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Evaluation of The Anticancer Activity of Extracts of Amomum Subulatum using Lung Cancer Cell Line (A549) by Invitro Methods

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ABSTRACT

The study was carried out to evaluate the anticancer effect of various extracts of leavesof *Amomum subulatum* in human lung cancerous cell line A549 using various *in vitro* models. Evaluation of Phytochemical analysis of ethyl acetate, ethanol and petroleum ether extractsof *Amomum subulatum revealed* the presence of proteins, alkaloids, tannins, steroids, phenolsand flavanoids. In this present study, Ethyl acetate extract of *Amomum subulatum* was found to possesspotent cytotoxic activity in human lung cancerous cell line A549 and was compared with standard drug 5 Fluorouracil. The Apoptotic effect was confirmed in treated cells by appearance of loss of membrane integrity, leakage of cytoplasmic contents and fragmentation of DNA in treated cells. Its anti cancer effect was further confirmed by reduced levels of proteins Phospho histidine phosphatase and C Reactive Protein in ethyl acetate extract of *Amomum subulatum* treated cells, whose elevated levels are said to possess a risk in cancer development. Its apoptotic and anti cancer effect may be due to up regulation of genes like p53 and TNF α and down regulation of genes like Bcl-2 and IL-6, which was confirmed by RT-PCR. These results show that ethyl acetate extract of *Amomum subulatum* possess anti cancer effect and for future perspective, it can be further confirmed by isolating the compounds responsible for the activity and studying the exact mechanism by which the plant possess this activity and confirm the results using *in vivo* animal models.

Keywords: Anticancer Activity, Amomum Subulatum, Lung Cancer Cell Line (A549), Invitro Methods

INTRODUCTION

The burden of cancer rose to 18.1 million new cases and 9.6 million deaths in 2018. With 36 different types, cancer mainly affects men in the form of colorectal, liver, lung, prostate, and stomach cancer and women in the form of breast, cervix, colorectal, lung, and thyroid cancer [1]. Treating cancer has become a whole new area of research. There are conventional as well as very modern techniques applied against cancers. A variety of techniques i.e., chemotherapy, radiation therapy, or surgery are used for treating cancer. However, all of them have some disadvantages [2]. The use of conventional chemicals bears side effects and toxicities [3]. But as the problem persists, new approaches are needed for the control of diseases, especially, because of the failure of conventional chemotherapeutic approaches. Therefore, there is a need for new strategies for the prevention and cure of cancer to control the death rate because of this disease.

Herbal medicine has become a very safe, non-toxic, and easily available source of cancer-treating compounds. Herbs are believed to neutralize the effects of diseases in a body because of various characteristics they possess[4]. For instance, among the many anticancer medicinal plants, Phaleria macrocarpa (local name: Mahkota dewa) and Fagonia indica (local name: Dhamasa) have been used

traditionally for the anticancer properties of their active ingredients[5,6]. Metabolites extracted from the plant material are used to induce apoptosis in cancer cells. Gallic acid as the active component was purified from the fruit extract of P. macrocarpa and has demonstrated a role in the induction of apoptosis in lung cancer, leukemia, and colon adenocarcinoma cell lines[7,8]. It is a polyhydroxy phenolic compound and a natural antioxidant that can be obtained from a variety of natural products i.e., grapes, strawberries, bananas, green tea, and vegetables[9]. It also plays a critical role in preventing malignancy transformation and the development of cancer [10]. Similarly, other compounds such as vinca alkaloids, podophyllotoxin, and camptothecin obtained from various plants are used for the treatment of cancer. Amomum subulatum Roxb (Zingiberaceae) is commonly known as greater cardamom. It is native to the Eastern Himalayan region particularly Bhutan, Nepal, and India. Also found in Srilanka (Anonymous, 1987). Major producers are Nepal (52%), India (37%) and Bhutan (11%). There was no report for the evaluation of its anticancer activity focusing on geneexpression levels and protein levels in cancerous cells along with apoptosis study. So the study was carried out to evaluate the anticancer effect of various extracts of leavesof *Amomum subulatum* in human lung cancerous cell line A549 using various *in vitro* models.

MATERIALS AND METHODS

Plant Collection

The plant *Amomum subulatum Linn* was collected from Telangana on July 2022 and was identified and authenticated.

Preparation of Extract

The Plant were shade dried at room temperature and was subjected to size reduction to a coarse powder by using dry grinder. 50grams of this coarse powder was packed into soxhlet apparatus and was subjected to extraction sequentially with 500ml of petroleum ether, ethyl acetate and ethanol. The extraction was continued until the colour of the solvent in the siphon tube became colourless. Extracts of ethyl acetate and ethanol were subjected toevaporation by using Rotary evaporator at 60°C.

The percentage of the yield from the plant *Amomum subulatum* using different solvents is givenin Table 1.

Table 1: The percentage of the yield from the plant *Amomum subulatum* using differentsolvents

EXTRACT	Plant material used for Extraction	Yield in (gm)	Percentage Yield(%)
Ethanol	50gm	8.6	17.2
Ethyl acetate	50gm	5.7	11.4
Petrolium ether	50gm	1.8	3.6
		TOTAL	**32.2**

Phytochemical studies

The freshly prepared extracts of ethyl acetate, ethanol and petroleum ether were subjected to phytochemical screening for the presence or absence of active phytochemical constituents by following methods

Test for alkaloids

Crude extract was treated with few drops of dilute hydrochloric acid and filtered. The filteratewas tested with various alkaloidal reagents such as

Mayer's reagent : Cream precipitate
Dragendroff's reagent : Orange brown precipitate
Wagner's reagent : Reddish brown precipitate

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

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 presenceof flavonoids.

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

Test for phenols and tannins

Crude extract was mixed with 2ml of 2% solution of ferric chloride. Appearance of violetcolour indicate the presence of phenolic compounds and tannins. Crude extract was 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 Proteins

Millions test:

Crude extract was mixed with 2ml of millions reagent. Appearance of white precipitatewhich turns red on gentle heating, indicates the presence of proteins.

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 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 Terpenoids

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

In vitro anti cancer activity

MTT Assay, Fluorescent microscopic studies, DNA fragmentation studies, cell cycle analysis, apoptosis, Gene expression studies including protein assay and RT-PCR were carried out.

Passaging of cell lines

MTT Assay

It is otherwise called as Tetrazolium Salt Assay/ Microculture tetrazolium test. MTT Assay is an *in vitro* method for anticancer drug screening, which has been internationally accepted.MTT is a yellow water soluble tetrazolium salt. MTT assay utilizes a colour reaction in measurement of viability of cells. Principle:Chemically MTT is 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide which contain tetrazolium ring. MTT salt colour is yellow. Living cells contain Mitochondrial enzyme succinate dehydrogenase that cleves the tetrazolium ring. This converts the MTT to an insoluble purple formazan. Amount of formazan formed is directly proportional to number of viable cells. DMSO has been used as a solubilisation solution, which dissolves serum as well as the purple formazan product. The absorbance at 570nm was measured with a UV-Spectrophotometer to measure the colour intensity.

