Anticancer, antioxidant potential and profiling of polyphenolic compounds of \textit{Wrightia tinctoria} Roxb. (R.Br.) bark

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\textbf{Abstract}

\textit{Wrightia tinctoria} Roxb. (R.Br.) is an Ayurvedic remedy, ethnomedically used in the treatment of various ailments. The present work was carried out to evaluate the anticancer and antioxidant activity as well as total phenolic and phytochemical contents of \textit{W. tinctoria} bark methanolic extract (WTBM) by high-performance liquid chromatography (HPLC)-diode array detector. Antiproliferative activity of WTBM was evaluated against MDA-MB-231 and MCF-7 cancer cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, colony formation, and Hoechst staining. In addition, the antioxidant potential was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation decolorization assay. Total phenolic content was assessed by Folin–Ciocalteu method. The results demonstrated that WTBM exhibited significant antiproliferative effect against MDA-MB-231 ($IC_{50} = 88.9 \pm 1.27 \mu g/ml$) and MCF-7 ($IC_{50} = 45.71 \pm 7.74 \mu g/ml$) cancer cells in time- and dose-dependent manner. WTBM significantly suppresses colony formation and induces apoptosis in both MDA-MB-231 and MCF-7 cells as evident by morphological assessment, clonogenic assay, and Hoechst staining. The total phenolic content of WTBM was found to be 30.3 gallic acid equivalent mg/g dry weight of bark extract while $IC_{50}$ value for DPPH and ABTS radical scavenging activity was $72.2 \pm 2.8 \mu g/ml$ and $45.16 \pm 1.95 \mu g/ml$, respectively. HPLC analysis showed the presence of gallic acid, rutin, and quercetin in WTBM. These findings demonstrated that WTBM significantly inhibited proliferation of breast cancer cells and induced apoptosis, suggesting the potential chemopreventive activity of \textit{W. tinctoria} bark.

\textbf{Key words:} Antioxidant, antiproliferative, breast cancer, high-performance liquid chromatography, \textit{Wrightia tinctoria}

\textbf{INTRODUCTION}

Cancer is one of the most life-threatening and complex diseases involving cell transformations, dysregulation of apoptosis, proliferation, invasion, angiogenesis, and

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Breast cancer is the second most common type of cancer diagnosed in developing countries and most common malignancy type diagnosed in developed countries. According to the International Agency for Research on Cancer in 2008, there were approximately 1.38 million new cases of breast cancer, and by the end of 2020, this figure is expected to escalate to 1.7 million.[3] Despite the recent advancements in currently available therapies including radiation, chemotherapy, immunomodulation, and surgery, there is an imperative need for alternative strategies for efficient cancer management.

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Phytochemical screening
Screening of WTBM for the presence of various phytochemical constituents was evaluated as standard method described by Trease and Evans, 1989.[6]

MATERIALS AND METHODS

Collection of plant material and extract preparation
The bark of W. tinctoria was collected from Ahraura, Mirzapur District, Uttar Pradesh, India, and identified by Dr. Sharad K. Srivastava, Department of Pharmacognosy, NBRI, Lucknow. The powdered plant material was percolated in 95% methanol; after 72 h, combined extract was filtered and concentrated under reduced pressure and controlled temperature on a rotatory evaporator. The residue obtained dried completely and named as WTBM and kept in a desiccator for further study.

Cell culture
Human breast cancer MDA-MB-231 (human breast adenocarcinoma, triple-negative, tumorigenic, and invasive) and MCF-7 (human breast adenocarcinoma, ER-positive, and invasive) cells were obtained from ATCC, USA, and cultured with Roswell Park Memorial Institute medium supplemented with 5% fetal bovine serum (FBS) and 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin, in a humidified 5% CO₂ incubator at 37°C.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
MDA-MB-231and MCF-7 (5000 cells per well) cells were seeded in 96-well culture plate at 37°C for 24 h, followed by treatment with different concentrations of WTBM and cisplatin (positive control) in triplicate. 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5.0 mg/ml in phosphate-buffered saline [PBS]) was added to each well and further incubated for 3–4 h. Medium containing MTT was gently aspirated and dimethyl sulfoxide (100 µl/well) was added to dissolve the formazan crystal and absorbance was measured at 540 nm.

Morphological analysis
Morphological changes in MDA-MB-231and MCF-7 cells after the treatment with WTBM were studied by phase-contrast inverted microscope. Cells shrinkage, membrane blebbing, chromatin condensation, and fragmentation were observed.

