Plant Science Research and Practices

Luteolin

Natural Occurrences, Therapeutic Applications and Health Effects

Alexis J. Dwight

Editor

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PLANT SCIENCE RESEARCH AND PRACTICES

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NATURAL OCCURRENCES, THERAPEUTIC APPLICATIONS AND HEALTH EFFECTS

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ALEXIS J. DWIGHT

EDITOR

New York

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PREFACE

Luteolin belongs to a group of naturally occurring compounds called flavonoids that are found widely in the plant kingdom. Luteolin is widely present in many fruits, vegetables and medicinal herbs. It has many beneficial health effects, including antioxidant, anti–inflammatory, anti–proliferative, proapoptotic, and anti–angiogenic. This book discusses the natural occurrences of luteolin, as well as the therapeutic applications and health effects.

Chapter 1 – Luteolin is a flavonoid found in several fruits and vegetables in significant amounts and number of biological properties of this phenolic was described, including the antioxidant activity. Several biological activities protect against the harmful action of reactive oxygen species (ROS), and here we focused our attention on the relationship between the biological activities of luteolin and their antioxidant properties. In this study, the antioxidant, antimicrobial, hemolytic and cytotoxicity activities were evaluated. Significant antioxidant activity was demonstrated and luteolin was effective against all bacteria and Candida strains tested, and showed an ability to inhibit hyphal formation. Non-hemolytic and antiproliferative activity could be demonstrated. The flavonoid showed a significant antioxidant activity, and it seems be essential for antibacterial, antifungal, non-hemolytic and antiproliferative activities.

Chapter 2 – Luteolin, a plant–derived polyphenolic flavonoid, is widely present in many fruits, vegetables and medicinal herbs. It has many beneficial health effects, including antioxidant, anti–inflammatory, anti–proliferative, proapoptotic, and anti–angiogenic activities. Observations published in the last ten years suggest that luteolin could be an anticancer agent for various cancers, and the anticancer property of luteolin is associated with the induction of apoptosis or autophagy, and inhibition of cell proliferation, metastasis and

angiogenesis. Furthermore, luteolin has also been reported to exhibit cardiovascular protection activities. For instance, luteolin reduces vascular smooth muscle cells' proliferation and migration, and prevents ischemia-reperfusion injury by reducing necrosis and apoptosis in rat cardiomyocytes. Our previous report also indicated the anti-hypertension effect of luteolin. In fact, the antioxidant and anti-inflammatory activity may be also linked to both the anticancer and cardiovascular protection property of luteolin. This chapter will discuss the potentially activities and mechanisms of luteolin as a candidate of antineoplastic drug or cardiovascular protective drug.

Chapter 3 – Natural antioxidants have become very important in recent decades because of their well known benefits to human health and the increasingly restricted use of synthetic antioxidants. Flavonoids are one of the groups of natural antioxidants widely produced by the plants as secondary metabolites. They are molecules composed of two benzene rings linked through a chain of three carbon atoms. Flavonoids are widely found in fruits, seeds and vegetables. Luteolin (3′,4′,5,7-tetrahydroxy-flavone; LUT) belongs to the subclass of flavonoids known as flavones and is rated as one of the most bioactive flavonoids. LUT has a resorcinol group in ring A, and a catechol group in ring B. LUT has beneficial effects on human health, such as cardiovascular protection, anti-allergic, and anticancer activities, anti-ulcer effects, and prevents cataracts. LUT also inhibits platelet aggregation by vasodilating action. LUT is a compound as active as tert-butyl hydroxyanisole (BHA) but more active than α-tocopherol. Different analytical methods have been reported for the determination of LUT. They include thin-layer chromatography, gas chromatography, high-performance liquid chromatography, and capillary electrophoresis, coupled to different detection techniques such as UV spectrophotometry, photo diode array, electrochemical array, etc. Even though these techniques have made possible the highly selective and sensitive quantification of LUT, they present some disadvantages such as high cost, high time consumption and reagents, and high complexity of operation. In recent years, electroanalytical techniques have become very important as analytical tools in the determination of different compounds of biological interest. Compared with chromatographic techniques, they require cheap equipment, short analysis times, low solvent consumption, etc.

In this chapter, we report the application of electroanalytical techniques to determine LUT in real matrices. Both, results obtained in our laboratory and those reported by other authors are included. We also discuss the application of chemometric tools in those cases where LUT is present in real samples in

the presence of other interfering electroactive species whose electrochemical signals show a strong overlap.

Chapter 4 - Luteolin belongs to a group of naturally occurring compounds called flavonoids that are found widely in the plant kingdom. Different studies are focused on their beneficial properties but little is known about the damage of this flavone in normal host cells. The studies conducted in order to evaluate the cyto-genotoxic effects of luteolin on normal animal and human cells showed diverse results that reveal that this flavone could have toxic effects depending on diverse factors as concentration, test carried out (*in vitro* or *in vivo*), time and mode of administration and type of cell. Because there are many biological activities attributed to luteolin, some of which could be beneficial or detrimental depending on various aspects, further studies are required to ensure the security of this flavonoid for potential future application in a host system.

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Chapter 1

THE ANTIOXIDANT PROPERTY OF LUTEOLIN AND ITS CORRELATION AMONG ANTIMICROBIAL, HEMOLYTIC, AND ANTIPROLIFERATIVE ACTIVITIES

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ABSTRACT

Luteolin is a flavonoid found in several fruits and vegetables in significant amounts and number of biological properties of this phenolic was described, including the antioxidant activity. Several biological activities protect against the harmful action of reactive oxygen species (ROS), and here we focused our attention on the relationship between the biological activities of luteolin and their antioxidant properties. In this study, the antioxidant, antimicrobial, hemolytic and cytotoxicity activities were evaluated. Significant antioxidant activity was demonstrated and luteolin was effective against all bacteria and Candida strains tested, and showed an ability to inhibit hyphal formation. Non-hemolytic and antiproliferative activity could be demonstrated. The flavonoid showed a

significant antioxidant activity, and it seems be essential for antibacterial, antifungal, non-hemolytic and antiproliferative activities.

1. Introduction

Reactive oxygen species are continuously produced in human cells [1,2]. Oxidative stress is now recognized as a major factor associated with the development of chronic diseases, including cancer and cardiovascular disease. In this sense, natural products have been attracting scientific interest due to their antioxidant and chemopreventive properties. It is well-known that one of the main characteristics responsible for the antioxidant activity of a plant extract is its high content of phenolic compounds. Phenolic compounds, like flavonoids, exhibit a wide range of other biological effects [3-7]. Flavonoids are the largest group of polyphenols present in many plants which are known to promote a number of physiological benefits, especially in scavenging free radicals [8]. This has led to the hypothesis that the beneficial effects of natural products could be largely explained by their high content of antioxidants [9,10]. Several antioxidants in plants have been suggested to contribute to the anticarcinogenic effect, and others such as flavanols have also been able to inhibit cancer cell proliferation in vitro.

A molecular frame of two phenyl rings linked by a three-carbon chain, making them good electron donors or acceptors characterize flavonoids. Their anti-oxidant capacity depends on this framework, the number and pattern of substitutions (primarily with hydroxyl groups), their ability to chelate with metal ions, and on their specific environment [11].

Luteolin (3,4,5,7-tetrahydroxyflavone) (Figure 1) is a flavonoid that is found in several fruits and vegetables in significant amounts, such as celery, carrots, chamomile tea and green pepper [12]. As other flavonoids, luteolin is most often found in plant materials in the form of glycosides. Luteolin plays effectively role as anti-inflammatory in vitro and in vivo, antibacterial, antifungal, anticancer, among others properties [11-14]. The biological activities of luteolin depend on a complex sum of individual properties, which can only partly be explained by its anti-oxidant activity and free radical scavenging capacities. Luteolin possesses the structures essential to flavonoid's antioxidant activity. Briefly, the ortho-dihydroxy structure in the B-ring and the 2,3-double bond in conjugation with the 4-oxo function of the C-ring provides a good anti-oxidant capacity of luteolin [14]. Furthermore, it is more lipophilic and may perform better in test systems with biological molecules or

membranes. Several biological activities protect against the harmful action of reactive oxygen species, and here we focused our attention on the relationship between the biological activities tested and the antioxidant properties of luteolin [12,15-17]. Thus, the aim of the present chapter was to evaluate the antioxidant, antimicrobial, hemolytic and human cancer cell antiproliferative activities of luteolin and demonstrate the correlation with its antioxidant property.

2. Materials and Methods

2.1. ABTS Radical Cation Scavenging Activity

The free radical scavenging activity of the luteolin was determined by ABTS radical cation decolorization assay [18]. It involved the generation of $ABTS^{\bullet+}$ chromophore by the oxidation of ABTS with potassium persulfate. The $ABTS^{\bullet+}$ radical cation was generated by reacting ABTS and potassium persulfate after incubation at room temperature in the dark for 12–16 h. The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$ solution with methanol, to obtain an absorbance of about 0.7 at 734 nm using the spectrophotometer. The reactive mixture (extract in different concentrations and $ABTS^{\bullet+}$) was allowed to stand at room temperature for 15 min and the absorbance was immediately recorded at 734 nm. Luteolin solutions were prepared under the same condition. The flavonoid quercetin was analysed in order to compare the two compounds. The results were expressed as 50% inhibitory concentration or IC50.

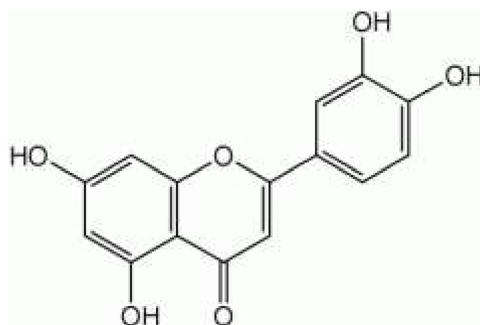

Figure 1. Chemical structure of luteolin.

2.2. Antimicrobial Activity

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method, according to the standard reference method [19,20]. The antifungal activity was evaluated against *C. albicans* (ATCC 18804 and NCPF 3153), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750) and the antibacterial activity was evaluated against *Staphylococcus aureus* (ATCC 25923), *Bacilus subtillis* (ATCC 19659), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella setubal* (ATCC 19196). The luteolin (Sigma®) were dissolved in 25% ethanol and water to initial concentration of extract of 1000 µg/mL. Then, a two-fold serial dilution was made in order to obtain concentration ranges of 7.8–1000 µg/mL. 100 µL of each concentration were added to 96-well microplates containing 80 µL of RPMI 1640 for yeast and Muller-Hinton broth for bacteria. The inocula of bacteria and yeast were standardized at 1.0×10^7 and 2.5×10^3 CFU/mL, respectively. Ciprofloxacin, fluconazole and 25% ethanol and water were used as positive and negative control. The plates were incubated at 37 °C for 24 h for bacteria and 48 h for yeast. The assay was repeated three times. The MIC of the samples was detected after the addition (50 µL) of resazurin solution (0.2 mg/mL) for bacteria and 2.0% triphenyl-tetrazolium chloride (TTC) solution for yeast, and incubated at 37 °C for 30 min. Growth of bacteria changes the blue dye resazurin to a pink color. The pink color indicates positive growth, and blue indicates growth inhibition. Yeast growth changes the colorless TTC to a red color. MIC was defined as the lowest sample concentration that prevented this change and exhibited inhibition of microorganism growth.

For the determination of minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC), a portion from each well that showed antibacterial activity and antifungal activity was plated on Muller-Hinton and Sabouroud agar, and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MBC/MFC [21].

2.2.1. Inhibition of Hyphal Growth

C. albicans (NCPF 3153) cells from a 48 h stationary phase culture were transferred to microplate with RPMI 1640 medium supplemented with fetal bovine serum (FBS) to a final concentration of 2.5×10^3 CFU/mL, and luteolin solution were added to the growth medium to final concentrations of 0.5 x

MIC, 1 x MIC and 2 x MIC, and the cultures were incubated for 12 and 24 h at 37 °C, 5% CO_2 [22]. The hyphal formation was observed under an inverted light microscope (Nikon TE 2000-U Eclipse) with the magnification of 400x. Amphotericin B solution (5 mg/L) was used as a positive control.

2.3. MTT and LDH Cell Viability Assay

Cell viability was determined by using the conversion of MTT to formazan via mitochondrial oxidation [23]. The human cervical adenocarcinoma cells line (HeLa—ATCC: CCL-2; 5000 cells/well) were treated with luteolin (0, 0.5 x MIC, 1 x MIC and 2 x MIC to *C. albicans*) for 24 h. Then, MTT solution was added to each well at a concentration of 10 mg/mL and the plates were incubated at 37 °C for another 4 h. After incubation, 0.2 mL DMSO was added to each well to dissolve the formazan and the absorbance was read at 570 nm using a spectrophotometric microplate reader. The viability was determined based on a comparison with untreated cells. The experiments were performed in triplicate and repeated at least three times.

Cell injury was quantitatively assessed by the measurement of LDH, released from damaged or destroyed cells, in the extracellular fluid 24 h after the experiment. An aliquot of bathing media was combined with NADH and pyruvate solutions. LDH activity is proportional to the rate of pyruvate loss. The quantity of LDH released by the cells into the medium was measured by the decrease in the absorbance at 340 nm for NADH disappearance within different times (0, 1, 2 and 3 min). The difference in absorbance per minute was determined and the average multiplied by the factor (10080).

The results were statistically analyzed by comparing the LDH and MTT values obtained at baseline with those obtained in different treatments by analysis of variance (ANOVA) followed by Dunnett test (multiple comparisons with one control) with $p < 0.05$.

2.4. Hemolytic Assay

Human erythrocytes from healthy individuals were collected in vacuum tubes containing heparin as anti-coagulant. The erythrocytes were harvested by centrifugation for 10 min at 2000 rpm and washed three times in phosphate buffered saline (PBS). To the pellet, PBS was added to yield a 10% (v/v)

erythrocytes/PBS suspension. The 10% suspension was then diluted 1:10 in PBS. 0.1 mL of this suspension was added in triplicate to 96-well microplates containing 0.1 mL of luteolin (1.9–250 µg/mL) serially diluted in PBS. Total hemolysis was achieved with 1% Triton X-100. The microplate was incubated for 1 h at 37 °C and then centrifuged for 10 min at 2000 rpm. The absorbance of the supernatant was measured spectrophotometrically at 450 nm [24]. The percentage of hemolysis was calculated and statistically analyzed by analysis of variance (ANOVA) followed by Dunnett test (multiple comparisons with one control) with $p < 0.05$.

3. Results

3.1. ABTS Radical Cation Scavenging Activity

Table 1 shows the concentrations of the luteolin and quercetin needed to decrease the initial ABTS concentration by 50% (50% Inhibitory Concentration, IC50). The IC50 value of the luteolin and quercetin showed significant antioxidant activity as was to be expected.

3.2. Antibacterial and Antifungal activities

Luteolin showed activity against all microorganisms tested. The results of MIC determinations (Table 2) showed noticeable MIC values for the luteolin against *Candida* species and Gram-positive bacteria. The results of MFC and MBC determination indicated that the fungicidal and bactericidal effect of the compound on the studied microorganisms could be expected. A close look at MFC and MIC values revealed that most MFC values correspond to MIC values.

Table 1. 50% Inhibitory Concentration (IC50) values of luteolin and quercetin obtained in the ABTS radical test

Samples	IC_{50} [a] Means ± SD
quercetin	1,074 ± 0,038 [b]
luteolin	1,208 ± 0,031 [b]

[a] Values in µg/mL; [b] p<0,001; SD: standard deviation.

Table 2. Antibacterial and antifungal activity of luteolin

Microorganism	MIC[a]	MBC/MFC[a]
C. albicans	125	500
C. krusei	250	250
C. parapsilosis	125	125
C. tropicalis	250	250
S. aureus	125	125
B. subtilis	62,5	125
E. faecalis	31,25	125
E. coli	250	250
P. aeruginosa	62,5	250
S. setubal	250	-

[a] Values given as µg/ml; (-) > 1000 µg/mL

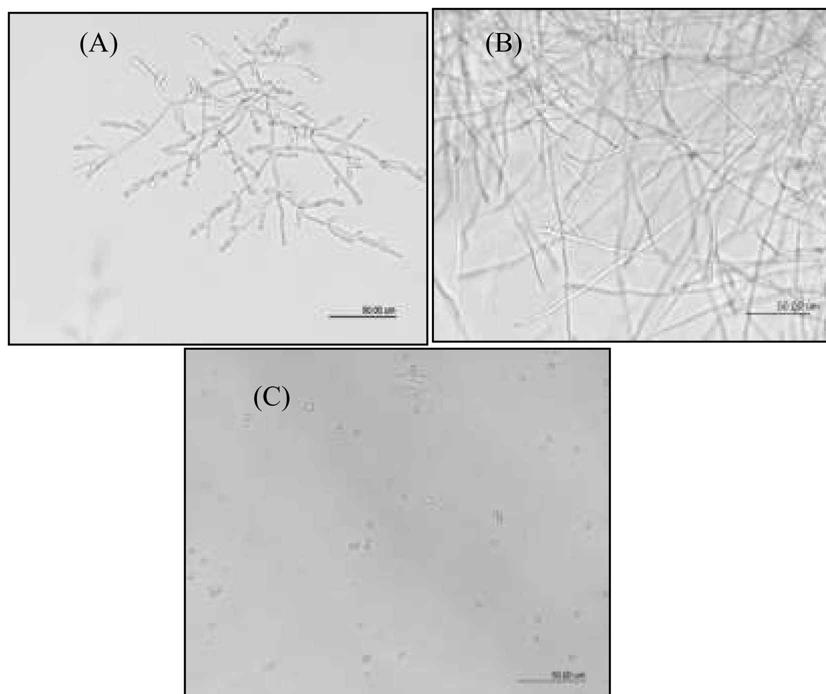

Figure 2. (Continued).

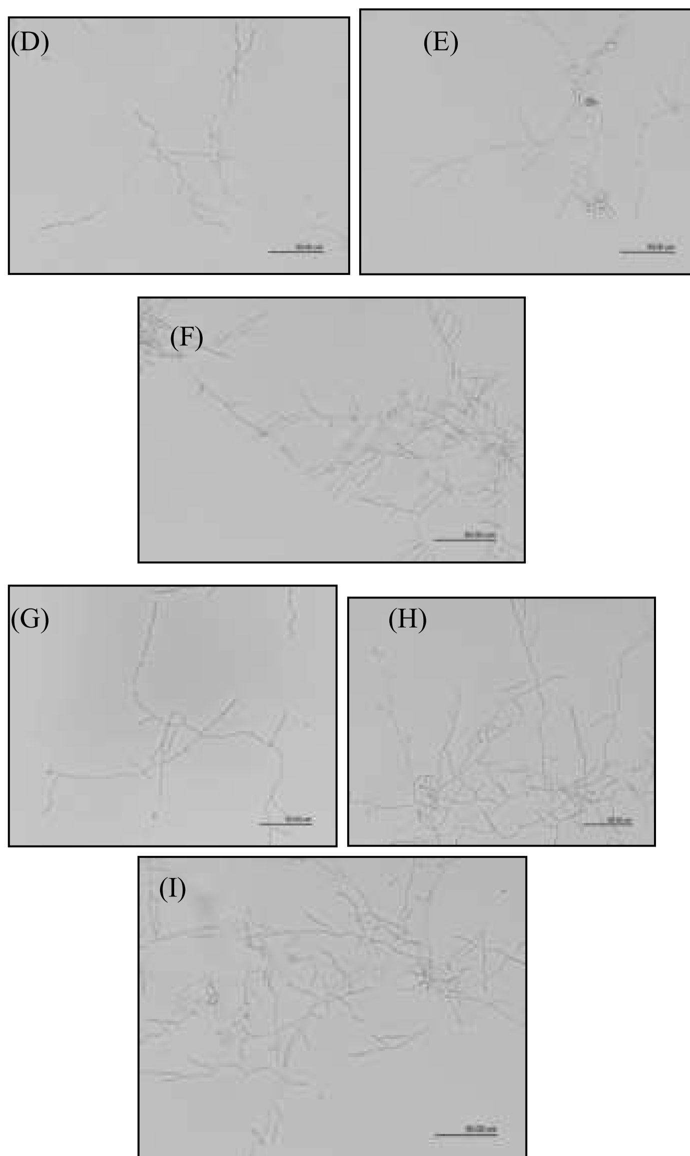

Figure 2. Hyphal formation of *C. albicans* NCPF 3153 cells. **(A)** 12 h normal growth; **(B)** 24 h normal growth; **(C)** *C. albicans* cells treated with ANF 5 µg/ml as the positive control. Hyphal formation of *C. albicans* cells was not inhibited by the luteolin at (**D**) 250 µg/ml; (**E**) 125 µg/ml; (**F**) 62,5 µg/ml at 12 h; (G) 250 µg/ml; (H) 125 µg/ml; (I) 62,5 µg/ml at 24 h, compared with (**A**) and (**B**). The black bar represents a length of 50 µm.

