International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Antitumor Activity of Chenopodium album flavonoids in HEPG2 Cell

Khalil A. Elhalfawy[1], El-fiky B. A.[2], Amna Dahy Radwan[3]

[1, 3]Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute,Sadat City University, Egypt

[2]Animal Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Egypt

Abstract: ***Chenopodium album (family: Chenopodiaceae) is an annual widely grown in Asia, Africa, Europe and North America. The leaves of C. album which has various pharmacological Properties; antifungal, anti-inflammatory, anti-allergic, antiseptic, Hepatoprotective activity and Immunomodulation activity. The flavonoids contained in C. album leaves were extracted, the results showed that anti tumor activity of Chenopodium album flavonoids (rutine, rutin hydrate and quercetin).***

Keywords: *Chenopodium album,* Chenopodiaceae, Flavonoids, Rutin, rutin hydrate and Quercetin.

1. Introduction

Chenopodium album is a wild neglected herb which has various pharmacological properties; antifungal, anti-inflammatory, anti-allergic, antiseptic, Hepatoprotective activity and Immunomodulation activity **(Cutillo et al., 2006).** The phytochemicals like flavonoids, isoflavonoid, polyphenol etc., has a great interest for their potential role in the maintenance of human health in particular, significant cancer risk reduction **(Penarrieta, et al., 2008).** Flavonoids, a subclass of polyphenols, are a group of phytochemicals that are among the most various activities such as antioxidants, anti inflammatory, anti cancer etc.

The aim of this paper was to evaluate the antitumor properties of Chenopodium album.

2. Materials and Methods

2.1 Chenopodium album Collection

Chenopodium album plant was collected from May - July 2016, in Menoufia governorates (Egypt) .The plant species were identified at Agriculture Research's Center (Giza, Egypt) as well as a comparison of standard specimen.

2.2 Processing of Leaves for Extraction

Green fresh leaves were rinsed in shaking water bath containing sterilized water, three periods each about 30 min. to remove dust. Then the leaves were spread evenly and dried in shadow for 3 to 4 days and the dried leaves was ground finely using a mixer grinder and stored in light resistance container for use.

2.3 Extraction

30g of leaves powder was extracted using 70 % methanol then ,the crude extract was transferred into a separatory funnel and finally extracted by different solvents with increasing polarities followed the sequence of petroleum ether, ethyl acetate , butanol and residual methanol fractions respectively. The extraction procedures was repeated in triplicate and.extracts was filtrated to reach concentrated dryness and condensed under pressure (150 - 250 ppm) using Buchner Rotavapor (R-114, Germany) at 60°C. The Petroleum ether, ethyl acetate, butanol and methanol crude extracts (1 g) was completely dissolved in 100 mL of its own solvents. It was prepared the stock solution. The obtained stock solution was used for phytochemical screening.

2.4. Phytochemical Screening

2.4.1. Determination of Flavonoids

The stock solution (1 mL) was taken in a test tube and added few drop of dilute NaOH solution. An intense yellow colour was appeared in the test tube. It became colourless when on addition of a few drop of dilute acid that indicated the presence of flavonoids. Total flavonoids were evaluated according to the method described by **Lamaison and carret (1990)**. 4 subfractions from methanolic extraction; A1: ether extraction, A2: ethyl acetate extraction, A3: methanolic extraction, A4: butanol extraction.

2.4.2. Preparation of Lyophilized Extracts

0.1 g of the extract was weighed and dissolved either in ethanol / water based on the solubility and made up to 1ml in Minimum essential medium (Sigma Aldrich) and made the final stock solution as 1mg/1ml by adjusting the pH to 7.0 with HCl or NaOH. The dissolved extract was filtered using 0.22 µm syringe filter.

2.4.3. High performance Liquid Chromatography (HPLC) analysis

Determination of polyphenols, total polyphenol content was determined by the Folin-Ciocalteau method as described by **Matthaus (2002).** HPLC analysis of the plant extract sampls A3 and A4 the hydrolyzed samples for HPLC were prepared according to the method of **Hertog *et al.* (1993).** A stock standard solution (100µg/ml) of each phenolic compound was prepared in methanol by weighing out approximately 0.005 g of the analyte into 50 ml volumetric flask. The mixed standard solution was prepared by dilution the mixed stock standard solutions in methanol to give a concentration of 20 µg/ml for each polyphenols all standard solutions were stored in the dark at 5°C and were stable for at least three months.

