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*Original Article*

# Myricitrin from *Physalis pubescens* L. leaves and frankincense decrease resistance of MCF-7 cells and ameliorate efficacy of epirubicin

[La miricitrina de las hojas *de Physalis pubescens* L. y la resina *frankincense* disminuyen la resistencia de las células MCF-7 y mejoran la eficacia de la epirrubicina]

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**Abstract**

*Context*: It was found that flavonoids and frankincense exert anti-cancer effect through their antioxidant and anti-inflammatory activities.

*Aims*: To evaluate the cytotoxic effect against MCF-7 cells of flavonoids isolated from *Physalis pubescens* L., frankincense ethanol extract and the combined therapy with epirubicin to reduce the resistance and the side effects.

*Methods*: MTT assay against MCF-7 was carried out for rutin, quercitrin, myricitrin and frankincense. The compound or extract with the best anti-cancer effect was tested against WI-38 cells. 50% inhibitory concentration ($IC_{50}$) of the different treatments and the combined therapy with epirubicin against MCF-7 was determined. Assessment the effect on expression of ABCB1, TGF-β1 and ATG7 genes was done by RT-qPCR.

*Results*: Isolation and identification of myricitrin from leaves of *Physalis pubescens* L. was carried out for the first time. $IC_{50}$ (µg/mL) regarding MCF-7 cells was of epirubicin (0.8 ± 0.052), rutin (350.16 ± 1.241), quercitrin (259.6 ± 1.45), myricitrin (114.0 ± 0.517), frankincense ethanol extract (86.8 ± 0.91), combined epirubicin + myricitrin (EM) (0.37 ± 0.087), combined epirubicin + frankincense (EF) (0.50 ± 0.1732). The $IC_{50}$ µg/mL regarding WI-38 cells was of epirubicin (1.26 ± 0.0057), myricitrin (462.0 ± 1.062) and frankincense (299.5 ± 1.32). All assays were done at 48 h time interval. Myricitrin and EM reduced ABCB1 expression and upregulated expression of TGF-β1 and ATG7 genes. Frankincense and EF downregulated expression of ABCB1, TGF-β1 and ATG7 genes.

*Conclusions*: Myricitrin and frankincense would be a promising adjuvant therapy to improve epirubicin anticancer activity with minimal adverse effect.

*Keywords*: ABCB1; ATG7; flavonoids; MTT assay; TGF-β1; WI-38.

**Resumen**

*Contexto*: Se encontró que los flavonoides y la resina frankincense ejercen un efecto anticancerígeno a través de sus actividades antioxidantes y antiinflamatorias.

*Objetivos*: Evaluar el efecto citotóxico frente a células MCF-7 de flavonoides aislados de *Physalis pubescens* L., extracto etanólico de resina frankincense y la terapia combinada con epirrubicina para reducir las resistencias y los efectos secundarios.

*Métodos:* Se realizó ensayo MTT contra MCF-7 para rutina, quercitrina, miricitrina e incienso. El compuesto o extracto con el mejor efecto anticancerígeno se probó contra células WI-38. Se determinó la concentración inhibidora del 50% ($IC_{50}$) de diferentes tratamientos y la terapia combinada con epirubicina contra MCF-7. La evaluación del efecto sobre la expresión de los genes ABCB1, TGF-β1 y ATG7 se realizó mediante RT-qPCR.

*Resultados*: Se realizó por primera vez el aislamiento e identificación de miricitrina de hojas de *Physalis pubescens* L. La $CI_{50}$ (µg/mL) con respecto a las células MCF-7 fue de epirrubicina (0,8 ± 0,052), rutina (350,16 ± 1,241), quercitrina (259,6 ± 1,45), miricitrina (114.0 ± 0,517), extracto de etanólico de resina frankincense (86,8 ± 0,910), epirrubicina combinada + miricitrina (EM) (0,37 ± 0,087), epirrubicina + incienso (EF) combinados (0,50 ± 0,1732). La $IC_{50}$ (µg/mL) con respecto a las células WI-38 fue de epirrubicina (1,26 ± 0,0057), miricitrina (462,0 ± 1,062) extracto de resina frankincense (299,5 ± 1,32). Todos los ensayos se realizaron en un intervalo de tiempo de 48 h. Myricitrin y EM redujeron la expresión de ABCB1 y aumentaron la expresión de los genes TGF-β1 y ATG7. El incienso y la EF redujeron la expresión de los genes ABCB1, TGF-β1 y ATG7.

*Conclusiones*: La miricitrina y el extracto frankincense serían una terapia adyuvante prometedora para mejorar la actividad anticancerosa de la epirrubicina con un efecto adverso mínimo.

*Palabras Clave*: ABCB1; ATG7; ensayo MTT; flavonoides; TGF-β1; WI-38.

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## INTRODUCTION

Natural products from plants always represent an endless source for anticancer drug discovery, for example not for counting, podophyllotoxin from *Podophyllum* species, alkaloids from *Vinca* species and colchicine from *Colchicum autumnale*. (Hosseini and Ghorbani, 2015).

Flavonoids among the different natural plant products were extensively investigated for anticancer activity against different cell lines. Their effectiveness was confirmed in many studies. Flavonoids include compounds of phenylalanine derivatives, flavonol glycosides are subclass of flavonoids which are represented in this study by quercitrin, rutin and myricitrin. The pronounced antiproliferative effects of flavonoids have triggered biological studies to figure out their molecular mechanism of action against tumor cells (Sak, 2014).

