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Efficient Potential Cytotoxic and Anti Proliferation Activities Of Cardiotonic Steroids in Urginea Maritima Extract in Human Malignant Neuroblastoma With Less Cytotoxicity Susceptibility Toward Neuron-Like Cells

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Abstract-Natural products have played a significant role in the conventional treatment and the innovation of new drugs for numerous illnesses, including malignant diseases. Urginea maritima is an herb of the Liliaceae family. It is commonly used in its region of origin in folk medicine as a remedy of cancers and other chronic disorders. Notwithstanding, there is a lack of evidence pertaining to the comprehension of the biological activities of Cardiotonic Steroids, as well as other Urginea maritima constituents contained within water-based extracts related to human neuroblastoma disorders. In this study, we sought to investigate the cytotoxicity potential and to evaluate its anti-proliferation effects on human malignances neuroblastoma SH-SY5Y cell line. Through the utilization of several in vitro techniques, the present work illustrates that constituents of the Urginea maritima aqueous extract trigger a series of toxic responses. The viability of neuroblastoma SH-SY5Y cells are selectively inhibited in a time-and-dose dependent manner. The estimated IC50 value was 10ng/ml in the MTS assay after an incubation of 72hrs with less susceptibility in neuron-like cells for neurotoxicity. The data obtained provided in vitro evidence that constituents of the Urginea maritima aqueous extract could be effective to control proliferation of the tumorigenic cells. Moreover, this emphasizes the probability for the use of this unusual natural product for curative applications as well as candidates for further drug design, particularly against human malignant neuroblastoma disorders.

I. INTRODUCTION

NEUROBLASTOMA is the most common extra-cranial solid tumor and it mostly affects infants. According to oncology, approximately 15% of the most deadly malignances of all predicates are associated with neuroblastoma [7]. It is an extreme heterogeneity tumor, whereas high-risk diseases are very aggressive metastatic tumors and are difficult to treat successfully. Even though the most intensive multimodal therapies available are currently being applied, new therapeutic strategies are still required [7]. Natural products with their great structural diversity have offered major opportunities for the identification of novel drugs that are active against a wide range of diseases [13]. Recently, significant extensive research was conducted in an attempt to identify new promising compounds that have anti-tumor potential from natural sources, including cardiac steroids (CTs) [7, 13 and 17].

Urginea maritima is classified as an herb of the Liliaceae family and is indigenous to the Mediterranean region. It has been well known as a medicinal herb since early times as far back as the ancient Egyptians [18]. Previous phytochemical analysis has identified Cardiac steroids (CTS; bufadienolides) as the major constituents of Urginea maritima bulbs [8]. Anthocyanins, flavonoids, polysaccharides and calcium oxalate are also present [1]. Consequently, Urginea maritima has a powerful digitalis-like cardiac effect. It is utilized as a source of natural cardiac steroid products in traditional remedial and veterinary medicine as well [18]. Conversely, the acute toxicity of these compounds has been ignored in related biological studies for several decades. Recently, several works provide scientific evidence concerning the efficiency and possession of this compound's unique character to target and attack cancer cells with less severe toxicity to non-malignant cells [15]. Furthermore, in vitro and in vivo epidemiological data suggested that plant derived cardiac steroids mediated anticancer activities through a regulated number of cellular processes such as proliferation, apoptosis and cell cycle arrest in various types of

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cancer cell lines including Human MCF-7, MDA-MB2311, prostate, melanoma, pancreatic, lung, colon cancer cell (HT19) and leukemia [5] and [6]. This evidence-augmented interest concerning the investigation of these compounds and natural resources to further elucidate their biological properties in order to discover the effectiveness of these natural compounds as effective management for various types of malignancy diseases [20]. On this basis, therapeutic strategies involving either the addition of or aggression of cardiac steroids are currently under development [2]. Urginea maritima has been well known for a long time with traditional ethno-pharmacological application. However, the anticancer biological activities of this medical plant are still largely unexplored. A literature survey on the Urginea maritima species indicates that only a few articles address the antitumor effect of Urginea maritima in solid tumors [3]. Therefore, this study aimed to investigate the cytotoxicity and anti-proliferation of Urginea maritima water-based extracts with the hope of identifying a new candidate that would be feasible for utilization in the management of human malignant neuroblastoma.

