COMPARATIVE *IN VITRO* STUDY OF THE ANTI-CANCER EFFECT OF APRICOT AND PEACH KERNEL EXTRACTS ON HUMAN COLON CANCER CELLS

WAGHEDA CASSIEM

COMPARATIVE *IN VITRO* STUDY OF THE ANTI-CANCER EFFECT OF APRICOT AND PEACH KERNEL EXTRACTS ON HUMAN COLON CANCER CELLS

by

WAGHEDA CASSIEM

Submitted in partial fulfilment for the degree

Magister Scientiae

Department of Medical Biosciences

University of the Western Cape

Bellville

Supervisor: Prof M. de Kock

Co-supervisor: Dr A. Mohammed

May 2015

DECLARATION

I, the undersigned, declare that **Comparative *in vitro* study of the anti-Cancer effect of the apricot and peach kernel extracts on HT -29 human colon cancer cell line** is my own work, that it has not been submitted before for any degree or assessment at any university, and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

UNIVERSITY of the
WESTERN CAPE

____________________ ____________________

W. Cassiem Date

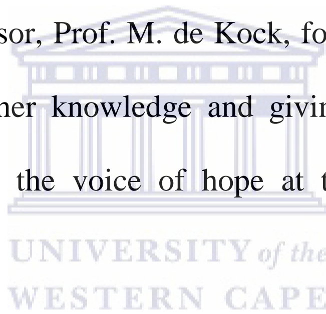
Acknowledgements

The completion of this thesis has been made possible by the efforts of many individuals and institutions whom I would like to acknowledge for their contribution.

I firstly acknowledge The Creator for His mercy and affording me the opportunity to realise this project under the guidance of those whom He has guided.

This study was carried out in the department of Medical Biosciences at the University of the Western Cape (UWC), the Chemistry department (UWC) laboratory, and the Health Sciences Faculty Immunology department at the University of Cape Town (UCT).

I would like to thank my supervisor, Prof. M. de Kock, for enthusiastically taking me under her wings, selflessly imparting her knowledge and giving of her time, being a pillar of support and encouragement and the voice of hope at the times of despair. It is much appreciated.



I would also like to thank:

- Dr Ahmed Mohammed, Department of Chemistry, University of the Western Cape, for his assistance as my co-supervisor.
- Ronnie Dreyer, University of Cape Town, for his expertise in flow cytometric analysis.
- The late Prof. Sedick Isaacs, for his invaluable comments and encouragement to pursue this study.
- Prof. G. van der Horst, Department of Medical Bioscience, University of the Western Cape, for his assistance with MedCalc.

- Colleagues and staff members at the School of Natural Medicine, University of the Western Cape, for their support and encouragement. A special mention of the HoD, Dr. J. Campbell and Chinese Medicine co-ordinator Dr. Ma Xuesheng.
- Students at the Department of Medical Bioscience, University of the Western Cape, for their lab assistance and timeous encouragement.
- Extended family, friends and members of the community who always kept my family and me in their prayers during this strenuous period of study.

To my father, Achmad, and late mother, Fatima, who were the inspiration for this study, husband Shafiek, children Mujahieda, Jawaad, Ali-Ammaar, Maseeh, Hadiyah, and brother Zubayr, thank you for all your support and endless encouragement. May you all be blessed abundantly.

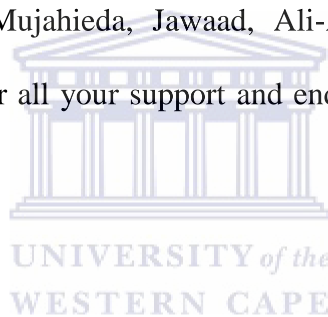


Table of Contents

Table of Contents i

List of Tables vi

List of Figures vii

List of Abbreviations xv

Summary xviii

Chapter 1: Literature review 1

1.1 Cancer 1

1.1.1 Colon Cancer 3

1.1.2 Pathogenesis 5

1.2 Chinese Medicine (CM) 7

1.2.1 Peach and Apricot Kernels in Chinese Medicine 7

1.2.2 Composition of the Apricot Bitter Kernel 9

1.2.3 Composition of the Peach Kernel 10

1.3 Specific active components of the apricot and peach kernels 12

1.3.1 Amygdalin 12

1.3.2 Laetrile and the "Krebs Hypothesis" 14

1.3.2.1 Theories for the possible mechanism of action of Laetrile 15

1.3.2.2 Rhodanese enzyme 19

1.3.3 Metabolism and Toxicity of Amygdalin 20

UNIVERSITY of the
WESTERN CAPE

1.3.3.1 Cyanide 23

1.3.3.1.1 Metabolism and Excretion of Cyanide 23

1.3.3.1.2 Toxicity of cyanide derived from cyanogenic glycosides in apricot kernels 26

1.4 Research of possible effects of Amygdalin 27

1.4.1 Cell growth / cell death studies 27

1.4.2 Antimicrobial properties of amygdalin 28

1.4.3 Antioxidant properties of amygdalin 28

1.4.4 Toxicity tests 29

1.5 Disclaimers 30

1.6 Overview of the cell cycle 32

1.6.1 The cell cycle phases 33

1.6.1.1 G_1 Phase 34

1.6.1.2 S Phase 36

1.6.1.3 G_2 phase 39

1.6.1.4 M phase 40

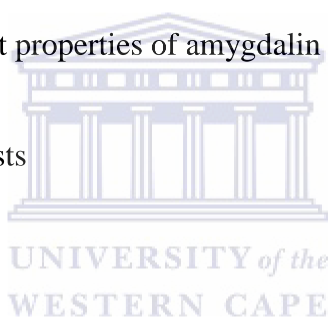
1.6.2 Cell cycle checkpoints 41

1.6.2.1 DNA damage checkpoint 42

1.6.2.2 Intra-S phase checkpoint 44

1.6.2.3 The spindle assembly checkpoint 46

1.7 Types of cell death 47


UNIVERSITY of the
WESTERN CAPE

1.7.1 Apoptosis 49

1.7.1.1 Extrinsic pathway / Cytoplasmic pathway 49

1.7.1.2.1 Intrinsic pathway / mitochondrial pathway 50

1.8 Significance to health care and or biomedical science 52

Chapter 2: Materials and Methods 55

2.1 Materials

2.1.1 Chemicals and Solutions used 55

2.1.2 Equipment 56

2.2 Methods

UNIVERSITY of the
WESTERN CAPE

2.2.1 General cell culture procedures 57

2.2.2 Cell counts 58

2.2.3 Preparation of extraction fractions from kernels 59

2.2.4 Organic ethanol / acetone extractions (including total, lipophilic and hydrophilic extractions) 59

2.2.4.1 Total extraction and filtration 59

2.2.4.2 Lipophilic extraction and filtration 60

2.2.4.3 Hydrophilic extraction and filtration 60

2.2.4.4 Evaporation of the Total, Lipophilic and

Hydrophilic extractions 60

2.2.5 Double boil decoction / Aqueous extraction 61

2.2.5.1 Freeze drying method of the aqueous extractions 62

2.2.5.2 CAK, CPK, TAK, SAK and SPK treatment 62

2.2.6 Cell growth and viability 62

2.2.6.1 Crystal Violet (CV) 62

2.2.7 Cell Morphology 64

2.2.7.1 Haematoxylin and Eosin Staining (H&E) 64

2.2.8 Cell cycle progression 65

2.2.8.1 Flow Cytometry 65

2.2.9 Hoechst 33342 fluorescent stain 67

2.2.10 Statistical analysis 67

Chapter 3: Results

3.1 The rationale for making the extractions used to treat the HT-29 colon cancer cells 69

3.1.1. Expected compounds in the various extractions 74

3.2 Cell Viability 77

3.2.1 Crystal violet method studying the effects of CAK, CPK, TAK, SAK and SPK organic and CAK, CPK, SAK and SPK aqueous extractions

UNIVERSITY of the
WESTERN CAPE

on the growth of HT-29 colon cancer cells 77

3.3 Morphological study 104

3.3.1 Haematoxylin and Eosin staining (H&E) 104

3. 4 Flow cytometry 112

3.4.1 Cell cycle progression after treatment with 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 113

3.4.2 Cell cycle progression over 24, 48 and 72 hours after treatment with 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 126

3.4.3 Cell cycle progression over 24, 48 and 72 hours after treatment with 100, 500 and 1000 µg/mL CAK, CPK, SAK and SPK aqueous extractions 143

3.5 Hoechst 33342 fluorescent stain 150

Chapter 4: Discussion 155

Annexure 170

References 208

UNIVERSITY of the
WESTERN CAPE

List of Tables

Table 2.1 Formula used to calculate percentage yield 61

Table 3.1 General description of the organic fractions of CAK, CPK, TAK, SAK and SPK 73

Table 3.2 Shows the percentage yield of CAK, CPK, TAK, SAK and SPK organic kernel extraction fractions 75

Table 3.3 Shows dry weight in grams of CAK, CPK, SAK, SPK powder of freeze dried aqueous kernel extraction fractions 76

Table 3.4 Summary presentation of extracts which showed the most significant inhibition on cell proliferation of HT-29 human colon cancer cells 101

Table 3.5 Summary of the organic kernel extractions that significantly altered cell cycle progression, increasing the number of cells in the S phase and decreasing the number of cells in the G_2 phase of the HT-29 colon cancer cells after 24, 48 and 72 hours exposure to 100, 500 and 1000 µg/mL 137

Table 3.6 Summary of the aqueous kernel extractions that significantly altered cell cycle progression, increasing the number of cells in the S phase and decreasing the number of cells in the G_2 phase of the HT-29 colon cancer cells after 24, 48 and 72 hours exposure to 100, 500 and 1000 µg/mL 148

UNIVERSITY of the WESTERN CAPE

List of Figures

Figure 1.1	Causes and Pathogenesis according to Chinese Medicine	6
Figure 1.2	Amygdalin	13
Figure 1.3	Laetrile	14
Figure 1.4	Graphic representation of the chemistry of nitrilosides in cancer	18
Figure 1.5	Hydrolysis of Amygdalin	21
Figure 1.6	Structures of common plant-derived cyanoglycosides and principle pathway of HCN formation	22
Figure 1.7	Basic processes involved in the metabolism of cyanide	25
Figure 1.8	Mammalian cell cycle	32
Figure 1.9	Cell cycle and its checkpoints	34
Figure 1.10	Cell cycle regulation of DNA replication	36
Figure 1.11	Signalling at G_2/M transition	39
Figure 1.12	Cell cycle control: G_1/S phase checkpoint	41
Figure 1.13	Intra-S-phase checkpoint	44
Figure 1.14	Spindle Assembly Checkpoint (SAC) principles	46
Figure 1.15	Apoptotic signalling pathways and their relevance to microRNA regulation in cancer	52
Figure 3.1	Shows the dried Chinese apricot kernel (CAK), Chinese peach kernel	

UNIVERSITY of the
WESTERN CAPE

(CPK), Turkish apricot kernel (TAK), South African apricot kernel (SAK) and South African peach kernel (SPK) which were removed from their respective pips 70

Figure 3.2 Shows pictures of the end product of the CAK, CPK and TAK organic extractions 71

Figure 3.3 24h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 µg/mL Chinese apricot kernel organic extractions 78

Figure 3.4 48h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 µg/mL Chinese apricot kernel organic extractions 78

Figure 3.5 72h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 µg/mL Chinese apricot kernel organic extractions 79

Figure 3.6 24h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 µg/mL Chinese peach kernel organic extractions 81

Figure 3.7 48h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 µg/mL Chinese peach kernel organic extractions 82

Figure 3.8 72h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 µg/mL Chinese peach kernel organic extractions 82

Figure 3.9 24h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to100, 500 and 1000 µg/mL Turkish apricot kernel organic extractions 84

Figure 3.10 48h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 µg/mL Turkish apricot kernel organic extractions 85

UNIVERSITY of the WESTERN CAPE

Figure 3.11 72h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500, 1000 μg/mL Turkish apricot kernel organic extractions 85

Figure 3.12 24h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 μg/mL South African apricot kernel organic extractions 87

Figure 3.13 48h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 μg/mL South African apricot kernel organic extractions 88

Figure 3.14 72h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 μg/mL South African apricot kernel organic extractions 88

Figure 3.15 24h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 μg/mL South African peach kernel organic extractions 90

Figure 3.16 48h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 μg/mL South African peach kernel organic extraction 91

Figure 3.17 72h Box-and-whisker plots illustrating the HT-29 colon cancer cells exposure to 100, 500 and 1000 μg/mL South African peach kernel organic extractions 91

Figure 3.18 24h Box-and-whisker plot illustrating the exposure of HT-29 colon cancer cell to 100, 500 and 1000 μg/mL CAK, CPK, TAK, SAK and SPK organic extractions 95

UNIVERSITY of the
WESTERN CAPE

Figure 3.19 48h Box-and-whisker plot illustrating the exposure of HT-29 colon cancer cell to 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 96

Figure 3.20 72h Box-and-whisker plot illustrating the exposure of HT-29 colon cancer cell to 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 97

Figure 3.21 24h, 48h, 72h:Box-and-whisker plot illustrating the exposure of HT-29 colon cancer cell to 100, 500 and 1000 µg/mL CAK, CPK, SAK and SPK aqueous extractions 100

Figure 3.22 – 3.31 show 24h, 48h and 72h untreated cells and cells exposed to CPK and CAK organic and aqueous extractions 105

Figure 3.32 – 3.36 show the effects SAK aqueous and organic extractions on HT-29 colon cancer cells 107

Figure 3.37 – 3.42 show the effects of some SAK organic extractions on HT-29 colon cancer cells using H&E staining 108

Figure 3.43 – 3.48 show the effects of some SPK aqueous and organic extractions on HT-29 colon cancer cells using H&E staining 109

Figure 3.49 – 3.54 show the effects of some SPK organic extractions on HT-29 colon cancer cells using H&E staining 110

Figure 3.55 – 3.56 show the effects of SPK-T organic extractions on HT-29 colon cancer cells using H&E staining 111

Figure 3.57 Flow cytometric analysis of HT-29 cells after 24 hour exposure to concentrations of 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 115

UNIVERSITY of the
WESTERN CAPE

Figure 3.58 Flow cytometric analysis of HT-29 cells after 48 hour exposure to concentrations of 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 116

Figure 3.59 Flow cytometric analysis of HT-29 cells after 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 117

Figure 3.60 Flow cytometric analysis of HT-29 cells after 24, 48 and 72hour exposure to concentrations of 100 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 120

Figure 3.61 Flow cytometric analysis of HT-29 cells after 24, 48 and 72hour exposure to concentrations of 500 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 121

Figure 3.62 Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions 122

Figure 3.63 Flow cytometric analysis of HT-29 cells after 24hour exposure to concentrations of 100, 500 and 1000 µg/mL total extractions of CAK, CPK, TAK, SAK and SPK 123

Figure 3.64 Flow cytometric analysis of HT-29 cells after 48h exposure to concentrations of 100, 500 and 1000 µg/mL total extractions of CAK, CPK, TAK, SAK and SPK 124

UNIVERSITY of the
WESTERN CAPE

Figure 3.65 Flow cytometric analysis of HT-29 cells after 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL total extractions of CAK, CPK, TAK, SAK and SPK 124

Figure 3.66 Flow cytometric analysis of HT-29 cells after 24 hour exposure to concentrations of 100, 500 and 1000 µg/mL lipophilic extractions of CAK, CPK, TAK, SAK and SPK 126

Figure 3.67 Flow cytometric analysis of HT-29 cells after 48 hour exposure to concentrations of 100, 500 and 1000 µg/mL lipophilic extractions of CAK, CPK, TAK, SAK and SPK 127

Figure 3.68 Flow cytometric analysis of HT-29 cells after 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL lipophilic extractions of CAK, CPK, TAK, SAK and SPK 127

Figure 3.69 Flow cytometric analysis of HT-29 cells after 24 hour exposure to concentrations of 100, 500 and 1000 µg/mL hydrophilic extractions of CAK, CPK, TAK, SAK and SPK 129

Figure 3.70 Flow cytometric analysis of HT-29 cells after 48 hour exposure to concentrations of 100, 500 and 1000 µg/mL hydrophilic extractions of CAK, CPK, TAK, SAK and SPK 130

Figure 3.71 Flow cytometric analysis of HT-29 cells after 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL hydrophilic extractions of CAK, CPK, TAK, SAK and SPK 130

Figure 3.72 Flow cytometric analysis of HT-29 cells after 24h, 48h and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic CAK extractions 132

UNIVERSITY of the
WESTERN CAPE

Figure 3.73 Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic CPK extractions 133

Figure 3.74 Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic TAK extractions 133

Figure 3.75 Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic SAK extractions 134

Figure 3.76 Flow cytometry analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic SPK extractions 135

Figure 3.77(a-n) Flow cytometric analysis of HT-29 cells after 24h, 48h and 72h exposure to concentration of 100 µg/mL aqueous extractions of CAK, CPK, SAK and SPK 140

Figure 3.78 Flow cytometric analysis of HT-29 cells after 24h, 48h and 72h exposure to concentration of 100 µg/mL aqueous extractions of CAK, CPK, SAK and SPK 144

Figure 3.79 Flow cytometric analysis of HT-29 cells after 24h, 48h and 72h exposure to concentration of 500 µg/mL aqueous extractions of CAK, CPK, SAK and SPK 144

Figure 3.80 Flow cytometric analysis of HT-29 cells after 24h, 48h and 72h exposure to aqueous extractions of CAK, CPK, SAK and SPK at concentration of 1000 µg/mL 145

UNIVERSITY of the
WESTERN CAPE

Figure 3.81 Flow cytometric analysis of HT-29 cells after 24h exposure to concentrations of 100, 500 and 1000 µg/mL aqueous extractions of CAK, CPK, SAK and SPK 146

Figure 3.82 Flow cytometric analysis of HT-29 cells after 48h exposure to aqueous extractions of CAK, CPK, SAK and SPK at concentrations of 100, 500 and 1000 µg/mL 147

Figure 3.83 Flow cytometric analysis of HT-29 cells after 72h exposure to concentrations of 100, 500 and 1000 µg/mL aqueous extractions of CAK, CPK, SAK and SPK 147

Figure 3.84(A)-(H) show fluorescent stained HT-29 colon cancer cells exposed for 24 and 48hours at 500 µg/mL to organic kernel extractions 152

Figure 3.85(A)–(D) show fluorescent stained HT-29 colon cancer cells treated with CAK, CPK, SAK and SPK aqueous kernel extractions at 24 and 48 hour 500 µg/mL 154

UNIVERSITY of the
WESTERN CAPE

List of Abbreviations

APC/C: Anaphase-Promoting Complex / cyclosome

BAD: Bcl-2 Antagonist of Cell Death

ATM: ataxia-telangiectasia mutated

ATR: **A**taxiaand Rad3 Related

BAK: Bcl-2 Homologous Antagonist/Killer

BAX: Bcl-2 Associated X Protein

BCL-2: B-Cell Lymphoma-2 Family Members

UNIVERSITY of the
WESTERN CAPE

BID: BH3-Interacting Domain Death Agonist

Bw: body weight

CAK: Chinese apricot kernel

CARD: Caspase-Recruiting Domain

Cdc: Cell Division Cycle

Cdc20: Cell Division Cycle 20

Cdc25A, B: Cell Division Cycle 25A, B

Cdk: Cyclin Dependent Kinases

Cdk2: Cyclin Dependent Kinase 2

Chk1, 2: Checkpoint 1, 2

CKI: Cdk-inhibitors

CM: Chinese Medicine

CHM: Chinese Herbal Medicine

Cip/Kip: Cyclin inhibiting protein / Kinase inhibiting protein

CPK: Chinese peach kernel

CV: Crystal Violet

MdM2: Mouse Double Minute-2

DMEM: Dulbeco's Modified Eagle's Medium

DMEM/F12: Dulbecos Minimum Essential Medium F12

DMSO: Dimethyl-Sulfoxide

DNA: Deoxyribonucleic Acid

ECETOC: European Centre for Ecotoxicology & Toxicology of Chemicals

FAP: Familial Adenomatous Polyposis

FBS: Fetal Bovine Serum

EFSA: European Food Safety Authority

G_1 phase: Gap 1 phase

G_2 phase: Gap 2 phase

GLOBOCAN: International Agency for Research on Cancer (IARC's) online database

H&E: Haematoxylin and Eosin

LC: Level codes

L-Glut: L-Glutamine

MMC: Mitomycin-c bladder cell line

MDM2: Mouse Double Minute -2 Homologue

MOMP: Mitochondrial Outer Membrane Permeabilisation

NOAEL: No-Observed Adverse Effect Level

PBS: Phosphate-buffered saline solution

PI: Propidium iodide

Rb: Retinoblastoma

RFB: Replication Fork Barriers

SAK: South African apricot kernel

UNIVERSITY of the
WESTERN CAPE

SPK: South African peach kernel

S phase: Synthesis phase

TAK: Turkish apricot kernel

TDI: Tolerable Daily Intake

Thr: Threonine

Tri NaCitrate: Tri Sodium Citrate

Tyr: Tyrosine

WEE-1: Wee1-Like Protein Kinase

WM: Western Medicine

ABSTRACT

Amygdalin, a controversial anti-cancer agent, is a cyanogenic glycoside plant compound found in apricot and peach kernels. Both amygdalin and its patented form, Laetrile®, have been promoted and sold as "vitamin B-17", although neither compound is a vitamin. No consensus on the efficacy of amygdalin regarding the treatment of different cancers has been reached. Cancer is now the third leading cause of death worldwide. More than 7.6 million deaths were estimated to have occurred in 2007 and by 2030 it is projected to increase to 17 million cancer deaths per year. Cancers of the lung, breast, colon/rectum, liver and prostate are no longer largely confined to Western industrialized countries but are among the most common cancers worldwide (Thun et al. 2010). In South Africa it is estimated that one in every four males and one in every five females will be affected by a cancer diagnosis in their lifetime. The most common cancers in males are prostrate, lung, oesophagus, bladder and colorectal and in females they are cervix, breast, colorectal, oesophagus and lung (Haggar & Boushey 2009). Colon cancer is one of the most prevalent cancers worldwide, especially in western societies and is nutrition dependent (Klenow et al. 2009). It is one of the leading causes of death in both men and women in industrialised western countries. Colon cancer development involves both hereditary factors and lifestyle factors which include absence of physical exercise, unbalanced nutrition and long term smoking (Forman et al. 2004; Heavey et al. 2004). Colon cancer is traditionally treated by the resection of the colon, chemotherapy, radium therapy, and pharmaceutical hormonal drugs (Willson et al. 1987; Padussis et al. 2004)). Epidemiological studies supports evidence that colon cancer is preventable by adjusting the diet (Forman et al. 2004) and a protective effect is attributable to polyphenols and foods such as fruits and vegetables (Araújo et al. 2011). It was reported by Ruan et al. (2006) that the addition of Chinese Herbal Medicine in conjunction with chemotherapy not only raised the efficacy of the chemotherapeutic drug, but also reduced the toxic side-effects.

UNIVERSITY of the
WESTERN CAPE

The aim of this research was to carry out a comparative *in vitro* study of the anti-tumour effect of the Chinese , South African and Turkish apricot (Xing ren / *Armeniacea Semen*) and Chinese and South African peach (Tao ren / *Persica Semen*) kernel extracts on the HT-29 colon cancer cell line.

All the extracts significantly reduced cell viability and inhibited proliferation in the HT-29 cancer cells after 24 hours with the lipophilic and total fractions of CAK being the most effective. After 72 hours, it is clear that the inhibitory effects have been abolished and replaced by a stimulatory effect as the cell viability is higher in the treated cultures than the untreated controls. Results show that the total and the hydrophilic fractions of all the kernels increased cell viability more than the lipophilic fractions. It cannot be said with certainty that it was the amygdalin metabolite cyanide that affected the cell viability or induced apoptosis on its own. If hydrolysis of amygdalin indeed happened and cyanide was produced, it would affect the cells by shutting down aerobic respiration. Since cancer cells have more β-glucosidases and less rhodanese than normal cells, it is a possibility that the HT-29 cancer cells had some rhodanese to convert cyanide into a relatively harmless compound thiocyanate. It could be that *in vitro* this conversion, in light of the low enzyme levels in the HT-29 cancer cells, happened slowly and that the effect was only seen after 48 hour. However, this does not explain the overall inhibition even by the lipophilic fractions that should not contain any amygdalin or the eventual stimulatory effect, observed from 48 hour onwards.

UNIVERSITY of the
WESTERN CAPE

The S phase block observed, was mostly seen after 24 hour exposure to organic extractions, with the SAK showing 86% of cells in the S phase in contrast to the aqueous extractions which only slightly increased the S phase fraction.

This could indicate that synergistic and/or additive effects between polyphenolic compounds may also be responsible for the reduction of cell viability, proliferation and apoptosis. All the kernels and the various fractions affected cell viability and to an extent cell cycle progression, but more studies is needed to establish the most effective kernel and specific fraction or signature active component.

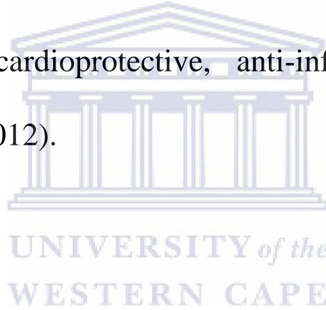
Inhibition of cell viability and proliferation and the induction of apoptosis could be an important preventive approach in chemoprevention. Understanding how dietary components regulate proliferation and cell survival could play a critical role in development of new enriched agents that can prevent and treat cancer with reduced risk of toxicity.

UNIVERSITY of the
WESTERN CAPE

Chapter 1

1. Literature Review

Food for the majority of people is the one form of medicine that can easily be controlled. “Let food be thy medicine; and medicine be thy food” – Hippocrates. The apricot (Xing ren / *Armeniacea Semen*) and peach (Tao ren / *Persica Semen*) kernels are normally consumed as a food supplement, anti-cancer nutritional treatment, or herb and may be taken in the form of a decoction or tincture as medicine. Literature has revealed that the apricot kernel has antimicrobial, anti-mutagenic, cardioprotective, anti-inflammatory, antinociceptive and antioxidant activities (Raj et al. 2012).


UNIVERSITY of the
WESTERN CAPE

1.1 Cancer

Cancer is one of the leading causes of morbidity and mortality worldwide. It has been predicted that by the year 2020, the number of new reported cancer cases in the world will have increased to an excess of 15 million with deaths increasing to 12 million (Kanavos 2006). In most developing countries cancer is the largest single cause of death in both men and women (Parkin et al. 1999).

Cancer is a disease of uncontrolled cell growth and may be caused by poor dietary habit, genetic predisposition and environmental carcinogenic agents. A large percentage of all cancers worldwide are caused by unhealthy dietary habits, and in the case of colon cancer, diet may account for 80% of the cases. When alcohol and smoking are added to the diet the

percentage may increase dramatically (Reddy et al. 2003). Genetic predisposition to cancer lends itself ~20% of cancer cases, thus the majority of cancers are associated with a host of environmental factors (Doll & Peto 1981). As the causes of cancer may be due to exposure to known suspected risk factors related to lifestyle or the environment provides a clear challenge to develop preventive strategies. For many cancers, curative treatment is generally not possible and this may be exacerbated by poverty, health and inappropriate facilities in poorer areas. Therefore, prevention of cancer in relation to diet is important to reduce the incidence, especially in areas where dietary deficiencies contribute to risk factors in cancer development. Dietary changes should thus be the focus on health care strategies that are cost effective.

Despite many therapeutic advances in medicine and the understanding of the process of carcinogenesis, overall incidence and mortality from cancer is still high (Ouédraogo et al. 2011). Natural or synthetic compounds are becoming more common that are used to block, reverse, or prevent the development of invasive cancers. The use of natural products as new chemotherapeutic agents need to be implemented more in order to make a difference in the mortality rate (Reddy et al. 2003). Cellular carcinogenesis forms the biologic basis for the identification of these preventive products, the assessment of their activity and ultimately the success or failure of a therapy (Reddy et al. 2003; Jemal et al. 2010).

UNIVERSITY of the
WESTERN CAPE

1.1.1 Colon Cancer

This is the third most commonly diagnosed cancer in males and the second in females with over 1.2 million new cases and 608,700 deaths estimated to have occurred in 2008 and is more prevalent in developed countries (Padussis et al. 2004; Jemal et al. 2010). Colon cancer development involves both hereditary factors and lifestyle factors which include absence of physical exercise, unbalanced nutrition and long term smoking (Forman et al. 2004; Heavey et al. 2004). Hereditary factors that increase risk include a personal or family history of colorectal cancer and/or polyps, a personal history of chronic inflammatory bowel disease (e.g. ulcerative colitis or Crohns disease), certain inherited genetic conditions (e.g. Lynch syndrome, also known as hereditary non-polyposis colorectal cancer, or familial adenomatous polyposis [FAP]), and type 2 diabetes (Roynette et al. 2004). The onset occurs in people 30 – 50 years and younger. A study based on a national database of 400,000 patients with colon or rectal cancer (Bailey et al. 2014) showed that for patients 20 to 34 years, the incidence rates of localised, regional and distant colon and rectal cancers have soared. Incidence rates today, per 100,000 people, are 3 for ages 20 to 34; 17 for ages 35 to 49; and 300 for people over 50. According to Bailey et al (2014) in 2030, the incidence rates for colon and rectal cancers will increase by 90% and 124,2%, respectively, for patients 20 to 34 years and by 27,7% and 46%, respectively, for patients 35 to 49 years. Approximately 95% of colorectal cancers are adenocarcinomas. Colon carcinomas arise within adenomatous polyps, and not all polyps develop into cancers; carcinomas are 10 fold more frequent in villous polyps than tubular polyps and hyperplastic lesions are rarely the site of carcinomas (Willson et al. 1987). Metastasis is normally around the focus areas such as the liver, pancreas, spleen, abdominal lymph nodes and lungs. Epidemiological studies supports evidence that colon cancer is

UNIVERSITY of the
WESTERN CAPE

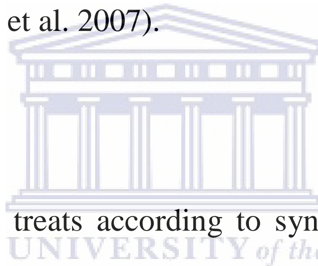
preventable by adjusting the diet (Forman et al. 2004) and a protective effect is attributable to polyphenols and foods such as fruits and vegetables (Araújo et al. 2011). The increased risk is associated with a high-fat and low fibre diet (Singh & Fraser 1998) as well as obesity, diet high in red or processed meat (Ferguson 2002) and alcohol consumption (Jemal et al. 2010).

There are disparities in this type of cancer incidence and mortality among races and ethnic groups where African-American populations have a higher incidence and mortality rate compared to other ethnic populations (Padussis et al. 2004). The reasons are not entirely known but it has been postulated that differences in access to high quality regular screening, timely diagnosis and treatment, lifestyle and dietary factors and socio-economic factors all play a role (Padussis et al. 2010). Age is a big risk factor with people aged 50 years having a higher mortality rate which further increases over 50 years (Padussis et al. 2010). Patients with a personal history of adenomatous polyps or a previous history of colorectal cancer are at an increased risk of developing colon cancer in the future. Size, number and histology of the polyps are important prognostic factors with a size of > 1 cm, villous or tubuvillous histology, and multiple polyps conferring a greater risk of colorectal cancer (Padussis et al. 2010). It is estimated that the incidence and mortality rates per 100 000 in Southern Africa for males vs. females was 235.9:161.0 and 172.1:108.1 respectively by 2008 (Jemal et al. 2011). The rate for all cancers (in both men and women) was 1.7 times higher in more developed countries than in less developed countries, with 14.1 million new cases and 8.2 million cancer-related deaths in 2012, and colorectal cancer accounting for 1.36 million of the most commonly diagnosed cancers (Ferlay et al. 2014; WHO/GLOBOCAN 2013).

UNIVERSITY of the
WESTERN CAPE

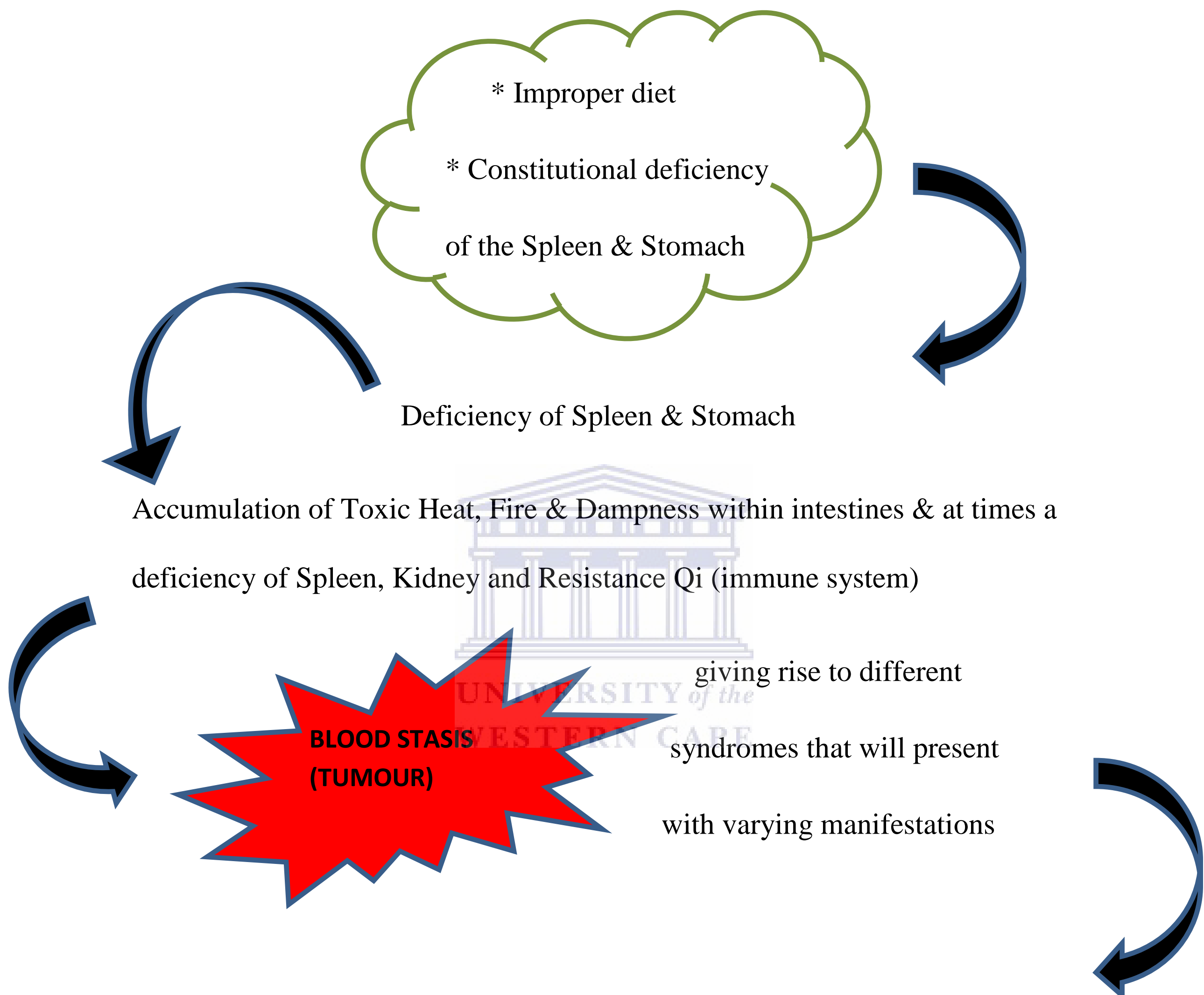
1.1.2 Pathogenesis

The aetiology of large intestine cancer is still unclear but is said to possibly be due to chronic inflammation, polyps, adenoma of the large intestine which is related to diet and environmental factors. According to Western Medicine pathology the aetiology includes inherited genetic factors and environmental factors with diet being the biggest contributing factor referring to the bacterial flora in the large intestine, bowel transit time, and the amount of cellulose, amino acids and bile acids in the bowel contents. It has also been linked to the high density protein CD 133 population, a marker of organ specific adult stem cells that can grow a new tumour (Ricci-Vitiani et al. 2007).



Chinese Medicine diagnoses and treats according to syndrome and pattern differentiation. The main patterns attributed to colon cancer according to Chinese Medicine are that of states of excess (i.e. blood stasis, damp-heat and accumulation of toxic materials) and states of deficiency (i.e. deficiency of Qi, Blood, Yin, and Yang of the spleen, liver and kidney). The tumour itself is seen mainly as Blood Stasis (see Figure 1.1).

Figure 1.1: Causes and Pathogenesis according to Chinese Medicine



UNIVERSITY of the
WESTERN CAPE

Syndromes:

- Agglomeration of Dampness and Heat
- Stagnation of Toxic Materials & Blood Stasis
- Deficiency of Stomach & Liver Yin
- Deficiency of Qi & Blood
- Deficiency of Spleen & Kidney Yang
- Deficiency of Liver & Kidney Yin

1.2 Chinese Medicine (CM)

CM, an ancient discipline of medicine, refers to the human body as having four vital substances, namely Qi, Blood, Body fluids and Essence, and as constituting of four levels i.e. Wei level (outer most and first energetic level also known as the defensive level), Qi level (second energetic level and relating more to the organs), Ying level (third and nutritive level) and lastly the Blood level (deepest level). A balance in Yin and Yang, a theoretical framework by which everything is classified, renders the body in a state of health, whereas an imbalance thereof will indicate a state of ill-health and thus an imbalance in any of the vital substances and which may manifest on any of the four levels. CM employs the philosophy that "Qi is the commander of Blood (it thus moves blood by providing it with the necessary force for it to course through our organs, vessels and meridians), and blood is the mother of Qi (it thus serves as the material foundation for Qi)" (Maciocia 2005). Chinese Herbal Medicine (CHM) is but one branch of CM and boasts over 4000 years of empirically based evidence. The lungs and large intestine are said to have an interior / exterior relationship in terms of their organ function and according to acupuncture meridian theory as paired meridians, and in this fashion is able to treat each other respectively (Maciocia 2005). The large intestine is the last area of body fluid reabsorption.

UNIVERSITY of the
WESTERN CAPE

1.2.1 Peach and Apricot Kernels in Chinese Medicine

In the Chinese Materia Medica by Bensky et al (2004) the Chinese apricot and peach kernels are shown to have an effect on the large intestine and lung meridians and organs. According to Zhang Bing-Cheng (Dan Bensky 2004a) the Chinese apricot and peach

kernels are similar in nature although "the one moves into the liver meridian at the Blood level (deepest level) and the other into the lung meridian at the Qi level (second level)". Thus the apricot and peach kernels together addresses both the Qi and Blood levels, and because the flow of Qi supports the movement of blood, a combination of apricot and peach kernels can be used when Blood stasis leads to pain in the chest, abdomen or throughout the body (Dan Bensky 2004a). Raj et al (2012) indicated that the apricot kernel (Xingren; *Prunus armeniaca* - native to northern China) according to Chinese Medicine has been traditionally prescribed for the treatment of asthma, constipation and cough.

In comparison to the peach kernel, the apricot's action is more focused on the upper part of the body whereas the peach kernel is more focused on the lower parts of the body despite both having a moistening effect on the intestines (Dan Bensky 2004a). Their status as fruit kernels implies that there is some life-giving generative force, which is expressed by the peach kernel's ability to assist in the generation of new blood following the expulsion of the old stagnant blood, and in combination herbal treatment is thus used in cancer treatment (Dan Bensky 2004a). The peach kernel thus has the ability to remove blood stasis and improve the circulation.

UNIVERSITY of the
WESTERN CAPE

The **apricot kernel** appears in the category of herbs which relieves coughing and wheezing. According to Chinese Materia Medica by Bensky et al (2004) it is used to treat the following symptoms: cough, wheezing, with or without constipation, heaviness of the head and body. Apricot kernels have the following properties: bitter, slightly warm, slightly toxic; enters the lung and large intestine channels and is said to direct the lung-Qi downwards hence stopping cough and facilitating peristalsis, disperse exterior wind cold and moisten the intestines. The

kernels are slightly toxic (Dan Bensky 2004a) but by virtue of its toxicity, it is able to treat sores and kill parasites (Dan Bensky 2004a). Due to the interior / exterior relationship of the lung and the large intestine, when the lung-Qi fails to descend (governing respiration) the large intestine also fails in its rhythmic movement (peristalsis) thus leading to constipation. However, apricot kernels may be used to treat any type of constipation. Investigations of differences between the bitter apricot kernel (beixingren) and the sweet apricot kernel (nánxingren) showed that the sweet apricot kernel is less toxic and more moistening and primarily used for alleviating coughing and wheezing (Femenia et al. 1995).

1.2.2 Composition of the Apricot Bitter Kernel

i) Glycosides: glucosidases, amygdalin, amygdalase, prunase, prunasin, mandelonitrile (secondary product)

ii) Fixed oils: oleic acid, linoleic acid, palmitic acid, stearic acid, linolenic acid, eicosatetraenoic acid

iii) Volatile oils: benzaldehyde, linalool, 4-terpenenol, α-terpineol (Dan Bensky 2004a).

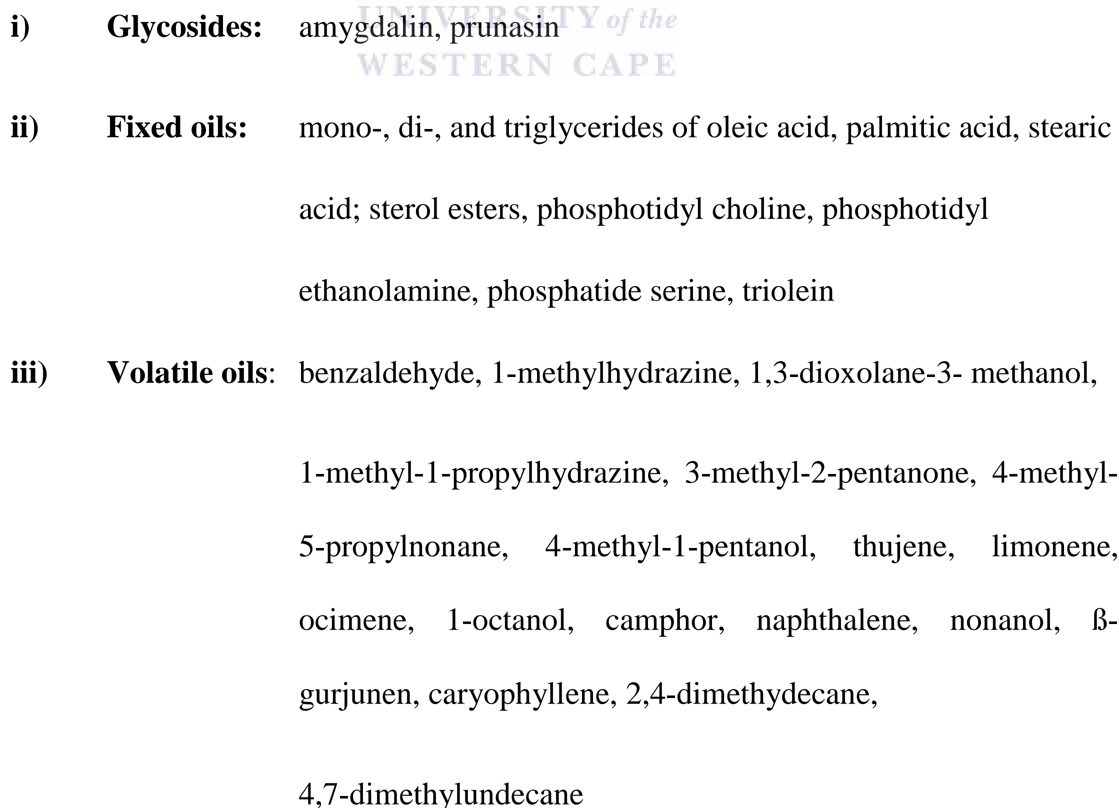
(See **Annexure 1** for the complete chemical composition of the Apricot kernel)

It must be noted that the **peach kernel** (Tao ren, *Prunus persica* - native to northern and central China) is more often spoken about in terms of cancer treatment than apricot kernels due to its ability to remove blood stasis.

UNIVERSITY of the
WESTERN CAPE

Experiments have shown that CHM plays an anti-cancer role by inducing apoptosis and differentiation, improving the immune system, inhibiting angiogenesis, reversing multidrug resistance, etc. (Ruan et al. 2006). According to Chinese Materia Medica (Dan Bensky 2004b), the peach kernel falls into the category of herbs that invigorate the blood. It has the ability to treat the following symptoms: menstrual disorders, abdominal pain, trauma, injury, lung and intestinal abscess with fixed mass, all with the characteristic blood stasis syndrome; and constipation due to dryness. It has the properties of being sweet, bitter and neutral; enters the heart, lung, liver and large intestine channels; it invigorates the blood and dispels blood stasis, moistens the intestines, stops coughing and wheezing.

1.2.3 Composition of the Peach Kernel



UNIVERSITY of the
WESTERN CAPE

i) **Glycosides:** amygdalin, prunasin

ii) **Fixed oils:** mono-, di-, and triglycerides of oleic acid, palmitic acid, stearic acid; sterol esters, phosphotidyl choline, phosphotidyl ethanolamine, phosphatide serine, triolein

iii) **Volatile oils**: benzaldehyde, 1-methylhydrazine, 1,3-dioxolane-3- methanol, 1-methyl-1-propylhydrazine, 3-methyl-2-pentanone, 4-methyl-5-propylnonane, 4-methyl-1-pentanol, thujene, limonene, ocimene, 1-octanol, camphor, naphthalene, nonanol, ß-gurjunen, caryophyllene, 2,4-dimethydecane, 4,7-dimethylundecane

iv) Flavanoids: (+) catechin, pruning, hesperitin-5-*0*-glucoside, naringenin, kaempferol, dihydrokaempferol, kaempferideglucoside, quercitinglucoside (Dan Bensky 2004b).

According to Bensky et al (2004), the apricot kernel is toxic due to its cyanogenic glycoside content; for application in a decoction 10 to 20 kernels is the toxic dose for children and 40 – 60 kernels for adults; ingestion of 50 - 120 kernels may cause death, however, within the normal dosage range and taken as a decoction, no toxic side effects are to be expected. It must be noted that the powdered kernel suspension is 4 – 5 times as toxic as the decoction of the kernels and peeled kernels are less toxic than unpeeled kernels (Dan Bensky 2004a). Dry-frying the apricot kernel mitigates their bitterness and reduces the oil content (Dan Bensky 2004a).

UNIVERSITY of the
WESTERN CAPE

According to the Phytochemical database of the American Department of Agriculture (Duke 1992), the seed contains some amounts of the hormones alpha-estradiol and estrone (E1). According to Risk Profile *Apricot Kernel oil (AKO)* CAS No. 72869-69-3 (31.05.2013) (Anon 2013), independent to the aforementioned database the author Ning DD et al. (1990) also reports that the seed contains beta-estradiol (E2) and E1. Risk Profile *Apricot Kernel oil (AKO)* CAS No. 72869-69-3 (31.05.2013) furthermore states that the seed contains both free and conjugated estradiol and E1 in a separate oestrogenic fraction that makes up 0.09% of the seeds weight as demonstrated in the Monograph on apricot in the Herbal Medicines (World Health Organisation 2004). There is about 200 apricot species mainly found in the northern

hemisphere; however, Prunus Africana is the only species native to Southern Africa. (Iziko Museum 2002).

1.3 Specific active components of the apricot and peach kernels

Various documents from the oldest civilizations such as Egypt and China, 2500 years before Christ, mention the therapeutic use of derivatives of the bitter almonds (Pulido 2000). Egyptian papyri from 5000 years ago mention the use of 'aqua amigdalorum' for the treatment of some tumours of the skin (Pulido 2000). A white crystalline substance, pure amygdalin, was first isolated in 1830 by two French chemists Roubiquet and Boutron-Charland (Halenár et al. 2013; Greenberg & Francisco 1975; Pulido 2000). In 1837, the German scientists von Liebig and Woehler found that in the presence of certain enzymes, amygdalin breaks down into glucose, benzaldehyde, and hydrogen cyanide (which is poisonous) (Cooke et al. 2009; South 1845)

UNIVERSITY of the
WESTERN CAPE

1.3.1 Amygdalin

Amygdalin (Figure 1.2), a controversial anti-cancer agent, is a cyanogenic glycoside plant compound that was initially isolated from bitter almonds (Halenár et al. 2013; Abtahi 2008; Milazzo et al. 2007). Amygdalin is found in many fruit pits and plants of the Rosacea family such as *P. persica* (peach) and *P. armeniaca* (apricot) and *P. amygdalusvaramara* (bitter almond) (Milazzo et al. 2009). It is a white, crystalline, inodorous powder that is slightly

soluble in cold water and very soluble in hot water, acetone, alcohol, but not in ether (Al Bakri et al. 2010). Recent high resolution Raman imaging has revealed that throughout the apricot seed there are local amygdalin "concentration spots" (Krafft et al. 2012). When pressed, the apricot kernel releases oil very chemically similar to the oil found in sweet almond and peach kernels. This oil contains olein, glyceride, linoleic acid, and a transparent, crystalline chemical compound, amygdalin (Dai & Mumper 2010). Although the oil from apricot seeds usually breaks down into a toxic substance capable of causing death of the human, which is the main cause for the controversy, there are also varieties of apricot seeds that are reported to be edible (Dai & Mumper 2010).

UNIVERSITY of the
WESTERN CAPE

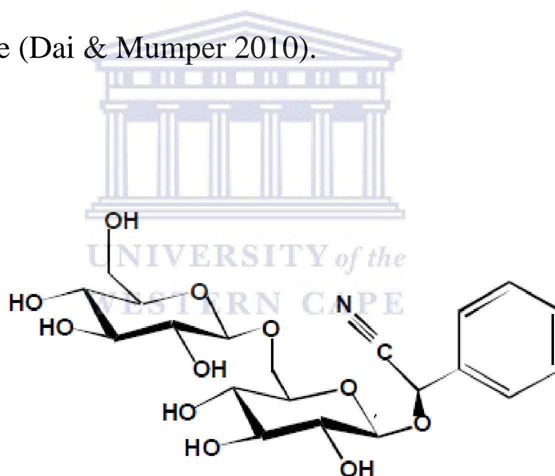


Figure 1.2: **Amygdalin**. CAS no. 29883-15-6.UIPAC name: [(6-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] (phenyl) acetonitrile CAS no. 29883-15-6, Chemical formula is C20H27NO11, MW=457.43 g/ mol (Anon 2013).

1.3.2 Laetrile and the “Krebs Hypothesis”

Laetrile (Figure 1.3), is the trade name for laevo-mandelonitrile-beta-glucuronoside, a substance allegedly synthesized by Ernst T. Krebs, Sr., M.D when he was a pharmacy student. He had theorized that "cancer proteins" could be broken down by this enzyme he had prepared. When the substance proved too toxic in animal experiments, he boiled it and obtained better results (Seers et al. 2012). In 1949, Krebs, Jr., modified his father's extraction process and named the result Laetrile that is registered with the U.S. Patent Office. This compound is chemically related to amygdalin. Most advocators of laetrile for the treatment of cancer use the terms "laetrile" and amygdalin interchangeably (Seers et al. 2012).

UNIVERSITY of the
WESTERN CAPE

Laetrile (laevo-D-mandelonitrile-B-glucoronide) is the patented purified, semi synthetic form of amygdalin which contains 2 sugars, a benzaldehyde, and hydrogen cyanide (Halenár et al. 2013). As a nitriloside, amygdalin resembles the B complex structures and after enforcement agencies began trying to ban Laetrile as a drug Krebs Jnr. claimed that Laetrile was a vitamin (“Vitamin B17”) and that cancer is caused by a deficiency of this vitamin (Fassa 2009).

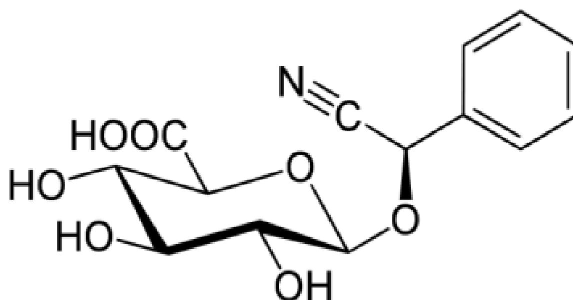


Figure 1.3: **Laetrile** (CAS no. 1332-94-1). MW=309.2714 g/mol. Laetrile is also classified as a cyanogenic glycoside (Anon 2013).

Vitamin B17 is also known as Laetrile or Amygdalin and these three names are being used interchangeably (Enculescu 2009). According to Krebs Jnr (Ernst T. Krebs 1975) this "newly designated vitamin B-17" (nitriloside) could account for:

1. The thiocyanate in the body fluids, blood, urine, saliva, sweat, and tears
2. Part of the benzoic acid (and subsequently hippuric acid) and salicylic acid isomers
3. The HCN that is used for the production of cyanocobalamin (Vitamin B_{12})

1.3.2.1 Theories for the possible mechanism of action of Laetrile

Four different theories have been posed regarding the anti-cancer activity of Laetrile (Cooke et al. 2009)

UNIVERSITY of the
WESTERN CAPE

i) **Trophoblast theory:** The trophoblast theory (Beard, 1911) suggests that all cancers arise from special cells which are randomly dispersed in the body during embryonic development (which would normally have become egg or sperm cells). Building on the trophoblast theory, Krebs (1970) suggested that the transformation of the rogue egg/sperm cells into a cancerous state could be prevented by ingesting laetrile. Trophoblast cancer cells are thought to have a different balance of enzymes in comparison to normal non-cancerous cells, that is, more beta-glucuronidase and less rhodanese than normal cells. Beta-glucuronidase breaks laetrile down and produces cyanide (which kills the cell by shutting down aerobic respiration); however, rhodanese can convert cyanide into a relatively harmless compound (thiocyanate). As cancerous cells

have less of rhodanese enzyme to convert cyanide to a harmless form they are more affected by cyanide than healthy cells.

Therefore, it is believed cancerous cells are more likely to be adversely affected by laetrile, while normal cells are thought to be unaffected. However, there is no experimental evidence to support the idea that normal and malignant cells differ in their concentrations of these two enzymes (Cooke et al. 2009)

ii) **Enzyme balance**: The second theory of action of laetrile is similar to the first in suggesting that cancerous cells have a different balance of enzymes. The main difference with this theory is that the trophoblastic explanation for cancer cell development is not used. This second approach states that cancer cells have more beta-glucosidase and less rhodanese enzymes than normal cells, and therefore laetrile negatively affects only cancerous cells as they are not protected by sufficient rhodanese. However, no experimental evidence exists to support this theory (Cooke et al. 2009)

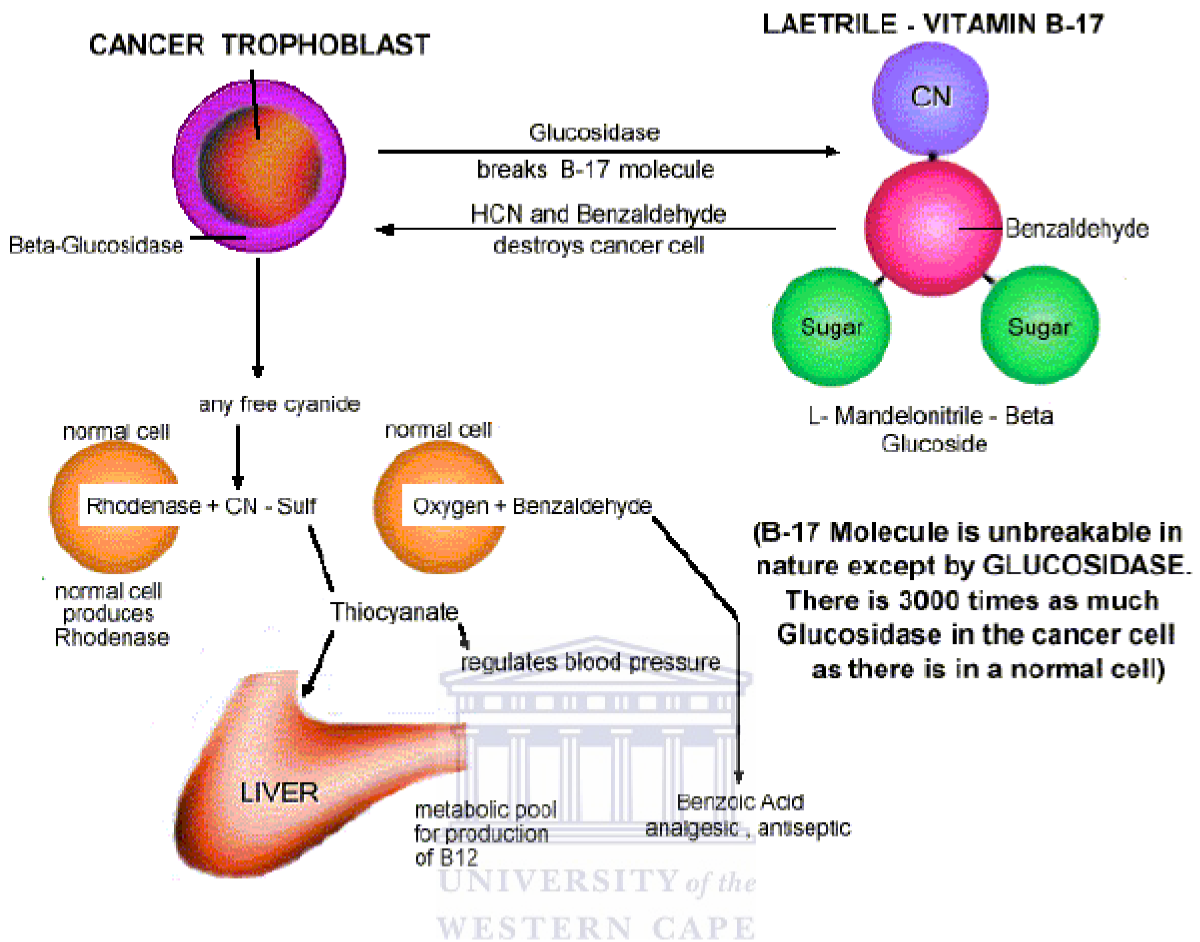
UNIVERSITY of the
WESTERN CAPE

iii) **Vitamin B17" deficiency:** The third theory is that cancer is a result of a metabolic disorder caused by a deficiency in so-called "vitamin B17". Dr Krebs (1970) used vitamin B17 as another name for laetrile, and it is thought that by restoring this missing "vitamin" in the body, health can be restored. Studies have shown that the vitamin status of an individual can determine the

development of cancer. However, there is no evidence that B17 or laetrile is needed for normal metabolism or that it even functions as a vitamin in humans or animals (Cooke et al. 2009)

iv) **Fourth Theory:** As well as disrupting aerobic respiration, the cyanide released by laetrile increases the acid content of tumours and leads to the destruction of lysosomes within the tumour cells. The lysosomes release their contents (i.e. enzymes which can break down other cellular molecules) thereby killing the cancer cell(s) and stopping the growth of the tumour. However, this theory is not supported by evidence (Cooke et al. 2009).

UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

Figure 1.4: Graphic representation of the chemistry of nitrilosides in cancer (Anon 2001)

Thus the entire rationale for using amygdalin/laetrile rests on the assumption that normal cells have higher levels of an enzyme, rhodanese, which "neutralizes" the amygdalin production of cyanide (Pulido 2000). In this way the amygdalin serves as a source of glucose to the healthy cells (see Figure 1.4) whilst killing malignant cells.

In 1980, the U.S. Supreme Court prohibited the use of Laetrile® as a cancer therapy or as a treatment for any other medical condition in the United States, but the compound continues to be manufactured and administered as an anticancer therapy, primarily in Mexico.

1.3.2 Rhodanese enzyme

Rhodanese is a multifunctional, mitochondrial, sulphur transferase that catalyzes the detoxification of cyanide by suphuration in a double displacement mechanistic reaction (Saidu 2004). With the aid of electron density map and gel electrophoresis, bovine liver rhodanese shows that it consists of a single polypeptide chain with molecular weight of 32,000 to 33,000 (Saidu 2004). The principal detoxification pathway of cyanide is that catalyzed by a liver mitochondrial enzyme, namely rhodanese (Cyanide: Thiosulphate Sulphur Transferase; E.C.2.8.1.1) (Saidu 2004). The physiological role in animal tissue of rhodanese (Cyanide: Thiosulphate Sulphur Transferase; E.C.2.8.1.1), a sulphur transferase, is to catalyze, *in vitro*, the formation of thiocyanate that is excreted by the kidneys from cyanide and thiosulphate; *in vivo* however, the enzyme is multifunctional (Saidu 2004).

