

Selective Cytotoxicity and Pro-apoptotic Activity of Stem Bark of *Wrightia tinctoria* (Roxb.) R. Br. in Cancerous Cells

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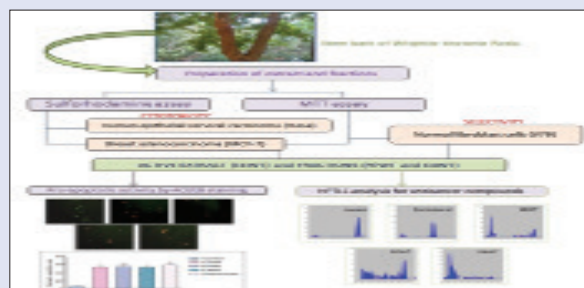
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ABSTRACT

Background: *Wrightia tinctoria* (Roxb.) R. Br. is a widely available shrub in India used traditionally in various ailments, including cancer. However, the anticancer activity of the bioactive fractions has not been validated scientifically. **Objective:** To investigate the anticancer potential of stem bark of *W. tinctoria* and establish its phytochemical basis. **Materials and Methods:** The ethanol extract and subsequent fractions, petroleum ether, ethyl acetate, n-butanol, and aqueous were prepared by standard methods. *In vitro* cytotoxicity was determined in MCF-7 (breast) and HeLa (cervical) adenocarcinoma cells, and V79 (nontumor fibroblast) cells and apoptogenic activity in MCF-7 cells by acridine orange (AO)/ethidium bromide (EB) staining. Additionally, the antioxidant potential was evaluated using suitable methods. High-performance thin layer chromatography (HPTLC) analysis was performed for identification of active phytoconstituents. **Results:** Petroleum ether and ethyl acetate fractions were most potent with IC₅₀ values of 37.78 and 29.69 µg/ml in HeLa and 31.56 and 32.63 µg/ml in MCF-7 cells respectively in the sulforhodamine B assay. Comparable results were obtained in HeLa cells in 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide assay and interestingly, the fractions were found to be safe to noncancerous fibroblast cells. Both fractions induced significant ($P < 0.05$) apoptotic morphological changes observed by AO/EB staining. Moreover, extract/fractions exhibited excellent inhibition of lipid peroxidation with the ethyl acetate fraction being most active (IC₅₀: 23.40 µg/ml). HPTLC confirmed the presence of two anti-cancer triterpenoids, lupeol, and β-sitosterol in active fractions. **Conclusion:** Extract/fractions of *W. tinctoria* exhibit selective cytotoxicity against cancerous cells that is mediated by apoptosis. Fractions are less toxic to noncancerous cells; hence, they can be developed as safer chemopreventive agents. **Key words:** β-sitosterol, anticancer, chemoprevention, high-performance thin layer chromatography, lupeol

SUMMARY

- Petroleum ether and ethyl acetate fractions were most active and exhibited dose-dependent cytotoxicity in HeLa and MCF-7 cells.
- Fractions were relatively less toxic to non-tumor fibroblast cells demonstrating its selectivity to cancer cells.
- Fractions exhibited pro-apoptotic activity in MCF-7 cells in AO/EB staining.
- Lupeol and β-sitosterol were identified as anticancer constituents by HPTLC.



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INTRODUCTION

Cancer is characterized by uncontrolled cell proliferation in which multiple signaling pathways are affected simultaneously. Irregularities in DNA repair, cell cycle, apoptosis, and redox balance are the cornerstones of cancer progression and therapeutic strategies targeting these pathways are being explored. Redox imbalance results in a condition known as "oxidative stress" that is an important mediator of cancer initiation. Several signal transduction factors of cellular survival and proliferation like nuclear factor (erythroid-derived-2), p53, activator protein-1, mitogen-activated protein kinase, and executors of cell death like caspases, B-cell lymphoma 2 (Bcl-2), and cytochrome C are sensitive to oxidative imbalance.^[1] Even a moderate increase in free radicals and reactive oxygen species (ROS) promote cellular proliferation, whereas excessive levels of ROS can lead to serious and irreversible oxidative injury to cellular bio molecules (DNA, proteins, lipids, and carbohydrates) altering the structural and functional integrity. Since, redox imbalance is essentially involved in cancer pathogenesis, restoring redox homeostasis is an attractive strategy to prevent cancer initiation and worsening of the disease.^[1-3]

