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Study on effect of *Tinospora cordifolia & Azadirachta indica* extract on human embryonic kidney cell line HEK 293

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Abstract:

Tinospora cordifolia & ***Azadirachta indica*** commonly known as Giloy & Neem are the holistic members of the Ayurveda family**. *T. cordifolia is often called*** as **"**Amrita" or the beverage of the immortality & ***A. indica*** is called as the omnipotent tree for its disease curing properties. The present study was pursued to determine the role of ***Tinospora cordifolia*** in combination with ***Azadirachta indica*** on human embryonic kidney cells (**HEK 293)** line. In this study the rate of proliferation using Trypan Blue dye exclusion assay, cytotoxicity using MTT Assay, cell migration employing Wound Healing/Scratch Assay and also the morphological changes on HEK 293 cells upon administration of *T.cordifolia* and *A.indica* extract combination under inverted phase contrast microscope were evaluated.

Keywords: Human embryonic kidney cells (**HEK 293),** Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS).

1. INTRODUCTION

We are living in a world where we expect everything to happen within a fraction of a second, unable to tolerate pain and so is the advantage of medicine-producing industries. Modern allopathic medicine offers significant benefits and drawbacks. It has saved lives and improved the quality of life for many but has diminished quality of lives and cost the lives of many. Intake of modern-day medicine which gives us relief instantly and makes our life easy and intake of medicine becomes a regular habit. Henceforth, we are making our immune system weak. Therefore, holistic practitioners believe that the emphasis on treating parts and diseases instead of

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

people is a flawed approach. The focus on parts, diseases, and symptoms has earned our modern medical system the title of disease care, rather than health care (Singh et al., 2014).

Ayurvedic medicine is one of the world's oldest holistic healing systems. It was developed more than 3,000 years ago in India. Ayurveda therapies have varied and evolved over more than 2 millennia. Therapies are typically based on complex herbal compounds like giloy, neem, minerals, and metal substances. These substances are known to cause observable benefits. Ayurveda's use of medicinal and culinary herbs draws upon India's incredible biodiversity with a variety that is unsurpassed by any medical system; yet, of all the herbs used, none has a status comparable to ***Tinospora cordifolia*** or giloy (Jagetia & Rao, 2006), (Jagetia et al., 1998).

Tinospora cordifolia commonly named "Guduchi" in Sanskrit belonging to the family Menispermaceae is a genetically diverse, large, deciduous climbing shrub with greenish-yellow typical flowers, found at higher altitudes (Saha & Ghosh, 2012). It's called the "Amrita," or the beverage of immortality. Recently, the plant has been of great interest to researchers across the globe because of its reported medicinal properties, such as anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, anti-stress, anti-leprotic, anti-malarial, hepatoprotective, immunomodulatory, and anti-neoplastic (Prince & Menon, 2001).

Azadirachta indica or neem is a member of the Meliaceae family and its role as a health-promoting effect is attributed to it is a rich source of antioxidants (Baligar et al., 2014). It's called the omnipotent tree because it holds the potential to cure the majority of diseases(Chundran et al., 2015). Earlier findings confirmed that neem and its constituents play a role in the scavenging of free radical generation and prevention of disease pathogenesis(Alzohairy, 2016). Numerous biological and pharmacological have been reported including anti-bacterial, anti-fungal, and anti-inflammatory. Earlier investigators have confirmed their role as anti-inflammatory, anti-arthritic, antipyretic, hypoglycemic, anti-gastric ulcer, anti-fungal, and anti-tumor activities (Patil et al., 2013).

As the effects of a combination of *Tinospora cordifolia* and *Azadirachta indica* extracts on the growth, cytotoxicity, cell migration, and viability of the HEK 293 cell line has not been studied previously hence in this study, the effects of a combination of *Tinospora cordifolia* and *Azadirachta indica* extracts on the growth, cytotoxicity, cell migration, and viability on the HEK 293 cell line, a model system for studying cellular responses were evaluated.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

2. MATERIALS AND METHOD

2.1 Reagents: All the chemicals used in the study were of tissue culture and molecular grade. Dulbecco's Phosphate Buffer Saline (DPBS) (TS1006), penicillin-streptomycin (Pen-strep) (A001-5X), phosphate-buffered saline (PBS), fetal bovine serum (FBS) (RM10832), Trypsin-EDTA (TCL007), MTT Reagent (RM1131), and Dulbecco's Modified Eagle's Medium (DMEM) (AL007A), Trypan blue dye (TCL005), were purchased from HI Media laboratories, Mumbai. ***Tinospora cordifolia*** extract was obtained from Patanjali company, ***Azdirachta indica*** **extract** was obtained from Kapiva company, Human embryonic kidney 293 (HEK 293) cell line was procured from National Cell Centre for Cell Science (NCSS, Pune, Maharashtra).

