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

Poorni Chanuka Rathnayake[1], Duvinika Chalani Senavirathna[1], Matheen Muhammadh Milhan[1], Sandani De Vass Gunawaradane[1], Nirwani Natasha Seneviratne[1], Dipun Nirmal Perera[1], Umapriyatharshini Rajagopalan[1], Kanishka Sithira Senathilake[1], Sameera Ranganath Samarakoon[1*]

[1]Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo
No. 90, Cumaratunga Munidasa Mawatha, Colombo 03, Sri Lanka.

***Corresponding author:**
Sameera R Samarakoon
Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo
No. 90, CumaratungaMunidasa Mawatha, Colombo 03, Sri Lanka.
Telephone: +94 11 2552528 (Ext- 318)
Fax: +94-11-2553683
Email: sam@ibmbb.cmb.ac.lk

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 NTERA-2 cl.D1 cancer stem-like model, a human embryonal carcinoma-derived pluripotent cell line. Gas Chromatography-Mass Spectrometry (GC-MS) was performed for phytochemical analysis. 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 Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) was performed to analyze the expression levels of apoptosis-related genes (*TP53, BIRC5*) and the autophagy-related gene *mTOR*. Further, the free-radical scavenging activity of Vernolac extract was assessed using 2,2-diphenyl-1-picrylhydrazyl and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay. The reactive oxygen species levels in NTERA-2 cl.D1 cells were quantified using the nitroblue tetrazolium assay. GC-MS analysis revealed 20 phytochemical constituents in the supercritical CO_2 extract. *In vitro* 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. RT-qPCR revealed the upregulation of tumor suppressor protein P53 while downregulating *BIRC5* and *mTOR*. Additionally, Vernolac extract inhibited the migration rate of NTERA-2 cl.D1 cells and elevated intracellular reactive oxygen species levels. These findings suggest that the supercritical CO_2 extract of Vernolac exerts potent anticancer properties against NTERA-2 cl.D1 cancer stem-like model, highlighting its therapeutic potential for targeting cancer stem cells.

Key words: Anti-cancer, Vernolac, Supercritical CO_2 extraction, Nutraceutical, Cancer Stem Cell like models

Introduction

Cancer is one of the most common causes of mortality worldwide [1]. Despite various cancer therapies being available, conventional treatments often show limited success due to the challenges, including treatment resistance and tumor recurrence, and adverse side effects [1,2]. 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 [3–6]. Different treatment strategies are being investigated to overcome the limitations of conventional treatment options. Natural pharmaceuticals are a treatment option currently being investigated for their favorable properties, such as fewer side effects, diverse modes of action, and reduced potential of treatment resistance [7–12]. Moreover, polyherbal extracts are 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 [13–15]. 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-like model phenotype behavior and evaluate therapeutic responses, serving as a validated *in vitro* platform for evaluating novel anticancer agents targeting stemness-associated pathways [14,16].

Commercially available nutraceutical 'Vernolac' is coined after the main constituent *Vernonia zeylanica.* Vernolac is 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 [8]. Natural phytochemicals in nutraceuticals reveal multiple advantages through their antioxidant, anti-inflammatory, and cancer prevention potentials [17–23]. Alpha-hedrin, Thymoquinone, and Vernolactone are some of the phytochemicals present in Vernolac formulation, which possess anticancer, antioxidant, and anti-inflammatory properties [15,24–30]. An *in vitro* study conducted by Abeysinghe et al. [15] and Mendis et al. [22] 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. In addition, Samarakoon et al. [23], Galhena et al. [25], and Pathiranage et al. [26] 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.

88 Although the anticancer effects of individual phytochemicals present in the constituent plants of
89 the Vernolac have been demonstrated in various studies [13,15,22,23,31–34], the integrative
90 anticancer effect of the complete polyherbal formulation extracted using the supercritical CO_2
91 method has not yet been evaluated, particularly on CSC-like cell populations. Therefore, the
92 present study was designed to investigate the anticancer potential of the supercritical CO_2 extract
93 of Vernolac against NTERA-2 cl.D1 cells, to elucidate its efficacy as a potential CSC-targeted
94 anticancer therapeutic.

95

96 **Materials and methods**

97 **Preparation of Vernolac extract**

98 A polyherbal formulation comprised of *V. zeylanica* aerial parts, *N. sativa* seeds, *H. indica* roots,
99 *S. glabra* rhizome, and *L. zeylanica* aerial parts was extracted using the supercritical fluid
100 extraction method. Plant materials were collected in September 2024 (Batch No. V24085). The
101 extraction was performed using supercritical carbon dioxide (CO_2) at 42°C, at 30 MPa pressure
102 and the extracts were collected at a CO_2 flow rate of 4 mL/min for 1 h.

103 **Cell culture**

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

110 **Determination of cell viability by SRB assay**

111 Briefly, cells were cultured in 96-well cell culture plates at a density of 7.5×10^3cells/well in 200
112 µL of medium for 24h. Cells were treated with varying concentrations of Vernolac extract (6.25,
113 12.5, 25, 50, 100, 200, and 400 µg/mL) with < 0.1 % DMSO and incubated for 24 and 48h. After
114 incubation, sulforhodamine B (SRB) assay was performed as described in Rajagopalan et al. [35]
115 with minor modifications. Absorbance was measured at 540 nm using a Synergy HT microplate
116 reader, BioTek Instruments, USA.

117 **Fluorescence microscopic analysis**

118 As described by Abeysinghe et al. [12], NTERA-2 cl.D1 cells (2×10^5 cells/mL) were cultured
119 on a cell culture-treated coverslip and incubated for 24h. The cells were treated with different doses
120 of Vernolac (5.0, 10, and 20 µg/mL) and incubated for 24h. Paclitaxel (1.0, 2.0, and 4.0 µg/mL)
121 was used as a positive control. At 24h post-treatment, cells were washed with ice-cold PBS and
122 fixed with 4% formaldehyde solution in the dark for 10 minutes. Cells were stained using Acridine
123 orange and Ethidium bromide (AO\EB) solution (20 µL). Excess stains were removed with PBS
124 (1 mL); cells were air-dried prior to mounting with anti-fading solution. The stained cells were
125 observed under the fluorescence microscope (BX51 TRF, Olympus Corporation, Tokyo, Japan).

126

127 **Caspase 3/7 luminescence assay**

128 NTERA-2 cl.D1 cells were cultured in a cell culture-treated 96-well plate (20,000 cells/ well in
129 100 µL of media) and incubated for 24h. Cells were treated with different concentrations of
130 Vernolac extract (2.5, 5.0, 10, and 20 µg/mL) and incubated for 24h. Caspase 3/7 Glo® reagent
131 (100 µL) was added to each treated well and incubated for 1h in the dark condition at 37°C as
132 described by Ediriweera et al. [36]. After incubation and 15 minutes in the shaker, Luminescence
133 was measured using a microplate reader (Synergy HT, Biotek Instruments, USA).

134

135

136 **Nitrobluetetrazolium Reactive Oxygen Species (NBT-ROS) assay**

137 NTERA-2 cl.D1 cancer cells (5×10^4 cells/well) were cultured in a 96-well plate using DMEM
138 growth medium with 10% FBS and incubated for 24h. The extract was treated with Vernolac
139 extract 2.5, 5.0, 10, and 20 µg/mL and incubated for 24h. At 24h post-incubation, the NBT-ROS
140 assay was performed according to Ediriweera et al. [37]. Using the Synergy HT microplate reader,
141 absorbance was measured at 560 nm, and ROS expression levels were analyzed as percentage
142 relative values of the untreated control.