Chemoprevention aids in the arrest or reversal of malignant progression by the exogenous supply of antioxidants to restore the redox balance of premalignant cells. Compounds derived from plants could serve as excellent chemopreventive agents by mitigating oxidative stress and redox imbalance impeding the progression of cancer. Moreover, natural compounds are known to act on multiple cancer targets simultaneously. Current chemotherapeutic agents have limited selectivity toward cancer cells as compared to normal cells mediated only through the rapid

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division of malignant cells. As a consequence, fast-dividing normal cells like the hematopoietic cells, hair cells, and cells of the gastrointestinal tract are also affected leading to unwanted adverse effects associated with chemotherapy.^[4,5] Lack of selectivity and modulation of multiple targets simultaneously are the key unmet challenges in cancer therapy. The synergistic effects of multiple plant constituents offer selectivity, safety, and efficacy by modulation of key cancer-causing mediators simultaneously and are definitely worth exploring.^[6-8]

Wrightia tinctoria (Roxb.) R. Br. belonging to the family Apocyanaceae is commonly known as Indrajau or Dudhi. It is a small deciduous medicinal plant used extensively in the traditional systems of medicine. Traditionally, the plant has been used for dysentery, piles, skin diseases, flatulence, bilious conditions,^[9] jaundice, inflammatory conditions,^[10] wound healing,^[11] and in the treatment of cancer.^[12] The bark is also used in the treatment of psoriasis, pyrexia, dysentery, diarrhea, hemorrhage, and as an antidote for snake poison.^[13] Phytoconstituents present in the plant include glycosides, steroids, triterpenoids, saponins, tannins, phenolics, and flavonoids.^[14] Triterpenes like α -amyrin, β amyrin, lupeol, and β -sitosterol have been isolated from the bark of *W. tinctoria*.^[15] A recent study also confirmed the anticancer activity of the alcohol extract in MCF-7 and HL-60 cells.^[12] However, to the best of our knowledge, the plant fractions have not been evaluated for their cytotoxicity and selectivity toward cancer cells.

Here, we investigate the cytotoxic activity of ethanol extract/fractions of the bark of *W. tinctoria* in human carcinoma cells, noncancerous fibroblasts and determined the possible mode of action of cell death. High-performance thin layer chromatography (HPTLC) analysis was performed to establish the possible phytochemical basis of anticancer activity. Additionally, free radical scavenging and antioxidant capacity were evaluated to strengthen its use as a chemo preventive agent.

MATERIALS AND METHODS

Collection of plant

The bark of *W. tinctoria* collected during September 2009 from Koppa, Karnataka, India was verified by Dr. M. M. Setty, Professor at Manipal College of Pharmaceutical Sciences, Manipal University, India. The voucher specimen (PP586) of the bark has been preserved in the herbarium of Manipal College of Pharmaceutical Sciences for future reference.

Extract preparation

The bark was oven dried at 40°C, coarsely powdered and extraction was carried out with 95% ethanol using a Soxhlet apparatus at 80°C. The extract was concentrated in a Rotavapor at 40°C under vacuum. The dried extract was suspended in distilled water, and solvent-solvent partitioning was carried out with petroleum ether (60–80°C), ethyl acetate, and n-butanol. The yield of ethanol extract was 8.1% w/w and subsequent fractions were petroleum ether (12.2%), ethyl acetate (8.2%), n-butanol (7.1%), and aqueous (68.2%) w/w.

Phytochemical analysis

Phytochemical screening was performed for the presence of sterols, terpenoids, alkaloids, phenols, flavonoids, glycosides, tannins, saponins, fixed oils, and fat by standard methods.^[16]

Cell viability assays

Maintenance of cell lines

MCF-7 (human breast adenocarcinoma), HeLa (human epithelial cervical adenocarcinoma) and V79 (nontumor Chinese hamster normal fibroblast) cells were obtained from NCCS, Pune, India. The cells were cultured in Dulbecco's Minimum Essential Medium with 10% fetal bovine serum and 50 μ g/ml gentamicin and incubated at 37°C in a CO₂ incubator (humidified 5% CO₂ and 95% air).

Sulforhodamine B assay

Exponentially growing HeLa and MCF-7 cells were seeded into 96 well microplate (10⁴ cells per well in 100 μ l media) and allowed to attach for 24 h. The extract/fractions were added to the cells at concentrations ranging from 25 to 200 μ g/ml for 48 h, followed by addition of 25 μ l of 50% trichloroacetic acid. Plates were flicked and washed with water, dried, stained with sulforhodamine B (SRB) (0.05% prepared in 1% acetic acid) for 30 min. The plate was again washed four times with 1% acetic acid to remove the unbound dye, dried completely, followed by addition of 100 μ l of 10 mM Tris base to solubilize the dye. The absorbance was recorded using microplate reader and percentage cell viability was calculated.

