# Part III

## Chapter 1

**Cytotoxcic activity of essential oil of *Ocimum basilicum* L.and *Pimpinella anisum* L.on normal peripheral blood cells (PBMCs), hepatocellular carcinoma (HepG-2) and colorectal adenocarcinoma (Caco-2).**

## Chapter 2

**Antioxidant activity of essential oil of *Ocimum basilicum* L.and *Pimpinella anisum* L.using DPPH assay.**

## Chapter 3

**Immunostimulant activity of essential oil of *Ocimum basilicum* L. and *Pimpinella anisum* L.**

# Chapter 1

## Cytotoxcic activity of essential oil of *Ocimum basilicum* L. and *Pimpinella anisum* L. on normal peripheral blood cells (PBMCs), hepatocellular carcinoma (HepG-2) and colorectal adenocarcinoma (Caco-2).

# INTRODUCTION

**Cancer**

Cancer is a complex genetic disease that is caused primarily by environmental factors.

The cancer-causing agents (carcinogens) can be present in food and water, in the air, and in chemicals and sunlight that people are exposed to. Since epithelial cells cover the skin, line the respiratory and alimentary tracts, and metabolize ingested carcinogens, it is not surprising that over 90% of cancers occur in epithelia [156].

The causes of serious ill-health in the world are changing; may be expressed in one of three ways, namely incorrect diet, via the environment, and genetic predisposition. At least 35%of all cancers in the world are caused by incorrect diet, especially in the case of colon cancer diet may account for 80% of the cases. Furthermore, cigarettes and alcohol when added to the diet, percentage may increase to 60% of cancer cases. Genetic predisposition represents 20% of cancer cases, thus leaving majority of cancers being associated with a host of environmental carcinogens [157].

When it comes to geographic differences in carcinogenic risks, they are especially stark in developing countries versus developed countries where populations are more homogenous in terms of their economic development and life style factors. Egypt is a developing country with 50% of its population living in major cities in Nile Delta Region (NDR) [158]. In recent studies, it was found a high incidence of breast [159,160] and uterine cancer [161] in urban areas of NDR.

Previously, it was suggested that hormonal exposures, probably to xenoestrogens, may be higher in urban than rural areas [160,162]. In continuing with research investigation, geographic differences in incidence was explored for other cancer sites in both men and women with the objective that these geographic differences may indicate probable differences in distribution to environmental risk factors of the respective cancer sites. This in turn will provide deeper insights into the etiology of various cancer sites [163].

**Carcinogens**

The majority of carcinogens are environmental; these include both natural and manmade chemicals, radiation, and viruses.

Carcinogens may divide into several classes, as shown in [TableXI]. (1) Genotoxic carcinogens, if they react with nucleic acids. These can be directly acting or primary carcinogens, if they are directly affecting cellular constituents. (2) Alternatively, they may be pro carcinogens that require metabolic activation to induce carcinogenesis. (3) Epigenetic carcinogens are those that are not genotoxic. Molecular diversity of cancer initiating compounds ranges from metals to complex organic chemicals, and their potency highly varies. The variation in structure and potency suggests that more than one mechanism is involved in carcinogenesis. Apart from exposure to these reported carcinogens, other factors are included in initiating cancer such as genetic predisposition. Thus, patients with *xeroderma pigmentosum*

are more susceptible to skin cancer. Furthermore, bladder cancer is more common in patients with slow acetylator phenotype, especially if they are exposed to aromatic amines [157].

Carcinogens in diet that trigger the initial stage include moulds and aflatoxins, nitrosamines, rancid food and cooking oils, alcohols, additives, and preservatives. A combination of food may have accumulative effect, and when incorrect diet is added to a polluted environment, smoking, UV radiation, free radicals, lack of exercise, and stress, the stage is set for DNA damage and cancer progression[157]. On the protective side, there is no doubt that diet rich in vegetables, fruits and fibers is associated with a reduced risk of cancer.

In 2013, WHO launched the Global Action Plan for the prevention and control of non -communicable diseases 2013-2030 that aims to reduce by 25% premature mortality from cancer, cardiovascular diseases, diabetes and chronic respiratory diseases [158].

Cell cycle

Cancer is a disease in which disorder occurs in the normal processes of cell division, which are controlled by the genetic material (DNA) of the cell. Viruses, chemical carcinogens, chromosomal rearrangement, tumor suppressor genes, or spontaneous transformation have been implicated in the cause of cancer. For a cell to replicate, it must (1) faithfully reproduce its DNA, (2) manufacture sufficient cellular organelles, membranes, soluble proteins, etc., to enable daughter cells to survive, (3) partition of the the DNA and cytoplasm containing organelles equally to form two daughter cells. This process requires a significant amount of feedback control to ensure that the steps are sequential and correctly orientated. Thus, "failure to control cell cycle process carries with it a high price" [163].

**Table XI. Types of carcinogens** [164]

| **Type** | **Example** |
|---|---|
| 1. Genotoxic carcinogens<br>Primary, direct- acting alkylating agents | Dimethyl sulphate, ethylene imine, β-propiolactonel |
| 2. Procarcinogens<br>Polycyclic aromatic hydrocarbons<br>Nitrosamines<br>Hydrazine<br>Inorganic | <br>Benzo[α]pyrene<br>Dimethylnitrosamine<br>1,2-dimethylhydrazine<br>Cadmium, plutonium |
| 3. Epigenetic carcinogens<br>Promoters<br>Solid state<br>Hormones<br>Immunosuppressant<br>Cocarcinogens | <br>Phorbol esters, saccharin, bile acids<br>Asbestos, plastic<br>Estrogens<br>Purine analogues<br>Catechol |
| 4. Unclassified | Clofibrate, phthalate esters |

**Carcinogenesis**

The transformation of a normal cell to a cancerous cell is believed to proceed through many stages over a number of years or even decades. The stages of carcinogenesis include initiation, promotion, and progression. The first stage involves a reaction between the cancer causing agent and DNA of the tissue cells. This stage may remain dormant, and subject is t at risk of developing cancer at a later stage. The second stage occurs very slowly over a period ranges from several months to years. During this stage, a change in diet or lifestyle may have a beneficial effect so that the person may not develop cancer during his or her lifetime. The final stage involves progression and spread of the cancer. Preventing initiation is an important anticancer strategy, as are the opportunities to inhibit cancer throughout the latter stages of malignancy. One of the important mechanisms contributing to cancer is considered to be oxidative damage to the DNA. If a cell containing a damaged DNA divides before DNA can be repaired, the result is likely to be a permanent genetic alteration constituting a first step in carcinogenesis [163].

So, body cells that divide rapidly are more susceptible to carcinogenesis because there is less opportunity for DNA repair before cell division [163].

Global cancer incidence and epidemiological studies

The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries.

Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Lung cancer is the leading cancer site in males, comprising 17% of the total new cancer cases and 23% of the total cancer deaths. Breast cancer is now also the leading cause of cancer death among females in economically developing countries, a shift from the previous decade during which the most common cause of cancer death was cervical cancer. Further, the mortality burden for lung cancer among females in developing countries is as high as the burden for cervical cancer, with each accounting for 11% of the total female cancer deaths [165].

Although overall cancer incidence rates in the developing world are half those seen in the developed world in both sexes, the overall cancer mortality rates are generally similar. Cancer survival tends to be poorer in developing countries, most likely because of a combination of a late stage at diagnosis and limited access to timely and standard treatment.

A substantial proportion of the worldwide burden of cancer could be prevented through the application of existing cancer control knowledge and by implementing programs for tobacco control, vaccination (for liver and cervical cancers), and early detection and treatment, as well as public health campaigns promoting physical activity and a healthier dietary intake. Clinicians, public health

professionals, and policy makers can play an active role in accelerating the application of such interventions globally [165].

In the present study, cytotoxic effect of essential oils extracted from calli of *O. basilicum* L. and *P. anisum* L. were examined *in vitro* using cancerous cell lines, HepG-2 and Caco-2, these are representing liver and colon cancer, respectively aiming to discover new candidates against the available cancerous cell lines in our laboratory.

**Hepatocellular carcinoma (HCC)**

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, perhaps even the most common. There is, however, striking geographic variation in its incidence throughout the world; in much of Asia, especially China, and sub-Saharan Africa it is one of the leading malignancies, while in most Western countries it is rare. There is a striking correlation between the incidence of HCC and the prevalence of the chronic hepatitis B virus (HBV) carrier state [166].

HBV infection accounts for about 60% of the total liver cancer in developing countries and for about 23% of cancer in developed countries [167]; the corresponding percentages for hepatitis C virus (HCV) infection are 33% in developing countries and 20% in developed countries [152]. Interaction of aflatoxin B1 (AFB) exposure with chronic HBV infection has been noted to increase liver cancer [168,169]. However, the contribution of AFB exposure to the liver cancer burden in parts of Africa and Asia, where the exposure is prevalent, is unknown [170]. In the United States and several other low-risk Western countries, alcohol-related cirrhosis and possibly nonalcoholic fatty liver disease, associated with obesity, are thought to account for the majority of liver cancer [171].

