Original Article

Lyophilized Fruit Juice of *Citrus sinensis* Triggers Mitochondria-mediated Apoptosis via Down-regulation of PI3K/AKT in MCF-7 Cell Line

Jayasindu Mathiyazhagan[1], John Arul Daniel[1], George Priya C. Doss[1], Rama Jayaraj[2], Kodiveri Muthukaliannan Gothandam[1,*]

[1]School of Bio Sciences and Technology, Vellore Institute of Technology (VIT), Vellore-14, Tamil Nadu, INDIA.
[2]Theme Lead, Flinders NT, Flinders University, Northern Territory 0909, AUSTRALIA.

ABSTRACT

Background: Breast cancer is prevalent cancer among women and a major cause of death worldwide due to various genetic and environmental factors. *Citrus sinensis* is widely taken fruit juice in most countries that are believed to reduce breast cancer risk. However, the mechanism through which it acts remains unclear. Our objective was to explore the mechanism through which *Citrus sinensis* acts as a cytotoxic agent. **Materials and Methods:** FT-IR and GC-MS analyses of lyophilzed orange juice (LOJ) were performed to find phytocompounds that were subjected to *in silico* docking analysis against phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway. The proteins that showed the low free energy of binding was taken for gene expression analysis. RT-PCR analysis was carried out to observe PI3K/AKT/mTOR genes inhibition in LOJ treated cells. Cytotoxicity, apoptosis, caspase 9 activity were performed against the human breast cancer cell line (MCF-7). **Results:** The docking analysis against PI3K/AKT/mTOR with LOJ compounds showed the best interactions. Further down-regulation of PI3K/AKT and moderate up-regulation of mTOR was confirmed using gene expression analysis. LOJ showed concentration-dependent cytotoxicity, apoptosis, decreased mitochondrial membrane stability and caspase 9 against the MCF-7 cell line. **Conclusion:** This study concludes that LOJ can induce apoptosis in MCF-7 cells via PI3K and AKT inhibition.

Key words: Lyophilized orange juice, Molecular docking, Apoptosis, MCF-7 cell line, PI3K/AKT/mTOR.

INTRODUCTION

In women, breast cancer accounts for the majority of cancer death around the globe.[1] Even though ongoing treatments have shown a better effect against breast cancer, it could not completely cure the nefarious activity of metastasis or advanced breast cancer stages.[2] Apoptosis or programmed cell death is a natural phenomenon to remove impaired and redundant cells. Apoptosis is triggered by various factors, including anticancer drugs, growth factors and oxidative stress.[3] There are two apoptotic pathways, namely, intrinsic or mitochondrial-induced pathway and extrinsic or receptor-induced pathway. Caspases are essential components of the apoptotic process, in which caspase 9 (intrinsic initiator) is a promising therapeutic target against proliferative diseases like cancer.[4]

Oxidative stress enacts a vital role in discrete human diseases, including cancer.[5] Oxidative stress is enhanced by reactive oxygen species (ROS) that act via various exogenous and endogenous stimuli, resulting in cell injury. ROS includes hydrogen peroxide, superoxide anion radicals and hydroxyl radicals controlled by oxidation and antioxidant systems under normal physiological conditions.[6] At the cellular level, PI3K/AKT/mTOR contributes to increased ROS

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Correspondence:
Dr. KM Gothandam
Professor, School of Bio Sciences and Technology, VIT University, Vellore-632 014, Tamil Nadu, INDIA.
Phone no: +91-9489337967
Email id: gothandam@gmail.com

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production by directly modulating the bioenergetics of mitochondria or indirectly as a metabolic by-product.[7] The down-regulation of this pathway activates caspase 9 dependent mitochondrial apoptosis.[8] The PI3K/AKT/mTOR pathway is a major signalling pathway involved in cell cycle regulation and progression. Hyperactivation of proteins in this pathway leads to inflated feedback inhibition of components in the signalling network, resulting in down-regulation of receptors and their signals, which led to the development of inhibitors for this pathway. It is necessary to develop an inhibitor of the PI3K/AKT/mTOR pathway with fewer side effects that act efficaciously in breast cancer cells.

