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NEW PROSPECTS OF URGINEA MARITIMA (L. BACKER): EFFICIENT PROLIFERATION INHIBITION AND INDUCED INTRINSIC APOPTOSIS PATHWAY AGAINST HUMAN LEUKAEMIA HL-60 CELL LINE

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Abstract:-

Natural products with their great structural diversity have offered major opportunities for identifying of new drugs against numerous of diseases, including malignant diseases. Leukaemia is a malignant disease that originates from a multistage process of accrual genetic alterations. These alterations destroy the slight sense of balance between cell proliferation, differentiation, and apoptosis. Conventionally, the chemotherapy, radiotherapy, and transplantation techniques are the major treatments for leukaemia diseases. However, these traditional approaches have drawbacks. Therefore, the identification of new effective therapeutic agents against leukaemia is an important topic. U. maritima is classified as an herb under Liliaceae family. It is renowned in traditional ethno pharmacological applications. Notwithstanding, there is a lack of evidence pertaining to the bioactivities of U. maritima to human leukaemia diseases. Therefore, the current study sought to investigate the potential proliferation inhibition and apoptotic inducing effects of U. maritima against HL-60 leukaemia cell. Through the utilization of several in vitro techniques, the present work revealed that the main phytochemical constituents of U. maritima reduced the proliferation of leukaemia HL-60 cells in a time and-dose dependent manner. Additionally, the rate of apoptosis efficiently induced as evidenced by Annexin-V/PI staining and analysis conducted by flow cytometry. Moreover, the data illustrate induced apoptosis mechanism, through intrinsic/ mitochondria apoptosis. The obtained finding provided in vitro for the first time that U. maritima could be an effective to control proliferation of the tumorigenic cells. Further, the data elucidate the probability afforded an interesting basis of this natural product as promising candidates for anti-leukemia curative applications.

Keywords:-*Promyelocytic leukaemia HL-60 cell line, Urginea maritima (L. Backer), Proliferation inhibition, Intrinsic apoptosis pathway.*

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INTRODUCTION:

Leukemia is a malignant disease that originates from hemopoietic stem cell. It is a multistage process, which involves the accrual of genetic alterations over a period [6, 23]. Conventionally, the chemotherapy, radiotherapy, and bone marrow transplantation techniques are the major treatments for leukemia diseases. However, these traditional approaches have the drawbacks, which include, drug resistance, toxicity and the low specificity of currently available cytotoxic drugs and expensive diagnosis [4, 15]. Therefore, the identification and development of new effective therapeutic agents for treatment of leukemia is an important topic. Natural products with their great structural diversity have offered major opportunities for the identification of new drugs that are active against a wide range of diseases [5, 3 and 18]. Recently, significant extensive research was conducted in an attempt to identify new promising compounds that have anticancer potential from natural sources, including cardiac steroids (CTs) [9, 13 and 26]. The term cardiac steroids (CTs) refer to a large family of natural mostly plant-derived compounds. The cardiac steroids (CTs) classes share the ability to operate as potent inhibitors of the plasma membrane Na+/K+-ATPase [2,19].*U. maritima*is classified as an herb of the Liliaceae family and is indigenous to the Mediter-ranean region. It has been well known as a medicinal herb since early times as far back as the ancient Egyptians [1, 9 and 14]. Previous phytochemical analysis has identified cardiac steroidsCTS; bufadienolides) as the major constituents of *U. maritima* bulbs [8, 10]. Anthocyanins, flavonoids, polysaccharides and calcium oxalate are also present [1, 11]. Recently, several works provide scientific evidence concerning the efficiency and possession of cardiac steroids compounds unique character to target and attack cancer cells with less severe toxicity to non-malignant cells [8, 13 and 16].

Significant *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 cancer cell lines including Human MCF-7, MDA-MB2311, prostate, melanoma, pancreatic, lung, colon cancer cell (HT19), neuroblastoma and leukemia [7, 20 and 21]. Thess evidences augmented interest concerning the investigation of these compounds and natural resources to elucidate their biological properties as effective management for various types of malig779 nancy diseases. On this basis, therapeutic strategies involving of cardiac steroids are currently under development [2, 17].*U. 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 *U. maritima*species indicates that only a few articles address the antitumor effect of *U. maritima*in malignant disorders [8, 9 and 14]. Therefore, this study aimed to investigate the anti-proliferation and apoptotic potential induce of *U. maritima* water-based extracts with the hope of identifying a new candidate that would be feasible for utilization in the management of leukemia disorder.

