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In vitro Studies on the Effect of *Citrus limon* Linn in Neuroblastoma Sh-Sy5y Cell Lines

Perala Sai Krishna, Dinesh Bhaskar and Balasubramanian Sathyamurthy

Abstract
The leaves of *Citrus limon* are commonly used in traditional medicine by many Asian and European Countries and they are also used to treat as clenceing agent, insecticide, dermatitis and many more. This leaves extract are found to have antioxidant and anticancer activities; limonene is the active principle which is responsible for these beneficial effects. This work was aimed to study the effect of *Citrus limon* on Neuroblastoma SH-SY5Y cell line, in which the Methanol extracts of *Citrus limon* leaves has phytochemical compounds having more inhibitory scavenging and high anticancer activities.

Keywords: *Citrus limon,* limonene, SH-SY5Y cell line.

Introduction
Cancer and heart diseases are the two main pathologies that cause death at very high Worldwide. Cancer is the second leading cause of death globally, and was responsible for 8.8 million deaths in 2015. Globally, nearly 1 in 6 deaths is due to cancer. Despite the ongoing development of synthetic drugs that represent the mainstay of pharmaceutical care, the plant kingdom still remains an attractive source of novel anti-cancer drugs. It provides biologically active molecules for use in pharmaceuticals applications, and it has been estimated that about 70% of anti-cancer drugs originate to some extent from natural sources [1]. Use of plants as a source of medicine has been inherited from the onset of human civilization and is an important component of the healthcare system [2]. Plants have been used for medicinal purposes long before recorded history. Much of the medicinal use of plants seems to be developed through observations of wild animals, and by trial and error. As time went on, each ethnic group added the medicinal power of herbs in their area to its knowledge base. They methodically collected information on herbs and developed well-defined herbal pharmacopoeias [3].
Globally, there has been an unprecedented growth in the plant-derived medicinally useful formulations, drugs and health-care products. It has a market covering more than 65% products derived from plant origin. India exhibits remarkable outlook in contemporary medicines that are based on natural products besides traditional system of Indian medicines. Almost, 75% of the contemporary medicines in India are derived from natural products. Medicinal plants play a central role not only as traditional medicines but also as trade commodities, meeting the demand of distant markets [4].

SH-SY5Y cell model

Organism	:	*Homo sapiens*, human
Tissue	:	Bone Marrow
Disease	:	Neuroblastoma
Age	:	Below 4 years
Gender	:	Female
Morphology	:	Epithelial
Growth Properties	:	Adherent and suspension

SH-SY5Y is a well-characterized and commonly used human NB cell line. It was originally established by Dr June Biedler and co-workers, and is a trice cloned (SK-N-SH -> SH-SY -> SH-SY5 -> SH-SY5Y) N-type sub-clone of the parental cell line SK-N-SH, originating from a bone marrow biopsy of a metastatic tumor from a 4-year old female patient. The large majority of cells in a SH-SY5Y culture show an adherent growth pattern, and keeping growth conditions strictly constant is an important issue when keeping cultures of SH-SY5Y cells to

avoid variations in the cells phenotype [4, 5].

SH-SY5Y cells express tyrosine hydroxylase (TH), low levels of NSE, and chromogranin proteins and can be used as a model of undifferentiated neuroblasts. As other sub clones from SK-N-SH, SH-SY5Y do not have MYCN amplification, but have abnormalities in chromosome 1q, 2p, 4 and 5q, 7t, 9 and 10p, 14q, 16q, 15, 17 and 22q and mutated ALK (F1174L). SH-SY5Y cells exhibits normal expression of p53, show no ras gene-mutations or over expression of the anti-apoptotic Bcl-2. Wild-type SH-SY5Y express low levels of neurotrophin recepors (NTRKs) but the cells can be transfected with exogenous TRKs and thus become responsive to NGF (Trk A), brain-derived neurotrophic factor (BDNF) (Trk B) and NT-3 (Trk C). SH-SY5Y cells can successfully be grown in 3D-cultures as spheroids overcoming some of the limitations with traditional tumor cell lines grown in monolayers and more closely mimic the phenotype of untransformed cells. SH-SY5Y cells can also be injected intramuscularly or subcutaneously in nude mice or rats to form human NB xenografts, and the histology and phenotype of the developing *in vivo* tumors are similar to the features of human NBs. SH-SY5Y cells can be morphologically and biochemically differentiated *In vitro* by several different agents including the phorbol ester 12-O-tetradecanoyl-13-phorbol-acetate (TPA) in presence of serum or defined growth factors and the vitamin A derivative all-trans retinoic acid (ATRA) [6].

