

Original Research Article

Effect of Carica seed extract on inhibitory kappa B kinase beta and mTOR mRNA expression in lung cancer cells(A549 cells).

Running title: Effect of Carica seed extract on lung cancer cells (A549 cells).

ABSTRACT-

Background: Lung cancer is considered as one of the leading causes of cancer related deaths globally. *Carica papaya* is one of the most well known traditional medicines to treat diseases, and is also known to treat cancer and help in cancer prevention. The aim of this experiment is to study the effect of Carica seed extract on inhibitory kappa B kinase beta and mTOR mRNA expression in lung cancer cells(A549 cells).

Methods: Cell viability test was done using MTT assay. mRNA expression of inhibitory kappa B kinase beta and mTOR mRNA was done by real-time PCR. The obtained data was analysed statistically by one way analysis of variance and Duncan multiple range test with graphpad prism version 5 software. $p < 0.05$ was considered significant.

Results: In this study it was observed that there was increased cell death at the end of 48 hours, showing maximum inhibition (50%) at 400-500 μ g/ml of Carica seed extract. It was also found that the fold change over control of mTOR mRNA expression was significant at 500 μ g/ml of *Carica* seed extract and the fold change over control of IKKB mRNA expression was significant ($p < 0.05$) at 400 μ g/ml in cancer cells treated with *Carica* seed extract.

Conclusion: Thus concluding that Carica seed extract has been found to have significant anticancer property on A549 lung cancer cell lines, and can be used as a natural product in combating lung cancer.

Keywords: lung cancer, Carica seed extract, inhibitory kappa B kinase beta, mTOR mRNA expression, innovative technique

INTRODUCTION-

Cancer is one among the foremost deadly diseases of this century. Cancer is a life aggressive metabolic disease having a high mortality rate and the incidence rate is also intensifying year by year. It is a life threatening deadly disease which is a major health concern worldwide. Carcinogenesis is a phenomenon that is induced by one or many agents. It proceeds in three steps such as initiation, promotion, and progression (1). It is usually characterized by uncontrolled cell growth and the spread of abnormal cells. Some of the important features of cancer are increase in proliferative growth signals, insensitive to growth-inhibitory signals, apoptosis evasion, angiogenesis induction, invasion leading to metastasis (2). These factors have led us to focus on pharmacological interventions in order to target the inflammatory pathways to inhibit growth of tumour and its progression (Lim JCW, 2012).

Lung cancer is considered as one of the leading causes of cancer related deaths globally. Lung cancer is presently the malignant tumor having the highest mortality rate worldwide, as it is usually not detected until there is substantial progression of the disease which leads to a huge reduction in quality of life of the patient (Luara de Sousa Monteiro et al 2014). Various factors are responsible for the cause of lung cancer such as cigarette smoking, exposure to air pollution, exposure to radiation and occupational exposure to agents like nickel, chromium, and arsenic etc. These Days, surgery, chemotherapy, radiation, hormones, and immunotherapy are some of the main approaches for the treatment of cancer often supplemented by alternative therapies such as herbal medicines. Lack of efficient cure, side effects and complications associated with available synthetic drugs, has enabled our research to focus on the search for new natural compounds having no or less side effects . Since natural compounds like plants have minimum toxicity, they can be considered under the focus of research to study its potential as anticancer agents. Plants are usually used for the treatment of various diseases.(3) Due to strong therapeutic effects, medicinal plants have been traditionally used to treat diseases (4)-(5). Herbal medicine is a well known source of new drugs that leads towards various health issues and the synthesis of new formulations(6) . Plants have been recognized for their anticancer properties for centuries (Lakshmi Priya.M et al). (7)

Carica papaya is one of a well known traditional medicine used to treat diseases. Different parts of the plant like the leaves, barks, roots, latex, fruit, flowers, and seeds have a wide range of medicinal applications.(8) It has been found that *C. papaya* is known for its anti protozoan, antibacterial, antifungal, antiviral, anti inflammatory, antihypertensive, hypoglycemic, wound healing, antitumor, free-radical scavenging, antisickling, neuroprotective, diuretic, abortifacient and antifertility activities. *Carica papaya* is also known to treat cancer and help in cancer prevention. The pharmacological properties of *Carica papaya* help in altering the growth of various cancer cell lines, thus having an anticancer effect (9).(10)

