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Bioactive Compounds from Mexican Lime (*Citrus aurantifolia*) Juice Induce Apoptosis in Human Pancreatic Cells

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Lime (*Citrus aurantifolia* Swingle) is one of the major citrus fruits and widely consumed, but there is limited evidence about its health-promoting properties. Hence, an investigation was conducted to understand the chemopreventive effects of lime juice on pancreatic cancer cells and the possible mechanism for induction of apoptosis using Panc-28 cells. Freeze-dried lime juice was extracted with different solvents, such as chloroform, acetone, MeOH, and MeOH/water (8:2). The chloroform extract showed the highest (85.4 and 90%) radical-scavenging activity by 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) methods at 624 μg/mL, whereas the MeOH/water extract showed the lowest (<20%) activity. The active components were identified by high-performance liquid chromatography (HPLC) using a C-18 column as rutin, neohesperidin, hesperidin, and hesperitin. Furthermore, the limonoids identified are limonexic acid, isolimonexic acid, and limonin. All of the extracts of lime juice inhibited Panc-28 cancer cell growth. The MeOH extract exhibited the maximum activity, with an IC_{50} value of 81.20 μg/mL after 72 h. The inhibition of Panc-28 cells was in the range of 73−89%, at 100 μg/mL at 96 h. The involvement of apoptosis in induction of cytotoxicity was confirmed by expression of Bax, Bcl-2, casapase-3, and p53. The results of the present study clearly indicate that antioxidant activity is proportionate to the content of flavonoids and proliferation inhibition ability is proportionate to the content of both flavonoids and limonoids.

KEYWORDS: Limonoids; hesperidin; Panc-28 cells; antioxidant activity; apoptosis; antiproliferation; caspases

1. INTRODUCTION

Pancreatic cancer is one of the most devastating of all malignancies, with the highest mortality compared to other cancers (*1*), and is the fourth leading cause of cancer death in the U.S.A. (*2*). Pancreatic cancer is known to exhibit resistance to most chemotherapeutic agents, such as 5-fluorouracil, taxol, doxorubicin, cisplatin, and camptothecin, and persist to be a therapeutic problem (*1*). Recently, new strategies for suppressing the pancreatic tumor cells with safe and naturally occurring dietary chemopreventive bioactive compounds are attracting attention because of their abilities in limiting the tumor cell resistance to apoptosis (*3*). Furthermore, epidemiological studies have demonstrated an inverse relation between the consumption of fruits and vegetables with the incidence of pancreatic cancer (*4*). A large number of case-control studies and a cohort study have also reported similar findings (*5*). The current available information suggest the potential of dietary constituents in the prevention of pancreatic cancer.

Limes are consumed throughout the world for their tart, tangy-flavored juice and especially for their characteristic aromas. Limes are popular for the preparation of juice and carbonated beverages and as a component of alcoholic drinks. In Asian countries, limes are used in pickling, culinary, and medical applications. Lime juice (100 mL) supplies 110−140 kJ (26 kcal) of energy, 50 mg of ascorbic acid (vitamin C), and a trace of dietary fiber (*6*). Further, the most widely consumed product of limes is juice, and it accounts for approximately more than 50% of the total mass of the whole fruit (*7*). Therefore, a study to understand the health benefits of bioactive compounds from the juice of limes is warranted.

Recently, several bioactive compounds were identified in lime, including ascorbic acid, flavonoids, limonoids, coumarins, and phytosterols, and were investigated (*8*). Among the flavonoids, hesperidin was found to be the most abounded in *Citrus reticulata* Blanco (6.76−12.0 mg/g of dried matter), *Citrus sinensis* (L.) Osbeck (6.98−10.8 mg/g of dried matter), and *Citrus limon* (L.) (3.58 mg/g of dried matter) (*9*). Hesperidin is known to act as anticancer agent through prostaglandin (*10*) and inhibition of

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chemical carcinogenesis (*11*). The other flavonoid reported in limes is rutin (quercetin-3-rutinoside) (*12*). Rutin has shown significant scavenging properties on oxidizing species, such as hydroxyl radical, superoxide radical, and peroxyl radical. Furthermore, it has shown anti-allergic, anti-inflammatory, anti-tumor, antibacterial, antiviral, and antiprotozoal properties (*13*), Among the limonoids, limonin, nomilin, obacunone, and their glucosides are found in most of the citrus species (*14*). Results from our laboratory and elsewhere have demonstrated several biological activities of citrus bioactive compounds, such as inhibition of breast (*15*), neuroblastoma (*16*) and prostate (*17*) cancer cells. Furthermore, results also indicated that certain citrus limonoids inhibit the colon cancer cell proliferation in both cell culture and animal studies (*18*, *19*). However, very little information is available on the effects of citrus bioactive compounds on pancreatic carcinoma cells. Therefore, the present study was focused to investigate radical-scavenging ability and proliferation inhibitory activity of lime juice extracts on human pancreatic cancer cells (Panc-28). In addition, the possible mechanisms involved in cell suppression were also explored. Moreover, the bioactive components, such as flavonoids and limonoids, were also identified and quantified by high-performance liquid chromatography (HPLC).

2. MATERIALS AND METHODS

2.1. Materials. Limes (*Citrus aurantifolia* Swingle) were collected from the Texas A&M University−Kingsville, Citrus Center Orchard, Weslaco, TX, during Dec 2006. Limes were hand-squeezed to obtain juice, and juice was freeze-dried using a Labconco freeze drier (Labconco Corporation, Kansas City, MO). Freeze-dried juice was stored at −20 °C until further use.

All of the solvents used for the extraction and quantification of bioactive compounds were ACS- and HPLC-grade and obtained from EDM Chemicals, Inc. (Gibbstown, NJ). The other chemicals, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent, Trolox, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulphate, ascorbic acid, and catechin, were obtained from Sigma Chemical Co. (St. Louis, MO). Rutin (RUT), hesperidin (HES), neohesperidin (NEH), didymin (DID), and hesperitin (HEP) were obtained from Chromadex, Inc. (Irvine, CA). All limonoids used in the present study for the quantification were purified and identified according our published methods (*20*−*23*). Reagents and media used for cell culture studies were of molecular biology grade and purchased from Hyclone (Logan, UT) and Mediatech, Inc. (Manassas, VA). The primary antibodies used for Bax, Bcl-2, casapase-3 (pro-caspase-3, 34 kDa), p53, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antisera was from Pierce Biotechnology, Inc. (Rockford, IL).

