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INVESTIGATING THE LUNG CYTOTOXICITY OF KINETIN PROLONGED TREATMENT; *IN VITRO* AND *IN VIVO* STUDY

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Cytokinins are plant hormones that regulate various aspects of plant growth and differentiation. Recent studies have revealed that they may also have pharmacological effects on mammals. Among the cytokinin family, kinetin (N6-furfuryladenine) is commonly used because of its effectiveness and low cost. Previous in vitro studies show that kinetin mediates protection effects at low concentrations but promotes cytotoxicity and genotoxicity at higher concentrations. There is, however, a need for further investigation into the cytotoxicity of kinetin after prolonged treatment time in lung cells.

Consequently, the present study examined kinetin's cytotoxicity in vitro by treating WI38 (normal lung fibroblast cells) and A549 (lung cancer cell line) in different concentrations for 48 hours with kinetin. Its effect was also studied in adult albino rats treated with different doses of kinetin for 10 days. Our in vitro and in vivo results confirm that kinetin at concentrations as high as 100 nM or 1 mg/kg has no cytotoxic effect on lung cells and can safely be used systemically without harming the lung tissues

Keywords: *Kinetin; lung cells; cytotoxicity; in vitro; in vivo*

INTRODUCTION

It has been well-established for many years that natural products have played an essential role in treating human diseases. Natural products constitute a large percentage of the current pharmaceutical industry, especially in the field of anticancer drugs and antibiotics[1].

Cytokinins have been used as an essential plant growth factor that regulates various aspects of plant growth and differentiation[2,3]. There have been numerous studies on cytokinins in animals and mammalian cells, which have revealed different pathways and processes relating to cytokinins. This knowledge has prompted several studies into their potential utility in treating diseases caused by dysfunctional cell proliferation and/or differentiation[4.5]. As a natural plant hormone, kinetin (N6-furfuryladenine) is widely used in cosmetic preparations; it possesses a wide range of pharmacological and health-promoting properties, including neuroprotective, immune-modulatory, and antiproliferative effects[6-10]. Kinetin was isolated and identified in 1955 as a degradation product of DNA that induces division of the plant cells, and it was thought to be an artificial cytokinin that results from the autoclaving of herring-sperm DNA or forms upon DNA storage over a long period[11,12]. In addition, Kinetin is found in freshly extracted DNA from human cells in culture, plant cell extract, and human urine[13.14].

Kinetin has previously been shown to have a biphasic effect at higher and lower concentrations. At lower concentrations, kinetin protects against oxidative stress in the mammalian cellular system, which helps in pathophysiological conditions by potentiating the Adenine Phosphoribosyl Transferase enzyme mechanism of cellular DNA repair. But at higher concentrations, it promotes cytotoxicity and genotoxicity[15-17]. However,

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further research is required to determine whether kinetin is toxic to lung cells after prolonged treatment. In this study, we examined the cytotoxicity effect of kinetin in vitro using WI38 cells (normal lung fibroblast cells) and A549 cells (lung cancer cells) treated for 48 hours with different concentrations of kinetin (0.5, 1, 10, and 50 nM), as well as adult male albino rats treated for 10 days with different doses of kinetin (0.25, 0.5 and 1 mg).

MATERIAL AND METHODS

Chemicals

Kinetin (K3378) and MTT kit (CT02) were purchased from Sigma-Aldrich (Dorset, Germany). RPMI-1640 medium was purchased from Lonza (12-115F) (USA). Cell Lines WI38 and A549 were purchased from VACSERA, Egypt. Penicillin-Streptomycin was purchased from Thermo scientific (15140-122) (USA). Fetal Bovine Serum was purchased from (10270-106) Gibco, USA.

Both cell lines WI38 and A549 were grown in 5% CO2 in RPMI 1640 with L-glutamine medium at 37˚C, supplemented with 10% fetal bovine serum, and 1% (wt/vol) antibiotics (50-U/mL penicillin and 50-mg/mL streptomycin). The cells were routinely sub-cultured twice per week.

MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) Assay

Cell proliferation was evaluated in cell lines by the MTT assay in triplicates. Briefly, cells were plated in a 96-well microtiter plate at a density of 1 × 104 cells per well in a final volume of 100 µL of culture medium. These cells were treated for 48 hrs with different concentrations (0.5-100 nm) of kinetin at 37 °C with 5% CO2. After treatment, the cells were immediately incubated with 10 µL MTT (5.0 mg/mL) for 4 hrs at 37 °C. The cells were lysed in 100 µL of lysis buffer (isopropanol, conc. HCl, and Triton X-100) for 10 min at room temperature and 300 rpm/min shaking. The enzymatic reduction of MTT to formazan crystals dissolved in DMSO was quantified by photometry at 570 nm.

