ANTICANCER, ANTIOXIDANT AND IMMUNO-MODULATORY EFFECTS OF "*ACACIA* HONEY" IN SOME CANCER CELL LINES

By

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CERTIFICATION

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DEDICATION

This piece of work is dedicated to the memory of my Father late Muhammad Abdullahi.

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ABSTRACT

Cancer is one of the leading causes of death worldwide. Cancer curative properties of honey have been documented. However, there is dearth of information on the exact mechanism of anticancer effect of honey. This study was designed to evaluate the antioxidant, immuno-modulatory and anticancer properties of "*Acacia* Honey" (AH).

The AH used was obtained from Achida, Sokoto State, Nigeria and authenticated by pollen grain analysis. Antioxidant and immuno-modulatory properties of 0.125-2.5 % ($^{v}/_{v}$) AH and its dichloromethane, ethylacetate and aqueous fractions were investigated in blood, neutrophils and macrophages by luminol and lucigenin-amplified chemiluminescence methods. The AH cytotoxic and anti-proliferative effects on lymphocytes were evaluated by cytochalasin B- blocked micronucleus and mitotic index assays, respectively. Anti-proliferative effects of 0.5-10 % ($^{v}/_{v}$) AH on NIH/3T3, PC-3 and NCI-H460 cells were conducted using MTT (3-[4, 5-dimethyl Thiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide), mitotic index and fluorescence-activated cell sorting methods. Cell cycle analysis and expressions of TNF-α, IL-1β and Prostate Specific Antigen (PSA) were done by flow cytometry and ELISA. Expressions of *p*53 and bcl-2 genes were done using real-time PCR. Male Wistar rats (112-200g) were randomly assigned to four groups of five (5) animals treated orally with distilled water (control), AH 20 % ($^{v}/_{v}$), Sodium Arsenite (SA) (5 mg/kg body weight), AH and SA daily for one week. The frequency of micronucleated polychromatic erythrocytes (mPCEs) was determined by microscopy. Levels of serum and tissue (brain and liver) lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were determined by spectrophotometry. Data were analyzed by one-way ANOVA and LSD at $p = 0.05$.

In blood, neutrophils and macrophages, fractions of AH caused pro-oxidant effect while the unfractionated sample elicited antioxidant effect with IC_{50} of < 0.25, 0.20 and < 0.125 % respectively. The cytotoxicity index for control was 0.00 ± 0.00. In 0.5 %, 1.0 %, 2.0 % and 4 % ($^{v}/_{v}$) AH treated, cytotoxicity index were 5.66 ± 0.02, 4.50 ± 0.01, - 1.22 ± 0.00 and - 4.79 ± 0.03 respectively. The mitotic, nuclear division and cytokinesis-block proliferation indices for the controls were 7.35 ± 0.64, 1.40 ± 0.02 and 1.38±0.01 respectively. These indices increased proportionately with increase in AH concentration. The AH exhibits cytotoxic effects on NIH/3T3, PC-3 and NCI-H460 cells with IC_{50} of 3.7, 1.9 and 7.5 % ($^{v}/_{v}$) respectively. Treatment with 2.0 %, 4.0 % and 8 % ($^{v}/_{v}$) AH significantly decreased PSA levels (430.0 ± 10.0, 425.1 ± 15.0, 420.1 ± 20.0 pg/mL respectively) relative to control (530.0 ± 0.01 pg/mL) in PC-3 cells. The AH (2.0 %, 4.0 % and at 8.0 % ($^{v}/_{v}$)) significantly and dose-dependently arrested G_0/G_1 in NCI-H460 and PC-3 cells. The AH significantly decreased TNF-α, *p*53, bcl-2 expressions while IL-1β was elevated in the cells. The SA significantly increased LPO (serum, brain and liver) and mPCEs levels while co-treatment with AH significantly decreased these levels with increased GSH, CAT and SOD.

Acacia honey shows anticancer property by eliciting cytotoxic and antiproliferative effects on cancer cells via activation of apoptotic pathways and antioxidant activity.

Key words: *Acacia* honey, Anticancer, Apoptosis, Antioxidant, Immune-modulation

Word count: 499

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LIST OF ABBREVIATIONS

ABT-737	-	Abbott Laboratories
ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase
AH	-	*Acacia* honey
ALD	-	Alcoholic liver disease
ANOVA	-	Analysis of variance
BBM	-	Brush border membrane
BChE	-	Butyrylcholinesterase
BCR-ABL	-	breakpoint cluster region Abelson
bFGF	-	Basic fibroblast growth factor
BRCA	-	Breast cancer-susceptibility gene
C5a	-	Activated complement
CAT	-	Catalase
CDK	-	Cyclin dependent kinase
CNS	-	Central nervous system
CS	-	Cigarette smoke
DISC	-	Death inducing signaling complex
DMEM	-	Dubellco modified eagle medium
DN	-	Diastase number
ECRP	-	Environmental control and research program
EGF	-	Epidermal growth factor
eNOS	-	Endothelial nitric oxide synthase
FADD	-	Fas associated dead domain
fMLP	-	N-formyl-met-leu-phe
GPx	-	Glutathione peroxidase
GSH	-	Reduced glutathione
GST	-	Glutathione-S-transferase
HBSS	-	Hanks buffered saline solution
HBV	-	Hepatitis B virus
HCC	-	Hepatocellular carcinoma
HCV	-	Hepatitis C virus

HER	-	Human epidermal receptor
HER	-	Human epidermal regulated factor
HER	-	Hydroxyethyl free radicals
HMF	-	Hydroxylmethyl furfural
HPV	-	Humanpapilloma virus
IARC	-	International agency of research in cancer
IL1β	-	Interleukin 1beta
KC	-	Kinatochore positive
LAF	-	Lymphocyte-activating factor
L-NAME	-	L-arginine methyl esther
MDA	-	Malondialdehyde
MN	-	Micronucleus
mPCEs	-	micronucleated polychromatic erythrocytes
MSCs	-	Mesenchymal stem cells
MTT	-	3-[4, 5-dimethyl thiazole-2-yl]-2, 5-diphenyl-tetrazolium romide
MYC	-	myelocytomatosis viral oncogene
MyD88	-	Myeloid differentiation primary response gene (88)
N	-	Nucleus
NBT	-	Nitroblue tetrazolium
NCI-H460	-	National cancer institute-human non-small lung cancer
NEM	-	N-methylmaleimide
NIH/3T3	-	National institute of health/ 3-day transfer, inoculum 3 x 10^5 cells
NTP	-	National toxicological programme
ORAC	-	Oxygen radical absorbance capacity
PC-3	-	Prostate cells
PDGF	-	Platelet derived growth factor
PGE	-	Prostaglandin E
PMA	-	Phobol-12-myristate-13-acetate
PMS	-	Phenazine methosulphate
PSA	-	Prostate specific antigen
PTEN	-	Phosphatase and tensin homolog

Q-PCR	-	Quantitative real time polymerase chain reaction
Rb	-	Retinoblastoma
RDI	-	Recommended daily intake
RET	-	Rearranged during transfection
RIP	-	Receptor interacting protein
RLU	-	Relative lights units
ROS	-	Reactive oxygen species
RPMI	-	Roswell park memorial institute
RT-PCR	-	Real time-polymerase chain reactions
SA	-	Sodium arsenite
SHR	-	Spontaneously hypertensive rats
TBA	-	Thiobarbituric acids
TGFβ	-	Transforming growth factor
TLR	-	Toll-like receptor
TNFR	-	Tumor necrosis factor receptor
TNFα	-	Tumor necrosis factor alpha
VEGF	-	Vascular endothelial growth factor
VHL	-	Von Hippel-Lindau

CHAPTER ONE

1.0 INTRODUCTION

1.1 Introduction

Cancer is one of the leading causes of death worldwide. The development of cancer is frequently associated with DNA damage, aneuploidy, and nonrandom chromosome aberrations all of which can result from exposure to environmental toxins such as arsenicals (Hermann, 2013). Inorganic arsenic is acutely toxic, and ingestion of large doses leads to gastrointestinal symptoms, disturbances of cardiovascular and nervous system functions, and eventually death (Hermann, 2013). Substances produced by honey bees (*Apis mellifera*); including propolis, honey, wax and venom has been used for their medicinal properties throughout history (Carnwath *et al.*, 2014). The cancer prophylactic and curative properties of other types honey have been documented (Mohammed *et al*., 2013). Honey has been used in the topical treatment for a variety of diseases (Naiara and Amer, 2014). It is a promising antitumour agent with pronounced anti-metastatic and anti-angiogenic effects (Hanaa and Shynaa, 2011). It also has antibacterial, anti-inflammatory, immune-stimulant, antiulcer and wound/burn healing effects (Fiorani *et al.*, 2006). Various signaling pathways, including stimulation of tumour necrosis factor-alpha (TNF-α) release, inhibition of cell proliferation, induction of apoptosis and cell cycle arrest, as well as inhibition of lipoprotein oxidation, mediate the beneficial effects exerted by honey (Gheldof *et al*., 2002; Mabrouk *et al*., 2002; Swellam *et al*., 2003; Tonks *et al*., 2001; Woo *et al*., 2004). Antioxidant compounds are biosynthesized by a large number of plants that may be used by honeybees to collect nectar; subsequently, a wide variety of free radical-scavenging phytochemicals can be transferred to honey via regurgitation by the bees (Alvarez-Suarez *et al.*, 2010). *Acacia*

is a genus of shrubs and trees belonging to the subfamily Mimosoideae of the family Fabaceae, first described in Africa by Swedish botanist Carl Linnaeus in 1973. They are also known as thorn trees, whistling or wattles. *Acacia modesta* and *Acacia nolitica* are the species that belongs to genus *Acacia.* Though they have certain similar features, they tend to differ based on the geographical region (Martin *et al*., 2004). *Acacia* honey (AH) is the sweetest honey due to very high contents of fructose. It is produced from *Acacia* flowers hence the name (Figure 1). Its phytochemical analysis revealed three (3) phenolic acids [p-hydroxybenzoic, ferullic and t-cinnamic acid] and five (5) free flavonoids [pinobanksin, apigenin, pinocembrin, chrysin and acacetin] (Liviu *et al*., 2010). AH and chrysin, the active principle, have been reported to reduced proliferation of melanoma cells through alterations in cell cycle progression *in vitro* (Elena *et al.*, 2010). Traditionally in Nigeria, honey has been used in various forms to treat various types of ailments such as: Bronchial cough feverish cough, colds, sore or irritated throats, ulcers in the mouth, tension, burns/wounds and earache (http://tribune.com.ng/index.php/natural-health/38771).

Cytokines are hydrosoluble extracellular polypeptides or glycoproteins ranging from 8 to 30 kDa (Caio *et al.*, 2011). They are produced by several types of cells, at the site of injury through mitogen-activated protein kinases. Some cytokines can have a pro- [Th1] or anti-inflammatory [Th2] actions, according to the microenvironment in which they are located (Caio *et al.*, 2011). For example, Th1 includes interleukins (IL) 1, 2, 6, 7, and tumour necrosis factor (TNF). Th2 cytokines include IL-4, IL-10, IL-13, and transforming growth factor (TGFβ). Interestingly, most of the pro-inflammatory cytokines tends to play a dual role in apoptosis and carcinogenesis especially at the level

of cancer progression. The divalent calcium cation Ca^{2+} is used as a major signaling molecule during cell signal transduction to regulate energy output, cellular metabolism, and phenotype as well as apoptosis via direct and indirect mechanisms (George *et al.*, 1999). Apoptosis, also called programmed cell death or physiological cell death, is an essential and evolutionarily conserved function involved in the maintenance of multicellular organisms and found in all species from worm to man (Hunot and Flavell, 2001). It is required in many fundamental biological processes including embryonic development, metamorphosis, hormone-dependent atrophy and chemically-induced cell death (Meier *et al.*, 2000).

Imbalance in the equilibrium of the generation of reactive oxygen species and the body battery defense system in favor of former is what actually leads to oxidative stress. Human aerobic respiration is accompanied by oxygen as an essential factor for the survival of humans. During the utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals (Yu, 1994; Halliwell and Gutteridge, 1988), like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the cell of human body rendering each cell to face about 10,000 oxidative hits per second (Lata and Ahuja, 2003). These ultimately stimulated the redox sensitive biomarkers for example; reduced glutathione, superoxide dismutase, catalase and generate lipid Peroxidation products like malondialdehyde. The role of arsenic in oxidative stress (Sanjib and Pallab, 2012), and the antioxidative potentiality of honey have been documented (Omotayo *et al.*, 2012).

Acacia modesta

Acacia modesta flowers

Apis mellifera

***Acacia nilotica* and its flowers**

Figure 1: *Acacia modesta/ nilotica*, its flowers and *Apis mellifera*
(http://commons.wikimedia.org/wiki/File:Flowers-Acacia_modesta)
(http://www.bidorbuy.co.za/item/22448218/10_Acacia_nilotica)
(http://www.naturephoto-cz.com)

1.2 Statement of Research Problem

Cancer is a disease caused by multifactorial processes. It affects most of the populace in both developed and under developed countries. It is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13 % of all deaths) in 2008. Lung cancer, which accounted for 1.37 million deaths, is the most common cause of cancer death world-wide (Alissa *et al*., 2013). Prostate cancer is the most common malignancy in men and the second leading cause of cancer-related deaths (Cooperberg *et al*., 2004). Deaths from cancer worldwide are projected to continue to rise to over 13.1 million in 2030 (http://www.who.int/medicentre/factssheets/fs297/en/).

Continuous exposure of humans to arsenicals through long-term ingestion of contaminated water and its attendant health problems has been widely reported (Sinha *et al.,* 2005). Water as one of the major ingredient in both traditional and modern beer fermentation process may be contaminated with arsenic due to poor quality control. As a result people are exposed to the end product which constituted arsenic compound. Induction of cancer is frequently associated with DNA damage, changes in ploidy of cells, and non-random chromosome aberrations which can result from exposure to arsenic (Sinha *et al.,* 2005). Arsenic can act as co-mutagen due to its ability to inhibit the activities of thiol containing enzymes (Mazumder, 2005), such as DNA ligase (Chattopadhyay *et al.,* 2001), resulting in defective DNA replication, repair, recombination and joining of single- and double-stranded DNA breaks (Sunderman, 1984).

1.3 Justification of Study

Failure of many anticancer drugs and their side effects has become an issue of major concern. There have been increased interests in natural products based-research. This is because they are relatively non-toxic and have been utilized as natural remedies since ancient times. Availability, accessibility and affordability of natural antioxidants are also among the key factors considered in choice of natural medicine. Honey has been proved to be beneficial both at preventive and curative levels. It is produced from many different floral sources and its biochemical and pharmacological activities vary depending on its origin and processing. Antioxidant activity of honey has been found to be as a result of the synergistic effects of wide range of compounds present in it (Gheldof *et al.*, 2002). Antiproliferative potential and the alteration of cell cycle progression due to *Acacia* honey (AH) in melanoma cells have been reported (Elena *et al.*, 2010). However, to best of our knowledge no such work has been carried out on NIH/3T3, PC-3 and NCI-H460 cell lines. The comparative assessment of redox sensitive biomarkers due to arsenic and AH administration have not been reported. Genotoxic, cytotoxic and immune-modulatory potential of AH on human peripheral blood lymphocytes and the relationship with its antioxidant potentials on whole blood, neutrophils and macrophages have not been reported.

1.4 Aim and Objectives

The aim of this study is to evaluate antioxidant, immuno-modulatory and anticancer properties of *Acacia* honey (AH) using *in vitro* and *in vivo* experimental models with reference to cellular and molecular studies.

The specific objectives are:

- To assess the quality of honey by assaying the diastase activity and determination of hydroxylmethylfurfural level
- To determine antioxidant potential in whole blood, Neutrophils and macrophages by chemiluminescence assay
- To determine anti-proliferative, genotoxic and cytotoxic effects on human peripheral blood lymphocytes by mitotic index and cytochalasin B block micronucleus assays, respectively.
- To determine cytotoxic effect of honey on normal and cancer cell lines by MTT assay
- To determine the effects on selected cancer cell lines proliferation through cell cycle, cytokines, calcium ion and expression of p53 & Bcl-2 genes analyses
- To determine the effects of honey daily consumption on biochemical and haematological parameters in rats
- To compare the antioxidant and anticlastogenic potential of AH of Pakistan and Nigeria sources by assessing oxidative stress biomarkers and frequency of micronuclei in rats

1.5 Research Questions

In order to achieve the aforementioned aim and objectives, an attempt was made to answer the following questions:

- Is Acacia honey (AH) of good quality compared to standard?
- Does fractionation process affect its antioxidant potentials?

- Does AH stimulate lymphocytes proliferation without causing genotoxic and cytotoxic effects while suppressing the production of reactive oxygen species?
- Is it cytotoxic to normal and/or cancer cells?
- Does it confer anti-proliferative effects on cancer cells?
- What effects can it daily consumption has in experimental rats?
- Does it have antioxidant and anticlastogenic potential in rats?

CHAPTER TWO

2.0 Literature Review

2.1 Cancer

Cancer is a complex disease characterised by abnormal cellular proliferation associated with resistance to normal growth inhibitory signals, uncontrolled activation of growth signals, impairment of apoptosis, promotion of angiogenesis (the development of new blood vessels to support tumour growth), invasion of surrounding tissues and metastasis i.e. spread of tumour cells to distant tissues (Hanahan and Weinberg, 2000; Doucas and Berry, 2006). The transformation of normal human cells into cancer involves acquiring certain molecular, biochemical, and cellular changes shared by many types of human cancer which result from alterations in genes that control various cellular functions leading to the disruption of mechanisms governing cell growth (Doucas and Berry, 2006). Since all mammalian cells regulate their proliferation, differentiation and death in a similar way, therefore, disruption of a limited number of cellular regulatory pathways is sufficient to cause cancer (Hanahan and Weinberg, 2000). There are more than 100 distinct types of human cancers but some common mechanisms usually underlie development of cancers of different origins even though there may be various molecular mechanisms leading to the development of specific tumour types. Basically, carcinogenesis is a slow process resulting from molecular changes affecting the tumour cells, failure of cellular differentiation and alterations in the interaction among cancer cells and the adjoining stromal tissues. Transformed cells take up glucose more rapidly than normal cells and derive most of their energy through aerobic glycolysis (Dang and Semenza, 1999).

2.1.1 Epidemiology of Cancer

Cancer causes 12% of the world's mortality and is the 2nd leading cause of death in the Western world after cardiac diseases and the 3rd leading cause in the developing world after cardiac and infectious diseases (Efferth *et al*., 2008). In the developed countries it is the 2nd most common cause of death among children under 15 years of age, due to leukemias (34 %), brain tumours (23 %), and lymphomas (12 %) (Kaatsch, 2010). It is apparent that lung and breast carcinomas are the most common cancers in men and women, respectively. Lung cancer is the most common cancer worldwide accounting for 12% of all new cancers, the commonest type of cancer in males (Youlden *et al*., 2008) and the leading cause of cancer-related deaths in both sexes (Smith and Khuri, 2004). In females, lung cancer is now the 4th most frequent cancer of women worldwide and the 2nd most common cause of death from cancer (12.8 % of the total). The highest incidence rate is observed in Northern America (where lung cancer is 2nd most frequent cancer in women), and the lowest in Middle Africa (15th most frequent cancer; IARC, 2008).

Breast cancer is the most frequent cancer among women in both developed and developing regions of the world with increasing age being the major risk factor for both breast cancer incidence and mortality (Taylor, 2010). However, incidence rates vary from 19.3 per 100,000 women in Eastern Africa to 89.9 per 100,000 women in Western Europe, and are high (greater than 80 per 100,000) in developed regions of the world (except Japan) and low (less than 40 per 100,000) in most of the developing regions. The mortality rate is much less (approximately 6-19 per 100,000) with breast cancer ranking as the 5th cause of death from cancer overall, but is still the most frequent cause

of cancer death in women in both developing and developed regions of the world (IARC, 2008).

Colourectal cancer is the third most common cancer in men and the second in women worldwide with almost 60 % of the cases occurring in developed regions. Prostate cancer is the second most frequently diagnosed cancer in men and nearly three-quarters of the registered cases occur in developed countries. Cervical cancer is the third most common cancer in women, and the seventh overall with more than 85% of the cases occurring in developing countries. Stomach cancer is currently the fourth most common malignancy in the world with more than 70 % of cases occurring in developing countries, and 50% in Eastern Asia (mainly in China, Japan and Korea). It is about twice as common in men as in women. Liver cancer is the fifth most common cancer in men and the seventh in women (6.5 % of the total), with 85% of the cases occurring in developing countries (IARC, 2008). Oral and pharyngeal cancer, grouped together, is the sixth leading cancer in the world while in high-risk countries such as Bangladesh, India, Pakistan and Sri Lanka; it may contribute up to 25 % of all new cases of cancer (Warnakulasuriya, 2010).

2.1.2 Aetiology of Cancer

Multiple factors are involved in the aetiology of cancer which results from the interaction between the environment and the genetic make-up of a person. Inherited factors are clearly involved in childhood and early adulthood cancers while it is also obvious that skin colour plays a large role in sun-associated cancers such as melanoma.

2.1.3 Genetic and Epigenetic Factors of Cancer

There are approximately 22,000 genes (Human Genome Project information, August 2006) in the human genome and more than 1% of these genes are involved in

carcinogenesis (Futreal *et al*., 2004). Two types of genes i.e. oncogenes (cancer-causing genes that increase cell proliferation) and tumour suppressor genes (that inhibit cell growth) play crucial roles in the development of cancer. Genetic damage to the cells results in the loss of the cellular regulation and causes mutations in oncogenes and tumour suppressor genes leading to cancer (Figure 2). Mutation is the change in the nucleotide sequence of DNA due to damage or by the incorporation of non-complementary nucleotides during DNA synthesis. The cancer cells possess several mutations, each of which overcomes a natural anticancer defense mechanism. These mutations continue to occur even after tumour development, which causes difficulties in their treatment (Figure 2).

DNA can be damaged by exogenous agents such as chemicals; viruses; and irradiation; reactive oxygen and nitrogen species generated by normal cellular processes; alkylation i.e. transfer of an alkyl group to DNA; depurination i.e. hydrolysis of the beta-N-glycosidic bond between a purine base (adenine or guanine) and the deoxyribose-phosphate backbone; cytidine deamination (Loeb, 1989). The number of depurinated sites generated daily and the number of alterations resulting from the generation of reactive oxygen species is more than ten thousand per cell (Lindahl, 1993; Ames, 1995). Alteration of genes occurs due to the effects of carcinogens or by errors in their copying and repair. Lack of repair of damaged DNA before cell replication can lead to mutations, translocations, amplifications, etc. in genes controlling cell growth and differentiation. For example, proto-oncogenes code for proteins that regulate cell growth and differentiation which can become oncogenes due to mutations or increased expression that cause the genes to become excessively active in growth promotion. Important among these are Ras, MYC, BCR-ABL; their activation induces release of mitogenic signals in cell cytoplasm (Hahn and Weinberg, 2002); and / or development

and maintenance of various malignancies (Weinstein, 2002; Weinstein and Joe, 2006; Felsher, 2008). Ras gene mutations have been found in many human tumours e.g. adenocarcinomas of the pancreas (90%), colon (50%), lung (30%); thyroid tumours (50%); and myeloid leukemia (30%) (Wogan, 1992). Inactivation of the transforming oncogene can partially revert the neoplastic phenotype (Weinstein and Joe, 2006; Huettner *et al*., 2000; Chin *et al* ., 1999).

The tumour suppressor genes include retinoblastoma (Rb; Weinberg, 1995), phosphatase and tensin homolog (PTEN; Cantley and Neel, 1999) and p53; their inactivation may occur by point mutations and / or chromosomal deletions and other structural changes. Mutation of the p53 tumour suppressor gene is the most frequently observed genetic lesion and is present in approximately 50% of all human tumours (Levine, 1997).

The childhood cancers are mainly due to inheriting a mutant cancer gene (Ponder, 2001). The genetically linked cancers are many including retinoblastoma, Wilms' tumour, familial adenomatous polyposis, and the Li-Fraumeni syndrome (Wogan, 1992). In Xeroderma pigmentosum, mutations in genes that repair UV-induced DNA damage result in skin cancer in individuals exposed to UV-irradiation (Wogan, 1992; Cleaver and Kraemer, 1989). Loss of von Hippel–Lindau (VHL) tumour suppressor protein function results in an autosomal-dominant cancer syndrome known as VHL disease, which manifests as angiomas of the retina, hemangioblastomas of the central nervous system, renal clear-cell carcinomas and pheochromocytomas (Chen *et al*., 1995).

Mutations in the RET proto-oncogene are responsible for the development of medullary thyroid carcinoma and papillary thyroid carcinoma, which originate from the

parafollicular C-cells and the follicular cells, respectively (Pützer and Drosten, 2004). HER-kinase activation deregulates growth, desensitizes cells to apoptotic stimuli and regulates angiogenesis; therefore HER-family tyrosine kinases take part in the pathogenesis of glioblastoma, breast cancer and other tumours (Chen *et al*., 2003). Insulin-like growth factor-1 (IGF-1) exerts mitogenic and anti-apoptotic effects and the defective regulation of the IGF network have been involved in the carcinogenesis of prostate cancer in both *in vitro* and *in vivo* studies (Li *et al*., 2003; Renehan *et al*., 2004).

The breast cancer-susceptibility genes 1 and 2 (BRCA1 and BRCA2) encode proteins that play important roles in the cellular response to DNA damage; inactivating mutations in these genes increase the susceptibility to breast and ovarian cancers, therefore women with BRCA1 and BRCA2 mutations have a very high risk for developing these cancers (Struewing *et al*., 1997). BRCA1 appears to be responsible for about one-half of all cases of early-onset breast cancer and for the majority of familial breast and ovarian cancers (Hall *et al*., 1990; Easton *et al*., 1993). Although BRCA1 and BRCA2 genes account for only 5% of breast cancer cases (Mazhar and Waxman, 2006), however, it is estimated that the risk of developing breast cancer associated with mutations in BRCA1 and BRCA2 gene exceeds 80% in women (Easton *et al*., 1995; Eeles and Kadouri, 1999) and 6% in men (Wolpert *et al*., 2000) by the time the carrier reaches age 70 years. BRCA1/2 mutation carriers are also at heightened risk for cancers of the prostate (Struewing *et al*., 1997), larynx (Easton *et al*., 1997), gastrointestinal tract and pancreas (Figer *et al*., 2001), lymphoma and liver (Ghadirian *et al*., 1995).

In addition to the genetic mutations, deregulated epigenetic mechanisms are also associated with cancer development (Figure 2). The term epigenetics includes all stable

changes of phenotypic traits that are not coded in the DNA sequence itself (Baylin, 2005; Feinberg *et al*., 2006). Epigenetic changes include (i) DNA methylation, (ii) histone modifications and (iii) RNA-mediated gene silencing. The transfer of a methyl group to the C5 atom of cytosine base by the DNA methyl transferase is called DNA methylation which is involved in the regulation of gene expression and genomic stability, and is biologically necessary for the maintenance of many cellular functions (Sadikovic *et al*., 2008). Histone modifications include (i) acetylation, (ii) methylation, (iii) phosphorylation, and (iv) ubiquitination, of core histones (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Histone marks play important roles in gene transcription and DNA repair. RNA-mediated gene silencing involves microRNAs (miRNAs), small noncoding RNA molecules that can also control gene expression (Calin and Croce, 2006). miRNAs may act as either tumour suppressors or oncogenes by affecting distinct genes involved in cell proliferation and differentiation. As epigenetic events may regulate important cellular processes, therefore epigenetic alterations may disrupt the control of fundamental cellular mechanisms leading to tumour formation. Many physical and chemical carcinogens and infectious agents in the environment are believed to promote neoplastic process through disruption of epigenetically maintained patterns of gene expression and chromatin structure and function. It has been reported that epigenetic deregulation can directly or indirectly affect most key processes found in cancer cells, such as silencing of tumour suppressor genes, activation of oncogenes, aberrant cell cycle processes, defects in DNA repair, and deregulation of cell death (Lambert and Herceg, 2008). The sites of DNA damage are continuously repaired by DNA repair systems, which include base-excision repair, nucleotide excision repair, transcription-coupled repair, and mismatch repair (Hahn and Weinberg, 2002).

2.1.4 Environmental Factors and Carcinogens

Human beings are 99.1% identical in their genetic sequence and therefore most of the chronic illnesses and diseases cannot be explained by differences in their genetic makeup and hence associated with the lifestyle and environmental factors (Wong, 2005). It has been observed that certain cancers are more common among people of some countries than others (Abidoye *et al*., 2007; Ziegler, 1993). For example, populations that migrate from low to high incidence countries often develop breast cancer rates that approximate those of the host country (McCracken *et al*., 2007). If one twin is suffering from breast cancer, the chance that the second twin will be diagnosed with breast cancer is only 20% (Locatelli *et al*., 2004). Therefore, the diet may be the environmental factor linked to the breast cancer and some other cancers like those of colon and stomach. It has been observed that populations that consume food rich in fruits and vegetables have a lower incidence of cancers (Reddy *et al*., 2003). Review of results from 206 human epidemiologic studies and 22 animal studies has shown an inverse relationship between consumption of vegetables and fruits and risk for cancers of the stomach, esophagus, lung, oral cavity and pharynx, endometrium, pancreas, and colon (Steinmetz and Potter, 1996).

Factors that cause the molecular damage leading to cancer are called carcinogens or initiators. Carcinogenesis can be activated either by the ionizing and the non-ionizing radiation; carcinogens: present in the environment such as industrial emissions, gasoline vapors; used for pleasure such as tobacco, cigarette smoke, betel nut, areca nut and present in the workplace e.g. naphthylamines, coal tar derivatives (Aggarwal and Shishodia, 2006). Co-carcinogens are factors that enhance the activity of carcinogens but can also become carcinogenic at high doses or in the presence of other co-

carcinogens or predisposing factors. Similarly, tumour promoters are not carcinogenic but enhance the activity of a carcinogen (Carbone and Pass, 2004).

Cigarette smoke is the most well established carcinogen. Cigarette smoking increases the risk of all histological types of lung cancer and in the United States it is estimated to account for 90% of lung cancer cases among men and 79% of lung cancer cases among women (Hecht, 2006). Approximately 80% of head and neck cancer cases among men and 61% of cases among women are attributed to smoking and alcohol drinking (Hecht, 2006). Cigarette smoking causes cancer of the oral cavity, and this risk is greatly increased by the use of smokeless tobacco or by alcohol consumption in combination with smoking. Cigarette smoking is causally associated with cancer of the esophagus; both squamous cell carcinoma and adenocarcinoma, as well as with hypopharyngeal, laryngeal and oropharyngeal cancers, and increases the risks for nasopharyngeal and sinonasal cancers. Furthermore, cigarette smoking causes cancers of the liver, pancreas and stomach as well as transitional cell carcinomas of the bladder, renal pelvis and ureter, and renal cell carcinoma. Finally, cigarette smoking is also a cause of squamous cell cervical carcinoma and myeloid leukaemia, and the risk of colourectal cancer can also be increased by smoking (IARC, 2004). Environmental tobacco smoke causes lung cancer while smokeless tobacco products are established causes of oral cavity cancer (IARC, 2007). Epidemiology studies indicate that daily supplements of 20–30 mg of β-carotene are associated with an increased risk of lung cancer in smokers who also drink alcoholic beverages (Omenn, 1998).

Alcohol drinking is associated with an increased risk for liver cancer (Boffetta and Hashibe, 2006); upper aero digestive tract cancers of the oral cavity and pharynx, esophagus and larynx (Jensen *et al*., 1996). Heavy alcohol consumption may be related

to the risk of pancreatic cancer (Silverman *et al*., 1995). There is also a positive association between moderate alcohol consumption and the risk of prostate cancer in older men (Sesso *et al*., 2001). The consumption of alcoholic beverages increases the risk of colourectal cancer especially of distal colon and rectum (Sharpe *et al*., 2002) and is the only established dietary risk factor for breast cancer (Tjonneland *et al*., 2003).

In 1960, the asbestos was first associated with the development of mesothelioma by Wagner and colleagues in South Africa (Wagner, 1960). Exposure to asbestos was responsible for about 4000 to 6000 deaths from lung cancer per year (Omenn *et al*., 1986). Many pesticides and antimicrobial agents used in agriculture are carcinogenic (Bailar and Travers, 2002). Carcinogenic nitrosamines are produced in the stomach from endogenously formed amines and dietary nitrates (Lijinsky, 1999).

Betel quid chewing is linked with the high incidence of oral leukoplakia, oral submucous fibrosis and oral cancer (Trivedy *et al*., 2002). Arecoline, the main areca alkaloid in betel quid, having cytotoxic, genotoxic and mutagenic effects in various cells, has been suggested as a possible carcinogen (Lee *et al*., 2006a). The risk factors for oral cancer can be summarized as tobacco (all forms of tobacco especially smokeless tobacco), excess consumption of alcohol (Hindle *et al*., 2000) and betel quid usage (IARC, 2004).

The established risk factors for hepatocellular carcinoma (HCC) include Hepatitis B or C virus infections, alcohol drinking, tobacco smoking and aflatoxin (Chuang *et al*., 2009). Hepatitis B (HBV) virus infections are responsible for its incidence in developing countries of sub-Saharan Africa and Asia, while Hepatitis C (HCV) virus infections cause most cases of HCC in developed countries (Kew, 1998). Mycotoxins, such as aflatoxin B_1, formed by fungi *Aspergillus flavus* and *Aspergillus parasiticus*

growing on poorly stored grain products, can be strong liver carcinogens, especially in individuals infected by the HBV (Wogan, 1992) and its contamination is particularly prevalent in Africa, South-East Asia and China (Chuang *et al*., 2009).

2.1.5 Chronic Infections

Approximately one in six human cancers is caused by a human tumour virus (Parkin, 2001). As mentioned above, HBV and HCV viruses are a major cause of chronic inflammation leading to HCC, which is one of the most common cancers in Asia and Africa (Monto and Wright, 2001). HBV virus interacts with other carcinogens like aflatoxin B1 in China and alcohol in USA and Europe to cause HCC (Carbone and Pass, 2004).

Human T-cell lymphotropic virus type 1 is the etiologic agent of adult T-cell leukemia (Hatta and Koeffler, 2002). Human papillomaviruses cause the vast majority of cervical carcinomas (Hausen, 2002). Epstein-Barr virus contributes to the development of Burkitt's lymphomas, post-transplant lymphomas and Hodgkin's lymphomas (Hammerschmidt and Sugden, 2004). *H. pylori* infection increases the risk of gastric cancer and a positive correlation exists between gastric cancer rates and prevalence of *H. pylori* infection (Uemura *et al*., 2001). The main known cause of cholangiosarcoma, second most common liver cancer, is infestation with the liver flukes, *Opistorchis viverrini* and *Clonorchis sinensis* (Chuang *et al*., 2009)

2.2.1.6 Pathogenesis of Cancer

2.1.6.1 Growth Factors

Normal cells require growth signals to begin proliferation while growth factors are the proteins which activate cellular proliferation and/or differentiation by binding to

receptors on the cell surface. These include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), transforming growth factors (TGF)-α and -β, erythropoietin (Epo), insulin-like growth factor (IGF), interleukin (IL)-1, 2, 6, 8, tumour necrosis factor (TNF), interferon-γ (INF-γ) and colony-stimulating factors (CSFs) (Hanahan and Weinberg, 2000). In cancer, there is over-expression of genes encoding proteins which take part in growth signaling and so the balance of pathways that regulate proliferation and differentiation in normal tissues is disturbed. Uncontrolled activation of growth signaling pathways leads to increased cell proliferation, impairment of apoptosis, invasion of surrounding tissues and metastasis. Most growth factors are made by one type of cells in order to stimulate the proliferation of their neighboring cells (paracrine signals) or distant cells (via systemic or endocrine signals). However, many cancer cells acquire the ability to synthesize growth factors to which they are responsive (autocrine signals) thereby reducing their dependence on stimulation from their normal tissue micro-environment. Examples include the production of PDGF and TGF-α by glioblastomas and sarcomas (Fedi *et al*., 1997; Hanahan and Weinberg, 2000).

2.1.6.2 Growth Factor Receptors

The growth factor receptors, often carrying tyrosine kinase activities in their cytoplasmic domains, are over expressed in many cancers which may enable the cancer cells to become hyper responsive to normal levels of growth factor that usually would not initiate proliferation, for example, the HER2/neu receptor is over expressed in breast carcinomas (Slamon *et al*., 1987). Other growth factor receptor networks such as the IGF-1 receptor and vascular endothelial growth factor (VEGF)-receptor are also implicated in carcinogenesis (Hahn and Weinberg, 2002).

2.1.6.3 Cell Cycle

In order to maintain homeostasis in healthy tissues cell cycle progression is tightly controlled by various check points ensuring that DNA replication and chromosome segregation are completed before the beginning of next stage. It is regulated by a group of proteins, the cyclins, and protein kinases, the cyclin-dependent kinases (cdks) (Stewart *et al*., 2003). The balance between the cyclins and the cyclin-dependent kinases ensures the tight control of the timing of entry of the cell in mitosis. The cdk binding to cyclin D1 is essential for the progression of cells through the first gap (G1) phase of the cell cycle (Baldin *et al*., 1993).

It is well known that the mitogenic signals commit the cells to enter into the cell cycle, which is divided into four phases S (synthesis) phase: synthesis of DNA; M (mitotic) phase: separation of two daughter cells; G2 (gap 2) phase: the time between the S and M phases which allows cells to repair errors that occur during DNA duplication in order to prevent the propagation of these errors to daughter cells and G1 (gap 1) phase: during which cells prepare for DNA duplication. In G1, S and G2 phases of the cell cycle, the DNA has a diffuse nuclear stain and on entering the mitotic phase the DNA condenses and organizes into chromosomes (Weinberg, 1995).

When regulation of the cell cycle check points is impaired, there is over activation of growth-promoting cell cycle factors such as cyclin D1 and cyclin-dependent kinases and cell proliferation occurs (Diehl, 2002). Within a normal tissue, anti-growth signals block proliferation either by (a) forcing cells out of the active proliferative cycle into the quiescent (G_0) state, or (b) by instructing cells to enter irreversibly into post-mitotic differentiated state. Normal cells respond to antiproliferative signals by regulating the cell cycle clock, specially the transit of the cell through the G1 phase possibly through

the phosphorylation of retinoblastoma protein (pRb). When in a hypo-phosphorylated state, pRb blocks proliferation by controlling the expression of genes essential for progression from G1 into S phases (Weinberg, 1995).

Most mammalian cells in culture have a finite life span and after a certain number of doublings, they stop growing, this phenomenon is termed as senescence (Hayflick, 1997). When their pRb and p53 tumour suppressor proteins are disabled, cultured human fibroblasts continue to multiply until they enter into the crisis state, which is characterised by massive cell death, karyotypic disarray associated with end-to-end fusion of chromosomes, and in some cases, immortalization; (a colony of cells acquire the ability to multiply without limit) (Wright *et al*., 1989). It has been observed that various normal human cell types have the capacity for 60–70 doublings when cultured, and the counting device for cell generations comprise of telomeres, the ends of chromosomes which are composed of several thousand repeats of a short 6 bp sequence element. Replicative generations are counted by the 50–100 bp loss of telomeric DNA from the ends of every chromosome during each cell cycle as DNA polymerases are unable to completely replicate the 3′ ends of chromosomal DNA during each S phase. The progressive shortening of telomeres through successive cycles of replication results in the loss of their ability to protect the ends of chromosomal DNA. Telomere maintenance is required for immortalization and continuous tumour cell growth (Foddis *et al*., 2002) and in 85-90% of malignant tumours, there is up-regulation of the telomerase (an enzyme that elongates telomere sequences) (Bryan and Cech, 1999).

2.1.6.4 Apoptosis

Cells undergo death either by (1) necrosis, in which there is primary damage to the metabolic or membrane integrity of the cell, or (2) apoptosis, a genetically controlled

process of programmed cell death. Apoptosis plays important roles in many normal processes, ranging from fetal development to adult tissue homeostasis (Reed, 2001), via (a) the extrinsic pathway, which is death-receptor-induced or (b) the intrinsic pathway, that is mitochondria-apoptosome-mediated (Hu and Kavanagh, 2003). Death receptors are cell surface receptors that belong to the tumour necrosis factor receptors superfamily. In the apoptotic process, the unwanted or severely damaged cells are removed with the help of caspases, a family of intracellular cysteine proteases, which are activated in a cascade of sequential cleavage reactions from their inactive zymogen precursors (Thornberry and Lazebnik, 1998). The caspases are divided according to the roles they play in inducing apoptosis, either into (i) initiator or (ii) effector caspases (Salvesen and Dixit, 1999). The initiator caspases include caspase-1, -8, -9, and are activated either by (i) cell death receptor-mediated pathway, Characterised by binding cell death ligands and cell death receptors, and subsequently activating caspase-8 and caspase-3 (Schempp *et al*., 2001); or (ii) mitochondria-mediated pathway, by the liberation of the cytochrome *c* from mitochondria to cytosol which activates caspase-9, and the latter then activates caspase-3 (Li *et al*., 1997). In both pathways activation of caspase-3 plays the central role in the initiation of apoptosis (Salvesen and Dixit, 1999). There is degradation of cellular proteins, cell shrinkage, DNA fragmentation, chromatin condensation, loss of plasma membrane potential and membrane blebbing (Don and Hogg, 2004).

Bcl-2 is the oncoprotein activated in B cell lymphoma and Bcl-2 gene family is composed of a group of related genes that either promote or prevent apoptosis. The commitment of the cell to apoptosis is dependent on the balance between pro-apoptotic (Bax, Bak, Bid, Bim) and anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W) members of the Bcl-2 family (Debatin *et al*., 2002; Green and Evan, 2002). The most commonly occurring

loss of a proapoptotic regulator through mutation involves the p53 tumour suppressor gene. The resulting functional inactivation of its product, the p53 protein, results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Harris, 1996). The p53 tumour suppressor protein can elicit apoptosis by up-regulating the expression of pro-apoptotic Bax in response to sensing DNA damage; Bax in turn stimulates mitochondria to release cytochrome C. If the anti-apoptotic members of the Bcl-2 family are over-expressed, they can maintain cell survival in spite of pro-apoptotic stimuli, whereas the over-expression of pro-apoptotic Bax can lead to apoptosis. Bcl-2 is over-expressed in the most of non-Hodgkin's lymphoma patients (Waters *et al*., 2000) and a reduced expression of Bax correlated with a poor prognosis in patients with breast cancer (Sjostrom *et al*., 1998).

The pro-apoptotic Bcl-2 family proteins form large channels in mitochondrial membranes (Debatin *et al*., 2002; Kuwana *et al*., 2002) whereas the anti-apoptotic proteins Bcl-2 and Bcl-XL antagonise the formation of these channels. The cells, deficient in pro-apoptotic proteins Bak and Bax, are resistant to apoptosis (Wei *et al*., 2001). Following p53 activation, a mitochondrial protein PUMA, (p53 upregulated modulator of apoptosis), is induced in cells, binds to Bcl-2 family proteins, including Bax, and promotes their translocation and multimerization in the mitochondria. A change in mitochondrial membrane potential causes the release of cytochrome *c* from the mitochondria into the cytosol, and a cascade of caspase activation, resulting in programmed cell death (Nakano and Vousden, 2001). Within 30–120 minutes, there is disruption of cellular membranes, the cytoplasmic and nuclear skeletons are broken, the cytosol is extruded, the chromosomes are degraded and the nucleus is fragmented. Within 24 hours the shriveled cell corpse is engulfed by phagocytosing cells and disappears (Wyllie *et al*., 1980).

Impairment of apoptosis is central to cancer development and is achieved either by (a) up-regulation of survival signals and induction of cell proliferation by the activation of (i) the serine–threonine kinase AKT pathway, or (ii) transcription factors activator protein-1 (AP-1) and/or nuclear factor kappa B (NF-κB) or (b) inactivation of the tumour-suppressor gene products, such as (i) p53, (ii) Rb (retinoblastoma) or (iii) PTEN (phosphatase and tensin homolog), a phospholipid phosphatase that normally attenuates the AKT survival signal (Cantley and Neel, 1999; Carbone and Pass, 2004; Don and Hogg, 2004). The down-regulation of NF-κB sensitizes the cells to induction of apoptosis whereas, its activation leads to cell proliferation by silencing the apoptosis pathway via up-regulation of NF-κB-regulated genes (Bcl-2, Bcl-XL, survivin etc., Aggarwal, 2004).

In case of cellular stress due to hypoxia, DNA damage, ionizing radiation etc., the p53 tumour suppressor protein is rapidly accumulated in cells (Sherr, 1998; Asker *et al*., 1999). When the DNA damage is mild, the p53 gene induces cell cycle arrest to repair the damage, but when the damage is severe, it can induce cell death (Li *et al*., 1997; Tron *et al*., 1998). The p53 protein activates the transcription of genes such as p21 (cyclin-dependent kinase 2 inhibitor), WAF1 and Bax to induce the apoptotic process (Vogelstein and Kinzler, 1992; el-Deiry *et al*., 1993).

2.1.6.5 Angiogenesis

Nearly all cells in a tissue reside within 100 μm of a capillary blood vessel for the supply of oxygen and nutrients essential for cell function and survival. The development of new blood vessels is an essential for supporting tumour growth and is achieved by (a)

angiogenesis (the development of new blood vessels from existing vessels), and (b) vasculogenesis (the development of new blood vessels from progenitor cells). These blood vessels penetrate into cancerous tissues, supply nutrients and oxygen and remove waste products. The cancer cells secrete molecules that send signals to the surrounding normal host tissue activating genes that encode proteins to encourage new blood vessel formation. Therefore, the local balance of proangiogenic factors and endogenous inhibitors of angiogenesis is disturbed leading to the initiation of development of new blood vessels (Folkman and Hanahan, 1991; Hanahan and Folkman, 1996). The proangiogenic molecules include growth factors e.g. acidic and basic fibroblast growth factors (FGF1/2), epidermal growth factor (EGF), granulocyte colony-stimulating factor (GC-SF), interleukin IL-8, platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF), adenosine and prostaglandin E (PGE). VEGF, an endothelial-specific mitogen and survival factor, is involved in both angiogenesis and vasculogenesis (Ferrara *et al*., 2003) and up-regulation of its expression in certain cell types is induced by the activation of Ras oncogene (Rak *et al*., 1995). VEGF and basic fibroblast growth factor (bFGF) are most commonly involved in sustaining tumour growth (Aggarwal and Shishodia, 2006).

Solid tumours are maintained by interactions between tumour cells and the tumour stromal which includes inflammatory cells, the vasculature, fibroblasts and the extracellular matrix. Stromal cells provide growth factors, blood supply, mechanical support and metalloproteases. The vascular endothelial growth factor-A and fibroblast growth factor-2 also induce the expression of matrix-degrading proteinases that lead to remodeling of the extracellular matrix to facilitate the invasion of new blood vessels (Hanahan and Folkman, 1996; Heissig *et al*., 2003).

The nitric oxide produced by the endothelial nitric oxide synthase (eNOS, an endothelial cell-specific isoform of nitric oxide producing enzyme) regulates blood vessel tone, platelet aggregation, vascular permeability, leukocyte-endothelial interaction and neo-vascularization (Fukumura *et al*., 1998). Both VEGF and nitric oxide also regulate angiogenesis during inflammation, wound healing and tumour growth (Fukumura *et al*., 2001). Apart from tumour cells, VEGF is also produced by tumour-associated macrophages and tumour-associated fibroblasts which are also involved in tumour-induced angiogenesis and tumour growth (Mantovani *et al*., 2002; Dong *et al*., 2004; Silzle *et al*., 2004).

2.1.7 Prevention and Treatment of Cancer

2.1.7.1 Cancer Prevention

In order to reduce the incidence of cancer, it is generally advisable to avoid smoking, increase the consumption of fruits and vegetables, avoid intense sun exposure, increase physical activity, control infections and reduce alcohol and possibly red meat consumption. As the process of oncogenesis is very long, therefore, its development can be arrested or slowed at various stages by developing agents that stop or slow inappropriate cell division, or induce apoptosis of damaged cells or lead to cellular senescence (Malumbres and Barbacid, 2001). Furthermore, increased survival of the patient can also be achieved by inhibiting angiogenesis or metastasis at later stages of carcinogenesis as metastases are the cause of 90% of human cancer deaths (Sporn, 1996).

Cancer development can also be prevented by the timely diagnosis and removal of the premalignant lesions, for example, cervical dysplasia, a precursor of carcinoma, is

recognized by the Pap test which was developed by Dr. Papanicolau (De May, 1996). These dysplastic lesions can be removed surgically before they become invasive cancers. As cervical cancer is caused by infection with human papillomaviruses (HPV) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (Munoz *et al*., 2003), vaccines have been developed for these HPV strains and thus their infection along with the associated cancers could be prevented. Additionally, adenomas, the pre-malignant lesions in the colon, can be identified and removed by colonoscopy. Furthermore, daily supplements of 25 mg of β-carotene can inhibit the recurrence of colourectal adenomas in nonsmokers who do not drink alcoholic beverages (Baron *et al*., 2003).

The prevention of degenerative diseases including cancer through dietary intervention, either by the use of specific diets or dietary supplements is termed as chemoprevention and is especially important for those healthy individuals who are at higher risk for cancer, people with precancerous conditions and patients who are at risk for a second primary cancer. There is a role of non-steroidal anti-inflammatory drugs in the prevention of colourectal, esophageal and gastric cancers (Husain *et al*., 2002). Hence, aspirin and other non-steroidal anti-inflammatory drugs may be used for preventing the development of esophageal cancer (Corley *et al*., 2003).

Cancer chemopreventive agents exert their effects mainly by inducing the expression of antioxidant and detoxification enzymes. It is well known that the reactive oxygen species generated either endogenously in the body or by exogenous chemicals present in food, water or air exert toxic or mutagenic effects on DNA (Ames, 1989). The DNA-damage can be prevented by phase I and phase II detoxification enzymes either by

directly scavenging ROS or by altering the metabolism of procarcinogenic molecules so that they are not converted to carcinogens (Figure 2). The phase I drug-metabolizing enzymes include the cytochrome P450-dependent monooxygenases which insert an atom of molecular oxygen into the substrate, resulting in increased hydrophilicity and elimination. However, this reaction can also result in the activation of the chemical to a toxic or mutagenic product which is usually detoxified by phase II conjugating enzymes (e.g. glutathione-S-transferases (GST), glucuronidases, quinone reductase and sulpho-transferases) (Hayes and Pulford, 1995; Wolf, 2001). Many dietary agents containing ascorbate, carotenoids and tocopherols possess antioxidant properties and/or the ability to induce these protective enzymes (Wolf, 2001).

Retinoids inhibit cancer development in a variety of tissue types (e.g. skin, lung and oral cavity in many animal models) and clinically they appear to reverse premalignant lesions and inhibit the development of second primary tumours throughout the respiratory epithelium (Dawson, 2004). They regulate the differentiation of airway epithelium *in vitro* and suppress carcinogenesis in animal models of lung cancer (Dawson, 2004; Dirami *et al*., 2004).

The development of cancer can also be prevented by removing the causes of cancer, for example, smoking cessation programs in North America led to the decline of lung cancer mortality after about 20 years (Franco *et al*., 2004). Moreover, the incidence of endometrial cancer also declined 5 years after the reduction in prescriptions of estrogens for hormone replacement therapy (Schottenfeld and Fraumeni, 1996).

Hepatitis B virus (HBV) vaccination can prevent the hepatocellular carcinomas (HCC) caused by HBV (Monto and Wright, 2001). In endemic areas, the incidence of HCC has decreased after HBV vaccination and the prevention of food crop contamination by aflatoxin B1 (Fong *et al*., 2001; Wogan *et al*., 2004). Control of hepatitis C virus infection, alcohol drinking and tobacco smoking are additional preventive measures for liver cancer (Chuang *et al*., 2009).

Adult T-cell leukemia, endemic in Japan, is usually transmitted to infants from their infected mothers' milk (Hino *et al*., 1985), therefore prenatal screening and refraining from breast-feeding has led to an 80% reduction in the level of vertical transmission in Japan (Bittencourt, 1998). Furthermore, administration of S-adenosyl-L-methionine may be effective in preventing alcohol-associated liver cancer (Lu and Mato, 2005). The probability of regression of gastric precancerous lesions increases by curing the *H. pylori* infection and by dietary supplementation with ascorbic acid and/or beta-carotene for 6 years (Correa *et al*., 2000). It has been suggested that at least three-quarters of oral cancers could be prevented by the cessation of tobacco smoking and a reduction in alcohol consumption (Warnakulasuriya *et al*., 2010).

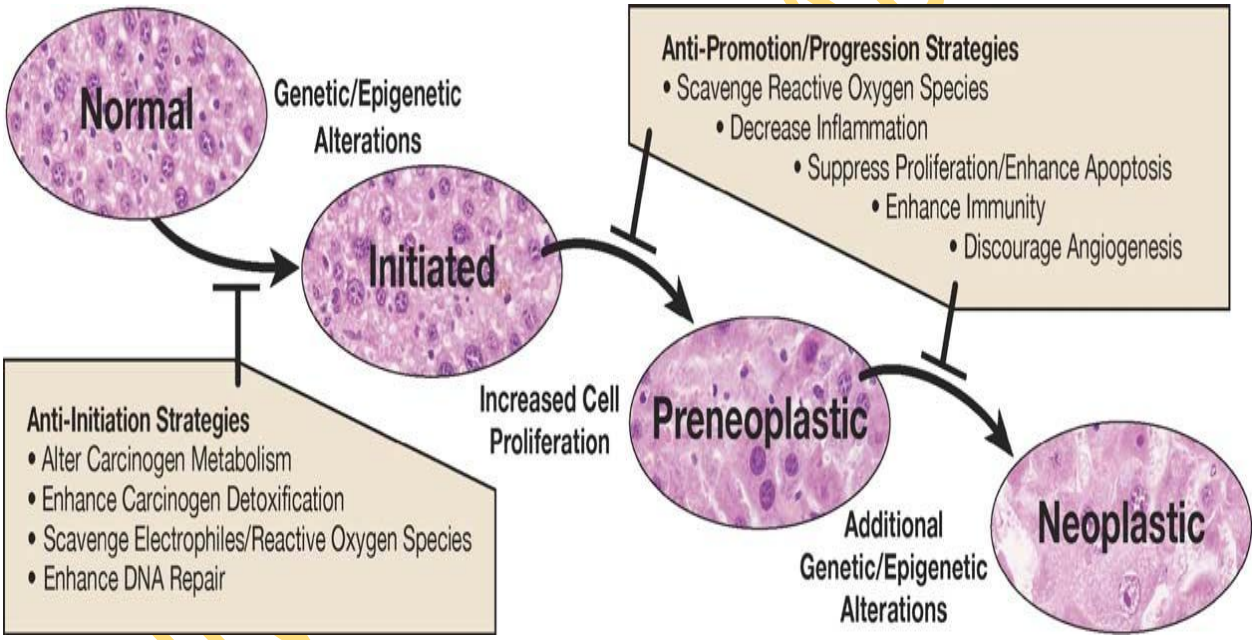


Figure 2: Carcinogenesis and chemoprevention (Stephen *et al*., 2005)

2.1.8 Cancer Treatment

Chemotherapy, surgery and radiation therapy are the three modalities of cancer treatment. The aim of cancer treatment is to eradicate cancer without causing damage to the patient. There has been a lot of improvement in the management of cancer in the last decade yet the cure from cancer is still not a reality for most patients mainly due to the toxicity of chemotherapy and development of drug resistance.

The cancer type, aggressiveness and location determine the treatment to be used. When the tumour is localized, surgery or radiation therapy is usually employed. If invasion or metastasis has already occurred, systemically administered chemotherapy has a better chance for reaching and destroying the cancer cells. Chemotherapy may be curative in some hematological malignancies while it is generally used in combination with surgery or radiation therapy for the treatment of solid tumours. The current chemotherapeutic drugs are very expensive and their exposure to normal cells increases the risks of severe adverse effects which reduce the quality of life. The search for new, potent and safer anticancer drugs with minimum toxicity is an ongoing process and natural products represent attractive targets for drug development.

2.1.8.1 Chemotherapeutic Drugs

The standard cytotoxic drugs are usually classified according to their mechanism of action and include (a) antimetabolites; which interfere with the synthesis of nucleic acids (DNA or RNA) (b) alkylating agents and anthracyclines; which directly interact and damage the nucleic acids (c) topoisomerase inhibitors; which also inhibit the synthesis of nucleic acids (d) antimitotic agents; which interfere with chromosome

separation during mitosis by inhibiting the function of the mitotic spindle (DeVita Jr. *et al*., 1997).

Antimetabolites are cytostatic agents which block the formation and use of nucleic acids required for DNA replication; majority are structural analogs of natural nucleosides e.g. 5-fluorouracil, 6-mercaptopurine, methotrexate and 6-thioguanine (Monsuez *et al*., 2010). They interfere with purine and pyrimidine nucleotide metabolism and nucleic acid synthesis. Alkylating agents interfere with DNA base pairing, leading to strand breaks and blockade of DNA replication e.g. cisplatin, cyclophosphamide, ifosfamide, mitomycin etc. (Monsuez *et al*., 2010). Inhibition of topoisomerase II blocks repair of disrupted DNA strands. Examples include doxorubicin, etoposide, teniposide etc.

The antimicrotubule interfering agents, also called spindle poisons, are classified into three (3) groups: (a) *Vinca (Catharanthus)* alkaloids, e.g. vinblastine, vincristine and vinorelbine that bind to tubulin dimer, depolymerize microtubules and prevent the formation of microtubules (Jordan, 2002) (b) Tubulin depolymerization inhibitors e.g. paclitaxel (Taxol) and docetaxel (Taxotere) result in stabilization of microtubules by inducing tubulin network polymerization (Honore´ *et al*., 2003). Paclitaxel prevents microtubules from breaking down and so cancer cells become so clogged with microtubules that they cease to grow and divide. (c) The third group binds to a common site on tubulin called the colchicine site e.g. combretastatin-A4 and its analogue AVE-8063 (Cragg and Newman, 2005). At low concentrations, these drugs suppress the dynamic instability of microtubules resulting in a failure of chromosome alignment

causing mitotic arrest that eventually leads to apoptosis (Mollinedo and Gajate, 2003; Zhou and Giannakakou, 2005).

Vinca alkaloids are widely used in combination chemotherapy regimens for the treatment of solid tumours and leukemia (Pourroy *et al*., 2004). Paclitaxel is the drug of choice for the treatment of many solid malignancies like that of breast, head and neck, lung and ovarian cancers, and leukemia (Aguirre *et al*., 2010). Doxorubicin (*Streptomyces* metabolite) is a potent anthracycline drug used to treat many cancers (Zordoky *et al*., 2010) and has multiple mechanisms of action including intercalation and alkylation of DNA (Young *et al*., 1981), inhibition of RNA and DNA polymerases (Zunino *et al*., 1975), and inhibition of topoisomerase II (Binaschi *et al*., 2001).

Most of the anticancer agents target cells undergoing cell division and hence the normal proliferative cells of bone marrow, epithelia, hair etc. are also affected. Moreover, the cancer cells rapidly develop resistance to these drugs and so limit their effectiveness, therefore new treatment strategies are being developed.

2.1.8.2 Novel Therapeutic Strategies

Conventional chemo- and/or radiotherapy are generally invasive and have adverse side-effects; therefore attempts are being made to design better and specific drugs which kill cancer cells only, and do not harm the normal tissues. But the targeted treatment can be ineffective by the development of small changes or mutations in the targeted molecule. Therefore, the combination of targeted and standard cytotoxic compounds is likely to be used in future (Eckhardt, 2006). Some of the new treatment strategies include (a)

inhibitors of activated tyrosine kinases; (b) tumour angiogenesis inhibitors and (c) restoration of altered apoptotic machinery (Figure 2) (Ross *et al*., 2004; Eckhardt, 2006).

2.1.8.3 Inhibition of Deregulated Tyrosine Kinases

Tyrosine kinase activity plays a vital role in the transmission of growth, differentiation, migration and apoptotic signals. Loss of the tyrosine kinase regulation in malignant cells results in spontaneously increased tyrosine kinase activity with an uncontrolled cell replication in about 70% of tumours (Monsuez *et al*., 2010). The receptor tyrosine kinases are important mediators of extracellular signals into the cells and hence useful anticancer targets. Monoclonal antibodies compete with the ATP-binding site of these receptor tyrosine kinases and hence interfere with their enzymatic activity (Noble *et al*., 2004). HER-2, also known as ErbB2 or the oncogene *neu*, is a transmembrane tyrosine kinase receptor which is overexpressed or amplified in 18-20% breast cancers (Fernandez *et al*., 2010). Herceptin (Trastuzumab) is a humanized monoclonal IgG1 antibody against HER2/neu that binds with high selectivity to the extracellular portion of the HER2/neu receptor and is successfully being used in the treatment of HER2/neu positive breast cancers (Fischer *et al*., 2003; Murphy and Fornier, 2010). Bevacizumab (Avastin) is an IgG1 humanized monoclonal antibody directed against VEGF receptor and is being used for the treatment of colourectal and non-small cell lung cancers (Monsuez *et al*., 2010).

Small molecule inhibitors which interfere with the intracellular downstream signaling pathway and block the function of these tyrosine kinases (Fischer *et al*., 2003; Bennasroune *et al*., 2004) have proven effective in the management of several

malignant diseases such as chronic myeloid leukemia, gastrointestinal stromal tumours and renal cell carcinoma (Monsuez *et al*., 2010). For example, imatinib mesylate (Gleevec) is used for the treatment of BCR–ABL positive chronic myeloid leukemia as it is a selective inhibitor of the BCR–ABL fusion protein, platelet derived growth factor receptor (PDGF-R) and stem cell ligand receptor (c-kit) tyrosine kinases (Druker *et al*., 2001). It is also used to reverse breast cancer resistance protein (BCRP)-mediated drug resistance since BCRP is an ATP-binding cassette transporter which is responsible for the development of resistance to various anticancer agents such as mitoxantrone and topotecan (Burger *et al*., 2004; Houghton *et al*., 2004).

Another example is gefitinib (Iressa), an inhibitor of the epidermal growth factor receptor kinase domain, used for the treatment of metastatic non-small cell lung cancer (Ross *et al*., 2004). Several tyrosine kinases initiate the intracellular signal transduction pathways (e.g. phosphatidylinositol 3-kinase) and by blocking these pathways their oncogenic function may be blocked (Bollag *et al*., 2003; Fingar and Blenis, 2004). The signaling of tyrosine kinases can also be blocked by interfering with receptor synthesis at the level of RNA e.g. RNA interference (Borkhardt, 2002).

2.1.8.4 Inhibition of Tumour Angiogenesis

Many angiogenesis inhibitors, such as anti-VEGF antibodies (Kerbel and Folkman, 2002), for example ZD6474 inhibits the tyrosine kinase activity of vascular endothelial growth factor receptor-2 and is given orally for the treatment of breast and non-small-cell lung cancers (Wedge *et al*., 2002; Bates, 2003). Since endothelial nitric oxide synthase (eNOS) is involved in both angiogenesis and vasculogenesis, therefore,

cavtratin which selectively inhibits eNOS, blocks tumour vessel hyper-permeability and stops tumour progression (Figure 2) (Gratton *et al*., 2003).

2.1.8.5 Oncoprotein Antagonism

ABT-737, an antagonist of Bcl-2, the oncoprotein activated in follicular lymphoma, is effective against certain lymphomas and solid tumours as a single agent and sensitizes many tumours to cytotoxic drugs (Cory and Adams, 2005). As RET oncoprotein is involved in the carcinogenesis of medullary thyroid carcinoma and papillary thyroid carcinoma, therefore, the inhibition of RET oncoprotein activity may be useful in their treatment. Although no selective drug that specifically targets the activity of oncogenic RET has been developed yet good results have been obtained using other available tyrosine kinase inhibitors (Pützer and Drosten, 2004).

2.1.8.6 Multi-drug Resistance

P-glycoprotein over-expression causes increased drug efflux leading to reduced intracellular accumulation of many anticancer drugs resulting in decreased tumour cell kill. Inhibition of P-glycoprotein activity can increase the sensitivity of malignant cells to anticancer agents by increasing drug accumulation (Oza, 2002).

2.1.8.7 Chemokines

In addition to directing migration of leukocytes, activating inflammatory responses, promoting or inhibiting angiogenesis, small chemotactic cytokines, the chemokines, are also involved in tumour progression and metastasis. Hence, disruption of their interactions may be useful in the treatment of cancer as in experimental models, the growth and/or spread of malignant tumour cells have been inhibited by using monoclonal antibodies against chemokine receptors (Tanaka *et al*., 2005).

2.1.8.8 Inhibition of Cell Migration

The primary mechanism of cell motility is the reorganization of the actin cytoskeleton which is regulated by Rho family small GTPases such as Rho, Rac and Cdc42. Cell migration is involved in tumour invasion and metastasis, hence, control of cell migration by inhibiting Rho family small GTPase signaling suppresses the invasion of cancer cells and possibly metastasis (Yamazaki *et al*., 2005).

