



Faculty of Science  
Zoology Department

**Evaluating and Comparing the Anti-tumour Effects of Alcoholic  
Extract and Some Bioactive Components of Sweet Marjoram  
(*Origanum majorana* L.) on Colon Cancer Cell Line**

**A Thesis**

Submitted for the Award of the  
Ph.D. of Science in Zoology

By

**Mai Mamdouh Hassan Hosni Zahra**

M.Sc. in Zoology (2011)

**Supervisors**

**Prof. Dr. Nadia Mohamed Abd El-Aziz El-Beih**

Professor of Physiology, Zoology Department,  
Faculty of Science, Ain Shams University

**Prof. Dr. Gamal Ramadan Shebl Ramadan**

Professor of Immunology, Zoology Department,  
Faculty of Science, Ain Shams University

**Prof. Dr. Khaled Mahmoud Mohamed Hanafi**

Professor of Biochemistry, Pharmacognosy Department,  
National Research Centre

**2018**

## **ACKNOWLEDGMENT**

I would like to express my special appreciation and thanks to **Prof. Dr. Nadia Mohamed Abd El-Aziz El-Beih** (Professor of Physiology, Department of Zoology, Faculty of Science, Ain Shams University) for designing the plan of this study, direct supervision, continuous and valuable criticism and guidance in the different fields related to this research, and for encouraging me to grow as a true researcher. What I have learned from her, not only in the scientific arena, but also in the daily life, which will greatly benefit my career and life in the future.

I also owe very special thanks to **Prof. Dr. Gamal Ramadan Shebl Ramadan** (Professor of Molecular and Cellular Immunology and Head of Physiology Division, Department of Zoology, Faculty of Science, Ain Shams University) for designing the plan of this study and the associated research, his perceptive comments and questions that made me extend my research from various points of view. In addition, he directed me to the right path to achieve the practical work of the thesis, supported me with statistical analysis program, gave me advice regarding the real good scientific writing, and reviewed all work line by line. I think without that this work could not have been completed and I could not have imagined having a better advisor and mentor for my Ph.D. than him.

Also, I would like to thank **Prof. Dr. Khaled Mahmoud Mohamed Hanafi** (Professor of Biochemistry and Head of Department of Pharmacognosy, National Research Centre) for using his laboratory and his continuous support and guidance regarding tissue culture facilities and techniques. Really, without his precious support, it would have not been possible to complete this research study.

Many thanks are also given to all staff members of Zoology Department (Faculty of Science, Ain Shams University) for continuous help and encouragement.

This study was partially supported by a fund from the Academy of Scientific Research and Technology (PI: Prof. Dr. Nadia Mohamed Abd El-Aziz El-Beih).



## **ABSTRACT**

**Zahra, Mai Mamdouh Hassan Hosni**

### **Evaluating and Comparing the Anti-tumour Effects of Alcoholic Extract and Some Bioactive Components of Sweet Marjoram (*Origanum majorana* L.) on Colon Cancer Cell line**

Ph.D. in Zoology, Faculty of Science, Ain Shams University, Cairo, 2018

**Keywords:** Apoptotic pathways - Carvacrol - Colorectal cancer - Cytotoxicity - Death receptors - HCT116 cell line - Marjoram - Morphological alterations - Oxidative stress - Ursolic acid.

Colorectal cancer (CRC) is one of the most frequent malignant tumours worldwide. Nowadays, complementary and alternative treatment strategies have become necessary to improve the survival rate of the CRC patients. Therefore, the present study investigated the anticancer effects of different concentrations of the Egyptian sweet marjoram (*Origanum majorana* L.) methanolic extract (ME) and some of its bioactive components (carvacrol, CA and ursolic acid, UA) on the HCT116 colon cancer cell line. The data of the present study indicated that ME, CA and UA were specifically able to inhibit proliferation and induce cytotoxicity in the HCT116 cells compared with the non-cancerous human skin cell line (BJ-1). Based on the IC<sub>50</sub> values and the selectivity index (SI), three concentrations (0.25, 0.5 and 1.0 of the IC<sub>50</sub> at 48 hours of cell culture incubation) were used in the present study to evaluate the possible anticancer molecular mechanisms of the used natural products. Our results proved that ME, CA and UA induced morphological alterations related to apoptosis, inhibited cell adhesion and migration, and reduced spheroids' volumes of the HCT116 cells after 48 hours of treatment in a concentration-dependent manner.

