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In our recent study, we have demonstrated certain limonoids isolated from citrus seeds have induced apoptosis in human pancreatic (Panc-28) cells. In the current study, limonin, nomilin and limonexic acid (LNA) were investigated for possible mode of cytotoxicity in cultured pancreatic cancer (Panc-28) cells. All three limonoids inhibited Panc-28 cell proliferation, with IC_{50} values less than 50 µM after 72 h of incubation. Induction apoptosis was confirmed through cleavage of caspase-3, decreased mitochondrial membrane potential and expression of apoptosis related proteins. The Bax/bcl_2 ratio was increased up to 11 fold in cells pre-treated with 60 µM limonoids for 48 h. Apart from this, limonoids also induced expression of p21, and exhibited anti-inflammatory activity through decreasing expression of cox-2, NF-κB and IL-6. Based on these results, we were interested in understanding the possible mode of inhibition by LNA, which had highest activity. Treatment of Panc-28 cells resulted in dose and time dependent induction of apoptosis inducible proteins. In addition, treatment of 60 µM of LNA has resulted in activation of Akt associated signals to induce apoptosis and the same was confirmed though influence of compound on pAkt, p53, VEGF and caspase proteins. The results of the present study has demonstrated cytotoxicity of limonoids to human pancreatic cancer cells through modulation of genes involved in proliferation and survival.

Introduction

Pancreatic cancer accounts for more than 2% of new cancer cases and 6% of cancer related deaths in US. It is estimated in 2018, approximately 458,918 new cases have been diagnosed globally and 432,242 deaths were reported due to pancreatic cancer (WHO, Globocon 2018 data). In The United States 57,600 Americans will be diagnosed and 47,050 patients will die of pancreatic cancer in 2020 [1]. Currently, there is no complete therapy or medication that can prevent all the symptoms of pancreatic cancer[2, 3]. Hence, it important to conduct research on alternative strategies for pancreatic cancer prevention and treatment and explore the potential of using naturally derived compounds in combinations with other drugs for

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treatment of this disease. Recent *in vitro* cell culture and *in vivo* studies suggest that some natural compounds can effectively inhibit pancreatic cancer growth and these include flavonoids [4], polyphenols [5], and more recently chemically modified derivatives of glycyrrhetinic and ursolic acid [6]. Results of animal studies and clinical investigation of curcumin on pancreatic cancer are promising [7] and demonstrate the ability of natural compounds in prevention therapy for pancreatic cancer [8, 9].

Citrus is one among the most widely consumed fruits in US and the Western world. Bitterness in citrus juice was a major concern for industry and loss of $ 8-10 million per year was estimated due to bitterness from California citrus products[10]. Interestingly, the same bitter compounds are gaining significance due to their potential health benefits. The putative compounds responsible for bitter taste have been identified as limonoids and these compounds are found to be beneficial in preventing certain types of cancers [11, 12]. Both purified limonoids and flavonoid are known for inhibition of carcinogen induced cancer growth in experimental animals by inducing glutathione-s transferase activity, which acts to detoxify carcinogens and decreases ROS [13, 14]. In addition, limonoids also induce apoptosis and activate anti-inflammatory pathway [15]. The synthetic triterpenoid and their derivatives inhibit pancreatic cancer cell and growth of tumour [16-19].

Research in our laboratory and elsewhere has clearly demonstrated that flavonoids and terpenoids present in citrus inhibit growth of different cancer cell lines[14, 20-24]. Citrus limonoids inhibit proliferation of leukemia (HL-60), ovary (SKOV-3), cervix , stomach (NCI-SNU-1), liver (Hep G2), breast (MCF-7) [25] , neuroblastoma [26] and colon cancer cells [27]. In addition, inhibition of carcinogen induced colon cancer and induction of phase-II enzymes in experimental animals has also been reported[15] .

Limonin ($C_{26}H_{30}O_8$) is an oxygenated triterpenoid present in citrus fruits. The citrus juice contain 20-30 ppm of limonin [28] and other parts of the fruit (juice industry waste consist of pulp, seed and peel) contain 0.5-3.5 mg g^{-1} of limonin on dry weight basis [29]. Nomilin ($C_{28}H_{34}O_9$) is present in different parts of fruits in the range of 0.5-3.0 mg g^{-1} dry weight during different stages of maturity [29]. We have also demonstrated that limonin can additively inhibit colon cancer cells when combined with curcumin, the principal compound of turmeric rhizome. The content of nomilin in oranges and

grapefruits ranges from 0.07-0.15 ppm and reconstituted white grapefruits concentrate contains 1.22 ppm of nomilin [30]. Nomilin is more potent than limonin as an inducer of phase-II enzyme in both liver and small intestine, in experimental animals treated with doses up to 10 mg/day/rat [31]. Limonexic acid (LNA) is another triterpenoid found in citrus and other Meliaceae plants [32]. This compound resembles limonin, except that the furan moiety attached to D ring at C-17 position of limonin is replaced with γ-hydroxybutenolide. LNA isolated from *Raulinoa echinata* has shown antinociceptive activity (reduction of pain through opioid receptors) in experimental mice. This action seems to be through GABA and nitroxidergic pathways [33]. Furthermore, LNA exhibits radical scavenging activity [34], however, very little is known on the anticancer activity of LNA compared to other limonoids.

We hypothesize that citrus limonoids induce programmed cell death in pancreatic cancer cells and this was evaluated in human pancreatic Panc-28 cells treated with citrus limonoids and subjected to biochemical analysis in order to understand the influence of limonoids on pathways/genes associated with specific cytotoxicity.

Materials and Methods

Materials and Methods

Cell culture and maintenance.

The Panc-28 cell line was gift from Dr. Paul Chiao, The University of Texas M.D. Anderson Cancer Center (Houston, TX). Cells were cultured in DMEM containing 10 % FBS (Fetal bovine serum) and maintained in a CO_2 incubator at 37°C and 85 ± 5% RH. Cells were used for experiments after attaining 70% of confluence with normal morphology. Panc-28 cells between passage number 5-12 were used in the current experiment.

Purification of limonoids from citrus.

