Republic of Iraq

Ministry of Higher Education and

Scientific Research

AL-Nahrain University

College of Science

Molecular Study of BCR-ABLGene and Genetic Variations in Chronic Myeloid Leukemia Patients in Iraq

A thesis

Submitted to the College of Science/AL-Nahrain University as a partial fulfillment of the requirements for the Degree Master of Science in Biotechnology.

By

Yasameen Ali Hadi AL-Yasiri

B.Sc. Biology/College of Science/Al-Mustanssryia University 2000

Supervised by

Dr. Saleh Ahmed wohaieb
professor

Dr. Bilal Kamil Sulaiman
Lecturer

November 2011 Thu Al-Haja 1432

Supervisors Certification

We, certify that this thesis entitled" **Molecular study of the BCR-ABL gene and genetic variations in Chronic Myeloid Leukemia patients in Iraq**" under our supervision at the College of Science/Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in **Biotechnology**.

Signature:
Supervisor: Dr.Saleh A. Wohaib
Scientific degree: professor
Date:

Signature:
Supervisor: Dr. Bilal K. Sulaiman
Scientific degree: Lecturer
Date:

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Signature:
Name: Dr. Majed Husein. Al-Gelawi
Scientific degree: Professor
Title: Head of Biotechnology Department
Date:

Committee Certification

We, the examining committee certify that we have read this thesis entitled "**Molecular study of the BCR-ABL gene and genetic variations in Chronic Myeloid Leukemia patients in Iraq**" and examined the student" Yasameen Ali Hadi "in its contents and that in our opinion, its accepted for the Degree of Master in Biotechnology.

Signature:
Name: Dr. Khulood W. Abood
Scientific Degree: Professor
Date:
(Chairman)

Signature:
Name: Dr. MushtakT.S. Al-Ouqaili
Scientific Degree: Ass. Professor
Date:
(Member)

Signature:
Name: Dr. Ayad M. Ali
Scientific Degree: Ass.Professor
Date:
(Member)

Signature:
Name: Dr. Saleh A. Wohaib
Scientific degree: professor
Date:
(Member and supervisor)

Signature:
Name: Dr. Bilal K.Sulaiman
Scientific degree: Lecturer
Date:
(Member and supervisor)

I hereby certify upon the decision of the examining committee.

Signature:
Name: Dr. Khulood Wahyeb. Abood
Scientific Degree: Professor
Title: Dean of the Collage of Science
Date:

Acknowledgment

First, ***I*** thank the merciful Allah for helping me to finish this work. ***I'd*** like to express my great respect and sincere gratitude to my supervisors Prof. Dr. Saleh Wohaieb and Dr. Bilal Kamil for their excellent guidance and encouragement; ***I*** wish them all the best. Also ***I*** would like to express my appreciation to the staff members of Biotechnology Research Center at Al –Nahrain University for their assistance and advice during the years of this study. Words of thanks and appreciation to the previous maneger of Biotechnology research center and current Dean of college of science Prof. Dr. Khulood Al-Samaraie for her unlimited support. Also, ***I*** would like to thank Dr. Majeed Arsheed, Dr. Farooq Abraheem, Dr. Saad Al- Neda, Mrs. Shamiaa Yousif, Miss. Ghassiq Tariq, Mr. Anas Noori, Mrs. Luma Burhan, Mr. Ghaith Lutfi, Mrs. Haybia Yonan and all the staff of Medical molecular Biotechnology dept. Thanks and appreciation to Dr. Adeeb Al-Shami, the manager of the National Center of hematology for his generous help and the staff of the center, mainly Mrs. Luma Amir, Miss Ban lateef and Miss Ahllam for their assistance and advices.

My thank goes to all the patients and people who participated in this study and made this project possible.

Most of all I am sincerely grateful to my parents, sisters and all my family members for their help and support throughout my study.

Yasameen

Dedication

To my father……..

For his unlimited care, guidance and

Sacrifices

To my lovely mother …….

For her unlimited love and

Kindness

To my dearest sisters……

Shaymaa, Basmaa, Sabreen and their family

I dedicate this work

In gratitude for your

Support

Patience

Understanding

Yasameen

2011

Summary

This study is conducted to investigate the possible antileuckemic activity of ethanolic olive leaves extract against a group of patients with chronic myeloid leukemia (CML), diagnosed using Real Time-PCR.

The response of those individuals to the treatment with Imatinib and the response of selected individuals to the treatment with olive leaves ethanolic extract were followed up using same method. Selected individuals of the chronic myeloid leukemia patients ere investigated for the genetic instability using Random Amplified Polymorphisum DNA-Polymerase chain reaction. Blood samples collected from 48 CML patients and 12 apparent healthy individual.

Mononuclear cells (MNCs) isolated from both patients and controls using ficoll, while RNA was extracted using the method of Trizol. The RNA quantity and quality have been detected using spectrophotometer and a garose gel electrophoresis, respectively. Extracted RNA was used for synthesis of cDNA, to be used in the Real Time –PCR.

Real Time-PCR results showed the presence of BCR-ABL fusion gene forming the P210 protein in 43 out of 48 CML patients. The other 5 patients may have expressed another type of fusion genes.

The active compounds in the olive leaves ethanolic extract were detected and analyzed using chemical methods and results showed the presence of flavonoids, terrpens, glycosides and steroids while no alkaloid appeared in the extract.

Three different concentrations (800, 1200, and 1450) µg/ml of the olive leaves extract applied on the MNCs of CML patients and controls, and

incubated at 37˚C for 48 hr. Then the treated cells were recollected and used for the RNA extraction followed by Real Time-PCR to verify the presence of any change in the quantity of the expressed fusion gene in comparison with values obtained prior to treatment with OLE.

Results showed no significant differences between the expression levels of the treated cells with that measured before the treatment for the three concentrations.

Because genetic instability is a known phenomenon associated with the development of cancer, RAPD-PCR is used in the study to investigate the presence of any genetic instability for group of CML patients and group of normal controls. Twelve universal primers selected for the above mentioned purpose, 5 of them were already selected based on preliminary experiments.

The results of RAPD-PCR revealed that some primers produced distinguished patterns in a group of patients with unique bands. This finding deserves further studies to assess its clinical significance, and whether or not they can be validated as a potential genetic marker for diagnosis of CML, or to assess the CML patient's response to antileukemic therapy.

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List of Abbreviation

ALL	**Acute lymphoblastic leukemia**
CLL	**Chronic lymphoblastic leukemia**
AML	**Acute myeloid leukemia**
CML	**Chronic myeloid leukemia**
ANL	**Acute non lymphoblastic leukemia**
AGL	**Acute granulocytic leukemia**
HCL	**Hairy cell leukemia**
PH	**Philadelphia chromosome**
ABL	**Abelson murine leukemia virus**
BCR	**Break point cluster region**
PCR	**Polymerase chain reaction**
RAPD	**Random amplified polymorphic DNA**
RT-PCR	**Reverse transcriptase polymerase chain reaction**
cDNA	**Complimentary deoxy nucleotide**
PB	**Peripheral blood**
Q-PCR	**Quantitative polymerase chain reaction**
OLE	**Olive leaves extract**
FBS	**Fetal calf serum**
MNCs	**Mononuclear cells**
RPMI	**Rose park media institute 1640**
TBE	**Tris-Borite –EDTA**

1.1 Introduction

Leukemia is a progressive, malignant disease of the blood forming organs characterized by distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow, and can be classified clinically on the basis's of duration and character of the disease into acute and chronic leukemia (Hoffbran *et al.,* 2006).In Iraq, Leukemia ranks second among ten types of cancer in 2004 (Ministry of Health , Iraqi Cancer Board , 2008), while in 1989,it ranked seven among the ten types of cancer (Ministry of Health , Iraqi Cancer Board , 1993).

One of the important types of leukemia **is chronic myeloid leukemia (CML),** which is the stem cell disorder with acquired chromosomal abnormality, Philadelphia chromosome (ph) which arises from the reciprocal translocation of part of the long arm of chromosome 9 in which protoonco gene ablson gene (ABL) is located to long arm of chromosome 22, in which break point cluster region BCR is located. (Mughal and Goldman, 2007).The BCR-ABL fusion gene can be detected using several molecular methods. Different kinds of PCR technique have been extensively utilized for the detection and characterization of mutation associated with tumor development (Wang *et al.*, 2001; Boultwood and Fiddler, 2002).

Reverse transcription- polymerase chain reaction (RT-PCR) is one of the most common techniques used for analysis of gene expression due to its simplicity, rapidity and sensitivity (Peter *et al.,* 2004; Dahii, 2010)

Random Amplified Polymorphic DNA-Polymerase chain reaction (RAPD-PCR) method can simply and rapidly used for the detection of genetic alterations in the entire genome without need of previous knowledge of specific

DNA sequence. It is also used as a mean for identifying the genetic alterations in human tumors (Wang *et al*., 2001).

Real time PCR or quantitative PCR is currently the most sensitive method to determine the amount of a specific DNA in complex biological samples (klein, 2002; Liss and Rober, 2004). Genomic analysis is carried out during ongoing amplification using fluorescent reporters (Wilhclm and Pingoud, 2003).

Herbal medicine has frequently been used in the prevention and treatment of cancer. A great deal of pharmaceutical research in advanced countries had considerably improved quality of the herbal medicine used in treatment of cancer**.** ***Olea europea*** **L.,** is well known plant in most countries and very useful in many clinical applications. The active compound of olive leaf is **oleuropein**, which exhibited potent antioxidant with anti-inflammatory properties (Benavente-Garcia *et al.,* 2006). It has pharmacological and health promoting properties including hypoglycaemic, anti mycoplasmal (Al-Azzawie and Alhamdani, 2006), anti viral (Micol *et al.*,2005), anti -tumour and antigenic activities(Hamdi and Castellon,2005) . Epidemiological studies, has correlated with the low incidence of some types of cancer (colorectal, breast cancer), atherosclerosis, coronary heart disease in the Mediterranean countries with olive consumption in the Mediterranean diet (Fki *et al.*, 2005).

Objectives of the study:

1. Isolation of Mononuclear cells from controls and CML patients
2. Detection and evaluation of the genetic alteration of CML patients using Reverase transcription-PCR and Real time PCR.
3. To investigate the possible effect of olive leaf ethanolic extract on the expression of BCR-ABL gene *in vitro*.
4. Studying the genetic variations of CML patients using Random Amplified Polymorphism- Polymerase chain reaction technique.

1.2 Literature

1.2.1. Leukemia

Leukemia is cancer of the blood or bone marrow characterized by an abnormal increase of blood cells usually leukocytes (white blood cells).The course of leukemia may vary from a few days or weeks to many years, depending on the type of disease (John, 2000; Chen *et al.*, 2010) figure(1-1).

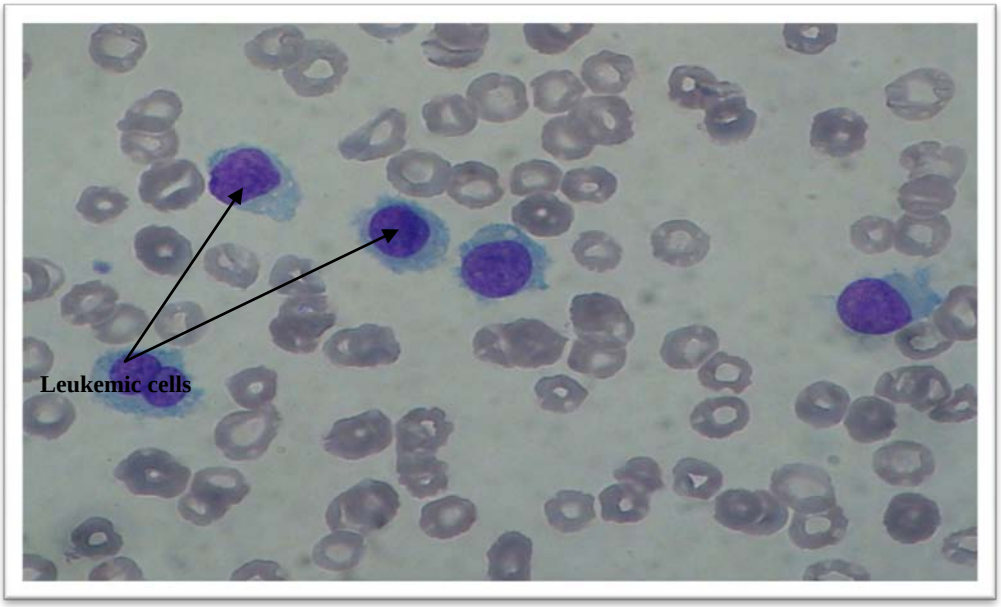

Figure (1-1): leukemic cells

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1.2.1.1 Classification of Leukemia

Leukemia is clinically and pathologically subdivided into a variety of large groups. The first sub- division is into an acute and chronic form (Mughal *et al.*, 2006):

- **Acute leukemia** is characterized by the rapid increase of immature blood cells. This crowding makes the bone marrow unable to produce healthy blood cells. Immediate treatment is required in acute leukemia due to the rapid progression and accumulation of malignant cells, which then spill over into the blood stream and spread to other organs of the body. Acute forms of leukemia are the most common forms of leukemia in children.
- **Chronic leukemia** is distinguished by the excessive build up of relatively mature, but still abnormal, white blood cells. Typically taking months or years to progress, the cells are produced at a much higher rate than normal cells, resulting in many abnormal white blood cells in the blood. Chronic leukemia mostly occurs in older people.

Additionally, leukemia's are subdivided according to the type of white blood cells affected, either into lymphocytic leukemia or myeloid leukemia (Goldman *et al*., 1999):

- **Acute lymphoblastic leukemia (ALL):** Childhood acute lymphoblastic leukemia (ALL) is a disease in which too many underdeveloped lymphocytes are found in a child's blood and bone marrow. ALL is the most common form of leukemia in children, and the most common kind of childhood cancer *(*Moorman *et al*., 2006).Acute lymphoblastic leukemia (ALL) represents nearly one third of all pediatric cancers. Annual incidence of ALL in USA is about 30 cases per million populations, with a peak incidence in patients aged 2-5 years. Although a small percentage of cases are associated with inherited genetic syndromes, the cause of ALL remains largely unknown (Jeffrey, 2005).
- **Chronic lymphocytic leukemia (CLL)*:*** is a disease in which mature lymphocytes become cancerous and gradually replace normal cells in lymph nodes. More than three fourths of the people who have chronic lymphocytic

leukemia (CLL) are older than 60. This type of leukemia affects men 2 to 3 times more often than women. CLL is the most common type of leukemia in North America and Europe. It is rare in Japan and Southeast Asia, which indicates that heredity plays some role in its development (Wierda *et al.*, 2007).

- **Acute myelogenous leukemia (AML):** is also known as acute myelotic leukemia, acute myeloblastic leukemia, acute none lymphocytic leukemia (ANLL) and acute granulocytic leukemia (AGL) (Heffner *et al.*, 2007). This type occurs more commonly in adults than in children, although it does occurs with some frequency in older ones. There appears to be two peaks in the disorder one at 15 to 20 years of age and another peak after 50 years of age (Lowenberg *et al*., 1999).
- **Chronic myelogenous leukemia (CML):** occurs mainly in adults. A very small number of children also develop this disease. Treatment is with imatinib (Gleevec) or other drugs.

