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Evaluation of Anticancer Activity of Various Extracts of Fruit of Garcinia Pedunculata UsingCervical Cancer Cell Line by Invitro Methods

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ABSTRACT

The present study was undertaken to investigate invitro anticancer activity of various extracts of *Garcinia pedunculata* using HeLA cell line. Phytochemical analysis of acetone, ethanol and aqueous extracts of *Garcinia pedunculata revealed* the presence of proteins, terpenoids, tannins, steroids, phenols and flavonoids. In this study, ethanolic extract of *Garcinia pedunculata showed* cytotoxic activity in cervical cancer cell line HeLa by MTT assay. The Apoptotic effect was confirmed by loss of membrane integrity, chromatin condensation, leakage of cytoplasmic contents and fragmentation of DNA by microscopic methods. Its apoptotic and anti cancer effect may be due to up regulation of genes like p53 anddown regulation of gene Bcl-2, which was confirmed by RT-PCR. The ethanolic extract of *Garcinia pedunculata possess* anti-cancer effect and for futureperspective, it can be further confirmed by isolating the compounds responsible for the activity and studying the exact mechanism by which the plant possess thisactivity and confirm the results using *in vivo* animal models.

Keywords: Anticancer Activity, Garcinia Pedunculata, Cervical Cancer, Invitro Methods

INTRODUCTION

Cervical cancer is the fourth most commonly diagnosed cancer in females worldwide, with 528,000 new cases and 266,000 deaths annually [1]. The incidence and mortality rates of cervical cancer have considerably reduced in developed countries through the application of cervical cancer screening tests for high-risk human papillomavirus and through Papanicolaou smear; however, the incidence rate of this disease remains high (i.e., 80%) in developing countries, where 70% of the patients are at the advanced stage of the disease, and the age at diagnosis of the patients is slightly decreasing [2, 3]. The recurrence rate of cervical cancer is still high in developing countries, and radical surgery affects the long-term recovery and survival rate of patients after curative resection [4]. Moreover, radioactive rays and most anticancer drugs suppress DNA duplication or damage DNA in order to kill cancer cells divided rapidly. Meanwhile, they also affect normal cells to cause adverse side effects, such as bone marrow function inhibition, nausea, vomiting, and alopecia [5, 6]. Therefore, new forms of therapy should be discovered to improve the clinical outcome of patients with cervical cancer.A HeLA cell is a cell type in an immortal cell line used in scientific research. It isthe oldest and most commonly used cell line. The cell line was derived from cervical cancer. HeLa cells, like other cell lines, are termed immortal in that they can divide an unlimited number of times in a laboratory cell culture plate as long as fundamental cell survival conditions are met (*i.e.*, being maintained and sustained in a suitable environment). In *Garcinia pedunculata* antioxidant activity has been proved, so present study was undertaken to investigate invitro anticancer activity of various extracts of *Garcinia pedunculata* using HeLA cell line.

METHODOLOGY

Extraction

It is defined as separation of medicinally active parts of plant

using selective solvents through standard procedure. Extraction procedure was carried out.

Plant parts used:

The fruits of *Garcinia pedunculata was* used for extraction which was collected in month of July 2022 at Tirupathi. It was examined and authenticated.

Solvents used

Based on polarity various solvents were used such as ethanol, acetone, and water.

Type of extraction

Hot continuous (soxhlet) extraction method.

Apparatus description

The apparatus consists of body of the extractor (thimble) attached with side tube, siphon tube, lower end attached with distillation flask, mouth of the extractor is fixed to the condenser by standard joints.

Principle

It is a process of continuous extraction method in which the solvent can be circulated through the extractor for several times. The vapours of solvent are taken to thecondenser and the condensed liquid is returned to the extract for continuous extraction.

Procedure

50 grams of fruit was packed into soxhlet apparatus and was subjected to extraction sequentially with 500ml of ethanol, acetone, and water. The extraction was continued until the colour of the solvent in the siphon tube became colourless. Extracts of acetone and ethanol were subjected to evaporation at room temperature till a semisolid mass was obtained. Aqueous extract was subjected to lyophilisation with the help of lyophiliser to a semisolid mass.

Phytochemical analysis

The freshly prepared extracts of acetone, ethanol and water were subjected to phytochemical screening for the presence or absence of active phytochemical constituents by following methods.

Test for Steroids:

Salkowskis test: Crude extract was mixed with 2ml of chloroform. Then 2ml of conc. Sulphuric acid was added carefully and shaken gently. Appearance of reddish brown colour ring indicated the presence of steroids

Test for Flavanoids:

Shinoda test: Crude extract was treated with 5 ml 95% ethanol, few drops concentrated hydrochloric acid and 5 grams magnesium turnings, appearance of pink colour indicated the presence of steroid

Lead acetate test: Crude extract was treated with few drops of lead acetate solution. Appearance of yellow colour precipitate indicate the presence of flavanoids

Alkaline reagent test: Crude extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavanoids.

