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Phytochemical Profiling and Anti-cancer Study of Lyophilized Pure Fruit Juice of *Citrus Limon* (L.) Osbeck against Human Breast Cancer (MCF-7) Cell Line

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Abstract. The present study aims at the phytochemical profiling of lyophilized pure fruit juice powder of *Citrus limon* and demonstrates its efficacy as an antitumor agent against human breast cancer cell line (MCF-7). The cytotoxicity was evaluated using the changes in cell morphology, cell viability, and DNA fragmentation, the percentage of cell viability was determined by MTT assay. Our results showed that lyophilized pure fruit juice powder of *Citrus limon* inhibited the proliferation of human breast cancer cell line MCF-7 with an IC_{50} 98.16 µg/ml at 48 h incubation, it was shown to promote apoptosis as seen as DNA fragmentation using ladder assay. These results suggest that lyophilized pure fruit juice powder of *Citrus limon* has antiproliferative effect against MCF-7 cell by suppressing its growth.

Key words: *Citrus limon* fruits, FT-IR, GC-MS, MTT, DNA fragmentation.

1 Introduction

Cancer is a multi-factorial, multi-faceted, and multi-mechanistic disease requiring a multi-dimensional approach for its treatment, control, and prevention. Cancer remains a major public health burden in developed as well as developing countries [1-4]. Cancer as a second cause of death after heart disease in the world has posed a great challenge to the field of medicine and immunology [5]. WHO has predicted that the number of new cases of cancer may increase up to 15 million in the year 2020 [6]. Cancer is a group of diseases that cause cells in the body to change and grow out of control. Most types of cancer cells eventually form a lump or mass called a tumor, and are named after the part of the body where the tumor originates [7].

Human breast cancer is the most commonly occurring cancer in females, rare in male. It accounts for 23% of newly occurring cancer worldwide and represents 13.7% of all cancer death. In both developed and developing countries, breast cancer is the most frequent cancer and most frequent cause of cancer deaths [8]. It's a serious global health problem being the second most common of all cancers and by far the most frequent cancer amongst women [9]. Although breast cancer is considered as one of the most chemo sensitive solid tumors, most initially responsive tumors relapse and develop resistance to a broad spectrum of drugs. Consequently, breast cancer becomes refractory to cytotoxic drugs and is typically incurable [10]. The vast majority of breast cancers begin in the parts of the breast tissue that are made up of glands for milk production, called lobules, and ducts that connect the lobules to the nipple. The remainder of the breast is made up of fatty, connective, and lymphatic tissues [7].

Chemotherapy is one of the commonly used strategies in breast cancer treatment. This therapy is usually associated with adverse side effects, ranging from nausea to bone marrow failure [11] and development of multi-drug resistance (MDR) [12]. The toxicity and resistance of traditional chemotherapeutic drugs makes it critical to develop new targets and novel drugs for cancer therapy. Despite extensive research and rapid progress in cancer treatment, there is a growing demand for new therapies. It is important to identify new agents and targets for the treatment of cancer [13, 14].

More than 60% of the chemotherapeutic drugs are developed from plants and their derivatives. Medicinal plants are potential sources of natural products exhibiting anti-proliferation and anti-metastatic properties [15]. In addition to synthetic drugs, immense use of natural products and its derivatives in the development of anti-cancer drugs are increasing all over the world because of lesser

side effects as compared to synthetic drugs [16-27]. In search of new agents to treat cancer with fewer or less side effects, a number of medicinal plants have been evaluated [28-35]. More than 50% of all modern drugs in clinical use are of natural products, many of which have been recognized to have the ability to include apoptosis in various cancer cells of human originals; there is an urgent need to develop much effective and less toxic drugs [36,37].

Citrus one of the important economically plants, but attention leaves and seeds the role of citrus not given importance in comparison to fruits despite the presence of phenols quantity that varies among species [38]. Majority of citrus fruits are preferably eaten fresh *e.g.*, oranges, mandarins, grapefruits, Clementines, and tangerines. Orange and grapefruit produce very palatable juice and hence are used to make nutritious and popular breakfast [39]. Lemon is an important medicinal plant of the family Rutaceae. It is cultivated mainly for its alkaloids, which are having anti-cancer activities [40]. Its fruits have peculiar fragrance partly due to flavonoids and limonoids present in the peel and these fruits are good sources of vitamin C and flavonoids [41].