Frankincense or olibanum is an aromatic oleo-gum resin. It was used from the ancient time in treatment of many ailments (Raffaelli et al., 2006). The volatile oil fraction is composed of terpenoids and traces of ethers, fatty esters and fatty alcohols. The oleo-resin is obtained from different species of *Boswellia* family *Burseraceae*, which are found in Africa, Arabian Peninsula, and the Indian subcontinent (DeCarlo et al., 2019). Resin fraction is composed mainly of rich terpenoids, among, which are the medicinally important group of boswellic acids (BAs) and their derivatives (Shah et al., 2009). *Boswellia* oleo-gum resin has different biological effects as antioxidative, anti-inflamma-tory (Siddiqui, 2011) and anti-cancer effects (Ahmed et al., 2014; Efferth and Oesch, 2020).

The most common type of cancer in women is the breast cancer, which is a heterogeneous disease on the molecular level. It is curable in 70-80% of patients with early- stage, non- metastatic disease. Breast cancer is a global problem, which led to major emphasis on how much we need to discover new chemotherapeutic drugs with minimum side effects (Harbeck et al., 2019). Anthracyclines are the cornerstone of chemotherapy used in both early and metastatic breast cancer because they offer a survival advantage when compared to other non-anthracycline containing adjuvant regimens. Doxorubicin and epirubicin are the most commonly used anthracyclines in clinical practice. They are essential in most of combination chemotherapy plans especially for early- stage cancer (Gianni et al., 2009; Khasraw et al., 2012). Despite the magnificent progress in chemotherapy in the last decades, tumor resistance against chemotherapeutic agents increased by time. The effective dose of anticancer drug need to be increased to overcome the tumor resistance with increasing risk of side effects and toxicity to normal tissue cells (Liang et al., 2010).

Epirubicin and other anthracycline drugs unfortunately can cause different side effects. The most clinically significant adverse reaction could be acute or delayed cardiotoxicity (Morris and Hudis, 2010).

The current study was conducted to investigate anti breast cancer effect of rutin, quercitrin (El-Sherbeni and Al-Ashmawy, 2020) and myricitrin isolated from *Physalis pubescens* L. leaves, family *Solanaceae* (Singh, 2010), on MCF-7, as well as frankincense oleo-gum resin ethanol extract. Frankincense resin was obtained from *Boswellia dalzielii* Hutch. family *Burseraceae*, which inhabits Nigeria and other countries from Chad to Mali (The International Plant Names Index, 2021). MTT viable cell assay was carried out to determine the most effective compound or extract through determination of $IC_{50}$. A combination between the most effective compound or extract and epirubicin was done to try to reduce the resistance of MCF-7 cells and the effective dose of epirubicin to lessen its cardiotoxicity and other side effects. The molecular mechanism of action could be deduced from expression of ABCB1, ATG7 and TGF-β1 genes by RT-qPCR.

## MATERIAL AND METHODS

### Plant and resin materials

Collection of *Physalis pubescens* L. leaves was carried out in July 2017 from Shibin el-Qanater

(30° 18' 47.88" N, 31° 19' 17.04" E) of Qalyubia governorate in Egypt. This plant was recognized by Prof. Dr. Mohammed Ibrahim Fotoh, professor of ornamental horticulture and landscape design. Frankincense resin (300 g) was purchased from an herbal store in May 2019 from Al Farwaniyah in Kuwait (29° 16' 39.00" N, 47° 57' 31.00" E), which was collected from *Boswellia dalzielii* Hutch. growing in Nigeria. The description of the trees was confirmed with that of literature of *Boswellia dalzielii* Hutch. (Jastor and The Herbarium Catalogue, 2021) Voucher specimens (PG00413) and (PG00416), respectively were deposited at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Tanta University.

### Cell lines and materials for MTT assay

The breast cancer cells (MCF-7) and the normal cell line (WI-38) were purchased from the Cell Culture Department of the Holding Company for Biological Products & Vaccines (Vacsera), Dokki-Giza, Egypt. Dulbecco's modified Eagle medium (DMEM), with glucose and L-glutamine (Lonza Verviers Sprl, Belgium), 10% fetal bovine serum (Sigma-Aldrich, USA), 1% of penicillin/ streptomycin/amphotericin B (Gibco, USA), ethanol 70% (BDH, England), trypsin (0.25%) (AMRESCO - USA), trypan blue solution 0.4% (Sigma-Aldrich, USA) and dimethyl sulfoxide (DMSO) (BDH, England), epirubicin hydrochloride (Epirubicin® vials, Mylan S.A.S., France), acidified isopropanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT solution 10×, Sigma-Aldrich, USA), sterilized saline solution (0.9%).

### Materials for RT-qPCR

RT-PCR primers used in this study were autophagy-related 7 or ATG7 (Cat. No. QT00008974), transforming growth factor beta 1 or TGF-β1 (Cat. No. QT00000728), ATP-binding cassette, subfamily B (MDR/TAP), member 1 or ABCB1 (Cat. No. QT00081928) and actin beta or ACTB (Cat. No. QT00095431) were all purchased from (QIAGEN, Germany). RNeasy® Mini Kit (QIAGEN, Germany) was used according to the manufacturer's protocol sheet. Complementary DNA (cDNA) was synthesized from the extracted RNA using QuantiTect® reverse transcription (QIAGEN, Germany). Synthesized cDNA was used as a template for amplification and quantification of ATG7, ABCB1, and TGF-β1 genes using QuantiTect® SYBR Green PCR Kit (QIAGEN, Germany).

### Materials for isolation of myricitrin

Methanol, methylene chloride, acetone (analytical grade, Fischer), ethanol 100% (Chem-Lab, Belgium), methanol and water (HPLC grade, Fischer) and deionized water. Column chromatography was performed on silica gel G 60 (70-230 mesh, Merck), ODS-C18 (Merck) and DIAION™ HP20SS (Mitsubishi Chemical, Japan). TLC was carried out using pre-coated TLC plates of silica gel 60 F254 (Merck) and silica gel 60 RP-18 F254 sheets (5 × 7.5 cm, Merck).