RESULTS

Phytochemical Analysis

Phytochemical analysis was carried out for petroleum ether, ethyl acetate and ethanolextracts of *Amomum subulatum* and the results are shown in table.

Phytochemical analysis of the various extracts of *Amomum subulatum*

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

Cytotoxicity test

MTT assay

MTT assay was carried out with Petrolium ether, ethyl acetate and ethanol extract of *Amomum subulatum* and with 5-Fluorouracil and the results are shown.

Table 2: MTT Assay of Ethanolic Extract of *Amomum subulatum*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.09	16.66
2	500	1:01	0.15	27.77
3	250	1:02	0.19	35.18
4	125	1:04	0.23	42.59
5	62.5	1:08	0.27	50
6	31.2	1:16	0.32	59.25
7	15.6	1:32	0.39	72.22
8	7.8	1 64	0.45	83.33
9	Cell control	-	0.54	100

IC50 concentration and %cell viability

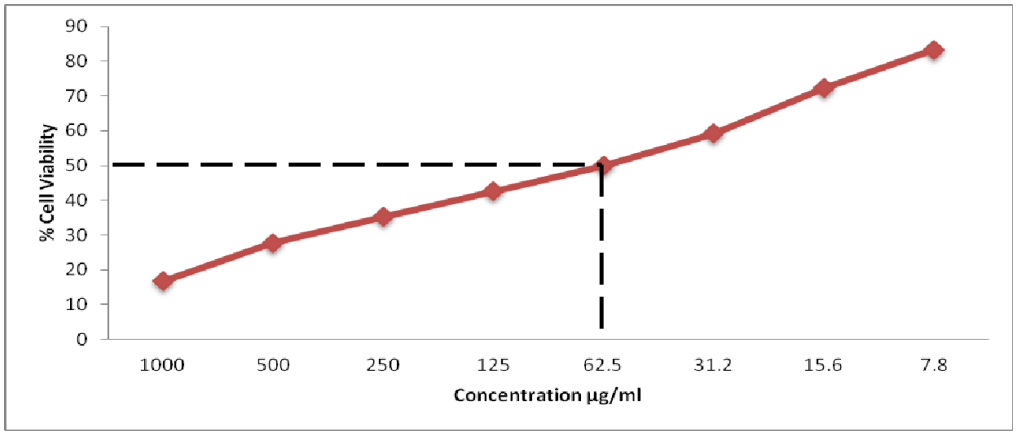


Fig 1: Graphical representation of % cell viability vs Concentration in µg/ml of ethanolic extract of *Amomum subulatum*

Table 3: MTT Assay of Petroleum ether Extract of *Amomum subulatum*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.15	27.77
2	500	1:01	0.19	35.18
3	250	1:02	0.26	48.14
4	125	1:04	0.29	53.70
5	62.5	1:08	0.32	59.25
6	31.2	1:16	0.36	66.66
7	15.6	1:32	0.39	72.22
8	7.8	1 64	0.45	83.33
9	Cell control	-	0.54	100

IC50 concentration and % cell viability

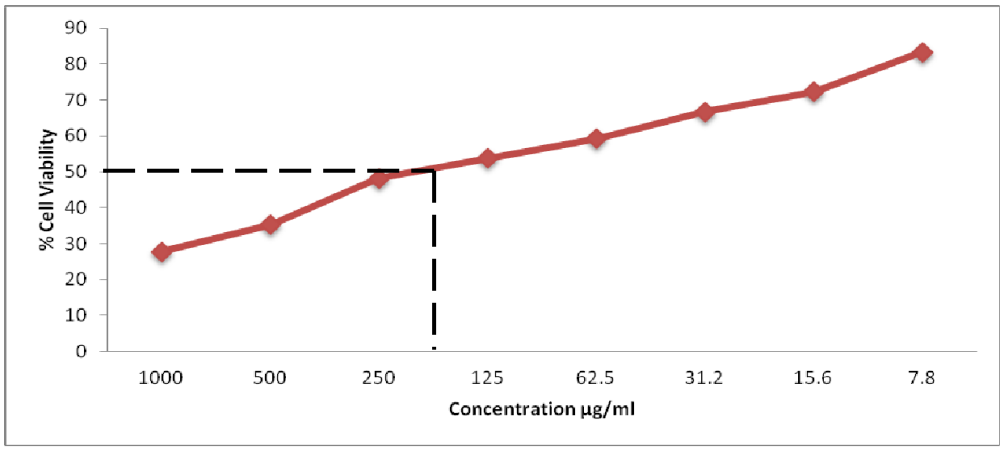


Fig 2: Graphical representation of % cell viability vs Concentration in µg/ml ofPetroleum ether extract of *Amomum subulatum*.

Table 4: MTT Assay of Ethyl acetate Extract of *Amomum subulatum*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.03	5.55
2	500	1:01	0.08	14.81
3	250	1:02	0.1	18.51
4	125	1:04	0.14	25.92
5	62.5	1:08	0.18	33.33
6	31.2	1:16	0.23	42.59
7	15.6	1:32	0.28	51.85

8	7.8	1 64	0.38	70.37
9	Cell control	-	0.54	100

IC50 concentration and % cell viability

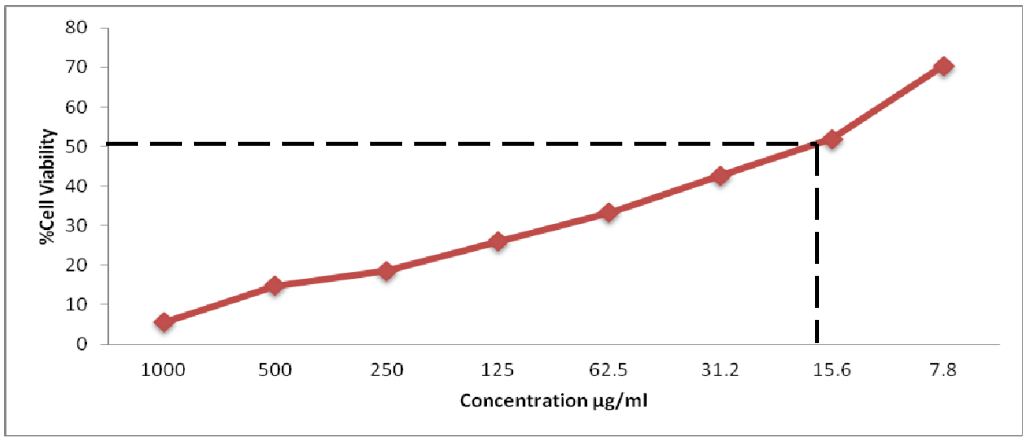


Fig 3: Graphical representation of % cell viability vs Concentration in µg/ml of Ethylacetate extract of *Amomum subulatum*.