Applications

NB cell lines are used as *In vitro* models for several different applications including studies of tumor biology, neuronal development, nervous tissue damage and regeneration, neurotoxicity, and as a tool for studies of differentiation and growth control in immature nerve cells. This makes NB cell lines model systems for discoveries of broad biological and medical significance. Frequently used cell lines for *In vitro* studies of human NB are LAN-2, LAN-5, IMR-32 and SH-SY5Y. SH-SY5Y is a common model for NB, and also frequently used as a model system for studies of Alzheimer disease and Parkinson disease [7].

Citrus limon

Lemon is a predominant medicinal plant of the family Rutaceae. It is cultivated mainly for its alkaloids, which are having antitumour activities and the antibacterial potential in unrefined extracts of different parts (viz., leaves, stem, root and flower) of Lemon. Citrus biflavonoids have a large range of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities. Flavonoids can function as direct antioxidants and free radical scavengers, and have the capacity to modulate enzymatic activities and inhibit cell proliferation. Plants seem to play a defensive role against invading pathogens, including bacteria, fungi and viruses. Flavonoids are generally present in glycosylated forms in plants, and the sugar moiety is an important factor determining their bioavailability [8].

It is indigenous to North India, but cultivated on a very large scale in countries like Sicily, Italy and Spain. It is also cultivated in India, Florida and California. In India, the cultivation is carried out in Uttar Pradesh, Madhya Pradesh, Punjab and Karnataka. Lemon grows on thorny, small trees which reaches a height of 10 - 20 feet. The colors of leaves of the lemon are dark green. The lemon has a fragrant, white flower with five petals. This specific flower comes from a lemon cultivar called 'Pink Lemonade. Leaves 6.5 to 100 mm, Fruits are ovoid or globose, berry, hesperidium, and yellow when ripe. Mature lemons turn green to yellow, weighs about 40 to 80 grams and measure about 5-8 cm in diameter. Flowers will male or bisexual. Petals have white and tinged purple colour. Stamens 20-30; Fruit oblong or ovoid mamillate, yellow when Ripe. Seeds of lemon nestle within the pulp near the center of each fruit. Their size and numbers vary according to variety, but most are white, wrinkled, hard, oval or elliptical and measure about 3/8inch long [9].

The constituents of *Citrus limon*

The leaves of *Citrus limon* essential oil contains eleven constituents they were identified and found to be dominated by citronellal (29.31 %), limonene(17.59 %), (E)-citral (12.71 %), 1,6-octadien-3-ol,3,7-dimethyl (10.91 %), biocyclo [3.1.0] hexane, 4-mehylene-1-(1-methyl) (8.80 %), 6-octen-1-ol,3,7-dimethl (7.95 %), 2,6-octadien-1-ol,3,7-dimethyl-, acetate, (Z) (6.29 %), 1,3-cyclohexadiene,5-(1,5-dimethyl-4-hexenyl)-2-methyl,[S(R*,S*)] (2.81 %), cyclohexene,3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*,S*)](1.64 %), bezene,1-(1,5-dimethyl-4-hexenyl)-4-methyl (1.10 %) and cyclohexene,1-methyl-4-(5-methyl-1-methyl-1-methylene-4-hexenyl)(0.88%). Two chemotypes were identified for lemon leaf oils: limonene/β-pinene/geranial/neral and linalool/linalyl acetate/α-terpineol [10].

