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Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Antiproliferative activity and gas chromatography–tandem mass spectrometry profiling of *Wrightia tinctoria* bark fractions

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Abstract

Breast and prostate cancers contribute substantially to cancer-related morbidity and mortality. Despite significant advancements in cancer treatment modalities, there remains a need to deepen mechanistic understanding and develop improved therapeutic strategies, given toxicity, resistance, and limited selectivity. Natural products have played a pivotal role in drug discovery. The structural diversity of natural compounds allows them to target multiple pathways, making them promising candidates in anticancer drug development. *Wrightia tinctoria* (WT), widely used in Ayurvedic and traditional medicine, has emerged as a plant of significant pharmacological interest. The present study focuses on identifying the bioactive constituents of WT bark through bioassay-guided fractionation, chromatographic techniques, and mass spectrometric profiling. In the present study, polarity-based fractions, including petroleum ether, hexane, chloroform, ethyl acetate, and methanol, were screened for their antiproliferative effects against MDA-MB-231, MCF-7, and PC-3 cancer cell lines. Among these, the petroleum ether fraction (PFWT) showed potent cytotoxic activity, with IC_{50} values of 59.73 ± 2.90, 97.06 ± 4.76, and 43.63 ± 2.68 µg/mL, respectively. The gas chromatography–tandem mass spectrometry analysis of sub-fractions from PFWT further tentatively identified 42 chemical compounds, out of which 27 compounds are reported for the first time. Overall, the study demonstrates that PFWT possesses strong and broad antiproliferative activity, reinforcing the value of integrating traditional medicinal knowledge with modern analytical and pharmacological approaches. These findings position WT as a promising and sustainable source of novel anticancer agents and warrant further in-depth investigation.

Keywords: *Wrightia tinctoria*; Antiproliferative activity; Gas chromatography–tandem mass spectrometry; Fractions; Bioactive compounds

1. Introduction

In recent decades, cancer has emerged as a leading cause of morbidity and mortality, a devastating major health challenge for human populations around the globe. The International Agency for Research on Cancer, based on results published from 115 countries, estimated that there were 20 million new cancer cases and 9.7 million deaths in 2022.[1] Breast and prostate cancers remain one of the most serious global health challenges due to high morbidity and mortality rates.[2] Breast carcinoma was the most diagnosed cancer in 2023.[3] As per the report of GLOBOCAN 2020[4], breast and prostate cancers are increasing worldwide and account for 6.9% and 3.8% deaths, respectively. Although treatment modalities, such as surgery, chemotherapy, radiation therapy, and hormonal therapy, are available, the efficacy of these treatments varies among patients due to their genetic polymorphisms, nature, and stage of cancer, as well as tumor heterogeneity.[5,6] Chemotherapy strategies for breast cancer target the functions of estrogen receptor alpha, progesterone receptor, and the epidermal growth factor receptor.[7] These targets have been reported to play a crucial role in the initiation and progression of breast cancer. However, resistance to these therapies and the lack of defined molecular targets in subtypes like triple-negative breast cancer continue to limit therapeutic success. Similarly, the treatment for prostate cancer relies mainly on androgen deprivation therapy and androgen receptor-targeted agents. Nevertheless, most patients eventually develop multiple complications due to mechanisms such as androgen receptor amplification, splice variants, and activation of the phosphoinositide 3-kinase/Akt pathway.[8,9] Moreover, approved drugs, including tamoxifen, trastuzumab, paclitaxel, gemcitabine, and cyclophosphamide, are clinically effective, but overall effectiveness is limited by toxicity, variable response rates, and the emergence of resistance.[10,11] These limitations and challenges in the management of different cancers underscore the need for improved multi-targeted and personalized therapeutic strategies.

Given the limited available treatment strategies, the scientific community worldwide is intensifying efforts to understand tumorigenesis, with an increasing focus on new anticancer agents and alternative therapies. In recent years, translating discoveries from natural compounds with anti-tumor potential into clinical therapies has been vital, underscoring the importance of preclinical research.[12] Medicinal plants have been a cornerstone of drug discovery, providing a rich source of bioactive compounds with therapeutic potential.[13] Researchers worldwide have isolated numerous natural compounds and synthesized a diverse range of naturally inspired molecules that play powerful roles in the management of different diseases, including cancer.[14] These compounds, due to their antioxidant and apoptogenic properties, can combat inflammation and angiogenesis and prevent the spread of cancer.[13–17]

Natural compounds and their structural analogs have been extensively explored as potential cancer therapies, demonstrating significant chemical diversity and promising therapeutic potential. The anticancer agents derived from natural products include vincristine, vinblastine, paclitaxel, docetaxel, camptothecin, and their derivatives. Topotecan and irinotecan have been used to treat various cancers.[18–20]

These promising discoveries in natural products have attracted significant attention from the scientific community, which is now investigating unexplored natural products and their phytochemicals, where effective treatments and management are currently unavailable.[21] The structural diversity and ability of natural compounds to interact with multiple molecular targets, such as modulation of key pathways[22], cell cycle arrest[23], induction of apoptosis[24], and inhibition of angiogenesis, make them promising candidates for drug design and development.[25]

Wrightia tinctoria R. Br., (WT; Apocynaceae) is a small deciduous tree distributed throughout the world. In India, WT is known locally by various vernacular names; the most common are *Indrajava*, *Svetkutaja*, *Krsnkutaja* (Sanskrit), *Kalakuada* (Marathi), and *Mithaindrajau* (Hindi).[26] The different parts of the plant are known to have specific medicinal properties and have a long history of use traditionally by Ayurveda, Siddha, Unani, and folk medicine.[27,28] Earlier, we reported that the methanolic crude extract of WT bark has significant antiproliferative activity against MDA-MB-231 and MCF-7 cancer cells and free radicals' inhibition potential in *in vitro* experiments.[29] The phytochemistry of WT bark comprises alkaloids, flavonoids, and phenolic compounds, which offer promise as antiproliferative agents.

The bioassay-guided isolation and identification of bioactive compounds is a commendable strategy for isolating active compounds from complex plant extracts. The polarity-based fractionation of plant crude facilitates the isolation of compounds with different polarities, such as phenolics, terpenoids, alkaloids, flavonoids, and sterols.[30] This study aims to systematically identify bioactive compounds from WT bark using a bioassay-guided approach. The novelty of this study lies in the dual approach of bioassay-guided fractionation and mass spectrometry (MS) to uncover the antiproliferative potential of WT bark and identify its components. Unlike prior studies that predominantly focused on the leaves or seeds of this plant, this research emphasizes the underexplored bark and its

potential as a source of novel anticancer compounds.

The findings of this research hold significant implications for new drug entities, particularly in the development of natural product-inspired anticancer agents. The isolation of bioactive compounds from natural products not only enriches the repository of potential therapeutics but also supports the sustainable use of traditional medicinal plants, which are well accepted by the human body. By identifying and characterizing antiproliferative compounds from WT bark, this study helps bridge the gap between traditional medicine and modern pharmacology, paving the way for future preclinical and clinical evaluations.