Three triterpenoids, limonin, nomilin and LNA were isolated from citrus seeds using successive solvent extraction and purified using column chromatography as previously described [35, 36]. Structures of limonoids used in the study are as shown in Figure.1 and the purity was determined using reversed phase HPLC [37]. Purity for limonin, nomilin and LNA were more than 98% based on HPLC analysis.

Effect of limonoids on cells viability.

2.3.1. MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide) assay: Panc-28 cells (5×10^3 cells/well) were treated with different concentrations (5-100 μM) of limonin, nomilin, LNA for 24, 48 and 72 h. Cell viability was measured spectrophotometrically by the MTT assay, DMSO was used to dissolve formazan and served as solvent control [38].

Proliferation assay by viable cell count: Based on the results of MTT assay, Panc-28 cells (20×10^3 cells/well) were treated with 20, 40 and 60 μM of limonin, nomilin and LNA and viable cells were counted after treatement for 48, 96 and 144 h using a Z_1 coulter particle counter (Beckman Coulter counter, Miami, FL). Results were expressed as mean ± SD for each determination and compared to DMSO treatment.

Flow-cytometer analysis of cells using Annexin V and PI probes.

Approximately 1×10^6 Panc-28 cells/well were grown in petri-plate and incubated with test compounds (60 μM limonin, nomilin, LNA or DMSO) for 12, 24 and 48 h. After incubation, cells were detached using Accutase (Innovative cell Technologies, Inc., CA, USA) and treated with Annexin V and PI as per the manufacturer's instruction. The stained cells were subjected to flowcytometic analysis using Becton-Dickinson FACS Calibur™ (BD, NJ, USA) instrument.

Effect of limonoids on apoptosis related markers.

Caspase-8 assay. Panc-28 cells were treated with 60 μM limonin, nomilin, LNA and 40 μM camptothecin (positive control). After 48 h, cells were analyzed for caspase-8 as per of the manufacturer's instructions. Activity of caspase-8 was calculated in terms of cleaved pNA spectroscopically at 405 nm using ELISA reader (Bio-Tek Instruments Inc., Winooski, VT) and quantified using pNA standard curve. Simultaneously, buffer and substrate blanks were also used under identical conditions to minimize errors.

Caspase-3 activity. Panc-28 cells were treated with 60 μM limonin, nomilin, LNA and 40 μM camptothecin for 48 h and total caspase-3 content was measured as per manufacturer's instructions. The AMC liberated as a result of caspase-3 activity was measured spectrofluorimetrically using excitation wavelength of 360 nm and emission wavelength of 460 nm using ELISA reader.

Measurement of mitochondrial membrane potential (Ψ). Mitochondrial membrane potential (MMP) was

monitored using fluorescent dye Rhodamine 123. Panc-28 cells were treated with limonin, nomilin, LNA (60 μM) and camptothecin (40 μM) for 12, 24 and 48 h. This was followed by incubation with 2.0 μM Rhodamine 123 for 30 min at 37°C. Cells were washed twice with PBS (phosphate buffered saline, pH7.4) and re-suspended in 100 μL sterile PBS and fluorescent intensity of cell suspensions were measured fluorometrically using excitation of 480 nm and an emission of 530 nm in ELISA reader [39].

Immunoblotting for measuring protein expression. Panc-28 cells were treated with 60 μM of limonin, nomilin and LNA and incubated for 24 and 48 h. After treatment, cells were washed in PBS and lysed in RIPA buffer (150 mM NaCl, 10 mM Tris-Cl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA) with protease inhibitor cocktail for 30 min on ice and centrifuged at 7826 x *g* for 15 min (4 °C) [40] . The protein content of the supernatant was measured using BSA standard curve. Samples equivalent to 50 μg of protein were denatured and resolved on 12% SDS-PAGE (for apoptosis and cell signaling related proteins) and 10% SDS-PAGE (for inflammatory related proteins separation) using Mini-PROTEAN® Tetra Electrophoresis System (Bio-Rad laboratories, Hercules, CA, USA). Protein were transferred to nitrocellulose membrane (0.45 μm, Trans-Blot, Transfer medium, Bio-Rad laboratories, Hercules, CA, USA) after separation using semi dry transfer system (TRANS-BLOT SD, Semidry transfer cell, Bio-Rad, Hercules, CA, USA) and membranes were blocked using dried fat free skimmed milk powder. This blocked membranes were probed with mouse monoclonal anti-AR antibody (1:2500 dilution) (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) at 4°C. Membranes probed with primary antibody were washed four times in TBST, followed by incubation with HRP-conjugated goat anti-mouse secondary antisera (1:25000 dilution). From the probed membranes protein bands were visualized using West femto maximum sensitivity substrate using LAS4000 mini imaging system.

Samples for immunoblotting of cytosolic cytochrome-c and NF-κB (p65) were prepared by using a cytosol fractionation kit (Bio-Vision incorporated, Mountain view, CA, USA). The cytosolic fraction equivalent to 50 μg of protein was used for western blotting as outlined above after separating the proteins on 10% SDS PAGE (for NF-κB) and 12% for cytochrome-c.

Statistical analysis.

Experiments were conducted in triplicate and results are expressed as means ± SD. Statistical analyses were performed using ANOVA and data were compared using Tukey's posttest analysis in GraphPad Prism software version-5.00.288.

Results

Citrus triterpenoids inhibit proliferation of pancreatic carcinoma cells

All three limonoids exhibited dose and time dependent inhibition of Panc-28 cell proliferation (Fig. 2A-C) and the IC_{50} values of 98.5, 52.3 and 41.5 µM were observed for limonin, nomilin and LNA, respectively after 24 h of treatment. Inhibitory effect of LNA was higher than observed for the other two limonoids, with an IC_{50} of 15.43 µM after treatment for 72 h (Table.1). After treatment for 2, 4 and 6 days, viable cells were counted using cell counter. The inhibition was found to be 71, 81 and 89%, for limonin, nomilin and LNA respectively after treatment or 6 days.

Translocation of phosphatidylserine (PS) from plasma membrane to cells surface and indication of apoptosis.