Also, they triggered the DNA fragmentation and increased the percentage of apoptotic cells in sub-G1 phase in the HCT116 cells, which accompanied by decreasing the total antioxidant capacity along with increasing the tumour suppressor p53 protein and the release of mitochondrial cytochrome C into the cytosol. In addition, they upregulated the expression of death receptors (Fas/CD95 and tumour necrosis factor receptor-1) and increased caspases 3, 8 and 9 in the HCT116 cells. All of these data indicated the ability of these natural products to significantly trigger both the intrinsic and extrinsic pathways of apoptosis in cancer cells. In general, the highest anticancer activity in the present study was achieved by UA followed by CA, rendering these compounds may become promising anticancer agents in treating CRC patients in the near future.

---

---

## **LIST OF TABLES**

---

---

<b>Table's Number &amp; Title</b>	<b>Page</b>
-----------------------------------	-------------

---

---

### **MATERIALS AND METHODS**

- |           |  |           |
|-----------|--|-----------|
| <b>I</b>  | Sequence of primers used for the real-time polymerase chain reaction (qPCR). | <b>42</b> |
| <b>II</b> | The conditions of the real-time polymerase chain reaction (qPCR).            | <b>42</b> |

### **RESULTS**

- |          |   |           |
|----------|---|-----------|
| <b>1</b> | The cytotoxic effects of different concentrations of ME, CA and UA on colon cancer cell line (HCT116) after 24, 48 and 72 hours of cell culture.  | <b>46</b> |
| <b>2</b> | The cytotoxic effects of different concentrations of ME, CA and UA on non-cancerous cell line (BJ-1) after 24, 48 and 72 hours of cell culture.   | <b>48</b> |
| <b>3</b> | Effects of different concentrations of ME, CA, and UA on the morphological changes (%), cell adhesion ( $\times 10^4$ cells), cell migration (%) and spheroid volume ( $\text{mm}^3$ ) of colon cancer cell line (HCT116) after 48 hours of incubation. | <b>56</b> |
| <b>4</b> | Effects of different concentrations of ME, CA, and UA on the total antioxidant capacity (mmol/L) of colon cancer cell line (HCT116) after 48 hours of incubation.   | <b>72</b> |
| <b>5</b> | Effects of different concentrations of ME, CA, and UA on DNA fragmentation (%) of colon cancer cell line (HCT116) after 48 hours of incubation.   | <b>76</b> |

- 6** Effects of different concentrations of ME, CA and UA on apoptotic cells (sub-G1, %) of colon cancer cell line (HCT116) after 48 hours of incubation. **82**
- 7** Effects of different concentrations of ME, CA, and UA on the caspase 3 concentration (ng/ml) of colon cancer cell line (HCT116) after 48 hours of incubation. **84**
- 8** Effects of different concentrations of ME, CA, and UA on the caspase 8 concentration (ng/ml) of colon cancer cell line (HCT116) after 48 hours of incubation. **88**
- 9** Effects of different concentrations of ME, CA and UA on the protein expression of the caspase 9 of colon cancer cell line (HCT116) after 48 hours of incubation. **92**
- 10** Effects of different concentrations of ME, CA, and UA on the protein expression of Bcl-2 and Bax as well as the Bax/Bcl-2 ratio of colon cancer cell line (HCT116) after 48 hours of incubation. **97**
- 11** Effects of different concentrations of ME, CA and UA on the cytochrome C concentration (ng/ml) of colon cancer cell line (HCT116) after 48 hours of incubation. **101**
- 12** Effects of different concentrations of ME, CA, and UA on the protein expression of tumour suppressor p53 of colon cancer cell line (HCT116) after 48 hours of incubation. **105**
- 13** Effects of different concentrations of ME, CA, and UA on the protein expression of Fas death receptor (CD95) of colon cancer cell line (HCT116) after 48 hours of incubation. **108**

