Antitumor Activity of Hesperidin Extracted from Citrus Limon

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Abstract

Cancer is a group of diseases characterized by uncontrolled development and spread of abnormal cells and may be lead to death. Although the exact cause of most cancers is not well understood but it has recognized that the genetic aspect contributes to 5 to 10% of cancers. Other reasons Included poor diets, sure infections, lack of bodily activity, obesity, smokers, and pollution. Phytochemical analysis for plant extract were possessed flavonoids with different contents of 341.66±83.33 mg/ml for of Citrus limon. The reductive ability was more effective than trolox observrd the all concentrations in 0.64 mg/ml recorded the highest reductive ability in C. limon was 0.82±0.02 mg/ml. Also, the radical scavenging (DPPH) was significantly more effective in all concenteration than vitamin C as positive control, the concentrations 0.500 mg/ml represented the highest DPPH in Citrus limon was 97.53±1.43mg/ml. The flavonoid purified from Citrus limon by HPLC, as a possible induction of apoptosis that initiated by hesperidin treated AMJ13 cells was assessed by acridine orange- propidium iodide dual fluorescent staining. The results revealed that hesperidin induced cell death through apoptosis which detected by acridine orange-propidium iodide dual fluorescent staining assay, further confirmed its anticancer effects, suggesting potential applications of hesperidin and its derivatives as anticancer therapy.

Keywords: Hesperidin; Apoptosis; breast cancer.

1. Introduction

Cancer refers to a collection of diseases in which certain type of human cells start growing and multiplying in disorder. It is a major public health issue around the world and is the second-largest cause of death in the United States [1]. The overall cancer death rate dropped continuously from 1991 to 2016 by a total of 27%. In 2017, cancer was the second-most common cause of death in the United States, comprising 22.5% of the total number of deaths; 591,699 people died from complications related to cancer in 2017 [2]. In 2019, 1,762,450 new cancer cases and 606,880 cancer deaths are projected to occur in the United States [3]. The most common cancers are: Lung (2.09 million cases), Breast (2.09 million cases), Colorectal (1.80 million cases), Prostate (1.28 million cases) Skin cancer (non-melanoma) (1.04 million cases) and Stomach (1.03 million cases), The most common causes of cancer death are cancers of Lung (1.76 million deaths), Colorectal (862 000 deaths), Stomach (783 000 deaths), Liver (782 000 deaths) and Breast (627 000 deaths) [4].

Hesperidin is a flavonoid amply found in citrus species including orange peel which is broadly used in Chinese herbal medicine. Previous reports have shown that hesperidin is nontoxic to normal cells, but it is an effective anticancer agent against several types of cancer including bladder cancer, prostate cancer, breast cancer, and hepatocellular carcinoma [5]. It has already been reported that hesperidin induces anticancer activity through the promotion of apoptosis [6]. A recent report showed that hesperidin also inhibits epithelial–mesenchymal transition (EMT) and the expression of members of the MMP family, and thereby inhibiting cell invasion and contributing to its anticancer activity [7]. However, the role of hesperidin in increasing of apoptosis, has yet to be investigated in breast cancer.

2. Materials and Methods

Preparation of Plant Extract

According to Adham [8] a methanolic extract of Citrus limon was made. The soxhlet equipment was used to extract 50 grams of plant leaf powder with 80 percent methanol (250 ml) at 65°C for 3 hours. The extract solution was dried in the oven at 37°C until it was used to make the needed concentrations [8].