Role of Limonene

Limonene (1-methyl-4-isopropyl-cyclohexene) is a cyclic monoterpene and isolated mainly in essential oils of orange, lemon, mandarin, lime, grapefruit and many other plants. It is listed in the Code of Federal Regulations as generally recognized as safe (GRAS) for a flavoring agent and can be found in common food items such as fruit juices, soft drinks, baked goods, ice cream and pudding. The epidemiological studies on citrus peel consumption show a potential preventive effect on squamous cell carcinoma. When limonene is induced to rodent tumor models, limonene has been showed to have Chemoprophylaxis activity at the initial and promotion/progression phases. Evidence from a phase I clinical trial showed that limonene as well tolerated and may have clinical activity in cancer patients.

Several mechanisms may account for the antitumor effect of limonene. The Chemoprophylaxis activity of limonene during the initial phase of carcinogenesis might be attributed to the initiation of phase I and phase II carcinogen-detoxifying enzymes, which are known to be responsible for detoxification of carcinogen. An earlier reports revealed that the current treatment of limonene repress the Ras-ERK signaling pathway, inflammation and oxidative stress as well as the succession of pro-apoptotic state in the TPA-mediated promotion of DMBA in induced skin cancer in a mouse model. *In vivo* and *In vitro* studies showed that the Chemoprophylaxis activity of limonene may be attributed to the induction of apoptosis. However limonene initiates apoptosis of human blood cancer, cells involves in the increase in Bax protein expression, the release of cytochrome *c* from mitochondria and the activation of caspases, suggesting that the mitochondria-mediated intrinsic death pathway may play a main role in limonene induced cell death.

The serine/threonine protein kinase (Akt, a member of the PI3K pathway) is involved widely in divergent cellular processes including cell death/apoptosis and cell proliferation. The abnormal activation of phosphoinositide 3-kinase (PI3K)/AKT has been documented as a frequent occurrence in

human cancer, including colorectal cancer. Furthermore, abnormal activation of the PI3K/AKT pathway restores these cells less sensitive to apoptosis stimulation and inhibition of this pathway should provide a therapeutic approach for cancer. Once this PI3K/AKT is activated, however, Akt enhance the cell survival partially by phosphorylation and suppression of several pro-apoptotic proteins, includes GSK-3, BAD and caspase-9. Cell death/apoptosis and autophagy is also initiated Geraniol, an acyclic dietary monoterpene by suppression of AKT signaling, and the effect of limonene on Akt signaling remains unclear. Phase 1 study reported that three individuals with colorectal carcinoma, while on limonene at dose of 0.5–1 g/m^2 per day, were able to suspend progression of the disease for over six months. This clinical trial strongly suggested that limonene could be an efficient therapeutic agent for colorectal cancer. In this study we are going to see the effect of limonene present in lemon leaf on SH-SY-5Y cell line by invitro method using MTT Assay [11].

Materials and Methods

Sample Collection

Matured leaves wear collected in the Ghandhi Krushi Vignana Kendra(GKVK), University of Agriculture campus, Bangalore. The plant samples were collected and identified by an agriculturist in the GKVK. The samples were allowed to dry at room temperature. The dry samples were then crushed in fine powder and stored in tightly sealed polyethylene bags.

Extraction procedure

Plant leaves were washed thoroughly with distilled Water. The leaves were dried under shade at room temperature. The dried leaves of *Citrus limon* were finely grinded using electrical grinder and stored in air tight containers for further use. A total of 250 g of the pulverized plant material was extracted using Methanol. The extracts were then filtered through Whatman's No. 1 filter paper and then condensed to dryness using rotary evaporator. The thick extracted mass was then dried at room temperature. Dried extract was collect [12].

