# Evaluation of In vitro-antioxidant and anticancer potential of Abutilon crispum Linn

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**ABSTRACT**
The present study evaluated the in vitro antioxidant and anticancer potential of ethanolic leaf extract of Abutilon crispum Linn. Leaves were Soxhlet-extracted with 90% ethanol to obtain the crude extract. Antioxidant activity was assessed by DPPH and nitric oxide (NO) radical scavenging assays at concentrations up to 100 µg/mL, using ascorbic acid as reference. The extract produced 93.89% DPPH scavenging and 72.34% NO scavenging at 100 µg/mL, compared with ascorbic acid's 95.34% and 86.84%, respectively, indicating strong free-radical scavenging capacity. Cytotoxicity against A549 human lung carcinoma cells was evaluated by MTT assay to determine GI50 and by trypan blue exclusion for cell viability across concentrations up to 1600 µg/mL; 5-fluorouracil served as a positive control. The extract showed dose-dependent growth inhibition with a GI50 of 255 µg/mL and induced complete cell death at 1600 µg/mL, whereas 5-fluorouracil produced >90% cytotoxicity at 50 µg/mL, demonstrating greater potency of the chemotherapeutic control. Apoptosis-related effects were investigated by Hoechst 33258 nuclear staining and DNA fragmentation analysis. Treated A549 cells exhibited nuclear condensation and fragmentation, and DNA laddering was observed, supporting apoptotic cell death. Collectively, these findings indicate that ethanolic leaf extract of Abutilon crispum possesses robust in vitro antioxidant activity and induces dose-dependent cytotoxicity in A549 cells via apoptosis. The results support subsequent in vivo studies and bioassay-guided isolation to identify active constituents and elucidate molecular mechanisms underlying the observed anticancer effects.

**Keywords:** Abutilon crispum, antioxidant activity, anticancer, apoptosis, A549, DPPH, DNA fragmentation

## Introduction:

Cancer is one of the leading causes of death globally, with an estimated 10 million deaths in 2020, according to the GLOBOCAN 2020 report. Lung, breast, colorectal, and pharyngeal cancers remain the most frequently diagnosed types worldwide. In India, the burden of cancer is also increasing, with approximately 1.5 million individuals affected, and the country showing some of the highest rates of oral, pharyngeal, and gall bladder cancers. The alarming rise in incidence and mortality has prompted the search for safer, more effective therapeutic alternatives, including those based on medicinal plants.

India is home to more than 6000 plant species that are used in traditional, folk, and herbal medicine systems, accounting for nearly 75% of the primary healthcare needs in developing countries. Herbal remedies have been employed for millennia for both preventive and curative purposes. With growing concern about the adverse effects and resistance associated with conventional chemotherapeutic agents, there is renewed interest in phytomedicines for cancer treatment and prevention.

Abutilon crispum Linn. (family: Malvaceae), also known as Herissantia crispa, Gayoides crispum, bladdermallow, or curly butilon, is a plant with documented ethnomedicinal applications. In India, tribal communities in Andhra Pradesh and Tamil Nadu traditionally use different parts of this plant to treat ailments such as diabetes, jaundice, piles, ulcers, bronchitis, and asthma. Pharmacological studies have reported that aqueous leaf extracts of A. crispum possess hepatoprotective, anti-inflammatory, analgesic, and diuretic properties. Notably, no signs of acute toxicity were

observed in animal models up to 72 hours after administration, indicating a favorable safety profile. Thin Layer Chromatography (TLC) has been employed for phytochemical fingerprinting and standardization of the plant.
Despite its traditional medicinal value, scientific studies exploring the antioxidant and anticancer potential of A. crispum are limited. In particular, its cytotoxic effects against human cancer cell lines have not been thoroughly evaluated. Considering its folk medicinal use and documented bioactivities Abutilon crispum can be a good source of natural compounds with antioxidant and anticancer activity. Therefore, the present study aims to investigate the in vitro antioxidant and anticancer activities of the ethanolic leaf extract of Abutilon crispum using standard models such as DPPH, nitric oxide scavenging, MTT assay, trypan blue exclusion, Hoechst 33258 staining, and DNA fragmentation assay.