143

144 **2,2-diphenyl-1-picrylhydrazyl (DPPH) assay**

145 Free-radical scavenging capacity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH)
146 reagent according to the method described by Ediriweera et al. [36]. Volume of 50 µL from each
147 concentration of Vernolac extract (0.01, 0.023, 0.046, 0.093, 0.1875, 0.375, 0.75, 1.5, and 3 µg/mL)
148 prepared with DMSO by the double dilution method. was added in 96-well plates. Samples were
149 further diluted with methanol (90 µL/well) and mixed with 60 µL of DPPH solution (2 mg/ mL)
150 in each well in a dark environment. Ascorbic acid was used as a positive control. The plates were
151 incubated at room temperature in the dark for 10 min, and the absorbance was measured at 517
152 nm using Synergy HT microplate reader. Free-radical scavenging capacity of each concentration
153 was calculated in percentage using the formula (Absorbance $_{control}$ – Absorbance $_{sample}$)/Absorbance
154 $_{control}$ × 100%, where control contains all the reagents used except Vernolac extract.

155

156

157 **2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay**

158 The 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of
159 Vernolac extract was measured described by num A dilution series of Vernolac extract (7.8125,
160 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL) was prepared in PBS prior to the assay.
161 Vernolac (160 µL) and ABTS (40 µL) were added to each well, and the plate was incubated for 10
162 min at room temperature. Ascorbic acid was used as a positive control. Absorbance of each well
163 was measured at 734nm using a microplate reader. The percentage radical scavenging activity was
164 calculated as (Abcontrol – Absample) /(Abcontrol) × 100.

165

166

167 **Cell migration assay**

168 NTERA-2 cl.D1 cells were seeded in 96-well plates at a density of $2x10^4$/200 µL and incubated
169 up to 100% confluence until a fully confluent cell monolayer was observed. As described by
170 Abeysinghe et al. [12], a vertical scratch was made on the monolayer formed in each well and
171 washed twice with PBS to remove detached cells and debris. Then, the cells were treated with

172 different concentrations of Vernolac extract (5, 10, 20, and 40 µg/mL) and paclitaxel as the positive
173 control in separate wells. Untreated cells were used as controls. Closure of the scratch made was
174 observed by a phase-contrast microscope at every 12h of incubation, and the area of closures was
175 calculated using the recorded images. The rate of migration affected by respective treatments was
176 calculated using the change in the cell-free area (wound) over time.

177

178

179 **Reverse Transcriptase quantitative polymerase chain reaction (RT-qPCR)**

180 NTERA-2 cl.D1 cells ($2.5x10^5$ cells/mL) were seeded into 25 cm^2 cell culture flasks and incubated
181 for 24h in 37 C and 5 % CO2 : 95 % air environment. Then the cells were exposed to different
182 concentrations (10 and 20 µg/mL) of Vernolac extract under the previously mentioned conditions.
183 Total RNA was isolated using PromegaSV Total RNA isolation system, USA (Cat. No: Z3100SV)
184 according to the manufacturer's instructions. Complementary DNA (cDNA) from isolated total
185 RNA was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase
186 system (Cat.no: M1701, Promega Corporation, Madisons, USA). Gene expression profiling of
187 *TP53*, *BIRC5,* and *mTOR* was carried out with *GADPH* gene as the internal control using
188 QuantiNova® SYBR® Green PCR Kit (Cat. No. 208052, Qiagen) and the qPCR reaction was
189 carried out with QuantStudio® 5dx Real-Time PCR Systems (Applied Biosystems, Massachusetts,
190 USA) on 96-well PCR plates. *GADPH* was used as the internal control. The relative gene
191 expression analysis was performed using the Livak method, where fold changes were calculated
192 using $2^{-\Delta\Delta Ct}$ equation [38]. Primers used for gene expression profiling are given in Table 1, and the
193 PCR cycle conditions were as follows: initial denaturation at 95°C for 10 min, and amplification
194 in three steps for 40 cycles (denaturation at 95˚C for 30 s; annealing at 58˚C for 1 min, and
195 extension at 60˚C for 30 s). Annealing temperature for gene *mTOR* was maintained at 52˚C.

196

197

198

199

200 Table 01: Primers used for expression of *P53, BIRC5, mTOR, GAPDH*

Gene	Forward & reverse primers
***P53*:**	Forward: 5′-TCTGGCCCCTCCTCAGCATCTT Reverse: 5′-TTGGGCAGTGCTCGCTTAGTGC
***BIRC5*:**	Forward:5′-TGGCCGCTCCTCCCTCAGAAAA Reverse:5′- GCTGCTGCCTCCAAAGAAAGCG
***mTOR*:**	Forward: 5′- TGGGACAGCATGGAAGAATA Reverse: 5′- TGTTGTGCCAAGGAGAAGAG
***GAPDH*:**	Forward: 5′- GGCATTGCCCTCAACGACCAC Reverse: 5′- ACATGACAAGGTGCGGCTCCCTA

201

202

203 **Gas chromatography and mass spectroscopic analysis (GC-MS)**

204 GC-MS analysis was conducted on an Agilent 7890 GC system coupled with a 5975 MS detector
205 (Agilent Technologies, Inc., Santa Clara, CA, USA). Separation was achieved on a J&W DB-5
206 MS capillary column (30.0 m × 250 µm, film thickness 0.25 µm; 5% phenyl methyl siloxane).
207 Ultra-high pure helium (99.999%) served as the carrier gas at a constant flow rate of 1 mL/min.
208 Injections were performed in split mode with a 1:10 ratio using a 1 µL sample volume. Electron
209 ionization was performed at 70 eV ionization energy, while injector and detector temperatures
210 were maintained at 260 °C and 320 °C, respectively. The oven temperature program started at 110
211 °C (held for 5 min), increased to 280 °C at 20 °C/min (held for 1 min), and then further ramped to
212 320 °C at 20 °C/min (held for 5 min). Data acquisition and instrument control were managed using
213 MS Solution software. Compound identification was achieved by comparing mass spectra with
214 those in the NIST (National Institute of Standards and Technology) library.

215

216 **Statistical analysis**

217 Data analysis was performed using GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego,
218 CA, USA). Each experiment was triplicated (n=3) for biological triplicates. One-way analysis of

219 variance (ANOVA) was used to compare means across multiple groups in the caspase 3/7 activity
220 assay and NBT-ROS assay, followed by Bonferroni's post hoc test for multiple pairwise
221 comparisons. For gene expression analysis, one-way ANOVA followed by Dunnett's post hoc test
222 was performed to compare treatment groups against the control group. Statistical significance was
223 set at $P < 0.05$.

224

225 **Results**

226

227 **Vernolac extract Characterization**

228

229 The Vernolac extract yielded 3% (Batch No. V24085). The GC–MS analysis revealed 20 distinct
230 compound peaks. Table 02 summarizes the principal phytochemicals identified in the extract.

