Original Research

# Evaluation of *in vitro* anti-cancer potential and apoptotic profile of ethanolic plant extract of *Wrightia tinctoria* against oral cancer cell line

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## Abstract

**Background:** For decades, *Wrightia tinctoria* (*W. tinctoria*) has been important in the field of traditional medicine. The identification and isolation of numerous chemically potent and pharmaceutically advantageous phytochemicals isolated from this plant extract has contributed to its resurrection into evidence-based modern medicine. This plant is inexpensive and indigenous to the Indian subcontinent.

**Materials and Method:** An *in vitro* study was carried out using KB (KERATIN-forming tumour cell line HeLa) oral cancer cell line. The control drug doxorubicin along with the test drug *W. tinctoria* was tested for anti-cancer potential by using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and for apoptotic activity by using DNA fragmentation assay and western blotting assay.

**Results:** The study was conducted using MTT assay to determine the cytotoxicity of plant extract against oral cancer cell line by determining the IC50 values at the end of 24 hours by the MTT calorimetric assay. The IC50 value of 48.89 and 9.62 was arrived for the *W. tinctoria* extract and doxorubicin, respectively, by using MTT calorimetric assay. DNA fragmentation assay showed a ladder pattern when treated with 59 µg/mL extract of *W. tinctoria*, and western blot analysis revealed the presence of cleaved caspase 3, confirming the apoptotic potential of the extract.

**Conclusion:** The current study demonstrated that the plant extract has effective anti-cancer and apoptotic potential and can be used as a natural source for the production of new anti-cancer and cytotoxic agents against oral cancer.

**Keywords:** Apoptosis, cancer therapeutics and oral cancer, cytotoxicity, *Wrightia tinctoria*

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## INTRODUCTION

*Wrightia tinctoria (W. tinctoria)* is known by the names 'pala indigo' and 'indrajao' and belongs to the Apocyanaceae family. It is extensively used in the treatment of skin diseases and liver disorders and possesses a broad spectrum of biological activities. Within India, it is found in most of the peninsular and central India except in the northern and north-eastern states. Traditionally, the plant is used to

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treat seizures, wounds, jaundice, leukaemia, gynaecological disorders, toothache, headache, dandruff, diarrhoea, and skin disorders (such as psoriasis, eczema, and scabies). Phytochemical studies have shown the presence of alkaloids, triterpenoids, steroids, flavonoids, lipids, and carbohydrates. *W. tinctoria* is found to have an active constituent of flavonoid, which is cytotoxic. Indirubin, (a flavonoid) is a constituent of *W. tinctoria*, an anti-leukaemia medicine used widely in China. Similarly, *W. tinctoria* has also shown anti-cancer activity against MCF-7 (Michigan Cancer Foundation-7), the human breast cancer cell line.[1-3]

Using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, the MTT reagent can pass through the cell membrane as well as the mitochondrial inner membrane of viable cells, presumably due to its positive charge as well as its lipophilic structure, and is reduced to formazan by metabolically active cells.[4-6] Caspases are central to the apoptosis mechanism because they are both the initiators and executioners. Three pathways exist for activating caspases. The two commonly described initiation pathways are the intrinsic (or mitochondrial) and extrinsic (or death receptor) pathways of apoptosis. Both pathways eventually lead to a common pathway or the execution phase of apoptosis. A third less well-known initiation pathway is the intrinsic endoplasmic reticulum pathway.[7-11]

The protease enzymes known as caspases can be categorised as initiator caspases (caspase-9) and effector caspases (caspase-3, caspase-6, and caspase-7), and they are essential apoptosis-related enzymes.[12] The main feature of apoptosis is DNA fragmentation, which serves as a marker of apoptosis. The DNA ladder assay uses the presence of the 'DNA ladder' pattern of DNA fragments occurring during apoptosis.[13,14] Thus the aim of the study was to investigate the anti-cancer activity and apoptotic profile of ethanolic plant extract *W. tinctoria* against KB cell line (human oral epidermoid carcinoma – K stands for Karolinska and B for Benzon cell line).

