

**“Study on the effects of Naringin and  
Clozapine combination in an experimental  
model of Schizophrenia in rats“**

*Thesis presented by*

*Mina Youssif George Youssif*

B.Sc. in pharmaceutical sciences and drug design (BPSDD),  
Ain Shams University (2013).

M.Sc in Pharmacology and Toxicology,  
Ain Shams University (2016).

Assistant lecturer of Pharmacology and Toxicology,  
Faculty of Pharmacy, Ain Shams University.

**Submitted for partial fulfillment of Ph.D. Degree in  
Pharmaceutical Sciences  
(Pharmacology and Toxicology)**

**Under the supervision of**

**Dr./ Ebtehal El-demerdash Zaki**

Professor of Pharmacology and Toxicology,  
Faculty of Pharmacy, Ain Shams University.

**Dr./ Marianne George Tadros**

Professor of Pharmacology and Toxicology,  
Faculty of Pharmacy, Ain Shams University.

**Dr./ Ahmed Esmat Abdel-razik**

Associate Professor of Pharmacology and Toxicology,  
Faculty of Pharmacy, Ain Shams University.

**Dr./ Esther Tharwat Menze**

Associate Professor of Pharmacology and Toxicology,  
Faculty of Pharmacy, Ain Shams University.

**Faculty of Pharmacy**

**Ain Shams University (2020)**

*First and foremost, no words can be ever said expressing my deep thanks to **GOD** for helping, supporting, encouraging, providing me the ability to complete this work and blessing me with generous professors while carrying out my research.*

*I would like to express my great appreciation and thanks to **Prof. Ebtehal El-demerash Zaki**, Professor of Pharmacology and Toxicology, Head of department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, who has helped me to make this work possible by her great effort, support and endless help from the scratch start till the end. I would like also to thank her for the great effort she did towards our department.*

*Most heartfelt thanks are due to **Prof. Marianne George Tadros**, Professor of Pharmacology and Toxicology, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, for her support, effort, encouragement, guidance and indispensable help. Her precious advices were always pushing me forward.*

*I would like to express my appreciation and gratitude to **Dr. Ahmed Esmat Abdel-razik**, Associate professor of Pharmacology and Toxicology, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, for his support and encouragement. I would like to thank him too for his continuous guidance and indispensable help throughout thesis writing and revision.*

---

Foremost, I would like to express my sincere gratitude to **Dr. Esther Tharwat Menze**, Associate professor of Pharmacology and Toxicology, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, for the continuous support of my PhD study, for her patience, motivation, enthusiasm, and immense knowledge. Her guidance helped me in all the time of research starting from the research question to writing of this thesis. In fact, she was more than a supervisor, she never stopped supporting and encouraging me.

It is my great pleasure to thank all members of Pharmacology and Toxicology Department, Faculty of Pharmacy, Ain Shams University, each one by name, for their continuous support and help. Really I enjoyed and still enjoying being a member of this department.

Finally, but of great importance, I wish to express my deep gratefulness and thanks to my father **Prof. Youssif George Youssif** and my brother **Eng. Michael Youssif George** for their support, continuous prayers for which my mere expression of thanks likewise does not suffice and for all what they endured to tolerate and uphold me in finishing this thesis.

*Mina Youssif George*

---

<b><i>Subject</i></b>	<b><i>Page NO.</i></b>
<b>Abstract</b>	<b>i</b>
<b>List of abbreviations</b>	<b>ii</b>
<b>List of figures</b>	<b>v</b>
<b>List of tables</b>	<b>x</b>
<b>Introduction</b>	<b>1</b>
I. Schizophrenia	1
A. Background	1
B. Epidemiology	1
C. Signs and symptoms	1
D. Pathophysiology	2
1. Neurotransmitters imbalance	3
2. Mitochondrial dysfunction and oxidative stress	6
3. Apoptotic signaling	6
E. Risk Factors	10
G. Current treatment options	11
II. Experimental Model	
A. Different experimental models	15
1. Amphetamine model	15
2. Knockout mice model	15
3. Ketamine model	16
III. GSK-3 $\beta$ /Akt and Wnt/ $\beta$ -catenin Pathways	18
IV. Naringin	24
A. Background	24
B. Structure and molecular weight	24
C. Pharmacokinetic properties	24
D. Pharmacodynamic properties	25
E. Adverse effects and toxicities	27
F. Clinical uses	27
<b>Aim of the work</b>	<b>29</b>