231 Table 02: GC/MS analysis of Vernolac Extract

	Compounds
GC/MS analysis	Alpha -Tocopherol acetate Alpha-pinene Beta-Myrcene Beta-pinene D-Limonene Dodecanoic acid 1,6-Octadien-3-ol, 3,7-dimethyl- Carvacrol Cycloartenol Thymoquinone 1,3,5-Trimethyl benzene Alpha terpinyl acetate Hexadecanoic acid Linalyl acetate Stigmasterol Octadecanoic acid Oleic acid Palmitic acid Lauric acid Acetic acid,phenylmethyl ester

232

233 Notably, several bioactive phytochemicals were identified, including α-Tocopherol acetate,
234 Thymoquinone, Carvacrol, Cycloartenol, Stigmasterol, and Oleic acid, which are known for their
235 antioxidant, anti-inflammatory, and therapeutic properties. Other detected compounds included
236 monoterpenes (α-Pinene, β-Myrcene, β-Pinene, D-Limonene, Linalyl acetate, α-Terpinyl acetate),
237 fatty acids (Lauric acid, Palmitic acid, Hexadecanoic acid, Octadecanoic acid), and additional
238 aromatic or ester derivatives (1,3,5-Trimethyl benzene, Acetic acid phenylmethyl ester).

239 **Cytotoxicity and cell proliferation assay**

240 As indicated by the results of the SRB assay (Table 03), Vernolac extract exerted a potential to
241 mediate dose- and time-dependent suppression of NTERA-2 cl.D1 cell proliferation. Both
242 Vernolac extract, and Paclitaxel showed a dose and time-dependent anti-proliferative activity
243 against NTERA-2 cl.D1 cells.

244 Table 03: IC_{50} values (µg/mL) of Vernolac extract and Paclitaxel in NTERA-2 cl.D1 and MCF-10
245 cells.

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

246

247

248 **Fluorescence microscopic observations**

249 Acridine Orange (AO)/Ethidium Bromide (EB) stained NTERA-2 cl.D1 cells were incubated 24h
250 after the treatment with different concentrations of Vernolac extract and Paclitaxel. By employing
251 Acridine orange/Ethidium Bromide staining, clear identification of apoptosis-associated
252 membrane alterations was achieved. The apoptotic changes of the cells were distinguished and
253 visualized by the fluorescence staining, yellow/orange fluorescence indicates early apoptotic cells
254 while red fluorescence indicates late apoptosis, demonstrating disruption of cellular membrane.
255 The induction of apoptosis was increased in Vernolac extract (5, 10 and 20 µg/mL) - and Paclitaxel
256 (1, 2, and 4 µg/mL) treated NTERA-2 cl.D1 cells in a dose-dependent manner (Figure 01).

257

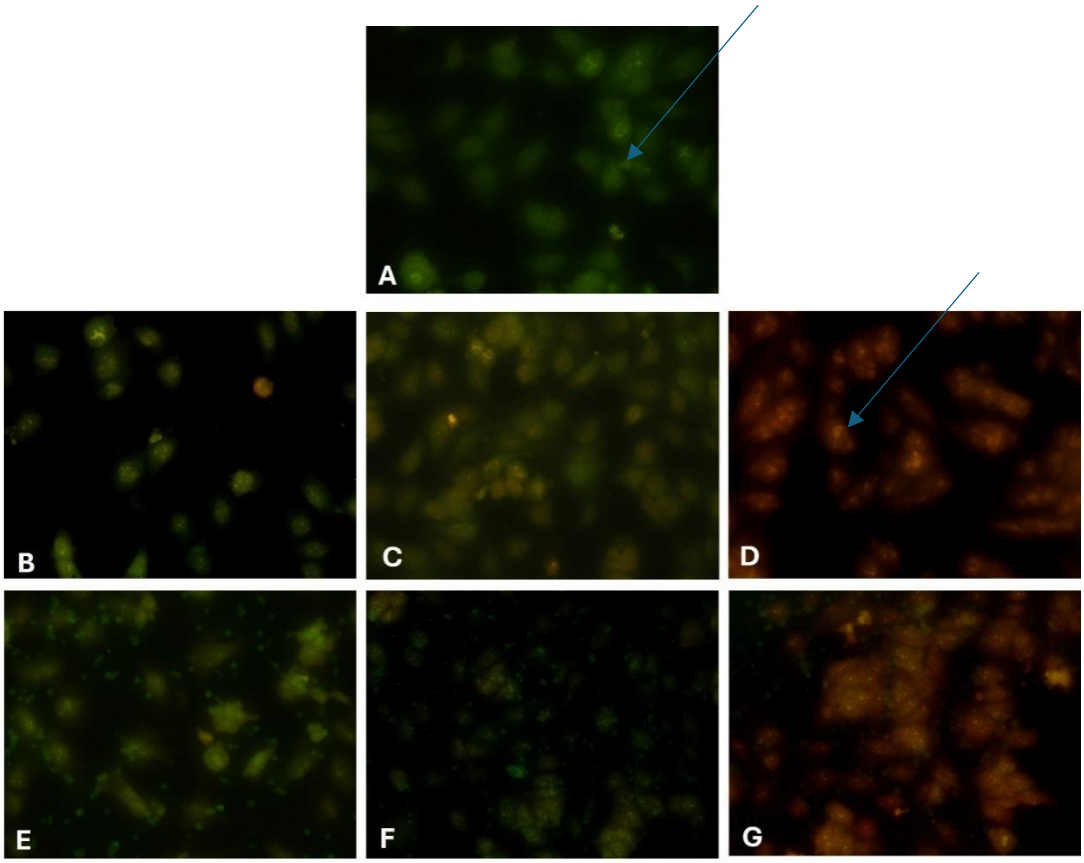


Figure 01: AO/EB stained Fluorescence microscopic observation of NTERA-2 cl.D1 cells under 200X magnification 24h post-treatment incubation. A – Untreated control, B – Paclitaxel (1 µg/mL), C – Paclitaxel (2 µg/mL), D – Paclitaxel (4 µg/mL), E –Vernolac extract (15 µg/mL).F– Vernolac extract - (30 µg/mL), G –Vernolac extract (60 µg/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.

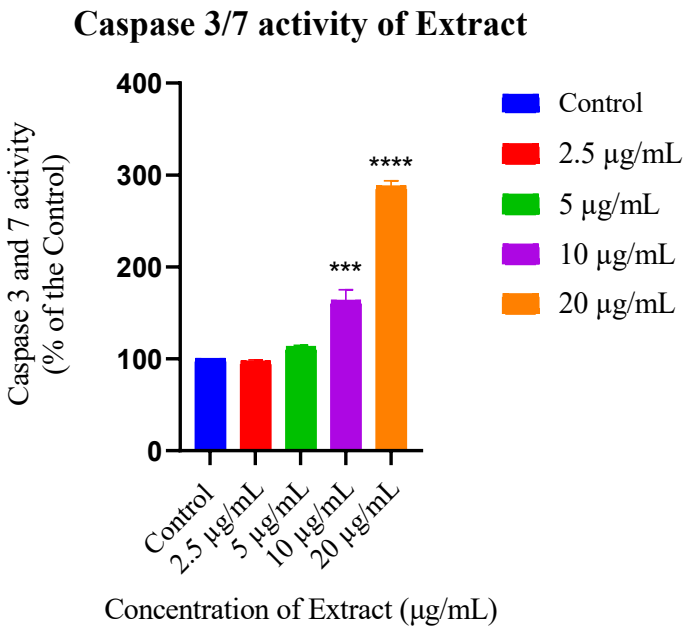


Figure 02: Expression of Caspase 3 and 7 in NTERA-2 cl.D1 cells after 24h post-treatment with Vernolac extract. (Results are indicated as mean ± standard deviation of duplicate determination. *** $P < 0.001$ and ****$P < 0.0001$ compared to the control).