1. Materials and Methods

1.1 Reagents and chemicals

Annexin V-FITC apoptosis detection kit, BD Pharmingen, USA, RPMI medium (Sigma, USA), non-essential amino acids (100×), L-glutamine (Sigma, USA), Gentamicin (10 mg/mL, fetal bovine serum (FBS), Cell proliferation assay solution (MTS, Promega, USA), CaspaTag™ In Situ fluorescent assay (Chemicon®, USA).

1.1 Preperation of *U. maritima* extraxct the bulbs of *U. maritima*medicinal plants were prepared to evaluate its antileukemia activity. Briefly,the filtered solution of *U. maritima* will lyophilize under sterile condition in freeze dryer system. The concentrated of *U. maritima*extract was dissolved in dimethyl sulphoxide (DMSO Sigma, USA) to get a stock solution. Then stored at 4^{O}C for further process.

1.2 Cell line and Culture Condition

Promyelocytic Leukeamia HL-60 cell Line were cultured in RPMI with 1% non-essential amino acids, 1% L-glutamine, 1% Gentamicin and supplemented with 10% FBS. HL-60 cells were maintained in an incubator at 37 ºC in a 5% CO_2 with 95% humidity.

1.3 Anti-proliferation (MTS) Assay

The anti- proliferation activitiesof *U. maritima* aqueous extract were performed by cell titer 96 aqueous assay, uses MTS (Promega, USA) and read at 490 nm byusing Glomax multidetection system (Promega,USA). In brief, cells (1×10^5 cells/ml) were seeded in 96-well plates and left to grow in 5% CO_2 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. The plates were incubated for selected period 24, 48 and 72hr, respectively. After the corresponding period cell proliferation was measured, 20µL of MTS solution (Sigma, USA) was added and incubated for 24hr. The percentage of proliferation was calculated by the following formula: % of proliferation = Absorbance of test wells /Absorbance of control wells ×100.

1.4 Apoptosis mechanism detection Annexin V-FITC apoptosis detection kit (BD Pharmingen, USA) was used to determine the rate of apoptosis in cultured HL-60 cells. In brief, cells in exponential growth counted and plated in six well

plates at cells density 1×10^6 cells/mL for 24 h. The cells then treated with 10 µg/ml, 1µg/ml and 100ng/ml of aqueous *U. maritima* extract for 24hours. Positive control (doxorubicin) culture with HL-60 was prepared simultaneously. Following the treatments, cells harvested, the cells re-suspended in binding buffer, and AnnexinV- FITC and Propidium Iodide (PI) (Sigma, USA) added. After 15 min incubation at room temperature in dark, the cells analyzed with flow cytometry (Beckman Coulter, USA). Each extract and control assayed in triplicate.

1.1 Caspase-3/ 7 In Situ fluorescent-based assay In order to gain further investigation into the intracellular event during induced apoptosis mechanism in Leukemia HL-60 cells upon exposure to IC_{50} of *U. maritima*aqueous extract, CaspaTag™ Caspase-3/ 7 In Situ fluorescent-based assay have been utilized. Leukemia HL60 cells ($\approx1\times10^6$ cells/ml) in exponential growth phase, with quality of ≥ 90 % viability, was seeded in 96well plates and left to grow in a an incubator containing 5% CO_2 and 90% humidity at 37ºC. The following days, fresh media containing 10µg/ml, 1µg/ml and 100ng/ml concentration of aqueous *U. maritima* extract were added. The plates were incubated for the selected period with the extract 24h, 48hrs, respectively. After the correspondding period, detection of active caspase-3/ 7 in cells undergoing apoptosis was then determined. According to the manufacturers. Each sample was measured using GlomaxMulti Detection System (Promega, USA).

