Mohamed Ahmed Zaki, Ph.D.

Department of Pharmacognosy, Faculty of Pharmacy, Beni Suef University, Beni Suef, Egypt.
Tel: 0020822362211 (Office) 00201102424231 (Mobile) Fax : 0020822317953
E-mail : m_abuzaki@yahoo.com
mohamed.zaki@pharm.bsu.edu.eg

General Summary of Ph. D. thesis

Chemical Studies and Bioactivities of *Eugenia rigida* DC.

A Thesis submitted by

Mohamed Ahmed Zaki Mohamed (M.Sc.)

In Fulfillment of PhD Degree in
Pharmaceutical Sciences
(Pharmacognosy)

Under Supervision of

Prof. Dr. Omar Abdel Salam Rashwan
Deceased Professor of Pharmacognosy
Cairo University

Prof. Dr. Mona H. Hetta

Professor of Pharmacognosy
Head of Pharmacognosy Department
Faculty of Pharmacy, Beni-Suef University

Dr. Rabab Mohammed

Associate Professor
Pharmacognosy Department
Faculty of Pharmacy, Beni-Suef University

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Faculty of Pharmacy

Beni-Suef University

Mohamed Ahmed Zaki, Ph.D.
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Family **Myrtaceae** is economically fairly important. Its plants are grown as ornamentals; others produce edible fruits rich in vitamins and are of medicinal value. The genus *Eugenia* is the largest in the Myrtaceae family with up to 2,000 species distributed from the south of Mexico, Cuba and the Antilles to Uruguay and Argentina, with a small number of species in Africa. *Eugenia rigida* DC. is a shrub or small tree containing characteristic brown and green fruits, which turn black when mature. It was used traditionally in Argentina for leukemia. This plant has not previously been subjected to either chemical or biological investigations, but the genus exhibits a wide array of secondary metabolites.

The work is composed of two parts

Part I: Chemical study

Part II: Biological study

Part I: Chemical study includes three chapters

Chapter 1: Phytochemical study

Chemical investigation of the constituents of *Eugenia rigida* DC. leaves afforded 16 compounds; among which five compounds were new in nature; and one of which **(Er-5)** was previously synthesized but for the first time from nature. The compounds are: two new flavanones; 5,7-dihydroxy-6-formylflavanone **(Er-1)** and 5,7-dihydroxy-8-formylflavanone **(Er-2)**; two stilbenes *Z*-3,4,3′,5′-tetramethoxystilbene **(Er-5)** and *E*-3,4,3′,5′-tetramethoxystilbene **(Er-6),** which

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were found to be positional isomers to each other, one new dihydrochalcone; 2′-methoxy- 3′-methyl - 4′,6′- dihydroxydihydrochalcone **(Er-3)**, and one chalcone: 2′,6′-dihydroxy-4′-methoxy-5′-methylchalcone **(Er-14).** In addition to ten known compounds were previously isolated from other plants and new in the species**:** **3,4′,5-trimethoxy-*trans*-**stilbene **(Er-4),** betulinal **(Er-7),** α-betulinic acid **(Er-8),** betulonic acid **(Er-9),** β-betulinic acid **(Er-10),** comptonin **(Er-11),** barbinervic acid **(Er-12),** diospyric acid **(Er-13),** 3′-formyl-2′,4′,6′-trihydroxy-5′-methyldihydrochalcone **(Er-15)** and 3′-formyl-2′,4′,6′-trihydroxydihydrochalcone **(Er-16).**