Cell viability was calculated according to the following equation

$$\% \text{ cell viability} = (Sample\ absorption \div Negative\ controle\ absorption)*100$$

Statistical analysis

Student T-test in Microsoft EXCEL was used. $P < 0.05$ is considered as significant.

Animals' model

Wistar Adult male rats weighing 140–160 g were obtained from the Animal Care Unit, Faculty of Agriculture, Minia University. The rats were kept in the standard animal house facility (12 h lighting cycle and 24 ± 2 °C) and were fed with standard food and tap water. The study conformed to the animal study procedures of the Animal Care Community, Minia University, Egypt (Permit Number: 54/2019). After 2 weeks of acclimatization period, rats were randomly divided into nine groups (n=6) as follows:

- **Group 1** (Control group): Rats received only the saline vehicle for 10 days
- **Group 2** (0.25 K): Rats received a single daily IP dose of 0.25 mg Kn/kg bw for 10 days
- **Group 3** (0.5 K): Rats received a single daily IP dose of 0.5 mg Kn/kg bw for 10 days
- **Group 4** (1.0 K): Rats received a single daily IP dose of 1.0 mg Kn/kg bw for 10 days

Histopathological analysis

Portions of the lung were fixed in 10 % buffered formalin and embedded in paraffin blocks. Paraffin blocks were sectioned at 4 µm thick with the use of microtome, deparaffinized, and stained with hematoxylin–eosin (H&E) for viewing histopathological lesion under a light microscope at a magnification of × 200[18]. The various tissue sections from the groups were evaluated and scored/ranked based on the extent of alterations as no changes (0), mild (1), moderate (2), and severe (3) according to their histopathological findings.

RESULTS AND DISCUSSION

Results

Nature is the biggest source of biologically active novel compounds. For many years, natural products have played an essential role in managing various disorders[19,20]. Cytokinins are a group of phytohormones influencing the entire plant construction plan, ranging from seed germination, cell division, flowering,

organogenesis, immunity, and communication until the senescence of the plant[10,21,22]. Cytokinins were assumed to be exclusive to the kingdom Plantae; however, their discovery in all life forms except Archaea has changed the former notion[23].

The small molecule adenosine N6-furfuryladenine (N6FFA: kinetin) is commonly used by the plant community as a low-priced proxy for natural cytokinins. Kinetin was first isolated from human urine decades ago[21]. Since its exact biogenesis in mammalian cells, its functional implications, and its toxicity effects had not been addressed until now; we focused our studies on the endogenous function of CKs and their mechanisms in mammalian systems[17,24,25].

In vitro and *in vivo* studies have evaluated kinetin's cytotoxicity and genotoxicity as significant aspects of addressing the safety of Kinetin in lung tissue[16,17,27].

As in vitro models, we tested cells with diverse potencies and functional capabilities such as HL60 (promyelocytic leukemia), HaCaT (human keratinocyte), NRK (rat epithelial kidney), MEF-WT (Mouse Embryonic Fibroblasts, Wild type) and human peripheral lymphocytes cells[17,26,27]; with double staining, MTT, DHE staining, comet, and micronucleus frequency assays. In all these cellular systems, kinetin concentrations of 500 nM and above induced a sort of cytotoxicity in the treated cells. However, the cytotoxicity of kinetin after prolonged treatment time in lung cells was not assessed in any previous studies. Therefore, the present study aimed to examine the cytotoxic effect of kinetin in 2 models; firstly, in vitro using WI38 and A549 treated with different concentrations of kinetin for 48 hrs., and secondly; in vivo using adult male albino rats treated with different doses of kinetin for 10 days.