Percentage of cell viability = $100 - [(X - Y)/X] \times 100$

where X = Absorbance of cells treated with 0.1% dimethyl sulfoxide (DMSO) medium, Y = Absorbance of cells treated with extracts. 0.1% (v/v) DMSO in the medium was used as negative control. IC₅₀ values were calculated using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). The experiment was performed in triplicate.^[17]

3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide assay

HeLa and nontumor V79 cells were plated and incubated in a similar way as mentioned in SRB assay. Cells were treated with extract/fractions at concentrations ranging from 25 to 200 μ g/ml in HeLa and 50 to 500 μ g/ml in V79 cells for 48 h, followed by addition of 20 μ l 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (5 mg/ml in PBS) followed by incubation at 37°C for 4 h. The medium was removed, followed by addition of 100 μ l DMSO and absorbance recorded using a micro plate reader at 540 nm followed by the calculation of percentage cell viability.

Percent cell viability = $100 - [(X - Y)/X] \times 100$

where X = Absorbance of cells treated with 0.1% DMSO medium, Y = Absorbance of cells treated with extracts. 0.1% (v/v) DMSO in the medium was used as negative control. IC₅₀ values were calculated using GraphPad Prism version 5.0. The experiment was performed in triplicate.^[18]

Apoptosis by staining

The apoptotic morphology of cells was examined using acridine orange (AO) and ethidium bromide (EB) stains simultaneously.^[19] MCF-7 cells (1 \times 10⁶) cells were treated with ethanol extract at 100 μ g/ml and active fractions, petroleum ether, and ethyl acetate at 30 μ g/ml for 48 h (concentrations were selected based on the IC₅₀ values in SRB assay). Methotrexate was used as positive control. Cells were trypsinized, washed with phosphate buffered saline, and fixed with 70% ethanol at 4°C for 12 h. The cells were treated with AO/EB at 100 μ g/ml for 2 min and visualized under a fluorescence microscope. Apoptosis was confirmed by nuclear condensation, membrane blebbing, and formation of round apoptotic bodies. The cells were counted in six different fields to determine the percentage of apoptotic cells. The experiment was performed in triplicate.

Antioxidant activity

The free radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH)^[20] and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)^[21] radicals were performed for the extract/fractions as per the methods described in literature.

Ferrous sulfate stimulated lipid peroxidation

To 0.5 ml of 10% rat brain homogenate (in potassium chloride), 1 ml of the extract/fractions were added, followed by addition of 1 ml of 100 μ M ferrous sulfate to stimulate lipid peroxidation. The mixture was incubated at 37°C for 30 min, followed by addition of ice cold TBA-TCA-HCl reagent to terminate the reaction. The solution was mixed thoroughly and

heated on a water bath for 15 min at a temperature not exceeding 80°C. The tubes were centrifuged at 2000 rpm to allow the floccules to settle. The absorbance of the supernatant was recorded at 530 nm.^[22] Curcumin was used as a standard. The experiment was performed in triplicate.

Total antioxidant capacity

The phosphomolybdenum method was employed to determine the total antioxidant capacity using ascorbic acid to construct the calibration curve.^[23]

High-performance thin layer chromatography

The presence of lupeol and β -sitosterol were identified and quantified in extract/fractions of *W. tinctoria* by a validated HPTLC method.^[24] Standard solutions of β -sitosterol and lupeol were prepared at 100 μ g/ml in methanol. The extract/fractions of *W. tinctoria* were prepared at 2 mg/ml in methanol and used for analysis. Sample solutions (10 μ L) were applied as 8 mm bands in duplicate under nitrogen gas flow on pre-coated silica gel GF₂₅₄ plates (20 cm \times 10 cm; 0.2 mm thickness) with a Linomat 5 applicator. Toluene: methanol: formic acid (7:2.7:0.3) was used as mobile phase and kept for saturation for 20 min in twin trough chamber. The plate was developed up to 75 mm from the point of application of test samples by linear ascending development and scanned with scanner-3 with slit dimensions of 6 mm \times 0.45 mm. After development, the plate was air-dried, and derivatization carried out with anisaldehyde sulfuric acid reagent at 110°C for 10 min and scanned at 600 nm. The peak areas and percentage of lupeol and β -sitosterol were calculated.