UNIVERSITY of the
WESTERN CAPE

1.3.3 Metabolism and Toxicity of Amygdalin

The proposed mechanism of action of amygdalin in healthy or diseased bodies has not been established yet (Greenberg & Francisco 1975; Cooke et al. 2009). It is known that cyanide is a by-product of the metabolism of amygdalin and is a toxic substance which may be lethal. The method of administration (oral vs. intramuscular vs. intravenous) is thus called into question as to the most suitable form of administration to reduce the toxic effects of the cyanide since there seems to be a link with intestinal bacteria and the release of cyanide (on oral administration). According to data collected by Jonathan Newmark (October 1981), the rat and human small intestine is rich in amygdalin and prunasin-hydrolyzing enzymes. This data is consistent with the findings of Moertel et al as cited by Park et al (2005) that amygdalin administered intravenously caused no side effects of cyanide toxicity, but that oral administration did. Amygdalin is hydrolyzed by the enzyme emulsion (β-glucosidase) (see Figure 1.5) to prunasin (D-mandelonitrilemonoglucoside); prunasin is then hydrolyzed to mandelonitrile by the same enzyme (β-glucosidase). Nitrilosides are hydrolysed to free hydrogen cyanide (HCN), benzaldehyde or acetone and glucose (Ernst T. Krebs 1970). Cyanogenic glycosides, which are monosaccharide or disaccharide conjugates of cyanohydrins, are widely present in plants where they are the principal precursors of hydrocyanic acid. Representatives of importance identified in edible plants are: amygdalin which is found in bitter almonds, apple pips, kernels of cherries, apricots and peaches (Genderen 1997). Formation of hydrogen cyanide in plants first requires the hydrolisation of glycosides by glycosidases to the cyanohydrins and mono- or – disaccharides (Genderen 1997). Cyanohydrins undergo further hydrolysis by lyases to hydrogen cyanide and carbonyl compounds involved (Genderen 1997).

UNIVERSITY of the
WESTERN CAPE

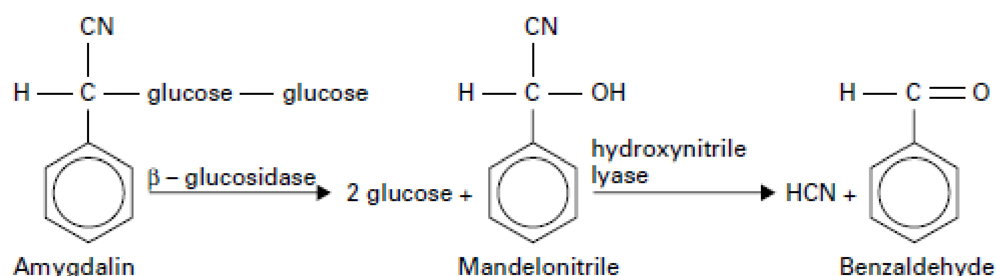
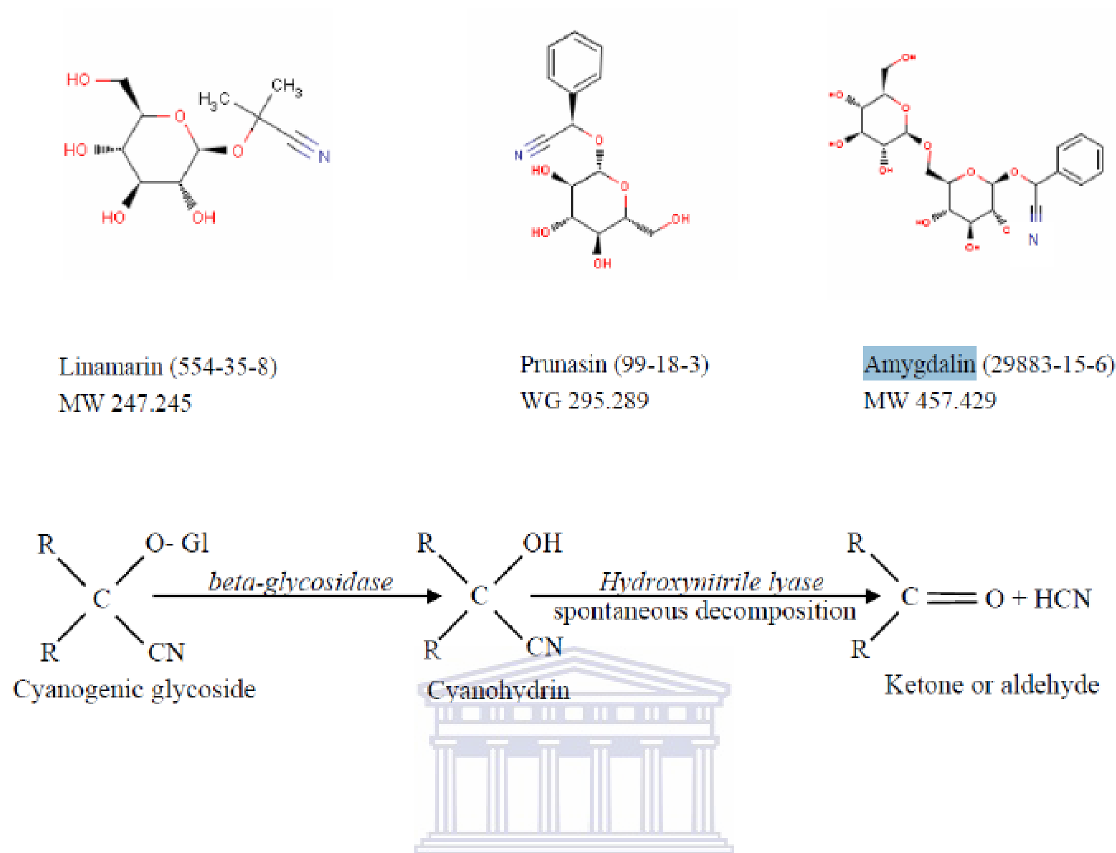


Figure 1.5: Hydrolysis of Amygdalin (Padmaja 1995)

Cyanogenic glycosides such as amygdalin (the predominant cyanogenic glycoside variant in apricot kernels) are considered non-toxic until cyanide (HCN) is released. This usually occurs as a result of enzymatic hydrolysis by β-glucosidases following grinding of plant tissue which activates intracellular β-glucosidases, or by the gut micro-flora. It can also occur, to a lesser degree, by glucosidases of the liver and other tissues (Padmaja 1995). This reaction can also result from chewing, which causes the enzyme and the cyanogenic glycosides stored in different compartments to combine (Chaouali et al. 2013). The reaction occurs rapidly in an alkaline environment, and the hydrolysis is complete in 10 min. Hydrolysis is possible in an acid solution but takes place slowly. The released HCN is readily absorbed and rapidly distributed in the body via the blood (EFSA 2004).

UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

Figure 1.6: Structures of common plant-derived cyanoglycosides and principle pathway of HCN formation. (Al Bakri et al. 2010; Femenia et al. 1995; Yildrim and Askin 2010).

Glycosides and salts are expected in total extractions. Glycosides are a class of molecules in which, a sugar molecule is bound to a "non-sugar" molecule (aglycone). Many plants store their secondary bioactive metabolites in the form of inactive glycosides. Once the glycoside is split into its two components (sugar and aglycone), the aglycone can exert its biological effects. The aglycone part in apricot and peach kernels contains a cyanide group, and some contain a sulphate group. Subjecting these compounds to hydrolysis using either aqueous medium or enzymes yield an aglycone which may contain the cyanide group (C=N), and sometimes the cyanide group can be split further into a hydrocyanic acid (HCN), which is a volatile compound. Boiling of any of the above mentioned starting materials in water

(aqueous extract) can decrease the cytotoxicity due to the volatility of HCN molecule, making it usable by human beings for medication. However, the organic extraction procedures may keep the glycosidic compounds without decomposition and could be more cytotoxic than aqueous extractions.

1.3.3.1 Cyanide

Cyanide acts through the inhibition of cytochrome-c oxidase in the respiratory electron transport chain of the mitochondria, impairing both oxidative metabolism and the associated process of oxidative phosphorylation, thereby causing death through energy deprivation and oxygen uptake (Chaouali et al. 2013). Cyanide causes intracellular hypoxia by reversibly binding to mitochondrial cytochrome oxidase a3 within the mitochondria (Chaouali et al. 2013). Cytochrome oxidase a3 is necessary for the reduction of oxygen to water (Chaouali et al. 2013). The toxicity of cyanide is largely attributed to the cessation of aerobic cell metabolism causing central nervous system and cardiovascular dysfunctions by cellular hypoxia (Chaouali et al. 2013).

UNIVERSITY of the
WESTERN CAPE

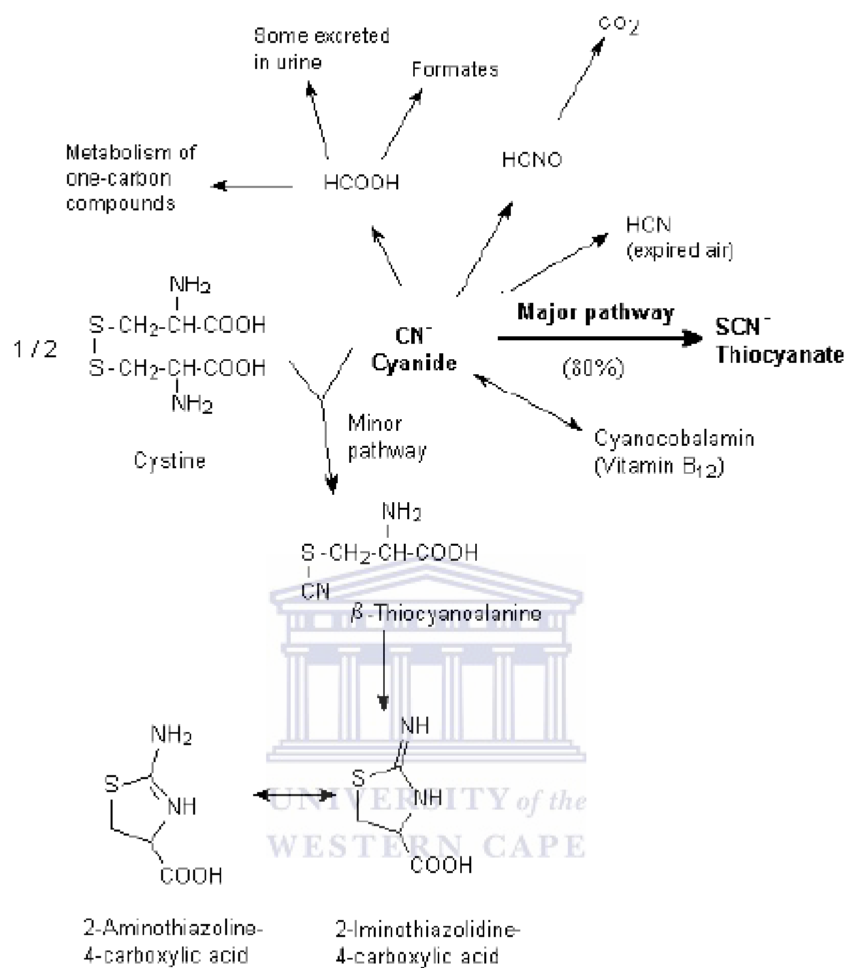
1.3.3.1.1 Metabolism and Excretion of Cyanide

Hydrogen cyanide has a pKa of 9.22; thus, at physiological pH (about pH 7), hydrocyanic acid is distributed in the body as hydrogen cyanide and is not present as the free cyanide ion. Hence, the form of cyanide to which exposure occurs, the salt or the free acid, does not influence distribution, metabolism, or excretion from the body (Simeonova & Fishbein 2004).

The major portion of cyanide in blood is sequestered in the erythrocytes, and a relatively small proportion is transported via the plasma to target organs. Cyanide is concentrated in red blood cells at a red blood cell to plasma ratio of 199:1, but levels in plasma reflect tissue levels better than levels in whole blood or erythrocytes (Simeonova & Fishbein 2004). The major route of metabolism for hydrogen cyanide and cyanides is detoxification in the liver by the mitochondrial enzyme rhodanese as discussed in section 1.3.2.2, which catalyses the transfer of the sulfanesulfur of thiosulfate to the cyanide ion to form thiocyanate (Figure1.6) (Saidu 2004). About 80% of cyanide is detoxified by this route.

The limiting factor in cyanide metabolism is the low concentration of the sulphur-containing substrates in the body, primarily thiosulfate, but also cystine and cysteine (Aminlari & Baghshani 2009). While rhodanese is present in the mitochondria of all tissues, the species and tissue distributions of rhodanese are highly variable. In general, the highest concentrations of rhodanese are found in the liver, kidney, heart, brain, and muscle, but the supply of thiosulfate is limited (Aminlari & Baghshani 2009). The half-time for hydrogen cyanide elimination is approximately 1 hour (World Health Organisation 2004).

UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

Figure 1.7: **Basic processes involved in the metabolism of cyanide** (Anon 2013).

1.3.3.1.2 Toxicity of cyanide derived from cyanogenic glycosides in apricot Kernels

Acute toxic effects: related to cyanide have occurred from consumption of stone fruit kernels (EFSA 2004). The lethal dose in humans is about 0.5 – 3.5mg/kg body weight (bw) (Bewertung 2007; EFSA 2004; Chaouali et al. 2013). The estimated no-effect level in adults is 5µg/kg bw, corresponding to the intake of a bitter apricot kernel (Bewertung 2007) and translates to a maximum daily limit of two kernels.

Food: Cyanogenic glycosides present in apricot kernels (and/or Laetrile™), as sources of hydrocyanic acid (HCN), are relatively non-toxic until HCN is released. This can occur as a result of enzymatic hydrolysis by β-glucosidases following maceration of plant tissue or by the gut microflora e.g. as part of the digestive process. Benzaldehyde is also produced by the hydrolysis of amygdalin in addition to sugar moieties and HCN.

UNIVERSITY of the
WESTERN CAPE

Fatal acute: (e.g. deaths) have occurred from consumption of stone fruit kernels, whereas chronic uptake of HCN in sub-acutely toxic doses may be involved in the disturbance of thyroid function and neuropathies. The systemic effects of an oil prepared from the seeds containing 94% unsaturated fatty acids, and oleic and linoleic acids were assessed in a 13-week feeding study in rats. The animals were fed a diet containing 10% oil. No toxic effects were observed and no macroscopic or microscopic lesions in any of the organs were found (Gandhi et al. 1997).

A study concluded that data on chronic toxicity were not adequate to establish a no-observed adverse effect level (NOAEL) or Tolerable daily intake (TDI) in humans (EFSA 2004).

1.4 Research of possible effects of Amygdalin

1.4.1 Cell growth / cell death studies

The first recorded use of amygdalin to treat cancer was reported in 1845 by T. Inosmetzeff, a professor at the Imperial University of Moscow. A young male cancer patient aged 20 years received approximately 46,000 mg of amygdalin over a period of 3 months, and was still alive 3 years later (South 1845). Furthermore, a woman aged 48 years, with extensive metastasis from a primary right ovarian tumour that received varying amounts of amygdalin over a period of years, survived 11 years at the time of the report (South 1845) and no sustained pharmacologic harm was seen in these patients.

UNIVERSITY of the
WESTERN CAPE

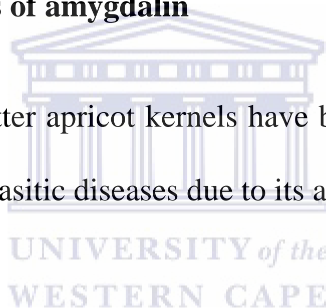
The apricot kernel was found to be antimutagenic against Mitomycin-c bladder cell line (MMC) but not inhibitory to the synthesis of DNA of 4 kinds of tumour cells, namely mouse metallothioneins (MT-II), murine L929 fibrosarcoma cells (L929), SKV 20 (Wei et al. 2002). Park et al (2005) showed that amygdalin down regulated cell-cycle related genes in SNU-C4 human colon cancer cells concluding that amygdalin could possibly be used as therapeutic anticancer drugs. Human DU145 and LNCaP prostate cancer cells exhibited several morphological characteristics of apoptosis after treatment with an aqueous apricot kernel extract of amygdalin, increasing Bax expression and caspase-3-enzyme activity, and decreasing Bcl-2 expression (Chang et al. 2006).

Syrigos et al (1998) demonstrated that amygdalin was cytotoxic to HT1376 bladder cancer cells only at high concentrations, whereas the combination of amygdalin with HMFG1-β-glucosidase enhanced the cytotoxic effect of amygdalin 36 fold. Fukuda (2003) reported amygdalinic acid to have anticancer properties upon oral administration to mice having

inoculated tumour cells (AC755). The inhibitory 12-*0*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr Virus early antigen (EBV-EA) activation tests in Raji cells results showed amygdalinic acid produced 65-80% inhibition of EBV-EA activation at a concentration of 500 mol ratio / TPA, without producing cytotoxicity (Fukuda et al. 2003). Amygdalin in aqueous solution is epimerized to neoamygdalin / inactive form (L-mandelonitrile-β-gentiobioside) which is inactive against cancer (Kwon 2003).

1.4.2 Antimicrobial properties of amygdalin

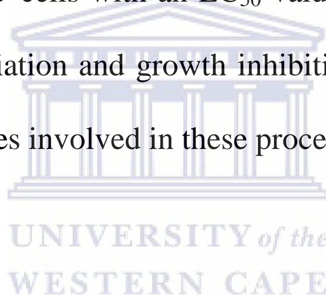
Abtahi et al (2008) stated that bitter apricot kernels have been used in folk medicine for the treatment of skin diseases and parasitic diseases due to its antimicrobial properties.


UNIVERSITY of the
WESTERN CAPE

1.4.3 Antioxidant properties of amygdalin

Yigit (2009) indicates that the sweet and bitter apricot kernel has antioxidant activities. Wu et al (2011) had found a high content of phenolic compounds (4.1593mg GAE/g) which contribute to the antioxidant activity in the kernels oil. The main fatty acids found in the peach kernel oil were oleic acid (61.87g / 100g in oil) and linoleic acid (29.07g / 100g oil). The HPLC analysis of the phenolic compounds found that ritun, (-)-epicatechingallate, hydrocinnamic acid, sinopinic acid, dithiothreitol and caffeic acid were major constituents. These results suggested that peach kernel oil is a good source of unsaturated fatty acid, phenolic compounds with strong antioxidant activity thus having the potential to be used as nutrient rich food oil (Wu et al. 2011).

Flavanoids, a polyphenolic antioxidant compound, occur alongside carotenoids (natural colouring with antioxidant activity), glutathione, vitamin C, tocopherols, ascorbate and other compounds in plants, fruits and vegetables. Antioxidants protect proteins, lipids and DNA from the damage of free radicals (Vardi et al. 2008). Vardi et al (2008) had shown that elevated myeloperoxide (MPO) levels contributed to Methotrexate (MTX)-induced oxidative small intestine injury and that apricot and beta-carotene caused a decrease in MPO activities. Vardi et al (2008) sites Ramos et al (2008) as saying that the apricot provides inhibition of lipid peroxidation, capillary permeability and platelet aggregation. Flavones were shown to reduce cell proliferation in HT-29 cells with an EC_{50} value of 54.8 ± 1.3 μM and to induce programmed cell death, differentiation and growth inhibition in transformed colonocytes by acting at the mRNA levels of genes involved in these processes (Wenzel et al. 2000).


UNIVERSITY of the
WESTERN CAPE

1.4.4 Toxicity tests

A comparative study for both pure amygdalin and the apricot kernel proved that both of them exhibit significant analgesic, anti-inflammatory and H_1 receptor blocking effects (Badr & Tawfik 2010). The toxicity results revealed that they are both safe up to a concentration of 100 mg / kg of the animal's body weight (Badr & Tawfik 2010).

The average lethal dose of HCN in humans has ranged from 50 – 60 mg /g to 1.52 mg /kg (106 mg for a 70 kg adult) and the lowest reported fatal dose was 0.56 mg / kg (Suchard et al. 1998).

A decrease in proliferation of human promyelocytic leukemia cells (HL-60) cells with peach kernel extract was observed after 48h and the IC_{50} was approximately 11 mg / mL. In the presence of ß-glucosidase, the LC_{50} was 6.4 mg / mL (Kwon et al. 2003).

1.5 Disclaimers

Many review articles on trials or research reports have disclaimed the anti-tumour effect of apricot kernels saying that the effectiveness of laetrile is not supported by clinical trials (Milazzo et al. 2009; Greenberg & Francisco 1975). According to the Committee on Toxicity report the total lethal dose (TDI) is not conclusive due to lack of evidence (Committee On Toxicity 2006). High doses chemotherapy of amygdalin, in murine P388 lymphocytic leukemia and P815 mast cell leukemia in BDF1 mice, at dosages of 200 mg / kg and 2000 mg / kg results showed no prolongation in the life-span of mice bearing either the P388 or P815 tumour (Chitnis et al. 1985).

UNIVERSITY of the
WESTERN CAPE

According to the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment's *Statement on Cyanogenic Glycosides in Bitter Apricot Kernels,* (Committee On Toxicity 2006), rats given drinking water containing up to 300 mg / L sodium cyanide for 13 weeks (equivalent to approximately 12.5 mg / kg bw / day cyanide) showed no apparent significant changes in haematology, clinical chemistry or urinary parameters. There were no treatment-related gross or histopathological changes in the rats. Slight changes were observed in the testes and spermatozoa of treated males. Comparable results were obtained from a 13 week study in mice. Testicular effects have also been observed in dogs fed a cassava or rice

plus cyanide diet. The lethal dose according to this study in humans is 0.5 – 3.5 mg / kg body weight.

In the studies mentioned, the favourable outcomes were obtained where lower doses were used. The latent period and toxic dose depends on the botanical species ingested, the type of processing (peeled kernels are less toxic than unpeeled), the method of administration (decoctions are far less toxic than ingestion of unprepared kernels; hot water blanching treatment of 20 min at 100 °C is enough to inactivate endogenous ß-glucosidase activity (Tuncel et al. 1998) as well as the length of time it is chewed and the pH of the gastric juice (Dan Bensky 2004a). Children under 5 years of age 5 –10 kernels are sufficient to cause side effects and 20 kernels can be lethal (Dan Bensky 2004a). From the dosages mentioned above one can deduce that it was too big in comparison to the size and weight of the rodents and standard dosages. Acute lethal dose of CN for mammals is 0.5 mg CN / kg of body weight; acute oral lethal dose of HCN for humans is 0.5 – 3.5 mg / kg body weight and the consumption of 50 bitter almonds is deadly for adults, and 5 - 10 kernels are fatal for young children (Chaouali et al. 2013).

UNIVERSITY of the
WESTERN CAPE

1.6 Overview of the cell cycle

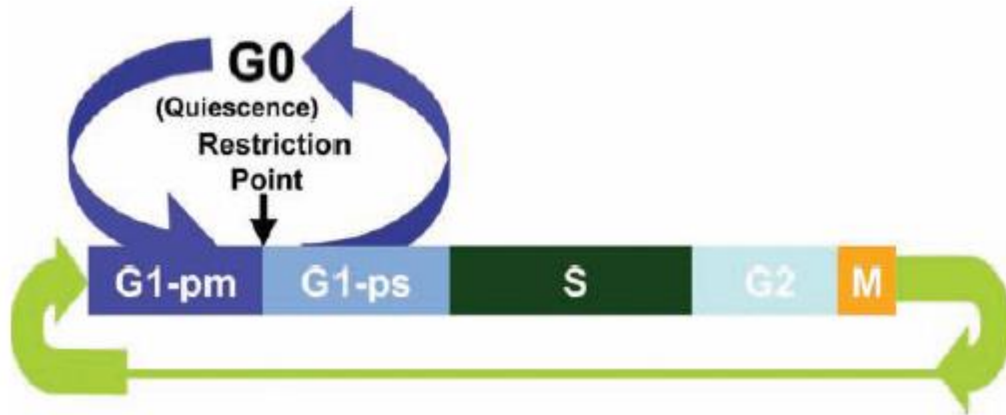
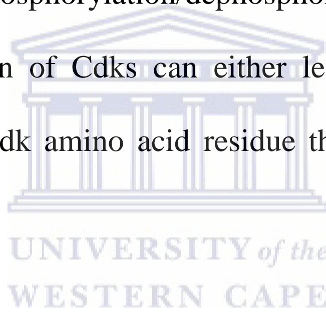


Fig 1.8: Mammalian cell cycle. Cell cycle consists of two parts: mitosis and interphase. During interphase, the genome is duplicated and was called S phase for synthesis. G_1 and G_2 were proposed as the gaps between mitosis and S phase and between S phase and mitosis, respectively. G_1 is usually where critical decisions are made as to whether to enter a resting quiescent stage known as G_0 or to continue cycling and commit to replicating the genome and mitosis. The point in G_1 where this growth factor-dependent decision is made is known as the restriction point (R). G_1 has been described as consisting of 2 parts on either side of R, where the first part of G_1 is known as G_1-pm (post-mitotic), and the second part is known as G_1-ps (pre-S) (Foster et al. 2010).

UNIVERSITY of the
WESTERN CAPE

The cell cycle consists of four phases which occur in sequence, namely gap1 (G_1), synthesis (S), gap2 (G_2) and mitotic (M) phase (Besson et al. 2008). During the G_1 and G_2 phases the cell ensures that all is ready for the process of DNA replication and of cell division, respectively. During the S phase, DNA replication takes place. The G_1, S and G_2 phases are collectively referred to as interphase. The M phase is the process of nuclear and cytoplasm division and it can be subdivided into prophase, metaphase, anaphase and telophase. The progression from one phase of the cell cycle to the next is mainly controlled by a family of serine/threonine protein kinases called cyclin dependent kinases (Cdks) (Masai et al. 2005; Suryadinata et al. 2010).

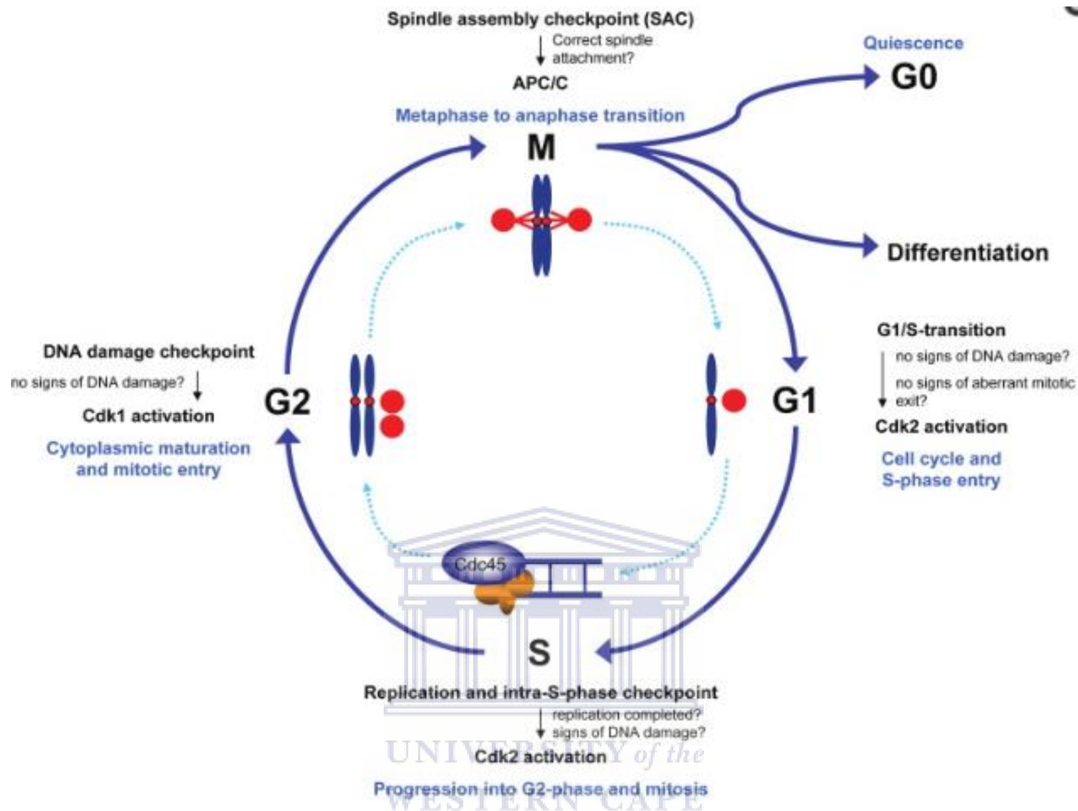
Cdks are present in an inactive form throughout the cell cycle within cells that have the potential to divide and only become activated at specific points in the cell cycle. The Cdks required during the different cell phases of the cell cycle are as follows: Cdk4 and Cdk6 are active during the G_1 phase; Cdk2 is active during the G_1 and S phase with Cdk1 active in both the G_2 and M phases. For their specific activation, Cdks require association with their specific regulatory subunits known as cyclins. When activated, Cdks phosphorylate selected proteins required at specific stages of the cell cycle. Cyclins are proteins whose synthesis is dependent on whether or not they are required at a specific phase of the cell cycle. The activities of the Cdks can also be regulated by phosphorylation/dephosphorylation events as well as by Cdk-inhibitors (CKI). Phosphorylation of Cdks can either lead to their activation or to their deactivation depending on the Cdk amino acid residue that is phosphorylated (Duronio & Xiong 2013).



1.6.1 The cell cycle phases

In order to progress from $G_0 \rightarrow G_1 \rightarrow S \rightarrow G_2 \rightarrow M$, the cells must meet the criteria of each cell cycle and the DNA must be intact. Checkpoints are of great importance to ensure that the integrity of the genetic material is protected. Failing to repair DNA damage and entering mitosis with faulty DNA, gives rise to dead, aneuploid, or mutant cells (Woollard & Nurse 1995). Aneuploidy and mutation can produce uncontrolled cell proliferation that gives rise to cancer.

1.6.1.1 G_1 Phase



UNIVERSITY of the
WESTERN CAPE

Figure 1.9: Cell cycle and its checkpoints. The human cell cycle can be divided into four phases – G_1, S, G_2 and M phase. Cells must proceed through the cell cycle in a unidirectional manner and cell cycle progression is restricted to cells that have fulfilled specific requirements to enter the next phase of the cell cycle. Whether requirements for cell cycle progression are met is supervised by the checkpoints which hold back cells at cell cycle transitions (Schnerch et al. 2012).

The G_1 phase is a period in which a cell decides whether it has received the necessary growth signals to proceed to the S phase. During this phase the cell receives signals from both the extracellular and intracellular environment (Jossen & Bermejo 2013; Masai et al. 2005; Willis & Rhind 2009). If the cell has not received the appropriate signals it will not pass through a point known as restriction point (R-point), it will either temporally stop or it will

exit the cell cycle and enter the phase known as the quiescence phase (G_0) (Ford & Pardee 1999; Bartek & Lukas 2001).

In early G_1 phase the levels of D-type cyclins increase due to appropriate growth signals and they bind with and activate Cdk4 and Cdk6 (Paternot et al. 2014). Following their complete activation (i.e. after cyclin binding and phosphorylation by Cdk-activating kinase, CAK) the cyclinD-Cdk4/6 complex phosphorylates the retinoblastoma protein (pRb) (Masai et al. 2005; Paternot et al. 2014b). Rb, a tumour-suppressor protein, which when unphosphorylated, binds to the elongation factor2 (E2F) (Harbour et al. 1999; Henley & Dick 2012; Zhang et al. 2000). E2F controls transcription of several genes implicated in DNA synthesis and in cell cycle progression (Zhang et al. 2000). Cyclin E is another cyclin which is induced during the G_1 phase. Cyclin E binds with Cdk2 to form a cyclinE-Cdk2 complex (Ford & Pardee 1999). CyclinE-Cdk2 participates in keeping Rb in the hyper-phosphorylated state and is important for transition from G_1 to S phase (Harbour et al. 1999; Masai et al. 2005; Paternot et al. 2014). Cyclin A is not expressed until the S phase where the CyclinA-Cdk complexes are important for maintaining phosphorylation of Rb during S phase (Lim & Kaldis 2013).

UNIVERSITY of the
WESTERN CAPE

The activities of Cdk4/6 andCdk2 can be regulated by cyclin kinase inhibitors (CKIs) (Henley & Dick 2012; Rhind & Russell 2012). Two groups of CKIs have been identified. The first group, the INK4, consist of $p15^{INK4a}$, $p16^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. These proteins function only at the G_1 phase; they form complexes with Cdk4/6 before they bind to cyclin D. Thus inhibiting their kinase activity and interfering with their cyclin D association (Besson et al. 2008). Another group of inhibitors, namely the Cip/Kip family consist of $p21^{waf1,\ Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (Lim & Kaldis 2013). Contrasting to the INK4 proteins the Cip/Kip

proteins serve as CKIs in all phases of the cell cycle and binds to both cyclins and Cdks. p27 is up-regulated in mitogen starved and quiescent state cells; is down-regulated as cells enter the cell cycle (Besson et al. 2008).

1.6.1.2 S Phase

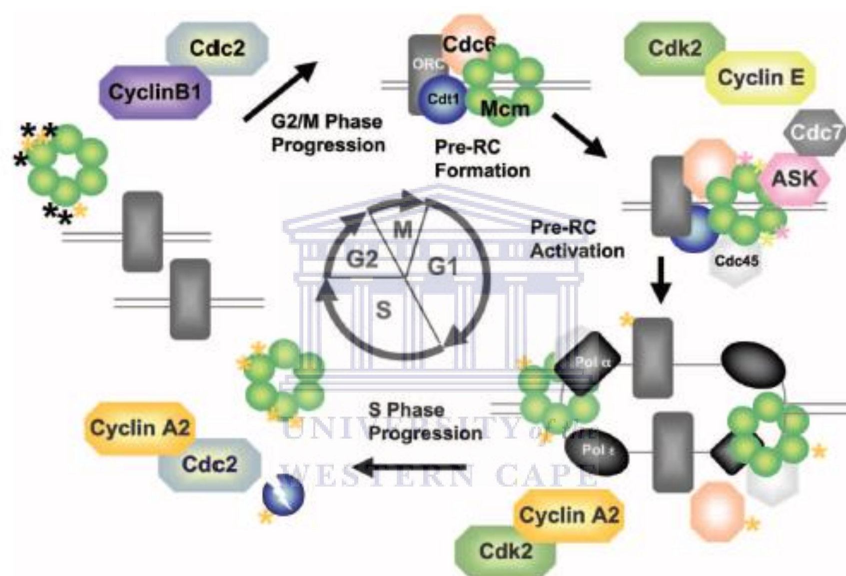


Figure 1.10: Cell cycle regulation of DNA replication. Assembly and disassembly of protein complexes for DNA replication during cell cycle progression; the stars represent phosphorylation mediated by Cdc7-ASK, Cdk2-CyclinE, Cdk2-CyclinA, and Cdc2-CyclinB, respectively (Masai et al. 2005).

Exogenous stressors which compromise the replication process by slowing down or stalling fork progression are known as replication fork barriers (RFB's). Examples of RFB's include: (1) DNA protein complexes (Anand et al. 2012), (2) alternative DNA metabolism (Lambert & Carr 2005), (3) secondary DNA structure (Branzei & Foiani 2010), (4) DNA damage

(Branzei & Foiani 2010), (5) Replication inhibitors (Lambert & Carr 2005). Cells with undamaged DNA that pass the G_1 restriction point proceed into S phase (Takeda & Dutta 2005). DNA replication takes place in S phase and is initiated at multiple origins of replication that are activated either during early, mid or late S phase.

Onset of S phase requires firstly the activation of cyclin E-Cdk2 and later cyclinA-Cdk2 complexes (Ford & Pardee 1999; Harbour et al. 1999; Sørensen & Syljuåsen 2012). Exit from the cycle requires down regulation of these complexes (Henley & Dick 2012). During the S phase of the cell cycle the entire DNA content of the nucleus must be replicated completely and precisely in a period of a few hours (Jossen & Bermejo 2013; Takeda & Dutta 2005). Thus S phase cytoplasm induces nuclei from non-proliferating cells to replicate their DNA. Progression from G_1 to S phase involves conversion of pre-RCs into replication forks, which are complex structures in which parenteral DNA is unwound to produce a single stranded DNA template (ssDNA) for replicative DNA polymerases. Progression thus requires origin unwinding, stabilization of single stranded DNA (ssDNA) and loading of replicative DNA polymerases (Jossen & Bermejo 2013; Takeda & Dutta 2005). The replication forks are fragile structures and prone to developing DNA breaks. Cyclin dependent kinases (CDKs) and Dbf dependent kinase (DDK)/Dbf4-Cdc7 regulated throughout S phase is also needed and initiates DNA replication (Lee et al. 2012). MCM helicase, CDK and DDK is needed for recruitment of Cdc45 which is important for origin unwinding and loading of replicative polymerases together with AND-1/CTF4 (Jossen & Bermejo 2013; Sørensen & Syljuåsen 2012; Takeda & Dutta 2005).

Three DNA polymerases are necessary for the replication of DNA namely, DNA pol-Ɛ, DNA pol-α and DNA pol-δ elongation factors which primarily synthesizes the lagging

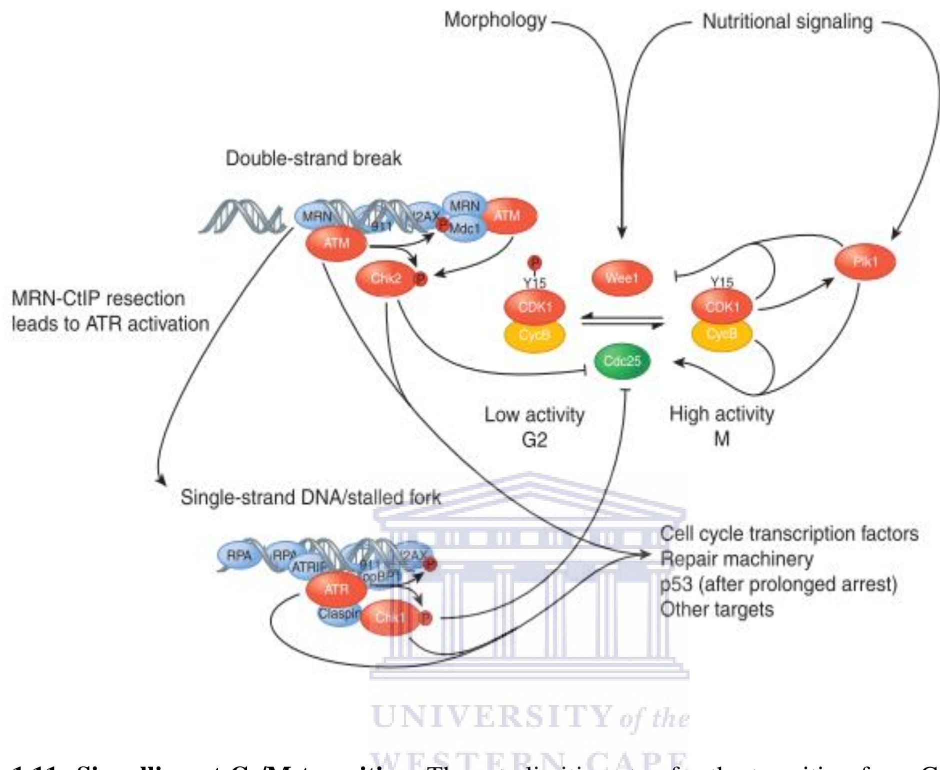
UNIVERSITY of the WESTERN CAPE

Strand (Jossen & Bermejo 2013; Takeda & Dutta 2005). Loading of these polymerases and accessory proteins (including a clamp and clamp loader) onto the chromatin and replication origins results in functional replication forks capable of synthesizing both strands (Masai et al. 2005). DNA pol-δ is mainly associated with DNA replication in the S phase and the lagging strand (Jossen & Bermejo 2013).

The length of S phase differs between species and between different developmental stages within species, but within any particular type of cell, the S phase is remarkably constant in length (Takeda & Dutta 2005). On completion of S phase the cyclinA-Cdk2 complex dissociates and the initiating signal is abolished, allowing activation of Cdk1 and entry into mitosis. A signal has to be sent to block mitosis in cells which fail to complete the S phase. This checkpoint (G_2/M) ensures that cells do not attempt to divide before their entire genomes become duplicated. Failure of this checkpoint results in 'catastrophic' mitosis of cells that have incompletely replicated DNA (Bartek et al. 2004). DNA replication occurs only during the S phase. In early S phase, cyclins D and E are targeted by ubiquitination to be degraded by proteasomes (Suryadinata et al. 2010). Cyclin A levels then increase activating Cdk2 and enabling S phase progression, whilst enzymes and other proteins increase in amount at the beginning of the S phase but are not rate limiting (Ford & Pardee 1999).

UNIVERSITY of the
WESTERN CAPE

1.6.1.3 G_2 phase



UNIVERSITY of the
WESTERN CAPE

Figure 1.11: Signalling at G_2/M transition. The rate limiting step for the transition from G_2 to mitosis is the dephosphorylation of Cdk1 on Tyr[15] and in some cases Thr[14]. This phosphorylation is catalysed by the Wee1 family of dual-specificity kinases and the phosphate is removed by Cdc25 phosphatases. Most of the many signalling pathways that affect the G_2/M transition regulate Wee1 or Cdc25. The DNA damage and replication checkpoints inactivate Cdc25; the morphogenesis and nutritional checkpoints activate Wee1. Cdk1 regulates its own activation as part of a feedback loop by directly phosphorylating Wee1 and Cdc25 or doing so indirectly through Plk1 (Rhind & Russell 2012).

During the G_2 phase cells assess whether all the genetic material and cellular structures are correctly duplicated and then get ready to undergo mitosis. Cells prepare for mitosis by increasing the levels of cyclin A. CyclinA-Cdk1 complex promotes progression into the M phase. Cyclin B (the main mitotic cyclin) also increases in this phase (Suryadinata et al. 2010).

Cyclin B forms a complex with Cdk1 and this complex are known as the M-phase maturation-promoting factor (MPF). MPF inactivation by Cyclin B degradation is required for exit from mitosis. CyclinB-Cdk1 complex is however held inactive by remaining in the cytoplasm and by inhibiting phosphorylation of Cdk1 at its Thr^{14} and Tyr^{15}amino acid residues by Wee 1 and Myt1 (Cdc25 phosphatase) (Rhind & Russell 2012). At the end of the G_2 phase, when the cell is ready to go into mitosis, the cyclinB-Cdkl complex is activated by phosphorylation at its Thr^{161} amino acid residue by phosphatases, Cdc25B and Cdc25C and is then transported into the nucleus (Rhind & Russell 2012; Sørensen & Syljuåsen 2012).

1.1.1.4 M phase

In the M phase of the cell cycle the chromosomes are pulled from the equator towards opposite poles of the cell and cytoplasmic cleavage also takes place. Entry into mitosis is induced by increased activity of cyclinB-Cdk1 complex. Activated cyclin B-Cdk1 complex phosphorylates many proteins essential for the M phase. CyclinB–Cdk1 complex activates the anaphase-promoting complex/cyclosome (APC/C) which is an E3 ubiquitin-protein ligase that regulates sister chromatid separation and exit from mitosis by targeting key proteins for degradation (Ibrahim et al. 2008; Lim & Kaldis 2013). APC/C promotes anaphase by degrading securin, the inhibitor of separase. Separase is a protease which cleaves cohesion and a protein that bind sister chromatids at the kinetochore, thus preventing their separation. APC/C also targets cyclins A and B for degradation; the degradation of cyclin B leads to the end of the M phase (Lim & Kaldis 2013).

UNIVERSITY of the
WESTERN CAPE

1.1.2 Cell cycle checkpoints

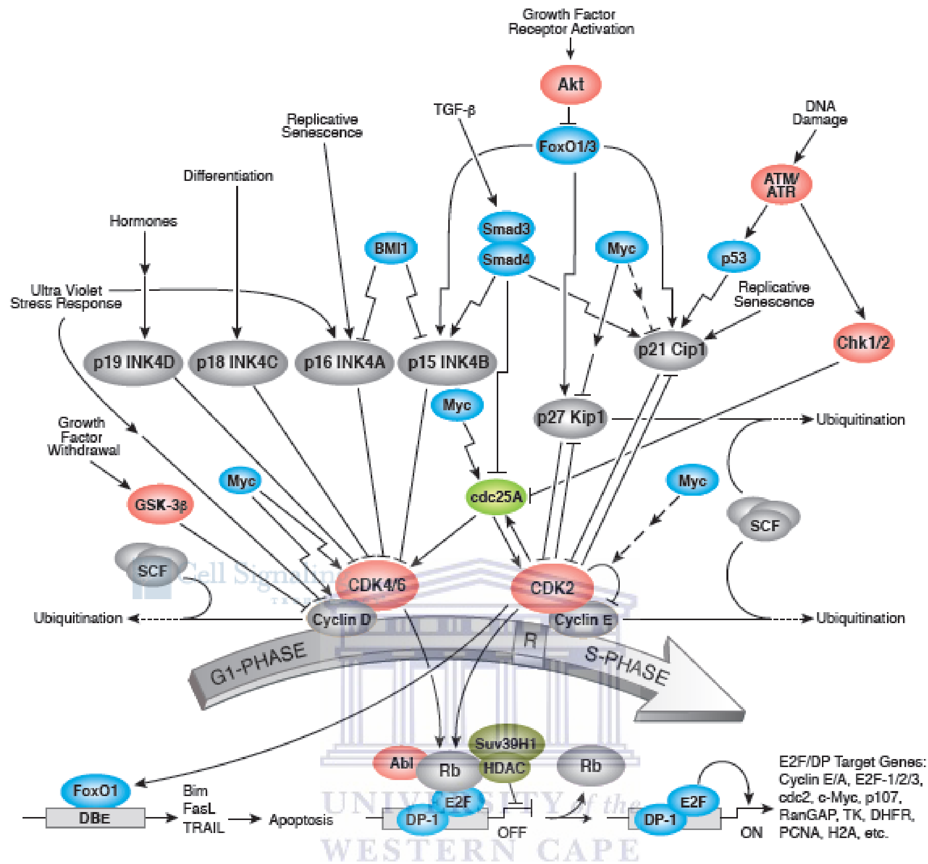


Figure 1.12: Cell cycle control: G_1/S phase checkpoint. The G_1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first 'gap' phase (G_1) into the DNA synthesis phase (S). Two cell cycle kinases, Cdk4/6-CyclinD and Cdk2-CyclinE, and the transcription complex that includes Rb and E2F are pivotal in controlling this checkpoint. During G_1 phase, the Rb-HDAC repressor complex binds to the E2F-DPI transcription factors, inhibiting the downstream transcription. Phosphorylation of Rb by Cdk4/6 and Cdk2 dissociates the Rb-receptor complex, permitting transcription of S-phase genes encoding for proteins that amplify the G_1 to S phase switch and that are required for DNA replication. Many different stimuli exert checkpoint control including TGFβ, DNA damage, contact inhibition, replicative senescence, and growth factor withdrawal. The first four act by inducing members of the INK4 or Cip/Kip families of cell cycle kinase inhibitors. TGFβ additionally inhibits the transcription of Cdc25A, a phosphatase that activates the cell cycle kinases. Growth factor withdrawal activates GSK3β, which phosphorylates cyclin D, leading to its rapid ubiquitination and proteosomal degradation. Ubiquitination, nuclear export, and degradation are mechanisms commonly used to rapidly reduce the concentration of cell cycle control proteins (Cell Signaling Technology 2014).

The term "cell cycle checkpoint" refers to the entire process of monitoring cell cycle events such as DNA replication and spindle assembly, generating signals in response to errors in these processes, and halting the cell cycle at a specific point (Murray 1995). Cells must induce the response rapidly to stop the cell cycle before the genome is irreversibly damaged and the checkpoint must amplify the initial signal generated by a small number of damaged molecules to a level at which it can halt the cell cycle (Murray 1995; Woollard & Nurse 1995). Checkpoint mechanisms delay mitosis due to DNA damage or replication stress (Magiera et al. 2014).

1.6.2.1 DNA damage checkpoint

UNIVERSITY of the WESTERN CAPE

The cell cycle DNA damage checkpoints occur in the G_1/S and in G_2/M transitions and may arrest the cells in the S or M phase. The G_1/S and G_2/M checkpoints prevent entry into S and M phases respectively (Willis & Rhind 2010).At the G_1/S checkpoint, p53, which is normally kept low through the inhibition of Mouse Double Minute-2 (Mdm2), is required for cell cycle arrest. p53 is a tumour suppressor protein that is phosphorylated by protein kinases such as ataxia-telangiectasia mutated (ATM), ataxia and Rad3 related (ATR) kinase P13k-like family in response to DNA damage (Jaehnig et al. 2013), oncogenic insult and hypoxia (Pan et al. 2011). p53 is thus stabilised and activated to induce cell cycle arrest, apoptosis, DNA damage repair, senescence and a number of other protective responses (Pan et al. 2011). ATM responds to double stranded breaks (DSB) and ATR to replication blocking lesions (Sørensen & Syljuåsen 2012; Willis & Rhind 2010). When p53 is activated it stimulates the

transcription of various genes such as p2l, Mdm2 and Bax. p21, one of the Cip/Kip family members as already mentioned, blocks cyclin-Cdk activities, thus preventing replication of damaged DNA. Mouse Double Minute-2 Homolog (Mdm2) is a negative regulator of p53; it inhibits p53 transcriptional activity and facilitates its ubiquitination. Over expression of Mdm2 has been shown to lead to the enhancement of tumorigenic potential.

If DNA damage cannot be repaired, p53 can induce cell death by activating apoptosis promoting genes such as Bax. When DNA damage occurs in G_2/M, cells can initiate cell cycle arrest irrespective of whether or not p53 is present (Schnerch et al. 2012). Entry into mitosis is prevented by keeping Cdk1 in its inactive form. This can be done by phosphorylation or by sequestration of components of the cyclinB-Cdk1complex. Two protein kinases that facilitate these processes are checkpoint kinase 1 and 2 (Chk1 and Chk2), which are triggered when there is DNA damage. ATM activates Chk2 which then phosphorylates and then degrades the phosphatase Cdc25A; ATR activates Chk1 leading to the degradation of Cdc25A (Sørensen & Syljuåsen 2012; Willis & Rhind 2009). Chk1 and Chk2 phosphorylation of Cdc25 also results in the inhibition of Cdc25 binding activity to l4-3-3 γ proteins. 14-3-3γ protein keeps the phosphatase in the cytoplasm and stops the removal of the inhibitory phosphorylation on Thr^{14} and Tyr^{15} of Cdk1, thereby maintaining cyclinB-Cdk1 in an inactive form (Sørensen & Syljuåsen 2012).

UNIVERSITY of the
WESTERN CAPE

1.6.2.2 Intra-S phase checkpoint

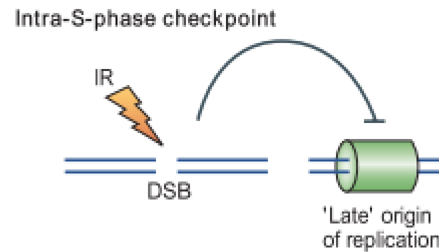


Figure 1.13: Intra-S-phase checkpoint. This represents the double strand break-induced (DSB) replication dependent intra-S phase checkpoint (Bartek et al. 2004).

UNIVERSITY of the
WESTERN CAPE

S phase is the genetically most vulnerable period of the cell division cycle and are more significant for preventing genetic instability than the G_1 or G_2 or mitotic-spindle checkpoints (Jossen & Bermejo 2013; Kaufmann 2009). Mre11-Rad50-Nbs1 (MRN) is required for S phase DNA damage checkpoint response (Kaufmann 2009; Liu et al. 2012). It works parallel to the Chk2-Cdc25A pathway which regulates origin firing (Willis & Rhind, 2009). There is 3 types of S phase checkpoints, namely i) DSB-induced replication dependent intra-S phase checkpoint, ii) replication dependent intra-S phase checkpoint / or replication checkpoint, iii) S-M checkpoint (Bartek et al. 2004). The intra-S phase checkpoint has sequential steps including DNA damage sensors, signal transducers and effectors (Liu et al. 2012).

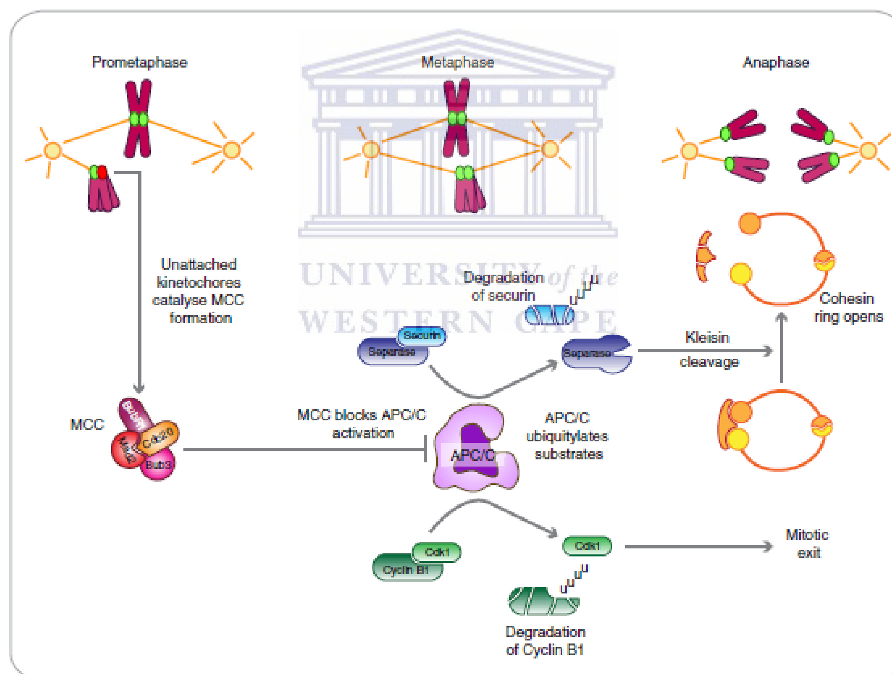
The intra-S phase checkpoint does not require active replication forks and prevents the firing of new replication forks by inhibiting initiation at licensed but unfired origins (Lambert & Carr 2005; Lee et al. 2012) whether early or late (Grallert & Boye 2008), resulting in a

reduction in the rate of progression through the S phase rather than an arrest of the S phase (Oakley & Hickson 2002). The intra-S-phase checkpoint is activated by double strand breaks (DSB) that are generated in the genomic loci outside the active replicons. None of the three S-phase checkpoints require p53, the target of G_1 checkpoint that arrests the cell cycle in G_1 phase (Bartek et al. 2004). Intra-S phase checkpoint activated by genotoxic insults causes only temporary, reversible delay in cell cycle progression, mainly by inhibition of new replicons initiation and thereby slowing down DNA replication (Bartek and Lukas 2001), but not permanently arrested (Oakley & Hickson 2002; Willis & Rhind 2009; Bartek et al. 2004).

A long term intra-S-phase block would limit the amount of sister chromatids and therefore reduce available templates for efficient repair by homologous recombination (Bartek & Lukas 2001). Complete inhibition of Cdks and prolonged intra-S-phase arrest may cause regaining of replication competence of already fired origins, making the recovery process prone to over-replication of at least parts of the genome (Bartek & Lukas 2001). Willis and Rhind (2009) suggest that this checkpoint may be more concerned with tolerating and accommodating damage during replication rather than repairing it. DSB sensors recruit ATM to damaged DNA. Activated ATM induces phosphorylation and activation of the checkpoint component Chk2 which then targets Cdc25A and BRCA1 and Nbs1 a component of MRN protein complex (Tasat & Yakisich 2010). Cdc25A, an unstable protein with a half-life of 20 – 30 minutes, requires ATR, claspin and Chk1 (Bartek et al. 2004). MRN works parallel to the Chk2-Cdc25A pathway which regulates origin firing (Willis & Rhind 2009). Cdc25A-degradation pathway may be involved in slowing down the ongoing S phase, thus the ATM-Chk2-Cdc25A-Cdk2-Cdc45 axis emerges as a key mechanism of not only the rapid prevention of S phase entry in the G_1 checkpoint but also in the intra-S-phase response

(Bartek & Lukas 2001). Inhibition of Cdk2 activity through the Cdc25A degradation leads to a several hour delay of S phase progression, which correlates with the intra-S-phase checkpoint response (Tasat & Yakasich 2010; Bartek & Lukas 2001; Willis & Rhind 2009). Interference with the Chk2-Cdc25A-Cdk2 cascade at any of the steps downstream of ATM results in radioresistant DNA synthesis (RDS) (Bartek & Lukas 2001).

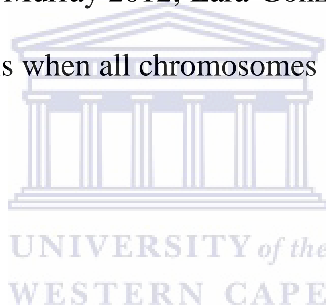
1.6.2.3 The spindle assembly checkpoint



UNIVERSITY of the WESTERN CAPE

Figure 1.14: Spindle Assembly Checkpoint (SAC) principles. During the early stages of mitosis (pro-metaphase), unattached kinetochores catalyse the formation of the mitotic checkpoint complex (MCC) composed of BubR1, Bub 3, Mad2 and Cdc20, leading to inhibition of the APC/C. Once all the chromosomes are aligned with their kinetochores attached to the spindle (metaphase), regeneration of the MCC ceases, allowing Cdc20 to activate the APC/C, leading to the ubiquitylation and degradation of securing and cyclin B1. Degradation of securin liberates separase which in turn cleaves the Scc1 kleis in subunit of the cohesion ring structure; this opens the ring, allowing the sister chromatids to separate (anaphase). Meanwhile, degradation of cyclin B1 inactivates Cdk1 leading to mitotic exit (Lara-Gonzalez et al. 2012).

The absence of microtubule attachment, or lack of tension at the kinetochore (because of chromosomes failing to attach to opposite poles) activates the spindle assembly checkpoint (SAC), arresting the cells at the metaphase-to-anaphase transition (Lau & Murray 2012). The SAC inhibits the commencement of anaphase until all kinetochores are attached properly to the mitotic spindle. The proteins that are activated during this checkpoint include the Mitotic arrest eficient2 (Mad2) and the budding uninhibited by benomyl (Bub) proteins. Mad2 prevents transition from metaphase to anaphase by binding to kinetochores thus preventing activation of, the E3 ubiquitin ligase, anaphase-promoting complex (APC) and its co-activator Cdc20 (APC/C) (Lau & Murray 2012; Lara-Gonzalez et al. 2012). This ensures that cells only progress through mitosis when all chromosomes are properly attached.


UNIVERSITY of the
WESTERN CAPE

1.7 Types of cell death

There are several types of programmed cell death (PCD) that have been identified, namely mitotic catastrophe, oncosis, necrosis, apoptosis and autophagy. There exists an intimate relationship between necrosis, autophagy and apoptosis. In this dissertation, only the one type of programmed cell death, apoptosis, will be discussed in detail since it has been shown to be closely related to anti-cancer therapy.

Mitotic catastrophe is a term used to indicate cell death from aberrant mitosis. It is a cell pathway that occurs during mitosis as a result of errors in the cell cycle checkpoint and cellular damage. It is associated with morphological features such as the presence of multinucleated and micronucleated cells, with all these characteristics occurring prior to cell

death. Therefore mitotic catastrophe guards cells against unnecessary aneuploidization (Vitali et al. 2011).

Oncosis, a passive form of cell death, occurs due to severe cellular damage as a result of cytotoxicity or failure of plasma membrane ion channels. It is characterized by cellular and organelle swelling, membrane blebbing as well as increased permeability of the plasma membrane, which leads to cell lysis. The lysed cell releases signalling molecules which in the end induces inflammation (Trump et al. 1997).

Autophagy (type II PCD) is a catabolic process every cell undergoes to recycle long-lived proteins and to eliminate damaged macromolecules and organelles; therefore it helps to maintain the cell's health (Bouzas-Rodríguez et al. 2012; Grasso & Vaccaro 2014). It is a survival mechanism in nutritionally deprived cells allowing them to live longer by recycling their components (Bouzas-Rodríguez et al. 2012). Autophagy also helps to prevent neurodegeneration by degrading miss-folded proteins (Bouzas-Rodríguez et al. 2012). It thus plays either a pro-death or pro-survival role (Ouyang et al. 2012).

UNIVERSITY of the
WESTERN CAPE

Necrosis (type III PCD) is a type of cell death that is commonly referred to as an 'accidental' type of cell death or premature cell death when cells are exposed to serious physical or chemical insults. Here the integrity of the cell membrane is disrupted, intercellular materials released into the extracellular milieu leading to an inflammatory response by the immune cells (Ouyang et al. 2012). The morphological features of necrosis thus include an increase in cell volume enlargement of cytoplasmic organelles, severe plasma membrane damage and loss of cellular contents (Ouyang et al. 2012).

1.7.1 Apoptosis

Apoptosis ('programmed / normal' cell death) (type I PCD) is a cellular mechanism by which unwanted or useless cells undergo death in response to signals originating from inside or outside the cell without eliciting inflammation. Apoptosis plays a significant role in cell growth during development and homeostasis (Roche Applied Science 2007).