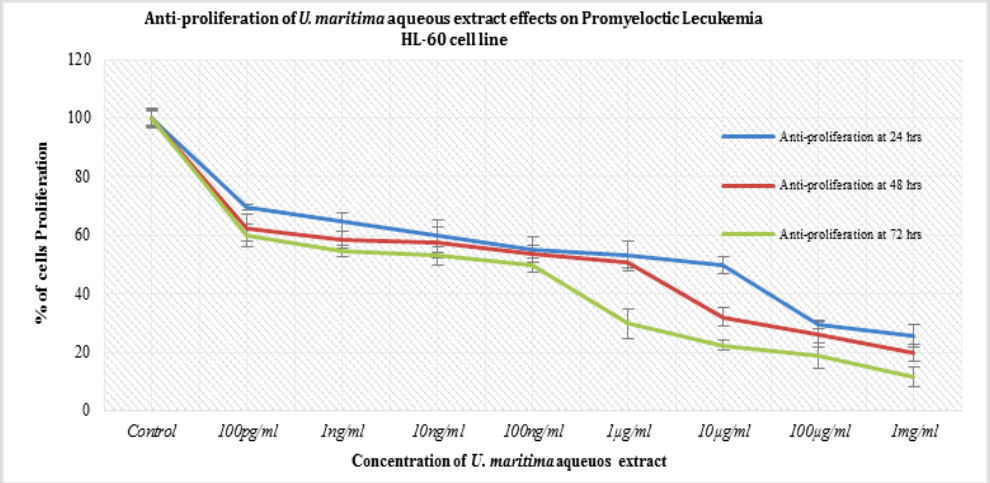
1.1 Statistical analysis

Statistical analysis performed using Graph Pad Prism (Version 5.01) statistical software. Analysis of variance (one- way ANOVA) was carried out when multiple comparisons were evaluated, *p<0.05, was defined significant.

2. Result

2.1 Result of anti-proliferation effects of *U. maritima*

Cellular proliferation of Promyelocytic leukemia HL-60 cells after incubation with 100pg/ml- 1mg/ml of *U. maritima* water-based extract experienced a significant decrease inproliferation at low concentrations of *U. maritima* ranging 100pg/ml- 1µg/ml, with an eventual decline at the highest concentrations tested 10µg/ml -1mg/ml. The estimated IC_{50} values of *U. maritima* extract (concentration causing death of 50% of the cells) ranged 10µg/ml, 1µg/ml and 100ng/ml after incubation of 24, 48 and 72hours, respectively.High concentrations of *U. maritima* extract from 10 µg/ml- 1mg/ml decreased the cell viability of leukeamia HL-60 cells (49%- 25%) in 24h. Subjected to the same conditions, the treated cells displayed a (32% - 19%) decrease in cell proliferation after 48h incubation. While, cell proliferation of treated cells declined in proliferation growth (11%) following the treatment 100µg/ml of *U. maritima* extract after 72hrs incubation. As



displayed in Fig. 3.2.

Figure 3.1: Strong anti-proliferation effects of U. maritima aqueous extract on treated HL-60 cells, after (24, 48 and 72hrs) incubation with diverse range of U. maritima (100pg/ml -1mg/m). Proliferation of HL-60 cells significantly reduced in a dose-time dependent manner compared with corresponding control cells by using one –way ANOVA analysis followed by Dunnett's M.C.T, (Graph Pad Prism 5.01 software). Each data represent the mean ±SD of three independent experiment.

This result clearly show that cell proliferation of *U. maritime*- treated leukeamia HL-60 groups reduced significantly (***P<0.001) in dose- time dependent manner compared with corresponding untreated leukaeamia HL-60 (negative control) group. Fig. 3.1.