<b>Materials and Methods</b>	<b>30</b>
I. Experimental design	30
II. Materials	35
1. Drugs	35
2. Animals	35
3. Chemicals and reagents	36
4. Apparatuses	44
III. Methods	51
1. Behavioral assessments	51
2. Body weight	59
3. Blood glucose	59
4. Hematology test	59
5. Lipid profile	60
6. Neurotransmitters	64
7. Total protein determination	73
8. Oxidative stress markers	75
9. Caspase-3 activity	83
10. Immunohistochemical detection	88
11. Western blotting	90
12. Statistical analysis	94
<b>Results</b>	<b>95</b>
<b>Discussion</b>	<b>163</b>
<b>Summary and Conclusion</b>	<b>170</b>
<b>References</b>	<b>175</b>
<b>Arabic summary</b>	<b>I</b>

---

Schizophrenia is a chronic, disabling and one of the major neurological illnesses affecting nearly 1% of the global population. Currently available antipsychotic medications possess several side effects. The current research aimed at investigating potential therapeutic add-on benefit to enhance the effect and cease the side effects of clozapine anti-schizophrenic. In this study, naringin was given to Wistar rats at a dose of 100 mg/kg orally, alone or in combination with clozapine 5 mg/kg i.p. To induce schizophrenia, ketamine was administered at a dose of 25 mg/kg i.p. for 14 consecutive days. Ketamine induced positive, negative and cognitive schizophrenia symptoms together with neurotransmitters' imbalance. In addition, ketamine administration caused significant glutathione depletion, lipid peroxidation and reduction in catalase activity. Naringin and/or clozapine treatment significantly attenuated ketamine-induced schizophrenic symptoms and oxidative injury. Additionally, ketamine provoked apoptosis via increasing Bax/Bcl2 expression, caspase-3 activity, and cytochrome-c and Akt protein expression while naringin/clozapine treatment significantly inhibited this apoptotic effect. Moreover, naringin activated the neurodevelopmental wnt/ $\beta$ -catenin signaling pathway evidenced by increasing pGSK-3 $\beta$  and reducing  $\beta$ -catenin protein expression. Furthermore, naringin was able to reduce main clozapine side effects including agranulocytosis, dyslipidemia, weight gain and hyperglycemia when added to clozapine treatment. Collectively, these findings may suggest that naringin possesses a potential therapeutic add-on effect against ketamine-induced schizophrenia.

**Keywords:** Naringin; ketamine-induced schizophrenia; clozapine side effects; antioxidant; apoptosis; Wnt/ $\beta$ -catenin.

## *List of Abbreviations*

<b>4-AAP</b>	4-amino-antipyrine
<b>ACh</b>	Acetylcholine
<b>AChE</b>	Acetylcholinesterase
<b>Akt</b>	Protein Kinase B
<b>AMPA</b>	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
<b>APC</b>	Adenomatous Polyposis Coli
<b>BAX</b>	Bcl-2 Associated X
<b>BCA</b>	Bicinchoninic acid
<b>Bcl-2</b>	B-cell lymphoma-2
<b>BDNF</b>	Brain-derived Neurotrophic Factor
<b>BVA</b>	Bovine Serum Albumin
<b>cAMP</b>	Cyclic Adenosine Monophosphate
<b>CAT</b>	Catalase
<b>CE</b>	Cholesterol Esterase
<b>CK1<math>\alpha</math></b>	Casein Kinase 1 $\alpha$
<b>CMC</b>	Carboxy Methyl cellulose
<b>CO</b>	Cholesterol Oxidase
<b>CPT-1</b>	Carnitine Palmitoyltransferase-1
<b>D2 Receptots</b>	Dopamine Receptors
<b>DAT</b>	Dopamine Transporter
<b>DCHBS</b>	3,5-dichloro-2-hydroxy benzene sulfonic acid
<b>DTNB</b>	5,5' dithiobis (2-nitrobenzoic acid)
<b>DTT</b>	Dithiotheritol
<b>Dvl</b>	Disheveled
<b>ECD</b>	Electrochemical Detector
<b>FZ</b>	Frizzled
<b>GABA</b>	Gamma Amino Barbituric Acid
<b>GluR</b>	Glutamate Receptors
<b>GlyT1</b>	Glycine Transporter 1
<b>GP<sub>x</sub></b>	Glutathione Peroxidase
<b>GR</b>	Glutathione Reductase
<b>GSH</b>	Reduced Glutathione
<b>GSK-3<math>\beta</math></b>	Glycogen Synthase Kinase -3 $\beta$
<b>H<sub>2</sub>O<sub>2</sub></b>	Hydrogen peroxide

