS and trypsinised. Thereafter, the cells were prepared in 0.1M PBS (20 000 cells in 25µl 0.1M PBS).

Three frosted slides per sample (control and FA) were prepared by adding three layers of low melting point agarose gel (LMPA, 37°C) with each layer being allowed to solidify (4°C, 10 min). The first layer consisted of 700µl 2% LMPA. The second layer consisted of 25µl cell suspension (20 000 cells in 0.1M PBS), 0.5µl Gel Red (catalogue no. 41003, Biotium Inc., Hayward, CA) and 175µl 1% LMPA and the third layer consisted of 200µl 1% LMPA. Thereafter, all slides were immersed in freshly prepared cold lysing solution [2.5M NaCl, 100mM EDTA, 1% Triton X-100, 10mM Tris (pH 10) and 10% DMSO] and incubated (1 hr, 4°C) to remove all cellular proteins. Following incubation, the slides were placed in electrophoresis buffer [300mM NaOH, 1mM Na_2EDTA; pH 13] for 20 min at RT to allow DNA unwinding. The slides were then electrophoresed (25V (300mA), 35 min, RT) using the Bio-Rad compact power supply. Following electrophoresis, the slides were washed three times with neutralisation buffer [0.4M Tris; pH 7.4] for 5 min each in order to remove excess detergents and alkali. Slides were viewed using the Olympus IX51 inverted fluorescent microscope with 510-560nm excitation and 590nm emission filters (Figure 2.1). Images of at least 50 cells and comets were captured per sample at 20x magnification and the comet tail lengths were measured

using the Soft Imaging System (Life Science – Olympus Soft Imaging Solutions version 5) and reported as μm.
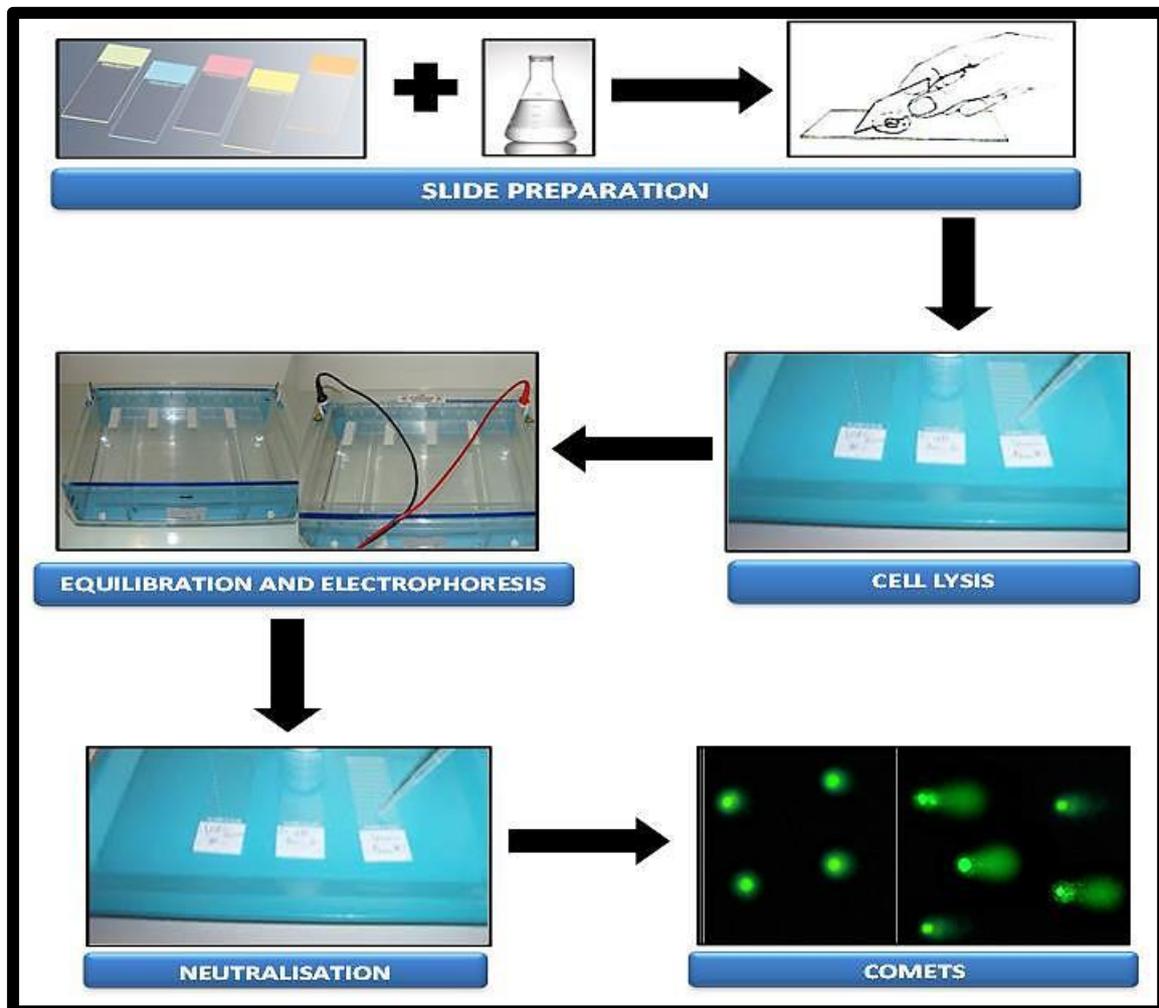
Figure 2.1: Overview of the comet assay (Prepared by author)

2.5. Hoechst 33342 Assay

2.5.1. Introduction

Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2, 5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a cell permeable fluorescent dye that binds specifically to the adenine-thymine (A-T) rich regions in the minor groove of DNA and emits an intense blue fluorescence at 350-490nm (Chazotte, 2011).

The Hoechst 33342 assay is a simple and rapid technique that allows easy visualization of the nucleus in interphase cells and chromosomes in mitotic cells (Chazotte, 2011). This allows one to observe cells at different phases of the cell cycle as well as those undergoing apoptosis.

2.5.2. Protocol

The Hoechst 33342 assay was used to determine the effect of FA on the cell cycle and apoptosis in HepG$_2$ cells. HepG$_2$ cells (500 000 cells/well) were seeded into a 6-well plate and allowed to adhere overnight. Thereafter, the CCM was removed; cells were rinsed three times using 0.1M PBS and treated with FA for 24 hrs. Three technical replicates were prepared. Following treatment, the cells were rinsed thrice in 0.1M PBS and fixed using 10% paraformaldehyde (500μl/well, 5 min, RT) to preserve the morphology and viability of the cells. Once fixed, the paraformaldehyde was removed and the cells were rinsed thrice in 0.1M PBS to remove excess paraformaldehyde. The Hoechst 33342 working solution (catalogue no. B2261, Sigma-Aldrich; 500μl/well, 5μg/ml) was added and the plate was incubated (15 min, 37°C). Thereafter, the cells were rinsed thrice in 0.1M PBS to remove any unbound stain and viewed using the Olympus IX51 inverted fluorescent microscope with 350nm excitation and 450nm emission filters. Three images per sample replicate were captured at 20x magnification (Figure 2.2).
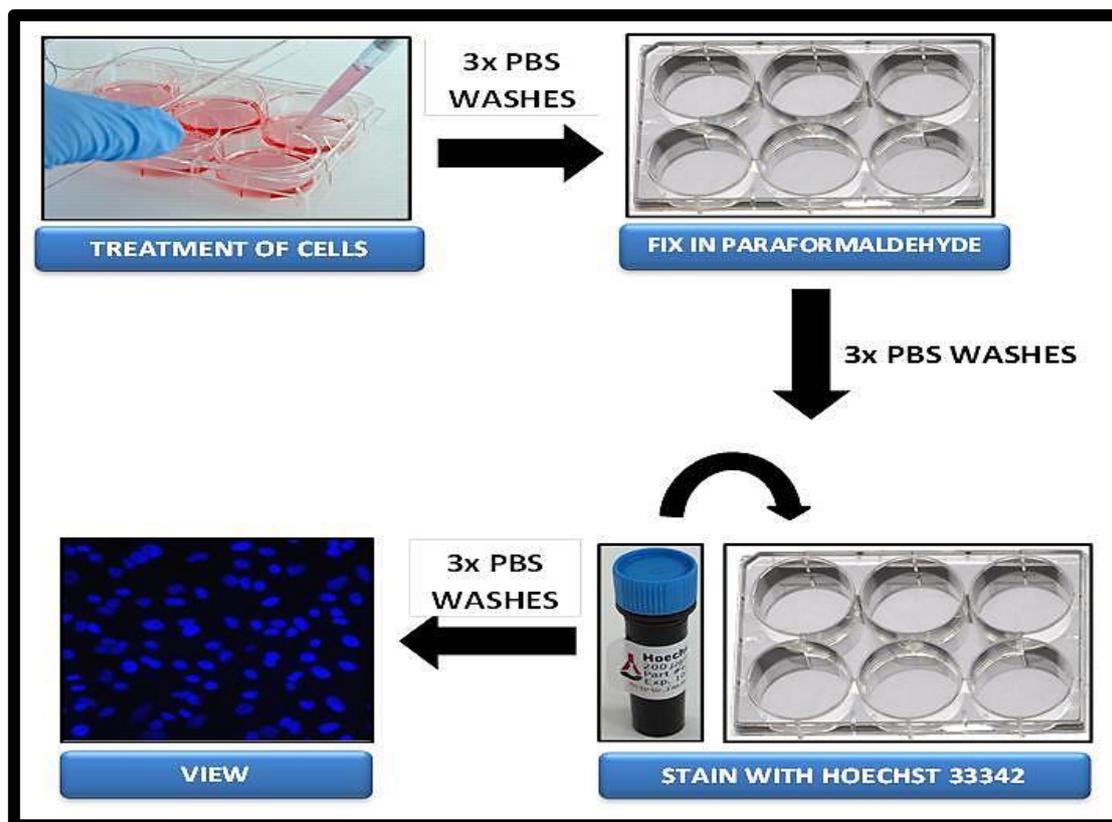
Figure 2.2: Overview of the Hoechst 33342 Assay (Prepared by author)

2.6. Western Blot

Western Blot is an analytical technique that utilises the principle of antigen-antibody binding to detect and quantify specific proteins in a given sample (Eslami and Lujan, 2010, Mahmood and Yang, 2012). First, proteins are separated according to their molecular weights in a process known as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE, a voltage is applied causing the negatively charged proteins to migrate towards the positively charged electrode through a gel matrix. This migration separates proteins according to their size with the higher molecular weight proteins migrating slower through the matrix and the lower molecular weight proteins migrating faster (Mahmood and Yang, 2012). Once electrophoresis is complete, the proteins are electro-transferred to a nitrocellulose membrane and incubated in blocking buffer to prevent the non-specific binding of proteins. The membranes are then immunoblotted with a specific primary and enzyme-conjugated secondary antibody to yield bands that are representative of protein expression. This enables target

proteins to be detected and quantified from a sample containing several different proteins (Mahmood and Yang, 2012).

2.6.1. Protein Isolation

2.6.1.1. Introduction

Total protein was isolated from HepG$_2$ cells using the Cytobuster™ Protein Extraction Reagent (catalogue no. 71009-4, Novagen, Bloemfontein, SA). Cytobuster is a gentle non-ionic reagent that consists of a variety of detergents that when combined with the mechanical scraping of cells disrupts cell membranes allowing for the efficient extraction of soluble proteins that are functionally active and expressed within cells. Loss of cell compartmentalization during protein isolation results in the release of several tightly regulated proteases and phosphatases that degrade and damage cellular proteins. In order to prevent the degrading properties of these enzymes, the lysis reagent is supplemented with protease and phosphatase inhibitors that inactivate endogenous proteolytic and phospholytic enzymes. This enables proteins to maintain their integrity and avoid becoming denatured.

2.6.1.2. Protocol

HepG$_2$ cells were treated with FA for 24 hrs. The supernatants were removed from the flask and the cells were rinsed three times in 0.1M PBS. Thereafter, Cytobuster reagent (200μl) (Novagen, catalogue no. 71009) supplemented with a cocktail of protease inhibitors (Roche, catalogue no. 05892791001) and phosphatase inhibitors (Roche, catalogue no. 04906837001) was added to the flask and the cells were kept on ice for 30 min. The protein from the untreated and treated cells was further extracted using a cell scraper. The lysed cells were transferred to 1.5ml micro-centrifuge tubes and centrifuged (10 000xg, 10 min, 4°C). The supernatants containing the crude protein extract was aspirated into fresh 1.5ml micro-centrifuge tubes and kept on ice until further quantification and standardisation (Figure 2.4). The pellet was discarded.

2.6.2. *Quantification and Standardisation of Proteins*

2.6.2.1. Introduction

The total crude protein was quantified using the Bicinchoninic Acid (BCA) Assay. The BCA assay was first invented by Paul K. Smith in 1985. It is a colorimetric assay based on the biuret reaction in which cupric (Cu^{2+}) ions are converted to cuprous (Cu^+) ions in an alkaline medium (Walker, 1996). First, the Cu^{2+} ions react with the peptide bonds in proteins and are reduced to form Cu^+ ions. The Cu^+ ions then react with two molecules of BCA to form a BCA-Cu^+ complex that has an intense purple colour and maximally absorbs light at a wavelength of 562nm (Figure 2.3 and Figure 2.4). The intensity of the purple colour produced is directly proportional to the number of peptide bonds participating in the reaction and is an indication of the amount of protein present in the sample (Walker, 1996).
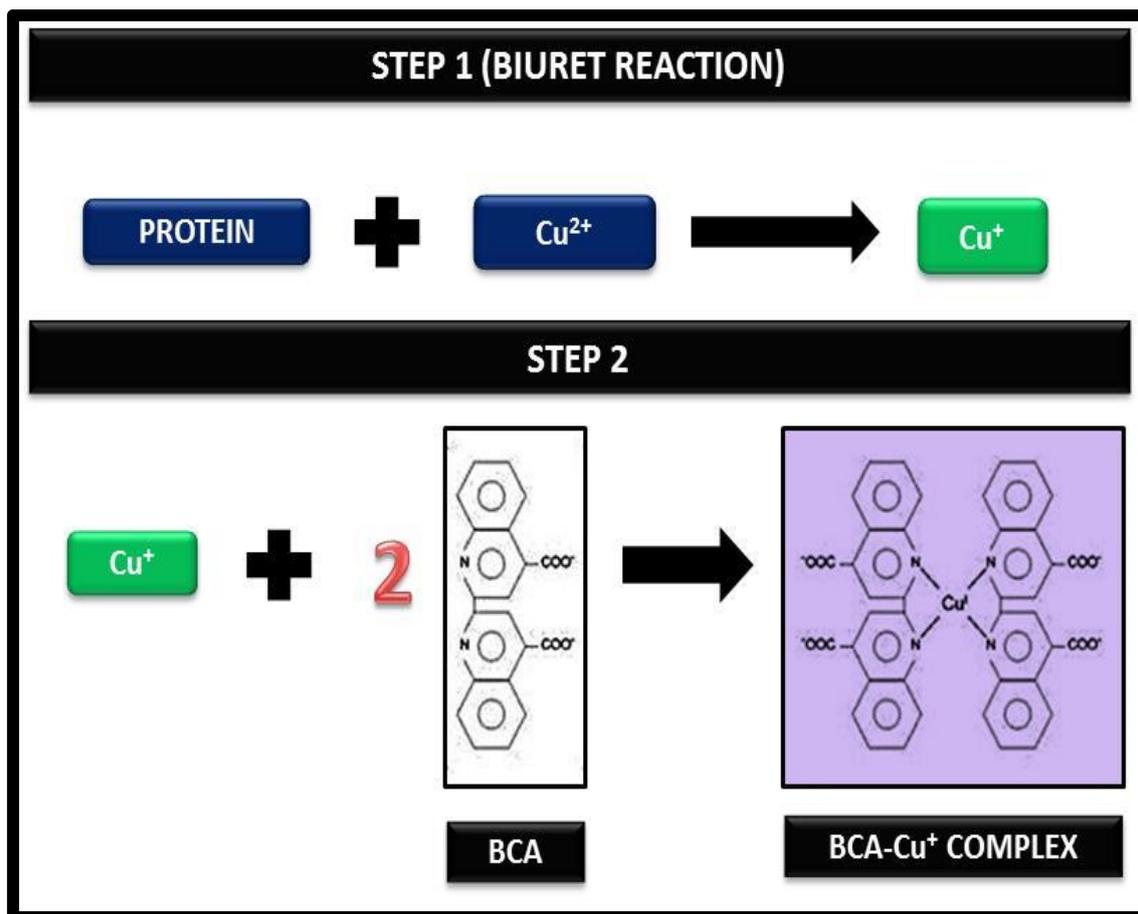
Figure 2.3: Principle of the BCA assay (Prepared by author)

2.6.2.2. Protocol

Bovine serum albumin (BSA) standards [0, 0.2, 0.4, 0.6, 0.8 and 1mg/ml] were prepared in distilled water. Thereafter, 25μl of each standard (triplicate) and protein sample (duplicate) was pipetted into a 96-well microtiter plate. A working solution (200μl) consisting of 198μl BCA and 4μl $CuSO_4$ was added to each well and the plate was incubated at 37°C for 30 min. The absorbance at 562nm was measured using a spectrophotometer (Bio-tek μQuant Plate Reader). The absorbances of the BSA standards were used to construct a standard curve from which the concentration of protein present in each sample was determined (Appendix B).