## MATERIALS AND METHODS

An *in vitro* study was conducted using an oral cancer cell line (KB cell line). The control drug doxorubicin and ethanolic extract of the test drug *W. tintoria* were tested for various properties, namely cytotoxicity, anti-cancer, and apoptosis. The leaves of the plant *W. tinctoria* were used in this study, and the whole crude extract was used to check for the anti-cancer potential. Ethanolic extract of *W. tinctoria* was preferred in this study because of its ability to dissolve a wide range of compounds, compatibility with the cell line, practical considerations, and cost-effectiveness. Ethical approval was obtained from the institutional ethical committee (Letter No. 2441/IEC/2021 dated 27th May 2021). Various procedures done to assess the anti-cancer and apoptotic properties are as follows,

### 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT assay)

A tetrazolium salt has been used to develop a quantitative colorimetric assay for cell survival and proliferation. The assay detects living but not dead cells; therefore, this method can be used to measure cytotoxicity, proliferation, or activation. Cytotoxicity of *W. tintoria* ethanolic extract was determined against cancer cell lines at 24 hours based on MTT colorimetric assay. Briefly, approximately $4 \times 10^3$ cells were seeded in 96-well plates and allowed to adhere for a period of 24 hours. After being cultivated, the cells were treated with *W.tinctoria* ethanolic extract ranging from 10 μg/mL to 160 μg/mL and doxorubicin at concentrations ranging from 2.5 μg/mL to 40 μg/mL for 24 hours [Figure 1]. At the end of the treatment, the media with the drug was discarded and 20 μL of freshly prepared phosphate-buffered saline (PBS) buffered MTT (5 mg/mL) was added to each well and incubated for 4 h in a $CO_2$ incubator. Next, the plate was dried by removing the liquor from the well, and the formazan formed was carefully dried and dissolved with 100 μL of dimethyl sulfoxide (DMSO). Absorbance was recorded on a microplate reader by using a test wavelength of 570 nm and a reference wavelength of 630 nm. The final concentration of 0.1% DMSO was used as a negative control in all experiments.

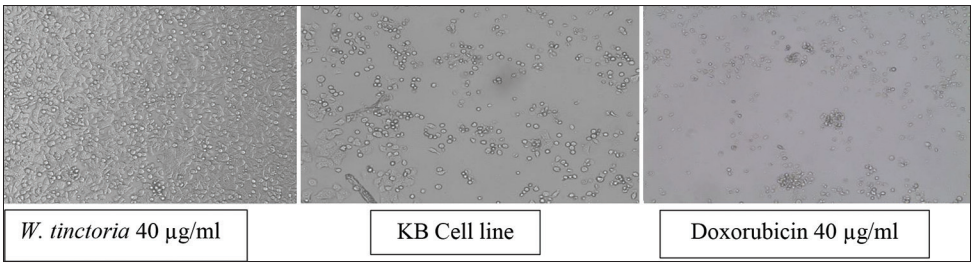


**Figure 1:** KB cell viability at 40-μg/mL concentration of *W. tinctoria* ethanolic extract. The IC50 value of 48.89 and 9.62 was arrived for the *W. tinctoria* extract and doxorubicin, respectively, based on MTT calorimetric assay

**Table 1: Viability of the cell at varying concentrations of doxorubicin**

| Concentration µg/mL | % Viability | | |
|---|---|---|---|
| | R1 | R2 | R3 |
| 2.5 | 89.895833 | 91.145833 | 91.458333 |
| 5 | 72.8125 | 71.875 | 73.28125 |
| 10 | 50.520833 | 50.104167 | 50.729167 |
| 20 | 23.697917 | 24.791667 | 23.072917 |
| 40 | 3.5416667 | 3.8020833 | 5.46875 |

**Table 2: Viability of the cell at varying concentrations of *W. tinctoria* ethanolic extract**

| Concentration µg/mL | % Viability | | |
|---|---|---|---|
| | R1 | R2 | R3 |
| 10 | 88.854167 | 90.260417 | 91.927083 |
| 20 | 78.177083 | 71.927083 | 75.9375 |
| 40 | 58.020833 | 60.520833 | 60.833333 |
| 80 | 33.645833 | 35.208333 | 33.385417 |
| 160 | 12.447917 | 13.177083 | 12.708333 |