3.2.1. Inhibition of Hyphal Formation

C. albicans cells were incubated for 12 and 24 h in the presence or absence of luteolin and amphotericin B (positive control), and then observed under an inverted light microscope (Figure 3). In the absence of the drugs, hyphal formation was observed, while in the different concentrations of luteolin were not able to inhibit hyphal formation. The normal hyphae formation was distorted by luteolin action and it is possible to observe an abnormal development of hyphae.

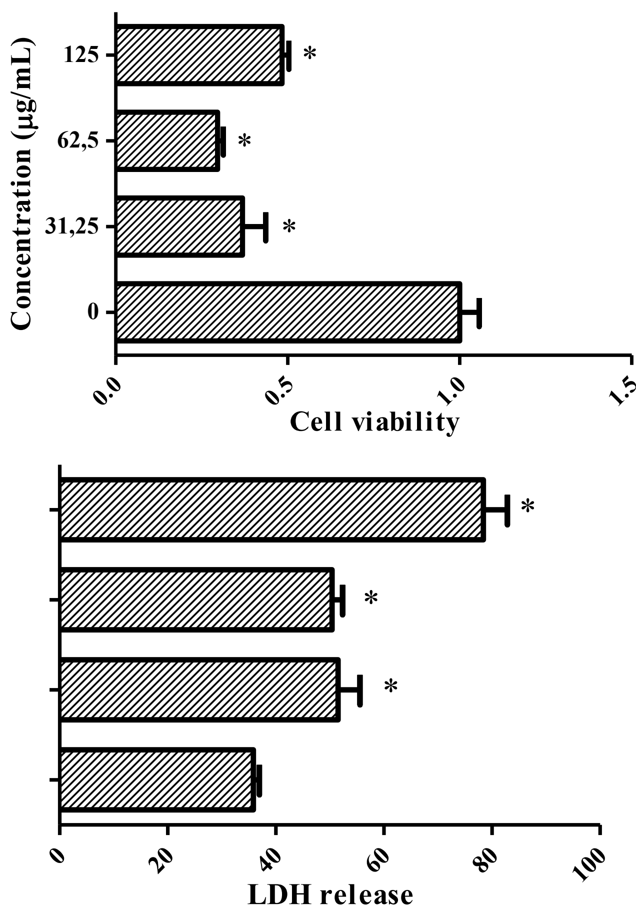

Figure 3. Anti-proliferative effect of the luteolin on HeLa cells. *Significantly different from the basal conditions.

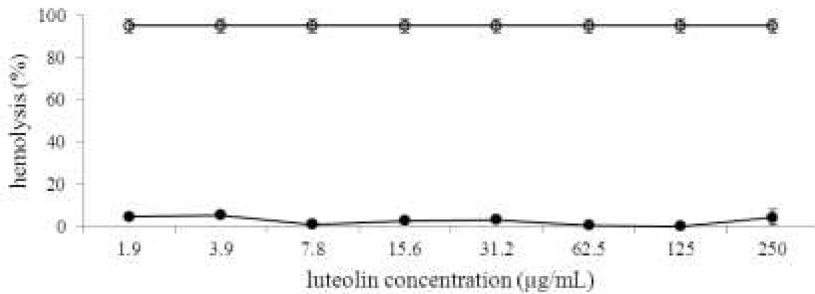

Figure 4. Hemolytic activity of luteolin (filled circle) and positive control Triton X-100 (open circle).

3.3. MTT and LDH Cell Viability

Figure 3 shows the effect of the luteolin on the cell viability of human cervical adenocarcinoma cells line (HeLa cells) after a 24 h incubation period. Luteolin significantly inhibited cell growth at the tested concentration ($p < 0.05$). Cell injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) release. After a 24 h incubation period, luteolin concentrations significantly increased cell LDH release ($p < 0.05$), when compared with the untreated control.

3.4. Hemolytic Assay

Figure 4 shows the hemolytic activity of the luteolin investigated by measuring the lysis of a 10% (v/v) human red blood cells suspension in a spectrophotometric assay. In this experiment, Triton X-100 1% (v/v) was used as a positive control and induced 95.0 ± 3.1% of red blood cell lysis. Luteolin showed no significant effect on red blood cells lysis.

4. DISCUSSION

The antioxidants block the generation of ROS and free radicals and present a beneficial role in preventing the free radical-related diseases. ROS and free radicals such as superoxide anion, hydrogen peroxide and hydroxyl radicals are considered to be implicated in degenerative processes related to aging, cancer and atherosclerosis, mainly because they can induce the

oxidative damage of cell membranes, DNA, and proteins [25]. Several studies have shown that phenolics are the bioactive compounds that offer more benefits to human health and many authors have reported a direct relationship between the phenolic compounds and antioxidant activity [26–28]. In agreement, in this study the IC50 values of luteolin obtained from the ABTS assay were significant, showing that this flavonoid presents an important antioxidant activity.

Multiple mechanisms may underlie the antioxidant effect of luteolin. Luteolin functions as a ROS scavenger through its own oxidation and possesses the essential structures to antioxidant activity of flavonoids: 3',4'-hydroxylation, the presence of a double bond between carbons 2 and 3 and a carbonyl group on carbon 4. The hydrogen atom from an aromatic hydroxyl group can be donated to free radicals. As an aromatic compound, luteolin can support unpaired electrons around the electron system. Moreover, luteolin inhibits ROS generating oxidases and may directly inhibit enzymes that catalyze the oxidation of cellular components [11,17,29].

The free radical scavenging activity of luteolin was determined by ABTS radical cation decolorization assay and the IC50 value showed significant antioxidant activity when compared to standard quercetin. The results of minimal inhibitory concentration (MIC) determinations showed noticeable MIC values for luteolin against all bacterial and fungi tested. The effect of luteolin on the cell viability of human cervical adenocarcinoma cells line (HeLa cells) after a 24 h incubation period significantly inhibited cell growth at the tested concentration ($p < 0.05$). Cell injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) release after a 24 h incubation period. Luteolin significantly increased cell LDH release ($p < 0.05$), when compared with the untreated control. The hemolytic activity was investigated by measuring the lysis of a 10% (v/v) human red blood cells suspension in a spectrophotometric assay, and luteolin showed no significant effect on red blood cells lysis.

Multiple mechanisms may underlie the antioxidant effect of luteolin. Luteolin functions as a ROS scavenger through its own oxidation and possesses the essential structures to antioxidant activity of flavonoids: 3',4'-hydroxylation, the presence of a double bond between carbons 2 and 3 and a carbonyl group on carbon 4. The hydrogen atom from an aromatic hydroxyl group can be donated to free radicals. As an aromatic compound, luteolin can support unpaired electrons around the electron system. Moreover, luteolin inhibits ROS generating oxidases and may directly inhibit enzymes that catalyze the oxidation of cellular components [29]. Luteolin showed

antimicrobial activity, and antioxidant activity may be important in the antimicrobial mode of action of this phenolic compound. A hypothesis is that during stress, microorganism respond by controlling secondary metabolite production and authors have also linked the antioxidant activity of phenolic compounds to their activity on metabolites biosynthesis [30]. Luteolin showed activity against all *Candida* species tested and the microscopy observations showed that this flavonoid was able to inhibited hyphal formation during the hyphal induction of *C. albicans*. *C. albicans* is a dimorphic yeast and its ability to switch from yeast cells to hyphae is considered to be important for the interactions with its host [31].

The luteolin showed very low hemolytic activity against human blood cells. On the other hand, flavonoids have been revealed to protect biological cell membranes against free radical-induced oxidative damage. They scavenge reactive oxygen species and, in some cases, their interaction with cellular proteins has been suggested, especially with heme proteins, which exert their physiological functions by the oxidation and reduction of heme iron [32,33].

Antioxidant activity is involved in cancer prevention at the initiation stage while antiproliferative activity is targeting cancer cells at the promotion and progression stages. Luteolin showed antiproliferative activity and the LDH leakage assay revealed that it has promoted rupture of the cell membrane in HeLa cells. This indicates that the flavonoid promptly initiated a series of cellular events leading to the inhibition of cell proliferation and/or the induction of cell death. Data from other cell culture studies strongly suggest that the mechanism whereby phenolic compounds modulate cell proliferation is remarkably dose-dependent [25-29].

CONCLUSION

The biological activity of luteolin depends on a complex sum of individual properties including its chemical, affinity for the target site, survival within the biological system, transport properties, and state of the target organism. The flavonoid showed a significant antioxidant activity, and it seems be essential for antibacterial, antifungal, non-hemolytic and antiproliferative activities. A better understanding of how antioxidant compounds inhibit microorganism growth and inhibit tumor cell proliferation will allow them to be more efficiently used and perhaps synergistically used with other antimicrobial, antiproliferative agents or their anti-hemolytic activity.

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Chapter 2

HEATH EFFECTS OF LUTEOLIN: NEW FINDINGS IN ANTICANCER AND CARDIOVASCULAR ACTIVITY

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ABSTRACT

Luteolin, a plant–derived polyphenolic flavonoid, is widely present in many fruits, vegetables and medicinal herbs. It has many beneficial health effects, including antioxidant, anti–inflammatory, anti–proliferative, proapoptotic, and anti–angiogenic activities. Observations published in the last ten years suggest that luteolin could be an anticancer agent for various cancers, and the anticancer property of luteolin is associated with the induction of apoptosis or autophagy, and inhibition of cell proliferation, metastasis and angiogenesis. Furthermore, luteolin has also been reported to exhibit cardiovascular protection activities. For instance, luteolin reduces vascular smooth muscle cells' proliferation and migration, and prevents ischemia-reperfusion injury by reducing necrosis and apoptosis in rat cardiomyocytes. Our previous report also indicated the anti-hypertension effect of luteolin. In fact, the antioxidant and anti-

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inflammatory activity may be also linked to both the anticancer and cardiovascular protection property of luteolin. This chapter will discuss the potentially activities and mechanisms of luteolin as a candidate of antineoplastic drug or cardiovascular protective drug.

1. INTRODUCTION

Flavonoids are ubiquitous in nature. They are widely presented in food, providing an essential link between diet and prevention of chronic diseases including cancer and cardiovascular disease. Luteolin (3', 4', 5, 7-tetrahydroxyflavone), a kind of naturally occurring flavonoid, is enriching in many natural plants [1]. Actually, luteolin and luteolin glycosides have a wide distribution in the plant kingdom and mainly exist in many plant families in higher plants and have been identified in Bryophyta, Pteridophyta, Pinophyta and Magnoliophyta [1], for example, *Eclipta prostrata* L. [2], *Coix lachryma-jobi* L. [3], *Sideritis scardica* [4], *Schizonepeta tenuifolia* Briq. [5], *Melampyrum pretense* [6], *Scutellaria baicalensis* [7], *Scutellaria immaculata* and *S. ramosissima* (Lamiaceae) [8], *Eminium spiculatum* (Blume) Kuntze (family Araceae) [9], *Sasa senanensis* Rehder [10], *Scutellaria barbata* [11], *Rosmarinus officinalis* [12], *Chionanthus retusus* [13], and *Carex folliculata* seeds [14]. Moreover, luteolin and its glycosides are often preferentially present in certain fruits and food items, especially in olive [15], honey [16] and red wine.

Luteolin possesses a variety of pharmacological activities, such as anti-inflammatory [17-20], anti-cancer ability [21-23], affecting bone remodeling [24], neuroprotective effect [25-27], anti-Hepatitis C Virus (HCV) activity [28], antioxidant activity [8,29], anti-platelet aggregation [9], inhibiting lung fibrosis [30], protection of renal injury [31], anti-diabetic properties [32,33], whitening activity [34], antidepressant-like effect [35] and other biological activities. In fact, luteolin is one of the most important active components in natural herbs, and anti tumor and cardiovascular are not surprising that the two main areas of research on the biological actions of luteolin.

Anticancer effects of luteolin and its glycosides depend on several factors: the chemical structure and concentration, the type of cancer, and also on the methylated degree of flavonoids [36]. Luteolin can be considered as promising candidate agents for treatment of breast cancer, prostate cancer and ovarian cancers, respectively, and it has good perspectives as potent antitumor agents for several types of cancers. In most cases, malignant cells from different

tissues reveal somewhat different sensitivity toward luteolin and, for example, the cytotoxic effect of luteolin on breast and prostate cancer cells are highly related to the expression of hormone receptors. Therefore, the preference of luteolin to various human cancer types is analyzed in this chapter.

Epidemiological and animal studies point to a possible protective effect of luteolin against cardiovascular diseases. Significantly, much of the activity of luteolin appears to impact blood and microvascular endothelial cells. Many epidemiological studies have shown that regular flavonoid intake is associated with a reduced risk of cardiovascular diseases. In coronary heart disease, the protective effect of luteolin mainly includes antithrombotic, anti-ischemic, anti-oxidant, and vasorelaxant. It is suggested that luteolin decrease the risk of cardiovascular disease by several major actions: inhibiting myocardial apoptosis, preventing the inflammatory process, improving coronary vasodilatation, decreasing the ability of platelets in the blood to clot, and preventing low-density lipoproteins (LDLs) from oxidizing.

Herein, we searched the PubMed database to summarize and investigate the mechanisms of action of luteolin to accelerate the discovery of anticancer or cardioprotective drugs derived from luteolin and its glycosides. We propose that the development of luteolin into new anticancer or cardioprotective agents has a bright future despite some difficulties.

2. ANTICANCER EFFECTS OF LUTEOLIN AND THE RELATED MECHANISMS

Multiple mechanisms may underlie luteolin's anticancer effect. The anti-tumor potential of luteolin to inhibit angiogenesis, to induce apoptosis and autophagy, to arrest cell cycle, to inhibit cell adhesion and invasion, to protect from carcinogenic agents in animal models, to inhibit fatty acid synthase (FAS), to reduce tumor growth in vivo and to sensitize tumor cells to the chemotherapy of some anticancer drugs suggests that this flavonoid has cancer chemopreventive and chemotherapeutic potential [37]. Modulation of ROS levels, inhibition of topoisomerases I and II, reduction of nuclear factor kappa B (NF-κB) and AP-1 activity, stabilization of p53, and inhibition of phosphatidylinositol 3’-kinase (PI3K), signal transducer and activator of transcription (STAT3), insulin-like growth factor I receptor (IGF1R) and human epidermal growth factor receptor 2 (HER2) are possible mechanisms involved in the biological activities of luteolin.

2.1. Luteolin on Tumor Proliferation

It is well demonstrated that luteolin inhibits cell proliferation and induces apoptosis in various types of cancer cells. Luteolin sensitizes cancer cells to therapeutic-induced cytotoxicity through suppressing cell survival pathways such as PI3K/Akt, NF-κB, and X-linked inhibitor of apoptosis protein (XIAP), and stimulating apoptosis pathways including those that induce the tumor suppressor p53. Choi et al. investigated the molecular mechanisms of the anti-cancer effect of luteolin in Neuro-2a mouse neuroblastoma cells, and suggested that luteolin induces apoptosis through ER stress and mitochondrial dysfunction in Neuro-2a mouse neuroblastoma cells. Luteolin also induced activation of mitogen-activated protein kinases (MAPK) such as jun N-terminal kinase (JNK), p38, and extracellular regulated protein kinases (ERK), and inhibitors of MAPK reduced luteolin-induced cell death, mitochondrial BCL2-associated X protein (Bax) translocation and cytochrome c release [38].

The anti-cancer cell proliferative potential of luteolin is also involved in its effect on cell cycle regulation. Luteolin inhibits cell proliferation through the activation of a $p16^{INK4A}$ -dependent cell cycle checkpoint signaling pathway orchestrated by UHRF1 and DNMT1 down-regulation [39]. Luteolin is also recognized as an inhibitor of cyclin-dependent kinase 9 (CDK9) and block phosphorylation of the carboxy-terminal domain of RNA polymerase II at Ser^2 in Polier and his coworker's research [7]. Luteolin inhibited cell cycle progression at G1 phase and prevented entry into S phase in a dose- and time-dependent manner. Luteolin treatment led to down-regulation of cyclin D1, leading to reduced cyclin-dependent kinase 4/6 (CDK4/6) activity and suppression of retinoblastoma protein (Rb) phosphorylation, and subsequently inhibition of the transcription factor E2F-1. Luteolin was also capable of suppressing Akt phosphorylation and activation, resulting in de-phosphorylation and activation of glycogen synthase kinase-3β (GSK-3β). Activated GSK-3β then targeted cyclin D1, causing phosphorylation of cyclin D1 at Thr(286) and subsequent proteasomal degradation. Taken together, luteolin was able to abrogate the effect of insulin on the Akt/GSK-3β/Cyclin D1 pathway, resulting in suppression of insulin-induced cell proliferation [40].

Luteolin triggers both endoplasmic reticulum stress-related apoptosis and non-canonical autophagy, which function as a cell death mechanism in NCI-H460 human lung cancer cells. It was shown that luteolin induces apoptotic cell death through modulating both the extrinsic pathway and intrinsic pathways, which are suppressed by z-VAD-fmk (Caspase Inhibitor), indicating that luteolin triggers caspase-dependant apoptosis. Furthermore, the α subunit

of the eukaryotic initiation factor 2/ CCAAT/enhancer-binding protein (eIF2α/C/EBP) homologous protein pathway, but not the JNK pathway, played a critical role in induction of apoptosis by luteolin. Notably, luteolin also increases the accumulation of microtubule-associated protein light chain-3 (LC3) II protein and induces Beclin-1-autophagy as a cell death mechanism [41].

Luteolin and its derivatives are also recognized as a kind of small molecule Bcl-2 protein inhibitor [42]. The SAR (structure-activity relationship) analysis results indicated that luteolin and its derivatives with a benzyl group introduced to the B ring, were new small molecule Bcl-2 protein inhibitors, and their anti-tumor activity was likely related to their effect on the Bcl-2 protein. Polier et al. found that anti-cancer flavones, e.g., wogonin, baicalein, apigenin, chrysin and luteolin enhance ABT-263-induced apoptosis in different cancer cell lines and in primary AML and ALL cells by down-regulation of Mcl-1 (a Bcl-2 family protein which can act as an apical molecule in apoptosis control, promoting cell survival) expression [43]..

Furthermore, luteolin is recognized as a radiotherapy and chemotherapy sensitizer. Pretreatment of BxPC-3 human pancreatic cancer cells with low concentrations of luteolin effectively aid in the anti-proliferative activity of chemotherapeutic drugs. Pretreatment of cells with 11-19 μM of either flavonoid for 24h resulted in 59-73% growth inhibition when followed by gemcitabine (10 μM, 36h). Luteolin (15 μM, 24h) pretreatment followed by gemcitabine (10 μM, 36h), significantly decreased protein expression of nuclear GSK-3β and NF-κB p65 and increased pro-apoptotic cytosolic cytochrome c [44]. Luteolin also have anti-proliferative and chemosensitizing effects on human gastric cancer AGS cells, the effects were involved in including Caspase-3, 6, 9, Bax, and p53, and decreased the levels of anti-apoptotic protein Bcl-2, thus shifting the Bax/Bcl ratio in favor of apoptosis [45]. Another result suggested that luteolin effectively blocked SMC3-induced NF-κB activation and expression of anti-apoptotic NF-κB targets, therefore, combination of SMC3 and luteolin is an effective approach for improving the anticancer value of SMC3, which has implications in cancer prevention and therapy [46].

The combination of luteolin and (-)-epigallocatechin-3-gallate (EGCG) has synergistic/additive growth inhibitory effects and provides important basis for future application of luteolin in chemoprevention trials of head and neck and lung cancers [47]. The combination of luteolin and EGCG at low doses (only induce minimal apoptosis for each single agents) synergistically increased the mitochondria-dependent apoptosis (3-5-fold more than the

additive level of apoptosis) of head and neck and lung cancer cell lines. It also significantly reduced the volume of xenografted tumors in nude mice by inhibition of Ki-67 expression and increase in TUNEL-positive cells in xenografted tissues. Moreover, luteolin promoted stabilization and ATM-dependent Ser (15) phosphorylation of p53 due to DNA damage, and ablation of p53 using shRNA strongly inhibited apoptosis as evidenced by decreased poly (ADP-ribose) polymerase and caspase-3 cleavage. In addition, the mitochondrial translocation of p53 was limited by treatment with luteolin or the combination of EGCG and luteolin.