Too much or too little of the apoptosis pathway can result in diseases such as cancer, autoimmune diseases and neurodegenerative disorders (Roche Applied Science 2007). Cells undergoing apoptosis can be recognized by the following morphological features: cell shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing and formation of apoptotic bodies. The morphological characteristics observed in cells undergoing apoptosis are due to the activities of a family of intracellular cysteine proteases known as cysteine-dependent aspartate-specific proteases (caspases). In order for apoptosis to take place, various cellular processes such as, early gene transcription and translation are activated, leading to the synthesis of specific apoptosis associated proteins (De Kock et al. 1994). The initiation of apoptosis can occur either via an extrinsic (death receptor pathway) or an intrinsic (mitochondrial pathway) pathway depending on the stimulus (Ouyang et al. 2012; Sayers 2011).

UNIVERSITY of the
WESTERN CAPE

1.7.1.1 Extrinsic pathway / Cytoplasmic pathway

The extrinsic pathway involves the binding of specific extracellular ligands to cell surface receptors known as death receptors (DRs). Death receptors are members of the tumour necrosis factor (TNF) receptor super-family which have apoptosis inducing activities (Sayers et al. 2011). The best described ligands and their matching death receptors comprise of FasL/FasR, TNF-α/TNF-Rl, Apo-3ligand(Apo-3L)/DR3, Apo-2ligand(Apo-2L)/DR4,

Apo2L/DR5 and DR6 (Ouyang et al. 2012; Sayers et al. 2011). They are predominantly produced by cells of the immune system.

After binding of an appropriate ligand to receptor death domain (DD) the receptor recruits adaptor proteins such as Fas-associated DD (FADD) or TNF receptor associated DD (TRADD) that also have a DD at their C-terminus and a second domain, a death-effector domain (DED) at their N -terminus. The DED of the adaptor protein binds to the DED of an inactive initiator caspases (procaspase 8/1 0) forming a complex that leads to the activation and release of active caspase 8 or 10. The activated initiator caspase then activates a series of effector caspases resulting in the cleavage of caspase substrates (e.g. structural and pro-apoptotic proteins), which in the end leads to apoptosis (Ouyang et al. 2012). The active initiator caspase can in addition cleave a B-cell lymphomaprotein2 (Bcl-2) family member termed Bid to truncated Bid (tBid), its active form. Bid promotes activation of the intrinsic pathway of apoptosis.

UNIVERSITY of the
WESTERN CAPE

1.7.1.2 Intrinsic pathway / mitochondrial pathway

The mitochondrial pathway is activated by intracellular events and depends on the release of pro-apoptotic factors released from the mitochondria (Sayers et al. 2011). The mitochondria play a central role in the transmission of death signals in response to the various forms of cellular stressors. In this pathway, outer mitochondrial membrane permeabilization (MOMP) is produced by the formation of pores in the mitochondrial membrane. The formation of pores is controlled by Bcl-2 family proteins which inhibit apoptosis. These proteins can be categorized into three sub-groups, namely the anti-apoptotic proteins (Bcl-2,Bcl-x, Bcl-XL,

Bcl-XS, Bcl-w and BAG) and the pro-apoptotic proteins (Bax, Bad, Bok, Bid, Bim, Bik, Noxa, Puma) (Ouyang et al. 2012; Sayers 2011).

Upon apoptosis induction, pro-apoptotic Bcl-2 proteins such as Bax and Bid translocates from the cytoplasm to the outer mitochondrial membrane where they work together with other pro-apoptotic Bcl-2 proteins to form pore-like structures in the membrane and inhibit the protective effect of Bcl-2 (Ouyang et al. 2012). This leads to the release of pro-apoptotic proteins such as cytochrome-c (Cyt-c), and second mitochondria-derived activator of caspase/ direct IAP-binding protein with low pI(Smac/DIABLO), and serine protease HtrA2/Omi, which cleaves and inactivates IAP, from the mitochondrial inter-membrane space into the cytosol (Sayers 2011). Cyt-c together with ATP binds to apoptotic protease-activating factor-l (Apaf-l) forming the apoptosome complex (Ouyang et al. 2012; Tun et al. 2007). The apoptosome recruits and activates procaspase-9 by the interaction of the caspase recruiting domain (CARD) of Apaf-l, with that of procaspase-9 which triggers the activation of the initiator caspase-9. Activated caspase-9 cleaves and then initiates caspase-3 and -7 creating an expanding caspase cascade of proteolytic activity and ultimately causing cell death (Ouyang et al. 2012).

UNIVERSITY of the
WESTERN CAPE

The release of mitochondrial Smac/DIABLO as well as the HtrA2/Omi into the cytoplasm promotes apoptosis by inhibiting the activity of apoptosis protein inhibitors (lAP) overrides this checkpoint (Sayers 2011; Tun et al. 2007). IAP’s are proteins which normally attach to caspases-3-7-9 through its IAP repeat domain to provide an important checkpoint to prevent caspase activation, thus inhibiting their activity; they have also been shown to promote the degradation of caspases (Sayers et al. 2011).

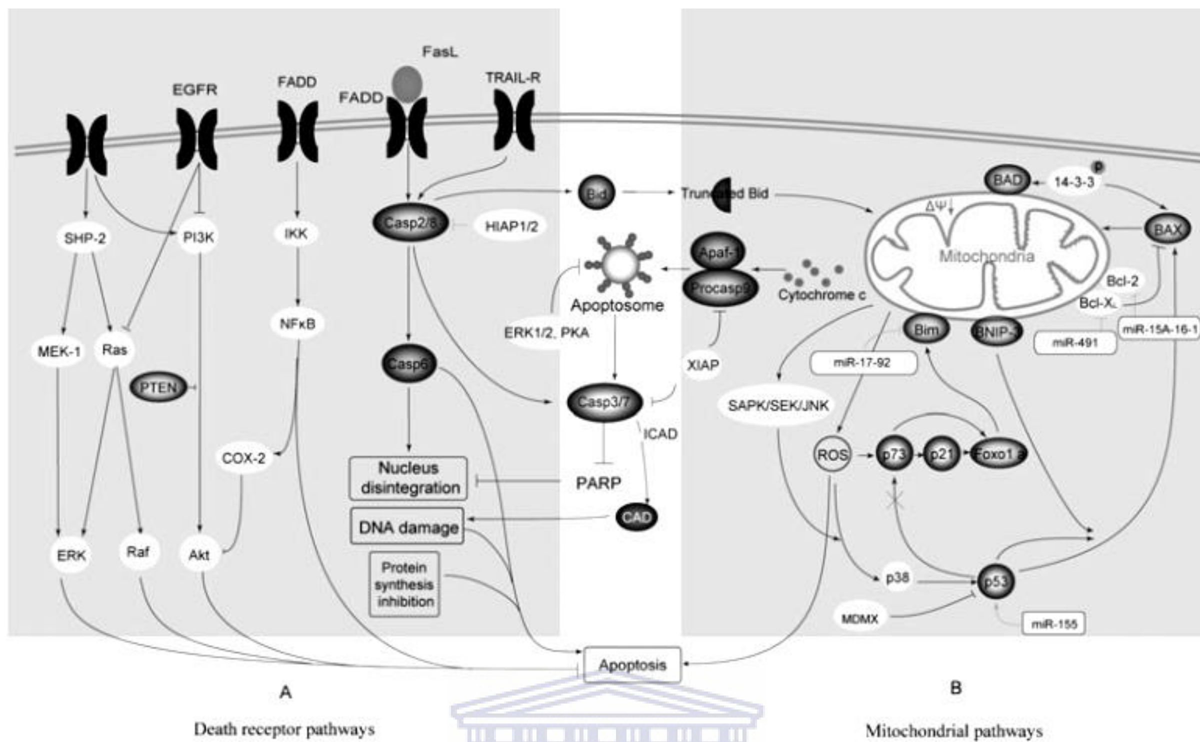


Figure 1.15: Apoptotic signalling pathways and their relevance to microRNA regulation in cancer (Ouyang et al. 2012).

UNIVERSITY of the
WESTERN CAPE

1.8 Significance to health care and or biomedical science

❖ There is a great demand for effective cancer treatment. In the hope that some benefit can be derived from the apricot and / or peach kernel preparations in the treatment of colon cancer, thus contributing to society at large in terms of medicines, and give credit to those who have used such protocols in the past, and who have been silenced in the name of big pharmaceuticals at the expense of the sick, suffering and dying patients and their family members.

- Chinese Medicine practitioners use these two kernels in herbal prescriptions, the peach kernel in particular in cancer treatment where there is Blood stasis in especially the colon and lungs complicated with constipation and cough. It would thus be beneficial to know if these kernels have this type of activity and such an effect at a cellular level.
- Pharmacological studies have been performed using amygdalin as an anti-cancer agent (Syrigos et al. 1998; Kwon et al. 2003). It was used as a pro-drug in antibody-directed enzyme pro-drug therapy (Syrigos et al. 1998; Kwon et al. 2003). It is therefore imperative that *in vitro* and *in vivo* studies on the effects of the mechanism of action of peach and apricot kernels be carried out to assist in the development of peach and apricot kernel extracts as drugs or pro-drugs.
- Clinical studies have shown that metastasis of colon carcinoma occurs after invasion of the bowel mucosa as a result of surgical resection (Willson et al. 1987). It would therefore be advantageous to find a cure for this type of cancer which may then not require surgical resection but rather an edible product which may have a direct effect, namely a food substance.

UNIVERSITY of the
WESTERN CAPE

The difference between Western Medicine and Chinese Medicine is that Chinese Medicine makes its diagnosis and treats in terms of syndromes and patterns. According to Chinese Medicine the onset of disease is as a result of the imbalance of Yin and Yang. Diagnosis is based on the premise of ‘one pattern, many syndromes; and one syndrome, many patterns’. It is not possible to know the syndrome or pattern of the sample specimen’s origin when doing

in vitro studies and thus will not be able to say whether or not the extractions are effective in a particular tissue sample according to a particular pattern or syndrome.

This research is a Western Medicine take of treatment of cancer cells. The goal of this research is specifically directed at testing whether the kernels, its various forms and extract preparations have any anti-tumour effect at all. Further studies may then focus on biopsied tissue samples from specific patients with specific patterns and syndromes for which these extracts and forms may be effective.

The aim of this study is:

To perform a comparative *in vitro* study of the effects of the apricot and peach kernel extracts on a human colon cancer cell line to determine:

UNIVERSITY of the
WESTERN CAPE

(i) Whether the apricot and peach kernels extracts induce any cytotoxic and or growth inhibitory effects in the HT-29 colon cancer cells.

(ii) Whether there are differences in the efficacy of the respective kernel preparations on the HT-29 colon cancer cells.

(iii) Whether the kernels from the various origins display effectiveness on the HT-29 cells including a time and dose difference.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Solutions used

Dulbecos Minimum Essential Medium F12 (+L-Glut, FCS, HEPES) (DMEM)

[GIBCO; Cat. No. 31331-028]

Crystal violet [Sigma-Aldrich; Cat. No. C3886]

Gluteraldehyde

Triton X-100 [Sigma-Aldrich]

UNIVERSITY of the
WESTERN CAPE

Phosphate Buffer Saline (1:9 PBS) [GIBCO; Cat. No. 20012-019]

Bouin's solution [Sigma-Aldrich; Cat. No. HT10132]

Eosin B [Sigma-Aldrich; Cat. No. 861006]

Xylene [Kimix]

Haematoxylin [Sigma-Aldrich; Cat. No. H9627]

Trypsin [Sigma-Aldrich; Cat. No. T4049]

Tri Sodium Citrate [Sigma-Aldrich]

Propidium Iodide [Sigma-Aldrich; Cat. No. 81845]

RNase A [Roche]

Trypan blue [Sigma-Aldrich; Cat. No. T6146]

Penicillin / Streptomycin [GIBCO; Cat. No. 15140-122]

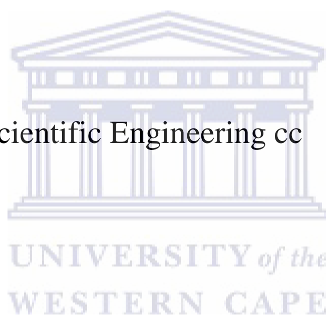
Fetal Bovine Serum (FBS) [GIBCO; Cat. No. 10500-056]

Dimethyl-Sulfoxide (DMSO) [Scharlau;Cat. No. SU0155]

Hoechst 33342 fluorescent stain [Sigma-Aldrich; B2261]

2.1.2 Equipment

Buchi water-bath B-480 Scientific Engineering cc


UNIVERSITY of the
WESTERN CAPE

Buchi rotavapor R-114

Bench top Freeze dryer

Glomax plate reader

ZEIS ICM 405 light microscope

Erlenmeyer soxlet apparatus

Buchi vac V-500

FACs Calibur DNA analysis flow cytometer

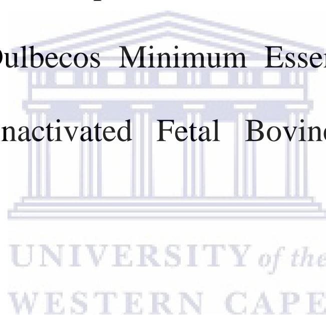
Nikon Eclipse 50i light microscope

All chemicals used were of analytical grade and obtained from Sigma. Sterile tissue culture consumables obtained from Greiner.

2.2 Methods

2.2.1 General cell culture procedures

HT-29 human colon cancer cells obtained from Dr Gelderblom, Medical Research Centre, Bellville, Cape Town, were grown in 25cm^2 and 75cm^2 tissue culture flasks and maintained in a 5% CO_2, 95% humidified air atmosphere at 37°C. The maintenance growth medium of the HT-29 cells consisted of Dulbecos Minimum Essential Medium F12 (DMEM/F12) supplemented with 5% heat inactivated Fetal Bovine Serum (FBS), penicillin and streptomycin (1000 µg/mL).


UNIVERSITY of the
WESTERN CAPE

The HT-29 human colon cancer cell line was originally isolated by Fogh and Trempe from a human carcinoma of the colon (Forgue-Lafitte et al. 1989). HT-29 human colon cancer cells can proliferate in a defined (serum – free) medium containing no added growth factors within 3 to 4 days or 2 to 3 days (on an autologous extracellular matrix) compared to 1 day in the presence of fetal calf serum (Forgue-Lafitte et al. 1989).

When the cells reached 80% confluency, they were washed with PBS, and then incubated with just enough trypsin to cover the monolayer for ± 3 minutes, at 37°C. The trypsin was then removed and the flask was tapped lightly to allow cells to detach from the surface of the flask. Equal amounts of medium were added to inhibit the action of the trypsin, thereafter the cells were centrifuged for 4 minutes at 2500 rpm. Cells were then resuspended in fresh

medium and divided into subcultures or used in experiments. Some sub-cultured cells were placed in cryovials in freeze medium and stored at -80°C to ensure a continuous supply of low passage HT-29 ells. The freeze medium used to store cells in consisted of 70% growth medium, 10% DMSO and 20% FBS.

2.2.2 Cell counts

Cells were harvested by trypsinization as described in 2.2.1. Cells were then transferred to a centrifuge tube, and centrifuged for 4 minutes at 2500 rpm. The supernatant was poured off, and cells were resuspended in 1mL growth medium. The resuspended cells were then counted by making use of a haemocytometer. A ratio of 1:1 (trypan blue: cell suspension) was used to determine the amount of viable cells. 10µL trypan blue was added to 10µL of the cell suspension in a micro Eppendorf. 10µL of this solution was then placed on each side of the slide and counted. Dead cells take up the dye, therefore stain blue, and are not included in the cell count. Viable cells do not take up the dye, so they remain clear and are counted.

UNIVERSITY of the
WESTERN CAPE

The number of cells per ml was determined by the following calculation:

$$\text{cells/mL} = \text{average number of cells x dilution factor x } 10^4$$

The cell suspension was then diluted with medium to give the required cell number and was seeded into the appropriate tissue culture plates.

2.2.3 Preparation of extraction fractions from kernels

Chinese apricot kernels and Chinese peach kernels were obtained from China in June 2012 via Dr Li Chunlan, Newlands, Cape Town. Turkish apricot kernels from Malatya were sponsored by Mrs Asma Kariem-Elitemiz in July 2012. South African apricot kernels were sponsored by Mr Dirk Versfeld in December 2012. South African peach kernels were donated by De Wit Industries Cape Town, South Africa in May 2014.

The various fractions prepared from the kernels include two main groups of extractions namely: organic and aqueous extractions. To simplify the format by which the kernels could be used to treat the cells organic extractions were fractionated into a total fraction, lipophilic fraction and a hydrophilic fraction.

UNIVERSITY of the
WESTERN CAPE

2.2.4 Organic ethanol / acetone extractions (including total, lipophilic and hydrophilic extractions)

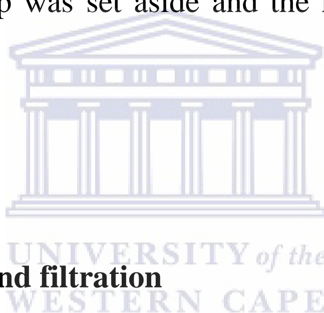
2.2.4.1 Total extraction and filtration

A specific amount of CAK, CPK, TAK, SAK, and SPK kernel types was used, grounded and placed into Erlenmeyer flasks respectively. The grounded kernels were soaked in 80% ethanol for 24hr then filtered. This first portion of filtrate was kept aside. The kernel pulp was soaked again in 80% ethanol and the process was repeated to provide a second portion of filtrate. This second portion of filtrate was added to the first amount to provide the total extraction fraction and the grounded pulp was discarded.

2.2.4.2 Lipophilic extraction and filtration

A specific amount of CAK, CPK, TAK, SAK, and SPK kernel types was used, grounded and placed into Erlenmeyer flasks respectively. The grounded kernels were soaked in 100% acetone for 24hr and then filtered. This first portion of filtrate was kept aside. The kernel pulp was soaked again in 100% acetol and the process was repeated to provide a second portion of filtrate. This second portion of filtrate was added to the first amount to provide the total extraction fraction.

This procedure was repeated for all the kernels. After each lipophilic extraction's second filtration was completed, the pulp was set aside and the hydrophilic extractions performed using this pulp.



2.2.4.3 Hydrophilic extraction and filtration

The pulp set aside from the lipophilic extractions was soaked in 80% ethanol solution for 48hour. It was filtered, the pulp kept aside and soaked again in 80% ethanol and left to stand for 48hour. Once filtered, this second filtrate was added to the first filtrate and the pulp discarded.

2.2.4.4 Evaporation of the Total, Lipophilic and Hydrophilic extractions

Each respective filtrate was poured into a round bottom flask and attached to a Buchi rotavapor. At 55ºC the flask was submerged into the warm water bath and the sample evaporated. An empty glass vial was weighed. Each respective oil sample fraction was

transferred to the vial and weighed and percentage yield calculated (see Table 3.2). The oil fractions were stored at -20ºC.

Table 2.1: Formula used to calculate percentage yield

$$\frac{(vial + sample) - sample}{100g} \times 100 = \%\, yield$$

2.2.5 Double boil decoction / Aqueous extraction

UNIVERSITY of the
WESTERN CAPE

A specific amount of Chinese apricot kernel (CAK), Chinese peach kernel (CPK), Turkish apricot kernel (TAK), South African apricot kernel (SAK) and South African peach kernel (SPK) were grounded separately using a domestic coffee grinder and placed into separate 1 litre beakers.

The grounded kernels were covered with boiled distilled water and placed onto a hot plate (DragonLab MS-H-Pro). It was brought to a boil at a maximum temperature of 400ºC, and the temperature then lowered to 200ºC and allowed to simmer. This process took approximately one hour forty minutes. The solution was filtered and the liquid removed and kept aside. The grounded substrate was placed back into the beaker and covered with 250mL boiled distilled water. The boiling and filtration process was repeated using the same method.

2.2.5.1 Freeze drying method of the aqueous extractions

The final liquid volume was divided into two round bottom flasks and freeze dried for approximately 48hr. The freeze dried powdered kernels were then weighed (g) (see Table 3.3) and placed into plastic jars and stored in a dry area at room temperature.

2.2.5.5 CAK, CPK, TAK, SAK and SPK treatment

To make up a final concentration of 100% organic stock solution of the respective CAK, CPK, TAK, SAK and SPK kernel extractions, 1g of the respective oils were added to 9mL of DMSO to make up a final volume of 10mL.

To make up a final concentration of 100% aqueous stock solution of CAK, CPK, TAK, SAK and SPK kernel extractions, 1g of freeze dried powdered kernel extract was added to 9mL of DMSO to make up a final volume of 10mL.

UNIVERSITY of the
WESTERN CAPE

Final treatment concentrations of 100µg/mL, 500µg/mL and 1000µg/mL respectively of each kernel extract was made by adding 10µg/mL, 50µg/mL and 100µg/mL respectively of stock solution to 10mL growth medium.

2.2.6 Cell growth and viability

2.2.6.1 Crystal Violet (CV)

Principle: Crystal violet is a triphenylmethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N, N-dimethyl-aniline) also known as gentian violet (or hexamethylpararosaniline chloride). Despite the fact that this colorimetric assay is much simpler, faster and more

objective than the classic colony formation assay, it generally gives an overestimation of the survival rate compared to the clonogenic assay since they measure dividing and non- dividing cells. The dye stains DNA and the colour of the dye depends upon the pH of the solution. Upon solubilisation, the amount of dye taken up by the monolayer and the intensity of the colour produced are proportional to the cell number.

Method: 5000 cells were seeded in each well of a 96 well culture plate and incubated at 37°C for 24hr to attach. Thereafter the medium was discarded and replaced with fresh medium and 100µL of each treatment (see Section 2.2.5.5) was added to the wells and incubated at 24hr, 48hr and 72hr intervals respectively.

The medium and treatment was discarded and the cells fixed by adding 100µL of 1% gluteraldehyde to each well and the plates incubated at room temperature for 15min. The gluteraldehyde was removed and 100µL of 0.1% crystal violet stain was added to each well and the plates incubated for 30min at room temperature. The plates were then washed under running water for 15min and left to dry overnight. When thoroughly dried, 200µL of 0.2% TritonX-100 was added to each well and incubated at room temperature for 30min to solubilise the cells. Thereafter, by using reverse pipetting technique, 100µL of the solubilised solution was transferred to non-treated 96 well plates and the absorbance of the specimens analysed at 570nm using a Glomax plate reader. This experiment was done in triplicate (n = 3).

UNIVERSITY of the
WESTERN CAPE

2.2.7 Cell Morphology

In this study we looked at the morphological changes within the cells after being exposed to the kernel extractions.

Method: The morphology of the cells, after treatment with CAK, CPK, TAK, SAK, SPK organic extractions and CAK, CPK, SAK and SPK aqueous extractions was evaluated using Haematoxylin and Eosin (H&E) staining.

2.2.7.1 Haematoxylin and Eosin Staining (H&E)

UNIVERSITY of the
WESTERN CAPE

Principle: The study of histology is based on microscopic analysis of the structure of tissues /cells. H&E yields excellent morphology, making it useful for visualizing structures of different composition, even under low magnification. H&E is able to differentiate cellular components based on the chemical nature of the cell. Cells are made up of negatively charged acidic components and positively charged basic components. H&E takes advantage of these properties, forming electrostatic linkages to ionizable radicals in the cell, leading to positively and negatively charged cell components to be termed acidophilic and basophilic, respectively, according to the properties of the stain they take up. In the case of H&E, haematoxylin is a basic dye that stains cell nuclei blue–purple, whereas eosin is an acidic dye that stains the cell cytoplasm, collagen, and erythrocytes red–pink. When these two stains are applied sequentially, they combine to provide more contrast between cellular components than either stain would alone.

Method: Cells were seeded on sterilized coverslips placed in 6-well plates and was incubated at 37°C for 24hr to attach. The medium was then removed and replaced with 2mL fresh medium and 2mL treatment (see Section 2.2.5.5). It was then incubated for 24hr, 48hr and 72hr intervals respectively.

The coverslips with attached cells were removed and fixed in Bouin's fixative for 30min. Thereafter it was placed in 70% ethanol for 20min and then rinsed in tap water till white. The cells were then stained in haematoxylin (Ehrlich) for 15min followed by another rinse in tap water for 1min. Thereafter the cells were stained in eosin for 20min followed by a dehydration procedure by placing the coverslips ina graded series of ethanol twice in each (70%, 90% and 100% ethanol) for 5min respectively and cleared in xylene. The coverslips were then mounted using DPX mountant onto microscope slides. The slides were analysed by viewing them under a Nikon Eclipse 50i light microscope. The cytotoxic effects of the various doses of the kernel extracts on HT-29 cells, including apoptotic body formation, were studied.

UNIVERSITY of the
WESTERN CAPE

2.2.8 Cell cycle progression

2.2.8.1 Flow Cytometry

Principle: Flow cytometry is a method which can determine multiple characteristics of single particles flowing in a single file down a stream of fluid. It aims to measure optical fluorescent characteristics of particles or cells. Among these properties is size and DNA, which corresponds to forward angle of light scatter and granularity or internal complexity which corresponds to side scatter and is often used to analyse cell cycle progression as the lightscatters off the cell and excites the fluorescent probe to fluoresce a certain colour

emission which can then be quantitatively determined by software. In this study propidium iodide was used to bind to double stranded DNA under appropriate staining conditions. The DNA stained in this manner will emit fluorescence in direct proportion to their DNA content. The flow cytometer measures the fluorescence from each stained cell as it passes through the laser beam. Flow cytometric analysis provides quantitative data and the ability to measure large numbers of cells rapidly. Flow cytometry was used to analyse the cell cycle in the HT-29 colon cancer cells. A combination of mirrors and filters was used to separate the green (FITC) and the Propidium iodide (PI).

Method: Equal numbers of cells were seeded in 25cm² flasks and incubated at 37°C for 24hr to attach. The cells were then exposed to the different treatments (see Section 2.2.5.5) for 24hr, 48hr and 72hr intervals respectively. Cells were harvested and pelleted by centrifugation at 3500 rpm/6min. The trypsin was poured off and the cells resuspended in 1ml cold PBS and centrifuged. To achieve permeability, 3mL of ice cold 95% ethanol was added to each tube in a drop wise manner whilst vortexing each tube. The specimens were stored at -20°C overnight. The ethanol was removed by centrifugation at 3500 rpm/6min and the cells washed with 1ml PBS at 6min intervals. The sediment was resuspended in 1mL of the hypotonic DNA staining buffer, PI / RNase, and stored at 4°C protected from light. 30min before reading the specimens using a FACS Calibre flow cytometer. For each sample at least 10 000 events were collected and aggregated cells were gated out.

(RNase solution: 250mL distilled water, 0.25g Tri NaCitrate, (750µL) 0.75mL Triton X-100, 0.025g PI and 0.005g RNase A)

UNIVERSITY of the WESTERN CAPE

2.2.9 Hoechst 33342 fluorescent stain

Principle: Hoechst 33342 fluorescent stain was used to assess apoptosis in HT-29 colon cancer cells. Hoechst 33342 is a UV light excitable nucleic acid binding dye that is a cell permeant. It stains highly condensed chromatin of apoptotic cells and lightly stains the looser chromatin structure of viable cells. The apoptotic cells may take the form of crescents around the periphery of the nucleus. Hoechst 33342 is excited by ultra violet rays at around 350nm and emits blue / cyan fluorescence light around an emission maximum at 460nm. It may be used on live or fixed cells.

UNIVERSITY of the
WESTERN CAPE

Method: Cells were seeded in growth medium on heat sterilised coverslips placed in 6-well plates and was given 24h to attach after which it was exposed to SAK and SPK hydrophilic, SPK lipophilic organic extractions and CAK, CPK, SAK and SPK aqueous extractions (see Section 2.2.5.5) for 24h and 48h at a concentration of 500 µg/mL. After 24h and 48h respectively the medium was removed and 1mL of Hoechst 33342 was added and incubated at 37ºC for 30 minutes in an incubator. Coverslips were mounted onto slides. Images were captured with a Nikon fluorescence microscope. Magnification of photographs was 40x.

2.2.10 Statistical analysis

Data obtained in the quantification of morphological lesions using crystal violet were analysed for statistical significance. MedCalc statistical software version 12 (as developed by Frank Schoonjans, Mariakerke, Belgium) was used to analyse data. In MedCalc, *Level Codes* (LC) is used to break-up the (ordinal) data in one variable into different sample subgroups.

As the results appeared to have non-parametric data distributions the Kruskal-Wallis test was employed. Data from independent experiments are shown as the mean ±SD and were statistically analysed for significance using the ANOVA one-way analysis of variance, to compare the control and treated groups. Means are presented in bar charts, with Box-and-Whisker referring to standard deviations. *P-values*<0.05 are regarded as statistically significant. Annexure2 – 20 represents true ANOVA and Kruskal-Wallis analysis and not just a repetition of a means comparison. This was done to ensure that results which were shown to differ significantly also differed on the basis of a between groups and within groups analysis. The point is that two or more sets of data may differ significantly on the basis of a Student-t-test, but on the basis of variance may not be comparable (the variance within groups too large and can therefore not be compared. SDs does not necessarily take care of this).

The Box-and-Whisker plots show aspects which are difficult to tabulate or demonstrate by means of histograms or other statistical treatments. In the figures where data is represented as box-and-whisker plots, each box displays the following parameters for a given distribution: (i) median (centre line of box), (ii) second and third quartile values (25 to 75 percentile) representing the middle 50% of the values (central box), (iii) range of data excluding data points lying outside the one and a half times interquartile range (T-bars) and (iv) data points lying outside the one and a half time (plotted with a square marker) or three times the interquartile range (plotted with a round marker).

UNIVERSITY of the
WESTERN CAPE

The flow cytometric DNA analysis of cell cycle progression of HT-29 cells was performed after 24, 48 and 72 hours of exposure to 100, 500, 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic and aqueous extractions. Manual DNA analysis using a FACs Calibur DNA analysis flow cytometer was doneto establish percentage values of cells in G_1, G_2 and S phase at a minimum of 10 000 cycle events.

Chapter 3

Results

3.1 The rationale for making the extractions used to treat the HT-29 colon cancer cells

The total extract of plant material contains apart from the active compounds, an uncounted number of secondary metabolites, which may work in an antagonistic or synergistic manner and may also contain inactive compounds against the biological target under study. The rationale to fractionate the organic extract into total, lipophilic and hydrophilic fractions was to simplify the format by which the kernels may be introduced into the cells. There is a difference in the manner in which the organic (total, lipophilic and hydrophilic fractions) and aqueous extractions may exert their effects. The lipophilic fraction contains the lipid soluble compounds, while the hydrophilic fraction contains the hydrophilic compounds. Whereas, the total extract contains relative amounts of both lipid soluble and hydrophilic (polar) compounds. The compounds of each of the fractions, when used as a whole, may counteract each other's effects, or increase each other's activity and therefore the cells were exposed to the total as well as the separate lipophilic and hydrophilic extractions and the results compared.

The aqueous extractions are similar but not identical to the hydrophilic extraction as it was obtained using water and not 80% ethanol as an extraction compound. It therefore, has a greater amount of polar compounds in comparison to the hydrophilic extraction.

UNIVERSITY of the WESTERN CAPE

The various extractions may therefore provide a means to simplify the investigation of the effects of the apricot and peach kernels on HT-29 colon cancer cells, resulting in a fractional analysis of the respective kernels, at the various concentrations and time periods.

Chinese medicine practitioners may prescribe the kernels as a single herb or in a combination formula. The method of administration may be in the form of a double boil / aqueous decoction. This aspect of the study will hence also provide relevant information to the practitioner in clinic in the treatment of colon cancer.

UNIVERSITY of the
WESTERN CAPE

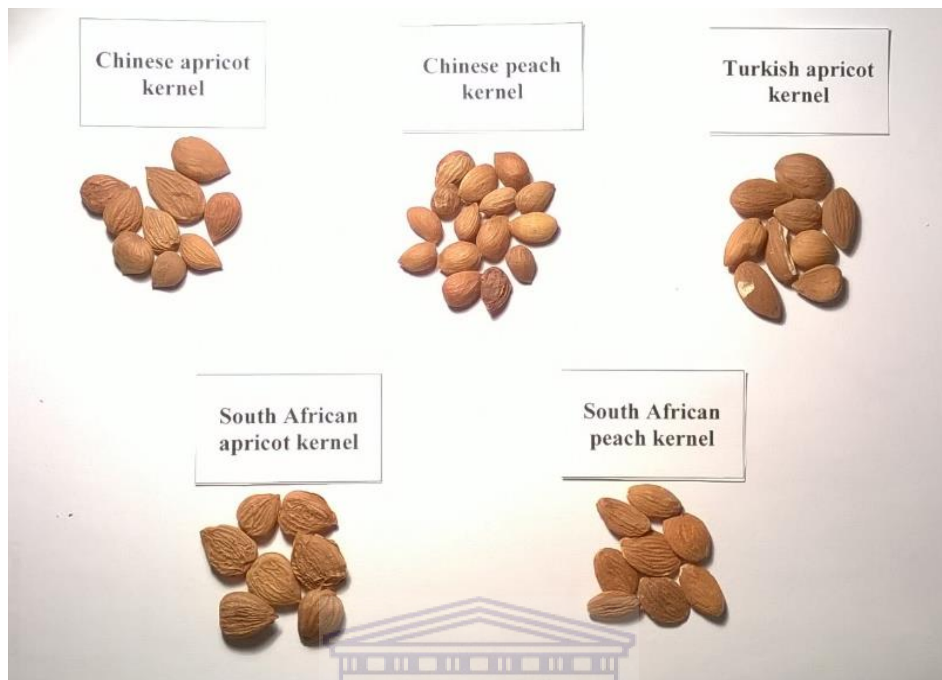
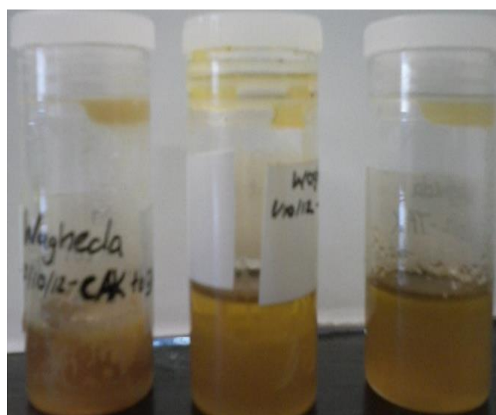


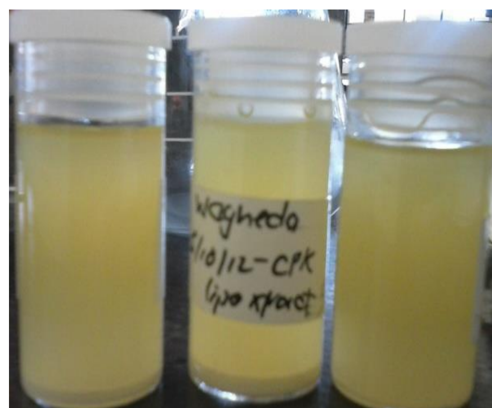
Figure 3.1: The dried Chinese apricot kernel (CAK), Chinese peach kernel (CPK), Turkish apricot kernel (TAK), South African apricot kernel (SAK) and South African peach kernel (SPK) which was removed from their respective pips.

UNIVERSITY of the
WESTERN CAPE

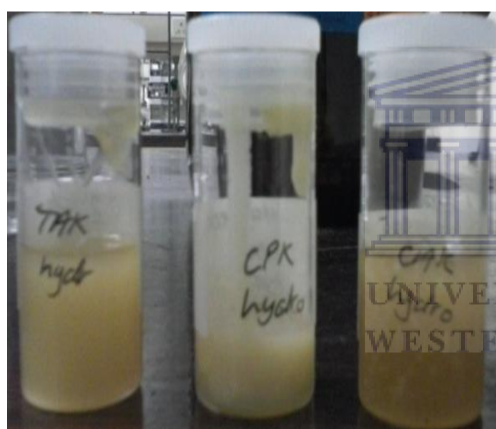
The raw, dried CAK, CPK, TAK, SAK and SPK are all light brown in colour, approximately 1cm in length and 0,5cm in width. All the kernels have a tear dropped / oval shape with mild ridges on the surface. Comparing the shape of the kernels, the CAK has a sharper edge, CPK has a rounder shape with sharp edges and TAK has rounded edges. The SAK is the biggest and widest in size and flat whilst the SPK is narrower than the SAK and flat (see Fig 3.1).



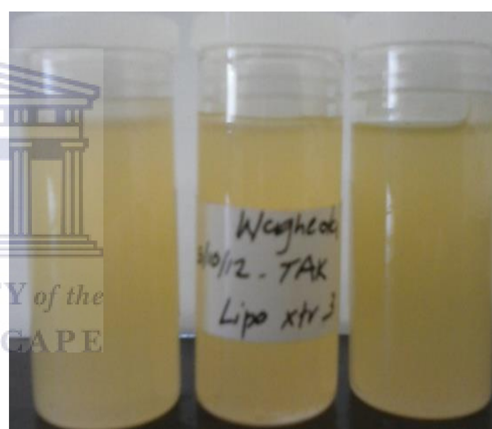
3.2A



3.2B



3.2C



3.2D

UNIVERSITY of the
WESTERN CAPE

Figure 3.2: Pictures of the end product of the CAK, CPK and TAK organic extractions. Figure 3.2A shows the oil of the CAK total fraction. Figure 3.2B shows the oil of the CPK lipophilic fraction. Figure 3.2C shows the oil of the TAK, CPK and CAK hydrophilic fractions with different consistencies. Figure 3.2D shows the oil of the TAK lipophilic fraction. See Table 3.1 for a description of the fractions.

Table 3.2 provides a general description of what the CAK, CPK, TAK, SAK and SPK organic fractions looked like after they were evaporated using a Buchirotavapor R-114 and allowed to settle at room temperature.

Table 3.1: General description of the organic fractions of CAK, CPK, TAK, SAK and SPK

Kernel Fraction	Description of Fractions
CAK-T; Chinese Apricot Kernel total extract	Brown-yellowish in colour; a creamy consistency
CAK-L; Chinese Apricot Kernel lipophilic extract	Yellowish in colour; semi-solid oil
CAK-H; Chinese Apricot Kernel hydrophilic extract	Yellowish in colour; a creamy consistency
CPK-T; Chinese Peach Kernel total extract	Light cream in colour; semi-creamy-oil consistency
CPK-L; Chinese Peach Kernel lipophilic extract	Light cream in colour, oil with fatty sedimentation
CPK-H; Chinese Peach Kernel hydrophilic extract	Light cream in colour; creamy consistency
TAK-T; Turkish Apricot Kernel total extract	Light yellow in colour; semi-cream-oil consistency
TAK-L; Turkish Apricot Kernel lipophilic extract	Light yellow in colour; oil
TAK-H; Turkish Apricot Kernel hydrophilic extract	Light yellow in colour; semi-cream-oil consistency
SAK-T; South African Apricot Kernel total extract	Dark yellow in colour; oil with fatty deposit on top
SAK-L; South African Apricot Kernel lipophilic extract	Yellow in colour; oil with fatty deposit at the bottom
SAK-H; South African Apricot Kernel hydrophilic extract	Light yellow in colour; oil
SPK-T; South African Peach Kernel total extract	Yellow in colour; liquid consistency
SPK-L; South African Peach Kernel lipophilic extract	Yellow in colour; oil with fatty deposit at bottom
SPK-H; South African Peach Kernel hydrophilic extract	Light yellow in colour; oil

UNIVERSITY of the
WESTERN CAPE

3.1.2. Expected compounds in the various extractions

According to Bensky et al (2004) the apricot and peach kernel have varying amounts of glycosides, fixed and volatile oils, with the peach kernel also containing flavanoids. The apricot and peach kernel total extraction was expected to yield relative amounts of fixed and volatile oils. The apricot and peach kernel lipophilic extraction was expected to yield a greater amount of fixed (e.g. linoleic acid) and volatile oils (e.g. benzaldehyde). The peach kernel lipophilic extraction was also expected to yield a small amount of flavanoids due to the use of acetone to perform the extraction. Trace amounts of flavanoids may be expected in the successive hydrophilic extractions which may result in dissolution.

UNIVERSITY of the
WESTERN CAPE

A percentage yield of lipophilic extractions (Table 3.2) of the various kernels achieved is related to the nutritional value. A percentage yield of 40% and more is an indication of the high nutritional value of the specific kernel. The nutritional value is the composition of the specific kernel and its extraction and its impact on the body. The low total extraction yields from the CAK, CPK and TAK (Table 3.2) could also be due to the procedure which required the evaporation of the CAK, CPK and TAK to be repeated twice as a result of a water residue. This may also be attributed to the nature of the compounds, as it is well known that lipophilic compounds are relatively difficult to be extracted using aqueous solvents. The exact chemistry and validation of the amount and types of glycosides, fixed, volatile oils and flavanoids is not within the scope of this study. The apricot and peach kernel hydrophilic extractions were expected to yield hydrophilic compounds and trace amounts of lipophilic compounds.

Table 3.2: The percentage yield of CAK, CPK, TAK, SAK and SPK organic kernel extraction fractions

Kernel type	Total Extraction* (% Yield)	Lipophilic Extraction** (% Yield)	Hydrophilic Extraction*** (% Yield)
CAK	***7.1***	40.2	10.7
CPK	***7.9***	46	7.6
TAK	***8.5***	45.6	11.1
SAK	43.3	51.1	5.7
SPK	45.2	61.3	6.6

UNIVERSITY of the
WESTERN CAPE

The total* extraction aimed at yielding relative amounts of fixed and volatile oils was performed with 80% ethanol. The lipophilic** extraction of the lipid molecules was performed with acetone, and was followed by a successive extraction of the hydrophilic*** fraction of the polar compounds with 80% ethanol. The low percentage yield of CAK, CPK and TAK total fractions is indicated in ***bold and italics***.

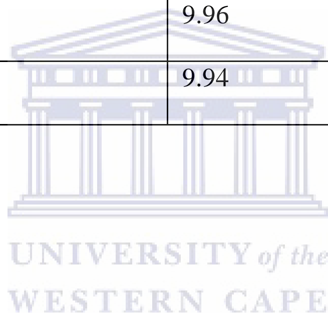
In contrast to the organic extraction which yielded oil, the aqueous extraction yield was a white-yellowish powder after the aqueous extraction was freeze dried using a bench top freeze dryer.

Aqueous extractions (Table 3.3) is said to have a greater amount of polar compounds (e.g. salts and carbohydrates) and a smaller amount of non-polar compounds. The apricot and peach kernel aqueous extractions are expected to yield a greater amount of glycosides,

including amygdalin and prunasin, salts, and relatively smaller amounts of fixed and volatile oils.

Table 3.3: Dry weight in grams of CAK, CPK, SAK, SPK powder of freeze dried aqueous kernel extraction fractions

Kernel Type	Dry Weight (g)
CAK	9.82
CPK	9.90
SAK	9.96
SPK	9.94


UNIVERSITY of the
WESTERN CAPE

The following data represents the results after the HT-29 colon cancer cells were exposed at 24, 48 and 72 hours to concentrations of 100, 500 and 1000 µg/mL of organic (total, lipophilic and hydrophilic) and aqueous extractions of the Chinese apricot kernel (CAK), Chinese peach kernel (CPK), Turkish apricot kernel (TAK), South African apricot kernel (SAK) and South African peach kernel (SPK). The Turkish peach kernel and the Turkish aqueous extract were not available at the time of the experiments.

3.2 Cell Viability

3.2.1 Crystal violet method studying the effects of CAK, CPK, TAK, SAK and SPK organic and CAK, CPK, SAK and SPK aqueous extractions on the growth of HT-29 colon cancer cells

The crystal violet staining method was used to determine the cell proliferation of HT-29 colon cancer cells after exposure to CAK, CPK, TAK, SAK and SPK organic and aqueous extractions at concentrations of 100, 500 and 1000 µg/mL for 24, 48 and 72 hours. These experiments were done in triplicate. Absorbance values of samples (LC) were read at 570nm using a Glomax spectrophotometer. See Annexure 3 to 21 for statistical analysis reports. In the figures colour coding is used to depict the control and various sample fractions: yellow (control), green (total fractions), red (lipophilic fractions) and blue (hydrophilic fractions).

UNIVERSITY of the
WESTERN CAPE

Quantification of the effects of the various organic extracts of the **Chinese apricot kernel** on cell proliferation of HT-29 colon cancer cells is shown in figures 3.3 – 3.5. The study showed that after 24 and 48 hours at concentrations of 100, 500 and 1000 µg/mL all the fractions inhibited cell proliferation. Whereas, after 72 hours (Fig 3.5) the cells show a recovery in cell viability and cell proliferation as no significant difference between the control and these treated samples was observed. See Annexure 3 – 5 for full Kruskal Wallis test report.

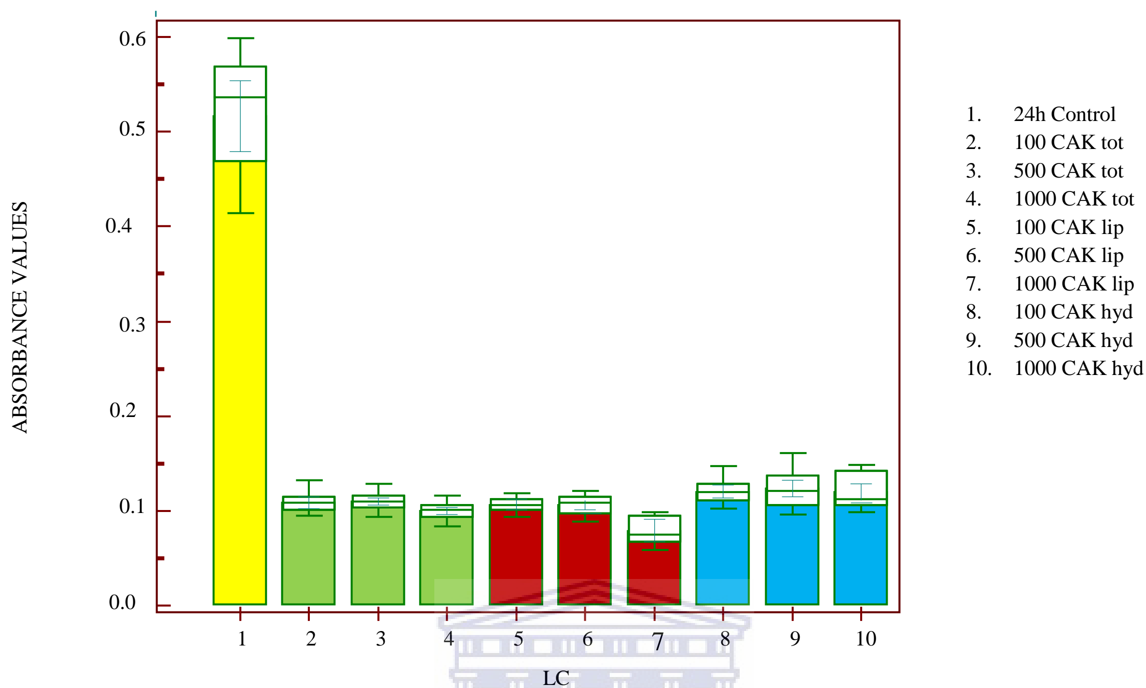


Fig 3.3: 24h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL Chinese apricot kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 1000 µg/mL CAK lip (LC7) showed the most outspoken growth inhibition (±20%) when compared to the control

UNIVERSITY of the
WESTERN CAPE

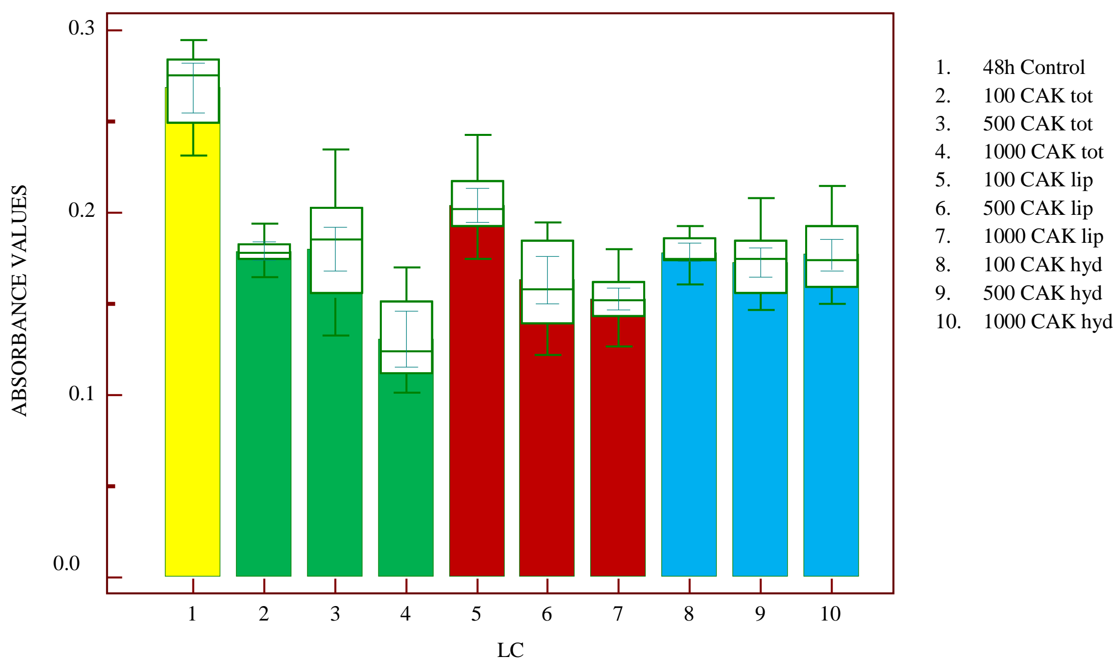


Fig 3.4: 48h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL Chinese apricot kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 1000 µg/mL CAK total (LC4) showed the most outspoken growth inhibition (±40%) when compared to the control

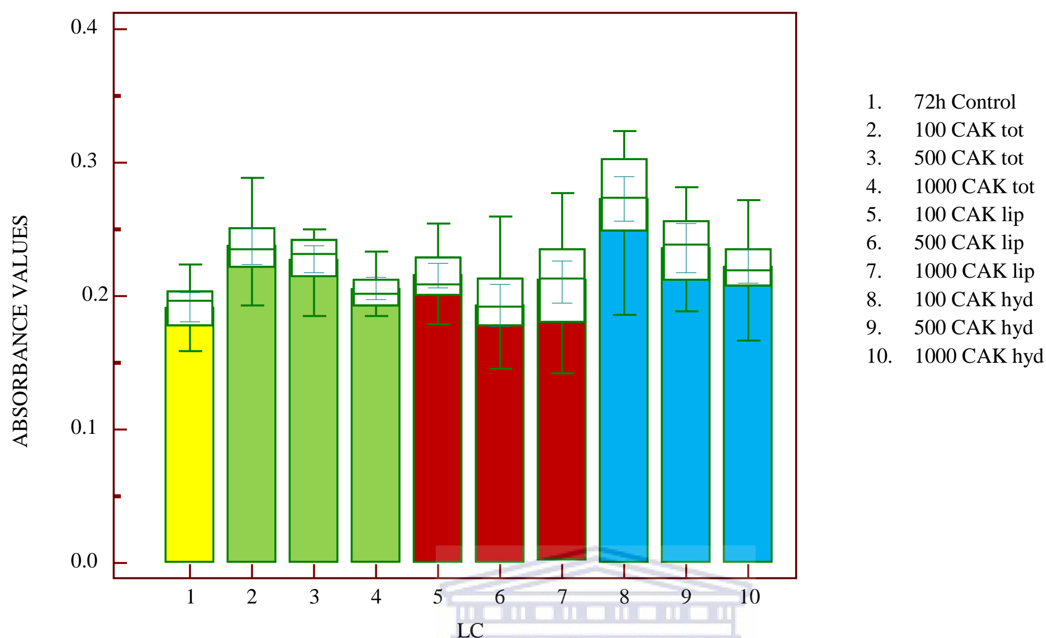


Fig 3.5: 72h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL Chinese apricot kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 100 µg/mL CAK hyd (LC8) showed the most outspoken increase in cell proliferation indicating a recovery in cell growth

UNIVERSITY of the
WESTERN CAPE

CAK treated samples in figures 3.3 – 3.5 differed from the control with a significant P-value of $p<0.001$. Whereas, samples differed from each other showing a significant P-value of $p<0.05$ (see Annexure 3 – 5 for full Kruskal Wallis test report).

At 24 hours all CAK treated samples differed from the control (Absorbance (A) = 0,575). The CAK fractions inhibited cell growth to approximately 20% to that of the control with the 1000 µg/mL CAK lipophilic extract (Fig 3.3, LC7) having the most outspoken growth inhibitory effect.

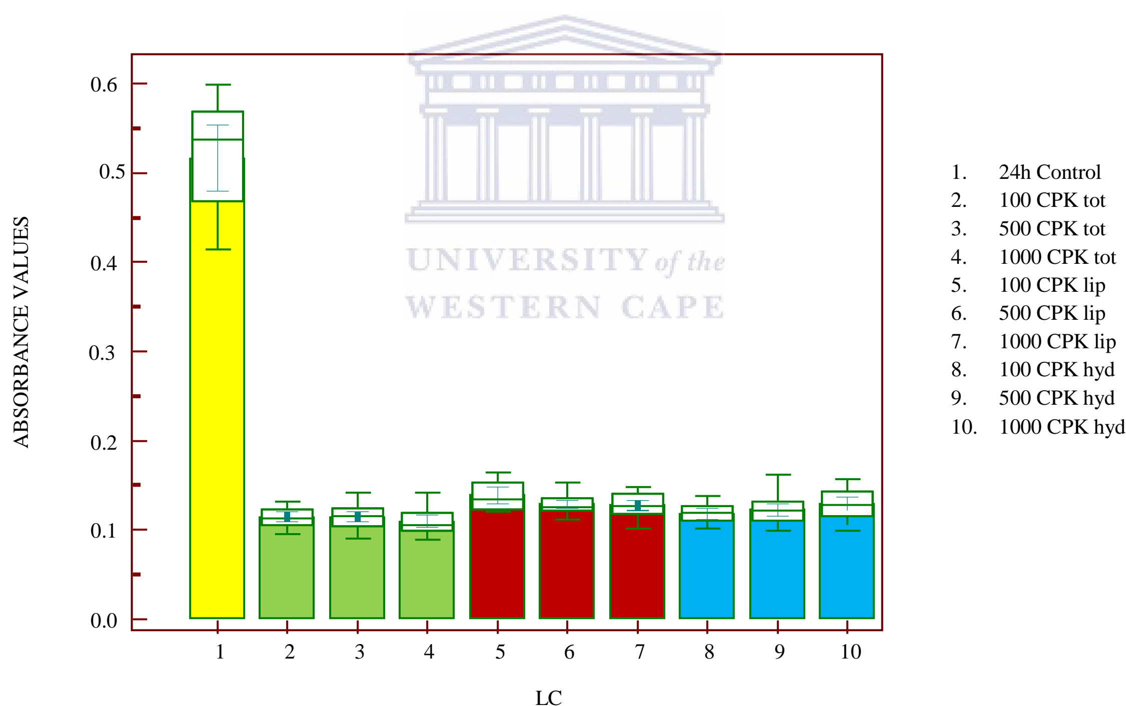
At 48 hours all CAK treated samples differed from the control (A = 0,287). The 1000 µg/mL CAK total extract (Fig 3.4, LC4) showed the greatest inhibitory effect on HT-29 colon cancer cell proliferation followed by the 1000 µg/mL CAK lipophilic extract (Fig 3.4, LC7).

No inhibitory effect was seen after 72 hours CAK exposure. Instead some treated samples showed an increase in cell proliferation (Fig 3.5, LC2, 3, 8 and 9) indicative of a recovery in cell proliferation in the HT-29 colon cancer cells.

In summary, the 24h1000 µg/mL CAK lipophilic extract (approximately 80% decrease) showed the greatest growth inhibitory effect, and after 48 hours the 1000 µg/mL CAK total extract (approximately 40% to that of the control) showed an outspoken inhibitory effect on cell proliferation.

UNIVERSITY of the
WESTERN CAPE

Quantification of the effects of the various organic extracts of the **Chinese peach kernel** on cell proliferation of HT-29 colon cancer cells is shown in figures 3.6 – 3.8. The study showed that after 24 and 48 hours at concentrations of 500 and 1000 µg/mL there was significant inhibition of cell proliferation. Whereas, after 72 hours at concentrations of 100, 500 and 1000 µg/mL the growth inhibitory effect was overcome and the cells show a recovery in cell proliferation.



UNIVERSITY of the WESTERN CAPE

Fig 3.6: 24h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL Chinese peach kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 1000 µg/mL CPK total (LC4) showed the most outspoken growth inhibition (±20%) when compared to the control

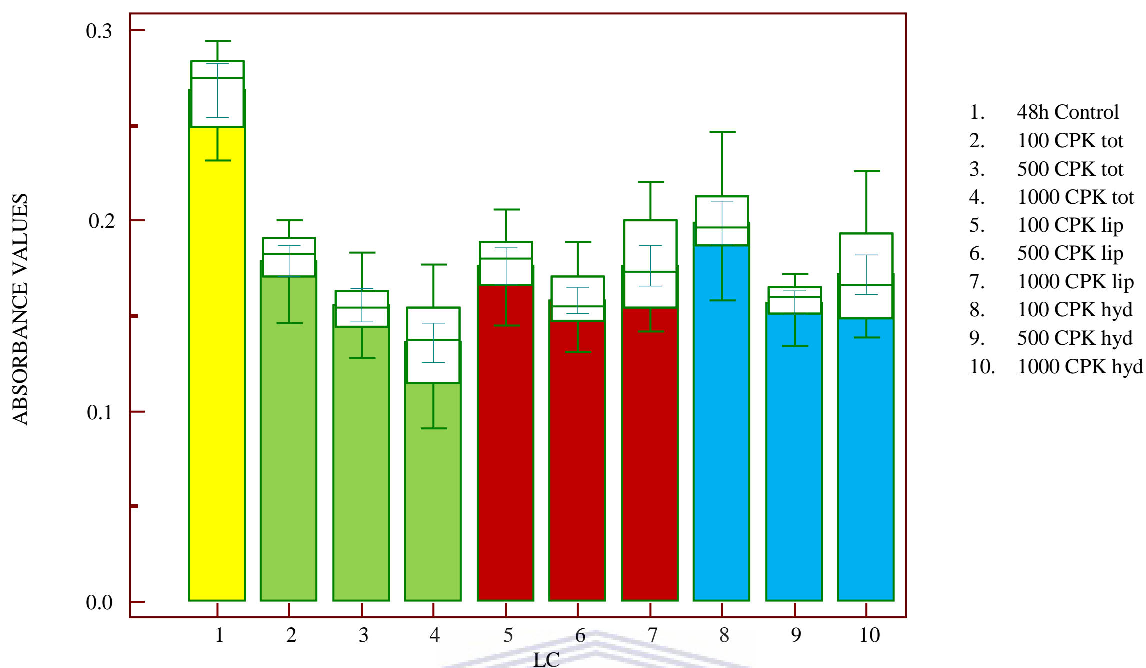


Fig 3.7: 48h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL Chinese peach kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 1000 µg/mL CPK total (LC4) showed the most outspoken growth inhibition (±40%) when compared to the control

UNIVERSITY of the
WESTERN CAPE

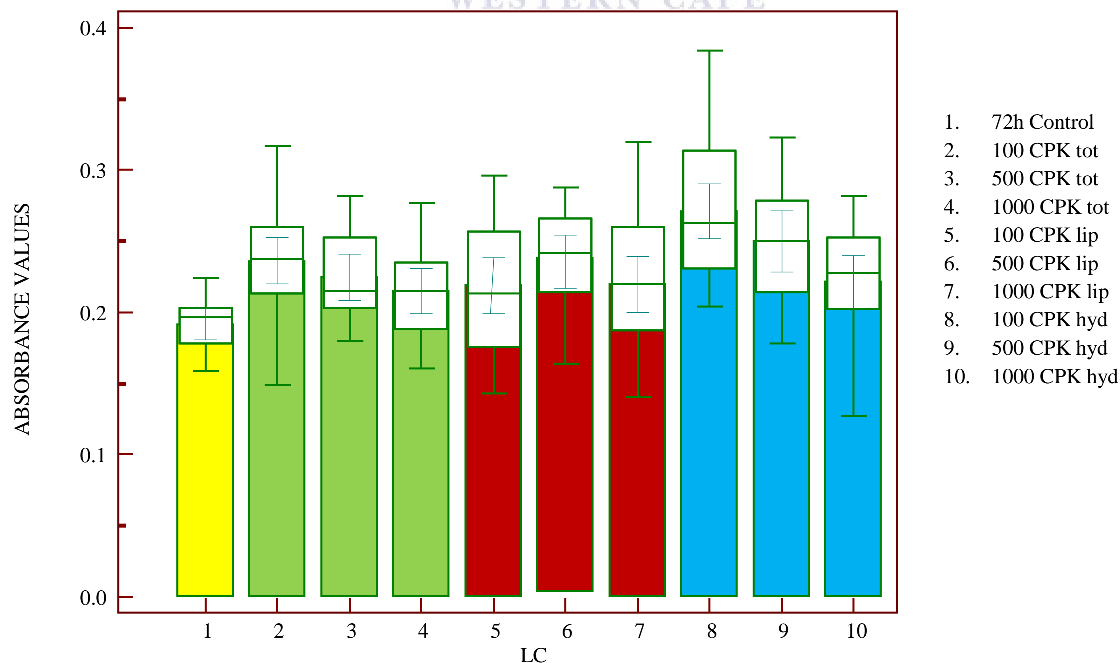


Fig 3.8: 72h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL Chinese peach kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 100 µg/mL CPK hyd (LC8) showed the most outspoken increase in cell proliferation indicating a recovery in cell growth

CPK treated samples differed from the control with a significant P-value of $p<0.001$. Whereas, samples differed from each other showing a significant P-value of $p<0.05$ (see Annexure 6 – 8 for full Kruskal Wallis test report).

