JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

SCREENING FOR ANTICANCEROUS ACTIVITY OF STEM BARK OF *NIMBA (AZADIRACHTA INDICA* A. JUSS.)-AN IN-VITRO STUDY

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ABSTRACT-

Introduction-Cancer is a major reason people die around the world. Malignant melanoma is the most aggressive form of skin cancer, with a high mortality rate. More than 98% of the skin cancer are non-melanoma skin cancer (squamous cell cancer and basal cell cancer) the rest 1-2% are Melanoma skin cancer. One of the main problems in cancer treatment is gradual resistance mechanism. Recently herbal medicines are providing better management and preventive measure for cancer. The preliminary studies on a number of promising herbal drugs having anticancer potential. The main purpose of this study is to look into how protective *Azadirachta indica* A. Juss stem bark is in-vitro. **Objectives-** To Evaluate and Compare the effect of aqueous extract and hydro-alcoholic extract of stem bark of *Nimba, Azadirachta indica* A Linn on skin carcinoma in vitro. **Methods and materials***:* Aqueous and hydro-alcoholic extract of Stem bark of *Nimba (Azadirachta indica* A. Juss**)** was evaluated for its anticancerous activity against A-431 skin cell lines by MTT -Assay. *Nimba* plant is attributed with various medicinal properties like *Kushtaghna*, *Kandughna, Raktashodhaka, Vranaropana and Shophahara*. Neem leaf extract have shown efficacy against a variety of human carcinoma

cell lines and Animal models. Chemical constituents in both Extracts of leaf and Stem bark of *Nimba* are identical to each other. **Result**-There was a significant increase in percentage of cell death with increasing concentration showed significant result in both aqueous and hydro- alcoholic extract. Hydro alcoholic extract 89% cell death more effective than Aqueous extract. However standard drug Cisplatin showed 0.6% of cell death at 1000 µg /ml. **Conclusion**-Hydro-alcoholic extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) is proven to have anti-cancerous activity, when tested on Skin cell line A-431 MTT assay.

Index Term- Skin cancer, *Nimba*, Aqueous and hydro-alcoholic extract, A-431 skin cell line, MTT -Assay

INTRODUCTION-

Nimba, Azadirachta indica A Linn is a fast-growing evergreen tree commonly found in India. Stem bark, root, leaves, flowers and fruits are useful parts. Neem plant is attributed with various medicinal properties like *Kushtaghna, Krimighna, Kandughna, Raktashodhaka, Vranaropana, Vishaghna and Shophahara* [1]. Neem leaf extract have shown efficacy against a variety of human carcinoma cell lines and Animal models. Chemical constituents in both Extracts of leaf and Stem bark of *Nimba* are identical to each other. Leaf and stem bark contains Nimbolide, Nimbiol, Azadirachtin, Limonoids, Gedunin and Terpenoids and have exhibited anticancerous activity [2].

Skin carcinoma is the abnormal growth of skin cells, most often develops on skin exposed to sunlight. The treatment includes a surgery, Tropical treatment, Photodynamic therapy, Chemotherapy, Radiation therapy and External beam radiation therapy which are found to be sensitive in few patients, chance of recurrence and side effects like skin irritation, blister, redness or peeling, hair loss, change in skin colour in rare cases new skin cancer lesions developing year later [3]. The incidence of both non-melanoma and melanoma skin cancers has been increasing over the past decades. One in every three cancers diagnosed is a skin cancer. In India, the incidence of melanoma of the skin in the North region is 1.62 for males and 1.21 for females for every 1,00,000 people. The overall incidence of nonmelanoma skin cancer is the highest in the Northeast region, going as high as 5.14 for men and 3.98 for women for every 1,00,000 people [4].

All five parts of *Nimba* (root, bark, leaf, flower, fruit) have been used separately or collectively (*Panchanga*) in various skin disorders. Neem limonoids such as Azadirachtin, Gedunin, and Nimbolide target multiple pathways in cancer by anti-proliferative, pro-apoptotic, anti-inflammatory, anti-angiogenic effects, and inhibition of tumor invasion [5]. Hence it is implicated that stem bark would possess Anti-cancerous activity, the present study is intended to screen stem bark of *Nimba* (*Azadirachta indica* A Linn) for its Anti-cancerous activity on skin carcinoma.