NF- kappaB is a mediator for lung cancer and is usually targeted for lung cancer prevention (11). Inhibitory kappa B kinase beta (IKK β / IkkB) helps in the activation of the NF-kB transcription factor family by the phosphorylation of IkkB inhibitors (12). The NF- kB transcription factors help in the balance between cell survival and regulation of cell proliferation and differentiation of many cell types. The changes in the activity of IkkB and NF- kB is said to be found in many diseases which include acute and chronic inflammation (13).The mTOR pathway has a very important role in cell metabolism, cell proliferation, cell cycle progression showing that it is one of the major survival pathways which is dysregulated in various cancer types. In most of the cancer cases this pathway is active leading to inhibition of PCD and promotes cell survival. Therefore inhibition of the mTOR pathway will be of great help in causing cell death associated with autophagy (Furlong Liu et al 2017). There is limited information on the medicinal value of *Carica* seed extract, particularly its cytotoxicity effect against cancer cell lines. Hence, the aim of the current study was done to evaluate the effect of *Carica* seed extract on inhibitory kappa B kinase beta and mTOR mRNA expression in lung cancer cells. This could help in better understanding of the underlying mechanisms of the anticancer potential of the *Carica* seed extract. Our team has extensive knowledge and research experience that has translated into high quality publications (14–16)(17–20),(21)(22),(23),(24)(25–29). In the present study, *Carica* seed extract was evaluated against lung cancer cell line (A549 cells) for its potential as an anticancer property.

MATERIALS AND METHODS

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Pvt Ltd, USA. Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, RPMI 1640 medium and phosphate buffered saline (PBS) were purchased from Gibco, Canada. (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolocarbo-cyanine iodide) and Real Time PCR kit was purchased TAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

Cell lines and cell culture

Human Lung cancer cell line (A549) was purchased from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM medium (Thermo Fisher Scientific, CA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, CA, USA) at 37°C with 5% CO₂.

Cell viability by MTT assay

Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert MTT, a tetrazolium compound into purple formazan crystals by mitochondrial reductases (Mosmann, 1983). Briefly, the cells (1×10^4 /well) were exposed to different concentrations of *carcica papaya* extract (100-500 µg/ml) with A549 cells for 48 h. At the end of the treatment, 100 µl of 0.5 mg/ml MTT solution was added to each well and incubated at 37 °C for an hour. Then, formed formazan crystals were dissolved in dimethyl sulfoxide (100 µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A_{570 nm} of treated cells/A_{570 nm} of control cells] × 100.

Gene expression analysis by Real Time-PCR

Samples from each group were submerged in 2 ml Trizol (Invitrogen, Carlsbad, CA, USA) for RNA extraction and stored at -80°C until further processed. cDNA synthesis was performed on 2 µg RNA in a 10 µl sample volume using Super Script II reverse transcriptase

(Invitrogen) as recommended by the manufacturer. Real-time PCR array analysis was performed in a total volume of 20 μ l including 1 μ l cDNA, 10 μ l qPCR Master Mix 2x (Takara, USA) and 9 μ l ddH₂O. Reactions were run on an CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using universal thermal cycling parameters (95°C for 5 min, 40 cycles of 15 sec at 95°C, 15 sec at 60°C and 20 sec at 72°C; followed by a melting curve: 5 sec at 95°C, 60 sec at 60°C and continued melting). For quality control purposes, melting curves were acquired for all samples. The specificity of the amplification product was determined by melting curve analysis for each primer pairs. The data were analyzed by comparative CT method and the fold change is calculated by $2^{-\Delta\Delta CT}$ method described by Schmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

Statistical analysis

The obtained data were analyzed statistically by one-way analysis of variance (ANOVA) and Duncan's multiple range test with a computer-based software (Graph Pad Prism version 5) to analyze the significance of individual variations among the control and experimental groups. The significance was considered at $p < 0.05$ level in Duncan's test.