At 24 hours all treated samples differed from the control (A = 0,575). The 1000 µg/mL CPK total extract (Fig 3.6, LC4) induced the most outspoken growth inhibition by approximately 20% to that of the control.

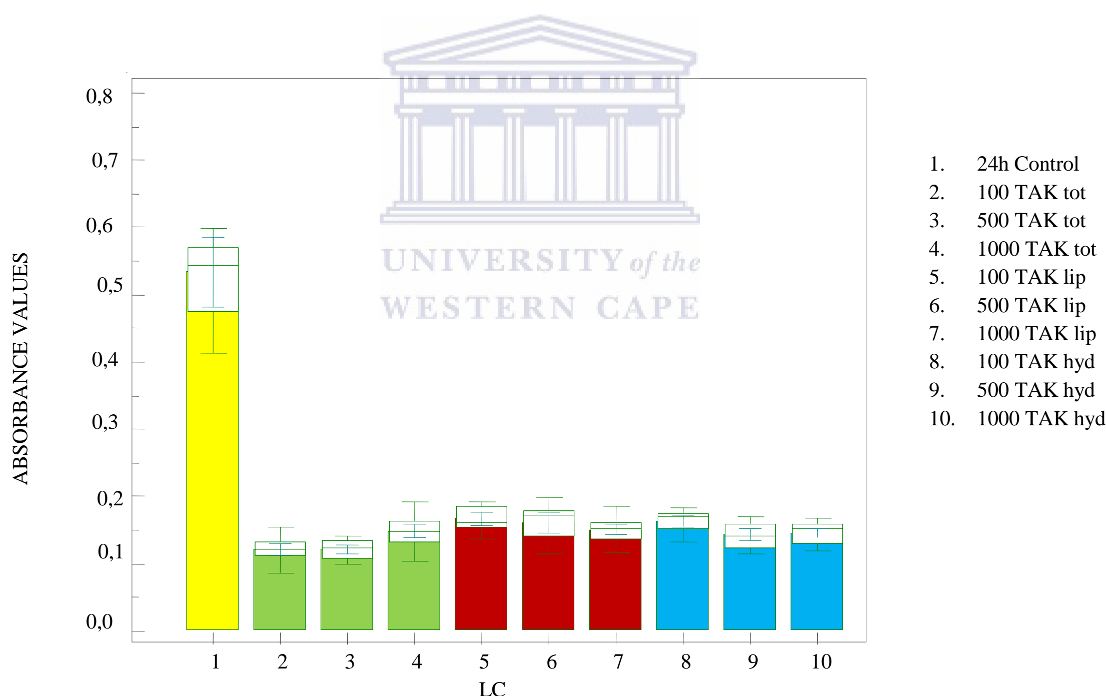
At 48 hours all treated samples differed from the control (A = 0,287). After 48 hours the 1000 µg/mL total extract (approximately 40% to that of the control) showed the greatest inhibitory effect (Fig 3.7, LC4). Followed by 500 µg/mL total extract which also showed a high inhibitory effect (Fig 3.7, LC3).

UNIVERSITY of the
WESTERN CAPE

At 72 hours no growth inhibition was observed which could be seen as an indicator of cell growth recovery. The 1000 µg/mL CPK hydrophilic extract did show the most outspoken increase in cell proliferation (Fig 3.8, LC8).

In summary, the CPK extracts which showed the greatest growth inhibitory effect was at 24h 1000 µg/mL total and after 48 hours at a concentration of 1000 µg/mL total extract. After 72 hours it would appear that the initial inhibitory effect of the CPK was abrogated and the cells resumed proliferation to an even greater extent than before the treatment.

Quantification of the effects of the various organic extracts of the **Turkish apricot kernel** on cell proliferation of HT-29 colon cancer cells is shown in figures 3.9 – 3.11. The study showed that after 24 and 48 hours at concentrations of 100, 500 and 1000 µg/mL there was significant inhibition of cell proliferation. Whereas, after 72 hours at concentrations of 100, 500 and 1000 µg/mL the growth inhibitory effect was overcome and the cells show a recovery in cell proliferation (see Kruskal Wallis for full test report - Annexure 9 – 11).



UNIVERSITY of the
WESTERN CAPE

Fig 3.9: 24h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL Turkish apricot kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 500 µg/mL TAK total (LC3) showed the most outspoken growth inhibition (±20%) when compared to the control

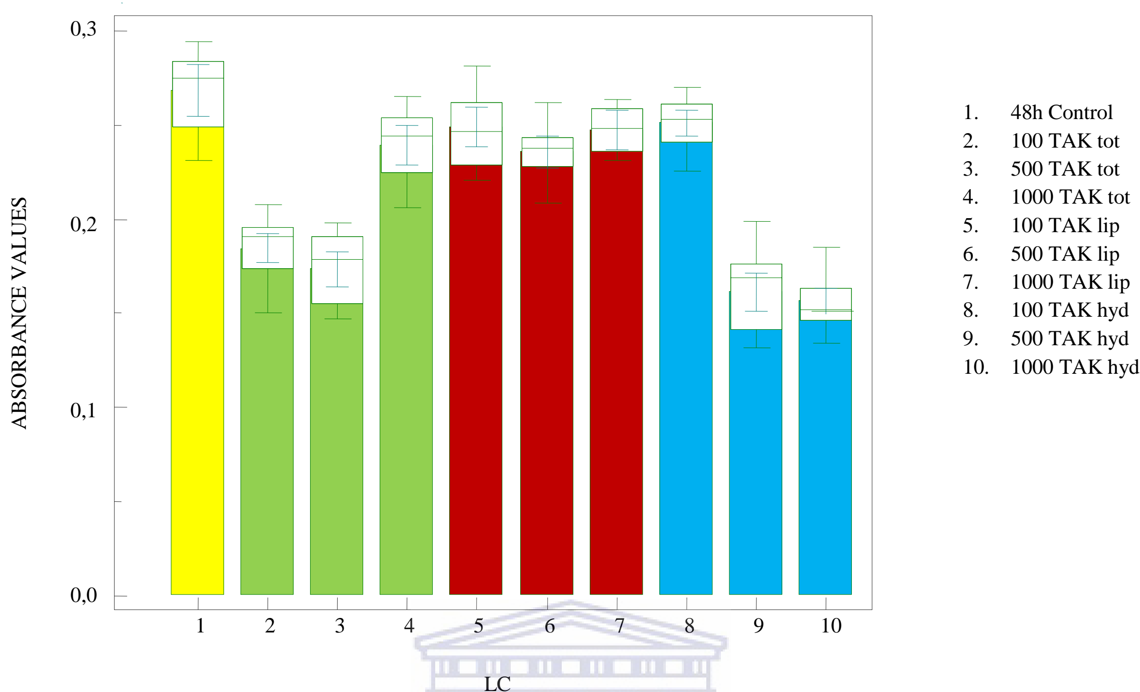


Fig 3.10: 48h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500, 1000 µg/mL Turkish apricot kernel organic extractions P<0,001). Samples differed between each other (p<0.05). 1000 µg/mL TAK hyd (LC10) showed the most outspoken growth inhibition (±56%) and 100 µg/mL TAK total (LC5) & 100 µg/mL TAK hyd (LC8) the most outspoken recovery in growth

UNIVERSITY of the
WESTERN CAPE

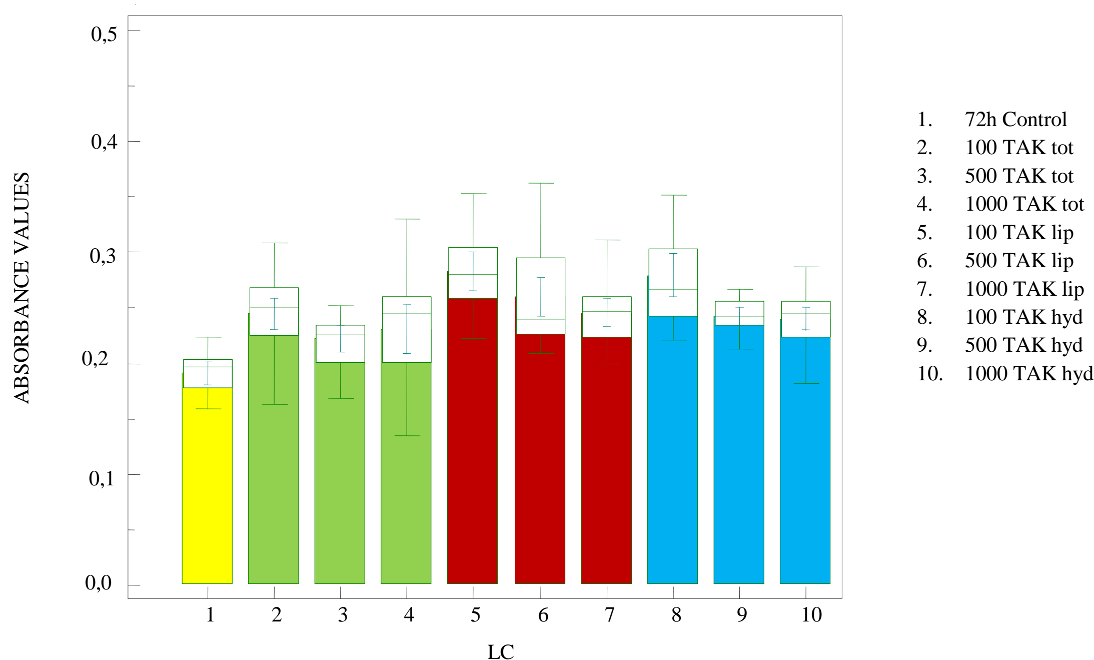


Fig 3.11: 72h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL Turkish apricot kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 100 µg/mL TAK lip & hyd (LC5 & LC8) showed the most outspoken increase in cell proliferation indicating a recovery in cell growth

The TAK treated samples differed from the control with a significant P-value of $p<0.001$. Whereas, samples differed from each other showing a significant P-value of $p<0.05$ (see Annexure 9 – 11 for full Kruskal Wallis test report).

At 24 hours all TAK treated samples differed from the control (A = 0,575). The 500 µg/mL total TAK extract showed a significant inhibitory effect on cell proliferation of the HT-29 colon cancer cells by approximately 20% to that of the control (Fig 3.9, LC3). This was followed by the 100 µg/mL total extract (Fig 3.9, LC2).

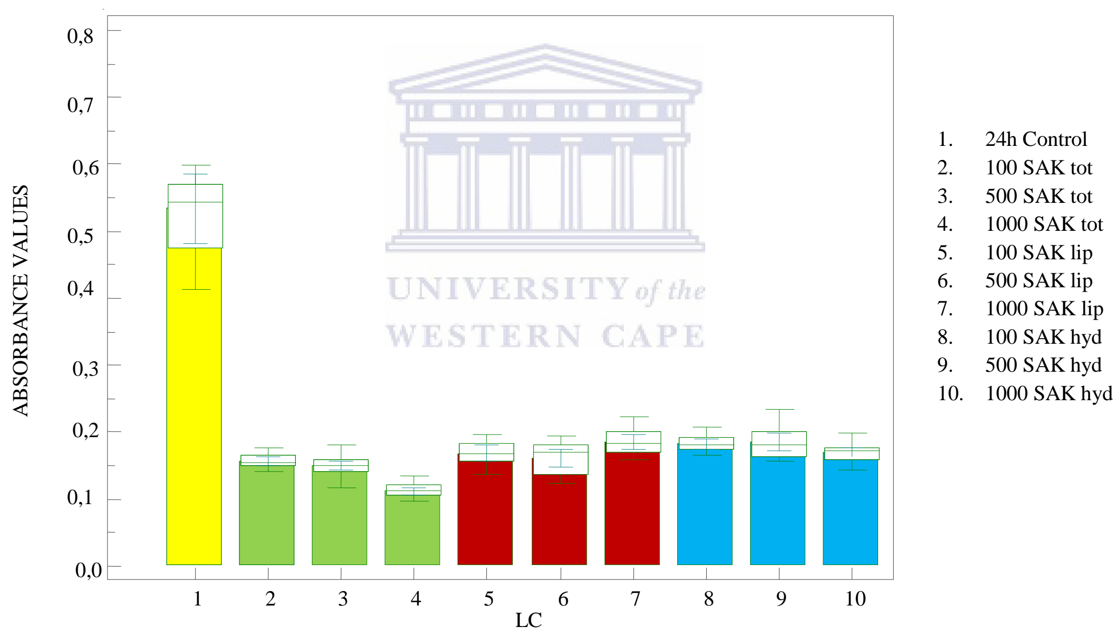
At 48 hours all TAK treated samples differed from the control (A = 0,287). The sample treated with 1000 µg/mL hydrophilic extract (Fig 3.10, LC10; Kruskal Wallis report Annexure 10) showed a significant growth inhibitory effect by approximately 56% to that of the control. However, there was an outspoken increase in cell proliferation of cells treated with 100 µg/mL TAK lipophilic and hydrophilic extract (Fig 3.10, LC5 and LC8 respectively).

UNIVERSITY of the
WESTERN CAPE

At 72 hours the 100, 500 and 1000 µg/mL treated samples showed no effect on growth inhibition on the HT-29 colon cancer cells which may indicate cell growth recovery (Fig 3.11). In fact, samples treated with 100 µg/mL TAK lipophilic and hydrophilic extracts once again showed an outspoken increase in cell proliferation.

In summary, the 24h100 µg/mL total TAK extract and 48h1000 µg/mL hydrophilic extract showed a significant growth inhibitory effect with no inhibitory effect seen at 72 hours.

Quantification of the effects of the various organic extracts of the **South African apricot kernel** on cell proliferation of HT-29 colon cancer cells is shown in figures 3.12 – 3.14. The study showed that after 24 and 48 hours at concentrations of 500 and 1000 µg/mL there was inhibition of cell proliferation. Whereas, after 72 hours at concentrations of 100, 500 and 1000 µg/mL total, lipophilic and hydrophilic extracts no significant inhibitory effect was observed (see Kruskal Wallis test report Annexure 14).



UNIVERSITY of the
WESTERN CAPE

Fig 3.12: 24h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL South African apricot kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 1000 µg/mL SAK total (LC4) showed the most outspoken growth inhibition (±30%) when compared to the control

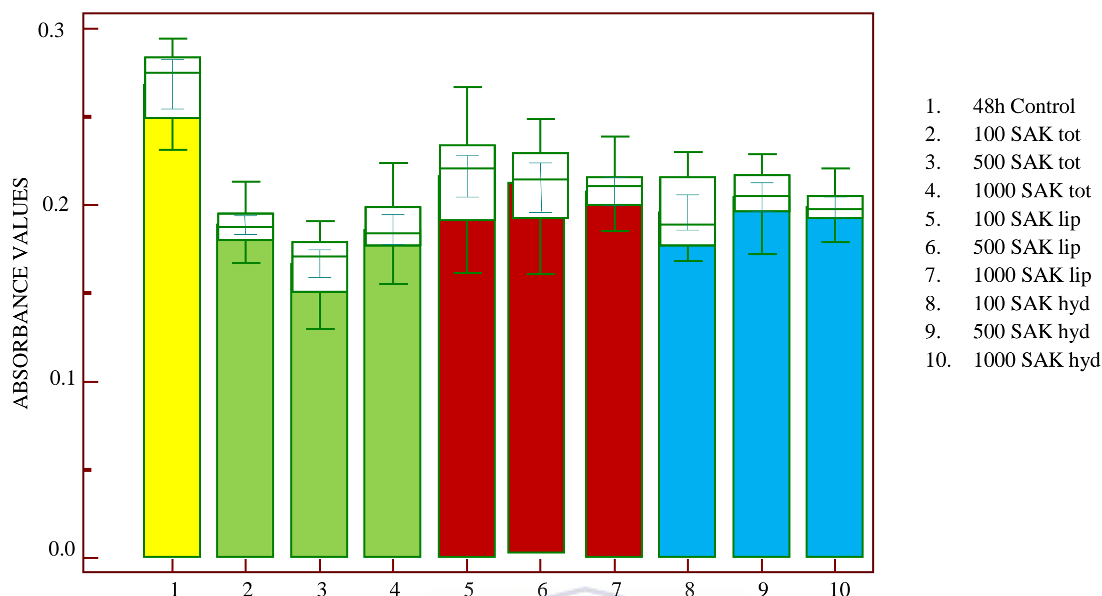


Fig 3.13: 48h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL South African apricot kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 500 µg/mL SAK total (LC3) showed the most outspoken growth inhibition (±60%) when compared to the control

UNIVERSITY of the
WESTERN CAPE

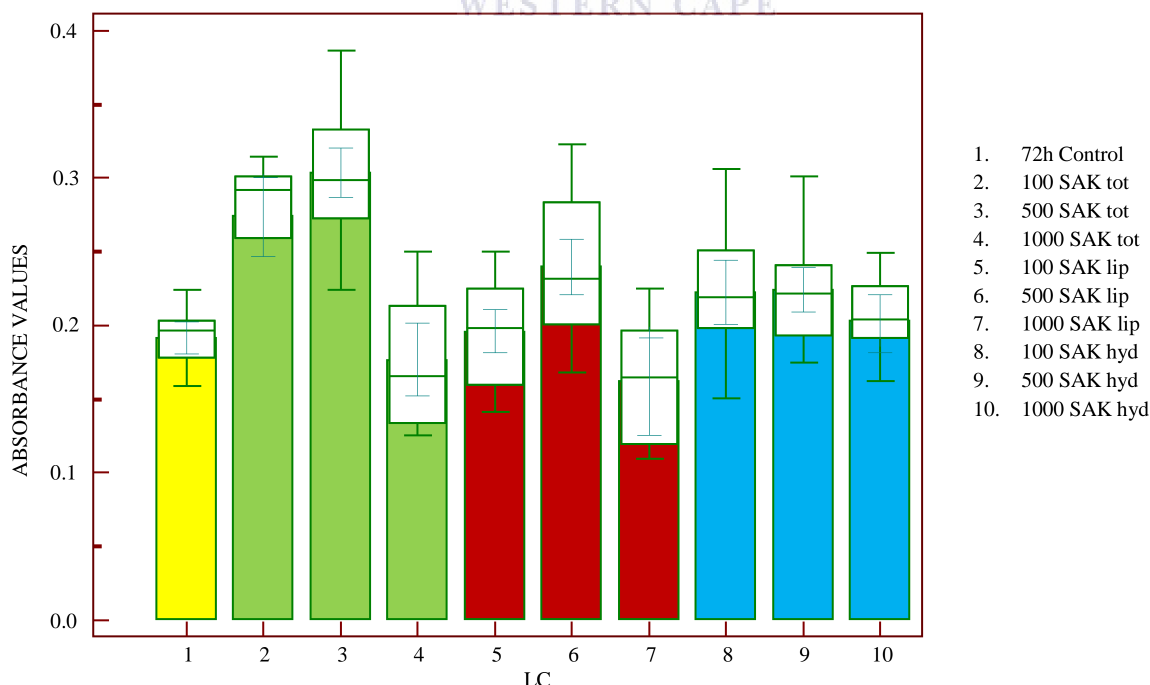


Fig 3.14: 72h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL South African apricot kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 500 µg/mL SAK total (LC3) shows the most outspoken increase in cell proliferation indicating a recovery in cell growth

The SAK treated samples differed from the control with a significant P-value of $p<0.001$. Whereas, samples differed from each other showing a significant P-value of $p<0.05$ (see Annexure 12 – 14 for full Kruskal Wallis test report).

At 24 hours all the SAK treated samples differed from the control (A = 0,575). The 24h1000 µg/mL total extract (Fig 3.12, LC4; Annexure 12) showed the most outspoken growth inhibitory effect by approximately 30% to that of the control.

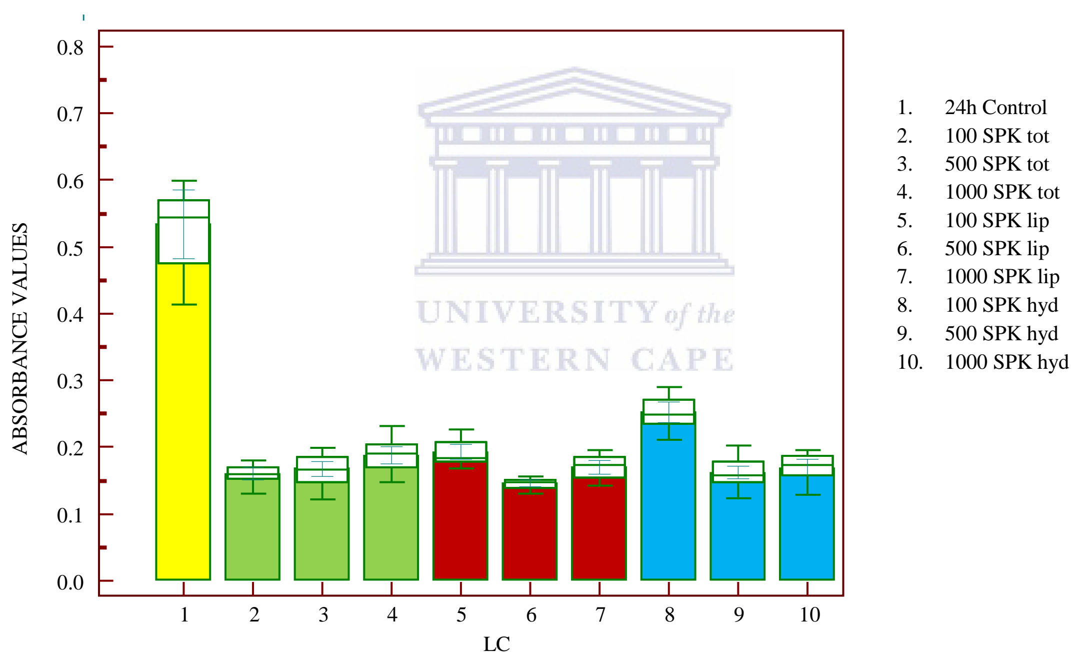
At 48 hours all the SAK treated samples differed from the control (A = 0.287). The 500 µg/mL total extract treated sample (Fig 3.13, LC3; Annexure 13) showed the most significant inhibition in cell proliferation by approximately 60% to that of the control.

At 72 hours (Fig.3.14; Annexure 14) no growth inhibitory effect was shown for the 100, 500 and 1000 µg/mL extracts. In fact the 500 µg/mL SAK total extract (LC3) showed an outspoken increase in cell proliferation (Fig 3.13).

UNIVERSITY of the
WESTERN CAPE

In summary, at 24 hours the 1000 µg/mL SAK total extract showed the most outspoken growth inhibitory effect. At 48 hours the 500 µg/mL total extract showed the most outspoken inhibition on cell proliferation and subsequently showed the most outspoken increase in cell proliferation at 72 hours. After 72 hours it would appear that the initial inhibitory effect of the SAK was abrogated and the cells recovered inducing cell proliferation to an even greater extent than before the treatment with SAK.

Quantification of the effects of the various organic extracts of the **South African peach kernel** on cell proliferation of HT-29 colon cancer cells is shown in figures 3.15 – 3.17. The study showed that after 24 hours and 48 hours there was significant inhibition of cell proliferation. Whereas, at 72 hours no inhibition in cell growth was shown which is indicative of a recovery in cell proliferation.



UNIVERSITY of the
WESTERN CAPE

Fig 3.15: 24h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL South African peach kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 500 µg/mL SAK lip (LC6) showed the most outspoken growth inhibition (±30%) when compared to the control

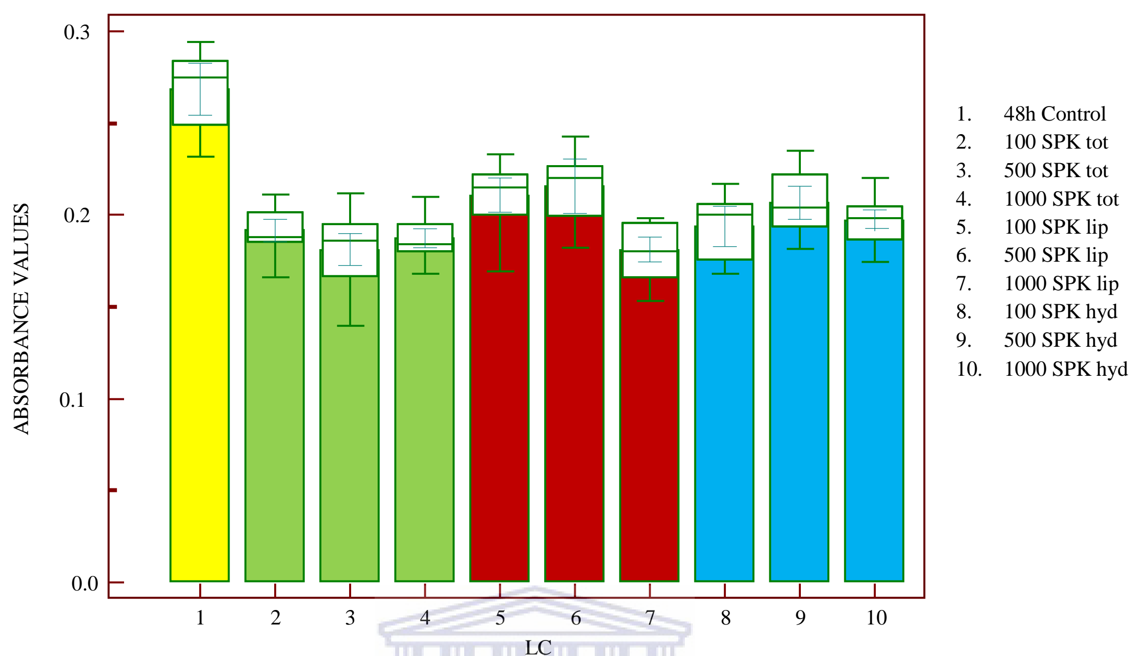


Fig 3.16: 48h Box-and-whisker plot illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL South African peach kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). 500 µg/mL SPK total (LC3) & 1000 µg/mL SPK lip (LC7) showed the most outspoken growth inhibition (±60%) when compared to the control

UNIVERSITY of the
WESTERN CAPE

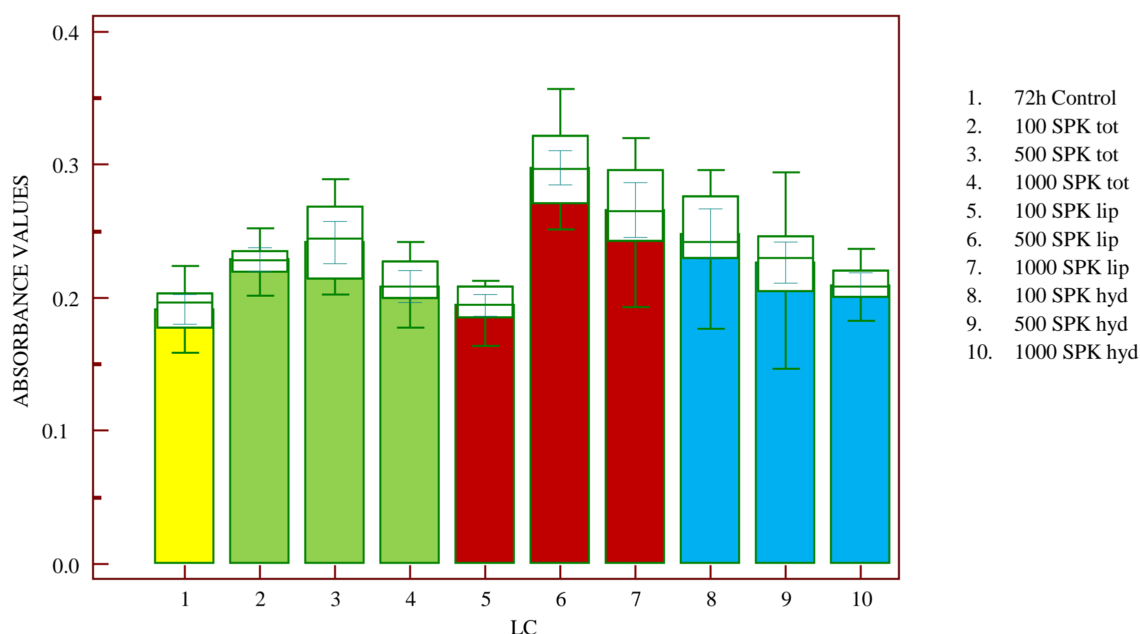


Fig 3.17: 72h Box-and-whisker plots illustrating the growth of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL South African peach kernel organic extractions (P<0,001). Samples differed between each other (p<0.05). No significant growth inhibition was shown. 500 µg/mL SPK lip (LC6) shows an increase in cell proliferation indicating a recovery in cell growth

The SPK treated samples differed from the control with a significant P-value of $p<0.001$. Whereas, samples differed from each other showing a significant P-value of $p<0.05$ (see Annexure 15 – 17 for full Kruskal Wallis test report).

At 24 hours all the SPK treated samples differed from the control (A = 0,575). The 500 µg/mL lipophilic extract (approximately 30% to that of the control) followed by 100 µg/mL total extract showed the most outspoken growth inhibitory effect (Fig 3.15, LC6 and LC2 respectively; Annexure 15).

At 48 hours all the SPK treated samples differed from the control (A = 0,287). The most outspoken inhibition on cell proliferation was seen with the 1000 µg/mL lipophilic extracts followed by the 500 µg/mL total extract (Fig 3.16, LC7 and LC3 respectively) by approximately 60% respectively to that of the control.

UNIVERSITY of the
WESTERN CAPE

At 72 hours no inhibition on cell proliferation was shown. However, 500 µg/mL and 1000 µg/mL lipophilic extracts showed an increase in cell proliferation which may be an indication of cell growth recovery (Fig 3.17, LC6 and LC7; Annexure 17).

In summary, the 24h500 µg/mL lipophilic extract showed the most significant growth inhibitory effect. While after 48 hours and at a concentration of 500 µg/mL, the total extract showed the most significant inhibition on cell proliferation.

Figs 3.18 – 3.20 compares all the organic kernel extractions after 24, 48 and 72 hours exposure. The organic extract treated samples differed from the control with a significant P-value of $p<0.001$. Whereas, samples differed from each other showing a significant P-value of $p<0.05$ (see Annexure 18 – 20 for full Kruskal Wallis test report).

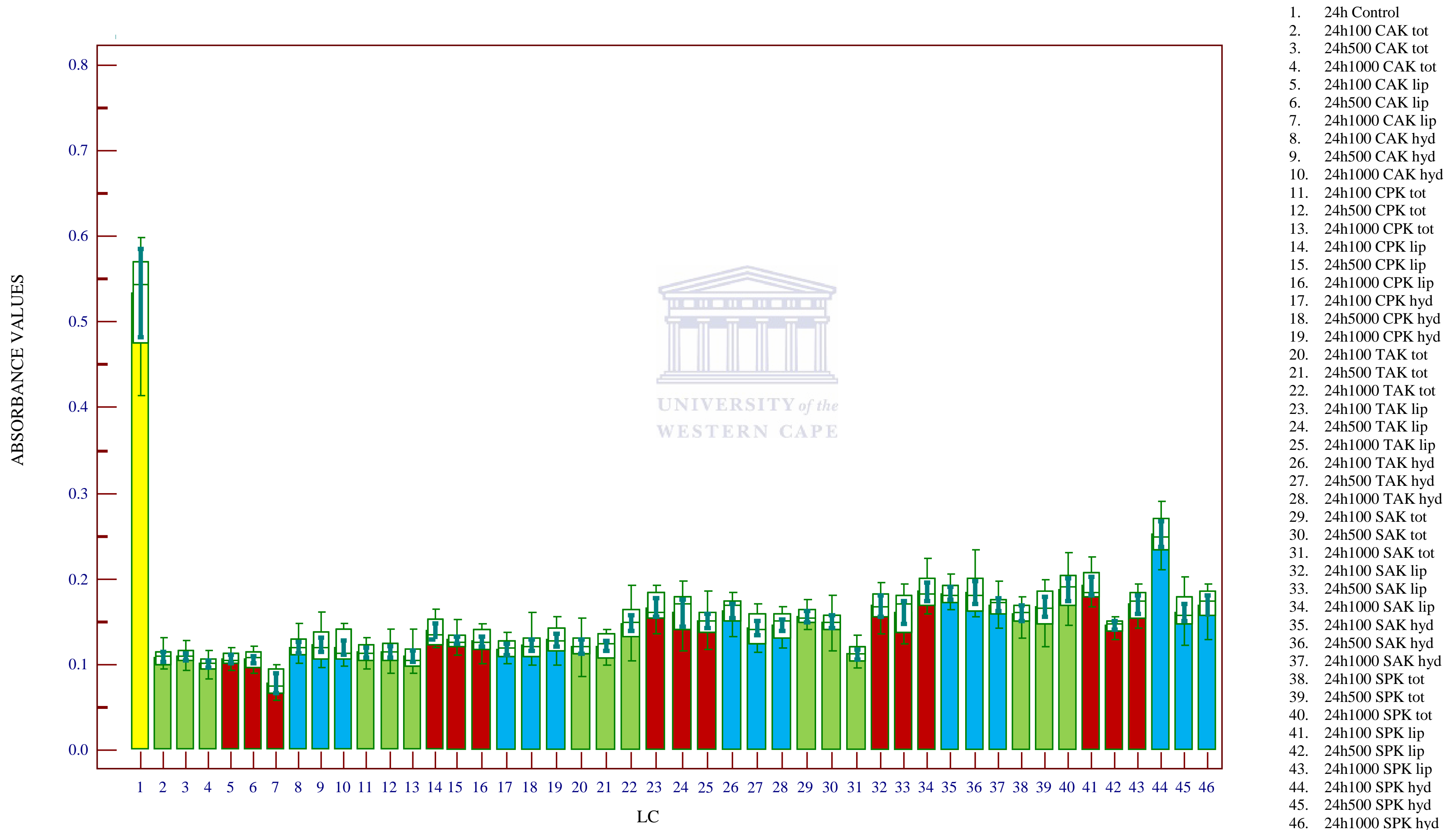
A significant decrease in the cell growth of the HT-29 colon cancer cells by all organic extractions after 24 hours was observed. After 24 hours, the overall most outspoken growth inhibitory effect by ±20% to that of the control was induced by the 1000 µg/mL CAK lipophilic extract followed by the 1000 µg/mL CAK total extract (Fig 3.18, LC7 and LC4 respectively) (see Kruskal Wallis test result for full report – Annexure 18 – 20).

After 48 hours the greatest growth inhibitory effect by approximately 40% to that of the control was seen in the cells treated with 1000 µg/mL CAK total extract followed by 1000 µg/mL CPK total extract (Fig 3.19, LC4 and LC13 respectively). The most outspoken recovery in cell proliferation after 48 hours was seen in samples treated with 100 µg/mL TAK lipophilic and hydrophilic extracts (Fig 3.19, LC23 and LC26 respectively). Growth inhibition was thus not to the same extent as observed after 24 hours.

After 72 hours a slight inhibition of cell proliferation was still seen with the 1000 µg/mL SAK lipophilic extract (Fig 3.20, LC34) whilst the most outspoken recovery in cell proliferation was shown in the sample treated with 500 µg/mL SAK total extract (Fig 3.20, LC30). No indication of any recovery effect was observed after 24 hours. Over time the treatment lost the ability to inhibit cell growth seemingly resulting in a recovery, thus the inhibitory effect was not cytotoxic at all in either of the kernels or fractions used to treat the cells.

There was an initial overall decrease in cell proliferation of approximately 80% of the HT-29 colon cancer cells at 24 hours and approximately 40% overall inhibition of cell growth after 48 hours and a further possible recovery in cell proliferation after 72 hours. This may indicate that in order for a sustained effect to be achieved that an individual patient may have to consume the kernel extracts on a daily basis.

UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

Fig 3.18: 24h Box-and-whisker plot illustrating the growth inhibition of HT-29 colon cancer cell after exposure to 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions (P<0,001). Samples differed between each other (p<0.05). 1000 µg/mL CAK total and lip extracts showed overall most outspoken inhibition on cell proliferation (LC7 & LC4) at 24 hours (±20%).

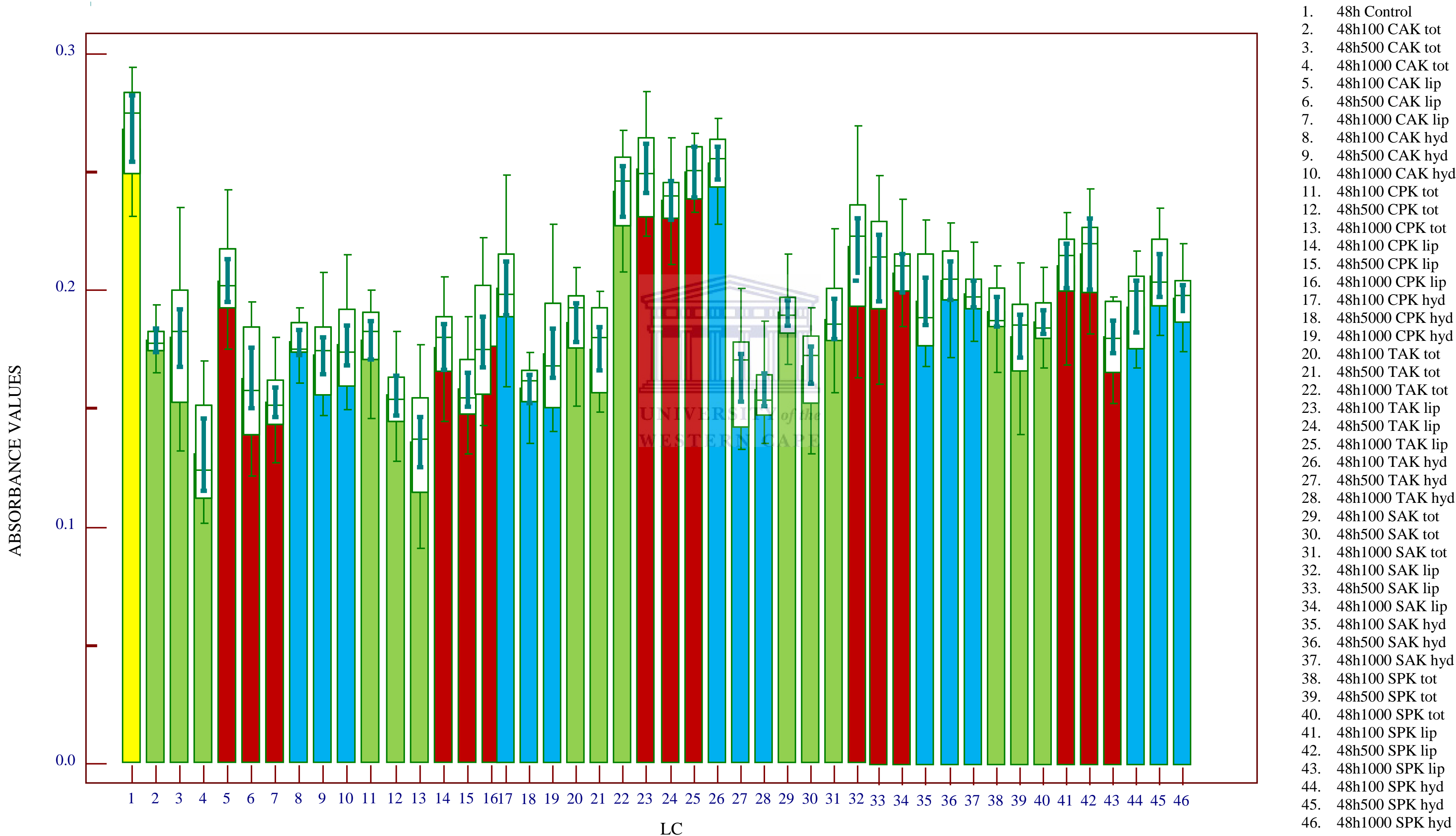


Fig 3.19: 48h Box-and-whisker plot illustrating the growth inhibition of HT-29 colon cancer cell exposure to 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions (P<0,001). Samples differed between each other (p<0.05). 1000 µg/mL CAK and CPK total extracts showed the most outspoken overall inhibition on cell proliferation (LC4 & LC13). 100 µg/mL TAK lip & hyd (LC23 & 26) showed most outspoken growth recovery

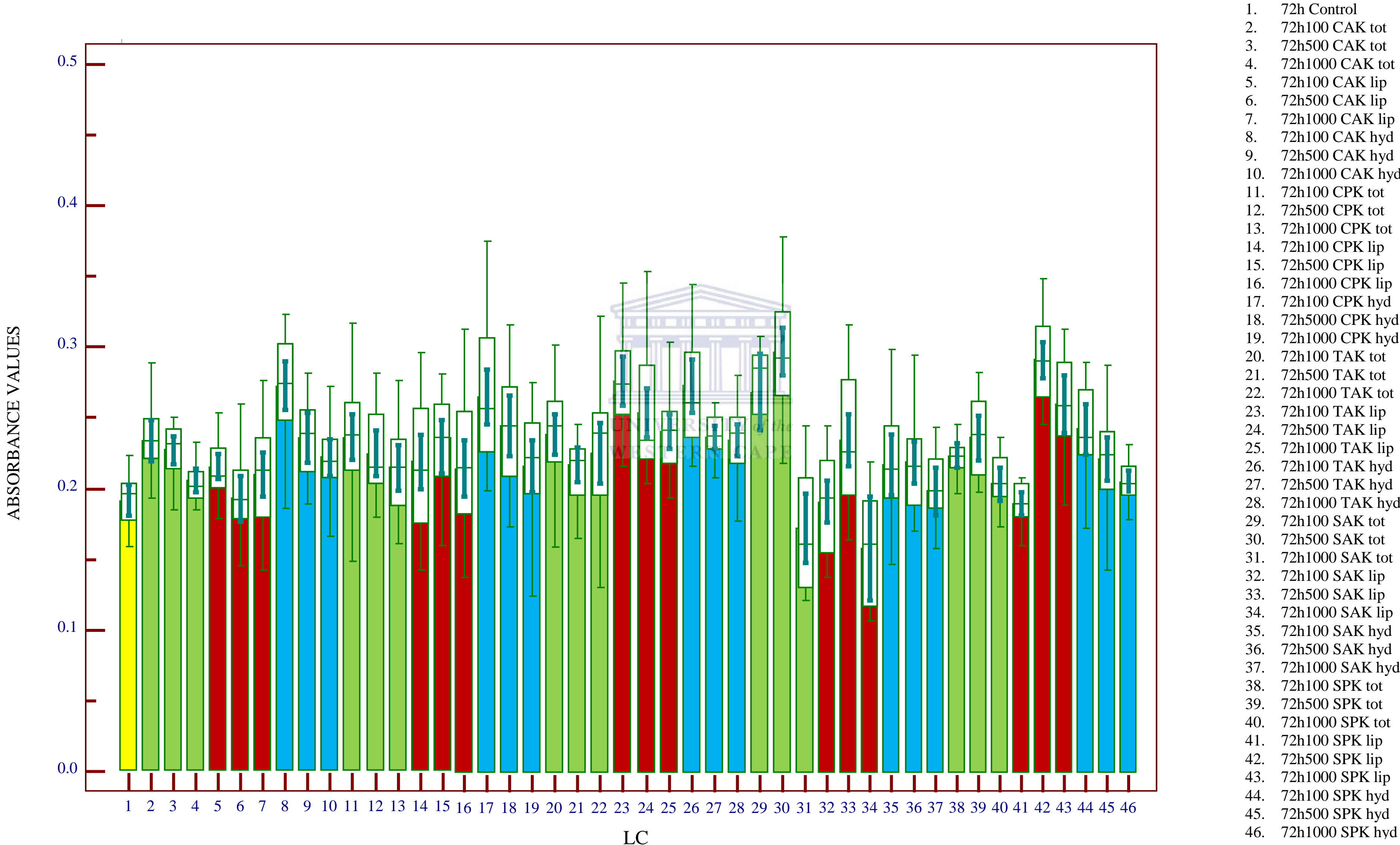


Fig 3.20: 72h Box-and-whisker plot illustrating the growth inhibition of HT-29 colon cancer cell after exposure to 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions (P < 0,001). Samples differed between each other (p<0.05). 1000 µg/mL SAK lip extract (LC34) showed the least cell growth recovery and 500 µg/mL SAK total (LC30) showed the most outspoken cell growth recovery

Fig 3.21 shows quantification of all CAK, CPK, SAK and SPK aqueous extractions for 24, 48 and 72 hours exposure. TAK aqueous extraction was not available at time of the experiment. A significant decrease in cell proliferation by all available extractions was seen after 24 and 48 hours with a slight increase in the cell proliferation after 72 hours which may indicate a recovery in cell growth.
All aqueous treated samples differed from the control with a significant P-value of $p<0.001$. The samples differed from each other showing a significant P-value of $p<0.05$ (see Annexure 21 for full Kruskal Wallis test report).

At 24 hours all the aqueous treated samples differed from the control (A = 0,575). All the aqueous kernel extractions induced a growth inhibitory effect to approximately 30% to that of the control. The 500 µg/mL CPK and CAK aqueous extracts induced the most outspoken growth inhibitory effect (Fig 3.21, LC6 and 3 respectively) at 24 hours.

UNIVERSITY of the
WESTERN CAPE

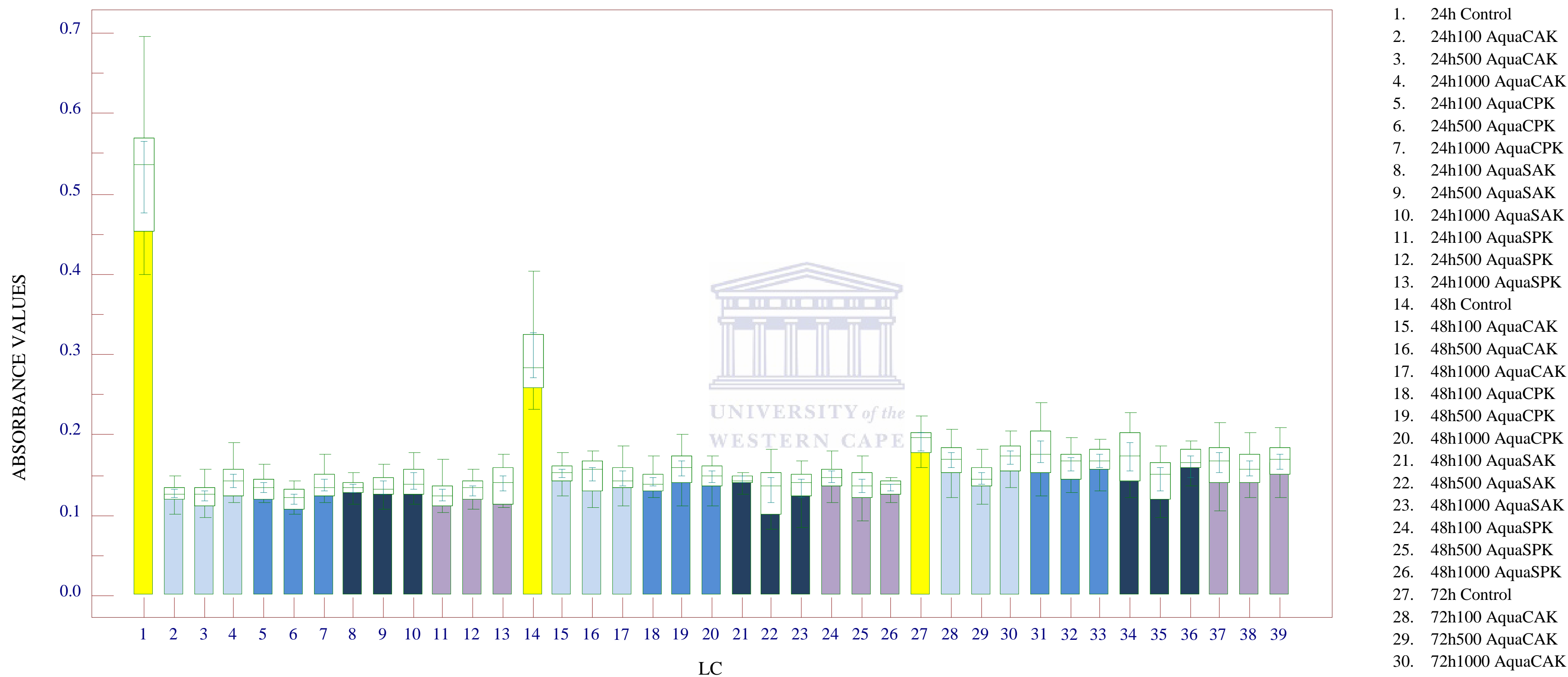
All the aqueous treated samples at 48 hours differed from the 48 hour control (A = 0,287). The 500 µg/mL SAK aqueous extract (Fig 3.21, LC22) induced the most outspoken inhibition on cell proliferation by approximately 50% to that of the control.

After 72 hours the 500 µg/mL SAK aqueous extract still induced the most outspoken inhibition on cell proliferation although to a much lesser degree compared at 48 hours (Fig 3.21, LC35). This was followed by 500 µg/mL CAK aqueous extract (Fig 3.21, LC29). It would appear that the inhibitory effect of the 100 and 1000 µg/mL CAK, CPK and SAK aqueous extracts may have been overcome inducing an increase in cell proliferation

(Fig 3.21, LC28, 30, 31, 33, 34 and 36 respectively). The SPK aqueous extract treated samples did not differ much from each other compared to the control.

In summary, the 500 µg/mL CPK aqueous extract had an overall growth inhibitory effect at 24 hours, whilst the 500 µg/mL SAK aqueous showed an overall growth inhibitory effect at 48 hours and 72 hours. At 24 hours no increase in cell proliferation is shown, whereas after 72 hours an increase in cell proliferation is observed. It may be concluded that the inhibitory effect of the aqueous extracts observed is thus not cytotoxic.

UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the WESTERN CAPE

Fig 3.21: 24h, 48h, 72h Box-and-whisker plot illustrating the growth inhibition of HT-29 colon cancer cells after exposure to 100, 500 and 1000 µg/mL CAK, CPK, SAK and SPK aqueous extractions. The proliferation of all treated samples differed markedly from the control (P<0,001). 24h 500 µg/mL CPK, 48h & 72h 500 µg/mL SAK aqueous extractions showed the most outspoken inhibition of cell proliferation (LC6, LC22 & LC35).

Table 3.4: Summary of the organic and aqueous extracts fractions that induced the most outspoken inhibition on cell proliferation of HT-29 human colon cancer cells

<table>
<tr><th>SAMPLE EXTRACT</th><th>24 HOURS</th><th>48 HOURS</th><th>72 HOURS</th><th>SAMPLE EXTRACT</th><th>24 HOURS</th><th>48 HOURS</th><th>72 HOURS</th></tr>
<tr><td rowspan="2">ORGANIC CAK</td><td rowspan="2">1000 µg/mL lip</td><td>1000 µg/mL lip</td><td rowspan="2">None</td><td rowspan="2">AQUEOUS CAK</td><td rowspan="2">500 µg/mL</td><td rowspan="2"></td><td rowspan="2">500 µg/mL</td></tr>
<tr><td>1000 µg/mL total</td></tr>
<tr><td rowspan="2">ORGANIC CPK</td><td rowspan="2">1000 µg/mL total</td><td>1000 µg/mL total</td><td rowspan="2">None</td><td rowspan="2">AQUEOUS CPK</td><td rowspan="2">500 µg/mL</td><td rowspan="2"></td><td rowspan="2">500 µg/mL</td></tr>
<tr><td>500 µg/mL total</td></tr>
<tr><td rowspan="2">ORGANIC TAK</td><td>100 µg/mL total</td><td rowspan="2">1000 µg/mL hyd</td><td rowspan="2">None</td><td rowspan="2" colspan="4">AQUEOUS TAK NOT AVAILABLE AT TIME OF EXPERIMENT</td></tr>
<tr><td>500 µg/mL total</td></tr>
<tr><td>ORGANIC SAK</td><td>1000 µg/mL total</td><td>500 µg/mL total</td><td>1000 µg/mL lip</td><td>AQUEOUS SAK</td><td></td><td>500 µg/mL</td><td>500 µg/mL</td></tr>
<tr><td rowspan="2">ORGANIC SPK</td><td>500 µg/mL lip</td><td>1000 µg/mL lip</td><td>None</td><td rowspan="2">AQUEOUS SPK</td><td rowspan="2" colspan="3">Experimental sample did not differ significantly from the control</td></tr>
<tr><td>100 µg/mL total</td><td>500 µg/mL total</td><td></td></tr>
</table>

UNIVERSITY of the
WESTERN CAPE

Table 3.4 tabulates a comparative summary of the organic and aqueous kernel extractions that displayed the most outspoken inhibition on cell proliferation at 24, 48 and 72 hours. At 24 and 48 hours all the organic and aqueous extractions induced a decrease in cell proliferation. Of the organic CAK extractions the 1000 µg/mL CAK lipophilic extract induced the most outspoken growth inhibitory effect at 24 hours and the 1000 µg/mL CAK total extract did so at 48 hours (Fig 3.3 and 3.4). Whereas the CPK total extract at 1000 µg/mL caused a growth inhibitory effect at 24 and 48 hours (Fig 3.6 – 3.8). After 72 hours the inhibitory effect caused by the CAK and CPK organic fractions after 24 and 48 hours was completely abrogated with some of the treated samples even showing an increase in cell viability / proliferation. The cells exposed to the CAK and CPK aqueous extractions showed the same tendency as with the organic extractions including the recovery in cell viability after 72 hours.

UNIVERSITY of the
WESTERN CAPE

The SAK total extract induced an outspoken growth inhibitory effect at 24h1000 µg/mL and 48h500 µg/mL (Fig 3.12 – 13). Whereas the SPK lipophilic extract at 24h500 µg/mL and 48h1000 µg/mL showed an outspoken growth inhibitory effect (Fig 3.16 – 17). The SAK aqueous extraction at 48h500 µg/mL and 72h500 µg/mL had induced an outspoken growth inhibitory effect (Fig 3.21).

It is observed that of the aqueous fractions the CAK, CPK and SAK 500 µg/mL seemingly had the most outspoken growth inhibitory effects (Table 3.4). Whereas, the total and lipophilic organic fractions of all the kernels are seen to have the most outspoken inhibition on cell proliferation. The Turkish apricot kernel has shown to be effective in decreasing cell proliferation at 24 and 48 hours and increasing cell proliferation at 72 hours (Fig 3.9 – 11, Table 3.4) which could indicate that the inhibitory effect is being overcome.

The Turkish apricot organic extractions therefore showed a similar result to that of the CAK, CPK, SAK and SPK organic extractions after 24 and 48 hours exposure.

Exposure of the HT-29 colon cancer cells to the organic and aqueous fractions produced similar results in that the 24 and 48 hour samples all show a decrease in cell proliferation and after 72 hours show an increase in cell proliferation indicative of a recovery in cell growth.

UNIVERSITY of the
WESTERN CAPE

3.3 Morphological study

3.3.1 Haematoxylin and Eosin staining (H&E)

Light microscopy was conducted to investigate the morphological effects exerted by 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic and aqueous extractions on the HT-29 colon cancer cell line after 24-, 48- and 72 hour exposure. The H&E staining was performed to visualise morphological changes in the cytoplasm and nucleus since haematoxylin stains the cell nucleus blue and the eosin stains the cytoplasm pink. H&E staining also visualises the cells undergoing mitosis as well as apoptotic and / or necrotic cells.

HT-29 colon cancer cells tend to form colonies with a characteristic round to elliptical shape depending on the confluency of cells in the flask. Round nuclei with 2 - 4 nucleoli are visible in the cells. The untreated cells were well rounded or elliptically shaped with uniform sized cells in each colony.

The morphological changes of the HT-29 colon cancer cells in response to the treatment with the various kernel extractions showed some cells displaying cell membrane blebbing possibly due to changes in membrane permeability, irregularly shaped cells, cellular shrinking and hypercondensed chromatin all features associated with cells undergoing apoptosis. These changes are illustrated by Figures 3.22 – Figures 3.56. The HT-29 colon cancer cells tend to form colonies displaying nuclei and prominent nucleoli. The treated HT-29 colon cancer cells grew in a sparse manner, more obvious clumping, smaller colonies and a decrease in cells numbers. After 72 hours however, the cells seem to resume their initial growth appearance, increase in colony size and cells.

UNIVERSITY of the WESTERN CAPE

The selection of the H&E figures is based on the results obtained by the crystal violet staining and the flow cytometric data analysis representing the various kernel extractions at the specific concentrations and time periods as those with possibly the most significant morphological changes when treated with the respective kernel fractions. The treatments are grouped according to the specific kernel types.

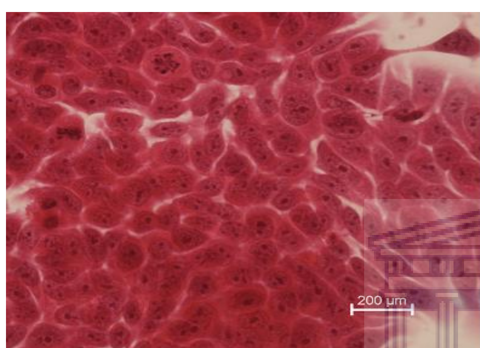


Figure 3.22: 24h Control

Figure 3.23: 48h Control

UNIVERSITY of the
WESTERN CAPE

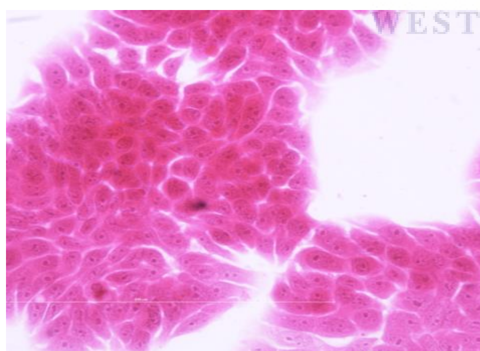


Figure 3.24: 72h Control

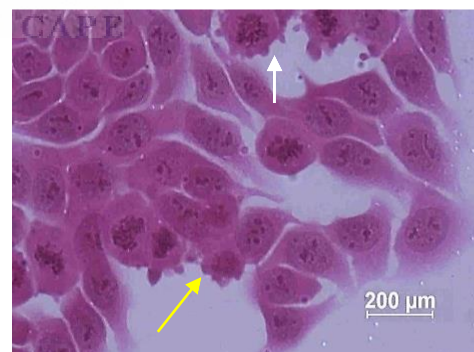


Figure 3.25: CPK-H 24h100 µg/mL

Figures 3.22 – 3.31 show 24h, 48h and 72h untreated cells and cells exposed to CPK and CAK organic and aqueous extractions. Fig 3.22 Untreated 24h control shows cells in interphase. Fig 3.23 shows a 48h control. Fig 3.24 shows a 72h control. Fig 3.25 shows cell membrane blebbing (white arrow) and irregular cell shape (yellow arrow) after exposure to CPK-H 24h100 µg/mL (magnification: 40x).