II. MATERIALS AND METHODS

A. Reagents and Chemicals

Cell Titer 96*AQueous Non-Radioactive Cell proliferation Assay Solution (MTS, Promega, USA), Essential Medium Eagle (EMEM) Sigma, USA, Hams-F12 Sigma, USA, non-essential amino acids (100 ×) PAA Laboratory GmbH, Austria, L-glutamine (200 mM) Sigma, USA, Gentamicin (10 mg/mL) PAA Laboratory GmbH, Austria, fetal bovine serum (FBS), PAA Laboratory GmbH, Austria Germany, Retinoic acid (RA), Sigma, USA. Dimethyl sulphoxide (DMSO, Sigma, USA).

B. Aqueous Extract of U. maritima

The bulbs of U. maritima medicinal plant will be collecting from numerous locate of green mountain towns in Libya. The botanical identification was done by Prof. Mohammed Alsharif Specimens are deposited at the herbarium section of the Biology Science, University of Garyouns Libya. An aqueous extract of U. maritima medicine plant will be prepared to evaluate its antitumor activity. Briefly the dry bulbs of U. maritima will be ground into fine powder.100g of this powder will be weight and mix with 1000mL boiling double sterile distilled water , the mixture will be shacked with horizontal shaker at 37°C , 250 r.p.m for 48hrs, after that the mixture will be centrifuged at 2735×g for 3 min. The supernatant will be collected and the residue will re-extract for two more time. Finally, all of supernatant will filter through filter paper (whatman no.4). The filtered solution of U. maritima will be lyophilized in freeze dryer system at <40 ◦C for about one week. The concentrated of U. maritima extract was dissolved in dimethyl sulphoxide (DMSO Sigma, USA) to get a stock solution of 10mg/ml. (the percentage of DMSO in the experiment should not exceed 0.5). The sub stock solution of 1.0 mg/mL was prepared, resulting in a series of final concentrations ranging from 100pg/ml - 1mg/ml. The sub stock solution was stored at 4-◦C stock for further process [10].

C. SH-SY5Y Neuroblastoma cells culture condition

Mazatulikhma Binti Mat Zain, Tissue Culture Research Laboratory, Institute of Science (IOS), University Technology MARA kindly provided human Malignant Neuroblastoma SH-SY5Y cell line. SH-SY5Y cells were cultured in 1:1 of Minimum Essential Medium Eagle: Nutrient mixture F12-Ham (EMEM : Hams-F12), with 1% non-essential amino acids ,1% L-glutamine (Sigma, USA), 1% gentamicin (10 mg/mL), and supplemented with 10% fetal bovine serum (FBS) SH-S5Y cells were maintained in an incubator (Contherm Scientific Ltd, New Zeland) at 37ºC in a 5% CO2 atmosphere with 95% humidity.

D. Neurotoxicity

Retinoic acid (Sigma, USA) will induce the differentiation of the neuroblastoma SHSY5Y cells to neuron phenotype characteristics that were confirmed microscopically. Approximately 2×104 cells per well were seeded in 96-well plate. After 24h, R.A was added at a final concentration of 10μM in complete EMEM-F12 media. The medium in plate was changed at day three with fresh RA and cultures were ready to be tested at day six, U. maritima aqueous extract tested for Neurotoxicity effects. The serial dilutions of extract in (EMEM-F12) were made fresh prior to test differentiated cells with final concentrations ranging from100pg/ml-1mg/ml .The wells were agitated lightly and incubated for 48 hrs. Cell viability of treated and untreated cultures was then determined using MTS assay.

E. MTS Cytotoxicity Assay

The cytotoxicity active of U. maritima aqueous extract were performed by Cell Titer 96 AQueous Assay, uses the novel tetrazolium compound (3-(4, 5dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-

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2H-tetrazolium, inner salt; MTS, Promega, USA) using Glomax multi detection system (Promega,USA) and read at 490 nm. In brief, Cells(1×105 cells/ml) were seeded in 96-well plates and left to grow over night in humidified atmosphere containing 5% CO2 at 37 ºC and 90% humidity incubator. The following days, media was aspirated off and replaced with 100µL of fresh media containing a serial dilution of final concentrations of U. maritima aqueous extract ranging from 100pg/ml - 1mg/ml in triplicates. The plates were incubated for selected period with the extract 24 h, 48 and 72hrs, respectively. After the Corresponding period cell viability was measured, 20µL of MTS solution (Sigma, USA) was added into each well in the 96-well plate and incubated for 2-4 hrs. Results were representative of at least three independent experiments. In addition, the percentage of proliferation was calculated by the following formula:
%Viability = Absorbance of test wells /Absorbance of control wells ×100.