- 14** Effects of different concentrations of ME, CA and UA on the gene expression of tumour necrosis factor receptor-1 (TNFR1) of colon cancer cell line (HCT116) after 48 hours of incubation. **111**
- 
-



---



---

## LIST OF FIGURES

---



---

Figure's Number & Title	Page
-------------------------	------

---

### LITERATURE REVIEW

<b>I</b>	Pathogenesis of CRC.	<b>7</b>
<b>II</b>	Apoptotic extrinsic and intrinsic pathways.	<b>11</b>
<b>III</b>	Chemical structure of carvacrol.	<b>18</b>
<b>IV</b>	Chemical structure of ursolic acid.	<b>21</b>

### MATERIALS AND METHODS

<b>V</b>	Amplification curve of the real-time polymerase chain reaction (qPCR).	<b>43</b>
<b>VI</b>	Dissociation curve of the real-time polymerase chain reaction (qPCR).	<b>43</b>

### RESULTS

<b>1</b>	The cytotoxic effect of different concentrations of <b>(I)</b> methanolic extract of <i>Origanum majorana</i> , ME, <b>(II)</b> carvacrol, CA, and <b>(III)</b> ursolic acid, UA, on the colon cancer cell line (HCT116) after 24, 48 and 72 hours of cell culture.	<b>47</b>
<b>2</b>	The cytotoxic effect of different concentrations of <b>(I)</b> methanolic extract of <i>Origanum majorana</i> , ME, <b>(II)</b> carvacrol, CA, and <b>(III)</b> ursolic acid, UA, on the non-cancerous cell line (BJ-1) after 24, 48 and 72 hours of cell culture.	<b>49</b>
<b>3</b>	The selective cytotoxicity of ME, CA and UA towards tumour (HCT116) cells as compared with non-cancerous (BJ-1) cells.	<b>50</b>
<b>4</b>	Some morphological changes of colon cancer cell line (HCT116) induced by different	<b>53</b>

---

	concentrations of ME after 48 hours of incubation (Magnification: 100X).	
<b>5</b>	Some morphological changes of colon cancer cell line (HCT116) induced by different concentrations of CA after 48 hours of incubation (Magnification: 100X).	<b>54</b>
<b>6</b>	Some morphological changes of colon cancer cell line (HCT116) induced by different concentrations of UA after 48 hours of incubation (Magnification: 100X).	<b>55</b>
<b>7</b>	Effects of different concentrations of ME, CA and UA on the morphological changes (%) of colon cancer cell line (HCT116) after 48 hours of incubation.	<b>57</b>
<b>8</b>	Effects of different concentrations of ME, CA and UA on the cell adhesion of colon cancer cell line (HCT116) after 48 hours of incubation (Magnification: 40X).	<b>58</b>
<b>9</b>	Effects of different concentrations of ME, CA and UA on the numbers of adherent cells ( $\times 10^4$ ) of colon cancer cell line (HCT116) after 48 hours of incubation.	<b>59</b>
<b>10</b>	Cell migration of the colon cancer cell line (HCT116); scale bar: 400 $\mu$ m.	<b>62</b>
<b>11</b>	Effects of different concentrations of ME on the cell migration of colon cancer cell line (HCT116) after 48 hours of scratching; scale bar: 400 $\mu$ m.	<b>63</b>
<b>12</b>	Effects of different concentrations of CA on the cell migration of colon cancer cell line (HCT116) after 48 hours of scratching; scale bar: 400 $\mu$ m.	<b>64</b>
<b>13</b>	Effects of different concentrations of UA on the cell migration of colon cancer cell line	<b>65</b>

