Republic of Iraq
Ministry of Higher Education
and Scientific Research
AL-Nahrain University
College of Science

Cytotoxic and Molecular Assessment of *Hymenocrater longiflorus* Plant in Human Carcinoma Cells

A thesis
Submitted to the College of Science, University of Al-Nahrain
as a partial fulfillment of the requirements for the Degree of
Doctor of Philosophy in Biotechnology

By

Rafal Shakeeb Abdul Wahab Alanee

B.Sc. Biotechnology 2002
M.Sc. Biotechnology 2006

Supervised by

Dr. Khulood W.Al Sammaraie
Professor

Dr. Ghassan M. Sulaiman
Assisst. Professor

September 2014 **Thul-Qida 1435**

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا
عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ

صدقَ الله العَظيم

سورة البقرة ألاية "٣٢"

Supervisors Certification

We, certify that this dissertation entitled **"Cytotoxic and Molecular Assessment of *Hymenocrater longiflorus* plant in Human Carcinoma Cells"** 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 Sammaraie
Scientific Degree: Professor
Date:

Signature
Dr. Ghassan M. Sulaiman
Scientific Degree: Assist. Professor
Date:

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

Signature:
Name: **Dr. Hameed M. Jasim**
Scientific Degree: Professor
Title: Head of Biotechnology Department
Date:

Committee Certification

We, the examining committee certify that we have read this dissertation entitled **''Cytotoxic and Molecular Assessment of *Hymenocrater longiflorus* plant in Human Carcinoma Cells''** and examined the student **''Rafal Shakeeb Abdul Wahab Alanee''** in its contents and that in our opinion; it is accepted for the Degree of Doctorate of Philosophy in Biotechnology.

(Chairman)
Signature:
Name: **Dr. Saad M. Shuker**
Scientific Degree: Professor
Date:

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

(Member)
Signature:
Name: **Dr. Abbas A. Mohammad**
Scientific Degree: Professor
Date:

(Member)
Signature:
Name: **Dr. Salman A. Ahmed**
Scientific Degree: Assist. Professor
Date:

(Member)
Signature:
Name: **Dr. Abdulwahid Shamkhi**
Scientific Degree: Assist. Professor
Date:

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

(Member / Supervisor)
Signature:
Name: **Dr. Ghassan M. Sulaiman**
Scientific Degree: Assist. Professor
Date:

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

Signature:
Name: **Dr. Hadi M. A. Abood**
Scientific Degree: Assist. Professor
Title: Dean of College of Science
Date:

Acknowledgments

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

First and foremost, I pay my heartfelt tribute to my supervisors Prof. Dr. Khulood W. Abood and Assist. Prof. Dr. Ghassan M. Sulaiman, 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.

I wish here to thank Dr. Hameed Al Dulaimi (Head of Biotechnology Department), and biotechnology department staffs.

I am very grateful to Ministry of higher education and scientific research for their facilities to finish this work.

The work presented here was performed at the Laboratory of Biology, Department of Structural and Functional Biology, In Naples Federico II University, Naples, Italy.

Sincere gratitude is also extended to Prof. Dr. Luigi Lania (Head of Biology Depatment), Prof. Dr. Barbara Majello, Dr. Giuliana Napolitano, Stefano, Christina, Susana and James for their 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 their personal support and their interest in my career, support that have been instrumental in helping me to realize my future undertaking. For this I am truly grateful. Indeed, I express my sincere gratitude to all the other colleagues, who were working at the College.

I am indebted to Dr. Ali H. Ad'hiah for his support. and to my friendship Mrs. Raghad Al Laheeby, Dr. Niyaff Al Shamary, Dr. Zaynab Yassin, Dr. Ahmed Fadhil for offering their support and encouragement.

In particular I am also eternally grateful to my father soul, my mother, my brothers and my sister. 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 Mother for her love and support over all these years, and the hard work and her prayers; I could achieve nothing without her, gods bless her.

Summary

The present study was conducted to evaluate the effect of *Hymenocrater longiflorus* methanolic extract on human osteosarcoma U2OS cancer cell line and colon RKO cancer cell line.

Chemical analysis of the crude methanolic extract of the plant was done using High Performance Liquid Chromatography – electrospray tandem mass spectrometry (HPLC-ESI /MS) analysis for the phenolic compounds (flavonoids, phenolic acids and their esters). Eleven compounds were detected in this extract, which were acacetin, apigenin, apigenin-7-O-glucoside,caffeic acid, ferulic acid, carnosic acid, crismartin, genistein, isorhamantin, N-carboxybenzyloxy-cysteinyl cystein and rosmaric acid.

The free radical scarvenging activity of *H. longiflorus* methanol extract was evaluated by using 2, 2,-diphenyl-1picrylhydrazyl (DPPH) assay. The results reveal that *H. longiflorus* has antioxidant activity, and this activity which is concentration dependent increases until reaching 86.226% antiradical activity (ARA) at concentration of 1000 µg ml^{-1}.

The *in vitro* study on cell viability reveals that the plant has an inhibitory effect on cancer cells (U2OS and RKO) using Dye exclusion assay and MTT assay which convert the yellow color to purple, the growth decreased and a significant effect was observed like decrease in the cell number as compared with non treated cells.

Through assessment of cell cycle arrest using propidium iodine (PI) by Fluorescence Activated Cell Sorter (FACS) analysis using flow cytometry, indicate a mild effect was observed at G2 phase of U2OS and at G1 phase of RKO cell line in comparison with non treated cells (control).

Phosphorylation of H2AX was induced in response to DNA double strand breaks originated from diverge origins of γH2AX including external damage, replication fork collision, apoptosis and dysfunctional telomeres using immunoflourescence assay, by this assay accumulation of damage foci was observed as compared with non treated cells in both U2OS and RKO cell line.

The study of the apoptosis using DAPI and Sulforhodamine stain, clarified decrease in cell size and shrinkage in both U2OS and RKO cell line, these results show the signs of apoptosis.

Proteins and DNA damage using Western blot analysis was observed, different proteins were used (p21, p53, p-p53, γ H2AX and parp), Proteins increased in size with cleavage of parp protein into two bands, which is an indication of apoptosis in addition to increase in p53 and γ H2AX proteins for both U2OS and RKO cell line.

From all obtained results, It is possible suggest that *H. longiflorus* methanol extract is a promising anticancer plant due to its effect on cell growth, cell cycle arrest DNA damage then lead to apoptosis, taking into consideration that RKO cell line was more sensitive than U2OS cell line.

List of Contents

Chapter Three: Materials and Methods

List of Tables

List of Figures

List of Abbreviations

$[M-H]^-$	Pseudo-Molecular Ion (Negative Mode)
Apaf-1	Apoptotic protease activity factor-1
Bax	Bcl2-associated X Gene
bcl-2	B Cell Leukemia/Lymphoma 2-like Gene
Caspase	Cystein- aspartic protease
CDKs	Cyclin-Dependent Kinases
CKI	Cyclin Kinase Inhibitor
CPT	Camptothecin
DCFDA	Dichloroflurores Cin diacetate
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DPPH	diphenylpicrylhydrazyl
FACS	Fluorescence-activated Cell Sorter
Flip	FLICE- like inhibitory protein
GC	Gas Chromatography
HPLC-ESI/MS	High Performance Liquid Chromatography Coupled to Electrospray Mass Spectrometry
IAPS	Inhibitor of Apoptosis Protein
IL	Interleukin
IRIF	Irradiation induced foci
53 BP1	P53 Binding Protein A
IR	Infrared
MS/MS	Mass Spectra

NT	Non Treated Cells (control)
PARP	Poly (ADP- ribose) polymerase
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PE	Plant Extract
PI	Propidium Iodide
pRB	Protein Retinoblastoma Phosphorylation
PS	Phosphatidylserine
RNase A	Ribonuclease A
RT	Retention Time
TGF	Transforming Growth Factor
TIC	Total Ion Current
TNF	Tumour Necrosis Factor
TP53	Tumor protein 53
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling
UV	Ultraviolet
v/v	Volume of Volume
ATM	Ataxia telangiectasia mutated
Mdc1	Mediator of DNA damage checkpoint protein
RNF8	Ring finger protein 8
DSB	DNA double strand break
Mdc1	Mediator of DNA damage checkpoint protein
RNF8	Ring finger protein 8

ASK1	Apoptosis signal regulating kinase
JNK	Jun N-terminal kinase
DDR	DNA damage response
ATR	Ataxia telangiectasia and Rad 3-related protein

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 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).

Cancer is a term describing conditions characterized by uncontrolled cellular proliferation and dedifferentiation (Slavov and Botstein., 2011). Hanahan and Weinberg identified four hallmark features of the cancer cell phenotype: disregard of signals to stop proliferating and to differentiate; a capacity for sustained proliferation; evasion of apoptosis; invasion and angiogenesis (Chia *et al*., 2013). The phenotypic changes which the cell undergoes in the process of malignant transformation are a reflection of the sequential acquisition of genetic alterations. This multistep process is not an abrupt transition from normal to malignant growth; but may take place over many years. The mutation of critical genes, including suppressor genes, oncogenes and genes involved in deoxyribonucleic acid (DNA) repair, cell-cycle control and angiogenesis leads to genetic instability and a progressive loss of differentiation. 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 and Nicewander, 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).

The principal modalities of therapy, i.e. surgery, radiotherapy and chemotherapy, may be utilized separately or in combination. Chemotherapy has been used in cancer treatment for more than 50 years, and has proved to be a very efficient strategy. Chemotherapeutics is similar to antibiotics in one property: both can kill certain living cells in the host organism. A basic expectation of antitumour agents is that their effect should be limited to cancer cells. The selective toxicity of the currently used agents is dependent on the rate of proliferation. In many cases, this selectivity is not adequate, resulting in low activity on slowly growing tumour tissues. On the other hand, a toxic effect on physiological rapidly dividing tissues frequently develops, including bone marrow suppression, deterioration of the gastrointestinal mucosa and hair loss (Hanahan and Weinberg, 2000). From these considerations, it seems obvious that an ideal anticancer drug would aim exclusively at the tumour cell instead of utilizing the high growth rate as a marker of targeted cells. Such agents would kill even slowly growing cancer cells without deteriorating normal tissues with a high turnover.

Throughout medical history, nature has long been shown to be an excellent and reliable source of new drugs, including anticancer agents. It is well established that plants have always been useful sources of antitumor or cancer prevention compounds (Reddy *et al*., 2003; Guo *et al*., 2010). Approximately more than 60% of currently used anticancer chemotherapeutic drugs are derived in one way or another from natural sources, including plants (Gragg and Newman, 2005; Tan *et al*., 2006). Hartwell (1982) listed more than 3000 plant species that have reportedly been used in

the treatment of cancer; Graham *et al*. (2000) had added another 350 species to Hartwell's list. Plants have been a major source of highly effective conventional drugs for the treatment of many forms of cancer, and while the actual compounds isolated from the plant frequently may not serve as the drug, they provide leads for the development of potential novel agents.

Large groups of different phenolic compounds from plants are important and essential anticancer agents (Duangmano *et al*. 2010; Yin *et al*., 2010). In many cases, they are much more effective and do not have large unintended consequences compared with synthetic drugs. In fact, they are much studied in order to explore their further use in pharmacy and medicine in the prevention and treatment of cancer.

The genus, *Hymenocrater* Fisch., belongs to the plant family Lamiaceae and named Gol-e-Arvaneh in the Persian language. It contains eleven shrub species with colorful calyxes based on Flora Iranica. Some of those species are endemic to Iran such as; *H. incanus* Bunge, *H. oxyodontus* Rech. , and *H. yazdianus* Rech. f. Among the *Hymenocrater* species, *H. calycinus* (Boiss.) Benth. is growing wildly in the north east of Iran and some parts of Turkmania as an endemic plant (Rechinger 1982, Mozaffarian 1996). No report however exists regarding the biological effects *H. longiflorus* registered in Iraq. There have been many attempts to validate biological effects of *Hymenocrater* genus and elucidate its composition. Chemical constituents of the essential oil of *H. longiflorus*, has been studied and the main components reported as 1, 8- cineole, and ß-caryophyllene (Mirza *et al*. 2001). The antibacterial and antifungal activity of *H. longiflorus* Benth., growing widely in Iran, has been determined (Zaidi and Crow 2005). Anti-oxidant potentials of *Hymenocrater logniflorus* have also been recorded (Ahmadi *et al*., 2010).

1-2 Aims of the study

Given the above information, the present study was designed to investigate the antioxidant and anti- tumour potentials of *Hymenocrater longiflorus* methanol extract growing in Iraq against two tumour cell lines which were human colon carcinoma (RKO) and human osteosarcoma (U2OS) cells. Accordingly, the following experiments were achieved:

1- Detection of some active compounds using high performance liquid chromatography coupled to electrospray mass spectrometry (HPLC-ESI/MS).
2- Free radical scavenging activity of *Hymenocrater longiflorus* extract.
3- *In vitro* assessments of growth inhibition of RKO and U2OS cell lines on different concentrations and times.
4- Effect on nuclear morphological aspects.
5- Assessment of γ-H2AX-histone formation by Immunoflourescence assay.
6- Western blot analysis for β-actin, actnin, PARP, p21, P53 and p53-p for RKO and U2OS cell lines.
7- Assessment of cell cycle arrest using FACS analysis.

Chapter Two

Literature review

2.1. *Hymenocrater*

Taxonomy
Kingdom: *Plantae*

Phylum: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Lamiales*

Family: *Lamiaceae*

Genus: *Hymenocrater*

Species: *Hymenocrater longiflorus*

http://www.gbif.org/species/3884311 on 2014-09-21

2.1.1 Description of *Hymenocrater longiflorus* Benth.

Hymenocrater longiflorus is a Perennial herb. Leaves ovate-oblong or lanceolate. Calyx with cylindrical tube and deeply 5 lobed, usually greatly enlarged after anthesis, corolla purple, violet or blue. Nutlets ovoid smooth or tuberculate (Figure 2-1).

Habitat, This plant is growing above timberline or in subalpine zone between the rocks, or among herb and grass land, and it's very rare species in Kurdistan Iraq. collected in May 2012 at full flowering stage .

Location, Hawraman Mountain, above Ballkha village, 1739 m asl., N 35 12 39, E 46 08 45.

Figure (2-1): Photo of *Hymenocrater longiflorus.*

2.1.2 History of *Hymenocrater*

Plants have the ability to produce tens of thousands of highly complex secondary metabolites to assist their survival in the environment, many of which protect the plant from predators. Man has exploited these compounds of self-defense as sources of medicinal agents (Mohanasa, 2001).

The earliest known medical document is a 4000 year old Sumerian clay tablet that recorded plant remedies for various illnesses. By the time of the ancient Egyptian civilization, a great wealth of information already existed on medicinal plants. Among the many remedies prescribed were mandrake for pain relief, and garlic for the treatment of heart and circulatory disorders (Kong *et al*., 2003). Medicinal plant represents a diverse group of herbs spread throughout the world with a high content of bioactive compounds possessing a variety of biological activities (Capek *et al*., 2009).

Lamiaceae, also called labiatae, the mint family of flowering plants, with 236 genera and more than 7,000 species, the largest family of the order lamiales. It is important to humans for herb plants useful for flavour, fragrance, or medicinal properties. Most members of the family have square stems; paired, opposite, simple leaves; and two-lipped, open-mouthed, tubular corollas (united petals), with five-lobed, bell-like calyxes (united sepals)(Anne and Victor, 2007).

In 2004, lamiaceae were divided into seven subfamilies with ten genera not placed in any of the subfamilies (Raymond *et al*., 2004). The unplaced genera are: *Tectona*, *Callicarpa*, *Hymenopyramis*, *Petraeovitex*, *Peronema*, *Garrettia*, *Cymaria*, *Acrymia*, *Holocheila*, and *Ombrocharis*. The subfamilies are symphorematoideae,vitiocoidae, ajugoideae, prostantheroideae, nepetoideae, scutellarioideae, and lamioideae. The subfamily viticoideae is probably not mnophyletic (Gemma *et al*., 2009).

Although lamiaceae occurring almost throughout the world, with the exception of the coldest Polar Regions, the lamiaceae are particularly well represented in tropical and temperate areas especially those with a seasonal climate, such as the Mediterranean region and in tropical upland savannas. While some species are characteristic of semi-arid conditions, many others are adapted to wet habitats, in seasonally flooded areas or along river banks in forest (Wagstaff and Olmstead, 1997).

The family of lamiaceae has an important role as a source of medicinal and aromatic plants of commercial importance (Satil *et al*., 2007). *Hymenocrater* is an important genius of lamiaceae family. *Hymenocrater* Fish and Mey. from stachyioideae subfamily, having numerous varieties of species, it is expanded from Iran to Iraq, Pakistan, Afghanistan (Jafari and Jafarzadeh, 2008). This genus has aromatic essential oil and antimicrobial effects (Firouznia *et al*., 2005).

Hymenocrater is represented by 11 species in Flora Iranica and two species in Flora of the USSR (Rechinger, 1982).

Hymenocrater genus has over 21 species in the world and the *H. longiflorus Benth* was firstly nominated by G. Bentham in 1848. The local name of *H. longiflorus* , is *Soor-Halale* (*Sǒǒr-HΛLΛLE*). The other names of this herb are: *Gole Arvaneh-Avarmani* and *SoorSanduo* (Bundy *et al*., 2001; Ebrahimzadeh *et al*., 2008). The *H. longiflorus* was utilizedas a medicinal herb in local and traditional medicine of Kermanshah. Aerial parts of this herb in crude or baked form was utilized as an anti-inflammatory, sedative,anti-skinallergic reaction (for skin diseases and insect bite) in folk medicine. Recently, this plant has drawn more attention due to the antimicrobial, antifungal and antioxidative effects (Ahmadi *et al*., 2010; Taherpour *et al*., 2011).

2.1.3 Biological potentials

Lamiaceae, also known as mint, is a family of flowering plants that includes 250 to 258 genera and approximately 6,000 to 6,970 species across the world (Mabberley, 1997).

The family has a cosmopolitan distribution and contains many plant species with culinary and medicinal purposes; examples of the former are basil, mint, rosemary, sage, savory, marjoram, oregano, thyme, lavender, and perilla (Naghibi *et al*., 2005). The Lamiaceae family of plants has been used since ancient times as folk remedies for various health problems such as common cold, throat infections, acaricidal, psoriasis, seborrheic eczema, hemorrhage, menstrual disorders, miscarriage, ulcer, spasm and stomach problems (Ribeiro *et al*., 2010). Their constituents, particularly diterpenoids and triterpenoids, have been found to have

antiseptic, antibacterial, anti-inflammatory, cytotoxic, cardio-active and other properties (Ulubelen, 2003).

The genus of *Hymenocrater*, with about nine species in Iran (Rechinger, 1982 and Akramian *et al*., 2008) is a member of lamiaceae family. Although *Hymenocrater* species are not abundant in the Iranica flora, four of them are endemic: *H. longiflorus* Benth., *H. platystegius* Rech.f., *H. yazdianus* Rech.f., and *H. incanus* Bunge (Naghibi *et al*., 2005). According to best knowledge some of the species belonging to *Hymenocrater* genus have been previously investigated from different points of view: identification of essential oils composition (Firouznia *et al*., 2005; Barazandeh, 2006; Akramian *et al*., 2008), anti-microbial effects (Zaidi and Crow, 2005) and anatomical and pollen ornamentation study (Jafari and Jafarzadeh, 2008).

Water-distilled essential oils from aerial parts of *H. calycinus* (Boiss.) Benth. collected from three different locations of Bojnourd (Iran) village of Yekeh-Shakh (A) , village of Nodeh (B) and Golestan forest(C) , were analyzed by GC and GC/MS. α-Pinene (10.5%) and sabinene (10.5%) were the major constituents of sample A. The main constituents found for sample B were spathulenol (35.4%) and abietatriene (13.4%). In sample C β-caryophyllene (32.8%) and caryophyllene oxide (16.1%) were the most abundant compounds (Firozinia *et al*., 2011).

The anti-microbial and antifungal activity of *H. longiflorus* extracts against several pathogenic microorganisms was studied by disc diffusion and minimum inhibitory concentration procedures. The results revealed that the essential oil and polar sub-fraction are effective mostly against *Staphylococcus aureus*, *Aspergillus niger* and *Candida albicans*. The antioxidant activity was also determined by 1,1′-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging, β-carotene linoleic acid assay and reducing power (Ahmedi *et al*.,2010).

2.1.3.1 Antioxidant activities

Plants are the most important sources of medicines. Today the large number of drugs in use is derived from plants. The important advantages for therapeutic uses of medicinal plants in various ailments are their safety besides being economical, effective and easy availability (El-Sayed and Hussin , 2013).

Medicinal plants have been playing a vital role on the health and healing of man since down of human civilization. In spite of tremendous development in the field of allopathic medicines during the 20^{th} century, plants still remain one of the major sources of drugs in modern as well as in traditional system of medicine (Krishnaiah *et al*., 2011). Medicinal plants are source of certain bioactive molecules which act as antioxidants and antimicrobial agents (Khalil *et al*., 2007; Sengul *et al*., 2009). Flavonoids are strong antioxidants that occur naturally in foods and can inhibit carcinogenesis in rodents (Hertog *et al*., 1993; Boots *et al*., 2008).

Research has shown culinary herbs and spices to be a dietary source of bioactive polyphenols (Hinneburg *et al*., 2006), which has stimulated the study of their phenolic composition and antioxidant properties. Several culinary herbs and spices are now known to have beneficial effects for human health, including digestive stimulant, anti-inflammatory, antimicrobial, antioxidant and anticarcinogenic activities (Zheng and Wang, 2001), which are attributed to the predominant polyphenol compounds in these plant materials.

Plants constitute an important source of active natural product which differs widely in terms of structures, biological properties and mechanisms of actions. Various phytochemical components, especially polyphenols (such as flavonoids, phyenyl propanoids, phenolic acids, tannins, etc) are known to be responsible for the free radical scavenging and antioxidant activities of plants. Polyphenols possess many biological effects; these effects are mainly attributed to their antioxidant activities in

scavenging free radicals inhibition of peroxidantion and chelating transition metals. In generally, polyphenols all share the same chemical patterns, one or more phenolic groups for which they react as hydrogen donors and in that way neutralize free radicals (Parejo *et al*., 2002; Lee *et al*., 2003; Miliauskas *et al*., 2004; Atoui *et al*., 2005; Capecka *et al*., 2005; Galvez *et al*., 2005; Melo *et al*., 2005).

It is well known that lamiaceae spices have potent antioxidant properties, mostly due to the polyphenolic compounds present in them (Moller *et al*., 2007). Recently, interest has increased considerably in naturally occurring antioxidant for use in foods as replacements for synthetic antioxidants such as Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), whose use is being restricted due to concerns over safety (Hossain *et al*., 2008).

Natural antioxidants can protect the human body from free radicals and could retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods (Kristinova *et al*., 2009). Oxidation of lipids in food not only lowers the nutritional value, but is also associated with cell membrane damage, aging, heart disease and cancer in living organisms (Babovic *et al*., 2010). Therefore the addition of natural antioxidants to food products has become popular as a means of increasing shelf life and to reduce wastage and nutritional losses by inhibiting and delaying oxidation. As previously state spices in the lamiaceae family are a well known source of antioxidant particularly polyphenols. Furthermore, spices have been used for many years to enhance the sensory attributes such as taste and aroma of foods (Herrero *et al*., 2010). There is an upsurge in demand of plant materials containing phenolics as they retard oxidative degradation of lipids and thereby improving quality and nutritional value of food (Landry, 1995; Rice-Evans *et al*, 1996).

The large generation of free radicals, particularly reactive oxygen species and their high activity plays an important role in the progression of a great number of

pathological disturbances like inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, Alzheimer's disease, etc (Mensor *et al*., 2001; Parejo *et al*., 2002; Hou *et al*., 2003; Orhan *et al*., 2003; Tepe *et al*., 2005; Ozgen *et al*., 2006). Therefore, the great interest has been recently focused on the natural foods, medicinal plants and phytocostituents due to their well-known abilities to scavenge free radicals (i.e. antioxidant power) (Hou *et al*., 2003; Galvez *et al*., 2005; Kukic *et al*., 2006).

2.1.3.2 Antitumor activities

Over the past few decades, cancer has remained as the largest cause of mortality worldwide and the number of individuals living with cancer is steadily expanding. Hence, a major portion of the current pharmacological research is involved with the anticancer drug design customized to fit new molecular targets (Xia *et al*., 2004). Due to the enormous propensity of plants, which synthesize a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of chemical constituents with antitumor and cytotoxic activities.

Traditionally various plants have long been used in the treatment of cancer (Kim *et al*., 2005; Kintzios, 2006; Indap *et al*., 2006; Bufalo *et al*., 2009). Dubick (1986) reported that the medical use of herbs is deeply rooted in human history and folklore, and incorporated into the historical medicine of virtually all human cultures. He describe the history of Gineseng and Garlic as two famous plants widely used –till now- in traditional medicine and proved to have many active constituents.

An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or combination) to block the development of cancer in humans. Plants, vegetables and herbs used in the folk and traditional medicine have been accepted currently as one

of the main source of cancer chemoprevention drug discovery and development (Abdullaev, 2001).

Due to the toxic and adverse side effects of synthetic drugs as well as conventional treatments are being failed to fulfill their objectives (tumor control), for these consequence herbal medicine has made a comeback to improve the fulfillment of our present and future health needs (Gennari *et al*., 2007; Harun *et al*., 2002). The development of efficient anticancer agents, such as vinblastine and vincristine isolated from *Catharanthus roseus* , provided convincing evidence that plants could be a source of novel cancer chemotherapeutic agents (Cragg *et al*., 1996 ; Liu *et al*., 2004; Hu and Kitts., 2004). Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer (Hengartner, 2000; Lee *et al*., 2003).

2.1.3.3 Viability and Cytotoxicity of Cells

A viability assay is an assay to determine the ability of cells or tissues to maintain or recover its viability (Henkelman *et al*., 2010). The measurement and monitoring of cells viability is an essential technique in any laboratory focused on cell-based research. This skill allows for the optimization of cell culture conditions as well as the determination of cytokinases, growth factors, or hormone activity. More importantly, the cytostatic nature of anticancer compounds in toxicology testing, the efficacy of therapeutic chemicals in drug screening, and cell-mediated cytotoxicity can all be accessed through the quantification and monitoring of cell viability and growth (Lecoeur , 2002).

Various assays are in use to determine the effect of a drug (broadly defined chemical or other inhibitory substance) on cells propagated *in vitro*. They range from

simple assays that measure cell viability after drug exposure, i.e., dye exclusion that measures membrane integrity and effect of the drug on cell growth (simply enumerating cells), to other assays that measure cell viability, indirectly, by assessing the ability of the cell to reduce compounds such as 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium MTS, Sulforhodamine B (SRB) , and AlamarBlue or to generate Adenosine-5'-triphosphate (ATP).

The advantages of these assays are that they are performed easily and, with the use of 96-well plates, many dilutions or many compounds can be tested rapidly. There are many methods summarizes as bellow (Alosi *et al*., 2010):

1. Cytolysis or membrane leakage assays: This category includes the lactate dehydrogenase assay, a stable enzyme common in all cells which can be readily detected when cell membranes are no longer intact. For examples:
    - Propidium iodide
    - Trypan blue
    - 7-Aminoactinomycin D
2. Mitochondrial activity or caspase assays: Resazurin and Formazan (MTT/XTT) can assay for various stages in the apoptosis process that foreshadow cell death.
3. Functional assays: Assays of cell function will be highly specific to the types of cells being assayed.
4. Genomic and protoemic assays: Cells can be assayed for activation of stress pathways using DNA microarrays and protein chips.

2.1.3.4 Apoptosis

Cell death occurs by two alternative and basically different modes: necrosis and apoptosis. Necrosis, the ”ordinary” cell death with the characteristics of a passive process, is traditionally associated with inflammation, occurs in response to severe forms of physical or chemical types of injury, or results from severe depletion of cell energy and nutrition stores (Kanduc *et al*.,2002). Apoptosis (or programmed cell death) is a fundamental and complex biological process that enables an organism to kill and remove unwanted cells during any stage of development, therefore maintaining normal homeostasis and eliminating infected or malignant cells. In the experimental development of new anticancer agents, apoptosis is considered the pre-eminent form of pathophysiological cell death (Simoni *et al*., 2001). Apoptosis is one of the most important regulatory functions whereby a living organism maintains homeostasis. In some diseases, this regulation is damaged, and the rate of apoptosis is pathologically increased (e.g. neurodegenerative diseases) or decreased (e.g. cancer).

1. Morphological hallmarks of apoptosis

The definition of apoptosis was first based on a distinct sequence of morphological features observed by electron microscopy, described by Kerr and colleagues in 1972 (Kerr *et al*., 1972). The onset of apoptosis is characterized by shrinkage of the cell and the nucleus, and by the condensation of nuclear chromatin into sharply delineated masses that become marginated next to the nuclear membranes. Later the nucleus progressively condenses and breaks up. The term budding has been coined for a process whereby the extensions separate and the plasma membrane seals to form a separate membrane around the detached solid cellular material. These apoptotic bodies are crowded with closely packed cellular organelles and fragments of the nucleus (Anita *et al*., 2014).

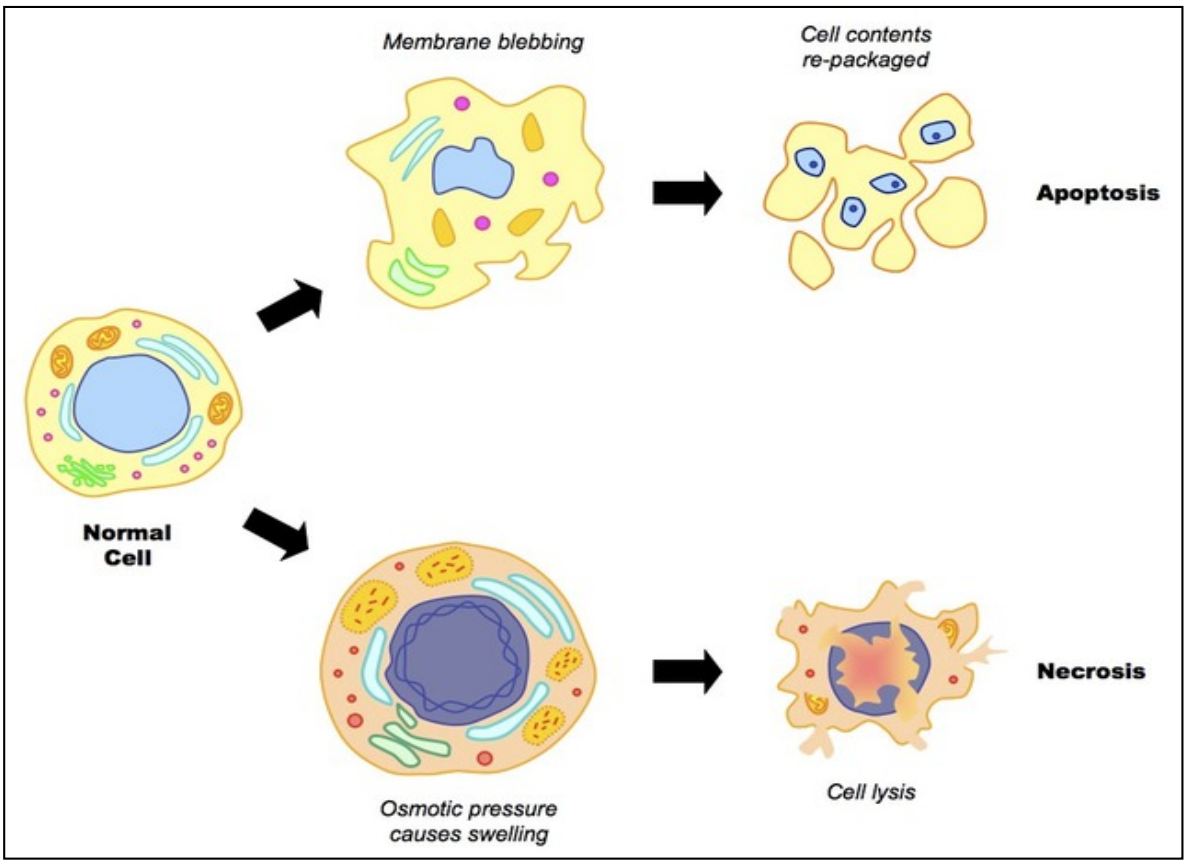


Figure (2- 2): Morphological changes during apoptosis and necrosis (http://www.vce.bioninja.com)

The apoptotic bodies are rapidly phagocytosed into neighbouring cells, including macrophages and parenchymal cells. Apoptotic bodies can be recognized inside these cells, but eventually they become degraded. If the fragmented cell is not phagocytosed, it will undergo degradation, which resembles necrosis, in a process called secondary necrosis (Kerr *et al*., 1994). Apoptotic shrinkage, disassembly into apoptotic bodies and engulfment of individual cells characteristically occur without associated inflammation; the release of intracellular contents into the tissues is therefore prevented.

2. Biochemical hallmarks of apoptosis

Biochemical features associated with apoptosis include internucleosomal cleavage of DNA, leading to an oligonucleosomal ”ladder”, phosphatidylserine (PS) externalization and proteolytic cleavage of a number of intracellular substrates (Martin *et al*., 1995) . Activated nucleases are responsible for DNA degradation, resulting in 180-200 base pairs (bp) fragments (Wyllie, 1980). As a consequence, some of the cells contain a decreased amount of DNA and can be detected as a subdiploid population in the cell cycle distribution by flow cytometry.