### DNA fragmentation assay

DNA fragmentation ladder assay was performed using the agarose gel electrophoresis method. Briefly, cancer cells were treated with respective $IC_{50}$ concentrations of the test drug and positive control doxorubicin in tissue-culture dishes. At the end of the 24/48-hour treatment period, bounded and unbounded cells from the culture dish were harvested and washed with ice-cold PBS. The cells were lysed with DNA lysis buffer by incubating the cells for 3 hours at 55°C in a cocktail of 50 mM Tris–HCL (Tris (hydroxymethyl) aminomethane hydrochloride-pH 8.0), 10 mM EDTA (Ethylenediamine tetraacetic acid), 0.5% N-lauroylsarcosine, and 2 mg/mL proteinase K, and then subjected to RNAse treatment for another 3 hours. The genomic DNA was extracted with a saturated phenol-chloroform-isoamylalcohol reagent. The extracted DNA was purified by treating DNA with 0.1 vol sodium acetate (pH 4.8) and 2.5 vol ethanol at 20°C for 30 minutes, and further, the DNA was precipitated with ice-cold 70% ethanol and pelleted by centrifugation. DNA samples isolated were electrophoresised in 2% agarose gel, and the DNA in the gel was visualised under UV-transilluminator after staining with 5 µL of Redsafe dye.

### Western blotting

To determine the protein level of cleaved caspases, the hallmark of apoptosis was evaluated by western blot assay on oral cancer cell line (KB cell line) against doxorubicin and ethanolic extract of *W.tinctoria*. Approximately 70%–80% of confluent human cancer cells were treated with doxorubicin and ethanolic extract of *W. tinctoria* for 24 hours. Total proteins were extracted using *Thermo Scientific* M-PER *Mammalian* Protein Extraction Reagent with protease inhibitor. Supernatants were collected and stored at − 80°C until use for western blot analysis. Thirty micrograms of protein of each test and control group were fractionated on 10% SDS-polyacrylamide gels [Bio-Rad, Hercules, CA]. The proteins were transferred to the Hybond-P polyvinylidene difluoride [PVDF] transfer membrane [Amersham, Pittsburgh, PA]. The membranes were then incubated with the cleaved caspases-3 primary antibodies followed by a horseradish peroxidase secondary antibody and developed with enhanced chemiluminescence reagent using a gel documentation system. The western blots were quantitated by densitometry analysis using gelQuant software/ImageJ software.

## RESULTS

### MTT assay

At the end of 24 hours, the IC50 value was determined by the MTT calorimetric assay by conducting the study at various concentrations of the test drug and the control drug. The mean IC50 values were obtained by triplicating the samples. The results obtained are tabulated [Tables 1 and 2].

## APOPTOTIC PROFILE ASSESSMENT

### DNA fragmentation assay

At the end of 24/48 hours, the DNA lysis was carried out and the DNA samples were studied by gel electrophoresis. DNA in the cell was visualised under a UV trans-illuminator after staining with 5µL of Redsafe dye. The assay revealed a typical ladder pattern when treated with 59 µg/mL extract of *W. tinctoria*; the pattern was similar to the pattern observed in the control drug doxorubicin [Figure 2].

### Western blot assay

The protein levels of caspase-3 were quantitated by densitometry analysis. The results revealed the expression of cleaved caspase-3, which was very significant at 59 µg/mL of the extract, confirming the apoptotic potential of the extract [Figure 3].

## DISCUSSION

Herbal extraction and analysis play a crucial role in the advancement of herbal formulations, including quality control and modernisation. Substantial evidence has highlighted the efficacy of herbal medicines and their natural-product chemical constituents as anti-inflammatory, anti-diabetic, anti-nociceptive, hepatoprotective, anti-bacterial, anti-fungal, anti-viral, anti-psoriatic, anti-cancerous, anthelmintic, aphrodisiac, analgesic, and anti-pyretic agents.[5,15-18]

Oral cancer has shown only a modest increase in survival rate over the past few decades despite considerable