Compared to synthetic drugs, natural products are preferable because they show low toxicity and decidedly fewer side effects.[9] Many phytocompounds wield chemopreventive activities against breast cancer. One of them is citrus fruits, which belong to the Rutaceae family grown throughout the world. It encompasses *Citrus sinensis* (sweet orange), *Citrus reticulate* (tangerine tree), *Citrus vitis* (grapefruit tree), *Citrus aurantifulia* (lime tree) and *Citrus limonum* (lemon tree). Owing to the phytonutrient contents, all citrus fruits hold similar therapeutic and dietetic properties.[10] Phytonutrients are plant nutrients that advocate human health and disease prevention through their specific biological activities. Myriads of biologically active products are provided by plant secondary metabolites, including flavonoids, phenolics and terpenoids.[11] Citrus fruits are a source of flavanones, flavones and polymethoxylated flavones that has myriad bioactivities.[12,13] Our preliminary data showed that lyophilized orange juice (LOJ) is rich in phenolic acids and flavonoids, thus scavenging ROS. The intracellular mechanism of cancer cell death was achieved by ROS induced mitochondria-mediated apoptosis *via* down-regulation of PI3K and AKT.

MATERIALS AND METHODS

Collection and preparation of pure *Citrus sinensis* juice powder

Fresh sweet oranges were purchased from a local market in Vellore district, Tamil Nadu, India. The skin was removed from each of the endocarp segments present in the orange. Pure juice was obtained from the orange by crushing the fruit using a mortar pestle. The juice was filtered, and the filtrate was then lyophilized to acquire a water-free sample. The lyophilized sample was then dissolved in methanol for further analysis.

Quantitative determination of phytochemicals

The total phenolic content (TPC) and the total flavonoid content (TFC) present in the LOJ were quantified using the Folin-Ciocalteu and aluminium chloride colorimetric methods.[14,15] The results were indicated mg/g GAE (gallic acid equivalent) weight for TPC and mg/g QE (quercetin equivalent) for TFC.

FT-IR and GC-MS analysis

The functional groups of the LOJ were established using Fourier-transform infrared spectroscopy analysis. 10 mg of the dried powder was added to 100 mg of potassium bromide pellet to produce pellucid sample discs. The sample was loaded in FT-IR spectroscopy in the range of 4000 to 400 cm^{-1}.

Gas chromatography-mass spectrometry analysis of LOJ was done to identify the phytoconstituents. The sample was loaded in Elite – 5MS capillary column with 30m X 0.25mm ID X 0.25µm df with helium as carrier gas at 1ml/min flow rate. Mass spectral patterns were matched with the NIST library for the determination of structures.

Antioxidant activity

The free radical scavenging activity of LOJ was achieved to find its antioxidant activity.[16] 1 mL of 0.3mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol was combined to 2.5 mL of different concentrations of the sample. Then the samples were stored in the dark for 30 min. The optical density of these samples was taken at 518 nm.

In silico docking analysis

Three-dimensional structures of compounds identified by GC-MS analysis were fetched from PubChem. The ligands that passed Lipinski's rule of five were taken for docking studies. The three-dimensional structures of PI3Kα (4JPS), AKT (3MV5) and mTOR (4JSX) proteins were retrieved from the Protein Data Bank and were used as the target for the phytocompounds.[17]

Autodock 4 was used for the docking simulation to run and analyse. Polar hydrogen and Kollman charges were included. The Lamarckian genetic algorithm was chosen to search the global optimised and the best conformation. The default parameters were used for docking studies. After ten runs, the best confirmation of the ligand was selected based on the lowest binding energy.

Cell cytotoxicity assay

Cell cytotoxicity was estimated in the MCF-7 cell line using MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide). MCF-7 cells were seeded onto a 96-well tissue culture plate and kept in 5% CO_2 at 37°C humidified incubator. After cell attachment, cells were treated with different concentrations of LOJ (10,

50, 75, 100, 150 and 200 µg/mL) for 24 h. Following the treatment period, 20 µl of MTT was added to the wells at a 2.5 mg/mL concentration. After 4 hrs MTT was removed from the wells, and metabolically active cells were measured using a microtiter plate reader at 570 nm wavelength. An inverted phase-contrast microscope was used to observe cell morphology changes in the MCF-7 cell line after treatment with LOJ. The cells were captured using the phase-contrast microscope for morphological changes (100X magnification).

AO/EB staining

LOJ induced apoptosis was studied by acridine orange (AO)/ethidium bromide (EB) dual staining. AO emits green fluorescence by taking up the nuclear DNA of live and damaged cells. EB emits red fluorescence by taking up nuclear DNA from damaged cells, which have entered the late apoptotic stage.[18] An equal amount of AO and EB (100 µg/mL) was added to the plates containing untreated control cells, and LOJ treated cells (IC_{50}=185.5 µg/mL). Apoptosis was observed under a fluorescent microscope (Lawrence and Mayo (India) Pvt. Ltd).