Colony formation assay
To determine the growth inhibition effects, MDA-MB-231 and MCF-7 cells were treated with different concentrations (25–200 µg/ml) of WTBM for 24 h. Colonies formed after 7 d were fixed with 4% paraformaldehyde, stained with 0.05% Coomassie blue, and counted under an inverted microscope. The following formula was used to calculate the colony inhibition rate:

Colony inhibition rate (%) = ([Colony number of control group − Colony number of treated group]/Colony number of control group) × 100)

Hoechst 33342 staining
Changes in nuclear morphology of MDA-MB-231 and MCF-7 cells after WTBM treatment were evaluated with the help of Hoechst 33342 staining. Briefly, cells (200 cells per well) were grown on coverslip in a six-well plate and treated with different concentrations (12.5–50 µg/ml) of WTBM for 18–20 h. Cells were harvested and fixed with 4% paraformaldehyde for 15 min at 4°C. Cells were then washed with ice-cold PBS three times and stained with Hoechst 33342 dye (1 µg/ml in PBS) and incubated for 2 min in the dark, at room temperature. Fixed cells were again washed three times with ice-cold PBS and observed under a phase-contrast and fluorescence inverted microscope with standard excitation filters.

Total phenolic content
Total phenolic content of WTBM was estimated by Folin–Ciocalteu reagent as determined by Singleton and Rossi with slight modifications.[7] In brief, 0.1 ml of WTBM (1 mg/ml) was added to 1 ml 10% Folin–Ciocalteu reagent and incubated for 5 min. After 5 min, 0.8 ml solution of Na₂CO₃ (10% w/v in distilled water) was added and

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incubated for 15 min; thereafter, absorbance was measured at 765 nm. The total concentration of phenolic content in the tested extract was determined as the gallic acid equivalent (GAE) mg/g of dry extract.

2,2-diphenyl-1-picrylhydrazyl and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical scavenging activity

Different working concentrations (12.5–800 µg/ml) of WTBM and standard (ascorbic acid) were prepared from stock solution (1.0 mg/ml) in 1.0 ml volume in a test tube. Then, 1.0 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.1 mM) was added to each test tube and was shaken gently. Mixture stands for 20–30 min and absorbance was taken at 517 nm using an ultraviolet-visible spectrophotometer.

The 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation (ABTS+) solution was prepared by the mixing of 7 mM ABTS and 2.45 mM potassium persulfate after incubation at 23°C in the dark for 16 h. 3.9 ml ABTS+ solution (absorbance = 0.700 ± 0.005) was added to 0.1 ml of the different concentrations of WTBM and incubated for 6 min; thereafter, absorbance at 734 nm was recorded. Rutin was used as positive control. The absorbance was recorded for each concentration, and percent quenching of DPPH and ABTS was calculated.

Radical scavenging activity (%) = ([A0 − A1/A0] × 100)

Where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance of the compound.

High-performance liquid chromatography analysis

The phenolic compounds (gallic acid, rutin, and quercetin) in WTBM were determined with the help of an HPLC equipped with an integrated system with Agilent 1260 Infinity HPLC-DAD. Data were analyzed using Agilent ChemStation revision B.04.01 software (Agilent Technologies, Santa Clara, CA 95051, United States). The chromatographic separation was achieved with the Agilent Zorbax C-18 (4.6 mm × 250 mm, 5 µm) column thermostated at 25°C. For gradient elution, two solvents were used; one of these consists of 0.3% HCOOH in water and the other was consists of only methanol. 10 mg of crude extract was dissolved in 10 ml methanol for each sample to achieve a concentration of 1 mg/ml. The injection volume and flow rate were 5 µl and 1.0 ml/min, respectively, and detection was carried at 280 nm by DAD.

RESULTS

Phytochemical analysis

The qualitative preliminary phytochemical analysis revealed that the WTBM showed a positive test for alkaloids, tannin, terpenes, steroids, coumarins, and flavonoids. However, glycosides and saponins were absent.