Lemons and limes can be used to make lemonades and pickles and their juices can be added to various food preparations as flavoring agents. Citrus fruits are rich sources of active compounds and beneficial for human health *e.g.*, vitamin C, carotenoids, flavonoids, limonoids, essential oils, acridone alkaloids, minerals, and vitamin B complex [42]. Flavonoids especially polymethoxyflavones, flavanone glycosides, and limonoids are natural secondary metabolite compounds of citrus [43]. Fruits have a lot of biological effective compounds, that have the ability to attack radical free and work as anti-natural oxidative stress, such as phenolic compounds (phenolic acids, flavonoids, and tannins) make them play an important role in reducing the risk of many diseases like cancers, cardiovascular and neurological diseases [44].

Therefore, the present investigation was aimed to analyze the phyto-constituents present in lyophilized fruit juice powder of *Citrus limon* and its anti-cancer and anti-apoptotic activity against human breast cancer (MCF-7) cell line.

2 Materials and Methods

2.1 Collection and Identification of Fruit

Fresh lemon fruits (Scientific name) were collected from Ammoor village, Vellore District, Tamil Nadu, India, and authentically identified as *C. limon* (L.) Osbeck, by Dr. P.T. Devarajan, Associate Professor of Plant Biology and Plant Biotechnology, Presidency College, Chennai-05.

2.2 Preparation of Pure Fruit Juice Powder

Fresh fruits were washed and the pure juice was taken from the fruit by squeezing them. The pure juice was filtered using Whatmann No.1 filter paper and the filtrate was lyophilized by subjecting it to freeze dryer. The yield of lyophilized powder was quantified and further subjected to biological activity.

2.3 Qualitative Phytochemical Analysis

Pure lyophilized juice powder of lemon fruit was subjected to preliminary phytochemical screening for its phytoconstituents according to Kokate (1988) method [45].

2.4 FT-IR Spectral Analysis

Lyophilized fruit juice powder was used for FT-IR analysis. Precisely, 10 mg of the dried powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered samples were loaded in FT-IR spectroscope (Shimadzu, IR Affinity 1, Japan), with a scan range from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. The output of the results in the form of graphs, was analyzed and the functional groups were identified by the peaks and the reference tables.

2.5 GC-MS Spectral Analysis

GC-MS spectral analysis was carried out to determine the presence of aromatic compounds in the powders. The model of the GC-MS used for mass spectral identification was an Agilent 7890 interfaced to a 240 mass selective detector with ion trap. The capillary column (30 m x 0.25 mm x 0.25 µm film thickness) was HP-5MS. The oven temperature was initially maintained at 80°C and then increased to 300°C. The carrier gas used was nitrogen (99.999%), at a flow rate of 1.0 ml/ min., and injection volume of 1.0 µL was employed (split ratio of 10:1). The electron-impact ionization of the mass spectrometry was operated at electron energy of 70 eV. Mass spectra were taken at 70 eV, a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time was 61 min.

2.6 Cell Viability Assay (MTT Assay)

Cell viability was assessed by the MTT method as described by Mosmann (1983) [46].

2.7 Cell Morphological Study

The morphological changes of pure juice powder-treated MCF-7 cell lines were assessed by using light microscopy. Cancer cells (1×10^6 cells/ml) were plated in 100 mm dishes and incubated for 24 h under controlled environment. Then, the spent medium was removed, followed by addition of fresh medium with or without juice powder at an inhibitory concentration and incubated for 48 h. After incubation, the cells were visualized under Radical inverted light microscope at 20X magnification.

2.8 DNA Fragmentation Assay

Low molecular weight genomic DNA was powdered using the previously described method of Yawata (1998) [47].

2.9 Statistical Analysis

The data of MTT assay with five replicates were subjected to statistical analysis and the mean value along with its respective standard error was calculated. The per cent change between control and experimental data was calculated. The data were analyzed statistically using Analysis of Variance (ANOVA). The data together with tables and graphs/bar diagrams are presented in appropriate places in the text.

3 Results

Lyophilized fresh pure juice of *C. limon* L. was dark brown in color and had powder texture which was used for the further studies.

3.1 Qualitative Phytochemical Analysis

The preliminary phytochemical analysis of lyophilized pure fruit juice powder of *C. limon* showed the presence of alkaloids, tannins, and proteins in trace amount, and the phenols, flavonoids, and acids in high amount (Table. 1).