Mitochondrial membrane staining

Rhodamine 123 is a cationic green-fluorescent dye that aggregates and accumulates in normal cells of the mitochondrial membrane. This dye binds only to the metabolically active mitochondria. The untreated control cells and LOJ treated cells were incubated with 5µg/ml of rhodamine 123 dye for 30 min. The stained cells were viewed under a fluorescent microscope at 505 nm excitation with 534 nm emission (Lawrence and Mayo (India) Pvt. Ltd) for mitochondrial membrane potential changes.

DCFH-DA method and caspase 9 activity

The 2',7'-dichlorofluorescein diacetate (DCFH-DA) method was used to measure ROS levels using flow cytometry. The cells were treated with the IC_{50} concentration of LOJ for 24 h and trypsinized. The cells were washed and incubated with 20 µM DCFH-DA for 30 min in darkness. ROS levels were then estimated using flow cytometry. The results were analyzed using cytexpert software (CytoFLEX, Beckman Coulter, USA). Caspase 9 activity was measured at an absorbance of 405 nm using a caspase 9 colorimetric assay kit (Biovision, CA, USA).

Real-time polymerase chain reaction (RT-PCR)

Total RNA was obtained from control and LOJ treated (185.5 µg/mL) MCF-7 cells using TRIzol reagent. The cDNA was synthesised using RNA and reverse transcribed using cDNA reverse transcription kit. The qRT-PCR was carried out with SYBR® Premix Ex Taq™ on the CFX96™ Real-Time System (BIO-RAD, California, USA). The primer sequences used were: β-actin, forward primer 5'-GTCATTCCAAATATGAGATGCGT-3' and reverse primer 5'-GCTATCACCTCCCCTGTGTG-3'; PIK3CA, forward primer 5'-ATTGTCGTGCATGTGG GATG-3' and reverse primer 5'-AATCTGGTCGC CTCATTTGC-3'; AKT, forward primer 5'- GGACA AGGACGGGCACATTA-3' and reverse primer 5'-CG ACCGCACATCATCTCGTA-3'; mTOR, forward primer 5'- TGCCTTTGAGCAGAAAAGGT-3' and reverse primer 5'- CTGGTTTCACCAAACCGTCT-3'. The comparative threshold cycle ($2^{-\Delta\Delta t}$) calculation was utilised to calculate gene expression.

Statistical analysis

The data of all the assays with three replicates are subjected to statistical analysis and are given as mean ± standard deviation (SD) using GraphPad Prism 7 software. The comparison between groups were analysed with a one-way analysis of variance (ANOVA) followed by the Tukey test. The significance was considered when $p<0.05$.

RESULTS

Quantitative and qualitative determination of phytochemicals

The TPC in LOJ was calculated from the calibration curve (R^2 = 0.99) that was estimated to be 1.233 ± 0.01 mg GAE/g dry weight and total flavonoid (R^2 = 0.99) was estimated to be 0.995 ± 0.04 mg QE/g dry weight. The antioxidant activity of LOJ using DPPH assay is shown in Figure 1. The scavenging activity of LOJ was 53% at 300 µg/mL concentration against ascorbic acid

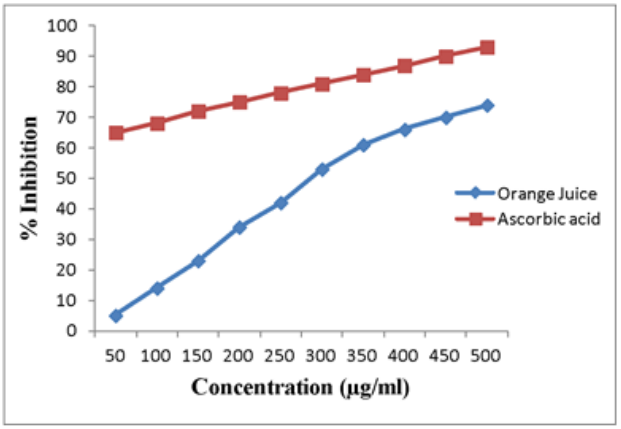

Figure 1: DPPH radical scavenging assay; LOJ against standard compound ascorbic acid.

(81% at 300 µg/mL). Numerous secondary metabolites like phenolics and flavonoids act as antioxidants that perform scavenging activity.[19] There was a significant positive correlation between total phenolics and antioxidant (R^2 = 0.988), as well as with total flavonoids and antioxidants (R^2 = 0.980).

The FT-IR peak values are shown in Table 1. The spectrum was used to find the functional groups in the LOJ. The powder showed the presence of phenols, acetates and formates. A total of 41 compounds from LOJ were identified by GC-MS, which belongs to families such as esters, ethers, aldehydes, alcohols and terpenes.

