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Cell Cycle Arrest in Different Cancer Cell Lines "Liver, Breast, and Colon" Induce Apoptosis under the Influence of the Chemical Content of *Aeluropus lagopoides* Leaves Extracts

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Abstract: Natural product especially secondary metabolites that produced by plants under the stressed condition shown to have a different pharmacological impact. *Aeluropus lagopoides* is one of the typical halophyte plants survivals under stressed conditions. It has been used for wound healing and as a painkiller. The bioactivity and the chemical composition of this plant have poorly investigated. Consequently, chemical components of *A. lagopoides* leaves were extracted using hexane (nonpolar), ethyl acetate (semi-polar), n-butanol (polar) to extract the most extensive variety of metabolites. The cytotoxicity and anticancer impact of extracted secondary metabolites evaluated against breast (MCF-7), colon (HCT-116), and liver (HepG2) cancer cell lines using SRB test. The mechanism of action verified by observing the appearance of apoptotic bodies using the fluorescent microscope while their antiproliferative impact had been evaluated using flow cytometer. Results revealed that secondary metabolites extracted using hexane and ethyl acetate were having the highest cytotoxicity and thus anticancer activity effect on HepG2 with IC_{50} (24.29 ± 0.85, 11.22 ± 0.679 µg/mL) respectively. Where apoptotic bodies observed, flow cytometer results exhibited that secondary metabolites can inhibit cell cycle in G0/G1 phase. Accordingly, *A. lagopoides* hexane and ethyl acetate extracts may consider as a candidate anti-cancer drug.

Key words: *Aeluropus lagopoides*; HepG2; HCT-116; MCF-7; Apoptosis; chemical composition.

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1. Introduction

Cancer, the multistage process in different aspects molecular and morphological become the second cause of death around the world[1]. In Saudi Arabia over 15.800 people were diagnosed with cancer[2]. The production of anticancer drug facing the challenges of cancer cells complications and resistance against the drug. Consequently, the perception of the pathways inside the cancer cell based on the role of the enzymes and their catalysis is the real way to keep cancer cells under control. Secondary metabolites which produced by different plants and organisms to adapt to their environment may have roles as toxins to initiate or to inhibit different pathways inside targeted cells. On the other hand, apoptosis is one of the critical pathways inside cancer and normal cells. So, it is - like other pathways- controls by some enzymes like caspase or cyclin which can be activated by many catalysts. Whatever, natural products are considered as the best candidate anticancer drugs due to their fewer side effects. Consequently, became the primary source of almost 80% of approved drugs from (FAD) [3]. Therefore, the global focus was on the isolation of bioactive compounds, identification and measuring their therapeutic potential in cancer prevention and treatment [4]. Plant, especially medicinal plants have used as a source of medicine since in early civilization for treating different diseases[5]. Plants have given famous names of drugs which used against cancer as anticancer or chemoprevention drugs. Such as taxol, vinca alkaloids, camptothecin and topoisomerase inhibitors [6-8]. Halophyte plants cultivated under stressed abiotic conditions [9]. Subsequently, they are the most candidate plants to develop different molecular, physiological, and morphological mechanisms for their survival [10]. Halophyte plants have investigated as sources of nutrition[11], fuel[12], bioremediation[13]. Medical halophytes are rich in bioactive secondary metabolites such as antioxidant, polyphenol, flavonoids compound these components showed biological activity as antimicrobial, antiviral, anticancer, anti-inflammatory where they are often nontoxic to normal cell lines [14-16]. *Aeluropus lagopoides*, member of halophyte plants from the family Poaceae. It is a perennial grass that tolerated to salinity, grows in the coastal salt marshes [17]. It is a productive plant used as a source of nutrition and livestock [18, 19]. It has been used for wound healing and as a painkilling in the Arabian countries [20]. To our knowledge, this is the first report on the GC\MS profiling and anticancer activity of *A.lagopoides* crud extracts against cancer cell lines. The current study is aimed to investigate the ability of *A. lagopoides* non-polar, semi-polar and polar leaves crude extracts to activate different necessary mechanisms to prevent cancer cell proliferation or to induce cancer cells to apoptosis.

2. Results:

2.1. Cytotoxicity

The prepared crude extracts tested against different cancer cell line MCF-7, HCT116, and HepG2. Results revealed that hexane and ethyl acetate extracts present a significant impact comparing to n-butanol extracts (Table.1). Ethyl acetate extract recorded the highest values of IC50 (11.22± 0.679 µg/ml) against HepG2 cell line while hexane extract recorded (24.29 ± 0.85 µg/ml) against the same cell line. However, n-butanol extract presented insignificant impact with (100<) on all cell lines. The results afford mentioned confirmed by cell viability curves (Figure.1 a, b and c).