	(HCT116) after 48 hours of scratching; scale bar: 400µm.	
<b>14</b>	Effects of different concentrations of ME, CA and UA on the cell migration (%) of colon cancer cell line (HCT116) after 48 hours of scratching.	<b>66</b>
<b>15</b>	Effects of different concentrations of ME, CA and UA on the tumour spheroids of colon cancer cell line (HCT116) after 48 hours of incubation; scale bar: 200 µm.	<b>68</b>
<b>16</b>	Effects of different concentrations of ME, CA and UA on the tumour spheroid volume (mm <sup>3</sup> ) of colon cancer cell line (HCT116) after 48 hours of incubation.	<b>69</b>
<b>17</b>	Effects of different concentrations of ME, CA and UA on the total antioxidant capacity (mmol/L) of colon cancer cell line (HCT116) after 48 hours of incubation.	<b>73</b>
<b>18</b>	Agarose gel for colon cancer cell line (HCT116) fragmentation.	<b>75</b>
<b>19</b>	Effects of different concentrations of ME, CA and UA on DNA fragmentation (%) of colon cancer cell line (HCT116) after 48 hours of incubation.	<b>77</b>
<b>20</b>	Flow cytometry analysis of cell cycle showing apoptotic cells (sub-G1) of colon cancer cell line (HCT116) treated with different concentrations of ME, CA and UA for 48 hours.	<b>81</b>
<b>21</b>	Effects of different concentrations of ME, CA and UA on apoptotic cells (sub-G1, %) of colon cancer cell line (HCT116) after 48 hours of incubation.	<b>83</b>
<b>22</b>	Effects of different concentrations of ME,	<b>85</b>

- CA and UA on the caspase 3 concentration (ng/ml) of colon cancer cell line (HCT116) after 48 hours of incubation.
- 23** Effects of different concentrations of ME, CA and UA on the caspase 8 concentration (ng/ml) of colon cancer cell line (HCT116) after 48 hours of incubation. **89**
- 24** Western blot analysis of caspase 9 protein expression (47 KDa) of colon cancer cell line (HCT116) treated with different concentrations of **(I)** ME, **(II)** CA and **(III)** UA for 48 hours. **91**
- 25** Effects of different concentrations of ME, CA and UA on the protein expression of caspase 9 of colon cancer cell line (HCT116) after 48 hours of incubation. **93**
- 26** Western blot analysis of Bcl-2 protein expression (28 KDa) of colon cancer cell line (HCT116) treated with different concentrations of **(I)** ME, **(II)** CA and **(III)** UA for 48 hours. **95**
- 27** Western blot analysis of Bax protein expression (20 KDa) of colon cancer cell line (HCT116) treated with different concentrations of **(I)** ME, **(II)** CA and **(III)** UA for 48 hours. **96**
- 28** Effects of different concentrations of ME, CA and UA on the protein expression of Bcl<sub>2</sub> and Bax as well as the Bax/Bcl<sub>2</sub> ratio (I-III) of colon cancer cell line (HCT116) after 48 hours of incubation. **98**
- 29** Effects of different concentrations of ME, CA and UA on the cytochrome C concentration (ng/ml) of colon cancer cell line (HCT116) after 48 hours of incubation. **102**

- 30** Western blot analysis of tumour suppressor p53 protein expression (53 KDa) of colon cancer cell line (HCT116) treated with different concentrations of **(I)** ME, **(II)** CA and **(III)** UA for 48 hours. **104**
- 31** Effects of different concentrations of ME, CA and UA on the protein expression of tumour suppressor p53 of colon cancer cell line (HCT116) after 48 hours of incubation. **106**
- 32** Western blot analysis of Fas death receptor (CD95) protein expression (48 KDa) of colon cancer cell line (HCT116) treated with different concentrations of **(I)** ME, **(II)** CA and **(III)** UA for 48 hours. **107**
- 33** Effects of different concentrations of ME, CA and UA on the protein expression of Fas death receptor (CD95) of colon cancer cell line (HCT116) after 48 hours of incubation. **109**
- 34** Effects of different concentrations of ME, CA and UA on the gene expression of tumour necrosis factor receptor-1 (TNFR1) of colon cancer cell line (HCT116) after 48 hours of incubation. **112**