In silico interaction analysis

Bioavailability is one of the fundamental pharmacokinetic properties of drugs. Bioavailability is the rate of absorption of a drug available at the site of physiological activity after administration. The ligands' bioavailability and drug likeliness were studied with the pkCSM software and Molinspiration tool (Table 2), respectively.[20] The compounds passing Lipinski's rule were chosen for further analysis. These screened compounds were taken for molecular docking analysis against the PI3K/AKT/mTOR pathway receptors. LOJ compounds were used for docking analysis with these target proteins to find their molecular interactions. The co-crystallized ligands were docked with their specific proteins, and the interactions were noted. The lowest binding affinities and best interactions of the ligands were compared with its specific inhibitor (Table 3).

In vitro analysis

The cell cytotoxicity assay results reported that the LOJ has concentration-dependent cytotoxicity activity against the MCF-7 cell line after 24 h of treatment with LOJ compared with the control. The cell viability is mentioned in Figure 2. The morphological examination of untreated control cells was elongated in shape and grown completely. Whereas the cells treated with LOJ (IC_{50}-185.5 µg/ml) showed a reduction in cell size, spheroid shape, shrinkage and detachment of cells, indicating cell death. The observed morphological changes are given in Figure 3a and 3b.

The images of AO/EB staining of MCF-7 cells with and without LOJ treatment are depicted in Figure 3c and 3d. The cells without treatment were green in colour, indicating the live cell population and the cells treated with LOJ had half of the cells turned completely orange

Table 1: FT-IR analysis to identify compound present in lyophilized orange juice.

S.no	Peak cm-1	Functional group
1	3267.41	Phenols, Intermolecular H Bonds
2	2927.94	=C-H
3	1714.72	Nonconjugated
4	1612.49	Conjugated
5	1408.04	CH2
6	1350.17	CH3
7	1240.23	Acetates
8	1101.35	Formates
9	1029.99	Acetates
10	987.55	Formates
11	921.97	-CH=CH2
12	501.49	Alkyl Halides (R-Br) C-Br stretch

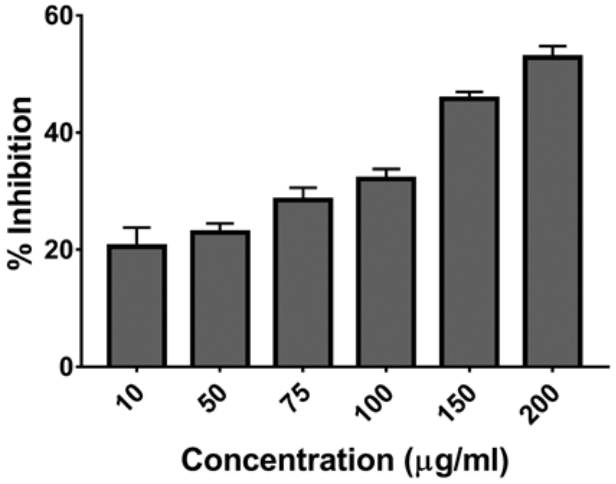

Figure 2: Effect of different concentrations of LOJ on Viability of MCF-7 cell lines. Cell cytotoxicity was observed after 24 hr of treatment with LOJ.

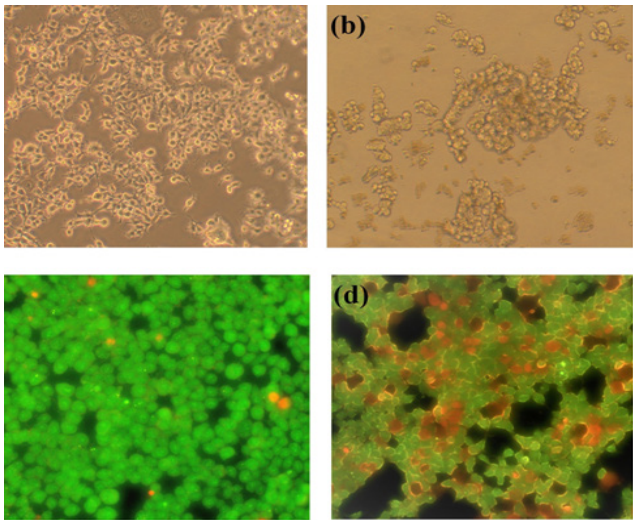

Figure 3: Morphology changes in MCF-7 cells (a) Control cells, (b) LOJ treated cells; AO/EB staining for the detection of apoptosis in fluorescence microscopy (200X magnification): (c) Control cells, (d) LOJ treated cells.

Table 2: ADMET properties of LOJ compounds.