Statistical analyses

The results were expressed as mean \pm standard error of the mean (SEM) of experimental values. Graphs were prepared using GraphPad Prism version 5 software. A one-way analysis of variance was employed to estimate the difference between means, followed by *post-hoc* Dunnett's test. The value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Phytochemical screening

Qualitative phytochemical tests revealed the presence of sterols, triterpenoids, alkaloids, phenols, flavonoids, tannins, and saponins as major components in ethanol extract. Petroleum ether and ethyl acetate fractions were strongly positive for sterols and triterpenoids whereas ethyl acetate and butanol fractions both exhibited the presence of phenols, flavonoids, and alkaloids. The butanol and aqueous fractions tested positive for saponins and tannins.

In vitro cytotoxicity assays

Sulforhodamine B assay

The ethanol extract, petroleum ether and ethyl acetate fractions were found to inhibit proliferation of HeLa [Figure 1] and MCF-7 cancer cells in a concentration-dependent manner [Figure 2]. The IC₅₀ values of petroleum ether and ethyl acetate fractions are summarized in Table 1.

3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide assay

The ethanol extract and fractions were found to inhibit the viability of HeLa cells in a concentration-dependent manner [Figure 3]. The IC₅₀ values in the MTT assay were found to be comparable to SRB assay. Interestingly, the extract and fractions were found to be less cytotoxic to normal fibroblast cells; V79 [Figure 4] hence demonstrating their selectivity to cancer cells [Table 2].

Butanol and aqueous did not show cytotoxicity in both SRB and MTT assays (data not shown) hence they were not taken for further studies.

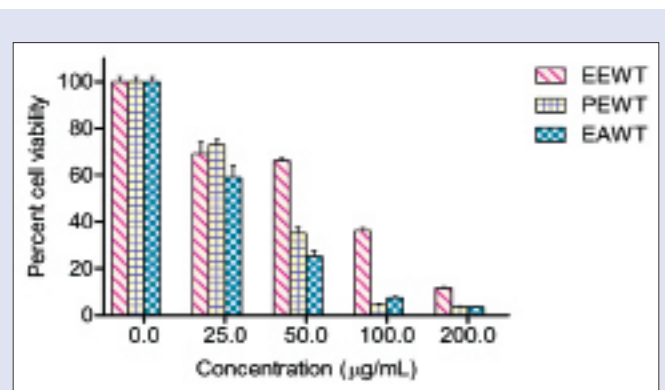


Figure 1: Percent cell viability of ethanol extract of *Wrightia tinctoria*, petroleum ether fraction of *Wrightia tinctoria* and ethyl acetate fraction of *Wrightia tinctoria* at concentrations in the range of 25–200 μ g/ml in HeLa (human epithelial cervical carcinoma) cells after 48 h incubation by sulforhodamine B assay. Data are expressed as mean \pm standard error of the mean. Experiment was done in triplicate

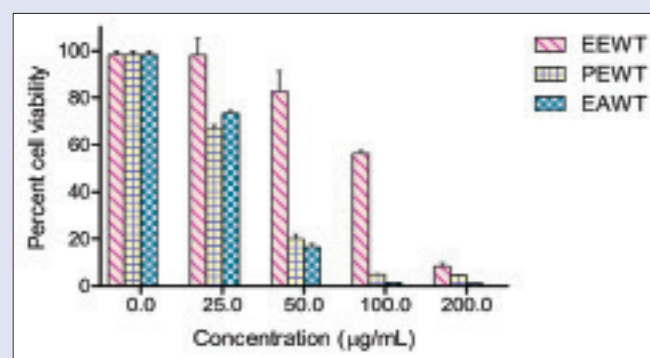


Figure 2: Percent cell viability of ethanol extract of *Wrightia tinctoria*, petroleum ether fraction of *Wrightia tinctoria* and ethyl acetate fraction of *Wrightia tinctoria* at concentrations in the range of 25–200 μ g/ml in MCF-7 (human breast adenocarcinoma) cells after 48 h incubation by sulforhodamine B assay. Data are expressed as mean \pm standard error of the mean. Experiment was done in triplicate

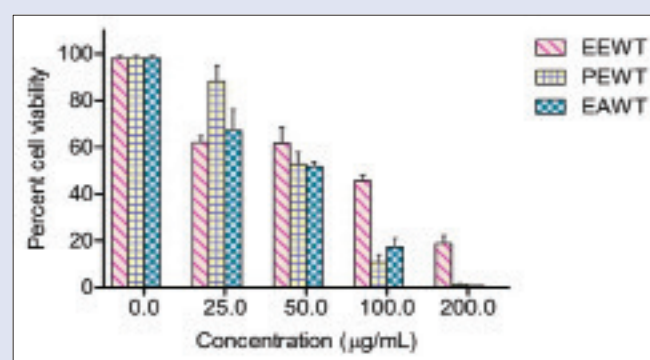


Figure 3: Percent cell viability of ethanol extract of *Wrightia tinctoria*, petroleum ether fraction of *Wrightia tinctoria* and ethyl acetate fraction of *Wrightia tinctoria* at concentrations in the range of 25–200 μ g/ml in HeLa (human epithelial cervical carcinoma) cells after 48 h incubation by 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide assay. Data are expressed as mean \pm standard error of the mean. Experiment was done in triplicate