The proteins were subsequently standardised to a concentration of 1.5mg/ml in Cytobuster reagent and the standardised samples were prepared in laemmli buffer [dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% β-mercaptoethanol and 1% bromophenol blue] by boiling for 5 min. Each component of the laemmli buffer has a specific function. Tris-HCl serves as the buffer and maintains pH. Glycerol adds weight to the samples allowing the samples to sink easily into the wells of the gel in SDS-PAGE (Mahmood and Yang, 2012). Bromophenol blue allows visualization of the samples as they migrate through the gel. SDS denatures proteins and imparts an overall negative charge enabling the proteins to be separated solely based on their size rather than charge and shape (Mahmood and Yang, 2012). The β-mercaptoethanol acts as a reducing agent to break disulphide bonds and denature the proteins. Following protein preparation, the samples were allowed to cool to RT before storing at -20°C until assayed.
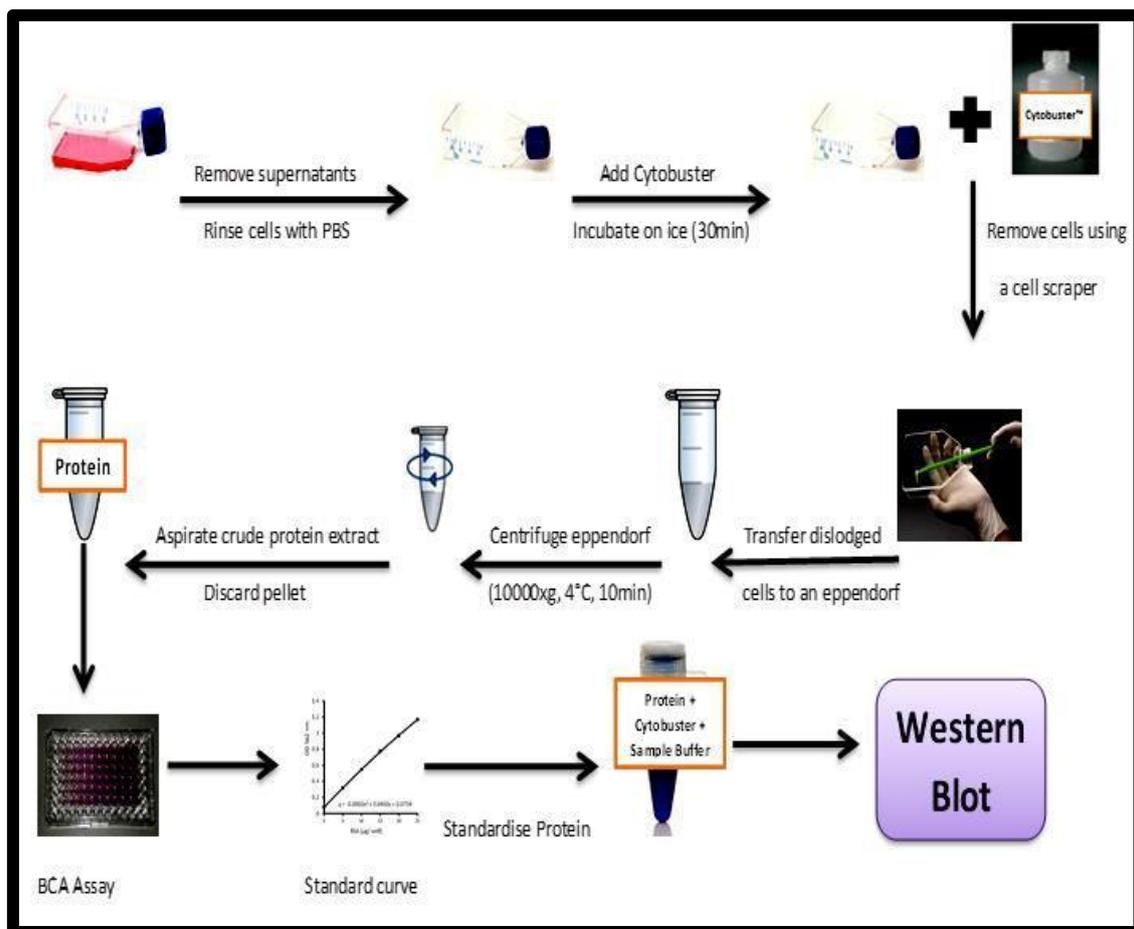
Figure 2.4: Overview of the isolation, quantification and standardisation of proteins (Prepared by author)

2.6.3. Preparation of Gels for SDS-PAGE

2.6.3.1. Introduction

Polyacrylamide gels are a 3D polymer network composed of acrylamide and a cross-linker known as N, N'-methylene bis-acrylamide under the catalyzation of ammonium persulfate (APS). SDS-PAGE utilises two types of agarose gels to separate proteins according to their molecular weights. The higher gel is known as the stacking gel and is slightly acidic (pH 6.8) with a low polyacrylamide content causing the gel to have large pores that poorly separate proteins (Mahmood and Yang, 2012). However, the stacking gel is important as it enables the protein to form thin sharply defined bands so that they can be separated by the lower resolving gel. The resolving gel is basic (pH 8.8) and has a high polyacrylamide content causing the gel to

have narrower pores which function to separate proteins according to their size (Mahmood and Yang, 2012).

2.6.3.2. Protocol

Gels for SDS-PAGE were prepared using the Mini-PROTEAN Tetra Cell casting stand (Figure 2.5) (Bio-Rad). A 10% resolving gel [dH_2O, 1.5M Tris (pH 8.8), 10% SDS, bis-acrylamide, 10% APS and TEMED] was prepared and allowed to polymerize for 1 hr. Thereafter, a 4% stacking gel [dH_2O, 0.5M Tris (pH 6.8), 10% SDS, bis-acrylamide, 10% APS and TEMED] was prepared and added on top of the resolving gel. A 1cm plastic comb was placed between the glass plates to enable the formation of wells for sample loading (Figure 2.5 and Figure 2.6) and the gel was allowed to set for approximately 40 min.
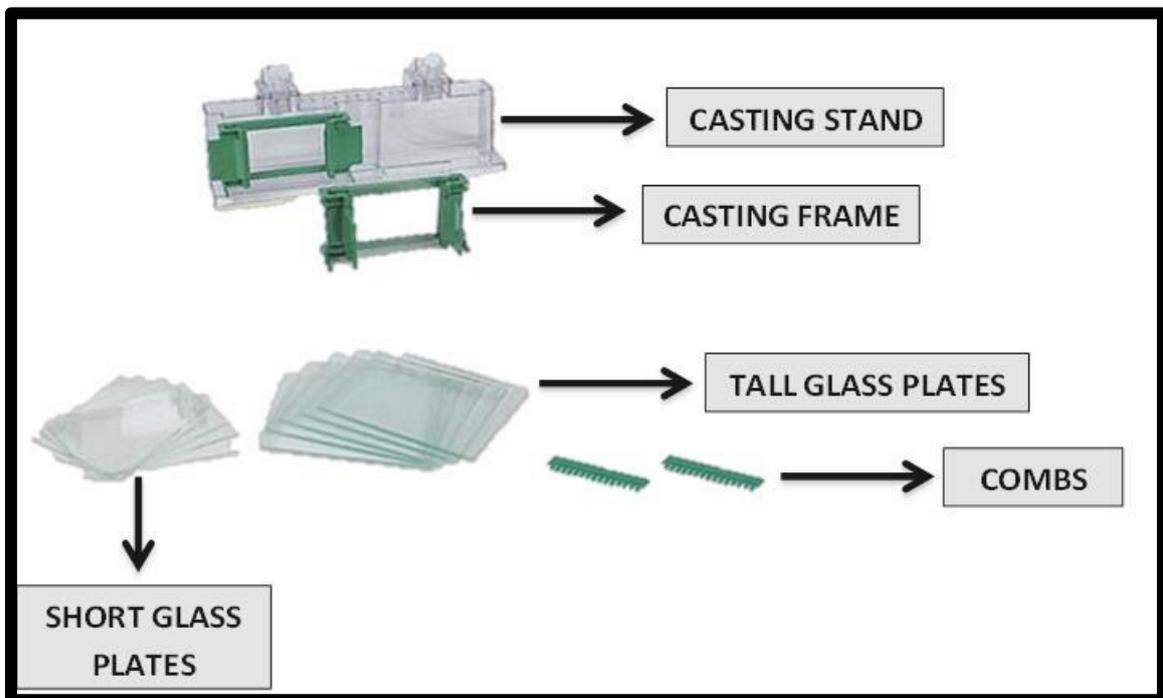
Figure 2.5: Equipment used to prepare SDS-PAGE gels (Prepared by author)
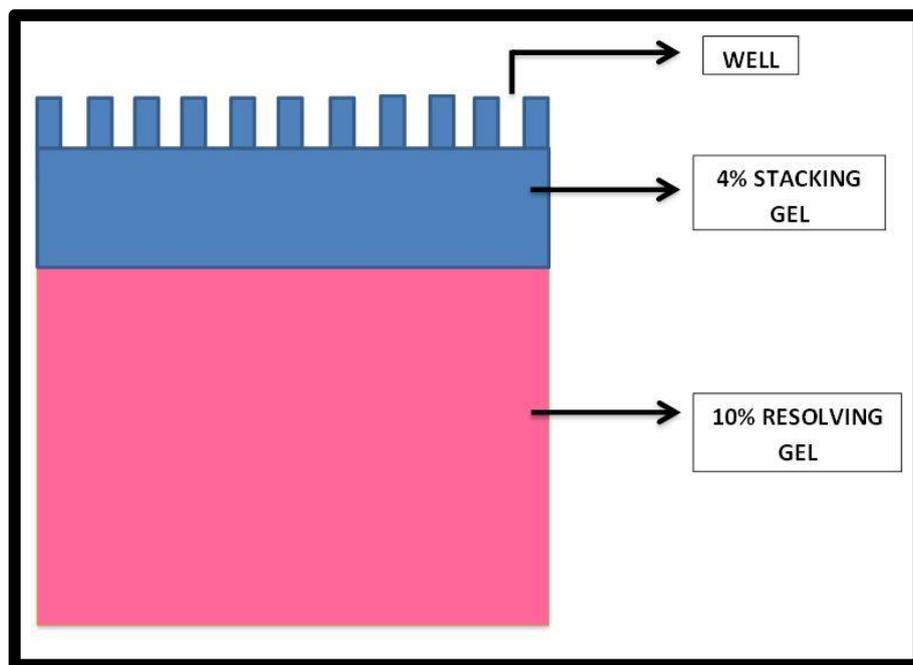
Figure 2.6: A typical example of an SDS-PAGE gel (Prepared by author)

2.6.4. SDS-PAGE

The gel cassettes were placed into the electrode assembly and the electrode tank (Mini-PROTEAN Tetra Cell System, Bio-Rad). The protein samples (25µl) along with a molecular weight marker (5µl) (Precision Plus Protein All Blue Standards, catalogue no. #161-0373, Bio-Rad) was loaded into the wells. The tank was filled with running buffer [25mM Tris, 192mM glycine and 0.1% SDS] and the samples were electrophoresed at 150V for 1 hr using the Bio-Rad compact power supply (Figure 2.7).
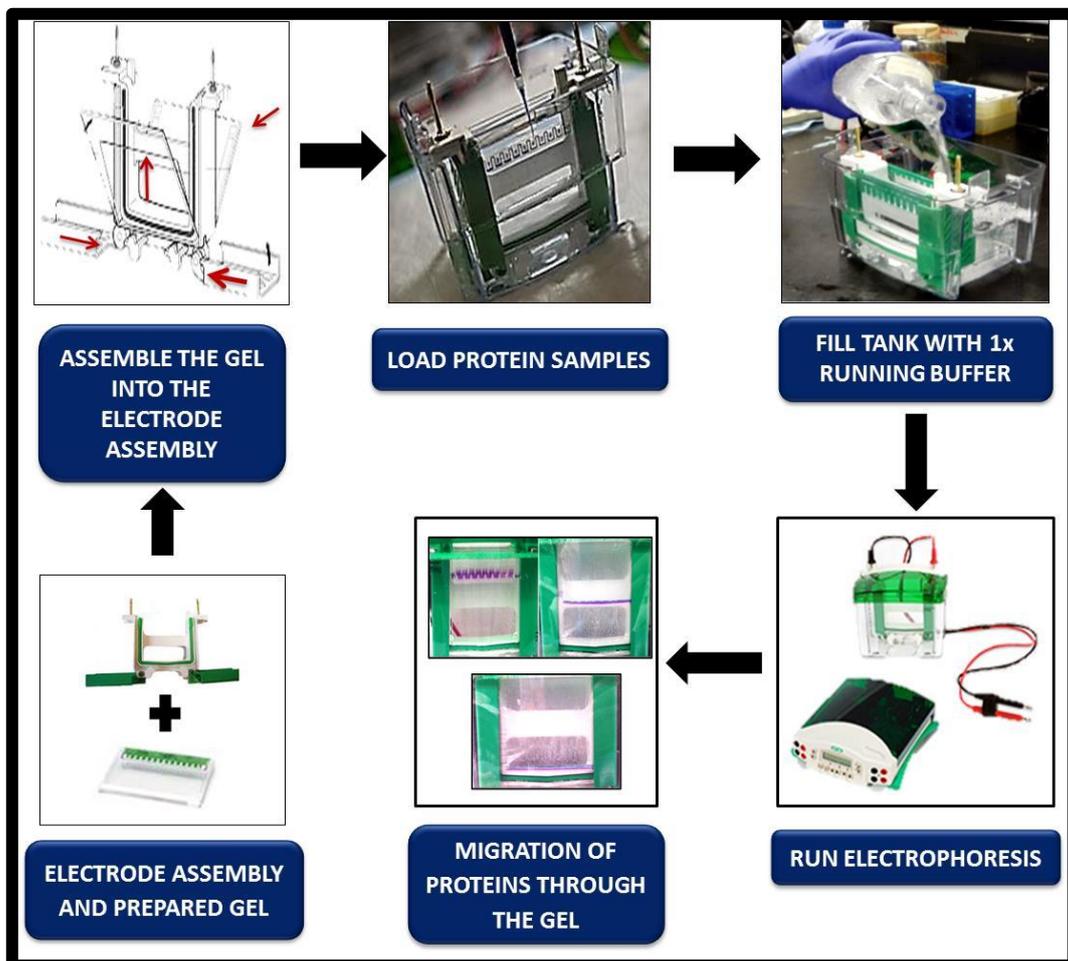
Figure 2.7: Process of SDS-PAGE (Prepared by author)

2.6.5. *Protein Transfer*

Once electrophoresis was complete, the separated proteins were transferred to a nitrocellulose membrane. The electrophoresed gels were gently removed from the glass plates and the stacking gel was removed. The electrophoresed gels, nitrocellulose membranes and two fibre pads were equilibrated in transfer buffer [25mM Tris, 192mM glycine and 20% methanol, pH 8.3] for 10 min. Thereafter, a gel sandwich consisting of a fibre pad, nitrocellulose membrane, gel and fibre pad (Figure 2.8) was assembled between the two electrodes in the transfer apparatus and the separated proteins were electro-transferred to the nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo Transfer System (30 min, 20V).