2.1.8.9 The p73 Protein

The p73 protein belongs to a family of p53-related nuclear transcription factors that includes p53, p73 and p63. p73 is induced in response to DNA-damaging agents and exerts its pro-apoptotic activity in a way that is distinct from that of p53. It has been suggested that p73 can induce tumour cell apoptosis in a p53-dependent as well as p53-independent manner. Therefore p73 can be particularly useful in treatment of tumours which exhibit resistance to the p53-dependent apoptotic program (Ozaki and Nakagawara, 2005).

2.1.8.10 Immunotherapy

T cells use cytokines, including interferon-γ and tumour necrosis factor-α, and cytotoxic molecules, such as perforin, to reject tumours. They can recognize tumour antigens on tumour and stromal cells and modulate the function of tumour-associated macrophages, fibroblasts and endothelial cells (Kammertoens, 2005).

2.1.8.11 Personalized Medicine

New specific immunotherapeutic strategies are being developed based on the knowledge about genes and proteins. Preventive/therapeutic vaccination using

patient-specific tumour antigenic peptide may present a new era of personalized treatment, i.e. treat the patients as directed by specific tests (Kanduc *et al*., 2003).

2.1.8.12 All-trans Retinoic Acid combined with Arsenic Trioxide and Chemotherapy

The all-trans retinoic acid, given in addition to chemotherapy and arsenic trioxide, has been successfully used for the treatment of acute promyelocytic leukaemia (Tallman and Altman**,** 2008). The retinoids can also be used for the treatment of cutaneous T-cell lymphoma and squamous or basal cell carcinoma (Dawson, 2004).

2.1.8.13 Plant Derived Anticancer Drugs

Phytochemicals and herbal products have gained popularity since early 1990s because of the general belief that naturally derived medicinal products are effective against various diseases while being safer than anthropogenic medicines (Patel *et al*., 2010). More than 5000 phytochemicals are known which can alter biological function at the organ, cellular, subcellular and molecular levels (Patel *et al*., 2010). About 47.1% of the total 155 clinically approved anticancer drugs have been derived from natural products (Newman and Cragg, 2007).

The protective effects of plant-based diets on cancer has been established in many epidemiologic studies and a diet rich in a variety of fruits, legumes, nuts, oils, vegetables and whole grains is recommended as they contain numerous bioactive compounds having beneficial health effects. For example, hydroxytyrosol, a phenolic compound present in olives and olive oil, is a potent antioxidant, while lycopene, a potent antioxidant carotenoid in tomatoes and other fruits possibly protects against

prostate and other cancers (Surh, 2003). Resveratrol, found in red grapes and red wine, has antioxidant, antithrombotic and anti-inflammatory properties, and inhibits carcinogenesis (Bhat and Pezzuto, 2002; Estrov *et al*., 2003). Organosulfur compounds in garlic and onions, isothiocyanates in cruciferous vegetables, and monoterpenes in cherries, citrus fruits and herbs have anti-carcinogenic actions in experimental models (Kris-Etherton *et al*., 2002).

Many substances derived from dietary plants are known to be effective chemo-preventive and antitumour agents in a number of experimental models of carcinogenesis and research on plants/natural products has resulted in the development of various anticancer drugs. For example, phytochemicals with anticancer properties have been identified in 187 plant species, belonging to 102 genera and 61 families. Among them, only 15 species (belonging to ten genera and nine families) have been utilized in cancer chemotherapy at a clinical level, whereas, the rest of the identified species are either active against cancer cell lines or exhibit chemotherapeutic properties on tumour-bearing animals under experimental conditions (Kintzios, 2006). There are many plant-derived anticancer drugs currently being used clinically including paclitaxel (against breast, lung and ovarian cancers, Srivastava *et al*., 2005), vincristine (against acute lymphocytic leukemia, Kersey, 1997) and vinblastine (against Hodgkin's disease, Bonadonna *et al*., 1975).

The first anticancer plant alkaloid camptothecin (CPT) was identified as the active antitumour ingredient extracted from the Chinese ornamental tree *Camptotheca acuminata* (Wall *et al*., 1966). Semisynthetic derivatives of CPT i.e. irinotecan and

topotecan are being used clinically (DeVita *et al*., 2008). The alkaloids vinblastine and vincristine were isolated from the plant *Catharanthus roseus* (Pourroy *et al*., 2004). Etoposide and teniposide are semisynthetic derivatives of podophyllotoxin (a bioactive lignan obtained from the North American Mayapple plant family (*Podophyllum peltatum*) (Srivastava *et al*., 2005). Paclitaxel (Taxol) was isolated from the bark of *Taxus brevifolia* (Rajagopal *et al*., 2003).

Several potential lead molecules, from plants that have exhibited cytotoxicity against many human cancer cell lines, are still being investigated. For example, betulinic acid, a pentacyclic triterpene, was initially identified as a melanoma-specific cytotoxic agent, but now it has been recognized as an anticancer agent with a broad specificity for multiple tumour types (Kessler *et al*., 2007). It is available from the bark of white birch trees (*Betula alba*) and is reportedly devoid of cytotoxic effects against healthy cells (Zuco *et al*., 2002). Turmeric, commonly used in Southeast Asia, contains a major polyphenolic compound, curcumin or diferuloylmethane. Solubilized curcumin blocked brain tumour formation in mice and also eliminated brain tumour cells (Purkayastha *et al*., 2009). Others include combretastatin A-4 (a stilbene isolated from the South African tree *Combretum caffrum* (Srivastava *et al*., 2005); flavopiridol, a synthetic compound, based on a flavone isolated from *Dysoxylum binectiferum* (Shapiro *et al*., 2001); genestein from soyabeans (Wang, 2000); indole 3·carbinol from cruciferous vegetables such as brussels sprout and broccoli (Shukla *et al*., 2004); epigallocatechin from green tea (Wang *et al*., 2009); and homoharringtonine from the Chinese tree, *Cephalotaxus harringtonia* (Kantarjian *et al*., 2001). Several of these molecules or their derivatives and many others are undergoing clinical trials for the treatment of various

cancers, such as combrestatin A4, curcumin, homoharringtonine, perillyl alcohol, phenoxodiol, protopanaxadiol and salvicine etc. (Pan *et al*., 2010).

2.2 Clastogens and Clastogenesis

A clastogen is a substance that can cause breaks in chromosomes, leading to sections of the chromosome being deleted, added, or rearranged. Therefore, clastogenicity is described as the microscopically visible damages or changes to chromosomes (Testoni *et al*., 1997). This can cause a mutation, and lead to cancer development, as cells that are not killed by the clastogen may become cancerous. Known clastogens include acridine yellow, benzene, ethylene oxide, arsenic, phosphine and mimosine (Testoni *et al*., 1997).

Clastogens generally have low molecular weight (< 10,000 Daltons). Apart from causing chromosomal breakage, they can cause gene mutation, sister chromatid exchanges and other chromosomal aberrations. They were first described in the plasma of irradiated persons, but they are also found in hereditary breakage syndromes and chronic reactions (Testoni *et al*., 1997). Some compounds have been identified to be carcinogenic, mutagenic as well as being clastogenic. For instance, Melq-2-amino-3,4-dimethylimidazo-(4,5-F) quinoline is a potent clastogen and carcinogen having been shown to develop fore-stomach and liver tumour (Ramsey *et al*., 1998). Also 2-acetylaminofluorene, besides acting as a mutagen has the capacity of causing chromosomal damage (Ames *et al*., 1972). Another potent and common clastogen is arsenic which may be present in water as contaminants.

During cell division, the genetic material replicates and then divides equally between the two daughter cells that are produced. This process is disrupted by clastogens, which also cause chromosomal damage. When this occurs, the genetic material is not incorporated into a new nucleus, and then may form its own micronucleus which is clearly visible with a microscope (NTP, 2001). A positive correlation between the degree of clastogeneticity and carcinogenicity has been established (Natarajan, 1984).

2.2.3 Arsenic

Arsenic, a naturally occurring element and by-product of copper, lead and other metals smelters, is the top environmentally hazardous substance, which was demonstrated to be a human carcinogen (Mohammad *et al*., 2012). It is a naturally occurring element found in any of four valence states: -3 (arsine), 0 (elementary arsenic), +3 (arsenites), and +5 (arsenates). Elementary arsenic is a gray metallic-looking crystalline powder; arsine is a colourless gas; arsenites and arsenates are white crystalline powders. Arsenic is a sulfhydryl-reactive metalloid, is a major environmental toxicant produced by the burning of arsenic contaminated coal and glass manufacturing (Hamid and Najmeh, 2012). The term “arsenic” is used when the valence state is not specified and generally refers to arsenite and/or arsenate. The alkali salts are highly soluble in water but the calcium and lead salts are not. The pentavalent form, As^{5+} (arsenate or organic form) is less toxic than trivalent form, As^{3+} (arsenite or inorganic form) based on lower solubility. Both forms are found in arsenic-contaminated water, and they are inter-convertible once absorbed. Arsenic belongs to the group V elements of the periodic table and has atomic number of 33 and mass number of 76 (Chemistry world, 2008). In chemistry, arsenite is a chemical compound containing an arsenic oxoanion where arsenic has oxidation state +3. Examples of arsenites include SA which contains a

polymeric linear anion, $[AsO_2\text{-}]_n$ silver arsenite which contain trigonal anion, sometimes called ortho-arsenite (Greenwood *et al.*, 1997). Compounds in this class are acutely toxic, carcinogenic, teratogenic, and mutagenic. They are readily absorbed by various body tissues through the skin, respiratory and intestinal tracts, and transplacentally. They may cause severe irritation of tissues (skin, eyes, mucous membranes, and lungs). All arsenic compounds are moderately toxic (arsine is highly toxic), mutagenic in some but not all test systems, and teratogenic (ECRP, 1988). Major uses of arsenic in various forms are as pesticides (insecticides, herbicides, and sheep and cattle dips) and in drugs. SA is used in the water gas shift reaction to remove carbon dioxide. Arsenic is a known human carcinogen and is able to induce malformations in male reproductive system (Hamid and Najmeh, 2012).

2.2.4 Forms and Occurrence of Arsenicals

Arsenic compounds are ubiquitous in nature and most metallic ores contain small quantities. Mispickel or arsenical pyrites ($FeS_2.FeAs_2$), the sulphides realgar (As_2S_2) and Orpiment (As_2S_3) form the natural sources of arsenicals (Clarke and Clarke, 1975). Arsenic trioxide is normally produced in the roasting of metallic ores. This may be carried as a dust along with the smoke, and hence serve as a contaminant of soil, herbage and waste. In drinking water, inorganic arsenics are found as pentavalent (arsenate) and trivalent (arsenite) forms. In the human body, pentavalent arsenate is reduced to trivalent arsenite and it has been shown that the toxicity of arsenite compounds is higher than arsenate (Wang *et al.*, 2007).

2.2.5 Arsenic Trioxide (As_2O_3)

This is most common in general use. It occurs as an amorphous or crystalline, tasteless and odorless, white powder. It is sparingly soluble, volatile on heating, forming distinct

glistering octahedral crystals on sublimation. It resembles flour and French chalk, which has led to several accidents. Copper arsenite, which was initially used as a cheap pigment for colouring wall-papers and artificial flowers, was stopped due to deaths that resulted from its use. However, it is being used as an insecticide antihelminthic and slug bait (Clarke and Clarke, 1975). Base on the American FDA recommendation, arsenic trioxide (arsenite) has been used for the treatment of relapsed or refractory of acute promyelocytic leukemia in 2000 (Mohammad *et al*., 2012).

2.2.6 Sodium Arsenite ($NaAsO_2$)

SA is a trivalent inorganic compound belonging to general class of compound called arsenicals. It has molecular weight of 129.91017 g/mol (Sigma, Research and Diagnostic Reagents Catalogue, 1994). It is toxic and the most effective agent of the arsenicals. SA is produced from arsenious acid (As_2O_3) by reacting with alkali such as arsenite and water (Lenihan and Fletcher, 1977).

$$As_2O_3 + H_2O \longrightarrow 2HAsO_2$$

$$As_2O_3 + 2NaOH \longrightarrow 2NaAsO_2 + H_2O$$

SA is structurally represented as;

$$O = As - O^- \quad Na^+$$

2.2.7 Sources of Arsenic Compounds/Exposure

Arsenic compounds are widely distributed in the biosphere and on the earth's crust (Lenihn and Fletcher, 1977). Arsenic is a common environmental toxicant found in soil, water and air. Significant exposure to arsenic occurs via both anthropogenic and natural sources. Arsenic is released into the air by volcanoes and is a natural contaminant of some deep water wells. Occupational exposure to arsenic is common in the smelting

industry (in which arsenic is a byproduct of ores containing lead, gold, zinc, cobalt and nickel) and is increasing in the microelectronics industry (in which gallium arsenite is responsible). Low level arsenic exposure continues to take place in the general population (as do some cases of high dose poisoning) through the commercial use of inorganic arsenic compounds in common products, such as wood preservatives, ant-killers, herbicides, fungicides, through the consumption of foods and smoking of tobacco treated with arsenic-containing pesticides and through burning of fossil fuels in which arsenic is a contaminant (Figure 3).

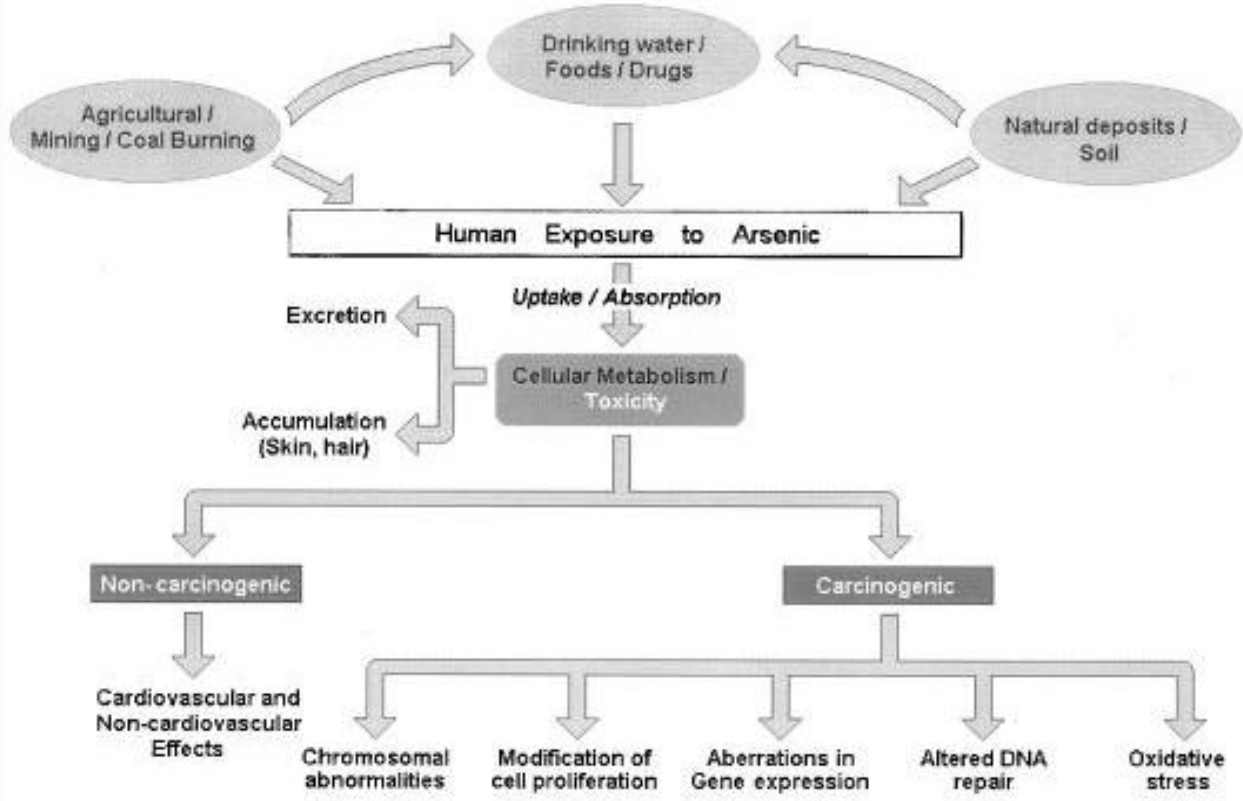


Figure 3: Sources of human exposure to arsenic and various modes of arsenic toxicity (Goering *et al.*, 1999)

2.2.8 Uses of Sodium Arsenite

SA is widely used as weed killer (Herbicides), dressing of grain, insect poisons (insecticides), cattle and sheep-dips and wood preservatives and debarking of trees. Reynolds (1999) reported that over 8,000 arsenic based compounds were used to treat asthma, malaria, tuberculosis, diabetes, skin diseases and sleeping sickness. The agricultural industry utilizes arsenic in the form of monosodium methylarsenite (MSMA), disodium methyl arsenite (DSMA), cacodylic acid (dimethyl arsenic acid) and arsenic acid (Armbrust and Bridges, 2002). They are used for the control of Johnson and nutsedge grass and weeds in cotton fields as herbicide application. The use of inorganic arsenicals (SA and arsenic trioxide) as herbicides has been reduced greatly because of livestock losses, environmental persistence, and their association with carcinogenesis. Arsenic derivatives continue to be available in other parts of the world in wood preservatives and insecticide formulations. These compounds can be hazardous to animals when used as recommended. Ruminants are apparently attracted to and lick plants poisoned with arsenite. The highly soluble organic arsenicals (methane arsonate, methyl arsonic acid) can concentrate in pools in toxic quantities after a rain has washed them from recently treated plants. Arsenicals are used as desiccants or defoliants on cotton, and residues of cotton harvest fed to cattle may contain toxic amounts of arsenic (Armbrust and Bridges, 2002).

2.2.2.9 Biological Effects

2.2.9.1 Arsenic Absorption and Fate

More than one hundred million people are at high risk of elevated arsenic exposure, mainly via drinking water, as well as by the air born metalloid in the areas with coal burning and industrial emissions. Consumption of the arsenic contaminated fishes

collected from the polluted waters might also contribute to bioaccumulation of arsenic in human beings (Randhir and Tarun, 2012). The rate of absorption of inorganic arsenicals from digestive tract depends upon their solubility. SA is readily soluble, rapidly absorbed and highly toxic (Calesnick *et al.*, 1966). The soluble arsenic compounds get easily absorbed by contact with intact skin and absorption from a fresh wound is very rapid. Accumulation of arsenic is seen in the liver with slow release and distribution to other tissues. Continued administration can cause its disappearance from soft tissues and its long term storage in bones, skin and keratinized tissues for example, hair and hoof (Grollman and Slaughter, 1947). Arsenic deposited in hair is irremovable and moves slowly along the hair as it grows (Figure 3). Arsenic is excreted in the urine, faeces, sweat and milk. The rate of excretion depends on the compound and it is inversely related to the toxicity (Sabeh *et al.*, 1993). Pentavalent arsenic is well absorbed through the gut, but the trivalent form is more lipids soluble. Toxicity results from the arsenite form (As^{3+}), especially by dermal absorption. Arsenic compounds are well absorbed parenterally within 24 hours. SA is readily soluble, rapidly absorbed and highly toxic (Sabeh *et al*., 1993). Arsenites and arsenates are absorbed by ingestion and parenteral injection; SA, arsenic trioxide, and arsine are absorbed by inhalation. SA and arsenate are absorbed through the placenta.

2.2.9.2 Distribution of Sodium Arsenite

SA is distributed to other tissues from the liver where it accumulates and excess can be stored in bone, skin, and keratinized tissues such as hair and hooves (Figure 3). Arsenic initially localizes in the blood bound to globulin. Redistribution occurs within 24 hours to the liver, lungs, intestinal wall, and spleen, where arsenic binds to sulfhydryl groups of tissue proteins only small amounts of arsenic penetrates the blood-brain barrier

(Winsk *et al*., 1995). There is also a significant accumulation of arsenate in the skeleton, presumably by exchange with phosphate (Landgren *et al*., 1983). Application of pentavalent arsenic to skin results first in an accumulation of arsenic in the skin, followed by distribution to other organs, followed by urinary excretion. Significant deposition in hair and nails has been demonstrated in man and animals.

2.2.9.3 Biotransformation of Sodium Arsenite

Arsenic is biotransformed *in vivo* by methylation to monomethyl and dimethyl arsenic. Dimethylarsenic is the principal transformation product and it appears to be the terminal metabolite which is rapidly formed. Dimethylarsenic promotes lung and skin tumour by way of the metabolic production of free radicals such as dimethyl arsenic peroxyl radical [$(CH_3)_2AsO$]. Dimethylarsenic may play an important role in arsenic carcinogenesis through induction of oxidative damage, particularly of base oxidation especially in the target organs of arsenic carcinogenesis; skin, lung, liver, and urinary bladder (Jayanthika *et al*., 2001). SA produces alteration in the RNA and DNA synthesis in cells, this inhibition is said to be dose-dependent (Goering *et al*., 1999). The possible mechanism of this cytotoxic effect of arsenite might be due to its reactivity with intracellular Sulfhydryl groups. The biotransformation of arsenic is based on several generally accepted steps (Figure 4 and 5). For methylation to occur arsenate (As^{5+}) species must be reduced to arsenite (As^{3+}) in a process that occurs through reactions involving glutathione (GSH). The arsenite is then methylated to monomethlarsonic acid (MMA). The MMA is then methylated to dimethylarsinic acid (DMA) (Goering *et al*., 1999).

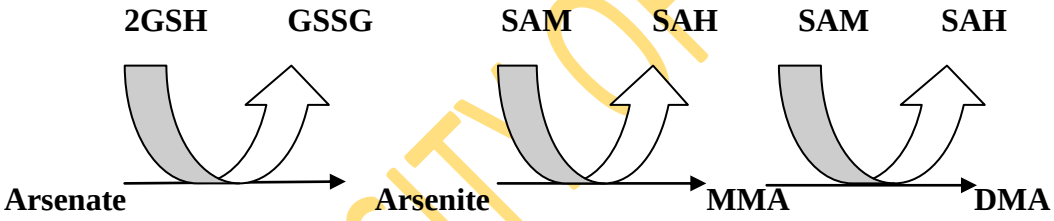


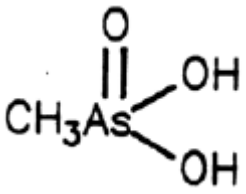
Figure 4: The biotransformation pathway for arsenic (Goering *et al.*, 1999)

Oxidative addition of methyl groups to arsenic occurs by methyltransferase enzymes, with S-adenosyl-methionine (SAM) as the methyl-donating cofactor (Aposhian *et al.*, 1997). In addition to the pentavalent metabolites, the trivalent metabolites, monomethylarsonous acid [MMA (111)] and dimethylarsinous acid [DMA (111)], have been identified as intermediary metabolites in the methylation of arsenic compounds and have been detected in cultured human cells treated with inorganic arsenic. Mandal *et al.*, (2001) reported the presence of MMA (III) and DMA (III) in the urine of people chronically exposed to inorganic arsenic via drinking water in West Bengal, India.

2.2.9.4 Excretion

The pathways of arsenic metabolism vary with the type of arsenic compound administered, route of administration, and animal species. Urinary and fecal excretion products are inorganic arsenic and the result of successive methylation to Monomethylarsonic Acid (MAA) and Dimethylarsonic Acid (DMA) in most species including man (Marafante *et al*., 1985).

Monomethylarsonic Acid (MMA)



Dimethylarsonic Acid (Cacodylic acid, DMA)

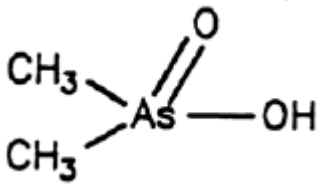


Figure 5: Structure of arsenic metabolism products.

2.2.9.6 Toxic Effects

Acute and chronic effects of arsenic intoxication in man have been reported (IARC, 1980). They include a burning sensation of mouth and throat; metallic, garlicky odor of breath and feces; difficulty in swallowing; vomiting; diarrhea; and cyanosis. Chronic effects include hyperpigmentation and keratosis (characteristics of prolonged treatment with Fowler's solution), vascular effects ("blackfoot disease"), cirrhosis of the liver, and effects on the hematopoietic system (leukopenia, anemia). The chief toxic effect of inhaled arsine is due to its binding to hemoglobin, resulting in extensive hemolysis and hematuria followed by jaundice; the usual cause of death is renal failure (Gullen *et al.*, 1995). Studies have shown that SA in short and long period of treatment caused reduction of rats Bone Marrow Mesenchymal Stem Cells (MSCs) viability and induced caspase dependent apoptosis (Mohammad *et al*., 2012).

2.2.9.7 Basis of Arsenite-Induced Toxicity

Arsenite interacts with thiol-containing amino acids, peptides and proteins (Winsk *et al*., 1995). Arsenite exerts its cellular toxicity by binding to sulflhydryl groups which results in enzyme inhibition. During arsenic metabolism, oxygen radical may be produced, possibly leading to damage of DNA, proteins, lipids and other molecules (Figure 3). There is a positive correlation between lipid peroxidation and arsenic tissue concentrations in the livers, kidneys and heart of arsenite treated rats (Ramos *et al.*, 1995). Arsenite induces the body's antioxidant activities in human firoblasts (Lee *et al*., 1995). It induces heme oxygenase, leading to the heme degradation iron release and decrease in the cytochrome p450 biotransformation enzymes important in both endogenous and xenobiotic metabolism (Albores *et al.*, 1989). Because of arsenite affinity for protein sulfhydryls, many side effects can occur from enzyme inhibition.

Chronic arsenite toxicity results in mitochondrial changes that block lipoic acid-dependent dehydrogenase, which in turn inhibits glycolysis and results in demand for glucose and subsequently hypoglycemia (Cobo *et al*., 1995). Arsenicals also inhibit pyruvate dehydrogenase in gluconeogenesis (Szinicz'L *et al*., 1998). Carbohydrate depletion caused by gluconeogenesis depletion may therefore aggravate arsenic toxicity. SA readily reacts with thiol group of enzymes, receptors or coenzymes which may inhibit important biochemical events that could alter cellular redox status and eventually lead to cytotoxicity (Jin *et al*., 2004; Liao *et al*., 2007). Increasing evidence indicates that arsenic acts on signaling pathways that regulate cell proliferation rather than causing direct DNA damage because arsenic exhibits its mutagenic activities only at concentrations high enough to also produce cell damage (Jacobson-Kram and Montalbano, 1985). Arsenic has been shown to modulate the mitogen-activated protein AP-1 (Cavigelli *et al*., 1996). AP-1 mediates many biological effects of tumour promoters and is an important regulator of cell growth. The ability of arsenic to interact with protein thiol groups on key regulatory proteins and subsequently alters their activities is likely to contribute to this effects (Cavigelli *et al*., 1996). Accordingly, it has been demonstrated that arsenic can induce a moderate increase in keratinocyte cell proliferation (Figure 3), as evidenced by increases in thymidine incorporation (Germolec *et al*., 1999), cell cycling (Klimecki *et al*., 1996), labeling of the proliferating cell marker Ki-67 (Klimecki *et al*., 1997), ornithine decarboxylase activity (Brown and Kitchin, 1996), and expression of oncogenes and growth factors such as c-*fos*, c-*jun*, c-*myc*, and transforming growth factor (Germolec *et al*., 1998).

2.2.9.8 Carcinogenic Effects

The International Agency for Research on Cancer classify arsenic as a carcinogen for which there is sufficient evidence from epidemiological studies to support a causal association between exposure and skin cancer. Chronic arsenic exposure has also been associated with a greatly elevated risk of skin cancer and possibly of cancer of the lung, liver, angiosarcoma, bladder, kidney and colon cancers (ECRP, 1988). There is strong evidence for a link between bladder cancer and exposure to arsenic (As) in drinking water at concentrations exceeding 300 – 500 µg/l (Silvia *et al*., 2012). The mechanism of arsenic-induced bladder cancer remains unclear. Arsenic inhibits indirectly sulfhydryl containing enzymes and interferes with cellular metabolism such as cytotoxicity, genotoxicity and inhibition of enzymes with antioxidant function (Anetor *et al*., 2007). On the other hand, the p53 protein may be involved in the development of bladder cancer. In a Taiwanese study, bladder tumours from people chronically exposed to As showed mutations in the p53 gene at codon 175 and transitions at points 9 and 10 (Tapio and Grosche, 2006). However, in a South American study, Moore *et al*., (2003) did not find evidence that As exposure was associated with an increased prevalence of p53 mutations or immunopositivity of p53 protein in bladder cancer. There is also evidence that the process of arsenic carcinogenesis may be modulated by genetic differences in DNA repair (Andrew *et al*., 2009). Andrew *et al* (2009), observed gene-environment interaction of 549 controls and 342 cases with arsenic exposure in relation to bladder cancer risk for a variant allele of the double-strand break repair gene. Chronic arsenic exposure may exert serious harmful effects including cancers, melanosis, hyperkeratosis, lung disease, peripheral vascular disease (Blackfoot disease), gangrene, diabetes mellitus, hypertension and ischemic heart disease (Hamid and Najmeh, 2012). They also detected elevation of serum globulins and development of esophaged varices

at follow up studies. There is also some evidence suggesting of changes in choloestatic function of the liver as shown by conjugated hyperbilirubinemia and elevated alkaline phosphatase activity, which directly relates to the concentration of total arsenic in urine (Ellenhern, 1997). Pulmonary findings of chronic arsenic toxicity include both obstructive and restrictive patterns of pulmonary function tests (Ellenhern, 1997). Arsenicals have been shown to cause enzymatic inhibition of the tricarboxylic acid (TCA) cycle (Webb, 1996). Pyruvate dehydrogenase system has been shown to be especially sensitive to trivalent arsenicals because of their apparent interaction with disulphdryl lipoic acid moiety of this system as shown in Figure 6.

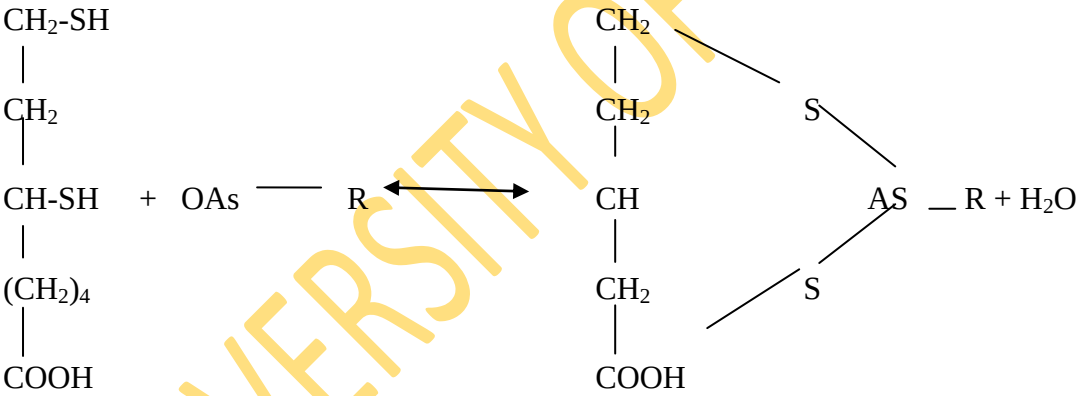


Figure 6: Enzymatic inhibition reaction of SA (Webb, 1966).

SA was found to inhibit methyl thymidine uptake in human cells *in vitro*, consistent suppression of DNA synthesis. Chromosomal aberrations were observed in human leucocyte exposed to SA (Figure 3). Arsenic has also been suggested to substitute for phosphorus in DNA, causing a weak bond in DNA chain (Petres *et al.*, 1970). The toxicity of trivalent arsenic to animals and human beings has also been thought to be caused by its binding to thiol ions, thus inhibiting enzymatic reactions. As late as 1980 it was believed that arsenic compounds were not carcinogenic in experimental animals, and this conclusion was drawn from a summary of largely negative results (IARC, 1980). Since that time evidence has appeared which indicates carcinogenicity in rats (Ivankovic *et al*., 1979). The evidence for carcinogenicity of arsenic compounds in man is more positive, and this has been reviewed (Landrigan, 1981; IRAC, 1980). Lung cancers have been noted in men involved in the production of arsenicals (Mabuchi *et al*., 1980).

2.2.10 Ethanol and Hepatotoxicity

Ethanol is a colourless liquid with pleasant smell and a molecular formula; C_2H_5OH. Alcohol-related liver diseases are complex, and ethanol has been shown to interact with a large number of molecular targets. Ethanol can interfere with hepatic lipid metabolism in a number of ways and is known to induce both inflammation and necrosis in the liver. Ethanol increases the formation of superoxide by Kupffer cells thus implicating oxidative stress in ethanol-induced liver disease (Ernest, 2004). Similarly pro-oxidants (reactive oxygen species) are produced in the hepatocytes by partial reactions in the action of CYP2E1, an ethanol-induced CYP isoform (Figure 7). The formation of protein adducts in the microtubules by acetaldehyde, the metabolic product formed from

ethanol by alcohol dehydrogenase, plays a role in the impairment of VLDL secretion associated with ethanol (Ernest, 2004).

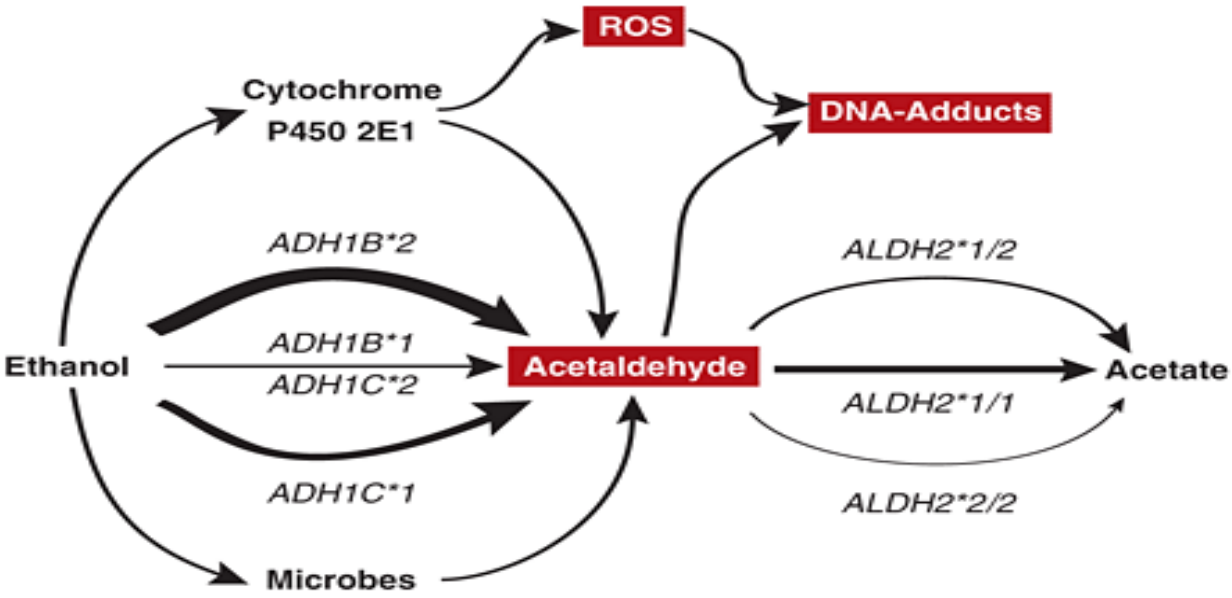


Figure 7: Pathways of ethanol metabolism and their role in carcinogenesis (http://www.pubs.niaaa.nih.gov/publications/arh301/38-47.htm)

2.2.10.1 Ethanol and Oxidative Stress

Oxidative stress is generally considered the result of an imbalance between two opposing, antagonistic forces, free radicals and antioxidants, in which the effects of the former predominate over the compensating action of the latter (Antero *et al*., 2013). Free radicals are molecules or fragments of molecules containing unpaired electron in their outermost orbitals (José, 2003).

The involvement of free radical mechanisms in the pathogenesis of Alcoholic Liver Disease (ALD) is demonstrated by the detection of lipid peroxidation markers in the liver and the serum of patients with alcoholism, as well as by experiments in alcohol-feed rodents that show a relationship between alcohol-induced oxidative stress and the development of liver pathology. Ethanol-induced oxidative stress is the result of the combined impairment of antioxidant defences and the production of reactive oxygen species by the mitochondrial electron transport chain, the alcohol-inducible cytochrome P450 (CYP) 2E1 and activated phagocytes (Emanuele, 2006). Furthermore, hydroxyethyl free radicals (HER) are also generated during ethanol metabolism by CYP2E1 (Figure 7). The mechanisms by which oxidative stress contributes to alcohol toxicity are still not completely understood. The available evidence indicates that, by favouring mitochondrial permeability transition, oxidative stress promotes hepatocyte necrosis and/or apoptosis and is implicated in the alcohol-induced sensitization of hepatocytes to the pro-apoptotic action of TNF-α. Moreover, oxidative mechanisms can contribute to liver fibrosis, by triggering the release of pro-fibrotic cytokines and collagen gene expression in hepatic stellate cells. However, the reactions of HER and lipid peroxidation products with hepatic proteins stimulate both humoral and cellular

immune reactions and favour the breaking of self-tolerance during ALD. Thus, immune responses might represent the mechanism by which alcohol-induced oxidative stress contributes to the perpetuation of chronic hepatic inflammation (Emanuele, 2006). The mechanisms underlying alcohol-related cancers are unclear but several factors have been suggested to play a role (Gyamfi and Wan 2010; Testino *et al*., 2012): local effect of ethanol, acetaldehyde (isoenzymes polymorphism), induction of cytochrome P450 2E1 (CYP2E1) (conversion of various xenobiotics), nutritional deficiencies, interactions with retinoids, changes in the degree of methylation, immune surveillance, angiogenesis. Alcohol may be important in the initiation of cancer, either by increasing the expression of certains oncogenes or by impiring the cell's ability to repair DNA, thereby, increasing the likelihood that oncogenic mutations will occur (Testino *et al*., 2012).

2.3 Antioxidants

Antioxidant-the word itself is magic (Shashi *et al.,* 2014). An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. In their definition of the term, Halliwell and Gutteridge (1989), state that an antioxidant is 'any substance that when present at low concentrations compared with that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate'. It is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electron from a substance to an oxidizing agent (Sangh and Sanjay, 2012). This definition includes compounds of a non-enzymatic as well as an enzymatic nature. Clearly, the diversity of antioxidants matches that of pro-oxidants (Sies, 1997). Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain

reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves (Sangh and Sanjay, 2012). As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Rahmat *et al*., 2012; Sies, 1997). An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed 'oxidative stress'. Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions. Antioxidant defence involves several strategies, both enzymatic and non enzymatic (Sangh and Sanjay, 2012). In the lipid phase, tocopherols and carotenes as well as oxy-carotenoids are of' interest, as are vitamin A and ubiquinols. In the aqueous phase, there are ascorbate, glutathione and other compounds. In addition to the cytosol, the nuclear and mitochondrial matrices and extracellular fluids are protected. Overall, these low molecular mass antioxidant molecules add significantly to the defence provided by the enzymes superoxide dismutase, catalase and glutathione peroxidase (Sies, 1997).

2.4 Cytokines

2.4.1 Tumour Necrosis Factor-alpha

Tumour necrosis factor (TNF, cachexin, or cachectin, and formerly known as tumour necrosis factor-alpha or TNF-α) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages, although it can be produced by other cell types as well like CD4+ lymphocytes and NK cells. The primary role of TNF is in the regulation of immune cells. TNF, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, sepsis (through IL1 and IL6 production), cachexia, inflammation,

and to inhibit tumourigenesis and viral replication. Dysregulation of TNF production has been implicated in a variety of human diseases, including Alzheimer's disease (Swardfager *et al.,* 2010), cancer (Locksley *et al.,* 2001), major depression (Dowlati *et al.,* 2010) and inflammatory bowel disease (Brynskov *et al.,* 2002). Additional beneficial functions of TNF-α include its role in the immune response to bacterial, and certain fungal, viral, and parasitic invasions as well as its role in the necrosis of specific tumours. Lastly it acts as a key mediary in the local inflammatory immune response. TNF-α is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. TNF-α secreted by the macrophage causes blood clotting which serves to contain the infection. Without TNF-α, mice infected with gram negative bacteria experience septic shock (Janeway *et al.,* 1999).

2.4.1.1 Discovery

The theory of an anti-tumoural response of the immune system *in vivo* was recognized by the physician William (1968), Dr. Gale A Granger from the University of California, Irvine, reported a cytotoxic factor produced by lymphocytes and named it lymphotoxin (Kolb and Granger, 1968). Credit for this discovery is shared by Dr. Nancy H. Ruddle from Yale University, who reported the same activity in a series of back-to-back articles published in the same month (Ruddle and Waksman, 1968**)**. Subsequently in 1975 Dr. Lloyd from Memorial Sloan-Kettering Cancer Center, New York, reported another cytotoxic factor produced by macrophages, and named it tumour necrosis factor (TNF, Carswell *et al.,* 1975). Both factors were described based on their ability to kill mouse fibrosarcoma L-929 cells. When the cDNAs encoding LT and TNF were cloned (Pennica *et al.,* 1984), they were revealed to be similar. The binding of TNF to its

receptor and its displacement by LT confirmed the functional homology between the two factors. The sequential and functional homology of TNF and LT led to the renaming of TNF as TNF-α and LT as TNF-β. In 1985, Bruce and Anthony discovered that a hormone that induces cachexia and previously-named cachectin was actually TNF (Beutler *et al.*, 1985). These investigators then identified TNF as a mediator of lethal endotoxin poisoning (Beutler *et al.*, 1985). Tracey and Cerami (1986) discovered the key mediator role of TNF in lethal septic shock, and identified the therapeutic effects of monoclonal anti-TNF antibodies.

2.4.1.2 Tumour Necrosis Factor-alpha and Signaling

Two complex models are shown in Figure 8. Upon ligation with TNF-α (within 10–20 min), TNFR I undergo trimerization and recruits various adapter molecules resulting in the activation of NF-κB, which induces several anti-apoptotic genes (Complex I formation) and survival signal. This is followed by (more than 2–3 hours) an endocytosis of receptor complex resulting in the dissociation of certain adapter proteins (TRAF-2, RIP) and recruitment of fas – associated death domain (FADD) and procasepase-8 to form death-inducing signaling complex (DISC). In the DISC, caspase-8 is activated and released into the cytoplasm where it activates effector caspases to induce apoptosis.

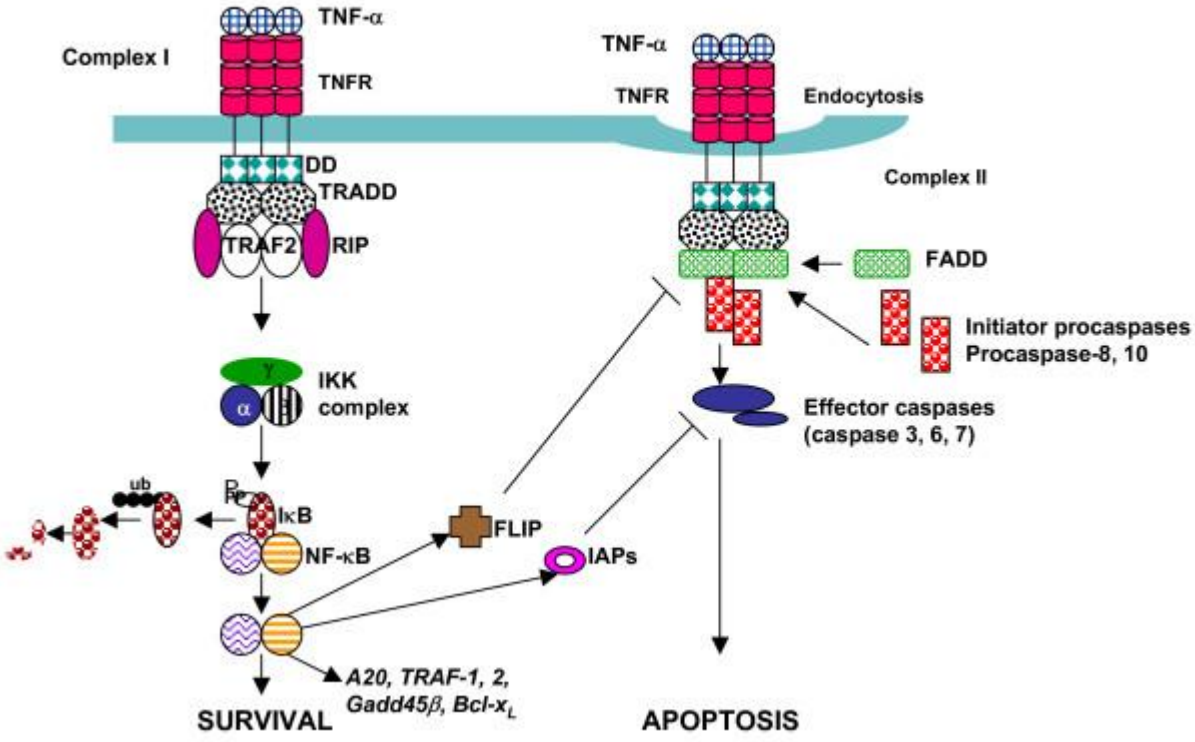


Figure 8: TNF-alpha receptor and signaling (Sudhir *et al.*, 2006).

2.4.2 Interleukin-1beta

Interleukin-1 beta (IL-1β) also known as catabolin, is a cytokine protein that in humans is encoded by the *IL1B* gene. IL-1β precursor is cleaved by caspase 1 (interleukin 1 beta convertase). Cytosolic thiol protease cleaves the product to form mature IL-1β. Molecular weight of the proteolytically processed IL1B is 17.5 kDa (Auron *et al.*, 1984; March *et al.*, 1985). Interleukin 1 was discovered by Gery in 1972. He named it lymphocyte-activating factor (LAF) because it was a lymphocyte mitogen. It was not until 1985 that interleukin 1 was discovered to consist of two distinct proteins, now called interleukin-1 alpha and interleukin-1 beta (March *et al.*, 1985). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The induction of cyclooxygenase-2 (PTGS2/COX2) by this cytokine in the central nervous system (CNS) is found to contribute to inflammatory pain hypersensitivity. This gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2 (Entrez Gene: IL1B interleukin 1, beta, HGNC: 5992; Caio *et al*., 2011).

2.4.2.1 Production and Release of IL-1β

Primary blood monocytes (Figure 9), tissue macrophages, or dendritic cells are activated by the cytokine IL-1β resulting in the formation of the IL-1 receptor complex (composed of IL-1RI and IL-1RAcP). The intracellular TIR domains of the IL-1 receptor complex recruit MyD88 and other signaling components leading to translocation of the transcription factor NF-kB into the nucleus. This results in transcription of the gene encoding the precursor of IL-1β and its translation into protein (Bocker *et al*., 2001). ATP released from activated monocytes (or from dying cells) accumulates outside the cell. As the extracellular levels of ATP increase, the P2X7

receptor is activated, triggering the efflux of potassium ions from the cell. Low intracellular potassium ion levels enable the components of the caspase- 1 inflammasome to assemble. Assembly results in the conversion of procaspase-1 to active caspase-1. There is evidence that the components of the inflammasome localize to secretory lysosomes together with the IL-1β precursor protein and lysosomal enzymes (Andrei *et al*., 2004). In the secretory lysosome, active caspase- 1 cleaves the IL-1β precursor, generating active mature IL-1β. Mature IL-1β is released along with the IL-1β precursor and the contents of the secretory lysosomes (Andrei *et al*., 2004). There is evidence that secretion of active IL-1β requires an increase in intracellular calcium ion levels (Kahlenberg and Dubyak, 2004). Processing of the IL-1β precursor can also take place in the cytosol independently of caspase-1 and the inflammasome (Brough and Rothwell, 2007). Studies have also shown that Rab39a, a member of the GTPase family, contributes to the secretion of mature IL-1β by helping IL-1β traffic from the cytosol into the vesicular compartment (Becker *et al*., 2009) from where it is secreted by exocytosis (Qu *et al*., 2007). Mature IL-1β can also exit the cell through a loss in membrane integrity (Laliberte *et al*., 1999) or exocytosis via small vesicles (MacKenzie *et al*., 2001).

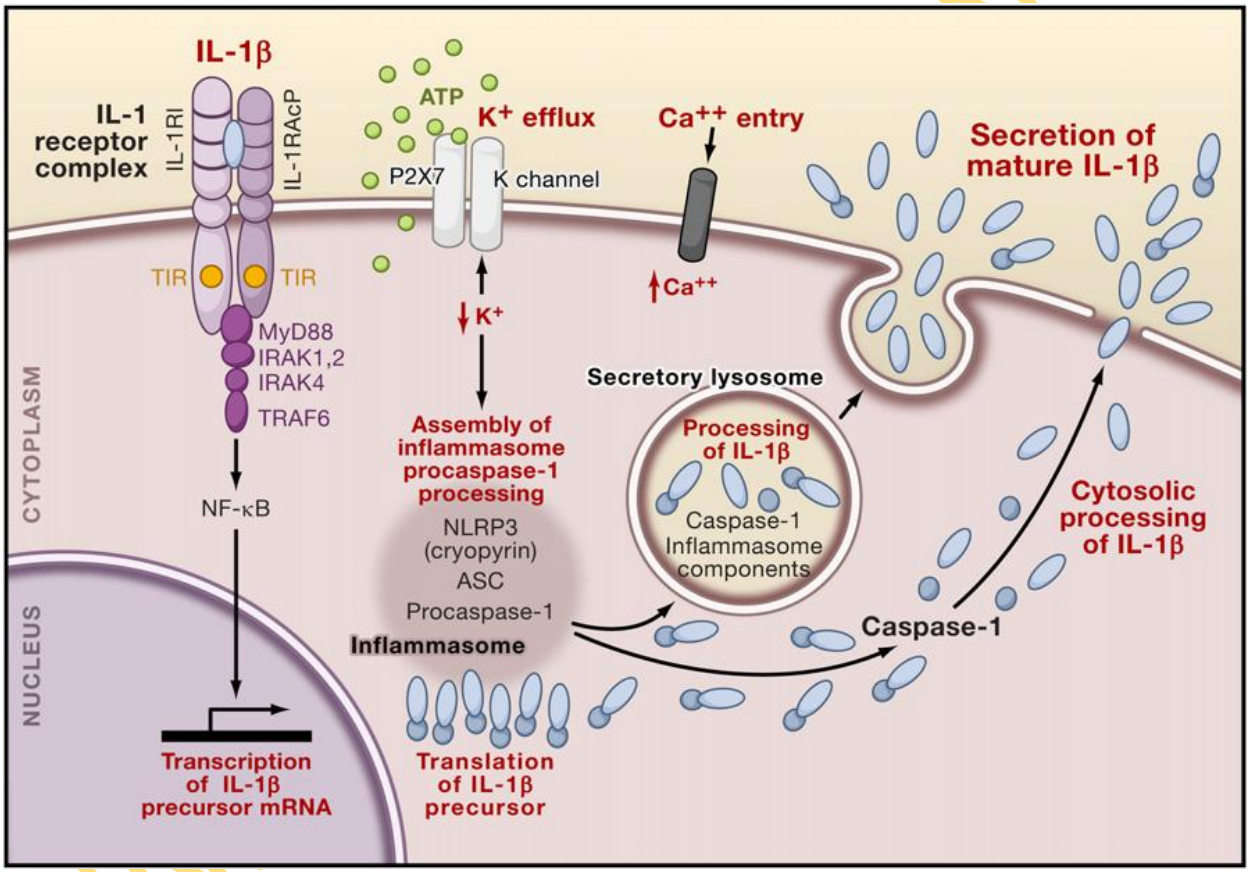


Figure 9: Production and release of IL-1β (Charles, 2010).

2.5 Cell Death

Cell death is a crucial process during development, homeostasis and immune regulation of multicellular organisms, and its dysregulation is associated with numerous pathologies. Cell death is often induced upon pathogen infection as part of the defense mechanism, and pathogens have evolved strategies to modulate host cell death. Basically four major types of cell death have been identified namely: programmed cell death (apoptosis), necrosis, autophagic cell death and pyroptosis (Linde *et al.*, 2009).

2.5.1 Apoptotic Cell Death

In 1972, the term ‘‘apoptosis’’ was used for the first time to describe a form of cell death associated with specific morphological features. Since then, apoptosis has been extensively studied and underlying signaling events are now well Characterised. It is a complex and highly regulated phenomenon (Megha *et al.*, 2014), playing a key role in the elimination of unnecessary or damaged cell and in a variety of normal biological processes such as cell proliferation and differentiation, tissue homeostasis, and aging (Wyllie, 2010; Ola *et al*., 2011; Mason and Rathmell, 2011; Natalia and Gennadii, 2012; Jean and Laurence, 2013). Morphologically, apoptosis is associated with cell shrinkage, membrane blebbing, and chromatin condensation. In mammals, there are two main apoptotic pathways (Figure 10a), extrinsic pathway (death receptor mediated pathway) and intrinsic pathway (mitochondrial mediated pathway). The extrinsic pathway is mediated by cell surface death receptors while the intrinsic pathway is mediated by different apoptotic stimuli (Samar *et al*., 2012; Jin and El-Deiry, 2005; Wang and Lenardo, 2000). It is a cell-intrinsic programmed suicide mechanism that results in the controlled breakdown of the cell into apoptotic bodies, which are subsequently recognized and engulfed by surrounding cells and phagocytes. Two main evolutionarily

conserved protein families are involved in apoptosis, namely the Bcl-2 family of proteins, which control mitochondrial integrity (Youle and Strasser, 2008), and the cysteinyl aspartate-specific proteases or caspases, which mediate the execution phase of apoptosis (Fuentes-Prior and Salvesen, 2004). However, molecular mechanism of apoptosis is depicted in Figure 10b and 10c. Stimulation of Tumour necrosis factor receptor (TNFR1) initiates the extrinsic pathway of apoptosis. Upon TNF stimulation, complex I is formed, resulting in NF-kB activation and subsequent transcription of anti-apoptotic genes. After endocytosis of TNFR1, complex II is formed, in which caspase-8 is recruited and activated. Subsequent activation of the executioner caspases leads to cleavage of their substrates and to cell death. The intrinsic pathway of apoptosis is activated upon intracellular stress signals at the mitochondrial level. Activation of Bax and Bak induces the release of several mitochondrial apoptotic mediators. The subsequent formation of the apoptosome results in the activation of caspase-9. In addition, caspase-8-mediated cleavage of Bid amplifies the extrinsic pathway of apoptosis by activating the mitochondrial pathway.

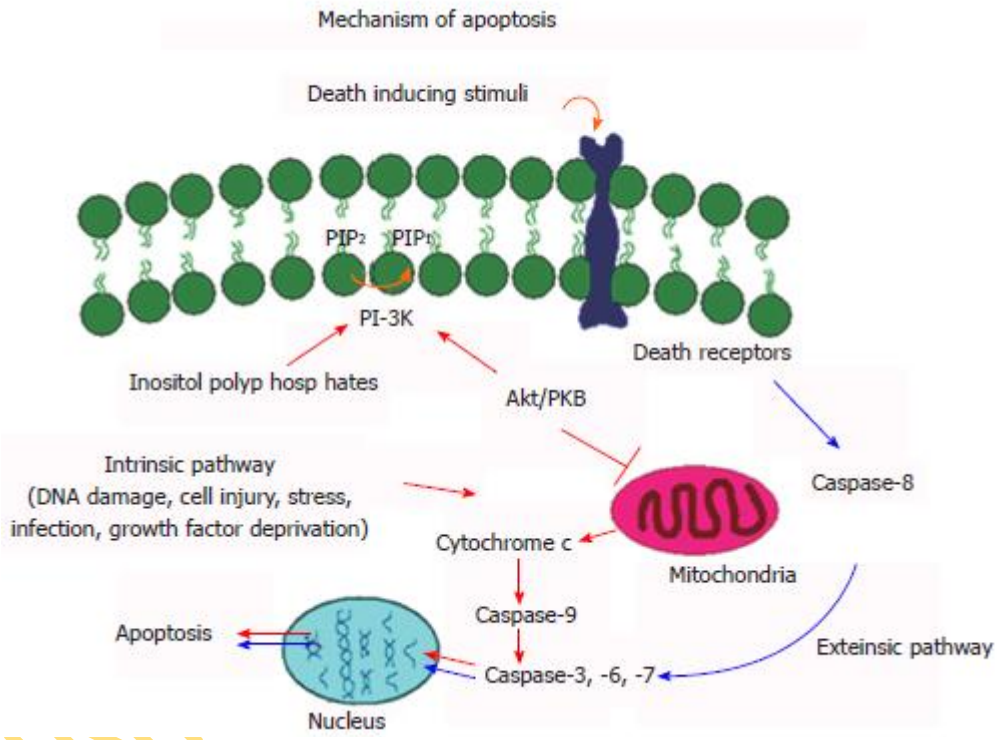


Figure 10a: Molecular signaling mechanisms of apoptosis (Samar *et al*., 2012).

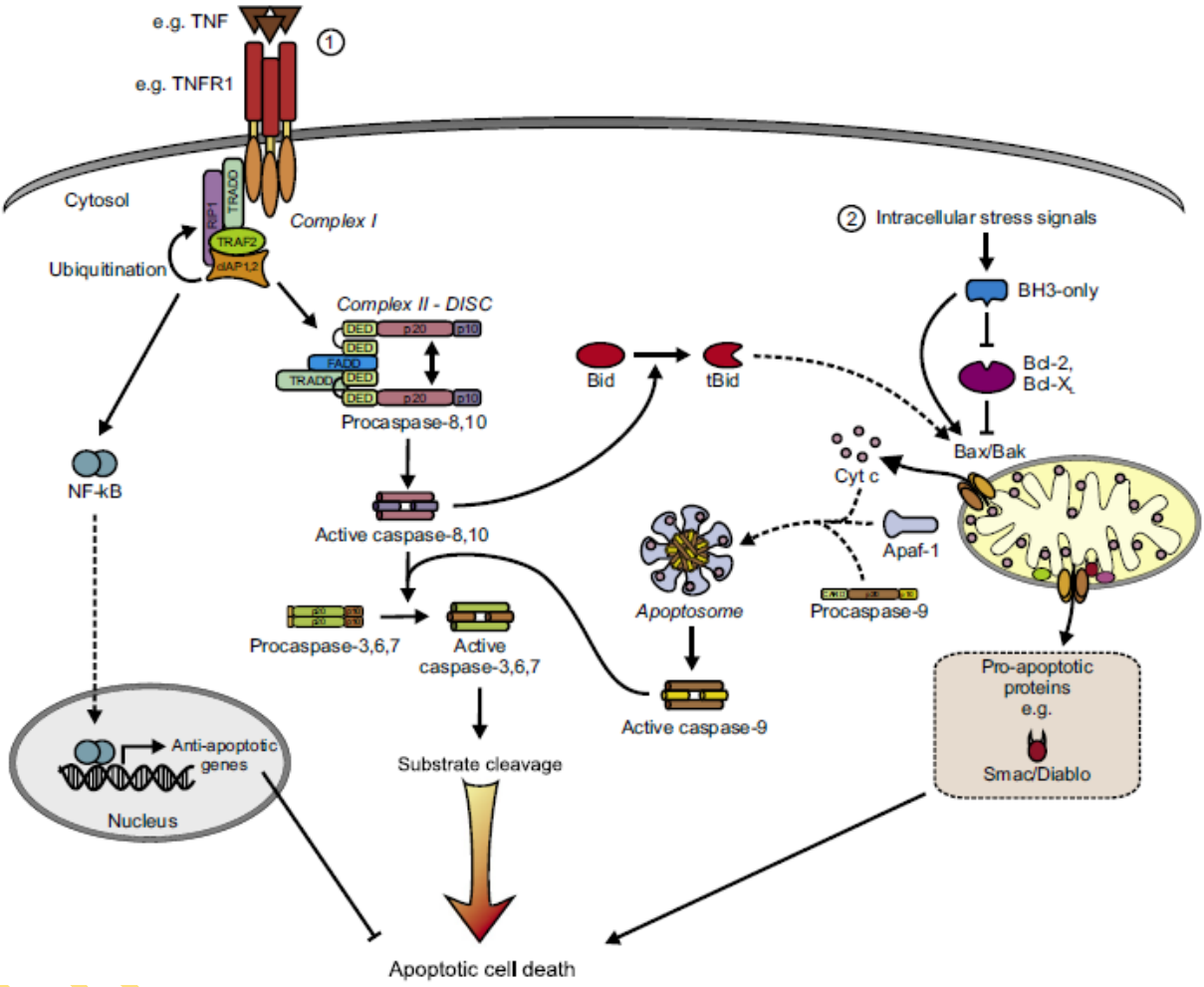


Figure 10b: Schematic representation of extrinsic and intrinsic apoptotic signaling (Linde *et al.*, 2009).

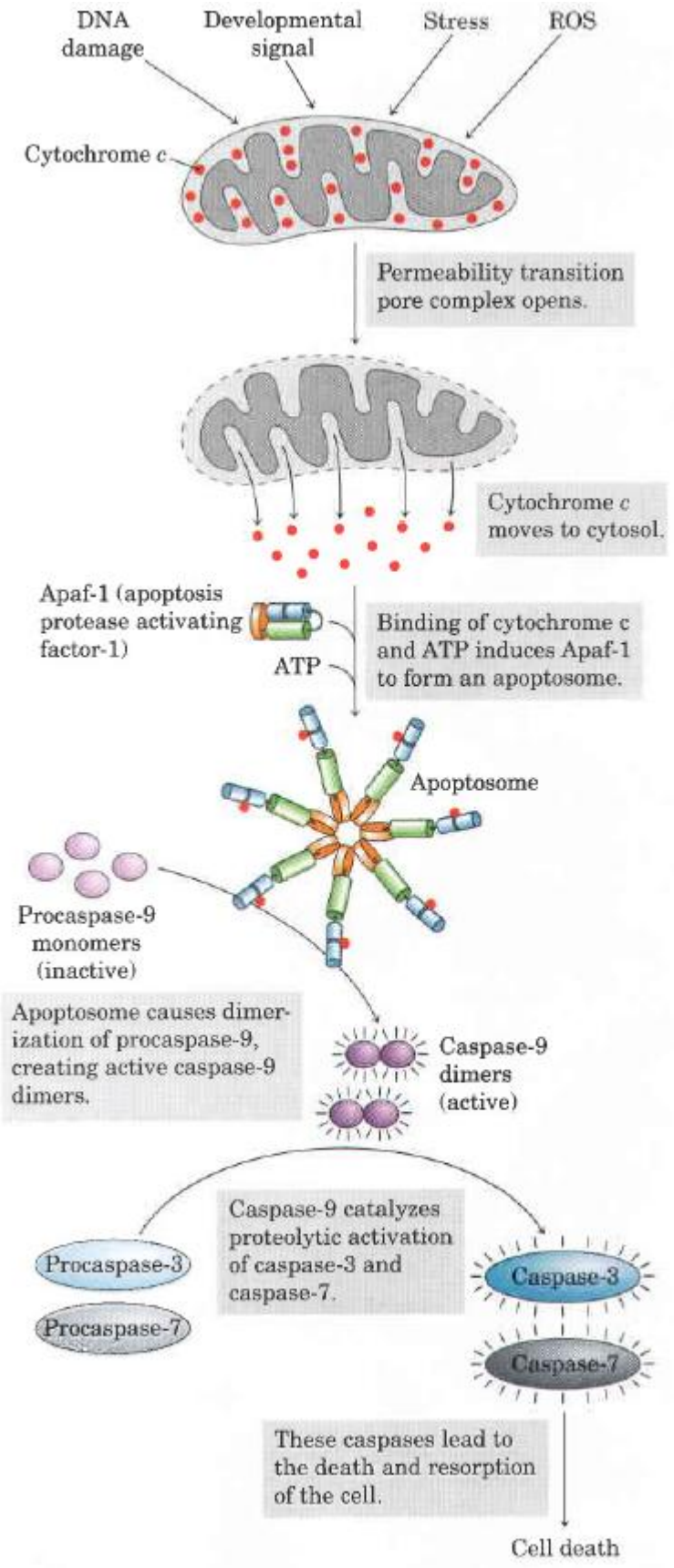


Figure 10c: The role of cytochrome c in apoptosis (David and Michael, 2008).

2.5.2 Necrotic Cell Death

For a long time, necrosis has been considered an accidental and uncontrolled form of cell death lacking underlying signaling events. This might be true for cell death resulting from severe physical damage, such as hyperthermia or detergent- induced cytolysis. However, accumulating evidence supports the existence of caspase-independent cell death pathways that can function even in a strictly regulated developmental context, such as interdigital cell death (Chautan *et al*., 1999). Necrotic cell death is Characterised by cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and release of the cellular contents into the surrounding extracellular space. However, molecular mechanism of necrosis is depicted in Figure 11. Stimulation of TNFR1 leads to the activation of receptor interacting protein (RIP1), which induces a pro-survival pathway by activating transcription factors, such as NF-kB and AP-1. In addition, RIP1 interacts with RIP3 and both are crucial initiators of death receptor-induced necrotic signaling. Through RIP1 kinase activity, a wide range of necrotic mediators are activated, such as ROS, calcium, calpains, cathepsins, phospholipases, NO and ceramide. The same mediators can be activated by DNA damage or by triggering of toll-like receptor (TLR-3, TLR-4) and NACHT/LRR/PYD-containing protein (Nalp-3).