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Table 1.The IC50 (µg\ml) of different extracts of A.lagopoides against different solid tumor cell lines.

Extract	MCF-7	HCT-116	HepG2
Hexane	32.7± 0.58	27.79± 0.71	24.29± 0.85
Ethyl acetate	28.03± 0.98	34.6 ± 0.82	11.22± 0.679
n-butanol	100<	100<	100<
Doxorubicin	0.6 ± 0.022	0.45 ± 0.0516	0.42±0.103

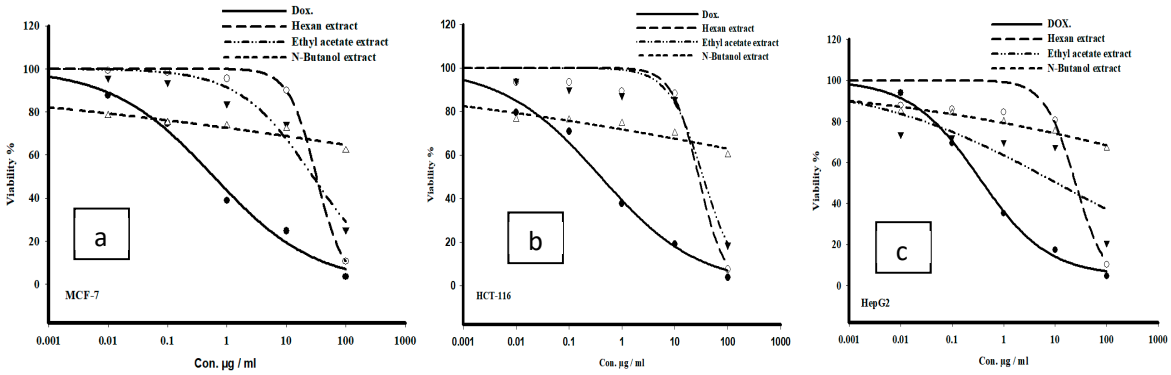

Figure 1. Dose-response curve of different extracts of *A.lagopoides* against solid tumor cell line (a).MCF-7, (b). HCT116, and (c). HepG2. Cells were exposed to the extracts for 72h. Cell viability was determined using SRB-U assay and data are expressed as mean±S.D. (n=3).

2.2. Fluorescence Microscopic Analysis of Cell Viability and Apoptosis

The results obtained from the fluorescence microscope revealed that hexane and ethyl acetate extracts have a high ability to induce apoptosis. (Figure.2). Cells exhibited different apoptotic morphological characteristics such as membrane blebbes, chromatin condensation in addition to apoptotic bodies formation.

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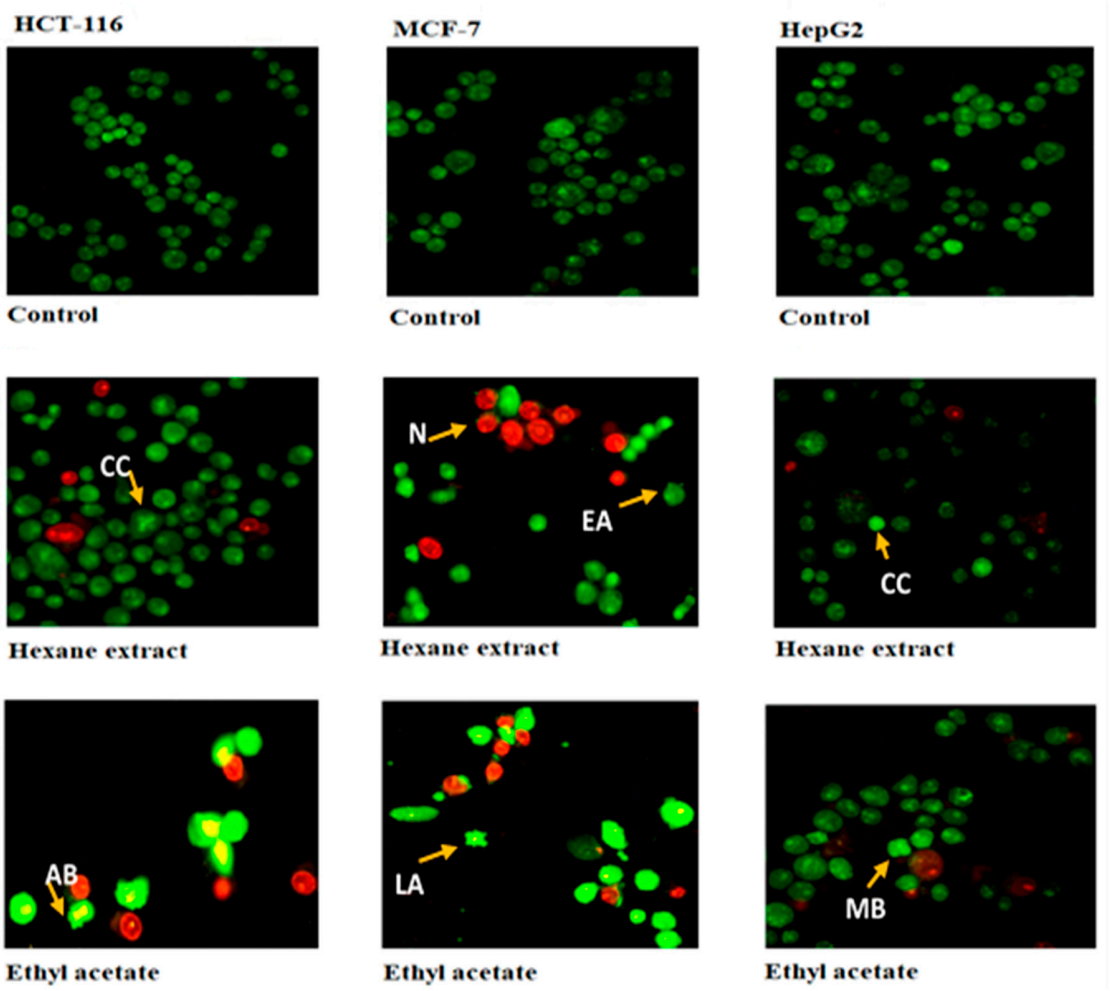