The application of the voltage generates an electric current perpendicular to the surface of the gel. This causes the negatively charged proteins to migrate out of the gel and onto the nitrocellulose membrane.
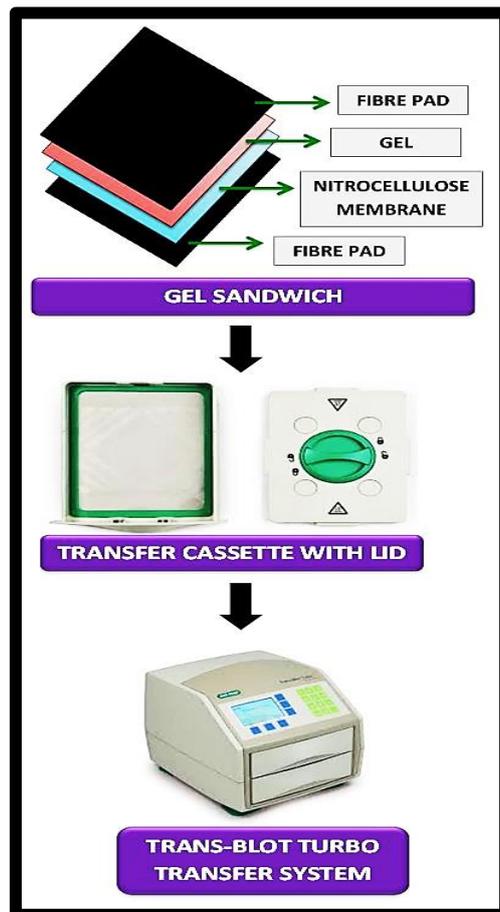
Figure 2.8: Transfer of proteins to the nitrocellulose membrane (Prepared by author)

2.6.6.   *Blocking and Antibody Incubation*

After transfer, the membranes were blocked in 5ml blocking buffer consisting of either 5% BSA or 5% non-fat dry milk (NFDM) in Tris buffered saline with 0.05% Tween 20 [TTBS; 150mM NaCl, 3mM KCl, 25mM Tris, 0.05% Tween 20, dH$_2$O, pH 7.5] for 1 hr at RT with gentle shaking to prevent the non-specific binding of proteins. The membranes were then immunoblotted with the specific primary antibody (Table 1) for 1 hr on the shaker at RT and then overnight at 4°C. The primary antibody is specific to the target protein and hence will bind only to the target protein. Following the overnight incubation, the membranes were equilibrated to RT and washed five times with TTBS (10 min each, RT) to remove unbound primary antibody. The membranes were then incubated with the respective horse-radish peroxidase (HRP)-conjugated secondary antibody [1:10 000 dilution in 5% BSA (goat anti-rabbit, sc-2004) and 1:10 000 dilution in 5% NFDM (goat anti-mouse, sc-2005)] for 2 hrs at RT with gentle shaking. The secondary antibody is specific to the primary antibody and hence will bind only to the bound primary antibody. Following incubation, the membranes were washed five times with TTBS (10 min each, RT) to remove any unbound secondary antibody.

Table 1: Antibody Dilutions

ANTIBODY	DILUTION	CATALOGUE NUMBER
Anti-MDM2	1:1 000 in 5% NFDM	M4308 (Sigma-Aldrich), mouse monoclonal
Anti-p53	1:1 000 in 5% BSA	Sc-6243 (Santa Cruz), rabbit polyclonal
Anti-a-K382-p53	1:500 in 5% BSA	#2525 (Cell Signalling Technology), rabbit polyclonal
Anti-p-Ser15-p53	1:1 000 in 5% BSA	#9284 (Cell Signalling Technology), rabbit polyclonal
Anti-HDAC1	1:1 000 in 5% BSA	Ab19845 (Abcam), rabbit polyclonal
Anti-a-CBP (K1535)/p300 (K1499)	1:1 000 in 5% BSA	#4771S (Cell Signalling Technology), rabbit polyclonal
Anti-p-Ser47-Sirt1	1:500 in 5% BSA	#2314L (Cell Signalling Technology), rabbit polyclonal

2.6.7. Imaging

The Clarity™ Western ECL Substrate Kit (catalogue no. #170-5060, Bio-Rad) consisting of a hydrogen peroxide substrate and an enhanced luminol solution was used for the antigen-antibody complex and the signal was detected using the ChemiDoc™ XRS+ Molecular Imaging System (Bio-Rad). The HRP conjugated to the secondary antibody reacts with the hydrogen peroxide (H_2O_2) substrate to yield oxygen radicals. The oxygen radical reacts with luminol causing the luminol to break down into aminophtalic acid which in turn reacts with enhancer molecules causing it to luminesce enabling the visualization of protein bands (Figure 2.9).
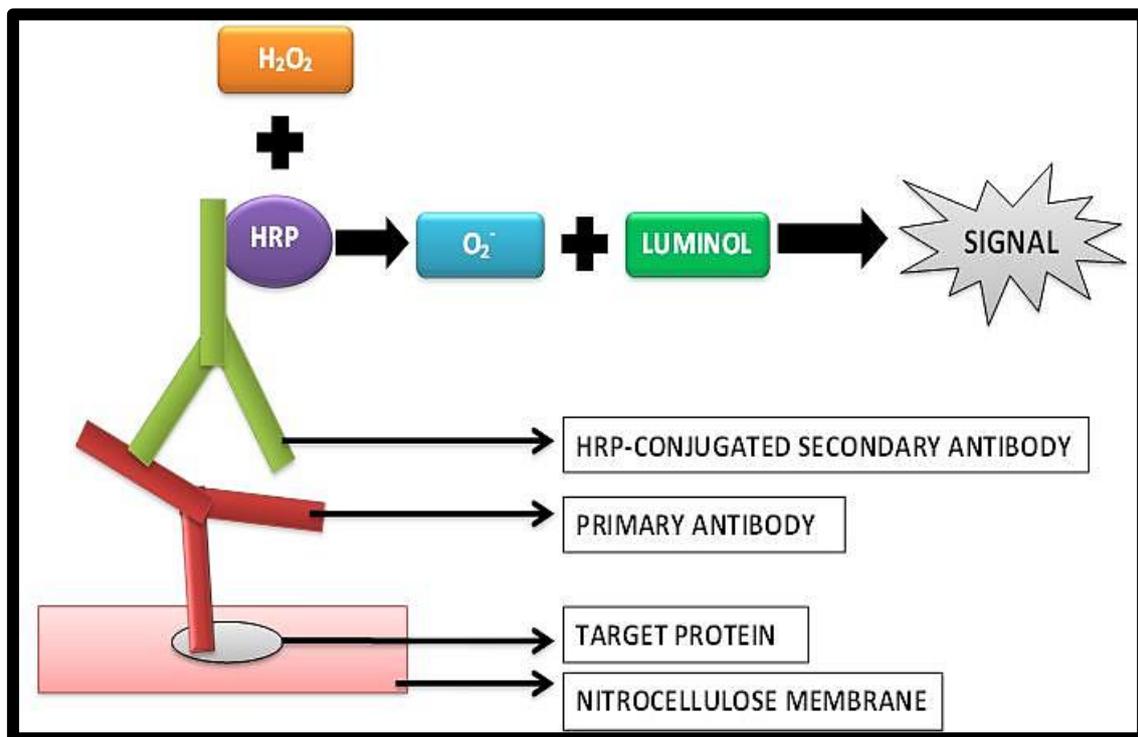
Figure 2.9: Principle of antigen-antibody binding and detection of proteins using the Clarity™ Western ECL Substrate Kit (Prepared by author)

2.6.8. *Quenching and Normalisation*

After detection, the membranes were quenched using 5ml H_2O_2 (30 min, 37°C), washed once with TTBS (10 min, RT), blocked in 5% BSA in TTBS (1 hr, RT) and probed with the housekeeping protein, anti-β-actin (catalogue no. A3854, Sigma-Aldrich, 1:5 000 dilution in 5% BSA, 30 min, RT) in order to normalise protein expression and correct for loading error.

Protein expression was analysed using the Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as relative band density (RBD) and fold-change. Protein expression was normalised by dividing the RBD of the protein of interest by the RBD of β-actin. The normalised protein expression of the treatment was then divided by the normalised protein expression of the control to determine a fold-change relative to the control.

2.7. Statistical Analyses

Microsoft Excel 2010 and GraphPad Prism version 5.0 (GraphPad Software Inc., California) was used to perform all statistical analyses. The unpaired t-test with Welch's Correction was used for all assays. All results were represented as the mean ± standard deviation unless otherwise stated. A value of *p<0.05* was considered statistically significant.

CHAPTER 3

RESULTS

3.1. Comet Assay

The comet assay was used to assess DNA integrity in the FA treated and untreated control HepG$_2$ cells at 24 hrs (Figure 3.1 A). The length of the comet tails were measured (µm) and are an indication of the amount of damaged DNA present within the cell (Figure 3.1 B).
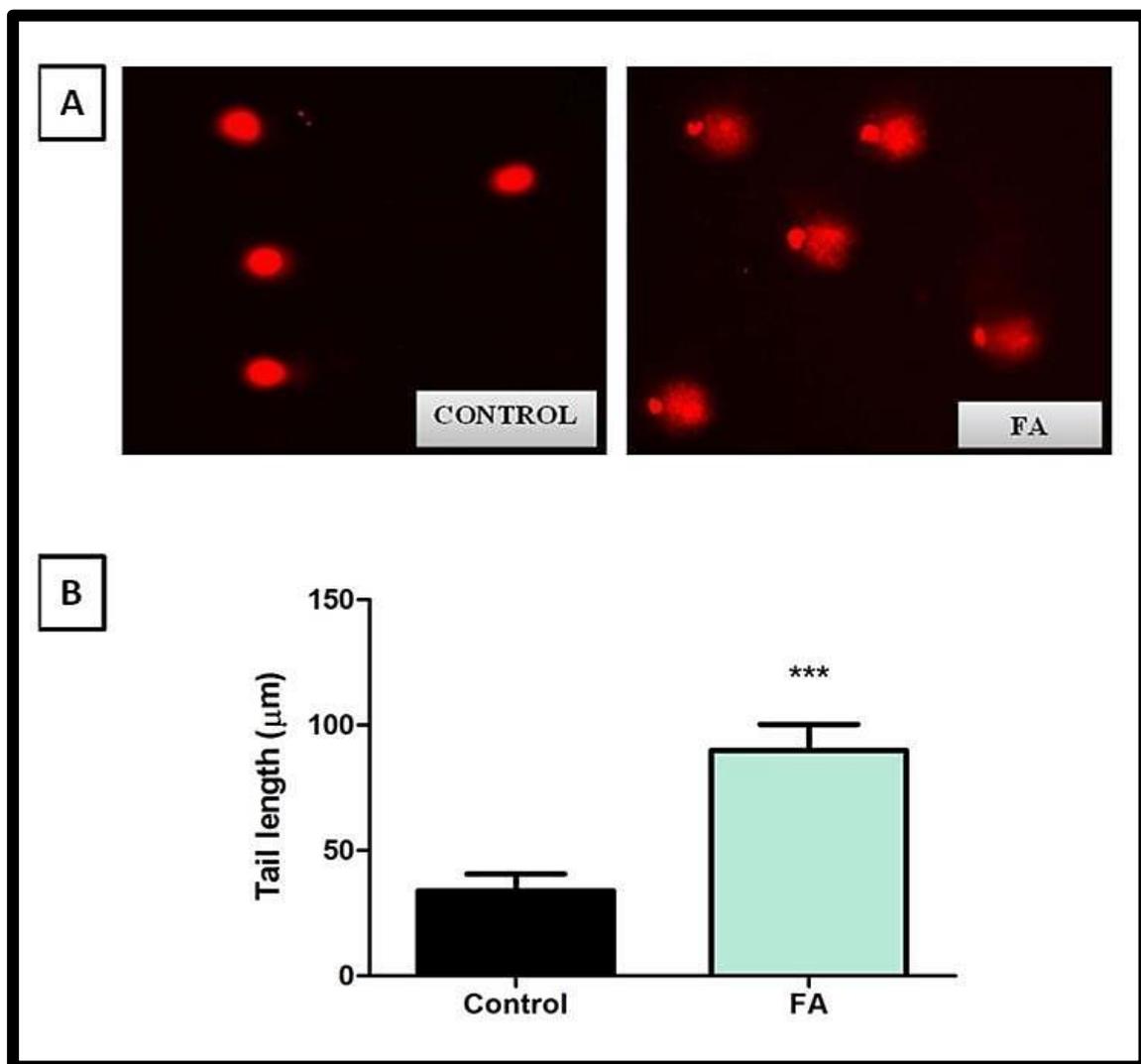
Figure 3.1: Comet tails of the FA treated and control HepG$_2$ cells (20x magnification) (A) Fusaric acid increased comet tail lengths in HepG$_2$ cells at 24 hrs (****p<0.0001*) (B).

Fusaric acid induced DNA damage in HepG$_2$ cells at 24 hrs. This is shown by the significant increase in comet tail lengths in the FA treatment [89.98 ± 10.36μm vs. 33.99 ± 6.76μm, *p<0.0001*] compared to the control (Figure 3.1).

3.2.   Western Blot

Western blot was used to determine the effect of FA on the post-translational modifications, phosphorylation and acetylation, of p53. The protein expression of p53, phosphorylated-Serine 15-p53 (p-Ser15-p53), acetylated-Lysine 382-p53 (a-K382-p53), acetylated-CBP (Lysine 1535)/p300 (Lysine 1499) (a-CBP (K1535)/p300 (K1499)), HDAC1, phosphorylated-Serine 47-Sirt1 (p-Ser47-Sirt1) and MDM2 in the FA treated and control HepG$_2$ cells at 24 hrs was assessed. The expression of the target proteins was normalised against the housekeeping protein, β-actin. Results are expressed as fold-change relative to the control (control = 1-fold).