In case of epidemiology of liver cancer in Egypt, The National Cancer Institute Pathology Registry indicated that liver cancer formed 11.75% of the malignancies of all digestive organs and 1.68% of total malignancy. This registry shows under estimation of liver tumors because most of them are diagnosed by non-histopathology means. Liver tumors were mostly hepatocellular carcinoma (70.48%), while hepatoblastoma constituted 10.24%, non-Hodgkin's lymphoma 4.21% of hepatic malignancies and adenocarcinoma unspecified 9.03% [172].

Occupational exposure to pesticides may have a contributory role in the etiology or progression of HCC. In a study, it was found that a major segment of Egyptian population is employed in agriculture and using pesticides routinely to control insects, weeds, rodents, and fungal infection of crops and livestock [173].

Once diagnosed with the disease, only 30-40% of patients are deemed eligible for curative intention with treatment modalities including surgical resection, liver transplantation, and chemoembolization. Eventually, most patients will receive some forms of chemotherapy in hope of prolonging life. Sorafenib is the first molecular inhibitor to be approved by the FDA for the treatment of advanced HCC. It is a tyrosine kinase inhibitor, targeting multiple molecular pathways[174].

Prior to the arrival of sorafenib, doxorubicin was routinely used as a single drug for advanced HCC, but has shown inefficacy, with a response rate of about 15-20%.

Other chemotherapy agents, such as epirubicin, cisplatin, 5-fluorouracil, etoposide and their combinations, demonstrate even lower efficacy. While being considered an advance over conventional chemotherapy, sorafenib only improves life expectancy approximately by 3 months over placebo. With that in mind, continuous efforts have been put into finding new targets and molecular pathways for possible new drug development [174].

**Colorectal cancer (CRC)**

Colorectal cancer (CRC) is cancer that starts in the colon or the rectum. These cancers can also be referred to separately as colon cancer or rectal cancer, depending on where they start. Colon cancer and rectal cancer have many features in common [175].

Colorectal cancer is the second most prevalent cancer and the third leading cause of cancer-related deaths in the world with almost 500,000 related deaths every year [175]. The American Cancer Society's estimates for the number of colorectal cancer cases in the United States for 2014 are 96,830 new cases of colon cancer and 40, 000 new cases of rectal cancer [175].

Modifiable risk factors for colorectal cancer include smoking, physical inactivity, overweight and obesity, red and processed meat consumption, and excessive alcohol consumption [176, 177] . Population-based colorectal screening programs are feasible only in economically developed countries, although future attention should also be focused in those areas of the world with an aging population and increasingly westernized lifestyle [178,179].

In Egypt, CRC is uncommon and represents only 3% of all malignant tumors (excluding tumors of the nervous system) [180]. It is the third most common tumor in males after urinary bladder and lymphohemopoietic malignancies, and in females it ranks fifth after breast, lymphohemopoietic , cervical, and urinary bladder cancers[181]. Recent interest in Egyptian CRC has been raised when personal observations and epidemiologic studies revealed a high incidence of the disease among the young Egyptian population [182].

While significant advances have been made, ongoing improvement is needed. Until the early 1990s, chemotherapy options were primarily limited to 5-fluorouracil (5-FU). The recent integration of oxaliplatin and irinotecan for the management of patients with advanced disease has produced meaningful benefits in survival. 5-FU remains the mainstay of treatment for adjuvant therapy, although efforts to evaluate the role of oxaliplatin and irinotecan in this setting are already underway. Leucovorin (LV) is used also for this type of cancer [183] .

**Natural drugs and defense against carcinogenesis**

At least 35% of all cancers worldwide are caused by incorrect diet. The plant kingdom has a great place in the treatment of diseases with no ill effect. Numerous plant products are now used for the remedy of cancer. According to WHO estimates, more than 80% people in developing countries depend on traditional medicine for their primary health needs. Consumption of large amount of vegetables and fruits can prevent the development of cancer. Several natural products are available as chemoprotective agents against various types of cancer. These chemoprotective agents are present in fruits, vegetables, plant extracts, herbs, microbes and marine organisms. A host of natural product constituents could be responsible for the protective effect against cancer. Although the mechanism of the protective effect is unclear, nevertheless, the consumption of natural products lowers the incidence of cancer. A major group of these products are the powerful antioxidants, others are phenolic in nature and the remainder includes reactive groups [184].

Carcinogenesis encompasses a prolonged accumulation of injuries at several different biological levels and includes both genetic and biochemical changes in the cells. At each of these levels, there are several possibilities of intervention in order to prevent, slow down or even halt the gradual march of healthy cells towards malignancy. Carotenoids, flavanoid polyphenols, isoflavones, catechins, and several other components that found in cruciferous vegetables are molecules that are known to protect against the deleterious effect of reactive oxygen species. A number of epidemiological and experimental studies have shown that vitamin C and E, Beta-carotene and the essential trace element selenium can reduce the risk of cancer. Consistent observations during the last few decades that cancer risk is reduced by a diet rich in vegetables, fruits, legumes, grains and green tea have encouraged research to identify several plant components especially phytochemicals that protect against DNA damage. Many of these substances block specific carcinogen pathways [185].

There are at least 250,000 species of plants out of which more than one thousand plants have been found to possess significant anticancer properties. While many molecules obtained from nature have shown wonders, there are a huge number of molecules that still either remains to be trapped or studied in details by the medicinal chemists. Taxol, one of the most outstanding agents, has been found beneficial in treatment of refractory ovarian, breast and other cancers. Another prominent molecule includes Podophyllotoxin. Synthetic modification of this molecule led to the development of Etoposide, known to be effective for small cell cancers of the lungs and testes. Camptothecin isolated from *Camptotheca acuminata* .Other important molecules include Vincristine, Vinblastine, Colchicine, Ellipticine and Lepachol along with Flavopiridol, a semi-synthetic analogue of the chromone alkaloid Rohitukine from India, a pyridoindole alkaloid from leaves of *Ochrosia* species and many more [186].

Elemene, isolated from the Chinese medicinal herb *Rhizoma zedoariae,* was shown to exhibit antitumor activity in human and murine tumor cells *in vitro* and *in*

*vivo*. This novel antineoplastic agent has substantial clinical activity against various tumors. The *in vitro* effect of elemene on the growth of leukemia cells was evaluated by MTT assay. The $IC_{50}$values of elemene for promyelocytic leukemia HL-60 cells and erythroleukemia K562 cells were 27.5 μg/mL and 81 μg/ml, respectively, while $IC_{50}$ for peripheral blood leukocytes (PBL) was 254.3 μg/ml. The inhibitory effect of elemene on proliferation of HL-60 cells was associated with cell cycle arrest from S to $G_2M$ phase transition and with induction of apoptosis. Allicin, a natural organosulfide from garlic,has also an inhibitory effects on proliferation of tumor cells that associated with the cell cycle blockage of $S/G_2M$ boundary phase and induction of apoptosis [187].

In a study *O. basilicum* and its essential oil were examined for their cytotoxic effects. The results revealed that this plant may belong to the methyl cinnamate and linalool chemotype. A methyl thiazol tetrazolium assay was used for *in vitro* cytotoxicity screening against the human cervical cancer cell line (HeLa), human laryngeal epithelial carcinoma cell line (HEp-2) and NIH 3T3 mouse embryonic fibroblasts. The $IC_{50}$ values obtained were 90.5 and 96.3 $\mu g\,ml^{-1}$, respectively, and the results revealed that basil oil has potent cytotoxicity [188].

In the preliminary experiments, toxic and mutagenic potential of essential oil (EO) from *O. basilicum* and pure substances: linalool, ß-myrcene and 1,8-cineole were tested using *Salmonella typhimurium* TA98, TA100 and TA102, with and without S9 mix (microsomal fraction of rat liver). No mutagenic effect of *O. basilicum* derivatives was detected in any tested strain. Antimutagenic effects of essential oil from *O. basilicum* and its pure constituents were further evaluated in the Ames test using *S. typhimurium* TA100. UVC irradiation and three chemical mutagens, 4-nitroquinoline-N-oxide (4NQO), 2-nitropropane (2-NP) and benzo(a)pyrene were used to induce mutagenesis. All tested *O. basilicum* derivatives significantly reduced UV-induced mutations. The maximum inhibition was in the range of 64–77 %. Inhibitory potential against direct acting model mutagen/carcinogen 4NQO was similar to UV (52–67 %). In the presence of S9, EO and 1,8-cineole showed moderate inhibition of 2-NP induced mutagenesis, while the remaining two substances had no effect. Linalool exhibited high co-mutagenic effect with benzo(a)pyrene, 1,8-cineole showed moderate inhibitory effect against benzo(a)pyrene -induced mutations, while EO and ß-myrcene were ineffective [44].