Test for terpenoids: 5 ml of each extract was mixed in 2 ml of chloroform. 3 ml of concentrated H2SO4 was then added to form a layer. A reddishbrown precipitate colouration at the interface formed indicated the presence of terpenoids.

Test for Proteins:

Millions test: Crude extract was mixed with 2ml of millions reagent. Appearanceof white precipitate which turns red on gentle heating, indicates the presence of proteins.

Test for Glycosides:

Liebermann's test: Crude extract was mixed with 2ml of chloroform and 2ml of acetic acid. Mixture was cooled in ice and conc. sulphuric acid was added. Colour change from violet to blue to green indicates the presence of steroidal nucleus

Test for Carbohydrates:

Fehling's test: Crude extract was treated with equal volume of Fehling A and Fehling B reagents and mixed together and gently boiled. Appearance of brick red precipitate at the bottom of the test tube indicate the presence of reducing sugars

Test for phenols and tannins: Crude extract was mixed with 2ml of 2% solution of ferric chloride. Appearance of violet colour indicates the presence of phenolic compounds and tannins. Crude extractwas dissolved in water and treated with 10% of lead acetate solution, appearance of white precipitate indicate the presence of tannins and phenolic compounds.

Test for alkaloids: Crude extract was treated with few drops of dilute hydrochloric acid and filtered. The filterate was tested with various alkaloidal reagents such as

Mayer's reagent – Cream precipitate Dragendroff's reagent – Orange brown precipitate Wagner's reagent–Reddish brown precipitate.

Cytotoxicity studymtt assay

It is a universally accepted in-vitro method for screening the drugs having cytotoxic activity. It was described by Mosmamm (1983) & Monks (1991). This assay is used to determine the IC50 of drugs or extracts.

Principle

The Tetrazolium salt, 3,-(4,5- dimethyl thiazol-2-yl)-2,5, diphenyl tetrazolium bromide is reduced into blue formazan product by the mitochondrial dehydrogenase enzyme of live or metabolically active cells. The intensity of blue or purple colored formazan produced is directlyproportional to cell viability.

Apoptotic dna fragmentation

Apoptosis is characterized by cleavage of chromosomal DNA into oligonucleosomal fragments. Irregularities in apoptosis have paved way for many diseases like cancer, autoimmune disease and neuronal degeneration. This cleavage of DNA or its fragmentation can be visualized by DNA laddering assay. Cleavage ofchromosomal DNA into oligo-nucleosomal fragments is a hallmark of apoptosis. This fragmentation of DNA in cancer cells after treatment with standard drug or test extract can be studied with the help of Agarose Gel Electrophoresis. Electrophoresis is a method of separating substances based on the rate of movement under the influence of electric field.

Real time reverse transcriptase polymerase chain reaction

Real time reverse transcriptase polymerase chain reaction is abbreviated as qRT- PCR. It is a technique where expression of RNA is studied by converting it into cDNA with the help of

enzyme reverse transcriptase and quantitatively measuring the amount of amplified target sequence from entire cDNA using fluorescent dye SYBR green in real time. Upon binding with DNA, SYBR green dye used will emit fluorescence and the fluorescence intensity is directly proportional to number of DNA copies or expression produced. The fluorescence which is emitted is analysed by detector with the help of LED source and it gives the relative expression of genes. The procedure was carried out as per Step 1 plus ABI protocol.

Primer synthesis

The primers synthesized were P53, Bcl2, along with house keeping gene GPDH. The primers were synthesized by Geno Rime with the help of Primer express software with the available primer sequence. The concentration of primers synthesized were 100pM/µl. It was diluted in the ratio of 1: 10 with water to get a concentration of 10pM/µl.

RESULTS

Table 1: Phytochemical analysis of various extracts of fruits of *Garcinia pedunculata.*

Constituents	Ethanol extract	Acetone extract	Aqueous extract
Proteins	Present	Present	Present
Carbohydrates	Present	Present	Present
Glycosides	Present	Present	Present
Triterpenoids	Present	Absent	Absent
Flavanoids	Present	Absent	Absent
Alkaloids	Absent	Absent	Absent
Steroids	Present	Present	Present
Phenols and tannins	Present	Present	Present

Table 2: Percentage yield of extracts

Extract	% yield
Ethanol	5.20
Acetone	3.45
Aqueous	6.40

Cytotoxicity test

MTT assay was carried out with ethanolic extract of *Garcinia pedunculata a n d* with 5 Fluorouracil and the results are shown in Table 3 & 4.