3.2.1. p53

The tumour suppressor protein, p53 is activated in response to DNA damage. Fusaric acid significantly decreased the protein expression of p53 in HepG$_2$ cells (0.73 ± 0.03-fold vs. 1.02 ± 0.01-fold, *p=0.0034*) compared to the control (Figure 3.2).
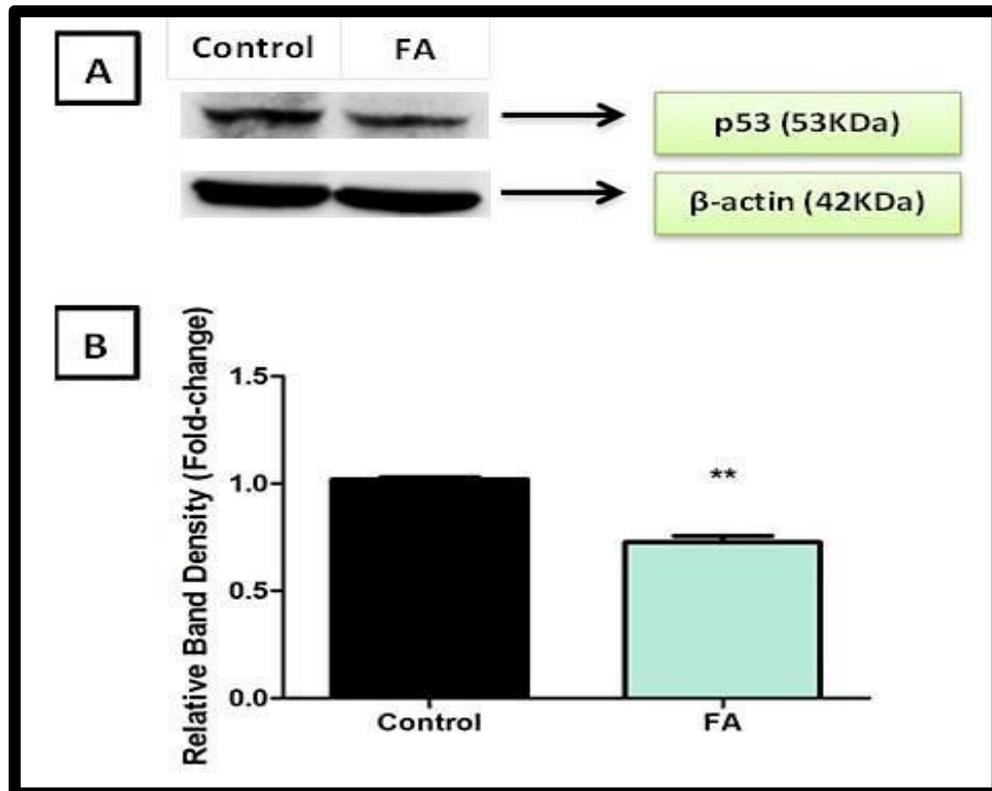
Figure 3.2: Western blot images (A) and relative fold-change (B) of p53 expression in the FA treated and control HepG$_2$ cells at 24 hrs (**$p<0.005$*).

3.2.2. Phosphorylated-Ser15-p53 and acetylated-K382-p53

The phosphorylation and acetylation of p53 are the most common post-translational modifications that occur during DNA damage. Fusaric acid significantly increased the phosphorylation of p53 on the Ser-15 residue in HepG$_2$ cells (9.76 ± 0.20-fold vs. 1.02 ± 0.01-fold, *$p=0.0002$*) compared to the control (Figure 3.3). Acetylated p53 on the K382 residue was significantly increased in the FA treatment compared to the control (1.75 ± 0.23-fold vs. 1.02 ± 0.01-fold, *$p=0.0329$*; Figure 3.4). These results indicate that FA not only alters the protein expression of p53, but also the phosphorylation and acetylation status of p53 in HepG$_2$ cells.
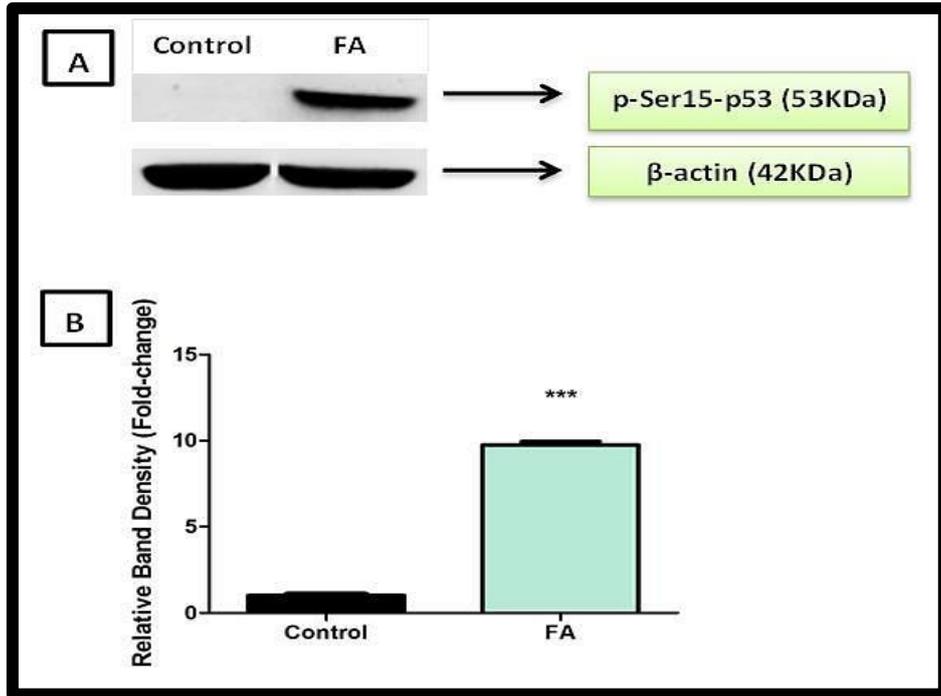
Figure 3.3: Western blot images (A) and relative fold-change (B) of p-Ser15-p53 expression in the FA treated and control HepG$_2$ cells at 24 hrs (****p<0.0005*).
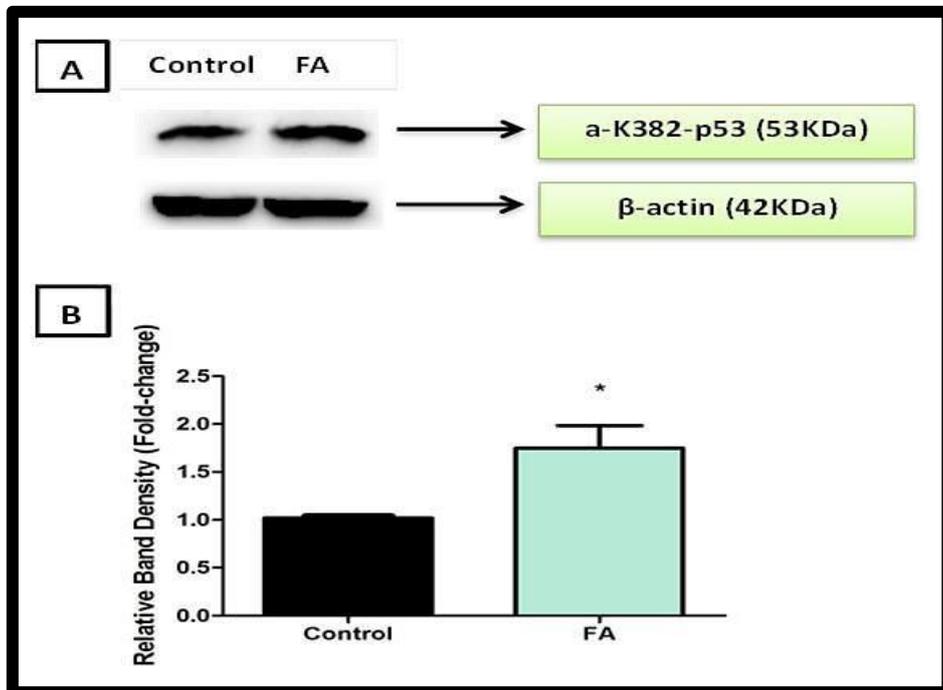
Figure 3.4: Western blot images (A) and relative fold-change (B) of a-K382-p53 expression in the FA treated and control HepG$_2$ cells at 24 hrs (**p<0.05*).

3.2.3. Acetylated-CBP (K1535)/p300 (K1499)

CREB-binding protein and p300 regulate the acetylation of p53 on the K382 residue. Fusaric acid significantly decreased the expression of a-CBP (K1535)/p300 (K1499) in HepG$_2$ cells compared to the control (0.58 ± 0.05-fold vs. 1.02 ± 0.01-fold, *p=0.0043*; Figure 3.5).
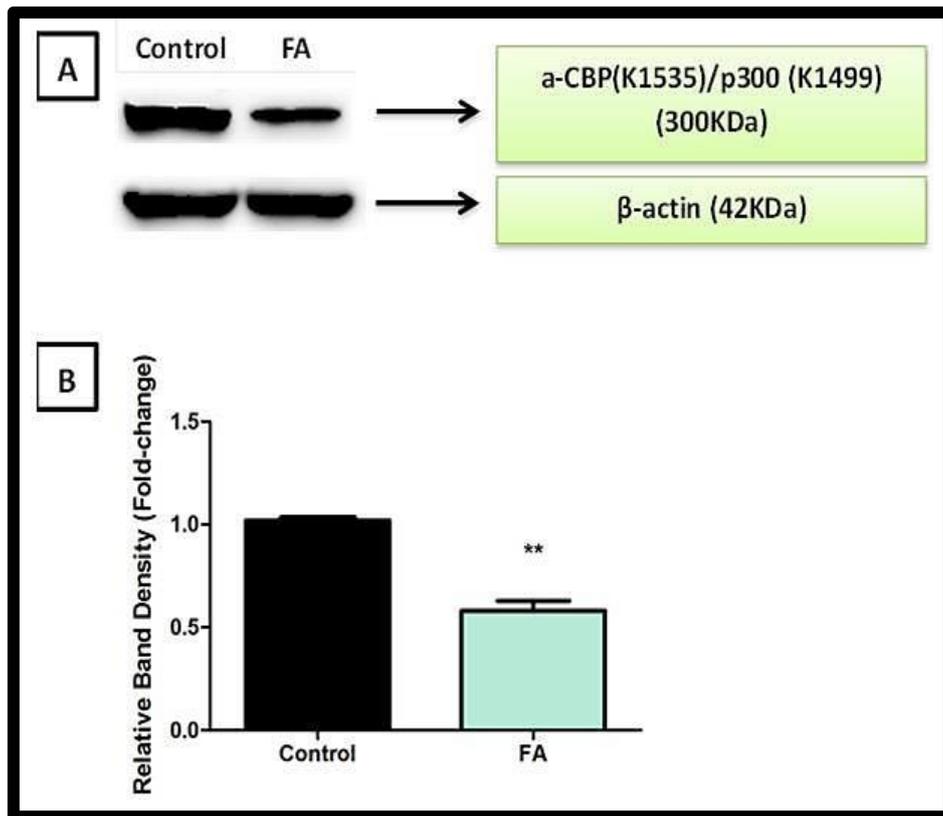
Figure 3.5: Western blot images (A) and relative fold-change (B) of a-CBP (K1535)/p300 (K1499) expression in the FA treated and control HepG$_2$ cells at 24 hrs (**p<0.005*).

3.2.4. HDAC1 and p-Ser47-Sirt1

The deacetylases, HDAC1 and Sirt1, are known to deacetylate p53 on K382. The expression of HDAC1 was significantly reduced in the FA treated HepG$_2$ cells compared to the control (0.84 ± 0.02-fold vs. 1.02 ± 0.01-fold, *p=0.0006*; Figure 3.6). However, the protein expression of p-Ser47-Sirt1 was higher in the FA treatment compared to the control (1.24 ± 0.04-fold vs. 1.02 ± 0.01-fold, *p=0.0127*; Figure 3.7).
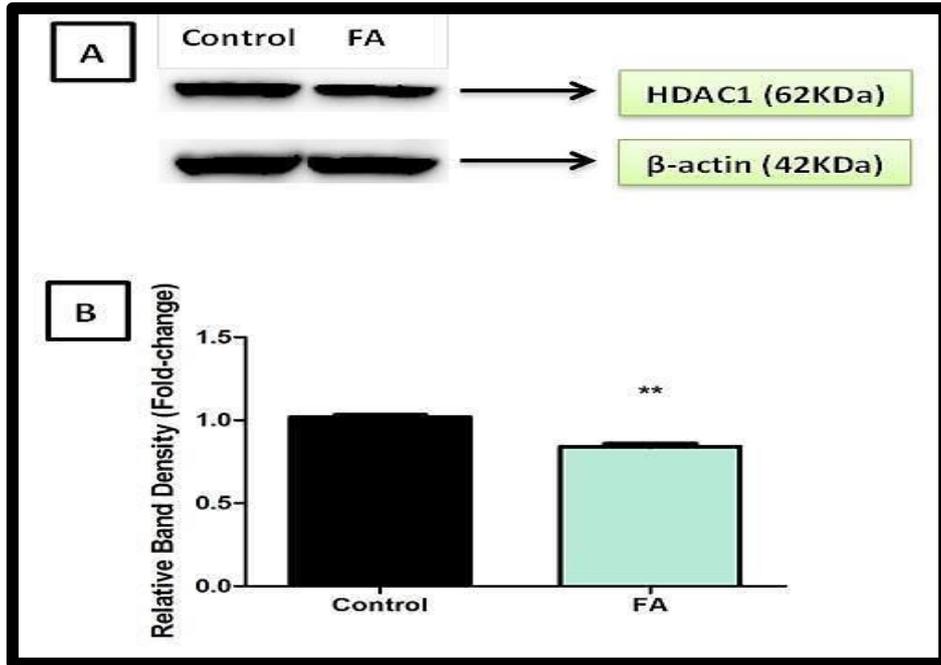
Figure 3.6: Western blot images (A) and relative fold-change (B) of HDAC1 expression in the FA treated and control HepG$_2$ cells at 24 hrs (**$p<0.005$).
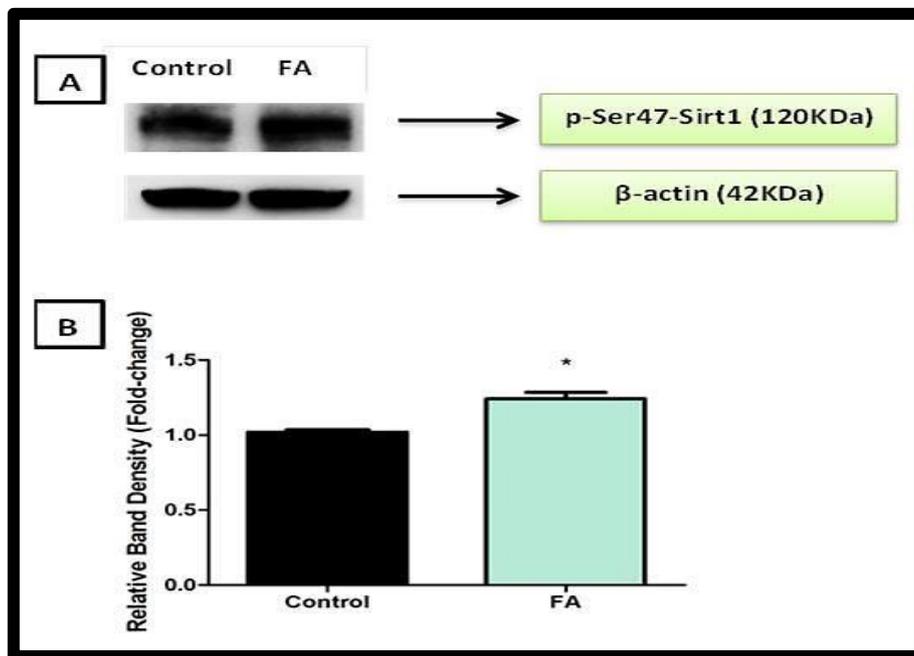
Figure 3.7: Western Blot images (A) and relative fold-change (B) of p-Ser47-Sirt1 expression in HepG$_2$ cells at 24 hrs (*$p<0.05$).

