Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University,
College of Science,
Biotechnology Department

Investigating the Anti-tumour Properties of Iraqi Propolis *in vitro* and *in vivo*

A Dissertation

Submitted to the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology

By

Ghassan Mohammad Sulaiman

B.Sc. Biology/ University of Baghdad /1995
M.Sc. Biology/ University of Baghdad / 2002

Supervised by

Dr. Khulood W.Al Sammarae
Professor

Dr. Ali.H.Ad'hiah
Assist. Professor

July 2010

Rajab 1431

بِسْمِ اللّهِ الرِّحْمَنِ الرِّحِيمِ

وَأَوْحَى رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ (٦٨) ثُمَّ كُلِي مِن كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلاً يَخْرُجُ مِن بُطُونِهَا شَرَابٌ مُّخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاء لِلنَّاسِ إِنَّ فِي ذَلِكَ لآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ (٦٩)

صدقَ الله العَظيم

سورة النحل

In the name of God Most Gracious, Most Merciful

(68) “And your Lord inspired the bees saying:”Take you habitations in the mountains and in the trees and what they erect. (69)”Then to eat of all fruits and follow the ways of your Lord made easy (for you). There comes forth their bellies, a drink of varying colour wherein is healing for men. Verily in this is indeed a sign for people who think”.

Holy Quran
Surah 16. An-Nahl

Supervisors Certification

We, certify that this dissertation entitled **"Investigating the anti-tumour properties of Iraqi propolis *in vitro* and *in vivo* "** under our supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology.

Supervisors

Signature

Dr. Khulood W. Al Sammarae

Scientific Degree: Professor

Date:

Signature

Dr. Ali H. Ad'hiah

Scientific Degree: Assist. Professor

Date:

In view of the available recommendations, I forward this dissertation for debate by the examining committee.

Signature: K. M. Ibrahim

Name: Dr. Kadhim M. Ibrahim

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date: 5/7/2010

Committee Certification

We, the examining committee certify that we have read this dissertation entitled **"Investigating the anti-tumour properties of Iraqi propolis *in vitro* and *in vivo*"** and examined the student **"Ghassan Mohammad Sulaiman"** in its contents and that in our opinion; it is accepted for the Degree of Doctorate of Philosophy in Biotechnology.

(Chairman)
Signature:
Name: Dr. Hussam Hasson Ali
Scientific Degree: Professor
Date: 11 . 7 . 2010

(Member)
Signature:
Name: Dr. Saleh Ahmed Waheeb
Scientific Degree: Professor
Date:

(Member)
Signature: A.H.M.Alfaisal
Name: Dr. Abdul Hussein Al-Faisal
Scientific Degree: Assist. Professor
Date:

(Member)
Signature:
Name: Dr. Amna Nsyif Jassim
Scientific Degree: Assist. Professor
Date:

(Member)
Signature:
Name: Dr. Mahfoodha Abass Umran
Scientific Degree: Assist. Professor
Date: 18/7/2010

(Member / Supervisor)
Signature:
Name: Dr. Khulood W. Al Sammarae
Scientific Degree: Professor
Date:

(Member / Supervisor)
Signature:
Name: Dr. Ali H. Ad'hiah
Scientific Degree: Assist. Professor
Date:

I, hereby certify upon the decision of the examining committee.

Signature:
Name: Dr. Laith Abdul Aziz Al-Ani
Scientific Degree: Assist. Professor
Title: Dean of College of Science
Date: 27 . 7 . 2010

Acknowledgments

At the beginning thanks to Almighty Allah who showered upon me all His blessings for the completion of this work

The work presented here was performed at the Laboratory of Cancer Pharmacology, Department of Oncology, Mario Negri Institute for Pharmacological Research, Milan, Italy.

First and foremost, I pay my heartfelt tribute to my supervisors Prof. Dr. Khulood W. Al Sammarae, Assist. Prof. Dr. Ali H. Ad'hiah and Dr. Maurizio D'Incalci (Head of Oncology Department) for giving me the best possible introduction to the world of science; their support, patience and motivation made this work not only possible, but also very enjoyable.

Sincere gratitude is also extended to Dr. Eugenio Erba (Head of Flow Cytometry Unit) for his innate sense of enthusiasm and encouragement, particularly in times of difficulty, proved a source of motivation and helped in remaining focused on the task at hand. I am particularly appreciative for his personal support and his interest in my career, support that has been instrumental in helping me to realize my future undertaking. For this I am truly grateful.

I have been lucky to work with an amazing group of people and I am very grateful to the members of Flow Cytometry Unit; Mr. Sergio Bernasconi, Miss Sarah Uboldi and Miss Michela Romano for their help and support over the duration of the research. Also, I express my thanks to Dr. Sergio Marchini for his patience, time and guidance through certain laboratory procedures. My sincere appreciation and respect goes to Dr. Renzo Bagnati (Head of Analytical Instrumentation Unit) for special attention, personal help, time and effort in instructing me for different chemical analysis programs. Of course, I am indebted to Dr. Massimo Zucchetti (Head of Cancer Clinical Pharmacology Unit) for his support, fun and friendship. I really thank Dr. Roberta Frapolli, Dr. Ezia Bello and

Miss Federica Zanzottera for help and support in taking care and management of laboratory nude mice. Indeed, I express my sincere gratitude to all the other colleagues, who were working at the institute, especially my flat mate Salman Mohammad (India) for its valuable aid and for patient sustaining in good and bad times. Special thanks to Dr. Farooq Ibrahim for all his help, technical support and advice with the immunohistochmical analysis (CEINGE centre, University of Naples, Italy). My sincere thanks go to Prof. Dr. Eugenio Scanziani (Director of Veterinary Medicine Faculty, University of Milan, Italy) for invaluable assistance, suggestions and evaluations of histopathology and immunohistochemistry part. I would also like to extend many thanks to the administrative staff of the Institute of Mario Negri where this work has been carried out, especially Mrs. Maura Montani and Ms. Simona Stupia. I also thank Prof. Dr Vassya Bankova (Head of Natural Product Chemistry Laboratory, Institute of Organic Chemistry, Bulgaria) for advices in understanding the chemical analysis.

I wish here to thank Prof. Dr. Kadhim M. Ibrahim (Head of Biotechnology Department), Prof. Dr Saleh Ahmed waheeb, Dr. Hameed Al Dulaimi, Dr. Maysaa Ch. Al-Yas, Mr. Mohammed Mahmood, Dr. Khalail Ibrahim, Mrs. Sura Ali, Dr. Usama Al-Rawi and Mr. Omar Khalid, for offering their support and encouragement .

In particular I would like to express my genuine thanks to Mr. Abdullah Khudair, whose generous help and support particularly in taking care of my family during the course of my research work abroad. I am also eternally grateful to my father, my mother, my uncles, my sister and her husband. Their love, unlimited support, guidance and prayers throughout my life kept the internal desire for knowledge at all times. Last but not least, an enormous thanks to my wife Nada for her love and support over all these years, and the hard work at home and for taking care of our kids; I could achieve nothing without you.

MARIO NEGRI
ISTITUTO DI RICERCHE FARMACOLOGICHE

ISTITUTO DI RICERCHE FARMACOLOGICHE MARIO NEGRI

Via Giuseppe La Masa, 19 - 20156 Milano MI - Italy - www.marionegri.it

tel +39 02 39014.1 - fax +39 02 354.6277 - mnegri@marionegri.it

TO WHOM IT MAY CONCERN

This is to certify that Dr. Ghassan Mohammad Sulaiman has been working in the Department of Oncology at the Mario Negri Institute for Pharmacological Research from December 1, 2008 to October 20, 2009. During his tenure he has utilized his time in learning research techniques and completing his doctorate thesis on the topic: **'investigating the anti-tumour properties of Iraqi Propolis *in vitro* and *in vivo*'**. The techniques that he mastered during his study are:

1- HPLC/MS-MS using LTQ Orbitrap XL mass spectrometer and data analyzing with LTQ-Orbitrap Xcalibur software system.
2- Cell lines and cell culture techniques.
3- Flow cytometric analysis using Fluorescence Activated Cell Sorter (FACS, Becton Dickinson -USA) for monoparametric and biparametric staining of DNA and data analyzing with CellQuest software system.
4- Mechanism of apoptosis assays.
   A. Gel electrophoresis assay.
   B. TUNEL assay.
   C. AnnexinV –PI assay.
   D. Determination of apoptosis by fluorescence microscopy.
   E. Flow cytometric assays for Bax, Bcl2 and p53 genes as well as to H2AX phosphorylation.
5- Xenograft tumour model with histopathological study for tumour sections.

His stay was beneficial in submitting some research papers with flow cytometry and analytical instrumentations units. We would like to wish him all the best in his research with the hope to have continued fruitful collaboration in the near future.

ISTITUTO RICERCHE FARMACOLOGICHE MILANO MARIO NEGRI

Maurizio D'Incalci, M.D.
Head, Department of Oncology

Prof . Silvio Garattini
Director

Milan, October 6, 2009

Summary

The present study was conducted to evaluate anti-oxidant, anti-mutagenic and anti-tumour potentials of propolis samples collected during the period 20-4-2007 to 1-7-2008 from five different geographical locations in Iraq (Baghdad1; B1, B2, Dahuk; D, Mosul; M and Salah ad-Din; S), and/or their phenolic compounds (caffeic acid; CA, chrysin; CH and caffeic acid phenethyl ester; CAPE) *in vitro* using one normal (human dermal fibroblast; HuFb) and two cancer (human promyeloid leukemia; HL-60 and human colon carcinoma; HCT-116) cell lines. Additionally, chemical analyses of propolis samples were carried out.

Phenolic compounds (flavonoids and phenolic acids and their ester) concentrations were estimated using high performance liquid chromatography that was coupled to electrospray mass spectrometry (HPLC-ESI/MS) analysis. Such compounds were further determined spectro-photometrically using Folin-Ciocalteu reagent. Accordingly, 38 different compounds were characterized and 33 of them were polyphenols. The others included four clerodane diterpenoids that have not been reported in temperate zones, while one compound was considered unknown. Quantitative assay of flavonoids showed that phenolic acids and their esters were the predominant constituents in propolis extracts, followed by flavones and flavonols, and then flavanones and dihydroflavonols. The highest concentrations of these compounds were observed in D and M samples.

The free radical scavenging activity of propolis was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The results revealed that propolis extracts exhibited strong free radical scavenging activity, but D and M samples had the highest activity, while CAPE has the highest anti-radical activity than other investigated compounds (CA or CH).

The results of *in vitro* growth inhibitory (GI) and growth inhibitory rate (GIR) against HL-60, HCT-116 and HuFb cells demonstrated that a treatment of cell cultures with B2, D, M or CAPE decreased the growth of cells significantly ($P \leq 0.05$) as compared to control cultures (untreated), and the effect was dose-, as well as, time-dependent. The M sample was less toxic against HuFb cells, while CH had no toxic effects against HL-60 cells.

The cell death was evaluated *in vitro* in tumour cell lines after a treatment with propolis extracts or their phenolic compounds through four types of assays, which were morphological aspects, gel electrophoresis, Annexin V/PI Flow cytometry and regulation of *Bcl-2*/*Bax* gene expression. Results revealed that the tested substances showed a potent inhibitory cytotoxic effect against the proliferation of HL-60 and HCT-116 cells through either apoptosis or necrosis, respectively. The apoptotic effect was associated with down-regulation of Bcl-2 and activation of Bax expression and the effect was markedly at 10 hours of treatment (22% and 33%, respectively). Such evaluations were further extended for M sample only and included p53 expression (HCT-116 cells), cell cycle perturbations through monoparametric and biparametric DNA evaluations and assessment of γ-H2AX-histone formation (HL-60 and HCT-116).

With respect to *p53* gene, the HCT-116 cells, a slight increased expression was observed after three hours of M sample treatment (4.80%), and such increase was also detected at six hours (5.57%), but after 10 hours it was reduced to reach no expression. However, after 24 hours, the expression started to increase again, and 27.98% of the cells were positive, but at 48 hours, it was decreased to reach 5.14%. After 72 hours, the positive expression was increased to reach 11.31%.

The monoparametric cell cycle distribution assay revealed that HL-60 cells treated with 5 µg ml^{-1} of M sample at 48 and 72 hours exhibited an increase in proportion of cells at S phase and a decrease in G1 phase, while no changes in cell cycle phases were observed at 24 hours. In cells treated with 15 or 25 µg ml^{-}

^{1} at 24 and 48 hours, there was an accumulation of cells in the S phase too, while a G2/M accumulation was apparent only after a treatment with 25 µg ml^{-1} at 72 hours. Furthermore, a sub-G1 peak was observed after a treatment with 15 or 25 µg ml^{-1} at different time intervals. On the other hand, most accumulations of HCT-116 cells occurred in G1 or G2/M phase in a time- and dose-dependent manner, while no sub-G1 peak was observed. In biparametric evaluation, the cells (HL-60 and HCT-116) progressed in the S phase more slowly than untreated cells, and in HL-60 cells, a fraction of new G1 positive BrdUrd cells was detected after 10 hours post-treatment. In both cell lines, the cells that were in G1 phase (negative BrdUrd) at the start of treatment remained blocked in this phase for less than 24 hours, and started to show S phase cell populations only after 24 hours.

The expression of γ-H2AX in HL-60 cells was clearly increased in a time-dependent manner, showing a fraction of cells with an increased γ-H2AX for the six investigated periods (3, 6, 10, 24, 36 and 48 hours post-treatment), but such increase was not observed in HCT-116 cells.

The anti-tumour evaluation (histopathologically and immunohisto-chemically for *p53* and *Ki-67* gene expressions) was further extended *in vivo* on tumours obtained from nude mice xenografted with HCT-116 tumour cells and treated orally (p.o.) or intrapertoneally (i.p.) with two doses (500 and 1000 mg kg^{-1}) of M sample for 20 (i.p.) or 27 days (p.o.). The M sample was significantly effective in reducing the tumour size in a dose-dependent manner, but the i.p. treatment was associated with adverse side effects. Histopathological examinations of tumour sections from p.o. treated mice revealed a decreased percentage of mitosis and increased endoreduplications of cells, as well as, necrosis, ulceration, degeneration and inflammatory cells infiltration. The *p53* gene showed a positive expression in 16% of vehicle tumour cells (control), while it showed an increased expression in tumour sections of animal treated orally with the dose 500 mg kg^{-1} (33.5%) and a further increase (40.8%) at the

dose 1000 mg kg^{-1}. With respect to *Ki-67*, the reverse picture was observed, in which, the gene was expressed in 85% of vehicle tumour section cells, while it was expressed in 47.0 and 46.8% of tumour sections obtained from mice treated with the dose 500 or 1000 mg kg^{-1}, respectively.

List of Contents

Chapter Four: Results

Chapter Five: Discussion

Conclusions and Recommendations

List of Tables

List of Figures

List of Abbreviations

26-L5	Murine Colon Carcinoma
A2058	Human Melanoma
ACF	Aberrant Crypt Foci
B1or 2	Baghdad1or 2
bax	Bcl2-associated X Gene
bcl-2	B Cell Leukemia/Lymphoma 2-like Gene
Bcl-w	Bcl-2-like 2
Bcl-xL	Bcl-2-like 1
Bfl-1	Bcl-2-Related Protein A1
BH	Bcl-2 Homology
BrdUrd	5-Bromo-2-deoxyuridine
CA	Caffeic Acid
CAPE	Caffeic Acid Phenethyl Ester
CD	Cluster of Differentiation
CDKs	Cyclin-Dependent Kinases
CH	Chrysin
CKI	Cyclin Kinase Inhibitor
CPT	Camptothecin
CTRL	Control
D	Dahuk
DAPI	4',6-Diamidino-2-Phenylindole
DDR	DNA Damage Response
DMH	1,2 Dimethylhydrazine
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DPPH	2, 2-Diphenyl-1-Picryl-Hydrazyl
DSB	DNA Double-Stranded Break
EDTA	Ethylene Diamine Tetra Acetic Acid
FACS	Fluorescence-activated Cell Sorter Flow Cytometer
FBS	Fetoel Bovine Serum
FITC	Fluorescein Isothiocyanate
FL	Fluorescence
FTMS	Fourier Transform Mass Spectrometry
GI	Growth Inhibitory
GIR	Growth Inhibitory Rate

H.P.F	High Power Field
HCT-116	Human Colon Carcinoma
HeLa	Human Cervical Carcinoma
HEp-2	Human laryngeal epidermoid carcinoma
HL-60	Human Promyeloid Leukemia
HLC-2	Human Lung Carcinoma
HPLC-ESI/MS	High Performance Liquid Chromatography Coupled to Electrospray Mass Spectrometry
HRP	Horse Radish Peroxidase
HT-1080	Human Fibrosarcoma
HuFb	Human Dermal Fibroblast
HUVEC	Human Umbilical Vein Endothelial Cell
IC	Inhibitory Concentration
i.p.	Intraperitonially Administration
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IMR-32	Human neuroblastoma
ISEL	*In Situ* End Labeling
ITMS2	Ion-trap Tandem Mass Spectrometry
LD	Lethal Dose
M	Mosul
M1	Marker1
MCF-7	Human Breast Cancer
$[M-H]^-$	Pseudo-Molecular Ion (Negative Mode)
MS/MS	Mass Spectra
NHOF	Normal Human Oral Fibroblast
NK	Natural Killer Cells
OEC-M1	Oral Epidermoid Carcinoma-Meng 1
p.o.	Oral Administration
p53	Tumour Suppressor Gene
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PEs	Propolis Extracts
PI	Propidium Iodide
pRB	Protein Retinoblastoma Phosphorylation
PS	Phosphatidylserine

RNase A	Ribonuclease A
RT	Retention Time
S	Salah ad-Din
SAS	Squamous Cell Carcinoma
SCC-25	Human Head and Neck Squamous Cell Carcinoma
TGF	Transforming Growth Factor
TIC	Total Ion Current
TNF	Tumour Necrosis Factor
TP3	TO-PRO-3 Iodide
TPA	Total Peak Area
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling
TVT	Canine Transmissible Venereal Tumour
v/v	Volume of Volume
VEGF	Vascular Endothelial Growth Factor
w/v	Weight for Volume
WEP	Water Extract Propolis
WHO	World Health Organization
WSP	Water-Soluble Propolis
XIC	Extracted Ion Chromatograms
γ-H2AX	H2AX Phosphorylation

Chapter One
Introduction

Chapter One
Introduction

1.1 Introduction

According to the World Health Organization (WHO), more than 11 million people are diagnosed with cancer every year and it is estimated that by 2020 there will be 16 million new cases per year, and moreover approximately 7 million people are died from cancer every year worldwide (WHO, 2006). In Iraq, it has been estimated that approximately 15000 people have been died of cancer in 2005, and such number represents 22.8% of the total deaths, moreover, it is projected that such percentage can be increased up to 35.4% in 2030 (WHO, 2005).

The aetiology of cancer is far from being perfectly defined, but it is well-understood that a malignant tumour is the result of a series of DNA alterations in a single cell, or clones of that cell, which lead to loss of normal function, aberrant or uncontrolled cell growth and often metastases (Brennan, 2002). Several of the genes, which are frequently lost or mutated, have been identified including those whose function is to induce cell proliferation under specific circumstances (for instance; *ras* and *myc* proto-oncogenes) and genes which are programmed to halt proliferation in damaged cells (for instance; *p53* and *APC* tumour suppressor genes) (Jakobisiak *et al*., 2003). Other mutations are also necessary, including genes involved in DNA repair, cell-cycle control, angiogenesis and telomerase production (Hanahan and Weinberg, 2000). The pattern of losses and mutations is complex, although mutation or loss of at least one proto-oncogene and one or more tumour suppressor genes in a single cell resulting in uncontrolled and unchecked cellular proliferation, is likely to occur in nearly all tumours (Vogelstein and Kinzler, 2004). Accordingly, a sporadic cancer may acquire mutations as a result of genotoxic exposure to external or internal agents (with the exception of rare familial cancers which are primarily

caused by a germline inheritance of a specific mutation) and a consequent DNA adduct formation (Schulz, 2005). The likelihood of a mutation occurring and persisting in subsequent clones may be heavily dependent on the efficiency with which potentially toxic exposures are metabolized and excreted, and also the efficiency with which small mistakes in DNA replication are rectified (Friedberg, 2003).

With these understandings, the researchers have developed anti-cancer strategies to overcome such fatal disease, and accordingly novel pharmacological paradigms have been developed which quickly and efficiently moves prospective anti-cancer drugs from the discovery phase through pharmacology testing and into therapeutic trial assessment. Some of these developments are based on natural products (Gordaliza, 2007). Screening of organisms in nature for medicines to counter human diseases has had a long and bountiful past from Ayurvedic medicine to modern ethical drugs, and over-the counter treatments have been widely applied (Swerdlow, 2000). Indeed, the majority of our prescription drugs, particularly in the case of cancer, and the vast majority of our nutraceuticals, has their origin in nature's products (Newman *et al*., 2003). Moreover, there is a large and over-expanding global population that prefers the use of natural products in treating and preventing various medical complications (Gautam *et al*., 2007). The worldwide upsurge in the use of natural product preparations and active ingredients have provided the pharmaceutical industry with up to 40% of modern drugs that are derived from natural resources. Furthermore, over a 100 new products are in a clinical development, particularly as anti-cancer agents and anti-infectives (Harvey, 2008). In this regard, bacteria and plants have been the major contributors of the contributing drugs like adriamycin and camptothecin, respectively (Newman and Cragg, 2004; Zhao *et al*., 2007), however, natural products of insects have also been added to the list, and their chemical constituents may have a bright potential for anti-cancer drug discovery. One of these products is propolis.

Propolis (bee glue) is the generic name for the resinous substance collected by honey bees (*Apis mellifera*) from various plant sources and used by bees to seal holes in their honeycombs, smooth out the internal walls, and protect the entrance against intruders (Benkovic *et al*., 2007). It is rich in biochemical constituents, and more than 300 compounds have been identified; including a mixture of polyphenols, flavonoids, phenolic acid and their esters, phenolic aldehydes and ketones, terpenes, sterols, vitamins, amino acids, and others, among which phenolics predominate (Lotfy, 2006; Trusheva, *et al*., 2007; Kumar *et al*., 2009). Healing properties of propolis are known in folk medicine from antiquity; however, recently, the interest in propolis as a harmless medicine is increasing. There have been many attempts to validate biological effects of propolis and elucidate its composition (Castaldo and Capasso, 2002). It has been shown that propolis and its constituents have strong anti-microbial effects, acting on viruses (Gekker *et al*., 2005), bacteria (Alencar *et al*., 2007) and fungi (Koc *et al*., 2005). It has also been demonstrated that propolis and some of its active substances have a pronounced cytostatic, anti-carcinogenic and anti-tumour effects both in *in vitro* and *in vivo* tumour models (Najafi *et al*., 2007; Inoue *et al*., 2008; Li *et al*., 2009). Anti-oxidant and immunomodulatory potentials of propolis have also been recorded (Ad'hiah *et al*., 2007; Naik *et al*., 2009; Syamsudin *et al*., 2009).

1.2 Aims of Study

Based on the forthcoming presentation, the present study was planned with the aim to evaluate the anti-oxidant, anti-mutagenic and anti-tumour potentials of propolis samples collected from five different geographical locations in Iraq, and/or their polyphenolic compounds (caffeic acid; CA, chrysin; CH and caffeic acid phenethyl ester; CAPE) *in vitro* and *in vivo* using one normal (human dermal fibroblast; HuFb) and two cancer (human promyeloid leukemia; HL-60

and human colon carcinoma; HCT-116) cell lines. Accordingly, the following assessments were achieved:

i. Extraction and chemical analysis of propolis samples using high performance liquid chromatography coupled to electrospray mass spectrometry (HPLC-ESI/MS).
ii. Free radical scavenging activity of propolis samples and their polyphenolic compounds.
iii. *In vitro* assessments of growth inhibition, clonogenic, apoptosis and necrotic potentials on the cell lines.
iv. Effect on nuclear morphological aspects.
v. Flow cytometric assessments of *bax, bcl-2* and *p53* gene expressions.
vi. Cell cycle perturbations through monoparametric and biparametric DNA evaluations.
vii. Assessment of γ-H2AX-histone formation.
viii. The anti-tumour evaluation was further extended *in vivo* on nude mice implanted with a xenograft tumour to assess the anti-tumour effects and follow-up the morphological changes, as well as, the immunohistochemical expression of *p53* and *Ki-67* genes.

Chapter Two
Literature Review

Chapter Two
Literature Review

2.1 Propolis

Propolis (CAS No. 9009-62-5) is a strongly adhesive and resinous substance (sometimes also referred to as a bee glue), which is collected by honeybees (*Apis mellifera* L.) from various plant sources, transformed and used by bees to seal holes in their honeycombs and smooth out the internal walls (Marcucci *et al*., 2001; Benkovic *et al*., 2007). The term "propolis" is derived from the Greek, πρω, *pro*, meaning "for" or "in defence of", and πολιζ, *polis*, "the community or city", and thus has the meaning of "for the defence of the community" (Salatino *et al.*, 2005). Honeybees collect the resin from cracks in the bark of trees and leaf buds. This resin is masticated, salivary enzymes added and the partially digested material is mixed with bee wax and used in the hive, and although propolis may contain some pollen, it is not pollen nor should it be confused with 'bee bread' and 'royal jelly', which are wholly different products of honeybees (Bankova *et al.*, 2000; Bankova, 2005). In order to manufacture propolis, bees may also use material actively secreted by plants, or exuded from wounds in plants; for instance lipophylic material on leaves, mucilages, gums, resins and lattices (Gomez-Caravaca *et al*., 2006).

2.1.1 History and Folkloric Applications

Propolis is a natural remedy that has been employed extensively since ancient times. Egyptians knew very well the anti-putrefactive properties of propolis and used it to embalm cadavers. Propolis was also recognized for its medicinal properties by Greek and Roman physicians; for instance, Aristoteles, Dioscorides, Pliny and Galen (Hegazi, 2000). The product was employed as an antiseptic and cicatrizant in wound treatment and as a mouth disinfectant, with

these uses being perpetuated in the Middle Ages and among Arab physicians (Abd El Hady and Hegazi, 2002).

The Holy Qur'an has also documented the health benefits of bees and their products in the "Sorat with the name Bees; An-Nahl". Furthermore, Abu Ali bin Sina (Avicenna) distinguishes two kind of wax (clean and black wax) in his well known book "The Canon of Medicine (Al-Qanun Fi'l-Tibb)". The clean wax is that which composes the comb wells where the bees rear the brood and store the honey, while the black wax represents the filth in the hive, but it is clear that the black wax was propolis because Avicenna's testimony has stated its characteristic of eliminating the spikes (Hegazi, 2000; Lotfy, 2006). Propolis was also recognized by other peoples unrelated to Old World civilizations; Incas employed propolis as an anti-pyretic agent, and the London Pharmacopoeias of the 17th century listed propolis as an official drug. Between the 17th and 20th century the drug became very popular in Europe on account of its anti-bacterial activity (Castaldo and Capasso, 2002; Ramos and Miranda, 2007).

Modern herbalists recommend propolis for its anti-bacterial, anti-fungal, anti-viral, hepatoprotective and anti-inflammatory properties, to increase the body's natural resistance to infections and to treat gastroduodenal ulcers. Today propolis is currently used as a popular remedy and is available in the form of capsules (either in pure form or combined with other constituents). It is also available commercially as purified product in which the wax has been removed (Alternative Health and Herbs Remedies, 2010).

Propolis is marketed by health food stores as a traditional medicine, and for its claimed beneficial effect on human health. Natural medicine practitioners use propolis for the relief of various conditions, including inflammations, viral diseases, ulcers, superficial burns or scalds (Farre *et al.*, 2004). Propolis is also believed to promote heart health, strengthen the immune system and reduce the chances of cataracts (Orhan *et al.*, 1999). Old beekeepers also recommend a piece of propolis kept in the mouth as a remedy for a sore throat (Krell, 1996).

Some of these claims are being clinically investigated and several studies have been published in the biomedical literature.

2.1.2 Chemical Constituents

In the evaluation of the quality of propolis, in addition to a visual inspection of its physical and organoleptical characteristics (appearance, consistency, origin, colour or smell; Table 2-1), which provide a subjective appreciation of the product and an indication of quality to a certain degree, the contents of its active principles should be determined, in order to provide a real and objective evaluation (Bankova, 2000; Farre *et al*., 2004).

Table 2-1: Characteristics that permit the quality evaluation of propolis.*

Parameter	Quality		
	Good	Average	Poor
Presentation	Flaky and granulated	In blocks or balls	Powder
Appearance	On cutting differences in external and internal colouring	Slight differences in external and internal colouring	No differences in external and internal colouring
Colour	Green, yellow, orange or shades of the same colour	Brown	Dark
Smell	Aromatic resin	Resinous	No smell
Test	Spicy or resinous	Slightly resinous	Insipid

***** Farre *et al*. (2004)

The chemical composition of propolis is still insufficiently characterized, but it is a resin being dark green or brown in colour with a pleasant flavour of poplar buds, honey, wax and vanilla, and it can also have a bitter taste. When burnt, it exhibits a smell of aromatic resins (Bankova, 2000). The chemical composition of propolis, as well as, its colour and aroma are changed according to the geographical zones. Its colour varies from yellowish-green to dark brown depending on its source, and it can be likened to aromatic glue. It is hard and

brittle when it is cold, but becomes soft and very sticky when it is warm (Salatino *et al*., 2005).

Over 300 compounds have been isolated from propolis (Kumar *et al*., 2009). Its main components are resins and balsams, which contain flavonoids and phenolic acids or their esters; highly variable wax contents; volatile oils; pollen and impurities. Additionally, it contains small quantities of terpens, tannins, traces of secretions from the salivary glands of bees and possible contaminants (Krell, 1996; Almedia, and Menezes, 2002). Propolis also contains some minerals such as magnesium, calcium, iodide, potassium, sodium, cupper, zinc, manganese and iron, as well as, some vitamins like B1, B2, B6, C and E, and a number of fatty acids (Table 2-2). In addition, it contains some enzymes; for instance succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase and acid phosphatase (Tikhonov and Mamontova, 1987).

Table 2-2: Average chemical composition of propolis.*

Class of Compound	Group of Components	Percentage
Resins	Flavonoids, phenolic acids and esters.	45-55
Waxes and Fatty Acids	Beeswax and plant origin.	23-35
Essential Oils	Volatiles.	10
Pollen	16 free amino acids (>1%); Arginine and proline together represent 46% of the total.	5
Other Organics and Minerals	14 trace minerals: iron and zinc most common; ketones, lactones, quinones, steroids, benzoic acid, vitamins and sugars	5

*After Krell (1996) and Almedia and Menezes (2002).

The active compounds are flavanoids (flavons, flavanols, flavonons, and flavononols) and phenolic compounds (phenolic acids and their esters). However, it should be pointed out that most of studies carried out have not been aimed at determining a complete chemical composition, but have merely

determined some of the components of interest, particularly the flavonoids (Popova *et al*., 2005; Silva *et al*., 2007a; Xu *et al*., 2009; Mello *et al*., 2010).

2.1.3 Biological Potentials

Propolis is a product of extraordinary interest, as much in the field of medicine as the pharmaceutical industry. It has been attributed with numerous properties: it is an anti-flammatory agent (Buyukberber *et al.*, 2009), an immunostimulant (Ad'hiah *et al.*, 2007; Syamsudin *et al.*, 2009), a hepatoprotector (El Khatib *et al.*, 2002), anti-oxidant (Naik *et al*., 2009), anti-tumour and a carcinostatic (Li *et al*., 2009), it has anti-microbial (Yaghoubi *et al*., 2007), anti-viral (Schnitzler *et al*., 2009), anti-fungal (Koc *et al*., 2007) and anti-parasitic (Duran *et al*., 2008) activities, and it is also an anesthetic and a tissue regenerator (Bogdanov, 2009).

The flavonoids (quercitin, apigenin, galangina, and others) and the phenolic acids (caffeic, isoferulic, cynammic and benzoic), in addition to being toxic to yeasts, inhibit the enzymatic activity of hyaluronidase; an enzyme that depolymerises polysachharides hyaluronic acid in the extracellular matrix of connective tissues (Moon *et al.*, 2009). Additionally, caffeic acid and the activity of dihydrofolate reductase could explain the similarity between some of its effects and those of some non-steroid anti-inflammatory medicines (Alfaris *et al.*, 2009).

2.1.3.1 Anti-oxidant Potentials

Active free radicals, together with other factors are responsible for cellular aging and many conditions such as cardiovascular diseases, cancer, diabetes, arthritis, Parkinson disease and Alzheimer (Najafi *et al.*, 2007). The anti-oxidant serves as a defensive factor against free radicals in the body. Enzymes such as superoxide dismutase, catalase and glutathione peroxidase are the main system that opposes oxidation. If free radicals production becomes more than the

capacity of enzymatic system, the second line of defence (vitamins) may come to action. Anti-oxidant such as vitamins C and E quench free radicals and become oxidized and inactive (Halliwell, 1994; Mohammadzadeh *et al.*, 2007).

Flavonoids and various phenolics are the most important pharmacologically active constituents in propolis and have been shown to be capable of scavenging free radicals and thereby protecting lipids and other compounds such as vitamin C from being oxidized or destroyed during oxidative damage (Hegazi and Abd El Hady, 2002). Propolis has also gained popularity and used extensively in drinks and foods to improve health and prevent diseases such as inflammation, heart disease, diabetes and even cancer (Satoshi *et al.*, 2005).

Because of such broad spectrum of biological properties and their different uses, there is a renewed interest in its anti-oxidant activities. Several investigations on propolis in Europe and South America have indicated that flavonoids concentrated from propolis are powerful anti-oxidants, which are capable to scavenge free radicals (Kumazawa *et al.*, 2004). Therefore, propolis is a natural source of anti-oxidants that protect serum lipoproteins from oxidation (Isla *et al.*, 2001). Its anti-oxidant properties are due to its anti-radical activity (alcoxi radicals and to a lesser extent, superoxide) and inhibiting effect on the cuprous ion, which is an initiator of oxidation of low density lipoproteins (Claus *et al.*, 2000).

In Brazilian and Chinese propolis, the aqueous extracts have shown greater anti-radical activity than the methanolic extracts. The contrary was found to be true of extracts from Dutch or Peruvian origin (Banskota *et al.*, 2000). Propolis has also been shown to exert anti-oxidant effects on the colon, reducing the concentration of lipid hydroperoxide concentration, and as some of its compounds are absorbed and enter into circulation, they act as hydrophilic anti-oxidants and increase tissue concentrations of vitamin C (Sun *et al.*, 2000).

2.1.3.2 Anti-tumour Potentials

Several researchers have reported the anti-tumoural properties of propolis *in vivo* and *in vitro*. Propolis antiproliferative activity on tumour cells has been demonstrated and some responsible compounds were isolated (Rao *et al*., 1995; Huang *et al*., 1996; Banskota *et al*., 2001).

Matsuno (1995) isolated an active substance from Brazilian propolis and characterized it as a new clerodane diterpenoid (namely PMS-1), which inhibited the growth of hepatoma cells and arrested the tumour cells at S phase. Matsuno *et al*. (1997a) also isolated a compound (PRF-1) from a water extract of propolis, which showed anti-oxidant activity and was cytotoxic to human hepatocellular carcinoma, human cervical carcinoma (HeLa) and human lung carcinoma (HLC-2) cells. Their group also isolated a tumouricidal compound identical to artepillin C, described as a constituent from *Baccharis* species, and its cytoxicity seemed to be partly attributable to apoptosis-like DNA fragmentation (Matsuno *et al*., 1997b). Kimoto *et al*. (1998) investigated the effects of artepillin C *in vitro*, verifying suppression of tumour growth, and *in vivo* there was an increase in the ratio of CD4/CD8 T cells, indicating that this compound activated the immune system.

Liao *et al*. (2003) demonstrated the inhibitory effect of propolis-isolated caffeic acid phenethyl ester (CAPE) on angiogenesis, tumour invasion and pulmonary metastatic capacity of CT26 cells. The CAPE also prolonged the survival of mice implanted with CT26 cells, suggesting its potential as an anti-metastatic agent. The same compound had a dose-dependent effect on the cytotoxicity of C6 glioma cells, reducing the viability to 42% in relation to control, and increasing the proportion of hypodiploid DNA, as an indication of apoptosis. It has also been found that CAPE increased the phosphorylation and expression of *p53* and *Bax* genes, which can form heterodimers with Bcl-2 in mitochondrial membrane and accelerate apoptosis (Lee *et al*., 2003). In agreement with such finding, Aso *et al*. (2004) reported that the anti-tumour

activity of propolis occurs through the induction of apoptosis via caspase pathways.

Caffeic acid phenethyl ester also interfered in cell cycle arrest. After incubation with CAPE for 24 hour, the cell percentage of C6 glioma cells in the G0/G1 phase increased to 85%, due to the inhibition of protein retinoblastoma (pRB) phosphorylation. The phosphorylation of pRB by the cyclin-dependent kinases (CDKs) /cyclins is believed to be a crucial event in the regulation of S-phase entry, and appears to define the restriction point in the late G1- phase. An *in vivo* study demonstrated that CAPE decreased the growth of the xenografts of C6 glioma cells in nude mice by inhibiting cell proliferation. Histochemical and immunohistochemical analysis revealed that CAPE treatment significantly reduced the number of mitotic cells and proliferating cell nuclear antigen (PCNA)-positive cells in C6 glioma (Kuo *et al*., 2005). Derivatives of CAPE were also investigated on oral cancer using cultured cancer cell lines (squamous cell carcinoma; SAS and oral epidermoid carcinoma-Meng 1, OEC-M1) and normal human oral fibroblast (NHOF), to examine their effects on cell growth pattern, cytotoxicity and changes in the cell cycle. The derivatives showed cytotoxic effects on tumour cells but not on NHOF cell line. Flow cytometric analysis showed OEC-M1 cell arrest at G2/M phase. Such differential effects on cancer and normal cells demonstrated that these compounds might be useful in oral cancer chemotherapy (Lee *et al*., 2005b).

Although direct carcinostatic effects of propolis or its isolated components have been demonstrated, an important question has been addressed whether propolis acts on the immunocompetent cells to enhance tumour cell destruction. Resistance to spontaneous tumour development has been associated with the cytotoxic activity of natural killer (NK) cells, found both in humans and experimental animals (Kaneno, 2005). Treatment with propolis for 3 days increased the cytotoxic activity of NK cells against murine lymphoma (Sforcin *et al*., 2002). Such finding confirmed a previous observation that propolis

administration over a short-term leads to better results concerning the immune system; increasing the immunological response (Scheller *et al*., 1988). The lack of seasonal effect of propolis activity was also observed in NK assays (Sforcin *et al*., 2002). Natural killer cells are under cytokines action, such as interferons (IFN-α, -β and -γ), tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β1, interleukin (IL)-1β, IL-2, IL-4, IL-10, IL-12, IL-13, IL-15, IL-21 and IL-23 (Kaneno, 2005). Although, the activation mechanism of these cells by propolis still remains obscure, it has been suggested that propolis-activated macrophages could produce cytokines, such as TNF-α and IL-12, which act on NK cells, increasing their cytotoxic activity (Sforcin, 2007).

Propolis potential in carcinogenesis and mutagenesis assays was also investigated. Propolis effect on the process of colon carcinogenesis and DNA damage in Wistar rats was evaluated, using the aberrant crypt foci (ACF) assay and comet test. Animals were treated with the carcinogen 1,2 dimethylhydrazine (DMH) and treated with ethanolic extract of propolis simultaneously or after DMH administration. Propolis given simultaneously to DMH did not suppress the development of ACF. These results indicated that propolis was not able to block or minimize the initiation step of DMH-induced colonic carcinogenesis. Since DMH is an indirect carcinogen, which has to be metabolized to exert its carcinogenic effect, it could be postulated that propolis has no interference on DMH metabolic pathways. On the other hand, when propolis was administered during 2 weeks after DMH treatment, there was a significant reduction in the number of ACF, which could reflect a suppression of the clonal expansion of the initiated cells that characterizes the promotion step of carcinogenesis. No anti-genotoxicity of propolis was observed in the comet assay, and DNA damage was seen in the peripheral blood cells (Bazo *et al*., 2002). However because of propolis solvent effect, new investigations have been carried out with an aqueous extract of propolis, verifying its protective effect on DMH-induced genotoxicity, as evidenced in the comet test, but it did not suppress the

development of ACF in the distal colon (Alves De Lima *et al*., 2005). Qualitative and quantitative variations in the composition of ethanolic or aqueous extract of propolis could explain these distinct responses.

Another approach to verify propolis anti-tumour action was to analyse its effects on canine transmissible venereal tumour (TVT), which is a contagious and sexually transmissible neoplasm with an unclear origin and affecting only canines (Silva *et al*., 2003). Propolis showed a time-concentration effect on TVT. With regards to TVT morphology, plasma cell-like TVT was more resistant to propolis action. The absence of propolis solvent effect suggested that the results were exclusively due to propolis components. In order to reduce the side effects of chemotherapy and considering that propolis possesses antitumour, anti-metastatic and immunomodulatory activities, its introduction as a therapeutic procedure *in vivo* could provide a new contribution to TVT treatment, as well as, to other neoplasia treatments (Bassani Silva *et al.*, 2007).

More recently, the effects of water-soluble propolis (WSP) on tumour cells *in vitro* were evaluated by measuring the intracellular uptake of ^{3}H-thymidine. ^{3}H-thymidine uptake was inhibited in accordance with the concentration of WSP. The minimum concentration of WSP necessary for ^{3}H-thymidine uptake inhibition was 1.0 $\mu g\ ml^{-1}$ and uptake was suppressed to 88% of the level in non-treated cells at this concentration. In a further experiment using tumour-bearing mice, oral administration of WSP was begun 24 hours after transplantation of S-180 cells. The orally administered WSP significantly inhibited the growth of transplanted tumours. Furthermore, histological findings revealed a significant reduction in mitotic cells and tumour invasion of the muscular tissue (Inoue *et al*., 2008).

Anti-angiogenic effects of water extract of a Brazilian green propolis (WEP) and its constituents; caffeoylquinic acid derivatives, against angiogenic processes in human umbilical vein endothelial cells (HUVECs) *in vitro* were evaluated (Chikaraishi *et al*., 2009). They also examined the anti-angiogenic

effects of WEP against retinal neovascularization in a murine oxygen-induced retinopathy model *in vivo*. The WEP and its constituents significantly suppressed vascular endothelial growth factor (VEGF)-induced HUVEC proliferation, migration, and tube formation *in vitro*. Furthermore, they suppressed VEGF-stimulated phosphorylation of mitogen-activated protein kinase in HUVECs (*versus* VEGF alone). Moreover, WEP significantly suppressed retinal neovascularization in the murine oxygen-induced retinopathy model. The data indicated that (i) WEP has angiostatic effects against angiogenic processes *in vitro* and in an *in vivo* model of murine oxygen-induced retinopathy and (ii) the inhibitory effects of WEP against *in vitro* angiogenesis were chiefly derived from its caffeoylquinic acid derivatives. The judging from these findings was that WEP and its caffeoylquinic acid derivatives may represent candidates for preventive or therapeutic agents against diseases caused by angiogenesis.

2.2 Present Study Parameters

To assess the anti-oxidant and anti-tumour potentials of propolis, different parameters (free radical scavenging activity, cancer growth inhibition, apoptosis and necrosis, *bax*, *bcl-2* and *p53* gene expression, cell cycle perturbations, assessment of γH2AX-histone formation and *in vivo* anti-tumour evaluation) were inspected in the present study.

2.2.1 Free Radical Scavenging Activity

Reactive free radicals, such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and peroxyl radical ($ROO^{\bullet}$), are particularly reactive and are known to be a biological product in reducing molecular oxygen (Williams and Jeffrey, 2000). Damages mediated by free radicals result in the disruption of membrane fluidity, protein denaturation, lipid peroxidation, oxidative DNA and alteration of platelet functions, which have generally been considered to be linked with many chronic

health problems such as cancers, inflammation, aging and atherosclerosis (Geronikaki and Gavalas, 2006). An anti-oxidant, which can quench reactive free radicals, can prevent the oxidation of other molecules and may therefore have health-promoting effects in the prevention of degenerative diseases (Astley, 2003). The interest in anti-oxidants has been increasing because of their high capacity in scavenging free radicals related to various diseases (Silva *et al*., 2007b).

There is a growing interest in natural anti-oxidants, present in medicinal and dietary plants that might help attenuate oxidative damage (Silva *et al*., 2005). These natural anti-oxidants not only protect food lipids from oxidation, but may also provide health benefits associated with preventing damages due to biological degeneration (Hu and Kitts, 2000). Several anti-inflammatory, hepatoprotective, neuroprotective, and anti-necrotic drugs have been shown to have an antioxidant and/or anti-radical scavenging mechanism as part of their activity (Lin and Huang, 2002). Experimental studies support the role of reactive oxygen species in cancer, in part by showing that dietary anti-oxidants (for instance; vitamin E, vitamin C, phenolic compounds, and other phytochemicals), as well as, endogenous anti-oxidants (for instance glutathione) that neutralize or trap reactive oxygen species; and acting as cancer preventive agents (Khan, 2002; Borek, 2004).

While the efficacy of anti-oxidants during cancer treatment is still being evaluated and clinical trials are ongoing or being set up, many cancer patients who are undergoing therapy take anti-oxidant supplements in an effort to alleviate treatment toxicity and improve long-term outcome (National Institutes of Health, 2004). Further data, however, have shown that some dietary anti-oxidants may show promise in cancer therapy by their palliative action, reducing painful side effects associated with treatment and have a potential as adjuvant to induce apoptosis in cancer cells while sparing normal cells. Other findings, in a model of metastatic growth, showed that anti-oxidants are an angiostatic factor

and may have a potential in aiding host resistance to tumour growth and invasiveness (Borek, 2004).

The free radical-scavenging activity of natural compounds has also been confirmed by using several methodologies (Chang *et al.*, 2007; Kumar and Kumar, 2009), and one of these is examining their ability to bleach the stable radical 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) *in vitro*. This assay provides information on the reactivity of test compounds with a stable free radical, because of its odd electron DPPH that gives a strong absorption band at 517 nm in visible spectroscopy (deep violet colour). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up (Moreira *et al*., 2008).

2.2.2 Cancer Growth

The progressing from normal to malignant cells involves multiple steps, during which several distinguishable properties are acquired. Pending normal development and throughout adult life, intricate genetic control systems regulate the balance between cell birth and death in response to growth signals, growth inhibiting signals, and death signals (Weinberg, 2007). Most importantly, cancer cells lose the ability to control growth, and can proliferate almost inevitably. They are able to promote angiogenesis and thus guarantee an unlimited supply of nutrients and oxygen. Furthermore, the telomerase enzyme is reactivated and maintains stable telomeres during repeated cycles of cell proliferation (Akouchekian, 2008).

The cancer forming process, called oncogenesis or tumourigenesis, is the interplay between genetic and environmental factors. Most cancers arise after genes are altered by carcinogens or by errors in the copying and repair of genes, and even if the genetic damage occurs only in one somatic cell, the division of cell will transmit the damage to the daughter cells giving rise to clone of altered

cells (de Lange, 1994). In this regard, cancer cells no longer respond to apoptotic signals (Rudin and Thompson, 1998), and in the final of progression, they are able to detach from their original site, migrate through blood vessels or lymphatics to new destinations, and form new colonies; a process called metastasis (Hanahan and Weinberg, 2000).

At the tissue level, cancers can be categorized on the basis of their origin. For example; sarcomas are derived from soft tissues and bones, whereas leukemias originate from blood cells (Schulz, 2005). Carcinoma, derived from epithelial tissue, is the most common type of cancer. It typically starts within a benign, well-differentiated tumour, which has a structure resembling that of normal tissue. The next stage is carcinoma *in situ* (local tumour), and after reaching the invasive stage, cancer is able to penetrate into the basal membrane, and infiltrate the underling tissue. Finally, the ability to metastasize is acquired (Akouchekian, 2008). The clinical stages of carcinoma are illustrated in figure 2.1.

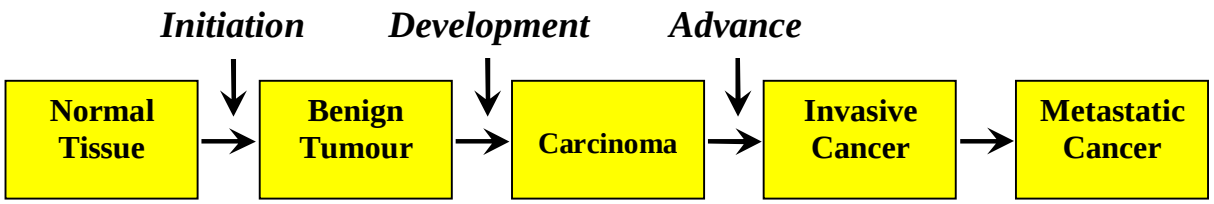


Figure 2-1: Clinical stages of carcinoma (Akouchekian, 2008).

During the multistage process of carcinogenesis, only rarely selected cells could proceed through a series of genetic and epigenetic changes from one stage to the next one along the pathway leading them ultimately to the formation of malignant tumour. At each stage, the pathway leading to malignancy is only one of the possible routes and at each stage there are natural anticancer defence mechanisms further diminishing cancer risk. The peculiarity of these defence mechanisms stems from the fact that actually the most effective of them seems

to operate at the very early stage, preceding the formation of the neoplastic cell (Jakobisiak *et al.,* 2003).

2.2.3 Apoptosis and Necrosis

The term apoptosis was first used by John Kerr in 1972 (Kerr, 2002). It is derived from the ancient Greek and means "the falling of petals from a flower" or "of leaves from a tree in the autumn" (Holdenrieder and Stieber, 2004). The term programmed cell death is often used interchangeably with apoptosis. However, it is important to note that other forms of programmed cell death have been described and other forms of programmed cell death may yet be discovered (Debnath *et al.*, 2005).

Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defence mechanism such as in immune reactions or when cells are damaged by a disease or noxious agents. Although there are a wide variety of stimuli and conditions, both physiological and pathological, that can trigger apoptosis, not all cells will necessarily die in response to the same stimulus (Norbury and Hickson, 2001).

In oncology, the interest in apoptosis originates from the observations that ionizing radiation or interactions of anti-tumour drugs with their respective intracellular targets often result in this mode of cell death, and that the efficacy of several anti-tumour drugs correlates with their ability to induce apoptosis. The possibility, therefore, of modulating this process can open new strategies for improving chemotherapy. Also, the ability to monitor early signs of apoptosis in samples from patient tumours may be predictive of the outcome of treatment protocols (Portugal e*t al.*, 2009).

In contrast to apoptosis, necrosis is a nonspecific mode of cell death, often caused by exposure to external factors or drastic changes in the physiological environment; such as heat, anoxia, loss or increase of ions, and toxic substances,

and it is usually a pathogenic process (van Cruchten and van den Broeck, 2002). In general, the trigger needs to be more massive to provoke necrosis instead of apoptosis. Apoptosis and necrosis not only differ in the triggers and the course of events, but also in the picture they present during this process (Martin, 2006).

The image of apoptosis is characterized by shrinkage of cell and cell nucleus, condensation of chromatin, DNA fragmentation, and blebbing of the cell membrane with the constriction of apoptotic bodies without losing the structural integrity and most of the plasma membrane function. Whereas necrosis is represented by swelling of cell and cytoplasmatic organelles and disrupture of the cell membrane leading to inflammation and thus causing further tissue damage (Elmore, 2007).

Apoptosis is a dynamic process, and the diverse methods of apoptosis assessment vary in their sensitivity and specificity for the different stages of it. As a result, different methods may yield varying results, even when the same specimen is analyzed at the same time (Steensma e*t al.*, 2003). However, there are a number of tissue-based assays for detecting apoptosis that are not based on flow cytometric principles. Simple morphologic analysis supplemented by electron microscopy can reveal apoptotic bodies, as well as, the typical late apoptotic findings of chromatin condensation and nuclear fragmentation (White and Cinti, 2004). DNA stains such as Hoechst 33258 and 4',6-diamidino-2-phenylindole (DAPI) can facilitate visualization of the condensed nuclear fragments that are typical of apoptosis (Saito e*t al.*, 2004; Nguyen e*t al.*, 2007).

Agarose gel electrophoresis of cells undergoing apoptosis may reveal the characteristic pattern of a “DNA ladder.” The DNA ladder is a bold visual display of DNA fragments of varying lengths, typically multiples of 180–200 base pairs; the rungs are fragments of nuclear DNA that have been degraded by caspases and other endonucleases, and DNA ladders, like apoptotic bodies, are a late finding in the apoptosis cascade (Steensma e*t al.*, 2003).

Other tissue-based methods such as *in situ* end labeling (ISEL) and the related *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) techniques detect the single- and double-strand DNA breaks that occur during intra-nucleosomal DNA degradation in early apoptosis (Huerta e*t al.*, 2007). The nick-ends can also be detected by flow cytometry, as described below. These methods can preserve tissue architecture and also allow quantification. However, counting cells in order to obtain an accurate apoptotic index must generally be done manually with ISEL and TUNEL (Yasuhara e*t al.*, 2003).

Flow cytometric methods of apoptosis detection offer several advantages over the above techniques. Flow cytometers make use of a focused beam of laser light to interrogate a single-cell suspension whose laminar flow through a chamber is tightly controlled. The light scattering and fluorescent properties of these cells in fluid suspension and any bound antibodies (which may be labeled with any of several fluorochromes) can be assayed via a series of filters and photomultiplier tubes. Flow cytometry can rapidly quantify and evaluate the properties of thousands of cells. This is particularly important when the cells of interest are interspersed in a heterogeneous tissue such as bone marrow or blood, as is the case in most hematopoietic malignancies. The flow cytometer can simultaneously assess apoptosis-associated properties, as well as, the presence of other cell characteristics (for instance; cluster of differentiation markers or other cell surface antigens), allowing isolation and study of specific cellular subspecies (“gating”) or correlation of apoptosis with other cellular features (Shapiro, 2003; Mc Carthy, 2007).

There are a series of differences between apoptotic cells and normal cells that can be exploited via flow cytometric techniques in order to allow apoptosis detection. These properties have recently been discussed by Wlodkowic *et al*. (2009), and they include changes in plasma membrane, changes in cellular light

scattering properties, changes in cellular DNA content and DNA sensitivity to denaturation and changes in cellular organelles.

2.2.4 Genes Involved in Tumour Progression

Three major groups of genes are involved in tumour progression: (i) oncogenes and tumour suppressor genes; (ii) genes involved in the control of tumour progression and metastasis; and (iii) genes encoding proteins that protect the organism from tumour cells. Each group contains numerous genes, and the discovery of new important genes is an exciting prospect in tumour research (Jakobisiak *et al*., 2003).

2.2.4.1 The *Bcl-2* genes family

The intrinsic pathway of apoptosis is regulated by members of the Bcl-2 (B cell leukemia/lymphoma 2-like proteins) family (Figure 2-2). This family is composed of pro- and anti-apoptotic proteins that share up four conserved regions known as Bcl-2 homology domains designated BH1, BH2, BH3 and BH4, which correspond to α-helical segments (Lessene *et al*., 2008).

In excess of 25, Bcl-2 family members have been identified in human (Almawi *et al*., 2004), and certain proteins share Bcl-2's ability to oppose programmed cell death; Bcl-2-like 1 (Bcl-xL), Bcl-2-like 2 (Bcl-w), myeloid cell leukemia-1 (Mcl-1), and Bcl-2-related protein A1 (Bfl-1) (Brunelle and Letai, 2009). These proteins share a sequence homology in 4 α-helical Bcl-2 homology (BH) regions; BH1-BH4 (Gustafsson and Gottlieb, 2007). Bax and Bak (Brunelle and Letai, 2009), which promote cell death, share only the BH1–BH3 domains. A third class of proteins is known as BH3-only proteins (Adams and Cory, 2007), which include Bid, Bad, Bik, Puma, Noxa, Bmf, and Hrk, and demonstrate homology only in the BH3 region (Lessene *et al*., 2008). Like Bax and Bak, the pro-apoptotic BH3-only proteins require an intact BH3 domain to promote apoptosis (Brunelle and Letai, 2009).

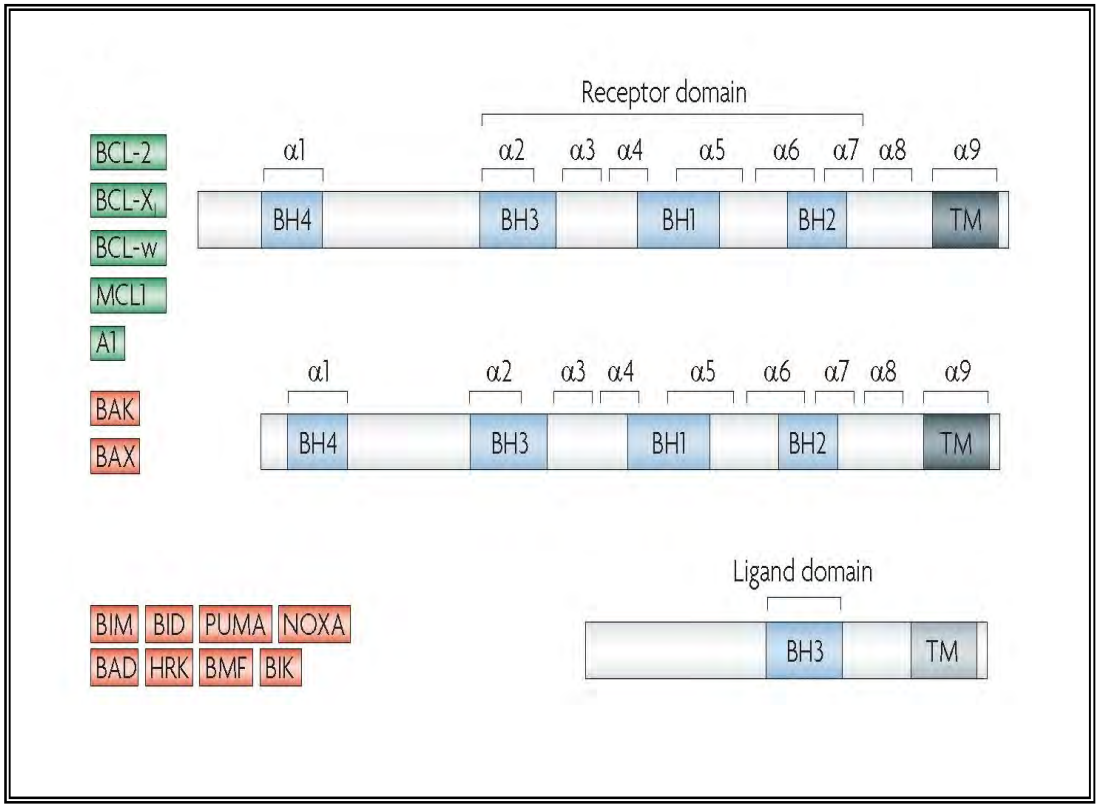


Figure 2-2: The extended Bcl-2 protein family (Lessene *et al.*, 2008).

Bax (Bcl2-associated X protein) and Bcl-2 are at the core of the apoptotic pathway located at the mitochondria where multiple cell death signals converge to trigger apoptosis (Cheng *et al.*, 2001; Yuan *et al.*, 2003). The product of the *bcl-2* gene is an anti-apoptotic protein that suppresses apoptosis in response to a wide variety of stimuli (Nemec and Khaled, 2008). *Bcl-2* proto-oncogene was discovered at the chromosomal breakpoint of the t (14; 18) bearing human lymphoma; an indolent B cell non-Hodgkin lymphoma (Tsujimoto *et al.*, 1985; Reed, 2008).

The Bcl-2 is a 26 kDa intracellular integral membrane protein found primarily in the nuclear envelope, endoplasmic reticulum and outer mitochondrial membrane (Hoetelmans *et al.*, 2000). While *Bcl-2* was initially identified as an oncogene in follicular lymphoma, its expression has been identified in many cancers; including melanoma, myeloma, small-cell lung cancer, and prostate and acute leukemias (Letai, 2003). Expression of other anti-

apoptotic proteins has been detected in many cancers, including Bfl-1 in diffuse large-cell lymphoma (Shipp *et al.*, 2002), Mcl-1 in myeloma (Derenne *et al.*, 2002), and Bcl-xL in lung adenocarcinoma (Berrieman *et al.*, 2005).

Conversely, Bax is a pro-apoptotic protein that can accelerate death, and it is localized in the cytosol prior to a death signals (Reed, 2008). It is a 21 kDa tumour suppressor protein that suppresses tumourigenesis and stimulates apoptosis *in vivo*. The ratio between Bcl-2/Bax dictates to a large extent the susceptibility or resistance to apoptotic signals, which may be imparted by growth factors (Pepper *et al.*, 1997), radiation (Lee *et al.*, 1999), and anticancer drugs (Thomas *et al.*, 1996) and frequently associated with conditions associated with unimpeded cell growth and proliferation or alternatively, with accelerated cell death. These include cancer (Scopa *et al.*, 2001), autoimmune disorders (Li *et al.*, 1999), human immunodeficiency virus infection, and ischemia-reperfusion injury (Wang *et al.*, 2002).

Whereas Bcl-2 and Bax proteins exhibited sequence and structural similarities, they functionally opposed each other and appeared to regulate apoptosis independently (Adams and Cory, 2007). Although the exact mechanisms leading to apoptosis are not fully understood, it is believed that the BH3-only-containing proteins Bad (and Bim), which are phosphorylated by protein kinase A (PKA) and Akt, initiate it. The subsequent translocation of Bax to the mitochondria involves a conformational change that exposes the N- and C-termini (Kim *et al.*, 2009), and its complexing with Bak (Bax:Bak) also requires a conformational change in Bak (Yao and Marassi, 2009). Bax:Bak complex in turn stimulates the release of cytochrome C via a mechanism that may include formation of mitochondrial pores (Almawi *et al.*, 2004). Cytochrome C then binds its adaptor, Apaf-1, leading to the activation of caspase 9, which subsequently activates effector caspases that are proteases which can cleave key cellular proteins (Marsden *et al.*, 2002; Letai, 2005). This leads to many of the morphological characteristics of apoptosis; including

condensed nuclei, DNA laddering and exposure of phosphatidylserine to the outer leaflet of the plasma membrane (Brunelle and Letai, 2009).

2.2.4.2 The Gene *p53*

The gene *p53* was the first tumour suppressor gene to be identified, and it was first described in 1979, and initially believed to be an oncogene. It is located on the short arm of chromosome 17 (17p13.1) with approximately 16-20 kilobase pairs of DNA (Wang *et al*., 1994). The gene encodes a 53-kilodaltons nuclear phosphoprotein that is made of 393 amino acids (Berns *et al*., 1998). This protein acts as a multifunctional transcription factor by playing a critical role in regulation of cell proliferation mainly through induction of growth arrest or apoptosis. So this gene maintains the integrity of the genome (Levine, 1997). Mutations in this gene are the most frequent genetic abnormalities found in human malignancies. The protein product of a mutated *p53* gene has a prolonged half-life within the cell nucleus, relatively to the wild-type p53 protein. This abnormal prolonged half-life enables the detection of *p53* expression by immunohistochemistry (IHC) using anti-p53 monoclonal antibodies (Imamura *et al*., 1994).

The response of cells to DNA-damaging agents is complex involving recognition and repair of the lesions in DNA to minimize the risk of genetic instability. Therefore, mutations that interfere with the function of key metabolic steps can predispose to genome instability, cancer and other pathologies (Kops *et al*., 2005). A central control in protecting the integrity of the genome is p53 (p53 gene or protein), which is present at low levels under unperturbed conditions but becomes rapidly stabilized and activated in response to a variety of stimuli including DNA damage (Lavin and Gueven, 2006).

The importance of *p53* role in maintaining genome stability is exemplified by the findings that this molecule is mutated in approximately 50% of tumours, and moreover, these tumours respond poorly to therapy (Hainaut, 1995; Fridman

and Lowe, 2003). The p53 exerts its control on the cell cycle primarily through the G1/S checkpoint but it has also been shown to regulate the G2/M checkpoint (Lavin and Gueven, 2006).

Although, the exact mechanism of *p53* stabilization remains unclear, it involves a series of post-translational modifications to both itself and MDM2 (mouse double minute), which facilitate the dissociation of the MDM2–p53 complex that is primarily responsible for keeping the levels of p53 in check (Moll and Petrenko, 2003). However, it is evident that a multitude of other proteins also influence the stability of p53. The capacity of p53 to induce specific proteins to regulate the passage of cells through the cell cycle is of a key importance in fulfilling its role in genome stability, but its involvement in nucleotide and base excision repair pathways may also contribute (Adimoolam and Ford, 2003; Gatz and Wiesmuller, 2006).