Table 3: MTT assay of ethanolic extract of *Garcinia pedunculata*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	-	0.07	11.86
2	500	1:1	0.13	22.03
3	250	1:2	0.19	32.20
4	125	1:4	0.22	37.28
5	62.5	1:8	0.28	47.45
6	31.2	1:16	0.34	57.62
7	15.6	1:32	0.39	66.10
8	7.8	1:64	0.44	74.57
9	**Cell control**	**-**	**0.59**	**100**

IC50 concentration and Percentage cell viability

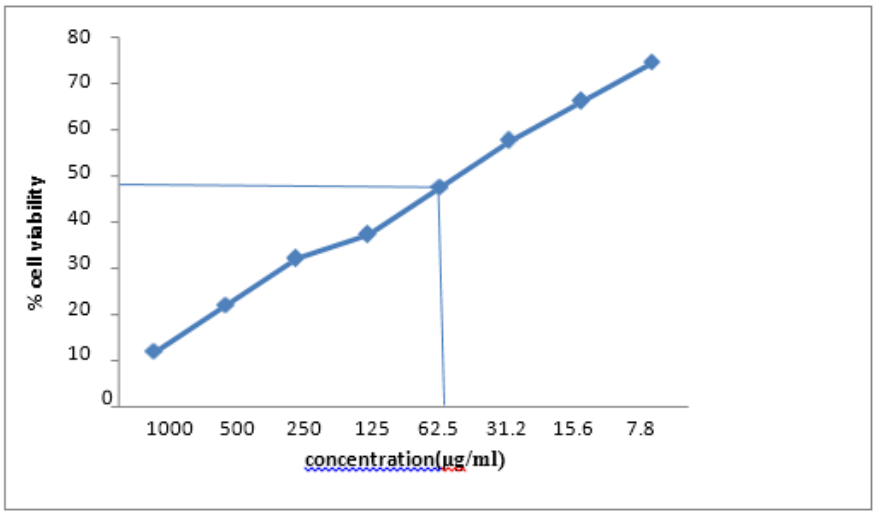


Fig 1: Graphical representation of % cell viability vs concentration (µg/ml) ofethanolic extract of *Garcinia pedunculata.* IC50 concentration and % cell viability of 5 Fluorouracil

Table 4.: MTT Assay of 5 Fluorouracil

S.No	Concentration(µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	-	0.01	1.85185185
2	500	1:01	0.03	5.55555556
3	250	1:02	0.06	11.1111111
4	125	1:04	0.1	18.5185185
5	62.5	1:08	0.13	24.0740741
6	31.2	1:16	0.17	31.4814815
7	15.6	1:32	0.2	37.037037
8	7.8	1 64	0.28	51.8518519
9	Cell control	-	0.54	100

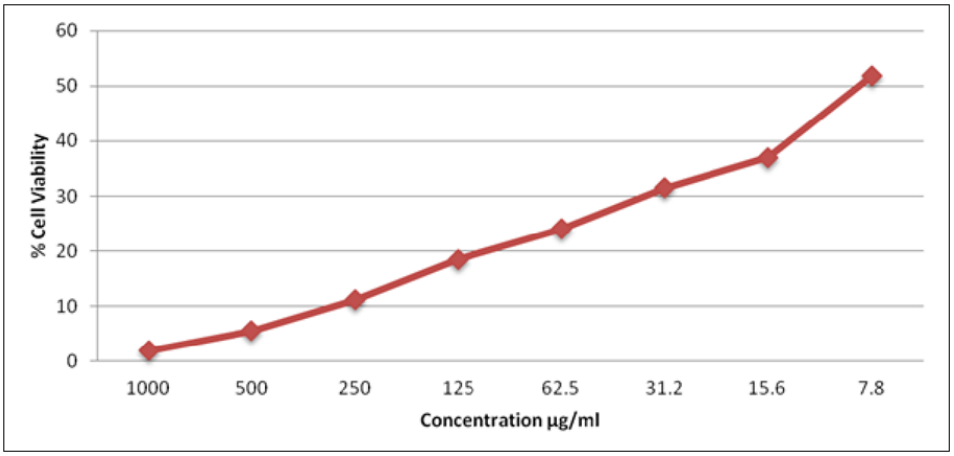


Fig 2: Graphical representation of % cell viability vs Concentration in µg/ml of5 Fluorouracil
Anticancer effect of Sample *on HeLa* Cell line using microscopeNormal *HeLa* cell line