1.2.1.2 Risk factors

Several factors have been identified and exposure to either one puts a person at a higher risk of developing leukemia. These factors include:

1.2.1.2.1 Genetic Factors: Leukemia, like other cancers, results from somatic mutations in the DNA. Certain mutations produce leukemia by activating oncogenes or deactivating tumor suppressor genes, and thereby disrupting the regulation of cell death, differentiation or division. These mutations may occur spontaneously or as a result of exposure to radiation or carcinogenic substances, and are likely to be influenced by genetic factors (Rowley, 2001). Some people have a genetic predisposition towards developing leukemia. This predisposition is demonstrated by family histories and twin studies. The affected people may

have a single gene or multiple genes in common. In some cases, families tend to develop the same kind of leukemia as other members; in other families, affected people may develop different forms of leukemia or related blood cancers (Arzanian *et al.,* 2006**;** Aoun *et al.,* 2007**).**

1.2.1.2.2 Environmental factors:

Ionizing radiation like gamma-ray was the first identified agent associated with induction of leukemia and this become apparent in the survivors of atomic bomb explosions in Hiroshima and Nagasaki (Henshaw *et al.,* 1990). In addition, patients who received radiotherapy for malignant disease may have a chance to develop leukemia (Goldman and Tarig, 1990). The fact that exposure to benzene may induce leukemia was proposed in the late nineteenth of century because of immunosuppressive effect for a range of lymphoid and myeloid malignancies (Rinsky and Young, 1981). Also, the exposure to paints, herbicides, pesticide and chemicals that are used in chemotherapy of various cancers may result in gene mutations, which lead to leukemia (korte, 2007). Viruses have also been linked to some forms of leukemia. Experiments on mice and other mammals have demonstrated the relevance of retroviruses in leukemia; human retroviruses have also been identified. The first human retrovirus identified was human T-lymphotropic virus or HTILV-1 which is known to cause adult T-cell leukemia (Butel, 2007).

1.2.2 Chronic myelogenosis leukemia:

Chronic myeloid leukemia (CML), also known as chronic myelogenous leukemia was first described in 1960 and was the first disease state to be associated with a specific cytogenetic abnormality (Nowell *et al.,* 1960) (figure 1-2).Chronic myeloid leukemia has been the showcase disease for hematologists for nearly 50 years since the discovery of the Philadelphia chromosome (Ph) as the hallmark of the disease in 1960 by Nowell and Hungerford and the first

instance of an acquired chromosomal change linked with a human malignancy. In 1973, Rowley recognized this as a reciprocal translocation, the first such description between chromosomes 9 and 22, juxtaposing the BCR and ABL genes onto the shortened derivative chromosome 22, producing a fusion protein with abnormal tyrosine kinase activity. BCR-ABL was identified in 1982 and, in 1990; cells transected with BCR-ABL produced a myeloproliferative disorder typical of CML in a mouse model (Daley *et al.*, 1990). Recently, CML has been the first human malignancy treated with a gene-targeted therapy, imatinib. The five-year survival rate is 90% (Mughal & Goldman, 2007; Joske, 2008) (figure 1-2).

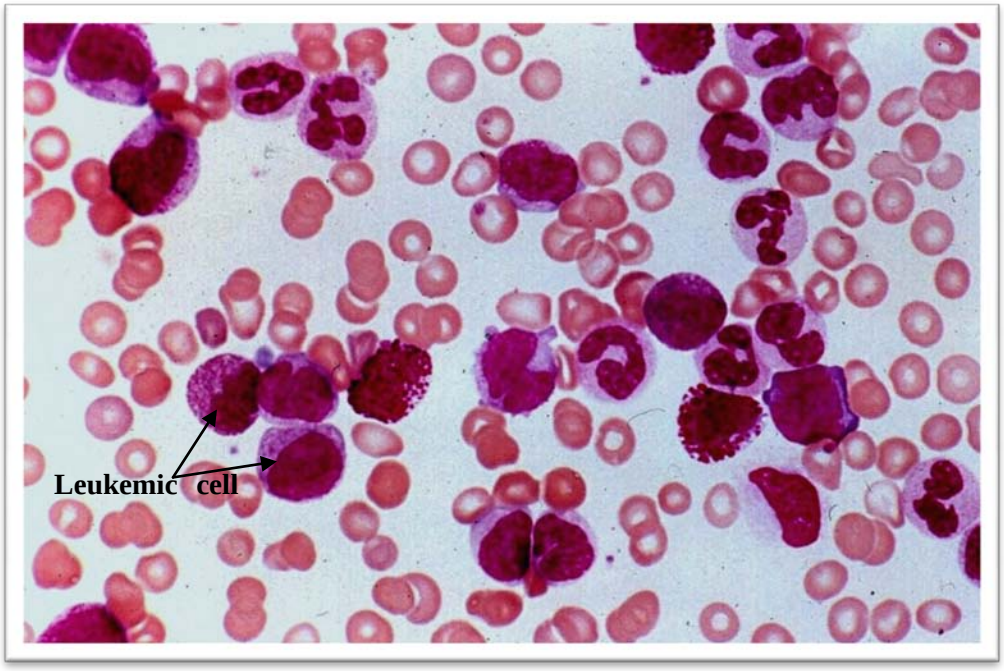

Figure (1-2): Chronic Myelogenosis Leukemia

From: http//:pathologystudent.com

1.2.2.1 Biology of CML

Examination of the chromosomal structure of cells of lymphoid, esinophil, or platelet lineage from the patient with CML shows that all these lineages contain the Ph chromosome. This indicates that CML is a stem cell disease. To define it more specifically, CML is a myeloproliferative disorder. It is a clonal expansion of the translocation of chromosomes 9 and 22. The consequence of this translocation is the creation of an unregulated tyrosine kinase activity. Chromosome 9 contains the tyrosine kinase gene, ABL, named for the Abelson murine leukemia virus. Chromosome 22 contains the BCR (for breakpoint cluster region) (Groffen *et al.*, 1984).

In CML a translocation occurs to form a different chromosome, and this chromosome encodes for an oncoprotein that has very strong tyrosine kinase activity. On the other hand, the BCR has multiple sites at which the breakpoint can occur. These breakpoints have different implications for the aggressiveness of the disease (Melo, 1999).

The p210 form of BCR-ABL is the most common form observed in patients with CML. The p230 form of BCR-ABL actually is associated with a more indolent disease. Finally, the p190 form of BCR-ABL is also diagnostic for the Ph chromosome, but this is most commonly seen in ALL as opposed to CML. The ABL gene region has just 1 breakpoint; thus, there are no variants that can affect the disease. The ABL protein normally is a regulated kinase with the usual constraints of a kinase. In contrast, the BCR-ABL kinase continues to phosphorylate downstream protein targets associated with cellular proliferation without constraint. These targets include RAS, RAF, and AKT, among others (Sawyers, 1999). In addition to dysregulating

proliferation, these phosphorylation events also create changes in the adherence properties of these cells as well as diminished apoptosis (programmed cell death). All these alterations then lead to the development of CML (Sessions, 2007).

1.2.2.2Classification of CML

CML is often divided into three phases based on clinical characteristics and laboratory findings. In the absence of intervention, CML typically begins in the *chronic* phase, and over the course of several years progresses to an accelerated phase and ultimately to a blast crisis. Blast crisis is the terminal phase of CML and clinically behaves like an acute leukemia. One of the drivers of the progression from chronic phase through acceleration and blast crisis is the acquisition of new chromosomal abnormalities in addition to the Philadelphia chromosome (Chen *et al.*2010).

- **Chronic phase**

Approximately 85% of patients with CML are in the chronic phase at the time of diagnosis. During this phase, patients are usually asymptomatic or have only mild symptoms of fatigue or abdominal fullness. The duration of chronic phase is variable and depends on how early the disease was diagnosed as well as the therapies used. Ultimately, in the absence of curative treatment, the disease progresses to an accelerated phase (Tefferi, 2006).

- **Accelerated phase CML:**

Is characterized by an increased number of immature cells (up to 20 percent), as well as other fluctuations in white blood cell and platelet counts. Patients in the accelerated phase may have symptoms including fever and weight loss (Deininger, 2007).

- **Blast phase CML**:

  The most advanced phase of the disease is an aggressive form of leukemia and typically means the immature cells have spread beyond the bone marrow to the tissues and organs. The number of immature cells found in the bone marrow or blood exceeds 20 percent and patients often experience symptoms of fever, weight loss and decreased appetite (Deininger, 2007).

1.2.2.3 Epidemiology of CML

Chronic myelogenous leukemia accounts for 15%of adult leukemia's, has an incidence of 1 to 2 cases per 100 000 population, and has a male-to-female ratio of 1.3 to 1. Incidence increases with age. The median age in one report is up to 67 years (Baranovsky *et al.,* 1986).

1.2.2.4 The Philadelphia Chromosome

The Philadelphia chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22—t (9; 22) (q34; q11) (Rowley 1973). It occurs in 90% of patients with chronic myelogenous leukemia, in 5% of children and 15% to 30% of adults with acute lymphoblastic leukemia, and in 2% of patients with acute myeloid leukemia (Specchia *et al.,* 1995). The translocation transposes a 39 segment of the ABL gene from chromosome 9q34 into the major breakpoint cluster region of the BCR gene on chromosome22q11.This event creates a hybrid BCR-ABL gene that is transcribed into chimeric BCR-ABL messenger RNA.The fusion messenger RNA is subsequently translated into a chimeric 210-kDa protein called p210 BCR-ABL (Lichtman & Liesveld, 2006) figure(1-3).

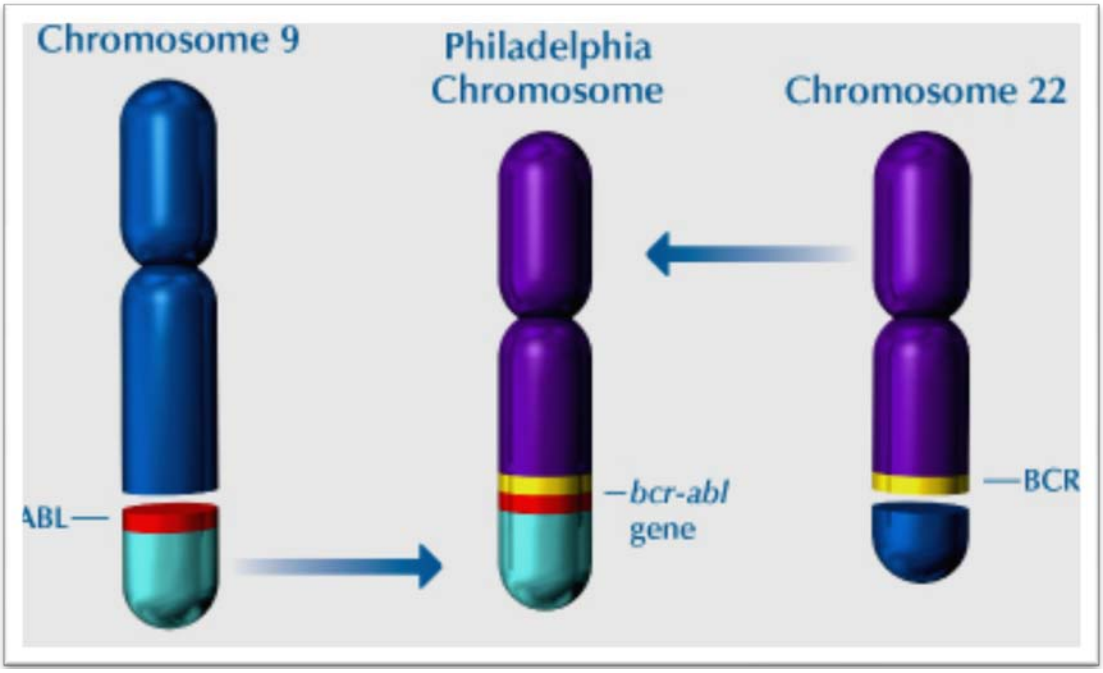

Figure (1-3): The Philadelphia chromosome (Lichman and Liesveld, 2006)

1.2.2.5 Treatment and Prognosis

In the 1920s, splenic irradiation was introduced for symoptomatic relief. Effective control of blood counts became feasible with busulfan (1959). Ten years later, the better- tolerated hydroxyurea became available, probably the first intervention with a (modest) prolongation of survival. A breakthrough was achieved in the mid -1970s when the Seattle group reported the disappearance of the Ph chromosome in CML patients who underwent allotransplant. Soon later, Interferon was found to induce durable complete cytogenetic responses and long term survival, although in only a small fraction of patients. In the pre- imatinib era, when drug therapy relied on interferon-alpha-based regiments, the median duration of the chronic phase was approximately five years, with few patients surviving longer than ten years (Cortes and Deiniger, 2007). However the disease has progressed

beyond the chronic phase, allotransplant is still the recommended treatment for all eligible patients (Deininger and Druker, 2003). In 1992, Alexender Levitzki proposed the use of ABL inhibitors to treat leukemias driven by ABL oncogenens. At about the same time, scientist at Ciba-Geigy had synthesized a potent inhibitor of ABL that was termed GCP57148B, and is currently known as imatinib. The majority of patients diagnosed with chronic phase CML can expect to have durable responses with good quality of life (Deininger *et al., 2003*).

1.2.2.5.1 Imatinib

Imatinib mesylate (Gleveec®) is a molecular therapeutic chemotherapy for oral administration tyrosine kinase inhibitor that was initially developed as a 2-phenylaminopyrimidine derivative. It was subsequently found to block the BCR-ABL kinase by competing with the ATP-binding site, inducing remission in most patients in chronic phases of chronic myelogenousis leukemia (Buchunger *et al.,* 2000; Druker *et al.,*2001; Mcphee *et al.,* 2006).Furthermore resistance to imatinib can involve amplification of BCR-ABl breakpoint as well as the development(or clonal expansion) of mutation in the ATP-binding pocket of BCR-ABL, which no longer allows binding to imatinib(Mcphee *et al.,* 2006).Imatinib may also have a role in the treatment of pulmonary hypertension. It has been shown to reduce both the smooth muscle hypertrophy and hyperplasia of the pulmonary vasculature in a variety of disease processes, including pulmonary hypertension (trapper *et al.,* 2009). In systemic sclerosis, the drug has been tested for potential use in slowing down pulmonary fibrosis. In laboratory settings, imatinib is being used as an experimental agent to suppress platelet-derived growth factor (PDGF) by inhibiting its receptor (PDGF-Rβ) (Scheinfeld, 2006). One of its effects is

delaying atherosclerosis in mice without (Bucher et al., 2003) or with diabetes (Lassila *et al.*, 2004)

1.2.2.5.1.1 Mechanism of action

In chronic myelogenous leukemia, the Philadelphia chromosome leads to a fusion protein of ABL with BCR. As this is now a constitutively active tyrosine kinase, imatinib is used to decrease BCR-ABL activity. The active sites of tyrosine kinases each have a binding site for ATP. The enzymatic activity catalyzed by a tyrosine kinase is the transfer of the terminal phosphate from ATP to tyrosine residues on its substrates, a process known as protein tyrosine phosphorylation. Imatinib works by binding close to the ATP binding site of BCR-ABL, thereby preventing the phosophorlation of tyrosine residues on substrate protein, thus preventing the activation of signal transduction pathway (growth factor receptor) that induce the leukemic transformation to CML, so enhancing apoptosis(Razelle *et al.*, 2003; Mcphee *et al.*2006; Wong and Mirshahid, 2011).