2.2. Luteolin on Tumor Differentiation

Inducing tumor differentiation is also involved in the luteolin's anti-tumor activity. Researchers provided the mechanisms about how luteolin attenuated the epithelial-mesenchymal transition (EMT) of A549 lung cancer cells induced by transforming growth factor-β1 (TGF-β1). Pretreatment of luteolin prevented the morphological change and downregulation of E-cadherin of A549 cells induced by TGF-β1. In addition, the activation of PI3K-Akt-IκBa-NF-κB-Snail pathway which leads to the decline of E-cadherin induced by TGF-β1 was also attenuated under the pretreatment of luteolin [48].

2.3. Luteolin on Tumor Metastasis

Available scientific evidence indicates that flavonoids are a ubiquitous dietary phenolics subclass and exert extensive in vitro anti-invasive and in vivo anti-metastatic activities. Flavonoids in dietary vegetable foods that are responsible for anti-invasive and anti-metastatic activities of tumors include luteolin, apigenin, myricetin, tangeretin, kaempferol, glycitein, licoricidin, daidzein, and naringenin [49]. Inside, luteolin has anti-metastatic effect in several cancer cell lines and may have considerable potential for development as an anti-metastatic agent. An in vivo mouse study showed that oral administration (10 or 50 mg/kg) of luteolin significantly inhibited tumor nodules and tumor volume of lung metastasis induced by intravenous injection of CT-26 cells. Mechanically, luteolin suppresses matrix metalloproteinase (MMP)-2 and -9 activities and invasion in murine colorectal cancer CT-26 cells. Western blot and kinase assay data revealed that luteolin inhibited Raf and PI3K activities and subsequently attenuated phosphorylation of MEK and

Akt. Result from pull-down assay also indicated that luteolin non-competitively bound with ATP to suppress Raf activity and competitively bound with ATP to inhibit PI3K activity [50]. Luteolin also prevent the migration of glioblastoma cells by affecting PI3K/AKT activation, modulating the protein expression of Cdc42 and facilitating their degradation via the proteaosome pathway [51].

Luteolin also reduced cellular adhesion, it was identified as an active inhibitors of ICAM-1 expression, and the important role of –OH group at positions 5 and 7 of A ring and at position 4 of B ring is noted [52]. The fundamental mechanisms of luteolin's strong anticancer effect are also related with its inhibition of angiogenesis. Luteolin inhibited HUVEC proliferation and vessel growth in CAM in vivo. In addition, the secretion of VEGF by human pancreatic carcinoma cells was downregulated by luteolin. In a co-culture system, pretreated pancreatic carcinoma cells with luteolin, could decrease the capillary-like structure formation by HUVEC [53].

2.4. Luteolin on Tumor Metabolism

As a plant-derived flavonoid, luteolin is thought to inhibit tumor growth by regulating the tumor cell metabolism. AMP-activated protein kinase (AMPK) is a metabolic sensor and may prevent carcinogenesis via modulation of signaling networks. Research indicated that AMPK is a novel regulator of NF-κB in luteolin-induced cancer cell death. AMPK activity is critical for the inhibition of cancer cell growth by luteolin in HepG2 hepatocarcinoma cell, possibly via modulation of NF-κB activity. The strong inhibition effects of luteolin in HepG2 cell proliferation and tumor growth in a tumor xenograft model were accompanied by AMPK activation by luteolin. Luteolin also had a strong inhibitory effect on NF-κB, and inhibition of AMPK activity restored luteolin-inhibited NF-κB DNA-binding activity. Furthermore, luteolin treatment causes the release of reactive oxygen species (ROS) and that these intracellular ROS in turn mediate AMPK-NF-κB signaling in HepG2 hepatocarcinoma cells [54].

As a glycolysis inhibitor, luteolin can sensitize breast cancer cells to doxorubicin (Dox) and might be a new adjuvant agent for chemotherapy. In vitro, luteolin could reverse tumor resistance to Dox and promote death of tumor cells under hypoxia. In vivo, luteolin could offer superior efficacy and lesser toxicity of Dox in 4T1 and MCF-7 bearing mice. Further study showed that luteolin was able to suppress glycolytic flux but did not affect glucose

uptake, the P-glycoprotein, anti-oxidative enzymes under hypoxia in vitro, and had not also effect on the intratumor Dox level in vivo. In addition, the activity of superoxide dismutase (SOD) and carnitine acylcarnitine translocase (CAT) was increased in serum and was decreased in tumor by luteolin in vivo [55].

Lipornetabolism is also abnormal in cancer cell. The expression and activity of Fatty Acid Synthase (FASN; the sole enzyme capable of the reductive de novo synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and nicotinamide adenine dinucleotide phosphate -NADPH-, a key lipogenic enzyme overexpressed in many human cancers) is extremely low in nearly all nonmalignant adult tissues, whereas it is significantly up-regulated or activated in many cancer types, thus creating the potential for a large therapeutic index. Naturally occurring flavonoids (i.e., luteolin, quercetin, and kaempferol) are identified as a kind of FASN inhibitors and limit cancer cell growth by influencing fat metabolism [56]. In addition, as a typical polyphenolic compounds, luteolin may exert its cancer-preventive and antineoplastic effects by the FASN inhibitory property. Koen Brusselmans et al. found that six flavonoids, epigallocatechin-3-gallate, luteolin, quercetin, kaempferol, apigenin, and taxifolin, markedly inhibited cancer cell lipogenesis. Interestingly, in both prostate and breast cancer cells, a remarkable dose-response parallelism was observed between flavonoid-induced inhibition of fatty acid synthesis, inhibition of cell growth, and induction of apoptosis [57].

2.5. Luteolin on Tumor Immunity and Inflammatory

Chronic inflammation can augment tumor development in various types of cancers, including prostate cancer and breast cancer. Researches indicated that the anti-inflammatory activity may be linked to its anticancer property. Reduction of inflammation is therefore an important anticancer therapeutic opportunity. Previous studies have shown that luteolin has cancer prevention and anti-inflammatory effects. Osman et al. investigated the effect of luteolin supplementation and Aspirin treatment in dimethylhydrazine (DMH)-induced carcinogenesis in rats. The supplementations of luteolin enhance the anti-inflammation effect to Aspirin in the treatment of DMH-induced carcinogenesis in rats [58]. DMH (20 mg/kg/body weight/week, i.g) injections induce colon polyps and renal bleeding in rats, significantly increasing carcinoembryonic antigen (CEA), cyclooxygenase-2 (COX-2), oxidative stress, and kidney function tests and reducing antioxidant markers. Either

Aspirin (50 mg/kg/body weight/week) or luteolin (0.2 mg/kg/body weight/day) gavages alone or combined for 15 weeks produce a significant decrease in colon polyp number and size, significantly decreasing CEA, COX-2, and oxidative stress and increasing antioxidant markers.

STAT3 is involved in many cellular functions. It regulates genes that are involved in cell growth and division, cell movement, and the self-destruction of cells (apoptosis). In the immune system, the STAT3 protein is involved in the regulation of inflammation, which is one way the immune system responds to infection or injury. Yang et al. used paclitaxel combined with luteolin to treat human breast cancer MDA-MB-231 cells. Luteolin alone demonstrated an anti-proliferative effect. Co-administration of luteolin and paclitaxel resulted in an increase in apoptosis compared with the treatment of paclitaxel alone as evidenced by the results of a diamidino-2-phenylindole (DAPI) stain and Annexin-V-based assay. Moreover, immunoblotting analysis also showed that the co-administration of luteolin and paclitaxel activated caspase-8 and caspase-3 and increased the expression of Fas. Furthermore, the increased expression of Fas due to co-administration was shown to be due to the blocking of STAT3. Finally, combination therapy with luteolin and paclitaxel significantly reduced tumor size and tumor weight in an orthotopic tumor model of MDA-MB-231 cells in nude mice [59].

2.6. Other Activities

Research shows that 23 percent of cancer patients take antioxidants. The use of antioxidants as an adjunct to conventional or as an integral part of alternative cancer therapy is an area of intense research. A major mechanism in the cellular defense against oxidative or electrophilic stress is activation of the Nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element signaling pathway. Nrf2 is over-expressed in many types of tumor, promotes tumor growth, and confers resistance to anticancer therapy. Hence, Nrf2 is regarded as a novel therapeutic target in cancer.

Luteolin was previously reported as a strong inhibitor of Nrf2 in vitro, it is also showed that luteolin reduced the constitutive expression of NAD (P) H quinone oxidoreductase 1 in mouse liver in a time- and dose-dependent manner. Further, luteolin inhibited antioxidant enzymes and glutathione transferases expression, limiting the reduced glutathione in the liver of wild-type mice under both constitutive and butylated hydroxyanisole-induced conditions. Luteolin inhibits the Nrf2 pathway in vivo and can serve as an

adjuvant in the chemotherapy of NSCLC. Oral administration of luteolin, either alone or combined with intraperitoneal injection of the cytotoxic drug cisplatin, greatly inhibited the growth of xenograft tumor from non-small-cell lung cancer (NSCLC) cell line A549 cells grown subcutaneously in athymic nude mice. Cell proliferation, the expression of Nrf2, and antioxidant enzymes were all reduced in tumor xenograft tissues [60].

Luteolin also inhibits Azoxymethane-induced colorectal cancer through activation of Nrf2 signaling. Colorectal cancer (CRC) was induced by administration of Azoxymethane (AOM) (15 mg/kg body weight) intraperitoneally (i.p.) once a week for three weeks. Luteolin administration (1.2 mg/kg body weight/day) significantly alleviated Phase1 enzymes in colon and liver, it increased the levels of phase 2 enzymes. Luteolin modulates the expressions of glutathione S-transferase (GST) -α, μ and also the expression of Nrf2. Collectively, results of our hypothesis shows that luteolin is a novel candidate for treating CRC [61].

3. The Effect of Luteolin on Different Human Cancer Types

Different malignant cells from different tissues are often show different sensitivity toward luteolin, and knowledge about the malignant tissue-specific anticancer effects of luteolin could be purposely applied both in chemoprevention as well as in cancer treatment.

3.1. Breast Cancer

Luteolin possesses potent estrogen agonist activity and could contribute to endocrine disruptors, it displays progesterone antagonist activity beneficial in a breast cancer model but deleterious in an endometrial cancer model [62]. Luteolin effectively suppresses MDA-MB-231 estrogen receptor (ER) negative breast tumor growth both in vitro and in vivo, and its anticancer activity may be partly derived from inhibitory effects on epidermal growth factor receptor (EGFR)-mediated cell survival. Study results showed that luteolin suppresses (3) H thymidine incorporation indicating cell growth inhibition, and this was accompanied by cell cycle arrest at the G2/M and S stages and apoptotic activity. Further analyses showed that luteolin exhibited

cell cycle arrest and apoptotic activity by decreasing Akt, polo-like kinase 1(PLK1), cyclin B, cyclin A, cell division control protein 2 (CDC2, also known as cyclin-dependentkinase 1, Cdk1), CDK2, and Bcl-xL expression and increasing p21 and Bax expression. Underlying mechanisms of action exerted by luteolin included the down-regulation. EGFR mRNA expression followed by the inhibition of EGF-induced MAPK activation, including the phosphorylation of ERK, p38 and Akt. Luteolin-supplementation at 0.01% or 0.05% significantly reduced tumor burden in nude mice inoculated with MDA-MB-231 cells. In conclusion, luteolin effectively suppresses MDA-MB-231 ER-negative breast cancer cell growth [63].

Luteolin (30mg/kg, p.o.), combined with cyclophosphamide (10mg/kg, i.p.) (LU+CYC) orally administered for 20 days provided antioxidant defense with strong chemopreventive activity against the genesis of 7, 12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in Wistar rats [64]. Luteolin showed the highest degree of cytotoxicity in the absence of considerable DNA damage, inhibited topoisomerase IIa and IIb gene expression to variable extents and with variable specificity in a canine macrophage/monocytic tumour cell line (DH82).

3.2. Liver Cancer

Numerous studies have demonstrated that luteolin inhibit the cell proliferation and tumor growth in liver cancers. Cell proliferation of liver cancer cell lines SMMC-7721 and BEL-7402 were inhibited by luteolin with dose-time-dependent manner. Luteolin could arrest the cells at G1/S stage, reduce mitochondrial membrane potential, and induce higher apoptosis rate and the typical apoptotic morphological changes of the liver carcinoma cells, and the mechanism maybe through enhancing Bax level, reducing anti-apoptotic protein Bcl-2 level, resulting in activating caspase-3 enzyme and decrease of mitochondrial membrane potential, and finally leading to cell apoptosis [65]. Otherwise, Proteomic analysis revealed that PRDX6 and PHB were key targets of luteolin that the mechanism of luteolin-induced apoptosis in Huh-7 cells is mediated through effects involving intracellular ROS [66]. The Inhibitory effect of luteolin on hepatocyte growth factor (HGF) /scatter factor-induced HepG2 cell invasion was also involving both MAPK/ERKs and PI3K-Akt pathways. Luteolin suppressed the phosphorylation of c-Met, the membrane receptor of HGF, as well as ERK1/2 and Akt, but not JNK1/2, which is activated by HGF [67].

3.3. Lung Cancer

Lung cancer is the top cause of cancer death in United States [68]. There are two main types of lung cancer, small cell lung cancer (SCLC) and NSCLC. NSCLC is one of the most commonly diagnosed malignancies in the world. Small-molecule inhibitors of the EGF receptor's tyrosine kinase domain (TKIs), including luteolin and quercetin, have been widely used for treating NSCLC. Unfortunately, nearly all patients after initially experiencing a marked improvement while on these drugs, eventually progress to acquire resistance to TKIs. Hong et al. discovered that luteolin may be a potential candidate for NSCLC therapy, especially for treatment of patients with acquired erlotinib-resistant NSCLC. Luteolin exerted significant anti-tumourigenic effects on the EGF receptor L858R/T790M mutation and erlotinib-resistant NSCLC both at the cellular and animal levels. Mechanistically, luteolin induced degradation of the EGF receptor by inhibiting the association of Hsp90 with the mutant EGF receptor, and, therefore, prevented PI3K/Akt/mTOR signalling, which resulted in NSCLC cell apoptosis [69].

Luteolin inhibited the growth of human alveolar basal epithelial cell A549 cells by inducing G1 phase cell cycle arrest and apoptosis. Furthermore, stress fiber assembly and cell migration in A549 cells was markedly suppressed by luteolin [70]. Leung et al.'s experiments found that luteolin-induced human lung squamous carcinoma CH27 cell apoptosis was accompanied by activation of antioxidant enzymes, such as SOD and catalase, but not through the production of ROS and disruption of mitochondrial membrane potential. Therefore, the effects of luteolin on CH27 cell apoptosis were suspected to result from the antioxidant rather than the prooxidant action of luteolin [71].

3.4. Prostate Cancer

Lin FM et al. indicated that luteolin from *Wedelia chinensis* could inhibit the proliferation of androgen receptor (AR)-dependent prostate cancer cells, and the 50% inhibition concentration value was 2.4 μM [72]. Chin-Hsien Tsai et al. indicated that exposure of prostate cancer cells to *W. chinensis* extract (enriched with wedelolactone, luteolin, and apigenin) induced apoptosis selectively in AR–positive prostate cancer cells and shifted the proportion in each phase of cell cycle toward G2-M phase in AR-negative prostate cancer cells. Oral administration of herbal extract (4 or 40 mg/kg/d for 24-28 days)

attenuated the growth of prostate tumors in nude mice implanted at both subcutaneous (31% and 44%, respectively) and orthotopic (49% and 49%, respectively) sites. Results indicated that tumor suppression effects of luteolin were associated with increased apoptosis and lower proliferation in prostate cancer cells as well as reduced tumor angiogenesis. The antitumor effect of *W. chinensis* extract was correlated with accumulation of the principle active compounds wedelolactone, luteolin, and apigenin in vivo [73].

Tsui et al. determined the antitumor function of luteolin via upregulation of prostate-derived Ets factor (PDEF) gene expression in human prostate carcinoma LNCaP cells, PDEF is a novel epithelium-specific Ets transcription factor, acts as an androgen-independent transcriptional activator of the prostate-specific antigen (PSA) promoter. Luteolin blocks PSA gene expression by downregulation of AR expression. Results revealed that luteolin treatments attenuated cell proliferation and arrested the cell cycle at the G1/S phase, and induced cell apoptosis in high concentration (30 μM). Luteolin upregulated PDEF but downregulated AR gene expression, which decreased PSA gene expression in LNCaP cells. Luteolin also enhanced gene expression of PDEF, B-cell translocation gene 2 (BTG2), N-myc downstream regulated gene 1 (NDRG1) and Maspin, which markedly attenuated in vitro cell proliferation and invasion of LNCaP cells [74].

3.5. Skin Cancer

Like other flavonoids, luteolin can strongly increases the resistance of normal keratinocytes, against UVB-induced apoptosis, and derivatives may be used in sun protection products for prevention of skin cancer and early skin ageing [75]. The flavonoids such as diosmetin, apigenin, acacetin and luteolin isolated from *Chrysanthemum morifolium* were found to be active in inhibiting c-Kit both at enzyme and cellular levels. In addition, these flavonoids attenuated SCF-induced proliferation of human primary melanocytes without toxicity and suppressed ultraviolet (UV)B irradiation-mediated melanin synthesis significantly, have potential to suppress SCF-/UVB-induced melanogenesis [76]. George et al. investigated the effects of luteolin in HaCaT (human immortalized keratinocytes) and A375 (human melanoma) cells, and suggested that luteolin inhibits cell proliferation and promotes cell cycle arrest and apoptosis in skin cancer cells with possible involvement of programmed cell death [77]. As a TKI, luteolin also inhibits the EGF-induced tyrosine phosphorylation, suppresses the cellular protein phosphorylation and secretion

of MMPs, leads to the retard of hman skin basal cell carcinoma A431 cell growth and metastasis. SAR results reveal that the double bond between C2 and C3 in ring C and the OH groups on C3' and C4' in ring B are critical for the biological activities [78].

3.6. Other Cancers

Luteolin is also a candidate for the prevention and treatment of other types of cancer, such as human cervical cancer, human esophageal squamous carcinoma cell, colonel cancer, ovarian cancer and oral squamous cancer. Luteolin induces growth inhibition and apoptosis of human esophageal squamous carcinoma cell line Eca109 cells in vitro. MTT assays showed that luteolin had obvious cytotoxicity on Eca109, the IC50 was 70.7±1.72 μM at 24 h. Luteolin arrested cell cycle progression in the G_0/G_1 phase and prevented entry into S phase in a dose- and time-dependent manner. Moreover, luteolin downregulated the expression of cyclin D1, survivin and c-myc, and it also upregulated the expression of p53, in line with the fact that luteolin was able to inhibit Eca109 cell proliferation [79].

Pandurangan et al. analyzed the in vitro anticancer and apoptosis-inducing property of luteolin using HCT-15 colon adenocarcinoma cells. Luteolin at the IC_{50} concentration (100 μM) decreased the expressions of non-P-β-catenin, phosphorylated (inactive) glycogen synthase kinase-3β, and cyclin D1 expressions in HCT-15 cells. Luteolin also promoted substantial cell cycle arrest at the G2/M phase in HCT-15 cells, and it induces apoptosis in HCT-15 cells by enhancing the expression of Bax and caspase-3, and suppressing the expression of Bcl-2 [80].

Epithelial ovarian cancer (EOC) is the leading cause of death among gynecological malignancies worldwide. Based on widely published in vitro and mouse-model data, some anti-inflammatory phytochemicals, such as luteolin, appear to exhibit activity in modulating the tumor microenvironment. Luteolin represses NF-κB and inhibit proinflammatory cytokines such as TNF-α and IL-6. Additionally, Luteolin stabilizes p53 protein, sensitizes TRAIL (TNF receptor apoptosis-inducing ligand) induced apoptosis, and prevents or delays chemotherapy-resistance. Recent studies further indicate that luteolin potently inhibits VEGF production and suppress ovarian cancer cell metastasis in vitro [81]. In addition, luteolin induces apoptosis in oral squamous cancer SCC-4 cells by decreasing the expression of CDKs, cyclins, and phosphor-Rb anti-apoptotic protein, but increased the expression of pro-apoptotic proteins

and activated caspase 9 and 3, with a concomitant increase in the levels of cleaved poly-ADP-ribose polymerase (PARP) [82]. Luteolin also shows a strong potent inhibitory activity in the proliferation of rat basophilic leukemia (RBL)-2H3 cells (IC_{50}=1.5 μg/mL) [3].