2. Materials and methods

2.1. Extraction and fractionation

The stem bark of WT was collected from Ahraura, Mirzapur district, Uttar Pradesh, India, and identified by Dr. Sharad K. Srivastava of the Department of Pharmacognosy, CSIR-National Botanical Research Institute, Lucknow. The collected plant material was air-dried and macerated with methanol. The crude methanolic extract was further fractionated into increasing-polarity fractions, including petroleum ether, hexane, chloroform, ethyl acetate, and methanol.

2.2. *In vitro* antiproliferative study

2.2.1. In vitro cell viability MTT assay

To assess cell viability, MDA-MB-231, MCF-7, PC-3, and HaCaT cells (5,000 cells/100 µL/well) were seeded in 96-well plates and incubated at 37 °C for 24 h. Following cell adhesion, different concentrations of the test fractions (6.25–400 µg/mL) were applied in triplicate, and cell viability was measured after 24, 48, and 72 h. Dimethyl sulfoxide (DMSO) was used as the vehicle control, and its final concentration in all wells was carefully maintained at ≤0.5% (v/v) to avoid any solvent-related effects on cells. After the treatment period, 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (prepared at a concentration of 5 mg/mL in PBS) was added to each well, and the plate was incubated for 3 to 4 h, allowing viable cells to convert MTT into purple formazan crystals. After incubation, the medium was gently removed from each well, and cells were washed with phosphate-buffered saline (PBS), then 100 µL of DMSO was added to each well to dissolve the formed formazan crystals. Then, the absorbance was recorded at 540 nm using a microplate reader (Synergy HT, BioTek, United States [US]). Cell viability was calculated using **Equation 1**:

$$Cell\ viability(\%) = \frac{OD\ of\ treated}{OD\ of\ control} \times 100 \quad (1)$$

2.2.2. Hoechst staining

Hoechst staining was carried out to examine changes in nuclear morphology in MDA-MB-231, MCF-7, and PC-3 cells following treatment with the different fractions (6.25–200 µg/mL). Approximately 2000 cells/well were seeded into six-well plates and allowed to adhere. The cells were then treated with different fractions and incubated for 18–20 h. Cells treated with 0.1% DMSO in complete growth medium served as the control. At the end of the treatment time, the cells were gently fixed with 10% neutral-buffered formalin for 15 min at 4 °C. The fixed cells were then washed with ice-cold PBS and stained with Hoechst 33342 (1 µg/mL in PBS), followed by a brief 2-min incubation in the dark at room temperature to visualize nuclear changes. The fixed cells were observed under a phase-contrast and fluorescence inverted microscope (ECLIPSE Ti-U, Nikon, Japan) equipped with standard excitation and emission filters (346 nm and 460 nm, respectively). Images were captured at 20× magnification using a charge-coupled device camera (Nikon Eclipse, Japan) and analyzed with NIS-Elements D 3.0 software (Nikon, Japan) across three randomly selected fields per well. Cells with condensed or fragmented nuclei were identified as apoptotic.

2.3. Identification of compounds through gas chromatography–tandem mass spectrometry analysis

Identification and characterization of phytochemicals were carried out using gas chromatography–tandem mass spectrometry (GC-MS/MS; Thermo TSQ Quantum XLS with Triplus autosampler and triple quadrupole, Thermo Fisher Scientific, US). Analysis was performed on an Elite-5MS column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness) with helium (99.99%) as the carrier gas at a flow rate of 1.1 mL/min in splitless mode. A 1 µL silylated sample (derivatized with 1.5 µL N,O-bis(trimethylsilyl) trifluoroacetamide [BSTFA]) was injected at 250 °C, with the transfer line maintained at 300 °C. The oven temperature was programmed from 65 °C (2 min hold) to 230 °C at 6 °C/min, then to 290 °C at 10 °C/min (20 min hold). Compounds were identified in full-scan mode by matching retention times and fragmentation patterns to the National Institute of Standards and Technology (NIST) MS 2.0 library.

2.4. Statistical analysis

All experiments were carried out in triplicate, and data are represented as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with GraphPad Prism 5.01 (GraphPad, USA). Results were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Effect of *Wrightia tinctoria* extract and fractions on cell viability

The antiproliferative activity of WT bark methanolic extract (MEWT) and its increasing polarity-wise fractions, namely petroleum ether fraction (PFWT), hexane fraction (HFWT), chloroform fraction (CFWT), ethyl acetate fraction (EFWT), and methanolic fraction (MFWT), showed a significant ($p < 0.05$) dose-dependent reduction in the viability of human breast and prostate cancer cells. PFWT showed consistent antiproliferative activity against all the tested MCF-7, MDA-MB-231, and PC-3 cancer cell lines, with the half maximal inhibitory concentration (IC_{50}) values of 59.73 ± 2.90, 97.06 ± 4.76, and 43.63 ± 2.68 µg/mL, respectively (Figures 1–4 and Tables 1 and 2). In the MCF-7 cancer cell line, CFWT showed the highest cell inhibitory activity, with an IC_{50} of 26.13 ± 6.99 µg/mL, followed by EFWT (IC_{50} = 39.37 ± 2.87 µg/mL). Meanwhile, CFWT showed moderate cytotoxic effect against MDA-MB-231 (IC_{50} = 84.16 ± 3.81 µg/mL) and PC-3 (IC_{50} = 133.33 ± 4.5 µg/mL) cancer cell lines.

The results were compared with the standard drug cisplatin, used as a positive control. It was observed that PFWT and CFWT exhibit potent antiproliferative activity, but with clearly higher IC_{50} values than that of cisplatin.[31,32] This finding indicates that cisplatin remains more potent under *in vitro* conditions. Nevertheless, PFWT and CFWT, along with their chemical diversity and selectivity profiles, warrant further investigation, including fraction purification, mechanism studies, and *in vivo* evaluation.

Selectivity index (SI)—ratio of IC_{50} calculated for normal and cancer cells[33,34]—was calculated to assess the cancer-specific cytotoxicity of the extract and fractions relative to HaCaT normal keratinocyte cells (Table 2). An SI value higher than 1 signifies desirable selectivity of the candidate against cancer cells.[35] The SI values showed differences in selectivity among the studied cancer cell lines, indicating the therapeutic potential of the different fractions. Among all fractions, CFWT exhibited the highest SI value, particularly against MCF-7 cells (SI = 15.01), demonstrating marked selectivity. The results further suggest that CFWT contains bioactive chemical constituents with a strong affinity to hormone-responsive breast cancer cells. Furthermore, CFWT showed comparatively high SI values against MDA-MB-231 (SI = 4.66) and PC-3 (SI = 2.94), supporting its broad-spectrum anticancer potential.

