***In vitro* anti-cancer properties of a commercially available polyherbal nutraceutical (Vernolac) capsule on cancer stem cell-like (NTERA-2 cl.D1) cells.**

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Abstract

Vernolac is a commercially available polyherbal nutraceutical formulation comprising *Vernonia zeylanica* aerial parts, *Nigella sativa* seeds, *Hemidesmus indica* roots, *Smilax glabra* rhizome, and *Leucas zeylanica* aerial parts. Previous studies have demonstrated anti-cancer activities of phytochemicals derived from these individual plant components. However, the anti-cancer properties of the supercritical CO_2 extract of Vernolac remain unexplored against cancer stem-like cell populations. The current study is focused on the anti-cancer potential of Vernolac extract on cancer stem-like (CSCL) NTERA-2 cl.D1 cells, a human embryonal carcinoma-derived pluripotent cell line. Several in vitro assays evaluated the anti-cancer properties of the Vernolac extract. Cytotoxicity was assessed using the Sulforhodamine B assay, and apoptosis induction was determined by Acridine Orange/Ethidium Bromide staining and the caspase 3/7 activity assay. Scratch assay was used to evaluate cell migration and quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed to analyze the expression levels of apoptosis-related genes (TP53, Survivin) and the autophagy-related gene mTOR. Further, the free-radical scavenging activity of Vernolac extract was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the reactive oxygen species (ROS) levels in NTERA-2 cl.D1 cells were quantified using the nitroblue tetrazolium assay. Results demonstrated that Vernolac extract exhibits significant anti-proliferative activity in NTERA-2 cl.D1 cells, with an IC_{50} of 41.12 µg/mL at 48 h, while exerting minimal effects on non-cancerous MCF-10A cells (IC_{50} > 1000 µg/mL). Fluorescence microscopy and caspase 3/7 assay showed that Vernolac extract leads to early apoptosis in NTERA-2 cl.D1 cells. Quantitative RT-PCR revealed the upregulation of tumor suppressor protein TP53 while downregulating Survivin and mTOR genes. Additionally, Vernolac extract inhibited the migration rate of NTERA-2 cl.D1 cells and elevated intracellular reactive oxygen species (ROS) levels. These findings suggest that the supercritical CO_2 extract of Vernolac exerts potent anticancer properties against NTERA-2 cl.D1 cancer stem-like cells, highlighting its therapeutic potential for targeting cancer stem cells.

Key words: Anti-cancer, Vernolac, Supercritical CO_2 extraction, Nutraceutical, cancer Stem cells

Introduction

Cancer is the second most common cause of mortality worldwide. Despite various cancer therapies being available, conventional treatments often show limited success due to the challenges including as treatment resistance and tumor recurrence, and adverse side effects (Bray et al., 2024; Siegel et al., 2025). These limitations are mainly due to a subpopulation of tumor-initiating cells known as cancer stem cells (CSCs), which possess self-renewal and multi-lineage differentiation capabilities, making them resistant to many conventional therapies, thereby sustaining tumor heterogeneity and recurrence(Pal and Ravindran, 2006). Different treatment strategies are being investigated in order to overcome the limitations of conventional treatment options. Natural pharmaceuticals are a treatment option currently being investigated for their favorable properties such as lesser side effects, diverse modes of actions and reduced potential of treatment resistance (Afkar et al., 2024; Asma et al., 2022; Garg et al., 2024; Naeem et al., 2022; Safe, 2024; Shaik et al., 2022). Moreover, polyherbal extracts is emerging as potential cancer treatment options, and numerous phytochemicals in traditional polyherbal formulations have been shown to exert anticancer effects, with several constituents targeting CSCs through various molecular pathways (Abeysinghe et al., 2019; Saliu et al., 2024; Tharmarajah et al., 2017). The NTERA-2 cl.D1 cell line, a well-characterized human pluripotent embryonal carcinoma cell line, is frequently used as a model to study CSC phenotype behavior and evaluate therapeutic responses, serving as a validated *in vitro* platform for evaluating novel anticancer agents targeting stemness-associated pathways (Borlongan et al., 2025; Tharmarajah et al., 2017).

Commercially available nutraceutical 'Vernolac' is termed after the main constituent *Vernonia zeylanica.* Vernolacis formulated using five different herbal plants, including *Vernonia zeylanica* aerial parts, *Nigella sativa* seeds, *Hemidesmus indica* roots, *Smilax glabra* rhizome and *Leucas zeylanica* aerial parts *(*Afkar et al., 2024*)*. Natural phytochemicals in nutraceuticals reveal multiple advantages through their antioxidant, anti-inflammatory, and cancer prevention potentials (Ali Abdalla et al., 2022; Anand et al., 2023; Atanasov et al., 2021; Guo et al., 2022; Mendis et al., 2018; Samarakoon et al., 2010a; Yun et al., 2012). Alpha-hedrin, Thymoquinone, and Vernolactone are some of the phytochemicals present in of Vernolac formulation, which possess anticancer, antioxidant, and anti-inflammatory properties (Abeysinghe et al., 2019; Daulagala, 2021; Galhena et al., 2012a; Pathiranage et al., 2020a; Ratnasooriya et al., 2007; Rukshala et al., 2021; Samarakoon et al., 2010b; Wu et al., 2022). An *in vitro* study conducted by Abeysinghe et al.