1. Phytochemical analysis

Phytochemical analysis of *Citrus limon* extracts were done using the protocols described by; segelman *et al* for the following.

Test for Sterols - Liebermann Burchard reaction
Tests for Alkaloids - Mayer's and Wagner's test
Tests for Tannins - Ferric chloride reagent test
Tests for Saponins - Foam test
Tests for Phenols - Ferric chloride reagent test
Tests for Flavonoids- Sodium hydroxide test
Test for Terpenoids- Salkowiski test
Test for Carbohydrate- Benedict's test
Test for protein- Biuret test

2. Nitric Oxide Radical scavenging assay

Principle

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be measured using Griess reagent at 546 nm spectro phtometrically [13].

Procedure

Nitric oxide scavenging assay is carried out as per the method of Sreejayan and Rao *et al*. In brief, 200µl of 10mM sodium nitroprusside and 200µl of test solution/reference standard of various concentrations are incubated at room temperature for 150 minutes. Add 500 µl Griess reagent and incubated for 10 min at room temperature. Measure the absorbance at 546 nm spectrophotometrically. Test substances are replaced by buffer solution for a control.

3. HPLC analysis of Quercetin

Plant Extraction

10gms plant powder was extracted with 50ml Methanol at 50^{o}C for 4 hours. The Methanol extracts were filtered through Whatmann No. 1 filter paper and filtrate was evaporated to dryness. Methanol extract (10mg/ml) was used for HPLC analysis.

Quercetin Standard: 100ug/ml prepared in Methanol

HPLC Condition:

Instrument	:	Shimadzhu LC- Prominence 20AT
Column particle	:	C18 column 250 mm x 4.6 mm, 5u
Mobile Phase	:	Linear
A	:	HPLC grade Acetonitrile (60%)
B	:	HPLC grade Water (40%)
Flow Rate	:	1.0 ml/min
Injection volume	:	10ul

Quantification of Quercetin in plant extracts

Concentration of Standard	:	100µg/ml
Sample concentration	:	10mg/ml

Formula used for quantification of quercetin in plant extract

Quercetin (Microgram/gram) = Sample area / Standard area X Standard concentration injected X Dilution factor.

Cytotoxicity studies using SHSY5Ycell line by MTT assay

SHSY5Ycell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD USA) (ATCC Number-CRL-2266). The steps and procedure for cell culture, Thawing, Revival and Propagation of Cells were followed as described by Kangas, L. *et al*. [13].

Procedure

The collected cells should reach about 70-80% confluency. Check the viability of the cells and centrifuge. Take about 50,000 cells / well and seed it in 96 well plates and incubate for 24 hrs at 37^{0}C, 5% CO_2 incubator. Add plant samples which is to be tested from 0 – 320µg/ml (2 fold variation) concentration in RPMI without FBS and are incubated for 24 hr. Add 100µl/well of the MTT (5 mg/10ml of MTT in 1X PBS) to incubated plant samples to the respective wells and incubated for 3to 4 hours. Discard the MTT reagent by using pipette without disturbing the cells and add 100 µl of DMSO to solubilize the formazan rapidly. Measure the Absorbance at 590 nm.

Calculating Inhibition

% Inhibition = 100 – (OD of sample/OD of Control) X 100.

Results and Discussion

1. Phytochemical analysis

Table 1: Phytochemical Analysis of *Citrus limon* leaves extracts

S. No	Tests	Observation	Inference
1	Froth formation test	Formation of stable froths was observed.	Presence of Saponins was confirmed.
2	Mayer's and Wagner's test	A brown color Precipitates was observed.	Presence of Alkaloid was confirmed.
3	Ferric Chloride test	Dark green color was developed.	Presence of Tannin was confirmed.
4	Liebermann-Burchard test	Formation of bluish green color was not observed.	Absence of Steroid was confirmed.
5	Sodium hydroxide test	Change from yellow color to colorless was observed.	Presence of Flavonoid was confirmed.
6	Ferric chloride test	Violet color was developed.	Presence of Phenol was confirmed.
7	Salkowski test	Reddish brown coloration was observed.	Presence of Terpenoid was confirmed.
8	Benedict's test	Formation of an orange red precipitate was observed.	Presence of reduceing sugar was confirmed.
9	Biuret test	Formation of pink colour in the extract layer was found.	Presence of protein was confirmed.