S.No	Ligands	Absorption-Intestinal absorption (human) %	Distribution-BBB Permeability (log BB)	Metabolism (Cytochrome enzymes)	Excretion-Total clearance (log ml/min/kg)	Toxicity-AMES/ Hepatotoxicity
1	Triacetone triperoxide	93.79	1.557	No	1.236	No/No
2	4-Tert-Butylcyclohexanecarboxylic Acid	100	0.305	No	0.966	No/No
3	Acetic Acid, 2-Acetoxymethyl-1,2,3-Trimethylbutyl Ester	97.21	0.585	No	1.189	Yes/No
4	N-Aminomorpholine	99.13	-0.268	No	0.684	No/No
5	2-(Aminooxy)- Propanoic Acid	81.4	-0.029	No	0.833	No/No
6	2-Formylhistamine	76.24	-0.912	No	1.003	No/No
7	3-Bromo-2-pentanol	93.48	0.064	No	0.432	Yes/No
8	5-Methoxy-3-Oxovalerate	99.15	-0.267	No	0.888	Yes/No
9	Trans-2-Chlorovinylacetate	100	-0.007	No	0.092	No/No
10	Sec-butyl isopropyl ether	96.16	0.567	No	1.465	No/No
11	Endo-2,3-O-Ethylidene-. Beta.-D- Erythrofuranose	100	-0.249	No	0.86	No/No
12	1,7-Octanediol	76.97	-0.123	No	0.571	No/No
13	3-Pentanone, 1,5-Dimethoxy- Butane	100	-0.181	No	0.899	No/No
14	1,2-Propanediol diformate	100	-0.26	No	0.729	No/No
15	L-2-Amino-4-Methyl-1-Pentanol	77.64	-0.244	No	0.725	No/No
16	Propionic Acid	93.86	-0.292	No	0.396	No/No
17	Acetal	96.97	0.193	No	0.84	Yes/No
18	5-Methylene-2,4,7,9-Tetraoxadecane	84.88	-0.208	No	0.354	No/No
19	(3-Methyl-Oxiran-2-Yl)-Methanol	97.32	-0.238	No	0.529	Yes/No
20	4-Hydro-L-2-Amino-3-Methyl-1- Pentanol	77.64	-0.244	No	0.725	No/No
21	Methyl Propyl Ether	100	0.08	No	0.675	No/No
22	Diglycolic Acid	65.78	-0.378	No	0.867	No/No
23	2-(2-Aminoethyoxy)-Ethanol	84.02	-0.301	No	1.124	Yes/No
24	Chloromethoxy methane	100	0.05	No	0.399	No/No

Table 3: Docking energy of LOJ ligands with PI3K/AKT/mTOR pathway targets.

S.No	Targets	PI3		AKT		mTOR	
	Ligands	Binding Energy (kcal/mol)	Hydrogen bond	Binding Energy (kcal/mol)	Hydrogen bond	Binding Energy (kcal/mol)	Hydrogen bond
1	Triacetone triperoxide	-6.63	VAL851	-6.27	GLU191, LYS179	-6.66	VAL2240
2	4-Tert-Butylcyclohexanecarboxylic Acid	-5.54	VAL851	-5.33	ALA230	-5.24	VAL2240
3	Acetic Acid, 2-Acetoxymethyl-1,2,3-Trimethylbutyl Ester	-4.81	VAL851	-4.07	LYS276, LEU295, GLY294	-4.87	VAL2240
4	N-Aminomorpholine	-4.5	-	-4.94	GLU234	-4.33	-
5	2-(Aminooxy)- Propanoic Acid	-2.97	VAL851, SER854	-3.23	LYS419, LYS289	-4.23	VAL2240, TRP2239, CYS2243
6	2-Formylhistamine	-5.51	ASP933, ASP810, TYR836	-6.43	ASP439, GLU234	-6.17	ASP2195, ASP2357
7	3-Bromo-2-pentanol	-4.19	VAL851	-3.48	GLY294	-4.29	GLY2238, VAL2240
8	5-Methoxy-3-Oxovalerate	-3.94	VAL851, ASP933	-3.9	GLY294, LEU295, LYS179	-4.01	VAL2240
9	Trans-2-Chlorovinylacetate	-4.16	VAL851	-4.52	THR312	-4.26	VAL2240
10	Sec-butyl isopropyl ether	-4.24	VAL851	-4.52	-	-4.64	VAL2240
11	Endo-2,3-O-Ethylidene-. Beta.-D- Erythrofuranose	-3.45	VAL850, GLU798, ARG852	-4.1	GLY294, LEU295, LYS179	-3.82	VAL2240
12	1,7-Octanediol	-4.27	VAL851, SER854, GLU849	-4.07	ALA230, LEU156, ASP439	-4.16	ASP2195, ASP2357, VAL2240
13	3-Pentanone, 1,5-Dimethoxy- Butane	-3.7	VAL851	-3.92	GLY294	-4.0	VAL2240
14	1,2-Propanediol diformate	-3.42	VAL851, ASP933	-4.3	LEU275, ALA317, TYR315	-3.56	VAL2240
15	L-2-Amino-4-Methyl-1-Pentanol	-4.78	ASP933, ASP810	-4.94	GLU234	-4.33	-
16	Propionic Acid	-3.04	LYS924	-2.52	HIS207	-2.99	LYS2171
17	Acetal	-3.61	VAL851	-3.75	GLY294, LEU295	-3.94	VAL2240
18	5-Methylene-2,4,7,9-Tetraoxadecane	-3.09	VAL851	-3.08	LEU295	-3.64	VAL2240
19	(3-Methyl-Oxiran-2-Yl)-Methanol	-3.55	LYS802, TYR836, ASP933	-4.01	ASP331, ARG273, LEU275	-3.49	VAL2240
20	4-Hydro-L-2-Amino-3-Methyl-1- Pentanol	-2.5	SER854, ASN853	-2.79	LYS179, GLY294, ASN279, ASP274	-3.42	CYS2243
21	Methyl Propyl Ether	-3.21	VAL851	-3.2	LEU295	-3.42	VAL2240
22	Diglycolic Acid	-2.87	ARG770, SER774	-3.75	ARG206, LYS214	-3.44	TRP2239, ARG2348, LYS2171