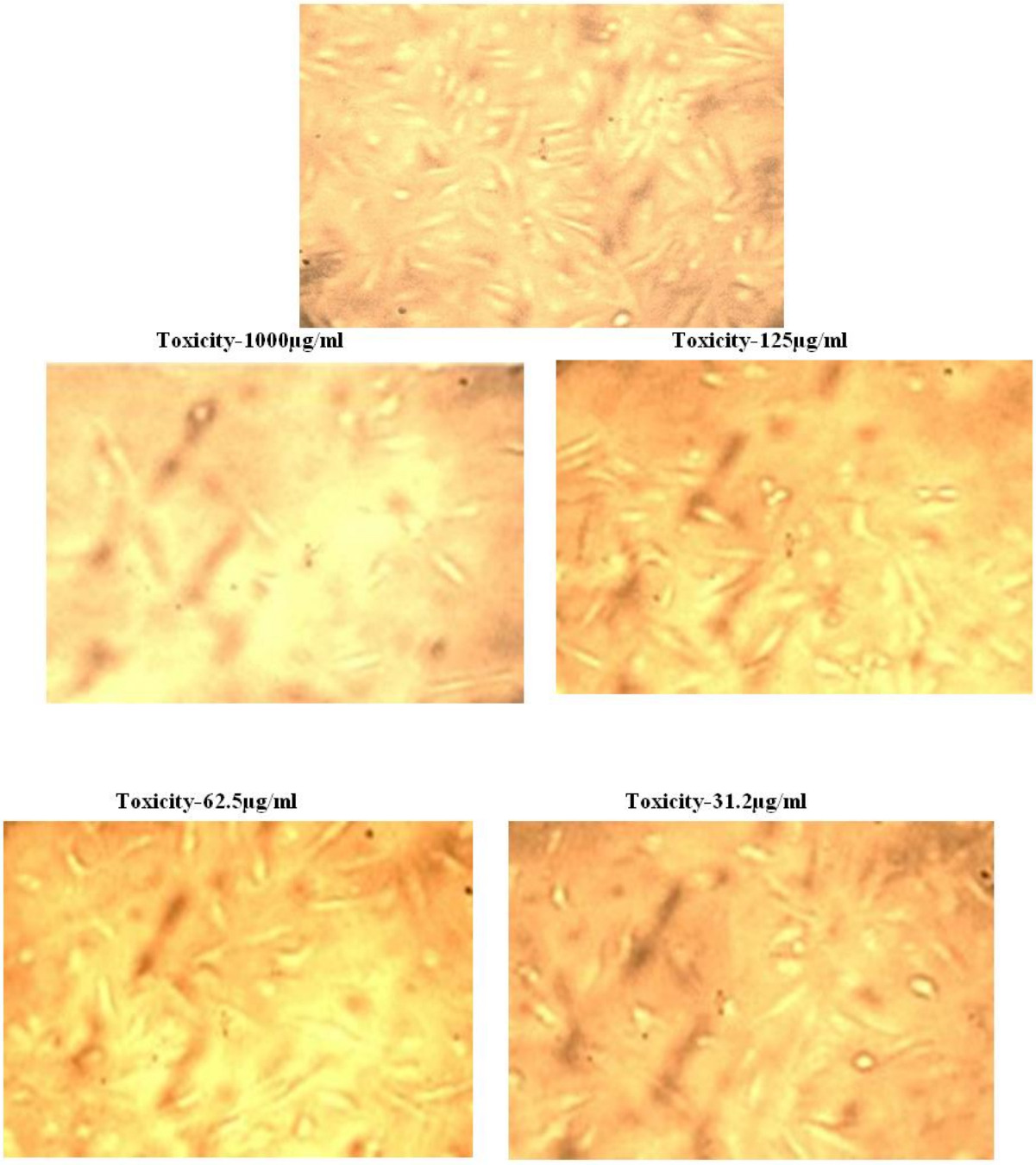
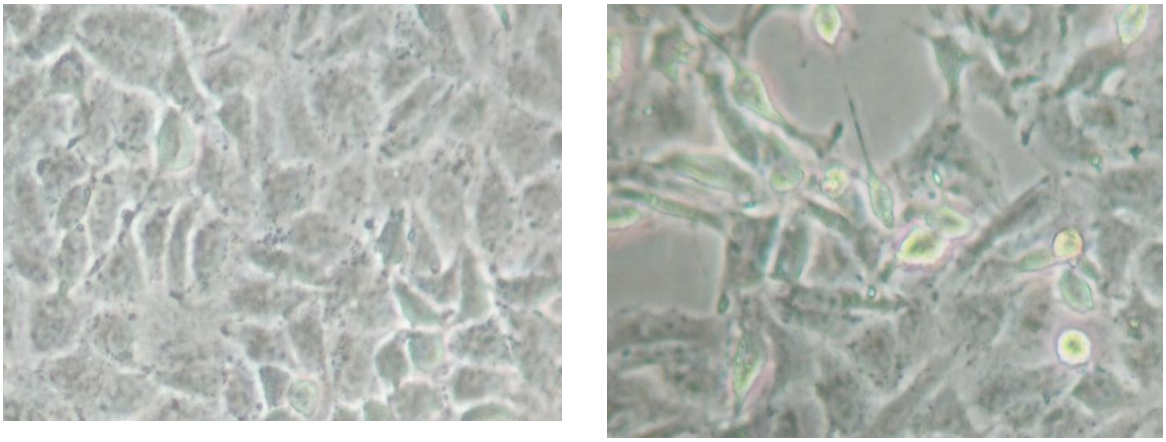