0.5 g of the dried plant material was taken in a flask with BHA (2g/L) and sonicated for 5 minutes in 40 ml of 65% methanol. 10 ml of 6 N HCl was added and nitrogen purged for 60 seconds. The mixture was then heated on a water bath at 90oC for 2 hr. stirring constantly then cooled, filtered and sonicated for 5 minutes and then used for HPLC analysis. The components were then determined by HPLC according to [7] **Siddhuraju and Becker (2003).** In brief, a gradient elution was employed for flavonoids other than catechins with a mobile phase consisting of 50 mM H3PO4, pH 2.5 (solution A), and acetonitrile (solution B) which is as follows: isocratic elution 95% A/5% B, 0-5 min; linear gradient from 95% A/5% B to 50% A/50% B, 5-55 min; isocratic elution 50% A/50% B, 55-65 min; linear gradient from 50% A/50% B to 95% A/5% B, 65-67 min; post-time 6 min before next injection. The system, WATERS 2487 is equipped with a C-18 column (Nova-Pak C18, 3.9 × 150 mm). 280 nm, 320 nm and 380 nm. Wave lengths were selected for the detection. The flow rate of the mobile phase was 0.8 mL/min, and the injection volume was 20 µl. The peaks were identified in comparison with authentic standards.

2.4.4 Maintenance of HepG2 cells

HepG2 cells (HepG2, ATCC No. 84113001) are cultured in MEM Medium supplemented with 10% heat inactivated fetal calf serum (FCS) (Sigma Aldrich, USA), The culture flasks were incubated in 5% CO_2 incubator with 95% humidity at 37°C.

2.4.5 Trypsinization of Cells

Confluent monolayer of HepG2 in 75-cm2 flasks were examined under inverted microscope to examine; viability of cells and being free of contamination. The old medium was aspirated off and 8 ml trypsin (1:250) pre-warmed at 37°C, added to each flask then incubated at 37°C for 10 min. Cell culture was examined via inverted microscope; if cells were detached; the action of trypsin was deactivated by the addition of equal amounts of growth medium supplemented with 10% FCS. Cell suspension was collected and cell viability was checked using 0.4% Trypan blue vital stain, then cell counting were made using THOMA haemocytometer.

2.4.6 Trypan blue exclusion

0.1 ml Trypan blue dye (0.4% in water) was mixed with cell suspension, 15 min incubation period. At the end of incubation period, the petri plates were carefully taken out and 1.0% Sodium dodecyal sulfate was added to each petri plates by pipetting up and down several times unless the contents get homogenized and the number of viable cells (not stained) counted using a haemocytometer. Viability was expressed as a percentage of control number of cells excluding Trypan blue dye. Although numbers of Trypan blue dye staining cells were not counted.

2.4.7 Cytotoxicity Study and LD50 determination.

Harvested monolayer cells of HepG2 resuspended at (1×106) Cell / ml MEM supplemented with 10% FCS. 10 ml of resuspended cells were added to sterile petri dish, pipette 100 µl cell suspension / well in 96 wells microtiter plats.Microtiter platsare incubated at 37ºC for 24 h. to reach complete monolayer, growth medium was aspirated off and 100µl growth medium supplemented with 2% FCS / well are added to each well of microtiter plates. 100µl of each tested sample A1, A2, A3 andA4 (0.1gm/100µl)was added to wells no.(1-8) as her sampel had 2well,well no. 9-10 had flavonoids standard and wells no.(11-12) no samples used as cell control , Serial dilution of tested samples are from A-H row, Titer plates are incubated at 37ºC for 24 h, to investigate LD50 dose, cytopathic effect .Cell suspension was collected and cell viability was checked using 0.4% trypan blue vital stain, then cell counting were made Using THOMA haemocytometer **(Bahgat. ELfiky *et al*., 2011)**. Sampels of leaves extraction A1 to A4 were added to well no. (1-8), as her sampel had 2well, well no (9-10) had flavonoids standard and well no (11-12) had no samples uesed as cell control.

2.4.8 Antitumor Activity

Harvested monolayer cells of HEPG2 resuspended at 3.5 x 10^5 cells / ml MEM supplemented with 10% FCS, and seeded in plate 2x3 wells cell culture plates, incubated at 37ºC in 5% CO_2 for 24 hr to reach complete monolayer. Sub lethal dose of tested extracts A1, A2, A3 and A4 were added, flavonoids standard. (Gallic acid, catechins, vanillin acid, Caffeic acid, rutin hydrate, rutin, cinnamic acid, quercetin and Kaempferol) (Sigma Aldrich, USA), as anti cancer natural drug added and wells had no treated samples were used as control. Cells plate was incubate at 37ºC in 5% CO_2 incubator for 24hr.

2.4.9 Cell proliferation study

Each well was accessed by observation under microscope after staining the cells with a 0.5% trypan blue vital stain. Then cell counting was made using THOMA haemocytometer.