In the early stages of apoptosis, plasma membrane alterations occur at the cell surface, and PS translocates from the inner side of the plasma membrane to the outer layer. PS and phosphatidylethanolamine are actively confined to the inner cytofacial leaflet of the plasma membrane by the aminophospholipid translocase. This has been identified as a trigger for stimulation of the phagocytosis of apoptotic cells by macrophages, thus preventing secondary necrosis and inflammation of the surrounding tissue (Vanags *et al*.,1996). Surface PS is detectable by flow cytometry using fluorescein isothiocyanate (FITC)-labeled annexin V, a Ca2+-dependent phospholipid-binding protein with high affinity for PS (Bratton *et al*., 1997).

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 *et 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 *et al*., 2006; Nguyen *et al*., 2009).

2.1.3.5 Apoptosis and Cancer

Apoptosis, as a way to eliminate unwanted cells, is crucial for development, organ morphogenesis, and tissue homeostasis. There is accumulating evidence showing that the accumulation of damaged cells in the tissue resulted from lacking of proper apoptosis is closely associated with tumorigenesis (Hanahan and Weinberg, 2000). The resistance to apoptosis of cancer cells is acquired through a variety of biochemical changes, including over-expression or low-expression of certain functional proteins relevant to apoptosis. Moreover, these changes also attribute to the responsiveness of cancer cells to anticancer therapy. Therefore, apoptosis regulatory molecules are legitimate targets for anticancer treatment.

Apoptosis is executed by activated intracellular proteases, known as caspases, which are responsible for the specific apoptotic biochemical and morphological changes. The activation of caspases is regulated by a fine balance with/between two opposite sides, i.e. proapoptotic signals that facilitate its activation and antiapoptotic signals that inhibit its activation. Changes at either side may perturb the balance and confer the cancer to be resistant or sensitive to apoptosis stimuli. For example, low expression of cell death receptors contributes to the resistance of some cancers (Wang and Deiry, 2003).

Apoptotic protease activity factor-1 (Apaf-1), a cell-death effector that acts with cytochrome C and caspase-9, is frequently inactivated in cancers such as malignant melanoma (Soengas *et al*., 2001). Another well known example is p53, a tumor suppressor protein, which activation in response to DNA damage induced by anticancer drugs will lead to cell cycle arrest or apoptosis. However, many cancers have mutant p53, which confers the cancer cell to be resistant to anticancer therapy

(Koechli, 1994). Thus activation of p53, for example by inhibiting its interaction with MDM2 (Vassilev, 2004), a p53 antagonist, or restoration of wide type p53, for example by introducing wide type p53 using gene therapy (Quist *et al*., 2004), have been proved to be effective in anti-cancer therapy. The cancer resistance can also be acquired by over-expression of one or several anti-apoptotic proteins, such as Bcl-2, survivin, FLIP and IAPs (Deveraux and Reed, 1999). Understanding of the molecular basics of cancer resistance also helps to locate proper targets for activating apoptosis in cancer therapy.

2.1.3.6 Western Blotting

Western blotting is a method in molecular biology, biochemistry and immunogenetics to detect protein in a given sample of tissue homogenate or extract. this technique include gel electrophoresis to separate denatured proteins by mass. The proteins are then transferred out of the gel and onto a membrane (typically nitrocellulose polyvinylidene difluoride (PVDF) membrane and combine with antibodies specific to the protein. The secondary antiboy can be stained and pictured by a film. The film with the protein binds can be kept for a long time and scanned any time it needs to quantity the protein levels. As a result, researchers can examine the amount of protein in a given sample and compare levels between several groups. Other techniques also using antibodies allow detection of proteins in tissues and cell (immunocytochemistry) (Ambroz, 2006; Hui *et al*., 2012).

The name Western blotting is a pun on the name Southern blotting, a technique for DNA detection and the detection of RNA is termed northern Blotting. Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for WB analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane in the same pattern as they separate on the SDS-PAGE. All sites on the membrane which do not contain

blotted protein from the gel can then be non-specifically "blocked" so that antibody (serum) will not non-specifically bind to them, causing a false positive result. Often the membrane is cut into strips to facilitate testing of a large number of samples for antibodies directed against the blotted protein (antigen) (Neill, 2006; Alegria *et al*., 2009).

To detect the antigen blotted on the membrane, a primary antibody (serum) is added at an appropriate dilution and incubated with the membrane. If there are any antibodies present which are directed against one or more of the blotted antigens, those antibodies will bind to the protein(s) while other antibodies will be washed away at the end of the incubation. In order to detect the antibodies which have bound, anti-immunoglobulin antibodies coupled to a reporter group such as the enzyme alkaline phosphatase are added (e.g. goat anti-human IgG- alkaline phosphatase). This anti-Ig-enzyme is commonly called a "second antibody" or "conjugate". Finally after excess second antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody bound to the protein (Watts *et al*., 2003; Ma and Shieh, 2006).

- **Types of Proteins used for the Western Blot**

1. p53 protein

The p53 protein, also called tumour protein 53 (or TP 53), is one of the best known tumour suppressor proteins encoded by the tumour suppressor gene TP53 located at the short arm of chromosome 17 (17p13.1). It is named after its molecular weights, i.e., 53 kDa (Levine *et al*.,1991). It was first identified in 1979 as atransformation-related protein and a cellular protein accumulated in the nuclei of cancer cells binding tightly to the simian virus 40 (SV40) large T-antigen. Initially, it was found to be weakly-oncogenic (Vikhanskaya *et al.*, 2007). It was later discovered that the oncogenic property was due to a p53 mutation, or what was later called a

"gain of oncogenic function" (Avery *et al.*, 2011). Since its discovery, many studies have looked into its function and its role in cancer. It is not only involved in the induction of apoptosis but it is also a key player in cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation and cellular senescence and is called the "guardian of the genome" (Wei *et al.*,2008).

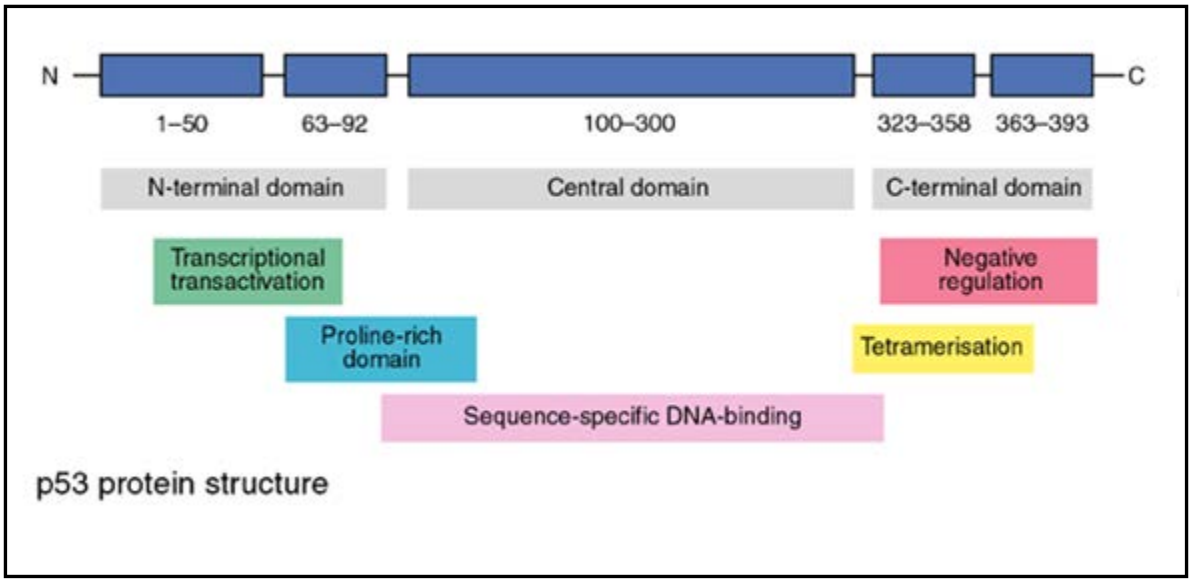


Figure (2-3): p53 protein structure. (Expert Reviews in molecular medicine ©2003 Cambridge University Press)

Defects in the p53 tumour suppressor gene have been linked to more than 50% of human cancers (Shen *et al.*, 2010). Recently, Avery-Kieida *et al* reported that some target genes of p53 involved in apoptosis and cell cycle regulation are aberrantly expressed in melanoma cells, leading to abnormal activity of p53 and contributing to the proliferation of these cells (Small *et al.*, 2010 ; Slatter *et al.*,2011).

2. p21 protein

Protein p21 is a cyclin-dependent kinase inhibitor, which is important in the response of cells to genotoxic stress and a major transcriptional target of p53 protein. In human is encoded by CDKN1A gene located in chromosome 6 (6p21.2). It is

named after its molecular weights, 21 KDa. Based on the localization, p21 protein executes various functions in the cell (Zhang *et al*., 2007). In the nucleus p21 binds to and inhibits the activity of cyclin dependent kinases Cdk1 and Cdk2 and blocks the transition from G1 phase into S phase or from G2 phase into mitosis after DNA damage. This enables the repair of damaged DNA. p21 was also found as an important protein for the induction of replication senescence as well as stress-induced premature senescence. In the cytoplasm, p21 protein has an anti-apoptotic effect. It is able to bind to and inhibit caspase 3, as well as the apoptotic kinases ASK1 and JNK (Bedelbaeva *et al*., 2010).

The function of p21 in response to a DNA damage probably depends on the extent of the damage. In the case of low-level DNA damage, the expression of p21 is increased; it induces cell cycle arrest, and performs also anti-apoptotic activities. However, after extensive DNA damage the amount of p21 protein is decreased and the cell undergoes apoptosis. Dual function of p21 was also observed in cancerogenesis (Klopfleisch *et al*., 2010). On the one hand, p21 acts as a tumor suppressor; on the other hand it prevents apoptosis and acts as an oncogene. Better understanding of the role of p21 (Cip1/Waf1) in various conditions would help to develop better cancer-treatment strategies (Dolezalova *et al.*, 2012).

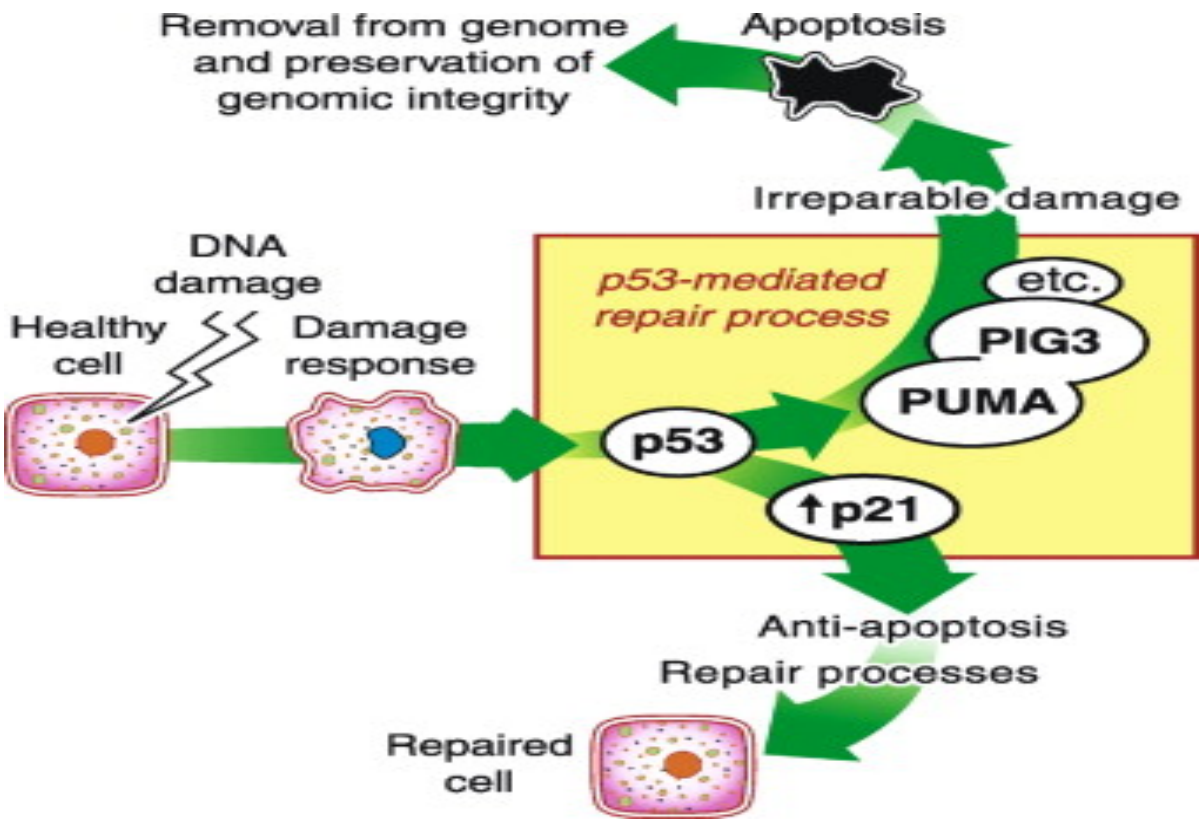


Figure (2-4): p21 determine the fate of DNA damaged cell (Weiss *et al*., 2003).

3. H2AX protein

Histone H2AX is a histone variant found in almost all eukaryotes. It makes a central contribution to genome stability through its role in the signaling of DNA damage events and by acting as a foundation for the assembly of repair foci (Goodarzi *et al.*, 2008). The H2AX protein sequence is highly similar and in some cases overlapping with replication-dependent canonical H2A, yet the H2AX gene and protein structures exhibit a number of features specific to the role of this histone in DNA repair (Collins *et al.*, 2008). The most well-known of these is a specific serine at the extreme C-terminus of H2AX which is phosphorylated by Phosphoinositide-3-Kinase-related protein Kinases (PIKKs) to generate the gamma H2AX mark (Nitiss, 2009). However, recent studies have demonstrated that phosphorylation,

ubiquitylation and other post-translational modifications are also crucial for function. H2AX transcript properties suggest a capability to respond to damage events. Furthermore, the biochemical properties of H2AX protein within the nucleosome structure and its distribution within chromatin also point to features linked to its role in the DNA damage response (Pommier and Cushman, 2009). In particular, the theoretical inter- nucleosomal spacing of H2AX and the potential implications of amino acid residues distinguishing H2AX from canonical H2A in structure and dynamics are considered in detail (Srivastava *et al*., 2008).

One of the key events that initiate DDR is phosphorylation at the Ser139 of histone H2AX, a chromatin-bound histone variant compromising up to 25% of the H2A. This phosphorylation process is catalyzed by the master regulator of DDR, ATM and ATR. Phosphorylation of H2AX at Ser139 is very rapid and this phosphorylated H2AX (γH2AX) serves as a platform, directly recruiting Mdc1 (mediator of DNA-damage checkpoint 1), and additional factors such as 53BP1, RNF8, and the BRCA1A complex to affected sites (Harper and Elledge; 2007).

Over the past decades, many studies have contributed and led to the outline of the molecular framework of DDR pathways. Histone variant H2AX is a key DDR component. It becomes rapidly (i.e. within minutes) phosphorylated at its carboxyl terminus to form the so-called cH2AX at DSB sites (Bonner *et al.*, 2008).

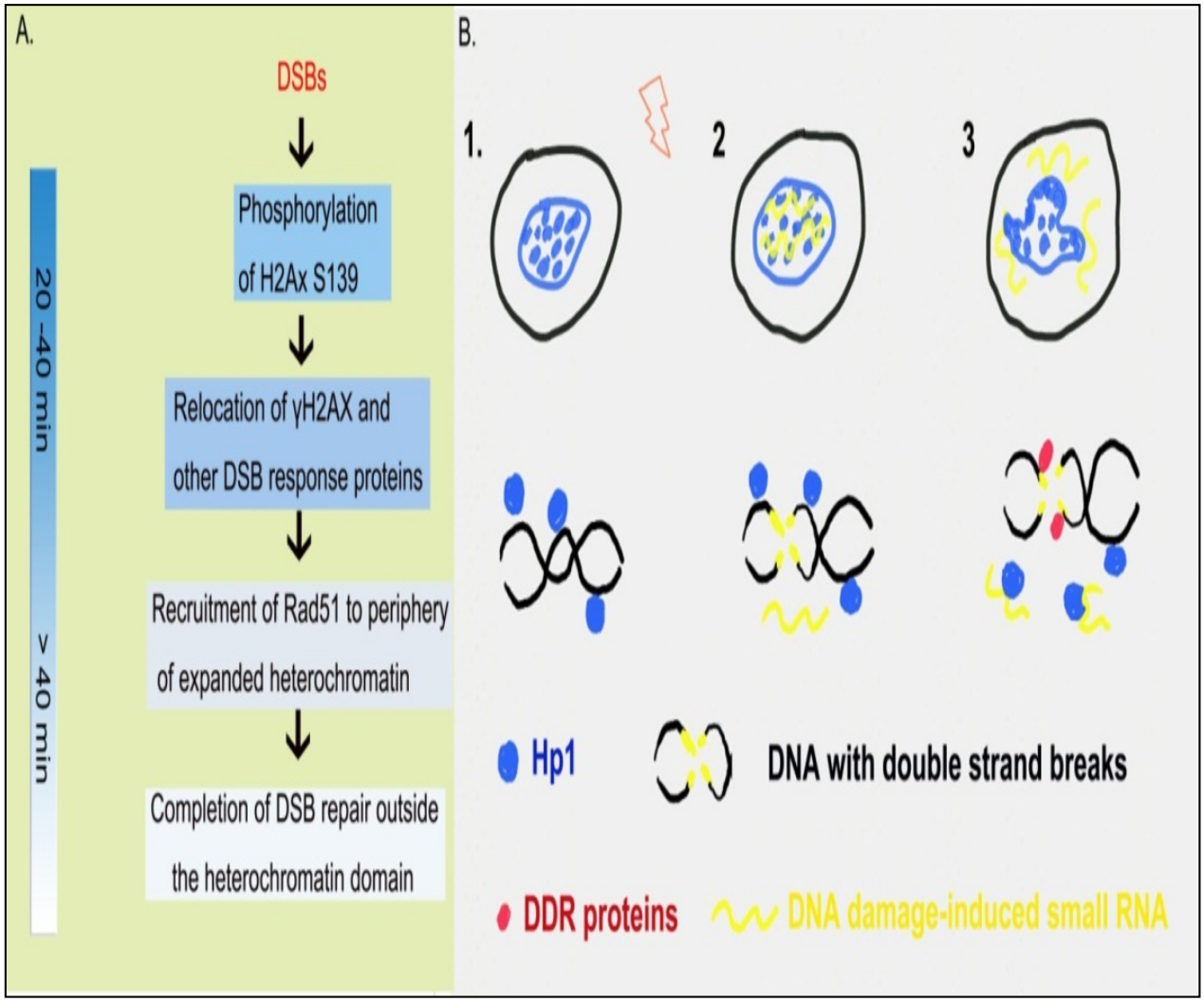


Figure (2- 5): **A**. Schematic diagram shows DNA damage response in heterochromatin. **B**. A potential model for DNA damage response in heterochromatin. (Chiolo *et al*., 2011).

4. PARP protein

Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in a number of cellular processes involving mainly DNA repair and programmed cell death. PARP is composed of four domains of interest: a DNA-binding domain, a caspase-cleaved domain, an auto-modification domain, and a catalytic domain. The DNA-binding domain is composed of two zinc finger motifs. In the presence of damaged DNA (base pair-excised), the DNA-binding domain will bind the DNA and

induce a conformational shift. It has been shown that this binding occurs independent of the other domains. This is integral in a programmed cell death model based on caspase cleavage inhibition of PARP. The auto-modification domain is responsible for releasing the protein from the DNA after catalysis. Also, it plays an integral role in cleavage-induced inactivation (Bernestein *et al.*, 2008).

PARP is found in the cell's nucleus. The main role is to detect and signal single-strand DNA breaks (SSB) to the enzymatic machinery involved in the SSB repair. PARP activation is an immediate cellular response to metabolic, chemical, or radiation-induced DNA SSB damage. Once PARP detects a SSB, it binds to the DNA, and, after a structural change, begins the synthesis of a poly (ADP-ribose) chain (PAR) as a signal for the other DNA-repairing enzymes such as DNA ligase III (LigIII), DNA polymerase beta (polβ), and scaffolding proteins such as X-ray cross-complementing gene 1 (XRCC1). After repairing, the PAR chains are degraded via Poly (ADP-ribose) glycohydrolase (PARG) (Isabelle *et al.*, 2010).

It is interesting to note that NAD+ is required as substrate for generating ADP-ribose monomers. The overactivation of PARP may deplete the stores of cellular NAD+ and induce a progressive ATP depletion, since glucose oxidation is inhibited, and necrotic cell death. In this regard, PARP is inactivated by caspase-3 cleavage (in a specific domain of the enzyme) during programmed cell death.

PARP enzymes are essential in a number of cellular functions, (Piskunova *et al.*, 2007) including expression of inflammatory genes: (Espinoza *et al.*, 2007) PARP1 is required for the induction of ICAM-1 gene expression by smooth muscle cells, in response to TNF (Zerfaoui *et al.*, 2008).

PARP can be activated in cells experiencing stress and/or DNA damage. Activated Parp can deplete the ATP of a cell in an attempt to repair the damaged DNA. ATP depletion in a cell leads to lysis and cell death (necrosis). PARP also has

the ability to induce programmed cell death, via the production of PAR, which stimulates mitochondria to release AIF (Yu *et al.*, 2006). This mechanism appears to be caspase-independent.

Cleavage of Parp, by enzymes such as caspases or cathepsins, typically inactivate Parp. The size of the cleavage fragments can give insight into which enzyme was responsible for the cleavage, and can be useful in determining which cell death pathway has been activated (Doetsch *et al.*, 2012).

2.1.3.7 Cell Cycle

In eukaryotic cells, cell proliferation proceeds through DNA replication followed by division of nucleus and separation of cytoplasm to yield two daughter cells. The sequential process, called cell cycle, contains 4 distinct phases biochemically. G1 phase is a period when cells decide whether to start proliferation or to stay quiescent. Once cells decide to proliferate, their DNA will be replicated during a DNA synthesis phase (S phase). The phase after DNA synthesis is called G2 phase which allows for the repair of DNA damage and replication errors. When there is no DNA damage or replication errors, the nucleus and cytoplasm will be equally divided into two and yield two daughter cells, which is call mitosis phase (M phase) (Massague, 2004). Cell cycle progression is timely regulated by cyclin-dependent kinases (CDKs) and their cyclin subunits (Ekholm and Reed, 2000). G1 progression and G1/S transition are regulated by CDK4-cyclin D, CDK6-cyclin D and later CDK2-cyclin E. While CDK2 controls S-phase when associated with cyclin A and G2/M transition is regulated by CDK1 in combination with cyclins A and B (Donjerkovic and Scott, 2000). Activation of CDKs is regulated by cyclin as well as CDK inhibitors (CKIs). Two families of mammalian CKIs have been identified: the INK4 family, which specifically inhibits CDK4 and CDK6, and the CIP/KIP family,

including p21cip1/waf1, p27kip1 and p57kip2, which have a broad range of inhibition (Ekholm and Reed, 2000).

Cell cycle checkpoints have been the targets for chemotherapeutic and chemopreventive agents. In several *in vitro* experiments, flavonoids have been found to inhibit the proliferation of many cancer cells by arresting cell cycle progression either at G1 or at G2/M phase (Lindenmeyer *et al.*, 2001). Cell division consists of two consecutive processes, mainly characterized by DNA replication and segregation of replicated chromosomes into two separate cells. Originally, cell division was divided into two stages: mitosis (M), i.e. the process of nuclear division; and interphase, the interlude between two M phases. Stages of mitosis include prophase, metaphase, anaphase and telophase. Under the microscope, interphase cells simply grow in size, but different techniques revealed that the interphase includes G1, S and G2 phases (Wang *et al.*, 2004).

Replication of DNA occurs in a specific part of the interphase called S phase. 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. G1, S, G2 and M phases are the traditional subdivisions of the standard cell cycle. Cells in G1 can, before commitment to DNA replication, enter a resting state called G0. Cells in G0 account for the major part of the non-growing, non-proliferating cells in the human body (Li *et al*., 2005).

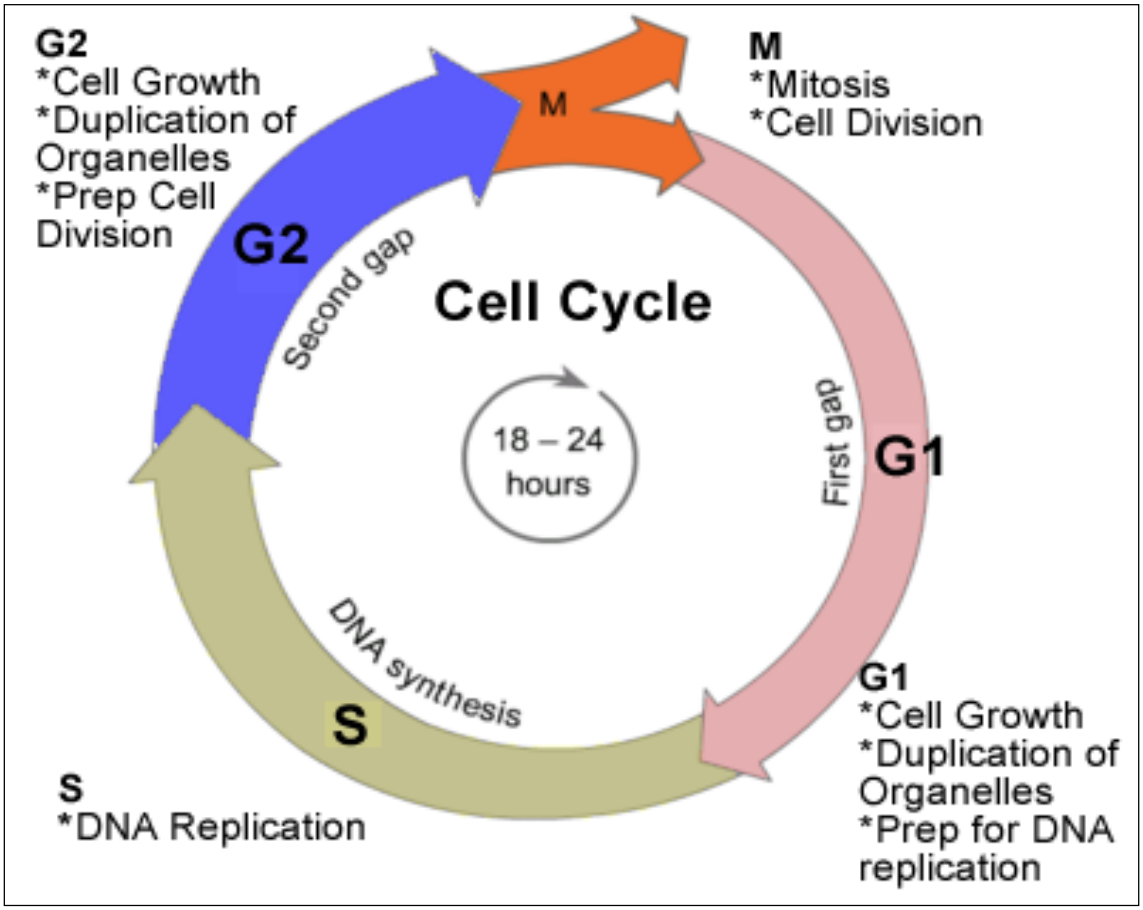


Figure (2- 6): Cell cycle checkpoints. (Dehay and Kennedy, 2007).

- **Cell cycle analysis**

Cell cycle analysis is a method in cell biology that employs flow cytometry to distinguish cells in different phases of the cell cycle. Before analysis, the cells are permeabilised and treated with a fluorescent dye that stains DNA quantitatively, usually propidium iodide (PI). The fluorescence intensity of the stained cells at certain wavelengths will therefore correlate with the amount of DNA they contain. As the DNA content of cells duplicates during the S phase of the cell cycle, the relative amount of cells in the G0 phase and G1 phase (before S phase), in the S phase, and in the G2 phase and M phase (after S phase) can be determined, as the fluorescence of

cells in the G2/M phase will be twice as high as that of cells in the G0/G1 phase (Wersto *et al*., 2001).

Cell cycle anomalies can be symptoms for various kinds of cell damage, for example DNA damage, which cause the cell to interrupt the cell cycle at certain checkpoints to prevent transformation into a cancer cell (carcinogenesis). The first protocol for cell cycle analysis using propidium iodide staining was presented in 1975 by Awtar Krishan from Harvard Medical School and is still widely cited today (Rai *et al*., 2014).

2.1.3.8 Cells lines

For decades, human immortal cancer cell lines have constituted an accessible, easily usable set of biological models with which to investigate cancer biology and to explore the potential efficacy of anticancer drugs (Chatterjee, 2007; Davis *et al*., 2010).

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. It is also used in drug screening and development, and large scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells (Masters; 2012).

In this study, there are two cell lines used which are:-

1. RKO cell line

Human colon carcinoma (RKO) is a poorly differentiated human colon carcinoma cell line developed by Michael Brattain. RKO cells contain wild-type p53 who is a gene

that is pivotal in maintaining genome integrity and in inducing apoptosis in cells damaged beyond repair, but lack endogenous human thyroid receptor nuclear receptor (h-TRbeta1). The level of p53 protein is higher in RKO (ATCC CRL-2577) cells than in RKO-E6 (ATCC CRL-2578) cells. The RKO cell line is the parental cell line (isogenic). It can be used as the control cell line for investigating the effects of p53 and gadd45 on cellular parameters (Toledo and Wahl, 2006).

2. U2OS cell line

Human osteosarcoma (U2OS) is a human osteosarcoma cell line expressing wild type p53 and Rb, but lacking p16. The U2OS cell line, originally known as the 2T line, was cultivated from the bone tissue of a fifteen-year-old human female suffering from osteosarcoma. Established in 1964, the original cells were taken from a moderately differentiated sarcoma of the tibia. U2OS cells exhibit epithelial adherent morphology and viruses were not detected in the line during co-cultivation with WI-38 cells or in CF tests against SV40, RSV, or adenoviruses (Grossel *et al*., 1999).

Chapter Three

Materials and Methods

3.1. Equipments and Apparatus

The following equipments and apparatus were used throughout the present study:

3.1.1. Specific equipments

- **Electrophoresis:** Bio Rad 2D protein Electrophoresis (Bio-Rad-USA).
- **HPLC-ESI /MS:** LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc.-USA) supplied with 1200 Series capillary LC pump and autosampler (Agilent Technologies -USA)**,** electrospray ionization Omni Spray (Prosolia -USA), Zorbax SB-C 18 column (Agilent Technologies -USA) and LTQ-Orbitrap Xcalibur software.
- **Flowcytometry:** Fluorescence Activated Cell Sorter (FACS, Becton Dickinson -USA) and CellQuest software syste Pro andModFit LT 3.0 softwares.
- **Fluorescence microscopy:** Fluorescent Microscope (Nikon- USA).
- **Bio-Rad Chemi-Doc MP Imager Station**: The light signal has been revealed through the Chemidoc (Bio-Rad) and acquired through the program "Quantity One" (Bio-Rad).

3.1.2. Other instruments

Spectrophotometer UV/VIS lambda 19 (Perkin Elmer-USA), Soxhlet (Electrothermal -England), Rotary evaporator (Heidolph -Germany), PH-Meter (PH-meter Mettler Toledo Seven Easy, Switzerland), Refrigerated Centrifuge 5702R (Eppendorf – Germany), Microfuge (Heraeus sepatech -Germany), Centrifuge Mikro 200 (Sartorius - UK), Multispeed Refrigerated Centrifuge PK121R (ALC- Italy), Electrical Balance (ISIC- India), Sensetive balance PM300 **(**Mettler-Switzerland), Incubator with CO_2 (Thermo electron corporation-USA), Laminar Flow compact (Sterile VBH- Italy), Fume Hood (Bio-Optica- Italy), Autoclave (FEDEGARI T-line,

Niagara), Light Microscope (Olympus - Japan), Oven (Heraeus sepatech - Germany), Magnetic stirrer MR2002 (Heidolph- Germany), Digital multi pipette (Eppendorf - Germany), Micropipette 2,10,100,200 and 1000 µl (Eppendorf - Germany), Thermo Shaker (Euroclone -Italy), Distillation unit (Millipore -France), Ice maker (Breama - Japan), Water bath (Gallen Kamp , England) ,Vortex (Heidolph Reax 2000- Germany) and Spectrophotometer Beckman Coulter (Beckman- USA).

3.1.3 Tissue culture vessels

3.1.3.1 Plasticwares

Disposable sterile plastic tissue culture flasks with different surface areas (T-25 cm^2 and T-75 cm^2, BD Falcon -USA), multi-well plates (96-well, BD Falcon-USA),sterile polypropylene conical tubes (15 ml and 50 ml), polystyrene conical tubes 15ml and polystyrene round -bottom tubes 5ml (BD Falcon -USA), single-use disposable sterile pipettes (2 ml, 5 ml, 10 ml, 25 ml and 50 ml, Sterilin -UK), syringes (1, 10 and 20ml, Ico - Italy), syringe driven filters (0.22 and0.45 µm, Millipore - Ireland) and Eppendorf tubes (100,200,500 and 2000 µl , Eppendorf - Germany).

3.1.3.2 Glasswares

Beakers (50,100,200,500 and 800 ml), Pasture pipettes, Funnels, Cylinders, Volumetric flasks (50,250 and 5000 ml) and bottles (75,200 and 1000 ml) were purchased from DISA- Italy. All glassware used was soaked overnight in detergent (Candiggena -Italy) and rinsed at least three times with tap water and three times with distilled water. All glassware was sterilized by autoclaving at 121°C and 30 psi on a liquid cycle for 30 minutes.