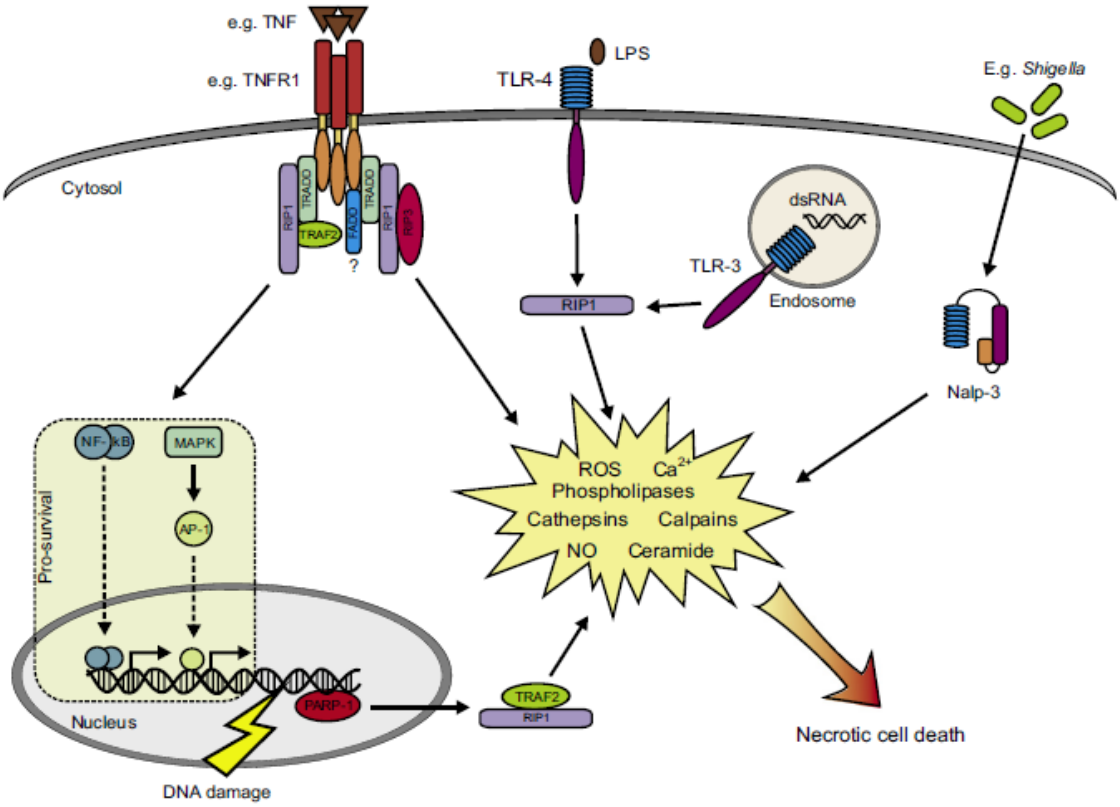


Figure 11: Schematic representation of necrotic Signaling (Linde *et al.*, 2009).

2.5.3 The p53–Bcl-2 Connection

The tumour suppressor p53 (Figure 12) and the proto-oncogene Bcl-2 (Figure 13) were two of the earliest identified cancer genes. Human p53 is a nuclear phosphoprotein of MW 53 kDa, encoded by a 20-Kb gene containing 11 exons and 10 introns (Lamb and Crawford, 1986), which is located on the small arm of chromosome 17 (Isobe *et al.*, 1986). This gene belongs to a highly conserved gene family containing at least two other members, p63 and p73**.** On the other hand, Bcl-2 protein contains four conserved structural homology domains. BH1, BH2 and BH3 are required for interaction with other Bcl-2 family members, whereas the BH4 domain mediates cell-cycle control functions and the association with c-Raf-1. The transmembrane domain (TM) is required for the subcellular localization of Bcl-2 and is of key importance for its function. The α5/α6 helix part of Bcl-2 displays some homology with the channel-forming part of the diphtheria toxin and is required for the full activity of Bcl-2. Bcl-2 and the related Bcl-xL contain all BH domains, whereas the pro-apoptotic members lack some BH domains. Based on their structure, the pro-apoptotic Bcl-2 family members are divided into Bax-like proteins and the BH3-only proteins (Belka, and Budach, 2002).

Mutant p53 proteins were first discovered in transformed murine cell lines (Linzer and Levine, 1979; Lane and Crawford, 1979), whereas Bcl-2 translocations were first identified in human follicular lymphoma (Tsujimoto, 1985; Bakhshi, 1985; Cleary and Sklar, 1985). Despite this shared cancer relevance, they were initially thought to have little else in common. p53 was proposed to activate cell cycle checkpoints (Korsmeyer, 1992), whereas Bcl-2 was shown to inhibit cell death. Additionally, Bcl-2 over expression was found mostly in hematopoietic cancers, whereas p53 mutations were

primarily found in solid tumours. However, recently the emergence of strong genetic and biochemical ties between these two proteins has become increasingly evident that signaling between p53 and Bcl-2 is of fundamental importance to cancer biology. p53 is a sequence-specific transcription factor that is activated by diverse forms of cellular stress (Ko and Prives, 1996). Early studies of p53 focused on the ability of tumour-derived p53 mutants to promote cell growth and transformation (Hemann and Lowe, 2006). Subsequently, p53 was Characterised as an essential mediator of cell cycle arrest in response to diverse cellular stresses (David and Michael, 2005). The first suggestion that p53 could promote apoptosis came more than 10 years after its discovery from experiments in which p53 was introduced into a p53-deficient leukemia cell line. These experiments showed that enforced p53 expression could induce cell death in cells deprived of pro-survival cytokines. Subsequently, work in p53 knockout mice demonstrated that p53 activity was essential for radiation-induced death in thymocytes and chemotherapy-induced apoptosis in fibroblasts expressing deregulated oncogenes (Hemann and Lowe, 2006). Importantly, the ability of p53 to promote cell death could be directly linked to its tumour suppressive function (David and Michael, 2005). Development of certain tumours in p53 null mice was associated with decreased cell death rather than increased cell cycle progression (Hemann and Lowe, 2006). Additionally, certain tumour-derived p53 mutants were shown to be impaired for apoptosis induction, but capable of promoting cell cycle arrest. Early studies also indicated that the pro-apoptotic activity of p53 correlated with its ability to function as a transcription factor, as tumour derived p53 mutants defective in their ability to bind DNA in a sequence-specific manner were also found to be impaired for apoptosis induction. Still, some studies suggested p53 may promote apoptosis through transcription-independent functions. It now appears that the primary action of p53 in

apoptosis is to directly and indirectly regulate the activity of the Bcl-2 family proteins (Belka and Budach, 2002; Hemann and Lowe, 2006).

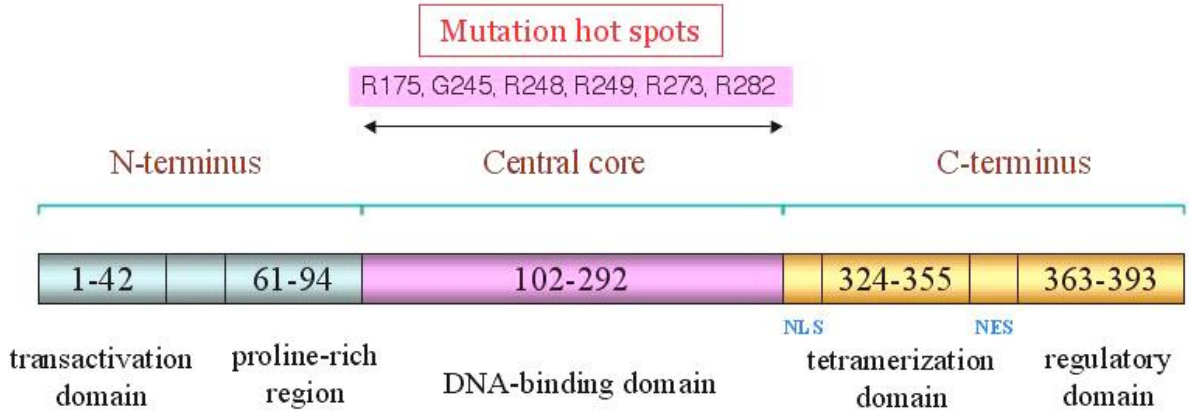


Figure 12: Schematic representation of the p53 structure, nuclear localization signal sequence (NLS) and nuclear export signal sequence NES; Ling and Wei-Guo, 2006).

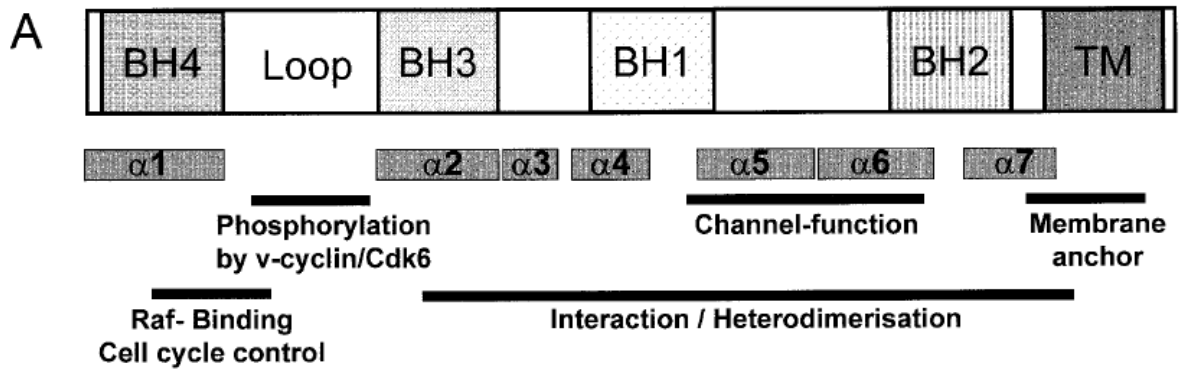


Figure 13: Structure of Bcl-2 protein (Belka and Budach, 2002).

Among various cellular responses induced by p53, most notable are the induction of cell cycle arrest and apoptosis. It appears that the ability of p53 to prevent cell growth is pivotal to its tumour suppressor functions. p53 can induce cell cycle arrest in the G1, G2 and S phases of the cell cycle (Agarwal *et al.,* 1995). The induction of cell cycle arrest at G1 and G2 by p53 provides additional time for the cell to repair genomic damage before entering the critical stages of DNA synthesis and mitosis. The arrested cells can be released back into the proliferating pool through p53's biochemical functions that facilitate DNA repair including nucleotide excision repair and base excision repair (Zhou *et al.,* 2001). A cyclin-dependent kinase (CDK) inhibitor, p21 is perhaps the best known downstream target of p53 among the various p53 target gene products identified. p21 is a primary mediator of p53-dependent G1 cell cycle arrest following DNA damage (Harper *et al.,* 1993; El-Deiry *et al.,* 1993; Xiong *et al.,* 1993). In response to cellular stresses (Figure 14), p53 up regulates endogenous p21 mRNA and protein levels (El-Deiry *et al.,* 1993). p21 binds cyclin-CDK complexes through the ZRXL motif (Adams *et al.,* 1996). Overexpression of p21 induces G1 arrest by blocking cyclin E/CDK2-mediated phosphorylation of Rb and release of E2F which functions to induce expression of genes required for S phase entry (Chen *et al.,* 1996).

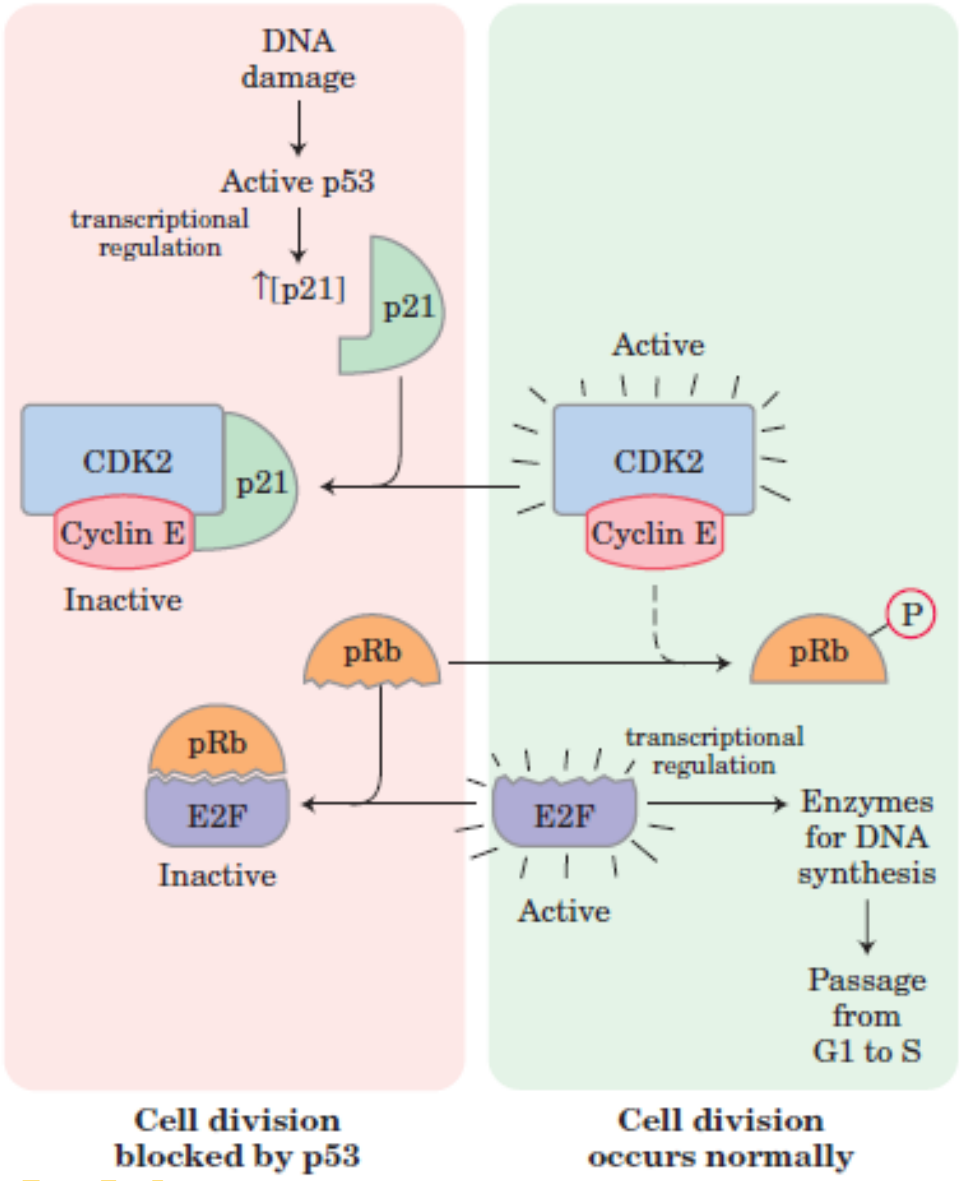


Figure 14: The role of p53 in cell cycle (David and Michael, 2005). pRb: retinoblastoma protein, CDK: cyclin dependent kinase.

As a cellular gatekeeper (Levine, 1997; Kinzler and Vogelstein, 1997), one of roles of p53 is to monitor cellular stress and to induce apoptosis as necessary (Hofseth *et al.*, 2004). In tissues where stressors generate severe and irrevocable damage, p53 can initiate apoptosis, thereby eliminating the damaged cells. p53 induces apoptosis mainly via two pathways: extrinsic and intrinsic pathways. The p53-associated extrinsic pathway is mainly executed by activating caspase 8 to induce apoptosis, whereas the p53- associated intrinsic pathway is almost executed by influencing mitochondrial proteins, by which activate caspase 9 to induce apoptosis. In addition, p53 may directly activate Apaf-1 (Figure 15) to induce apoptosis (Ling and Wei-Guo, 2006).

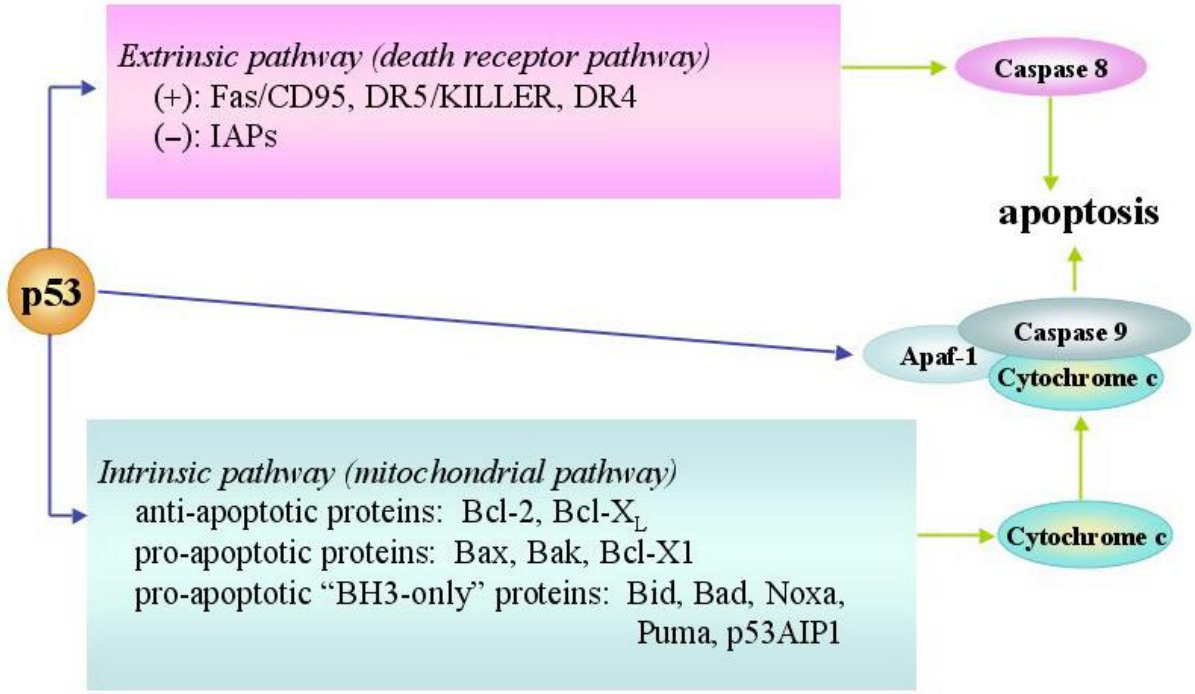


Figure 15: The p53-associated genes and pathways involved in apoptotic cell death (Ling and Wei-Guo, 2006).

In addition to its ability to promote the transcription of Bcl-2 antagonists, p53 employs additional strategies to regulate Bcl-2. For example (Figure 16), p53 can act to repress Bcl-2 transcription in some settings (Hemann and Lowe, 2006). Hence, introduction of p53 into certain p53 null cell lines results in repressed Bcl-2 gene expression, and induction of p53 using gamma-irradiation leads to reduced Bcl-2 expression in leukemia cells. Although the mechanism for such repression is not completely clear, the Bcl-2 promoter contains a p53-negative response element raising the possibility that Bcl-2 may be a direct target of p53-mediated transrepression (Hemann and Lowe, 2006). p53 may also directly impact Bcl-2 activity as part of a transcription-independent program of cell death. In this process, cytoplasmic p53 binds to pro-apoptotic Bcl-2-family proteins, leading to permeabilization of mitochondria and apoptosis. Consequently, p53, itself, may act like a BH3-only protein and antagonize Bcl-2 function. Interestingly, structural studies have shown that the DNA-binding domain of p53 is required for direct p53–Bcl-2 interaction. Thus, tumour-derived p53 mutants impaired for DNA binding may also be impaired for Bcl-2 interaction (Ling and Wei-Guo, 2006).

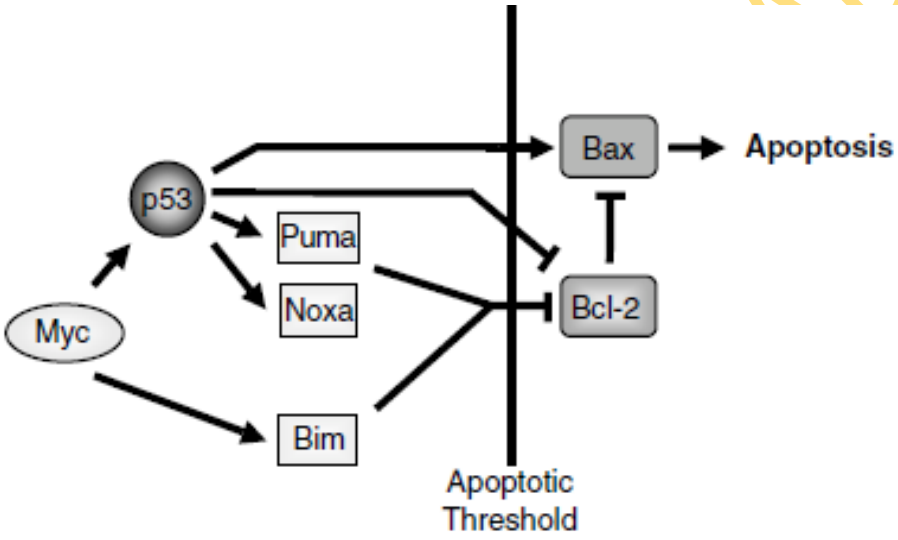


Figure 16: Overcoming the antiapoptotic threshold set by Bcl-2 family members (Hemann and Lowe, 2006).

2.6 Honey

Honey, a natural product formed from nectar by honeybees, has been a subject of renewed research interest in the last few years. It is a natural sweetener, but it is not just a sweetener it's a nature's gift to mankind. Its production is basically through a regurgitation mechanism from nectar or from the secretion of living part of plants. It is composed of at least 300 compounds and is basically a solution supersaturated in sugars, with fructose and glucose being the most concentrated. In addition, there is a great variety of minor components, including phenolics acids and flavonoids, different enzymes, carotenoids, organic acids and proteins (Anklam, 1998; Gheldof *et al.*, 2002; Tchoumboue *et al.*, 2007). The pure honey contains alkaloids, authraquinone glycosides, cardiac glycosides, flavonoids and reducing Compounds (Rakhi *et al*., 2010). Honey was the most popular Egyptian drug being mentioned 500 times in 900 remedies (Grossman, 1986). Whilst Hippocrates (3rd and 4th centuries BC) made little use of drugs in treatment he prescribed a simple diet, favouring honey given as oxymel (vinegar and honey) for pain, hydromel (water and honey) for 'thirst' (Guthrie, 1958; Adams, 1939).

2.6.1 Types of Honey

Honey are generally named after their floral source and these include: (i) Manuka honey (New Zealand and Australia), (ii) Pasture honey (New Zealand), (iii) Jelly bush honey (Australia), (iv) Jungle honey, (v) Chestnut honey, (vi) Rhododendron honey (New Zealand), (vii) Blossom honey (Australia and spain),(viii) AH (Asian and African continents; Hatice *et al*., 2010). Others are Neem honey (India, Nigeria) and Sage honey (USA).

2.6.2 Nutritional Aspects of Honey

2.6.2.1 Carbohydrates in Honey

The main sugars in honey are the monosaccharides fructose and glucose (Figure 17). Additionally, about 25 different oligosacharides have been detected (Doner, 1977; Siddiqui, 1970). The principal oligosaccharides in blossom honey are the disaccharides sucrose, maltose, trehalose and turanose, as well as some nutritionally relevant ones such as panose, 1-kestose, 6-kestose and palatinose. The amount of glucose, fructose and sucrose in % present in honey is 30.31 (White, 1980), 38.38 (White, 1980) and 1.31 respectively and amount of Reducing Sugars in %- 76.75 (White, 1978). Compared to blossom honey honeydew honey contains higher amounts of the oligosaccharides melezitose and raffinose. In the process of digestion after honey intake the principal carbohydrates fructose and glucose are quickly transported into the blood and can be utilized for energy requirements by the human body. A daily dose of 20 g honey will cover about 3% of the required daily energy (Singh *et al*., 2012).

2.6.2.2 Proteins, Enzymes and Amino Acids in Honey

Honey contains roughly 0.5% proteins, mainly enzymes and free amino acids. The contribution of that fraction to human protein intake is marginal. The three main honey enzymes are diastase (amylase), decomposing starch or glycogen into smaller sugar units, invertase (sucrase, α-glucosidase), decomposing sucrose into fructose and glucose, as well as glucose oxidase, producing hydrogen peroxide and gluconic acid from glucose. The amount of true protein is 168.6 mg/100g (White, 1962).

2.6.2.3 Vitamins, Minerals and Trace Compounds

The amount of vitamins and minerals is small and the contribution of honey to the recommended daily intake (RDI) of the different trace substances is marginal. It is known that different unifloral honeys contain varying amounts of minerals and trace elements (Bengsch, 1992). From the nutritional point of view chromium, manganese and selenium are important, especially for 1 to 15 years old children. The elements sulphur, boron, cobalt, fluoride, iodide, molybdenum and silicon can be important in human nutrition too, although there are no RDI values proposed for these elements (White, 1975; Conti, 2000; Terrab *et al*., 2004; Iskander, 1995; Rodriguez-Otero *et al*., 1994; Golob *et al*., 2005; Yilmaz and Yavuz, 1999; Bogdanov and Matzke, 2003; Heitkamp, 1984; Crane *et al*., 1984). Honey contains 0.3–25 mg/kg choline and 0.06 to 5 mg/kg acetylcholine (Heitkamp, 1984). Choline is essential for cardiovascular and brain function as well as for cellular membrane composition and repair, while acetylcholine acts as a neurotransmitter (Singh *et al*., 2012).

2.6.2.4 Aromatic Compounds, Taste-Building Compounds and Polyphenols

There is a wide variety of honey with different tastes and colours, depending on their botanical origin (Crane *et al*., 1984). The sugars are the main taste-building compounds. Generally, honey with a high fructose content (e.g. *acacia*) are sweeter compared to those with high glucose concentration (e.g. rape). The honey aroma depends also on the quantity and type of acids and amino acids present. In the past decades extensive research on aroma compounds has been carried out and more than 500 different volatile compounds were identified in different types of honey. Indeed, most aroma building compounds vary in the different types of honey depending on its botanical origin (Bogdanov *et al*., 2007). Honey flavour is an important quality for its application in

food industry and also a selection criterion for the consumer's choice. Polyphenols are another important group of compounds with respect to the appearance and the functional properties of honey. 56 to 500 mg/kg total polyphenols were found in different honey types (Al-Mamary *et al*., 2002; Gheldof and Engeseth, 2002). Polyphenols in honey are mainly flavonoids (e.g. quercetin, luteolin, kaempferol, apigenin, chrysin, galangin), phenolic acids and phenolic acid derivatives (Tomas-Barberan *et al*., 2001). These are compounds known to have antioxidant properties. The main polyphenols are the flavonoids, their content can vary between 60 and 460 µg/100 g of honey and was higher in samples produced during a dry season with high temperatures (Kenjeric *et al*., 2007).

2.6.2.5 Glycemic Index and Fructose

The impact of carbohydrates on human health has been the subject of controversy, especially the understanding of how the carbohydrates of a given food affect the blood glucose level. Today, the dietary significance of carbohydrates is often indicated in terms of the glycemic index (GI). Carbohydrates with a low GI induce a small increase of glucose in blood, while those with a high GI induce a high blood glucose level. The only comprehensive data on honey GI are the one presented in, based mainly on data of different Australian honeys (Arcot and Brand-Miller, 2007; Foster-Powell *et al*., 2002). There is a significant negative correlation between fructose content and GI, probably due to the different fructose/glucose ratios of the honey types tested. It is known that unifloral honeys have varying fructose content and fructose/glucose ratios (Persano and Piro, 2004). Some honeys, e.g. acacia and yellow box, with relatively high concentration of fructose, have a lower GI than other honey types. The GI values of four (4) honeys found in one study varied between 69 and 74 (Ischayek and Kern, 2006),

while in another one the value of a honey unidentified botanical origin was found to be 35 (Kreider *et al*., 2000). As the GI concept claims to predict the role of carbohydrates in the development of obesity (Ludwig, 2000), low GI honeys might be a valuable alternative to high GI sweeteners. In order to take into consideration the quantity of ingested food, a new term, the glycemic load, was introduced. It is calculated as follows: the GI value is multiplied by the carbohydrate content in a given portion and divided by 100. Values lower than 10 are considered low, between 10 and 20 are intermediate and above 20 belong to the category high. For an assumed honey portion of 25 g the glycemic load of most honey types is low and some types are in the intermediate range. The GI concept was developed to provide a numeric classification of carbohydrate foods, assuming that such data are useful in situations where the glucose tolerance is impaired (Singh *et al*., 2012). Therefore, food with a low GI should provide benefits with respect to diabetes and to the reduction of coronary heart disease (Jenkins *et al*., 2002). The consumption of honey types with a low GI e.g. AH might have beneficial physiological effects and could be used by type-2 diabetes patients (Al-Khalidi *et al*., 1980).

2.6.3 Experimental Evidence on the Novel Antioxidant Potential of Honey

2.6.3.1 Evidence from *in vitro* Studies

The *in vitro* antioxidant properties of natural or synthetic agents are measured in the form of antiradical activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, oxygen radical absorbance capacity (ORAC) assay and ferric reducing antioxidant power (FRAP) assay (Gheldof *et al*., 2002; Hussein *et al*., 2011). Using these tests, different varieties of honey from various countries and geographical regions have been shown to exhibit high antioxidant properties. Turkish red pine honey

produced by *Marchalina hellenica* was reported to scavenge DPPH effectively, suggestive of its antiradical activities (Akbulut *et al*., 2009). Some Saudi Arabian honey samples were demonstrated to exhibit antioxidant activities (Al-Hindi *et al*., 2011). Similar antioxidant properties were also reported for Peruvian honey (Rodríguez-Malaver *et al*., 2009). Australian honey produced by the stingless bees *Trigona carbonaria* was reported to have high antioxidant properties (Oddo *et al*., 2008). Malaysian tualang honey produced by the giant Asian bees *Apis dorsata* has been shown to exhibit good antioxidant and antiradical activities (Mohamed *et al*., 2009; Khalil *et al*., 2011; Kishore *et al*., 2011). Antioxidant activities have also been documented for American buckwheat honey (Van den Berg *et al*., 2008), Croatian oak honeydew honey (Jerkovic *et al.,* 2010), Spanish honey (Pérez *et al.,* 2007), Portugal honey (Estevinho *et al.,* 2008), Cuban honey (Alvarez-Suarez *et al.,* 2010), Venezuelan honey (Vit *et al.,* 2009) and Ecuadorian honey (Guerrinia *et al.,* 2009). The antioxidant activity of honey is generally attributed to its phenolic compounds and flavonoids (Khalil *et al*., 2011; Van den Berg *et al*., 2008; Beretta *et al.,* 2007; Kishore *et al.,* 2011). Findings from a recent study suggest that gamma irradiation may increase the antioxidant capacities and total phenolic contents in honey (Hussein *et al*., 2011). The main phenolic and flavonoids compounds in honey include (Figure 18) ellagic acid, gallic acid, syringic acid, benzoic acid, cinnamic acid, ferulic acids, myricetin, chlorogenic acid, caffeic acid, hesperetin, coumaric acid, isoramnetin, chrysin, quercetin, galangin, luteolin and kaempferol (Kassim *et al.,* 2010; Hussein *et al*., 2011; Eraslan *et al.,* 2010; Petrus *et al.,* 2011). While some of these bioactive compounds such as alangin, kaempferol, quercetin, isorhamnetin and luteolin are found in most honey samples, others such as hesperetin and naringenin are found in few honey varieties (Petrus *et al.,* 2011). Among some Malaysian honey samples investigated, catechin was

found to be common flavonoids (Khalil *et al*., 2011). By and large, the avalanche of data on the *in vitro* antioxidant activities of honey indicate that honey is not only an antioxidant but also a rich source of antioxidants (Omotayo *et al*., 2012).

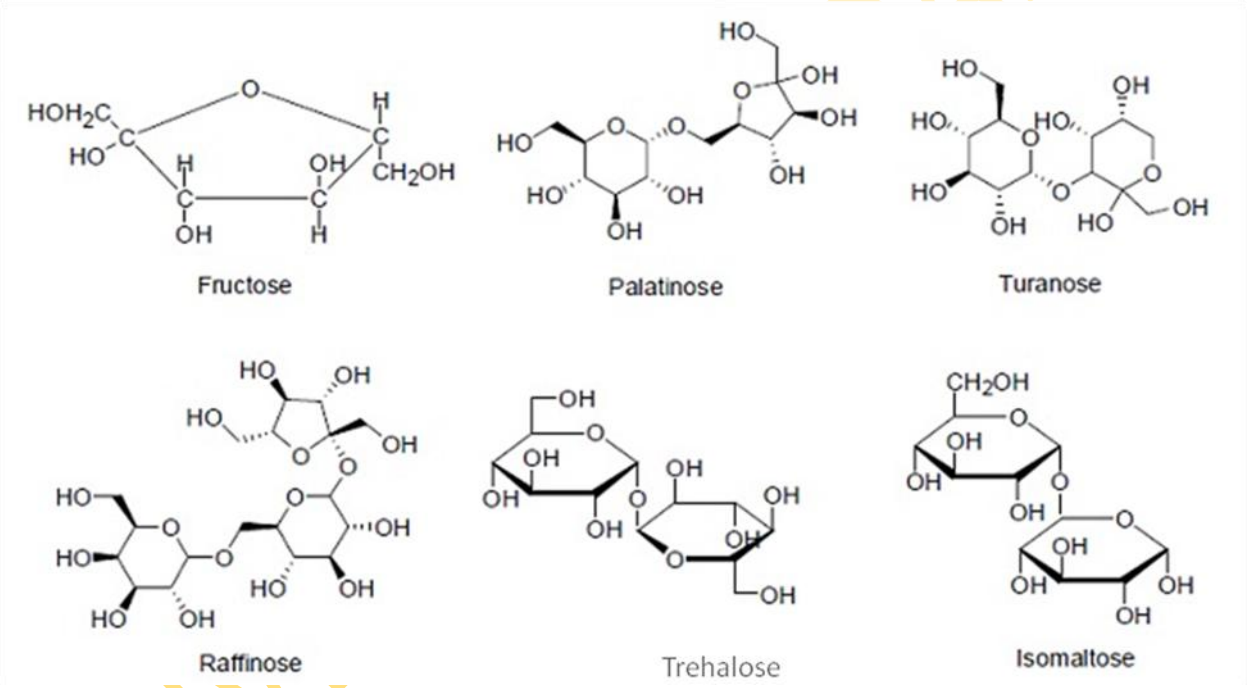


Figure 17: Structures of fructose and oligosaccharides found in honey (Omotayo *et al*., 2012).

Furthermore, it is paramount to note that most of phenolics and/or flavonoids (Figure 18) are basically required in low concentration because they are antioxidants in nature. At higher concentration, they attend maximum stability by resonance delocalisation (Yu, 1994; Halliwell and Gutteridge, 1988).

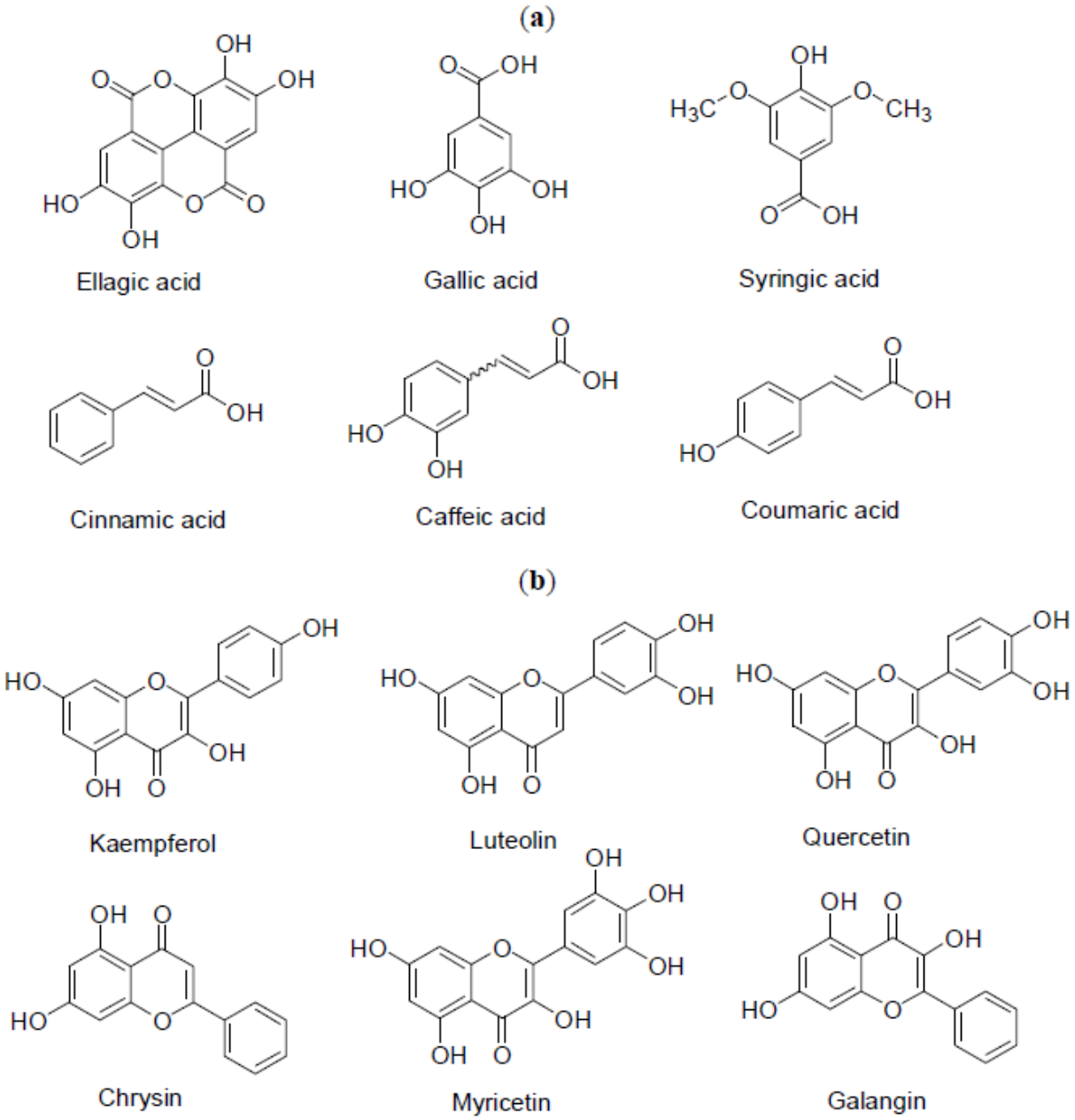


Figure 18: Phenolics and flavonoids isolated from honey (Omotayo *et al*., 2012)

2.6.3.2 Evidence from *in vivo* Studies

Honey, like any other antioxidant agents, confers a protection against damage or injury. This protective effect of honey is partly mediated via amelioration of oxidative stress in tissues such as GIT, liver, kidney, pancreas, eye, plasma, red blood cells and reproductive organs (Gharzouli *et al.*, 2002; Al-Waili *et al.*, 2006; Mohamed *et al.*, 2011; Zaid *et al.*, 2011; Erejuwa *et al.*, 2010; Kassim *et al.*, 2010).

2.6.3.2.1 Gastro Intestinal Tract (GIT)

Evidence indicates that in diseased conditions such as diabetes mellitus, the GIT is susceptible to oxidative stress, which may impair the brush border membrane (BBM) fluidity (Bhor and Sivakami, 2003). The intestinal uptake of substances, molecules or mineral ions is also known to be modulated or influenced by the redox state of the transporter (Faria *et al.*, 2006). These transporters can be regulated rapidly through alteration of their trafficking or affinities in response to oxidative stress (Faria *et al.*, 2009). The beneficial effect of GSH, an antioxidant, on these transporters has been demonstrated (Faria *et al.*, 2009). It is suggested that such modification of redox or oxidative state might influence the bioavailability of essential macronutrients and drugs (Faria *et al.*, 2009). Antioxidant vitamin E was reported to increase the activities of CAT and SOD and reduce lipid peroxidation and protein oxidation in small intestine of diabetic rats (Shirpoor *et al.*, 2007). At the moment, there is no evidence to suggest that honey exerts an antioxidant effect in the intestine. However, considering the reported antioxidant effect of honey in different cells or tissues such as pancreas, serum, kidney and liver, it is possible that honey might also ameliorate intestinal oxidative stress (Omotayo *et al.*, 2012). Hence, it may not be a surprise that co-administration of hypoglycemic agents and honey further improves glycemic control in diabetic rats.

(Erejuwa *et al.*, 2011). This might be due to modification of intestinal redox or oxidative state which influences the bioavailability of hypoglycemic drugs (Faria *et al.*, 2009). Available evidence indicates that honey exerts gastroprotective effect in rodents administered indomethacin, ethanol, aspirin or ammonia (Gharzouli *et al.*, 2002; Ali *et al.*, 1990; Gharzouli *et al.*, 2001; Mobarok, 2003). Although the sugars such as fructose, glucose, sucrose and maltose present in honey may play a role in its gastroprotective effect (Gharzouli *et al.*, 2002; Gharzouli *et al.*, 2001), there is a possibility that the antioxidant effect of honey may also contribute to its gastroprotective effect. This assertion is based on the evidence which indicates that increased mucosal lipid peroxide and reduced GSH levels exacerbate gastric hemorrhagic ulcer in diabetic rats (Hung, 2005). Another study also reported increased lipid peroxidation and impaired antioxidant enzymes (increased SOD and decreased CAT activities) in gastric mucosa of rats with cold restraint stress-induced gastric ulceration (Tandon, 2004). A similar increase in oxidative stress level was also demonstrated in patients with peptic ulceration and gastric carcinoma (Tandon, 2004). Additional evidence in support of the role of antioxidant effect of honey in mediating its gastroprotective effect is provided by Kim (Kim, 2005). The author found that oxidative stress is one of the mechanisms by which *Helicobacter pylori* induces gastric injury (Kim, 2005). Interestingly, honey also inhibits the growth of *Helicobacter pylori*, which causes gastric and duodenal ulcers (Ali *et al.*, 1991; Nzeako and Al-Namaani, 2006). Considering the prolonged gastric ulcer healing, the prevalence of silent gastric ulcer or erosions and the highly oxidative milieu in diabetes mellitus (Harsch *et al.*, 2003; Naito *et al.*, 2009; Boehme *et al.*, 2007), the antioxidant-mediated gastroprotective effect of honey might be beneficial in diabetes mellitus. Honey might also be valuable in ulcerative colitis (Medhi *et al.*, 2008). In trinitrobenzosulphonic acid (TNBS)-induced ulcerative colitis model in rats,

honey combined with an antibiotic (sulfasalazine) enhanced antioxidant defense system and reduced oxidative damage and colonic inflammation (Medhi *et al.*, 2008). Similarly, in rats with chemically (TNBS)-induced colitis, honey supplementation significantly reduced the colonic mucosal malondialdehyde (MDA) content (Bilsel *et al.*, 2002). In a nutshell, amelioration of oxidative stress as a result of honey administration might restore impaired intestinal brush border membrane (BBM) fluidity and redox or oxidative state. This might modify or modulate the bioavailability of vital macronutrients and drugs. This antioxidant effect in the GIT might also contribute to the gastroprotective effect of honey.

2.6.3.2.2 Liver

The liver plays an important role in many metabolic processes such as glycemic control, detoxification of xenobiotics, synthesis of lipoproteins, hormones and enzymes (Klip and Vranic, 2006). In diabetes mellitus, the liver is associated with abnormalities such as elevations in serum Aspartate aminotransferase, alkaline phosphatase and alanine aminotransferase (Leeds *et al.*, 2009). Available evidence suggests that the liver is susceptible to oxidative stress and damage; and the beneficial effect of antioxidants on hepatic oxidative stress has been documented (Dias *et al.*, 2005; Gumieniczek, 2005). In the liver of young and middle-aged rats, honey supplementation was reported to restore activities of CAT and GPx (Yao *et al.*, 2011). In male BALB/c mice administered trichlorfon, oral supplementation with pine honey restored the activities of hepatic GPx (significantly) and SOD and CAT (moderately) and reduced hepatic damage (Petrus *et al.*, 2011). Similar hepatoprotective effect of honey was also reported in STZ-induced diabetic rats (Erejuwa *et al.*, 2012) and sheep administered carbon tetrachloride (CCl_4; Al-Waili, 2003). In rats with obstructive jaundice, usually associated with increased

hepatic ROS formation, oxidative stress and inflammation (Celebi *et al.*, 2004; Liu *et al.*, 2001), honey supplementation significantly reduced the levels of MDA and increased GSH content in the liver (Kilicoglu *et al.*, 2008). The amelioration of oxidative stress, as a result of honey administration, was accompanied by significant reductions in the size of enlarged hepatocytes and edema, restoration of bile canaliculi dilatation and reduced number of apoptotic cells (Kilicoglu *et al.*, 2008). Similar hepatoprotective effect of honey was also reported in rats with obstruction of the common bile duct (Erguder *et al.*, 2008). In rats with N-ethylmaleimide (NEM)-induced liver injury, honey supplementation significantly restored the levels of hepatic glutathione, ameliorated the (NEM)-induced congestion and mononuclear cell infiltration in the liver (Korkmaz and Kolankaya, 2009). Supplementation with honey and ginseng was reported to protect against CCl_4-induced hepatotoxicity in rats by reducing lipid peroxidation and enhancing antioxidant capacity (El Denshary *et al.*, 2012). These findings, generally, suggest that amelioration of oxidative stress in the liver may contribute to the hepatoprotective effect of honey.

2.6.3.2.3 Pancreas

The pancreatic β-cells are highly vulnerable to oxidative stress as a result of their intrinsically low expressions and activities of free radical scavenging enzymes (Grankvist *et al.*, 1981). The beneficial effect of antioxidants in protecting the pancreas against oxidative stress and damage is well documented (Palsamy and Subramanian, 2010). Honey has a potential to protect pancreas against oxidative stress and damage. Honey supplementation significantly reduced elevated levels of MDA and restored the activities of SOD and CAT in pancreas of diabetic rats (Erejuwa *et al.*, 2010a). The pancreas of diabetic rats treated with the combination of glibenclamide and honey had

increased CAT activity and restored the elevated GPx activity and levels of MDA (Erejuwa *et al.*, 2011). In a related study, the combination of glibenclamide and metformin ameliorated oxidative stress only partially (Erejuwa *et al.*, 2010b). This was evidenced by the attenuation of GPx activity only while no significant effect on other antioxidant enzymes and lipid peroxidation was observed in the pancreas of diabetic rats (Erejuwa *et al.*, 2010b). In contrast, the combination of glibenclamide, metformin and honey significantly increased CAT activity while GPx activity was down-regulated. The combination with honey also considerably prevented lipid peroxidative damage (Erejuwa *et al.*, 2010b). These data clearly suggest that the combination of hypoglycemic agents with honey markedly restores antioxidant enzymes, ameliorates oxidative stress and protects the pancreas against oxidative damage.

2.6.3.2.4 Hypertension

The role of oxidative stress in hypertension is a subject of much research interest. Oxidative stress is implicated in the pathogenesis of hypertension (Rodrigo *et al.*, 2011), while some evidence also indicates that hypertension generates oxidative stress (Lopes *et al.*, 2011). These lines of evidence support a role of oxidative stress as an important determinant in the imbalance between vasoconstrictor and vasodilator mechanisms (Rodrigo *et al.*, 2011; Lopes *et al.*, 2011; Erejuwa *et al.*, 2012). The beneficial effects of antioxidants in ameliorating oxidative stress and suppressing or reducing elevated blood pressure in experimental and clinical studies further corroborate the role of oxidative stress in hypertension (Houston, 2011). In a recent study, it was reported that honey supplementation in spontaneously hypertensive rats (SHR) restored the elevated antioxidant defenses (GST, TAS and CAT) in kidney of SHR (Erejuwa *et al.*, 2012). Honey administration also prevented the formation of MDA in the kidney of SHR

(Erejuwa *et al.,* 2012). The amelioration of oxidative stress was accompanied by suppressed elevations in blood pressure in SHR (Erejuwa *et al.,* 2012).

2.6.3.2.5 Reproductive Organs

The exposure to cigarette smoke (CS) causes apoptosis and damage in the testis (Rajpurkar *et al.,* 2002). Evidence implicates the role of oxidative stress in CS-induced testicular damage (Rajpurkar *et al.,* 2000). A number of studies have demonstrated the beneficial effects of antioxidants in preventing or ameliorating testicular damage in rodents (Agarwal *et al.,* 2005). A study investigated the effect of honey in the testis of rats exposed to CS (Mohamed *et al.*, 2011). It was found that honey supplementation for 13 weeks markedly reduced the level of lipid peroxidation (Mohamed *et al.,* 2011). Honey administration also increased the reduced TAS and restored the activities of SOD, GPx and CAT. This antioxidant effect of honey was associated with amelioration of testicular damage as evidenced by higher Leydig cell count, reduced percentage of tubules with germ cell loss, larger seminiferous tubules diameter and epithelial height (Mohamed *et al.*, 2011). These data suggest that honey may protect or ameliorate CS-induced testicular damage in rats via its antioxidant effect. The authors in one of their previous studies also reported that honey supplementation in normal rats improved spermatogenesis (Mohamed *et al.,* 2011). A recent study also demonstrated the beneficial effects of honey on sperm motility and morphology in rats (Asiyah *et al.*, 2011). A study by Abdul-Ghani and colleagues also indicated that honey supplementation in rats caused increased epididymal sperm count and improved the activity of testicular marker enzymes for spermatogenesis, as evidenced by increased sorbitol dehydrogenase and reduced lactate dehydrogenase (Abdul-Ghani *et al.,* 2008). Available data in ovariectomised female rats also suggest that honey may produce

beneficial effects in female reproductive organs (Zaid *et al.*, 2011). A similar beneficial effect of honey on oxidative stress was also reported in human subjects (Tartibian *et al.*, 2011). A study investigated the effects of 8-week honey supplementation on seminal plasma cytokines, oxidative stress and antioxidants in male road cyclists during intensive cycling training (Tartibian *et al.*, 2011). The study found that honey supplementation significantly increased the concentrations of seminal SOD, CAT and TAS. This antioxidant effect of honey was also associated with lower elevations in the seminal IL-1beta, IL-6, IL-8, TNF-alpha, ROS and MDA levels (Tartibian *et al.*, 2011).

2.6.3.2.6 Eye

Visual impairment caused by alkali burns of the corneal and conjunctival surface is considered one of the most devastating injuries to the eye (Gakhramanov, 2005; Gakhramanov *et al.*, 2006). These chemical burns-induced eye injuries are accompanied by increased oxidative stress (Gakhramanov, 2005; Gakhramanov *et al.*, 2006). The beneficial effects of antioxidants in the treatment of these eye injuries have been reported (Gakhramanov, 2005; Gakhramanov *et al.*, 2006). A study investigated and compared the antioxidant effects of honey and conventional treatment in alkali injury on the eyes of New Zealand White rabbits (Bashkaran *et al.*, 2011). The study did not find any significant difference in TAS and MDA levels in aqueous humour, vitreous humour and serum of rabbits treated with honey and conventional treatment (Bashkaran *et al.*, 2011). The lack of significant effect of honey on oxidative stress parameters might be a result of the short duration of the study (7 days). Other possible explanations include non-inclusion of control and small sample size. Therefore, considering the previous data on the beneficial effects of antioxidants in chemical burns-induced eye injuries in animals (Gakhramanov, 2005; Gakhramanov *et al.*, 2006), together with the limitations

of this recent study (Bashkaran *et al.*, 2011), it is premature to conclude that honey is not beneficial in the treatment of chemical burns-induced eye injuries in animals. This is in view of the fact that honey has been shown to be beneficial in other eye diseased conditions such as in human patients with dry eye syndrome (Jankauskiene *et al.*, 2007) or endophthalmitis (Cernak *et al.*, 2011).

2.6.3.2.7 Inflammation

Oxidative stress and inflammation are frequent manifestations and play an important role in the etiology of many diseases and disorders (Kilicoglu *et al.*, 2008; Korkmaz *et al.*, 2009; Peake *et al.*, 2007). Evidence indicates that they are intimately interrelated as each can cause the other (Kilicoglu*et al.*, 2008; Korkmaz *et al.*, 2009; Peake *et al.*, 2007). In rats with inflammatory bowel disease, intra-rectal honey administration significantly reduced myeloperoxidase (MPO) activity (Bilsel *et al.*, 2002). This was associated with lower levels of colonic MDA with no change in NO content (Bilsel *et al.*, 2002). A recent study that investigated the effects of honey and its extracts in rat models of inflammation reported that honey and its extracts inhibited NO and prostaglandin E (2) production (Kassim *et al.*, 2011). The authors also found that honey and its extracts reduced edema and pain in inflammatory tissues. The inhibition of edema and pain was found to correlate with the inhibition of nitric oxide and prostaglandin E (2) (Kassim *et al.*, 2011). Another study investigated the effects of various doses of honey on acute and chronic inflammations in rats using carrageenan, cotton pellet and formaldehyde methods and NO production by administering NG-nitro-L-arginine methyl ester (L-NAME) and L-arginine (Owoyele *et al.*, 2011). Honey supplementation was found to reduce the paw size, the granuloma weight and the arthritis in the carrageenan, the cotton pellet and formaldehyde methods, respectively

(Owoyele *et al.*, 2011). Additional evidence in support of the anti-inflammatory effect of honey was demonstrated by inhibition of paw oedema by L-NAME and the loss of anti-inflammatory effect of honey following the administration of L-arginine (Owoyele *et al.*, 2011). These data indicate that honey can exert an anti-inflammatory effect via inhibition of NO and prostaglandin E (2) production and release (Owoyele *et al.*, 2011). This antioxidant effect may contribute to its anti-inflammatory effect.

2.7 White Blood Cells

White blood cell**,** also called leukocyte or white corpuscle, a cellular component of the blood that lacks hemoglobin, has a nucleus, is capable of motility, and defends the body against infection and disease by ingesting foreign materials and cellular debris, by destroying infectious agents and cancer cells, or by producing antibodies (LaFleur-Brooks, 2008). A healthy adult human has between 4,500 and 11,000 white blood cells per cubic millimetre of blood. Fluctuations in white cell number occur during the day; lower values are obtained during rest and higher values during exercise. Intense physical exertion may cause the count to exceed 20,000 per cubic millimetre. White cell count also may increase in response to convulsions, strong emotional reactions, pain, pregnancy, labour, and certain disease states, such as infections and intoxications. Although white cells are found in the circulation, most occur outside the circulation, within tissues, where they fight infections; the few in the bloodstream are in transit from one site to another. As living cells, their survival depends on their continuous production of energy. The chemical pathways utilized are more complex than those of red blood cells and are similar to those of other tissue cells. White cells, containing a nucleus and able to produce ribonucleic acid (RNA), can synthesize protein (Gartner and Hiatt, 2007). White cells are highly differentiated for their specialized functions,

and they do not undergo cell division (mitosis) in the bloodstream; however, some retain the capability of mitosis. On the basis of their appearance under a light microscope, white cells are grouped into three major classes—lymphocytes, granulocytes, and monocytes—each of which carries out somewhat different functions (http://www.britannica.com/EBchecked/topic/337728/white-blood-cel)

2.7.1 Granulocytes

Granulocytes, the most numerous of the white cells, rid the body of large pathogenic organisms such as protozoans or helminths and are also key mediators of allergy and other forms of inflammation (Henson, 1972). These cells contain many cytoplasmic granules, or secretory vesicles, that harbour potent chemicals important in immune responses. They also have multilobed nuclei, and because of this they are often called polymorphonuclear cells. On the basis of how their granules take up dye in the laboratory, granulocytes are subdivided into three categories: neutrophils, eosinophils, and basophils. The most numerous of the granulocytes—making up 50 to 80 percent of all white cells—are neutrophils. They are often one of the first cell types to arrive at a site of infection, where they engulf and destroy the infectious microorganisms (Malech *et al.*, 2014) through a process called phagocytosis (Jacobs *et al.*, 2010). Eosinophils and basophils, as well as the tissue cells called mast cells, typically arrive later. The granules of basophils and of the closely related mast cells contain a number of chemicals, including histamine and leukotrienes that are important in inducing allergic inflammatory responses. Eosinophils destroy parasites and also help to modulate inflammatory responses ((http://www.britannica.com/EBchecked/topic/337728/white-blood-cel).

2.7.2 Monocytes

Monocytes, which constitute between 4 and 8 percent of the total number of white blood cells in the blood, move from the blood to sites of infection, where they differentiate further into macrophages (Swirski *et al*., 2009).These cells are scavengers that phagocytose whole or killed microorganisms and are therefore effective at direct destruction of pathogens and cleanup of cellular debris from sites of infection (http://www.britannica.com/EBchecked/topic/337728/white-blood-cel). Neutrophils and macrophages are the main phagocytic cells of the body, but macrophages are much larger and longer-lived than neutrophils. Some macrophages are important as antigen-presenting cells, cells that phagocytose and degrade microbes and present portions of these organisms to T lymphocytes, thereby activating the specific acquired immune response (Rita *et al*., 2011). Specific types of cells are associated with different illnesses and reflect the special function of that cell type in body defense. A fall in white cell count, which is called leukopenia, occurs in states such as debilitation, anaphylaxis, and overwhelming infection. In general, newborns have a high white blood cell count that gradually falls to the adult level during childhood. An exception is the lymphocyte count, which is low at birth, reaches its highest levels in the first four years of life, and thereafter falls gradually to a stable adult level (http://www.britannica.com/EBchecked/topic/337728/white-blood-cel).

2.7.3 Lymphocytes

Lymphocytes, which are further divided into B and T cells, are responsible for the specific recognition of foreign agents and their subsequent removal from the host. B lymphocytes secrete antibodies, which are proteins that bind to foreign microorganisms in body tissues and mediate their destruction. Typically, T cells recognize virally

infected or cancerous cells and destroy them, or they serve as helper cells to assist the production of antibody by B cells (Sahar *et al*., 2011). Also included in this group are natural killer (NK) cells, so named for their inherent ability to kill a variety of target cells. In a healthy person, about 25 to 33 percent of white blood cells are lymphocytes (www.britannica.com/EBchecked/topic/337728/white-blood-cel).

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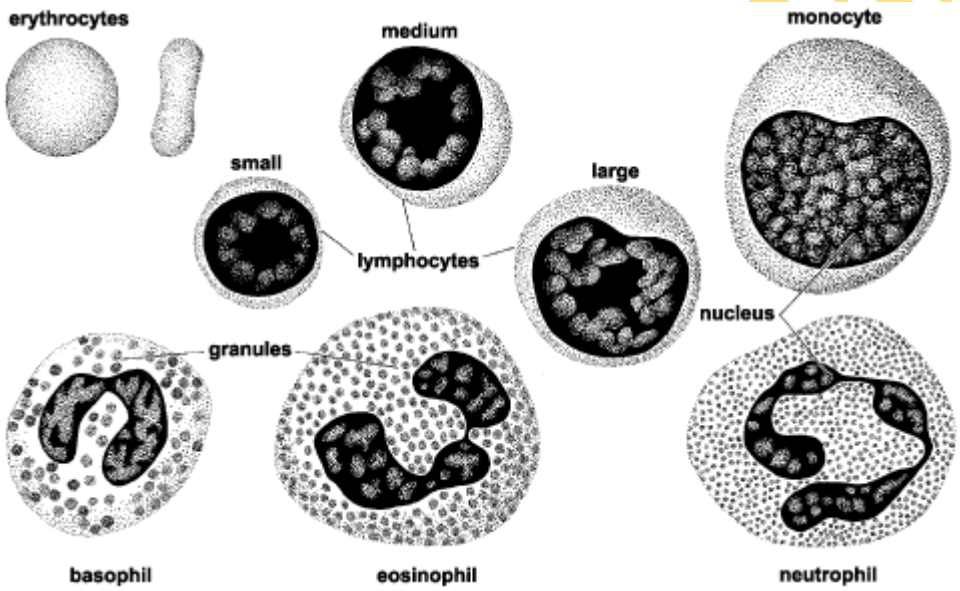

Figure 19: Diagrammatic representation of human blood cells (http://www.answers.com/topic/blood).

2.7.4 Reactive Oxygen Species Generation in the Blood

Oxidative stress refers to pathological condition of variety of cells in which overproduction of reactive oxygen species (ROS) may occur. The function of ROS production is to kill or oxidize harmful pathogens, however imbalance of ROS levels may damages the cell components including proteins, lipids and DNA resulting cell death (Halliwell, 1989). ROS includes superoxide anions ($O_2^{\cdot-}$) hydrogen peroxide (H_2O_2), hydroxyl free radical (·OH) and hypochlorite (HOCl) (Firdovich, 1978). The ROS formation occurs mainly in white blood cells (neutrophils, monocytes and macrophages) and catalyzed by membrane bound NADPH oxidase and myeloperoxidase (MPO) however; it can also be catalyzed by exposure to UV light or heat. NADPH oxidase, xanthine oxidase and cytochrome P-450 donate an electron to the O_2 (oxygen molecule) to form superoxide anions ($O_2^{\cdot-}$) which is unstable and spontaneously converted into hydrogen peroxide (H_2O_2) and hydroxyl free radical (·OH). Myloperoxidase released by lysosomal granules coverts H_2O_2 into hypochlorite (HOCl) which is most toxic molecule and leads to pathology of many inflammatory diseases via peroxidation of lipids, proteins and DNA (Helfand *et al*., 1982). Reactive oxygen species ROS are believed to be mediators of inflammation and responsible for the pathogenesis of acute as well as chronic inflammatory diseases (Halliwell and Gutteridge, 1999) aging and cancer (Waris and Ahsan, 2006), Parkinson's disease (Jenner and Olanow, 1996) Alzheimer's disease (Manton *et al*., 2004), atherosclerosis (Halliwell, 1989).

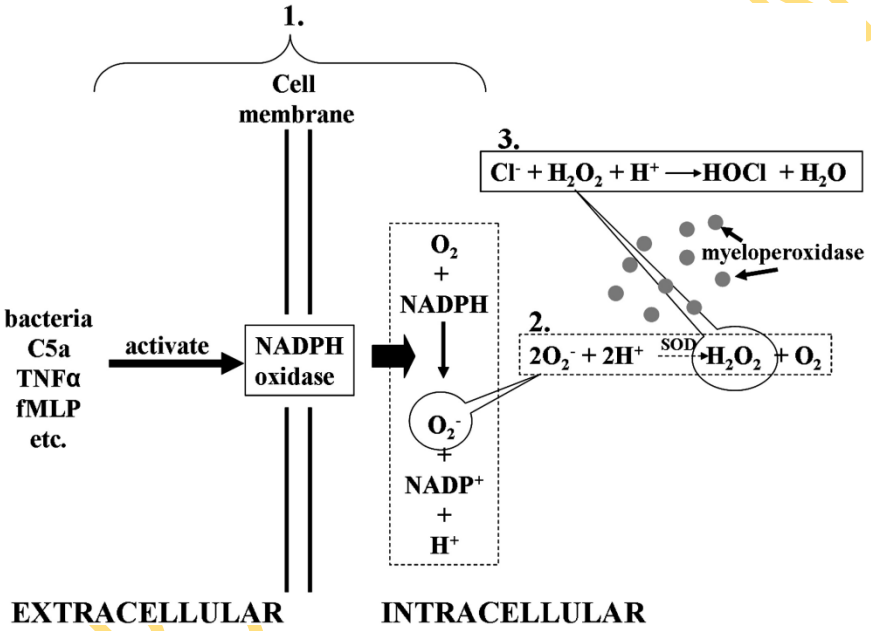

Figure 20: Diagrammatic representation for the production of reactive oxygen species in the blood (Fung and Silliman, 2009). Activated complement 5 (C5a); N-formyl-Met-Leu-Phe (fMLP); tumour necrosis factor alpha (TNF-α).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Samples Collection

Honey sample was collected from the Northwest frontier of Pakistan (N.W.F.P) during the month of February, 2012 or from Achida town, Sokoto State, Nigeria during December, 2012 from *Acacia modesta/nolitica* flower, identified and maintained at 4^{o}C until analysis at the industrial analytical centre, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan or Department of Biochemistry, Ahamadu Bello University Zaria, Nigeria. Human blood samples were collected from apparently healthy volunteers (aged 25-35 years) based on the ethical clearance. The volunteers were well informed about the significance of the study and without exposure to higher concentration of honey prior to the commencement of the experiment. The cell lines (NCI-H460, PC-3 and NIH/3T3) were generously provided by Dr. Ahmed M. Mesaik and Mr. Salman A. Khan all from Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan.

3.1.1 Liquid-Liquid Fractionation of Honey

Exactly 50 g of honey was weighed and dissolved in 500 mL distilled water, thereafter partitioned by liquid-liquid fractionation using dichloromethane and ethylacetate separately based on the distribution ratio/partition coefficient. The fractions were collected and concentrated using rotary evaporator. The aqueous fraction was later freeze-dried. The % yield of aqueous, dichloromethane and ethyl acetate fractions were 26, 5 and 3 % respectively. The fractions were maintained at -20 ^{o}C until analysis.