23	2-(2-Aminoethyoxy)-Ethanol	-4.11	ASP933, LYS802, ASP810	-4.57	ASP439, GLU234	-4.35	ASP2357, ASP2195
24	Chloromethoxy methane	-2.58	VAL851	-2.89	LEU295	-2.71	VAL2240
25	PI3K inhibitor (Alpelisib (BYL719))	-10.8	VAL851, GLN859, SER854	-	-	-	-
26	AKT inhibitor **(**(3R)-1-(5-methyl-7H-pyrrolo[2,3-d] pyrimidin-4-yl)pyrrolidin-3-amine**)**	-	-	-8.49	GLU228, ALA230	-	-
27	mTOR inhibitor (Torin 2)	-	-	-	-	-9.82	VAL2240, THR2245

and partially orange that indicates cells undergoing apoptosis and late apoptosis.

Mitochondrial membrane potential (MMP) changes in cells with and without LOJ treatment were stained with Rhodamine 123, and the results showed the accumulation of dye in untreated cells, which shows good mitochondrial membrane potential. A significant reduction in dye accumulation in treated cells was observed that shows the loss of MMP (Figure 4a, 4b).

ROS levels were measured by flow cytometry analysis (Figure 4c, 4d). The cellular esterases convert nonpolar DCFH-DA into polar DCFH. A nonfluorescent DCFH oxidized to fluorescent DCF by intracellular ROS. Thus, the increase in ROS levels shows high fluorescence. Control cells showed a 0.10% ROS level, whereas LOJ treated cells showed 56.51% ROS levels. As shown in the results, we observed high ROS levels in LOJ treated cells. Mitochondria-mediated apoptosis was further confirmed by quantification of caspase 9 levels in LOJ treated cells. The mitochondria-mediated apoptosis pathway involved in the loss of MMP and cytochrome C release that activates pro-caspase 9 cleavage.[21] As indicated (Figure 4e), increased caspase 9 levels were detected in LOJ treated cells than in the untreated control cells.

To further support LOJ induced apoptosis, the mRNA levels of PI3K/AKT/mTOR were quantified by qRT-PCR. The PI3K/AKT/mTOR pathway is correlated with apoptosis, cell growth and proliferation. Thus, inhibition of this pathway could drive cancer cell apoptosis and prevent proliferation as well. We observed down-regulation of mRNA levels of PI3K (fold change - 8.26) and AKT (fold change - 5.54) but moderate up-regulation (fold change – 1.40) of mTOR in LOJ treated MCF-7 cells related to the control cells (Figure 5), confirming cancer cell apoptosis *via* down-regulation of PI3Kα and AKT genes.