Compounds isolated from *Eugenia rigida* DC. Leaves

Code	Nature	IUPAC name	Common name	Isolation
Er-1	natural	5,7-dihydroxy-6-formylflavanone	6-formylpinocembrin	new in nature
Er-2	natural	5,7-dihydroxy-8-formylflavanone	8-formylpinocembrin	new in nature
Er-3	natural	2′-methoxy- 3′-methyl - 4′,6′-dihydroxydihydrochalcone	-	new in nature
Er-4	natural	3,4′,5-trimethoxy-*trans*-stilbene	-	New in species
Er-5	natural	*Z*-3,4,3′,5′-tetramethoxystilbene	-	New in nature but previously synthesized
Er-6	natural	*E*-3,4,3′,5′-tetramethoxystilbene	-	New in species
Er-7	natural	3β-Hydroxy-lup-20(29)-en-28-al	Betulinal	New in species
Er-8	natural	3α-Hydroxy-lup-20(29)-en-28-oic acid	α- Betulinic acid	New in species
Er-9	natural	Lup-20(29)-en-3-on-28-oic acid	Betulonic acid	New in species

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Er-10	natural	3β-Hydroxy-lup-20(29)-en-28-oic acid	β- Betulinic acid	New in species
Er-11	natural	7-Hydroxy-5-mthoxy-6-methylflavanone	Comptonin	New in species
Er-12	natural	3α,19-dhydroxyurs-12-en-24,28-dicarboxyliic acid	Diospyric acid	New in species
Er-13	natural	3α,19,24-trihydroxyurs-12-en-28-oic acid	Barbinervic acid	New in species
Er-14	natural	2′,6′-dihydroxy-4′-methoxy-5′-methylchalcone	-	New in nature
Er-15	natural	3′-formyl-2′,4′,6′-trihydroxy-5′-methyldihydrochalcone	-	New in species
Er-16	natural	3′-formyl-2′,4′,6′-trihydroxydihydrochalcone	-	New in species
5S	synthetic	(S) 5,7-dihydroxy-6-formylflavanone	(S) 6-formylpinocembrin	-
5R	synthetic	(R) 5,7-dihydroxy-6-formylflavanone	(R) 6-formylpinocembrin	-
6S	synthetic	(S) 5,7-dihydroxy-8-formylflavanone	(S) 8-formylpinocembrin	-
6R	synthetic	(R) 5,7-dihydroxy-8-formylflavanone	(R) 8-formylpinocembrin	-
7	synthetic	2′,4′,6′-trihydroxydihydrochalcone	-	-
7a	synthetic	3′-formyl-2′,4′,6′-trihydroxydihydrochalcone	-	-
8	synthetic	2′,4′,6′-trihydroxychalcone	-	-
8a	synthetic	3′-formyl-2′,4′,6′-trihydroxychalcone	-	-
1a	synthetic	6-formyl-5,7-dihydroxyflavone	6-formylchrysin	-
1b	synthetic	8-formyl-5,7-dihydroxyflavone	8-formylchrysin	-

Chapter 2: Chemical modification of 18 flavonoid derivatives

Each of the two flavanone derivatives 6-formylpinocembrin **(Er-1)** and 8-formylpinocembrin **(Er-2)**, flavanone derivatives, each has a chiral center at C_2,

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and was isolated during the phytochemical study of *Eugenia rigida* DC. leaves as a racemic mixture. They exhibited antifungal activity, herein, the synthesis of their enantiomers *(R)* and *(S)* of both **Er-1** and **Er-2** along with other products **(**2′,4′,6′-trihydroxydihydrochalcone **(7),** 3′-formyl-2′,4′,6′-trihydroxydihydrochalcone **(7a),** 6-formylchrysin **(1a),** 8-formylchrysin **(1b),** 2′,4′,6′-trihydroxychalcone **(8),** 3′-formyl-2′,4′,6′-trihydroxychalcone **(8a)** has been achieved.

Chapter 3: Analytical study

- ***Analysis of photoisomerization of E isomer (Er-6) to Z isomer (Er-5) of 3,4,3′,5′-tetramethoxystilbene***

In order to incresase the low yield of the sterically hindered *Z*-isomer of 3,4,3′,5′-tetramethoxystilbene (**Er-5**), an additional quantity of this compound was prepared from the more stable *E* isomer (**Er-6)** by photoisomerization at λ_{254} nm. A time dependent UV irradiation was carried out to study the conversion of *E*- (**Er-6)** to *Z*- (**Er-5**) isomers over a time range of 0 - 1000 min, where the yield of product **Er-5** was quantified by UHPLC/APCI-MS.