In unstressed cells, p53 levels are kept low through a continuous degradation of the protein. A protein called MDM2 (also called HDM2 in humans) binds to p53, preventing its action and transports it from the nucleus to the cytosol. Also MDM2 acts as ubiquitin ligase and covalently attaches ubiquitin to p53 and thus marks p53 for degradation by the proteasome. However, a mutant p53 protein often does not induce MDM2, and is thus able to accumulate at very high concentrations. Furthermore, mutant p53 protein itself can inhibit normal p53 protein levels. In a normal cell, p53 is inactivated by its negative regulator, MDM2. Upon DNA damage or other stresses, various pathways will lead to the dissociation of the p53 and MDM2 complex. Once activated, p53 will either induce a cell cycle arrest to allow repair and survival of the cell or apoptosis to discard the damaged cell (Figure 2-3) (reviewed by Soussi and Wiman, 2007).

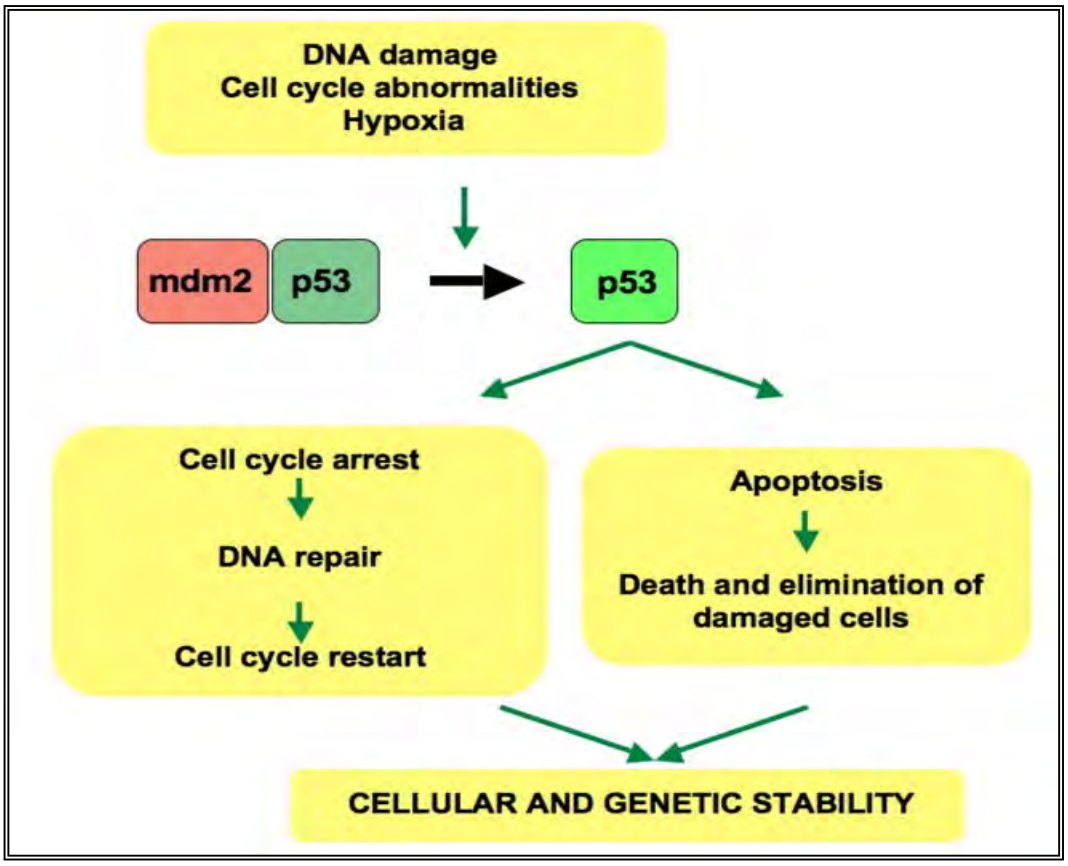


Figure 2-3: Pathways of p53 interactions (Soussi and Wiman, 2007).

The gene product of *p53* is involved in anti-cancer mechanisms, which are outlined in the following as suggested by Vousden and Lu (2002):

- It can activate DNA repair proteins when DNA has sustained damage.
- It can induce growth arrest by holding the cell cycle at the G1/S regulation point on DNA damage recognition.
- It can initiate apoptosis if the DNA damage proves to be irreparable.

In the case of a genetic damage, which is induced by ionizing radiations or by chemical causes, a rapid increase in the p53 level and its activation are occurred. This activation of *p53* induces cell cycle arrest by p21; a CDK inhibitor, and this interval in the cell cycle allows the cell to induce a state of repair. In the case of irreparable genetic damage, p53 induces apoptosis by the activation of a pro-apoptotic gene; *bax* (Sa and Das, 2008). In cells with

mutations or homozygous loss of *p53*, there is no repair or apoptosis of genetically damaged cells because p53 can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the 'stop signal' for cell division. Therefore, cells divide uncontrollably, and formation of transformed cell and finally development of a neoplasm is occurred (Abdulamir *et al*., 2008).

2.2.4.3 The Gene *Ki-67*

Ki-67 was identified by Gerdes and co-workers in 1991(Gerdes *et al.*, 1991) as a nuclear non-histone protein, shortly after the corresponding antibody was described by the same group (Gerdes *et al.*, 1983) in the city of Kiel (hence "Ki") after immunization of mice with the Hodgkin's lymphoma cell line L428 (67 refers to the clone number on the 96-well plate in which it was found). The absence of *Ki-67* in quiescent cells and its universal expression in proliferating tissues created great interest on its potential role as a marker of cell proliferation. A large number of studies have confirmed this feature, and the expression of *Ki-67* in resting cells has rarely been reported (Scholzen and Gerdes, 2000; Urruticoechea *et al.*, 2005).

The *Ki-67* gene is located on the long arm of human chromosome 10 (10q25) (Fonatsch *et al.*, 1991). In 1993, Schluter *et al*. published the complete sequence of the cDNA encoding for the protein (Schluter *et al.*, 1993). Two alternative mRNA species resulting from alternative splicing encode two isoforms of the protein. The "large" Ki-67 protein isoform has a calculated molecular mass of 359 kD, while the "small" isoform has a mass of 320 kD. The presence or absence of the sequence encoded by exon 7 of the gene differentiates one isoform from the other. The most outstanding feature when the sequence is analyzed is the presence of 16 repeated elements, the *Ki-67* repeats, in the large exon 13 that makes up 70% and 79% of the open reading frame of the large and small species, respectively. These are concatenated sequences

depicting an important similarity with 43% to 62% identical amino acids. Within these repeats, a sequence of 22 amino acids, the so-called *Ki-67* motif, can be found. This motif is highly conserved between species and includes the epitope (FKEL) targeted by the original Ki-67 antibody. There is some evidence derived from murine and human cell lines that more post-splicing variants may exist with a consequent larger range of protein isoforms (Scholzen and Gerdes, 2000).

The expression of Ki-67 varies in intensity throughout the cell cycle, and this has raised concern that it could lead to a misclassification of cycling cells as resting ones (van Dierendonck *et al.*, 1989). An overall evidence indicates that levels of Ki-67 are low during G1- and early S-phases and progressively increase to reach a maximum during mitosis. A rapid decrease in expression starts during anaphase and telophase (Lopez *et al.*, 1991). Some authors have found that the degree of expression in G1 can be minimal (Bruno and Darzynkiewicz, 1992) and may represent a handicap to Ki-67's accuracy in identifying cells in this phase. This irregular presence of Ki-67 in G1 has been explained as the result of the differences between G1-phase in a previously dormant cell and G1 immediately following a previous cycle or even as a marker of different conditions of growth (Urruticoechea *et al.*, 2005).

The half-life of Ki-67 protein has been estimated at around 60 to 90 minutes (Heidebrecht *et al.*, 1996). Differences of expression during the cell cycle do not seem to be due to the accumulation of non-degraded protein; rather, they seem largely to reflect variable *de novo* synthesis (Urruticoechea *et al.*, 2005).

The cellular appearance and location of the Ki-67 protein throughout the cell cycle is not homogeneous. During early G1, it is found as generally weakly staining discrete foci throughout karyoplasms (Kill, 1996). It progressively condenses during late G1 in larger peri-nucleolar granules (Heidebrecht *et al.*, 1996). During S- and G2-phases, it is mainly found associated with the nucleolar region in larger foci, as well as, with some heterochromatin regions. When the

nuclear membrane disrupts during early mitosis, *Ki-67* shows an intense expression associated with the surface of condensed chromosomes in the cytoplasm. This intensity rapidly disappears in anaphase-telophase (Urruticoechea *et al.*, 2005).

Despite all this information about the nature, location, and sequence of Ki-67 protein, there is a little known of its function beyond its being a protein phosphorylated via serine and threonine (Urruticoechea *et al.*, 2005) with a critical role in cell division. This has been concluded from the arrest of cell proliferation when Ki-67 is blocked either by microinjection of blocking antibodies or by inhibition of dephosphorylation (Nabi *et al.*, 2008).

2.2.5 Cell Cycle

Cell cycle is a mechanism by which cells divide, and it is controlled by numerous mechanisms to ensure correct cell division and consists of two consecutive processes; DNA replication and segregation of replicated chromosomes into two separate cells. These processes occur through two stages, which are interphase and mitosis (M) (Stewart *et al.*, 2003). Replication of DNA occurs in a specific part of the interphase (S phase). The S-phase is preceded by a gap called G1 during which the cell is preparing for DNA synthesis and is followed by a gap called G2 during which the cell prepares for mitosis (Vermeulen *et al*., 2003). Different cellular proteins regulate the transition from one cell cycle phase to another. Key regulatory proteins are the CDK, a family of serine/threonine protein kinases that are activated at specific points of the cell cycle. When activated, CDKs induce downstream processes by phosphorylating selected proteins. The CDK protein levels remain stable during the cell cycle, in contrast to the cyclins. Cyclin protein levels rise and fall during the cell cycle and in this way they periodically activate CDK (Cooper and Hausman, 2009).

Defects in cell cycle regulation are a characteristic feature of tumour cells and mutations in the genes involved in controlling the cell cycle are extremely

common in cancer (Bowen and Wylie, 2005). Mutations mainly occur in two classes of genes; proto-oncogenes and tumour suppressor genes. In normal cells, the products of proto-oncogenes act at different levels along the pathways that stimulate cell proliferation, and mutated versions of proto-oncogenes or oncogenes can promote tumour growth, while inactivation of tumour suppressor genes like *pRb* and *p53* results in dysfunction of proteins that normally inhibit cell cycle progression (Alfieri *et al*., 2009). Therefore, cell cycle deregulation associated with cancer can occur through mutation in genes involved in coding for proteins important at different levels of the cell cycle. In cancer, mutations have been observed in genes encoding CDK, cyclins, CDK-activating enzymes, cyclin kinase inhibitor (CKI), CDK substrates, and checkpoint proteins (Vermeulen *et al*., 2003).

Monitoring dysfunctional cell cycle regulation is thus the focus of intense interest, since it provides an opportunity to discover new targets for anti-cancer drugs and improved therapeutics. The cell cycle, then, can be used as a tool to explore the mechanisms of various toxins and drugs (Cope *et al*., 2009). A variety of markers have been employed to determine the cell cycle status, including DNA ploidy, bromodeoxyuridine (BrdUrd) incorporation, tritiated thymidine (^{3}H-thymidine) incorporation, *Ki-67* expression, PCNA expression, cyclin expression, CDK expression, and CDK phosphorylation status. The use of these markers can range from the simplest measurements, such as the relative proportion of cells in a population in the cell cycle to the most complex, such as the pinpointing of the location of a given cell in the cycle or the determination of cell cycle kinetics (Beresford *et al.*, 2006).

2.2.5.1 Single-Parameter DNA Histogram Analysis

Single-parameter DNA histogram (DNA ploidy) is one of the measurements to study drug effects on cell cycle kinetics and one of the earliest applications of flow cytometry (Brockhoff and Knuechel, 2003). The frequency

distribution of a population of cells stained for DNA content has a distinct shape if the population is growing exponentially. Figure 2-4 provides an example of a DNA frequency distribution typical for an asynchronously growing exponential population of cells (Traganos, 2004). The initial peak at the lowest fluorescence value represents post mitotic G1 (and under some circumstance, quiescent G0) cells that have divided but have not yet initiated DNA synthesis. The second peak is at approximately twice the fluorescence of the first that contains cells that have doubled their DNA content and have yet to divide (G2) or are in the process of dividing (i.e. undergoing mitosis; G2/M), while the distribution between the two peaks contains cells with increasing levels of DNA, and represent DNA synthesizing or S-phase cells (Davies, 2008).

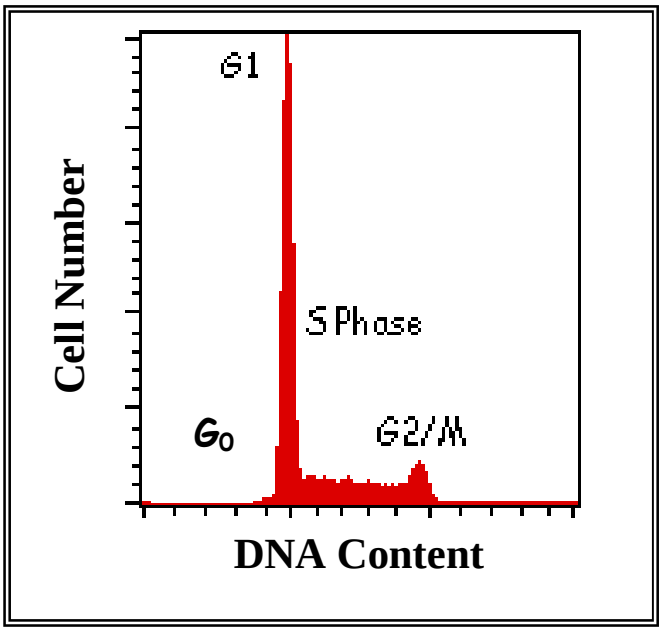


Figure 2-4: DNA frequency distributions typical for exponentially growing cells. (Davies, 2008).

2.2.5.2 Measuring DNA Synthesis Using 5-Bromodeoxyuridine

Historically, the study of cell cycle kinetics entailed the use of radioactively labeled thymidine to detect cell synthesizing DNA, and by using autoradiography, radioactive thymidine incorporation allows for the measurement of the labeling index and the fraction of or percentage of labeled mitoses. The labeling index then can be used to determine the fraction of cells

synthesizing DNA (depending on S-phase duration), although the changing percentage of labeled mitoses curve provides information on the progression of labeled cells through the cycle (Terry and White, 2001).

These tedious techniques have now been supplemented by the use of fluorescence-labeled halogenated pyrimidines as a replacement for radioactive thymidine. The most notable and widely used is BrdUrd (Traganos, 2004). Detection of incorporation of these fluorescence-labeled analogs by flow cytometry allows for thousands or ten thousands of cells to be analyzed in details providing a statistical data. In virtually all cases, incorporation of halogenated pyrimidines is detected by tagging them with fluorescence-labeled monoclonal antibodies (Rosato *et al.*, 2001). Initially, however, techniques for detecting BrdUrd incorporation into DNA involved the fact that certain DNA stains quenched in its presence. The decrease in fluorescence of the quenched dye could be combined with a DNA-specific dye whose fluorescence emission is unaffected by the presence BrdUrd. Therefore, the binding analysis of both dyes provides a more precise quantitation of the S-shape population in the cell cycle distribution (Darzynkiewicz *et al.*, 2004).

This approach allows for measurement of a number of parameters and is especially useful because more than one cell cycle can be analyzed in the same measurement (Darzynkiewicz *et al.*, 2001). As noted, detection of BrdUrd incorporation into DNA is based on immunocytochemical techniques, and in this approach, partial DNA denaturation by strong acid or heat is required to make the incorporated halogenated pyrimidine accessible to the monoclonal antibody probe, which can be either directly or indirectly labeled with fluorescein. The non-denaturated DNA can then be stained, typically with an intercalating flourochrome such as propidium iodide (PI). The combination of PI red fluorescein and fluorescein green fluorescence provide a bivariate plot of cellular DNA content *versus* incorporated BrdUrd (Darzynkiewicz and Juan, 2001).

In induced DNA breaks by physical or chemical agents at the sites of BrdUrd incorporation, the enzyme terminal deoxynucleotide transferase is used to add many fluorchrome-labeled deoxynucleotides (typically BrdUrd) to each DNA break site (Traganos, 2004). This means that the label is not on the antibody attempting to bind the single molecule of BrdUrd incorporated into DNA, but that the fluorochrome is attached to one of many molecules being added at the site of DNA by denaturation or digestion in a way that would affect binding of intercalating dye PI (Darzynkiewicz *et al.*, 2006). As can be seen in figure 2-5, there is an approximately 10 times increase in fluorescence intensity of the cells incorporating BrdUrd compared to control cells grown in the absence of the label (reviewed by Traganos, 2004).

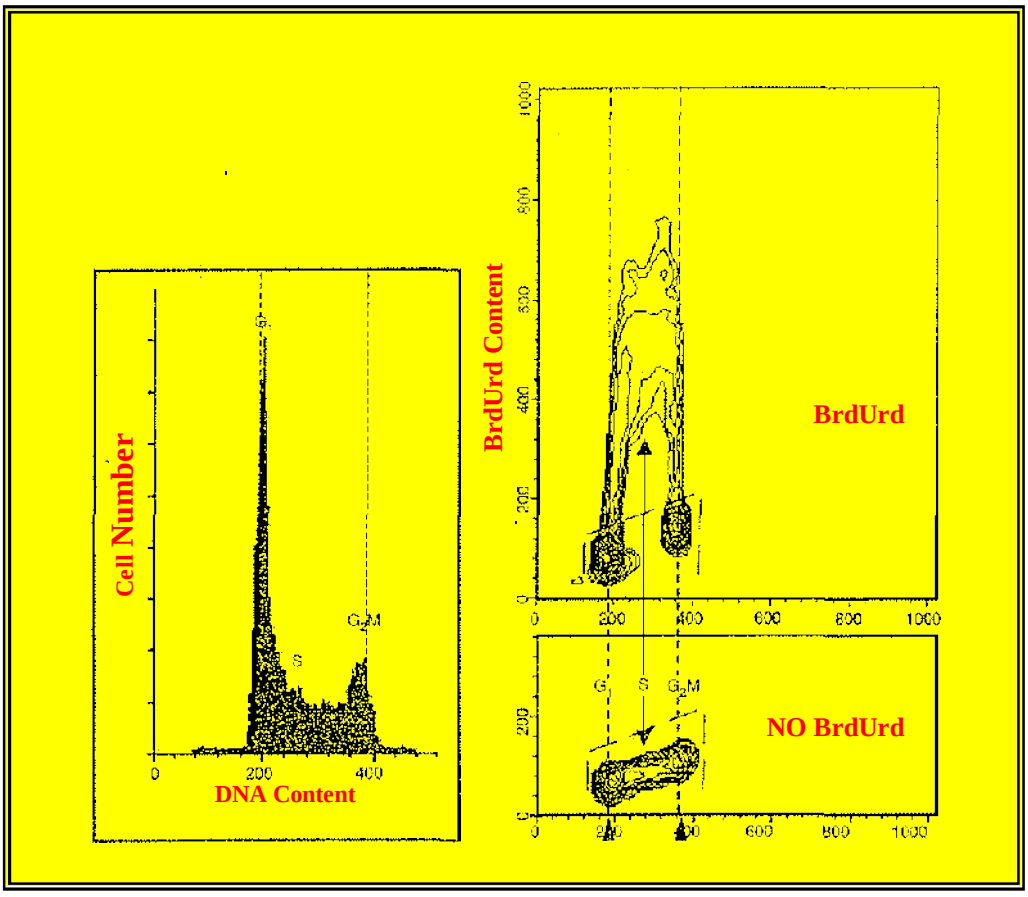


Figure 2-5: Bivariate analysis of DNA content *versus* incorporation of BrdUrd into DNA of S-phase cells. Left: DNA frequency distribution of exponentially growing cells. Right: Bivariate distribution in which S-phase cells have incorporated the thymidine analog BrdUrd into DNA. (Traganos, 2004).

2.2.6 H2AX-Histone Phosphorylation Formation

The introduction of double-strand breaks into DNA triggers a complex set of responses in eukaryotic cells; including cell cycle arrest, relocalization of DNA repair factors, and in some cases apoptosis (Jackson, 2002). Failure to arrest cellular functions can lead to a high level of genomic instability, which is linked to increased probabilities of oncogenic transformation (Prindull, 2008).

Several factors known to be involved in DNA repair or in signaling the presence of damage have been shown to accumulate in large nuclear domains (foci) after a double-strand DNA breakage (Riballo *et al*., 2004). This response is not yet fully understood, but it has been suggested to be a visual indication of DNA repair centres (Nakamura *et al.*, 2010). One of the first cellular responses to the introduction of double-strand breaks is the phosphorylation of H2AX to create γ-H2AX, which functions to recruit DNA damage response factors to sites of DNA damage (Shrivastav *et al.*, 2008).

In eucaryotes, DNA is packaged into nucleosomes, which are in turn arranged in various higher order structures to form chromatin (Fernandez-Capetillo *et al.*, 2004). The crystallographic structure of nucleosome has been elucidated. Two copies of each histone protein; H2A, H2B, H3 and H4, are assembled into an octamer that has 145-47 base pairs (bp) of DNA wrapped around it to form a nucleosome core (Bilsland and Downs, 2005). In mammals, each histone family is encoded by multiple genes, which (with few exceptions) are expressed in concert with replication (Redon *et al.*, 2002). The various members of the H4, H3 and H2B families differ in few if any amino acid residues. In contrast, the H2A family includes three subfamilies whose members contain characteristic sequence elements that are conserved independently throughout eucaryotic evolution (Bilsland and Downs, 2005). The three H2A subfamilies are H2A-Bpd, macro-H2A, H2AZ and H2AX (Zlatanova and Thakar, 2008). H2AX constitutes a major H2A species, and its levels vary from 2-25% of the mammalian histone H2A pool depending on the cell line or tissue

examined (Kinner *et al.*, 2008). Serine 139 in the unique carboxy-terminal tail of H2AX is phosphorylated within 1 to 3 minutes after damage, and the number of H2AX molecule phosphorylation increases linearly with the severity of the damage (Takahashi and Ohnishi, 2006). An antibody specific to the γ-H2AX has been produced and used to show that γ-H2AX is localized in large chromatin domains at the sites of DNA damage. The γ-H2AX can be detected visually as microscopic foci (Cowell *et al.*, 2007). Other effective techniques include immunoprecipitation, Western blotting laser scanning cytometry and flow cytometry, but each method having its own merits and drawbacks (Kataoka *et al.*, 2006).

Measurement of γ-H2AX immunofluorscence by Multiparameter laser-scanning or flow cytometry is particularly advantageous (Olive, 2004). The major benefit of the cytometric approach stems from the fact that H2AX phosphorylation *in situ* in chromatin of individual cells, can be measured with high sensitivity and accuracy and the expression of γ-H2AX can be directly correlated, within the same cells, with their DNA content, induction of apoptosis or any other cell attribute of interest (Huang *et al.*, 2005). Large cell numbers, thus, may be rapidly analysed and the data provide information on the extent of H2AX phosphorylation with respect to their cell cycle phase, commitment to die in response to DNA damage and their surface immunophenotype amongst others (Tanaka *et al.*, 2007). Cytometry also allows one to analyse intercellular variability in H2AX phosphorylation within cell populations and to identify rare cell subpopulations, otherwise they are undetectable by Western blotting (Tanaka *et al.*, 2009).

2.2.7 Tumour and Normal Cell Lines

2.2.7.1 HL-60 Cell Line

The HL-60 cell line was established in 1977 from a female patient with acute myeloid leukaemia. The cells largely resemble promyelocytes but can be

induced to differentiate terminally *in vitro*. Some reagents cause HL-60 cells to differentiate to granulocyte-like cells, others to monocyte/macrophage-like cells (Birnie, 1988). The HL-60 cell genome contains an amplified *c-myc* proto-oncogene; therefore *c-myc* mRNA levels are correspondingly high in undifferentiated cells but decline rapidly following induction of differentiation (Shimizu *et al.,* 1994). These features have made the HL-60 cell line an attractive model for studies of human myeloid cell differentiation.

2.2.7.2 HCT-116 Cell Line

The HCT-116 cell line was established from a primary colon carcinoma of an adult man. Cells were described to carry a *RAS* mutation in codon 13 and to be tumourgenic in nude mice (Shirasawa *et al.,* 1993). These cells have a wild-type TP^{53} genotype, which is a somatic frame shift mutation in exon 3 of the *Bax* gene (De Angelis *et al.,*1998), DNA-mismatch repair-deficiency due to lack of *hMHL1* expression (Hawn *et al.,*1995), have two different thymidylate synthase enzymes (Hughey *et al.,*1993) and a near-diploid DNA complement (De Angelis *et al.,*2004).

2.2.7.3 HuFb Cell Line

The HuFb cell line was derived from the dermis of a normal human neonatal foreskin or adult skin (Rittie and Fisher, 2005). Fibroblasts are extensively used for a wide range of cellular and molecular studies. This is mainly because they are one of easiest types of cells to grow in culture, and their durability makes them amenable to a wide variety of manipulations ranging from studies employing gene transfection to microinjection (Mansbridge, 2001; Tamanini, 2007).

Chapter Three
Materials and Methods

Chapter Three
Materials and Methods

Different laboratory methods were employed to achieve the aims of the present study; therefore each method together with its chemicals, solutions and kits were given separately. The exception was general equipments and instruments that were given in a single section.

3.1 General Equipments and Apparatuses

The following general equipments and apparatuses were used throughout the study:

- Autoclave (Vapromatic770, Italy).
- Caliper (Italy).
- Cell counter with multisizer 3 software (Beckman coulter, USA).
- Centrifuges 5810 and 5810R (Eppendorf, Germany).
- Concentrator 5301R (Eppendorf, Germany).
- Digital multi-pipette (Eppendorf, Germany).
- Distillation unit (Millipore, France).
- Electrophoresis set: Agarose gel tank (Whatman Biometra, Göttingen, Germany) with a Bio-Rad model 200/0.2 power supply (Bio-Rad, USA) and UV transilluminator (Image Quant 400, USA).
- Entry Level Image System (Immagini and computer, Italy).
- Flowcytometry: Fluorescence activated cell sorter (FACS, Becton Dickinson, USA) and CellQuest software system.
- Fluorescence microscopy: BX 41TF and CKX41 fluorescent microscope (Olympus, Japan).
- Fume hood (Bio-optica, Italy).
- Glasswares: Beakers (50, 100, 200, 500 and 800ml), Pasture pipettes, funnels, cylinders, volumetric flasks (50, 250 and 5000ml) and bottles

(75, 200 and 1000ml) were purchased from DISA, Italy. All glasswares used were soaked overnight in detergent (Candiggena, Italy) and rinsed at least three times with tap-water and three times with distilled water. Finally, they were sterilized either by autoclave (121°C, 1.5 pounds/ $inch^2$ for 30 minutes) or oven (160°C for 3 hours).

- Histopathology and Immunohistochemistry: Autostainer (DAKO, USA), rapid microwave histoprocessor histos 5 (Milestone, USA), paraffin cold plate EG1150C, heated paraffin dispensing module EG 1150H, microtome RM2255 and mounting system CV5020 (Leica, Germany).
- HPLC-ESI/MS: LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc., USA) supplied with 1200 series capillary LC pump and auto-sampler (Agilent Technologies, USA)**,** electrospray ionization Omni spray (Prosolia, USA), Zorbax SB-C 18 column (Agilent Technologies, USA) and LTQ-Orbitrap Xcalibur software.
- Ice maker (Breama, Japan).
- Incubator (Bio-concept, Italy).
- Incubator with CO_2 supply (Thermo Electron Corporation, USA).
- Laminar air flow (Steril-CTH72C2, Italy).
- Light microscope (Olympus, Japan).
- Lyophilizer (Labconcco, England).
- Magnetic stirrer (LabincoL-71, Netherland).
- Microfuge (Heraeus sepatech, Germany).
- Microfuge 5417R (Eppendorf, Germany).
- Micropipettes; 2, 10, 100, 200 and 1000 µl **(**Eppendorf, Germany).
- Oven (Memmert, Germany).
- pH-Meter GIP22 (Crison, Spain).
- Plasticwares: Disposable sterile plastic tissue culture flasks with different surface areas (T-25 cm^2, T-75 cm^2 and T-150 cm^2; Iwaki, Japan), multi-

well plates (6-well, 12-well and 24-well; BD Falcon, USA), sterile polypropylene conical tubes (15ml and 50ml), polystyrene conical tubes 15ml and polystyrene round-bottom tubes 5ml (BD Falcon, USA), disposable sterile pipettes (2, 5, 10, 25 and 50ml; Sterilin, UK), syringes (1, 10 and 20ml; Ico, Italy), syringe driven filters (0.22 and 0.45µm; Millipore, Ireland) and Eppendorf tubes (100, 200, 500 and 2000µl; Eppendorf, Germany).

- Rotary evaporator (Heidolph, Germany).
- Sensitive balance (Gibertini E.505, Italy).
- Shaker (Stuart, UK).
- Soxhlet (Electrothermal, England).
- Spectrophotometer UV/VIS lambda 19 (Perkin Elmer, USA).
- Thermo-mixer (Eppendorf, Germany).
- Vortex (Stuart, UK).
- Water bath (Stuart, UK).

3.2 Collection and Extraction of Propolis Samples

3.2.1 Collection

The Iraqi propolis raw samples were collected from different Iraqi geographical regions during the period 20-4-2007 to 1-7-2008, by scraping the sample off from the frames of beehives. The locations of hives were Baghdad (two samples: B1 and B2), Dahuk (D), Mosul (M) and Salah ad-Din (S) (Table 3-1). The samples of propolis were kept in the dark and stored in the freezer (-20ºC). After that, they were transported in cooled box by air to the Department of Oncology, Mario Negri Institute for Pharmacological Research, Milan, Italy, at which the extraction, chemical analysis and the other laboratory investigations were carried out.

Table 3-1: Investigated propolis samples presented by their codes, region of collection, appearance, form, colour and odour.

Sample Code	City (Position of Hives)	Appearance and Form	Colour and Odour
B1	Baghdad Centre	Gummy, Waxy	Light brown, Aromatic
B2	North east Baghdad	Gummy, Waxy	Dark brown, Aromatic
D	Dahuk	Gummy, Waxy	Dark green, Aromatic
M	Mosul	Powder	Dark green, Aromatic
S	Salah ad-Din	Waxy	Dark brown

3.2.2 Extraction

- **Solutions**

i. Absolute methanol (Carlo Erba, Italy).

ii. Ethanol (96%) (Carlo Erba, Italy).

iii. L-lysine (8%): It was prepared by dissolving 8 grams of L-lysine (Sigma, Germany) in 100 ml of distilled water (Nikolov *et al*., 1987).

- **Method**

The propolis exract (PE) was prepared according to a method presented by Nikolov *et al*. (1987) as illustrated in figure 3-1. Accordingly, 25 grams of propolis were cut into small pieces and extracted with 250 ml of absolute methanol for 60 minutes at 60°C using the soxhlet, and then the PE solution was filtered (Whatman filter paper No. 3) and collected. A further two extractions were carried out in a similar manner. After the third extraction, the three extract solutions were combined and then stored overnight at 4°C to induce the crystallization of dissolved waxes. The resultant extract solution was filtered, and the precipitate was washed with cold 96% ethanol. The filtrate was evaporated to dryness at 50ºC, giving a resinous brown product, which was then

dissolved in 25 ml of L-lysine solution at 50°C for 30 minutes using a vortex supplemented with heater. The obtained solution was lyophilized for 24 hours, and the final yield was yellow-brown powder, which represented the PE. The PE was stored under sterile conditions, protected from light in a dry and cool place at -20°C until use (a maximum of nine months).

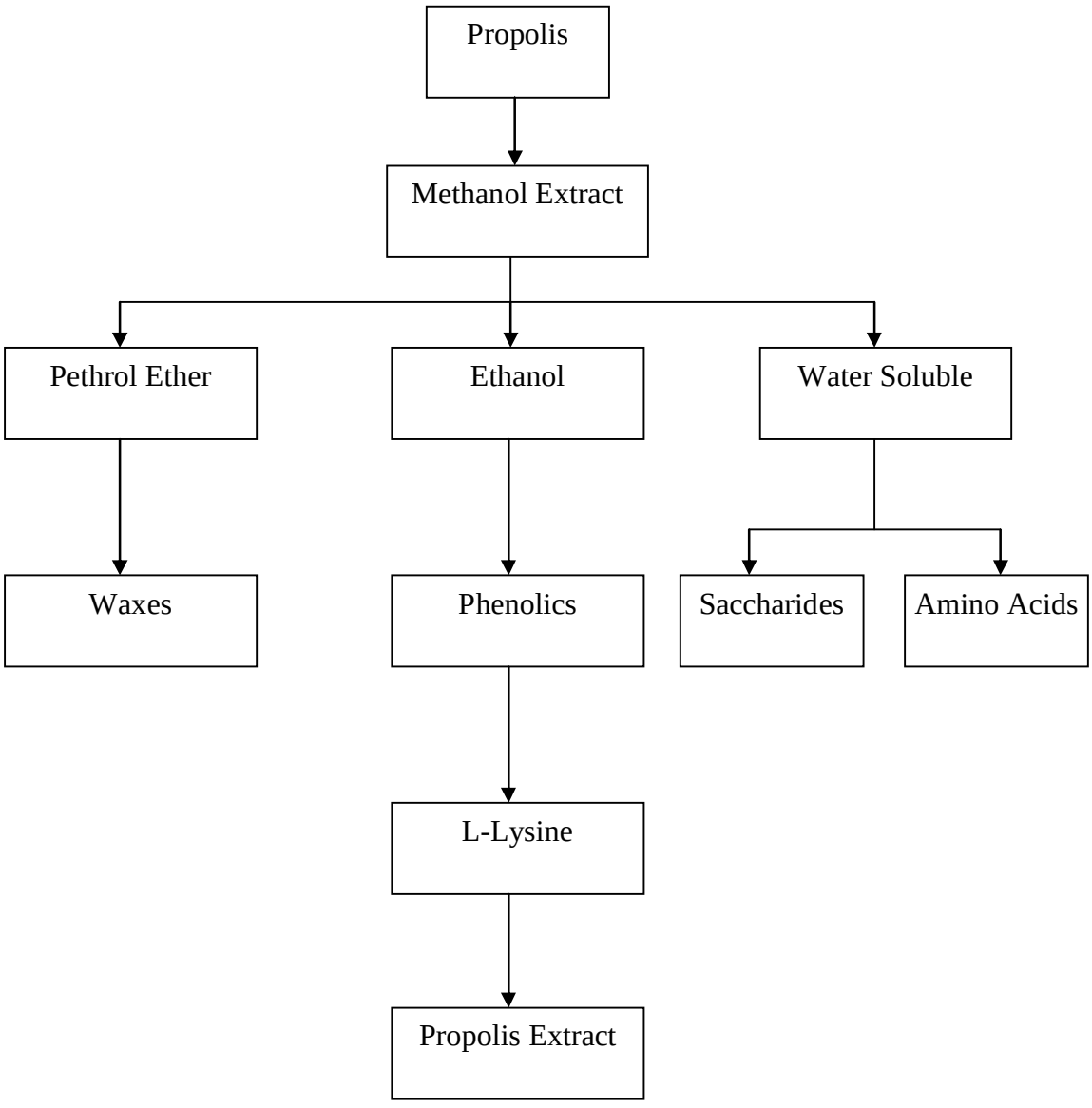
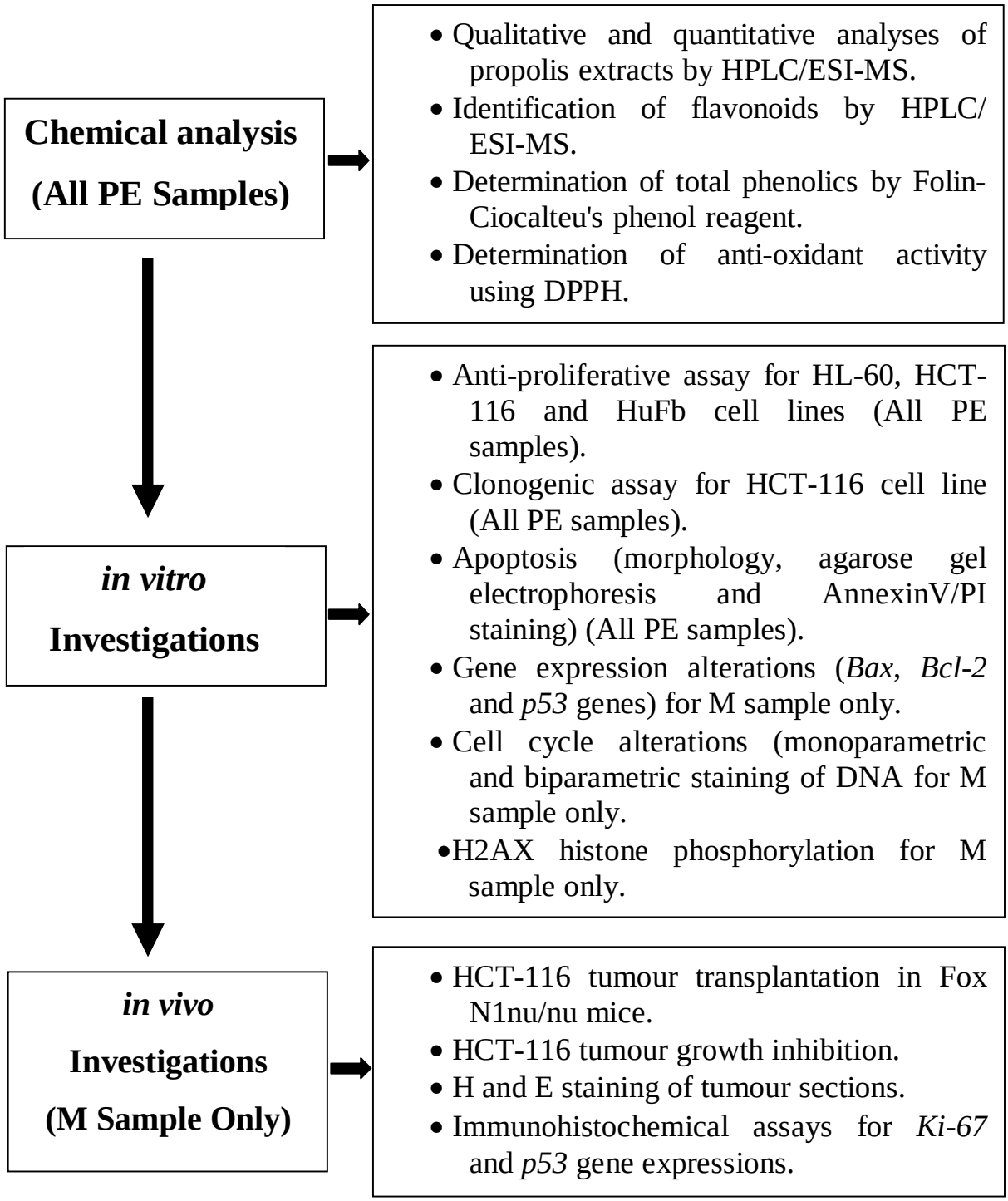


Figure 3-1: Schematic presentation of propolis extraction (Nikolov *et al*., 1987).

3.3 Laboratory Investigations

Three main laboratory investigations were carried out to achieve the aims of the present study. They are outlined in the following:


Chemical analysis
(All PE Samples)
• Qualitative and quantitative analyses of propolis extracts by HPLC/ESI-MS.
• Identification of flavonoids by HPLC/ ESI-MS.
• Determination of total phenolics by Folin-Ciocalteu's phenol reagent.
• Determination of anti-oxidant activity using DPPH.
in vitro
Investigations
• Anti-proliferative assay for HL-60, HCT-116 and HuFb cell lines (All PE samples).
• Clonogenic assay for HCT-116 cell line (All PE samples).
• Apoptosis (morphology, agarose gel electrophoresis and AnnexinV/PI staining) (All PE samples).
• Gene expression alterations (*Bax*, *Bcl-2* and *p53* genes) for M sample only.
• Cell cycle alterations (monoparametric and biparametric staining of DNA for M sample only.
•H2AX histone phosphorylation for M sample only.
in vivo
Investigations
(M Sample Only)
• HCT-116 tumour transplantation in Fox N1nu/nu mice.
• HCT-116 tumour growth inhibition.
• H and E staining of tumour sections.
• Immunohistochemical assays for *Ki-67* and *p53* gene expressions.

3.3.1 Chemical Analysis

3.3.1.1 Qualitative and Quantitative Analyses of Propolis Extracts

- **Materials and Solutions**

i. **Propolis sample preparation:** The PE samples (B2, D, M and S) were dissolved at a concentration of 10 mg ml^{-1} in DMSO (Sigma, USA): distilled water (2:1v/v), while B1 sample was dissolved in distilled water only. Such preparations were based on a series of experiments that determined the optimal solubility, and they were considered as stock solutions.

ii. **Solvent A:** It was prepared by diluting 0.05 ml of acetic acid (Carlo Erba, Italy) in 99.95 deionized distilled water (Medana *et al*., 2008).

iii. **Solvent B:** It was ready used acetonitrile solution (CH_3CN, DuPont, USA).

- **Method**

The forthcoming five prepared propolis samples were diluted 1:200 (v/v) with a diluting solution consisted of solvent B: distilled water (1:4 v/v). Qualitative and quantitative analyses of PE solutions were done by HPLC-ESI/MS System Thermo Electron Corporation Instrument, which consisted of a 1200 series capillary LC pump equipped with autosampler and coupled to LTQ Orbitrap XL mass spectrometer. The ion source was a desorption electrospray ionization Omni Spray, which was used in the nano-electrospray mode for negative and positive ions (Figure 3-2).

The HPLC separation was obtained with a zorbax SB-C 18 column (100 × 0.5 mm; 5 µm particle size), using an elution mixture composed of solvent A and solvent B. The injection volume was 1-2 µl and the flow rate was 10 µl $minute^{-1}$. The elution gradient of solvent B was from 20 to 100% in 40 minutes (hold at 100% for 6 minutes) at a total flow rate of 10 µl min^{-1}. The separation was performed at room temperature (20-25°C). Samples (1 µl) were directly

injected into the HPLC column, which was directly coupled to the ion source spray capillary by a liquid junction (Figure 3-3).

Figure 3-2: The HPLC-ESI/MS system thermo electron corporation instrument.

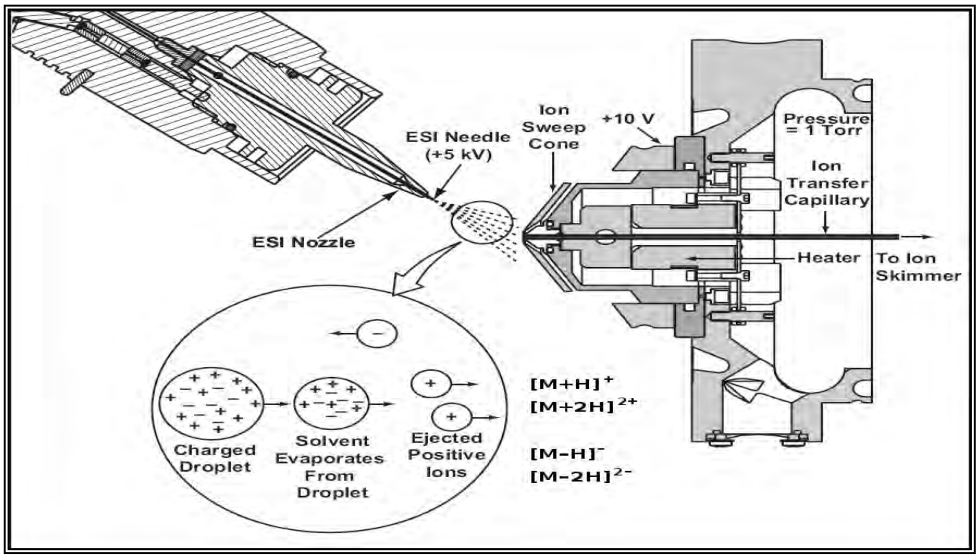


Figure 3-3: The mechanism of HPLC separation.

The MS data were acquired with the Orbitrap analyzer at 60000 resolutions for full scan MS spectra and with the LTQ analyzer (Linear Ion Trap) at unit resolution for MS2 spectra, which were automatically obtained in a single

chromatographic run, using real time data dependent acquisition that was based on the characteristics of the previously acquired MS spectra. Data processing and calculations were done using the LTQ-Orbitrap Xcalibur 1.4 software.

3.3.1.2 Biologically Active Compound Analyses

I- Identification of Flavonoids by HPLC-ESI/MS

- **Material and Solutions**

  i. **Standards and propolis samples preparation:** The PE samples (B2, D, M and S) and standard compounds were dissolved at a concentration of 10 mg ml^{-1} in DMSO (Sigma, USA): distilled water (2:1v/v), while B1 sample was dissolved in distilled water only. The standard compounds included stock solutions of flavonoids (chrysin; Aldrich, Germany); tectochrysin, apigenin and acacetin (flavones); quercetin, galangin and kaempferide (flavonols); pinocembrin, pinostrobin and sakuranetin (flavanones) (Genay, France); caffeic acid and caffeic acid phenethyl ester (Sigma, Germany).

  ii. **Solvent A:** It was prepared as in section **3.3.1.1**

  iii. **Solvent B:** It was prepared by diluting one part of acetonitrile solution (CH_3CN; DuPont, USA) in three parts of deionized distilled water.

- **Method**

Solutions of each PE sample (10 mg ml^{-1}) were diluted 1:1000 (v/v) with a diluting solution consisted of solvent B: distilled water (1:3 v/v). Standard solutions of chrysin (CH), tectochrysin, apigenin, acacetin, quercetin, galangin , kaempferide , pinocembrin, pinostrobin , sakuranetin , caffeic acid (CA) and caffeic acid phenethyl ester (CAPE) (0.1 μg ml^{-1}) were used as references for qualitative and quantitative calculations. All samples were analyzed by HPLC-ESI/MS using a Thermo Electron Corporation LTQ-Orbitrap XL instrument, with the same conditions reported in section **3.3.1.1.**

II- Determination of total phenolics spectrophotometrically

- **Materials and Solutions**

i. **Sodium carbonate solution:** It was prepared by dissolving 7.5 grams of sodium carbonate (BDH, England) in 100 ml of deionized distilled water (Chen *et al.*, 2004).

ii. **Folin-Ciocalteu's phenol reagent:** Ready used Folin-Ciocalteu's phenol reagent (Sigma, Germany).

iii. **Samples preparation:** The PE and CA solutions were prepared as in section **3.3.1.2**.

- **Method**

Total phenolic compound concentrations were determined spectro-photometrically (Chen *et al.*, 2004). The PE (0.1 ml) was diluted with deionized distilled water (7.9 ml) at a concentration of 10 mg ml $^{-1}$. Folin-Ciocalteu reagent (0.5 ml) was then added, and the contents were mixed thoroughly. After 1 minute, 0.2 ml of sodium carbonate solution was added, and the mixture was mixed thoroughly. The absorbance of blue colour produced solution was measured at 765 nm. Total phenolic content (µg ml^{-1}) was estimated using a standard curve for CA concentration range 100-1000 µg ml^{-1} (Figure 3-4). For each extract, three measurements were performed and the mean was adopted.

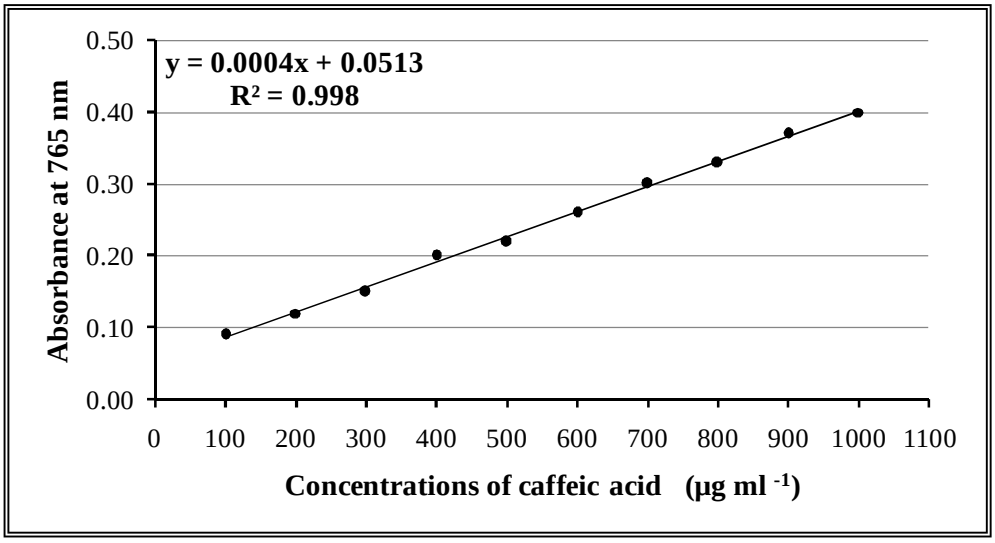


Figure 3-4: Standard curve of caffeic acid concentrations.

3.3.1.3 Stable Free Radical Scavenging Capacity

- **Materials and Solutions**

i. **Ethanol:** Absolute ethanol (Carlo Erba, Italy) was used.

ii. **DPPH solution:** It was prepared by dissolving 2.366 mg of 2, 2-diphenyl-1-picrylhydrazyl (DPPH; Sigma, Germany) in 100 ml of absolute ethanol to obtain 60 μM DPPH (Kumar *et al*., 2008).

iii. **Polyphenolic compounds and propolis samples preparation:** They were prepared as in section **3.3.1.2**.

- **Method**

The free radical scavenging capacity of PE or its polyphenolic compounds (CA, CH and CAPE) were measured with DPPH assay (Kumar *et al*., 2008). The DPPH radical has a deep violet color due to its unpaired electron and radical scavenging capability can be followed spectrophotometrically by absorbance loss at 517 nm when the pale yellow non-radical form is produced. Based on this assay, equal volumes (0.5 ml) of DPPH (60 μM) and each PE (1, 10 or 100 μg ml^{-1}) were mixed in a cuvette and allowed to stand for 30 minutes at room temperature. Then, the absorbance was read at 517 nm in a UV/VIS Lambda 19 spectrophotometer. The absorbance of control (DPPH solution) was also read. The percentage of DPPH decolouration of the sample was calculated according to the formula:

$$\text{Percentage of Decolouration} = \left(\frac{\text{Control Absorbance - Sample Absorbance}}{\text{Control Absorbance}} \right) \times 100$$

3.3.2 *in vitro* Investigations

3.3.2.1 Cell Lines and Cell Cultures

- **Materials and Solutions**

The solutions were prepared according to methods given by Erba *et al.* (2001) and Tavecchio *et al*. (2008a).

i. **Polyphenolic compounds and propolis samples preparation:** They were prepared as in section **3.3.1.2**.
ii. **RPMI-1640 medium (Medium A)**: Ready used solution (Lonza, Belgium), but in the establishment of cultures, it was supplemented with 10% heat-inactivated (water bathed for 30 minutes at 56ºC) foetal bovine serum (FBS) and 1% L-glutamine (Sigma, Germany).
iii. **IMDM medium (Medium B)**: Ready used solution (Lonza, Belgium), but in the establishment of cultures, it was supplemented with 10% FBS and 1% L-glutamine (Sigma, Germany).
iv. **RPMI-1640 medium (Medium C)**: Ready used solution (Lonza, Belgium), but in the establishment of cultures, it was supplemented with 20% FBS (Hyclone, USA) and 1% L-glutamine (Sigma, Germany).
v. **Phosphate buffered saline (PBS)**: It was prepared by dissolving the content (50 grams) of Instamed PBS Dulbecco (Biochrom AG, Germany) in 5 liters of sterilized distilled water.
vi. **Washing and stop buffer:** It was PBS supplemented with 5% FBS.
vii. **Trypsin/Ethylene-diamine-tetra-acetic-acid**: A ready trypsin/EDTA solution (Sigma, Germany) was used to detach the HCT-116 and HuFb cell lines.
viii. **Crystal violet solution:** It was prepared by dissolving 10 grams of crystal violet stain powder and 4 grams of phenol (BDH, England) in 1000 ml of distilled water and kept at 25ºC.

A. Cell lines

Human promyeloid leukemia (ATCC CCL-240; HL-60), Human colon carcinoma (ATCC CCL-247; HCT-116) and Human dermal fibroblast (ATCC PCS-201-010; HuFb) cell lines were purchased from the Resource Bank of Mario Negri Institute for Pharmacology Research (Italy). Cells were recovered from the cell bank by rapid thawing at 37ºC in a water bath, centrifuged at 800

rpm for 10 minutes at room temperature, resuspended in 5 ml culture medium (medium A, B or C) and transferred to a T-25 tissue culture flask, which was incubated at 37ºC for 24 hours, and then, the medium was removed and a new fresh medium was added.

B. Cell culture

The HL-60 cells were suspended in medium A and grown and propagated in T-75 culture flasks in a humidified atmosphere supplemented with 5% CO_2 and 95% air at 37ºC. On the other hand, HCT-116 cells were maintained in medium B, while HuFb cells were maintained in medium C and sub-cultured under the same conditions of HL-60 cells. The grown HCT-116 and HuFb cells were detached from the surface of flasks and collected by trypsin/EDTA solution. The trypsin activity was stopped by adding stop buffer. To maintain the cell lines in an expontial growth, the cultures were sub-cultured every three days. The Cells were rinsed with PBS, counted (Coulter Counter provided with multisizer 3 programme) and seeded at the required density.

C. Cell growth and cytotoxicity assays

For cell growth and cytotoxicity assays, the HCT-116, HuFb and HL-60 cells were seeded in 6, 12 and 24-well flat-bottom culture plates, respectively at a density of 2×10^4, 2×10^4 and 1.5×10^5 cells ml^{-1}, respectively and incubated for 48 hours in humidified atmosphere supplemented with 5% CO_2 and 95% air at 37ºC. The HCT-116 and HL-60 cells were treated with three concentrations (5, 15 or 25 μg ml^{-1}) of PE (B2, D, M, S)or polyphenolic compounds (CA, CH and CAPE) for additional 24, 48 and 72 hours (in triplicates) for B1 sample, the corresponding concentrations were 50, 150 or 250 μg ml^{-1}.With respect to HuFb cells, they were treated with the same concentrations, but they were investigated for one incubation period (72 hours). After each incubation period, the cells were counted and the viability was assessed using the Coulter Counter (Lee *et al*., 2000).

D. Clonogenicity of HCT-116 cells

To investigate the inhibition of colony formation, HCT-116 cells were seeded at a density of 1000 cells ml $^{-1}$ in 6-well flat-bottom culture plates (2 ml per well) and incubated for 48 hours in a humidified atmosphere supplemented with 5% CO_2 and 95% air at 37ºC. Cells were treated with different concentration of PE or its polyphenolic compounds, and incubated for additional 96 hours (in five replicates).

When the control colonies (not treated) were readily visible under the microscope as a confluent monolayer, the medium B (supplemented with 1% Pen-Strep) was removed and the wells were washed with 2 ml of filtered PBS. Then, the colonies were stained with 200 µl per well of crystal violet for 2 minutes. After removing the stain, an extensive washing in tap water for 10 minutes and additional two washes in distilled water for 10 minutes were carried out. Then, the plates were tapped on towel paper and air-dried, and the number of stained colonies was counted by the Entry Level Image System and the results were presented as a percentage of clonogencity (Tavecchio *et al*., 2008b).

3.3.2.2 Determination of Apoptosis by Fluorescence Microscopy

Sulforhadamine101-DAPI method was used to observe nuclear morphologic aspects by fluorescence microscopy using 365 nm filter. The HL-60 and HCT-116 cells were treated with two different concentration systems of PE or polyphenolic compounds (CA, CH and CAPE) for 24 hours.

In the first, it was 5 µg ml^{-1} for B2, D, M, S, CA, CH and CAPE, and 50 µg ml^{-1} for B1, while in the second, it was 15 µg ml^{-1} for CAPE and 25 µg ml^{-1} for B2, D, M, S, CA and CH, and it was 250 µg ml^{-1} for B1. Camptothecin (CPT, 10µM) was used as a positive control.

- **Materials and Solutions**

i. **Polyphenolic compounds and propolis samples preparation:** They were prepared as in section **3.3.1.2**.

ii. **RPMI-1640 medium:** It was ready solution (Lonza, Belgium), but it was supplemented with 10% heat-inactivated FBS, 1% L-glutamine (Sigma, Germany) and 1% Pen-Strep (Bio Source International, Belgium) before use (Erba *et al.*, 2001).

iii. **IMDM medium:** It was ready solution (Lonza, Belgium), but it was supplemented with 10% FBS, 1% L-glutamine (Sigma, Germany) and 1% Pen-Strep (Bio Source International, Belgium) before use (Tavecchio *et al.*, 2008b).

iv. **Camptothecin:** It was prepared as a stock solution at a concentration of 10 mM by dissolving 2.9 mg of camptothecin (CPT; Sigma, Germany) in 830 µl of DMSO (Sigma, USA), stored at -20ºC and freshly diluted in RPMI-1640 medium before treatment (Czuwara-Ladykowska *et al*., 2001).

v. **Fixative I:** It was freshly prepared by diluting 10 ml of formaldehyde free methanol (Polyscience, USA) up to 120 ml with PBS, and the pH was adjusted to 7.4 before reaching the final volume (Bergamaschi *et al*., 1999).

vi. **Fixative II:** It was 96% ethanol (Carlo Erba, Italy).

vii. **Permeabilisation solution:** It was freshly prepared by diluting 0.5 ml of Triton X-100 (Merck, Germany) up to 100 ml of filtered PBS (Tavecchio *et al*., 2008a).

viii. **TRIS-HCl solution:** It was prepared by dissolving 12 grams of TRIS-base (BDH, England) and 6 grams of NaCl (Carlo Erba, Italy) in 100 ml of distilled water. Then, 54 ml of HCl (1N) (Carlo Erba -Italy) were added, and the volume was brought-up to 1000 ml with distilled water.

The pH was adjusted to 8.0 before reaching the final volume (Cancer Pharmacology Laboratory Protocol, Mario Negri Institute, Italy).

ix. **DAPI (4, 6-Diamidine-2-phenylindole dihdrochloride) solution:** It was prepared by dissolving 3.5 mg of DAPI (GmbH, Germany) in 100 ml of distilled water(Cancer Pharmacology Laboratory Protocol, Mario Negri Institute, Italy).

x. **Sulforhodamine101 solution:** It was prepared by dissolving 3 mg of Sulforhodamine101 (Sigma, Germany) in 100 ml of TRIS-HCl solution (pH 8.0) (Cancer Pharmacology Laboratory Protocol, Mario Negri Institute, Italy).

xi. **Sulforhodamine101-DAPI eluent:** It was prepared by mixing equal volumes of Sulforhodamine101 and DAPI solutions (Cancer Pharmacology Laboratory Protocol, Mario Negri Institute, Italy).

xii. **Mounting medium:** Entellan mounting medium (Merck, Germany) was used.

- **HL-60 staining method**

Cells at a density 5 x 10^5 of HL-60 cell suspension were taken after 24 hours treatment with different concentrations of PE or polyphenolic compounds (CA, CH and CAPE). The cell suspension was centrifuged at 800 rpm for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 1 ml of filtered PBS, centrifuged at 800 rpm for 5 minutes, and the supernatant was discarded. Then, the pellet was resuspended in 1 ml of fixative I for 15 minutes, and after that, the cell suspension was centrifuged at 800 rpm for 5 minutes. The pellet was resuspended in 1 ml of filtered PBS, centrifuged at 800 rpm for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 30 µl of filtered PBS, and the cell suspension was dropped on a poly-L-lysine slide and left for air-drying at 25ºC. The slide was rinsed in filtered PBS for 2-3 minutes and transferred to permeabilisation solution for 5 minutes. The slides

washed with filtered PBS and stained with sulforhadamine101-DAPI solution for 30-60 minutes. After staining the slide was rinsed with distilled water, air-dried and covered with a cover-slip. The morphological aspects of nuclei were inspected for nuclear changes under 400X power of a fluorescence microscope (Bergamaschi *et al*., 1999).

- **HCT-116 staining method**

HCT-116 cells were seeded at a density 5 x 10^3 in 6 multi-well plates containing sterile cover-slip on the bottom of each well and allowed to adhere for 48 hours in a humidified atmosphere supplemented with 5% CO_2 and 95% air at 37ºC. Then the cells were treated for 24 hours with different concentrations of PE or polyphenolic compounds (CA, CH and CAPE). After that, the medium was discarded by aspiration and cells were washed with filtered PBS. The PBS was removed and cells were fixed with fixative II for 30-60 minutes. Then, the fixative was removed and distilled water was added for an additional washing and the cells were stained with sulforhadamine101-DAPI solution for 30-60 minutes. After that, the stain was removed and the cover-slips were washed with distilled water, air-dried and applied on a slide. The morphological aspects of nuclei were inspected for nuclear changes under 40X power of a fluorescence microscope (Powolny *et al*., 2001).

3.3.2.3 Analysis of DNA Fragmentation

- **Materials and Solutions**

i. RPMI-1640 medium, IMDM medium and CPT were prepared as in section **3.3.2.2**,Trypsin/EDTA was prepared as in section **3.3.2.1**, while propolis samples were prepared as in section **3.3.1.2**.

ii. **Isolation buffer:** It was prepared by mixing 0.5 ml Tris-HCl (1M), 0.8 ml EDTA (2.5M) (Fluka, Switzerland), 0.5 ml Triton-X 100 (Merck, Germany) and 50 ml of distilled water, and the pH was adjusted to 8.0,

and then the volume was made up to 100 ml with distilled water (Bestwick and Milne, 2006).

iii. **Ribonuclease A -Protease free:** It was prepared by dissolving 10 mg of RNase A (Cat. 556746, Calbiochem, Germany) in 1 ml of sterilized distilled water (Cancer Pharmacology Laboratory Protocol, Mario Negri Institute, Italy).

iv. **Proteinase k:** It was prepared by dissolving 10 mg of proteinase k (Cat. A4392, AppliChem GmbH, Germany) in 1 ml of sterilized distilled water (Cancer Pharmacology Laboratory Protocol, Mario Negri Institute, Italy).

v. **Extraction solution I:** It was phenol that was saturated with Tris buffer (Cat. 100997, Roche, Germany).

vi. **Extraction solution II**: It was prepared by diluting one part of isomyl alcohol (Carlo Erba, Italy) with 24 parts of chloroform (Sigma, Germany) as given by Cancer Pharmacology Laboratory Protocol, Mario Negri Institute, Italy.

vii. **Sodium acetate:** 0.3M sodium acetate (Cat. S8388, Sigma, Germany) was used.

viii. **Nuclease free water:** Nuclease free water ready solution (Promega, USA) was used.

ix. **Orange Loading Dye Solution (6X):** It was ready-used solution (Fermantas, Germany).

x. **Electrophoresis buffer solution (1x TAE):** It was ready-used solution (Fermantas, Germany).

xi. **DNA ladders:** 1Kb and 50bp DNA ladders (Fermantas, Germany) were used.

xii. **Gel preparation:** It was prepared by dissolving 1.5 gram of ultra pure agarose (Sigma, Germany) in 100 ml of distilled water. The solution was heated in a microwave and boiled until all the agarose was dissolved. Then, it was cooled to approximately 50ºC. After cooling, 10 µl of

ethidium bromide (10mg ml^{-1}, Fluka, Switzerland) was added, and the agarose solution was casted in the running plate system. After gelling, the plate was kept at 4ºC before use (approximately two hours) as given by Cancer Pharmacology Laboratory Protocol, Mario Negri Institute, Italy.