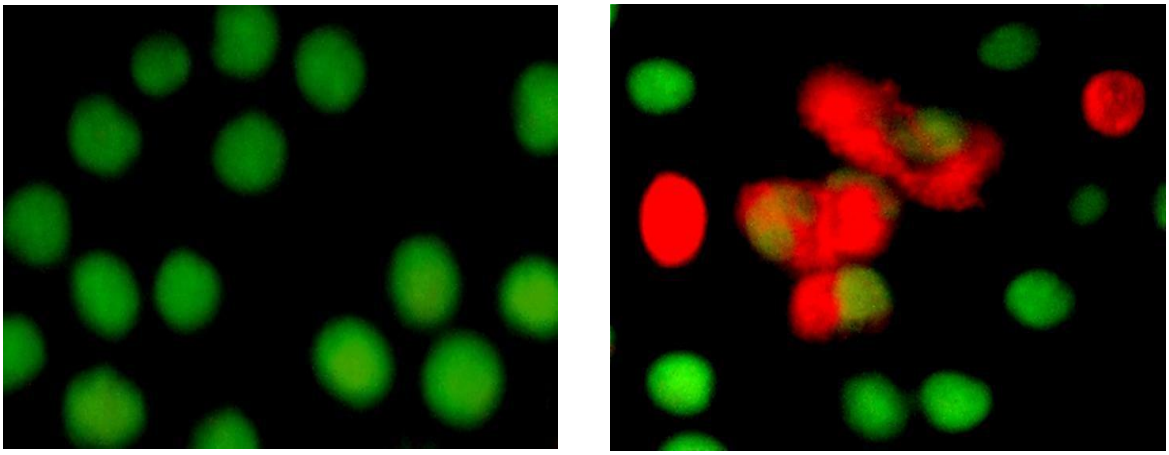
Fig 3: When HeLa cell line was treated with plant extract the cell death was more at1000µg/ml and the least was at the concentration of 31.2µg/ml

Microscopic studies for apoptosis



A).HeLa cells – Control B).HeLa cells after treatment with ethanolic extract of *Garcinia pedunculata* indicating decrease in cell population, chromatin condensation and destruction ofmonolayer

Fig 4: Light Microscopic study images



A). HeLa cells – Control, indicting viable cells stained green in colour
B). HeLa cells after treatment with Ethanolic extract of *Garcinia pedunculata* showing dead cells-stained orange in colour with loss of membrane integrity andcytoplasmic contents leaking out of the cell.

Fig 5: Flourescent Microscopic Images

DNA Fragmentation

- In the control HeLa cells, there was no fragmentation observed in agarose gel. Fragmentation was observed in HeLa cells treated with IC50 concentration of standard 5 fluorouracil and ethanolic extract of *Garcinia pedunculata.*
- This fragmentation of DNA in ethanolic extract treated cells indicated the characteristics of apoptotic cells.
- Results obtained in fragmentation studies are shown in figure 8.