Table 5:MTT Assay of 5-Fluorouracil

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.01	1.85
2	500	1:01	0.03	5.55
3	250	1:02	0.06	11.11
4	125	1:04	0.1	18.51
5	62.5	1:08	0.13	24.07
6	31.2	1:16	0.17	31.48
7	15.6	1:32	0.2	37.03
8	7.8	1 64	0.28	51.85
9	Cell control	-	0.54	100

IC50 concentration and % cell viability

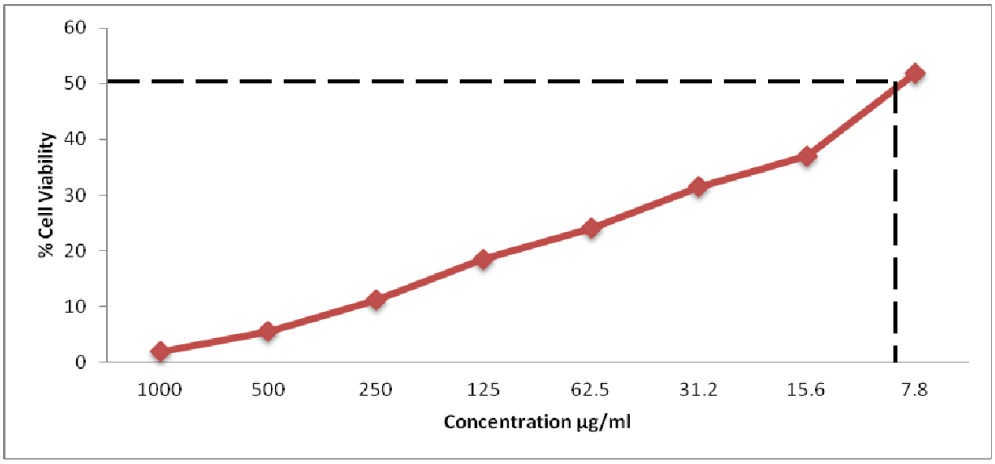
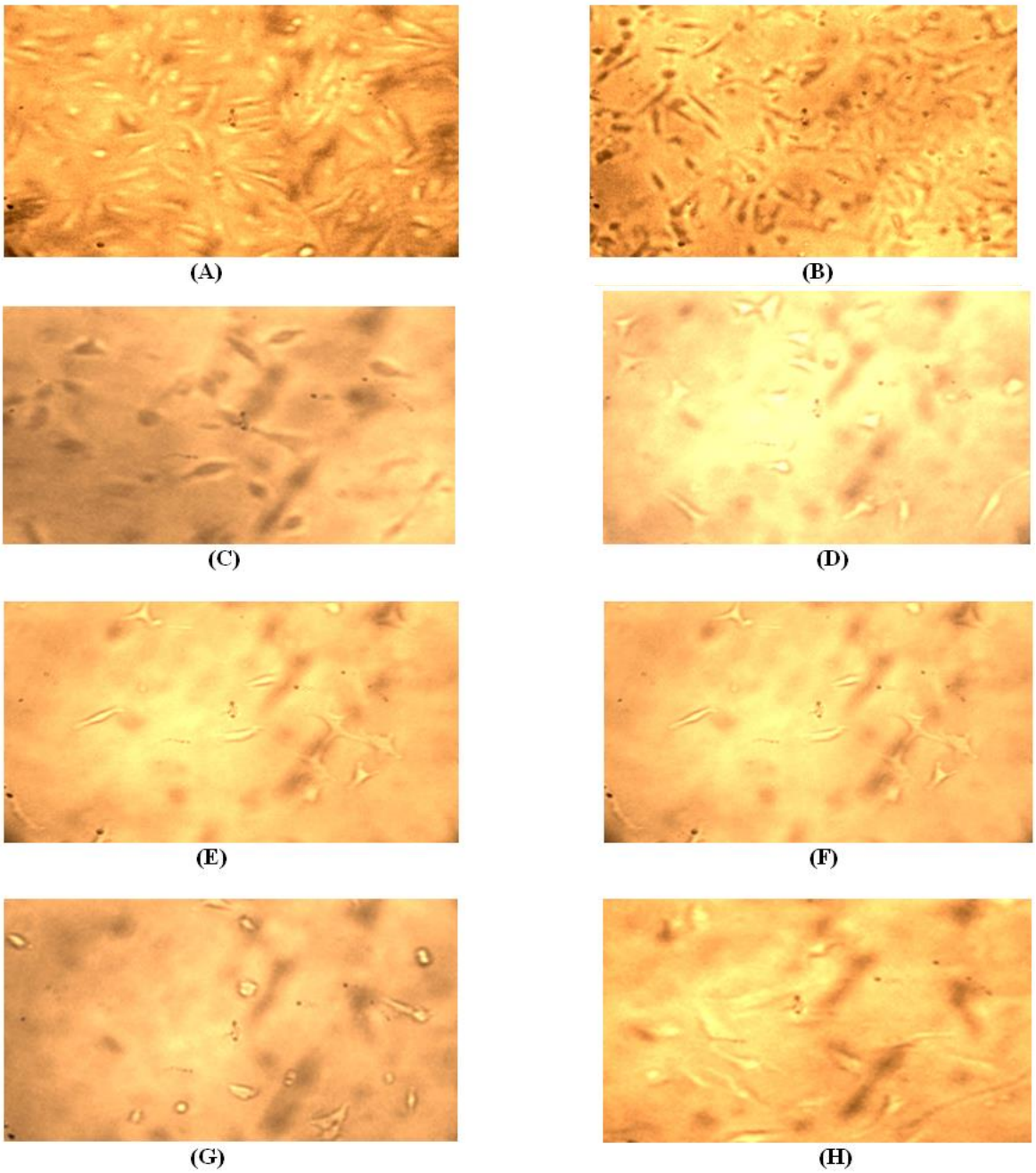


Fig 4: Graphical representation of % cell viability vs Concentration in *µ*g/ml of 5Fluorouracil



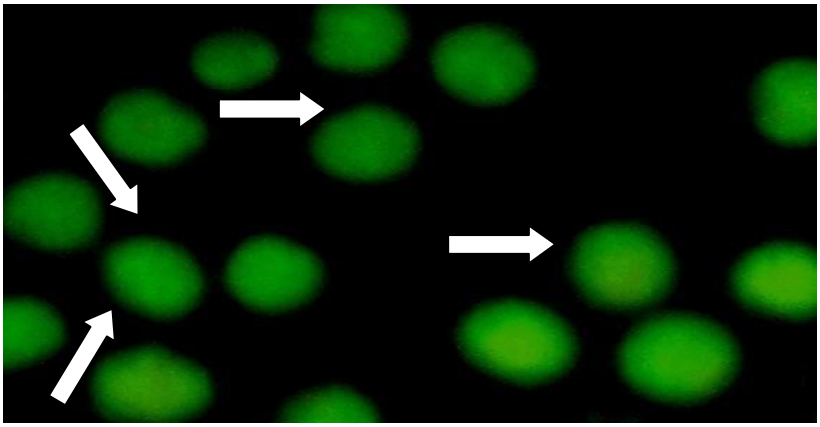
(A)Control, (B)7.8 µg/ml, (C)15.6 µg/ml, (D)31.2 µg/ml, (E)62.5 µg/ml , (F)125 µg/ml, (G) 250 µg/ml, (H)1000 µg/ml.