3.2 Collection and extraction of *Hymenocrater* sample

The Iraqi *Hymenocrater* plant was collected from north of Iraq (Kurdistan) during May 2012. The plant material was identified by Dr. Saman Abdulrahman Ahmad, department of Field Crop, College of Faculty of Agriculture Science, University of Sulaimania /Iraq. The fresh aerial parts were washed thoroughly with tap water at room temperature. These washed aerial parts were dried in the shade at room temperature for seven days. Then, they were crushed into final powder. One hundred powder was placed once in the Soxhlet hot extractor using 70% methanol (500 ml) as solvent for two hours at 40 °C, then the plant extract (PE) solution was filtered by Whatman filter paper No. 3. The PE was concentrated to dryness in rotary evaporator under reduced pressure at 45°C. Then, the resulting extract was stored, protected from light in a refrigerator at -20°C in a glass container until use. For *in vitro*, studies *H. longiflorus* was dissolved at a concentration of 10 mg mL^{-1} in DMSO: D.W (2:1v/v). Two main laboratory experiments were carried out with plant extract to achieve the aims of the present study. They are outlined in figure 3-1 (Mukherjee, 2004).

3.3. Chemical Analysis

3.3.1. Qualitative Analysis of *Hymenocrater longiflorus* Extract

1. Materials and Solutions

i. ***Hymenocrater* sample preparation:** The plant extract was dissolved at a concentration of 10 mg ml^{-1} in DMSO (Sigma, USA).

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).

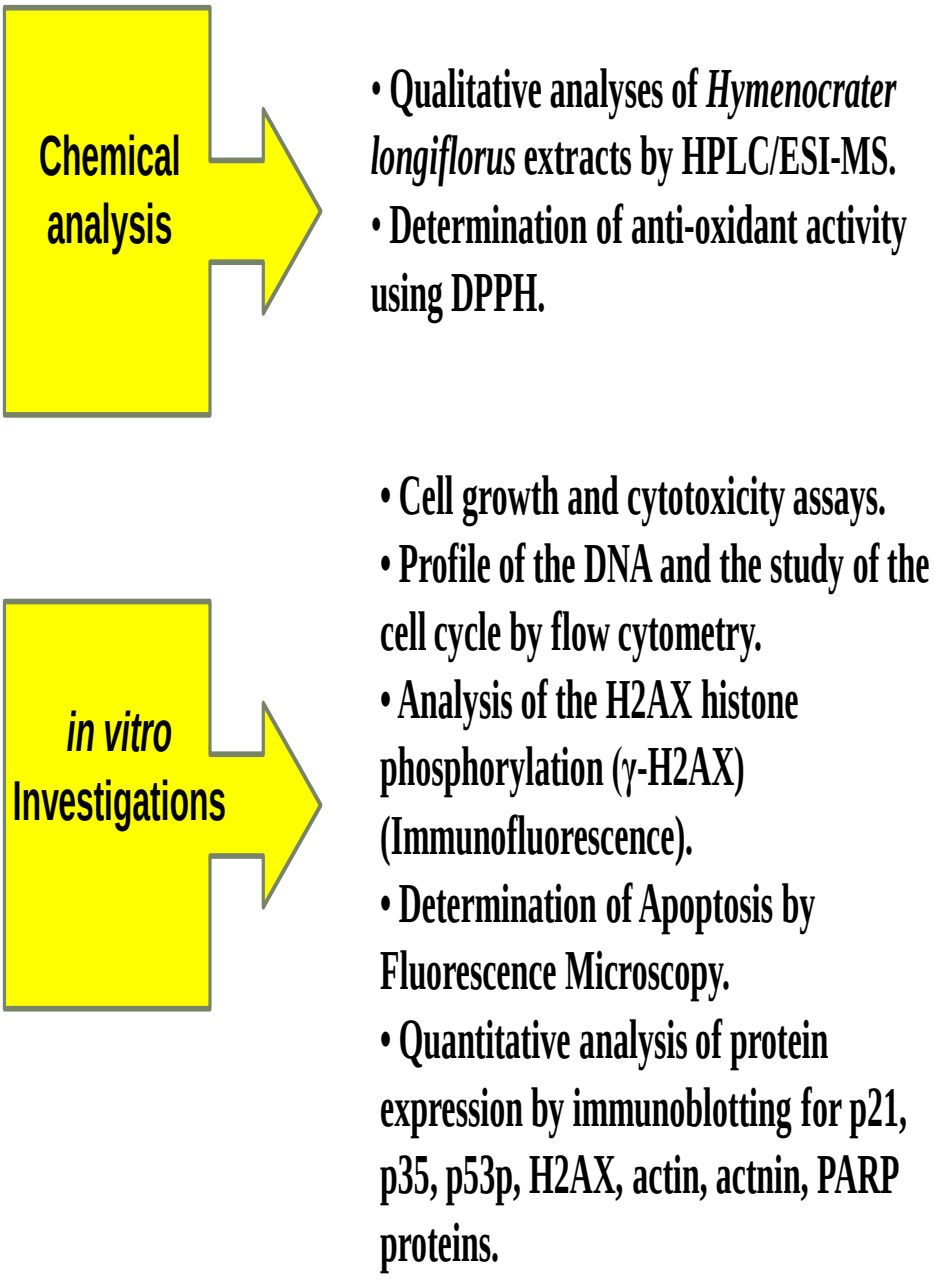


Figure (3-1): Schematic presentation of the main laboratory experiments.

2. Methods

The *Hymenocrater* sample was diluted 1:100 (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).

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

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). Sample (1 µL) was directly injected into the HPLC column, which was directly coupled to the ion source spray capillary by a liquid junction.

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.

3.3.2. Stable Free Radical Scavenging Capacity

1. **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).

2. **Method**

The free radical scavenging activities of plant extract was 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 (10, 20, 40,100,500 and 1000 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:

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

3.4. *In vitro* Experiments

3.4.1. Cell culture

1. Materials and Solutions

i. **Dulbecco's modified Eagle's medium (DMEM):** Its high glucose media from (Euro clone) and provided with 10 % fetal bovine serum (50 mL) from (Euro clone) and 1% streptomycin / penicillin (5.5 mL) (Sigma, Aldrich).

ii. **Versene:** it was prepared by adding 0.2 g EDTA, 8 g NaCl, 0.2 g KCl, 1.45 g Na_2HPO_4, 0.2 g KH_2PO_4 and H_2O to complete the volume to 1 L.

iii. **Trypsin:** the stock is 2.5% (Euro clone) and the work is 0.25% this means it was prepared by taking 5mL of trypsin and 45mL of versene.

2. Method

Human osteosarcoma (U2OS) and human colon carcinoma (RKO) cells were purchased from the Resource Bank of Department of Structural and Functional Biology, University of Naples Federico II, Naples, Italy. The cells were recovered from cell bank by rapid thawing at 37ºC in a water bath. Aliquot 500 mL of DMEM culture medium, containing glucose, sodium pyruvate and L-glutamine, provided with fetal bovine serum (10%) was added to the cells drop by drop with well mixing. After that the volume was brought-up to 9.5 ml with the same medium, mixed again and centrifuged at 1200 rpm for 4 min. After centrifugation the supernatant was discarded.

The cells were resuspended with 10 ml of DMEM medium, mixed and distributed to Petri dishes with 5 ml, the volume was brought-up to 15 ml with the same medium and incubated for monolayer confluent in a humidified atmosphere supplemented with 5% CO2 and 95% air at 37ºC.

For sub cultures, the U2OS and RKO cells were grown in adherent plates in DMEM medium provided with 0.1 % of antibiotics (penicillin and streptomycin), kept in incubator in a humidified atmosphere supplemented with 5% CO2 and 95% air at 37ºC. Upon reaching confluence, the cells were transferred into new plates twice a week to maintain the cell line in an exponential growth, a dilution to both cell lines were 1:5 v/v.

This operation involves removal of the exhausted medium from the plates and washing each plate with 5 mL of Versene. Then 1 mL of diluted trypsin with 0.25% Versene was added. Further mechanical step using Stripette was also used to detach all cells in plate. After harvesting, 10 mL of DMEM medium was added to cells and counts.

3.4.2. Cell Count

1. **Materials and Solutions**
   i. **Trypan Blue dye 0.4 %: already prepared (**Sigma, Aldrich).
2. **Method**

For each experiment conducted in this work, the cells were seeded in petridish and allow cells to growing well. The number of cells was estimated using a Burker chamber, constituted by a rectangular glass slide and a cover slip objects. The Burker chamber has two cells with a depth of 1/10 mm and the plane of each cell is divided into 9 squares of side equal to 1mm, for which the average counts in each square by the cell concentration to 0.1 μL. Then take 20 μL of the harvested cell suspension. This volume is diluted 5-fold by the addition of 80 μL of Trypan Blue, a dye that penetrates the membrane of dead cells of blue coloring them, while the live cells appear clear, then cells and trypan blue solution are placed between the bedroom and the coverslip Burker objects. It then proceeds to the cell counts in at least three square

and calculate an average. This number is multiplied by the dilution factor and 10^4, in order to obtain the estimate of the number of cells mL^{-1}.

3.4.3. Drug and treatments

1. Materials and Solutions

i. **Camptothecin:** Camptothecin (CPT) 12 µM (Sigma, Aldrich).

2. Method

The cells were subjected to different treatments; CPT drug was added directly to the media, at the final concentration of 12 µM. CPT is an alkaloid that exerts cytotoxic activity by binding to topoisomerase I, which introduces a breakthrough in a single strand of DNA and this involves the complete fragmentation of the double helix of DNA during replication (Chung *et al*., 2006).

3.5. Cell growth and cytotoxicity assay (viability assay)

For cell growth and cytotoxicity assays, the U2OS and RKO cells were seeded in 12-well flat bottom culture plates, at density 8×10^4 cells mL^{-1} and incubated for 24 hours in humidified atmosphere supplemented with 5% CO_2 and 95% air at 37°C. The U2OS cells were treated with four concentrations (50, 100, 150 and 200 µg mL^{-1}) of plant extract for 24, 48 and 72 hours; while for RKO cell two concentrations were used (80 and 100 µg mL^{-1}) at 3 and 6 hrs. And further four concentrations (10, 20, 40 and 80 µg mL^{-1}) at (24 and 48 hrs), after each incubation period, the cells were counted and the viability was assessed using Haemocytometer (Louis and Siegel, 2011).

- **MTT assay**

1. Materials and Solutions

i. Phosphate buffer saline (PBS). It was prepared by dissolving 80 g NaCl , 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 100 mL of deionized distilled water, and the pH was adjusted to 7.0, and then the volume was made up to 1000 mL.

ii. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, Germany) was a yellow tetrazole) is reduced to purple formazan in living cells (Figure 3-3) MTT was dissolved in PBS at 5 mg mL^{-1} and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT and kept in dark place at 4°C (Freshney, 2005). This solution is stable for several weeks. For the experiments, 1.5 mL of MTT stock was added to 13.5 mL of DMEM medium.

iii. 0.01N Acidic Isopropanol: It was prepared by mixing 0.5mL of concentrated HCL (Sigma, Germany) with 14.5 mL of concentrated isopropanol (Sigma, Aldrich).

2. Method

U2OS and RKO Cells were seeded at 10000 cells /well (100µL) and incubated overnight in humidified atmosphere supplemented with 5% CO_2 and 95% air at 37°C. In U2OS different concentrations of plant extract (10, 20, 40, 80, 100 and 150µg mL^{-1}) were used and incubated for 2, 4, 8, 24, 48 and 72 hrs. While for RKO cells the concentrations 10, 20, 40, 80 and 100 µg mL^{-1} were used and incubated for 2 and 4 hrs. Then, MTT was added to each well and incubated for 60 minutes in the dark. After that, MTT has been removed and add 100µl of 0.01 N acidic isopropanol were added to each well to dissolve the formazan crystals. After 5 min. incubation the culture plate was placed on a Biotex model micro-plate reader, and the absorbance was measured at 570 nm. The amount of color produced is directly proportional to the

number of viable cells. Each experiment was done in triplicate (Ferrari *et al*., 1990). The relative cell viability (%) of the control wells containing the cell culture medium only as a vehicle was calculated as follow:

$$\text{Percentage of cell viability (\%)} = \left(\frac{\text{Sample Absorbance}}{\text{Control Absorbance}} \right) \times 100$$

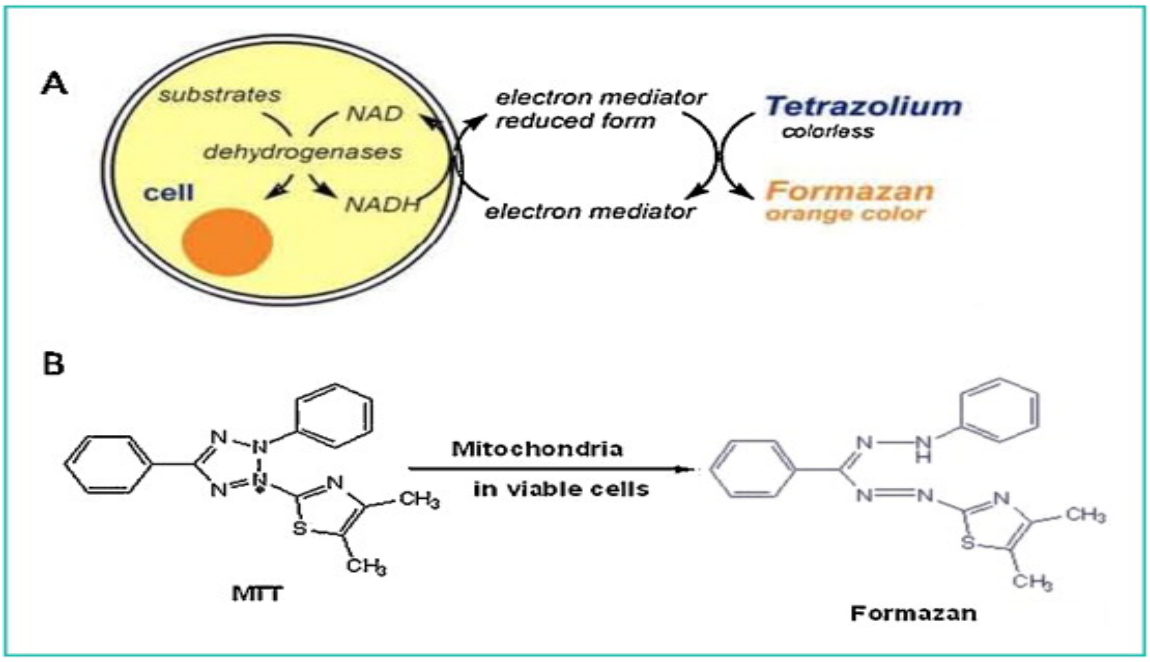


Figure (3-3) A. MTT assay principle. B. Chemical structures of MTT and Formazan. (http://www.sciencedirect.com).

3.6. DNA Profile and Cell Cycle by Flow Cytometry

1. Materials and Solutions

i. **PBS:** It was prepared as in section 3.5.

ii. **Hypotonic solution:** It was prepared by dissolving 0.1% of Na-citrate, 50 µg RNase and 50 µg propidium iodide in one milliliter of PBS.

iii. **Absolute Ethanol:** it's already prepared (Sigma, Germany).

2. Method

Analysis of the cell cycle was performed according to Erba *et al* (2001) method with slight modifications.The cells were counted and plated at a concentration of 1 × 10^6 cells per 100 mm plate, allowed to grow for 24 hrs and subjected to different treatments. For U2OS cell line, the treatment was performed at two concentrations of plant extract (50 or 100µg mL^{-1}) and for RKO, the concentrations were 40 or 80 µg mL^{-1}. In both experiments, negative control (non treated cells) was also performed. The controls and treated cells were harvested at a density of 1 × 10^6 after 3 or 6 hrs of treatment.

The cells were centrifuged (2000) rpm at (4) °C for (2) minutes. Then the superntant was removed and the cells were washed twice by PBS, fixed in cold methanol and kept at -20 ° C for at least 30 minutes. Then add 500µL PBS×1 mix by hand then the fixed cells were collected by centrifugation at 1200 rpm at 4°C for 5 minutes. The cells were suspended in PBS mix very well by micropipete and leave at 30 minutes on ice Then, the cells were permeabilised for 20 minutes at room temperature with (500µL) hypotonic solution containing Na-citrate (that allows permeabilization of the membranes), RNase (which digests the cellular RNA, in a way that does not interfere with the analysis) and propidium iodide (which intercalates into the double helix of DNA emitting fluorescence in red).

Fluorescence emitted from the PI-DNA complex was quantified after excitation of the fluorescent dye by Fluorescence-activated cell sorter flow cytometer FACS calibur (Figure 3-4). The distribution of DNA content was expressed as G1, S, and G2/M phases (Napolitano *et al*., 2013). The data were analyzed with CellQuest and ModFit 3.0 softwares.

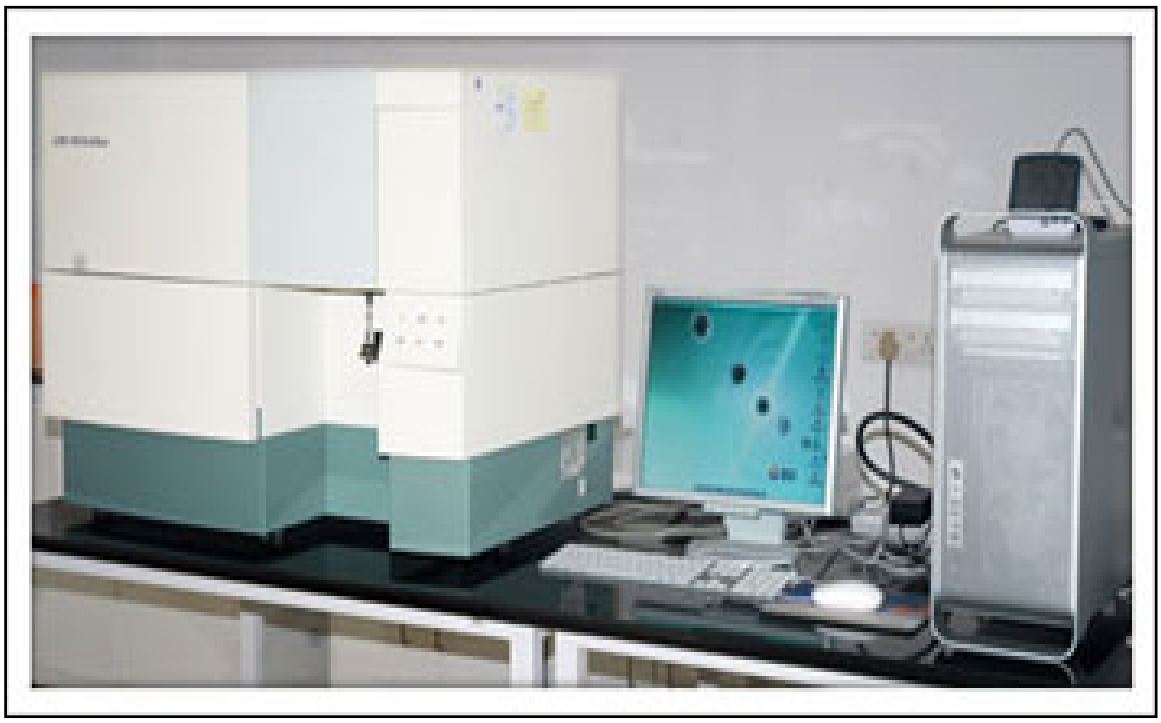


Figure (3-4): Fluorescence-activated cell sorter flow cytometer (FACS, Becton Dickinson).

3.7. Quantitative analysis of protein expression

3.7.1. Preparation of cell lysates

1. Materials and Solutions

i. Isotonic buffer: It was prepared as:-

Buffer A: It was ready used solution containing 0.2 Mm EDTA, 10 mM Hepes, 10mM KCL, MgCl2 1.5mM, NaCl 150 /200mM, NP-40 0.5% and H_2O dd to volume then add PhosStop and complete mini EDTA –free.

2. Method

Whole cell extracts for the Western Blot analysis were isolated using cell lysis according to Michels *et al*., 2003. Briefly, U2OS and RKO cells were seeded into cell

culture dishes at density 1×10^6 cells/dish. For U2OS, the cells were treated with 50 or 100 µg ml^{-1} of PE and incubated in humidified atmosphere supplemented with 5% CO_2 and 95% air at 37°C for 3, 6, 16 and 24hrs. While, for RKO the cells were treated with 40 or 80 µg ml^{-1} of *H. longiflorus* and incubated in humidified atmosphere supplemented with 5% CO_2 and 95% air at 37°C for 3 and 6 hrs. Following incubation, medium was removed and cells washed twice in PBS (isotonic buffer) prior to lysis. Cells were lysed in 400 µL lyses buffer (Buffer A) in ice for 15 to 20 minutes and were centrifuged at 13000 rpm at 4 ° C to remove the cellular debris. All samples were assayed immediately after lysis.

3.7.2. Bradford Assay

1. Materials and Solutions

i. **Bradford Reagent:** 5X solution from (Sigma, Aldrich).

ii. **Bovine serum albumin:** It was already prepared from Bio-Rad (Sigma,Aldrich) (1µg µL^{-1} to use as standard)

2. Method

Total protein concentrations were measured from the cell lysates (prepared in section 3.7.1). The Bradford protein quantification assay was performed in accordance with the manufacturer's instructions. Brifly, this assay is based on a shift in the absorbance maximum when Coomassie® Brilliant Blue G-250 dye associates with proteins. The Lambert-Beer's Law is applied for quantification of protein by selecting an appropriate ratio of dye volume to sample concentration. At the assay pH, the dye molecules are doubly protonated and are present as the red cationic dye form. Binding of the dye to protein stabilizes the blue anionic dye form, detected at 595 nm. Dye binding requires a protein containing active basic or aromatic residues. 1 mL of Bradford reagent (Bio-Rad) was mixed with 2 µL of the sample, incubated for 5 min

at room temperature and absorbance was determined at 595 nm. A calibration curve was established each time a protein assay was performed with bovine serum albomin dilutions of known concentrations (2, 4 and 6µg mL^{-1}). Using the standard curve, the concentration of each sample was determined according to its absorbance by interpolation (Bradford, 1976). All samples were measured in duplicate.

3.7.3. Estimation of protein volume

1. **Materials and Solutions**

   i. **Laemmli 5X:** It was prepared by adding 2.5 g SDS, 3.86 g DTT, 0.025 g blue dye Bromophenol to 25 mL of glycerol, then 20 mL of 1 M Tris HCl was added and the pH was adjusted to 6.8.

   ii. **Dithiothreitol (DTT):** Prepare a 1M stock solution and store at -20ºC. The working concentration of DTT is 50mM (Sigma. Aldrich).

   iii. **Sodium-dodecyl sulfate (SDS)**: it's already prepared (Sigma, Aldrich).

2. **Method**

After protein concentrations are determined, it is necessary to calculate the volume of protein to be load on a polyacrylamide gel. For the experiments described below 50 µg of total proteins were loaded on the gel. To ensure that lose their native structure, the proteins are denatured thermally in the presence of Dithiothreitol (DTT), a reducing agent that breaks the disulfide bonds, and sodium-dodecyl sulfate (SDS), which interferes with the non-covalent bonds. In addition, the SDS, with its apolar portion, interacts with the peptide chain conferring a negative charge proportional to its mass. This causes the repulsion between the charges adjacent denaturated proteins with a linear structure and, therefore, that the differences in migration are not due to the structure, but only to the molecular weight. Fifty micrograms of total protein extracts were denatured chemically in buffer A and 1X Laemmli, containing SDS and DTT, by heating at 95 °C for 5 minutes using heater. After denaturation the samples

were placed on ice for 2 minutes and subjected to brief centrifugation 5000 rpm, before being loaded on the gel.

3.7.4. Separation of proteins by SDS-PAGE

1. **Materials and Solutions**

   i. **Tris HCl 1 M pH 6.8:** It is prepare by dissolving 60.57 g of Tris, HCl (37%) in 100 mL of deionized distilled water, and the pH was adjusted to 6.8, and then the volume was made up to 500 mL.

   ii. **Tris HCl 1.5 M pH 8.8:** It is prepare by dissolving 181.71 g of Tris, HCl in 100 mL of deionized distilled water, and the pH was adjusted to 8.8, and then the volume was made up to 500 mL.

   iii. **Lower layer:** for preparation of SDS-PAGE 10% (final volume 10mL) (Sigma, Aldrich) by adding ddH_2O 3.96mL, Tris pH 8.8 1.5 M 2.5 mL, Acrylamide/bis 30% 3.33 mL, SDS 10% 100 µL, APS 10% 100 µL and Temed 10 µL.

   iv. **Stacking layer:** for preparation of SDS-PAGE 5% (final volume 5mL) by adding ddH_2O 3.74 mL, Tris pH 6.8 1M 325 mL, Acrylamide/bis 30% 830 µL, SDS 10% 50 µL, APS 10% 50 µL and Temed 5 µL.

   v. **N, N methylenebisacrylamide:** it's already prepared 99.9% (Sigma, Aldrich).

   vi. **Ammonium persulfate (APS):** its prepared by adding 0.5 g Ammonium persulfate and up to 5.0 mL H2O (stable frozen) **(**Sigma, Aldrich).

   vii. **Tetra-ethylene-diamine (TEMED):** Its odor is remarkably similar to that of fish its already prepared (Sigma, Aldrich).

   viii. **Iso-propanol:** it was already prepared at concentration 99% (Sigma, Aldrich).

ix. **Bromophenol Blue:** it's already prepared (Sigma, Aldrich).

x. **10% SDS:** (sodium dodecyl sulfate) was prepared by adding 5 g SDS (Sigma, Aldrich) and up to100 mL ddH_2O.

2. Method

The electrophoretic procedure described by (Fraignier *et al*., 1995) was followed with some modifications. The SDS-PAGE vertical electrophoresis is conducted on a poly-acrylamide gel, which allows the separation of proteins exclusively on the basis of their molecular weight. The polymerization reaction occurs between the acrylamide and N, N methylenebisacrylamide in the presence of ammonium persulfate (APS), which allows the formation of cross links producing a three-dimensional lattice, and is catalyzed by Tetra-ethylene-diamine (TEMED). The poly-acrylamide gel is formed by two parts, a lower gel, which the separation will take place protein, and an upper gel, that having a larger mesh gel stroke, does not separate the proteins, but rather focuses in a thin band in such a way that they enter the lower gel all at the same time. The principle is based on the difference of the buffers used. When generating the electric potential, the ions Cl-in the Tris-HCl buffer pH 6.8 of stacking gel form a band fast that directs downward, followed by a band of glycinate ions, dissolved in the running buffer, slower. These two bands behave as "bearings", among which are to find the protein, when stacked. These three bands can then enter the separation gel, where they find a pH 8.8: here the ions buffer assume a greater charge, migrate much faster than proteins and consequently the gradient and the effect of stacking are eliminated. At the same time, the separation gel has a pore size much smaller and allows the separation of proteins. The porosity of the separation gel is expressed in percentage of acrylamide, and depends on the proteins that they want to observe.

The gel polymerizes inside two glass plates separated by appropriate spacers, in this case of 1.5 mm, which are arranged vertically on the support of polymerization and sealed by appropriate pliers. Poured the solution for the Lower gel, it adds a layer of iso-propanol which, having lower density, form a phase overlying shaping the front of the gel and ensures the absence of oxygen, required for the polymerization.

A polymerization completed, removes the iso-propanol and prepares the solution for the stacking gel (upper layer) with the same mode; pour over the separation gel and insert the comb that determines the upper gel formation of the wells for the loading of protein samples. At the end of the polymerization the gel moves in a electrophoresis, contenette the solution of stroke, or Running Buffer, removing the comb and proceeds with the loading of the samples and of the marker, ie, a mixture of proteins with known molecular weight. The marker used in the experiments described is the Precision Plus Protein Dual Color Standards of Bio-Rad.

Connecting the end the bedroom with the electrophoretic current generator, it triggers the run at 30 mA constant until the blue line of Bromophenol Blue is not few millimeters above the edge of the gel.

3.7.5. Western blotting

1. **Materials and solutions**

   i. **Transfer Buffer 1X:** add 100 mL Tris-Glycine 10X, 20% methanol and ddH_2O complete volume to 1 L.

   ii. **Nitrocellulose membrane**: is a high quality membrane ideal for blotting of proteins and nucleic acids. Composed of 100% pure nitrocellulose to provide high-quality transfer

iii. **TBS-T 10X**: add 100 ml of Tris HCl 1M the pH was adjust to 8 then 300 mL NaCl 5 M, 100 mL Tween 20 and ddH_2O complete volume to 1 L.

iv. **Running Buffer 1X:** add 100 mL Tris- glycine 10X then 10% SDS and dd H_2O complete volume to 1 L.

v. **Ponceau S**: it was prepared by adding 0.1% Ponceau S red color (Sigma, Aldrich) and 5% acetic acid.

vi. **Anti-p53:** Anti-p53 monoclonal antibody (Clone sc-126, Santa Cruz Biotechnology, USA) was used at concentration 1: 5000.

vii. **Anti-p21:** Anti-p21 polyclonal antibody produced in rabbit (Clone sc-397, Santa Cruz Biotechnology, USA) was used at concentration 1: 5000.

viii. **Anti-actinin:** Anti- actinin monoclonal antibody (Clone sc-10750, Santa Cruz Biotechnology, USA) was used at concentration 1:5000.

ix. **Anti-P-p53-ser15:** Anti-P-p53-ser15 monoclonal antibody produced in rabbit (Clone 9286, Cell Signaling Technology) was used at concentration 1: 5000.

x. **Anti- Parp:** Anti- Parp monoclonal antibody produced in mouse (Clone sc-53643, Santa Cruz Biotechnology, USA) was used at concentration 1: 2500.

xi. **Anti- γH2AX:** Anti- γH2AX monoclonal antibody in rabbit (Clone 2577, Cell Signaling technology) was used at concentration 1: 5000.

xii. **Anti- Actin:** Anti- Actin monoclonal antibody in goat from (Clone sc-1616, Santa Cruz Biotechnology, USA) was used at concentration 1:5000.

2. Methods

According to Michels *et al*., 2003, the protein extracts were loaded onto a 10% polyacrylamide gel and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (prepared in section 2.6.1) using a Bio-Rad apparatus. The running time was approximately one hour in an

electric potential of 100 V at 4 ° C. The gel was transferred to a nitrocellulose membrane for 90 minutes using a Bio-Rad gel transfer apparatus (Figure 3-5). The membranes were immediately stained by a working solution of Ponceau S to show loading and transfer efficiency of proteins and were photographed.Following the transfer, the nitrocellulose membrane was immersed in blocking buffer (5% powdered milk in TBS-T) for 30 minutes. The membrane was then subjected to an immunoblotting procedure, by adding primary monoclonal antibodies to p53, p21, actinin , P-p53-ser15, PARP, γH2AX and β-actin diluted with 5% powdered milk in TBS-T, as indicated by manufacturer and incubating for three hours and half at room temperature or overnight at 4°C with slow constant shaking. This was followed by 15 minutes washes with TBS-T prior to one hour and half incubation with the secondary antibody (goat anti-mouse IgG horse radish peroxidase conjugated).

The membrane was washed again for 15 minutes as above before subjecting the membrane to the development procedure. The membrane was visualized by enhanced chemiluminescence substrate using the ECL (Amersham Corporation, Arlington Heights, IL, USA) system. Quantification was performed by acquiring an image with a CCD camera of Chemidoc (Bio-Rad; Figure 3-6), which allowed densitometry with computer software (Quantity One software; Bio-Rad). The results are normalized to the CPT treated either U2OS or RKO positive control before comparisons are made (Amente *et al*., 2009).

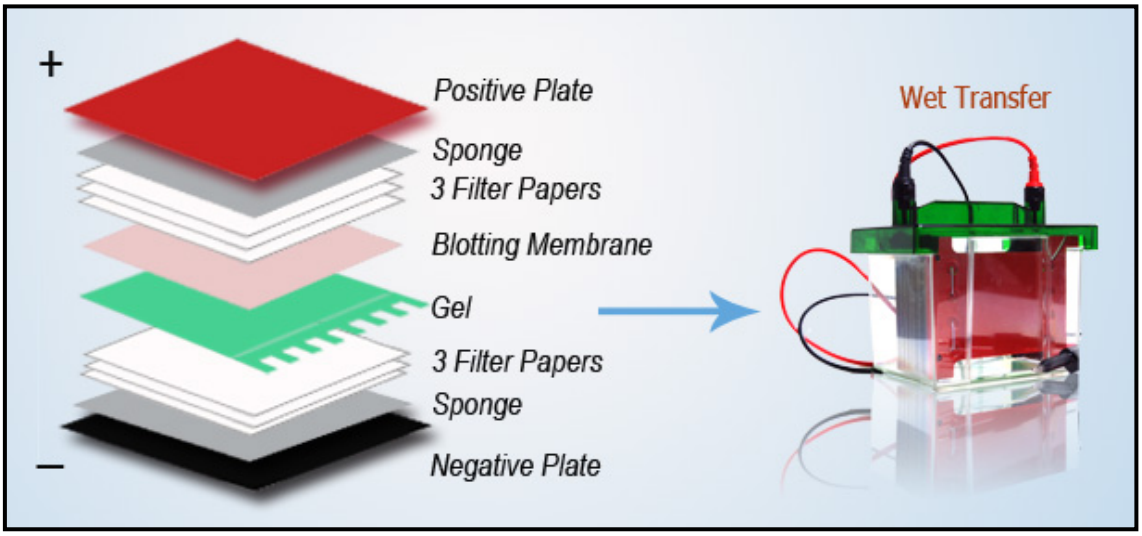


Figure (3-5) Western blot transfer buffer.