### Instruments

ELISA microplate reader (Bio-RAD micro plate reader, Japan) for MTT assay, UV spectra were recorded on Shimadzu UV/Vis spectrophotometer (UV-1800, Japan). Mass spectrum was obtained by Thermo Scientific TSQ Quantum Access MAX triple quadrupole system, USA. $^{1}$H- and $^{13}$C-NMR spectral data were carried out using Bruker High performance Digital FT-NMR spectrophotometer Avance III (400 MHz), Germany and $CD_3OD$ was used as solvent. Water deionization was done by Mirae ST Co., Ltd, Korea.

### Preparation of frankincense extract

Frankincense (300 g) was extracted with one liter of 100% ethanol for one week by cold maceration and evaporated using rotary evaporator to obtain 17.6 g of semisolid yellow residue.

### Isolation of myricitrin from *Physalis pubescens* L. leaves

Leaves of *P. pubescens* L. were air dried at room temperature and powdered to give 2 kg. Extraction was carried out by cold maceration for three times using 6 L of methanol 95% each time and evaporated under vacuum to obtain 226 g dry methanol extract residue (11.3% yield). Methanol dry residue (200 g) was suspended in aqueous

methanol and fractionation was done successively by four different solvents. First solvent was 4 L of petroleum ether to yield after evaporation 38 g, then 5 L of methylene chloride (44 g yield), then 6 L of ethyl acetate and 4 L of *n*-butanol to yield 55 and 53 g, respectively.

A suspension of *n*-butanol fraction (30 g) in deionized water was applied to mixed bed diaion (HP-20) column (100 g, 3.5 x 35 cm). Elution was started with 100% deionized water, then 50% methanol in deionized water, then 100% methanol. Fraction, which was eluted with 100% methanol was evaporated under reduced pressure to afford 4.5 g dry residue. The dry residue was chromatographed on silica gel column (160 g, 3.5 × 50 cm) using 100% methylene chloride then increase polarity gradually by adding methanol to methylene chloride.

Fraction eluted at (70:30:2.5) of methylene chloride:methanol:water was applied to another silica gel column (40 g, 2 × 20 cm) using methylene chloride and methanol as previously mentioned.

Fractions, which were collected at ratio of methylene chloride:methanol (85:15) were subjected to chromatography using reversed phase ODS-18 column (10 g, 1.5 × 6 cm), starting with water and decrease polarity with methanol, to afford 9 mg of pure substance in yellow powder form. This pure substance was obtained at 35% methanol in water.

**MTT cell viability assay**

MCF-7 breast carcinoma and WI-38 normal cell line were routinely cultured in a standard medium consisting of Dulbecco's modified Eagle medium (DMEM) with glucose and L-glutamine containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin/ amphotericin B and incubated at 37°C in a 5% $CO_2$ prior to use (Lanucara and Eyers, 2011). The medium was then replaced with fresh DMEM-10% FBS. The cells were maintained by subculturing them after arriving at an acceptable confluence. The cells were seeded into 96-well cell culture plates at a concentration of $1 \times 10^4$ cells/mL and incubated 24 h at standard condition to reach exponential growth. The cells were treated with different concentrations (listed in Table 1) of drugs (quercitrin, rutin, myricitrin and frankincense). At the end of the incubation time (48 h), the medium was removed and 5 mg/mL of MTT reagent was added to each plate and left to incubate for 3-4 h. The formazan crystals were dissolved in 100 µL acidified isopropanol and read at 630 nm by an ELISA microplate reader (Alley et al., 1988).

Cell viability % was calculated by the following equation [1]:

$$\text{Cell viability (\%)} = (\text{Abs s} / \text{Abs c}) \times 100 \quad [1]$$

Where: Abs s and Abs c were absorbance of the cells incubated with samples, and without sample, respectively. Detection of the potential cytotoxicity and determination of the half maximal inhibitory concentration ($IC_{50}$) were carried out. MTT assay was performed for combination treatments as well.

**Experiment design of MTT cell viability assay on MCF-7 and WI-38 cell lines**

This experiment was done in two phases. Phase one was planned to determine the cytotoxic effect of rutin, quercitrin and myricitrin isolated from *Physalis pubescens* L. leaves, as well as frankincense resin ethanol extract (FEE).

Rutin, quercitrin and myricitrin and frankincense ethanol extract (FEE) in DMSO were tested at 31.25, 62.5, 125, 250 and 500 µg/mL on MCF-7 cells. Compound and/or extract with best anticancer effect (lowest $IC_{50}$) was tested on WI-38 normal cell line to assess safety profile. Selectivity index (SI) was determined through dividing $IC_{50}$ of epirubicin, myrcitrin and frankincense, on WI-38 cells, by $IC_{50}$ of them on MCF-7 (Badisa et al., 2009).

Phase two was designed to assess the best compound and/or extract as adjuvant therapy with epirubicin to study if the effective dose of epirubicin could be reduced. Combination ratios were determined by using $IC_{50}$ of best effective compound and/or extract with different concentrations of epirubicin below and above its $IC_{50}$ value against MCF-7.