2.2 Cell Culture: HEK 293 cell line was maintained as per supplier's protocol. The cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin (full growth medium (FGM)) at 37 °C in a 5% CO_2 incubator and media were replaced by FGM after every 2–3 days. The cells were grown till 80-90% confluency; after which they were used for *in vitro* experimental studies

2.3 Dose and time-dependent effect of *T. cordifolia* and *A. indica* extract on the morphology of HEK293

For *in vitro* experimental studies HEK 293 cells were grown at 80% confluency in the T25 flask. Followed by trypsinization the cells were counted using haemocytometer and 50000 cells/2ml/well in FGM (were plated in a 6-well plate. The 6 well plates were then kept in the humidified incubator at 37^{o}C, 5% CO_2 allowing the cells to attach and spread on the surface and form a confluent monolayer for 24hr.

Subsequently, cells were washed with 2ml Dulbecco's Phosphate Buffer Saline (DPBS) twice and then FGM (2ml) containing 3 different doses of *T. cordifolia* (T.c) and *A. indica* (A.i) were added in the following combinations:-

1st well = 5µl (T.c (2.5µl) + A.i (2.5µl)

2nd well = 10µl (T.c (5µl) + A.i (5µl)

3rd well = 20 µl (T.c (10µl) + A.i (10µl)

and their effect was determined by observing morphology of cells under the microscope at day 0, 1, 2 and 3.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

2.4 Scratch assay – The HEK 293 cells at the cell density 50,000 cells/2ml/well were plated in 6 well plate in 3 wells. The plate was incubated for 24 hrs at 37ºC and 5% CO_2 allowing the cells to attach and spread properly on the plate. A confluent layer of HEK 293 cells was wounded/scraped in 6 well plates with microtip. After scratching, cells were washed with fresh DPBS, and full growth media containing 3 different doses of *T. cordifoli*a and *A. indica* was added. Photographs were taken.

3 different doses of *T. cordifoli*a (T.c) and *A. indica* (A.i) were added in the following combinations:-

1st well = 5µl (T.c (2.5µl) + A.i (2.5µl)

2nd well = 10µl (T.c (5µl) + A.i (5µl)

3rd well = 20 µl (T.c (10µl) + A.i (10µl)

To obtain the same field during the image acquisition, markings were made using a permanent marker which was to be used as a reference point close to the scratch, and the plate was placed in a tissue culture incubator at 37˚C for 72 hrs. Cells were observed consecutively for 3 days.

2.5 Time-Dependent Effect of *T. cordifoli*a and *A. indica* extract on proliferation of HEK 293 cells by Growth Curve Analysis using Trypan Blue Exclusion Test (Cell Viability)

In order to determine the effects of *T. cordifoli*a and *A. indica* extract on the proliferation of these HEK 293 cells, the growth curve analysis was performed. Cells (20000 cells/ml/well) in 1ml FGM were plated in 3 different 24–well plates and were allowed to grow until 80 – 90 % confluency was achieved. The cells were then treated with 1ml FGM containing 10µl of combination mixture of *T. cordifolia* and *A. indica* extract (10µl dose = T.c (5µl) + A.i (5µl)) and the control cells were untreated followed by incubation in a tissue culture incubator at 37˚C.

Plate 1 was observed after 24 hours. Media was discarded followed by washing with 1ml DPBS and then cells were trypsinized and centrifuged at 4000rpm for 5mins. The pellet was resuspended in 1ml Full growth medium. 10µl of this cell suspension was taken and added to 10µl Trypan blue exclusion dye. 10µl of this mixture was then added to Neubauer's haemocytometer for cell counting under the microscope. The same procedure was repeated after 48 hours and 72 hours for Plate 2 and 3 respectively. The percentage of viable cells was calculated as follows: -

Percentage of viable cells = number of unstained cells x100/total number of cells.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

2.6 MTT Assay- The *in vitro* cytotoxicity is measured using MTT assay. This assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis leading to the reduction in cell viability. It is based on the principle that for most viable cells, mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt into formazan crystals, which can be solubilized for homogenous measurement. Thus, any increase or decrease in the viable cell number can be detected by measuring formazan concentration reflected in optical density (OD 570,650 nm). For this $1x10^4$ HEK 293 cells/100µl in 100 µl Full growth media were added to each well of the 96- well plate and incubated for the next 24 hours in tissue culture incubator at 37°C. Next day the cells were washed twice with DPBS buffer and then treated with FGM (100µl) each containing the following 3 Different doses of the mixture of *T. cordifolia* and *A. indica* :-

1^{st} well = 5µl (T.c (2.5µl) + A.i (2.5µl)

2^{nd} well = 10µl (T.c (5µl) + A.i (5µl)

3^{rd} well = 20 µl (T.c (10µl) + A.i (10µl)

Cells were then incubated in the humidified incubator at 37°C for 24 hrs. Then 100µl (0.5mg/ml) of MTT reagent was added into the wells and incubation was done for 3 hrs in dark at room temperature until purple precipitates were visible. 100µl of DMSO was added after 3 hrs of incubation. Optical density readings were taken in Nandropscanning spectrophotometer at wavelength of 570 nm and 650 nm. The cell viability was calculated.