(2019) and Mendis et al. (2018) demonstrated that Vernolactone in *V. zeylanica* triggers the antiproliferative activity and apoptosis in NTERA-2 cl.D1 cells, as well as the triads of breast cancer cells (MDAMB-231, MCF-7, SKBR-3) in a time and dose-dependent manner(Abeysinghe et al., 2019; Mendis et al., 2018). In addition, Samarakoon et al.(2012), Galhena et al. (2017),and Pathiranage *et al*.(2020) have reported that a polyherbal mixture comprised of *N. sativa seeds, H. indicus* roots and *S. glabra* rhizome contain possible anticancer and anti-inflammatory effects (Galhena et al., 2017; Pathiranage et al., 2020; Samarakoon et al., 2012a, 2012b).

Although the anticancer effects of individual phytochemicals present in the constituent plant of the Vernolac have been demonstrated in various studies (Abeysinghe et al., 2019; Galhena et al., 2012b; Mendis et al., 2018; Pathiranage et al., 2020; Saliu et al., 2024; Samarakoon et al., 2012c, 2012b, 2010a).The integrative anticancer effect of the complete polyherbal formulation extracted using the supercritical CO_2 method has not yet been evaluated particularly on CSC-like cell populations. Therefore, the present study was designed to investigate the anticancer potential of the supercritical CO_2 extract of Vernolac against NTERA-2 cl.D1 cells, with the aim of elucidating its efficacy as a potential CSC-targeted anticancer therapeutic.

Materials and methods

Preparation of Vernolac extract

A polyherbal formulation comprised of *V. zeylanica* aerial parts, *N. sativa* seeds, *H. indica* roots, *S. glabra* rhizome and *L. zeylanica* aerial parts was extracted using supercritical fluid extraction method. The extraction was performed using supercritical carbon dioxide (CO_2) at 42°C, at 30 MPa pressure and the extracts were collected at a CO_2 flow rate of 4 mL/min for 1 h.

Cell culture

All the cell lines were cultured and maintained according to American Type Culture Collection (ATCC) guidelines. NTERA-2 cl.D1 (ATCC CRL-1973) cells were cultured in Dulbecco's Modified Eagle medium (DMEM) and MCF-10A cells were cultured in Mammary Epithelial Cell Basal Medium (MEBM). Both the media types were supplemented with 10%, fetal bovine serum (FBS), 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were maintained in a humidified incubator with at 5% CO_2 at 37°C.

Determination of cell viability by SRB assay

Briefly, cells were cultured in 96 well cell culture plates at a density of 7.5×10^3cells/well in 200 µL of medium for 24 h. Cells were treated with varying concentrations of Vernolac extract (6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL) with < 0.1 % DMSO and incubated for 24 and 48 h. After incubation, sulforhodamine B (SRB) assay was performed as described in Rajagopalan *et al.,* 2018, with minor modifications (Rajagopalan et al., 2018). Absorbance was measured at 540 nm using a Synergy HT microplate reader, BioTek Instruments, USA.

Fluorescence microscopic analysis

As described by Abeysinghe *et al.* (2019), NTERA-2 cl.D1 cells (2×10^5 cells/mL) were cultured on a cell culture-treated coverslip and incubated for 24 h. The cells were treated with different doses of Vernolac (5.0, 10, and 20 µg/mL) and incubated for 24 h. Paclitaxel (1.0, 2.0, and 4.0 µg/mL) was used as positive control. At 24 h post-treatment, cells were washed with ice-cold PBS and fixed with 4% formaldehyde solution in the dark for 10 minutes. Cells were stained using Acridine orange and Ethidium bromide (AO\EB) solution (20 µL). Excess stains were removed with PBS (1 mL); cells were air-dried prior to mounting with anti-fading solution. The stained cells were observed under the fluorescence microscope (BX51 TRF, Olympus Corporation, Tokyo, Japan).

Caspase 3/7 luminescence assay

NTERA-2 cl.D1 cells were cultured in a cell culture-treated 96-well plate (20,000 cells/ well in 100 µL of media) and incubated for 24 h. Cells were treated with different concentrations of Vernolac extract (2.5, 5.0, 10, and 20 µg/mL) and incubated for 24 h. Caspase 3/7 Glo® reagent (100 µL) was added to each treated well and incubated for 1 h in the dark condition at 37°C as described by Ediriweera *et al.*2016 (Ediriweera et al., 2016). After incubation and 15 minutes in the shaker, Luminescence was measured using a microplate reader (Synergy HT, Biotek Instruments, USA).

Nitrobluetetrazolium Reactive Oxygen Species (NBT-ROS) assay

NTERA-2 cl.D1 cancer cells (5×10^4 cells/well) were cultured in a 96-well plate using DMEM growth medium with 10% FBS and incubated for 24 h. The extract was treated with Vernolac extract 2.5, 5.0, 10 and 20 µg/mL and incubated for 24 h. At 24 h post-incubation, NBT-ROS assay was performed according to Ediriweera *et al*. 2017. Using Synergy HT microplate reader, absorbance was measured at 560 nm and ROS expression levels were analyzed as percentage relative values of the untreated control (Ediriweera et al., 2017).