The flow cytometry analysis of Panc-28 cells treated with limonoids for 12, 24 and 48 h suggests the clear evidence of apoptosis induction after 12 h of treatment. Amount of early apoptosis cells which were stained with annexin-V were 1.7, 2.4 and 2.4% after 12 h of treatment with 60 µM of limonin, nomilin and LNA, respectively. On the other hand, percentages of cells stained with PI were more after 24 and 48h of treatment. The PI stained cells were 13.7, 11.0 and 16.2% after 48 of treatment with limonin, nomilin and LNA, respectively (Table 2). There was significantly reduced number of live cells with treatment of limonoids for 48 h suggesting the cytotoxic nature of the compound. The percentage of cells stained with both annexin V and PI were 12.5, 16.7 and 12.8%, respectively with treatment of limonin, nomilin and LNA for 24 h (Fig.2D).

Limonoids modulate biochemical markers associated with apoptosis in Panc-28 cells

Pre-treatment of Panc-28 cells with limonoids (60µM) significantly enhanced the activation of caspase-3 after 48 h (Fig 3A). The total caspase-3 activity was increased 2.2, 2.1 and 2.6 fold, after treatment with limonin, nomilin and LNA, respectively. Camptothecin (positive control) increased the caspase-3 activity by more than 4 fold However, there was no significant influence on the activity of caspase-8 either by camptothecin or the Limonoids (Fig 3B).

The effect of limonoids on mitochondrial membrane potential (MMP) was determined in the cells treated with rhodamine dye and the three limonoids (60 µM each) for 12, 24 and 48 h. The difference in the fluorescent intensity was used to access the loss of mitochondrial membrane integrity and between 12 and 48 h of treatment, the loss of MMP ranged from 20-39% (Fig.3C).

Limonoids alter apoptosis related proteins in Panc-28 cells

Expression of p21 (cyclin-dependent kinase inhibitor) was increased in Panc-28 cells following treatment with limonin, nomilin and LNA by 2.5, 4.6 and 6.6 fold, respectively for 24 h. Treatment of Panc-28 cells with limonin, nomilin and LNA also increased expression of Bax up to 80% after treatment for 48 h, whereas expression Bcl_2 was decreased by 70-80% over the same period (Fig.4A). Ratio of Bax to Bcl_2 was significantly increased by 3.2 and 3.5 fold in the cells treated with limonin and nomilin, respectively for 48 h. The increase in the Bax/Bcl2 ratio was 4.5 fold in cells treated with LNA for 48 h (Fig.4C). Decrease expression of full length (inactive) caspsae-3 (34 kDa) in cells treated with limonoids for 24 and 48 h supports the enzyme activity. Expression of cytosolic cytochrome-c was increased by 15-25% after 24 h and up to 30% after treatment with limonoids for 48 h. This result was consistent with decreased MMP and loss of mitochondrial cytochrome-c.

Limonoids down regulate expression of inflammatory proteins.

The cytosolic content of nuclear factor-κB (NFκB- p65) expression was decreased by 10-28% and 20-48% after treatment with limonoids for 24 and 48 h, respectively. This was also accompanied by a 76, 61 and 77% decreased expression of cyclooxygenase (Cox) -2 after treatment with either limonin, nomilin or LNA, respectively for 48 h. The levels of interleukin-6 (IL-6), one of the downstream products of NF- κB activation was also decreased by 39-58% and 60-85%, after 24 and 48 h of treatment (Fig. 4B), demonstrating the anti-inflammatory effects of limonoids.

Limonoids influences key markers of cell death

In addition to apoptosis and inflammation, limonoids used in the current study also activated cell signalling. Expression of Akt and pAkt clearly suggest the ability of all the three limonoids to activate cell signalling. Tumor suppressor protein (p53) expression was elevated by 19, 55 and 70% in the cells treated with limonin, nomilin and

LNA, respectively for 24 h and was increased after 48 h. Limonoids also decreased expression of full length (inactive) caspase 8 and 9. In addition, expression of vascular endothelial growth factor was down regulated by 50-60% after treatment of limonoids for 48 h (Fig. 5). The result of current study demonstrates that limonoids tested in the current study are known to activate multiple pathways involved in activation of programmed cells death specifically in pancreatic cancer cells.

Discussion

Citrus fruits and juice are widely consumed natural products and it has been associated with various health benefits. Limonoids such as, limonin, nomilin and limonexic acid are linked with the bitterness of citrus. However, these compounds exhibits a range of biological activities and undoubtedly contribute to the health benefits of citrus products [41, 42]. Phytochemicals of citrus such as, flavonoids, limonoids and pectin are known for inhibition of cancer cells and tumors [25, 43-45]. Limonin, a common limonoid in citrus plants is known for inhibition of different cancer cells proliferation and tumor markers of colon cancer [36, 46, 47]. Limonin is also known to induce phase-II detoxifying enzymes in carcinogen challenged as well as normal animals [48, 49], similar activity is observed with chemical derivatives of limonin[14]. Nomilin has shown inhibition of colon, breast and lung cancer cells proliferation and suppression of tumour in chemically induced colon cancer model [25, 44]. Recent report from our laboratory demonstrates inhibition of colon cancer cells proliferation and cell cycle arrest by LNA[50]. Additionally, we have demonstrated that LNA induces apoptosis in Panc-28 cells through the activity of Bax and bcl2 [51]. Based on the available information and our previous results, In the study we have used 3 limonoids limonin, nomilin and LNA as models to investigate and compare their effects on growth inhibition and induction of apoptosis in Panc-28 cells

The effect of limonin, nomilin and LNA on inhibition of cell proliferation of Panc-28 cell was determined using both the MTT and cell counting assays. Growth inhibitory IC_{50} values after treatment of Panc-28 cells with limonin, nomilin and LNA for 72 h were 22.2, 21.1 and 15.4 µM, respectively as measured by MTT assay (**Table 1**) and the cell counting assay gave similar results (Fig 2A-C). These data are consistent with previous *in vitro* studies in cancer cell lines, which show that limonoids inhibit growth of cancer cell lines[47, 52, 53]. Treatment of Panc-28 cells with limonin, nomilin and LNA also enhanced the annexin-V staining after 12 h of

treatment and this is due to translocation of the membrane phospholipid phosphatidylserine (PS) from the inner face of the plasma membrane to the outer surface (**Fig. 2D**). Further % of cells stained with only annexin V were highest after 12 h and those stained with both annexin V and PI were higher at both 24 and 48 h of treatment. This suggest the possible onset of apoptosis after 12 h, and this was further analysed after treatment for 24 and 48 h. This cellular marker of apoptosis was accompanied by induction of p21, activation of caspase-3 and increases Bax/Bcl2 ratio, confirming the induction of apoptosis in Panc-28 cells. We also observe that the limonoids decreased MMP and enhanced cytosolic cytochrome-c and this is consistent with induction of the intrinsic apoptosis pathway. Limonin, nomilin, obacunone, and deacetylnomilin, isolated from molasses of citrus fruits have induced apoptosis in colon (Caco-2) and neuroblastoma (SH-SY5Y) through activation of caspase 3/7 [47]. Another study suggests that the extracts of *Dictamnus dasycarpus* bark containing limonin induced apoptosis in concanavalin A-activated T cells through casaspe-8 cleavage [54], which is on similar agreement with results of our study.