Figure 2. Morphological changes of HCT-116, MCF-7 and HepG2 cells induced by the IC_{50} concentration of *A.lagopoides* hexane and ethyl acetate extract, stained with AO/EB. The images were taken using fluorescence microscopy at 20×. **MB: membrane blebbing; CC: chromatin condensation; EA: early apoptosis; LA: late apoptosis; AB: an apoptotic body; N: necrosis.**

2.3. Cell cycle analysis

In order to identify which phase in the cell cycle was affected by *A.lagopoides* hexane and ethyl acetate crude extracts, the current study used flow cytometer. Results revealed that the hexane and ethyl acetate extracts have similar impact mechanism on the cancer cell types. Thus, the result manifests that the impact of the hexane and ethyl acetate extracts showed in the G0-G1 phase (Table 2.) (Fig.3-5).

Table 2. Effect of Hexane and ethyl acetate extracts of *A.lagopoides* on the cell cycle distribution of three tumor cell lines for 24 h and compared with control cells.

Tumor cell line	Compound	Cell cycle phase		
		G0-G1	S	G2-M
HCT-116	Control	44.8±0.85	51.79±0.59	3.41±0.29

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	Hexane extract	69.59±0.38	26.28±0.52	4.12±0.89
	Ethyl acetate extract	67.57±0.58	26.6±0.58	5.8±0.89
MCF-7	Control	45.4±0.97	46.2±0.81	8.34±0.93
	Hexane extract	61.8±0.9	25.91±0.59	12.21±0.31
	Ethyl acetate extract	70.82±0.49	25.3±0.8	3.8±0.75
HepG2	Control	50.48±0.62	43.3±0.71	6.21±0.36
	Hexane	72.79±0.6	26.91±0.9	0.3±0.51
	Ethyl acetate	78.3±0.40	21.5±0.5	0.19±0.33