Fig 5: A549 cells treated with Ethyl acetate extracts.

Apoptosi

Flourescence microscopic observation

Control cells without any drug or extract treated were bright green in colour. Cells treated with ethyl acetate extract of *Amomum subulatum* were bright orange incolour withloss of membrane integrity and cytoplasmic contents leaking out of the cell. The observations of light microscopic studies and fluorescent microscopic studies aredepicted in figures.



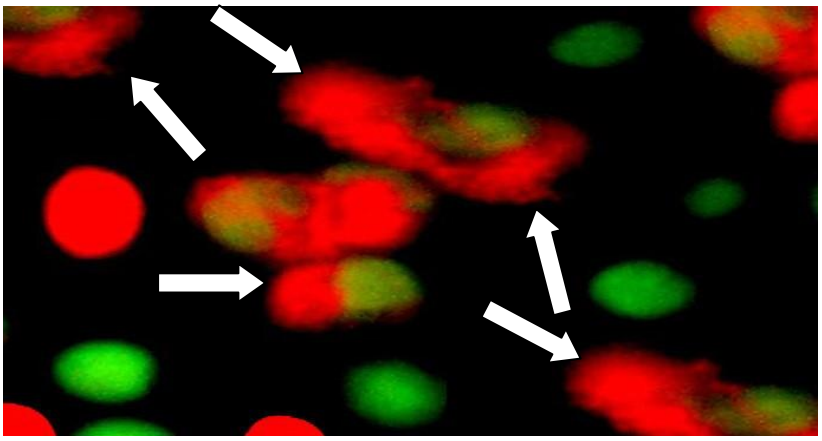


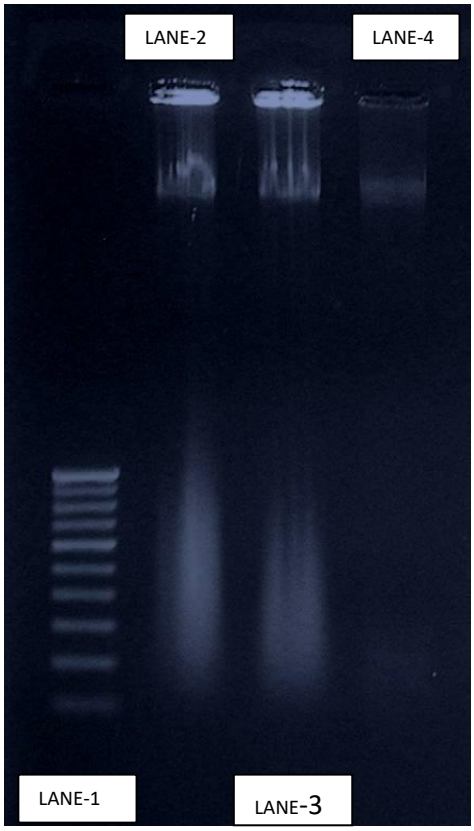
Fig 6: A549 cells – Control, indicting viable cells stained green in colour

Fig 7: A549 cells after treatment with Ethyl acetate extract of *Amomum subulatum*

Fig 7 showing dead cells stained orange in colour with loss of membrane integrity andcytoplasmic contents leaking out of the cell.

DNA fragmentation

In the control A549 cells, there was no fragmentation observed in agarose gel. Fragmentation was observed in A549 cell treated with IC50 concentration of standard 5-Fluorouacil and ethyl acetate extract of Amomum subulatum . This Fragmentation of DNA in ethyl acetate extract treated cells indicated the characteristics of apoptotic cells. Results obtained in fragmentation studies are shown in figure.



Lane 1: 100 base pair DNA marker; Lane 2: A549 cells treated with 5-Fluorouracil; Lane 3: A549 cells treated with ethyl acetate extract of *Amomum subulatum;* Lane 4: A549 cells without any treatment.

Fig 8: DNA Fragmentation in A549 cells.

Protein assay

Quantification of proteins, C reactive protein and Phospho histidine phosphatase present in A549 cells treated with IC50 concentration of ethyl acetate extract of *Amomum subulatum* and5-fluorouracil and also from cells without any treatment were analysed using Bio analyzer. The results obtained were given in Table and in Figures.

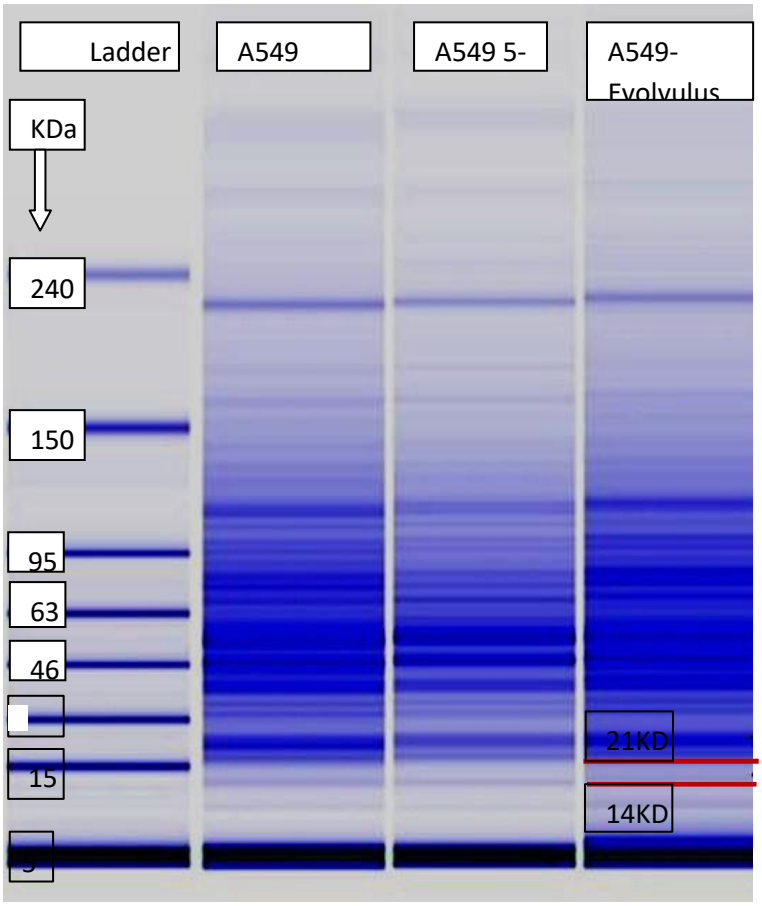


Fig 9: Gel Image of Protein analysis

Quantification of Protein:C-Reactive Protein

Table 6: Quantification of Protein- C-Reactive protein

Protein	Treatment	Size (KDa)	Concentration (pg/ml)
Reactive Protein	A549- Control	20.2	420.5
	A549-5-Flourouracil	21.4	330.7
	A549 Ethyl acetate extract of *Amomum subulatum*	20.8	354.6