- **Method**

Analysis of genomic DNA fragmentation was performed according to Bestwick and Milne (2006) method with slight modifications. The HL-60 and HCT-116 cells were treated with different concentrations of PE and CPT for 24 hours in a humidified atmosphere supplemented with 5% CO_2 and 95% air at 37ºC. Cells were then harvested at a density of 1 x 10^7 and suspended in ice-cold PBS. The cell suspension was centrifuged (1200 rpm) at 4°C for 10 minutes. The supernatant was discarded and the pellet was frozen (-20°C) overnight. The cell pellet was thawed at room temperature and suspended in 0.4 ml of isolation buffer and incubated for 10 minutes in an ice bath. The resultant lysate was centrifuged at 12000 rpm for 20 minutes at 4°C and the supernatant was transferred carefully into Eppendorf tubes. The supernatant was incubated for 1 hour at 37°C after the addition of 4 µl RNase A. After incubation, 4 µl proteinase k was added and a further incubation (2 hours) at 50°C was carried out. The soluble fragmented DNA was extracted with an equal volume of extraction solution I, centrifuged at 12000 rpm for 10 minutes at 4°C and re-extracted with an equal volume of extraction solution II and centrifuged at 12000 rpm for 10 minutes at 4°C. The fragmented DNA was precipitated with 0.3M of sodium acetate and 75% ethanol, and the precipitation was allowed to proceed overnight at -20°C. This step was shortened by putting samples in a bath of ethanol/dry ice for 30 minutes and the solution was centrifuged at 12000 rpm for 30 minutes at 4°C. The pellets were washed by adding to each tube 450 µl of ice-cooled 70% ethanol and centrifuged at 12000 rpm for 5 minutes at 4°C. The ethanol was discarded by aspiration, and any drops or

fluid remained adherent to the wall of the tube was carefully removed with a concentrator. The pellet was suspended in 25 µl of nuclease free water and kept for 48 hours at 4°C. Samples of DNA were mixed with 5 µL loading buffer (6X) containing orange dye. The addition of loading buffer to samples allows to load gel wells more easily and to monitor the run of samples. Approximately 20 µl DNA was loaded onto agarose gel wells and electrophoresis was carried out at 40V for approximately 2 hours in TAE 1x buffer. DNA bands were visualized under UV light with UV transilluminator. Two gel loading markers were loaded in lines parallelled to that of the samples.

3.3.2.4 Annexin V/PI Double-Staining Analysis

- **Materials and Solutions**

  i. RPMI-1640 medium, IMDM medium and CPT were prepared as in section **3.3.2.2**,Trypsin/EDTA and PBS were prepared as in section **3.3.2.1**, while propolis samples were prepared as in section **3.3.1.2**.

  ii. **Annexin V-FITC kit contents (BD Phamingen, USA)**

  a. **Annexin-binding buffer (1x):** It was ready-used solution (Cat. 556454).

  b. **Green fluorescence:** It was ready-used FITC-labeled annexin V (Cat. 556419) in vial contained 1 ml.

  c. **Propidium iodide solution:** It was ready-used solution (Cat. 556463), in which, the concentration of propidium iodide was 50 µg ml^{-1} in vial contained 2 ml.

- **Method**

The annexin V-FITC-labeled apoptosis detection kit was used to detect and quantify apoptosis by flow cytometry according to the manufacturer's instructions. Accordingly, HCT-116 and HL-60 cells were seeded in 6 and 24 multi- well plates, respectively at a density of 2×10^4 and 1×10^5 cells ml^{-1}, respectively, and cultured for 48 hours in a humidified atmosphere

supplemented with 5% CO_2 and 95% air at 37ºC. Then, cells were treated with two concentrations of PE for 6 hours. After that they were harvested in ice-cooled PBS and collected by centrifugation for 10 minutes at 1200 rpm. Cells were then resuspended at a density of 5×10^5 in binding buffer and stained simultaneously with 10 µl of FITC-labeled annexin V and 5µl of propidium iodide (PI) for 15 minutes at room temperature. After that, 600 µl of binding buffer was added and mixed gently. Cells were analyzed with a fluorescence-activated cell sorter flow cytometer (FACS; Figure 3-5).

The fluorescence emission was at 530 nm (Fluorescent 1; FL1) and 580 nm (FL2). The data were analyzed with CellQuest software. Dual staining with Annexin V and a DNA-binding dye such as PI can distinguish four cell populations: live cells (Annexin negative and PI negative), early apoptotic cells (Annexin positive and PI negative), late apoptotic (Annexin positive and PI positive) and dead cells (PI positive). CPT (10 µM) was used as a positive control.

Figure 3-5: Fluorescence-activated cell sorter flow cytometer (FACS, Becton Dickinson).

3.3.2.5 Gene Expression of *Bcl-2* in HL-60 Cells

- **Materials and Solutions**

i. RPMI-1640 medium and CPT were prepared as in section **3.3.2.2,** PBS and washing buffer solutions were prepared as in section **3.3.2.1**, while propolis sample (Only M sample was tested at a concentration 25µg ml^{-1}) was prepared as in section **3.3.1.2**.

ii. **GM saline:** It was prepared by dissolving 1.1 gram glucose (Sigma, Germany), 8 grams NaCl (Carlo Erba, Italy), 0.4 gram KCl, 0.2 gram $Na_2HPO_4.2H_2O$, 0.15 gram KH_2PO_4 and 0.2 gram EDTA (Fluka-Switzerland) in 500 ml of sterilized distilled water, and then the volume was made-up to 1000 ml (Erba and Sen, 1996).

iii. **Fixative I:** It was 1% formaldehyde-methanol free solution (Polyscience, USA) (Tavecchio *et al.*, 2008a).

iv. **Fixative II**: It was prepared by mixing one part of GM saline with 3 parts of iced-cooled 96% ethanol (Carlo Erba, Italy) (Tavecchio *et al.*, 2008a).

v. **Permeabilisation solution:** It was prepared by dissolving 1 gram of bovine serum albumin (BSA) in 20 ml of filtered PBS, and then 0.5 ml of Tween 20 (Sigma, Germany) was added. The final volume was made up to 100 ml with filtered PBS (Erba *et al.*, 2001).

vi. **Mouse Anti-human Bcl-2 Kit Contents (BD Phamingen, USA**)

   a. **FITC mouse anti human Bcl-2 antibody:** It was prepared by diluting 20 µl of FITC mouse anti human Bcl-2 antibody (Clone Bcl-2/100, Cat. 556357) with 50 µl of permeabilisation solution.

   b. **FITC labeled mouse IgG1 isotype:** It was prepared by diluting 20 µl of FITC labeled mouse IgG1 isotype (Clone MOPC-21, Cat. 556357) with 50 µl of permeabilisation solution.

- **Method**

Detection of *Bcl-2* gene expression in HL-60 cells by flow cytometry was performed using FITC conjugated mouse anti-human Bcl-2 kit. The staining was

performed according to the manual producer. The control and treated HL-60 cells were harvested at a density of 1×10^6 after 3, 6, 10 or 24 hours of treatment. After treatment, the cells were centrifuged (1200 rpm) at 4°C for 10 minutes. Supernatant was discarded and the pellets were fixed with 1 ml of ice-cooled fixative I for 30 minutes. The fixed cells were resuspended in fixative II drop-wise with a continuous vortexing and incubated at 4°C overnight. Then, the cell pellets were collected by centrifugation at 1200 rpm for 10 minutes. The fixed cells were suspended in washing buffer and collected by centrifugation at 1200 rpm for 10 minutes, and permeabilised with permeabilisation solution for 15 minutes at room temperature. Then cells were incubated in 100 µl of FITC conjugated mouse anti-human Bcl-2 antibody for 60 minutes in the dark at room temperature. The cells were washed with washing buffer, centrifuged at 1200 rpm at 4°C for 10 minutes and resuspended in 1 ml of PBS, and then they were analyzed with FACS instrument. The fluorescence emission was at 530 nm (FL1), and data were analyzed with CellQuest software. Non-specific labeling was prepared by incubation of non-treated cells with 100 µl of FITC labeled mouse IgG1 isotype according to the manufacturer instructions. The CPT (10 µM) was used as a positive control.

3.3.2.6 Gene Expression of *Bax* in HL-60 Cells

- **Materials and Solutions**

  i. RPMI-1640 medium and CPT were prepared as in section **3.3.2.2**, propolis sample (Only M sample was tested at a concentration 25µg ml^{-1}) was prepared as in section **3.3.1.2**, PBS and washing buffer solutions were prepared as in section **3.3.2.1**, while GM saline and fixative II were prepared as in section **3.3.2.5**.

  ii. NaOH (1N): Forty grams of NaOH (BDH, England) were dissolved in 500 ml distilled water and the volume was made up to 1000 ml.

iii. **Paraformaldehyde solution (4%):** It was prepared by dissolving 4 grams of paraformaldehyde (Polyscience, USA) in 80 ml distilled water by stirring at 70ºC in fume hood, and then few drops of 1N NaOH were added until dissolving the paraformaldehyde. The solution was cooled to room temperature and the pH was adjusted to 7.5. Ten ml of PBS was then added and the volume was made up to 100 ml with distilled water, filtered, divided into aliquots and stored at -20ºC (Save *et al.*, 2004).

iv. **Fluorescein FITC-conjugated goat anti-mouse IgG:** It was prepared by diluting one part of Alexa flour 488 $F(ab')_2$ fragment of goat anti-mouse IgG (Cat.A-11017, Molecular Probe, USA) with 99 parts of washing buffer.

v. **Anti- human Bax Kit Contents (MBL, Japan**)

   a. **Clear Back:** It was ready-used human Fc receptor blocking reagent (Cat. MTG-001).

   b. **Anti-human Bax monoclonal antibody:** It was prepared by diluting one part of anti-human Bax monoclonal antibody (Clone 4F11, Cat. M010-3) with 99 parts of washing buffer.

- **Method**

Detection of *Bax* gene expression in HL-60 cells by flow cytometry was performed using the anti-human Bax kit. The staining was performed according to the manufacturer producer. The control and treated HL-60 cells were harvested at density 5×10^5 after 3, 6, 10 or 24 hours of treatment. The cells were centrifuged (1200 rpm) at 4°C for 10 minutes. Supernatant was discarded and the pellet was fixed with 1 ml of ice-cooled paraformaldehyde 4% for 30 minutes. The fixed cells were resuspended in 2 ml of fixative II drop-wise with a continuous vortexing and incubated at -20°C for 30 minutes. The cell pellets were collected by centrifugation at 1200 rpm for 5 minutes. The collected cells were washed with 1 ml washing buffer and collected by centrifugation at 1200

rpm for 5 minutes and incubated with 10 µl of human Fc receptor blocking reagent for 5 minutes at room temperature. Then, the cells were incubated with 40 µl of anti-human Bax antibody for 30 minutes in the dark at room temperature. The cells were washed with 1 ml washing buffer, centrifuged (1200 rpm) at 4°C for 5 minutes. The supernatant was removed by careful aspiration, and the cells were incubated with 30 µl of fluorescein FITC-conjugated Alexa flour 488 $F(ab')_2$ fragment goat anti-mouse IgG for 15 minutes in the dark at room temperature. Cells were resuspended in 1 ml of PBS and analyzed with FACS instrument. The fluorescence emission was at 530 nm (FL1), and data were analyzed with CellQuest software. Non-specific labeling was prepared by incubation of untreated cells with 40 µl of washing buffer according to the manufacturer instructions. The CPT (10 µM) was used as a positive control.

3.3.2.7 Gene Expression of *p53* in HCT-116 Cells

- **Materials and Solutions**

i. IMDM medium and CPT were prepared as in section **3.3.2.2**., propolis sample (Only M sample was tested at a concentration 25µg ml^{-1}) was prepared as in section **3.3.1.2**, Trypsin/EDTA, PBS, washing and stop buffer solutions were prepared as in section **3.3.2.1**, while fixative II and permeabilisation solutions were prepared as in section **3.3.2.5**.

ii. **Anti-p53 monoclonal antibody:** It was prepared by diluting one part of anti-p53 monoclonal antibody (Clone DO-1, Cat. SC126, Santa Cruz Biotech., USA) with 9 parts of permeabilisation solution.

iii. **Fluorescein FITC-conjugated goat anti-mouse IgG:** It was prepared by diluting one part of Alexa flour 488 $F(ab')_2$ fragment of goat anti-mouse IgG with 799 parts of washing buffer.

iv. **Ribonuclease A-Protease free:** It was prepared by dissolving 1 mg of RNase A (Cat. 556746, Calbiochem, Germany) in 1 ml of sterilized distilled water and kept at -20 ^{o}C.

v. **TO-PRO-3 iodide reagent**: TO-PRO-3 iodide (Cat. T3605, Ivetrogen, USA) was diluted to 0.75 μM m^{-1} in PBS from a stock solution (1mM) in DMSO.

• **Method**

The control and treated HCT-116 cells were harvested by trypsin-EDTA at a density 1-2 × 10^6 after 3, 6, 10, 24, 48 or 72 hours of treatment. The cells were fixed in 1 ml of fixative II drop-wise with a continuous vortexing and incubated in ice bath for 30 minutes, and further incubation at 4°C was carried out for overnight. After that, the cell pellet was collected by centrifugation at 1200 rpm for 10 minutes. The fixed cells were suspended in 1 ml washing buffer and collected by centrifugation at 1200 rpm for 10 minutes and permeabilised with permeablisation solution for 15 minutes at room temperature. Then, cells were washed with 1ml washing buffer and collected by centrifugation at 1200 rpm for 10 minutes and incubated with 100-200 μl of anti-p53 (DO-1) monoclonal antibody for 60 minutes in the dark at room temperature. After removing the free anti-p53 by washing the cells with permeabilisation solution for 15 minutes at room temperature, the cells were incubated with 200 μl of FITC-conjugated Alexa flour 488 $F(ab')_2$ fragment goat anti-mouse IgG for 60 minutes in the dark at room temperature. Finally, the cells were resuspended in 1-2 ml of a solution containing 0.75 μM of TO-PRO-3 iodide in PBS and 12.5 μl RNAse and stained overnight at 4°C in the dark. Cells were analyzed with FACS instrument, and the fluorescence emission was at 530 nm (FL1) and 665 nm (FL4), and data were analyzed with CellQuest software. A blank sample was prepared by incubation of untreated cells with 100 μl of permeabilisation solution instead of anti-p53 (Bonsing *et al.*, 1997). The CPT (10 μM) was used as a positive control.

3.3.2.8 Cell Cycle Distribution Assay

- **Materials and Solutions**

  i. RPMI-1640 medium and IMDM medium were prepared as in section **3.3.2.2**, propolis sample (Only M sample was tested at a concentrations 5, 15 or 25µg ml^{-1}) was prepared as in section **3.3.1.2**, Trypsin/EDTA, PBS, washing and stop buffer were prepared as in section **3.3.2.1**, Fixative II was prepared as in section **3.3.2.5**, while Ribonuclease A-Protease free was prepared as in section **3.3.2.7**.

  ii. **Propidium iodide solution:** It was prepared by dissolving 25 mg of PI (Calbiochem, Germany) in 1000 ml of PBS and kept at 4°C (Erba *et al.*, 2001).

- **Method**

Analysis of the cell cycle was performed according to Darzynkiewicz and Huang (2004) method with slight modifications. Accordingly, HL-60 and HCT-116 cells were seeded in 24 and 6 multi-well plates, respectively at a density 1×10^5 and 2×10^4 cells ml^{-1}, respectively, and cultured for 48 hours. The Cells were treated with different concentration of M sample for 24, 48 or 72 hours. Cells were harvested at a density 2×10^6 and transferred to polypropylene conical tube and centrifuged at 1200 rpm for 10 minutes and the supernatant was discarded. The pellet was fixed with fixative II drop-wise with a continuous vortexing and incubated in ice bath for 30 minutes. The tube was kept overnight in 4°C, and after that, the cell pellet was collected by centrifugation at 1200 rpm for 10 minutes and resuspended in 1 ml of washing buffer. A further centrifugation at 1200 rpm for 10 minutes was carried out to collect the cell pellet, and then 2 ml of PI solution and 25µl RNase A were added, and the mixture was allowed to stand overnight in the dark at 4°C. Fluorescence emitted from the PI-DNA complex was quantified after excitation of the fluorescent dye by FACS. At least 10000 cells were examined for each sample, and fluorescence

emission was at 610 nm (FL3). All measurements were done under the same instrument settings. The distribution of DNA content was expressed as G1, S, and G2/M phases. Cells with DNA content less than G1 were distributed as pre-G1 (hypodiploid cells) and expressed as the apoptotic phase. The data were analyzed with CellQuest software.

3.3.2.9 BrdUrd-DNA Analysis of HL-60 and HCT-116

- **Materials and Solutions**

i. RPMI-1640 medium and IMDM medium were prepared as in section **3.3.2.2**, propolis sample (Only M sample was tested at a concentration 25µg ml^{-1}) was prepared as in section **3.3.1.2**, Trypsin/EDTA, PBS, washing and stop buffer were prepared as in section **3.3.2.1**, Fixative II and permeabilisation solutions were prepared as in section **3.3.2.5**, while Ribonuclease A-Protease free and TO-PRO-3 iodide reagent were prepared as in section **3.3.2.7**.

ii. **5-bromo-2-deoxyuridine (BrdUrd):** 20 µM solution of BrdUrd (BD Biosciences, USA) was used.

iii. **2N HCl:** It was prepared by diluting 20.13 ml of concentrated HCl (36.23%, Carlo Erba, Italy) up to 100 ml with deionized distilled water.

iv. **3N HCl:** It was prepared by diluting 30.18 ml of concentrated HCl (36.23%, Carlo Erba , Italy) up to 100 ml with deionized distilled water.

v. **Sodium tetrabrate solution:** It was prepared by dissolving 38.137 grams of sodium tetraborate (BDH, England) in 100 ml of filtered PBS. The volume was made up to 1000 ml with filtered PBS after adjusting the pH to 8.5 (Erba *et al*., 2001).

vi. **Anti-BrdUrd monoclonal antibody:** It was prepared by diluting one part of mouse anti-BrdUrd monoclonal antibody (Clone B44, Cat.347580, BD Bioscience, USA) with nine parts of permeabilisation solution.

vii. **Fluorescein FITC-conjugated goat anti-mouse IgG:** It was prepared by diluting one part of Alexa flour 488 $F(ab')_2$ fragment of goat anti-mouse IgG with 499 parts of permeabilisation solution.

- **Method**

For detection of 5-bromo-2-deoxyuridine (BrdUrd) incorporation into DNA during exponential phase of growing cells with the presence 25 μg ml^{-1} of propolis M sample, the HL-60 and HCT-116 cells were seeded in T-25 culture flasks at a density 1.5×10^5 and 2×10^4, respectively, and they were allowed to grow for 48 hours, and then 20 μM BrdUrd (6 μl ml^{-1}) was added to all flasks. After 15 minutes, the medium was removed, the cells were washed twice with PBS and a fresh medium was provided. Then, cells were treated for 2, 4, 6, 8, 10, 24, 36 or 48 hours, and after each period of treatment, the cells were harvested (trypsin-EDTA was used for HCT-116). After that, 2×10^6 cells were transferred to polypropylene conical tube and centrifuged at 1200 rpm for 10 minutes and the supernatant was discarded. The pellets were fixed with fixative II drop-wise with a continuous vortexing and incubated in ice bath for 30 minutes. The tube was kept overnight in 4°C, and then the cell pellet was collected by centrifugation at 1200 rpm for 10 minutes, resuspended in 1 ml of washing buffer and a further centrifugation at 1200 rpm for 10 minutes was carried out to collect the cell pellet. The DNA was denaturated with 1 ml of 2N and 3N HCl, respectively for 30 minutes at room temperature to allow the anti-BrdUrd monoclonal antiboday (MAb) to react with the BrdUrd incorporated in the DNA chain. DNA denaturation was stopped by adding 4 ml of 0.1 M sodium tetraborate, and after centrifugation, the pellet was incubated with 1ml of permeabilisation solution for 15 minutes at room temperature. After centrifugation the incorporated BrdUrd was visualized by incubating the cells with 150 μl of monoclonal antibody anti-BrdUrd for 60 minutes in the dark at room temperature. After removing free anti-BrdUrd antibody by washing the

pellet with 1 ml of ice-cooled washing buffer, the pellet was collected by centrifugation at 1200 rpm for 10 minutes. After centrifugation the pellet was incubated with 1ml permeabilisation solution for 15 minutes at room temperature. Then, the cells were incubated with 150 µl of FITC-conjugated Alexa flour 488 $F(ab')_2$ fragment goat anti-mouse IgG for 60 minutes in the dark at room temperature. Finally, the cells were resuspended in 2 ml of a solution containing 0.75 µM of TO-PRO-3 iodide in PBS and 12.5 µl RNAse and incubated for 20 minutes in the dark. Cells were analyzed with a FACS instrument. The fluorescence emission was at 530 nm (FL1) and 665 nm (FL4) for green and red fluorescence, respectively, and the data were analyzed with CellQuest software. A blank sample was prepared by incubation of untreated cells with 150 µl of permeabilisation solution (Erba *et al*., 2001).

3.3.2.10 γ-H2AX Histone

- **Materials and Solutions**

i. RPMI-1640 medium and IMDM medium were prepared as in section **3.3.2.2**, propolis sample (Only M sample was tested at a concentration 25 µg ml^{-1}) was prepared as in section **3.3.1.2**, Trypsin/EDTA, PBS, washing and stop buffer were prepared as in section **3.3.2.1**, Fixative I and fixative II were prepared as in section **3.3.2.5**, while Ribonuclease A-Protease free, fluorescein FITC-conjugated goat anti-mouse IgG and TO-PRO-3 iodide reagent were prepared as in section **3.3.2.7**.

ii. **Permeabilisation solution:** It was prepared by dissolving 1 gram of BSA in 20 ml of filtered PBS, and 0.2 ml of Ttiton X-100 was then added to the solution. The volume made up to 100 ml with filtered PBS (Darzynkiewicz and Huang, 2004).

iii. **Anti-H2AX (SER 139)monoclonal antibody:** It was prepared by diluting 1 µl of (1 mg ml^{-1}) anti-H2AX (SER 139) monoclonal antibody (Clone JBW301, Cat.05-636, Millipore, France) in 0.625 µl of

permeabilisation solution and then one part of this stock solution was diluted up to 32 parts with the same permeabilisation solution to prepare 0.05 µg ml $^{-1}$ of anti-H2AX antibody.

- **Method**

Analysis of the H2AX histone phosphorylation (γ-H2AX) was performed according to Tanaka *et al*. (2009) method with slight modifications. Accordingly, HL-60 and HCT-116 cells treated with 25 µg ml^{-1} of M sample were harvested at a density 2×10^6 and suspended in ice-cooled PBS. The cells were centrifuged (1200 rpm) at 4°C for 10 minutes. Supernatant was discarded and the pellet was fixed with 1 ml of ice-cooled 1% paraformaldehyde for 30 minutes. The fixed cells were suspended in fixative II drop-wise with a continuous vortexing and incubated at 4°C. After being left to stand for overnight, the cell pellet was collected by centrifugation at 1200 rpm for 10 minutes. The fixed cells were suspended in 1 ml ice-cooled 5% FBS-PBS and collected by centrifugation at 1200 rpm for 10 minutes and permeabilised with permeabilisation solution for 15 minutes at room temperature. Then, cells were washed with washing buffer and collected by centrifugation at 1200 rpm for 10 minutes and incubated with 200 µl of anti-H2AX (SER 139) monoclonal antibody overnight in the dark at 4°C. After removing the free anti-H2AX antibody by washing the cells with 1 ml of washing buffer, the cells were incubated with 150 µl of FITC-conjugated Alexa flour 488 $F(ab')_2$ fragment goat anti-mouse IgG for 60 minutes in the dark at room temperature. Finally, the cells were resuspended in 1 ml of a solution containing 0.75 µM of TO-PRO-3 iodide in PBS and 12.5 µl RNAse and incubated for 30 minutes in the dark at room temperature. Cells were analyzed with FACS instrument, and the fluorescence emission was at 530 nm (FL1) and 665 nm (FL4) and data were analyzed with CellQuest software. A blank sample was prepared by incubation of untreated cells with 200 µl of permeabilisation solution.

3.3.3 *in vivo* Investigations

3.3.3.1 Laboratory Animals

Fox N1-nu/nu nude female mice were used in the transplantation of HCT-116 tumour cells. They are genetically lack thymus and are unable to produce T-lymphocytes; therefore, the animals are immunodeficient. The animals were purchased from Harlan Italy Company, Bresso, Italy, and their age at the beginning of experiments was 6 weeks, and their weight range was 18-22 grams. They were maintained in a controlled animal house at 25°C, 60-70% humidity, 12-hour of artificial lighting (8:00 to 20:00) and under specific pathogen free (SPF) environment in cages with a filter paper cover (Figure 3-6) and supplied with sterilized food and water *ad libitum*.

Figure 3-6: Ventilated mouse cage provided with air inlet and exhaust system with automatic shut-off devices.

Procedures involving animals and their care were conducted in a conformity with the institutional guidelines that are in the compliance with the national (D.L. n. 116, G.U., Suppl. 40, 18 Febbraio, 1992; Circolare No. 8, G.U.,

14 Luglio, 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). Before carrying out experiments, the animals were kept for at least 5 days prior to dosing in the animal room at the animal house of Mario Negri Institute, to allow for their acclimatization for the laboratory conditions (5 mice in each cage).

3.3.3.2 Tumour Transplantation

- **Materials**

  i. IMDM medium was prepared as in section **3.3.2.2**, while trypsin/EDTA, PBS, washing and stop buffer were prepared as in section **3.3.2.1.**

  ii. Saline (NaCl**):** It was ready-used solution **(**SALF, Italy), and the concentration was 0.9% (w/v).

- **Method**

HCT-116 cells were seeded at a density of 2×10^4 cells ml^{-1} in T-150 culture flasks and incubated for 7 days in a humidified atmosphere supplemented with 5% CO_2 and 95% air at 37ºC. Four days later, the medium was removed and the cells were supplemented with a new medium. After the incubation period, the cells were harvested by trypsinization (i.e. 5 ml of trypsin/EDTA were added to each flask, and after two minutes 15 ml stop medium was added). The collected cells were centrifuged (800 rpm) for 10 minutes, and the cell pellet was then suspended in saline, counted and their density was adjusted to 25×10^6 cells ml^{-1}. To implement the tumour cells, each mouse was injected subcutaneously with 0.2 ml of cell suspension by micro-syringe into the right flank (Figure 3-7). When the tumour was palpable (approximately 90-100 mg weight, which attained roughly 7 days after transplantation), the animals were weighed, identified through ear rings and

randomly divided into six groups of ten mice each (total number of mice was 60).

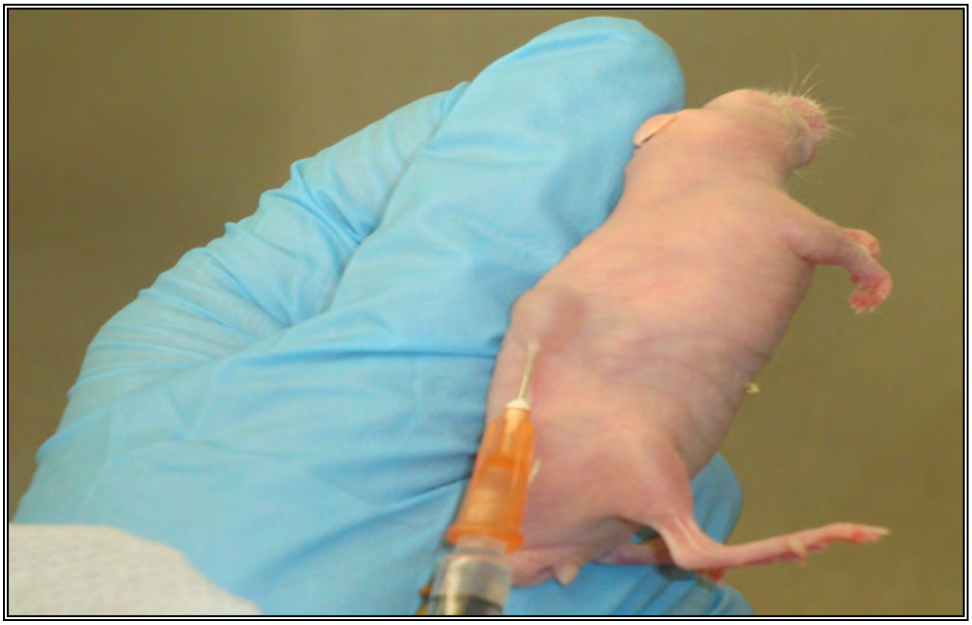


Figure 3-7: Nude female mouse injected subcutaneously with 0.2 ml of HCT-116 cell suspension in the right flank.

3.3.3.3 Dosage and Administration of Propolis M Sample

- **Materials and Solutions**

  i. **Saline:** 0.9% saline was used.

  ii. **Diethylene glycol monoethyl ether (Transcutol CG 20%):** it was prepared by diluting 20 ml of Transcutol CG (Indena, Italy) with 80 ml of 0.9% saline.

  iii. **Propolis sample preparation:** Only M sample was used, and the dose was either 500 or 1000 mg kg^{-1}. The propolis suspension was prepared immediately before use by dissolving it in 20% of transcutol CG and then was diluted with saline to prepare the required concentrations.

- **Method**

After the establishment of transplanted tumour in mice (section 3.3.3.2), they were treated with propolis M sample. Two propolis doses were used; 500 or

1000 mg kg^{-1}, which were administered daily (single dose per day; 0.20-0.25 ml) for three weeks, and the route was either intraperitoneum (i.p.) or oral by gavage (p.o.). Therefore, four groups of animals were investigated each with 10 mice. Two further groups (intraperitoneal and oral controls) were included, and the transplanted mice were treated with the solvent of propolis sample (20% of transcutol CG in saline).

3.3.3.4 Assessment of Tumour Growth and Inhibition

The tumour volume was assessed every three days, and for up to 21 days (i.p. treatment) or 28 days (p.o. treatment). The volume was estimated by a caliper measurement of two dimensions (Figure 3-8) using the following equation:

$$\text{Tumour volume } (mm^3) = (a \times b^2) \div 2$$

Where a and b are the tumour length and width (mm), respectively (Orsolic *et al*., 2006). The effect on tumour growth was expressed as the tumour-growth inhibition rate (TIR; %), which was assessed according to the following equation:

$$\text{Tumour inhibition rate } (\%) = [(C\text{-}T) \div C] \times 100$$

Where T is the tumour volume of propolis-treated group and C is the tumour volume of the corresponding control group (Orsolic *et al*., 2006).

To assess the propolis toxic effects, the mice were weighed at intervals that were applied to the tumour growth assessments. After 28 (i.p. groups) or 35(o.p. groups) days, the mice were sacrificed, and the tumours were excised, weighed and the tumour index (%) was determined using the following equation:

$$\text{Tumour index } (\%) = (TW \div MW) \times 100$$

Where TW is the tumour weight and MW is the mouse weight (Orsolic *et al*., 2006).

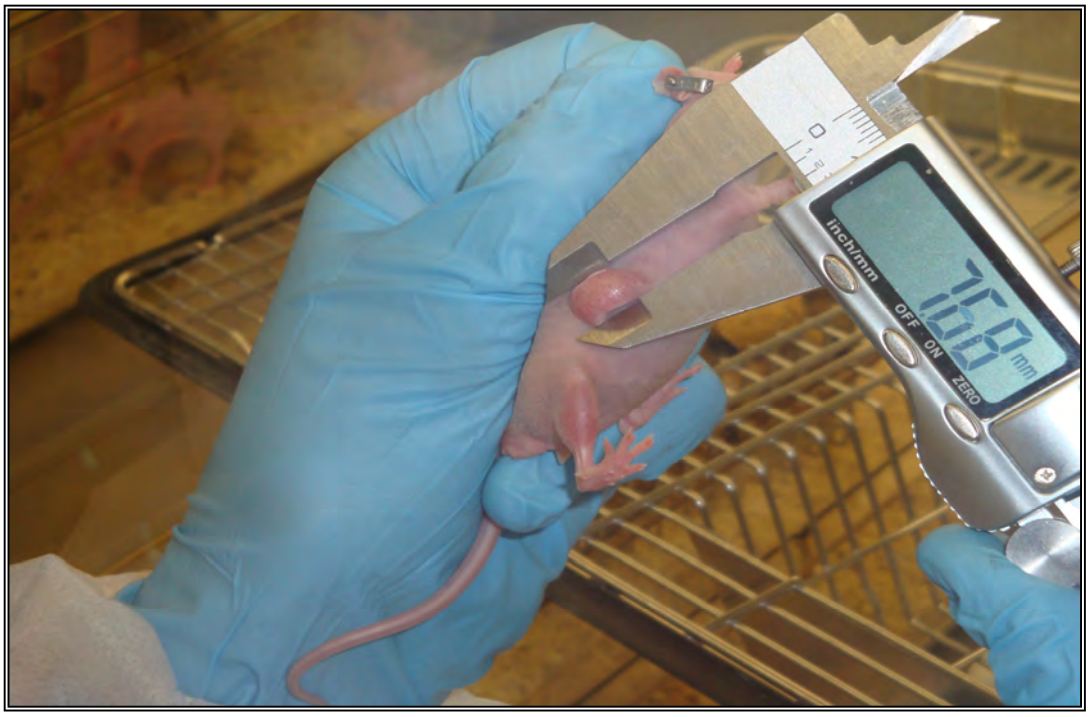


Figure 3-8: Measurement of tumour length and width by a caliber.

3.3.3.5 Histopathology of Tumours

- **Materials and Solutions**

  i. **Buffered formalin:** 10% buffered formalin was purchased from BD Phamingen, USA.

  ii. **Paraffin wax:** Paraffin wax with a melting point ranged 56-58ºC (Merck, Germany) was used.

  iii. **Ethanol:** 70, 80, 95 and 100% concentrations of ethanol were used (Carlo Erba, Italy).

  iv. **Xylene:** It was purchased from Carlo Erba, Italy**.**

  v. **Haematoxylin:** Mayer's haematoxylin solution was purchased from Fluka, Switzerland.

  vi. **Eosin:** It was prepared by dissolving 5 grams of eosin Y (Merck, Germany) in 100 ml of distilled water.

- **Method**

At the end of treatment period (section 3.3.3.3), tumours of oral administration groups were excised, fixed in buffered formalin (10%) for 24 hours, embedded in paraffin, and 5-µm sections were prepared by a microtome. Subsequently, sections were placed on microscopic polysine slides and left overnight at room temperature. After that, processing and staining of sections were performed according to a standard procedure used in histopathological laboratories (Hood *et al.*, 2005).

Briefly, the sections were de-paraffinized twice (15 and 5 minutes) with xylene. Then, the sections were transferred to gradual descending concentrations of ethanol (100, 95, 80 and 70%) for 5 minutes in each concentration, and finally in distilled water for 5 minutes. After hydration, the sections were stained with haematoxylin (H) for 15 minutes, washed with running tap water for 5 minutes, followed by counterstaining with eosin (E) for 3 minutes. Then, the slides were washed with distilled water for 5 seconds. Subsequently, sections were dehydrated with gradual ascending concentrations of ethanol (70, 80, 95 and 100%) for 30 seconds in each concentration, and then they were transferred to a further 100% ethanol for 5 minutes. After dehydration of sections, they were cleared with xylene for 15 minutes, and the slides were mounted with Entellan mounting medium using Leica CV5020 mounting system. The stained slides were air-dried at room temperature in a fume hood and examined under a light microscope to assess the histological features of tumour sections.

3.3.3.6 Immunohistochemical Evaluations of Tumours

- **Materials and solutions**

i. Ethanol, xylene and haematoxylin were prepared as in section **3.3.3.5**.
ii. **Liquid Blocker:** Super pap pen (Daido Sangyo, Japan) was used.

iii. **Anti-p53 monoclonal antibody:** It was prepared by diluting one part of anti-p53 monoclonal antibody (Clone DO-7, Cat.M7001, Dako Cytomation, USA) with forty nine parts of antibody diluent.

iv. **Anti-Ki67 monoclonal antibody:** It was prepared by diluting one part of anti-Ki67 monoclonal antibody (Clone MIB-1, Cat.M7240, Dako Cytomation, USA) with twenty four parts of antibody diluent.

v. **Staining Kit contents (EnVision™ FLEX, Dako, Denmark)**

   a. **Target retrieval solution**: It was ready-used citrate buffer with high pH (pH = 9 for p53) and low pH (pH = 6 for Ki67) and diluted 1:50 with distilled water for each.

   b. **Washing buffer:** It was ready-used solution that contained 1% Tween 20 in PBS and diluted 1:20 with distilled water.

   c. **Peroxidase-blocking reagent:** It was ready-used solution.

   d. **Antibody diluent:** It was ready-used Tris buffer, pH 7.2, containing 15 mmol/L of NaN_3, and protein.

   e. **Substrate buffer:** It was ready-used buffered solution containing hydrogen peroxide and preservative.

   f. **Horse radish peroxidase (HRP):** It is dextran coupled with peroxidase conjugated to goat anti-mouse immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent.

   g. **DAB-Chromogen:** It is 3, 3'-diaminobenzidine tetrahydrochloride in organic solvent. The color of this reagent may vary from strong violet to colorless without having any influence on the performance of the kit and used by adding one drop to 1ml substrate buffer.

- **Method**

For immunohistochemical (IHC) evaluations of tumours, the sections were deparaffinized and rehydrated in graded alcohols in similar conditions that were applied for H and E histological method. These sections were subjected to IHC

evaluations using two monoclonal antibodies, which were anti-Ki67 and anti-p53. The instructions of manufacturer were followed with slight modifications.

For antigen retrieval, the sections were incubated in target retrieval solution of pH 6.0 (for Ki-67) or pH 9.0 (for p53) for 1 hour in a water bath at 97°C .The slides were cooled for 20 minutes at room temperature, and then the edges surrounding the sections were marked by a liquid blocker pap pen to avoid the distribution of the materials out of the sections during the run of the IHC staining. Then, the slides were transferred quickly to the auto-stainer racks to avoid drying of samples. The auto-stainer was programmed depending on the number of the slides during the run (maximum 48 slides) and the number of the drops that the machine will drop it on the slide depending on the section area (each drop approximately 100 µl). After staining, the slides were washed with washing buffer for 1 minute and then incubated with peroxidase-blocking reagent for 10 minutes. This reagent contains H_2O_2, which is the substrate for peroxidase, because some cells or tissues contain endogenous peroxidase. This is reasoned by the fact that using HRP conjugated antibody may result in a high, non-specific background staining, and such non-specific background can be significantly reduced by pre-treatment of cells or tissues with H_2O_2 prior to incubation with HRP conjugated antibody.

The slides were washed vigorously with washing buffer, protein blocking reagent (antibody diluent) was added for 30 minutes to block the excess site of another proteins, washed with washing buffer, then the determined amounts (100 µl) of anti-ki67 or anti-p53 antibodies were added to the slides and incubated for 60 minutes. Then, the slides were washed with washing buffer, and after that, the HRP (100 µl) was added and the slides were incubated for 40 minutes and washed vigorously. After washing, the DAB-Chromogen solution (100 µl) was added and the slides were incubated for 10 minutes (the addition and incubation time of DAB is very important and it was previously optimized to avoid the background formation in the stained slides that will interfere with

the results) and washed vigorously two times with washing buffer. The H stain solution was added as a counter stain for 4 minutes, and the slides were washed with distilled water, transferred to dehydration jars, mounted, and finally examined under a light microscope and photographed in a similar manner as in H and E histological method.

3.4 Statistical Methods

Data were presented as mean ± standard error (S.E.) . To get such data, the individual values were tabulated in a sheet of the statistical programme GraphPad Prism version 5.01 (GraphPad software, Inc., La Jolla, CA, USA). The difference between means was assessed by Duncan's test, in which $P \leq 0.05$ was considered significant.

Chapter Four
Results

Chapter Four
Results

4.1 Chemical Analysis and Characterization of Propolis

4.1.1 Qualitative and Quantitative Analysis by HPLC-ESI/MS

The chemical composition of five propolis samples, which were collected from five different geographical regions in Iraq (Baghdad1; B1, B2, Dahuk; D, Mosul; M and Salah ad-Din; S), was identified by HPLC-ESI/MS analysis. Table 4-1 shows the results of such analysis, in which 38 different compounds were characterised. Thirty three (86.8%) of these compounds were polyphenols and four (10.5%) were clerodane diterpenoids, while one compound was considered unknown. The total peak area (TPA) of such analysis showed different distributions in the five samples of propolis. The highest TPA was observed in D sample (4.166×10^6), followed by samples from M (3.778×10^6), B2 (3.203×10^6), S (2.566×10^6) and finally B1 (0.192×10^6). Accordingly, the total ion current (TIC) showed different percentage for each compound that was identified, but the prenyl caffeate (polyphenol compound) showed the highest TIC in the five samples investigated. However, each sample showed different TIC of prenyl caffeate. The highest TIC was observed in B2 sample (22.41%), followed by S (20.65%), M (18.52%), B1 (18.47%) and finally D (10.35%). As these values represented the percentage of the total TPA for each sample, which were different in each sample investigated, therefore the TIC of prenyl caffeate or other identified compounds was not correlated positively with its corresponding concentration for each sample.

The compounds labeled as clerodane terpenoids were found in three propolis samples (D, M and S), and such compounds were identified for the first time in samples collected from temperate zones (Iraq), while in propolis sample coded as B2, the TIC was very low (0.001%), and in B1 sample, it was zero (Table 4-1).

Table 4-1: Chemical compounds identified by HPLC-ESI /MS in propolis samples extracts that were collected from five geographical regions in Iraq.

Compound	RT min	Molecular Formula	Molecular Weight	$[M-H]^-$	Total Ion Current (%)				
					B1	B2	D	M	S
Caffeic acid	4.17	$C_9H_8O_4$	180.0412	179.0339	14.29	0.85	1.02	1.67	0.69
Coumaric acid	6.20	$C_9H_8O_3$	164.0463	163.0390	6.37	0.45	0.54	0.84	0.42
Vanillin	6.85	$C_8H_8O_3$	152.0463	151.0390	0.00	0.01	0.02	0.04	0.02
Methyl caffeate	6.90	$C_{10}H_{10}O_4$	194.0568	193.0495	1.44	0.20	1.27	0.69	0.51
Ferulic acid	7.45	$C_{10}H_{10}O_4$	194.0568	193.0495	9.01	1.09	0.55	1.73	1.05
Ferulic acid methyl ester	11.70	$C_{11}H_{12}O_4$	208.0730	207.0652	10.31	1.50	2.13	1.59	1.51
Luteolin	13.16	$C_{15}H_{10}O_6$	286.0467	285.0394	0.00	0.32	0.34	0.46	0.34
Quercetin	13.70	$C_{15}H_{10}O_7$	302.0416	301.0343	0.00	0.18	0.43	0.50	0.38
Sakuranetin	14.05	$C_{16}H_{14}O_5$	286.0830	285.0757	3.27	3.46	8.45	4.67	5.26
Methyl quercetin	14.60	$C_{16}H_{12}O_7$	316.0578	315.0499	0.16	1.11	3.00	2.25	2.00
Cinnamic acid	14.80	$C_9H_8O_2$	148.0514	147.0441	0.95	0.08	0.14	0.15	0.11
Tectochrysin	15.80	$C_{16}H_{12}O_4$	268.0725	267.0652	0.04	0.80	0.88	0.47	0.49
Apigenin	16.10	$C_{15}H_{10}O_5$	270.0513	269.0440	0.29	2.04	1.12	2.21	1.97
Naringenin	16.28	$C_{15}H_{12}O_5$	272.0674	271.0601	0.24	0.28	0.73	0.46	0.45
Pinobanksin	16.80	$C_{15}H_{12}O_5$	272.0674	271.0601	12.71	5.50	3.85	6.74	5.98
Kaempferol	16.80	$C_{15}H_{10}O_6$	286.0467	285.0394	0.00	0.87	0.46	0.87	0.85
Kaempferide	17.60	$C_{16}H_{12}O_6$	300.0628	299.0550	0.36	2.03	1.49	1.83	1.75
Bis-methylated quercetin	18.00	$C_{17}H_{14}O_7$	330.0734	329.0656	0.13	1.32	3.95	2.63	2.75
Acacetin	18.60	$C_{16}H_{12}O_5$	284.0674	283.0601	0.00	0.46	1.14	0.82	0.53
Hesperetin	19.10	$C_{16}H_{14}O_6$	302.0780	301.0707	0.02	0.08	0.27	0.16	0.20
Prenyl caffeate	**21.40**	**$C_{14}H_{16}O_4$**	**248.1038**	**247.0965**	**18.47**	**22.41**	**10.53**	**18.52**	**20.65**
Benzyl caffeate	21.75	$C_{16}H_{14}O_4$	270.0881	269.0808	0.23	0.66	1.68	1.46	1.19
Chrysin	22.17	$C_{15}H_{10}O_4$	254.0568	253.0495	1.70	11.58	9.19	8.80	9.21
Pinocembrin	22.75	$C_{15}H_{12}O_4$	256.0725	255.0652	6.77	12.11	8.05	8.59	7.69
Galangin	22.85	$C_{15}H_{10}O_5$	270.0513	269.0440	0.59	5.41	3.99	5.08	4.30
Caffeic acid phenetyl ester	22.92	$C_{17}H_{16}O_4$	284.1038	283.0965	1.83	6.64	6.86	6.70	7.16
Pinobanksin-3-acetate	23.40	$C_{17}H_{14}O_6$	314.0785	313.0707	8.44	12.80	6.91	10.83	11.66
Isopentyl caffeate	23.70	$C_{14}H_{18}O_4$	250.1200	249.1121	0.47	1.21	0.47	0.84	1.30
Unknown	**24.05**	**$C_{18}H_{32}O_4$**	**312.2295**	**311.2217**	**0.48**	**0.34**	**1.65**	**0.63**	**0.81**
Isoprenyl coumarate	25.20	$C_{14}H_{16}O_3$	232.1094	231.1016	0.11	1.08	1.37	1.27	1.07
Isoprenyl ferulate	25.75	$C_{15}H_{18}O_4$	262.1200	261.1121	0.04	1.69	2.85	1.56	1.68
Pinobanksin-3-propionate	26.05	$C_{18}H_{16}O_6$	328.0941	327.0863	0.15	1.25	3.51	1.44	1.98
Clerodane diterpenoid I	**26.58**	**$C_{20}H_{32}O_3$**	**320.2346**	**319.2268**	**0.00**	**0.001**	**4.70**	**1.76**	**1.93**
Clerodane diterpenoid II	**27.35**	**$C_{20}H_{32}O_3$**	**320.2346**	**319.2268**	**0.00**	**0.001**	**2.93**	**1.02**	**1.15**
Pinostrobin	**26.85**	**$C_{16}H_{14}O_4$**	**270.0881**	**269.0808**	**0.00**	**0.00**	**2.39**	**0.22**	**0.52**
Clerodane diterpenoid dihydro	**28.80**	**$C_{20}H_{34}O_3$**	**322.2502**	**321.2424**	**0.00**	**0.00**	**0.44**	**0.32**	**0.14**
Clerodane diterpenoid dehydrated	**38.05**	**$C_{20}H_{30}O_2$**	**302.2240**	**301.2162**	**0.00**	**0.00**	**0.52**	**0.22**	**0.10**
Palmitic acid	45.50	$C_{16}H_{32}O_2$	256.2392	255.2319	1.13	0.10	0.08	0.08	0.16
Total Peak Area (TPA) × 10^6					**0.192**	**3.203**	**4.166**	**3.778**	**2.566**

RT min: Retention time; **$[M-H]^-$**: Pseudo-molecular ion performed in negative ion mode; **B1**, **B2**, **D**, **M** and **S**: propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively.

The data presented in table 4-1 were based on information obtained from figure 4-1. The figure illustrates the TIC chromatograms and retention time (RT min) under negative ion mode obtained by LTQ-Orbitrap XL analyzer for the five investigated propolis samples. As evident that the propolis extracts showed approximately the same TIC profile in B2, D, M and S samples, and such similarity appear to be due to the presence of the same molecular substances. Full scan spectra (m/z =1450000 - 8000000) and the corresponding MS/MS spectra (not shown) were in favour of a high number of phenolic compounds in propolis samples. In contrast, B1 sample at the same full scan spectra showed very less numbers of phenolic compounds and a peculiar TIC profile.

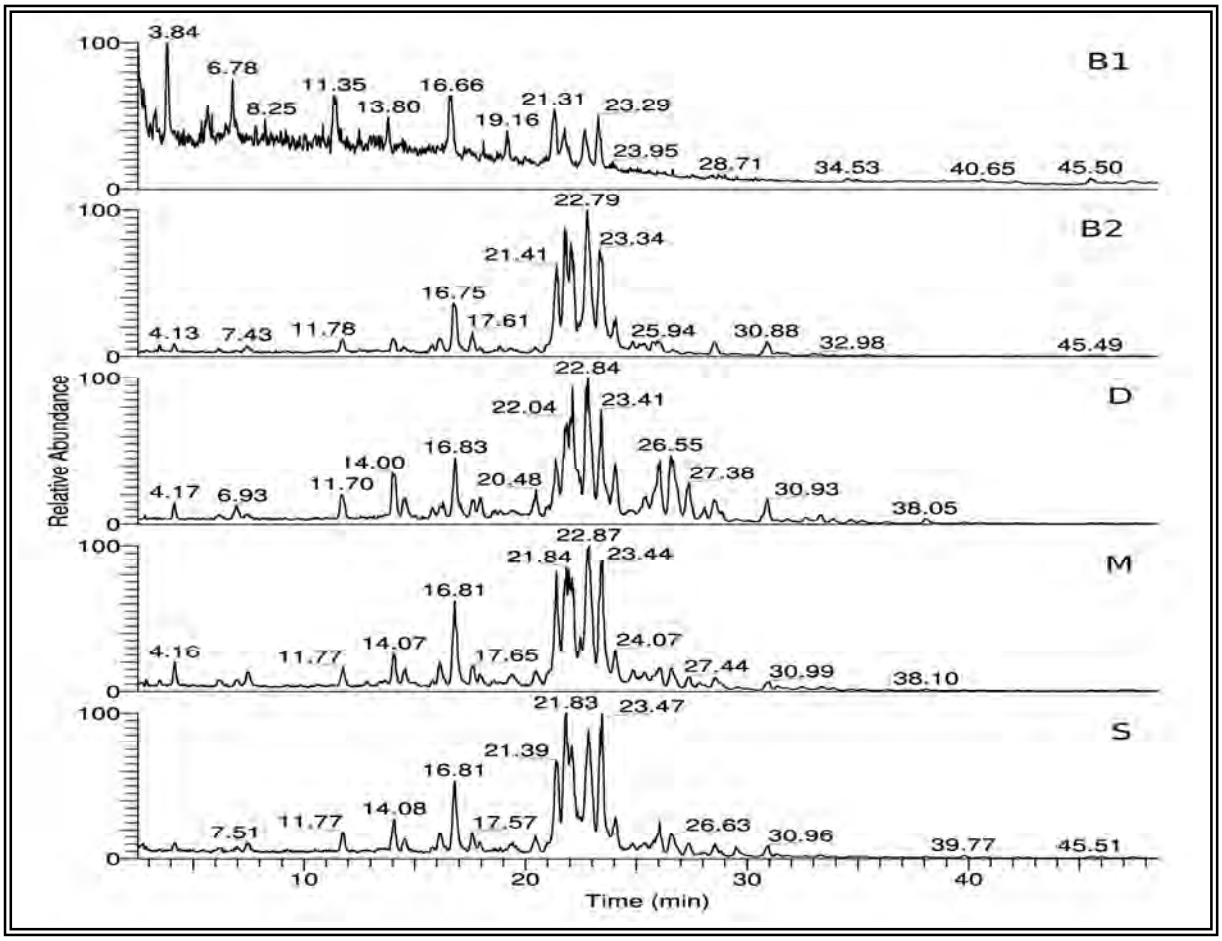


Figure 4-1: Total ion current chromatograms and retention time (RT min) under negative ion mode obtained by LTQ-Orbitrap XL analyzer for the five investigated propolis samples (B1, B2, D, M and S: propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively).

Figures 4-2 shows an example of HPLC-ESI/MS extracted ion chromatograms (XIC) and negative mass spectra of three compounds identified in propolis extract (PE) of M sample. These compounds were chrysin (CH), caffeic acid (CA) and caffeic acid phenetheyl ester (CAPE). Furthermore, a high resolution full scan spectra of Fourier transform mass spectrometry (FTMS) was acquired with the orbitrap analyzer, while the corresponding low resolution MS/MS of spectra ion-trap tandem mass spectrometry (ITMS2) was acquired by the linear ion trap analyzer.

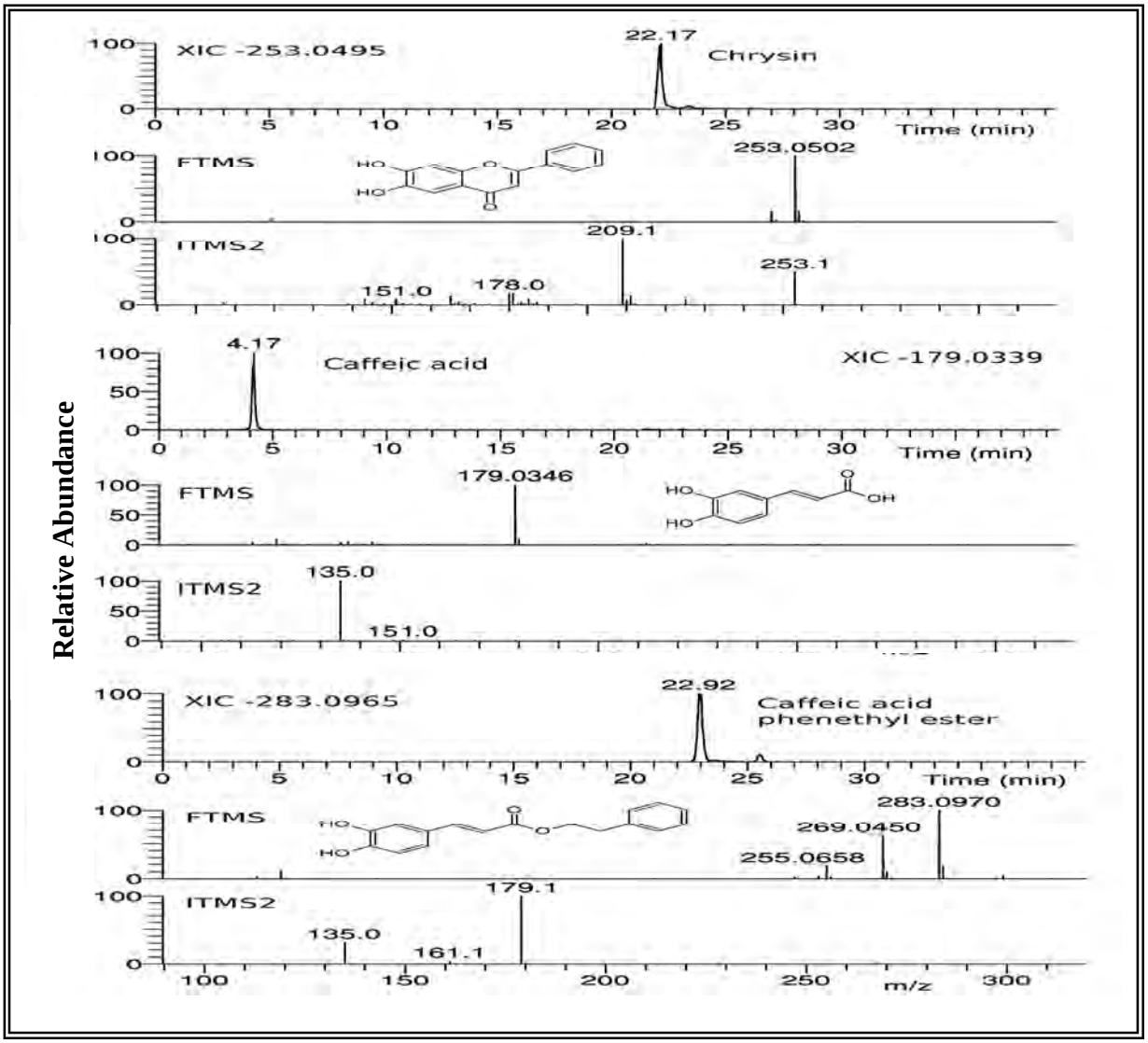


Figure 4-2: HPLC-MS extracted ion chromatograms (XIC) and mass spectra of three compounds (chrysin, caffeic acid and caffeic acid phenetheyl ester) identified in propolis extract of M sample.

Figure 4-3 shows the HPLC-MS extracted ion chromatograms (XIC) for negative (Panel A) and positive (Panel B) mass spectra of two isomers of clerodane diterpenoids found in PE of D sample. A high resolution full scan spectra (FTMS) was also acquired with the orbitrap analyzer, while the corresponding low resolution MS/MS spectra (ITMS2) was acquired by the linear ion trap analyzer.

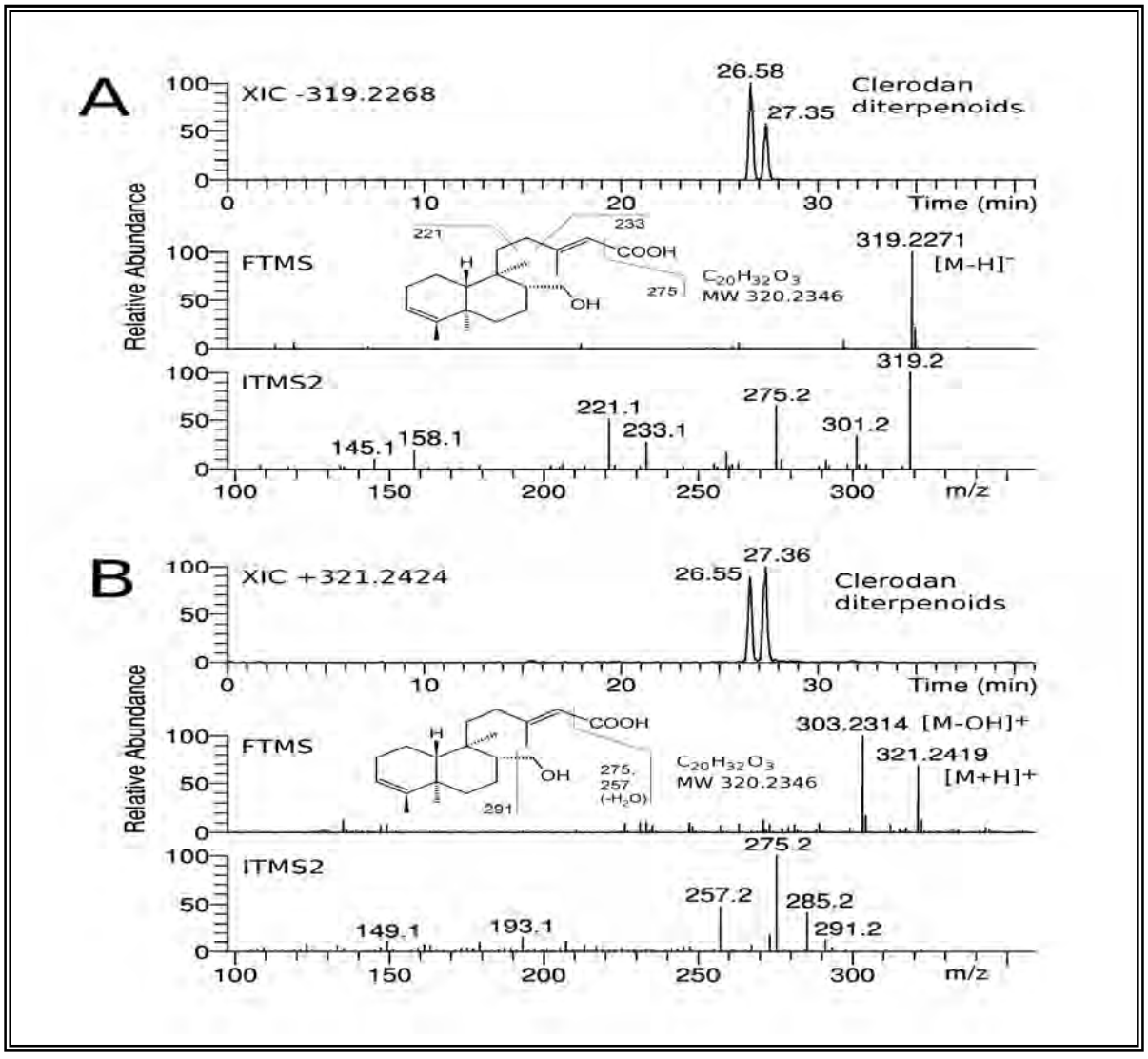


Figure 4-3: HPLC-MS extracted ion chromatograms (XIC) and mass spectra of two isomers of clerodane diterpenoids found in propolis extract of D sample. Panel A: ESI negative ionization; Panel B: ESI positive ionization.

Different phenolic acids and esters were identified in propolis samples, and they are given in Table 4-2. Their total concentration was sample-dependent. The highest total concentration was observed in M sample (4282.44 μg ml^{-1}), followed by B2 (4278.49 μg ml^{-1}), D (3287.08 μg ml^{-1}), S (1071.68 μg ml^{-1}) and finally sample B1 (818.69 μg ml^{-1}). With respect to the individual identified compounds, the highest concentration was reported for prenyl caffeate, and it was different in each sample investigated. The highest concentration was observed in B2 (2044.55 μg ml^{-1}), followed by M (1781.52 μg ml^{-1}), D (1103.10 μg ml^{-1}) and S (515.91 μg ml^{-1}), while in sample B1, CA recorded the highest concentration (278.04 μg ml^{-1}) among the other compounds. Furthermore, most samples shared a low concentration of vanillin, which was also not detected in B1 sample (Table 4-2).

Table 4-2: Phenolic acids and esters concentrations assessed by HPLC-ESI /MS in propolis sample extracts collected from five geographical regions in Iraq.

Compound	Concentration of phenolics acids and esters (μg ml^{-1})				
	B1	B2	D	M	S
Caffeic acid	**278.04**	355.50	197.31	421.59	58.35
Coumaric acid	123.95	187.99	104.73	211.19	36.06
Vanillin	**0.00**	**5.93**	**4.24**	**10.03**	**1.87**
Methyl caffeate	9.74	18.14	133.17	66.20	12.62
Ferulic acid	175.28	458.39	106.34	436.23	89.82
Ferulic acid methyl ester	69.89	137.22	223.47	153.32	37.74
Cinnamic acid	18.41	33.37	28.02	38.94	9.31
Prenyl caffeate	125.24	**2044.55**	**1103.10**	**1781.52**	**515.91**
Benzyl caffeate	1.54	60.37	176.01	140.23	29.77
Caffeic acid phenethyl ester	12.43	613.60	718.77	644.07	178.90
Isopentyl caffeate	3.16	110.71	49.07	106.49	32.56
Isoprenyl coumarate	0.72	98.38	143.80	122.30	26.85
Isoprenyl ferulate	0.29	154.34	299.05	150.33	41.92
Total phenolic acid and ester concentrations	**818.69**	**4278.49**	**3287.08**	**4282.44**	**1071.68**

B1, B2, D, M and S: Propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively.

Twelve flavones and flavonols compounds were identified in propolis samples, and they are given in table 4-3. The total concentration of these compounds was also sample-dependent. The highest concentration of flavones and flavonols was observed in sample D (3727.85 μg ml^{-1}), followed by B2 (2981.95 μg ml^{-1}), M (2937.71 μg ml^{-1}), S (705.17 μg ml^{-1}) and finally sample B1 (52.28 μg ml^{-1}). The highest concentration of each compound was dependent on the sample investigated. It was pinocembrin in B1 (39.75 μg ml^{-1}) and B2 (1329.13 μg ml^{-1}), while in samples D, M and S, CH recorded the highest concentration (1505.33, 1195.58 and 317.78 μg ml^{-1}, respectively) (Table 4-3).

Table 4-3: Flavone and flavonol concentrations assessed by HPLC-ESI/MS in propolis sample extracts collected from five geographical regions in Iraq.

Compound	Concentration of flavones and flavonols (μg ml^{-1})				
	B1	B2	D	M	S
Luteolin	0.00	33.19	55.87	62.42	11.67
Tectochrysin	0.25	87.75	143.86	63.20	16.75
Apigenin	0.32	16.37	30.36	35.02	5.72
Acacetin	0.00	50.15	187.20	112.02	18.40
Chrysin	10.00	1270.73	**1505.33**	**1195.58**	**317.78**
Pinocembrin	**39.75**	**1329.13**	1319.59	1166.42	265.23
Quercetin	0.00	3.22	15.60	11.42	2.19
Methyl quercetin	0.25	19.85	109.54	51.74	11.58
Kaempferol	0.00	15.51	16.82	19.95	4.91
Kaempferide	0.57	36.13	54.21	42.12	10.14
Bis-methylated quercetin	0.19	23.49	144.15	60.59	15.90
Galangin	0.95	96.43	145.32	117.23	24.90
Total flavone and flavonol concentrations	**52.28**	**2981.95**	**3727.85**	**2937.71**	**705.17**

B1, B2, D, M and S: Propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively.