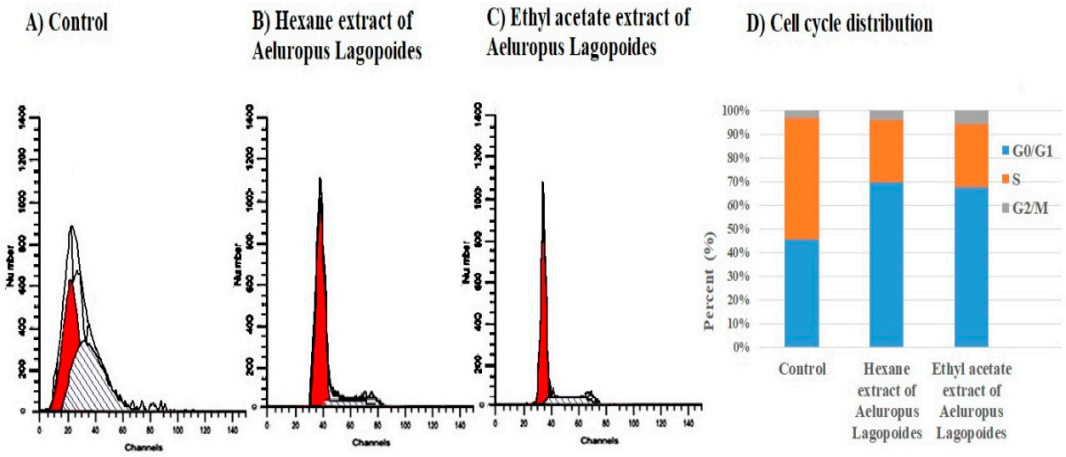

Figure 3. Effect of A.lagopoides extracts on the cell cycle distribution of HCT-116 cells. Cells were exposed to Hexane extract (B); Ethyl acetate extract (C) for 24 h and compared with cell control (A). Cell cycle distribution was determined using DNA cytometry analysis and different cell phases were plotted (D) as a percent of total events (n = 3).

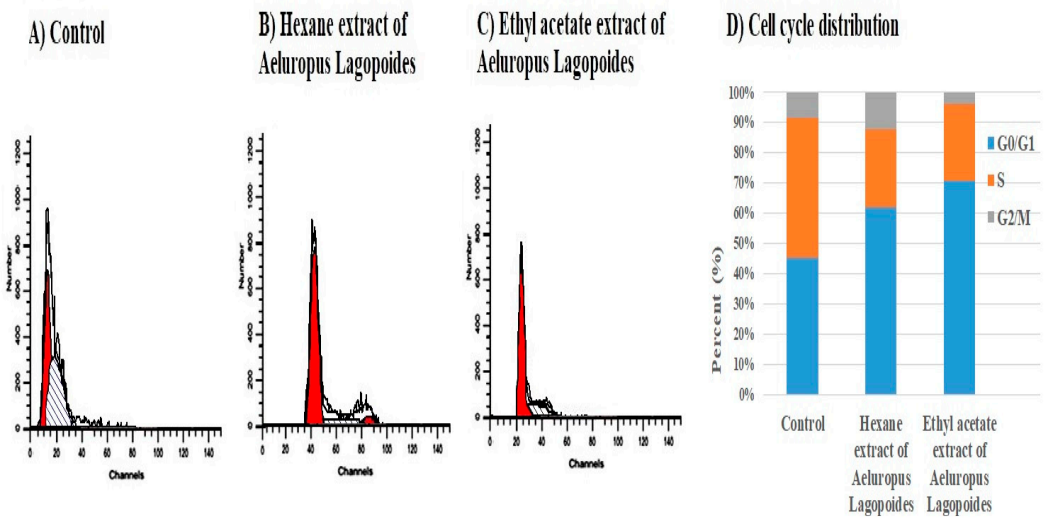

Figure 4. Effect of of *A.lagopoides* extracts on the cell cycle distribution of MCF-7 cells. Cells were exposed to Hexane extract (B); Ethyl acetate (C) for 24 h and compared with cell control (A). Cell cycle distribution was determined using DNA cytometry analysis and different cell phases were plotted (C) as a percent of total events (n = 3).

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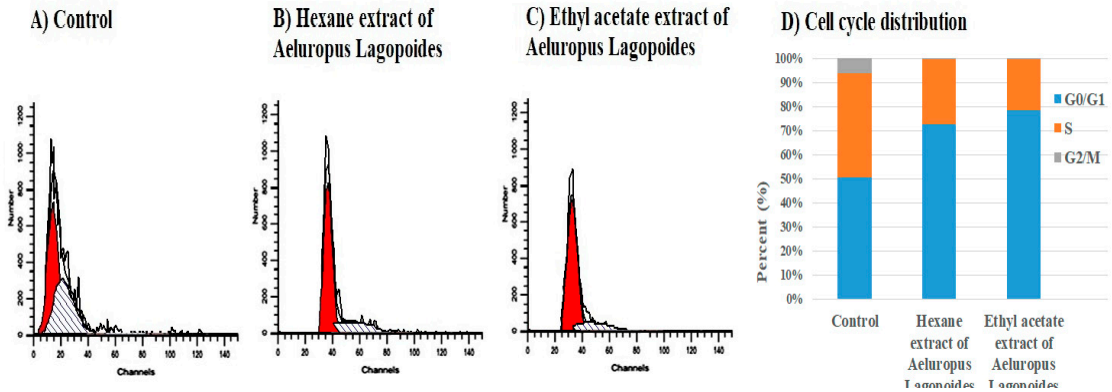

Figure 5. Effect of *A.lagopoides* extracts on the cell cycle distribution of HepG2 cells. Cells were exposed to Hexane extract (B); Ethyl acetate (C) for 24 h and compared with cell control (A). Cell cycle distribution was determined using DNA cytometry analysis and different cell phases were plotted (C) as a percent of total events (n = 3).