Figure (3-6) Bio-Rad Chemi-Doc MP Imager Station

3.8. Immunofluorescence of H2AX histone phosphorylation (γ-H2AX)

1. Materials and solutions

i. **4% PFA:** It was prepared by adding 2,739 mL of Formaldehyde solution (36.5%) or 1g of Formaldehyde powder (Sigma Aldrich, Europe) to 25mL of PBS X 1 to obtain final concentration 4% FA or 4% PFA respectively.

ii. **Ethanol:** It was 96% ethanol (Carlo Erba, Italy).

iii. **Permeabilisation solution:** It was prepared by adding 1 mL of Triton (Applichem) to 10 mL of PBS (Triton X-100, 10%). This solution is diluted 20 fold in 1X PBS to obtain the working solution (0.5% final).

iv. **Blocking solution:** It was prepared by dissolving 5 g of milk in 100 mL TBS-T (TBS-T 10X: Tris HCl 100mM, NaCl 1.5M, Tween 1%, to be diluted 10 fold with bi-distilled water to obtain the working solution).

v. **Anti-γH2AX (SER 139) polyclonal antibody (primary antibody):** It was prepared by diluting 1 µL of (1 mg mL^{-1}) anti-γH2AX (SER 139) polyclonal antibody (JBW301, Cat.05-636, Millipore, France***)*** in 50µL of blocking solution.

vi. **Secondary antibody:** It was prepared by diluting 1µL of anti-rabbit fluorescence monoclonal antibody (goat anti-rabbit Cy3, Jackson Lab) in 400µL of blocking solution.

vii. **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.

viii. **Mounting medium:** MOWIOL 4-88 mounting medium (Sigma, Aldrich) was prepared according manufacturer and used.

2. Method

U2OS and RKO cells were counted and seeded at density at 5×10^4 cells in 12 multi-well plates containing sterile cover-slip on the bottom of each well and allowed to adhere for 18 hours in a humidified atmosphere supplemented with 5% CO_2 and 95% air at 37ºC. U2OS cells were treated for 4 hours with Hymenocrater longiflorus extract at 80 or 100 µg mL^{-1} at 4hrs, while for RKO 50 µg mL^{-1} at 8 and 24 hrs while for concentration 80 µg mL^{-1} at 24hrs. After treatment, medium was removed and cells washed with PBS 1X (2 or 3 times). Then cells were fixed with cooled 4% PFA for 15 minutes. The cells were washed with 1 ml of filtered PBS X1 ml of cooled 70 % ethanol was added and left for at least 6 hrs or over-night

Then, cells were permeabilized in 0.5 % triton X-100 in PBS for 10-15 minutes at room temperature, washed with PBS and subjected to blocking with blocking solution for at least 30 minutes at room temperature. After blocking cells were incubated with 20µl of anti-γH2AX (SER 139) polyclonal antibody diluted 1:100 for 1 hour in the dark at 37°C. After removing the free anti-γH2AX antibody by washing the cells with 1 mL of TBS-T the cells were incubated with 20 µL of anti-rabbit Cy3 conjugated secondary antibody 45 minutes in the dark at room temperature. After that the slides were washed with TBS-T (three times) and then in PBS (twice) and stained with DAPI X 1 (10 min at room temperature). Finally, slides were mounted onto coverslips using MOWIOL, dried at room temperature and then, cells were inspected under fluorescence microscope (TE Eclipse 2000, Nikon) Images were acquired with Vico software (Napolitano *et al*., 2002).

3.9. Determination of Morphological Aspects

Sulforhadamine101-DAPI method was used to observe nuclear morphologic aspects by fluorescence microscopy using 365 nm filters. For U2OS cell line, two concentrations of PE were used (50 and 100µg mL^{-1}) at 6 hrs treatment. While for

RKO the concentration 40 and 80µg mL^{-1} were used at 3 and 6 hrs treatment. Camptothecin (CPT, 12µM) was used as a positive control in addition to non treated cells.

1. **Materials and solutions**

i. **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).

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

iii. **Permeabilisation solution:** It was freshly prepared by diluting 0.5 mL of Triton X-100 (Merck, Germany) up to 100 ml of filtered PBS (Takeuchi *et al*., 2007).

iv. **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.

v. **DAPI (4, 6-Diamidine-2-phenylindole dihdrochloride) solution:** It was prepared as same section of 3.8.

vi. **Sulforhodamine101 solution:** It was prepared by dissolving 3 mg of Sulforhodamine101 (Sigma, Germany) in 100 mL of TRIS-HCl solution (pH 8.0).

vii. **Sulforhodamine101-DAPI eluent:** It was prepared by mixing equal volumes of Sulforhodamine101 and DAPI solutions.

viii. **Mounting medium:** MOWIOL as above describe.

2. U2OS and RKO staining method

U2OS and RKO cells were seeded at a density 5 x 10^4 in 6 multi-well plates containing sterile cover-slip on the bottom of each well and allowed to adhere for 18 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 .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 fluorescence microscope (Powolny *et al*., 2001).

3.10. Statistical analysis

Mean and standard error were determined for all experiments. The Statistical Analysis Duncan 1955 test was used to significant compare between means in this study.

Chapter Four

Results and Discussions

Chapter Four

Results and Discussions

4.1. Qualitative Analysis of *Hymenocrater longiflorus* by HPLC-ESI/MS

The chemical composition of methanolic extract of *Hymenocrater longiflorus* sample, which were collected from the north of Iraq (Kurdistan) was identified by HPLC-ESI/MS analysis. Table 4-1 shows the results of such analysis, in which 11 different compounds were characterized.

Table (4-1): Chemical compounds analyzed by HPLC-ESI/MS under negative mode obtained by LTQ-Orbitrap XL analyzer in *Hymenocrater longiflorus* methanolic extract.

Compound	RT –ve Min	Peak Area -ve	Molecular Formula	Molecular Weight
Isorhamnetin	21.63	27621294	$C_{16}H_{12}O_7$	316.26
Ferulic acid	21.40	35969442	$C_{10}H_{10}O_4$	194.18
Caffeic acid	14.58	53495455	$C_9H_8O_4$	180.0412
Carnosic acid	36.69	60324640	$C_{20}H_{28}O_4$	332.42
Acacetin	32.37	73250463	$C_{16}H_{12}O_5$	284.26
N-Carbobenzyloxy-Cysteinylcysteine	22.90	77731838	$C_{14}H_{18}N_2O_5S_2$	358.43
Apigenin	26.04	106470766	$C_{15}H_{10}O_5$	270.24
Genistein	26.04	106470766	$C_{15}H_{10}O_5$	270.24
Apigenin-7-O-Glucoside	19.44	421579156	$C_{15}H_{10}O_5$	270.24
Rosmarinic acid	19.21	708259070	$C_{18}H_{16}O_8$	360.31
Cirsimaritin	29.66	787837315	$C_{17}H_{14}O_6$	314.29
Total Peak Area (TPA)		246×10^7		

The data presented in table 4-1 were based on information obtained from figure 4-1. The figure illustrates the Total ion current (TIC) chromatograms and retention time (RT min) under negative and positive ion mode obtained by linear quadrupole ion trap-orbitrap (LTQ-Orbitrap XL) analyzer for the investigated *Hymenocrater* extract. Figure 4-2 shows the chemical structure of phenolic compounds identified in *H. longiflorus* extract. These compounds were Cirsimaritin, Rosmarinic acid, Apigenin-7-O-Glucoside Genistein and apiginein were the highest compounds followed by N-Carbobenzyloxy-Cysteinylcysteine, Acacetin, Carnosic acid , Caffeic acid , Ferulic acid and finally Isorhamnetin.

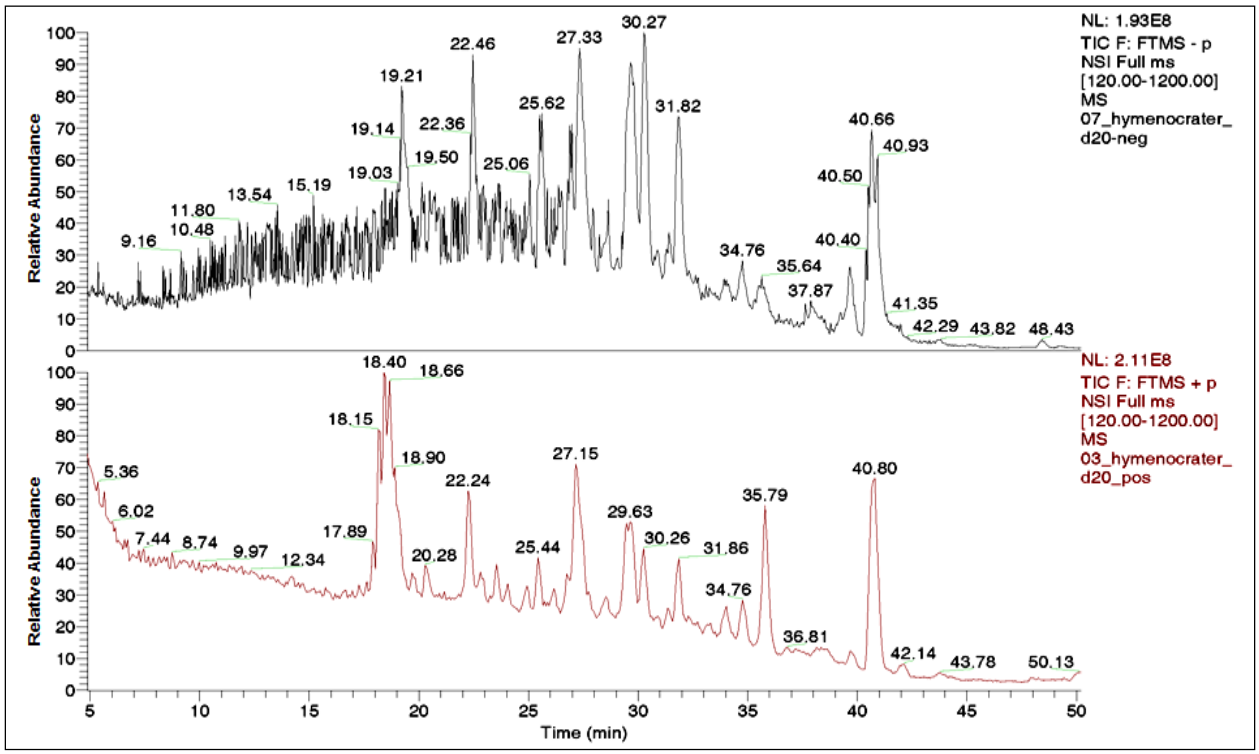
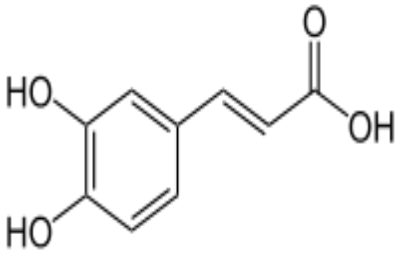
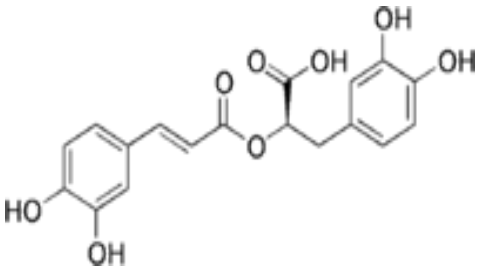


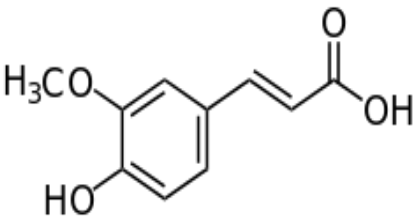
Figure (4-1): Total ion current chromatograms and retention time (RT min) under negative (upper lane) and positive (lower lane) ion modes obtained by LTQ-Orbitrap XL analyzer for *Hymenocrater* extract.



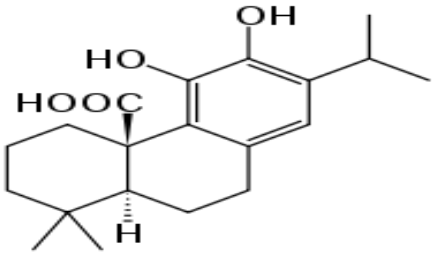
Caffeic acid



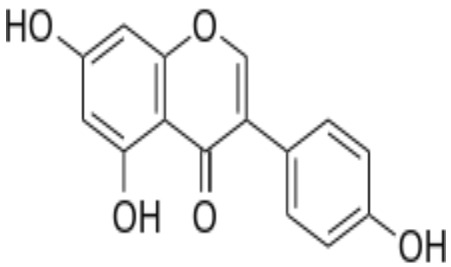
Rosmarinic acid



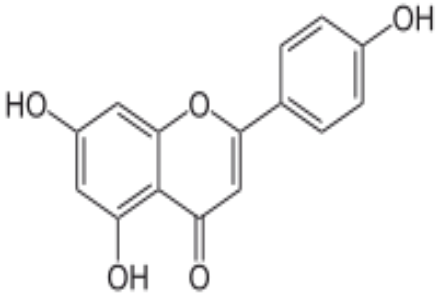
Ferulic acid



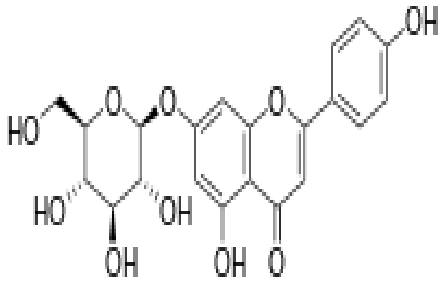
Carnosic acid



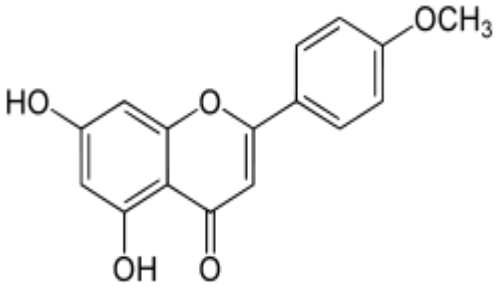
Genistein



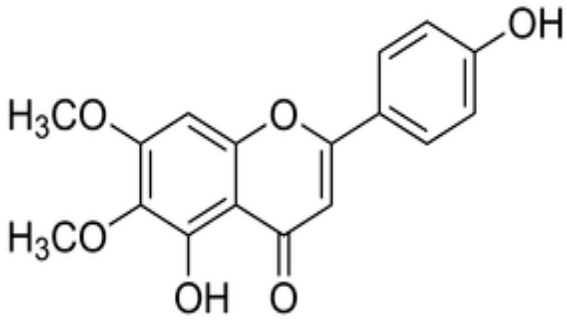
Apigenin



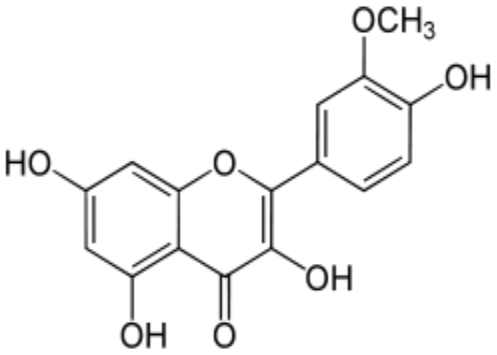
Apigenin -7- 0- glucoside



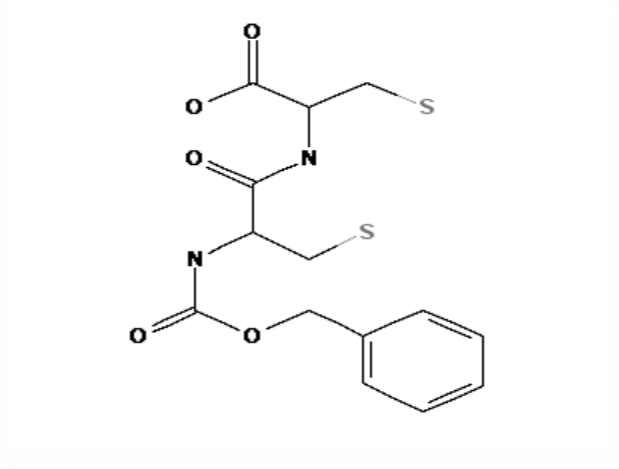
Acacetin



Cirsimaritin



Isorhamnetin



N-Carbobenzyloxy-Cysteinylcysteine

Figure (4-2): Chemical structure of some active compounds of *Hymenocrater longiflorus*.

Secondary metabolites can be classified on the basis of chemical structure (for example, having rings, containing a sugar), composition (containing or nitrogen or not), their solubility in various solvents, or the pathway by which they are synthesized (e.g., phenylpropanoid, which produces tannins). A simple classification includes three main groups: the terpenes (made from mevalonic acid, composed almost entirely of carbon and hydrogen), phenolics (made from simple sugars, containing benzene rings, hydrogen, and oxygen), and nitrogen-containing compounds (extremely diverse, may also contain sulfur) (Lee *et al*., 2004; Liu *et al*., 2004).

Polyphenols have been classified by their source of origin, biological function, and chemical structure. Also, the majority of polyphenols in plants exist as glycosides with different sugar units and acylated sugars at different positions of the polyphenol skeletons. MS-HPLC in this experiment was detected on some Polyphenols that have been classified according to their chemical structures of the aglycones. They are Phenolic Acids and flavinoids. Phenolic acids are non-flavonoid polyphenolic compounds which can be further divided into two main types, benzoic acid and cinnamic acid derivatives based on C1–C6 and C3–C6 backbones, in *H. longiflorus* there are (**Caffeic acid** and **Ferulic acid)**, both of them are hydroxycinnamic acid, polyphenolic diterpene (**Rosmaric acid)** is caffeic acid ester and 3, 4-dihydroxyphenyllactic acid, and (**Carnosic acid),** who is also phenolic compound diterpene. While flavinoids have the C6–C3–C6 general structural backbone in which the two C6 units (Ring A and Ring B) are of phenolic nature, most of the flavonoids are glycosides, which are conjugated with sugars. In this experiment There are flavones (**Apigenin**, **Apigenin 7-O-beta D-glucoside, Acacetin** and **Crisimaritin**), flavonol (**Isorhamnetin**), isoflavones (**Genistein**) as shown in figure (4-2).

A study by (Ahmedi *et al*., 2010), which they mentioned that eighty seven volatile compounds from the essential oil in *H. longiflorus*, were identified by gas chromatography–mass spectrometry (GC–MS). These compounds are mainly monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygenated monoterpenes and oxygenated sesquiterpenoids compounds. In addition the total phenol of essential oil (54.6 ± 1.2), polar sub-fraction (50.0 ± 1.4) and non-polar sub-fraction (64.7 ± 2.0) were determind.

Chemical constituents of the essential oil of *H. incanus* (from Iran), has been studied and the main components reported as 1, 8-cineole, and ß-caryophyllene (Mirza *et al*.2001). Masoudi *et al*., 2012 study on the leaves oil of *H. yazdianus* fifty five component were characterized, representing 95.1% of the total components detected. The major constituents were identified as 1,8-cineole (17.6%), beta-caryophyllene (13.9%), alpha-pinene (10.6%) and caryophyllene oxide (10.4%).

Another study on *Hymenocrater* was done by Barazandeh , 2011 whose study was on *H. elegans* collected from Firoozkooh in northeast Iran. Dried leaves of this plant were steam distilled for 45 min. The yield of the oil produced was 0.1% (based on dry weight). GC and GC/MS analysis of this oil revealed the presence of 33 compounds, among which germacrene D (10.2%), β-caryophyllene (9.7%), α-humulene (9.6%), β-bourbonene (7.1%) and germacrene B (6.9%) were the major constituents.

4.2. Free radical scavenging ability

For measuring free radical scavenging ability, the method used is 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay**.** The stable free radical DPPH method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compounds or plant extracts (Ebrahimzadeh *et al*., 2008). DPPH is commercialized

in the radical form due to its stability. This radical shows a strong absorption maximum at 517 nm (purple). In the presence of antioxidants, the color turns from purple to yellow as shown in Figure 4-3.

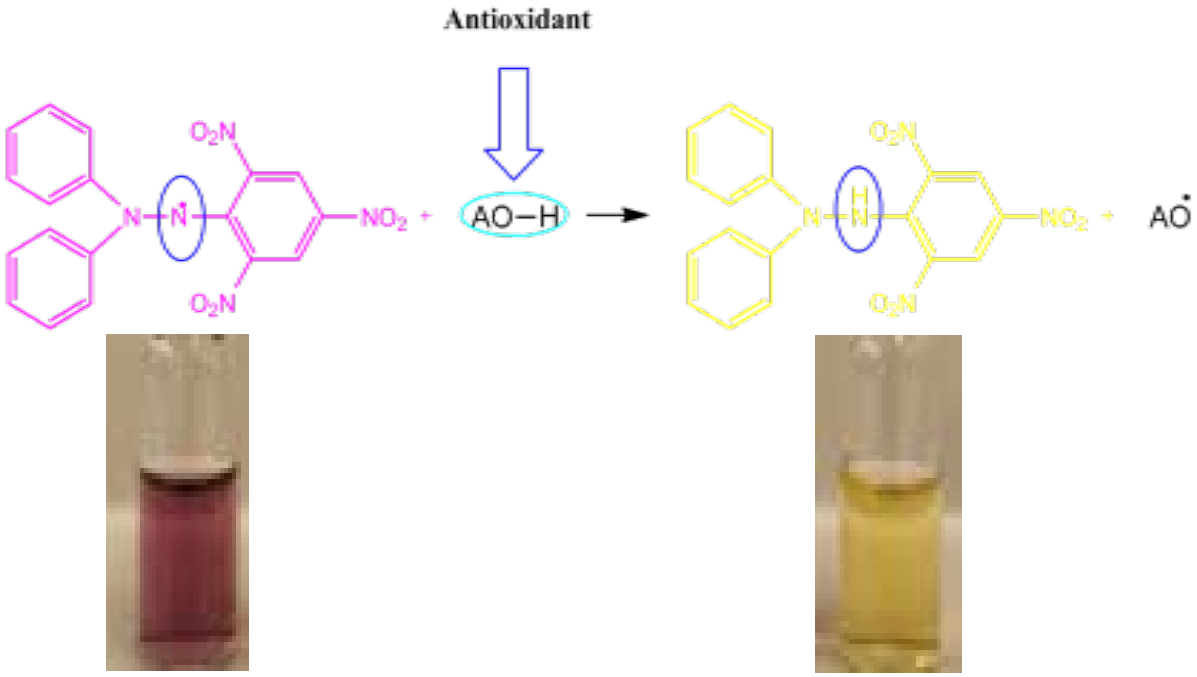


Figure (4-3): Photograph showing changing of DPPH color from purple to yellow after adding the *Hymenocrater longiflorus* extract.

Therefore the sole equipment needed for the assay is a UV-Vis spectrophotometer. Initially, DPPH radical was thought to be reduced to the corresponding hydrazine when it reacted with the donating hydrogen substances. However, more recent studies have shown that what occurs is mainly a fast electron transfer from the sample to DPPH radical. The abstraction of hydrogen from the sample by DPPH radical is marginal, because it occurs very slowly and depends on the hydrogen-bond accepting solvent. Methanol and ethanol, solvents generally used for antioxidant ability assays, are strongly hydrogen bond-accepting; therefore the hydrogen-abstracting reaction occurs very slowly (Miguel, 2010).

In present study, the free radical scavenging effect of *H. longiflorus* extract in DPPH free radical system was determined, and given as a percentage of anti-radical activity (ARA) for six concentrations 10, 20, 40, 100, 500 and 1000 µg mL^{-1} and all these concentrations are read in triplicate. In general, a concentration-dependent ARA was observed, and 1000 µg mL^{-1} was significantly better than the other concentrations which show highest at 86.226% antiradical activity and followed by 83.471%, 78.374%, and finely with concentration 10 µg mL^{-1} which shared approximated 51.51% as shown in (Table 4-2).

Table (4-2) shows that the mean of absorbance start to decrease when the concentration increased and there are significant increase at concentrations 100, 500 and 1000 µg mL^{-1} which shared approximated (0.0523 ± 0.001 C, 0.040 ± 0.002 C and 0.033 ± 0.002 C) , respectively. Such potent anti-oxidant activity seemed to be correlated with the total polyphenolic compounds.

Table (4-2): DPPH free radical scavenging activity on different concentrations of *Hymenocrater longiflorus* extract.

Sample Concentrations	**Mean ± S.E. (Three absorbance reading) Absorbance 517 nm**	**ARA**
DPPH	0.242 ± 0.035 A	0
10 µg/mL	0.1173 ± 0.001 B	51.515
20 µg/mL	0.1043 ± 0.002 B	56.887
40 µg/mL	0.0913 ± 0.004 BC	62.259
100 µg/mL	0.0523 ± 0.001 C	78.374
500 µg/mL	0.040 ± 0.002 C	83.471
1000 µg/mL	0.033 ± 0.002 C	**86.226**

Control absorbance: DPPH = 0.242 ± 0.035 A; ARA**:** Anti-radical activity (%); Different upper letters: significant difference (P≤0.05) between means of absorbance.

The isolated flavonoids from thyme (Lamiaceae family) are tested for their antioxidant scavenging effects on DPPH radical and compared their activity to a quercetin standard used as antioxidant reference. The results obtained are expressed as the percentage of the scavenging activity of DPPH. The result of experiment demonstrated that quercetin possesses high antioxidant activity, luteolin , apigenin and kaempferol have a moderate activity, while the chrysin has low radical scavenging activity (Takeuchi *et al.*, 2007 ; Bazylco and Strzelecka, 2007).

Flavonoids are reported to be the most abundant and most effective anti-oxidant compounds. Our findings suggest that apigenin and the related flavonoids are potentially useful for anti-oxidant and therapeutic treatments of diseases. Ferulic acid, like many natural phenols, is an antioxidant *in vitro* in the sense that it is reactive toward free radicals such as reactive oxygen species (ROS). ROS and free radicals are implicated in DNA damage, cancer, accelerated cell aging (Peng *et al*., 2012). polyphenols are known to have anti-oxidant and radical scavenging properties, thus, they play a role in the prevention against cell- and DNA-damaging diseases like cancer, atherosclerosis, and neurodegenerative ones.

Recently, there has been growing awareness of the importance of a high dietary content of phenolic compounds, such as flavonoids and hydroxycinnamic acids, because of their apparent multiple biological effects, including metal chelation, free-radical scavenging, inhibition of cellular proliferation, modulation of enzymatic activity and signal transduction pathways (Del Rio *et al*., 2013).

Isorhamnetin which is one of the primary active flavonol aglycones of *H. rhamnoides*, was found to show inhibitory activity on Farnesyl Protein Transferase (FPTase) (Oh *et al*., 2005). It has been reported that isorhamnetin has antioxidant activity (Echeverry *et al*., 2004).

Rosmarinic acid found in the extract of *H. longiflorus* may have neural protection effect; *in vitro* study, rosmarinic acid significantly attenuated induced ROS generation and apoptotic cell death of human dopaminergic cell line (Lee *et al*., 2008). Also Genistein exhibits antioxidant properties and has been reported to induce differentiation of numerous cell types (Power *et al*., 2006). Inhibition of glutathione reductase, cytochrome P450, and topoisomerase I DNA relegation may be caused by Acacetin detect in the *Hymenocrater* extract in a study by Doostdar *et al*., 2000.

Generally the lamiaceae family demonstrated antioxidant and anti-inflammatory activity. It shows they have pharmacological properties. So this plant supposed to be a promising one for treatment of diseases such as aging, cancer and atherosclerosis and cardiovascular disease (Ma *et al*., 2011).

4.3 *In vitro* Investigations

4.3.1 Viability of U2OS and RKO tumor cell lines

In this experiment, the cell viability by cell counter was used for U2OS and RKO cell lines. For viability of U2OS cells, four concentrations of *H. longiflorus* methanolic extract were used. The concentrations were 50, 100, 150 and 200 μg mL $^{-1}$ and cells were treated for three incubation periods (24, 48 and 72 hrs) as shown in table (4-3). The result demonstrated that the treatment of cells with *H. longiflorus* extract decreased the viability of U2OS cells; the decrement was in a significant concentration-, as well as, time- dependent manner. Increasing the concentration to 200 μg mL^{-1} at 48 hrs and 150 and 200 μg mL^{-1} at 72 hrs showed no cells growth. In contrast, there was an increase in cell viability for the non treated cells at 24, 48 and 72 hrs (241666 ± 112.62, 393750 ± 254.37 and 575000 ± 432.42), respectively as shown in table (4-3).

The percentage of growth inhibition for U2OS cells was clarified in table (4-4). For non treated cells the percent was 100 % at 24 hrs of incubation. The percentage of inhibition started to decrease at concentrations 50, 100,150 and 200 µg ml^{-1} to reach 82.76 ± 3.62, 20.68 ± 2.04, 17.24 ± 1.58 and 6.89 ± 0.71%, respectively. No growth was observed at concentrations 150 µg mL^{-1} at 72 hrs and 200 µg mL^{-1} for both 48 and 72 hrs.

Table (4-3): Mean of cell number of U2OS cells treated with different concentrations of *H. longiflorus* extract.

Concentrations µg mL^{-1}	**Number of cells (Mean ± S.E)**		
	24hrs	**48hrs**	**72hrs**
Control	**241666 ± 112.6 Ac**	**393750 ± 254.3 Ab**	**575000 ± 432.4 Aa**
50	**200000 ± 450.5 Ba**	**158333 ± 164.7 Bb**	**83333 ± 205.3 Bc**
100	**50000 ± 188.9 Ca**	**16666 ± 136.3 Cb**	**8333 ± 0.5 Cc**
150	**41666 ± 126.4 Da**	**8333 ± 67.4 Db**	**Nil**
200	**16666 ± 90.3 Ea**	**Nil**	**Nil**

Nil: no growth; Different upper case letters: significant difference (P≤0.05) between means of columns; Different lower case letters: significant difference (P≤0.05) between means of rows.

Table (4-4): growth percentage of U2OS cells treated with different concentrations of *H. longiflorus* extract.

Concentrations µg mL^{-1}	**growth percentage (Mean ± S.E)**		
	24hrs	**48hrs**	**72hrs**
Control	**100 ± 0 Aa**	**100 ± 0 Aa**	**100 ± 0 Aa**
50	**82.76 ± 3.62 Ba**	**40.21 ± 2.18 Bb**	**14.49 ± 0.84 Bc**
100	**20.68 ± 2.04 Ca**	**4.23 ± 0.26 Cb**	**1.45 ± 0.07 Cb**
150	**17.24 ± 1.58 CDa**	**2.12 ± 0.06 Cb**	**Nil**
200	**6.89 ± 0.71 Da**	**Nil**	**Nil**

Nil: no growth; Different upper case letters: significant difference (P≤0.05) between means of columns; Different lower case letters: significant difference (P≤0.05) between means of rows.

For RKO cell line, two different concentration systems of extract were performed. In the first, the concentrations were 80 and 100 μg mL^{-1} and the cells were treated for 3 and 6 hrs (table 4-5). While in the second, the concentrations were10, 20, 40 and 80 μg mL^{-1} and the cells were treated for 24 and 48 hrs (table 4-5).

The same effect of inhibition was also observed in these cell lines. The treatment of cells with *H. longiflorus* extract decreased the viability of RKO cells and the decrement was in a significant concentration-, as well as, time- dependent manner. Increasing the concentration to 100 μg mL^{-1} at 3 hrs and 80 or 100 μg mL^{-1} at 6 hrs showed no cells growth. Also no growth was observed when cells were treated with 40 and 80 μg mL^{-1} at 24 hrs or 20, 40 and 80 μg mL^{-1} at 48 hrs. In contrast, there was an increasing in cell viability for the non treated cells at 3, 6, 24 and 48 hrs (33333 ± 237.5, 33333 ± 237.5, 91666 ± 137.4, and 108000 ± 213.9), respectively as shown in table (4-5).

Dependind on the results in table (4-6) showing the percentage of growth inhibition for RKO cells. The non treated cells appeared with the percent 100 % at 3, 6, 24 and 48 hrs of incubation. The percentage of inhibition was decreased at concentrations 80 μg ml^{-1} to reach 24.99 ± 2.16 % at 3 hrs of treatment. No growth was observed at concentrations 80 μg mL^{-1} at 6 hrs or 100 μg mL^{-1} for both 3 and 6 hrs. The same effect was observed at 24 and 48 hrs (table 4-6). The findings of present study showed that RKO cells line more sensitive to *H. longiflorus* extract than U2OS cells.

Table (4-5): Mean of cell number of RKO cells treated with different concentrations of *H. longiflorus* extract.

Concentrations µg mL^{-1}	Number of cells (Mean ± S.E)			
	3hrs	6hrs	24hrs	48hrs
Control	33333 ± 237.5 Ac	33333 ± 237.5 Ac	91666 ± 137.4 Ab	108000 ± 213.9 Aa
10	ND	ND	33333.0 ± 237.5 Ba	16000.0 ± 175.3 Bb
20	ND	ND	8333 ± 102.6 Ca	0.00 ± 0
40	ND	ND	0.00 ± 0	0.00 ± 0
80	8333 ± 102.6 Ba	0.00 ± 0	0.00 ± 0	0.00 ± 0
100	0.00 ± 0	0.00 ± 0	ND	ND

ND: not determined; Different upper letters: significant difference (P≤0.05) between means of columns; Different lower letters: significant difference (P≤0.05) between means of rows.