Effect of Wrightia tinctoria bark methanolic extract on proliferation of breast cancer cells

To assess antiproliferation activity, MDA-MB-231 and MCF-7 cells were exposed to increasing dose of WTBM for 24, 48, and 72 h. We observed dose- and time-dependent cytotoxic effects of WTBM on cells viability. WTBM caused 2.1 ± 0.6–68.16 ± 0.65% and 9.21 ± 2.6–69.41 ± 1.39% growth inhibition in MDA-MB-231 and MCF-7 cells, respectively, at the concentration ranged from 6.25 to 400 µg/ml after 24 h and exposure. However, IC50 for MDA-MB-231 cells was 88.9 ± 1.27 µg/ml and for MCF-7 cells was 45.71 ± 7.74 µg/ml after 24 h of exposure [Figure 1]. We observed a decrease in IC50 values for MDA-MB-231 and MCF-7, 60.7 ± 8.4 µg/ml and 37.03 ± 4.62 µg/ml, respectively, after 48 h of WTBM treatment, while standard cisplatin showed the IC50 of 41.91 ± 2.13 µg/ml and 13.5 ± 1.39 µg/ml, respectively, for MDA MB-321 and MCF-7 cells.

Effect of Wrightia tinctoria bark methanolic extract on morphological changes by phase-contrast inverted microscope

Morphological changes of MDA-MB-231 and MCF-7 cells upon exposure using WTBM was observed. Usually,
cells undergoing apoptosis display a similar pattern of morphological changes including blebbing, loss of cell membrane symmetry and attachment, chromatin condensation and fragmentation, and cell shrinkage; however, untreated cells did not show the apparent apoptotic morphological changes. As the concentration increased, enlargement of cells was conspicuously observed [Figure 2].

**Effect of* Wrightia tinctoria* bark methanolic extract on colony formation of breast cancer cells**

To further investigate the antiproliferative effect of WTBM, the colony formation assay was used to assess the survival of tumorigenic cells. At the concentration range of 25 μg/ml to 400 μg/ml, colony formation was significantly reduced in WTBM treated cells when compared to control/untreated cells [Figure 3]. A significant decrease of 17.63%–72.02% and 15.4%–98.16% were observed after the treatment of WTBM against MDA-MB-231 and MCF-7 cells showing dose-dependent antiproliferative activity.

**Effect of* Wrightia tinctoria* bark methanolic extract on morphological changes and quantification of apoptosis by Hoechst 33342 staining by fluorescence microscope**

Hoechst 33342 staining in MDA-MB-231 and MCF-7 cells revealed that both cells possess marked morphological changes in a dose-dependent manner after exposure to WTBM. In untreated cells, slightly blue and homogeneous cells with intact normal nuclei were observed [Figure 4], while in the case of treated cells, the blue fluorescence was much brighter due to nuclear morphological changes. Significant changes such as cell shrinkage, chromatin

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**Figure 2:** Morphological changes of MDA-MB-321 cells and MCF-7 cells after treatment with *Wrightia tinctoria* bark methanolic extract for 24 h and imaged by inverted phase-contrast microscope

**Figure 3:** *Wrightia tinctoria* bark methanolic extract inhibited colony formation in MDA-MB-321 and MCF-7 cells. Cells were seeded into 6-well plate, treated with various concentrations of *Wrightia tinctoria* bark methanolic extract for 24 h, fixed and stained with 4% paraformaldehyde and 0.05% Coomassie blue.
condensation and fragmentation, and abnormal morphology in cells were observed.

**Total phenolic content**
Total phenolic content was determined in comparison with standard gallic acid, and the results were expressed in terms of mg GAE/g dry extract. Total phenolic content (TPC) was found to be 30.3 GAE mg/g dry weight of bark extract.

**Effect of *Wrightia tinctoria* bark methanolic extract on 2,2-diphenyl-1-picrylhydrazyl and 2,2- azino-bis-3-ethylbenzothiazoline-6-sulfonic acid-free radicals**
The results of DPPH radical scavenging activity are expressed as percentage inhibition of DPPH radical. At the concentration ranged from 12.5 to 800 µg/ml, the scavenging activity of WTBM was 4.37%–85.6%. The WTBM exhibited high free radical scavenging activity with an IC$_{50}$ value of 72.2 ± 2.8 µg/ml [Figure 5]. The efficiency of inhibition was compared with standard ascorbic acid with an IC$_{50}$ value of 2.04 ± 0.1 µg/ml.

The scavenging activity of ABTS radical by WTBM is shown in Figure 5. The data clearly indicated that WTBM is an effective scavenger of ABTS radical and this activity is comparable to rutin. At the concentration range of 12.5–800 µg/ml, the ABTS radical scavenging activity of WTBM was found to be 12.49%–83.24%. The IC$_{50}$ value of rutin was found to be 4.31 ± 0.56 µg/ml while IC$_{50}$ value of WTBM was 45.16 ± 1.95 µg/ml.