First, to evaluate the effect of kinetin on both WI38 and A549 lung cell lines, an MTT vitality test was performed. Cells were treated with different concentrations of kinetin (0.5,1,10,50, and 100 nM) for 48hr, followed by quantification of viable and dead cells. Different concentrations of kinetin did not show a cytotoxicity effect in both cell lines **(Fig. 1&2)**; our results are in agreement with our previous findings with other mammalian cell lines[17,26], although, in the present study, we treated the cells for a longer time. In normal lung fibroblast cell line, 10nM kinetin showed a statistically significant difference than control group which may be optimum concentration for this cell line. Whereas, in cancer lung cell line, 0.5nM kinetin showed a statistically significant difference than control group which may be optimum concentration for this cell line. These results prove the safety of Kinetin when used in concentrations up to 100nM for 48 hours on either normal or cancer lung cells and that it has no anticancer effect. Two previous experiments presented the cytoprotective effect of Kinetin in different mammalian systems in a biphasic response of protection (Kinetin concentrations up to 100nM did not cause toxicity, whereas Kinetin concentrations of 500nM reduce cell viability[16,17].

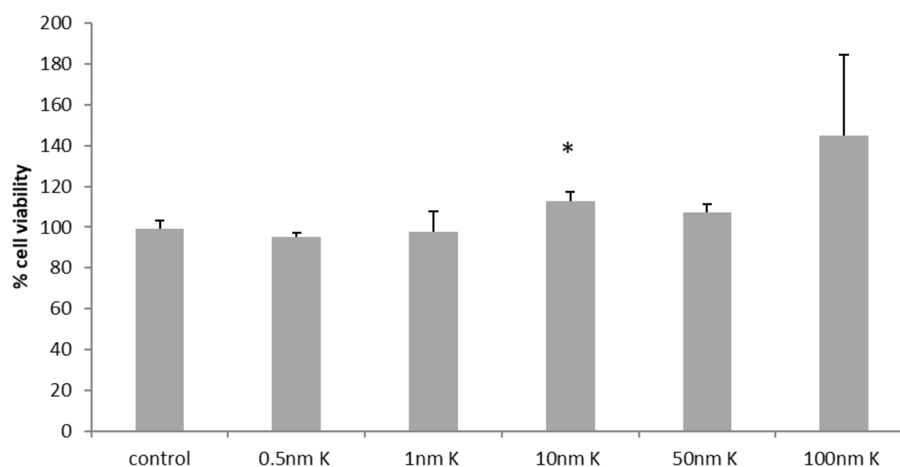

Fig. 1: % of cell viability in Wi38 cell line measured by MTT assay after 48 hrs treatment with different concentrations of kinetin; data are expressed as mean ± SD, *: $P < 0.05$ compared to control.

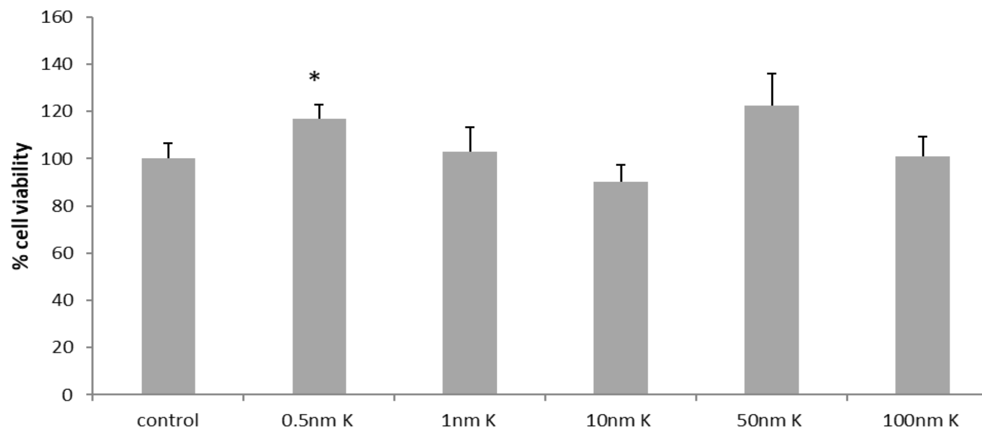

Fig. 2: % of cell viability in A549 cell line measured by MTT assay after 48 hrs treatment with different concentrations of kinetin; data are expressed as mean ± SD, *: $P < 0.05$ compared to control.