Apoptosis by acridine orange/ethidium bromide staining

To determine whether the anti-proliferative activity was due to apoptosis, we carried out staining assay in MCF-7 cells after treatment with extract/fractions for 48 h. AO dye is taken up by all cells and makes nuclei fluoresce green while EB is taken up by dead cells that appear orange. Moreover, EB overcomes AO stain. Hence, live cells fluoresce green while early apoptotic cells appear yellow and late apoptotic cells appear orange in color.^[19] Ethanol extract, petroleum ether, and ethyl acetate fractions were found to induce apoptosis in MCF-7 cells as observed under the fluorescence microscope. In the control group, uniform green cells were observed with normal morphology. However, cells treated with

extract/fractions showed yellow early apoptotic cells with condensed chromatin and red apoptotic bodies [Figure 5a]. The extract/fractions significantly ($P < 0.05$) increased the apoptotic index in MCF-7 cells as compared to cells treated with 0.1% DMSO in media alone [Figure 5b].

Antioxidant assays

The ethanol extract/fractions of *W. tinctoria* showed dose-dependent scavenging of DPPH and ABTS free radicals. The ethyl acetate fraction exhibited most potent free radical scavenging activity (IC_{50} : 98.70 and 35.50 $\mu\text{g/ml}$ in DPPH and ABTS assays respectively). The ethanol extract/fractions showed excellent dose-dependent inhibition of ferrous sulfate-stimulated lipid peroxidation in rat brain homogenate. The ethanol extract was most potent with an IC_{50} of 4.18 $\mu\text{g/ml}$ that might be due to the synergistic activity of phytoconstituents in the parent extract. Similar to other assays, ethyl acetate fraction demonstrated the highest inhibition of lipid peroxidation with ethyl acetate being most active (IC_{50} : 23.40 $\mu\text{g/ml}$) followed by the butanol fraction (IC_{50} : 27.06 $\mu\text{g/ml}$). Moreover, ethyl acetate fraction exhibited the highest total antioxidant capacity by phosphomolybdenum method (1586.54 μg ascorbic acid equivalents/mg extract) which correlates with the presence of phenols and flavonoids in the ethyl acetate fraction [Table 3].

Detection and quantitation of lupeol and β -sitosterol by high-performance thin layer chromatography

We identified lupeol and β -sitosterol as active components in the ethanol extract and active fractions of *W. tinctoria* by HPTLC. The ethanol

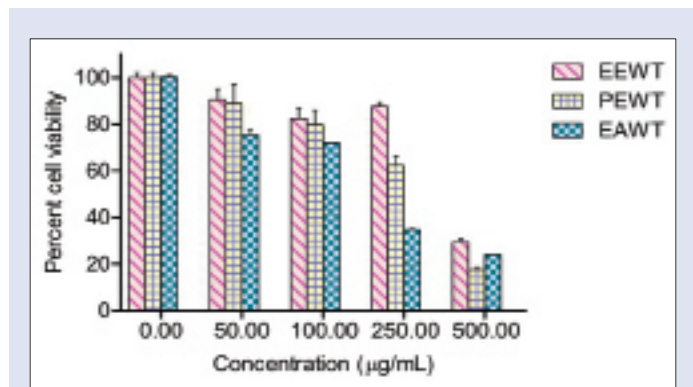


Figure 4: Percent cell viability of ethanol extract of *Wrightia tinctoria*, petroleum ether fraction of *Wrightia tinctoria* and ethyl acetate fraction of *Wrightia tinctoria* at concentrations in the range of 50–500 $\mu\text{g/ml}$ in V79 (nontumor Chinese hamster normal fibroblast) after 48 h incubation by 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide assay. Data are expressed as mean \pm standard error of the mean. Experiment was done in triplicate. The extract/fractions were relatively less toxic to normal V79 cells as compared to cancer cells

Table 1: IC_{50} values of extract/fractions of *Wrightia tinctoria* in HeLa (human epithelial cervical carcinoma) and MCF-7 (human breast adenocarcinoma) cells by SRB assay after 48 h incubation with different concentrations of extract/fractions