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Free-radical scavenging capacity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent according to the method described by Ediriweera *et al*. (2016). Volume of 50 µL from each concentration of Vernolac extract (0.01, 0.023, 0.046, 0.093, 0.1875, 0.375, 0.75, 1.5 and 3 µg/mL) prepared with DMSO by double dilution method. was added in 96-well plates. Samples were further diluted with methanol (90 µL/well) and mixed with 60 µL of DPPH solution (2 mg/ mL) to each well in a dark environment. Ascorbic acid was used as a positive control. The plates were incubated at room temperature in the dark for 10 min, and the absorbance was measured at 517 nm using Synergy HT microplate reader. Free-radical scavenging capacity of each concentration was calculated in percentage using the formula $(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})/\text{Absorbance}_{\text{control}} \times 100\%$, where control contains all the reagents used except Vernolac extract (Ediriweera et al., 2016).

Cell migration assay

NTERA-2 cl.D1 cells were seeded in 96-well plates at a density of $2x10^4$/200 µL and incubated up to 100% confluence until a fully confluent cell monolayer was observed. As described by Abeysinghe *et al.* (2018), a vertical scratch was made on the monolayer formed in each well and washed twice with PBS to remove detached cells and debris. Then, the cells were treated with different concentrations of Vernolac extract (5, 10, 20, and 40 µg/mL) and paclitaxel as the positive control in separate wells. Untreated cells were used as controls. Closure of the scratch made was observed by a phase-contrast microscope at every 12 h of incubation, and the area of closures was

calculated using the recorded images. The rate of migration affected by respective treatments was calculated using the change in the cell-free area (wound) over the time.

Real time - polymerase chain reaction (qPCR)

NTERA-2 cl.D1 cells ($2.5x10^5$ cells/mL) were seeded into 25 cm^2 cell culture flasks and incubated for 24 h in 37 C and 5 % CO2 : 95 % air environment. Then the cells were exposed to different concentrations (10 and 20 µg/mL) of Vernolac extract under previously mentioned conditions. Total RNA was isolated using PromegaSV Total RNA isolation system, USA (Cat. No: Z3100SV) according to the manufacturer's instructions. Complementary DNA (cDNA) from isolated total RNA was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase system (Cat.no: M1701, Promega Cooperation, Madisons, USA). Gene expression profiling of *TP53*, *Survivin,* and *mTOR* was carried out with *GADPH* gene as the internal control using QuantiNova® SYBR® Green PCR Kit (Cat. No. 208052, Qiagen) and the qPCR reaction was carried out with QuantStudio® 5dx Real-Time PCR Systems (Applied Biosystems, Massachusetts, USA) on 96 well PCR plates. *GADPH* was used as the internal control. The relative gene expression analysis was performed using Livak method, where fold changes were calculated using $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001). Primers used for gene expression profiling are given in Table 1 and the PCR cycle conditions were as follow initial denaturation at 95°C for 10 min, and amplification in three steps for 40 cycles (denaturation at 95˚C for 30 s; annealing at 58˚C for 1 min and extension at 60˚C for 30 s). Annealing temperature for gene *mTOR* was maintained at 52˚C.

Table 01: Primers used for expression of *TP53, Survinin, mTOR,GAPDH*

Gene	Forward & reverse primers	Amplicon size/bp
***TP53*:**	Forward:5′- TCCAGGATCGAGCAGGGCGAA Reverse: 5′- CGATCCGCTTGAGACACTCGCT	369 bp
***Survinin*:**	Forward:5′-TGGCCGCTCCTCCCTCAGAAAA Reverse:5′- GCTGCTGCCTCCAAAGAAAGCG	190 bp
***mTOR*:**	Forward: 5′- TGGGACAGCATGGAAGAATA Reverse: 5′- TGTTGTGCCAAGGAGAAGAG	110 bp

***GAPDH*:**	Forward: 5′- GGCATTGCCCTCAACGACCAC Reverse: 5′- ACATGACAAGGTGCGGCTCCCTA	283 bp

Statistical analysis

Data analysis was performed using GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Each experiment was replicated. Statistical analysis was performed for caspase 3/7 activity assay and NBT-ROS assay by using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A p-value of < 0.05 was considered statistically significant.

Results

Cytotoxicity and cell proliferation assay

As indicated by the results of the SRB assay (Table 02), Vernolac extract exerted a potential to mediate dose- and time-dependent suppression of NTERA-2 cl.D1 cell proliferation. Both Vernolac extract, and Paclitaxel showed a dose and time-dependent anti-proliferative activity against NTERA-2 cl.D1 cells.

Table 02: IC_{50} values (µg/mL) of Vernolac extract and Paclitaxel in NTERA-2 cl.D1 and MCF-10 cells.

Cell type	Vernolac extract (µg/mL)		Paclitaxel (µg/mL)	
	24 h	48 h	24 h	48 h
NTERA-2 cl.D1 cells	64.5	41.12	4.96	1.79
MCF-10A cells	1075	803.5	11.90	6.70

Fluorescence microscopic observations

Acridine Orange (AO)/Ethidium Bromide (EB) stained NTERA-2 cl.D1 cells were incubated 24 h after the treatment with different concentrations of Vernolac extract and Paclitaxel. By employing Acridine orange/Ethidium Bromide staining, clear identification of apoptosis-associated membrane alterations was achieved. The apoptotic changes of the cells were distinguished and visualized by the fluorescence staining, yellow/orange fluorescence indicates early apoptotic cells while red fluorescence indicates late apoptosis, demonstrating disruption of cellular membrane. The induction of apoptosis was increased in Vernolac extract (5, 10 and 20 µg/mL) - and Paclitaxel (1, 2, and 4 µg/mL) treated NTERA-2 cl.D1 cells in a dose-dependent manner (Figure 01).