Second, to confirm the observed result in in vivo model, Wistar Adult male rats were injected via the IP route with different doses of kinetin for 10 days, and at the end of the experiment, lung tissues were collected and examined for any histopathological changes. Pulmonary section of control and Kinetin-treated rats in all groups revealed normal histological structure showed normal empty alveoli with bilayer lining epithelium without thickening or alveoli edema or interstitial tissue congestion, no inflammatory cellular infiltrate as shown in **fig. (3,4,5 and 6)**. No pathological changes were observed in the four groups and the control, and the Kinetin groups revealed normal empty alveoli with bilayer epithelium (arrow), and the four Kinetin groups were in (0) score, as shown in **Fig. 7**. The present results are in agreement with our previous investigation, which reported the safety of using kinetin on different other tissues, such as the liver, testes, and lymphocytes[24,25]. A previous study investigated the safety of systematic use of Kinetin in adult male albino rats proved a significant dose-dependent toxicity response of Kinetin on vital organs such as the kidney and liver, and the acute toxicity was only for a dose of 5mg/kg Kinetin[27]. Other previous in vivo studies demonstrated that Kinetin concentration (up to 5mg/kg) maintains spleen cells' vitality and biological function[28].

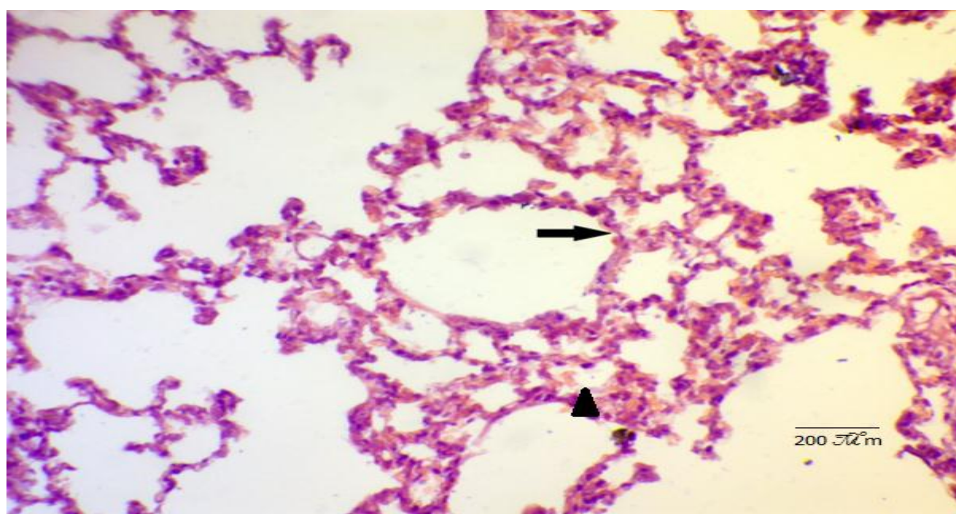

Fig. 3: Negative control (200 x): Lung sections showed normal empty alveoli with bilayer lining epithelium without thickening or intraalveolar edema (black arrow). Interstitial tissue showed mild congestion, and no inflammatory cellular infiltration (arrow head).

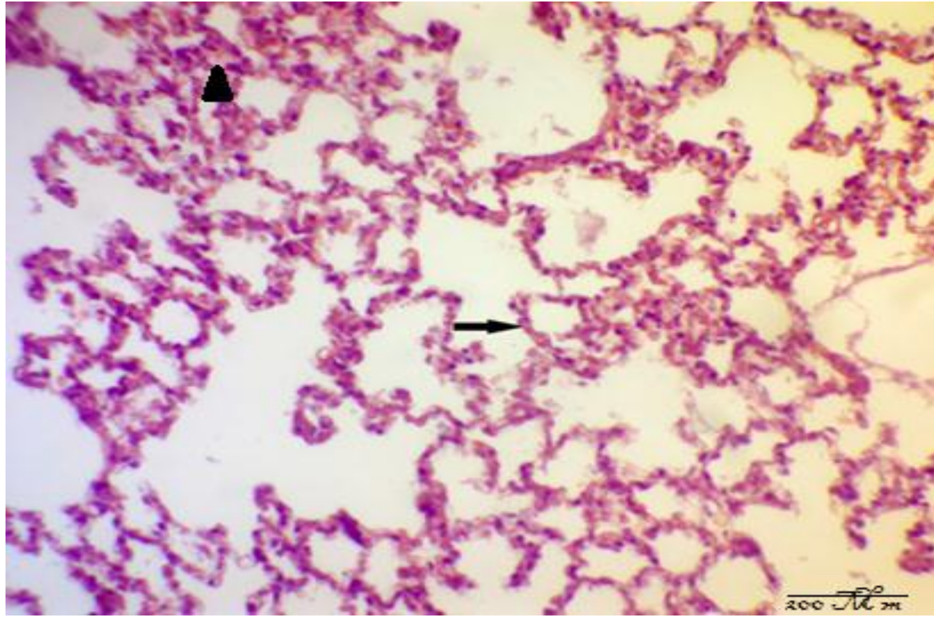

Fig.4 : Kinetin 0.25 mg/kg (200x): lung sections showed normal empty alveoli with bilayer lining epithelium without thickening and intra-alveolar slough (arrow) . Interstitial tissue mild congestion with scattered RBCs without inflammatory cellular infiltrates (arrow head).