2.2. Extraction of Bioactive Compounds. Freeze-dried lime juice powder (462.4 g) was extracted using a Soxhlet-type apparatus with 2000 mL of chloroform at 55−60 °C for 8 h. The extract was filtered through Whatman NO1 filter paper in a Büchner funnel to remove particles. The residue from the funnel and Soxhlet apparatus was re-extracted with other solvents, such as acetone, MeOH, and MeOH/water (8:2), successively for 8 h each. All of the extracts were concentrated under reduced pressure using a rotary evaporator (Büchi, Switzerland) at 40 °C to recover almost 95% of the solvent. The concentrate was freeze-dried and stored at −20 °C until further use.

2.3. Determination of Total Phenolics. The concentration of total phenolics in the lime juice extracts was determined using a spectrophotometer (*24*) with a slight modification, and the results were expressed as catechin equivalents. The freeze-dried chloroform extract was dissolved in acetone, and other extracts were dissolved in methanol and water (5:5, v/v). Different concentrations (10, 20, 30, 40, 50, 75, and 100 μg) of standard (+)-catechin and extracts were taken in tubes, and the volume was adjusted to 10 mL using Millipore water. A total of 1 mL of 10-fold diluted Folin−Ciocalteu reagent and 0.8 mL of 7.5% sodium carbonate solution were added to all of the tubes. After 30 min of incubation at 25 °C, the absorbance was measured at 765 nm using a spectrophotometer. The concentration of phenolics in the samples was calculated using the catechin regression equation and dilution factor. The estimation of total phenolics in all of the extracts was carried out in triplicates and expressed as catachin equivalents.

2.4. Quantification of Bioactive Compounds by HPLC Analysis. *2.4.1. Limonoids.* Most of the citrus limonoids are not available commercially in the market. Hence, we have isolated and identified them from different varieties of citrus according to our published methods (*20*−*23*). The lime extracts were dissolved in MeOH, filtered with a 0.45 μm filter, and subjected to HPLC analysis. The HPLC system consisted of an Agilent HPLC 1200 series (Foster City, CA) system consisting of a degasser, quaternary pump, autosampler, column oven, and diode array detector. Limonoids were separated on a C_{18} Phenomenex Gemini series column (Torrence, CA), 5 μm particle size (250 × 4.6 mm), and detected at 210 nm. The binary solvent system used was 3 mM phosphoric acid (solvent A, pH 3.0) and acetonitrile (solvent B); other parameters were maintained as per the published method from our laboratory (*22*). The compounds were quantified using CHEMSTATION software.

2.4.2. Flavonoids. The HPLC system consisted of a Waters 1525 binary HPLC pump coupled with a Waters 717 plus autosampler and Waters 2996 photodiode array detector (Waters Corporation, Milford, MA). Flavonoids were separated on a C_{18} Waters XBridge column, 3 μm particle size (150 × 4.6 mm), and detected at 280 nm. The binary solvent system used was water/acetic acid (96:4, v/v, solvent A) and acetonitrile (solvent B), and the elution of the binary solvent was conducted in gradient fashion, starting at 85% solvent A, reducing to 50% after 35 min, and completing the gradient at 85% at the end of 40 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before the next run. The flow rate was kept at 1.0 mL/min. The calibration curve for each flavonoid was prepared by plotting the concentration of the flavonoid versus the peak area (average of three runs).

2.5. Radical-Scavenging Activity Using the DPPH Method. Various concentrations (10, 20, 30, and 40 μL) equivalent to 208, 416, 624, and 832 μg/mL of lime freeze-dried extracts along with ascorbic acid were pipetted into 96-well plates. The total volume of the samples and standards were adjusted to 40 μL by the addition of MeOH. A total of 200 μL of solution of DPPH (100 μM) was added and shaken gently (*25*). A control was prepared as described above without samples or standards. The changes in the absorbance of all of the samples and standards were measured at 517 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT) after 20 min. Radical-scavenging activity was expressed as the inhibition percentage and calculated using the following formula:

$$\text{percent radical-scavenging activity} = [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100$$

2.6. ABTS Assay. ABTS was prepared by the reaction of 7 mmol/L aqueous ABTS solution and 2.45 mmol/L potassium persulfate solution (*26*). The mixed solution was stored in the dark for 16 h; the radical cation solution was further diluted in MeOH until the initial absorbance value of 0.7 at 734 nm. Solutions of four extracts of lime juice and ascorbic acid were pipetted into 96-well plates, as mentioned in the DPPH assay. Diluted ABTS radical solution (200 μL) was added to each well, and the readings were recorded after 15 min at 734 nm using a microplate reader with an interval of 3 min. The radical-scavenging activity was calculated using the formula mentioned in the DPPH method. All of the measurements were conducted in triplicate, and the results were averaged.

2.7. Cell Lines and Cell Culture. Pancreatic cancer cell lines (Panc-28) were received from Dr. Paul Chiao, Department of Molecular and Cellular Oncology, The University of Texas, M. D. Anderson Cancer Center, Houston, TX. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in a CO_2 incubator at 37 °C and 85 ± 5% relative humidity (RH). These cells were subcultured, and the stock culture obtained was used for the experiment after 4−5 passage upon observing a normal multiplication pattern.