**Fig no: 1 Abutilon crispum (Linn).**

The aim of the present investigation was to evaluate the antioxidant and anticancer activity of Abutilon crispum.[4]
Roots extract of Potentilla fulgens proven that it has strong anticancer activity against A549 cell line. It has another gateway for plant based therapeutic agents against cancer cell line which encourage to explore the cytotoxicity study of abutilon crispum against A549 cell lines. Therefore A549 cell line was used for the study.
Reference

## Materials And Methods

**Requirements:**
Shade dried coarse powder of leaves of Abutilon crispum (Linn), Soxhlet apparatus. Petroleum ether, Ethanol 95% v/v and Distilled water.

**Collection and authentication of plant material:**
The leaves of Abutilon crispum Linn. were collected from the foot hills Kodaikannal, Dindigul district in the month of Nov-2015. The plant was then taxonomically identified and authenticated by the Botanist Dr. V. Chellathurai. The authenticated plant material was used for preparation of extracts.

**Preparation of the extract:**
Abutilon crispum (Linn) leaves that had been shade-dried for 72 hours at 60 to 80 degrees Celsius and ground into a coarse powder. After passing through sieve number 40, the powder was kept in an airtight container for the extraction process. In the Soxhlet device, 400g of powdered material was equally packed and Petroleum ether was used for extraction. Following the extraction process, the defatted extracts were heated and filtered using Whatmann filter paper (No. 10) to eliminate any remaining contaminants. After reducing the volume to 1/10 using vacuum distillation, the concentrated extract was moved to a 100 ml beaker, and the leftover solvent was evaporated on a water bath. An extract with a dark greenish yellow was produced. Following the drying of the petroleum ether extraction, the marc was extracted for 72 hours using 95% v/v ethanol at 75–78$^{0}$C. Following the extraction process, distillation was used to get rid of the solvent. The extract was then stored in a desiccator to remove the excessive moisture. For future research, the dried extracts were stored in an airtight glass container Extracts were used for phytochemical evaluation. Ethanolic extract revealed higher content of secondary metabolites so ethanolic extract was used for further studies.

**In-vitro antioxidant studies:**
**DPPH Scavenging activity (1, 1 diphenyl 2, picryl hydrazyl) [5]:**
Using the Blois method, the hydrogen donating capacity or DPPH scavenging activity was measured in the presence of a stable DPPH radical. In short, 0.05 ml of test chemicals dissolved in methanol at varying concentrations (20–100µg/ml) were added to a methanolic solution of DPPH (100µM, 2.95 ml). After stirring the reaction mixture, absorbance was measured using spectrophotometry (spectramax plus384, Molecular Devices, USA) at 517 nm at 30-second intervals

for five minutes, and the reading was recorded for twenty minutes. The standard was ascorbic acid. The extract's scavenging effectiveness is indicated by the degree of discolouration. The following formula was used to determine the radical scavenging activity, which was expressed as a drop in DPPH absorbance. The results were depicted in Table no.2

Scavenging effect (%) = (1-B/A) X 100

Where,

A = Absorbance of DPPH control with solvent (517nm)

B = Absorbance of decolorized DPPH in presence of test sample (517nm)

**Nitric oxide scavenging assay**[6]

In order to conduct the nitric oxide scavenging experiment, sodium nitroprusside was used. The Griess Illosvoy reaction can be used to ascertain this. 0.5 ml of extract/sub-fraction at different concentrations was combined with 2 ml of 10 mM sodium nitroprusside in 0.5 ml of phosphate buffer saline (pH 7.4), and the combination was incubated at 25°C for 150 minutes. 0.5 ml of the incubated mixture was removed and added to 1.0 ml of sulphanilamide solution (0.33% in 20% glacial acetic acid). The combination was then incubated for an additional five minutes at room temperature. Lastly, 1.0 ml of 0.1% w/v naphthyl ethylenediamine dihydrochloride was combined and allowed to sit at room temperature for half an hour. At 546 nm, the absorbance was measured.

The percentage inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = \left(1 - \frac{A_1}{A_0}\right) \times 100$$

Where,

$A_l$= Absorbance of the extract or standard,

A0 = Absorbance of the control

**Anticancer studies:**

The ethanolic extract of leaves of Abutilon crispum (Linn) was used for the evaluation of in-vitro anticancer activity. The cell line A549, which is an human lung adenocarcinoma cell line, is available from the National Centre for Cell Science (NCCS), Pune, India. NCCS is a national cell repository and research center offering cell culture and related services.