Table 1. Phytochemical screening of lyophilized powder of *C. limon*

Primary Phytochemicals	Presence/Absence
Alkaloids	+
Anthraquinones	-
Carbohydrates	-
Flavonoids	+++

Glycosides	-
Phenols	+++
Quinones	-
Saponins	-
Tannins	+
Triterpenoids	-
Steroids and Phytosteroids	-
Proteins	+
Acids	+++

Note: +++ - Highly present; + - Trace; - - Absent

3.2 FT-IR Spectral Analysis

The FT-IR spectrum was used to identify the functional group of the active components based on the peak value in the region of infrared radiation. The results of FT-IR peak values and functional groups were presented in Table 2 and Figure 1. The lyophilized lemon juice powder revealed the presence of alkyl halides, amines, carboxylic acids, phenols, and alcohols.

Table 2. FT-IR spectral peak values and functional groups (4000 to 400 cm-1) obtained lyophilized powder of C. limon

S. No.	Peak cm^{-1}	Intensity	Peak Area	Functional Group
1	401.19	0.3	13.316	Alkyl halides (R-I) C-I stretch
2	412.77	0.09	29.59	
3	430.13	0.079	27.701	
4	435.91	0.206	19.491	
5	453.27	0.067	15.343	
6	462.92	0.138	14.235	Alkyl halides (R-Br) C-Br stretch
7	474.49	0.162	23.862	
8	487.99	0.105	20.959	
9	499.56	0.122	27.067	
10	507.28	0.047	32.717	
11	520.78	0.16	20.363	Amines (R-NH_2) Intensity (w-m) N-H bond
12	530.42	0.15	33.632	
13	547.78	0.489	34.162	
14	574.79	0.883	31.219	
15	590.22	1.087	26.153	
16	621.08	1.368	28.236	
17	667.37	1.713	16.88	
18	680.87	1.829	16.667	
19	1608.63	1.809	52.177	
20	1645.28	1.329	14.296	Carboxylic acids Intensity (S broad) O-H stretch
21	2692.63	1.977	19.677	
22	2742.78	1.822	79.69	Carboxylic acids Intensity (S broad) O-H stretch
23	2769.78	1.732	47.249	
24	2802.57	1.624	51.326	
25	2846.93	1.409	80.7	
26	2872.01	1.334	39.533	
27	2891.3	1.26	40.039	
28	2902.87	1.234	18.35	

S. No.	Peak cm^{-1}	Intensity	Peak Area	Functional Group
29	2916.37	1.168	25.933	
30	2937.59	1.159	22.369	Functional Group
31	2949.16	1.151	22.389	
32	2964.59	1.156	22.391	
33	2995.45	1.137	22.437	
34	3018.6	1.112	26.304	
35	3039.81	1.073	26.55	
36	3061.03	1.047	22.832	Carboxylic acids Intensity (S broad) O-H stretch
37	3091.89	0.996	23.108	Phenols and alcohols Hydrogen-bonded O-H Stretch
38	3105.39	0.967	23.245	
39	3120.82	0.92	35.106	
40	3134.33	0.93	11.739	
41	3149.76	0.88	23.696	
42	3169.04	0.846	31.761	
43	3196.05	0.795	28.142	
44	3207.62	0.778	24.346	
45	3228.84	0.727	24.622	
46	3242.34	0.736	20.889	
47	3261.63	0.721	20.619	
48	3275.13	0.693	24.831	
49	3296.35	0.667	20.889	
50	3305.99	0.648	21.03	
51	3327.21	0.611	46.61	
52	3354.21	0.611	25.565	
53	3375.43	0.605	21.32	
54	3402.43	0.585	25.795	
55	3429.43	0.596	25.684	
56	3626.17	1.504	34.093	