3.2.5. *Murine Double Minute 2 (MDM2)*

The E3 ubiquitin ligase, MDM2, negatively regulates p53 expression by adding ubiquitin molecules to the C-terminal lysine residues of p53 targeting it for degradation by the 26S proteasome. The ubiquitination of p53, mediated by MDM2, is an important post-translational modification that occurs in the absence of cellular stress. The expression of MDM2 was significantly elevated in the FA treated HepG$_2$ cells compared to the control (5.63 ± 0.78-fold vs. 1.02 ± 0.01-fold, *p=0.0094*; Figure 3.8).
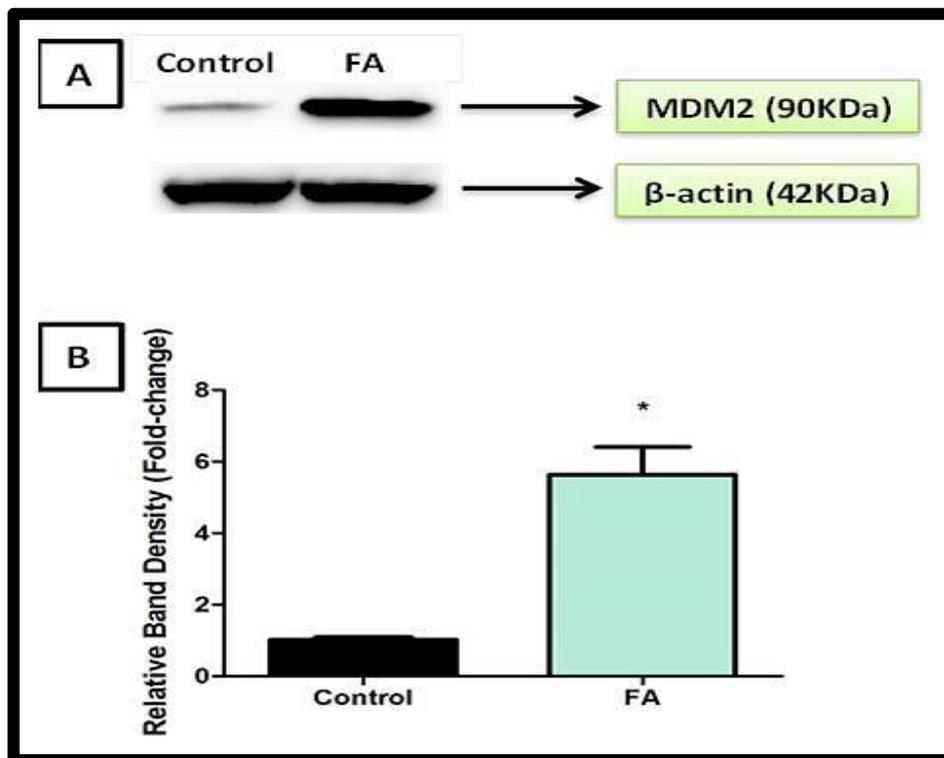
**Figure 3.8:** Western blot images (A) and relative fold-change (B) of MDM2 expression in FA treated and control HepG$_2$ cells at 24 hrs (**p<0.05*).

3.3. Hoechst 33342 Assay

The Hoechst 33342 stain is a cell permeable fluorescent stain that binds specifically to the A-T rich regions of DNA enabling visualization of the different stages of cell division as well as gradations of nuclear damage. The Hoechst 33342 assay was used to determine the effect of FA on cell proliferation, cell cycle arrest and apoptosis in HepG$_2$ cells at 24 hrs (Figure 3.9). Hoechst analysis of FA treated HepG$_2$ cells at 24 hrs (Figure 3.9 B) displayed fewer cells with the absence of dividing cells and the presence of several apoptotic bodies compared to the control cells (Figure 3.9 A). Therefore, FA inhibited cell proliferation, caused cell cycle arrest and induced apoptosis in HepG$_2$ cells.
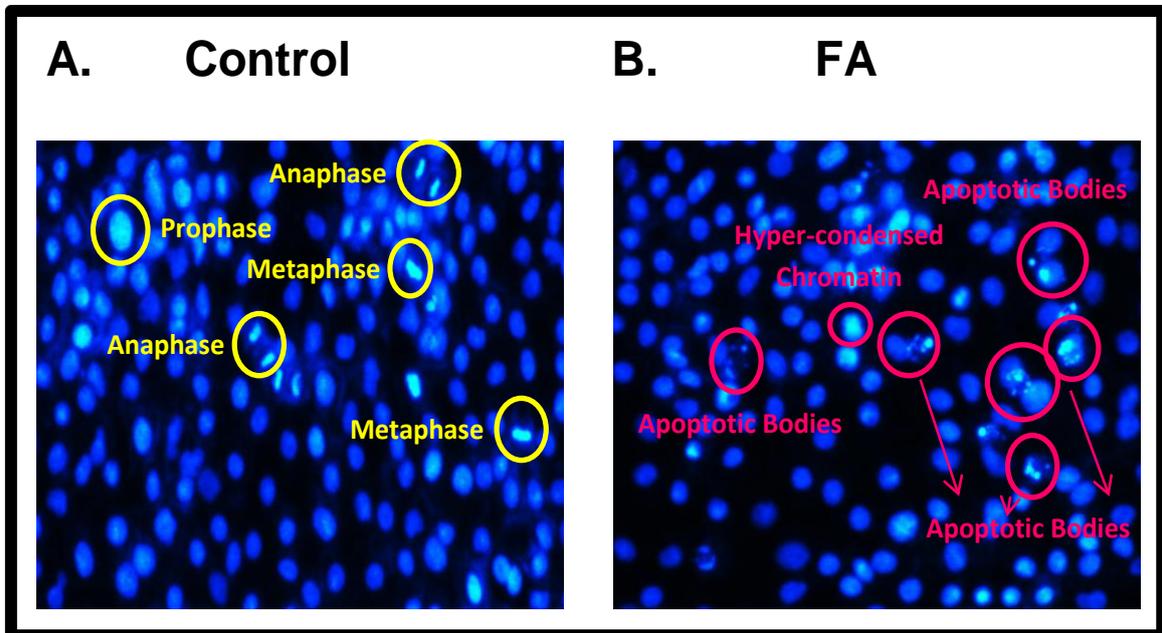
Figure 3.9: Hoechst 33342 staining of the control (A) and FA treated (B) HepG$_2$ cells at 24 hrs (20x magnification).

CHAPTER 4

DISCUSSION

Fusaric acid is a common mycotoxin produced by the *Fusarium* species and a natural contaminant of several food commodities, especially maize (Bacon et al., 1996). These foods form an integral part of local diets and the consumption of FA contaminated foods may be harmful to humans and animals.

Fusaric acid is toxic to plants (D'Alton and Etherton, 1984, Diniz and Oliveira, 2009, Pavlovkin et al., 2004, Pirayesh et al., 2015, Rani et al., 2009) and animals (Bacon et al., 1995, Bungo et al., 1999, Dowd, 1988, Fairchild et al., 2005, Hidaka et al., 1969, Pirayesh et al., 2015, Rani et al., 2009, Terasawa and Kameyama, 1971, Voss et al., 1999). However, the mechanism by which this mycotoxin exerts its toxicity is currently unknown. Numerous studies have shown FA to exert its toxicity by elevating oxidative stress (Abdul et al., 2016, D'Alton and Etherton, 1984, Diniz and Oliveira, 2009, Sapko et al., 2011). However, others show that FA is a potent chelator of divalent cations and thus may exert its toxicity by removing essential metal ions (Fernandez-Pol et al., 1993, Rani et al., 2009, Stack et al., 2004).

This study aimed to determine a possible mechanism of FA induced toxicity in HepG$_2$ cells at 24 hrs. The ability of FA to cause DNA damage, post-translationally modify the tumour suppressor protein, p53, and induce cell death was determined.

Due to its continuous replication, DNA is highly susceptible to damage by both endogenous and exogenous agents (Sancar et al., 2004). Damage to cellular DNA disrupts normal functioning of the cell and can lead to death of the cell or malignant transformation. The longer comet tail lengths observed in the FA treated cells compared to the control (Figure 3.1 A) indicate that FA increased DNA damage in HepG$_2$ cells (Figure 3.1 B). The increase in DNA damage may be a result of an increase in oxidative stress as studies show that FA elevates oxidative stress (Abdul et al., 2016, Sapko et al., 2011). Oxidative stress refers to an increase in reactive oxygen species and a decrease in antioxidants (Södergren, 2000). Reactive oxygen species are highly reactive molecules that contain oxygen including superoxide radical (O$_2^{\cdot}$), hydroxyl radical (HO$^{\cdot}$) and hydrogen peroxide (H$_2$O$_2$); that are capable of causing damage to cellular macromolecules (Södergren, 2000). Reactive oxygen species damage cellular DNA by oxidising DNA bases, causing breaks in the DNA molecule (single- and double-strand breaks) and by forming highly toxic and mutagenic DNA lesions (Salmon et al., 2004).

p53 is a major stress response protein that is activated in response to DNA damage (Brooks and Gu, 2003). Extensive DNA damage increases the steady-state level and activity of p53 leading to cell cycle arrest, inhibition of cell proliferation and apoptosis (Blattner, 2008). Fusaric acid significantly decreased the protein expression of p53 in HepG$_2$ cells (Figures 3.2). p53 is a metalloprotein that requires a zinc atom in order to maintain structural stability. The zinc ion is coordinated by a histidine (His179) and three cysteine side chains (Cys176, Cys238 and Cys242) (Joerger and Fersht, 2007) and is essential for maintaining the wild-type conformation and stability of the p53 protein (Ostrakhovitch et al., 2006). The removal of the zinc ion by a metal chelating agent such as FA substantially destabilizes the p53 protein resulting in local structural perturbations and loss of sequence-specific DNA binding (Joerger and Fersht, 2007).

p53 is subjected to a variety of post-translational modifications that enhance its stability and activity as a transcription factor (Brooks and Gu, 2003). During DNA damage, the kinases ATM, ATR and DNA-PK are activated to phosphorylate p53 on Ser-15 (Barlev et al., 2001, Blattner, 2008, Brooks and Gu, 2003, Dai and Gu, 2010, Reed and Quelle, 2014). Fusaric acid significantly increased the expression of p-Ser15-p53 in HepG$_2$ cells (Figure 3.3).

The phosphorylation of p53 on Ser-15 primes p53 for acetylation. This occurs by increasing the interaction between p53 and histone acetyltransferases and decreasing the interaction with MDM2 (Barlev et al., 2001, Kang et al., 2015, Lambert et al., 1998, Tang et al., 2008, Zhang et al., 2015). The histone acetyltransferases, CBP and p300 acetylate p53 on K382 (Barlev et al., 2001, Dai and Gu, 2010, Das et al., 2014, Zhang et al., 2015). Fusaric acid significantly increased the expression of a-K382-p53 in HepG$_2$ cells (Figure 3.4) despite the significant decrease in the expression of a-CBP (K1535)/p300 (K1499) (Figure 3.5). The acetylation of CBP on K1535 and p300 on K1499 is known to enhance the histone acetyltransferase activity of CBP and p300 (Das et al., 2014, Thompson et al., 2004). These transcriptional co-activators function by directly binding to a variety of transcriptional regulators where they recruit additional CBP/p300 molecules to acetylate histones and non-histone proteins thereby, activating their transcriptional activity (Nyborg and Peersen, 2004). The structure of CBP/p300 plays a major role in its HAT function (Das et al., 2014, Park et al., 2013). CBP/p300 contain several well conserved domains, three of which bind zinc and are required for the formation of a stable folded structure (Park et al., 2013, Nyborg and Peersen, 2004). The decrease in the expression of a-CBP/p300 observed in the FA treatment may be attributed to the removal of the zinc ions from the CBP/p300 protein leading to structural destabilization. Similar results were obtained by Nyborg et al. (2004) in which the zinc-chelating agent, EDTA, caused unfolding and disruption of the CBP/p300 protein structure. CBP/p300 are auto-acetylated in the presence

of acetyl-CoA (Black et al., 2008) and a decrease in CBP/p300 will ultimately lead to a decrease in a-CBP (K1535)/p300 (K1499).

The decrease in a-CBP (K1535)/p300 (K1499) did not affect the acetylation of p53 as the phosphorylation of p53 on Ser-15 enables a-CBP/p300 to bind to p53 with a high affinity leading to C-terminal acetylation and enhanced transcriptional activity of p53 (Blattner, 2008).

The acetylation of p53 promotes p53 mediated gene activation which ultimately determines the cells response to stress in the form of senescence, cell growth arrest and apoptosis (Barlev et al., 2001, Blattner, 2008). Hoechst analysis of HepG$_2$ cells showed that FA inhibited cell growth and possibly induced apoptosis, as shown by the absence of dividing cells and the presence of apoptotic bodies (Figure 3.9). This is in accordance with the study by Abdul et al. (2016) in which FA induced apoptosis in HepG$_2$ cells by elevating the activity of the executioner caspases-3/7. Another study conducted by Ogata et al. (2001) showed that FA also effectively induced apoptosis in HL-60 (human promyelocytic leukaemia cells) cells.

The deacetylases, Sirt1 and HDAC1 deacetylate and inactivate p53 (Brooks and Gu, 2003). HDAC1 is known to deacetylate p53 on several lysine residues (K320, K373 and K382). Fusaric acid significantly decreased the protein expression of HDAC1 and increased the expression of p-Ser47-Sirt1 in HepG$_2$ cells (Figure 3.6 and 3.7 respectively). Sirt1 is an NAD+-dependent deacetylase that is responsible for deacetylating p53 specifically on K382 (Wang et al., 2008). Sirt1 catalyses the deacetylation of lysine residues in a unique reaction that utilises NAD+ and generates nicotinamide and O-acetyl ADP-ribose (Avalos et al., 2005). Nicotinamide is a non-competitive inhibitor of Sirt1 activity (Avalos et al., 2005) and the inhibition of Sirt1 activity has been reported to suppress cell growth as well as induce cell cycle arrest and apoptosis *in vitro* (Wang et al., 2013). The function of Sirt1 is regulated by phosphorylation. Several protein kinases have been shown to phosphorylate Sirt1; however, whether or not Sirt1 is activated by phosphorylation is largely dependent on the protein kinase that phosphorylates it (Sasaki et al., 2008). For example, the phosphorylation of Sirt1 on Ser-47 by JNK1 (c-Jun kinase) promotes its nuclear localization and enzymatic activity (Conrad et al., 2016, Nasrin et al., 2009) whereas the phosphorylation of Sirt1 on Ser-47 by mTOR (mammalian target of rapamycin) was found to inhibit Sirt1 deacetylase activity (Back et al., 2011).

Sirt1 also contains a multifunctional domain known as the C-pocket that is capable of directly binding NAD+ and is involved in mediating NAD+ cleavage, base-exchange activity and nicotinamide regulation (Avalos et al., 2005). Pyridine derivatives are able to undergo exchange

reactions with the nicotinamide moiety of NAD+ thereby, forming NAD+ analogues (Bochner et al., 1980, Fernandez-Pol et al., 1977). Fusaric acid is a pyridine derivative and nicotinic acid related compound that may bind to the C-pocket of Sirt1 and inhibit its activity by preventing NAD+ from binding to the C-pocket and catalysing the deacetylation reaction. Fusaric acid may also mimic the structure of nicotinamide, bind to the C-pocket of Sirt1 and inhibit Sirt1 activity. Therefore, although FA elevated the expression of p-Ser47-Sirt1, it may not necessarily be active as an increase in a-K382-p53 was also observed.