3.2 Experimental Animals

Male Wistar albino rats weighing 112 – 200 g and mice 25-30 g were used for the present investigation. They were reared at the animal house of the Department of Pharmaceutical Sciences, Faculty of Pharmacy, Ahmadu Bello University, Zaria, Nigeria or the International Center for Chemical and Biological Sciences, ICCBS, University of Karachi, Karachi, Pakistan with the approval of animal rights review committee. They were acclimatized for 1 week on normal diet of pelletized mouse chow, with water given *ad libitum* at room temperature within a 12-hrs light and dark cycle before the commencement of each experiment.

3.2.1 Collection of Brain and liver from Normal Rats for *in vitro* Studies

The normal male albino rats were collected from the animal house of International Centre of Chemical and Biological Sciences, University of Karachi, Pakistan with the approval of Animal right review committee. They were humanely sacrificed by cervical dislocation. Brain and liver tissues were collected, homogenized in 1:5 of 0.15 M Tris-KCl buffer (pH 7.4). The supernatant was collected after centrifugation at 3500 ×g at -4^{o}C in a refrigerated centrifuge for 5 mins and kept at – 80 ^{o}C until analysis. α-tocopherol at the concentration of 0.5 mg/mL as positive control and 4 % (v/v) AH were used for lipid peroxidation, Superoxide Dismutase (SOD) and Catalase activity assays.

3.2.2 Collection of Tissues and Blood Samples for *in vivo* Studies

Twenty four (24) hrs after the last administration and overnight fasting, the rats were humanely sacrificed using 60 mg/kg body weight of sodium pentothal. Blood, brain and liver tissues were collected. Tissues were homogenized in 1:5 of 0.9 % sodium chloride/phosphate buffer (pH 7.4). The supernatant and blood serum were collected after centrifugation at 3500 ×g at 4^{o}C in a refrigerated centrifuge for 10 mins and kept at

– 80 ^{o}C until analysis. Part of the whole blood samples were placed in EDTA-container for complete blood counts. Each time the supernatant/serum was outside the freezer, it was kept in ice bags. The total protein content of the serum and study tissues were determined using Auto Analyzer Hitachi Roche 7020 (902), Japan Inc. according to manufacturer's protocols.

3.3 Isolation of Neutrophils from Human Whole Blood

Venous blood was collected from apparently healthy adult volunteers and neutrophils were isolated by dextran sedimentation and density gradient centrifugation as described by El Benna *et al*., (1994); and Dewas *et al*., (2000). Erythrocytes were removed by hypotonic lysis. Following isolation, the cells were resuspended in appropriate medium, such as Hank's balanced salt solution (HBSS). The viability of the cells was assess using 0.4 % trypan blue for 5min and percent cell viability was calculated as: Cell viability (%) = (Number of viable cells/Total no. of cells) × 100 (Fernandez-Botlan and Vetvick, 1995). The viability was >90 %.

3.4 Isolation of Macrophages from Mice

The method of Ray and Dittel (2010) was adopted. Mice weighing 25-30 g were collected from the Animal house of International Centre for Chemical and biological sciences (ICCBS). Animals were kept under standard conditions in plastic cages, receiving a commercial standard diet and water was given *ad libitum*. 1mL heat inactivated fetal bovine serum (FBS) was injected into the peritoneum cavity of mice using sterile 1 mL syringe and thereafter, they were kept for 72 hour. At 72 hour the animals were humanely sacrificed by cervical dislocation and the whole body sterilized by dipping into 70 % ethanol. 10 mL of 10% RPMI 1640 media without antibiotic was injected into the peritoneum cavity and massage for two mins. The lower part of the

peritoneum cavity was cut and the injected RPMI 1640 media was collected using a sterilized syringe and kept on ice. The mixture were centrifuged at 400 × g for 20 mins at 4^{o}C, supernatant discarded and the pellet washed with the media and further centrifuged at 300 × g for 10 mins at 4 ^{o}C. The pellet was then re-suspended in 1 mL RPMI1640/HBSS. The viability of the cells was assess using 0.4 % trypan blue for 5min and percent cell viability was calculated as: Cell viability (%) = (Number of viable cells/Total no. of cells) × 100 (Fernandez-Botlan and Vetvick, 1995). The viability was >90 %.

3.5 Quality Assessment of Honey

Principle

This is based on the reaction between hydroxylmethylfurfural and $NaHSO_3$ in a particular food substance like honey which gives information about adulteration and storage (AOAC, 2006).

Reagents

Carrez I and Carrez II solutions, $NaHSO_3$, soluble starch, concentrated HNO_3, HCl, methanol, propanol, helium gas, 5 % diphenyl/95 % dimethyl polysiloxane, 10 % aluminum chloride, 1 M potassium acetate, quercetin, garlic acid, Folin-Ciocalteau and 20 % sodium carbonate were purchased from Sigma Aldrich, USA.

Determination of Hydroxylmethylfurfural (HMF)

Procedure

Five gram (5 g) of honey was dissolved in 25 mL of distilled water, treated with a clarifying agent (0.5 mL of Carrez I and 0.5 mL of Carrez II solutions) and volume made up to 50 mL. The solution was filtered, and the first 10 mL discarded. The

absorbance of the filtered solution was measured at 284 and 336 nm against an aliquot of the filtered solution treated with $NaHSO_3$. HMF was determined as:

mg of HMF/100g of honey = $(A_{284nm} - A_{336nm}) \times 14.97 \times 5$/g of test sample

Principle

This based on the ability of the honey sample's diastase activity/number (DN) to hydrolyse starch. The diastase is normally calculated using the time taken for the absorbance to reach 0.235, and the results were expressed in Gothe degrees as the amount (mL) of 1 % starch hydrolyzed by an enzyme in 1 g of honey in 1hour.

Determination of Diastase Activity/Number (DN)

Procedure

Diastase activity was determined using a buffered solution of soluble starch and honey incubated in a thermostatic bath at 40 ^{0}C. Thereafter, 1 mL aliquot of this mixture was removed at 5 min intervals and the absorption of the sample was followed at 660 nm. The DN was calculated as follows:

DN (units/g of honey) = 28.2 × change in A_{660nm} + 2.64

Determination of Sugar Contents

The serial dilution of 5.0, 10.0 and 20.0 % (v/v) of honey were made and the contents of the sugar were determined using refractometer based on the application of Snell's law. The average was calculated.

Determination of Mineral Elements

The mineral elements calcium, iron, potassium, magnesium and zinc were determined using Atomic Absorption Spectrometry (AAS) machine according to manufacturer's

protocol. Briefly, to 1 g of honey concentrated HNO_3 (3 mL): HCl (1 mL) was added and mixture was allowed to boil to almost dryness. Thereafter, 25 mL of deionized water was added followed by filtration. The filtrate was transferred to 100 mL volumetric flask and made up to 100 mL with deionized water. Aliquots were taken for AAS to analyze the aforementioned mineral elements.

Determination of Vitamins (A, C and E)

Vitamins A, C and E were determined in the honey after adopting the methods of Maciej and Krzysztof (2007), and Dahot *et al.* (1990), respectively. Briefly, 5 mL of methanol (Vitamin C), propanol (vitamin A and E) was added to 2 mL of honey. The mixture was boiled (airtight) for 30 mins at 40 ^{0}C and thereafter, filtered. The UV-spectrometric determination (vitamin A at 325 nm, vitamin C at 478.50 nm and vitamin E at 800 nm) was done on the filtrate.

Gas chromatography-mass spectrometry Analysis on AH

Principle

This is based on the separation of sample into relative 'pure' eluents and subsequently followed by the combination of ionization, fragmentation and separation processes which results in characteristic 'mass spectra.'

Procedure

The analysis was carried out on a Shimadzu GCMS-QP2010 PLUS Gas Chromatography/ Mass Spectrometry (GC-MS) Japan using helium gas and 5 % diphenyl/95 % dimethyl polysiloxane as mobile and stationary phases, respectively. The oven and injection temperature were 100 ^{0}C and 250 ^{0}C. The injection mode was split and helium at 1.75 mL/min was used as carrier gas. Mass spectra were recorded in

electronic impact (EI) mode at 70 eV, scanning the 50–700 *m/z* range. The source and interphase temperature were 200°C and 250 °C, respectively.

Determination of pH

The serial dilution of 5.0, 10.0 and 20.0 % (v/v) of honey were made, thereafter, the pH of each dilution was measured using pH meter. This followed by calculating the average for the three (3) dilutions.

Determination of Total Flavonoids

Principle

This is based on the complex formed as a result of an interaction between Aluminum chloride and flavonoids ring structures.

Procedure

Aluminum chloride colorimetric method with slight modification was used for flavonoids determination (Chang *et al*., 2002). The aliquot 0.5mL of 0.5 mg of AH was dissolved in 1 mL of methanol was separately mixed with 0.1 mL of 10 % aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. It remained at room temperature for 30 mins; the absorbance of the reaction mixture was measured at 415 nm. The standard curve was prepared making quercetin solution at concentration of 0.2-1.0 mg/mL in methanol. Total flavonoids content was calculated as quercetin equivalent (QE) in mg per 100 g of honey.

Determination of Total Phenolics

Principle

This is based on the complex formed as a result of an interaction between Folin-Ciocalteau reagent phenol reagent and polyphenols/phenolics compounds.

Procedure

The total phenolics content of the AH was determined using Folin-Ciocalteau reagent phenol reagent as described (McDonald *et al*., 2001) with slight modification. An aliquot of 0.5 mL of 0.5 mg of AH was dissolved in 1mL of methanol, transferred in to test tubes and made up to 0.5 mL with distilled water. 0.25 mL of Folin-Ciocalteau reagent (10 % v/v in distilled water) and 1.25 mL of 20 % sodium carbonate solution were added. The tubes were vortexed and allowed to stand for 40 mins before the absorbance was taken at 725 nm. The standard curve was prepared using 0.2-1.0 mg/mL solution of garlic acid in methanol and the phenolic content was expressed as garlic acid equivalent (GAE) in mg per100 g of honey.

3.6 Antioxidant Activity Assays

Reagents

Sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), thiobarbituric acid (TBA), tris HCl (Flukar analytical), ethanol (BDH, Prolabo), potassium chloride (BDH laboratories, pooled England), SA (BDH laboratories, pooled England), $NaHSO_3$ (BDH laboratories, Pooled England), Sodium Nitroprusside (BDH laboratories, Pooled England) and dichromate (Hopkins & Williams, England).

Preparations

0.05M Carbonate buffer (pH 10.2)

Exactly 3.58 g of $Na_2CO_3.10H_2O$ and 1.05 g of $NaHCO_3$ were dissolved in 200 mL of distilled water. The pH was adjusted to 10.2 and then made up to 250 mL with distilled water.

0.3mM Adrenaline

Exactly 0.01 g of adrenaline (epinephrine) was dissolved in 200mL-distilled water. Always prepare fresh when needed.

5 % $K_2Cr_2O_7$ (Dichromate Solution)

Five (5) gram of $K_2Cr_2O_7$ was dissolved in 80mL of distilled water and made up to 100 mL with same.

0.2M H_2O_2 (Hydrogen peroxide)

Exactly 11.50 mL of 30% (w/w) H_2O_2 was diluted with distilled water in a volumetric flask and the solution made up to 500 mL.

Dichromate/acetic acid

This reagent was prepared by mixing 5 % solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) and could be used indefinitely.

30% Trichloroacetic acid (TCA)

Nine (9) gram of TCA (CCl_3COOH) was dissolved in distilled water and made up to 30mL with same.

0.75% Thiobarbituric acid (TBA)

This was prepared by dissolving 0.23 g of TBA in 0.1M HCl and made up to 30mL with same.

0.15 M Tris-KCl buffer (pH 7.4)

Exactly 1.12 g of KCl and 2.36 g of Tris base were dissolved separately in distilled water and made up to 100mL with same. The pH was then adjusted to 7.4.

Phosphate buffer (0.01 M, pH 7.4)

Exactly 3.58 g of Na_2HPO_4 $12H_2O$ and 1.19 g NaH_2PO_4 $2H_2O$ dissolved in 900 mL of distilled water. The pH adjusted to 7.0 and distilled water added to make up to 1 litre.

GSH working standard

Exactly 40 mg GSH (Sigma, Mol. Weight 307.3g) was dissolved in 100 mL of 0.1M phosphate buffer, pH 7.4, and then stored in the refrigerator.

Ellman Reagent [5′, 5′-Dithiobis- (2-nitrobenzoate) DTNB]

This was prepared by dissolving 40 mg (0.04 g) of Ellman reagent in 0.1M Phosphate buffer and made up to 100 mL.

Precipitating Agent

Exactly 4 % Sulphorsalicyclic acid ($C_7H_6S.2H_2O$ mol. Wt. 254.22) was prepared by dissolving 4 g of sulphorsalicyclic acid in 100mL of distilled water. This is stable for approximately three weeks at 4°c.

Estimation of reduced glutathione (GSH) level *in vivo*

Principle

This method is based upon the development of a relatively stable (yellow) colour when 5′, 5′–dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds (Figure 79). The chromophoric product resulting from the reaction of Ellman reagent with the reduced glutathione, 2 – nitro-5-thiobenzoic acid possesses a molar absorption at 412nm.

Procedure

The method of Beutler *et al*., (1963) was followed in estimating the level of reduced glutathione (GSH). Briefly, an aliquot of the sample was deproteinated by the addition of an equal volume of 4% sulfosalicyclic acid. This was centrifuged at 4,000×g for 5mins. Thereafter, 25µL of the supernatant was added to 225µL of Ellman reagent and the absorbance read at 412nm.

GSH

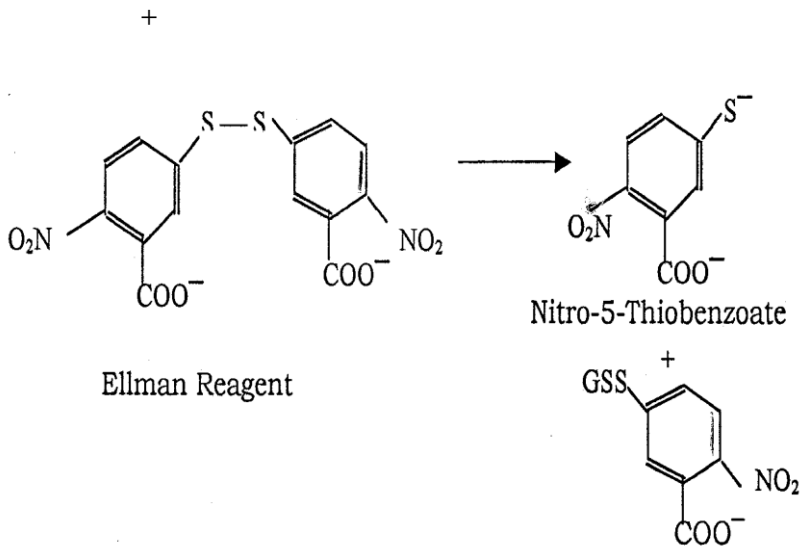

Figure 79: Reaction of reduced GSH with Ellman Reagent

Lipid Peroxidation Assay

Principle

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation (Ohkawa *et al*., 1979). This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde (Figure 80): an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532 nm and which is extractable into organic solvents such as butanol. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982).

***In vitro* Assay**

Lipid peroxidation assay was carried out by the modified method of Ohkawa *et al*. (1979). Briefly, 100 µL of the sample (tissues) was mixed with a reaction mixture containing 30 µL of 0.1 M Tris-HCl buffer (pH 7.4), sample (100 µL) and 30 µL of the pro-oxidant (7µM Sodium Nitroprusside). The volume was made up to 300 µL with water before incubation at 37°C for 2 h. The colour reaction was developed by adding 300 µL of 8.1 % SDS (Sodium dodecyl sulphate) to the reaction mixture, followed by the addition of 600 µL of acetic acid/HCl (pH 3.4) and 600 µL of 0.8 % TBA (Thiobarbituric acid). This mixture was incubated at 100 °C for 1h. The absorbance of TBARS (Thiobarbituric acid reactive species) produced were measured at 532 nm in an ELISA plate reader. The percentage inhibition of MDA (Malondialdehyde) produced was calculated using the following formula:

$$\text{Inhibition rate \%} = \left[1-\left(\frac{A_1-A_2}{A_0}\right)\right]\times 100$$

Where A_0 = absorbance of control (without the honey); A_1 = absorbance of mixture with honey, A_2 = absorbance of mixture without control and honey

***In vivo* Assay**

Briefly, an aliquot of 40 µL of the sample was mixed with 16 µL of Tris-KCl buffer to which 50 µL of 30% TCA was added. Then 50 µL of 0.75 % TBA was added and placed in a water bath for 45 mins at 80 ^{o}C. This was then cooled in ice and centrifuged at 3000 ×g. The clear supernatant was collected and absorbance measured at 532 nm.

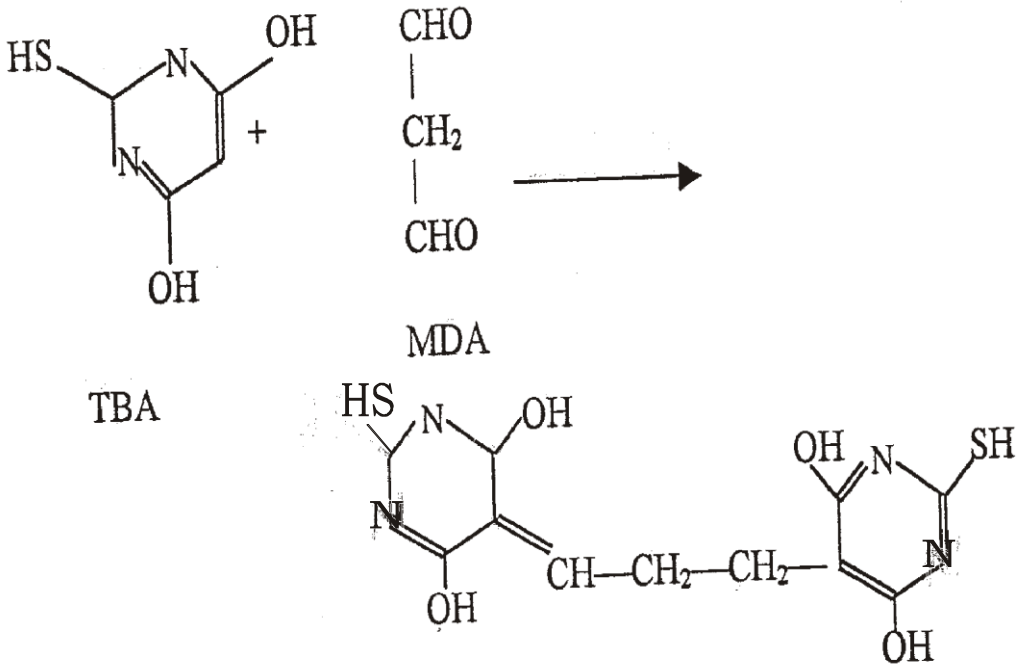

Figure 80: MDA reaction in lipid peroxidation assay (Adam-Vizi and Seregi 1982).

Determination of Catalase Activity

Principle

This method is based on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2) with the formation of perchromic acid as an unstable intermediate. The Catalase activity of the tissues was determined by adopting the method of Brannan *et al*., (1981) with some slight modification.

***In vitro* Assay**

Briefly, 200 µL of tissue sample was diluted in 1800 µL of distilled water. An aliquot of 100 µL of the diluted sample was pipetted into a 96-well plate. 30 µL of the pro-oxidant (7µM SNP) was added to the sample, after which 100 µL of the 4 % honey was added and incubated at 37 ^{0}C for 2 hrs. 50 µL of 1.2 mM H_2O_2 (in 0.05 M phosphate buffer [pH 7.0]) was added to the reacting mixture and allowed to stand for 25 mins. The reaction was stopped by the addition of 40 µL of dichromate/acetic acid to give a red colored compound and absorbance measured at 505 nm using an ELISA plate reader. The enzyme activity was calculated using the molar extinction coefficient of 40.00 M^{-1} cm^{-1} expressed as unit/mg protein

***In vivo* Assay**

Briefly, 200µL of the sample was diluted in 1800 µL of distilled water. An aliquot of 100 µL of the diluted sample was pipette into a 96-well plate. 50 µL of 1.2 mM H_2O_2 (in 0.05 M phosphate buffer [pH 7.0] was added to the reacting mixture and allowed to stand for 25 min. The reaction was stopped by the addition of 40 µL of

dichromate/acetic acid to give a red colored compound and absorbance measured at 505 nm using an ELISA plate reader.

Determination of Superoxide Dismutase Activity

Principle

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide ($O_2^{\bullet-}$) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per $O_2^{\bullet-}$ introduced increased with increasing pH (Valerino and Mc Cormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide ($O_2^{\bullet-}$) radical and hence inhibitable by superoxide dismutase. The level of SOD activity was determined by the method of Misra and Fridovich, (1972) with some slight modification.

***In vitro* Assay**

Briefly, 100 µL of tissue sample was diluted in 900 µL of distilled water to make a 1 in 10 dilution. An aliquot of 200 µL of the diluted sample was added to 2.5 mL of 0.05M carbonate buffer (pH 10.2). 60 µL of the pro-oxidant (7 µM SNP) was added to the mixture, after which 200 µL of the 4 % honey was added and incubated at 37 ^{0}C for 2 hrs. 300 µL of freshly prepared 0.3mM adrenaline was added to the mixture. The blank contained 2.5mL buffer, 300 µL of substrate (adrenaline) and 200 µL of water. The absorbance was read at 480nm using ELISA plate reader. SOD activity was measured as the percentage inhibition of adrenaline oxidation using the formula:

$$\text{Inhibition rate \%} = \left[1-\left(\frac{A_1-A_2}{A_0}\right)\right]\times 100$$

Where A_0 = absorbance of control (without the honey); A_1 = absorbance of mixture with honey; A_2 = absorbance of mixture without control and honey.

***In vivo* Assay**

The level of SOD activity was determined by the method of Misra and Fridovich, (1972). Briefly, 100 µL of the sample was diluted in 900 µL of distilled water to make a 1 in 10 dilution. An aliquot of 20 µL of the diluted sample was added to 250 µL of 0.05M carbonate buffer (pH 10.2). This followed by the addition of 30 µL of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The blank contained 250 µL buffer, 30 µL of substrate (adrenaline) and 20 µL of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Determination of DPPH Radical Scavenging Activity

Principle

This is based on the ability of a subsatance to reduce the DPPH free radicals.

Reagents

0.135 mM DPPH and methanol were purchased from Sigma-Aldrich, USA.

Procedure

The method of Liyana-Pathiranan and Shahidi (2005) was adopted to evaluate the scavenging activity of DPPH free radical of the honey (0.5, 1.0, 1.5, 2.0 and 2.5 % (v/v) in methanol. A solution of 0.135 mM DPPH (Sigma-Aldrich) in methanol was prepared and 100 µL of this solution was mixed with 100 µL of honey. The reaction mixture

vortexes thoroughly, and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability of the honey to scavenge DPPH radical was calculated by the equation:

DPPH radical scavenging activity = {(Abs control – Abs sample)/(Abs control)} × 100

Where Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + honey.

Reducing Power Assay

Principle

This is based on the reduction of ferric ion to ferrous ion which invariably indicates the reducing power.

Reagents

1 % potassium ferricyanide, phosphate buffer (0.2 M, pH 7.4), 10 % tetrachloroacetic acid solution and 0.1 % ferric chloride.

Procedure

This was determined according to the method of Oyaizu (1986). The honey was taken in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 % v/v) in phosphate buffer (0.2 M, pH 7.4) and incubated with 1 % potassium ferricyanide at 50^{o}C for 20 mins. The reaction was terminated by adding 10 % tetrachloroacetic acid solution, centrifuged at 3000 rpm for 10 min and the supernatant was mixed with 0.1 % ferric chloride, the absorbance was measured at 700 nm.

Superoxide Radical Scavenging Activity Assay

Principle

This based on the ability of the radical scavenging agents to reduce of superoxide anion generated by phenazine methosulphate.

Reagents

60 µM phenazine methosulphate (PMS), 156 µM nitroblue tetrazolium (NBT) and 468 µM NADH were purchased from Sigma Aldrich, USA.

Procedure

This was evaluated by adopting the method of Robak and Gryglewski (1998) with slight modifications. 0.5, 1.0, 1.5, 2.0 and 2.5 % (v/v) of honey were used. In addition, 60 µM phenazine methosulphate (PMS), 156 µM nitroblue tetrazolium (NBT) and 468 µM NADH were used. All solutions were prepared in 100 mM phosphate buffer (pH 7.4). Equal volume of honey, PMS, NBT and NADH were added and vortexes thoroughly, thereafter incubated at 25 ^{o}C for 5 mins which followed by measuring the absorbance at 560 nm after addition of 100 µL PMS.

Superoxide radical scavenging activity = {(Abs control – Abs sample)/(Abs control)} × 100

Where Abs control is the absorbance of PMS + NBT + NADH; Abs sample is the absorbance of PMS + NBT + NADH + honey.

3.6.1 Chemiluminiscence Assay

Principle

Blood phagocytes respond to particulate or soluble stimuli by the oxidative burst associated with increased production of Reactive Oxygen Species (ROS). Emission of photons which takes place during ROS production can be measured easily as chemiluminescence (Helfand *et al*., 1982)..

Reagents

Hank's buffered saline solution (HBSS), hydrogen peroxide (H_2O_2), lucigenin (Bis-N-methyl-lacridinium nitrate), phorbol-12-myristate-13-acetate (PMA), luminol (3-aminophthalhydrazide) (Alfa Aesar Germany), luminol (G-9382 Sigma-Aldrich), luminometer (Lab system Luminoskan RS, Helsinki, Finland), Zymosan (Sigma-Aldrich, Buchs, Switzerland), ficoll paque and zymosan A were purchased from MP France.

Luminol/Lucigenin-amplified Chemiluminescence Assay

Luminol/Lucigenin-amplified Chemiluminescence assay was used as described by Helfand *et al*., (1982). Briefly, neutrophils (1×10^6 / mL), macrophages (1×10^6 / mL) and whole blood (1:20) suspended in modified Hank's solution (MHS) were incubated with serial concentrations of honey (0.5, 1.0, 1.5, 2.0, and 2.5 % v/v in PBS for whole blood and 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 % v/v in PBS for neutrophils and macrophages); 0.25, 1.25 and 2.5 mg/mL of the fractions for 30 min. MHS with cells and no honey was run as control. Then Zymosan (1 mg/mL) was added, followed by 25 µL (10^{-5}M) of luminol. For the lucigenin-amplified chemiluminescence, luminol and Zymosan were replaced with lucigenin (0.64 mg/mL) and phorbol-12-myristate-13-

acetate (PMA) (0.2 µg/mL) respectively. Total chemiluminescence (CL) was recorded with the Luminometer. The Luminometer was set to measure the resulting light emission in 96-well plate for a period of 50 mins in repeated scan mode with 50 scans, 30 seconds interval, and one second point measuring time. This method was adopted due to its sensitivity, reproducibility and availability.

Percent reduction of reactive oxygen species was calculated using following formula:

Percent reduction ROS = 100 - (RLUs sample mean)/ RLUs control mean × 100

RLUs: relative light units

3.7 Cell Culture

Reagents

The dimethyl sulfoxide (DMSO), ethylene-diamine-tetraacetic acid (EDTA), fetal bovine serum (FBS), gentamycin sulphate, L-glutamine penicillin streptomycin solution (GPSS), Roswell Park Memorial Institute-1640 (RPMI-1640), amphotericin B (Forme pharma), trypan blue and trypsin-EDTA were obtained from Sigma Aldrich (USA).

Human Peripheral Blood Lymphocyte culture and Treatments

Blood was collected by venipuncture from apparently healthy donors aged from 25-35 years with no previous known contact with high concentration of honey. Whole blood cultures were set up as described by Surrallés *et al*., (1992) in five sterilized tubes in triplicates. Briefly, 0.5mL of blood was added to 4.5 mL culture medium containing RPMI 1640, 10 % heat inactivated fetal bovine serum, antibiotics, L-glutamine and 1 % Phytohaemagglutinin. The contents were gently mixed and incubated in a slanted position in humidified 5 % CO_2 at 37 ^{o}C for 24 h. 0.5, 1.0, 2.0, and 4.0 % v/v of Honey was made using RPMI 1640 media.

3.7.1 *In vitro* Mitotic Index Assay in Cells

Principle

Mitotic Index assay is used to characterize proliferating cells and to identify compounds/chemicals that inhibit or induce mitotic progression.

Reagents

RPMI 1640 media, colcemid solution (10 µg/mL), 0.075 M KCl, methanol: acetic acid (3:1) and 5 % Giemsa stain.

Human Peripheral Human Blood Lymphocytes

At the 24 h the various concentrations of the honey were added to the four tubes except fifth tube which served as control but RPMI 1640 media was added to normalize the condition. After mixing the contents of each culture tube by shaking gently, they were incubated in a slanted position at 37 °C for 72 hrs. After 70 h of incubation, a 0.1 mL colcemid solution (10 µg/mL) was added to each culture tube and mixed by shaking gently. After 72 hrs of incubation, cells were harvested by centrifugation using hypotonic treatment (0.075 M KCl) and fixing in a fresh fixative solution (methanol: acetic acid, 3:1). The fixation step was repeated three times. Slides were air-dried and stained with 5 % Giemsa (Ozkul *et al*., 2005). Mitotic Index was calculated as the proportion of metaphase for 1000 cells as shown below:

Percent mitotic index = Number of metaphase × 100/Total number of cells

Cancer Cell Lines (NCI-H460 and PC-3)

The experiment was conducted for 48 hrs incubation period. Exponentially growing cells were harvested, counted with hemocytometer and diluted with a medium. Cell culture with the concentration of 1×10^5 cells/mL was prepared and introduced

1mL/well. 24 hrs after, the various concentrations of the honey were added to the wells except the control that was given only the medium in order to normalized the condition. After 70 hrs of incubation, a 0.1 mL colcemid solution (10 µg/mL) was added to each culture tube and mixed by shaking gently. After 72 hrs of incubation, cells were harvested by centrifugation, by hypotonic treatment (0.075 M KCl) and fixing in a fresh fixative solution (methanol: acetic acid, 3:1). The fixation step was repeated three times. Slides were air-dried and stained with 5 % Giemsa (Ozkul *et al*., 2005). Mitotic Index was calculated as the proportion of metaphase for 1000 cells as shown below:

Percent mitotic index = Number of metaphase × 100/Total number of cells

3.7.2 *In vitro* Cytochalasin B- block Micronucleus Assay on Human Peripheral Human Blood Lymphocytes

In cytokinesis-block method, micronuclei are scored only in the cells which have been inhibited from undergoing cytokinesis, hence called cytokinesis blocked cells. They are recognized as binucleated cells that have completed nuclear division but not cytoplasmic division. These cells can be obtained in lymphocyte culture by adding cytochalasin B (Cyt-B) after 44 hrs of culture initiation (Fenech and Morley, 1985; Fenech, 2000). For Micronucleus analysis, the peripheral lymphocytes were incubated at 37 ºC for 72 hrs. The cells were treated with various concentrations of the honey. Cytochalasin B was added at 44 h of incubation at a final concentration of 5 µg/mL to block cytokinesis. At the end of incubation at 37 ºC, cells were harvested by centrifugation. The Micronucleus staining was performed according to Ozkul *et al*., (2005). 1000 cells were observed and the criteria suggested by Scarpato and Migliore (1996) for recognizing Micronucleus were followed. The extent and progression of nuclear division were measured by counting the number of cells with one, two, three

and four nuclei in a total of 1000 cells per sample as nuclear division index (NDI) = (M1+2M2+3M3+4M4)/N. The cytokinesis-blocked proliferation index (CBPI) was calculated as follows: CBPI = [M1 + 2M2 + 3(M3 + M4)]/N (Surrallés *et al.*, 1995), where M1, M2, M3 and M4 are the number of cells in first metaphase, second metaphase, third metaphase and fourth metaphase, respectively (Holland *et al.*, 2002). However, cytotoxicity index (CI) was also calculated as follows: CI =100 – 100 ($CBPI_T$ –1/$CBPI_C$ – 1) where T and C stand for sample and control, respectively.

Maintenance of Cell Lines

The adherent human cancer cell lines viz PC-3, NCI-H460 and NIH/3T3 (normal) cells were maintained according to the protocols as described by Celis (1998) and Freshney (2000). Since, these cell lines were received in a frozen state in cryovials, they were thawed and mixed with culture medium followed by centrifugation and determination of cell concentration and viability as described in subsequent sections.

Thawing of Cell lines

The cryovials containing the cell lines were placed in water bath at 37 ^{o}C for 2-3 mins and contents were immediately transferred into centrifuge tubes (15mL) containing washing medium (RPMI-1640/ DMEM: 98 %, FBS: 1 %, GPSS: 1 %). After gentle mixing, the tubes were balanced and centrifuged for 10 mins at 1000 rpm. The supernatant was discarded; the cell pellet was dislodged by gentle tapping and re-suspended in washing medium (1 mL) for counting and determination of viability. All the cell lines were placed individually in flasks (25 cm^2) in culture medium {(RPMI-1640/ DMEM supplemented with heat-inactivated FBS (10 %), L-glutamine (2 mM), streptomycin (100 µg/mL), penicillin (100 U/mL) and amphotericin-B (2.5 µg/mL)} and placed in a humidified atmosphere of CO_2 (5 %) at 37^{o}C, after loosening the caps

for the exchange of gases and to maintain the desired pH (7.2-7.4), for the formation of monolayer. The normal color of the culture medium was pink because of phenol red. When the medium became acidic, the color changed to orange and dark yellow due to the production of pyruvic, lactic acids and CO_2 as the byproducts of normal metabolism of the cells. The cells were examined daily for any change in their morphology, color of the medium and contamination and the culture medium was changed after every 2-3 days.

Trypsinization of Monolayer

The culture medium was discarded and 1x PBS (phosphate buffer saline, 10 mL) was added to remove serum and dead cells followed by addition of trypsin-EDTA (0.05 %, 5 mL for 25 cm^2 flask) at room temperature. The time of cell detachment is depended on the nature of cell line used (2-6 mins). The trypsin was inactivated by the addition of washing medium (7 mL) for 25 cm^2 flask. The cell suspension was centrifuged at 1000 rpm for 10 mins at room temperature and the cell pellet was resuspended in washing medium (1 mL).

Cell Viability

The cell viability was assessed by trypan blue dye exclusion method (Rosengard and Cochrane, 1983) using hemocytometer. Trypan blue solution (10 µL, 0.4 % in 0.9 % saline) was mixed gently with the cell suspension (10 µL) and the sample (10 µL) was examined within 30 seconds under the light microscope at 10x magnification. The blue stained cells were counted as non-viable while the unstained, bright and translucent cells as viable. The concentration of cells was calculated as follows:

Cells per mL = N x 5 x D.F. / volume

Where, N is the total number of cells counted in 5 squares, D.F. is the dilution factor and volume is the depth of the counting chamber (0.1 mm^3 = $1x10^{-4}$). Therefore, cells / mL = N x 5 x D.F. x 10^4. Dilution factor was calculated by dividing the total volume used (20 µL) with the cell suspension volume (10 µL) so the dilution factor was 2. The percent cell viability was determined as follows:

Viable cells x 100 / Total number of cells

3.8 Cell Culture and Treatments for Cell lines

PC-3 and NIH/3T3 cells were grown in DMEM medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 1 % (v/v) L-glutamine, 100 U penicillin and 100 µg/mL streptomycin at 37 ˚C in a humidified atmosphere with 5 % CO_2 whereas NCI-H460 cells were grown in RPMI-1640 medium with the same supplementation. The cells were passage twice weekly. Whole honey was initially dissolved in serum-free culture medium at a final concentration of 10 % ($^v/_v$) and the mixture was filter-sterilized using 0.22 µm syringe filter unit (Millipore, USA). The honey mixture was freshly prepared before added to cell cultures. AH was added to cells in complete medium after 24 hrs of growth. Honey was diluted at a final concentration of 2.0, 4.0, 6.0, 8.0 and 10.0 % (v/v) for normal cell lines and 0.5, 1.0, 2.0, 4.0 and 8.0 % (v/v) for cancer cell lines. The experiments were in triplicates/duplicates and repeated at least three times along with untreated cells serving as control.

3.8.1 Cytotoxicity Assays

Reagents

Giemsa stain (Sigma), acetic acid (Lab scan, Ireland), glacial acetic acid, ELISA kits for specific cytokines (TNF-α and IL-1β) (R&D systems, USA and Canada), micro plate

reader (spectra 340, softmax PRO 4.3.1.LS, Molecular device, USA), phosphate buffer saline tablets, o-cresolphthalein (MP Biomedicals, LLC, France), Colchimid (invitrogen), Cytochalasin B (sigma USA) , NaCl (BDH laboratories, pooled England), colchicines and May-Grunwald Stain (Sigma Aldrich USA).

Determination of Tumor Necrosis factor-alpha, Interleukin-1Beta and Prostate Specific Antigen (TNF-α, IL-1β and PSA) Expression from Cultured Supernatant

Principle

This is test that uses antibodies and colour change to identify and quantify a substance by coupled assay due to antigen-antibody interactions.

Procedure

The experiment was conducted for 24 hrs and or 48 hrs incubation period according to the manufacturer's protocol (Figure 44). Each sample was tested in duplicate in each of three or more replicate experiments. Briefly, 100 µL of capture antibody (anti-human TNF-α/ IL-1β/PSA) was added to the well plates and incubate overnight. This followed by washing three times, addition of 300 µL reagent diluents and incubation at room temperature for 1 hr. The plates were washed again and 100 µL of the sample was added to each well. The plates were then incubated for 2 hrs and thereafter washed and detection antibody (biotinylated goat anti-human TNF-α/ IL-1β/PSA) was added and incubated for another 2 hrs. The plates were then washed and 100 µL working solution of streptavidin conjugated to horseradish-peroxidase was added (in dark) to each well followed by 20 mins incubation. The plates were then washed and 100 µL of substrate solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine) was added (in dark) and allowed for 20 mins incubation. 50 µL of stop solution (2 N H_2SO_4) was added, gently tap and absorbance reads at 450nm.

The absorbance readings were converted to pg/mL based upon standard curves obtained with recombinant cytokine in each assay. The advantage of this technique is that it is cheaper, reproducible and highly sensitive.

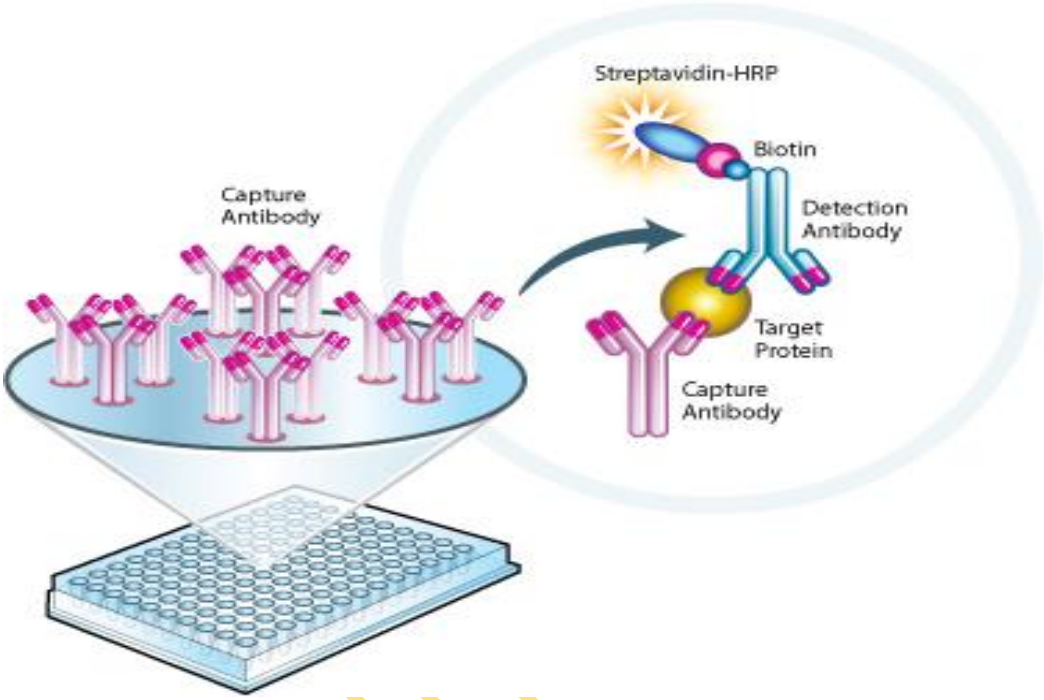

Figure 44: The General principle of ELISA.

(http://www.abcam.com/index.htmL?pageconfig=resource&rid=13855)

Determination of Calcium ion (Ca^{2+}) from Cultured Supernatant

Principle

The calcium ion level in was quantified according to manufacturer's protocols using an automatic analyzer. The principle was based on the complex formed between calcium ion and o-cresolphthalein complexone in alkaline solution. The absorbance was read at 450 nm.

Procedure

The experiment was conducted for 24 hrs and or 48 hrs incubation period. The cell lines were treated with honey as previously described and the cultured supernatant was collected for the assay. Each sample was tested in duplicate in each of three or more replicate experiments.

MTT (3-[4, 5-dimethyl thiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) Assay

Principle

The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dye, giving a purple color (Figure 45). A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth.

Procedure

Anticancer/cytotoxicity of AH on PC-3, NCI-H460 and NIH/3T3 cells was evaluated in 96-well flat-bottomed micro plates by using standard MTT (3-[4, 5-dimethyl thiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colorimetric assay (Mosmann, 1983). Exponentially growing cells were harvested, counted with hemocytometer and diluted

with a medium. Cell culture with the concentration of 1×10^4 cells/mL were prepared and introduced (100 µL/well) into 96-well plates. After overnight incubation, medium was removed and 200 µL of fresh medium was added with different concentrations of AH. After 48 hrs, 200 µL MTT (2 mg/mL) was added to each well after aspiration of media and incubated further for 4 hrs. Subsequently, 100 µL of DMSO was added to each well after aspiration of media. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 570 nm, using a micro plate reader (spectra 340, softmax PRO 4.3.1.LS, Molecular device, USA). The same procedure was followed checking the anticancer activity of AH on PC-3 cells after 24 hrs incubation with AH and another plates 24 hrs after the replacement of AH with just the medium. The anticancer/cytotoxicity activity was recorded as concentration causing 50% growth inhibition (IC_{50}) for PC-3, NCI-H460 and NIH/3T3 cells. The percent inhibition was calculated by using the following formula:

% inhibition = 100 - (mean of Absorbance of sample/mean of Absorbance of control) × 100

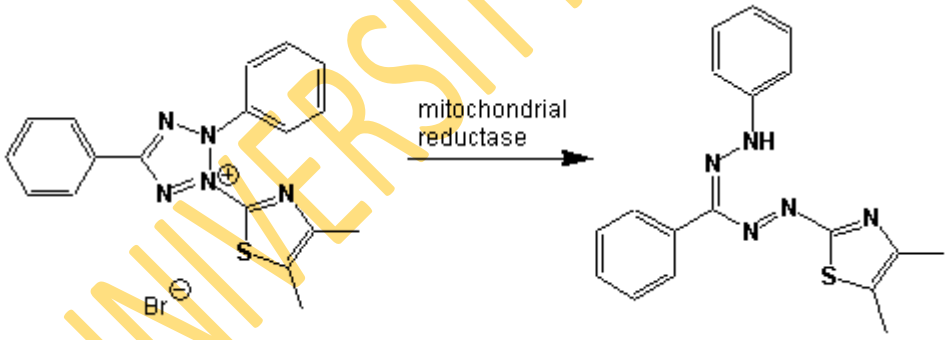

Figure 45: MTT assay reaction (Mossman, 1983).

Cell Viability Analysis by Fluorescence-activated Cell Sorting (FACS)

Principle

Live cells with intact membranes are distinguished by their ability to exclude dyes that easily penetrate dead or damaged cells as described in Figure 46. This causes the release of nuclear materials from the cells which when bound to a dye like propidium iodide increases it fluorescence.

Procedure

NCI-H460 cells were seeded at density of 22 x 10^4 cells/well in 24-well plates. After 24 h cells were treated with honey as previously described. After 48 hrs treatment and incubation at 37 ˚C and 5 % CO_2, cells were washed with PBS harvested with trypsin and centrifuged. Cells pellet were resuspended in propidium iodide (PI) buffer (0.2 % BSA and 0.1 % sodium azide in PBS) and kept at 4^{o}C in dark until analysis. 1 µg/mL PI was added and Cell viability analyzed by flow cytometry on a FACSCalibur. PI was excited at 488 nm, and fluorescence analyzed at 610 nm. A total of 10,000 events in each sample were acquired. Using CellquestPro software, the percentages of live and dead cells were determined.

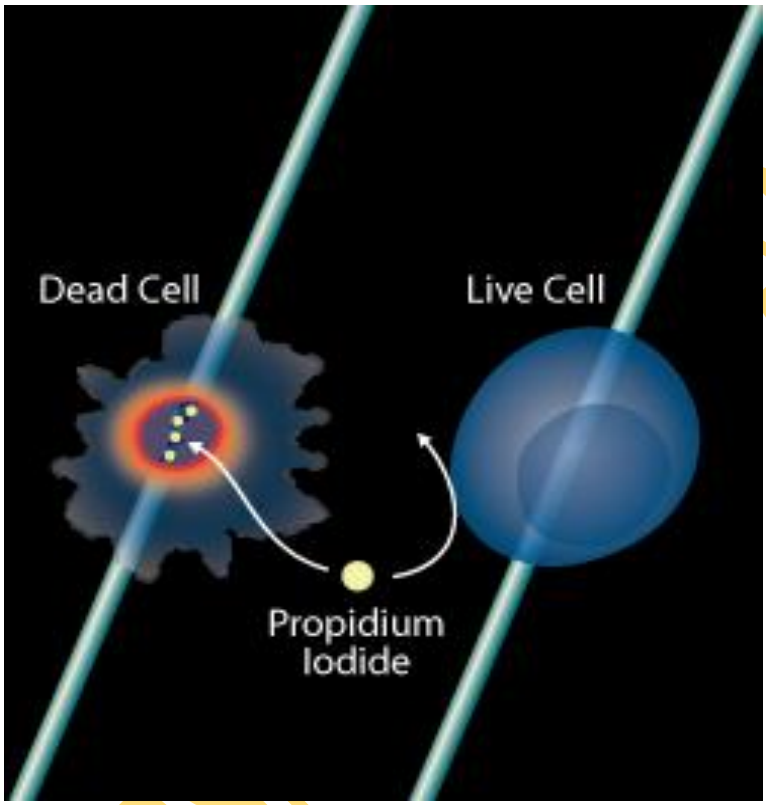

Figure 46: Principle of cell viability analysis. (http://www.rndsystems.com/literature_flow_cytometry_analysis_propidium_iodide.as)

3.8.2 Cell Cycle Analysis by Fluorescence-activated Cell Sorting (FACS)

Principle

This analysis is based on the ability to stain the cellular DNA in a stoichiometric manner. The amount of stain is directly proportional to the amount of DNA within the cell (Figure 47). A variety of dyes are available to serve this function, all of which have high binding affinities for DNA. The location to which these dyes bind on the DNA molecule varies with the type of dye used.

Reagents

Sodium citrate (Merck, Germany), sodium azide (BDH laboratories, pooled England), propidium iodide (Flukar analytical) and ribonuclease A (SERVA, Germany).

Procedure

NCI-H460/PC-3 cells were seeded at density of 1 x 10^6 cells/well in 6-well plates. After 24 h cells were treated with honey as previously described. After 48 hrs treatment and incubation at 37˚C and 5 % CO_2, cells were washed with PBS harvested with trypsin and centrifuged, fixed with ice-cold 100 % ethanol overnight. They fixed cells were centrifuged for 1 hr at 300 ×g and washed with PBS twice. The cells were then treated with 125 µL of 500 U/mL RNase A and incubated at 37^{o}C for 15 mins and thereafter, 125 µL of 5 mg /100mL Propidium iodide in 1.12 % sodium citrate was added and allowed to stand at room temperature for 30 mins. Cell cycle distribution was analyzed by flow cytometry on a FACSCalibur. PI was excited at 488 nm, and fluorescence analyzed at 610 nm. A total of 10,000 events in each sample were acquired. Using CellquestPro software, the percentages of cells at different phases of the cell cycle were determined.

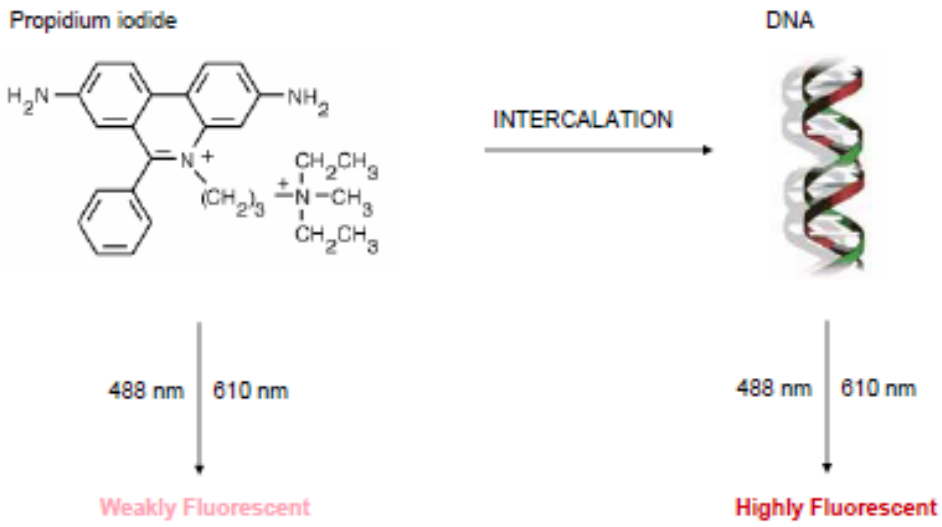

Figure 47: Principle of cell cycle analysis (http://www.metroflow.org/archive/2008Jul01/presentation/Jennifer).

3.8.3 Gene Expression Studies

Principle of Real-time Polymerase Chain Reaction

This is based on the PCR technique, which is used to amplify and simultaneously quantify a targeted DNA molecule (Figure 59). For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

Reagents

Trypsin-EDTA solution, RNA isolation reagent, Chloroform, isopropanol, ethanol, RNAse free H_2O, cDNA synthesis kit and 2×SYBR Green were purchased from Sigma Aldrich, USA.

RNA Isolation from NCI-H460 Cell Lines

Cells were seeded at the density of 1 x 10^6 cells/mL in 6-well plates. The isolation was carried out according to the manufacturer's protocol, with slight modification. The wells were washed twice with PBS, and incubated with trypsin-EDTA solution at 37 °C. From each well the cells were harvested and centrifuged at 1000 rpm for 5 mins at 4 °C. To the cell pellet 0.5 mL RNA isolation reagent was added and incubated at 15-30 ˚C for 5 mins. Chloroform was added and centrifuged at 12000 rpm. The supernatant was removed and mixed with an appropriate volume of isopropanol. After centrifugation at 12000 rpm for 10 mins at 4 °C, the pellet was washed with ethanol. The RNA pellet was air-dried and re-suspended in RNAse free H_2O, subjected to 55-60 ˚C thermal block incubation. The RNA fraction was quantified by using the Nanodrop 2000, spectrophotometer and kept at -80 °C until analysis.

Complementary DNA (cDNA) Synthesis

The isolated RNA was transcribed in to complementary DNA using the cDNA synthesis kit. The protocol was followed as per manufacturer's instructions for the 20µL reverse transcriptase reaction mixture. The RNA samples were mixed with random hexamer for one cycle of 65 °C for 5 mins in a PCR mastercycler. The addition of reaction buffer, RNase inhibitor and DNTPs were made. Following cycles were then run: 5 mins at 25 °C, 42 °C for 60 mins and 5 mins for 70 °C. After incubation, samples were diluted and stored at -20 °C.

Primer Design

The primer sequence was designed using the primer 3 software [Primer3 Input 0.4.0 (primer3-web/htdocs/input-040.htm)] program for GAPDH, Bcl-2 and p53 genes.

Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)

Q-PCR was performed using a Light Cycler system. Each sample was tested in duplicate and GAPDH was used as an internal control. Primers for p53, bcl-2 and GAPDH are listed in table 1. The 20 µL reaction mixture containing 2 µL of complementary DNA, 10 µL of 2×SYBR Green PCR Master Mix, 0.2 µL each primer pair, constituting of 1:199 dilution in the final reaction and 7.8 µL of RNAse free water was made. Thermo cycler conditions consisted of 1 cycle, 50 °C for 30 mins, and 95 °C for 10 mins. 40 cycles, 95 °C for 14 seconds, 60 °C for 1 minute, and 72 °C for 45 seconds, and finally 1 cycle, 95 °C for 14 seconds, 60 °C for 14 seconds, and 95 °C for 14 seconds. A dissociation curve was obtained for each quantitative PCR run.

The real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ relative quantization method following the manufacturer's instructions using the formula: Relative fold change/ratio = $(E_{target})^{\Delta Ct}{}_{target} / (E_{reference})^{\Delta Ct}{}_{reference}$

Whereas $\Delta Ct_{target} = Ct_{control} - Ct_{treatment}$ and $\Delta Ct_{reference} = Ct_{control} - Ct_{treatment}$

E = efficiency = 2

Ratio = $2^{-\Delta\Delta Ct}$ Whereas $\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{target}$

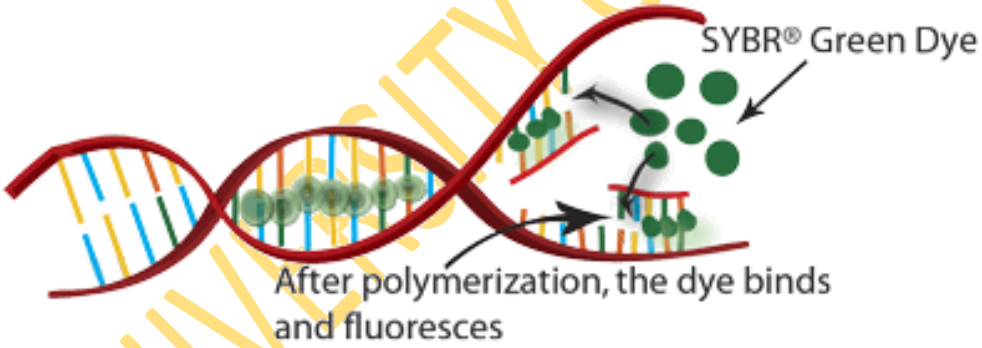

Figure 59: Principle of SBYR green real time PCR (Edwards *et al.*, 2004).

3.9 Determination of Biochemical Parameters from Serum

The biochemical parameters were determined by using Auto Analyzer Hitachi Roche 7020 (902), according to manufacturer's protocols. All analyses were based on the following principles as follows:

Alanine Amino Transferase Activity (ALT)

α-ketoglutarate + L-Alanine $\xrightarrow{\text{ALT}}$ L. Pyruvate + glutamate

Alanine aminotransferase was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine (Reitman and Frankel, 1957).

Aspartate Amino Transferase Activity (AST)

α-ketoglutarate + L-aspartate $\xrightarrow{\text{AST}}$ L. glutamate + Oxaloacetate

Aspartate aminotransferase was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine (Mohun and Cook, 1957).

Alkaline Phosphatase Activity (ALP)

p-nitrophenylphosphate + H_2O $\xrightarrow{\text{ALP}}$ phosphate + p-nitrophenol (Tietz *et al.*, 1983).

Total Protein

Cupric ions (Cu^{2+}) in the biuret reagent complex with the groups involved in the peptide bond. In the presence of alkaline media i.e. NaOH and at least two peptide bonds a violet colored chelate is formed (Gornall *et al.*, 1949).

Albumin

Serum Albumin binds with the bromocresol green (BCG) indicator in an acid medium to form a green BCG complex, the amount of which produced is directly proportional to the albumin concentration present in the sample (Doumas *et al.*, 1971).

Globulin

Globulin is calculated by subtracting the amount of albumin from total protein

Urea

Urea is hydrolyzed by urease to form CO_3^{2-} and ammonium

Urea + H_2O $\xrightarrow{\text{urease}}$ $2NH_4^+ + CO_3^{2-}$

α-ketoglutarate + $2NH_4^+$ + NADH $\xrightarrow{\text{GLDH}}$ L-glutamate + $NAD^+ + H_2O$

the decrease in absorbance due to consumption of NADH is measured kinetically (Talke and Schubert, 1965).

Creatinine

Creatinine + picric acid $\xrightarrow{\text{alkaline solution}}$ Creatinine-picric acid complex (Bartels *et al.*, 1972).

Glucose

Glucose present in the plasma is oxidized by the enzyme glucose oxidase (GOD) to gluconic acid with the liberation of hydrogen peroxide, which is converted to water and oxygen by the enzyme peroxidase (POD). 4 aminophenazone, an oxygen acceptor, takes up the oxygen and together with phenol forms a pink coloured chromogen which can be measured at 515mm (Trinder, 1969; Barham and Trinder, 1972).

Total Cholesterol

This is based on the determination of Δ^4-cholestenone after enzymatic cleavage of the cholesterol ester by cholesterol esterase, conversion of cholesterol by cholesterol oxidase, and subsequent measurement by the Tinder reaction of hydrogen peroxide formed (Liebermann, 1985; Burchard *et al.*, 1989).

High Density Lipoprotein (HDL)

This involved the use of polyethylene glycol-modified (PEG) enzymes and dextran sulfate. When cholesterol esterase and cholesterol esterase enzymes are modified by PEG, they show selective catalytic activities towards lipoprotein fractions, with the reactivity increasing in the order: LDL< VLDL=chylomicrons< HDL (Sugiuchi *et al.*, 1995).

Low Density Lipoprotein (LDL)

When cholesterol esterase and cholesterol esterase enzymes are modified by adding detergent, they show selective catalytic activities towards lipoprotein fractions, with the reactivity increasing in the order: HDL< chylomicrons< VLDL< LDL (Rifai *et al.*, 1992).

Very Low Density Lipoprotein (VLDL)

This calculated by dividing total triglyceride by five (5).

Triglyceride

$$\text{Triglyceride} \xrightarrow[\text{glycerol kinase}]{\text{lipoprotein lipase}} \text{glycerol + fatty acids}$$

Glycerol + ATP $\xrightarrow{Mg^{2+}}$ glycerol-3-phosphate + ADP

Glycerol-3-phosphate

glycerol-3-phosphate + O_2 $\xrightarrow{\text{oxidase}}$ dihydroxi-acetonephosphate + H_2O_2

H_2O_2 + amino-4-antipyrine + ESPAS $\xrightarrow{\text{peroxidase}}$ red derivative of quinone+ 4 H_2O

ESPAS: N-ethyl-N-sulfopropyl-m-anizidine (Giovanni and Harold, 1973).

Determination of Acetylcholinesterase Activity *in vivo*

The activities of AChE (EC 3.1.1.7) in the brain homogenates was determined spectrophotometrically by the method of Ellman's *et al.*, (1961) as modified by Srikumar *et al.*, (2004) using acetylthiocholine iodide as appropriate substrate and 5-5'-dithiobis (2- nitrobenzoic) acid DTNB as a chromogen. Briefly, 177.5 µL of 100mM sodium phosphate buffer (pH 8), 2.5 µL of the sample were mixed. The reaction was initiated by the addition of 10 µL DTNB and 10 µL ACh. The hydrolysis of this substrate was monitored spectrophotometrically by the formation of yellow 5-thio- 2 – nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide at a wavelength of 412nm. The specific activity of AChE was expressed as µmole/gram of tissue/minute/mg protein.

3.9.1 Determination of Haematological Parameters

Complete blood counts were determined in the blood samples (in EDTA) collected from the experimental animals using Coulter HmX Hematology Analyzer according to

manufacturer's protocols. The parameters include: Packed cell volume (PCV), Red blood cell count (RBC), white blood cell counts (WBC), hemoglobin (Hb) and platelet (PT).

The principle

The Coulter method accurately counts and sizes cells by detecting and measuring changes in electrical resistance when a particle (such as a cell) in a conductive liquid passes through a small aperture. Each cell suspended in a conductive liquid (diluent) acts as an insulator. As each cell goes through the aperture, it momentarily increases the resistance of the electrical path between the submerged electrodes on either side of the aperture. This causes a measurable electronic pulse. The number of pulses correlates to the number of particles. The height of the electrical pulse is proportional to the cell volume.

3.9.2 Bone Marrow Micronuclei Assay

Principle

The purpose of the micronucleus assay is to identify test substance(s) that cause micronuclei formation (Figure 81) as a result of lagging of chromosome fragments (clastogenicity) or whole chromosomes (aneugenicity), generally in rodent bone-marrow erythropoietic cells (Gopala and Makoto, 2000).

Reagents

5 % Giemsa Stain

Five (5) gram of Giemsa was dissolved in phosphate buffer (pH 6.8) and the volume made up to 100 mL. It was used for micronucleus assay.

0.04 % Colchicine

Exactly 0.04 g Colchicine was dissolved in distilled water and the volume made up to 100 mL. It was used to inject the rats (1mL/100g body weight) two hours prior to the sacrifice to arrest the metaphase.

0.4 % May-Grunwald Stain 1

Exactly 0.4 mL of May-Grunwald stain was dissolved and made up to 100mL with absolute methanol.

0.4 % May-Grunwald Stain 2

Exactly 1:1 dilution was made using stain 1 and distilled water. They were used in staining of slides.

Micronuclei Assay

Clastogenic effects were evaluated in the rat bone marrow using the micronucleus assay as described by Heddle and Salmone, (1981) and modified by Heddle, *et al*, (1981). Bone marrow cells removed using 0.9 % (w/v) NaCl from both femurs were used for preparing slides. The slides were fixed with methanol, air-dried and pretreated with/without May-Grunewald solution. They were then stained with 5% Giemsa solution. The slides were scored for the presence of micronucleated polychromatic erythrocytes (mPCEs) in 1000 cells according to standard procedure.

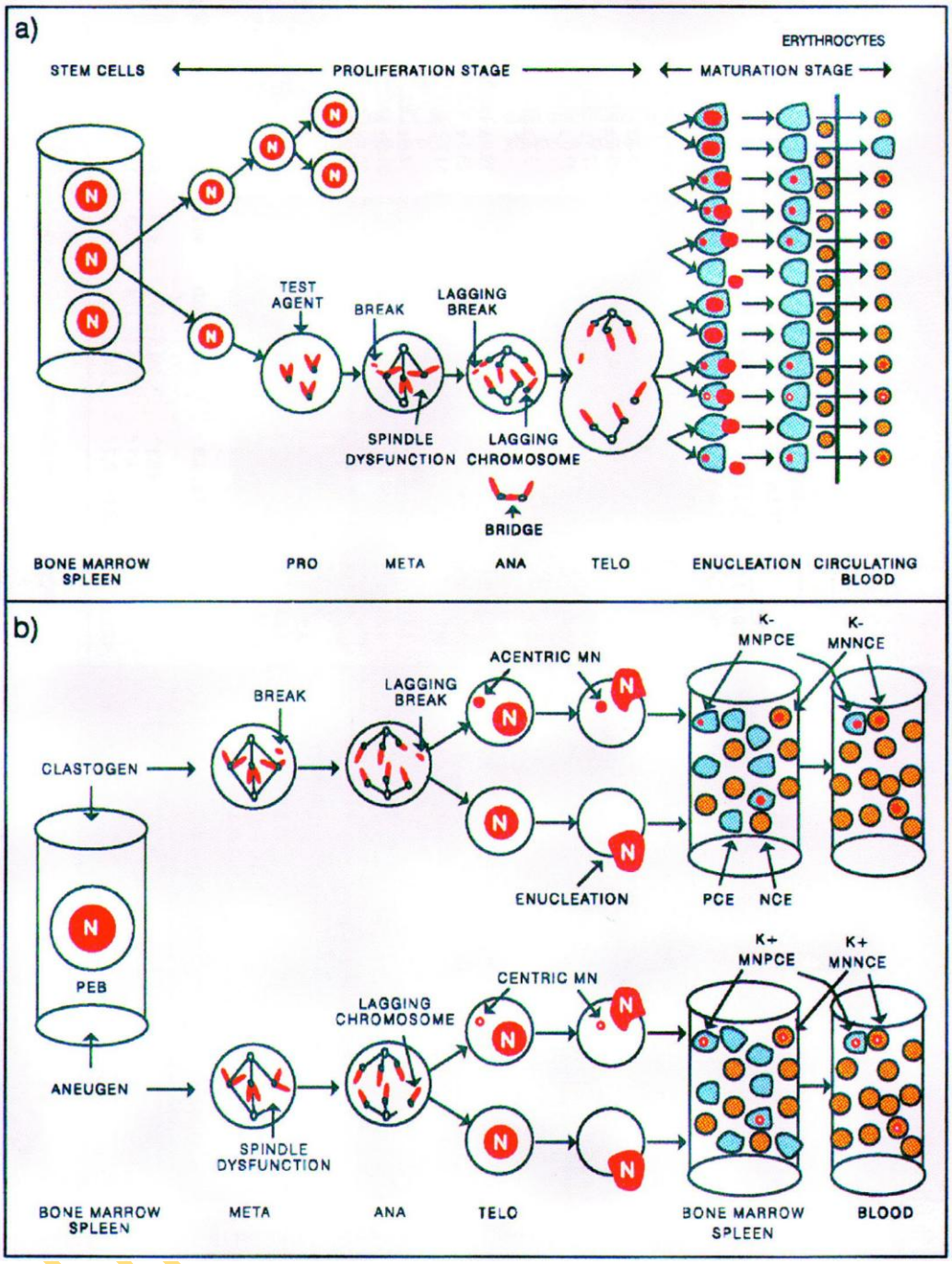

Figure 81: (a) The process of erythropoiesis in vivo; (b) the mechanism of micronucleus formation in the polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). Also classification of kinetochore-positive (KC) and kinetochore-negative (K−) erythrocytes. N, nucleus; PEB, proerythroblast; MN, micronucleus (Gopala and Makoto, 2000).

