

Effect of kabasura kudineer extract on inhibitory kappa B kinase beta and m TOR mRNA complex expression in lung cancer cells (A549 cell)

RUNNING TITLE :

To find the effect of kabasura kudineer effect on IKKB and mTOR mRNA complex in lung cancer cell lines.

ABSTRACT :

BACKGROUND : Lung cancer has the highest mortality rate and highest rate of metastasis. Kabasura kudineer was used widely during the Covid period. The aim of the study is to find the effect of kabasura kudineer extract on inhibitory kappa B kinase beta and mTOR mRNA complex expression in lung cancer cells.

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

RESULTS : Kabasura kudineer caused a marked increase in cell death in dose dependent manner. At the end of 48 hours, maximum inhibition was at 300 and 400 $\mu\text{g/ml}$. Kabasura kudineer has inhibited the mRNA expression of inhibitory kappa B Kinase and mTOR in lung cancer cell lines (A549 cell).

CONCLUSION : The study indicates that kabasura kudineer extract has anti cancer activity on the lung cancer cell line (A549 cell).

KEYWORDS :

Kabasura kudineer, lung cancer, inhibitory kappa B kinase, mTOR, Novel method

INTRODUCTION

Siddha medicines is a traditional system of medicine which is widely used by the South Indian population. It originated more than 10,000 years ago. Siddha medicine is used for various diseases like psoriasis, STD, diseases in liver and gastrointestinal tract, arthritis and urinary tract infections (1). It believes that food is the principle medicine. The efficiency of the medicines have also been examined scientifically in respiratory and other ailments (2)■

Kabasura Kudineer and Novel Herbal Preparation which are commonly used in treating viral infections and respiratory infections and will be effective against the pandemic novel coronavirus SARS-CoV-2 (3). Kabasura kudineer is a part of siddha medicine which is extracted from herbs. It is mostly used for respiratory diseases. It is reported to boost up the humoral immunity in the body. It has many phytoconstituents (4). Kabasura kudineer have many pharmacological activities such as anti asthmatic, anti oxidant, anti immuno modulatory activity. Recently kabasura kudineer is used in COVID - 19 because of its antioxidant property (4,5). Due to strong therapeutic effects, the medicinal plants have been traditionally used to treat diseases(6)(7)(8).

It is a compound formulation consisting of fifteen herbal ingredients. It's commonly used for the treatment of fever with or without respiratory tract infection. It has been widely used during the epidemic of swine influenza as a prophylactic and media reports gave a rebirth to the present official Siddha formulation. Siddha medicinal preparations are classified as 32 internal and 32 external medicinal forms and choornam is one of the interior medicinal forms. The drug is further classified as kudineer choornam which suggests a drug to be made into liquid form and consumed (9). Kabasura Kudineer is the name given to the Siddha formulation during which the entire plant or the actual part of the plant is grinded into powder. The obtained powder is named 'choornam'. it's then made into a kudineer by adding water and heated till water reduces to 1/4th or 1/8th of its volume. it's then filtered and filtrate is employed. Dose of the kudineer is usually 30 ml before food, three to fourfold each day. Lifetime of a prepared kudineer is 3 hours (10). The way of preparation of kudineer is a simple process and therefore the phytoconstituents doesn't undergo any major change while processing and preparation, unlike other traditional formulations.

Lung cancer carcinoma cases and deaths are rising (11). In 2018, GLOBOCAN estimated 2.09 million new cases (11.6% of total cancer cases) and 1.76 million deaths (18.4% of total cancer

deaths), above 2012 reported rates (1.8 million new cases and 1.6 million deaths), making it the foremost frequent cancer and explanation for cancer death in men and ladies combined and in women, the third common cancer type and therefore the second common explanation for cancer death (12).

Owing to the strong therapeutic effects, the medicinal plants are being traditionally used to treat several diseases (8) (13) (14). Different parts of medicinal plants have numerous nutraceutical values and are enriched with proteins, carbohydrates, vitamins, fibre, potassium, calcium and also the presence of phytoconstituents contributes to its significant medicinal property (15–17) (18) (15–17).

Between countries, significant variation in carcinoma incidence and demographic distribution are evaluated, and tobacco smoking rates and stage of economic development influence these patterns. Although cancer statistics in the developing countries are less reliable, carcinoma incidence is predicted to grow more in developing regions. Aim of the study is to evaluate the effect of Kabasura kudineer extract on inflammatory cytokines in the lung cancer cell line. Our team has extensive knowledge and research experience that has translated into high quality publications (19–21) (22–27), (28) (29), (30) (31) (32–36). The ingredients of kabasura kudineer may have anti cancer effects, hence the study was intended to investigate its effect on lung cancer cells.