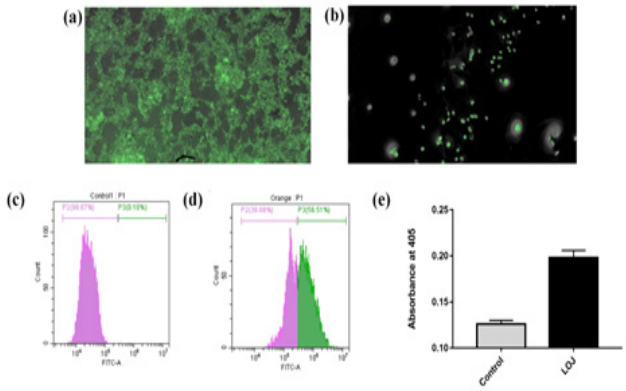

Figure 4: Rhodamine 123 staining for mitochondrial membrane potential to find the apoptosis in mitochondria (100X magnification): (a) Control cells, (b) LOJ treated cells; Measurement of ROS levels by flow cytometry: (c) Control cells, (d) LOJ treated cells; (e) caspase 9 activity of control and LOJ treated cells.

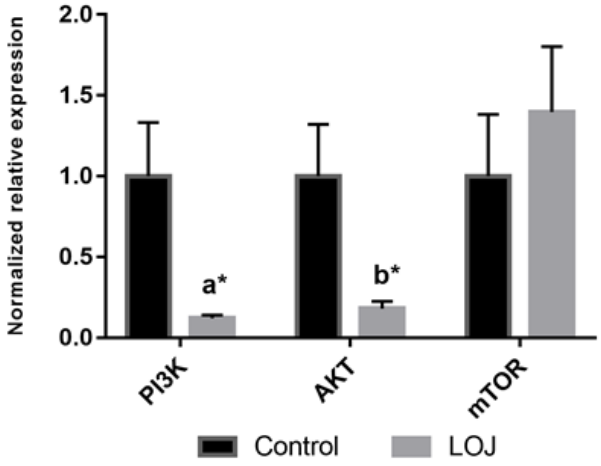

Figure 5: mRNA expression levels of PI3K/AKT/mTOR were quantified by RT-PCR using β- Actin as a reference gene. The reduction in PI3K and AKT expressions were observed in cells treated with LOJ. *=p<0.05; a = Comparison of PI3K control with PI3K in LOJ treatment, b = Comparison of AKT control with AKT in LOJ treatment.

DISCUSSION

Appropriate food, balanced nutrition and regular exercise can prevent cancer by at least 30 to 40%.[22] Most of the food components are biologically active and prevent cancer that is yet to be elucidated scientifically. These components are fatty acids, phytochemicals (flavonoids, isothiocyanates and retinoids) and selenium.[23] It is proven that diet is an epigenetic modulator that changes the gene expression pattern.[24] Flavonoids have an important role in epigenetic remodelling and improve human health.[25] Flavonoids also target other major pharmacological pathways, such as anti-angiogenesis, anti-apoptotic and anti-inflammatory.[26,27] Though the *in vitro* and *in vivo* studies on flavonoids are still limited and yet to be elucidated.[28] In this study, the chemopreventive effect of lyophilised orange juice powder and its mechanism through which it acts has been reported. Phenolic compounds are a diverse class of phytocompounds that show chemotherapeutic and chemopreventive effects. The redox properties of phenolic compounds function as antioxidants in the prevention of cancer.[29] Flavonoids, also called Vitamin P, the plant secondary metabolites giving characteristic colours to plants, also play a pivotal role in anti-inflammatory, anticancer and antioxidant activities.[30] Earlier reports have already shown the concentration-dependent polyphenols and flavonoids activity in cancer prevention.[31] Radical scavenging activity is essential to protect the biological system from free radicals. The experimental data indicated radical scavenging of LOJ showed high antioxidant activity. The correlation between TPC and TFC with antioxidant activity showed a positive correlation.

In most human cancers, the PI3K/AKT/mTOR pathway holds a significant role in cancer progression and proliferation.[32] This pathway is also correlated with apoptosis and cell growth. The PI3K/AKT/mTOR inhibition could lead to apoptosis of cancer cells and also prevent proliferation.[33] Hence, this pathway is considered as one of the major therapeutic targets in anticancer drug discovery. The PI3K subunit is mutated in 18-40% of breast cancers.[34] Thus, it is an ideal target for drug development. Inhibition of the breast cancer cell line could be attributed to the ameliorative effects of phytocompounds present in the orange juice, such as phenols, flavonoids, alcohols and acids. A previous study showed that breast cancer cells' inhibition by polymethoxyflavones (PMFs) from *C. sinensis* and a clinical study involving postmenopausal breast cancer patients showed that a high amount of flavones in orange juice might reduce mortality.[35,36] Decreased mitochondrial membrane potential, and increased ROS levels are linked to apoptosis. The loss of MMP activates cleavage of caspase 9. The mitochondrial membrane is an essential parameter for assessing mitochondrial function.[37] Our results depicted that MCF-7 cells with LOJ treatment had reduced mitochondrial membrane potential, increased ROS levels and increased caspase 9 activities compared to control cells. Also, suppression of the PI3K/AKT pathway is associated with mitochondria-mediated apoptosis.[38] In line with that, qRT-PCR results showed down-regulation of the PI3K and AKT genes responsible for ROS induced caspase 9-mediated apoptosis.