2.4. Qualitative phytochemical analysis

The quantitative phytochemical analysis of *A.lagopoides* leaves hexane, ethyl acetate, and butanol extracts show the presence of a different chemical compound (Table 3).

Table 3. Qualitative phytochemical analysis of A.lagopoides leaves different crude extract

Compound	**Test**	**The crude extract of *Aeluropus lagopoides***		
		Hexane	**Ethyl acetate**	**n-butanol**
Alkaloid	***Mayer's test***	-	-	-
	Wagner's test	-	-	+
Flavonoids	***Alkaline reagent***	-	+	+
Phenolic	***Ferric chloride***	-	-	+
	Lead acetate	-	-	+
Tannins	***Ferric chloride***	-	-	+
Terpenoids	***Liebermann-Burchard test***	-	-	+
	Salkowski test	+	+	+
Phyto steroid	***Liebermann-Burchard test***	+(steroids nucleus)	+ (steroids nucleus)	-
	Salkowski test	+ (steroid ring)	+ (steroid ring)	+ (steroid ring)
Cardio glycoside	***Keller-Killiani test***	+	-	-
Saponin	***foam test***	+	-	+
Fixed oils and fats	***stain test***	+	+	-

2.5. GC\MS

GC\MS profiling for the different crude extracts indicate the presence of different molecules belonging to a different chemical group (Table 4)

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Table 4. GC/MS profiling for the chemical composition of different crude extracts from the leaves of A.lagopoides

Hexane extract					
No	**Time**	**Compound name**	**REF**	**M.W**	**Formula**
1	39.434	2-Methoxy-4-vinylphenol	94.2%	150	C9H10O2
2	45.160	2-Propenoic acid, 3-phenyl-trans-cinnamic acid	99.2%	148	C9H8O2
3	52.387	2-Pentadecanone,6,10,14-trimethyl	90.4%	268	C18H36O
4	53.298	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	94.4%	276	C17H24O3
5	53.779	Eicosanoic acid	91.5%	312	C20H40O2
6	54.831	Tetratriacontane	96.8%	618	C44H90
7	55.372	Phytol	97.8%	297	C20H40O
8	56.320	Z-8-methyl-9-tetradecaene-1-ol acetate	83.7%	268	C17H32O2
Ethyl acetate extract					
1	50.413	2,2'-Biphenylene dicinnamate	87.1%	446	C30H22O4
2	52.396	2-Pentadeccanone, 6,10,14-trimehyl-	89.9%	268	C18H36O
3	52.300	3,7,11,5-Tetramethyl-2-hexadecen-1-ol	95.5%	296	C20H40O
4	53.300	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	88.6%	276	C17H24O3
5	53.781	Eicosanoic acid	85.8%	312	C20H40O2
6	54.084	Hexadecanoic acid, ethyl ester	92.3%	284	C18H36O2
7	54.818	Tritetracontane	93.2%	604	C43H88
8	55.366	Phytol	97.8%	296	C20H40O
9	55.899	9, 12, 15-Octadecatrienomic acid, ethyl ester (z,z,z)	92.7%	309	C20H34O2
10	56.321	Propenoic acid, 3-phenyl-, 2-(3-nitrophenyl)-2-oxoethyl ester	80%	311	C17H13O5N
n-butanol extract					
1	21.998	Butanoic acid, butyl ester	96.4%	144	C8H16O2
2	52.345	1-Hexyl-2-nitrocyclohexane	85.7%	213	C12H23O2N
3	54.196	Vitamin E	95.9	430	C29H50O2
4	54.825	Tetratriacontane	95.1%	618	C44H90
5	55.247	.Alpha.-tocopherol quinone	69.7%	446	C29H50O3
6	57.069	Eicosane, 9-octyl-	82.9%	394	C28H58
7	57.839	1,3-Benzene dicarboxylic acid, bis(2-ethylhexyl) ester	84%	390	C24H38O4