- ***Quantification of 10 standard compounds in Eugenia rigida DC. plant samples***

UPLC method was applied for the quantification of the ten selected compounds (**Er-1, Er-2, Er-4, Er-5, Er-6, Er-8, Er-9, Er-10, Er-14** and **Er-**

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17) using two types of detectors: UV and MS. Results indicated that **Er-6** *(E)*-3,3´,4´,5 tetramethoxystilbene is the major component in both *Euginia rigida* DC. leaves (4.98 µg/mg plant sample, methanol extract) and stems (0.35 µg/mg plant sample, methanol extract). The contents of the other compounds are in the range of (0.06-3.56 µg/mg leaves, methanol) and (0.05-0.35µg/mg stems, methanol). Compounds **Er-4**, **Er-5**, **Er-8**, and **Er-14** were not detected in the stems. The low recovery rate of **Er-8**, **Er-9** and **Er-10** might be due to the low solubility of these compounds in methanol.

Part II: Biological study

The isolated and synthetic compounds were subjected to screening for potential antimicrobial, cytotoxic and antioxidant activities.

- **Antimicrobial activity**

The new compounds **Er-1** and **Er-2** were isolated in minute amounts, showed high antifungal activity against *Candida glabrata*, therefore synthesis of certain derivatives, in addition to their isomers was carried out to be able to study their structure-activity relationship. The results revealed that both the addition of a formyl group affected the antifungal activity of the tested compounds.

- **Cytotoxic activity**

. The results showed cytotoxic activity of **Er-5** against HL-60, of KB, BT-459, HeLa and SK-MEL cell lines with IC_{50} values of 4.3, 4.3, 4.0, 3.6 and 4.3

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μM, respectively. Compound **Er-6** on the other hand, was weakly active against HL-60, HeLa and SK-MEL (IC_{50} 33.3, 8.0 and 60.0 μM) . In addition, **Er-4** was also weakly active as cytotoxic towards KB, BT-459, Hela and SK-MEL cells (IC_{50} 17.7, 15.6, 13.3 and 12.2 μM, respectively). Compound, **Er-5** was inactive against a noncancerous cell line (monkey kidney fibroblast; VERO) up to 33.3 μM, thus exhibiting selectivity toward the tumor cells.

Er-5 was more potent than **Er-6** as inhibitor of Stat3, Smad2/3, myc, Ets, Notch, and Wnt signaling. Ap-1 and NF-κB signalings were inhibited by both compounds **Er-5** and **Er-6** similarly and neither of them inhibited E2F or Hedgehog pathway activation at the tested concentrations. Similarly, the activation of the apoptotic mediator FoxO was not observed with either compound. Compound **Er-5** was similar in potency to resveratrol for inhibiting signaling mediated by Stat3, Smad2/3, myc, Ets, and Notch while resveratrol was more potent for NF-κB and Hedgehog. Compound **Er-4** was also tested and found to be inactive up to 100 μM. None of the compounds at the tested concentrations inhibited luciferase expression driven by the minimal thymidine kinase promoter (pTK), indicating the lack of general cytotoxicity or luciferase enzyme inhibition. Activity of synthetic compounds revealed that both presence and position of the formyl group in the flavanone compound is crucial for the activity, but there is no difference in activity between the different enantiomers.

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- **Antioxidant activity:**

The inhibition of intercellular generation of reactive oxygen and nitrite species was measured to determine the potentials of the test compounds against oxidative and inflammatory stress in the cellular environment. The results obtained showed that compound **Er-6** (the *E*-isomer of 3,4,3′,5′-tetramethoxystilbene) exhibited an inhibition of 50% in ROS generation at 33.3µM in PMA-induced HL-60 cells, while the *Z*-isomer did not exhibit any effect at the dose tested (100 µM), which might be due to the enhanced conjugation of aromatic ring in *E*, compared to *Z*, olefinic bond. However, they did not show any effect on *i*NOS activity in LPS induced macrophages (RAW264.7) up to 33.3 µM.