2.8. 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) Assay. Cell viability was determined using a MTT

Table 1. Percentage of Extractable Yield, Phenolics, Limonoids, and Flavonoids from Lime Juice

solvents	yield (g/100 g)	phenolics (catechin equivalents) (g/100 g)[a]	LG	LNA	ILNA	LIM	total limonoids[a]	RUT	HES	DID	HEP	total flavonoids[a]
chloroform	4.39	3.65 ± 0.21	ND	53.56 ± 1.53	134.98 ± 4.07	4.25 ± 0.03	192.79 ± 5.63	464.89 ± 7.20	46.18 ± 3.43	44.14 ± 1.25	23.87 ± 2.30	579.08 ± 14.18
acetone	16.20	0.69 ± 0.19	35.20 ± 0.63	67.59 ± 2.17	ND	ND	102.79 ± 2.8	ND	104.29 ± 2.42	ND	ND	104.29 ± 2.42
MeOH	8.58	4.20 ± 0.16	ND	ND	ND	ND	ND	ND	386.24 ± 3.87	ND	ND	386.24 ± 3.87
MeOH/water (80:20)	3.33	0.66 ± 0.03	ND	ND	ND	ND	ND	ND	206.83 ± 5.89	ND	ND	206.83 ± 5.89

[a] Average of three independent experiments ($n = 3$).

assay according to a previously described protocol (*27*). To detect the cytotoxicity of Panc-28, cells were treated with extracts of lime juice at different concentrations of 6.25, 12.5, 25, 50, 100, and 200 μg/mL and incubated for 24, 48, and 72 h. Temoxifen citrate was used as a positive control along with limonin, LG, rutin, and hesperdin for comparison purposes. The control group was treated with an equivalent amount of vehicle dimethyl sulphoxide (DMSO; a maximum of 0.2% of the assay mixture was used). The intensity of formazan, reduced product of MTT after the reaction with active mitochondria of live cells, was determined by measuring the absorbance in the 96-well microplate reader (Bio-Tek, Winooski, VT) at a wavelength of 550 nm. Results were expressed as percent inhibition considering that the absorbance of control cells with DMSO was considered as 100% viable.

2.9. Cell Proliferation Using a Cell Count Assay. This assay was performed for all of the extracts; 2×10^4 cells/well were cultured in 12-well sterile plates and incubated for 24 h. After ensuring the growth and condition of cells, media were replaced by 1.0 mL of fresh DMEM containing different concentrations of extracts (25, 50, and 100 μg/mL) and temoxifen acetate (50 μg/mL). After 48, 96, and 144 h of treatment, the viable cells were counted using a Z_1 coulter particle counter (Beckman Coulter, Miami, FL). Results were expressed as percent inhibition with respect to the control. Apart from these extracts, major bioactive compounds, such as LG, LNA, ILNA, limonin, and rutin, were also tested for their inhibitory potential at 50 μg/mL and a mixture of the pure compounds (both limonoids and flavonoids) present in the extracts were also tested at 25, 50, and 100 μg/mL to know the difference in the effects.

2.10. Protein Expression Analysis. The Panc-28 cells (2×10^6 cells/100 mm dish) were seeded in DME medium overnight, prior to the treatment of samples. Cells were treated with medium containing 100 μg/mL lime juice (freeze-dried extracts) for 24 h. The protein expression was measured using 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), as mentioned in our previous publication (*28*).

2.11. Statistical Analysis. Results are expressed as means ± standard error of the mean (SEM). The data were analyzed by one-way analysis of variation (ANOVA) followed by a Tukey−Kramer multiple comparison test using GraphPad Prism software (version 5.00.288). Differences were considered significant for $p < 0.05$.

3. RESULTS AND DISCUSSION

The current study was focused on understanding the potential role of lime juice bioactive compounds in the prevention of human pancreatic cancer, using a cell culture model. The content of bioactive compounds responsible for the radical-scavenging activity and proliferation inhibition were quantified, and also the possible mode of cytotoxicity induction was elucidated. This study provided the valuable information regarding the role of flavonoids and limonoids from lime juice for the induction of apoptosis-mediated cytotoxicity to human pancreatic cancer cells.

3.1. Yield and Phenolic Content of Lime Juice. Freeze-dried lime juice was extracted with four different solvents, such as chloroform, acetone, MeOH, and MeOH/water. The yield phenolic content, total limonoids, and total flavonoids of various extracts from lime juice are depicted in **Table 1**. Among the solvents used, acetone extraction (16.20%) gave the maximum yield. However, the highest phenolics were found in the MeOH extract (4.20%), and the lowest phenolics were found in the MeOH/water extract (0.66%). The results suggest that extracts of lime juice differ in their phenolic content with respect to each solvent. The phenolic content of lime juice extracts ranged from 0.66 to 4.20% (w/w) on a dry weight basis. The MeOH solvent was able to extract most of the phenolics because of its high polarity. Similar results, with 0.48% (w/w) phenolic content, have been reported from Valencia juice (*29*). The total polyphenol content in orange fruits ranged from 0.5 to 1.0% (w/w) in fresh