METHOD AND MATERIALS

The study was conducted in following phases.

a. Pharmacognostical study

b. Invitro antimicrobial study

a. **Pharmacognostical study**: The stem bark of *Nimba* was collected from natural habitat of channarayapatna, Hassan and authentified by the department of Dravyaguna, SDM College of Ayurveda and Hospital, Hassan. The stem bark of *Nimba* was subjected for macroscopic-macroscopic evaluation, physicochemical evaluation, phytochemical evaluation and MTT Assay.

b. **In vitro antimicrobial study**: The study was undertaken for the following test samples.

a. Aqueous extract of stem bark of *Nimba*

b. Hydro-alcoholic extract of stem bark of *Nimba*

METHOD OF SAMPLE PREPARATION

Table No 1-Ingredients for Preparations of Extracts

Sl no	Sanskrit name	Botanical name	Parts used	Quantity used
1.	*Nimba*	Azadirachta indica A Juss (Aqueous extract)	Stem Bark	10 gm coarse powder 100 ml distilled water
2.	*Nimba*	Azadirachta indica A Juss (hydro-alcoholic extract)	Stem Bark	10 gm coarse powder 50 ml ethanol + 50 ml distilled water

METHODS

Following steps are involved in the experimental procedure:

- Preparation of medium for cell line study.
- Growing, trypsinization of confluent cells and cell seeding to 96 well plates.
- Screening of anti-cancer activity using various concentrations of *Nimba* stem bark against A431 cell lines for 48 hours followed by addition of MTT Dye.
- Calculation of percentage of viable cells with the following formula.

% of viable cells = [(Test sample-blank)/(Control-blank)] x 100

MATERIALS REQUIRED

- Microplate Reader with 450-490 nm filter
- 96 well microplate, sterilized clear plate for cell assay
- Pipette and pipette tips for 10-100µl
- CO2 incubator

- Hematocytometer
- Centrifuge
- Cell culture media
- Material to be tested
- PBS (Phosphate buffer saline) or other buffers

PREPARATION OF MINIMUM ESSENTIAL MEDIUM (MEM):

- Take 9.5gms of AT017A and suspended in 900 ml sterile cell culture grade water with constant, gentle stirring until the powder was completely dissolved.
- Check the pH and it was adjusted to 4.0 before autoclaving.
- Medium was autoclaved at 121°C at 15psi for 15 minutes.
- After removing the medium from the autoclave, it was allowed to cool at room temperature.
- Add 29.3 ml of 7.5% sodium bicarbonate solution and 10 ml of 200 mM L- glutamine solution.
- The pH was checked and it was adjusted to range of pH 7.2 to 7.4 and volume was adjusted to IL. The medium was filtered through filtration unit having cellulose nitrate Membrane (0.22 micron).
- Finally add 10 ml of 100 X antibiotic solution (A001-100ML) to the liquid medium and stored at 2-8°C and in dark till use.

PREPARATION OF REAGENT:

Phosphate Buffer Saline (PBS): NaCl (8 gms), Na_2HPO4 (1.44 gms), KH2PO4 (0.24 gms), KCI (0.2 gms) is weighed and dissolved in distilled water. The pH was adjusted to 7.4 and volume was made up to 1000 ml. The above solution was filtered through Whatman No I filter paper and autoclaved at standard condition and stored at 2-8°C till use.

CELL LINE AND EXTRACTS USED -

We obtained the human Skin cancer cell line A-431 from the National Centre for Cell Science (NCCS) in Pune. The cells were cultured in Minimum essential medium with 10% fetal bovine serum (FBS). At 37°C, the cells were kept in 95% air, 100% humidity, and 5% CO2. Every week, maintenance cultures were changed, and the medium was changed twice a week.