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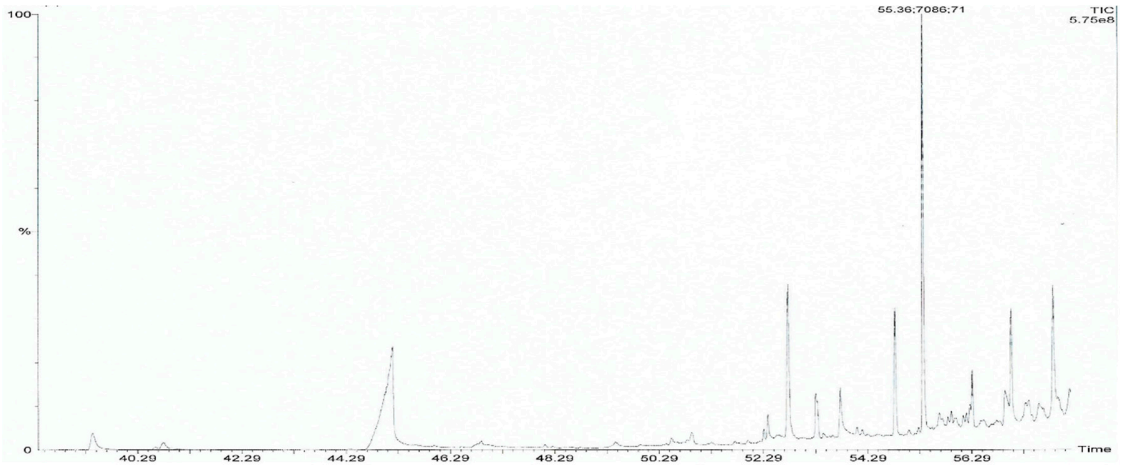

Figure 6. Typical gas chromatograph of the chemical constituents of hexane extract from the leaves of *A.lagopoides*

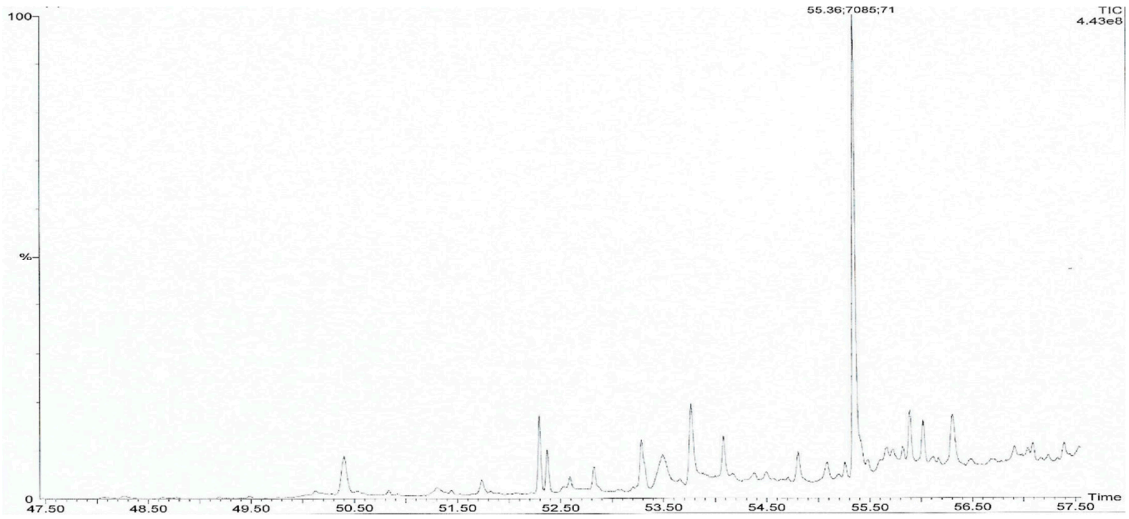

Figure 7. Typical gas chromatograph of the chemical constituents of ethyl acetate extract from leaves of *A.lagopoides*

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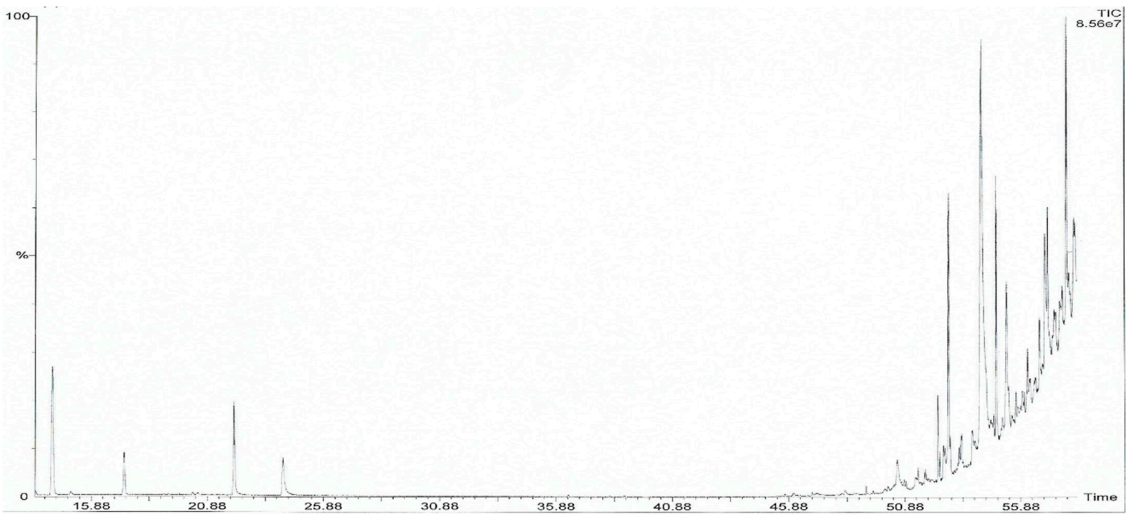