MDM2 plays the most central role in the regulation of p53. MDM2 binds and ubiquitinates p53 targeting it for degradation by the 26S proteasome (Brooks and Gu, 2003). This enables p53 to be maintained at low levels in the absence of cellular stress. However, excessive p53 activation can increase the expression of MDM2 in a negative feedback loop to decrease p53 expression and activation (Brooks and Gu, 2003). Fusaric acid significantly increased the expression of MDM2 in $HepG_2$ cells (Figure 3.8). The acetylation of p53 on K382 indicates an increase in p53 stability and activity (Barlev et al., 2001, Brooks and Gu, 2003, Shieh et al., 1997, Sakaguchi et al., 1998). Therefore, the increase in MDM2 expression may occur in response to the increase in a-K382-p53 (increase in p53 activity) observed in the FA treated $HepG_2$ cells. The decrease in p53 protein levels may not be attributed to the increase in MDM2 expression as the phosphorylation of p53 on Ser-15 prevents the interaction between p53 and MDM2 thereby, inhibiting its ubiquitination and degradation in the presence of DNA damage (Brooks and Gu, 2003, Shieh et al., 1997).

In response to DNA damage, ATM and DNA-PK are capable of phosphorylating both p53 and MDM2 (Shi and Gu, 2012). DNA-PK and ATM phosphorylate MDM2 on Ser-17 and Ser-395 respectively. The phosphorylation of MDM2 on Ser-17 prevents MDM2 from binding and ubiquitinating p53 (Mayo et al., 1997) whereas the phosphorylation of MDM2 on Ser-395 inhibits the export of p53 from the nucleus to the cytoplasm (Maya et al., 2001). This prevents p53 degradation and may provide a possible explanation for how p53 trans-activates the *MDM2* gene but is not initially inhibited by the resulting increase in MDM2 protein (Mayo et al., 1997, Maya et al., 2001).

A summary of the effect of FA on DNA integrity and the post-translational modifications of p53 in $HepG_2$ cells is represented in Figure 4.1.
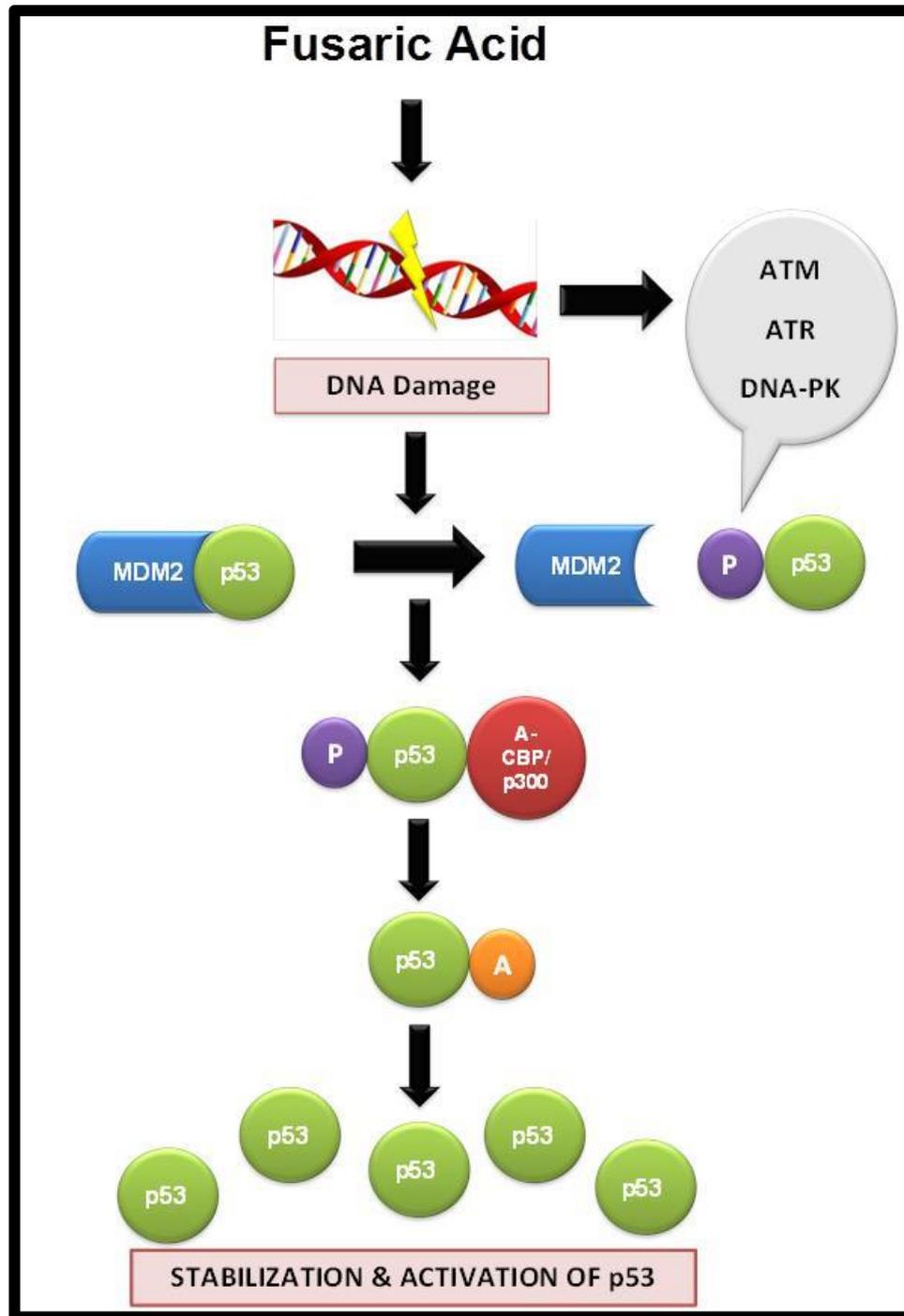
Figure 4.1: Mechanism of FA induced toxicity in the HepG$_2$ cell line. FA induces DNA damage in HepG$_2$ cells. The protein kinases ATM, ATR and DNA-PK are activated in response to DNA damage. These protein kinases phosphorylate p53 on Ser-15. The phosphorylation of p53 alleviates the interaction between p53 and MDM2 and increases the interaction between p53 and a-CBP/p300. This leads to the acetylation of p53. The acetylation of p53 stabilises and activates p53 leading to cell growth arrest and apoptosis (Prepared by author).

CHAPTER 5

CONCLUSION

The contamination of foods and feeds with mycotoxins is a serious problem that results in major economic losses and a huge impact on human and animal health (Milićević et al., 2010, Bennett and Klich, 2003, Zain, 2011, Yiannikouris and Jouany, 2002). The application of modern agricultural practices and the presence of legislatively regulated food processing help curb the extent of mycotoxin contamination; however, it does not totally eradicate it. Of major concern is the often neglected *Fusarium* produced mycotoxin, FA. It is well established that FA is a common contaminant of several food commodities and several studies have shown FA to be toxic (Abdul et al., 2016, Bacon et al., 1995, Bungo et al., 1999, Dowd, 1988, Fairchild et al., 2005, Ogunbo et al., 2007, Pavlovkin et al., 2004, Smith and MacDonald, 1991, Smith et al., 1997, Terasawa and Kameyama, 1971, Voss et al., 1999). However, limited scientific data is available on the mechanism by which FA exerts its toxic effects.

Studies evaluating the mechanism of FA induced toxicity are important in understanding the risks of FA contamination to human and animal health. This is particularly relevant in developing countries and poverty stricken areas where exposure to mycotoxins is high due to the continuous consumption of contaminated agricultural commodities.

This study, for the first time, provides a possible mechanism of FA induced toxicity in the human liver using the $HepG_2$ cell line. The activation of p53 occurs in response to various cellular stressors such as DNA damage and may provide the most likely mechanism of FA induced toxicity. The results indicate that FA is genotoxic and post-translationally modified (increased the phosphorylation and acetylation) p53 leading to cell growth arrest and apoptosis of $HepG_2$ cells. These findings correspond with the hypothesis which stated that FA induced DNA damage and post-translationally modified p53 in $HepG_2$ cells. Interestingly, the results also indicate a control for p53 acetylation and highlight the importance of the regulation between p53 and MDM2 for cell development, proper protection from DNA damage, cell cycle control and growth. Furthermore, the results indicate that Ser-15 phosphorylation is required for p53 function and this may suggest a possible universal role in promoting p53-transcriptional activity and determining cell fate. Nevertheless, future studies are required to validate the mechanism of FA induced toxicity using an *in vivo* model. It would also be interesting to determine possible synergistic effects of FA with other *Fusarium* produced mycotoxins.

REFERENCES

ABDUL, N. S., NAGIAH, S. & CHUTURGOON, A. A. 2016. Fusaric acid induces mitochondrial stress in human hepatocellular carcinoma (HepG2) cells. *Toxicon,* 119**,** 336-344.

AVALOS, J. L., BEVER, K. M. & WOLBERGER, C. 2005. Mechanism of sirtuin inhibition by nicotinamide: altering the NAD+ cosubstrate specificity of a Sir2 enzyme. *Molecular Cell,* 17**,** 855-868.

BACK, J. H., REZVANI, H. R., ZHU, Y., GUYONNET-DUPERAT, V., ATHAR, M., RATNER, D. & KIM, A. L. 2011. Cancer cell survival following DNA damage-mediated premature senescence is regulated by mammalian target of rapamycin (mTOR)-dependent Inhibition of sirtuin 1. *Journal of Biological Chemistry,* 286**,** 19100-19108.

BACON, C., PORTER, J., NORRED, W. & LESLIE, J. 1996. Production of fusaric acid by Fusarium species. *Applied and Environmental Microbiology,* 62**,** 4039-4043.

BACON, C. W., PORTER, J. K. & NORRED, W. P. 1995. Toxic interaction of fumonisin B1 and fusaric acid measured by injection into fertile chicken egg. *Mycopathologia,* 129**,** 29-35.

BARLEV, N. A., LIU, L., CHEHAB, N. H., MANSFIELD, K., HARRIS, K. G., HALAZONETIS, T. D. & BERGER, S. L. 2001. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. *Molecular Cell,* 8**,** 1243-1254.

BENCHIMOL, S. 2001. p53-dependent pathways of apoptosis. *Cell Death and Differentiation,* 8**,** 1049-1051.

BENNETT, J. W. & KLICH, M. 2003. Mycotoxins. *Clinical Microbiology Reviews,* 16**,** 497-516.

BERGSTROM, G. 2014. *NY Wheat Producers: Make Your Fungicide Decisions Soon!* [Online]. Available: https://blogs.cornell.edu/ccefieldcropnews/2014/05/page/2/ [Accessed 15 September 2016].

BLACK, J. C., MOSLEY, A., KITADA, T., WASHBURN, M. & CAREY, M. 2008. The SIRT2 deacetylase regulates autoacetylation of p300. *Molecular Cell,* 32**,** 449-455.

BLATTNER, C. 2008. Regulation of p53: the next generation. *Cell Cycle,* 7**,** 3149-3153.

BOCHNER, B. R., HUANG, H.-C., SCHIEVEN, G. L. & AMES, B. N. 1980. Positive selection for loss of tetracycline resistance. *Journal of Bacteriology,* 143**,** 926-933.

BOUARAB, K. 2009. *Molecular plant-microbe interactions, edited by Kamal Bouarab, Normand Brisson, Fouad Daayf*, edition 1, 319-335.

BROOKS, C. L. & GU, W. 2003. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Current Opinion in Cell Biology,* 15**,** 164-171.

BUNGO, T., SHIMOJO, M., MASUDA, Y., CHOI, Y. H., DENBOW, D. M. & FURUSE, M. 1999. Induction of food intake by a noradrenergic system using clonidine and fusaric acid in the neonatal chick. *Brain Research,* 826**,** 313-316.

CHAO, C., HERGENHAHN, M., KAESER, M. D., WU, Z., SAITO, S. I., IGGO, R., HOLLSTEIN, M., APPELLA, E. & XU, Y. 2003. Cell type-and promoter-specific roles of Ser18 phosphorylation in regulating p53 responses. *Journal of Biological Chemistry,* 278**,** 41028-41033.

CHAZOTTE, B. 2011. Labeling nuclear DNA with hoechst 33342. *Cold Spring Harbor Protocols,* 2011**,** 83-85.

CHENG, H.-L., MOSTOSLAVSKY, R., SAITO, S. I., MANIS, J. P., GU, Y., PATEL, P., BRONSON, R., APPELLA, E., ALT, F. W. & CHUA, K. F. 2003. Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. *Proceedings of the National Academy of Sciences*, 100, 10794-10799.

CHIPUK, J. & GREEN, D. 2006. Dissecting p53-dependent apoptosis. *Cell Death & Differentiation,* 13**,** 994-1002.

COLLINS, A. R. 2004. The comet assay for DNA damage and repair. *Molecular Biotechnology,* 26**,** 249-261.

CONRAD, E., POLONIO-VALLON, T., MEISTER, M., MATT, S., BITOMSKY, N., HERBEL, C., LIEBL, M., GREINER, V., KRIZNIK, B. & SCHUMACHER, S. 2016. HIPK2 restricts SIRT1 activity upon severe DNA damage by a phosphorylation-controlled mechanism. *Cell Death & Differentiation,* 23**,** 110-122.

D'ALTON, A. & ETHERTON, B. 1984. Effects of fusaric acid on tomato root hair membrane potentials and ATP levels. *Plant Physiology,* 74**,** 39-42.

DAI, C. & GU, W. 2010. p53 post-translational modification: deregulated in tumorigenesis. *Trends in Molecular Medicine,* 16**,** 528-536.

DARWISH, W. S., IKENAKA, Y., NAKAYAMA, S. M. & ISHIZUKA, M. 2014. An overview on mycotoxin contamination of foods in Africa. *The Journal of Veterinary Medical Science,* 76**,** 789-797.

DAS, C., ROY, S., NAMJOSHI, S., MALARKEY, C. S., JONES, D. N., KUTATELADZE, T. G., CHURCHILL, M. E. & TYLER, J. K. 2014. Binding of the histone chaperone ASF1 to the CBP bromodomain promotes histone acetylation. *Proceedings of the National Academy of Sciences,* 111**,** E1072-E1081.

DEHN, P., WHITE, C., CONNERS, D., SHIPKEY, G. & CUMBO, T. 2004. Characterization of the human hepatocellular carcinoma (hepg2) cell line as an in vitro model for cadmium toxicity studies. *In Vitro Cellular & Developmental Biology-Animal,* 40**,** 172-182.

DELCUVE, G. P., KHAN, D. H. & DAVIE, J. R. 2012. Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. *Clinical Epigenetics,* 4**,** 1-13.

DINIZ, S. & OLIVEIRA, R. 2009. Effects of fusaric acid on Zea mays L. seedlings. *Phyton (Buenos Aires),* 78**,** 155-160.

DONG, X., LING, N., WANG, M., SHEN, Q. & GUO, S. 2012. Fusaric acid is a crucial factor in the disturbance of leaf water imbalance in Fusarium-infected banana plants. *Plant Physiology and Biochemistry,* 60**,** 171-179.

DOWD, P. F. 1988. Toxicological and biochemical interactions of the fungal metabolites fusaric acid and kojic acid with xenobiotics in Heliothis zea (F.) and Spodoptera frugiperda (JE Smith). *Pesticide Biochemistry and Physiology,* 32**,** 123-134.

ESLAMI, A. & LUJAN, J. 2010. Western blotting: sample preparation to detection. *JoVE (Journal of Visualized Experiments),* 44**,** e2359-e2359.

FAIRBAIRN, D. W., OLIVE, P. L. & O'NEILL, K. L. 1995. The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology,* 339**,** 37-59.

FAIRCHILD, A., GRIMES, J., PORTER, J., CROOM JR, W., DANIEL, L. & HAGLER JR, W. 2005. Effects of diacetoxyscirpenol and fusaric acid on poults: Individual and combined effects of dietary diacetoxyscirpenol and fusaric acid on turkey poult performance. *International Journal of Poultry Science,* 4**,** 350-355.

FAVALORO, B., ALLOCATI, N., GRAZIANO, V., DI ILIO, C. & DE LAURENZI, V. 2012. Role of apoptosis in disease. *Aging (Albany New York),* 4**,** 330-349.