## *List of Abbreviations*

<b>HRP</b>	Horseradish Peroxidase
<b>IAP</b>	Inhibitors of Apoptosis Protein
<b>IL-6</b>	Interleukin-6
<b>iNOS</b>	Inducible Nitric Oxide Synthase
<b>IκB</b>	Inhibitor of kappa B
<b>K<sub>2</sub>HPO<sub>4</sub></b>	Dipotassium hydrogen phosphate
<b>KH<sub>2</sub>PO<sub>4</sub></b>	Anhydrous potassium dihydrogen phosphate
<b>Lrp5/6</b>	Low Density Lipoprotein 5/6
<b>mAChRs</b>	Muscarinic Acetylcholine Receptors
<b>MAO</b>	Monoamine Oxidase
<b>MDA</b>	Malondialdehyde
<b>mTORC2</b>	mammalian target of rapamycin complex 2
<b>nAChRs</b>	Nicotinic Acetylcholine Receptors
<b>NF-κB</b>	Nuclear Factor kappa B
<b>NMDA</b>	N-Methyl D- Aspartate
<b>OD</b>	Optical Density
<b>PDK1</b>	3-phosphoinootide-dependent protein kinase 1
<b>PI3K</b>	Phosphatidylinositol 3-kinase
<b>PI3P</b>	Phosphatidylinositol 3,4,5 trisphosphate
<b>pNA</b>	p-nitroaniline
<b>PPI</b>	PrePulse Inhibition
<b>PVDF</b>	Polyvinylidene Fluoride
<b>ROS</b>	Reactive Oxygen Species
<b>SDS</b>	Sodium dodecyl sulfate
<b>SDS-PAGE</b>	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
<b>SP</b>	Sucrose Preference
<b>SREBP</b>	Sterol Regulatory Element-binding Proteins
<b>SOD</b>	Superoxide Dismutase
<b>TBA</b>	Thiobarbituric acid
<b>TBS</b>	Tris Buffered Saline
<b>TBARS</b>	Thiobarbituric Acid Reactive Substances
<b>TBS</b>	Tris Buffered Saline
<b>TCA</b>	Trichloroacetic Acid
<b>TCF/LEF</b>	T-cell-specific transcription factor/lymphoid

## *List of Abbreviations*

---

	enhancer-binding factor
<b>TEMED</b>	Tetramethylethylenediamine
<b>TGs</b>	Triglycerides
<b>TNF-<math>\alpha</math></b>	Tumor Necrosis Factor- $\alpha$
<b>UCP-2</b>	uncoupling protein-2

<b>Figure No.</b>	<b>Figure title</b>	<b>Page No.</b>
i	Distribution of population with schizophrenia worldwide	2
ii	Circuitry of dopamine, glutamate, and GABA neurotransmitters in healthy and schizophrenic brains	5
iii	Intrinsic and extrinsic apoptotic signaling pathways in schizophrenia	9
iv	Effect of genetic variations and age on schizophrenia incidence	10
v	Relation between AKT and GSK-3 $\beta$ (Phosphorylation and dephosphorylation)	20
vi	Wnt signaling pathways	22
vii	Canonical wnt signaling pathway	23
viii	Structure of (i) flavonoids, (ii) Naringenin and (iii) Naringin	24

<b>Figure No.</b>	<b>Figure title</b>	<b>Page No.</b>
1	Study design	31
2	Experimental design including treatments and behavioral experiments performed.	32
3	Locomotor activity detector	44
4	Startle reflex apparatus	45
5	Step-through passive avoidance apparatus	46
6	Morris water maze apparatus design	47
7	BioRad mini protein electrophoresis separation unit and electrophoresis power supply	48
8	BioRad Trans-Blot Turbo apparatus	49
9	Standard calibration curve for glutamate content	66
10	Standard calibration curve for GABA content	67
11	Standard calibration curve for dopamine content	68
12	Standard calibration curve for serotonin content	69
13	Standard calibration curve of reduced glutathione level	79
14	Standard calibration curve of MDA level	82
15	Standard calibration curve for pNA	87
16	Effect of naringin/clozapine treatment on locomotor activity against ketamine-induced schizophrenia in rats	104
17	Effect of naringin/clozapine treatment on startle response training against ketamine-induced schizophrenia in rats	105
18	Effect of naringin/clozapine treatment on %PPI training against ketamine-induced schizophrenia in rats	106