1.2.2.6 Diagnosis of Chronic myelogenous leukemia

The diagnosis of CML is initially based on the results of a simple blood test. The test may show an abnormally high white blood cell count. In blood samples examined under a microscope, less mature white blood cells, normally found only in bone marrow, are seen .Tests that analyze chromosomes (cytogenetic or molecular genetics) are needed to confirm the diagnosis by detecting the Philadelphia chromosome (Emil *et al.*,2008).

1.2.2.7 Molecular investigation:

1.2.2.7.1 Polymerase chain reaction (PCR)

The polymerase chain reaction is an *in vitro* technique which allows the amplification of a single or a few copies of a piece of DNA, and rapidly produce thousands to millions of copies of a particular DNA sequence in a matter of only few hours (Mcperson and MØller, 2001). PCR invented by Mullis in 1983, has revolutionized the science of a molecular biology. This process takes its name from DNA polymerase, on enzyme that carries out DNA replication in a cell (Leland *et al.,* 2000).

PCR is a sensitive, rapid, and is not limited by the quality of DNA. It is widely used in different fields of biological sciences and genetic disease studies, like in biomedical research. Detection of infectious agents and genetic defects, the study of molecular evolution, medical epidemiology and forensic medicine besides environmental monitoring (Ou, 1990). With its exquisite sensitivity and high selectivity, PCR has been used in wartime human identification and validation in crime labs for mixed –sample forensic cases. Environmental food pathogens can be quickly identified and quantified at high sensitivity in complex matrices with simple sample preparation techniques (Chen and Jans, 2002)

The PCR processes require a repetitive series of the three fundamental steps that defines one PCR as cycle shown in figure (1-4). Each cycle starts with heating of samples to 94-96 ˚C to achieve full denaturation of double stranded template DNA, and complete separation of the two strands is essential for a successful PCR. Step 2 starts with annealing of two oligonucleotide primers to single –stranded template, that starts when the sample are cooled down to 50-68˚C. Third step start with enzymatic extension which occurs at 72˚C for most templates. The denaturation, primer annealing, and primer extension processes,

lead to doubling the number of target DNA copies (Saiki, 1989; Innes *et al.;* 1990).

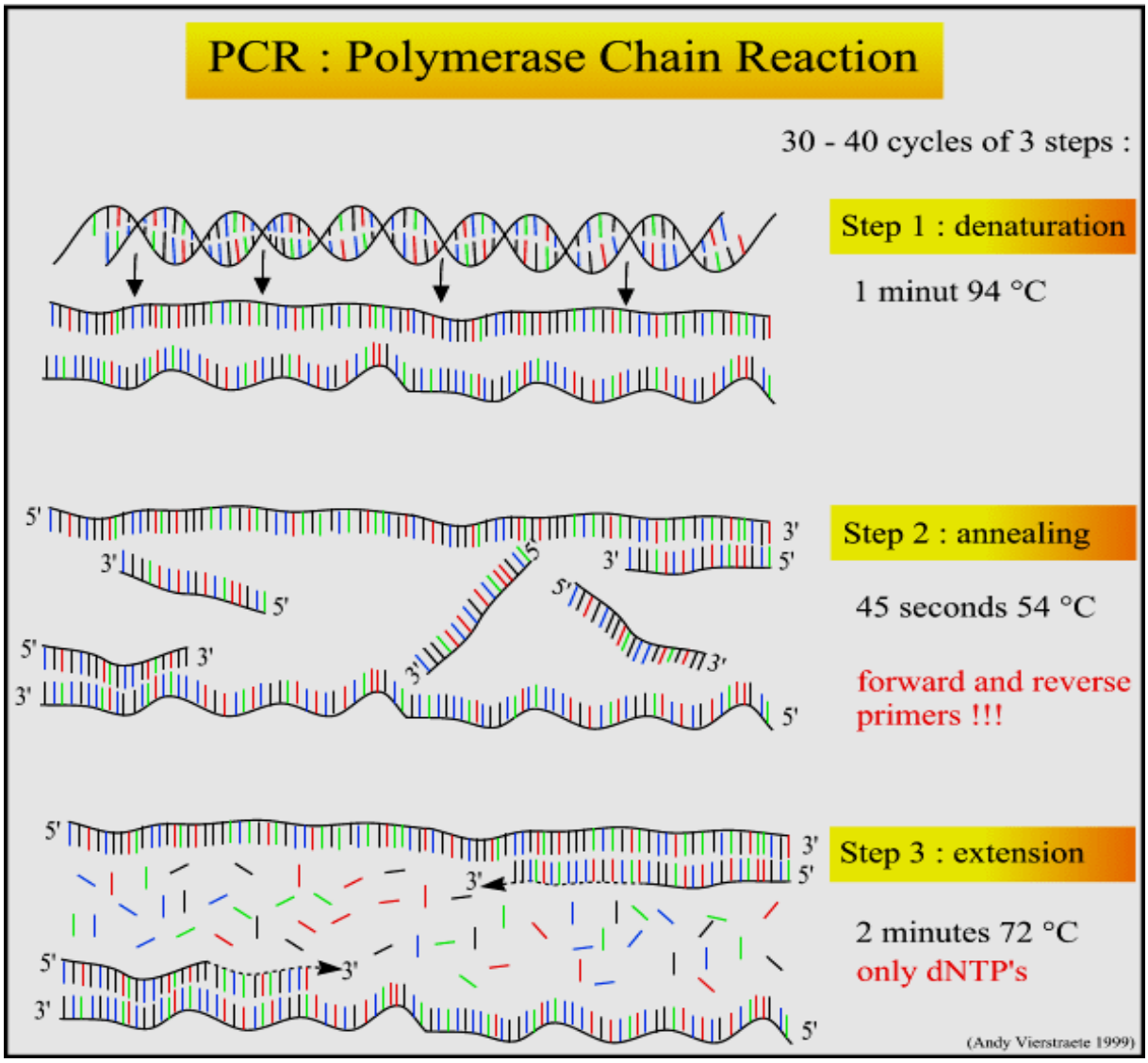

Figure (1-4): polymerase chain reaction

From http://commons.wikimedia.org/wiki

1.2.2.7.2 Types of PCR techniques.

1. Random Amplified Polymorphic DNA -PCR (RAPD-PCR)

Random Amplified Polymorphic DNA (RAPD) is a PCR technique based on polymerase chain reaction (PCR) amplification of random fragments of genomic DNA with single short primer of arbitrary nucleotides sequence usually 10-bases (Williams *et al.*, 1990).

Genetic variation between individuals appears when primers match to complementary sequence result in different patterns of amplified DNA fragments with different molecular sizes. Amplification products can be detected through examination of ethidium bromide- stained agarose gels (Mcpherson and MØller, 2001).

The RAPD technique can simply and rapidly detect genetic alterations the entire genome without knowledge of specific DNA sequence information and can work effectively with tiny amount of DNA. RAPD-PCR become a particularly popular technique and thus was applied in genetic mapping, for plant and animal breeding application, study on population genetics, DNA fingerprinting, epidemiological studies and typing of micro- organisms and for detection of pathogenic strains of bacteria and fungi (Williams *et al.*, 1990).

It is also utilized for detection abnormal DNA sequences in human cancers. Genomic fingerprints of DNA from normal and tumor tissue can be used to evaluate or generate DNA sequence in cancer cells. RAPD was used to detect genetic instability in tumors (Luceri *et al.*, 2000).

2. Reverse Transcription polymerase chain reaction (RT-PCR)

In RT-PCR, an RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR (Hunt, 2006). The RT step can be performed either in the same tube with

PCR (one-step PCR) or in a separate one (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used (Bustin, 2002).

RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites and, if the genomic DNA sequence of a gene is known, to map the location of exons and introns in the gene (Trout*et al*., 1992).

3. Real time PCR or quantitative PCR (Q-PCR):

Real time –PCR is a modification of the PCR used to rapidly measure the quantity of DNA, cDNA or RNA present in a sample. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule, which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes).

Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves resources and time. These advantages of the fluorescence based real time PCR technique have completely revolutionized the approach to PCR-based quantification of DNA and RNA. Real time (Q-PCR) has an additional cycle of reverse transcription that leads to formation of a DNA molecule from a RNA molecule. This is done because RNA is less stable as compared to DNA (Ding and Cantor, 2004).

1.2.2.7.3 Requirements of PCR reaction

There are many factors that may affect the reaction of PCR (Samboork and Russell, 2001). These factors include:

1. Template: that contains the DNA, cDNA or RNA fragment (target) to be amplified.
2. Deoxy nucleotide triphosphate (dNTPS): the building blocks from which the DNA polymerases synthesize a new DNA strand.
3. Primers: which are complementary to the DNA regions at the 5' and 3' ends of the DNA region.
4. PCR buffer: This provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.
5. DNA polymerase: such as *Taq polymerase* or another DNA polymerase with a temperature optimum at around 70°C. PCR performance is often related to the DNA polymerase, so enzyme selection is critical to success. The reverse transcriptase (RT) is as critical to the success of qRT-PCR as the DNA polymerase. It is important to choose an RT that not only provides high yields of full-length cDNA, but also has good activity at high temperatures.
6. Magnesium concentration: magnesium chloride ions ($MgCl_2$) which is a critical factor that exist dNTPs-$MgCl_2$ complexes that interact with sugar backbone of nucleic acids influencing the activity if of the Tag DNA polymerase.

1.2.3. Olive tree (*Olea Europea* L.*)*

The olive tree (*Olea Europea L.*; Oleaceae) is one of the most important trees in Mediterranean countries. It grows through the entire Mediterranean and in most of southern European countries (Gundia, 2004). *Olea Europaea*

L. wildly studied for its alimentary use. The fruits and oil are important components in the daily diet of a large part of the world's population, where as the leaves are important for their contents of secondary metabolites (figure1-5).They consist of phenolic compounds, flavonids and volatiles oils (Hansen *et al.*, 1996; Altarejos *et al.*, 2005).

Figure (1-5): *olea europea*

From http: //www. dermaxime.com

1.2.3.1. Plant description and classification

The plant follows the following classification (Srivastava, 1985):

Kingdom: Plantae

Phylum: Anthophyta

Class: Dicotyledones

Order: Scrophulariales

Family: Oleaceae

Genus: Olea

Species: *Olea europaea* L.

This plant is related to Oleaceae family**.** The medicinal parts are the dried leaves, the oil extracted from the ripe fruits, and the fresh branches with leaves and clusters of flowers. The flowers are in small auxiliary clustered inflorescence. The leaves are opposite, entire, stiff, curvaceous, narrow elliptical to lanceolate or cordite with thorny tips. The upper surface is dark green, glabrous and underside shimmers silver with hairs (Wagner *et al.*, 1999).

1.2.3.2 Chemical components

The leaves contain secoiridoids - including oleuropein, as well as ligustroside and oleacein. It further contains triterpenoids (oleanolic acid and uvaol), sterols, flavonoids (chrysoeriol, apigenin and luteolin glycosides) and various other phenolic acids. Other ingredients found in the leaves extract are luteolin, rutin, apigenin and diosmetin (Meirinhos *et al.,* 2005) as well as 1,5-Anhydroxylitol(Campeol *et al.*,2004).

1.2.3.3 Medicinal uses and pharmacological action

1. **Cardiovascular effects:** The hypotensive action of the olive tree leaves is due to the oleuropeoside. It has been reported that oleuropein had anti-arrhythmic and vasodilating effects in addition to hypotensive activity

(Petkov and Manolovo1992; Gruenwald *et al.,* 1998; Khayyl *et al.,* 2002). The body metabolizes oleuropein to calcium elenolate, which appears to be responsible for many of the pharmacological effects of olive leaf.

2. **Diabetes:** Oran and Al-Eisawi (1998) illustrated the uses of *O. europea* leaves as astringent, hypotensive, antidiabetic.
3. **Antioxidant effects:** Phenolic compounds derived from the leaves, fruits and oil of the olive tree (*O. europea* L.) have long been known to have anti-oxidative properties (O'Brien *e tal.*,2006; Bouaziz *et al.,* 2008; Wang *et al.,* 2011). Le Tutour and Guedon (1992) demonstrated that oleuropein, hydroxytyrosol, and in particular, extracts of *O. europea* leaves were more potent antioxidants than vitamin E. Another *in vitro* study showed that oleuropein significantly inhibited copper sulphate-induced oxidation of low density lipoprotein (LDL) extracted from normal human plasma (Visioli and Galli, 1994). Olive leaves extract protected those blood vessels from damage, and has been shown to be effective in protecting the heart from coronary occlusion (Samovar *et al.,* 2003). When taken over an extended period of time, it is believed to reverse arteriosclerosis (Fki *et al.,* 2005).
4. **Antimicrobial effect:** Oleuropein, was considered to be the source of the olive tree's powerful disease-resistant properties. Fredickson (2000) tested the effects of olive leaves extract on a range of viruses, and reported a potent antiviral activities against herpes, hepatitis virus, rotavirus, bovine rhinovirus, and feline leukemia virus. Recently, Zahar (2007) reported that olive leaves extract inhibited *in vitro* infectivity of infection laryngiotracheties virus, suggesting its potential use as a natural antiviral agent**.**
5. **Anti cancer activity:** Plant have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer (Cragg and

Newman, 2006; Mazzio and Soliman, 2009). In many instances, the active compound isolated from the plant may lead to the production of potential novel drugs capable of inhibiting the action of, or interacting with, several key proteins involved in the mechanism of carcinogensis. Cancer protection associated with the consumption of olive products is well established (Omar, 2010) but not for leukemia. Incubating human leukemia HL-60 cells with olive leaf extracts for several hours resulted in DNA fragmentation, nitroblue tetrazolium reduction (a marker of differentiation) and significant inhibition of HL-60 cells. These findings suggest the presence of olive leaves extract mediated cell differentiation (Abaza *et al.,* 2007). Olive-leaves crude extracts were also found to inhibit cell proliferation of human breast adenocarcinoma (MCF-7), human urinary bladder carcinoma (T-24) and bovine brain capillary endothelial (BBCE) cells. The dominant compound of the extracts was oleuropein; phenols and flavonoids were also identified. These phytochemicals demonstrated strong antioxidant potency and inhibited cancer and endothelial cell proliferation at low micromolar concentrations, which is a significant finding considering their high abundance in fruits and vegetables (Goulas *et al.,* 2009). Furthermore, Mazzio and Soliman (2009) evaluated 374 natural extracts for their relative tumoricidal potency *in vitro* using a murine malignant neuroblastoma cell line. Their findings indicated that olive leaf extracts showed a weak to moderate anticancer activity with lethal concentration (LC50) =2.72 mg/ml. Recently, Mijatovic *et al.(*2011) indicated that dry olive leaf extract possessed strong antimelanoma potential when incubated with B16 mouse melanoma cell line *in vitro*.