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-tetraethylbenzimidazolcarbocyanine iodide) and Real Time PCR kit was purchased TAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

CELL LINE AND CELL STRUCTURE :

Human lung cancer cell line (A549) was purchased from the 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 × 10⁴/well) were exposed to different concentrations of Kabasura kudineer 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. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100 µl) and incubated at 37—°C. Then the intensity of color which was developed is 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 extract free medium. In control medium cell viability without any treatment was represented as 100%. The cell viability is calculated by 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 Superscript II reverse transcriptase (Invitrogen) as recommended by the manufacturer. Real-time PCR array analysis was performed in a total volume of 20 µl including 1 µl cDNA, 10 µl qPCR Master Mix 2x (Takara, USA) and 9 µl 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, for all samples melting curves were acquired. The specificity of the amplification product was determined by melting curve analysis for each primer pair. 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 KABASURA KUDINEER ON THE CELL VIABILITY :

Kabasura kudineer was evaluated against lung cancer cell line (A549) and cell viability was determined after administering the different doses of kabasura kudineer, It was found to exhibit inhibition of cancer cells by decreasing the percent viability of cancer cells in dose dependent manner. It was found that maximum inhibition of cell growth was at maximum concentration (300-400 μ g/ml) used in this study when compared to control. (Figure 1)

EFFECT OF KABASURA KUDINEER ON THE mRNA EXPRESSION OF INHIBITORY KAPPA B KINASE BETA:

The activity of IKKB alpha was assessed in a dose dependent manner. In the treated cancer lines, There was no significant reduction in mRNA expressions of inhibitory kappa B kinase beta at 400 and 500 μ g/ml. ($p>0.05$)(Figure 2).

EFFECT OF KABASURA KUDINEER ON THE mRNA EXPRESSIONS OF mTOR:

The mRNA expression of mTOR was assessed in a dose dependent manner. The cancer cells were significantly inhibited and it was found that there was significant reduction in mRNA expression of mTOR when compared to control at a dose of 400 and 500 μ g/ml ($p<0.05$). Thus interestingly, the decrease was in dose dependent manner (37) (Figure 3).

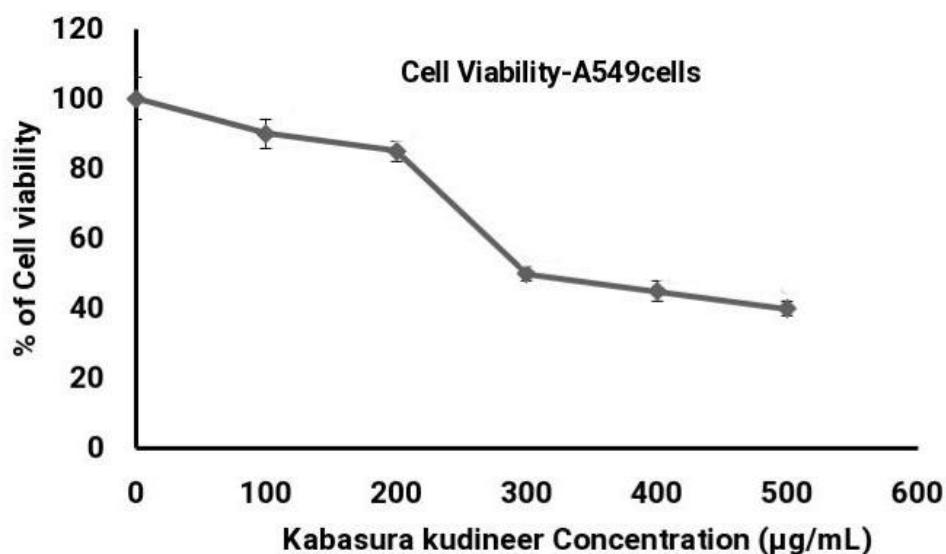


Figure 1 shows the Effect of kabasura kudineer extract on cell viability in A549 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$.

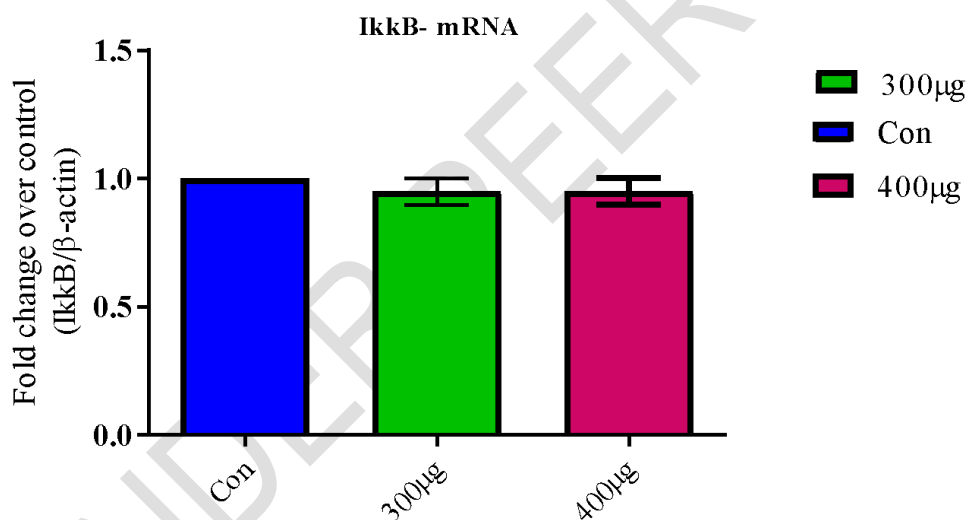


Figure 2: Effect of kabasura kudineer extract on IL-6 mRNA expression in A549 cells. Each bar represents a mean \pm SEM of 6 observations. The X-axis represents - different concentrations of Kabasura kudineer and the Y axis represents the fold change over control. Statistically there was no significant difference between the control and treated groups ($p > 0.05$)