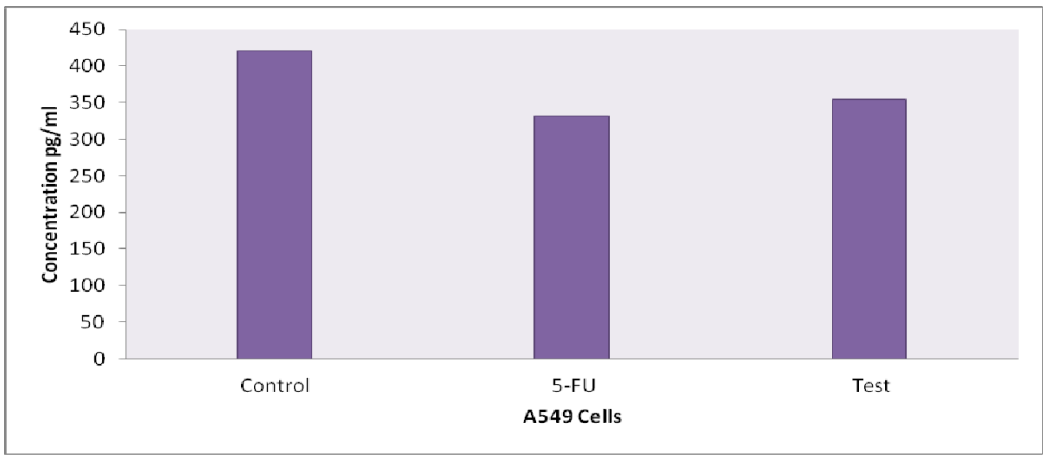


Fig 10: Graphical representation of concentration of C Reactive Protein in A549 cells before treatment and after treatment with ethyl acetate extract of *Amomum subulatum* and5 fluorouracil.

Phospho histidine phosphatase

Table 7: Quantification of Protein- Phospho histidine phosphatase

Protein	Treatment	Size (KDa)	Concentration (pg/ml)
Phospho histidine phosphatase	A549- Control	14.0	128.0
	A549-5-Flourouracil	13.9	65.2
	A549 Ethyl acetate extract of *Amomum subulatum*	13.8	86.4

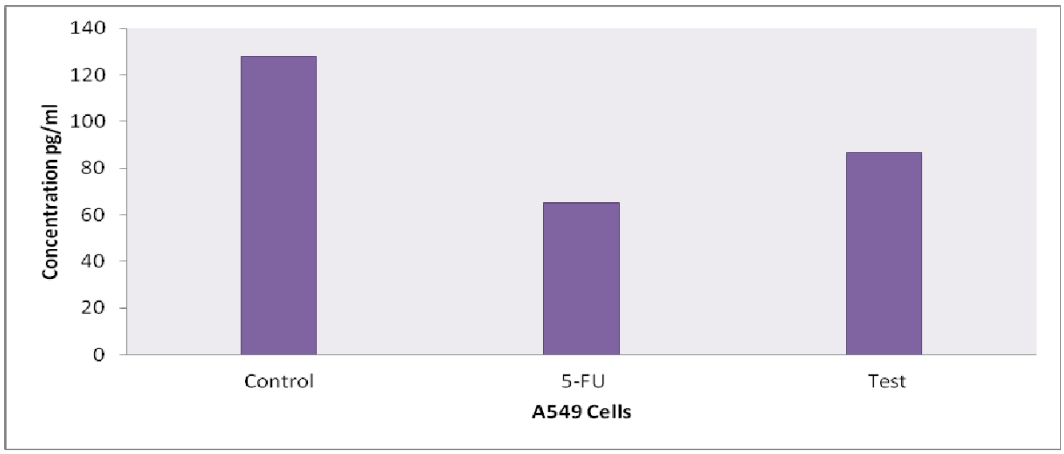
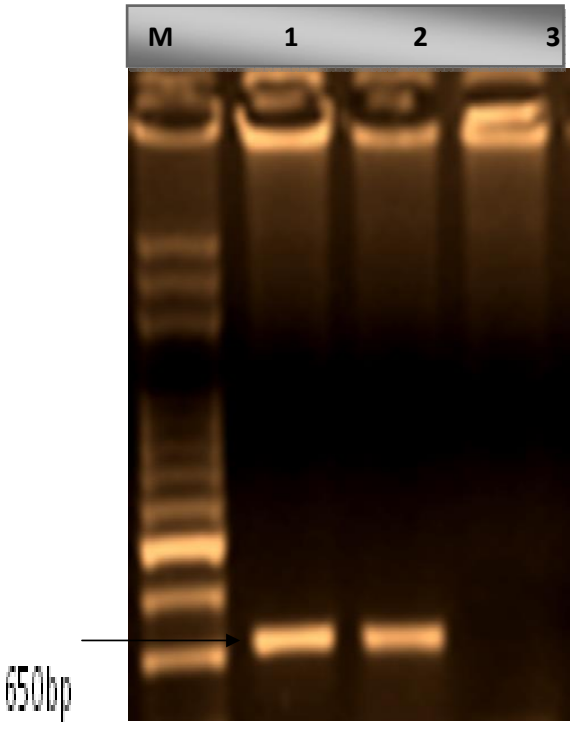


Fig 11: Graphical representation of concentration of Phospho histidine phosphatase inA549 cells before treatment and after treatment with ethyl acetate extract of *Amomum subulatum* and 5 fluorouracil.

Gene Expression Studies:P53 Gene in A549 Cell Line



M:Marker- 1 kb DNA Ladder ; 1: Standard- 5-Flourouracil; 2: Test- IC50 concentration of Ethyl acetate extract of Amomum subulatum; 3: Control- Without any treatment

Fig 12: p53 Expression

Expression levels of P-53

Table 8: Expression levels of P-53

P-53	Relative Quantitation	Standard Error
Standard- 5 Fluorouracil	5.23	0.1
Ethyl acetate Extract	4.62	0.12
Control	1.41	0.03