Table (4-6): growth percentage of RKO cells treated with different concentrations of *H. longiflorus* extract.

Concentrations µg mL^{-1}	Growth percentage (Mean ± S.E)			
	3hrs	6hrs	24hrs	48hrs
Control	100 ± 0.00 Aa	100 ± 0.00 Aa	100 ± 0.00 Aa	100 ± 0.00 Aa
10	ND	ND	36.36 ± 1.68 Ba	14.81 ± 0.93 Bb
20	ND	ND	9.09 ± 0.85 Ca	0.00 ± 0
40	ND	ND	0.00 ± 0	0.00 ± 0
80	24.99 ± 2.16 Bb	0.00 ± 0	0.00 ± 0	0.00 ± 0
100	0.00 ± 0	0.00 ± 0	ND	ND

ND: not determined; Different upper letters: significant difference (P≤0.05) between means of columns; Different lower letters: significant difference (P≤0.05) between means of rows.

In vitro cytotoxicity of *H. longiflorus* extract in both cell lines by MTT method was also performed for conformational test. The MTT assay is a widely used test to measure cell proliferation, cell viability/survival, or drug toxicity (Mosmann , 1983). In the recent studies, the use of MTT has been described as one of the major techniques for testing tumor cell sensetivity to anticancer agents. This assay is based on the ability of live cells to cleave the tetrazoliam ring and convert

it to formazan crystals. However, various concentrations of plant extract (10, 20, 40, 80, 100 and 150 µg mL^{-1}) and times 2, 4, 8, 24, 48 and 72 hrs were examined for U2OS cells. While for RKO cell line the concentrations 10, 20, 40, 80,100,150 and 200µg mL^{-1} at 3 and 6 hours and 10, 20, 40, 80 and 100µg mL^{-1} at 24 hours were examined. The antitumor drug, doxorubicin was used as a positive control for U2OS cell lines.

As shown in figures (4-4) and (4-5), U2OS cell line appeared with no or mild effects after 2 and 4 hrs for (10, 20, 40, 80, 100 and 150) µg mL^{-1}. After 8 hrs the effect was seen at concentration 150 µg mL^{-1} as compared with non treated (- ve control) and doxorubicin (+ ve control) (Figure 4-6). Figures (5- 7; 8 and 9) also showed effects of *H. longiflorus* crude extract at concentrations 80, 100 and 150µg mL^{-1} as compared them with doxorubicin and this appeared clearly. From statistical analysis of these figures there were no significant effect of plant extract at concentration 10 and 20µg mL^{-1}, while at concentration 40, 80, 100 and150µg mL^{-1} showed significant effects.

It is found that organic extracts and fractions of four plants; *Inula viscosa, Ormenis eiriolepis (Asteraceae), Retama monosperma (Fabaceae) and Marrubium vulgare (Lamiaceae)* not exerted a significant activity on cell activity of U2OS cell line at the tested concentrations, except *Retama monosperma* (42, 3%). This could be explained by the fact that U2OS cell line is chromosomally highly altered (Belayachi *et al*., 2013). The same conditions also done for PC-3 Prostate cancer cell line, cells which were exposed to the same conditions results that only *Inula viscosa* (1,2%) and *Ormenis eiriolepis* (8,2%) were active against PC- 3 cells, although they both induced a dramatic effect on these cells.

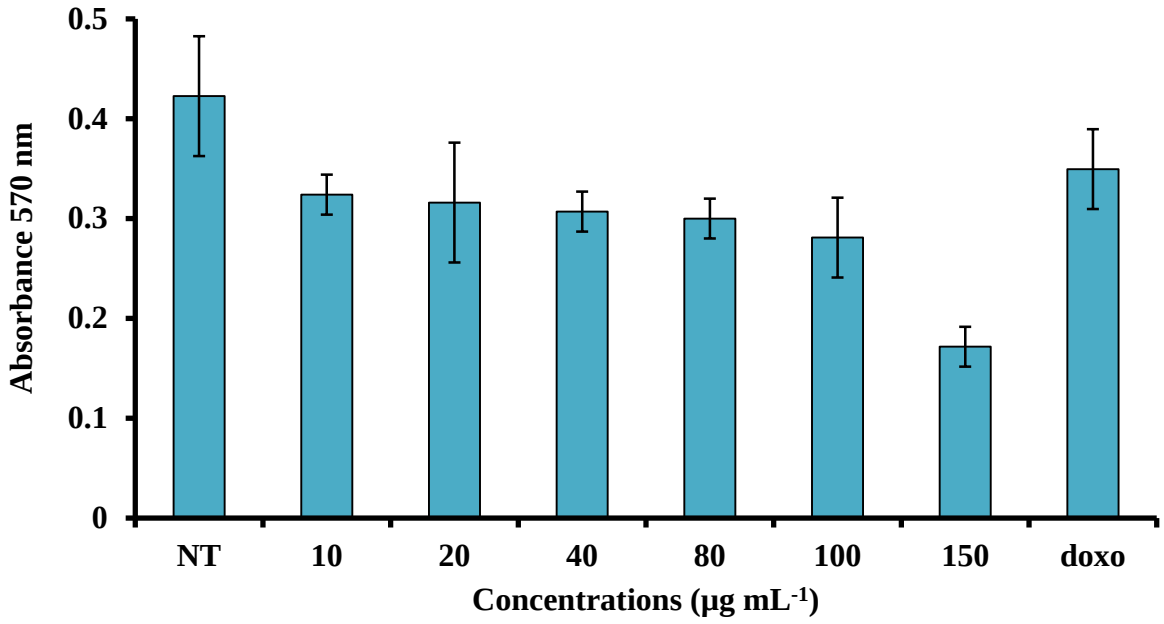


Figure (4-4): MTT results of U2OS cells at 2 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated; doxo: doxorubicin).

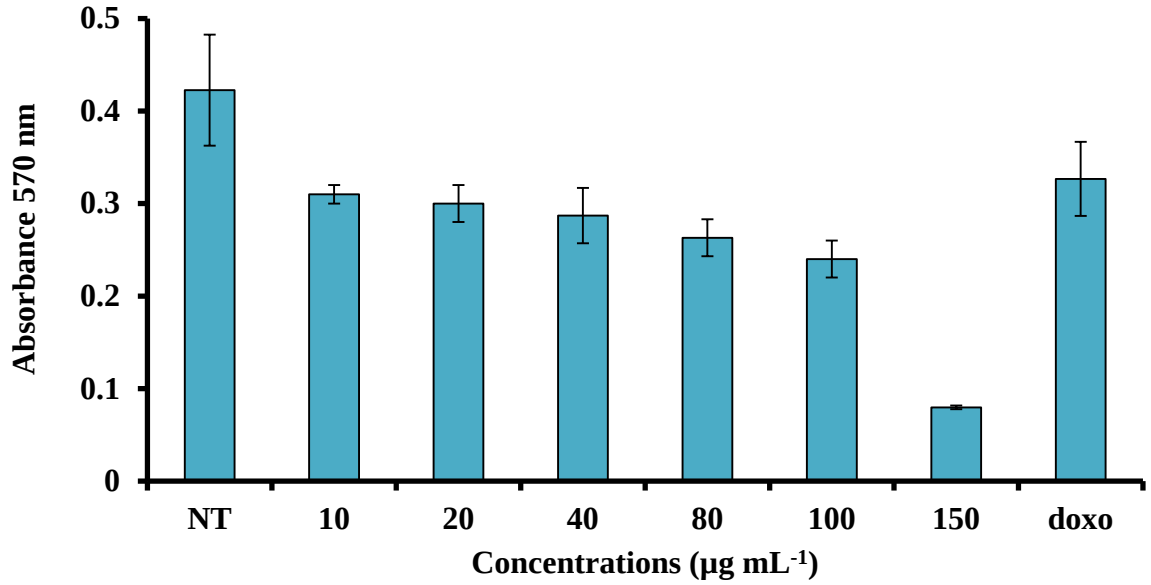


Figure (4-5): MTT results of U2OS cells at 4 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated; doxo: doxorubicin).

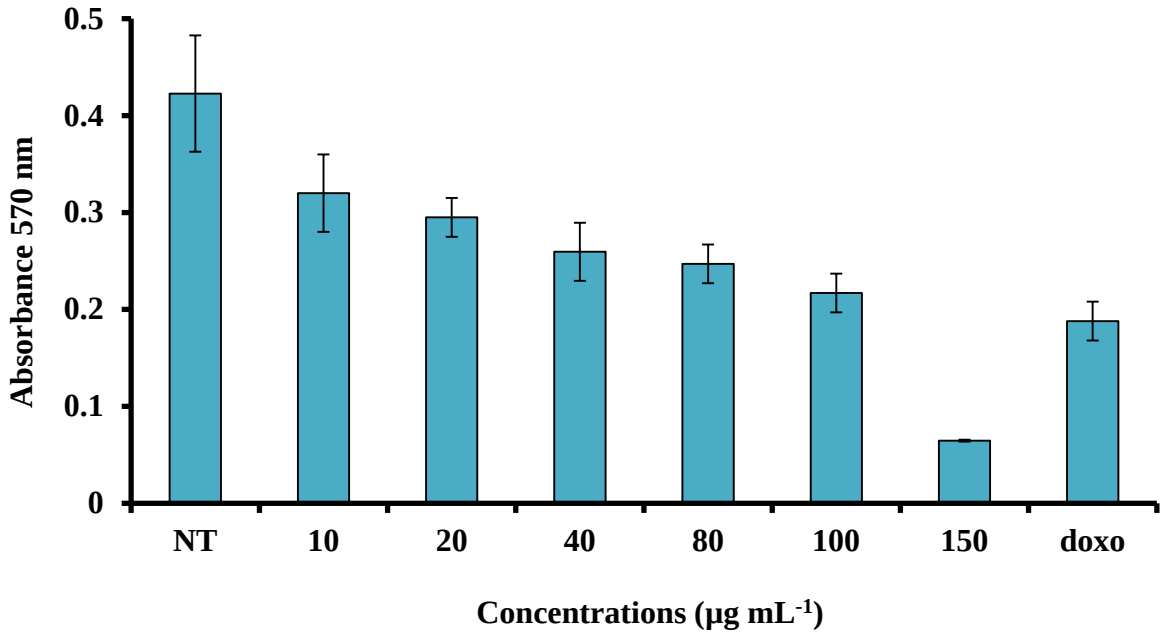


Figure (4-6): MTT results of U2OS cells at 8 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated; doxo: doxorubicin).

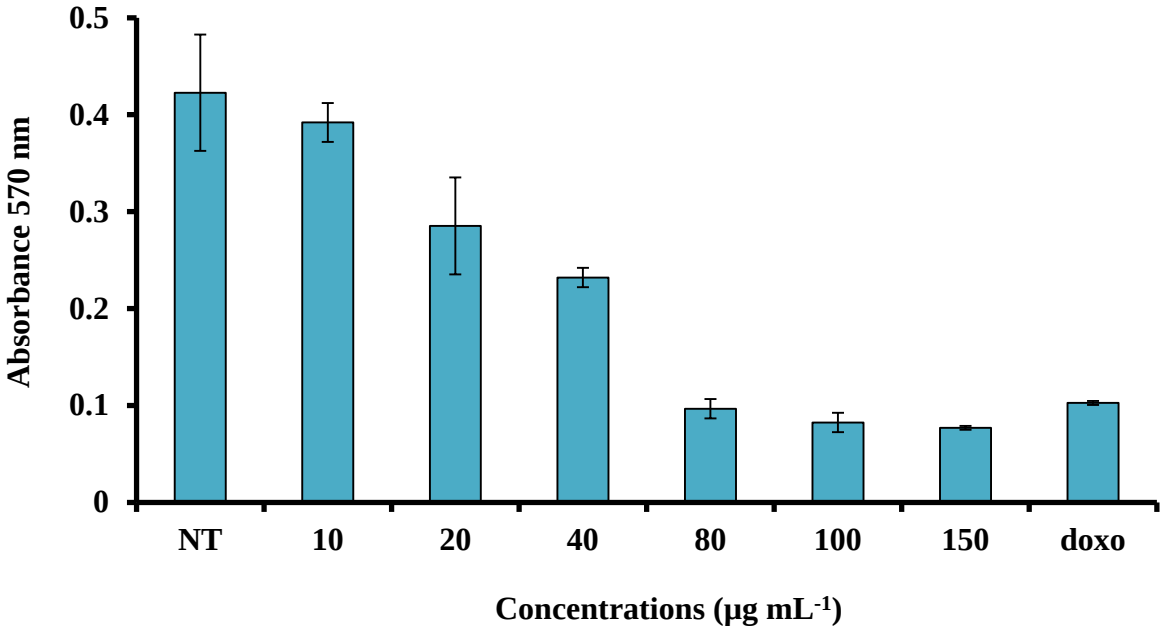


Figure (4-7): MTT results of U2OS cells at 24 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated; doxo: doxorubicin).

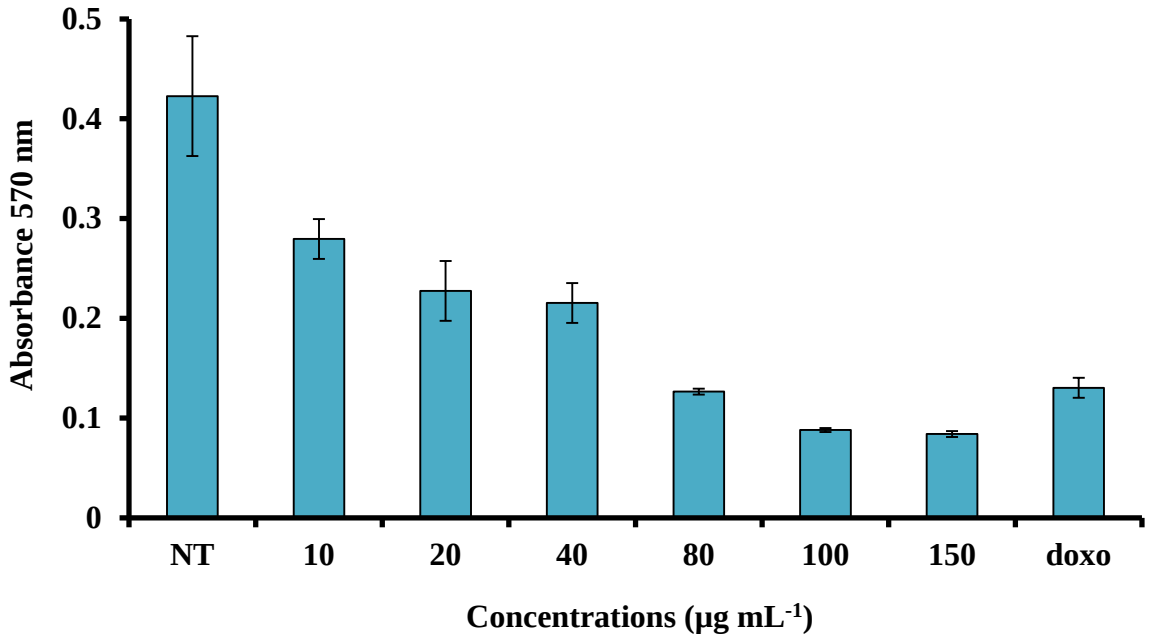


Figure (4-8): MTT results of U2OS cells at 48 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated; doxo: doxorubicin).

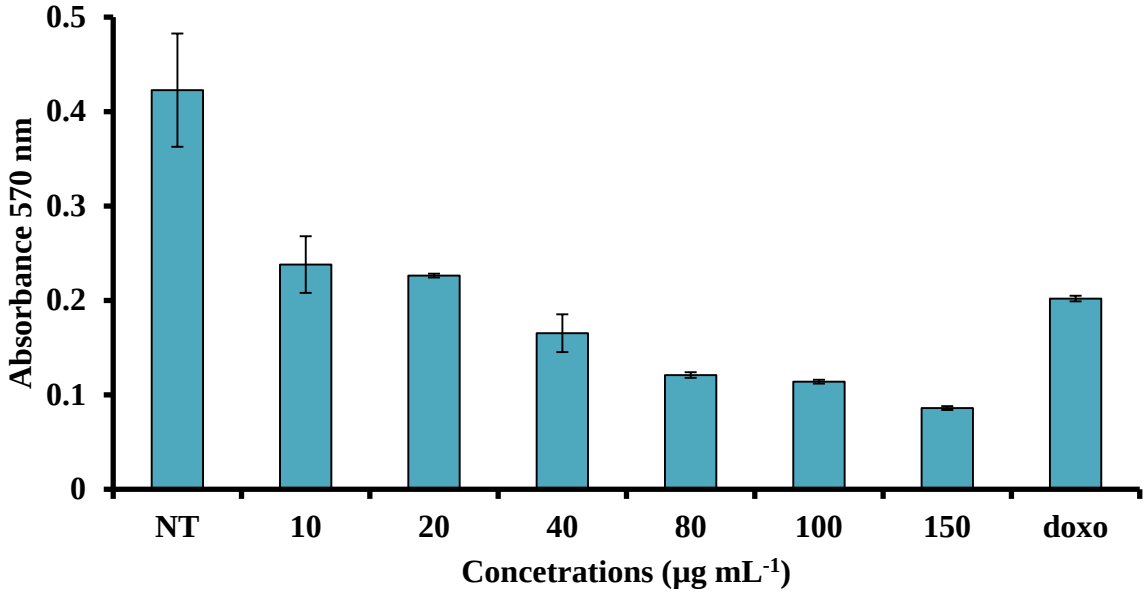


Figure (4-9): MTT results of U2OS cells at 72 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated; doxo: doxorubicin).

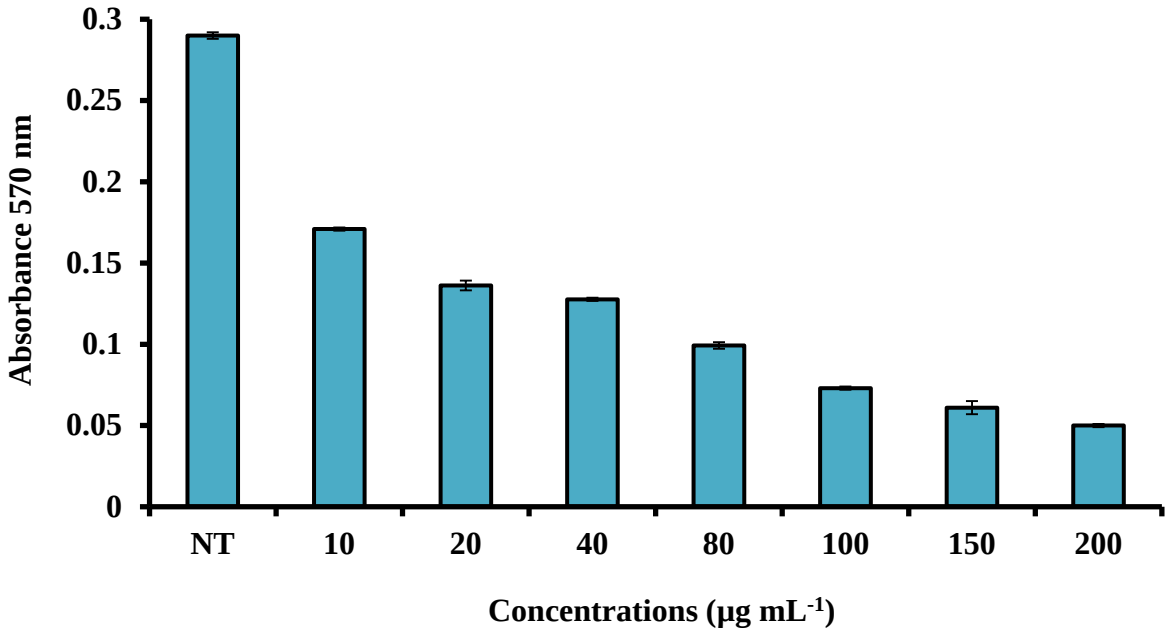


Figure (4-10): MTT results of RKO at 3 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated).

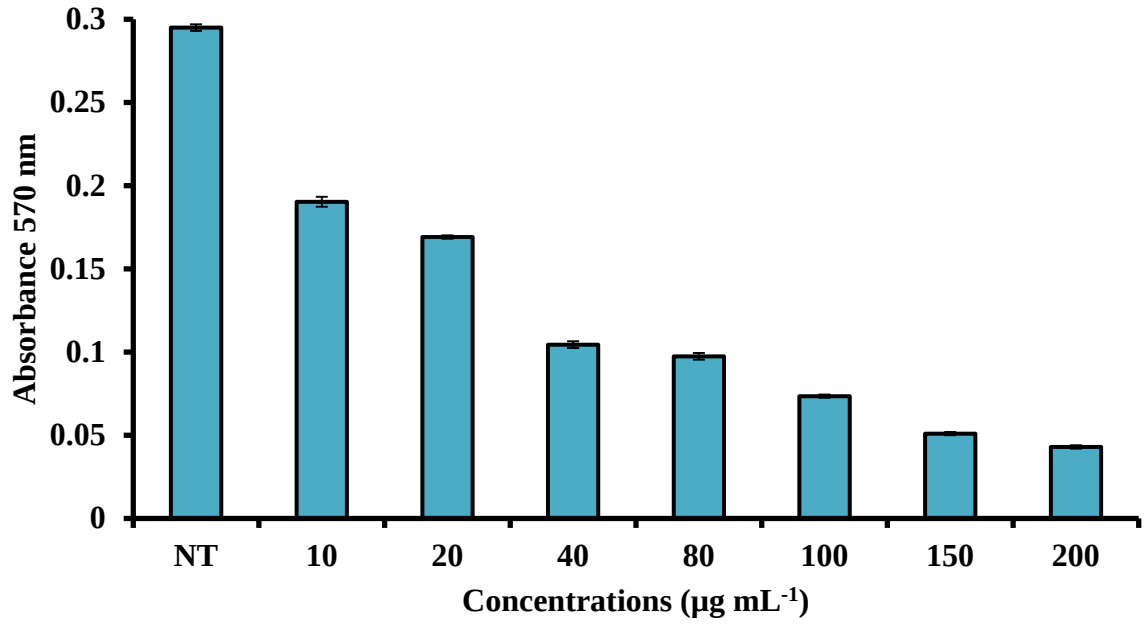


Figure (4-11): MTT results of RKO at 6 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated).

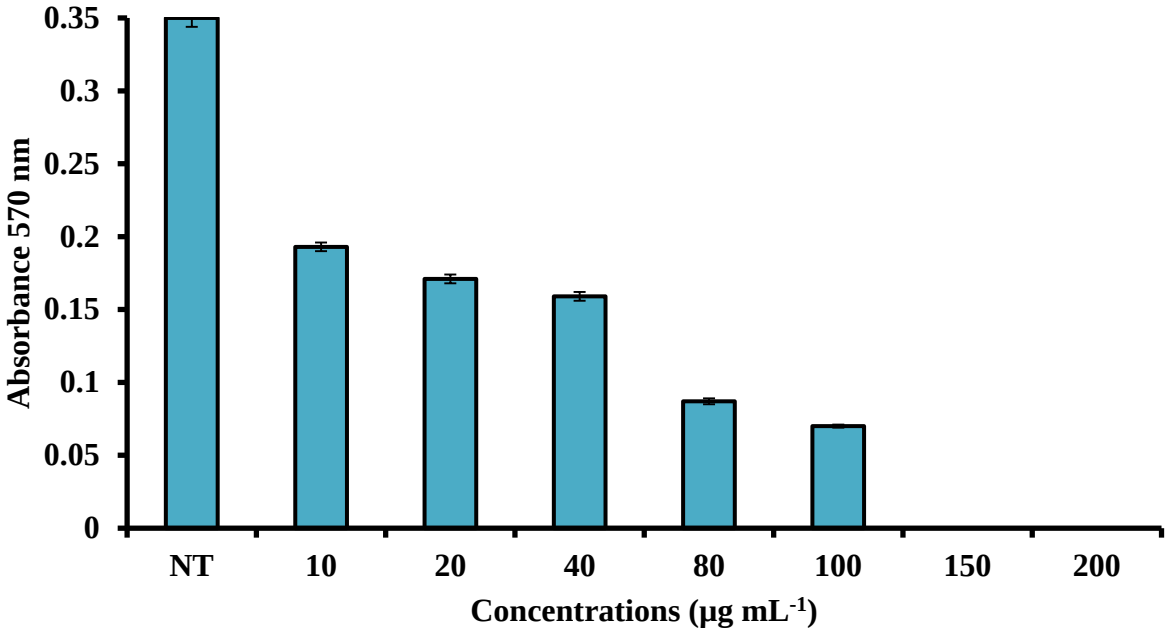


Figure (4-12): MTT results of RKO at 24 hours of treatment with different concentrations of *H. longiflorus* extract. (NT: Non treated).

The Cytotoxicity of *H. longiflorus* extract was belong to it is active compounds like polyphenols which were detected by HPLC-ESI/MS analysis and also seemed correlated with the antioxidant activity of plant extract against DPPH. In the present study, it was observed that RKO colon cancer cell line was more sensitive to *H. longiflorus* extract than U2OS cells during 3, 6 and 24 hrs (figure 4-10;11 and 12).

Therefore, it is possible to suggest that the chemical constituents of *H. longiflorus*, especially phenolic acids and phenols including flavonoids, may have a selective cytotoxic effect against cells, and such effect is determined by the type of cells under investigation. 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).

Nowadays, flavonoids are considered potentially important constituents of the human diet for the chemoprevention of cancer because of their biological activities at the cell level, and several hypotheses have been proposed to explain their antitumoral activities. These include their potential cytotoxic activity, the inhibition of cell proliferation, and their effect on cell differentiation and angiogenesis processes. Various mechanisms have been proposed to explain these hypotheses in different murine and human melanoma lines, such as their anti-invasive and antimetastasic potential, the induction of apoptosis by negative retroregulation of the protein Bcl-2 or inhibition of the transmembrane transport of glucose and the promotion of Bax expression in both cases , the inhibition of the kinases CDK1 and CDK2 by the positive retroregulation of inhibitors of p27 and p21, or phosphorylation of the residues Tyr 15 of kinase (Hui *et al*.,2012 ; Bertero *et al*., 2013).

It is found that rosmarinic acid, which is widely distributed in Lamiaceae herbs, inhibited both PDGF and TNF-α induced mesangial cell proliferation. Since rosmarinic acid inhibited mesangial cell proliferation induced by different mitogens, the inhibitory effects may involve mechanisms independent of the mitogens and their trans-receptor signalling modes. Cell proliferation is a complex phenomenon involving the interaction of growth factors with cell membranes, the successive phosphorylation of various proteins in the cytoplasm, and the regulation of the cell cycle at the nuclear level (Erpel and Courtneidge, 1995).

Different in vitro studies suggest that ferulic acid may have direct antitumor activity against breast cancer (Bouftira *et al*., 2012) and liver cancer (Gelinas and Mckinnon, 2006). Phenolics such as ferulic, caffeic, gallic acids and curcumin were tested for their potential anti proliferative and cytotoxic properties in human breast cancer cell line (MCF-7) as well as on a spontaneous mammary adenocarcinoma tumor. As a single agent, caffeic acid showed substantial growth inhibitory activity (Barkume *et al*., 2003).

4.3.2 Fluorescence Activated Cell Sorter (FACS) analysis

To determine whether the *H. longiflorus* induced growth delay via an arrest in any specific cell cycle phases. The cell cycle histograms for U2OS osteosarcoma cell line were performed by FACS analysis at 3 and 6 hrs with 50 or 100 µg mL^{-1} of plant extract as shown in figures (4-13; 4-14).

In cells treated with 50 µg mL^{-1}/3 hrs, 50 µg mL^{-1}/6 hrs, 100 µg mL^{-1}/3 hrs and 100 µg mL^{-1}/6 hrs, a mild arrest of cells in G2 phase was observed (45.95%, 47.02 %, 49.36 % and 46.35 %, respectively) as compared with non treated cell (44.70 %). Many anticancer agents from plants that have been prescribed for treating malignancies nowadays inhibit cancer cell growth through cell cycle regulation including the G2/M accumulation. It is well known that agents affect the G2/M phase cell cycle arrest interact by targeting tubulin protein or disrupting the tubulin-microtubule equilibrium (Hadfield et al., 2003). According to our results, *H. longiflorus* cause G2 cell cycle arrest, thus it should interact with tubulin to the same extent as the plant - derived chemotherapeutic agents. However, the antiproliferative activities of this plant might be possibly dependent on cell types.

The mild activity of *H. longiflorus* on U2OS cell line may be attributed to the crude extract of this plant which contained many compounds like phenolic acid

(caffeic, ferulic and rosmaric acid), phenolic diterpenes (carnosic acid), flavonol (isorhamnetin), isoflavones (genistein) in spite of flavone compound (apigenin).

Most flavonoids studied to date have been shown to target the cell cycle. Flavonoid treatment results in a wide range of cell cycle alterations including inhibition of cyclin-dependent kinases and cyclins, or up-regulation of cdk-inhibitors of the cip/kip family. Alterations in the regulators of G1 to S transition including retinoblastoma (RB) and E2 factor (E2F) proteins have also been demonstrated. Flavonoids have also been shown to regulate cell cycle checkpoint pathways, in particular the DNA damage response pathway (Haddad, 2008).

The S phase checkpoint insures proper replication of DNA and that the genetic material has been duplicated only once per cycle before cell division. In addition, this checkpoint monitors correct duplication of the centrosomes. After progression to G2, the cells can still assess the condition of the replicated DNA and halt the cycle if required. Lastly, the mitosis checkpoint, also referred to as spindle point checkpoint, monitors the attachment of chromosomes to the mitotic spindle. Any negative signal from an unattached kinetochore blocks progression to anaphase and delays the cycle (Gassmann *et al*., 2010).

In contrast, the present study also recorded an arrest in G1-phase of RKO colon cancer cell line at 40 µg mL^{-1}/3hrs, 40 µg mL^{-1}/6hrs, 80 µg mL^{-1}/3hrs and 80 µg mL^{-1}/6hrs (29.07%, 34.54 %, 38.00 % and 56.10 %, respectively) as compared with non treated cells (26.69 %)(figures 4-15; 4-16). In this study the comparison between U2OS and RKO cell line shows that U2OS was more resistant than RKO to *H. longiflorus* extract and this consistent with cells viability and MTT results.

Therefore, it is possible to suggest that *H. longiflorus* extract may show different effects against different cell lines. Thus, prediction of the type of cell cycle arrest by *H. longiflorus* or its phenolic compounds is difficult, and probably

depends on several factors, and further studies against different cell lines are needed.

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).

Until now there is no report to ensure this result because this is the first study on this plant, only effects as antioxidant and antimicrobial was studied (Ahmadi *et al*., 2010), as mentioned before this extract was crude not purified and contain many compounds differ from others in mechanisms and mode of actions.

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 (Liu *et al*., 2013). Further studies are underway to explore the molecular mechanisms of the novel flavonoids identified in this study, and to determine their properties *in vivo*.

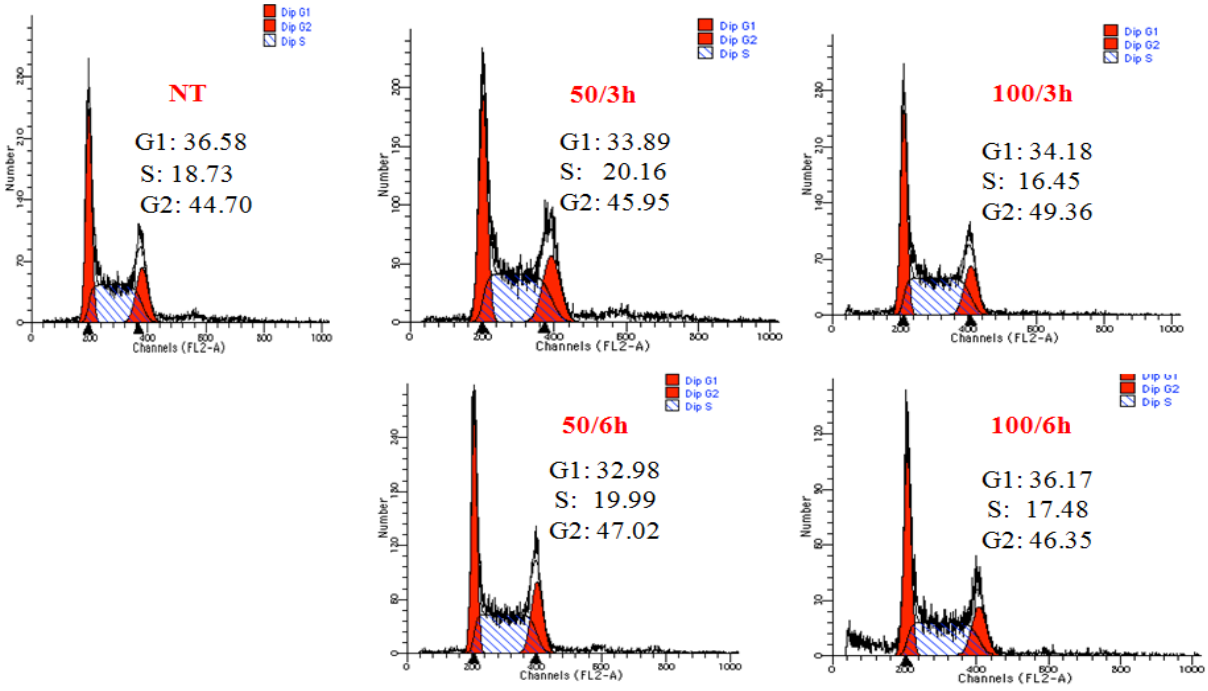


Figure (4-13): Cell cycle histogram for U2OS cell line performed by FACS analysis. (NT: Non treated).