In pancreatic cancer, factors associated with inflammation are known to cause genomic instability and enhance cellular proliferation, favoring malignant transformation. Inflammatory pathways in cancer cells are associated with activated cytokines, reactive oxygen species and inflammatory mediators such as NF-κB and cyclooxygenase-2 [55]. Therefore, agents that inhibit NF-κB and Cox-2 are being developed as chemotherapeutics for prevention and therapy. In the current study, the cytoplasmic expression of NF-κB was decreased by 10-35% and 18-38% after treatment of limonoids for 24 and 48 h, respectively (**Fig 4B**). Interleukin-6 (IL-6) expression is regulated by NF-κB and potent mediator of several responses including cell proliferation and survival [56]. Limonoids depleted activity of IL-6 by 60% and 85% after treatment for 24 and 48 h and this was consistent results of NF-κB expression. Cox-2 is another NF-κB dependent protein and key mediator of inflammation. In the current study, limonoids also decreased Cox-2 protein level by 90%, after 48 h of treatment. These results demonstrate for the first time that limonoids specifically target anti-inflammatory mediators in Panc-28 cells and this activity will be important for the potential cancer chemopreventive and chemotherapeutic effects of these compounds. Results of the current study demonstrate that the citrus

terpenoids are also capable of inhibiting VEGF expression up to 50%. In addition, these compounds have demonstrated the ability to activate p53, BAD, Caspae-8 and 9 in Panc-28 cells (**Fig. 5**). Based on the association of these key markers with inhibition of angiogenesis and support apoptosis of cancer cells specifically, it may be worth to explore the mechanism of these natural compounds for benefit in cancer using animal models.

Conclusions

In summary, the results of this study show that limonoids which are commonly found in citrus significantly inhibit Panc-28 cells growth. Cellular events including translocation of phosphatidylserine from plasma membrane to cell surface, Induction of caspase-3 and alteration of mitochondrial membrane potential indicate that induction of apoptosis is a major contribution in the cytotoxicity of the compounds. Further analysis of key proteins expression like Bax, Bcl2 indicates that limonoids induce apoptosis, regulate cell cycle (p21), inhibit inflammation (NFkB, Cox-2, IL-6) and enhance tumour suppressor p53. Among the three limonoids tested in the current study, the order of cytotoxic potency was LNA> nomilin> limonin. Further studies will investigate the in vivo activity of individual limonoids and their mixture and possible chemopreventive and chemotherapeutic agents for treatment of pancreatic cancer

Conflicts of interest

No author has any conflicts of interest to declare

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Notes and references

Legends of Tables and figures.

Table 1. IC_{50} values (μM) of three limonoids study in this research. The values were calculated based on the proliferation inhibition activity was measured by MTT assay.

Table 2. Staining of Panc-28 cells treated with limonoids with annexin-V and PI. Values are mean of two independent experiments each treatment and analysis were done in quadruplicate (n=8). * represents significance between control and treatment groups at $P<0.05$ level using Tukey's posttest analysis.

Fig. 1 Chemical structure of three purified triterpenoid aglycones used for study **(a)** limonin, **(b)** nomilin **(c)** LNA

Fig.2 Antiproliferative activity of limonin(**A**), nomilin(**B**) and LNA(**C**) on Panc-28 cells as measured by cell counting following the treatment (Values are Mean ± SE, n=6). Results expressed as % inhibition with reference to control cells treated with DMSO. (**D**)**.** Effect of limonoids on annexinV binding and PI staining of Panc-28 cells, values were presented as percentage. A representative image of flowcytometry of 24 h treatment is shown in figure (experiments were performed in duplicate and each treatment were done in quadruplicate). ** indicate highly significant ($p<0.01$) and

Fig.3 (A) Effect of limonoids on content of caspase-3 (values are mean ± SE, n=4), measured based on the fluorescent intensity (360/460 nm) of 7-amino-4-methylcoumarin from a specific substrate Ac-DEVD-AMC after 48 h of treatment [N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methyl coumarin)].Values are compared with control, [*-significant $p<0.05$ and ** highly significant $p<0.01$]. (**B)** Effect of limonoids on caspase-8 content of Panc-28 cells (values are Mean ± SE, n=4), caspase-8 measured spectroscopically at 405 nm after interaction with specific substrate Ac-IETD-pNA (benzyloxycarbonyl-Ile-Glu-Thr-Asp-*p*Na) after 48 h of treatment. (**C**)**.** Effect of limonoids on mitochondrial membrane potential of Panc-28 cells (values are Mean ± SE, n=6). The Panc-28 cells incubated with limonoids for 12, 24 and 48 h were incubated with rhodamine123 at 2.0 μM for 30 min at 37° C and cells were washed twice with PBS and fluorescent intensity of cells were measured after suspending in 100μL PBS at 480nm (excitation) and 530 nm (emission).

Fig. 4. (**A)** Expression of apoptosis related proteins by treatment of limonin, nomilin and LNA (60 μM) for 24 h. Proteins from treated cells were separated on 12% SDS-PAGE and transferred in to nitrocellulose membrane. Immunoblotting using β- actin, Bax, bcl_2, cytochrome-c, pro-caspase-3 and caspase-8 antibodies were performed as described in methods. (**B**) Expression of inflammation related proteins in Panc-28 cells incubated with limonin, nomilin and LNA (60 μM) for 24 h Immunoblotting using β- actin, cox-2, IL-6 and NFκB (p65) antibody were performed as explained in methods. (**C).** Ratio of expression of Bax/Bcl_2 in cells treated with limonoids (values are in terms of relative abundance based on the densitometry scores).