Graphical representation of p53 expression

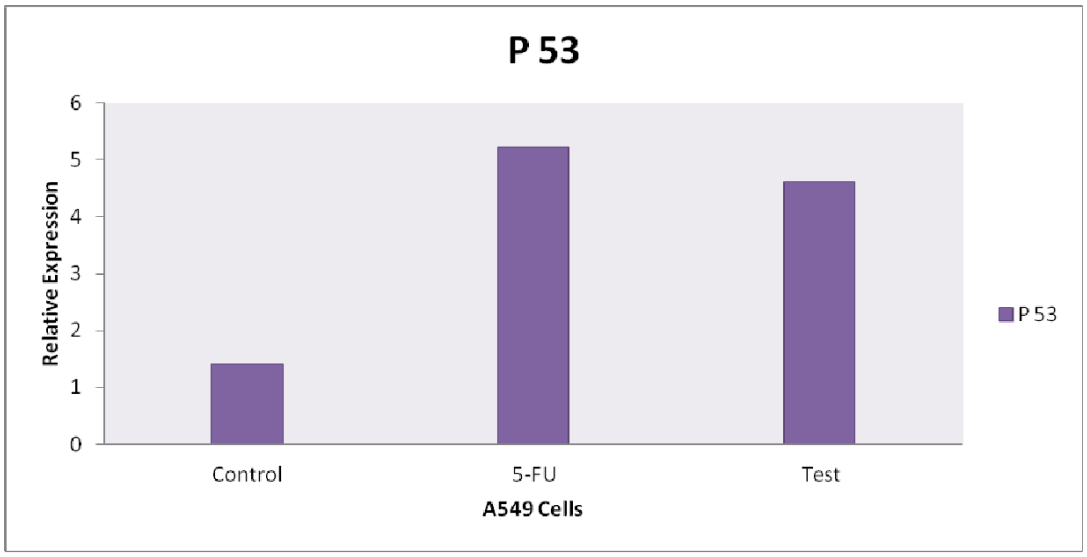
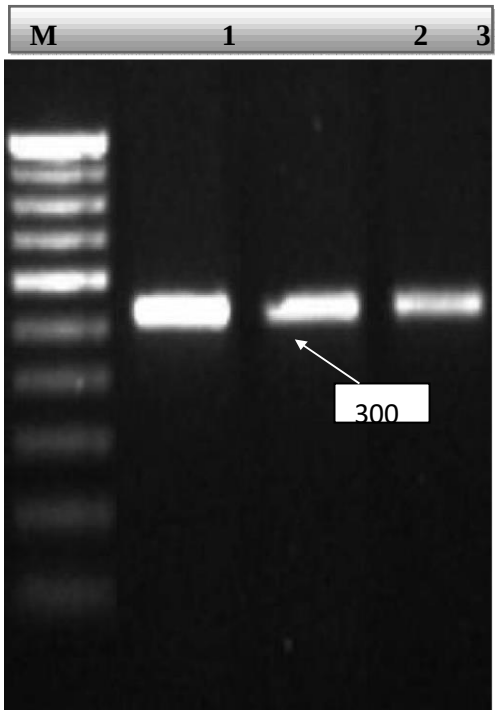


Fig 13: Graphical representation of p53 expression

Bcl2 Gene in A549 Cell Line



M: Marker DNA; 1: Control - Without any treatment2: Standard- 5-Fluorouracil; 3:Test- IC50 concentration of Ethyl acetate extract of Amomum subulatum

Fig 14: Bcl2 Expression

Expression levels of Bcl2

Table 9: Expression levels of Bcl2

Bcl2	Relative Quantitation	Standard Error
Control	6.17	0.12
5-Fluorouracil	3.62	0.106
Ethyl acetate Extract	3.81	0.09

Graphical representation of Bcl2 expression

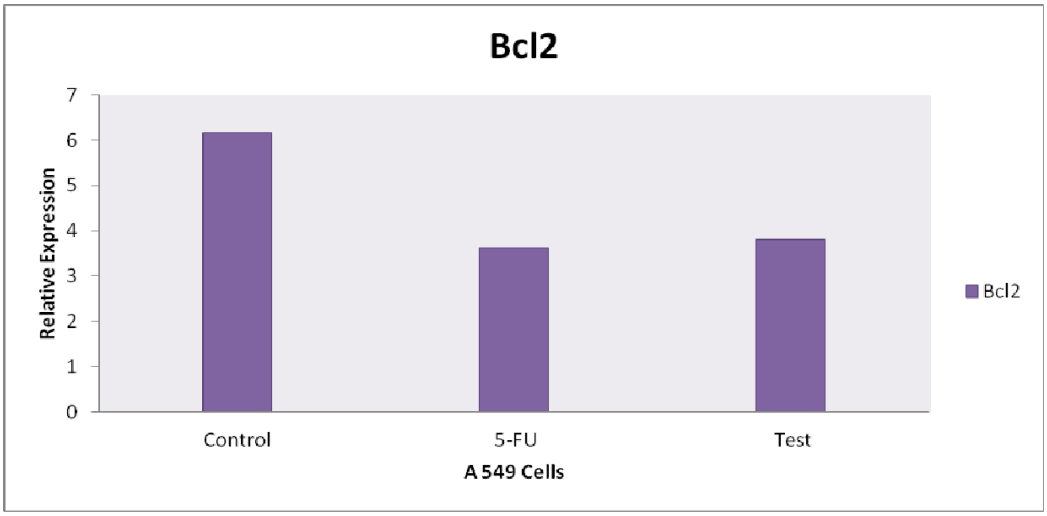
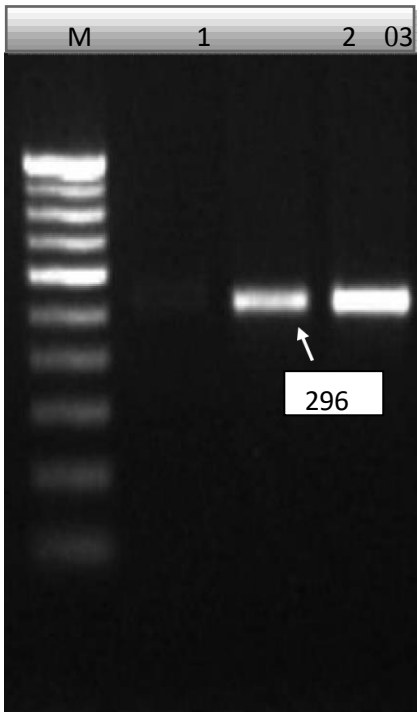


Fig 15: Graphical representation of Bcl2 expression.