4. Cardiovascular Protection Effects of Luteolin and the Related Mechanisms

Chinese medicine has numerous applications in the cardiovascular system, and it is important to find new, efficient, and affordable medications with low toxicity. It has been reported that intake of flavonoids, plant polyphenolic compounds abundant in fruits and vegetables, provide protective effects against oxidative stress, apoptosis and atherosclerosis (AS) [83-85]. Luteolin is an important plant compound postulated to be one of the constituents responsible for the biochemical benefits of eating vegetables and fruits, and has been reported to possess a variety of biological and pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, anti-allergic, anti-platelet, and a number of other activities [1, 21, 86]. One of the most well-known benefits of luteolin is for cardiovascular health [87].

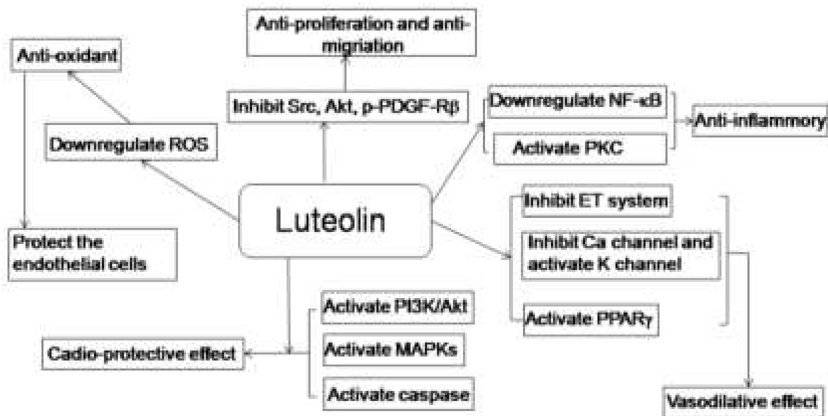

Figure 1. Diagram shows cardiovascular protection effects of luteolin and the related mechanisms.

As shown in figure 1, previous studies have shown that the anti-inflammatory effects of luteolin may result in the favorable outcomes in the

treatment of atherosclerosis [88], acute coronary syndrome [89], and atrial arrhythmias [90]. The anti-oxidant effects of luteolin may also account for ameliorating the development of atherosclerosis [88]. Furthermore, it has been reported that luteolin exerted anti-amnesic effects against $A\beta_{25\text{-}35}$-induced in mice [91]. The protective effects of luteolin on the cardiovascular system have been previously described and its use as a therapeutic agent to mitigate cardiovascular disease and other vascular dysfunctions are currently being investigated. Here, we focus mainly on the effects of luteolin on the cardiovascular system.

4.1. Luteolin on VSMCs Proliferation and Migration

Previous studies had shown that luteolin could inhibit vascular smooth muscle cells (VSMCs)' proliferation and migration, and had the capacity to interact with multiple molecular targets, which involved diverse intracellular pathways.

It has been reported that up-regulations of platelet-derived growth factor (PDGF) ligand and receptor gene expression are associated with the development and progression of proliferative cardiovascular diseases, including hypertension [92] and atherosclerosis [93, 94]. Kim et al. tested the effects of luteolin on rat VSMCs in culture and aimed to explore the mechanisms by which the cell cycle was affected by luteolin [95,96]. MAPK is an important player in the early intracellular mitogenic signal transduction for cell growth. Among the MAP kinase family, ERK1/2 has been implicated in proliferation of various cell types [97]. Therefore, they examined whether the anti-proliferation of luteolin could act through down regulation of ERK1/2 cascade. Luteolin inhibited the PDGF-BB-induced ERK1/2 activation. The data showed that the blocking of ERK1/2 might be important in the anti-proliferating activity of luteolin. In addition, they also determined the phosphorylated phospholipase C (PLC)-γ1 and Akt, both of which were also activated by PDGF-BB. Moreover, luteolin showed a marked decrease in the PDGF-BB induced phosphorylation of PLC-γ1 and ERK1/2. These data indicated that PDGF receptor beta (PDGF-Rβ) might be a potential target for luteolin. Similarly, Lamy and coworkers found that luteolin inhibited PDGF-BB–dependent tyrosine phosphorylation of PDGF-Rβ in smooth muscle cells and that this inhibitory effect was associated with an impairment of downstream signaling events triggered by this receptor, such as phosphorylation of the $p42^{MAPK}$ and $p44^{MAPK}$ forms (ERK-1/2) and of Akt

[98]. The inhibitory effect of luteolin on the PDGF-BB-induced proliferation and migration of VSMCs may be mediated by blocking phosphorylation of PDGF-Rβ. The Akt signaling pathway is involved in the regulation of multiple biological processes, including cell survival, proliferation, migration and glycogen metabolism [99]. Akt is essential for VSMCs' proliferation and migration, and the ablation of Akt leads to a severe lesion in atherosclerosis and occlusive artery disease [100]. It has been found that luteolin inhibit the migration and proliferation of VSMCs or human umbilical vein endothelial cells (HUVECs) induced by Angiotensin II (Ang II) through the down-regulation of the PI3k/Akt signaling pathway [101, 102]. In the experiments, luteolin could suppress the Ang II-induced up-regulation of Nox4, p-Src, p-Akt and vascular endothelial growth factor (VEGF) expression in ECs. Similarly, another study reported that the activation of Src, phosphoinositide-dependent kinase-1 (PDK-1), Akt in the luteolin-treated group, which was significantly lower than that seen in the hydrogen peroxide (H_2O_2) group. It showed that luteolin suppressed H_2O_2-directed migration and proliferation in VSMCs partially due to down-regulation of the Akt and Src signalling pathways [103]. These results have demonstrated that luteolin's inhibition of VSMCs' proliferation and migration induced by H_2O_2 or Ang II is partially due to suppressing the Akt signaling pathways. To sum up, in response to the stimulation, such as H_2O_2, PDGF-BB and Ang II, the reactive oxygen species (ROS) can be produced by VSMCs and stimulate MAPKs and Akt. However, luteolin inhibits VSMCs' proliferation and migration induced by H_2O_2 or Ang II partially due to suppressing the Src and Akt signaling pathways. Meanwhile, the inhibitory effect of luteolin on the PDGF-BB-induced proliferation and migration of VSMCs may be mediated by blocking phosphorylation of PDGF-Rβ.

4.2. Luteolin on Endothelial Cells Dysfunction

Previous studies had reported that luteolin could prevent endothelial dysfunction induced by oxidative stress, which may be through inhibiting cell apoptosis and adhesion and protecting the endothelium.

Oxidant stress modulates pathologic processes, which is fundamental to development and progression of endothelial dysfunction, such as the oxidation of LDL, the loss of bioavailable nitric oxide (NO), and the vascular inflammatory response. Thereby therapeutic strategies to reverse endothelial dysfunction have begun to focus on agents with the ability to ameliorate

oxidant stress [104]. It has been found that luteolin can inhibit endothelial cell injuries induced by H_2O_2 [105-107]. Some studies reported it might be related with anti-oxidation due to increasing the concentration of NO [106, 107]. Chen and coworkers showed that luteolin activated Nrf2 signaling pathway to prevent oxidative-induced injury [105]. In addition, increased ROS caused the deterioration of the cerebral defense system and the blood-brain barrier (BBB) disruption [108], which also was proved by Liu and coworkers. They founded that luteolin inhibited ROS generation, increased SOD activity and preserved cerebral microvascular endothelial cells (CMECs) barrier function [109].

The apoptosis of endothelial cells is an important feature of endothelial dysfunction associated with the pathogenesis of vascular diseases and their complications, such as hypertension, atherosclerosis, diabetes, ischemia–reperfusion injury, and heart failure [110, 111]. Superoxide stress, caused by superoxide anion and its derivatives, ROS, is the most widely recognized source of dysfunction and apoptosis in the endothelium and the related pathogenesis.

He et al. showed that luteolin was an effective agent for the protection of endothelial cells from superoxide stress-induced apoptosis via the MAPK signaling pathway [112]. Song and coworkers reported that luteolin's apoptosis-suppressive effect was mediated through the blockage of a calcium/mitochondria/caspases- dependent pathway [113].

Endothelial dysfunction that initiates with the induction of cell adhesion molecules is one of the early events that lead to the development of the inflammatory reaction associated with atherosclerosis [114, 115]. It has been demonstrated that oxidative stress up-regulates cell adhesion molecule expression that is inhibited by antioxidant systems [116-119]. Jenong et al. revealed that luteolin inhibited THP-1 cell adhesion onto oxidised LDL-activated HUVEC and significantly abolished HUVEC expression of vascular cell adhesion molecule-1, E-selectin and lectin-type oxidized LDL receptor 1(LOX-1) enhanced by oxidized LDL [120].

In summary, luteolin can suppress oxidant stress, which induces cell apoptosis and adhesion, to exert the protection in endothelium and improve endothelial dysfunction.

4.3. Luteolin on Vasodilation

Previous studies had shown that luteolin had the vasorelaxant effect and owned the capacity to interact with multiple molecular targets, which involved

diverse intracellular pathways. Luteolin can exert vasodilative effect through NO production and release, ET-1 expression and secretion, and the inhibition of sarcolemmal calcium channels.

Blood pressure is controlled by the tonic constriction and dilation of blood vessels, vascular dysfunction and arterial vasodilation are associated with the development and progression of hypertension [121]. Enhanced production or attenuated degradation of ROS leads to oxidative stress, a process that affects endothelial and vascular function, and contributes to vascular disease. Moreover, NO, a product of the normal endothelium, is a principal determinant of normal endothelial and vascular function [122]. The endothelium is critically involved in modulating vascular relaxation through release of endothelial-derived NO [123].

In many experiments, aortic rings were pre-contracted with phenylephrine (PE) to investigate the vasoactive effects of luteolin and its mechanisms of action on the rat thoracic aorta [124-128]. They all founded that treatment with luteolin markedly inhibited the impairment of PE-induced endothelium dependent contraction in aortic rings. This mechanism of vasodilative effect of luteolin has been confirmed with the following researches. Qian et al. had reported that luteolin significantly inhibited the increase of ROS level and hydroxy radical ($OH^{-\bullet}$) formation, and the decrease of NO level, NOS and SOD activity caused by high glucose and improved endothelium-dependent vasorelaxation in diabetic rat aortic rings, which may be mediated by reducing oxidative stress and enhancing activity in the NOS–NO pathway [125]. Another study reported that luteolin protected resistance arteries from injury through reducing oxidative stress [126]. Other researches showed that luteolin could directly act on vascular endothelial cells (ECs), leading to NO production and subsequent vascular relaxation [124,129]. Moreover, Zhang et al. found that luteolin exerted the vasodilative effect through suppressing inflammatory inducible nitric oxide synthase (iNOS) expression [128]. In addition, suppressing the production of the vasoconstrictor peptide endothelin-1(ET-1) is another mechanism of luteolin's vasodilative effect. Takaharu and coworkers reported that luteolin could potently inhibit the secretion and gene expression of ET-1 in porcine aortic endothelial cells [130].

Peroxisome proliferator-activated receptor γ (PPARγ) is most abundantly expressed in adipose tissue and facilitate differentiation of fibroblasts into adipocytes [131]. PPARγ is also involved in the regulation of lipid metabolism, as ligand-dependent activation leads to an increase in genes that regulate fatty acid uptake and storage [132]. Over time, PPARγ was discovered to play an important role in the cardiovascular system. ET-1 is a

vasoconstrictive protein that can also regulate VSMC proliferation. PPARγ ligands attenuate both ET-1 expression and secretion in endothelial cells by blocking AP-1 signaling [133]. Conversely, endothelial cell–derived NO is a molecule that is a key participant in vasodilatory activity [134]. Subsequent studies showed that PPARγ ligands increase NO production and release [135]. Zhou et al. found that luteolin could prevent the impaired of endothelium dependent vasorelaxation of mice aorta induced by high fat diet, this effect might be due to the activation of PPARγ [136]. Therefore, we conclude that luteolin may exert vasodilative effect through the production of NO in endothelial cells due to activativing PPARγ.

There are two kinds of Ca^{2+} channels in the vascular smooth muscle: receptor-operated Ca^{2+} channels (ROCC) and voltage-dependent Ca^{2+} channels (VDCC), which can be activated by PE and high extracellular K^+, respectively. Influx of extracellular Ca^{2+} through ROCC and VDCC and release of Ca^{2+} from the sarcoplasmic reticulum result in increased intracellular Ca^{2+} concentration, which causes vascular smooth muscle contraction. PE caused aortic contraction through release of Ca^{2+} from the sarcoplasmic reticulum and by Ca^{2+} influx through ROCC. K^+ channels play important roles in the regulation of muscle contractility and vascular tone. In many instances, the vasodilation mediated by membrane hyperpolarization is attributed to a rise in K^+ permeability [137]. Direct activation of K^+ channels on arterial smooth muscle cells normally hyperpolarizes the cell membrane and thus inhibits Ca^{2+} influx through VDCC, which results vasorelaxation. The results show that luteolin induces relaxation in the rat thoracic aorta via a series of mechanisms, including the inhibition of sarcolemmal calcium channels, release of intracellular Ca^{2+} stores and activation of K^+ channels [127, 138].

In conclusion, luteolin can exert vasodilative effect through NO production and release induced by PPARγ activation or decline of ROS. Moreover, the vasoconstrictor peptide ET-1 suppressed by luteolin contributes to vasoreaxation. Furthermore, the inhibition of sarcolemmal calcium channels, release of intracellular Ca^{2+} stores and activation of K^+ channels by luteolin induce vascular relaxation.

4.4. Luteolin on Cardiomyocyte Apoptosis and Injury

To date, considerable research has indicated that apoptosis is involved in myocardial ischemia reperfusion injury (IRI) and that luteolin can play an

important anti-apoptotic role during IRI [139,140]. Until now it has been thought that myocardial apoptosis during ischemia/reperfusion (I/R) is mainly involved in the following signaling pathways: PI3K/Akt, MAPKs, caspase, janus kinase/signal transducer and activator of transcription(JAK/STAT), cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) and LOX-1 [141].

The PI3K/Akt signaling transduction pathway is considered to be the most important signaling pathway involved in controlling cell survival. As a critical regulator of PI3K, Akt can transduce the anti-apoptotic signals in cardiomyocytes, and activated Akt can inhibit cardiomyocyte apoptosis during I/R. It is assumed that the phosphorylation of Akt is down-regulated in myocardial IRI [141]. In recent years, a large number of experiments have confirmed that the anti-apoptotic effect of luteolin shown in myocardial IRI is related to the activation of PI3K/Akt signaling pathway [142-144]. Fang and coworkers confirmed that luteolin improved I/R-induced cardiomyocyte contractive function through the PI3K/Akt pathway during I/R [142, 143]. Cardiomyocyte shortening amplitude was indexed as the percentage reduction of cell length after stimulation, and luteolin prevented IRI by reducing necrosis and apoptosis in rat cardiomyocytes. Moreover, the cardioprotective effect of luteolin treatment was indeed mediated by the PI3K/Akt pathway. Inhibition of Akt activity markedly diminished the luteolin-induced positive contraction and inhibition of apoptosis in cardiomyocate following I/R.

The MAPK signaling pathway is believed to regulate the apoptosis of myocardial cells. MAPKs are serine/threonine protein kinases that are activated by phosphorylation on both a threonine and tyrosine residues. The kinase family has three members, including ERK, JNK /stress-activated protein kinases (JNK/SAPK) and the protein kinase p38 [141]. Type 1 protein phosphatase (PP1a) is present in the sarcoplasmic reticulum (SR) and it is the main phosphatase that dephosphorylates phospholamban (PLB), thereby impacting the activity and activity of cardiac sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) [145]. As a consequence, PP1a may also be a critical regulator of cardiac contractility. Wu et al. reported that pretreatment with luteolin or JNK inhibitor SP600125 significantly improved the contraction of the isolated heart and cardiomyocytes, reduced infarct size and lactate dehydrogenase (LDH) activity, decreased the rate of apoptosis and increased the Bcl-2/Bax ratio. However, pretreatment with ERK1/2 inhibitor PD98059 alone before I/R had no effect on the above indexes. Further, these consequences of luteolin pretreatment were abrogated by coadministration of PD98059. They also found that pretreatment with PD98059 caused a

significant increase in JNK expression, and SP600125 could cause ERK1/2 activation during I/R. In addition, luteolin affected PP1a expression, which resulted in the up-regulation of the PLB, thereby relieving its inhibition of SERCA2a. These results showed that luteolin improved cardiomyocyte contractile function after I/R injury by an ERK1/2-PP1a-PLB-SERCA2a mediated mechanism independent of JNK signaling pathway [146].

The caspase signaling pathway includes the mitochondria, death receptor and endoplasmic reticulum approaches [147]. The Bcl-2 family plays an important role in cell apoptosis, this family consists of both cell death promoters, such as Bax, Bad, and Bak, as well as cell death inhibitors, such as Bcl-2, Bcl-X, and ced-9. The relative ratio of anti-apoptotic proteins to pro-apoptotic proteins plays a key role in determining cell survival or death.[88] Many studies have showed that pretreatment with luteolin can promote the expression of Bcl-2 and Bad while down-regulating the expression of Bax thereby decreasing apoptosis. Meanwhile, the up-regulation of Bcl-2 expression can suppress the expression of caspase-8 and caspase-3, resulting in anti-apoptosis [142-144, 146, 148].

Besides the above mentioned common signaling pathways, more and more evidences have shown that there are other signaling pathways involved in the myocardial apoptosis during I/R, including NF-κB and ROS pathways. Despite these signaling pathways seldom being mentioned in the literature, their effects on I/R myocardial apoptosis have been confirmed [144, 149]. Evidence from various investigations of the myocardium suggests that ROS [150], which are formed within the ischemic myocardial cells and in the first few moments of reperfusion, are known to be cytotoxic to surrounding cells [151-155]. In addition, NO can inhibit mitochondria function and break single-stranded DNA. Moreover, NO and superoxide radicals can rapidly combine to form a strong reactive metabolite, peroxynitrite, which is a potent oxidant that can potentially cause membrane lipid peroxidation and lead to myocardial dysfunction [156, 157]. Liao et al. had reported that luteolin also down-regulated inducible NO synthase protein and mRNA expression, but did not significantly alter neuronal NO synthase or endothelial NO synthase expression. The results suggest that luteolin is capable of protecting the myocardium against IR injury. The actions of luteolin are at least partly mediated through down-regulation of NO production and its own antioxidant properties [149].

To sum up, luteolin can exert cardio-protection through suppressing anti-apoptosis during I/R, mediated with several of signaling pathways, such as PI3K/Akt, MAPKs and caspase.

4.5. Luteolin and Anti-inflammatory Effect

Previous studies had shown that luteolin could exert its anti-inflammatory effect, which involve several mechanisms. The following mechanisms can account for the effect.

Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) have emerged as significant contributors to myocardial dysfunction [158]. TNF-α is implicated in the pathogenesis of cardiovascular diseases, including congestive heart failure, acute myocardial infarction, myocarditis, and dilated cardiomyopathy and sepsis-associated cardiac dysfunction. Compelling evidence has identified the NF-κB as a key regulator of TNF-α gene activation [159,160]. Lv et al. founded that luteolin protected against lipopolysaccharide (LPS)-induced TNF-α expression via inhibition of the NF-κB signaling pathway, suggesting that luteolin may be a potential therapeutic agent for the treatment of inflammation related myocardial diseases [87].

During obesity, pro-inflammatory cells (M1 macrophages) accumulate largely, which increases the release of pro-inflammatory cytokines [161], such as TNF-α, monocyte chemoattractant protein 1 (MCP1) and interleukin-6 (IL-6) [162]. Meanwhile, anti-inflammatory cells (M2 macrophages) are decreasing. The imbalances make adipose tissue appear inflammatory [163], which induces the development of metabolic syndrome, such as diabetes and cardiovascular disease. As zhang et.al reported, dietary luteolin reduced high-fat-diet -induced adipose tissue macrophage (ATM) infiltration and mRNA levels of M1 macrophage marker genes. In LPS-stimulated conditions, luteolin inhibited the expression of pro-inflammatory cytokines and M1 marker genes in RAW264.7 macrophages. In contrast, the expression of M2 marker genes was enhanced by luteolin. However, these effects of luteolin were aborted by protein kinase C (PKC) activator PMA. They concluded that luteolin could inhibit obesity-associated ATM and RAW264.7 macrophage polarization in LPS-stimulated conditions through PKC pathway [164].