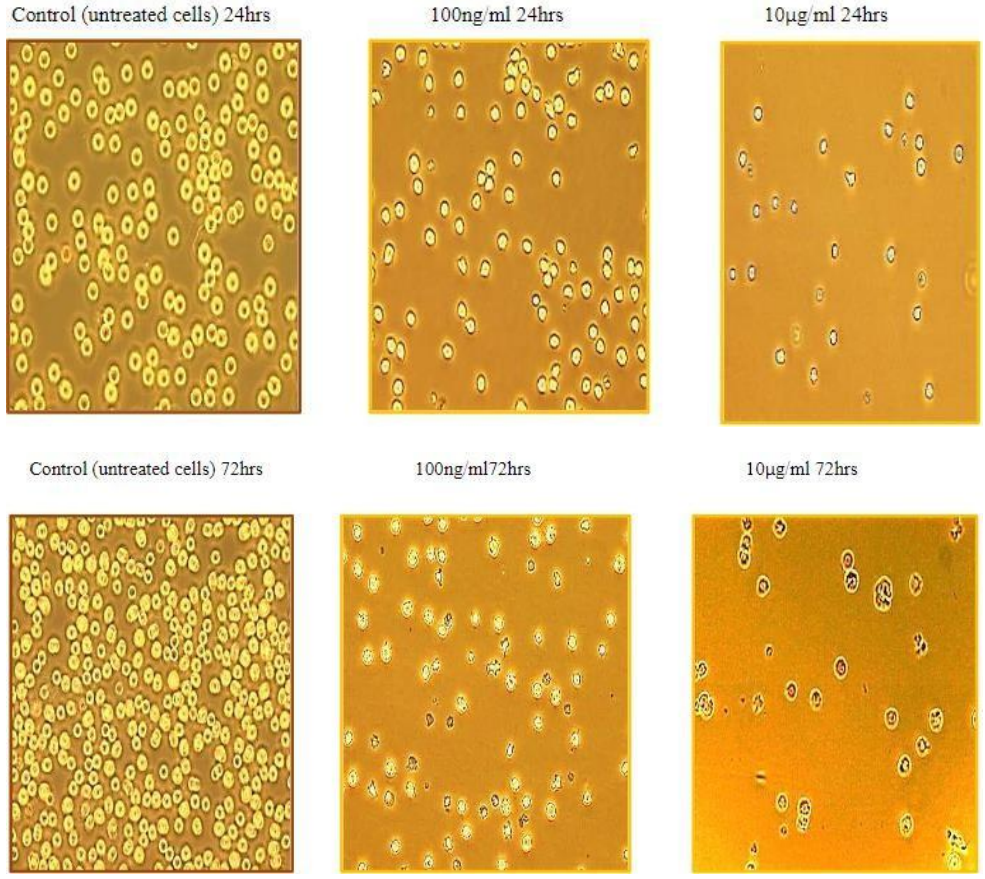


Figure 3.2: Representative photos to show Morphological features effect of U. maritima water extract (100ng/ml,1µg/ml & 10µg/ml) treated HL—60 cells after 24hrs and 72hrs treatment using Phase contrast inverted Microscopy (20 x).

1.1 Result of Apoptosis Studies

Annexin V-FITC assay conducted to confirm the ability of phytochemical composition of *U. maritima*aqueous extract to mediate apoptosis mechanism in human Leukemia HL-60 cells. In this study flow cytometryperformed to detect the cells in apoptosis condition. The cells undergoing early apoptosis rupture the phospholipid asymmetry of their plasma membrane, thus causing rapid alterations in the organization of phosphatidylserine (PS) in the cells.

The phosphatidylserine exposure represents a hallmark of early apoptotic [6]. The late apoptotic and necrotic cells with disrupted cell membranes can identify by using the vital dye Propidium Iodide (PI). Human Leukemia HL-60 cells treated with *U. maritima*water-based extracts demonstrated potential apoptotic proportions significantly increase in a dose- time dependent manner Fig: 3.3.The treated HL-60 cells with 100 ng/ml, 1µg/ml, and 10 µg/ml ofconcentration of *U. maritima* extract have recorded apoptotic proportions approximately to 44%, 47%, and 56% with High significate (***P< 0.001) at 24h respectively, compared with corresponding negative controlgroup. By contrast, the viability proportion of HL-60 cells reaches 47%, 45%, 29% under the same condition of treatment and incubation. The positive control doxorubicin (5 µM) induced an early apoptotic in human Leukemia HL-60 cell that is approximately (27 %). The appearance of the treated *U. maritima* HL-60 cells at early apoptotic condition signifying that the cytotoxic effects observed in response to the studied extract are associated with the induction of apoptotic cell death mechanism. As shown in figure 3. 3.