2.1 Subjects

The forty eight patients were included in this study which was chronic myeloid leukemia (CML), which was referred to National center for Hematology, Al-Mustansryia University. The patients were their mean age ± SE was (39.72± 2.033). All of them were under treatment with imatinib, 400mg for variable duration (yrs). A further twelve normal healthy individual (control group) were also investigated. They were staff of Biotechnology Research Center, Al-Nahrain University and had no history or signs of leukemia, their mean age was (36.75± 2.14). All of them (controls and patients) were collected from January 2010- October 2010.

2.2. Materials

2.2.1: Apparatus

Table (2-1) Apparatus used and their manufactures

No.	Name of equipments	company/Origin
1.	Cool micro Centrifuge	Eppendroff /Germany
2.	Electrophoresis casting tray	Mettle /Switzerland
3.	Eppendorff tubes	Eppendroff/ Germany
4.	Gel documentation	Smart / Germany
5.	Hemocytometer	Neubaure/ Germany
6.	Laminar air flow	Biotech / U.K
7.	Micropipette	Eppendroff / Germany

8.	Oven	Chilipison/U.K
15.	Real time PCR	BioRad/Ital,Applied Biosystmes-Singpore Smart cycler-cephied-USA
9.	Rotary evaporator	Buchi / Switzerland
10.	Spectrophotometer	Shimadzue/ Japan
11.	Thermal cycler	Eppendroff / Germany
12.	Uv-transilluminator	Consort /England
13.	Vortex	Sturat scientific/ U.K
14.	Water bath	Memmert / Germany

2.2.2 Chemicals

Table (2-2) Chemicals materials used in the present study

NO.	Material	Company/Origin
1.	Agarose	Sigma /U.S.A
2.	BCR-ABL M-bcr quant kit	Sacace/Italy
3.	Chlorophrom	BDH/England
4.	DNA ladder	Promega/ U.S.A
5.	Ethidum bromide	Sigma /U.S.A

6.	**Ferric chloride**	**BDH/England**
7.	**Fetal calf serum**	**Sigma/U.S.A**
8.	**Ficol-Histopacue**	**Sigma /U.S.A**
9.	**HCL**	**BDH/England**
10.	**KOH**	**BDH/England**
11.	**Loading dye**	**Promega /U.S.A**
12.	**Reverta-L kit**	**Sacace/Italy**
13.	**Rio-Zoil-D kit**	**Sacace /Italy**
14.	**RPMI-1601**	**Sigma/U.S.A**
15.	**Sulfuric acid**	**BDH/England**
16.	**Tris-Borite-EDTA**	**Promega /U.S.A**
17.	**Wizarid genomic kit**	**Promega /U.S.A**

2.2.3 Preparation of solutions and buffers

2.2.3.1 Solutions used in detection of active compounds in ethanolic olive leaf extract

1. **Ferric chloride:** It was Prepared by dissolving 1g of ferric chloride in 100ml distilled water.
2. **Potassium hydroxide**: The mixture was prepared by dissolving 50 g of potassium hydroxide in 50ml of D.W. Then the volume was complete to 100 ml with D.W.

3. **Dragindroff reagent**: The reagent was prepared by dissolving 8 g of Bi $(NO_3)_{3.}H_2O$ in 30% w/v HNO_3 and 27.2 g KI was dissolved in 50 ml D.W. The solution stood for 24 h, filtered the reagent and was equivalent to 100ml with water (Silva *et al.,* 1998)

2.2.3.2 Solutions used in tissue culture techniques

1. **Phosphate buffer saline (PBS)**: The solution was prepared by dissolving the following chemicals Sodium chloride (NaCl) 8gm, Potassium chloride (KCL) 0.2 gm, Sodium phosphate, hydrate (NaHPO4) 1.15 gm in an appropriate amount of D.W, then pH was adjusted to (7.2) and the solution was sterilized by autoclaving and stored at 4˚C (Hudson and Hay, 1989).
2. **Trypan blue:** One gram of trypan blue powder was dissolved in 100 ml of PBS. The stain solution was filtered (Whatman paper NO.3) before use (Ad hiah, 1990).

2.2.3.3 Solutions used in Agarose gel electrophorsis

1. **Tris-borite (TBE) buffer:** To prepare 10 x TBE solution, the components used are: 108 gm of Tris-Base, 55gm of Borric acid, 40ml of 0.5M EDTA (pH=8.0) in an appropriate amount of D.W, pH was adjusted to 7.8 and volume completed to 1 liter with D.W. The solution was sterilized by autoclave and stored at room temperature (Sambrook *et al;* 1989).
2. **Etidium bromide solution (10mg/ml):** It was prepared by dissolving 1gm of Ethidium bromide powder in 100 ml of a sterile D.W., and the bottle kept in a dark (Maniatis *et al.*, 1982).
3. **Loading buffer**: It was prepared by dissolving 0.25gm of bromo phenol blue –xylene cyanole dye in 50 ml D.W,30ml of glycerol was added,volume complete with D.W to 100ml and stored at 4˚C (Manaitis *et al.*,1982).

4. **Bench top PCR marker:** The DNA marker (1Kb) is a convenient marker for determine the size of DNA or RNA. It consists of 15 fragments that range from 250 to 10000 bp. The recommended amount of marker to load on agarose gel is 1µg/ lane.

2.3 Methods

2.3.1 Blood sample collection

Peripheral blood (5-10 ml) was obtained under aseptic conditions from each subject by a vein puncture using a disposable syringe pre coated with heparin. The blood sample was placed in a cool box and transferred to the laboratory, where it was kept at 4˚C and processed within 24h (Dhot *et al.,* 2003; Yaghmaie *et al;* 2008).

2.3.2 Separation of mononuclear cells (MNCs)

The mononuclear cells (MNCs) were separated from blood sample by density gradient centrifugation according to the protocol described by Erices *et al.* (2000) with some modification as follow figure (2-1):

1. Blood was diluted in a proportion of 1:1 in a phosphate buffer saline.
2. Carefully layer the diluted blood to heprinized tube containing Ficol –histo Paque solution.
3. In order to isolate MNCs, Centrifuged the mixture at 4°C at 2200 rpm for 25 min.
4. The upper layer was drew off using a clean Pasteur pipette, the MNCs rich zone called (Buffy coat) was isolated, transferred into a new 10 ml tube and washed twice with culture medium through centrifugation at 2000 rpm for 8 min and 1000 rpm for 10 min, respectively. The final pellet was re-suspended

with culture medium supplemented with 10% Fetal calf serum and was considered ready for cell count viability.

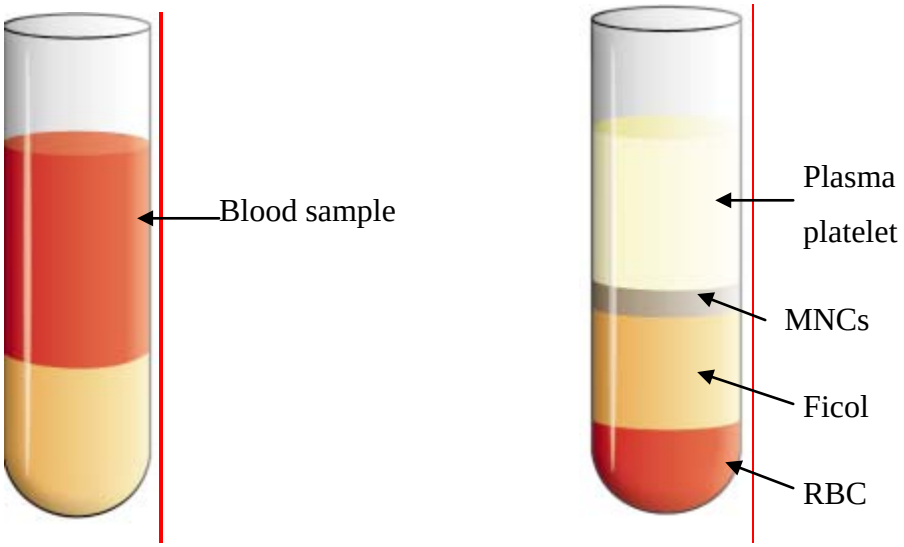

Figure (2-1): Isolation of MNCs from blood sample

2.3.3 Determination of cell number and viability

The cell count and viability can be performed by using a haemocytometer. About 100µL of re-suspended cells diluted 1:1 with trypan blue solution to determine the cell viability. A drop of cells suspension added to either side of the haemocytometer taking care not to over fill it and making sure that the cover slip was firmly in place, using the light microscope at low power. Total numbers of cells (viable and dead) were calculated in the four Neumbaur hemocytometer chamber as following (Doyle and Griffiths, 1998):

Viable Cells (%) = [No. of viable cells/ total No. of cells (dead and viable) x100

2.3.4 Molecular detection of BCR-ABL mutation gene (ratio of gene copy).

2.3.4.1 RNA extraction on using (Ribo –Ziol-D kit, Italy):

- 1.5 ml polypropylene tubes were used.
- 400 µL of solution D was added to each tube.
- 100 µL of cells was pipette.

1. Controls was prepared as follows:

a) 400 µl of solution D was added and 50 µl of negative control to the tubes labeled C neg and C pos(1 or 2)

b) 10 µl of pos2 bcr-abl was added (or pos1 bcr-abl) to the tube labeled Cpos.

2. To lysing samples with solution D, 40 µl of solution E was added, then vortex and centrifuged for 5-7 sec.
3. To all tubes 400 µl of solution A was added then vortex and centrifuged for 5-7 sec.
4. All tubes 130 µl of solution B was added, vortex for 1-2 min.
5. Incubated all the tubes at -20 ˚C for 10 min.
6. Centrifuged all the tubes for 10 min at 13000-16000Xg.
7. Prepared during the centrifugation new 1.5 ml tubes for each sample and add to all tubes 400 µl of solution C.
8. 10 µl of tRNA was added to the tubes Cneg and Cpos.
9. Then transferred to appropriate tubes with solution C approximately 400 µl of supernatant obtained after the sample centrifugation. Vortex and centrifuge for 5-7 sec.
10. All the tubes were incubated at -20 ˚C for 20 min.
11. The tubes were centrifuged for 10 min at 14000-16000Xg and carefully removed and discard supernatant from each tube without disturbing the pellet. If the pellet isn’t visible leave in the tube 20 µl of supernatant.

12. 400 µl of cold washing solution 3 was added, Vortex vigorously and centrifuge for 10min at 14000-16000 Xg, by using a micropipette, carefully removed and discard supernatant from each tube without disturbing the pellet.
13. All tubes were incubated with open cap for 6-7 min at 56˚C.
14. The pellet re-suspended in 30 µl of RNA-eluent BCR-ABL, then incubated for 2-3min at 56 ˚C. Vortex and centrifuged the tubes for 1min at maximum speed (12000-16000Xg).The supernatant contained RNA ready for amplification and can be stored at -70 ˚C for 1 year.

2.3.4.2. Estimation of RNA concentration using spectrophotometer

The concentration of RNA was measured using a spectrophotometer method (Sambrook *et al.*, 1989). In principle of this method depends on the amount of U.V irradiation absorbed by the nitrogen bases composed in RNA. The method was performed by adding 10 µl of RNA sample to 490 µl of Depec water. Then the optical density was determined at 260 nm in a UV spectrophotometer using Depec water as a blank, the concenatrion of RNA was calculated using the formula:

RNA concentration (mg/ml) =OD260× 40 × Sample Dilution

To determine the degree of purity of the RNA, an additional measurement was made at 280 nm. This provides a method of gauging RNA purity using the ratio of measurements at OD260/OD280.

2.3.4.3 Agarose Gel Electrophoresis for RNA extracted sample

In order to separate fragments of RNA, agrose gel of 1-2 % concentration was prepared, gel were run horizontally in 1x TBE buffer (Bartlett, 1996). Generally, samples (mix 10 µl from sample with 3 µl from loading dye) were loaded into the well of the gel, the working TBE buffer added up to the level of horizontal gel surface and gel was run for 1.5-2

hours at 10 v/cm. Agarose gel were stained with eithdium bromide by immersing the gel in the distilled water containing the final concentration of ethidium bromide (0.5 mg/ml) for 30-45 min or by adding it to the gel and electrophoresis buffer. A known amount of DNA or RNA ladder loaded alongside your RNA sample as a standard (Bartlett, 1996).RNA bands were visualized by U.V transillmuminator at 365 nm.Gel documentation system was used for the documentation of the results and printed directly using thermal printer or stored on computer to undergo further processing later using suitable computer programs (Sambrook *et al.*, 2001).

2.3.4.4 cDNA synthesis from RNA extracted sample using Reverta-L kit (Sacace, Italy)

1. To Prepared reaction mixture: for 1 sample, 0.41 µl RT-G-mix-1was added into the tube containing 10.4 µl of RT-mix and vortex for at least 5-10 seconds, centrifuged briefly. This mix was stable for 1 month at-20˚C.
2. 0.5 µl Revetase was added into the tube with Reagent mix, mixed for 3 sec, centrifuged 5-7 sec.
3. 10 µl of reaction mix was added into each sample tube.
4. RNA samples (15µl) pipette to the appropriate tube.
5. The mixture was Placed into thermal cycler and programmed as the following –temperature profile (2-3):

Table (2-3): Reverse transcription of cDNA from RNA.

Step	Temperature	Time
1	**50 ˚C**	**15 min**
2	**95 ˚C**	**3 min**

2.3.4.5. Real Time PCR for cDNA samples using (BCR-ABL M-bcr quant kit, Italy):

1. reaction mix for M-bcr-abl detection and N-abl detection was prepread as it in the following table(2-4):

Table (2-4): pipeting scheme for the quantity of reagents for N sample.

Quantitave test	
Mixture for M-bcr-abl detection	Mixture for N-abl detection
(n+7)*7µl PCR mix-1M bcr-abl (n+7)*7.5µl PCR –buffer (n+7)*0.5Tag Fpolymerase	(n+5)*7µl PCR mix-1N-abl (n+5)*7.5µl PCR –buffer (n+5)*0.5µlTagFpolymerase

2. Each sample tube for Mix –bcr-abl detection was added 15 µl of prepared reaction mix M-bcr-abl and added mix M-bcr-abl and in each sample tube for N-abl detection 15 µl of prepared reaction mix N-abl (prepared in step 1).
3. 10 µl of cDNA sample piptte to the appropriate tube with mix M-bcr-abl and 10 µl of cDNA sample to the appropriate tube with mix N-abl.
4. For M-bcr-abl mix was prepred 5 standards (QS1, QS2, QS3, QS4, QS5) and negative control (DNA buffer) by added 10 µl of these reagents to the appropriate tube.
5. For N-abl mix prepread 3 standards (QS1, QS2, and QS3) and 1negtive control (DNA buffer) by added 10µlof these standards to the appropriate tube.

6. The tubes was transferred them to the real time instrument, and then Programmed the real time instrument according to table (2-5):

Table (2-5): programming of real time for amplification.

Step	Temperature	Time	Fluorescence detection	Repeats
1	95˚C	15min	_	1
2	95˚C	20sec	_	47
	60˚C	55sec	Joe /Hex	

Quantitative format:

The following formula is used for the calculation of the normalized concentration of RNA M-bcr-abl in the clinical and control samples:

Ratio =number of cDNA M-bcr-abl copies /number of cDNA N-abl copies.