Cell viability (%) = (treated cells/control) x 100, where treated cells and control cells are the absorbance of treated and untreated cells.

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3. RESULTS

3.1 Time and Dose-Dependent Effect of Mixture of *Tinospora cordifolia* and *Azadirachta indica* on the Morphology of HEK 293 cells

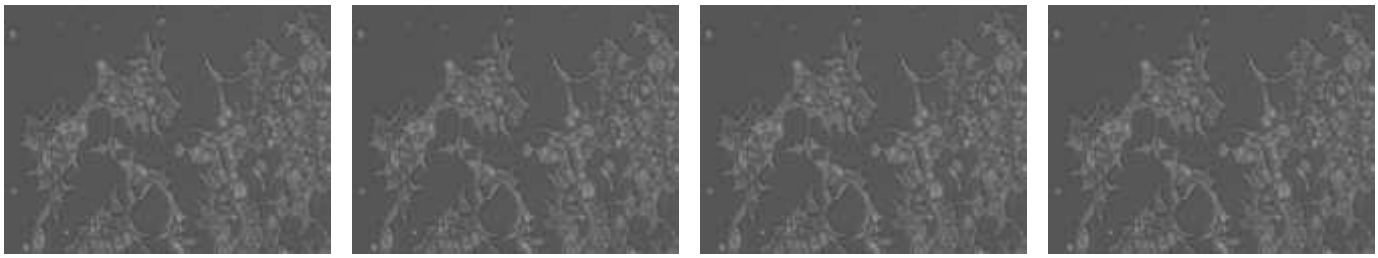
To determine the effects of different combinations of *Tinospora cordifolia* and *Azadirachta indica* extract on the morphology of cultured HEK 293 cells, the morphological examination of cells was done at different time intervals. To carry out this experiment, low to high doses of combination mixture *T. cordifolia* and *A. indica* extract i.e., 5 µl/ml, 10 µl/ml and 20 µl/ml were added to all the 3 wells marked as A, B and C respectively.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

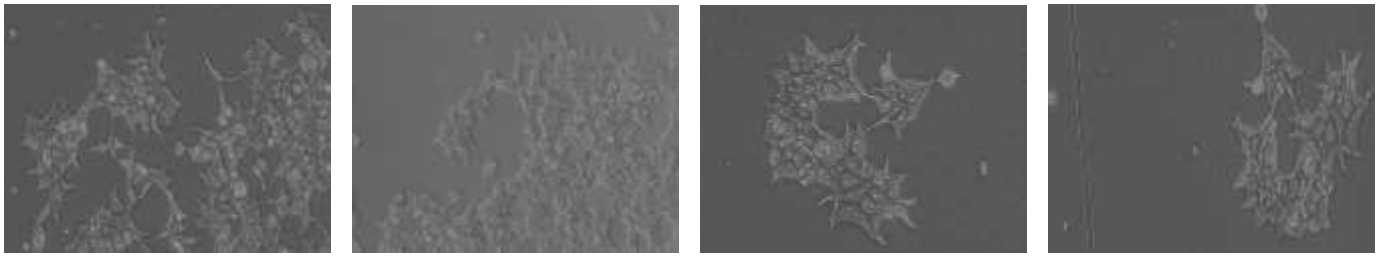
Cells were seeded in 6 well plate and were allowed to attain 80 - 90% confluency. Subsequently, the low to high doses of extract were added to cells cultured in 6 well plate to check morphological changes occurring in the cells at day 1, 2 and 3 and the control remained untreated throughout the experiment. The representative pictures of the cells for control, for days 0, 1, 2 and 3 are Fig.1 (a, e, i, m) respectively. The representative pictures of cells treated with 5 µl/ml dose for days 0, 1, 2 and 3 are Fig.1 (b, f, j, n) respectively. The representative pictures of cells treated with dose 10 µl/ml dose for days 0, 1, 2 and 3 are Fig.1 (c, g, k, o) respectively. The representative pictures of cells treated with 20 µl/ml dose for days 0, 1, 2 and 3 are Fig.1 (d, h, l, p) respectively. Cells were observed at 10 x magnification and photomicrographs were taken. It was observed that there were no morphological changes in the cells supplemented with the combination mixture *T. cordifolia* and *A. indica* extract doses as compared to control at all the time points. Cells showed normal typical fibroblastic morphology. Upon morphological examination under microscope, no changes in morphology of cells were noted on day 0, 1, 2, and 3 respectively.