Methanolic extract of *O. basilicum* was fractionated into petroleum ether soluble (PE-S) and insoluble (PE-I) fractions. These were evaluated on HT-144, MCF-7, NCI-H460 and SF-268 cell lines using Sulforhodamine B assay. Immunofluorescence microscopy was employed to study their effects on the cytoskeleton and nuclei of MCF-7 cells. Fractionation of PE-I (GI50: 5 μg/ml; LC50: 71μg/ml against MCF-7) led to the isolation of four compounds, mainly ursolic acid (LC50: 18.6 μg/ml). Ursolic acid (100 μM) induced a significant decrease in the percentage of cells in anaphase/telophase stages along with F-actin aggregation and mitotic spindle distortion. These results support anti-proliferative activity of *O. basilicum* extract against MCF-7 cells which may partly be due to effects of ursolic acid on F-actin and microtubules [45].

## Materials and methods

Materials

### Media, reagents and plastic wares

i. Ficoll-Hypaque(density 1.077 g/l, Lonza, USA)
ii. Trypan blue (Sigma, USA)
iii. RPMI 1640 medium(Lonza, USA)
iv. DMEM medium, (Lonza, USA)
v. HEPES buffer (Lonza, USA)
vi. L-glutamine (Lonza, USA)
vii. Fetal Bovine Serum (FBS; Lonza, USA)
viii. Neutral Red (Bio Basic Inc., Canada)
ix. Fixation buffer (0.5% formalin with 1% calcium chloride (Sigma, USA)
x. Extraction buffer (destaining buffer, 50% ethanol (Fisher Scientific, USA)with 1% glacial acetic acid (Sigma, USA) in distilled water)
xi. Dimethyl Sulfoxide (DMSO) Fisher Scientific, USA)
xii. 0.2 µm syringe filter (TPP, Switzerland)
xiii. Heparinized blood Collection Tube, 4 ml (Vacuette®, USA)
xiv. Syringes 5 ml and 10 ml (MASCO, Egypt)
xv. Falcon tubes 50 ml and 15 ml (Corning, USA)
xvi. Polystyrene 96-well plate (TPP Techno Plastic Products, Switzerland)
xvii. Disposable pipettes 2 ml and 10 ml (Sorfa, Zhejiang, China (Mainland))

Biological

### Cell lines obtained from American Tissue Culture Collection (ATCC, USA)

Caco-2 (ATCC® Number: HTB-37™) cell line
HepG-2 (ATCC® Number: HTB-8065™) cell line

Instruments

i. Vertical laminar airflow belonging to S2 safety grade, Telstra, Terrazza, Spain
ii. Centrifuge, GmbH, Hamburg, Germany
iii. Water jacketed $CO_2$ incubator, New Brunswick, NJ, USA
iv. Microplate reader, SPECTROstar Nano, BMG LABTECH, Germany
v. Micropipettes (Gilson, France)
vi. Multichannel micropipette (Gilson, France)

**Method**

A- *In vitro* cytotoxicity assay

*In vitro* cytotoxicity assay was performed to assess the viability of normal cells (Human peripheral blood mononuclear cells (PBMCs) after incubation for 72 hours with prepared extracts of the volatil oils of *P. anisum* and *O. basilicum*. Viability of cells was measured using neutral red uptake assay as described by Borenfreund and Puerner [189] to determine the non cytotoxic concentration of each extract. This assay depends on the fact that neutral red dye can be incorporated into the lysosomes of living cells [190] providing a quantitative assay to the cytotoxic effects.

Cytotoxicity assay involved three main steps. First, the isolation of PBMCs from freshly collected blood sample, then incubating PBMCs with different concentrations of the extracts and finally, measuring cells' viability using neutral red uptake assay.

PBMCs were isolated from heparinized healthy volunteer peripheral blood by density gradient centrifugation technique as described by Boyum [191].Blood samples were freshly collected into heparinized sterile tubes. Blood was diluted using equal volume of RPMI-1640 medium supplemented with 25 mM HEPES buffer and 4mM L-glutamine. Diluted blood was layered over equal volume of Ficoll-Pague™ Plus (density 1.077 g/l) (lymphocyte Separation Medium LSM) and centrifuged at 2000 rpm for 30 minutes with no acceleration and break at room temperature. The buffy mononuclear cell layer was collected using sterile Pasteur pipette into 50 ml sterile Falcon tube and washed twice in phosphate buffered saline (PBS) using centrifugation at 1650 rpm for five minutes. The isolated PBMCs viability was assessed by hemocytometer count using the trypan blue exclusion method. The PBMCs were re-suspended at 1 x $10^6$ cells/ml in RPMI-1640 medium containing 25 mM HEPES, 4mM L-Glutamine supplemented with 10% heat-inactivated FBS.

Preparation of essential oil extracts

A 150 µl of essential oil extracted from calli of *O. basilicum* and *P . anisum* was dissolved in DMSO/ethanol mixture to obtain a stock concentration 20. 3125 µl/ml The desired concentrations (20.3125, 10.125, 5.15625, 0.31250 and 0.15625µl/ml) were prepared using serial dilution in a 96-well plate.

Tested extracts' wells were prepared by adding 100 µl of suspended cells at 1 x $10^6$ cells/ml (final cell number/well was equal to $1\times10^5$ PBMCs in 100 µl culture media). Parallel concentrations of the solvent were prepared to be used as controls. Control wells were prepared by adding 100 µl culture media to a suspension of $1\times10^5$ PBMCs in 100 µl culture media. Blank wells contained 200µl of culture media only (without cells or compound solution). Each set of samples was pipetted in duplicate. The plate was then gently shaken then incubated at 37 ºC, 5% $CO_2$ for 72 hours.

After incubation, the plate was centrifuged (2000 rpm for ten minutes). The media were discarded by inversion over absorbent filter paper. Neutral red working solution (80µg/ml) stain (Bio Basic Inc., Canada) was prepared, and 100 µl of this solution was added to each well, then the plate was gently shaken. Followed by incubation at 37 ºC in humidified 5% $CO_2$ for three hours, and then centrifuged (2000 rpm for ten minutes). Excessive dyes were discarded, and cells were washed 3times using PBS and centrifugation at 2000 rpm for 5 min. The stained cells were fixed with

100 µl fixing solution (0.5% formalin with 1% calcium chloride for one minute. Cells were destained in 100 µl destaining solution (50% ethanol with 1% glacial acetic acid for five minutes by shaking. The stain intensity was assayed using automated microplate reader spectrophotometer adjusted at 540 nm.

Viable cell fraction was calculated according to the following equation of cell viability:

Cell viability (%) = (E / C) * 100

Cell inhibition (%) by blank =100 – [(B / C) *100]

Actual cell viability (%) = Cell viability (%) + cell inhibition (%) by blank

Where:

**E**: The mean absorbance of extract/compound treated wells.
**B**: The mean absorbance of blank control wells.
**C**: The mean absorbance of control wells.

Results were interpreted to calculate both the effective concentration that kills 50% of cells ($EC_{50}$) and the maximum safe concentration that keep 100% ($EC_{100}$) cell viability for each oil using GraphPad InStat3.0 software [192].

B- *In vitro* anticancer screening against liver and colon cancer cell lines

Oil extracts were screened for their potential anticancer activity against hepatocellular carcinoma cell line HepG-2and human epithelial colorectal adenocarcinoma cell line Caco-2.

**Experimental:**

HepG-2 cells were routinely maintained as adherent cells in RPMI-1640 medium while Caco-2 cells were maintained in DMEM medium both supplemented with 10% FBS at 37 ºC in a humidified air incubator containing 5% $CO_2$. Cells were subcultured for two weeks before assay. Cell viability was assessed using trypan blue exclusion method.

Caco-2, HepG-2 cells were washed twice in its respective medium supplemented with 4 mM L-glutamine and 25 mM HEPES buffer. The HepG-2 cells were suspended at 3 x $10^3$cells/ml in RPMI culture medium (RPMI supplemented medium and 10% FBS) while Caco-2 cells were suspended at 4 x $10^3$ cells/ml in DMEM culture medium (DMEM supplemented medium and 10% FBS). The appropriate number of cells (seeding cell density) was chosen to be 3 x $10^3$ cells/well (100 µl of the prepared suspension) for HepG-2 cells and 4 x $10^3$ cells/well (100 µl of the prepared suspension) for Caco-2 .The cells were left to adhere on the polystyrene 96-well plates in by incubator at 37 ºC, 5% $CO_2$ and 95% humidity for 24 hours.