**High-performance liquid chromatography analysis**
Among all the polyphenols analyzed, concentration of rutin (2.181 ± 0.09 µg/gm of dry weight) was highest, followed by quercetin (1.861 ± 0.06 µg/g of dry weight) and gallic acid (0.28 ± 0.2 µg/g of dry weight) in WTBM [Figure 6].

**DISCUSSION**
The management of triple-negative breast cancer is ever challenging due to its highly aggressive nature, limited treatment options, and extremely poor prognosis. The

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**Figure 4:** *Wrightia tinctoria* bark methanolic extract induced apoptosis cell death in MDA-MB-231 and MCF-7 cells. Detection of morphological apoptosis with Hoechst 33342 staining. Cell morphology was observed under a fluorescence microscope. Arrows indicate the cells with DNA fragmentation.

**Figure 5:** Percent inhibition of 2,2-diphenyl-1-picrylhydrazyl free radical and 2,2- azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical after treatment with *Wrightia tinctoria* bark methanolic extract. The data were represented as the mean ± standard deviation (n = 3)
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apoptotic and antiproliferative effects of WTBM on triple-negative and highly aggressive MDA-MB-231 cells are intriguing. We observed typical morphological change such as cell shrinkage, nuclear condensation, and formation of round apoptotic bodies in both MDA-MB-231 and MCF-7 cells after exposure with WTBM. These findings suggest the involvement of apoptotic pathways behind antiproliferative property; however, more in-depth studies are needed to understand molecular cascades and apoptotic signaling induced by \textit{W. tinctoria}. Although we have not tested the cytotoxic effect of \textit{W. tinctoria} on non-tumorigenic cells. However, this plant has been widely used in India for the treatment of various disorders without any observed toxicity as clearly indicated by a study on acute oral cytotoxicity by Jain and Bari 2011, using a very high oral dose of 2500 mg/kg crude extract showed no mortalities or adverse effects in mice.\cite{8}

Natural chemopreventive agents exhibiting antiproliferative and apoptotic effects on cancer cells usually have antioxidant property that targets signaling molecules, protecting or preventing cells from further damage or transforms into cancerous cells.\cite{9} Free radical scavenging assay demonstrated that \textit{W. tinctoria} exhibited the potent inhibitory property of DPPH and ABTS free radicals in a concentration-dependent manner. There are many reports demonstrating the protective role of antioxidants in cancer. It has been reported that supplementation with antioxidants and vitamins decreases incidence of cancer, and dietary phytochemicals slow down progression of cancer.\cite{10} Due to its potent antiradical property, \textit{W. tinctoria} may act as an external antioxidant and prevent propagation of reactive oxygen species-initiated cellular damage. There is accumulating evidence indicating that natural compounds not only offer protection against oxidative reactions but also suppress cell proliferation in both \textit{in vivo} and \textit{in vitro} preclinical models.\cite{11}

Phenols are most important antioxidants due to their idyllic structure chemistry for free radical scavenging activity; they can act as reducing agents, metal chelators, and free radical quenchers either by donating a hydrogen atom or electron to free radicals or by quenching singlet or triplet oxygen or decomposing peroxides.\cite{12} The high amount of TPC present in \textit{W. tinctoria} (30.3 mg/g) may contribute to its antiproliferative and free radical scavenging properties. The phytochemical analysis of \textit{W. tinctoria} revealed the presence of three major groups of polyphenolic compounds, i.e. phenols, flavonoids, and tannins, which may provide a synergistic effect to enhance its anticancer properties. Gallic acid selectively induces apoptosis in various cancer cells.\cite{13} Rutin is a bioflavonoid that inhibits carcinogenic processes and a potent chemotherapeutic agent.\cite{14} Quercetin also has been reported to possess antiproliferative activity against different cancer cells.\cite{15} The presence of gallic and other flavonoid compounds (rutin and quercetin) in \textit{W. tinctoria} bark significantly contributes its anticancer and antioxidant properties.

**CONCLUSION**

In conclusion, our study provides compelling evidences that \textit{W. tinctoria} bark extract showed significant inhibitory potential against breast cancer cells. The antiproliferative activity of \textit{W. tinctoria} is highly concurrent with apoptosis induction in breast cancer. On the basis of our results it may be stated that the cell inhibiting potential and apoptotic properties of \textit{W. tinctoria} in breast cancer cells may be due to the presence of different phytochemicals and phenolic contents. However, further studies are needed to isolate...
and identify *W. tinctoria* active compounds and to unveil its mechanism involved in breast cancer inhibition

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

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