With respect to flavanones and dihydroflavonols, seven compounds were identified in propolis samples, and the concentration was also sample-dependent. The highest concentration of flavanones and dihydroflavonols was observed in

sample D (955.23 μg ml^{-1}), followed by M (565.42 μg ml^{-1}), B2 (416.74 μg ml^{-1}), S (150.87 μg ml^{-1}) and finally sample B1 (38.45 μg ml^{-1}). Also, the highest concentration of each compound was dependent on the sample investigated. It was pinobanksin in B1 (20.53 μg ml^{-1}), sakuranetin in D (311.60 μg ml^{-1}), while in B2, M and S samples, pinobanksin-3-acetate recorded the highest concentration (228.27, 249.80 and 67.51 μg ml^{-1}, respectively) (Table 4-4).

Table 4-4: Flavanone and dihydroflavonol concentrations assessed by HPLC-ESI/MS in propolis sample extracts collected from five geographical regions in Iraq.

Compound	Concentration of flavanones and dihydroflavonols (μg ml^{-1})				
	B1	B2	D	M	S
Sakuranetin	3.63	61.80	**311.60**	107.83	30.50
Naringenin	0.39	4.99	26.61	10.63	2.60
Hesperetin	0.03	1.40	9.68	3.69	1.14
Pinostrobin	0.00	0.00	87.19	5.06	3.02
Pinobanksin	**20.53**	98.04	140.20	155.32	34.64
Pinobanksin-3-propionate	0.24	22.24	128.10	33.09	11.46
Pinobanksin-3-acetate	13.63	**228.27**	251.85	**249.80**	**67.51**
Total flavanone and dihydroflavonol concentrations	**38.45**	**416.74**	**955.23**	**565.42**	**150.87**

B1, B2, D, M and S: Propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively.

Taken the forthcoming results in a collective theme, the qualitative assay of flavonoids by HPLC-ESI/MS showed that the phenolic acids and their esters were the predominant class of substances in PEs, followed by flavones and flavonols, and then flavanones and dihydroflavonols (Tables 4-2, 4-3 and 4-4).

The total phenolic compounds were also confirmed using a spectrophotometric method with Folin–Ciocalteu reagent. As mentioned early in this section, the concentration of these compounds was sample-dependent. Samples D and M shared a similar concentration (9333.3 and 9033.3 μg ml^{-1},

respectively), while lowers concentrations were observed in samples B2, S and B1 (6933.3, 1400.0 and 700.0 µg ml^{-1}, respectively), and the differences were significant ($P \leq 0.05$) (Table 4-5).

Table 4-5: Total phenolics in Iraqi propolis sample extracts collected from five geographical regions in Iraq.

Compound	Mean ± S.E.(Number = 3 measurements)*				
	B1	B2	D	M	S
Total phenolic compound concentration (µg ml^{-1})	700.0 ± 1.2^{d}	6933.3 ± 6.8^{b}	9033.3 ± 68.9^{a}	9333.3 ± 74.7^{a}	1400.0 ± 51.27^{c}

B1, B2, D, M and S: Propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively.
*Different letters: Significant difference ($P \leq 0.05$) between means.

4.1.2 Stable Free Radical Scavenging Capacity

The free radical scavenging effect of five propolis samples and three polyphenolic compounds (CA, CH and CAPE) in DPPH free radical system was determined, and given as a percentage of anti-radical activity (ARA) for three concentrations 1.0, 10.0 and 100 µg ml^{-1} of each extract or compound. In general, a concentration-dependent ARA was observed, and 100 µg ml^{-1} was significantly better than the other two concentrations for most of the investigated samples and compounds. The exception was CH, in which the ARA values were almost similar (13.3, 16.6 and 20.0%, respectively) and no significant difference between them was observed, and furthermore, this compound recorded the lowest ARA as compared with other samples or compounds (Table 4-6).

Although the concentration 100 µg ml^{-1} recorded the best ARA, the activity was sample or compound-dependent. In this regard, CAPE was the compound with the highest ARA (93%) followed by B2, D, M and CA, which shared approximated ARAs (80, 83.3, 83.3 and 80%, respectively) in these samples or compounds (Table 4-6).

Table 4-6: The DPPH free radical scavenging activity of five Iraqi propolis extracts and their polyphenolic compounds.

Samples and Compounds	Mean ± S.E. (Three Absorbance Readings)*					
	1.0 μg ml^{-1}		10.0 μg ml^{-1}		100.0 μg ml^{-1}	
	Absorbance	ARA	Absorbance	ARA	Absorbance	ARA
B1	0.24 ± 0.04^{Aa}	20.0	0.22 ± 0.03^{Bb}	26.6	0.18 ± 0.01^{Bc}	40.0
B2	0.17 ± 0.03^{Ca}	43.0	0.11 ± 0.02^{Cb}	63.3	0.06 ± 0.02^{Dc}	**80.0**
D	0.13 ± 0.01^{Da}	56.6	0.07 ± 0.03^{Db}	76.6	0.05 ± 0.01^{Db}	**83.3**
M	0.11 ± 0.03^{Ea}	63.3	0.07 ± 0.03^{Db}	76.6	0.05 ± 0.03^{Db}	**83.3**
S	0.20 ± 0.03^{Ba}	33.3	0.11 ± 0.01^{Cb}	63.3	0.10 ± 0.01^{Cb}	66.6
CA	0.13 ± 0.03^{Da}	56.6	0.10 ± 0.03^{Cb}	66.6	0.06 ± 0.03^{Dc}	**80.0**
CH	0.26 ± 0.03^{Aa}	**13.3**	0.25 ± 0.03^{Aa}	**16.6**	0.24 ± 0.03^{Aa}	**20.0**
CAPE	0.08 ± 0.03^{Fa}	73.3	0.05 ± 0.01^{Db}	83.3	0.02 ± 0.01^{Ec}	**93.3**

ARA: Anti-radical activity (%); Control absorbance: 0.30±0.03.
*Different capital letters: Significant difference ($P \leq 0.05$) between means of columns.
*Different lower case letters: Significant difference ($P \leq 0.05$) between means of rows.
B1, B2, D, M and S: Propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester.

4.2 *In Vitro* Investigations

4.2.1 Growth Inhibition of Tumour and Normal Cell Lines

The *in vitro* growth inhibitory (GI) effects were assessed for three concentrations (5, 15 and 25 μg ml^{-1}) of four PE samples (B2, D, M and S) and three phenolic compounds (CA, CH and CAPE) against two tumour cell lines (HL-60 and HCT-116) after three incubation periods (24, 48 and 72 hours). For B1 sample, the corresponding concentrations were 50, 150 and 250 μg ml^{-1}. The GI was also investigated in a normal cell line (HuFb) for the former three concentrations, but for one incubation period (72 hours), and data was also given in terms of growth inhibitory rate (GIR) rather than GI. The clonogenic effect of PE samples and compounds against HCT-116 cells was also investigated.

The results demonstrated that a treatment of cell cultures with B2, D, M or CAPE decreased the growth of HL-60 cells significantly ($P \leq 0.05$) as compared to control cultures (untreated), and the effect was dose-, as well as, time-dependent (i.e. the highest concentration showed the highest GI at 72 hours incubation). In contrast, there was a less GI after a treatment with B1 or S samples. The 15 and 25 μg ml^{-1} concentrations of CA showed mild effects after 72 hours only, while CH had no effects (Figure 4-4).

As shown in figure 4-5, the B2, D and M samples of propolis or its polyphenolic compounds; CH and CAPE, decreased the growth of HCT-116 in a significant dose-, as well as, time-dependent manner. The CH and CAPE were more effective than the other samples or compounds in this regard, while no significant GI was observed after a treatment with B1, S or CA.

Inhibitory effects of propolis samples and three phenolic compounds on the growth rate of HL-60, HCT-116 and HuFb cells *in vitro* was assessed after 72 hours. The samples B2, D, M and CAPE were the more effective against proliferation rates of HuFb, HCT-116 and HL-60. However, the proliferation rate demonstrated less cytotoxic effects on HuFb after a treatment with M sample, but such sample recorded a significant inhibition proliferation rate in HL-60 and HCT-116 cells (Figure 4-6).

Colony formation assay was performed to test whether the propolis or polyphenolic compounds can inhibit the clonogenicity of the HCT-116 cells. The control group (untreated cells) formed colonies after seven days of incubation at 37ºC, while the clonogenicity of the treated cells showed a significant dose-dependent decrease, but the inhibitory concentration $(IC)_{50}$ was different for the investigated samples. It was 300 μg ml^{-1} for B1, 4 μg ml^{-1} for B2, D and M, and 50 μg ml^{-1} for S (Figure 4-7). It is worth to mention that the clonogenic effect was assessed for 12 concentrations (data not shown), but the results were presented for 5 concentrations only, in which IC_{50} was based.

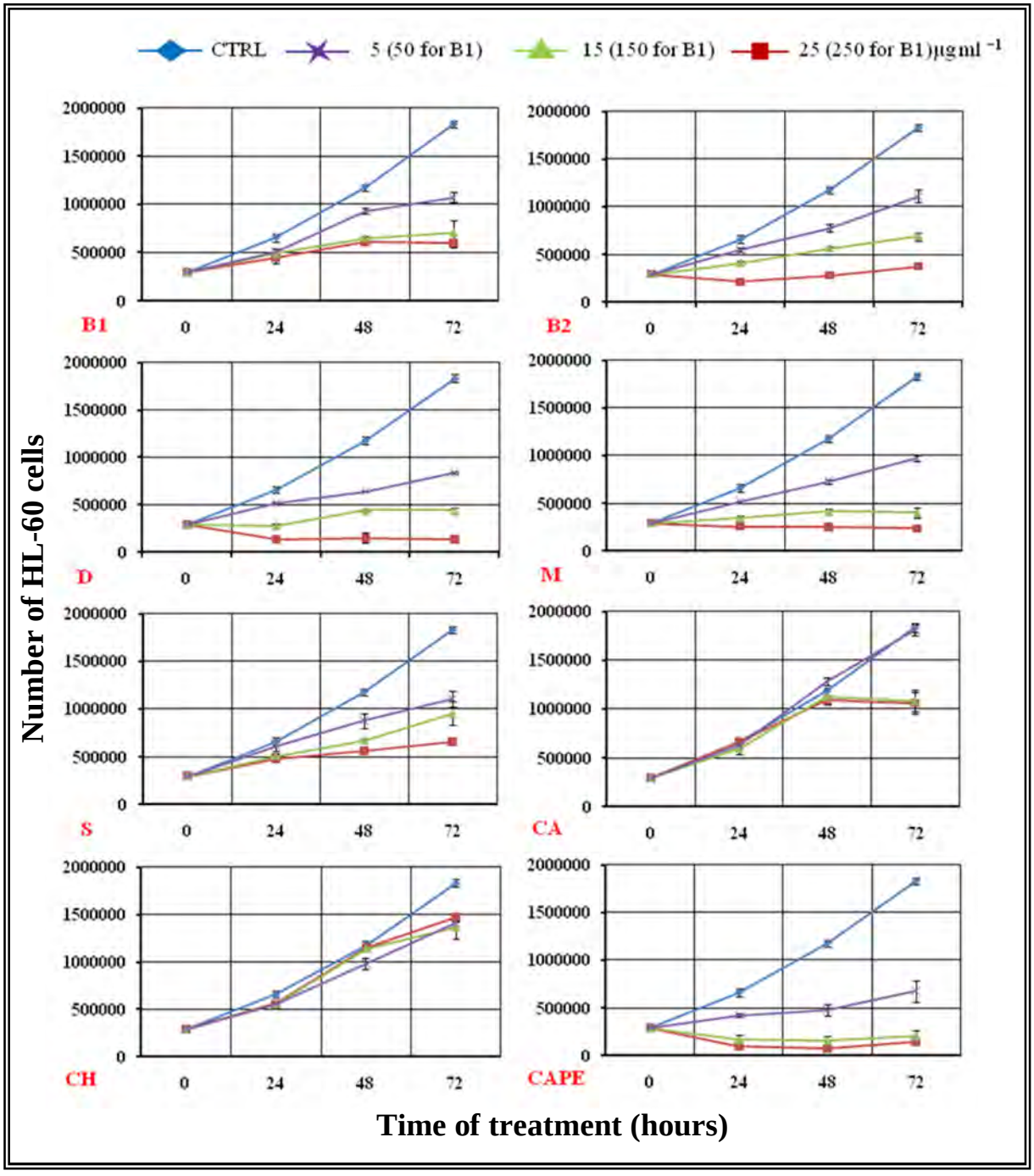


Figure 4-4: Growth inhibition of HL-60 cells treated with different concentrations of Iraqi propolis extracts or polyphenolic compounds. The values were mean ± S.E. from three experiments. B1, B2, D, M, S: Propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester; CTRL: Control.

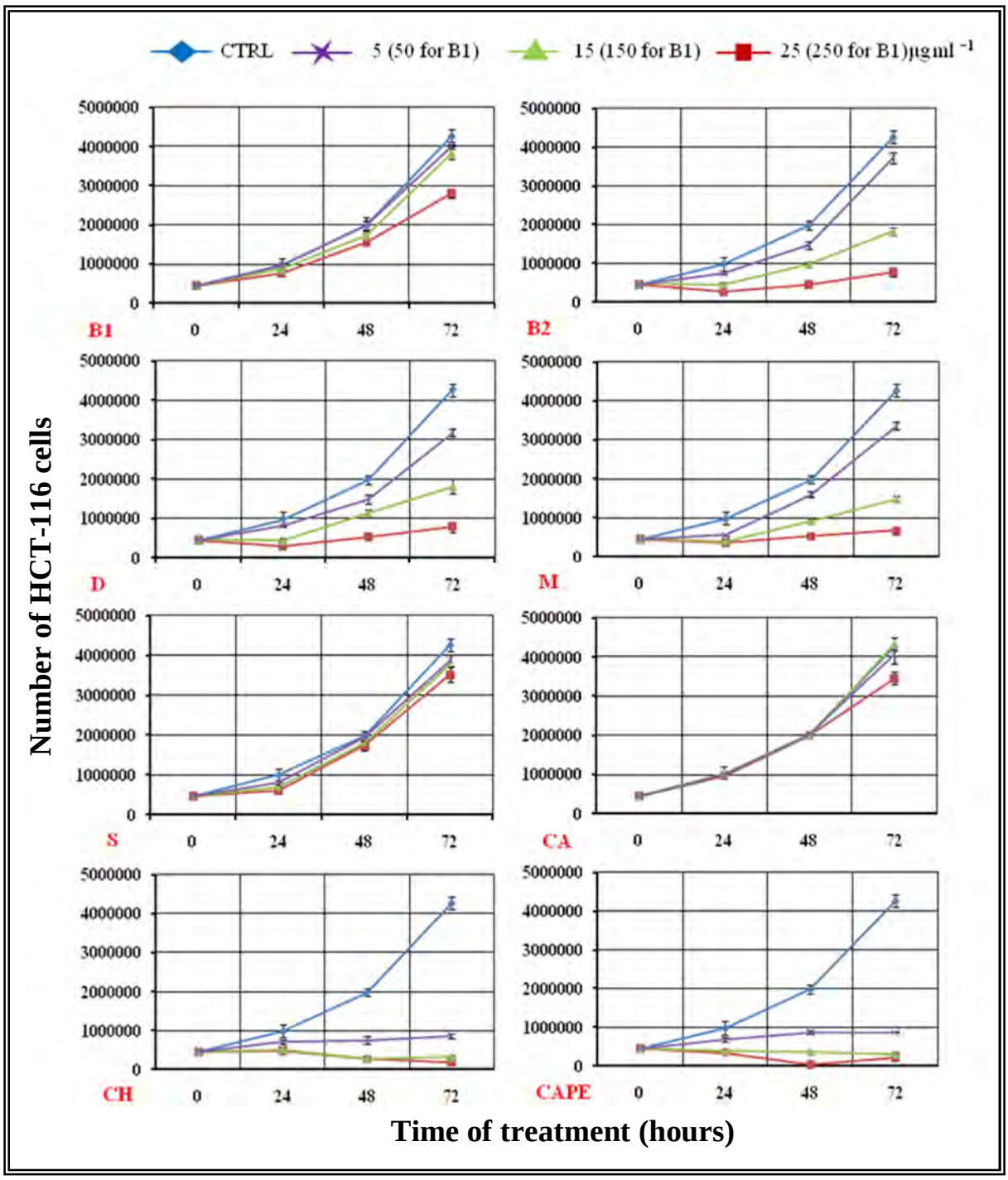


Figure 4-5: Growth inhibition of HCT-116 cells treated with different concentrations of Iraqi propolis extracts or polyphenolic compounds. The values were mean ± S.E. from three experiments. B1, B2, D, M, S: Propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester; CTRL: Control.

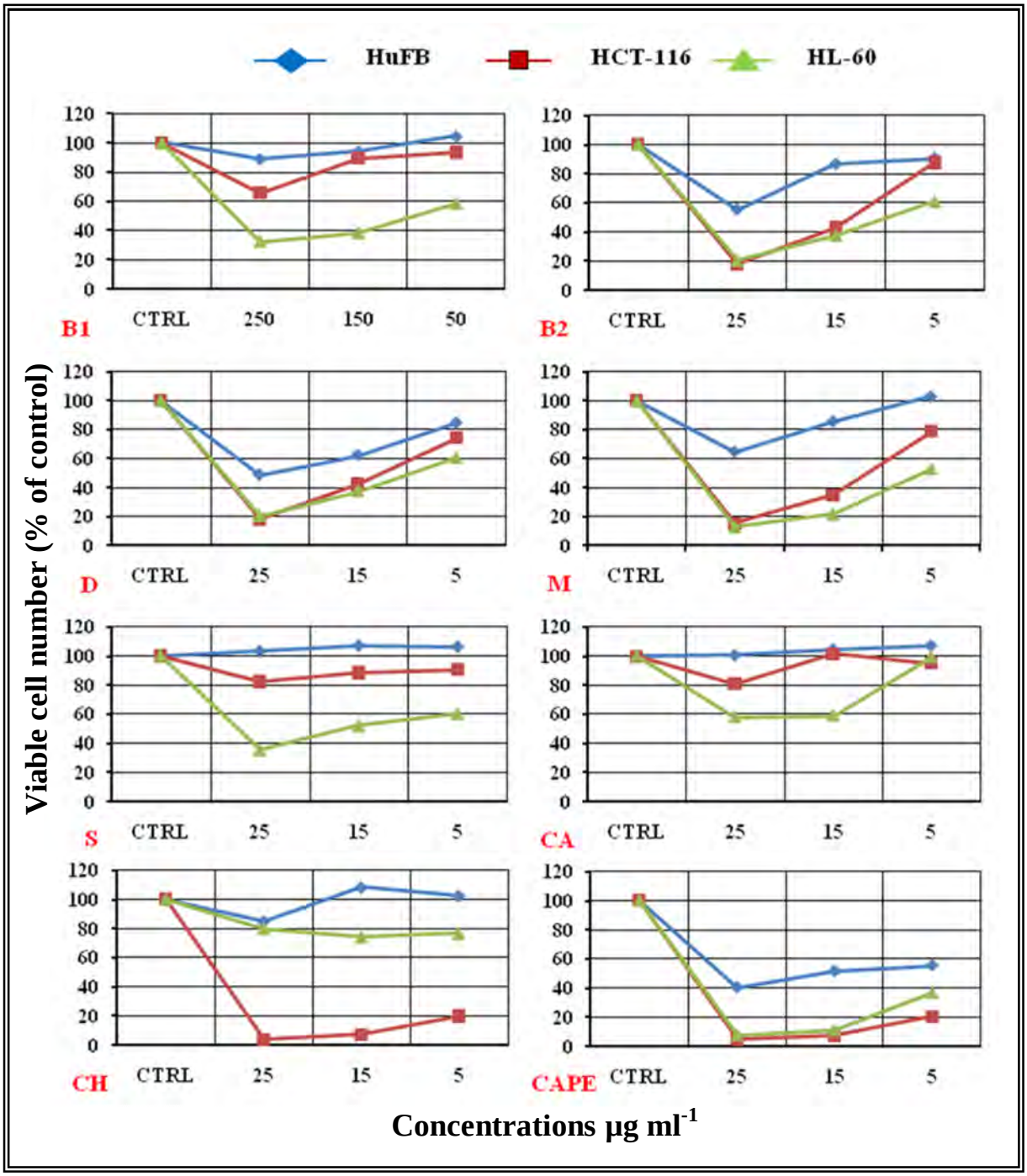


Figure 4-6: Growth inhibitory rate of different concentrations of Iraqi propolis extracts or polyphenolic compounds on HuFb, HCT-116 and HL-60 after 72 hours. The values were mean ± S.E. from three experiments. B1, B2, D, M, S: Propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester; CTRL: Control.

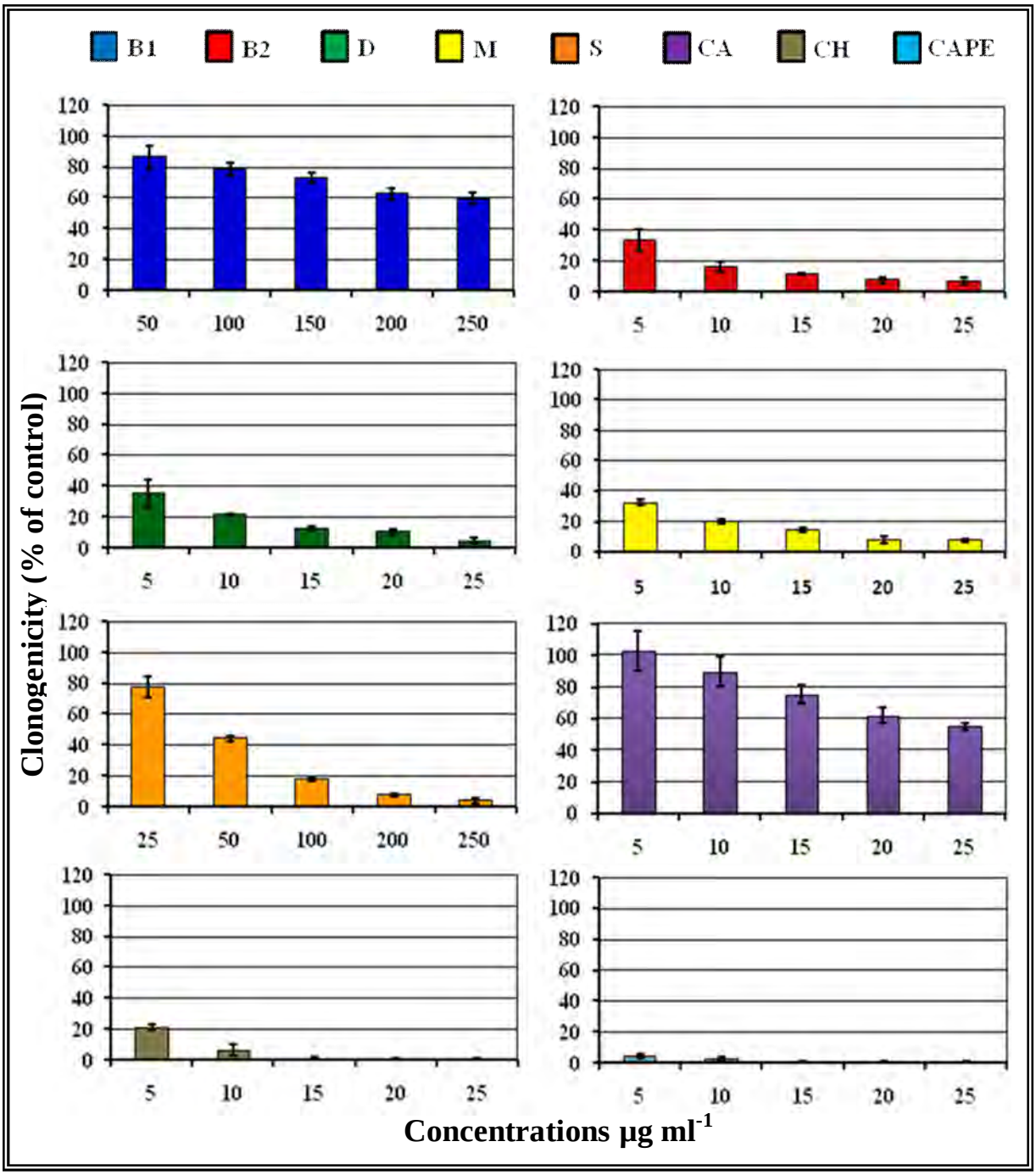


Figure 4-7: Colony inhibition of HCT-116 cells treated with different concentrations of Iraqi propolis extracts or polyphenolic compounds. The values were mean ± S.E. from five experiments. B1, B2, D, M, S: Propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester; CTRL: Control.

4.2.2 Assessment of Apoptosis in Tumour Cell Lines

The apoptosis was assessed in tumour cell lines after a treatment with PEs or their phenolic compounds through four types of assays, which were morphological aspects, gel electrophoresis, Annexin V/PI Flow cytometry and regulation of *Bcl-2*/*Bax* gene expression.

4.2.2.1 Morphological Aspects

To evaluate whether the observed GI in HL-60 and HCT-116 cells was related to apoptosis or necrosis, the morphological changes were examined by subjecting the cells to two different concentration systems. In the first, it was 5 µg ml^{-1} for B2, D, M, S, CA, CH and CAPE, and 50 µg ml^{-1} for B1, while in the second, it was 15 µg ml^{-1} for CAPE and 25 µg ml^{-1} for B2, D, M, S, CA and CH, and it was 250 µg ml^{-1} for B1. The cells were treated for 24 hours, and then stained with sulforhadamine101-DAPI stain. Negative (CTRL), as well as, positive (CPT; Camptothecin) controls were also included, and the results are presented in figures 4-8, 4-9, 4-10, and 4-11.

The HL-60 cells exposed to the second concentration system of PE samples or phenolic compounds (B1, B2, D, M, S and CAPE) exhibited apoptotic features, such as nuclear condensation and fragmentation, while the phenolic compound CH did not show such effects (Figure 4-9).

In HCT-116, the second concentration system revealed effects, but they were necrotic features in B2, D, M, CH and CAPE, while no effect with B1, S and CA was observed. The size of cells was dramatically increased by swelling of cytoplasm and loss of membrane integrity, which lead to cell rupture and release of cellular contents (Figure 4-11).

In both cell lines, concentrations employed in the first system of PE extracts or their phenolic compounds were not clearly effective in inducing apoptosis or necrosis in HL-60 (Figure 4-8) or HCT-116 (Figure 4-10) cells.

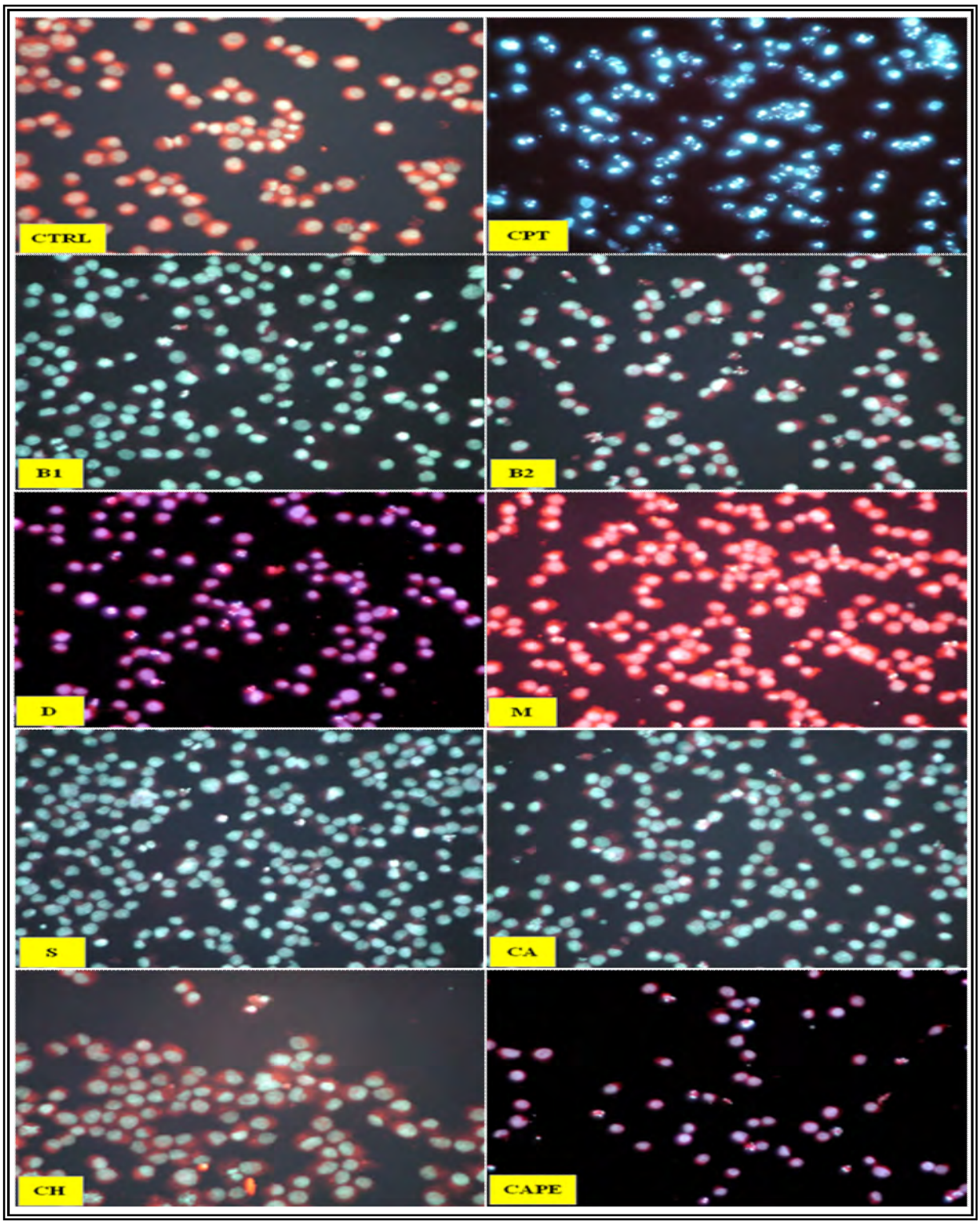


Figure 4-8: Morphological aspects of HL-60 cells treated with the first concentration system. B1, B2, D, M, S: Propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester; CTRL: Negative control; CPT; Camptothecin (positive control). (400X).

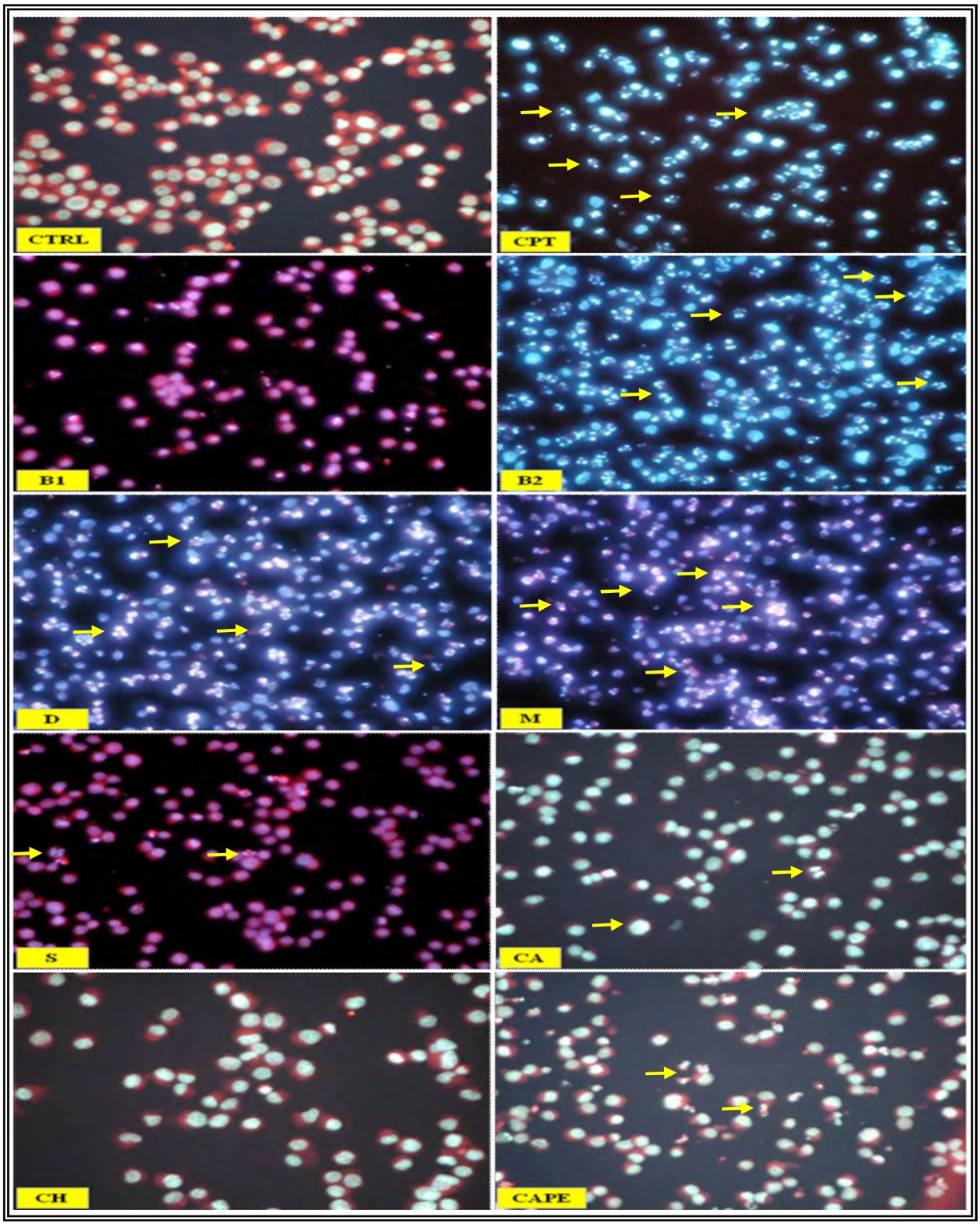


Figure 4-9: Morphological aspects of HL-60 cells treated with the second concentration system. B1, B2, D, M, S: Propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester; CTRL: Negative control; CPT; Camptothecin (positive control). (400X).

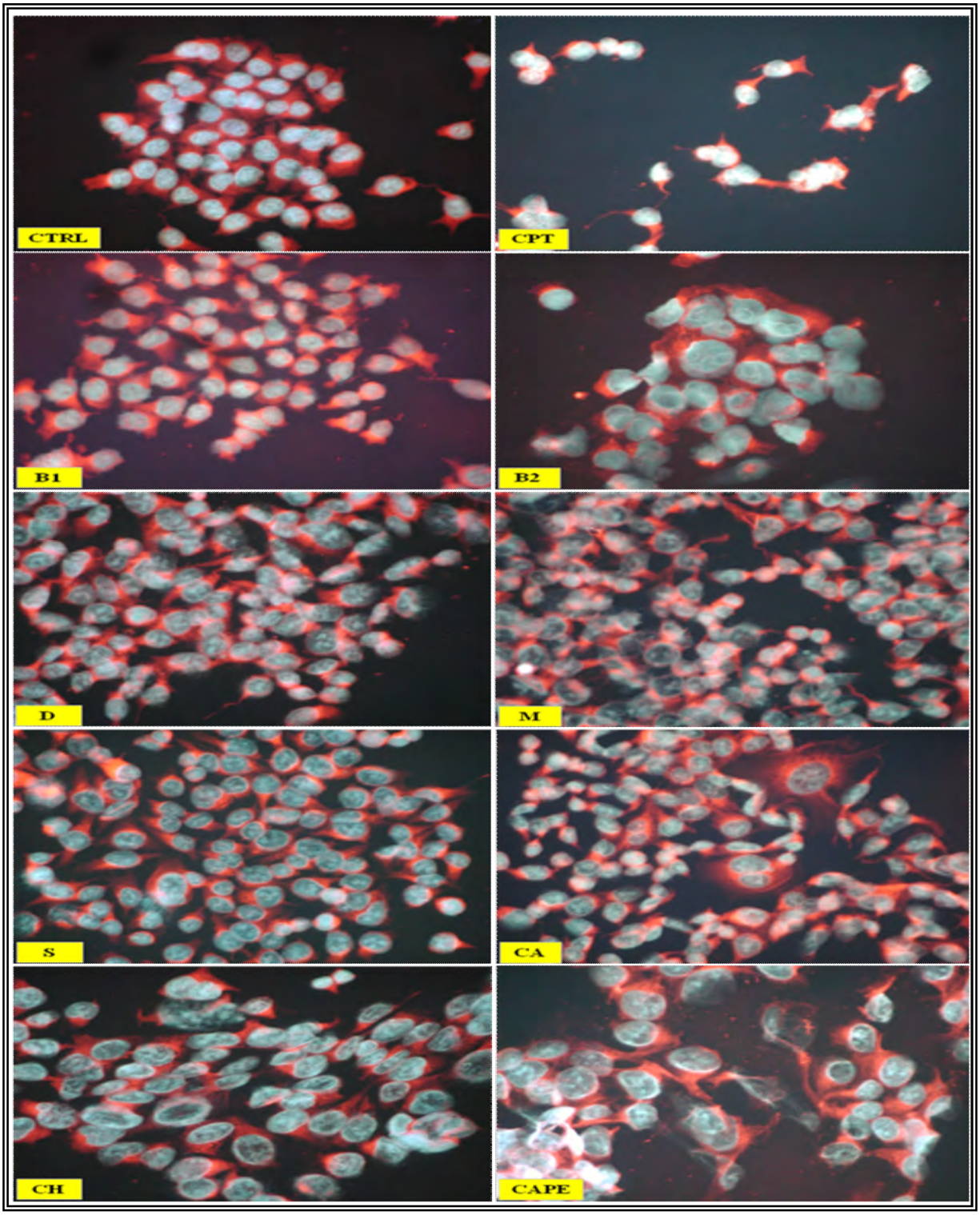


Figure 4-10: Morphological aspects of HCT-116 cells treated with the first concentration system. B1, B2, D, M, S: Propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester; CTRL: Negative control; CPT; Camptothecin (positive control). (400X).

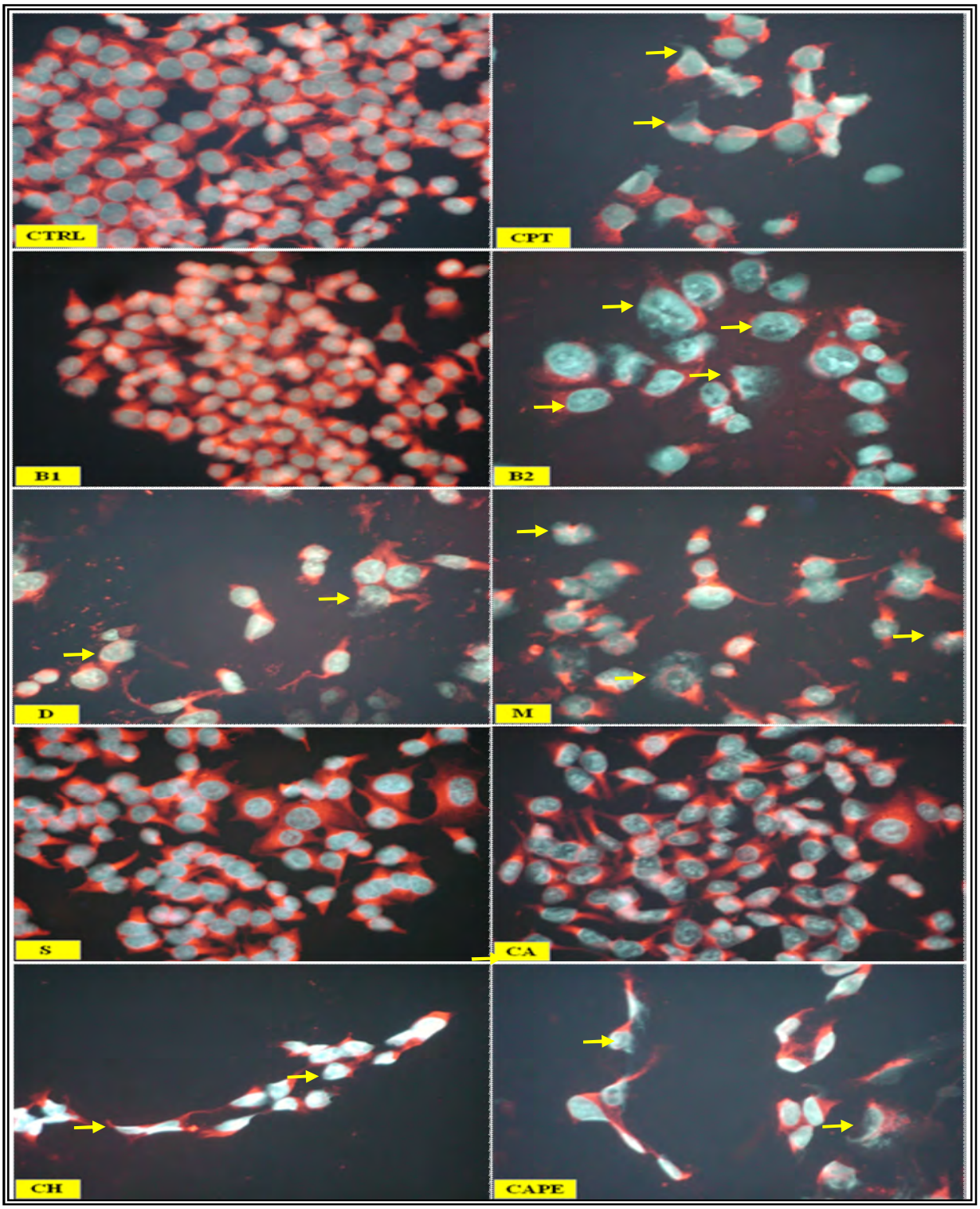


Figure 4-11: Morphological aspects of HCT-116 cells treated with the second concentration system. B1, B2, D, M, S: Propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din; CA: Caffeic acid; CH: Chrysin; CAPE: Caffeic acid phenetheyl ester; CTRL: Negative control; CPT; Camptothecin (positive control). (400X).

4.2.2.2 Gel Electrophoresis

To investigate whether DNA fragmentation induced by propolis samples was the result of apoptosis, agarose gel electrophoresis of DNA extracted from HL-60 and HCT-116 cells that were treated with 25 µg ml^{-1} of B2, D, M or S sample, 250 µg ml^{-1} for B1 sample or two concentrations of CPT1 and CPT2 (10 and 20µM, respectively; positive control) for 24 hours. As shown in figure 4-12A, an apoptotic DNA ladder was observed in HL-60 cells when treated with PEs, while DNA fragmentation was not observed in HCT-116 cells (Figure 4-12B).

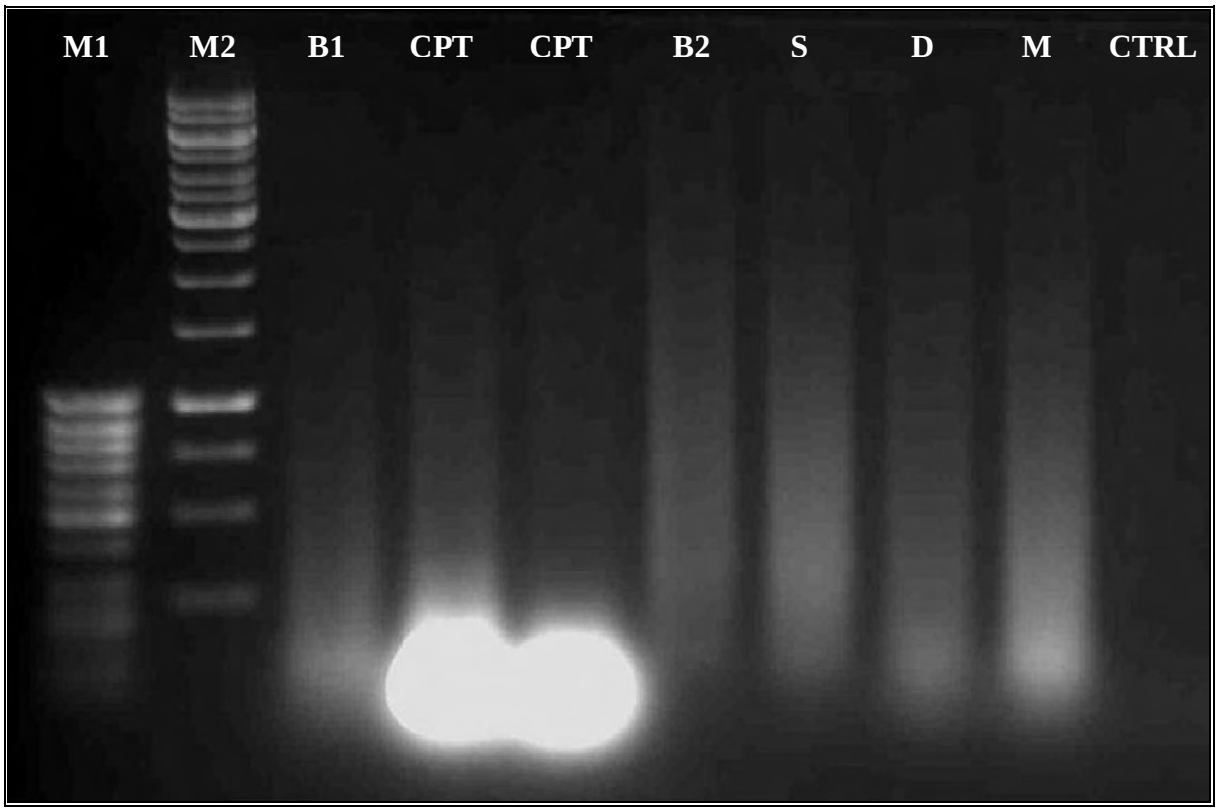


Figure 4-12A: Detection of apoptosis by agarose gel electrophoresis in HL-60 cell line. B1, B2, D, M and S: propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din. Two DNA markers (M1 and M2; 50 bp and 1 Kb, respectively) and two concentrations of camptothecin (CPT1 and CPT2; 10 and 20µM, respectively) were used.

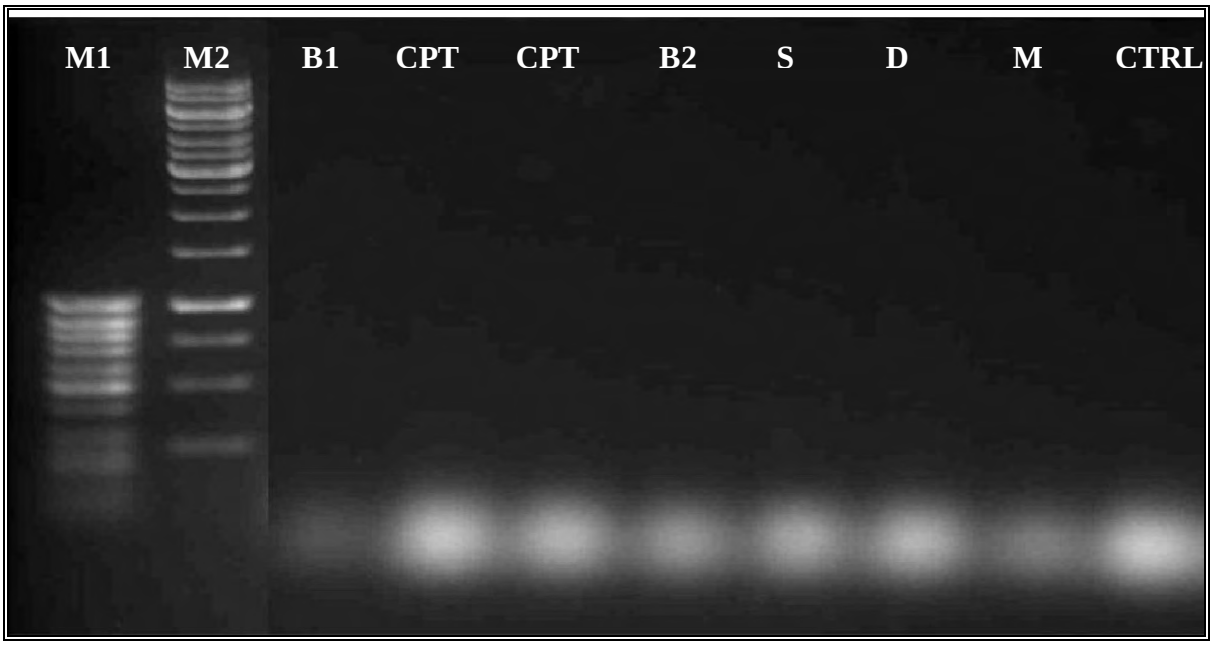


Figure 4-12B: Detection of apoptosis by agarose gel electrophoresis in HCT-116 cell line. B1, B2, D, M, S: propolis samples from Baghdad, Dahuk, Mosul, Salah ad-Din. Two DNA markers (M1 and M2; 50 bp and 1 Kb, respectively) and two concentrations of camptothecin (CPT1 and CPT2; 10 and 20µM, respectively) were used.

4.2.2.3 Annexin V/PI by Flow Cytometry

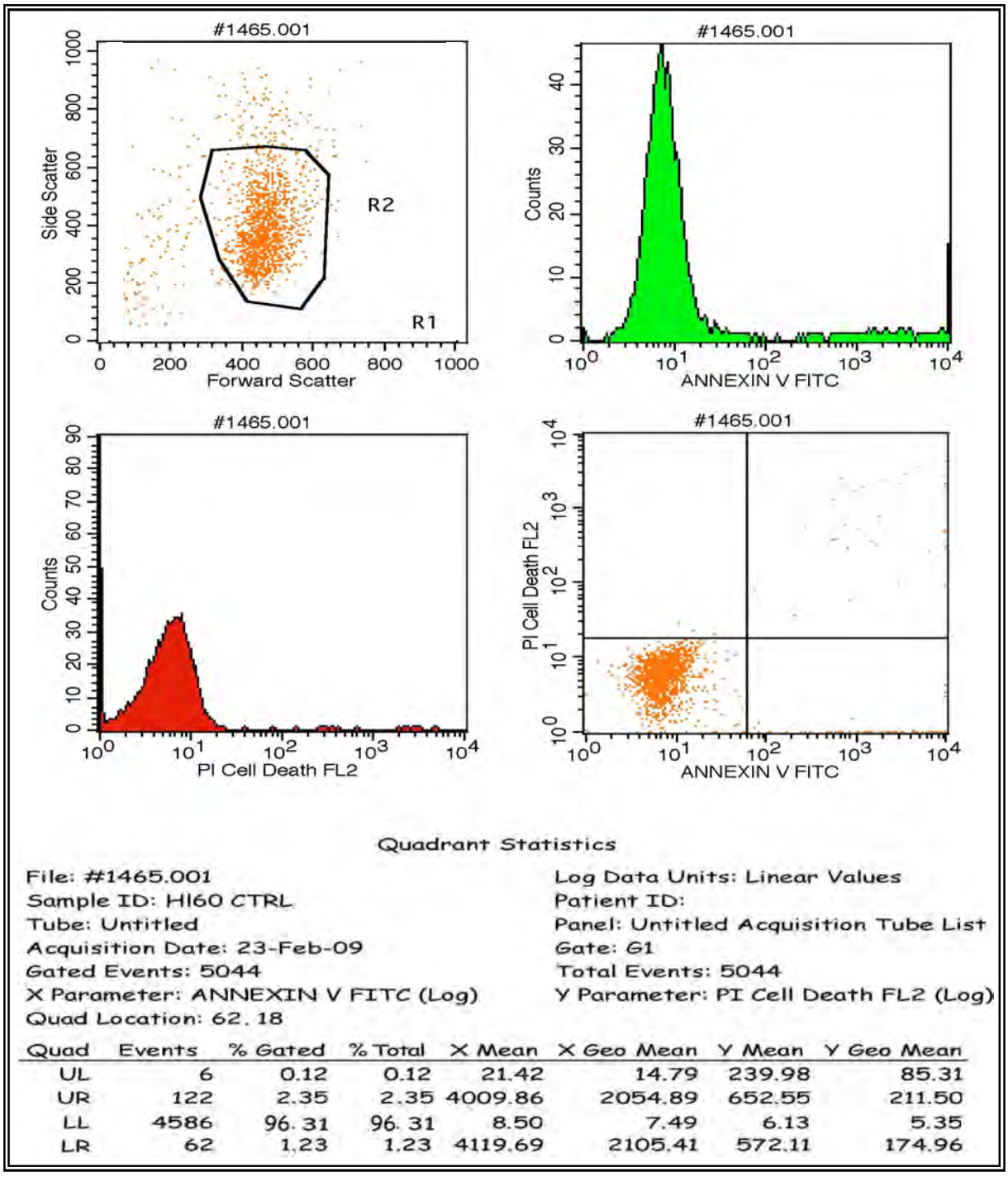
To determine whether propolis-induced cell death was due to apoptosis, the HL-60 and HCT-116 cells were treated with two different concentrations of B1, B2, D, M or S propolis sample in each cell line. For HL-60 cells, the concentrations were 25 µg ml^{-1} or 50 µg ml^{-1} for B2, D, M or S sample and 250 µg ml^{-1} or 500 µg ml^{-1} for B1 sample, while in HCT-116, the concentrations were 15 µg ml^{-1} or 25 µg ml^{-1} for B2, D, M or S sample and 150 µg ml^{-1} or 250 µg ml^{-1} for B1 sample. Then the cells were stained with FITC-conjugated Annexin V plus PI. The analysis was done on at least 10000 cells for each sample by fluorescence-activated cell sorter flow cytometer (FACS) Calibur instrument, and fluorescence pulses were detected using a band pass filter 530 and 580 nm for green and red fluorescence, respectively (Figure 4-13).

In untreated HL-60 cells, the early apoptotic changes appeared in only 1.23% of cells, while late apoptotic changes were almost doubled (2.35%) after

6 hours of incubation. The corresponding figures in CPT-treated cells (positive control) were 51.22 and 14.35%, respectively. After six hours treatment with propolis, all five samples induced early and late apoptosis, but with different percentages, and there was concentration-, as well as, sample-dependent effective. The 50 µg ml^{-1} concentration was more effective than 25 µg ml^{-1} in inducing apoptosis. With respect to propolis samples and at the same concentration, the highest early apoptotic features were observed in M and B2 samples (15.88 and 14.95%, respectively), while cells at a late apoptosis were more frequently observed after a treatment with D sample (15.34%), followed by M (8.92%) sample. The B1 sample also induced apoptotic changes at the two concentrations (250 and 500 µg ml^{-1}), but they were mostly observed at the earlier stages (7.81 and 8.60%, respectively) of apoptosis (Figure 4-14).

The forthcoming findings were based on HL-60 cells that showed a distinct population of early apoptotic cells, which were stained only with FITC-Annexin V, but not with PI (lower right quadrant). When cells were stained with both Annexin V and PI (upper right quadrant), they represented later stages of apoptosis (Figure 4-14).

For HCT-116 cell line, no apoptotic cells were observed, but cells treated with D (9.59 and 19.03%, respectively) and M (7.59 and 12.25%, respectively) samples showed increased necrotic changes at the tested concentrations (15 and 25 µg ml^{-1}) as compared with other samples (B2: 1.90 and 2.66%; S: 0.76 and 0.79%, respectively), or negative (0.76%) and positive (1.30%) controls. For B1 sample, the corresponding concentrations were 10 times higher than the forthcoming concentrations, but the necrotic changes were still a less frequent (0.81 and 0.66%, respectively). The cells were considered dead (necrotic cells), because they were only stained with PI (upper left quadrant); an observation that occurs when the indicated cells show disrupted membranes more frequently (Figure 4-15).



Quadrant Statistics

File: #1465.001
Sample ID: Hl60 CTRL
Tube: Untitled
Acquisition Date: 23-Feb-09
Gated Events: 5044
X Parameter: ANNEXIN V FITC (Log)
Quad Location: 62, 18

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 5044
Y Parameter: PI Cell Death FL2 (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	6	0.12	0.12	21.42	14.79	239.98	85.31
UR	122	2.35	2.35	4009.86	2054.89	652.55	211.50
LL	4586	96.31	96.31	8.50	7.49	6.13	5.35
LR	62	1.23	1.23	4119.69	2105.41	572.11	174.96

Figure 4-13: AnnexinV/PI analysis page of untreated HL-60 cells performed on at least 10000 cells using FACS Calibur instrument. Fluorescence pulses were detected using green laser emitting at 530 nm and red diode laser at 580 nm for PI emitted fluorescence detection. Quad: Quadrant; UL: Upper left; UR: Upper right; LL: Lower left; LR: Lower right.

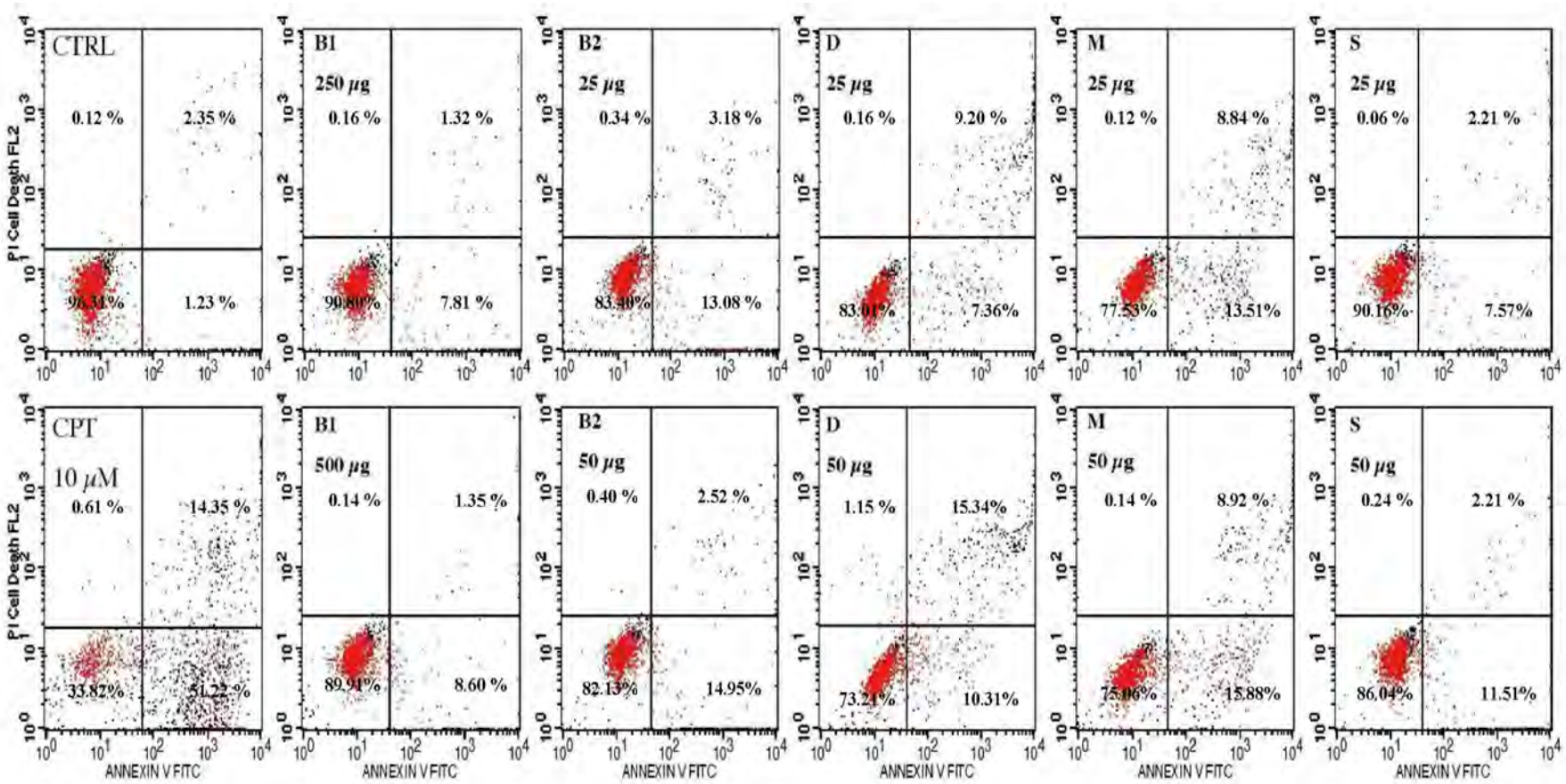


Figure 4-14: Detection of apoptosis in HL-60 by Annexin V-PI assay. The horizontal (Annexin V-FITC; FL1) and vertical (PI - FL2) axes represent labeling with Annexin V and cell number, respectively. B1, B2, D, M and S: Propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively. In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, cells in the upper right are in late apoptosis, and cells in the upper left quadrant are dead. Percentage of total signal within the quadrant is indicated.

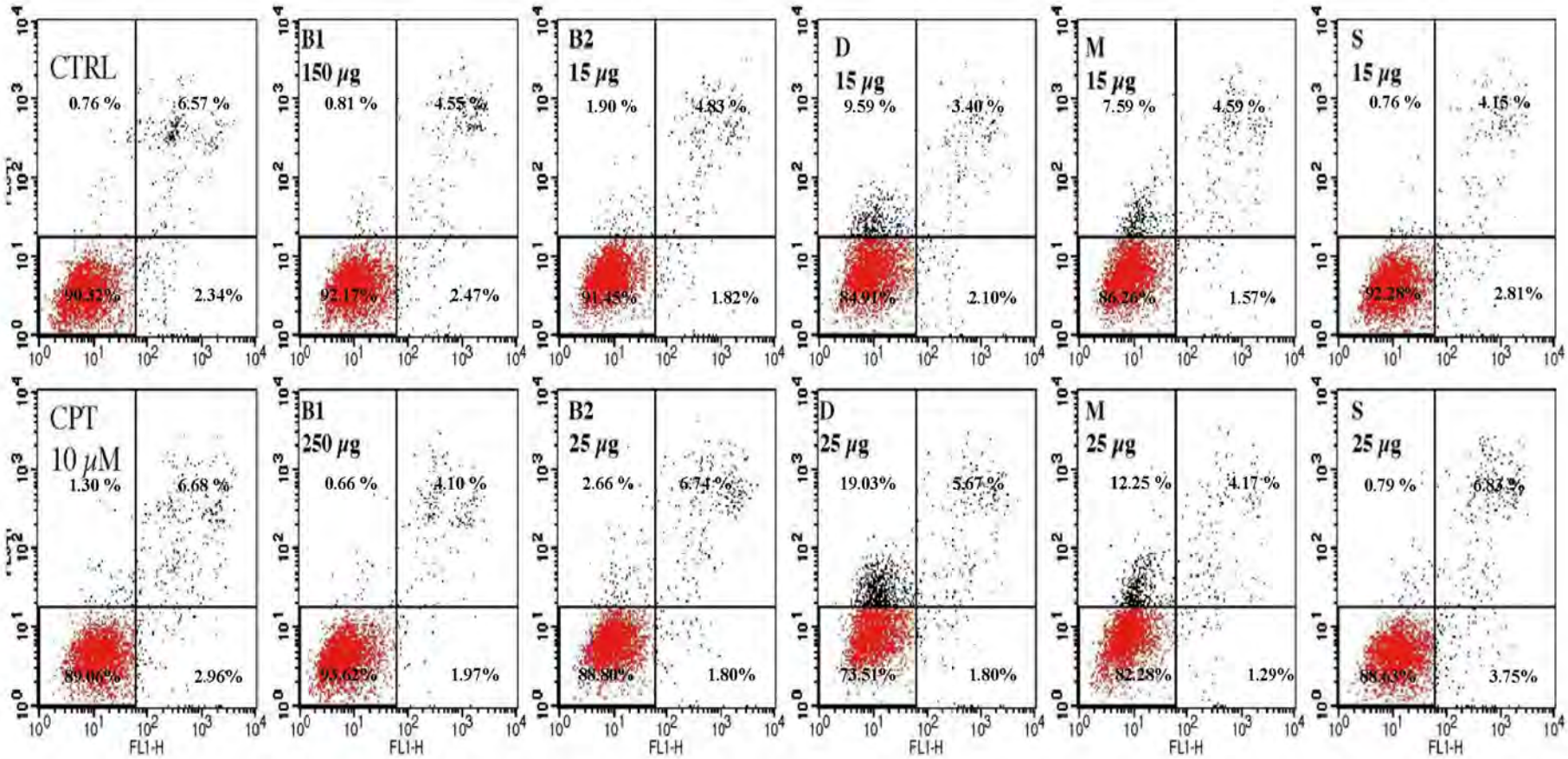


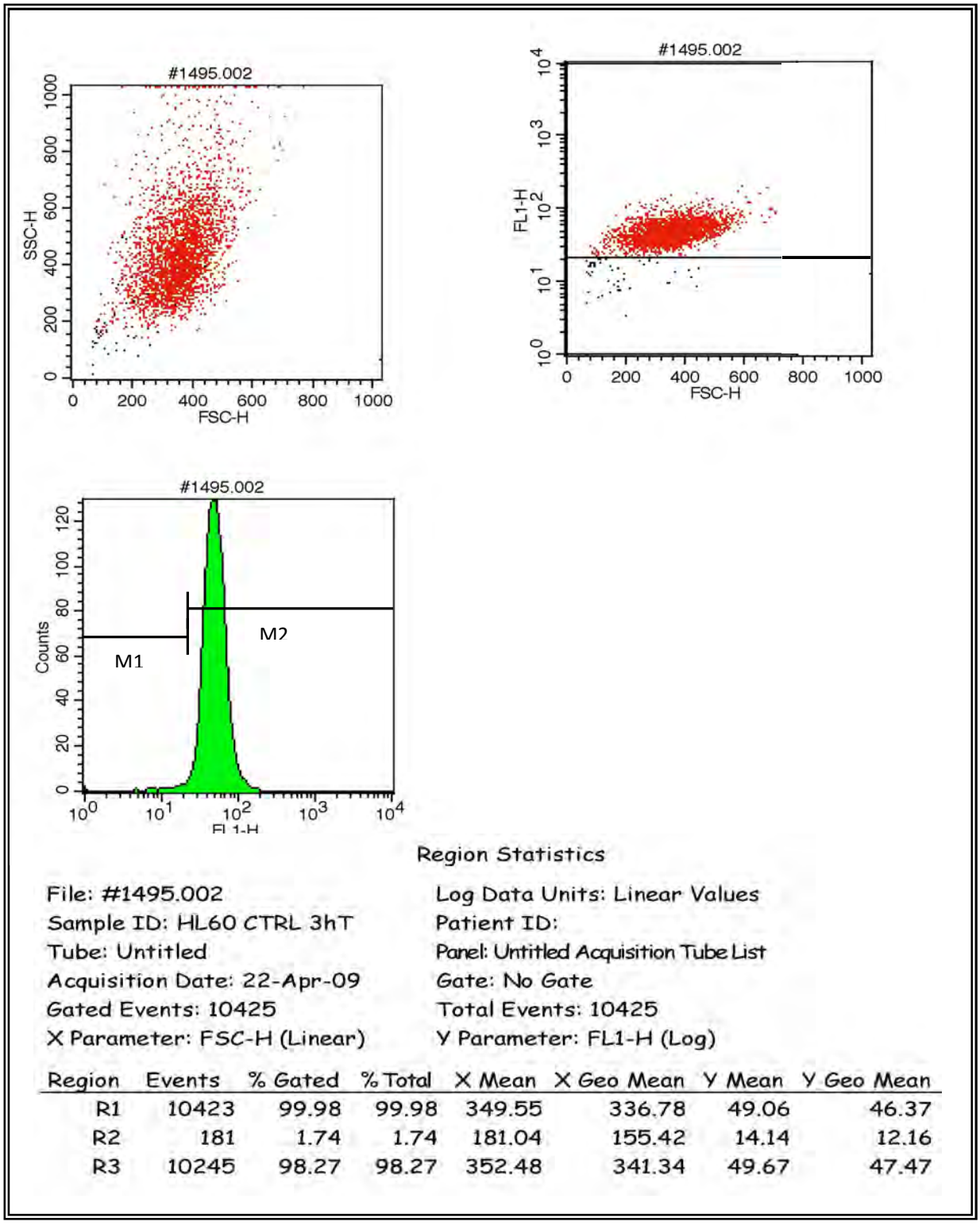
Figure 4-15: Detection of apoptosis in HCT-116 by Annexin V-PI assay. The horizontal (Annexin V-FITC; FL1) and vertical (PI -FL2) axes represent labeling with Annexin V and cell number, respectively. B1, B2, D, M and S: Propolis samples from Baghdad, Dahuk, Mosul and Salah ad-Din, respectively. In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, cells in the upper right are in late apoptosis, and cells in the upper left quadrant are dead. Percentage of total signal within the quadrant is indicated.