A 20 µl/ml stock solution of each extract was prepared in DMSO/ethanol mixture. The desired concentrations (10, 5, 2.5, 1.25, and 0.625 µl/ml) were prepared using serial dilutions in a 96-well plate. Tested extracts' wells were prepared by adding 100 µl of the previously prepared concentrations to a 100 µl of cancer cells' suspension. Parallel concentrations of the solvent were prepared to be used as controls. Mitomycin C (MCC)(0.09 µg/ml)was used as a positive anti cancer drug control. Control wells were prepared by adding 100 µl culture media to a 100 µl of cancer cells suspension. Blank wells contained 200 µl of culture media only (without cells or compound solution). Each set of samples was pipetted in duplicate.

The plate was gently shaken, then incubated at 37 ºC, 5% $CO_2$ for 72 hours. Cancer cells viability was measured using neutral red uptake assay as described previously.

The half maximal inhibitory concentration ($IC_{50}$) values were determined from the *GraphPadInStat3.0* software. Percent cell inhibition was calculated from the following equation:

Cell inhibition (%) =100- [(E-B/C-E)] X 100]

Where:
**E**: The mean absorbance of cells exposed to extract.
**B**: The mean absorbance of blank control.
**C**: The mean absorbance of control cells.

# Results

**Table XII. $EC_{50}$and $EC_{100}$ of essential oils of *O. basilicum* L. and *P. anisum* L.**

| Essential oil | $EC_{100}$(µl/ml) | $EC_{50}$(µl/ml) |
|---|---|---|
| *O. basilicum* L. | 175.615 | 467 |
| *P .anisum* L. | 26.465 | 6.9 |

**Table XIII. The Percentage of cell viability for normal PBMCs of the tested essential oils of *O. basilicum* L. and *P. anisum*L.**

| Conc. / E.oil | % Cell viability | | | | |
|---|---|---|---|---|---|
| | 0.15625µl/ml | 0. 3125µl/ml | 5.15625µl/ml | 10.3125µl/ml | 20.3125µl/ml |
| *O. basilicum* L. | 89.835 | 93.524 | 103.114 | 108.196 | 143.442 |
| *P. anisum* L. | 42.713 | 62.214 | 62.285 | 80.642 | 95.214 |

**Table XIV. $IC_{50}$ of essential oils of *O. basilicum* L. and *P. anisum* L. on Caco-2, HepG-2 and normal PBMCs.**

| | $IC_{50}$ (µl/ml) | | |
|---|---|---|---|
| Cell line | Caco-2 | HepG-2 | PBMCs |
| *O. basilicum* L. | $10.5\pm0.001^{b}$ | $19.3\pm0.02^{b}$ | $467\pm2.485^{b}$ |
| *P. anisum* L. | $4.7\pm0.001^{a}$ | $1\pm0.001^{a}$ | $6.9^{a}\pm0.042^{a}$ |

*All values are expressed as Mean± SEM( Standard error mean) ($p< 0.05$)

**a**: the cytotoxic activity of *O. basilicum* is statistically significant compared to its value for *P. anisum.*

**b**: the cytotoxic activity of *P. anisum* is statistically significant compared to its value for *O. basilicum*.

**Table XV. Percentage inhibition (%Inhibition) of Caco-2 and HepG-2 by essential oil of *O. basilicum* L. at different concentrations.**

| | % Inhibition | |
|---|---|---|
| Concentration(µl/ml) | Caco-2 | HepG-2 |
| 10 | 62.420 | 44.852 |
| 5 | 7.634 | 9.708 |
| 2.5 | 9.177 | 9.448 |
| 1.25 | 8.919 | 10.327 |
| 0.625 | 3.144 | 5.038 |

**Table XVI. Percentage inhibition (%Inhibition) of Caco-2 and HepG-2 by essential oil of *P. anisum* L. at different concentrations.**

| | % Inhibition | |
|---|---|---|
| Concentration(µl/ml) | Caco-2 | HepG-2 |
| 10 | 66.277 | 47.478 |
| 5 | 73.140 | 73.109 |
| 2.5 | 56.269 | 71.481 |
| 1.25 | 36.329 | 58.166 |
| 0.625 | 20.174 | 37.576 |

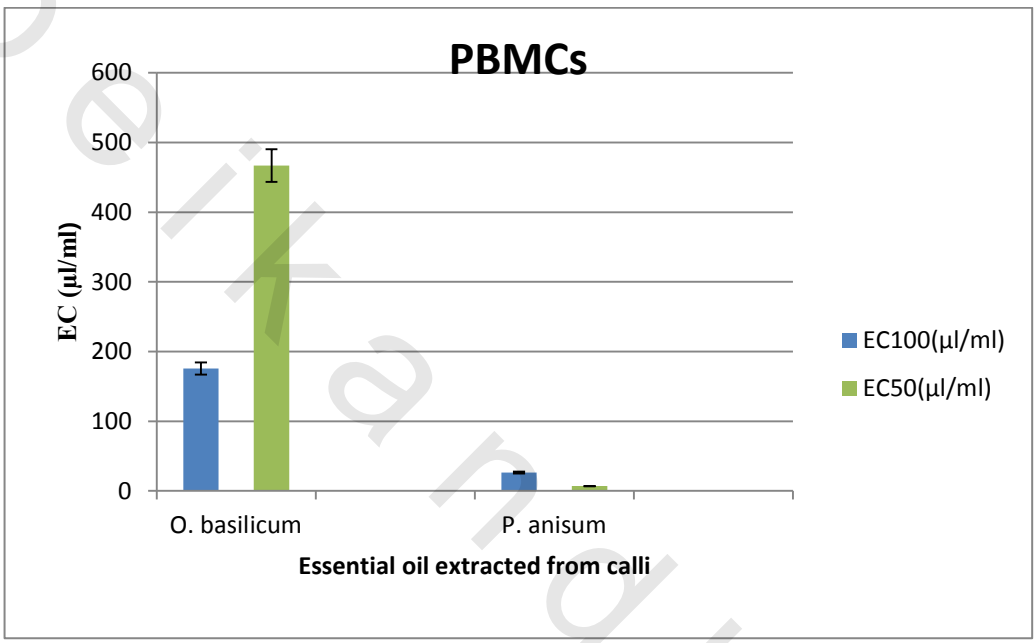


**Figure 34. $EC_{50}$ and $EC_{100}$ of essential oils of calli of *O. basilicum* L. and *P. anisum* L.**

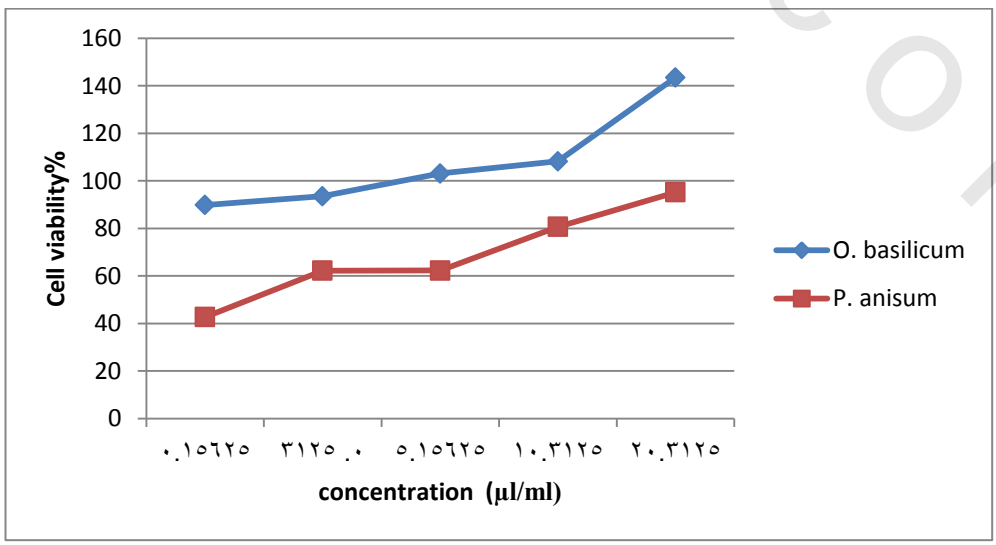

**Figure 35. The % cell viability for PBMCs tested with essential oils of *O. basilicum* L. and *P. anisum* L. at different concentrations.**