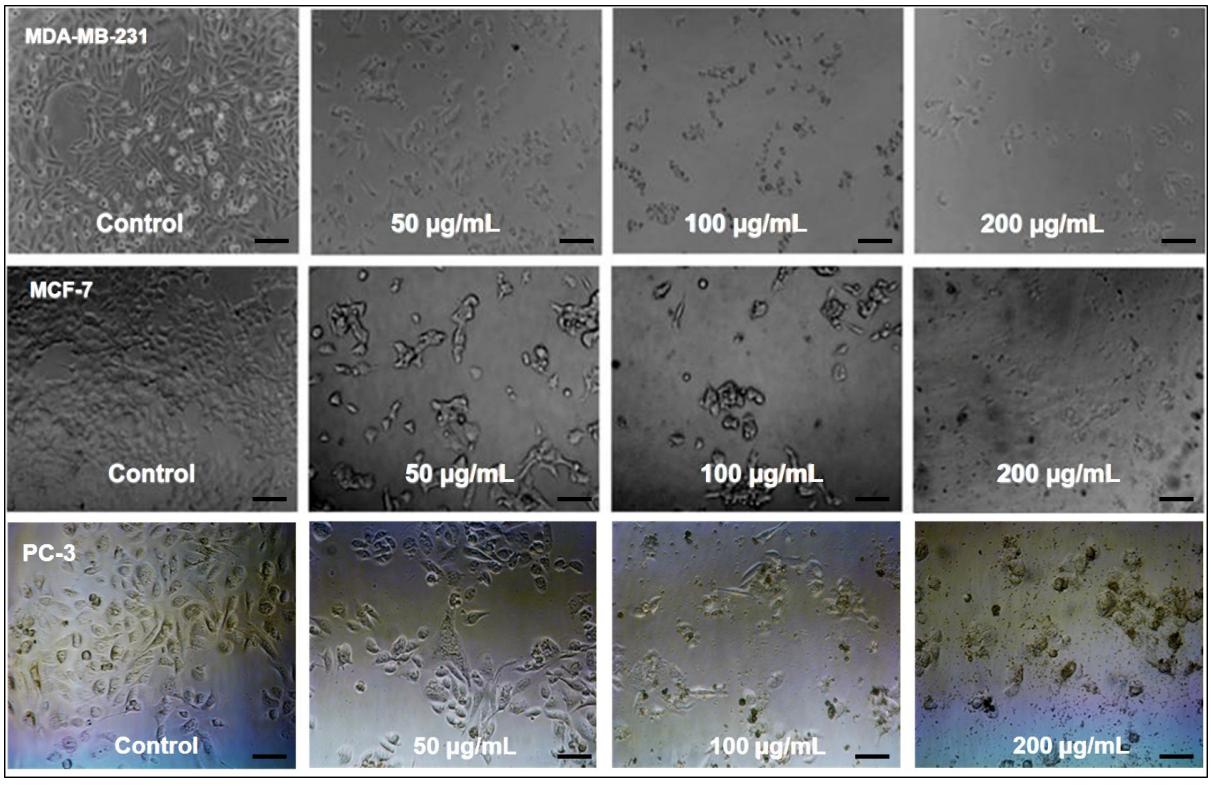


Figure 1. Effect of petroleum ether fraction on morphological changes of breast (MDA-MB-231 and MCF-7) and prostate cancer (PC-3) cell lines. Scale bars: 100 µm; magnifications: 10×.

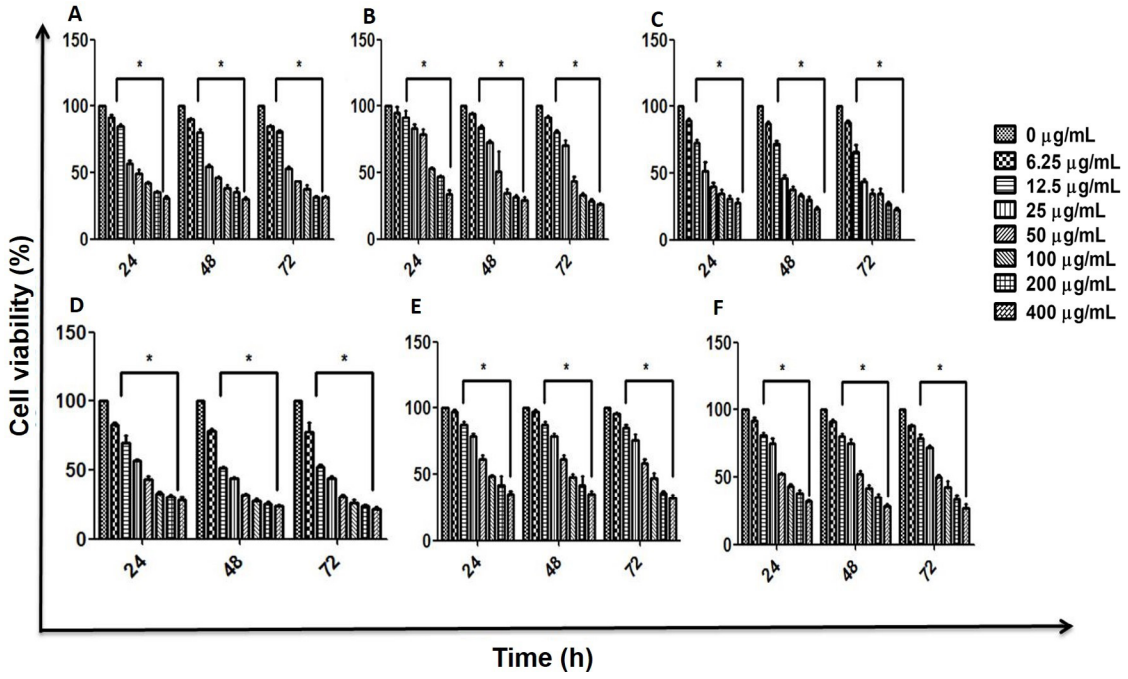


Figure 2. Effects of *Wrightia tinctoria* (WT) bark fractions on cell viability of human breast cancer (MCF-7) cell line, after incubation for 24 h, 48 h, or 72 h at the concentration of 6.25–400 µg/mL: (A) WT bark methanolic extract, (B) hexane fraction, (C) chloroform fraction, (D) ethyl acetate fraction, (E) methanolic fraction, and (F) petroleum ether fraction. After the treatment, cell viability was measured using the MTT assay.
Notes: Data are presented as mean ± SD from three independent experiments conducted in triplicate. *$p < 0.05$.