Before being passed on to new media, the cells had been subcultured in DMEM with 10% FBS and cryopreserved in DMEM with 10% FBS and 10% dimethylsulfoxide (DMSO). Cells were adapted to FBS-free media directly or progressively. Briefly, cells were plated into uncoated T75 flasks and cultured in a humidified atmosphere with 5% CO2 at 37 C. medium was replaced every 2–3 days. About twice a week, when 80–90% confluency was reached, cells were passed with TrypLE, an animal-free recombinant cell dissociation enzyme

**In-vitro anti-cancer studies:**

Various in-vitro models were used to evaluate the anti-cancer activity. Among those we have undergone with Trypan Blue, MTT Assay, Hoechst Staining Assay and DNA fragmentation assay

In-Vitro Cytotoxicity (Trypan blue method)7:

Using A549 lung cancer cell lines, short-term in-vitro cytotoxicity was evaluated by incubating various EEAC doses for three hours at 37°C. Using an insulin syringe, the tumor cells were extracted from the peritoneal cavity of tumour-bearing mice and placed in a test tube filled with isotonic saline. After that, the cells were rinsed in regular saline, and the cell count was measured with a hemacytometer and adjusted to 10x106 cells/ml. Different extract concentrations (25–1600 µg/ml) were applied to each tube for the cytotoxicity test, and the final volume was adjusted to one milliliter using regular saline. Saline, tumor cells, and drug-free control tubes were maintained. For a period of three hours, each tube was incubated at 37°C. Following incubation, 0.1 ml of 0.4% trypan blue dye in isotonic saline was added to each tube, and a hemocytometer was used to count the number of living (unstained) and dead (stained) cells. Every samples were triplicated to obtain mean value.

$$\% \text{ Dead cells} = \frac{\text{Total cells counted} - \text{total viable cells}}{\text{Total cells counted}} \text{ x } 100$$

**Cytotoxic studies - MTT assay[8]:**

**Formazan-based viable cell Mass Assay (MTT assay):**

EEAC and 5-FU (1–0.039µM) were applied to FHC and A-549 cells.Using the MTT assay, cell viability was assessed at 24 hours. In a nutshell, the cells were planted at a density of 4×103 cells/well in a 96-well plate and left to adhere for the entire night. Following the removal of the medium, 200 µL of fresh media containing 10 mmol/L HEPES (pH 7.4) was added to each well. Following the addition of 50 µL MTT to each well, the plate was incubated for two to four hours at 37°C in the dark. After removing the medium, the wells were filled with 25 µL of Sorensen's glycine buffer and 200 µL of DMSO. An ELISA plate reader set to 570 nm was used to detect absorbance.

**Hoechst staining assay[9]**

DNA nick generation was analyzed by fluorescence microscopy after staining the treated and untreated cells separately with Hoechst 33258(10µM). These stains are preferentially bound with nicked DNA. Cells (10cells/ml) were cultured for 24h on well plates. The cells were incubated for their respective time points with and without the drug. The control received no drug. Following treatment, cells were fixed in 2% paraformaldehyde, stained with Hoechst 33258 (10µM), and rinsed with PBS. Then the cells were analyzed under fluorescence microscope and representative photographs were taken for further qualitative analysis.

**DNA fragmentation Assay[10]:**

225 µg of ethanolic extract of abutilon crispum were treated with A-549 cells which were grown in 10cm petri dishes. Phosphate buffer saline in an icecold condition was used to wash the attached and unattached cells thrice to make it clear. 100 µL DNA lysis buffer consists of Tris-Hydrochloric acid (50Mm at pH 8.0), EDTA (10Mm), N-lauroylsarcosine (0.5%) and proteinase K (2 mg/mL) was used for dissolving cells. The solutions were incubated for 3hr at 55°C, and RNAse A was added and then again, the solutions were incubated for 3hrs again. Equal amounts of phenol and chloroform-isoamyl alcohol (24:1 v/v) were used twice to extract the DNA. Following an overnight precipitation with 0.1 ml of sodium acetate (pH 4.8) and 2.5 ml ethanol at 20°C, the DNA was pelleted for one hour at 12,000g. DNA was detected by EtBr staining after samples were electrophoresed in a 1.5% (w/v) agarose gel.