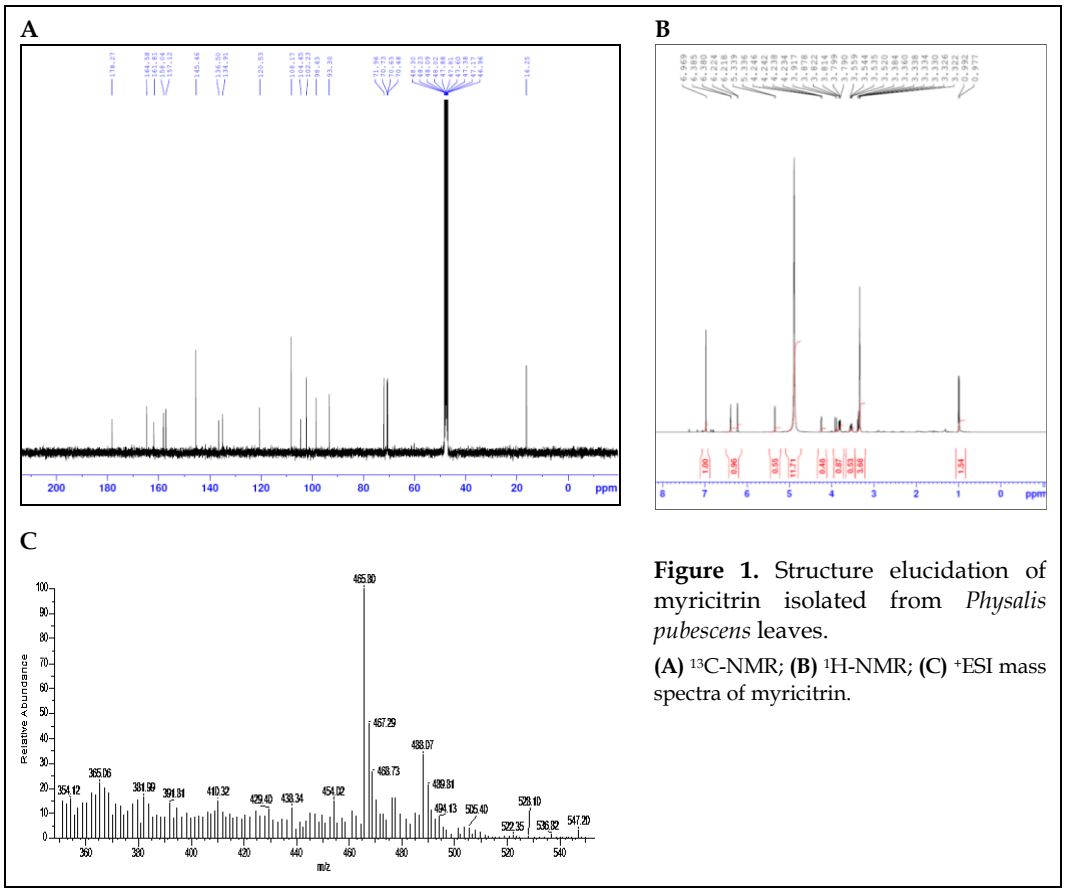


**Figure 1.** Structure elucidation of myricitrin isolated from *Physalis pubescens* leaves.

**(A)** $^{13}$C-NMR; **(B)** $^{1}$H-NMR; **(C)** $^{+}$ESI mass spectra of myricitrin.

### Real time quantitative PCR

The expression of ABCB1, ATG7 and TGF-β1 genes in harvested cells were determined by real time quantitative polymerase chain reaction (RT-qPCR) (Rao et al., 2013). The threshold cycle (Ct) values of target genes were compared to the Ct value of the housekeeping gene, β actin, and expressed as fold change. The real-time PCR program was set to start with initial heat activation for 15 min at 95°C, followed by 45 cycles (15 s at 94°C, 30 s at 55°C, and 30 s at 72°C) using Rotor-Gene Q 5plex (QIAGEN, USA). The expression fold change of genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

### Statistical analysis

Data analysis was achieved using Excel (Microsoft 365, 2019) and GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). Results were expressed as mean ± standard error of mean (SEM) and standard deviation (SD). Statistically significant difference was determined by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The p-value < 0.05 was considered significant.

## RESULTS

### Structure elucidation of the isolated compound from *Physalis pubescens* L. leaves

Light yellow powder; UV, $\lambda_{max}$ nm 256, 364 (MeOH); positive ESI-MS displayed $[M+H]^+$ at *m/z* 465.80; $^{1}$H-NMR (400 MHz, $CD_3OD$): *δ*H 6.22 (1H, d, *J*= 2.4 Hz, H-6), 6.38 (1H, d, *J*= 2 Hz, H-8), 6.96 (2H, s, H-2', 6'), 5.33 (1H, d, *J*=1.2 Hz, H-1''), 4.23 (1H, dd, *J*=3.2, 1.6 Hz, H-2'') 3.79 (1H, dd, *J*=9.4, 3.4 Hz, H-3''), 3.32~3.33 (m, H-4''), 3.52~3.55 (m, H-5''), 0.97 (3H, d, *J*=6 Hz, H-6''); $^{13}$C-NMR (100 MHz, $CD_3OD$): δC 158.0 (2), 134.9 (3), 178.2 (4), 161.8 (5), 98.4 (6), 164.5 (7), 93.3 (8), 157.1 (9), 104.4 (10), 120.5 (1'), 108.1 (2', 6'),145.4 (3', 5'), 136.5 (4'), 102.2 (1''), 70.4 (2''), 70.7 (3''), 71.9 (4''), 70.6 (5''), 16.2 (6'') (Fig. 1).

### MTT cell viability assay of different compounds and frankincense extract

$IC_{50}$ could be determined for different drugs in single and combined forms as shown in Table 1. Selectivity index (SI) was determined by dividing $IC_{50}$ of WI-38 cells by $IC_{50}$ of MCF-7 cells. Selectivity index of epirubicin, myricitrin and frankincense was 1.6, 4.1 and 3.5, respectively.

Myricitrin and frankincense exerted the best cytotoxic effect, $IC_{50}$ was 114 and 86.8, respectively against MCF-7 cells (human breast cancer cells), while they had no cytotoxic effect on WI-38 normal cells at their $IC_{50}$ on MCF-7 cells. The $IC_{50}$ of myricitrin and frankincense against WI-38 normal cells was 462.0 and 299.5, respectively with good selectivity index. This led to the possibility to use them as adjuvant therapy with epirubicin. MTT cell viability assay showed that they could reduce $IC_{50}$ of epirubicin (fewer side effects) with more efficacy. $IC_{50}$ was significantly different, using Tukey's multiple comparison test, of rutin, quercitrin, myricitrin and frankincense compared to epirubicin. $IC_{50}$ of combined treatment of myricitrin + epirubicin and frankincense + epirubicin was not significant compared to epirubicin at $p < 0.05$. $IC_{50}$ was expressed as mean ± SEM of three independent experiments (Fig. 2).

