Journal of Applied Pharmaceutical Science Vol. 8(09), pp 114-118, September, 2018
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2018.8917
ISSN 2231-3354

Effect of *Kirganelia reticulata* extract on Human Embryonic Kidney cells with evidence on morphological changes

Madhurima Ghosh[1], Jhinuk Chatterjee[2], V. Krishnamurthy[2]

[1]Department of Biotechnology, PES Institute of Technology, Bangalore - 560085, India.
[2] Department of Biotechnology, PES University, Bangalore - 560085, India.

ARTICLE INFO

Article history:
Received on: 12/03/2018
Accepted on: 13/08/2018
Available online: 30/09/2018

Key words:
Phytochemicals, *Kirganelia*, HEK-293, Cell morphology, Anti-Proliferative.

ABSTRACT

Kirganelia reticulata has been proven to have cytotoxic activities. The aim of this study is to demonstrate the anti-proliferative activity of the methanolic extract of *K. reticulata* on Human Embryonic Kidney cells (HEK-293). Changes in cellular morphology of HEK-293 cells were evident at higher concentration and with an increase in time, which was supported by our experimental data. Our preliminary data suggest that *K. reticulata* methanolic extract has anti-proliferative activity.

INTRODUCTION

Phytochemicals refer to an extensive diversity of nonessential nutritive compounds with protective or disease preventive properties (Russo *et al*., 2010), produced by plants to protect themselves, and are not required by the human body for sustaining life. Recent research demonstrates that these chemical compounds can also protect humans against diseases. (Suresh *et al*., 2007). Some of the more commonly known phytochemicals include beta-carotene, ascorbic acid (vitamin C), folic acid, and vitamin E. Phytochemicals are promoted for the evidence that certain phytochemicals prevent and treat many health conditions, including heart disease, diabetes and high blood pressure (Michaud *et al*., 2000), and also prevent the formation, block the action on their target organs or tissue, act on cells to suppress substances that cause cancer development (Mehta and Pezzuto, 2002).

This framework has been valuable for the development of research on many different types of secondary plant metabolites that show biological activity in mammals, and it continues to provide a valuable approach for the classification of these compounds and their effects. The genus *Phyllanthus L*., one of the largest genera of Euphorbiaceae, is diverse in the morphology and chemical constituents. It is divided into 11 subgenera including *Kirganelia*, *Cicca*, *Emblica*, etc. The plants of this genus have been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infection, diabetes and hepatitis B (Unander *et al*., 1990). Some laboratory studies in cell cultures and animals have shown that certain phytochemicals have some activity against cancer cells or tumors (Lampronti *et al*., 2003). Researchers have also shown much interest in phytochemical supplements. However, despite the promising results from experimental studies, only a limited number of clinical trials are ongoing to assess the therapeutic efficacy of these molecules.

Kirganelia reticulata, is a locally available plant in Savanadurga forest of Karnataka. This plant is also found in tropical areas of Indian sub-continent and China (Ghani, 2003). According to the folklore, the plant has essentially been a part traditional medication for the treatment of gastric complaints (Joshi *et al*., 1991), diuretic and cooling medicine, to cure diarrhea in infants, treat sore eyes and superficial burns (Chopra

*Corresponding Author
Dr. Jhinuk Chatterjee, Assistant Professor, Department of Biotechnology, PES University, Bangalore-560085. E-mail: jhinukchatterjee @ pes.edu

et al., 1956). The anti-diabetic activity was also claimed and validated in tribal areas (Kumar *et al*., 2008). Morphological and microscopic characteristics of the leaves of *Kirganelia reticulata* were established, and anti-bacterial potential of the leaf extract has been evaluated (Shruthi *et al*., 2010). This plant has proved to potentially possess cytotoxic and antitumor activities. This was demonstrated using brine shrimp lethality assay and crown gall tumor inhibition assay (Reddy *et al*., 2014).

Numerous cell lines have been used as a model cell line for the detection of cytotoxicity in cells. HEK-293, Caco-2, HT-29, A549, has been majorly used for detection of morphological anomalies. Changes in cellular morphology, cell viability, cell membrane integrity, reactive oxygen species levels, mitochondrial membrane potential, cell death (apoptosis and necrosis), and the DNA damage were observed (Selvaraj *et al*., 2014; Kataria *et al*., 2011). Dose-dependent inhibition on proliferation and viability of the cells shows the cytotoxic activity of the active compound. In-vitro verification of cytotoxic effects on HEK-293 and inhibitory effects on the proliferation has been taken as a method to validate the present study, where we aim to evaluate and investigate methanolic leaf extract of *K. reticulata* for anti-proliferative effect in adherent cells (HEK-293).