Antioxidant activity of Vernolac Extract

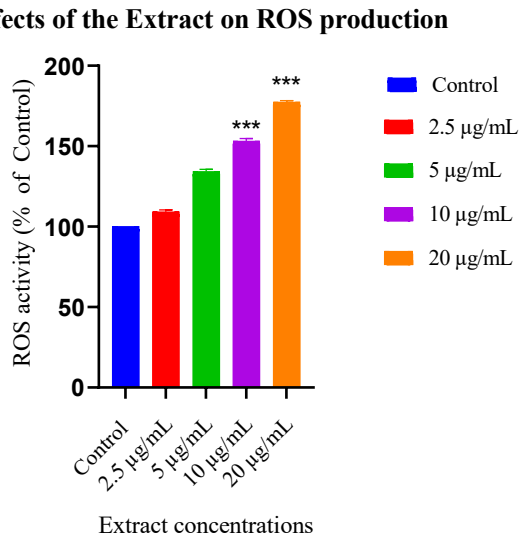
The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were performed to evaluate the antioxidant activity of Vernolac extract. Ascorbic acid was used as a positive control in both assays. Vernolac extract showed a low free radical scavenging activity (EC_{50} > 1000 µg/mL) in both ABTS and DPPH assays. The obtained EC_{50} values of free radical scavenging activity of Vernolac extract and Ascorbic are summarized in Table 04.

Table 04: 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

284 **Effect of the Vernolac extract on ROS levels**

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



289

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

293

294

295 **Analysis of the wound healing rate of NTERA-2**

296 The NTERA-2 cl.D1 cells were incubated until they created a confluent monolayer at the bottom
297 of the well. An artificial gap was made on the confluent monolayer, and both Vernolac extract and
298 Paclitaxel were treated separately according to a concentration gradient. After 24h of drug
299 treatment, untreated control cells have migrated at a rate of 62.126 µm/h. The cell migration rate
300 of Vernolac extract and Paclitaxel is graphically represented as shown in Figure 04. The
301 morphological representation of cell migration at 0h and after the gap of control closure is shown
302 in Figure 05.

303

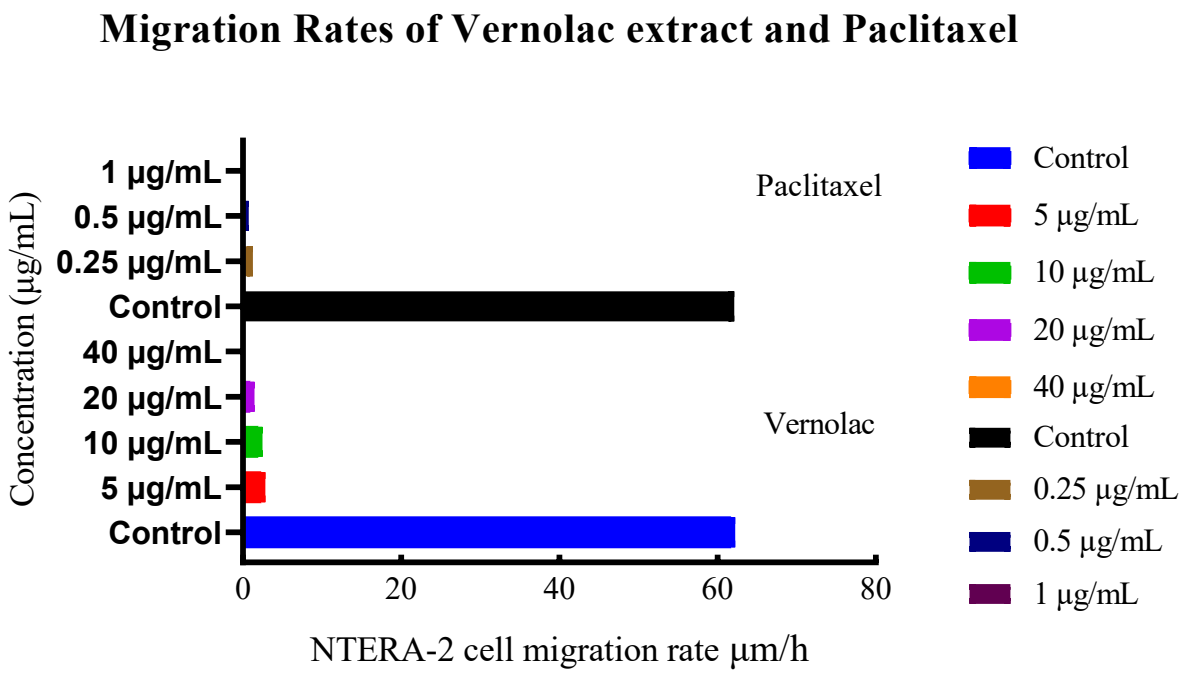


Figure 04: Cell migration rates of Vernolac extract with the concentrations of 5, 10, 20, and 40 µg/mL and Paclitaxel 0.25, 0.5, and 1 µg/mL treated NTERA-2 cl.D1 cells compared to untreated control under different concentrations.