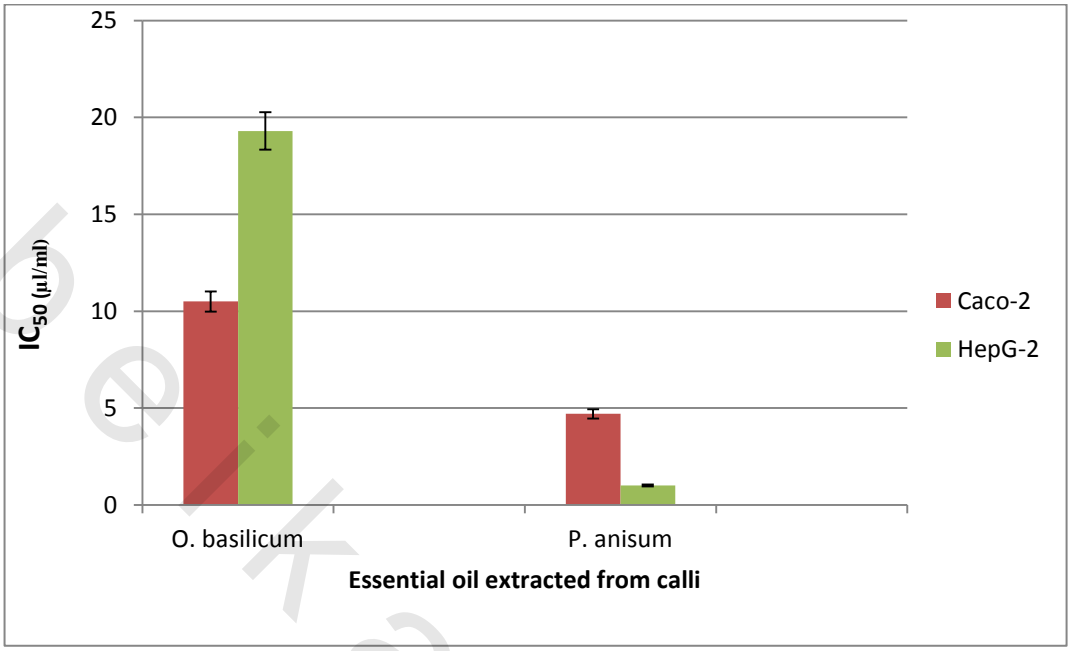


**Figure 36. $IC_{50}$ of essential oils of *O. basilicum* L. and *P. anisum* L. calli on Caco-2 and HepG-2.**

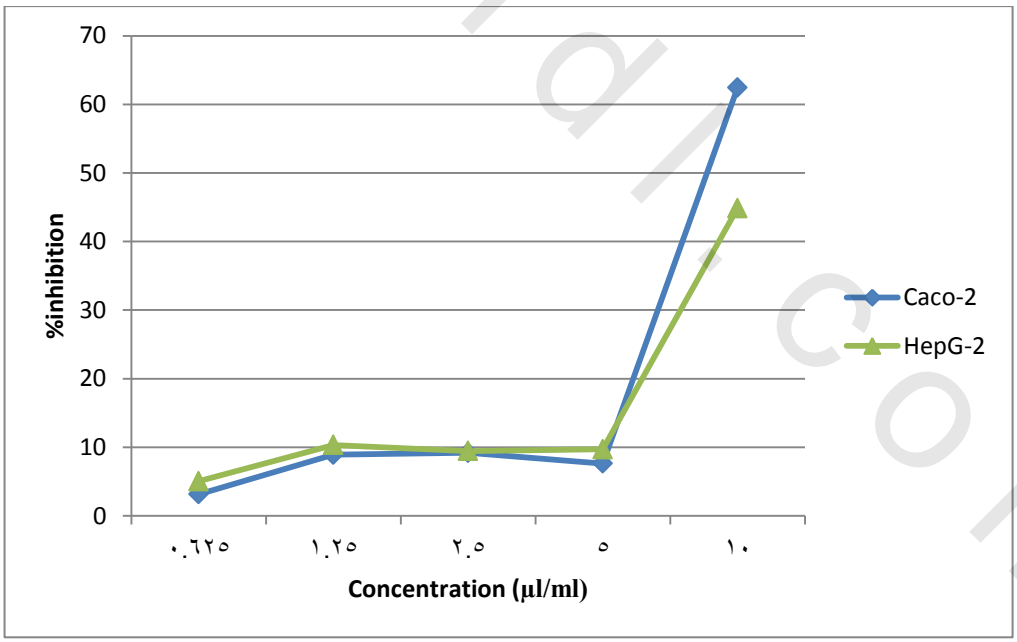


**Figure 37. Cytotoxic activity of essential oil of *O. basilicum* L. on Caco-2 and HepG-2.**

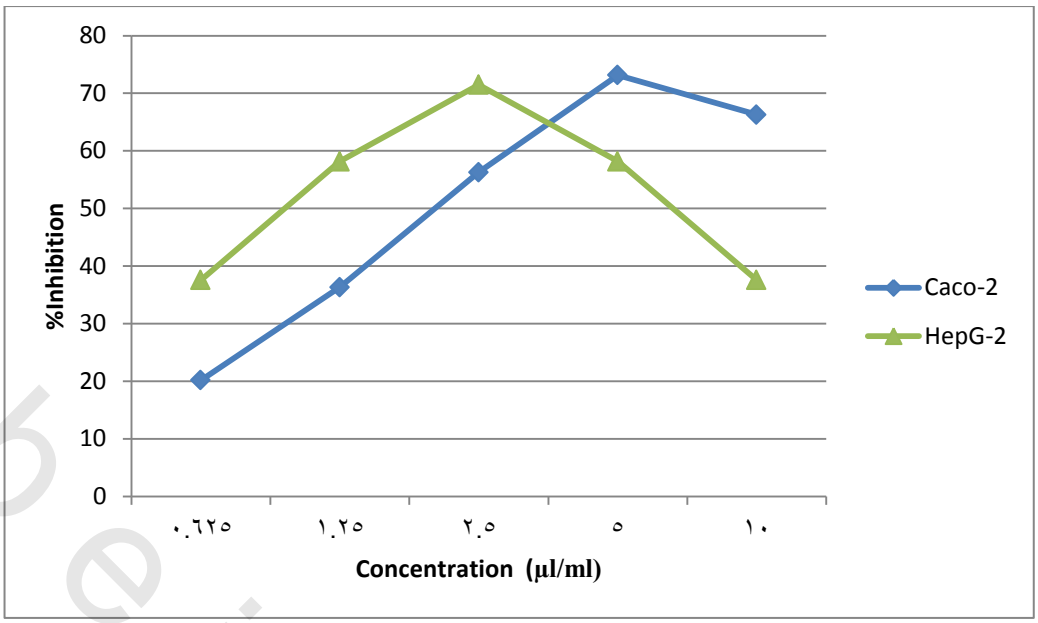


**Figure 38. Cytotoxic activity of essential oil of *P. anisum* L. on Caco-2 and HepG-2.**

## Results and discussion

Results of cytotoxicity study of essential oils extracted from calli of *O. basilicum* and *P. anisum* on normal peripheral blood mononuclear cells (PBMCs) and cancerous cell lines; HepG-2, and Caco-2 are presented in [Tables XII-XVI] and [Figures 34-38]. The cytotoxicity of the test compounds were expressed in terms of $IC_{50}$ which is a measure of the effectiveness of a compound in inhibiting biological or biochemical function in terms of % inhibition of cell viability.

Both The effective concentration that keeps 50% of cells ($EC_{50}$) and the maximum safe concentration that keep 100% ($EC_{100}$) cell viability on normal PBMCs were measured for essential oils extracted from calli of *O. basilicum* and *P. anisum*, both oils showed no toxicity on the normal PBMCs as viability of cells increase with increasing concentration of oils.

The % inhibition of both cancerous cells increased with the increase of concentration of *O. basilicum* essential oil ranging from (0.625-10µl/ml) and with a sharp increase at concentration 10µl/ml, therefore the cell viability of cancerous cells was significantly ($P< 0.05$) decreased with increase of concentration of essential oil.

$IC_{50}$ for essential oil of *O. basilicum* against Caco-2 was 10.5 µl/ml and19.3 µl/ml for HepG-2, so essential oil of *O. basilicum* is more effective as anticancer agent for colorectal cancer than hepatocellular carcinoma.

Linalool (as the main component of essential oil of *O. basilicum*) was tested by using WST-1 analysis; results showed that linalool has a good inhibitory effects against colorectal and liver cell cancers, $IC_{50}$ values for those cancer cell types were 22µM and 290 µM respectively. Cell cycle analysis confirmed that linalool can lead to apoptosis. Furthermore, by using cytokine arrary analysis, results showed that linalool con stimulate IFN-1, IL-13, IL-2, IL- 21R, IL- 4, IL-4, IL-6sR and TNF-α secretion [193]. These results may hold great potential for essential oil of *O. basilicum* use in cancer therapy.

The % inhibition of both cancerous cells increased gradually with the increase of concentration of *P. anisum* essential oil ranging from (0.625- 2.5µl/ml), but at higher concentrations of 5 µl/ml and 10 µl/ml % inhibition of both cancerous cells decreased. So, anticancer activity of essential oil of *P. anisum* doesn't increase in a dose dependant manner.

$IC_{50}$ for essential oil of *P. anisum* against Caco-2 was 4.7µl/ml and1µl/ml for HepG-2, so essential oil of *P. anisum* is more effective as anticancer agent for hepatocellular carcinoma than colorectal cancer.