MATERIAL AND METHODS

Materials and reagents

All chemicals were obtained from Sigma Aldrich (St. Louis, Missouri, USA) unless otherwise indicated.

RPMI-1640, Trypsin and Antibiotic-Antimycotic (100X) were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). 0.22-micron syringe filters were obtained from Millipore Ireland Ltd. Cell Culture plastic ware was obtained from Greiner Bio-One International GmbH, Austria.

Cell line

Human Embryonic Kidney cell line, HEK-293 was obtained from National Centre for Cell Science, Pune, India (tested as Mycoplasma negative), and was cultured in a T-25 and T-75 cell culture flask using RPMI-1640 medium supplemented with 10% FBS and antibiotics (1X Antibiotic-Antimycotic). The cells were incubated at 37°C in a humidified incubator containing 5% CO_2 and subcultured when the confluence of the flask reaches 80% or every 72 hours, whichever is early using a starting inoculum of 0.3×10^5 cells/ml.

The design of the experiment was set up for 24-well tissue culture plate in triplicate, and each well was seeded with 0.3×10^5 HEK-293 cells in 1 ml RPMI-1640 media supplemented with 10% FBS. The tissue culture plate was incubated in a CO_2 incubator for 24 hours for the HEK-293 cells to completely adhere to the plate surface and to confirm the sterility of the culture plate. The HEK-293 cells were found to be completely attached with 60% confluency after 24 hours. The tissue culture plate was ready for the treatment with different concentrations of crude extract of *Kirganelia reticulata*.

Preparation of *Kirganelia reticulata* leaf extract

10 grams of ground leaf powder from *Kirganelia reticulata* was subjected to 150 ml of 70% methanol extraction in a Soxhlet extractor at 70˚C for 4 hours. Post filtration, the solvent was evaporated using a rotary vacuum evaporator under a reduced pressure condition and at a temperature below 50˚C. The dried methanolic extracts were stored in a refrigerator for future phytochemical analysis (Reddy *et al*., 2014). 24 hours prior to the set-up of the experiment, the extract was dissolved in RPMI-1640 at a stock concentration of 2 mg/ mL, filtered through a 0.2-micron syringe filter and a sample was tested for sterility over-night in a CO_2 incubator. Once sterility was confirmed, the extract was used for the study.

Experimental design for determination of antiproliferative activity

In the present study, we have determined the antiproliferative activity of the extract on HEK-293 cells based on confluency percentage. For adherent cells, the confluency is the percentage of area covered by the cells. For example, 50% confluency means, the area covered by the cells and the empty spaces in the plate look similar under the microscope. 100% confluency means, there won't be any spaces in between the cells under microscopic observation. Once the sterility of the stock (2 mg/ml) of the methanolic extract of *K. reticulata* was confirmed and the HEK-293 cells were found to be at ~60% confluency with expected morphology, the design of experiment was set up in triplicates in 24-well tissue culture plates. The seeding density of cells was 0.3×10^5 cells/ml per well. The cells were treated with seven different concentrations from 0 (control, with no extract added) to 1000 µg/mL of the methanolic extract of *K. reticulata* and observed over a period of 1, 2 3 and 24 hours. The concentrations of the methanolic extract that the cells were tested for are 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml. The volume of extract added per well was calculated in reference to the stock concentration. The confluence of HEK-293 cells was observed under 20X magnification. The proliferation of the HEK-293 cells was assessed by confluency percentage.

Cell morphology analysis

Morphological changes of HEK-293 cells were closely monitored, noted and images were captured. Under the phase-contrast microscope, the untreated cells exhibited typical growth patterns and a smooth, flattened morphology with normal nuclei. When treated with plant extract at different concentrations, the cells exhibit apoptotic morphological changes with cytoplasmic blebbing in some cases, and detachment from the surface (Merlin *et al*., 2010).