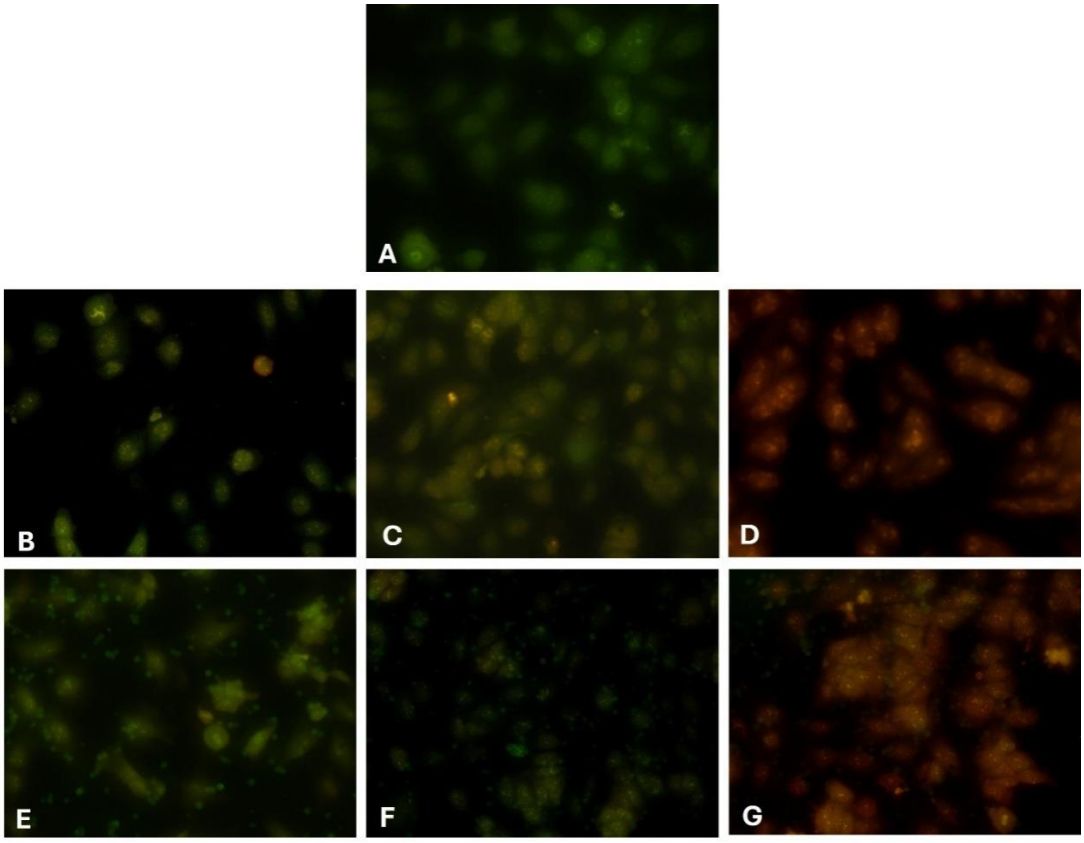

Figure 01: Fluorescence microscopic observation of NTERA-2 cl.D1 cells under 200X magnification after 24 h of Paclitaxel and Vernolac extract treatment and stained with AO/EB (A – Untreated control, B – Paclitaxel (1 µg/mL), C – Paclitaxel(2 µg/mL), D – Paclitaxel(4 µg/mL), E –Vernolac extract (5µg/mL).F–Vernolac extract - (10µg/mL), G –Vernolac extract (20µg/mL).

Expression of caspase 3 and Caspase 7 activity in NTERA-2 cl.D1 cells

Activities of caspase 3 and 7 were assessed using the Caspase Glo®3/7 assay kit (Promega, USA) according to the manufacturer's guidelines. Results are presented as the mean ± standard deviation values. Vernolac extract significantly increased the activities of caspase 3 and caspase 7 in

NTERA-2 cl.D1 cells at the concentrations of 10 µg/mL ($P < 0.001$) and 20 µg/mL ($P < 0.0001$) as shown in Figure 02.

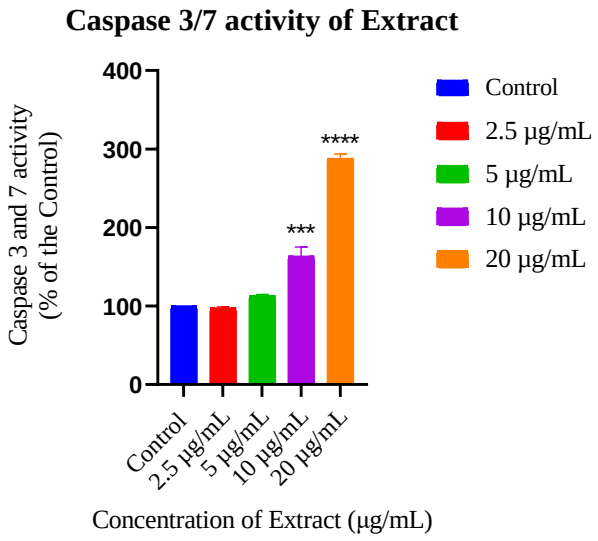

Figure 02: Expression of Caspase 3 and 7 in NTERA-2 cl.D1 cells after 24 h post-treatment with Vernolac extract. (Results are indicated as mean ± standard deviation of duplicate determination. *** $P < 0.001$ and ****$P < 0.0001$ compared to the control).