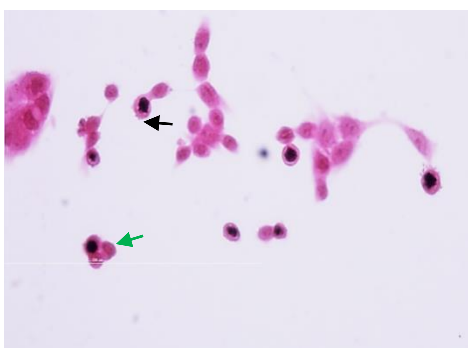


Figure 3.26: CAK aqueous 24h100 µg/mL

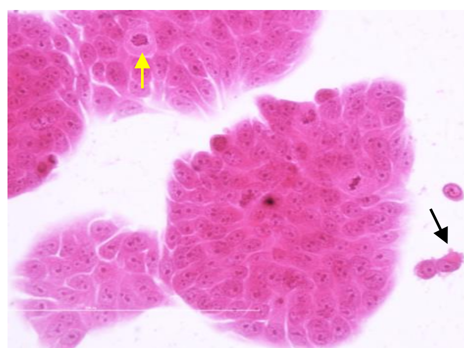


Figure 3.27: CAK aqueous 24h500 µg/mL

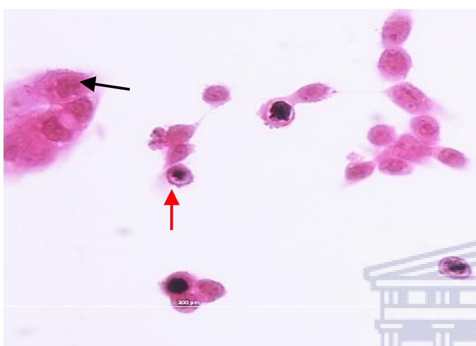


Figure 3.28: CAK aqueous 72h100 µg/mL

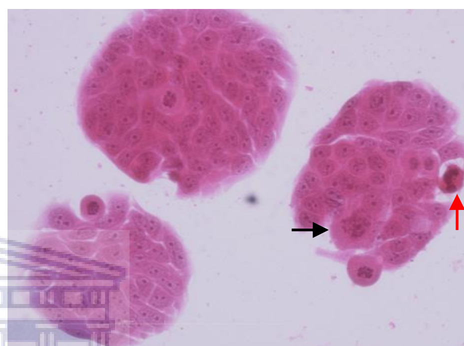


Figure 3.29: CPK aqueous 24h1000 µg/mL

UNIVERSITY of the
WESTERN CAPE

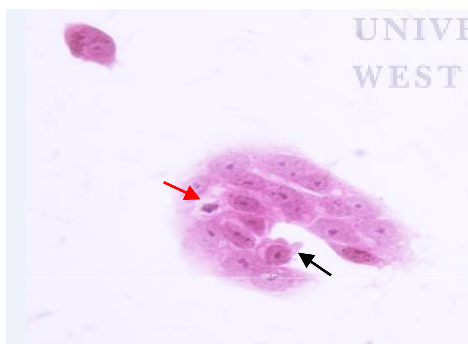


Figure 3.30: CPK aqueous 48h500 µg/mL

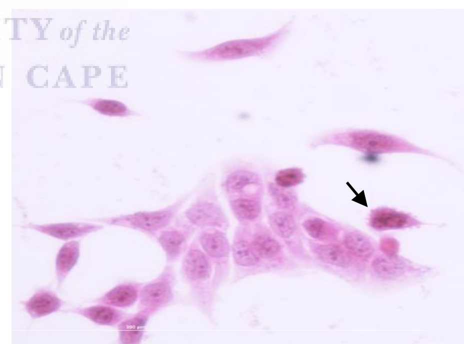


Figure 3.31: CPK aqueous 48h1000 µg/mL

Fig 3.26 shows small rounded cells displaying cell membrane blebbing (black arrow) and possible apoptotic cell (green arrow) after exposure to CAK aqueous 24h100 µg/mL. Fig 3.27 shows an enlarged cell (yellow arrow) and cellular shrinkage (black arrow) after exposure to CAK aqueous 24h500 µg/mL. Fig 3.28 shows membrane blebbing (black arrow) and hypercondensed chromatin (red arrow) after exposure to CAK aqueous 72h100 µg/mL. Fig 3.29 shows a cell with irregular shape (black arrow) and possible apoptotic cell (red arrow) after exposure to CPK aqueous 24h1000 µg/mL. Fig 3.30 shows membrane blebbing (black arrow) and possible apoptotic cell (red arrow) after exposure to CPK aqueous 48h500 µg/mL. Fig 3.31 shows membrane blebbing (arrow) after exposure to CPK aqueous 48h1000 µg/mL (magnification: 40x).

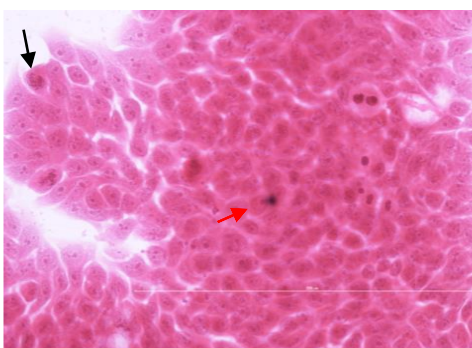


Figure 3.32: SAK aqueous 72h100 µg/mL

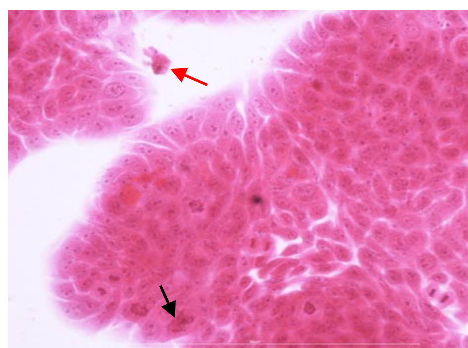


Figure 3.33: SAK aqueous 72h500 µg/mL

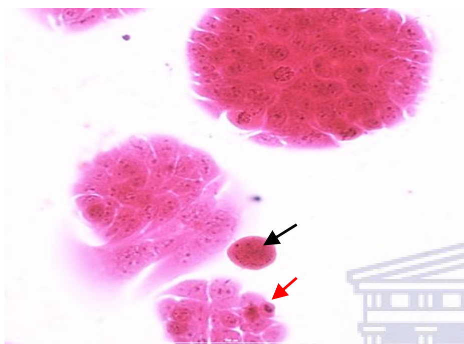


Figure 3.34: SAK-H 24h100 µg/mL

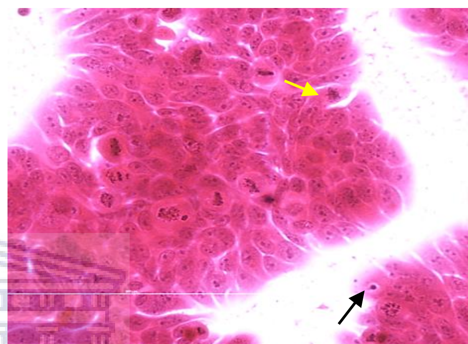


Figure 3.35: SAK-H 24h500 µg/mL

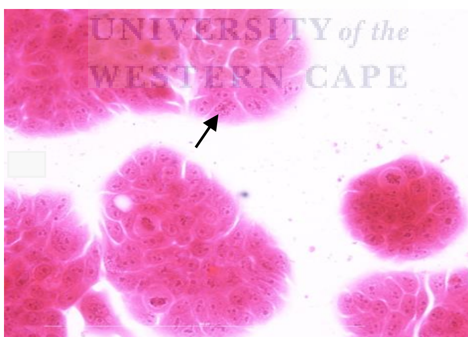


Figure 3.36: SAK-H 24h1000 µg/mL

UNIVERSITY of the
WESTERN CAPE

Figure 3.32 – 3.36 show the effects of SAK aqueous and organic extractions on HT-29 colon cancer cells. Fig 3.32 shows an irregular cell (black arrow) and dense hyperchromatin (red arrow) after exposure to SAK aqueous 72h100 µg/mL. After 72 hours the cells start overcome the effect of the extractions and resume their shape and colony formation as seen with the controls. Fig 3.33 an irregular shaped cell (black arrow) and possible apoptotic cell (red arrow) after exposure to SAK aqueous 72h500 µg/mL is seen. Fig 3.34 shows possible apoptosis (red arrow) and an enlarged cell with granulation (black arrow) after exposure to SAK-H 24h100 µg/mL. Fig 3.35 shows cells with dense hyperchromatin (black arrow) and membrane blebbling (yellow arrow) after exposure to SAK-H 24h500 µg/mL. Fig 3.36 shows an irregular shaped cell (arrow) after exposure to SAK-H 24h1000 µg/mL. (magnification: 40x).

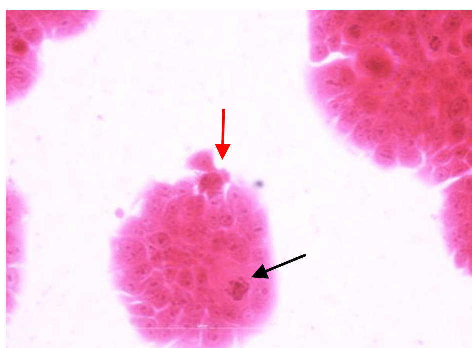


Figure 3.37: SAK-L 24h100 µg/mL

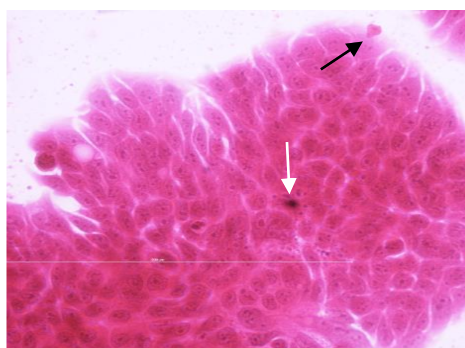


Figure 3.38: SAK-L 72h100 µg/mL

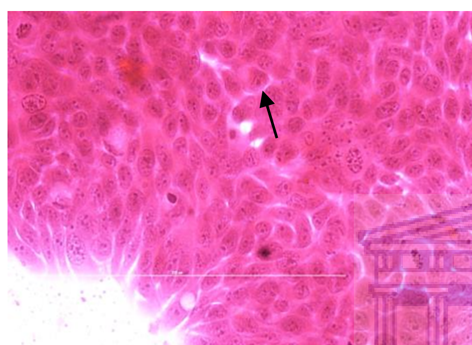


Figure 3.39: SAK-L 48h500 µg/mL

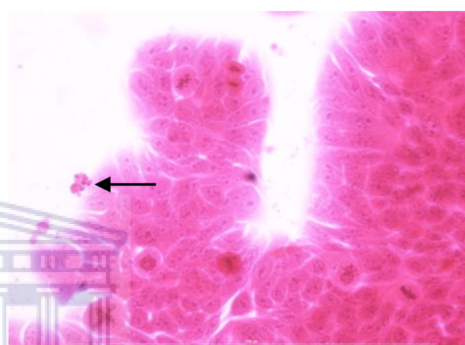


Figure 3.40: SAK-L 48h1000 µg/mL

UNIVERSITY of the
WESTERN CAPE

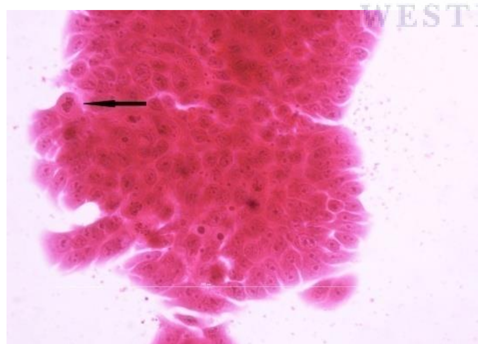


Figure 3.41: SAK-T 24h100 µg/mL

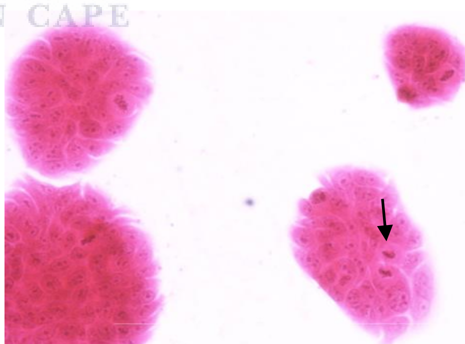


Figure 3.42: SAK-T 24h1000 µg/mL

Figure 3.37 – 3.42 show the effects of some SAK organic extractions on HT-29 colon cancer cells. Fig 3.37 abnormal cell with irregular shape (red arrow) and membrane blebbing (black arrow) after exposure to SAK-L 24h100 µg/mL is seen. Fig 3.38 an irregularly shaped cell (black arrow) and possible apoptotic cell (white arrow) after exposure to SAK-L 72h100 µg/mL can be observed. Fig 3.39 shows an irregularly shaped cell (arrow) after exposure to SAK-L 48h500 µg/mL. Fig 3.40 displays possible apoptotic cells (black arrow) after exposure to SAK-L 48h1000 µg/mL. Fig 3.41 abnormal cell with an enlarged irregularly shaped cell (arrow) after exposure to SAK-T 24h100 µg/mL can be observed. Fig 3.42 shows possible apoptotic cell (arrow) after exposure to SAK-T 24h1000 µg/mL (magnification: 40x).

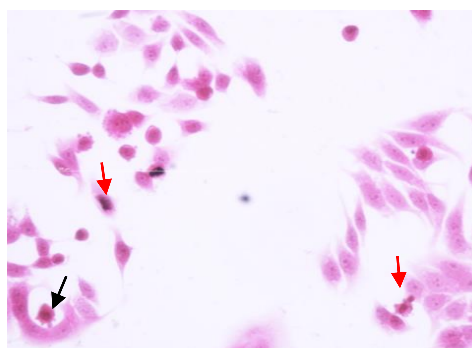


Figure 3.43: SPK aqueous 72h100 µg/mL

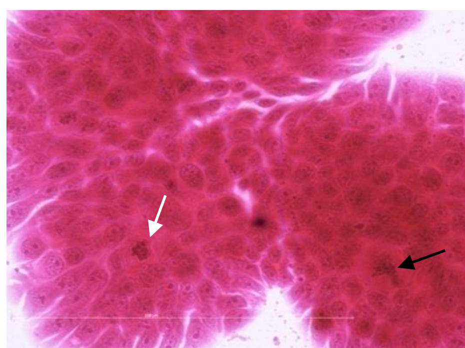


Figure 3.44: SPK-H 24h500 µg/mL

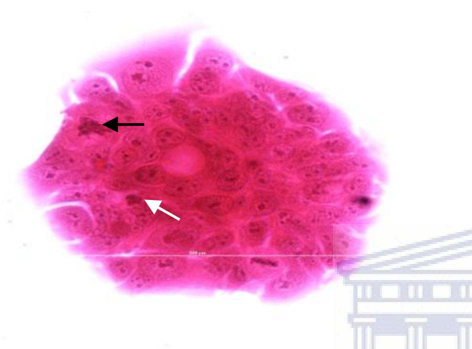


Figure 3.45: SPK-H 24h1000 µg/mL

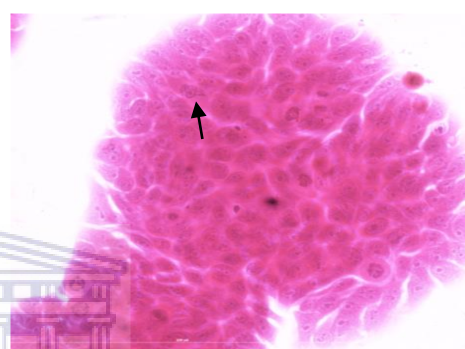


Figure 3.46: SPK-H 48h100 µg/mL

UNIVERSITY of the
WESTERN CAPE

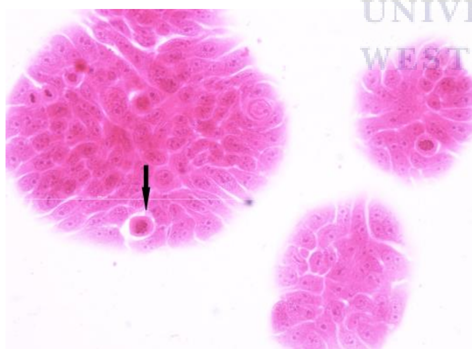


Figure 3.47: SPK-H 48h500 µg/mL

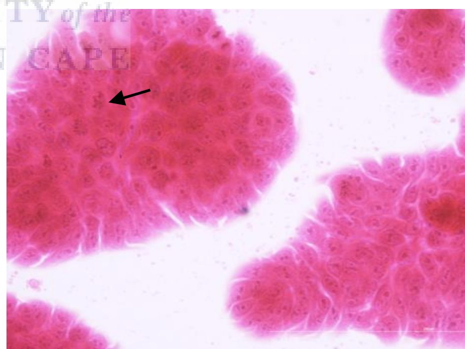


Figure 3.48: SPK-L 24h100 µg/mL

Figure 3.43 – 3.48 show the effects of some SPK aqueous and organic extractions on HT-29 colon cancer cells using H&E staining. Fig 3.43 shows possible apoptotic cells (red arrows) and blebbing (black arrow) after exposure to SPK aqueous 72h100 µg/mL. Fig 3.44 shows abnormal cell with irregular cell shape (black arrow) and hypercondensed chromatin (white arrow) after exposure to SPK-H 24h500 µg/mL. Fig 3.45 shows irregularly shaped enlarged cell (black arrow) and possible apoptotic cell (white arrow) after exposure to SPK-H 24h1000 µg/mL. Fig 3.46 an irregularly shaped cell after exposure to SPK-H 48h100 µg/mL is observed. Fig 3.47 shows an enlarged and detached cell (arrow) after exposure to SPK-H 48h500 µg/mL. Fig 3.48 irregularly shaped cell (arrow) after exposure to SPK-L 24h100 µg/mL is seen (magnification: 40x).

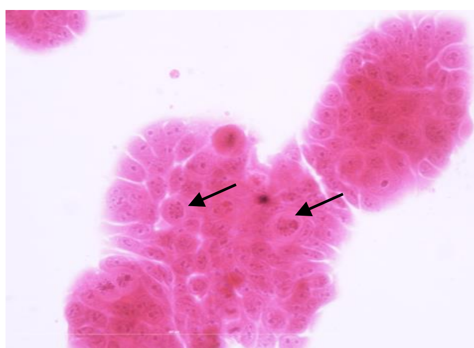


Figure 3.49: SPK-L 24h1000 µg/mL

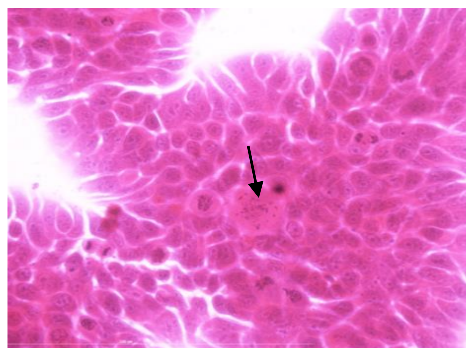


Figure 3.50: SPK-L 48h100 µg/mL

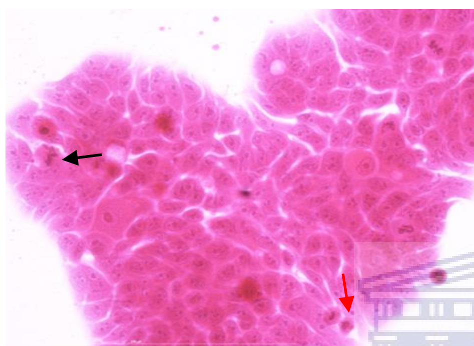


Figure 3.51: SPK-L 48h500 µg/mL

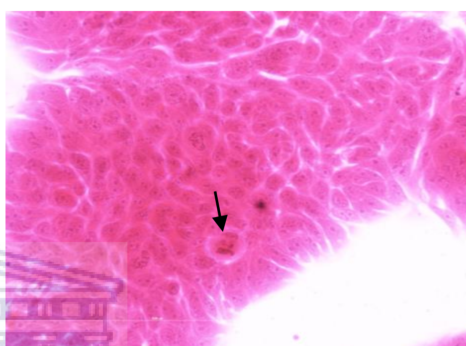


Figure 3.52: SPK-L 48h1000 µg/mL

UNIVERSITY of the
WESTERN CAPE

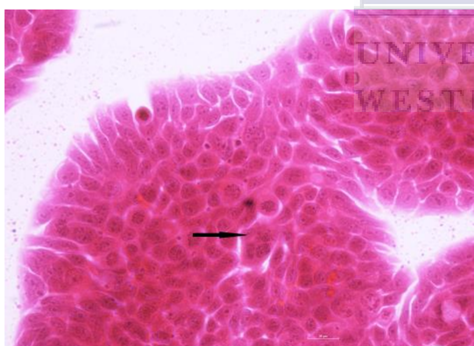


Figure 3.53: SPK-L 72h100 µg/mL

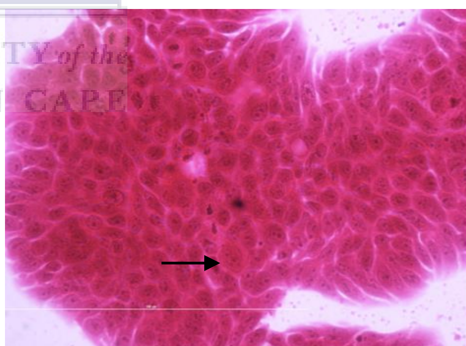


Figure 3.54: SPK-L 72h500 µg/mL

Figure 3.49 – 3.54 show the effects of some SPK organic extractions on HT-29 colon cancer cells using H&E staining. Fig 3.49 shows abnormal cells after exposure to SPK-L 24h1000 µg/mL. Fig 3.50 shows cells with granulation and irregular shape (arrow) after exposure to SPK-L 48h100 µg/mL. Fig 3.51 a cell with membrane blebbing (black arrow) and possible apoptotic cell (red arrow) after exposure to SPK-L 48h500 µg/mL is observed. Fig 3.52 an irregular shaped cell (black arrow) after exposure to SPK-L 48h1000 µg/mL is seen. Fig 3.53 shows abnormal cell with loss of internal cellular integrity (arrow) after exposure to SPK-L 72h100 µg/mL. Fig 3.54 shows irregular shaped cell (arrow) after exposure to SPK-L 72h500 µg/mL (magnification: 40x).

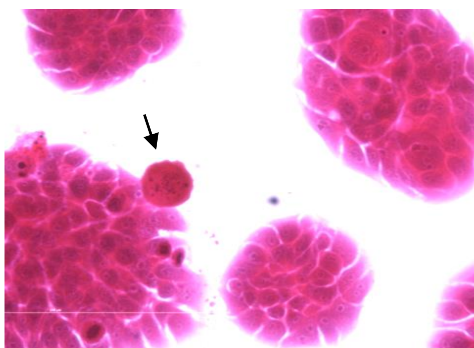


Figure 3.55: SPK-T 48h100 μg/mL

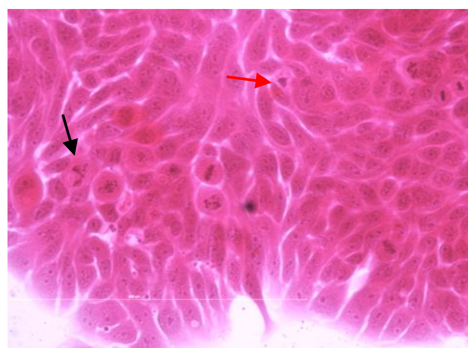


Figure 3.56: SPK-T 72h1000 μg/mL

Figures 3.55 – 3.56 show the effects of SPK-T organic extractions on HT-29 colon cancer cells using H&E staining. Fig 3.55 shows an abnormal enlarged cell with membrane blebbing after exposure to SPK-T 48h100 μg/mL. Fig 3.56 shows possible apoptotic cells (red arrow) and abnormal cell with irregular internal cellular structure (black arrow) after exposure to SPK-T 72h1000 μg/mL. Cells seemingly overcome the effects of the kernel fractions after 72 hours and an increase in cell growth is seen (magnification: 40x).

UNIVERSITY of the
WESTERN CAPE

3. 4 Flow cytometry

Propidium iodide staining detected by flow cytometry was utilised to investigate the effects of 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic and aqueous extractions on cell cycle progression. This allowed for the quantification of DNA content in the respective samples. Cell cycle distribution and an S phase block were revealed (See Annexure 2 for DNA analysis data).

Inhibition of cell cycle progression with an increase in the amount of cells in the S phase (during which DNA synthesis occurs) and subsequent decrease of cells in the G_2 phase could be an indication of the activation of an intra-S phase block. The slowing down of cell cycle progression in the S phase may not be permanent and often a recovery in cell proliferation and the progression of DNA into the G_2 phase may be seen. If DNA damage is too severe then the likelihood that cells would progress to the G_2 phase is decreased and thus programmed cell death may occur. A G_2 value of zero percent (0%) may indicate that the cells were blocked in the S phase or the cells may in fact have undergone programmed cell death. An increase of cells in the G_1 phase after 72 hours and a decrease of cells in the S phase may indicate a complete repair of DNA damage resulting in a continuation of cell cycle progression.

UNIVERSITY of the
WESTERN CAPE

3.4.1 Cell cycle progression after treatment with 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions

Figure 3.57 - 3.62 show time and dose related histograms of the respective 24, 48 and 72 hour exposures of HT-29 colon cancer cells to 100, 500 and 1000 µg/mL **CAK, CPK, TAK, SAK and SPK organic extractions**. All organic extractions influenced cell cycle progression differently depending on the type of kernel extraction, duration and concentration. The first three figures 3.57 – 3.59 compare effects of the various concentrations of the fractions on cell cycle progression for specific time periods. The next three figures (3.60 – 3.62) compares the effect of a specific concentration of the fractions for the three exposure times.

UNIVERSITY of the
WESTERN CAPE

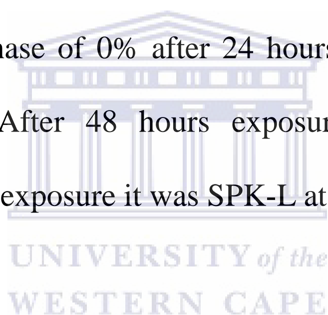
Figure 3.57 show cell cycle progression after 24 hours. 100, 500 and 1000 µg/mL SAK hydrophilic extracts (SAK-H) increased the number of cells in the S phase to 86,4%, 65,57% and 74,47% respectively. A corresponding decrease of cells in the G_2 phase of 0% was observed (Annexure 2). 100 µg/mL SPK lipophilic extract (SPK-L) increased the number of S phase cells to 66,8%, 500 µg/mL SAK total extract (SAK-T) to 60,13% and 1000 µg/mL SAK-T to 60,19%. The sample treated with 100 µg/mL CAK lipophilic extract (CAK-L) had the least amount of cells in the S phase (3,76%) and 1,44% cells in the G_2 phase (Fig 3.57, Annexure 2). Cell cycle progression of most samples treated with 1000 µg/mL fractions showed an increase in the number of cells in the S phase to some extent.

After 48 hours (Fig 3.58) cells exposed to 100 µg/mL and displaying an outspoken increase of cell numbers in the S phase were SPK-H (S=76,08%) and SPK-L (S=52,94%). A peak in the S phase of cells exposed to 500 µg/mL SAK-L (S=70,74%) and

SPK-H (S=41,88%), and SPK-T (S=77,62%) and SPK-H (S=46,54%) at a concentration of 1000 µg/mL were also observed (Annexure 2). All the concentrations of the SPK-H fraction affected cell cycle progression after 48 hours in this manner.

After 72 hours (Fig 3.59) a minor effect on cell cycle progression was seen except for 100 µg/mL SPK-L and SAK-L that induced a peak in the number of cells in the S phase to 63,44% and 29,61% respectively, 500 µg/mL CPK-H and SPK-L to 56,73% and 37,14% respectively, and 1000 µg/mL SPK-T to 33,16% (Fig 3.59; Annexure 2).

In summary, the fraction (s) which was most consistent at inducing an increased number of cells in the S phase and a G_2 phase of 0% after 24 hours for all three concentrations was SAK-H followed by SAK-T. After 48 hours exposure it was SPK-H for all three concentrations and after 72 hours exposure it was SPK-L at 100 µg/mL.


UNIVERSITY of the
WESTERN CAPE

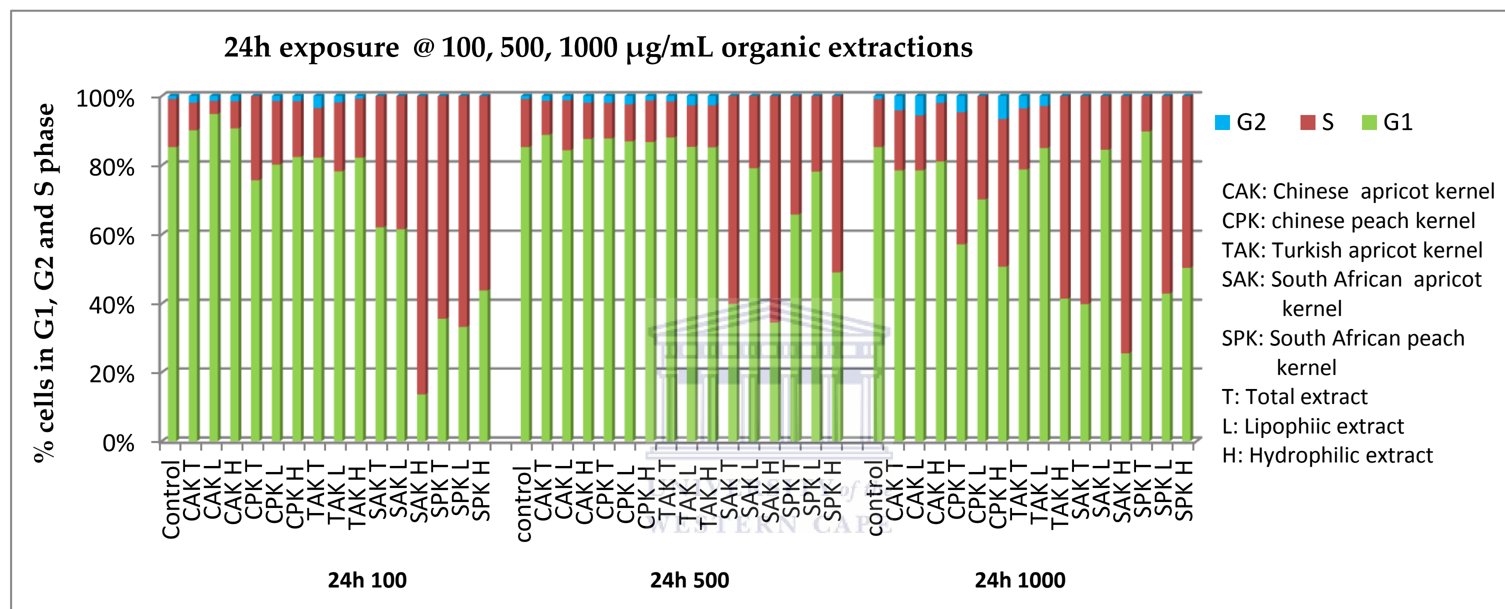


Figure 3.57: Flow cytometric analysis of HT-29 cells after 24 hour exposure to concentrations of 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions. Cell cycle analysis show an outspoken increase in cells in S-phase and decrease in cells in G_2 phase when treated with 100µg/mL SAK-H (S=86,4%, G_2=0%), SPK-L (S=66,8%, G_2=0%); at 500 µg/mL SAK-H (S=65,57%, G_2=0%), SAK-T (S=60,13%, G_2=0%); at 1000 µg/mL SAK-H (S=74,47%, G_2=0%), SAK-T (S=60,19%, G_2=0%) (Annexure 2).

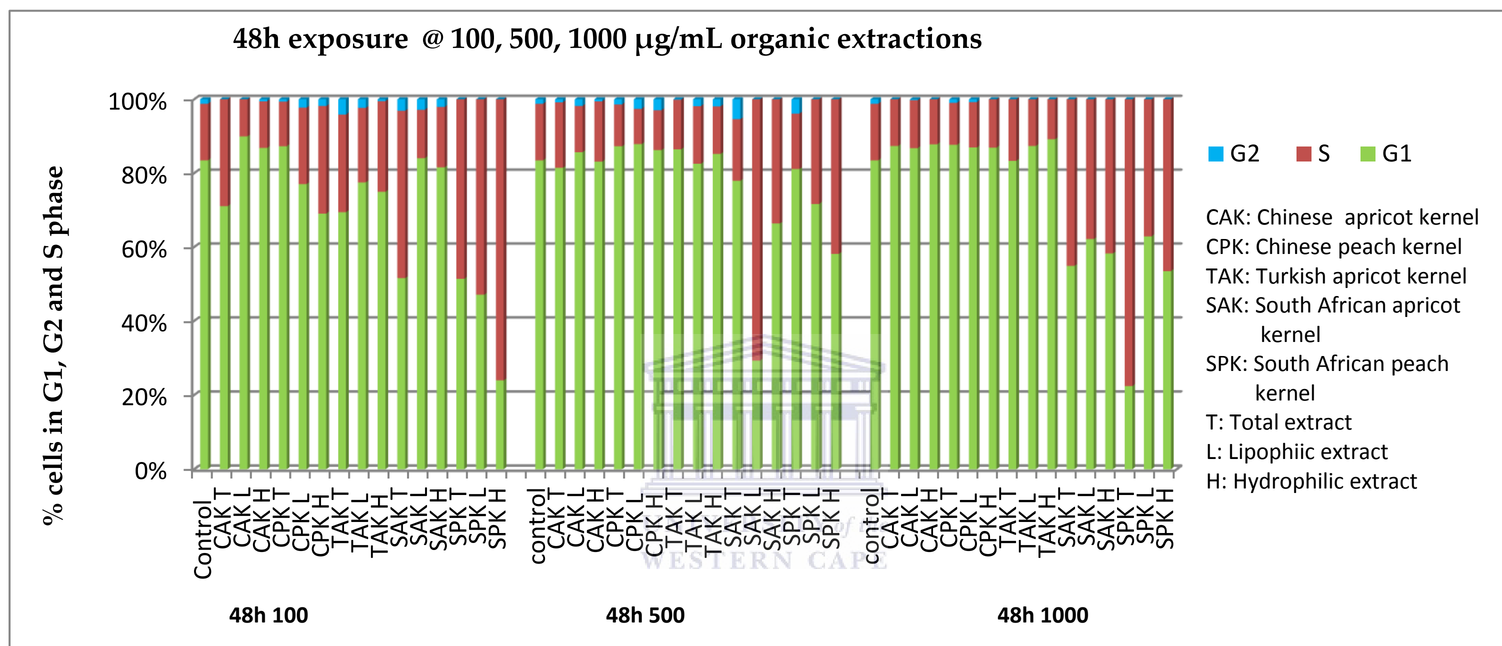


Figure 3.58: Flow cytometric analysis of HT-29 cells after 48 hour exposure to concentrations of 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions. After exposure to 500 and 1000 µg/mL cells show a tendency to recover which is shown by the decrease in cells in the S phase and an increase in cells in the G_2 phase. Cells show an outspoken increase in cells in S-phase and decrease in cells in G_2 phase indicating a growth inhibitory effect when treated with 100 µg/mL SPK-H (S=76,08%, G_2=0%) and SPK-L (S=52,94%, G_2=0%); at 500 µg/mL SAK-L (S=70,74%, G_2=0%), SPK-H (S=41,88%, G_2=0%); and 1000 µg/mL SPK-T (S=77,62%, G_2=0%) and SPK-H (S=46,54%, G_2=0%) (see Annexure 2 for data).

72h exposure @ 100, 500, 1000 µg/mL organic extractions

Figure 3.59: Flow cytometric analysis of HT-29 cells after 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions. Cell cycle analysis show an increase in cells in G_1 and G_2 phase, as well as a decrease in cells in S phase indicating a recovery in cell proliferation. Cells treated with the following fractions yielded 0% cells in the G_2 phase and the highest S phase peak namely at 100 µg/mL SPK-L (S=63,44%); at 500 µg/mL CPK-H (S=56,73%) and at 1000 µg/mL SPK-T (S=33,16%) (see Annexure 2 for data).

3.4.2 Cell cycle progression over 24, 48 and 72 hours after treatment with 100, 500 and 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions

Figure 3.60 - 3.62 show time related histograms comparing the same concentration for all the fractions over time. Figure 3.60 show 100 µg/mL over 24, 48 and 72 hours. Exposure to 100 µg/mL at 24, 48 and 72 hours showed that all the SPK organic extractions affected cell cycle progression by inducing a peak in the number of cells in the S phase with SPK-L inducing an increase in all three time periods. However, the most effective 100 µg/mL kernel fraction was SAK-H inducing the biggest S phase peak, 86,4%, after 24 hours (Fig 3.60, Annexure 2).

500 µg/mL SPK organic extraction affected the cell cycle progression by inducing a peak in the number of cells in the S phase and 0% in the G_2 phase after 24, 48 and 72 hours with SPK-H being the more consistent fraction having an effect. 500 µg/mL SAK-L after 48 hours however, showed the most outspoken effect by increasing the S phase to 70,74% (Fig 3.61, Annexure 2).

UNIVERSITY of the

1000 µg/mL SPK organic extraction consistently induced a peak in the number of cells in the S phase after the three exposure times with 1000 µg/mL SPK-T at 48 hours inducing the highest peak in the S phase (S=77,62% and G_2=0%) (Fig 3.62, Annexure 2).

Therefore the 100 µg/mL SAK-H had the greatest effect on cell cycle progression by inducing an increase in the number of cells in the S phase to 86,4% with no cells in the G_2 phase at 24 hours. The SPK total, lipophilic and hydrophilic organic extractions increased the number of cells in the S phase consistently in all the exposure times. After 72 hours, for all three concentrations of CAK, CPK and TAK, no effect on cell cycle progression was observed. It follows that the S phase block observed mostly after 24 and 48 hours had been

overcome and the cell resumed cell cycle progression and thus cell proliferation (Fig 3.60 – 3.62).

.

24h , 48h and 72h exposure @ 100 µg/mL organic extractions

G2 S G1

CAK: Chinese apricot kernel
CPK: Chinese peach kernel
TAK: Turkish apricot kernel
SAK: South African apricot kernel
SPK: South African peach kernel
T: Total extract
L: Lipophiic extract
H: Hydrophilic extract

UNIVERSITY of the
WESTERN CAPE

Figure 3.60: Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure at a concentration of 100 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions. Cell cycle analysis showed an increase in cells present in S-phase as well as a marked decrease in the cells present in G_2 phase (G_2=0%) when treated at 24h100 µg/mL SAK-H (S=86,4%), SPK-L (S=66,8%); 48h100 µg/mL SPK-H (S=76,08%), SPK-L (S=52,94%) and at 72h100 µg/mL SPK-L (S=63,44%) and SAK-L (S=29,61%) (see Annexure 2 for complete flow cytometric data).

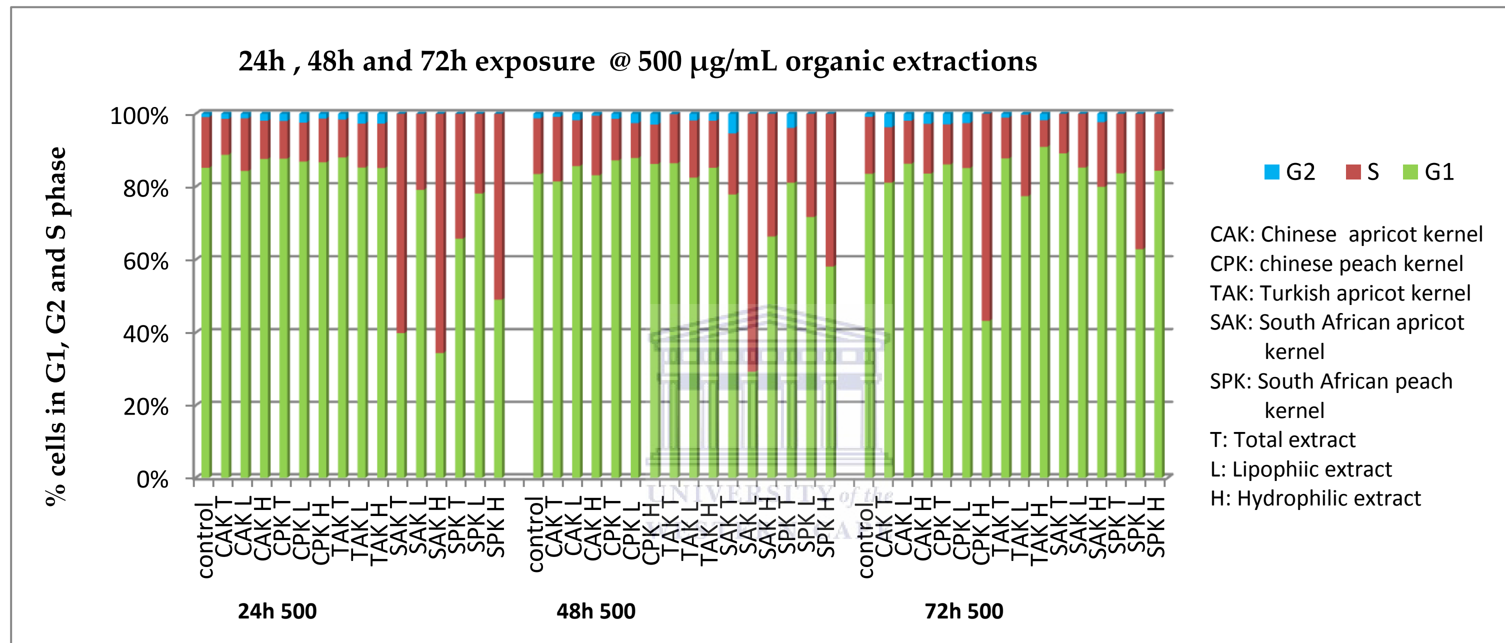


Figure 3.61: Flow cytometric DNA analysis of HT-29 cells after 24, 48 and 72 hour exposure to a concentration of 500 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions. An increase in the number of cells in S-phase as well as a decrease in cells present in G_2 phase (G_2=0%) when treated at 24h500 µg/mL SAK-H (S=65,57%) and SAK-T (S=60,13%); 48h500 µg/mL SAK-L (S=70,74%) and SPK-H (S=41,88%) and 72h500 µg/mL CPK-H (S=56,73%) and SPK-L (S=37,14%) (see Annexure 2). An increase in the number of cells in the G_1 phase of the treated samples indicates a recovery of the damaged DNA and thus a progression of the cell cycle.

24h , 48h and 72h exposure @ 1000 µg/mL organic extractions

% cells in G1, G2 and S phase

G2 S G1

CAK: Chinese apricot kernel
CPK: chinese peach kernel
TAK: Turkish apricot kernel
SAK: South African apricot kernel
SPK: South African peach kernel
T: Total extract
L: Lipophiic extract
H: Hydrophilic extract

24h 1000 48h 1000 72h 1000

UNIVERSITY of the
WESTERN CAPE

Figure 3.62: Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure to a concentration of 1000 µg/mL CAK, CPK, TAK, SAK and SPK organic extractions. The occurrence of an S-phase peak as well as a decrease in cells in G_2 phase (G_2=0%) was observed when treated at 24h1000 µg/mL SAK-H (S=74,47%) and SAK-T (S=60,19%); 48h1000 µg/mL SPK-T (S=77,62%) and SPK-H (S=46,54%); and 72h1000 µg/mL SPK-T (S=33,16%). An increase in the number of cells in the G_1 phase indicates a recovery in cell cycle progression of the treated samples.

To evaluate and compare the specific kernel extractions with one another the following figures 3.63 - 3.71 display dose related histograms of specific fractions over time. **Figure 3.63 – 3.65** show 100, 500 and 1000 µg/mL of CAK, CPK, TAK, SAK and SPK **total extractions** for the three time periods. The total extractions show an increase in the number of cells in the S-phase with a decrease in cells in the G_2 phase at 24 and 48 hour exposure with a minor effect on cell cycle progression seen after 72 hours (Fig 3.65).

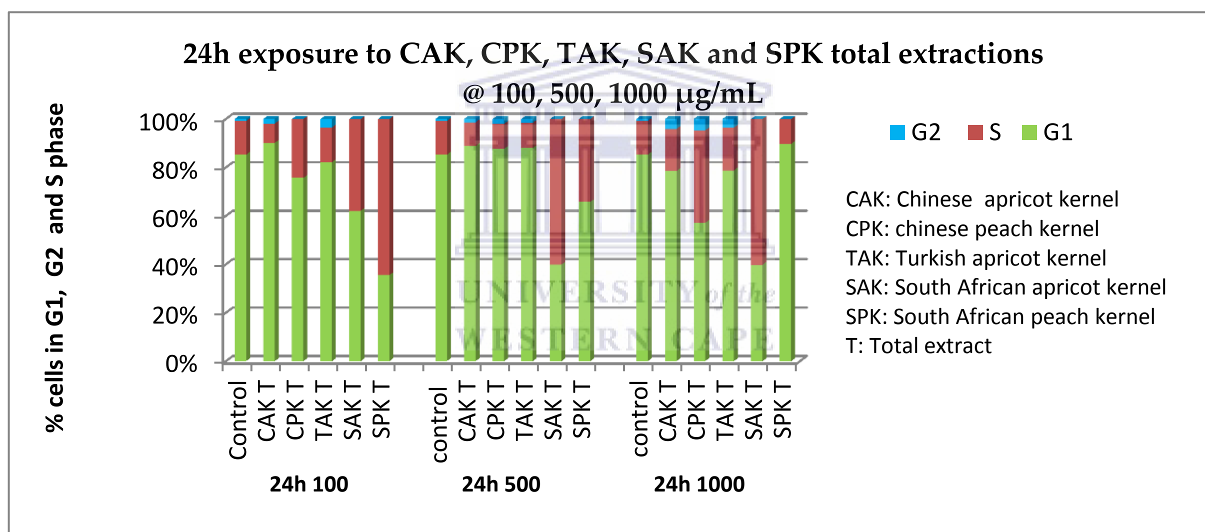


Figure 3.63: Flow cytometric analysis of HT-29 cells after 24 hour exposure to concentrations of 100, 500 and 1000 µg/mL total extractions of CAK, CPK, TAK, SAK and SPK. Cell cycle analysis showed an increase in the number of cells present in the S-phase as well as a decrease in the number of cells present in the G_2 phase (G_2=0%) when treated with 100 µg/mL SPK-T (S=64,47%), SAK-T (S=37,92%) and CPK-T (24,32%); 500 µg/mL SAK-T (S=60,13%) and SPK-T (S=34,25%); 1000 µg/mL SAK-T (S=60,19%).

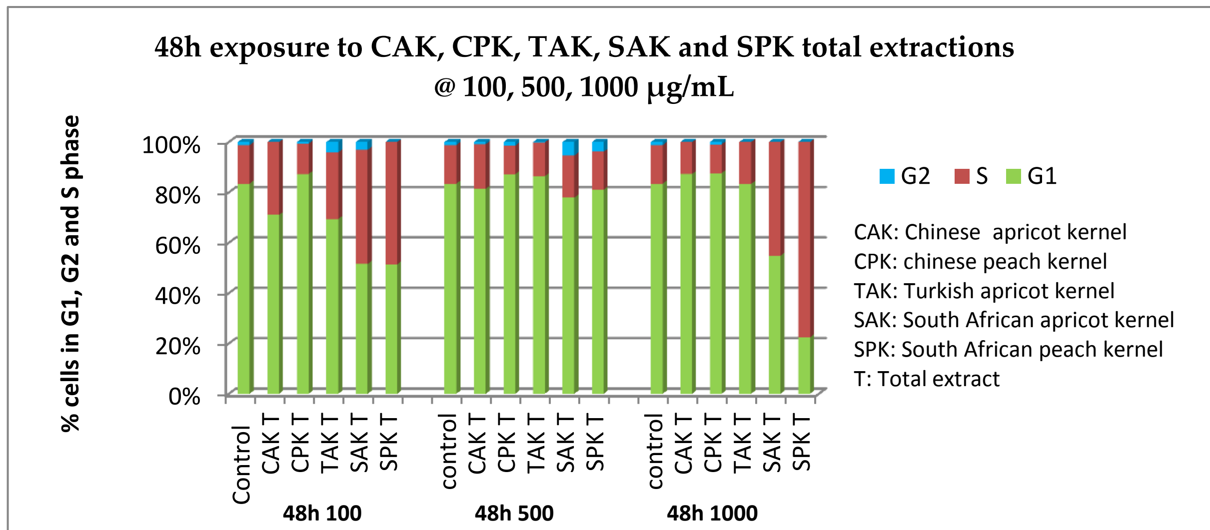


Figure 3.64: Flow cytometric analysis after 48 hour exposure to 100, 500 and 1000 µg/mL total extractions of CAK, CPK, TAK, SAK and SPK. Analysis showed a peak in the number of cells in the S-phase and a decrease in cells present in G_2 phase (G_2=0%) when treated with 1000 µg/mL SPK-T (S=77,62%). Treated samples separately showing a peak in cells present in the S phase and a G_2=0% included 100 µg/mL CAK-T (S=28,95%) and SPK-T (S=48,61%), 1000 µg/mL TAK-T (S=16,67%) and SAK-T (S=45,13%).

UNIVERSITY of the WESTERN CAPE

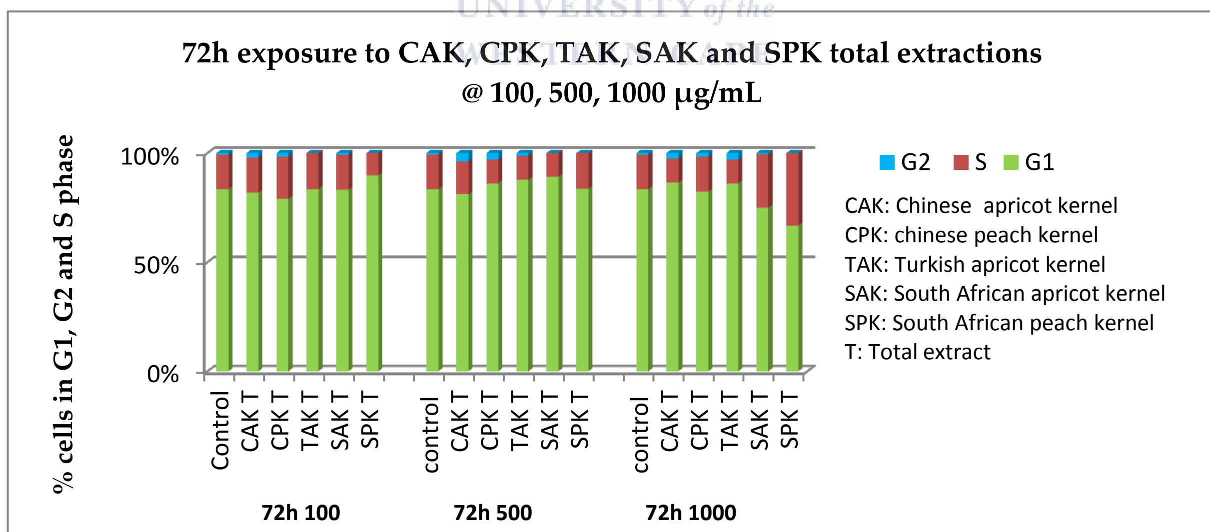
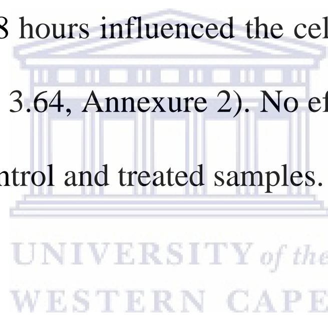


Figure 3.65: Flow cytometric analysis after 72 hour exposure to 100, 500 and 1000 µg/mL total extractions of CAK, CPK, TAK, SAK and SPK. A peak in the number of cells in the S-phase and a decrease in cells present in G_2 phase (G_2=0%) when treated with 100 µg/mL TAK-T (S=16,44%; SPK-T (S=16,36%); and 1000 µg/mL SPK-T (S=33,16%). After 72 hours no difference from the control is observed indicating that effect of the treatment is no longer present and cells resume progression of the cell cycle (Annexure 2).

Figures 3.63 - 3.65 compared the total fractions of all the kernels to establish the specific kernel and its organic total fraction that had the most outspoken effect on cell cycle progression by inducing a peak in the S phase.

The total fractions inducing an increase in the number of cells in the S phase was 100 µg/mL SPK-T after 24 hours (Fig 3.63) to 64,47%, 1000 µg/mL SPK-T after 48 hours (Fig 3.64) to 77,62% and 1000 µg/mL SPK-T after 72 hour exposure (Fig 3.65) to 33,16%.

In comparison to the effect induced by the 100 and 1000 µg/mL total fractions, none of the 500 µg/mL total fractions after 48 hours influenced the cell cycle progression by influencing either the S phase nor the G_2 (Fig 3.64, Annexure 2). No effect after 72 hours is seen as there was no difference between the control and treated samples.


UNIVERSITY of the
WESTERN CAPE

Figures 3.66 - 3.68 show histograms comparing the **lipophilic extractions** inducing a peak in the S-phase and a decrease in cells present in the G_2 phase with little difference in the G_1 phase seen over 24, 48 and 72 hour periods.

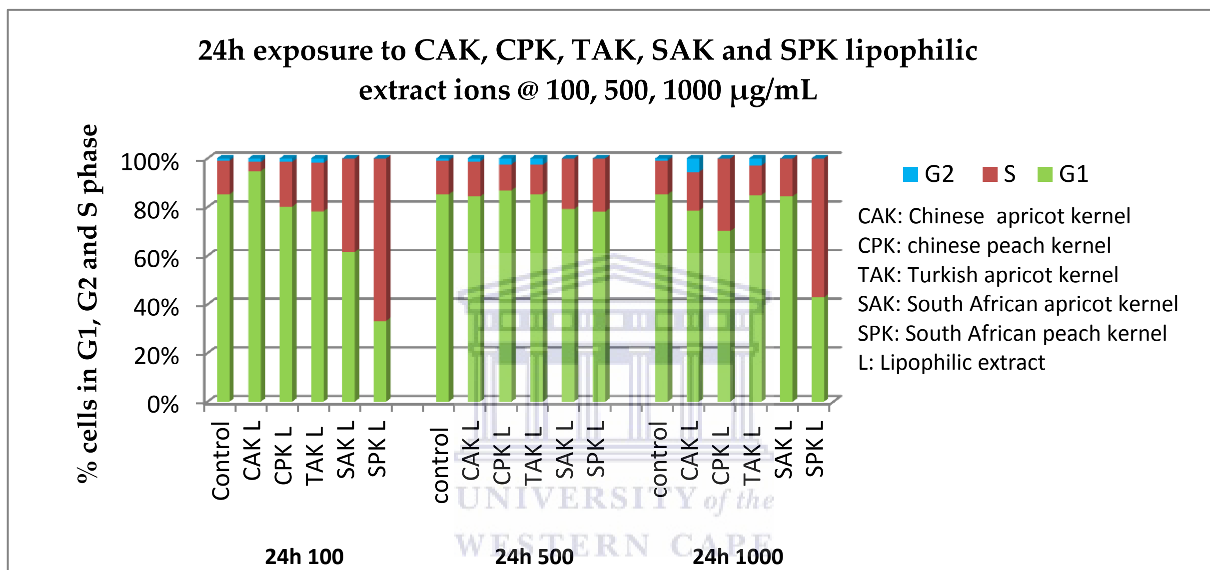


Figure 3.66: Flow cytometric analysis of HT-29 cells after 24 hour exposure to 100, 500 and 1000 µg/mL lipophilic extractions of CAK, CPK, TAK, SAK and SPK. Cell cycle analysis show the highest increase in cells present in the S-phase as well as a decrease in cells present in G_2 phase (G_2=0%) when treated with 100 µg/mL SPK-L (S=66,8%); 500 µg/mL SPK-L (S=21,88%); and 1000 µg/mL SPK-L (S=57,12%) (Annexure 2 for complete data report).

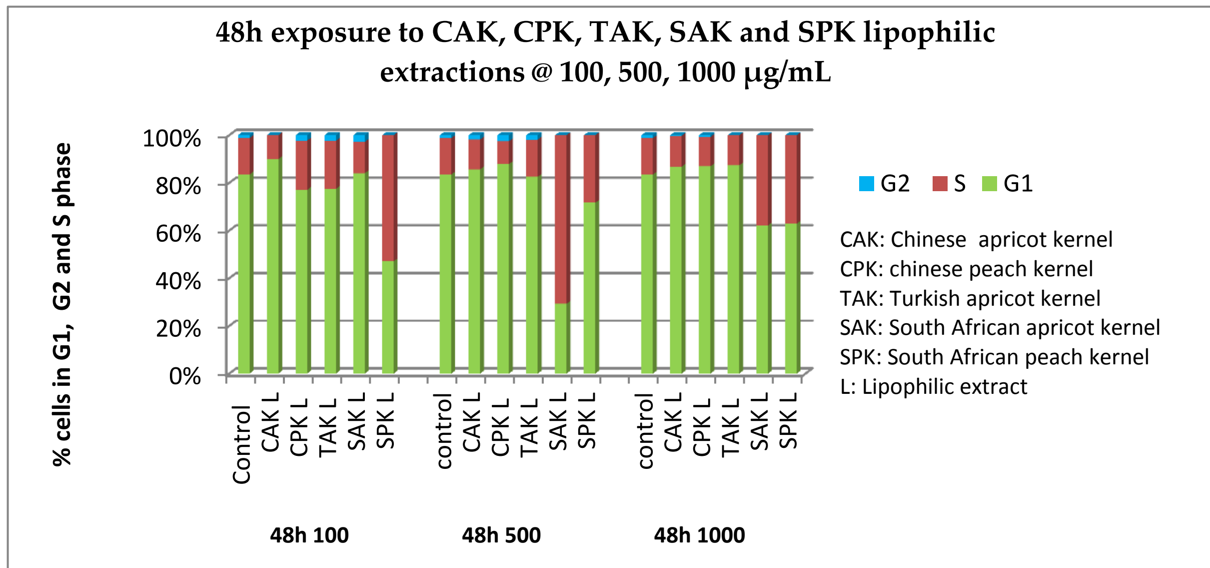


Figure 3.67: Flow cytometric analysis of HT-29 cells after 48 hour exposure to 100, 500 and 1000 µg/mL lipophilic extractions of CAK, CPK, TAK, SAK and SPK. Analysis showed a peak in the S-phase as well as a decrease in cells present in the G_2 phase (G_2=0%) when treated with 100 µg/mL SPK-L (S=52,94%); 500 µg/mL SAK-L (S=70,74%); and 1000 µg/mL SAK-L (S=37,87%) (Annexure 2 for complete data report).

UNIVERSITY of the
WESTERN CAPE

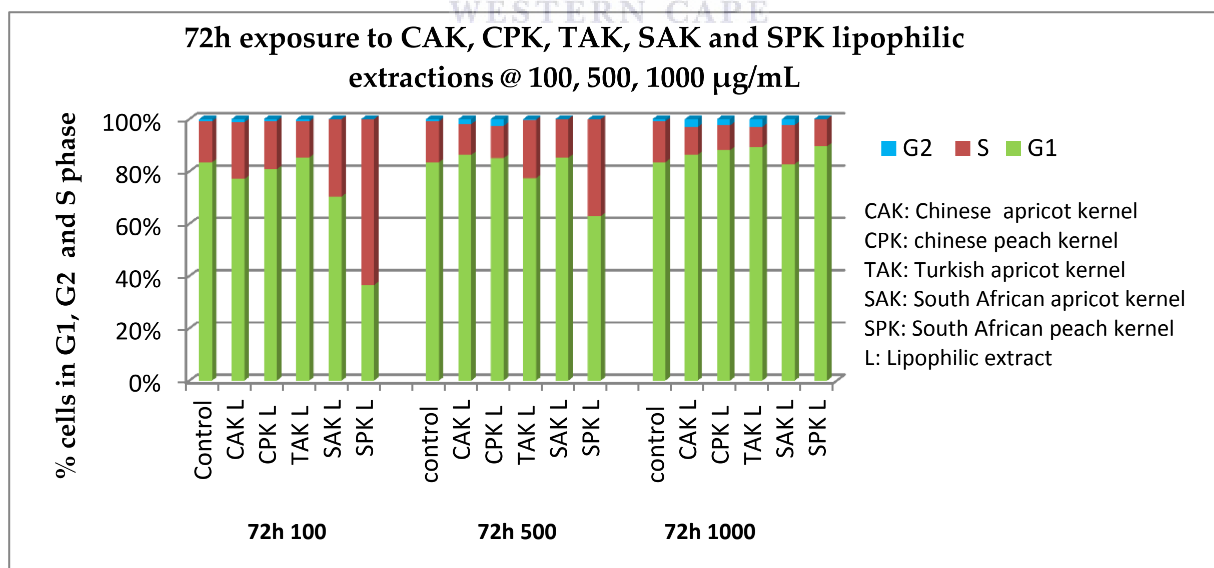


Figure 3.68: Flow cytometric analysis of HT-29 cells after 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL lipophilic extractions of CAK, CPK, TAK, SAK and SPK. Cell cycle analysis showed an increase in cells in the S-phase and a decrease in cells in G_2 phase (G_2=0%) when treated with 100 µg/mL SPK-L (S=63,44%); and 500 µg/mL SPK-L (S=37,14%) (Annexure 2 for complete data report).

The lipophilic fractions that induced an occurrence of an S phase peak were 100 µg/mL SPK-L (S=66,8%) after a 24 hour exposure (Fig 3.66), 500 µg/mL SAK-L (S=70,74%) after 48 hours (Fig 3.67), and 100 µg/mL SPK-L (S=63,44%) after 72 hours (Fig 3.68).