Extract/fractions	IC_{50} ($\mu\text{g/ml}$)	
	HeLa	MCF-7
EEWT	63.06	102.5
PEWT	37.78	31.56
EAWT	29.69	32.63

EEWT: Ethanol extract of *Wrightia tinctoria*; PEWT: Petroleum ether fraction of *Wrightia tinctoria*; EAWT: Ethyl acetate fraction of *Wrightia tinctoria*; SRB: Sulforhodamine B

Table 2: IC_{50} values of extract/fractions of *Wrightia tinctoria* in HeLa (human epithelial cervical carcinoma) and V79 (nontumor Chinese hamster normal fibroblast) cells by MTT assay after 48 h incubation with different concentrations of extract/fractions

Extract/fractions	IC_{50} ($\mu\text{g/ml}$)	
	HeLa	V79
EEWT	62.93	400.7
PEWT	51.31	267.3
EAWT	43.97	170.8

EEWT: Ethanol extract of *Wrightia tinctoria*; PEWT: Petroleum ether fraction of *Wrightia tinctoria*; EAWT: Ethyl acetate fraction of *Wrightia tinctoria*; MTT: 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide

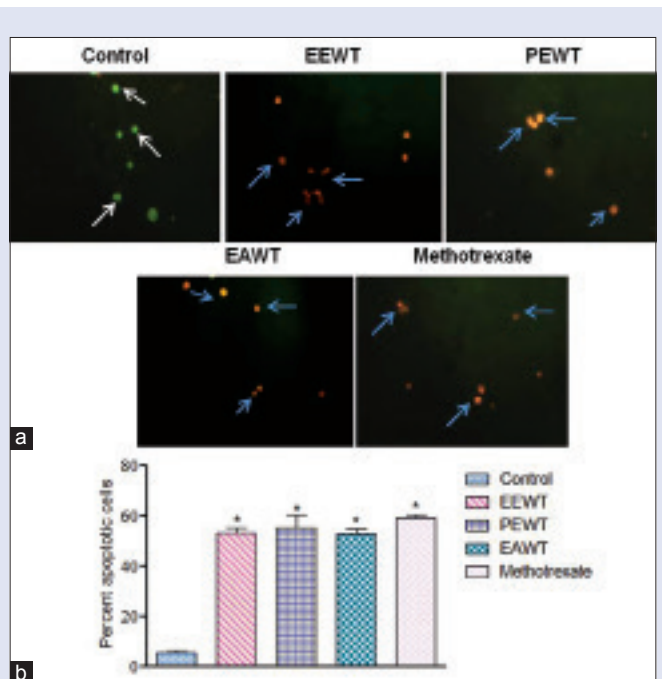


Figure 5: (a) Ethanol extract of *Wrightia tinctoria*, petroleum ether fraction of *Wrightia tinctoria* and ethyl acetate fraction of *Wrightia tinctoria* induce apoptosis in MCF-7 cells analyzed by acridine orange/ethidium bromide staining. Representative images of cells after treatment for 48 h. Viable cells appear green indicated by white arrows; apoptotic cells appear yellow/orange indicated by blue arrows. (b) Percent of apoptotic cells were determined by counting apoptotic cells in six different fields. Results are expressed as mean \pm standard error of the mean. * $P < 0.001$ compared with negative control. Treatments were carried out in triplicate

extract was found to contain lupeol (1.73%) and β -sitosterol (0.28%) w/w. Petroleum ether fraction was found to be significantly enriched with lupeol (3.05% w/w) whereas ethyl acetate fraction showed the presence of relatively lesser, 0.76% w/w lupeol. β -sitosterol content was higher in ethyl acetate (1.2% w/w) as compared to petroleum ether fraction (0.95% w/w) [Figure 6]. The results are in accordance with prior reports of lupeol as the major component and β -sitosterol as a minor component in stem bark of *W. tinctoria*.^[15]

DISCUSSION

In this study, we made an attempt to determine the anticancer and pro-apoptotic potential of the extract/fractions of *W. tinctoria* in breast

Table 3: Free radical scavenging and antioxidant capacity of extract and fractions of *Wrightia tinctoria*

Extract/ fraction	IC ₅₀ (μ g/ml)			TAC (μ g AAE/ mg extract)
	DPPH scavenging	ABTS scavenging	Lipid peroxidation	
EEWT	213.50	118.70	4.18	784.40
PEWT	>1000	>1000	ND	409.17
EAWT	98.70	35.50	23.40	1586.54
BUWT	126.20	35.80	27.06	1506.54
AQWT	852.10	292.20	51.13	688.21
Ascorbic acid	6.76	1.97	-	-
Curcumin	-	-	25.21	-