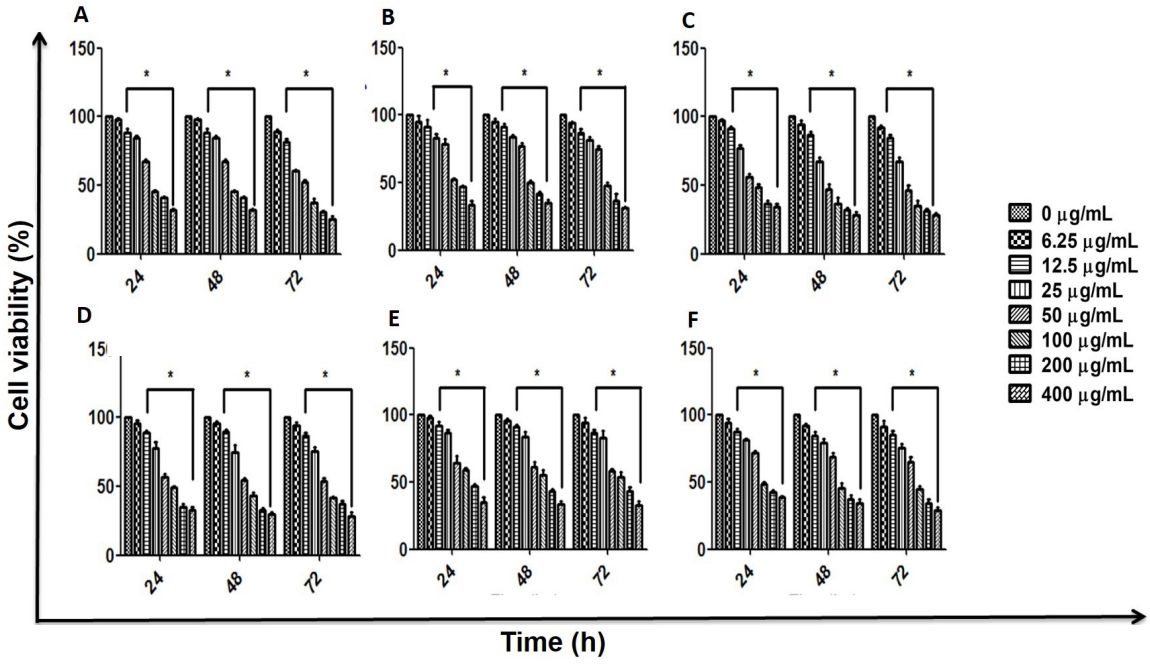


Figure 3. Effects of *Wrightia tinctoria* (WT) bark fractions on cell viability of human breast cancer (MDA-MB-231) cell line, after incubation for 24 h, 48 h, or 72 h at the concentration of 6.25–400 µg/mL: (A) WT bark methanolic extract, (B) hexane fraction, (C) chloroform fraction, (D) ethyl acetate fraction, (E) methanolic fraction, and (F) petroleum ether fraction. After the treatment, cell viability was measured using the MTT assay.
Notes: Data are presented as mean ± SD from three independent experiments conducted in triplicate. *$p < 0.05$.

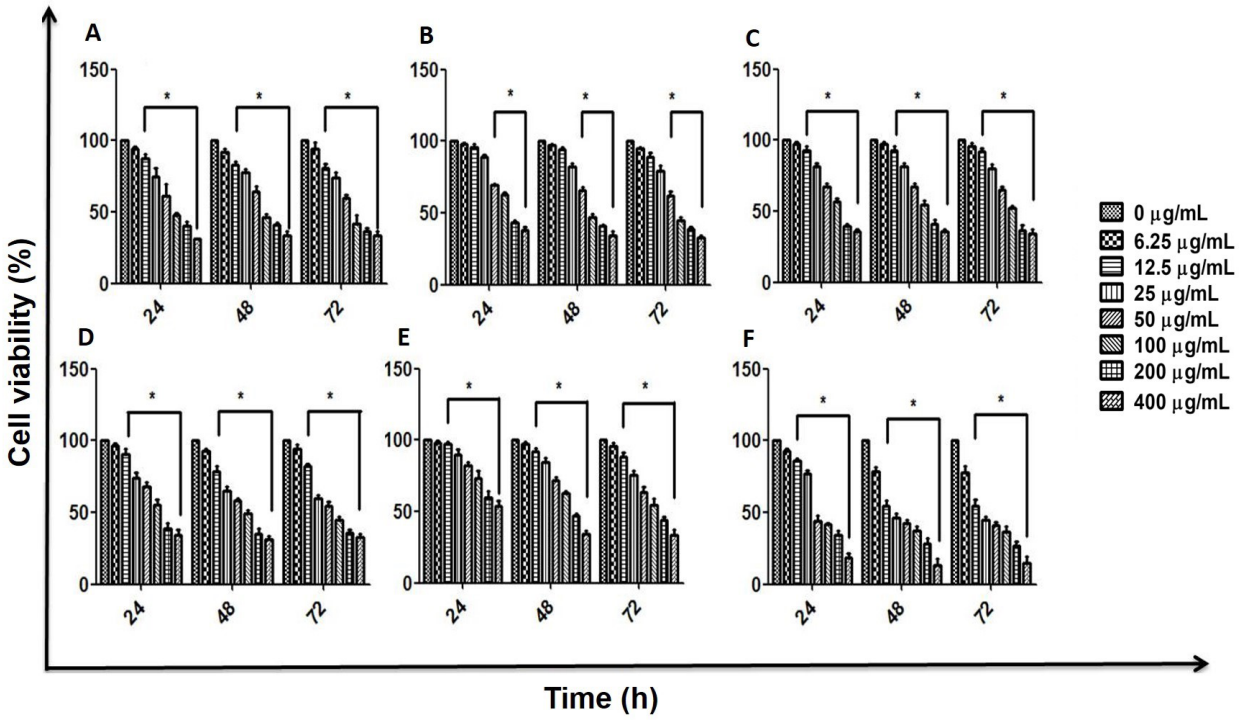


Figure 4. Effects of *Wrightia tinctoria* (WT) bark fractions on cell viability of human prostate cancer (PC-3) cell line, after incubation for 24 h, 48 h, or 72 h at the concentration of 6.25–400 µg/mL: (A) WT bark methanolic extract, (B) hexane fraction, (C) chloroform fraction, (D) ethyl acetate fraction, (E) methanolic fraction, and (F) petroleum ether fraction. After the treatment, cell viability was measured using the MTT assay.
Notes: Data are presented as mean ± SD from three independent experiments conducted in triplicate. *$p < 0.05$.

Table 1. IC_{50} of *Wrightia tinctoria* crude extract and its fractions against breast cancer (MDA-MB-231 and MCF-7), prostate cancer (PC-3), and normal (HaCaT) cell lines

Extract/ fraction	IC_{50} (µg/mL)			
	MCF-7	MDA-MB-231	PC-3	HaCaT
MEWT	45.71 ± 7.74	88.90 ± 1.27	85.00 ± 7.73	298.65 ± 0.64
HFWT	47.90 ± 1.25	140.30 ± 3.21	163.00 ±3.51	348.00 ± 8.35
CFWT	26.13 ± 6.99	84.16 ± 3.81	133.33 ± 4.50	392.04 ± 9.61
EFWT	39.37± 2.87	98.13 ± 3.52	126.33 ± 8.50	278.57 ± 6.24
MFWT	92.10 ± 6.60	173.66 ± 5.85	>400	398.50 ± 6.76
PFWT	59.73 ± 2.90	97.06 ± 4.76	43.63 ± 2.68	187.68 ± 8.24
Standard (Cisplatin)	13.50 ± 1.39	41.91 ± 2.13	3.40 ± 1.25	64.64 ± 6.50

Notes: Data represent the mean ± SD from three experiments conducted in triplicate. All values are significantly different ($p < 0.05$) compared with cisplatin.