DAY – 0 (Fig-1.1)



(a) CONTROL **(b) DOSE - 5µl** **(c) DOSE - 10µl** **(d) DOSE - 20µl**

DAY – 1 (Fig – 1.2)

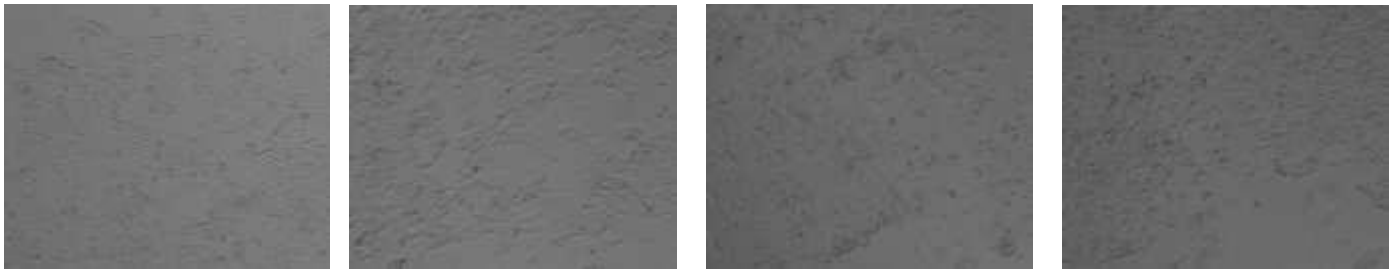


(e) CONTROL **(f) DOSE - 5µl** **(g) DOSE - 10µl** **(h) DOSE - 20µl**

DAY – 2 (Fig – 1.3)

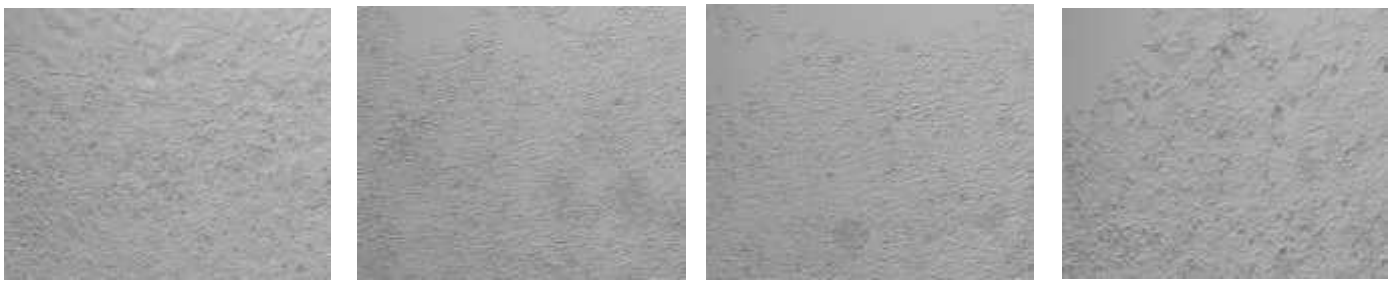
ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022



(i) CONTROL **(j) DOSE - 5µl** **(k) DOSE - 10µl** **(l) DOSE - 20µl**

DAY – 3 (Fig – 1.4)



(m) CONTROL **(n) DOSE - 5µl** **(o) DOSE - 10µl** **(p) DOSE - 20µl**

Fig 1- (a), (e), (i) and (m) are control for day 1,2,3 respectively, containing HEK 293 cells without dose.
(b), (f), (j) and (n) are wells containing 5µL dose for days 0, 1, 2, 3 respectively.
(c), (g), (k) and (o) are wells containing 10µL dose for days 0, 1, 2, 3 respectively.
(d), (h), (l) and (p) are wells containing 20µL dose for days 0, 1, 2, 3 respectively.

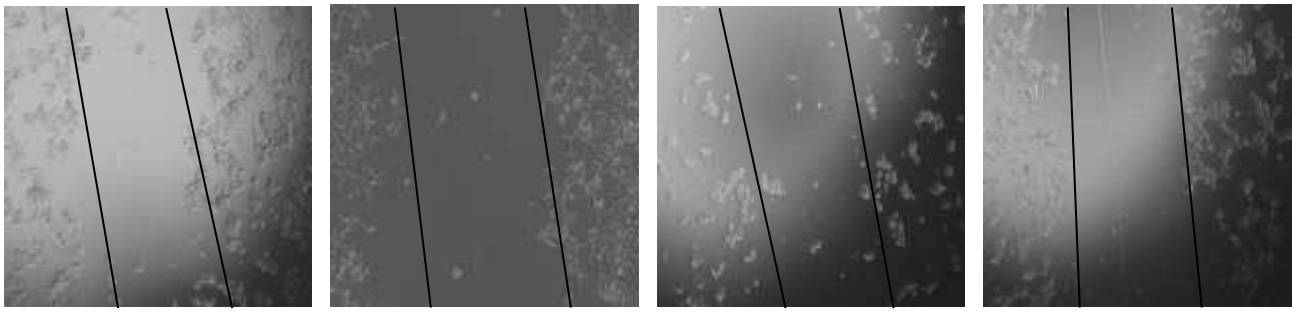
3.2 Time and Dose-Dependent Effect of Combination Mixture of *Tinospora cordifolia* and *Azadirachta indica* Extract on the Migration by Scratch Assay