1. **MTT ASSAY** [6]

Principle of MTT ASSAY:

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay is a simple colorimetric assay for screening cell viability, depends on cellular NAD (P) H oxidoreductase enzymes of oral cells. The mitochondrial succinate dehydrogenase from live cells which reduces yellow 3-(4, 5-dimethythiazo12-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to an insoluble, coloured (dark purple) formazan. Further these formazan crystals are solubilized with suitable solvent and measured between 500 and 600 nm by a spectrophotometer. Since reduction of MTT can only occur in metabolically cells the level of activity is a measure of the viability of the cells (Mosmann 1983).

Procedure

Cell proliferation will be determined using a mitochondrial dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-Diphenyl tetrazolium bromide (MTT) to a purple formazan product. Pre-confluent cell lines will be seeded in 96-well plates at a density of3, 000-10,000 cells/well and incubated for 24 hrs. After cells will be treated with different concentrations of aqueous extract of and incubated for 24hrs or 48 hrs. at 37°C in CO2 incubator. Further MTT at the concentration of 5mg/ml will be added to all wells and incubated for 4h in dark. At the end of the experiment, the insoluble formazan product will be dissolved in using DMSO and MTT reduction will be quantified by measuring the absorbance at 570nm and 630nm in Spectrophotometer.

Experiment No 1:

The anticancer activity of aqueous and hydro-alcoholic extract of *Nimba* stem bark against A431 cell line using by MTT assay (Mosmann, T. 1983).

Culture of cells and drug treatment:

Around 70-80% confluent A431 cell line flask was taken and medium from the culture flask was removed.

- The cells were washed twice with sterile Phosphate buffer saline (PBS) without disturbing the cells. The wash solution from the culture flask was removed.
- Around 50-100 µl of trypsin (0.25 %) was added to flask and uniformly spread over the cells and culture flask was incubated in incubator at standard condition for approximately 2-5 minutes until cell starting detached from the flask.
- After completion of incubation time, the excess trypsin was removed and flask was gently tapped and observed under inverted microscope to check the activity of trypsin on cells.
- Once the cells are detached from the flask, around 1-2 ml of fresh medium (medium with 10% fetal bovine serum) was added to the flasks.
- Based on the cell density around 1 to 2 ml of medium containing cells transferred to 15 ml sterile centrifuge tube and centrifuged at around 800 to 1000 rpm for 5 minutes.
- After centrifugation, the pellet was carefully washed twice with PBS and re Suspended with growth medium (medium with 10% FBS).
- About 100 µl of trypan blue (0.04 %) was pipetted to a vial and equal volume of cell suspension was added. Both are mixed carefully and loaded to hemocytometer and counted under inverted microscope.
- After counting the cells, seed the cells to 96 well plate so that, each well having around 10,000 cells/well in 100 µl of medium.
- After completion of seeding the 96 well plate was incubated in CO2 incubator or 24 hours
- After 24 hours, the old medium from 96 well plate was carefully discarded.
- Cells were carefully washed once with PBS using multichannel pipette.
- Aqueous and hydro-alcoholic extract of *Nimba* stem bark was dissolved in serum free medium and different concentration of *Nimba* stem bark (1-1,000 ug/ml) was added to different test groups and incubated for 48 hours respectively. Control cells are supplemented with routine growth medium.

- Treat the cells with Cisplatin (500 and 1000 ug/ml) separately as a positive control.
- After completion of incubation time 20 µL of MTT dye (5mg/mL in PBS) was
- Added to all wells in dark. Plate was covered with aluminum foil and incubated in CO2 incubator at 37° C for 4 hours.
- After 4 hours, 100 µL of 0.4 N HCl and isopropanol (1:24) was added to all the wells and mixed carefully to dissolve the crystals. By using multi plate reader, the absorbance was recorded at 570 nm and 640 nm. Reference range.
- The percentage of viable cells were calculated using the formula:

% of viable cells = [(Test sample-blank)/(Control-blank)] x 100.

Observation and Results

The observations and results of the present study has been presented in the following three points.