3.9.3 Statistical Analysis

To address the biological variability and stability of the samples, each and every experiment was repeated at least three times and the results were expressed as mean ± Standard deviation. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS) software, SPSS Inc., Chicago, Standard version 10.0.1. P-values< 0.05 were considered statistically significant for differences in mean using the least of significance difference (LSD).

CHAPTER FOUR

4.0 EXPERIMENTS AND RESULTS

4.1 Experiment 1: Quality Assessment of Honey

Introduction

Honey, a natural product formed from nectar by honeybees, has been a subject of renewed research interest in the last few years. Its production is basically through a regurgitation mechanism from nectar or from the secretion of living part of plants (Anklam, 1998). After harvesting, the honey may be intentionally or not be adulterated for commercial purpose which may/may not affects it biological activity due to decreased in nutritional values. It was against this background that we evaluated the quality of our sample (*Acacia* honey) before further experiments.

Procedure

The sugar, pH, minerals, vitamins, phytochemicals, diastase activity and hydroxylmethylfurfural levels were determined in the honey as described in 'Materials and Methods' (section 3.5, page 117-121). The experimental design is presented in Figure 21.

Results

The diastase activity and hydroxylmethylfurfural level were 2.7 units/g of AH and 0.44 mg/100 g of AH. The pH and sugar contents were 4.33 and 87 %. The flavonoids and phenolics contents are shown in Figure 22 with flavonoids being higher than phenolics. As seen (table 3), are the proposed active principles along with the similarity index of AH that were identified by GC-MS analysis. Some important phenolics like p-hydroxybenzoic acid, cinnamic acid, 9-octadecanoic acid-2-hydroxy-1-(hydroxymethyl)

ethyl ester and flavonoids like chrysin were identified. Others include 2-hydroxypentadecanone, Hydrofol acid, 1, 6-anhydro-beta-D-glucofuranose, 5-hydroxymethylfurfural, Pyrazol-3-one and 2, 4-dihydroxy-5-methylpyrimidine was also determined. However, Figure 23 shows the vitamin contents of AH with vitamin A being higher in contents followed by C and lastly E. Important mineral elements (Figure 24) of physiological importance like Calcium, iron, magnesium, potassium and zinc were quantified with iron being higher in contents followed by magnesium, potassium, calcium and zinc.

Conclusion

It was observed that the *Acacia* honey (AH) is of good quality for further experiments due to high vitamins and phenolics contents.

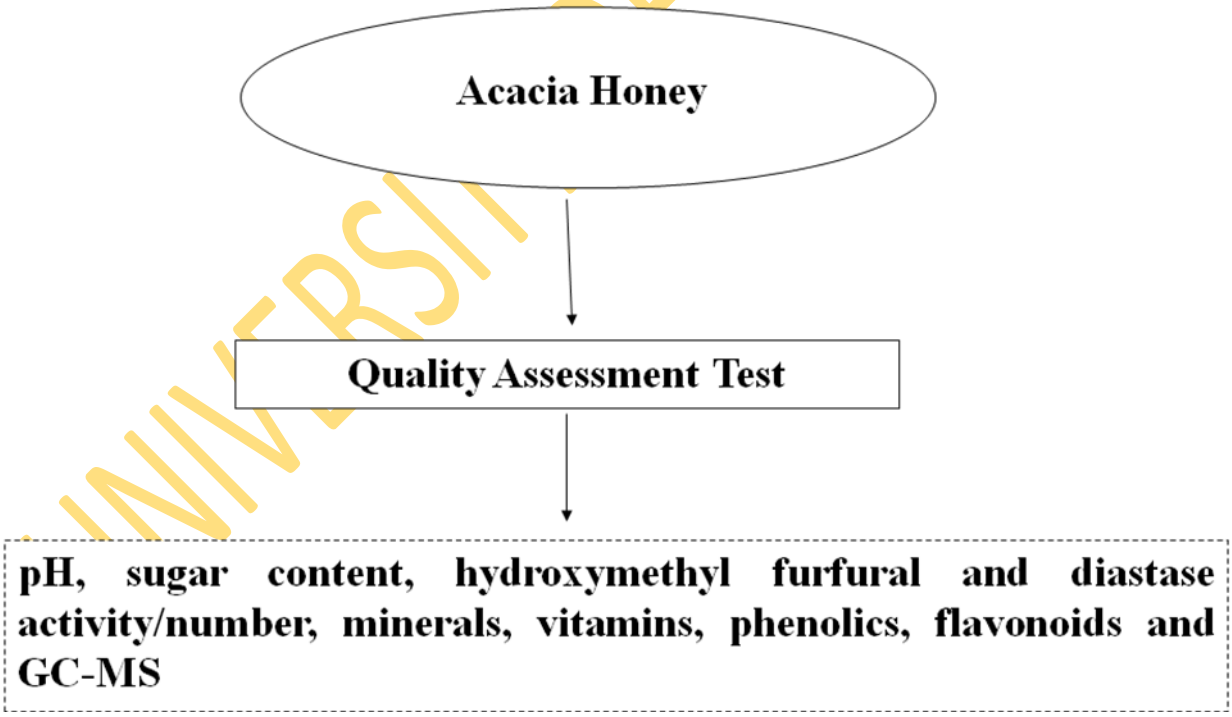

Figure 21: Quality assessment of *Acacia* Honey

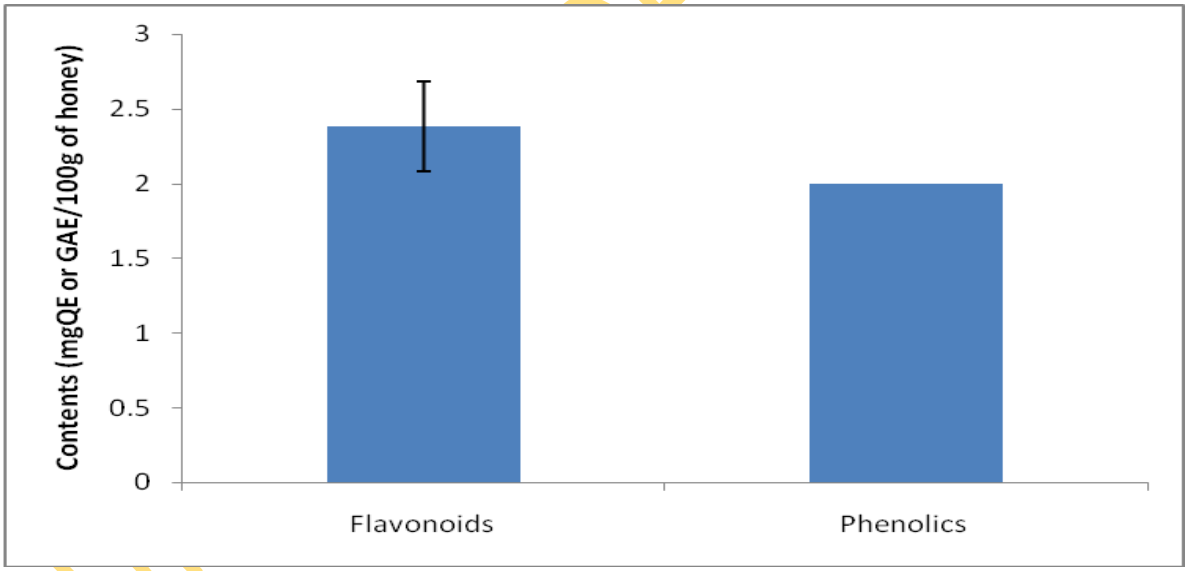

Figure 22: The Flavonoids and Phenolics Contents of AH

Table 3: Proposed compounds identified by GC-MS analysis

S/N	Proposed compounds	Similarity index (%)
1	2,4-dihydroxy-5-methylpyrimidine	77
2	Pyrazol-3-one	76
3	glycerol	89
4	5-hydroxymethylfurfural	92
5	2-propylthiopene	85
6	furanmethanol	81
7	D-allose	94
8	1,6-anhydro-beta-D-glucofuranose	92
9	n-hexadecanoic acid	92
10	Hydrofol acid	94
11	9-octadecenal	85
12	9-octadecanoic acid-2-hydroxy-1-(hydroxymethyl) ethyl ester	81
13	9-octadecanoic acid, 1,2,3-propane triyl ester	82
14	p-hydroxybenzoic acids	84
15	cinnamic acid	82
16	2-hydroxypentadecanone	90
17	chrysin	82

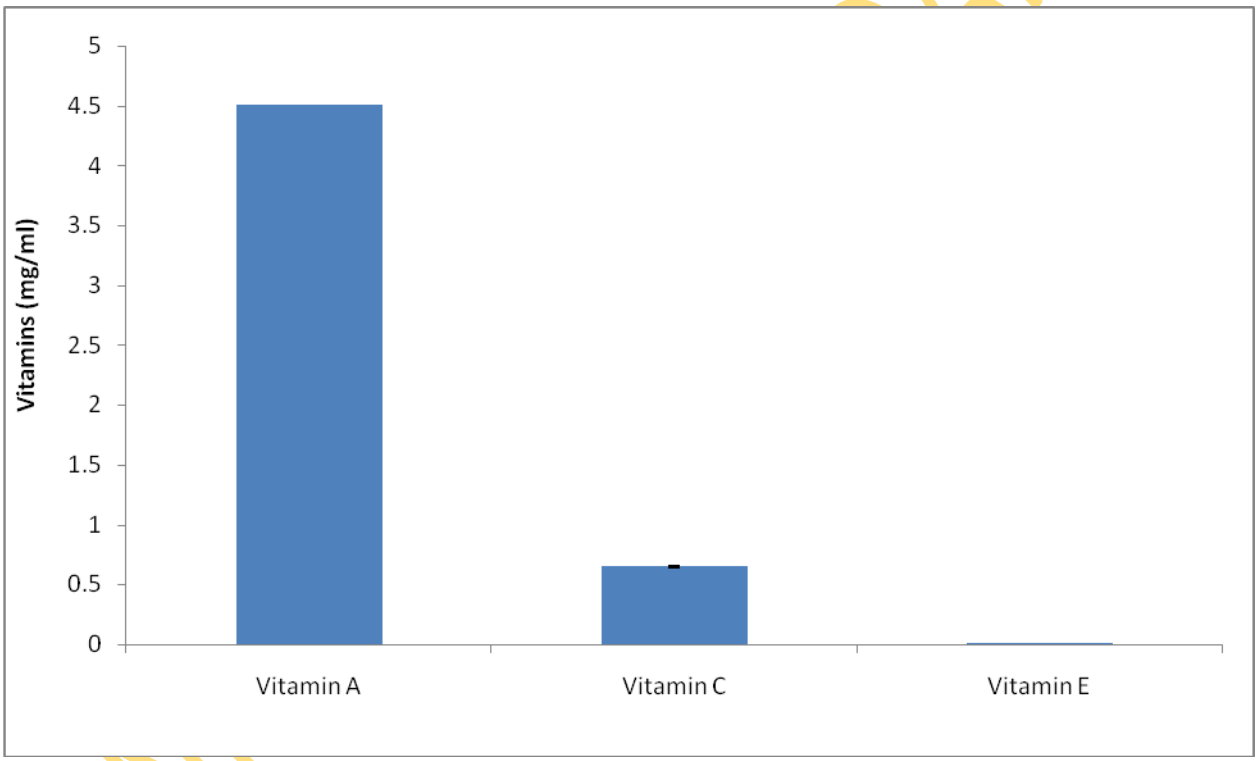

Figure 23: The vitamins contents of AH

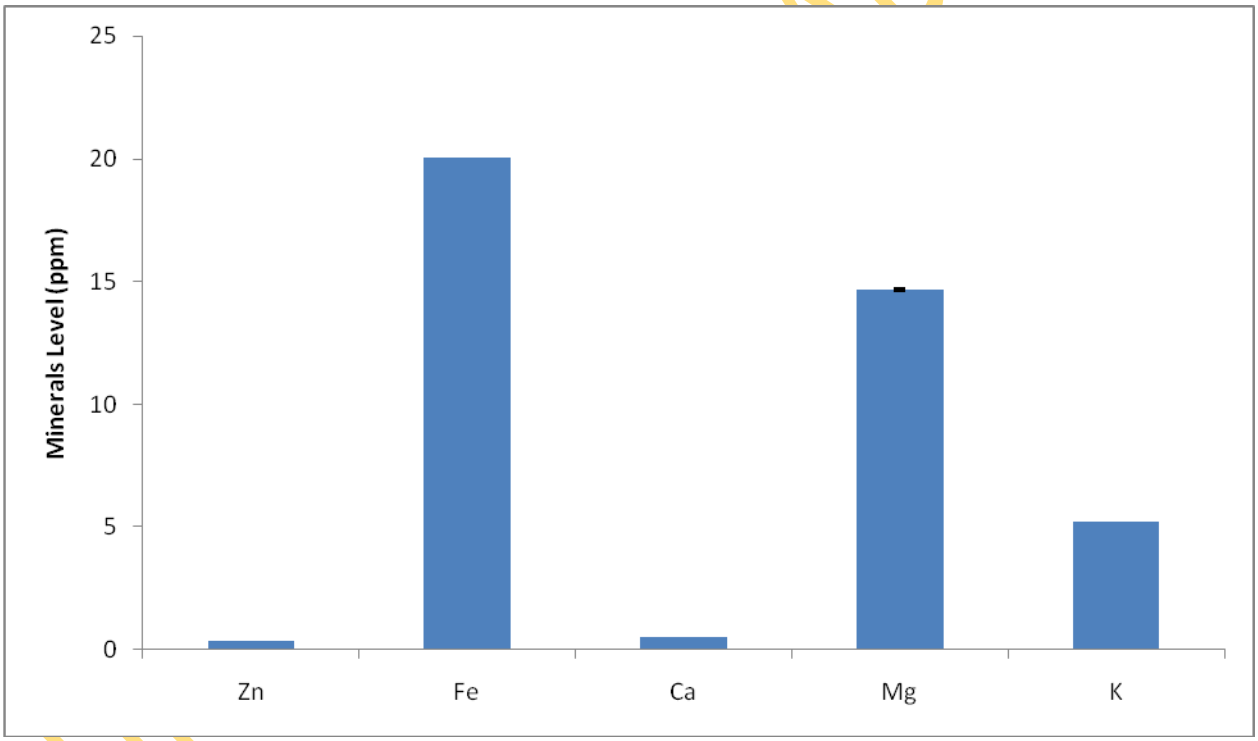

Figure 24: The mineral elements contents of AH.

4.2 Experiment 2: Effects of Fractionation of AH on its *in vitro* Antioxidant Potential

Introduction

It has been reported that the antioxidants activity of most types of honey is due to synergistic effects of wide range of compounds present in them (Gheldof *et al.*, 2002). These wide ranges of compounds include phenolics, peptides, organic acids, enzymes, Millard reaction products, and possibly other minor components and that the phenolics compounds contribute significantly to the antioxidant capacity of honey but are not solely responsible for it (Nada, 2009). It was against this background that the effect of fractionation process on the antioxidant activity of AH was evaluated.

Procedure

The honey sample was fractionated as described under 'Materials and Methods' (section 3.1.1, page 114) and thereafter, the antioxidant activity on the human blood and normal rat's tissues (brain and liver) after induction of oxidative stress was evaluated against the unfractionated sample as described (section 3.2.1. page 115; section 3.6. page 121-131; section 3.6.1. page 134). The experimental design is presented in Figure 25.

Results

As seen in Figure 26, dichloromethane and ethyl acetate fractions showed better potency in the suppression of reactive oxygen species production than aqueous fraction. None of the concentration of the fraction was able to cause 50 % inhibition. In fact it was observed that the aqueous fraction stimulated ROS generation greater than the control probably due to generation of H_2O_2 as result of sugar oxidation. To see whether

the decreased antioxidant activity was as result of fractionation, pure AH was used as depicted in Figure 27. There was a significant ($p<0.05$) inhibition in a dose-dependent manner with an IC_{50} of <0.25 % (v/v). By implication this suggests that the fractionation had negatively affected the antioxidant activity, thus suggesting a synergetic activity of the whole AH. To further confirm our observation, 4 % (v/v) of AH was used to see its effect on the antioxidant activities of brain and hepatic tissue after inducing the oxidative stress with sodium nitroprusside. The result (Figure 28) shows that AH was able to induce SOD activity by about 43 and 42 % for hepatic and brain tissues respectively as compared with α - tocopherol. Similarly, in Figure 29, significant ($p<0.05$) catalase activity was observed in both tissues. However, the study could not detect appreciable inhibition of lipid peroxidation in both brain and hepatic tissues as depicted in Figure 30. This is not surprising due to earlier induction of SOD and Catalase activity. This can be attributed to the fact that catalase reduces H_2O_2 to H_2O and O_2, thereby making H_2O_2 unavailable for Fenton and Haber Weiss reactions which could generate hydroxyl radical leading to lipid peroxidation. From Figure 31, AH was found to be less toxic as it has less superoxide dismutase-catalase ratio as compared with α – tocopherol.

Conclusion

The fractionation process seems to affect the antioxidant potential of AH signifying synergism.

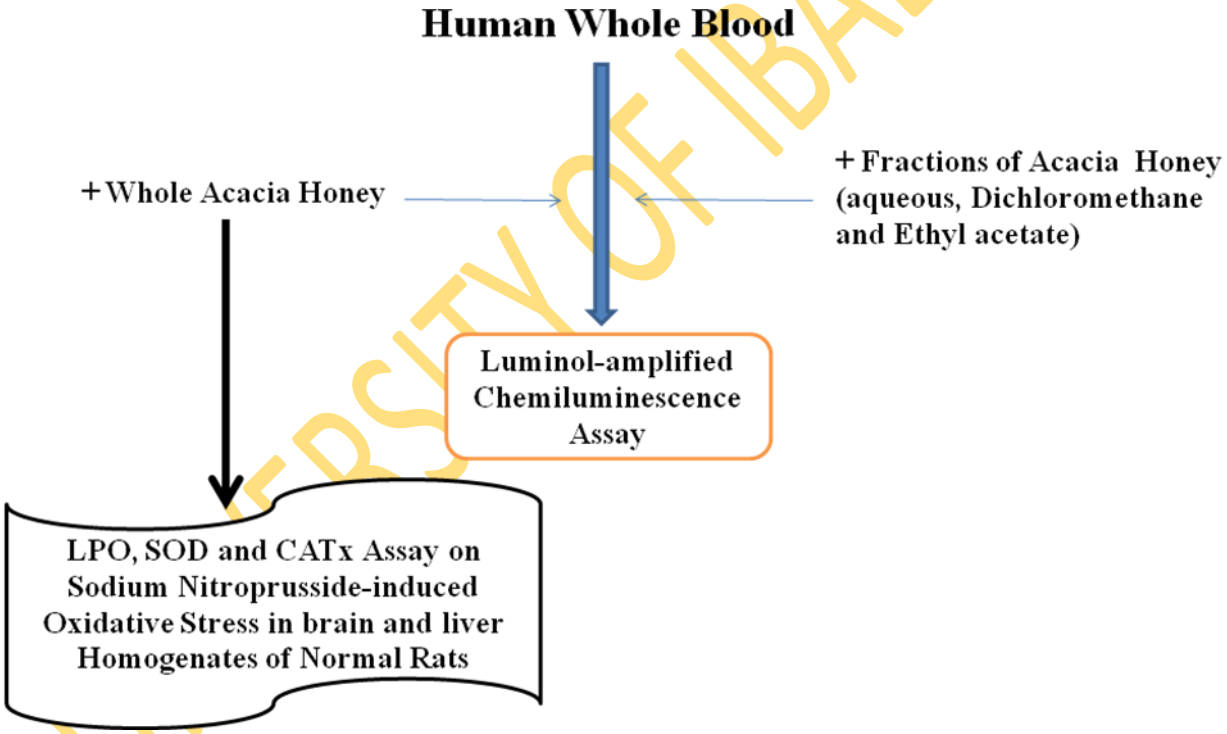

Figure 25: Antioxidant effects of AH and its fractions on whole Human blood, brain and liver tissues (Normal Rats) after induction of an oxidative stress.

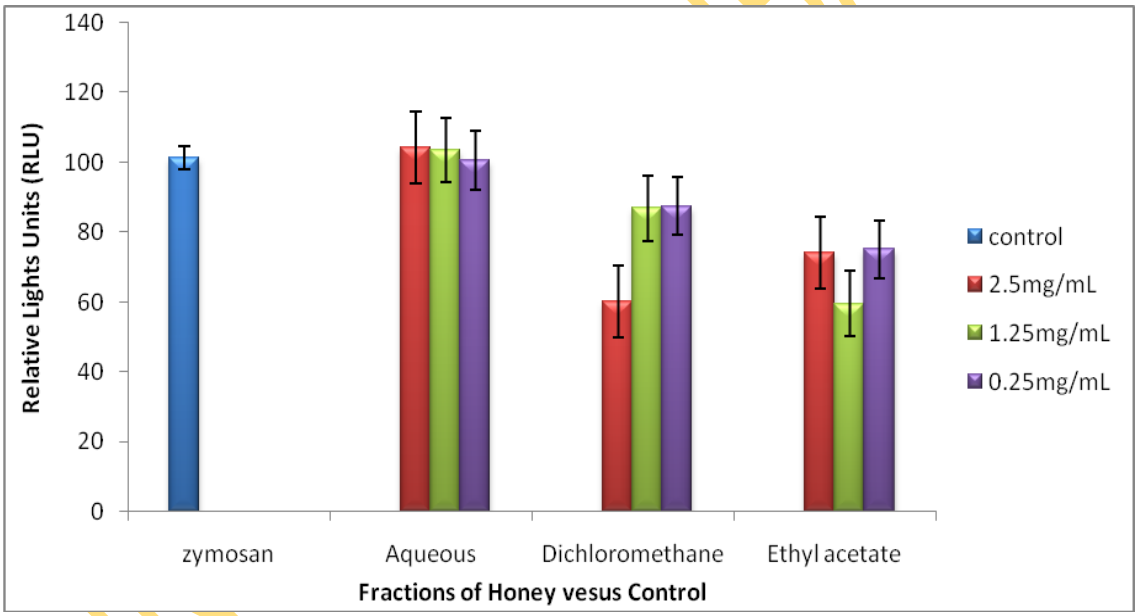

Figure 26: Luminol-amplified chemiluminescence assay results from whole blood using different fractions of AH

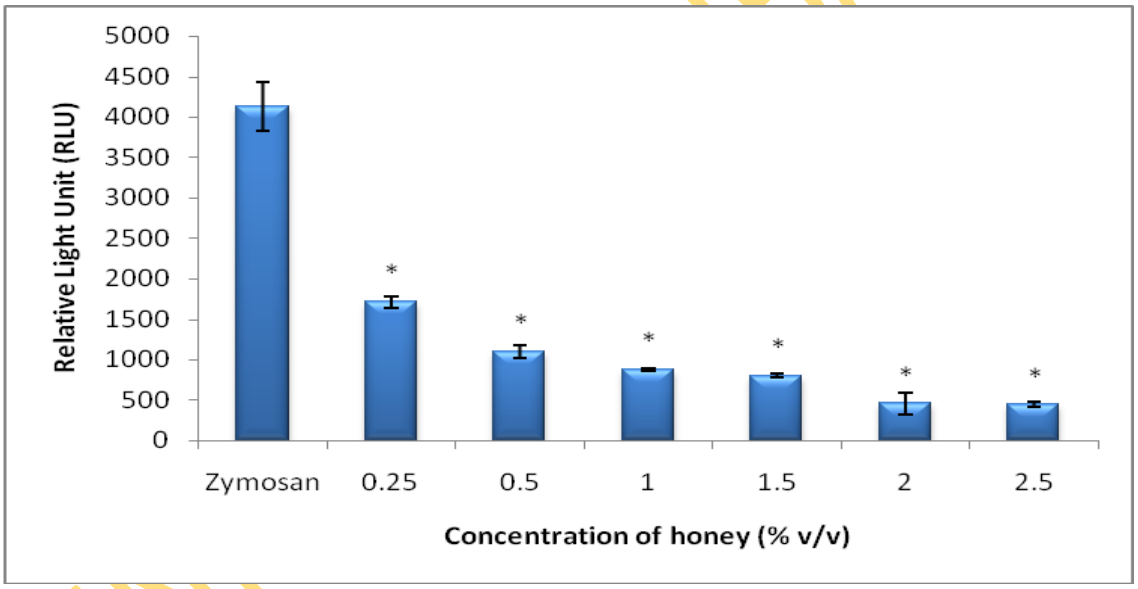

Figure 27: Luminol-amplified chemiluminescence assay results from whole blood using different concentration of AH. *statistical significant ($p < 0.05$) as compared with control

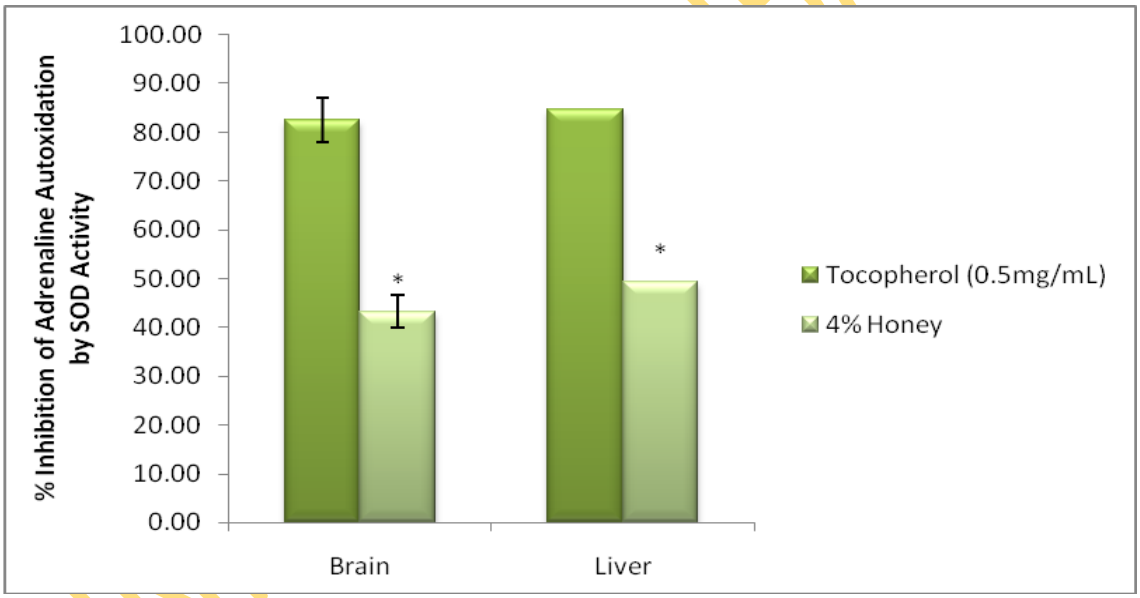

Figure 28: Percentage (%) inhibition of adrenaline autoxidation by superoxide dismutase (SOD) in the presence of AH and tocopherol in brain and liver homogenate. *statistical significant ($p < 0.05$) as compared with tocopherol

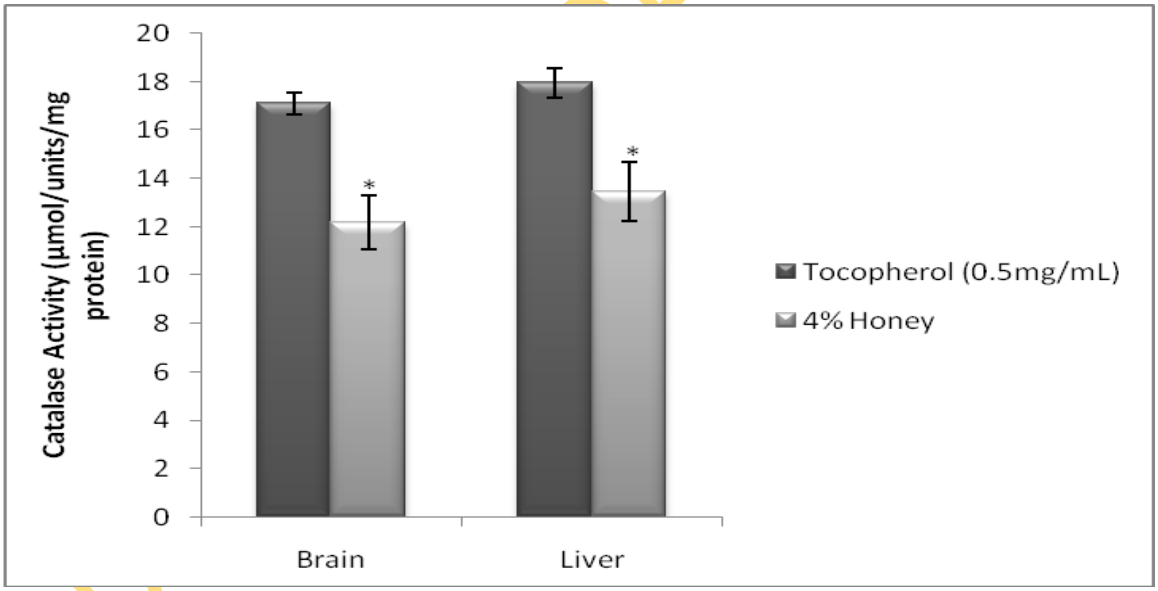

Figure 29 Catalase activity in brain and liver homogenate due to AH and tocopherol.

*statistical significant ($p < 0.05$) as compared with tocopherol

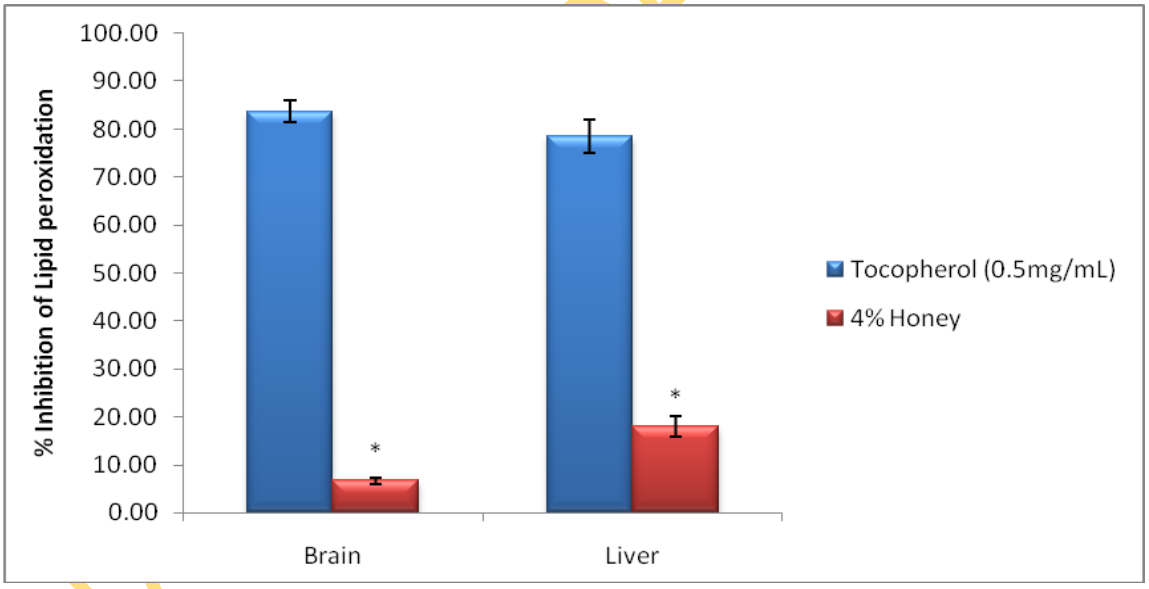

Figure 30: Percentage (%) inhibition of lipid peroxidation by tocopherol and AH in brain and liver homogenate. *statistical significant ($p < 0.05$) as compared with tocopherol

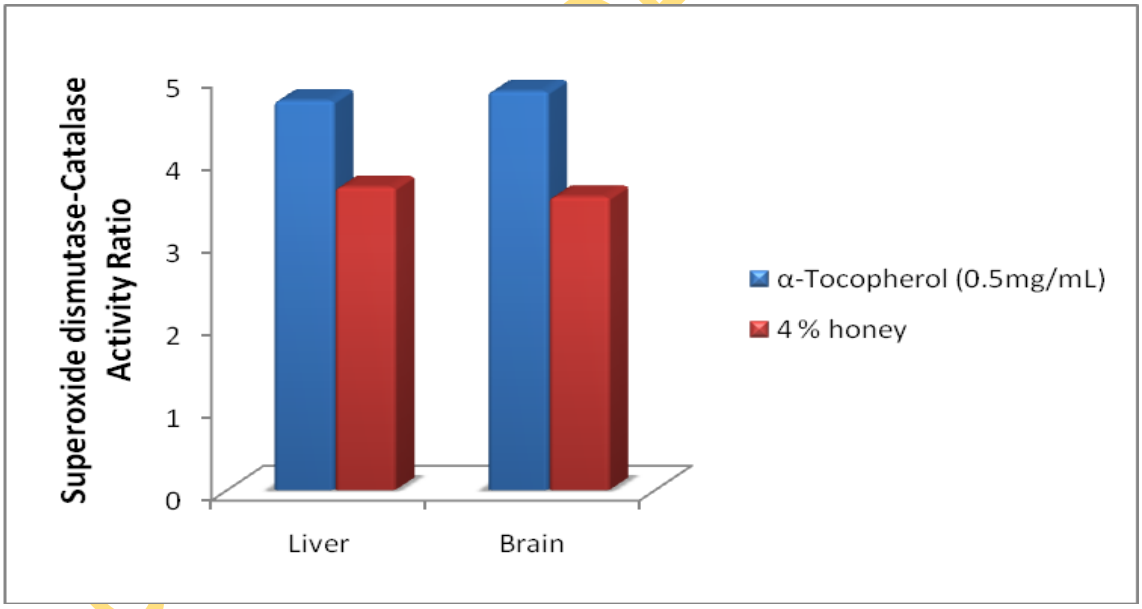

Figure 31: Superoxide dismutase (SOD)-catalase activity ratio as toxicity index of AH and tocopherol

4.3 Experiment 3: Antioxidant, mitogenic and immuno-modulatory potentials of AH

Introduction

High dose of antioxidants may increase the chances of making them pro-oxidants instead; as a result they may induce damaging effects on macromolecules like nucleic acids and protein. For example high doses of flavonoids are reported to be clastogenic in contrast to their potential to reduce oxidative DNA damage, retard growth of leukemia cells, obstruct cell signal transduction and induce cellular differentiation in cancer (Sanjeev *et al*., 2006). It has also been reported that the anti-mutagenic/pro-mutagenic and anti-oxidant/pro-oxidant activity of substance largely depends upon the levels consumed as well as the physiological conditions of the body (Christine and Smith, 2000). It was against this background that we determined the anti-proliferative, genotoxic and cytotoxic effects of AH on human peripheral blood lymphocytes by mitotic index and cytochalasin B block micronucleus assays, respectively.

Procedure

The anti-proliferative, genotoxic and cytotoxic effects of AH on human peripheral blood lymphocytes by mitotic index and cytochalasin B block micronucleus assays were determined as described under ‘Materials and Methods’ (section 3.6.1. page 134; section 3.7.1 page 136; section 3.7.2. page 137).

Results

A significant dose-dependent inhibition of the intensity of luminol and lucigenin-amplified Chemiluminescence was observed with different concentration of AH versus control; 0.125, 0.25, 0.5, 1, 1.5, 2 and 2.5% v/v for neutrophils and macrophages. Figure

33 and 34, illustrates the inhibition of the generation of Reactive Oxygen Species due to Zymosan stimulations for neutrophils with AH showing significant ($p<0.05$) inhibition based on the luminol-amplified pathway than the lucigenin-amplified pathway ($p>0.05$) as compared to control with IC_{50} of 0.2% v/v. As seen in Figure 35 and 36 from macrophages, significant ($p<0.05$) suppression of oxidants production in the presence of AH as compared with control being more active intracellular than extracellular from 1.5 to 2.5% v/v. The IC_{50}s were 0.24 and <0.125% v/v respectively. For mitotic index and cytochalasin B-block micronucleus assay, the concentration of AH was increased in order to see whether or not it has proliferative, cytotoxicity and genotoxicity effects. As seen in Figure 37, mitotic index decreased significantly ($p<0.05$) in a dose-dependent manner except from 2 to 4% v/v as compared with control. Furthermore, one micronucleus was detected at 0.5% v/v which is comparable with the control (Figure 38). Cytotoxicity index (Figure 38) was significantly ($p<0.05$) reduced in a concentration dependent manner when compared with the control. Nuclear division and cytokinesis-block proliferation indices were having similar trend with mitotic index (Figure 39). To verify and ascertain our results on the antioxidant potential observed, we attempted other *in vitro* experimental models which interestingly further corroborate with the other results. As depicted in Figure 40, AH possess a significant ($p < 0.05$) dose dependent inhibition of DPPH and superoxide anion radical with more pronounced effect against DPPH radical. Invariably, from Figure 41, the reducing power of AH was found to increase with increased in concentration.

Conclusion

It was observed that the Acacia honey possesses antioxidant, mitogenic and immuno-modulatory potentials.

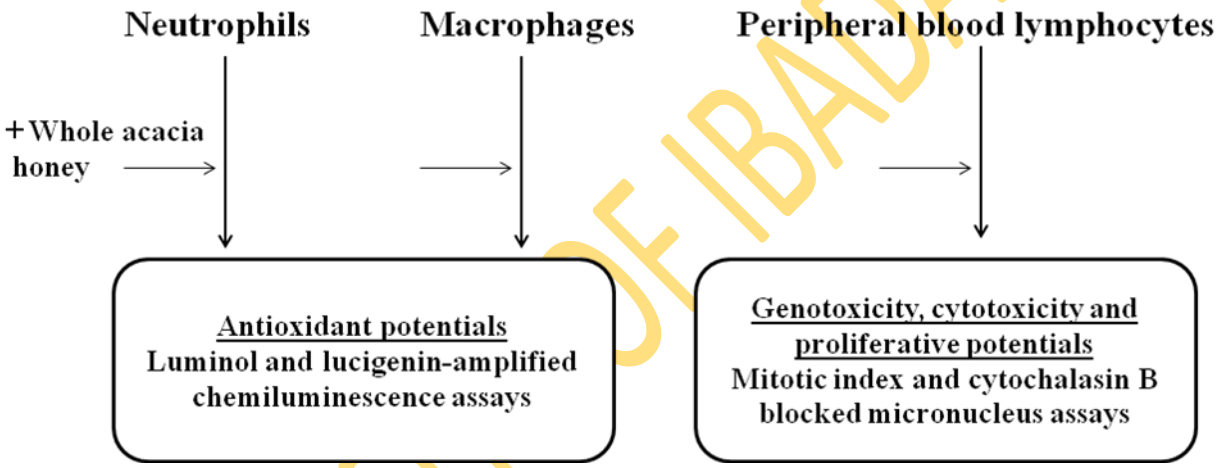

Figure 32: Antioxidant, mitogenic and immuno-modulatory potentials of AH

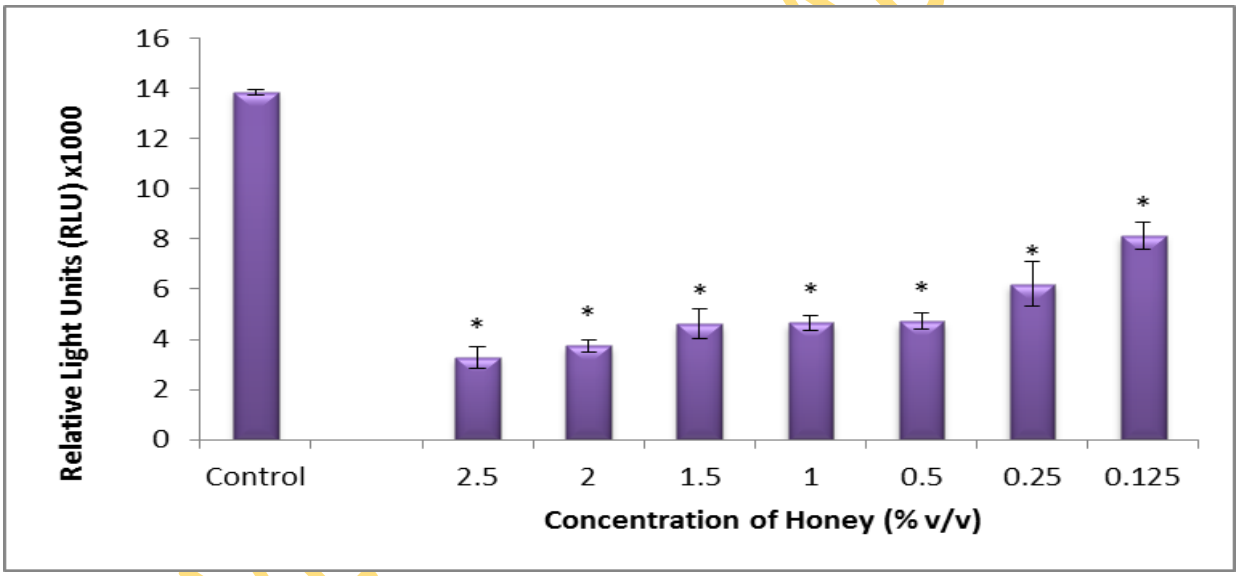

Figure 33: Luminol-amplified chemiluminescence assay results from neutrophils (1×10^6 cells/mL) after exposure to AH. *Statistical significant ($p<0.05$) as compared with a control.

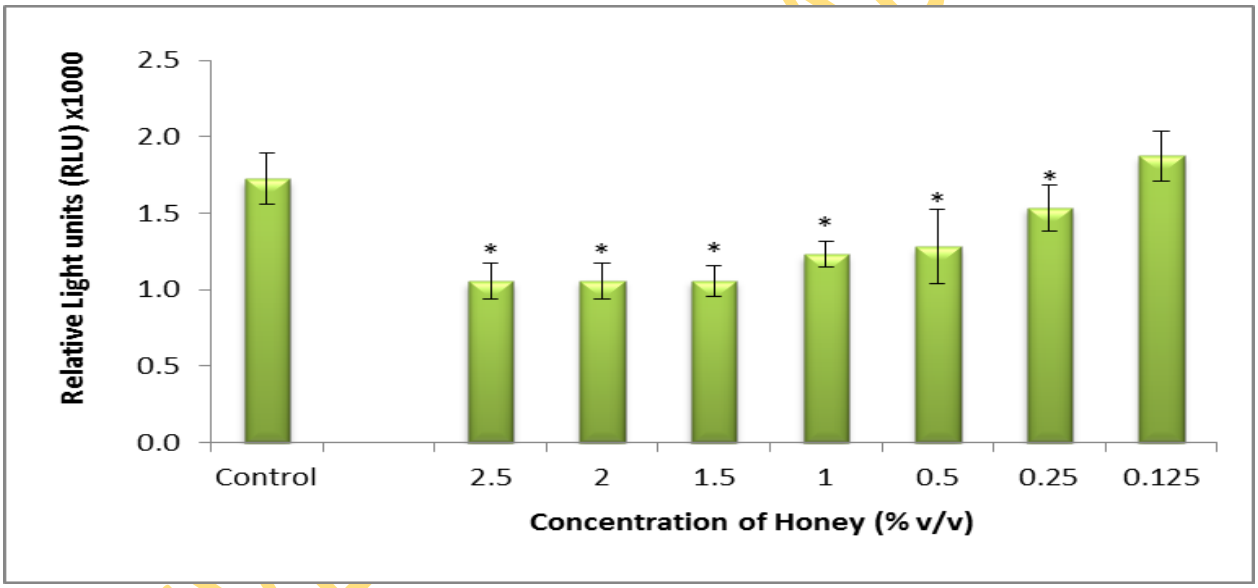

Figure 34: Lucigenin-amplified chemiluminescence assay results from neutrophils (1×10^6 cells/mL) after exposure to AH. *Statistical significant ($p<0.05$) as compared with a control.

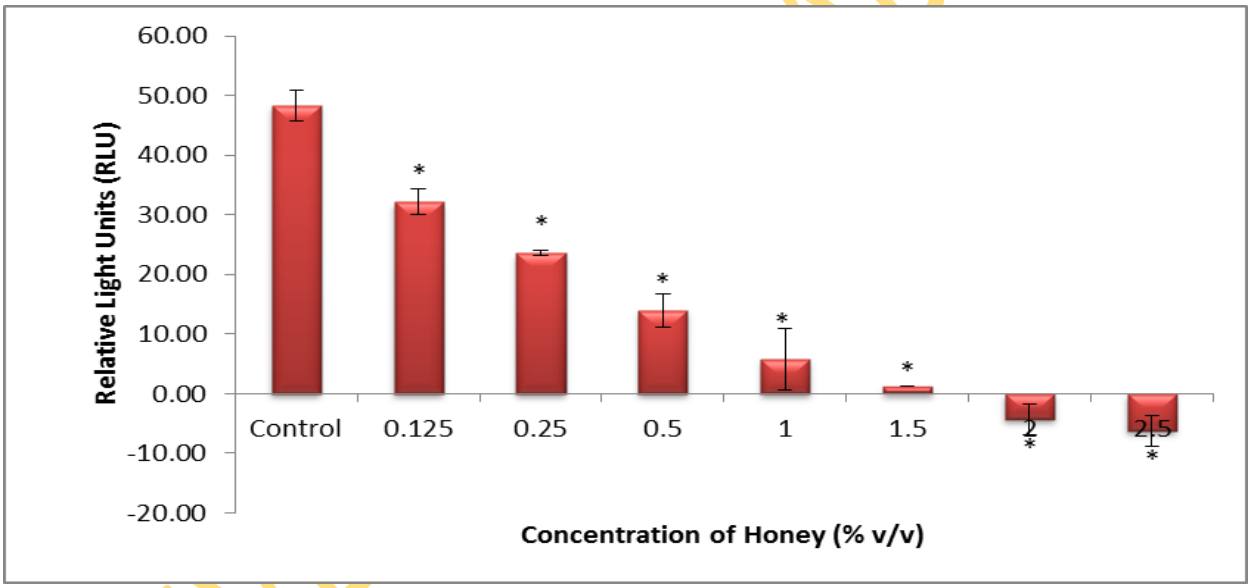

Figure 35: Luminol-amplified chemiluminescence assay results from Mouse macrophages (1×10^6 cells/mL) after exposure to AH. *Statistical significant ($p<0.05$) as compared with a control.

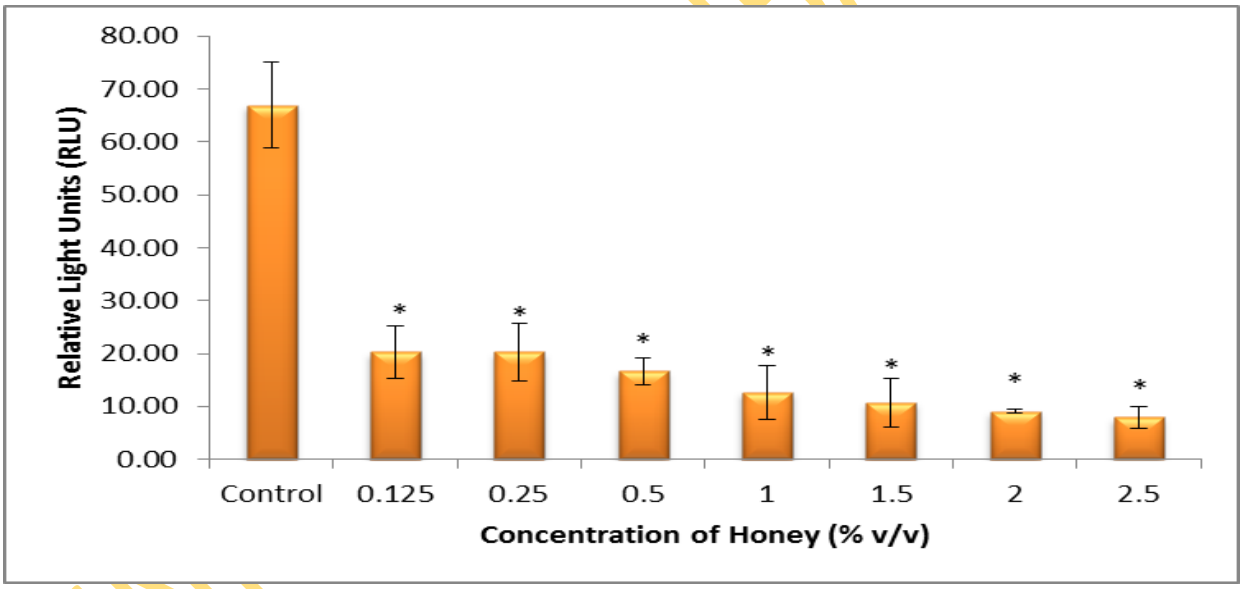

Figure 36: Lucigenin-amplified chemiluminescence assay results from Mouse macrophages (1 × 10^6 cells/mL) after exposure to AH. *Statistical significant ($p<0.05$) as compared with a control.

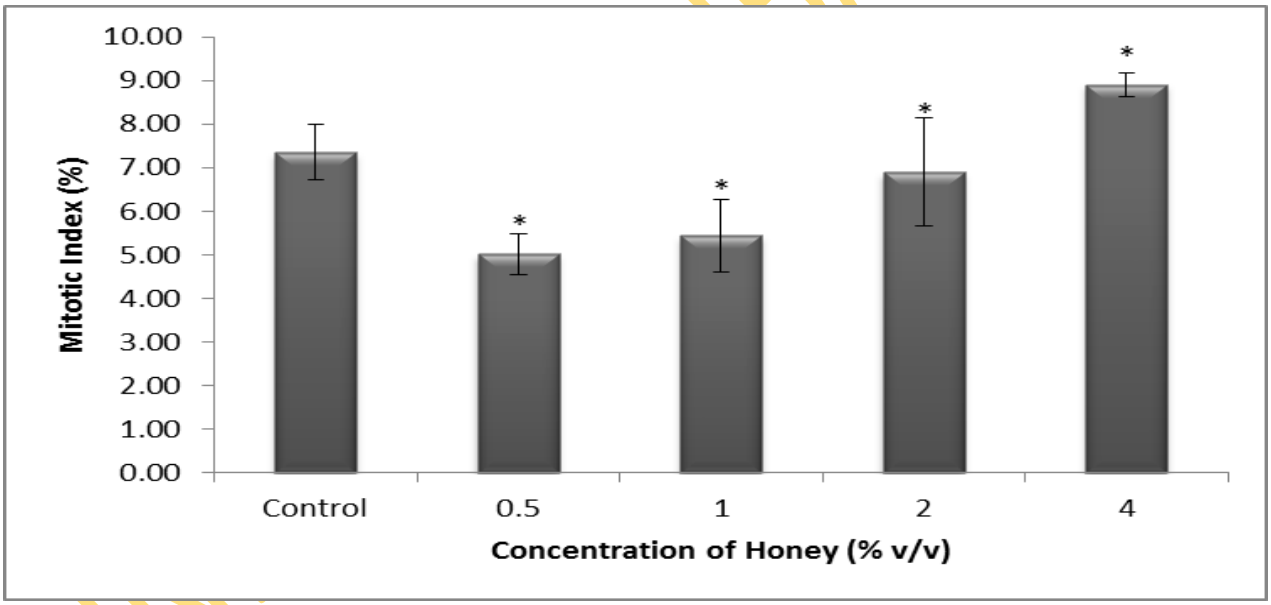

Figure 37: Results of mitotic index assay of Human peripheral blood lymphocytes after exposure to AH. *Statistical significant ($p<0.05$) as compared with a control.

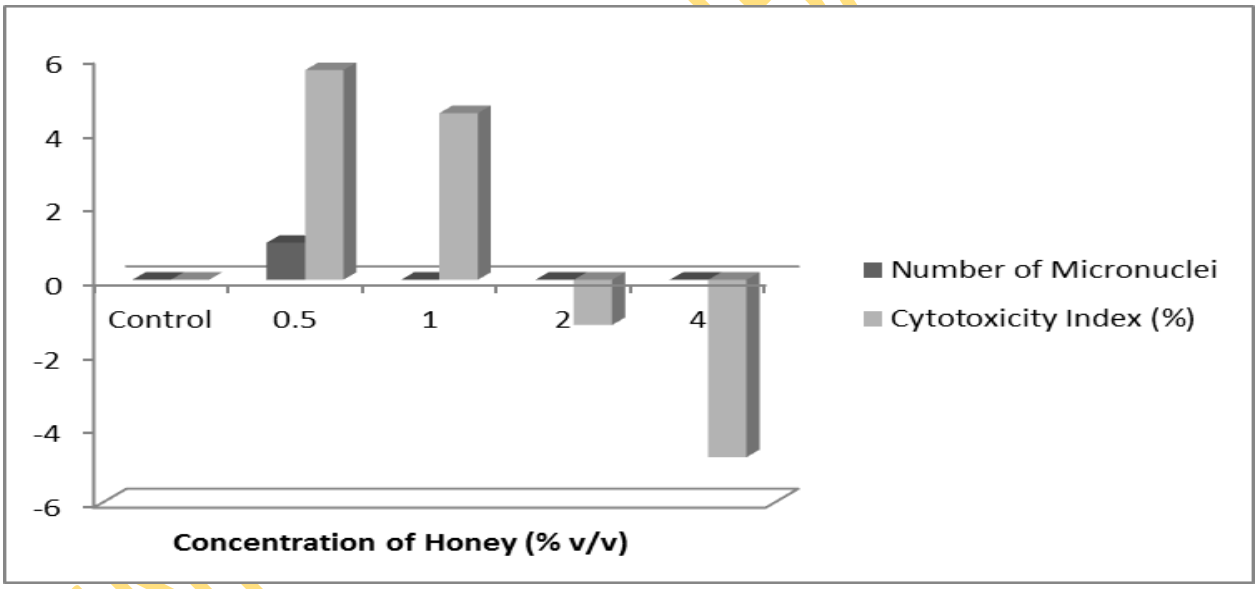

Figure 38: Number of micronuclei and cytotoxicity index from cytochalasin B-blocked micronucleus assay of Human peripheral blood lymphocytes after exposure to AH.

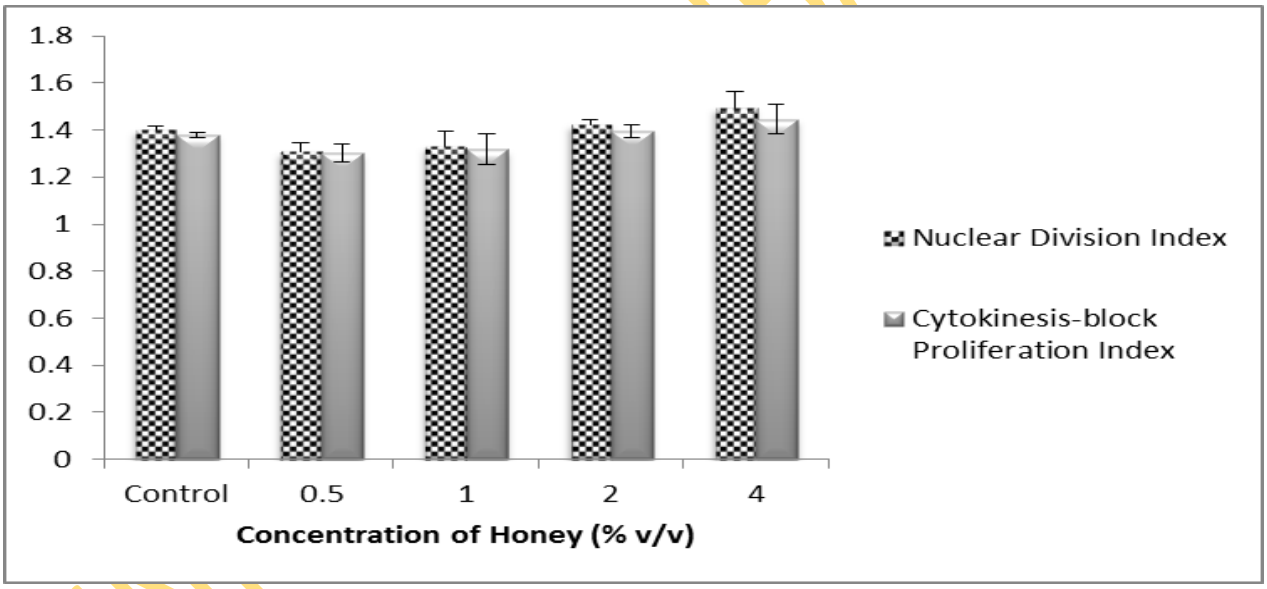

Figure 39: Nuclear division and cytokinesis-block proliferation index from cytochalasin B-block micronucleus assay of Human peripheral blood lymphocytes after exposure to AH.

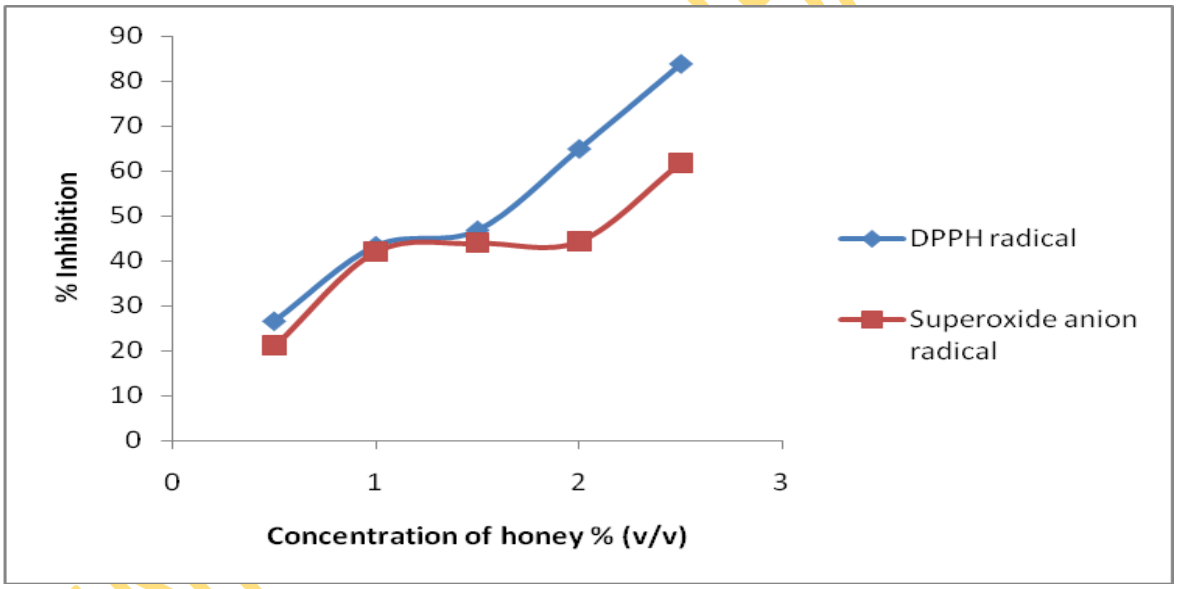

Figure 40: Percentage (%) inhibition of DPPH and superoxide anion radical by AH.

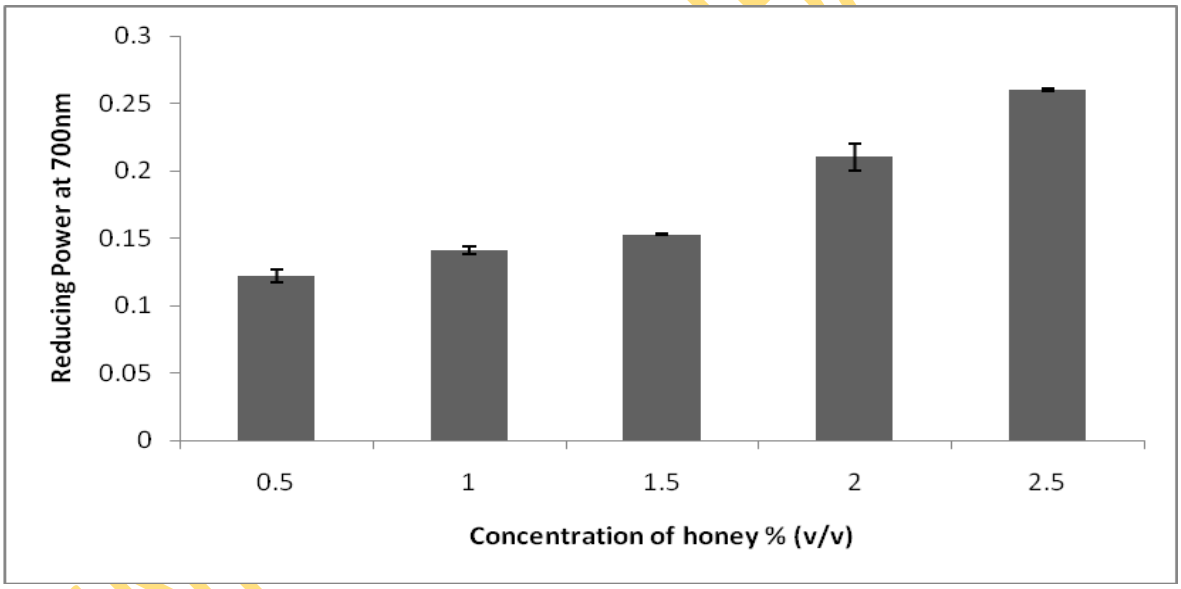

Figure 41: The Reducing power of AH at different concentration.

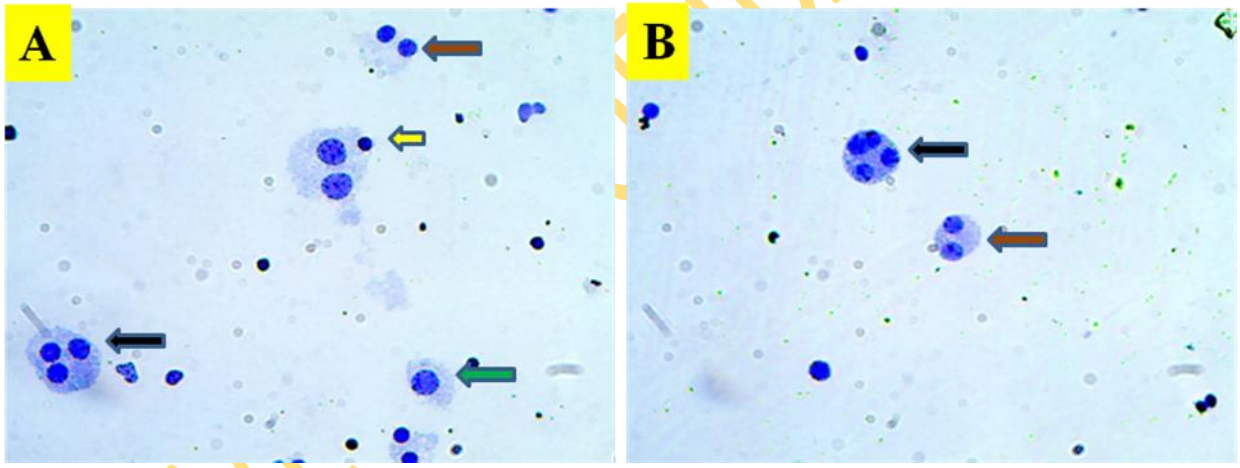

Figure 42: **A**, Plate showing mononucleated (green arrow), binucleated (orange arrow), trinucleated (black arrow) and binucleated cell with micronucleus (yellow arrow) from cultured Human peripheral blood lymphocytes after exposure to AH (0.5%). **B**: Plate showing the same thing without micronucleus (control) using cytochalasin B-block micronucleus assay (× 20 magnifications, Giemsa stain).