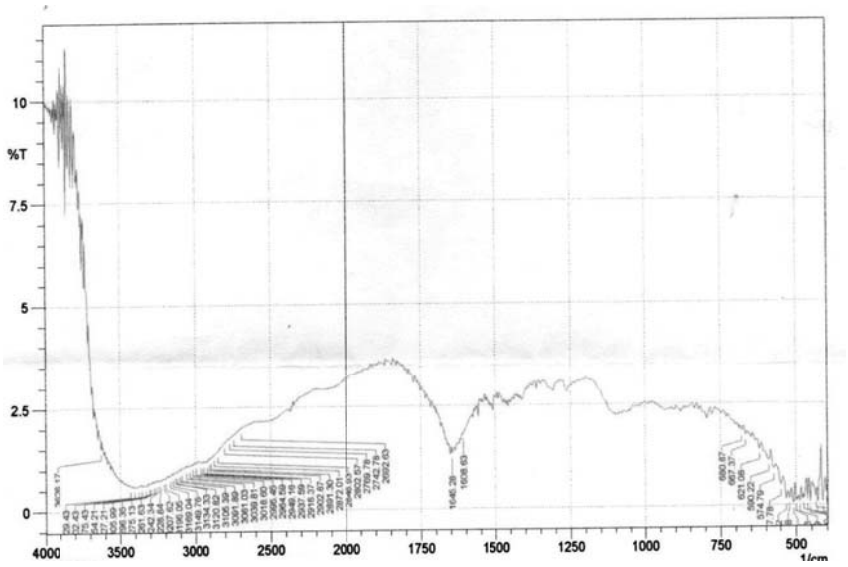

Figure 1. FT-IR spectra of various functional groups (4000 to 400 cm^{-1}) obtained for lyophilized powder of *C. limon*

3.3 GC-MS Spectral Analysis

GC-MS spectra of lyophilized powder of *C. limon* contain three active compounds such as 1,3-Dioxane-2-propanol, 2-methyl (RT-1.98), Methyl-hexadecyl ether (RT-19.92), D asicarpidan-1-methanol, acetate (ester) (RT-29.87). The peaks are given in Figure 2 and the individual mass peak and NIST match are given in Figure 3a to 3c.