RESULTS-

Effect of *C.papaya* on cell viability in A549 cells.

In the present study, *Carica papaya* extract significantly increased ($p < 0.05$) inhibiting the growth of the lung cancer cells dose-dependently compared to untreated control cells. However, 400 to 500 μ g/ml concentration of the extract showed maximum inhibition of the viability of the lung cancer cells suggesting that *C.papaya* has cytotoxic effect in A549 cells, as shown in (Fig. 1).

Effect of *C.papaya* on IkkB mRNA expression in A549 cells.

In untreated control cells, IkkB mRNA expression was found to be increased. Treatment with 400 and 500 $\mu\text{g}/\text{ml}$ concentration of *Carica papaya* extract reduced the expression of IkkB mRNA when compared to control cells ($p < 0,05$). As shown in (Fig. 2).

Effect of *C.papaya* on mTOR mRNA expression in A549 cells.

In untreated control cells, mTOR mRNA expression was found to be significantly increased. Treatment with 400 and 500 $\mu\text{g}/\text{ml}$ concentration of *Carica papaya* extract reduced the expression of mTOR mRNA when compared to control cells ($p < 0.05$). As shown in (Fig. 3).

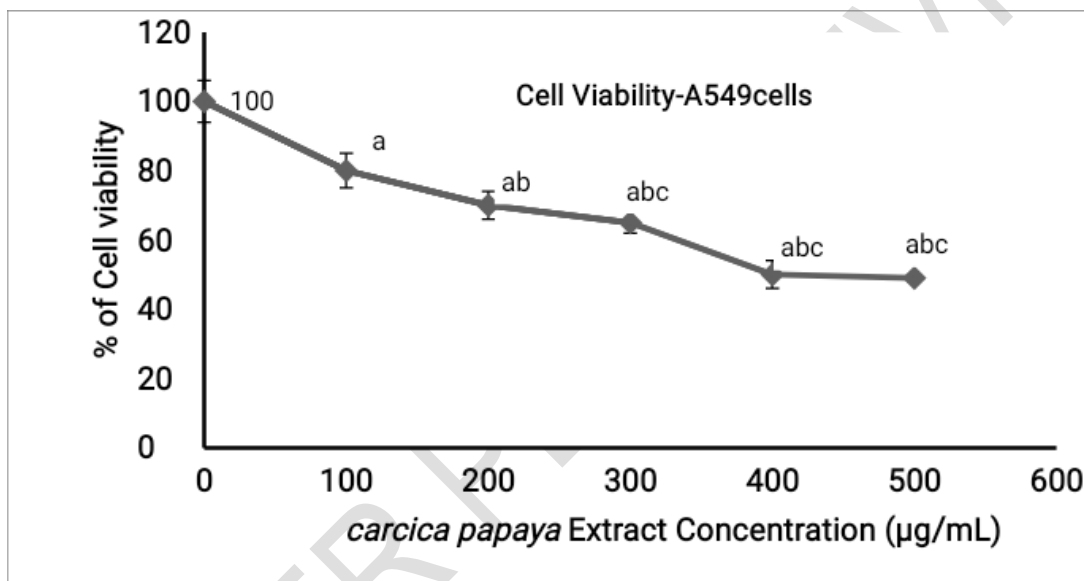


Fig. 1: Effect *carcica papaya* seed extract on cell viability in A549cells. Each bar represents mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 1nM treated A549 cells.