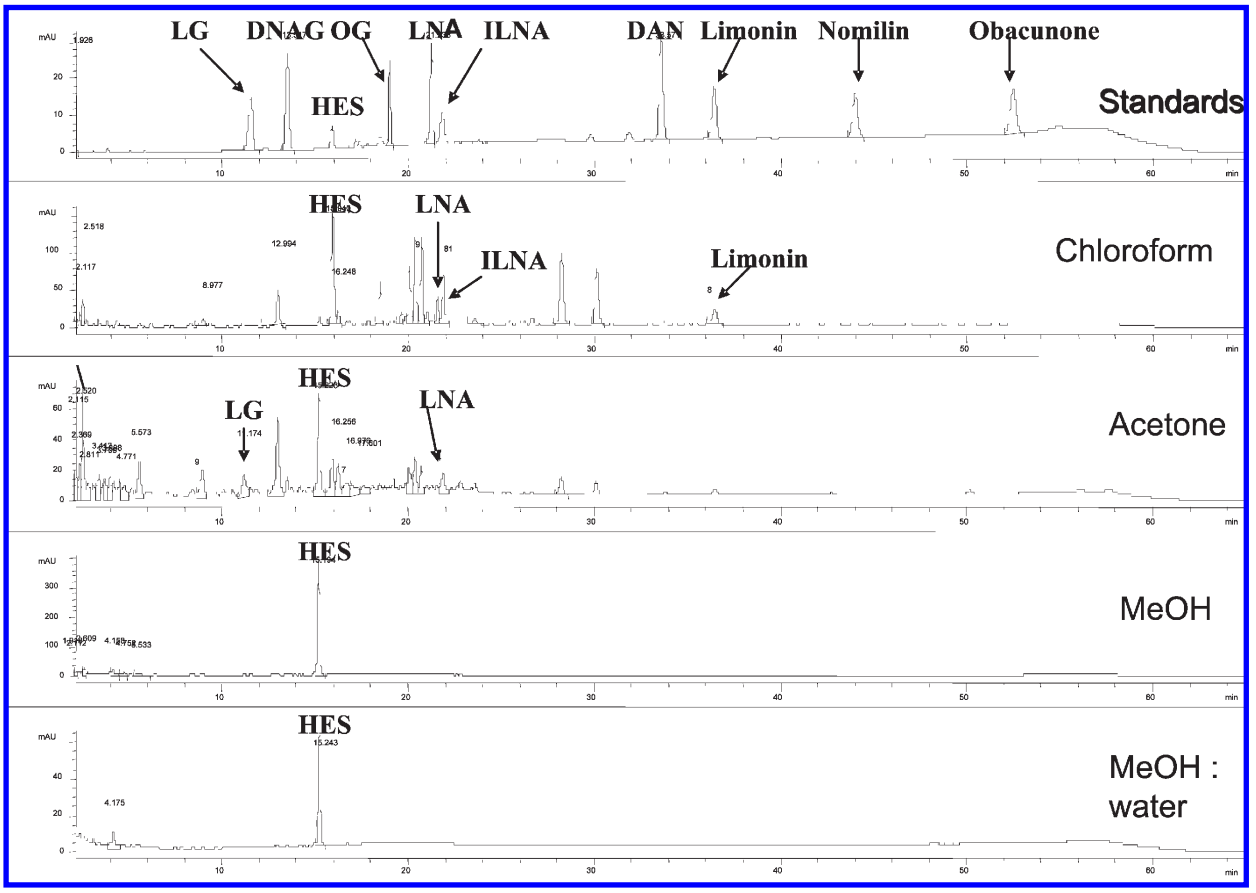

Figure 1. HPLC chromatograms of standard limonoids (LG, limonin glucoside; DNAG, deacetyle nomilinic acid glucoside; HEP, hesperidin; LNA, limonexic acid; ILNA, isolimonexic acid; DAN, deacetyl nomilin; limonin; nomilin; and obacunone) and different extracts from lime juice using a C-18 column.

juice (*30*). Furthermore, total polyphenols in three commercially available orange, Jaffa orange, and Florida orange ranged from 0.5 to 0.75% (w/w).

3.2. Quantification of Limonoids in Lime Juice. HPLC analysis of lime juice indicted that the chloroform extract contained four aglycones and one glucoside (**Table 1** and **Figure 1**). The aglycones were ILNA, LNA, and limonin, while LG (35.20 mg/100 g) was the only glucoside found in the lime juice. However, maximum LNA (67.6 mg/100 g) and LG (35.20 mg/100 g) were found in the acetone extract.

The other important groups of bioactive compounds in citrus are limonoids. Previous studies indicate that the commercial citrus juice contained a high concentration of limonoid glucosides (LGs), and the content was 320, 190, and 82 ppm in orange, grapefruit, and lemon juices, respectively. The major glucoside in citrus juice was limonin 17-*O*-β-D-glucopyianoside (LG), which constituted over 50% of the total limonoid glucosides in the juices (*31*). Furthermore, LG was reported as a major glucoside in a variety of citrus juices tested. Orange juice contains an average of 180 ppm or 56% of the total limonoids. Grapefruit juice contain 120 ppm, which accounts for 63% of the total limonoids, while lemon juice is known to contain about 54 ppm, which was 66% of the total limoniod content (*31*). However, in the current study, the major limoniods, such as LNA and ILNA, accounted for 40 and 45%, respectively.

3.3. Quantification of Flavonoids in Lime Juice. The freeze-dried juice analysis indicated that limes have flavanones and flavonols (**Figure 2** and **Table 1**). Flavanones from lime juice consist of hesperidin, hesperitin, and didymin, while only one flavonol (rutin) was found in lime juice. Among the solvent used for the extraction, chloroform fraction has maximum flavonoids, and it may be due to the presence of 8−10% moisture present in the sample. This moisture content improved the extraction efficiency, and more flavonoids were observed.

Citrus plants accumulate large amounts of flavonoids; however, limonoids are more specific to citrus. In the current study, hesperidin was the most abundant flavonone quantified [58.66% (w/w) of total flavonoids], followed by rutin [36.67% (w/w) of total flavonoids]. The other minor flavonoids were didymin [3.46% (w/w) of total flavonoids] and hespertin [1.87% (w/w) of total flavonoids] on a dry weight basis. It has been reported that the most prominent flavanone from limes was hesperidin. The hesperidin and eriocitrin are found to be prominent flavonones of lemon, whereas only hesperidin is in major concentrations in limes (*32*). In another study, flavonone contents of Mexican lime were quantified and the results indicated the presence of hesperidin (92.4%, w/w), eriocitrin (1.8%, w/w), and narirutin [2.0% (w/w) of total flavonoids] (*33*). In addition to flavanone, limes also contain flavonol, such as rutin (*33*). The rutin, didymin, and hesperitin are the other flavonoids found in lime juice. The presence of rutin, along with other classes of flavonoids, makes lime juice unique in nature compared to other citrus juices (*34*).

3.4. Antioxidant Activity. The radical-scavenging potential of extracts from lime juice and ascorbic acid was tested by DPPH and ABTS methods. The chloroform extract exhibited significantly higher (40−88%) free-radical-scavenging activity at all of the tested concentrations using the DPPH method, which may be attributed to a higher flavonoid content compared to other extracts. On the other hand, low radical-scavenging activity was observed in the MeOH/water extract (20% at 832 μg/mL), which has recorded the lowest phenolic content. Results suggest that the