Abbreviations: CFWT: Chloroform fraction; EFWT: Ethyl acetate fraction; HFWT: Hexane fraction; MEWT: *Wrightia tinctoria* bark methanolic extract; MFWT: Methanolic fraction; PFWT: Petroleum ether fraction.

Table 2. Selectivity index of *Wrightia tinctoria* crude extract and its fractions against breast (MDA-MB-231 and MCF-7) and prostate cancer (PC-3) cell lines

Extract/Fraction	Selectivity index		
	MCF-7	MDA-MB-231	PC-3
MEWT	6.53	3.36	3.51
HFWT	7.26	2.48	2.14
CFWT	15.01	4.66	2.94
EFWT	7.08	2.84	2.21
MFWT	4.33	2.29	>0.99
PFWT	3.14	1.93	4.30

Abbreviations: CFWT: Chloroform fraction; EFWT: Ethyl acetate fraction; HFWT: Hexane fraction; MEWT: *Wrightia tinctoria* bark methanolic extract; MFWT: Methanolic fraction; PFWT: Petroleum ether fraction.

On the other hand, PFWT showed strong selectivity against PC-3 cells, with an SI of 4.30, suggesting promising prostate-specific cytotoxicity. In contrast, it showed a lower SI value against breast cancer cells than CFWT.

Taken together, the SI value suggests that CFWT is the most promising broad-spectrum candidate, with distinct selectivity for breast cancer (MCF-7). On the other hand, PFWT showed targeted potential for prostate cancer (PC-3). EFWT and HFWT also emerge as potent and relatively safe fractions, mostly for breast cancer cells. The findings collectively support the therapeutic potential of these fractions and warrant further studies. Although CFWT and PFWT demonstrated notable selectivity toward specific cancer cell lines, the clinical significance of this limited selectivity remains to be established. Further *in vivo* studies and detailed toxicity studies are warranted to determine whether the observed selectivity translates into a therapeutic advantage.

3.2. Effect of the petroleum ether fraction on nuclear morphology of cancer cells

A preliminary cell viability assay showed a significant growth-inhibitory effect of the WT crude extract and its fractions against human breast and prostate cancer cells; therefore, we examined whether this activity was due to apoptosis. We performed Hoechst 33342 staining to evaluate the apoptotic properties of the active fractions. The MDA-MB-231, MCF-7, and PC-3 cell lines were exposed to different concentrations of PFWT. The results revealed that, after exposure, the treated cancer cells exhibited distinct apoptotic features across all tested cancer cell lines compared with untreated cells. Fluorescence microscopy of treated cells versus untreated cells confirmed typical features of apoptosis, including chromatin condensation, cell shrinkage, membrane blebbing, chromatin cleavage, and loss of cell membrane symmetry or attachment (Figure 5). As the treatment concentration increased from 6.25 to 200 µg/mL, distortion in cell morphology was clearly observed after 24 h of incubation. In contrast, the control cells maintained their normal morphology.

These results indicated that WT, a traditionally used medicinal plant in the Indian system of medicine, has therapeutic potency. Thiagarajan *et al.*[36] demonstrated that WT leaf ethanolic extract shows effective anticancer and apoptotic potential against the oral cancer KB cell line. Additionally, a semi-purified fraction, DW-F5, from the dichloromethane extract of WT leaves exhibited significant anti-melanoma activity, inhibiting metastasis and angiogenesis in non-obese diabetic severe combined immunodeficient mice.[37]

To the best of our knowledge, this is the first investigation using WT polarity-wise fractions in MCF-7, MDA-MB-231, and PC-3 cell lines. Taken together, these findings support the view that natural products have always been a valuable source of alternative therapeutic approaches and novel medicines.[38]

3.3. Identification of compounds from *Wrightia tinctoria* bark

The PFWT and CFWT samples were potently active against MDA-MB-231, MCF-7, and PC-3 cancer cells. To identify and isolate the active compounds of PFWT and CFWT, column chromatography was performed. PFWT was chromatographed over silica gel 60–120 mesh, then packed in a glass column. The column was eluted with a gradient of hexane:ethyl acetate (100:0 to 0:100%). Repeated column chromatography yielded two active sub-fractions (PFSF1 and PFSF2) and three pure compounds (Wrightia Petroleum Ether [WPE]1–3) (data not shown for WPE1, WPE3, and CFWT compound isolation). Thin-layer chromatography examination of PFSF1 and PFSF2 indicates the presence of unsaturated and low polarity-based compounds, which were subjected to gas chromatography coupled with triple quadrupole mass spectrometer profiling.

3.4. Gas chromatography–tandem mass spectrometry analysis of sub-fractions of *Wrightia tinctoria*

The GC-MS/MS chromatogram of PFSF1 showed 14 prominent peaks, indicating the presence of a diverse range of chemical compounds (Figure 6). The compounds