2.4.10 Apoptosis Study

Apoptotic cells were identified by AnnexinVFITC Apoptosis Detection Kits (Beyotime Institute of Biotechnology, China). Cells undergoing apoptosis were identified by binding of Annexin V protein to exposed phosphatidylserine (PS) residues at cell surface **(Jiang *et al*., 2008)**. After exposure to Dryocrassin ABBA (0, 25, 50 and 75 µg/mL) for 48 h, cells were collected, washed twice with PBS, gently resuspended in annexinV-binding buffer, and incubated with annexinV-FITC and PI in dark for 10 min. FITC fluorescence was analyzed by flowCytometry (BD FACSCalibur).

2.4.11 Quantitative RT-PCR of BCL2 and P53 gene exepression:

HepG2 cells were seeded in 2x3 wells plate at a density of at 3.5 x 10^5 cells/well in 3 ml medium and treated with sub lethal dose of samples and flavonoid standard ,incubated for 24 hr.

Total RNA was isolated with (RNA extraction Qiagen kit) , and oligo (dT)-primed RNA (1 µg) was reverse-transcribed using iScriptTM One-Step RT-PCR Kit with SYBR® Green.

The obtained cDNA was used to determine the mRNA expression levels of Bcl-2 and P53 by PCR analysis. GAPDH was used as an internal control. All primers were

synthesized by Sangon Biotech (Shanghai, China).The primer sequences used for the amplification of Bcl-2, P53and GAPDH were as follows:

Bcl-2 forward,5′-TCCGCATCAGGAAGGCTAGA-3′
reverse,5′- AGGACCAGGCCTCCAAGCT -3′
P53 forward,5′-TGCAGCTGTGGGTTGATTCC-3′
reverse,5′- AAACACGCACCTCAAAGCTGTTC -3′
GADPH forward, 5′- TGCACCACCAACTGCTTAGC -3′
reverse, 5′- GGCATGGACTGTGGTCATGAG-3′

The reaction included 1 µl cDNA, 2 µl 10X Taq Buffer, 1.2 µl 25 mM MgCl2, 0.4 µl 10 mM dNTP, 0.8 µl 1U/µL Taq polymerase, 1 µl each primer and DEPC water up to 20 µl. The PCR conditions were as follows: 95°C for 3 min and 30 cycles at 95°C for 30 sec, 56°C for 40 sec and 72°C for 40 sec. **(Bustin, 2005 and kubista *et al*., 2006).**

3. Results

3.1 Phytochemical Screening

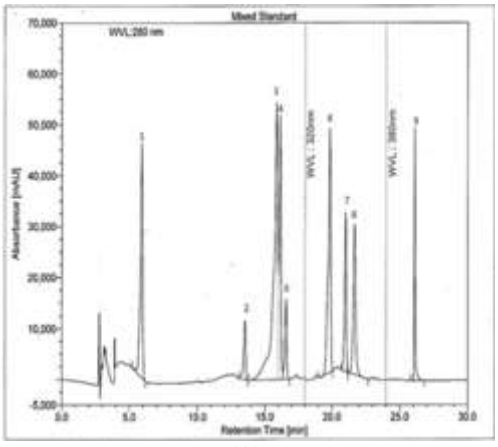
Phytochemical screening of butanol and methanol extracts of Chenopodium album leaves showed high expression of flavonoids in methanol and butanol and no expression in petroleum ether and ethyl acetate (Table 1).

Table 1: Phytochemical Screening of Different *Chenopodium album* Extracts

Constituents	Methanol	Butanol
Flavonoids	+++	+++

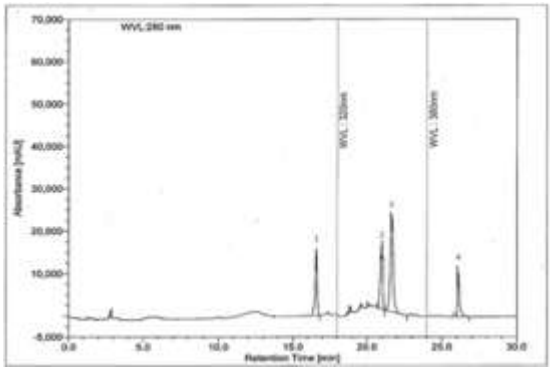
3.2 High Performance Liquid Chromatography (HPLC) analysis

Methanol and butanol fractions extract of Chenopodium album active components were analyzed by HPLC. HPLC chromatogram of methanol fraction leaves extract detected rutin hydrate, cinnamic acid, quercetin and Kaempferol. HPLC chromatogram of butanol fraction leaves extract. Detected rutin hydrate, rutin and quercetin.