Agarose gel electrophoresis

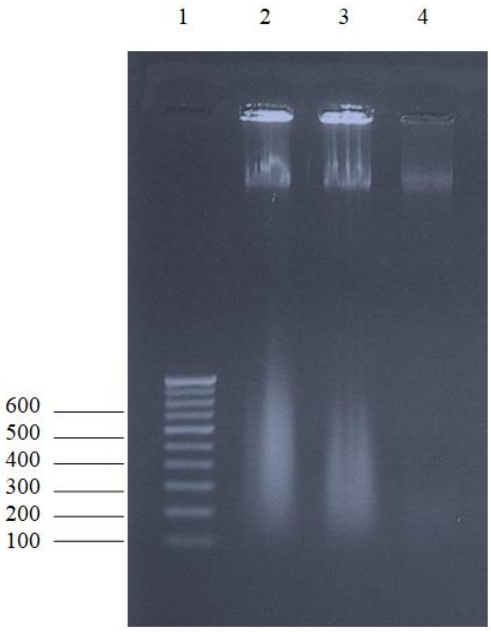


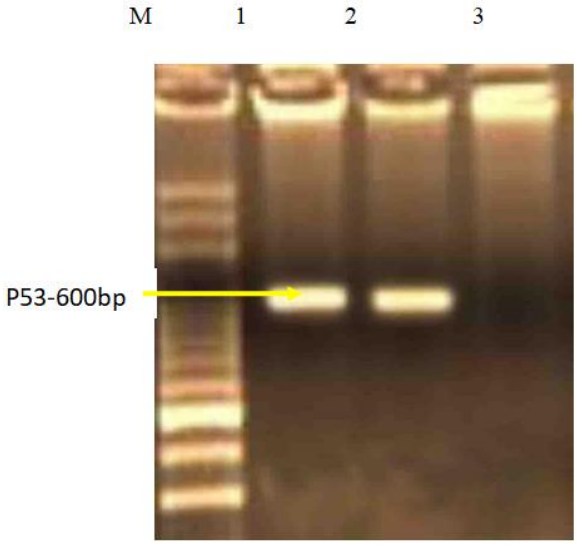
Fig 6: DNA Fragmentation in HeLa cells.

DNA Fragmentation in HeLa cells.
Lane 1: 100 base pair DNA marker
Lane 2: HeLa cells treated with 5 Fluorouracil
Lane 3: HeLa cells treated with ethanolic extract of *Garcinia pedunculata*
Lane 4: HeLa cells without any treatment.

Real time PCR

The expression levels of p53, Bcl-2 were studied using RT-PCR and the resultsare shown in **Figure 9 and 11.**

P_{53} Gene in HeLa Cell Line by gel electrophoresis



M- 100 bp DNA Ladder, Lane 1 and 2: Samples in duplicate

Fig 7: P53 gene expression

Table 5: Expression levels of p53

P 53	Relative Quantification (ng/µl)	Standard error
Control	2.25	0.1
5 fluorouracil	6.15	0.12