### **DISCUSSION**

- 35** Possible apoptotic mechanisms induced by ME, CA and UA in the colon cancer cell line (HCT116). **129**
- 
-

## **LIST OF ABBREVIATIONS**

<b>A<sub>B</sub></b>	Absorbance of blank
<b>ANOVA</b>	Analysis of variance
<b>Apaf1</b>	Apoptotic protease activating factor-1
<b>A<sub>S</sub></b>	Absorbance of sample
<b>ATCC</b>	American Type Culture Collection
<b>A<sub>VNC</sub></b>	Averages of the absorbance of the negative control
<b>A<sub>VS</sub></b>	Averages of the absorbance of the sample
<b>Bax</b>	Bcl-2 associated X protein
<b>Bcl-2</b>	B-cell lymphoma-2
<b>Bcl-xL</b>	Bcl extra-large
<b>Bid</b>	BH3 interacting-domain death
<b>bp</b>	Base pair
<b>BSA</b>	Bovine serum albumin
<b>CA</b>	Carvacrol
<b>CCD</b>	Charge-coupled device
<b>CCM</b>	Complete culture media
<b>Cdks</b>	Cyclin-dependent kinases
<b>cDNA</b>	Complementary DNA
<b>Cox</b>	Cyclooxygenase
<b>CRC</b>	Colorectal cancer
<b>Ct</b>	Threshold cycle
<b>d</b>	Diameters
<b>DMEM/F12</b>	Dulbecco's Modified Eagle Medium: Nutrient Mixture F12
<b>DMSO</b>	Dimethylsulfoxide
<b>DNA</b>	Deoxyribonucleic acid
<b>dNTPs</b>	Deoxynucleotide triphosphates
<b>DPA</b>	Diphenylamine assay

<b>EDTA</b>	Ethylene-diamine-tetra-acetic acid
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>Fas</b>	First apoptosis signal (CD95 death receptor)
<b>FasL</b>	Fas Ligand
<b>FBS</b>	Foetal bovine serum
<b>GAPDH</b>	Glyceraldehydes-3-phosphate dehydrogenase
<b>GRAS</b>	Gained generally regarded as a safe
<b>H<sub>2</sub>O<sub>2</sub></b>	Hydrogen peroxide
<b>hr</b>	hours
<b>HRP</b>	Horseradish peroxidase
<b>JNK</b>	Jun N-terminal kinase
<b>MAPK</b>	Mitogen-activated protein kinase
<b>ME</b>	Methanolic extract of Egyptian sweet marjoram
<b>MMP</b>	Matrix metalloprotease
<b>M-MuLV</b>	Moloney murine leukaemia virus
<b>MTT</b>	Tetrazolium dye
<b>NF-<math>\kappa</math>B</b>	Nuclear factor kappa-light-chain-enhancer of activated B-cells
<b>PAGE</b>	Polyacrylamide gel electrophoresis
<b>Akt</b>	Protein kinase B
<b>PARP</b>	Poly (ADP ribose) polymerase
<b>PBS</b>	Phosphate buffered saline
<b>PI3K</b>	Phosphatidylinositol-3-kinase
<b>poly-HEMA</b>	Poly-2-hydroxyethyl methacrylate
<b>PVDF</b>	Polyvinylidene difluoride
<b>qPCR</b>	Quantitative (real-time) polymerase chain reaction
<b>RIPA</b>	Radioimmunoprecipitation assay
<b>RNA</b>	Ribonucleic acid
<b>ROS</b>	Reactive oxygen species