Anticancer and Apoptotic Activity in Neuroblastoma SK-N-SH using Phospholipid Extract from Bone of Scomberomorus niphonius

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Abstract

Among various types children’s health challenges, Neuroblastoma is the most serious solid neoplasm forming outside the cranium. *S. niphonius* is a valuable edible fish that has been widely used for a meal. In this study, we obtained phospholipid extract from the bone of *S. niphonius* with the supercritical CO$_2$ extraction method and tested anticancer activity with a cell viability assay. The phospholipid showed anticancer activity on neuroblastoma SK-N-SH cells, and the anticancer activity was presented with an IC$_{50}$ of 710.25 ± 28.31 µg/ml, but didn't show a significant toxicity on HUVEC cell lines. Westernblot was used to detect signaling proteins; Bak, caspase-9, caspase-8, caspase-3, Bax and IκBα were increased, whereas IKKβ and NFκB were downregulated in experimental groups compared to untreated groups. Gene expression was revealed by RT-qPCR, and the fold ratio of Apaf-1, cytochrome-c, caspase-9, caspase-3 and Bax genes’ expression was raised in treated groups, implying apoptosis. Gel electrophoresis revealed that the experimental groups had more fragmented DNA than the control group. The study shows that a phospholipid extract from *S. niphonius's* bone could be used as a biological origin of anticancer activity in neuroblastoma SK-N-SH cells.

Introduction

Cancer is currently one of the world's most serious health problems and the top cause of mortality. The predominant etiological factors in the incidence of cancer include environmental/physical, some virus infections/biological, and diets/chemical carcinogens (1). Neuroblastoma is a solid extracranial tumor that has a wide range of clinical manifestations in children (2) and the third most common pediatric cancer (12). It is a neuroblastic tumor of the primordial neural crest(3). Neuroblastoma is a cancer that develops as a result of abnormal sympathetic nervous system maturation (4). It is a postganglionic sympathetic nervous system embryonal cancer that mostly develops in the adrenal gland. And it's the most prevalent cancer observed in children under the age of one (5). Tumors can regress on their own due to triggering of apoptosis or division, or they might be exceedingly malignant with limited cure chances (3). Neuroblastoma is known for its clinical variability, with the chance of tumor growth varied greatly depending on the patient's age and anatomic level at the time of diagnosis (2). Neuroblastoma presents an extensive range of clinical symptoms that are dependent on the location, size, and physiological characteristics of the main tumor, as well as the existence of distant metastatic illness. Abdominal distension, generalized skeletal pain or even arthritis type complaints, effects of hormone production and non-specific findings from bone marrow involvement, such as weight loss, malaise, anemia, fever and irritability, can be encountered (6). The cancers develop in sympathetic nervous system tissues, most commonly in the adrenal medulla or paraspinal ganglia, and can manifest as tumor nodules in the neck, chest, abdomen, and pelvis (4). One of the most common symptoms associated with cervical neuroblastomas is a neck lump, stridor, or difficulty swallowing (7). The diagnosis of neuroblastoma may come as early as the neonatal stage in certain cases. (2). Prior to recent discoveries, there was a paucity of information on the genetic underpinnings of this illness (4). This might be an underestimate if certain genetic variants are more likely to coincide with fatal birth abnormalities or if the
undiscovered tumor is more likely to go into remission on its own without medical intervention (8). There is limited evidence to establish a causal relationship between maternal consumption of tobacco during pregnancy and the development of neuroblastoma (9). It is widely recognized that drinking alcohol while pregnant causes disturbance in the normal development of the neural pathways in the brain. Early exposure to ethanol prevents neuronal cells from migrating and multiplying normally, which can lead to the death of neuronal cells (10)–(11). Case studies that identified the tumor as co-occurring with fetal alcohol syndrome prompted researchers to speculate about the potential of a connection between neuroblastoma and the consumption of alcohol. On the other hand, the illness has the greatest spontaneous regression rate of any malignancy (8). The condition is identified in 90% of children who have it within the first five years of their lives (13). The majority of children who have neuroblastoma appear with a palpable abdominal mass between the ages of 1 and 5 years old, with the median age being 2 years (14). The spectrum of endeavors may be useful to stratify the most appropriate prevention and management with this disease.