EEWT: Ethanol extract of *Wrightia tinctoria*; PEWT: Petroleum ether fraction of *Wrightia tinctoria*; EAWT: Ethyl acetate fraction of *Wrightia tinctoria*; BUWT: Butanol fraction of *Wrightia tinctoria*; AQWT: Aqueous fraction of *Wrightia tinctoria*; ND: Not determined; TAC: Total antioxidant capacity; AAE: Ascorbic acid equivalents; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

and cervical cancer cells. Previous reports exist on the cytotoxic potential of alcohol extract of *W. tinctoria*. However, the activity of the fractions and mode of action of cell death has not been evaluated until date. To further validate its use as an antioxidant and chemopreventive agent, we determined the free radical scavenging activity and phytochemical constituents that could be responsible for the biological activities.

In cytotoxicity assays, petroleum ether and ethyl acetate fractions exhibited the highest activity in MTT and SRB assays. Both the fractions showed significant concentration-dependent cytotoxicity in human breast adenocarcinoma and cervical carcinoma cells in the SRB assay. Comparable results were observed in the MTT assay in HeLa cells after 48 h incubation. Butanol and aqueous fractions were not cytotoxic to HeLa and MCF-7 cells hence not taken up for further studies.

Chemotherapy is largely limited by its toxicity to normal cells, and selective killing of cancer cells is a highly desired mode of action for chemotherapeutic drugs. Interestingly, all the fractions were found to be relatively safer to a nontumor cell line; V79 fibroblast cells hence demonstrating their selective cytotoxicity to cancer cells.

To determine whether the extract/active fractions induce apoptosis in cancer cells, we carried out AO/EB staining in MCF-7 cells and observed the morphological changes under the microscope after treatment with the active fractions. Apoptosis is the preferred mode of action of cell death by anticancer agents since it spares excessive damage to normal cells. The treatments induced apoptotic cell-like morphology with cellular shrinkage, nuclear condensation and formation of round apoptotic bodies clearly indicating the pro-apoptotic effect of fractions in MCF-7 cells that is a desirable attribute of anticancer agents.

Moreover, increased formation of deleterious free radicals and consequent oxidative stress leading to a pro-oxidant status is a characteristic feature of cancer cells. The altered redox environment plays a key role in tumor

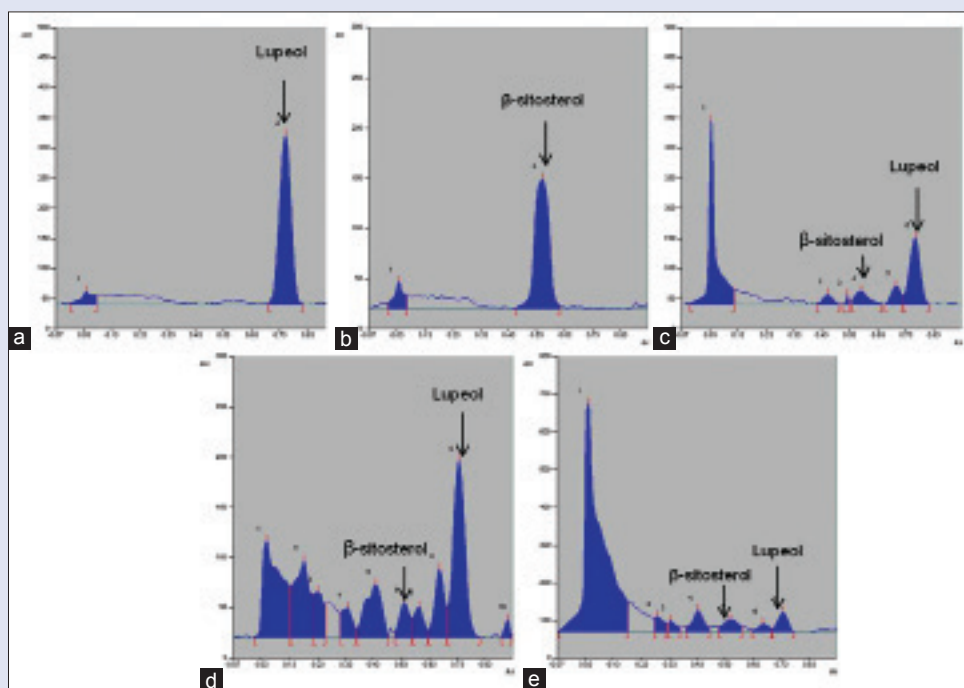


Figure 6: High performance thin layer chromatography chromatograms of: (a) Standard lupeol ($R_f = 0.75$), (b) Standard β -sitosterol ($R_f = 0.55$), (c) Ethanol extract of *Wrightia tinctoria*, (d) Petroleum ether fraction of *Wrightia tinctoria*, (e) Ethyl acetate fraction of *Wrightia tinctoria*. R_f value of 0.75 ± 0.02 indicates lupeol and 0.55 ± 0.02 indicates β -sitosterol. Mobile phase used was toluene: Methanol: Formic acid (7:2.7:0.3). Detection was carried out at 600 nm after spraying with anisaldehyde sulfuric acid reagent