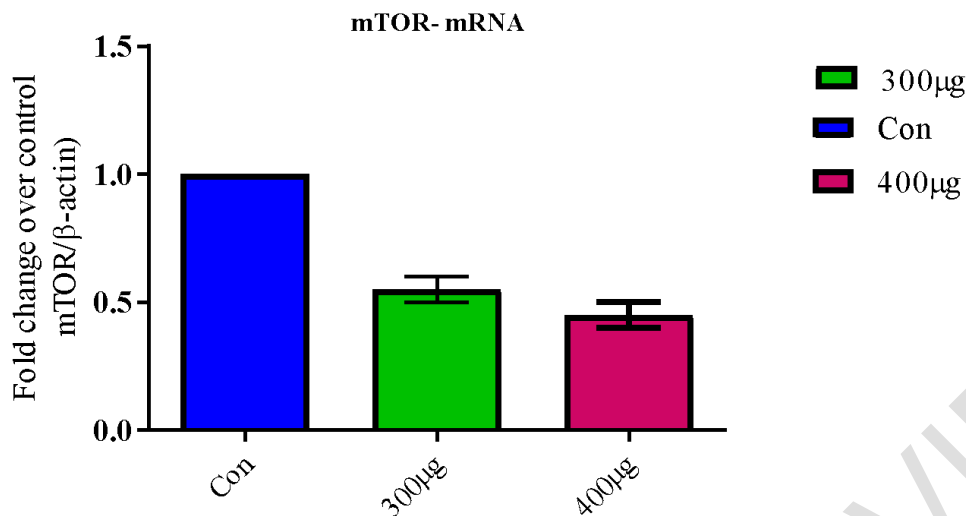


Figure 3: Effect of kabasura kudineer extract on TNF alpha mRNA expression in A549 cells. Each bar represents a mean \pm SEM of 6 observations. The X-axis represents - different concentrations of Kabasura kudineer and the Y axis represents the fold change over control. a- compared with untreated control cells. There is a statistically significant difference between the control and treated groups ($p < 0.05$).

DISCUSSION:

Kabasura kudineer can be used as a home remedy to boost up immunity and stop from viral infections. Kabasura kudineer is found to be more cost effective than allopathic medicine which may have side effects and used as a self medication to impress our body from resisting all kinds of viral and fungal infection (38). People find that kabasura kudineer has less side effects compared to allopathic medicine since it's found to have natural products. The necessity to extend research where the efficiency, safety and potential of Siddha medicines are to be tested using clinical trials. Such studies will be a benchmark for other developing nations to enhance their stance within the health care sector (38,39). The present study found that kabasura kudineer had a significant cytotoxic effect against lung cancer cell lines and is dose dependent at the concentration 300-400µg/ml. Previously the studies have been conducted on ginger, one of the components of kabasura kudineer and it was reported that it has an anticancer effect by inhibiting mTOR pathway and this is similar to our study (40).

NF- kappaB may be a mediator for carcinoma and is typically targeted for carcinoma prevention (Chen et al., 2011). Inhibitory kappa B kinase beta (IKBKB/ IkkB) helps within the activation of the NF-kB transcription factor family by the phosphorylation of Ikb inhibitors (Bai et al., 2011). The NF- kB transcription factors help within the balance between cell survival and regulation of cell proliferation and differentiation of the many cell types. The changes within the activity of IkkB and NF- kB is claimed to be found in many diseases which include acute and chronic inflammation (41), mTOR(the mechanistic target of rapamycin) is a serine/threonine protein kinase in the PI3K based kinase (42). The mTOR pathway integrates growth factor signals and nutritional status. The mTOR pathway is an intracellular signalling network which is often hyperactivated in many types of cancer. It is known to have prototypic functions in cellular proliferation, growth, differentiation and survival. Therefore, the mTOR signalling pathway is a promising tool against cancer (43). This study shows that mTOR shows inhibitory properties when cancer cell lines were treated with kabasura kudineer. This is an in vitro study but to have a conclusive result further studies can be conducted in animal models. Further studies can be carried out in the future in kabasura kudineer to exactly find out about the underlying mechanisms.

CONCLUSION :

This study showed that kabasura kudineer has cytotoxic effect against lung cancer cell lines through inhibition of Inhibitory kappa B kinase beta and mTOR mRNA expression. This effect could be attributed to the presence of phytochemicals present in the extract.

NOTE:

The study highlights the efficacy of " Siddha medicines" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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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