Free radical Scavenging activity of Vernolac Extract

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed to evaluate the free radical scavenging activity of Vernolac extract. Ascorbic acid was used as a positive control. The obtained EC_{50} values of free radical scavenging activity of Vernolac extract and Ascorbic are summarized in Table 03.

Table 03: EC_{50} values of free radical scavenging activity for Vernolac extract and Ascorbic acid.

Sample	EC_{50} values (µg/mL)
Vernolac extract	> 1000
Ascorbic acid	53.678

Effect of the Vernolac extract on ROS levels

NBT-ROS assay was performed to analyze the ROS production in NTERA-2 cl.D1 cells after 24 h of Vernolac extract. A P<0.001 was considered to indicate a strong statistically significant result. As seen in the bar graph (Figure 03), treatment with 10 µg/mL or 20 µg/mL of Vernolac extract caused a major increase in ROS activity compared to the non-treated control group (P< 0.001).

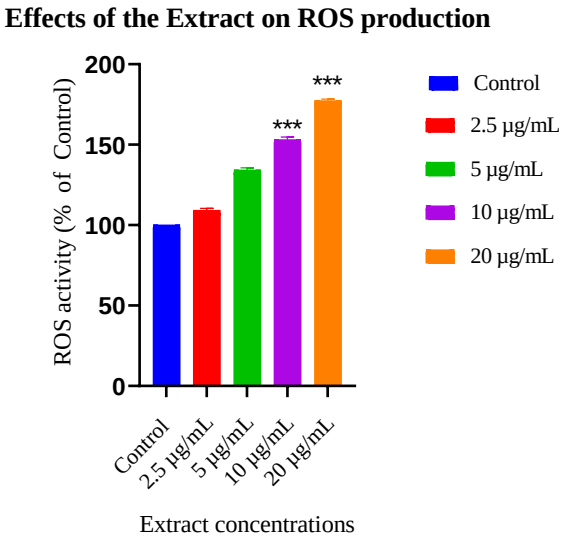

Figure 03: Levels of reactive oxygen species (ROS) detected in NTERA-2 cl.D1 cells after 24 h incubation with Vernolac extract treatment under different concentrations (Results are indicated as mean ± Standard deviation of triplicate determination. ***P < 0.001 compared to the control.

Analysis of the wound healing rate of NTERA-2

The NTERA-2 cl.D1 cells were incubated until they created a confluent monolayer at the bottom of the well. An artificial gap was made on the confluent monolayer, and both Vernolac extract, and Paclitaxel were treated separately according to a concentration gradient. After 24 h of drug treatment, untreated control cells have migrated at a rate of 62.126 µm/h. The cell migration rate

of Vernolac extract and Paclitaxel is graphically represented as shown in Figure 04. The morphological representation of cell migration at 0 h and after the gap of control closure is shown in Figure 05.

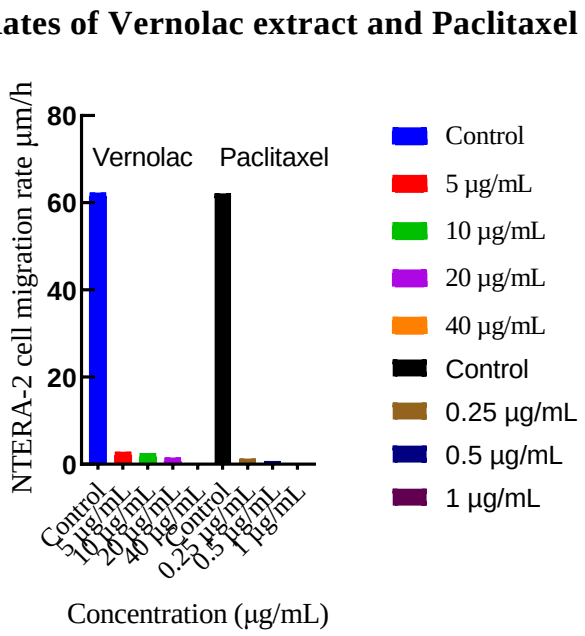

Figure 04: Cell migration rates of Vernolac extract with the concentrations of 5-, 10-, 20-, 40-µg/mL and Paclitaxel (0.25-, 0.5- and 1 µg/mL) treated NTERA-2 cl.D1 cells compared to untreated control under different concentrations.