The expression of iNOS has been found in several human tumor tissues and inflammatory disorders [165], with the extent of NO production by iNOS often reflects the degree of inflammation [166]. In addition to iNOS, macrophages will overproduce inducible COX-2, which is involved in transforming arachidonic acid to prostaglandin H_2, a precursor of prostaglandin E2 (PGE_2), prostacyclin and thromboxane upon endotoxin stimulation. When living cells are exposed to an inflammatory stimulus, the production of PGE_2 is associated with the expression of COX-2 [167]. Compounds interfering with both iNOS and COX-2 generally act as potential

inhibitors of NF-κB activation [168]. The increasing researches have reported that luteolin can exert anti-inflammatory effect via suppressing the expression of COX-2 to down-regulate the expression of PGE_2 [167,169,170]. In addition, Zhang et al. showed that anti-inflammatory effect of luteolin was attributed to decrease of COX-2 due to the suppression of NF-κB activation [171].

To sum up, luteolin down-regulates NF-κB, resulting in a decrease in the expression of TNF-α and COX-2, which inhibits the development of inflammatory. Meanwhile, luteolin can suppress obesity-associated ATM polarization through PKC pathway.

4.6. Luteolin and Antioxidant Effect

The ROS include partially reduced forms of molecular oxygen, such as hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{-\bullet}$), H_2O_2, lipid peroxides, and hypochlorous acid (HClO). An overproduction of ROS from mitochondria by oxidative stress can cause damage to the lipids, the proteins and the DNA, which is implicated in the pathogenesis of several human diseases including atherosclerosis, cancer, neurodegenerative diseases and aging [172]. All kinds of cells have developed their own defensive mechanisms to counteract the ROS generation through the induction of intracellular phase II enzyme activities [173].

Among this family of enzymes, heme oxygenase-1 (HO-1) has exhibited a critical role in maintaining a cellular redox homeostasis against the oxidative stress [174]. In advance of the HO-1 protein induction, transcription is regulated by an inducible transcription factor, a nuclear factor-erythroid 2 p45-related factor 2 (Nrf2). Nrf2 is activated in response to various extracellular stimuli, including the oxidative stress [173]. In addition, the nuclear translocation of Nrf2 requires the activation of the upstream signaling molecules, such as MAPKs and PI3K [175].

Song et al. founded that luteolin potently strengthened the HO-1-mediated anti-oxidative potential through the modulation of the Nrf2/MAPK signaling pathways. Luteolin significantly up-regulated the HO-1 expression at 40 μM for 12h. The nuclear translocation of Nrf2 was initiated when RAW 264.7 cells were exposed against 40μM of luteolin and the Nrf2 activation was constantly increased by an uninterrupted exposure, which was in accordance with the accelerated HO-1 expression. This study further investigated whether the activation of the p38 and JNK MAPKs signaling pathways was a inevitable

event for the HO-1 expression through a pharmacological study applied to four well-known selective inhibitors, including U0126 (for ERK), SP600125 (for JNK), SB202190 (for p38) and LY294002 (for PI3K). The results showed that the addition of SB202190 and SP600125 significantly abolished the HO-1 protein expression induced by luteolin. These results suggest that the Nrf2 mediated HO-1 expression by luteolin is regulated by the p38 and JNK signaling pathway in RAW 264.7 cells, respectively [176].

5. THE EFFECT OF LUTEOLIN ON DIFFERENT CARDIOVASCULAR DISEASES

The preclinical studies of luteolin show that luteolin can prevent cardiovascular complication, such as hypertension, diabetes, atherosclerosis, IRI and arrhythmia, which were summarized in figure 2.

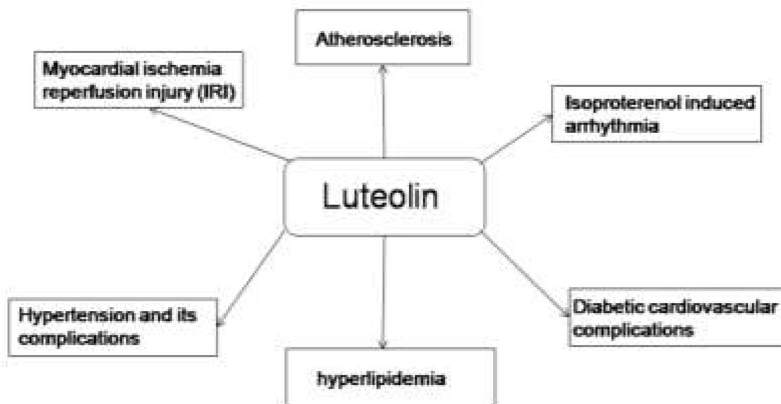

Figure 2. Diagram shows the effect of luteolin on different cardiovascular diseases.

5.1. Myocardial Ischemia Reperfusion Injury (IRI)

In recent years, luteolin has been shown to play an important role in the cardioprotection of IRI. Myocardial ischemia refers to a clinical state characterized by low coronary blood flow arising from various causes, but resulting in a lack of myocardial oxygen supply which can damage myocardial

structure and heart function. In most cases, the damaged structure can be repaired and heart function can be restored to its basal condition through ischemia/reperfusion (I/R) [153,177]. However, in some instances, I/R has the opposite effect, which not only fails to improve heart function but instead exacerbates cardiac function and worsens structural damage. On the basis of the resultant aggravation in myocardial ischemic tissue damage or even irreversible damage after blood flow is increased, this phenomenon is known as myocardial ischemia reperfusion injury [178-180].

Fang and coworkers founded that luteolin significantly not only restored contractility of the left ventricle, but also reduced the infarct size and lactate dehydrogenase leakage during I/R. In addition, luteolin pretreatment significantly improved cardiomyocyte shortening amplitude and decreased the apoptotic rate [142,143].

In addition, Liao et al. showed that administration of luteolin at 1 or 10 μg/kg significantly suppressed the incidence and duration of ventricular tachycardia and ventricular fibrillation and completely prevented mortality during myocardial IR injury. Pretreatment with luteolin decreased carotid blood LDH levels, which served as an indicator of cellular damage, during the same period of IR. Moreover, in animals subjected to 1h of coronary artery occlusion and 3h of reperfusion, the cardiac infarct zone was reduced by pretreatment with luteolin [149].

Similarly, Sun et al. reported that luteolin reduced incidence of arrhythmia, reduced LDH release and decreased infarct size of diabetic rats subjected to I/R injury. They also demonstrated that luteolin significantly improved the left ventricular function via increasing ±dp/dt max, Left ventricular ejection fraction (LVEF) and limiting the increase of Left ventricular end-systolic volume (LVESV) and Left ventricular end-diastolic volume (LVEDV) [144].

Wu et al. founded that pretreatment with luteolin significantly attenuated I/R-induced cardiomyocyte death, LDH leakage and infarct size. Further, it also improved the systolic/diastolic function of single cardiomyocytes and whole heart. Moreover, luteolin effectively improved the degeneration of cell shortening amplitude and cell death induced by I/R, with a maximal effect observed at a dose of 8mmol/L [146]. Furthermore, Rump and coworkers reported that luteolin significantly enhanced the left ventricular pressure, the global coronary flow and the relative coronary flow. Luteolin significantly diminished epicardial NADH-fluorescence area and intensity [181]. These results suggest that luteolin may actually exert anti-IRI effects in myocytes.

5.2. Isoproterenol Induced Arrhythmia

Arrhythmias can be the result of a variety of structural changes or ion channel dysfunction. Regardless of the mechanism, the manifestation of the arrhythmias continues to be a major contributor to human morbidity and mortality. Unstable ventricular arrhythmias secondary to channelopathies significantly increase the risk of sudden cardiac death [182]. Many causal factors participate in deteriorating cardiac arrhythmias. Oxidative stress has been reevaluated as an important etiological event implicated in life threatening arrhythmias [183,184]. In addition, evidence suggests that oxidative stress, which is caused by the over-accumulation of intracellular ROS, is one of the leading factors triggering myocyte apoptosis [185]. Myocyte apoptosis can largely induce the development of the myocardial injury [186]. Hence, the modulation of intracellular ROS levels and regulation of apoptotic cascade are considered crucial therapeutic strategies for treating cardiovascular diseases. The rat model of isoproterenol(ISO)-induced relative myocardial ischemia or hypoxia is a well-known standardized model for evaluating cardio-protective drugs and cardiac functions [187]. Sun et al. examined the protective effects of luteolin against ISO-mediated myocardial injury in vivo, and demonstrated that luteolin administration could protect against H_2O_2-induced apoptosis in H9c2 cardiomyoblasts. In the experiment, pretreatment with luteolin groups prevented marked cardiomyocytic vacuolization induced by ISO and significantly reduced ST-segment elevation was detected. Moreover, luteolin enhanced activities in antioxidant enzymes SOD, glutathione peroxidase (GSH-Px), and HO-1 and decreased the levels of malondialdehyde (MDA) and ROS in heart tissues, suggesting that luteolin protected the heart from injury by scavenging free radicals and also improving the endogenous antioxidant system in ISO-treated rats [148].

5.3. Hypertension and Its Complications

Hypertension is a major risk factor for coronary artery disease, stroke, heart failure and renal failure [188]. It is well established that hypertension is associated with changes at the vascular and myocardial tissue level caused by various mechanisms, including endothelial dysfunction and coronary arteriolar constriction with resultant ischemia, as well as increased inflammatory changes and ensuing apoptosis and fibrosis [189]. Although older anti-hypertensive drugs, such as diuretics and β-blockers, are helpful in controlling

blood pressure, they have the potential for adverse metabolic effects that may exacerbate the metabolic syndrome. Natural products, especially medicinal plants, could be an ideal source to develop safe and effective agents for hypertension [190]. Plants rich in luteolin have been used in Chinese traditional medicine for treating hypertension [190-193].

The renin–angiotensin–aldosterone system (RAAS) is a hormone system that regulates blood pressure and water (fluid) balance. In the RAAS, rennin acts on angiotensinogen to release angiotensin I which reacts with angiotensin converting enzyme to form Ang II [190]. Ang II appears to frequently elicit enhanced vasoconstrictor responses in essential hypertension [194]. It is classically described as a vasoconstrictor agent. Moreover, altered vascular tone in hypertension is associated with impaired endothelium-dependent vasodilation due, in large part, to reducing NO signaling [123]. Enhanced production of the vasoconstrictor peptide ET-1 is another mechanism whereby abnormal endothelial function may contribute to vasospasm, blood pressure elevation and complications of hypertension [195]. Therefore, NO production within endothelial cells as a protective mechanism against the action of vasoconstrictor compounds, including ET-1. Gui-Yuan Lv and coworkers founded that the combined used of luteolin and buddleoside could inhibit the blood pressure and the serum levels of ANG, aldosterone (ALD) and ET, but increase serum NO concentration on spontaneously hypertensive rats (SHR). The combined used of luteolin and buddleoside showed a dose dependent anti-hypertension activity, and its mechanism should be related with the RAAS and ET systems [190].

Aldose reductase is a member of the aldo-keto reductase superfamily [196], which is implicated in proliferation of smooth muscle cell (SMC) growth in a model of vascular repair [197]. The abnormal proliferation and migration of VSMCs have been implicated to play a key role in a number of cardiovascular pathologies, such as in the pathogenesis of hypertension [198,199]. Aldose reductase has been extensively studied in relation to hypertension and its complications. It has been found that luteolin can inhibit the activation of Myocardial tissue's aldose reductase and Ang II on SHR. Moreover, the activation of aldose reductase is correlated with the concentration of Ang II [191-193]. We conclude that luteolin can suppress the expression of aldose reductase through inhibiting Ang II signaling pathway.

In conclusion, luteolin can prevent the development of hypertension and related cardiovascular disease through suppressing Ang II signaling pathway and ET systems.

5.4. Diabetic Cardiovascular Complications

Diabetic complications are the major causes of morbidity and mortality in patients with diabetes. Microvascular complications include retinopathy, nephropathy and neuropathy, which are leading causes of blindness, end-stage renal disease and various painful neuropathies; whereas macrovascular complications involve atherosclerosis related diseases, such as coronary artery disease, peripheral vascular disease and stroke. Diabetic complications are the result of interactions among systemic metabolic changes, such as hyperglycemia, local tissue responses to toxic metabolites from glucose metabolism, and genetic and epigenetic modulators. Chronic hyperglycemia is recognized as a major initiator of diabetic complications [200]. Previous studies have shown that luteolin can significantly decrease plasma glucose concentration via multiple molecular targets involving diverse intracellular pathways, which inhibits the development of diabetic complications.

Increased oxidative stress has been associated with the pathogenesis of chronic diabetic complications, including cardiomyopathy [201]. It has been suggested that NO pathway is involved in augmenting oxidative stress [202]. NO is produced by a set of three NOS isozymes: endothelial NOS (eNOS), iNOS, and neuronal NOS (nNOS). These enzymes convert L-arginine to L-citrulline, leading to the generation of the free radical NO. Many studies have shown that eNOS and iNOS play an essential role in the pathogenesis of cardiovascular complications in diabetes [203]. As luteolin has a striking anti-oxidant property, it may have preventive effective on the development of cardiovascular complications in diabetes.

Abbasi et al. reported that luteolin at low concentrations, as well as vitamin C, decreased intracellular ROS and increased NO release during high glucose treatment and restored the balance between NO and ROS formation. The results showed that the protective effects of luteolin against hyperglycemic stress in HUVEC might be attributed to its inhibition of intracellular ROS formation, restoration of NO production [204]. In contrast, El-Bassossy and coworkers founded that luteolin markedly inhibited the elevations in basal levels of intracellular NO and ROS in IR animals [205]. Such excessive ROS caused by IR damaged cellular proteins, membrane lipids, and nucleic acids, and eventually resulted in cell death. More importantly, NO readily reacts with superoxide, generating the highly reactive oxidant molecule peroxynitrite (ONOO-).

ROS can directly stimulate vasoconstriction or potentiate the constriction due to vasoactive agents by increasing intracellular calcium in smooth muscle

and endothelial cells [206]. The association between elevated serum uric acid level and type 2 diabetes mellitus (T2D) has been established for years [207,208]. Ding et al. found that the protective effect of luteolin on uric acid-induced dysfunction of β-cells was through the NF-κB-iNOS-NO signalling pathway via regulation of MafA expression. It should be mentioned that luteolin did not affect cell viability at its effective concentration in Min6 cells, suggesting that luteolin could provide benefit for protection of pancreatic β-cells without causing cytotoxicity. Therefore, this study provided a better understanding of the molecular mechanism underlying the protective effect of luteolin on T2D and suggested that the compound might be of benefit for the treatment of T2D in hyperuricemic patients [209].

The PI3K/Akt signaling transduction pathway is considered to involve the decrease of plasma glucose concentration. AKT, also known as protein kinase B (PKB), is a serine/threonine kinase and downstream effectors of PI3K. It is activated via phosphorylation. AKT plays a central role in the metabolic actions of insulin including glucose transport, and the synthesis of glycogen [210,211]. Azevedo and coworkers reported luteolin was an insulin sensitizer, leading to an increase in insulin receptorβ autophosphorylation and a subsequent activation of downstream PI3K signaling pathway. The activation of this pathway would result in phosphorylation and inactivation of GSK3, leading to an increase glycogen synthase activation and glycogen synthesis [212]. Similarly, Wang et al. founded that diabetes led to reduce expression of p-Akt without affecting levels of Akt, while luteolin partially restored streptozotocin-induced loss of Akt phosphorylation, which suggested that luteolin improved cardiac function through enhancing Akt/PKB phosphorylation in diabetic hearts [213].

The PPARγ signaling transduction pathway is considered to involve the Insulin Resistance (IR). IR is observed in patients with not only type 2 diabetes and obesity, but also type 1diabetes. In addition to its important role for maintaining glycemic control, insulin has many vasotropic actions. Insulin resistance in vascular tissues is associated with endothelial dysfunction, leading to cardiovascular diseases [200]. PPARs are ligand-activated transcription factors belonging to the group of nuclear hormone receptors like the vitamin D or steroid receptor [214]. PPARs modulate genes that regulate lipid and glucose metabolism as well as gene expression in vascular cells [215]. PPARγ is expressed in the endothelium and smooth muscle in the blood vessel wall [216]. The importance of PPARγ is originally recognized in adipogenesis and insulin sensitivity. Xu and coworkers reported that the

selective PPARγ regulation of luteolin might reduce high-fat-diet-induced adipogenesis, adipose tissue inflammation, and insulin resistance in mice, which suggested a new therapeutic and interventional approach for obesity and insulin resistance [217]. Similarly, El-Bassossy et al. also founded that luteolin alleviated vascular complications associated with IR mainly through PPAR-dependent pathways [205].

Besides the above mentioned common signaling pathways, the increasing evidences have shown that inflammatory involved in the development of hyperglycemia, which is a key feature in diabetes. High glucose levels induce the release of pro-inflammatory cytokines. Kim et al. reported that while hyperglycemic conditions significantly induced histone acetylation, NF-κB activation, and pro-inflammatory cytokine (IL-6 and TNF-α) release from THP-1 cells, luteolin suppressed NF-κB activity and cytokine release. Luteolin also significantly reduced CREB-binding protein/p300 (CBP/p300) gene expression, as well as the levels of acetylation and histone acetyltransferase (HAT) activity of the CBP/p300 protein, which is a known NF-κB coactivator. These results suggested that luteolin inhibited hyperglycemic-induced cytokine production in monocytes, through epigenetic changes involving NF-κB, which suggested that luteolin might be a potential candidate for the treatment and prevention of diabetes and its complications [218]. Moreover, Xu et al. also founded that luteolin inhibited mast cell-derived IL-6 expression, which was a key cytokine that contributed to mast cell-associated metabolic derangements, to ameliorate diet-induced obesity [217].

In summary, luteolin can suppress the development of diabetes and its complications, mediated with various of signalling pathways, such as oxidative stress, PI3K/Akt, and PPARγ.

5.5. Hyperlipidemia

Elevated serum lipid levels have been identified as one of the modifiable risk factors in the aetiology of cardiovascular disease. The INTERHEART study established that elevated lipid level was the greatest contributor to the development of myocardial infarction worldwide [219]. The fact that hyperlipidemia is a strong risk factor for cardiovascular disease is well established. Hyperlipidemia refers to elevated cholesterol, elevated triacylglycerol (TG) or both. In addition, hypercholesterolemia is a risk factor

for atherosclerosis [220]. Previous studies had shown that luteolin could effectively decrease the blood lipid level and oxidative stress level.

The increasing studies show that luteolin can significantly reduce the total cholesterol (TC), TG, LDL-cholesterol (LDL-C) and MDA and increase the high density lipoprotein cholesferol (HDL-C) and NO [221-223]. Liu and coworkers founded that luteolin enhanced the phosphorylation of AMPKα and its primary downstream targeting enzyme, acetyl-CoA carboxylase (ACC), up-regulated gene expression of carnitine palmitoyl transferase 1 (CPT-1), which was the rate-limiting enzyme in mitochondrial fatty acid β-oxidation, and down-regulated sterol regulatory element binding protein 1c (SREBP-1c) and fatty acid synthase mRNA levels in the absence and presence of palmitate. In addition, luteolin significantly decreased ROS production and ameliorated lipid accumulation in HepG2 cells caused by palmitate. Furthermore, intracellular TG measurement indicated that the luteolin-mediated reduction of enhanced TG caused by palmitate was blocked by pretreatment with the AMPK inhibitor, compound C. The results suggested that the lipid-lowering effect of luteolin might be partially mediated by the up-regulation of CPT-1 and down-regulation of SREBP-1c and FAS gene expression, possibly by activation of the AMPK signaling pathway, and partially might be through its antioxidative actions [224]. On the other hand, Nekohashi et al. reported that luteolin reduced high blood cholesterol levels by specifically inhibiting intestinal cholesterol absorption mediated by Niemann–Pick C1-Like 1 (NPC1L1) [225].

In conclusion, luteolin can significantly reduce the level of TC, TG, LDL and MDA, and increase the level of HDL and NO. We conclude the lipid-lowering effect of luteolin may be through its anti-oxidative actions.

5.6. Atherosclerosis

As one of the leading causes of death and disability in industrialized society, atherosclerosis is a multistage and multi-factorial disease involving genetic, environmental, psychosocial and other factors. Some features of atherosclerosis remain to be studied [226]. Inflammation has been linked to atherogenesis and plaque disruption and it is well established that atherosclerosis is a chronic inflammatory process [227]. Atherosclerotic plaques consist of lipids, heavy infiltrates of inflammatory cells of macrophages and T-lymphocytes, smooth muscle cells and extracellular matrix. Cell adhesion molecules is one of the early events that lead to the

development of the inflammatory reaction associated with atherosclerosis [114,115].