**Table 1.** $IC_{50}$ of different compounds and extract against MCF-7 and WI-38 cells.

| Compound or extract (µg/mL) | MCF-7 cells $IC_{50}$ (µg/mL) | WI-38 normal cell line $IC_{50}$ (µg/mL) |
|---|---|---|
| Epirubicin (0.21, 0.42, 0.85, 1.7, 3.4) | 0.8 ± 0.052 (1.6)[a] | 1.26 ± 0.0057 |
| Rutin (31.25, 62.50, 125, 250, 500) | 350.16 ± 1.241 | - |
| Quercitrin (31.25, 62.50, 125, 250, 500) | 259.6 ± 1.45 | - |
| Myricitrin (31.25, 62.50, 125, 250, 500) | 114.0 ± 0.517 (4.1)[a] | 462.0 ± 1.062 |
| Frankincense resin ethanol extract (31.25, 62.50, 125, 250, 500) | 86.8 ± 0.91 (3.5)[a] | 299.5 ± 1.32 |
| Myricitrin $IC_{50}$ (114) + epirubicin (0.2, 0.4, 0.6, 0.8, 1, 1.4) | 0.37 ± 0.087 | - |
| Frankincense $IC_{50}$ (86.8) + epirubicin (0.2, 0.4, 0.6, 0.8, 1, 1.4) | 0.5 ± 0.1732 | - |

$IC_{50s}$ ± SEM of different flavonoids (rutin, quercitrin and myricitrin) and ethanolic extract of frankincense oleo-gum resin were determined by MTT cytotoxic assay. The best cytotoxic effect was for myricitrin and frankincense. $IC_{50}$ of combined treatment of myricitrin or frankincense with epirubicin was assessed, $IC_{50}$ of epirubicin decreased while efficacy increased. [a]Selectivity index. $IC_{50}$ is half maximal concentration. MCF-7 is human breast cancer cells. WI-38 is human lung fibroblasts. $IC_{50}$ was significantly different, using Tukey's multiple comparison test, of rutin, quercitrin, myricitrin and frankincense compared to epirubicin. $IC_{50}$ of combined treatment of myricitrin + epirubicin and frankincense + epirubicin was not significant compared to epirubicin at $p < 0.05$. $IC_{50}$ was expressed as mean ± SEM of three independent experiments.