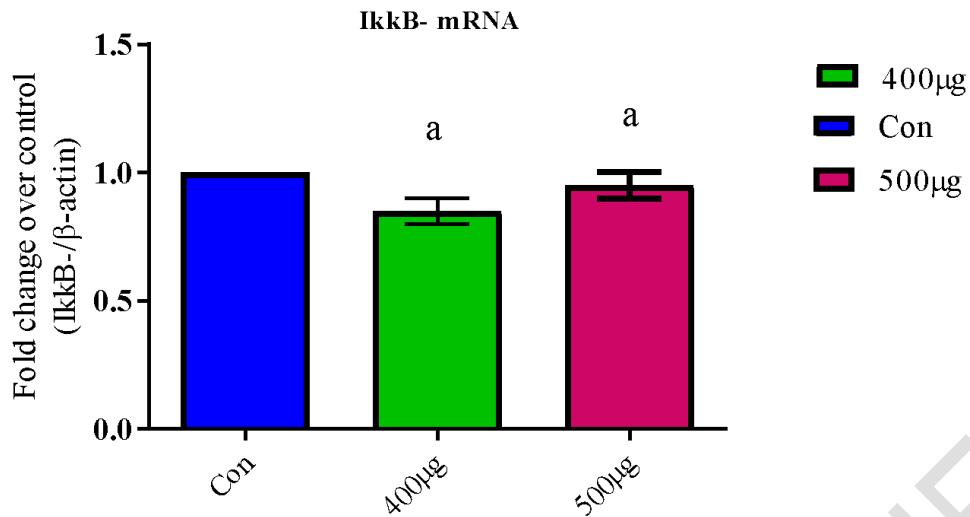


Fig. 2: Effect of *carcica papaya* seed extract on IkkB mRNA expression in A549 cells. Each bar represents the mean \pm SEM of 6 observations. X axis represents - different concentration of Carica papaya and the Y axis represents the fold change over control of IkkB. There is a statistically significant difference between the control and treated groups with p value < 0.05 . a-compared with untreated control cells.

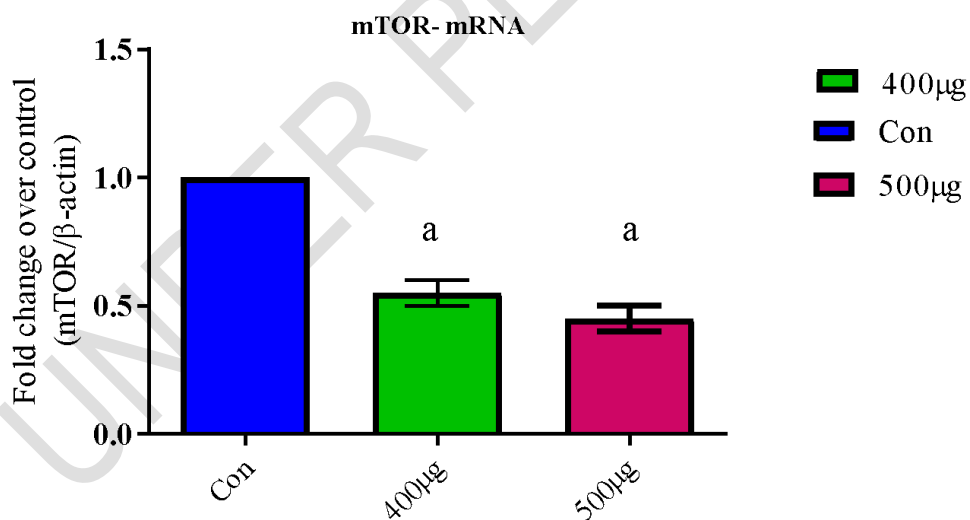


Fig. 3: Effect of *carcica papaya* seed extract on mTOR mRNA expression in A549 cells. Each bar represents the mean \pm SEM of 6 observations. X axis represents - different concentration of Carica papaya and the Y axis represents the fold change over control of

IkKB. There is a statistically significant difference between the control and treated groups with p value < 0.05. a-compared with untreated control cells.