initiation and progression.^[25] Free radicals stimulate cell proliferation, genetic instability and damage to cellular macromolecules like proteins, lipids, DNA, and carbohydrates that enable cancer cells to maintain their malignancy.^[26] A redox imbalance of the cells leads to dysregulated antioxidant defense mechanisms illustrated by altered levels of glutathione, SOD, catalase, and lipid peroxidation.^[27,28] Therefore, exogenous antioxidants can be used to maintain the oxidant-antioxidant balance in cells as chemopreventive agents. The extract/fractions showed moderate free radical scavenging activity. However, they exhibited excellent inhibition of lipid peroxidation quantified as thiobarbituric acid reactive substance. Free radicals generated by oxidative stress react with polyunsaturated fatty acids of lipid membranes forming lipoperoxyl radicals and secondary products which after cyclization produce aldehydes like malondialdehyde that react with DNA causing genetic mutations and further carcinogenesis. Additionally, the end products of lipid peroxidation further act as secondary messengers of oxidative stress that have the ability to diffuse to distant sites causing further damage.^[1,29]

The results from the *in vitro* studies appeared promising hence we further examined the chemical nature of the fractions. HPTLC analysis revealed the presence of lupeol and β -sitosterol in ethanol extract, petroleum ether and ethyl acetate fractions. Both these compounds are naturally occurring triterpenes that exhibit significant antitumor activity in various cancer cells. The probable mechanisms of action for both compounds involve the modulation of cell cycle arrest and apoptotic pathways. Previous reports suggest that lupeol exerts cytotoxic activity against HeLa, MCF-7, MDA-MB-231 cells in concentration and time-dependent manner.^[30,31] Similar results were observed with a lupeol-enriched petroleum ether fraction in our study in MCF-7 cells. Also, lupeol induces G_2/M arrest in prostate cancer cells through the modulation of the cyclin-B pathway.^[32] In DMBA-induced carcinogenesis, lupeol induced G_2/M arrest and apoptosis by over expression of Bax and caspase-3 and under expression of Bcl-2 and survivin genes.^[33] Moreover, β -sitosterol is also reported to be cytotoxic to MDA-MB-231 cancer cells by induction of apoptosis through up-regulation of Bax and caspases and down-regulation of Bcl-2 and IAP family.^[34] Moreover, another group confirmed the cytotoxic and apoptogenic activity of β -sitosterol in MDA-MB-231 cells^[35] and chemical-induced colon cancer^[36] demonstrating the chemopreventive efficacy of β -sitosterol.^[35] To summarize, lupeol and β -sitosterol exert multi-targeted action causing key molecular alterations in cancer cells that are implicated in different cancer types. Also, they possess the capacity to up regulate antioxidant defense capacity of cells maintaining the redox balance and reducing deleterious free radicals that are determinants of cancer initiation and progression.^[31,36] As mentioned elsewhere in the article, ethyl acetate exhibited the highest content of β -sitosterol that could have contributed to the anticancer potential. This is the first study demonstrating the anticancer activity of *W. tinctoria* in cervical and breast cancer with higher selectivity toward cancer cells. In addition, our results partially explain the phytochemical basis of anticancer and antioxidant activities of the plant. Both fractions are currently under investigation in our laboratory to identify the active constituents and explore their mode of action of cell death in breast and cervical cancer.

CONCLUSION

To summarize, our study demonstrates the antioxidant and anticancer activity of *W. tinctoria* in breast and cervical cancer. The extract/fractions are pro-apoptotic in breast cancer cells that could partly be attributed to the anticancer triterpenoids, lupeol, and β -sitosterol. Moreover, the fractions exhibit higher cytotoxicity to cancer cells as compared to normal fibroblast cells demonstrating their selectivity toward cancer cells. Further studies to isolate bioactive compounds in the active fractions and decipher the molecular mechanisms are in progress in our laboratory.

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Conflicts of interest

There are no conflicts of interest.

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