The discovery of novel medications to treat neuroblastoma with a high risk of recurrence has been the focus of a large body of research and the efforts of several groups (4). Anti-tumor compounds work through a variety of mechanisms, including inducing apoptosis via DNA cleavage mediated by topoisomerase I or II inhibition, mitochondrial permeabilization, inhibition of key enzymes involved in signal transduction or cellular metabolism, and inhibition of tumor-induced angiogenesis (15). The conventional treatments for cancer, such as surgery, radiation, and chemotherapy, all have undesirable side effects. As a consequence of this, there is a growing need for natural substances that have fewer adverse effects, are less toxic, and have a higher degree of effectiveness in the prevention and treatment of cancer (16). Almost fifty percent of the most popular medications are natural or derived from natural ingredients (17). Marine sources are used in functional foods and medicines, accomplish a wide range of biological functions, and provide a significant contribution to items that promote health. However, the ocean is home to a diverse array of living forms teeming with natural substances having potential therapeutic use. Because marine exploration is still a relatively recent phenomena, the marine life that exists is still a largely untapped area of possibility (15). They have an enticing nutritional content and numerous classes of bioactive compounds, such as alkaloids, steroids, polyphenols, polysaccharides, fatty acids, micronutrients, minerals, vitamins, and a great deal of other types of bioactive compounds (18)(19)(20). *S. niphonius* is one of the marine species that includes numerous compounds that are ideal for a healthy diet. Some examples of these compounds are high-quality protein and polyunsaturated fatty acids (docosahexaenoic, eicosapentaenoic, and linolenic acid), essential amino acids, vitamins, and minerals (21). Marine phospholipid carrying mainly omega-3 fatty acid. Phospholipids are amphipathic lipids. Furthermore, phospholipids are essential component for the synthesis of unsaturated fatty acids, which are required as precursors for the synthesis of eicosanoids (22). In vitro, the expansion of neurites drives up the need for phospholipids, and the activity of nerve growth factor stimulates the manufacture of phosphatidylcholine in response to this demand (23). From the vast number of investigations, it has become obvious that dietary phospholipids have a good influence on a number of disorders, presumably without serious adverse effects. Moreover,
they have been proven to lessen the adverse effects of certain medications. Due to their ability to transfer fatty acid residues to cells implicated in many illnesses, it is possible to explain both of these findings, e.g. Immune or cancer cells. Researchers have investigated the effectiveness of phospholipids in inhibiting the growth of cancer in several ways. Numerous studies have shown that phospholipids possess qualities that are both anti-cancer and anti-metastatic (22).

Various marine species and their extracts, notably S. niphonius and phospholipid, have been the subject of countless scientific study thus far. This research aims to ascertain the neuroblastoma SK-N-SH cells’ in vitro apoptotic pathway and anticancer activity in response to the phospholipid extract of S. niphonius.

Materials And Methods

Phospholipid extraction

Removal of oil by SC-CO$_2$ extraction: For oil extraction, a supercritical fluid extraction method was applied. In particular, 100 g of freeze-dried, crushed fish bone was added to a 200 mL stainless steel extractor. Using SC-CO$_2$ extraction at 45°C and 25 MPa pressure for 3 hours, the oil was extracted from the sample. Completion of oil extraction was ensured by repeated weighing of the extracted oil for 3 consecutive times at 5min intervals which showed consistent weight. Around 35.15 ± 1.14% oil was recovered at this stage. Then, the residues were left in the extractor so that SNPL could be extracted using SC-CO$_2$ and ethanol as a co-solvent. Organic solvent was utilized to extract SNPL from the de-oiled residue.