TNF-α Gene in A549 Cell Line



M: Marker DNA; 1: Control - Without any treatment2: Standard- 5-Fluorouracil; 3:Test- IC50 concentration of Ethyl acetate extract of Amomum subulatum

Fig 16: TNF alpha Expression

Expression levels of TNF-α

Table 10: Expression levels of TNF-*α*

P-53	Relative Quantitation	Standard Error
Control	1.21	0.11
5-Fluorouracil	4.12	0.041
Ethyl acetate Extract	3.42	0.03

Graphical representation of TNF-α expression

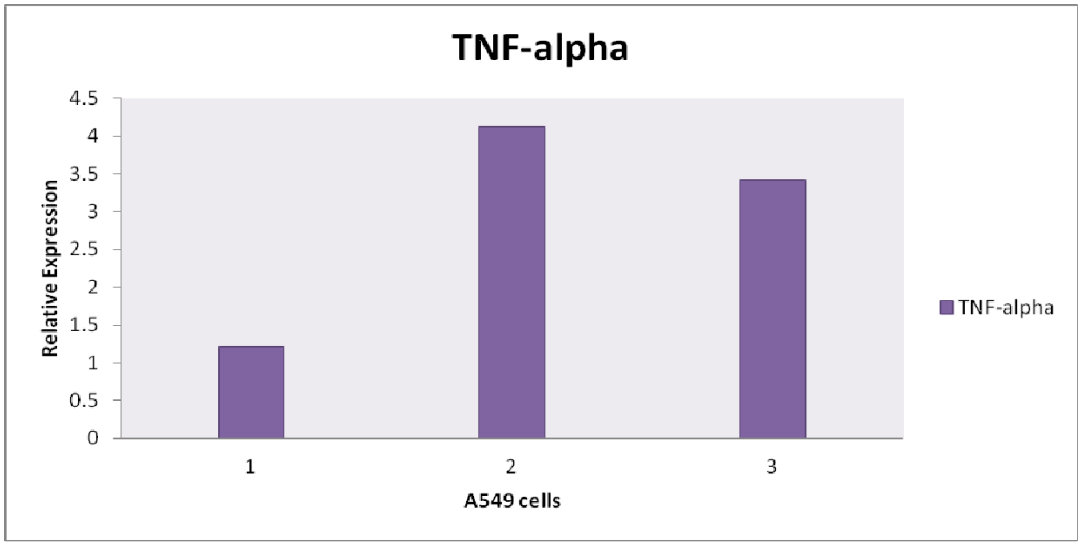
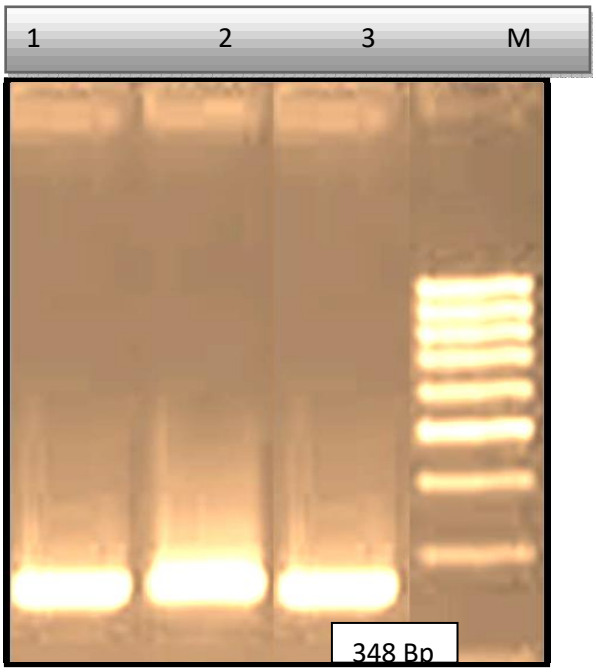


Fig 17: Graphical representation of TNF-*α* expression

IL-6 Gene in A549 Cell Line



M: Marker DNA; 1: Standard- 5-Fluorouracil; 2: Control - Without any treatment; 3:Test- IC50 concentration of Ethyl acetate extract of Amomum subulatum

Fig 18: IL-6 Expression

Expression levels of IL-6

Table 11: Expression levels of IL-6

P-53	Relative Quantitation	Standard Error
Control	8.62	0.01
5-Fluorouracil	6.42	0.06
Ethyl acetate Extract	6.12	0.03

Graphical representation of IL-6 expression

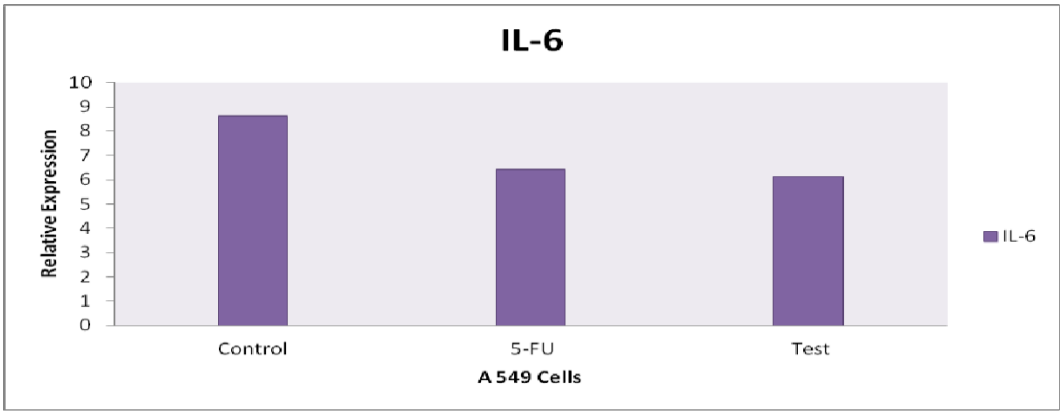


Fig 19: Graphical representation of IL-6 expression

DISCUSSION

Cancer is considered as a serious health problem worldwide. Lung cancer remains a major global health problem and leading cause of cancer mortalities in most of the countries in the world with approximately 1.3 million new cases and 300000 deaths each year estimated by World Health Organisation. Lung cancer has been classified into two groups namely small cell and non small cell lung cancer, with the latter being more prevalent accounting for nearly 80% oflung cancer cases.

With increase in mortality rates among patients suffering from cancer and with limited success being achieved in clinical therapies including radiation, chemotherapy, immune modulation and surgery in treating cancer patients, there arises a need for new way of cancer management.

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.

The mechanism of inhibition of tumour progression by natural phytochemicals rangefrom 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.

Discovery of effective herbs and elucidation of their underlying mechanisms could lead to development of an alternative and complimentary method for cancer prevention and treatment.

Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries around world. Currently more than 3000 plants around world have been reported to possess anti cancer properties.

Screening of different plant components in search of anti cancer drugs is one of the main research activities throughout the world. Vinca alkaloids and cytotoxic podophyllotoxins were discovered in the 1950's as first anticancer agents from plants. In addition to several plants extracted compounds such as camptothecin, vincristine, vinblastine, taxol and podophyllotoxin , many of the natural compounds were structurally modified to result in stronger anti cancer analogues with less adverse effects.

Amomum subulatum plant used traditionally to treat bowel problems and to promote conception. The entire plant was considered astringent and useful for treating haemorrhages, and there are a variety of other medical applications, including use as an adaptogenic, antiphlogistic, antipyretic, antiseptic, aphrodisiac, febrifuge, stomachic, tonic, and vermifuge, in the treatment of asthma, bronchitis, scrofula, syphilis, or in "controlling night emissions" and to promote wound healing.

Phytochemical analysis has revealed the presence of alkaloids, tannins, steroids and flavanoids. It has been reported with adaptogenic, anxiolytic, anti-amnesic, antioxidant, antiulcer, anticatatonic, anti Diarrhoeal, anti-inflammatory, antipyretic and immunomodulatory activity.There was no previous study to prove its anti cancer activity. So the study was carried out to evaluate the anticancer effect of extracts of leaves of *Amomum subulatum* and the gene expression levels playing a role in cancer pathology using *in vitro* models.