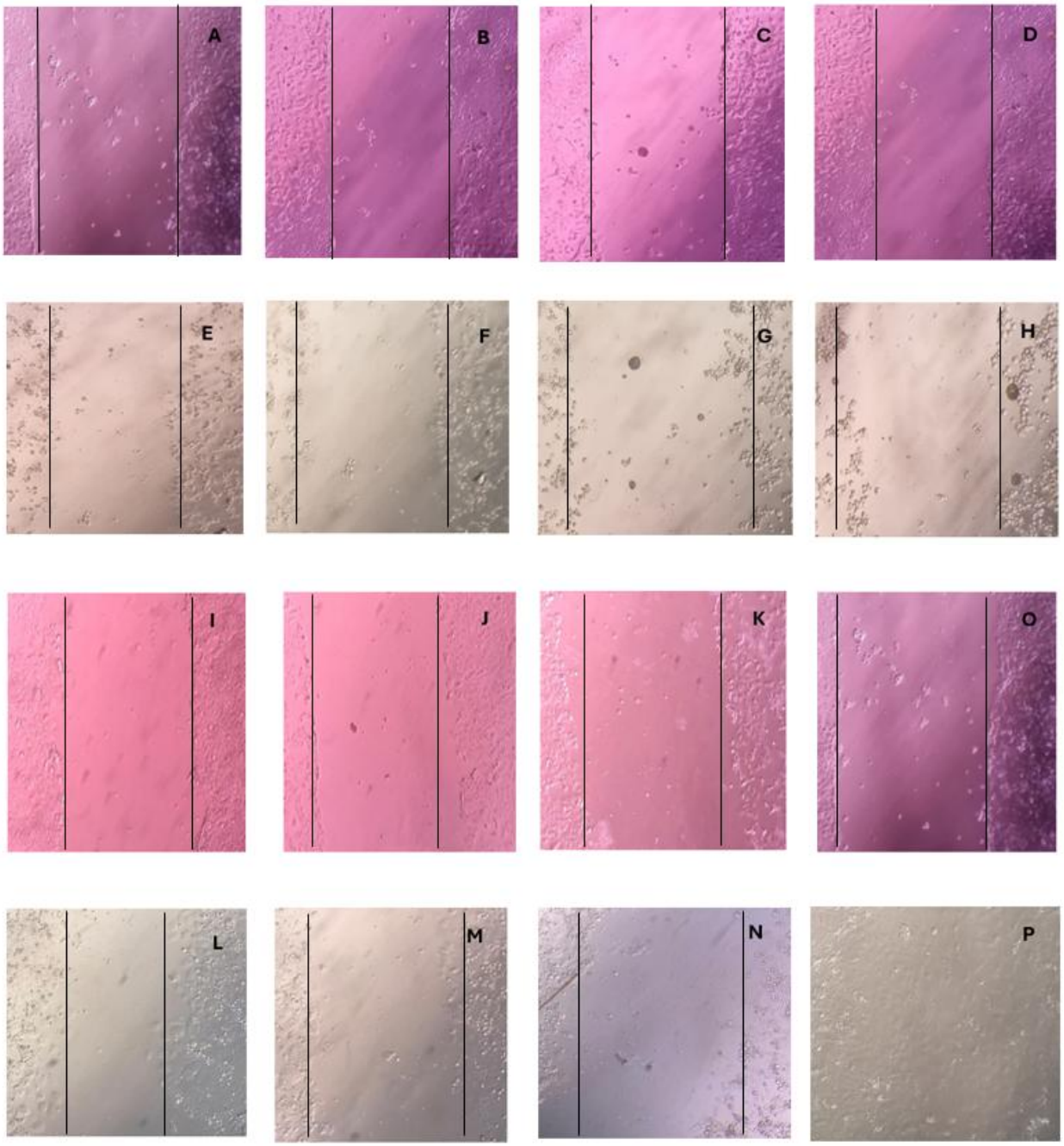
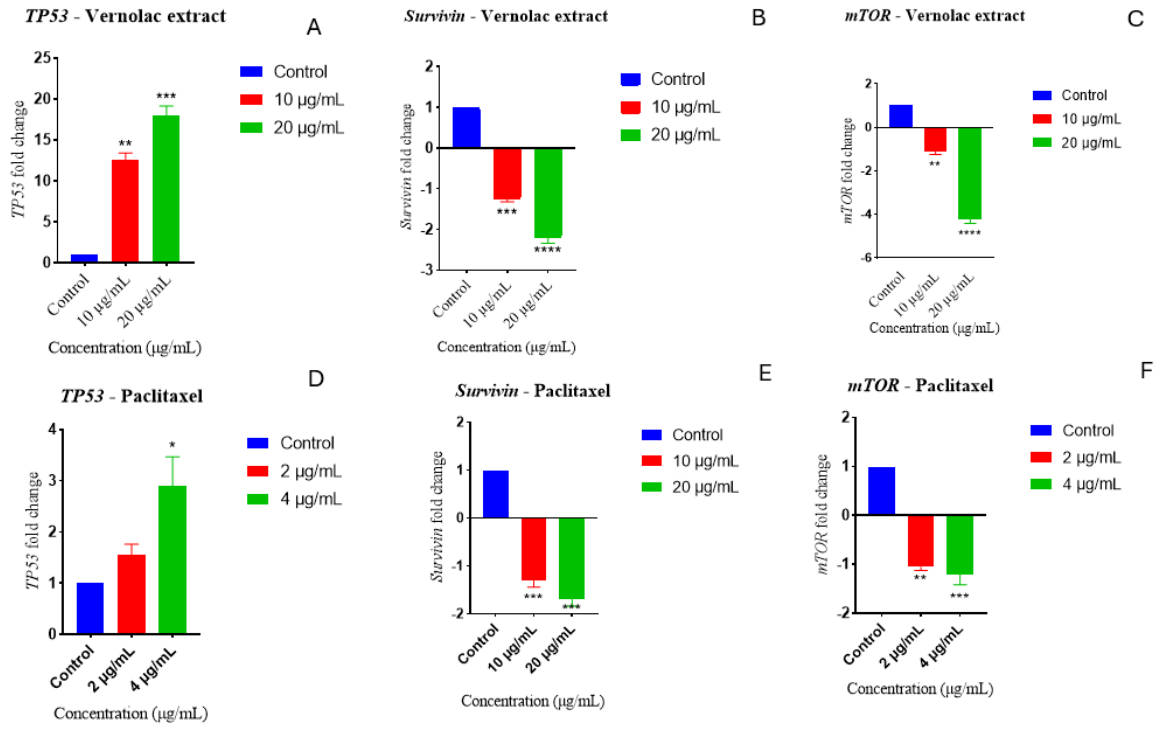


Figure 05: Phase contrast light microscopic images under 10× magnification captured after 0h of creating the gap in NTERA cl.D1 cell monolayer, followed by Vernolac extract with 5, 10, 20, and 40 µg/mL concentrations (A-D), paclitaxel with 0.25, 0.5, and 1 µg/mL concentrations (I–K), and the Untreated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

315 **Effect of Vernolac extract on the expression of *P53*, *BIRC5*, and *mTOR***

316 Results of the RT- qPCR (Figure 06) demonstrated that the Vernolac extract enhanced *TP53* mRNA
317 expression in human NTERA-2 cl.D1 cells in a significant ($P < 0.001$) dose-dependent manner.
318 Furthermore, treatment with all concentrations of supercritical Vernolac extract and Paclitaxel
319 resulted in a significant downregulation ($P < 0.05$) of the expression of *mTOR* and *BIRC5*.

320



321

322 Figure 06: mRNA expression of selected genes on Vernolac extract and Paclitaxel treatment. (A-
323 Vernolac extract on *TP53* expression, B - Vernolac extract on *BIRC5* expression, C - Vernolac
324 extract on *mTOR* expression, D- Paclitaxel on *TP53* expression, E - Vernolac extract on *BIRC5*
325 expression, F - Vernolac extract on *mTOR* expression). (Results are indicated as mean ± standard
326 deviation, duplicate determination. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.
327 compared to the control).

328

329

330

331 **Discussion**

332 Previous studies have shown that traditional polyherbal formulation demonstrates significant anti-
333 proliferative activities against several cancer cell lines, including MCF-7, HepG2, and NCI-H292
334 [23,26,32,39,40]. Sailu et al. [13] and Abeysinghe et al. [15] have elucidated that phytochemicals
335 such as alpha-hederin and Vernolactone present in the constituents of Vernolac have proven
336 anticancer activity against cancer stem-like cells. Chemical analysis using GC/MS identified 20
337 compounds in all the biological Triplicates of Vernolac extract (Table 02). The present *in vitro*
338 investigation employed supercritical fluid extraction with liquid CO_2, a superior extraction
339 methodology that preserves thermolabile compounds while eliminating organic solvent residues,
340 to evaluate the anticancer efficacy of Vernolac extract against the pluripotent human testicular
341 embryonal carcinoma (NTERA-2 cl.D1) cell line.

342 Consistent with these earlier findings, as shown by Abeysinghe et al. [12], the profiles obtained
343 through SRB assay of this study demonstrated dose and time-dependent cytotoxicity against
344 NTERA-2 cl.D1 cells. Vernolac extract showed an anti-proliferative effect against the cells with
345 an IC_{50} value of 41.12 µg/mL after 48h treatment, while demonstrating significantly lower
346 cytotoxicity against non-tumorigenic epithelial MCF-10A cells (IC_{50} value of 1075 µg/mL),
347 suggests selective cytotoxicity towards cancer stem cells (Table 03). 'Vernolactone', which is an
348 active compound of the polyherbal isolated from *V. Zeylanica*, has been shown a significant
349 cytotoxicity against NTERA-2 cl.D1 (0.4808 µg/mL at 72h) cells with Paclitaxel [15]. Similarly,
350 Mendis et al. [22], Samarakoon et al. [23] and Pathiranage et al. [26] have also reported that a
351 polyherbal mixture, including several constituents of Vernolac (*N. sativa, H. indicus* and *S.*
352 *glabra,*) displayed a dose-dependent inhibition of cell proliferation in treated cancer cell lines
353 (MCF-7, MDA-MB-231, HepG2), aligning with the findings.