Scratch assay was performed to assess the migration of the cells and the wound healing. Cells were established in 6 - well plate and were allowed to grow until 80 % confluency was achieved. Using a microtip, the plate was scratched carefully and low to high doses i.e., 5 µl, 10 µl and 20 µl were added as described in Materials and Methods. Photographs were taken on days 0, 1, 2 and 3 respectively. Results are described as in Fig 2.

DAY – 0

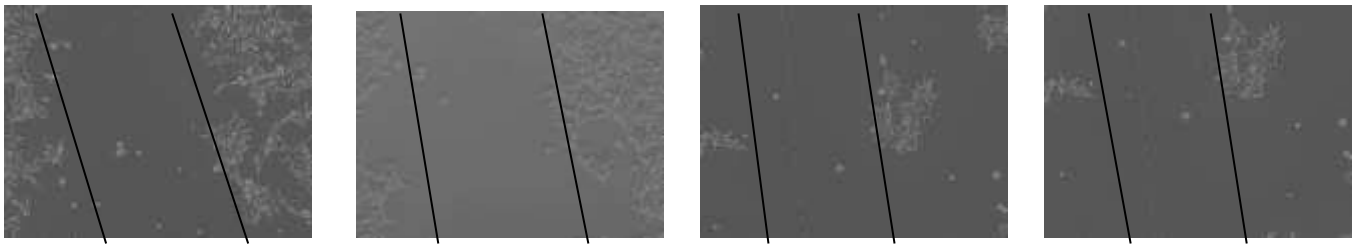
ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022



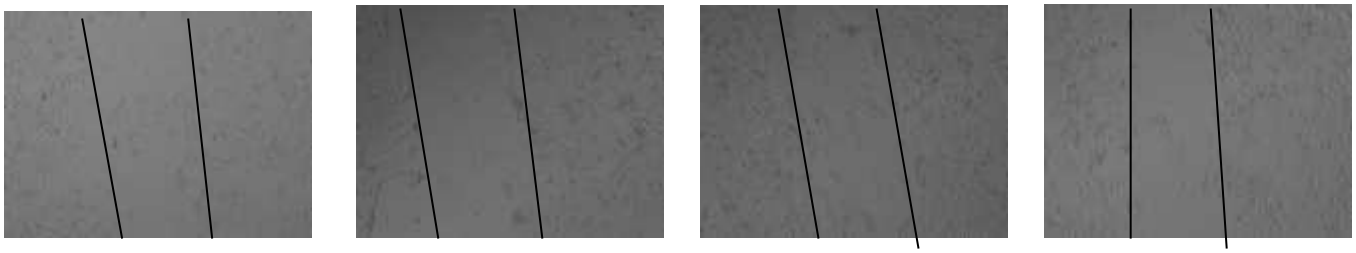
(a)CONTROL **(b) DOSE - 5µl** **(c) DOSE – 10 µl** **(d) DOSE – 20 µl**

DAY -1



(e) CONTROL **(f) DOSE - 5µl** **(g) DOSE – 10 µl** **(h) DOSE – 20 µl**

DAY – 2

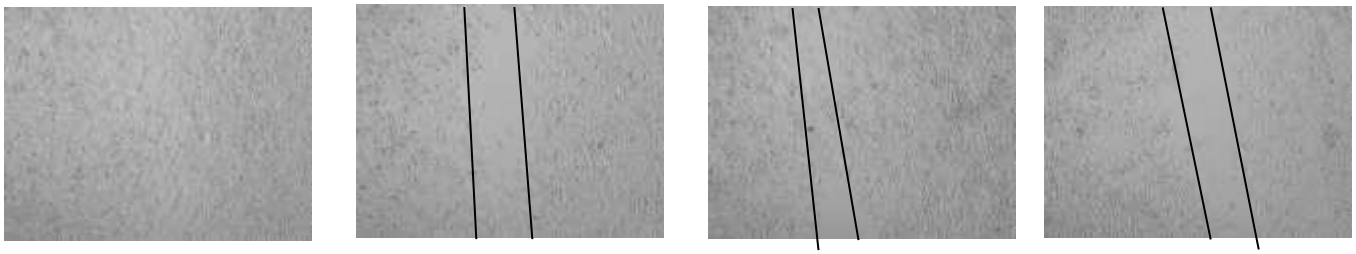


(i) CONTROL **(j) DOSE - 5µl** **(k) DOSE – 10 µl** **(l) DOSE – 20 µl**

DAY – 3

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022



(m)CONTROL **(n) DOSE - 5µl** **(o) DOSE – 10 µl** **(p) DOSE – 20 µl**

Fig 2- (a), (e), (I), (m) are control for day 0,1,2,3 respectively, in which scratch was induced containing HEK cells.