Dried whole plant of *Amomum subulatum* were extracted with solvents like petroleum ether, Ethyl acetate and Ethanol. Cytotoxic activity was carried out in lung cancerous cell line A549 with extracts of ethyl acetate, ethanol and was compared with standard drug 5 Fluorouracil. Test for cytotoxicity was carried out by MTT assay and among the three extracts evaluated, the effective

extract was found to be ethyl acetate extract with a IC50 value of 15.6µg/ml followed by ethanol and petroleum ether extract with IC50 value of 62.5 µg/ml and 250 µg/ml respectively. 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.

Ethyl acetate extract was found to be more effective of all three extracts by carrying out MTT assay and further studies were carried out with extract of ethyl acetate. Apoptotic studywas carried out by Microscopic analysis and DNA fragmentation.

Apoptotic effect of IC50 concentration of ethyl acetate extract of *Amomum subulatum* treated A549 cells were further confirmed with the help of fluorescence microscopy 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 ethyl acetate extract showed reddish or orange colour with loss of membrane integrity and leakage of cytoplasmic contents representing dead cells and and the obtained results were similar to those reported by Shahrul Hisham Zainal Ariffin et al in Hep G2 cells. This led to confirmation that ethyl acetate extract of *Amomum subulatum* showed apoptotic effect in lung cancerous cell line A549.

DNA fragmentation study was carried out by extracting DNA from the cells after treatment with IC50 concentration of ethyl acetate extract of *Amomum subulatum* and standard 5 Fluorouracil for 48 hours and also from cells without any treatment. Apoptosis is characterized by cleavage of chromosomal DNA into oligonucleosomal fragments. Cleavage of chromosomal DNA into oligonucleosomal fragments is a hallmark of apoptosis and apoptotic cells often produce nucleotide fragments at an interval of 180-200 base pairs, visualized by DNA agarose gel electrophoresis. On examination, DNA fragmentation appeared in A549 cells treated with ethyl acetate extract and 5 fluorouracil when compared to cells without any treatment, which did not show any fragmentation. The results obtained were similar to the results shown by Abhimanyu kumar Jha et al using SiHa cell line. This fragmentation of DNA indicated the characteristics of apoptotic cells. Thus ethyl acetate extract of *Amomum subulatum* causes DNA damage in A549 cells, thereby inducing apoptosis.

Protein levels in cancer cells before and after treatment with extract and 5 Flourouracil were studied using bio analyzer. C Reactive Protein is a 21KDa protein, a systemic biomarker of inflammation whose levels were found to be high in malignancies indicating a close linkage between inflammation and malignancy. It was reported that C reactive protein levels were high in patients suffering from lung cancer and there was a strong association of elevated levels of CRP with tobacco related lung cancer. CRP lowering agents may have promising role in prevention and therapy of malignancies in future. Another protein associated with lung cancer is 14KDa phosphohistidine phosphatase which was reported to play a role in lung cancer cell migration and invasion.

The levels of these two proteins were quantified in A549 cells after treatment with ethyl acetate extract of *Amomum subulatum* and standard for 48 hours. C reactive protein present in A549 cells without any treatment were found to be 420.5 pg/μl, whereas in cells treated with ethyl acetate extract and standard were found to be 354.6 pg/μl and 330.7 pg/μl respectively. Similarly Phospho histidine phosphatase present in A549 cells without any treatment were foundto be 128 pg/μl, whereas in cells treated with ethyl acetate extract and standard were found to be 86.4 pg/μl and 65.2 pg/μl. This marked reduction in protein levels which are said to be associatedwith lung cancer risk in cells after treatment shows that the ethyl acetate extract of *Amomum subulatum* posses the anti cancer effect.

Cancer DNA markers like p53, Bcl2, TNF-α and immune response marker IL-6 plays a major role in cancer pathology and their expression levels determine the progression of the disease. These gene expression levels were studied in cells treated with IC50 concentration of ethyl acetate extract of *Amomum subulatum* and 5-Fluorouracil by RT-PCR methodology using SYBR green. The expression levels of p53 was found to be increased in cells treated with ethyl acetate extract of *Amomum subulatum* and in cells treated with 5-Fluorouracil when compared to cells without any treatment, indicating the ability of ethyl acetate extract to up regulate p53 and promote apoptosis. The expression levels of TNF-α was found to be increased in cells treated with ethyl acetate extract and in cells treated with 5 Fluorouracil when compared to cells without any treatment, indicating the ability of ethyl acetate extract to induce apoptosis by increasing the expression of TNF-α. The results obtained agreed with those obtained by Azizi et al and Ali Alshehri.

High expression of anti-apoptotic members like Bcl-2 found in human cancers leads to neoplastic cell expansion by interfering with normal cell death mechanism. Decrease in expression of Bcl-2 leads to apoptosis. The expression levels of Bcl-2 in ethyl acetate 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 A549 lung 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 in Hela cells.

Immune response marker IL-6 plays a role in cancer. High serum IL-6 levels were detected in patients with lung, breast, prostate, colorectal, gastric, pancreatic, ovarian and renal cell cancers. High serum IL-6 levels were associated with progressive diseases and poor survival.Expression of IL-6 in control cells were found to be high when compared to cells treated with ethyl acetate extract and cells treated with standard 5 Fluorouracil indicating that apoptosis in cells of A549 may be due to decreased expression of IL-6. These gene expression levels indicates that ethyl acetate extract of *Amomum subulatum* exhibits apoptotic effect by over expression of p53, TNF-α and down regulation of Bcl-2 and immune response marker IL-6.

CONCLUSION

Evaluation of Phytochemical analysis of ethyl acetate, ethanol and petroleum ether extracts of *Amomum subulatum revealed* the presence of proteins, alkaloids,

tannins, steroids, phenolsand flavanoids. In this present study, Ethyl acetate extract of *Amomum subulatum* was found to possesspotent cytotoxic activity in human lung cancerous cell line A549 and was compared with standard drug 5 Fluorouracil. The Apoptotic effect was confirmed in treated cells by appearance of loss of membrane integrity, leakage of cytoplasmic contents and fragmentation of DNA in treated cells. Its anti cancer effect was further confirmed by reduced levels of proteins Phospho histidine phosphatase and C Reactive Protein in ethyl acetate extract of *Amomum subulatum* treated cells, whose elevated levels are said to possess a risk in cancer development. Its apoptotic and anti cancer effect may be due to up regulation of genes like p53 and TNF α and down regulation of genes like Bcl-2 and IL-6, which was confirmed by RT-PCR. These results show that ethyl acetate extract of *Amomum subulatum* possess anti cancer effect and for future perspective, it can be further confirmed by isolating the compounds responsible for the activity and studying the exact mechanism by which the plant possess this activity and confirm the results using *in vivo* animal models.

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