354 The fluorescent and phase-contrast microscopic data revealed that Vernolac extract induces dose-
355 dependent apoptosis-related cytomorphological changes in NTERA-2 cl.D1 cells (Figure 01),
356 including cell membrane blebbing and cell shrinkage. Furthermore, compared to the untreated cells
357 (control), Vernolac extract significantly ($P < 0.0001$) enhanced the enzymatic activities of
358 executioner caspases, caspase 3, and caspase 7 at a 20 µg/mL concentration, indicating activation
359 of the terminal phase of programmed cell death (Figure 01). Concentration gradient for the Caspase
360 3/7 assay was obtained after several dose optimizations, and depending on the Caspase 3/7 activity

361 results, the concentration of Vernolac extract for the other core assays, including NBT-ROS and
362 RT-qPCR has been decided. These findings align with previous research done by Abeysinghe et
363 al. [15], who reported that Vernolactone significantly ($P < 0.0001$) upregulated caspase 3/7 activity
364 at both 2 µg/mL and 4 µg/mL concentrations when compared to the untreated NTERA-2 cl.D1
365 cells (untreated control). A traditional polyherbal mixture formulated using *N. sativa*, *H. indicus*
366 and *S. glabra* has also demonstrated an elevated caspase 3/7 activation against several cancer cell
367 lines (HepG2, MCF-7, MDA-MB-231, SKBR-3), suggesting a conserved apoptotic mechanism
368 among these phytotherapeutic agents [22,26,32]. Further, extracts of *N. sativa* seed extract
369 demonstrated an elevated caspase 3 activity on the MDA-MB-231 breast cancer cell line.

370 In this study, the DPPH and ABTS free radical scavenging assays revealed that Vernolac extract
371 contains a low antioxidant activity (EC_{50} value > 1000 µg/mL) compared to the positive control:
372 Ascorbic acid (EC_{50} = 53.678 µg/mL) (Table 03). The obtained result aligns with a previous study
373 demonstrating that Vernolactone possesses low antioxidant activity (free radical scavenging
374 capacity) through the DPPH and ABTS assays [15]. However, multiple investigations have
375 indicated that *N. sativa, H. indicus,* and *S. glabra* extracts have shown free radical scavenging
376 activity through multiple analyses [26,31]. Comprehensive antioxidant profiling using
377 complementary analysis is recommended to fully characterize the antioxidant capacity of Vernolac
378 extract. Despite, extract of Vernolac having a minimal antioxidant activity, a significantly ($P <$
379 0.001) elevated ROS level was determined in NTERA-2 cl.D1 at the concentrations of 10 and 20
380 µg/mL (Figure 03). This pro-oxidant effect in cancer cells, contrasting with limited antioxidant
381 capacity in cell-free systems, suggests a selective modulation of redox homeostasis within the
382 cellular microenvironment, potentially contributing to the observed cytotoxicity [41].

383 The effect of Vernolac extract on the migration of NTERA-2 cl.D1 cells was evaluated using a
384 wound healing assay. A dose-dependent migration rate (< 3 µm/h across all tested concentrations)
385 (Figure 04), demonstrated minor or no reduction in the wound width throughout the experiment in
386 Vernolac-treated cells compared to the untreated NTERA-2 cl.D1 cells (Figure 05), indicates
387 significantly reduced migratory capacity. Similarly, paclitaxel also restricted cellular migration to
388 rates below 1.5µm/h for all tested concentrations. Low serum concentrations in the cell medium
389 were maintained to ensure the suppression of cell proliferation [42]. This pronounced inhibition

390 of cellular motility indicates impairment of cytoskeletal reorganization and extracellular matrix
391 interaction, critical processes in tumor cell invasiveness and metastatic potential [43].

392 Moreover, RT-qPCR was performed to evaluate the mRNA expression of several autophagy and
393 apoptosis-related genes (*TP53*, *BIRC5,* and *mTOR*). The tumor suppressor *TP53* is a key gene that
394 is essential for DNA repair, apoptosis, and triggering of cell cycle arrest, and plays a role in
395 autophagy [44]. Moreover, *p53* involves suppression of *BIRC5* at both mRNA and protein
396 expression levels to promote apoptosis [43]. Lower doses of Vernolac extract and paclitaxel were
397 used to maintain the cells in viable conditions until the cells were under the early apoptotic stage
398 at the time of RNA extraction. Vernolac extract led to a significant ($P < 0.001$) upregulated tumor
399 suppressor *TP53* expression, with a 9.67-fold change at 10 $\mu g/mL$ and 13.29 fold change at 20
400 $\mu g/mL$ compared to the untreated control (Figure 06). Concurrently, Vernolac extract-treated
401 NTERA-2 cells resulted in significant ($P < 0.05$) downregulation of mammalian target of
402 rapamycin (*mTOR*) and *BIRC5* mRNA expression at both 10 and 20 µg/mL concentrations(Figure
403 06). According to Abeysinghe et al. [15], it was shown that Vernolactone in *V. zeylanica*
404 significantly induced apoptosis and autophagy in NTERA-2 cl.D1 cells through modulation of the
405 PI3K/Akt/mTOR pathways. Key phytochemicals of *Nigella sativa*; Beta catenin, Alpha-hederin
406 and Thymoquinone, have been reported to inhibit DNA synthesis, induce cell cycle arrest, and
407 promote apoptosis via both p53-dependent and p53-independent mechanisms [13,33,34,45].
408 Collectively, the above-mentioned studies indicate that active compounds in the constituents of
409 Vernolac extract exert their anticancer effects through coordinated modulation of apoptotic and
410 autophagy-related pathways.

411 Vernolac extract comprises *V. zeylanica* aerial parts, *N. sativa* seeds, *H. indica* roots, *S. glabra*
412 rhizome, and *L. zeylanica* aerial parts investigated in the present study, exerting anti-proliferative
413 and anti-cancer effects via apoptosis, autophagy, and cell cycle arrest through upregulation of
414 mRNA expression of *TP53*and downregulation of *BIRC5* and *mTOR* mRNA expression. The
415 observed cytotoxic and apoptotic effects likely result from the action of a single active compound
416 and/or synergistic interactions among various phytochemicals within the extract, potentially
417 offering advantages over single-compound therapies [13,22,35,45]. Furthermore, in vitro
418 investigations are necessary to demonstrate and confirm the precise molecular mechanisms
419 underlying the anticancer activity, and subsequent *in vivo* studies are essential to evaluate its

420 efficacy and the biological effects of Vernolac extract within living organisms (physiologically
421 relevant systems), particularly its safety profile.

422 **Conclusion**

423 This study demonstrates that Vernolac extract showed selective antiproliferative effects on
424 NTERA-2 cl.D1, cancer stem-like cells by inducing apoptosis and autophagy, while exhibiting
425 minimal cytotoxicity toward noncancerous epithelial cells (MCF-10A). These findings support the
426 potential of Vernolac extract as a promising therapeutic candidate targeting cancer stem cells.
427 Limitations of this study include the absence of assays such as CSC markers, autophagy flux
428 measurements, apoptosis validation, and migration controls. These limitations will be addressed
429 in future investigations on Vernolac.