**Statistical Analysis**

All of the assays were performed in final, and triplicate data were presented as mean standard deviation. Different concentrations of the extracts of the plant were tested, and then half-maximal (IC50) inhibitory concentration values for all the experiments were computed. Statistical difference of mechanistic studies (Hoechst 33342 Stain) was compared using ANOVA (one-way analysis of variance), and was followed by Tukey's multiple tests. Differences were considered significant at $p < 0.05$.

## Result

The herb was gathered in and around the Kodaikannal foothills, according to research on ethanopharmacology. A botanist identified and verified the plant that was collected. Using a cold maceration procedure and a continuous hot percolation process with a Soxhlet apparatus, the phytoconstituents were extracted using a variety of solvents with increasing polarity. Extractive values were presented in Table no: 1

**Table no: No.1: Extractive values of leaves of Abutilon crispum Linn.**

| Plant Name | Part Used | Method of extraction | Yield in percentage% | |
|---|---|---|---|---|
| | | | [illegible] | [illegible] |
| Abutilon crispum linn | Leaves | Continuous Hot Percolation and Cold Maceration | 5.8 | 11 |

**Evaluation Of In vitro-Antioxidant Activities:**

**DPPH Method:**

Every extract was tested for its ability to scavenge free radicals using DPPH. The extracts were tested at concentrations of 20,40,60,80,100 µg/mL. The plant extract has shown 93.3% inhibition of the DPPH radical at 100 µg/mL concentration, whereas the standard (Ascorbic acid) has shown 95.34% inhibition at the same concentration. 15.21 µg/mL is IC 50 DPPH activity The results are shown in Table no: 2 and fig no.2

**Table No.2: Free radical scavenging activity of ethanolic extracts of leaves of Abutilon crispum Linn. by DPPH method**

| S.no | **Concentration µg/ml** | **% Inhibition** | |
|---|---|---|---|
| | | **EAC** | **Ascorbic Acid** |
| 1 | 20 | 65.74 ± 0.35 | 71.50 ± 0.50 |
| 2 | 40 | 69.36 ± 0.37 | 76.33 ± 0.33 |
| 3 | 60 | 75.13 ± 0.15 | 81.50 ± 0.43 |
| 4 | 80 | 85.40 ± 0.23 | 91.17 ± 0.40 |
| 5 | 100 | 93.89 ± 0.29 | 95.34 ± 0.20 |

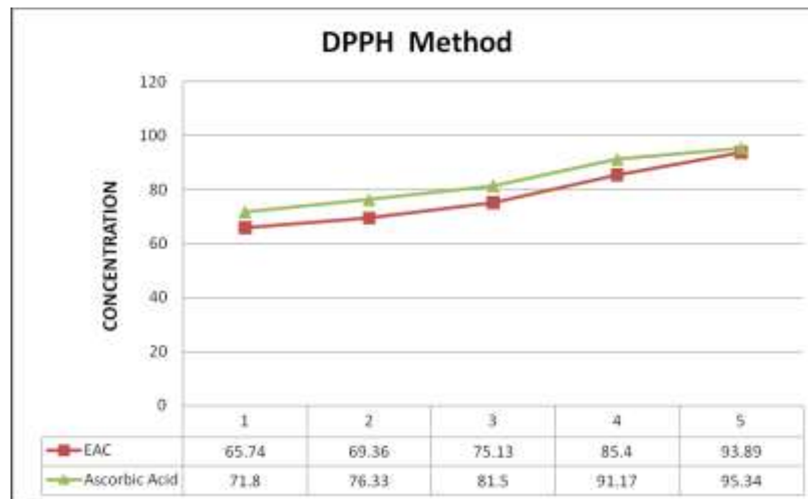



**Fig. no. 2: Free radical scavenging activity of ethanolic extracts of leaves of Abutilon crispum Linn. by DPPH method**