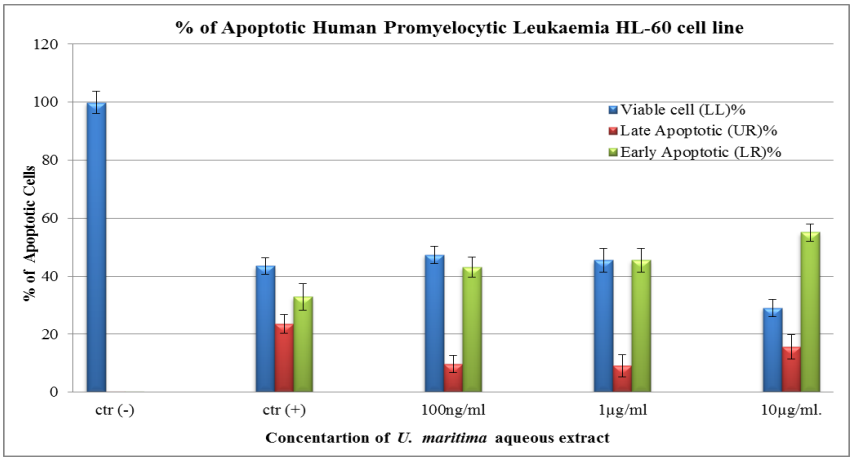


Figure 3.3: Bar diagrams to the apoptotic proportion of HL-60 cells in Annexin V-FITC/PI. Significantly increase of apoptotic proportional and viability decrease in dose-time dependent manner compared with corresponding control cells (**P<0.01 and ***P<0.001) by using One-way ANOVA analysis followed by Dunnett's M.C.T. (Graph Pad Prism5.01 software). Each data represent of three independent experiments.

2.2 Caspase-3/ 7 In Situ fluorescent-based assay

The experimental human Promyelocytic Leukemia HL-60 cells exposed to IC_{50}, 100ng/ml, 1µg/ml, 10µg/ml-concentrationsof *U. maritima* extract at time incubation 24hr, were selected for examination the caspase-3 and -7 activities. The level of caspases 3 / 7 activity wasa significantly enhanced increased in treated HL-60 cells within IC_{50} of *U. maritima* aqueous extract involving compeared with untreated HL-60 group. The Caspase-3/ 7 activity were calculated as follows:

(Mean fluorescence intensity of the FAM-DEVD-–treated cells) / (Mean fluorescence intensity of unstained cells).

Among the experimental *U. maritima* tested dose, 10µg/ml treated Leukemia HL-60 cells, at time point incubation 24hours appearing with more significant increased portion of activation caspases 3/ 7 than other treated groups, with estimated percentage value was 47.010 ±2.000. However, the percentage of activation the caspases 3/ 7 level of the same dose.

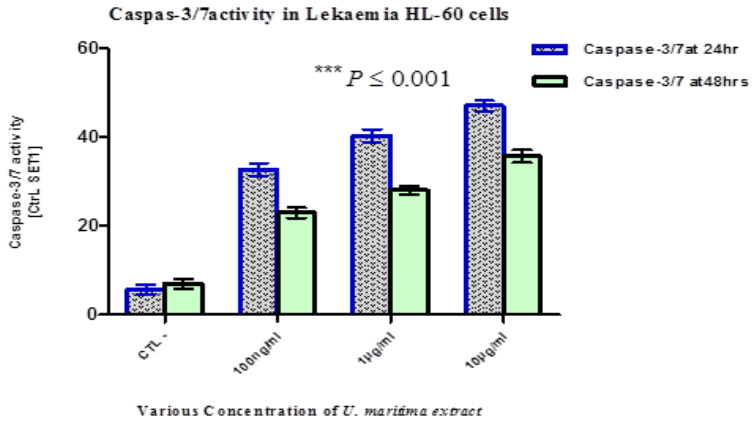


Figure 3.4: Bar diagrams shown the mean percentage of the FAM-DEVD in promyelocytic leukemia HL -60 cell line after incubation with IC_{50} (100ng/ml, 1µg/ml, 10µg/ml) of *U. maritima* aqueous extract for the time duration 24, 48. The data exhibited a significant stimulus of the caspases 3/ 7 activity compared to the control groups ***P< 0.001. By using One-way ANOVA analysis followed by Dunnett's M.C.T. (Graph Pad Prism 5.01 software). Each date represents the mean ±SD of three independent experiments.

However, the percentage of activation the caspases 3/ 7 level of the same dose. 10 µg/ml- was displaying decreased at 48hours. As shown in Figure 3. 4. Concurrently, the activating caspase-3 level of both 100ng, and 1 µg/ml treating Leukemia HL-60 cells groups resulted mediate portion of activation caspases 3/ 7 in comparison to untreated population (a negative control) group at the same incubation condition. On the other hand, the present study data exhibited a great significant stimulus of the caspases 3/7 activity of the completely experimental testing concentration of*U. Maritime* extract. Thereby, the phytochemical compounds, which closely related to *U. maritima* aqueous extract possesses capability to induces-apoptosis mechanism in human Leukemia HL-60 cells through activating the caspase-3/ 7 death dependent pathway.