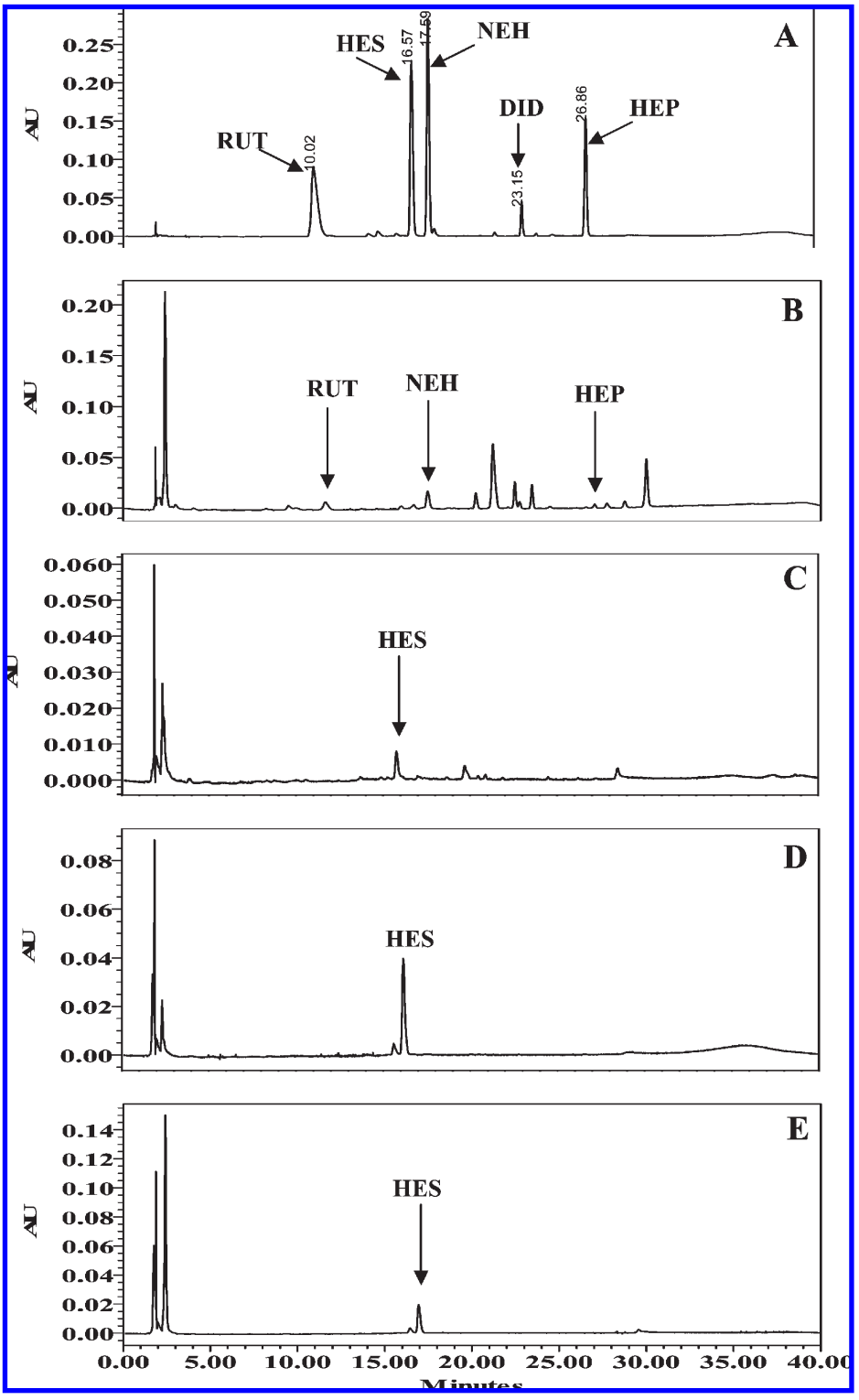

Figure 2. HPLC separation of (A) standards of flavonoids (RUT, rutin; HES, hesperidin; NEH, neohesperidin; DID, didymin; HEP, hesperitin) and lime juice extracts of (B) chloroform, (C) acetone, (D) MeOH, and (E) MeOH/water.

radical-scavenging activity is mainly due to flavonoids. The results of ABTS values from the same juice and different solvent extracts were similar to DPPH-scavenging potential. The chloroform extract showed a significant reduction of ABTS radicals (95%) compared to ascorbic acid (92%), which was followed by MeOH (68%), MeOH/water (31%) and acetone (29%) extracts at 836 μg/mL (**Figure 3**). Recently, natural antioxidants of plant origin are becoming more in demand in developing dietary supplements (*35*). Hence, efforts were made to determine the possible antioxidant components from lime juice. Further, the antioxidant activity from citrus extracts is primarily attributed to its proton-donating capacity (*36*). Evidence from current research indicates that the antioxidant activity may also be due to the presence of flavonoids, phenolics, and ascorbic acid in limes.

3.5. Correlation between the Radical-Scavenging Potential and Phenolic and Flavonoid Contents of Extracts. The contribution of total phenolics and flavonoids to the observed radical-scavenging