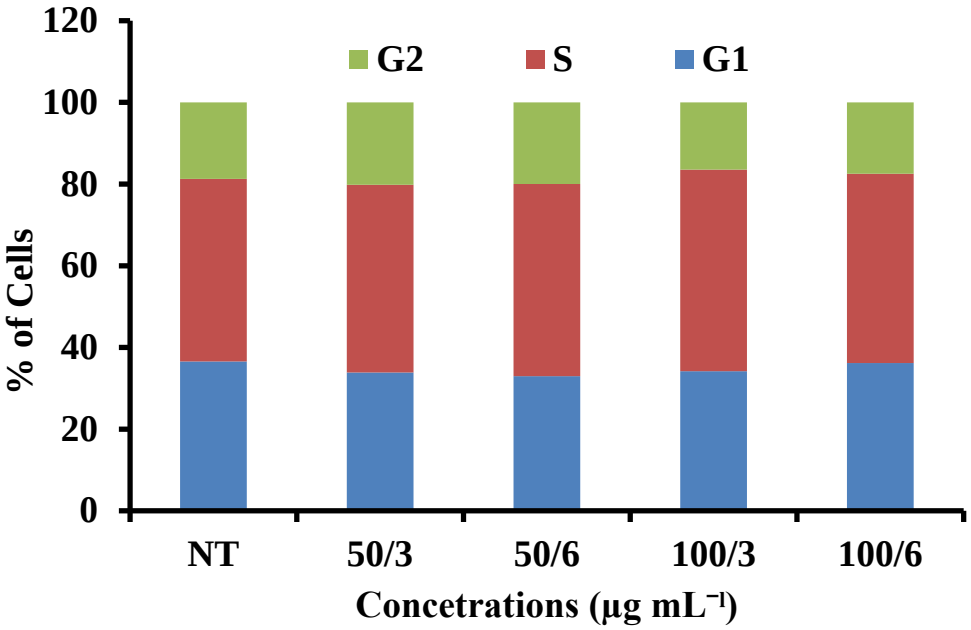


Figure (4-14): Cell cycle for U2OS cell line at concentration 50 and 100 µg mL^{-1} at 3 and 6hrs. (NT: Non treated).

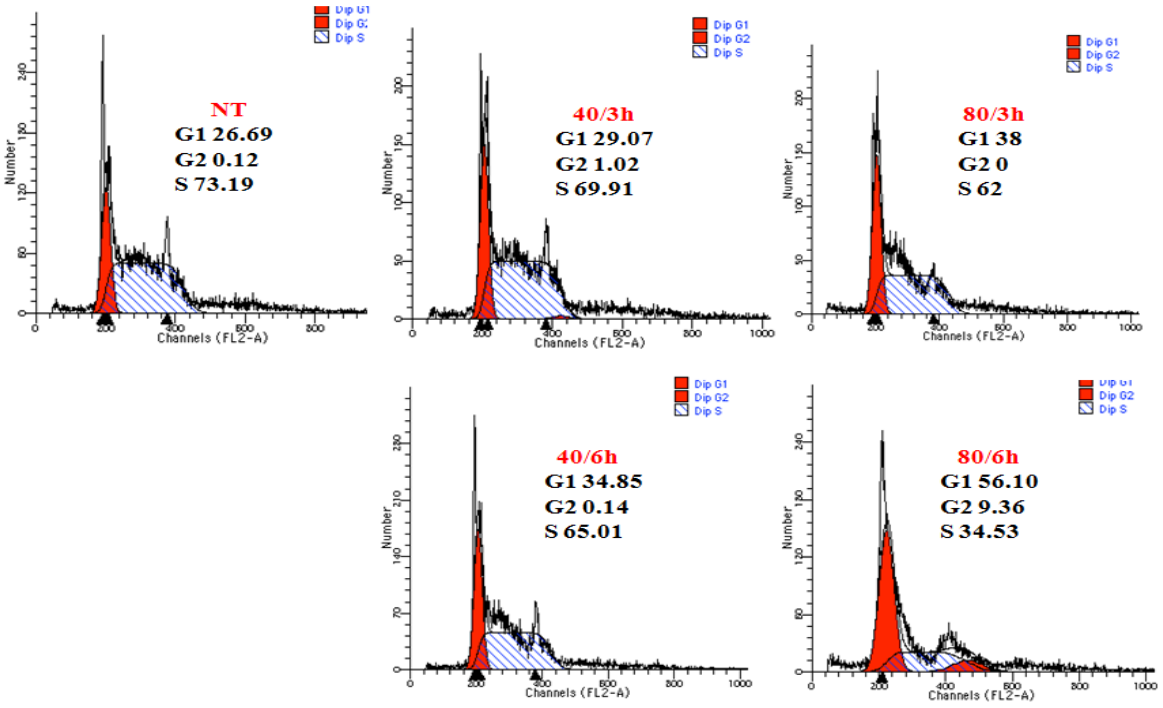


Figure (4-15): Cell cycle histogram for RKO cell line performed by FACS analysis. (NT: Non treated).

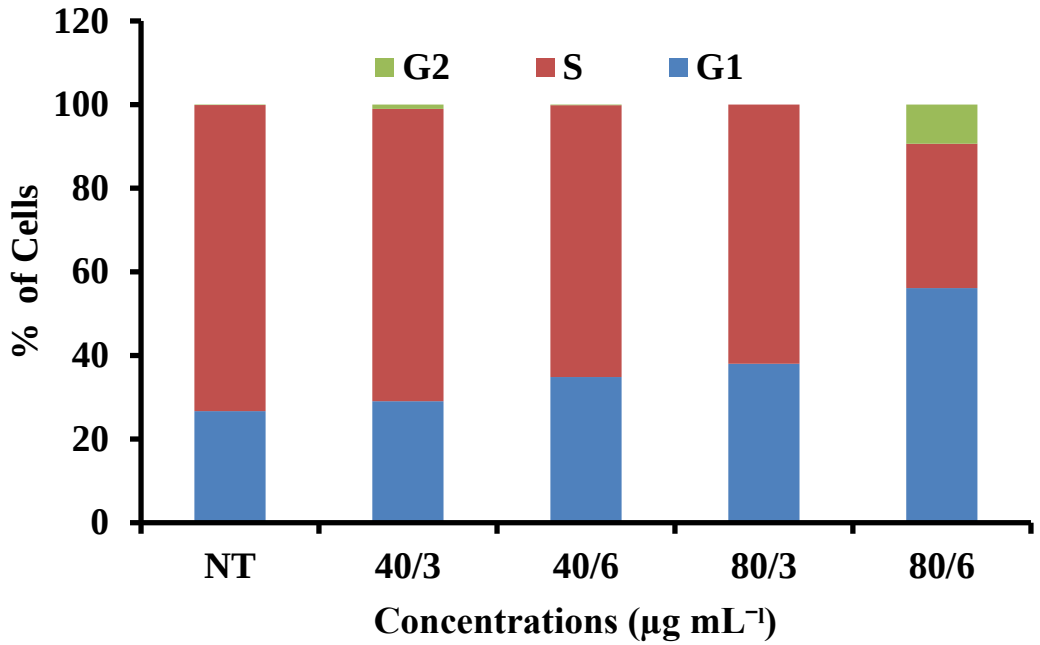


Figure (4- 16): Cell cycle for RKO cell line at concentration 40 and 80 µg mL^{-1} at 3 and 6 hrs. (NT: Non treated).

4.3.3 Immunoflouresence staining for γ-H2AX

Depending on the results of evaluating the effects of *H. longiflorus* sample on H2AX phosphorylation (γ-H2AX), this task has not been targeted in *Hymenocrater* worldwide. As shown in figure 4-17, the result of U2OS cell line treated with *H. longiflorus* at both concentrations (50 and 80µg mL^{-1}) used in present study, ultra violet and Camptothecine (CPT) (positive control) showed accumulation of damage foci as compared with non treated cells (negative control). The same effects were observed with RKO cell line at concentrations 80 and 100 µg mL^{-1} (Fig. 4-18) which showed also accumulation of damage foci.

DNA damage is a potential end point that might predict tumor cell survival after cytotoxins are administered *in vitro*. Several factors known to be involved in DNA repair and signaling the presence of damage have been shown to accumulate in large nuclear domains (foci) after DNA double-strand breakage. This response is not yet fully understood but has been suggested to be a visual indication of DNA repair centres. Interestingly, several studies have demonstrated that H2AX S139 phosphorylation is important for foci formation under a vast range of conditions where DSBs are formed (Furuta *et al*., 2003).

Induction of DNA double-strand breaks causes phosphorylation of histone H2AX at serine 139 (Bhatia *et al*., 2013), and the resulting regions covering megabases flanking each DNA double strand break appear as spots or foci when examined microscopically after antibody staining. Those foci slowly resolve as DNA repair proceeds, yet residual foci can be observed hours to days after treatment and may represent sites of incomplete repair associated with drug/radiation sensitivity. In addition to the classic double-strand break inducing agents such as ionizing radiation, bleomycin, and neocarcinostatin, serine 139 γ-H2AX is also formed after exposure to UV, camptothecin,

methylmethanesulfonate, high-dose hydrogen peroxide, doxorubicin, etoposide, and 3-amino-1,2,4-benzotriazine-1,3-dioxide (tirapazamine) (Banath and Olive,2003; Liu *et al*., 2003).

Following H2AXS139 phosphorylation, the product of the *BRCA1* tumor suppressor gene and later, the Rad50 and Rad51 repair factors colocalize with phospho-H2AX foci (Jha *et al*., 2013). DNA double strand breaks (DSBs) generated by either a laser scissors apparatus or a 137Cs source of IR, show that the initial pattern of phospho- H2AX molecules formed in the nucleus corresponded to Brca1, Rad50 and Rad51 IR (ionizing radiation) induced foci (IRIF), which appear subsequently in the recovery. Interference with H2AX phosphorylation by the use of the PIKK inhibitor wortmannin or the use of a kinase defective cell line (DNA-PK absent and ATM at extremely low levels), inhibits the initiation of focus formation (Singh *et al*., 2013), proving further evidence for the role of H2AX phosphorylation in the process.

H2AX works with mediator of DNA damage checkpoint protein 1 (MDC1) to promote the recruitment of repair proteins to the sites of DNA breaks, besides controlling the damage induced cell-cycle arrest checkpoints (Goldberg *et al*.,2003). H2AX is also important for both MDC1 and 53BP1 damage-induced phosphorylation; and peptides representing the C-tail of H2AX specifically recruit MDC1 and 53BP1 proteins in a phosphorylation-dependent manner. Interestingly, depleting cells of MDC1 protein by siRNA, significantly affected H2AX phosphorylation and phospho-H2AX foci formation, after exposure to both IR and UV (Wang *et al*., 2014). Therefore, in response to DNA damage, MDC1 and H2AX appear to form a complex at sites of DNA lesions and are phosphorylated in a mutually dependent fashion. Surprisingly, H2AX is actually dispensable for the initial recruitment of DNA repair factors to sites of DSB (Celeste *et al*., 2003).

H2AX is important, however, for the retention and the subsequent increase in the concentration of repair factors at sites of DNA damage, which can be visualized as IRIF. Although the physiological function of IRIF is not clear, the concentration of DNA DSB repair and signaling factors in the vicinity of a DSB is thought to facilitate DNA repair and amplify the damage induced checkpoint signal (Jackson and Bartek , 2009).

γ-H2AX forms before the appearance of internucleosomal DNA fragments and the externalization of phosphatidylserine to the outer membrane leaflet. γ-H2AX formation is inhibited by N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone and the inhibitor of caspase-activated DNase, and it is induced when DNase I and restriction enzymes are introduced into cells, suggesting that any apoptotic endonuclease is sufficient to induce γ-H2AX formation. These results indicate that γ-H2AX formation is an early chromatin modification following initiation of DNA fragmentation during apoptosis (Rogakou *et al*., 2000). Preliminary support for apoptosis possibility is shown in Figures (4-17 and 4-18) the undergoing apoptosis cells revealed morphology changes to circular shapes and this may lead to cell shrinkage, chromatide condensations then to apoptosis.

There are suggestions for γ -H2AX studies, like examine the changes in 53BP1 and RAP80 ionizing radiation induced foci (IRIF) besides controlling the damage induced cell-cycle arrest checkpoints, as these are factors that restrict resection. another study the depleting cells of MDC1 protein by siRNA which affected H2AX phosphorylation and phospho-H2AX foci formation, after exposure to both IR and UV and may other suggestions as mentioned above.

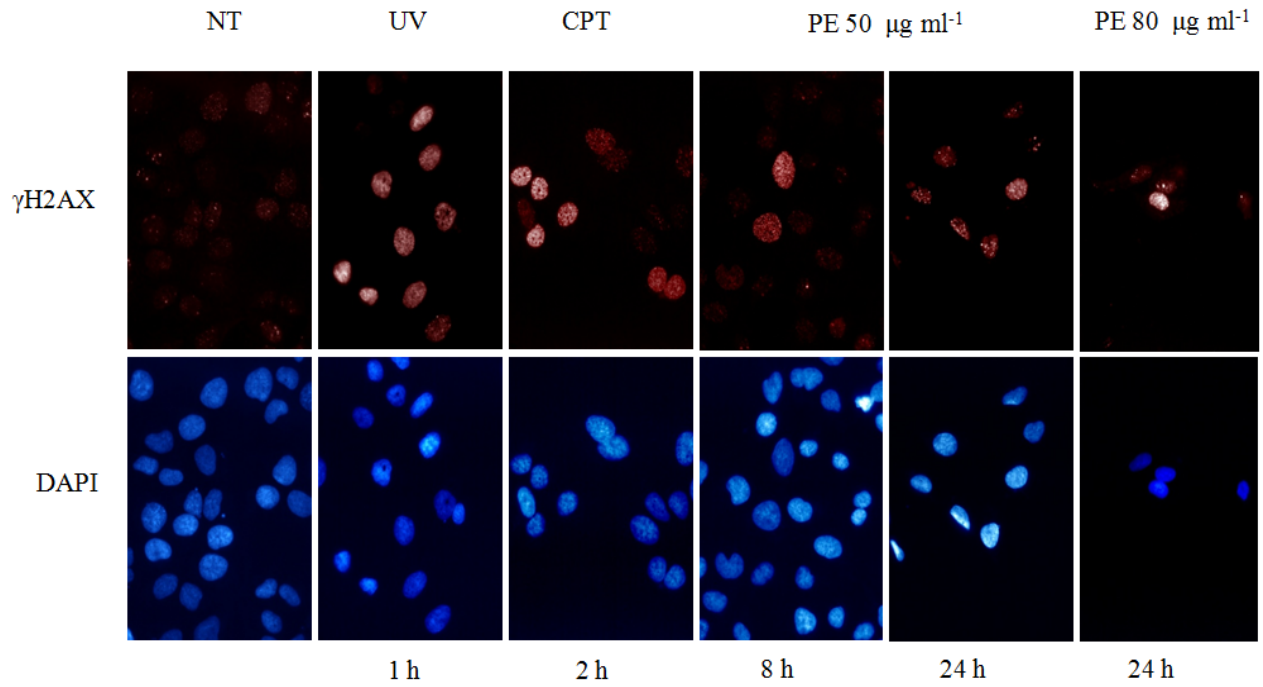


Figure (4-17): Immunoflourescence staining for γ-H2AX of U2OS cell line.

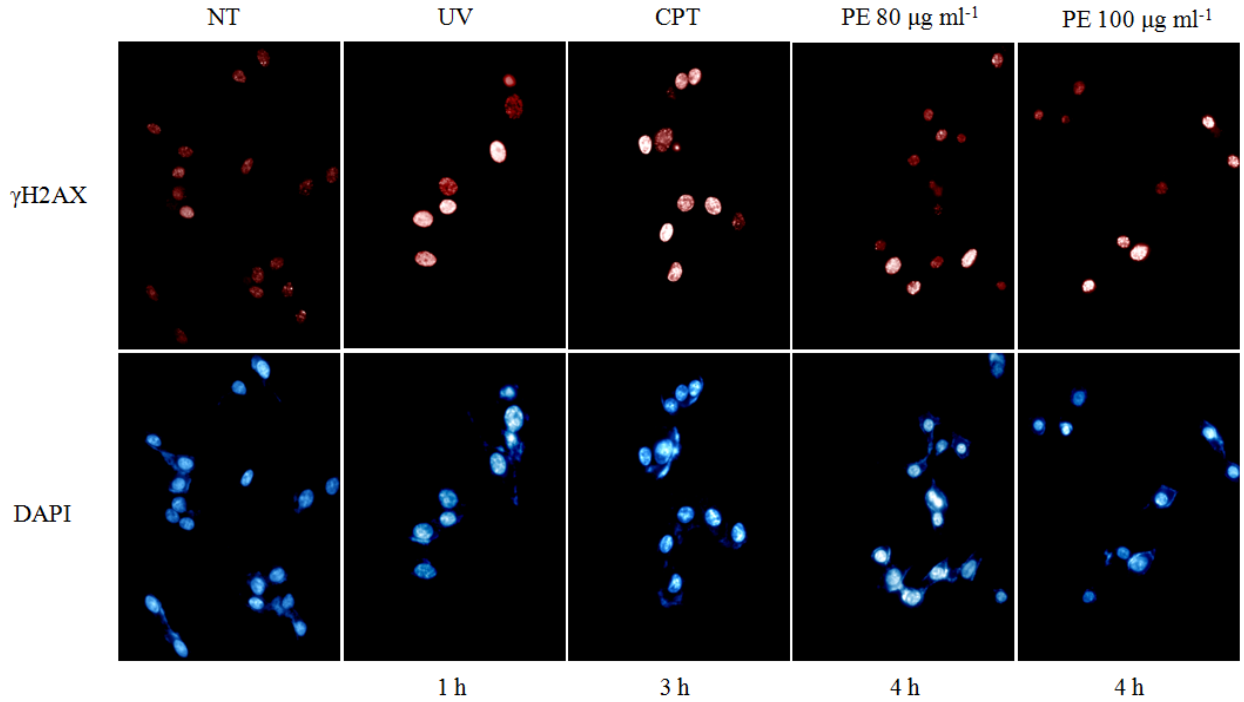


Figure (4-18): Immunoflourescence staining for γ-H2AX of RKO cell line.

4.3.4 Assessment of hallmarks and nuclear morphologies

When all repair mechanisms fail or damage is too severe, the cell will initiate a suicidal program termed apoptosis. Apoptotic cells show distinct morphological alterations such as cellular rounding, shrinkage, autophagy, blebbing, chromatin condensation and nuclear fragmentation. Apoptosis is a form of controlled self-destruction, in which a set of cutting enzymes, also known as caspases are sequentially activated (Nagata *et al*., 2003). As shown in figure (4-19), the results revealed changing in cellular morphology with the concentrations 50 and 100μg mL^{-1} as compared with non treated cells (negative control), indicative of increased apoptosis. However, the same result obtained from RKO cell line (result not shown).

Willingham (1999) noted that cellular morphology changes in cells which undergo apoptosis are: 1) a loss of adhesion to substratum, resulting in cell rounding; 2) shrinkage of the cells and 3) cell blebbing. Shrinkage of the cells (decreased in size) was one of the morphological changes mentioned match with our cells morphological results. Typical nuclear changes found in pre-apoptotic cells include increased chromatin condensation and nuclear deformation. By visualizing chromatin using a DNA intercalating dye (DAPI), we found a significant increase of aberrant nuclei in cell cultures.

Various mechanisms have been proposed to account for the antioxidant ability of phenolic compounds, which include their radical scavenging ability, metal chelating property and hydrogen donating ability. A correlation between the antioxidant property and radioprotection by phenolic compounds was first proposed by Shimoi *et al*., 1994. In principle, plant metabolites delay the oxidation of bio-molecules by inhibiting the initiation and propagation of oxidizing chain reactions, thereby interfering with the initiation of apoptosis.

Polyphenols are contained in fruits, vegetables, seeds and drinks and are classified as stilbenes, flavonoids, tannins, phenolic acids and their analogues, lignans and others depending on their chemical structures (Huang *et al*., 2010).

These polyphenolic compounds that taken by diet show their effects through similar or different molecules in the cells. As a result of these effects, they may lead to inhibition of telomerase activity while inducing apoptosis in cancer cells (Avci *et al*., 2011 and Cosan *et al*., 2011). The possible explanation of such better effects is their richness in polyphenols, which were significantly higher in this sample. We have been found that polyphenols interacts with a variety of proteins and modifies their expression and activity, and is thus a good candidate for preventive agents and/or therapeutic agents of cancer. And this may be one reason for apoptosis in U2OS and RKO cell lines. Until today there is no result on effect of *H. longiflorus* on RKO and U2OS or even on any cancer cell line this is the first study on *H. longiflorus* in these cell lines, and further studies against different cell lines are also needed.

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, multiple molecular pathways may be involved. These include pro-and anti-apoptotic Bcl-2 family proteins, mitochondrial membrane potential, mitochondrial cytochrome C and caspase-3 (Song *et al*., 2009).

Caffeic acid, one of the phenolic constituents of honey, inhibited the colon cancer cell proliferation in a dose-dependent manner. Caffeic acid treatment resulted in increasing accumulation of cells at sub-G1 phase of cell cycle indicating apoptosis. Induction of apoptosis was accompanied by increased ROS generation as indicated by DCFDH-DA staining. Further mitochondrial membrane potential fall was also observed in the treated cells. Dose- and time-dependent staining by Yo-pro-1 demonstrated increasing accumulation of apoptotic cells after

caffeic acid treatment. Photomicrograph images depicted the membrane blebbing and shrinkage of treated cells. Hence, caffeic acid can be considered as a potential candidate for inducing apoptosis in colon cancer cells through ROS and mitochondrial mediated mechanism (Jaganathan, 2012). However, further experiments in preclinical and clinical settings will promote this as a likely candidate for chemoprevention of colon cancer.

It has been shown that the *in vitro* growth inhibitory effects of flavonoids correlate with variations in chemical structure. Flavonoids are characterized by a benzene ring (A) fused with a pyrone ring (C), that carries a phenyl ring (B) in position 2 or 3. Previous studies have shown that the C ring with oxo function at position 4, and C2-C3 double bond correlate with antiproliferative activity (Wang *et al*., 2004). Future developments of this work include the purification and study of active compounds, as a mean to better understand the relationship between mechanisms of death action pathways using *in vivo* models.

The presence of the C2-C3 double bond strongly correlated with the anti-survival effect in NUB-7 and LAN-5. Naringenin , which is identical to apigenin (4-1) but lacks the C2-C3 double bond, had no effects on survival or PARP cleavage, further suggesting that the presence of the C2-C3 double bond is necessary for activation of apoptosis. In addition, the presence of hydroxy or methoxy substitutions on the flavone ring structure have been shown to correlate with increased inhibitory activity , as also shown here by the lack of growth-inhibitory activity of nonhydroxylated flavone and the strong anti-survival effects of hydroxylated apigenin, quercetin, diosmetin, and chrysin (Torkin *et al*., 2005).

Exposure to curcumin (lamiaceae family) which is rich with falvonoid, progressive decrease to mitochondrial membrane potential and release of cytochrome C into the cytosol were also observed in U2OS cells. It has been noticed in many *in vitro* systems that apoptosis is associated with a loss of

mitochondrial membrane potential, which may correspond to the opening of an outer membrane pore. Thus, it has been suggested that this event is responsible for cytochrome C release into the cytosol from mitochondria (Puthalakath and Strasser; 2002) and this may be one apoptosis result found in increase p53 and p21 protein during treatment with *H. longiflorus* extract.

Multiple molecular pathways are involved in curcumin (Lamiaceae family) which is a natural phenol induced apoptosis of human U2OS cells. These include pro-and anti-apoptotic Bcl-2 family proteins, mitochondrial membrane potential, mitochondrial cytochrome C and caspase-3 (Song *et al*., 2009).

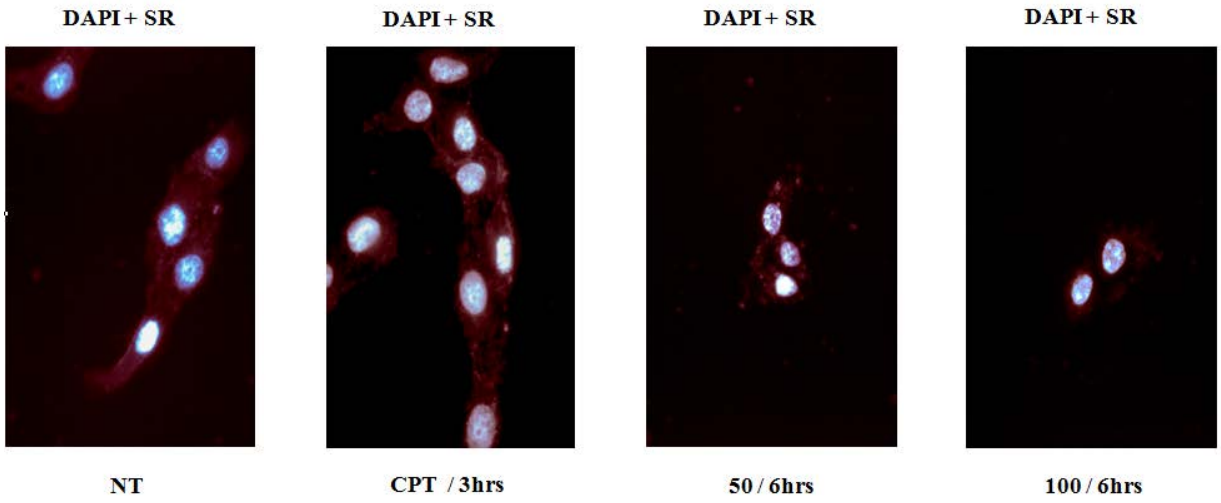


Figure (4-19): Morphological aspects of U2OS cells treated with *H. longiflorus* methanolic extract.

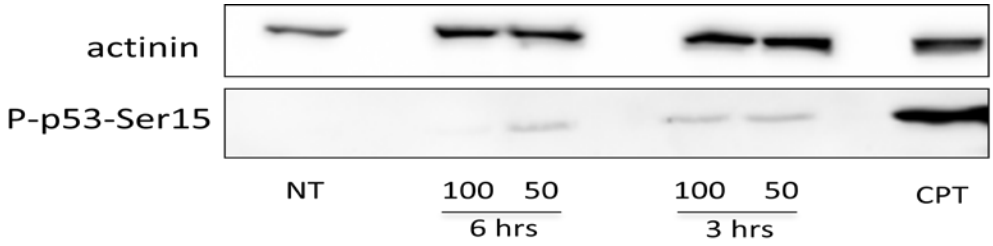
4.3.5 Western blot analysis

To evaluate the observed cell cycle arrest in U2OS and RKO cell lines due to the activation of H2AX, P21, P53, PARP, Actin and Actinin proteins, following treatments with *H. longiflorus* extract. Western blotting is an important technique used in cell and molecular biology. By using a Western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. accomplish this task: the technique include three elements (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody to visualize (Hang *et al*., 2012).

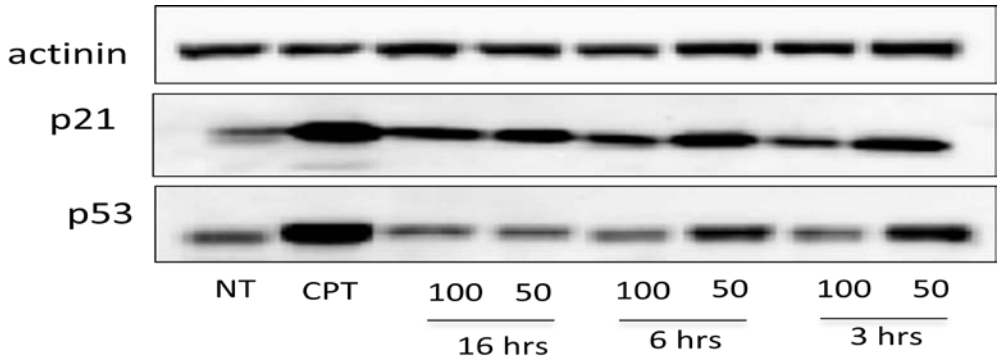
- **Treatments with *H. longiflorus* extract lead to activation of p53 pathway**

In the present assay, osteosarcoma U2OS cell line shows expressions of actinin, p21, P-p53-ser and p53 proteins (Figure 4-20; A and B). As shown in figure 4-20 (A), a mild increase in P-p53-ser protein was observed in both concentrations after 3 hrs of treatment with *H. longiflorus*. In p53 and p21, a significant increase in protein expression was recorded and the expression was concentration and time dependent (Figure 4-20; B). The expression of actinin not affected by induction of DNA damage as compared with non treated cells (Figure 4-20; A and B).

In RKO cell line, increasing of P-p53-ser was observed in a significant levels and the expression was concentration and time dependent (Figure 4-21; A). In figure (4-21; B), the observed increasing in p21 and p53 was also present at recommended concentration as compared with non treated cells. The expression of actinin not affected by induction of DNA damage as compared with non treated cells (Figure 4-21; A and B).



(A)



(B)

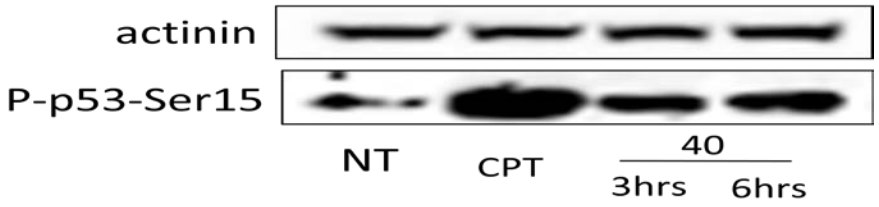
Figure (4-20): Western blot analysis for U2OS cell line.

(A): Western blot shows actinin (upper panel), and P-p53-ser 15 (lower panel).

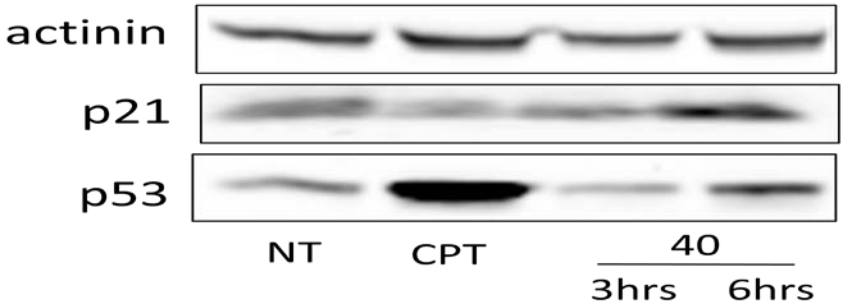
(B): Western blot shows actinin (upper panel), P21 (middle panel) and p53 (lower panel).

NT: Non treated cells (control).

CPT: Camptothecin.



(A)



(B)

Figure (4-21): Western blot analysis for RKO cell line.

(A): Western blot shows actinin (upper panel), and P-p53-ser 15 (lower panel).

(B): Western blot shows actinin (upper panel), P21 (middle panel) and p53 (lower panel).

In both cell lines, the results showed an increase of both onco-suppressor proteins, and accumulation of the phospho-Ser15-p53, thus meaning that accumulation of p53 is due to the increase of its stress-activated form. These data suggest that the observed cell cycle arrest is in part due to *H. longiflorus* extracts-dependent activation of p53-p21 pathway.

The tumor suppressor p53 can induce cell cycle arrest or apoptosis in response to a variety of stress signals, such as DNA damage, oncogenic stimuli, or hypoxia. Activation of p53 occurs by several mechanisms including protein stabilization and modification of the protein by phosphorylation and acetylation.

P53 is a transcription factor that recognizes specific binding sites within numerous target genes including *mdm2*, *cyclin G*, *bax*, and p21/WAF1/CIP1. While multiple downstream targets are involved in the mediation of apoptotic effects, the main target for p53-induced cell cycle arrest seems to be the p21 gene (Chen *et al*., 2011).

Phosphorylation of p53 mostly occurs in the N-terminal activation domain at the Ser6, Ser9, Ser15, Thr18, Ser20, Ser33, Ser37, Ser46, Thr55, and Thr81 residues, with some phosphorylation occurring in the C-terminal linker and basic regions at Ser315, Ser371, Ser376, Ser378, and Ser392. Phosphorylation on most of these sites is induced by DNA damage, with some, such as Thr55 and Ser376, being repressed upon genotoxic stress (Holmberg *et al*., 2002). How these individual residues contribute to p53 stabilization and activation is still not fully understood, and, at times, has been the subject of debate.

However, it has been shown that apigenin and genistein modulate the effects of deregulated cell cycle checkpoints and are there by believed to contribute to the prevention of cancer. They appear to elicit their beneficial effects not only through cell cycle arrest and the induction of cyclin−dependent kinase inhibitors (p15, Cip−1/p21 and Kip−1/p27) but also through down−regulation of anti−apoptotic gene products (Bcl−2, Bcl−xL) and induction of pro−apoptotic p53 and Bax.24, 25,29,47,49 Apoptosis, or programmed cell death, is an important mechanism in normal development and in anticancer surveillance. The process is regulated by various oncogenes /proteins, including the important pro−apoptotic p53, the anti−apoptotic and cell survival Bcl−2 and the caspase cascade (Meeran and Katiyar, 2008).