Fig. 5 Expression of proteins associated with angeogenesis and cell death in Panc-28 cells treated with limonoids. Proteins from treated cells were separated on 12% SDS-PAGE and transferred in to nitrocellulose membrane. Immunoblotting of the expression of specific proteins were performed as described in methods.

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Table1. IC_{50} Values (μM) of limonoids, values were calculated based on the proliferation inhibition activity measured by MTT assay.

Limonoids	**IC_{50} ± SD**		
	24 h	**48 h**	**72 h**
limonin	98.56 ± 1.07	36.29 ± 1.08$^{\Delta}$	22. 20 ± 0.84*$^{\Delta}$
nomilin	52.29 ± 0.91	24.78 ± 2.08*$^{\Delta}$	21.11 ± 1.19*$^{\Delta}$
limonexic acid	41.49 ± 1.45	28.66 ± 1.9*$^{\Delta}$	15.42 ± 1.45**$^{\Delta}$

Note: Values are mean of three independent experiments (n=9); [**- Highly significant ($p<0.01$ and *-Significant ($p<0.05$) compared to respective treatment at 24 h and $^{\Delta}$- significant compared to control]

Table 2. Staining of Panc-28 cells treated with limonoids with Annexin-V and PI. Values are mean of two independent experiments each treatment and analysis were done in quadruplicate (n=8). * represents significance between control and treatment groups at $P<0.05$ level using Tukey's posttest analysis. $^{\Delta}$ - Indicate values were significant compared to 12 h treatment of the same compound.

Treatment	Duration of treatment	Live	Early apoptosis	Necrosis	Late apoptosis
Control	NA	94.2 ± 5.10	1.0 ± 0.9	0.9 ± 0.16	1.26 ± 0.26
Limonin	12 h	93.6 ± 3.60	1.7 ± 0.68	2.0 ± 0.80	2.66 ± 0.44
	24 h	74.3 ± 2.80*	1.5 ± 0.39	11.5 ± 0.70*	12.5 ± 0.10*
	48 h	62.2 ± 1.35$^{\Delta}$*	1.1 ± 0.73	13.7 ± 1.10*	20.3 ± 0.35$^{\Delta}$*
Nomilin	12 h	93.1 ± 4.13	2.4 ± 0.26*	2.2 ± 0.35	2.7 ± 0.35
	24 h	70.4 ± 3.45	1.8 ± 0.39	7.5 ± 0.65*	16.7 ± 0.85*
	48 h	59.2 ± 2.35$^{\Delta}$	1.6 ± 0.70	11.0 ± 0.75*	23.3 ± 1.62$^{\Delta}$*
Limonexic acid	12 h	92.4 ± 3.10	2.4 ± 0.31*	1.5 ± 0.15	2.9 ± 0.75
	24 h	55.9 ± 3.15*	2.2 ± 0.23*	12.9 ± 0.75*	12.8 ± 0.8*
	48 h	24.2 ± 1.80$^{\Delta}$*	1.6 ± 0.56	16.2 ± 1.4$^{\Delta}$*	26.8 ± 2.0$^{\Delta}$*