Extraction of phospholipid by SC-CO$_2$ with ethanol as co-solvent: Phospholipid was extracted from the residue left behind after de-oiling fish bones using SC-CO$_2$ with ethanol as the co-solvent. This process was carried out at 45°C and between 22.5 and 30 MPa, and varied CO$_2$ densities were maintained throughout. Several studies have proposed the temperature and pressure required for phospholipid extraction. The flow rate of carbon dioxide was maintained at 27 g per minute during the whole 2.5 hours that it took to complete the extraction process. With the help of a solvent delivery pump, the flow rate of the co-solvent ethanol was pre-optimized and kept at 3 mL/min throughout the process (Model: M930, Young Lin Co., Korea). After using a rotary evaporator heated to 45 °C to remove the solvent from the ethanol-phospholipid combination, the SNPL that was extracted was kept at a temperature of -20 °C.

Cell culture

This study utilized SK-N-SH (ATCC, Manassas, VA, USA) and HUVEC (ATCC, Manassas, VA, USA) cell lines, which were maintained in a humidified environment containing 5% CO$_2$ at 37°C in MEM (Mediatech, Manassas, VA, USA) and EBM-2 medium, respectively. The cells were then subcultured until they had reached passage-3 and were ready for the experiment when they attained a confluence of around 80%. EBM-2 (Lonza, Walkersville, MD, USA) was supplemented with EGM-2 kit (Lonza, Walkersville, MD, USA)
and MEM was supplied with 10% heat inactivated FBS and 1.1% antibiotic antimycotic (Mediatech, Manassas, VA, USA).

**Solution preparation**

After dissolving 30 mg of SNPL in 150 µl of DMSO, the final concentration of the SNPL stock was brought up to 200 µg/ml. To treat SK-N-SH and HUVEC cells, stock was diluted in the appropriate medium and then prepared at a range of concentrations (600, 700, 800, and 900 µg/ml).

**Cell viability assay**

To assess the cytotoxicity of SNPL, control and experimental group cells were sown in a 96-well plate at a density of $1 \times 10^4$ cells/well; and blank wells containing 100 µl of medium and no cells was incubated for 24 hours at 37°C with 5% CO2. SK-N-SH and HUVEC experimental group cells were loaded with varying doses (600, 700, 800, and 900 µg/ml) of the SNPL and then incubated for another 24 hours. Next, media was changed and replaced with fresh media containing EZ-Cytox solution (10 µl) in blank, control and experimental group wells, also protected from light and incubated for 2 hours at 37°C 5% CO2. Next, the media in the blank, control, and experimental group wells were removed and replaced with new media containing EZ-Cytox solution (10 µl). These wells were likewise shielded from light and incubated at 37°C for 2 hours. Using an ELISA plate reader at 460 nm, the cell viability was computed (Varioskan Lux, Vantaa, Finland). Cell viability calculated as $(\text{experimental} - \text{blank}) ÷ (\text{control} - \text{blank}) \times 100$. This experiment was conducted through repetition.

**Protein extraction and Western blot**

A total of 20 mL of MEM constituted SK-N-SH cells (adjusted: $1 \times 10^5$ cells/mL) was prepared, then 5 ml were put into the 100mm cell culture plate and incubated at 37°C with 5% CO2 for 24 h. The media were subsequently switched, and the control group received no treatment, while the experiment group received 600, 700, and 800 g/ml of SNPL and was incubated at 37°C with 5% CO2 for 12 hours. After 12 hours, the cells were washed and scraped with cold PBS buffer and then transferred into 15 ml tubes. Followed by 5 min of centrifugation at 1400 RPM. By adding 35 µl lysis buffer to the centrifuged cells, the cells are lysed (iNtRON BIOTECHNOLOGY, Cat. No. 17081, Korea). After 10 minutes of incubation on ice, lysates were obtained and cleared by centrifuging at 14000 rpm for 20 minutes at 4°C. Using a 2 mg bovine albumin standard and Bradford reagent for an ELISA microplate reader at 595 nm, protein concentration was determined. 12% SDS-PAGE was used to separate aliquots of whole cell lysates or cytosolic fractions, which were subsequently transferred to nitrocellulose membranes. PBST (PBS buffer with 0.5% Tween-20) with 5% skim milk was used to block the membranes. Following the blocking of any non-specific sites, the membranes were probed with primary antibodies (Cell Signaling Technology), washed three times in PBST, and then subjected to an incubation period of one hour with horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG as secondary antibodies (Cell Signaling Technology). The membranes were then rinsed with PBST and observed using an improved chemiluminescent detection
solution (Abfrontier, Lot.QJN28, Seoul, Korea) and apparatus (Thermo Fisher Scientific, iBrightCL1000, Waltham, MA, USA). Adobe Photoshop CS6 was used to conduct the measurement of the band density.