1. The anti-cancer activity of **Aqueous extract of *Nimba* stem bark** against A431 cell line by MTT assay
2. The anti-cancer activity of **Hydro-alcoholic extract of *Nimba* stem bark** against A431 cell line by MTT assay
3. The anti-cancer activity **of Aqueous and Hydro-alcoholic extract of *Nimba* stem bark** against A431 cell line by MTT assay

Table No 2-The anti-cancer activity of Aqueous extract of *Nimba* stem bark against A431 cell line by MTT assay

Conc. (µg / mL)	Mean
Control	100
1	85.276
2	80.909
4	76.669
8	73.526
10	71.649
20	67.543
40	66.027
80	64.951
100	62.802
200	60.076

400	55.425
500	53.182
800	40.169
1000	30.440
Cisplatin (500)	2.268
Cisplatin (1000)	1.249

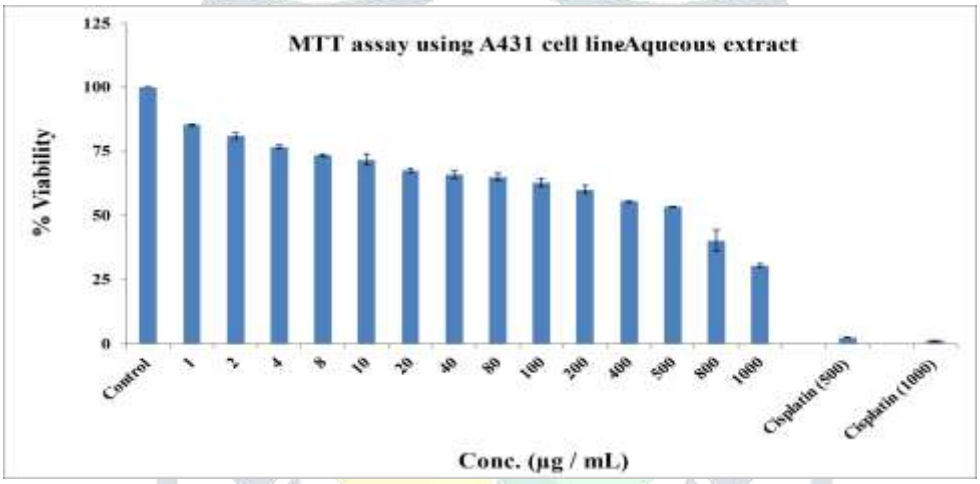

Fig 1- The anti-cancer activity of aqueous extract of *Nimba* stem bark against A431 cell line by MTT assay

Table No 3 The anti-cancer activity of **Hydro-alcoholic extract of *Nimba* stem bark** against A431 cell line by MTT assay

Conc (µg / ml)	Mean
Control	100
1	69.814
2	67.003
4	64.575
8	45.774
10	43.665
20	42.086
40	35.831
80	34.730
100	32.864
200	31.897
400	30.440
500	23.432
800	19.386
1000	10.817
Cisplatin (500)	1.354
Cisplatin (1000)	0.606

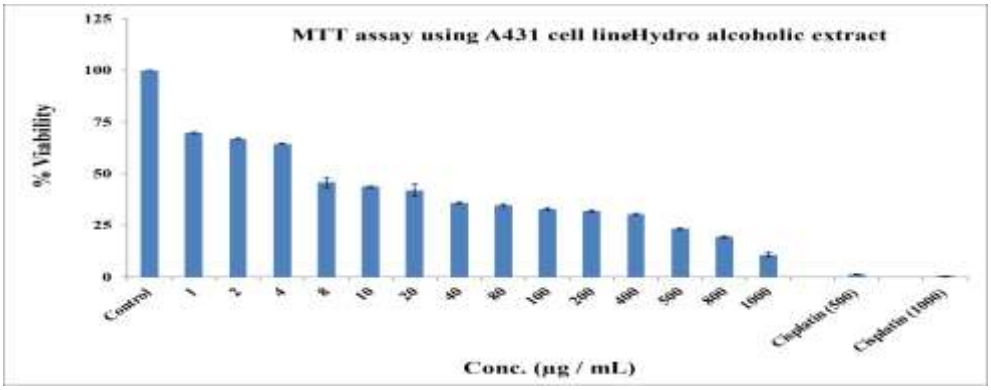

Fig 2- **The anti-cancer activity of Hydro-alcoholic extract of *Nimba* stem bark against A431 cell line by MTT assay**