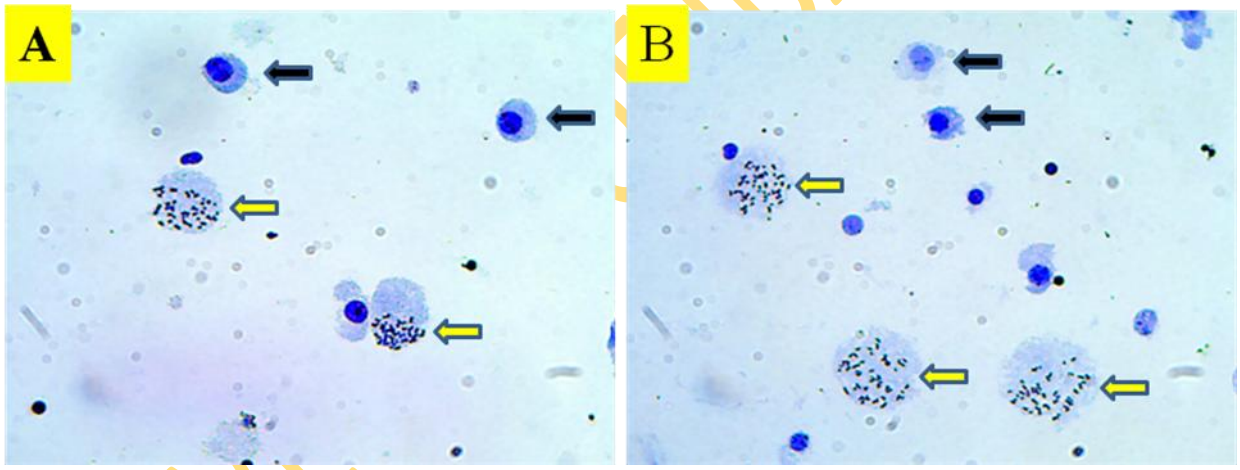

Figure 43: **A** (control) and **B** (in the presence of AH) Plates showing cells at the interphase (black arrow) and metaphase stages (yellow arrow) of mitosis from cultured Human peripheral blood lymphocytes after exposure to AH using mitotic index assay (× 20 magnification, Giemsa stain).

4.4 Experiment 4a and 4b: Anticancer Effects of Acacia Honey in some Cancer Cell lines

Introduction

Cancer is one of the leading causes of death worldwide (Hermann, 2013). Cancer curative properties of honey have been documented (Carnwath *et al.*, 2014; Mohammed *et al*., 2013). However, there is dearth of information on the exact mechanism of anticancer effect of honey. It was based this that the effects of AH on selected cancer cell lines proliferation through cell cycle, cytokines, calcium ion and expression of p53 & Bcl-2 genes analyses was evaluated in the present study.

Procedure

Cytotoxicity, cytokines, calcium ions, cell cycle and expressions of genes (p53 & Bcl-2) analyses were assayed for as described under 'Materials and Methods' (section 3.8. page 140; 3.8.1 page 140-148; section 3.8.2. page 149-150; section 3.8.3. page 151-154). The designs for experiment 4a and 4b are presented in Figure 48 and 58, respectively.

Results for Experiment 4a

As seen in Figure 49, AH was able to significantly ($p < 0.05$) inhibit the growth of 3T3 cell line in a concentration dependent manner with an IC_{50} of 3.7 % (v/v). 2, 4, 6, 8 and 10 % (v/v) were used in order to evaluate the cytotoxicity of AH on normal cell lines and from our results; it indicated that the AH is cytotoxic at about 4 % (v/v). Probably, this might be as a result of the generation of hydrogen peroxide due to oxidation of monosaccharide present in AH which has been implicated in initiating apoptosis. Figure 50 shows the anticancer activity of AH on PC-3 cell line with an IC_{50} of 1.9 % (v/v).

This indicates that it is more cytotoxic to cancer cells than the normal ones. However, in Figure 51, AH was only able to confer its anticancer activity after 24 hrs incubation with an IC_{50} of 4.43 % (v/v). On replacement of AH with just a medium, there was a reverse of anticancer activity with an IC_{50} >8.0 % (v/v) which was the highest concentration used. By implication, AH seems to be more active against PC-3 cells with increased in incubation period. As depicted in Figure 52, there was a significant ($p < 0.05$) increase in calcium ion level from 24 to 48 hrs incubation periods with AH. However, the level declined after replacement of the AH with the medium. This indicates that there is a calcium ion involvement on the anticancer activity of AH providing an evidence of apoptotic role as calcium ion is a mediator of programmed cell death. In Figure 53, TNF-α level increased significantly ($p < 0.05$) after 24 hrs incubation at the lowest concentration. However, the level declined with increase in concentration of AH. After 48 hrs incubation, the level was drastically reduced as compared to 24 hrs. This suggests that AH was able to modulate TNF-α expression by acting as an anti-inflammatory agent. This means that more TNF-α is being utilized for apoptotic pathway rather than inflammation which is a key factor for the survival of cancer cells. Furthermore, from Figure 54, IL-1β level increases significantly ($p < 0.05$) at the lowest concentration and start declining with increase in concentration after 24 hrs incubation indicating anti-inflammatory role. Surprisingly, the level elevated after 48 hrs incubation with increase in concentration of AH. This might be as a result of the fact that much of TNF-α has been utilized in the production of IL-1β which also believed to play a key role in the release of calcium ion from endoplasmic reticulum. The sudden increase observed in control might be as a result of the cytokine's role in keeping the cancer cells alive. Therefore, it is propose that AH modulates this cytokine towards apoptotic rather than inflammatory role by virtue of the increased in calcium

ion level. From Figure 55, the level of prostate specific antigen decreased from 2-8% (v/v) of AH indicating the effect on its expression. Similarly in Figure 56, mitotic index of PC-3 cell line decreases significantly ($p < 0.05$) in a concentration dependent manner. In fact we could not detect appreciable number of cells at 8 % (v/v). This is not surprising because the IC_{50} was 1.9 % (v/v). Arrest of cell cycle phases is one of the important steps towards anticancer activity. From the results (Figure 57), it indicates that AH was able to arrest G0/G1 phase significantly ($p < 0.05$) in a concentration dependent with concomitant decreased in % S phase and % G2/M phase as compared with control.

Conclusion for Experiment 4a

Apoptotic role of acacia honey on PC-3 Cell line may be due to G0/G1 arrest, modulation of Pro-inflammatory Cytokines, Calcium ions secretion and down regulation of Prostate specific antigen *in vitro*.

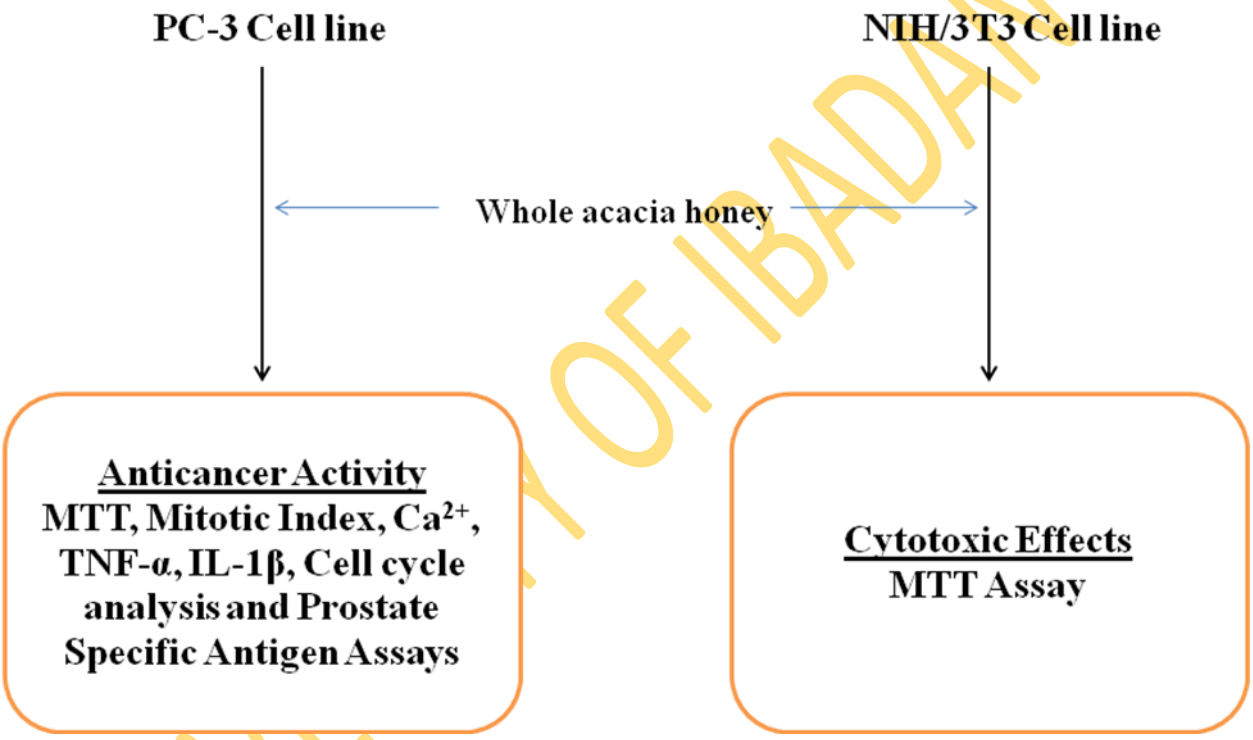

Figure 48: Anticancer activity of AH on PC-3 cell line and its cytotoxic effects on NIH/3T3 cell line

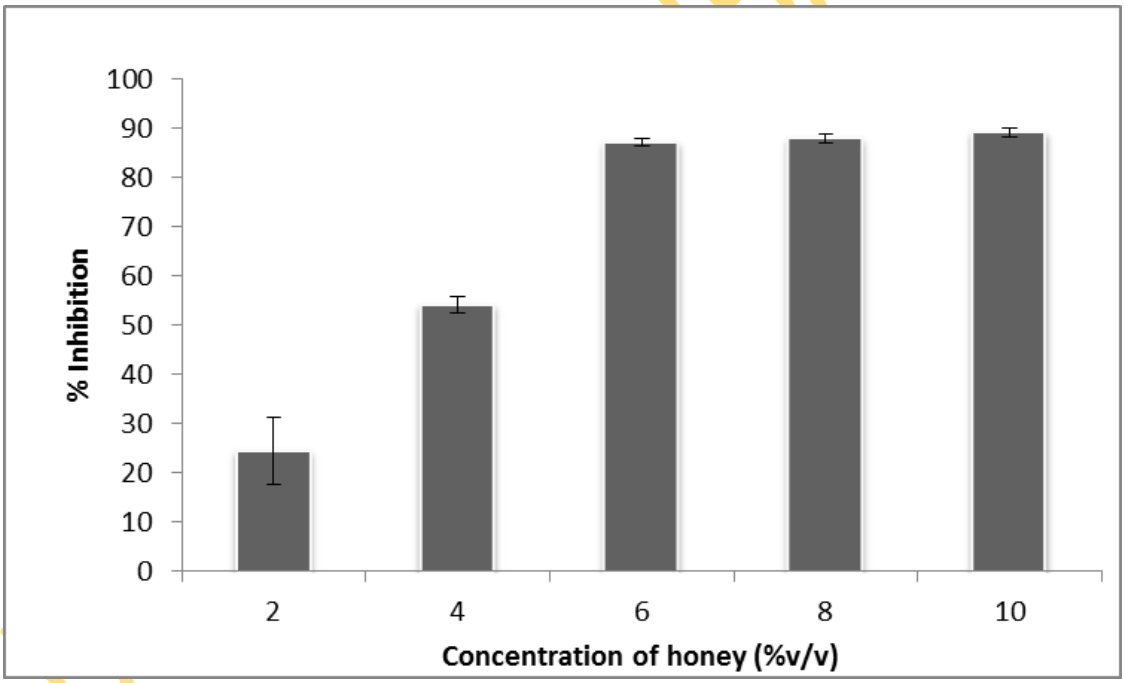

Figure 49: Percentage (%) inhibition on the cytotoxicity of AH on NIH/3T3 normal cell line

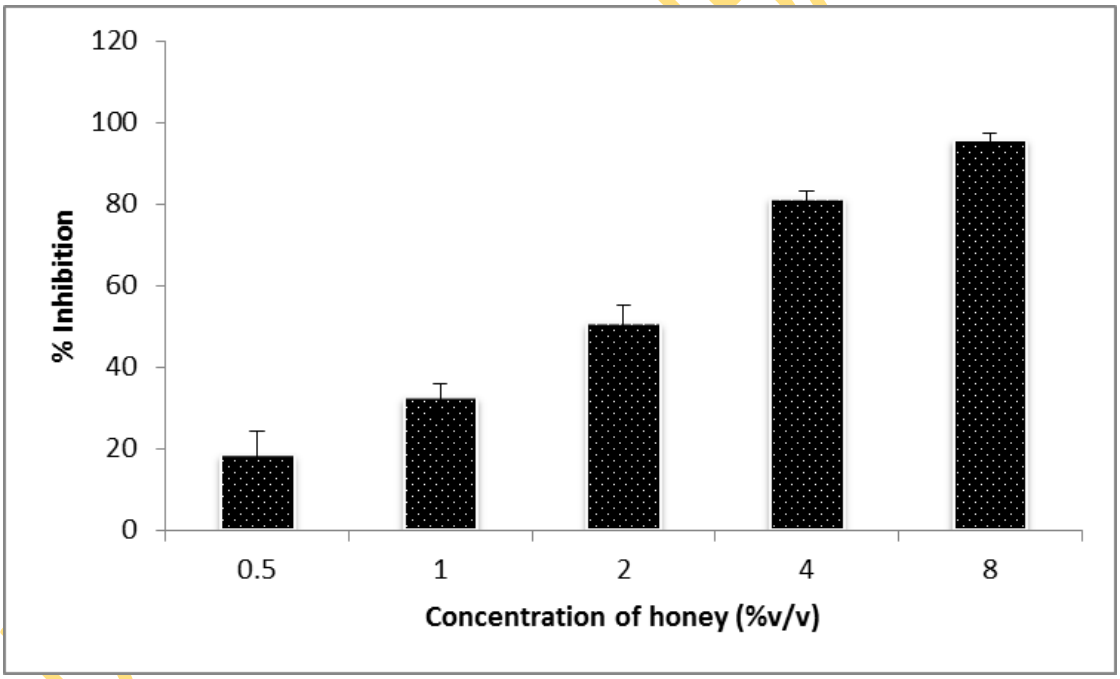

Figure 40: Percentage (%) inhibition on the cytotoxicity of AH on PC-3 cell line after 48 hrs incubation period.

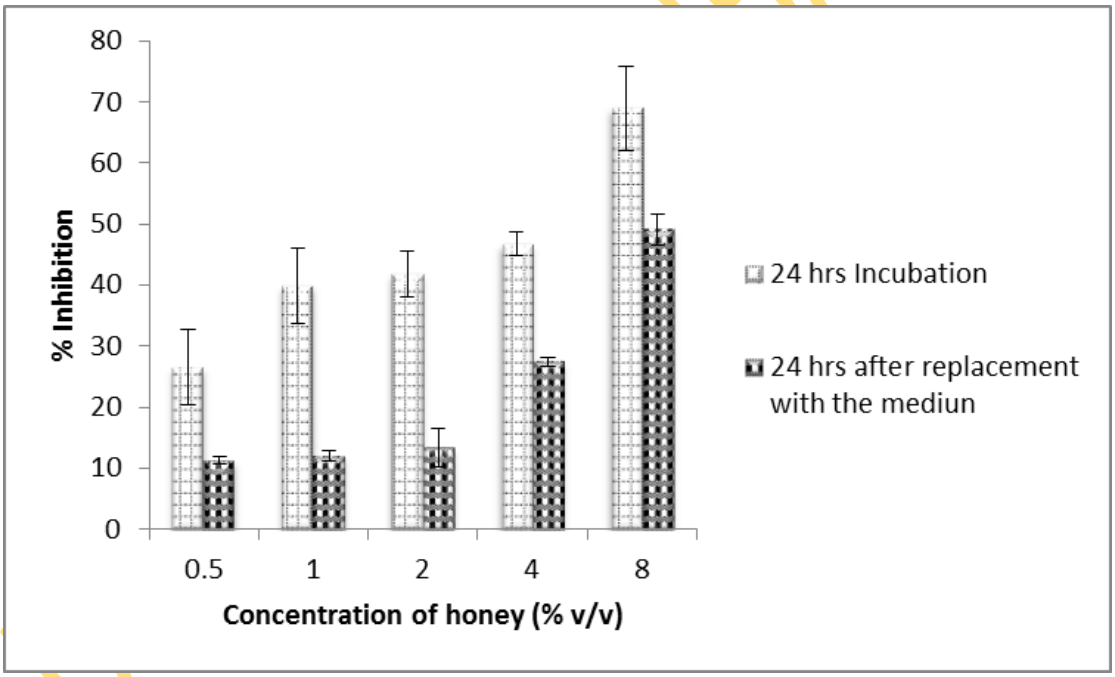

Figure 51: Percentage (%) inhibition on the cytotoxicity of AH on PC-3 cell line for 24 hrs incubation and after replacement with medium

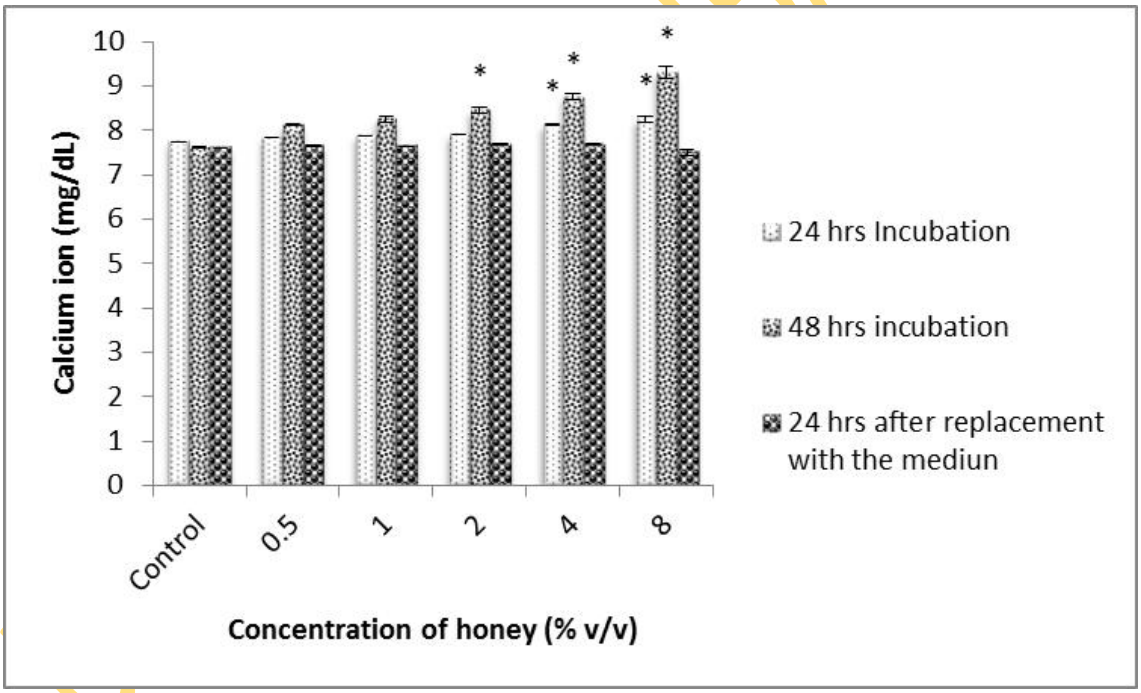

Figure 52: Calcium ion level from cultured supernatant of PC-3 cell line at different incubation time and replacement of AH with fresh medium. *statistical significant ($p < 0.05$) as compared with control

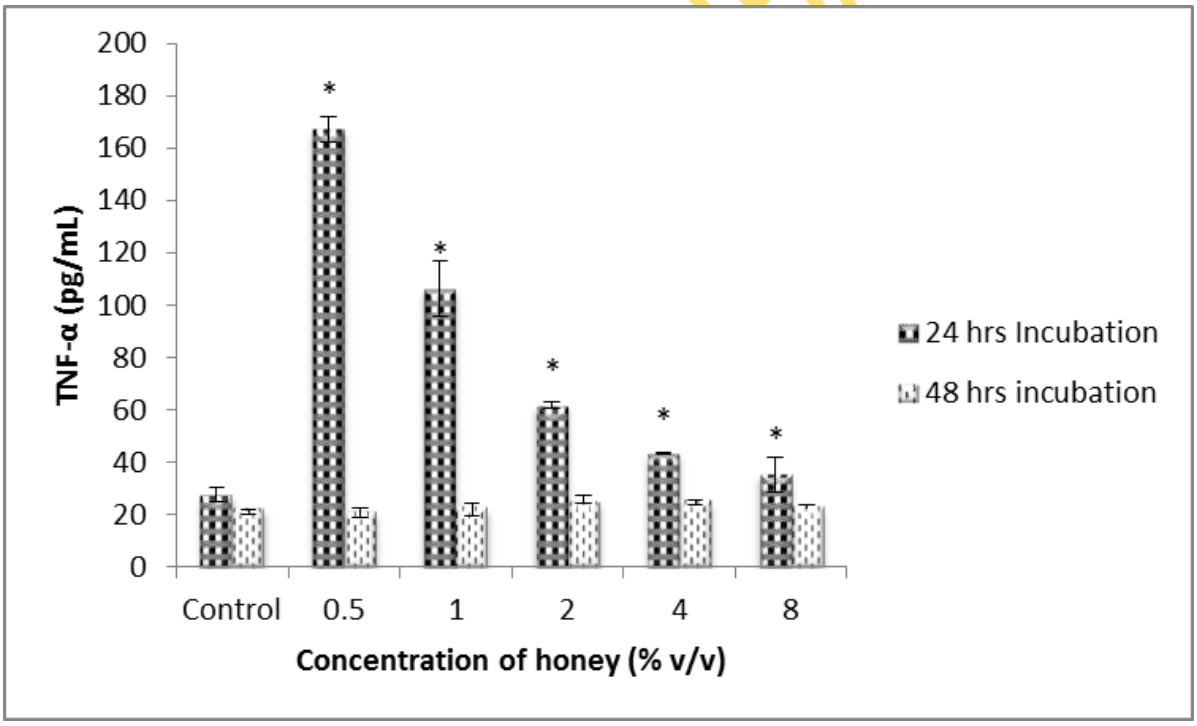

Figure 53: TNF-α level from cultured supernatant of PC-3 cell line after 24 and 48 hrs incubation periods. *statistical significant ($p < 0.05$) as compared with 48hrs incubation

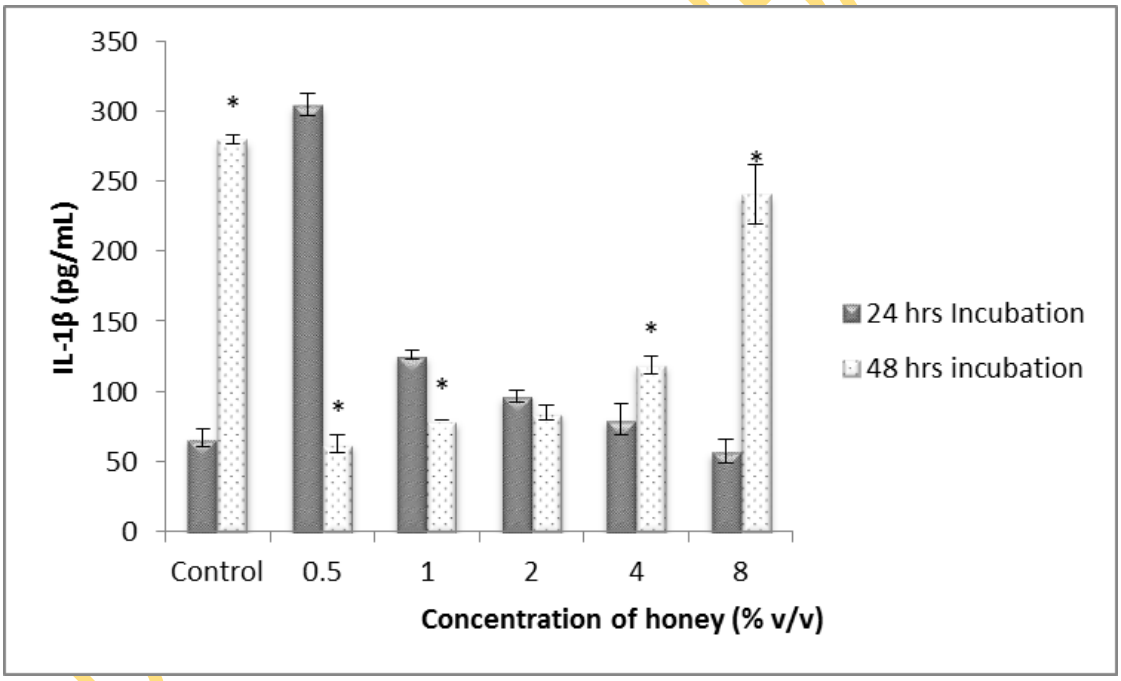

Figure 54: IL-1β level from cultured supernatant of PC-3 cell line after 24 and 48 hrs incubation periods. *statistical significant ($p < 0.05$) as compared with 24hrs incubation

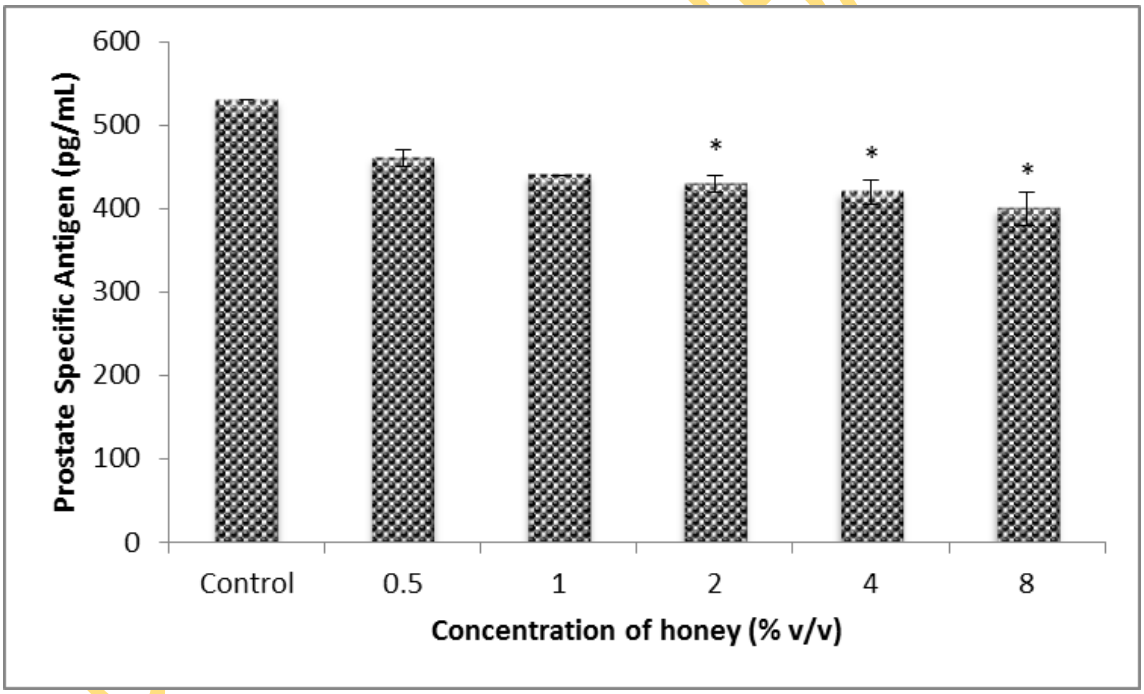

Figure 55: Prostate specific antigen level from cultured supernatant of PC-3 cell line after 48 hrs incubation period. *statistical significant ($p < 0.05$) as compared with control

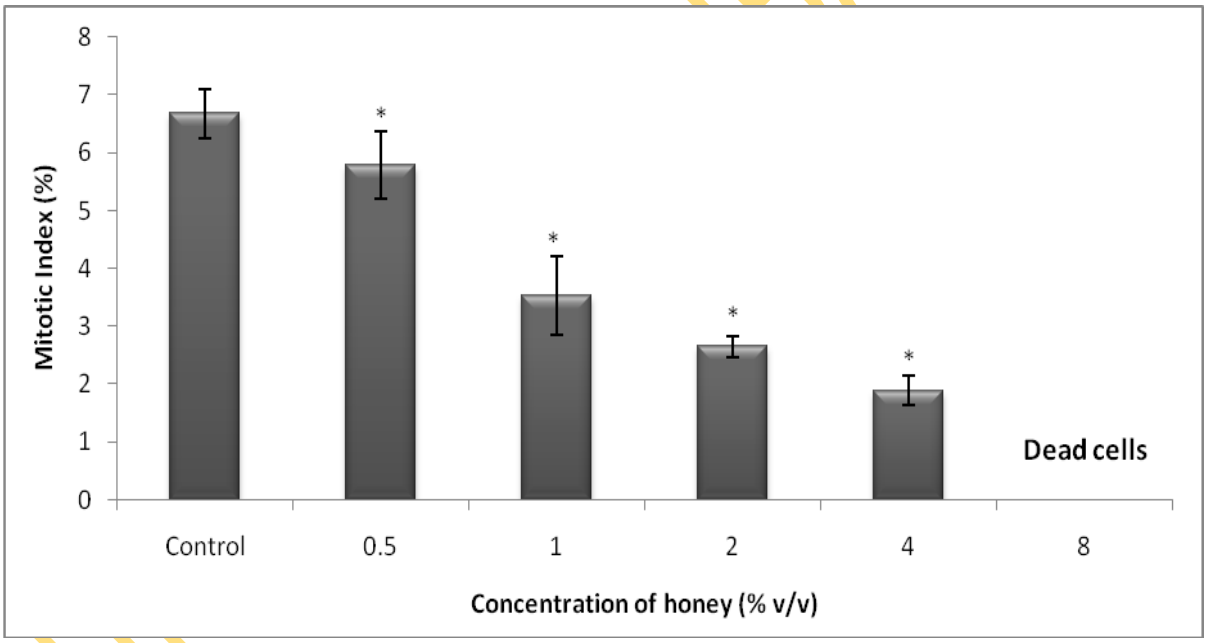

Figure 56: Percentage (%) mitotic index of PC-3 cell line after 48 hrs treatment with different concentration of AH. *statistical significant ($p < 0.05$) as compared with control

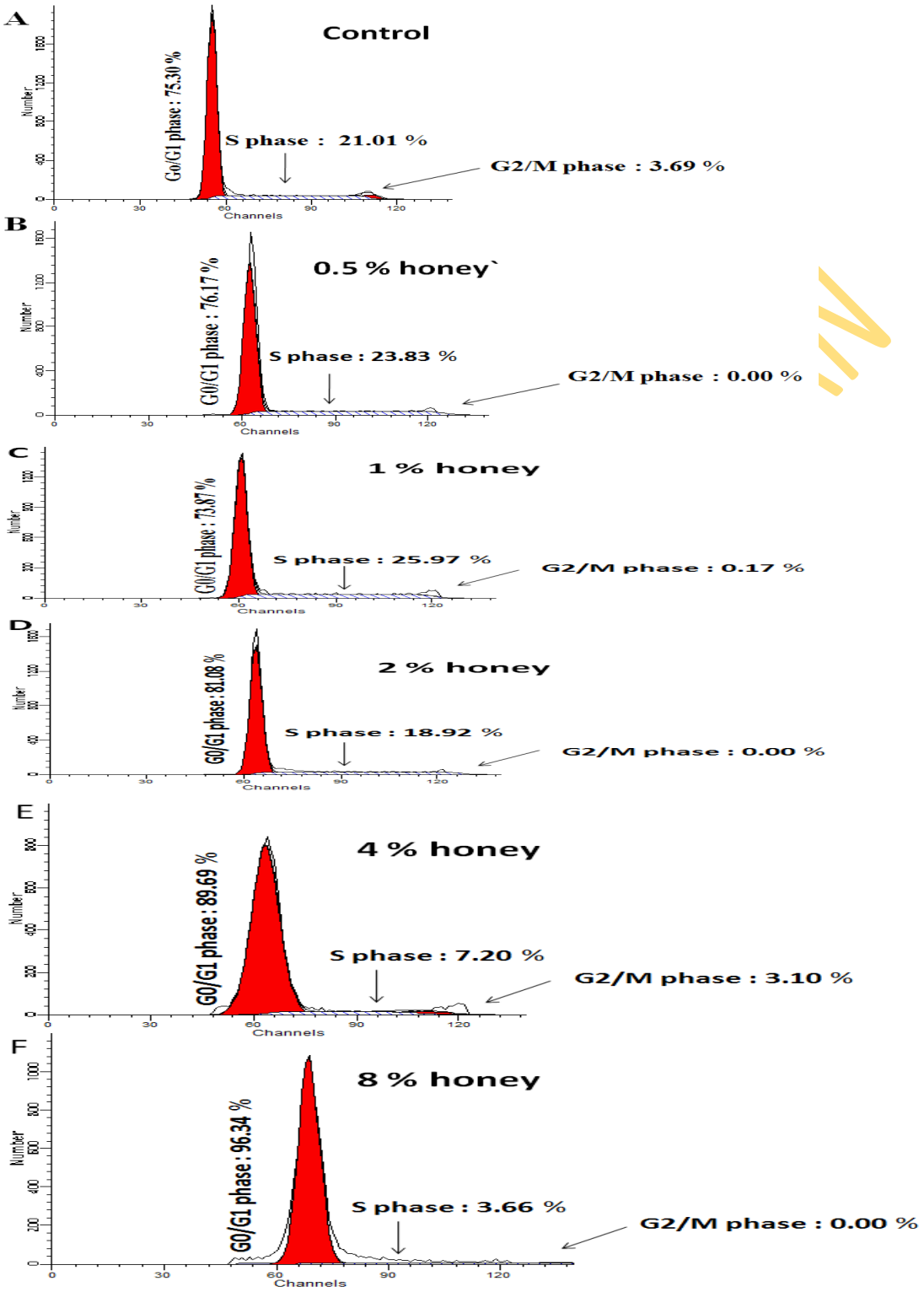

Figure 57: Results of cell cycle analysis on PC-3 cell line in the presence of AH

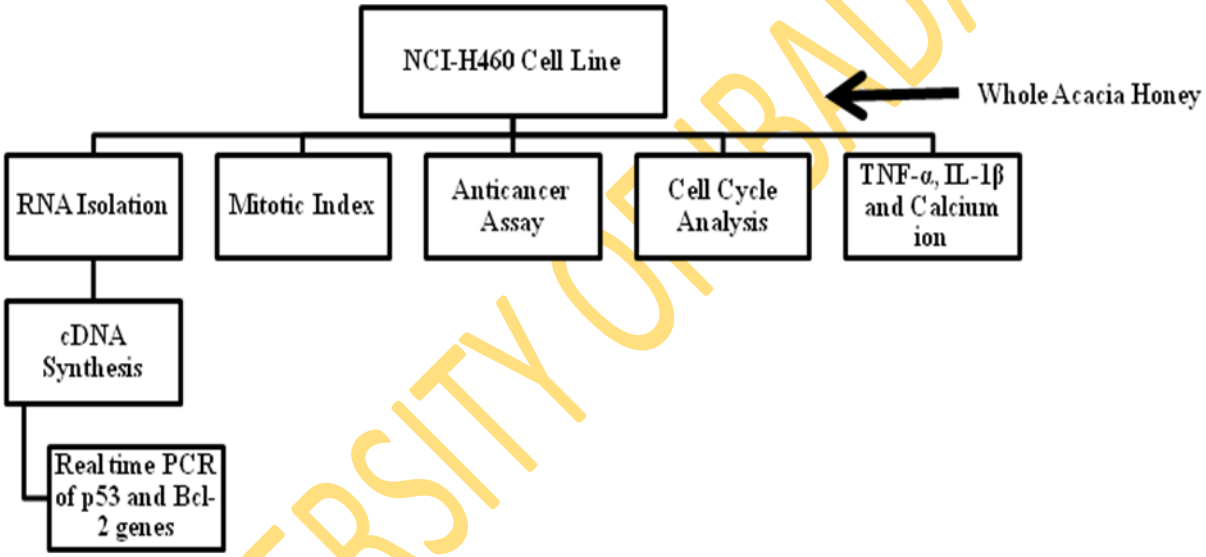

Figure 58: Anti-proliferative activity of AH on NCI-H460 cell line

Results for Experiment 4b

As seen in Figure 60, the % inhibition of AH on NCI-H460 cells was found to be significant ($p<0.05$) only at 8 % (v/v) with an IC_{50} of 7.69 % (v/v). This shows that the potency of the AH as an anticancer was only at higher concentration. However, we went further (Figure 61) to investigate the anticancer activity using fluorescence- activated cell sorting analysis which invariably showed the potency at the same concentration with more than 90 % inhibition. The variation might be due to sensitivity and sophistication of flow cytometry techniques. In order to confirm our observation we conducted mitotic index assay on the cells in the presence of AH. Our findings (Figure 62) indicated a significant ($p<0.05$) anti-proliferation potential of AH with increasing concentration as compared with control. From Figure 63 and 64, AH increased the production of TNF-α and IL-1β at the lowest concentration which starts declining from 2-4 % (v/v) as compared with control. Surprisingly, the level of these cytokines went up significantly ($p<0.05$) at the highest concentration which may explain the reason behind the anticancer activity at the same concentration as these cytokines play an important role in apoptosis. The increased observation at the lowest concentration is likely due to initial response of the cells towards the AH since these cytokines are immuno-modulators. Calcium ion is an apoptotic mediator and based on this it was quantified in the cultured supernatant. As seen in Figure 65, the concentration of AH was directly proportional to the level of calcium ions. These observations might provide a supportive evidence of the apoptotic role of AH because cytokines like IL-1β plays a role in the release of calcium ion from endoplasmic reticulum which can initiate apoptosis via direct and indirect mechanisms. Arrest of cell cycle phases is one of the important steps towards anticancer activity. The results (Figure 66) indicate that AH was able to arrest G0/G1 phase significantly ($p<0.05$) in a concentration dependent with concomitant

decreased in % S phase and G2/M phase as compared with control. In fact at higher concentration (4 and 8 %) appreciable number of cells in G2/M phase were not detected which corroborates the anticancer activity observed at the said concentrations. NCI-H460 cell is a p53 stable cell line with resistant to apoptosis due to up regulation of Bcl-2 gene. This means p53 gene here, promotes cell proliferation, survival and adhesion. Moderate (2 %) and higher concentrations (4 and 8 %) were used. From Figure 67, our research evidently from quantitative real time PCR indicates the ability of AH to down regulate the expression of p53 and Bcl-2 genes in a concentration dependent manner. By implication, this confirmed the anti-proliferative and apoptotic role of AH on NCI-H460 cell line. Figure 97 shows the proposed mechanism of action of anti-proliferation potential of AH on NCI-H460 cell line. p53 and Bcl-2 promote the growth of NCI-H460 cells and Bcl-2 also inhibits the action of TNF-α and IL-1β. AH suppressed the expression of p53, Bcl-2 gene and stimulates the production of TNF-α and IL-1β. IL-1β stimulates the release of calcium ion from endoplasmic reticulum. TNF-α and IL-1β as well as calcium ion stimulate apoptosis.

Conclusion for Experiment 4b

Molecular mechanism of the anti-proliferation potential of acacia honey on NCI-H460 cell lines may be due to cell cycle arrest, stimulation of cytokines and calcium ion as well as down regulation of Bcl-2 and p53 gene.

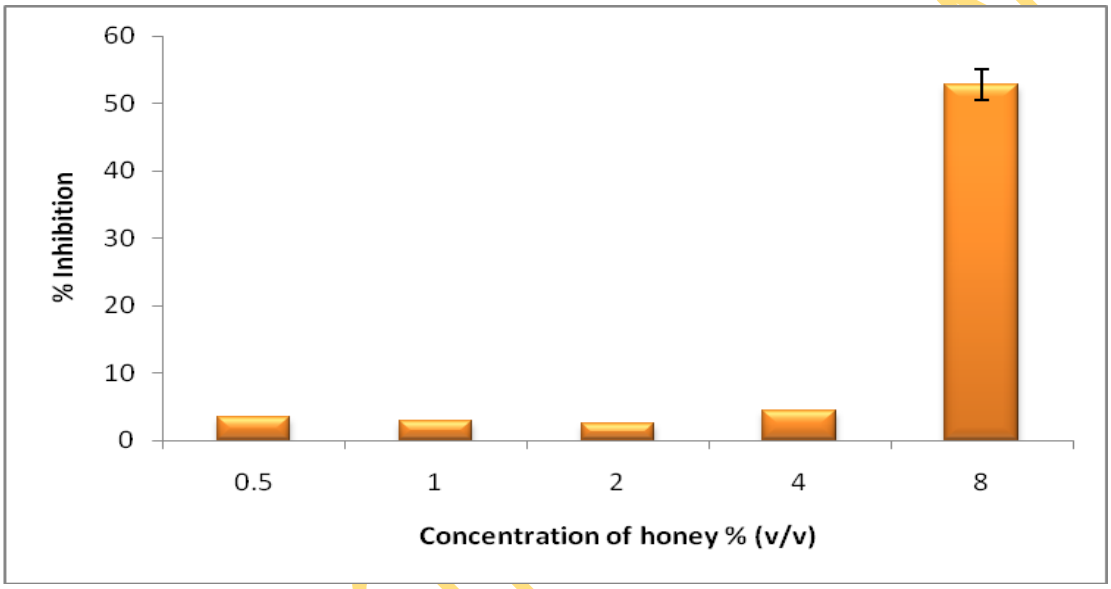

Figure 60: Percentage (%) inhibition on the proliferation of NCI-H460 cell line

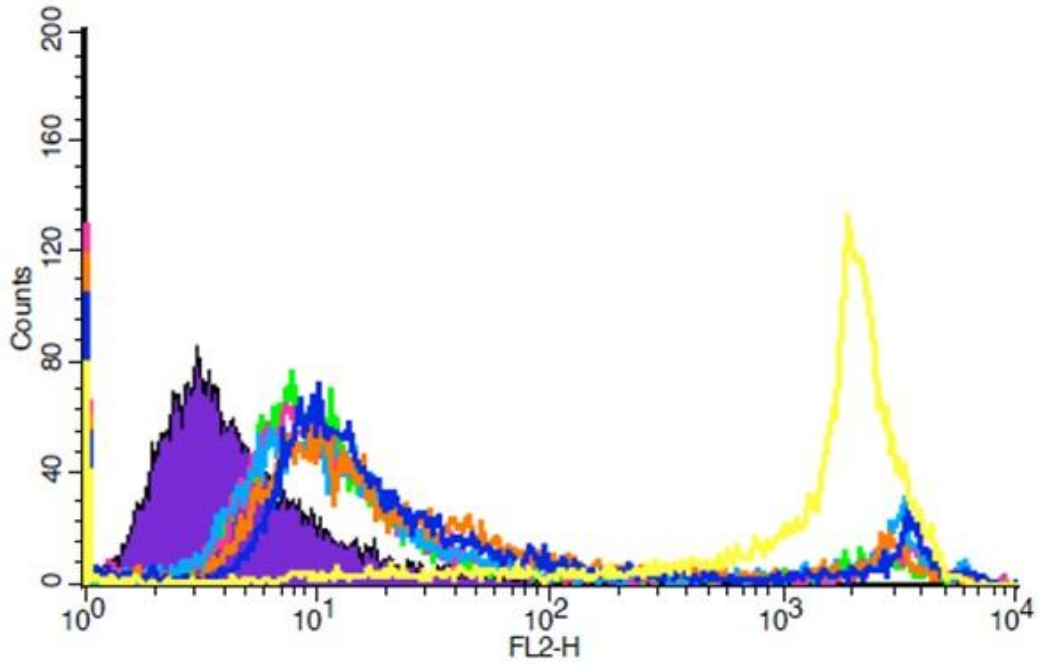

Figure 61: Decrease in the viability of NCI-H460 cell lines due to AH. Control before adding PI (purple), control after adding PI (green), 0.5% AH (pink), 1% AH (sky blue), 2% AH (orange), 4% AH (blue) and 8% AH (yellow).

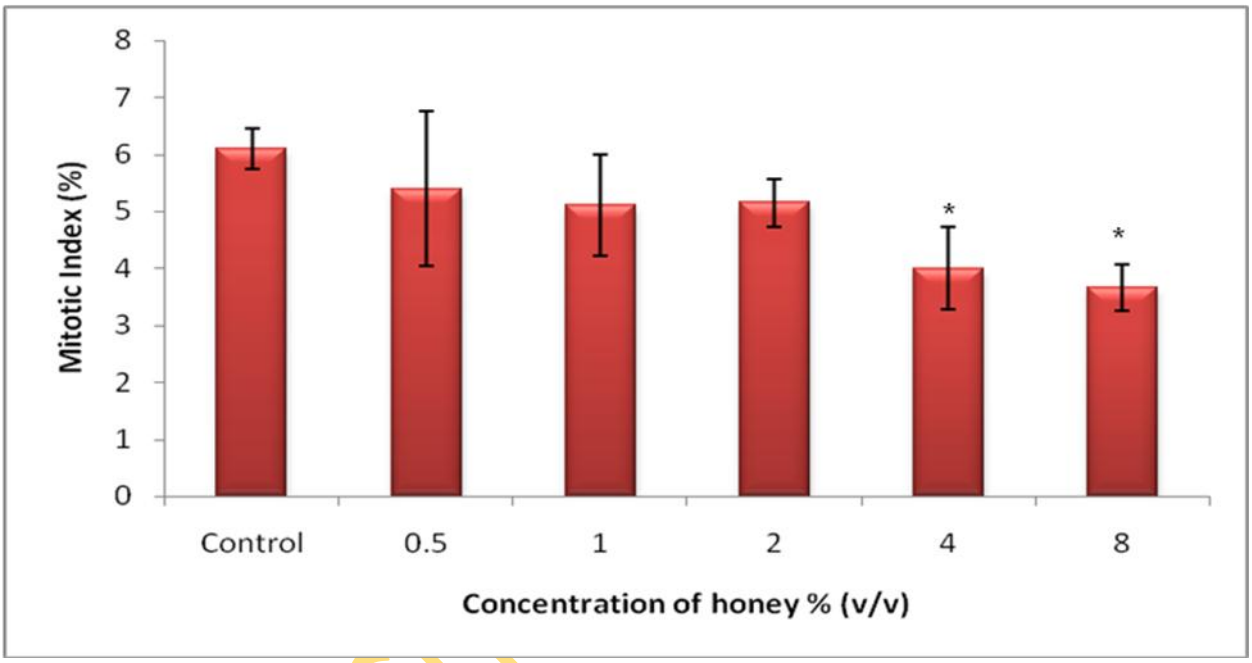

Figure 62: Mitotic index of NCI-H460 cell line after treatment with AH. * Statistical significant ($p < 0.05$) as compared with control.

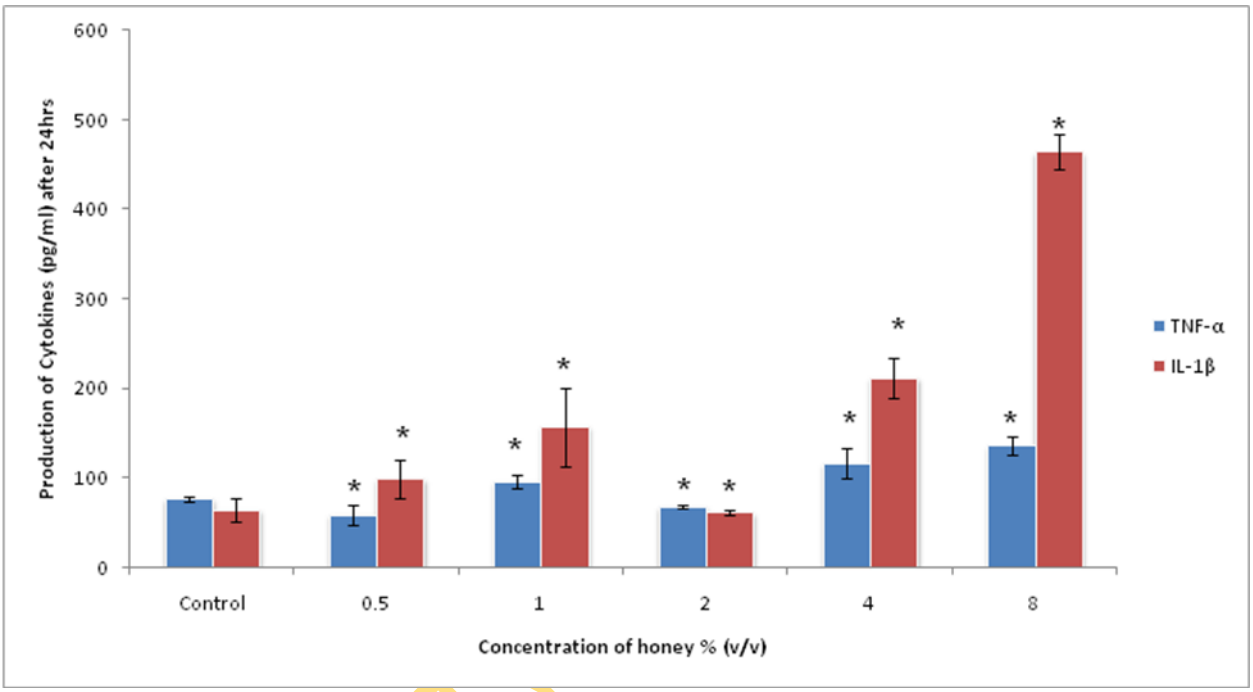

Figure 63: The level of cytokines from NCI-H460 cell line after treatment with AH for 24hrs. *statistical significant ($p < 0.05$) as compared with control

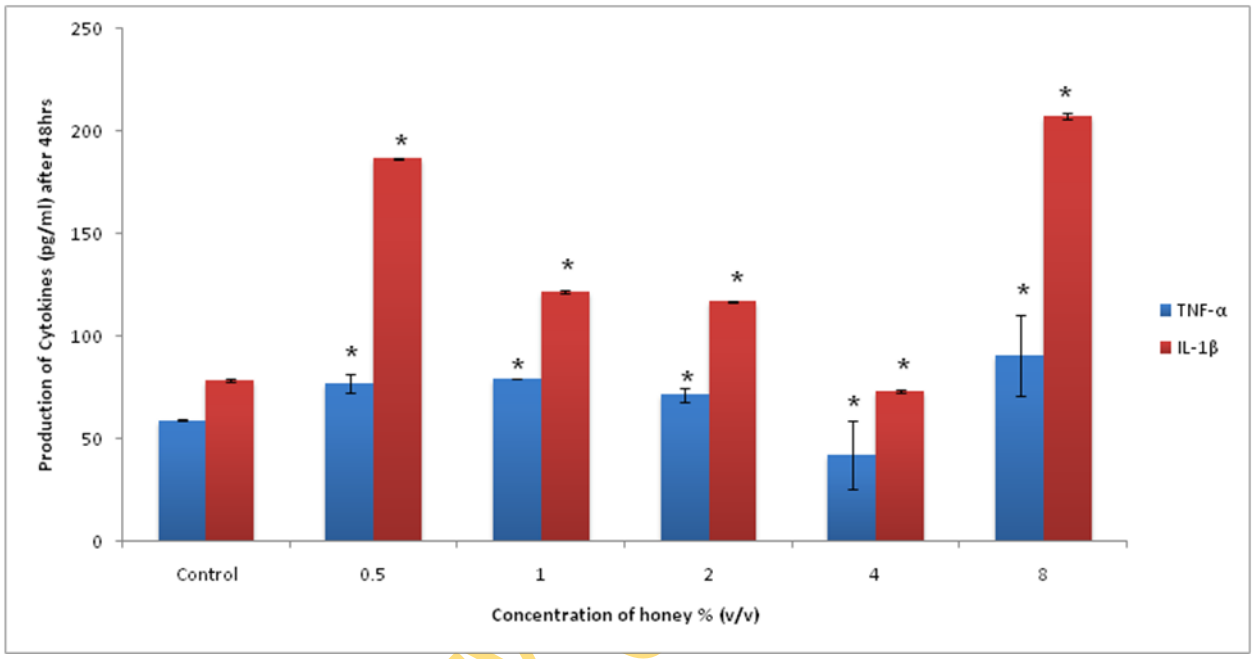

Figure 64: The level of cytokines from NCI-H460 cell line after treatment with AH 48hrs. *statistical significant ($p < 0.05$) as compared with control

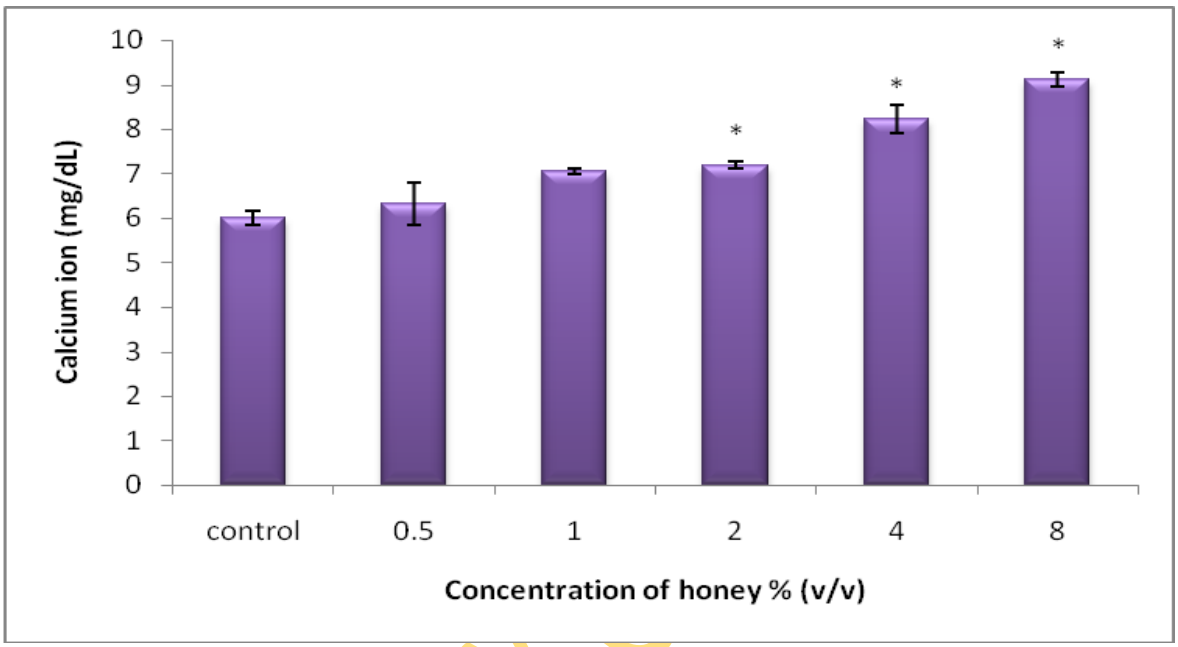

Figure 65: Calcium ion level of NCI-H460 cell lines in the presence of AH. *statistical significant ($p < 0.05$) as compared with control

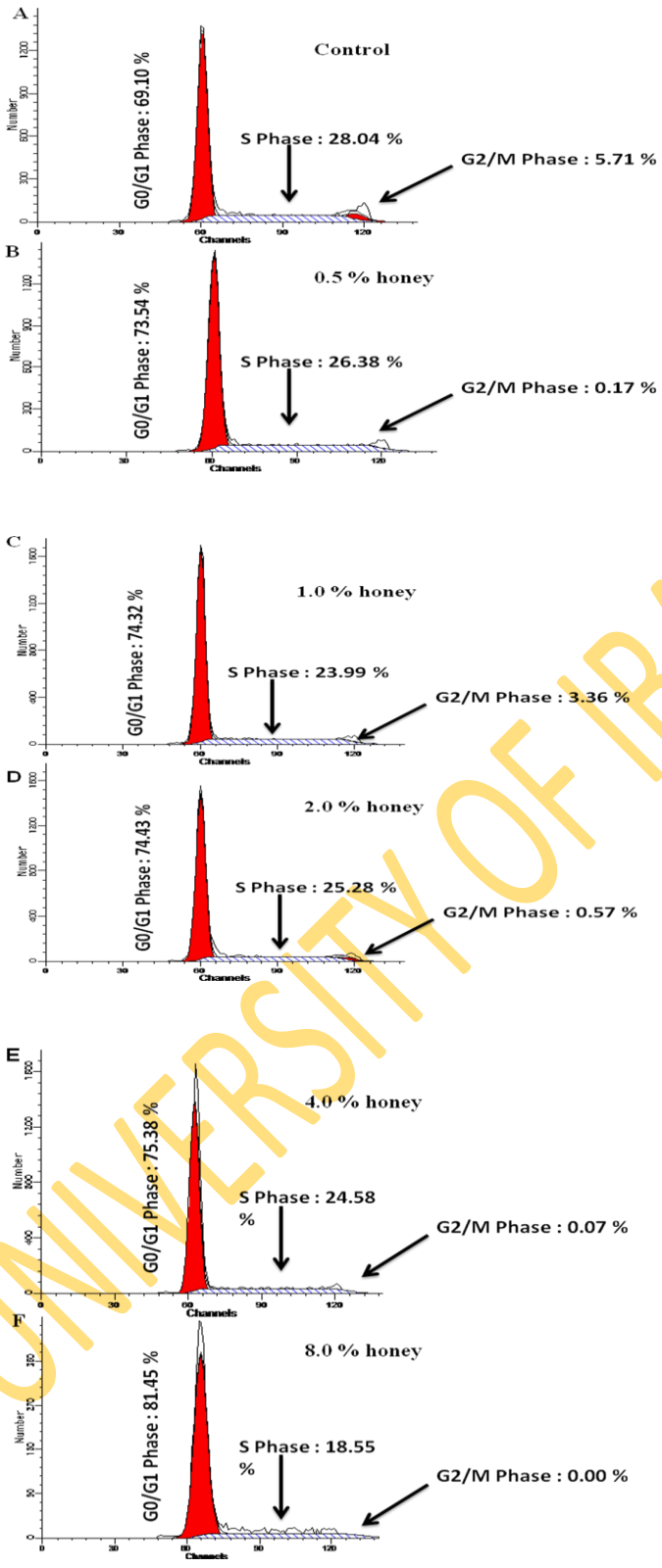

Figure 66: Cell cycle phase distribution of NCI-H460 cell line of after treatment with AH.

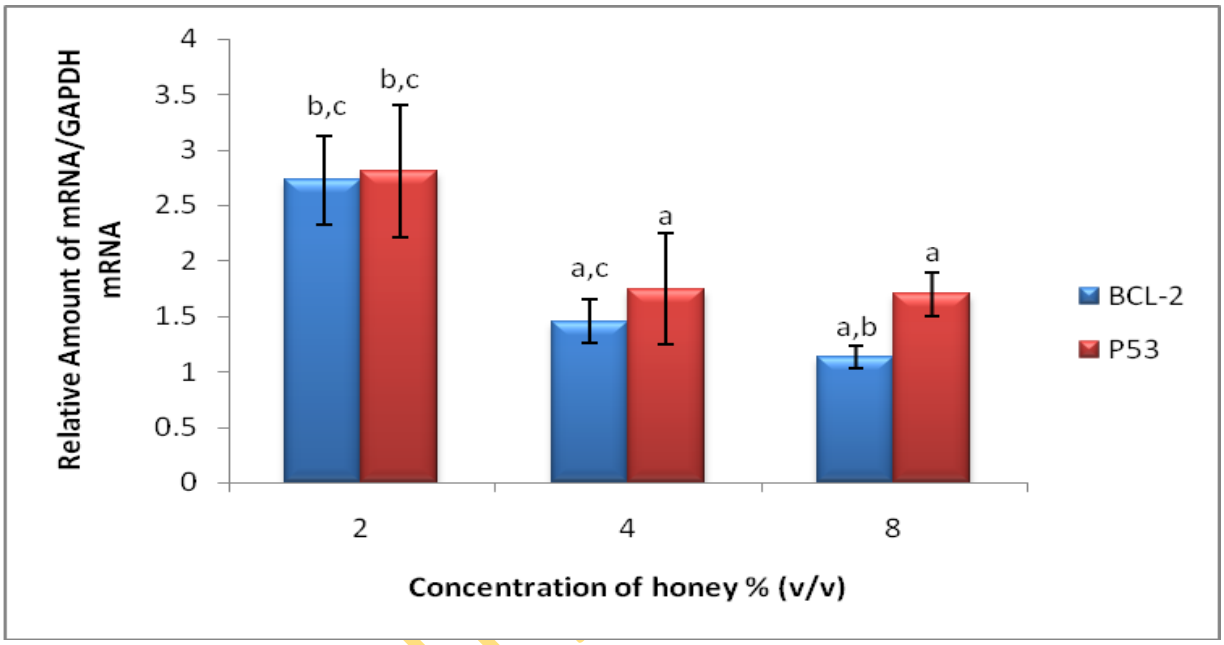

Figure 67: Expression of Bcl-2 and p53 gene of NCI-H460 Cell line in the presence of AH. a: statistical significant ($p<0.05$) as compared with 2 %, b: statistical significant ($p<0.05$) as compared with 4 %, c: statistical significant ($p<0.05$) as compared with 8 %.

4.5 Experiment 5: Effects of honey daily consumption on biochemical and haematological parameters in rats

Introduction

The main uses of honey are in cooking, baking, as a spread on bread, and as an addition to various beverages, such as tea, and as a sweetener in some commercial beverages. It consists of primarily sugars such as monosaccharides, disaccharides, oligosaccharides and polysaccharides (Bogdanov *et al.*, 2008; Erejuwa *et al.*, 2012). It contains enzymes such as glucose oxidase, diastase, invertase, catalase and peroxidase (Bogdanov *et al.*, 2008). Honey also contains other bioactive constituents such as organic acids, ascorbic acid, trace elements, vitamins, amino acids, proteins and Millard reaction products (Bogdanov *et al.*, 2008). It was based on this facts that the effects of AH daily consumption in Wistar rats was determined.

Procedure

Clinical, biochemical and haematological parameters were evaluated as described under 'Materials and Methods' (section 3.9. page 155-158; section 3.9.1. page 158-159). The experimental design is presented in Figure 68.

Results

Figure 69 shows the results of body weight of the experimental animals administered with AH. Significant ($p < 0.05$) increase in body weight was observed as the administration progressed except on 1st and 2nd days at 20% (v/v). Also within the treatment group, the body weight increased in a concentration dependent manner from 10% to 20% but started decreasing from 50% to 100% (v/v). There were no significant ($p < 0.05$) difference across the treatment groups based on the glucose (Figure 70).

However, there was a significant ($p < 0.05$) increased in ALP and ALT activity (Figure 70) in a concentration dependent manner, but surprisingly the activity dropped at 100% (v/v). Significant ($p < 0.05$) decrease in creatinine level was observed from 20% to 100% (v/v) treated groups indicating protection on kidney (Figure 71). The various treatment does not seem to have a significant ($p > 0.05$) effect on urea total protein, albumin, globulin and albumin/globulin ratio as shown in Figure 71. Similarly, from Figure 72, there were also no significant ($p > 0.05$) effects on cholesterol, HDL, LDL and cholesterol HDL ratio. However, significant ($p < 0.05$) decrease in triglyceride and VLDL were observed, indicating the fact that the treatments might not be having lipogenic effects. Figure 73 shows the effect on haematological parameters. No significant ($p > 0.05$) effect on Hb and RBC were observed with concomitant significant ($p < 0.05$) increased in PCV and WBC at 20% (v/v) treated group. However, the levels dropped with increasing concentration. Significant ($p < 0.05$) increase in PLT in a concentration dependent manner was also observed.

Conclusion

Daily consumption of Acacia honey might have some positive and negative effects on body weight, biochemical and hematological parameters depending on the concentration. Therefore, 20 % (v/v) AH was then selected as the optimal dose for subsequent experiments.