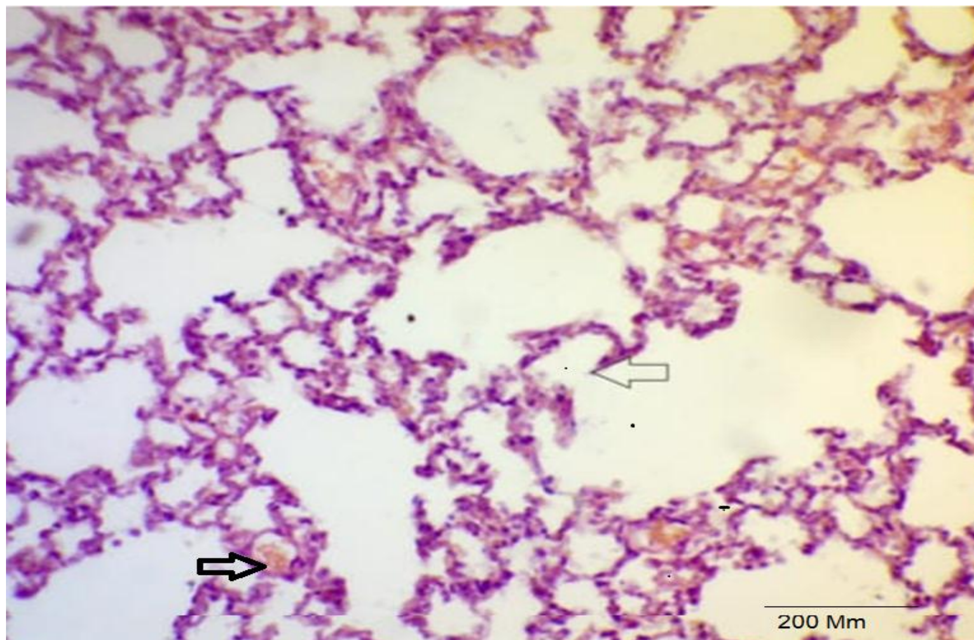

Fig. 5: Kinetin 0.5 mg/kg (200x): Lung sections showed normal empty alveoli with bilayer lining epithelium without thickening (white arrow). Interstitial tissue mild intravascular congestion and no inflammatory cellular infiltrate (black arrow).