Table No 4-The anti-cancer activity **of Aqueous and Hydro-alcoholic extract of *Nimba* stem bark** against A431 cell line by MTT assay

	Mean % viability (A431 cell lines)	
	Aqueous extract of *Nimba* stem bark	**Hydroalcoholic extract of *Nimba* stem bark**
Control	**100**	**100**
1 µg / ml	**85.276**	**69.814**
2 µg / ml	**80.909**	**67.003**
4 µg / ml	**76.669**	**64.575**
8 µg / ml	**73.526**	**45.774**
10 µg / ml	**71.649**	**43.665**
20 µg / ml	**67.543**	**42.086**
40 µg / ml	**66.951**	**35.831**
80 µg / ml	**64.802**	**34.730**
100 µg / ml	**62.802**	**32.864**
200 µg / ml	**60.076**	**31.897**
400 µg / ml	**55.425**	**30.440**
500 µg / ml	**53.182**	**23.423**
800 µg / ml	**40.169**	**19.386**
1000 µg / ml	**30.440**	**10.817**
Cisplatin 500	**2.268**	**1.354**
Cisplatin 1000	**1.249**	**0.606**

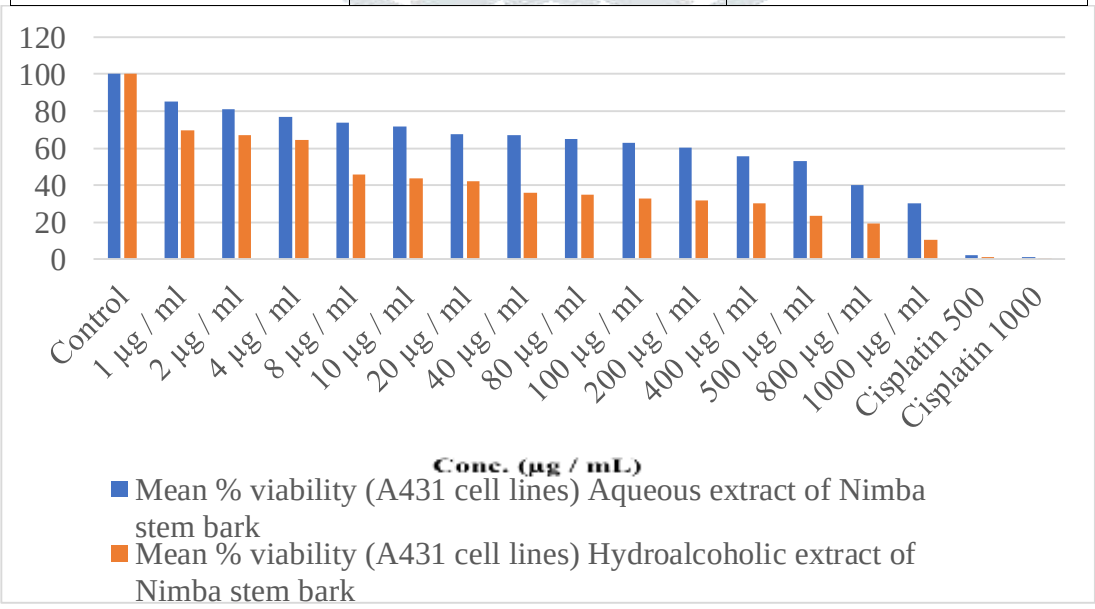

Fig 3- The anti-cancer activity of Aqueous and Hydro-alcoholic extract of *Nimba* stem bark against A431 cell line by MTT assay

DISCUSSION BASED ON OBSERVATION

➢ During MTT assay the cell line A-431shows the absorbance value in spectrophotometer for various concentrations (1, 2, 4, 8, 10, 20, 40, 80, 100, 200, 400, 500, 800 and 1000 µg /ml) used. The absorbance in MTT assay is carried out in duplets further the absorbance decreases with the increase in the concentration i.e. maximum absorbance in 1µg and minimum in 1000µg, which indicates cell number decreases as the concentration of the extracts increases. This may be due to the reason that at high drug concentrations other death mechanisms such as necrosis and increased autophagy are more prevalent than apoptosis.