Figure 8. Typical gas chromatograph of chemical constituents of n-butanol extract from leaves of *A.lagopoides*

3. Discussion

Control of biological processes within the cell such as cell division or transcription-translation of protein or apoptosis requires activation of many biological pathways performed by a complicated enzyme system, which requires the presence of many cofactors and coenzymes[21]. For this reason, predicting the activation of pathways via pure compound may not be accurate; hence the use of the crude extract may enhance synergistically impact between secondary metabolites. Consequently, crude extract had been chosen as a bio-pathways inducer in the current study to induce apoptosis which considered as the most likely pathway for the cancer therapy strategy [22]. For that, our study focused on the isolation of active ingredients and secondary metabolites from *Aeluropus lagopoides* plant leaves. To determine the cytotoxic, apoptotic and antiproliferative active chemical groups the mechanism of action had investigated from different points of view. Consequently, extracts analyzed and connected to their observed effect. Our results showed that hexane and ethyl acetate extracts have the highest cytotoxic activity compared to polar extracts (Table1) indicating that non-polar components of *A. lagopoides* tend to activate one or more antiproliferative pathways. However, this is directly related to the phase in which the antiproliferative achieved because each phase has a different definition for which mechanism that has been activated by excluding inactive mechanisms. Results revealed that hexane and ethyl acetate extract at equipotent concentrations arrested the cell cycle in G0-G1 phase with high value almost 65-78 % in all cancer cell lines. That indicates that all extracts ingredients are not clastogenic agents because cell cycle will arrest at G2 after exposure to clastogenic agents to repair DNA damages[23]. Depending on that, cells arrested in the cell cycle should choose to activate one of the suggested pathways like apoptosis, necrosis or differentiation. Whereas cancer cells have lost their ability to return to differentiation, thus they must go to apoptosis or necrosis. Hence, to suggest the mechanism of action of different extracts components, the cellular morphology changes had been screened by fluorescent dyes mix (Ethidium Bromide: Acridine Orange) to distinguish apoptotic symptoms from necrotic signs (Figure2). In this point of view, both extracts look to induce apoptotic that may activate via cyclin E or D, R point or one of the chick points that

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present in G0-G1 phase[24, 25]. On the other hand, the qualitative chemical analysis (Table 3) indicates that both hexane and ethyl acetate extracts contain hydrophobic bioactive compounds such as phytosteroid, terpenoids, cardioglycoside and fixed oil, which had confirmed their antiproliferative activity in previous studies[8, 26]. In the light of affording mentioned and observed results; the suggested mechanism of action of hydrophobic molecules extracted from *A. lagopoides* is related to their capability to flow through the phospholipid bilayer membrane of the cell easily, this changes the internal cellular homeostasis, which leads to arrest cell division. However, the interesting point here is all different pathways will induce –in the end- apoptosis which is the preferred mechanism to eliminate cancer cells.

4. Materials and Methods

4.1. Cell lines, chemicals, and biochemicals

Ethanol, methanol and SRB stain were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals obtained from Gibco / Life Technologies Co, (Carlsbad, CA, USA) unless mentioned otherwise. While cell culture vessels usually replenished from Nunc Co. (Roskilde, Denmark). Human colon (HCT 116), Human liver (HepG-2) and Human breast (MCF-7) cancer cell lines acquired from Vacsera (Giza, Egypt). Cells routinely maintained in RPMI 1640 cell culture media supplemented with 1 mM sodium pyruvate, 2 mM L. glutamine, 100 units/ml penicillin-streptomycin and 10% fetal bovine serum. Subsequently, they incubated in a humidified, 5% CO2 at 37 ºC.