Determination of Total Flavonoids

Total flavonoids content was spectrophotochemically determined in the methanolic extract of *citrus limon as* rutin (flavonoids standard) equivalent by aluminium chloride colorimetric method. The methanolic extract of all plant (3.2 mg) were dissolved in 5 ml of 50% methanol, followed by the addition of 1 ml of a 5% (w/v) sodium nitrite solution. After 6 min, 1 ml of a 10% (w/v) aluminium chloride solution was added and the mixture was allowed to stand for a further 5 minutes before 10 ml of a 10% (w/v) NaOH solution was added. The mixture was made up to 50 ml with distilled water and mixed well. Then the absorbance was measured at 450 nm with a spectrometer after 15 min. A similar procedure was applied to six concentrations (2.5, 5, 10, 20, 40 and 80 µg/ml) of rutin, and a standard curve was prepared (figure1). The total flavonoids content was determined using a curve-fitting equation of the standard curve.

Reductive Ability

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The method was adopted to evaluate the reductive ability, in which 1 ml of each concentration of the seven plants extract (0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mg/ml) was mixed with 1ml of 0.2M phosphate buffer (pH 6.6) and 1.5 ml of 1% potassium ferricyanide, then incubated at 50°C for 20 minutes. Also, 1ml of 10% trichloroacetic acid was added to the mixture to stop the reaction. The mixture was centrifuged for 10 minutes at 3000 rpm, and 2.5 ml of the supernatant was mixed with 2 ml of distilled water and 0.5 ml of freshly prepared 1% Ferric chloride. After that, the absorbance was measured at 700nm. The same procedure was applied to the Trolox solutions (standards). All tests were done in triplicates.

DPPH Radical Scavenging Activity

The antioxidant activity of plant methanolic extract and standard (vitamin C) were assessed on the basis of the radical scavenging effect of the stable DPPH free radical. An aliquots of 0.1 ml of the extract or standard (0.625, 0.125, 0.250 and 0.500 mg/ml) was added to 3.9 ml of DPPH solution in a test tube. After incubation at 37°C for 30 minutes, the absorbance of each solution was determined at 517nm using spectrophotometer. All measurements were made in triplicates. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity}\,(\%) = \left(1 - \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}}\right) \times 100$$

HPLC Analysis of *Citrus Peels* Methanolic Extract

HPLC sample preparation

Fifty mg of citrus peels methanol crude extract was diluted in 10 ml of (80:20) v/v methanol: water to examine the phytochemical contents of Citrus peels methanol extract, which had the highest inhibitory potency against glutaminase enzyme following docking results. The extract was ultrasonicated for 25 minutes at 60 percent duty cycles at 25°C in an ultrasonicator. Then, at 7500 rpm, centrifuged for 15 minutes. Prior to evaporation under vacuum, the clear supernatant of samples was treated with charcoal to remove colors (Buchi Rotavapor Re Type). By vortexing, dried samples were re-suspended in 1.0 ml HPLC grade methanol, which was then filtered through a 0.22 or 0.45 m disposable filter and kept at 4°C for the following stages. Then, 20 l of the sample was loaded into an HPLC system using the same optimum separation conditions that had been established earlier using genuine pure standard. (Sigma, U.K).[9].

Assay procedure

The eluted peaks were identified using a UV-Vis 10 A-SPD spectrophotometer and a high-performance liquid chromatography Shimadzu 10AV-LC with binary delivery pump model LC-10A Shimadzu. FLC (Fast Liquid Chromatographic (Phenomenex C-18, 3 m particle size; 50 x 2.0 mm I.D) column was employed under ideal conditions. In the mobile phase, the solvent was a linear gradient of 0.1 percent formic acid, and the solvent B was (6:3:1, v/v) acetonitrile: methanol: 0.1 percent formic acid, respectively. A gradient program from 0 percent B to 100 percent B was run for 8 minutes at a flow rate of 1.2 ml/min. At 25°C, elution was detected at UV 280 nm. [9].

3. Calculation

The sample concentrations were determined using the equation below.

(Area of sample / Area of standard) Concentration of standard dilution Factor = sample concentration (g/ml).