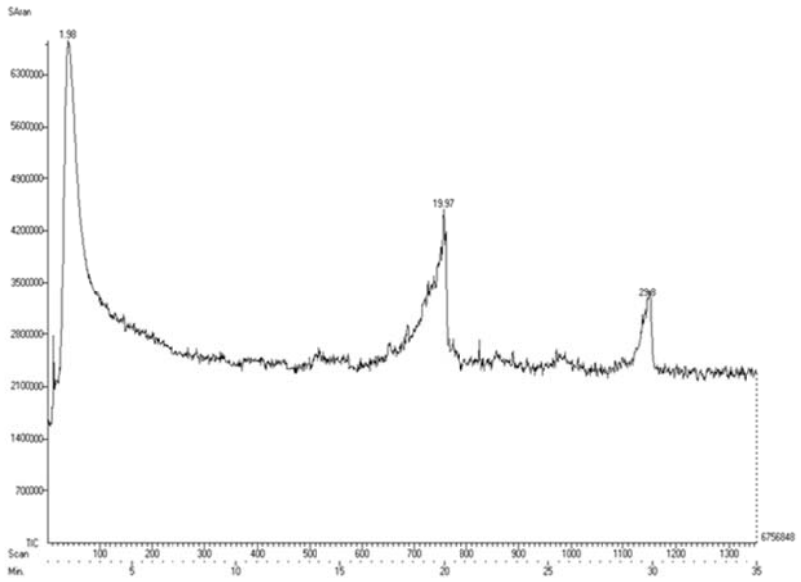

Figure 2: GC-MS chromatogram of C. limon lyophilized powder

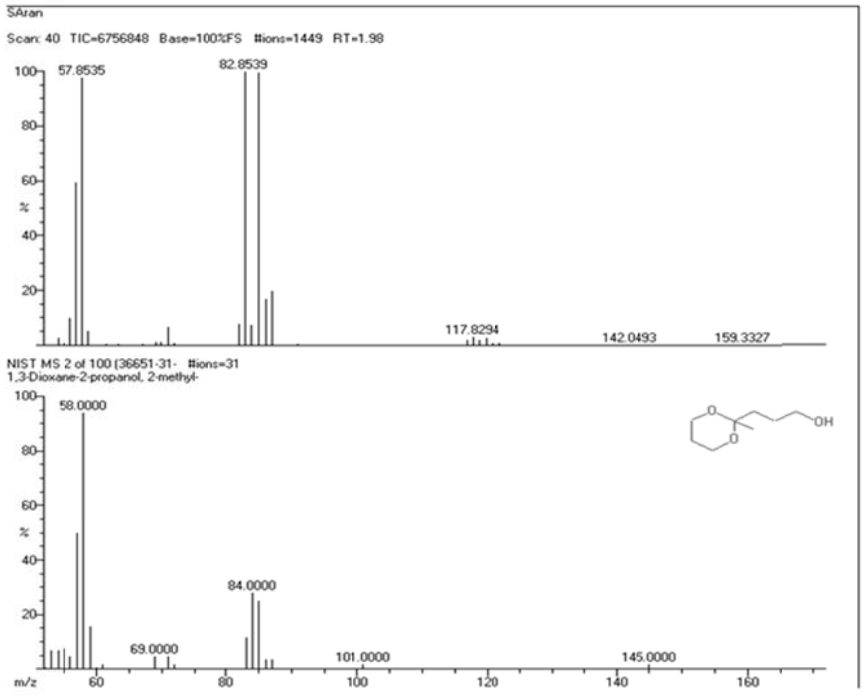

Figure 3a. Mass peak of RT 1.98

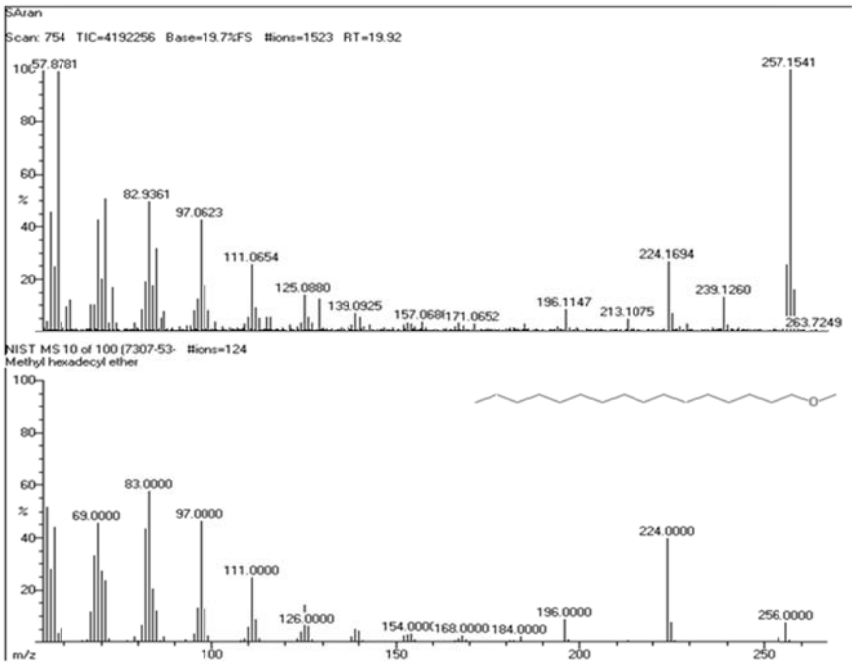

Figure 3b. Mass peak of RT 19.92

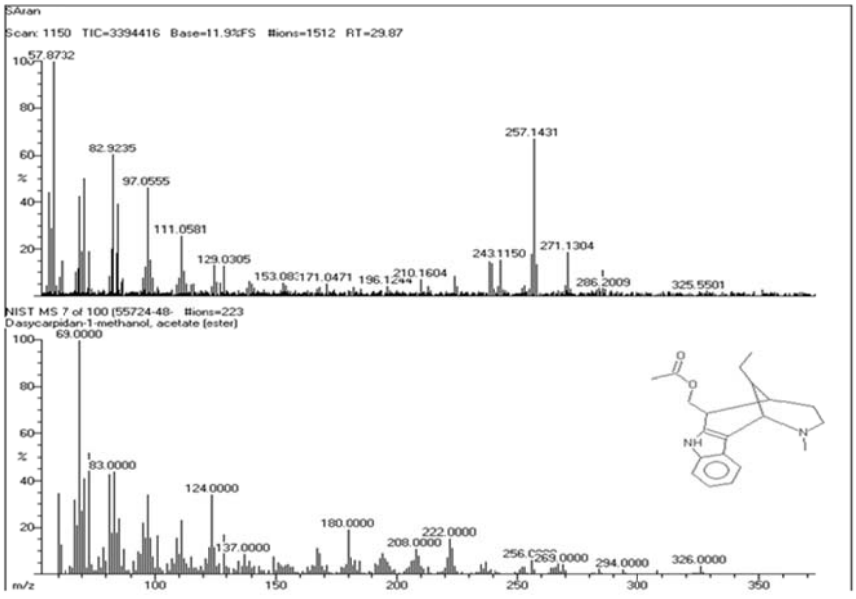

Figure 3c. Mass peak of RT 29.87

3.4 MTT Assay

Anti-proliferation of the cells was assessed by MTT assay for 48 h in the lemon juice powder and the data are presented in Table 3. The data revealed that anti-proliferative activity was seen in the MCF-7 cells when treated with different concentrations of fruit juice powder; the cell anti-proliferation being directly proportional to concentration. Statistical treatment of the data by two-way ANOVA showed that all the values were significant at 5% level. Per cent cell viability of MCF 7 cells was assessed for 48 h in the pure fruit juice powder at varying concentrations. The control cells were 100% viable and the viability decreased significantly with increase in concentration of the fruit juice powder. The per cent

decrease in cell viability was indirectly proportional to the concentration of *C. limon* powder. ANOVA analysis revealed that all the values were significantly different.

Table 3. Per cent cell viability of MCF-7 cells for 48 h when treated with pure fruit juice powder of C. limon.

Concentration of lyophilized fruit juice powder	Percentage of cell viability	
Control	100	
25 µg/ml	92.45 ± 0.25* (-7.55)	
50 µg/ml	78.29 ± 0.41* (-21.71)	
75 µg/ml	63.57 ± 0.42* (-36.43)	$IC_{50} = 98.16$