4.2. Extraction and crude extracts preparation:

The fresh leaves of Aeluropus lagopoides collected from Al- Hridha beach at (17.7644488,41.9261616), Asser region, Saudi Arabia on 17 July 2017. For the crude extract preparation, 100 grams of fresh leaves were washed with distilled water and ground via grinder with 500 ml of aqueous ethanol 80%. Then immersed in 1L of aqueous ethanol 80% and left for seven days with stirring at room temperature (18-24^{0}C). The ethanol extract was filtered using filter paper and concentrated to dryness under reduced pressure using a rotary evaporator at 37^{0}c (Ikia -Germany). The concentrated crude extract weight 10g, and the extraction yield (10%). The crude extract was then reconstituted in 400 ml of distilled water and extracted with different solvents according to the polarity (hexane, ethyl acetate, and n-butanol) non-polar, semipolar and polar respectively using the liquid-liquid extraction method. After that, the solvent phase was separated and reevaporated using rotary evaporator. Then, left for complete evaporation of the solvent at room temperature (20-26^{0}C) for five days. Hexane crud extract weight 1.3721, and the extraction yield (10% from the ethanol yield), ethyl acetate 0.9816, and the extraction yield (9.8% from the ethanol yield), and n-butanol 3.723 g, and the extraction yield (30% of the ethanol yield) respectively. Further, 0.01 gram of each crude extracted diluted in 1 ml of (DMSO) dimethyl sulfoxide as a stock solution for bioactivity assay. At 4^{0}C the crude extracts were stored for further studies.

4.3. Cytotoxicity activity of *A.lagopoides* crude extracts

The cytotoxicity and anticancer activities of prepared *A.lagopoides* leaves crude extracts were tested against Human breast (MCF-7), Human colon (HCT 116), and Human liver (HepG-2) cancer cell lines using SulphoRhodamine-B (SRB) assay described by Skehan *et al.* [27]. Different cancer cell lines exposed to a range of concentrations (0.01 to 100 µg/ml) of hexane, ethyl acetate, and n-butanol crude extracts then incubated in 5% CO_2 humidified incubator at 37 °C for 72 h. Doxorubicin used as a positive control. Treated cells were fixed with TCA (10%) for 1h at 4°C. Subsequently, to remove TCA, cells were washed with water many times, and then 0.4% SRB

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solution was used to stain cells in a dark place for 10 min. Stained cells washed with 1% glacial acetic acid. Finally, to dissolve SRB-stained cells, Tris–HCl was used. After drying overnight, the color intensity of remained cells was measured at 540nm by Elisa.

4.4. Detection activity signals of apoptosis.

For apoptotic bodies detection, treated cells were washed using PBS washing buffer twice and then collected using 0.25% trypsin–EDTA. After that, the cells were stained using Ethidium bromide (EtB) and Acridine Orange (AO) 1:1 concentration cells were transferred to slide. Stained apoptotic bodies were detected and photographed under a Nikon Fluorescent microscope (Japan).

4.5. Cell cycle distribution using DNA flow-cytometry

Adherent cancer cells exposed to IC_{50} equivalent concentrations of extract solutions for 48 h. Cells were then suspended using 0.25% trypsin-EDTA, washed with ice-cold PBS, and re-suspended in 0.5 ml of PBS. Cells were then fixed in 70% ice-cold ethanol at 4 ℃ for 1 h before transferred to -20 ℃ until required for analysis. Upon analysis, fixed cells were washed with ice-cold PBS and re-suspended in 1 ml of PBS containing 50 µg/ml RNase A and 10 µg/ml propidium iodide (PI). After 20 min incubation at 37 ℃, cells were analyzed for DNA contents by FACSVantageTM (Becton Dickinson Immunocytometry Systems, San Jose, CA). For each sample, 10,000 events were acquired. Cell cycle distribution was calculated using CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA)[28].

4.6. Statistical Analysis

The IC_{50} calculation was performed using Sigma Plot version 12.0.

4.7. Qualitative phytochemical analysis of the different crude extract

The crude hexane, ethyl acetate, and butanol of *A.lagopoides* leaves investigated for the qualitative presence of alkaloids, flavonoids, phenols, tannins, terpenoids, Phyto steroid, cardio glycoside, saponin, and fats. The presence represented by (+) and the absence with (-) as described in [29, 30].

4.8. GC\MS for plant extract

The different crude extracts from the leaves of *A.lagopoides* were analyzed using a Perkin Elmer GC–MS (Model Perkin Elmer Clarus 500, USA) equipped with a fused silica capillary column (30 m × 0.25 i.d., film thickness 0.25 µm) coupled with a Perkin Elmer Clarus 600C MS as described in [31]. Identification of phytochemical compound conducted using the database of National Institute Standard and Technology (NIST) library.

5. Recommendation

This study considered as the first report investigates the importance *Aeluropus lagopoides* plant leaves chemical constituents in the view of the impact on cancer cells and thus opened the door widely by reducing the effort and time required for searching of the most effective and influential compounds found in the proposed chemical groups. On the other hand, it concluded that hydrophobic compounds could change the internal homeostasis of cells, thereby stimulating the cell division arrest which encouraged us to suggest further research on the molecules that regulate current phases like cyclin E and D in addition to p53.

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