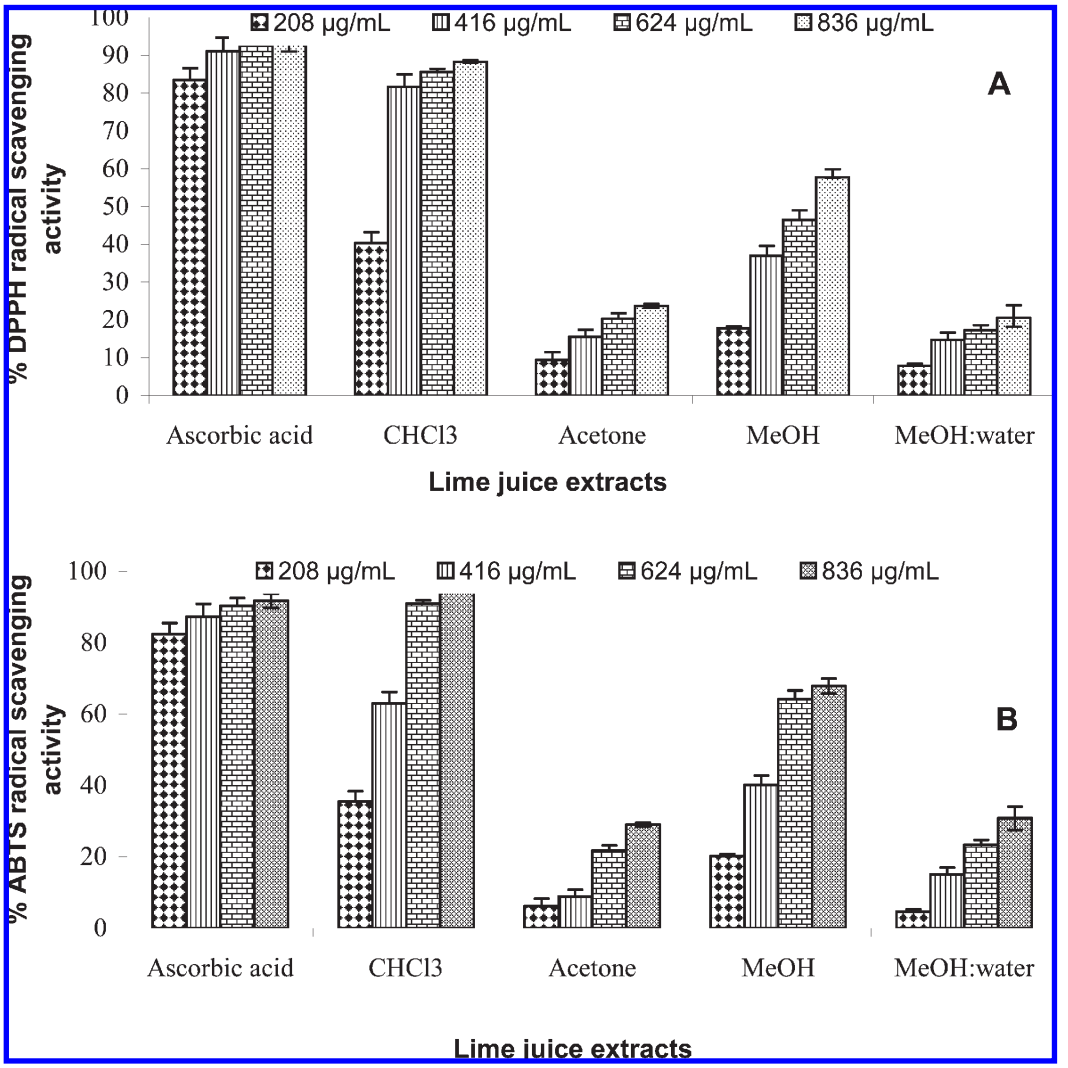

Figure 3. Radical-scavenging activity of lime juice (freeze-dried) by the (A) DPPH and (B) ABTS assays. Values are the mean of three independent experiments ($n = 9$).

Table 2. Pearson Correlation Coefficients between the Radical-Scavenging Activity of Lime Juice Extracts and Total Phenolics and Flavonoids[a]

	ABTS	DPPH	total phenolics	total flavonoids
ABTS	1.000	0.792	0.624	0.832
DPPH			0.539	0.900
total phenolics				0.477

[a] Correlation is significant at the 0.05 level (two-tailed).

Table 3. IC_{50} Values (μg/mL) of Extracts from Lime Juice Showing Inhibition against Panc-28 Cells by the MTT Assay[a]

extract/compounds	24 h	48 h	72 h
chloroform	>200	198.48 ± 8.45	123.31 ± 4.0
acetone	>200	161.69 ± 4.28	129.32 ± 5.84
MeOH	>200	109.67 ± 4.15	81.20 ± 5.75
MeOH/water	>200	121.82 ± 1.02	101.60 ± 5.30
limonin	>200	106.0 ± 1.81	89.31 ± 1.72
LG	116.0 ± 3.40	66.34 ± 2.46	31.69 ± 1.23
rutin	187.20 ± 5.58	49.47 ± 6.73	41.73 ± 3.25
hesperidin	147.28 ± 5.73	26.29 ± 0.48	16.68 ± 0.81

[a] Values are means of three biological replicas.

activity was investigated. The total phenolic content and flavonoids of the extracts seems to have a strong positive correlation with their DPPH and ABTS radical-scavenging capacity. The correlation coefficients of 0.900 and 0.832, respectively, indicating the activity exhibited by lime juice, are mainly due to the high flavonoid content (**Table 2**). The results of correlation studies in the current study are in agreement with the previous results of positive correlations between the Trolox equivalent antioxidant capacity (TEAC) and photochemiluminescence (PCL) assays and total phenolics (*37*). The other studies reported a linear relationship between total phenolic content and antioxidant capacity in berry crops and herbs (*38*).

3.6. MTT Assay. The IC_{50} value at 72 h of lime juice extracts seems to suggest that these treatments are capable of inducing cytotoxicity of Panc-28 cells at lower concentrations. The IC_{50} value of all the extracts were > 200 μg/mL at 24 h. Results of 48 and 72 h have shown that IC_{50} value of MeOH extract was minimum followed by MeOH/water and acetone. The MeOH (109.67 μg/mL) showed a minimum value at 48 h. However, the IC_{50} values of the pure compounds after 72 h of incubation were in the 16.7−89.3 μg/mL. Among the pure compounds tested, the IC_{50} values of flavonoids, such as rutin (41.73 μg/mL) and hesperidin (16.7 μg/mL), were lower compared to those of limonoids, such as limonin (89.3 μg/mL) and LG (31.7 μg/mL), suggesting the potential of certain flavonoids from lime juice in suppressing the pancreatic cancer cells (**Table 3**).

Table 4. Effect of Lime Juice Extracts and Major Phytochemicals Found in Lime Juice on the Proliferation Inhibition of Panc-28 Cells Measured by Cell Count[a]

extracts	concentration (μg/mL)	48 h	96 h	144 h
chloroform	25	40.69 ± 1.88	45.83 ± 2.77	72.60 ± 1.43
	50	75.27 ± 1.54	77.78 ± 1.67	95.77 ± 0.21
	100	82.45 ± 1.57	89.42 ± 1.50	98.32 ± 0.53
acetone	25	13.10 ± 1.03	15.68 ± 1.29	67.04 ± 1.71
	50	42.07 ± 0.54	49.57 ± 2.81	83.38 ± 1.08
	100	46.45 ± 2.15	73.02 ± 0.83	90.06 ± 1.41
methanol	25	13.56 ± 0.38	16.38 ± 0.62	77.90 ± 2.14
	50	45.21 ± 1.50	52.42 ± 2.79	90.32 ± 0.61
	100	55.50 ± 1.87	74.28 ± 1.02	94.16 ± 0.36
MeOH/water	25	14.89 ± 0.75	16.77 ± 1.50	81.75 ± 0.25
	50	41.76 ± 0.38	52.14 ± 1.81	96.03 ± 1.36
	100	46.28 ± 1.50	76.87 ± 2.70	97.77 ± 0.31
temaxofin acetate	50	91.13 ± 1.34	93.70 ± 1.28	98.28 ± 0.71
LG	50	44.94 ± 2.64	55.41 ± 3.86	91.25 ± 2.52
LNA	50	43.49 ± 3.53	58.34 ± 5.32	88.92 ± 2.13
ILNA	50	21.87 ± 9.59	68.26 ± 2.74	90.58 ± 1.51
hesperidin	50	61.89 ± 5.80	74.97 ± 2.90	91.56 ± 3.31
rutin	50	83.51 ± 5.01	94.63 ± 2.36	98.40 ± 0.39
multimix	25	50.30 ± 7.19	69.02 ± 1.91	94.87 ± 1.24
	50	58.30 ± 5.57	89.90 ± 2.39	99.19 ± 0.18
	100	67.56 ± 7.28	94.37 ± 1.53	99.65 ± 0.22
limonin	20	02.44 ± 0.55	60.45 ± 1.18	70.96 ± 4.50
	40	13.93 ± 1.50	64.70 ± 2.23	71.27 ± 2.82
	60	21.19 ± 1.65	69.35 ± 3.49	71.46 ± 3.43

[a] Values are in percent inhibition comparison to control cells and are means of three biological replicas.