Anethole (as the main component of essential oil of *P. anisum*) at a concentration below 1 mM has been shown to be *in vitro* a potent inhibitor of tumour necrosis factor (TNF)-induced cellular responses, such as activation of nuclear factor (kappa B) (NF-kB) and other transcription factors, and also to block TNF-induced activation of the apoptotic pathway. This might explain the cytotoxic activity of *P. anisum* essential oil [194].

# Chapter 2

## Antioxidant activity of essential oil of *Ocimum basilicum* L. and *Pimpinella anisum* L. using DPPH assay.

# INTRODUCTION

Free radicals and related species have attracted a great deal of attention in recent years. They are mainly derived from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS), and are generated in our body by various endogenous systems, exposure to different physicochemical conditions or pathophysiological states. Free radicals can adversely alter lipids, proteins and DNA and have been implicated in aging and a number of human diseases. Lipids are highly prone to free radical damage resulting in lipid peroxidation that can lead to adverse alterations. Free radical damage to protein can result in loss of enzyme activity. Damage caused to DNA, can result in mutagenesis and carcinogenesis. Redox signaling is a major area of free radical research that is attracting attention [195].

Nature has endowed us with protective antioxidant mechanisms- superoxide dismutase (SOD), catalase, glutathione, glutathione peroxidases and reductase, vitamin E (tocopherols and tocotrienols), vitamin C etc., apart from many dietary components. There are epidemiological evidences correlating higher intake of foods with antioxidant abilities to lower incidence of various human mortalities [195].

Natural products from dietary components such as Indian spices and medicinal plants are known to possess antioxidant activity. Newer and future approaches include gene therapy to produce more antioxidants in the body, genetically engineered plant products with higher level of antioxidants, synthetic antioxidant enzymes (SOD mimics), novel biomolecules and the use of functional foods enriched with antioxidants [195] .

The relation between free radicals and disease can be explained by the concept of "oxidative stress". In a normal healthy human body, the generation of pro-oxidants in the form of ROS and RNS are effectively kept in check by the various levels of antioxidant defense. However, when it gets exposed to adverse physicochemical, environmental or pathological agents such as atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, toxic chemicals, overnutrition and advanced glycation end products (AGEs) in diabetes, this delicately maintained balance is shifted in favor of pro-oxidants resulting in 'oxidative stress'. It has been implicated in the etiology of several (>100) of human diseases and in the process of ageing [195].

The idea of growing crops for health rather than for food or fiber is slowly changing plant biotechnology and medicine. Rediscovery of the connection between plants and health is responsible for launching a new generation of botanical therapeutics that include plant-derived pharmaceuticals, multicomponent botanical drugs, dietary supplements, functional foods and plant-produced recombinant proteins. Among polyphenols, flavonoids constitute the most important single group, including more than 5000 compounds that have been thus far identified [196].

Apart from nutrient components such as β-carotene, vitamins C and E, and selenium, compounds such as phenols, flavonoids, isoflavones, isothiocyanates, diterpenes, methylxanthines, dithiols, and coumarins appear to be important in cancer prevention through their role on the inhibition of tumor production [197].

*Allium cepa* (Onion), *Allium sativum* (Garlic), *Aloe vera* (Indian aloe), *Amomum subulatum* (cardamom), *Camellia sinensis* (Green tea), *Cinnamomum verum* (Cinnamon), *Curcuma longa* (Turmeric), *Nigella sativa* (Black cumin), and *Ocimum sanctum* (Holy basil) are examples of commonly known medicinal plants rich in antioxidants [199].

The antioxidant potential of essential oil and oleoresins from *P. anisum* seeds was studied. The antioxidant activities were assessed by inhibition of linoleic acid peroxidation, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, $Fe^{3+}$ reducing power, and various lipid peroxidation assays. The findings showed that the anise oil and its methanol oleoresin showed highest antioxidant activity, even higher than Butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT). However, the antioxidant activities of other oleoresins were somewhat lower [67].

*O. basilicum* contains high levels of phenolic acids that contribute to its strong antioxidant capacity and the substantial concentrations of rosmarinic acid, in particular, have been associated with the herb's medicinal qualities [34]. Rosmarinic acid is noted in the literature to be the most prevalent basil phenol component [35]. However, other caffeic acid derivatives, such as chicoric acid, have been also found in high concentrations [36]. The antioxidant activity of ethanol extract of *O. basilicum* was investigated by electrochemical measurements [37].

For the evaluation of antioxidant capacities for both *O. basilicum* free volatile aglycones and its essential oil, two different methods were performed: the 2,2′-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH) and ferric reducing/antioxidant power assay (FRAP). DPPH method shows that free volatile aglycones possess good antioxidant properties comparable with that of the essential oil and well-known antioxidant BHT, but less than pure eugenol. The results obtained by FRAP method show that these compounds are less effective antioxidants than essential oil and BHT [199].

The essential oil of *O. basilicum* was analyzed by means of gas chromatography mass spectrometry and assayed for its antioxidant activity. The antioxidant activity was evaluated as a free radical scavenging capacity (RSC), together with effects on lipid peroxidation (LP). RSC was assessed measuring the scavenging activity of the essential oil on $DPPH^{\bullet}$ and $OH^{\bullet}$ radicals. Effects on LP were evaluated following the activity of essential oil in $Fe^{2+}$/ascorbate and $Fe^{2+}/H_2O_2$ systems of induction. Essential oil exhibited very strong RSCs, reducing the DPPH radical formation $IC_{50}$ was 0.39μg/ml. The tested essential oil strongly inhibited LP, induced either by $Fe^{2+}$/ascorbate or by $Fe^{2+}/H_2O_2$ [200].

*O. basilicum* essential oil obtained from hydro distillation exhibited good antioxidant activity as measurements by DPPH free radical-scavenging ability, bleaching β -carotene in linoleic acid system and inhibition of linoleic acid oxidation [201].

# EXPERIMENTAL

## Materials and methods

1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), Sigma Chemical Co. (St. Louis, Mo, USA)

### DPPH assay

The antioxidant capacity was determined using a test based on the redox potential of DPPH (1,1-diphenyl-2-picrylhydrazyl radical).This molecule is a stable free radical with a delocalised spare electron showing an absorption band in methanol at 520 nm yielding a deep violet color. In the presence of an antioxidant, DPPH is reduced with consequent loss of the violet colour.

## Method

A 1 mM DPPH solution (0.394 mg/ml) in methanol was prepared and then diluted 1:10 to obtain a 100 µM solution (Abs at λ = 515nm is 0.5-0.6). 500 µl of the sample and 500µl of 100 µM DPPH solution were mixed in a cuvette and a negative control with 500 µl of methanol and 500µl of DPPH solution was also prepared. Both solutions were incubated in the dark at room temperature for 15min. Absorbance was read at λ = 515 nm using methanol as blank.

Results were expressed as the percentage of reduction of the radical absorbance [202].

$$\frac{\text{Abs max (negative control)} - (\text{Abs sample+DPPH}) \times 100}{\text{Abs max}}$$

# Results

**Table XVII. Percentage of absorbance reduction against different concentrations of *O. basilicum* L. essential oil.**

| Concentration | 12.5 µl/ml | 25 µl/ml | 50 µl/ml | 100 µl/ml | 200 µl/ml |
|---|---|---|---|---|---|
| % reduction of the radical absorbance | 19.477 | 19.995 | 23.651 | 22.913 | 16.706 |

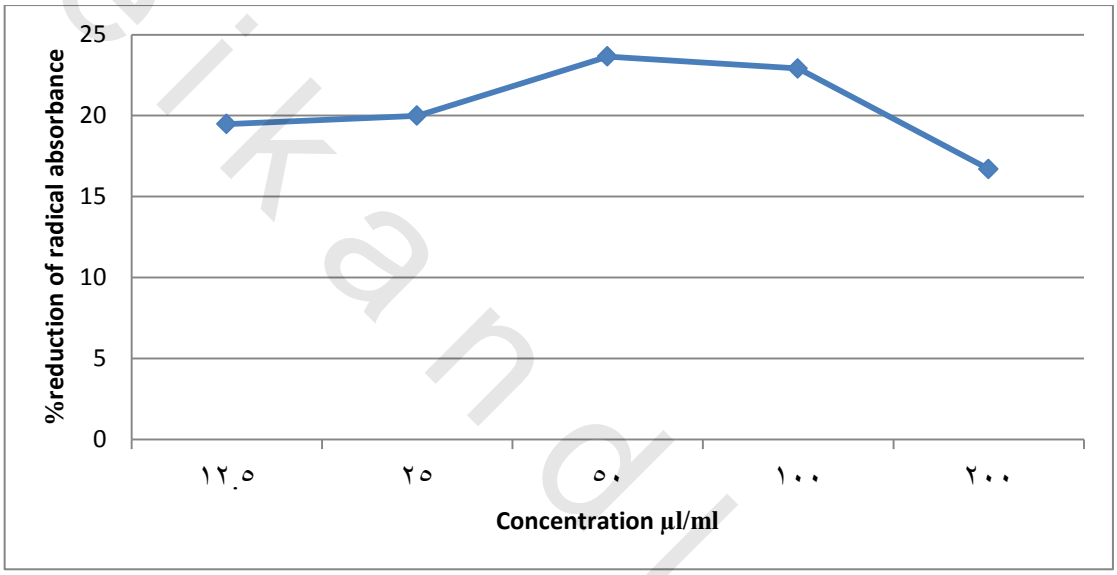


**Figure 39. Antioxidant activity of essential oil of *O. basilicum* L. using DPPH assay.**

**Table XVIII. Percentage of absorbance reduction against different concentrations of *P. anisum* L. essential oil.**

| Concentration | 1.25µl/ml | 2.5 µl/ml | 5 µl/ml | 10 µl/ml | 20 µl/ml |
|---|---|---|---|---|---|
| % reduction of the radical absorbance | 23.577 | 30.374 | 35.654 | 49.544 | 46.737 |