Some studies have showed that luteolin can inhibit THP-1 cell adhesion to suppress the development of the inflammatory reaction associated with atherosclerosis [120,228]. However, the abnormal proliferation and migration of VSMCs have been implicated to play a key role in a number of cardiovascular pathologies, such as in the progression of atherosclerosis [198,199]. Previous studies already had showed that luteolin could inhibit the exaggerated and uncontrolled VSMCs proliferation to induce the development of atherosclerosis. Aggregated platelets could release PDGF which stimulates VSMCs migration and proliferation and in turn stimulates the initiation and proliferation of atherosclerosis. Kim et al. showed that pretreatment with luteolin significantly inhibited PDGF-Rβ tyrosine phosphorylation induced by PDGF-BB in a concentration-dependent manner [95,96]. Similarly, Lamy and coworkers found that luteolin inhibited PDGF-BB–dependent tyrosine phosphorylation of PDGFR-β in smooth muscle cells and that this inhibitory effect is associated with an impairment of downstream signaling events triggered by this receptor, such as phosphorylationof the $p42^{MAPK}$ and $p44^{MAPK}$ forms (ERK-1/2) and of Akt [98].

Moreover, in atherosclerotic plaques, cell apoptotic occurs together with proliferation [229,230]. It has been well established that elevated plasma concentrations of lipids are associated with accelerated atherogenesis and that LDL plays a critical role in the pathogenesis of atherosclerosis. LDL is oxidized to form oxidized LDL (ox-LDL) in the subendothelial space of the arterial wall. Lysophosphatidylcholine (LPC) is one of the major bioactive components of ox-LDL, which seems to be a key factor in the development of atherosclerosis. Song et al. founded that luteolin reduced not only LPC-induced cell death but also LDH leakage. Luteolin inhibited LPC-induced apoptosis in endothelial cells, which demonstrated its protection against the cytotoxicity of LPC. LPC-induced apoptosis was characterized by a calcium-dependent mitochondrial pathway, involving calcium influx, activation of calpains, cytochrome C release and caspases activation [113]. Therefore, luteolin is a potential therapeutics for the atherosclerosis.

CONCLUSION

Up to now, the multiple therapeutic effects of luteolin have been extensively investigated, particularly in the treatment of cancer and

cardiovascular diseases. Although there is growing evidence that luteolin has a potential role in the protection against many various types of cancer and cardiovascular diseases, the mechanisms of action of luteolin on the cancer and cardiovascular diseases therapy has just started to be addressed and a wide diversity of questions still remain open. More extensive research regarding the effect of luteolin on suppressing cancer and preventing cardiovascular diseases in both animals and humans are warranted.

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Chapter 3

ELECTROANALYTICAL DETERMINATIONS OF LUTEOLIN

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ABSTRACT

Natural antioxidants have become very important in recent decades because of their well known benefits to human health and the increasingly restricted use of synthetic antioxidants. Flavonoids are one of the groups of natural antioxidants widely produced by the plants as

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secondary metabolites. They are molecules composed of two benzene rings linked through a chain of three carbon atoms. Flavonoids are widely found in fruits, seeds and vegetables.

Luteolin (3′,4′,5,7-tetrahydroxy-flavone; LUT) belongs to the subclass of flavonoids known as flavones and is rated as one of the most bioactive flavonoids. LUT has a resorcinol group in ring A, and a catechol group in ring B. LUT has beneficial effects on human health, such as cardiovascular protection, anti-allergic, and anticancer activities, anti-ulcer effects, and prevents cataracts. LUT also inhibits platelet aggregation by vasodilating action. LUT is a compound as active as tert-butyl hydroxyanisole (BHA) but more active than α-tocopherol.

Different analytical methods have been reported for the determination of LUT. They include thin-layer chromatography, gas chromatography, high-performance liquid chromatography, and capillary electrophoresis, coupled to different detection techniques such as UV spectrophotometry, photo diode array, electrochemical array, etc. Even though these techniques have made possible the highly selective and sensitive quantification of LUT, they present some disadvantages such as high cost, high time consumption and reagents, and high complexity of operation.

In recent years, electroanalytical techniques have become very important as analytical tools in the determination of different compounds of biological interest. Compared with chromatographic techniques, they require cheap equipment, short analysis times, low solvent consumption, etc.

In this chapter, we report the application of electroanalytical techniques to determine LUT in real matrices. Both, results obtained in our laboratory and those reported by other authors are included. We also discuss the application of chemometric tools in those cases where LUT is present in real samples in the presence of other interfering electroactive species whose electrochemical signals show a strong overlap.

1. Introduction

1.1. Flavonoids

Plants produce different substances necessary for all the natural life cycles through the photosynthesis process. Therefore, carbohydrates, proteins, fats and vitamins are synthesized from complex biochemical mechanisms. These compounds are known as primary metabolites and are present in all organisms. Plants are the basis of the food chain and support all other life forms on our planet.

There are other substances, known as secondary metabolites, which have not a direct role on growth or reproduction, but their absence could affect survival. Alkaloids, the steroidal saponins, essential oils, terpenoids and flavonoids, among others, belong to this group.

The main function of flavonoids is based on their antioxidant capacity, which the ability to protect organisms from damage caused by various oxidizing agents such as ultraviolet radiation, environmental pollution, chemicals in food, etc. (Aherne and O'Brien, 2002). In addition, they possess anti-aggregating, anti-allergic, anti-ischemic, anti-inflammatory, anti-bacterial, anti-fungal and anti-tumor properties (Chin and Nagaratnam, 1994; Butkovic et al., 2004).

In addition, flavonoids have various functions such as the stimulation of communication through the gap junctions, the impact on the regulation of cell growth and induction of detoxifying enzymes such as cytochrome dependent mono P-450, among others (Stahl et al., 2002).

1.2. Luteolin

1.2.1. Structure

Flavonoids are derivatives of benzo-γ-pyrone, with phenolic and piranics rings in their molecular structure. They have a well-defined chemical structure (Figure 1). Flavonoids have two benzene rings (A and B) linked through a chain of three carbon atoms (ring C, when it forms a cycle). Considering that each benzene ring possesses 6 carbon atoms, flavonoids are referred as a C6C3C6 compound. The carbon atoms in rings A and C are numbered from 5 to 10 and those of ring B from 2' to 6' (Kühnau, 1976).

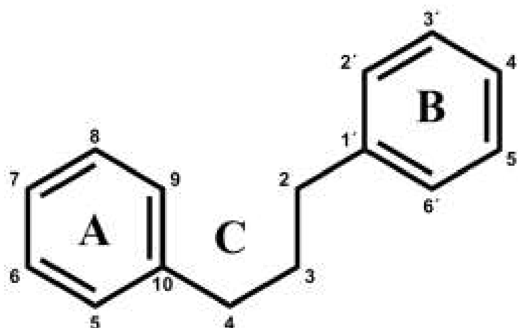


Figure 1. Basic structure of flavonoids.

Three structural features of flavonoids are important for the antioxidant activity and/or free radical scavenging (Letan, 1966):

a) The presence of the o-dihydroxy or catechol group in ring B
b) The presence of a double bond in the position 2,3
c) The presence of hydroxyl groups in positions 3 and 5

Nowadays, more than 6400 natural flavonoids are known, which have been classified into several classes according to structural variations of the C3 chain. Therefore, flavonoids are classified into several groups: chalcones, flavones, flavonols, flavanones, anthocyanidins, catechins, epicatechin, aurones, isoflavonoids, pterocarpans, carotenoids, etc. (Pokorny et al., 2001).

Flavonoids possess many trivial names. These names were assigned by the investigators as they were discovered in the nature. They are commonly used, but are not very useful when systematic information about these substances is required.

1.2.2. History

Flavonoids (from Latin flavus, "yellow") were named so since the first that was isolated. The first flavonoid isolated was "citrine", which has a yellow color. It was isolated from lemon peel in 1930 by the Nobel Prize Szent-György. Flavonoids were first called vitamin P (from permeability) when Rusznyak and Szent-Györgi (Rusznyak and Szent-Györgi, 1936) proposed that flavonoids were an essential dietary factor, contributing to vascular permeability. They were also known as vitamin C2 due to it has been found that some flavonoids had similar properties to vitamin C. However, it has never been shown that flavonoids act as vitamins and neither is thought to be related to the vascular permeability. For these reasons both denominations were abandoned in the 1950s (Singleton, 1981). Currently, it is well known that flavonoids have different colors and even some are colorless. In addition, they can be found in all tissues of higher plants (White and Xing, 1997) and about 90% are found as their glycoside derivatives. The bond with sugar is usually made through the C3 and less frequently through C7 of ring A (Ioku et al., 1995).

1.2.3. Occurrence

LUT was isolated from the aromatic plant *Salvia tomentosa* of Lamiaceae mint family (Ulubelen et al., 1979). It can be found in *Terminaliachebula*, ragweed pollen and flower clover (Mann, 1992).

LUT can be found in thyme, peppermint, parsley, celery, green pepper, perilla leaves, chamomile tea, peanut hulls, broccoli, dandelion, carrots, olive oil, rosemary, navel oranges, and oregano (Craker and Simon, 1996; Shimoi et al., 1998; Pokorny et al., 2001; López-Lázaro, 2009).

LUT can also be obtained from seeds of the palm *Aiphanesaculeata* (Lee et al., 2001).

2. Analytical Determinations of Luteolin

2.1. Overview

Given the structural complexity of the plants, the extraction of samples from them is a critical step in any analysis. Many techniques provide a real possibility for the sample preparation prior to the analysis with sufficient specificity. However, there is nota standard available technique for the preparation and for the sample procedure. The liquid extraction is, in many cases, the first step in the preparation process of the sample.

The application of thin layer chromatography (TLC) to identify different components of plant extracts is described as an analytical method in various Pharmacopoeias.

Through this technique, the chromatographic "fingerprint" of a plant extract can be obtained, which is very convenient for identification purposes.

To analyze the flavonoid, the gas chromatography (GC) cannot replace to HPLC, which can overcome the specific problems of separation, especially when aglycones and glycosides are present in the same sample.

The possibility to connect multiple detection devices to HPLC has made this technique a valuable and essential tool for the separation of polyphenols. However, this does not exclude the use of GC for the same purposes.

Ultraviolet (UV) detection is currently the preferred technique used in liquid chromatography (LC) and, even today, it is used with multi-wavelengths or photo diode array (PDA). It is a conventional tool in studies associated with detection, quantification and classification of subgroups. The development of liquid chromatography coupled to mass spectroscopy (LC-MS) has extended the field of application of MS, allowing the analysis and identification of compounds from natural products.

The particular advantage of LC-MS is its ability to identify both free and conjugated forms of flavonoids. However, for a further elucidation of the structure of the conjugates, it is essential the supplementary information given

by LC coupled to nuclear magnetic resonance (LC-NMR). In this application area, LC-NMR outperforms other techniques for identification purposes, but not in terms of quantification. For high throughput analysis, the development of techniques such as ultra-efficiency liquid chromatography (UPLC) coupled to MS has emerged as an alternative to the traditional HPLC technique.

The relevance of UPLC technology is its ability to separate and identify compounds with significant gains in resolution, sensitivity and marked reduction in the overall analysis time. The capillary electrophoresis (CE) coupled to MS detection can provide structural information of analytes present in real matrixes. However, compared to phenols analysis by LC, CE does not show a significant difference in analysis times, and limited sample and solvent consumptions do not appear to balance the low repeatability of the retention/ migration times and restricted sensitivity.

2.2. Chromatographic Methods

Liu et al. (1995) developed a chromatographic method for the determination of LUT and their metabolites in rat urine and bile. The method was based on GC coupled to MS. Recoveries of LUT were in the ranges 66.7–91.4%, when urine samples were spiked with 2.50, 5.00, 10.00, 25.00 and 50 μg mL^{-1} LUT solutions, with a relative standard deviation (RSD) less than 7.20% for three independent measurements. The limit of detection (LOD) was 500 ng mL^{-1}.

De Souza et al. (2002) proposed a method based on LC coupled to a UV detector (set at 362 nm) for the determination of flavonoids. Quercetin (QC), LUT and 3-O-methylquercetin (MQC) were flavonoids determined in preparations of *Achyroclinesatureioides*. Flavonoids were analyzed using a Shim-pack column CLC-ODS (M) RP-18. The mobile phase consisted of a mixture of methanol and phosphoric acid 0.16 M (53:47, v/v). Calibration curves showed a linear regression between 1 and 10 μg mL^{-1} for QC and LUT. LOD were 0.32 and 0.31 μg mL^{-1} for QC and LUT, respectively. Limits of quantification (LOQ) were 0.92 and 0.96 μg mL^{-1} for QC and LUT, respectively. The RSD for QC, MQC and LUT were less than 0.9, 2.3 and 10.2%, respectively.

Li et al. (2005) developed a reversed-phase (RP)-HPLC method for the simultaneous determinations of LUT and apigenin (APG) in dog plasma. These authors used a C_{18} column and UV detector, which was set at 350 nm. Before performing measurements, LUT and APG were extracted with ethyl

acetate. Calibrations curves were linear over the range of 38.5-4350 ngmL^{-1} for LUT, with a linear correlation coefficient (r) of 0.9996. LOD and LOQ of LUT were 1.82 and 7.84 ng mL^{-1}, respectively. Recoveries for LUT were in the range 102.7-104.5 %, with a RSD of 7.9 %.

Li et al. (2006) proposed another very interesting method. They used a RP-HPLC coupled to an UV detector to demonstrate the application of this system for the simultaneous analysis of LUT and APG in human urine after oral administration of tablets of *Chrysanthemum morifolium* extract. Calibration curves were linear in the range 0.00975-7.800 and 0.1744-13.95 µg mL^{-1} for LUT and APG, respectively (with r>0.9990). The intra-day and inter-day RSD for APG were less than 2.8 and 5.4%, respectively, and for LUT were below than 2.2 and 4.0 %, respectively. This method had a LOQ of 39.20 ng mL^{-1} for LUT and 31.45 ng mL^{-1} for APG, and recoveries of 85.7 % for both compounds.

Zhang et al. (2007)described a fast and interesting methodology based on gradient HPLC coupled to photo diode array detector for the analysis of chlorogenic acid, linarin and LUT in *Flos Chrysanthemi Indici* suppository (which is a Chinese medicine used for treatment of prostatitis and chronic pelvic inflammation). The mobile phase was a mixture of acetonitrile and water/acetic acid (99:1 v/v, pH 2.8). UV detection was recorded between 210 and 400 nm. These authors reported a LOQ for chlrogenic acid, linarin and LUT of 0.32, 0.08 and 0.05 µg mL^{-1}, respectively. The method yielded very satisfactory results when it was applied to *Flos Chrysanthemi Indici* suppository, with a RSD less than 3.0 %.

Chi et al. (2009) detected kaempferol (KAP), APG, rutin (RUT), QC, LUT and ferrulic acid (FA) in Chinese herbal tea using capillary zone electrophoresis coupled to amperometric detection. Effects of the detection potential, buffer pH, and the concentration on the peak current were studied. Calibration curves for these compounds were linear over the range of 0.008-2.4 µg L^{-1}. LOD of LUT was 0.113 µg L^{-1}, with a RSD of peak currents of 3.2 %. This method showed recoveries up to 91.3 % for all compounds studied.

Chen et al. (2011) developed two methods for the determination of 5-hydroxymethylfuraldehyde, geniposidic acid, chlorogenic acid, paeoniflorin, 20-hydroxyecdysone, coptisine, berberine, LUT, and glycyrrhizic acid in "samgiumgagambang" (SGMX) (an herbal medicinal preparation). HPLC and capillary electrophoresis (CE) methods were developed to determine the quality of SGMX. The HPLC (coupled to a UV- photo diode array detector, set at 250 nm) method used acetonitrile and water containing formic acid as the mobile phase and anEclipse-DB-C18 analytical column for separation.

The CE method was performed using a HP^{3D}CE (Hewlett Packard, Germany) equipped with a photo diode array detector set at 230 nm. The analysis was achieved using fused-silica capillary with a buffer solution of 70 mM $Na_2B_4O_7$ and 10% methanol.

Samples were injected at 50 mbar by 5 s for a 25 kV constant voltage applied. Calibration curves for LUT were linear over the range of 1-25 and 10-50 mg L^{-1} for HPLC and CE, respectively. LOD and LOQ for LUT by HPLC were 0.5 and 1 mg L^{-1}, respectively. LOD and LOQ for LUT by CE were 2 and 5 mg L^{-1}, respectively.

Villela et al. (2011) developed a RP-HPLC (coupled to a UV detector) method for the determination of the following flavones: luteolin-7,3′-O-diglucoside (LDG), luteolin-7-O-glucoside (LMG) and LUT in *Reseda luteola* samples. *Reseda luteola* were a popular source of yellow dyes for textiles. Mobile phases were a pH 3 buffer of 160 mM formic acid, 40 mM ammonium formiate and 0.04 mMof EDTA in water (phase A) and methanol (phase B). A linear gradient was applied: 85–60% phase A (0–35 min), 60–36% phase A (35–47 min), 36–23% phase A (47–60 min), 23–0% phase A (60–65 min), 0% phase A (65–70 min). LOD were 1.1, 0.7 and 0.25 ng g^{-1} for LDG, LMG and LUT, respectively. LOQ were 3, 2.2 and 0.8 ng g^{-1} for LDG, LMG and LUT, respectively.

Godoy-Caballero et al. (2012) proposed a method based on LC coupled to UV–vis and fluorescence (FLD) detections for the determination of phenolic compounds in virgin olive oil. Calibration curves of LUT were linear over the range of 0.500-100 µg mL^{-1}, with r = 0.9990. The LOD and LOQ for LUT were 0.1 and 0.2 µg mL^{-1}, respectively. Recoveries of LUT were 91% for both detection systems, with RSD from 2.2 to 6.3%.

Wei et al. (2013) proposed a method of heat-reflux and ultrasound assisted extraction combined with subsequent determination by RP-HPLC (coupled to an UV-visible detector) of flavonoids content in *Scutellaria barbata D. Don* (a traditional Chinese medicinal herb).

The principal bioactive flavonoids contained in *S. barbata D. Don* were APG, LUT and baicalein (BAC). Calibration curves were linear in the range 5-400, 6-180 and 6-200 µg mL^{-1} for APG, BAC and LUT, respectively (with $r \geq 0.9990$). LOD were 0.061, 0.064 and 0.078 µg mL^{-1} for APG, BAC and LUT, respectively; while LOQ were 0.192, 0.198 and 0.244 µg mL^{-1} for APG, BAC and LUT, respectively.

2.3. Electroanalytical Methods

2.3.1. A Short Review

In order to understand the relationship between the redox behavior and the biological activity of flavonoids, the electrochemical behavior of fifteen representative and structurally related flavonoids has been studied by cyclic voltammetry (CV) on glassy carbon electrodes (GCE) in aqueous solutions under physiological conditions (Hodnick et al., 1988). Thus, cyclic voltammograms were recorded in 0.1 M phosphate buffer solutions of pH 7.5, containing 2.5% (v/v) dimethyl sulfoxide. Under these experimental conditions, a value of $\Delta E_p = 0.055$ V for the difference between the anodic and cathodic peak potentials–was obtained for LUT, suggesting a quasi-reversible single-electron charge transfer. However, the interpretation of the experimental data was not easy, because it was observed that currents decreased when consecutive potential sweeps were performed, modifying the peak position and hindering the analysis. These results evidenced the formation of an insulating film, which would poison the electrode surface.

The electrochemical properties of four structurally related flavonoids, i.e., quercetin, queritrina, rutin (RUT) and LUT were studied on GCE by CV and rotating disk voltammetry (Hendrickson et al., 1994). These authors showed that flavonoids that have a catechol group in their molecular structures exhibit a reversible electrochemical behavior, whereas those without the catechol group generally show an irreversible electrochemical behavior. Thereby, all flavonoids that possess a 3',4'-dihydroxy group (catechol group) have a first oxidation peak that can be assigned to the reversible electro-oxidation of the catechol group, involving the transfer of 2 e^- and 2 H^+. Cyclic voltammogram of LUT exhibited two oxidation waves. The first wave corresponds to reversible oxidation of the substituent 3',4'-dihydroxy at ring B, and the second corresponds to the irreversible oxidation of 5,7-dihydroxy substituent in ring A (Figure 1). This latter behavior is typical of meta-substituted dihydroxy phenols (resorcinol group) or simple phenols, which are more difficult to oxidize and undergo electron transfer reactions complicated by coupled chemical reactions.