F. Statistical Analysis

All statistical analysis was performed using Graph Pad Prism (Version 5.01) statistical software. The results were represented as means ±standard error (S.D).Analysis of variance (one –way ANOVA) was carried out when multiple comparisons were evaluated, followed by Dunnett's multiple compare-son vs. control test and, Bonferroni's Multiple Comparison all pairs test. *p<0.05, was defined significant.

III. RESULTS

A. Result of Cytotoxicity effects of Urginea maritima

Cellular viability of neuroblastoma SH-SY5Y and neuron-like cells were assessed by MTS assay after incubation with a concentration range of 100pg/ml-1mg/ml of Urginea maritima water-based extract. Neuroblastoma SH-SY5Y cells experienced a significant decrease in viability at low concentrations of Urginea maritima 100pg/ml-1µg/ml with an eventual decline at the highest concentrations tested 10µg/ml-1mg/ml. The estimated IC50 values of Urginea maritima extract (concentration causing death of 50% of SH-SY5Y cells) ranged 1µg/ml, 100ng/ml and 10ng/ml after incubation of 24, 48 and 72 hours, respectively Fig: 1.

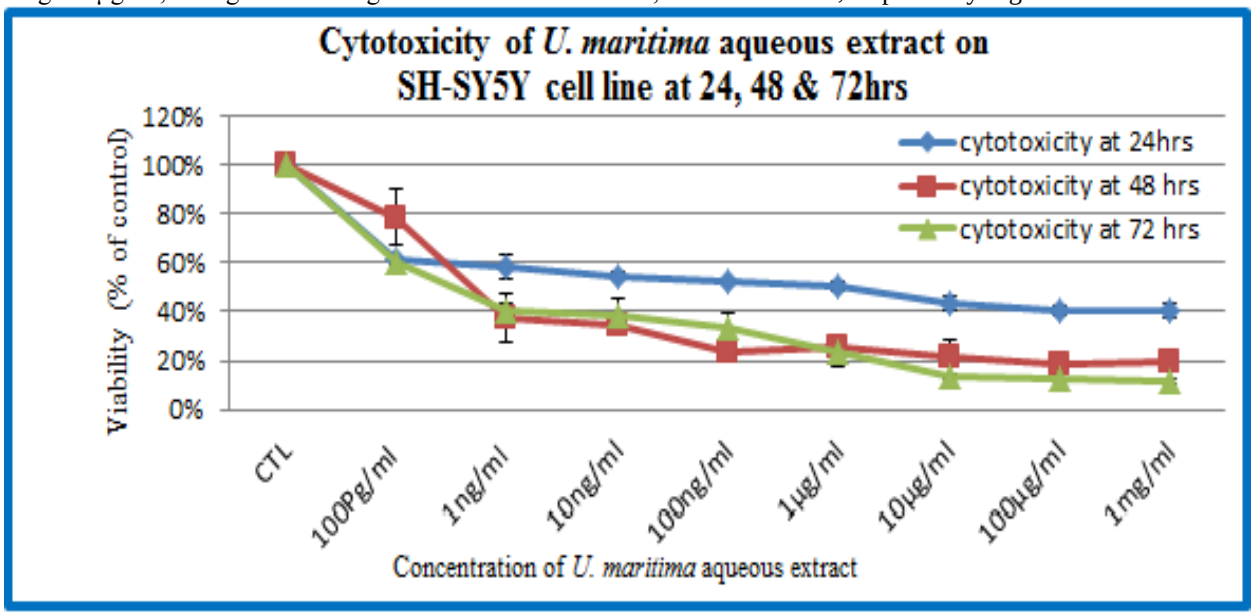

Fig: 1 Strong anti-proliferation activity of *U. maritima* aqueous extract on SH-SY5y cells after (24, 48 and 72hrs) incubation with diverse range of *U. maritima* 100pg/ml -1mg/m. SH-SY5Y viability significantly decreased in a dose-time dependent manner compared with correspond-ding Each date represent the mean ±SD of three independent experiment n=6.