**RNA Extraction**

After preparing 15 ml of MEM containing a constituted HeLa cell suspension (with an adjusted concentration of $1 \times 10^5$ cells/mL), a volume of 5 ml was plated onto each 100 mm cell culture dish, and the dishes were placed in an incubator at $37^0C$ containing 5% CO$_2$ for 24 hours. The medium was then switched out, the control group did not get any of the extract, and the experimental groups were given 700 and 800 μg/ml of SNPL before being incubated at $37^0C$ with 5% CO$_2$ for 12 hours. After a period of 24 hours, the media was discarded. Rinsing both the control and experimental groups (SNPL 700 and 800 μg/ml) with 1 ml of cold PBS in each plate, scraping, and collecting the cells in 1.5 ml Eppendorf tubes respectively were the next steps. 10 min were spent centrifuging at 8,000 RPM while the temperature was maintained at 4°C. For extraction of RNA, the RNeasy® Mini Kit (50; QIAGEN, Hilden, Germany) was used.

**Reverse Transcriptase PCR**

cDNA was synthesized using extracted RNA using a PCR machine (SampliAmp Thermal cycler, Singapore) using a SuPrimeScript RT Premix (2X with oligo dT; GeNet Bio, Global Gene Network, Daejeon, Korea) with the appropriate amount of RNA and RNase-free water. PCR condition: (50 °C for 60 min, then 70 °C for 10 min) 1-cycle. Eventually maintained at 4 °C.

**Real Time qPCR**

The RT-qPCR method was utilized in order to ascertain the relative fold change in gene expression in HeLa cells. In each qPCR tube, 7 μl distilled water, 1 μl forward and 1 μl reverse primer (Table-1) (Bioneer, Daejeon, Korea), 1 μl cDNA, and 10 μl qPCR master-mix were mixed, then spun down. The experiment was conducted three times employing an RT-qPCR equipment (Exicycler™ 96; Bioneer, Korea). The $2^{\Delta \Delta C_T}$ approach(24) was used to determine the relative quantification result.

Table-1. List of designed primers used for RT-qPCR.
<table>
<thead>
<tr>
<th>Genes</th>
<th>Product size</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>102 bp</td>
<td>Forward 5’-CTGTGAACCCTGCATTTGGC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’-ACTTCGGAAGCTGAACCTGG-3’</td>
</tr>
<tr>
<td>Cytochrome-c</td>
<td>104 bp</td>
<td>Forward 5’-TGGCTTAATGTGTTGCCT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’-AAGCCCAAGCAAAGGGAA-3’</td>
</tr>
<tr>
<td>Bax</td>
<td>102 bp</td>
<td>Forward 5’-ACGAGGGTGATAGGTGTTACA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’-TGTTCTTCCCTACACACG-3’</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>101 bp</td>
<td>Forward 5’-TGGGTGACTGACCTTTGCTTT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’-GTCTGTGAGATTCCCAGTG-3’</td>
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<td>Caspase-9</td>
<td>105 bp</td>
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<td></td>
<td></td>
<td>Reverse 5’-ATTGCACACGCACGTTCACAC-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>104 bp</td>
<td>Forward 5’-TCTTCCAGCCTTGCTTCCTG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’-GGTGTACAGGTCTTTGCGGA-3’</td>
</tr>
</tbody>
</table>