RESULTS

Kirganelia reticulata extract exhibit anti-proliferative activity

Post 1-hour treatment with >250 µg/ml of *K. reticulata,* the HEK-293 cells were found to round off and detach from the adherent surface. Also at the end of 24 hours, it was observed that the confluence levels have gone down to <10% as against control (0 µg/ml) which is at 90% confluency. When incubation time was prolonged to 24 hours, the HEK-293 cells looked completely healthy <50 µg/ml with confluency at >80%. This has been clearly illustrated in Fig. 1.

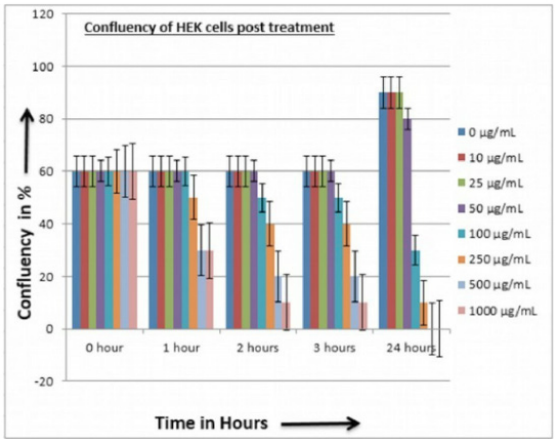

Fig. 1: Confluency of HEK-293 cells post treatment with *K. reticulata* extract.

Morphological changes in HEK-293

Cell morphology is an amicable visual tool to assess the toxicity in cells. HEK-293 cells–untreated and treated with different concentrations of *K. reticulata*, showed morphological changes. After completion of the treatment periods, the HEK-293 cells were observed under 20X magnification and images were taken for further analysis. Comparing the morphology of the control untreated HEK-293 cells with a lower concentration of 10–50 µg/ml up to 3 hours appeared similar. With the increase in concentration and increase in time (at 24 hours), cell shrinkage and decreased regularization of cells were observed. When treatment was more than 2 hours and the concentration was more than 100 µg/ml, cellular disintegration and a decrease in cell number and confluency was observed. Fig. 2 demonstrates the microscopic study, that with an increase in time and concentration, the gaps between HEK-293 cells increased.

