**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 most common causes for cancer-related death 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 study investigates 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 assessed using MTT assay. mRNA expression of inhibitory kappa B kinase beta and mTOR mRNA was analyzed by real-time PCR. The results was analysed statistically by ANOVA and Duncan multiple range test with graphpad prism version 5 software. p<0.05 was considered significant.

**Results:** It was observed that there was maximum inhibition (50%) at 400-500µg/ml of Carica seed extract. It was also found that the fold change over control of mTOR mRNA expression was significant at 500µg/ml of *Carica* seed extract and the fold change over control of IKKB mRNA expression was significant (p<0.05) at 400µg/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). Uncontrolled cell proliferation and the spread of abnormal cells are its common characteristics. Increased proliferative growth signals, insensitivity to growth-inhibitory signals, apoptosis evasion, induction of angiogenesis activation, and invasion leading to metastasis are some of the key characteristics of cancer (2). These characteristics have prompted us to concentrate on pharmaceutical therapies aimed at inhibiting tumour development and progression by targeting inflammatory pathways to inhibit growth of tumour and its progression.

Lung cancer is considered as one of the causes of cancer related deaths globally. Lung cancer has the highest mortality rate worldwide. 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. Lack of efficient cure, side effects and complications associated with existing therapeutic approaches for cancer, 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 (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, antihypertensive, hypoglycemic, antibacterial, antifungal, wound healing, antitumor, free-radical scavenging, antiviral, anti-inflammatory, 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 (IKBKB/ IkkB) helps in the activation of the NF-kB transcription factor family by the phosphorylation of IKB 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. The availability of limited information on the medicinal value of Carica seed extract, particularly its cytotoxicity effect against cancer cell lines has lead 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 mechanisms underlying the anticancer potential of the Carica seed extract. Our team has extensive knowledge and research experience that has translated into high quality publications (14-29). 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-tetraethylbenzimidazolocarbocyanine 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**

The ability of live cells to convert MTT, a tetrazolium chemical, into purple formazan crystals via mitochondrial reductases was used to determine cell viability. The cells (1 ×10⁴/well) were treated to various concentrations of *Carica papaya* extract (100-500μg/ml) for 48 hours with A549 cells. After the treatment, each well was filled with 100 l of 0.5 mg/ml MTT solution and incubated for an hour at 37°C. The produced formazan crystals were then dissolved in 100µl of dimethyl sulfoxide and incubated for an hour in the dark. The intensity of the colour created was then measured at 570 nm using a Micro ELISA plate reader. The percentage of viable cells was used to represent the quantity of viable cells.

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 ×10⁴/well) were exposed to different concentrations of *Carica papaya* extract (100-500μg/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 percentage of control cells cultivated in serum-free medium was used to calculate the number of viable cells. Cell viability in the control media was indicated as 100%. The formula for calculating cell viability is: % cell viability = [A570 nm of treated cells/A570 nm of control cells] x 100.

**Gene expression analysis by Real Time-PCR**

Gene expression was analyzed using real time PCR using the standard protocol

**Statistical analysis**

The results were analyzed statistically by 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 ($p<0.05$) inhibited the growth of the lung cancer cells dose-dependently compared to untreated control cells. However, 400 to 500 $\mu$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 $\mu$g/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 $\mu$g/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 *carica papaya* seed extract on cell viability in A549 cells. Significance at p < 0.05, a-compared with untreated control cells, b-compared with 1nM treated A549 cells.

Fig. 2: Effect of *Carica papaya* seed extract on IkkB mRNA expression in A549 cells. 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 *Carica papaya* seed extract on mTOR mRNA expression in A549 cells. Each bar represents the mean ± SEM of 6 observations. 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. The 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.

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 (100-500 microgram/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 400 microgram/ml in cancer cells treated with Carica seed extract. Previous studies showed that the phosphorylation of IKKα/β, IκBα, and NF-κB p65 was significantly reduced upon treatment with P. deltoides Leaf Extract. This result suggests that PLE effectively suppresses the NF-κB-associated inflammatory response (33). Another study on the effect papaya leaves on breast cancer cells for antiproliferative activity showed a decrease of NF-κB. 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 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.

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REFERENCES