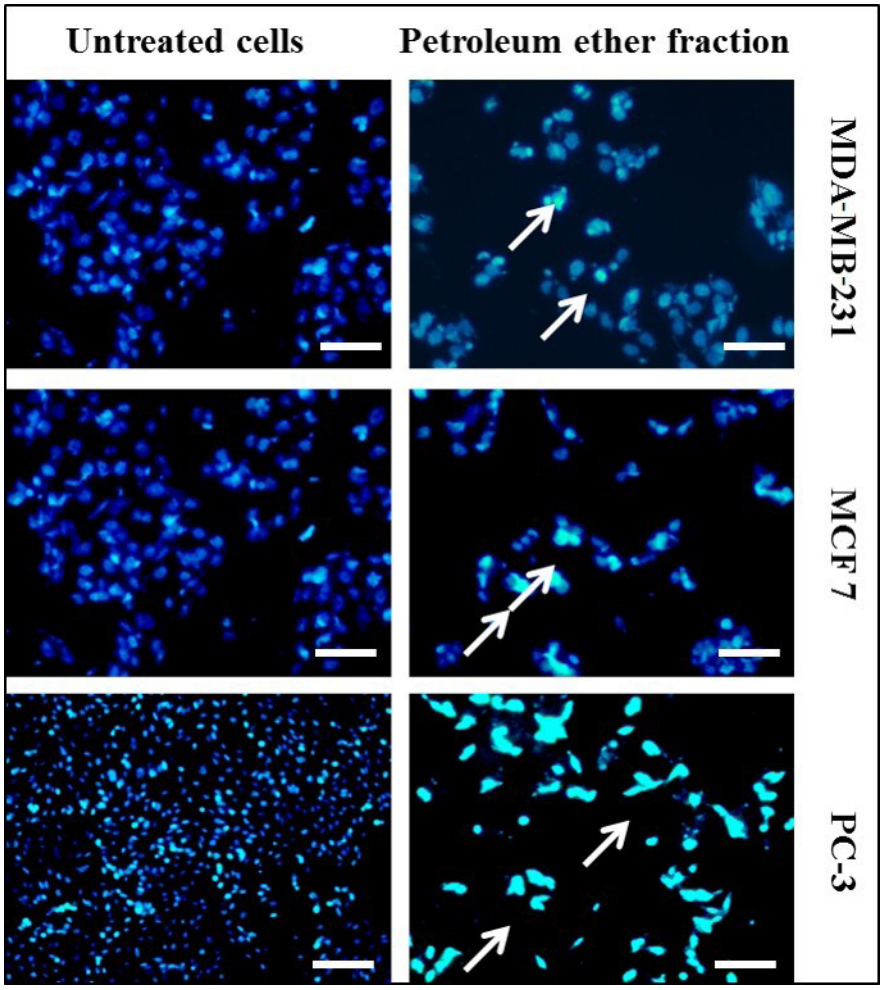


Figure 5. Apoptotic effects of petroleum ether fraction from *Wrightia tinctoria* bark methanolic extract on human breast (MDA-MB-231 and MCF-7) and prostate (PC-3) cancer cells. Detection of morphological apoptosis using Hoechst 33342 staining. Cell morphology was observed under a fluorescence microscope. White arrows indicate the cells with DNA fragmentation. Scale bars: 100 μm; magnifications: 10×.

present in PFSF1 were tentatively identified based on their retention times and fragmentation patterns, compared with the NIST MS 2.0 library. GC-MS/MS profiling enabled tentative identification of constituents, providing insights into the chemical composition of the fraction. The analysis suggested that PFSF1 contains a complex mixture of bioactive molecules across multiple classes, including terpenoids, flavonoids, and phenolic compounds, which may collectively contribute to its biological activities. Specifically, the chromatogram showed four prominent peaks in the retention time range 27.30–43.70 min. The largest peak at 29.87 min was due to the presence of cis-6-octadecenoic acid, trimethylsilyl ester (WPE-8), peak at 27.30 min was due to hexadecanoic acid, trimethylsilyl ester (WPE-7), the third significant peak at 43.7 min was characteristic of lup-20(29)-en-3-ol, acetate, (3á) (WPE-15), and the last major peak at 35.22 min was due to 2,4,6-tris(3,5-dimethylpyrazol-1'-yl)-5,6-difluoropyridine (WPE-9), which is a compound first-time reported in WT bark. The minor compounds identified in PFSF1 were (3S,4R)-3,4-O-isopropylidene-5-cyano-1-pentene (WPE-4), 3,7-dioxa-2,8-disilanonane,2,2,8,8-tetramethyl-5-[(trimethylsilyl) oxy]- (WPE-5), tetradecanoic acid, trimethylsilyl ester (WPE-6), campesterol trimethylsilyl (WPE-10), silane, trimethyl[[(3á)-stigmast-5-en-3-yl]oxy]- (WPE-11), 4-[2-[2-[N-(6-benzyloxy-2,5,7,8-tetramethylchroman-2-ylmethyl)-N-methylamino]ethoxy]benzaldehyde (WPE-12), silane, trimethyl[[(3á)-olean-12-en-3-yl]oxy]- (WPE-13), 2',4"-diethoxy-2",6'-dinitro-m-quaterphenyl (WPE-14), 27-Nor-9,19-cyclolanost-25-yne, 24-methyl-3-(t-butyldimethylsilyloxy)-, (20R,24R,14á) (WPE-16), and

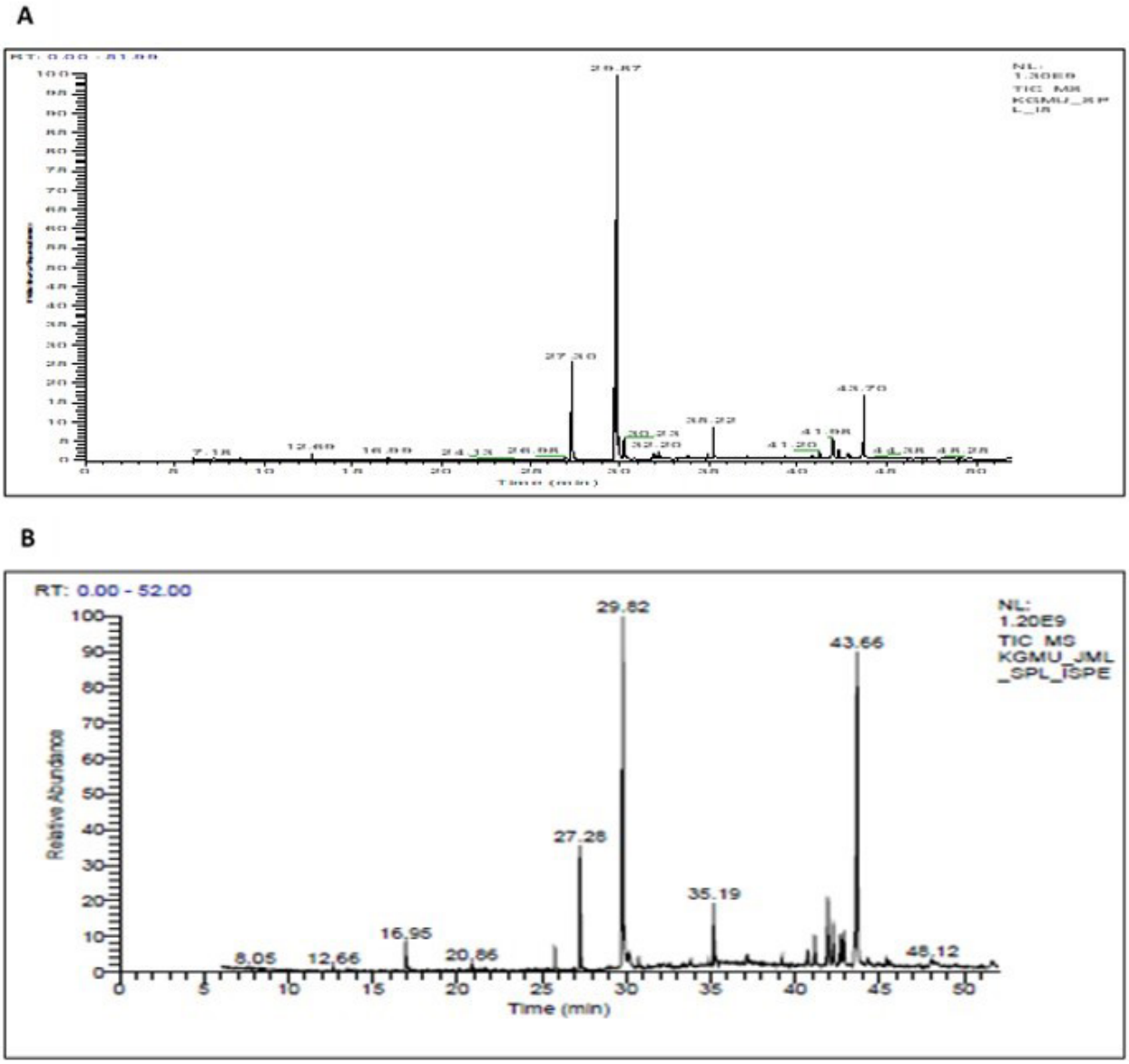


Figure 6. Gas chromatography–tandem mass spectrometry spectra of (A) petroleum ether sub-fraction 1 (PFSF1) and (B) PFSF2

benzoic acid, 3,4,5-tris[(trimethylsilyl)oxy]-propyl ester) (WPE-17). Overall, PFSF1 contains 14 compounds (Table 3), of which 5 compounds were tentatively identified for the first time in WT bark.