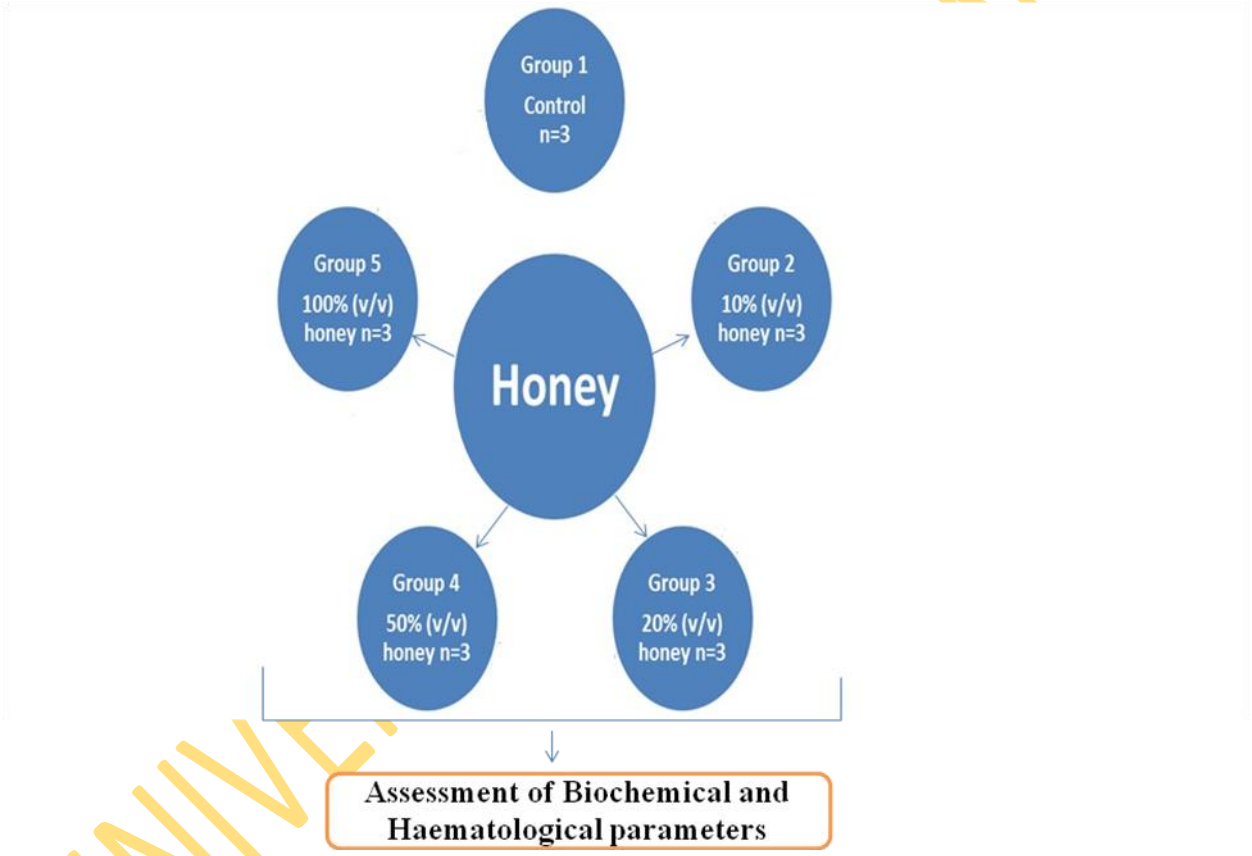

Figure 68: Effects of daily consumption of AH on male Wistar albino rats

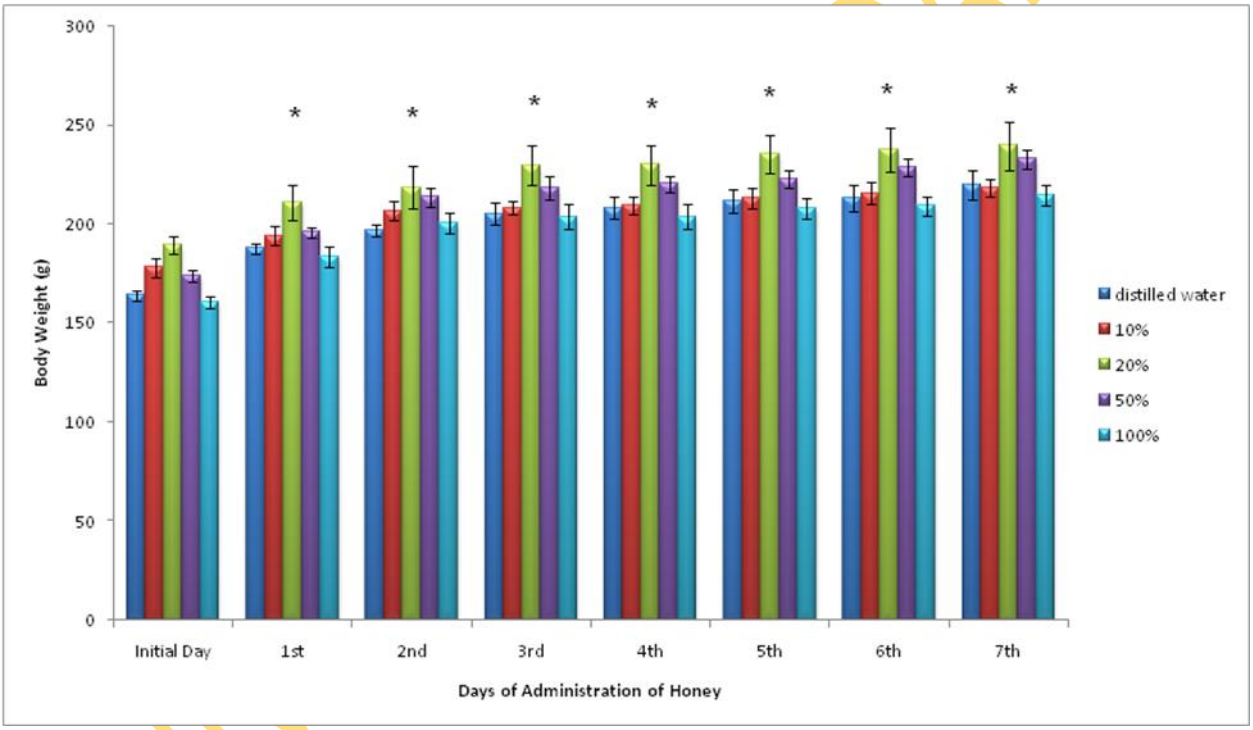

Figure 69: Results of body weight (g) of rats administered with AH for a period of one week. *statistical significant ($p<0.05$) as compared with initial body weight

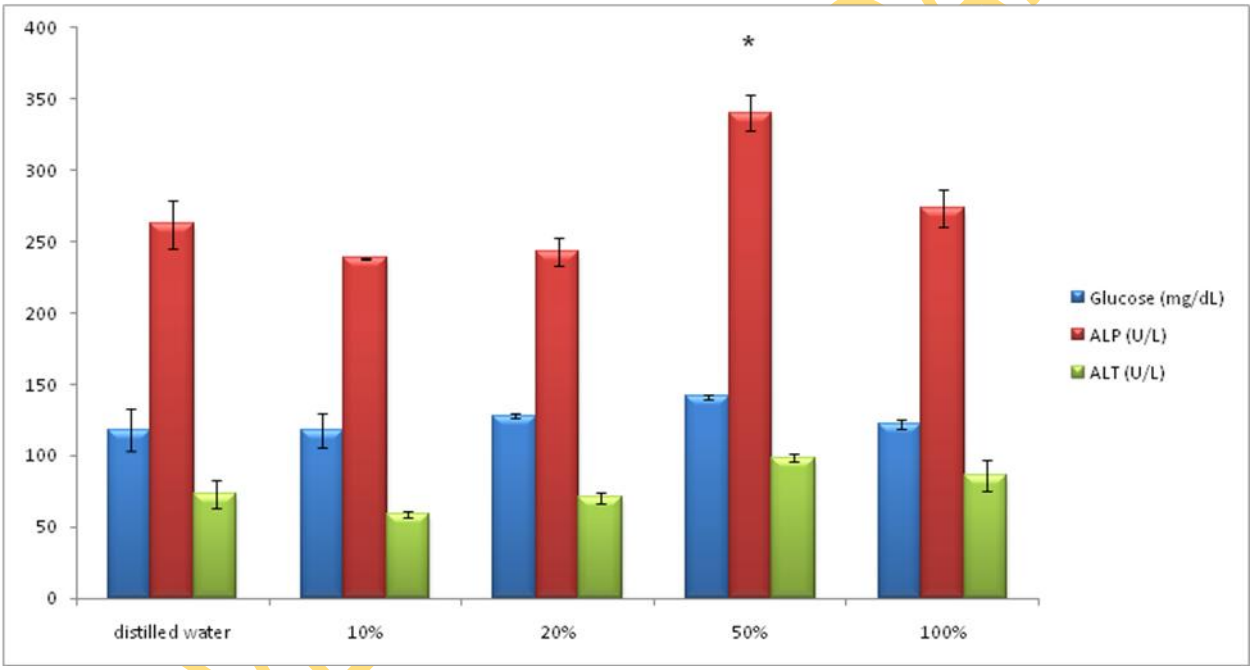

Figure 70: Glucose level and liver enzymes activity due to AH administration in male Wistar rats. *statistical significant ($p<0.05$) as compared with control

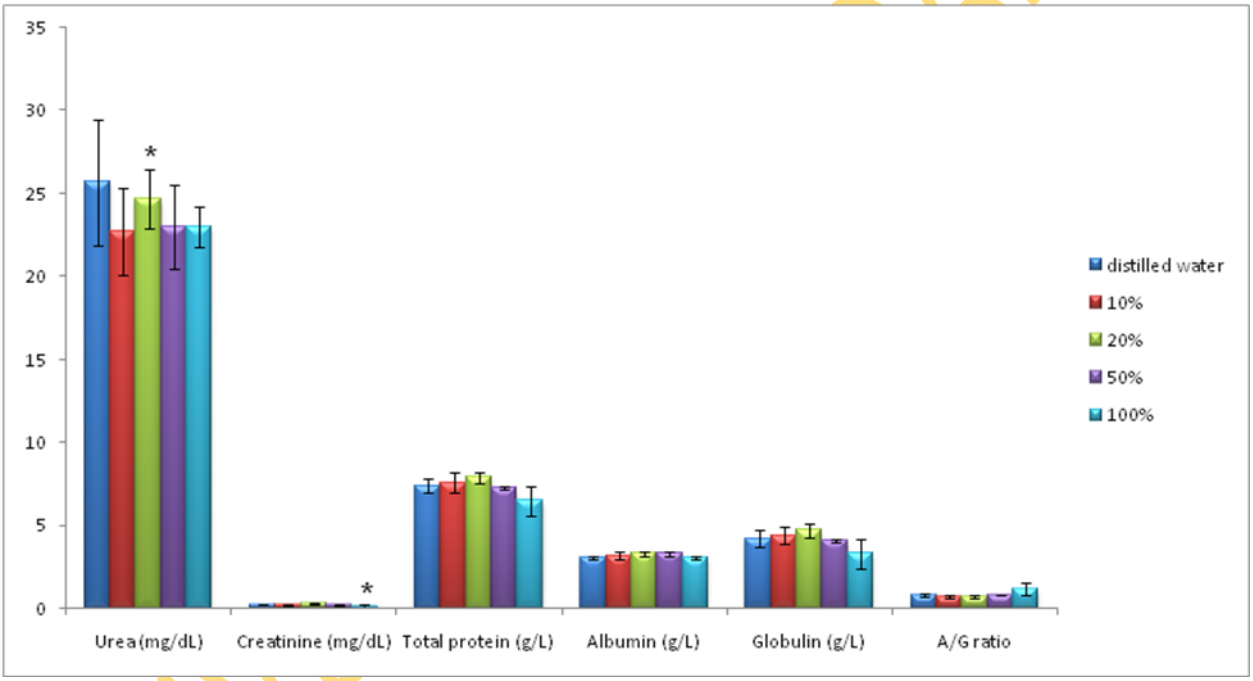

Figure 71: Protein profile, urea and creatinine level due to AH administration in male Wistar rats. *statistical significant ($p<0.05$) as compared with control

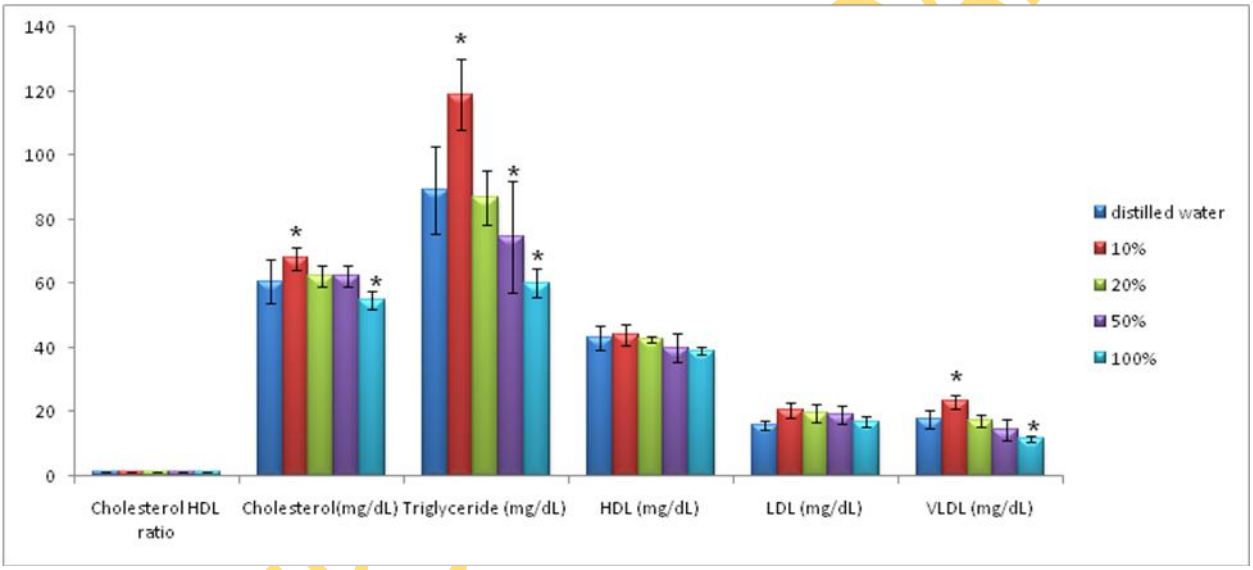

Figure 72: Lipid profile due to AH administration in male Wistar rats. *statistical significant ($p<0.05$) as compared with control

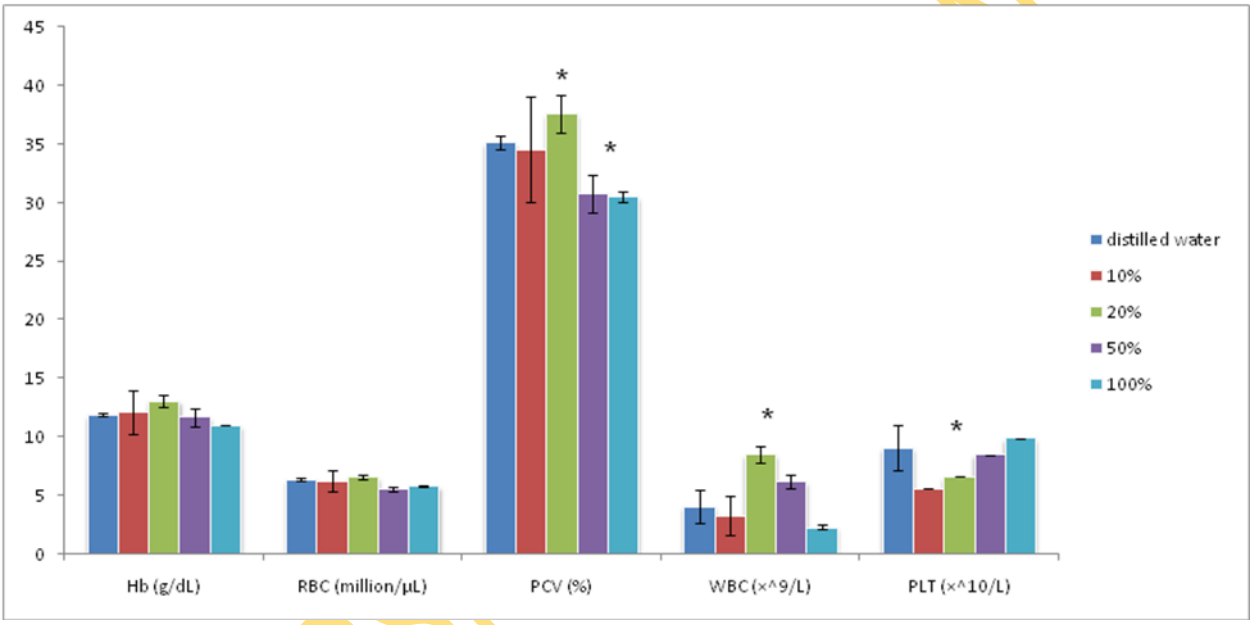

Figure 73: Hematological parameters due to AH administration in male Wistar rats.

*statistical significant ($p<0.05$) as compared with control

4.6 Experiment 6: Effects of Administration and Co-Administration of SA and Ethanol in Male Wistar Albino Rats

Introduction

Continuous exposure of humans to arsenicals through long-term ingestion of contaminated water and its attendant health problems has been widely reported (Sinha *et al.,* 2005). Water as one of the major ingredient in both traditional and modern beer fermentation process may be contaminated with arsenic due to poor quality control. As a result people are exposed to the end product which constituted arsenic compound. It was against this background that the interaction studies (sodium arsenite and ethanol) were conducted on Wistar albino rats.

Procedure

Clinical, biochemical and haematological parameters were evaluated as described under 'Materials and Methods' (section 3.9. page 155-158; section 3.9.1. page 158-159).The experimental design is presented in Figure 74.

Results

The body weight results from Figure 75, shows a significant ($p<0.05$) decrease in % weight change in a concentration dependent manner with concomitant significant ($p<0.05$) increase in the groups treated with SA and ethanol together. Based on the activity of the liver enzymes (ALP, ALT and AST) from Figure 76, the interaction of ethanol and SA significantly ($p<0.05$) decrease the activity of the liver enzymes as compared with the groups treated with SA, ethanol alone. This might suggest that ethanol is suppressing the effect of SA. As depicted in Figure 77, it was observed that administration of ethanol and SA significantly ($p<0.05$) decreased the number of

micronuclei/1000PCE as compared with the groups treated with SA, ethanol respectively, indicating that ethanol administration suppressed the clastogenic effect of SA. This might further suggest an interaction between SA and ethanol which led to the observed anticlastogenic activity as SA is a known clastogen. From Figure 77 the activity of acetylcholinesterase in the brain was significantly ($p<0.05$) inhibited by SA in a concentration dependent manner. At low concentration ethanol did not significantly ($p>0.05$) inhibit the activity of the enzyme but did at a higher concentration. However, the co-administration of SA and ethanol led to an increased activity of acetylcholinesterase suggesting that the interaction might have caused the enzyme induction. However, from Figure 78, most of the hematological parameters PCV, Hb and RBC were significantly ($p<0.05$) decreased with increased SA and ethanol concentration but these were observed to start increasing from group 6 to 9. Furthermore, in terms of WBC it was only group 8 that showed a significant ($p<0.05$) decrease while others showed no remarkable increase in WBC. This signifies that simultaneous administration of the toxicants in question has a counteracting effect, thus suggesting that the interaction is possibly having anti-anemic effect.

Conclusion

Ethanol suppresses the effects of sodium arsenite in male Wistar albino rats. The sodium arsenite at 5mg/kg body weight was then selected for subsequent experiments.

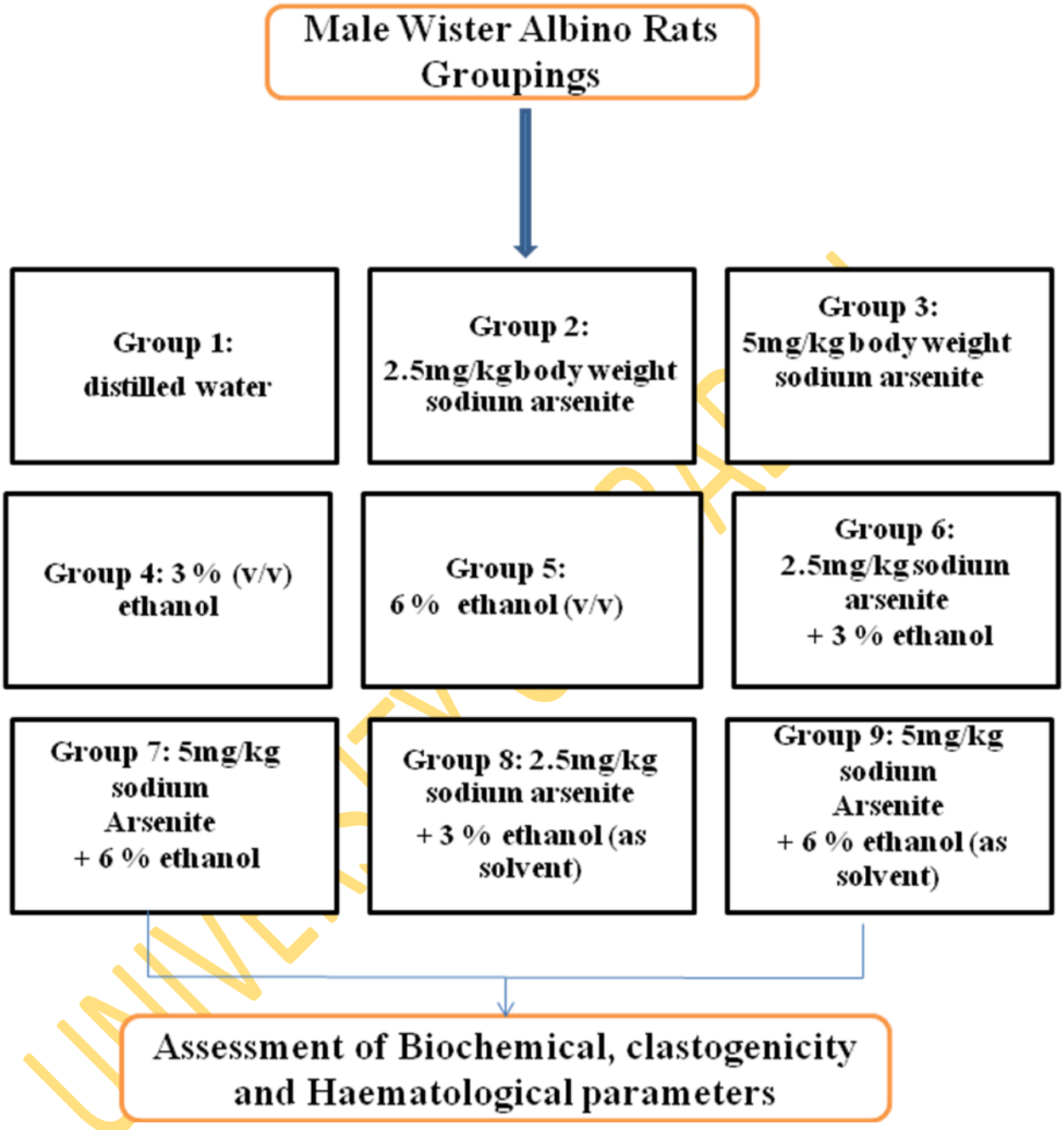

Figure 74: Effects of administration and Co-administration of SA and ethanol on male Wistar albino rats

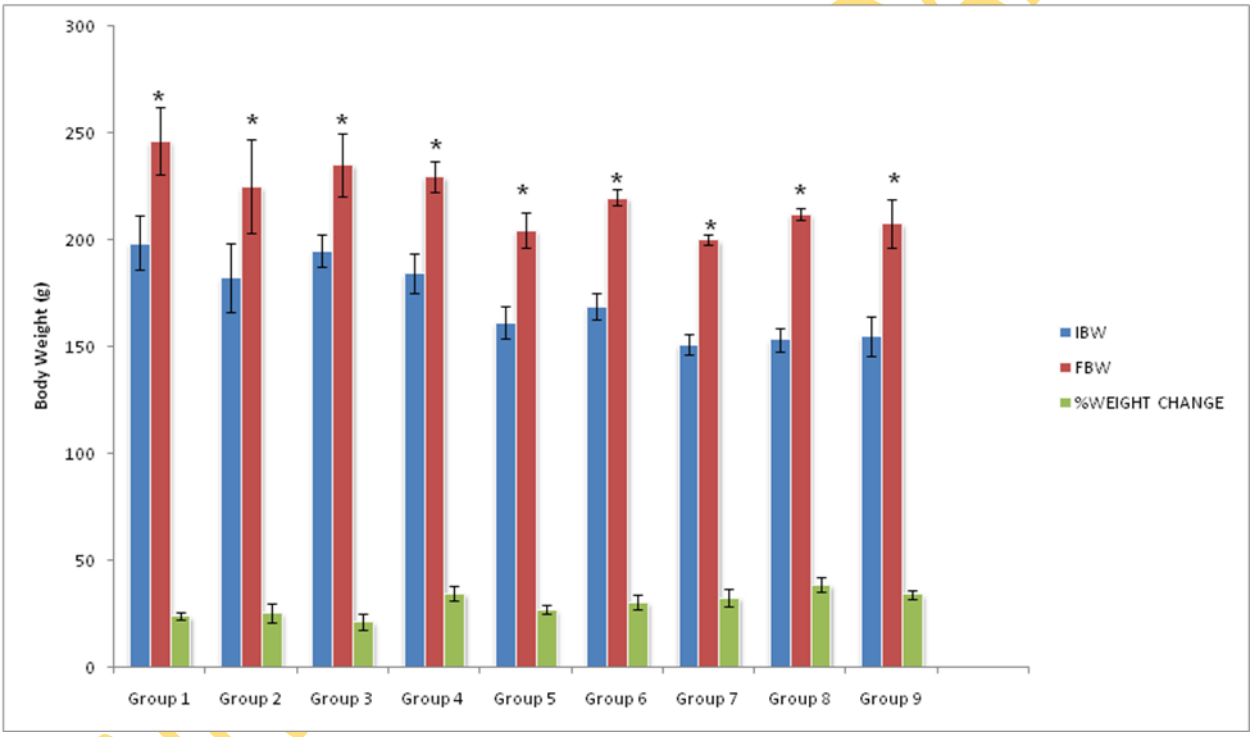

Figure 75: Body weight (g) of the experimental animals before and after exposure to SA and ethanol. *statistical significant ($p<0.05$) as compared with initial body weight (IBW)

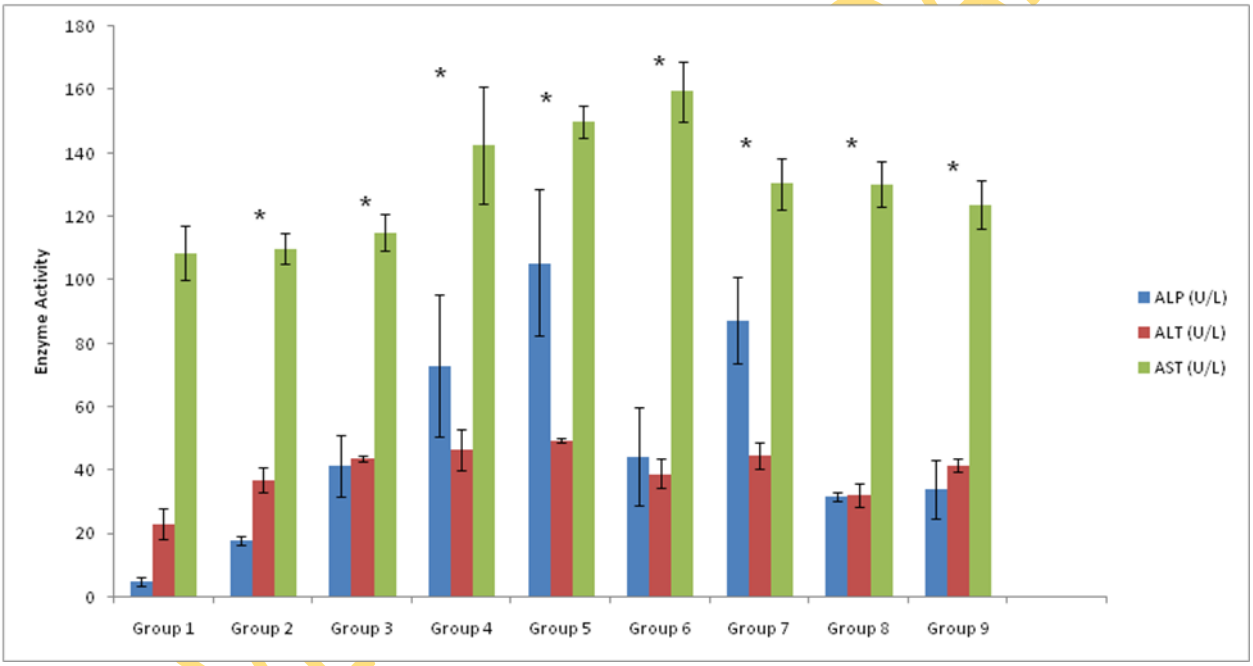

Figure 76: Serum level of aspartate amino transferase, alanine amino transferase and alkaline phosphatase in sera of rats. * Statistical significant ($p<0.05$) as compared with control

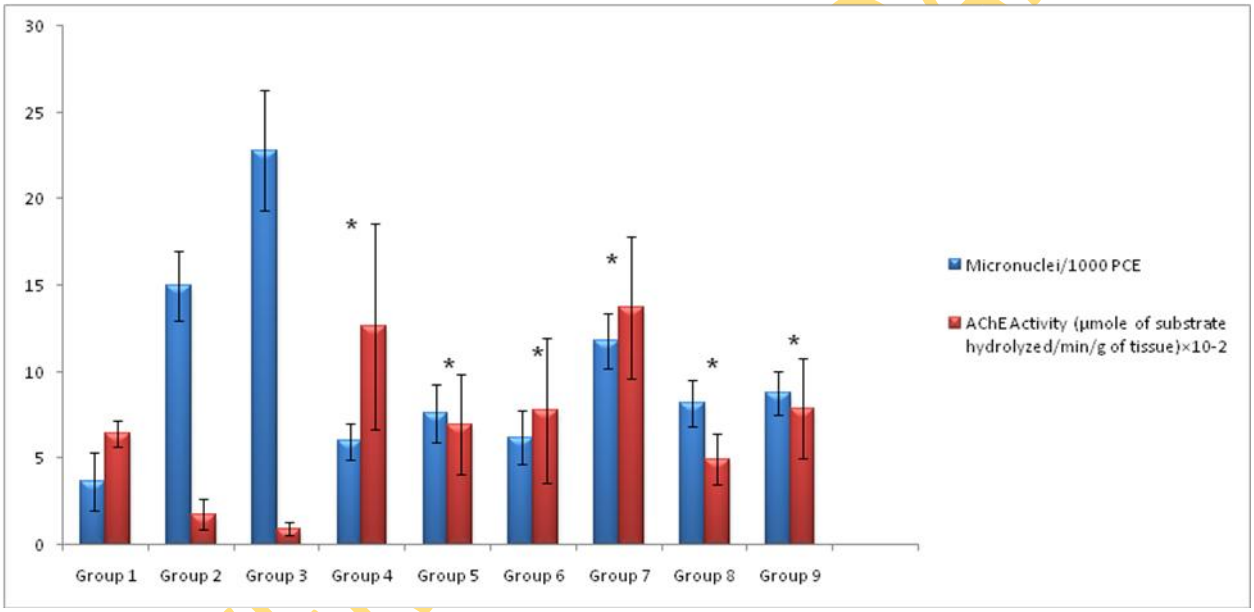

Figure 77: Acetylcholinesterase activity/micronuclei in the brain, bone marrow cells of the rats after exposure to SA and ethanol. *statistical significant ($p<0.05$) as compared with control

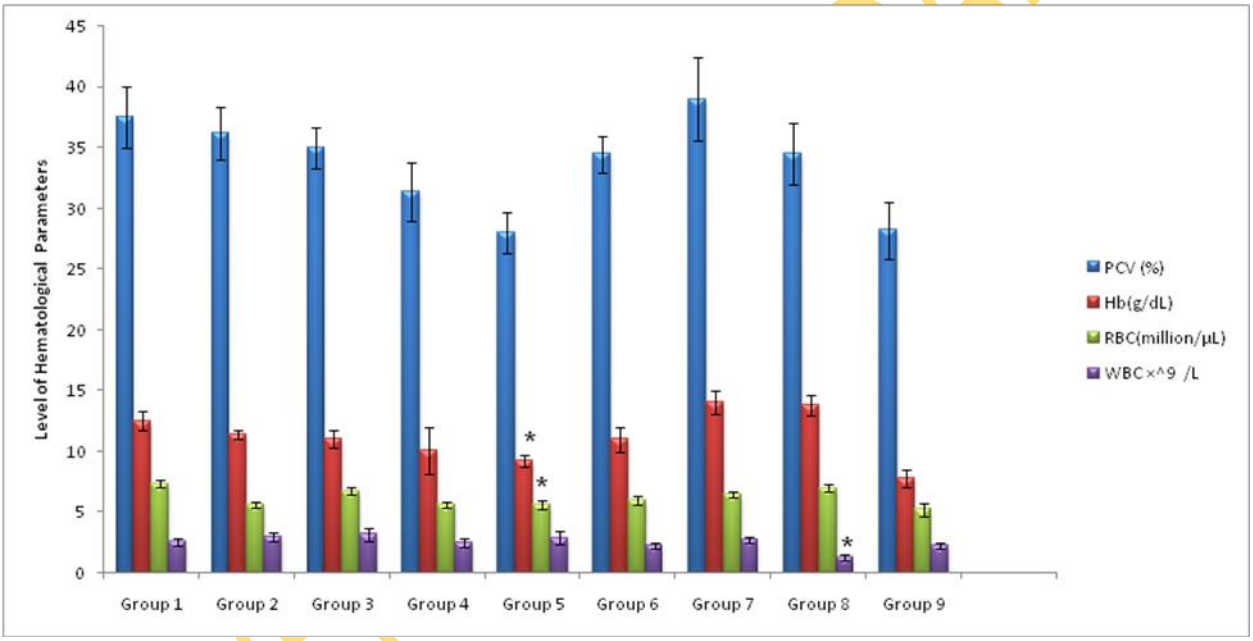

Figure 78: Hematological parameters of rats after exposure to SA and ethanol. *statistical significant ($p<0.05$) as compared with control

4.7 Experiment 7: Comparative Biological Activity of AH of Nigeria and Pakistani sources

Introduction

As a result of the participation of oxidative processes in the onset and development of degenerative diseases, much attention has been paid to the antioxidant properties of foods rich in polyphenols (Chiva-Blanch and Visioli, 2012), vitamins and minerals. Clastogen is a substance that can cause breaks in chromosomes, leading to sections of the chromosome being deleted, added, or rearranged. This causes mutation and leads to cancer development because cells that are not killed by the clastogen may become cancerous. Sodium arsenite, a known clastogen, has been reported to induce chromosomal breakage (Muhammad *et al*., 2012; Owumi *et al*., 2012). It was based on this facts that in the present study, we compare the antioxidant and anticlastogenic potential of AH of Pakistan and Nigeria sources by assessing oxidative stress biomarkers and frequency of micronuclei formation on sodium arsenite-induced toxicity in rats.

Procedure

The levels of oxidative stress biomarkers and the frequency of the formation of micronuclei in Wistar rats were assayed for as described under 'Materials and Methods' (section 3.6. page 121-131; section 3.9.2. page 159-161). The experimental design is presented in Figure 82.

Results

As depicted in Figure 83, there was a significant ($p < 0.05$) increase in body weight as the administration progresses with group 2 having the greater increase. Between the

groups, administration of AH lead to significant ($p < 0.05$) body weight increase while the reverse was observed in SA as compared with control. Simultaneous administration of the two significantly ($p < 0.05$) increases the body weight when compared with SA treated group. The same scenario surfaced with AH from Nigeria except for group 2 with shows decreased in body weight (Figure 84). Bearing in mind that SA is a carcinogen and thus, biotransformed to generate ROS and AH being a potential source of antioxidants, the level of reduced glutathione (GSH) was estimated because of its role in preventing damage to important cellular components as a result of ROS such as free radicals and peroxides. As shown in Figure 85, significant ($p < 0.05$) decreased in GSH was observed in the SA treated which was reversed in AH treated group. However, combined administration led to significant ($p < 0.05$) increase in GSH concentration in brain and liver except serum when compared with control group. From the same table, comparison of GSH levels across the serum, brain and liver tissues showed higher concentration in the liver, this was followed by the brain and then serum in the simultaneous administration groups. Whereas in AH treated group the trend is from brain, liver and then serum. Administrations of SA negatively affect the GSH level in serum follow by liver and then brain. The same scenario surfaced with AH from Nigeria (Figure 86). Dismutation of superoxide in to oxygen and hydrogen peroxide is a pre-requisite to catalase activity, Fenton and Haber Weiss reactions because hydrogen peroxide is a substrate to these reactions which are central to antioxidants and ROS generation. Here, we assayed for SOD activity as a function of adrenaline auto-oxidation inhibition (Figure 87). AH significantly ($p < 0.05$) increases SOD activity better than the SA. However, co-administration led a higher activity although less than the control group in brain and liver tissues. Whereas in serum SA stimulate the activity higher than the AH treated group as compared with control. Furthermore, the enzymatic

activity was higher in liver follow by serum and then brain. The same scenario surfaced with AH from Nigeria (Figure 88). Catalase is one of the important antioxidative enzymes that participate in the decomposition of hydrogen peroxide to water and oxygen thereby rendering it unavailable for Fenton and Haber Weiss reactions which are believed to utilize it for the generation of hydroxyl radical. From Figure 89, AH was able to significantly ($p < 0.05$) induces catalase activity much higher than the SA as compared with control group in brain, serum and liver. Co-administration of the two significantly ($p < 0.05$) increases the activity even higher than the control in brain and liver. Similarly, the induction trend was from brain follow by serum and lastly the liver. The same scenario surfaced with AH from Nigeria (Figure 90). Lipid peroxidation leads to oxidative degradation of lipids via the process in which free radicals remove electrons from polyunsaturated fatty acids in cell membrane. This brings about cellular damage as a result of malondialdehyde (MDA) and β-hydroxyl acrolein that have been implicated in the formation of DNA and protein adducts which can negatively affect the three dimensional structure of these biomolecules with ultimate instability in structure-function relationship. We determined MDA level as an index of lipid peroxidation (Figure 91). SA significantly ($p < 0.05$) increased the MDA level in the brain and liver tissues, which was suppressed by AH as observed in the co-administered group. There was no significant ($p > 0.05$) difference among other groups when compared with control in brain, serum and liver. However, AH was more active against lipid peroxidation with the trend of liver follow by brain and then serum. The same scenario surfaced with AH from Nigeria (Figure 92). To assess on whether or not our treatment is toxic, we determined clastogenic effects of AH and SA in bone marrow cells. As depicted in Figure 93, SA significantly ($P < 0.05$) induces the formation of micronuclei which was suppressed in the co-administered group corroborating the anti-clastogenic

potential of AH even though a slight increase was also observed in group 2 as compared with group 1. The same scenarios surfaced with AH from Nigeria (Figure 94) except that AH from Pakistan (group 2) seem to be more toxic as compared with the control group. As depicted in Figure 100 sodium arsenite when ingested is biotransformed to mono and dimethyl arsenic by S-adenosyl methionine (SAM)-dependent methylase which are more polar and easily excreted. However, when the level of dimethyl arsenic becomes high, its spontaneously react with superoxide radical to generates dimethyl arsenic peroxyl radical which can negatively affect the antioxidant system via a covalent binding to thiol group of protein. This leads to generation of ROS that can directly affect protein and DNA or indirectly via lipid peroxidation initiating carcinogenesis. Antioxidants from AH (AAH) might inhibit the spontaneous reaction of dimethyl arsenic and superoxide radical as well suppressing the generation of reactive oxygen species.

Conclusion

Acacia honey from two sources (Nigeria and Pakistan) showed strong *in vivo* antioxidant potentials as well as anticlastogenicity.

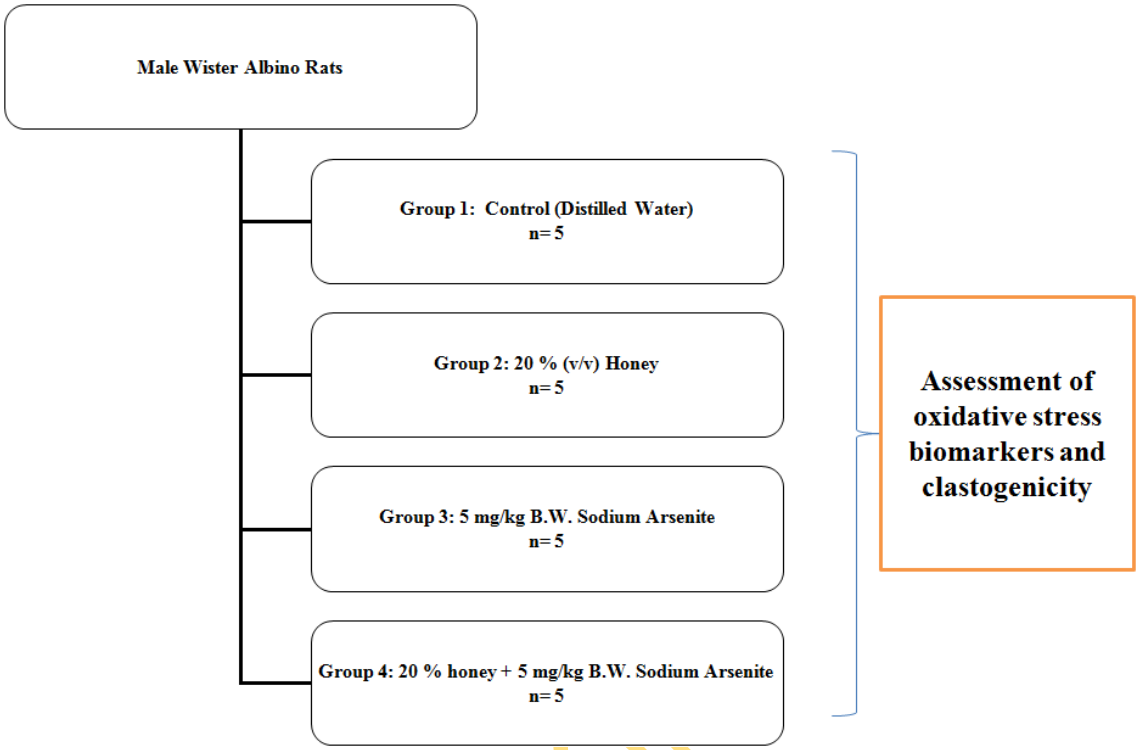

Figure 82: Effects of administration and co-administration of AH and SA on male Wistar albino rats

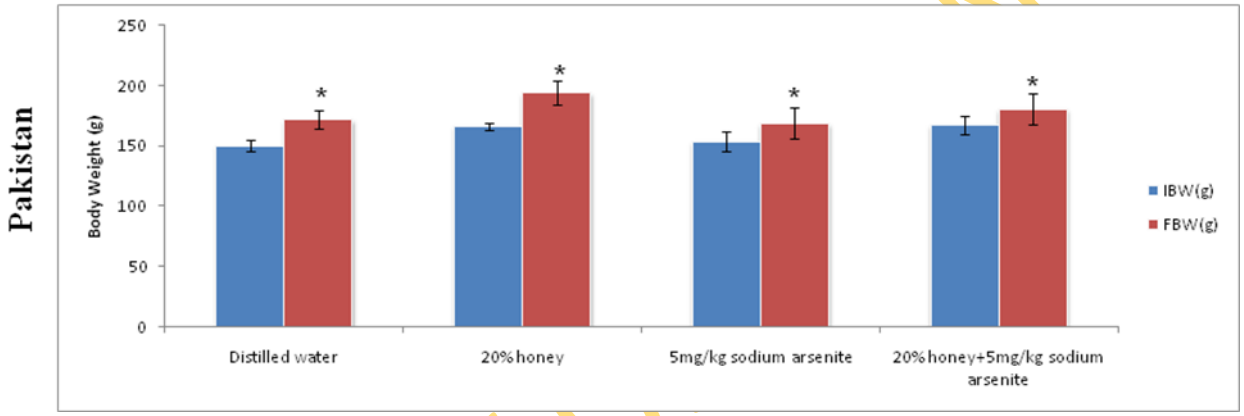

Figure 83: Initial and final body weight of male Wistar albino Rats. *statistical significant ($p<0.05$) as compared with initial body weight (IBW)

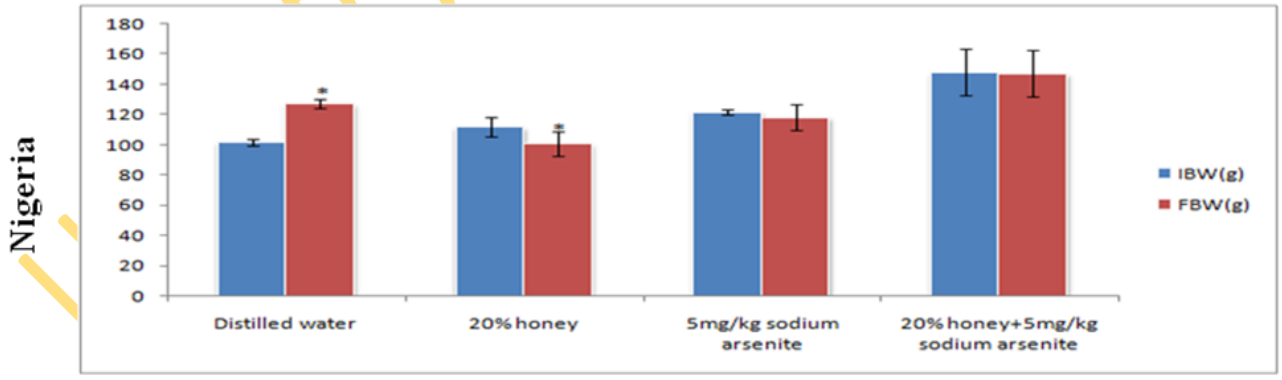

Figure 84: Initial and final body weight of male Wistar albino Rats. *statistical significant ($p<0.05$) as compared with initial body weight (IBW)

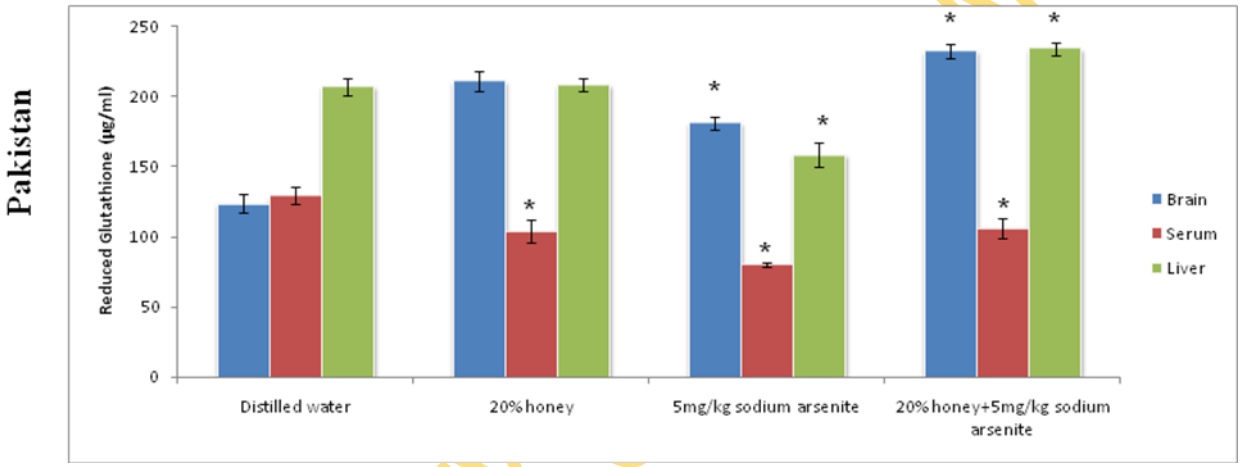

Figure 85: Reduced glutathione (GSH) level due to AH and SA administration. *statistical significant ($p<0.05$) as compared with control

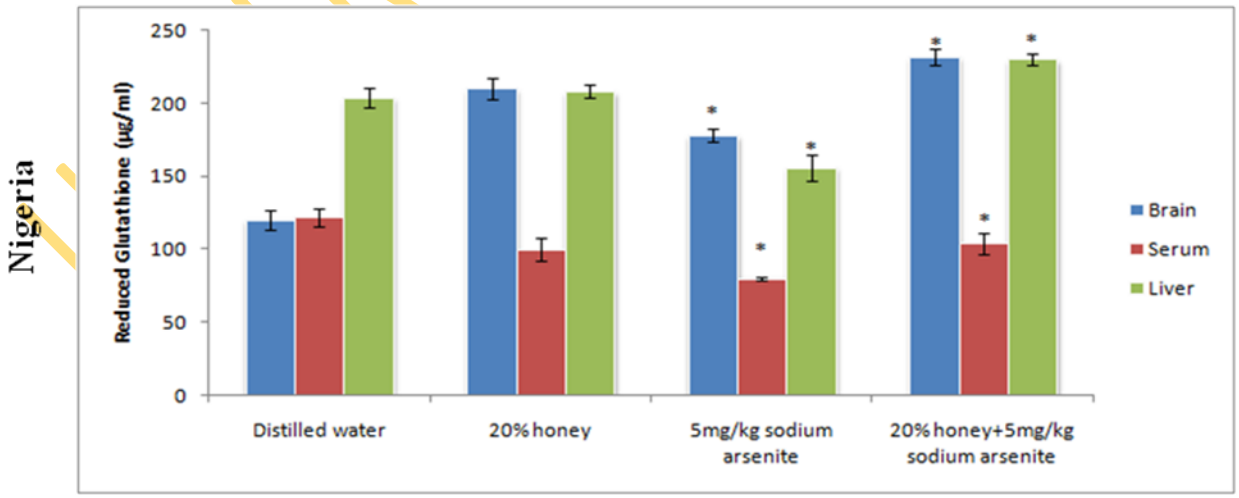

Figure 86: Reduced glutathione (GSH) level due to AH and SA administration. *statistical significant ($p<0.05$) as compared with control

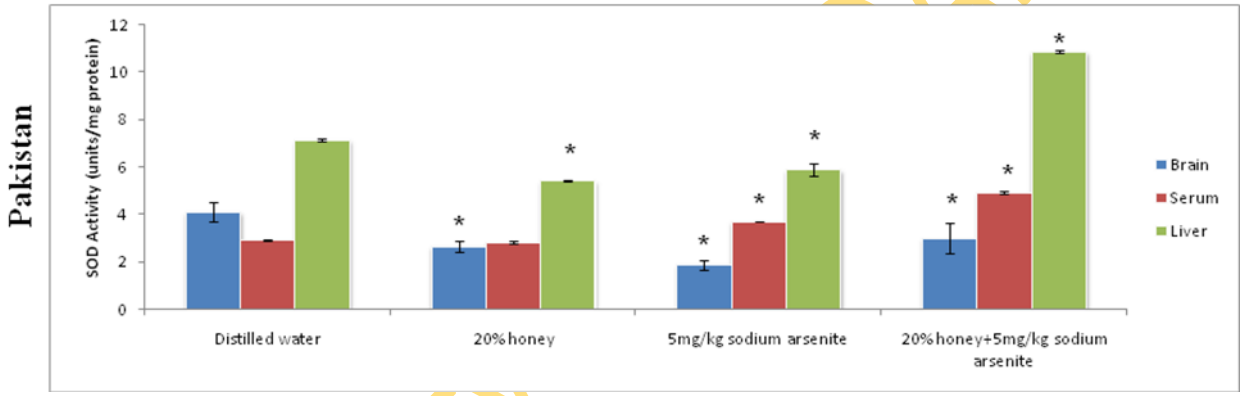

Figure 87: Superoxide dismutase (SOD) activity due to AH and SA administration.

*statistical significant ($p<0.05$) as compared with control

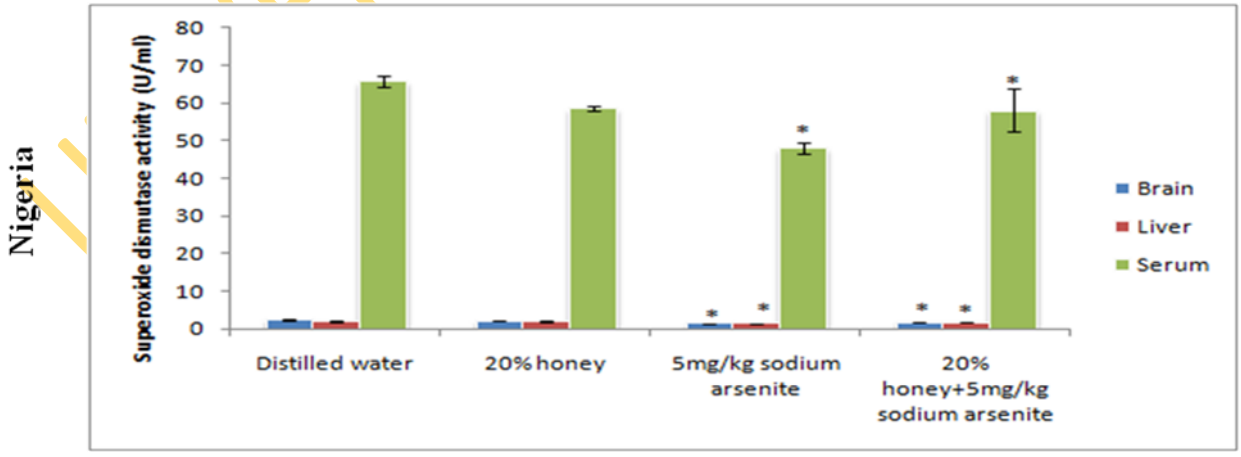

Figure 88: Superoxide dismutase (SOD) activity due to AH and SA administration.

*statistical significant ($p<0.05$) as compared with control

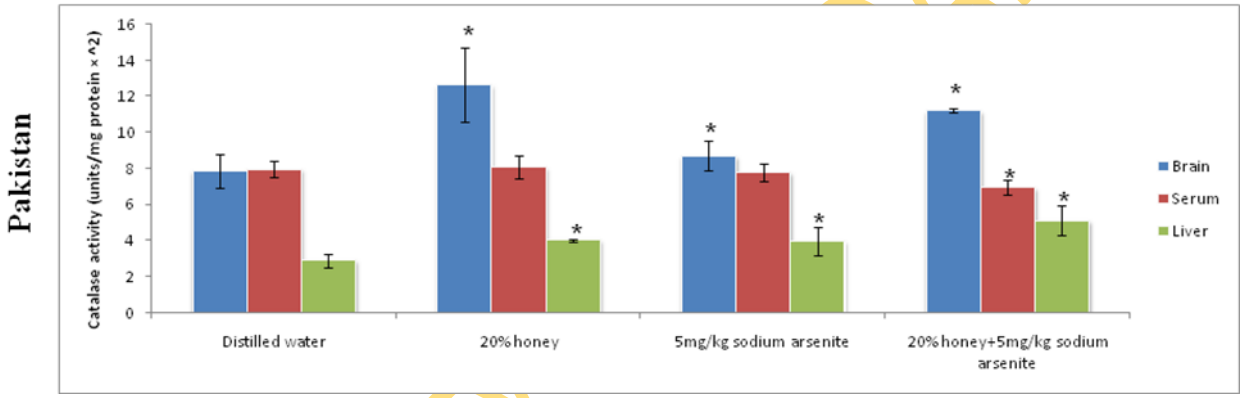

Figure 89: Catalase activity due to AH and SA administration. *statistical significant ($p<0.05$) as compared with control

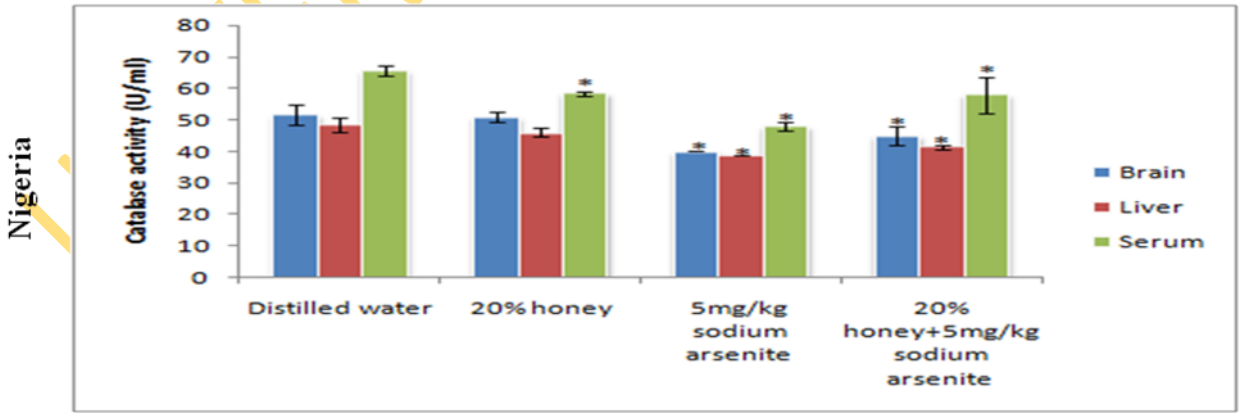

Figure 90: Catalase activity due to AH and SA administration. *statistical significant ($p<0.05$) as compared with control

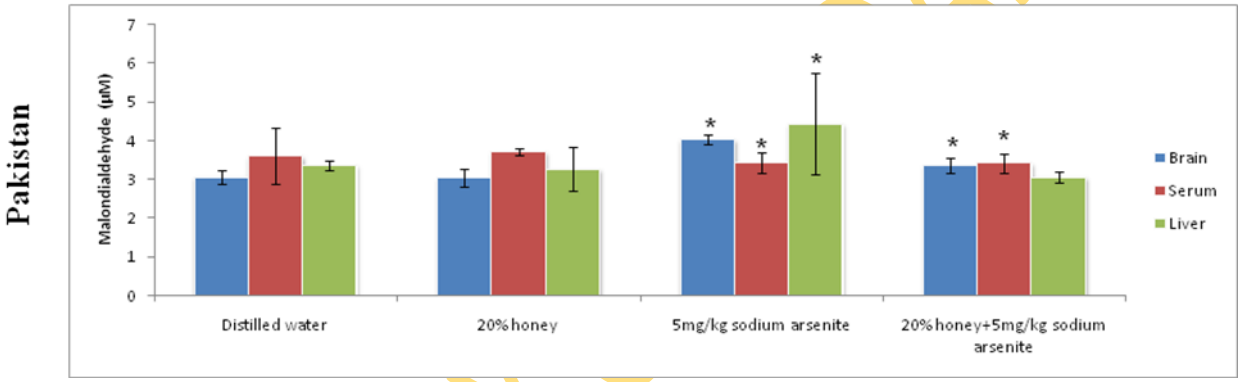

Figure 91: Malondialdehyde level (lipid peroxidation) due to AH and SA administration. *statistical significant ($p<0.05$) as compared with control

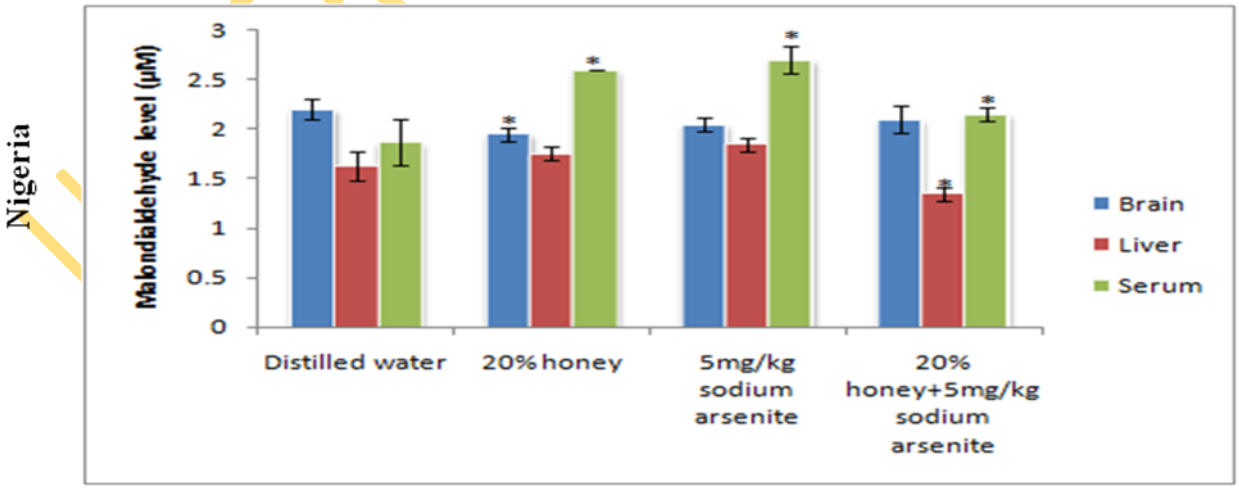

Figure 92: Malondialdehyde level (lipid peroxidation) due to AH and SA administration. *statistical significant ($p<0.05$) as compared with control

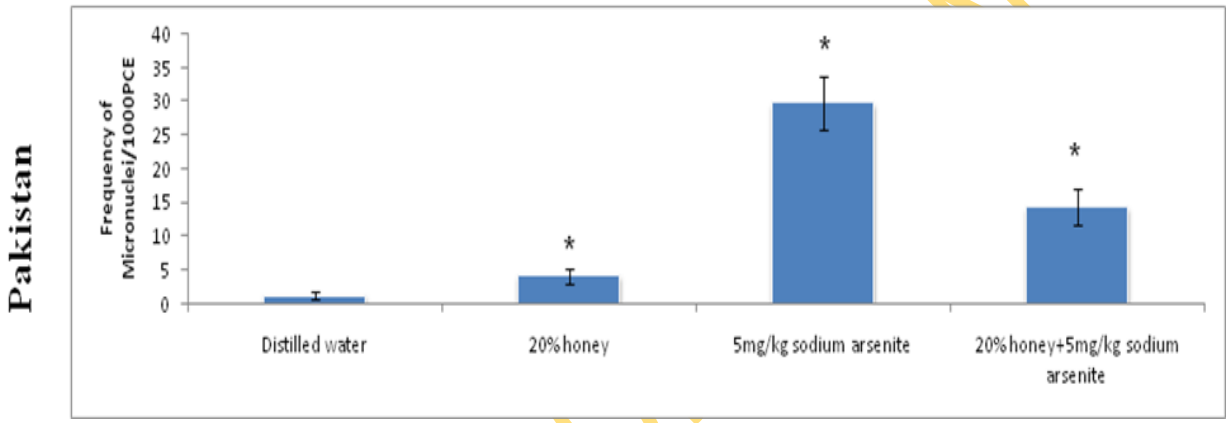

Figure 93: Induction of micronuclei in bone marrow cells due to AH and SA administration. *statistical significant ($p<0.05$) as compared with control

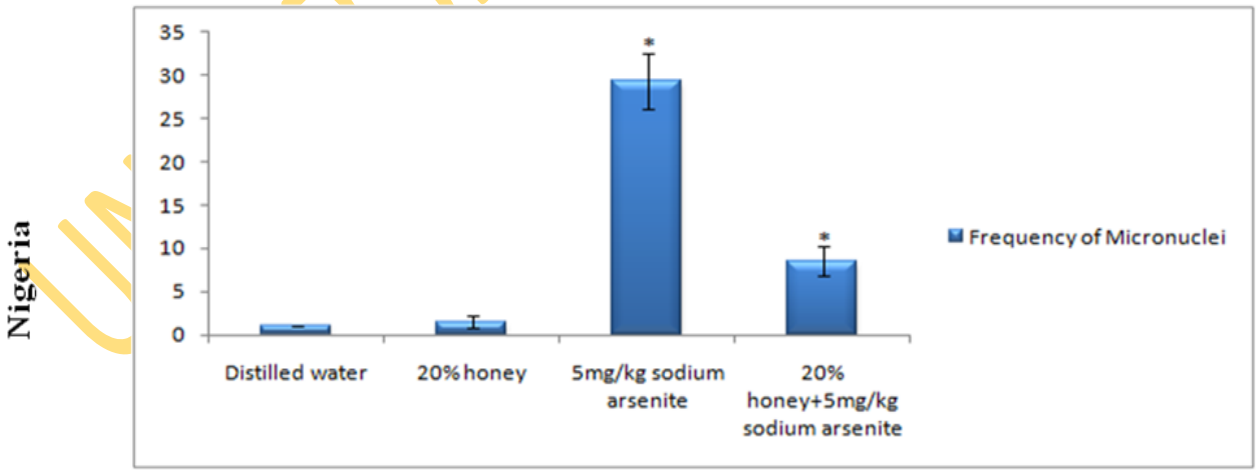

Figure 94: Induction of micronuclei in bone marrow cells due to AH and SA administration. *statistical significant ($p<0.05$) as compared with control

CHAPTER FIVE

5.0 DISCUSSION

Functional foods are characterized by having health-promoting and diseases-preventing effects apart from being nutritional and a typical example of such foods is honey, due to its prophylactic and curative properties. Blood phagocytes respond to particulate or soluble stimuli by the oxidative burst associated with increased production of Reactive Oxygen Species (ROS). NADPH oxidase located in the plasma membrane and in the membranes of specific granules produces superoxide anions from which other free radicals arise. ROS are released into the surrounding medium or into a membrane-enclosed subcellular organelle (Martina and Lukas, 2004). Dismutation of $O_2^{\bullet -}$, either spontaneously or catalyzed by superoxide dismutase, results in the formation hydrogen peroxide (H_2O_2), which acts as a substrate for the myeloperoxidase system (MPO) and this H_2O_2 converts to hypochlorous acid (HOCl) (Arnhold *et al.,* 2004). Emission of photons which takes place during ROS production can be measured easily as chemiluminescence. This effect can be amplified by the use of chemiluminescent probes, i.e. luminol (Allen, 1986). An important and a sensitive method of measuring the generation of these metabolites is chemiluminescence assays as described by Allen *et al.*, (1972), luminol-amplified chemiluminescence. Luminol is specific for the detection of hypochlorous acid, hydroxyl free radical etc., which are primarily produced at the later phase of oxidative burst by phagocytic myeloperoxidase (Allen *et al.,* 1972; Edwards, 1987; Gerber *et al.,* 1996; McNally and Bell, 1996; Dahlgren *et al.,* 1985; Costa *et al.,* 2006). Our findings clearly indicate that none of the fractions of AH was able to confer a significant antioxidant activity. This supports the findings from other types that the antioxidants activity of honey is due to synergistic effects of wide range

of compounds present in it (Gheldof *et al.*, 2002). These wide ranges of compounds include phenolics, peptides, organic acids, enzymes, Millard reaction products, and possibly other minor components and that the phenolics compounds contribute significantly to the antioxidant capacity of honey but are not solely responsible for it (Nada, 2009). However, the whole AH without being fractionated shows good antioxidant activity by decreasing the level of ROS generated from whole blood due to Zymosan stimulation in a dose-dependent manner. The results of this present study support previous data which reported that honey caused scavenging and quenching of ROS (Al-Mamary *et al.*, 2002; Gheldof *et al.*, 2002; Henriques *et al.*, 2006). Moreover, honey has been shown to decrease the Zymosan-stimulated human monocyte cell line-based ROS production (Tonks *et al.*, 2001). By implication our study has clearly indicate that the interactions between different components found in AH is important towards its antioxidant activity. Reactive Oxygen Species spontaneously reacts with nitric oxide (NO) generated by sodium nitroprusside producing cytotoxic reactive nitrogen species capable of nitrating proteins and damaging other molecules like lipids and nucleic acids. We demonstrated that whole AH stimulated the activity of SOD and Catalase as well as inhibition of lipid peroxidation against sodium nitroprusside-induced oxidative stress in brain and liver homogenates. In the liver of young and middle-aged rats, honey supplementation was reported to restore activities of catalase (Yao *et al.*, 2011), SOD (Petrus *et al.*, 2011) and inhibition of lipid peroxidation (El Denshary *et al.*, 2011), which invariably have supported our findings.