**DNA Fragmentation**

15 mL of MEM containing a suspension of SK-N-SH cells (1 x 10⁵ cells/mL) was made. Then, 5 mL of the suspension was put on each 100 mm culture dish and left to grow in the incubator for 24 hours. The medium was then changed, and the control and experimental groups containing 700 and 800 µg/ml SNPL were incubated for 48 hours. Kits (AccuPrep® Genomic DNA Extraction Kit; Bioneer, Korea) were used to extract genomic DNA from the control and experimental groups. An imaging device (Thermo Fisher Scientific, iBrightCL1000, USA) is used to assess for DNA fragmentation after gel electrophoresis.

**Statistical analysis**

The presentation and display of all data is done as Mean ± SE. Microsoft Excel was used for the statistical analysis, which included a comparison of each treatment group’s mean value to that of the control group using an ANOVA and a T-test. The p-value was utilized to determine statistical significance.

**Results**

Cell viability was done on SK-N-SH and HUVEC cell lines *in vitro*; results obtained and expressed as 50% inhibitory concentration (IC₅₀) of SNPL for SK-N-SH was 710.25 ± 49.04 µg/ml (Figure 1) when compared with control group and didn't show a significant sign of toxicity on HUVEC cells. (Figure 2) depicts a Western blot analysis of the signaling protein expression variations responsible for the difference in proliferation between the treated and control groups, Bak, Bax, caspase-3, caspase-8, caspase-9 and IκBα.
were upregulated; IKKβ and NFκB were down-regulated, related to dose dependent manner. RT-qPCR (Figure 3) was also used to look at the degree to which the genes that cause apoptosis, or cell death, are expressed, and the fold change ratio of genes expression for control, 700 and 800 µg/ml treated SK-N-SH cells was; 1.01 ± 0.11, 4.82 ± 0.45 and 18.44 ± 6.41 for caspase-3; 1.03 ± 0.19, 5.38 ± 0.61 and 24.02 ± 6.84 for Apaf-1; 1.03 ± 0.16, 5.29 ± 0.91, 22.52 ± 10.6 for cytochrome-c; 1.03 ± 0.16, 6.43 ± 0.88 and 35.54 ± 7.14 for caspase-9; 1.30 ± 0.68, 6.13 ± 1.73 and 32.02 ± 12.88 for Bax, respectively. Apoptotic cell death may be identified by a number of telltale characteristics, one of which is the fragmentation of nuclear DNA into nucleosomal pieces. Gel electrophoresis revealed that the experimental groups treated with 700 and 800 µg/ml had more fragmented DNA than the control group (Figure 4).

Discussion

This study is being conducted with the intention of assisting and serving mankind in the process of finding an acceptable and efficient therapeutic source for cancer. This research focuses on employing phospholipid extract from the bone of *S. niphonius* as a natural substance to treat and cure neuroblastoma/nerve cancer. Neuroblastoma cancer is a kind of cancer that affects youngsters and is both invasive and life-threatening. Neuroblastoma consists of neuroblasts, which are immature, undifferentiated small, round-shaped sympathetic cells, with little cytoplasm, dark nuclei and small indistinct nucleoli(6). The presence of typical histopathologic features, in addition to elevated urine levels of one of the catecholamines, is required to make a diagnosis of neuroblastoma (14). It has been shown that extracts from natural sources exhibit a wide variety of biological activities, one of which is the ability to fight cancer, and research reports on this topic are also growing. The phospholipid extract derived from the bone of *S. niphonius* own anticancer activity on SK-N-SH. Anticancer activity of the phospholipid extract on SK-N-SH cell was observed by causing death of 50% of the cells at 710.25 ± 28.31 µg/ml concentration amounts *in vitro*. The cytotoxic and antiproliferative effects of the phospholipid on SK-N-SH cells were shown by this finding.