The electrochemical behavior of LUT was studied on GC electrodes by CV and differential pulse voltammetry (DPV) in phosphate buffer solutions of pH 4.0 (Liu et al., 2008). The results showed that the first LUT peak oxidation is a quasi-reversible and well defined peak, which involves 2e^- and 2 H^+, and that the process was controlled by adsorption. The anodic charge transfer coefficient was determined by measuring the width of the peak at half height

in CV, using the peak obtained at v = 0.1 V s^{-1}, and a value of (1-α) = 0.66 was determined. The relation between the peak current and LUT concentration using DPV was linear in the range from 1 x 10^{-8} to 1 x 10^{-6} M, and a LOD of 5 x 10^{-9} M was obtained. The electroanalytical method based on DPV experiments, was successfully applied for the determination of LUT in tablets of pharmaceutical formulations.

A simple and sensitive electrochemical method was developed to determine LUT at trace levels. The method was based on the use of modified GC electrodes with multi-walled carbon nanotubes using CV and DPV as electrochemical techniques (Zhao et al., 2011). A surface quasi-reversible redox couple was observed by CV at potentials between 0.2 and 0.8 V (vs. saturated calomel electrode (SCE)). Cyclic voltammograms recorded with modified electrodes showed a considerable increase in the currents when compared to those obtained on bare electrodes. Different experimental variables such as pH, potential and accumulation times were optimized. Optimal conditions were a pH value of 4.5, an accumulation potential of 0.4 V (vs. Ag/AgCl, 3 M NaCl) and an accumulation time of 210 s. The quantitative determination of LUT was performed by DPV, obtaining a linear response between the peak current and the concentration of substrate in the range from 2 x 10^{-10} to 3 x 10^{-9} M, with a LOD of 6 x 10^{-11} M (for a signal to noise ratio of 3:1). This methodology was then applied to the determination of LUT in peanut shells, obtaining a value of 3.26 ± 0.04 mg g^{-1}, being this in agreement with that determined by HPLC (3.50 ± 0.06 mg g^{-1}).

Metal wire microelectrodes electrically heated, were implemented in the early 1990s (Gründler et al., 1993). Recently, lead pencil disk electrode, indirectly heated by application of a direct current has been manufactured (Wu et al., 2013). These authors showed a strong effect of temperature on the LUT adsorptive accumulation on the electrode. A LOD of 1.0 x 10^{-9} M was obtained for a signal to noise ratio of 3:1 with a heated electrode at 44°C, which was lower by more than an order of magnitude to the LOD obtained with the electrode at room temperature.

The heated electrode was then used to determine LUT in simulated urine samples with good accuracy. This electrode showed numerous advantages for analytical applications, such as easy fabrication, low cost, high thermal stability, high sensitivity and good reproducibility.

Pang et al. (2014) developed an electrochemical method based on GCE modified with nanocomposite of grapheme nanosheets (GNs) and hydroxyapatite (HA) to determine LUT in peanut hulls using DPV. With optimized experimental parameters, they found that the peak current increased

linearly with the concentration in the range from 2.0×10^{-8} to 1.0×10^{-5} M, with a LOD of 1.0×10^{-8} M for a signal to noise ratio of 3:1. Studies in real samples were performed at peanut shells, obtaining recovery percentages between 97.8 and 102.7%.

Finally, In_2O_3 nanoparticles modified glassy carbon paste electrode (In_2O_3NPs/GCPE) was developed by Ibrahim and Temerk (2015). It was used for selective and sensitive electrochemical determination of LUT using square wave voltammetry (SWV). The modified electrode showed electrocatalytic activity on the LUT oxidation. These authors obtained, with optimized experimental conditions, a linear range from $9.98x10^{-9}$ M to $8.84x10^{-8}$ M, with a LOD of $1.99x10^{-10}$ M. The analytical performance of this proposed sensor was evaluated for the detection and quantification of LUT in fortified human biological fluids. The recovery of LUT in a urine sample was 101.51%, and recovery values between 101.46 and 101.83% were obtained in thyme samples.

2.3.2. Use of Modified Glassy Carbon Electrodes for the Determination of Luteolin

Square wave voltammograms obtained in 10% ethanol + 90% 1 M $HClO_4$ aqueous solutionat bare glassy carbon electrodes and at modified glassy carbon electrodes with polyethylenimine (GCE/PEI), after 20 min of accumulation in a solution of 1 μMLUT in the same reaction medium,showed that, in both cases, the current response is poor, but that the modified surface is more electroactive than the bare surface. Moreover, when the electrode was modified with multi-walled carbon nanotubes (GCE/MWCNT-PEI) the signal current observed at 0.680 V (Ag/AgCl, 3 M NaCl) increased, with a net peak current ($I_{n,p}$) of 22.14uA. It is clear that the signal is not observed in the absence of a previous adsorption step, which shows that the adsorption is promoted not only by electrostatic interactions between LUT and PEI but also by hydrophobic interactions between the structure of LUT and MWCNT.

Results obtained from SWV in 1μM of LUT in 10% ethanol + 90% 1 M $HClO_4$ aqueous solution at a GC/MWCNT-PEI electrode clearly suggest the adsorption of LUT and the quasi-reversible nature of the surface redox couple (Figure 2).

SWV is a fast and sensitive technique to quantify the amount of adsorbed substrate considering the ability to discriminate the measured signal from the capacitive currents (Osteryoung et al., 1987). Due to increased signal achieved with this technique (compared with the CV), SWV was chosen to perform the quantitative determination of LUT (Tesio et al., 2014a).

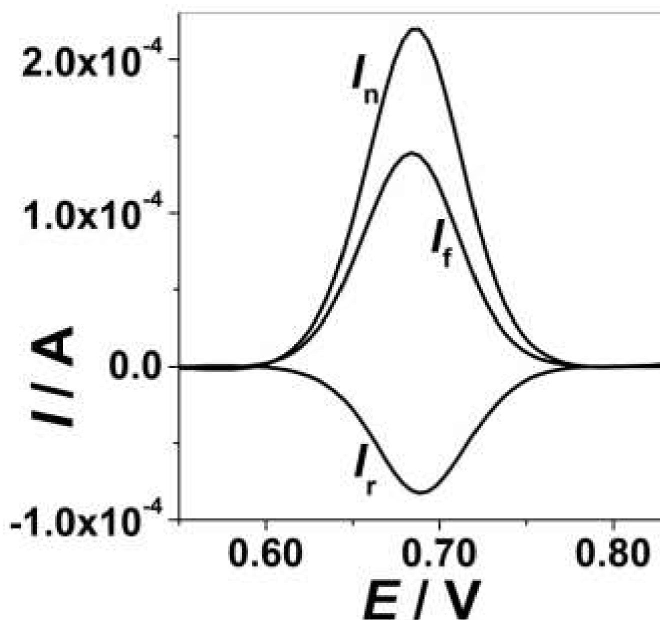


Figure 2. Square wave voltammogram registered subsequent to the accumulation of 1μM of LUT in 10% ethanol + 90% 1 M $HClO_4$ aqueous solution at GCE/MWCNT-PEI. t_{acc} =20 min, E_{acc} = 0.55 V, f = 40 Hz, ΔE_{sw} = 0.025 V, ΔE_s = 0.005 V.

The effect of adsorption time on the LUT oxidation signal at a concentration of 1.75 μM was evaluated at a potential of 0.55 V. The oxidation current increased almost linearly with the adsorption time up to 20 min, and then stabilized, as expected for adsorption behavior due to the presence of PEI and the aromatic rings of the MWCNT.

Therefore, an accumulation time of 20 min was selected to get a good compromise between sensitivity, reproducibility and analysis time.

The effect of the accumulation potential on the oxidation signal was evaluated for the adsorption of LUT at different potentials between -0.30 to 0.80 V (with an accumulation time of 20 min). The oxidation current was independent of the adsorption potential between -0.30 and 0.40 V, with a maximum response at 0.55 V. Thus, an adsorption potential of 0.55 V was selected.

Under these experimental conditions, a linear relationship between net peak currents ($I_{p,n}$) and LUT concentrations up to 1.75 μM was found. The sensitivity was 124 μA nM^{-1} with r =0.9980. LOD and LOQ were 0.5 nM and 1.5 nM, respectively (using 10σ/S, (Miller and Miller, 1993) (Figure 3).

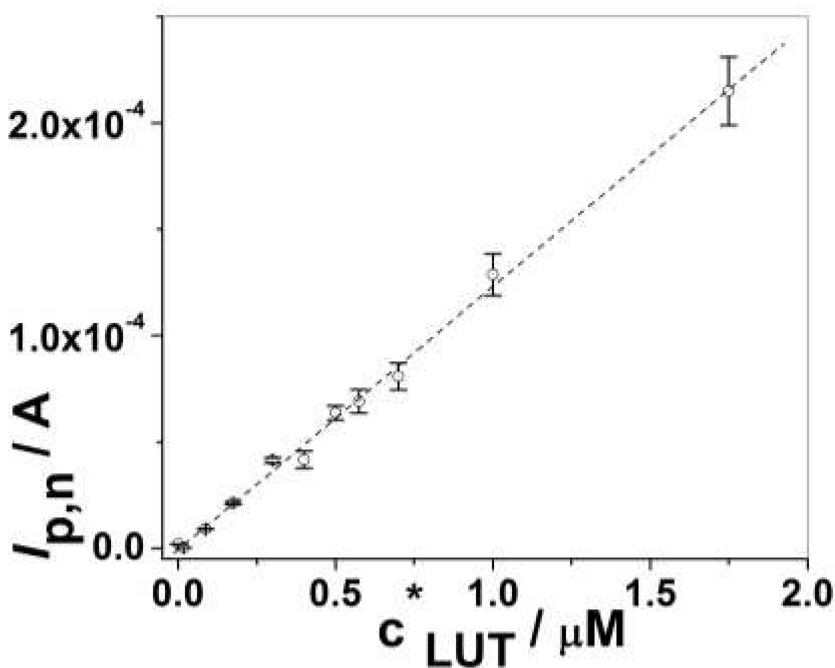


Figure 3. LUT calibration curve obtained with modified electrodes (GCE/MWCNT-PEI).

Reproducibility of MWCNT-PEI dispersions was 8.0 %, (using six electrodes modified with six different dispersions). Repeatability assays were also performed, obtaining a RSD value of 7.3% (using five electrodes modified with the same dispersion). These very good results indicate that the overall process is highly reproducible.

The GCE/MWCNT-PEI electrodes were then used for the quantification of LUT in peanut hulls samples (Tesio et al., 2014a). Before performing electrochemical determinations, LUT was extracted from peanut hulls using the following methodology: peanuts hulls were finely ground in an analytical mill and then 2.5 g of this fine powder was placed in a flask with 50 mL of methanol, at room temperature with a constant stirring for 24 h. Finally, the solution was filtered through Whatman filter paper, Grade 1 (Pin-Der y col., 1992).

Two different samples of peanut hulls were analyzed following the method previously described. Values of LUT obtained in both samples were

1.18 and 1.47 g/100 g peanut hulls. These values are in agreement with those values found by Yen et al. (1993).

These authors reported the LUT content in peanut hulls between 0.19 and 2.86 g/100 g peanut hulls, which is highly dependent on the ripeness thereof.

In addition, the LUT content in peanut hulls samples was determined by HPLC under the same conditions, using a binary mixture as mobile phase, which consisted of water:acetic acid (99:1) (solvent A) and methanol: acetonitrile:acetic acid (89:10:1) (solvent B). The separation column was reversed phase (Luna C18). The flow was kept constant at 0.2 mL min^{-1} and a elution gradient was used, starting with 60% of solvent A and 40% solvent B and was changed linearly, reaching values of 0% solvent A and 100% of solvent B in 30 min. LUT showed a very sharp and well-defined peak at a retention time of 28.5 min approximately. Values obtained by both methods were in a very good agreement.

2.3.3. Electroanalytical Determinations of Luteolin in Complex Mixtures Using Artificial Neural Networks

2.3.3.1. Introduction

In multivariate calibration, it is used experimental data measured using more than one sensor for the simultaneous determination of two or more analytes or an analyte in the presence of interferents. A well known example of a multisensor data is the absorption data from an absorption spectrum where the instrumental signal is absorbance, and the sensors are wavelengths.

Thus, methods of multivariate calibration based on the processing data of the vectorial type, i.e., spectra or other data like voltammograms are called first order, whereas those using arrays or other arrangements, of second order or higher order (Olivieri and Goicoechea, 2007). In this sense, univariate calibration is classified as zero order.

On the other hand, several chemometric tools have been described to resolve systems where signals appear overlapped (Olivieri and Goicoechea, 2007). Particularly in the field of electrochemistry, the most popular chemometric tools are multivariate calibrations based on different regression methods such as the multivariate curve resolution-alternating least square (MCR-ALS) (Brown et al., 2009), principal component regression (PCR) (Zhong et al., 2012), partial least squares regression (PLS-1) (Ni et al., 2006), and artificial neural networks (ANNs) (Olivieri and Goicoechea, 2007; Robledo et al., 2013).

We propose the simultaneous determination of LUT and rutin (RUT) in a pharmaceutical formulation (LutiMax®) at GCE modified with multi-walled carbon nanotubes (MWCNTs) dispersed in polyethylenimine (PEI) in 10% ethanol + 1 M $HClO_4$ aqueous solutions using SWV. Considering that surface voltammetric signals of LUT and RUT show a high overlapping, we used PLS-1 and ANNs methodologies and an experimental design to build the model calibration and quantify the LUT and RU content in the pharmaceutical formulation.

2.3.3.1.1. PLS-1

PLS-1 is a well-known first-order multivariate calibration methodology. It has been widely applied for different instrumental data (i.e., spectroscopic, electrochemical or chromatographic) with satisfactory results (Martens and Naes, 1989; Haaland and Thomas, 1988).

This method involves a calibration step where the relation between instrumental signal and reference component concentrations is established from a set of standard samples, and the prediction step, in which the calibration results are employed to estimate the component concentrations in unknown samples from its instrumental profile (Martens and Naes, 1989; Wold et al., 2001).

In the PLS-1 version, all model parameters are optimized for the determination of one analyte at a given time. During the model-training step, the calibration data are decomposed by an iterative algorithm, which correlates the data with the calibration concentrations using a so-called inverse model (Haaland and Thomas, 1988). This provides a set of loadings (p, size J×A, where J is the number of sensor and A the number of latent PLS variables), weight-loadings (W, size J×A) and regression coefficients to be applied to a new sample (v, size A×1). Given the profile of an unknown sample X_u (size J×1), the latter is projected onto the space of the loadings and weight-loadings to provide the test sample scores (t_u).

The sample scores are then multiplied by the regression coefficients to estimate the analyte concentration y_u.

2.3.3.1.2. ANNs

Artificial neural networks (ANNs) are computing systems made up with a large number of simple highly interconnected processing elements (neurons) that abstractly emulate the structure and operation of the biological nervous system. There are different types of networks. One of the networks used is a

multilayer perceptron network (MLP) with back propagation supervised learning method (Graupe, 2007).

The architecture of the ANNs based on MLP network, has an input, a hidden and an output layer.

The input layer has neurons with no activation function and is only used to distribute the input data. The hidden layer (which can be more than one) has neurons with continuously differentiable non-linear activation function. Finally, the output layer has neurons with either linear or non-linear activation functions. The data entered to the network move through it towards the output layer where the results are obtained.

These outputs are compared with expected values, and if a difference exists, then the connection weights between neurons are changed according to the rules of some learning error algorithm (Graupe, 2007).

The ANNs processing systems can be performed using a conventional set of independent analyses. However, it could be advantageous to replace this with a statistically designed experimental procedure in which several factors are simultaneously varied. This multivariate approach reduces the number of experiments and improves statistical interpretation of results (Tarley et al., 2009).

2.3.3.1.3. DWT-ANNs

The processing of raw voltammograms by ANNs has been reported in the literature. Tesio et al. (2014b) used ANNs for calibration with voltammograms acquired from aqueous solution binary mixtures of phenolic compounds, where no data reduction was performed, so one input neuron was required for each point of the voltammogram.

However, it is often necessary to reduce the length of input data to an ANNs in order to gain advantages such as the reduction in training time and avoiding of repetition and redundancy of input data. This can potentially yield more accurate networks, since successful data compression may improve the generalization ability of the ANNs, may enhance the robustness and may simplify the model representation (Gholivand et al., 2014).

On the other hand, Robledo et al. (2013) studied the electrochemical oxidation of three tocopherol isomers (α, γ, and δ) at a carbon fiber (CF) disk UME in benzene/ethanol (2:1) + 0.1 M H_2SO_4 by SWV, whose anodic voltammetric signals was overlapped.

Therefore, to solve this, the methodology of ANN was applied. In this case, four parameters obtained from voltammetric responses in order to reduce the dimensionality were used. Thus, input data were the voltammetric signal

area (A), the sum of absolute values of maximum and minimum of the first derivate of voltammetric signal (D), the net peak current ($I_{p,n}$), and the net peak potential ($E_{p,n}$) of each SW voltammogram recorded.

However, the most popular method for data compression in Chemometrics is the principal component analysis (PCA). When voltammograms are compressed by PCA, one must be aware of some theoretical limitations. PCA is a linear projection method that fails to preserve the structure of a non-linear data set (Gholivand et al., 2014).

If there is some non-linearity in voltammograms, this can appear as a small perturbation on a linear solution and will not be described by the first PCsas in a linear case (Despagne and Massar, 1998). Alternatively, it is possible to use discrete wavelet transform (DWT) to pre-process voltammetric signals before ANN modeling. The coefficients obtained from DWT decomposition, which are extracted from voltammograms, will inputs to an ANNs to obtain calibration models (Cocchi et al., 2003; Moreno-Barónet al, 2006). The pre-processing by DWT reduces the size of the data set being input to an ANNsand also its noise content. Thus, combining wavelets and neuronal networks could result in networks more efficient and capable of handle data of large dimensions (Zhang, 1997).

2.3.3.2. Results and Discussion

The pharmaceutical formulation (LutiMax®) contains mainly two flavonoids, i.e., LUT and RUT. Thus, the problem presented in this study was one case of generalization, trying to predict concentrations of LUT and RUT based on SW voltammograms.

Standard solutions were prepared by mixing aliquots of different concentrations of LUT (C^*_{LUT}) and RUT (C^*_{RUT}) in 10% ethanol + 1 M $HClO_4$ aqueous solutions. Ten samples were designed to obtain the calibration set. Their concentrations were established through an experimental design for two factors, "central composite" of type "2^2 + star", rotatable, and with two central points in the concentration range from 2.0 x 10^{-5} to 1.0 x 10^{-4} M for both substrates.

Nine samples were designed to obtain the validation set. Concentration values were chosen using of a factorial design of 3-levels with two factors (3^2) in a concentration range from 5.0 x 10^{-6} to 7.5 x 10^{-5} M for both substrates.

On the other hand, cyclic and square wave voltammograms recorded in an equimolar mixture of LUT and RUT also showed one single peak (results not shown). The overlapping of voltammetric signals can be explained considering

that RUT structure differs of LUT by only one extra glucoside in the ring C (Figure 1).

Current vs. concentration relationships of mixtures for both flavonoids were first analyzed by PLS-1 through MVC1 (Olivieri et al., 2004).

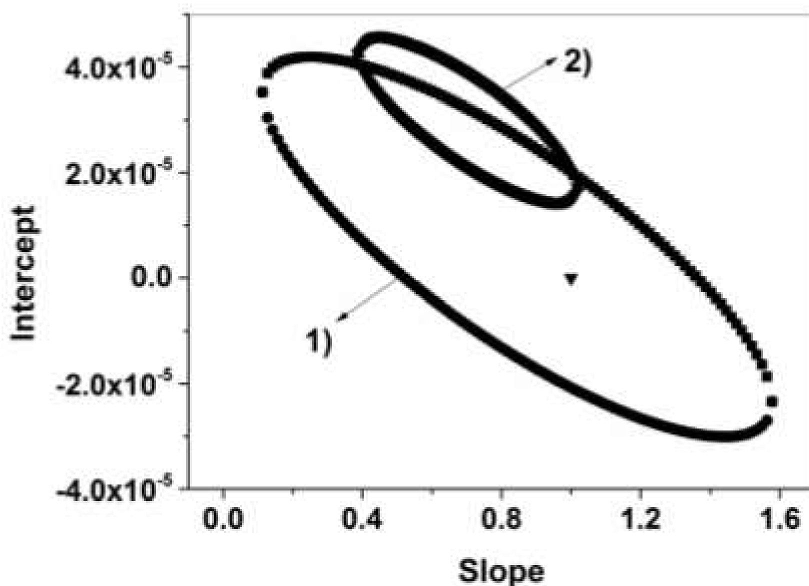


Figure 4. Elliptical regions of the joint confidence obtained by PLS-1 from the validation set for 1) LUT and 2) RUT. (▼) is the ideal point, (1,0).