The citrus limon leaf extract was taken for qualitative analysis and examined for phytochemical compounds which includes alkaloids, terpenoids, phenols, tannins, reducing sugars, sponins, flavonoids, proteins and steroids. The results have shown that expect steroids all other phytochemicals mentioned above were present.

2. Nitric Oxide Radical scavenging assay

Table 2: Nitric Oxide Radical scavenging assay

Plants Name	Concentration (µg/ml)	Absorbance 546nm	% Inhibition	IC_{50}
Control	0.0	0.5403	0.00	
Standard (Curcumin)	6	0.4992	7.61	25.52
	12	0.4172	22.78	
	25	0.3128	42.11	
	50	0.1680	68.91	
	100	0.1190	77.98	
	200	0.0380	92.97	
Citrus limon	5	0.5164	4.42	21.52
	10	0.4573	15.36	
	20	0.3586	33.63	
	40	0.2933	45.72	
	80	0.2472	54.25	
	160	0.1652	69.42	

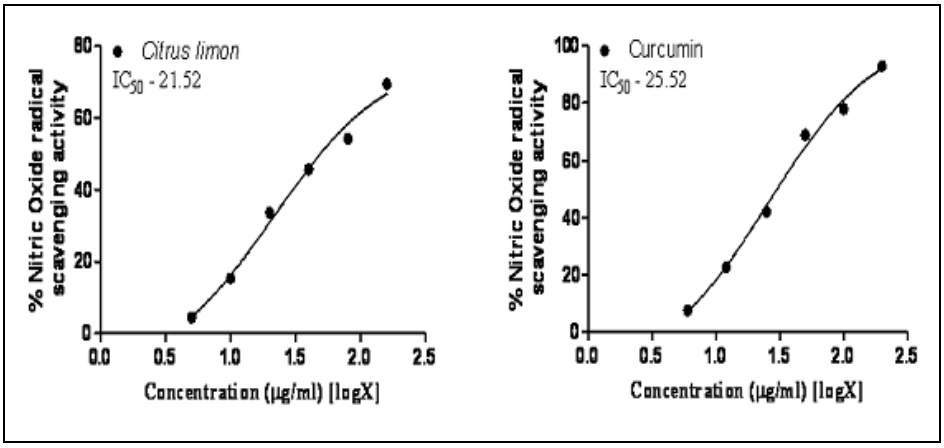

Fig 1: Nitric oxide scavenging assay of Curcumin and *Lantana Camara*

From Table 2 and Figure 1 shows the studies on nitric oxide radical scavenging assay using curcumin as standard and compared the results with IC_{50} values of the citrus limon leaf extracts were found more in curcumin than the citrus limon leaf extracts. Hence it is understood that the citrus (IC_{50}:21.25ug/ml) had more inhibitory concentration when compare to curcumin (IC_{50}: 25.52ug/ml). It may also due to the presence of phytochemicals which shows radical scavenging property.