4.2.2.4 Regulation of *Bcl-2*/*Bax* Gene Expression

To further analyse of the possible mechanisms underlying the M propolis sample-induced apoptosis in HL-60 cells, the expression of two apoptotic markers, *Bcl-2* and *Bax* genes, was examined by FACS Calibur instrument on at least 10000 cells for each examination, and fluorescence pulses were detected using green laser (FL1-H) emitting at 530 nm (Figures 4-16 and 4-17, respectively).

Expressions of the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were inspected at 3, 6, 10 and 24 hours after treating the cells with M sample (25 µg ml^{-1}). As shown in figure 4-18, the percentage of cells that were expressing Bax protein showed a gradual increase after 3, 6 and 10 hours incubation (16, 31 and 33%, respectively), while after 24 hours treatment, the percentage was decreased to 10%. The corresponding percentages in positive control were 11, 35, 31 and 59%, respectively, and in negative control, it was 3% (Figure 4-18, right lane).

With respect to the anti-apoptotic protein Bcl-2, the opposite outcome was observed. In untreated cells, it was negatively expressed in only 2% of cell population after three hours incubation periods. This percentage was increased in propolis-treated cells, but the effect was time-dependent. After three hours treatment, a slight increased percentage was observed (5%), but as the treatment time was progressing, the percentage of cells that showed a negative expression of Bcl-2 were increasing, and they represented 12, 22 and 21%, respectively at the time periods 6, 10 and 24 hours. The corresponding percentages in cells treated with CPT (positive control) were 56, 45, 9 and 6%, respectively. The latter findings (gradual decreased percentages that paralleled the incubation time) contrast the manner of effect (gradual increased percentages that paralleled the incubation time) that was recorded in cells treated with M propolis sample extract (Figure 4-18, left lane).



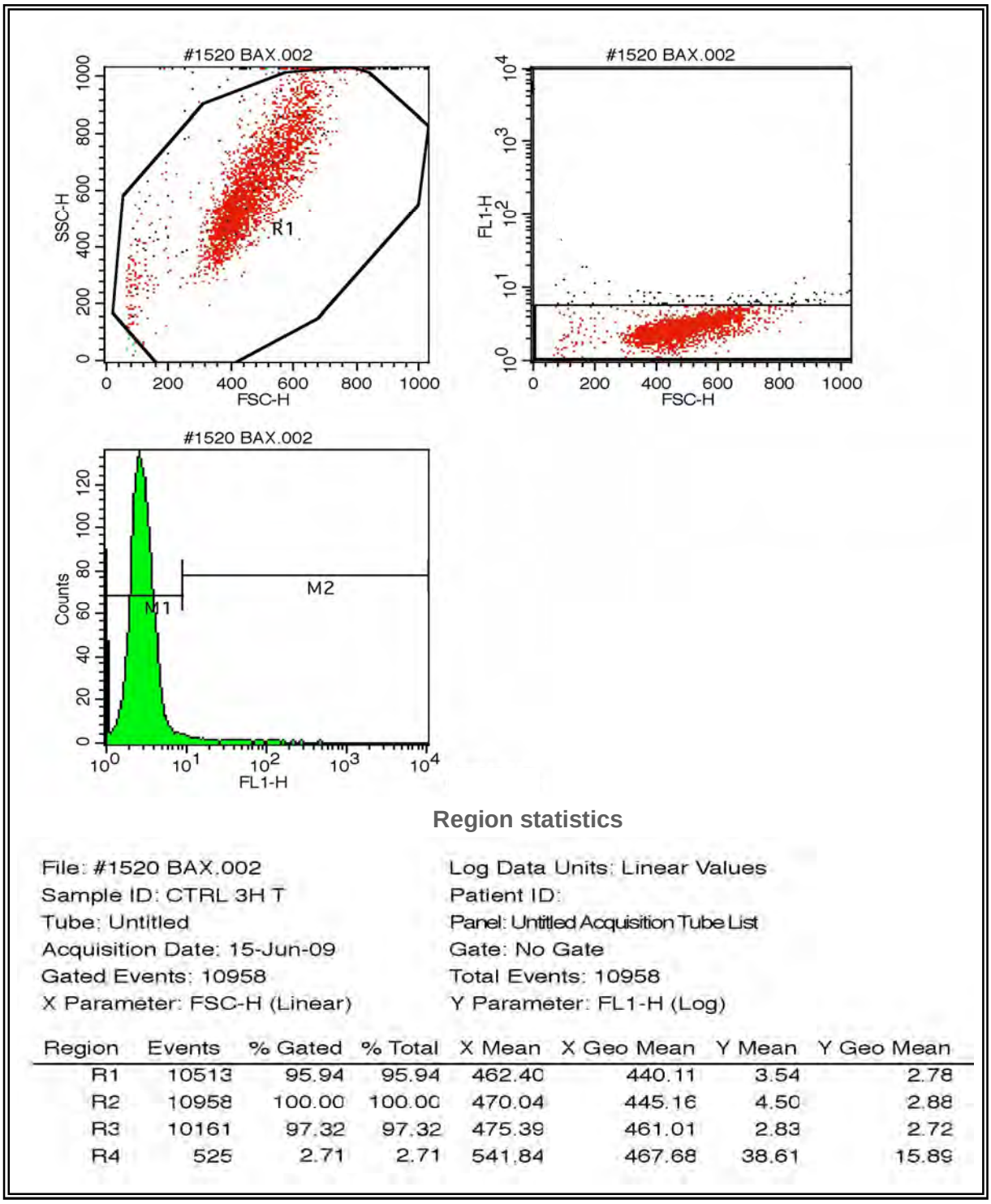
Region Statistics

File: #1495.002
Sample ID: HL60 CTRL 3hT
Tube: Untitled
Acquisition Date: 22-Apr-09
Gated Events: 10425
X Parameter: FSC-H (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 10425
Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	10423	99.98	99.98	349.55	336.78	49.06	46.37
R2	181	1.74	1.74	181.04	155.42	14.14	12.16
R3	10245	98.27	98.27	352.48	341.34	49.67	47.47

Figure 4-16: Bcl-2 analysis page of untreated HL-60 cells performed on at least 10000 cells using FACS Calibur instrument. Fluorescence pulses were detected using green laser emitting at 530 nm. FSC-H: Forward scatter height; SSC-H: Side scatter height; FL1-H: bandpass 530 nm.



Region statistics

File: #1520 BAX.002
Sample ID: CTRL 3H T
Tube: Untitled
Acquisition Date: 15-Jun-09
Gated Events: 10958
X Parameter: FSC-H (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 10958
Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	10513	95.94	95.94	462.40	440.11	3.54	2.78
R2	10958	100.00	100.00	470.04	445.16	4.50	2.88
R3	10161	97.32	97.32	475.39	461.01	2.83	2.72
R4	525	2.71	2.71	541.84	467.68	38.61	15.89

Figure 4-17: Bax analysis page of untreated HL-60 cells performed on at least 10000 cells using FACS Calibur instrument. Fluorescence pulses were detected using green laser emitting at 530 nm. FSC-H: Forward scatter height; SSC-H: Side scatter height; FL1-H: bandpass 530 nm.

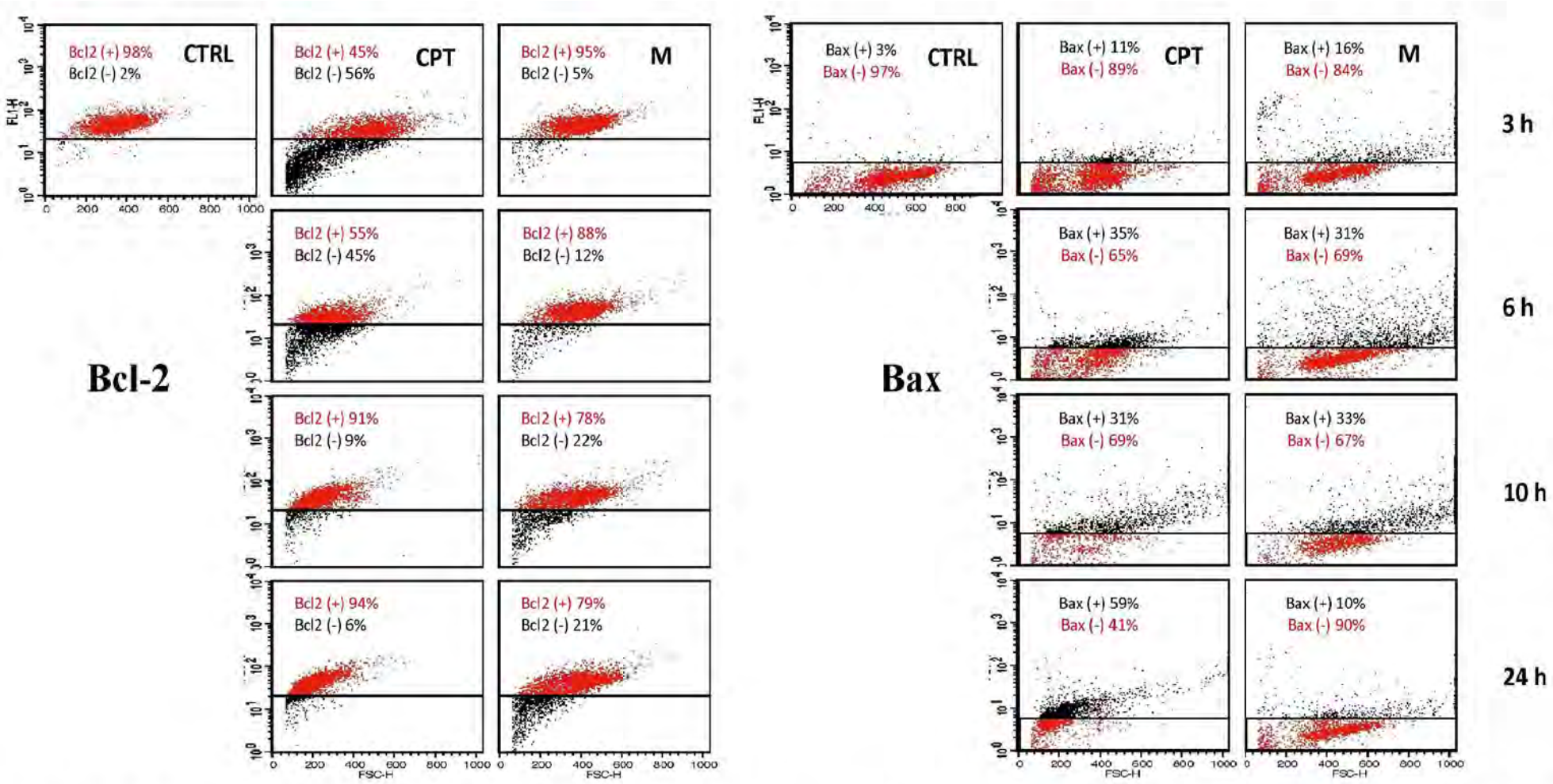


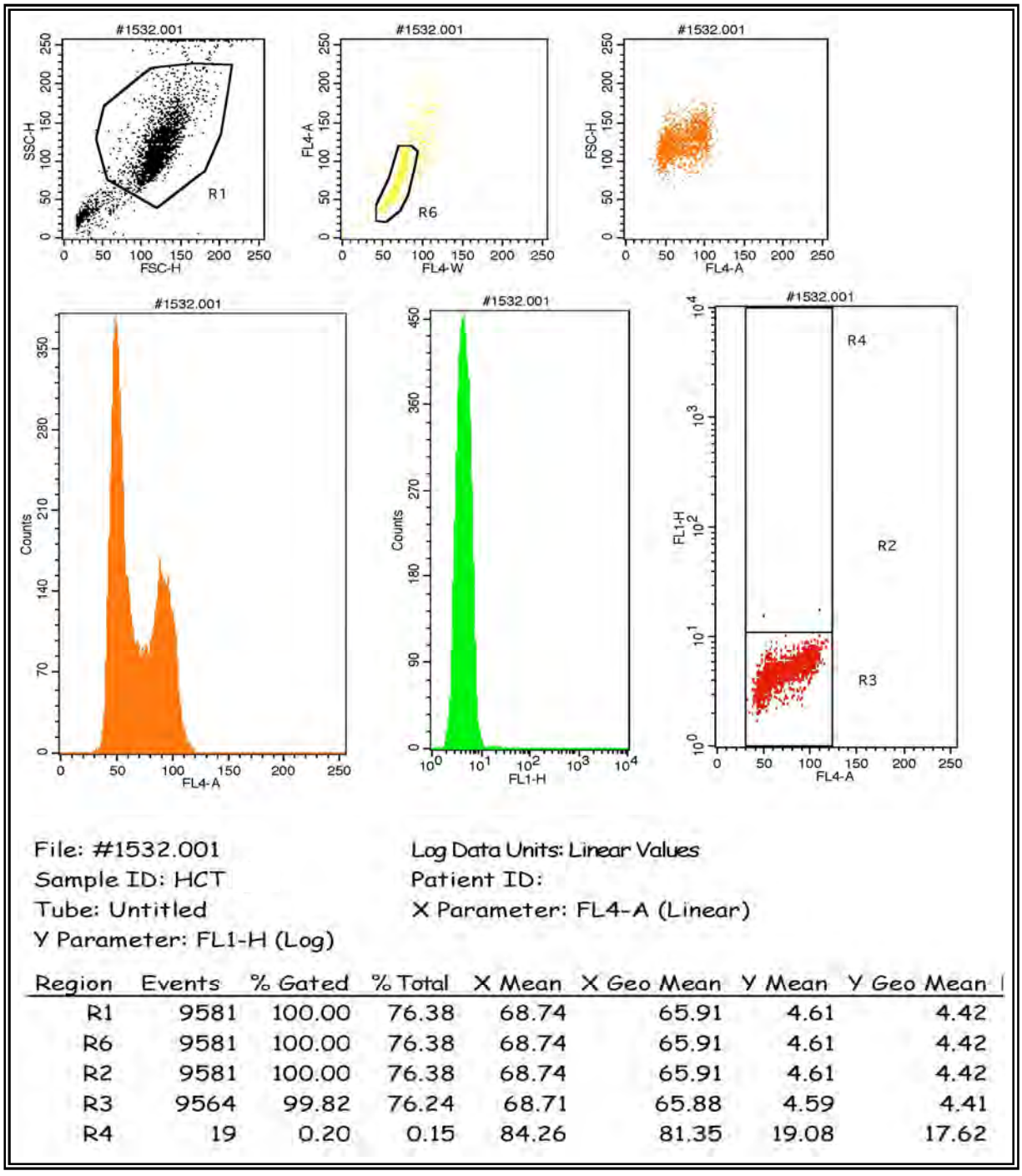
Figure 4-18: Flow cytometric analysis of Bcl-2 (left lane) and Bax (right lane) expressions in HL-60 cells. In both Bcl-2 and Bax lanes, the upper side shows the positive (+) expression while the lower side shows the negative (-) expression (percentage of expression). M: propolis sample from Mosul; CPT: Camptothecin; CTRL: Negative control.

4.2.3 Expression of *p53* Gene

In order to evaluate the cytotoxic effect of M propolis sample in relation to *p53* gene in HCT-116 cells, the p53 expression was examined by FACS and the analysis was carried out after 3, 6, 10, 24, 48 and 72 hours of treatment with M propolis sample extract (25µg ml^{-1}), and performed on at least 10000 cells in each analysis period. The fluorescence pulses were detected using green laser emitting at 530 nm (FL1) and red diode laser at 665 nm (FL4) for TO-PRO-3 iodide **(**TP3) emitted fluorescence detection (Figure 4-19).

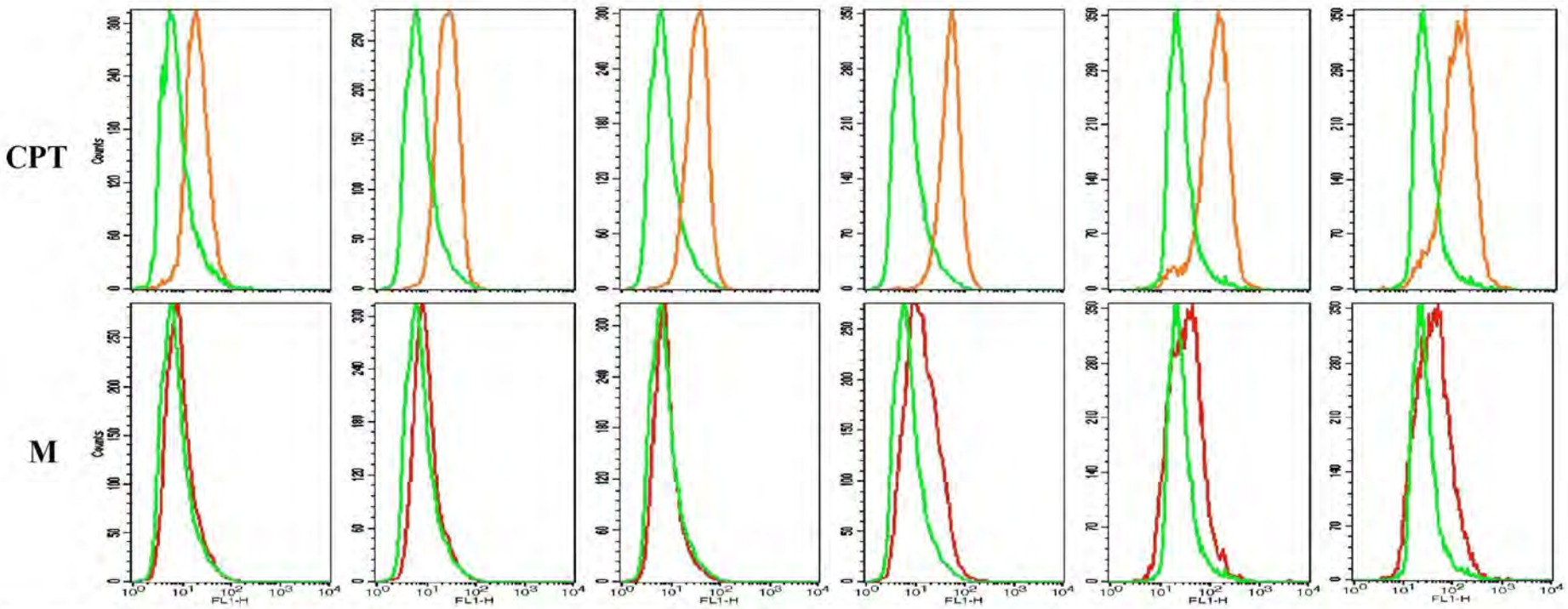
As shown in figure 4-20 (upper lane), the CPT (positive control; orange line) caused a gradual increased expression of p53 during the first four treatment periods (58.27, 75.09, 82.93 and 86.86%, respectively), and then the expression was gradually decreased at 48 and 72 hours treatments (71.79 and 63.50%, respectively).

With respect to M sample treated cells (red line), a slight increased expression of p53 was recorded after three hours treatment (4.80%), and such increase was also detected at six hours (5.57%), but after 10 hours it was reduced to reach no expression as compared to the p53 expression of untreated HCT-116 cells (Figure 4-20). However, after 24 hours treatment, the *p53* expression started to increase again, and 27.98% of the cells showed a positive expression, but at 48 hours, it was decreased to reach 5.14%. After 72 hours of treatment, the cells that showed positive expression of p53 were increased to reach 11.31% (Figure 4-20, lower lane).



Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	9581	100.00	76.38	68.74	65.91	4.61	4.42
R6	9581	100.00	76.38	68.74	65.91	4.61	4.42
R2	9581	100.00	76.38	68.74	65.91	4.61	4.42
R3	9564	99.82	76.24	68.71	65.88	4.59	4.41
R4	19	0.20	0.15	84.26	81.35	19.08	17.62

Figure 4-19: p53 analysis page of untreated HCT-116 cells performed on at least 10000 cells using FACS Calibur instrument. Fluorescence pulses were detected using green laser emitting at 530 nm and red diode laser at 665 nm for TP3 emitted fluorescence detection. FSC-H: Forward scatter height; SSC-H: Side scatter height; FL1-H: bandpass 530 nm; FL4- A and W: bandpass 665 Area and Width, respectively.



HCT-116 (%)	3 Hours		6 Hours		10 Hours		24 Hours		48 Hours		72 Hours	
	CPT	M	CPT	M	CPT	M	CPT	M	CPT	M	CPT	M
Total	99.86	99.97	99.93	99.87	99.72	99.83	99.85	99.86	99.96	100.00	99.72	100.00
Negative p53	41.59	95.17	24.84	94.30	16.79	99.83	12.99	71.88	28.17	94.86	36.22	88.69
Positive p53	58.27	4.80	75.09	5.57	82.93	0.00	86.86	27.98	71.79	5.14	63.50	11.31

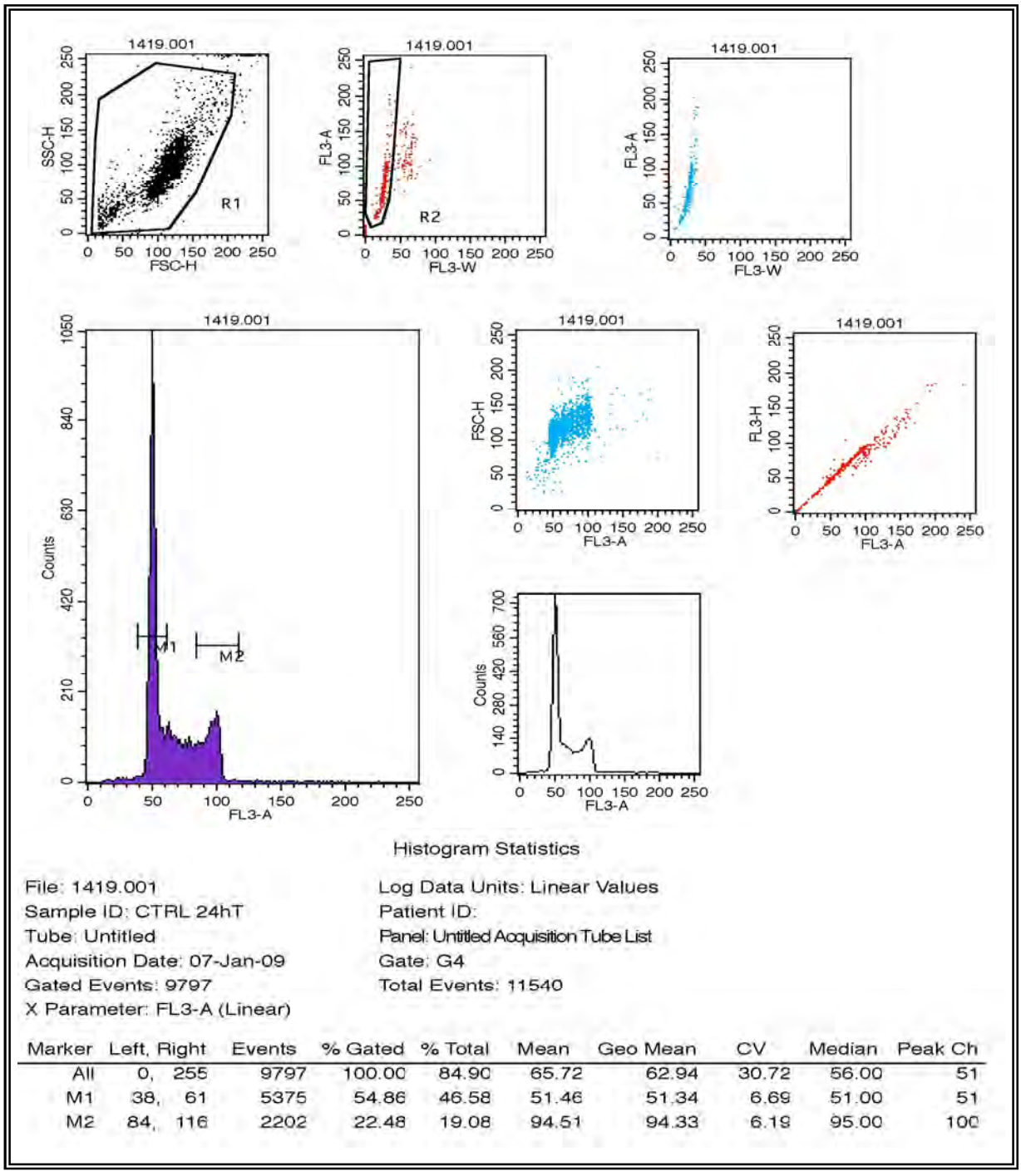
Figure 4-20: Flow-cytometric analysis of p53 expression in HCT-116 cells at various times (3, 6, 10, 24, 48 or 72 hours) after treatment with CPT (upper lane) and M (lower lane). In all panels, the green line shows the p53 expression in negative control, orange line shows the p53 expression in CPT (positive control) and red line shows the p53 expression in M. M: Propolis sample from Mosul; CPT: Camptothecin.

4.2.4 Monoparametric Cell Cycle Distribution Assay

To determine whether the M sample-induced growth delay was due to an arrest in any specific cell cycle phase, flow cytometric analysis was performed at 24, 48 and 72 hours post-treatment with different concentrations of M extract (5, 15 or 25 µg ml^{-1}). The analysis was done on at least 10000 cells and fluorescence pulses were detected using red diode laser at 610 nm for PI emitted fluorescence detection (Figure 4-21).

As shown in figure 4-22 (left panel), the HL-60 cells treated with 5 µg ml^{-1} at 48 and 72 hours exhibited an increase in the proportion of cells at the S phase (57 and 58%, respectively) and a decrease in G1 phase (38 and 34%, respectively), while no changes in cell cycle phases were observed at 24 hours (G1:44%; S: 52%; G2/M: 4%) as compared with untreated cells (G1: 44%; S: 52%; G2/M: 4%). In cells treated with 15 (58 and 60%, respectively) or 25 µg ml^{-1} (62 and 51%, respectively) at 24 and 48 hours, there was an accumulation of cells in the S phase too, while a G2/M accumulation (23%) was apparent only after a treatment with 25 µg ml^{-1} at 72 hours. Furthermore, a sub-G1 peak, in which DNA content was less than G1 peak and usually considered as an apoptotic cells, was observed after a treatment with 15 or 25 µg ml^{-1} at different time intervals (Figure 4-22; left panel). The cells exhibited reduced forward scatter, which was indicative of a decreased relative size, and enhanced side scatter, which was indicative of an increased internal complexity.

On the other hand, the HCT-116 cells treated with 5 µg ml^{-1} exhibited an accumulation of cells in G1 phase at 24, 48 or 72 hours (31, 49 and 54%, respectively) as compared with untreated cells (22, 44, and 44%, respectively). In cells treated with 15 or 25 µg ml^{-1}, an accumulation of cells in the G1 was observed at 24 and 72 hours, while the main accumulation of G2/M phase was at 48 hours. Additionally, the sub-G1 peak was not observed in HCT-116 cells during the investigated time intervals (Figure 4-22; right panel).



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 255	9797	100.00	84.90	65.72	62.94	30.72	56.00	51
M1	38, 61	5375	54.86	46.58	51.46	51.34	6.69	51.00	51
M2	84, 116	2202	22.48	19.08	94.51	94.33	6.19	95.00	100

Figure 4-21: Monoparametric DNA cell cycle analysis page of untreated HL-60 cells performed on at least 10000 cells using FACS Calibur instrument. Fluorescence pulses were detected using red diode laser at 610 nm for PI emitted fluorescence detection. FSC-H: Forward scatter height; SSC-H: Side scatter height; FL3-A and W: bandpass 610 nm Area and Width, respectively.

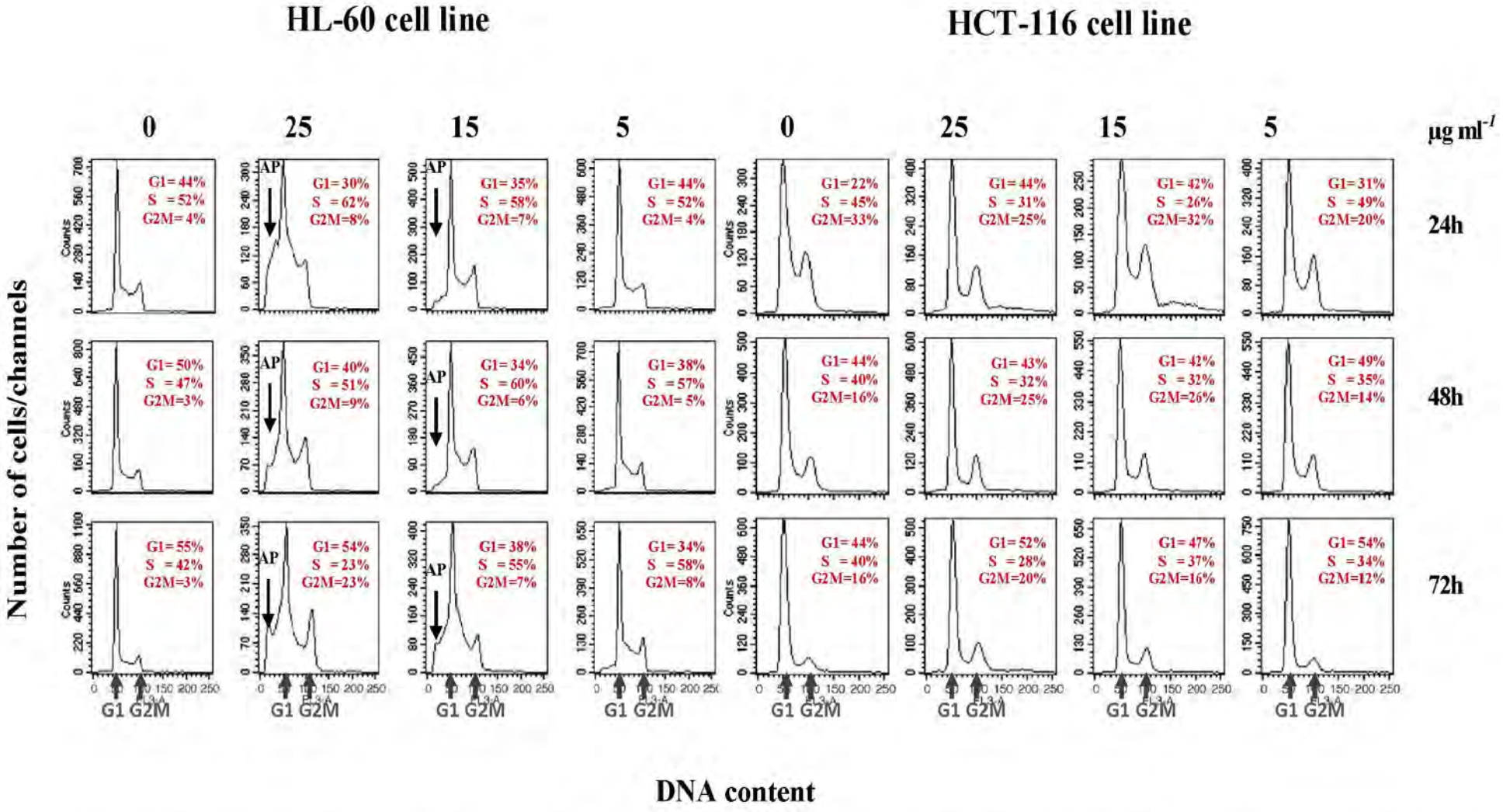


Figure 4-22: Cell cycle perturbations induced in HL-60 (left side) and HCT-116 (right side) cell lines by different concentrations of M sample performed after 24, 48 and 72 hours. AP: Apoptotic cells.

4.2.5 Biparametric BrdUrd/DNA Cell Cycle Assay

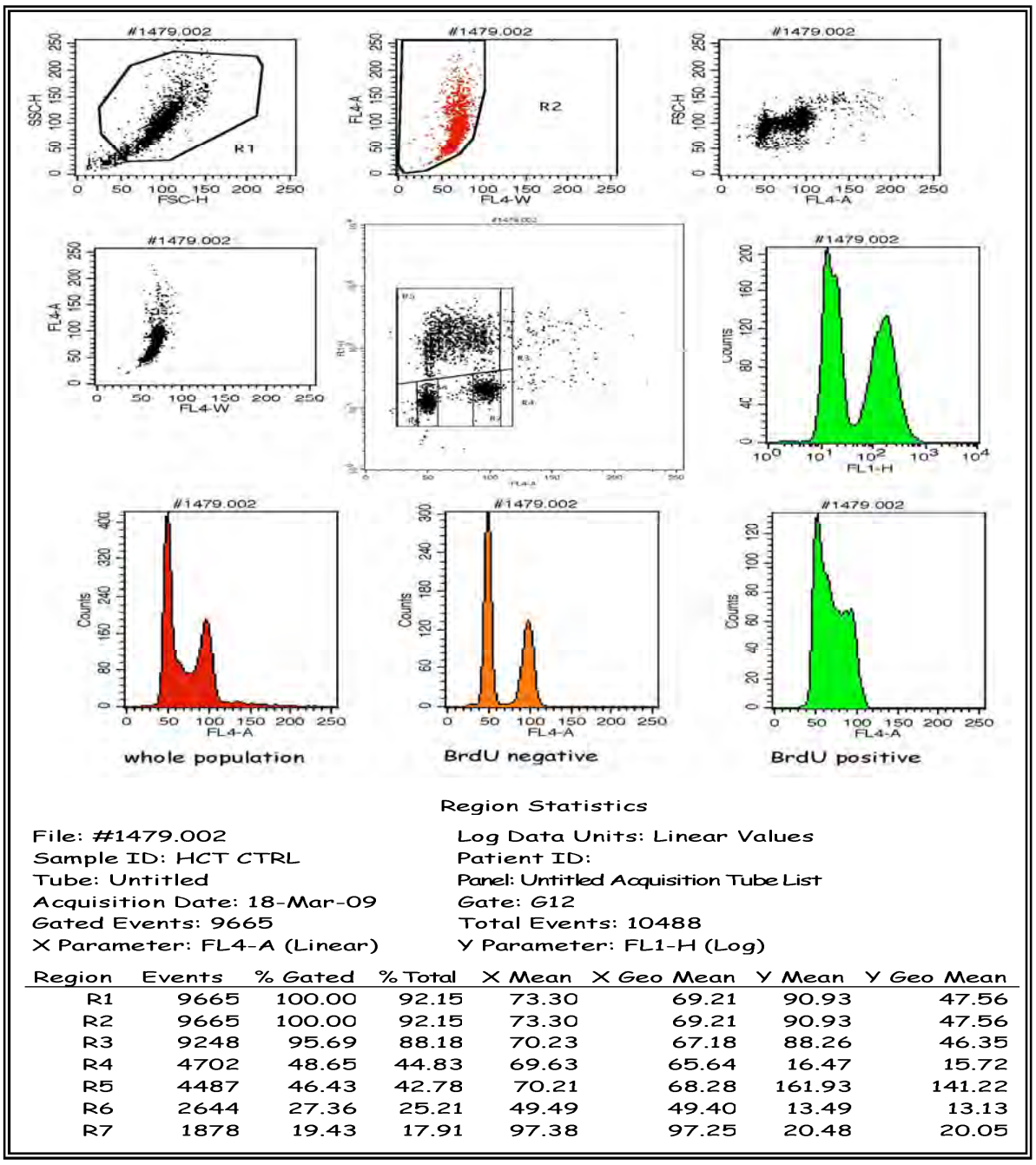
Biparametric BrdUrd/DNA cell cycle analysis was examined on at least 20000 cells using FACS Calibur instrument, and fluorescence pulses were detected using green laser emitting at 530 nm (FL1) and red diode laser at 665 nm (FL4) for TP3 emitted fluorescence detection (Figure 4-23). By applying this protocol, it was possible to obtain a distinct evaluation of the cell cycle perturbations in cells that were in S phase (BrdUrd-positive cells) or in G1 or G2/M phase (BrdUrd-negative cells).

The BrdUrd/DNA cell cycle assay for HL-60 (Figures 4-24 and 4-25) and HCT-116 (Figures 4-26 and 4-27) were performed at 0, 2, 4, 6, 8, 10, 24, 36 and 48 hours after a treatment with 25 µg ml^{-1} of M propolis extract. In figures 4-24 and 4-26, panels A show the biparametric BrdUrd/DNA cytograms of control and M-treated cells. The cells (HL-60 and HCT-116) in S phase (BrdUrd-positive cells) at the start of treatment progressed through this phase of the cell cycle more slowly than untreated cells. In HL-60 cell line, a fraction of new G1 BrdUrd positive cells was detected in the control cells at 4 hours, while in M-treated cells; this cell population was detectable only after 10 hours post-treatment. Although this block was found in both cell lines, but it was more evident in HL-60 cells than HCT-116 cells. In both cell lines, the cells that were in G1 phase (BrdUrd negative cells) at the start of treatment remained blocked in this phase for less than 24 hours, and started to show S phase cell population only after 24 hours.

Panels B in figures 4-24 and 4-26 show the cell growth histogram, which demonstrates the growth inhibition of exponential growing cells evaluated at different time intervals. Also, both cell lines showed a reduction in number of cells and the effect was time-dependent, but the highest reduction was recorded at eight hours treatment.

Figures 4-25 and 4-27 show the DNA frequency histograms of whole population; BrdUrd-negative and BrdUrd-positive of untreated cells and treated

cells. The same picture was drawn with both cell lines and recorded that M extract caused a block of the cells in the G1 phase of the cell cycle, with different kinetic for BrdUrd-positive and negative cells.



Region Statistics

File: #1479.002
Sample ID: HCT CTRL
Tube: Untitled
Acquisition Date: 18-Mar-09
Gated Events: 9665
X Parameter: FL4-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G12
Total Events: 10488
Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	9665	100.00	92.15	73.30	69.21	90.93	47.56
R2	9665	100.00	92.15	73.30	69.21	90.93	47.56
R3	9248	95.69	88.18	70.23	67.18	88.26	46.35
R4	4702	48.65	44.83	69.63	65.64	16.47	15.72
R5	4487	46.43	42.78	70.21	68.28	161.93	141.22
R6	2644	27.36	25.21	49.49	49.40	13.49	13.13
R7	1878	19.43	17.91	97.38	97.25	20.48	20.05

Figure 4-23: Biparametric BrdUrd/DNA cell cycle analysis page of untreated HCT-116 cells performed on at least 20000 cells using FACS Calibur instrument. Fluorescence pulses were detected using green laser emitting at 530 nm and red diode laser at 665 nm for TP3 emitted fluorescence detection. FSC-H: Forward scatter height; SSC-H: Side scatter height; FL1-H: bandpass 530 nm; FL4-A and W: bandpass 665 nm Area and Width, respectively.

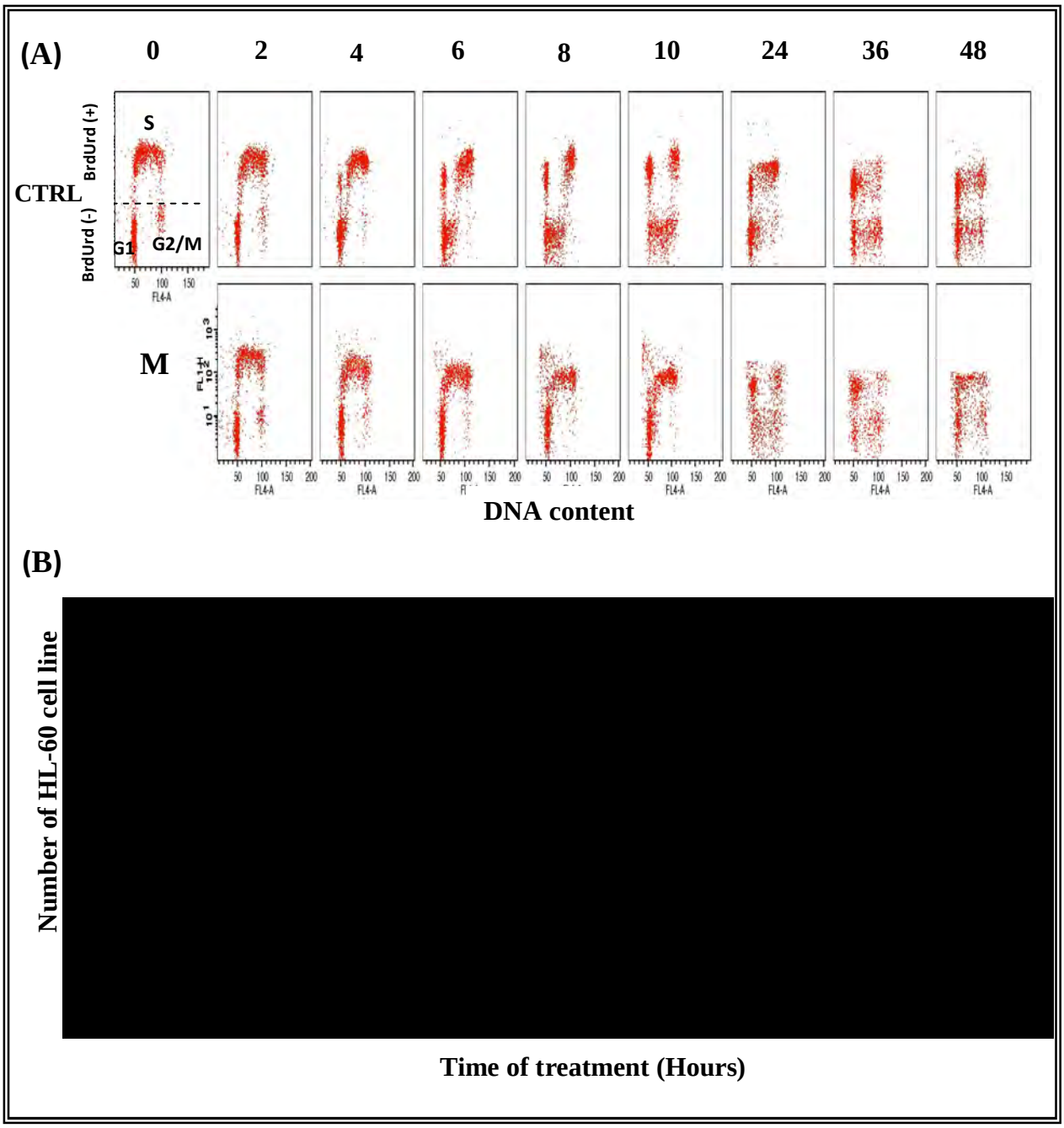


Figure 4-24: Effects of M propolis sample on cell cycle distribution of HL-60 cells evaluated at 0, 2, 4, 6, 8, 10, 24, 36 and 48 h. (A) Biparametric BrdUrd/DNA analysis of untreated (CTRL) and treated cells. (B) Cell growth histogram shows the growth inhibition of exponential growing cells evaluated at different time intervals. M: Propolis sample from Mosul. BrdUrd: 5-bromo-2-deoxyuridine.

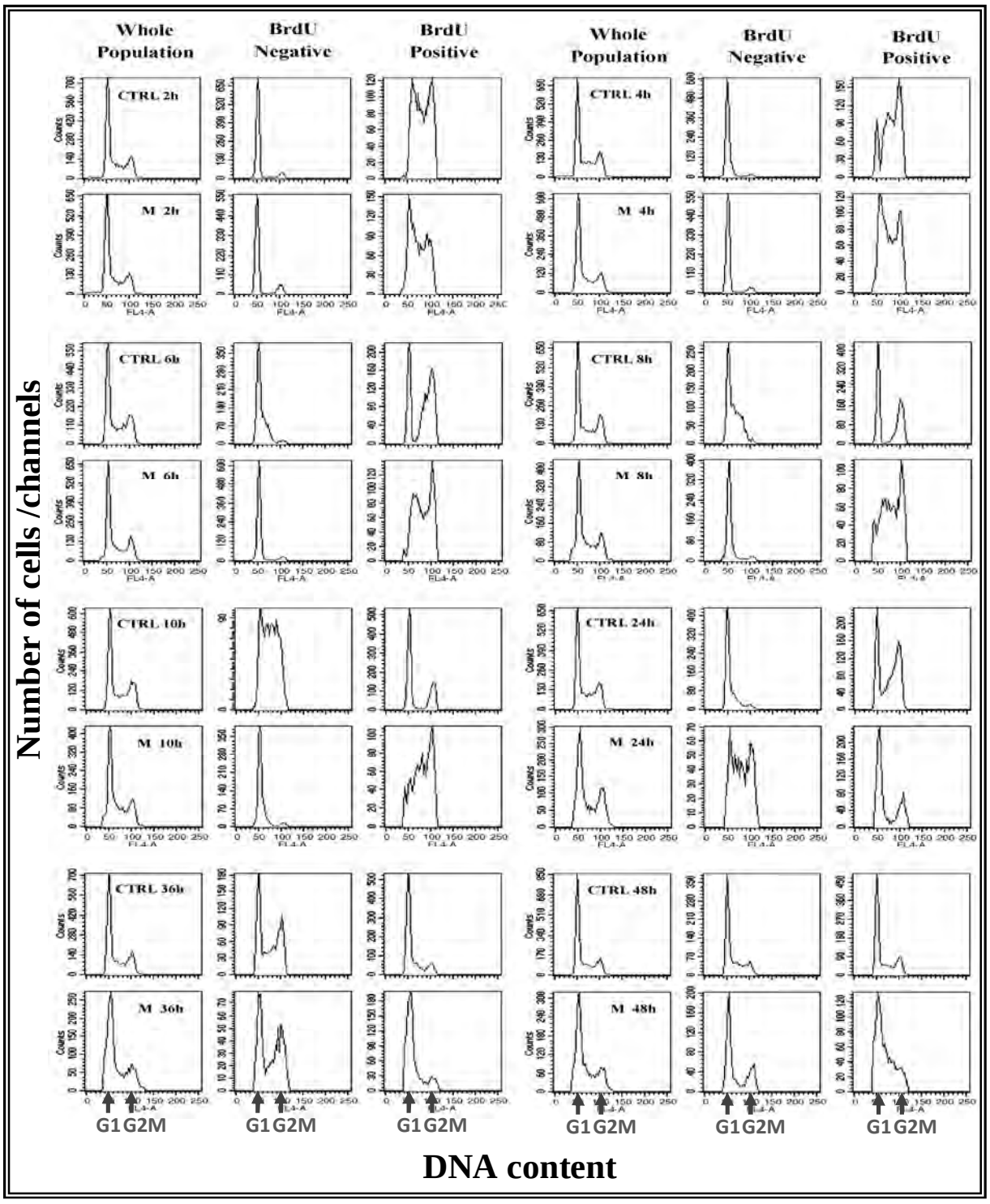


Figure 4-25: DNA frequency histograms of whole population; BrdUrd-negative and BrdUrd-positive of HL-60 untreated (CTRL) cells and cells treated with 25 µg ml^{-1} of M sample at different time intervals. M: Propolis sample from Mosul. BrdUrd: 5-bromo-2-deoxyuridine.

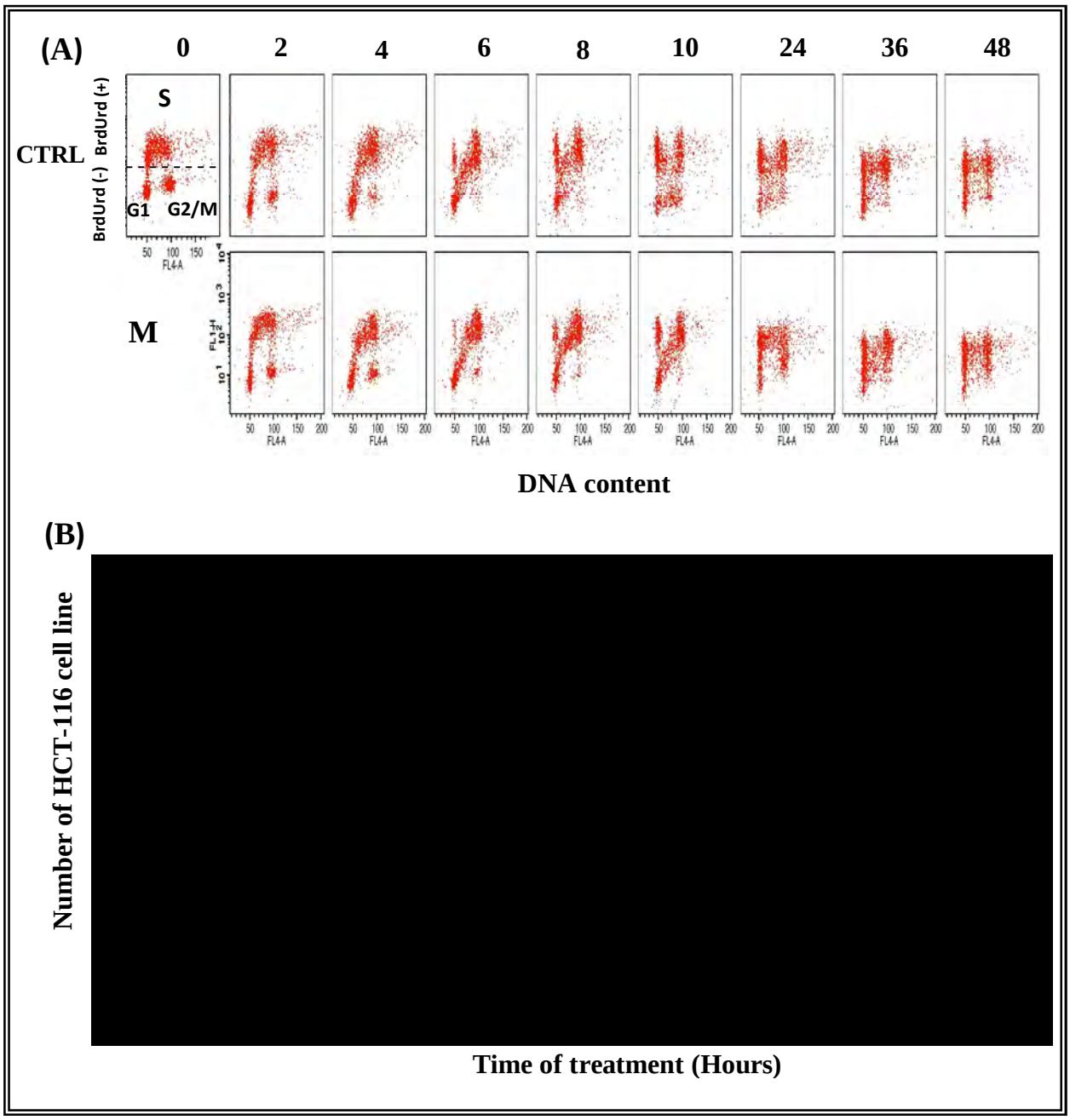


Figure 4-26: Effects of M propolis sample on cell cycle distribution in HCT-116 cells evaluated at 0, 2, 4, 6, 8, 10, 24, 36 and 48 hours. (A) Biparametric BrdUrd/DNA analysis of untreated (CTRL) and treated cells. (B) Cell growth histogram shows the growth inhibition of exponential growing cells evaluated at different time intervals. M: Propolis sample from Mosul. BrdUrd: 5-bromo-2-deoxyuridine.

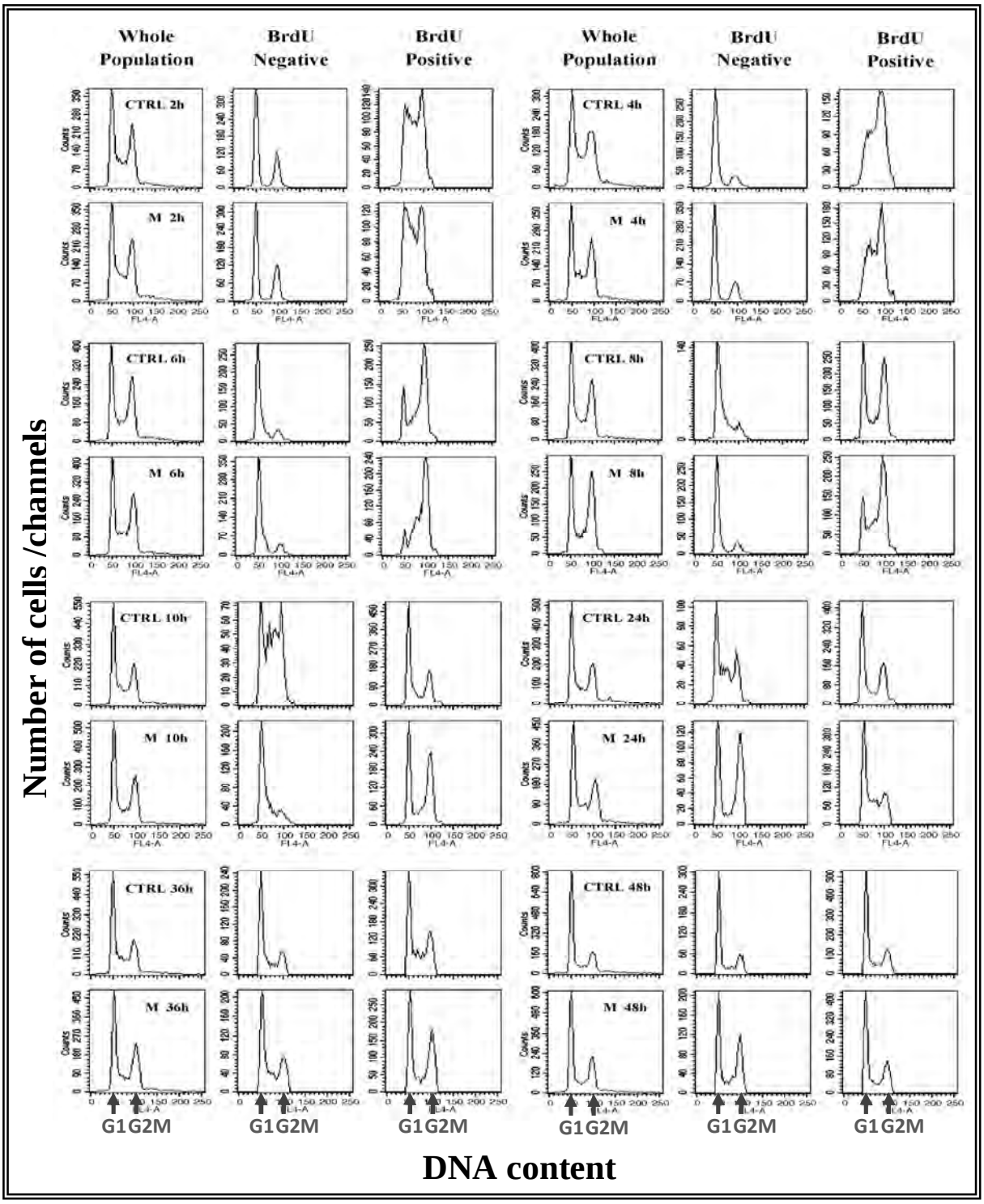
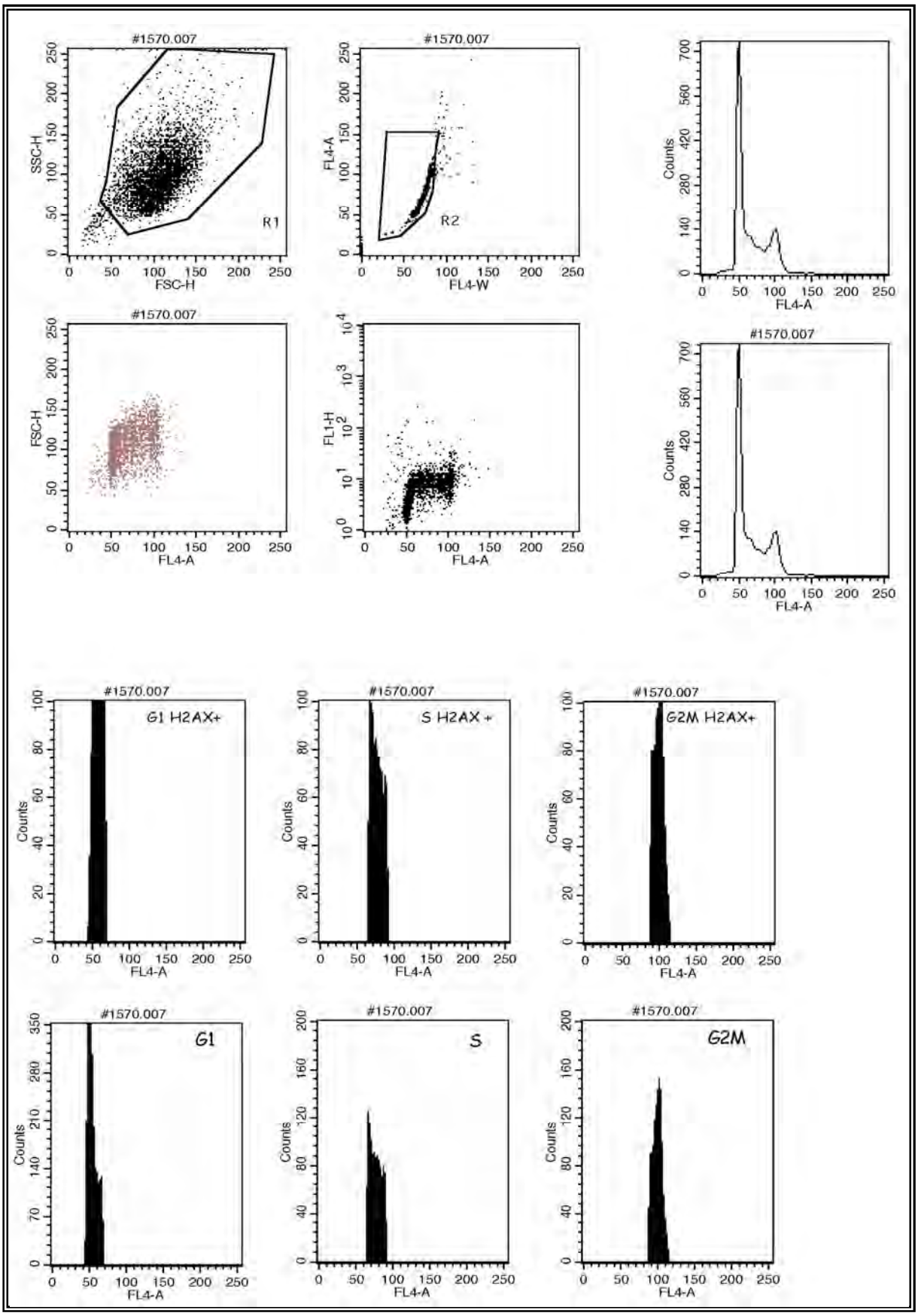


Figure 4-27: DNA frequency histograms of whole population; BrdUrd-negative and BrdUrd-positive of HCT-116 untreated (CTRL) cells and cells treated with 25 µg ml^{-1} of M sample at different time intervals. M: Propolis sample from Mosul. BrdUrd: 5-bromo-2-deoxyuridine.