These findings highlight the chemical richness of PFSF1, which is dominated by fatty acid derivatives, triterpenoids, and several structurally distinct compounds. Notably, the detection of five previously unreported constituents, including the novel heterocyclic compound WPE-9, emphasizes the unexplored phytochemical diversity present in WT bark. The presence of both major bioactive triterpenes and heterocycle constituents suggests potential multifunctional biological effects. Such diversity may underlie the fraction's antiproliferative activity and indicates that WT remains a promising source for identifying new chemical scaffolds with therapeutic relevance. Further structural confirmation and biological evaluation of these newly reported compounds are warranted.

The GC-MS/MS profiling of PFSF2 revealed the presence of several major bioactive compounds, along with numerous minor constituents (Figure 6). A total of 28 compounds were tentatively identified from PFSF2

Table 3. Identification of phytochemicals from petroleum ether sub-fraction 1 (PFSF1)

Compound ID	Retention time (min)	Compound detected	Molecular formula	Molecular mass
WPE-4[a]	7.18	(3S,4R)-3,4-O-isopropylidene-5-cyano-1-pentene	$C_9H_{13}NO_2$	167
WPE-5	12.69	3,7-dioxa-2,8-disilanonane,2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]	$C_{12}H_{32}O_3Si_3$	308
WPE-6	24.14	Tetradecanoic acid, trimethylsilyl ester	$C_{17}H_{36}O_2Si$	300
WPE-7	27.3	Hexadecanoic acid, trimethylsilyl ester	$C_{19}H_{40}O_2Si$	328
WPE-8	29.87	Cis-6-octadecenoic acid, trimethylsilyl ester	$C_{21}H_{42}O_2Si$	354
WPE-9[a]	35.22	2,4,6-tris(3,5-dimethylpyrazol-1'-yl)-5,6-difluoropyridine	$C_{20}H_{21}F_2N_7$	397
WPE-10	40.80	Campesterol trimethylsilyl	$C_{31}H_{56}OSi$	472
WPE-11	41.20	Silane, trimethyl[[(3á)-stigmast-5-en-3-yl]oxy]	$C_{32}H_{58}OSi$	486
WPE-12[a]	42.29	4-[2-[2-[N-(6-benzyloxy-2,5,7,8-tetramethylchroman-2-ylmethyl)-N-methylamino]ethoxy]benzaldehyde	$C_{31}H_{37}NO_4$	487
WPE-13	42.73	Silane, trimethyl[[(3á)-olean-12-en-3-yl]oxy]	$C_{33}H_{58}OSi$	498
WPE-14[a]	42.93	2',4"-diethoxy-2",6'-dinitro-m-quaterphenyl	$C_{28}H_{24}N_2O_6$	484
WPE-15	43.70	Lup-20(29)-en-3-ol, acetate, (3á)	$C_{32}H_{52}O_2$	468
WPE-16	44.38	27-Nor-9,19-cyclolanost-25-yne,24-methyl-3-(t-butyldimethylsilyloxy)-,(20R,24R,14á)	$C_{36}H_{62}OSi$	538
WPE-17[a]	48.25	Benzoic acid, 3,4,5-tris[(trimethylsilyl)oxy], propyl ester	$C_{19}H_{36}O_5Si_3$	428

Note: [a]Reported for the first time in *Wrightia tinctoria*.
Abbreviation: WPE: Wrightia Petroleum Ether.

(Table 4). Among them, 22 compounds were identified for the first time in WT bark. Results showed that PFSF2 contains 2,4-di(cyclopenta-2,4-dien-1-ylidene)-1,1,3,3-tetramethylcyclobutane (WPE-19), benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester (WPE-28), hexadecanoic acid, trimethylsilyl ester (WPE-7), cis-6-octadecenoic acid, trimethylsilyl ester (WPE-8), 2,4,6-tris (3,5-dimethylpyrazol-1'-yl)-5,6-difluoropyridine (WPE-9), à-*D*-galactopyranoside, methyl2,3-bis-O-(trimethylsilyl)-, cysilane, trimethyl[[(3á,22E)-stigmasta-5,22-dien-3-yl]oxyclic phenylboronate (WPE-36), and betulin (WPE-37).

Taken together across all analyzed WT fractions, 42 chemical compounds were tentatively identified, 27 of which were reported for the first time in this plant. These findings underscore the remarkable phytochemical diversity of WT, encompassing a wide range of chemical classes, including alkaloids, flavonoids, terpenoids, and phenolics. This diversity symbolizes the plant's broad pharmacological potential and its ability to modulate various biological pathways.

The identification of a broad spectrum of chemical entities may underpin the biological activities observed in the fractions, which are potentially the result of synergistic interactions rather than a single molecule. Newly detected constituents, including novel heterocyclic derivatives and triterpenoids, are also present in other plants with anticancer, antioxidant, or anti-inflammatory properties, and may collectively contribute to the antiproliferative effects observed in the present study. The tentative identification of these novel compounds not only enriches the phytochemical database of WT but also provides a foundation for further exploration of their biological activities. These compounds are potentially contributors to the plant's well-documented pharmacological properties, including its anticancer, anti-inflammatory, and antimicrobial activities. Future studies focusing on the bioactivity and mechanism of action of these newly identified compounds could lead to the development of novel therapeutic agents derived from WT.