In summary, the 500 µg/mL SAK-L extraction after 48 hours induced the highest S phase peak of 70,74% and a G_2=0%.

UNIVERSITY of the
WESTERN CAPE

Figures 3.69 - 3.71 show histograms comparing the **hydrophilic extractions**. The hydrophilic extractions show an increase in the number of cells in the S phase and a decrease in cells present in the G_2 phase at 24 and 48 hours. An increase in the number of cells in the G_1 phase and a decrease in the number of cells in the S and G_2 phases after 72 hours indicate that cell resumed cell cycle progression.

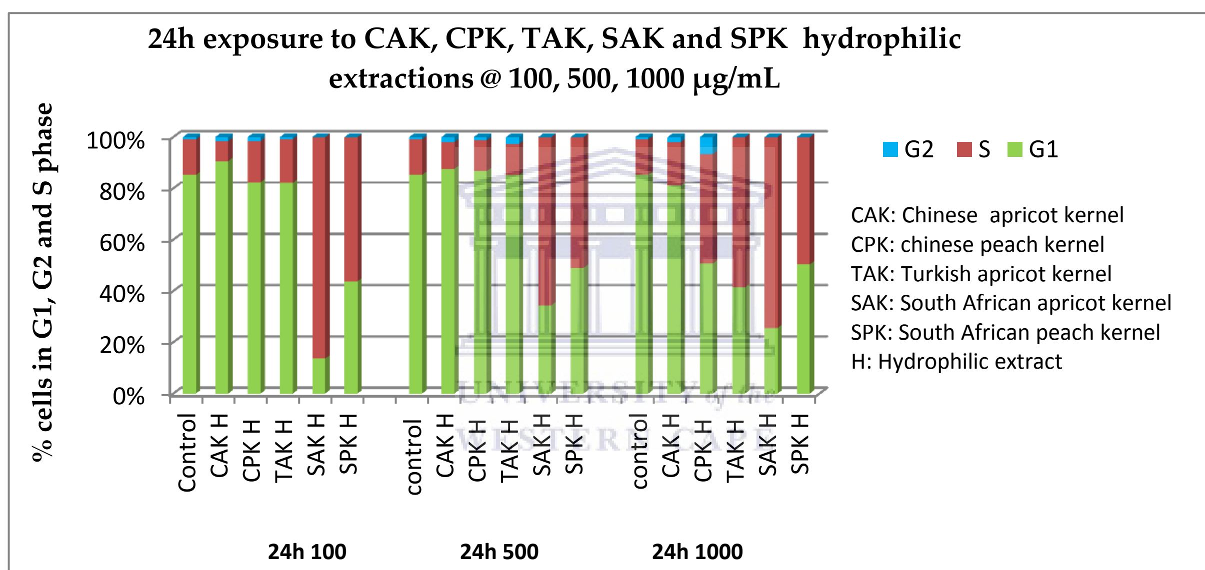


Figure 3.69: Flow cytometric analysis of HT-29 cells after 24 hour exposure to 100, 500 and 1000 µg/mL hydrophilic extractions of CAK, CPK, TAK, SAK and SPK. An increase in the number of cells present in the S-phase as well as a decrease in cells present in the G_2 phase (G_2=0%) was observed when samples were treated with 100 µg/mL SAK-H (S=86,4%) and SPK-H (S=56,25%); 500 µg/mL SAK-H (S=65,57%); and 1000 µg/mL SAK-H (S=74,47%) and TAK-H (S=58,62%). CAK-H and CPK-H show no inhibitory effect on the cell cycle progression (Annexure 2 for complete data report).

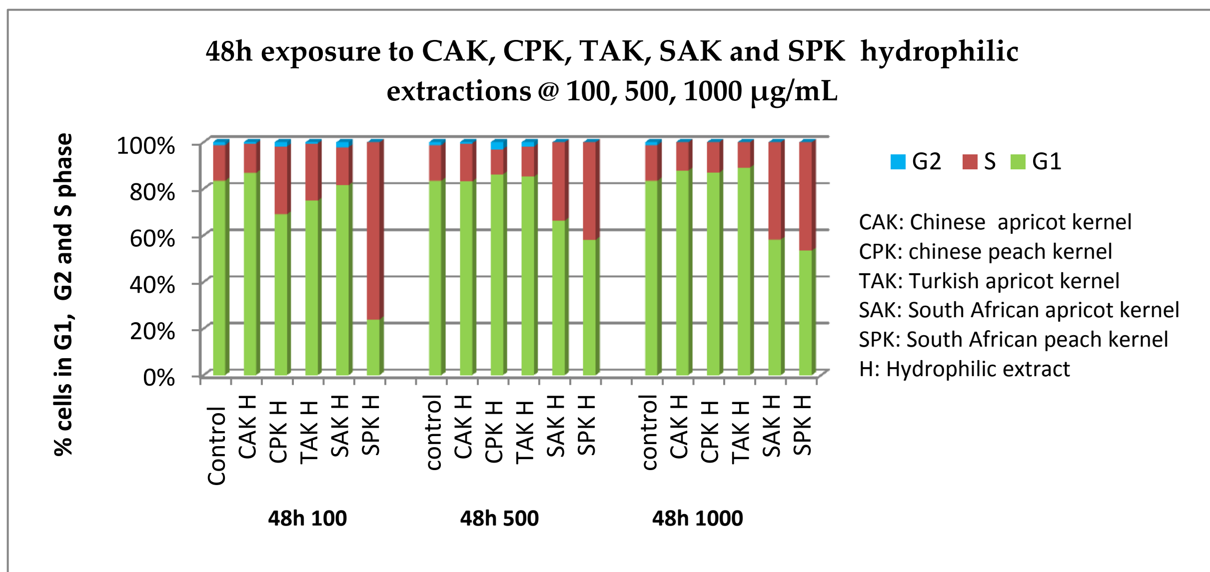


Figure 3.70: Flow cytometric analysis after 48 hour exposure to 100, 500 and 1000 µg/mL hydrophilic extractions of CAK, CPK, TAK, SAK and SPK. An increase in the number of cells in the S-phase as well as a decrease in cells present in the G_2 phase (G_2=0%) when treated with 100 µg/mL SPK-H (S=76,08%); 500 µg/mL SPK-H (S=41,88%); and 1000 µg/mL SPK-H (S=46,54%) was observed.

UNIVERSITY of the WESTERN CAPE

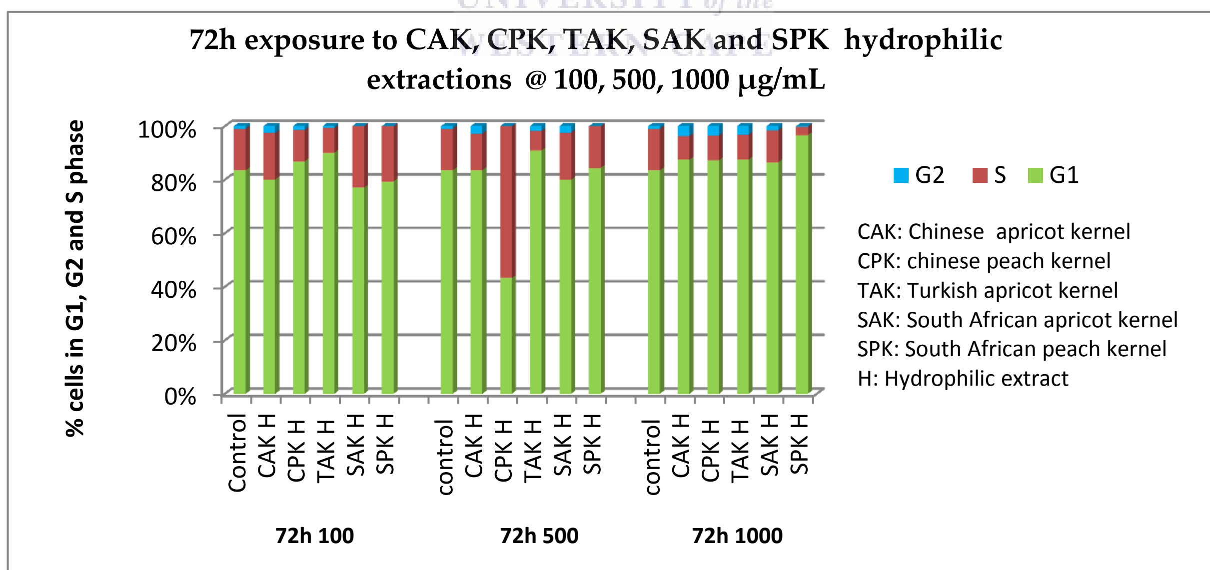


Figure 3.71: Flow cytometric analysis of HT-29 cells after 72 hour exposure to 100, 500 and 1000 µg/mL hydrophilic extractions of CAK, CPK, TAK, SAK and SPK. Analysis showed an outspoken increase in cells in the S-phase and a decrease in cells in G_2 phase (G_2=0%) when treated with 500 µg/mL CPK-H (S=56,73%). An increase in the number of cells in the G_1 phase and decrease in S phase indicates a recovery in cell cycle progression.

The exposure of the HT-29 colon cancer cells to the hydrophilic kernel extractions influenced the progression of the cell cycle by inducing an outspoken increase in the number of cells in the S phase with: 100 µg/mL SAK-H after 24 hour to 86,4% (Fig 3.69, Annexure 2), to 100 µg/mL SPK-H to 76,08% after 48 hours exposure (Fig 3.70) and to 56,73% with 500 µg/mL CPK-H after 72 hours (Fig 3.71). The CAK-H, CPK-H and TAK-H had shown very little effect on cell cycle progression, whilst a resumed progression in cell cycle is seen after 72 hours at 100 and 1000 µg/mL.

UNIVERSITY of the
WESTERN CAPE

Figures 3.72 - 3.76 show histograms comparing HT-29 colon cancer cells exposure to the respective CAK, CPK, TAK, SAK and SPK **organic extractions** after 24, 48 and 72 hour exposure at 100, 500 and 1000 µg/mL. The total and hydrophilic extractions show a more prevalent effect than the lipophilic extractions for the respective kernels.

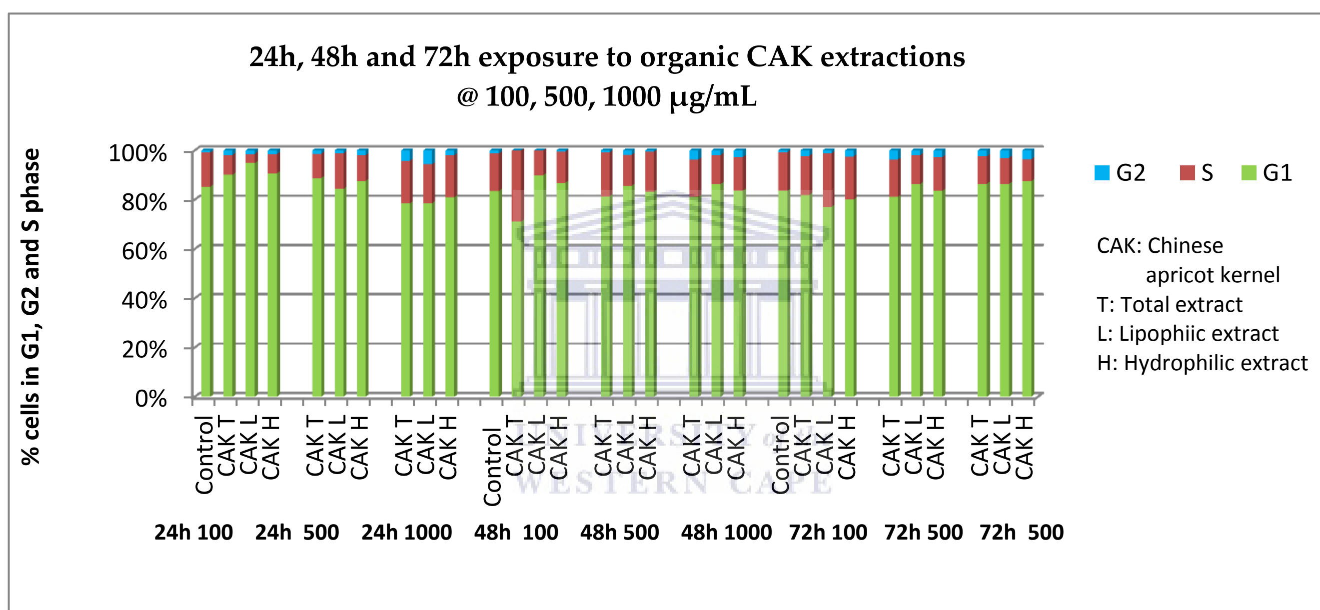


Figure 3.72: Flow cytometric analysis of HT-29 cells after 24h, 48h and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic CAK extractions. Cell cycle analysis showed an increase in cells in the S-phase and a decrease in cells in the G_2 phase (G_2=0%) when treated at 48h100 µg/mL CAK-T (S=28,95%). (Annexure 2 for complete data analysis).

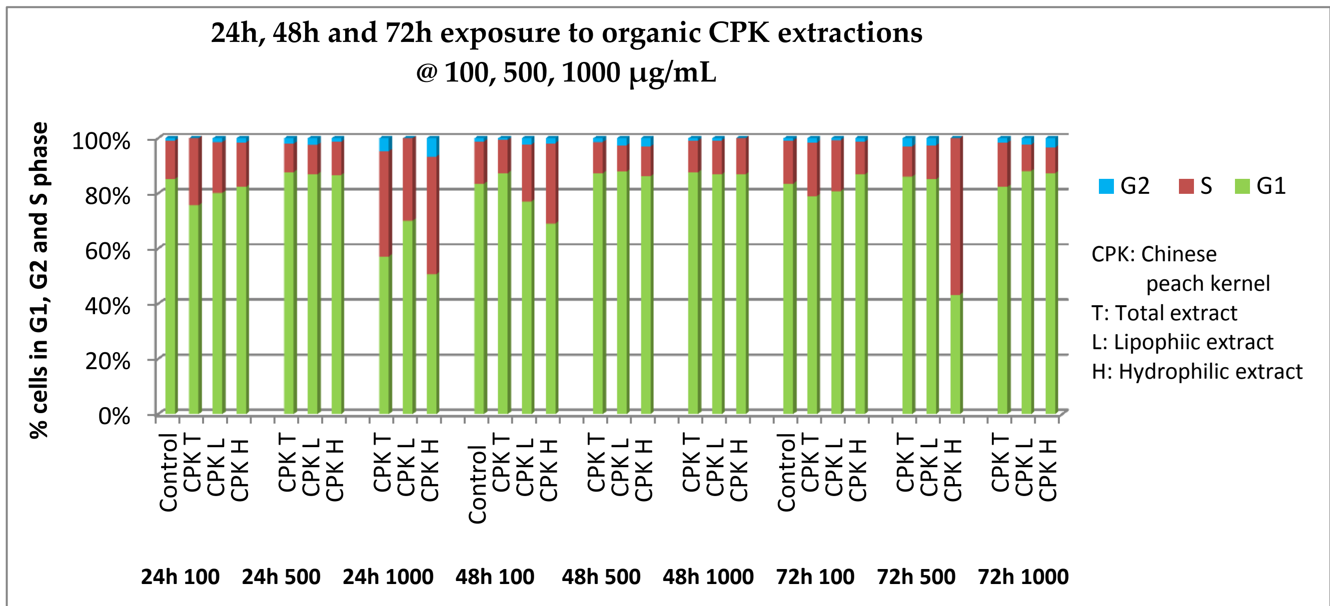


Figure 3.73: Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic CPK extractions. Cell cycle analysis showed an increase in cells in the S-phase as well as a marked decrease in cells in the G_2 phase (G_2=0%) when treated at 24h100 µg/mL CPK-T (S=24,32%), 24h1000 µg/mL CPK-L (S=29,9%) and 72h500 µg/mL CPK-H (S=56,73%).

UNIVERSITY of the
WESTERN CAPE

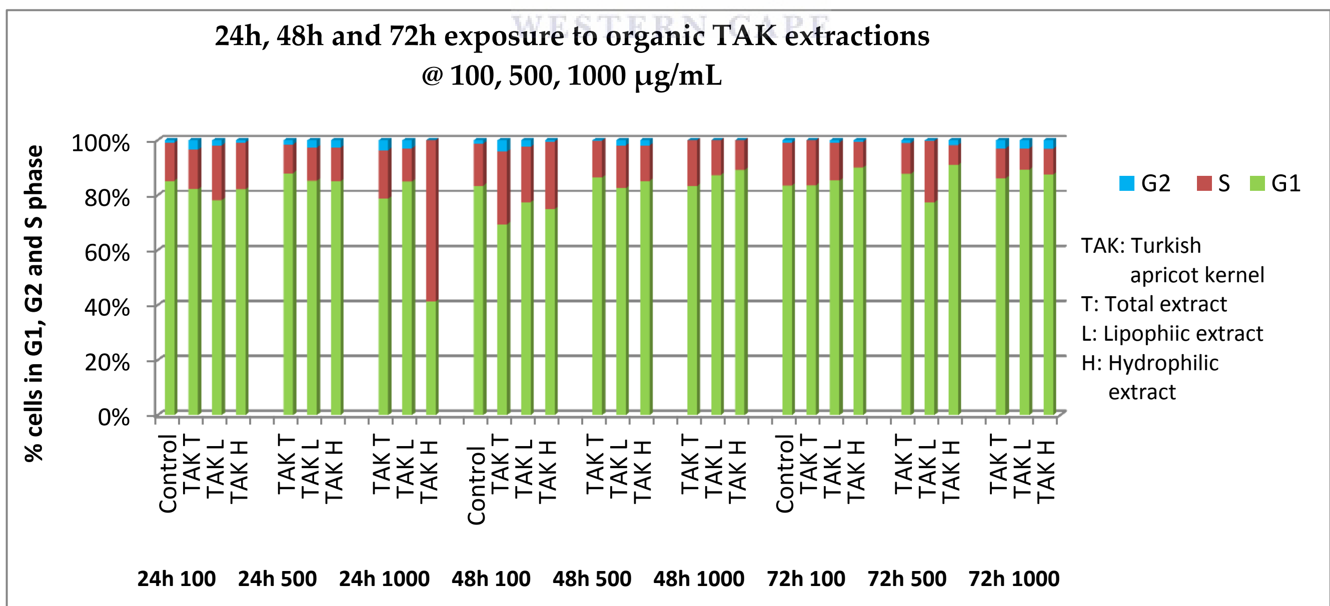


Figure 3.74: Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic TAK extractions. An outspoken increase in cells in the S-phase as well as a marked decrease in cells in the G_2 phase (G_2=0%) when treated at 24h1000 µg/mL TAK-H (S=58,62%). The rest of the fractions had no effect on cell cycle progression (Annexure 2).

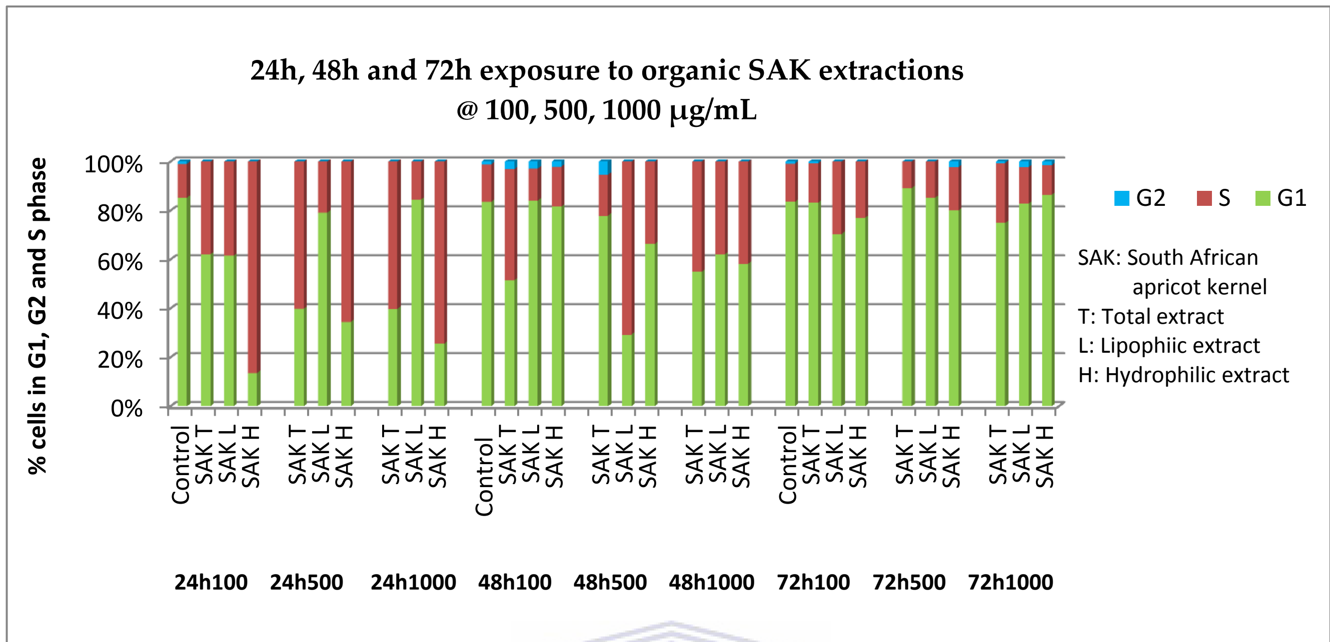


Figure 3.75: Flow cytometric analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic SAK extractions. Cell cycle analysis showed an outspoken increase in cells in the S-phase as well as a marked decrease in cells in the G_2 phase (G_2=0%) when treated at 24h100 µg/mL SAK-H (S=86,4%); 24h500 µg/mL SAK-H (S=65,57%) and SAK-T (S=60,13%); 24h1000 µg/mL SAK-H (S=74,47%) and SAK-T (S=60,19%); 48h500 µg/mL SAK-L (S=70,74%), SAK-H (S=33,66%); 48h1000 µg/mL SAK-T (S=45,13%); 72h100 µg/mL SAK-L (S=29,61%). No SAK fractions at 72h1000 µg/mL showed an effect on cell cycle progression and thus cells resume progression of the cell cycle after 72 hours (Annexure 2).

UNIVERSITY of the
WESTERN CAPE

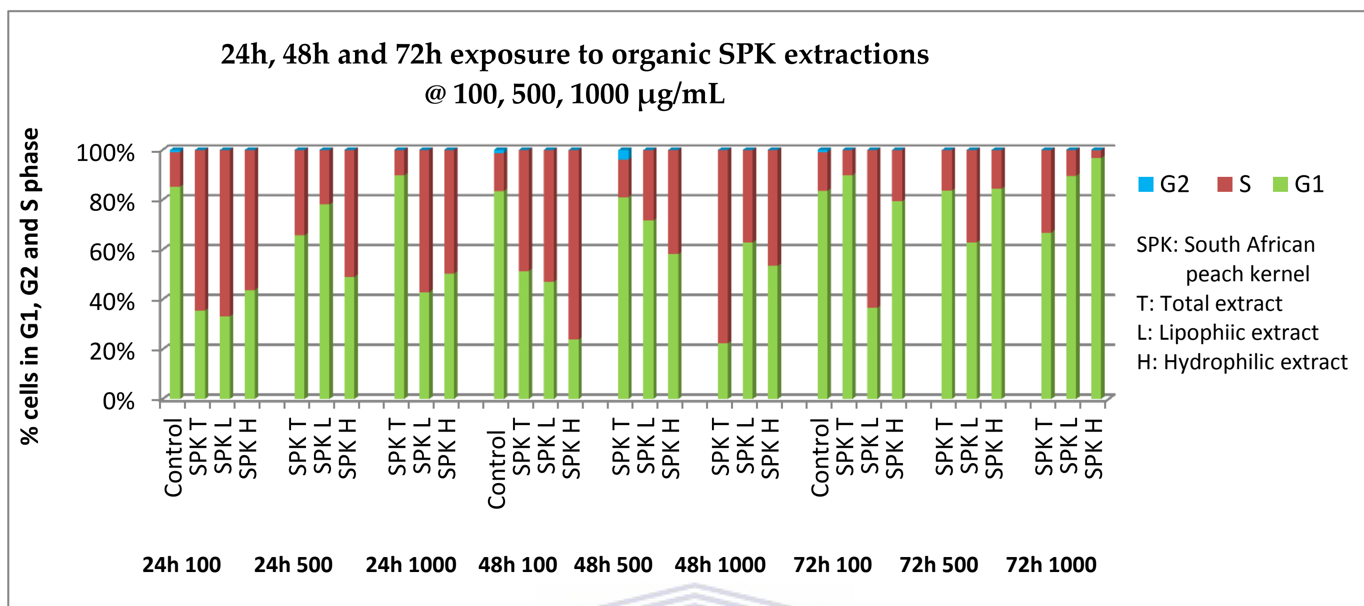


Figure 3.76: Flow cytometry analysis of HT-29 cells after 24, 48 and 72 hour exposure to concentrations of 100, 500 and 1000 µg/mL organic SPK extractions. Cell cycle analysis showed a significant increase in cells in the S-phase as well as a marked decrease in cells in the G_2 phase (G_2=0%) when treated at 24h100 µg/mL SPK-L (S=66,8%), SPK-T (S=64,47%), SPK-H (S=56,25%); 24h500 µg/mL SPK-H (S=50,98%); 24h1000 µg/mL SPK-L (S=57,12%); 48h100 µg/mL SPK-H (S=76,08%), SPK-L (S=52,94%); 48h500 µg/mL SPK-H (S=41,88%); 48h1000 µg/mL SPK-T (S=77,62%); 72h100 µg/mL SPK-L (S=63,44%); 72h500 µg/mL SPK-L (S=37,14%); and 72h1000 µg/mL SPK-T (S=33,16%). Cells resume cycle progression after 48 hours and 72 hours exposure to the SPK fractions (Annexure 2 for complete DNA analysis).

UNIVERSITY of the
WESTERN CAPE

DNA analysis of the respective kernel fractions at the relevant time periods and concentrations show that none of the CAK fractions show a significant inhibition of the cell cycle (Fig 3.72, Annexure 2).

The CPK and TAK fractions had a minor effect on the cell cycle where CPK-H at 72h500 µg/mL and TAK-H at 24h 1000 µg/mL showed the highest increase in the number of cells in the S phase (S=56,73% and S=58,62% respectively).

Most of the SAK and SPK fractions showed an effect on cell cycle progression with the SAK-H fraction at 24h100 µg/mL (S=86,4%) and the SPK-T fraction at 48h1000 µg/mL (S=77,62%) showing the most outspoken increase in the number of cells in the S phase (Fig 3.75 and 3.76, Annexure 2). A complete repair and recovery in the damaged DNA after 72 hours exposure to the SAK and SPK fractions is however seen with a decrease in the number of cells in the S phase.

Table 3.5 shows a summary of the flow cytometric data analysis indicating the organic kernel extractions that caused an increase in the number of cells in the S phase and a decrease in cells in G_2 phase. The period of exposure, concentration and kernel extraction is shown.

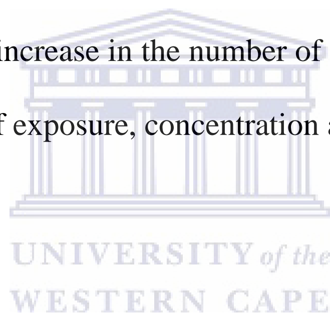

UNIVERSITY of the
WESTERN CAPE

Table 3.5: Summary of the organic kernel extractions that significantly altered cell cycle progression, increasing the number of cells in the S phase and decreasing the number of cells in the G_2 phase of the HT-29 colon cancer cells after 24, 48 and 72 hours exposure to 100, 500 and 1000 µg/mL.

KERNEL TYPE	KERNEL EXTRACTION (in order of significance)	TIME of EXPOSURE (hours)	CONCENTRATION (µg/mL)
CAK	Total	48h	100
CPK	Total	24h	100
	Lipophilic	24h	1000
	Hydrophilic	72h	500
TAK	Hydrophilic	24h	1000
	Total	72h	100
SAK	Hydrophilic	24h	100
	Lipophilic	24h	100
	Total	24h	100
	Hydrophilic	24h	500
	Lipophilic	24h	500
	Total	24h	500
	Hydrophilic	24h	1000
	Lipophilic	24h	1000
	Total	24h	1000
	Lipophilic	48h	500
	Hydrophilic	48h	500
	Total	48h	1000
	Hydrophilic	48h	1000
	Lipophilic	48h	1000
	Lipophilic	72h	100

UNIVERSITY of the
WESTERN CAPE

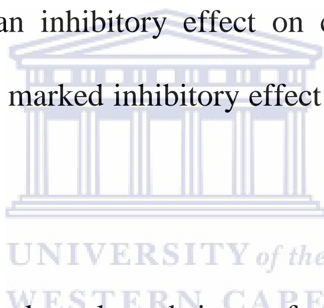
	hydrophilic	72h	100
SPK	Total	24h	100
	Lipophilic	24h	100
	Hydrophilic	24h	100
	Hydrophilic	24h	500
	Lipophilic	24h	500
	Total	24h	500
	Lipophilic	24h	1000
	Hydrophilic	24h	1000
	Hydrophilic	48h	100
	Lipophilic	48h	100
	Total	48h	100
	Hydrophilic	48h	500
	Lipophilic	48h	500
	Total	48h	1000
	Hydrophilic	48h	1000
	Lipophilic	48h	1000
	Hydrophilic	72h	100
	Lipophilic	72h	100
	Lipophilic	72h	500
	Total	72h	500
	Total	72h	1000

UNIVERSITY of the
WESTERN CAPE

All the organic kernel extractions affected cell cycle progression by producing an S phase block to varying degrees of exposure to the type of kernel extraction, duration and concentration. The CAK, CPK and TAK organic extracts had a minimal effect on cell cycle progression with the total and hydrophilic fractions reflecting more frequently.

The most marked and consistent effect on cell cycle progression by increasing the number of cells in the S phase was seen by all the SAK and SPK organic fractions to varying degrees of efficacy. All SAK organic fractions at the respective concentrations after 24 hours, and all the SAK organic fractions at 500 and 1000 µg/mL after 48 hours, as well as SAK-L and SAK-H at 100 µg/mL after 72 hours showed an effect on cell cycle progression. SAK-H at 24h100 µg/mL showed the most outspoken increase in cells in the S phase (86,4%) (Annexure 2).

All SPK organic fractions at the respective concentrations after 24 and 48 hours, as well SPK-L and SPK-H at 100 µg/mL, SPK-T and SPK-L at 500 µg/mL and SPK-T at 1000 µg/mL after 72 hours induced an inhibitory effect on cell cycle progression. SPK-T at 48h1000 µg/mL showed the most marked inhibitory effect by inducing a peak in the S phase to 77,625% (Annexure 2).


UNIVERSITY of the
WESTERN CAPE

Of all the organic fractions of the kernels and times of exposure it was SAK-H at 24h100 µg/mL that showed the biggest effect on cell cycle progression by inducing the highest peak in the S phase (86,4%) (Annexure 2).

Figure 3.77 (a) – (n) show a diagrammatic representation of the cell cycle analysis of HT-29 human colon cancer cells grown in the presence of organic and aqueous kernel extractions for 24h, 48h and 72h at various concentrations.

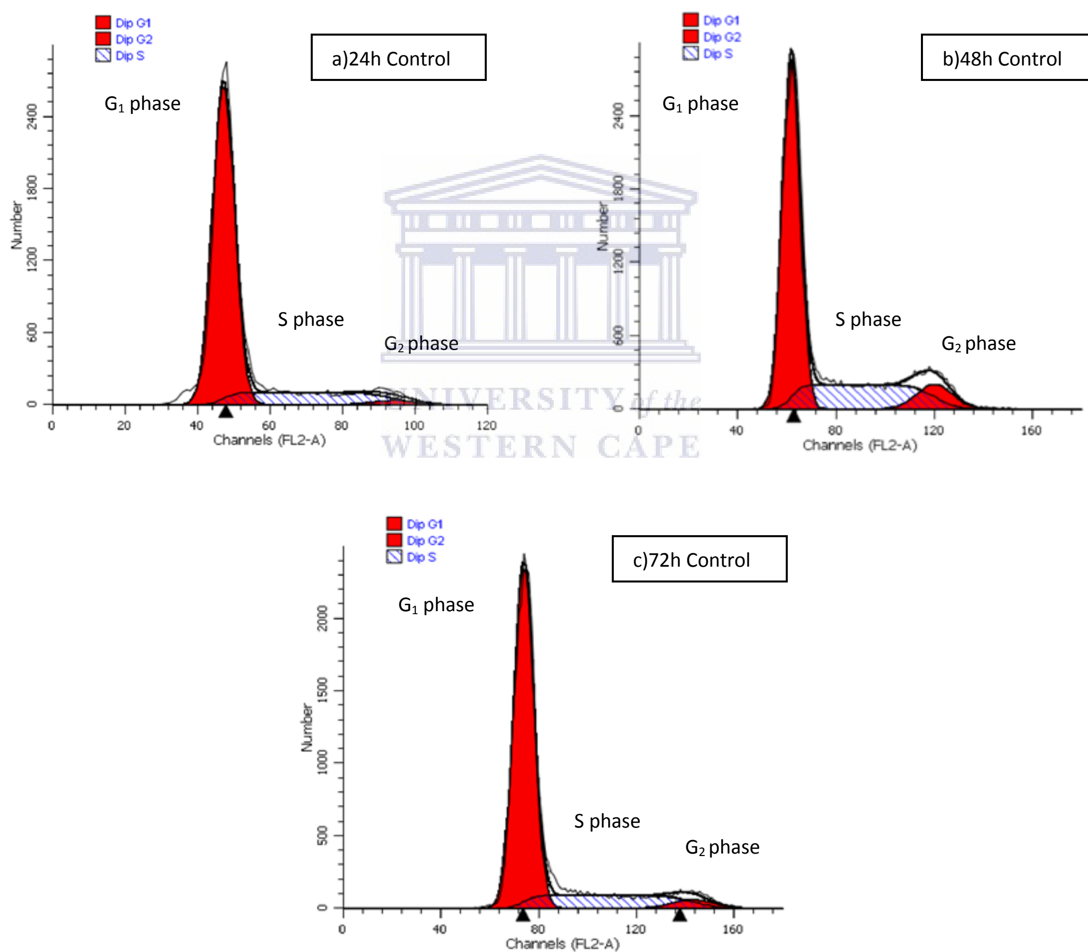
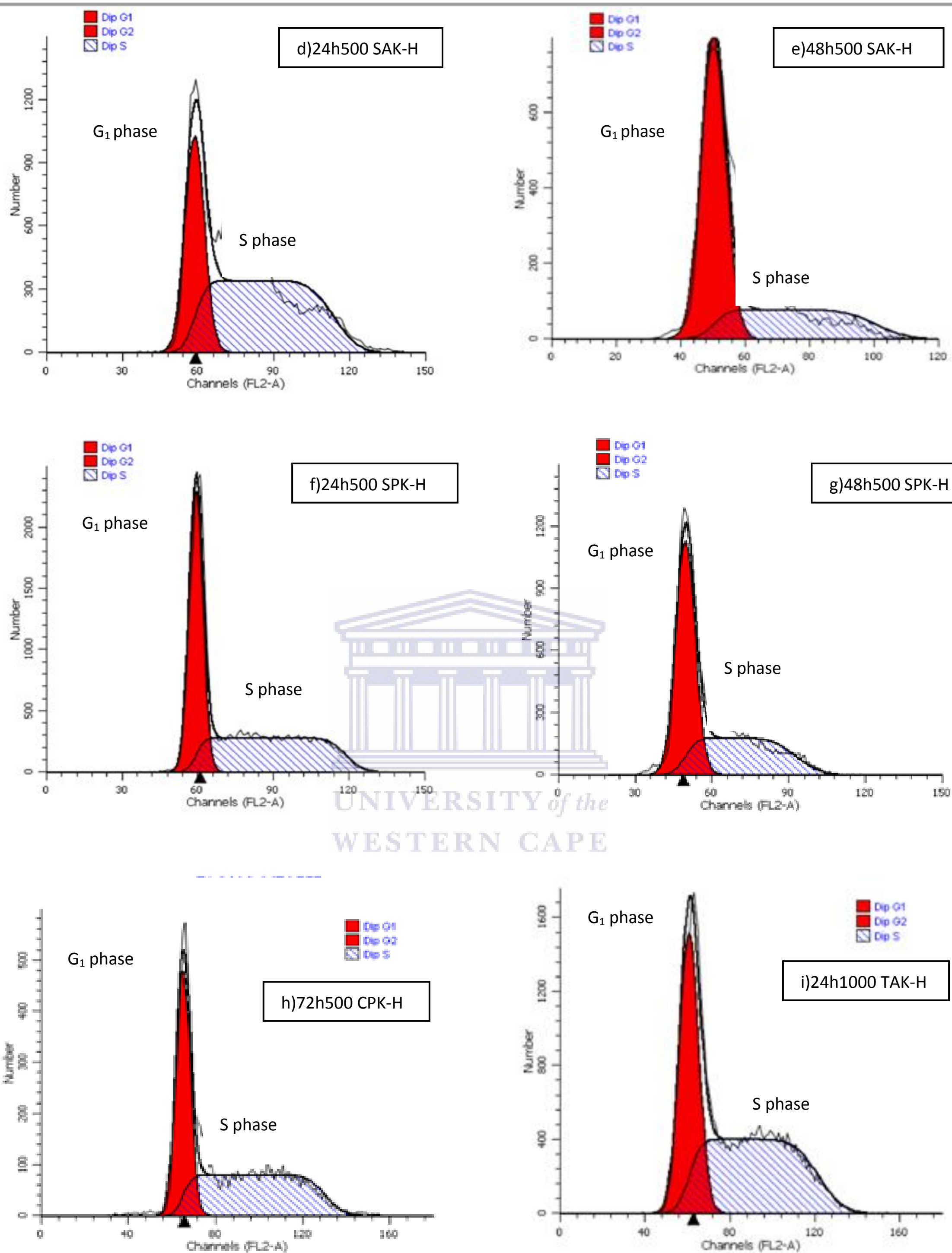
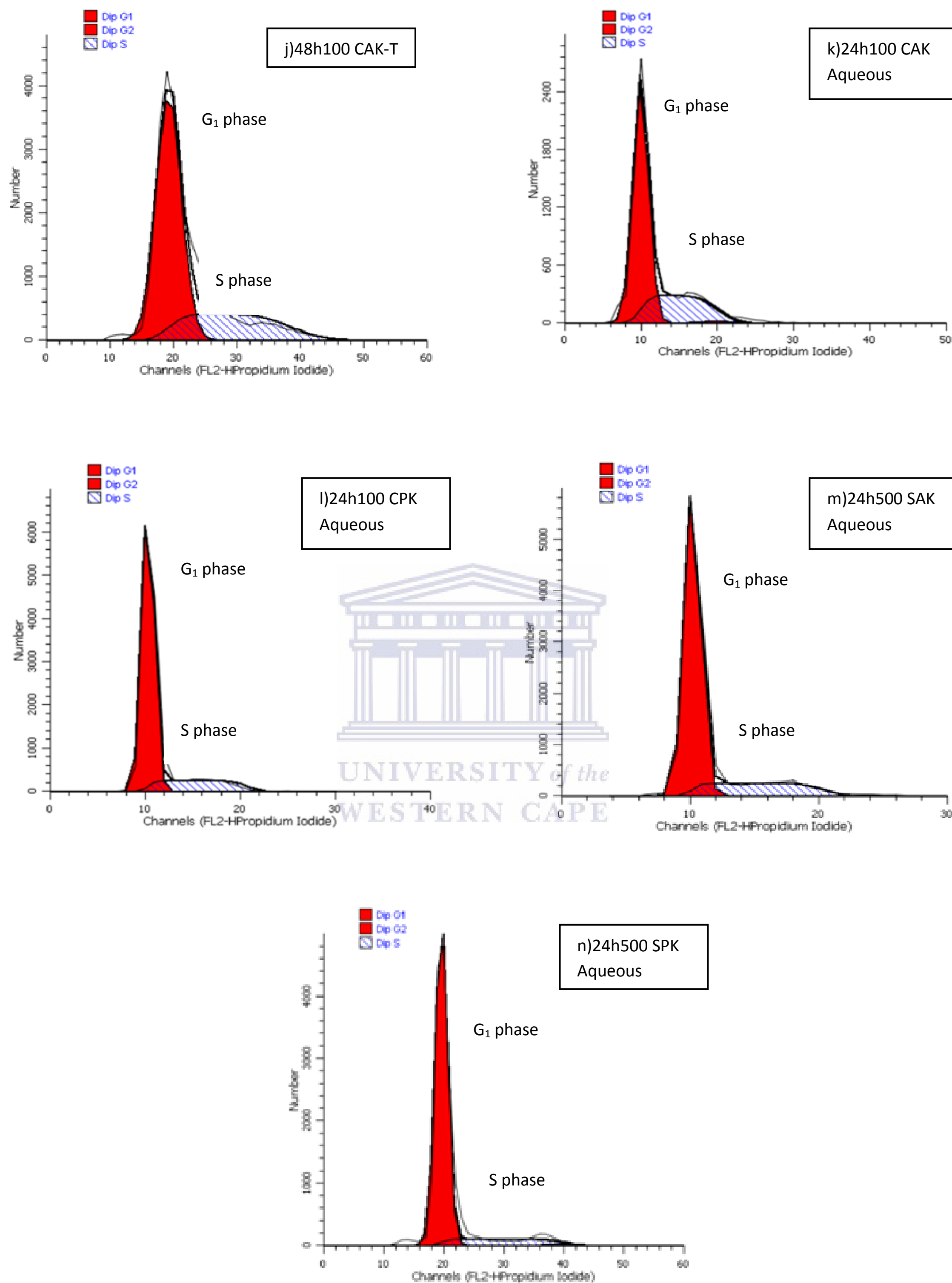


Figure 3.77 (a) – (c) show the 24h, 48h and 72 controls. Normal G_1, S and G_2 phases are seen after calibration to the Nile red beads used for calibration.



UNIVERSITY of the
WESTERN CAPE

Figure 3.77 (d) – (i) illustrates HT-29 colon cancer cells exposed to organic extractions of SAK-H, SPK-H, CPK-H and TAK-H at different times and concentrations. 24h and 48h exposure of HT-29 colon cancer cells to 500 µg/mL SAK-H, SPK-H show an increase in cells in S phase and no cells in the G_2 phase (d – g). a similar effect was observed after treating the cells with 72h500 µg/mL CPK-H, 24h1000 µg/mL TAK-H and 48h100 µg/mL CAK-T (h – j).



UNIVERSITY of the
WESTERN CAPE

Figure 3.77 (k) – (n) show HT-29 colon cancer cells exposed to 500 µg/mL aqueous extractions of CAK, CPK, SAK and SPK at 24h and 48h. An increase in cells in the S phase and a decrease in cells in the G_2 phase compared to the controls is seen after treatment with 24h100 µg/mL CAK and CPK and 24h500 µg/mL SAK and SPK aqueous extractions (k –n).

3.4.3 Cell cycle progression over 24, 48 and 72 hours after treatment with 100, 500 and 1000 µg/mL CAK, CPK, SAK and SPK aqueous extractions

Figures 3.78 - 3.83 show time and dose related histograms of the respective 24, 48 and 72 hour exposure of HT-29 colon cancer cells to the respective 100, 500 and 1000 µg/mL of **CAK aqueous (CAK-Aqu), CPK aqueous (CPK-Aqu), SAK aqueous (SAK-Aqu) and SPK aqueous (SPK-Aqu) extractions**. All the aqueous extractions influenced cell cycle progression differently depending on the time of exposure and concentration of the extract. The first three figures (3.78 – 3.80) compares the effects of the specific concentrations of the aqueous extractions for the three exposure times, while figures 3.81 – 3.83 compare the effects of all the concentrations of the aqueous extractions over a specific time of exposure on cell cycle progression.

UNIVERSITY of the
WESTERN CAPE

Figure 3.78 show cell cycle progression after 100 µg/mL exposure at 24, 48 and 72 hours respectively. The 100 µg/mL CAK-Aqu extract induced the highest increase in the number of cells in the S phase to 30.68% after 24 hours and again at 72 hours (35,77%). After 48 hours no outspoken changes in cell cycle progression was observed.

Looking at the 500 µg/mL aqueous concentrations (Fig 3.79), CAK-Aqu caused an S phase peak after 24 hours (33,83%) while SAK-Aqu increased the cell numbers in the S phase after 72 hours (32,81%).

The 1000 µg/mL CPK-Aqu concentration influenced cell cycle progression after 24 hours and 72 hours (Fig 3.80). No outspoken effect on cell cycle progression was observed with the other kernels at this high concentration.

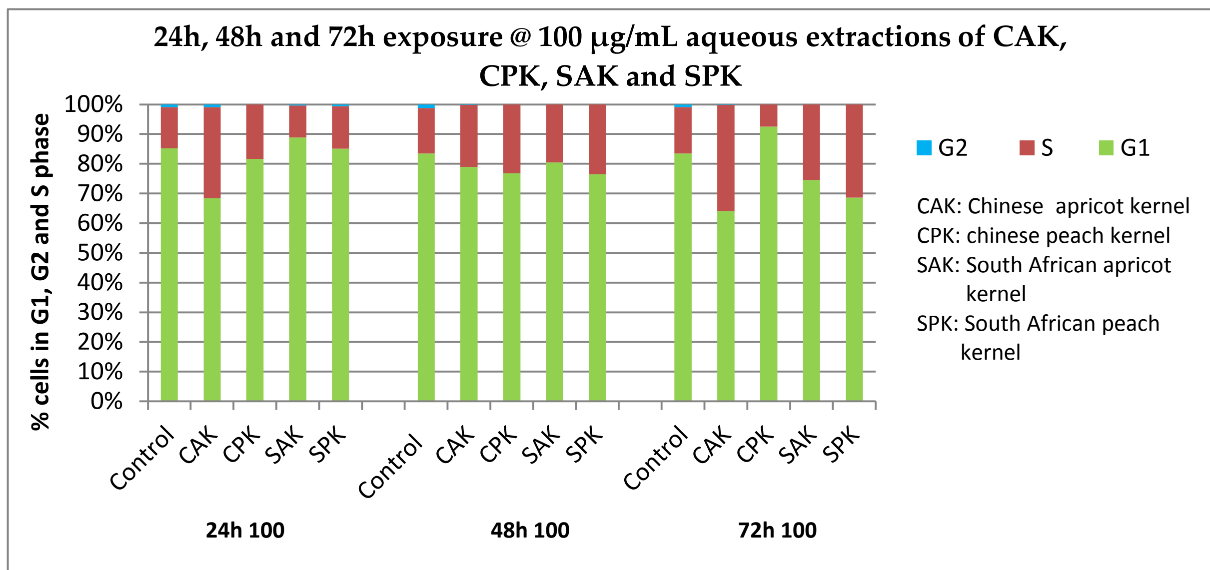


Figure 3.78: Flow cytometric analysis of HT-29 cells after 24h, 48h and 72h exposure to concentration of 100 µg/mL aqueous extractions of CAK, CPK, SAK and SPK. Analysis showed an increase in cells in the S-phase as well as a marked decrease in cells in the G_2 phase (G_2=0%) when treated at 24h100 µg/mL CAK-Aqu extract (30,68%) and again after 72h100 µg/mL (35,77%) (Annexure 2 for complete data analysis report).

UNIVERSITY of the WESTERN CAPE

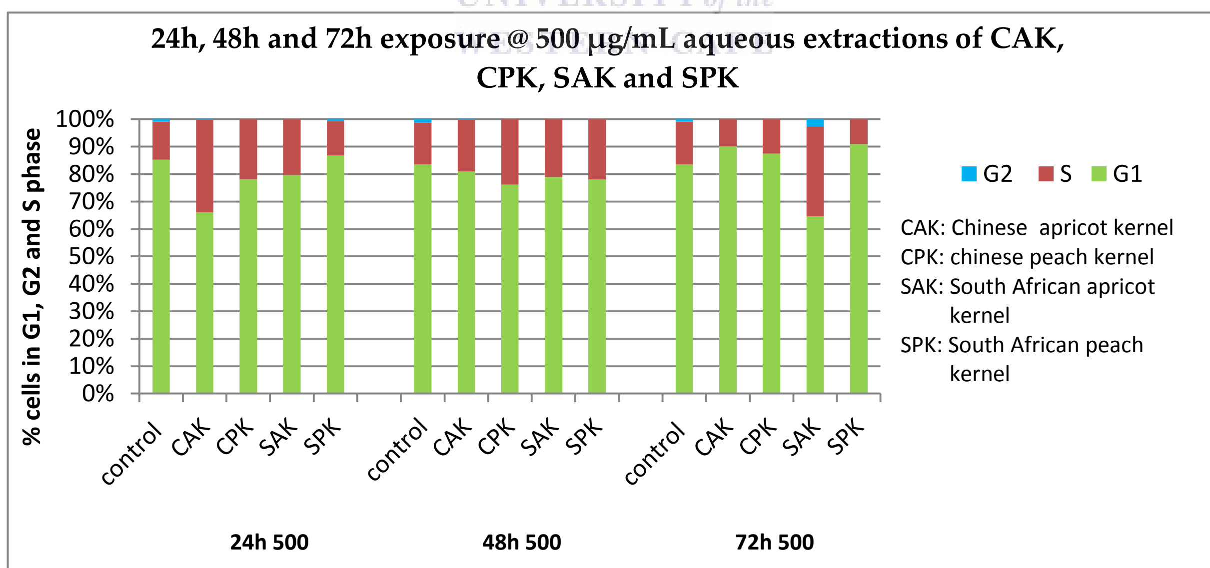


Figure 3.79: Flow cytometric analysis of HT-29 cells after 24h, 48h and 72h exposure to concentration of 500 µg/mL aqueous extractions of CAK, CPK, SAK and SPK. The highest peak in the S-phase was observed after 24h500 µg/mL CAK-Aqu (S=33,83%) and after 72h500 µg/mL SAK-Aqu extraction (32,81%) (Annexure 2).

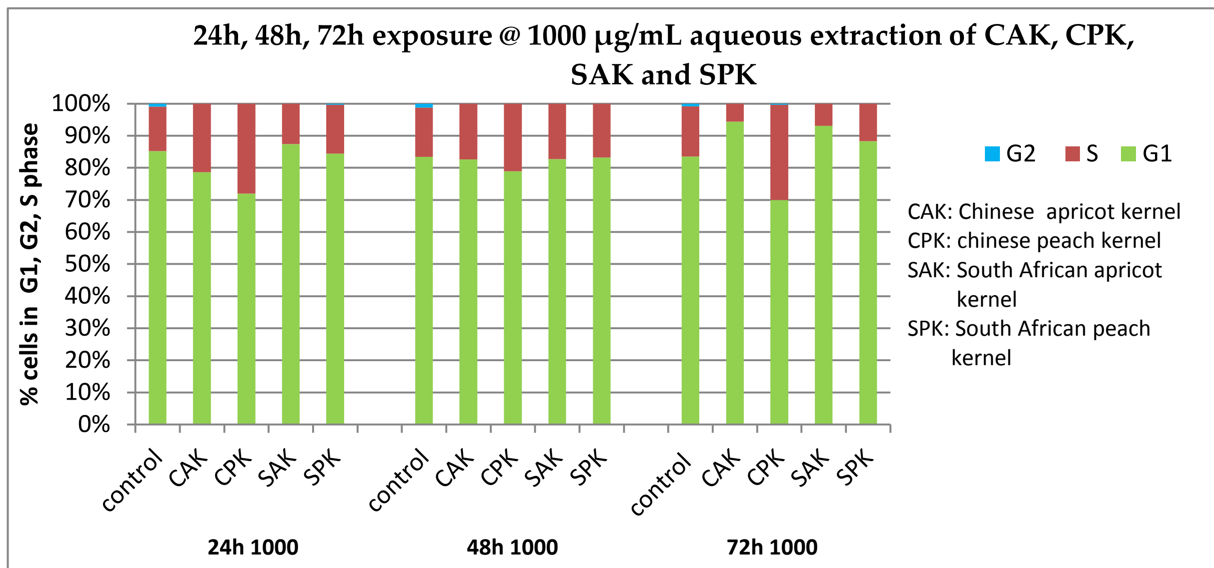


Figure 3.80: Flow cytometric analysis of HT-29 cells after 24h, 48h and 72h exposure to aqueous extractions of CAK, CPK, SAK and SPK at concentration of 1000 µg/mL. Cell cycle analysis showed that an increase in the number of cells in the S-phase as well as a marked decrease in cells in the G_2 phase (G_2=0%) was seen when cells were exposed to 1000 µg/mL CPK-Aqu extract after 24 hours (S=28,03%) and again after 72 hours (S=29,66%). No further effect was observed at this high concentration for the other kernels (Annexure 2).

UNIVERSITY of the
WESTERN CAPE

Figures 3.81 - 3.83 show dose related histograms comparing a specific time of exposure to all the aqueous extractions concentrations. After a 24 hour exposure all three concentrations of the CAK-Aqu (S=30,68%, 33,83% and 21,38% respectively) and CPK-Aqu extraction (S=18,39%, 21,88% and 28,03% respectively) showed an increase in the number of cells in the S phase (Fig 3.81). In comparison, after 72 hours exposure the 100 µg/mL CAK-Aqu, SAK-Aqu and SPK-Aqu extracts showed an increase in cell number in the S phase to 35,77%, 25,48% and 31,4% respectively (Fig 3.83), while only the 1000 µg/mL CPK-Aqu extract induced an S phase increase to 29,66%.

At a 48 hour exposure to the respective aqueous extractions however, only a minimal increase in the number of cells in the S phase was seen (Fig 3.82).

UNIVERSITY of the
WESTERN CAPE

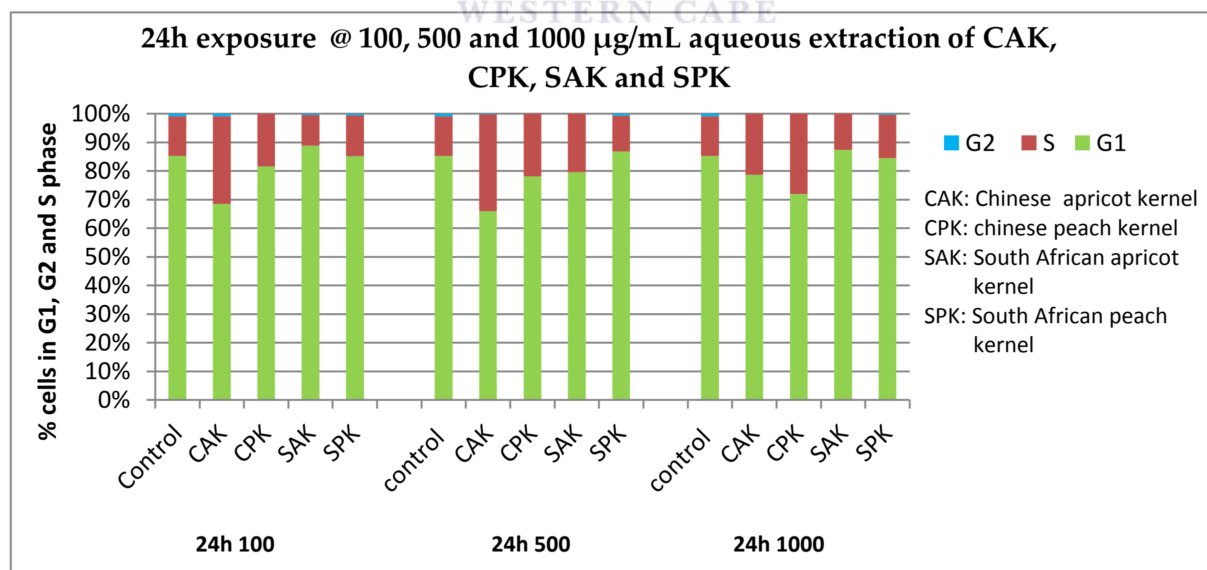


Figure 3.81: Flow cytometric analysis of HT-29 cells after 24h exposure to concentrations of 100, 500 and 1000 µg/mL aqueous extractions of CAK, CPK, SAK and SPK. Analysis showed the highest increase in cells in the S-phase when treated with 100 µg/mL CAK-Aqu extract (S=30,68%); 500 µg/mL CAK-Aqu extract (S=33,83%); and 1000 µg/mL CPK-Aqu extract (S=28,03%).

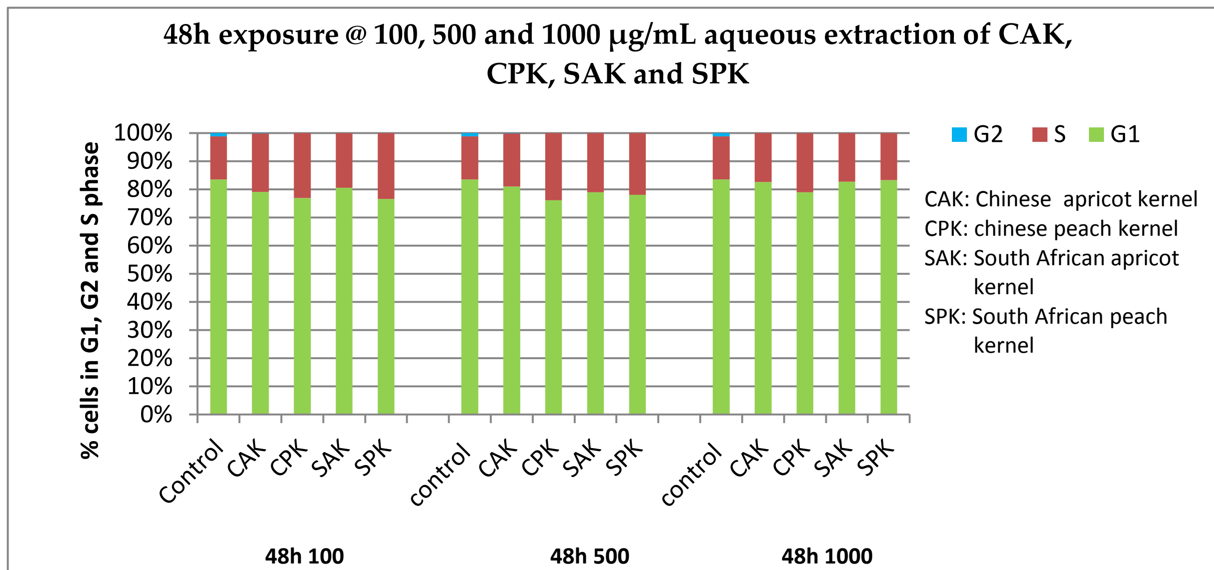


Figure 3.82: Flow cytometric analysis of HT-29 cells after 48h exposure to aqueous extractions of CAK, CPK, SAK and SPK at concentrations of 100, 500 and 1000 µg/mL. Cell cycle analysis showed a limited increase in cells in the S-phase after 48 hour exposure (Annexure 2).

UNIVERSITY of the
WESTERN CAPE

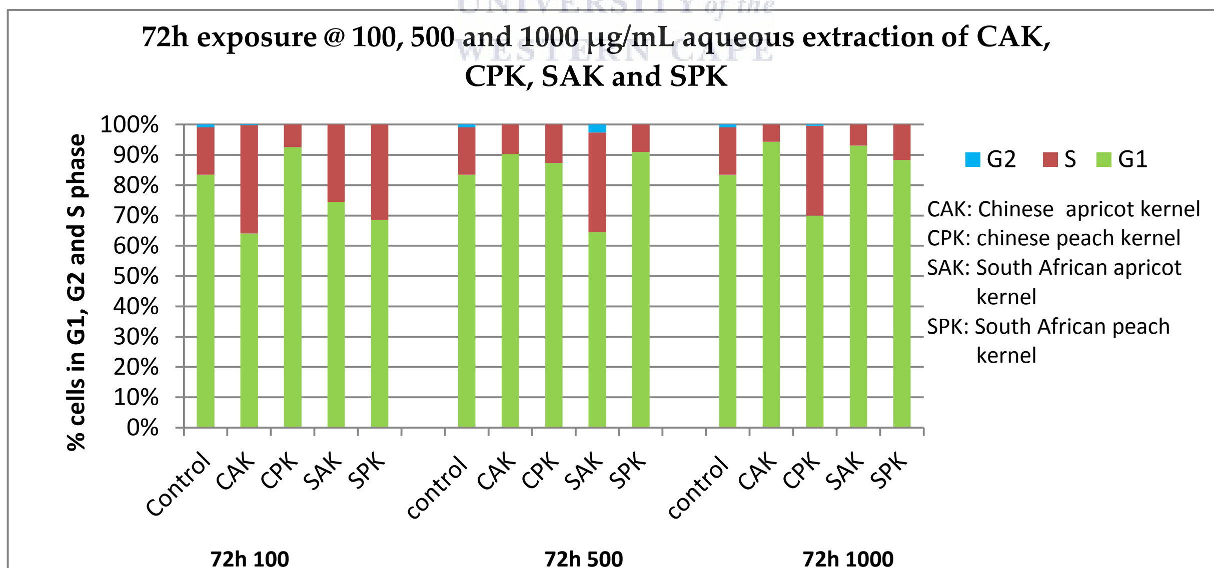


Figure 3.83: Flow cytometric analysis of HT-29 cells after 72h exposure to concentrations of 100, 500 and 1000 µg/mL aqueous extractions of CAK, CPK, SAK and SPK. Cell cycle analysis showed the most outspoken increase in cells in the S-phase after 72 hours when treated with 100 µg/mL CAK-Aqu extract (S=35,77%); 1000 µg/mL CPK-Aqu extract (S=29,66%).