HPLC analysis of Quercetin and*Citrus limon*

Table 3: HPLC analysis of Standard Quercetin

S. No.	Retention. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.933	373.177	22.881	20.5	14.0	0.20
2	3.107	92.433	3.352	5.1	2.0	0.49
3	3.487	1296.195	133.916	71.3	81.6	0.14
4	4.207	55.054	3.869	3.0	2.4	0.22
	Total	1816.859	164.018	100.0	100.0	

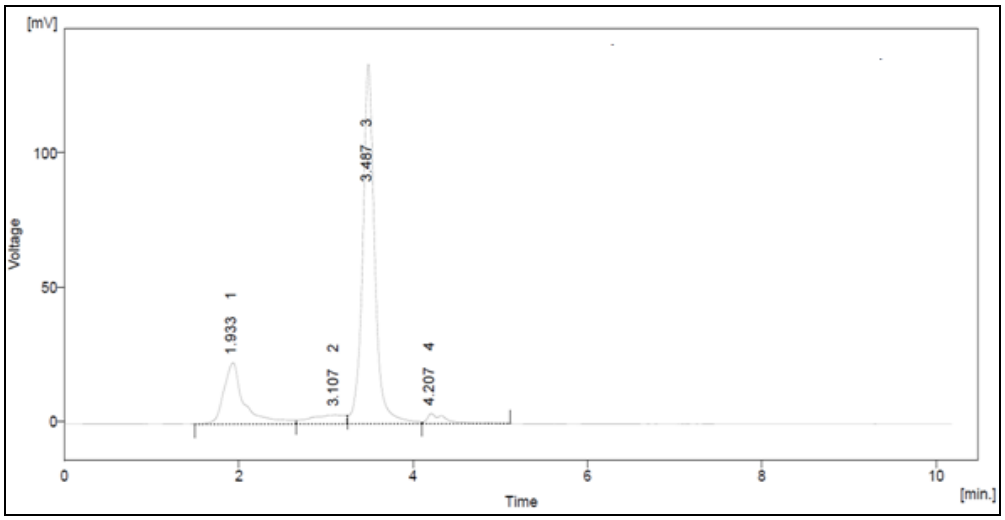

Fig 2: HPLC analysis of standard Quercetin

From Table – 3 and Figure – 2, the flavonoids were quantified at 254nm using peak area by comparison with a calibration curve derived from the quercetin.

Table 4: HPLC analysis of Quercetin content in *Citrus limon*

S. No.	Retention. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	4.787	349.686	34.676	85.1	87.9	0.12
2	5.397	25.086	2.519	6.1	6.4	0.12
3	5.833	17.969	1.287	4.4	3.3	0.17
4	6.480	14.194	0.712	3.5	1.8	0.17
5	14.330	3.954	0.272	1.0	0.7	0.24
	Total	410.888	39.466	100.0	100.0	