Ethanolic extract	3.62	0.09

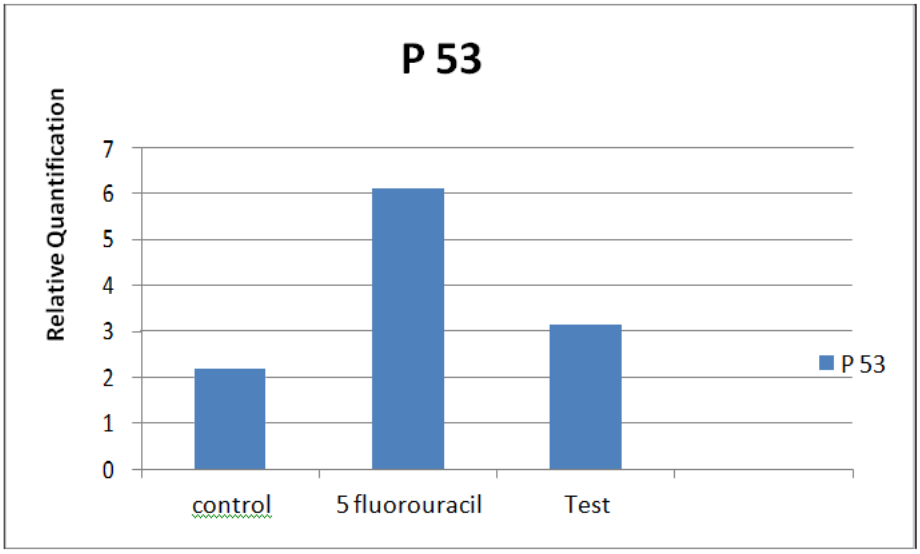


Fig 8: Graphical representation of p53 gene expression

BCl_2 Gene in HeLa Cell Line by gel electrophoresis

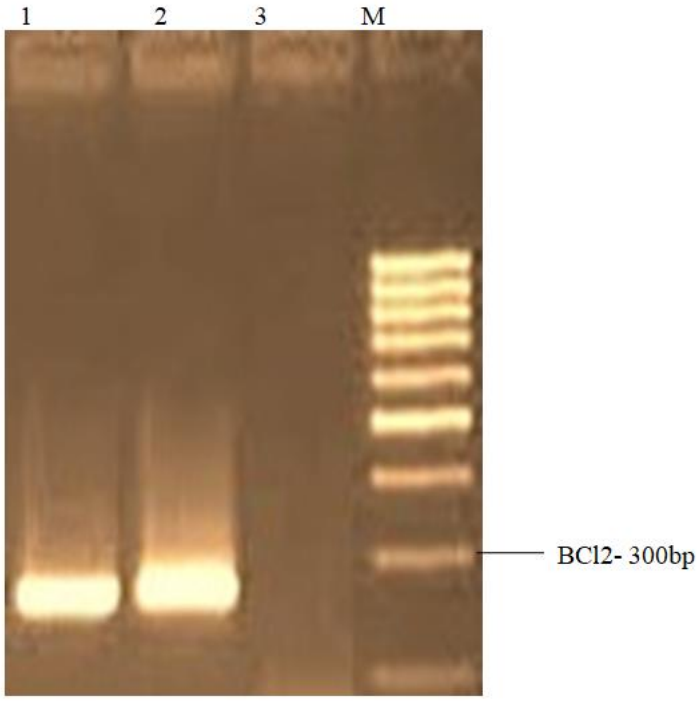


Fig 9: M- 100 bp DNA Ladder, Lane 1 and 2 : Samples in duplicate

Table 6: Expression levels of Bcl-2

Bcl2	Relative Quantification(ng/µl)	Standard error
Control	6.68	0.106
5 Fluorouracil	2.15	0.096
Ethanolic extract	3.84	0.131

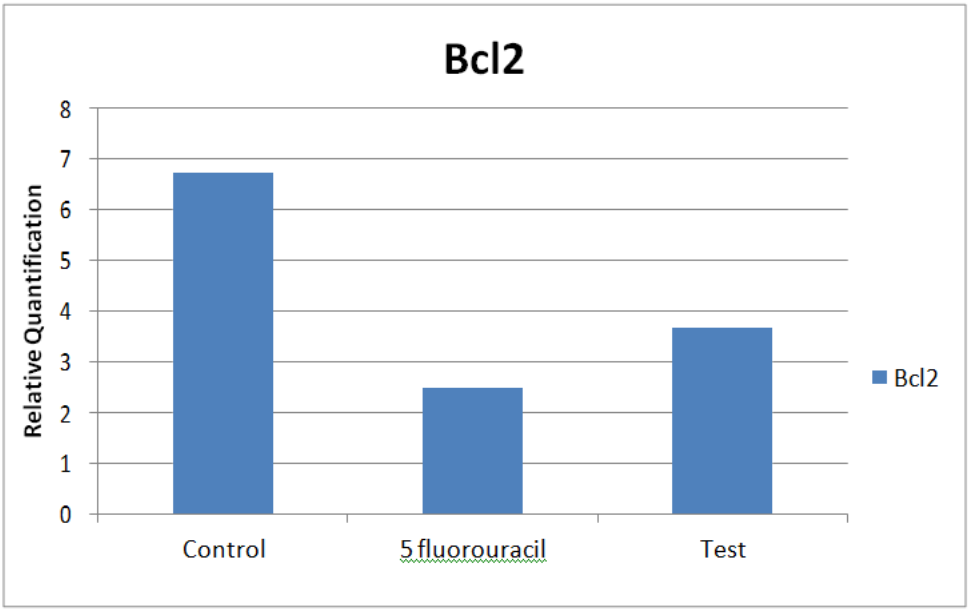


Fig 10: Graphical representation of Bcl-2 gene expression

DISCUSSION

Cancer is considered as a serious health problem worldwide. Cervical cancer is the third most common cancer in women. It is one of the leading cause of cancer mortalities in most of the countries in the world with approximately 2,88,000 deaths every year.

Natural phytochemicals derived from medicinal plants have attained a greater significance in potential management of several diseases including cancer. Several researches have been carried out in evaluation of plant extracts as prophylactic agents which offer greater potential to inhibit carcinogenic process.[53,54]

The mechanism of inhibition of tumour progression by natural phytochemicals range from inhibition of genotoxic effects, increased anti-inflammatory and anti-oxidant effect, inhibition of cell proliferation, protection of intracellular communications to modulate apoptosis and signal transduction pathways. [55]

The essential oil, obtained from the steam distillation of the crushed seeds of Larger Cardamom, contain cineole. Cineole gives the herb its aroma as well as its digestive and appetizer properties. Its antioxidant properties havealso been reported. It has been reported to have anti diabetic, anti fungal, anti oxidant, anti microbial, anti-inflammatory activity. There was no previous study to prove its anti cancer activity with special focus to apoptosis and gene expression study in spite of presence of flavonoid and triterpenoid (1,8 cineole) which is considered as an agent responsible for anti cancer activity. So the study was carried out to evaluate the anticancer effect of ethanolic extract of fruit of *Garcinia pedunculata* and the gene expression levels to determine its role in cancer pathology using *in vitro* methods.