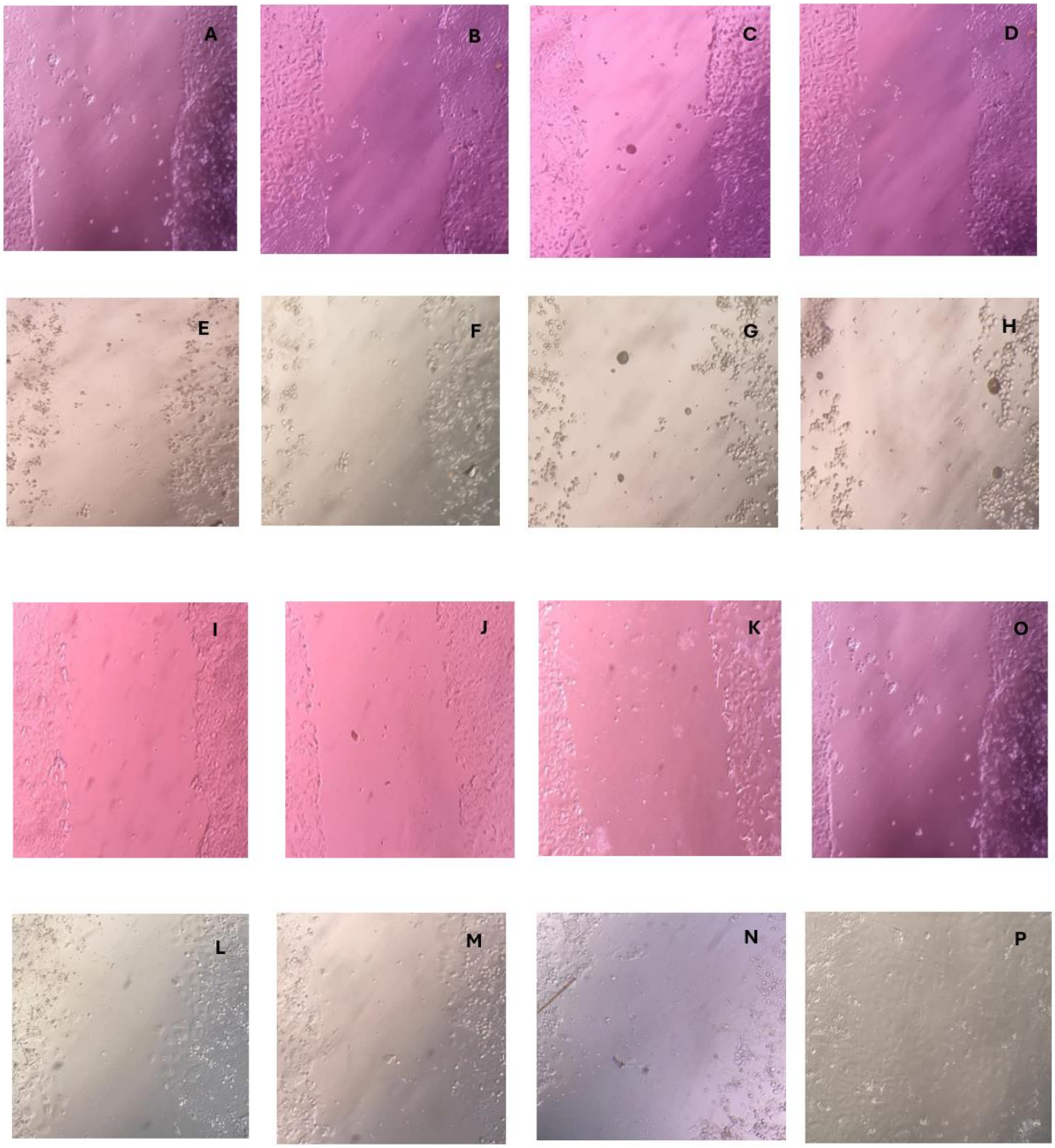

Figure 05: Phase contrast light microscopic images under 10× magnification captured after 0 h of creating the gap in NTERA cl.D1 cell monolayer, followed by Vernolac extract with 5-, 10-, 20-, 40- µg/mL concentrations (A-D), paclitaxel with 0.25-, 0.5-, and 1 µg/mL concentrations (I–K) and the Untreated control (O). Cell migration after 22 h of creating the gap, followed by Vernolac extract (E-H), paclitaxel (L–N), and Untreated control (P).

Effect of Vernolac extract on the expression of *TP53*, *Survivin,* and *mTOR*

Results of the qPCR (Figure 06) demonstrated that the Vernolac extract enhanced *TP53* mRNA expression in humanNTERA-2 cl.D1 cells in a significant ($P < 0.001$) dose-dependent manner. Furthermore, treatment with all concentrations of supercritical Vernolac extract and Paclitaxel resulted in a significant downregulation ($P < 0.05$) of the expression of *mTOR* and *Survivin*.