Apoptosis Assay (AO/EtBr)

The AO/EtBr (Sigma–Aldrich, USA) staining method was used to determine the mortality of AMJ13 cells caused by (hespirdin). Briefly, cells were seeded in 12-well plates 24 hours later and treated with IC50 of (hespirdin) before being incubated for another 20 hours. Phosphate-buffered saline was used to wash the cells twice. At an equivalent volume of cells, dual fluorescent dyes (10 L) were applied to the wells. Finally, fluorescence microscopy was used to examine the cells. [10].

4. Statistical Analysis

The MiniTab18 software and excel Microsoft are used to analysis all results. ANOVA was used to test for significant variation. [11].

5. Results and discussion

Assessment of Total Flavonoids

The total flavonoids content in *C. limon* was measured was based on reaction with AlCl3 and spectrophotometrical technique. All determinations were performed in triplicates. Results were expressed as mg/ml rutin equivalents. Upon detection for total flavonoids content for seven plants extract. the results indicated that total flavonoid in *C. limon* was found to be 341.66±83.33 mg/ml flavonoids.

Reductive Ability

In all concentration tested (0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mg/ml), the absorbance of *C. limon* methanolic extract was significantly higher than trolox (vitamin E), and such findings suggest that plant extract are more effective than trolox in the reductive ability, with concentrations-dependent. Since 0.64 gave the highest concenteration and 0.02 showed the lowest as showed figure (2). *C. limon* had reductive ability 0.5 ±0.02 at 0.02 mg/ml and increased significantly ($P \leq 0.05$) to 0.88±0.02 at 0.64 mg/ml.

DPPH Radical Scavenging Activity

Methanolic extract *C. limon* was significantly more effective in DPPH radical scavenging activity than vitamin C as positive control at the four concentrations tested (0.0625, 0.125, 0.250 and 0.500 mg/ml). (figure 3). *C. limon* represented the highest DPPH radical scavenging activity than other plant with an activity of (70.5±3.85) at 0.0625 mg/ml and (97.65 ±1.42) at 0.500 mg/ml

Plant essential oil have been reported to scavenge the free radicals that lead to cell death and tissue damage and the development of chronic diseases, citrus EOs have antioxidant activity which can delay or prevent cell damage caused by physiological oxidants by inhibiting or

eliminating the initiation or propagation of excess reactive species and reduce the risk of potential health effects in humans related to oxidative stress or free radicals [12].

HPLC of Citrus species peels methanol extract

Identification and quantification of hesperidin in peel

HPLC analysis for *Citrus limon* resulted in (figure 4), that showed presence of the highest content hesperidin in peel at 14.20 ppm of sample. Hesperidin was detected in peel of analyzed citrus fruits by comparing their retention times and UV spectra with standards.

The content of hesperidin in *Citrus lemon* diffenent according to part of plant and *Citrus species* as in juice the hesperidin was (23, 20.5 mg/100 mL), while (17, and16 mg/100 mL in edible fruit or juice. Hesperidin content in *Citrus medica* reported by other workers but quantitative determination of hesperidin content in *Citrus medica* by HPLC method was conducted for the first time [9].

Apoptosis (Acridine orange–ethidium bromide (AO/EtBr) double staining).

The fact that our results for hesperidin content in citrus fruits differed from those of other reps could be explained by the HPLC settings. Apoptosis is a type of planned cell death that occurs throughout the development and homeostasis of mammalian tissues and involves the elimination of aberrant cells. Apoptosis induction is considered a conventional and preferred method of cancer treatment. Apoptosis has a number of distinct characteristics, such as chromatin condensation, nucleus fragmentation, cell shrinkage, and membrane blebbing [13]. To confirm the observed changes in the morphologies of the nuclei of the treated AMJ13 cell line, the researchers used acridine orange/ethidium bromide (AO/EtBr) dual staining and fluorescence microscopy. In comparison to untreated cells, hespirdin-treated cells suffered significantly more damage to membrane integrity. Cells treated with hesperidin had less side effects (figure 5). The untreated nuclei