Dried fruits of *Garcinia pedunculata* were powdered and extracted with solvents like acetone, ethanol and water. Phytochemical analysis of acetone, ethanol and aqueous extract of *Garcinia pedunculata* showed the presence of proteins, carbohydrates, flavanoids, terpenoids, steroids, phenols and tannins. Cytotoxic activity was carried out in cervical cancer cell line HeLa with ethanolic extract and was compared with standard drug 5 Fluorouracil. Test for cytotoxicity was carried out by MTT assay and IC50 value of ethanolic extract was found to be 62.5 µg/ml. IC50 value of 5 Fluorouracil was found to be 7.8 µg/ml. Linearity was expressed with the help of graph plotted in Microsoft excel.[55]

Apoptotic study was carried out by Microscopic analysis and DNA fragmentation. HeLa cells after incubation with IC50 concentration of ethanolic extract for 48 hours were subjected to microscopic studies. On light microscopic observation of ethanolic extract of *Garcinia pedunculata* treated HeLa cells, typical morphological features of apoptosis like destruction of monolayer, reduction of HeLa cell population, reduction of cell volume, loss of integrity of membrane which resulted in crooked and vesicle shape of the membrane and chromatin condensation were observed when compared to cells without any treatment.

Apoptotic effect of IC50 concentration of ethanolic extract of *Garcinia pedunculata* treated HeLa cells were further confirmed with the help of fluorescencemicroscopy using acridine orange and ethidium bromide. Acridine orange is a vital dye capable of staining both dead and live cells, where as ethidium bromide will stain only cells that have lost their membrane integrity. On examination of cells without any treatment under fluorescent microscope, the cells were stained green in colour representing viable or live cells, whereas examination of cells after treatment with ethanolic extract showed reddish or orange colour with loss of membrane integrity and leakage of cytoplasmic contents representing dead cells and the obtained results were similar to those reported by Shahrul Hisham Zainal Ariffin et al[50] in Hep G2 cells. This led to confirmation that ethanolic extract of *Garcinia pedunculata* showed apoptotic effectin cervical cancer cell line HeLa.

DNA fragmentation study was carried out by extracting

DNA from the cells after treatment with IC50 concentration of ethanolic extract of *Garcinia pedunculata* and standard 5 Fluorouracil for 48 hours and also from cells without any treatment. DNA agarose gel electrophoresis showed cleavage of chromosomal DNA into oligonucleosomal fragments in HeLa cells treated Ethanolic extract of *Garcinia pedunculata* and 5 fluorouracil, there was no fragmentation seen in control. This showsthat Ethanolic extract of *Garcinia pedunculata* has anticancer activity. The results obtained were similar to the results shown by Abhimanyu kumar Jha et al[51] using SiHa cell line. This fragmentation of DNA indicated the characteristics of apoptotic cells. Thus ethanolic extract of *Garcinia pedunculata* causes DNA damage in HeLa cells,thereby inducing apoptosis.

Cancer DNA markers like p53, Bcl2, plays a major role in cancer pathology and their expression levels determine the progression of the disease. The expression levels of p53 and Bcl2 were studied in cells treated with IC50 concentration of ethanolic extract of*Garcinia pedunculata* and 5 Fluorouracil by RT-PCR methodology using SYBR green.

The expression levels of p53 was found to be increased in cells treated with ethanolic extract of *Garcinia pedunculata* and in cells treated with 5 Fluorouracil when compared to cells without any treatment, indicating the ability of ethanolic extract to up regulate p53 and promote apoptosis. The results obtained was similar to those obtainedby Azizi et al.

Bcl-2 and its family of proteins play a significant role in regulation of Apoptosis. Bcl-2 plays a major role in cancer and its resistance thereby interfering with therapeutic action of chemotherapeutic drugs. High expression of anti-apoptotic members like Bcl-2 found in human cancers leads to neoplastic cell expansion by interfering with normal celldeath mechanism. Decrease in expression of Bcl-2 leads to apoptosis. In this study, the expression levels of Bcl-2 in ethanolic extract treated cells and 5 Fluorouracil treated cells was found to be decreased when compared to expression in cells without any treatment which implies that apoptosis in HeLa cancerous cells may be due to decreased expression of Bcl-2. The results obtained were similar to those reported by Gul Ozcan Arican et al[39] in Hela cells.

CONCLUSION

Phytochemical analysis of acetone, ethanol and aqueous extracts of *Garcinia pedunculata revealed* the presence of proteins, terpenoids, tannins, steroids, phenols and flavonoids. In this study, ethanolic extract of *Garcinia pedunculata showed* cytotoxic activity in cervical cancer cell line HeLa by MTT assay. The Apoptotic effect was confirmed by loss of membrane integrity, chromatin condensation, leakage of cytoplasmic contents and fragmentation of DNA by microscopic methods. Its apoptotic and anti cancer effect may be due to up regulation of genes like p53 anddown regulation of gene Bcl-2, which was confirmed by RT-PCR. The ethanolic extract of *Garcinia pedunculata possess* anti-cancer effect and for futureperspective, it can be further confirmed by isolating the compounds responsible for the activity and studying the exact mechanism by which the plant possess thisactivity and confirm the results using *in vivo* animal models.

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