(b), (f), (j), (n) are wells containing 5µL dose for days 0, 1, 2, 3 respectively.

(c), (g), (k), (o) are wells containing 10µL dose for days 0, 1, 2, 3 respectively.

(d), (h), (l), (p) are wells containing 20µL dose for days 0, 1, 2, 3 respectively.

As shown in Fig. 2, controls for scratch assay on days 0, 1, 2, 3 depicts the migration of cells into the area of the scratch. It might show adequate wound healing capacity of cells. On day 1, there is not much migration of cells in all doses as compared to the control. On day 2, each dose treated cells show similar migration as observed for control. However on day 3, 5µL and 20µL treated cells showed significant decrease in migration as compared to control whereas 10µL dose treated cells showed significant migration of cells almost covering the area of wound as compared to the cells treated with both 5µL and 20µL of the dose as well as the control. This indicated that the combination mixture of *T. cordifolia* and *A. indica* extract at 10 µL dose promoted cell proliferation and hence promoted wound healing capacity of HEK cells on day 3.

3.3 Time-Dependent Effect of Combination Mixture of *Tinospora cordifolia* and *Azadirachta indica* on Proliferation of HEK 293 cells by Growth Curve Analysis using Trypan blue dye

To check out the effects of the combination mixture of *T. cordifolia* and *A. indica* extract on the proliferation of these cells, the growth curve analysis was performed. Cells were plated in 3 different 24 well plates (one plate for each day 1, 2 and 3 respectively) and were allowed to grow until 80 – 90 % confluency was achieved. The cells were then treated with the 10µl of combination mixture of *T. cordifolia* and *A. indica* extract (T.c (5µl) + A.i (5µl)) and the control cells were untreated.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

Plate 1 was observed after 24 hours. Media was discarded and PBS washing was done and then cells were trypsinized for detachment. 10µl of cells were taken into a micro centrifuge and 10µl Trypan blue exclusion dye was added to it. 10µl of this mixture was pipetted and added to Neubauer's hemocytometer for cell counting under the microscope. The same procedure was repeated after 48 hours and 72 hours.

As shown in Fig. 3, On day 1, 70 – 80 % cell confluency was observed at the dose of 10µl similar to control. There was significant increase in cell number. On day 2, 100 % cell confluency was seen at the dose of 10µl similar to control. However, the cell proliferation was found to be slightly higher on 3rd day i.e. after 72 hours at the dose of 10µl as compared to control.

The results demonstrated that the combination mixture of *T. cordifolia* and *A. indica* extract administration is time dependent and leads to cell proliferation.

DAY – 0

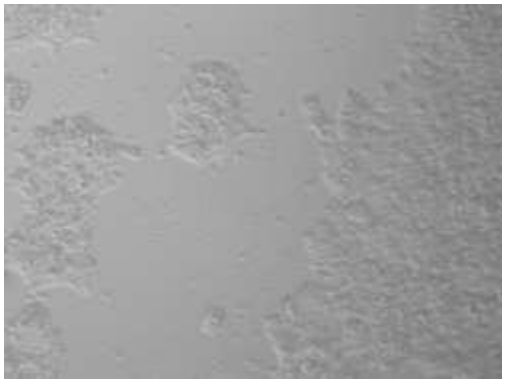
(A) CONTROL

(B) DAY – 0

DAY -1

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022



(C)CONTROL

(D) DOSE – 10 µl

DAY- 2

(E) CONTROL

(F) DOSE – 10 µl

DAY – 3

(G) CONTROL

(H) DOSE – 10 µl

Fig 3- (A), (C), (E) and (G) are control for days 0, 1, 2 and 3 respectively. (HEK 293 cells)
(B), (D), (F) and (H) are control for days 0, 1, 2 and 3 respectively. (HEK 293 cells + 10 µl)