Their viability significantly decreased in a dose-time dependent manner compared with corresponding control cells. As displayed in Fig: 1, high concentrations of Urginea maritima extract 10 µg/ml, 100 µg/ml and 1mg/ml decreased the cell viability of SH-SY5Y (50%-40%) in 24h. Subjected to the same conditions, these cells displayed a (25%-19%) decrease in cell growth after a 48h incubation Fig: 1. While, cell viability of the Neuroblastoma SH-SY5Y cells declined in cell growth to (23% and 12%) following the treatment of 1µg/ml, 10µg/ml, 100µg/ml & 1mg/ml of Urginea maritima after a 72h incubation Fig:1. Cell viability of the neuroblastoma SH-SY5Y cells decreased significantly (***P<0.001) compared to untreated cells.

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Morphological features effect of Urginea maritima water extract 10ng/ml, 100ng/ml & 1µg/ml treated SH-SY5Y cells after 24h and 72h treatment observation using Phase contrast microscopy, cells treated had detached, rounding and shrinkage of cells, reduction in the cells numbers were compared to untreated cells, condensed was seen at the higher concentration of extract. Cell debris was evident and some necrotic-looking cells were seen Fig:2.

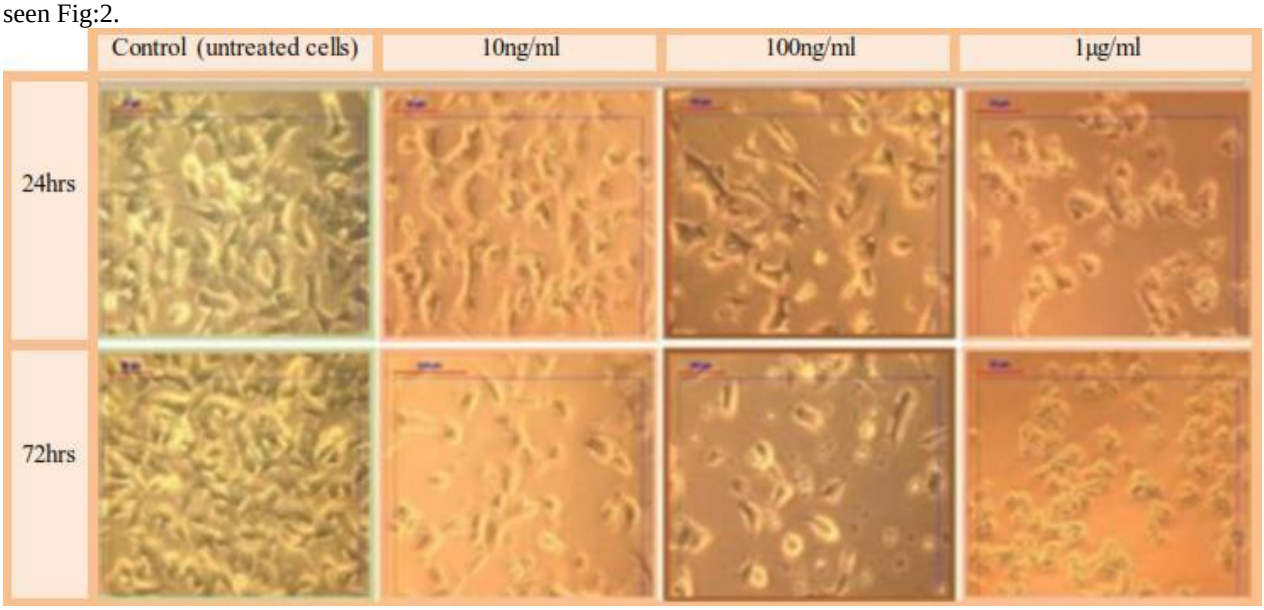

Fig: 2 Representative photos to show Morphological features effect of U. *maritima* water extract (10ng/ml, 100ng/ml &1µg/ml) treated SH-SY5Y cells after 24 and 72hrs. Using Phase contrast microscopy (100 xs). Cells treated had detached from their culture flasks, rounding and shrinkage of cells. Reduced in the cells numbers were comparable untreated cells and appeared condensed was seen at the higher concentration of extract 1µg/ml.