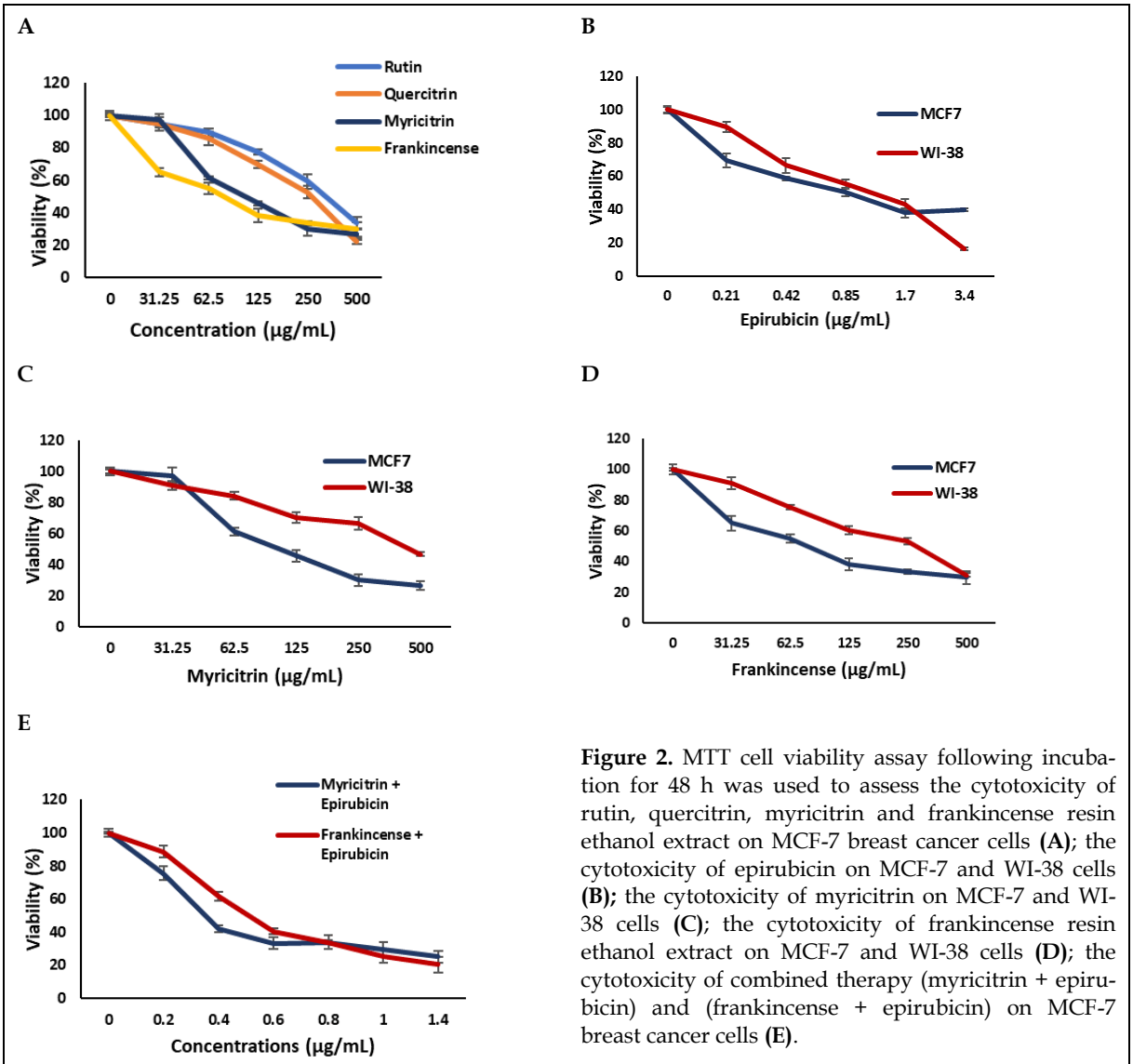


**Figure 2.** MTT cell viability assay following incubation for 48 h was used to assess the cytotoxicity of rutin, quercitrin, myricitrin and frankincense resin ethanol extract on MCF-7 breast cancer cells **(A)**; the cytotoxicity of epirubicin on MCF-7 and WI-38 cells **(B);** the cytotoxicity of myricitrin on MCF-7 and WI-38 cells **(C)**; the cytotoxicity of frankincense resin ethanol extract on MCF-7 and WI-38 cells **(D)**; the cytotoxicity of combined therapy (myricitrin + epirubicin) and (frankincense + epirubicin) on MCF-7 breast cancer cells **(E)**.

Values were expressed as mean ± SD of three independent experiments. 50 % inhibitory concentrations were determined for different treatments of breast cancer cells MCF-7and lung fibroblast cells WI-38. Myricitrin and frankincense showed the best cytotoxic effect against MCF-7 cells, while they exerted minimal toxic effect on WI-38 cells. $IC_{50}$ of combined treatment of myricitrin or frankincense with epirubicin was assessed. $IC_{50}$ of epirubicin decreased (reduced side effect) while efficacy increased in the combination treatment.

## Effect of myricitrin, frankincense, epirubicin and their combinations on genes expression by RT-qPCR

Fig. 3 showed effects of different treatments on different genes expression in MCF-7 cancer cells. Upregulation effect of epirubicin was detected in all genes expression of ABCB1, TGF-β1 and ATG7. Fig. 3A shows the ABCB1 gene expression relative to β-actin, the housekeeping gene, showed that myricitrin and combined treatment myricitrin with epirubicin (EM) exerted downregulation effect on the gene expression. Their effect was significant to epirubicin at $p < 0.05$. The observed effect of frankincense and combined treatment with epirubicin (EF), was significant ($p < 0.05$) compared to epirubicin. Frankincense, single treatment, showed reduction of ABCB1 gene expression.

Fig. 3B demonstrates the gene expression of TGF-β1 relative to β-actin the reference gene, it showed the upregulation effect of epirubicin, the significant downregulation effect of frankincense and EF to epirubicin as well as significant ($p < 0.05$) effect of myricitrin and EM to epirubicin. Fig. 3C exhibits the gene expression of ATG7 in relation to β-actin, the housekeeping gene, A significant increase of gene expression by myricitrin single treatment and combined treatment (EM) to both control and epirubicin was observed. The opposite reduction effect was observed for frankincense alone and the combined treatment (EF). This effect was significant ($p < 0.05$) compared to epirubicin in case of frankincense.

## DISCUSSION

Malignancy of breast cancer is the common leading cause of cancer death in women (Jemal et al., 2011). Flavonol glycosides are group of compounds belong to flavonoids, the largest and most vital natural constituents in plants, which exert strong antioxidant effect. They could scavenge the ROS, which are free radicals that cause oxidative damages and could change normal cells to cancer cells. Flavonol glycosides could prevent tumor initiation, growth and metastasis (Dai and Mumper, 2010). Rutin, quercitrin (El-Sherbeni and Al-Ashmawy, 2020) and myricitrin are flavonol glycosides, isolated from *Physalis pubescens* L. leaves. They were subjected to MTT cell viability assay to determine which of them showed the best anti-cancer effect with the least $IC_{50}$.

Myricitrin was isolated for the first time from *Physalis pubescens* L. leaves. Physical and spectral data compared to reference data confirmed its structure. Structure elucidation of the isolated compound was deduced from its UV absorption at $\lambda_{max}$ nm 256 and 364 in methanol, which indicated the flavonol structure. The positive ESI-MS analysis demonstrated $[M+H]^+$ peak at *m/z* 465.8. $^1$H-NMR spectrum showed different signals of meta