The response to DNA damage results in either cell cycle arrest, to allow the lesions to be repaired, or apoptosis. p53 is essential in both pathways. Specifically,

in cell cycle arrest at G1, p53 enhances p21 transcription, which in turn inhibits cyclin- dependent kinase (cdk) activity. This prevents pRb from derepressing E2F1, inhibiting progression from G1 to S phase (Harris and Levine, 2005). Another way p21 leads to G1 arrest is by binding to proliferating cell nuclear antigen (PCNA) and inhibiting its function in replication (Moldovan *et al*., 2007).

Loss of p21 results in replication defects and may lead to cell death after treatment with anticancer drugs (Liu and Lozano, 2005.). Progression to S phase after p53/p21-mediated arrest is achieved by proteosome-dependent degradation of p21 (Gottifredi *et al*., 2004).The p53/p21 pathway can also lead to a G2 arrest, preventing cells with damaged DNA from entering mitosis (Taylor and Stark, 2001). Cells may proceed to mitosis again by degradation of p21 (Amador *et al*., 2007).

The mechanism of p21 degradation has been controversial, with some reports claiming that it is ubiquitin-dependent and some others showing the opposite (Bloom *et al*., 2003). Although the increase of p21 levels is a well established pathway in DNA repair, a report by Bendjennat *et al* (Bendjennat *et al*., 2003) showed that p21 is degraded after low-dose UV-irradiation. This response was shown to be essential for effective DNA repair through PCNA binding to chromatin. Recent studies further support the existence of this new pathway of DNA repair that involves degradation of p21 (Suh *et al*., 2006; Meng *et al*., 2007).

Genistein activated stress signaling pathways that phosphorylated p53 and ATM, leading to p21 induction and γH2AX formation (Ye *et al*., 2004). Further, genistein modulated cyclin-dependent kinase Cdc2 activity through the protein phosphatase Cdc25C, thereby activating ATM and causing G_2/M arrest in hepatoma cells (Chang *et al*. 2004).

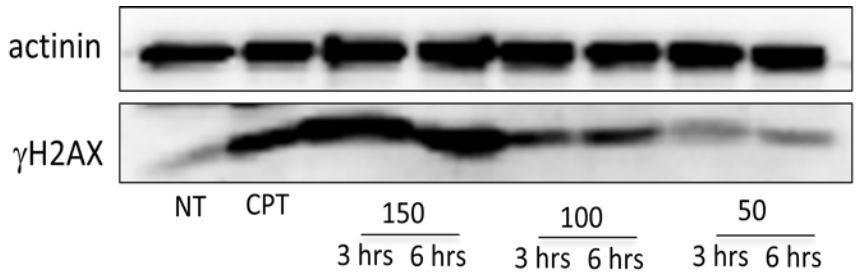
- **Treatments with *Hymenocrater longiflorus* extracts lead to DNA damage and PARP-1 cleavage.**

Activation of p53 transcriptional pathway, in the presence of cellular stress as induced by *H. longiflorus* extracts, leads to arrest of cell cycle. This arrest let the cells to repair the damage, otherwise, if the damage cannot be repaired, cells undergo apoptosis.

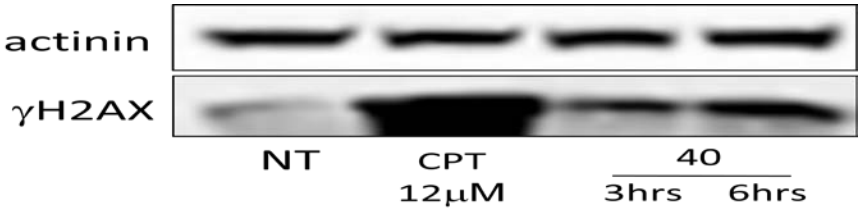
Accordingly, the present study based its aim on the hypothesis that *H. longiflorus* extracts could determine DNA damage in treated cell lines. To test this hypothesis, we performed immunofluorescence analyses probing fixed cells with antibody directed to the ser139-phosphorylated histone H2AX (called γ-H2AX) that accumulates on DNA double strand lesions (forming foci), and hence is a marker of DNA damage. As shown in Figure (4-17; 4-18), the accumulation of damage foci in treated cells was observed. Furthermore, γ-H2AX accumulation by Western blot analyses was also confirmed (Figure 4-22). Data showed indicate that *H. longiflorus* extract induce DNA damage, that is responsible of p53 activation and cell cycle arrest.

Double-strand breaks in mammalian DNA lead to rapid phosphorylation of C-terminal serines in histone H2AX (γ-H2AX) and formation of large nuclear γH2AX foci. After DNA repair these foci disappear, but molecular mechanism of elimination of γ-H2AX foci remains unclear. H2AX protein can be phosphorylated and dephosphorylated *in vitro* in the absence of chromatin (Polo and Jackson, 2011). Γ-H2AX formation by phosphorylation of the histone variant H2AX is the key process in the repair of DNA lesions including those arising at fragile sites under replication stress (Jungmin *et al* .,2013). The poly (ADP-ribosyl)ation catalyzed by poly(ADP-ribose)pomerase-1 and -2 (PARP-1 and PARP-2) is one of the earliest event in the signaling of DNA damage. Following DNA damage, PARP-1 and PARP-2 modify their own primary sequences (automodification) as

well as other cellular proteins (heteromodification) to allow DNA damage repair. In the case DNA damage exceeds the cellular repair capacity; PARP-1 undergoes cleavage by caspases into two fragments of 89 Kda and 24 Kda, starting apoptosis.



(A)



(B)

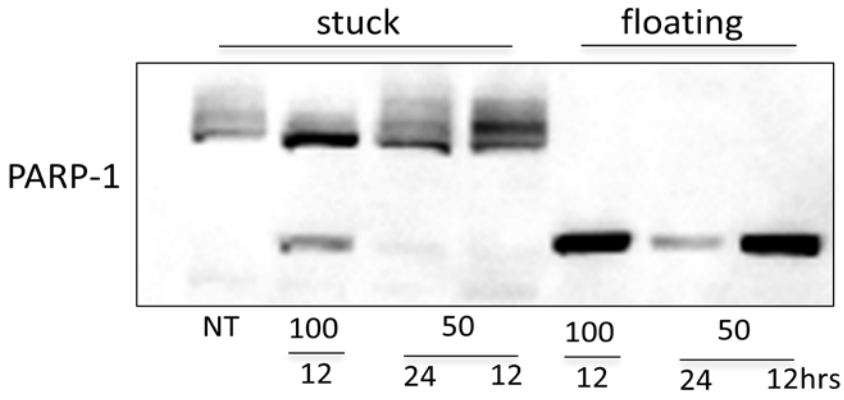
Figure (4-22): Western blot analysis

(A): Western blot in U2OS cell line shows actinin (upper panel) and γ-H2AX (lower).

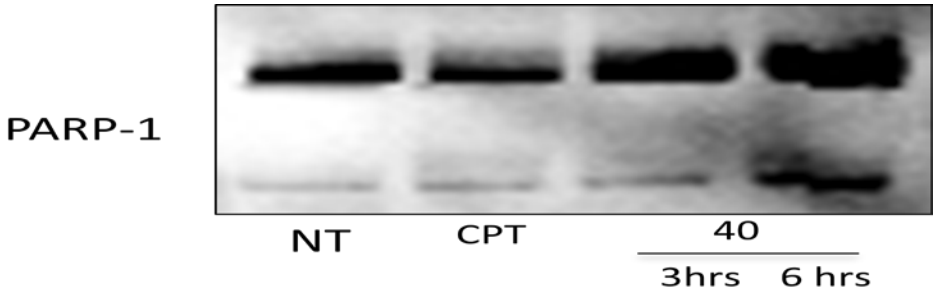
(B): Western blot in RKO cell line shows actinin (upper panel) and γ-H2AX (lower).

To evaluate if treatment with *H. longiflorus* extracts could induce PARP-1 cleavage, we treated U2OS cell line with 50 μg mL^{-1} at 12 and 24 hrs and for 100μg mL^{-1} for 12 hrs to the stuck (live cells) and for RKO 40 μg mL^{-1} at 3 and 6 hrs and probed cellular extracts with antibody anti-PARP-1. As shown in the Figure (4-23), administration of plant extract to cells induces PARP-1 cleavage, thus suggesting that cells start the apoptotic program. PARP belongs to a group of

nuclear enzymes that play a critical role in DNA damage repair through PAR production. It has been known that PAR formation is an energetically expensive process, causing failure in cellular ATP production, rapid depletion of NAD^+, and eventually cell death (Huang *et al*., 2009). On the contrary, PARP plays a protective role against ROS-induced cell death through LKB1-AMPK-mediated autophagy activation (Huang *et al*., 2009). In the present study, we showed for the first time that *H. longiflorus* activated p21, p53, p53-p,γ-H2AX and PARP proteins.



(A)



(B)

Figure (4-23): Western blot analysis

(A): Western blot in U2OS cell line shows PARP-1protein revealed the stack (live cells) and floating (dead cells).

(B): Western blot in RKO cell line shows PARP-1 protein.

Conclusions and Recommendations

Conclusions and Recommendations

I. Conclusions

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

1- *H. longiflorus* methanolic extract contains different phenolic active compounds include flavonoids (Apigenin, Apigenin 7-O-beta D-glucoside, Acacetin Crisimaritin, Isorhamnetin and Genistein) and phenolic acids (Caffeic acid, Ferulic acid, Rosmaric acid and Carnosic acid). The qualification of these compounds was detected using HPLC-ESI/MS analysis.

2- *H. longiflorus* extract showed anti-oxidant activity and this activity was dose dependent.

3- Significant differences in cell viability were obtained under the effect of *H. longiflorus* extract using cells counter and MTT.

4- Assessment of cell cycle arrest using FACS analysis, mild effect was observed in S phase for U2OS and G1phase for RKO cell line.

5- Phosphorelation of serine 139 and DNA damage foci were signs obtained during immunoflourescence assay via γ-H2AX protein.

6- Using sulforhodamine dye by fluorescent microscope shrinkage of cells was observed and this lead to apoptosis.

7- p21, p53, p-p53 and γ-H2AX proteins were increased for both cell lines treated with plant extract in addition to cleavage of parp protein. these results obtained by Western blot analysis are indicator of DNA damage and apoptosis.

References

II. Recommendations

1- Further phytochemical and pharmacological investigation on other active compounds found in *H. longiflorus* extract which may have anti-cancer activity.

2- Advanced methodologies are needed to study the anti-tumor effects of *H. longiflorus* extract such as commet assay, DNA microarray, immunohistochemical and *in situ* hybridization.

3- Further studies are needed on other proteins which can induce apoptosis like Bcl-2/ Bax and CDK9, under the effect of *H. longiflorus*.

4- Study the other plant extract materials (hot water or hot alcohol extract) for different part of *Hymenocrater longiflorus* plant (bark, root, stem, etc) and identified the stage of growth for this plant.

5- In vivo studies of the *Hymenocrater longiflorus* plant effect on cnacer cells by histopathological studies.

References

- Abdullaev, F.I. (2001). Plant-drived agents against cancer. In: Gupta SK, editor. Pharmacology and therapeutics in the new millennium. Narosa Publishing House: New Delhi, India., p 345-354.
- Ahmadi, F.; Sadeghi, S.; Modarresi, M.; Abiri, R. ; Mikaeli, A. (2010).Chemical composition, in vitro anti-microbial, antifungal and antioxidant activities of the essential oil and methanolic extract of *Hymenocrater longiflorus* Benth., of Iran. Food. Chem. Toxicol. 48:1137-1144.
- Akramian, M.; Ebrahimi, S.N.; Joharchi, M.R. (2008). Essential Oil Composition of Hymenocrater platystegius Rech. f. from Iran. J. Essential Oil-Bear Plants., 11: 199-202.
- Alegria-Schaffer, A.; Lodge, A.; Vattem, K. (2009). Performing and optimizing Western blots with an emphasis on chemiluminescent detection. Methods Enzymol., 463:573-599.
- Alosi, J.A.; McDonald, D.E.; Schneider, J.S.; Privette, A.R.; McFadden, D.W. (2010). Pterostilbene inhibits breast cancer in vitro through mitochondrial depolarization and induction of caspase-dependent apoptosis. J. Surg Res., 161: 195–201.
- Amador, V.; Ge, S.; Santamaría, P.G.; Guardavaccaro, D.; Pagano,M. (2007). APC/C (Cdc20) controls the ubiquitin-mediated degradation of p21 in prometaphase. Mol. Cell., 27: 462-473.
- Ambroz, K. (2006). Improving quantification accuracy for western blots. Analysis image.

- Amente,S.; Gargano,B.; Napolitano,G.; Lania,L.; Majello,B.(2009). Camptothecin release P-TEFb from the inactive 7SK snRNP complex. Cell cycle. 8: 1249- 1255.
- Anita, H. P. ;Sharma, . ; Patnaik, A.,(2014). Apoptosis (programmed cell death) - A Review. SJIF., 3:1854-1872.
- Anne, C. S.; Victor, A. A. (2007). Nomenclatural and taxonomic changes within the *Leucas clade* (Lamioideae; Lamiaceae). Syst. Geography Plants 77: 229-238.
- Atoui, A.K.; Mansouri, A.; Boskou, G.; Kefalas, P. (2005). Tea and herbal infusions: their antioxidant activity and phenolic profile. Food Chem., 89: 27-36.
- Avci, C.B.; Yilmaz, S.; Dogan, Z.O.; Saydam, G.; Dodurga, Y.; Atakan, E.H.; Kartal, M.; Sahin, F. Baran, Y.; Gunduz, C. (2011). Quercetin-induced apoptosis involves increased hTERT enzyme activity of leukemic cells. Hematology., 16: 303-307.
- Avery, K.A.; Bowden, N.A.; Croft, A.J.; Scurr, L.L.; Kairupan, C.F.; Ashton, K.A.; Talseth, B.A.; Rizos, H.; Zhang, X.D.; Scott, R.J.; Hersey, P. (2011). P53 in human melanoma fails to regulate target genes associated with apoptosis and the cell cycle and may contribute to proliferation. BMC Cancer. , 11: 203.
- Babovic, N.; Djilas, S.; Jadranin, M.; Vajs, V.; Ivanovic, J.; Petrovic, S. ; Zizovic, I. (2010). Supercritical carbon dioxide extraction of antioxidant fractions from selected Lamiaceae herbs and their antioxidant capacity. Innov. Food Sci. Emerg. Technol., 11: 98-107.
- Banath, J.P.; Olive, P.L. (2003). Expression of phosphorylated histone H2AX as a surrogate ofcell killing by drugs that create DNA double-strand breaks. Cancer Res., 63: 4347–4350.

- Barazandeh, M.M. (2006). Essential oil composition of Nepeta menthoides Bioss. Et Bushe from Iran. j. Essential oil Res., 18:144-145.
- Barkume, M.S.; Tayade, P.T.; Vavia, P.R.; Indap, M.A. (2003). Antiproliferative effect of Aspirin and its inclusion complex on human MCF-7 and K-562 cancer cells in vitro Indian J. Pharm Sci., 65: 486-491.
- Bazylko, A.; Strzelecka, H. (2007). A HPTLC densitometric determination of luteolin in Thymus vulgaris and its extracts. Fitotherapia., 78: 391-395.
- Bedelbaeva, K.; Snyder, A.; Gourevitch, D.; Clark, L.; Zhang, X.M.; Leferovich, J.; Cheverud, J.M.; Lieberman, P.; Heber-Katz, E.(2010). Lack of p21 expression links cell cycle control and appendage regeneration in mice. Proc. Natl. Acad. Sci. USA., 107: 5845-5850.
- Belayachi, L.; Aceves-Luquero, C.; Merghoub, N.; Bakri, Y.; Fernández de Mattos, S.; Amzazi, S.; Villalonga, P.(2013). Screening of North African Medicinal Plant Extracts for Cytotoxic Activity against Tumor Cell Lines, European. Med. Plants., 14: 310-332.
- Bendjennat, M., Boulaire, J.; Jascur, T.; Brickner, H.; Barbier, V.; Sarasin, A.; Fotedar, A.; Fotedar, R. (2003). UV irradiation triggers ubiquitin-dependent degradation of p21 (WAF1) to promote DNA repair. Cell., 114: 599-610.
- Bergamaschi, B. A.; Walters, J. S.; Hedges, J. (1999). Distributions of uronic acids and *O*-methyl sugars in sinking and sedimentary particles in two coastal marine environments; Geochim. Cosmochim. Acta., 63: 413-425.

- Bernstein, H.; Payne, C.M.; Bernstein, C.; Garewal, H.; Dvorak, K. (2008). Cancer and aging as consequences of un-repaired DNA damage. In: New Research on DNA Damages (Editors: Honoka Kimura and Aoi Suzuki) Nova Science Publishers, Inc., New York, Chapter 1, pp. 1- 47.
- Bertero, T.; Gastaldi, C.; Bourget-Ponzio,I.; Mari, B.; Meneguzzi, G.; Barbry,P.; Ponzio, G.; Rezzonico, R. (2013). CDC25A targeting by miR-483-3p decreases CCND-CDK4/6 assembly and contributes to cell cycle arrest. Cell Death Differ., 20: 800-811.
- Bhatia, A.; Dey , P.; Goel, S.; Singh, G. (2013). Expression of γH2AX may help in defining a genetically more stable subtype of infiltrating ductal carcinoma of breast. Indian J Med Res., 137: 759-766.
- Bloom, J.; Amador, v.; Bartolini, F.; DeMartino, G. ; Pagano, M. (2003). Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. Cell., 1: 71-82.
- Bonner, W.M.; Redon, C.E.; Dickey, J.S.; Nakamura, A.J.; Sedelnikova, O.A.; Solier, S.; Pommier, Y. (2008) GammaH2AX and cancer. Nat. Rev. Cancer., 8: 957-967.
- Boots, A.W., Haenen, G.R.; Bast, A. (2008). Health effects of quercetin: From antioxidant to nutraceutical. Eur. J. Pharmacol., 585: 325-337.
- Bouftira, I.; Chedly, A.; Souad, S. (2012). Antioxidant and Antibacterial Properties of *Mesembryanthemum crystallinum* and *Carpobrotus edulis* Extracts. Adv. Chem. Eng. Sci., 2: 359-365.
- Bradford, M.M. (1976). A dye binding assay for protein. Anal. Biochem., 72: 248-254.
- Bratton, D.L.; Fadok, V.A.; Richter, D.A.; Kailey, J.M.; Guthrie, L.A. ; Henson, P.M.(1997). Appearance of phosphatidylserine on apoptotic cells

requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J. Biol. Chem ., 272: 26159-26165.

- Bufalo, M.C.; Candeias, J.M.G. ; Sforcin, J.M. (2009).In vitro Cytotoxic Effect of Brazilian Green Propolis on Human Laryngeal Epidermoid Carcinoma (HEp-2) Cells., 6: 483-487.
- Bundy, J.G.; Morriss, A.W.J.; Durham, D.G.; Campbell, C.D.; Paton, G.I. (2001). Development of QSARs to investigate the bacterial toxicity and biotransformation potential of aromatic heterocylic compounds. Chemosphere., 42: 885-892.
- Capecka, E.; Mareczek, A. ; Leja, M. (2005). Antioxidant activity of fresh and dry herbs of some Lamiaceae species. Food Chem., 93: 223-226.
- Capek, P.; Machova, E.; Turjan, J. (2009). Scavenging and antioxidant activities of immune modulating polysaccharides isolated from *Salvia officinalis*. Int. J. Bio. Macromol., 44: 75-80.
- Celeste, A.; Fernandez-Capetillo, O.; Kruhlak, M.J.; Pilch, D.R.; Staudt, D.W.; Lee, A.; Bonner, R.F.; Bonner, W.M.; Nussenzweig, A. (2003). Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat. Cell Biol., 5: 675-679.
- Chiolo, I.; Minoda, A.; Colmenares, S.U.; Polyzos, A.; Costes, S.V.; Karpen, G.H. (2011). Doublestrand breaks in heterochromatin move outside of a dynamic HP1a domain to complete recombinational repair. Cell.,144: 732-744.
- Chang, K.L.; Kung, M.L.; Chow, N.H.; Su, S.J. (2004).Genistein arrests hepatoma cells at G2/M phase: involvement of ATM activation and upregulation of p21waf1/cip1 and Wee1. Bioche. Pharmacol., 67**:** 717-726.

- Chatterjee, R. (2007). Cell biology. Cases of mistaken identity. Science., 315: 928-931.
- Chen, Y.; Zhang, L.; Jones, K.A. (2011). SKIP counteracts p53-mediated apoptosis via selective regulation of p21Cip1 mRNA splicing. Genes and Dev., 25: 701-716.
- Chia, C. C.; Ivan M. K.; Cheng L.W. (2013). Complete microscale profiling of tumor microangiogenesis: a microradiological methodology reveals fundamental aspects of tumor angiogenesis and yields an array of quantitative parameters for its characterization. Biotechnol. Adv., 31: 396-401.
- Chung , M. K.; Han ,S. S.; Kim, J. C. (2006). Evaluation of the toxic potentials of a new camptothecin anticancer agent CKD-602 on fertility and early embryonic development in rats. Regulatory Toxicology and Pharmacology 45: 273- 281.
- Collins, A. R.; Oscoz, A. A. ; Oscoz, A. A. (2008).The comet assay: topical issues, Mutagenesis., 23: 143-151.
- Cosan, D.T.; Soyocak, A.; Basaran, A.; Degirmenci, I.; Gunes, H.V.; Sahin, F.M. (2011). Effects of various agents on DNA fragmentation and telomerase enzyme activities in adenocarcinoma cell lines. Mol. Biol., 38: 2463-2469.
- Cragg, G.M.; Simon, J.E.; Jato, J.G.; Snader, K.M. (1996). Drug discovery and development at the National Cancer Institute: Potential for New Pharmaceutical Crops. In: Progress in New Crops, Ed., Janick, J. ASHS Press., 554-560.
- Darzynkiewicz, Z.; Huang, X. (2004).Analysis of cellular DNA content by flow cytometry. Current Protocols in Immunology., 60: 5.7.1-5.7.18.

- Davis, A.; Theodosopoulos,G.; Atkin,I.; Drexler, H.G. ; Kohara, A.; MacLeod, R.A ; Masters, J.R.; Nakamura, Y. (2010). Check your cultures! A list of cross-contaminated or misidentified cell lines. Int. J. Cancer., 127: 1-8.
- Dehay,D.; Kennedy,H.(2007). Box 2- Structure and regulation of the cell cycle. Nature Reviews Neuroscience., 8: 438- 450.
- Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J. P.; Tognolini, M.; Borges, G.; Crozier, A. (2013). Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal., 18: 1818-1892.
- Deveraux, Q.L. ; Reed, J.C. (1999). IAP family proteins-suppressors of apoptosis. Genes Dev., 13: 239-252.
- Doetsch, M.; Gluch, A.; Poznanović, G.; Bode, J.; and Vidaković, M. (2012). YY1-binding sites provide central switch functions in the PARP-1 gene expression network. PLoS ONE 7 (8): e44125.
- Dolezalova, D.; Mraz, M.; Barta, T.; Plevova, K.; Vinarsky, V.; Holubcova, Z.; Jaros, J.; Dvorak, P.; Pospisilova, S.; Hampl, A. (2012). MicroRNAs regulate p21 (Waf1/Cip1) protein expression and the DNA damage response in human embryonic stem cells. Gene Dev., 30: 1362-1372.
- Donjerkovic, D.; Scott, D.W. (2000), Activation-induced cell death in B lymphocytes. Cell Res., 10: 179-192.
- Doostdar, H.; Burke, M.D.; Mayer, R.T. (2000). Bioflavonoids selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1. Toxicology., 144: 31-38.

- Duangmano, S.; Dakeng, S.; Jiratchariyakul, W.; Suksamrarn, A.; Smith, D.R.; Patmasiriwat, P. (2010). Antiproliferative effects of cucurbitacin B in breast cancer cells: down-regulation of the c Myc/ hTERT/ Telomerase pathway and obstruction of the cell cycle. Int. J. Mol. Sci., 11: 5323-5338.
- Dubick, M.A. (1986). Historical perspectives on the use of herbal preparations to promote health. J. Nutr., 116: 1348-1354.
- Ebrahimzadeh, M.A.; Pourmmorad, F.; Hafezi, S. (2008). Antioxidant activities of Iranian corn silk. Turk. J. Biol., 32: 43-49.
- Echeverry, C.; Blasina, F.; Arredondo, F.; Ferreira, M.; Abin-Carriquiry, J.A.; Vasquez, L. (2004). Cytoprotection by neutral fraction of tannat red wine against oxidative stress-induced cell death. J. Agric. Food Chem., 52: 7395-7399.
- Ekholm, S. V.; Reed, S. I. (2000). Regulation of G1 cyclin-dependent kinases in the mammalian cell cycle. Curr. Opin. Cell Biol., 12: 676-684.
- El-Sayed, M.W.; Hussin, W.A. (2013). Antimutagenic and antioxidant activity of novel 4-substituted phenyl-2, 2′-bichalcophenes and aza analogs. J. Drug Des. Dev. Ther., 7: 73–81.
- Erpel, T.; Courtneidge, S.A. (1995). Src family protein tyrosine kinases and cellular signal transduction pathways. Curr. Opin Cell Biol; 7: 176-182.
- Espinoza, L.A.; Smulson, M.E.; Chen, Z. (2007) Prolonged poly (ADP-ribose) polymerase-1 activity regulates JP-8-induced sustained cytokine expression in alveolar macrophages. Free Radic. Biol. Med., 42: 1430-1440.
- Ferrari, M.; Chiara, F. M.; Isetta, A. M. (1990). MTT colorimetric assay for testing macrophage cytotoxic activity *in vitro*. J. Immunol. Methods., 131:165-172.

- Firouznia, A.; Rustaiyan, A.; Nadimi, M.; Masoudi, S.; Bigdeli, M. (2005). Composition of the essential oil of *Hymenocrater calycinus* (Boiss.) Benth. From Iran. J. Essen. Oil Res., 7: 527-529.
- Fraignier, M. P.; Marques, L.; Fleuriet, A.; Macheix, .J.J. (1995). Biochemical and immunochemical characteristics of polyphenol oxidases from different fruit of Prunus. J. Agric. Food. Chem., 43: 2375-2380.
- Freshney, R.I. (2005). Culture of animal cells: a manual of basic techniques. 5th Ed. New York: Wiley-Liss., 120-128.
- Friedberg, E.C. (2003). DNA damage and repair. Nature., 421: 436-440.
- Furuta, T.; Takemura, H.; Liao, Z.Y. (2003). Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes. J. Biol. Chem., 278: 20303-20312.
- Galvez, M.; Martin-Cordero, C.; Houghton, P.J.; Ayuso, M.J. (2005). Antioxidant activity of methanol extracts obtained from Plantago species. J. Agric. Food Chem., 53: 1927-1933.
- Gassmann, R.; Andrew, J.; Holland, D., Wan, X.; Civril, F.; Don, W.; Oegema, K.; Salmon, D.; Desai, A. (2010). Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells. Genes Dev., 24: 957-971.
- Gelinas, P.; McKinnon, C. M. (2006). Effect of wheat variety, farming site, and bread-baking on total phenolics. Int. J. Food Sci. Tech., 41: 329-332.

- Gemma, L.C.; Bramley, C.; Felix, F.; Rogier, P.J. (2009). Troublesome tropical mints: re-examining generic limits of Vitex and relations (Lamiaceae) in South East Asia. Taxon., 58: 500-510.
- Gennari, C.; Castoldi, D.; Sharon, O. (2007). Natural products with taxol-like anti-tumor activity: Synthetic approaches to eleutherobin and dictyostatin. Pure Appl. Chem., 79: 173-180
- Goldberg, M. E.; Gorn, G. J.; Peracchio, L. A.; Bamossy, G. (2003).Understanding materialism among youth. J. Consum. Psycho., 13: 278-288.
- Goodarzi, A. A. ; Noon, A. T. ; Deckbar, D.; Shiloh, Y.; Lobrich, M.; Jeggo, P. A. (2008) . ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. Mol. Cell., 31: 167-177.
- Graham, J.G.; Quinn, M.L.; Fabricant, D.S.; Farnsworth, N.R. (2000). Plants used against cancer, an extension of the work of Jonathan Hartwell. J. Ethnopharmacol., 73: 347-377.
- Grossel, M.J.; Baker, G.L.; Hinds, P.W. (1999). cdk6 can shorten G(1) phase dependent upon the N-terminal INK4 interaction domain. J. Biol. Chem., 274: 29960-29967.
- Guo, X.; Zhu, K.; Zhang, H.; Yao, H. (2010). Anti-tumor activity of a novel protein obtained from Tartary Buckwheat. Int. J. Mol. Sci., 11: 5201-5211.
- Haddad, A.Q. (2008). The cellular and molecular properties of flavonoids in prostate cancer chemoprevention. Ph.D Thesis, Institute of Medical Science, University of Toronto.

- Hadfield, J.A.; Ducki, S.; Hirst, N.; McGown, A.T. (2003).Tubulin and microtubules as targets for anticancer drugs. Progr. Cell Cycle Res., 44: 309-325.
- Hanahan, D.; Weinberg, R.A. (2000). The Hallmarks of Cancer. Cell., 100: 57-70.
- Hang, L.H.; Yang, J.P.; Yin, W.; Wang, L.N.; Guo, F.; Ji, F.H.; Shao, D.H. (2012).Activation of spinal TDAG8 and its downstream PKA signaling pathway contribute to bone cancer pain in rats. Eur. J. Neurosci., 36: 2107-2117.
- Harper, J.W.; Elledge, S.J. (2007). The DNA damage response:ten years after. Mol. Cell., 28: 739-745.
- Harris, S.L.; Levine, A.J. (2005). The p53 pathway: positive and negative feedback loops. Oncogene., 24: 2899-2908.
- Hartwell, J.L. (1982). Plants used against cancer. Lawrence, MA. Quarterman Publications., 438-439.
- Harun-ur-Rashid, M.; Gafur, M.K.; Sadik,G. M. ; Rahman, M.A. (2002). Biological activities of a new acrylamide derivative from Ipomoea turpithum. Pakistan J. Biol. Sci., 5: 968-969.
- Hengartner, M. O. (2000). The biochemistry of apoptosis. Nature., 407: 770-776.
- Henkelman, S.; Lagerberg, J. W.; Graaff, R.; Rakhorst, G.; Van Oeveren, W. (2010). The effects of cryopreservation on red blood cell rheologic properties. Transfusion., 50: 2393-2401.
- Herrero, M.; Plaza, M.; Cifuentes, A.; Ibanez, E. (2010). Green processes for the extraction of bioactives from Rosemary: Chemical and functional characterization via ultra performance liquid chromatography-tandem

mass spectrometry and in-vitro assays. J. Chromatogr. A., 1217: 2512-2520.

- Hertog, M.G.; Hollman, .P.C.; Katan, M.B.; Kromhout, D. (1993). Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr. Cancer., 20: 21-29.
- Hinneburg, I., Damien Dorman, H. J.; Hiltunen, R. (2006). Antioxidant activities of extracts from selected culinary herbs and spices. Food Chem., 97: 122-129.
- Holmberg, C. I.; Tran, S.E.F.; Eriksson, J. E.; Sistonen, L. (2002). Multisite phosphorylation provides sophisticated regulation of transcription factors. Trends Biochem. Sci., 27: 619-627.
- Hossain, M. B.; Brunton, N. P.; Barry-Ryan, C.; Martin-Diana A. B.; Wilkinson, M. (2008). Antioxidant activity of spice extracts and phenolics in comparison to synthetic antioxidants. Rasayan J. Chem., 1: 751-756.
- Hou, W. C.; Lin, R.D.; Cheng, K.T.; Hung, Y.T.; Cho, C.H.; Chen, C.H.; Hwang, S.Y.; Lee, M.H. (2003). Free radical scavenging activity of Taiwanese native plants. Phytomedicine., 10: 170-175
- Hu, C.; Kitts, D. D. (2004). Luteolin and luteolin-7-O-glucoside from dandelion flower suppress iNOS and COX-2 in RAW264.7 cells. Mol. Cell Biochem., 265: 107-113.
- Huang, Q.; Wu, Y.T.; Tan, H.L.; Ong, C.N.; Shen, H.M. (2009). A novel functions of poly (ADP-ribose) polymerase-1 in modulation of autophagy and necrosis under oxidative stress. Cell Death Differ., 16: 264-277.
- Huang, J.Y.; Zhong, L.; Wang, C.M.; Sullivan, J.P.; Xu, W.; Qiang, L. (2010). In Situ Observation of the Electrochemical Lithiation of a Single SnO_2 Nanowire Electrode. Science., 330: 1515-1520.

- Hui, Z.; Cheng, X.; West, M.G.; Roberts, J.M. (2012). Loss of Cyclin-Dependent Kinase 2 (CDK2) Inhibitory Phosphorylation in a CDK2AF Knock-In Mouse Causes Misregulation of DNA Replication and Centrosome Duplication. American Society for Microbiology. Cell. Biol., 32: 1421-1432.
- Indap, M.A.; Radhika, S.; Motiwale, L.; Rao, K.V. (2006). Quercetin: antitumour activity and pharmacological manipulations for increased therapeutic gains. Indian J. Pharm. Sci., 68: 465-469.
- Isabelle, M.; Moreel, X.; Gagné, J.P.; Rouleau, M.; Ethier, C.; Gagne, P.; Hendzel, M.J.; Poirier, G.G. (2010). Investigation of PARP-1, PARP-2, and PARG interactomes by affinity-purification mass spectrometry. Proteome Sci., 8: 22-24.
- Jackson, S.P.; Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature., 461: 1071-1078.
- Jafari, A.; Jafarzadeh, F. (2008). Anatomical and pollen ornamentation study on Hymenocrater species in North East of Iran. Pakistan J. Biol. Sci., 11: 2149-2153.
- Jaganathan, S.K. (2012). Growth Inhibition by Caffeic Acid, One of the Phenolic Constituents of Honey, in HCT 15 Colon Cancer Cells. The Sci. World J., Volume 2012, Article ID 372345, 8 pages.
- Jha, H.C.; Upadhyay, S.K.; Prasad, A.J.; Jie, L.u.; Cai, Q.; Saha, A.; Robertson, S. (2013). H2AX Phosphorylation Is Important for LANA-Mediated Kaposi's Sarcoma-Associated Herpesvirus Episome Persistence. ASM. J. Virol., 87: 5255-5269.
- Jungmin, O.; Heonjoo, J.; Cho, A.R.; Kim, S.J.; Han, J. (2013). Antioxidant and antimicrobial activities of various leafy herbal teas. Food Control., 31: 403-409.

