



Faculty of Science  
Department of Biochemistry

# **Role of Autophagy in the Antitumor Activity of Aloin in Breast Cancer Cells**

*Thesis Submitted by*

***Asmaa Kamal Mansour Mohamed El-Gendy***

*M.Sc. in Biochemistry (2008)*

*Award of the Degree of Doctor of Philosophy in Biochemistry*

*Supervised by*

***Prof. Dr. Amr Youssef Esmat***

*Prof. of Biochemistry*

*Faculty of Science*

*Ain Shams University*

***Prof. Dr. Hala M. Ghanem***

*Prof. of Biochemistry*

*Faculty of Science*

*Ain Shams University*

***Prof. Mahmoud M. Said***

*Prof. of Biochemistry*

*Faculty of Science*

*Ain Shams University*

***Prof. Dr. Mahmoud Nour El-  
Din El-Rouby***

*Prof. of Immunology & Virology*

*National Cancer Institute*

*Cairo University*

***Dr. Emad K. Ibrahim***

*Assistant Prof. of Biochemistry*

*Faculty of Science*

*Ain Shams University*

**2020**



(قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا  
عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ)

صَدَقَ اللهُ الْعَظِيمُ

سورة البقرة (الأيه ٢٣)



**Faculty of Science**

**Department of Biochemistry**

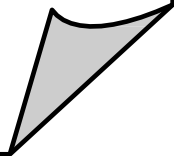
## ***Biography***

<b>Name</b>	Asmaa Kamal Mansour Mohammed Elgendy
<b>Date of Graduation and Education</b>	May 2001, Faculty of Girls for Arts, Science and Education
<b>Degree Awarded</b>	M.Sc in Biochemistry and Nutrition 2008
<b>Occupation</b>	Chemist at the General Organization of Empor and Export- Ministry of Trade and Industry

## ***Declaration***

***This thesis has not been submitted for  
a degree at this or any other university***

*Asmaa Kamal Mansour Mohamed Elgendy*



## ***Acknowledgements***

*First and foremost, many thanks are due to **Almighty Allah**, for giving me the knowledge, ability and opportunity to undertake this research study and to complete it satisfactorily. Without His blessings, this achievement would not have been possible.*

*I would like to express my deep gratitude and sincere appreciation towards **Prof. Dr. Amr Youssef Ezz El-Din Esmat**, Professor of Biochemistry, Faculty of Science, Ain Shams University, for suggesting the point, for his perpetual guidance, creative thinking, valuable suggestions, fruitful discussion and profound revision of the thesis.*

*I'm greatly indebted to express my special thanks and great appreciation to **Prof. Dr. Hala Mostafa Ghanem** for giving me the opportunity to work under her supervision. This thesis would have not been possible without her valuable guidance, continuous support and patience throughout this thesis.*

*My sincere gratitude is also extended to **Prof. Dr. Mahmoud Mohamed Said**, Professor of Biochemistry, Faculty of Science, Ain Shams University, for his help,*

*profound revision of the thesis, tremendous effort with the statistical analysis, constant guidance, sincere encouragement, valuable advice and criticism.*

*My sincere gratitude is also extended to **Prof. Dr. Mahmoud Nour El-Din El-Rouby**, Professor of Immunology and Virology, Cancer Biology Department, National Cancer Institute for providing me laboratory facilities and his tutorial assistance with the practical part of this work, as well as his valuable comments on the thesis and his useful advice throughout this work and for his fatherly attitude.*

*My appreciation goes also to **Dr. Emad Khairy Ibrahim**, Ass. Prof. of Biochemistry, Faculty of Science, Ain Shams University, for his supervision, fruitful discussion, cooperation, understanding, patience, personal guidance and great help in writing the thesis.*

*Deepest thanks and gratitude are due to **Prof. Dr. Hala Galal Mohamed El-Tantawi**, Prof. of cell and tissue biology, Zoology department, Faculty of Science, Ain Shams University, for her great help with the transmission electron microscope examination and comments.*

*I am deeply grateful and I would like to extend my heartfelt thanks to my family and my friends for their unconditional love and support. They all have been a constant source of motivation, encouragement and inspiration.*

## List of Contents

<b>Abstract</b>	i
<b>List of Abbreviations</b>	iii
<b>List of Tables</b>	vi
<b>List of Figures .....</b>	vii
<b>Chapter I. Introduction and Aim of the Work ..</b>	1
<b>Chapter II. Review of Literature .....</b>	7
• Breast Cancer .....	7
• Breast Cancer Classification .....	7
• Autophagy.....	15
• Molecular Mechanism of Autophagy .....	20
• Cell Signaling-Regulated Autophagy Process- Autophagy and m-TOR Signaling.....	25
• Autophagy and Cancer .....	37
• Doxorubicin.....	42
• Modes of Action of Doxorubicin as an Anticancer Therapeutic Agent .....	45
• Aloe Plant.....	52
• Aloin .....	54
<b>Chapter III. Materials and Methods .....</b>	63
<b>A-Materials .....</b>	63
• Cell Lines.....	63
• Compounds used .....	64
• Preparation of Doxorubicin and Aloin .....	64
• Cell lines Propagation .....	65
<b>B-Methods .....</b>	66