Apoptotic cells feature nuclei that are red to orange in color, with varying degrees of condensation or fragmentation. Early apoptotic cells, on the other hand, had yellow nuclei with condensation and a red hue, whereas late apoptotic cells had red nuclei with condensation. The promotion of cell death via apoptotic rather than necrotic pathways is revealed by changes in the shape of cells treated with hespirdin. Hespirdin activation of apoptotic pathways in breast cancer cells was validated by these findings. Sulaiman et al. [14] revealed that hesperidin can trigger apoptosis in prostate cancer cells, which is consistent with the current findings [14]. An earlier study suggested that boosting intracellular ROS generation could be responsible for the anticancer medicines killing action, at least in part [15].

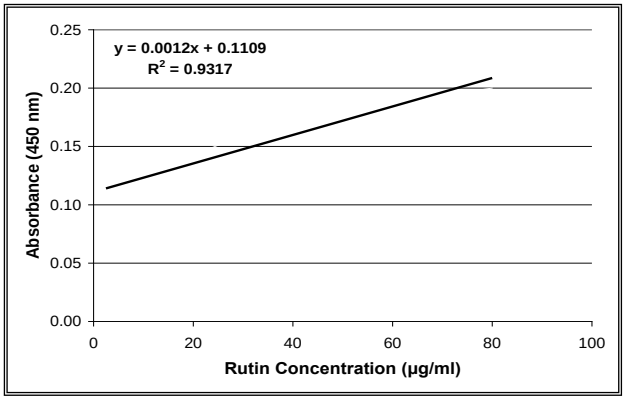

Figure 1: Standard curve for determination of rutin concentration.

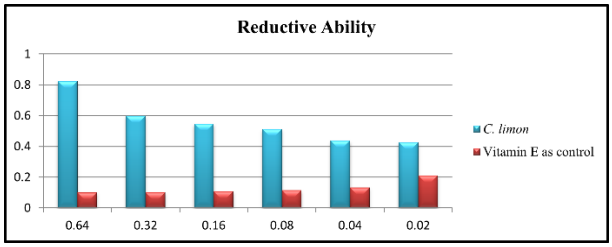

Figure 2: Reductive Ability of C. limon methanolic extract and vitamin E.

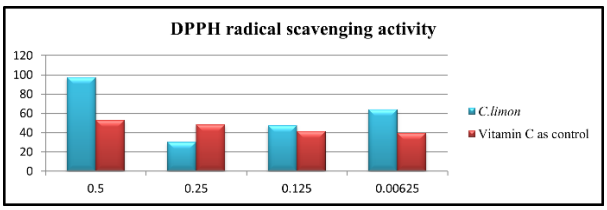

Figure 3: DPPH radical scavenging activity of C. limon methanolic extract and vitamin C.

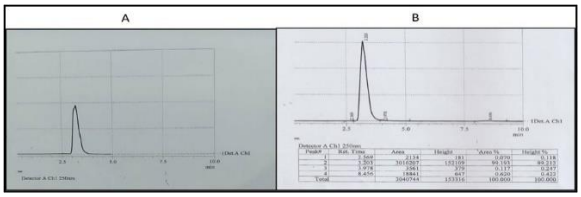

Figure4: HPLC chromatogram of

A. standard

B. hesperidin

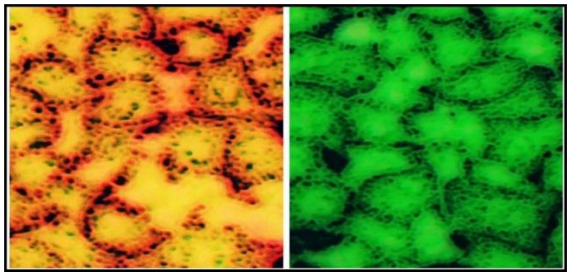

Figure 5: In AMJ13 cells, hesperidin and CB-839 cause apoptosis.

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