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

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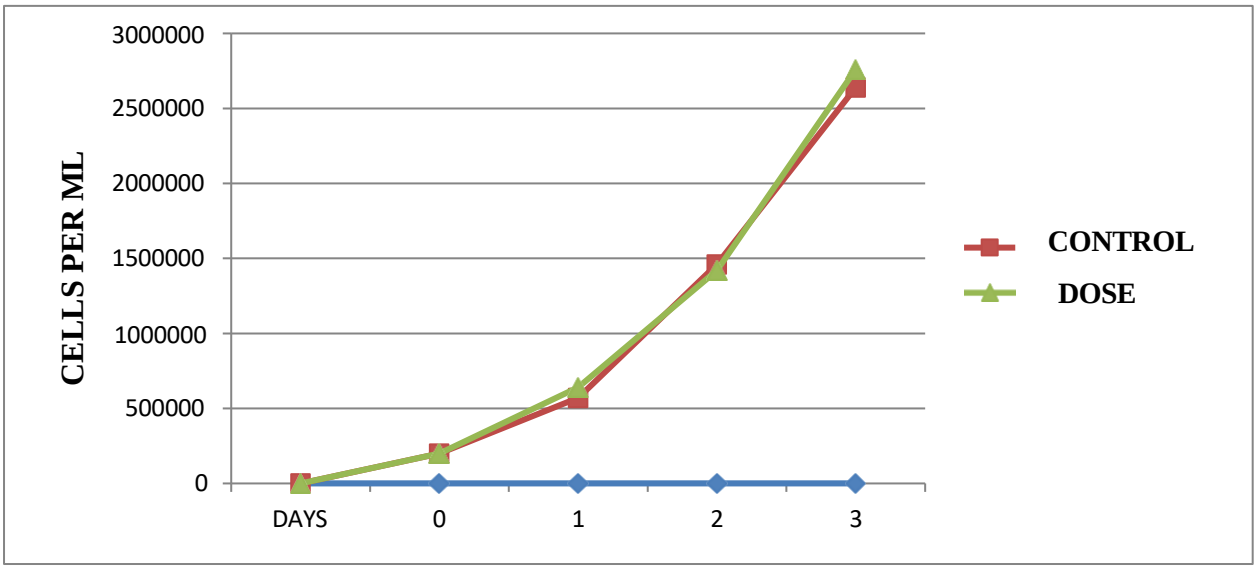

Fig.4- Growth curve for trypan blue exclusion dye test using the 10 µl dose containing combination mixture of *T. cordifolia* and *A. indica* extract ((T.c (5µl) + A.i (5µl)) on HEK 293.

DAYS	CONTROL (No. of Cells/ml)	DOSE Treated cells (No. of Cells/ml)
0	200000	200000
1	570000	640000
2	1460000	1420000
3	2640000	2760000

Cell proliferation data for the Growth curve

3.4 MTT Assay for combination mixture of *T. cordifolia* and *A. indica* extract on HEK 293

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of cell population's response to external factors. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in viability, i.e., cytotoxicity. It was observed that the administration of combination mixture of *T. cordifolia* and *A. indica* extract at low to high dose exhibited a dose dependent effect on the viability of HEK 293 cells after 24 hours

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

as there was high viability at 10 µl dose as compared to control, 5 µl and 20 µl doses. The percentage for the 10 µl dose was 136.8% whereas percentage for the 5 µl dose was 115.6% and for 20 µl dose was 125.5%. Hence, the most effective dose was observed to be 10 µl dose of the combination mixture of *T. cordifolia* and *A. indica* extract whose cell viability value was 136.8% in comparison to control.