DISCUSSION

Carica papaya seed extract is known for its anticancer effect and also contains various pharmacological properties which help in altering the growth of cancer cell lines. *Carica papaya* is known to have a wide range of phytochemicals including enzymes, carotenoids, alkaloids, phenolics, leaves, glucosinolates. It has been found that more than 5000 compounds from plants have been identified to be associated with anticancer properties. Three groups of bioactive compounds- phenolics, carotenoids, and glucosinolates are found to possess considerable interest in anticancer studies. Pure compounds of these three groups have been extensively studied in vivo and in vitro on many types of cell lines for their potential effects in cancer prevention and treatment. These compounds act via multiple mechanisms like cancer cell signaling, proliferation, apoptosis, migration, invasion, as well as angiogenesis and carcinogen elimination to exhibit in vitro and in vivo anticancer activities. (Thao T. T. Nguyen et al 2013)

In this study it was found that *Carica* seed extract which is used as a traditional medicine had a cytotoxic effect on human lung cancer cells(A549 cells). The cells were briefly exposed to *Carica* seed extract in different concentrations(100-500microgram/ml) for 48 hours. Maximum inhibition (50%) was found at 400 -500 microgram/ml concentrations of *Carica* seed extract. *Carica* seed extract increased cell death in a dose dependent manner at the end of 48 hours. Previous studies show that *C.papaya* water extract was shown to have no cytotoxicity to C6/36 cells within the range of concentrations tested, whereas methanol extract was more tolerable by the cells compared with ethanol extract(17). CC50 is the concentration of the tested sample able to cause the death of 50% of the cells and can be predictive to the degree of cytotoxic effect. A high IC50 value indicated that the extract was less toxic to the cells (30). Previous studies showed that pure lycopene and papaya juice inhibited the viability of liver cancer cell line HepG2 (IC50 = 22.8 -g/mL and 20 mg/mL, respectively) (31). This is similar to the present study showing that *Carica* seed extract has a cytotoxic effect on the human lung cancer cells (A549 cells).

In this study it was found that the fold change over control of mTOR mRNA expression was significant at 500 microgram/ml of *Carica* seed extract . Previous studies have found that

mTOR protein and mRNA expression levels were reduced upon treatment with apocynum leaf.(26) It was assumed that the AMPK/mTOR pathway may be the target by which apocynum leaf extract inhibits the progression of arterial atherosclerosis. Therefore, stating apocynum extract has the ability to inhibit AMPK/ mTOR signaling pathway activity (32). This is similar to the present study showing that *Carica* seed extract has inhibited the mTOR mRNA expression in lung cancer cells.

In this study it was found that the fold change over control of IKKB mRNA expression was significant at 400micro microgram/ml in cancer cells treated with *Carica* seed extract. Previous studies showed that the phosphorylation of IKK α/β , I κ B α , and NF- κ B p65 was significantly reduced upon treatment with *P. deltoides* Leaf Extract. This result suggests that PLE effectively suppresses the NF- κ B-associated inflammatory response (33). Another study on the effect papaya leaves on breast cancer cells for antiproliferative activity showed a decrease of NF- κ B. The presence of flavonoids in papaya extracts caused the reduction of RO3 which impacted the MCF-7 , BCL-2, BCL-XL that in turn inhibited the proliferation of breast cancer cell lines (MCF- 7) (34). This is in accordance with the present study stating that *Carica* seed extract has inhibited the expression of inhibitory kappa B kinase beta expression in lung cancer cells.

To our knowledge we performed this study to demonstrate the effect of Carica seed extract on lung cancer cells (A549 cells). Additional studies are required using other lung cancer cell lines of different origin to validate the specificity of Carica seed extract. In this study it was found that Carica seed extract has an anti cancer property on the A549 lung cancer cell line, thus indicating that Carica seed extract can be used as a natural product in combating lung cancer. Therefore these studies will be essential to pave the way for successful treatment of lung cancer.

CONCLUSION-

In this study it was observed that there was increased cell death at the end of 48 hours, showing maximum inhibition (50%) at 400-500 microgram/ml of Carica seed extract. Thus concluding that Carica seed extract has been found to have significant anticancer property on A549 lung cancer cell lines by the inhibition of the expression of inhibitory kappa B kinase

beta and mTOR mRNA expression , and can be used as a natural product in combating lung cancer.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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