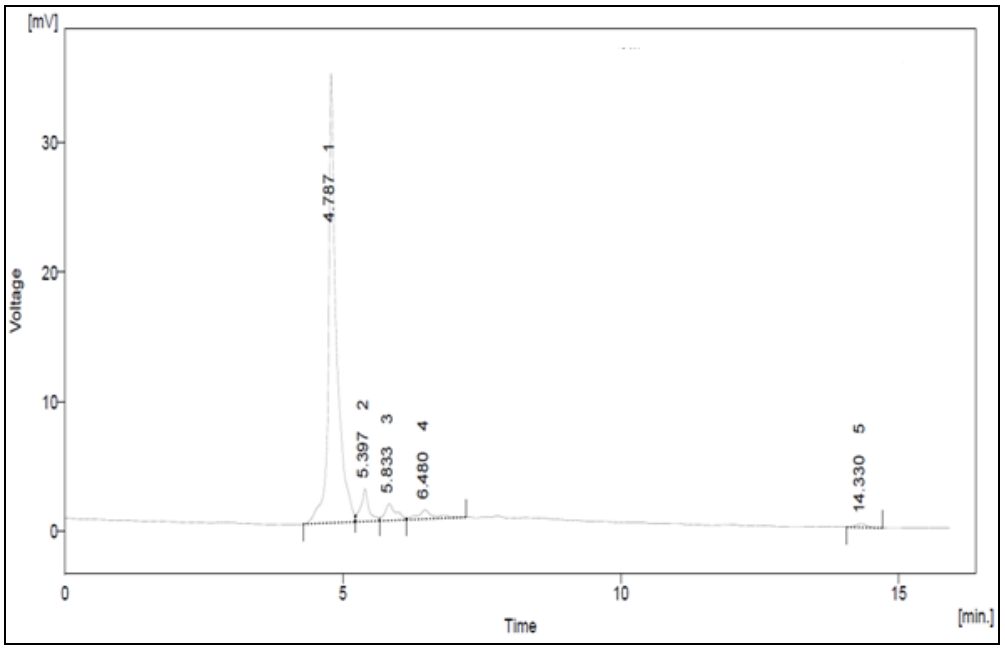

Fig 3: HPLC analysis of Quercetin content in*Citrus limon*

From Table – 4 and Figure – 3, the HPLC chromatograms of *Citrus limon* leaves extract shows the main difference was in peak eluted at 3.4min. External flavonoids were already analysed using HPLC method in various plant extracts.

The peaks in this study shown marked decreased in peak area when compared with standard quercetin.

From the calibration curve results, the amount of Quercetin, in the sample injected was calculated. *Citrus limon* leaves contain no quercetin. Other peaks (#1) in both the HPLC chromatogram *Citrus limo* leaves extracts indicated the presence of other chemical constituents. The present method was applicable for determining quercetin in any aerial part of plant material using HPLC technique.

Cytotoxicity studies using SHSY5Y cell line on *citrus limon* leaf extract by MTT assay

Table 5: cytotoxic study of*Citrus limon*

Plants name	Conc. µg/ml	OD at 590 nm	% Inhibition	IC_{50}
Control		0.6263	0.00	
Citrus limon	10	0.6066	3.15	70.52
	20	0.5366	14.32	
	40	0.4784	23.61	
	80	0.3592	42.65	
	160	0.2811	55.12	
	320	0.2018	67.78	

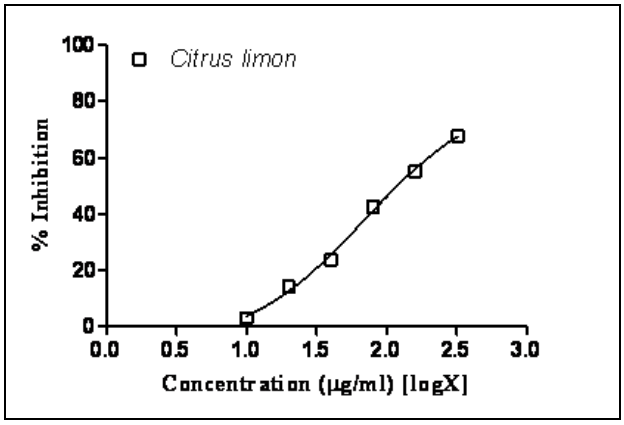

Fig 4: Cytotoxic study of *Citrus limon*

In Cytotoxic study of *Citrus limon* (from Table 5 and Figure 4) on SHSY5Y cancer cell line using MTT assay showed IC_{50} value 70.52ug/ml after completing sub cell culture collected the cells when they reach about 70 – 80% confluency suggests that limio leaf extract shown significant dose-dependent inhibition of growth of SHSY5Y cells. Hence the limion leaf extract was found to be a powerful anti-cancerous component. This inhibits the growth of SHSY5Y cell lines.

Conclusion

Our results in accordance with the above findings, shows that *Citrus limon* leaves possess good radical scavenging activity. The maximum Nitric oxide (NO) inhibition percentage and IC50 valve of standard cucumin and *Citrus limon* is 92.97%and 69.42%, 25.52 and 21.51 respectively. From HPLC analysis we came to know that the leaves of *Citrus limon* dose not contain any *cucurmin* content. The MTT assay found that there were cytotoxic effects with increasing concentration on SHSY5Y cell line from 10µg to 320µg concentration when compared to the untreated SHSY5Y cells. The reported IC value is 70.52.

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