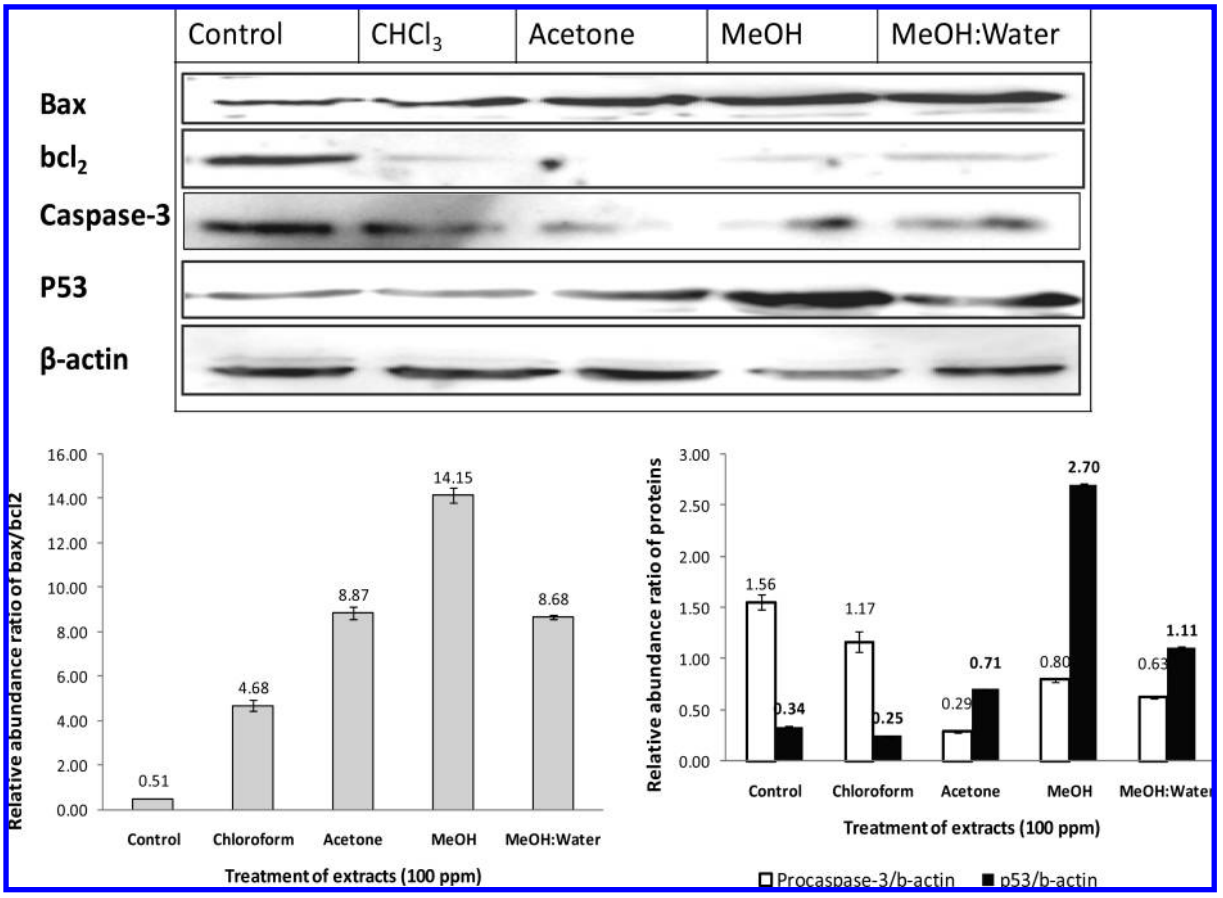

Figure 4. Effect of lime juice extracts on expression levels of apoptosis-related proteins of Panc-28 cells. Cells were treated with a 100 μg/mL concentration of extracts and incubated for 24 h. The total protein was separated on 10% SDS−PAGE. The separated protein band was incubated with specific primary antibodies overnight at 4 °C and tagged with secondary HRP-conjugated anti-mouse antibody for 2 h. The binding efficiency was detected using a Super Signal west femto-maximum sensitivity substrate, and the chemiluminescence image was captured using a LAS4000 image analyzer at −30 °C. β-Actin was used as a reference to ensure uniform protein. Bar graphs represent the relative abundance ratio of Bax/Bcl-2 (left) and expression of pro-casapase-3 (34 kDa) and p53 (right). The relative abundance was normalized with β-actin expression. The experiment was performed in duplicate, and the best of the two images was represented with a respective densitogram value (histograms are an average of three densitometry values of the blot).

The MTT assay of the cell viability assay is a colorimetric analysis to estimate the number of live cells after treatment with the test compound, and the extent of cell death is compared to the control treated with a respective concentration of vehicle treatment under similar conditions. The yellow-colored MTT will be converted to purple-colored water-insoluble dye known as formazan by the active mitochondria of live cells. The intensity of formazan is measured after dissolving it in DMSO. The intensity of formazan is directly proportional to the number of viable cells. The results of the MTT assay suggest that lime juice extracts exhibited significant inhibition of proliferation. The ability of extracts to inhibit proliferation may be attributed to either flavonoids or limonoids. Recent results from our laboratory and elsewhere have demonstrated that citrus limonoids are found to inhibit proliferation of several cancer cells (*39*). Studies have also shown that the major cause for inhibition of the proliferation of colon and neuroblastoma cells by citrus limonoids is due to apoptosis (*40*). Until now, very little information has been available with respect to the inhibition of pancreatic cells by bioactive compounds from lime juice. On the other hand, flavonoids present in these extracts are also known to inhibit the proliferation of a number of human cancer cells (*41*, *42*). Further, to confirm these results, a viable cell count assay was performed, which is based on the counting of viable cells after incubating the cells with test compounds and repeated dosing of test compounds after every 48 h.