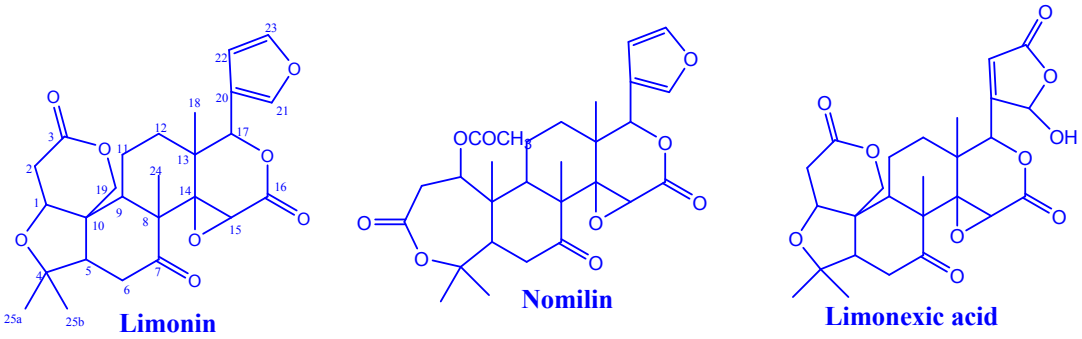


Fig.1

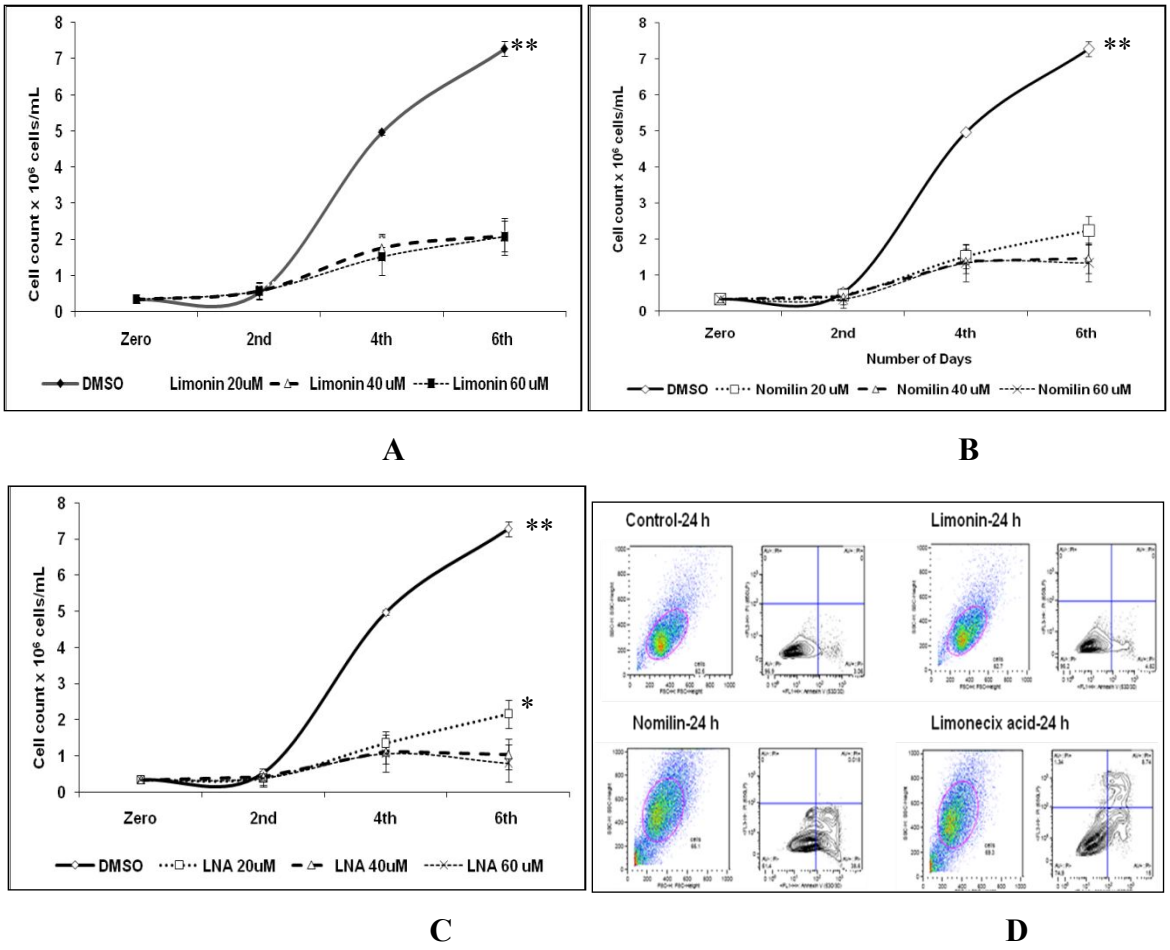

Cell count x 10⁶ cells/mL
Zero
2nd
4th
6th
**
DMSO
Limonin 20uM
Limonin 40 uM
Limonin 60 uM
A
Number of Days
Nomilin 20 uM
Nomilin 40 uM
Nomilin 60 uM
B
*
LNA 20uM
LNA 40uM
LNA 60 uM
C
Control-24 h
Limonin-24 h
Nomilin-24 h
Limonecix acid-24 h
D

Fig.2

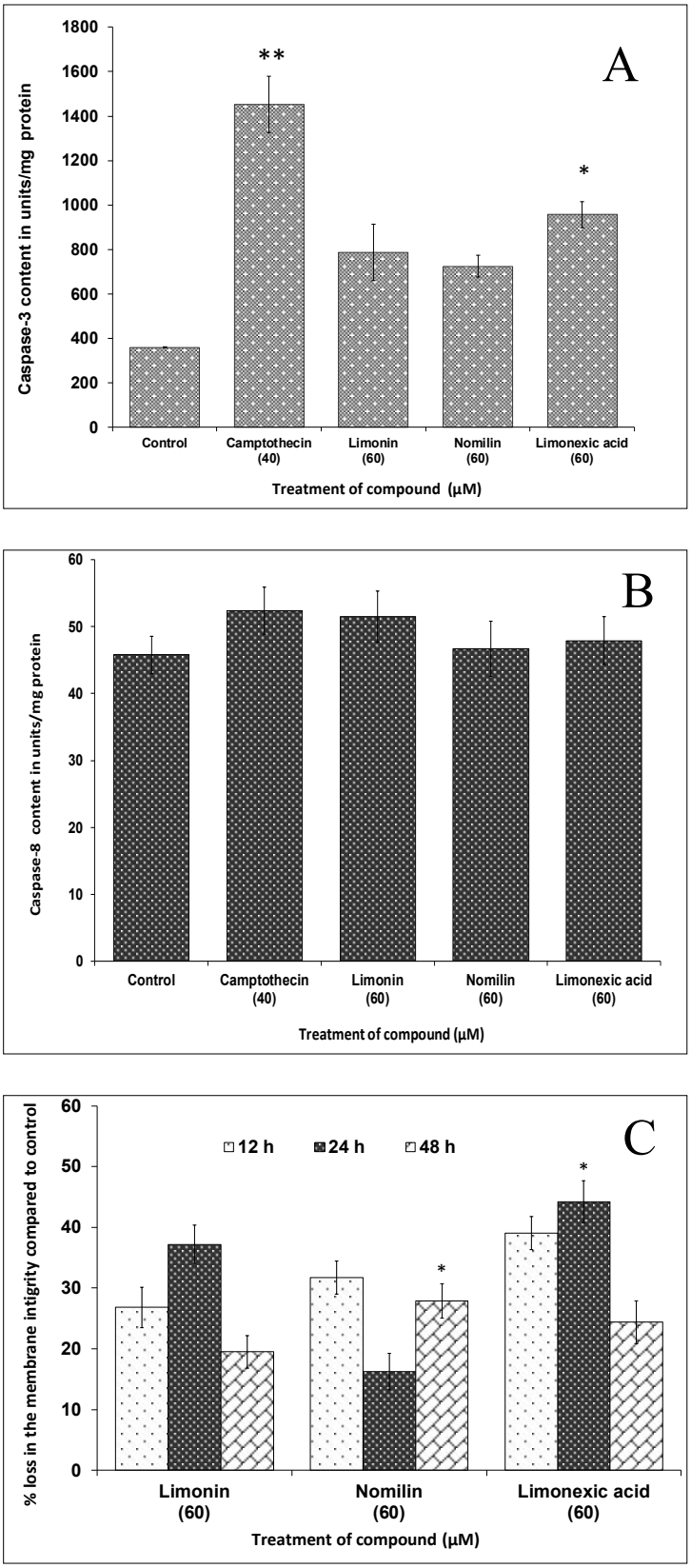
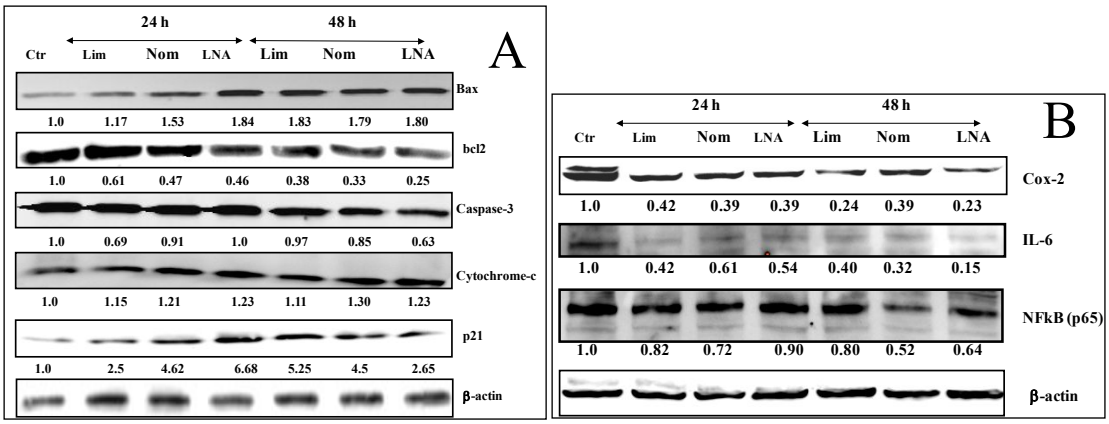