Table 3.6: Summary of the aqueous kernel extractions that significantly altered cell cycle progression, increasing the number of cells in the S phase and decreasing the number of cells in the G_2 phase of the HT-29 colon cancer cells after 24, 48 and 72 hours exposure to 100, 500 and 1000 µg/mL.

KERNEL TYPE	KERNEL EXTRACTION (in order of significance)	TIME of EXPOSURE (hours)	CONCENTRATION (µg/mL)
CAK	Aqueous	24h	100
		24h	500
		24h	1000
		48h	100
		48h	500
		48h	1000
		72h	100
CPK	Aqueous	24h	100
		24h	500
		24h	1000
		48h	100
		48h	500
		48h	1000
		72h	1000
SAK	Aqueous	24h	500
		48h	100
		48h	500
		48h	1000
		72h	100
		72h	500
SPK	Aqueous	24h	100
		24h	1000

UNIVERSITY of the
WESTERN CAPE

		48h	100
		48h	500
		48h	1000
		72h	100

Table 3.6 shows that all the aqueous kernel extractions showed an inhibitory effect on cell proliferation to varying degrees of exposure to the kernel type, duration and concentration.

CAK-Aqu extract affected cell cycle progression at all concentrations after 24 and 48 hours and at only 100 µg/mL after 72 hours (Annexure 2) which also showed the highest increase in cells in the S phase (35,77%) for all aqueous kernel treatments. Whereas CPK-Aqu extract affected cell cycle progression at all concentrations after 24 and 48 hours but only with 1000 µg/mL concentration (29,66%) after 72 hours (Annexure 2).

UNIVERSITY of the
WESTERN CAPE

The 500 µg/mL SAK-Aqu extraction after all exposure times and 100 µg/mL concentration after 48 and 72 hours induced a peak in the S phase with the most outspoken increase shown after 72h500 µg/mL exposure. While the 100 µg/mL SPK-Aqu extract after all exposure times, 1000 µg/mL after 24 and 48 hours, and only 500 µg/mL after 48 hours induced an increase in the number of cells in the S phase. SPK-Aqu after 72h100 µg/mL exposure induced the highest increase in the number of cells in the S phase (31,4%).

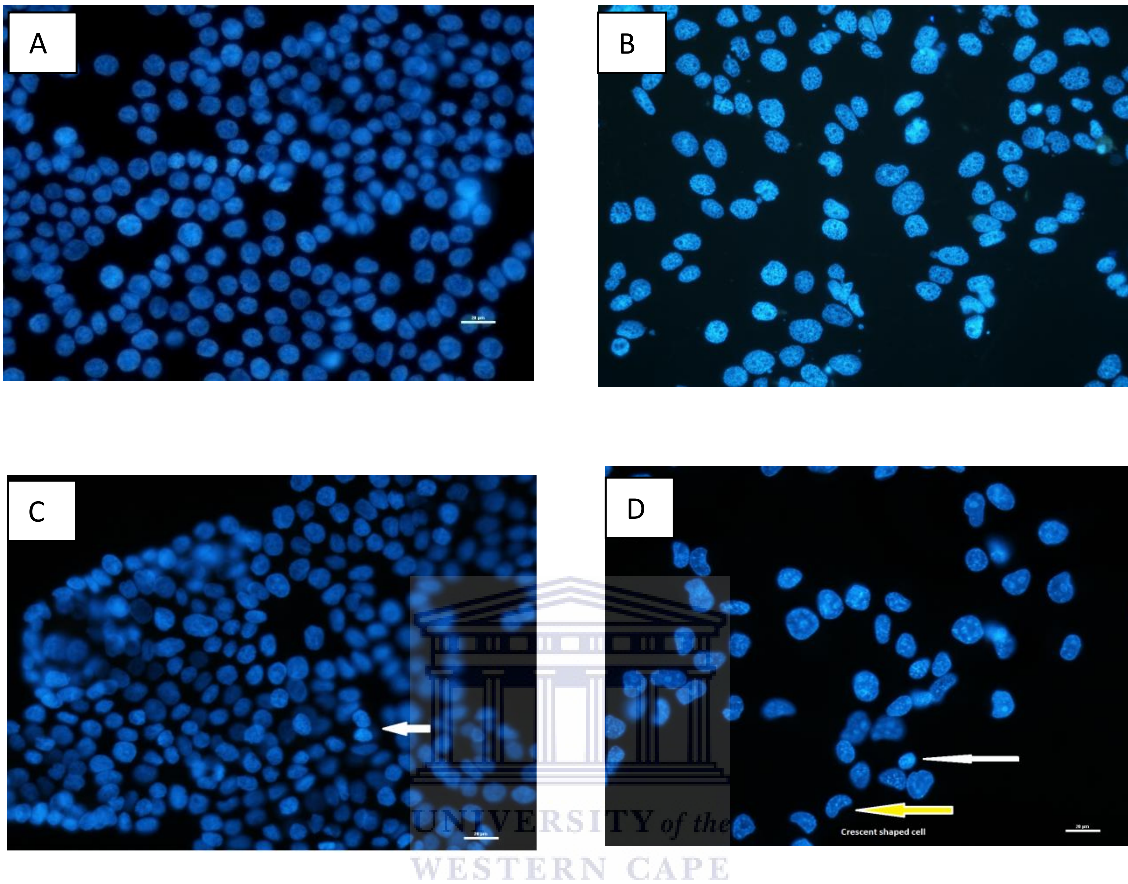
In summary, all the aqueous kernel extractions induced an S phase peak after 48 hours at all concentrations whilst the aqueous extract that induced the most outspoken increase in the numbers of cells in the S phase was the 100 µg/mL CAK-Aqu extract after 72 hours.

The increase in the number of cells in the S phase could be indicative of an S phase block. DNA synthesis occurs during the S phase and exogenous stressors can compromise the replication process by slowing down or stalling fork progression. The results show that the organic as well as the aqueous extractions affect DNA synthesis temporarily by inducing the S phase peaks mostly after 24 and 48 hours. After 72 hours most of the cells resume cell cycle progression and no difference between control and treated samples are seen.

3.5 Hoechst 33342 fluorescent stain

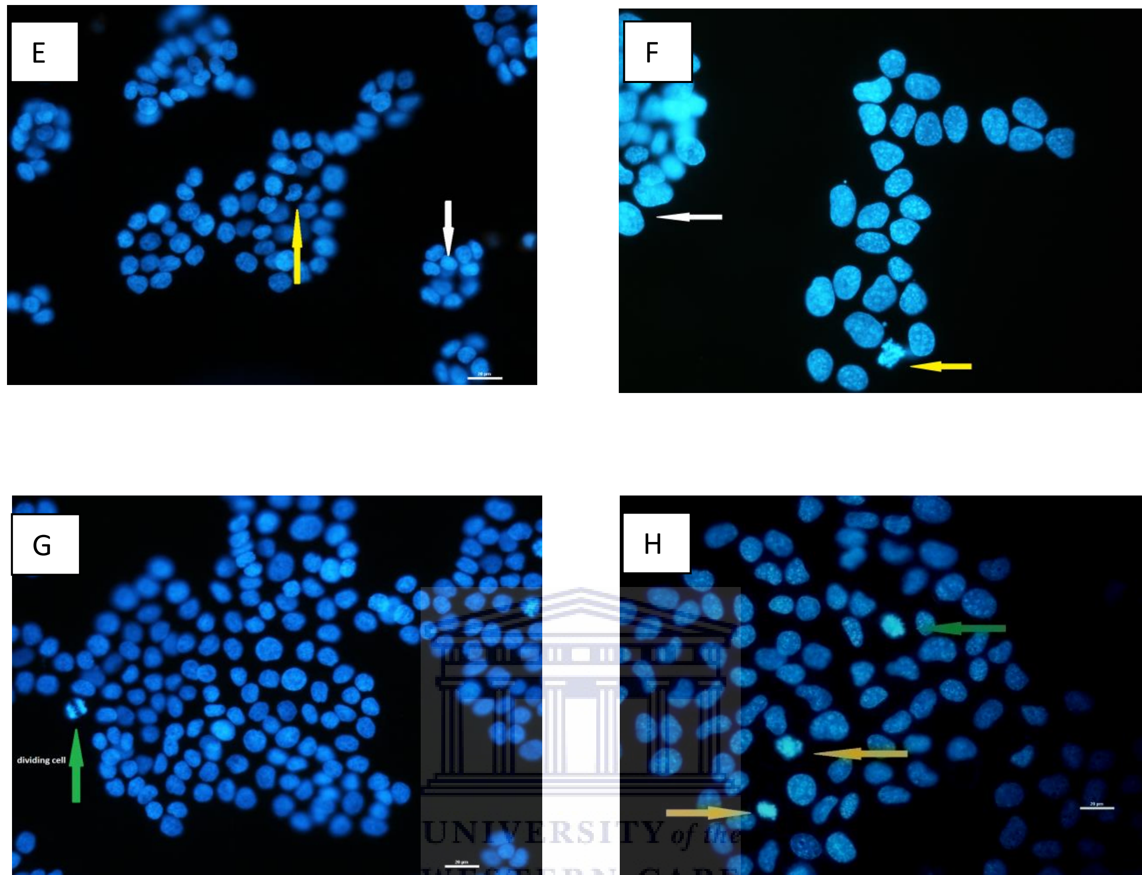
Normal cell cycle progression is important for cell function. A change in signalling pathways may result in a change in cell morphology and thus cell function. A morphological study is important for determining changes in signalling pathways to verify morphological changes and the presence of apoptotic cells. The cells were stained with Hoechst 33342 to assess apoptosis in HT-29 colon cancer cells by staining highly condensed chromatin of apoptotic cells and lightly staining the looser chromatin structure of viable cells. Treatment of the HT-29 human colon cancer cell with 500 µg/mL organic kernel extractions of SAK-H, SPK-H and SPK-L at 24 and 48 hours, and 500 µg/mL aqueous kernel extractions of CAK, CPK, SAK and SPK at 24 and 48 hours was done based on the results of the flow cytometric analysis which showed the HT-29 colon cancer cells as being significantly influenced by these kernel extractions. By studying the cell nucleoli we are able to observe structural changes, cell viability including cytotoxic effects leading to apoptosis. Figure 3.84 (A) – (H) show fluorescent stained HT-29 human colon cancer cells treated with organic kernel extractions at 24 and 48 hour 500 µg/mL.

UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

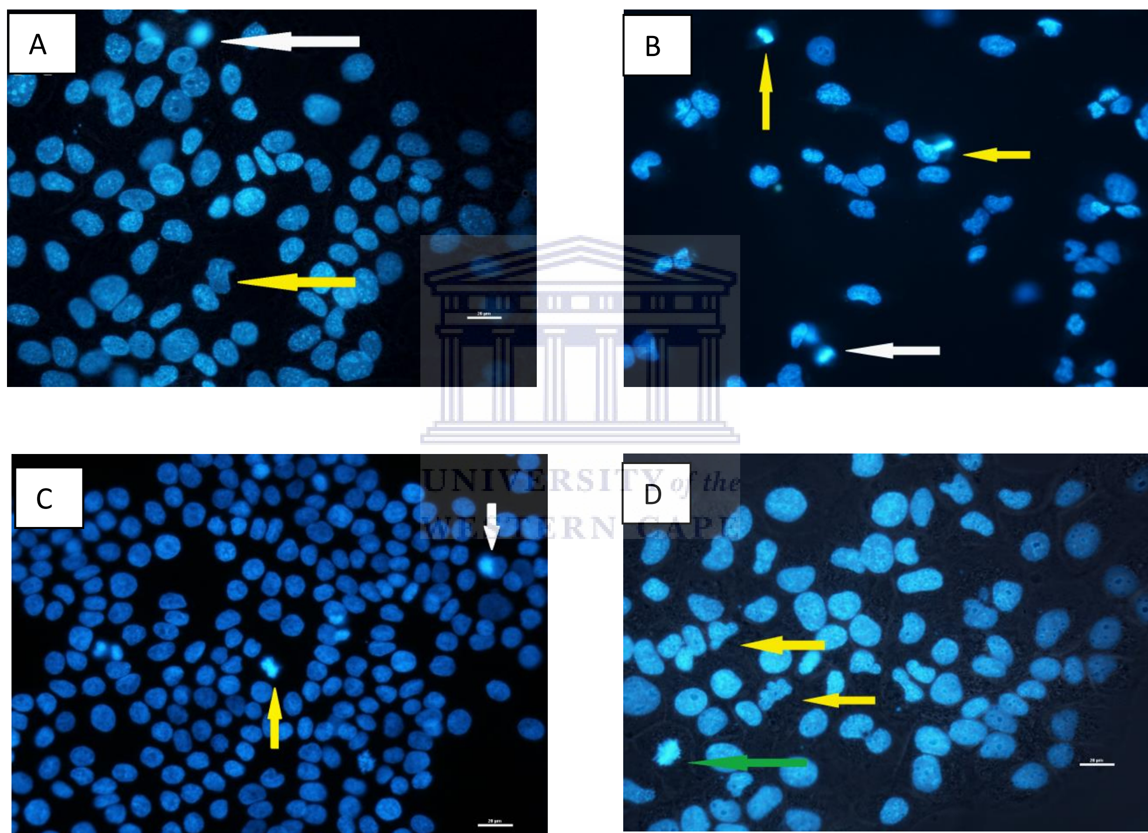
Figure 3.84 (A) - (H) show fluorescent stained HT-29 colon cancer cells exposed for 24 and 48 hours at 500 µg/mL to organic kernel extractions. Figures (A) 24h control and 48h control (B) show untreated fluorescent stained HT-29 colon cancer cell. Figure 3.84 (C) show cells after 24h500 µg/mL and (D) 48h500 µg/mL SPK-H exposure. Hypercondensed chromatin (white arrows) and in (D) a crescent shaped cell (yellow arrow) is seen (magnification: 20x).



UNIVERSITY of the
WESTERN CAPE

Figure 3.84 (E) shows cells after 24h500 µg/mL and (F) 48h500 µg/mL SPK-L exposure. Hypercondensed chromatin (white arrows) and apoptotic cells (yellow arrows) is seen. Figure 3.84 (G) and (H) show after 24h and 48h500 µg/mL respective SAK-H exposure dividing cells (green arrows) is seen and in (H) apoptotic cells (yellow arrows) are seen (magnification: 20x).

Figure 3.85 (A) – (D) show fluorescent stained HT-29 colon cancer cells treated with aqueous kernel extractions at 24 and 48 hour 500 µg/mL. Apoptotic body formation and hypercondensed nuclei are seen after all treatments indicating a definite morphological change however, a normal dividing cell is seen after 48h500 µg/mL SPK aqueous exposure.



UNIVERSITY of the
WESTERN CAPE

Figure 3.85 (A) – (D) show fluorescent stained HT-29 colon cancer cells treated with CAK, CPK, SAK and SPK aqueous kernel extractions at 24 and 48 hour 500 µg/mL. Figure 3.85 (A) show apoptosis (yellow arrow) and hypercondensed nuclei (white arrow) after 24h500 µg/mL CAK aqueous exposure. Figure 3.85 (B) show apoptotic body formation (yellow arrows) and hypercondensed nuclei (white arrow) after 24h500 µg/mL CPK aqueous exposure. Figure 3.85 (C) apoptosis formation (yellow arrow) and hypercondensed nuclei (white arrow) is seen after 48h500 µg/mL SAK aqueous exposure. Figure 3.85 (D) a dividing cell (green arrow) and increased apoptotic body formation (yellow arrow) is seen after 48h500 µg/mL SPK aqueous exposure (magnification: 20x).

Chapter 4

Discussion

Colon cancer is one of the most prevalent cancers worldwide, especially in western societies and is nutrition dependent (Klenow et al. 2009). It is one of the leading causes of death in both men and women in industrialised western countries. In America, colon cancer has a low incidence in the black African population compared to the white African population (Cronjé et al. 2009). However, in South Africa, Cronjé et al (2009) found over the past decade the opposite to be true, namely an increase in colon cancer in young black males.

A colorectal polyp growth (adenoma) is considered premalignant and during this stage can be cured by surgical removal. However, when the polyp shows severe cellular abnormalities and becomes invasive, it is considered malignant and the search for optimal treatment is still ongoing (Nel 2007). While elucidating the underlying mechanisms of cancer development, it is important to identify compounds that are able to interfere with one or more steps in carcinogenesis. Epidemiological studies revealed that colon cancer is preventable by adjusting the diet leading to a reduction in the incidence, especially in areas where dietary deficiencies contribute to risk factors in cancer development. In this regard identification of chemopreventive compounds in commonly consumed natural dietary sources such as fruits, vegetables and tea, is of particular importance in that it provides a means of everyday cancer prevention (Wattenberg 1996). The term cancer chemoprevention was first defined by Sporn et al (1976) and entails the use of agents to slow the progression of carcinogenesis, reverse or inhibit it, with the aim of lowering the risk of developing an invasive or a clinically significant disease. Epidemiological and laboratory studies have indicated a positive association between cancer reduction and intake of dietary plant foods and this has been

UNIVERSITY of the
WESTERN CAPE

attributed to the presence of phytochemicals (Greenwald et al. 2001). Due to their safety, low toxicity and general acceptance, these natural compounds have been targeted for use in chemoprevention. One group of phytochemicals that has shown potential in chemoprevention studies are plant polyphenols (Amin et al. 2009).

The apricot and peach kernels are composed of varying amounts of glycosides (flavonoids occur in plants and most foods as glycosides), fixed and volatile oils (Williamson 2004) thus potentially providing protection against cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases. The peach kernel displays a stronger antioxidant activity than apricot oils possibly due to the higher total phenolic component in the peach kernels (Sanhita et al. 2012). Flavonoids (such as amygdalin) are polyphenolic compounds that are ubiquitous in nature and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones (Williamson 2004).

UNIVERSITY of the
WESTERN CAPE

The antioxidant potential of polyphenols depends on the chemical structure, particularly the relative positions of the hydroxyl groups (Rice-Evans et al. 1996). The antioxidant effects are mediated by the scavenging of free radicals, chelation and stabilization of divalent cations, and the modulation of endogenous antioxidant enzymes (Araújo et al. 2011). However, although the anticancer properties of the polyphenolic constituents of apricot and peach kernels have been demonstrated against prostate and bladder cancer promotion (Chang et al. 2006; Syrigos et al. 1998), the underlying mechanisms involved are still not clear. To further elucidate the possible anticancer mechanisms involved, the present study was designed to investigate the *in vitro* chemopreventive properties of different extraction fractions of the

particular kernels. The choice was based on the fact that CAK and CPK kernels are regularly prescribed by Chinese Medicine practitioners, TAK because Turkey is the world's biggest producer of apricot kernels (Durmaz & Alpaslan 2007), and the South African kernels, SAK and SPK, to establish comparative or dissimilar effects from the other kernels.

A bitter taste is usually an indication of the presence of cyanide hence the respective raw kernels were tasted. All the kernels were to varying degrees bitter in taste which was confirmed through tasting of the samples by two individuals. The test was done blind without the individuals being aware of the sample type. The South African apricot and peach kernel used in this study had an immediate yet subtle bitter taste compared to the Chinese apricot and peach kernels whose bitter taste was less immediate. TAK proved to be more bitter in taste compared to CAK which had a subtle bitter taste. CPK had a delayed yet very potent bitter taste compared to the other kernels. According to ISO 2164-1975 NT standard, if a leguminous plant contains a lower rate than 10mg/kg then the sample is free of cyanide (Chaouali et al. 2013).

UNIVERSITY of the
WESTERN CAPE

The CAK and CPK, which was purchased from a Chinese Medicine practitioner, was in a treated (i.e. baked) form which mitigates any toxic side effect, thus have less volatile oils compared to TAK, SAK and SPK (non-baked form), and which makes it suitable for use in high dosages. It would therefore be expected that the CAK and CPK organic extractions would be less effective than the TAK, SAK and SPK. CPK is expected to have a slightly more favourable result than CAK as according to Bensky et al (2004) who categorised the Chinese peach kernel as a blood invigorating herb that is used more commonly than the Chinese apricot kernel in cancer formulations. SAK, SPK and TAK were purchased directly

from the source of harvest still in its hard shell and thus have not undergone the same preparation at the time of purchase. As mentioned previously, aqueous preparation of the kernels decreases its cytotoxicity whilst the organic preparations keep the glycosidic compounds without it decomposing thus retaining the toxicity.

Fractionating the organic preparation of the CAK, CPK, TAK, SAK and SPK kernels provided a means to simplify the evaluation of the kernel extractions effects on the HT-29 colon cancer cells. The lipophilic kernel extractions of the CAK, CPK, TAK, SAK and SPK kernels yielded percentage values of more than 40% indicating a high nutritional value which refers to the composition of the kernels and its impact on the body (see Table 3.2). The total extractions comprised of fixed and volatile oils and none or minimal amounts of glycosides whilst the aqueous extractions consisted of glycosides and a small amount of fixed and volatile oils. The hydrophilic extractions have glycosides with little to none fixed and volatile oils. The kernels are known to have a cyanogenic glucoside present namely amygdalin which is known to release cyanide when metabolised. However, food related cyanide intoxications are rare (Sanchez-Verlaan et al. 2010). At a pH of 7, hydrocyanic acid is distributed as hydrogen cyanide and not as free cyanide ion, thus it does not influence distribution, metabolism or excretion from the body (Simeonova & Fishbein 2004). A small proportion of hydrogen cyanide is transported to target organs such as the liver via the plasma.

UNIVERSITY of the
WESTERN CAPE

Cell viability study

Malignant cells are characterized by excessive proliferation, the inability to terminally differentiate or perform apoptosis under normal conditions, and an extended or immortalized life span. Thus, any perturbation of cell cycle specific proteins by polyphenols can potentially affect and/or block the continuous proliferation of these tumorigenic cells. Natural polyphenols have been reported to induce cell cycle arrest by the down-regulation of cyclins and cyclin-dependent kinases (CDKs) or by indirectly inducing the expression of p21, p27 and p53 genes (Ramos 2008). A study conducted by Hsu and Liu (2004), showed that the flavonoid acacetin, inhibited the proliferation of lung cancer cells by inducing apoptosis and blocking cell cycle progression in the G_1 phase. The isoflavone, genistein reduced proliferation and induced a G_2/M phase arrest and apoptotic death in colon cancer HT-29 cells (Hsu & Liu 2004). The flavonoids may inhibit the formation and growth of tumours by induction of cell cycle arrest and apoptosis (Wenzel et al. 2000). As both the organic (total, hydrophilic and lipophilic) and aqueous extracts effectively inhibited cell proliferation in the colon cancer cells it is presumed that the inhibitory action was due to the flavonoids in the kernels.

The crystal violet cell viability staining and flow cytometric analysis methods were used to measure cell proliferation and cell cycle progression of HT-29 colon cancer cells after exposure to the various kernel extractions. A time (24-, 48- and 72 hours) and dose (100, 500 and 1000 µg/mL) study was conducted.

The results show that all organic and aqueous kernel extractions inhibited cell proliferation after 24 and 48 hours. These effects were overcome after 72 hours with the cells showing a recovery in cell proliferation except for the SAK-L 500 µg/mL and the SAK-H 100, 500 and 1000 µg/mL treated samples that suppressed the proliferation of the colon cancer cells even after 72 hours. Cell viability tests showed that CAK-L, CPK-T, TAK-T, SAK-T and SPK-L after 24 hours, and CAK-T and -L, CPK-T, TAK-H, SAK-T, SPK-T and –L after 48 hours greatly inhibited cell proliferation. The total and lipophilic extractions more commonly showed an inhibitory effect which was probably due to the presence of glycosides, fixed and volatile oils. The kernel that displayed the greatest inhibitory effect on cell proliferation of the HT-29 colon cancer cells after 24 hours was 1000 µg/mL CAK-L extract followed by the CAK-T extract, and after 48 hours the 1000 µg/mL CAK-T extract followed by the 1000 µg/mL CPK-T extract.

UNIVERSITY of the
WESTERN CAPE

The CAK and CPK kernel extractions were expected to have a reduced effect on cell proliferation due to its prepared form. Although the SAK and SPK organic fractions affected cell growth more prominently than CAK and CPK, it was the CAK-T and –L extracts that had the most outspoken inhibitory effect despite the fact that the kernels were pre-treated by the vendor. This brings about the question as to whether the Chinese kernels were in fact more potent than the other kernels due to its components, or whether its pre-treated form rendered it more potent or whether the method of extraction may have enhanced its potency.

Cell cycle progression study

The natural progression of cells through the cell cycle is governed by signal pathways which ensure the successful replication and duplication of cells within a certain time period. During the S phase, DNA damage can elicit an intra S-phase block that slows down DNA replication. An arrest in the S phase may be as a result of minor DNA damage which may be repaired or major DNA damage that result in apoptosis or autophagy (Stewart et al. 2003; Yuan et al. 2014). The results of numerous studies in cell culture suggest that flavonoids may affect chronic disease by selectively inhibiting kinases involved in cell signalling, including DNA synthesis (Yuan et al. 2014).

UNIVERSITY of the
WESTERN CAPE

The study showed that the organic and aqueous extracts affected cell cycle progression differently. The CAK, CPK and TAK organic kernel extracts had a minor effect on cell cycle progression with the total and hydrophilic extracts being more effective. All the SAK organic extracts at especially 24 hours and all the SPK organic extracts at especially 24- and 48 hours induced an S phase block by showing a peak in the S phase cell population. In contrast all aqueous kernel extracts and respective concentrations affected cell cycle progression after 48 hours. After 72 hours however, similar to what was observed in the cell viability study, the cells overcame the inhibitory effect of the kernel extractions and resumed cell cycle progression except for the 72h100 μg/mL CAK aqueous extract that induced an outspoken peak in the S phase after 72 hours.

These results may be due to an activation of the intra-S phase checkpoint. Genotoxic insults cause only a temporary, reversible delay in cell cycle progression, mainly by inhibition of the initiation of new replicons and thereby slowing down DNA replication (Bartek & Lukas 2001).

The phenolic flavonoid structure, specifically the phenolic hydroxyl groups and their structural arrangements confer the antioxidant activity through their hydrogen donating properties. Pro-oxidative properties and the subsequent disruption in the function of the mitochondria could play a role in reduction in cell viability. Inhibition of cell growth occurred possibly via mechanisms that involve the delay of cell cycle progression, preventing the cells from exiting the S phase (Yuan et al. 2014). The activated Cdc25A-degradation pathway slows the S phase, making the ATM-Chk2-Cdc25A-Cdk2-Cdc45 axis the key mechanism of the intra-S-phase response (Bartek & Lukas 2001). Inhibition of Cdk2 activity through the Cdc25A degradation leads to a several hour delay of S phase progression (Bartek & Lukas 2001; Willis & Rhind 2009). It would seem that even though the cell viability is decreased after 24- and 48h and the cells are stalled in the S phase, the compound concerned, is either eliminated via a metabolic pathway leading to a full recovery in cell viability after 72 hours or it could be that the cells simply overcame the temporary S phase block and continued in the cell cycle.

UNIVERSITY of the
WESTERN CAPE

No significant differences between the apricot and peach kernel extractions for both the organic and aqueous extractions were seen even though the peach kernels have more flavonoids than the apricot kernels. However, both kernels contain a cyanogenic glycoside, amygdalin. Amygdalin is considered non-toxic until cyanide (HCN) is released, following enzymatic hydrolysis by β-glucosidases after grinding of plant tissue which activates intracellular β-glucosidases, or by the gut micro-flora (Cooke et al. 2009). Preparation of the extractions used in this study should not have caused activation of β-glucosidases, but freeze

drying or addition to the culture media could possibly result in hydrolysis and the production of cyanide which affects the cell by shutting down aerobic respiration.

Cancer cells are thought to have a different balance of enzymes in comparison to normal non-cancerous cells, that is, more β-glucosidases and less rhodanese than normal cells (Cooke et al. 2009). Rhodanese convert cyanide into a relatively harmless compound thiocyanate.
Cancerous cells such as the HT-29 colon cancer cells have less rhodanese enzyme to convert cyanide to a harmless form, thus they are more affected by cyanide than healthy cells. In this study, since the cells all recovered from the various treatments over time and in some instances even showed increased cell viability, it is questionable as to whether cyanide is indeed formed or that the inhibitory effects are induced by cyanide. The intra-S phase block is a temporary block and more studies on specific compounds will have to be done to establish if the same compound is affecting cell viability and inducing the cell cycle block.

UNIVERSITY of the
WESTERN CAPE

Furthermore, another question that needs to be answered is if it is the induction of this S phase block that is causing and or reducing cell viability?
It may be assumed that the preparation of the treatment, aqueous extraction process, addition to the culture media and the freeze drying process, may have contributed to the release of the cyanide.

Morphological studies

Morphological changes such as cell membrane blebbing, hypercondensed chromatin, changes in growth patterns and cell shape were studied in H&E stained colon cancer cells after 24-, 48- and 72h exposure to organic and aqueous extractions.

Apoptosis plays an important role in the maintenance of tissue homeostasis. It is important for getting rid of damaged cells, and suppressed apoptosis contributes to development of cancer (Lowe et al. 2004). Apoptosis is carried out by the coordinated actions of several caspases (Green 2006). Many flavonoids, including glycosides, causing apoptosis target the mitochondria and trigger the execution phase by the activation of caspases, which play an important role in signal transduction cascades (Salleh et al. 2011). It has been proposed that the reduction in cell viability could be attributed to the disruption in the function of the mitochondria which leads to the activation of the mitochondria-mediated apoptotic pathways.

UNIVERSITY of the
WESTERN CAPE

HT-29 colon cancer cells grow densely in colonies with multiple nucleoli and rounded to elliptical shapes. The 24- and 48 hours exposure of the cells to organic and aqueous kernel extractions showed fewer cell colonies, with changes in cell shape (irregular) and size (shrunken) as well as possible apoptotic bodies. After 72 hours though the cells tend to resume their pre-treatment growth pattern. The exposure of HT-29 colon cancer cells to CAK-Aqu and CPK-Aqu extractions at 24h500 µg/mL, and SAK-Aqu and SPK-Aqu extractions at 48h500 µg/mL show the presence of irregularly shaped cells, hypercondensed chromatin and apoptotic bodies (Fig 3.85). Even though all CAK organic extractions may

have shown an inhibitory effect on cell proliferation after 24 hours, it did not effect any prominent morphological changes.

Hoechst 33342 fluorescent stain

The Hoechst 33342 staining method was done to determine the presence or absence of apoptotic cells after exposure of the HT-29 colon cancer cells to the various kernel extractions. Apoptosis is distinguished from necrosis or accidental cell death by characteristic morphological and biochemical changes including compaction and defragmentation of nuclear chromatin, cytoplasm shrinkage and loss of membrane symmetry.

The morphological features described as chromatin condensation, membrane blebbing, cell shrinkage and apoptotic bodies were confirmed by staining cells with Hoechst 33342. Cell shrinkage and apoptotic bodies, although very few, were seen after treatment with 500 µg/mL SAK-H, SPK-H, SPK-L organic extracts and CAK, CPK, SAK and SPK aqueous extracts after 24 and 48 hours. The decrease in cell proliferation can therefore not be ascribed to apoptosis only.

UNIVERSITY of the
WESTERN CAPE

According to Suzuki et al (1998), HT-29 cells show an apoptosis-resistant phenotype in response to micro-environmental stresses. It is reported that bufalin (isolated from a traditional Chinese medicine) did not cause caspase-dependant cell death in colon cancer cells, instead, bufalin activated an autophagy pathway, as characterized by the accumulation of LC3-II and the stimulation of autophagy flux (Xie et al. 2011). Evidence exist that most cellular systems that have defects in the apoptotic signalling pathway had autophagy contribute to cell death (Chiu et al. 2009). For future studies, it would be of great value to

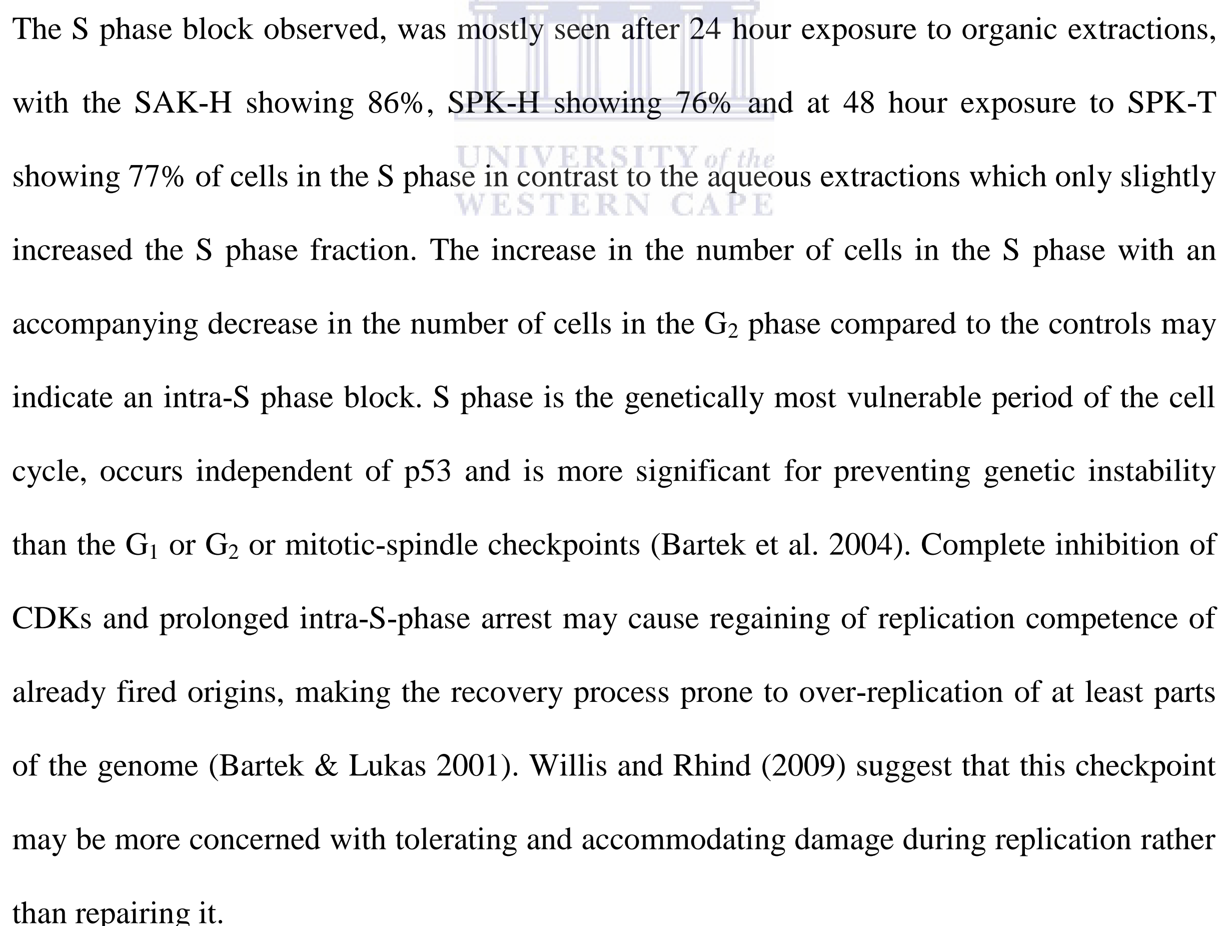
apply the triple staining method that distinguishes between apoptotic cells and cells undergoing autophagy in the cultures showing decreased viability or cell numbers, without apoptosis.

Thus, treatment lowered cell viability and in some instances induced an S phase block as seen with SAK-T and SPK-L after 24- and 48 hours at all concentrations. Some cells did die via apoptosis probably due to major DNA damage. Virtually all treated samples show a recovery in cell proliferation after 72 hours. Cells exposed to all concentrations of CAK treatment after 72 hours showed a complete recovery in cell proliferation, leading in some instances to an increased cell growth. Thus, not only a complete reversal of action / effect ensued, a different signalling mechanism was also stimulated. Either repair of damaged DNA resulting in continuation of cell cycle progression happened, or programmed cell death was switched on, or removal / absence of a metabolite ensured a switch from growth inhibition to growth stimulation.

UNIVERSITY of the
WESTERN CAPE

In summary, all the extracts significantly reduced cell viability and inhibited proliferation in the HT-29 colon cancer cells after 24- and 48 hours with the lipophilic and total fractions of CAK being the most effective. After 72 hours, it is clear that the inhibitory effects have been abolished and replaced by a stimulatory effect as the cell viability was higher in the treated cultures than the untreated controls. Results show that the total and the hydrophilic fractions of all the kernels increased cell viability more than the lipophilic fractions. Amygdalin is the major glycoside (flavonoids) in all the kernels but it cannot be said with certainty that it was the amygdalin metabolite cyanide that affected the cell viability or induced apoptosis on its own. If hydrolysis of amygdalin indeed happened and cyanide was produced, it would affect

the cells by shutting down aerobic respiration. Since cancer cells have more β-glucosidases and less rhodanese than normal cells (Cooke & Seers 2009), it is a possibility that the HT-29 cells had some rhodanese to convert cyanide into a relatively harmless compound thiocyanate. In the body this conversion usually takes place within an hour but, it could be that *in vitro* this conversion, in light of the low enzyme levels in the HT-29 cells, happened slowly and that the effect was only seen after 48 hours. However, this does not explain the overall inhibition even by the lipophilic fractions that should not contain any amygdalin or the eventual stimulatory effect, observed from 48 hours onwards.



The S phase block observed, was mostly seen after 24 hour exposure to organic extractions, with the SAK-H showing 86%, SPK-H showing 76% and at 48 hour exposure to SPK-T showing 77% of cells in the S phase in contrast to the aqueous extractions which only slightly increased the S phase fraction. The increase in the number of cells in the S phase with an accompanying decrease in the number of cells in the G_2 phase compared to the controls may indicate an intra-S phase block. S phase is the genetically most vulnerable period of the cell cycle, occurs independent of p53 and is more significant for preventing genetic instability than the G_1 or G_2 or mitotic-spindle checkpoints (Bartek et al. 2004). Complete inhibition of CDKs and prolonged intra-S-phase arrest may cause regaining of replication competence of already fired origins, making the recovery process prone to over-replication of at least parts of the genome (Bartek & Lukas 2001). Willis and Rhind (2009) suggest that this checkpoint may be more concerned with tolerating and accommodating damage during replication rather than repairing it.

Synergistic and or additive effects between polyphenolic compounds may also be responsible for the reduction of cell viability, proliferation and apoptosis. The apricot kernels have high levels of unsaturated fatty acids (linoleic acid, linolenic acid and eicosapentaenoic acid) that could contribute to the antioxidant activity of these kernels. A metabolite of linoleic acid, gamma-linoleic acid (18:3n-6), has been shown *in vitro*, to suppress growth and induce apoptosis of cervical cancer and to induce a cell cycle block (G_1/S) in osteogenic sarcoma cells *in vitro* (De Kock et al. 1994). All the kernels and the various fractions affected cell viability and to an extent cell cycle progression, but more studies are needed to establish the most effective kernel and specific fraction or signature active component.

Inhibition of cell viability and proliferation and the induction of apoptosis could be an important preventive approach in chemoprevention. Understanding how dietary components regulate proliferation and cell survival could play a critical role in development of new enriched agents that can prevent and treat cancer with a reduced risk of toxicity.

UNIVERSITY of the
WESTERN CAPE

Future studies should target the effect of selected pure signature compounds that can be investigated to provide more information about the possible active polyphenolic constituents.

It would also be of value to do quantitative apoptosis and autophagic studies to determine the extent of cell death after treatment.

The organic kernel extractions more than the aqueous kernel extractions had thus induced a growth inhibitory effect of the HT-29 colon cancer cells with minimal cytotoxic effect as the treated samples had shown to overcome the inhibitory effect of the kernel fractions.

All kernel fractions had shown to induce a growth inhibitory effect of the cells to varying degrees at different times of exposure and concentrations.

Kernels of all three origins affected the cells in different ways by both inhibiting cell proliferation and blocking cell cycle progression or by stimulating cell growth recovery. I am of the opinion that should untreated CAK and CPK kernels have been used, that it may have had a more pronounced effect on the HT-29 colon cancer cells. The CAK and CPK organic extracts showed an effect on cell proliferation mostly after 48 hours compared to the SAK and SPK organic extracts after 24 hours already (Table 3.5). Otherwise all kernels, irrespective of their origin, showed an effect on the HT-29 colon cancer cell's growth and cell cycle progression.

The results may definitely inform clinical practice and thus be of value to the Chinese Medicine Practitioner wishing to have a better understanding of the application of these herbs in a prescription, but also to the general public in search of a nutritional preventive and intervention in the treatment of cancer. A link between the daily consumption of plant foods and cancer reduction has definitely been established.

UNIVERSITY of the
WESTERN CAPE

REFERENCES

Abtahi, H. et al, 2008. Antimicrobial activities of water and methanol extracts of bitter apricot seeds. *Journal of Medical Science*, 8(4), pp.433–436.

Amin, A.R.M.. et al., 2009. Perspectives for cancer prevention with Natural Compounds. *Clinical Oncol.*, pp.27, 2712–2725.

Aminlari, M. & Baghshani, H., 2009. Comparison of rhodanese distribution in different tissues of Japanese quail, partridge and pigeon. *Comparative Biochemistry and Physiology,* 18, pp.217–220.

Anand, R.P. et al., 2012. Overcoming natural replication barriers: Differential helicase requirements. *Nucleic Acids Research*, 40(3), pp.1091–1105.

Anon, 2013. *Risk Profile Apricot Kernel oil (AKO) CAS No. 72869693*, Available at: http://www.mattilsynet.no/kosmetikk/stoffer_i_kosmetikk/risk_profile_apricotekerne.11323/binary/Risk Profile ApricoteKerne.

Anon, 2001. *The ultimate guide to vitamin B-17 metabolic therapy* 1st ed., Worldwithoutcancer.org.uk. Available at: http://www.ivonazivkovic.net/laetril.pdf.

Araújo, J., Gonçalves, P. & Martel, F., 2011. Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutr. Research*, 31(1), pp.77–87.

Badr, J. & Tawfik, M.K., 2010. Analytical & Pharmacological Investigation of Amygdalin in Punus armeniae L. kernels. *Journal of Pharmacy Research*, 3(9), pp.2134–2137.

Bailey, et al, 2014. Increasing Disparities in the Age-Related Incidences of Colon and Rectal Cancers in the United States, 1975-2010. *JAMA Surg.*

Al Bakri, Salih A, et al, 2010. Antibacterial activity of apricot kernel extract containing Amygdalin. *Iraqi Journal of Science*, 51(4), pp.571–576.

Bartek, J., Lukas, C. & Lukas, J., 2004. Checking on DNA damage in S phase. *Nature reviews. Molecular Cell Biology*, 5, pp.792–804.

Bartek, J. & Lukas, J., 2001. Mammalian G1- and S-phase checkpoints in response to DNA damage. *Current Opinion in Cell Biology*, 13(6), pp.738–747.

Besson, A., Dowdy, S.F. & Roberts, J.M., 2008. CDK Inhibitors: Cell Cycle Regulators and Beyond. *Developmental Cell*, 14(2), pp.159–169.

Bewertung, G. Der, 2007. Verzehr von bitteren Aprikosenkernen ist gesundheitlich bedenklich. *Bewertung, Gegenstand Der*, 3(014), pp.4–7.

Bouzas-Rodríguez, J. et al., 2012. The Nuclear Receptor NR4A1 induces a form of cell death dependent on autophagy in mammalian cells. *PLoS ONE*, 7(10).

Branzei, D. & Foiani, M., 2010. Maintaining genome stability at the replication fork. *Nature reviews. Molecular Cell Bology*, 11(3), pp.208–219.

Brouquet, A. et al., 2011. A model of primary culture of colorectal cancer and liver metastasis to predict chemosensitivity. *The Journal of Surgical Research*, 166(2), pp.247–54.

Cell Signaling Technology, 2014. Cell Cycle Control: G1/S Checkpoint. p.1. Available at: www.cellsignal.com [Accessed January 1, 2014].

UNIVERSITY of the
WESTERN CAPE

Chang, H.-K. et al., 2006. Amygdalin induces apoptosis through regulation of Bax and Bcl-2 expressions in human DU145 and LNCaP prostate cancer cells. *Biological & Pharmaceutical Bulletin*, 29(8), pp.1597–602.

Chaouali, N. et al., 2013. Potential Toxic Levels of Cyanide in Almonds (Prunus amygdalus), Apricot Kernels (Prunus armeniaca), and Almond Syrup. *ISRN Toxicology*, pp.1–7.

Chitnis, M., Adwankar, M. & Amonkar, A., 1985. Studies on highd-dose chemotherapy of Amygdalin in Murine P388 Lymphocytic Leukaemia and P815 Mast Cell Leukaemia. *Journal of Cancer Research Clinical Oncology*, 109, pp.208–209.

Chiu, H.-W. et al., 2009. Combination treatment with arsenic trioxide and irradiation enhances autophagic effects in U118-MG cells through increased mitotic arrest and regulation of PI3K/Akt and ERK1/2 signaling pathways. *Autophagy*, 4(5), pp.472–483.

Committee On Toxicity, 2006. *Statement On Cyanogenic Glycosides In Bitter Apricot Kernels*. pp.1-8. Available at: http://cot.food.gov.uk/sites/ default/files/cot/ cotstatementapricot200615.pdf.

Cooke, H., Seers, H. & Consortium, C.CAM., 2009. Laetrile. , pp.1–8. Available at: www.cam-cancer.org.

Cronjé, L., Paterson, A.C. & Becker, P.J., 2009. Colorectal cancer in South Africa: A heritable cause suspected in many young black patients. *South African Medical Journal*, 99(2), pp.103–106.

Dai, J. & Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules (Basel, Switzerland)*, 15(10), pp.7313–52.

UNIVERSITY of the
WESTERN CAPE

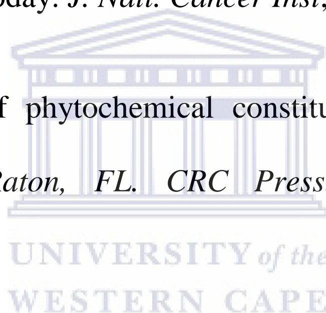
Dan Bensky, E.S., 2004a. *Chinese Medicine Materia Medica* 3rd ed., Eastland Press, pp.437-440.

Dan Bensky, E.S., 2004b. *Chinese Medicine Materia Medica* 3rd ed., Eastland Press, pp.624-627.

De Kock, M., Lottering, M.L. & Seegers, J.C., 1994. Differential cytotoxic effects of gamma-linolenic acid on MG-63 and HeLa cells. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, 51(2), pp.109–120.

Doll, R. & Peto, R., 1981. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst*, 66, pp.1191–1308.

Duke, J.A., 1992. Handbook of phytochemical constituents of GRAS herbs and other economic plants. *Boca Raton, FL. CRC Press.* Available at: http://www.ars-grin.gov/duke/.


UNIVERSITY of the
WESTERN CAPE

Durmaz, G. & Alpaslan, M., 2007. Antioxidant properties of roasted apricot (Prunus armeniaca L.) kernel. *Food Chemistry*, 100(3), pp.1177–1181.

Duronio, R.J. & Xiong, Y., 2013. Signaling pathways that control cell proliferation. *Cold Spring Harbor Perspectives in Biology*, 5(3), pp.1–13.

EFSA, 2004. Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on hydrocyanic acid in flavourings and other food ingredients with flavouring properties. *EFSA JOURNAL*, (105), pp.4–5.

Enculescu, M., 2009. Vitamin B 17 / Laetrile / Amygdalin (a Review). *Bulletin UASVM Animal Science and Biotechnologies*, 66(11), pp.20–25.

Ernst T. Krebs, 1975. *Anatomy of a coverup: Successful Slaon-Kettering Amygdalin (Laetrile) animal studies*, California: The Committee For Freedom of Choice in Cancer Therapy, Inc.

Ernst T. Krebs, J., 1970. The Nitrilosides (Vitamin B-17) - their nature, occurrence and metabolic significance (antineoplastic Vitamin B-17). *Journal of Applied Nutrition*, 22(3-4), pp.1–18.

Fassa, P., 2009. Apricot seeds kill cancer cells without side effects. *Citizen*, pp.1–5.

Femenia, A. et al., 1995. Chemical composition of bitter and sweet Apricot kernels. *J. Agric. Food Chem.*, 43, pp.356–361.

Ferguson, L.R., 2002. Natural and human-made mutagens and carcinogens in the human diet. *Toxicology*, 181-182, pp.79–82.

Ferlay, J. et al., 2014. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer*, 1, pp.1–28.

Ford, H. & Pardee, A., 1999. Cancer and the Cell Cycle. *Journal of Cellular Biochemistry Supplements*, 32/33, pp.166–172.

Forgue-Lafitte, M.E. et al., 1989. Proliferation of the human colon carcinoma cell line HT29: autocrine growth and deregulated expression of the c-myc oncogene. *Cancer Research*, 49(23), pp.6566–6571.

Forman, M. et al., 2004. Nutrition and cancer prevention: a multidisciplinary perspective on human trials. *Annu. Rev. Nutr*, 24, pp.223–254.

UNIVERSITY of the WESTERN CAPE

Foster, D.A. et al., 2010. Regulation of G1 Cell Cycle Progression: Distinguishing the Restriction Point from a Nutrient-Sensing Cell Growth Checkpoint(s). *Genes & Cancer*, 1(11), pp.1124–1131.

Fukuda, T. et al., 2003. Anti-tumor promoting effect of glycosides from Prunus persica seeds. *Biological & Pharmaceutical Bulletin*, 26(2), pp.271–3.

Gandhi, V.M. et al., 1997. Safety evaluation of wild apricot oil. *Food and Chemical Toxicology*, 35(6), pp.583–587.

Genderen, H. Van, 1997. Adverse effects of naturally occurring nonnutritive substances. In J. de Vries, ed. *Food Safety and Toxicity*. pp. 147–162.

Grallert, B. & Boye, E., 2008. The multiple facets of the intra-S checkpoint. *Cell Cycle*, 7(15), pp.2315–2320.

Grasso, D. & Vaccaro, M., 2014. Macroautophagy and the oncogene-induced senescence. *Frontiers in Endocrinology*, 5(157), pp.1–7.

Green, D., 2006. At the gates of death. *Cancer Cell*, pp.328–330.

Greenberg, D.M. & Francisco, S., 1975. Special Article: The Vitamin fraud in cancer quackery. *West J \Med*, 122, pp.345–348.

Greenwald, P., Clifford, C.. & Milner, J.., 2001. Diet and cancer prevention. *Eur. J. Cancer Oxf. Engl.*, 37(8), pp.948–965.

Haggar, F.A. & Boushey, R.P., 2009. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clinical Colon Rectal Surg.*, 22(4), pp.191–7.

UNIVERSITY of the WESTERN CAPE

Halenár, M. et al., 2013. Amygdalin and its effects on animal cells. *Journal of Microbiology, Biotechnology and Food Sciences*, 2, pp.2217–2226.

Harbour, J.W. et al., 1999. Cdk Phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell*, 98(6), pp.859–869.

Heavey, P., McKenna, D. & Rowland, I., 2004. Colorectal cancer and the relationship between genes and the environment. *Nutr. Cancer*, 48(2), pp.124–41.

Henley, S.A. & Dick, F.A., 2012. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell Division*, 7(1), p.10.

Hsu, Y.-L. et al., 2004. Acacetin-induced cell cycle arrest and apoptosis in human non-small cell lung cancer A549 cells. *Cancer letters*, 212(1), pp.53–60.

Ibrahim, B. et al., 2008. Mad2 binding is not sufficient for complete Cdc20 sequestering in mitotic transition control (an in silico study). *Biophysical Chemistry*, 134(1-2), pp.93–100.

Iziko Museum, 2002. Genus: Prunus (Almond, Apricot, Cherry, Nectarine, Peach and Plum genus). *Iziko, Museums of Cape Town*. Available at: http://www.biodiversityexplorer.org/ plants/rosaceae/prunus.htm.

Jaehnig, E.J. et al., 2013. Checkpoint kinases regulate a global network of transcription factors in response to DNA damage. *Cell Reports*, 4(1), pp.174–188.

UNIVERSITY of the
WESTERN CAPE

Jemal, A. et al., 2010. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiology, Biomarkers & Prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 19(8), pp.1893–907.

Jemal, A., Bray, F. & Ferlay, J., 2011. Global Cancer Statistics. *Cancer Journal for Clinicians*, 61(2), pp.69–90.

Jossen, R. & Bermejo, R., 2013. The DNA damage checkpoint response to replication stress: A game of Forks. *Frontiers in Genetics*, 4(26), pp.1–14.

Kanavos, P., 2006. The rising burden of cancer in the developing world. *Annals of Oncology*, 17(8), pp.15–23.

Kaufmann, W.K., 2009. The human intra-S checkpoint response to UVC-induced DNA damage. *Carcinogenesis*, 31(5), pp.751–765.

Khandekar, J.D. & Edelman, H., 1979. Studies of amygdalin (laetrile) toxicity in rodents. *JAMA : The Journal of the American Medical Association*, 242(2), pp.169–71.

Klenow, S., Pool-Zobel, B.. & Glei, M., 2009. Influence of inorganic and organic iron compounds on parameters of cell growth and survival in human colon cells. *Toxicol. Vitro Int. J. Publ. Assoc*, 23(3), pp.400–7.

Krafft, C. et al., 2012. Distribution of Amygdalin in apricot (Prunus armeniaca) seeds studied by Raman Microscopic Imaging. *Applied Spectroscopy*, 66(6), pp.644–649.

Kwon, H.-Y. et al., 2003. Apoptosis induction of Persicae Semen Extract in Human Promyelocytic Leukemia (HL-60) Cells. *Arch Pharm Res*, 26(2), pp.157–161.

UNIVERSITY of the
WESTERN CAPE

Lambert, S. & Carr, A.M., 2005. Checkpoint responses to replication fork barriers. *Biochimie*, 87(7), pp.591–602.

Lara-Gonzalez, P., Westhorpe, F.G. & Taylor, S.S., 2012. The spindle assembly checkpoint. *Current Biology*, 22(22), pp.966–80.

Lau, D.T.C. & Murray, A.W., 2012. Mad2 and Mad3 cooperate to arrest budding yeast in mitosis. *Current Biology*, 22(3), pp.180–90.

Lee, A.Y.L. et al., 2012. Dbf4 is direct downstream target of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) protein to regulate intra-S-phase checkpoint. *Journal of Biological Chemistry*, 287(4), pp.2531–2543.

Lim, S. & Kaldis, P., 2013. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development (Cambridge, England)*, 140(15), pp.3079–93.

Liu, S. et al., 2012. Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress. *Nucleic Acids Research*, 40(21), pp.10780–10794.

Lowe, S., Cepero, E. & Evan, G., 2004. Intrinsic tumour suppression. *Nature*, 432(7015), pp.307–315.

Maciocia, G., 2005. The Foundations of Chinese Medicine A Comprehensive Text for Acupuncturists and Herbalists. In *Diagnosis in Chinese Medicine A Comprehensive Guide*. Churchill Livingstone, p. 196.

UNIVERSITY of the
WESTERN CAPE

Magiera, M.M., Gueydon, E. & Schwob, E., 2014. DNA replication and spindle checkpoints cooperate during S phase to delay mitosis and preserve genome integrity. *Journal of Cell Biology*, 204(2), pp.165–175.

Masai, H., You, Z. & Arai, K., 2005. Control of DNA replication: regulation and activation of eukaryotic replicative helicase, MCM. *IUBMB Life*, 57(4-5), pp.323–35.

Milazzo, S. et al., 2009. Laetrile treatment for cancer (Review). *The Cochrane Collaboration*, John Wiley and Sons, Ltd, pp.1-22.

Milazzo, S., Lejeune, S. & Ernst, E., 2007. Laetrile for cancer: a systematic review of the clinical evidence. *Supportive Care in Cancer: official Journal of the Multinational Association of Supportive Care in Cancer*, 15(6), pp.583–95.

Murray, A.W., 1995. The genetics of cell cycle checkpoints. *Current Opinion in Genetics & Development*, 5(1), pp.5–11.

UNIVERSITY of the
WESTERN CAPE

Nel Amanda, 2007. *MSc*; *Clinically relevant ex vivo fatty acid profiles from a lipid model for colorectal adenocarcinoma*. University of the Free State, Bloemfontein, South Africa.

Newmark, J. et al., 1981. Amygdalin (Laetrile) and prunasin beta-glucosidases: distribution in germ-free rat and in human tumor tissue. *Proceedings of the National Academy of Sciences of the United States of America*, 78(10), pp.6513–6.

Oakley, T.J. & Hickson, I.D., 2002. Defending genome integrity during S-phase: Putative roles for RecQ helicases and topoisomerase III. *DNA Repair*, 1(3), pp.175–207.

Ouédraogo, M. et al., 2011. An overview of cancer chemopreventive potential and safety of proanthocyanidins. *Nutr. Cancer*, 63(8), pp.1163–73.

Ouyang, L. et al., 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation*, 45(6), pp.487–98.

Padmaja, G., 1995. Cyanide detoxification in cassava for food and feed uses. *Critical Reviews in Food Science and Nutrition*, 35(4), pp.299–339.

Padussis, J.C. et al., 2004. Neoplasms of the Small Intestine, Vermiform Appendix, and Peritoneum, and Carcinoma of the Colon and Rectum. In *Cancer Medicine*. People's Medical Publishing House, pp. 1172–1193.

Padussis, J.C. et al., 2010. Neoplasms of the Small Intestine, Vermiform Appendix, and Peritoneum, and Carcinoma of the Colon and Rectum. In *Cancer Medicine*. People's Medical Publishing House, pp. 1172–1193.

Pan, W., Issaq, S. & Zhang, Y., 2011. The in vivo role of the RP-Mdm2-p53 pathway in signaling oncogenic stress induced by pRb inactivation and Ras overexpression. *PLoS ONE*, 6(6), pp.1–10.

Park, H.-J. et al., 2005. Amygdalin inhibits genes related to cell cycle in SNU-C4 human colon cancer cells. *World Journal of Gastroenterology*, 11(33), pp.5156–61.

Parkin, D., Pisani, P. & Ferlay, J., 1999. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int. J. Cancer*, 80(6), pp.827–84.

Paternot, S. et al., 2014. The CDK4/CDK6 inhibitor PD0332991 paradoxically stabilizes activated cyclin D3-CDK4/6 complexes. *Cell Cycle*, 13(18), pp.2879–2888.

UNIVERSITY of the
WESTERN CAPE

Pulido, J.E.C., 2000. Amygdalin (Laetrile) B-17 Monographic Summary. pp.1–8. Available at: http://worldwithoutcancer.org.uk/studies-research-more-documented-evidence/ amygdalin-laetrile -b-17/ [Accessed May 8, 2015].

Raj, V., Jian, A. & Chaudhary, J., 2012. Prunus Armeniaca (Apricot): An Overview. *Journal of Pharmacy Research*, 5(8), pp.3964–3966.

Ramos, S., 2008. Cancer chemoprevention and chemotherapy: Dietary polyphenols and signalling pathways. *Mol. Nutr. Food Res*, 52(5), pp.507–526.

Reddy, L., Odhav, B. & Bhoola, K.D., 2003. Natural products for cancer prevention: a global perspective. *Pharmacology & Therapeutics*, 99(1), pp.1–13.

Rhind, N. & Russell, P., 2012. Signaling pathways that regulate cell division. *Cold Spring Harbor Perspectives in Biology*, 4(10), pp.1–15.

Ricci-Vitiani, L. et al., 2007. Identification and expansion of human colon-cancer-initiating cells. *Nature*, 445(7123), pp.111–5.

Rice-Evans, C., Miller, N. & Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.*, 20(7), pp.933–956.

Roche Applied Science, 2007. *Apoptosis, Cell Death and Cell Proliferation* I. F. Hans-Jürgen Rode, Ph. D., Doris Eisel, ed., Roche Diagnostics GmbH. Available at: http://download.bioon.com.cn/upload/201308/14100155_8995.pdf.

Roynette, C.E. et al., 2004. n-3 polyunsaturated fatty acids and colon cancer prevention. *Clin Nutr*, 23(2), pp.139–151.