FERNANDEZ-POL, J., BONO, V. H. & JOHNSON, G. S. 1977. Control of growth by picolinic acid: differential response of normal and transformed cells. *Proceedings of the National Academy of Sciences,* 74**,** 2889-2893.

FERNANDEZ-POL, J. A., KLOS, D. J. & HAMILTON, P. D. 1993. Cytotoxic activity of fusaric acid on human adenocarcinoma cells in tissue culture. *Anticancer Research,* 13**,** 57-64.

FULDA, S. 2010. Evasion of apoptosis as a cellular stress response in cancer. *International Journal of Cell Biology,* 2010**,** 2-6.

GEORGE, P. 2011. p53 How crucial is its role in cancer. *International Journal of Current Pharmaceutical Research,* 3**,** 19-25.

GRANT, R., COGGAN, S. & SMYTHE, G. 2009. The physiological action of picolinic acid in the human brain. *International Journal of Tryptophan Research,* 2**,** 71-79.

GREEN, D. R. & KROEMER, G. 2009. Cytoplasmic functions of the tumour suppressor p53. *Nature,* 458**,** 1127-1130.

GRIESSLER, K. 2008. *Fumonisin Mycotoxins* [Online]. Mycotoxins. Available: http://en.engormix.com/MA-mycotoxins/articles/fumonisins-mycotoxins-increasing-importance-t1109/255-p0.htm [Accessed 6 November 2016].

GROSS, A., MCDONNELL, J. M. & KORSMEYER, S. J. 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes and Development,* 13**,** 1899-1911.

GU, H., WANG, X., RAO, S., WANG, J., ZHAO, J., REN, F. L., MU, R., YANG, Y., QI, Q. & LIU, W. 2008. Gambogic acid mediates apoptosis as a p53 inducer through down-regulation of mdm2 in wild-type p53-expressing cancer cells. *Molecular Cancer Therapeutics,* 7**,** 3298-3305.

GU, W. & ROEDER, R. G. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell,* 90**,** 595-606.

HAWKINS, M. 2013. *Harvest 2013: It's quality, not just quantity, that counts* [Online]. http://www.wattagnet.com/articles/17719-harvest-2013-it-s-quality-not-just-quantity-that-counts: Wattagnet.com. [Accessed 3 August 2016.

HAWKINS, M. 2014. *Harvest analysis completed: All samples contained mycotoxins* [Online]. http://www.progressivedairycanada.com/topics/feed-nutrition/harvest-analysis-completed-all-samples-contained-mycotoxins: Progressive DairyMan. [Accessed 3 August 2016].

HENGARTNER, M. O. 2000. The biochemistry of apoptosis. *Nature,* 407**,** 770-776.

HIDAKA, H., NAGATSU, T., TAKEYA, K., TAKEUCHI, T., SUDA, H., KOJIRI, K.,
MATSUZAKI, M. & UMEZAWA, H. 1969. Fusaric acid, a hypotensive agent
produced by fungi. *The Journal of Antibiotics,* 22**,** 228-230.

HONGMEI, Z. 2012. *Extrinsic and intrinsic apoptosis signal pathway review*, Apoptosis and
Medicine.

ITO, A., KAWAGUCHI, Y., LAI, C. H., KOVACS, J. J., HIGASHIMOTO, Y., APPELLA, E.
& YAO, T. P. 2002. MDM2–HDAC1-mediated deacetylation of p53 is required for its
degradation. *The European Molecular Biology Organization (EMBO) Journal,* 21**,**
6236-6245.

JOERGER, A. & FERSHT, A. 2007. Structure–function–rescue: the diverse nature of common
p53 cancer mutants. *Oncogene,* 26**,** 2226-2242.

JOERGER, A. C. & FERSHT, A. R. 2010. The tumor suppressor p53: from structures to drug
discovery. *Cold Spring Harbor Perspectives in Biology,* 2**,** 1-21.

KANG, M., KIM, M.-S., KIM, J. Y., SHIN, Y.-J., SONG, J.-Y. & JEONG, J.-H. 2015. A novel
pyrido-thieno-pyrimidine derivative activates p53 through induction of phosphorylation
and acetylation in colorectal cancer cells. *International Journal of Oncology,* 46**,** 342-
350.

KERR, J. F., WINTERFORD, C. M. & HARMON, B. V. 1994. Apoptosis. Its significance in
cancer and cancer therapy. *Cancer,* 73**,** 2013-2026.

LAMBERT, P. F., KASHANCHI, F., RADONOVICH, M. F., SHIEKHATTAR, R. &
BRADY, J. N. 1998. Phosphorylation of p53 serine 15 increases interaction with CBP.
Journal of Biological Chemistry, 273**,** 33048-33053.

LEE, C.-W., WONG, L. L.-Y., TSE, E. Y.-T., LIU, H.-F., LEONG, V. Y.-L., LEE, J. M.-F.,
HARDIE, D. G., NG, I. O.-L. & CHING, Y.-P. 2012. AMPK promotes p53 acetylation
via phosphorylation and inactivation of SIRT1 in liver cancer cells. *Cancer Research,*
72**,** 4394-4404.

LI, C., ZUO, C., DENG, G., KUANG, R., YANG, Q., HU, C., SHENG, O., ZHANG, S., MA, L. & WEI, Y. 2013. Contamination of bananas with beauvericin and fusaric acid produced by Fusarium oxysporum f. sp. cubense. *Public Library of Science (PloS) One,* 8**,** 1-11.

LI, D. W., LIU, J., SCHMID, P., SCHLOSSER, R., FENG, H., LIU, W., YAN, Q., GONG, L., SUN, S. & DENG, M. 2006. Protein serine/threonine phosphatase-1 dephosphorylates p53 at Ser-15 and Ser-37 to modulate its transcriptional and apoptotic activities. *Oncogene,* 25**,** 3006-3022.

LUO, J., LI, M., TANG, Y., LASZKOWSKA, M., ROEDER, R. G. & GU, W. 2004. Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 2259-2264.

MAHMOOD, T. & YANG, P.-C. 2012. Western blot: technique, theory, and trouble shooting. *North American Journal of Medical Sciences,* 4**,** 429-434.

MAY, H. D., WU, Q. & BLAKE, C. K. 2000. Effects of the Fusarium spp. mycotoxins fusaric acid and deoxynivalenol on the growth of Ruminococcus albus and Methanobrevibacter ruminantium. *Canadian Journal of Microbiology,* 46**,** 692-699.

MAYA, R., BALASS, M., KIM, S.-T., SHKEDY, D., LEAL, J.-F. M., SHIFMAN, O., MOAS, M., BUSCHMANN, T., RONAI, Z. E. & SHILOH, Y. 2001. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes and Development,* 15**,** 1067-1077.

MAYO, L. D., TURCHI, J. J. & BERBERICH, S. J. 1997. Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Research,* 57**,** 5013-5016.

MILIĆEVIĆ, D. R., ŠKRINJAR, M. & BALTIĆ, T. 2010. Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. *Toxins,* 2**,** 572-592.

MOMAND, J., JUNG, D., WILCZYNSKI, S. & NILAND, J. 1998. The MDM2 gene amplification database. *Nucleic Acids Research,* 26**,** 3453-3459.

NASRIN, N., KAUSHIK, V. K., FORTIER, E., WALL, D., PEARSON, K. J., DE CABO, R. & BORDONE, L. 2009. JNK1 phosphorylates SIRT1 and promotes its enzymatic activity. *Public Library of Science (PloS) One,* 4**,** 1-9.

NYBORG, J. K. & PEERSEN, O. B. 2004. That zincing feeling: the effects of EDTA on the behaviour of zinc-binding transcriptional regulators. *Biochemical Journal,* 381**,** e3-e4.

O'BRATE, A. & GIANNAKAKOU, P. 2003. The importance of p53 location: nuclear or cytoplasmic zip code? *Drug Resistance Updates,* 6**,** 313-322.

OGATA, S., INOUE, K., IWATA, K., OKUMURA, K. & TAGUCHI, H. 2001. Apoptosis induced by picolinic acid-related compounds in HL-60 cells. *Bioscience, Biotechnology, and Biochemistry*, 65, 2337-2339.

OGUNBO, S., BROOMHEAD, J., LEDOUX, D., BERMUDEZ, A. & ROTTINGHAUS, G. 2007. The individual and combined effects of fusaric acid and T-2 toxin in broilers and turkeys. *International Journal of Poultry Science,* 6**,** 484-488.

OKADA, H. & MAK, T. W. 2004. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nature Reviews Cancer,* 4**,** 592-603.

OSTRAKHOVITCH, E. A., OLSSON, P.-E., JIANG, S. & CHERIAN, M. G. 2006. Interaction of metallothionein with tumor suppressor p53 protein. *Federation of European Biochemical Societies (FEBS) Letters,* 580**,** 1235-1238.

PARK, S., MARTINEZ-YAMOUT, M. A., DYSON, H. J. & WRIGHT, P. E. 2013. The CH2 domain of CBP/p300 is a novel zinc finger. *Federation of European Biochemical Scocieties (FEBS) Letters,* 587**,** 2506-2511.

PAVLOVKIN, J., MISTRIK, I. & PROKOP, M. 2004. Some aspects of the phytotoxic action of fusaric acid on primary Ricinus roots. *Plant Soil and Environment,* 50**,** 397-401.

PERAICA, M., RADIC, B., LUCIC, A. & PAVLOVIC, M. 1999. Toxic effects of mycotoxins in humans. *Bulletin of the World Health Organization,* 77**,** 754-766.

PINOTTI, L., OTTOBONI, M., GIROMINI, C., DELL'ORTO, V. & CHELI, F. 2016. Mycotoxin Contamination in the EU Feed Supply Chain: A Focus on Cereal Byproducts. *Toxins,* 8**,** 1-24.

PIRAYESH, S., ZAMANIZADEH, H. & MORID, B. 2015. Comparison of the production of fusaric acid produced by Fusarium oxysporum f. sp. lycopersici in different cultures with HPLC method. *International Journal of Biosciences* 6**,** 355-359.

POCHAMPALLY, R., FODERA, B., CHEN, L., SHAO, W., LEVINE, E. A. & CHEN, J. 1998. A 60 kd MDM2 isoform is produced by caspase cleavage in non-apoptotic tumor cells. *Oncogene,* 17**,** 2629-2636.

PRIVES, C. & HALL, P. A. 1999. The p53 pathway. *The Journal of Pathology,* 187**,** 112-126.

RANI, T., RAJAN, S., LAVANYA, L. & KAMALALOCHANI, S. 2009. An overview of Fusaric acid production. *Advanced Biotechnology,* 8**,** 18-22.

REDDY, R. V., LARSON, C. A., BRIMER, G. E., FRAPPIER, B. L. & REDDY, C. S. 1996. Developmental toxic effects of fusaric acid in CD1 mice. *Bull Environ Contam Toxicol,* 57**,** 354-60.

REED, S. M. & QUELLE, D. E. 2014. p53 acetylation: regulation and consequences. *Cancers,* 7**,** 30-69.

REGO, A., VESCE, S. & NICHOLLS, D. 2001. The mechanism of mitochondrial membrane potential retention following release of cytochrome c in apoptotic GT1-7 neural cells. *Cell Death and Differentiation,* 8**,** 995-1003.

SAKAGUCHI, K., HERRERA, J. E., SAITO, S. I., MIKI, T., BUSTIN, M., VASSILEV, A., ANDERSON, C. W. & APPELLA, E. 1998. DNA damage activates p53 through a phosphorylation–acetylation cascade. *Genes and Development,* 12**,** 2831-2841.

SALMON, T. B., EVERT, B. A., SONG, B. & DOETSCH, P. W. 2004. Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae. *Nucleic Acids Research,* 32**,** 3712-3723.

SANCAR, A., LINDSEY-BOLTZ, L. A., ÜNSAL-KAÇMAZ, K. & LINN, S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annual Review of Biochemistry,* 73**,** 39-85.

SAPKO, O., UTARBAEVA, A. S. & MAKULBEK, S. 2011. Effect of fusaric acid on prooxidant and antioxidant properties of the potato cell suspension culture. *Russian Journal of Plant Physiology,* 58**,** 828-835.

SASAKI, T., MAIER, B., KOCLEGA, K. D., CHRUSZCZ, M., GLUBA, W., STUKENBERG, P. T., MINOR, W. & SCRABLE, H. 2008. Phosphorylation regulates SIRT1 function. *Public Library of Science (PloS) One,* 3**,** 1-13.

SHI, D. & GU, W. 2012. Dual roles of MDM2 in the regulation of p53 ubiquitination dependent and ubiquitination independent mechanisms of MDM2 repression of p53 activity. *Genes and Cancer,* 3**,** 240-248.

SHIEH, S.-Y., IKEDA, M., TAYA, Y. & PRIVES, C. 1997. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell,* 91**,** 325-334.

SINGH, N. P., MCCOY, M. T., TICE, R. R. & SCHNEIDER, E. L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, 175, 184-191.

SMITH, T. & MACDONALD, E. 1991. Effect of fusaric acid on brain regional neurochemistry and vomiting behavior in swine. *Journal of Animal Science,* 69**,** 2044-2049.

SMITH, T. K., MCMILLAN, E. G. & CASTILLO, J. B. 1997. Effect of feeding blends of Fusarium mycotoxin-contaminated grains containing deoxynivalenol and fusaric acid on growth and feed consumption of immature swine. *Journal of Animal Science,* 75**,** 2184-2191.

SMITH, T. K. & SOUSADIAS, M. G. 1993. Fusaric acid content of swine feedstuffs. *Journal of Agricultural and Food Chemistry,* 41**,** 2296-2298.

SÖDERGREN, E. 2000. Lipid Peroxidation in vivo: Evaluation and application of methods for measurement. *Clinical Nutrition ACTA Universitatis Upsaliensis Uppsala,* Doctor of Medical Sciences in Geriatrics, 749**,** 1-76.

SOLDATOW, V. Y., LECLUYSE, E. L., GRIFFITH, L. G. & RUSYN, I. 2013. In vitro models for liver toxicity testing. *Toxicology Research,* 2**,** 23-39.

SOLOMON, J. M., PASUPULETI, R., XU, L., MCDONAGH, T., CURTIS, R., DISTEFANO, P. S. & HUBER, L. J. 2006. Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Molecular and Cellular Biology,* 26**,** 28-38.

ŠROBÁROVÁ, A., EGED, Š. & DA SILVA, J. T. 2009. The use of Bacillus subtilis for screening fusaric acid production by Fusarium spp. *Czech Journal of Food Science,* 27**,** 203-209.

STACK, B. C., HANSEN, J. P., RUDA, J. M., JAGLOWSKI, J., SHVIDLER, J. & HOLLENBEAK, C. S. 2004. Fusaric acid: a novel agent and mechanism to treat HNSCC. *Otolaryngology--Head and Neck Surgery,* 131**,** 54-60.

STACK JR, B. C., YE, J., WILLIS, R., HUBBARD, M. & HENDRICKSON, H. P. 2014. Determination of oral bioavailability of fusaric acid in male Sprague-Dawley rats. *Drugs in Research and Development,* 14**,** 139-145.

STREIT, E., SCHWAB, C., SULYOK, M., NAEHRER, K., KRSKA, R. & SCHATZMAYR, G. 2013. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins,* 5**,** 504-523.

SWINBURNE, T. R. 2012. *Iron, siderophores, and plant diseases*, Springer Science and Business Media.

TANG, Y., ZHAO, W., CHEN, Y., ZHAO, Y. & GU, W. 2008. Acetylation is indispensable for p53 activation. *Cell,* 133**,** 612-626.