A
Caspase-3 content in units/mg protein
1800
1600
1400
1200
1000
800
600
400
200
0
**
*
Control
Camptothecin (40)
Limonin (60)
Nomilin (60)
Limonexic acid (60)
Treatment of compound (μM)
B
Caspase-8 content in units/mg protein
60
50
40
30
20
10
0
Control
Camptothecin (40)
Limonin (60)
Nomilin (60)
Limonexic acid (60)
Treatment of compound (μM)
C
% loss in the membrane intigrity compared to control
12 h
24 h
48 h
60
50
40
30
20
10
0
*
*
Limonin (60)
Nomilin (60)
Limonexic acid (60)
Treatment of compound (μM)

Fig. 3


A
24 h
48 h
Ctr
Lim
Nom
LNA
Lim
Nom
LNA
Bax
1.0 1.17 1.53 1.84 1.83 1.79 1.80
bcl2
1.0 0.61 0.47 0.46 0.38 0.33 0.25
Caspase-3
1.0 0.69 0.91 1.0 0.97 0.85 0.63
Cytochrome-c
1.0 1.15 1.21 1.23 1.11 1.30 1.23
p21
1.0 2.5 4.62 6.68 5.25 4.5 2.65
β-actin
B
24 h
48 h
Ctr
Lim
Nom
LNA
Lim
Nom
LNA
Cox-2
1.0 0.42 0.39 0.39 0.24 0.39 0.23
IL-6
1.0 0.42 0.61 0.54 0.40 0.32 0.15
NFkB (p65)
1.0 0.82 0.72 0.90 0.80 0.52 0.64
β-actin

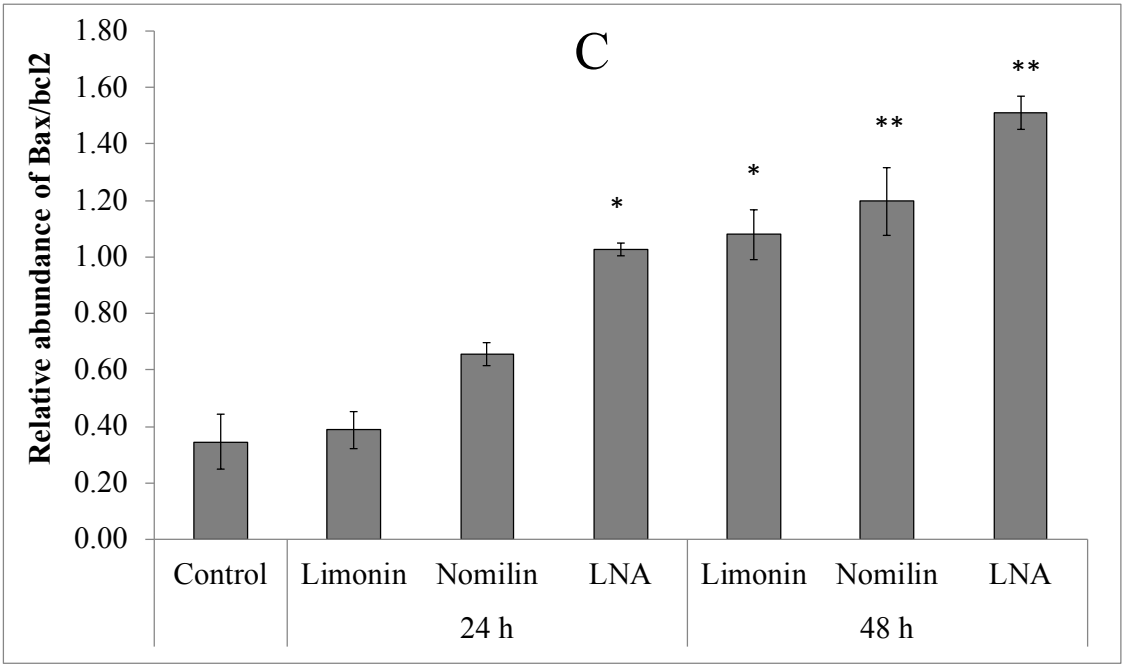

C
Relative abundance of Bax/bcl2
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
*
**
**
Control
Limonin
Nomilin
LNA
Limonin
Nomilin
LNA
24 h
48 h