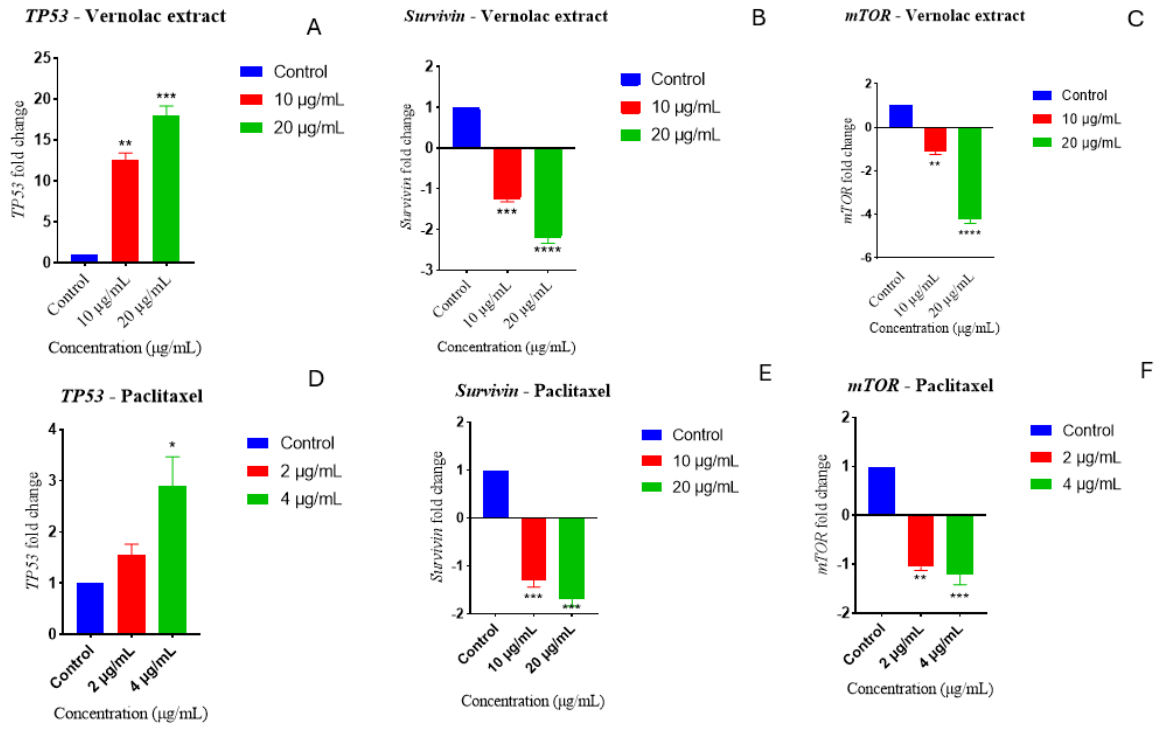

Figure 06: Expression of selected genes on Vernolac extract and Paclitaxel treatment. (A- Vernolac extract on *TP53* expression, B - Vernolac extract on *Survivin* expression, C - Vernolac extract on *mTOR* expression, D- Paclitaxel on *TP53* expression, E - Vernolac extract on *Survivin*expression, F - Vernolac extract on *mTOR* expression). Results are indicated as mean ± standard deviation, duplicate determination. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. compared to the control).

Discussion

Previous studies have shown that traditional poly herbal formulation demonstrates significant anti proliferative activities against several cancer cell lines, including MCF-7, HepG2, and NCI-H292 (Samarakoon et al., 2010a, 2012c; Sakthi Saravanan et al., 2015; Bhatt et al., 2017; Pathiranage et al., 2020). Sailu *et al*. (2024) and Abeysinghe *et al.* (2019) have elucidated that phytochemicals such as, alpha-hederin and Vernolactone present in the constituents of Vernolac have proven anticancer activity against cancer stem-like cells (Abeysinghe et al., 2019; Saliu et al., 2024). The present *in vitro* investigation employed supercritical fluid extraction with liquid CO_2, a superior extraction methodology that preserves thermolabile compounds while eliminating organic solvent residues, to evaluate the anticancer efficacy of Vernolac extract against the pluripotent human testicular embryonal carcinoma (NTERA-2 cl.D1) cell line.

Consistent with these earlier findings as shown by Abeysinghe et al. (2019), the profiles obtained through SRB assay of this study demonstrated dose and time dependent cytotoxicity against NTERA-2 cl.D1 cells (Abeysinghe et al., 2019). Vernolac extract showed an anti-proliferative effect against the cells with an IC_{50} value of 41.12 µg/mL after 48 h treatment, while demonstrating significantly lower cytotoxicity against non-tumorigenic epithelial MCF-10A cells (IC_{50} value of 1075 µg/mL), suggests selective cytotoxicity towards cancer stem cells (Table 02). 'Vernolactone', which is an active compound of the polyherbal isolated from *V. Zeylanica*, has been shown a significant cytotoxicity against NTERA-2 cl.D1 (0.4808 µg/mL at 72 h) cells with Paclitaxel (Abeysinghe et al., 2019). Similarly, Pathiranage et al. (2020) and Samarakoon et al. (2012) have also reported that a polyherbal mixture, including several constituents of Vernolac (*N. sativa, H. indicus* and *S. glabra,*) displayed a dose-dependent inhibition of cell proliferation in treated cancer cell lines (MCF-7, MDA-MB-231, HepG2), aligning with the findings (Samarakoon et al., 2012c; Pathiranage et al., 2020).

The fluorescent and phase contrast microscopic data revealed that Vernolac Extract induces dose-dependent apoptosis-related cytomorphological changes in NTERA-2 cl.D1 cells (Figure 01), including cell membrane blabbing and cell shrinkage. Furthermore, compared to the untreated cells (control), Vernolac extract significantly ($P < 0.0001$) enhanced the enzymatic activities of executioner caspases, caspase 3, and caspase 7 at a 20 µg/mL concentration, indicating activation of the terminal phase of programmed cell death (Figure 01). These findings align with previous

research done by Abeysinghe et al. (2019), who reported that Vernolactone significantly ($P < 0.0001$) upregulated caspase 3/7 activity at both 2 µg/mL and 4 µg/mL concentrations when compared to the untreated NTERA-2 cl.D1 cells (untreated control) (Abeysinghe et al., 2019). A traditional polyherbal mixture formulated using *N. sativa*, *H. indicus* and *S. glabra* has also demonstrated an elevated caspase 3/7 activation against several cancer cell lines (HepG2, MCF-7, MDA-MB-231, SKBR-3), suggesting a conserved apoptotic mechanism among these phytotherapeutic agents (Samarakoon et al., 2012c ; Mendis et al., 2018; Pathiranage et al., 2020). Further, extracts of *N. sativa* seed extract demonstrated an elevated caspase 3 activity on MDA-MB-231 breast cancer cell line.