Apoptosis is a sort of planned cell death that may be produced by either activation of death receptors, which is referred to as the extrinsic apoptotic pathway, or by intrinsic apoptosis, which occurs naturally inside the cell (25). The molecular mechanisms that lead to programmed cell death in mammals include regulation of apoptotic signals by Bcl-2 family of proteins and initiator caspases, cytochrome-c release, downstream caspase activation, chromatin condensation, DNA fragmentation and phagocytosis of dead cells by scavenger cells(26). The proteasome quickly phosphorylates, ubiquitinates, and degrades IκB, and the liberated NFκB dimer is then transported to the nucleus, where it may control particular genes (27). As crucial procedures in the control of NFκB complexes, the phosphorylation and degradation of IκB have attracted a lot of interest (28). In order to prevent NFκB from moving to the nucleus following treatment, a number of molecules maintain large levels of IκB protein in the cytoplasm (29). Some of these compounds stimulate IκB production, others inhibit IκB ubiquitination, and yet others prevent IκB breakdown (30). As a result, inhibitors of any stage of the ubiquitin-proteasome decrease NFκB activation by stabilizing IκB (31). IKK protein induces the phosphorylation of IκBα at Ser 32/36 (32). Innate immune regulators like inflammatory cytokines and DNA transcription are both controlled by the heterodimer
protein complex known as NFκB, which is essential for cell survival (33). Because of its fundamental effect on cellular proliferation and differentiation in cancers, an increasing body of data suggests that activation of NFκB is related with resistance to apoptosis, production of angiogenic proteins, and carcinogenesis (34). A potential strategy for increasing tumor cells' susceptibility to TNF-induced apoptosis is inhibition of the NFκB pathway. (35). The down-regulation of NFκB protein was seen in a dose-dependent way in this investigation, and this finding revealed one of the apoptotic possibilities. The study's findings suggest that the overexpression of IκBα and the downregulation of IKKβ and NFκB were connected in a dose-dependent way.

The Bcl-2 family proteins, which include both pro- and anti-apoptotic members, are important players in apoptosis; interactions between them may control mitochondrial integrity and play a crucial role in controlling the release of cytochrome-c from mitochondria (36). The intracellular Bcl-2 family of molecules is crucial for transmitting the apoptotic signal. Early in neural development, apoptosis-suppressing genes like Bcl-2 and Bclx are abundantly expressed. In the majority of neuroblastoma cell lines and actual tumors, the level of Bcl-2 expression is negatively correlated with the proportion of cells undergoing apoptosis and the level of cellular differentiation (37). The outer mitochondrial membrane's holes are considered to be created by the homo-oligomerization of Bax and Bak (38). The release of cytochrome c results from the oligomerization of Bax on mitochondria. Apoptosis is brought on by a variety of intracellular stresses that cause the release of cytochrome-c from the mitochondria into the cytoplasm. In the intrinsic route, cytochrome-c that has been liberated from the mitochondria interacts with the adaptor protein Apaf-1 to start the caspase-9 activation process that leads to the activation of caspase-3 via the apoptosome. Apaf-1 is a crucial protein in the intrinsic mechanism of apoptosis, also known as the mitochondrial pathway. It oligomerizes in reaction to the release of cytochrome-c and forms a huge complex that is known as the apoptosome. The apoptosome recruits and activates procaspase-9, a mitochondrial pathway initiator caspase, which results in the processing of caspase-3 downstream. Executioner caspases like caspase-3 need to be activated after caspase-8 or caspase-9 have been activated in order to carry out apoptosis (39). The activation of certain proteases known as caspases seems to be linked to the beginning of all intracellular activities. Caspase-3 seems to be involved in how apoptosis is carried out in this process. If the membrane potential is lowered, it is anticipated that the expression of the caspase-3 protein would change in drug-treated cells. Caspase-3, which actively participates in the proteolytic cleavage of the PARP (poly-ADP ribose polymerase) protein, is the most significant of the several caspases. The level of DNA degradation is increased when PARP is expressed more often. Additionally, throughout the apoptosis process, caspases break more than 100 proteins. They are proteins for DNA replication, transcription, or translation, cytoskeletal proteins, and kinases and phosphatases (40). Caspase-3 is consequently regarded to be an important marker of apoptotic cells. An adjustment to the Bcl-2 family, such as an uptick in pro-apoptotic protein Bax expression and a downtick in anti-apoptotic protein Bcl-2 expression, coincides with an increase in caspase-3 activation (36). The expression of apoptotic genes, Apaf-1, Bax, caspase-3, caspase-9 and cytochrome-c up-regulation were observed after treating SK-N-SH cells with SNPL. These data show that apoptosis occurred inside the cells in response to the therapy. Because these genes that produce apoptosis have a unique character
and capacity to generate apoptotic cell death signaling. SNPL treatment initiates intrinsic signaling for apoptosis induction.