2.3.5 Molecular analysis of genomic DNA and genetic instability of CML sample using Random amplified polymorphism DNA technique

2.3.5.1. Genomic DNA extraction from blood (promega DNA Wizard):

1. 900 µl of cell lysis solution was added to a sterile 1.5 ml microcentrifuge tube that contained 300 µl of blood sample.
2. Gently the tube of the blood was rocked during process of mixing; then the blood transferred to the tube containing the cell lyses solution. Inverted the tube for 5-6 times to mix.

3. The mixture was incubated for 10 minutes at room temperature (invert 2-3 times) during the incubation) to leys the red blood cell. The preparation centrifuge at 13000xg for 20 seconds at room temperature.
4. Discard as much as possible without disturbing the visible white pellet. Approximately 10-20 µl of residual liquid will remain in the tube.
5. The tube was vortex vigorously (10-20 times) until the white blood cells re-suspend.
6. 300 µl from Nuclei lysis solution was added to the tube contained the re-suspend cells. Pipetted the solution 5-6 times to lyse the white blood cell. The solution should become very viscous. If clumps were still visible after mixing incubated at 37 ˚C; clumps were disrupted.
7. Optional: RNase solutions (1.5 µl for 300 µl sample volume) was added and incubated at 37 ˚C for 15min and then cool to room temperature.
8. Protein precipitation solution (100 µl for 300 µl sample volume) was added to the nuclear lysate and vortex vigorously for 10-20 seconds, small protein clumps might be visible.
9. The preparation Centrifuged at 13000-16000Xg for 3min at room temperature.
10. The solution gently mixed by inversion until the white –thread like strands of DNA formed a visible mass.
11. The preparation centrifuged at 13000-16000 xg for 1 min at room temperature .The DNA would be visible as a small white pellet.
12. The supernatant was Decanted and 100 µl of 70% ethanol was added to the DNA pellet, gently inverted to the tube several times to wash the DNA pellet
13. The ethanol carefully was aspirated by using pipette tip and the tube was inverted.
14. DNA rehydration solution (100 µl) was added to the tube and rehydrate the DNA by incubating the solution at 65 ˚C for 1hour or by incubating overnight at 4˚C.

2.3.5.2. Estimation of DNA concentration by spectrophotometer.

Five microliters of each sample were added to 495 µl of Distilled water and mixed well to determine the DNA concentration and its purity by using spectrophotometer at optical density 260 nm and 280 nm (Sambrook *et al;* 1989). The concentration of DNA was calculated according to the formula:

DNA concentration (µl/ml) =O.D 260 nm x 50 x Dilution factor

The purity ratio of DNA was also estimated using this formula:

DNA purity ratio =O.D 260/ O.D 280

This ratio was used to detect DNA contamination in protein preparation. (Mainiatis *et al.,* 1989).

2.3.5.3. Agarose gel electrophorsis of genomic DNA

To separate DNA fragments, agrose gel as 0.8% concentration was used. Gel was run horizontally in 0.5x TBE buffer. Samples of DNA were mixed with loading buffer (loading buffer: DNA 2/7(v/v) and loaded into wells on the gel in checking step of total DNA. Electrophoresis buffer was added to cover the gel and run 1-2h at 5 v/cm, and then stained with ethidium bromide (0.5µg/ml for 20-30min). The bands of DNA were visualized by U.V transilluminator (Maniatis *et al.,* 1989).

2.3.5.4. RAPD PCR for DNA extraction (PCR reaction)

In this study, PCR reactions were performed using the following:

1. **universal primers:** Random sequence decamer primers were used, synthesizes by Alpha DNA-Canada from different series originally designed by operon tech-Inc A, C, D, B,GB and I in lyophilized form, and

were dissolved in a sterile D.W to give a final concentration of (10 pmol/µl) as recommended by provider .

2. **Go Tag ® Green Master Mix(2x):**Go Taq®Green Mater Mix is ready to use mixture that contains Taq DNA polymerase, $MgCl_2$, pure deoxynucleotied (dNTPs),reaction buffer and two dyes(blue and yellow) that allow monitoring of progress during electrophoresis, with concentration (2X).Go Taq ®Green Master Mix was provided by (promega-USA).
3. **Distilled water:** to achieve homogenisity of reagents and complete the reaction to the required volume, the D.W was added.
4. **DNA template.**

However, to prepare master mix for setting the RAPD-PCR reaction, the following reagents in table (2-6) were mixed in 0.5 ml eppendorff.

Table (2-6): Component required for RAPD-PCR reaction.

Addition Order	Component	Volume	Final con	Company
1	Promega green master mix 2X	12.5µl	1X	Promega
2	Sterile D.W	8.5µl		Promega
3	Primer	2µl	10 pmol/µl	Alpha DNA
4	DNA	2µl	100ng	
Total	25µl			

Negative control reaction was run in parallel; 23µl of master mix reaction was aliquot into 0.2 ml eppendorff tubes. 2µl of DNA template was also added and mixed gently; tubes then were run in PCR machine. The entire process was performed on ice aseptic condition using laminar air flow hood. The amplification reaction program was run as in table (2-7):

Table (2-7): The amplification reaction programe of RAPD PCR

Initial denaturation	Temp: 95Ċ	Time : 4min
No .of cycle =45 cycles		
Denaturation	Temp :92 ˚C	Time:1min
Annealing	Temp :36 ˚C	Time:1min
Extension	Temp :72 ˚C	Time:2min
Final extension	Temp :72 ˚C	Time: 5min

2.3.5.5. Agarose gel of RAPD PCR product

Approximately 20µl of PCR amplified products were separated by electrophoresis in 1.2% agrose gels (2 h, 5 v/cm, 0.5 x TBE buffer). Gels were stained with ethidium bromide, visualized by U.V transilluminator, and then imaged by gel documentation system (Hashmi *et al.*,2009). The size of PCR product estimated by comparing it with the marker 1Kb DNA ladder (250-10000) bp.

2.3.6: Collection of Olive leaves

Olive leaves were collected from Al-Jadrhia garden and classified as *olea europaea* L.by the Prof. Dr. Ali AL-Mossawy, the Biology department, college of science, Baghdad University.

2.3.6.1: preparation of Olive leaves extract

One kg of leaves is immersed with 3.8 L of 70% ethanol solution in water, and covered at 20˚C for 48h. At the end of this period, the alcohol is drained and another 3.8 L portion of ethanol is used to cover the leaves. The procedure was repeated twice more, and after the fourth cover, all extracts are combined, then distilled at 70 ˚C using a soxcelater to produce a concentrated alcoholic solution containing about 30 to 40 %solids, and the resultant crude extract was dried at 70˚C using evaporator to obtain a dry powder extract (Duke,2007). To be used in subsequent analyses, the powdered extract was dissolved in distillated water, and then sterilized by filtration.

2.3.6.2: Detection of some active compounds for olive leaves extract:

(I): Detection of tannins:

This was done according to the methods described by Harborne (1991). A few drops of ferric chloride was added to the diluted extract in test tube and shaken. The development of blue color was an indication of the presence of tannins.

(IV): Detection of Glycosides:

This was done according to the method described by shihata (1951). An equal amount of ethanolic extract of olive leaves was mixed with Fehling reagent in test tube, and then boiled in water for 10 minutes; red precipate would be an induction of the presence of glycosides.

(V): Terpenoid and Steroid:

This was done according to the method described by Al-Abid (1985). One gram of ethanolic extract was participating in a few drops of chloroform; a drop of acetic anhydride and a drop of concentrated sulphuric acid. A brown precipitate appeared which representing the presence of terpene, while the steroids represented the dark blue color in the extract.

(VI): Detection of Flavonoids:

Ten milliliters of 50% ethyl alcohol were mixed with 10 ml of 50% potassium hydroxide solution, and then added equal amount of volume of plant extract, the appearance of a yellow color is an indicator of the presence of flavonoids (Jaffer *et al.*, 1983).

(VII): Detection of Alkaloids:

This was done according to the method described by Silva *et al.*, (1998). Equal amounts of olive leaves extract and Dragindroff reagent were mixed; if alkaloids are present an orange- brownish precipitate will appear.

2.3.6.3: Effect of olive leaf extract on BCR-ABL genes ratio in MNCs *in vitro*.

Two samples of mononuclear cells from six blood samples of CML patients were randomly selected, to undergo either, pretreatment or post-

treatment analysis. BCR-ABL gene ratio was measured in each MNCs sample prior to treatment with different concentrations of OLE (800, 1200, 1450 µg/ml) for 48 h at 37˚C *in vitro* (Zahar, 2007). After treatment, BCR-ABL gene ratio was also measured for each sample. Comparison between pre-and post-treatment values for each MNCs sample were made in order to assess the anti- leuckemic potential of OLE.

2.3.7 Data analysis

2.3.7.1. Data Analysis of RAPD-PCR (Molecular Weight Estimation)

Molecular weight was calculated by using computer software M.W. Detecting program, Photo –Capture M.W. program from Consort, and based on comparing the PCR product with known size of DNA fragments of a Bench top PCR markers (which consist of 14 bands from 250 to 10,000 bp).

2.3.7.2. Data Analysis for Real time-PCR

The results were interpreted with software of Real Time -PCR instruments through the presence of crossing fluorescence curve with the threshold line and in accordance with instruments instruction .The cDNA M-bcr-abl is detected in the tubes with PCR-mix-1M-bcr-abl and cDNAof gene normalizer/internal control(IC) abl are detected in the tubes with PCR-mix-1N-abl, than the ratio was calculated.

2.3.7.3. Statistical analyses:

Data were given as means $\pm$ SE, and significant differences between means were assessed by student t-test using the computer programme SPSS version .13.

3. Results and discussions

3.1 Subjects

The current study included 48 CML patients with age range of 16-72yr, and the median age of (39.5 years), and 12 normal controls with the age range of (19-65yrs) and the median age of (35years). The highest age incidence was between (31-40), since (27.08%) of the incidence of the disease occurred among this age groups, with higher incidence in male than in female (1.8:1) (Figure 3-1). This finding agrees with that of Shbeeb (2008) who reported that the highest incidence of age was between (31-40). Furthermore, Hoffbrand and co-workers (2002) reported that the ratio of incidence of male to female (1.4:1) and the highest incidence occurs also frequently in middle age.

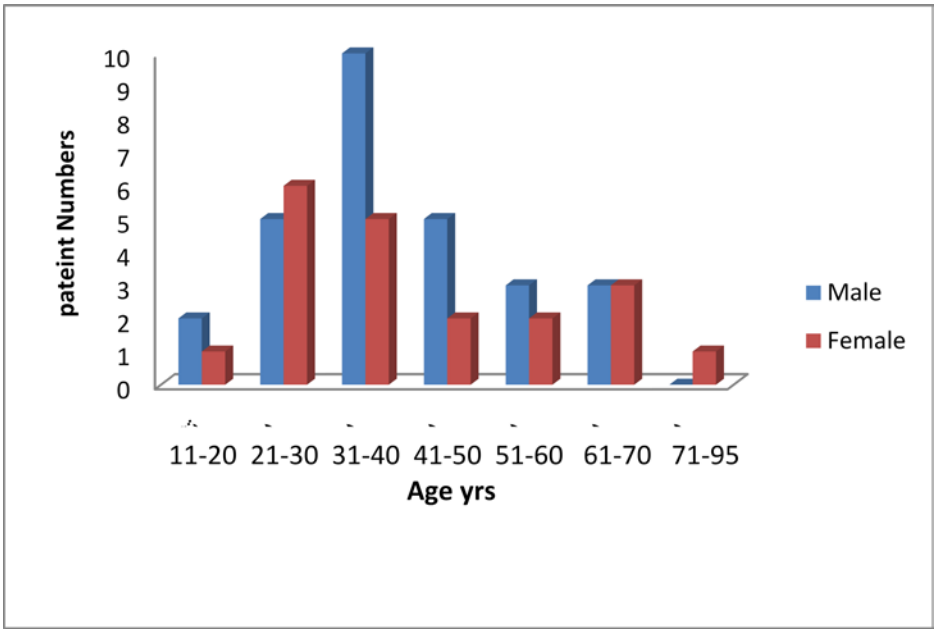

Figure (3-1): Age distribution of CML patients of the current study

3.1.1Viable cell counting:

The cell viability of the samples was determined before studying of the effect of *O. europea* extracts on MNCs. The cells with 90% viability were ready to test, and those with less than 90% cell viability were discarded.

3.1.2 Molecular detection of BCR –AB L gene in CML patients

3.1.2.1 Isolation of RNA from MNCs and its reverse transcription into cDNA

Patients and normal controls were screened for the detection and quantification of BCR-ABL (p210) fusion transcript. Total RNA was extracted from almost 10^{6} MNCs of blood using Trizol method (Yaghmaie, 2008). The integrity of RNA was determined by electrophoresis prior to reverse transcription and three bands were revealed as shown in figure (3-2). This result agrees with that of Abass *et al.* (2010) who reported that total RNA extracted from mammalian cells should give three bands (23S, 16S, 5 S). The purity of RNA was (1.5-1.7) for CML patients and (1.4-1.9) for healthy control using spectrophotometer. Agarose gel electrophoresis of cDNA which was generated from total RNA, gave a single band as shown in figure (3-3). This result agrees with that of Dahii (2010) who reported comparable RNA purity of (1.7) in CML patients. This could be attributed to the changes in the number of W.B.C (the only nucleated cells in the blood) because of the development of the disease. The high increase in the number of W.B.C as starting material could be negatively affect the quantity and quality of the extracted nucleic acids.

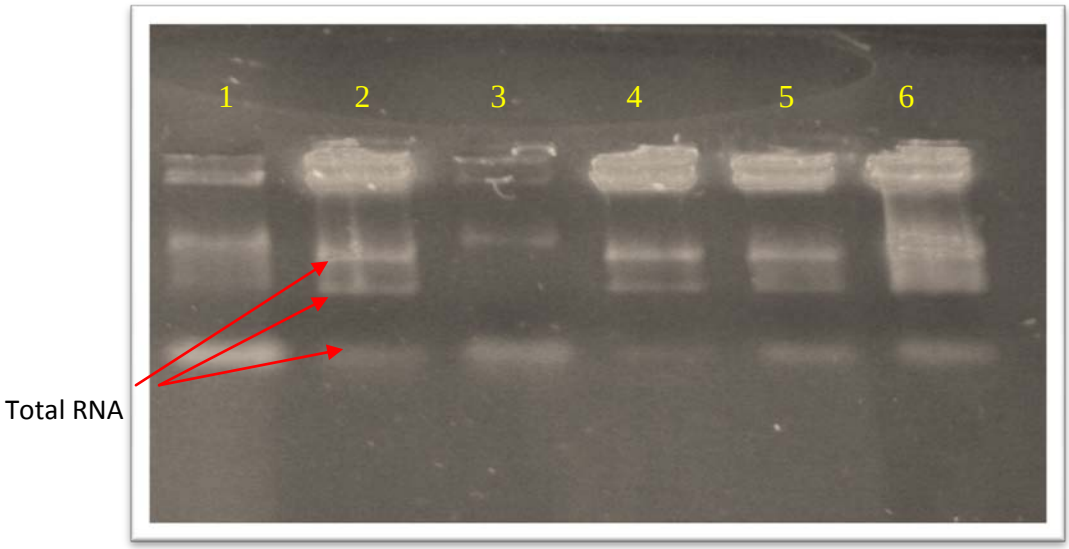

Figure (3-2): Agarose gel electrophoresis of the total RNA sample. Samples lanes (1-6) were fractionated by electrophoresis on 1% agarose gel (1 h, 10 V/cm, 0.5X TBE) and visualized under U.V light after staining with ethidium bromide.