DISCUSSION

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 [6, 17 and 21]. In parallel, several studies have screened phytochemical of medicinal plants with anti-leukemia activities, including cardiac steroids [5, 7]. Generally, cardiac steroids recognized as antiarrhythmic drugs that function by inhibiting Na+/K+-ATPase [19, 24]. Cardiac steroids (CTS; bufadienolides) have identified as the major constituents of *U. maritima* extracts [6, 8, 11 and 12]. The scientific work of the past few years, built on the foundation of the alterations in The Na (+), K (+) 785

ATPase function have been associated with apoptosis mechanism [22, 19 and 20]. In this preliminary study, we have focused our interest on medical plant *U. maritima*aqueous extract to evaluate their anti-proliferation and apoptosis induce potentials. 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 [6, 8 and 14]. 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 IC**50** value ≤ 30 µg/ml [15]. In line with these, the precious findings of the current study demonstrated that active ingredients of *U. maritima* aqueous extracts displayed significant dose and time dependent inhibitory proliferation activities against treated leukemia HL-60- cells (**P<0.01 and ***P<0.001). Whereas, the estimated IC**50** value were 10µg/ml, 1µg/ml and 100ng/ml after incubation 24h, 48hrs and 72hrs respectively (Fig; 3.1 and 3. 2). Thus,*U. maritime* aqueous extract may be considered as a potential cancer chemotherapy agent. In particular, there is a mounting body of evidence demonstrated 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, 23 and 24]. Previous study conducted by Diamandis and Prassas had reported that cardiac steroids inhibit cell growth in diverse cancer cells with the threshold concentration around nano-molar level, which is similar to the therapeutic plasma concentration in patients treated with cardiac steroids [7]. Our results seem to agree with these findings. Whereby the present work also displays that, the *U. maritima* water-based extract has great cytotoxic potential on treated Leukemia HL-60 cell line with the threshold concentration around micro and nanolevel. Interestingly, the activation of the plant extract at most concentrations tested produced significant anti-proliferation responses. Extensive research has been provide that cytotoxicity induced by cardiac steroids includes a series of morphological and biochemical changes that are characteristic for apoptosis, such as phosphati-dylserine externalization as an early event in the dynamics of cellular apoptosis. Additionally several studies have documented evidence that various cardiac steroids in nontoxic concentrations have shown to induce apoptosis in diverse malignant cell lines, *in vitro* [10, 14 and 27].

Similar, these characteristics obtained after exposure of Promyelocytic Leukemia HL-60 cells to the *U. maritima* extract constituents, which approve through annexinV-FITC/ PI stain. Recent immune assay reports of bufadienolides, determined that bufalin-like substances are very potent Na/K-ATPase inhibitor within malignant cells induction of apoptosis in cancer cells compared with normal physiological conditions [17, 20 and 25].

Accordingly, the low concentrations of *U. maritima* aqueous extract induced visible phosphatidylserine exposure after 24 hours of treatment (Fig3. 3). From the clinical viewpoint cardiac steroids might be ideal drugs for the treatment of leukemia since there is preferential killing of cancers cells by the apoptosis mechanism in contrast to necrosis, hence it does not induce inflammation. In line, caspase-3 is an "effector" caspase linked with the origination of the "death cascade". It serves as a master player for diverse signaling pathways; therefore, it activities are an appropriate biomarker of apoptosis events.

In the present study, the susceptibility of IC_{50} of *U. maritima*extract increased the percentage of caspase 3/7 level in treating Leukemia HL-60 cells were observed and the result revealed a significant stimulus of *U. maritima*- induced apoptosis through activation the caspases 3/ 7 (Fig 3. 4) in compared to the portion of the untreated Leukemia HL60 cells group. Our present workfindings elucidates evidence of highly anti-proliferation and efficient induce apoptosis effects of the active ingredients of *U. maritima* extract on Leukemia HL-60 cell line.

Conclusion

Along with increased recognition of apoptosis in a wide range of cancer disease treatment. The obtained data indicates *in vitro* that constituents of *U. maritima*extract have revealed for the first time to fulfil fundamental criteria of an effective pharmaceutical candidate in human Promyelocytic Leukemia HL-60 cells through mediated intrinsic (mitochondrial) apoptosis pathway. Thus, the active principle of ingredients of medicinal herb*U. maritima*could open an interesting basis area of medical application for development new drugs to combat leukemia disease.

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