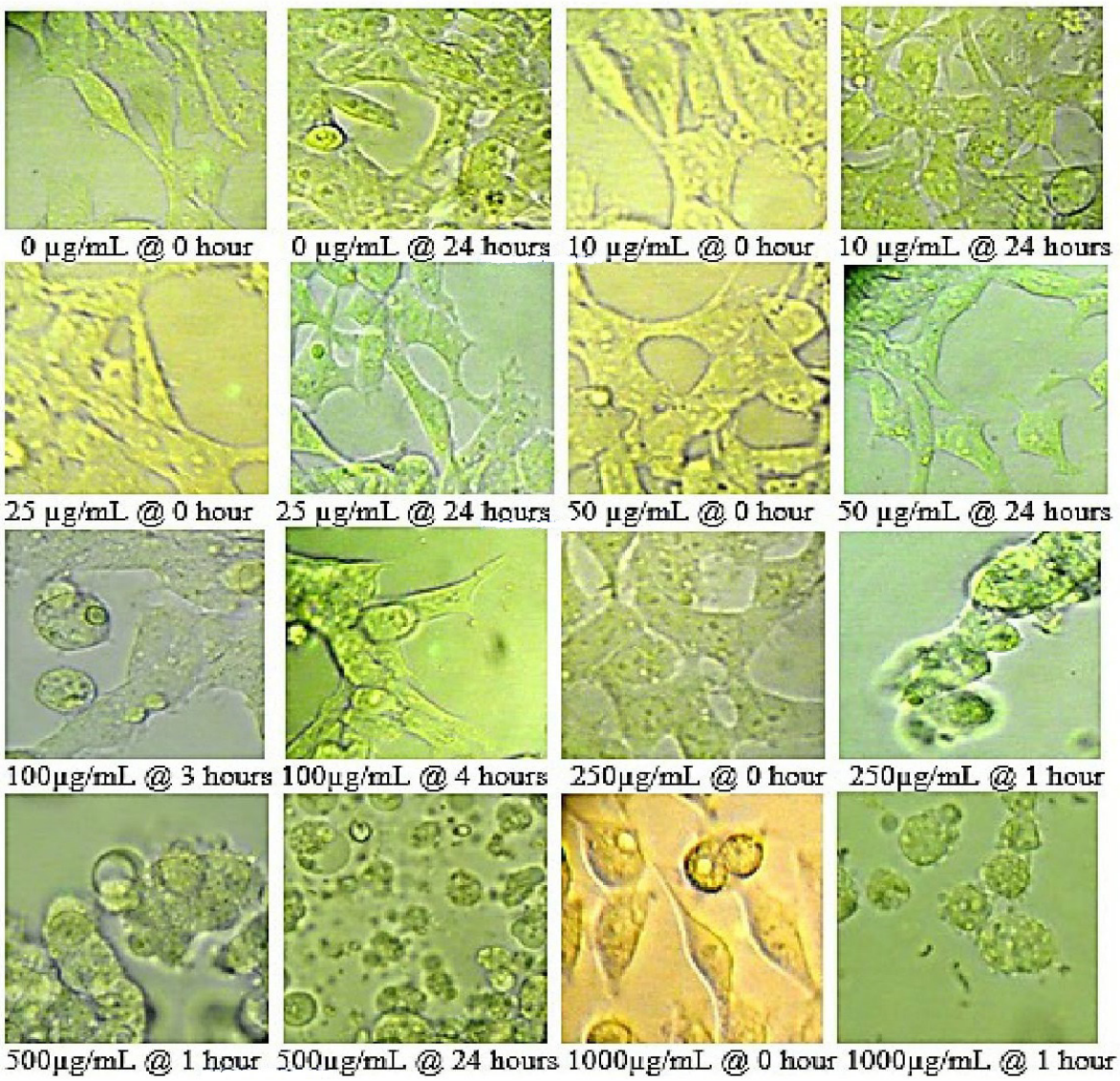

Fig. 2: Morphological changes of HEK-293 cells when exposed at various concentrations of *K. reticulata* extract for 24 hrs.

DISCUSSION

The success of drugs against cancer is solely on the mechanism of apoptosis of cells. The search for more effective drugs with antiproliferative activity have interested researchers in the traditional medicines of different parts of the world. A strategical and thoughtfully induced anti-proliferation by inducing apoptosis of cancer cells is the new age therapy for tumor cells and cancerous cells (Lampronti *et al*., 2003; Haass-Koffler *et al*., 2012).

Kirganelia reticulata was successfully tested and proven to be cytotoxic and antitumor using crown gall inhibitory assay (Reddy *et al*., 2014). In the present study, the exposure of methanolic extract of *K. reticulata* significantly reduced the proliferation of cells.

Our study demonstrates the exposure of methanolic extract of *K. reticulata* causes morphological changes in HEK-293 cells at a higher concentration more than 50 µg/mL and with an increase in time. The loss of normal morphology began with 250 µg/mL within 1 hour of treatment, where the cells were found to round off and lose the integrity (Haghparast *et al*., 2015). With the increase in exposure time and concentration of the extract, the cells retracted into a spherical shape and formed clusters in media after detachment from the surface (Guan *et al*., 2012; Joshi *et al*., 2013).

Thus, these findings add a new dimension on the methanolic extract of *K. reticulata* and t explore more on the potential of this phytochemical with anti-proliferative active compound towards cancer prevention (Sandercock *et al*., 2015).

CONCLUSION

In the present study, the exposure of methanolic extract of *Kirganelia reticulata* significantly reduced the proliferation of HEK-293 cells and causes morphological changes in cells at a higher concentration more than 50 µg/mL and with an increase in time.

ACKNOWLEDGMENT

The authors gratefully acknowledge Mr. Ravikumar A Reddy of Nitya Genomics Pvt Ltd, for providing the methanolic extract of *Kirganelia reticulata,* for conducting the study.

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How to cite this article:
Ghosh M, Chatterjee J, Krishnamurthy V. Effect of Kirganelia *reticulata* extract on Human Embryonic Kidney cells with evidence on morphological changes. J App Pharm Sci, 2018; 8(09): 114-118.

SUPPLEMENTARY INFORMATION

Data summary showing confluency percentage of HEK-293 cells post-treatment with *K. reticulata* extract.

	0 hours	**1 hour**	**2 hours**	**3 hours**	**24 hour**
0 µg/mL	60	60	60	60	90
10 µg/mL	60	60	60	60	90
25 µg/mL	60	60	60	60	90
50 µg/mL	60	60	60	60	80
100 µg/mL	60	60	50	50	30
250 µg/mL	60	50	40	40	10
500 µg/mL	60	30	20	20	0
1000 µg/mL	60	30	10	10	0