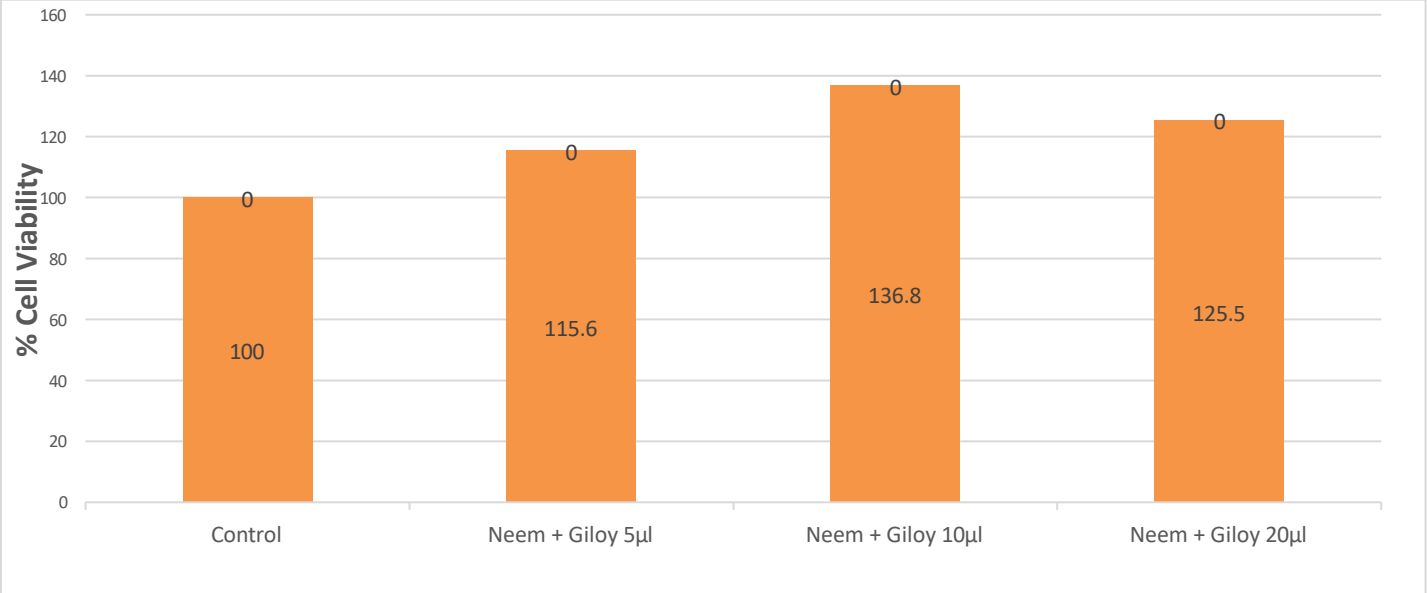

Fig. 5- MTT Assay for combination mixture of *T.cordifolia* and *A.indica* extract on HEK 293.

To our knowledge, this is the first demonstration of the effect of combination of *Tinospora cordifolia* and *Azadirachta indica* extract administration on the proliferation, viability, cytotoxicity and cell migration potential on Human Embryonic Kidney cell or HEK 293 cells.

4. DISCUSSION

In the present work, Human Embryonic Kidney cell line or HEK 293 cell line was used as a model system to study the effect of combination of *Tinospora cordifolia* and *Azadirachta indica* extract on the growth, cytotoxicity, cell migration and the viability of HEK 293 cell line. HEK 293 is a transformed cell line established in 1973 by exposing primary stage human embryonic kidney cells to culture to mechanically sheared DNA of type 5 adenovirus and can be cultured using appropriate media and environmental conditions (Grahm et al., 1977). Therefore, Human Embryonic Kidney cells or HEK 293 were administered with

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

combination mixture of *Tinospora cordifolia* and *Azadirachta indica* extract at 3 different doses i.e 5µL, 10µL, 20µL. The following results were obtained:

Firstly, the effect of the combination mixture of *Tinospora cordifolia* and *Azadirachta indica* extract for three doses 5 µL, 10 µL and 20 µL on the morphology of HEK 293 cells was studied for 3 days. Upon morphological analysis, the characteristic fibroblast- like morphology of HEK 293 cells was observed. No significant change was observed in the shape of the cells at all doses at day 1,2 and 3. Next the cell migration rate was morphologically analysed by using a scratch or wound healing assay. The results revealed that the combination mixture of *T. cordifolia* and *A. indica* extract administration at dose concentration of 5µL and 20µL showed a minimal number of cell migration into the scratch in the initial days as compared to control and 10µL dose on all 3 days. However, the scratch area was almost covered by cells at last day i.e day 3 at 10 µL dose, thereby indicating that the combination mixture of *T. cordifolia* and *A. indica* extract at 10 µL did not inhibit the migration of the cells and hence might promote wound healing capacity and hence cell proliferation of HEK cells at this dose (10 µl) on day 3. Further the effect of the combination mixture of *Tinospora cordifolia* and *Azadirachta indica* extract for the dose 10 µL was observed on cell proliferation by growth curve analysis by trypan blue dye exclusion assay. The results demonstrated that the combination mixture of *T. cordifolia* and *A. indica* extract at 10 µL dose had led to cell proliferation as the cell proliferation was observed to be higher on 3rd day i.e. after 72 hours at 10 µL dose. Additionally, the cytotoxicity of combination mixture of *T. cordifolia* and *A. indica* extract was evaluated using the MTT assay. The results obtained in the *in vitro* MTT assay demonstrated that the 10 µL dose of the combination mixture of these extracts promoted cell proliferation as the percentage cell viability for the cells treated with 10 µl dose was 136.8% as compared to the control cells after 24 hours.

CONCLUSION

Hence, this study investigated the effects of a combined *Tinospora cordifolia* and *Azadirachta indica* extract on HEK 293 cells. The extract induced cell proliferation, with no observed morphological changes. The scratch assay results indicated that lower extract concentrations initially reduced cell migration, while 10 µL dose of the combination mixture of these extracts promoted near-complete scratch closure, suggesting potential cell proliferation. Further, 10 µL dose of the combination mixture of these extracts promoted cell proliferation as assessed by MTT assay as well as by Growth curve analysis by trypan blue assay. In conclusion it is apparent that even though the observational studies provide suitable information for hypothesis formulation but certainly it is premature to generate a conclusive suggestion on *T. cordifolia* and *A. indica* and

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 12, Dec 2022

its therapeutic potential. Therefore, further tests are warranted to confirm the therapeutic potential of *T. cordifolia* and *A. indica* extract and its bioactive constituents on fundamental processes of cell proliferation which might prove to be naturally – derived potent therapeutic agent for various diseases.

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Conflict of interest

The authors state no conflict of interest.

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