100 µg/ml	48.92 ± 0.44* (-51.08)	
150 µg/ml	39.27 ± 0.97* (-60.73)	

Values are mean ± S.E. of five individual observations.
Values in parentheses are per cent change over control.
- Denotes per cent decrease over control.
* Denotes that values are significant at P<0.05.

At 100 µg/ml concentration of the lyophilized powder of *C. limon*, at the end of 48 h, -51.08% viability of the cells was observed. The median inhibiting concentration (IC_{50}) value was 98.16 µg/ml, where 50% of the cells were viable. From the results it is pragmatic that pure lemon fruit juice powder has profound effect in controlling MCF-7 cell proliferation. The data altogether depict that the *C. limon* lyophilized powder significantly controls cell proliferation of MCF-7 cells even at low concentrations. The graphical data are presented in Figure 4.

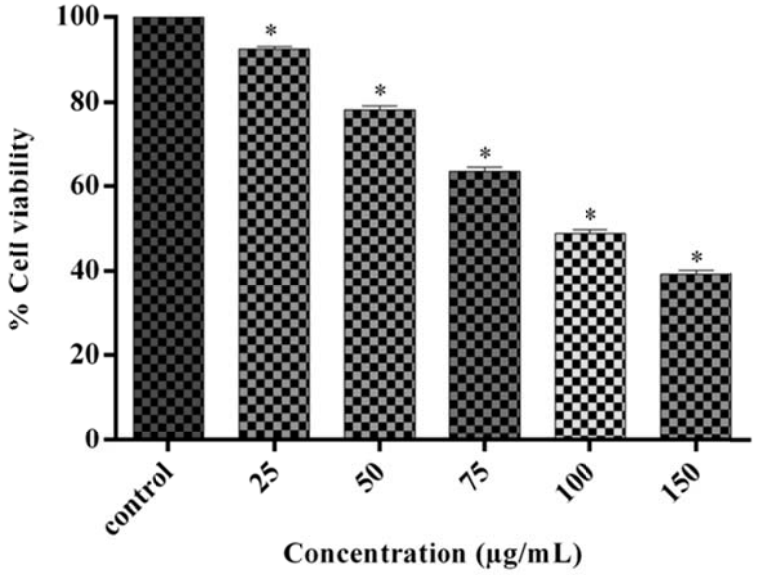

Figure 4. Bar diagram showing decrease in per cent viability of MCF-7 cells for 48 h when treated with pure fruit juice powder of C. limon.

3.5 Cell Morphological Studies

For morphological observations, MCF-7 cells were photographed in 10X magnification. The control cells showed irregular and confluent aggregates with rounded and polygonal cell morphology. But in the cells treated with pure *C. limon* fruit juice powder, after 48 h of incubation, the appearance of polygonal cells began to shrink and became spherical in shape (Figure 5) and the cell shrinkage increased progressively; the increase being dose and time dependent.