- Kanduc, D.; Mittelman, A.; Serpico, R.; Sinigaglia, E.; Sinha, A.A.; Natale, C.; Santacroce, R.; Di Corcia, M. G.; Lucchese, A.; Dini, L.; Pani, P.; Santacroce, S.; Simone, S.; Bucci, R.; and Farber, E.(2002). Cell death: apoptosis versus necrosis. Int. J. Oncol., 21: 165-170.
- Kerr, J.F.; Winterford, C.M.; Harmon, B.V. (1994). Apoptosis. Its significance in cancer and cancer therapy. Cancer., 73: 2013-2026.
- Kerr, J.F.; Wyllie, A.H. and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Cancer., 26: 239-257.
- Khalil, M.Y.; Moustafa, A.A.; Naguib, N.Y. (2007). Growth, phenolic compounds and antioxidant activity of some medicinal plants grown under organic farming condition. World J. Agricult. Sci., 3: 451-457.
- Kim, J.B.; Koo, H.N.; Joeng, H.J.; Lyu, Y.S.; Park, S.G.; Won, J.H. (2005). Induction of apoptosis by Korean medicine Gagam-whanglyunhaedoktang through activation of caspase-3 in human leukemia cell line, HL-60 cells. J. Pharmacol. Sci., 97: 138-145.
- Kintzios, S.E. (2006). Terrestrial plant derived anticancer agents and plants used in anticancer research. Crit. Rev. Plant Sci., 25: 79-113.
- Klopfleisch, R.; von Euler, H.; Sarli, G.; Pinho, S.S.; Gärtner, F.; Gruber, A.D. (2010). Molecular Carcinogenesis of Canine Mammary Tumors: News from an Old Disease. Veterinary Pathology., 228: 91-96.
- Koechli, O.R.; Perras, J.P.; Sevin, B.U. (1994) ATP-cell-viability-assay methodolgy: in both fresh gynecologic tumors and cell lines. In: Contributions to gynecology and obstetrics (series ed) Keller PJ, Zador G (eds) Koechli OR, Sevin BU, Haller U: Chemosensitivity testing in gynecologic malignancies and breast cancer. Karger, Basel, 108-121.

- Kong, J.; Khang, K.G.; Chia, L.S.; Chia, T.F. (2003). Recent advances in traditional plant drugs and orchids. Actapharmacol. Sci., 24: 7-21.
- Krishnaiah, D.; Sarbatly, R.; Nithyanandam, R. (2011). A review of the antioxidant potential of medicinal plant species. J. Food Bioprod. Process., 89: 217-233.
- Kristinova, V.; Mozuraityte, R.; Storro, I.; Rustad, T. (2009). Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants. J. Agric. Food Chem., 57: 10377-10385.
- Kukic, J.; Petrovic, S.; Niketic, M. (2006). Antioxidant activity of four endemic Stachys taxa. Biol. Pharm. Bul., 29: 725-729.
- Kumar, K.S.; Ganesan, K.; and Subba Rao, P. V. (2008). Antioxidant potential of solvent extracts of *Kappaphycus alvareii* (Doty) Doty –An edible seaweed. Food Chemistry., 107: 289-295.
- Landry, L. G.; Chappie, C.C.; Last, R.L. (1995). Arabidopsis mutants lacking phenolic sunscreens exhibit enhanced UVB injury and oxidative damage. Plant Physiology., 109: 1159-1166.
- Lecoeur, H. (2002). Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. Exp. Cell Res., 277: 1-14.
- Lee, H.J.; Cho, H.S.; Park, E.; Kim, S.; Lee, S.Y.; Kim, C.S.; Kim, K.;Kim, S.J.; Chun, H. S. (2008). Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis. Toxicology., 250: 109-115.
- Lee, K.W.; Kim, Y.J.; Lee, H.J.; Lee, C.Y. (2003). Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J. Agric. Food Chem., 51: 7292-7295.
- Lee, L.T.; Huang, Y.T.; Hwang, J. J.; Lee, A. Y. L.; Ke, F.C.; Huang, C. J.; Kandaswami, C.; Lee, P.P. ; Lee, M.T. (2004). Transinactivation of the

epidermal growth factor receptor tyrosine kinase and focal adhesion kinase phosphorylation by dietary flavonoids: effect on invasive potential of human carcinoma cells. Biochem. Pharma., 67: 2103-2114.

- Levine, A.J.; Momand, J.; Finlay, C.A. (1991). The p53 tumour suppressor gene. *Nature*, 351: 453-456.
- Li, W. X.; Cui, C. B.; Cai, B.; Wang, H. Y.; Yao, X. S. (2005). Flavonoids from Vitex trifolia L. inhibit cell cycle progression at G2/M phase and induce apoptosis in mammalian cancer cells. J. Asian Natr. Prod. Res., 7: 615-626.
- Lindenmeyer, F.; Li, H.; Menashi, S.; Soria, C.; Lu, H. (2001). Apigenin acts on the tumor cell invasion process and regulates protease production. Nutr. Cancer., 39: 139-147.
- Liu, G.; Ge, G.; Zhao, M.; Zhou, Z. (2013). Design, Synthesis and Cytotoxic Activities of Novel Aliphatic Amino-Substituted Flavonoids. Molecules., 18: 14070-14084.
- Liu, G.; Lozano, G. (2005). P21 stability: linking chaperones to a cell cycle checkpoint. Cancer Cell., 7: 113-114.
- Liu, J.S.; Kuo, S.R.; Beerman, T.A. (2003). Melendy T. Induction of DNA damage responses by adozelesin is S phase-specific and dependent on active replication forks. Mol. Cancer Ther., 2: 41-47.
- Liu, R. H. (2004). Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. J. Nutr., 134: 3479-3485.
- Louis, K.S.; Siegel, A.C. (2011). Cell viability analysis using trypan blue: manual and automated methods. Methods Mol. Biol. 740: 7-12.
- Ma, C.; Kavalier, A.R.; Jiang, B. (2011). Metabolic profiling of *Actaea* species extracts using high performance liquid chromatography

coupled with electrospray ionization time-of-flight mass spectrometry. J. Chromatogr A., 1218: 1461-1476.

- Ma, H.; Shieh, K. (2006).Western Blotting Method. J. American Sci., 2: 23-27.
- Mabberley, D.J. (1997). The Plant Book. A Portable Dictionary of the
- Martin, S.J.; Reutelingsperger, C.P.; McGahon, A. J.;Rader, J.A.; van Schie, R.C.; LaFace, D.M.; Green, D.R.(1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med.,182: 1545-1556.
- Masoudi, S.; Rustaiyan, A.; Mohebat, R.; Mosslemin, M.H. (2012). Composition of the essential oils and antibacterial activities of *Hymenocrater yazdianus*, Stachys obtusicrena and Nepeta asterotricha three Labiatae herbs growing wild in Iran. Natr. Prod. Commun., 7: 117-120.
- Massague, J. (2004). G1 cell-cycle control and cancer. Nature., 432: 298-306.
- Masters, J.R. (2012). Cell-line authentication: End the scandal of false cell lines. Nature., 492:186.
- Medana, C.; Carbone, F.; Aigotti, R.; Appendino, G.; Baicchi, C. (2008). Selective analysis of phenolic compounds in propolis by HPLC-MS/MS. Phytochem. Anal., 19: 32-39.
- Meeran, S.M.; Katiyar, S.K. (2008). Cell cycle control as a basis for cancer chemoprevention through dietary agents. Front Biosci., 13: 2191-2202.
- Melo, E.A.; Filho, J.M.; Guerra, N.B. (2005). Characterization of antioxidant compounds in aqueous coriander extract (Coriander sativum L.). LebensmWiss. Technol., 38: 15-19

- Meng, L.H., Koch, K.W.; Pommier, Y. (2007). Dose-response transition from cell cycle arrest to apoptosis with selective degradation of Mdm2 and p21WAF1/CIP1 in response to the novel anticancer agent, aminoflavone (NSC 686,288). Oncogene., 17: 4806-4816.
- Mensor, L.L.; Menezes,F. S.; Leitao, G.G.; Reis, A.S.; dos Santos, T. C.; Coube, C.S.; Leitao, S.G. (2001). Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res., 15: 127-130.
- Michels, A.A.; Nguyen, V.T.; Fraldi, A.; Labas, V.; Edwards, M.; Bonnet, F.; Lania, L.; Bensaude, O. (2003) MAQ1 and 7SK RNA interact with CDK9/Cyclin T complexes in a transcription-dependent manner dans. Mol. Cell. Biol., 23: 4859−4869.
- Miguel, M.G. (2010). Antioxidant activity of medicinal and aromatic plants. Flavour Fragr. J., 25: 291- 312.
- Miliauskas, G.; Venskutonis, P.R.; van Beek, T.A. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem., 85: 231-237.
- Mirza, M.; Ahmadi, L.; Tayebikhorrami, M. (2001). Volative constituents of Hymenocrater incanus Bunge, an Iranian endemic species. J. Flavor and Fragrance., 16: 239-240.
- Mohanasan, D.J. (2001). Screening of medical plants. Indian J. Pharmacol., 33: 460-462.
- Moldovan, G.L.; Pfander, B.; Jentsch, S. (2007). PCNA, the maestro of the replication fork. Cell., 129: 665-679.
- Moller, N.; Hempel, M.; Philipp, B.; Gross, E. (2007) Degradation of gallic acid and hydolysable polyphenols is constitutively activated in the

freshwater plant-associated bacterium *Matsuebacter* sp. FB25. Aqua. Microbial Eco., 47: 83-97.

- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods.,65: 55-63.
- Mozaffarian, V. (1996). A Dictionary of Iranian Plant Names, Tehran, Farhang-e Moaser., pp. 228-230.
- Mukherjee, K.P. (2004). Quality Control of Herbal Drugs. Business Horizons, New Delhi, pp. 405- 406.
- Nagata, M.; Fujita, H.; Ida, H.; Hoshina, H.; Inoue, T.; Seki, Y.; Ohnishi, M.; Ohyama, T.; Shingaki, S.; Kaji, M.; Saku, T.; Takagi, R.(2003). Identification of potential biomarkers of lymph node metastasis in oral squamous cell carcinoma by cDNA microarray analysis. Int. J. Cancer., 106: 683-689.
- Naghibi, F.; Mosaddegh, M.; Motamed, S.M. and Ghorbani, A., (2005). Labiatae family in folk medicine in Iran, from ethnobotany to pharmacology. Iran J. Pharmaceutical Res., 2: 63-79.
- Napolitano, G.; Licciardo, P.; Carbone, R.; Majello,B.; Lania, L. (2002). CDK9 has the intrinsicproperty to shuttle between nucleus and cytoplasm, and enhanced expres-sion of cyclin T1 promotes its nuclear localization, J. Cell. Physiol., 192:209- 215.
- Napolitano, G.; Amente, S.; Lavadera, M.L.; Palo, G.; Ambrosio, S.; Lania, L.; Dellino, G.; Pelicci, P.; Majello, B. (2013). Sequence-specific double strand breaks trigger P-TEFb-dependentRpb1-CTD hyperphosphorylation. Mutat. Res., 749: 21-27.

- Neill, R.A. (2006). Isoelectric focusing technology quantifies protein signaling in 25 cells. Proc. Natl. Acad. Sci. USA., 103: 16153-16158.
- Nguyen, T.; Nioi, P.; Pickett, C. B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J. Biol Chem., 284: 13291-13295.
- Nitiss, J.L. (2009). DNA topoisomerase II and its growing repertoire of biological functions. Nat. Rev. Cancer., 9: 327-337.
- Oh, H.M.; Kwon, B.M.; Baek, N.I.; Kim, S.H.; Chung, I.S.; and Park, M.H. (2005). Inhibitory activity of isorhamnetin from Persicaria thunbergii on Farnesyl Protein Transferase. Arch. Pharm. Res., 28: 169-171.
- Orhan, I.; Aydin, A.; Colkesen, A.; Sener, B.; Isimer, A.I. (2003). Free radical scavenging activities of some edible fruit seeds. Pharm. Biol., 41: 163-165.
- Ozgen, U.; Mavi, A.; Terzi, Z.; Yildirim, A.; Coskun, M.; Houghton, P. J. (2006). Antioxidant properties of some medicinal Lamiaceae species. Pharm. Biol., 44: 107-112.
- Parejo, I.; Viladomat, F.; Bastida, J.; Rosas-Romero, A.; Flerlage, N.; Burillo, J.; Codina, C. (2002). Comparison between the radical scavenging activities and antioxidant activity of six distilled and nondistilled maditerranean herbs and aromatic plants. J. Agric. Food. Chem., 50: 6882-6890.
- Peng, D.F.; Hu, T.L.; Schneider, B.G.; Chen, Z.; Xu, Z.K.; El-Rifai, W. (2012). Silencing of glutathione peroxidase 3 through DNA hypermethylation is associated with lymph node metastasis in gastric carcinomas. PloS One., 7(10):e46214.

- Piskunova, T.S.; Yurova, M.N.; Zabezhinski, M.A.; Ansimov, V.N. (2007). Poly (ADP-rybosa) polymerase-the relationsips with life span and carcinogenesis. Adv. Geronto., 20: 82-90.
- Plochmann, K.; Korte, G.; Koutsilieri, E.; Elke Richling, E.; Riederer, P.; Rethwilm, A.; Peter Schreier, P.; Scheller, C. (2007) Structure–activity relationships of flavonoidinduced cytotoxicity on human leukemia cell. Arch. Biochem. Biophys., 460: 1-9.
- Polo, S.E.; Jackson, S.P. (2011). Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes Dev., 25: 409-433.
- Pommier, Y.; Cushman, M. (2009). The indenoisoquinoline noncamptothecin topoisomerase I inhibitors: update and perspectives. Mol. Cancer Ther., 8:1008-1014.
- Power, K.A.; Ward, W.E.; Chen, J.M.; Saarinen, N.M.;Thompson, L.U. (2006).Genistein alone and in combination with the mammalian lignans enterolactone and enterodiol induce estrogenic effects on bone and uterus in a postmenopausal breast cancer mouse model. Bone., 39: 117-124.
- Powolny, A.; Xu, J.; Loo, G. (2001). Deoxycholate induces DNA damage and apoptosis in human colon epithelial cells expressing either mutant or wild-type p53. Int. J. Biochem. Cell Biol., 33: 193-203.
- Puthalakath, H.; Strasser, A. (2002). Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ., 9: 505-512.
- Quist, S.R.; Wang-Gohrke, S.; Kohler, T.; Krienberg, R.; Runibaum, I.B. (2004). Cooperative effect of adenoviral p53 gene therapy and standard chemotherapy in ovarian cancer cells independent of the endogenous p53 status. Cancer Gene Ther., 11: 547-554.

- Rai, R.; Kiran, L.; Sharma, S.; Misra, S.; Kumar, A.; Mittal, B. (2014). Death Receptor (DR4) Haplotypes Are Associated with Increased Susceptibility of Gallbladder Carcinoma in North Indian Population. PLoS One., 9(2): e90264.
- Raymond, M.H.; Sandy, A.; Andrey, L.B.; Philip, D.C.; Barry, J.C.; Renee, J.G.; Madeline, M.H.; Rogier, P.J.; Tatyana, V.K.; Ramon, M.; Alan, J.P. Olof , R. (2004). Labiatea. In: Klaus K (ed.) the families and genera of vascular plants. Vol. VII. Springer-Verlag, Berlin, Germany.167-275.
- Rechinger, K.H. (1982). Hymenocrater Fisch & CA Mey. In K.H. Rechinger (Ed.), Flora Iranica, Labiatae 150: 495-504. Graz, Austria: Akademische Druck-u Verlagsanstalt.
- Rechinger, K.H. (1982). Hymenocrater Fisch & CA Mey. In: Rechinger KH (ed) Flora Iranica, Labiatae, No 150. Akademische Druck-u Verlagsanstalt, Graz (Austria), 239-250.
- Reddy, L.; Odhav, B.; Bhoola, K. (2003). Natural products for cancer prevention: global perspective. Pharmacol. Ther., 99: 1-13.
- Ren, W; Qiao, Z.; Wang, H.; Zhu, I.; Zhang, L. (2003). Flavonoids: Promising AnticancerAgents. Med. Res. Rev., 234: 519-534.
- Ribeiro, L.H.; Prota, C.; Gomes, C.M. (2010). Long-term effect of early postoperative pelvic floor biofeedback on continence in men undergoing radical prostatectomy: a prospective, randomized, controlled trial. J. Urol., 184: 1034-1039.
- Rice-Evans, C. A.; Millar, N.J.; Bolwell, P.G.; Bramley, P.M.; Pridham, J. B. (1996). The relative antioxidant activities of plant derived polyphenolic flavonoids. Free Radical Res., 22: 375-383.

- Rogakou, P.; Neira, N.W.; Boon,C.; Pommier,Y.; William M. (2000). Initiation of DNA Fragmentation during Apoptosis Induces Phosphorylation of H2AX Histone at Serine 139. jbc., 275: 9390-9395.
- Saito, K.; Meyer, R.; Warner, A.; Basu, R.B.; Ray, R. (2006). Ray Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein J. Virol., 80: 4372-4379.
- Satil, F.; Unal, M.; Hopa, E. (2007). Comparative Morphological and Anatomical Studies of Hymenocrater bituminosus Fisch. (Lamiaceae) in Turkey. Turk J. Bot., 31: 269-275.
- Schulz, E.M.; Nicewander, W.A. (2005). Grade Equivalent and IRT Representations of Growth. J.E.M., 34: 293-372.
- Sengul, M.; Yildiz, H.; Gungor, N.; Cetin, B.; Eser, Z.; Ercisli, S. (2009). Total phenolics content, antioxidant and antimicrobial activities of some medicinal plants. Pak. J. Pharm. Sci., 22: 102-106.
- Shen, X.G.; Wang, C.; Li, Y.; Wang, L.; Zhou, B.; Xu, B.; Jiang, X.; Zhou, Z.G.; Sun, X.F.(2010). Downregulation of caspase-9 is a frequent event in patients with stage II colorectal cancer and correlates with poor clinical outcome. Colorectal Dis., 12:1213-1218.
- Shimoi, K.; Masuda, S.; Furugori, M.; Esaki, S.; Kinae, N.(1994). .Radioprotective effect of antioxidative flavonoids in gamma-ray irradiated mice. Carcinogenesis., 15: 2669-2672.
- Simoni, D.; Tolomeo, M.; Retinoids (2001). Apoptosis and cancer. Curr. Pharm. Des., 7: 1823-1837.
- Singh, V.V.; Dutta, D.; Ansari, M.A.; Chandran , S.D. (2013). Kaposi's Sarcoma-Associated Herpesvirus Induces the ATM and H2AX DNA

Damage Response Early during *De Novo* Infection of Primary Endothelial Cells, Which Play Roles in Latency Establishment. J. Virol., 88: 2821-2834.

- Slatter, T.L.; Hung, N.; Campbell, H.; Rubio, C.; Mehta, R.; Renshaw, P.; Williams, G.; Wilson, M; Engelmann, A.; Jeffs, A.; Royds, J.A.; Baird, M.A.; Braithwaite; A.W. (2011). Hyperproliferation, cancer, and inflammation in mice expressing a 133p53-like isoform. Blood., 117: 5166-5177.
- Slavov, N.; Botstein, D. (2011). Coupling among Growth Rate Response, Metabolic Cycle and Cell Division Cycle in Yeast. J. Mol. Biol. Cell., 22: 1997-2009.
- Small, S.; Keerthivasan, G.; Huang, Z.; Gurbuxani, S.; Crispino, J.D. (2010).Overexpression of survivin initiates haematologic malignancies in vivo. Leukaemia., 24: 1920-1926.
- Soengas, M.S.; Capodieci, P.; Polsky, D.; Mora, J.; Esteller, M.; Opitz-Araya, X. (2001) Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature., 409: 207-211.
- Song, Y.; Cook, N.R.; Albert, C.M.; Van Denburgh, M.; Manson, JE.(2009). Effect of homocysteine-lowering treatment with folic Acid and B vitamins on risk of type 2 diabetes in women: a randomized, controlled trial. Diabetes., 58: 1921-1928.
- Srivastava, R.K.; Chen, Q.; Siddiqui, I.; Sarva, K.; Shnkar, S. (2008). Linkage of curcumin-induced cell cycle arrest and apoptosis by cyclin-dependent kinase inhibitor p21WAF1/CIP1. Cell Cycle., 6: 2953-2961.
- Steensma, D.; Timm, M.; Witzig, T. (2003). Flow Cytometric Methods for Detection and Quantification of Apoptosis. Methods Molec. Med., 85: 323-332.

- Suh, E.J.; Kim, T.Y.; Kim, S.H. (2006). PP2Cgamma-mediated S-phase accumulation induced by the proteasome-dependent degradation of p21 (WAF1/CIP1). FEBS Lett., 580: 6100-6104.
- Sulaiman, G.M.; Sammarrae, K.W.A.; Ad'hiah.A.H . (2011). Chemical characterization of Iraqi propolis samples and assessing their antioxidant potentials. Food Chem. Toxicol., 49: 2415-2421.
- Szliszka, E.; Czuba, Z. P.; Mazur, B.; Sedek, L.; Paradysz, A.; Krol, W. (2009). Chalcones enhance TRAIL-induced apoptosis in prostate cancer cells. Int. J. Mol. Sci., 11: 1-13.
- Taherpour, A.; Maroofi, H.; Changizi, M.; VafaeiShoushtari, R.; Larijani, K.; Kazempour, A. (2011).Chemical compositions of the essential oil and calculation the biophysicochemical coefficients of the Components of *Hymenocrater longiflorus* Benth, of Iran. Nat. Sci., 3: 104- 108.
- Takeuchi, H.; Lu, Z.G.; Yu, O. (2007). Distinct crucial roles of flavonoids during legume nodulation. Trends in Plant Sci. 12: 282-283.
- Tan, G.; Gyllenhaal, C.; Sorjarto, D.D. (2006). Biodiversity as a source of anticancer drugs. J. Curr. Drug Targets., 7: 265-277.
- Taylor, W. R.; Stark, G. R. (2001). Regulation of the G2/M transition by p53. Oncogene., 20: 1803-1815.
- Tepe, B.; Sokmen, M.; Akpulat, H. A.; Sokmen, A. (2005). In vitro antioxidant activities of the methanol extracts of four Helichrysum species from Turkey. Food Chem., 90: 685-689.
- Teras, L.R.; Goodman, M.; Patel A.V.; Diver W.R.; Flanders, W.D. and Feigelson H.S. (2011). Weight loss and postmenopausal breast cancer in a prospective cohort of overweight and obese US women. J. Cancer Cause Control., 22: 573-579.

- Toledo, F.; Wahl, G.M. (2006). Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat. Rev. Cancer., 6: 909-923.
- Torkin, R.; Lavoie, j.; Kaplan, D.R.; Yeger, H. (2005). Induction of caspase-dependent, p53-mediated apoptosis by apigenin in human neuroblastoma. Mol. Cancer Ther., 4: 1-11.
- Ulubelen, A. (2003). Cardioactive and antibacterial terpenoids from Salvia species. Phytochemistry., 64: 395-399.
- Vanags, D.M.; Porn-Ares, M.I.; Coppola, S.; Burgess, D.H.; Orrenius, S. (1996).Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. J. Biol. Chem., 271: 31075-31085.
- Vassilev, A. (2004). Cadmium induced changes in chloroplast lipids and photosystem activities in barley plants. Biologia Plantarum., 48: 153-156.
- Vikhanskaya, F.; Lee, M.K.; Mazzoletti, M.; Broggini, M; Sabapathy, K. (2007) .Cancer-derived p53 mutants suppress p53-target gene expression-potential mechanism for gain of function of mutant p53.Nucl. Acids Res., 35: 2093-2104.
- Vogelstein, B., Kinzler, K.W. (2004). Cancer genes and the pathways they control. Nat. Med., 10: 789-799.
- Wagstaff, S. J.; Olmsread ,R. G. (1997). Phylogeny of the Labiatae and Verbenaceae inferred from rbcL sequences. Syst. Botany., 22: 165-179.
- Wang, Q.; Goldstein, M.; Alexander, P.; Timothy, P.; Sun, T.; Feng, J.; Lou, Z.; Michael, B. ; Wang, X. (2014). Rad17 recruits the MRE11-RAD50-NBS1 complex to regulate the cellular response to DNA double-strand breaks. J. EMBO., 33: 529-664.
- Wang, W.; El-Deiry, W.S. (2003). Cancer Biol. Ther., 2: 196-202.

- Wang, W.; Van Alstyne, P.C.; Irons, K.A. (2004). Individual and interactive effects of apigenin analogs on G2/M cell-cycle arrest in human colon carcinoma cell lines. Nutr. Cancer., 48:106 -114.
- Watts, M.; Pankhurst, N.W.; Pryce, A.; Sun, B. (2003). Vitellogenin isolation, purification and antigenic crossreactivity in three teleost species. Comparative Biochemistry and Physiology, Part B, Biochemistry and molecular biology., 134: 467-476.
- Wei, Y.; Fan, T.; Yu, M. (2008). Inhibitor of apoptosis proteins and apoptosis. Acta. Biochim. Biophys. Sin., 40: 278-288.
- Weiss, R.H.; Marshall, D.; Howard, L.; Corbacho, A.M.; Cheung, A.T.; Sawai, E.T. (2003). Suppression of breast cancer growth and angiogenesis by an antisense oligodeoxynucleotide to p21(Waf1/Cip1). Cancer Lett., 189: 39-48.
- Wersto, R.P; Chrest, F.J.; Leary, J.F.; Morris, C; Stevenson, S.M.A.; Gabrielson, E. (2001). Doublet discrimination in DNA cell-cycle analysis. Cytometry., 46: 296-306.
- White, M. K.; Cinti, C. (2004). A morphologic approach to detect apoptosis based on electron microscopy. Methods Mol. Biol., 285: 105-111.
- Willingham, M.C. (1999). Cytochemical methods for the detection of apoptosis. J. Histochem. Cytochem., 47:1101–1109.
- World Health Organization, (2005). Public Health Agency of Canada. Preventing chronic diseases: a vital investment. Geneva, World Health Organization.
- World Health Organization, Cancer, (2006).
- World Health Organization, UNICEF (2006). Meeting the MDG drinking-water and sanitation target: the urban and rural challenge of the decade.

Geneva, World Health Organization and UNICEF Joint Monitoring Programmed for Water Supply and Sanitation.

- World Health Organization: Cancer, (2005).
- Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature., 284: 555-556.
- Xia, M.; Wang, D.; Wang, M.; Tashiro, S.; Onodera, S.; Minami, M. (2004). Dracorhodin percholarate induces apoptosis via activation of caspases and generation of reactive oxygen species. J. Pharmacol. Sci., 95: 273-283.
- Ye, R.; Goodarzi, A.A.; Kurz, E.U.; Saito, S.; Higashimoto, Y.; Lavin, M.F.; Appella, E.; Anderson, C.W.; Lees-Miller, S.P. (2004). The isoflavonoids genistein and quercetin activate different stress signaling pathways as shown by analysis of site-specific phosphorylation of ATM, p53 and histone H2AX. DNA Repair., 3: 235-244.
- Yin, B.K.; Ling, S.C.; Balaram, P. (2010). Apoptotic effects of chrysin in human cancer cell lines. Int. J. Mol. Sci. 11: 2188-2199.
- Yu, S.W.; Andrabi, S.A.; Wang, H.; Kim, N.S.; Poirier, G.G.; Dawson, T.M.; Dawson, V.L. (2006). Apoptosis-inducing factor mediates poly (ADP-ribose) (PAR) polymer-induced cell death. Proc. Natl. Acad. Sci. U.S.A., 103: 18314-18319.
- Zaidi, M.A.; Crow, S.J. (2005). Biollogically active traditional medicinal herbs from Balochistan, Pakistan. J. Ethnopharmacol., 96: 331-334.
- Zerfaoui, M.; Suzuki, Y.; Naura, A.S.; Hans, C.P.; Nichols, C.; Boulares, A.H. (2008). Nuclear translocation of p65 NF-kappaB is sufficient for

VCAM-1, but not ICAM-1, expression in TNF-stimulated smooth muscle cells: Differential requirement for PARP-1 expression and interaction. Cell. Signal., 20: 186-194.

- Zhang, A.J.; Zheng, M. Y. ; Zou, H. J. ; Luo, X. M. ; Jiang, H. L. (2007). 3D-QSAR study of 20 (S)-camptothecin analogs. Acta. Pharmacologica Sinica., 28: 307-314.
- Zheng, W.; Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. J. Agri. Food Chemistry., 49: 5165-5170.

الخلاصة

أجريت هذه الدراسة لتقييم تأثير المستخلص الميثانولي الخام لنبات ال *Hymenocrater longiflorus* على الخط الخلوي لعظمية الإنسان U2OS و الخط الخلوي لسرطان قولون الانسان RKO.

تم التحليل الكيميائي للمستخلص الميثانولي باستخدام تحليل MS-ESI/HPLC لمركبات الفينول (الفلافونويد، الفينول واسترات الأحماض). تم الكشف عن أحد عشر مركبا في هذا النبات وهي:-

Acacetin و Apigenin وCrismartin وCaffeic acid و Ferulic acid وCarnosic acid

apigenin-7-O-glucoside و Genistein و Isorhamantin و N-Carboxybenzyloxy Cysteinyl Cystein وRosmaric acid .

تم تقييم فعالية ازالة للجذور الحرة ل *H. longiflorus* باستخدام (DPPH). أظهرت النتائج أن *H. longiflorus* لديه نشاط مضاد للأكسدة، وهذا النشاط يعتمد على زيادة تركيزالنبات حتى يصل إلى 86.226٪ ARA في تركيز 1000 ميكروغرام في الملتر الواحد.

كشفت دراسة أجريت في المختبر على حيوية الخلايا أن النبات له تأثير مثبط على خلايا السرطان (U2OS وRKO) باستخدام التريبان الأزرق مع Haemocytometer وMTT الفحص التي تحول اللون الأصفر إلى اللون الأرجواني،حيث لوحظ تاثير كبير لانخفاض النمو وانخفاض ترقيم الخلايا بالمقارنة مع الخلايا غير المعالجة.

من خلال تقييم اعتقال دورة الخلية باستخدام صبغة PI من قبل الإسفار المنشط فارز الخلية (نظام مراقبة الأصول الميدانية) التحليل باستخدام التدفق الخلوي، حيث لوحظ وجود تأثير معتدل في المرحلة G2 لخلايا U2OS وفي المرحلة G1 لخلايا RKO وكان المستحث الفسفرة من H2AX الكسر المزدوج على الحامض النووي فواصل حبلا مزدوجة نشأت من أصول تتباعد γH2AX بما في ذلك الأضرار الخارجية ، واستنساخ شوكة التضاعف Replication fork للنهايات و موت الخلايا المبرمج باستخدام فحص Immunoflourescence ، من خلال هذا التراكم فحص الضرر ومقارنته مع الخلايا غير المعالجة في كل من U2OS و RKO .

دراسة الخلايا باستخدام صبغة DAPI و صبغة Sulforhodamine ، أوضحت انخفاض في حجم الخلية وانكماش الخلايا لكلا من U2OS و RKO ، وهذه النتائج هي من علامات موت الخلايا المبرمج.

ولوحظ البروتين و الحمض النووي من التلف باستخدام تحليل لطخة غربية ، واستخدام عدة بروتينات (P21 و P53 و P- P53 و γH2AX و PARP) ، لوحظ زيادة البروتينات في الحجم مع الانقسام من البروتين PARP في شريطين ، والذي يعد مؤشرا على موت الخلايا المبرمج بالإضافة إلى زيادة البروتين p53 والبروتينات γ H2AX لكلا من U2OS و RKO .

من جميع النتائج التي تم الحصول عليها ، يمكننا القول ان *H. longiflorus* هو نبات مضاد للسرطان نظرا لتأثيره على نمو الخلايا ، ضرر في الحمض النووي DNA وتوقف دورة حياة الخلية والتي تؤدي إلى موت الخلايا المبرمج ، مع الأخذ بعين الاعتبار أن خط الخلية RKO كان أكثر حساسية من خط خلية U2OS مقارنة مع الخلايا غير المعاملة (السيطرة).

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

دراسة السمية الخلوية والتقييم الجزيئي لنبات ال *Hymenocrater longiflorus* في الخلايا السرطانية للانسان

اطروحة مقدمة إلى كلية العلوم /جامعة النهرين

وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في

علوم التقانة الاحيائية

من قبل

رفل شكيب عبد الوهاب العاني

بكالوريوس تقانة احيائية 2002

ماجستير تقانة احيائية 2006

بأشراف

ا.د خلود وهيب السامرائي **ا.م.د غسان محمد سليمان**

ذو القعدة 1435 **ايلول** 2014