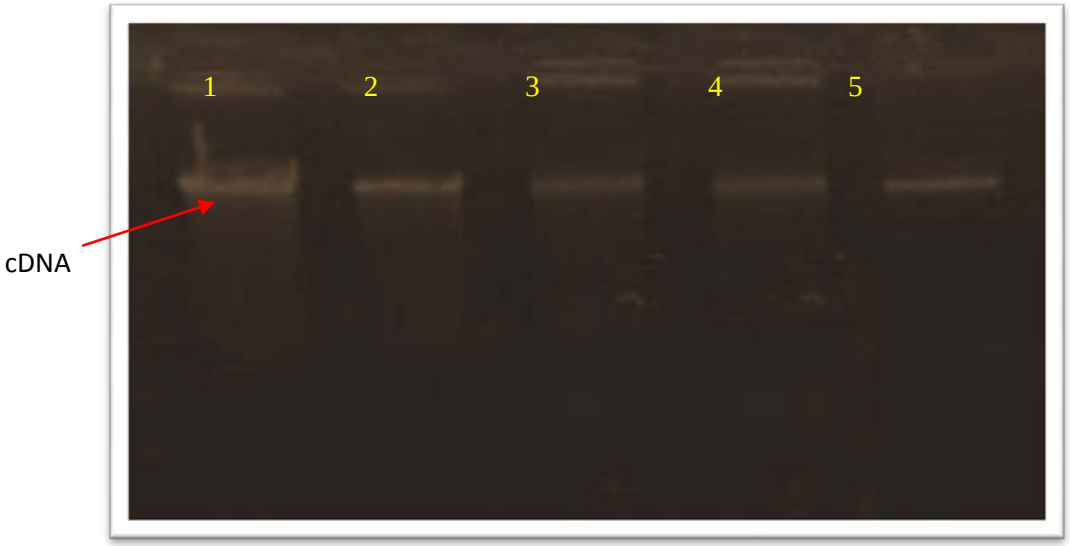

Figure (3-3): Agarose gel electrophoresis of the cDNA sample. Samples lanes (1-5) were fractionated by electrophoresis on 0.8% agarose gel (1 h, 10 V/cm, 0.5X TBE) and visualized under U.V light after staining with ethidium bromide.

3.1.2.2 Quantification of BCR-ABL gene using Real Time- PCR

Patients with CML have an abnormal Philadelphia chromosome, which creates an abnormal gene called BCR/ABL. The BCR/ABL gene leads to the production of a type of enzyme called tyrosine kinase, which induce the marrow to make too many white blood cells. Quantification of BCR-ABL p210 transcripts in the blood samples of Chronic Myeloid Leukemic patients and the normal individuals were done according to the instruction of BCR-ABL M-bcr Fusion*Quant*® Kit (Sacace, Italy). The tests were performed by RT-PCR Applied Bio system7300. Ratio of BCR-ABL gene was calculated using the following equation:

Number of copies of fusion gene (No. of copies of cDNA M_bcr_abl) / number of copies of normal gene (No. of copies of N-abl) as shown in (table 3-2).

Table (3-1): Effect of imatinib treatment duration on the ratio of BCR-ABL fusion gene

Duration of treatment (year)	Number of Patients.	Ratio of BCR-ABL gene
>2	20	2.14 ± 0.36^{a}
2-4	11	0.24 ± 0.02^{b}
5-7	12	$0.006 \pm 8.6*10^{-4}$ c

-values are expressed as mean ±SE

-values with different letters are significantly different (P<0.05)

The present results demonstrated that the longer the duration of treatment with anti leukemic drug (imatinib), the more drastic the decrease in the ratio of BCR-ABL gene, suggesting the presence of a relationship between treatment duration and BCR-ABL gene ratio. This finding is interesting, yet does not provide a conclusive evidence of such relationship for several reasons. First, the numbers of patients are too small to draw strong conclusion from them. Second, such relationship has not yet been reported in the available literature. Further studies are needed to investigate this finding or a large number of patients to test whether or not it could be utilized as a marker to assess the CML patients' response to imatinib. The treatment of CML was revolutionized by the introduction of imatinib, a BCR-ABL tyrosine kinase inhibitor; its use has resulted in a significantly improved prognosis, response rate, and overall survival in CML patients (O' Brien *et al.*, 2003, An *et al.*, 2010; Chen *et al.*, 2010). The majority of patients (43 out of 48 i.e 90%) on imatinib thereby achieved a marked decline in BCR-ABL gene ratio, but others (5 out of 48 i.e 10%) may have expressed another mutant form of gene (Gora- Taybor and Robak, 2006). Whether or not the latter finding may reflect a suboptimal response to imatinib (Hughes and Branford, 2009) is not clear at present.

3.2.1. Detection of active compounds

Plants are a natural source of a wide range of chemical compounds that make them of medicinal importance. These compounds are divided, depending on their activity, into two types of active constituents, inert and active constituents. The inert compounds are defined as compounds with no physiological effects; for instance, cellulose, lignin and subrine, while the active constituents defined as compounds with different medicinal and physiological activities. The active constitutes, in turn are divided into several types depending on their chemical and physical characteristic such

as alkaloid, tannins, glycoside, saponines, steroids and flavonoides (Hussein, 1981). In this study the active compounds of olive leaves ethanolic extract were analyzed chemically to illustrate its general active constituents. The ethanolic extract was positive for flavoindes, terrpenes, glycosides, and steroid tannins and negative for alkaloid (Table 3-1).

Table 3-2: Chemical analysis of olive leaves ethanolic extract

Chemical compounds	Reagents	Indication	Results
Flavonoids	KOH	Yellow	Positive
Glycosides	Fehling reagent	Red precipitate	Positive
Terrpenes	Acetic anhydride, chloroform, Concentrated sulfuric acid	Brown precipitate	Positive
Steroids	Acetic anhydride, chloroform, Concentrated sulfuric acid	Dark blue color	Positive
Tannins	Ferric chloride	Blue	Positive
Alkaloid	Drangindroff reagent	Orange-brownish precipitate	Negative

3.2.2 Investigation of the effect of olive leaves extract on BCR-ABL gene ratio in MNCs.

Table (3-3) showed that exposing of MNCs cells of CML patients to the three OLE concentrations (800, 1200, 1450 µg/ml) had no effect on the BCR-ABL gene fusion ratio, suggesting that OLE has no effect on the expression of BCR-ABL gene in CML patients. BCR-ABL oncogene has been shown to induce endogenous reactive oxygen species (ROS) that result in chronic oxidative DNA damage, double- strand breaks in S and G_2/ M cell- cycle phases, and mutagenesis (Nowicki *et al.*, 2004). Evidence is available which suggest that the BCR-ABL mediated ROS generation in combination with aberrant regulation of DNA repair pathways in CML cells contribute to genomic instability that results in cytogenetic abnormalities (Gardama and Cortes, 2009).

Table (3-3): Effect of ethanolic olive leaves extract on BCR-ABL gene ratio in mononuclear cells of Chronic Myeloid Leukemia patients

BCR-ABL gene Ratio		
OLE concentration	**Pretreatment**	**Post treatment**
800µg/ml	**1.38±0.45**	**1.34±0.46**
1200µl/ml		**1.25±0.42**
1450µg/ml		**1.32±0.31**

Values are expressed as mean + SE of 6 patients:

MNCs from each patient were analyzed in triplicates.

On the other hand, several studies have demonstrated that OLE possesses potent antioxidant activities against oxidative stress induced by ROS (O' Brien *et al.,* 2006; El and Karakaya, 2009; Wang *et al.,* 2011), which in turn may be responsible for the protective activity of OLE against radiation- induced skin damage and carcinogensis in hairless mice(Kimura and Sumiyoshi, 2009), human breast cancer cells (Goulas *et al.,* 2009; Fu *et al.,* 2010; Bouallaqui *et al.,* 2011), and HL 60 human leukemia cells (Abaza *et al.,* 2007; Anter *et al.,* 2011).

Therefore, it would seem reasonable to hypothesize that the ROS induced by BCR-ABL oncogene may be scavenged by OLEs, thereby exerting a protective effect against oxidative stress induced by these ROS. This was not the picture revealed in table 3-3. Although the mechanism for this lack of effect of OLEs is not clear at the moment, several possibilities may be raised. First, experimental conditions may be suboptimal in terms of concentrations of OLE used, method of extraction of olive leaves, and most importantly the type of cells which underwent in vitro treatment, i.e, MNCs tested. While the concentrations of OLE and methods of extraction can be easily manipulated in future studies by either increasing OLE concentration or using water extract instead of alcoholic extract, yet the use of MNCs was an un orthodox approach in the present study. No comparable report in the available literature indicated that MNCs from CML patients were used *in vitro* to test the antileukemic effect of OLEs. Instead, we have two reports using HL-60 leukemia cells treated with OLE (Abaza *et al.,* 2007; Anter *et al.,* 2011).

Furthermore, these MNCs were obtained from patients continuously treated with imatinib for considerable period of time. This fact may complicate the interpretation of the results and makes it difficult to differentiate between effects attributed to OLE from those caused by

imatinib. Indeed, our primary objective was scheduled to investigate effects of OLE on human myelogenous leukemia K562 together with MNCs. However, all our attempts to purchase these cells were futile because of the poor shipping and storage conditions, which damaged all of them.

Accordingly, our results suggest that, under the present experimental condition, OLE failed to exhibit any appreciable effect on BCR-ABL gene ratio in CML patients. Further studies are needed to investigate the effect of olive leaf extracts (water and/or alcoholic) on K562 cells *in vitro* using wide range of concentrations and for variable periods of incubation.

3.3 Molecular analysis of genetic instability using RAPD PCR

3.3.1 DNA isolation

To investigate the genomic instability, DNA was successfully extracted from peripheral blood of nine CML patients and five normal individuals using promega DNA Wizard kit. The range of purity of extracted DNA was 1.10-1.60 for CML patients, and 1.05-1.61 for normal controls. The range of DNA concentration for CML was (1.25- 3.88) µg/ml and the range for controls was (2.95-4.21)µg/ml. Because of the high specificity and sensitivity of PCR technique it does not requires high quantities of DNA (Saiki, 1989). Hence, the resulted DNA concentration and purity were suitable for amplification using RAPD-PCR technique as shown in figure (3-4). Other investigators reported similar results (Neri *et al.*, 2004; Tan *et al.*, 2006) of DNA purity.

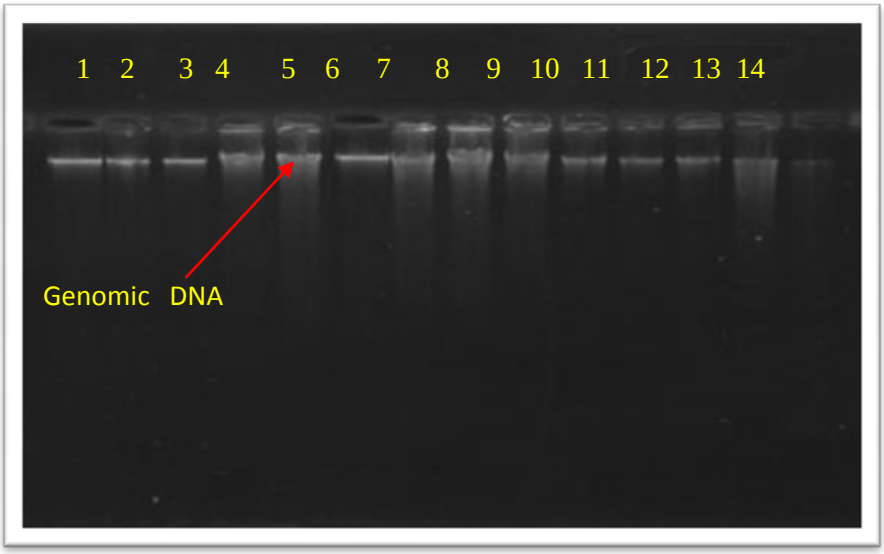

Figure (3-4): Agarose gel electrophoresis of the total genomic DNA. Lane (1-9) the extracted DNA from CML patients. Lanes (10-14) the extracted DNA from healthy individual. Extracted DNA sample were fractionated by electrophoresis on 0.8% agarose gel (1 h, 5 V/cm, 0.5 X TBE) and visualized under U.V light after staining with ethidium bromide.

3.3.2 RAPD –PCR

In this study, RAPD –PCR technique was used to reveal DNA polymorphism in DNA of CML patients and control sample. Our objective is to search for the source of differences that could be used as a DNA marker specific for the CML patients, and to investigate the presence of any genomic instability that could be resulted from carcinogenesis.

RAPD-PCR reaction was repeated on a set of DNA samples with several different primers, under PCR optimum conditions of programming and reagent concentration that result in a different RAPD patterns for each primer (Williams *et al.*, 1993). The primers used in this study were screened randomly, and to select the suitable universal primer, several primers had been tested at first for giving amplified products and polymorphism in DNA samples. In the first experiment 12 primers had been tested with same DNA sample under standard or optimum conditions.

The random primers that give results in term of amplification and/ or polymorphism include (OPA-09, OPA-07, OPC-08, OPN-16, OPO-04 (Table 3-4). In the second category, no amplified products were detected. In spite of repeating the experiment, similar results were obtained using 7 different primers. These included OPA-11, OPA-13, OPB-07, OPC-12, OPD-20, OPI-01and GB8 (Table 3-5). The failure of these primers to amplify the genomic DNA of healthy individual and CML patients may be attributed to the absence of suitable priming sites for these primers in the genomes of tested leukemic patients and normal individuals. In other words, there are no complementary sequences for these primers in leukemic patients and normal individual's genomes. Similar results have been reported by Papadopoulos and his co- worker (2002) who identified a number of RAPD primers that didn't show amplification products in breast cancer and uveal melanoma.