TELLES-PUPULIN, A., DINIZ, S., BRACHT, A. & ISHII-IWAMOTO, E. 1996. Effects of fusaric acid on respiration in maize root mitochondria. *Biologia Plantarum,* 38**,** 421-429.

TERASAWA, F. & KAMEYAMA, M. 1971. The clinical trial of a new hypotensive agent," fusaric acid (5-butylpicolinic acid)": the preliminary report. *Japanese Circulation Journal,* 35**,** 339-357.

THAKUR, V. S., GUPTA, K. & GUPTA, S. 2012. Green tea polyphenols increase p53 transcriptional activity and acetylation by suppressing class I histone deacetylases. *International Journal of Oncology,* 41**,** 353-361.

THOMPSON, P. R., WANG, D., WANG, L., FULCO, M., PEDICONI, N., ZHANG, D., AN, W., GE, Q., ROEDER, R. G. & WONG, J. 2004. Regulation of the p300 HAT domain via a novel activation loop. *Nature Structural and Molecular Biology,* 11**,** 308-315.

TICE, R., AGURELL, E., ANDERSON, D., BURLINSON, B., HARTMANN, A., KOBAYASHI, H., MIYAMAE, Y., ROJAS, E., RYU, J. & SASAKI, Y. 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis,* 35**,** 206-221.

VAKHRUSHEVA, O., BRAEUER, D., LIU, Z., BRAUN, T. & BOBER, E. 2008. Sirt7-dependent inhibition of cell growth and proliferation might be instrumental to mediate tissue integrity during aging. *Journal of Physiology and Pharmacology,* 59**,** 201-212.

VAN LEEUWEN, I. M., HIGGINS, M., CAMPBELL, J., MCCARTHY, A. R., SACHWEH, M. C., NAVARRO, A. M. & LAÍN, S. 2013. Modulation of p53 C-terminal acetylation by mdm2, p14ARF, and cytoplasmic SirT2. *Molecular Cancer Therapeutics,* 12**,** 471-480.

VOSS, K., PORTER, J., BACON, C., MEREDITH, F. & NORRED, W. 1999. Fusaric acid and modification of the subchronic toxicity to rats of fumonisins in F. moniliforme culture material. *Food and Chemical Toxicology,* 37**,** 853-861.

WALKER, J. M. 1996. *The protein protocols handbook*, Springer Science & Business Media.

WANG, R.-H., SENGUPTA, K., LI, C., KIM, H.-S., CAO, L., XIAO, C., KIM, S., XU, X., ZHENG, Y. & CHILTON, B. 2008. Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. *Cancer Cell,* 14**,** 312-323.

WANG, T., CUI, H., MA, N. & JIANG, Y. 2013. Nicotinamide‑mediated inhibition of SIRT1 deacetylase is associated with the viability of cancer cells exposed to antitumor agents and apoptosis. *Oncology Letters,* 6**,** 600-604.

WILD, C. P. & GONG, Y. Y. 2010. Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis,* 31**,** 71-82.

WILKENING, S., STAHL, F. & BADER, A. 2003. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metabolism and Disposition,* 31**,** 1035-1042.

WOODLE, E. S. & KULKARNI, S. 1998. Programmed cell death. *Transplantation,* 66**,** 681-691.

YE, J., MONTERO, M. & STACK JR, B. C. 2013. Effects of fusaric acid treatment on HEp2 and docetaxel-resistant HEp2 laryngeal squamous cell carcinoma. *Chemotherapy,* 59**,** 121-128.

YI, J. & LUO, J. 2010. SIRT1 and p53, effect on cancer, senescence and beyond. *Biochimica et Biophysica Acta-Proteins and Proteomics,* 1804**,** 1684-1689.

YIANNIKOURIS, A. & JOUANY, J.-P. 2002. Mycotoxins in feeds and their fate in animals: a review. *Animal Research,* 51**,** 81-99.

YIN, E. S., RAKHMANKULOVA, M., KUCERA, K., DE SENA FILHO, J. G., PORTERO, C. E., NARVAEZ-TRUJILLO, A., HOLLEY, S. A. & STROBEL, S. A. 2015. Fusaric acid induces a notochord malformation in zebrafish via copper chelation. *Biometals,* 28**,** 783-9.

ZAIN, M. E. 2011. Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society,* 15**,** 129-144.

ZHANG, J., SHEN, L. & SUN, L.Q. 2015. The regulation of radiosensitivity by p53 and its acetylation. *Cancer Letters,* 363**,** 108-118.

Methyl Thiazol Tetrazolium (MTT) Assay

The MTT assay is a colorimetric assay used to measure cell viability by determining mitochondrial activity. Mitochondrial activity is a common indicator of cell viability and is significantly reduced in dead and dying cells. The principle of the MTT assay is based on the reaction in which live metabolically active cells reduce the yellow tetrazolium salt (MTT; 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) to a purple insoluble formazan product ((E, Z)-5-(4, 5-dimethylthiazol-2-yl)-1, 3-dimethylformazan) with the action of dehydrogenase enzymes. These enzymes utilise reducing equivalents such as NADH and $FADH_2$ produced in the citric acid cycle to cleave the tetrazolium ring of MTT and produce the formazan product which can be solubilised and quantified using spectrophotometry (Mosmann, 1983). The intensity of the formazan produced is proportional to the number of viable cells.

Fusaric acid dose-dependently decreased the viability of $HepG_2$ cells at 24 hrs (Figure 5). This indicates that FA is cytotoxic to $HepG_2$ cells. The concentration of FA causing death of 50% of $HepG_2$ cells (IC_{50}) was determined to be 104μg/ml. This concentration was used as the treatment concentration for all subsequent assays.
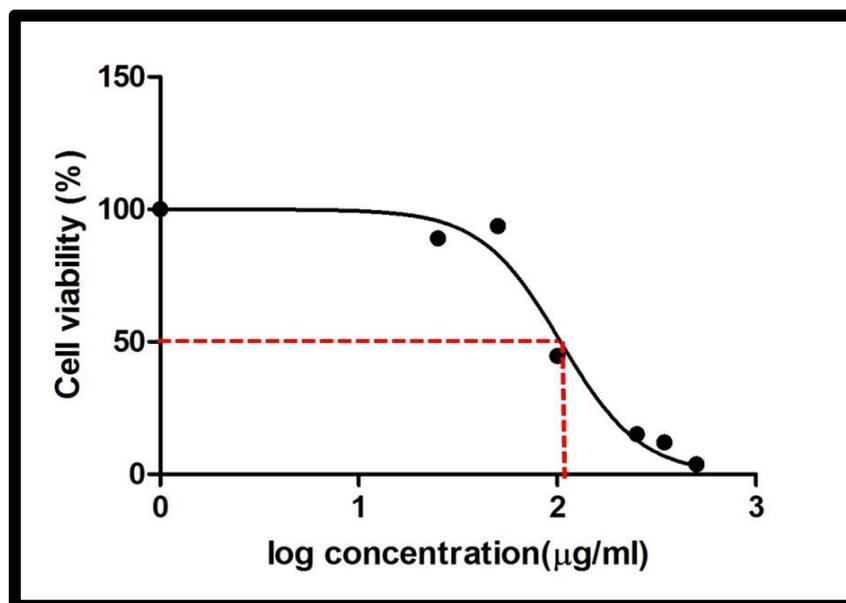

Figure 5: IC_{50} graph displaying the dose-dependent decline in $HepG_2$ cell viability (%) at 24 hrs (IC_{50} = 104μg/ml)

APPENDIX B

**Bicinchoninic Acid (BCA) Assay**
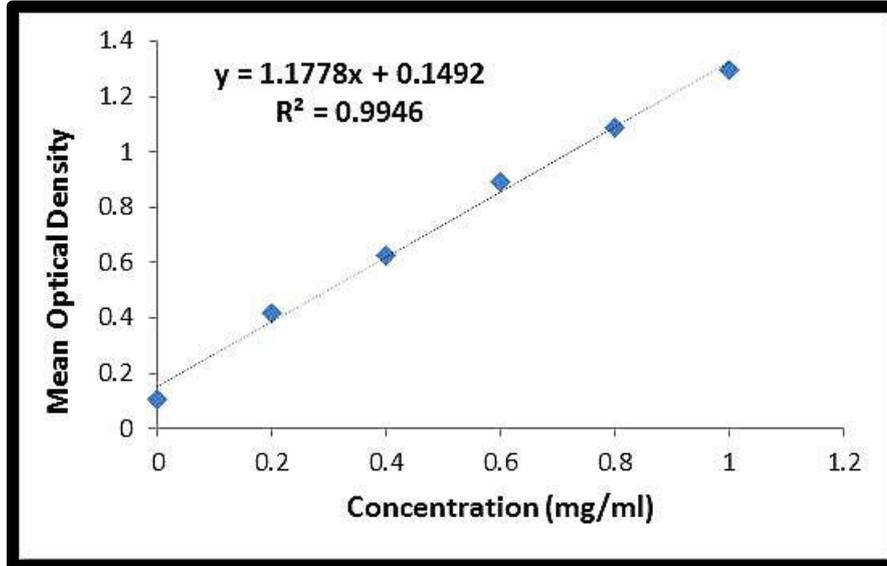
Figure 6: Standard curve displaying known concentrations of bovine serum albumin (BSA) used to determine the concentration of protein present in each sample

Table 2: Standardisation of proteins (Final volume = 400µl; Concentration = 1.5mg/ml)

Sample	Mean Absorbance	Protein Concentration (mg/ml)	Volume of Protein Stock (µl)	Volume of Cytobuster (µl)	Volume of Laemmli Buffer (µl)
Control	3.596	2.926	205	195	100
FA Treatment	2.523	2.015	298	102	100

Western Blot

Expression of p-ser15-p53 and a-K382-p53 relative to the total p53 expressed

The expression of p-Ser15-p53 and a-K382-p53 were divided by the expression of total p53 in order to determine the ratio of p53 acetylated and phosphorylated relative to the total p53 expressed within the cell.

p-Ser15-p53/p53

The ratio of p-Ser15-p53 to total p53 was significantly higher in the FA treated $HepG_2$ cells compared to the control [13.42 ± 0.32-fold vs. 1.02 ± 0.01-fold, *p=0.0002*; Figure 7.1].
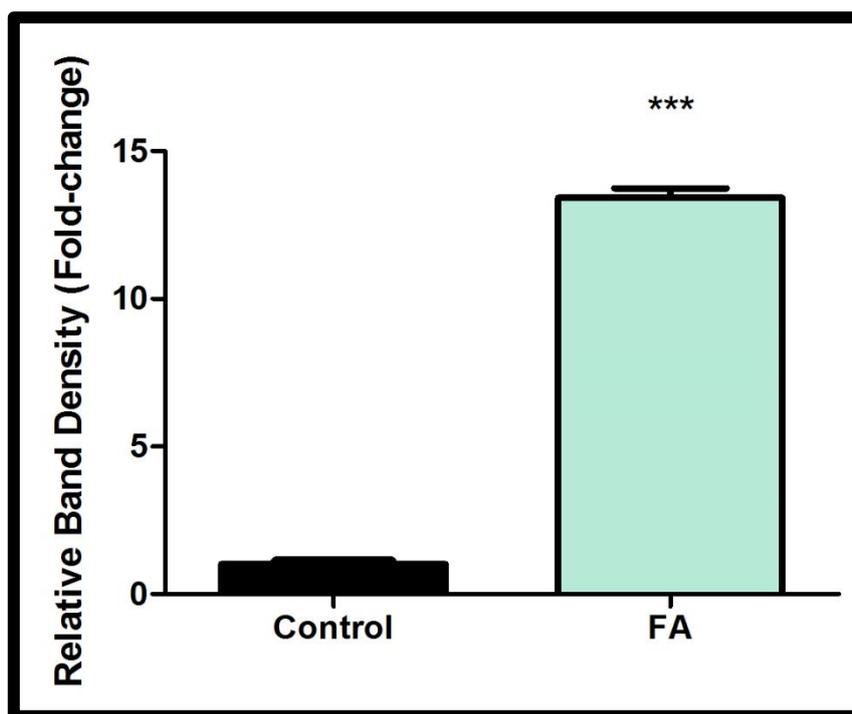

Figure 7.1: Fold-change of the expression of p-Ser15-p53 relative to total p53 (****p<0.0005*).

a-K382-p53/p53

The ratio of a-K382-p53 to total p53 was significantly higher in the FA treated HepG$_2$ cells compared to the control [2.41±0.33-fold vs. 1.02±0.01-fold, *p=0.0181*; Figure 7.2].
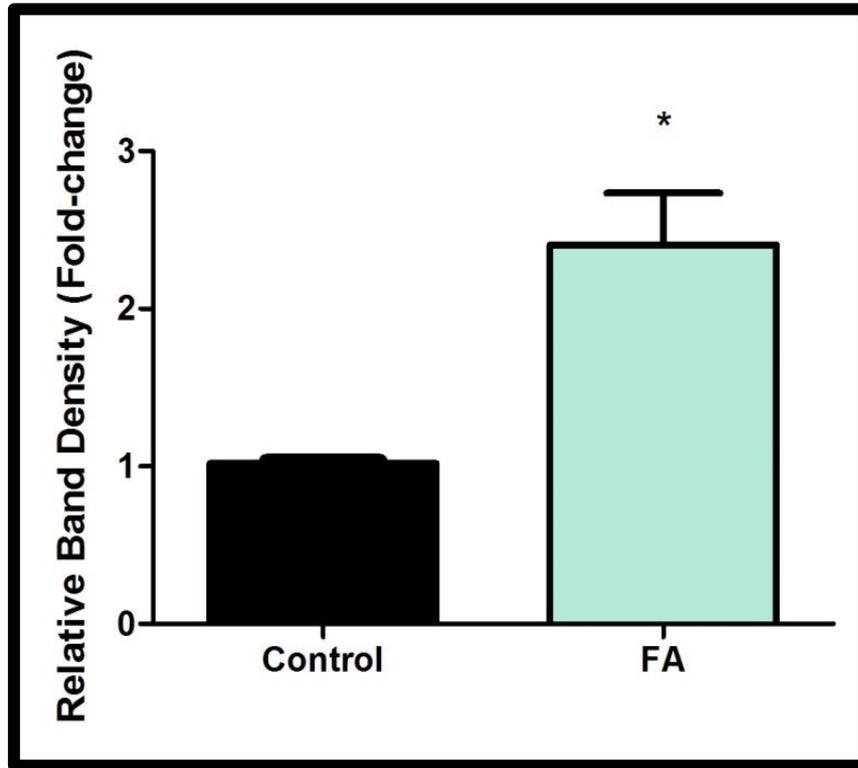

Figure 7.2: Fold-change of the expression of a-K382-p53 relative to total p53 (**p<0.05*).

APPENDIX D

Comet Assay

Table 3: Raw data/tail lengths (μm) obtained from the comet assay

Control	Fusaric Acid Treatment
45.59	76.11
44.86	76.88
43.87	77.12
43.88	77.34
43.78	77.40
42.57	77.66
42.57	78.65
41.28	78.69
42.50	78.69
41.28	78.76
38.62	79.87
39.99	79.98
39.99	81.27
40.78	81.27
38.70	81.56
38.70	81.69
37.41	82.09
34.54	82.56
37.41	83.67
35.12	83.85
36.19	84.78
34.43	85.14
37.12	85.14
35.83	87.72
34.83	89.01
33.54	90.30
32.25	90.97
34.34	91.59
32.25	91.78
33.56	92.88
32.25	94.17
30.96	95.46
32.24	95.46
29.67	96.74

29.67	96.75
28.35	98.04
28.45	98.05
27.12	98.19
28.09	98.24
27.08	98.46
25.80	99.33
25.80	99.76
25.74	100.62
25.95	100.62
27.80	101.43
24.51	104.49
24.59	104.89
25.51	105.78
20.58	108.36
21.64	119.97