Fig.4

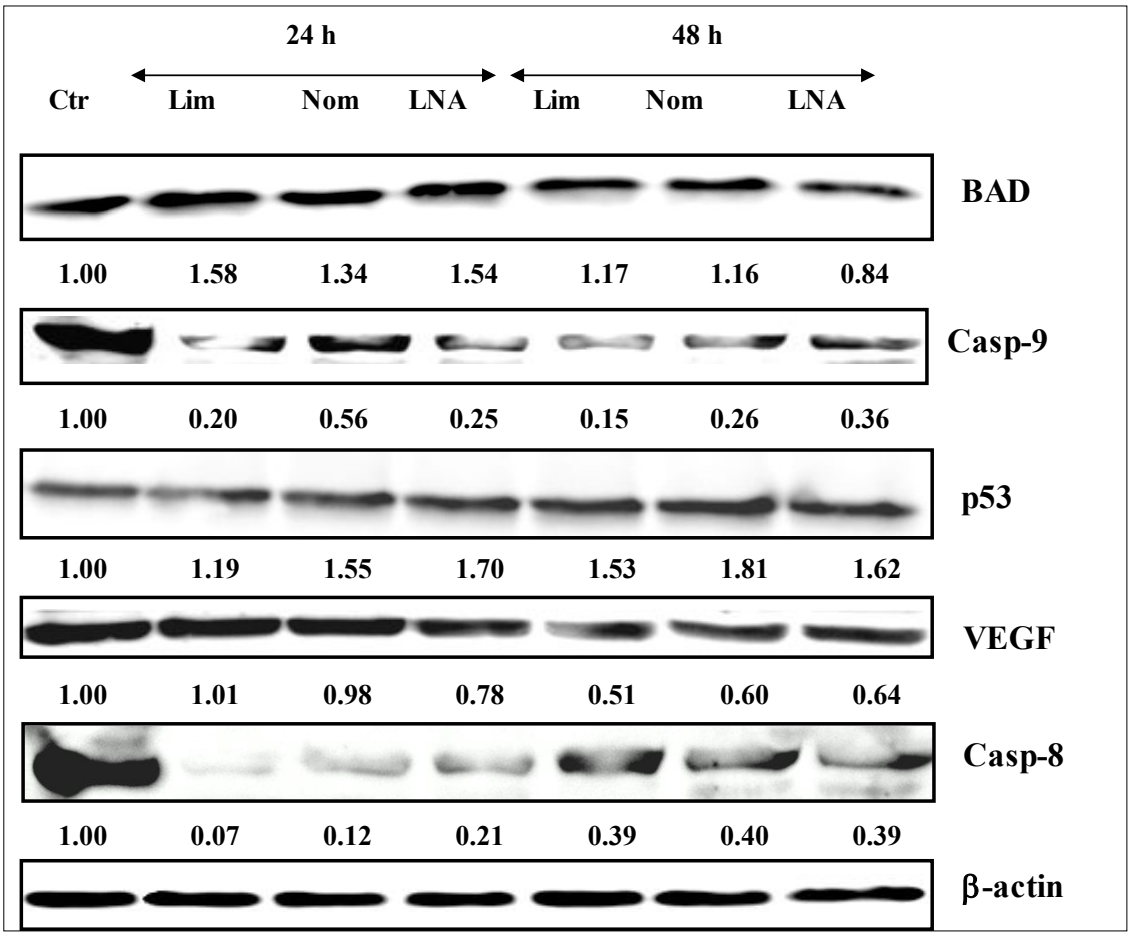
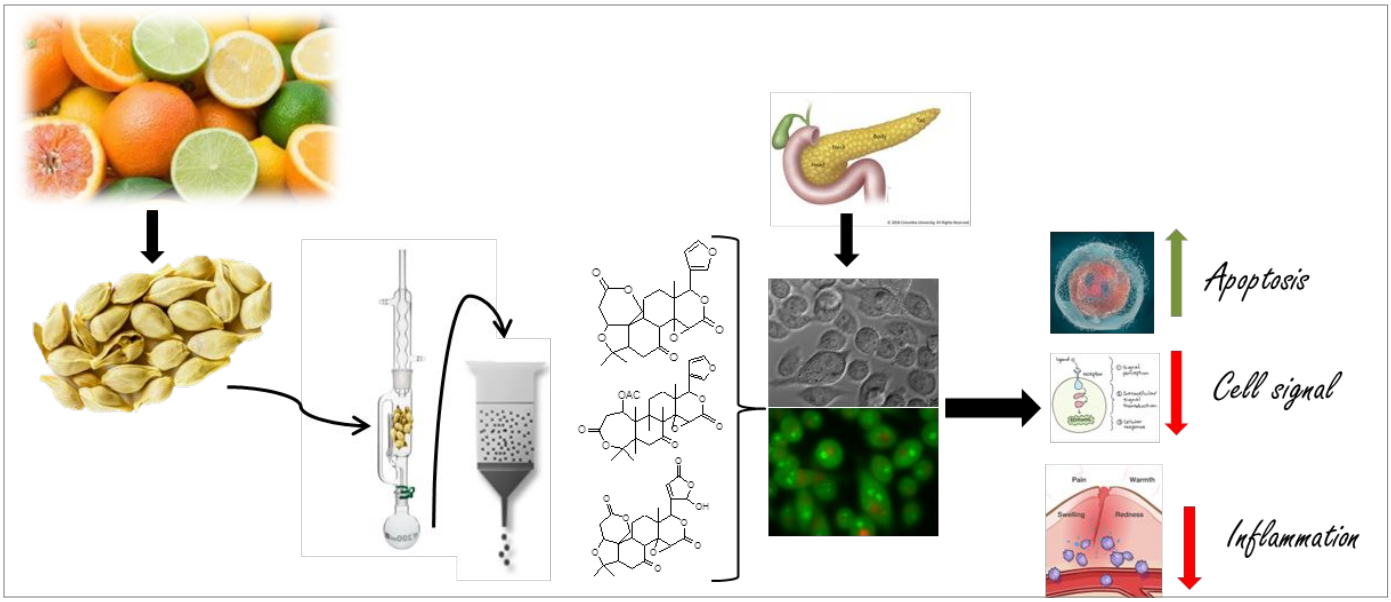

24 h
48 h
Ctr
Lim
Nom
LNA
Lim
Nom
LNA
BAD
1.00 1.58 1.34 1.54 1.17 1.16 0.84
Casp-9
1.00 0.20 0.56 0.25 0.15 0.26 0.36
p53
1.00 1.19 1.55 1.70 1.53 1.81 1.62
VEGF
1.00 1.01 0.98 0.78 0.51 0.60 0.64
Casp-8
1.00 0.07 0.12 0.21 0.39 0.40 0.39
β-actin

Fig.5



‘Limonoids found in citrus fruits are known to inhibit Human Pancreatic Cancer (Panc-28) cells’: an in vitro study based evidence.