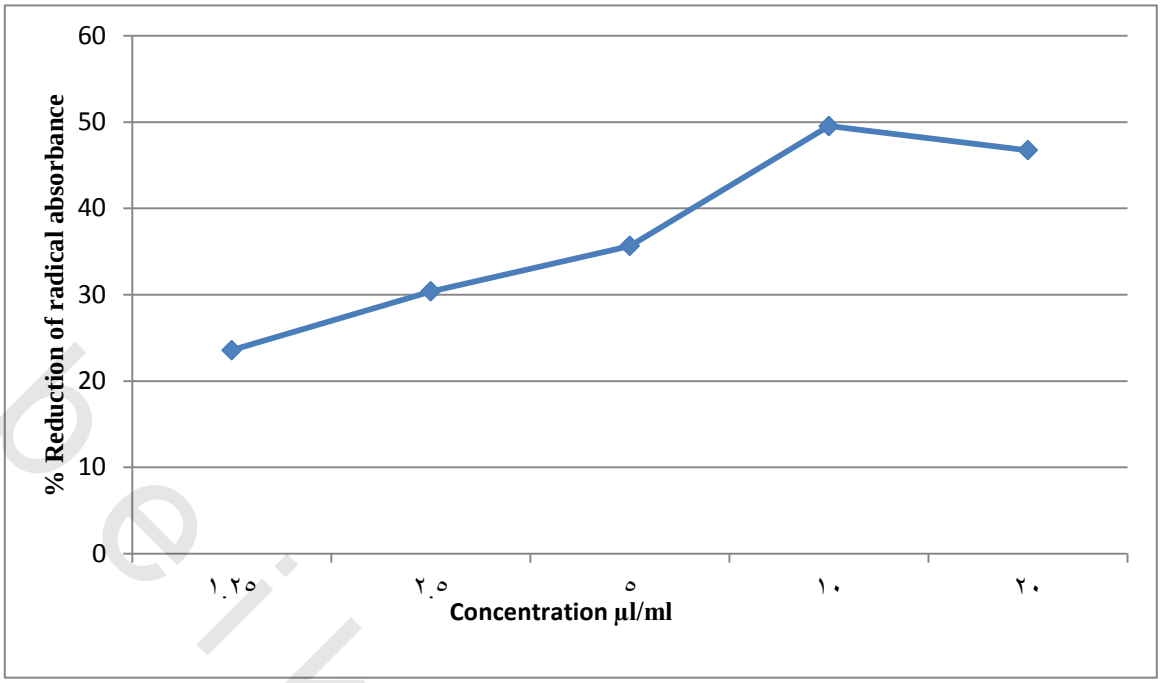


**Figure 40. Antioxidant activity of essential oil of *P. anisum* L. using DPPH assay.**

**Table IXX . $IC_{50}$ for antioxidant activity of essential oils of *O. basilicum* L. and *P. anisum*L.**

| Essential oil | $IC_{50}$(µl/ml) |
|---|---|
| *O. basilicum* L. | 26.52± 0.23 |
| *P. anisum* L. | 19.12±0.45 |

*Values represent the mean of two replicates and ± standard error mean (SEM).
**There is no significant difference between antioxidant activity of essential oil of *O. basilicum* and that of essentialoil of *P. anisum* ($P$>0.05).

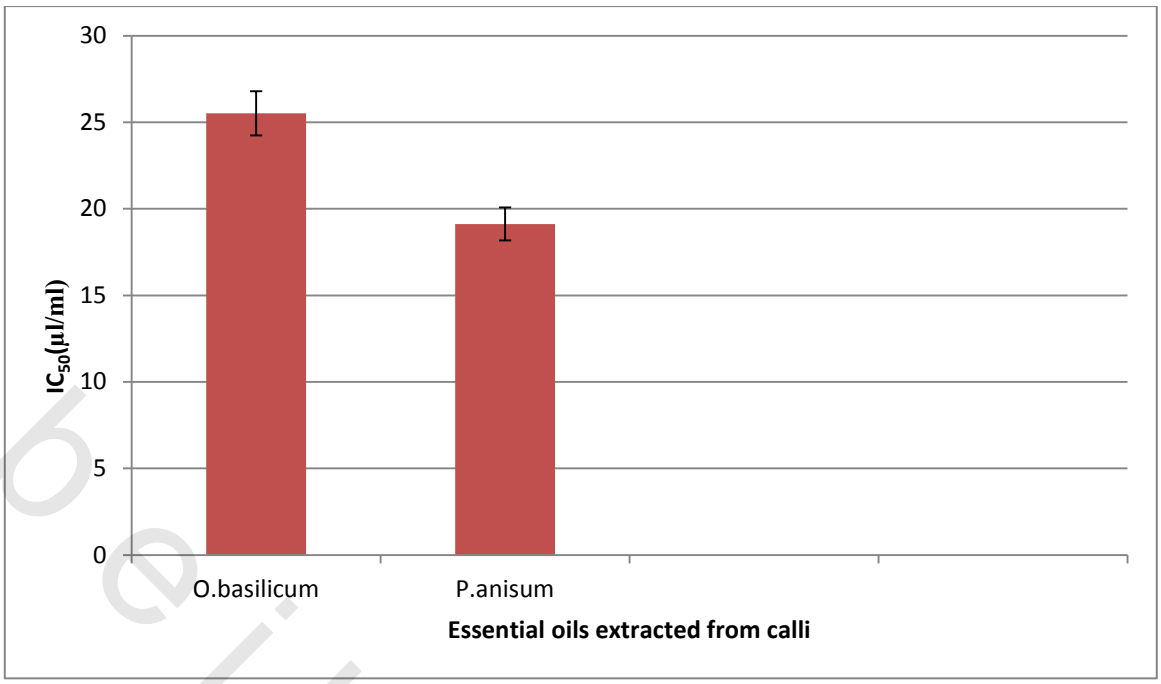


**Figure 41. $IC_{50}$ for antioxidant activity of essential oils of *O. basilicum* L. and *P. anisum* L.**

# Results and discussion

There are two main approaches for determining the *in vitro* antioxidant activity of essential oils [203] :

i. The inhibition of lipid oxidation in different systems (oils, solutions of lipids in organic solvents, oil-in-water emulsions, micelles, liposomes, *etc.*)
ii. The ability to scavenge free radical species.

In the present study our assay (DPPH assay) depends on the second approach for determination of antioxidant activity of the tested oils.

Results of antioxidant activity for essential oils of *O. basilicum* and *P. anisum* were expressed as % reduction in absorbance of free radical as shown in [Tables XVII-IXX] and [Figures 39-41].

The % reduction in absorbance of free radical is directly proportional to the increase in concentration of essential oil of *O. basilicum* except the last value there was a decrease in antioxidant activity. $IC_{50}$ of antioxidant activity for essential oil of *O. basilicum* was 25.52 µl/ml which indicates a good antioxidant activity. This data are in agreement with the earlier published data about antioxidant activity of *O. basilicum* essential oil [199-201].

Regarding essential oil of *P. anisum*, % reduction in absorbance of free radical is directly proportional to the increase in concentration with only a slight decrease in antioxidant activity at a concentration of 20µl/ml. $IC_{50}$ of antioxidant activity for essential oil of *P. anisum* was
19. 12 µl/ml and that indicates that essential oil of *P. anisum* may act as a promising free radical scavenger. Antioxidant activity of *P. anisum* essential oil is mainly correlated to its high content of *trans*- anethole which considered as effective antioxidant with a relative low $IC_{50}$value [204].

It is quite known that the antioxidant activity in these oils is correlated mainly to their total phenolic content [205]. From GC/MS data of the tested essential oils, we propose that their phenolic content was exposed to a sharp drop for unknown reasons, but they still can be regarded as beneficial and promising antioxidants.