B. Result of Neurotoxicity (neuron-like cells) effects of Urginea maritima

The incubation of neuron-like cells with concentration 1ng/ml, 10ng/ml, 100ng/ml and 1µg/ml in 48hrs, maintained viability (88.9%, 86.3%, 65.6% and 84%) respectively. In contrast, the viability of neuroblastoma SH-SY5Y cells reached (37%, 34%, 23% and 25%) respectively, at the same time points of incubation Fig: 3. Higher concentrations of the Urginea maritima extract 10µg/ml, 100µg/ml and 1mg/ml decreased the viability of both cells neuroblastoma and neuron-like cells progressively Fig: 3. The presence of Urginea maritima aqueous extract in the medium illustrated a diverse behavior of the tumorigenic and non-tumorigenic cells. The susceptibility of the neuroblastoma cells was significantly higher than that of neuron-like cells, suggesting evidence that Urginea maritima aqueous extract could be effective to control cell proliferation, particularly against neuroblastoma SH-SY5Y cells.

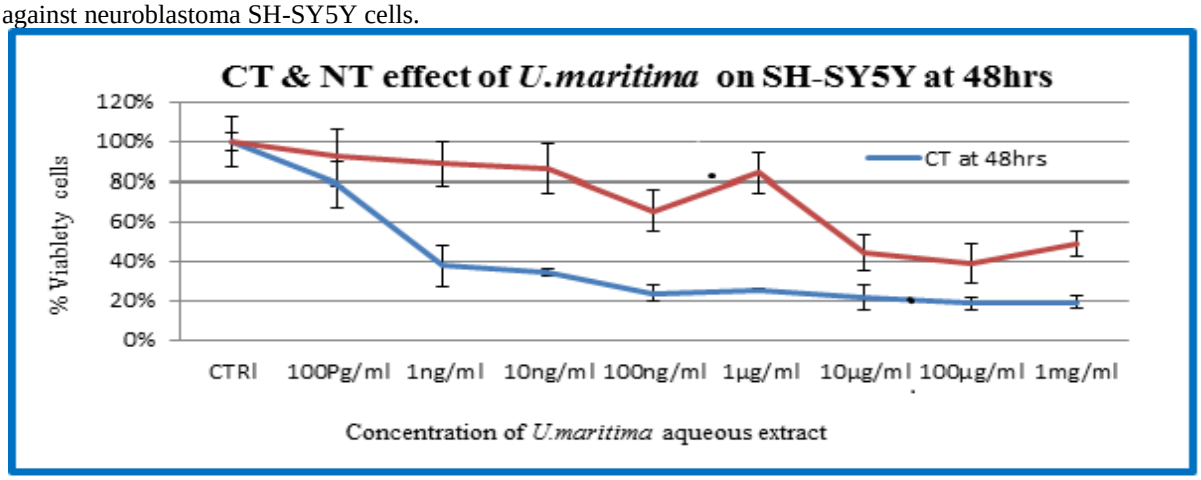

Fig: 3 U. *maritima* aqueous extracts selectively inhibited human neuroblastoma SH-SY5Y cell line Cytotoxicity (CT), and with less susceptibility in neuron-like cells Neurotoxicity (NT)

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Whereas the proportion of cell viability resulted strongly significant difference (***P<0.001) at concentrations of 10ng/ml, 100ng/ml and1µg/ml in contrast of (CT) of these concentration at the same incubation time (48hrs) for MTS assay. Results represent means ±S.D of three independent experiments with, n=6. By using one- way ANOVA analysis, followed, Bonferroni's Multiple Comparison test (Graph Pad Prism 5.01 software).