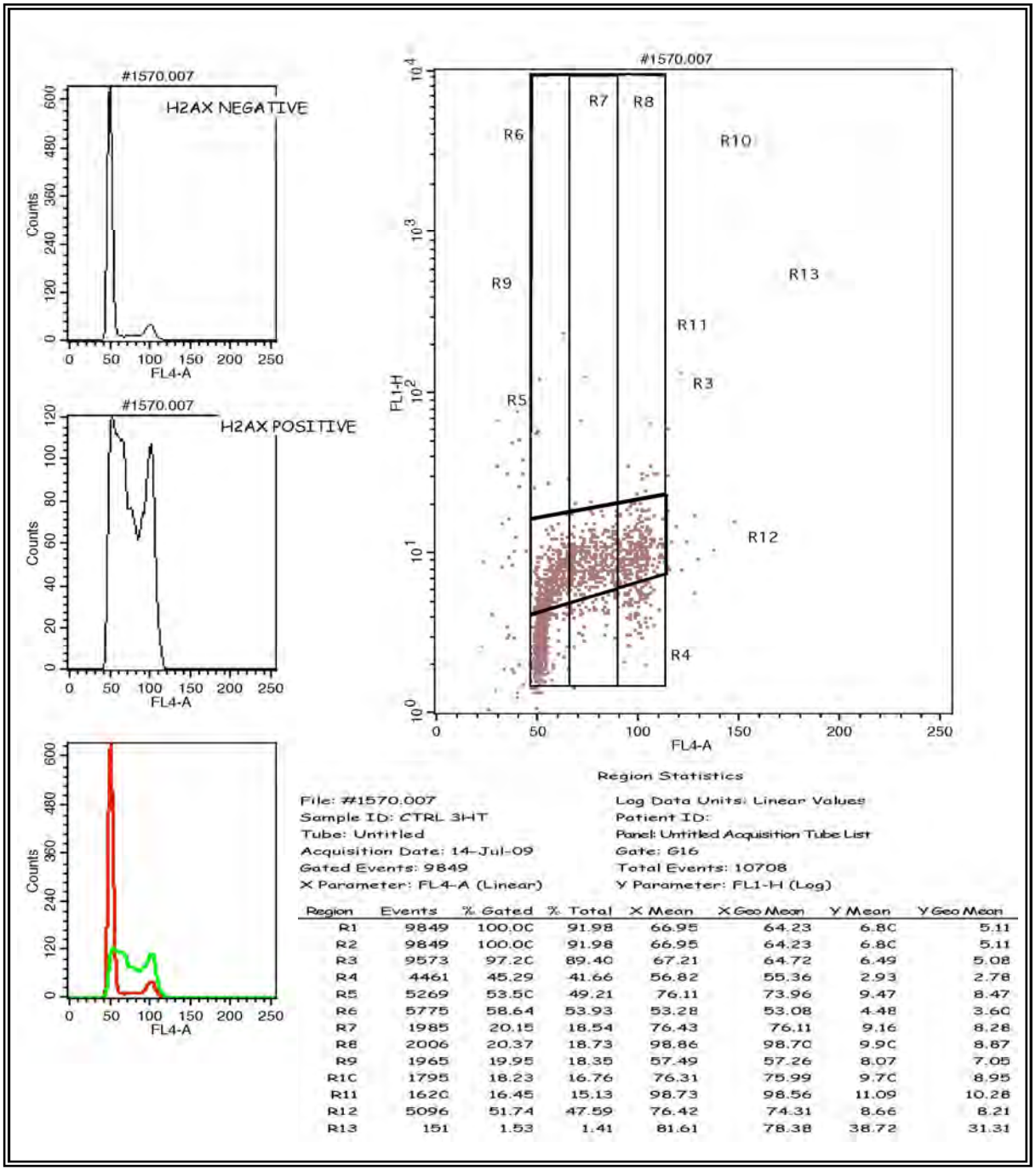
4.2.6 γ-H2AX Histone

In a further assay, it was tested whether 25 µg ml^{-1} of M sample can induce H2AX phosphorylation in HL-60 and HCT-116 cells at 3, 6, 10, 24, 36 and 48 hours post-treatment. As shown in figure 4-28, the analysis was performed on at least 10000 cells using FACS Calibur instrument and fluorescence pulses were detected using green laser emitting at 530 nm (FL1) and red diode laser at 665 nm (FL4) for TP3 emitted fluorescence detection.

The expression of γ-H2AX was measured concurrently with cellular DNA content by flow cytometry (Figures 4-29 and 4-30), and data are presented as log γ-H2AX expression histogram with positive (green line), negative (red line) (lane A) and dot plot of bivariate γ-H2AX *versus* DNA content distributions (lane B). As shown in figure 4-29, the degree of γ-H2AX staining in treated HL-60 cells was clearly increased in a time-dependent manner, showing a fraction of cells with an increased γ-H2AX immunofluorescence (11.0, 10.0, 11.5, 13.5, 12.4 or 16.5%, respectively) for the six investigated periods. Such increase was not observed in HCT-116 cells (Figure 4-30).


#1570.007
SSC-H
FSC-H
R1
#1570.007
FL4-A
FL4-W
R2
#1570.007
Counts
FL4-A
#1570.007
FSC-H
FL4-A
#1570.007
FL1-H
FL4-A
#1570.007
Counts
FL4-A
#1570.007
G1 H2AX+
Counts
FL4-A
#1570.007
S H2AX +
Counts
FL4-A
#1570.007
G2M H2AX+
Counts
FL4-A
#1570.007
G1
Counts
FL4-A
#1570.007
S
Counts
FL4-A
#1570.007
G2M
Counts
FL4-A

Continued



Region Statistics

File: #1570.007
Sample ID: CTRL 3HT
Tube: Untitled
Acquisition Date: 14-Jul-09
Gated Events: 9849
X Parameter: FL4-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G16
Total Events: 10708
Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	9849	100.00	91.98	66.95	64.23	6.80	5.11
R2	9849	100.00	91.98	66.95	64.23	6.80	5.11
R3	9573	97.20	89.40	67.21	64.72	6.49	5.08
R4	4461	45.29	41.66	56.82	55.36	2.93	2.78
R5	5269	53.50	49.21	76.11	73.96	9.47	8.47
R6	5775	58.64	53.93	53.28	53.08	4.48	3.60
R7	1985	20.15	18.54	76.43	76.11	9.16	8.28
R8	2006	20.37	18.73	98.86	98.70	9.90	8.87
R9	1965	19.95	18.35	57.49	57.26	8.07	7.05
R10	1795	18.23	16.76	76.31	75.99	9.70	8.95
R11	1620	16.45	15.13	98.73	98.56	11.09	10.28
R12	5096	51.74	47.59	76.42	74.31	8.66	8.21
R13	151	1.53	1.41	81.61	78.38	38.72	31.31

Figure 4-28: γ-H2AX histone analysis page of untreated HL-60 cells performed on at least 1000 cells using FACS Calibur instrument. Fluorescence pulses were detected using green laser emitting at 530 nm and red diode laser at 665 nm for TP3 emitted fluorescence detection. FSC-H: Forward scatter height; SSC-H: Side scatter height; FL1-H: bandpass 530 nm; FL4- A and W: bandpass 665 nm Area and Width, respectively.

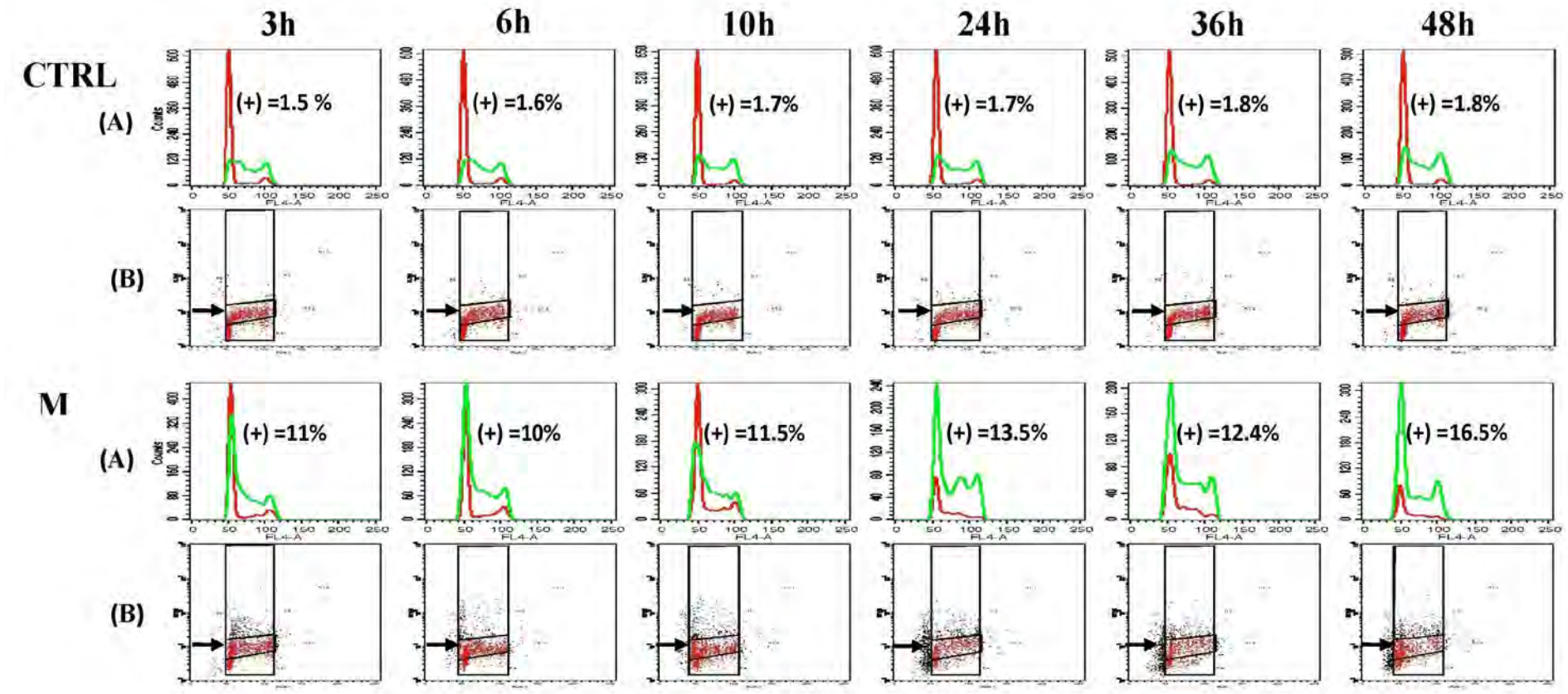


Figure 4-29: Induction of γ-H2AX expression in HL-60 cells after treatment with M propolis extract. The expression of γ-H2AX was measured concurrently with cellular DNA content by flow cytometry, and data are shown as log γ-H2AX expression histogram with positive (green line), negative (red line) (A) and dot plot of bivariate γ-H2AX *versus* DNA content distributions (B). Over 98% of the cells from the untreated (CTRL) cells expressed γ-H2AX inside the threshold box marked with black arrows. Percentage of positive expression is indicated at different time intervals.

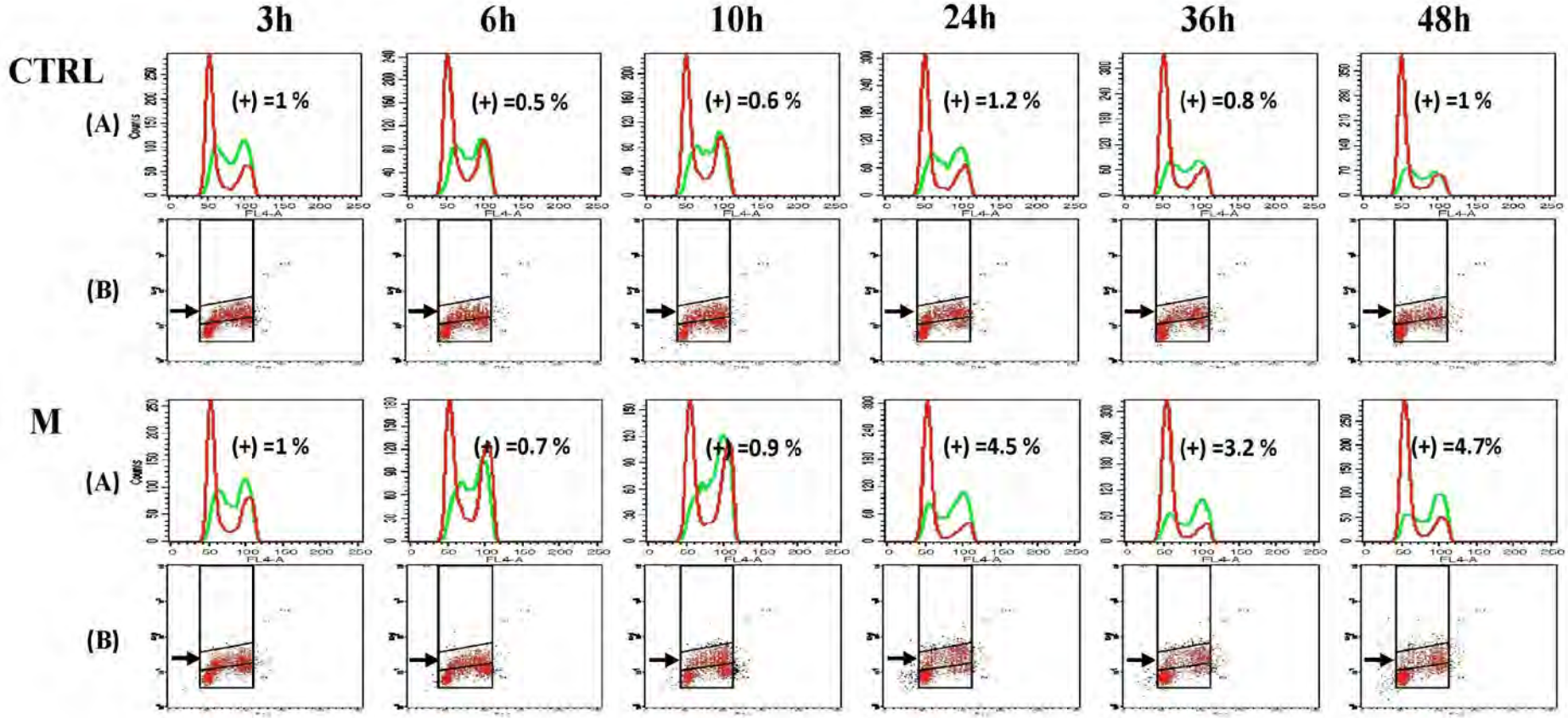


Figure 4-30: Induction of γ-H2AX expression in HCT-116 cells after treatment with M propolis extract. The expression of γ-H2AX was measured concurrently with cellular DNA content by flow cytometry, and the data are shown as log γ-H2AX expression histogram with positive (green line), negative (red line) (A) and dot plot of bivariate γ-H2AX *versus* DNA content distributions (B). Over 98% of the cells from the untreated (CTRL) cells expressed γ-H2AX inside the threshold box marked with black arrows. Percentage of positive expression is indicated at different time intervals.

4.3 *In Vivo* Investigations

4.3.1 Assessment of Anti-tumour Potential

To evaluate the anti-tumour potential of M propolis extract, 60 Fox N1nu/nu nude mice were xenografted with HCT-116 tumour cells. When the tumours reached 94 mm^3 on day 7, the animals were divided into six groups as mentioned in chapter three. In orally (p.o.) treated animals, the tumour size was measured on days 8, 12, 14, 16, 19, 22, 26, 28, 30, 33 and 35, while the corresponding days in intrapertoneally (i.p.) treated animals were 8, 12, 14, 16, 19, 22, 24, 26 and 28. However, the data were presented for four periods only as shown in tables 4-7 and 4-8. Mouse-bearing tumours for p.o. or i.p. groups are given respectively in figures 4-31 and 4-32.

In p.o. groups, the vehicle mice (controls) showed a tumour size of 94.4 mm^3 on day zero of treatment (i.e. day 8 post-tumour cell implantation), and then the size of tumour increased gradually during the investigated period and reached 985.9, 1205.9 and 1262.8 mm^3 on days 22, 25 and 27, respectively (i.e. days 30, 33 and 35 post-tumour cell implantation). Such differences were significant ($P \leq 0.05$), with the exception of days 25 and 27, in which no significant difference between them was observed. Treating tumour-bearing mice with 500 mg kg^{-1} of M propolis extract was associated with a decreased size of the xenografted tumours, and the corresponding sizes were 834.1, 916.0 and 921.9 mm^3, respectively, but a significant difference was reached on day 27 (921.9 *vs*. 1262.8 mm^3) (Table 4-7). The corresponding tumour inhibition rates were 15.39, 24.03 and 26.99%, respectively (Figure 4-33). Applying the dose 1000 mg kg^{-1} was involved in a further reduction of tumour size, and the corresponding figures were 683.4, 733.6 and 847.8 mm^3, respectively (Table 4-7). Such reductions were significant as compared to the corresponding sizes in vehicle mice, and demonstrated tumour inhibition rates of 30.68, 39.16 and 32.86%, respectively (Figure 4-33). Based on these findings, the calculated

tumour index on day 27 (day 35 post-tumour cell implantation) was much lower in animals treated with the two doses of propolis (500 and 1000 mg kg^{-1}) as compared with vehicle animals (2.38 and 2.33%, respectively *vs*. 4.00%) (Figure 4-34).

Intrapertoneally-treated animals were evaluated on days 16, 18 and 20 post treatment (i.e. days 24, 26 and 28 post-tumour cell implantation), and this earlier evaluation was reasoned by the fact that some animals started to die (one mouse on day 16 for each dose and a further one on day 18 for the dose 1000 mg kg^{-1}). The tumour in these animals also showed a reduced size, which was dependent on time, as well as, dose. In dose 500 mg kg^{-1}, the tumour size was 433.1, 573.2 and 633.7 mm^3 on days 16, 18 and 20, respectively, while in dose 1000 mg kg^{-1}, it was 511.8, 581.4 and 567.6 mm^3, respectively. The corresponding sizes in vehicle mice were 501.3, 689.1 and 822.9 mm^3, respectively. However, the tumour inhibition rate was less than that observed in p.o. treated mice, and it was 13.61, 16.81 and 22.98%, respectively at the dose 500 mg kg^{-1}, while it was -2.0, 15.62 and 31.02%, respectively at the dose 1000 mg kg^{-1} (Table 4-8 and Figure 4-35).

To evaluate the deleterious effects of tumour implantation and/or M propolis extract treatments on mice throughout the investigated period, the body weight was considered as a parameter for this regard. The p.o treated animals (vehicle or the two doses of propolis) did not show a significant weight variation (Table 4-7). However, i.p. mice contradicted such observation, and instead, animals treated with either doses of propolis started to have a weight loss, which paralleled the progression of investigated period. In this regard, the dose 1000 mg kg^{-1} was most effective, and the highest loss was observed on day 20 (day 28 post-tumour cell implantation)as compared to mice of vehicle group or the dose 500 mg kg^{-1} (Table 4-8).

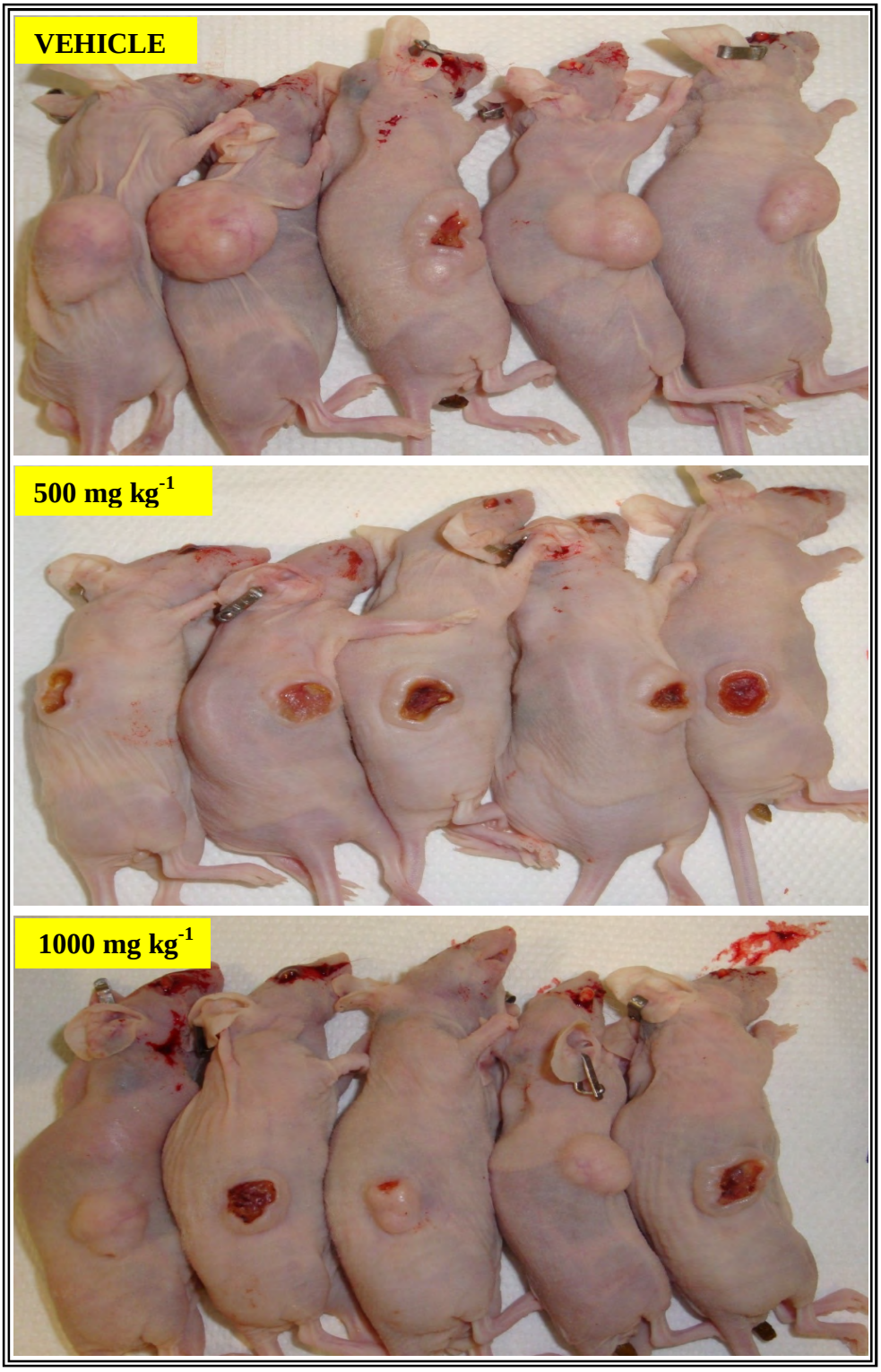


Figure 4-31: Tumour mass of orally treated-nude mice xenografted with HCT-116 cells. Vehicle: Untreated mice; 500 and 1000 mg kg^{-1}: Mice treated with two doses of M propolis extract.

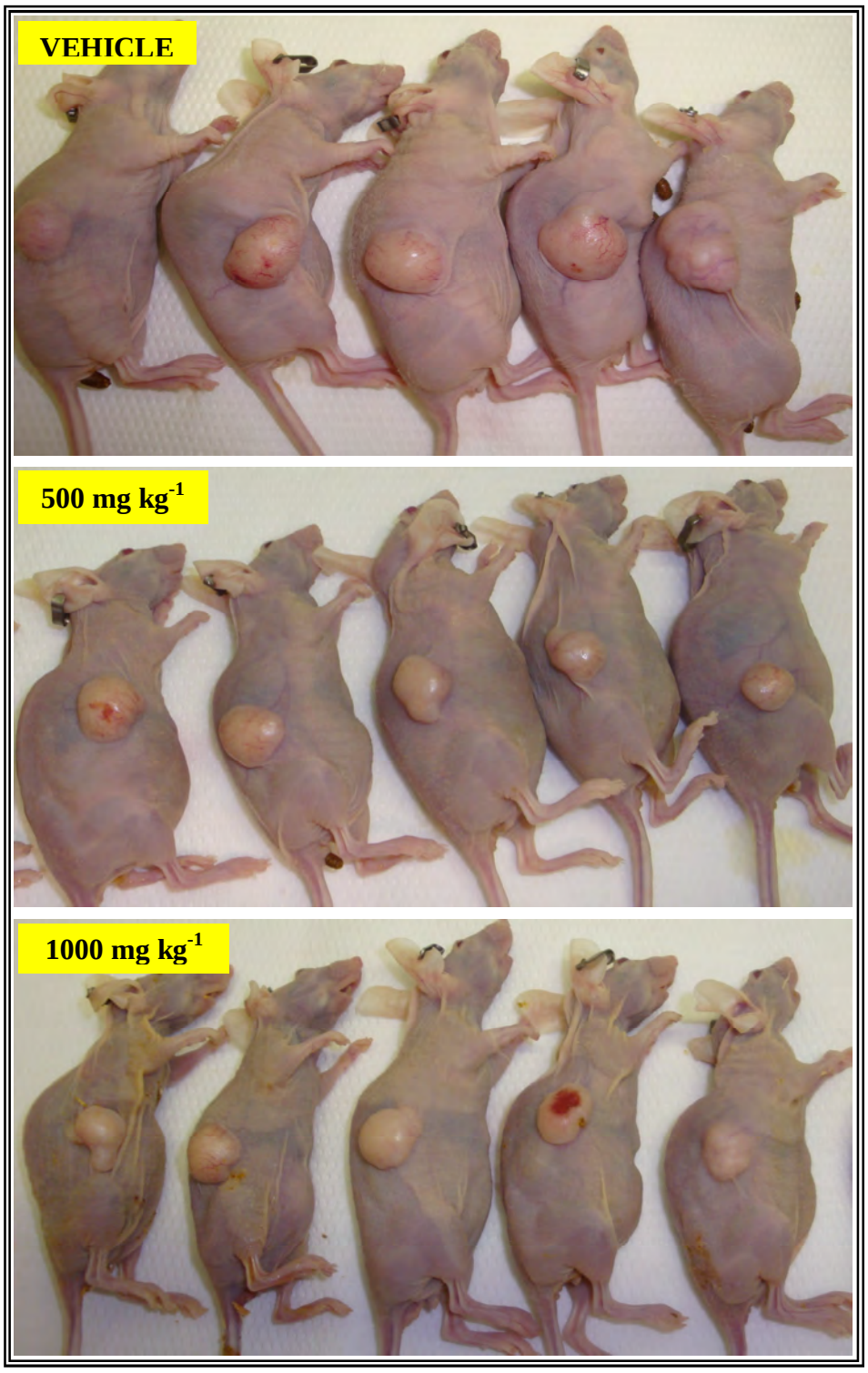


Figure 4-32: Tumour mass of intraperitoneally treated -nude mice xenografted with HCT-116 cells. Vehicle: Untreated mice; 500 and 1000 mg kg^{-1}: Mice treated with two doses of M propolis extract.

Table 4-7: Tumour volume in tumour-bearing nude mice treated orally with M propolis extract.

Groups	Dose	No. of Mice	Day*	Tumour Size** Mean ± S.E. (mm^3)	Mouse Weight** Mean±S.E. (gram)
Vehicle	0.0	10	0+	94.4 ± 9.0^{E}	21.4 ± 0.5^{A}
		10	22	985.9 ± 100.9^{B}	21.5 ± 0.6^{A}
		10	25	1205.9 ± 156.3^{AB}	21.2 ± 0.7^{A}
		10	27	1262.8 ± 160.1^{A}	21.5 ± 0.7^{A}
M Propolis Extract Treated Mice	500 mg kg^{-1}	10	0+	94.0 ± 10.0^{E}	21.6 ± 0.2^{A}
		10	22	834.1 ± 108.2^{CD}	23.2 ± 0.7^{A}
		10	25	916.0 ± 119.5^{BCD}	22.3 ± 0.7^{A}
		10	27	921.9 ± 127.8^{BC}	22.7 ± 0.8^{A}
	1000 mg kg^{-1}	10	0+	94.1 ± 7.9^{E}	21.7 ± 0.6^{A}
		10	22	683.4 ± 113.3^{D}	22.7 ± 0.6^{A}
		10	25	733.6 ± 126.6^{D}	22.8 ± 0.5^{A}
		10	27	847.8 ± 137.7^{CD}	22.3 ± 0.7^{A}

*Days post-M sample treatment. + Day 8 post-tumour cells implantation.

**Different letters: Significant difference ($P \leq 0.05$) between means of the same column.

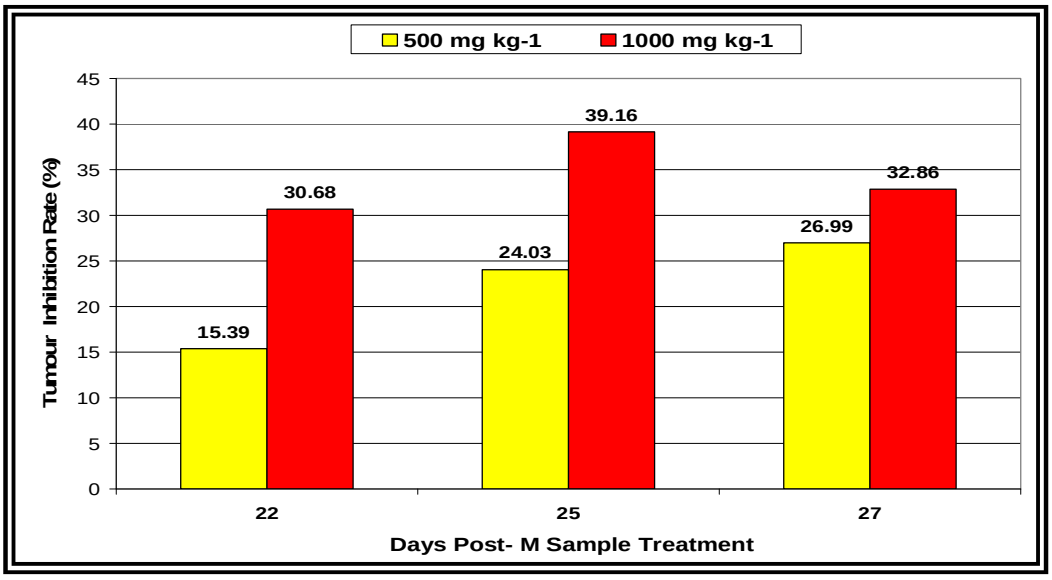


Figure 4-33: Tumour inhibition rate in tumour-bearing nude mice treated orally with M propolis extract.

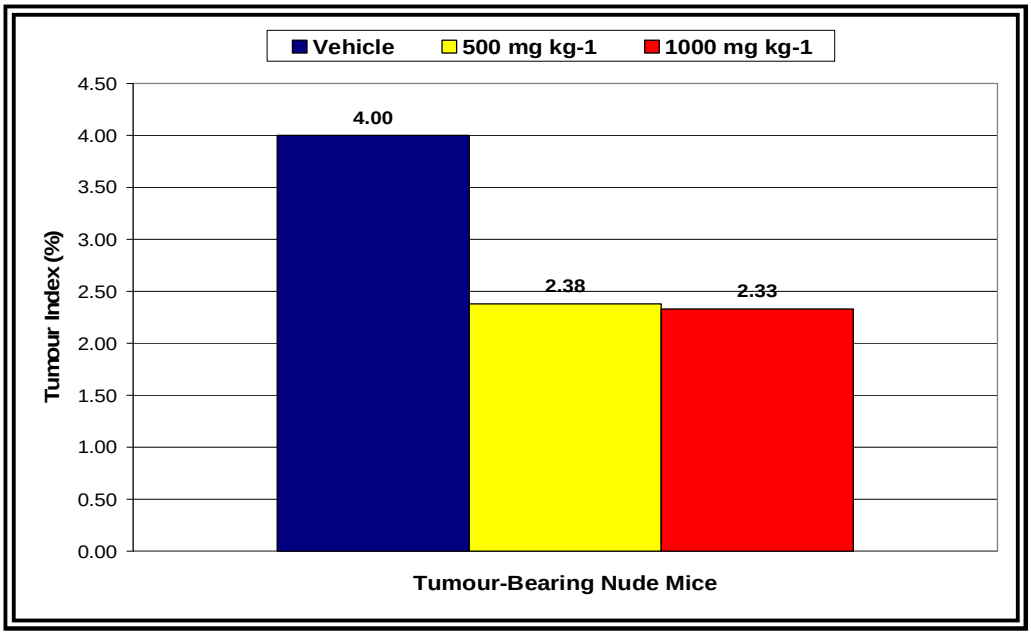


Figure 4-34: Tumour index in vehicle (untreated) and M propolis extract orally-treated tumour-bearing nude mice.

Table 4-8: Tumour volume in tumour-bearing nude mice treated intra-peritoneally with M propolis extract.

Groups	Dose	No. of Mice	Day*	Tumour Size** Mean ± S.E. (mm^3)	Mouse Weight** Mean±S.E. (gram)
Vehicle	0.0	10	0+	94.0 ± 8.1^{E}	22.0 ± 0.6^{AB}
		9	16	501.3 ± 52.5^{B}	21.5 ± 0.6^{BC}
		9	18	689.1 ± 77.1^{AB}	21.0 ± 0.6^{BC}
		9	20	822.9 ± 101.4^{A}	21.1 ± 0.7^{BC}
M Propolis Extract Treated Mice	500 mg kg^{-1}	10	0+	94.1 ± 8.1^{E}	23.8 ± 0.5^{A}
		9	16	433.1 ± 63.7^{D}	21.2 ± 0.6^{BC}
		9	18	573.2 ± 60.0^{BC}	20.4 ± 0.4^{C}
		9	20	633.7 ± 97.9^{AB}	20.5 ± 0.5^{C}
	1000 mg kg^{-1}	10	0+	94.2 ± 9.5^{E}	22.4 ± 0.5^{AB}
		9	16	511.8 ± 33.6^{BC}	20.8 ± 0.5^{C}
		8	18	581.4 ± 55.5^{B}	19.3 ± 0.5^{DC}
		8	20	567.6 ± 59.8^{B}	18.8 ± 0.6^{D}

*Days post-M sample treatment. + Day 8 post-tumour cells implantation.

**Different letters: Significant difference ($P \leq 0.05$) between means of the same column

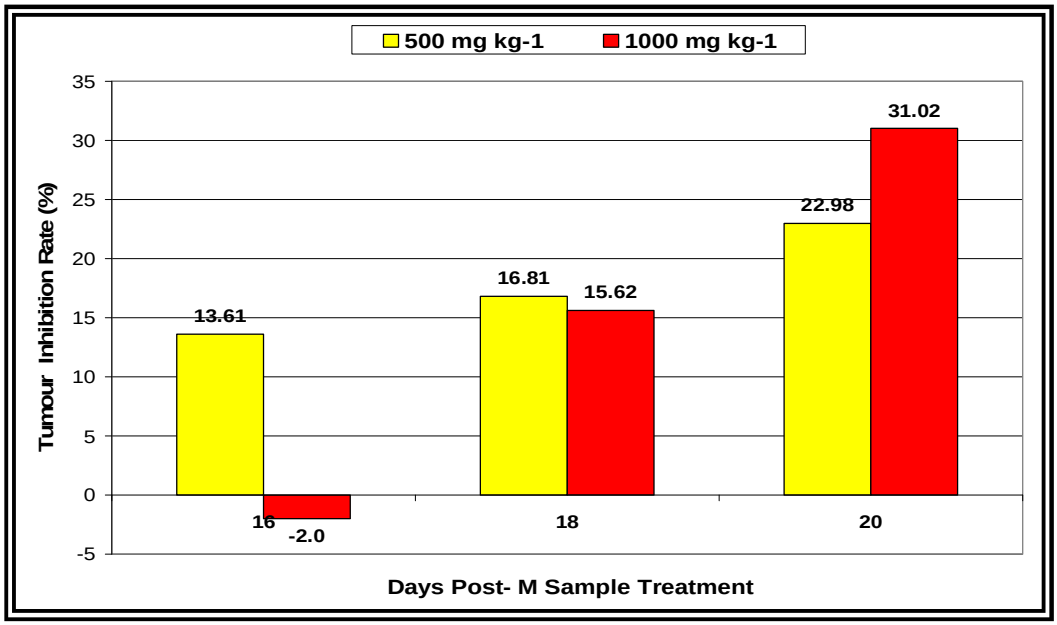


Figure 4-35: Tumour inhibition rate in tumour-bearing nude mice treated intra-peritoneally with M propolis extract.

4.3.2 Histological Evaluations of Tumours

Only tumours excised from p.o. groups (vehicle, 500 mg kg^{-1} and 1000 mg kg^{-1}) were subjected to histopathological and immunohistochemical examinations, while tumours from i.p. animals were frozen in liquid nitrogen for a pharmacokinetic assessment that has not been carried out.

4.3.2.1 Histopathological Examination

Tumour sections stained with haematoxylin and eosin were evaluated in terms of mitosis, necrosis, ulceration, degeneration, inflammation, undifferentiation, stroma and granulation tissue. Scoring of these features was based on examinations of four (Vehicle, 500 mg kg^{-1} dose and 1000 mg kg^{-1} dose) sections, each with three high power fields (H.P.F.: 400X), therefore, the given score was the mean of four H.P.F. (Table 4-9 and Figures 4-36, 4-37 and 4-38). These evaluations were based on a report given by the histopathologist Professor Eugenio Scanziani (Department of Veterinary pathology, Faculty of

Veterinary Medicine, University of Milan, Milan, Italy), and the findings are presented in the following:

- **Mitosis**: In vehicle mice, the mean of mitosis was 11.50 ± 1.84 mitotic cells/H.P.F., while it was 5.75 ± 0.75 and 4.50 ± 0.50 mitotic cells/H.P.F in tumour sections treated with 500 mg kg^{-1} or 1000 mg kg^{-1} dose, respectively. The latter two values were significantly not different, but they were significantly ($P \leq 0.05$) lower than the value that was reported in vehicle sections (Figure 4-36).
- **Necrosis**: A prominent central area of necrosis was present in each case even if in some of them the necrosis was not only central, however, the vehicle and 500 mg kg^{-1} sections shared approximated percentages of necrosis (65.0 and 60.0%, respectively), but it was significantly less frequent (26.3%) in tumour sections of mice treated with the dose 1000 mg kg^{-1} (Figure 4-37 A).
- **Ulceration**: No ulceration was observed in tumour sections of vehicle mice, but it was present in 100.0% of mice treated with the dose 500 mg kg^{-1} sections, and at the higher dose (1000 mg kg^{-1}), it was dropped to 50.0% (Figure 4-37 B). In the latter cases, it was possible to identify an ulceration area with a large amount of inflammatory cells (granulocytes) (Figure 4-37 C). In some of them there was an associated area of granulation tissue composed of fibroblast and newly formed blood vessels.
- **Degeneration**: The degeneration was scored by the histopathologist as (-), (+), (++) or (+++), which corresponded to absent, slight, moderate or severe necrosis, respectively. In vehicle mice, two sections were reported to be -, while the other two sections were (+). For mice treated with the dose 500 mg kg^{-1}, one section was considered -, two sections were (+), while the fourth section was (++). In animals treated with dose 1000 mg kg^{-1}, three sections were scored as (+++), while the fourth one was (+). It was also possible to notice signs of degeneration of neoplastic cells (cytoplasmic enlargement,

cytoplasmic vacuolization, light eosinophilic cytoplasm and well-demarcated membrane) in sections with the scores (+) or (++).

- **Inflammation**: In almost all cases, a peri-tumoural inflammation characterized by presence of inflammatory cells was present, but the type of cells that were involved and the severity of inflammation were different. In vehicle mice, three sections were reported to be (+), in which the inflammatory cells were lymphocytes, monocytes and granulocytes, while one section was (++), and the inflammatory cells were lymphocytes, monocytes, granulocytes and plasma cells. For mice treated with the dose 500 mg kg^{-1}, three sections were scored as (+++), in which the inflammatory cells were lymphocytes, monocytes and granulocytes, while one section was (++) with similar inflammatory cells, which were also observed in mice treated with the dose 1000 mg kg^{-1}, but the score (++) was given to three sections, and one section was considered (+++).
- **Undifferentiation**: The same score of degeneration was applied to undifferentiation. In vehicle mice, three sections were reported to be (+), while the fourth one was (-). For mice treated with the dose 500 mg kg^{-1}, the four sections were reported as (++), and a similar outcome was observed in animals treated with dose 1000 mg kg^{-1}, but the fourth section was scored as (+++). In some positive cases, tumour cells showed signs of severe undifferentiation characterized by a marked anisocytosis, anisokaryosis, presence of very prominent nucleoli and multinucleated cells (Figure 4-38), and a complete loss of lobular structure.
- **Stroma**: No differences could be noticed among the three investigated groups, although, a well-demarcated dermal solid mass composed of tumour cells supported by a small amount of fibrous stroma was present in some cases.
- **Granulation Tissue**: In vehicle mice, three sections were reported to be (+)**,** while in the other two sections, granulation was absent. For mice treated

with the dose 500 mg kg^{-1}, one section was scored as (+++), and a further one as (-), while the other two sections were (++). In mice treated with the dose 1000 mg kg^{-1}, two sections were reported to be (-), while the other two sections were (++). The presence of granulation tissue could be correlated with ulcerations that were observed in tumour sections of treated animals.

Table 4-9: Summary of histological findings in tumour sections of vehicle mice and mice treated orally with two doses (500 or 1000 mg kg^{-1}) of M propolis extract.

Groups	Histological Features							
	MI (H.P.F.)	NE (%)	UL	DE	IN	UD	ST	GR
Vehicle	11	75.0	-	-	+ (L, M)	+	+	-
	12	65.0	-	+	+ (L, M)	-	+	-
	16	60.0	-	+	++ (L, P, G)	+	-	+
	7	60%	-	-	+ (L, G)	+	+	+
Mean ± S.E.*	11.5 ± 1.84^{A}	65.0 ± 3.5^{A}						
Dose 500 mg kg^{-1}	7	80.0	+	-	+++ (G, L)	++	-	-
	4	45.0	+	+	+++ (G)	++	-	++
	5	75.0	+	+	++ (G, M, L)	++	+	+
	7	40.0	+	++	+++ (L, G)	++	+	++
Mean ± S.E.*	5.75 ± 0.75^{B}	60.0 ± 10.2^{A}						
Dose 1000 mg kg^{-1}	6	30.0	-	++	+++ (L, M)	++	+	-
	4	20.0	-	++	++ (L, M)	++	+	-
	4	30.0	+	+	++ (L, G)	++	-	++
	4	25.0	+	++	++ (L, M)	+++	+	++
Mean ± S.E.*	4.50 ± 0.50^{B}	26.3 ± 2.4^{B}						

MI: Mitosis; NE: Necrosis; UL: Ulceration; DE: Degeneration; IN: Inflammation; UD: Undifferentiation; ST: Stroma: GR: Granulation tissue; L: lymphocyte; M: Monocyte; G: Granulocyte; P: Plasma cell; H.P.F.: High Power Field; -: Absent; +: Slight; ++: Moderate; +++: Severe.

*Different letters: Significant difference ($P \leq 0.05$) between means of the same column.

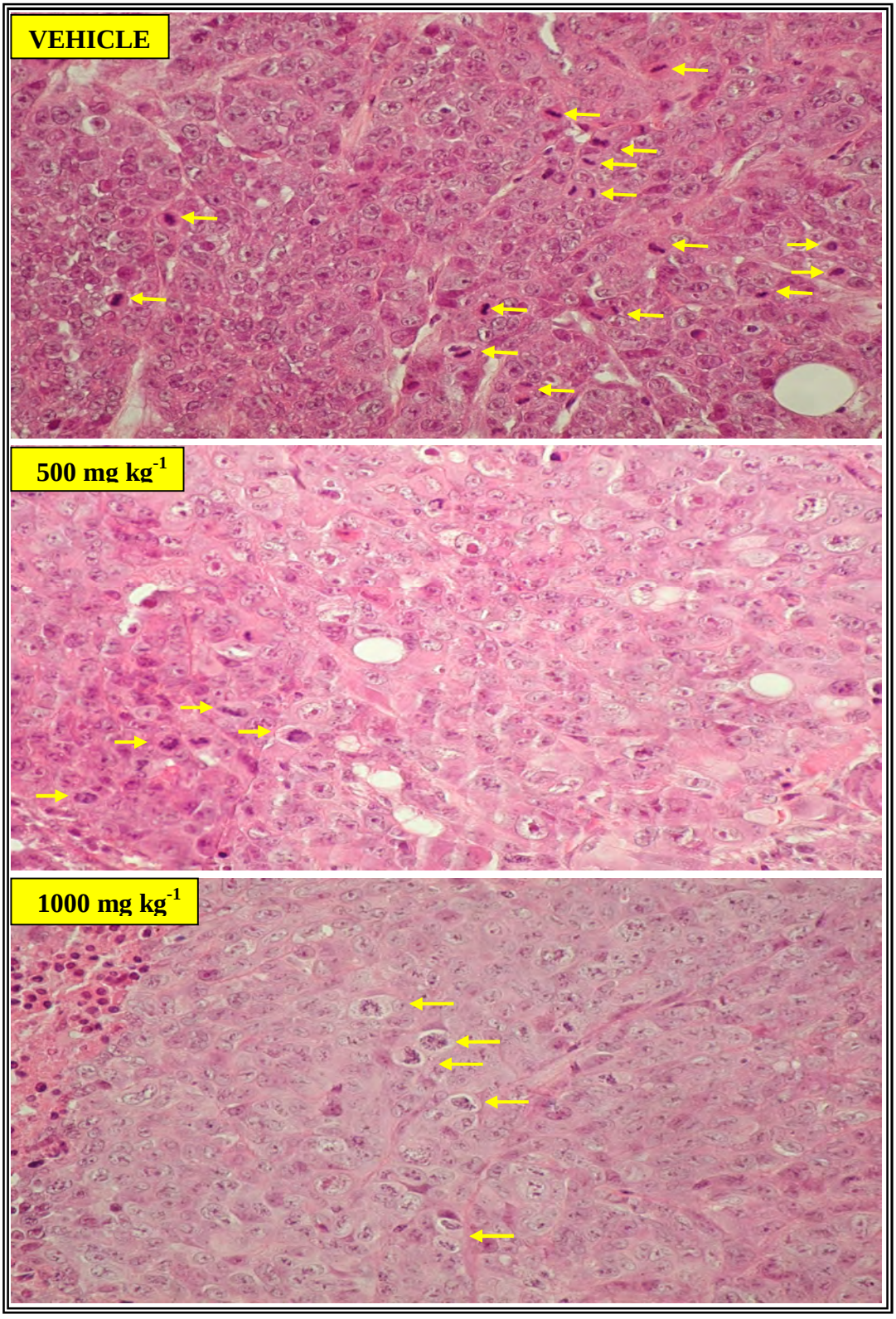


Figure 4-36: Tumour sections of vehicle mice and mice treated orally with M propolis extract showing mitosis (H and E; 400X).

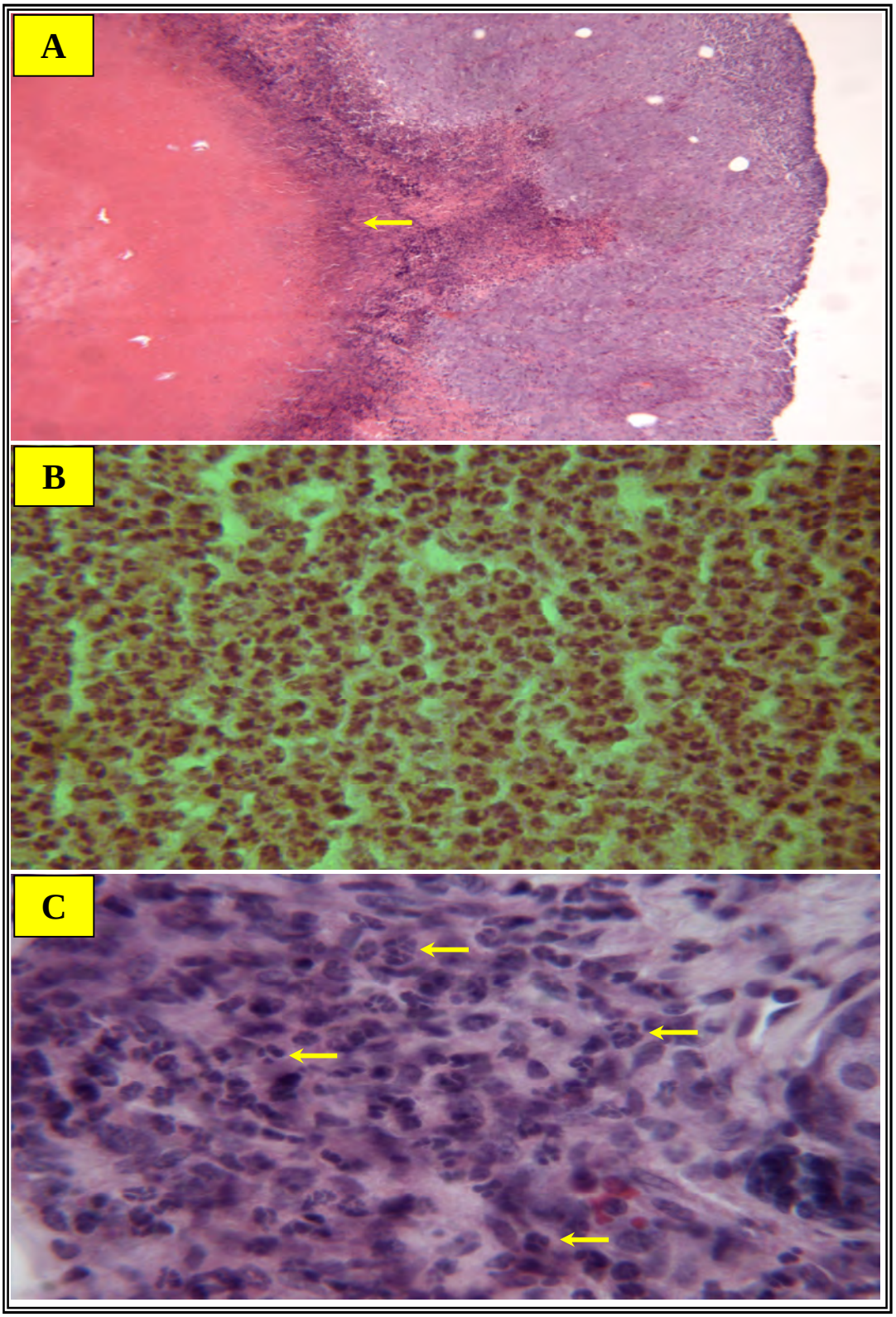


Figure 4-37: Tumour sections of vehicle mice and mice treated orally with M propolis extract showing necrosis (A), ulceration (B) and granulocytes (C) (H and E; 100,400,400X, respectively).

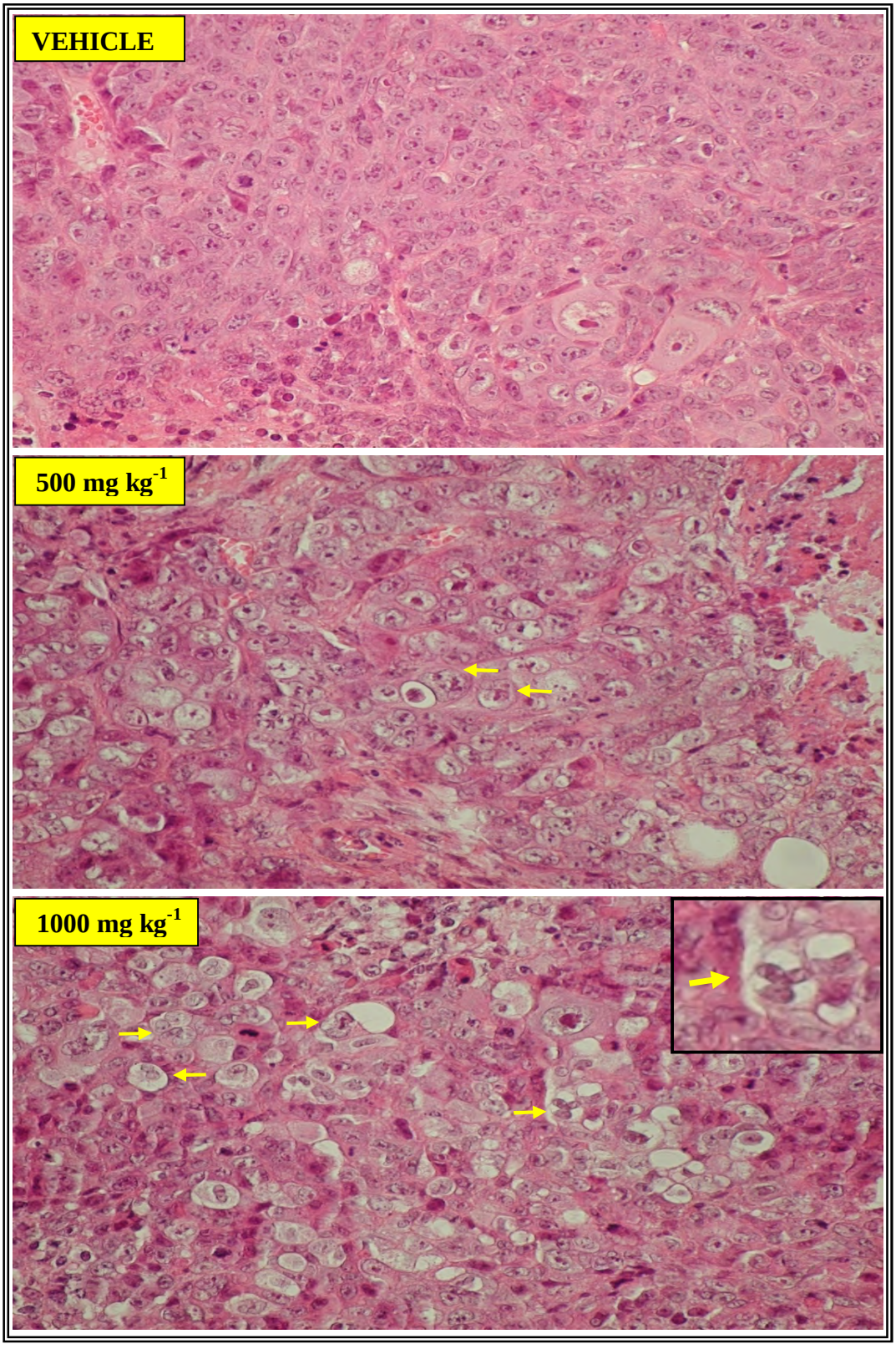


Figure 4-38: Tumour sections of vehicle mice and mice treated orally with M propolis extract showing multinucleated cells (H and E; 400X).

4.3.2.2 Immunohistochemical Examination of *p53* and *Ki-67* Genes

The p53 gene showed a positive expression in 16% of vehicle tumour cells, while it showed an increased expression in tumour sections of animal treated orally with the dose 500 mg kg^{-1} (33.5%) and a further increase (40.8%) at the dose 1000 mg kg^{-1} (Table 4-10 and Figure 4-39). The latter two values were significantly higher than the value of vehicle.

With respect to Ki-67 expression, the reverse picture was observed, in which, the gene was expressed in 85% of tumour section cells, while it was expressed in 47.0 and 46.8% of tumour sections obtained from mice treated with the dose 500 mg kg^{-1} or the dose 1000 mg kg^{-1}, respectively (Table 4-10 and Figure 4-40).

Table 4-10: Mean percentage of cells expressing *p53* or *Ki-67* genes in tumour sections of vehicle mice and mice treated orally with M propolis extract.

Gene	Percentage of Gene Expression (Mean ± S.E.)*		
	Vehicle	M Propolis Extract	
		500 mg kg^{-1}	1000 mg kg^{-1}
p53	16.00 ±1.95^{B}	33.50 ± 4.05^{A}	40.80 ± 2.59^{A}
Ki-67	85.00 ±1.70^{A}	47.00 ± 5.83^{B}	46.80 ± 3.73^{B}

*Different letters: Significant difference ($P \leq 0.05$) between means of the same row.

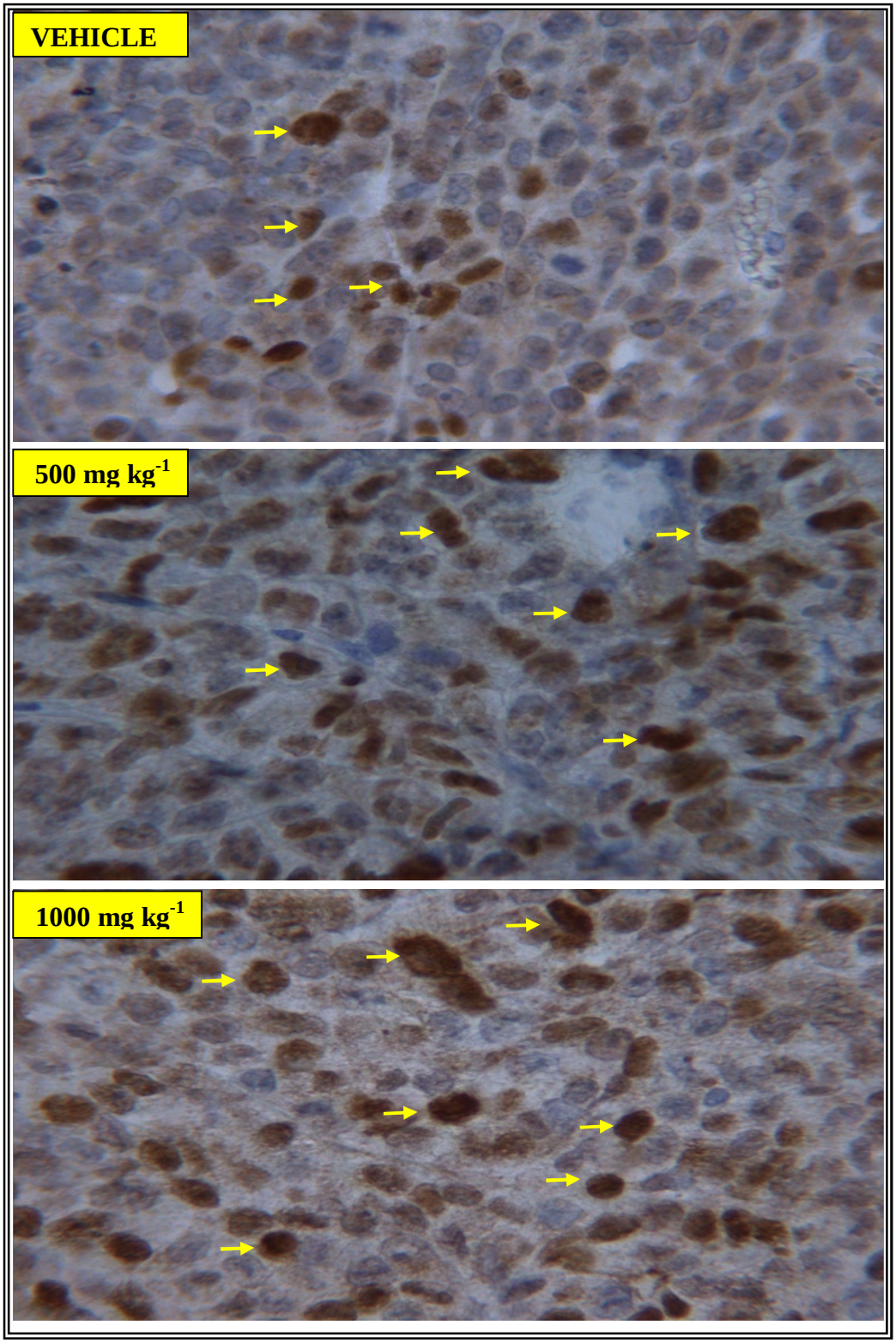


Figure 4-39: Immunohistochemical staining of p53 expression in tumour sections of vehicle mice and mice treated orally with two doses (500 or 1000 mg kg^{-1}) of M propolis sample extract (400X).

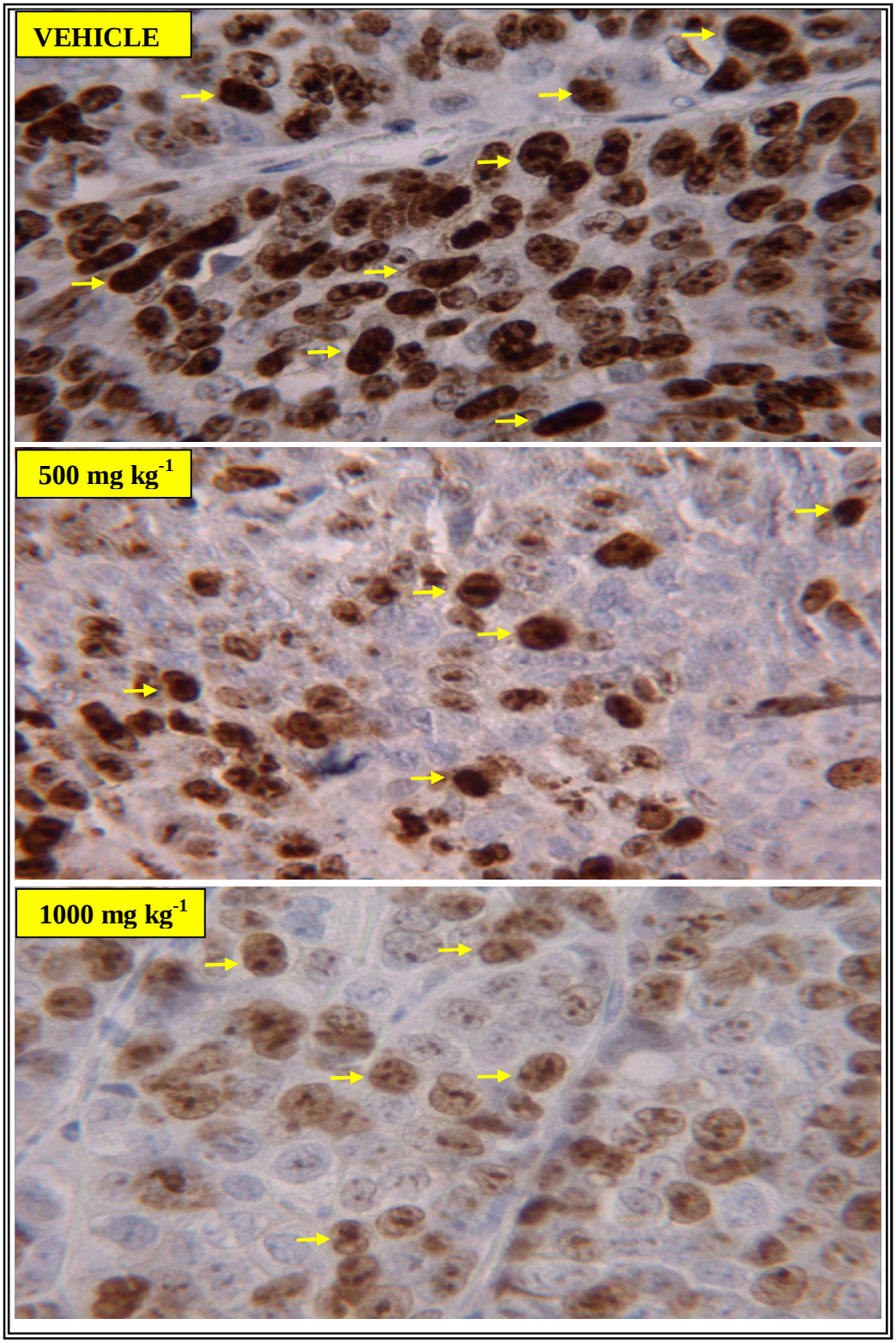


Figure 4-40: Immunohistochemical staining of Ki-67 expression in tumour sections of vehicle mice and mice treated orally with two doses (500 or 1000 mg kg^{-1}) of M propolis sample extract (400X).

Chapter Five
Discussion

Chapter Five
Discussion

5.1 Chemical Analysis

An accurate assessment of the contents of bioactive compounds in extract samples requires the validation of certain analytical parameters such as precision, recovery, linearity and limit of detection (Volpi and Bergonzini, 2006). To fulfill these obligations, the five samples of propolis extract (PE) were subjected to high-performance liquid chromatography (HPLC) coupled to mass spectrum (MS). For many years, however, the detailed analysis of main volatile and semi-volatile components of propolis was carried out by MS in conjunction with gas chromatography (GC-MS), but due to the fact that propolis contains many components that are not volatile enough for a direct GC-MS analysis even upon derivatization or high-temperature GC-MS, the HPLC-MS was employed (Gomez-Caravaca *et al*., 2006).

High-performance liquid chromatography represents the most popular and reliable analytical technique for the characterization of polyphenolic compounds as judged by the number of published papers in this subject (Gomez-Caravaca *et al*., 2006). HPLC coupled to MS has the advantage in improving the analysis of non-volatile compounds and allows establishing a more definitive chemical structure of the identified compounds (Sawaya *et al.*, 2004). Furthermore, electrospray ionisation (ESI) permits the direct ionization and transference of molecules, especially if two mass spectrometers (MS1 and MS2 spectra) are used, because such manipulation can extend the applicability of MS for a variety of new classes of molecules that have thermal instability, high polarity and high mass (Medana *et al*., 2008). Accordingly, coupling HPLC column to a hybrid MS instrument in the present study permitted a chemical analysis of propolis samples with high sensitivity and specificity as demonstrated by the obtained results.

The hybrid MS instrument, which is composed of a fast low resolution Linear Ion Trap Quadrupole (LTQ) followed by a high resolution Orbitrap analyzer, allowed the simultaneous acquisition of both MS and MS/MS spectra. Following such lead, all the chromatographic peaks of different substances were clearly separated by combining retention times and high resolution extracted ion chromatograms of pseudo-molecular ions. Moreover each peak was characterized by a measured exact mass of its pseudo-molecular ion (with an error equal or less than 5 ppm) and by its unique acquired MS/MS spectrum (Bagnati, R.; personal communication). Such analysis was further presented in term of $[M-H]^-$ ion peak to quantify the identified flavonoids in propolis extracts. Under this negative mode and at an energy level of 100% and a drying temperature at 350^oC, the flavonoids were identified as the major $[M-H]^-$ ion peaks, thereby allowing these ions to be selected for use in the quantitative analysis (Volpi and Bergonzini, 2006). Accordingly, this method can be considered very sensitive and accurate in achieving linearity over a wide range of concentrations and the findings of the present study concentration range (0.1 - 2000 $\mu g\ ml^{-1}$) can justify such conclusion.