.

# Chapter 3

## Immunostimulant activity of essential oil of *Ocimum basilicum* L.and *Pimpinella anisum* L

# INTRODUCTION

During the last two decades intensive investigations were carried out on the preparation, experimental and clinical characteristics of one relatively new category biologically active substances so called immune stimulants. They are products of natural or synthetic origin with different chemical characteristics and mechanisms of action [206, 207] .

Immunostimulants can activate different elements and mechanisms of the immune system of humans and animals; they reinforce a body's natural resistance in order to cope successfully with various viral and bacterial infections or to help in the treatment of other pathogenically related with suppressed immune system conditions such as cancer (malignant) diseases, AIDS, and SARS [208].

Immunostimulants created the base of the active and successful development and implementation in the clinical practice of the nonspecific immunotherapy and the nonspecific immune prevention by stimulating the main factors of the immune system. They can be implicated in many serious clinical conditions; (1) the increased multi resistance of the bacteria to antibiotics, which creates in some regions in the world dramatic situation as more than 40% of the circulating bacterial strains are resistant to available antibiotics; (2) the allergic reactions that develop to antibiotics and chemotherapeutics in patients and in medical personnel what restricts their use; (3) the cases of the antibiotics that proved immunosuppressive effect. They kill bacteria but in the same time diminish the natural resistance of the organism to cope with them;(4) the lack of activity of the antibiotics in viral infections and the lack of specific treatment or vaccines for the greatest part of the viral infections, including HIV/AIDS, SARS, avian flu and some others newly emerging or intentionally spread bacterial and viral infections [209].

## Immunostimulant activity of natural products

Lately the interest of researchers has grown towards the plant-kingdom as a possible source for new immunostimulants. This is why since1980 the majority of substances that have been tested as immune stimulants are extracted from plants [210-212].

Natural products and natural product derivatives have a traditional history as immune -stimulants. Emerging evidence indicates that herbal plants exert their beneficial effects on animal immune system mostly by plant secondary metabolites [210]. The immune stimulating activities of many of these components have been most widely studied in mice, chicken and human cell lines [214-216]. These pharmacological effects are of extensive range. For example, *Ginseng* with its steroidal saponine, has immune-stimulating properties including cytokine production (IL-2, IL6, TNF-α, and INF-γ), macrophage activation and lymphocyte activity [214]. Conversely, flavonoids and terpenes from *Ginkgo biloba* can mediate production of inflammatory cytokines [215]. Saponins have the ability to stimulate the cell-mediated immune system, as well as to enhance antibody production [219]. Saponins reportedly induced production of cytokines such as interleukins and interferons [220, 221] . Meyer saponins [222], Quillaia saponins [223] and the butanol extract of *Lonicera japonica* [224]and de-acylated

saponin-1 administered on the nasal mucosa [225] , all stimulated the immune responses *in vivo*.

The immune modulatory activity of aqueous and ethanolic extracts of leaves of *O. basilicum* in mice was reported. The aqueous and ethanolic extract of leaves of *OB* was administered orally at the dosage levels of 400 mg/kg/day body weight in mice. The assessment of immunomodulatory activity on specific and nonspecific immunity were studied by haemagglutination antibody (HA) titer, delayed type hypersensitivity (DTH), neutrophil adhesion test and carbon clearance test. The study demonstrates that *OB* triggers both specific and non-specific responses to a greater extent. From the results obtained and phytochemical studies the immunostimulant effect of *OB* could be attributed to the flavonoid content [226].

# Experimental

## Materials and methods

### Reagents for biological assay

i. Lymphocyte separation medium (Ficoll-Paque premium, density 1.077 g/ml, GE Healthcare, USA).
ii. Cell culture medium: RPMI 1640 medium (Lonza, USA) supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Lonza), 4 mM L-glutamine (Lonza), 100 U of penicillin and 100 µg streptomycin (Cambrex) and 10% FBS (Lonza).
iii. Fetal Bovine Serum (FBS) (F2442, Sigma Chemical, USA).
iv. Methylene blue (200-515-2, Panreac Quimica S.A., Spain).
v. Trypan blue stain 0.5% (w/v) in saline (Biochrome, Germany).

### Special apparatus

i. Lyophilizer
ii. Water jacketed $CO_2$ incubator (Model CO28IR, New Brunswick, NJ, USA).
iii. Shaker incubator (New Brunswick, NJ, USA).
iv. Vertical Laminar Airflow belonging to the S2 safety grade ( Telstra, Terrazza, Spain).
v. Inverted microscope (1X70, Olympus, Japan) with digital camera ( Cammedia C-4040 zoom) and an image analysis system Analysis 3.1 Soft Imaging System SIS (GmbH, Hammer Str.Münster, Germany).
vi. Eaid pipette (Jencons scientific, Ltd, Bds, UK).
vii. Eppendorf centrifuge 5804 (Eppendorf-Netheler-Hinz. GmbH, Hamburg, Germany).
viii. Vacuum pump (Telstra, Terrazza, Spain).
ix. Autoclave SA-300VF (STRDY- Industrial Co, Taiwan)
x. pH meter model 215 (Denver Instrumental company, Aviada, Colorado, U.S.A)
xi. ELISA reader (Anthos 2000 and ADAP software).

## Method

Peripheral blood (5 ml) was collected from healthy individuals in heparinized tubes and PBMCs were separated with the help of sterile lymphocyte separation medium using density gradient centrifugation method. Cell count and cell viability were determined using trypan blue exclusion test [227] . Finally, the separated lymphocytes were suspended at $1.0x10^6$ cell/ml in RPMI 1640 culture medium and subsequently incubated with different concentrations (0.1562, 0.3125, 5.1562, 10.3125, 20.3125 µl/ml) of the extracted oils and each sample was assayed in duplicates. Proliferation was determined using neutral red uptake assay [228] after incubation for 72 hrs at 37 $^oC$, 5% $CO_2$, and 95% humidity.

Data output was presented as Stimulation Index, which is the ratio of the optical density of lymphocytes stimulated by the tested material to the optical density of control unstimulated lymphocytes. A value higher or equals to 1.5 indicates a significant immune stimulant activity.

**Table XX: The Stimulation index of essential oils of *O. basilicum* L. and *P. anisum* L. at different concentrations.**

| | Stimulation Index | | | | |
|---|---|---|---|---|---|
| Essential oil | 0.1562µl/ml | 0. 3125µl/ml | 5.1562µl/ml | 10.3125µl/ml | 20.3125µl/ml |
| *O.basilicum* L. | 0.992789 | 1.033514 | 1.139493 | 1.585145 | 1.19565 |
| *P. anisum* L. | 0.541666 | 0.788949 | 0.789855 | 1.022645 | 1.20742 |

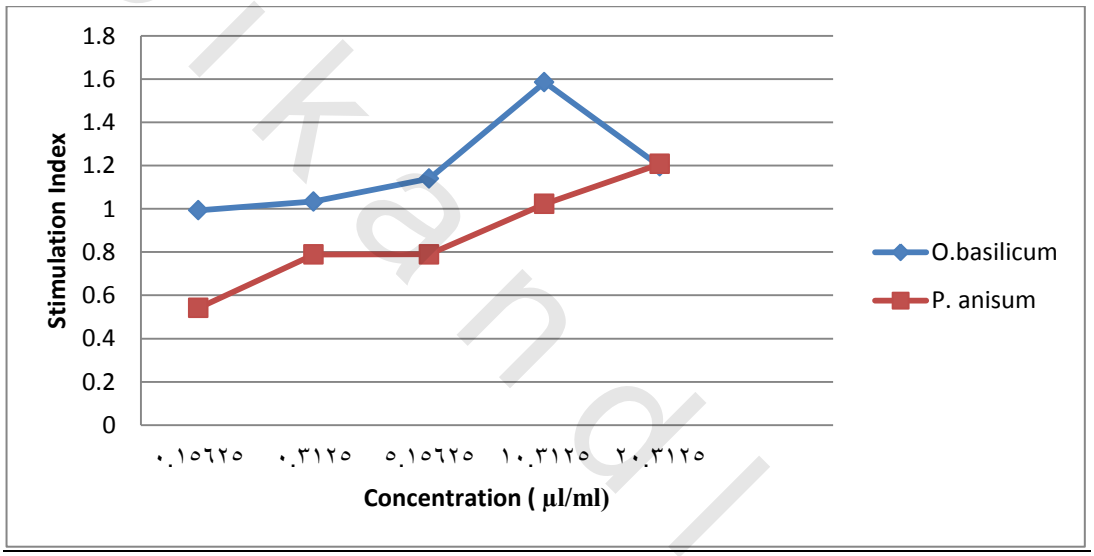


**Figure 42. The stimulation index of essential oils of *O. basilicum* L. and *P. anisum* L. at different concentrations .**