Table (3-4): Universal primers that gave results of polymorphism

Primers	Monomorphisum	Polymorphisum	Sequence
OPA-07	+	+	5'-AAGTCCGCTC-3'
OPA-09	+	+	5'-GGGTAACGCC-3'
OPC-08	+	+	5'-TGGCGGTG-3'
OPN-16	+	+	5'-CAAGGTGGGT-3'
OPO-04	+	+	5'-AAGTCCGTC-3'

Table(3-5): Universal primers that did not give results of polymorphism

Primers	Monomorphisum	Polymorphisum	Sequence
OPA-11	+	_	5'-CAATCGCCGT-3'
OPA-13	+	_	5'-CAGCACCCAC-3'
OPB-07	+	_	5 '-GTAGACCCGT-' 3
OPD-20	+	-	5'-TGTCATCCCC-3'
GB8	+	-	5'-GTCCACACGG-3'
OPI-01	+	-	5'-ACCTGGACA-3'
OPC-12	+	-	5'-TGTCATCCCC-3'

High levels of polymorphism were generated in this study among CML patients and healthy persons using the primers (OPA-07, OPA-09, OPC-08, OPN-16, andOPO-04). Other primers generate unique bands that could be used as DNA marker to distinguish CML from healthy person. In some instance, the reasons behind DNA polymorphism among samples may a single base changes in genomic DNA. Other sources of polymorphism may include deletion of a priming site, insertions that render priming sites too distant to support amplification, or insertion that change the size of a DNA segment without preventing its amplification (Madea *et al.*, 1999; Ong *et al.*, 1998; Papadopoulos *et al.*, 2002; Xian *et al.*, 2005). Furthermore, Williams *et al.* (1990) have reported that single nucleotide changes in a primer sequence caused a complete change in the pattern of amplified DNA segment.

3.3.2.1 Primer OPA-07

RAPD-PCR amplification of the total genomic DNA extracted from both CML patients and healthy individual using the primer OPA-07 which has the sequence (5' AAGTCCGCTC 3') gave a total of 26 bands with molecular weight ranged between (269-854 bp) (figure 3-5). The number of amplified bands ranged between (2-3) across lanes.

The biggest band with molecular weight 854 bp was a unique band, is absent in all CML patients and controls except in subject no.10. The band number two with molecular weight 760 bp was present in Patients no.1, 2, 3 and in normal individual no. 11 and 14, while absent in others CML patients and individual controls.

The band with molecular weight 725 bp was absent in CML and individual control except patients no.1, 3 and healthy controls no.11 and 14. The band number four with molecular weight 354 bp was observed as a monomorphic band in all CML patients and individual controls.

The band with molecular weight 269 bp present in Patients no. 2 and 3. On the other hand, it was absent in most CML patients and in individual controls (figure 3-5).

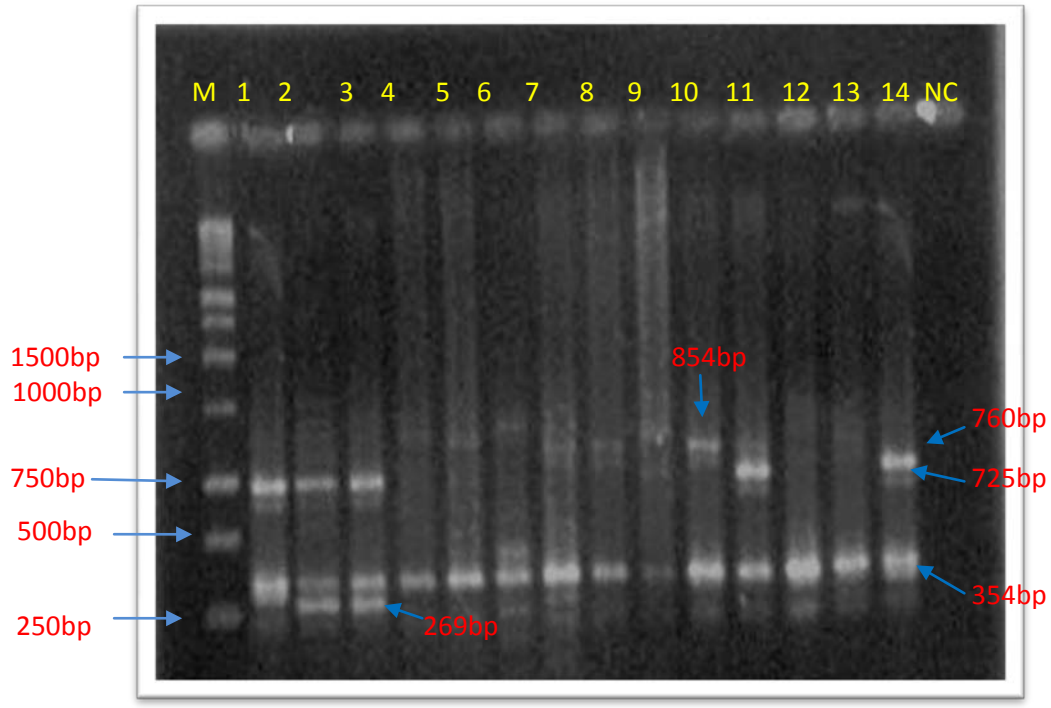

Figure (3-5): RAPD-PCR analysis of band patterns of CML patients obtained with OPA-07 primer. Electrophoresis was performed on (1.5%) agarose gel and run with 5 volt/cm for two hours. The lanes (1-9) represent CML patients, lanes (10-14) represent normal individual. M: marker ladder, NC: negative control.

3.3.2.2 Primer OPA-9

RAPD-PCR amplification performed using the primer OPA-09, with the sequences of (GGGTAACGCC), gave various numbers and patterns of bands in both CML patients and individual controls as seen in the(figure 3-6).

The number of amplified bands detected was 111 and ranged from 7-11 bands for the tested samples. The molecular weight of these bands ranged from (139-2195). The loss or gain of amplified band in CML patients when comparing with control is clearly detectable. For example the biggest band obtained using this primer with molecular weight 2195bp lost in Patients no.1, 3, 4, 5, 6 and 9 and in normal individual control no.

12,13,14 While this band was present in others normal individual and others CML patients.

The two bands with a molecular weights 1674 bp, 1402 bp were present in all CML patients and normal individuals. The other band with a molecular weight of 1287 bp was present in all CML patients and normal individuals except healthy controls no.13 and 14.

A band with a molecular weight of 1084 bp appeared in patient's no.5 and 8, but this band absent in normal individual's person and others patients. The other bands with molecular weight 916 bp present in Patients no. 2, 3,4 , 6, 7 and normal individuals control no. 11, 13 and 14.

The other band with molecular weight 841 bp were appeared as monomorphic in all others CML patients and normal individuals. The unique band with molecular weight 579 bp which appeared in Patient 6 while absent in all patients and normal individual.

The band with molecular weight 509 bp was revealed as monomorphic band in all patients and control. The other band with molecular weight 432, 331appeared in patient 6 while absent in all other CML patients and normal controls. The two small bands with molecular weight 211, 195 were present in all CML patients and normal control.

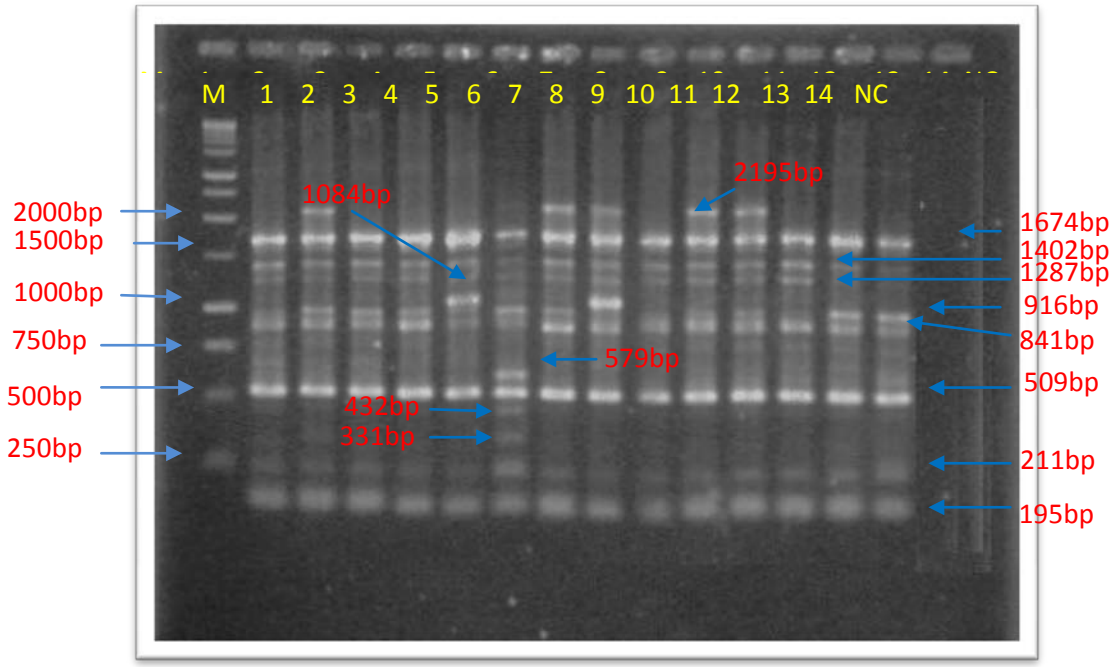

Figure (3-6): RAPD-PCR analysis of band patterns of CML patients obtained with OPA-09 primer. Electrophoresis was performed on (1.5%) agarose gel and run with 5 volt/cm for 2 hr. The lanes (1-9) represent CML patients; lanes (10-14) represent normal individuals. M: marker ladder, NC: negative control.

3.3.2.3 Primer OPC-08

Genomic DNA of CML patients and individuals controls were amplified by using primer C8 with sequences5'-TGGCGGTG-3', the result showed a total number of bands of 72, ranged from (418-2123) bp.

In regard to polymorphic bands, the biggest bands with molecular weight 2123, 1422 bp were present in all CML and in individual controls with different intensity. The band with molecular weight 1356 bp present in all CML except patients 6, 7 and present in all individual controls except no.11, 14. Another amplified band with molecular weight 1067 bp was present in all CML patients and in all individual controls. A band with molecular weight 667 bp appeared in all CML patients and normal controls except patient no. 8. Two Bands with molecular Weights 520, 476 bp were absent in Patients no.1, 4, 5, 8 ,9 and in healthy control no. 10 (Figure 3-7).

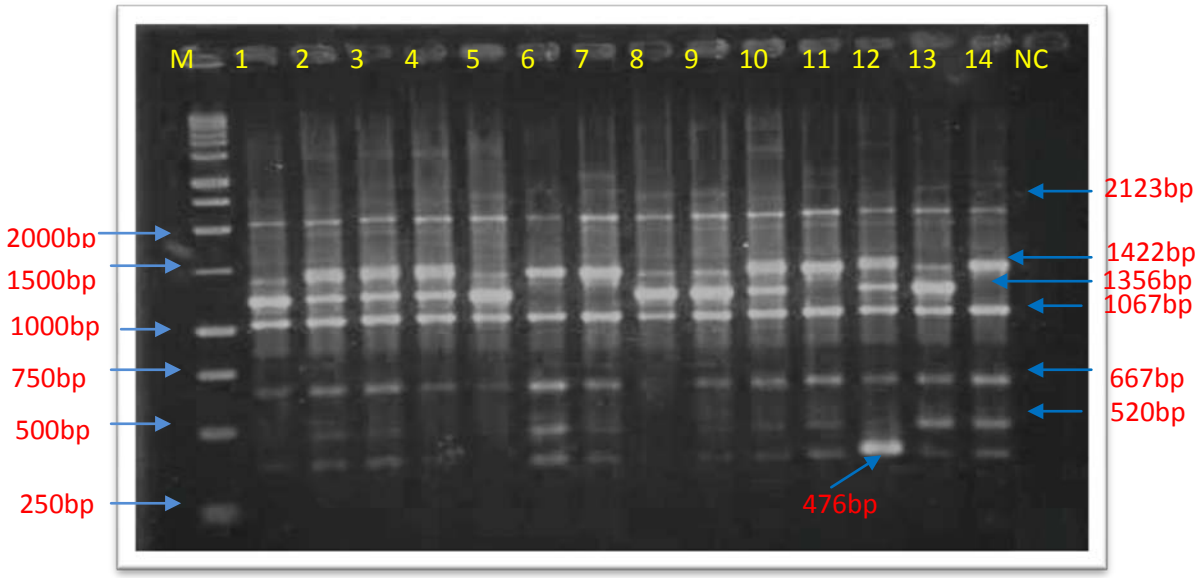

Figure (3-7): RAPD-PCR analysis of band patterns of CML patients obtained with C-08 primer. Electrophoresis was performed on (1.5%) agarose gel and run with 5v volt/cm for two hours. The lanes (1-9) represent CML patients, lanes (10-14) represent normal individuals, M: Ladder DNA, NC: negative control.

3.3.2.4 Primer OPN-16

RAPD-PCR analysis with primer N16 which has the sequence (5'-CAAGGTGGGT-3') resulted a total number of 62 bands, and number of amplified bands ranging between (3-8) across tested samples as shown in the (figure 3-8). The molecular weight of these bands ranged between (130-2196 bp). The biggest bands with molecular weights 2196, 1867, 1695, 1323 bp were absent in all of CML except 5, and in normal controls. Bands with molecular weights 930, 853, 670 bp were absent in Patient no. 1 but were present in all others CML and controls. The other band with molecular weight 509 was present in Patients1, 2, 3, 4, 5, 8, 9, and normal individual no.11, 12, 13, while absent in other normal controls and patients. The band with molecular weight 483 bp was present in Patients1, 3 and absent in all others CML patients and controls.

The other band with molecular weight 439 bp was absent in all CML patients and others individual controls except in one normal control no.14.The band with molecular weight 383 bp was present in all CML except Patient no.1 and in all controls. The band with molecular weight 130 bp was present in Pateints1and 2, but absent in others CML patients and in individual controls (figure 3-8).

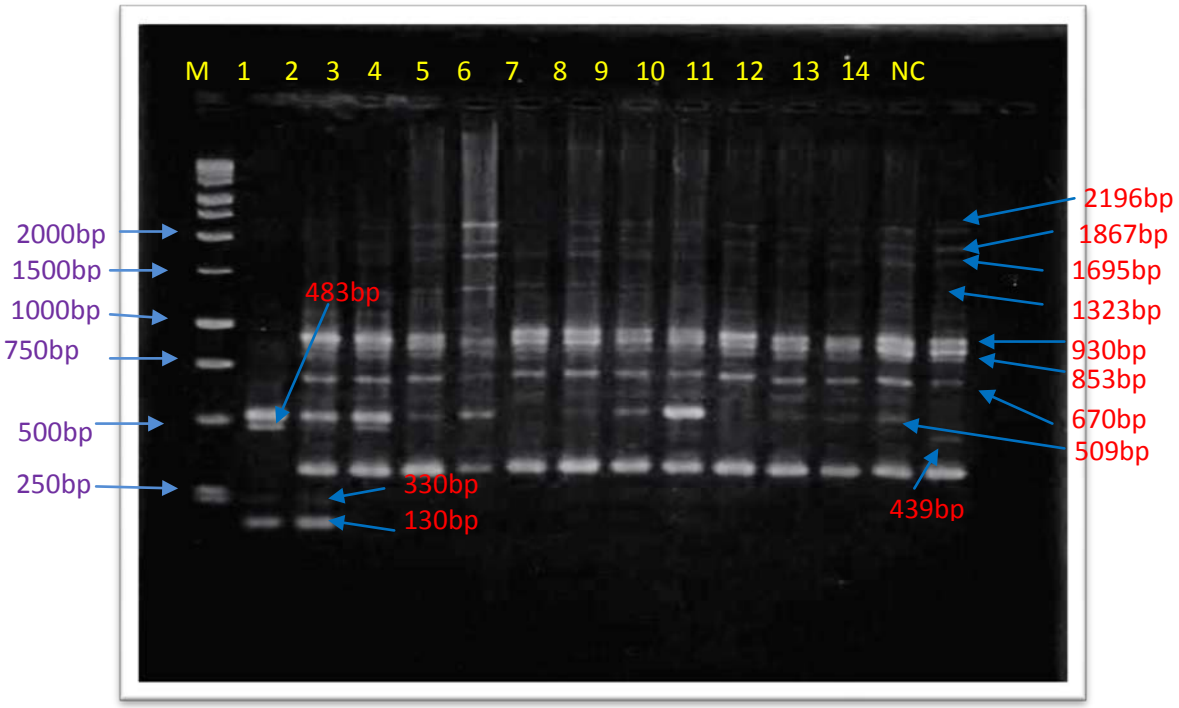

Figure (3-8): RAPD-PCR analysis of band patterns of CML patients obtained with OPN-16primer. Electrophoresis was performed on (1.5%) agarose gel and run with 5 volt/cm for two hours. The lanes (1-9) represent CML patients, lanes (10-14) represent normal individuals, M: ladder DNA, NC: negative control.