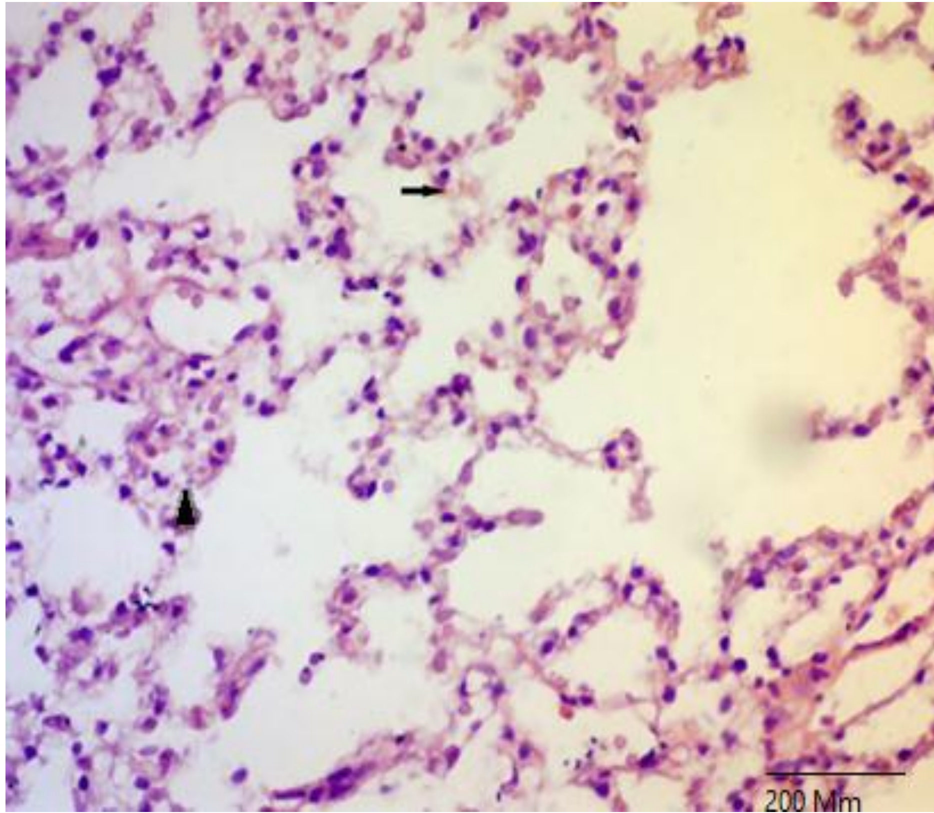

Fig. 6: Kinetin 1.0 mg/kg (200x): Lung sections showed normal empty alveoli with bilayer lining epithelium without thickening or alveoli edema (arrow). Interstitial tissue without congestion and no inflammatory cellular infiltrate (arrow head).

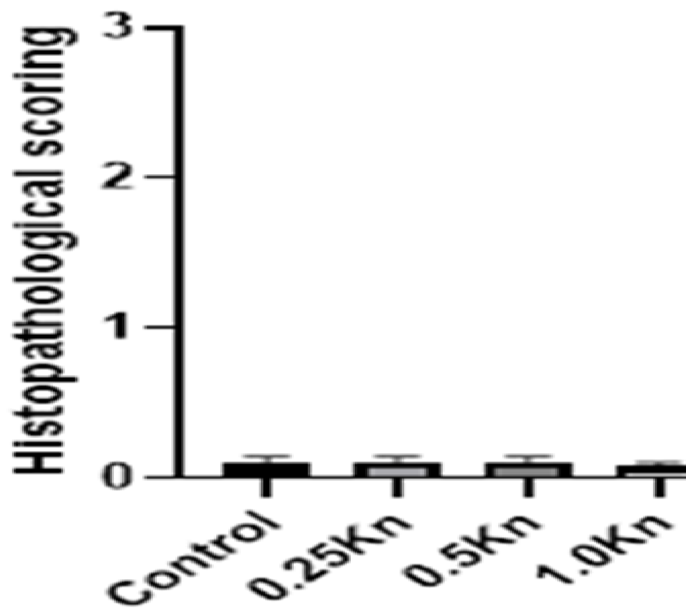

Fig. 7: Values of scoring are expressed as mean ± SEM.

Conclusion

Based on our in vitro and in vivo investigation, we suggest that kinetin at concentrations up to 100 nM or 1mg/kg does not show any cytotoxic effect on lung cells and that it can be safely used systemically without having any adverse effects on lung tissue.

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تقييم سمية الكينيتين في خلايا الرئة بعد العلاج المطول في المختبر وفي الجرذان

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السيتوكينينات هي هرمونات نباتية تنظم مختلف جوانب نمو النبات وتمايزه. كشفت الدراسات الحديثة أنه قد يكون لها أيضًا تأثيرات دوائية على الثدييات. من بين عائلة السيتوكينين ، يشيع استخدام الكينيتين بسبب فعاليته وانخفاض تكلفته. وفقًا للدراسات السابقة في المختبر ، فإن الكينيتين يتوسط تأثيرات الحماية بتركيزات منخفضة ولكنه يعزز السمية الخلوية والسمية الجينية بتركيزات أعلى. ومع ذلك ، هناك حاجة لمزيد من التحقيق في السمية الخلوية للكينيتين بعد وقت طويل من العلاج في خلايا الرئة ، ونتيجة لذلك ، فحصت الدراسة الحالية السمية الخلوية للكينيتين في المختبر عن طريق علاج الخلايا الليفية الرئوية الطبيعية و خلايا سرطان الرئة بتركيزات مختلفة لمدة ٤٨ ساعة بالكينتين.

وكذلك تمت دراسة التأثير أيضًا في الجرذان البالغة التي عولجت بجرعات مختلفة من الكينتين لمدة ١٠ أيام. تؤكد نتائجنا في المختبر وفي الجرذان أن الكينيتين بتركيزات تصل إلى ١٠٠ نانومولار أو ١ مجم / كجم ليس له تأثير سام للخلايا على خلايا الرئة ، ويمكن استخدامه بأمان بشكل منهجي دون الإضرار بأنسجة الرئة.