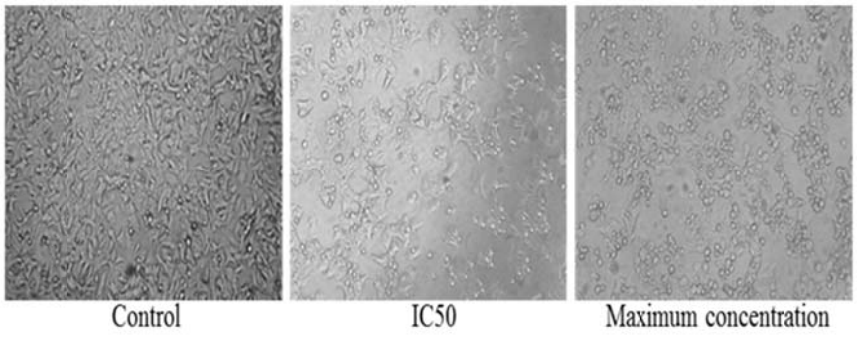

Figure 5. Morphological changes induced by MCF-7 cells induced by lyophilized powder of C. limon.

3.6 DNA Fragmentation Study

The lyophilized powder at 100 µg/ml concentration showed the induced DNA fragmentation. The fragments were observed from 500 bp to 600 bp, which indicate that MCF-7 cells underwent apoptosis by the induction of lyophilized powder (Figure 6).

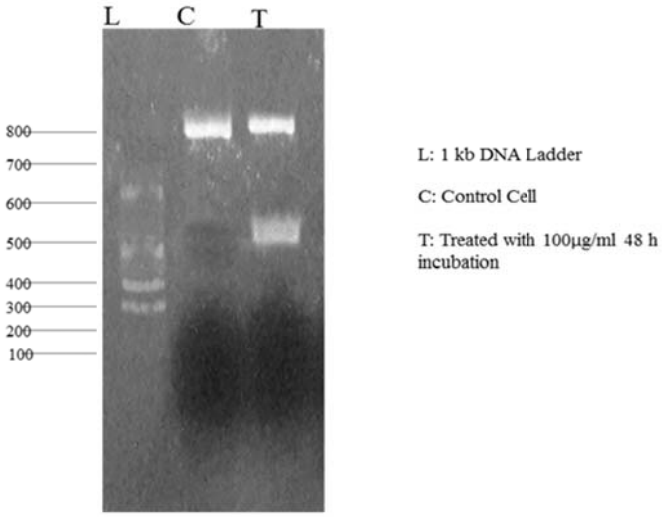

Figure 6. DNA fragmentation in MCF-7 cells induced by lyophilized powder of C. limon.

4 Discussion

According to Merina *et al.* (2012) [48], in recent times, medicinal plants occupy an important position for being the paramount sources of drug discovery, irrespective of its categorized groups such as herb, shrub, or tree. Plants have been indispensable in treating diverse forms of diseases including cancer. Natural products are formulated to generate different types of effective drugs to enhance anti-cancer activities. Proper understanding of the complex synergistic interaction of various constituents of anti-

cancer herbs, would help in formulating the design to attack the cancerous cells without harming the normal cells of the body as proved by the works of [49-67].

Among other herbal products, citrus fruits have been collected and used by man for centuries for medicinal, herbal, and agricultural purposes [68]. In citrus fruits, the lemon fruit *C. limon* is used in Siddha practice for curing giddiness, vomiting, nausea, thirst, scurvy, and in febrile and inflammatory conditions. Externally it is useful in whitlow, the juice of the fruit with sugar cures excessive thirst, it is processed into various forms and when taken for 6 months, helps in curing ascites, lethargy, expression and also helps in removing grey hair, and thus it acts as an elixir, coral prepared as white calyx is given with the adjuvant of lime juice helps in curing chronic and excessive diarrhea and dysentery [69]. Keeping this in mind, this study was planned to explore the phytochemical constituents of C. *limon* and also to anti-cancer potential against MCF-7 breast cancer cell line.

Phytochemicals are already a part of our diet through vegetables and fruits. Citrus fruits are found to be rich in phytoconstituents [70, 71]. Citrus flavonoids have a large spectrum of biological including anti-bacterial, anti-fungal, anti-diabetic, anti-cancer, and anti-viral activities [72,73]. Orange peel is medically used against fungi [74]. Limonene present in *Citrus sinensis* (sweet orange) is known as a significant chemo preventive agent with potential value as a dietary anti-cancer tool in humans [75,76]. The peel of *Citrus* fruits is a rich source of flavonoids glycosides, coumarins, β and γ-sitosterol, glycosides, and volatile oils as reported by Shahnah *et al.* (2007) [77]. Many polymethoxylated flavones have several important bioactivities, which are very rare in other plants as stated by Ahmad *et al.* (2006) [78]. In addition, the fiber of citrus fruit also contains bioactive compounds, such as polyphenols, the most important being vitamin C (Ascorbic acid), and they certainly prevent and cure vitamin C deficiency-the cause of scurvy [79].