➢ **At 500 µg/ml** the % of viable cells in the aqueous extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) was found to be 53.182% and the % of viable cells in the hydro-alcoholic extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) was found to be 23.432%.

➢ **At 800 µg/ml** the % of viable cells in the aqueous extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) was found to be 40.169% and the % of viable cells in the hydro-alcoholic extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) was found to be 19.386%.

➢ **At 1000 µg/ml** the % of viable cells in the aqueous extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) was found to be 30.440% and the % of viable cells in the hydro-alcoholic extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) was found to be 10.817%.

THE STANDARD DRUG

➢ **Cisplatin at 500µg/ml** the % of viable cells in the **aqueous extract of cisplatin** was found to be **2.268%** and the% of viable cells in the **hydro-alcoholic extract of** cisplatin was found to be **1.354%.**

➢ **At 1000µg/ml the %** of viable cells in **the aqueous extract of cisplatin** was found to be **1.249%** and the**% of viable cells in the hydro-alcoholic extract of cisplatin** was found to be **0.606 %.**

INTERPRETATION

Hydro alcoholic extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) was found to be Highly significant than Aqueous extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) all intervals.

Hydro-alcoholic extract of *Nimba* bark at the dose of **1000µg/ml**, more than **89%** of cell death was observed which is the criteria for significance of the test. Hence hydro-alcoholic extract of *Nimba* stem bark was found to be highly significant and more effective when compared to the Aqueous extract of *Nimba* stem bark.

At a dose of 1000 µg/ml of hydro-alcoholic extract of stem bark of *Nimba* (*Azadirachta indica* A Linn) **10.817%** of the cells were alive, indicating that **89.183%** of the cells died. Hence Hydro-alcoholic extract stem bark of *Nimba* (*Azadirachta indica* A Linn) was found to be highly significant and more effective when compared to the aqueous extract of stem bark *Nimba* (*Azadirachta indica* A Linn).

Comparison b/w cisplatin and *Nimba* (*Azadirachta indica* A Linn) in MTT assay:

At 500µg/ml

The % of viable cells in the aqueous extract of cisplatin was found to be **2.268%** Whereas the **% of viable cells in the aqueous extract of stem bark *Nimba*** (*Azadirachta indica* A Linn) was found to be **53.182%.**

The % of viable cells in the hydro-alcoholic extract of cisplatin was found to be **1.354%** whereas the **% of viable cells in the hydro-alcoholic extract of Stem bark *Nimba*** (*Azadirachta indica* A Linn) was found to be **23.432%.**

At 1000µg/ml

The % of viable cells in the aqueous extract of cisplatin was found to be **1.249** %whereas the **% of viable cells in the aqueous extract** of stem bark *Nimba* (*Azadirachta indica* A Linn) was found to be **30.440%.**

The % of viable cells in the hydro-alcoholic extract of cisplatin was found to be **0.606%** whereas **the % of viable cells in the hydro-alcoholic extract of stem bark *Nimba*** (*Azadirachta indica* A Linn) was found to be **10.817%.**

Hence, we can say that Cisplatin extract was more significant than the *Nimba* (*Azadirachta indica* A Linn) extract

CONCLUSION

The following conclusions can be drawn -

- In the current study, Nimba (Azadirachta Indica A. Juss) stem bark extract (both aqueous extract and hydro-alcoholic extract) on Skin carcinoma cell lines {A431} showed anti-cancerous activity based on % survival of the cell in both MTT assay.
- Comparison over the concentrations showed better results at higher concentrations when compared to the lower concentrations.
- Effect of Hydro-alcoholic extract of Nimba stem Bark was found to be Significant when compared to Aqueous extract of Nimba bark.
- Hence in the current study research Hypothesis holds good which states that - "Nimba (Azadirachta Indica A. Juss.) Stem Bark extract (aqueous and hydro- alcoholic) possess Anti-cancerous activity on skin carcinoma cell lines {A431}".

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