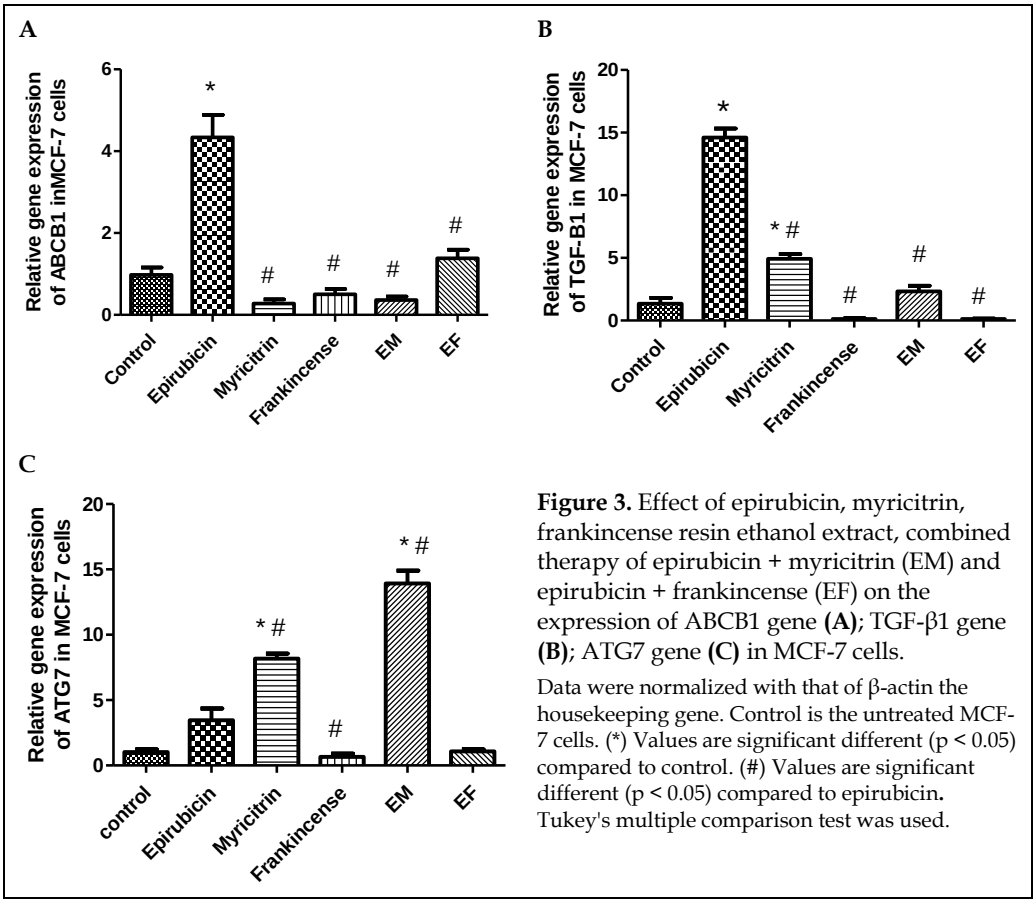


**Figure 3.** Effect of epirubicin, myricitrin, frankincense resin ethanol extract, combined therapy of epirubicin + myricitrin (EM) and epirubicin + frankincense (EF) on the expression of ABCB1 gene **(A)**; TGF-β1 gene **(B)**; ATG7 gene **(C)** in MCF-7 cells.

Data were normalized with that of β-actin the housekeeping gene. Control is the untreated MCF-7 cells. (*) Values are significant different ($p < 0.05$) compared to control. (#) Values are significant different ($p < 0.05$) compared to epirubicin. Tukey's multiple comparison test was used.

coupled protons H-6 and 8, which showed two douplets at *δ*H 6.22 and 6.38, respectively with coupling constant consistent with meta coupling. Signal of anomeric proton at *δ*H 5.33 with coupling constant = 1.2 Hz which is characteristic for alpha linkage configuration between sugar moiety and the aglycone. The different signals of sugar moiety (2'', 3'', 4'', 5'', 6'') resonating at *δ*H 4.23, 3.79, 3.32, 3.52 and 0.97, respectively, are suggested for rhamnose. $^{13}$C-NMR signals of different carbon atoms ascertain the previous suggestion of rhamnose glycoside of flavonol compound. Downfield signal at δC 178.2 is for carbonyl group. Signals at δC 161.8, 164.5, 145.4, 136.5 are for aromatic carbon atoms attached with hydroxyl groups at 5, 7, 3', 5`and 4'. Signals of carbon atoms of rhamnose sugar moiety showed anomeric carbon atom at 102.2 and other atoms resonating at 70.4, 70.7, 71.9, 70.6 as well as the deoxy carbon atom at 16.2 of C-6''. From this data, which was compared to that reported in research (Hwang and Chung, 2018) we could reveal that this structure is myricetin-3-*O*-α-L-rhamnoside or myricitrin (Fig. 4). Myricitrin, which was rarely investigated as anti-cancer agent, so it was noteworthy to assess its effect against breast cancer and to try to deduce its potential molecular mechanism of action.

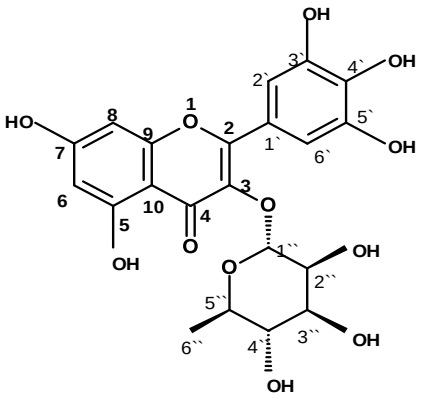


**Figure 4.** Myricitrin chemical structure.

The oleo-gum resins of *Boswellia* species known as frankincense have been used for ages in traditional medicine in India and the Arabian world (DeCarlo et al., 2019). Frankincense exerts strong antioxidant, anti-inflammatory activity and modulates immunity. It was found that there is close connection between chronic inflammation and carcinogenesis (Elinav et al., 2013). Frankincense acts as anticancer by multiple mechanisms, for example by the inhibition of leukotriene synthesis, cyclooxygenase, lipoxygenase, oxidative stress, and induction of apoptosis. It modulates signaling transduction responsible for cell cycle arrest inhibition of tumor growth, angiogenesis, invasion and metastasis (Efferth and Oesch, 2020).

Besides, drug resistance is a common problem, which needs new treatment options, the usage of natural products is one of these options, frankincense and flavonoids could be used as adjuvant therapy to the established anti-cancer chemotherapy. They could increase chemosensitivity of resistant cancer cells towards anti-cancer drugs and reduce side effects (Efferth and Volm 2017). It was reported that they are active against different forms of carcinoma (fibrosarcoma, carcinoma of prostate, pancreas, breast, cervix, liver, lung and bladder, glioblastoma, and meningioma) as well as hematopoietic tumors (leukemia and multiple myeloma).

Epirubicin, an anthracycline analogue, intercalates into DNA strands and blocking DNA and RNA synthesis. It is used as a therapeutic agent for a wide spectrum of tumors including breast, cervical, lung and ovarian malignancy (Yamaguchi et al., 2015).