Apoptotic features can be created in the cell in a number of ways, depending on the apoptosis inducing compounds used. One of the characteristics of apoptotic cell death and the most efficient method of cell death is the breakdown of nuclear DNA into nucleosomal fragments (40). After treatment with SNPL, chromosomal DNA is cleaved into oligonucleosomal-sized fragments, indicating apoptosis in SK-N-SH. DNA fragmentation may hasten the process of cell death in response to apoptotic stimuli, which may also induce cell death without causing DNA degradation. DNA fragmentation occurred in the later stages of the apoptotic process in SK-N-SH. In addition, caspase family is primarily involved in apoptotic signaling, which leads to DNA fragmentation. Following the treatment, SK-N-SH may undergo apoptotic DNA fragmentation in a manner that is either caspase-dependent or caspase-independent. In this experiment, the DNA ladder gel electrophoresis technique was carried out (Figure 4), and the results showed that the SNPL treated groups had much more DNA fragmentation than the control group had.

**Conclusion**

After treating SK-N-SH cells with the phospholipid extract from the bone of *S. niphonius*, this research found that there were discernible changes detected on cell survival, genes expression, proteins expression, and the outcomes of DNA fragmentation studies. These significant results help to support the conclusion that the phospholipid extract possesses natural therapeutic potential in the treatment of neuroblastoma by inducing intrinsic suicidal apoptotic signals, inhibiting transcription factors that control DNA survival and proliferation, and fragmenting DNA, which stops the growth and metastatic potential of the cancerous cells.

**Abbreviations**

- Apaf-1: Apoptotic protease activating factor-1
- HUVEC: Human umbilical vein endothelial cells
- MEM: Minimum Essential Medium
- SNPL: Phospholipid extracts from the bone of *Scomberomous niphonius*
- WHO: World Health Organization

**Declarations**

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Compliance with Ethical Standards: Disclosure of potential conflicts of interest.

Conflict of Interest: The authors declare that they have no conflict of interest.

References


Figures
Figure 1

Cell viability (%) results of SK-N-SH cells; control group (C = 100% viability) was received no treatment; and various concentrations were administered to the experimental groups. (600, 700, 800 and 900 µg/ml) of SNPL. IC\textsubscript{50} of SNPL for SK-N-SH was 710.25 ± 28.31 µg/ml. The data is given as Mean ± SE. Significant Mean difference (*p < 0.05) from control.
Figure 2

The results of the Western blot analysis of the protein signals produced by SK-N-SH cells in the control (C) and test groups incubated with varying amounts (600, 700 and 800 µg/ml) of SNPL for 12 h.
Figure 3

RT-qPCR results of relative gene expressions by the control (0 µg/ml) and SNPL (700 and 800 µg/ml) treated SK-N-SH cell lines for 12 h. The data are shown as Mean ± SE. Mean significant difference (*p < 0.05) compared to control.
After being exposed to SNPL (700 and 800 g/ml) for 24 hours, the genomic DNA from SK-N-SH cells from the control (C) and experimental groups was extracted, and the 1.5 % agarose gel with 50V electrophoresis was used to separate the DNA for 30 minutes.

Figure 4