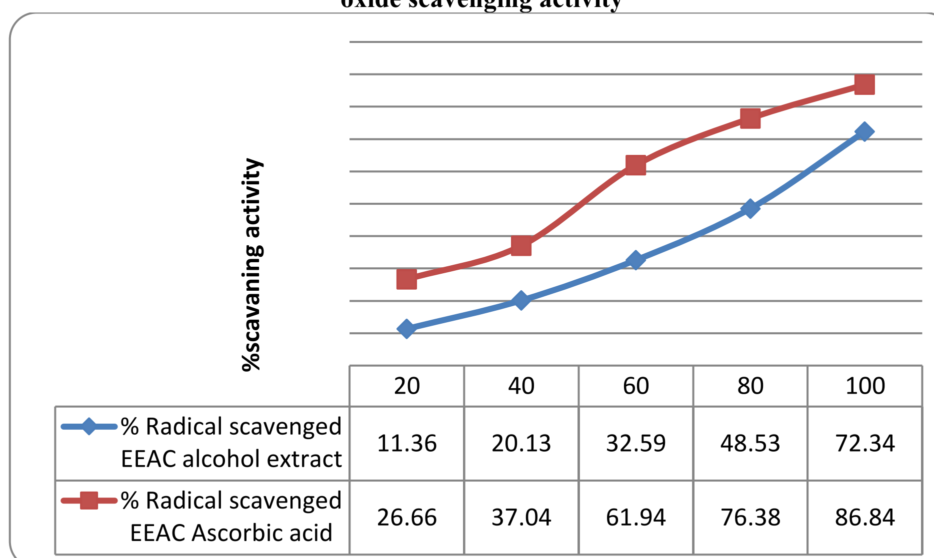
**Nitric oxide scavenging activity:**

The alcohol extract demonstrated 72.34% inhibition of the nitric oxide radicals in this nitric oxide scavenging activity at the maximum concentration of 100µg/mL, in contrast to 86.64% inhibition of the reference chemical, ascorbic acid. 81.23 µg/mL is IC 50 value of the nitric oxide scavenging activity. The results are shown Table no: 3 and Fig no:3.

**Table.No.3: Free radical scavenging activity of ethanolic extracts of leaves of Abutilon crispum Linn. by nitric oxide scavenging activity**

| S. No | Concentration (µg/mL) | % Free Radical scavenged | |
|---|---|---|---|
| | | EEAC | Ascorbic acid |
| 1 | 20 | 11.36±2.09 | 26.66±1.59 |
| 2 | 40 | 20.13±1.27 | 37.04±2.86 |
| 3 | 60 | 32.59±1.68 | 61.94±1.89 |
| 4 | 80 | 48.53±1.62 | 76.38±1.63 |
| 5 | 100 | 72.34±1.57 | 86.84±1.44 |

**Fig. no. 3: Free radical scavenging activity of ethanolic extracts of leaves of Abutilon crispum Linn. by nitric oxide scavenging activity**




**In vitro Anticancer Studies**:

**Trypan Blue Exclusion Assay:**

The effects of alcoholic extracts of the leaves of Abutilon crispum Linn. were evaluated in vitro using the MTT(3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay technique on A549, or human lung cancer cell

lines' epithelial cells.
The anticancer potential of EEAC against A549 cell lines was assessed using the trypan blue exclusion method in vitro cell viability assay.
The findings, which were displayed in Table No. 4 and Figure No. 4, demonstrated that EEAC effectively suppressed the proliferation of A549 tumor cells, with an ED50 value, or 50% of tumor cell death, at 400µg.

**Table. No:4 Effect of ethanolic extract of leaves of Abutilon crispum Linn. on A549 Cell lines by Trypan blue method**

| S.no | Concentration µg | Cell viability | | % of cell death (EEAC) | % Cell Death (5-FU) |
|---|---|---|---|---|---|
| | | Live cells | Dead cells | | |
| 1 | 25 | 197 | 37 | 15.8 | 12.30 |
| 2. | 50 | 184 | 46 | 19.49 | 25.72 |
| 3. | 100 | 168 | 79 | 31.98 | 43.65 |
| 4 | 200 | 135 | 100 | 42.55 | 60.86 |
| 5 | 400 | 95 | 145 | 59.18 | 76.21 |
| 6 | 800 | 38 | 172 | 81.9 | 91.59 |
| 7 | 1600 | 20 | 216 | 91.52 | 94.85 |

**Since p = 0.0079 < 0.05, the difference in % cell death between EEAC and 5-FU is statistically significant.The 95% confidence interval does not include 0, further confirming significance.5-FU is significantly more cytotoxic than EEAC at corresponding concentrations.**