I. Cytotoxicity Studies.....	66
1- <i>In vitro</i> Cell Proliferation Assay .....	66
2- Clonogenic Assay .....	72
II. Monitoring of Autophagy Formation.....	76
1-Transmission Electron Microscopy .....	76
2- Detection of Acid Vesicular Organelles (AVOs) with Acridine Orange by Confocal Fluorescence Microcopy.....	81
3- Quantification of Acidic Vesicular Organelles (AVOs) using Fluorescence Activated Cell Sorting (FACS).....	83
4- Protein Expression of Some Autophagy Markers by Western Blot .....	85
<b>Satistical Analysis.....</b>	<b>98</b>
<b>Chapter IV. Results.....</b>	<b>99</b>
<b>Chapter V. Discussion.....</b>	<b>158</b>
• <b>Conclusions.....</b>	<b>199</b>
• <b>Recommendations.....</b>	<b>201</b>
• <b>Summary.....</b>	<b>202</b>
• <b>References.....</b>	<b>208</b>



---

## **Role of Autophagy in the Antitumor Activity of Aloin in Breast Cancer Cells**

---

*Asmaa Kamal Mansour Mohamed Elgendy*

### **ABSTRACT**

Aloin is a natural bioactive anthraquinone extracted from *Aloe* sp. and has the potential of tumor regression by inhibition of cell proliferation and induction of cell apoptosis in different human cancer cell lines. The ability of cancer cells to evade apoptosis, which often limits the efficacy and accounts for the resistance to chemotherapy, strives the search of autophagy process as an alternative target to promote cell death. The present study was undertaken to verify the autophagy process as a probable mechanism for the antitumor activity of aloin in 2 types of breast cancer cell lines; estrogen receptor positive (T47D) and triple negative (MDA-MB231), compared to an anthraquinone analog, doxorubicin. Initially, the cytotoxicity of increasing concentrations of aloin and doxorubicin were assessed using MTT and clonogenic assays at 2 exposure periods (24 and 72h) to determine the half maximal inhibitory concentration ( $IC_{50}$ ) of aloin and doxorubicin in both types of cell lines. The formation of autophagy process in the treated tumor cells was initially detected by using transmission electron microscope (TEM). Emphasis of autophagy process was then achieved by monitoring the formation of acidic vesicular organelles (AVOs) qualitatively by confocal fluorescence microscope, and quantitatively by fluorescence activated cell sorting (FACS). Finally, the protein expression levels of some

autophagy-related genes were determined by Western blotting. Results obtained from this study revealed that aloin inhibited the cell growth of both T47D and MDA-MB231 cells, with a more pronounced effect in the 72 exposure regimen. TEM of tumor cells treated with IC<sub>50</sub> of aloin revealed the presence of autophagosomes, as early and autolysophagosomes, as late autophagic compartments. The autophagic activity of aloin was then emphasized by the accumulation of acidic vesicular organelles (AVOs) in the treated tumor cells and the up-regulation in the protein expression of some autophagy-related genes, such as microtubule-associated proteins 1A/1B light chain 3B (LC3B II), beclin1, phosphorylated AKT (p-PKB), and in contrast down-regulation of p62 and phosphorylated mammalian target of rapamycin kinase (p-mTOR). These findings concluded that autophagy is regarded as one of the modes of the cytotoxic action of aloin in T47D and MDA-MB231 breast cancer cells via modulating mTOR cell signaling pathway.

---

**Keywords:** Aloin, Doxorubicin, Breast cancer cells, Autophagy, Transmission electron microscopy, Confocal fluorescence microscopy, Flow cytometry and Western blotting.

## List of Abbreviations

<b>AVOs</b>	Acid Vesicular Organelles
<b>AMPK</b>	Adenosine monophosphate-activated kinase
<b>AL</b>	Aloin
<b>ATCC</b>	American Type Culture Collection
<b>AO</b>	Acridne orange
<b>ATG</b>	Autophagy related genes
<b>BRCA</b>	Breast cancer gene
<b>ECM</b>	Extracellular matrix
<b>CMA</b>	Chaperone-mediated autophagy
<b>JNK</b>	C-jun N-terminal kinase
<b>CLSM</b>	Confocal laser scanning microscopy
<b>DARK</b>	Death-associated protein kinase 1
<b>DMSO</b>	Dimethyl sulfoxide
<b>Dox</b>	Doxorubicin
<b>ER</b>	Estrogen receptor
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>HER2</b>	Human epidermal growth factor receptor 2
<b>EREs</b>	Estrogen receptor elements
<b>4EBP1</b>	Eukaryotic initiation factor 4E binding protein
<b>ERK</b>	Extracellular signal-regulated kinase
<b>FACS</b>	Fluorescence activated cell sorting
<b>FBS</b>	Fetal bovine serum
<b>FOXO1</b>	Forkhead box O protein O1
<b>GFs</b>	Growth factors