Epirubicin could cause early and delayed cardiotoxicity, which are common to happen within two months to year after finishing treatment with anthracycline drugs (Conte et al., 2000) The cytotoxic effect of myricitrin and frankincense oleogum resin on WI-38 normal cell line indicated that $IC_{50}$ were 462.0 ± 1.062 and 299.5 ± 1.320 µg/mL, respectively. These results figured out their safety against non-target normal cells.

In the current study it was found that myricitrin and frankincense exhibited the best anti MCF-7 breast cancer effect, $IC_{50}$ was 114 ± 0.517 and 86.8 ± 0.91 µg/mL, respectively. It was found that the $IC_{50}$ of epirubicin was reduced from 0.8 (epirubicin alone) to 0.37 and 0.5 µg/mL when combined with myricitrin and frankincense, respectively. This

emphasizes the possibility of using the natural products as adjuvant therapy.

Multidrug resistance (MDR) is a phenomenon in which cancer cells exhibit a cross-resistant phenotype against multiple unrelated drugs with different structure and/or function that may diverse molecular targets. MDR represents a crucial factor in the failure of many forms of chemotherapy. Cancer cells may exhibit intrinsic or primary MDR or they may acquire MDR during chemotherapy. The most extensively characterized MDR membrane transporters include P-glycoprotein (P-gp/ABCB1) (Saraswathy and Gong, 2013). The ATP-binding cassette subfamily B member 1 (ABCB1) gene, a multidrug resistance protein 1 (MDR1) gene, encodes p-glycoprotein (P-gp), which plays an important role in drug bioavailability and response to drugs.

Recently, several studies have documented the ability of polyphenols, including flavonoids, to increase the sensitivity of cancer cells to anticancer drugs and to reverse MDR by the inhibition of ABC transporters (Michalak and Wesolowska, 2012). They may improve the efficacy of conventional cancer chemotherapy through additive or synergistic effects with traditional chemotherapeutics or by inducing chemosensitization in resistant cancer cells. They may decrease cancer therapy-induced toxicity and the undesirable side effects (Lecumberri et al., 2013). Increased expression of the ABCB1 gene in cancer cells is usually connected with occurrence of multidrug resistance (MDR) and poor prognosis.

Transforming growth factor beta 1 or TGF-β1 is a polypeptide member of the transforming growth factor beta superfamily of cytokines. One of the most remarkable aspects of TGF- β1 lies within its apparent dichotomy. In normal cells, TGF-β1 functions as a potent tumor suppressor, however conversely in malignant cells, it behaves as a tumor promoter and fosters a more aggressive phenotype (Li et al., 2005). Most cancer cells secrete larger amounts of TGF- β1 than their normal cell counterparts, and this overexpression is strongest in the most advanced stages of malignancies (Glynne-Jones et al., 1994). The tumor-promoter activity of overexpression of TGF- β1 has been attributed to several causes other than its direct effect on cancer cells such as: the stimulation of angiogenesis, and the immunosuppressive effects of TGF- β1, all of which can exacerbate the malignant phenotype and lead to increased invasion and subsequent metastasis (Bian et al., 2002)

Autophagy related 7 is a protein in humans encoded by ATG7 gene. It acts as an essential protein for cell degradation and its recycling. Autophagy (from the Greek, "auto" oneself, "phagy" to eat) refers to any cellular degradative pathway. At least three forms have been identified - chaperone-mediated autophagy, microautophagy, and macroautophagy - that differ with respect to their physiological functions and the mode of cargo delivery to the lysosome (Kroemer and Jaattela, 2005). The role of autophagy in cancer is complex, as demonstrated by studies describing situations in which autophagy can either promote or inhibit tumorigenesis (Kimmelman, 2011). Autophagy has a prosurvival mechanism for epirubicin treated MCF-7 cells, it facilitates the development of epirubicin acquired resistance. This indicates that the combination of epirubicin and an autophagy inhibitor might be a suggested as a therapeutic strategy, especially for epirubicin resistant breast cancers (Sun et al., 2011).

RT-qPCR was used to study the effect of myricitrin, frankincense, the combined EF and EM treatments on genes expression of ABCB1, TGF-β1 and ATG7. Myricitrin significantly upregulates TGF-β1 and ATG7 when compared to control and to epirubicin, myricitrin and EM reduced expression of ABCB1 gene significantly to epirubicin. The potential mechanism of action concerning myricitrin as adjuvant therapy was through reduction of breast cancer cells resistance towards epirubicin. Ethanol extract of frankincense oleo-gum resin downregulated expression of ABCB1, TGF-β1 and ATG7, significantly, compared to epirubicin, which indicated its anticancer effect as a single treatment via inhibition of multidrug resistance, tumor invasion, metastasis, and autophagy. Frankincense effect as adjuvant therapy to epirubicin could be explained through expression reduction effect of EF on TGF-β1 and ATG7. The possible

molecular mechanism of action could be through reduction of tumor invasions, metastasis, and autophagy. Frankincense could be used as autophagy inhibitor combined to epirubicin to reduce resistance of breast cancer cells to epirubicin chemotherapy.

## CONCLUSIONS

The results of this study showed that myricitrin and frankincense exerted the best cytotoxic activity on MCF-7 cells, while they exerted minimal cytotoxic effect on WI-38 normal cells (lung fibroblast cells), regarding the selectivity index. These outcomes suggest that these natural products could be used as a supplementary or adjuvant chemotherapy. This combination of therapy could improve efficacy of epirubicin ($IC_{50}$ reduction) and reduce its unwanted side effects. The reversal of drug resistance mechanism of breast cancer cells towards established chemotherapy (epirubicin) by downregulating ABCB1 and ATG7 genes expression, may qualify frankincense and myricitrin as good partners in modern combination therapy regimens. Animal and clinical trials should be done to complete the scientific knowledge about them and to make their medicinal use to be applicable.

## CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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|---|---|---|
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