Quantitative estimations (concentrations) of the identified compound were made by comparing the peak areas of extracted chromatograms of pseudo-molecular ions for the five injected propolis samples with the injected available reference standards (chrysin, tectochrysin, apigenin, acacetin, quercetin, galangin, kaempferide, pinocembrin, pinostrobin, sakuranetin, caffeic acid and caffeic acid phenethyl ester). Whereas, compounds for which a reference standard was not available were quantified by comparing with the peak area of the most similar standard (based on chemical structure similarities). Then, the identification of unknown peaks was accomplished by combining the information obtained from two sources. In the first, the measured exact mass (or the most probable chemical formula that can be calculated from it) was compared with reference compounds available online in different chemical

database websites (www.chemspider.com, www.chemfinder.com and pubchem.ncbi.nlm.nih.gov). The second source was based on a comparison of acquired MS/MS spectra (obtained in the present study) with reference libraries or literature that have reported the corresponding spectra. In some cases, when matching compounds could not be found, the structure of the corresponding component was proposed on the basis of its retention time, exact mass and MS/MS fragmentation.

The five assessed samples of propolis were of different geographical origins. Although, propolis colour may be cream, yellow, green, red, light or dark brown, the present samples had different presentations, in which some samples showed a light or dark brown colour (B1, B2 and S samples), while other samples were dark green (D and M samples), furthermore, the appearance showed similar variations, in which B1, B2 and D samples shared the presentation of gummy and waxy, while M sample was powder and S sample was waxy. Such differences were probably arisen as a result of natural seasonal changes, local flora, phenology of the plants and vegetation at the site of collection, however, phenological differences in plant chemistry may be considered an important factor that contributes for the divergences of propolis presentation (Bankova, 2005). Additionally, the main source of propolis is although bud exudates from poplar (*Populus* spp.), exudates are also collected from other trees available at the areas of collection, which may show different plant flora and this can effect colour and/or appearance of propolis (Salatino *et al*., 2005).

The variation in colour and appearance of the present propolis samples was also applied to the chemical constituents of the samples investigated, in which different chemical compounds (mainly polyphenols) were identified with different concentrations. In this context, the forthcoming argument of colour and appearance can be also held true, and propolis samples from different regions of the world have shown variations in their chemical constituents, and their

concentrations were also regionally different (Chen *et al*., 2008). In agreement with such presentation, there was a new finding regarding the present propolis samples, in which the compounds labeled as clerodane terpenoid were identified for the first time in a temperate zone (D, M and S propolis samples), because such compounds are normally present in propolis samples from South America and more precisely Brazil (Matsuno, 1995; Mitamura *et al.*, 1996; Matsuno *et al*., 1997c; Salatino *et al*., 2005). In this regards, constituents of propolis from tropical zones (South America) appear to be different from those of temperate zones because of the difference in vegetation. There are no poplar buds in tropical areas, and the resins exuded by *Clusia minor, Clusia major* (Guttiferae), *Araucaria heterophylla* (Compositae) and different *Baccharis* spp. (Compositae) were reported to be the dominant sources of components found in tropical propolis from Venezuela and Brazil (Tomas-Barberan *et al*., 1993; Bankova *et al*., 1996; Banskota *et al*., 1998; Bankova *et al*., 2000). These plants are rich in polyprenylated benzophenones and various diterpenes‹ which are reported from tropical propolis. A clerodane and several labdane-type diterpenoids, which are virtually absent in propolis from temperate zones, were reported to be present in propolis from tropical regions, and vegetation sharing may account for the existence of such compounds in non-tropical regions (Bankova, 2005). However Silici and Kutluca (2005) presented a further point of view, in which the honeybee race may determine the propolis source as these races may use neither the same nor the single propolis source.

5.2 Free Radical Scavenging Activity

When the anti-oxidant activity of Iraqi propolis samples and their polyphenolic compounds were tested by the DPPH assay, all propolis samples marked differences in radical scavenging activity, but B2, D and M samples were the best in this regard. They shared the activity reported for Egyptian propolis samples with a slight variation (Hegazi and Abd El Hady, 2002), but

the three samples were more potent than those of Bulgaria, Hungary, New Zealand or China (Kumazawa *et al.*, 2004), suggesting that propolis region of collection is important in defining its free radical scavenging activity. Such potent activity seemed to be correlated with the total polyphenolic compounds, because the strong anti-oxidant activity occurs in propolis samples that are rich in phenolic compounds, and flavonoids are reported to be the most abundant and most effective anti-oxidant compounds found in propolis (Kumazawa *et al.*, 2004). Nieva Moreno *et al*. (2000) and Isla *et al*. (2001) have also previously investigated the anti-oxidant activity of Argentinean propolis, and reported a significant correlation between flavonoid contents and antioxidant activity. Furthermore, cinnamic acid derivatives including CAPE and artepillin C (flavonoid compounds), in addition to some diterpenoids from propolis were also reported to have interesting antioxidant activity (Banskota *et al*., 2001).

The anti-oxidant activity of propolis and its ability to sequester reactive oxygen species have been investigated by Simoes *et al*. (2004), who studied the biological effects of different extracts and fractions of green propolis. A correlation was observed between the anti-oxidant activity and chemical composition of its different fractions, with special emphasis on the presence of flavonoids and p-coumaric acid derivatives. The study concluded that the components of propolis act by different mechanisms sequestering reactive oxygen species. Additionally, several studies have confirmed that the pharmacological properties of propolis are attributed mainly to the presence of favonoids as a result of their action against free radicals (Vennat *et al*., 1995; Mirzoeva and Calder, 1996; Vynograd *et al*., 2000), and these polyphenols interfere not only with the propagation but also with the formation of free radicals both by chelating transition metals and by inhibiting enzymes involved in the initiation reaction (Russo *et al*., 2002).

The naturally occurring polyphenols are expected to help reduce the risk of various life-threatening diseases, including cancer and cardiovascular diseases,

due to their antioxidant activities. Thus propolis with antioxidant activity may protect humans from deleterious oxidative processes. Banskota *et al*. (2002) also reported that the anti-oxidative activity of propolis is due to its phenolic constituents, which also possess anti-tumour and anti-hepatotoxic activities. However, polyphenols include many compounds; for instance, caffeic acid (CA), chrysin (CH) and caffeic acid phenethyl ester (CAPE), and each compound may have different scavenging activity. Accordingly, these three compounds were assessed and CA or its ester (CAPE) showed a more potent antioxidant activity than CH. Overall, these results were in agreement with the findings of Banskota *et al*. (2002) who reported that both caffeates (CA and CAPE) have potent anti-oxidant activities and a weak DPPH radical scavenging activity of CH.

5.3 Cytotoxicity of Propolis and its Polyphenols

The *in vitro* cytotoxicity assessments of propolis samples or their three polyphenolic compounds (CA, CH and CAPE) in two human cancer lines (HL-60 and HCT-116) and one normal cell line revealed that the effects were dose- and time-dependent, and furthermore, the propolis sample or its polyphenolic compounds and target cell line were also interfered with these effects. In general B2, D and M propolis extracts shared the most effective cytotoxicity, while B1 and S samples were less effective in this regard. Such findings can be interpreted in the ground of their polyphenolic contents, because the highest concentrations of polyphenolics were observed in B2, D and M samples. Therefore, the cytotoxicity of propolis samples seemed to be positively correlated with both polyphenolic compounds, and the results of antioxidant activity evaluation support such correlation. In agreement with such theme, it has been reported that flavonoids, phenolics and aromatics are the most important pharmacological active constituents of propolis, and it is well known that these products are powerful anti-oxidants. For this reason, propolis is considered as being a natural

source of anti-oxidants, and the anti-oxidant activity of propolis compounds may play an important role in their anti-proliferative activities (Padmavathi *et al*., 2006).This view has been confirmed using propolis samples of Brazilian and Chinese origins, in which it has been demonstrated that propolis was effective in inducing growth inhibition and apoptosis in human melanoma (A2058), human breast cancer (MCF-7), human neuroblastoma (IMR-32), HL-60, human laryngeal epidermoid carcinoma (HEp-2), human fibrosarcoma (HT-1080), human colon carcinomas (HCT-116, HT29 and SW480), rat glioma (C6) and murine colon carcinoma (26-L5) cell lines (Chen et *al*., 2004; Bufalo *et al*., 2007; Ishihara *et al*., 2009). Thus, the anti-proliferative effects of Iraqi propolis samples could be related to an overall effect of the phenolic compounds present in the extracts, which were detected by HPLC-ESI/MS analysis.

The cytotoxic effects of phenolic compounds may depend on lipophilicity of the compound in question, which is very important for the penetration into the target cells. For instance, the maximum solubility of apigenin in culture medium is less than that of kaempferol and quercetin (Plochmann *et al*., 2007). On the other hand, lipids and proteins present in biological membranes facilitate the solubility of polyphenols, and differences in cell membrane structures and metabolic activation of chemicals can also affect the activity of polyphenols (Szliszka *et al*., 2009). However, the growth inhibition (GI) by propolis or its compounds can result in apoptosis or necrosis, and such outcome may be subjected to the cell line under investigation. In HL-60 cells, propolis extracts induced apoptosis while HCT-116 cells manifested the GI as necrosis. Whereas, the investigated propolis extract were significantly less effective against normal cells (HuFb cell line). Therefore, it is possible to suggest that the chemical constituents of propolis, especially phenolic acids and flavonoids, may have a selective cytotoxic effect against cells, and such effect is determined by the type of cells under investigation. Furthermore, and as propolis was less cytotoxic to normal cells, it is also possible to consider propolis a potential anti-tumour agent

that has no side effects. However, the latter evaluation can be bestly defined on the ground of *in vivo* studies, which are discussed later in this chapter. In agreement with such augmentation, related reports have indicated that some specific chemicals present in propolis such as CA, CH, CAPE and naringenin can inhibit growth of cancer cells without harming or affecting normal cells (Chen *et al*., 2003; Orsolic *et al*., 2009).

The selectivity of propolis compounds against cells may also determined by the types of tumour cell line, and in this regard Banskota *et al*. (2002) reported that benzyl, phenethyl and cinnamyl caffeates of a Netherlands propolis components showed potent anti-proliferative activities toward HT-1080 and B16-BL6 melanoma cell lines and most selectively toward 26-L5 carcinoma. Additionally, the study demonstrated that neither CA nor its corresponding alcohols (benzyl, phenyl and cinnamyl) showed any anti-proliferative activity against the tested cell lines, except for cinnamyl alcohol, which had a moderate anti-proliferative activity toward 26-L5 carcinoma and B16-BL6 melanoma. With respect to CA, the present study shares Banskota's findings and the CA showed no anti-proliferative activity toward HL-60 and HCT-116 cell lines, but the caffeates as a whole seem to be necessary for the anti-proliferative effects.

5.4 Propolis *in vitro* Apoptotic and Necrotic Potentials

Inhibition of apoptosis is an important mechanism for tumour formation and progression, and therefore, several chemotherapeutic compounds have been reported to induce apoptosis and this may be the primary mechanism for their anti-tumour activity (Tait, 2008). Accordingly, the present study based its aim on the hypothesis that propolis and/or its polyphenolic compounds might have anti-tumour activity, and the pathway of effect is through the induction of apoptosis. To test this hypothesis, the cell death was evaluated *in vitro* in tumour cell lines after a treatment with PEs or their phenolic compounds through four types of assays, which were morphological aspects, gel electrophoresis, Annexin

V/PI Flow cytometry and regulation of *Bcl-2*/*Bax* gene expression. The results revealed that the tested substances showed a potent inhibitory cytotoxic effect against the proliferation of HL-60 and HCT-116 cells through either apoptosis or necrosis, respectively.

Treated HL-60 cells showed a clear apoptotic manifestations characterized by morphological changes (chromatin condensation and DNA fragmentation), DNA laddering, plasma membrane alternations, and down-regulation of *Bcl-2* and up-regulation of *Bax* gene expressions. In contrast, treated HCT-116 cells showed no apoptotic features, and instead the size of cells was dramatically increased by swelling of cytoplasm and losing of membrane integrity, and cell rupture and release of cellular contents were clearly detected by fluorescence microscopy. Condensation of chromatin and inter-nucleosomal DNA fragmentation, together with cell shrinkage and shedding of apoptotic bodies ('blebbing'), are widely recognized as hallmarks of apoptosis (Nagata *et al*., 2003).

In the early stages of apoptosis, changes occur in the cells and a structural alteration of plasma membrane is one of the earliest features, which resulted as a consequence of phosphatidylserine (PS) translocation from the inner side of the plasma membrane to the outer layer, and by which PS becomes exposed at the external surface of the cell (Tait *et al*., 2006). This molecular event can be detected with annexin V; a calcium-dependent phospholipid binding protein that has a high affinity for negatively charged phospholipids such as phosphatidylserine (Gerke and Moss, 2002). Therefore, this protein can be used as a sensitive probe for PS exposure upon the cell membrane. During apoptosis the cells react to annexin V as soon as chromatin condenses but before the plasma membrane loses its ability to exclude PI. Hence by staining cells with a combination of fluorescenated annexin V and PI it is possible to detect non-apoptotic live cells, early apoptotic cells and late apoptotic or necrotic cells (Telford *et al*., 2004). The latter features were encountered in the present study,

but they were dependent on the sample investigated and its concentration, incubation period and the target cell line. With respect to the first dependent factor, propolis samples D and M were the most effective in inducing apoptosis in HL-60 cells. The possible explanation of such better effects is their richness in polyphenols, which were significantly higher in these two samples. Polyphenols have been shown to induce apoptosis in some cancer cell lines and although the molecular mechanisms by which flavonoids induce apoptosis have not yet been clarified, several mechanisms may be involved, including inhibition of DNA topoisomerase I/II activity (Bailly, 2000), regulation of heat shock proteins expression (Rong *et al*., 2000), decrease of reactive oxygen species (Khan, 2002), modulation of signaling pathways (Kim *et al*., 2005), release of cytochrome C with a subsequent activation of caspase-9 and caspase-3 (Michels *et al*., 2005), downregulation of Bcl-2 and Bcl-X(L) expression but promotion of Bax and Bak expression (Lee *et al*., 2005a), nuclear transcription factor kappaB (NF-kappaB) (Kanno *et al*., 2006), activation of endonuclease (Kook *et al*., 2007), and suppression of myeloid cell leukaemia-1 (Mcl-1) protein (Siegelin *et al*., 2008). More recently, preliminary evidence suggested that apoptosis induced by flavonoids in HL-60 cells may be associated with activation of caspase-3 and mediated through Fas and cytochrome C pathways, as well as, regulated through the inactivation of NF-kappaB (Naoghare *et al*., 2010).

The apoptotic effects of D and M propolis extracts were better at the higher doses, and therefore the dose can be considered as a further factor in the induction of apoptosis. In this regard, it has been demonstrated that the anti-tumour activity of some polyphenol compounds is highly dependent on dose, and accordingly it has been suggested that the higher doses of polyphenols can interfere with cell processes such as enzyme and glutathione levels, and this may induce cells death or apoptosis (Ramos, 2008).

Based on the forthcoming presentation, the M propolis sample, which possessed the highest potency in inhibiting growth of HL-60 and HCT-116

cancer cell lines, was selected for the evaluation of cell death markers. Markers that evaluate the effects of cell death are numerous, and in HL-60, the expression of two particular apoptotic markers, BAX and Bcl-2 was inspected. Up-regulation of the pro-apoptotic protein Bax was observed, while a down-regulation of the anti-apoptotic protein Bcl-2 was detected as a result of M sample treatment, especially at the 10 hours incubation period. In general, Bcl-2/Bax regulation has been regarded as a key factor in apoptosis induction (Wang, 2001), but such regulation effect has been demonstrated to be of different types. For instances, low level of Bcl-2 and Bax expression as it was demonstrated in human head and neck squamous cell carcinoma cell line (SCC-25) treated with H_2O_2 (Kowaltowski *et al*., 2001), decreased expression of Bcl-2 and unaltered BAX expression as was the case in leukaemic cell line U937 treated with propolis (Motomura *et al*., 2008) and down-regulation of Bcl-2 and up-regulation of Bax, which was observed in HL-60 cells treated with the present M propolis sample. The latter finding is consistent with observations made on other cell lines treated with different polyphenolic compounds of propolis (Chen *et al*., 2001; Chang *et al*., 2005; Orsolic *et al*., 2005; Onori *et al*., 2009). These collective observations may qualify the M sample as a potent apoptosis-inducing agent, and its action is through Bcl-2/Bax regulation, but it has to be confirmed on *in vivo* based studies.

5.5 Cell Cycle Perturbations

In HL-60 cells, M sample induced an accumulation of cells in the S phase at the highest concentration suggesting a block in the transition from S to G2-phase. These results are consistent with other studies that demonstrated the ability of some propolis constituents to induce growth inhibition coupled with cell cycle arrest; for instance, Bestwick *et al*. (2007) reported that treating HL-60 cells with a propolis flavonoid compound (kaempferol) inhibited their proliferation *in vitro*. After 5 hours treatment, the proportion of cells in S-phase

increased, and it was paralleled by a decrease in cells attending the G1-phase, while an initial accumulation of cells in S-phase and then G2/M was induced especially when the incubation period was progressed from 48 to 96 hours. Likewise, HL-60 cells treated with myricetin (propolis flavonoid compound) exhibited alterations in the number of cells in G0/G1 and S-phase, and an induction of apoptosis was markedly observed (Rusakc *et al.*, 2005). In the pesent study, it was interesting to note that a reduction of HL-60 cells in G1 phase was notice but it was accompanied by the appearance of a sub-G1 peak; an observation that marks an apoptotic feature. The induction of apoptotic cell death by M sample was supported by typical morphological and molecular hallmarks including chromatin fragmentation and phosphatidylserine exposure. These events were also observed in U937 (Aso *et al.*, 2004), human melanoma (Chen *et al.*, 2007) and HeLa (Szliszka *et al.*, 2009) cells, which also exhibited a DNA apoptotic laddering. The M sample-treated HL-60 cells exhibited reduced forward scatter, which was indicative of a decreased relative size, and enhanced side scatter, which was indicative of an increased internal complexity. Both exhibitions were targeted by a flow cytometer that has photometric tubes for the detection of laser-generated photons scattered from cells (Darzynkiewicz and Bedner, 2000; Ormerod, 2008). During apoptosis, cells shrink and decrease their forward scatter, but chromatin condensation and other intracellular changes may actually result in an increased side scatter early on in the apoptotic cascade. In late apoptosis, however, both forward and side scatter decrease as cells continue to shrink and as chromatin and organelles are sloughed off as apoptotic bodies (Ormerod, 2002; Allen and Davies, 2007).

The present study also recorded mixed accumulations in the G1 or G2/M-phase of HCT-116 cells treated with M sample, but the sub-G1 peaks were not induced. In support of such finding, it has been shown that CAPE induce accumulation of HCT-116 cells at the G1-phase (Wang *et al.*, 2005), while an arrest at G2/M phase was observed in oral epidermoid carcinoma-Meng 1(OEC-

M1) cells (Lee *et al*., 2005b). Therefore, it is possible to suggest that propolis may show different effects against different cell lines. Thus, prediction of the type of cell cycle arrest by propolis or its phenolic compounds is difficult, and probably depends on several factors, and further studies against different cell lines are needed. However, to overcome such obstacle and shed more light on the cell cycle, a biparametric assessment of cell cycle was introduced using 5-bromo-2-deoxyuridine (BrdUrd)/DNA cell cycle assay for both HL-60 and HCT-116 cells. This methodology recorded that M sample caused a block of the cells in the G1 phase of the cell cycle, with different kinetics for BrdUrd positive and negative cells, but they were similar in both cell lines. For the best knowledge of the investigator, there has been no a previous biparametric BrdUrd/DNA cell cycle analysis on kinetics of cells treated with propolis, but a general conclusion can be reached, in which cell cycle phases of propolis treated cells were perturbated.

Perturbations in cell cycle progression may account for the anti-carcinogenic effects of flavonoids, because a treatment of cultured cells with flavonoid has been demonstrated to outcome in a wide range of cell cycle alterations including inhibition of cyclin-dependent kinases (CDKs) and cyclins, or up-regulation of CDK-inhibitors of the cip/kip family (Haddad, 2008). Flavonoids have also been shown to regulate cell cycle checkpoint pathways, in particular the DNA damage response pathway, and in this regard, apigenin (a flavone compound) has been studied in three prostate cancer cell lines; PC3, LNCaP and DU145, and was able to induce G1 cell cycle arrest (Gupta *et al*., 2001). The molecular mechanisms behind the apigenin cell cycle arrest were found to include down-regulation of cyclins (D1, D2 and E), and inhibition of CDKs 2, 4 and 6. Such arrest was associated with up-regulation of p21 and p27 and a reduction in phosphorylated retinoblastoma (RB) protein (Shukla and Gupta, 2007). Checkpoints at both G1/S and G2/M of cell cycle in cultured cancer cell lines have also been found to be perturbed by flavonoids such as

quercetin, luteolin, kaempferol, apigenin, and epigallocatechin 3-gallate (Casagrande and Darbon, 2001). Flavonoids thus have a direct impact on key cell cycle regulatory mechanisms that ordinarily exist to suppress the proliferation of cells with tumourigenic potential. These properties of flavonoids may explain their chemopreventive effect highlighted in epidemiologic studies (Ren *et al*., 2003).

A key question with respect to flavonoids is the relationship between their structure and activity. Are the different flavonoids likely to act by different pathways/mechanisms, or by a common pathway? In fact, the answer is rather a complex task, with evidence suggesting that flavonoids have both similar and differing actions depending on structure. Flavonoids of a broadly similar structure act through similar pathways. On the other hand, flavonoids with major differences in functional side-groups have widely varying mechanisms of action (Haddad, 2008). Therefore, flavonoids show significant variation in their anti-proliferative potency, which is dependent on structural basis. It has been suggested, that the potency of flavonoids in perturbing cell cycle and inducing apoptosis, may be dependent on C2-C3 double bonds and number of hydroxyl groups in the 2-phenyl ring, which are important structural requirements for cytostatic effects of flavonoids (Rusakc *et al.*, 2005).

5.6 Expression of *p53* Gene

The p53 is widely considered to be the major sensor of a genotoxic stress and is a critical link between DNA damage, cell cycle arrest and apoptosis (Efeyan and Serrano, 2007). In present study, it was found that the death of HCT-116 was not accompanied by expression of p53 *in vitro*, therefore, M sample caused cytotoxic or growth inhibitory effects in these cells via a mechanism that was independent of p53, however, contradictory results were obtained after a treatment with camptothecin (CPT; a natural anti-tumour agent), in which the cytotoxicity was associated with p53 expression. Both

demonstrations have been documented to be responsible for the cytotoxicity of several anti-tumour drugs. For instance, alkylating agents mediate their genotoxic effect in a p53-dependant mechanism, while other synthetic anti-tumour drugs (e.g. lonidamine, alpha-bromoacryloyl-distamycin, the novel vitamin D3 analog EB1089 and Zoledronic acid) exert their cytotoxicity through a p53-independent mechanisms (Del Bufalo *et al*.,1996; Marchini *et al*., 1999; Pepper *et al*., 2003; Kuroda *et al*., 2004). However, it is augmented that the p53-independent pathway is the most important in inducing cytotoxic effects in tumour cells, although the type of tumour is crucial in this regard (Li *et al*., 1998; Abeysinghe *et al*., 2001; Pepper *et al*., 2003). Identifying drugs that induce a p53-independent cell death is therefore important and this may qualify propolis as a potential source for the development of cancer chemoprevention. Such conclusion must be interpreted with caution, because it was built-up on the ground of *in vitro* assessment and *in vivo* testing may question such conclusion.

Accordingly, p53 can be considered as important in terms of either a target for anti-cancer drugs or such drugs may target other gene products that modify the action of p53. In this regard, MDM2 is an important negative regulator of p53, and its hyperactivity may inhibit the function of p53 and lead to the development of a wide variety of cancers. For example, 30% of human sarcomas show no p53 mutations, but have an overexpressed MDM2 gene (Oliner *et al*., 1992). It is believed that inhibiting the E3 activity of MDM2 and blocking the interaction of p53 with MDM2 are potential effective strategies for killing certain tumour cells selectively by restoring the function of wild type p53 (Yang *et al*., 2004). Therefore, many studies have focused on the p53-MDM2 interaction as the basis of a drug development strategy. A series of small molecule inhibitors have been developed, and some of these can bind to MDM2 and block its interaction with p53, including peptides that have been shown to elevate the levels of p53 protein and its transcriptional activity and trigger p53-dependent apoptosis in tumour cells (Chene *et al*., 2000; Midgley *et al*., 2000).

A class of small molecules named nutlins have been identified to block p53/MDM2 interaction *in vitro* and *in vivo*. Treatment of tumour cells with nutlins resulted in induction of p53 and its target genes and triggering of apoptosis. More recently, a novel series of benzodiazepinedione antagonists of the p53/MDM2 interaction have been discovered which increase the transcription of p53 target genes and decrease proliferation of tumour cells expressing wild type p53 (Grasberger *et al*., 2005). One study suggested that anti-sense oligodeoxynucleotides targeted against MDM2 and p21Waf1/Cip1 could be employed in a potential therapeutic strategy sensitizing tumour cells to certain anti-neoplastic agents (Sato *et al*., 2000). One of the major concerns about blocking the p53/MDM2 interaction for use in treatment of cancer is the idea that activation of p53 might be toxic to normal tissues. However, certain data suggest that the mechanisms governing p53 activity in tumour cells and normal cells are quite different, so the different effects of p53 in reactivating different molecules in tumour cells and normal cells might provide a molecular basis for a therapy without the need for tumour targeting (Selivanova, 2004). Propolis may hold such promise and its action on tumour cells of the present study may justify such optimism.

5.7 Phosphorylation of H2AX Histone

The present study was designed with the aim to evaluate effects of M propolis sample on H2AX phosphorylation (γ-H2AX), and for the best knowledge of the investigator, this task has not been targeted in propolis worldwide. The present results demonstrated that the level of γ-H2AX was increased in HL-60 cells showing a fraction of cells expressing γ-H2AX at the investigated periods, while HCT-116 cells failed to show such response. As the growth inhibition exerted by M sample on the two types of cells was caused by two different mechanisms (apoptosis in HL-60 and necrosis in HCT-116),

therefore, the manifested γ-H2AX may be a consequence of apoptotic progress that occurred in HL-60 cells due to a propolis-treatment.

The maintenance of genome stability requires efficient DNA double-stranded break (DSB) repair mediated by phosphorylation of multiple histone H2AX molecules near the break sites (Kinner *et al*., 2008). The γ-H2AX molecules form foci covering many megabases of chromatin. The formation of γ-H2AX foci is critical for efficient DNA damage response (DDR) and for the maintenance of genome stability, and although the mechanisms of protein organization in foci is largely unknown (Nakamura *et al*., 2010), a role for γ-H2AX has been demonstrated in DNA repair, cell cycle checkpoints, regulated gene recombination events, and tumour suppression (Fernandez-Capetillo *et al*., 2004). When γ-H2AX levels are reduced to 50% of the wild-type levels, the resultant decrease in γ-H2AX formation is insufficient to maintain genomic stability, and leads to increased levels of chromosomal aberrations, reduced growth rates and radiation sensitivity (Celeste *et al*., 2003). In this regard, γ-H2AX has been shown to be a sensitive indicator of DNA double strand breaks produced by ionizing radiation and by drugs that cause double-strand breaks such as etoposide, doxorubicin and tirapazamine (Kuo and Yang, 2008).

Internucleosomal DNA fragmentation that leads to a multiplicity of DSBs is the hallmark of apoptosis, and during apoptotic DNA fragmentation, the generated DSBs can trigger γ-H2AX (Tanaka *et al*., 2007). Therefore, γ-H2AX can be considered a rather early event of apoptosis, occurring concurrently with the activation of caspase-3 and appearance of nucleosomal/oligonucleosomal DNA fragments. The latter appearance can be detected by gel electrophoresis (‘‘DNA laddering’’) and with externalization of phosphatidylserine at the outer leaflet of plasma membrane (Huang and Darzynkiewicz, 2006; Darzynkiewicz *et al*., 2008).

The recruitment of DNA damage signaling and repair proteins to sites of genomic damage constitutes a primary event triggered by DNA damage. Many

components of the DNA damage response, including ATM, BRCA1, 53BP1, MDC1, RAD51 and the MRE11/RAD50/NBS1 complex, form ionizing radiation-induced foci that co-localize with γ-H2AX foci as mentioned earlier in this section. These nuclear micro-domains are thought to contain hundreds to thousands of molecules that accumulate in the vicinity of a DSB (Fernandez-Capetillo *et al*., 2004). The localization of γ-H2AX foci is in agreement with their putative role in DSB repair and genomic instability. Therefore, an immunocytochemical assay recognizing the presence of γ-H2AX could theoretically be used as a sensitive test for the detection of potential carcinogens (Albino *et al*., 2004), as well as, testing the potential of natural products (e.g. propolis) as anti-carcinogenic agents. However, the present preliminary data are far from reaching a final conclusion, and further investigations are required to define the precise mechanisms that can explain how chromatin structure is modified by the phosphorylation of H2AX, and how this modification is linked to DNA damage repair and genomic instability, or apoptosis.

5.8 Anti-tumour Assessment *in vivo*

The anti-tumour potentials of propolis, which were assessed *in vitro*, were extended for a further *in vivo* evaluation in nude mice xenografted with HCT-116 cells and treated orally (p.o.) or intraperitoneally (i.p.) with M propolis extract. In these *in vivo* experiments, a dose-dependent inhibition of tumour growth was observed with a significant tumour regression at dose 1000 mg kg^{-1}, but such dose was well-tolerated in p.o treatment, while it caused some side effects in i.p. treatment. In p.o. mice the well-toleration was observed and manifested by lack of animals death or body-weight lose, while i.p. mice showed body weight lose, which was approximately 16% of the mean weight on day zero of propolis-treatment, in addition to death of some animals (two out of 10 mice; 20%), and such consequences made the investigator to end the observation period on day 20. The unusual results caused losing of mice weight

and death in an i.p. groups were not clarified in this experiment but may be related to use high doses of propolis since the bioavailability using i.p. injection is different of those in oral administration. The latter side effects may be also related to the route of administration (i.p.), because vehicle, as well as, 500 mg kg^{-1} propolis treated mice also showed 10% animal death. Therefore, the observed side effects may be not related to toxic effects of propolis only, especially if it is not given intraperitoneally for a long time because such side effects were observed in the last days of treatment. However, the investigators have demonstrated that propolis is not toxic to humans or mammals unless it exceed the recommended dose. According to Burdock (1998), it was stated that propolis is non-toxic, and its LD_{50} ranges from 2 to 7.3 gram kg^{-1} in mice, and for human the suggested dose was 1.4 mg kg^{-1} per a day. These findings were confirmed in rats treated with different doses (1, 3 and 6 mg kg^{-1} per day) of propolis extracts (aqueous or ethanolic) for varying periods of time (30, 90 and 150 days) (Mani *et al*., 2006). Their results demonstrated no significant alterations in lipid profile, live function enzymes or body weight. Furthermore, Cuesta *et al*. (2005) did not observe mortality or growth rate alteration after daily intake of propolis in the diet of fish during 6 weeks period.

As mentioned earlier, the investigated propolis sample was involved in an *in vivo* reduction of the xenografted tumour. To shed light on such potential and understand the pathway of tumour degeneration, a histological examination of tumours on p.o groups was carried out and revealed that a treatment with M propolis sample was associated with several histopathological changes that are generally considered as signs of tumour degeneration. The most important encountered changes were reduced mitosis and multinucleated cells (endoreduplication).

The mitotic index in tumour sections of propolis-treated mice was significantly decreased, and the effect was dose-dependent. In 500 mg kg^{-1} the reduction was 50.0%, while in dose 1000 mg kg^{-1} it reached 61% of the control

(vehicle) value. Therefore, such cells were arrested at the metaphase stage of mitosis, and such arrest may be related to propolis effects on the mitotic-spindle microtubules of dividing cells. Microtubules are extremely important in the process of mitosis, during which the duplicated chromosomes of a cell are separated into two identical sets before cleavage of the cell into two daughter cells. Their importance in mitosis and cell division makes microtubules an important target for anti-cancer drugs and their dynamics are the targets of a chemically diverse group of anti-mitotic drugs (with various tubulin-binding sites) that have been used with great success in the treatment of cancer (Jordan and Wilson, 2004; Zhou and Giannakakou, 2005; Schmidt and Bastians, 2007). In view of the success of this class of drugs, it has been argued that microtubules represent the best cancer target to be identified so far, and it seems likely that drugs of this class will continue to be important chemotherapeutic agents, even as more selective approaches are developed (Jackson *et al*., 2007). Microtubules seem to be a favourite target of naturally occurring, presumably self-protective, toxic molecules that are produced by a large number of plants and animals and most microtubule-targeted compounds have been discovered in large-scale screens of natural products (Schmidt and Bastians, 2007).

The end-stage of metaphase arrest is halting the cell cycle in mitosis, and when this occurs, cells may follow either of two possible pathways: commitment to a genetic program that results in apoptotic cell death, or a pathway involving the skipping of anaphase, initiating a new cell cycle (endocycle), and forming polyploid or endoreduplicated cells. Cells taking the latter pathway undergo a further round of DNA replication, with the result that at the next mitosis they produce metaphases made up of diplochromosomes (Cortes and Pastor, 2003; Cortes *et al*., 2003). Endoreduplication in eukaryotes is a process that involves DNA amplification without corresponding cell division. While the molecular mechanisms responsible for endoreduplication are poorly understood, it is a useful endpoint for assessing failure in the proper decatenation of replicated

chromosomes before mitosis (Pastor *et al.*, 2005). The latter outcome was observed as histopathological changes in tumour sections of mice treated with propolis, and accordingly, the investigated propolis extract may be rich in compounds that are able to induce such effects. One of the most encountered constituents in M propolis sample was polyphenols (flavonoids and phenolic acid and its esters). In this regard it has been demonstrated that flavonoids are very strong inducers of endoreduplication as compared with other investigated chemicals (Cantero *et al.*, 2006), and accordingly the authors suggested that the most likely hypothesis for the induced endoreduplication is that the flavonoids action on both DNA and the enzyme topoisomerase II itself results in a highly negative effect on the enzyme performance for chromosome segregation.

The histopathological examinations was extended further but in terms of immunohistochemical evaluation for the expression of two cell cycle regulatory genes, which were *p53* and *ki-67*.The first one showed a significant increased expression in tumour sections of mice treated with M propolis sample, while the second gene showed a significant decreased expression. Both deviated expressions have impact on the cell cycle progression, which is always a target for chemotherapies, although the pathway of effected mechanisms has not been well defined and its subjected to the type of tumour cells, nature of compounds employed in the treatment and others (Koelbl *et al.*, 2001). With respect to p53, one of the most dramatic responses to it is the induction of apoptosis. Apoptotic signals can engage two main pathways (which are also interconnected). These are the extrinsic pathway, which is induced through the activation of cell-surface receptors, and the intrinsic pathway, which responds to stress signals (Green, 1998). Although p53 has been implicated in both pathways, it predominantly seems to influence the intrinsic pathway. This apoptotic pathway leads to a perturbation of mitochondrial membrane potential, and so the release of apoptogenic factors from the mitochondrial intermembrane space into the

cytoplasm. This triggers a cascade of events leading to caspase activation and cell death (Danial and Korsmeyer, 2004).

A family of proteins with structurally conserved domains, known as the Bcl-2- homology (BH) domains, have a central role in the intrinsic apoptotic pathway as it was demonstrated in the *in vitro* evaluations of the present study. Two of these BH-domain proteins, Bax and Bak, function to promote apoptosis by regulating mitochondrial membrane potential. Anti-apoptotic BH2 proteins, such as Bcl-2 and Bcl-xL, negatively regulate Bax and Bak, whereas a further group of these proteins, the Bcl-2- homology domain-3 (BH3)-only proteins, control these survival proteins (Strasser *et al*., 2000; Cory and Adams, 2002). One of the key contributions of p53 to apoptosis is the induction of the expression of genes that encode apoptotic proteins, functioning in both extrinsic and intrinsic pathways. Many potential apoptotic target genes of p53 have been described, including those that encode the BH3-domain proteins Noxa and Puma (Jeffers *et al*., 2003). Deletion of many of the described apoptotic targets of p53 has little effect on the sensitivity of the cell to stress-induced apoptosis, possibly reflecting the multitude of other apoptotic signals that can be induced by p53. The dramatic effect that loss of Puma has on the sensitivity of several different cell types to p53-induced cell death is therefore particularly telling (Villunger *et al*., 2003), indicating that Puma is a crucial mediator of apoptosis in response to p53. Interestingly, Puma has been proposed to function to release cytoplasmic p53 from inhibitory interactions with anti-apoptotic BH3-domain proteins; allowing p53 to function in a transcriptionally independent manner as a BH3-only protein (Vousden, 2005).

The other gene is *ki-67*, which is expressed as a protein under the name nuclear antigen Ki-67. It is a proliferation marker expressed only in cycling cells, and a strong correlation between S-phase fraction and Ki-67 index has been demonstrated (Scholzen and Gerdes, 2000). Consequently, quantitative assessment of Ki-67 staining on paraffin embedded tumour sections can provide

an estimate of the proliferation status of treated tumours. Furthermore, immunohistochemical measurement of proliferation by measuring Ki-67 expression has been widely used as a method for assessment of tumour biological behaviour (Urruticoechea *et al*., 2005). Ki-67 antigens are present in proliferating cells (in G1, S, G2, and mitosis cell division phases) while quiescent or resting cells do not express Ki-67 protein. Determination of levels of Ki-67 expression can be of particular importance in cancers in which the clinical course is difficult to predict by histological criteria alone (Mitic and McKay, 2005). In the present study, Ki-67 expressing-cells were present in control and treated xenograft sections, but their percentages were different. It showed a decreased expression in M sample treated animals as compared to controls. Therefore, propolis may have important potential in regulating the expression of *Ki-67* gene in tumour cells, but the available data are not sufficient to draw a final conclusion, especially if we consider that Ki-67 protein is not just a proliferating marker but evidences in breast cancer revealed that is associated with worse outcomes (Cheang *et al*., 2009).

In addition to the forthcoming potentials of M propolis sample, its anti-tumour effects may follow other directions; for instance, inflammatory responses and the observed inflammatory cells in tumour sections may justify such suggestion. Therefore, propolis may have immunomodulatory effects. In agreement with such presentation, administration of a water soluble derivative (WSD) of propolis to mice prevented the cyclophosphamide effects and enhanced the survival rate of the animals (Dimov *et al*., 1991). These authors also suggested that propolis modulates the non-specific immunity via macrophage activation, propolis stimulated cytokines production, such as IL-1β and TNF-α, by peritoneal macrophages of mice (Moriyasu *et al*., 1994). Propolis was also able to modulate both *in vivo* and *in vitro* productions of C1q by macrophages, as well as, the complement receptor function either directly or via cytokines (Dimov *et al*., 1992). *In vitro* assays also showed that WSD of

propolis inhibited the classical and alternative pathways of the complement system, and C3 was one of the targets of propolis action, and flavonoids and phenolic compounds were pointed out as its major anti-complementary compounds (Ivanovska *et al*., 1995; Georgieva *et al*., 1997). In this regard, it was demonstrated that six isolated compounds of propolis, identified as caffeoylquinic acid derivatives, enhanced the motility and spreading of macrophages (Tatefuji *et al*., 1996). Macrophages play an important role in anti-tumour response, through secretion of inhibitory cytokines for tumour growth, and production of reactive oxygen and nitrogen intermediates (Kaneno, 2005). Additionally, an ethanolic extract of Iraqi propolis sample has been shown to increase the phagocytic activity of mice peritoneal phagocytes, and it was able to modulate the immune suppression effects of mitomycin C (Ad'hiah *et al*., 2007).

5.9 Concluding Remarks

At the end of this discussion, it is possible to address the following question: *Is it possible to use propolis as a biological therapy in the form of a natural supplement in cancer treatment*? It is difficult to answer such question, but the present obtained data may augment the view for an understanding of the biological potential of propolis. This can be firstly scoped in a ground of the identified chemical constituents, which are compounds that were presented with important anti-oxidant and anti-tumour potentials both *in vitro* and *in vivo*. If we consider such scene in a more prospective thinking, it is possible to augment the view that propolis is a promising anti-tumour agent, but the *in vivo* evaluation may limit such optimism, because the inhibition rate did not reach 100%, and in best evaluation, it was approximately 40%, which can not be ignored as well. Other evaluations of the implemented tumours may however direct our attention to the pathway of action for propolis to reach its 40% inhibition rate, it is the immune modulation, and taking such evaluation with other reported evaluations

in regard of modulating the pathway of chemotherapy (mitomycin C), it is possible to introduce propolis as an adjuvant in chemotherapy enhancing the anti-tumour non-specific immunity.

A further insight on the limited propolis anti-tumour activity can also be discussed in terms of minimal systemic bioavailability in addition to its poor aqueous solubility; a view that may revolutionize the anti-tumour potentials of propolis. In this regard, it has been recently demonstrated that administrating propolis as a nanofood enhanced its anti-tumour activity, and it inhibited pancreatic cell growth in murine xenograft models. These effects were accompanied by a potent anti-angiogenic response, and therefore should facilitate the eventual clinical translation of this well-known but under-utilized therapeutic agent (Kim *et al*., 2008). Thus, modifying propolis, chemically or physically, may open up avenues for systemic therapy of human cancers wherein beneficial effects of propolis have been propounded.

Conclusions and Recommendations

Conclusions and Recommendations

I. Conclusions

Based on the obtained results, it is possible to reach the following conclusions:

1. The Iraqi propolis samples are rich in chemical constituents that have important biological potentials; for instance, anti-oxidant and anti-tumour. Among these are flavonoids and phenolic acids and their esters, which were the most abundant compounds in the propolis samples. Clerodane diterpenoids were also detected, and it was for the first time in propolis samples collected from a temperate region. Furthermore, a compound with unknown chemical identity was spotted.
2. The use of a capillary HPLC column coupled to a hybrid MS instrument allowed the analysis of propolis samples very sensitive and accurate in achieving linearity over a wide range of concentrations.
3. The anti-oxidant activity of Iraqi propolis samples was positively correlated with the total of polyphenols, because the strongest anti-oxidant activity was observed in samples that were rich in these compounds.
4. The propolis samples that had a powerful antioxidant activity were the most cytotoxic against the investigated tumour cell lines (HL-60 and HCT-116), while they were less cytotoxic against normal cell line (HuFb), and this may demonstrate the selectivity of propolis compounds against cells.
5. The inhibitory effect of propolis samples against the proliferation of HL-60 and HCT-116 cells followed two different pathways of cell death, either apoptosis or necrosis, and M propolis sample possessed the highest potency in inhibiting growth of cancer cell lines.
6. The M propolis sample is a potent apoptosis-inducing agent against HL-60 cells, and its action was through Bcl-2/Bax regulation.

7. The growth inhibitory effect of M propolis sample was coupled with cell cycle arrest and the data were confirmed using both monoparametric and biparametric DNA cell cycle analysis. The biparametric BrdUrd/DNA cell cycle analysis recorded that M sample caused a block of cells in G_1 phase of cell cycle in HL-60 and HCT-116 cell lines, with different kinetic for BrdUrd positive and negative cells.
8. Assessing γ-H2AX by an immunocytochemical method revealed the anti-mutagenic potentials of M propolis sample, and such assay can be further employed in the detection of potential carcinogenic effects and the apoptotic process.
9. The M propolis sample was involved in reducing the progression of xenografted tumour *in vivo* using two administration route (orally and intrapertoneally), and the oral demonstration was the best in this regard with out any significant adverse effects or mortality.
10. The M propolis sample was significantly *in vivo* effective in increasing the expression of *p53* gene and decreasing the expression of *ki-67* gene in tumour sections, and such effects accompanied with a decreased frequency of mitosis, an increased endoreduplication, enhanced inflammation, ulceration and necrosis in tumour cells and tissue.
11. The obtained results from *in vitro* and *in vivo* evaluations suggested the selectivity of propolis against cancer cell lines, and it is possible to speculate that propolis is promising substance as a dietary supplement for reducing cancer risk because of their anti-proliferative potential and their cytotoxic effects against cancer cells.

II. Recommendations

1. The active ingredients responsible for the observed effects and the specific molecular signaling pathways involved in the cell cycle perturbations, angiogenesis and induction of cell death is remained to be identified.
2. To get a more reproducible view about the anti-mutagenic effects of propolis, future studies are recommended to use DNA microarray to examine the gene expression profile in propolis-treated tumour cells, *in vitro* and *in vivo*, especially the evaluation considers other tumour cell lines.
3. Further investigations are required to define the precise mechanisms that can explain how chromatin structure is modified by the phosphorylation of H2AX, and how this modification is linked to DNA damage repair and genomic instability, or apoptosis.
4. The immune regulatory effects of propolis has to be characterised on the basis of functional immunological properties that are based on evaluations of the innate and adaptive immune responses employing CD markers (CD4 and/or CD8) and cytokines as tools for such evaluations.
5. To enhance the biological potentials of propolis, it is recommended to introduce methodological modifications and evaluate such modifications in the treatment of xenografted tumours separately or in conjunction with chemotherapies used in cancer treatment.

References

Abd El Hady, F. K. and Hegazi, A. G. (2002). Egyptian propolis: 2. Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. Z. Naturforsch., 57: 386-394.

Abdulamir, A. S.; Hafidh, R. R.; Mahdi, L. K.; Aljeboori, T.; Abubakar, F. and Abbas, K. A. (2008). The Interplay between p53 and p21 tumor suppressor proteins in the transformation of colorectal adenoma to carcinoma. Am. J. Immunol., 4: 14-22.

Abeysinghe, R. D.; Greene, B. T.; Haynes, R.; Willingham, M. C.; Turner, J.; Planalp, R. P.; Brechbiel, M.W.; Torti, F. M. and Torti, S. V. (2001). p53-independent apoptosis mediated by tachpyridine, an anti-cancer iron chelator. Carcinogenesis, 22: 1607-1614.

Adams, J. M. and Cory, S. (2007). The Bcl-2-regulated apoptosis switch: mechanism and therapeutic potential. Curr. Opin. Immunol., 19: 488-496.

Ad'hiah, A. H.; Sulaiman, G. M. and Jaber, M. S. (2007). Some Immunological evaluations of Iraqi propolis extract in albino male mice. J. Fac. Med. Baghdad, 49:121-125.

Adimoolam, S. and Ford, J. M. (2003). p53 and regulation of DNA damage recognition during nucleotide excision repair. DNA Repair, 2: 947-954.

Akouchekian, M. (2008). Investigation of human mitochondrial DNA abnormality in colon cancer. Ph.D. Thesis, University of Hannover, Germany. Available at online http://dnb.ddb.de.

Albino, A. P.; Huang, X.; Jorgensen, E.; Yang, J.; Gietl, D.; Traganos, F. and Darzynkiewicz, Z. (2004). Induction of H2AX phosphorylation in pulmonary cells by tobacco smoke: a new assay for carcinogens. Cell Cycle, 3: 1062-1068.

Alencar, S. M.; Oldoni, T. L. C.; Castro, M. L.; Cabral, I. S. R.; Costa-Neto, C. M.; Cury, J. A.; Rosalen, P. L. and Ikegaki, M. (2007). Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis. J. Ethnopharmacol., 113: 278-283.

Alfaris, A. A.; Abdulsamad, R. K. and Swad, A. A. (2009). Comparative studies between propolis, dexametason and gentamycin treatments of induced corneal ulcer in rabbits. Iraqi J. Veter. Sci., 23:75-80.

Alfieri, R.; Barberis, M.; Chiaradonna, F.; Gaglio, D.; Milanesi, L.; Vanoni, M.;

Klipp, E. and Alberghina, L. (2009). Towards a systems biology approach to mammalian cell cycle: modeling the entrance into S phase of quiescent fibroblasts after serum stimulation. BMC bioinformatics, 10: S16.

Allen, p. and Davies, G. (2007). Apoptosis detection by flow cytometry: In: Flow cytometry: principles and applications. Macey, M.G. Ed., Humana Press, pp.: 147-164.

Almawi, W. Y.; Melemedjian, O. K. and Abou Jaoude, M. M. (2004). On the link between Bcl-2 family proteins and glucocorticoid induced apoptosis. J. Leukocyte Biol., 76:7-14.

Almedia, E. C. and Menezes, H. (2002). Anti-inflammatory activity of propolis extracts: a review. J. Venom. Anim. Toxins, 8: 191-212.

Alternative Health and Herbs Remedies (2010). Bee propolis, Available online at http://www.healthherbs.com.

Alves De Lima, R. O.; Bazo, A. P.; Said, R. A.; Sforcin, J. M.; Bankova, V.; Darros, B. R. and Salvadori, D. M. F. (2005). Modifying effect of propolis on dimethylhydrazine-induced DNAdamage but not colonic aberrant crypt foci in rats. Environ. Mole. Mutagen., 45: 8-16.

Aso, K.; Kanno, S.; Tadano, T.; Satoh, S. and Ishikawa, M. (2004). Inhibitory effect of propolis on the growth of human leukemia U937. Biol. Pharm. Bull., 27:727-730.

Astley, S. B. (2003). Dietary antioxidants - past, present and future?. Trends Food Sci. Technol., 14: 93-98.

Bailly, C. (2000). Topoisomerase I poisons and suppressors as anticancer drugs. Curr. Med. Chem., 7: 39-58.

Bankova, V. (2000). Determininig quality in propolis samples. J .Am. Apither. Soc.; vol. 7, Nº 2.

Bankova, V. (2005). Recent trends and important developments in propolis research. eCAM., 2:29-32.

Bankova, V.; De Castro, S. L. and Marcucci, M. C. (2000). Propolis: recent advances in chemistry and plant origin. Apidologie, 31: 3-15.

Bankova, V.; Marcucci, M. C.; Simova, S.; Nikolova, N.; Kujumgiev A. and

Popov, S. (1996). Antibacterial diterpenic acids from Brazilian propolis. Z. Naturforsch., 51c: 277-280.

Banskota, A. H.; Nagaoka, T.; Sumioka, L. Y.; Tezuka, Y.; Awale, S.; Midorikawa, K.; Matsushige, K. and Kadota, S. (2002). Antiproliferative activity of the Netherlands propolis and its active principles in cancer cell lines. J. Ethnopharmacol., 80: 67-73.

Banskota, A. H.; Tezuka, Y.; Adnyana, I.; Midorikawa, K.; Matsushige, K. and Message, D. (2000). Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China. J. Ethnopharmacol., 72: 239-246.

Banskota, A. H.; Tezuka, Y. and Kadota, S. (2001). Recent progress in pharmacological research of propolis. Phytother. Res., 15: 561-571.

Banskota, A. H.; Tezuka, Y.; Prasain, J. K.; Matsushige, K.; Saiki, I. and Kadota, S. (1998). Chemical constituents of Brazilian propolis and their cytotoxic activities. J. Nat. Prod., 61: 896-900.

Bassani Silva, S.; Sforcin, J. M.; Amaral, A. S.; Gaspar, L. F. J. and Rocha, N. S. (2007). Propolis effect *in vitro* on venereal transmissible canine tumor. R. P. C. V., 102: 261-265.

Bazo, A. P.; Rodrigues, M. A. M.; Sforcin, J. M.; Camargo, J. L. V.; Ribeiro, L. R. and Salvadori, D. M. F. (2002). Protective action of propolis on the rat colon carcinogenesis. Teratog. Carcinog. mutag., 22: 183-194.

Benkovic ,V. H.; Knezevic, A. H.; Brozovic, G.; Knezevic, F.; Dikic, D.; Bevanda, M.; Basic, I. and Orsolic, N.(2007). Enhanced antitumor activity of irinotecan combined with propolis and its polyphenolic compounds on Ehrlich ascites tumor in mice. Biomed. Pharmacother., 61: 292-297.

Beresford, M. J.; Wilson, G. D. and Makris, A. (2006). Measuring proliferation in breast cancer: practicalities and applications. Breast Cancer Res., 8: 216.

Bergamaschi, D.; Ronzoni, S.; Taverna, S.; Faretta, M.; De Feudis, P.; Faircloth, G.; Jimeno, J.; Erba, E. and D'Incalci, M. (1999). Cell cycle perturbations and apoptosis induced by isohomohalichondrin B (IHB), a natural marine compound. Br. J. Cancer, 79: 267-277.

Berns, E.; Klijn, J.; van Putten, W.; de Witte, H.; Look, M.; Meijer-van Gelder, M.; Willman, K.; Portengen, H.; Benraad, T. and Foekens, J. (1998). p53

protein accumulation predicts poor response to tamoxifen therapy of patients with recurrent breast cancer. J. Clin. Oncol., 16: 121-127.

Berrieman, H. K.; Smith, L.; O'Kane, S. L.; Campbell, A.; Lind, M. J. and Cawkwell, L. (2005). The expression of Bcl-2 family proteins differs between nonsmall cell lung carcinoma subtypes. Cancer, 103: 1415-1419.

Bestwick, C. S. and Milne, L. (2006). Influence of galangin on HL-60 cell proliferation and survival .Cancer Lett., 243: 80-89.

Bestwick, C.S.; Milne, L. and Duthie, S.J. (2007). Kaempferol induced inhibition of HL-60 cell growth results from a heterogeneous response, dominated by cell cycle alterations. Chem. Biol. Interact., 170: 76-85.

Bilsland, E. and Downs, J. A. (2005). Tails of histones in DNA double-strand break repair. Mutagenesis, 20: 153-163.

Birnie, G. D. (1988). The HL60 cell line: A model system for studying human myeloid cell differentiation. Br. J. Cancer, 58: 41-45.

Bogdanov, S. (2009). Functional and biological properties of bee products. Bee products science. Available online at www.bee-hexagon .net.

Bonsing, B. A.; Corver, W. E.; Gorsira, M. C.; van Vliet, M.; Oud, P. S. and Cornelisse, C. J. (1997). Specificity of seven monoclonal antibodies against p53 evaluated with Western blotting, immunohistochemistry, confocal laser scanning microscopy, and flow cytometry. Cytometry, 28: 11-24.

Borek, C. (2004). Dietary Antioxidants and Human Cancer. Integr. Cancer Ther., 3: 333-341.

Bowen, W. and Wylie, P. (2005). A high throughput, high content assay for cell cycle analysis. TTP LabTech Ltd, 1-3.

Brennan, P. (2002).Gene-enviroment interaction and aetiology of cancer: what does it mean and how can we measure it?. Carcinogenesis, 23: 381-387.

Brockhoff, G. and Knuechel, R. (2003). Flow cytometric DNA analysis in oncology: from single to multiparametric measurements. Laboratoriums Med., 27: 167-174.

Brunelle, J. K. and Letai, A. (2009). Control of mitochondrial apoptosis by the Bcl-2 family. J. Cell Sci., 122: 437-441.

Bruno, S. and Darzynkiewicz, Z. (1992). Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Prolif., 25: 31-40.

Bufalo, M. C.; Candeias, J. M. G. and Sforcin, J. M. (2007). *In vitro* Cytotoxic effect of Brazilian green propolis on human laryngeal epidermoid carcinoma (HEp-2) cells. eCAM., 1-5.

Burdock, G. A. (1998). Review of the biological properties and toxicity of propolis. Food Chem. Toxicol., 36: 341-363.

Buyukberber, M.; Savas, M. C.; Bagci, C.; Koruk, M.; Gulsen, M. T.; Tutar, E.; Bilgic, T.; Deveci, R. and Kucuk, C. (2009). The beneficial effect of propolis on cerulein-induced experimental acute pancreatitis in rats. Turk. J. Gastroenterol., 20: 122-128.

Cantero, G.; Campanella, C.; Mateos, S. and Cortes, F. (2006). Topoisomerase II inhibition and high yield of endoreduplication induced by the flavonoids luteolin and quercetin. Mutagenesis, 21: 321-325.

Casagrande, F. and Darbon, J. M. (2001). Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: Regulation of cyclin-dependent kinases CDK2 and CDK1. Biochem. Pharmacol., 61: 1205-1215.

Castaldo, S. and Capasso, F. (2002). Propolis, an old remedy used in modern medicine. Fitoterapia, 73: S1-S6.

Celeste, A.; Difilippantonio, S.; Difilippantonio, M. J.; Fernandez-Capetillo, O., Pilch, D. R.; Sedelnikova, O. A.; Eckhaus, M.; Ried, T.; Bonner, W. M. and Nussenzweig, A.(2003). H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. Cell, 114: 371-383.

Chang, H. Y.; Ho, Y. L.; Sheu, M. J.; Lin, Y. H.; Tseng, M. C.; Wu, S. H.; Huang, G. J.; and Chang, Y. S.(2007). Antioxidant and free radical scavenging activities of *Phellinus merrillii* extracts. Botanical Studies, 48: 407-417.

Chang, J. S. ; Hsu, Y. ; Kuo, P. L.; Kuo, Y. C., Chiang, L. C. and Lin, C. C. (2005). Increase of Bax/ Bcl-XL ratio and arrest of cell cycle by luteolin in immortalized human hepatoma cell line. Life Sci., 76: 1883-1893.

Cheang, M. C. U.; Chia, S. K.; Voduc, D.; Gao, D.; Leung, S.; Snider, J.; Watson, M.; Davies, S.; Bernard, P. S.; Parker, J. S.; Perou, C. M.; Ellis, M. J. and Nielsen,T. O. (2009). Ki67 Index, HER2 Status, and Prognosis of Patients with luminal B breast cancer. J. Natl. Cancer Inst., 101: 736-750.

Chen, C. N.; Weng, M. S.; Wu, C. L. and Lin, J. K. (2004).Comparison of radical scavenging activity, cytotoxic effects and apoptosis induction in human melanoma cells by Taiwanese propolis from different sources. eCAM., 1: 175-185.

Chen, C. N.; Wu, C. L. and Lin, J. K. (2007). Apoptosis of human melanoma cells induced by the novel compounds propolin A and propolin B from Taiwenese propolis. Cancer lett., 245: 218-231.

Chen, C. N.; Wu, C. L.; Shy, H. S. and Lin J. K. (2003). Cytotoxic prenylflavones from Taiwanese propolis. J. Nat. Prod., 66: 503-506.

Chen, Y.J.; Shiao, M.S and Wang, S.Y. (2001). The antioxidant caffeic acid phenethyl ester induces apoptosis associated with selective scavenging of hydrogen peroxide in human leukemic HL-60 cells. Anticancer Drugs, 12: 143-149.

Chen, Y. W.; Wu, S. W.; Ho, K. K.; Lin, S. B.; Huang, C. Y. and Chen, C. N. (2008). Characterisation of Taiwanese propolis collected from different locations and seasons. J. Sci. Food Agric., 88: 412-419.

Chene, P.; Fuchs, J.; Bohn, J.; Garcia-Echeverria, C.; Furet, P. and Fabbro, D. (2000). A small synthetic peptide, which inhibits the p53-hdm2 interaction, stimulates the p53 pathway in tumour cell lines. J. Mol. Biol., 299: 245-253.

Cheng, E. H.; Wei, M. C.; Weiler, S.; Flavell, R. A.; Mak, T. W.; Lindsten, T. and Korsmeyer, S. J. (2001). BCL-2, BCL-xL sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol. Cell, 8: 705-711.

Chikaraishi, Y.; Izuta, H.; Shimazawa, M.; Mishima, S. and Hara, H. (2009). Angiostatic effects of Brazilian green propolis and its chemical constituents. Mole. Nutr. Food Res., 54: 566 - 575.

Claus, R.; Kinscherf ,R. ;Gehrke, C.; Bonaterra, G.; Basnet, P. and Metz, J. (2000). Antiapoptotic effects of propolis extract and propel on human macrophages exposed to minimally modified low density lipoprotein. Arzn.

Forsch. Drug Res., 50: 373-379.

Cooper, G. M. and Hausman, R. E. (2009). The Cell: A Molecular Approach. 5th ed., Co-published by ASM Press and Sinauer Associates, Inc., p.: 771.

Cope, T.; Lupton, C.; Bradford, J.; Hung, J. and Bowen, W. (2009). Cell cycle analysis using microplate cytometry: A comparison of laser and dye combinations. TTP LabTech Ltd, 1.

Cortes, F. and Pastor, N. (2003). Induction of endoreduplication by topoisomerase II catalytic inhibitors. Mutagenesis., 18: 105-112.

Cortes, F.; Pastor, N.; Mateos, S. and Dominguez, I. (2003). Roles of DNA topoisomerases in chromosome segregation and mitosis. Rev. Mutat. Res., 543: 59-66.

Cory, S. and Adams, J. M. (2002).The Bcl2 family: regulators of the cellular life-or-death switch. Nature Rev. Cancer, 2: 647-656.

Cowell, I. G.; Sunter, N. J.; Singh, P. B.; Austin, C. A.; Durkacz, B. W. and Tilby, M. J. (2007). γ-H2AX foci form preferentially in euchromatin after ionising-radiation. PLoS ONE, 2: e1057.

Cuesta, A.; Rodriguez, A.; Esteban, M. A. and Meseguer, J. (2005). *In vivo* effects of propolis, a honeybee product, on gilthead seabream innate immune responses. Fish Shelfish Immunol., 18: 71-80.

Czuwara-Ladykowska, J.; Makiela, B.; Smith, E. A.; Trojanowska, M. and Rudnicka, L. (2001). The inhibitory effects of camptothecin, a topoisomerase I inhibitor, on collagen synthesis in fibroblasts from patients with systemic sclerosis. Arthritis Res., 3: 311-318.

Danial, N. N. and Korsmeyer, S. J. (2004).Cell death: critical control points. Cell, 116: 205-219.

Darzynkiewicz, Z. and Bedner, E. (2000). Analysis of apoptotic cells by flow and laser scanning cytometry. Methods Enzymol., 322:18-39.

Darzynkiewicz, Z.; Bedner, E. and Smolewski, P. (2001). Flow cytometry in analysis of cell cycle and apoptosis. Semin. Hematol. , 38: 179-193.

Darzynkiewicz, Z.; Crissman, H. A. and Jacobberger, J. W. (2004). Cytometry of cell cycle: Cycling through history. Cytometry, 58A: 21-32.

Darzynkiewicz, Z.; Galkowski, D. and Zhao, H. (2008). Analysis of apoptosis by cytometry using TUNEL assay. Methods, 44: 250-254.

Darzynkiewicz, Z. and Huang, X. (2004). Analysis of cellular DNA content by flow cytometry . In: Current Protocols in Immunology. Coico, R., Ed., Curr. Protoc.,Wiley-Liss, New York, 5.7.1-5.7.18.

Darzynkiewicz, Z.; Huang, X. and Okafuji, M. (2006). Detection of DNA strand breaks by flow and laser scanning cytometry in studies of apoptosis and cell proliferation (DNA replication). Methods Mol. Biol., 314: 81-93.

Darzynkiewicz, Z. and Juan, G. (2001). Analysis of DNA content and BrdU incorporation. Chapter 7: Unit 7.7., Curr. Protoc. Cytom., PubMed ID: 18770736.

Davies, D. (2008). Cell Cycle Analysis. Avaliable at online http://science.cancerresearchuk.org/sci/facs/facs_major_apps/cell_cycle_analysis/?version=1.

De Angelis, P. M.; Kravik, K. L.; Tunheim, S. H.; Haug, T. and Reichelt, W. H (2004). Comparison of gene expression in HCT116 treatment derivatives generated by two different 5-fluorouracil exposure protocols. Mol. Cancer, 3: 11. Avalaible online at http://www.molecular-cancer.com/content/3/1/11.

De Angelis, P. M.; Stokke, T.; Thorstensen, L.; Lothe, R. A. and Clausen, O. P. (1998). Apoptosis and expression of Bax, Bcl-x, and Bcl-2 apoptotic regulatory proteins in colorectal carcinomas, and association with p53 genotype/phenotype. Mol. Pathol., 51: 254-261.

Debnath, J.; Baehrecke, E. H., and Kroemer, G. (2005). Does autophagy contribute to cell death?. Autophagy, 1: 66-74.

De Lange, T. (1994). Activation of telomerase in a human tumor. Proc. Natl. Acad. Sci. U S A, 91: 2882-2885.

Del Bufalo, D.; Biroccio, A.; Soddu, S.; Laudonio, N.; D'Angelo, C.; Sacchi, A. and Zupi, G. (1996). Lonidamine induces apoptosis in drug-resistant cells independently of the p53 gene. J. Clin. Invest., 98: 1165-1173.

Derenne, S.; Monia, B.; Dean, N. M.; Taylor, J. K.; Rapp, M. J.; Harousseau, J. L.; Bataille, R. and Amiot, M. (2002). Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x (L) is an essential survival protein of human

myeloma cells. Blood, 100: 194-199.

Dimov, V.; Ivanovska, N.; Manolova, N.; Bankova, V.; Nikolov, N. and Popov, S. (1991). Immunomodulatory action of propolis. Influence on anti-infections protection and macrophage function. Apidologie, 22: 155-162.