## List of Abbreviations (Cont.)

<b>NSCLC</b>	Human non-small cell lung carcinoma
<b>Hsc70</b>	Heat shock protein
<b>HUVECs</b>	Human umbilical vascular endothelial cells
<b>IC50</b>	Inhibitory concentration that kill 50%
<b>ICF</b>	Inhibition of colony formation
<b>IRS1</b>	Insulin receptor substrate 1
<b>IRS2</b>	Insulin receptor substrate 2
<b>LC3B</b>	Microtubule-associated proteins 1A/1B light chain 3B
<b>mTORC1</b>	Mammalian target of rapamycin complex 1
<b>MTD</b>	Maximum tolerated dose
<b>MDR</b>	Multidrug resistance
<b>MAPK</b>	mitogen-activated protein kinase
<b>MTT</b>	3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide
<b>NRF2</b>	Nuclear factor erythroid-2–like 2
<b>SQSTM1/p62</b>	Sequestosome-1 (ubiquitin-binding protein p62)
<b>PDK1</b>	Pyruvate dehydrogenase kinase 1
<b>P-gp</b>	P-glycoprotein
<b>PCD</b>	Programed cell death
<b>PTEN</b>	Phosphatase and tensin homolog
<b>PBS</b>	Phosphate-buffered saline
<b>PIP3</b>	Phosphatidylinositol 3, 4, 5 trisphosphate
<b>PI3K</b>	Phosphoinositol-3-kinase
<b>PARP</b>	Poly ADP-ribose polymerase
<b>PR</b>	Progesterone receptor
<b>PKB/AKT</b>	Protein kinase B

### List of Abbreviations (Cont.)

<b>ROS</b>	Reactive oxygen species
<b>RTKs</b>	Receptor tyrosine kinases
<b>RPMI</b>	Complete RPMI-1640 medium
<b>siRNA</b>	small interfering RNA
<b>SPF</b>	S-phase fraction
<b>STAT</b>	Signal transducers and activators of transcription
<b>TNF-<math>\alpha</math></b>	Tumor necrosis factor alpha
<b>Topo II</b>	Topoisomerase II
<b>TEM</b>	Transmission Electron Microscopy
<b>TNBC</b>	Triple-negative breast cancers
<b>TSC</b>	Tuberous sclerosis complex
<b>ULK1</b>	51-like serine threonine kinase complex
<b>WHO</b>	World health organization

## List of Tables

Table No.	Title	Page
4.1	Effect of 24h exposure regimen to multiple concentrations of aloin on the percentage of cell viability of T47D cells.	101
4.2	Effect of 72h exposure regimen to multiple concentrations of aloin and doxorubicin on the percentage of cell viability of T47D cells.	103
4.3	Effect of 24h exposure regimen to multiple concentrations of aloin and doxorubicin on the percentage of cell viability of MDA-MB 231cells.	107
4.4	Effect of 72h exposure regimen to multiple concentrations of aloin and doxorubicin on the percentage of cell viability of MDA-MB231cells.	109
4.5	<i>In vitro</i> sensitivity of T47D cells to aloin and doxorubicin.	117
4.6	<i>In vitro</i> sensitivity of MDA-MB231 cells to aloin and doxorubicin.	119
4.7	Flow cytometric analysis of acridine orange positive T47D cells (%).	137
4.8	Flow cytometric analysis of acridine orange positive MDA- MB231 cells (%) after treatment with aloin or doxorubicin for 72h.	140

## List of Figures

<b>Fig. No.</b>	<b>Title</b>	<b>Page</b>
2.1	ER $\alpha$ acting as a transcription factor	13
2.2	Description of the process of autophagy	15
2.3	Different types of autophagy	17
2.4	Autophagy substrates (non-selective or selective forms of autophagy)	19
2.5	The autophagy process	24
2.6	Molecular composition and upstream regulators of mTORC1 and mTORC2	27
2.7	Regulation of ULK1 complex by mTORC1	29
2.8	Regulation of various steps of autophagy by mTORC1	30
2.9	Autophagy is induced by deprivation of nutrients, hormones, and energy	35
2.10	Autophagy regulation in response to stress	36
2.11	The two facets of autophagy in cancer	42
2.12	Structure of doxorubicin	43
2.13	The potential mechanisms of doxorubicin-mediated cell death	45
2.14	Structure of the doxorubicin-DNA	47
2.15	Proposed mechanisms of ROS formation by anthracyclines	50
2.16	A cross section illustration of Aloe leaf	54
2.17	Structure of aloin A, aloin B and aloemodin	55
3.1	Standard curve for bovine serum albumin	89