IV. DISCUSSION

One of the most prominent natural product research efforts is in the field of antineoplastic drugs in an attempt to discover a product that more susceptibly affects the signaling pathways in tumorigenic cells than non-tumorigenic cells. Recently, many researchers have become interested in further related investigations, which have sparked the use of cardiac steroids as healthcare and pharmaceutical agents to treat large panels of cancer cells of different histological origin [16] and [20]. In this preliminary study, we have focused our interest on crude plant extracts of Urginea maritima to evaluate the cytotoxic and anti-proliferation properties of Urginea maritima aqueous extracts. Since cardiac steroids are drugs with a narrow range of therapeutic safety, it is now a challenge to establish that cardiac glycosides can induce apoptosis and inhibit the growth of cancer cell lines at concentrations less sensitive to normal cells [5] and [29]. In line with these, the precious findings of the current study demonstrate that aqueous Urginea maritima extracts induce strong significant anti-proliferation activities against neuroblastoma SH-SY5Y cell line with minimal toxicity toward neuron-like cells. The American National Cancer Institute (NCI) assigns a significant cytotoxic effect of a good drug candidate and promising anticancer agents are required to have an IC50 value ≤ 30 µg/ml [11]. As the estimated IC50 value against SH-SY5Y cell lines was 10ng/ml after a 72-hour incubation period, thus Urginea maritima aqueous extract may be considered as a potential cancer chemotherapy agent. The cytotoxic effects induced in SH-SY5Y cells might be triggered by the Cardiac steroids structure. In particular, there is a mounting body of evidence that extremely low concentrations of cardiotonic steroids are able to initiate several signaling pathways, which may be extremely important for a variety of cell functions [2] and [30]. An interesting study was performed by Winnicka et al., who evaluated the cytotoxic effects of various Cardiac steroids in both MCF-7 and MDA-MB-231 breast cancer cells and they demonstrated that the compounds emphasized the potential usefulness of cardiac steroids as an anticancer agent [31]. Another study conducted by Prassas and Diamandis had reported that cardiac steroids inhibit cell growth in various cancer cells with the threshold concentration around nano-molar level, which is similar to the therapeutic plasma concentration in patients treated with cardiac glycosides [20]. Our results seem to agree with these findings. The present work, also displays that the Urginea maritima water-based extract has great cytotoxic potential on the neuroblastoma SH-SY5Y cell line with the threshold concentration around nano-level. Interestingly, the activation of the plant extract at most concentrations tested produced significant anti-proliferation responses. Recent works in this area provide compelling evidence that now endorses that malignant cells in general are more susceptible to the effects of cardiac steroids than normal cells [30, 31 and 34]. This might be due to many cases, Na+, K+ ATPase activity is different in tumor or transformed cells compared to their normal counterparts [34] and [35]. Recently, pre-clinical studies of (Anvirzel) have established that hot water extracts of oleander leaves' extracts contain cardiac glycoside and polysaccharides with immune modulatory potential [19] and [32]. This oleander extract has strong activity against a variety of human malignant cell lines including melanoma, breast and lung cancer [12] and [16]. In addition, the US Food and Drug Administration (FDA) accepted a phase 1 study of Anvirzel in patients with advanced solid tumors [19]. In this investigation, the results obtained indicate that the aqueous extracts of Urginea maritima were shown to induce great significant time-and-dose dependent inhibitory proliferation activities against human neuroblastoma SH-SY5Y cell lines and less sufficiently sensitive against neuron-like cells. Our present work illustrates evidence of a tumor specific cytotoxic effect of the active ingredients of Urginea maritima extract on the SH-SY5Y cell line. Moreover, this data opens an interesting basis for a widespread area of medical application of cardiac steroids for the development of new drugs to combat cancer disorders.

V. CONCLUSION

The discovery of a product that more susceptibly affects the signaling pathways in tumorigenic cells more than non-tumorigenic cells is still a critical target needed in most malignant diseases. Consequently, there is a lack of evidence pertaining to the comprehension of the biological activity properties of cardiac steroids, as well as other Urginea maritima constituents contained within water-based extracts related to the human neuroblastoma SH-SY5Y cell line. Therefore, this investigation has evaluated the cytotoxicity potential of Urginea maritima aqueous extracts on the neuroblastoma SH-SY5Y cell line. The resultant data indicates that Urginea maritima aqueous extract has been shown for the first time to fulfill the fundamental criteria of an effective therapeutic

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agent against human neuroblastoma SH-SY5Y cell line with minimal toxicity toward normal cells (neuron-like cells). The results provide evidence in vitro of the potential role of the studied extract as specific antitumor natural constituents as well as candidates for further drug development, which might lead to new structures in drug design to combat the cancer disease particularly toward human neuroblastoma disorders.

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