CONCLUSION

Lyophilised orange juice (LOJ) showed promising antioxidant and cytotoxic activity against breast cancer cell lines *via* mitochondria-mediated apoptosis and down-regulation of PI3K and AKT genes. The moderate non-significant up-regulation of mTOR was observed. Moreover, the overall interaction of LOJ compounds with the PI3K/AKT/mTOR protein targets was predictably high. This concludes that LOJ possibly acts via PI3K/AKT inhibition, leading to mitochondria-mediated apoptosis and anti-proliferation in breast cancer cells.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ABBREVIATIONS

LOJ: Lyophilized orange juice; **ROS:** Reactive oxygen species; **PI3K:** Phosphatidylinositol-3-kinase; **AKT:** Protein kinase B; **mTOR:** Mammalian target of rapamycin; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **DMEM:** Dulbecco's Modified Eagle's Medium; **MTT:** (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide); **AO/EB:** Acridine orange/ Ethidium bromide.

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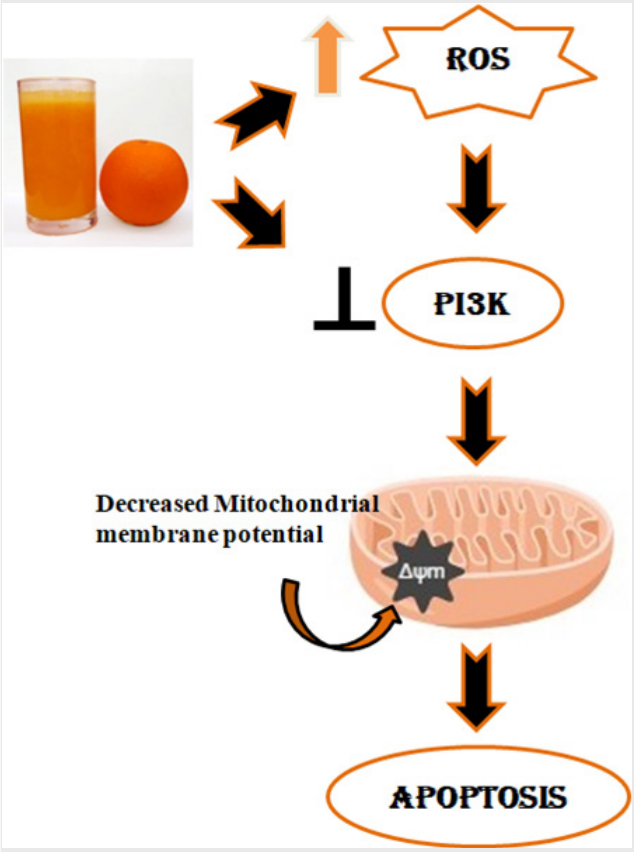
SUMMARY

Citrus fruits are a source of flavanones, flavones and polymethoxylated flavones that has myriad bioactivities. Our data showed that lyophilized orange juice (LOJ) is rich in phenolic acids and flavonoids, thus scavenging reactive oxygen species. LOJ acts via PI3K/AKT inhibition, leading to mitochondria-mediated apoptosis and anti-proliferation in breast cancer cell line (MCF-7).

PICTORIAL ABSTRACT



About Authors

Dr. Jayasindu Mathiyazhagan pursued Ph.D in School of Bio Sciences and Technology, Vellore Institute of Technology (VIT), working on phytotheraphy and diseases. She is interested in metabolism, epigenetics, phytomedicines and molecular biology

Mr. Arul Daniel is currently pursuing Ph.D in School of Bio Sciences and Technology, Vellore Institute of Technology (VIT), working in the field of biomedicine and therapy. He is skilled in nanomedicine, animal studies and molecular biology.

Dr. George Priya Doss is an Assistant Professor at Vellore Institute of Technology (VIT), India. His research interests include Bioinformatics, Biosciences and Technology and Nanobiotechnology.

Dr. Rama Jayaraj is a veterinarian and applied biotechnologist. He is working as a Theme Lead - Advanced Studies at Flinders University, Australia. He is skilled in Research Design, Meta-analysis, Science, Statistics, and Research.

Dr. K.M. Gothandam is a Professor at Vellore Institute of Technology (VIT), India. His research interests include Plant molecular biology, Functional genomics and phytotherapy.

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