430

431

432

433

434

435

436

437

438

439

440

441

442

443

444

Acknowledgments

The authors gratefully acknowledge the support provided by the Institute of Biochemistry, Molecular Biology and Biotechnology (IBMBB), University of Colombo. The authors gratefully acknowledge Fadna Life Science Pvt. Ltd for kindly providing the Vernolac extract and supporting its chemical characterization, which greatly contributed to the advancement of this research.

Disclosure statement.

The authors report there are no competing interests to declare.

Source of Funding

This work was funded by the Master of Science project, Institute of Biochemistry Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.

CRediT author statement

PCR: Investigation, Formal analysis, Writing – original draft, Writing – review and editing ; **DCS, MMM**: Writing – original draft, Writing – review and editing ; **SDVG, NNS, DNP**: Writing – review and editing ; **UR,KSS**: Supervision, Writing – review and editing ; **SRS**: Conceptualization, Writing – review and editing, Supervision, Project administration.

References

[1] Siegel RL, Kratzer TB, Giaquinto AN, Sung H, Jemal A. Cancer statistics, 2025. CA Cancer J Clin 2025. https://doi.org/10.3322/caac.21871.

[2] Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2024;74(3):229–63. https://doi.org/10.3322/caac.21834.

[3] Pal R, Ravindran G. Assessment of pluripotency and multilineage differentiation potential of NTERA-2 cells as a model for studying human embryonic stem cells. vol. 39. 2006.

[4] Khan SU, Fatima K, Aisha S, Malik F. Unveiling the mechanisms and challenges of cancer drug resistance. Cell Communication and Signaling 2024;22(109):1-25. https://doi.org/10.1186/s12964-023-01302-1.

[5] Chu X, Tian W, Ning J, Xiao G, Zhou Y, Wang Z, et al. Cancer stem cells: advances in knowledge and implications for cancer therapy. Signal Transduct Target Ther 2024;9:1-70. https://doi.org/10.1038/s41392-024-01851-y.

[6] Roszkowska M. Multilevel Mechanisms of Cancer Drug Resistance. Int J Mol Sci 2024;25. https://doi.org/10.3390/ijms252212402.

[7] Garg P, Malhotra J, Kulkarni P, Horne D, Salgia R, Singhal SS. Emerging Therapeutic Strategies to Overcome Drug Resistance in Cancer Cells. Cancers (Basel) 2024;16(13): 1-25. https://doi.org/10.3390/cancers16132478.

[8] Afkar A, Afolabi BO, Piyathilake PC, Perera DN, Senathilake K, Wijerathne SK, et al. Anticancer Therapeutic Potential of Natural Products. Journal of Desk Research Review and Analysis 2024;2(1):67–91. https://doi.org/10.4038/jdrra.v2i1.29.

[9] Safe S. Natural products as anticancer agents and enhancing their efficacy by a mechanism-based precision approach. Exploration of Drug Science 2024:408–27. https://doi.org/10.37349/eds.2024.00054.

[10] Naeem A, Hu P, Yang M, Zhang J, Liu Y, Zhu W, et al. Natural Products as Anticancer Agents: Current Status and Future Perspectives. Molecules 2022;27. https://doi.org/10.3390/molecules27238367.

[11] Shaik BB, Katari NK, Jonnalagadda SB. Role of Natural Products in Developing Novel Anticancer Agents: A Perspective. Chem Biodivers 2022;19(11):1-13. https://doi.org/10.1002/cbdv.202200535.

[12] Asma ST, Acaroz U, Imre K, Morar A, Shah SRA, Hussain SZ, et al. Natural Products/Bioactive Compounds as a Source of Anticancer Drugs. Cancers (Basel) 2022;14. https://doi.org/10.3390/cancers14246203.

[13] Saliu TP, Seneviratne NN, Faizan M, Rajagopalan U, Perera DC, Adhikari A, et al. In silico identification and in vitro validation of alpha-hederin as a potent inhibitor of Wnt/β-catenin signaling pathway in breast cancer stem cells. In Silico Pharmacol 2024;12(31):1-15. https://doi.org/10.1007/s40203-024-00199-z.

[14] Tharmarajah L, Samarakoon SR, Ediriweera MK, Piyathilaka P, Tennekoon KH, Senathilake KS, et al. In Vitro Anticancer Effect of Gedunin on Human Teratocarcinomal (NTERA-2) Cancer Stem-Like Cells. Biomed Res Int 2017;2017(1):1-9. https://doi.org/10.1155/2017/2413197.

[15] Abeysinghe NK, Thabrew I, Samarakoon SR, Ediriweera MK, Tennekoon KH, Pathiranage VPC, et al. Vernolactone Promotes Apoptosis and Autophagy in Human Teratocarcinomal (NTERA-2) Cancer Stem-Like Cells. Stem Cells Int 2019;2019. https://doi.org/10.1155/2019/6907893.

[16] Borlongan MC, Rodriguez T, Putthanbut N, Wang H, Lee JY. Modeling of cancer stem cells and the tumor microenvironment Via NT2/D1 cells to probe pathology and treatment for cancer and beyond. Discover Oncology 2025;16. https://doi.org/10.1007/s12672-025-02158-2.

[17] Anand U, Dey A, Chandel AKS, Sanyal R, Mishra A, Pandey DK, et al. Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics. Genes Dis 2023;10:1367–401. https://doi.org/10.1016/j.gendis.2022.02.007.

515 [18] Ali Abdalla YO, Subramaniam B, Nyamathulla S, Shamsuddin N, Arshad NM, Mun KS, et
516 al. Natural Products for Cancer Therapy: A Review of Their Mechanism of Actions and
517 Toxicity in the Past Decade. J Trop Med 2022;2022. https://doi.org/10.1155/2022/5794350.

518 [19] Guo M, Jin J, Zhao D, Rong Z, Cao LQ, Li AH, et al. Research Advances on Anti-Cancer
519 Natural Products. Front Oncol 2022;12. https://doi.org/10.3389/fonc.2022.866154.

520 [20] Atanasov AG, Zotchev SB, Dirsch VM, Orhan IE, Banach M, Rollinger JM, et al. Natural
521 products in drug discovery: advances and opportunities. Nat Rev Drug Discov
522 2021;20:200–16. https://doi.org/10.1038/s41573-020-00114-z.

523 [21] Yun BW, Yan Z, Amir R, Hong S, Jin YW, Lee EK, et al. Plant natural products: History,
524 limitations and the potential of cambial meristematic cells. Biotechnol Genet Eng Rev
525 2012;28:47–60. https://doi.org/10.5661/bger-28-47.

526 [22] Mendis AS, Thabrew I, Ediriweera MK, Samarakoon SR, Tennekoon KH, Adhikari A, et
527 al. Isolation of a New Sesquiterpene Lactone From Vernonia Zeylanica (L) Less and its
528 Anti-Proliferative Effects in Breast Cancer Cell Lines. Anticancer Agents Med Chem
529 2018;19:410–24. https://doi.org/10.2174/1871520619666181128163359.

530 [23] Samarakoon SR, Thabrew I, Galhena P, De Silva D, Tennekoon K. A comparison of the
531 cytotoxic potential of standardized aqueous and ethanolic extracts of a polyherbal mixture
532 comprised of Nigella sativa (seeds), Hemidesmus indicus (roots) and Smilax glabra
533 (rhizome). Pharmacognosy Res 2010;2:335–42. https://doi.org/10.4103/0974-8490.75451.