The importance of natural bioactive compounds has led to the development of a large and potential market for natural sources in pharmaceutics and food products, polyphenols in the plants are considered to be free natural radical defenses that were acknowledged to be beneficial for human health as an anti-oxidant, anti-tumor, and anti-microbial agent as reported [80.81]. *C. limon* (lemon) is also a vital medicinal plant of family *Rutaceae*. It is cultivated chiefly for its alkaloids contents, which are having anti-cancer activities**,** its peel show strong anti-microbial activity as reported by Dhanavade *et al.* (2011) [40]. Many studies have been reported on the anti-microbial and anti-oxidant effect of edible part as well as non-edible part of citrus fruits [35].

Therefore, the present study was undertaken to determine the anti-cancer effect of *C. limon* lyophilized pure fruit juice powder against human breast cancer (MCF-7) cells. In our study, the lyophilized pure fruit juice powder of *C. limon* caused cell growth inhibition of MCF-7 cells. This might be due to the presence of phytoconstituents such as alkaloids, tannins, phenols, flavonoids, and acids in the lyophilized pure fruit juice powder. Moreover, the presence of functional groups as detected by FT-IR study of *C. limon* lyophilized pure fruit juice powder like alkyl halides, amines, carboxylic acids, phenols, and alcohols and the presence of three active compounds such as 1,3-Dioxane-2-propanol 2-methyl (RT-1.98), Methyl-hexadecyl ether (RT-19.92), D asicarpidan-1-methanol acetate (ester) (RT-29.87) when analyzed by GC-MS spectra of lyophilized pure fruit juice powder of *C. limon* might have also acted either alone or synergistically in combination, thus inhibiting the growth and viability of the MCF-7 cells.

Likewise, morphological analysis of the cells showed shrinkage of MCF-7 cells treated with lyophilized pure fruit juice powder of *C. limon*. This shrinkage may be due to the inhibitory effect of the phytoconstituents and/or functional groups and/or active compounds present in the lyophilized pure fruit juice powder of *C. limon*. The above work finds support from similar observations on the effect of cladribine [82], *Antrodia camphorata* [19], selenium and vitamin E [83], zoledronic acid [84], doxorubicin [85], metronidazole [86], gemcitabine [87], fruit peel extracts [81], triazole linked N-(pyrimidin-2-yl) banzol [d] thaizol-2-amine [5] on MCF-7 cells.

In our study, in 100 µg/ml concentration of lyophilized pure fruit juice powder of *C. limon* caused -51.08% viability of MCF-7 cells, at the end of 48 h. The IC_{50} value of the lyophilized pure fruit juice powder was 98.16 µg/ml. In our *in vitro* studies of MCF-7 cells treated with lyophilized pure fruit juice powder of *C. limon,* the growth inhibitory effects of this pure fruit juice powder prove its anti-cancer effects. Our preliminary studies on major constituents of *C. limon* lyophilized pure fruit juice powder confirm that phytochemicals are important constituents and they possess anti-cancer and anti-apoptotic activity. Similar results were also recorded which were followed by the author [89].

DNA fragmentation study in the present investigation showed that the lyophilized pure fruit juice powder of *C. limon* at 100 µg/mL concentration induced DNA fragmentation. DNA fragment was observed from 500 bp to 600 bp, which indicates that MCF-7 cells underwent apoptosis which was induced by the lyophilized pure fruit juice powder. Similar observations were also recorded by Yang *et al.* (2006) [19] in MCF-7 cell lines, when treated with *Antrodia camphorata*, a Chinese herbal medicine.

4 Conclusion

In conclusion, the lyophilized pure fruit juice powder of *C. limon* fruit demonstrates promising anti-cancer and anti-apoptotic properties against human breast cancer (MCF-7) cells by *in vitro* method. Increasing awareness, promotion, and utilization of this fruit for public benefits are highly encouraged and identification of active phytoconstiuents in the juice will serve as a natural cytotoxic agent against various cancers.

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