UNIVERSITY of the
WESTERN CAPE

Ruan, W., Lai, M. & Zhou, J., 2006. Anticancer effects of Chinese herbal medicine, science or myth? *Journal of Zhejiang University Science B*, 7(12), pp.1006–14.

Saidu, Y., 2004. Physicochemical features of rhodanese : A review. *African Journal of Biotechnology*, 3(4), pp.370–374.

Salleh, S. et al., 2011. In vitro chemopreventive activity of an ethyl acetate fraction derived from hot water extract of orthosiphon stamineus in HepG2 cells. *J. Med. Plants Res.*, 5(10), pp.1892–1899.

Sanchez-Verlaan, P. et al., 2010. An unusual cause of severe lactic acidosis: cyanide poisoning after bitter almond ingestion. *Intensive Care Medicine*, 37, pp.168–169.

Sanhita, P., Chandra, K.J. & Chandra, S.G., 2012. Important findings on plants having antioxidant property: A Review. *International Journal of Pharmacy*, 3(5), pp.72–75.

Sayers, T.J., 2011. Targeting the extrinsic apoptosis signaling pathway for cancer therapy. *Cancer Immunology, Immunotherapy*, 60(8), pp.1173–1180.

Schnerch, D. et al., 2012. Cell cycle control in acute myeloid leukemia. *American Journal of Cancer Research*, 2(5), pp.508–28.

Seers, H., Cooke, H. & CAM-Cancer Consortium, 2012. Laetrile. *CAM-Cancer*, pp.1–6. Available at: http://www.cam-cancer.org/layout/set/print/CAM-Summaries/Dietary-approaches/Laetrile [Accessed March 20, 2015].

Simeonova, F.P. & Fishbein, L., 2004. *Concise International Chemical Assessment Document 61:- Hydrogen Cyanide and Cyanides: Human Health Aspects*, Virginia: World Health Organisation.

UNIVERSITY of the
WESTERN CAPE

Singh, P.N. & Fraser, G.E., 1998. Dietary risk factors for colon cancer in a low-risk population. *American Journal of Epidemiology*, 148(8), pp.761–774.

Sørensen, C.S. & Syljuåsen, R.G., 2012. Safeguarding genome integrity: The checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. *Nucleic Acids Research*, 40(2), pp.477–486.

South, J., Laetrile - the answer to Cancer. , pp.1–11. Available at: http://www. medicine nuove.net/cancro/JAMES_SOUTH.pdf.

Sporn, M. et al., 1976. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Fed. Proc.*, 35(6), pp.1332–1338.

Stewart, Z., Westfall, M. & Pietenpol, J., 2003. Cell-cycle dysregulation and anticancer therapy. *Trends Pharmacol Sci.*, 24(3), pp.139–145.

Suzuki, H., Tomida, A. & Tsuruo, T., 1998. A novel mutant from apoptosis-resistant colon cancer HT-29 cells showing hyper-apoptotic response to hypoxia, low glucose and cisplatin. *Jpn. J. Cancer Res. Gann*, (89), pp.1169–1178.

Suchard, J.R., Wallace, K.L. & Gerkin, R.D., 1998. *Acute Cyanide Toxicity Caused by Apricot Kernel Ingestion*. *Annals of Emergency Medicine*, 32(6), pp.742–744.

Suryadinata, R., Sadowski, M. & Sarcevic, B., 2010. Control of cell cycle progression by phosphorylation of cyclin-dependent kinase (CDK) substrates. *Bioscience Reports*, 30(4), pp.243–255.

UNIVERSITY of the
WESTERN CAPE

Syrigos, K.N., Rowlinson-Busza, G. & Epenetos, A., 1998. In vitro cytotoxicity following specific activation of amygdalin by beta-glucosidase conjugated to a bladder cancer-associated monoclonal antibody. *International Journal of Cancer.*, 78(6), pp.712–9.

Takeda, D.Y. & Dutta, A., 2005. DNA replication and progression through S phase. *Oncogene*, 24(17), pp.2827–43.

Tasat, D.R. & Yakisich, J.S., 2010. Intra S-Phase Ccheckpoint. In A. E. Thomas, ed. *DNA Damage Repair, Repair Mechanisms and Aging*. Nova Science Publishers, Inc., pp. 71–96.

Thun, M.J. et al., 2010. The global burden of cancer: priorities for prevention. *Carcinogenesis*, 31(1), pp.100–110.

Trump, B.E. et al., 1997. The Pathways of Cell Death: Oncosis, Apoptosis, and Necrosis. *Toxicologic Pathology*, 25(1), pp.82–88.

UNIVERSITY of the
WESTERN CAPE

Tun, C. et al., 2007. Activation of the extrinsic caspase pathway in cultured cortical neurons requires p53-mediated down-regulation of the X-linked inhibitor of apoptosis protein to induce apoptosis. *Journal of Neurochemistry*, 102(4), pp.1206–1219.

Tuncel, G., Nout, M.J.R. & Brimer, L., 1998. Degradation of cyanogenic glycosides of bitter apricot seeds (Prrcnus armeniaca) by endogenous and added enzymes as affected by heat treatments and particle size. *Food Chemistry*, 63(1), pp.65–69.

Vardi, N. et al., 2008. Potent protective effect of apricot and beta-carotene on methotrexate-induced intestinal oxidative damage in rats. *Food and Chemical Toxicology*, 46(9), pp.3015–22.

Vitali, I. et al., 2011. Mitotic catastrophe: a mechanism for avoiding genomic instability. *Molecular Cell Biology*, Volume 12(1), pp.1–8.

Wattenberg, L., 1996. Chemoprevention of cancer. *Prev. Med*, 25, pp.44–45.

Wei, G., Zhao, Z.Z. & Shan, B., 2002. Report on the Antimutagenicity of 6 kinds of TCM herbs. *Europe Pubmed Central*, 14(2), pp.94–98.

Wenzel, U., Kuntz, S. & Brendel, M.D., 2000. Dietary Flavone Is a Potent Apoptosis Inducer in Human Colon Carcinoma Cells. *Cancer Research*, pp.3823–3831.

Williamson, G., 2004. Common features in the pathways of absorption and metabolism of flavonoids. In *Phytochemicals*. CRC Press, pp. 21–33.

Willis, N. & Rhind, N., 2009. Regulation of DNA replication by the S-phase DNA damage checkpoint. *Cell Division*, 4, p.13.

UNIVERSITY of the
WESTERN CAPE

Willis, N. & Rhind, N., 2010. The fission yeast Rad32(Mre11)-Rad50-Nbs1 complex acts both upstream and downstream of checkpoint signaling in the S-phase DNA damage checkpoint. *Genetics*, 184(4), pp.887–897.

Willson, J.K. V et al., 1987. Cell Culture of Human Colon Adenomas and Carcinomas. *Cancer Research*, 47, pp.2704–2713.

Woollard, A. & Nurse, P., 1995. G1 regulation and checkpoints operating around START in fission yeast. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, 17(6), pp.481–490.

World Health Organisation, 2004. WHO monographs on selected medicinal plants. In *WHO Monographs On Selected Medicinal Plants*. pp. 64–76.

World Health Organisation/GLOBOCAN, 2013. Latest world cancer statistics. *International Agency for Research on Cancer*, pp.1–3. Available at: http://www.uicc.org/iarc-release-latest-world-cancer-statistics [Accessed October 17, 2014].

Wu, H. et al., 2011. Essential oil extracted from peach (Prunus persica) kernel and its physicochemical and antioxidant properties. *LWT - Food Science and Technology*, 44(10), pp.2032–2039.

Xie, C.M. et al., 2011. Bufalin induces autophagy-mediated cell death in human colon cancer cells through reactive oxygen species generation and JNK activation. *Free Radic. Biol. Med.*, (51), pp.1365–1375.

Yiğit, D., Yiğit, N. & Mavi, A., 2009. Antioxidant and antimicrobial activities of bitter and sweet apricot (Prunus armeniaca L.) kernels. *Brazillian Journal of Medical and Biological Research*, 42(4), pp.346–52.

Yildrim, Fatima and Askin, M.A., 2010. Variability of amygdalin content in seeds of sweet and bitter apricot cultivars in Turkey. *African Journal of Biotechnology*, Vol 9(39), pp.6522 – 6524.

Yuan, L. et al., 2014. Isoorientin induces apoptosis and autophagy simultaneously by reactive oxygen species (ROS)-related p53, PI3K/Akt, JNK, and p38 signaling pathways in HepG2 cancer cells. *J. Agric. Food Chem.*, 62, pp.5390–5400.

Zhang, H.S. et al., 2000. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell*, 101(1), pp.79–89.

UNIVERSITY of the
WESTERN CAPE

Annexure 1

This following overview as to the chemical composition of the Apricot kernel (seed) is taken from the phytochemical database of the American Department of Agriculture. It can be retrieved on the Internet at the address: http://www.ars-grin.gov/duke/

CHEMICAL	PART	Lo ppm	Hi ppm	REFERENCE
Alpha-estradiol	seed			HHB
Amygdalin	seed		8000	HHB
Ash	seed	1000	30 000	HHB
Beta-carotene	seed		0	CRC
Beta-glucosidase	seed			CAN
Beta-sitosterol	seed			DUKE 1992A
Calcium	seed	930	1522	SMO
Campesterol	seed			DUKE 1992A
Carbohydrates	seed		140 000	DUKE 1992A
Cholesterol	seed			CAN
Copper	seed	1	16	SMO USA
Cyanide	seed	20	2000	CAN
Delta-24-Cholesterol	seed			HHB
Dextrose	seed	81000	116 000	DUKE 1992A
EO	seed	800	16000	DUKE 1992A
Estrone	seed			HHB
Fat	seed	400 000	514 000	HHB
Fibre	seed		33000	DUKE 1992A
Linoleic acid	seed	56 000	411 200	HHB
Magnesium	seed		1750	SMO
Neo-Chlorogenic acid	seed			CAN
Oleic acid	seed	248 000	411 200	HHB JAD
Pangamic acid	seed			HHB

UNIVERSITY of the WESTERN CAPE

Phosphorus	seed		3000	DUKE 1992A
Potassium	seed	4180	7783	DUKE 1992A
Protein	seed		315 000	DUKE 1992A

Annexure 2: Flow cytometry data

The following represents the data as collected after a 24, 48 and 72 hour exposure of the HT-29 colon cancer cells to 100, 500 and 1000 µg/mL organic and aqueous kernel extractions. FACs Calibre DNA analysis was performed and manually read to obtain the data.

ORGANIC EXTRACTIONS FLOW CYTOMETRY RESULTS

		G1	S	G2			G1	S	G2			G1	S	G2
24hr100	**Control**	85,2	13,88	0,91	**48hr100**	**Control**	83,47	15,3	1,24	**72hr100**	**Control**	83,51	15,61	0,88
	CAK T	90,09	7,99	1,92		**CAK T**	71,05	28,95	0		**CAK T**	81,88	15,92	2,2
	CAK L	94,8	3,76	1,44		**CAK L**	89,91	10,09	0		**CAK L**	77,11	21,72	1,17
	CAK H	90,64	7,81	1,55		**CAK H**	86,82	12,63	0,55		**CAK H**	80,06	17,43	2,51
	CPK T	75,68	24,32	0		**CPK T**	87,31	12,02	0,67		**CPK T**	79,07	19,34	1,59
	CPK L	80,17	18,41	1,42		**CPK L**	77,04	20,71	2,25		**CPK L**	80,85	18,43	0,72
	CPK H	82,36	16,12	1,52		**CPK H**	69,05	29,12	1,83		**CPK H**	86,88	11,81	1,31
	TAK T	82,16	14,37	3,46		**TAK T**	69,4	26,47	4,14		**TAK T**	83,56	16,44	0
	TAK L	78,25	19,87	1,88		**TAK L**	77,5	20,15	2,35		**TAK L**	85,32	13,8	0,89
	TAK H	82,15	17,06	0,79		**TAK H**	74,94	24,52	0,54		**TAK H**	90,12	9,25	0,62
	SAK T	62,08	37,92	0		**SAK T**	51.56	45.32	3.12		**SAK T**	83,19	16,04	0,76
	SAK L	62,13	38,87	0		**SAK L**	84,06	13,12	2,83		**SAK L**	70.39	29.61	0

	SAK H	13.60	86.40	0		SAK H	81,57	16,33	2,1		SAK H	77	23	0
	SPK T	35.53	64.47	0		SPK T	51,39	48,61	0		SPK T	89,78	10,22	0
	SPK L	33.20	66.80	0		SPK L	47,06	52,94	0		SPK L	36,56	63,44	0
	SPK H	43.75	56.25	0		SPK H	23.92	76.08	0		SPK H	79.39	20.61	0
24h500	control	85,2	13,88	0,91	48h500	control	83,47	15,3	1,24	72h500	control	83,51	15,61	0,88
	CAK T	88,78	9,83	1,4		CAK T	81,41	17,74	0,85		CAK T	81,14	15,16	3,71
	CAK L	84,3	14,43	1,27		CAK L	85,68	12,53	1,79		CAK L	86,3	11,78	1,92
	CAK H	87,6	10,45	1,94		CAK H	83,12	16,33	0,55		CAK H	83,58	13,66	2,75
	CPK T	87,69	10,31	2		CPK T	87,25	11,34	1,4		CPK T	86,09	10,99	2,92
	CPK L	86,9	10,67	2,43		CPK L	87,88	9,56	2,56		CPK L	85,08	12,34	2,57
	CPK H	86,69	11,98	1,33		CPK H	86,21	10,82	2,97		CPK H	43,27	56,73	0
	TAK T	88,02	10,41	1,57		TAK T	86,43	13,43	0,15		TAK T	87,74	11,19	1,07
	TAK L	85,28	12,04	2,68		TAK L	82,52	15,63	1,85		TAK L	77,4	22,32	0,28
	TAK H	85,13	12,2	2,67		TAK H	85,18	12,9	1,91		TAK H	90,95	7,26	1,8
	SAK T	39.87	60.13	0		SAK T	77,9	16,71	5,39		SAK T	89,14	10,86	0
	SAK L	79,13	20,87	0		SAK L	29,26	70,74	0		SAK L	85.23	14.77	0
	SAK H	34.43	65.57	0		SAK H	66,34	33,66	0		SAK H	79.97	17.73	2.29
	SPK T	65,75	34,25	0		SPK T	81,08	15,06	3,86		SPK T	83,64	16,36	0
	SPK L	78,12	21,88	0		SPK L	71,66	28,34	0		SPK L	62,86	37,14	0
	SPK H	49.02	50.98	0		SPK H	58,12	41,88	0		SPK H	84.42	15.58	0
24h1000	control	85,2	13,88	0,91	48h1000	control	83,47	15,3	1,24	72h1000	control	83,51	15,61	0,88
	CAK T	78,47	17,38	4,15		CAK T	87,34	12,66	0		CAK T	86,43	11,13	2,45
	CAK L	78,51	15,88	5,61		CAK L	86,79	12,96	0,25		CAK L	86,39	10,57	3,04
	CAK H	81,06	16,95	1,99		CAK H	87,83	12,17	0		CAK H	87,47	8,86	3,67
	CPK T	57,11	38,17	4,72		CPK T	87,65	11,41	0,94		CPK T	82,31	16	1,69

	CPK L	70,1	29,9	0		**CPK L**	86,93	12,3	0,77		**CPK L**	88,12	9,49	2,39
	CPK H	50,67	42,67	6,65		**CPK H**	86,92	13,08	0		**CPK H**	87,31	9,24	3,45
	TAK T	78,74	17,64	3,62		**TAK T**	83,33	16,67	0		**TAK T**	86,13	10,86	3
	TAK L	84,96	12,14	2,9		**TAK L**	87,34	12,66	0		**TAK L**	89,27	7,76	2,97
	TAK H	41,38	58,62	0		**TAK H**	89,15	10,85	0		**TAK H**	87,51	9,34	3,15
	SAK T	39,81	60,19	0		**SAK T**	54,87	45,13	0		**SAK T**	74.90	24.54	0.56
	SAK L	84,43	15,57	0		**SAK L**	62,13	37,87	0		**SAK L**	82,83	14,9	2,27
	SAK H	25.53	74.47	0		**SAK H**	58.27	41.73	0		**SAK H**	86,51	11,97	1,53
	SPK T	89,71	10,29	0		**SPK T**	22,38	77,62	0		**SPK T**	66,84	33,16	0
	SPK L	42.88	57.12	0		**SPK L**	62,89	37,11	0		**SPK L**	89,59	10,41	0
	SPK H	50,33	49,67	0		**SPK H**	53,46	46,54	0		**SPK H**	96,66	3,23	0,11

UNIVERSITY of the
WESTERN CAPE

AQUEOUS EXTRACTIONS FLOW CYTOMETRY RESULTS														
		G1	S	G2	TIME-DOSE	KERNEL	G1	S	G2	TIME-DOSE	KERNEL	G1	S	G2
24hr100	Control	85,2	13,88	0,91	48hr100	Control	83,47	15,3	1,24	72hr100	Control	83,51	15,61	0,88
	CAK	68,46	30,68	0,87		CAK	79,01	20,84	0,16		CAK	64,1	35,77	0,13
	CPK	81,61	18,39	0		CPK	76,85	23,15	0		CPK	92,58	7,42	0
	SAK	88,82	10,77	0,41		SAK	80,5	19,5	0		SAK	74,52	25,48	0
	SPK	85,11	14,35	0,54		SPK	76,51	23,49	0		SPK	68,6	31,4	0
24h500	control	85,2	13,88	0,91	48h500	control	83,47	15,3	1,24	72h500	control	83,51	15,61	0,88
	CAK	65,99	33,83	0,18		CAK	80,96	18,83	0,21		CAK	90,18	9,82	0
	CPK	78,12	21,88	0		CPK	76,1	23,9	0		CPK	87,42	12,58	0
	SAK	79,6	20,4	0		SAK	78,97	21,03	0		SAK	64,57	32,81	2,62
	SPK	86,8	12,49	0,71		SPK	78,06	21,94	0		SPK	90,96	9,04	0
24h1000	control	85,2	13,88	0,91	48h1000	control	83,47	15,3	1,24	72h1000	control	83,51	15,61	0,88
	CAK	78,62	21,38	0		CAK	82,62	17,38	0		CAK	94,36	5,64	0
	CPK	71,97	28,03	0		CPK	78,95	21,05	0		CPK	70,01	29,66	0,33
	SAK	87,41	12,59	0		SAK	82,69	17,31	0		SAK	93,04	6,96	0
	SPK	84,45	15,23	0,32		SPK	83,22	16,78	0		SPK	88,3	11,7	0

UNIVERSITY of the WESTERN CAPE

Annexure 3

Kruskal-Wallis test

Data	24hCAKorganic
Factor codes	LC1
Sample size	230
Test statistic	79,5168
Corrected for ties Ht	79,5333
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	14	220,00	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	24	126,52	(1)(3)(4)(6)(7)
(3) 3	24	86,35	(1)(2)(4)(5)(8)(9)(10)
(4) 4	24	51,58	(1)(2)(3)(5)(7)(8)(9)(10)
(5) 5	24	135,65	(1)(3)(4)(6)(7)
(6) 6	24	82,02	(1)(2)(5)(8)(9)(10)
(7) 7	24	92,96	(1)(2)(4)(5)(8)(9)(10)
(8) 8	24	143,52	(1)(3)(4)(6)(7)
(9) 9	24	128,54	(1)(3)(4)(6)(7)
(10) 10	24	131,40	(1)(3)(4)(6)(7)

UNIVERSITY of the WESTERN CAPE

Annexure 4

Kruskal-Wallis test

Data	48hCAKorganic
Factor codes	LC2
Sample size	174
Test statistic	88,6423
Corrected for ties Ht	88,6567
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	11	168,73	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	11	93,68	(1)(4)(5)(7)
(3) 3	23	95,52	(1)(4)(5)(6)(7)
(4) 4	11	19,68	(1)(2)(3)(5)(6)(8)(9)(10)
(5) 5	20	136,00	(1)(2)(3)(4)(6)(7)(8)(9)(10)
(6) 6	17	66,09	(1)(3)(4)(5)(7)(10)
(7) 7	20	40,75	(1)(2)(3)(5)(6)(8)(9)(10)
(8) 8	13	91,42	(1)(4)(5)(7)
(9) 9	24	80,87	(1)(4)(5)(7)
(10) 10	24	89,04	(1)(4)(5)(6)(7)

UNIVERSITY of the WESTERN CAPE

Annexure 5

Kruskal-Wallis test

Data	72hCAKorganic
Factor codes	LC3
Sample size	190
Test statistic	68,7815
Corrected for ties Ht	68,7879
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	15	43,87	(2)(3)(5)(7)(8)(9)(10)
(2) 2	23	122,57	(1)(4)(5)(6)(7)(8)
(3) 3	16	111,25	(1)(4)(6)(7)(8)
(4) 4	16	65,31	(2)(3)(8)(9)(10)
(5) 5	23	86,54	(1)(2)(6)(8)(9)
(6) 6	16	50,47	(2)(3)(5)(7)(8)(9)(10)
(7) 7	24	80,12	(1)(2)(3)(6)(8)(9)
(8) 8	21	159,14	(1)(2)(3)(4)(5)(6)(7)(9)(10)
(9) 9	12	120,54	(1)(4)(5)(6)(7)(8)
(10) 10	24	97,23	(1)(4)(6)(8)

UNIVERSITY of the WESTERN CAPE

Annexure 6

Kruskal-Wallis test

Data	24hCPKorganic
Factor codes	LC4
Sample size	196
Test statistic	78,1837
Corrected for ties Ht	78,1978
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	14	189,50	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	16	63,63	(1)(5)(6)(7)(10)
(3) 3	24	66,50	(1)(5)(6)(7)(10)
(4) 4	19	48,79	(1)(5)(6)(7)(9)(10)
(5) 5	13	138,85	(1)(2)(3)(4)(8)(9)
(6) 6	23	116,15	(1)(2)(3)(4)(8)(9)
(7) 7	24	110,25	(1)(2)(3)(4)(8)
(8) 8	15	76,73	(1)(5)(6)(7)(10)
(9) 9	24	89,96	(1)(4)(5)(6)
(10) 10	24	111,65	(1)(2)(3)(4)(8)

UNIVERSITY of the WESTERN CAPE

Annexure 7

Kruskal-Wallis test

Data	48hCPKorganic
Factor codes	LC5
Sample size	188
Test statistic	91,8134
Corrected for ties Ht	91,8247
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	11	182,82	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	16	117,47	(1)(3)(4)(6)(8)(9)
(3) 3	16	64,69	(1)(2)(4)(5)(7)(8)(10)
(4) 4	24	36,29	(1)(2)(3)(5)(6)(7)(8)(9)(10)
(5) 5	15	110,60	(1)(3)(4)(6)(8)(9)
(6) 6	24	69,85	(1)(2)(4)(5)(7)(8)(10)
(7) 7	24	106,02	(1)(3)(4)(6)(8)(9)
(8) 8	18	147,11	(1)(2)(3)(4)(5)(6)(7)(9)(10)
(9) 9	16	69,00	(1)(2)(4)(5)(7)(8)(10)
(10) 10	24	97,40	(1)(3)(4)(6)(8)(9)

UNIVERSITY of the
WESTERN CAPE

Annexure 8

Kruskal-Wallis test

Data	72hCPKorganic
Factor codes	LC6
Sample size	198
Test statistic	39,5476
Corrected for ties Ht	39,5505
Degrees of Freedom (DF)	9
Significance level	P = 0,000009

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	15	44,60	(2)(3)(5)(6)(7)(8)(9)(10)
(2) 2	24	111,21	(1)(8)
(3) 3	16	92,72	(1)(8)
(4) 4	16	79,03	(8)(9)
(5) 5	24	85,98	(1)(8)(9)
(6) 6	15	110,17	(1)(8)
(7) 7	24	87,92	(1)(8)(9)
(8) 8	24	146,19	(1)(2)(3)(4)(5)(6)(7)(10)
(9) 9	16	126,41	(1)(4)(5)(7)
(10) 10	24	94,08	(1)(8)

UNIVERSITY of the
WESTERN CAPE

Annexure 9

Kruskal-Wallis test

Data	24hTAKorganic
Factor codes	LC7
Sample size	192
Test statistic	97,3666
Corrected for ties Ht	97,3890
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	14	185,50	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	16	37,75	(1)(4)(5)(6)(7)(8)(9)(10)
(3) 3	24	39,02	(1)(4)(5)(6)(7)(8)(9)(10)
(4) 4	24	94,90	(1)(2)(3)(5)(6)(8)
(5) 5	15	135,33	(1)(2)(3)(4)(7)(9)(10)
(6) 6	14	121,36	(1)(2)(3)(4)(9)(10)
(7) 7	21	101,93	(1)(2)(3)(5)(8)
(8) 8	16	130,38	(1)(2)(3)(4)(7)(9)(10)
(9) 9	24	84,31	(1)(2)(3)(5)(6)(8)
(10) 10	24	88,92	(1)(2)(3)(5)(6)(8)

UNIVERSITY of the
WESTERN CAPE

Annexure 10

Kruskal-Wallis test

Data	48hTAKorganic
Factor codes	LC8
Sample size	161
Test statistic	130,1313
Corrected for ties Ht	130,1489
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	11	142,00	(2)(3)(4)(5)(6)(7)(9)(10)
(2) 2	19	57,63	(1)(4)(5)(6)(7)(8)(9)(10)
(3) 3	18	45,25	(1)(4)(5)(6)(7)(8)(9)(10)
(4) 4	15	112,07	(1)(2)(3)(9)(10)
(5) 5	18	120,19	(1)(2)(3)(6)(9)(10)
(6) 6	16	105,16	(1)(2)(3)(5)(8)(9)(10)
(7) 7	8	121,06	(1)(2)(3)(9)(10)
(8) 8	15	126,77	(2)(3)(6)(9)(10)
(9) 9	19	31,08	(1)(2)(3)(4)(5)(6)(7)(8)
(10) 10	22	26,45	(1)(2)(3)(4)(5)(6)(7)(8)

UNIVERSITY of the WESTERN CAPE

Annexure 11

Kruskal-Wallis test

Data	72hTAKorganic
Factor codes	LC9
Sample size	221
Test statistic	68,6915
Corrected for ties Ht	68,6952
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	15	25,47	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	24	117,08	(1)(3)(5)(8)
(3) 3	24	67,83	(1)(2)(4)(5)(6)(7)(8)(9)(10)
(4) 4	24	98,73	(1)(3)(5)(8)
(5) 5	16	172,25	(1)(2)(3)(4)(6)(7)(9)(10)
(6) 6	24	129,15	(1)(3)(5)
(7) 7	23	114,22	(1)(3)(5)(8)
(8) 8	24	157,98	(1)(2)(3)(4)(7)(9)(10)
(9) 9	23	110,28	(1)(3)(5)(8)
(10) 10	24	105,46	(1)(3)(5)(8)

UNIVERSITY of the WESTERN CAPE

Annexure 12

Kruskal-Wallis test

Data	24hSAKorganic
Factor codes	LC10
Sample size	152
Test statistic	99,3956
Corrected for ties Ht	99,4143
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	14	145,50	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	12	55,04	(1)(4)(5)(7)(8)(9)(10)
(3) 3	23	47,26	(1)(4)(5)(6)(7)(8)(9)(10)
(4) 4	16	9,13	(1)(2)(3)(5)(6)(7)(8)(9)(10)
(5) 5	12	78,54	(1)(2)(3)(4)(7)(8)(9)
(6) 6	17	69,12	(1)(3)(4)(7)(8)(9)
(7) 7	14	102,89	(1)(2)(3)(4)(5)(6)(10)
(8) 8	13	102,69	(1)(2)(3)(4)(5)(6)(10)
(9) 9	16	99,09	(1)(2)(3)(4)(5)(6)
(10) 10	15	81,27	(1)(2)(3)(4)(7)(8)

UNIVERSITY of the WESTERN CAPE

Annexure 13

Kruskal-Wallis test

Data	48hSAKorganic
Factor codes	LC11
Sample size	186
Test statistic	86,9082
Corrected for ties Ht	86,9220
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	11	179,09	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	22	70,23	(1)(3)(5)(6)(7)(9)(10)
(3) 3	22	28,41	(1)(2)(4)(5)(6)(7)(8)(9)(10)
(4) 4	23	64,96	(1)(3)(5)(6)(7)(9)(10)
(5) 5	22	127,61	(1)(2)(3)(4)(8)(10)
(6) 6	16	117,28	(1)(2)(3)(4)(8)
(7) 7	16	115,66	(1)(2)(3)(4)(8)
(8) 8	19	86,13	(1)(3)(5)(6)(7)
(9) 9	16	108,59	(1)(2)(3)(4)
(10) 10	19	97,29	(1)(2)(3)(4)(5)

UNIVERSITY of the WESTERN CAPE

Annexure 14

Kruskal-Wallis test

Data	72hSAKorganic
Factor codes	LC12
Sample size	184
Test statistic	88,3241
Corrected for ties Ht	88,3298
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	15	54,40	(2)(3)(6)(8)(9)
(2) 2	18	138,75	(1)(4)(5)(6)(7)(8)(9)(10)
(3) 3	24	158,31	(1)(4)(5)(6)(7)(8)(9)(10)
(4) 4	15	48,73	(2)(3)(6)(8)(9)
(5) 5	24	63,85	(2)(3)(6)(8)(9)
(6) 6	24	105,65	(1)(2)(3)(4)(5)(7)(10)
(7) 7	8	34,75	(2)(3)(6)(8)(9)(10)
(8) 8	19	91,47	(1)(2)(3)(4)(5)(7)
(9) 9	22	92,25	(1)(2)(3)(4)(5)(7)
(10) 10	15	70,83	(2)(3)(6)(7)

UNIVERSITY of the WESTERN CAPE

Annexure 15

Kruskal-Wallis test

Data	24hSPKorganic
Factor codes	LC13
Sample size	147
Test statistic	93,2899
Corrected for ties Ht	93,3064
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	14	140,50	(2)(3)(4)(5)(6)(7)(9)(10)
(2) 2	12	48,29	(1)(4)(5)(6)(8)
(3) 3	18	60,33	(1)(4)(5)(6)(8)
(4) 4	15	87,00	(1)(2)(3)(6)(7)(8)(9)(10)
(5) 5	14	92,79	(1)(2)(3)(6)(7)(8)(9)(10)
(6) 6	14	22,25	(1)(2)(3)(4)(5)(7)(8)(9)(10)
(7) 7	14	62,93	(1)(4)(5)(6)(8)
(8) 8	13	126,31	(2)(3)(4)(5)(6)(7)(9)(10)
(9) 9	20	49,40	(1)(4)(5)(6)(8)
(10) 10	13	63,00	(1)(4)(5)(6)(8)

UNIVERSITY of the
WESTERN CAPE

Annexure 16

Kruskal-Wallis test

Data	48hSPKorganic
Factor codes	LC14
Sample size	165
Test statistic	74,6429
Corrected for ties Ht	74,6667
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	11	159,64	(2)(3)(4)(5)(6)(7)(8)(9)(10)
(2) 2	15	71,93	(1)(5)(6)(7)(9)
(3) 3	23	52,98	(1)(5)(6)(8)(9)(10)
(4) 4	21	57,67	(1)(5)(6)(9)(10)
(5) 5	16	116,94	(1)(2)(3)(4)(7)(8)(10)
(6) 6	9	122,78	(1)(2)(3)(4)(7)(8)(10)
(7) 7	21	46,90	(1)(2)(5)(6)(8)(9)(10)
(8) 8	13	78,08	(1)(3)(5)(6)(7)(9)
(9) 9	16	106,72	(1)(2)(3)(4)(7)(8)
(10) 10	20	87,35	(1)(3)(4)(5)(6)(7)

UNIVERSITY of the WESTERN CAPE

Annexure 17

Kruskal-Wallis test

Data	72hSPKorganic
Factor codes	LC15
Sample size	166
Test statistic	102,1537
Corrected for ties Ht	102,1628
Degrees of Freedom (DF)	9
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	15	30,00	(2)(3)(4)(6)(7)(8)(9)(10)
(2) 2	14	84,36	(1)(4)(5)(6)(7)(10)
(3) 3	16	96,34	(1)(4)(5)(6)(7)(10)
(4) 4	17	54,65	(1)(2)(3)(5)(6)(7)(8)(9)
(5) 5	16	32,34	(2)(3)(4)(6)(7)(8)(9)
(6) 6	24	146,35	(1)(2)(3)(4)(5)(7)(8)(9)(10)
(7) 7	15	120,23	(1)(2)(3)(4)(5)(6)(9)(10)
(8) 8	14	105,39	(1)(4)(5)(6)(9)(10)
(9) 9	21	80,93	(1)(4)(5)(6)(7)(8)(10)
(10) 10	14	53,64	(1)(2)(3)(6)(7)(8)(9)

UNIVERSITY of the WESTERN CAPE

Annexure 18

Kruskal-Wallis test

Data	24h_All_organic_extractions
Factor codes	LC_2
Sample size	803
Test statistic	608,2704
Corrected for ties Ht	608,3045
Degrees of Freedom (DF)	45
Significance level	P < 0,000001

UNIVERSITY of the WESTERN CAPE

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	14	796,50	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(42)(43)(45)(46)
(2) 2	15	140,67	(1)(7)(8)(9)(10)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(3) 3	23	148,02	(1)(4)(7)(8)(9)(10)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(4) 4	23	77,93	(1)(3)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)

			(45)(46)
(5) 5	12	122,17	(1)(7)(8)(9)(10)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(6) 6	22	122,16	(1)(7)(8)(9)(10)(12)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(7) 7	9	14,06	(1)(2)(3)(5)(6)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(8) 8	16	243,31	(1)(2)(3)(4)(5)(6)(7)(13)(14)(15)(19)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(9) 9	20	268,40	(1)(2)(3)(4)(5)(6)(7)(13)(14)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(10) 10	18	234,36	(1)(2)(3)(4)(5)(6)(7)(13)(14)(15)(16)(19)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(11) 11	16	193,81	(1)(4)(7)(14)(15)(16)(19)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(12) 12	24	200,56	(1)(4)(6)(7)(14)(15)(16)(19)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(13) 13	19	152,29	(1)(4)(7)(8)(9)(10)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(14) 14	13	407,19	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(16)(17)(18)(19)(20)(21)(23)(24)(25)(26)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(15) 15	23	327,70	(1)(2)(3)(4)(5)(6)(7)(8)(10)(11)(12)(13)(17)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(16) 16	24	312,77	(1)(2)(3)(4)(5)(6)(7)(10)(11)(12)(13)(14)(17)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(17) 17	15	228,07	(1)(2)(3)(4)(5)(6)(7)(14)(15)(16)(19)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(18) 18	24	266,77	(1)(2)(3)(4)(5)(6)(7)(13)(14)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(19) 19	24	322,94	(1)(2)(3)(4)(5)(6)(7)(8)(10)(11)(12)(13)(14)(17)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)

UNIVERSITY of the WESTERN CAPE

(20) 20	16	261,56	(1)(2)(3)(4)(5)(6)(7)(13)(14)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(21) 21	24	259,56	(1)(2)(3)(4)(5)(6)(7)(13)(14)(15)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(22) 22	24	471,31	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(15)(16)(17)(18)(19)(20)(21)(23)(26)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(23) 23	15	599,97	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(25)(27)(28)(30)(31)(34)(35)(40)(41)(42)(44)
(24) 24	14	544,71	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(27)(28)(31)(34)(35)(36)(40)(41)(44)
(25) 25	21	495,33	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(23)(26)(31)(32)(34)(35)(36)(37)(39)(40)(41)(43)(44)(46)
(26) 26	16	578,97	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(25)(27)(28)(30)(31)(34)(35)(36)(40)(41)(42)(44)
(27) 27	24	431,67	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(15)(16)(17)(18)(19)(20)(21)(23)(24)(26)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(28) 28	24	458,54	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(15)(16)(17)(18)(19)(20)(21)(23)(24)(26)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(29) 29	12	539,83	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(27)(31)(34)(35)(36)(40)(41)(44)
(30) 30	23	489,41	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(23)(26)(31)(32)(34)(35)(36)(37)(39)(40)(41)(43)(44)(45)(46)
(31) 31	16	178,16	(1)(4)(7)(9)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(32) 32	12	611,71	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(25)(27)(28)(30)(31)(41)(42)(44)
(33) 33	17	555,88	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(27)(28)(31)(34)(35)(36)(40)(41)(42)(44)
(34) 34	14	688,21	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(33)(38)(39)(42)(44)(45)
(35) 35	13	689,77	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(33)(38)(39)(42)(44)(45)
(36) 36	16	677,28	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(24)(25)(26)(27)(28)(29)(30)(31)(33)(38)(39)(42)(44)(45)

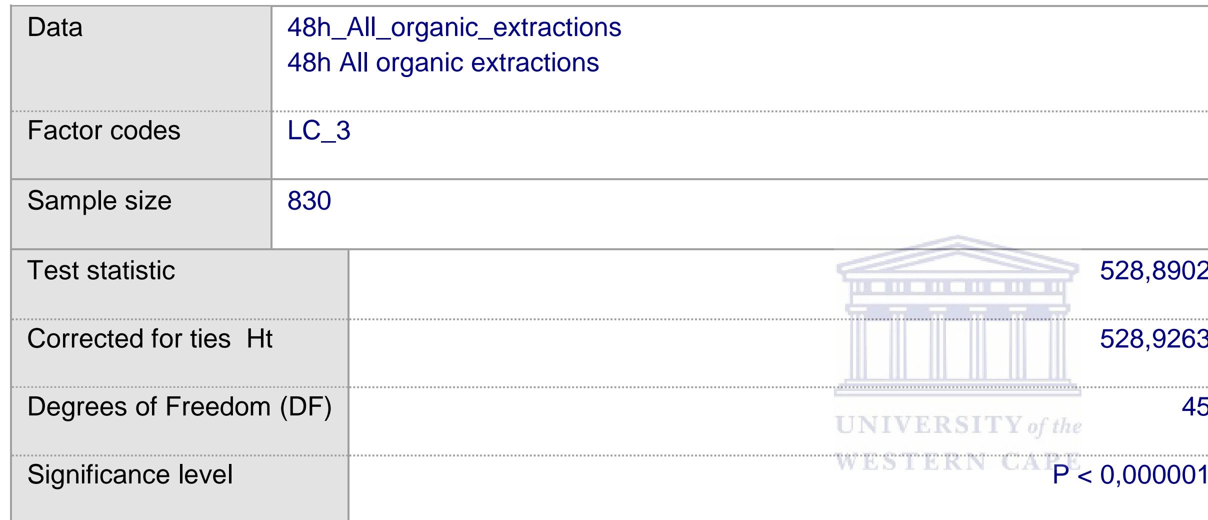
UNIVERSITY of the

(37) 37	15	623,83	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(25)(27)(28)(30)(31)(41)(42)(44)
(38) 38	12	564,67	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(27)(28)(31)(34)(35)(36)(40)(41)(42)(44)
(39) 39	18	597,92	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(25)(27)(28)(30)(31)(34)(35)(36)(40)(41)(42)(44)
(40) 40	15	687,53	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(33)(38)(39)(42)(44)(45)
(41) 41	14	714,39	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(37)(38)(39)(42)(43)(45)(46)
(42) 42	14	464,79	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(15)(16)(17)(18)(19)(20)(21)(23)(26)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(43) 43	14	619,86	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(25)(27)(28)(30)(31)(41)(42)(44)
(44) 44	13	781,73	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(42)(43)(45)(46)
(45) 45	20	561,37	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(27)(28)(30)(31)(34)(35)(36)(40)(41)(42)(44)
(46) 46	13	606,77	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(25)(27)(28)(30)(31)(41)(42)(44)

UNIVERSITY of the
WESTERN CAPE

Annexure 19

Kruskal-Wallis test



Data	48h_All_organic_extractions 48h All organic extractions
Factor codes	LC_3
Sample size	830
Test statistic	528,8902
Corrected for ties Ht	528,9263
Degrees of Freedom (DF)	45
Significance level	P < 0,000001

UNIVERSITY of the
WESTERN CAPE

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	11	808,27	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(2) 2	11	338,41	(1)(4)(5)(7)(12)(13)(15)(17)(18)(22)(23)(24)(25)(26)(27)(28)(32)(33)(34)(35)(36)(37)(38)(41)(42)(44)(45)(46)
(3) 3	23	373,67	(1)(4)(5)(6)(7)(12)(13)(15)(17)(18)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(35)(36)(37)(38)(41)(42)(44)(45)(46)
(4) 4	11	63,23	(1)(2)(3)(5)(6)(8)(9)(10)(11)(14)(15)(16)(17)(19)(20)(21)(22)(23)(24)(25)(26)(27)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45) (46)

(5) 5	20	567,65	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(39)(40)(43)
(6) 6	17	238,56	(1)(3)(4)(5)(7)(10)(11)(13)(16)(17)(20)(22)(23)(24)(25)(26)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(7) 7	20	134,87	(1)(2)(3)(5)(6)(8)(9)(10)(11)(14)(16)(17)(19)(20)(21)(22)(23)(24)(25)(26)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(8) 8	13	332,92	(1)(4)(5)(7)(12)(13)(15)(17)(18)(22)(23)(24)(25)(26)(27)(28)(29)(32)(33)(34)(35)(36)(37)(38)(41)(42)(44)(45)(46)
(9) 9	24	296,58	(1)(4)(5)(7)(12)(13)(15)(17)(18)(20)(22)(23)(24)(25)(26)(28)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(44)(45)(46)
(10) 10	24	335,33	(1)(4)(5)(6)(7)(12)(13)(15)(17)(18)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(40)(41)(42)(44)(45)(46)
(11) 11	16	354,53	(1)(4)(5)(6)(7)(12)(13)(15)(17)(18)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(35)(36)(37)(38)(41)(42)(44)(45)(46)
(12) 12	16	163,56	(1)(2)(3)(5)(8)(9)(10)(11)(14)(16)(17)(19)(20)(21)(22)(23)(24)(25)(26)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(13) 13	24	85,17	(1)(2)(3)(5)(6)(8)(9)(10)(11)(14)(15)(16)(17)(19)(20)(21)(22)(23)(24)(25)(26)(27)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(14) 14	15	329,50	(1)(4)(5)(7)(12)(13)(15)(17)(18)(22)(23)(24)(25)(26)(27)(28)(29)(32)(33)(34)(35)(36)(37)(38)(40)(41)(42)(44)(45)(46)
(15) 15	24	180,48	(1)(2)(3)(4)(5)(8)(9)(10)(11)(13)(14)(16)(17)(19)(20)(21)(22)(23)(24)(25)(26)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(16) 16	24	335,08	(1)(4)(5)(6)(7)(12)(13)(15)(17)(18)(22)(23)(24)(25)(26)(27)(28)(29)(30)(32)(33)(34)(35)(36)(37)(38)(40)(41)(42)(44)(45)(46)
(17) 17	18	521,64	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(30)(31)(32)(39)(41)(42)(43)
(18) 18	16	161,19	(1)(2)(3)(5)(8)(9)(10)(11)(14)(16)(17)(19)(20)(21)(22)(23)(24)(25)(26)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(19) 19	24	298,33	(1)(4)(5)(7)(12)(13)(15)(17)(18)(20)(22)(23)(24)(25)(26)(28)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(44)(45)(46)
(20) 20	19	417,18	(1)(4)(5)(6)(7)(9)(12)(13)(15)(17)(18)(19)(21)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(36)(37)(41)(42)(45)(46)

UNIVERSITY of the
WESTERN CAPE

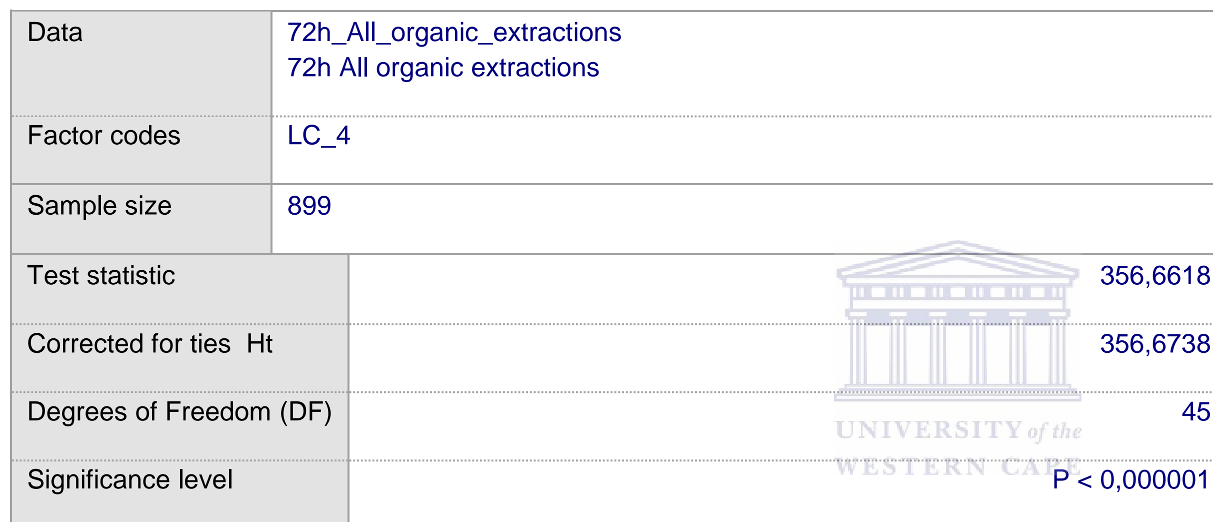
(21) 21	18	313,14	(1)(4)(5)(7)(12)(13)(15)(17)(18)(20)(22)(23)(24)(25)(26)(27)(28)(29)(31)(32)(33)(34)(35)(36)(37)(38)(40)(41)(42)(44)(45)(46)
(22) 22	15	753,43	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(23) 23	18	777,86	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(24) 24	16	747,66	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(25) 25	8	782,50	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(26) 26	15	790,17	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(27) 27	19	208,97	(1)(2)(3)(4)(5)(8)(10)(11)(13)(14)(16)(17)(20)(21)(22)(23)(24)(25)(26)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(28) 28	22	165,70	(1)(2)(3)(5)(8)(9)(10)(11)(14)(16)(17)(19)(20)(21)(22)(23)(24)(25)(26)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(29) 29	22	444,73	(1)(4)(5)(6)(7)(8)(9)(10)(12)(13)(14)(15)(16)(18)(19)(21)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(36)(37)(41)(42)(45)
(30) 30	22	243,80	(1)(3)(4)(5)(7)(10)(11)(13)(16)(17)(20)(22)(23)(24)(25)(26)(29)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46)
(31) 31	23	413,48	(1)(4)(5)(6)(7)(9)(12)(13)(15)(17)(18)(19)(21)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(36)(37)(41)(42)(45)(46)
(32) 32	22	629,48	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(35)(38)(39)(40)(43)(44)(46)
(33) 33	16	596,03	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(35)(38)(39)(40)(43)(44)
(34) 34	16	607,28	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(35)(38)(39)(40)(43)(44)
(35) 35	19	493,89	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(21)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(39)(41)(42)(43)(45)
(36) 36	16	584,16	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(38)(39)(40)(43)

UNIVERSITY of the
WESTERN CAPE

(37) 37	19	546,37	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(39)(40)(43)
(38) 38	15	478,13	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(21)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(36)(41)(42)(43)(45)
(39) 39	23	384,54	(1)(4)(5)(6)(7)(9)(12)(13)(15)(17)(18)(19)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(35)(36)(37)(41)(42)(44)(45)(46)
(40) 40	21	428,69	(1)(4)(5)(6)(7)(9)(10)(12)(13)(14)(15)(16)(18)(19)(21)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(36)(37)(41)(42)(45)(46)
(41) 41	16	624,91	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(35)(38)(39)(40)(43)(44)(46)
(42) 42	9	652,44	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(23)(26)(27)(28)(29)(30)(31)(35)(38)(39)(40)(43)(44)(46)
(43) 43	21	376,74	(1)(4)(5)(6)(7)(12)(13)(15)(17)(18)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(35)(36)(37)(38)(41)(42)(44)(45)(46)
(44) 44	13	486,12	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(21)(22)(23)(24)(25)(26)(27)(28)(30)(32)(33)(34)(39)(41)(42)(43)(45)
(45) 45	16	595,50	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(35)(38)(39)(40)(43)(44)
(46) 46	20	525,38	(1)(2)(3)(4)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(30)(31)(32)(39)(40)(41)(42)(43)

Annexure 20

Kruskal-Wallis test



Data	72h_All_organic_extractions 72h All organic extractions
Factor codes	LC_4
Sample size	899
Test statistic	356,6618
Corrected for ties Ht	356,6738
Degrees of Freedom (DF)	45
Significance level	P < 0,000001

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	15	166,33	(2)(3)(5)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(35)(36)(38)(39)(42)(43)(44)(45)
(2) 2	23	477,63	(1)(4)(5)(6)(7)(8)(13)(17)(23)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(43)(46)
(3) 3	16	412,03	(1)(4)(6)(8)(17)(23)(24)(26)(29)(30)(31)(32)(34)(37)(41)(42)(43)
(4) 4	16	239,13	(2)(3)(8)(9)(10)(11)(15)(16)(17)(18)(19)(20)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(35)(36)(38)(39)(42)(43)(44)(45)

(5) 5	23	318,70	(1)(2)(8)(9)(11)(15)(17)(18)(20)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(33)(34)(39)(41)(42)(43)(44)
(6) 6	16	193,19	(2)(3)(8)(9)(10)(11)(12)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(35)(36)(38)(39)(42)(43)(44)(45)
(7) 7	24	312,56	(1)(2)(8)(9)(11)(15)(17)(18)(20)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(33)(34)(39)(41)(42)(43)(44)
(8) 8	21	675,45	(1)(2)(3)(4)(5)(6)(7)(9)(10)(11)(12)(13)(14)(15)(16)(19)(20)(21)(22)(25)(27)(28)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(45)(46)
(9) 9	12	469,21	(1)(4)(5)(6)(7)(8)(17)(23)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(43)(46)
(10) 10	24	372,48	(1)(4)(6)(8)(17)(18)(20)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(34)(41)(42)(43)(44)
(11) 11	24	477,87	(1)(4)(5)(6)(7)(8)(13)(14)(17)(23)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(43)(46)
(12) 12	16	382,53	(1)(6)(8)(17)(18)(20)(23)(24)(25)(26)(27)(29)(30)(31)(32)(34)(41)(42)(43)(44)
(13) 13	16	323,06	(1)(2)(8)(11)(15)(17)(18)(20)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(33)(34)(39)(41)(42)(43)(44)
(14) 14	24	360,17	(1)(6)(8)(11)(17)(18)(20)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(34)(39)(41)(42)(43)(44)
(15) 15	15	470,10	(1)(4)(5)(6)(7)(8)(13)(17)(23)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(43)(46)
(16) 16	24	371,33	(1)(4)(6)(8)(17)(18)(20)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(34)(41)(42)(43)(44)
(17) 17	24	639,25	(1)(2)(3)(4)(5)(6)(7)(9)(10)(11)(12)(13)(14)(15)(16)(19)(21)(22)(28)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(45)(46)
(18) 18	16	545,34	(1)(4)(5)(6)(7)(10)(12)(13)(14)(16)(19)(21)(23)(26)(29)(30)(31)(32)(34)(35)(36)(37)(40)(41)(42)(46)
(19) 19	24	400,81	(1)(4)(6)(8)(17)(18)(20)(23)(24)(25)(26)(27)(29)(30)(31)(32)(34)(37)(41)(42)(43)(44)

UNIVERSITY of the

(20) 20	24	533,13	(1)(4)(5)(6)(7)(8)(10)(12)(13)(14)(16)(19)(21)(23)(26)(29)(30)(31)(32)(34)(35)(36)(37)(40)(41)(42)(46)
(21) 21	24	367,27	(1)(6)(8)(17)(18)(20)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(34)(39)(41)(42)(43)(44)
(22) 22	24	454,71	(1)(4)(5)(6)(7)(8)(13)(17)(23)(24)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(43)(46)
(23) 23	16	729,78	(1)(2)(3)(4)(5)(6)(7)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(24)(25)(27)(28)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(44)(45)(46)
(24) 24	24	589,42	(1)(3)(4)(5)(6)(7)(10)(12)(13)(14)(16)(19)(21)(22)(23)(30)(31)(32)(34)(35)(36)(37)(38)(40)(41)(42)(45)(46)
(25) 25	23	532,61	(1)(4)(5)(6)(7)(8)(10)(12)(13)(14)(16)(19)(21)(23)(26)(29)(30)(31)(32)(34)(35)(36)(37)(40)(41)(42)(46)
(26) 26	24	685,19	(1)(2)(3)(4)(5)(6)(7)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(25)(27)(28)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(45)(46)
(27) 27	23	527,87	(1)(4)(5)(6)(7)(8)(10)(12)(13)(14)(16)(19)(21)(23)(26)(29)(30)(31)(32)(34)(35)(36)(37)(40)(41)(42)(46)
(28) 28	24	503,19	(1)(4)(5)(6)(7)(8)(10)(13)(14)(16)(17)(21)(23)(26)(29)(30)(31)(32)(34)(36)(37)(40)(41)(42)(43)(46)
(29) 29	18	690,14	(1)(2)(3)(4)(5)(6)(7)(9)(10)(11)(12)(13)(14)(15)(16)(18)(19)(20)(21)(22)(25)(27)(28)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(45)(46)
(30) 30	24	787,98	(1)(2)(3)(4)(5)(6)(7)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(24)(25)(27)(28)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(31) 31	15	175,03	(2)(3)(5)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(35)(36)(38)(39)(42)(43)(44)(45)
(32) 32	24	231,96	(2)(3)(8)(9)(10)(11)(12)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(35)(36)(38)(39)(42)(43)(44)(45)

UNIVERSITY of the

(33) 33	24	473,19	(1)(4)(5)(6)(7)(8)(13)(17)(23)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(43)(46)
(34) 34	8	109,94	(2)(3)(5)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(35)(36)(38)(39)(42)(43)(44)(45)
(35) 35	19	387,66	(1)(4)(6)(8)(17)(18)(20)(23)(24)(25)(26)(27)(29)(30)(31)(32)(34)(41)(42)(43)(44)
(36) 36	22	380,23	(1)(4)(6)(8)(17)(18)(20)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(34)(41)(42)(43)(44)
(37) 37	15	261,50	(2)(3)(8)(9)(11)(15)(17)(18)(19)(20)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(38)(39)(42)(43)(44)(45)
(38) 38	14	422,82	(1)(4)(6)(8)(17)(23)(24)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(43)
(39) 39	16	502,06	(1)(4)(5)(6)(7)(8)(13)(14)(17)(21)(23)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(46)
(40) 40	17	276,21	(2)(8)(9)(11)(15)(17)(18)(20)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(38)(39)(42)(43)(44)(45)
(41) 41	16	172,25	(2)(3)(5)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(35)(36)(38)(39)(42)(43)(44)(45)
(42) 42	24	791,48	(1)(2)(3)(4)(5)(6)(7)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(24)(25)(27)(28)(31)(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(43)(44)(45)(46)
(43) 43	15	640,10	(1)(2)(3)(4)(5)(6)(7)(9)(10)(11)(12)(13)(14)(15)(16)(19)(21)(22)(28)(30)(31)(32)(33)(34)(35)(36)(37)(38)(40)(41)(42)(45)(46)
(44) 44	14	550,71	(1)(4)(5)(6)(7)(10)(12)(13)(14)(16)(19)(21)(23)(30)(31)(32)(34)(35)(36)(37)(40)(41)(42)(46)
(45) 45	21	416,76	(1)(4)(6)(8)(17)(23)(24)(26)(29)(30)(31)(32)(34)(37)(40)(41)(42)(43)(46)
(46) 46	14	270,39	(2)(8)(9)(11)(15)(17)(18)(20)(22)(23)(24)(25)(26)(27)(28)(29)(30)(33)(39)(42)(43)(44)(45)

WESTERN CAPE

Annexure 21

Kruskal-Wallis test

Data	24h_48h_72h_All_aqueous_extractions
Factor codes	LC_9
Sample size	855
Test statistic	391,3315
Corrected for ties Ht	391,3953
Degrees of Freedom (DF)	38
Significance level	P < 0,000001

UNIVERSITY of the
WESTERN CAPE

Post-hoc analysis

Factor	n	Average Rank	Different (P<0,05) from factor nr
(1) 1	15	847,93	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(2) 2	24	191,79	(1)(4)(7)(10)(13)(14)(15)(16)(17)(18)(19)(20)(21)(23)(24)(25)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(3) 3	24	183,81	(1)(4)(7)(10)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)

(4) 4	2 4	371,06	(1)(2)(3)(6)(11)(12)(14)(15)(19)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(5) 5	2 4	273,35	(1)(6)(14)(15)(16)(17)(19)(20)(21)(24)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(6) 6	2 4	138,69	(1)(4)(5)(7)(8)(9)(10)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(7) 7	2 3	308,65	(1)(2)(3)(6)(11)(14)(15)(16)(19)(20)(24)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(8) 8	1 6	266,75	(1)(6)(14)(15)(16)(17)(19)(20)(21)(24)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(9) 9	1 6	273,87	(1)(6)(14)(15)(16)(17)(19)(20)(21)(24)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(10) 1 0	1 6	365,41	(1)(2)(3)(6)(11)(14)(15)(19)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(11) 1 1	2 4	196,85	(1)(4)(7)(10)(13)(14)(15)(16)(17)(18)(19)(20)(21)(23)(24)(25)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(12) 1 2	2 3	247,54	(1)(4)(6)(14)(15)(16)(17)(18)(19)(20)(21)(24)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(13) 1 3	2 4	339,27	(1)(2)(3)(6)(11)(14)(15)(16)(19)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(14) 1 4	1 6	832,50	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)

UNIVERSITY of the WESTERN CAPE

(15) 15	24	484,90	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(18)(22)(23)(25)(26)(27)(28)(30)(31)(33)(39)
(16) 16	24	456,27	(1)(2)(3)(5)(6)(7)(8)(9)(11)(12)(13)(14)(22)(23)(25)(26)(27)(28)(30)(31)(32)(33)(34)(39)
(17) 17	21	404,83	(1)(2)(3)(5)(6)(8)(9)(11)(12)(14)(19)(26)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(18) 18	24	356,23	(1)(2)(3)(6)(11)(12)(14)(15)(19)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(19) 19	24	522,23	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(17)(18)(21)(22)(23)(25)(26)(27)(29)(30)(31)
(20) 20	24	428,77	(1)(2)(3)(5)(6)(7)(8)(9)(11)(12)(14)(22)(23)(25)(26)(27)(28)(30)(31)(32)(33)(34)(36)(37)(39)
(21) 21	28	393,29	(1)(2)(3)(5)(6)(8)(9)(11)(12)(14)(19)(26)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(22) 22	20	299,90	(1)(3)(6)(14)(15)(16)(19)(20)(24)(27)(28)(29)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(23) 23	20	317,87	(1)(2)(3)(6)(11)(14)(15)(16)(19)(20)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(24) 24	24	421,62	(1)(2)(3)(5)(6)(7)(8)(9)(11)(12)(14)(22)(26)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(25) 25	24	318,08	(1)(2)(3)(6)(11)(14)(15)(16)(19)(20)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)

UNIVERSITY of the

(26) 26	23	281,57	(1)(6)(14)(15)(16)(17)(19)(20)(21)(24)(27)(28)(29)(30)(31)(32)(33)(34)(35)(36)(37)(38)(39)
(27) 27	15	747,93	(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(28)(29)(32)(33)(34)(35)(36)(37)(38)(39)
(28) 28	24	606,69	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(20)(21)(22)(23)(24)(25)(26)(27)(29)(35)
(29) 29	24	410,63	(1)(2)(3)(5)(6)(8)(9)(11)(12)(14)(19)(22)(26)(27)(28)(30)(31)(32)(33)(34)(36)(37)(38)(39)
(30) 30	24	635,33	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(29)(35)(38)
(31) 31	23	651,78	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(29)(35)(38)
(32) 32	24	573,87	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(16)(17)(18)(20)(21)(22)(23)(24)(25)(26)(27)(29)(35)
(33) 33	24	617,60	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(20)(21)(22)(23)(24)(25)(26)(27)(29)(35)
(34) 34	16	589,66	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(16)(17)(18)(20)(21)(22)(23)(24)(25)(26)(27)(29)(35)
(35) 35	18	416,25	(1)(2)(3)(5)(6)(8)(9)(11)(12)(14)(26)(27)(28)(30)(31)(32)(33)(34)(36)(37)(39)
(36) 36	18	560,56	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(17)(18)(20)(21)(22)(23)(24)(25)(26)(27)(29)(35)

UNIVERSITY of the

(37) 37	24	557,44	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(17)(18)(20)(21)(22)(23)(24)(25)(26)(27)(29)(35)
(38) 38	24	528,31	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(17)(18)(21)(22)(23)(24)(25)(26)(27)(29)(30)(31)
(39) 39	24	597,19	(1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(20)(21)(22)(23)(24)(25)(26)(27)(29)(35)