534 [24] Samarakoon SR, Thabrew I, Galhena P, De Silva D, Tennekoon K. A comparison of the
535 cytotoxic potential of standardized aqueous and ethanolic extracts of a polyherbal mixture
536 comprised of Nigella sativa (seeds), Hemidesmus indicus (roots) and Smilax glabra
537 (rhizome). Pharmacognosy Res 2010;2:335–42. https://doi.org/10.4103/0974-8490.75451.

538 [25] Galhena PB, Samarakoon SR, Thabrew MI, Weerasinghe GAK, Thammitiyagodage MG,
539 Ratnasooriya WD, et al. Anti-inflammatory activity is a possible mechanism by which the
540 polyherbal formulation comprised of Nigella sativa (seeds), Hemidesmus indicus (root), and
541 Smilax glabra (rhizome) mediates its antihepatocarcinogenic effects. Evidence-Based
542 Complementary and Alternative Medicine 2012;2012.
543 https://doi.org/10.1155/2012/108626.

544 [26] Pathiranage VC, Thabrew I, Samarakoon SR, Tennekoon KH, Rajagopalan U, Ediriweera
545 MK. Evaluation of anticancer effects of a pharmaceutically viable extract of a traditional
546 polyherbal mixture against non-small-cell lung cancer cells. J Integr Med 2020;18:242–52.
547 https://doi.org/10.1016/j.joim.2020.02.007.

548 [27] Daulagala PWHKP. The Journal of the Society for Tropical Plant Research Medicinally
549 important Leucas zeylanica (L.) R. Br.: A review 2021.
550 https://doi.org/10.22271/tpr.2021.v8.i2.015.

551 [28] Wu H, Wang Y, Zhang B, Li YL, Ren ZX, Huang JJ, et al. Smilax glabra Roxb.: A Review
552 of Its Traditional Usages, Phytochemical Constituents, Pharmacological Properties, and

553 Clinical Applications. Drug Des Devel Ther 2022;16:3621–43.
554 https://doi.org/10.2147/DDDT.S374439.

555 [29] Ratnasooriya WD, Deraniyagala SA, Peiris SKJS. Antinociceptive potential of the Sri
556 Lankan endemic plant Vernonia zeylanica. Pharm Biol 2007;45:525–32.
557 https://doi.org/10.1080/13880200701215042.

558 [30] Rukshala D, de Silva ED, Ranaweera BVLR, Fernando N, Handunnetti SM. Anti-
559 inflammatory effect of leaves of Vernonia zeylanica in lipopolysaccharide-stimulated RAW
560 264.7 macrophages and carrageenan-induced rat paw-edema model. J Ethnopharmacol
561 2021;274. https://doi.org/10.1016/j.jep.2021.114030.

562 [31] Galhena PB, Samarakoon SR, Thabrew MI, Weerasinghe GAK, Thammitiyagodage MG,
563 Ratnasooriya WD, et al. Anti-inflammatory activity is a possible mechanism by which the
564 polyherbal formulation comprised of Nigella sativa (seeds), Hemidesmus indicus (root), and
565 Smilax glabra (rhizome) mediates its antihepatocarcinogenic effects. Evidence-Based
566 Complementary and Alternative Medicine 2012;2012.
567 https://doi.org/10.1155/2012/108626.

568 [32] Samarakoon SR, Thabrew I, Galhena PB, Tennekoon KH. Modulation of apoptosis in
569 human hepatocellular carcinoma (HepG2 cells) by a standardized herbal decoction of
570 Nigella sativa seeds, Hemidesmus indicus roots and Smilax glabra rhizomes with anti-
571 hepatocarcinogenic effects. BMC Complement Altern Med 2012;12.
572 https://doi.org/10.1186/1472-6882-12-25.

573 [33] Pathiranage VC, Thabrew I, Samarakoon SR, Tennekoon KH, Rajagopalan U, Ediriweera
574 MK. Evaluation of anticancer effects of a pharmaceutically viable extract of a traditional
575 polyherbal mixture against non-small-cell lung cancer cells. J Integr Med 2020;18:242–52.
576 https://doi.org/10.1016/j.joim.2020.02.007.

577 [34] Samarakoon SR, Thabrew I, Galhena PB, Tennekoon KH. Effect of standardized decoction
578 of Nigella sativa seed, Hemidesmus indicus root and smilax glabra rhizome on the
579 expression of p53 and p21 genes in human hepatoma cells (HepG2) and mouse liver with
580 chemically-induced hepatocarcinogenesis. Tropical Journal of Pharmaceutical Research
581 2012;11:51–61. https://doi.org/10.4314/tjpr.v11i1.7.

582 [35] Rajagopalan U, Samarakoon SR, Tennekoon KH, Malavige N, Silva ED. Screening of five
583 sri lankan endemic plants for anti-cancer effects on breast cancer stem cells isolated from
584 mcf-7 and mda-mb-231 cell lines. Tropical Journal of Pharmaceutical Research
585 2018;17:1825–32. https://doi.org/10.4314/tjpr.v17i9.21.

586 [36] Ediriweera MK, Tennekoon KH, Adhikari A, Samarakoon SR, Thabrew I, De Silva ED.
587 New halogenated constituents from Mangifera zeylanica Hook.f. and their potential anti-
588 cancer effects in breast and ovarian cancer cells. J Ethnopharmacol 2016;189:165–74.
589 https://doi.org/10.1016/j.jep.2016.05.047.

[37] Ediriweera MK, Tennekoon KH, Samarakoon SR, Thabrew I, De Silva ED. Induction of Apoptosis in MCF-7 Breast Cancer Cells by Sri Lankan Endemic Mango (Mangifera zeylanica) Fruit Peel through Oxidative Stress and Analysis of its Phytochemical Constituents. J Food Biochem 2017;41. https://doi.org/10.1111/jfbc.12294.

[38] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 2001;25:402–8. https://doi.org/10.1006/meth.2001.1262.

[39] Sakthi Saravanan V, Selvam P, Parthiban P, Chandra Bose Ps, Paul Raj S. Anticancer activity of Polyherbal formulation anti cancer activity of polyherbal formulation. International journal of pharmacy and pharmaceutical analysis 2015;02(03):01-05.

[40] Bhatt D, Vaghela K, Jethva K, Patel S, Zaveri M. Development and standardization of Polyherbal formulation for the management of breast cancer. International Journal of Pharmaceutical Science and Research 2017;2:2455–4685.

[41] Petronek MS, Stolwijk JM, Murray SD, Steinbach EJ, Zakharia Y, Buettner GR, et al. Utilization of redox modulating small molecules that selectively act as pro-oxidants in cancer cells to open a therapeutic window for improving cancer therapy. Redox Biol 2021;42. https://doi.org/10.1016/j.redox.2021.101864.

[42] Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. Journal of Investigative Dermatology 2017;137:11–6. https://doi.org/10.1016/j.jid.2016.11.020.

[43] Brábek J, Mierke CT, Rösel D, Veselý P, Fabry B. The role of the tissue microenvironment in the regulation of cancer cell motility and invasion. 2010.

[44] Abdollahzadeh N, Tafrihi M. Exploring in vitro anticancer potential of Ferula gummosa gum on PC-3 cells: insights from cellular and molecular studies. Egyptian Journal of Basic and Applied Sciences. 2025;12(1): 86-93. doi: 10.1080/2314808X.2024.2448403.

[45] Ndreshkjana B, Çapci A, Klein V, Chanvorachote P, Muenzner JK, Huebner K, et al. Combination of 5-fluorouracil and thymoquinone targets stem cell gene signature in colorectal cancer cells. Cell Death Dis 2019;2019;379(10): https://doi.org/10.1038/s41419-019-1611-4.