3.7. Cell Growth Inhibition by Lime Juice Extracts. The viable cell count assay was conducted after incubating with samples for 48, 72, and 144 h. The results indicated an inhibition of 13−40% of cells after incubation for 48 h with extracts of lime juice at 25 μg/mL. The chloroform extract of lime juice induced 40, 75, and 82% inhibition of pancreatic human cancer cells at 25, 50, and 100 μg/mL, respectively, at 48 h (**Table 4**). The chloroform extract at 48 h inhibited cells significantly (82.45%), while the acetone extract induced the lowest cell death induction (46.5%) as compared to other extracts at 100 μg/mL. However, in addition to temoxifen citrate, a positive control, the prominent bioactive compounds (rutin, hesperidin, LG, LNA, and ILNA) found in these extracts were also used for this assay along with a mixture of these compounds (multimix). The treatments with temoxifen citrate and rutin showed 91.1 and 83.51% inhibition, respectively, at 50 μg/mL (**Table 4**). Furthermore, the chloroform extract at 100 μg/mL and temoxifen citrate and rutin at 50 μg/mL showed more than 98% inhibition after 144 h. It is possible that lime juice may possess potential in the prevention of pancreatic cancer.

The results of these assays further confirm the proliferation inhibitory potentials of lime juice extracts. The viable cell count also indicated that the proliferation inhibition ability of extracts is significantly higher than individual limoniods and flavonoids. This indicates the possible additive or synergistic inhibition ability of compounds.

3.8. Apoptosis-Related Protein Expression of Panc-28 Cells. To understand the possible mechanism of cytotoxicity, immunoblotting studies were conducted to determine the level of major apoptotic-related proteins. The protein level of p53 (tumor suppressor protein) has exhibited higher levels in the cells treated with lime juice extracts (**Figure 4**). The level of p53 protein was the highest in cells treated with the MeOH extract, followed by the MeOH/water and acetone extracts. Furthermore, the protein level of pro-apoptotic Bax and anti-apoptotic Bcl-2 has demonstrated that lime juice favors the induction of apoptosis. The protein level of Bax was found to be elevated in cells treated with all of the extracts. Interestingly, the protein level of Bcl-2 was downregulated in cells treated with lime juice extracts. The ratio of the relative abundance of Bax/Bcl-2 using the MeOH extract had the highest ratio (27.75-fold), followed by cells treated with acetone (17.4-fold), MeOH/water (17.0-fold), and chloroform (9.18-fold) extracts compared to the control. The protein level of caspase-3 in cells treated with juice extract was on the order of acetone > chloroform > MeOH > MeOH/water. Results of the immunoblotting assay seem to indicate an evidence of apoptosis involvement in the induction of cytotoxicity by lime juice extracts. The induction of the tumor suppressor protein (p53) in cells treated with different extracts of lime juice suggests the potential of lime juice in p53-mediated apoptosis induction. The induction were in the order of MeOH > MeOH/water > acetone. Mutations of these proteins or dysfunctions are one of the major causes for malignant transformation (*43*). Furthermore, failure of the p53 tumor suppressor protein is a causal incident in the pathogenesis of a large portion of human malignancies (*44*). p53 is a transcription factor that coordinates cellular responses to stress, such as DNA damage and oncogene activation after induction. p53 alters the expression of a huge set of target genes, leading to cell-cycle arrest, apoptosis, increased DNA repair, and/or inhibition of angiogenesis (*45*). Thus, expression of p53 is one of the major proliferation activities.

Apoptosis is an evolutionarily conserved process, which is also known as programmed cell death, that plays an essential role in organism development and tissue homeostasis. Recent research uses this process of programmed cell death as an ultimate weapon to treat cancer. Apoptosis is controlled by the complex interaction between regulatory proteins from the Bcl-2 family. The death-inducing intrinsic pathway of apoptosis are controlled by these pro- and anti-apoptotic proteins (*46*). In the current study, the certain lime juice extracts have significantly inhibited the Bcl-2 protein level, while the Bax protein level was increased. These results seem to suggest that lime juice induced apoptosis by affecting Bax/Bcl-2.

To further understand the mechanism(s), we examined the involvement of caspases in the cell death. Results seem to indicate that the pro-caspase-3 (34 kDa) protein level was decreased in cells treated with lime juice extracts compared to the control. Caspase-3, an executor caspase, is believed to serve as a general mediator of apoptosis in the pathway and is activated early during apoptosis. A decrease in the level of pro-caspase-3 indicated cleavage of the protein to induce apoptosis. Activated caspase-3 is often considered the key executioner of apoptosis because of its ability to cleave a vast array of proteins. The results of the immunobloting have provided clear evidence of apoptosis involvement in the induction of cytotoxicity by the lime juice extracts. However, a further study on different doses and time points will provide additional information, and such studies are in progress in the laboratory.

The results of current study have revealed that hesperidin and rutin are the major flavonoids and LNA and ILNA are the most prominent limonoids of lime juice. Furthermore, lime juice seems to have the capacity of scavenging free radicals and inhibition of human pancreatic cancer cells. Furthermore, the induction of cytotoxicity was found to be through the p53-mediated intrinsic apoptosis pathway. To conclude these potential benefits, further studies using individual purified compounds are essential in both *in vitro* and *in vivo* systems.

ABBREVIATIONS USED

ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); AR, androgen receptor; Bax/Bcl-2, associated X-protein; Bcl-2, β cells lymphoma/leukemia-2; DID, didymin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EGCG, epigallocatechin gallate; ER, estrogen receptor;

EtOAc, ethyl acetate; HPLC, high-performance liquid chromatography; HEP, hesperidin; ILNA, isolimonexic acid; LG, limonin glucoside; LNA, limonexic acid; MeOH, methanol; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; NEH, neohesperidin, RUT, rutin, SG, β sitosterol glucoside.

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