19	Effect of naringin/clozapine treatment on active interaction time and latency for first interaction in social interaction testing against ketamine-induced schizophrenia in rats	107
20	Effect of naringin/clozapine treatment on sucrose preference training and testing against ketamine-induced schizophrenia in rats	108
21	Effect of naringin/clozapine treatment on step-through passive avoidance against ketamine-induced schizophrenia in rats	110
22	Effect of naringin/clozapine treatment on Moris water maze training and testing against ketamine-induced schizophrenia in rats	111
23	Effect of treatment with naringin/clozapine on prefrontal cortical glutamate content against ketamine-induced schizophrenia in rats	118
24	Effect of treatment with naringin/clozapine on prefrontal cortical GABA against ketamine-induced schizophrenia in rats	119
25	Effect of treatment with naringin/clozapine on striatal dopamine against ketamine-induced schizophrenia in rats	120
26	Effect of treatment with naringin/clozapine on striatal serotonin content against ketamine-induced schizophrenia in rats	121
27	Effect of treatment with naringin/clozapine on striatal, prefrontal cortical and hippocampal AChE activity against ketamine-induced schizophrenia in rats	124
28	Effects of ketamine and naringin/clozapine on striatal, prefrontal cortical and hippocampal catalase in schizophrenia-induced rats	130

29	Effects of ketamine and naringin/clozapine on striatal, prefrontal cortical and hippocampal reduced glutathione in schizophrenia-induced rats	131
30	Effects of ketamine and naringin/clozapine on striatal, prefrontal cortical and hippocampal malondialdehyde in schizophrenia-induced rats	132
31	Effects of ketamine and naringin/clozapine on striatal, prefrontal cortical and hippocampal caspase-3 activity in schizophrenia-induced rats	135
32	Expression of the pro-apoptotic protein Bax in the striata, prefrontal cortices and hippocampi by immunohistochemical staining	138
33	Expression of the anti-apoptotic protein Bcl2 in the striata, prefrontal cortices and hippocampi by immunohistochemical staining	139
34	Expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl2 in the striata, prefrontal cortices and hippocampi by immunohistochemical staining	140
35	Expression of cytochrome-c (cyt-c) in the striata, prefrontal cortices and hippocampi by immunohistochemical staining	143
36	Expression of cytochrome-c (cyt-c) in the striata, prefrontal cortices and hippocampi by immunohistochemical staining	144
37	Western blot analysis of the striatal, prefrontal cortical and hippocampal total and phosphorylated Akt	147
38	Western blot analysis of the striatal, prefrontal cortical and hippocampal total and phosphorylated GSK-3 $\beta$	151
39	Western blot analysis of the striatal, prefrontal cortical and hippocampal total and phosphorylated $\beta$ -catenin	152

40	Effect of naringin/clozapine treatment on body weight and percent change in body weight of rats.	157
41	Effect of naringin/clozapine treatment on percent change in blood glucose in rats	159
42	Effect of naringin/clozapine treatment on serum total cholesterol and triglycerides in rats	160
43	Effect of naringin/clozapine treatment on total leukocyte count in rats.	162

<b>Table No.</b>	<b>Table title</b>	<b>Page No.</b>
<b>Materials and Methods</b>		
1	Chemicals used, their source and use	36
2	Antibodies used, their source and use	39
<b>Results</b>		
3	Effect of treatment with clozapine/naringin combination on sucrose consumption in rats	99
4	Effect of treatment with clozapine/naringin on behavioral tests in ketamine-induced schizophrenia in rats	102
5	Effect of treatment with clozapine/naringin on neurotransmitters in ketamine-induced schizophrenia in rats	116
6	Effect of treatment with clozapine/naringin on acetylcholinesterase activity in ketamine-induced schizophrenia in rats	123
7	Effect of treatment with clozapine/naringin on oxidative stress markers in ketamine-treated rats	128
8	Effect of treatment with clozapine/naringin on caspase-3 activity in an experimental model of ketamine-induced schizophrenia in rats	134
9	Effect of treatment with clozapine/naringin on Bax/Bcl-2 in an experimental model of ketamine-induced schizophrenia in rats	137
10	Effect of treatment with clozapine/naringin on cytochrome-c in an experimental model of ketamine-induced schizophrenia in rats	142
11	Effect of treatment with clozapine/naringin on phosphorylated and total Akt protein expression in an experimental model of ketamine-induced schizophrenia in rats	146
12	Effect of treatment with clozapine/naringin on phosphorylated and total GSK-3 $\beta$ protein expression in an experimental model of ketamine-induced schizophrenia in rats	149
13	Effect of treatment with clozapine/naringin on phosphorylated and total GSK-3 $\beta$ protein expression in an experimental model of ketamine-induced schizophrenia in rats	150
14	Effect of treatment with clozapine/naringin combination on clozapine-induced side effects in rats	155