Despite the promising phytochemical variety and antiproliferative activity observed, the limitations of the study should be acknowledged. GC-MS/MS tentatively identified the compounds based on retention time and mass fragmentation patterns. However, the isolation and characterization of pure compounds should be confirmed using advanced structural techniques, such as 1D and 2D nuclear magnetic resonance, infrared spectroscopy, and high-resolution MS. Additionally, cytotoxic effects were evaluated only under *in vitro* conditions, and therefore,

Table 4. Identification of phytochemicals from petroleum ether sub-fraction 2 (PFSF2)

Compound	Retention time (min)	Compound detected	Molecular formula	Molecular mass
WPE-5	12.66	3,7-dioxa-2,8-disilanonane,2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]	$C_{12}H_{32}O_3Si_3$	308
WPE-18[a]	13.51	2,5,5-trimethyl-2-(butylthio) cycloheptatriene	$C_{14}H_{22}S$	222
WPE-19[a]	16.95	2,4-di(cyclopenta-2,4-dien-1-ylidene)-1,1,3,3-tetramethylcyclobutane	$C_{18}H_{20}$	236
WPE-20[a]	18.28	4,8-cis-8b,8c-cis-2,2,4,6,6,8-hexamethylperhydro-3a,4a,7a,8a-tetraazacyclopentano[def]fluorene	$C_{16}H_{30}N_4$	278
WPE-21[a]	19.37	1à-18o-1,25-dihydroxycholecalciferol	$C_{27}H_{44}O_3$	416
WPE-22[a]	20.12	Cis-4,7,10,13,16,19-docosahexaenoic acid, tert-butyldimethylsilyl ester	$C_{28}H_{46}O_2Si$	442
WPE-23[a]	20.86	11-isopropylidenetricyclo [4.3.1.1(2,5)] undec-3-en-10-one	$C_{14}H_{18}O$	202
WPE-24[a]	21	1,1'-dimethoxy-1,1',2,2'-tetrahydro-ψ,ψ-carotene	$C_{42}H_{64}O_2$	600
WPE-25[a]	22.27	ç-selinene	$C_{15}H_{24}$	204
WPE-26[a]	22.58	Per-trimethylsilyldehydro derivative of glucose	$C_{18}H_{42}O_5Si_4$	450
WPE-27[a]	23.92	5,7,9(11)-androstatriene, 3-hydroxy-17-oxo-	$C_{19}H_{24}O_2$	284
WPE-28[a]	25.79	Benzoic acid, 3,4,5-tris(trimethylsiloxy), trimethylsilyl ester	$C_{19}H_{38}O_5Si_4$	458
WPE-7	27.28	Hexadecanoic acid, trimethylsilyl ester	$C_{19}H_{40}O_2Si$	328
WPE-8	29.82	Cis-6-octadecenoic acid, trimethylsilyl ester	$C_{21}H_{42}O_2Si$	354
WPE-29[a]	30.2	2,6-dinitro-4,4'-di-tert-butylbiphenyl	$C_{20}H_{24}N_2O_4$	356
WPE-30[a]	31.93	3-(2-{[tert-butyl(diphenyl)silyl]oxy}ethyl)-3,4a,7,7,10a-pentamethyl-2,3,4a,6a,7,8,9,10,10a,10b-decahydro-1hbenzo[f]chromen-1-one	$C_{36}H_{50}O_3Si$	558
WPE-31[a]	33.84	1,3-bis(2-methoxy-3-nitrophenyl) tetrahydro-2-pyrimidinone	$C_{18}H_{18}N_4O_7$	402
WPE-9	35.2	2,4,6-tris(3,5-dimethylpyrazol-1'-yl)-5,6-difluoropyridine	$C_{20}H_{21}F_2N_7$	397
WPE-32[a]	37.1	1-[bis(trimethylsilyl)amino]-1-fluoro-3,3,3-trimethyl-1-[(trimethylsilyl)amino] disiloxane	$C_{12}H_{37}FN_2OSi_5$	384
WPE-33[a]	37.35	2,4,6-decatrienoic acid,1a,2,5,5a,6,9,10,10a- octahydro-5,5a-dihydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-11-oxo-1H-2,8a-methanocyclopenta[a]cyclopropa[e]cyclodecen-6-yl ester	$C_{30}H_{40}O_6$	496
WPE-34[a]	39.21	Silane, [[(3á)-cholest-5-en-3-yl]oxy]trimethyl	$C_{30}H_{54}OSi$	458
WPE-35[a]	41.15	Silane, trimethyl[[(3á,22E)-stigmasta-5,22-dien-3-yl]oxy]	$C_{32}H_{56}OSi$	484
WPE-36[a]	41.92	à-*D*-galactopyranoside, methyl2,3-bis-O-(trimethylsilyl)-, cysilane, trimethyl[[(3á,22E)-stigmasta-5,22-dien-3-yl]oxyclic phenylboronate	$C_{19}H_{33}BO_6Si_2$	424
WPE-37	43.66	Betulin	$C_{30}H_{50}O_2$	442
WPE-38[a]	44.29	4'-apo-ψ,ψ-caroten-4′-oic acid	$C_{35}H_{46}O_2$	498
WPE-39[a]	45.4	Prosta-5,13-dien-1-oic acid,9,11,15-tris[(trimethylsilyl)oxy]-, trimethylsilylester, (5Z,9à,11à,13E,15S)	$C_{32}H_{66}O_5Si_4$	642
WPE-40[a]	48.4	Methylsulfinato[2,3,7,8,12,13,17,18-octaethylporphyrinato] indium	$C_{37}H_{47}InN_4O_2S$	726
WPE-41[a]	51.67	Acetic acid,4,4,6a,6b,8a,11,12,14b-octamethyl-14-oxo-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-eicosahydropicen-3-yl ester	$C_{32}H_{50}O_3$	482

Note: [a]Reported for the first time in *Wrightia tinctoria*.
Abbreviation: WPE: Wrightia Petroleum Ether.

the biological relevance, pharmacokinetics, and safety of these fractions cannot be inferred for *in vivo* conditions. The complex mixture of compounds within each fraction makes it difficult to determine the specific constituents responsible for the observed activity or the extent of their synergistic interactions. Future studies involving compound isolation, mechanistic assays, and *in vivo* validation are necessary to strengthen these findings and fully assess the therapeutic potential of WT.

In summary, natural products have shown potent biological activities[39,40] and have always played an important role in the discovery of new chemical entities for therapeutic application, serving as promising lead compounds for the development of newer and more effective drugs through strategic structural modifications and optimization.[41] Consequently, the molecules from natural origin are supposed to be better candidates for further drug development.[42] The present study demonstrated the antiproliferative and phytochemical potential of WT stem bark against human breast and prostate cancer cells *in vitro*. The data from the in vitro studies appeared promising; therefore, it is warranted to further explore the target-based studies of WT fractions and their constituents.

4. Conclusion

Based on the results, the present study highlights the significant antiproliferative and apoptotic potential of the WT bark methanolic extract and its polarity-based fractions against human breast (MCF-7 and MDA-MB-231) and prostate (PC-3) cancer cell lines. Among the tested fractions, PFWT showed the most potent cytotoxicity, inducing distinct apoptotic features, including chromatin condensation and membrane blebbing. Furthermore, bioassay-guided fractionation and GC-MS/MS analysis of PFWT identified 42 compounds, 27 of which were reported for the first time in WT, encompassing diverse chemical classes, including alkaloids, flavonoids, terpenoids, and phenolics. These findings underscore the rich phytochemical diversity and pharmacological potential of WT bark, suggesting its constituents as valuable lead compounds for further mechanistic studies. The study establishes a scientific basis for the traditional use of WT. It warrants further exploration through target-based mechanistic studies.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The data are available from the corresponding author upon reasonable request.

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