In this study, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay revealed that Vernolac extract contains a low antioxidant activity (EC_{50} value > 1000 µg/mL) compared to the positive control: Ascorbic acid (EC_{50} = 53.678 µg/mL) (Table 03). The obtained result aligns with previous study demonstrating that Vernolactone possesses low antioxidant activity (free radical scavenging capacity) through the DPPH assay (Abeysinghe et al., 2019). However, multiple investigations have indicated that *N. sativa, H. indicus* and *S. glabra* extracts have shown free radical scavenging activity through multiple analyses (Galhena et al., 2012b; Pathiranage et al., 2020a). Comprehensive antioxidant profiling using complementary analysis, such as ABTS and FRAP assays, are recommended to fully characterize the antioxidant capacity of Vernolac extract. Despite, extract of Vernolac having a minimal antioxidant activity, a significantly ($P < 0.001$) elevated ROS level was determined in NTERA-2 cl.D1 at the concentrations of 10 and 20 µg/mL (Figure 03). This pro-oxidant effect in cancer cells, contrasting with limited antioxidant capacity in cell-free systems, suggests a selective modulation of redox homeostasis within the cellular microenvironment, potentially contributing to the observed cytotoxicity(Petronek et al., 2021).

The effect of Vernolac extract on the migration of NTERA-2 cl.D1 cells was evaluated using a wound healing assay. A dose-dependent migration rate (< 3 µm/h across all tested concentrations) (Figure 04), demonstrated minor or no reduction in the wound width throughout the experiment in Vernolac treated cells compared to the untreated NTERA-2 cl.D1 cells (Figure 05), indicates significantly reduced migratory capacity. Similarly, paclitaxel also restricted cellular migration to rates below 1.5µm/h for all tested concentrations. This pronounced inhibition of cellular motility

indicates impairment of cytoskeletal reorganization and extracellular matrix interaction, critical processes in tumor cell invasiveness and metastatic potential (Brábek et al., 2010).

Moreover, Real-time PCR (qPCR) was performed to evaluate the expression of several autophagy and apoptosis-related genes (*TP53*, *Survivin,* and *mTOR*). The tumor suppressor *TP53* is a key gene that is essential for DNA repair, apoptosis, and triggering of cell cycle arrest, and plays a role in autophagy. Moreover, *p53* is involved suppression of *Survivin* at both mRNA and protein expression levels to promote apoptosis (Brábek et al., 2010). Lower doses of Vernolac extract and paclitaxel were used to maintain the cells in viable conditions until the cells were under the early apoptotic stage at the time of RNA extraction. Vernolac extract led to a significant ($P < 0.001$) upregulated tumor suppressor *TP53* expression, with 9.67 fold change at 10 $\mu g/mL$ and 13.29 fold change at 20 $\mu g/mL$ compared to untreated control(Figure 06).Concurrently, Vernolac extract-treated NTERA-2 cells resulted in significant ($P < 0.05$) downregulation of mammalian target of rapamycin (*mTOR*) and *Survivin* expression at both 10 and 20 µg/mL concentrations(Figure 06). According to Abeysinghe et al. (2019), it was shown that Vernolactone in *V. zeylanica* significantly induced apoptosis and autophagy in NTERA-2 cl.D1 cells through modulation of the PI3K/Akt/mTOR pathways(Abeysinghe et al., 2019). Key phytochemicals of *Nigella sativa*; Beta catenin, Alpha-hederin and Thymoquinone, have been reported to inhibit DNA synthesis, induce cell cycle arrest, and promote apoptosis via both p53-dependent and p53-independent mechanisms (Ndreshkjana et al., 2019; Pathiranage et al., 2020b; Saliu et al., 2024; Samarakoon et al., 2012b). Collectively, these above-mentioned studies indicate that active compounds in the constituents of Vernolac extract exert their anticancer effects through coordinated modulation of apoptotic and autophagy-related pathways.

Vernolac extract comprises *V. zeylanica* aerial parts, *N. sativa* seeds, *H. indica* roots, *S. glabra* rhizome and *L. zeylanica* aerial parts investigated in the present study exert anti-proliferative and anti-cancer effects via apoptosis, autophagy and cell cycle arrest through upregulation of *TP53*and downregulation of *Survivin,* and *mTOR*. The observed cytotoxic and apoptotic effects likely result from the action of a single active compound and/or synergistic interactions among various phytochemicals within the extract, potentially offering advantages over single-compound therapies(Galhena et al., 2017; Mendis et al., 2018; Rajagopalan et al., 2018; Saliu et al., 2024). Further, *in vitro* investigations are warranted to elucidate and confirm the precise molecular

mechanisms underlying the anticancer activity, and subsequent *in vivo* studies are essential to evaluate its efficacy and the biological effects of Vernolac extract within living organisms (physiologically relevant systems), especially the safety profile.

Conclusion

This study demonstrates that Vernolac extract comprises *Vernonia zeylanica* aerial parts, *Nigella sativa* seeds, *Hemidesmus indica* roots, *Smilax glabra* rhizome and *Leucas zeylanica* aerial parts exerts selective antiproliferative effects on NTERA-2 cl.D1, cancer stem-like cell by inducing apoptosis and autophagy, while exhibiting minimal cytotoxicity toward noncancerous epithelial cells (MCF-10A). These findings support the potential of Vernolac extract as a promising therapeutic candidate targeting cancer stem cells.

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