Emission of photons which takes place during ROS production can be measured easily as chemiluminescence. This effect can be amplified by the use of chemiluminescent

probes, i.e. luminol/lucigenin (Allen, 1986; Faulkner and Fridovich, 1993). An important and a sensitive method of measuring the generation of these metabolites is chemiluminescence assays as described by Allen *et al*., (1972), which are luminol and lucigenin-amplified chemiluminescence. Lucigenin detects superoxide radical (O_2 •−) which is the initial event in the formation of ROS by an MPO-independent mechanism. On the other hand, luminol is specific for the detection of hypochlorous acid, hydroxyl free radical etc., which are primarily produced at the later phase of oxidative burst by phagocytic myeloperoxidase (MPO) (Edwards, 1987; Faulkner and Fridovich, 1993; Gerber *et al.*, 1996; McNally and Bell, 1996; Dahlgren *et al.*, 1985; Costa *et al.*, 2006). Our findings indicates that honey was able to act as an antioxidant by decreasing the level of ROS generated from whole blood, neutrophils and macrophages due to Zymosan and PMA stimulations in a dose-dependent manner. The results of this present study support previous data which reported that honey caused scavenging and quenching of ROS (Al-Mamary *et al.*, 2002; Gheldof *et al.*, 2002; Henriques *et al.*, 2006). Human consumption of honey has also been reported to increase plasma antioxidant levels (Schramm *et al.*, 2003). Moreover, honey has been shown to decrease the Zymosan-stimulated human monocyte cell line-based ROS production (Tonks *et al.*, 2003). The discovered honey natural products are oligosaccharides, flavonoids, isoflavones, glycosides, phenolics, peptides/proteins, waxes, pollen grains etc. (Merken and Beecher, 2000; Martos *et al.*, 2000; Jimenez *et al.*, 2000; Scarselli *et al.*, 2005; Chow, 2002; Wang and Gibson, 1993; Sanz *et al.*, 2004; Postmes *et al.*, 1995) and they exhibit a wide range of biological effects, including antibacterial, anti-inflammatory and antithrombotic action. Therefore, it is not surprising that our studies revealed a very significant ($p<0.05$) dose-dependent suppression of the production of ROS in the presence of honey. In this study, cytogenetics effects of honey were investigated in

cultured human lymphocytes. The peripheral lymphocytes are the best materials for the determination of cytogenetic effects. Mitotic index, nuclear division index and cytokinesis-block proliferation index are used as indicators of adequate cell proliferation biomarkers. Mitotic index measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be considered as cellular death or delay in the cell proliferation kinetics (Rojas, 1993). The mitotic index is used to characterize the proliferating cells and to identify compounds that inhibit or induce mitotic progression (Holland *et al*., 2002). It depends on two (2) factors: first the proportion of the cell population that participates in the whole cycle of interphase leading to division; second the relative lengths of interphase and recognizable mitotic stages (Walker, 1952). In the present study, we were able to demonstrate the fact that honey induces nuclear division and cell proliferation in a concentration dependent manner as compared with the control although some degree of inhibition was observed at low concentration demonstrating anti-proliferative potentials. Lymphoid cells from all animal species can be stimulated *in vitro* to synthesize DNA and undergo blastogenic transformation upon exposure to mitogens or specific antigens (Sharon and Lis, 1972; Oppenheim and Rosenstreish, 1976). Proliferative potentials of honey on B and T-lymphocytes have been reported (Nizar *et al*., 1999) which further corroborate with our findings. Mabrouk *et al*., (2002) demonstrated the ability of the honey to inhibit cell proliferation and this might explain the observed level of inhibition at low concentration. On why there are both stimulatory and inhibitory effects, we propose that honey behaves differently based on the morphological and physiological stage of the cells for example cancer and normal cells. This present study also made an attempt to investigate the possibility of honey inducing genotoxic and cytotoxic effects in cultured human peripheral blood lymphocytes based on cytochalasin B-block micronucleus assay. Basically, micronuclei formed as a result

of acentric chromosomal fragments or as whole chromosomes that fail to reach the poles. Micronuclei can also be recognized by the presence of some characters; they are small, round, DNA/chromatin-containing interphase structures occasionally found in the cytoplasm of cells. The morphology of micronucleus is similar to nucleus, but smaller in size (Rao *et al.*, 2008), hence named as micronucleus (Fenech, 2000). At telophase, a nuclear envelop will form around chromosomal fragments and the lagging chromosomes that are unable to travel to the spindle poles during mitosis, those steps will lead to the formation of micronuclei. Therefore, micronuclei are used as an index of chromosome breakage and chromosome loss (Fenech, 2000). Our findings indicate that honey was not able to induce chromosomal damage in lymphocytes because only one micronucleus was observed among all the concentration used. This might be attributed to some failure in the normal metabolism of nucleic acids because even at higher concentration we could not detect micronuclei. However, the cytotoxicity index was found to be decreasing in a dose-dependent manner suggesting a protective role of honey which may due to significant antioxidant activity observed from the present study.

The diversity and concentration of the components in honey are largely dependent on the botanical and geographical origin. Moreover, it is also influenced by the variation in climate and availability of plant sources for the honeybees to harvest nectar within a specific region (Anklam, 1998). Therefore, honeys originating from different floral sources differ in their chemical compositions. Our finding shows that AH was able to act as an anti-proliferative agent by its ability to inhibit the growth of PC-3 and NCI-H460 cell lines *in vitro*. This is in support with reports that AH reduces the proliferation of other cell lines like melanoma cells (Elena *et al.*, 2010). Basically, various polyphenols are reported in honey. Some of which are Caffeic acid, Caffeic acid phenyl

ester, Chrysin, Galangin, Quercetin, Acacetin, Kaempferol, Pinocembrin, Pinobanksin and Apigenin have evolved as promising pharmacological agents in treatment of cancer (Ozkul *et al.,* 2005; Saravana *et al.,* 2009). Characterization of AH revealed three (3) phenolic acids [p-hydroxybenzoic, ferullic and t-cinnamic acid], abscisic acid and five (5) free flavonoids [pinobanksin, apigenin, pinocembrin, crysin and acacetin] (Liviu *et al.,* 2010). Polyphenols and phenolics have been reported to inhibit cancer-related pathways and processes including lung and prostate cancer (Chan *et al.,* 2005; Von Low *et al.,* 2007). Inhibition of apoptosis can be seen as the basis of abnormal cell growth and calcium ion has been implicated to play a vital role via direct and indirect apoptotic pathways. The involvement of Ca^{2+} in cell death has been recognized very early in the history of programmed cell death, with the demonstration *in vitro* that Ca^{2+} ionophores, which are the molecules capable of transporting Ca^{2+} across membranes down its electrochemical gradient, are highly toxic to cells (Rosario *et al.,* 2003). In the present study we demonstrate that anti-proliferative effects of AH is being accompanied by stimulation of calcium ion and thus, implicating its anticancer activity in relation to the induction of apoptosis via the calcium ion dependent pathway. Pro-inflammatory cytokines are one of the major key players during chronic inflammation which favored the survival of cancer cells and of such type are TNF-α and IL-1β. It has been reported that pro-inflammatory cytokines are capable of promoting proliferation, invasion and angiogenesis of prostate cancer, and of several other solid tumors (Ricote *et al.,* 2004; Salazar-Onfray *et al.,* 2007; Lou *et al.,* 2000). Our results show the anti-inflammatory role of AH by its ability to down regulate the level of TNF-α. This support the anti-inflammatory role of honey as previously described (Fiorani *et al.,* 2006). The level of IL-1β was observed to be high in our present study. However, it has been established that TNF-α stimulates other cytokines including IL-1β which up regulates the level of

some protein kinases such as extracellular regulated kinase [ERK]. ERK has been found to phosphorylate the calcium ion channel of the endoplasmic reticulum thereby causing the depolymerisation of cytoskeletons leading to calcium ion release. The released calcium ion activates calpain which further activates procaspase-12 that will ultimately stimulates the execution caspases of an apoptotic pathway like procaspase 3 and 7 (Sten *et al.,* 2003; Min-Hsiung *et al.,* 2008; Michael *et al.,* 2011). We therefore propose that, AH modulates TNF-α towards the production of IL-1β which corroborates with an increased in calcium ion level and anticancer activity against cell lines in a concentration dependent manner. Cell cycle arrest especially at G0/G1 phase induces apoptosis. In this study, we observed that AH induced a dose-dependent G0/G1 phase arrest. The inhibition of cell growth could be a result of the induction of apoptosis that may be mediated by cell cycle arrest. Therefore we hypothesize that AH mediated inhibitory effects of cell growth of PC-3 and NCI-H460 cells might be due to perturbation in the cell cycle, which may possibly lead to programmed cell death. The accumulation of cell population in G0/G1 phase was also observed in other cancer cell lines after honey treatment (Elena *et al.,* 2010; Swellam *et al.,* 2003). However, the anti-proliferative and apoptotic role of AH was confirmed at the molecular level by its ability to down regulates the expression of p53 and Bcl-2 gene. Apart from it apoptotic, growth arrest and DNA repair role, p53 also plays an important role in cell survival, proliferation, migration, adhesion and angiogenesis (Jean-Marie *et al.,* 2007) which supports our findings on why honey suppressed its expression as NCI-H460 cell line is p53 stable. It has been reported that the Bcl-2 and Bcl-xL protein suppressed the production of IL-1β (Stephen *et al.,* 2005) which may explain the increased in IL-1β observed. In apoptosis, one pathway of caspase activation is triggered by cytochrome c following its release from mitochondria into the cytoplasm (Liu *et al.,* 1996). This

mitochondria-dependent mechanism of caspases activation has been called the 'intrinsic' pathway of apoptosis. Bcl-2 blocks the release of intermembrane space molecules from mitochondria (Kluck *et al.*, 1997; Yang *et al.*, 1997), and as such inhibiting the apoptotic pathway. This supports our findings as AH was able to suppress the expression of Bcl-2 gene thereby inducing the apoptosis. Prostate-specific antigen (PSA), a member of the kallikrein protein family, is widely used as a surrogate marker in the early diagnosis and management of prostate cancer (Diamandis, 1998). PSA is a serine protease with chymotrypsin-like activity (Armbruster, 1993), and is largely found in prostatic tissue and seminal plasma. In prostate cancer, excess PSA spills into circulation where it exists as a mixture of various molecular forms, including free-PSA and several PSA–protease inhibitor complexes (Zhang *et al.*, 1999). Our finding indicates that AH was able to decrease the level of PSA in a concentration dependent manner. PSA has been established to play a significant role in prostate tumor growth by regulating various pro-angiogenic and anti-angiogenic growth factors (Bindukumar *et al.*, 2005). It has also been reported to stimulate the generation of reactive oxygen species in prostate cancer (Xiao-Ya *et al.*, 2011). This further supports our findings that show the antioxidant potentials of AH due to the down regulation of PSA *in vitro*.

The main uses of honey are in cooking, baking, as a spread on bread, and as an addition to various beverages, such as tea, and as a sweetener in some commercial beverages. It consists of primarily sugars such as monosaccharides, disaccharides, oligosaccharides and polysaccharides (Bogdanov *et al.*, 2008; Erejuwa *et al.*, 2012). It contains enzymes such as glucose oxidase, diastase, invertase, catalase and peroxidase (Bogdanov *et al.*, 2008). Honey also contains other bioactive constituents such as organic acids, ascorbic acid, trace elements, vitamins, amino acids, proteins and Millard reaction products (Bogdanov *et al.*, 2008). Being rich in carbohydrates like glucose and fructose when

ingested could be metabolized to generate energy and help in tissue repair. We report the effect of honey on heamatological and biochemical parameters at various concentrations in normal albino rats. Our study indicates an increase in body weight among all the experimental animals as the administration progressed from day 1 to day 7. Within the same treatment groups, there was an increase from 10% to 20% (v/v) but this was observed to start decreasing as the concentration increased. The observed increase in body weight could be due to the androgenic properties of the honey since androgens possess anabolic activity (Johnson *et al.,* 1988). Moreover, decrease in body as a result of increased in concentration might be because of the fact that honey triggers a small spike in insulin levels (the glucose in honey stimulates in a small insulin release), and insulin stimulates the release of tryptophan in the brain. Tryptophan is converted to serotonin, which in the dark is converted to melatonin. Melatonin in return inhibits the release of insulin, thus further stabilizing blood sugar levels during the night (Ron, 2007) and this by implication down regulate the aerobic glycolytic pathway that is believed to play a vital role in lipogenesis which will ultimately lead to an increase in body weight. The observed decrease in the level of creatinine, ALP and ALT activities is a clear indication that the honey is having hepatoprotective and renal protective properties and this is in accordance with the fact that consumption of honey conferred the aforementioned effects (Wilson *et al.,* 2011). There were also no effects on cholesterol, HDL, LDL and cholesterol HDL ratio. However, the observed decreased level of triglyceride and VLDL indicates the fact that the treatments might not be having lipogenic effects. This further confirms why there was a decreased in body weight as the concentration increased. Honey administration tends to have stabilizing effects as there were no effects on total protein, albumin, globulin and albumin/globulin ratio as well as hemoglobin, red blood cell counts. Furthermore, an increased in packed cell volume and

white blood cell counts at 20% (v/v) treated group corroborate the honey as an anti-anemic and immune-stimulant agent (Fiorani *et al.,* 2006). This is in accordance of the fact that exclusive honey feeding significantly modifies the hematological parameters (Noori *et al.,* 2006).

Exposure to arsenite has been linked to diverse defects in both experimental animals and in humans (Longnecker and Daniels, 2001; Tseng, 1977; Klaassen, 1990; Waalkes *et al.,* 2003; Shukla and Pandey, 1984). Liver is an important target organ for arsenic toxicity (Mazumder, 2005). Arsenic has been claimed to be of clinical utility in the treatment of syphilis, amoebiasis, and certain other tropical diseases (Klaassen, 1990) and also has been used in Fowler solution in the treatment of arthritis (Klaassen, 1990), but recently arsenic intoxication in experimental animals has been associated with hepatic tumours (Waalkes *et al.,* 2003), the inhibition of testicular steroidgenic function (Chattopadhyay *et al.,* 2001), and spermatogenesis (Shukla and Pandey, 1984), as well as with severe metabolic disorders such as diabetes in humans (Longnecker and Daniels, 2001; Tseng, 1977). It is known that (SA) can act as comutagen due to its ability to inhibit the activities of thiol containing enzymes (Mazumder, 2005), such as DNA ligase (Chattopadhyay *et al.,* 2001) resulting in defective DNA replication, repair, recombination and joining of single- and double-stranded DNA breaks (Sunderman, 1984). Ethanol-induced oxidative stress is as a result of the combined impairment of antioxidant defenses and the production of reactive oxygen species by the mitochondrial electron transport chain, the alcohol-inducible Cytochrome P450 (CYP) 2E1 and activated phagocytes (Dey and Cederbaum, 2006). Furthermore, Hydroxyethyl Radicals (HERs) are also generated during ethanol metabolism by CYP2E1. The available evidence indicates that, by favouring mitochondrial permeability transition, oxidative stress promotes hepatocyte necrosis and/or apoptosis and is implicated in the alcohol-

induced sensitization of hepatocytes to the pro-apoptotic action of TNF-α. Moreover, oxidative mechanisms can contribute to liver fibrosis, by triggering the release of pro-fibrotic cytokines and collagen gene expression in hepatic stellate cells (Li and Rossman, 1989). We examined the effect of co-exposure to SA and ethanol on male Wistar albino rats. The interaction between SA and ethanol seem to reverse the effect of decreased body weight. This might be attributed to the fact that ethanol induces fatty liver with enhanced lipogenesis that ultimately lead to an increase in weight (Lasko *et al.,* 1990). The results of the present study clearly demonstrate that administration of SA and ethanol respectively, significantly ($P < 0.05$) induced the formation of micronuclei in the Polychromatic Erythrocytes (PCEs) of the rat bone marrow cells. However, the reversal of that happened when both toxicants were co-administration. This is may be due to the fact that arsenite generates free radicals that can attack DNA leading to chromosomal breakage. In addition, acetaldehyde the end product of ethanol metabolism can form DNA adducts which might also explain why groups treated with SA and ethanol respectively were able to induced clastogenicity. The results obtained from the assessment of the serum activities of ALP, ALT and AST shows that their activities increased in a concentration dependent manner. Interestingly, the activities were decreased in the groups of co-administration providing a clue that the chemical interaction between SA and ethanol is having a reversal effect. Exposure to SA had been shown to induce ALP, AST and ALT activity (Albano, 2006), which is clearly an indication of hepatotoxicity and oxidative stress. Acetylcholine (ACh) is a neurotransmitter that functions in conveying nerve impulses across synaptic clefts within the CNS (Tripathi and Srivastava, 2008). Following the transmission of an impulse across the synapse by the release of ACh, AChE is released into the synaptic cleft (Horton *et al.,* 2006). This enzyme hydrolyzes ACh to choline and acetate and

transmission of the nerve impulse is terminated (Liesener *et al.*, 2007). The same scenario surfaced on acetylcholinesterase activity being suppressed as concentration of the toxicants increased and elevated in the co-administered groups; probably the interaction might have initiated an enzyme induction as the activity of AChE is vital to neurological functions. Similarly, based on the hematological parameters single and co-administered groups were depicting an antagonistic effect. This suggests the possibility of an interaction between SA and ethanol to have a stabilizing effect on the levels of hematological parameters as the toxic compounds like CCl_4, arsenic and ethanol has been found to negatively affect the levels of these parameters (Al-Waili *et al.*, 2006). It is against this background we propose that the reaction between SA and ethanol in the presence of water generates dimethyl hydroxyl arsenous acid which ultimately make it more polar and as such easily excreted without necessarily causing harm to the system.

Redox sensitive biomarkers could be seen as those enzymatic and non-enzymatic antioxidants/non-antioxidant (like MDA) that are stimulated either at cellular or molecular level in response to oxidation-reduction reactions during metabolisms. Honey is a novel antioxidant because of the presence of flavonoids and phenolics as part of its condiments. In the present study, we reported the comparative assessment of redox sensitive biomarkers due to AH and SA administration *in vivo.* Our findings indicate that AH stimulate the increase in body weight of the rats as against the SA. This is in accordance with the findings of Muhammad *et al* (2012) that daily consumption of honey significantly increases the body weight of male Wistar albino rats. This might be as a result of androgenic substance present in honey since androgens has anabolic function (Muhammad and Amusa, 2005). Oxidative stress has been suggested in both experimental and clinical studies to play a major role in the pathogenesis of so many diseases like cardiovascular disease and cancer. Arsenite interacts with thiol-containing

amino acids, peptides and proteins (Winsk and Carter, 1995). Arsenite exerts its cellular toxicity by binding to sulfurhydryl groups which results in enzyme inhibition. During arsenic metabolism, oxygen radical may be produced, possibly leading to damage of DNA, proteins, lipids and other molecules. There is a positive correlation between lipid peroxidation and arsenic tissue concentrations in the livers, kidneys and heart of arsenite treated rats (Ramos *et al.*, 1995). SA-induced ROS, such as superoxide anions and hydroxyl radicals, exert effects directly or indirectly on cellular material (Poli *et al.*, 2004). It was also reported that arsenic induced oxidative stress by multiple mechanisms (Sanjib *et al.*, 2012). Chronic arsenic exposure through drinking water to humans leads to carcinogenesis of almost all organs in the human system (Guha, 2008; Kapaj *et al.*, 2006). Our results clearly show that SA administration stimulate lipid peroxidation with concomitant negative effects on the part of enzymatic and non-enzymatic antioxidants which was ameliorated in the presence of AH. The antioxidative potentiality of honey is has been reported (Omotayo *et al.*, 2012). It is generally attributed to its phenolic compounds and flavonoids (Khalil *et al.*, 2011; Van den Berg *et al.*, 2008; Beretta *et al.*, 2007; Kishore *et al.*, 2011). Basically, various polyphenols are reported in honey. Some of which are Caffeic acid, Caffeic acid phenyl ester, Chrysin, Galangin, Quercetin, Acacetin, Kaempferol, Pinocembrin, Pinobanksin and Apigenin have evolved as promising pharmacological agents in treatment of cancer (Liviu *et al.*, 2010). It is accepted that flavonoids and their metabolites, thanks to their both hydrophilic and relatively lipophilic properties, may interact with plasma proteins as well as the polar surface region of phospholipid bilayers in lipoproteins and cell membranes (Alvarez-Suarez *et al.*, 2012). It is these chemical interactions they may confer a protection against damages caused by ROS. Available data indicate that honey, like other antioxidant agents, does protect against damage or injury. This protective effect of

honey is partly mediated via amelioration of oxidative stress in tissues such as GIT, liver, kidney, pancreas, eye, plasma, red blood cells and reproductive organs (Gharzouli *et al.*, 2002; Al-Waili *et al.*, 2006; Mohamed *et al.*, 2011; Zaid *et al.*, 2011; Erejuwa *et al.*, 2010; Kassim *et al.*, 2010). A clastogen is a material that can cause breaks in chromosomes, leading to sections of the chromosome being deleted, added, or rearranged. Therefore, clastogenicity is described as the microscopically visible damages or changes to chromosomes (Testoni *et al.*, 1997). This can cause a mutation, and lead to cancer development, as cells that are not killed by the clastogen may become cancerous. Known clastogens include acridine yellow, benzene, ethylene oxide, arsenic, phosphine and mimosine (Sunderman, 1984). However, the ability of SA to induce chromosomal breakage has been reported (Aliyu *et al.*, 2012; Owumi *et al.*, 2012). It is also known that arsenic can act as co-mutagen due to its ability to inhibit the activities of thiol containing enzymes (Li and Rossman, 1989), such as DNA ligase resulting in defective DNA replication/repair, recombination and joining of single- and double-stranded DNA breaks (Lasko *et al.*, 1990). These literatures clearly supported our findings on the higher rate of micronuclei induction observed by SA as an index of clastogenicity. However, administration of AH was able to significantly reduce the number of micronuclei confirming its anti-clastogenic potentials. This supports the findings from the study with other type honey (Odunola *et al.*, 2005).

CHAPTER SIX

6.0 SUMMARY OF FINDINGS, CONCLUSION AND CONTRIBUTIONS TO KNOWLEDGE

6.1 SUMMARY OF FINDINGS

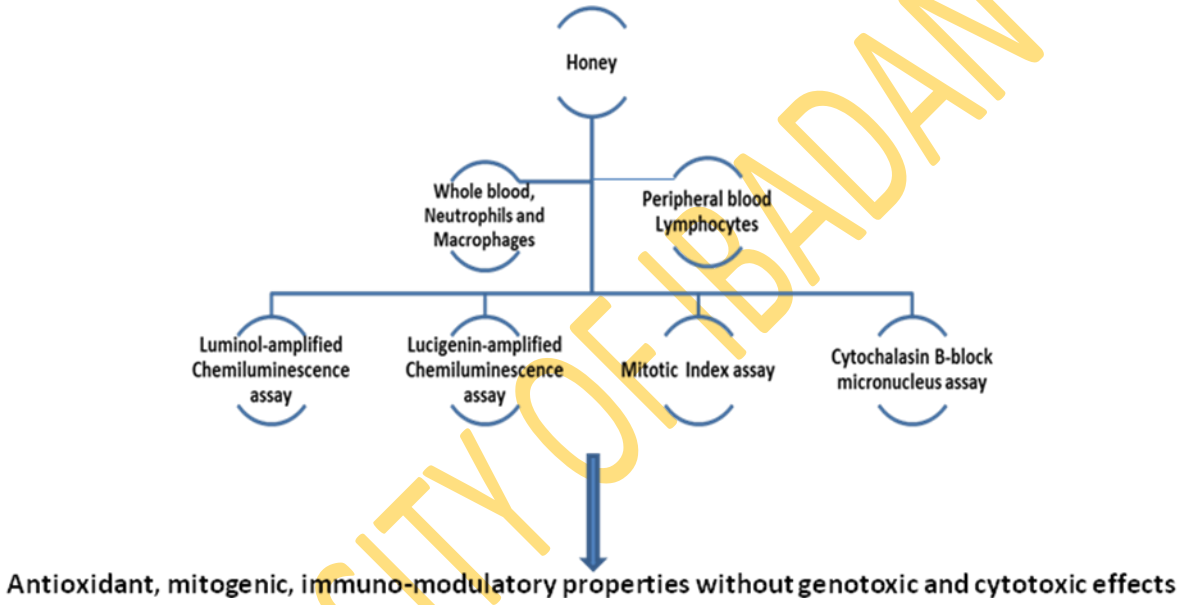

Figure 95: The summary of findings from blood and its components.

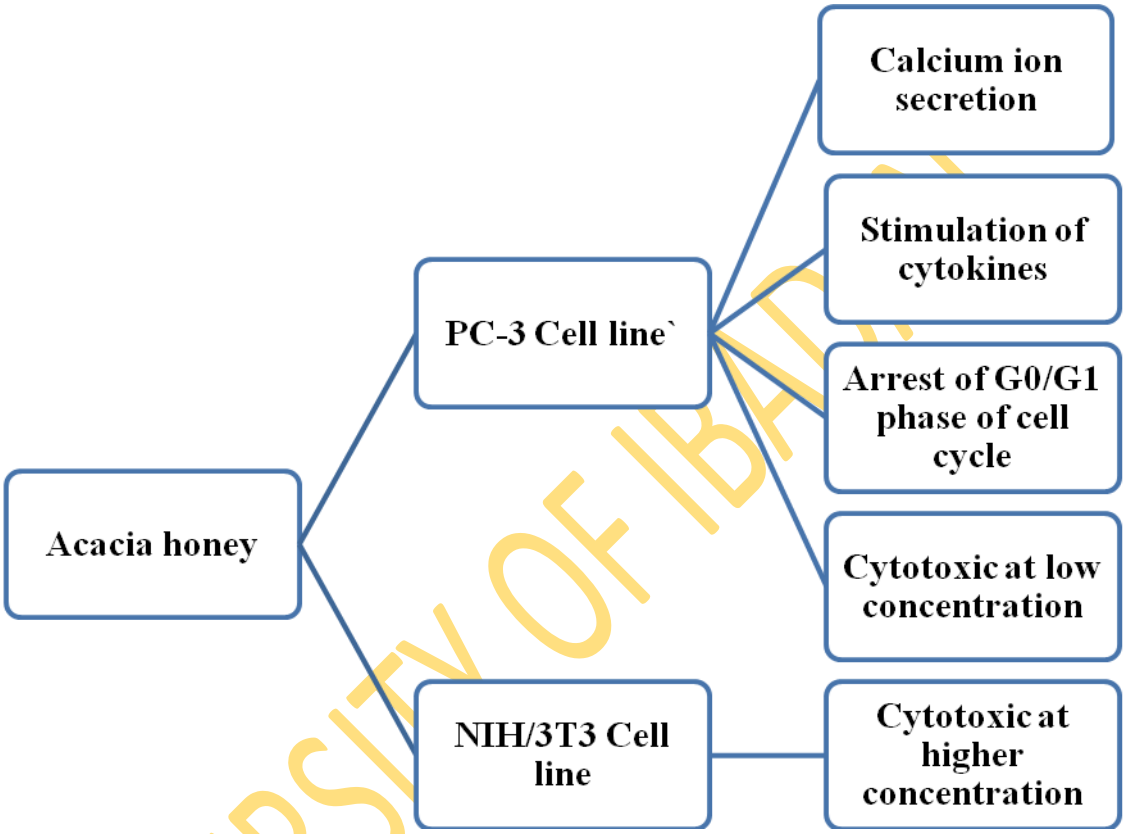

Figure 96: The summary of findings on PC-3 and 3T3 cell lines.

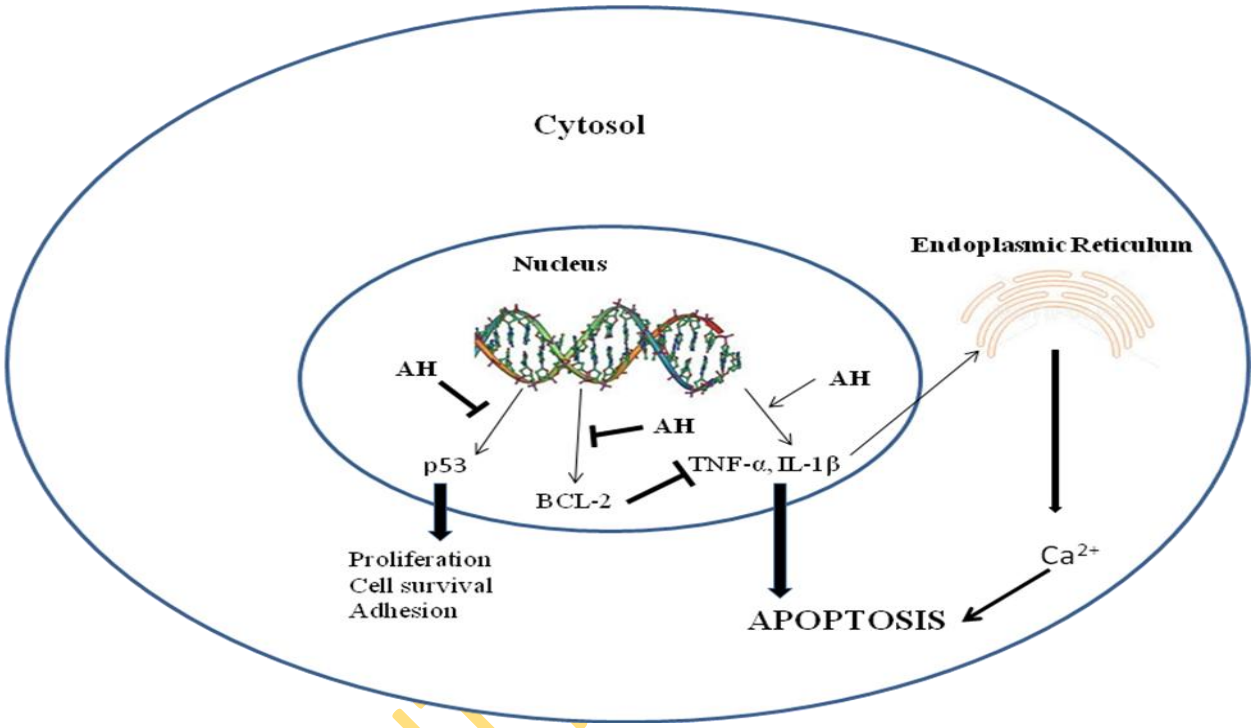

Figure 97: Proposed molecular mechanism of anti-proliferation potential of AH on NCI-H460 cell line.

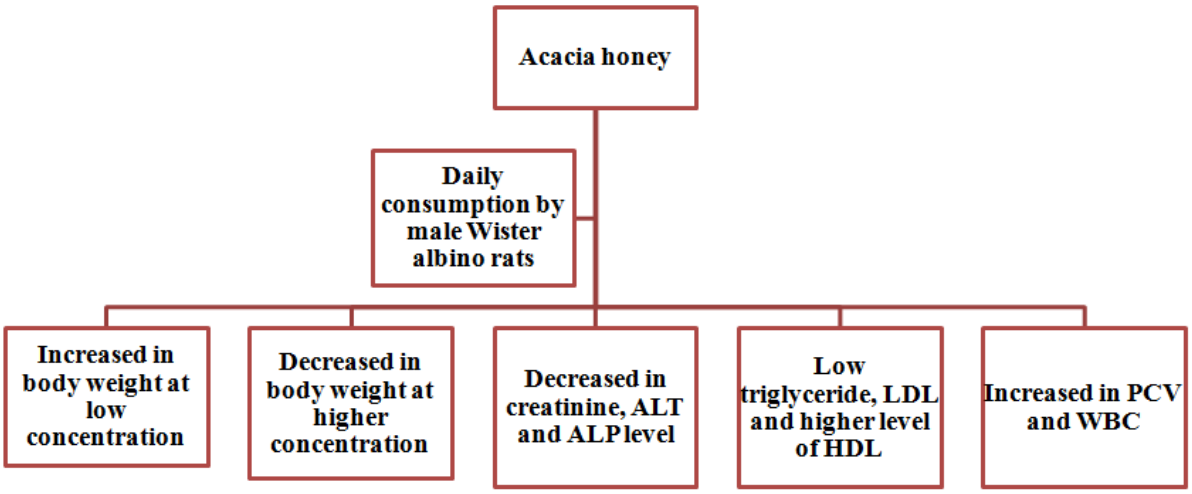

Figure 98: The summary of findings on daily consumption of AH in male Wistar albino rats.

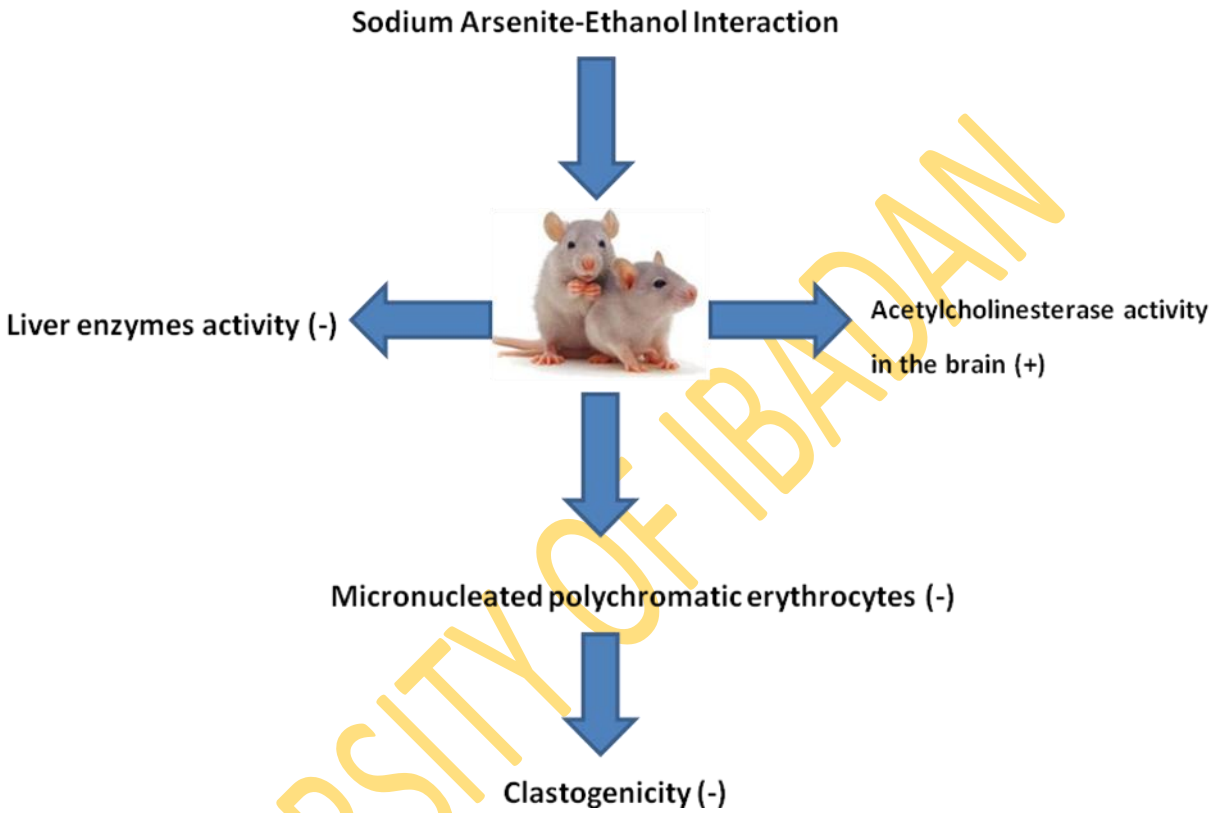

Figure 99: The summary of findings on the administration and co-administration of SA and ethanol. + Increase, - decrease.

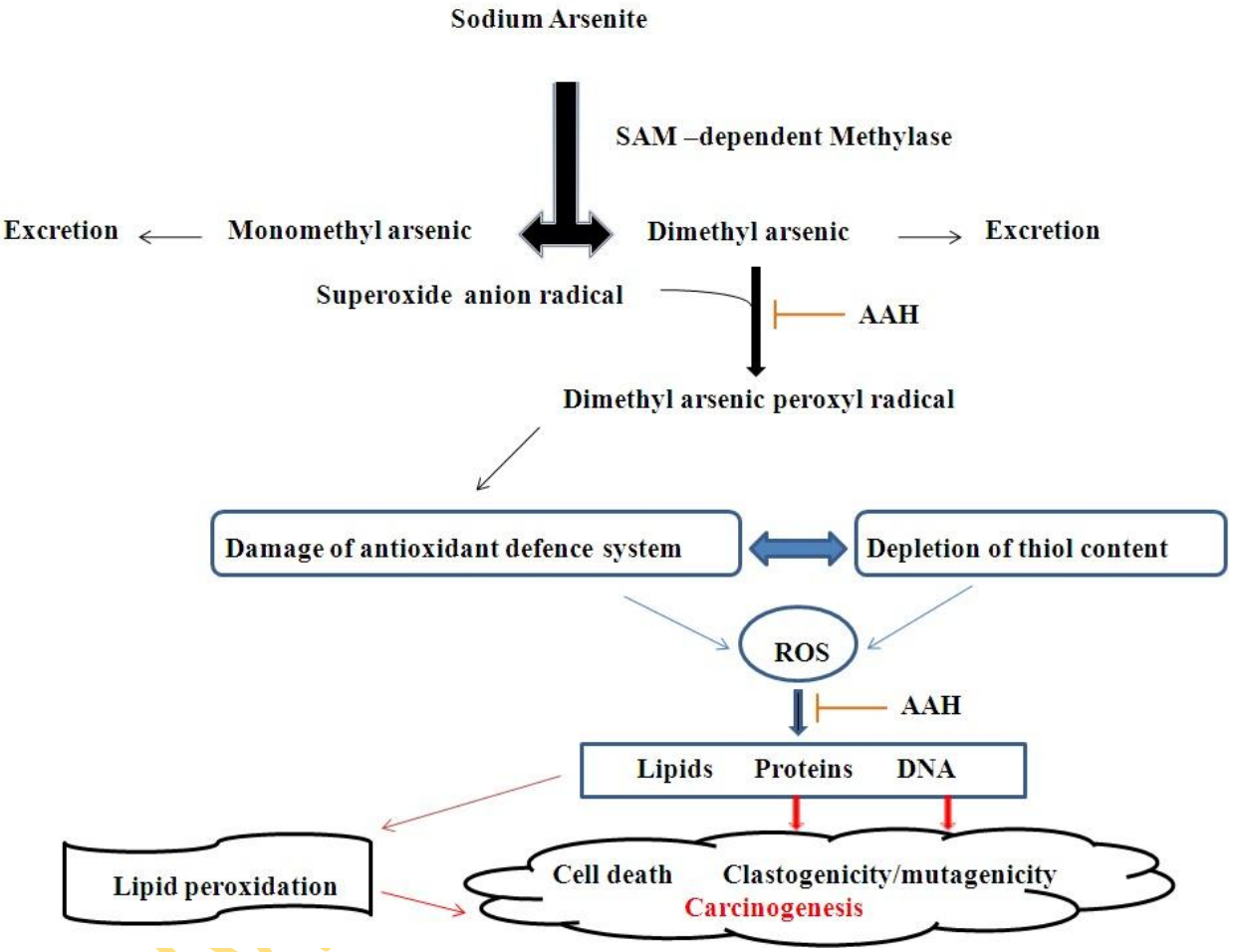

Figure 100: The summary of findings on the comparative assessment of redox biomarkers.

6.2 CONCLUSION

Acacia Honey possesses antioxidant, mitogenic and immuno-modulatory potentials *in vitro*. Apoptotic role of AH on PC-3 cell line may be due to G0/G1 arrest, modulation of pro-inflammatory cytokines and calcium ions secretion. The anti-proliferation potential of AH on NCI-H460 cell line may be due to similar reason as well as the down regulation of Bcl-2 and p53 genes. AH from Nigeria and Pakistan mitigate SA induced-oxidative stress and clastogenicity in rats, with their antioxidant properties more pronounced in liver tissues.

6.3 CONTRIBUTIONS TO KNOWLEDGE

Contributions to knowledge from the research findings are as follows:

1) AH is a potential source of antioxidants
2) It is an immuno-modulatory agent
3) It has strong anticancer activity
4) It is an apoptotic inducing agent
5) It may be use in the management of obesity
6) AH of Pakistan and Nigerian sources seem to have similar biological activity

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APPENDICES

LIST OF PUBLICATIONS

- Muhammad Aliyu, Oyeronke A. Odunola, Ahsana D. Farooq, Ahmed M. Mesaik, Muhammad I. Choudhary, Ochuko L. Erukainure, Shahida Perveen and Almas Jabeen, Fractionation of AH affects its Antioxidant Potential In vitro Journal of Acute Diseases, doi: 10.1016/S2221-6189(13)60070-0.

- Muhammad Aliyu, Oyeronke A. Odunola, Ahsana D. Farooq, Ahmed M. Mesaik, Muhammad I. Choudhary, Mudassar Azhar, Muhammad Asif and Ochuko L. Erukainure (2014), Antioxidant, Mitogenic and Immuno-modulatory Potentials of AH In vitro, Nutritional Therapy and Metabolism (Accepted).

- Muhammad Aliyu, Oyeronke A. Odunola, Ahsana D. Farooq, Ahmed M. Mesaik, Muhammad I. Choudhary, Beenish Fatima, Tariq A. Qureshi and Ochuko L. Erukainure (2013), AH Modulates Cell Cycle Progression, Pro-inflammatory Cytokines and Calcium ions Secretion in PC-3 Cell line http://dx.doi.org/10.4172/1948-5956.1000174.

- Muhammad Aliyu, Oyeronke A. Odunola, Ahsana D. Farooq, Huma Rasheed, Ahmed M. Mesaik, Muhammad I. Choudhary, Iffat S. Channa, Salman A. Khan and Ochuko L. Erukainure (2013), Molecular Mechanism of Anti-proliferation Potential of AH on NCI-H460 Cell Line In vitro Nutrition and Cancer, 65:2, 296-304.

- Oyeronke A. Odunola, Muhammad Aliyu, Ahsana D. Farooq, Kourosh Dalvandi, Huma Rasheed, Muhammad I. Choudhary, and Ochuko L. Erukainure (2013), Comparative Assessment of Redox Sensitive Biomarkers due to AH and SA Administration In vivo Mediterr J Nutr Metab DOI 10.1007/s12349-013-0127-1.

- Muhammad Aliyu, Oyeronke A. Odunola, Michael A. Gbadegesin, Ayodeji M. Adegoke, J. Olorunjuwon Olugbami and Ndidi S. Uche. (2014). Modulatory Role of AH from North-West Nigeria on Sodium Arsenite-induced Clastogenicity and Oxidative Stress in Male Wistar Rats, Natural Products Research, Taylor and Francis (published).

Consumables and Equipment

Adherent human cancer/normal cell lines: large cell lung cancer (NCI-H460), (NCI, USA), prostate cancer cell (PC-3), and normal cell from mouse fibroblast (NIH/3T3). Balance analytical (Precisia XB-120 A), Balance milligram (XB-320 B), cell culture boats, cell culture flasks (25cm^2 and 75 cm^2), 6, 24 and 96-bottom well plates, cell counter, centrifuge (D-37520, Kendro Lab Products, Germany), centrifuge tubes (1, 2, 5,15 and 50 mL), culture plates (96-well, clear flat-bottom) (Falcon), (VWR), gloves, incubator CO_2 (50049916 / b, Kendro Lab Products, Germany), Laminar hood / Safety cabinet class 2 (D-63450, Heraeus Germany), microcentrifuge tubes (1.5 mL) (Kartel, Italy), Microscopes: Inverted TS-100 and light E-400 (Nikon), multiwell microplate reader (Stat fax 2100), multiwell plate shaker (PMS 1000, Grant instruments, England)**,** neubauer's chamber (0.1mm and 0.0025mm^2, HBG, Germany), pipette tips (1-200µl, 200-1000µl), printer (Epson, LQ 300), P^H meter (Precisia 900), serological pipette (1, 5 and 10 mL), syringe filters (0.22 µm, Pyrex), Ultrasonic bath (MXB6, Grant), refractometer (GMK-701R, G-Wou HITECH; Co LTD), Mastercycler pro S, Eppendorf Scientific (NY, USA), cDNA synthesis kit (Fermentas Incorporation, Vilnius, Lithuania), RT-PCR machine (Agilent technologies stratagene Mx 3000P, USA), flow cytometer FACSCalibur (Becton Dickinson, Canada, Inc), Auto Analyzer Hitachi Roche 7020 (902), (Japan Inc.), Coulter HmX Hematology Analyzer (Beckman Coulter Inc.), Cobas kits, Nanodrop 2000, spectrophotometer (Thermo scientific, Delaware, USA), cDNA synthesis kits (Fermentas Incorporation, Vilnius, Lithuania), PCR Mastercycler (Mastercycler pro S, Eppendorf Scientific, NY, USA), Light Cycler system (Agilent technologies Stratagene Mx 3000P, USA), electrolyte analyzer (ion selective electrode, China).

Lipid Peroxidation Assay Calculation

Lipid peroxidation was computed with a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$

$$\text{MDA} = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532nm} \times \text{volume of sample}}$$

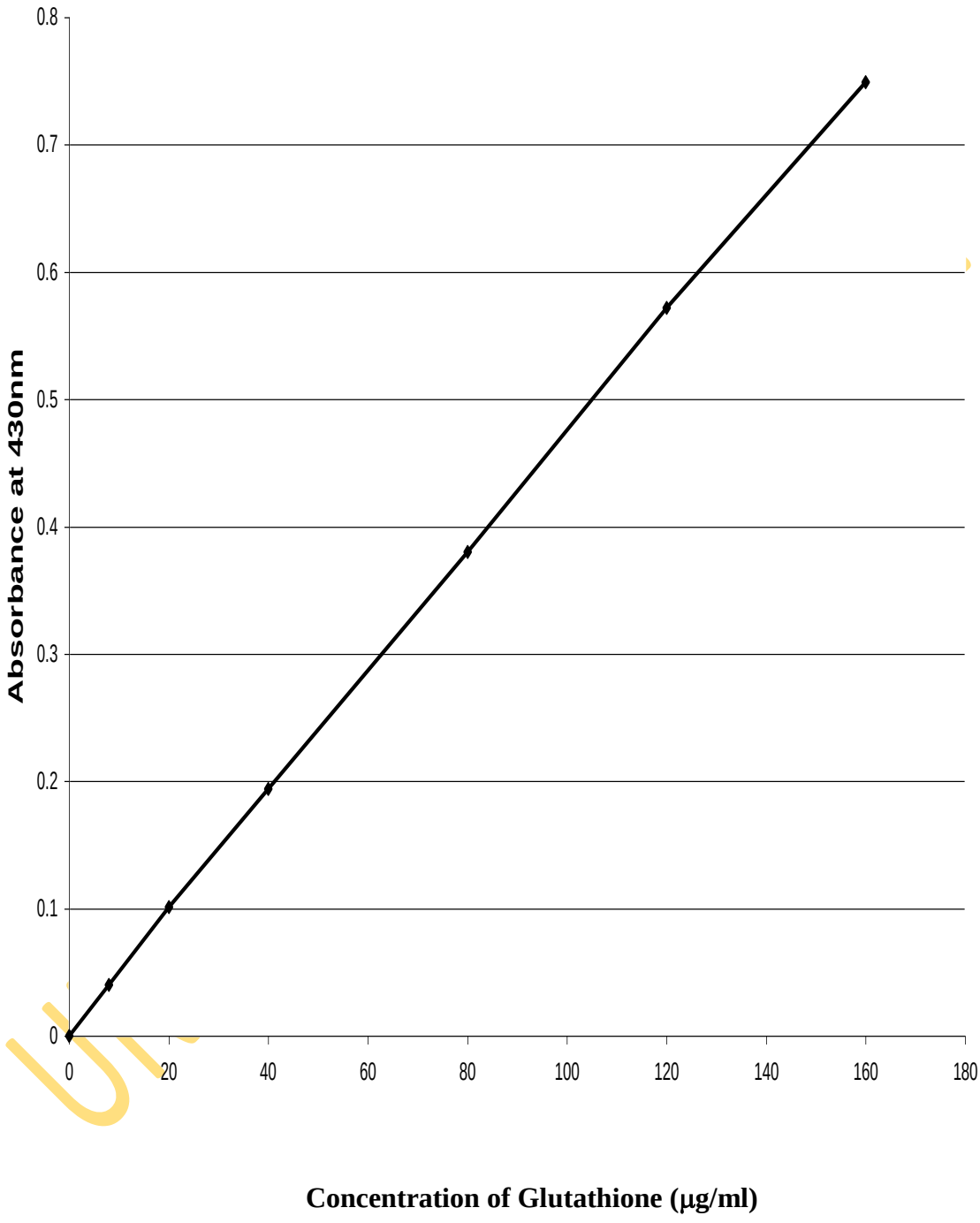

Figure 101: Standard curve for reduced glutathione

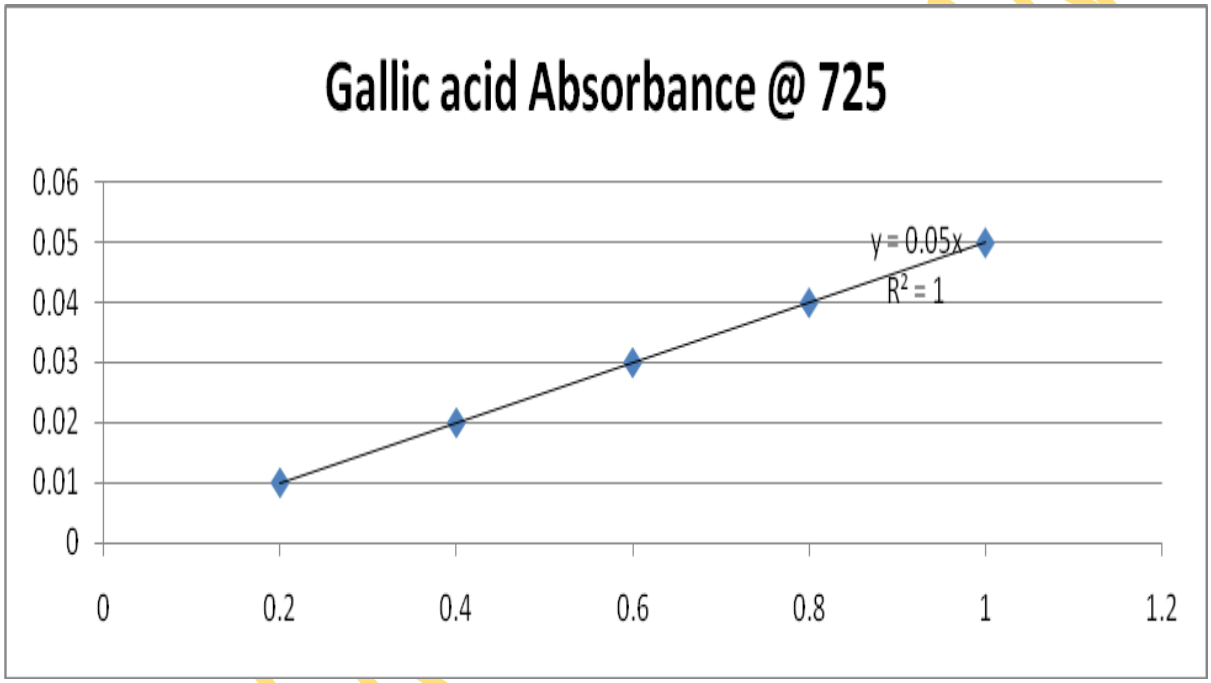

Figure 102: Standard curve for gallic acid

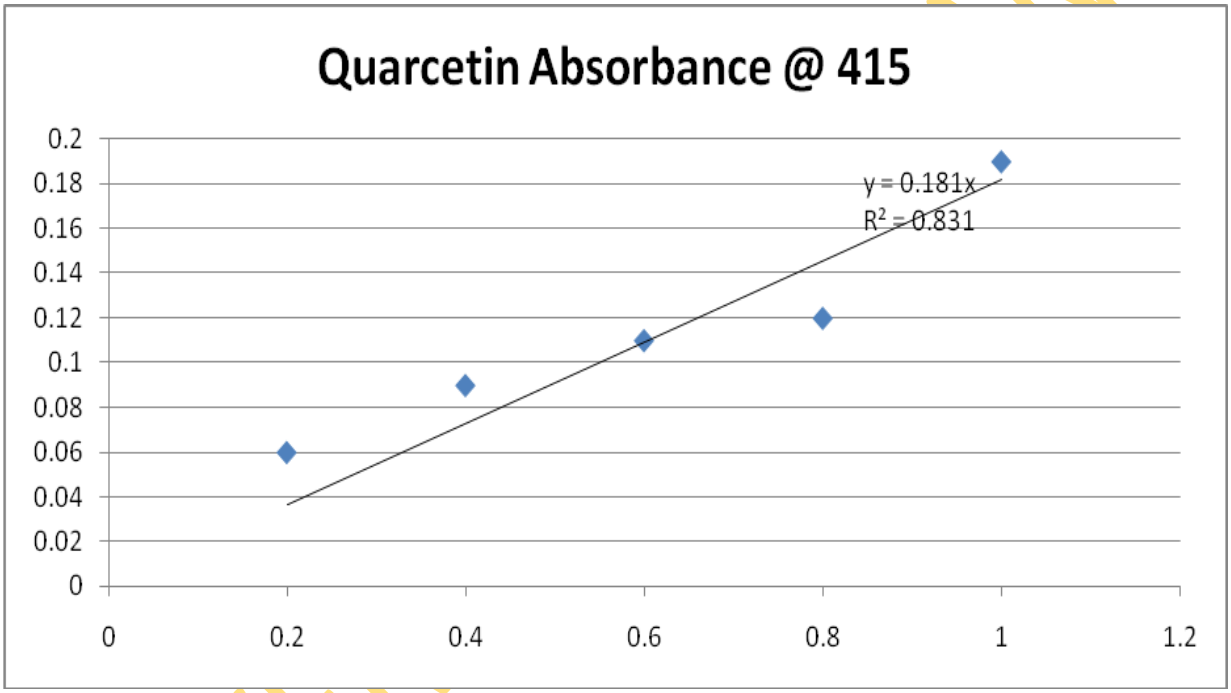

Figure 103: Standard curve for quercetin

Calculation of SOD Activity

Increase in absorbance per minute = $\frac{A_3 - A_0}{2.5}$

Where A_0=absorbance after 30 seconds

A_3=absorbance after 150 seconds

% inhibition =100——100 X $\frac{\text{increase in absorbance for substrate}}{\text{Increase in absorbance for blank}}$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

Calculation

Catalase activity was calculated by plotting the standard curve and the concentration of the remaining H_2O_2 was extrapolated from the curve.

H_2O_2 consumed =1200 µmoles – H_2O_2 remaining

Catalase activity = $\frac{H_2O_2 \text{ consumed}}{\text{mg protein}}$

Table 1: List of Primers used in this Study

Gene	Left Primer Sequence	Right Primer Sequence	Accession Number
GAPDH	GTCAGTGGTGGACCTGACCT	AGGTGGAGGAGTGGGTGTC	NM_002046
p53	CCAGTGTGATGATGGTGAGG	GTGGAAGGAAATTTGCGTGT	NM_001126114.2
Bcl-2	GGTGGAGGAGCTCTTCAGG	GCCGGTTCAGGTACTCAGTC	NM_000633

Table 2: Concentration of RNA after isolation from NCI-H460 cell line

Treatment(% v/v)	**Absorbance 260nm/280nm Ratio**	**RNA (µg/mL)**
control	1.88	1169.7
2	1.77	506.7
4	1.88	268.9
8	1.87	347.4

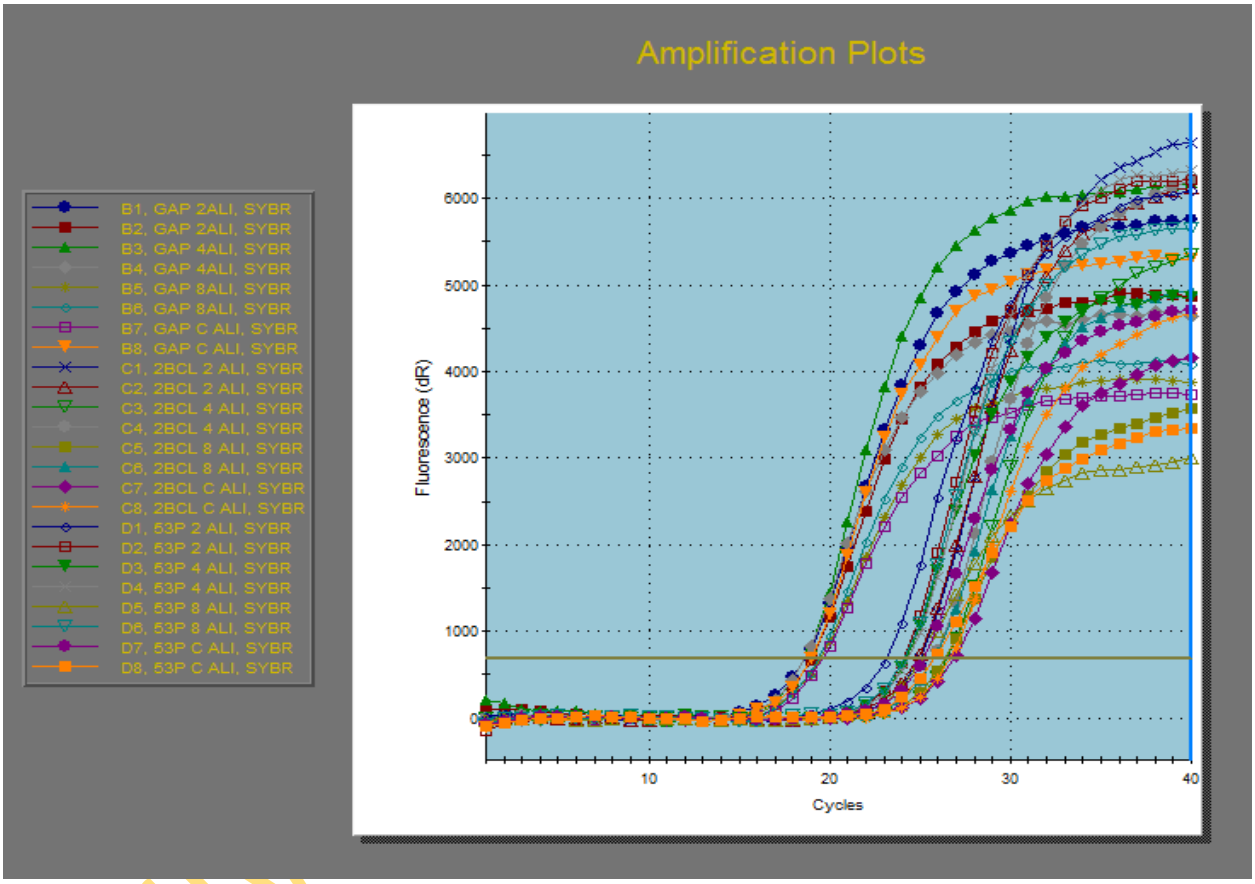

Figure 104: The amplification plots of RT-PCR on some genes (p53, Bcl-2 and GAPDH) from NCI-H460 cell line.

Table 4: Protocol for the estimation of hydrogen peroxide

Test tube	1	2	3	4	5	6	7
H_2O_2 (ml)	0.05	0.10	0.15	0.20	0.30	0.40	0.50
Dichromate/acetic acid (ml) `	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (ml)	0.95	0.90	0.85	0.80	0.70	0.60	0.50
H_2O_2 concentration (μ moles)	10	20	30	40	60	80	100
Absorbance (570nm)	0.049	0.095	0.145	0.195	0.291	0.385	0.484

Table 5: Preparation of GSH working Standard

Stock (ml)	PO_4	Ellman's Reagent	Absorbance (412nm)	GSH Conc. (ug/ml)
0.02	0.48	4.50	0.040	8
0.05	0.45	4.50	0.101	20
0.10	0.40	4.50	0.194	40
0.20	0.30	4.50	0.380	80
0.30	0.20	4.50	0.572	120
0.40	0.10	4.50	0.749	160

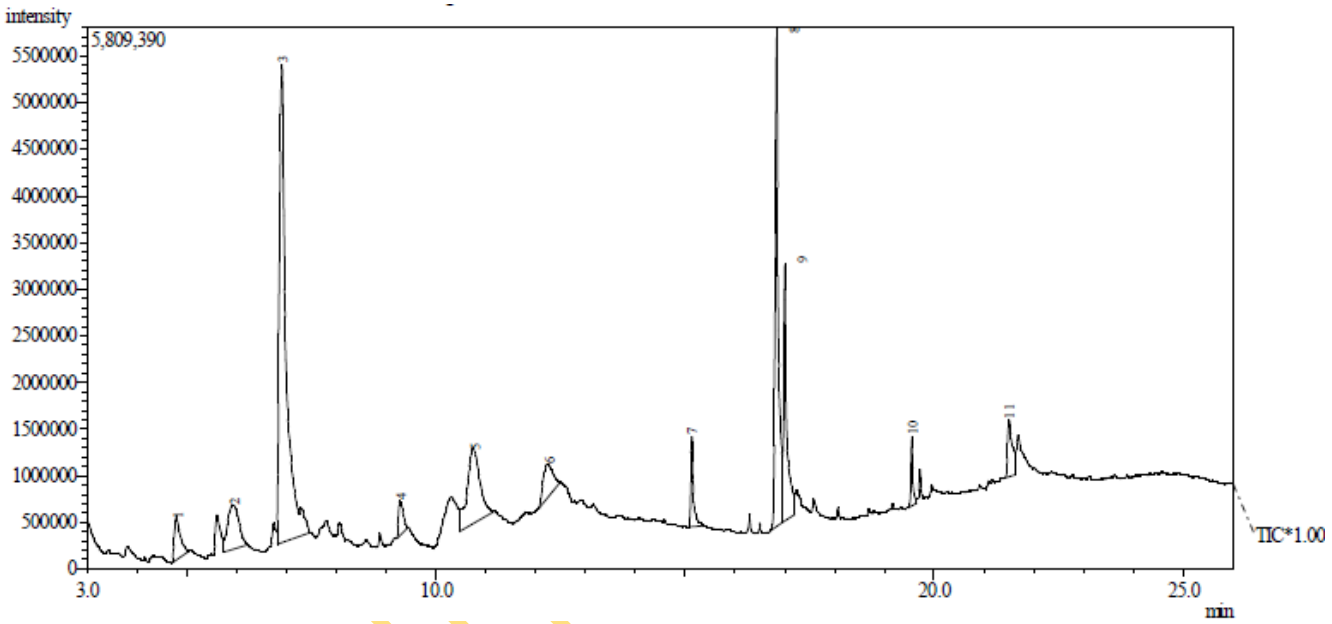

Figure 105: GC-MS analysis results of AH (Parkistan)