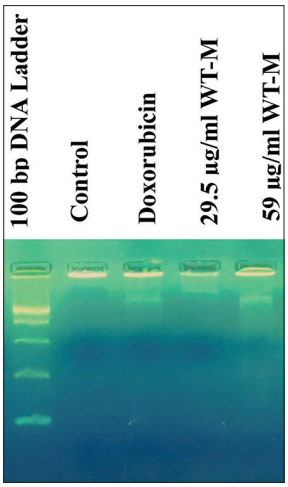


**Figure 2:** Represents DNA fragmentation assay showing ladder pattern from DNA extracted KB cells treated with doxorubicin and *W. tinctoria* extract

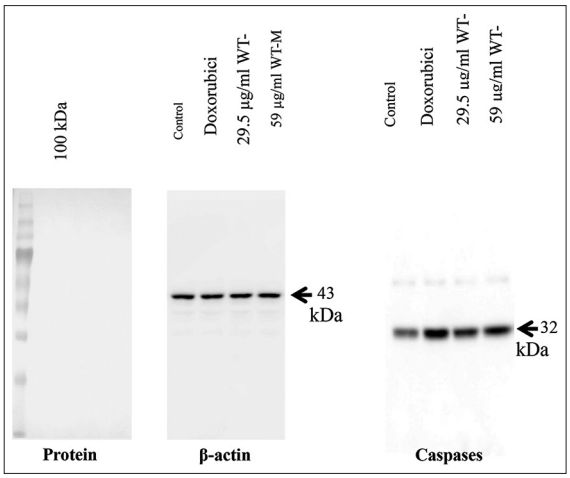


**Figure 3:** Caspase-3 protein expression by western blotting assay

modifications in the management and treatment choices for these neoplasms. According to the most recent data available, oral cancer has approximately 45% 5-year survival rate. This pattern necessitates dynamic approaches to treat the cause of the cancer with traditional methods using plant-based medicines.[19]

The extensive literature survey revealed that *W. tinctoria* is an important medicinal plant with a diverse pharmacological and phytochemical spectrum. The plant extract has already been proven to exhibit anti-melanoma activities, preventing metastasis and angiogenesis. The extract induces significant cytotoxicity in melanoma cells.[20] The plant shows the presence of many chemical constituents such as steroids, triterpenoids, saponins, tannins, phenols, flavonoids, glycosides, carbohydrates, alkaloids, and polyphenols, which are responsible for various pharmacological and medicinal properties.[6,21]

To our knowledge, this is the first investigation using *W. tinctoria* plant extract on the oral cancer cell line for its cytotoxic effects and apoptotic potential. In the present study, the ethanolic extracts demonstrated low OD and IC50 values when treated with oral cancer cell lines. The cell viability was reduced to 12.70% when treated with 160 µg/mL of the ethanolic extract of *W. tinctoria* explaining its cytotoxicity against the oral cancer cell line. This finding supports the fact that natural products, such as medicinal extracts, crude compounds, or pure standardised extracts, could provide a valuable source for new medicines due to the availability of bioactive compounds, accessibility, safety, and low costs as suggested by Cos *et al*.[13] An accessible technique for quickly identifying apoptotic changes in cell populations is the DNA ladder test. Working with cell lysates is made possible by this procedure, which also does not require any specialised lab equipment.[22]

The current study's findings demonstrated that even for plant-based drug analysis, this approach can be regarded as a helpful way to analyse DNA damage and fragmentation without the requirement for time-consuming laboratory procedures or costly kits or equipment.[23] DNA fragmentation assay showed a ladder pattern when treated with 59 µg/mL extract of *W. tinctoria*. This suggests that the degradation of genomic DNA due to the activation of endonuclease is one of the early events of cell degradation. Western blot assay suggests that activation of the apoptotic process can be efficiently done by ethanolic extract of *W. tinctoria*. Thus, the above-mentioned finding confirms the role of *W. tinctoria* as an apoptotic agent.

## CONCLUSION

The findings of this study demonstrate the effective anti-cancer and cytotoxic effects of *W. tinctoria* plant extract that can be used as a natural source for the production of new anti-cancer and cytotoxic agents against oral cancer. However, further experimental and clinical studies are required to isolate and analyse their chemical compositions for final evaluation.

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### Conflicts of interest
There are no conflicts of interest.

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