From merit figures, and error indicator based on deviations of net signal linearity, and sensor selection could be inferred a behavior away from linearity.

In addition, a procedure to verify if the point (1,0) is contained in the confidence elliptical region of the slope and the intercept of the fitted curve between nominal and estimated concentrations was used to evaluate whether or not these concentrations differ statistically (Riu and Rius, 1996; Olivieri and Goicoechea, 2007).

Thus, Figure 4 shows plots of ellipses obtained from the validation set, from the implementation PLS-1in order to evaluate whether or not concentrations estimated by the method differ statistically from nominal concentrations. Results obtained for LUT include the ideal point, whereas for RUT is not included. Thus, the PLS-1 method is not adequate as a calibration

model to determine both substrates in the real sample. In Table 1 are summarized statistical parameters obtained.

Based on these results, we applied the DWT - ANNs methodology to determine LUT and RUT in the pharmaceutical formulation.

Table 1. Error estimation obtained between nominal concentrations and estimated concentrations by PLS-1

Flavonoid	Optimal region (V)	PLS-components	RMSE, P[a]	REP, P[a] (%)	R^2
LUT	0.50 – 0.70	2	1.854×10^{-5}	30.7	0.58
RUT	0.55 – 0.85	3	2.138×10^{-5}	35.4	0.44

[a]prediction set.

For this purpose, we compared different architectures to build and validate the predictive model of the network, which consisted of an input layer, one hidden and one output. The number of neurons of the input layer was equal to the number of independent variables entered into the model, in this case, the matrix data after the DWT pre-processing from voltammetric responses, and the number of neurons in the output layer corresponded to the number of model output variables, i.e., concentrations of LUT and RUT, considering one analyte at a time. On the other hand, the number of neurons in the hidden layer was that obtained from the best architecture of DWT - ANNs.

According to the theory, the number of neurons in the hidden layer of the DWT-ANN should be equal to that of wavelet base. If the number is too small, DWT-ANN may not reflect the complex function relationship between input data and output value. On the contrary, a large number may create such a complex network that might lead to a very large output error caused by over-fitting of the training sample set. Therefore, the following procedure was applied: a) we created an ANNs with a number N of neurons in the hidden layer. b) It was defined the type of training and the transfer function. c) The network was trained with the calibration data set after the DWT pre-processing, considering a 70% of learning, a 15% for monitoring, and another 15% to test the network. d) The network was validated using the validation data set (independent set of calibration data), and e) Root mean square error (RMSE) and relative error prediction (REP%) were calculated by applying equations (1) and (2), respectively, to evaluate the quality of quantitative predictions of concentrations obtained from the DWT – ANNs.

After obtaining the errors for different architectures, we selected the best amount of the hidden layer neurons, the transfer function, and the training type based on the least error.

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{c}_i - c_i)^2}{n}} \tag{1}$$

$$\mathrm{REP}(\%) = \frac{100}{c_{mean}}\sqrt{\frac{\sum_{i=1}^{n}(\hat{c}_i - c_i)^2}{n}} \tag{2}$$

where $\hat{c}_i$ and c_i are estimated and nominal concentrations, respectively, and C_{mean} is the mean of nominal concentrations.

In addition, a procedure to verify if the point (1,0) is contained in the confidence elliptical region of the slope and the intercept of the fitted curve was used to evaluate whether or not these concentrations differ statistically.

Therefore, SW voltammograms of LUT and RUT solutions were recorded at GCE/MWCNTs-PEI electrodes.

Figure 5a shows SW voltammograms obtained for samples after subtracting the blank currents, which were used to obtain the calibration model. Figure 5b shows SW voltammograms recorded for two ethanol LutiMaxextracts at different concentrations. Therefore, Figure 6 shows the SW voltammograms after the DWT pre-processing.

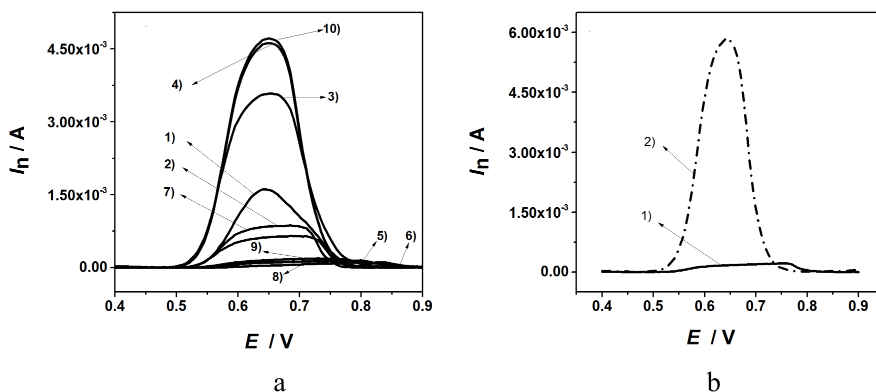
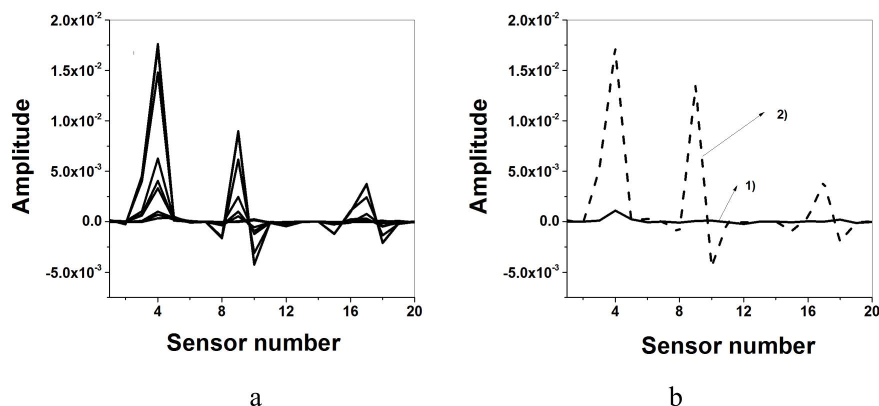


Figure 5. SW voltammograms of a) solutions used in the calibration set, and b) recorded for two ethanol LutiMax extracts at different concentrations of the extract. 1) LutiMax 1, and, 2) LutiMax 2 (see Table 4). ΔE_{sw}= 0.025 V, ΔE_s= 0.005 V, f = 100 Hz.



a b

Figure 6. SW voltammograms after the DWT pre-processing of a) solutions used in the calibration set, and b) recorded for two ethanol LutiMax extracts at different concentrations of the extract. 1) LutiMax 1, and 2) LutiMax 2 (see Table 4). ΔE_{sw}= 0.025 V, ΔE_{s}= 0.005 V, f = 100 Hz.

Table 2. Optimized parameters of DWT – ANNs

Flavonoid	LUT	RUT
Arquitecture	20-5-1	20-5-1
Number of iterations	30	25
Hidden layer transfer function	Tansing	Tansing
RMSETr, C[a]	1.95 x 10-11	3.85 x 10-11
RSMEM, C[b]	1.38 x 10-10	6.73 x 10-10
RSMET, C[c]	1.51 x 10-10	5.42 x 10-10

[a,b,c]are RMSE for training, monitoring, and test for the calibration set, respectively.

Table 3. Errors estimated for the simultaneous determination of LUT and RUT by DWT-ANNs

	LUT (M)	RUT (M)
RMSE,C[a]	2.15×10^{-6}	7.82×10^{-6}
RMSE,P[b]	3.14×10^{-6}	4.88×10^{-6}
REP (%)	5.2	9.6

[a,b]are RMSE for calibration and prediction sets, respectively.

Then, we applied the methodology previously explained to find the most suitable network architecture for the resolution of measured signals. Thus, the best network model was obtained using a 20-5-1 architecture, i.e., 20 neurons

in the input layer obtained from voltammetric responses after the DWT pre-processing, 5 in the hidden layer, and 1 in the output layer. We used Tansig sigmoid transfer function in the hidden layer, and the Purelin linear function for the output layer.

The most appropriate algorithm in the training stage was that of Levenberg-Marquardt (Mathworks, 2010).

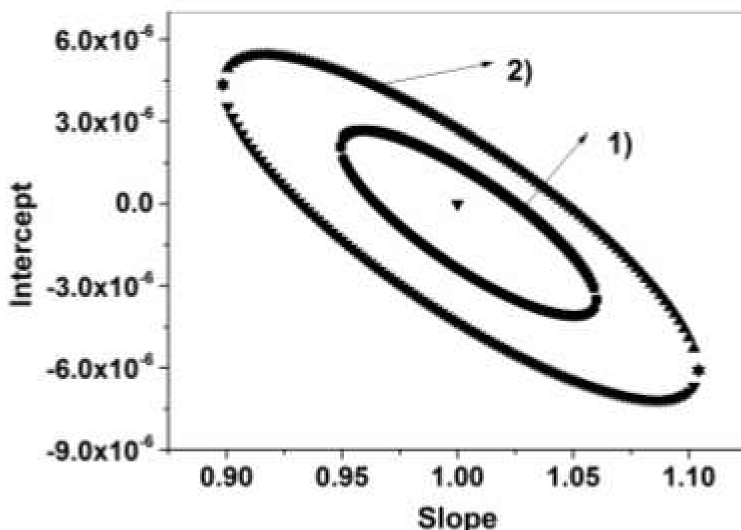


Figure 7. Elliptical regions of the joint confidence obtained by DWT-ANN from the validation set for 1) LUT and 2) RUT. (▼) is the ideal point, (1,0).

Optimized parameters and errors estimated from ANNs methodology are shown in Tables 2 and 3, respectively.

After optimizing the DWT - ANNs with the set of calibration, we used a separate set of voltammograms to perform the validation step through the analysis of accuracy and precision. Thus, Figure 7 shows plots of ellipses obtained from the validation set, in order to evaluate whether or not concentrations estimated by the method differ statistically from nominal concentrations. Results obtained for LUT and RUT by the DWT - ANNs method include the ideal point, exhibiting a comparable accuracy for both substrates, and a greater precision for LUT. Thus, the DWT - ANNs method is adequate as a calibration model to determine LUT and RUT in real samples.

Finally, we calculated concentrations of LUT and RUT in two different LutiMax ethanol extracts (LutiMax 1 and LutiMax 2) (Figure 5).

Results obtained are summarized in Table 4. We used two ethanol extracts concentrations to demonstrate that the results obtained were independent of the concentration levels of the substrates.

Calculated values for LUT and RUT concentrations in the pharmaceutical formulation were (94.3 ± 0.3) and (93 ± 1) mg per tablet, respectively. These results are close to the content of LUT and RUT declared by the manufacturer, with a difference of – 5.7, and – 7 % for LUT and RU, respectively.

Table 4. Results obtained in the determination of LUT and RUT in the pharmaceutical formulation

	Nominal concentrations		Estimatedconcentrations	
	LutiMax 1	LutiMax 2	LutiMax 1	LutiMax 2
C^*_{LUT}(M)	$9.00x10^{-5}$	$4.00x10^{-5}$	$8.51x10^{-5}$	$3.78x10^{-5}$
C^*_{RUT}(M)	$4.20x10^{-5}$	$1.87x10^{-5}$	$3.86x10^{-5}$	$1.67x10^{-5}$

Thus, adsorptive Stripping SW Voltammetry coupled to the DWT - ANN methodology were successfully used to determine the LUT and RUT content in a pharmaceutical formulation, following a very simple extraction procedure.

In addition, results obtained with these methodologies were in excellent agreement with those obtained using the chromatographic method (not shown). This good analytical performance allowed us to determine the content of LUT and RUT in a pharmaceutical formulation, providing an advantageous alternative to existing methods for the determination of LUT and RUT in real samples.

CONCLUSION

In this chapter, an overview of the current techniques for the determination of luteolin in real samples has been developed, putting emphasis on the chromatographic and electroanalytical methods.

Thus, it shows in a greater degree new electrochemical technique for luteolin quantification carried out in our laboratory.

The outstanding sensitivity and broad linear range of electrochemical detection have caught the attention in the last years. The attractiveness of these voltammetric techniques is the low cost, high sensitivity, simplicity, short measurement time and the possibility of miniaturization. The electrochemical

methods, especially square wave voltammetry, make possible to reduce time analysis compared to chromatographic methods.

In our knowledge, the use of the above methodology to determine the content of luteolin in real matrix, such as peanut hull or a dietary supplement, appears as a valuable alternative to chromatographic techniques.

Finally, we expect, for the near future, develop low cost intelligent sensors for the detection of different analytes in real time that suit the needs, mainly, of the food industry.

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Chapter 4

LUTEOLIN: CYTO-GENOTOXIC EFFECTS ON NORMAL CELLS

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ABSTRACT

Luteolin belongs to a group of naturally occurring compounds called flavonoids that are found widely in the plant kingdom. Different studies are focused on their beneficial properties but little is known about the damage of this flavone in normal host cells. The studies conducted in order to evaluate the cyto-genotoxic effects of luteolin on normal animal and human cells showed diverse results that reveal that this flavone could have toxic effects depending on diverse factors as concentration, test carried out (*in vitro* or *in vivo*), time and mode of administration and type of cell. Because there are many biological activities attributed to luteolin, some of which could be beneficial or detrimental depending on various

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aspects, further studies are required to ensure the security of this flavonoid for potential future application in a host system.

Keywords: Flavonoids, Luteolin, cytotoxicity, genotoxicity, normal cells

INTRODUCTION

Luteolin is one of the most common flavonoids that are found widely in the plant kingdom. This flavonoid is present in vegetables and fruits such as celery, parsley, broccoli, onion leaves, carrots, peppers, cabbages, apple skins, and chrysanthemum flowers. It was demonstrated that luteolin is heat stable and losses due to cooking are relatively low [1, 2]. Luteolin belong to the flavone group of flavonoids and its chemical structure consists of two benzene rings, a third, oxygen-containing ring, and a 2–3 carbon double bond, it also consists of hydroxyl groups at carbons 5, 7, 3′, and 4′ positions (Figure 1). The hydroxyl moieties and 2−3 double bond are important structure features in luteolin that are associated with its biochemical and biological activities [3].

Luteolin exhibits versatile biological effects in animal studies. This is likely to be attributable to the fact that luteolin is a molecule with many targets. Preclinical studies have shown that luteolin possesses a variety of biological and pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, anti-allergic, anti-platelet, among others. The anticancer property of luteolin is associated with inducing apoptosis, which involves redox regulation, DNA damage, and protein kinases in inhibiting proliferation of cancer cells and suppressing metastasis and angiogenesis [4].

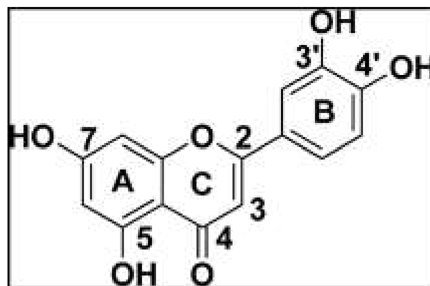

Figure 1. Chemical structure of luteolin.

While the most researches with luteolin are focused on their beneficial properties, little is known about the damage that this flavone could cause in normal host cells. Then, some reports on the cyto-genotoxic effects of luteolin are described.

Cytotoxic Effects of Luteolin

The researches related to the toxic effects of luteolin on normal animal and human cells show diverse results.

Many studies have been performed with peripheral blood mononuclear cells (PBMCs) because these cells are primary effector of acquired immunity and the key in the regulation of the homeostasis of the immune system. The immune system can be a target for many chemicals including environmental pollutants and natural or synthetic drugs, so it is extremely important evaluate the toxic effects of substances that purport to have a future application in human or animals.

López-Posadas et al., (2008) [5], determined the *in vitro* effects of luteolin on the viability of normal lymphocytes isolated from the spleen of female Wistar rats by trypan blue dye exclusion and MTT assays. The authors demonstrated that this flavone at concentration of 15μg/mL induced disruption of the plasma membrane in about 20% of cells, considered as a weak cytotoxic effect, while cell viability decreased sharply by altering the mitochondrial function.

We have evaluated the toxic effects of luteolin in peripheral blood lymphocytes of male and female Wistar rats and observed by trypan blue dye exclusion method that luteolin was also weakly toxic by altering the cytoplasmic membrane at a higher concentration (200μg/mL). However, in our study, no alteration of mitochondrial function was observed [6].

Moreover, Hougee et al., (2005) [7], investigated the toxic effects of luteolin on human PBMCs by apoptosis assay using Annexin V. The results obtained by flow cytometry revealed that luteolin at 5μg/mL induced a selective cytotoxic effect on monocytes without affecting the viability of the T or B cells after 18 h incubation. Rusak et al., (2010) [8], also evaluated *in vitro* luteolin toxic effects on human cells, using the method of fluorescent dye exclusion. These authors showed that luteolin at 3μg/mL did not alter the lymphocyte viability at 2 or 4 h post-treatment. However, after 18 h of incubation observed a slight decrease in cell viability (about 20%). We have also evaluated the toxic effects of luteolin on human PBMCs 24 h post-

treatment and demonstrated that concentrations <10 µg/mL did not affect the human PBMCs viability [9].

The luteolin ability to induce cell damage has also been investigated in other normal cells. We have investigated the effect of luteolin on viability of Vero cells and observed that this flavone (from 100µg/mL) showed slight toxicity on Vero cells causing a decrease of viability of approximately 20% by alteration of mitochondrial and lysosomal function [10].

Tsuji and Walle (2008) [11], showed that luteolin at 10µg/mL induced *in vitro* a slight inhibition of normal trout hepatocytes growth after 24 h of treatment, withoutstatistically significant differences respect to cell control. Other investigation demonstrated that luteolin inhibit the prostate normal cell growth from mice after oral treatment for two weeks with a concentration of 20 mg/kg [12].

Another study found that this flavone not exert toxicity on kidney cells of C57BL/6 mice after 3 days treatment at concentration of 50 mg/kg [13]. Matsuo et al., (2005) [14], investigated the *in vitro* capacity of luteolin to induce damage in human embryonic lung fibroblasts (TIG-1) and endothelial cells from human umbilical vein (HUVE) and demonstrated that this flavone inhibited the growth cell at 30 and 16µg/mL, respectively.

Genotoxic Effects of Luteolin

Given the negative impact on the genetic material that may have substances that are being investigated for possible application in animals or humans, global regulatory authorities require the most information regarding the genotoxic potential of these as part of the assessment process their safety. Because of this the genotoxicity assays have become an integral component of the regulatory requirements [15].

We have determined the genotoxic effects of luteolin by the micronucleus test in Balb/c mice.

After 24 h of intraperitoneal injection, none clinical signs of behavioral toxicity or mortality were observed in the animals treated with luteolin at 2.5, 5 and 10 mg/kg bw.

An increases of micronuclei (MN) in polychromatic erythrocytes (PCE) in luteolin group at higher doses tested (5 and 10 mg/kg bw) was observed. In addition, luteolin did not result cytotoxic in bone marrow cells due to this not alter the PCE/normochromatic erythrocytes (NCE) ratio [16].

On contrary, Shaheen and Nagarajan (1996) [17], determined by the micronucleus test in Wistar rats of both sexes that luteolin did not increase the frequency of MN.

Other *in vitro* studies demonstrate that this flavone induces genotoxic effects in different cells [8, 18].

It is known that flavonoids may exhibit a variety of effects on different biological systems. While it has been shown that they possess anti-carcinogenic, it is also known that depending on the test carried out, either *in vivo* or *in vitro*, under certain conditions, and dose of administration, time and type of target cell can exert genotoxic activity by production of reactive oxygen intermediates [19].

CONCLUSION

Most studies with luteolin support the consumption of this flavone due their protective effect in diverse diseases and multiple beneficial properties for health, other studies demonstrate no effect or potential harm. Studies conducted by us showed that low concentrations of luteolin not produce toxic effects on different cell types. However, because there are many biological activities attributed to luteolin, some of which could be beneficial or detrimental depending on various aspects, further studies are required to ensure the security of this flavonoid for potential future application in a host system.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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