Dimov, V.; Ivanovska, N.; Manolova, N.; Bankova, V.; Nikolov, N. and Popov, S. (1992). Immunomodulatory action of propolis. IV. Prophylatic activity against Gram-negative infections and adjuvant effect of the water-soluble derivate. Vaccine, 10: 817-823.

Duran, G.; Duran, N.; Culha, G.; Ozcan, B.; Oztas, H. and Ozer, B. (2008). *In vitro* anti- leishmanial activity of Adana propolis samples on *Leishmania tropica*: a preliminary study. Parasitol. Res., 102: 1217-1225.

Efeyan, A. and Serrano, M. (2007). p53: guardian of the genome and policeman of the oncogenes. Cell Cycle, 6: 1006-1010.

El Khatib, A.; Agha, A.; Mahran, L. and Khayyal, M. (2002). Prophylactic effect of aqueous propolis extract against acute experimental hepatotoxicity. Z. Naturforsch., 57: 379-85.

Elmore, S. (2007). Apoptosis: A review of programmed cell death. Toxicol. Pathol., 35: 495-516.

Erba, E.; Bergamaschi, D.; Bassano, L.; Damia, G.; Ronzoni, S.; Faircloth, G.T. and D'Incalci, M. (2001). Ecteinascidin-743 (ET-743), a natural marine compound, with a unique mechanism of action. E. J. C., 37: 97-105.

Erba, E. and Sen, S. (1996). Synchronization of cancer cell lines with methotrexate *in vitro*. Methods Cell Sci., 18: 149-163.

Farre, R.; Frascquet, I. and Sanchez, A. (2004). Propolis and human health. Ars Pharm., 45: 21-43.

Fernandez-Capetillo, O.; Lee, A.; Nussenzweig, M. and Nussenzweig, A. (2004). H2AX: the histone guardian of the genome. DNA Repair, 3: 959-967.

Fonatsch, C.; Duchrow, M.; Rieder, H.; Schluter, C.; Gerdes, J. (1991). Assignment of the human Ki-67 gene (MKI67) to 10q25-qter. Genomics, 11: 476-477.

Fridman, J. S. and Lowe, S. W. (2003). Control of apoptosis by p53. Oncogene, 22: 9030-9040.

Friedberg, E. C. (2003). DNA damage and repair. Nature, 421: 436-440.

Gatz, S. A. and Wiesmuller, L. (2006). p53 in recombination and repair. Cell Death Differ., 13: 1003-1006.

Gautam, R.; Saklani , A. and Jachak, S. M. (2007). Indian medicinal plants as a source of anti-rnycobacterial agents. J. Ethnopharmacol., 110: 200-234.

Gekker, G.; Hu, S.; Spivak, M.; Lokensgard, J. R. and Peterson, P. K. (2005). Anti-HIV-1 activity of propolis in CD4+ lymphocyte and microglial cell cultures. J. Ethnopharmacol., 102: 158-163.

Georgieva, P.; Ivanovska, N.; Bankova, V. and Popov, S. (1997). Anticomplement activity of lysine complexes of propolis phenolic constituents and their synthetic analogs. Z. Naturforsch., 52c: 60-64.

Gerdes, J.; Li, L.; Schlueter, C.; Duchrow, M.; Wohlenberg, C.; Gerlach, C.; Stahmer, I.; Kloth, S.; Brandt, E. and Flad, H. D. (1991). Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am. J. Pathol., 138: 867-873.

Gerdes, J.; Schwab, U.; Lemke, H. and Stein, H. (1983). Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer, 31: 13-20.

Gerke, V. and Moss, S.E. (2002). Annexins: from structure to function. Physiol. Rev., 82: 331-371.

Geronikaki, A. A. and Gavalas, A. M. (2006). Antioxidants and inflammatory disease: synthetic and natural antioxidants with anti-inflammatory activity. Comb. Chem. High Throughput Screen., 9: 425-442.

Gomez-Caravaca, A. M.; Gomez-Romero, M.; Arraez-Roman, D.; Segura-Carretero A. and Fernandez-Gutierrez, A. (2006). Advances in the analysis of phenolic compounds in products derived from bees. J. Pharm. Biomed. Anal., 41: 1220-1234.

Gordaliza, M. (2007). Natural products as leads to anticancer drugs. Clin. Transl. Oncol., 9: 767-776.

Grasberger, B. L.; Lu, T.; Schubert, C.; Parks, D. J.; Carver, T. E.; Koblish, H. K.; Cummings, M. D.; LaFrance, L. V.; Milkiewicz, K. L.; Calvo, R. R.; Maguire, D.; Lattanze, J.; Franks, C. F.; Zhao, S.; Ramachandren, K.; Bylebyl, G. R.; Zhang, M.; Manthey, C. L.; Petrella, E. C.; Pantoliano, M. W.; Deckman, I. C.; Spurlino, J. C.; Maroney, A. C.; Tomczuk, B. E.; Molloy, C. J. and Bone, R. F. (2005). Discovery and cocrystal structure of benzodiazepinedione HDM2 antagonists that activate p53 in cells. J. Med. Chem., 48: 909-912.

Green, D. R. (1998). Apoptotic pathways: the roads to ruin. Cell, 94: 695-698.

Gupta, S.; Afaq, F. and Mukhtar, H. (2001). Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. Biochem. Biophys. Res. Commun., 287: 914-920.

Gustafsson, A. B. and Gottlieb, R. A. (2007). Bcl-2 family members and apoptosis, taken to heart. Am. J. Physiol. Cell Physiol., 292: 45-51.

Haddad, A. Q. (2008). The Cellular and molecular properties of flavonoid in prostate cancer chemoprevention. Ph.D. Thesis, University Toronto, Canada, pp.: 1-244.

Hainaut, P. (1995). The tumor suppressor protein p53: a receptor to genotoxic stress that controls cell growth and survival. Curr. Opin. Oncol., 7: 76-82.

Halliwell, B. (1994). Free radicals and antioxidants, and human disease: curiosity, cause, or consequence. Lancet, 344: 721-724.

Hanahan, D. and Weinberg, R. A. (2000) .The Hallmarks of Cancer. Cell, 100: 57-70.

Harvey, A. L. (2008). Natural products in drug discovery. Drug Discov. Today, 13: 894-901.

Hawn, M. T.; Umar, A.; Carethers, J. M.; Marra, G.; Kunkel, T. A.; Boland, C. R. and Koi, M. (1995). Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. Cancer Res., 55: 3721-3725.

Hegazi, A. G. (2000). Propolis: An overview. Congreso Internacional de propoleos. Durante los dias 1 y 2 de Septiembre de 2000 en Buenos Aires - Argentina. http://www.apinetla.com.ar/ congreso 2000.

Hegazi, A. G. and Abd El Hady F. K. (2002). Egyptian Propolis: 3. Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands. Z. Naturforsch., 57c: 395-402.

Heidebrecht, H. J.; Buck, F.; Haas, K.; Wacker, H. H. and Parwaresch, R. (1996). Monoclonal antibodies Ki-S3 and Ki-S5 yield new data on the "Ki-67" proteins. Cell Prolif., 29: 413-425.

Hoetelmans, R. W. M.; van Slooten, H. J.; Keijzer, R.; Erkeland, S.; van de Velde, C. J. H. and van Dierendonck, J. H. (2000). Bcl-2 and Bax proteins are present in interphase nuclei of mammalian cells. Cell Death Diff., 7: 384-392.

Holdenrieder, S. and Stieber, P. (2004). Apoptotic markers in cancer. Clin. Biochem., 37: 605-617.

Hood, B. L.; Darfler, M. M.; Guiel, T. G.; Furusato, B.; Lucas, D. A.; Ringeisen, B. R.; Sesterhenn, I. A.; Conrads, T. P.; Veenstra, T. D. and Krizman, D. B. (2005). Proteomic analysis of formalin-fixed prostate cancer tissue. Mol. Cell. Prot., 4: 1741-1753.

Hu, C. and Kitts, D. D. (2000). Studies on the antioxidant activity of *Echinaceae* root extract. J. Agric. Food Chem., 48:1466-1472.

Huang, M. T.; Ma, W.; Yen, P.; Xie, J. G.; Han, J.; Frenkel, K.; Grunberger, D. and Conney, A. H. (1996). Inhibitory effects of caffeic acid phenethyl ester (CAPE) on 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion in mouse skin and the synthesis of DNA, RNA and protein in HeLa cells. Carcinogenesis, 17: 761-765.

Huang, X. and Darzynkiewicz, Z. (2006). Cytometric Assessment of Histone H2AX Phosphorylation: A Reporter of DNA Damage. Methods Mol. Biol., 314: 73-80.

Huang, X.; Halicka, H. D.; Traganos, F.; Tanaka, T.; Kurose, A. and Darzynkiewicz, Z. (2005). Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis .Cell Prolif., 38: 223-243.

Huerta, S.; Goulet, E. J.; Huerta-Yepez, S. and Livingston, E. H. (2007). Screening and detection of apoptosis. J. Surg. Res., 139: 143-156.

Hughey, C. T.; Barbour, K. W.; Berger, F. G. and Berger, S. H. (1993). Functional effects of a naturally occurring amino acid substitution in

human thymidylate synthase. Mol. Pharmacol., 44: 316-323.

Imamura, J.; Miyoshi, I. and Koeffler, H.P. (1994). p53 in hematologic malignancies. Blood, 84: 2412-2421.

Inoue, K.; Saito, M.; Kanai, T.; Kawata, T.; Shigematsu, N.; Uno, T.; Isobe, K.; Liu, C. and Ito, H. (2008). Anti-Tumor effects of water-soluble propolis on a mouse sarcoma cell line *in vivo* and *in vitro*. Am. J. Chin. Med., 36: 623-632.

Ishihara, M.; Naoi, K.; Hashita, M.; Itoh, Y. and Suzui, M. (2009). Growth inhibitory activity of ethanol extracts of Chinese and Brazilian propolis in four human colon carcinoma cell lines. Oncol. Rep., 22: 349-354.

Isla, M. I.; Nieva Moreno, M. I.; Sampietro, A. R. and Vattuone , M. A. (2001). Antioxidant activity of Argentine propolis extracts. J. Ethnopharmacol., 76: 165-170.

Ivanovska, N. D.; Dimov, V. B.; Pavlova, S.; Bankova, V. and Popov, S. (1995). Immunomodulatory action of propolis. V. Anticomplementary activity of a water-soluble derivative. J. Ethnopharmacol., 47: 135-143.

Jackson, J. R.; Patrick, D. R.; Dar, M. M. and Huang, P. S. (2007). Targeted antimitotic therapies: can we improve on tubulin agents?. Nat. Rev. Cancer, 7: 107-117.

Jackson, S. P. (2002). Sensing and repairing DNA double-strand breaks. Carcinogenesis, 23: 687-696.

Jakobisiak, M.; Lasek, W. and Golab, J. (2003). Natural mechanisms protecting against cancer. Immunol. Lett., 90: 103-122.

Jeffers, J. R.; Parganas, E.; Lee, Y.; Yang, C.; Wang, J.; Brennan, J.; Mac Lean, K. H.; Han, J.; Chittenden, T.; Ihle, J. N.; McKinnon, P. J.; Cleveland, J. L. and Zambetti, G. P.(2003). Puma is an essential mediator of p53-dependent and-independent apoptotic pathways. Cancer Cell, 4: 321-328.

Jordan, M. A. and Wilson, L. (2004). Microtubules as a target for anticancer drugs. Nat. Rev. Cancer, 4: 253-265.

Kaneno, R. (2005). Role of natural killer cells in antitumor resistance. Ann. Rev. Biomed. Sci., 7: 127-148.

Kanno, S. I.; Tomizawa, A.; Ohtake, T.; Koiwai, K.; Ujibe, M. and Ishikawa, M. (2006). Naringenin-induced apoptosis via activation of NF-κB and necrosis involving the loss of ATP in human promyeloleukemia HL-60 cells. Toxicol. Lett., 166: 131-139.

Kataoka, Y.; Bindokas, V. P.; Duggan, R. C.; Murley, J. S.; Grdina, D. J. (2006). Flow cytometric analysis of phosphorylated histone H2AX following exposure to ionizing radiation in human microvascular endothelial cells. J. Radiat. Res., 47: 245-257.

Kerr, J. F. (2002). History of the events leading to the formulation of the apoptosis concept. Toxicology, 181-182: 471-474.

Khan, N. A. (2002). Antioxdants and cancer. The Sciences, 2: 38-43.

Kill, I. R. (1996). Localisation of the Ki-67 antigen within the nucleolus: Evidence for a fibrillarin-deficient region of the dense fibrillar component. J. Cell Sci., 109: 1253-1263.

Kim, A. R.; Cho, J. Y.; Zou, Y.; Choi, J. S. and Chung, H. Y. (2005). Flavonoids differentially modulate nitric oxide production pathways in lipopolysaccharide-activated RAW264.7 cells. Arch. Pharm. Res., 28: 297-304.

Kim, D. M.; Lee, G. D.; Aum, S. H.; and Kim, H. J. (2008). Preparation of propolis nanofood and application to human cancer. Biol. Pharm. Bull., 31: 1704-1710.

Kim, H.; Tu, H.; Ren, D.; Takeuchi, O.; Jeffers, J. R.; Zambetti, G. P.; Hsieh, J. J. D. and Cheng, E. H. Y. (2009). Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. Mol. Cell, 36: 487-499.

Kimoto, T.; Arai, S.; Kohguchi, M.; Aga, M.; Nomura, Y.; Micallef, M. J.; Kurimoto, M. and Mito, K. (1998). Apoptosis and suppression of tumor growth by artepillin C extracted from Brazilian propolis. Cancer Detec. Prev., 22: 506-515.

Kinner, A.; Wu, W.; Staudt, C. and Iliakis, G. (2008). γ-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucl. Acids Res., 36: 5678-5694.

Koc, A. N.; Silici, S.; Ayangil, D.; Ferahbas, A. and Cankaya, S. (2005).

Comparison of *in vitro* activities of antifungal drugs and ethanolic extract of propolis against *Trichophyton rubrum* and *T. mentagrophytes* by using a micro-dilution assay. Mycosis, 48: 205-210.

Koc, A. N.; Silici, S.; Mutlu-Sariguzel, F. and Sagdic, O. (2007). Antifungal activity of propolis. Food Technol. Biotechnol., 45: 57-61.

Koelbl, O.; Rosenwald, A.; Haber, M.; Muller, J.; Reuther, J. and Flentje, M. (2001). p53 and Ki-67 as predictive markers for radiosensitivity in squamous cell carcinoma of the oral cavity? an immunohistochemical and clinicopathologic study. Int. J. Rad. Oncol. Biol. Phys., 49: 147-154.

Kook, S. H.; Son, Y. O.; Chung, S. W.; Lee, S. A.; Kim, J. G.; Jeon, Y. M. and Lee, J. C.(2007). Caspase-independent death of human osteosarcoma cells by flavonoids is driven by p53-mediated mitochondrial stress and nuclear translocation of AIF and endonuclease G. Apoptosis, 12: 1289-1298.

Kops, G. J.; Weaver, B. A. and Clevand, D. W. (2005). On the road to cancer: aneuploidy and the mitotic checkpoint. Nat. Rev. Cancer, 10: 773-785.

Kowaltowski, A. J.; Castilho, R. F. and Vercesi, A. E. (2001). Mitochondrial permeability transition and oxidative stress. FEBS Lett., 495: 12-15.

Krell, R. (1996). Value-Added Products from Beekeeping. FAO Agricultural Services Bulletin No. 124. Rome: Food and Agriculture Organization of the United Nations. Available online at www.fao.org/docrep/w0076e/w0076e00.htm#con.

Kumar, N.; Mueen A. K. K.; Dang, R. and Husain, A. (2008). Antioxidant and antimicrobial activity of propolis from Tamil Nadu zone. J. Med. Plant. Res., 2: 361-364.

Kumar, N.; Mueen, A. K. K.; Dang, R.; Shivananda, T. N. and Das, K. (2009). GC-MS analysis of propolis of Indian origin. Pharmacognosy, 1: 46-48.

Kumar, S. and Kumar, D. (2009). Antioxidant and free radical scavenging activities of edible weeds. A. J. F. A. N. D., 9: 1174-1190.

Kumazawa, S.; Hamasaka, T. and Nakayama, T. (2004). Antioxidant activity of propolis of various geographic origins. Food Chem., 84: 329-339.

Kuo, H. C.; Kuo, W. H.; Lee, Y. J.; Lin, W. L.; Chou, F. P. and Tseng, T. H.

(2005). Inhibitory effect of caffeic acid phenethyl ester on the growth of C6 glioma cells *in vitro* and *in vivo*. Cancer Lett., 20: 1-10.

Kuo, L. J. and Yang, L. X. (2008). γ-H2AX - A novel biomarker for DNA double-strand breaks. *In Vivo*, 22: 305-309.

Kuroda, J.; Kimura, S.; Segawa, H.; Sato, K.; Matsumoto, S.; Nogawa, M.; Yuasa, T.; Kobayashi, Y.; Yoshikawa, T.; Ottmann, O. G. and Maekawa, T. (2004). p53-independent antitumor effects of the nitrogen-containing bisphosphonate zoledronic acid. Cancer Sci., 95: 186-192.

Lavin, M. F. and Gueven, N. (2006). The complexity of p53 stabilization and activation. Cell death diff., 13: 941-50.

Lee, H. J.; Wang, C. J.; Kuo, H. C.; Chou, F. P.; Jean, L. F. and Tseng, T. H. (2005a). Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. Toxicol. Appl. Pharmacol., 203: 124-31.

Lee, J. U.; Hosotani, R.; Wada, M., Doi; R., Kosiba, T.; Fujimoto, K.; Miyamoto, Y.; Tsuji, S.; Nakajima, S.; Nishimura, Y. and Imamura, M. (1999). Role of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-X) on cellular susceptibility to radiation in pancreatic cancer cells. Eur. J. Cancer, 35: 1374-1380.

Lee, S. H.; Kim, D. C.; Lee, J. Y.; Cho, M. and Hwang, W. I. (2000). A study on the anticancer activity of propolis. J. Food Sci. Nutr., 5: 54-57.

Lee, Y. J.; Kuo, H. C.; Chu, C. H.; Wang, C. J.; Lin, W. C. and Tseng, T. H. (2003). Involvement of tumor suppressor protein p53 and p38 MAPK in caffeic acid phenethyl ester-induced apoptosis of C6 glioma cells. Biochem. Pharmacol., 66: 2281-2289.

Lee, Y. T.; Don, M. J.; Hung, P. S.; Shen, Y. C.; Lo, Y. S.; Chang, K. W.; Chen, C. F. and Ho, L. K. (2005b). Cytotoxic of phenolic acid phenethyl esters on oral cancer cells. Cancer Lett., 223: 19-25.

Lessene, G.; Czabotar, P. E. and Colman, P. M. (2008). BCL-2 family antagonists for cancer therapy. Nat. Rev. Drug Discov., 7: 989-1000.

Letai, A. (2003). BH3 domains as BCL-2 inhibitors: prototype cancer therapeutics. Expert Opin. Biol. Ther., 3: 293-304.

Letai, A. (2005). Pharmacological manipulation of Bcl-2 family members to control cell death. J. Clin. Invest., 115: 2648-2655.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell, 88: 323-331.

Li, F.; Awale, S.; Tezuka, Y. and Kadota, S. (2009). Cytotoxic constituents of propolis from Myanmar and their structure–activity relationship. Biol. Pharm. Bull., 32: 2075-2078.

Li, Q.; Sun, B.; Matteson, D. M.; O'Brien, T. P. and Chan, C. C. (1999). Cytokines and apoptotic molecules in experimental melanin-protein induced uveitis (EMIU) and experimental autoimmune uveoretinitis (EAU). Autoimmunity, 30: 171-182.

Li, R.; Sutphin, P. D.; Schwartz, D.; Matas, D.; Almog, N.; Wolkowicz, R.; Goldfinger, N.; Huiping, P.; Prokocimer, M. and Rotter, V. (1998). Mutant p53 protein expression interferes with p53-independent apoptotic pathways. Oncogene, 16: 3269-3277.

Liao, H. F.; Chen, Y. Y.; Liu, J. J.; Hsu, M. L.; Shieh, H. J.; Liao, H. J.; Shieh, C. J.; Shiao, M. S. and Chen,Y. J.(2003). Inhibitory effect of caffeic acid phenethyl ester on angiogenesis, tumor invasion, and metastasis. J. Agric. Food Chem., 51: 7907-7912.

Lin, C. C. and Huang, P. C. (2002). Antioxidant and hepatoprotective effects of *Acathopanax senticosus*. Phytother. Res., 14: 489-494.

Lopez, F.; Belloc, F.; Lacombe, F.; Dumain, P.; Reiffers, J.; Bernard, P. and Boisseau, M. R. (1991).Modalities of synthesis of Ki67 antigen during the stimulation of lymphocytes. Cytometry, 12: 42-49.

Lotfy, M. (2006). Biological activity of bee propolis in health and disease. Asian Pac. J. Cancer Prev., 7: 22-31.

Mani, F.; Damasceno, H. C. R.; Novelli, E. L. B.; Martins, E. A. M. and Sforcin, J. M. (2006). Propolis: effect of different concentrations, extracts and intake period on seric biochemical variables. J. Ethnopharmacol., 105: 95-98.

Mansbridge, J. (2001). Dermal fibroblast .In: Human Cell Culture. Koller, M. R.; Palsson, B. and Masters, J. R. W. Eds., vol. (5), Kluwer academic publishers, pp.: 125-172.

Marchini, S.; Ciro, M.; Gallinari, F.; Cozzi, P.; D'Incalci, M. and Broggini, M. (1999). Bromoacryloyl derivative of distamycin A (PNU 151807): a new non-covalent minor groove DNA binder with antineoplastic activity. Br. J. Cancer, 80: 991-997.

Marcucci, M. C.; Ferreres, F.; Garcia-Viguera, C.; Bankova, V. S.; De Castro, S. L. and Dantas, A. P. (2001). Phenolic compounds from Brazilian propolis with pharmacological activities. J. Ethnopharmacol., 74: 105-112.

Marsden, V. S.; O'Connor, L.; O'Reilly, L. A.; Silke, J.; Metcalf, D.; Ekert, P. G.; Huang, D. C. S.; Cecconi, F.; Kuida, K.; Tomaselli, K. J.; Roy, S.; Nicholson, D. W.; Vaux, D. L.; Bouillet, P.; Adams, J. M.; Strasser, A. (2002). Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. Nature, 419: 634-637.

Martin, K. R. (2006). Targeting apoptosis with dietary bioactive agents. Exp. Biol. Med., 231: 117-129.

Matsuno, T. (1995). A new clerodane diterpenoid isolated from propolis .Z. Nauturforsch 50c: 93-97.

Matsuno, T.; Chen, C. and Basnet, P. (1997a). A tumoricidal and antioxidant compound isolated from an aqueous extract of propolis. Med. Sci. Res., 25: 583-584.

Matsuno, T.; Jung, S. K.; Matsumoto, Y.; Saito, M. and Morikawa, J. (1997b). Preferential cytotoxicity to tumor cells of 3, 5-diprenyl-4-hydroxycinnamic acid (artepillin C) isolated from propolis. Anticancer Res., 17: 3565-3568.

Matsuno, T.; Matsumoto, Y.; Saito, M. and Morikawa, J. (1997c). Isolation and characterization of cytotoxic diterpenoid isomers from propolis. Z. Naturforsch., 52c: 702-704.

Mc Carthy, D. A. (2007). Fluorochromes and fluorescence .In: Flow cytometry: principles and applications. Macey, M. G. Ed., Humana Press, pp.: 59-112.

Medana, C.; Carbone, F.; Aigotti, R.; Appendino, G. and Baicchi, C. (2008). Selective analysis of phenolic compounds in propolis by HPLC-MS/MS. Phytochem. Anal., 19: 32-39.

Mello, B. C. B. S.; Petrus, J. C. C. and Hubinger, M. D. (2010). Concentration of flavonoids and phenolic compounds in aqueous and ethanolic propolis

extracts through nanofiltration .J. Food Eng., 96: 533-539.

Michels, G.; Watjen, W.; Niering, P.; Steffan, B.; Tran Thi, Q. H.; Chovolou, Y.; Kampkotter, A.; Bast, A.; Proksch, P. and Kahl, R. (2005). Pro-apoptotic effects of the flavonoid luteolin in rat H4IIE cells. Toxicology, 206: 337-348.

Midgley, C. A.; Desterro, J. M.; Saville, M. K.; Howard, S.; Sparks, A.; Hay, R. T. and Lane, D. P. (2000). An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination *in vitro* and can activate p53 *in vivo*. Oncogene, 19: 2312-2323.

Mirzoeva, O. K. and Calder, P. C. (1996). The effect of propolis and its components on eicosanoid production during the inflammatory response. P. L. E. F. A., 55: 441-449.

Mitamura, T.; Matsuno, T.; Sakamoto, S.; Maemura, M .; Kudo, H.; Suzuki, S.; Kuwa, K .; Yoshimura, S.; Sassa, S.; Nakayama, T . and Nagasawa, H . (1996). Effects of a new clerodane diterpenoid isolated from propolis on chemically induced skin tumors mice. Anticancer Res., 16: 2669-2672.

Mitic, T. and McKay, J. S. (2005). Immunohistochemical analysis of acetylation, proliferation, mitosis, and apoptosis in tumor xenografts following administration of a histone deacetylase inhibitor-a pilot study. Toxicol. Pathol., 33: 792-799.

Mohammadzadeh, S.; Sharriatpanahi, M.; Hamedi, M.; Amanzadeh, Y.; Ebrahimi, S. S. E. and Ostad, S. N. (2007). Antioxidant power of Iranian propolis extract. Food Chem., 103: 729-733.

Moll, M. and Petrenko, O. (2003). The MDM2-p53 Interaction. Mol. Cancer Res., 1: 1001-1008.

Moon, S. H.; Kim , K. T.; Lee , N. K.; Han, Y. S.; Nah, S. Y.; Cho, S. G.; Park, Y. S. and Paik, H. D.(2009). Inhibitory effects of naringenin and its novel derivatives on hyaluronidase .Food Sci. Biotechnol., 18: 267-270.

Moreira, L.; Dias, L. G.; Pereira, J. A.; Estevinho, L. (2008). Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal. Food Chem. Toxicol., 46: 3482-3485.

Moriyasu, J.; Arai, S.; Motoda, R. and Kurimoto, M. (1994). *In vitro* activation of mouse macrophage by propolis extract powder. Biotherapy, 8: 364-365.

Motomura, M.; Kwon, K. M.; Suh, S. J.; Lee, Y. C.; Kim, Y. K.; Lee, I. S.; Kim, M. S.; Kwon, D. Y.; Suzuki, I. and Kim, C. H. (2008). Propolis induces cell cycle arrest and apoptosis in human leukemic U937 cells through Bcl-2/Bax regulation. Environ. Toxicol. Pharmacol., 26 : 61-67.

Nabi, U.; Nagi, A. H. and Sami, W. (2008). Ki-67 Proliferating index and histological grade, type and stage of colorectal carcinoma. J. Ayub. Med. Coll. Abbottabad, 20: 44-48.

Nagata, S.; Nagase, H.; Kawane, K.; Mukae, N. and Fukuyama, H. (2003). Degradation of chromosomal DNA during apoptosis. Cell Death Differ., 10: 108-116.

Naik, D. G.; Vaidya, H. S. and Behera, B. C. (2009). Antioxidant properties of Indian propolis. J. Api. Pro. Api Med. Sci., 1: 110-120.

Najafi, M. F.; Vahedy, F.; Seyyedin, M.; Jomehzadeh, H. R. and Bozary, K. (2007). Effect of the water extracts of propolis on stimulation and inhibition of different cells. Cytotechnology, 54: 49-56.

Nakamura, A. J.; Rao, V. A.; Pommier, Y. and Bonner, W. M. (2010). The complexity of phosphorylated H2AX foci formation and DNA repair assembly at DNA double-strand breaks. Cell Cycle, 29: 1-9.

Naoghare, P. K.; Ki, H. A.; Paek, S.; Tak, Y. K.; Suh, Y.; Kim, S. G.; Lee, K. and Song, J. M. (2010). Simultaneous quantitative monitoring of drug-induced caspase cascade pathways in carcinoma cells. Integr. Biol., 2: 46-57.

National Institutes of Health (2004). Information on clinical trials. Available on line at: http://clinicaltrials.gov.

Nemec, K. N. and Khaled, A. R. (2008). Therapeutic modulation of apoptosis: targeting the BCL-2 family at the interface of the mitochondrial membrane. Yonsei Med. J., 49: 689 - 697.

Newman, D. J. and Cragg, C. M. (2004). Marine natural products and related compounds in clinical and advanced preclinical trials. J. Nat. Prod., 67: 1216-1238.

Newman, D. J.; Cragg, C. M. and Snader, K. M. (2003).Natural products as sources of new drugs over the period 1981–2002. J. Nat. Prod., 66: 1022-1037.

Nguyen, S. M.; Lieven, C. J. and Levin, L. A. (2007). Simultaneous labeling of projecting neurons and apoptotic state. J. Neurosci. Methods., 161: 281-284.

Nieva Moreno, M. I.; Isla, M. I.; Sampietro, A. R. and Vattuone, M. A. (2000). Comparison of the free radical scavenging activity of propolis from several regions of Argentine, J. Ethnopharmacol., 71: 109-114.

Nikolov, N.; Marekov, N.; Bankova, V.; Popov, S.; Ignatova, R. and ladimirova, I. (1987). Method for the preparation of water-soluble derivative of propolis. Bulgarian Patent Application N 79903/28.05.1987.

Norbury, C. J. and Hickson, I. D. (2001). Cellular responses to DNA damage. Annu. Rev. Pharmacol. Toxicol., 41: 367-401.

Oliner, J. D.; Kinzler, K. W.; Meltzer, P. S.; George, D. L. and Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature, 358: 80-83.

Olive, P. L. (2004). Detection of DNA damage in individual cells by analysis of histone H2AX phosphorylation. Meth. Cell Biol., 75: 355-373.

Onori, P.; De Morrow, S.; Gaudio, E.; Franchitto, A.; Mancinelli, R.; Venter, J.; Kopriva, S.; Ueno, Y.; Alvaro, D. ; Savage, J. ; Alpini, G. and Francis, H. (2009). Caffeic acid phenethyl ester decreases cholangiocarcinoma growth by inhibition of NF-kB and induction of apoptosis. Int. J. Cancer., 125: 565-576.

Orhan, H.; Marol, S.; Hepen, I. F. and Sahin, G. (1999). Effects of some probable antioxidants on selenite-induced cataract formation and oxidative stress-related parameters in rats. Toxicology, 139: 219-232.

Ormerod, M.G. (2002). Investigating the relationship between the cell cycle and apoptosis using flow cytometry. J. Immunol. Methods, 265: 73-80.

Ormerod, M.G. (2008). Flow Cytometry. A basic introduction. A valiable online at http://www.denovosoftware.com/site/introtoflowormerod.shtml.

Orsolic, N.; Saranovic, A. B. and Basic, I. (2006). Direct and indirect mechanism(s) of antitumour activity of propolis and its polyphenolic compounds. Planta Med., 72: 20-27.

Orsolic, N.; Stajcar, D. and Basic, I. (2009). Propolis and its flavonoid

compounds cause cytotoxicity on human urinary bladder transitional cell carcinoma in primary culture. Period. Biol., 111: 113-121.

Orsolic, N.; Terzic, S.; Mihaljevic, Z.; Sver, L. and Basic, I. (2005). Effects of local administration of propolis and its polyphenolic compounds on tumor formation and growth. Biol. Pharm. Bull., 28: 1928-1933.

Padmavathi, R.; Senthilnathan, P.; Chodon, D. and Sakthisekaran, D. (2006). Therapeutic effect of paclitaxel and propolis on lipid peroxidation and antioxidant system in 7, 12 dimethyl benz(a)anthracene-induced breast cancer in female Sprague Dawley rats. Life Sci., 78: 2820-2825.

Pastor, N.; Cantero, G.; Campanella, C. and Cortes, F. (2005). Endoreduplication induced in cultured Chinese hamster cells by different anti-topoisomerase II chemicals: Evidence for the essential contribution of the enzyme to chromosome segregation. Mutat. Res. Genet. Toxicol. Environ. Mutagen., 582: 11-19.

Pepper, C.; Hoy, T. and Bentley, D. P. (1997). Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with *in vitro* apoptosis and clinical resistance. Br. J. Cancer, 76: 935-938.

Pepper, C.; Thomas, A.; Hoy, T.; Milligan, D.; Bentley, P. and Fegan, C. (2003). The vitamin D3 analog EB1089 induces apoptosis via a p53-independent mechanism involving p38 MAP kinase activation and suppression of ERK activity in B-cell chronic lymphocytic leukemia cells *in vitro*. Blood, 101: 2454-2459.

Plochmann, K.; Korte, G.; Koutsilieri, E.; Richling, E.; Riederer, P.; Rethwilm, A.; Schreier, P. and Scheller, C. (2007). Structure-activity relationships of flavonoid induced cytotoxicity on human leukemia cells. Arch. Biochem. Biophys., 460: 1-9.

Popova, M.; Silici, S.; Kaftanoglu, O. and Bankova, V. (2005). Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. Phytomedicine, 12: 221-228.

Portugal, J.; Bataller, M. and Mansilla, S.(2009). Cell death pathways in response to antitumor therapy. Tumori, 95: 409-21.

Powolny, A.; Xu, J. and Loo, G. (2001). Deoxycholate induces DNA damage and apoptosis in human colon epithelial cells expressing either mutant or wild-type p53. Internat. J. Biochem. Cell Biol., 33: 193-203.

Prindull, G. (2008). Final checkup of neoplastic DNA replication: evidence for failure in decision-making at the mitotic cell cycle checkpoint G (1)/S. Exp. Hematol., 36: 1403-1416.

pubchem.ncbi.nlm.nih.gov

Ramos, A. F. N. and Miranda, J. L. (2007). Propolis: A Review of its anti-inflammatory and healing actions. J. Venom. Anim. Toxins incl. Trop. Dis., 13: 697-710.

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

Rao, C. V.; Desai, D.; Rivenson, A.; Simi, B.; Amin, S. and Reddy, B. S. (1995). Chemoprevention of colon carcinogenesis by phenylethyl-3-methylcaffeate.Cancer Res., 55: 2310-2315.

Redon, C.; Pilch, D.; Rogakou, E.; Sedelnikova, O.; Newrock, K. and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ. Curr. Opin. Genet. Dev., 12: 162-169.

Reed, J. C. (2008). Bcl-2–family proteins and hematologic malignancies: history and future prospects. Blood, 111: 3322-3330.

Ren, W.; Qiao, Z.; Wang, H.; Zhu, L. and Zhang, L. (2003). Flavonoids: promising anticancer agents. Med. Res. Rev., 23: 519-534.

Riballo, E.; Kuhne, M.; Rief, N.; Doherty, A.; Smith, G. C.; Recio, M. J.; Reis, C.; Dahm, K.; Fricke, A.; Krempler, A.; Parker, A. R.; Jackson, S. P.; Gennery, A.; Jeggo, P. A. and Lobrich, M. (2004). A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to γ-H2AX foci. Mol. Cell, 16: 715-724.

Rittie, L. and Fisher, G. J. (2005). Isolation and culture of skin fibroblasts. Methods Mol. Med., 117: 83-98.

Rong, Y.; Yang, E. B.; Zhang, K. and Mack, P. (2000). Quercetin-induced apoptosis in the monoblastoid cell line U937 *in vitro* and the regulation of heat shock proteins expression. Anticancer Res., 20: 4339-4345.

Rosato, M. T.; Jabbour, A. J.; Ponce, R. A.; Kavanagh, T. J.; Takaro, T. K.; Hill, J. P.; Poot, M.; Rabinovitch, P. S. and Faustman, E. M. (2001). Simultaneous analysis of surface marker expression and cell cycle

progression in human peripheral blood mononuclear cells. J. Immunol. Methods, 256: 35-46.

Rudin, C. M. and Thompson, C. B. (1998). Apoptosis and cancer. In: The Genetic Basis of Human Cancer. Vogelstein, B. and Kinzler, K. W. Eds. McGraw-Hill Companies Inc., USA, 193-204.

Rusakc, G.; Gutzeita, H. O. and Mullerb, J. L. (2005). Structurally related flavonoids with antioxidative properties differentially affect cell cycle progression and apoptosis of human acute leukemia cells. Nutr. Res., 25: 143-155.

Russo, A.; Longo, R. and Vanella, A. (2002). Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. Fitoterapia, 1: S21-S29.

Sa, G. and Das, T. (2008). Anti cancer effects of curcumin: cycle of life and death. Cell Div., 3: 1-14.

Saito, M.; Kobayashi, M.; Iwabuchi, S.; Morita, Y.; Takamura, Y. and Tamiya, A. (2004). DNA condensation monitoring after interaction with Hoechst 33258 by atomic force microscopy and fluorescence spectroscopy. J. Biochem., 136: 813-823.

Salatino, A.; Teixeira, E. W.; Negri, G. and Message, D. (2005). Origin and Chemical Variation of Brazilian Propolis. eCAM., 2: 33-38.

Sato, N.; Mizumoto, K.; Maehara, N.; Kusumoto, M.; Nishio, S.; Urashima, T.; Ogawa, T. and Tanaka, M. (2000). Enhancement of drug-induced apoptosis by antisense oligodeoxynucleotides targeted against Mdm2 and p21WAF1/CIP1. Anticancer Res., 20: 837-842.

Satoshi, M.; Yoshikazu, I.; Yukio, N.; Shozo, O.; Takashi, S.; Yoko, A.; Kazu-Michi, S.; Yukihiro, A. and Yoshinori, N. (2005). Identification of caffeoylquinic acid derivatives from Brazilian propolis as constituents involved in induction of granulocytic differentiation of HL-60 cells. Bioorg. Med. Chem., 13: 5814-5818.

Save, V.; Hall, P. A. and Coates, P. J. (2004). Detection and quantifying apoptosis in tissue sections. In: Apoptosis methods and protocols. Brady, H. J. M. Ed., vol. (282). Humana Press Inc., Totowa, NJ, p: 74.

Sawaya, A. C. H. F.; Tomazela, D. M.; Cunha, I. B. S.; Bankova, V. S.; Marcucci, M. C.; Custodio, A. R. and Eberlin, M. N. (2004). Electrospray

ionization mass spectrometry fingerprinting of propolis. Analyst, 129: 739-744.

Scheller, S.; Gazda, G.; Pietsz, G.; Gabrys, J.; Szumlas, J.; Eckert, L. and Shani, J. (1988). The ability of ethanol extract of propolis to stimulate plaque formation in immunized mouse spleen cells. Pharmacol. Res. Commu., 20: 323-328.

Schluter, C.; Duchrow, M.; Wohlenberg, C.; Becker, M. H. G.; Key, G.; Flad, H. D. and Gerdes, J. (1993). The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. J. Cell. Biol., 123: 513-522.

Schmidt, M. and Bastians, H. (2007). Mitotic drug targets and the development of novel anti-mitotic anticancer drugs. Drug Resist. Updates, 10: 162-181.

Schnitzler, P.; Neuner, A.; Nolkemper, S.; Zundel, C.; Nowack, H.; Sensch, K.H. and Reichling, J. (2009). Antiviral activity and mode of action of propolis extracts and selected compounds. Phytother. Res., 24: S20 - S28.

Scholzen, T. and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. J. Cell. Physiol., 182: 311-322.

Schulz, W. A. (2005). Molecular biology of human cancers: An advanced student's text book. Dordrecht. Springer Science and Business Media, Inc. USA. pp.: 1-489.

Scopa, C. D.; Vagianos, C.; Kardamakis, D.; Kourelis, T. G.; Kalofonos, H. P. and Tsamandas, A. C. (2001). bcl-2/bax ratio as a predictive marker for therapeutic response to radiotherapy in patients with rectal cancer. Appl. Immunohistochem. Mol. Morphol., 9: 329-334.

Selivanova, G. (2004). p53: fighting cancer. Curr. Cancer Drug Targets, 4: 385-402.

Sforcin, J. M. (2007). Propolis and the immune system: a review J. Ethnopharmacol., 113: 1-14.

Sforcin, J. M.; Kaneno, R. and Funari, S. R. C. (2002). Absence of seasonal effect on the immunomodulatory action of Brazilian propolis on natural killer activity. J. Venom. Anim.Toxins, 8: 19-29.

Shapiro, M. H. (2003). Practical flow cytometry.4th ed., Welly-Liss, Inc. New York. pp.:1-727.

Shimizu, N.; Nakamura, H.; Kadota, T.; Kitajima, K.; Oda, T.; Hirano, T. and Utiyama, H. (1994). Loss of amplified c-myc genes in the spontaneously differentiated HL-60 cells. Cancer Res., 54: 3561-3567.

Shipp, M. A.; Ross, K. N.; Tamayo, P.; Weng, A. P.; Kutok, J. L.; Aguiar, R. C. T.; Gaasenbeek, M.; Angello, M.; Reich, M.; Pinkus, G. S.; Ray, T. S.; Koval, M. A.; Last, K. W.; Norton, A.; Lister, T. A.; Mesirov, J.; Neuberg, D. S.; Lander, E. S.; Aster, J. O. N. C. and Golub, T. R. (2002). Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat. Med., 8: 68-74.

Shirasawa, S.; Furuse, M.; Yokoyama, N. and Sasazuki, T. (1993). Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science, 260: 85-88.

Shrivastav, M.; De Haro, L. P. and Nickoloff, J. A. (2008). Regulation of DNA double-strand break repair pathway choice. Cell Res., 18: 134-147.

Shukla, S. and Gupta, S. (2007). Apigenin-induced cell cycle arrest is mediated by modulation of MAPK, PI3K-Akt, and loss of cyclin D1 associated retinoblastoma dephosphorylation in human prostate cancer cells. Cell cycle, 6: 1102-14.

Siegelin, M. D.; Reuss, D. E.; Habel1, A.; Herold-Mende, C.; and von Deimling, A. (2008). The flavonoid kaempferol sensitizes human glioma cells to TRAIL-mediated apoptosis by proteasomal degradation of surviving. Mol. Cancer Ther., 7: 3566-3574.

Silici, S. and Kutluca, S. (2005). Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. J. Ethnopharmacol., 99: 69-73.

Silva, B. A.; Ferreres, F.; Malva, J. O. and Dias, A. C. P. (2005). Phytochemical and antioxidant characterization of Hypercom *perforatum* alcoholic extracts. Food Chem., 90: 157-167.

Silva, B. B.; Rosalen, P. L.; Cury, J. A.; Ikegaki, M.; Souza, V. C.; Esteves A. and Alencar, S. M. (2007a). Chemical composition and botanical origin of red propolis, a new type of Brazilian propolis. eCAM., 1-4.

Silva, E. M.; Souza, J. N. S.; Rogez, H.; Rees, J. F. and Larondelle, Y. (2007b). Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region. Food Chem., 101: 1012-1018.

Silva, S. B.; Amaral, A. S.; Gaspar, L. F. J.; Rocha, N. S.; Portela, R. F.; Spin, J. S.; Teixeira, C. R. and Sforcin, J. M. (2003). Tumor venereo transmissivel-revisao. Pet Food Health Care, 2: 77-82. Brazilian.

Simoes, L. M.; Gregorio, L. E.; Da Silva Filho, A. A.; de Souza, M. L.; Azzolini, A. E.; Bastos, J. K. and Lucisano-Valim, Y. M. (2004). Effect of Brazilian green propolis on the production of reactive oxygen species by stimulated neutrophils. J Ethnopharmacol., 94: 59-65.

Soussi, T. and Wiman, K. G. (2007). Shaping genetic alterations in human cancer: the p53 mutation paradigm. Cancer Cell, 12: 303-312.

Steensma, D. P.; Timm, M. and Witzig, T. E. (2003). Flow cytometric methods for detection and quantification of apoptosis. In: Methods in Molecular Medicine: Novel anticancer drug protocols. Buolamwini, J. K. and Adjei, A. A. Eds., Humana Press Inc., Totowa, NJ., vol.(85) pp.: 323-332.

Stewart, Z. A.; Westfall, M. D. and Pietenpol, J. A. (2003). Cell-cycle dysregulation and anticancer therapy. Trends Pharmacol. Sci., 24: 139-145.

Strasser, A.; Puthalakath, H.; Bouillet, P.; Huang, D. C.; O'Connor, L.; O'Reilly, L. A.; Cullen, L.; Cory, S. and Adams, J. M. (2000). The role of bim, a pro-apoptotic BH3-only member of the Bcl-2 family in cell death control. Ann. N. Y. Acad. Sci., 917: 541-548.

Sun, F.; Hayami, S.; Haruna, S.; Ogiri, Y.; Tanaka, K. and Yamada, Y. (2000). *In vivo* antioxidative activity of propolis evaluated by the interaction with vitamins C and E and the level of lipid hydroperoxidases in rats. J. Agric. Food Chem., 48: 1462-1465.

Swerdlow, J. L. (2000). Nature's medicine. plants that heal. National Geographic, Washington, D.C.

Syamsudin, Dewi, R. M. and Kusmardi, (2009). Immunomodulatory and *in vivo* antiplasmodial activities of propolis extracts. Am. J. Pharmacol. Toxicol., 4 : 75-79.

Szliszka, E.; Czuba, Z. P.; Domino, M.; Mazur, B.; Zydowicz, G. and Krol, W. (2009). Ethanolic extract of propolis (EEP) enhances the apoptosis-

inducing potential of TRAIL in cancer cells. Molecules, 14: 738-754.

Tait, J. F. (2008). Imaging of apoptosis. J. Nucl. Med., 49: 1573-1576.

Tait, J. F.; Smith, C.; Levashova, Z.; Patel, B.; Blankenberg, F. G. and Vanderheyden, J. (2006). Improved detection of cell death *in vivo* with Annexin V radiolabeled by site-specific methods. J. Nucl. Med., 47: 1546-1553.

Takahashi, A. and Ohnishi, T. (2006). Does γ-H2AX foci formation depend on the presence of DNA double strand breaks?. Cancer Lett., 236: 155-6.

Tamanini, F. (2007). Manipulation of mammalian cell lines for circadian studies. In: Circadian rhythms: Methods and protocols. Rosato, E. Ed., vol. (362), Humana Press Inc., pp: 433-452.

Tanaka, T.; Halicka, D.; Traganos, F. and Darzynkiewicz, Z. (2009). Cytometric analysis of DNA damage: Phosphorylation of histone H2AX as a marker of DNA double-strand breaks (DSBs). In: Chromatin Protocols, Chellappan, S. P. Ed., 2nd vol. (523), Humana Press, a part of Springer Science pp.: 161-168.

Tanaka, T.; Huang, X.; Halicka, H. D.; Zhao, H.; Traganos, F.; Albino, A. P.; Dai, W. and Darzynkiewicz, Z. (2007). Cytometry of ATM activation and histone H2AX phosphorylation to estimate extent of DNA damage induced by exogenous agents. Cytometry, 71A: 648-661.

Tatefuji, T.; Izumi, N.; Ohta, T.; Arai, S.; Ikeda, M. and Kurimoto, M. (1996). Isolation and identification of compounds from Brazilian propolis which enhance macrophage spreading and mobility. Biol. Pharm. Bull., 19: 966-970.

Tavecchio, M.; Simone, M.; Bernasconi, S.; Tognon, G.; Mazzini, G. and Erba, E. (2008 a). Multi-parametric flow cytometric cell cycle analysis using TO-PRO-3 iodide (TP3): Detailed protocols. Acta Histochem., 110: 232-244.

Tavecchio, M.; Simone, M.; Erba, E.; Chiolo, I.; Liberi, G.; Foiani, M.; D'Incalci, M. and Damia, G. (2008 b). Role of homologous recombination in trabectedin-induced DNA damage. E. J. C., 44: 609-618.

Telford, W. G.; Komoriya, A. and Packard, B. Z. (2004). Multiparametric analysis of apoptosis by flow and image cytometry. In: Methods in Molecular Biology: Flow Cytometry Protocols. Hawley, T. S. and Hawley,

R. G. Eds., 2nd ed., Humana Press, pp.141-159.

Terry, N. H. and White, R. A. (2001). cell cycle kinetics estimated by analysis of bromodeoxyuride incorboration. Meth. cell biol., 63: 355-474.

Thomas, A.; El Rouby, S.; Reed, J. C.; Krajewski, S.; Silber, R.; Potmesil, M. and Newcomb, E. W. (1996). Drug-induced apoptosis in B-cell chronic lymphocytic leukemia: relationship between p53 gene mutation and bcl-2/bax proteins in drug resistance. Oncogene, 12: 1055-1062.

Tikhonov, A. I.; Mamontova, I. N. S. (1987). Production and study of a lyophilized phenolic polysaccharide preparation from propolis. Farmatsevtichnii Zhurnal, 3: 67-68.

Tomas-Barberan, F. A.; Garcia-Viguera, C.; Vit-Olivier, P.; Ferreres F. and Tomas-Lorente, F. (1993). Phytochemical evidence for the botanical origin of tropical propolis from Venezuela. Phytochem., 34: 191-196.

Traganos, F. (2004). Mechanism of antitumor drug action assessed by cytometry. In: Method in Cell Biology, Cytometry: New developments. Darzynkiewicz, Z.; Roederer, M. H. and Tanke, J. Eds., vol. (75), 4th ed., Elsevier Inc., pp: 257-305.

Trusheva, B.; Trunkova, D. and Bankova, V. (2007). Different extraction methods of biologically active components from propolis: a preliminary study. Chem. Central J., 1: 13.

Tsujimoto, Y.; Cossman, J.; Jaffe, E. and Croce, C. (1985). Involvement of the Bcl-2 gene in human follicular lymphoma. Science, 228: 1440-1443.

Urruticoechea, A.; Smith, I. E. and Dowsett, M. (2005). Proliferation marker Ki-67 in early breast cancer. J. Clinic. Oncol., 23: 7212-7220.

van Cruchten, S. and van den Broeck, W. (2002). Morphological and biochemical aspects of apoptosis, oncosis and necrosis. Anat. Histol. Embryol., 31: 214-223.

van Dierendonck, J. H.; Keijzer, R.; van de Velde, C. J. H. and Cornelisse, C. J. (1989). Nuclear distribution of the Ki-67 antigen during the cell cycle: Comparison with growth fraction in human breast cancer cells. Cancer Res., 49: 2999-3006.

Vennat, B.; Arvouet–Grand, A.; Gross, D. and Pourrat, A. (1995).Qualitative

and quantitative analysis of flavonoids and identification of phenolic acids from a propolis extract. J. Pharm. Belg., 50: 438-444.

Vermeulen, K.; Van Bockstaele, D. R. and Berneman, Z. N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif., 30: 131-149.

Villunger, A.; Michalak, E. M.; Coultas, L.; Mullauer, F.; Bock, G.; Ausserlechner, M. J.; Adams, J. M. and Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins Puma and Noxa. Science, 302: 1036-1038.

Vogelstein, B. and Kinzler, K. W. (2004). Cancer genes and the pathways they control. Nat. Med., 10: 789-799.

Volpi, N. and Bergonzini, G. (2006). Analysis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry. J. Pharm. Biomed. Anal., 42: 354-361.

Vousden, K. H. (2005). p53 and PUMA: a deadly duo. Science, 309: 1685-1686.

Vousden, K. H. and Lu, X. (2002). Live or let die: the cell's response to p53. Nat. Rev. Cancer, 2: 594-604.

Vynograd, N.; Vynograd, I. and Sosnowski, Z., (2000). A comparative multicentre study of the efficacy of propolis, acyclovir and placebo in the treatment of genital herpes. Phytomedicine, 7: 1-6.

Wang, D.; Xiang, D. B.; He, Y. J.; Li, Z. P.; Wu, X. H.; Mou, J. H.; Xiao, H. L. and Zhang, Q. H. (2005). Effect of caffeic acid phenethyl ester on proliferation and apoptosis of colorectal cancer cells *in vitro* .World J. Gastroenterol., 11: 4008-4012.

Wang, P.; Reed, M.; Wang, Y.; Stenger, J. E.; Anderson, M. E.; Schwedes, J. F. and Tegtmeyer, P. (1994). p53 domains: structure, oligomerization, and transformation. Mol. Cell Biol., 14: 5182-91.

Wang, T. D.; Chen, W. J.; Su, S. S.; Lo, S. C.; Lin, W. W. and Lee, Y. T. (2002). Increased cardiomyocyte apoptosis following ischemia and reperfusion in diet-induced hypercholesterolemia: relation to Bcl-2 and Bax proteins and caspase-3 activity. Lipids, 37: 385-394.

Wang, X. (2001). The expanding role of mitochondria in apoptosis. Genes Dev., 15: 2922-2933.

Weinberg, R. A. (2007). The biology of cancer. Garland Science. New York, pp.:1-679.

White, M. K. and Cinti, C. (2004). A morphological approach to detect apoptosis based on electron microscopy. In: Methods in Molecular Biology: Cell cycle control and dysregulation protocols. Giordano, A. and Romano, G. Eds., Humana Press Inc., Totowa, NJ., Vol (285) pp.: 105-111.

Williams, G. M. and Jeffrey, A. M. (2000). Oxidative DNA damage: Endogenous and chemically induced. Regulatory Toxicol. Pharmacol., 32: 283-292.

Wlodkowic, D.; Skommer, J. and Darzynkiewicz, Z. (2009). Flow cytometry-based apoptosis detection. In: Apoptosis, Methods in Molecular Biology. Erhardt, P. and Toth, A. Eds., Humana Press, a part of Springer science and business media, p.: 559.

World Health Organization: Cancer, 2005

World Health Organization: Cancer, 2006

www.chemfinder.com

www.chemspider.com

Xu, Y.; Luo, L.; Chen, B. and Fu, Y. (2009). Recent development of chemical components in propolis. Front. Biol. China, 4: 385-391.

Yaghoubi, S. M. J.; Ghorbani, G. R.; Soleimanian, Z. S. and Satari, R. (2007). Antimicrobial activity of Iranian propolis and its chemical composition. DARU, 15: 45-48.

Yang, Y.; Li, C. C. and Weissman, A. M. (2004). Regulating the p53 system through ubiquitination. Oncogene, 23: 2096-2106.

Yao, Y. and Marassi, F. M. (2009). BAX and BAK Caught in the Act. Mol. Cell, 36: 353-354.

Yasuhara, S.; Zhu, Y.; Matsui,T.; Tipirneni, N.; Yasuhara, Y.; Kaneki, M.; Rosenzweig, A. and Martyn, J. A. J.(2003). Comparison of Comet assay,

electron microscopy, and flow cytometry for detection of apoptosis. J. Histochem. Cytochem., 51: 873-885.

Yuan, J.; Lipinski, M. and Degterev, A. (2003). Diversity in the mechanisms of neuronal cell death. Neuron, 40: 401- 413.

Zhao, J.; Zheng, X.; Xing, W.; Huang, J. and Li, G. (2007). Electrochemical Studies of Camptothecin and Its Interaction with Human Serum Albumin. Int. J. Mol. Sci., 8: 42-50.

Zhou, J. and Giannakakou, P. (2005). Targeting microtubules for cancer chemotherapy. Curr. Med. Chem., Anticancer Agents, 5: 65-71.

Zlatanova, J. and Thakar, A. (2008). H2A.Z: View from the Top. Structure, 16: 166-179.

خلاصة

اجريت الدراسة الحالية بهدف تقييم الفعاليه المضاده للاكسدة والمضاده للتطفير والمضاده للورم لعينات من العكبر جمعت خلال الفتره من 20-4-2007 الى 1-7-2008 من خمس مواقع جغرافيه عراقية متباينة (بغداد:B2, B1 ودهوك: D والموصل: M وصلاح الدين: S) و/ أو مركباتها الفينولية (caffeic acid; CA, chrysin; CH and caffeic acid phenethyl ester; CAPE) خارج الجسم الحي وعلى خط خلوي طبيعي واحد (human dermal fibroblast; HuFb) وخطين خلويين سرطانيين (human promyeloid leukemia; HL-60 و human colon carcinoma; HCT-116)، وفضلا عن ذلك فقد اجريت اختبارات كيميائيه على عينات العكبر.

قدرت تراكيز المركبات الفينوليه (الفلافونويدات والاحماض الفينوليه واستراتها) بطريقة كروموتوكرافيا السائل عالية الكفاءة والمجهزة بطيف الكتله ذاتي الحقن (HPLC-ESI/MS)، كما تم تقدير تراكيز نفس المركبات طيفيا باستخدام كاشف Folin-Ciocalteu . وعلى هذا الاساس تم الكشف عن 38 مركبا مختلفا، وكان 33 منها عبارة عن فينولات متعدده. اما المركبات الاخرى فضمت اربع مركبات clerodane diterpenoids والتي شخصت لاول مرة في المناطق المعتدلة الحراره ومركب واحد غير مشخص. اظهر القياس الكمي للفلافونويدات بان الاحماض الفينولية واستراتها كانت هي المركبات السائده في مستخلصات العكبر تلتها مركبات الفلافون والفلافونول ثم مركبات الفلافونون وثنائي هايدرو فلافونول. ولوحظت اعلى التراكيز من هذه المركبات في عينتي D و M .

قيمت فعاليه العكبر في كسح الجذور الحره باستخدام فحص 2, 2-diphenyl-1-picrylhydrazyl (DPPH) واظهرت النتائج بان مستخلصاته لها قابليه عاليه في كسح هذه الجذور، ولكن كانت عينتي D و M هي الاكثر فعالية في ذلك وكان المركب CAPE هو الاكثر فعالية بالنسبه لباقي المركبات المدروسه الاخرى (CA وCH).

اشارت نتائج تثبيط النمو (GI) ونتائج معدل تثبيط النمو (GIR) خارج الجسم الحي على خطوط الخلايا HL-60 وHCT-116 وHuFb بان المعاملة بالعينات B2 وD وM أو المركب CAPE قد ادت الى حصول تثبيط معنوي في نمو هذه الخلايا (أ $\leq$ 0.05) مقارنة بمزارع السيطره (غير المعامله) وكان التأثير معتمدا على كل من الجرعة ووقت المعاملة وكانت العينة M هي الاقل سمية على خلايا HuFb بينما لم يظهر المركب CH اي تاثيرات سميه على خلايا HL-60 .

قيم موت الخلية المبرمج خارج الجسم الحي في خطوط الخلايا السرطانيه بعد المعامله بمستخلصات العكبر او مركباتها الفينوليه بأربع طرائق والتي كانت دراسة التغيرات المظهريه ودراسة الترحيل على الهلام و دراسة التدفق الخلوي (Annexin V/PI) و دراسة تنظيم التعبير الجيني للجينين *Bcl-2/Bax*. أشارت النتـائج إلى إن المركبات المدروســـة قد تسببت بتأثير سمي تثبيطي فعال في نمو خلايا الـخطيــن HL-60 و HCT-116 من خلال الموت الخلوي المبرمج او النخر الخلوي على التوالي. تصاحبت نتائج الموت الخلوي مع انخفاض في تعبير الجين *Bcl-2* وتنشيط في تعبير الجين *Bax* وكان التاثير بدرجة كبيره عند 10 ساعات من مدة المعاملة (22% و 33% ، على التوالي). اتسعت تلك التقييمات لتشمل تعبير الجين *p53* (HCT-116) واضطرابات دورة الخلية من خلال تقييمات الدنا أحادية وثنائية العامل وكذلك تكون γ-H2AX في خلايا كل من HL-60 و HCT-116.

فيما يتعلق بالجيـن *p53* فقد لوحظت زيادة في تعبيـره بعـد ثلاث ساعــات مـن المعامــله بالعينـه M (4,80 %) ولوحظت مثل هذه الزيادة ايضا عند ست ساعات (5,57 %) ولكن بعد مرور 10 ساعات انخفض التعبير الى المستوى الطبيعي. وعلى أية حال وبعد مرور 24 ساعة بدأ التعبير في الزيادة مرة ثانية حيث وصلت نسبة الخلايا الموجبة الى 27,98 % ولكن بعد مرور 48 ساعة حصل انخفاض فيها ليصل إلى 5,14 % وبعد مرور 72 ساعة وصلت الزيادة في التعبير لتصل الى 11,31 %.

أظهر قياس اضطراب دورة الخلية الاحادية العامل في خلايا HL-60 والمعاملة بالتركيز 5 مايكروغرام مليليتر$^{-1}$ عند 48 ساعة و 72 ساعة الى زيادة في نسبة الخلايا في طور S وانخفاض في طور G1 بينما لم يكن هناك تغيير في اطوار دورة الخلية عند 24 ساعة. أظهرت الخلايا المعاملة بالتركيزيين 15 أو 25 مايكروغرام مليليتر$^{-1}$ عند 24 و 48 ساعة الى حصول تجمع للخلايا في طور S أيضا بينما ظهر تجمع للخلايا في طور G2/M فقط بعد المعاملة بالتركيز 25 مايكروغرام مليليتر$^{-1}$ عند 72 ساعة. وفضلاً عن ذلك فقد تم ملاحظة القمة تحت G1 بعد المعاملة بالتركيز 15 أو 25 مايكروغرام مليليتر$^{-1}$ وعند فترات المعاملة المختلفة. من ناحية اخرى كانت معظم تجمعات خلايا HCT-116 في طور G1 أو G2/M والتي كانت معتمده على الوقت والجرعة وبالإضافة إلى ذلك فلم تلاحظ القمة تحت G1.

أظهرت التقييمـات ثنائيـة العامل ان خلايــا HL-60 و HCT-116 قد تقدمت ببطيء خلال طور S أكثر من تلك الخلايا غير المعاملة، ولوحظ في خلايا HL-60 ظهور قليل في الخلايا الموجبة لمادة BrdUrd في طور G1 بعد عشر ساعات من فترة المعاملة، وفي كلا الخطين الخلويين بقيت الخلايا في طور G1 (سالبة لمادة BrdUrd) والمشاهدة عند بداية المعاملة متوقفة في هذا الطور لاقل من 24 ساعة، في حين ظهرت الخلايا في طور S فقط بعد 24 ساعة.

اظهر تعبير γ-H2AX في خلايا HL-60 حصول زيادة واضحة فيه ومعتمدة على الوقت للفترات الست المدروسة (3 و6 و6 و8 و10 و24 و48 ساعة من مدة المعاملة)، في حين لم تلاحظ مثل هذه الزيادة في خلايا HCT-116.

أمتد تقييم الفعل المضاد للورم (الأنسجة المرضية وكيمياء الأنسجة المرضية للتعبير الجيني لكل من *p53* و*Ki-67*) للعينة M داخل الجسم الحي في الفئران Nude المزروعة بخلايا الخط الورمي HCT-116 وبعد التجريع فمويا (27 يوم) أو بالحقن في غشاء الخلب (20 يوم) بجرعتين من المستخلص 500 و 1000 مليغرام كيلوغرام$^{-1}$. كان المستخلص فعال معنويا في اختزال كتلة الورم وكان التاثير معتمد على الجرعة، ولكن رافقت المعاملة في غشاء الخلب تأثيرات جانبية. أشارت اختبارات الأنسجة المرضية لمقاطع الورم في الفئران المعاملة فمويا الى انخفاض في نسبة الانقسام الخلوي وزياده في التضاعف الداخلي للخلايا، بالإضافة إلى نخر وتقرح وضمور وترشح بالخلايا الالتهابية. أظهر جين *p53* تعبير موجب في 16% من الخلايا الورمية للناقل (السيطرة)، بينما أظهرت المقاطع الورمية للحيوانات المعاملة فمويا بـالجرعة 500 مليغرام كيلوغرام$^{-1}$ زيادة في التعبير(33,5 %) ولوحظت هذه الزيادة (40,8 %) أيضا في الجرعة 1000 مليغرام كيلوغرام$^{-1}$. بينما كانت هناك صورة معاكسة بخصوص*Ki-67* ، حيث اظهر الجين تعبيرا في 85% من خلايا المقطع الورمي للناقل بينما كان تعبيره 47% و46,8 % في مقاطع الورم المأخوذة من الفئران المعاملة بالجرعتين 500 أو 1000 مليغرام كيلوغرام$^{-1}$، على التوالي.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

دراسة خواص العكبرالعراقي المضادة للورم خارج وداخل الجسم الحي

اطروحة

مقدمة الى مجلس كلية العلوم - جامعة النهرين

وهي جزء من متطلبات نيل درجة دكتوراه فلسفه في التقانة الاحيائية

من قبل

غسان محمد سليمان

بكالوريوس علوم حياة/ جامعة بغداد/ 1995
ماجستير علوم حياة/ جامعة بغداد/ 2002

باشراف

د. خلود وهيب السامرائي
أستاذ

د. علي حسين أدحيه
أستاذ مساعد

تموز 2010

رجب 1431