3.3.2.5 Primer OPO-04

RAPAD –PCR pattern generated from the tested CML and normal individuals using the primer OPO-04, which has the sequences of (5'-AAGTCCGTC-3'), gave the total number of 65 bands. These bands ranged between (3-5) bands in each tested samples we can see in the (figure 3-9).

This primer gave bands with molecular weights ranged between (125-927 bp). The first band with molecular weight 927 bp was present in all CML patients except patients' no. 8, and in normal individual. The second band with molecular weight 876 bp was present in all CML patients except Patient no.1 and in other normal individuals.

The third band with molecular weight 715 bp was absent in Patients no.1 and 6, while present in other CML patients and in normal individuals.

The fourth band with molecular weight 526 bp was present in Patients no.1, 2, 3, and 6; while it was absent in other CML patients and in normal individuals.

A band with molecular weight 494 bp was absent in most of CML patients except in Patients no.1 and 2.patients and also absent in normal controls except no.14.

Another band with molecular weight of about 330 bp appeared as monomorphic band in CML patients and in normal controls.

The last band with a molecular weight of about 125 bp was present in Patients no.1, 2, 3and 6, but absent in other CML patients and in normal individuals.

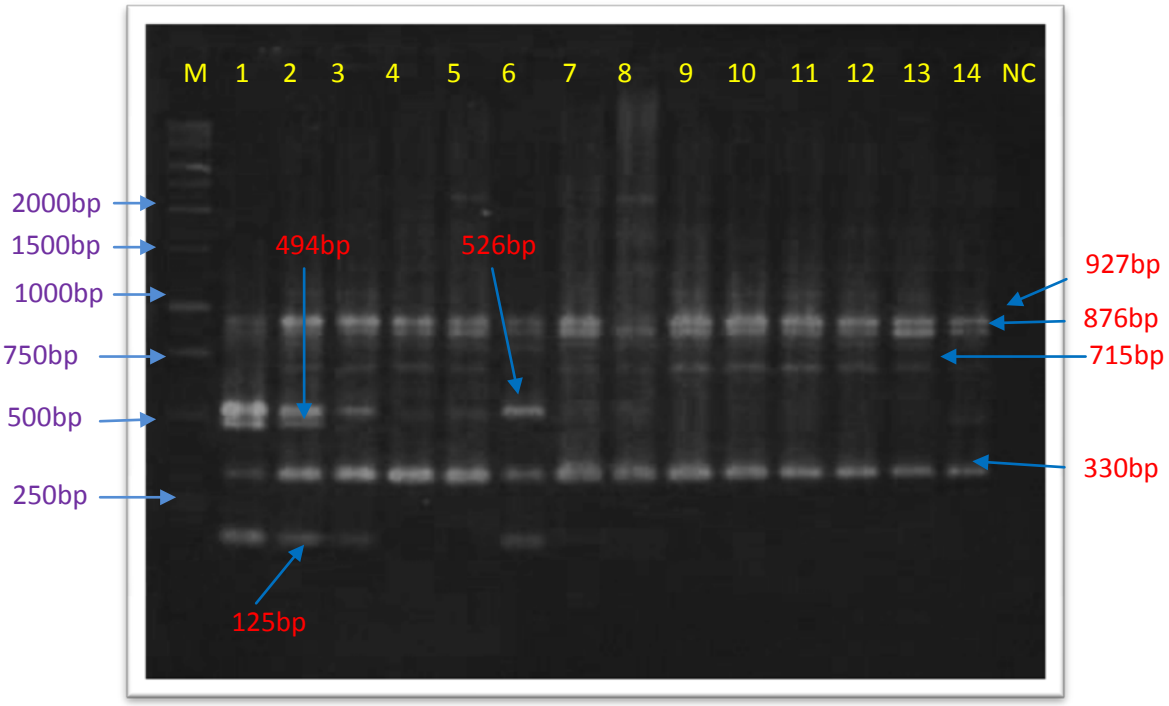

Figure (3-9): RAPD-PCR analysis of band patterns of CML patients obtained with OPO-04 primer. Electrophoresis was performed on (1.5%) agarose gel and run with 5 volt/cm for two hours. The lanes (1-9) represent CML patients, lanes (10-14) represent normal individuals, M: ladder DNA, NC: negative control.

3.3.2.6 Interpretation of RAPD-PCR analyses of primers (Table 3-6)

In this study, the results of RAPD –PCR analyses of five primers had revealed DNA polymorphism in genomic DNA from CML patients and normal individuals. The polymorphism resulted from differences in number of bands or in molecular weight of amplified bands.

The results showed differences in patterns of band profile produced by each primer. The observevation of variations in the number of bands in any one sample was possible; for example, the highest number of bands in any one sample were observed using primer OPA-9 (11 bands), while the lowest number of bands (2 bands) were observed using primer OPA-07. Other primers showed bands number ranging between >2 and<11. The variation in the number of amplified bands is influenced by the primer binding sites with

genomic DNA .Also there was a difference in the molecular weight of amplified band; the highest molecular weight of amplified band was that of 2196 bp using the primer OPN-16, and the lowest molecular weight of amplified band was 125 bp when using primer OPO-04. Polymorphism in the molecular weight of amplified bands demonstrated the differences in the length between the primer binding sites and the genomic DNA, and resulted in a mobility shift of bands (Ong *et al.*, 1998; Papadopoulos *et al.*, 2002; Xian *et al.*, 2005).

The efficiency value of polymorphism was measured by dividing the number of polymorphic bands on the total number of total amplified bands produced by the designated each primer (Hassan, 2002). The highest efficiency value was obtained when using the primer OPN-16 (0.193) and the lowest value was observed in the using primer OPC-08 (0.05). Accordingly, the primer efficiency indicates the ability of primer to yield the highest polymorphism among the CML patients and normal individual. Therefore, among (62) amplified bands appeared using primer OPN-16, the number of polymorphic bands were (12), indicating a highest efficiency value. On other hand, the primer OPC-8 produced the lowest efficiency value manifested by the fact that among a total number of 72 amplified bands, the polymorphic band was only (4) (table 3-7). In this regard, Shebeeb (2008) also revealed the efficiency of different types of primers used in leukemic patients. Furthermore, it was possible to observe a decrease or increase in the relative intensities of the amplified bands obtained in the RAPD analysis of genomic DNA of CML patients in comparison with normal individuals (Maeda *et al.*, 1999; Xian *et al.*, 2005).

Table (3-6): Analysis of RAPD-PCR results of CML patients and individual control.

Primer	Range no. of amplified bands	The range of molecular weights of amplified band	No. of polymorphic bands	Total no. of amplified bands	Efficiency
OPA-07	2-3	269- 854	4	26	0.153
OPA-09	7-11	139-2195	7	111	0.063
OPC-08	5-6	418-2123	4	72	0.05
OPN-16	3-5	130-2196	12	62	0.193
OPO-04	5-8	125-927	6	65	0.0923

Some primers were able to amplify a band in certain type of CML such band was absent in normal persons. For example, the result of primer OPA-07 showed the band number five with molecular weight 269 bp present in 22 % of CML while absent in other CML patients and in normal individual persons .The result of polymorphism of primer OPA-9 showed the band number five with molecular weight 1084 bp appeared in 22% of CML while absent in normal person and others CML. Furthermore the band number with molecular weight 579bp was appeared in 11% of CML and absent in all normal person and in others CML patients. The primer OPN-16 showed the band number eight with molecular weight 509 present in 77% of CML patients and absent in all normal person. Also the band number nine with molecular weights 483 bp appeared in 22% of CML and absent in all normal individual and in other CML patients. Another band with molecular weights

439 bp obtained in 11% in normal individual controls. On other hand, the result of primer OPO-4 showed a band with molecular weight 927 absent in 11% of CML while appeared in normal individual and in others CML patients. The band with molecular weight 125 bp appeared in 44% of CML while absent in normal individual and in others CML patients.

Based on the above mentioned finding, the amplified bands profile which appeared in response to primers had produced a variety of some unique patters in CML patients. By no means, those bands can be relied upon and considered as a genetic marker for CML. Yet, their significance can not the ruled out as well, and further studies are needed to investigate whether or not these bands could be utilized clinically as a potential markers for the diagnosis of CML, or to assess the response of patients to therapy. One approach could be utilization of further universal primers on a large number of patients undergoing therapy for various durations of time.

4.1 Conclusions:-

From the results of this study we conclude the following:

1. Real time PCR is very efficient and important tool in the identification of CML and the follow up of patients receiving treatment.
2. RAPD-PCR could be used as a simple and applicable technique in the study of chronic myeloid leukemia at the molecular level.
3. The crude ethanolic extract has no effect on the expression of BCR-ABL gene under the present experimental conditions.

4.2 Recommendations:

Further studies are needed to investigate the following topics:

1. The significance of the specific amplified bands which appeared in RAPD-PCR obtained with the universal primers. Investigating whether or not those bands can be unitized as a potential marker for CML diagnosis and /or assessing the response of patients to therapy, is very much needed by using other universal primers and study the quality of the produced amplified bands by RAPD-PCR.
2. The effect of OLE (water and alcoholic) in various doses and durations of incubation on human myelogenous leukemic (k562) cells, or human leukemia (HL-60) cells, and study the antioxidant and antileukemic activities of those extracts. Factors such as complete identification of active constituents of the extract, effective dose, and the duration of treatment with OLE must be taken in consideration.

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الخلاصة

اجريت الدراسة الحالية لغرض التحري عن فعالية المستخلص الكحولي لاوراق الزيتون على مجموعة من مرضى سرطان ابيضاض الدم النخاعيني المزمن Chronic myeloid leukemia (CML) الذين تم الكشف عن اصابتهم بالمرض باستخدام تقنية Real Time –PCR ومتابعة استجابتهم للعلاج (Imatinib) من جهة واستجابة مجموعة منتخبة منهم للمستخلص الكحولي لاوراق الزيتون ومن ثم اخضاع مجموعة منهم لدراسة عدم الثبات الوراثي باستخدام طريقة RAPD-PCR.

جمعت عينات الدم ل ٤٨ مريضا بسرطان ابيضاض الدم النخاعيني المزمن لمرضى من المركز الوطني لبحوث امراض الدم مصابين بالمرض من خلال الفحوصات المختبرية و ١٢ عينة دم من الاصحاء لاتظهر عليهم اي علامات مرضية، عزلت خلايا البيضاء احادية النواة Mononuclear cells (MNCs) بطريقة (Ficol) حيث تم استخلاص RNA من الخلايا المعزولة من المجموعتين باستخدام طريقة ال (Trizol) بالكشف عن كمية ونقاوة ال RNA المستخلص باستخدام مقياس الكثافة الضوئية والترحيل على هلام الاكاروز على التوالي.تم استخدام RNA للمستخلص لغرض تصنيع cDNA المكمل لغرض استخدامه لاحقا في تقنية Real Time-PCR. اظهرت نتائج فحص Real Time-PCR اصابة ٤٣ من المرضى بسرطان ابيضاض الدم النخاعيني المزمن CML الناتج عن اندماج جين BCR-ABL المنتج لبروتين (p 210). بينما كانت (٥) من المرضى لا يمثلون هذا الجين المدمج.

حيث تم الكشف عن المواد الفعالة في المستخلص الكحولي لأوراق الزيتون تم الكشف عنها وكانت النتائج ايجابية بالنسبة للفلافونيدات و التربينات والكلاكوسيدات والستيرويدات بينما كانت سالبة للقلويدات.استخدمت ثلاثة تراكيز من المستخلص الكحولي هي (٨٠٠، ١٢٠٠، ١٤٥٠) مايكروغرام /مل على الخلايا الاحادي النواة (MNCs) المعزولة من مرضى ابيضاض الدم النخاعيني المزمن وحضنت على درجة ٣٧ م° لمدة ٤٨ ساعة بوجود نموذج السيطرة.اعيد جمع الخلايا المعاملة وخلايا السيطرة واستخلص ال RNA منها واعيد تفاعل Real time -PCR عليها. لم تظهر النتائج وجود اي فرق معنوي بين الخلايا قبل وبعد المعاملة بالمستخلص وبالتراكيز الثلاثة.

ونظرا لكون حالة عدم الاستقرار الوراثي اصبحت ظاهرة معروفة ومصاحبة لكثير من الامراض السرطانية فقد تم استخدام طريقة ال RAPD-PCR للتحري عن عدم حالة الاستقرار الوراثي في بعض العينات المرضى المدروسة واستخدام في بعض عينات الاصحاء كسيطرة .

اختيرت (١٢) بادئا عشوائية لغرض استخدامها في التحري عن عدم الثبات الوراثي وانتخبت منها افضل (٥) بادئات اعطت نتائج تضاعف في التحري الاول.

اظهرت البادئات المنتخبة وجود بعض البادئات التي اعطت انماطا مميزة في مجموعة من المرضى مع وجود حزم نادرة مميزة لهم عما موجود في الاصحاء.ا لا ان النتائج لم تشر الى وجود بادئ معين يمكن الاعتماد عليه في التمييز مابين المرضى والاصحاء.لذا يتوجب اجراء دراسات مستقبلية للتعمق في هذه النتائج ومعرفة امكانية استخدامها في تشخيص المرض، او متابعة استجابة المريض للعلاج الخاص.

بِسْمِ اللّهِ الرَّحْمَنِ الرَّحِيمِ

(قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا
عَلَّمْتَنَا إِنَّكَ أَنْتَ العَلِيمُ الحَكِيمُ)

صدقَ اللّهُ العظيمُ

سورة البقرة الاية (32)

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

قسم التقانة الاحيائية

دراسة وراثية لجين ب س ر- أ ب ل والتغيرات الوراثية في مرضى سرطان ابيضاض الدم النخاعيني المزمن في العراق

رسالة

مقدمة الى كلية العلوم- جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

ياسمين علي هادي الياسري

بكالوريوس علوم حياة / الجامعة المستنصرية 2000

بأشراف

د.صالح احمد وهيب

استاذ

د.بلال كامل سليمان

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تشرين الثاني 2011

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