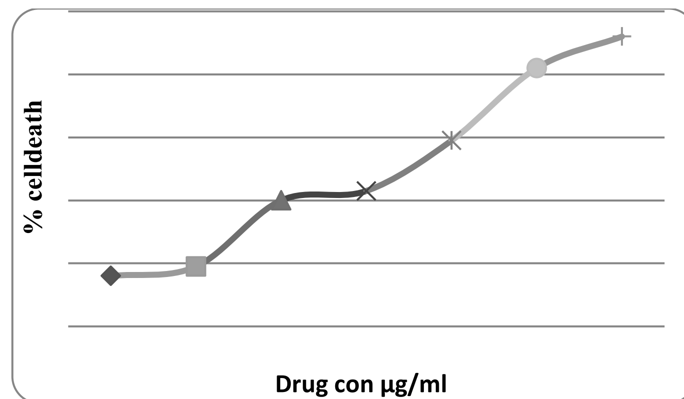



**Fig.No:4 Shows the result of the A549 cell by Trypan blue method**

**MTT ASSAY:**

The absorbance of the corresponding incubated cells in the 96-well plate was used to determine the percentage of survived cells. The results are shown in Table no: -5 fig no.6 and 7.

**Table. No:5 Effect of ethanolic extract of leaves of Abutilon crispum Linn. on A549 cell lines by MTT assay**

| S.no | Concentration µg/ml | % cell viability (EEAC) | % cell viability (5-FU) |
|---|---|---|---|
| 1 | 25 | 95 | 70 |
| 2 | 50 | 78 | 45 |
| 3 | 100 | 70 | 0 |
| 4 | 200 | 58 | - |
| 5 | 400 | 38 | - |
| 6 | 800 | 18 | - |
| 7 | 1600 | 0 | - |

**The p-value (0.0072) is statistically significant (p < 0.05), indicating a significant difference between the two treatments.The 95% confidence interval for the difference in cell viability is positive and does not include zero, further confirming the significance.EEAC shows significantly higher cell viability than 5-FU at the same concentrations (i.e., 5-FU is more cytotoxic).**

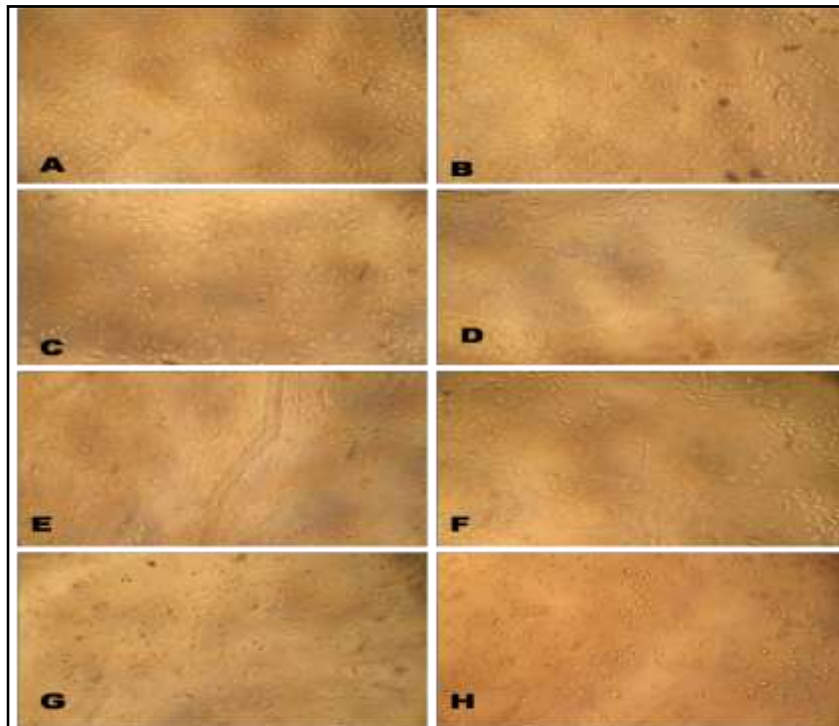



**Fig no - 6: Effect of ethanolic extract of leaves of Abutilon crispum Linn. on crystal formation of A549 cell by MTT assay**

EEAC's impact on A549 cells as assessed by the MTT test. For 24 hours, the cells were treated with various drug concentrations (ranging from 25 to 1600 µg) alterations in cell shape and biochemistry brought on by the EEAC. Only 0.1% DMSO was given to the control group. Under an inverted light microscope, cells were visible. 20X magnification. cell viability represented graphically.

Note: A) control, B) 25 µg, C) 50 µg, D) 100 µg, E) 200 µg, F) 400 µg, G) 800 µg and H) 1600 µg.

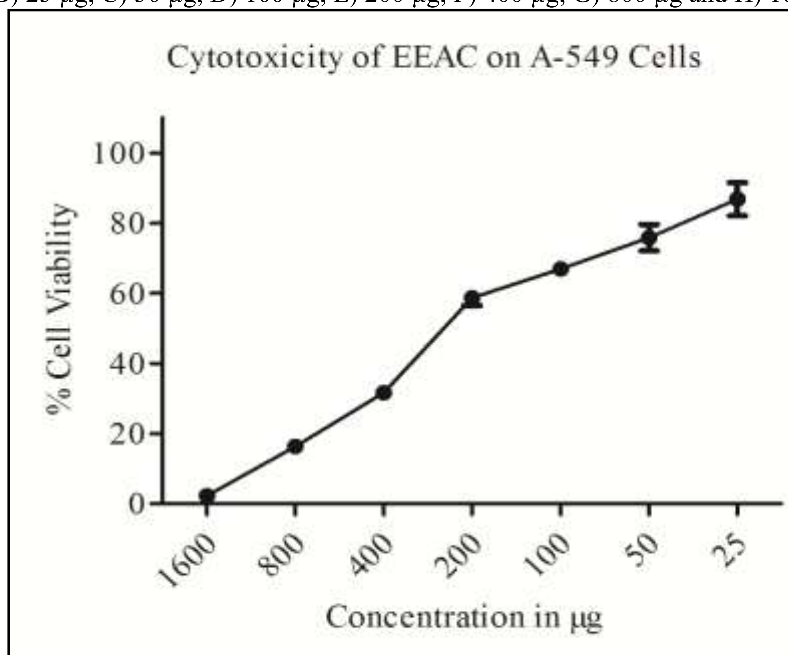



**Fig. No 7: Effect of ethanolic extract of leaves of Abutilon crispum Linn. in % viability on A549 cell at 24hrs by MTT assay method**

**Hoechst dye test:**

Hoechst 33258 staining was used to detect the EEAC at 20 µM for 24 hours, using HCPT as a positive control. Cells treated with the negative control were often blue, as seen in Figure 3. The negative group's cells were a typical shade of blue. The HCPT group, on the other hand, seemed crescent-shaped and compact. The cells' intense blue fluorescence demonstrated the hallmarks of apoptosis. The EEAC-treated cells had transformed, and their nuclei

seemed to be crescent-shaped and extremely condensed. These results show that EEAC could cause A549 cell lines to undergo apoptosis. Figure No. 8 displays the findings.

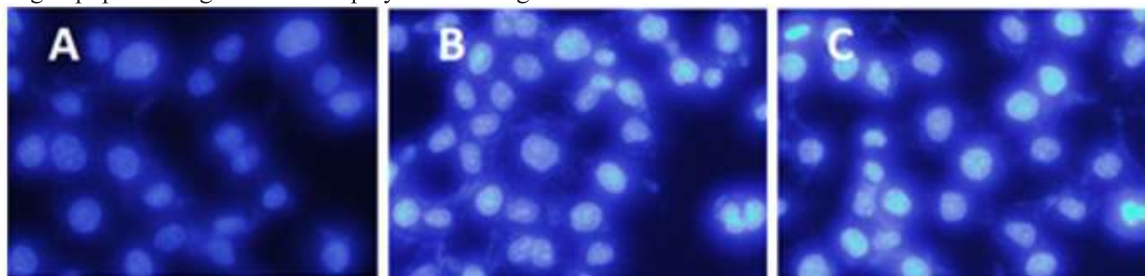



**Fig. No: 8 Effect of ethanolic extract of leaves of Abutilon crispum Linn. on A549celllines by Hoechst 33258 staining.**

**Group A- negative control; Group B - positive control, treated with HCPT (20 µM) for 48 h; Group C treated with EEAC (20 µM) for 24.**

**DNA Fragmentation Assay:**

When A549 cells were exposed to EEAC 255µg for 24 hours, the chromosomal DNA was broken down into smaller fragments, forming a ladder-like pattern of DNA that may indicate the presence of apoptopic cells with obvious nuclear fragmentation (Fig. No. 12). The results are displayed in Fig. No. 9 when compared to the control group.

**Fig. No: 9 Effect of ethanolic extract of leaves of Abutilon crispum Linn. on A549 cell line by DNA fragmentation assay**

## Discussion

The in vitro antioxidant and anticancer activities of the ethanolic extract of Abutilon crispum Linn. leaves (EEAC) were investigated in the current study. Our data prove that the extract shows strong antioxidant activity and strong cytotoxic and pro-apoptotic activities against A549 lung carcinoma cells.

**Antioxidant Activity**

The DPPH and nitric oxide scavenging assays validated the extract's ability to quench free radicals in a dose-dependent fashion. The $IC_{50}$ values (43 µg/mL for DPPH and 62 µg/mL for nitric oxide) indicate potent antioxidant activity, which is similar to that of ascorbic acid. This concurs with previous reports on other Abutilon species, which found phenolic compounds, flavonoids, and alkaloids to be responsible for antioxidant activity.4 These phytochemicals have the potential to block oxidative DNA damage, hence chemoprevention.

**Cytotoxic and Anticancer Activity**

The EEAC exhibited dose-dependent cytotoxicity against both the trypan blue exclusion and MTT assays with $GI_{50}$ and $IC_{50}$ between 325–400 µg/mL. The latter reflects moderate cytotoxic potency and merits further study. That this exceeds 5-FU, which was employed as a positive control, leads to a supposition that EEAC might be acting via an alternative mechanism to elicit anticancer activity, such as by oxidative stress modulation or mitochondrial impairment.

**Apoptosis Induction**

Both Hoechst 33258 staining and DNA fragmentation assays supported apoptosis in A549 cells treated with EEAC. The morphological features of apoptosis nuclear condensation, chromatin fragmentation, and apoptotic bodies were seen. DNA laddering established internucleosomal cleavage, the characteristic of programmed cell death. These findings are consistent with research on other extracts of medicinal plants with proven ability to induce apoptosis through caspase-dependent or mitochondrial pathways.[19]

In plant extract anticancer research, Hoechst 33342 staining has been used to detect apoptosis changes in A549 cells. The dye can bind to DNA and viewed under a fluorescence microscope for nuclear condensation and fragmentation of apoptotic features. In the example of Sesbania grandiflora and Simarouba glauca extracts, studies have reported such apoptotic features in treated A549 cells.[24-29]

Additionally, DNA fragmentation assays have been employed to confirm apoptosis. These involve the extraction of the DNA from treated cells followed by agarose gel electrophoresis for detecting the characteristic "ladder" pattern of apoptotic fragmented DNA. Such a pattern has been observed in A549 cells treated with various plant extracts, confirming the induction of apoptosis.

**Phytochemical Considerations and Mechanistic Insight**

Earlier phytochemical studies of Abutilon crispum have reported flavonoids, terpenoids, and polyphenols. These have been reported to have anticancer activities through ROS modulation, DNA intercalation, and induction of apoptosis. It is probable that the bioactivity seen in this study is a result of interactions among these constituents. LC-MS or HPLC profiling would be helpful to determine the actual compounds involved.

## Conclusion

The current study illustrates that the ethanolic extract of Abutilon crispum Linn. leaves (EEAC) is a potent in vitro antioxidant and anticancer agent. The extract exhibited high free radical scavenging activity in DPPH as well as nitric oxide assays, and significant cytotoxicity against A549 lung carcinoma cells in MTT and trypan blue exclusion assays. Moreover, apoptosis was established by nuclear condensation (Hoechst staining) and DNA fragmentation.

These findings indicate that Abutilon crispum could have bioactive.

phytoconstituents that can induce apoptosis as well as suppress the proliferation of cancer cells. Nevertheless, to gain a complete insight into its medicinal value, additional research should be conducted to isolate and characterize active compounds, reveal molecular mechanisms of action, and determine efficacy and safety in vivo.

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