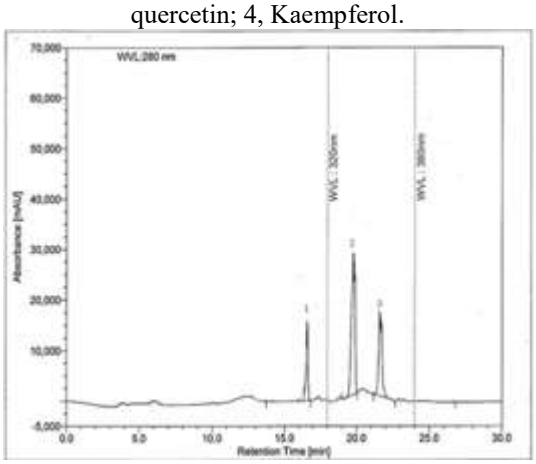
(A)

Figure 1: HPLC chromatogram of a standard mixture of compounds. Peaks: 1, gallic acid; 2, catechin; 3, vanillic acid; 4, Caffeic acid; 5, rutin hydrate; 6, rutin; 7, cinnamic acid; 8,quercetin; 9,Kaempferol .



(B)

Figure 2: HPLC chromatogram of methanol fraction leaves extract. Peaks: 1, rutin hydrate; 2, cinnamic acid; 3, quercetin; 4, Kaempferol.



(C)

Figure 3: HPLC chromatogram of butanol fraction leaves extract. Peaks: 1, rutin hydrate; 2, rutin 3, quercetin.

3.2.1. Cytotoxicity Study and LD50 determination

The cytopathic effect of extracts increased from sample (A1, A2) on cell lines compared to control. LD50 exhibited of sample (A1, A2) was 0.1(0.01 µ/ml) of the acute dose. Flavonoids standard LD50 was 0.001 of acute dose (0.0001 µ/ml).

3.3. Antitumor Activity

3.3.1. Cell proliferation study

3.3.1.1. Cell viability detected by 0.4% Trypan blue vital stain:

Cell viability decreased from A1and A2 compared to flavonoids standard control (Gallicacid, catechin, vanillic acid, Caffeic acid, rutin hydrate, rutin, cinnamic acid, quercetin and Kaempferol).

3.3.1.2. Cell counting by THOMA haemocytometer detected:

Table 2: A1: methanolic extract, A2: butanol extract.

Sample	Cells count
Control	(3.5×10^5) cells / ml.
Standard	(1.0×10^3) cells / ml
A1 (methanolic extract)	1.5×10^3) cells / ml
A2 (butanol extract)	(1.0×10^3) cells / ml

Counting of viable cells decrease from A1 and A2 respectively. Anti-tumor activity decreased from sample A2

and A1 respectively compared to flavonoids standard as natural drug anticancer control.

3.3.1.3. Morphological observation

The cells were observed under inverted microscope after 24 hr. to record the changes in morphology the induction of apoptosis resulted in cell shrinkage, leading to the formation of apoptotic bodies, cells got detached from the substratum shown by flagging of the cells and clump formation.

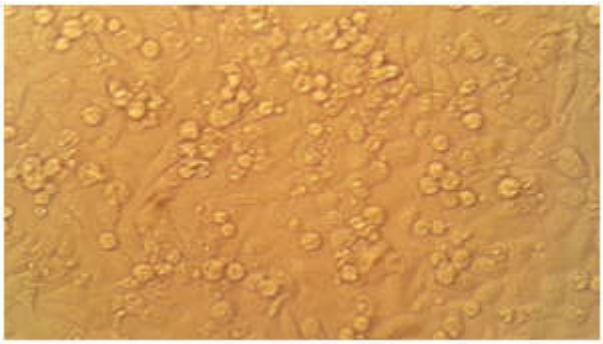


Figure 4: HepG2 Control showed complete monolayer, attachment, viable and cells proliferation appearing. (X:200)

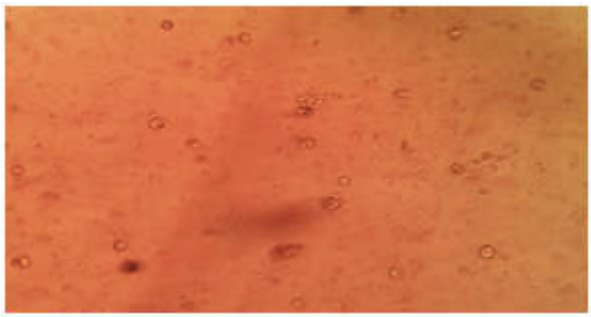


Figure 5: Flavonoids Standard showed cells detached, shrinkage and apoptosis (X:200)

Figure 6: A1 (methanol extract) sample showed cell shrinkage and apoptotic cells. (X:200)

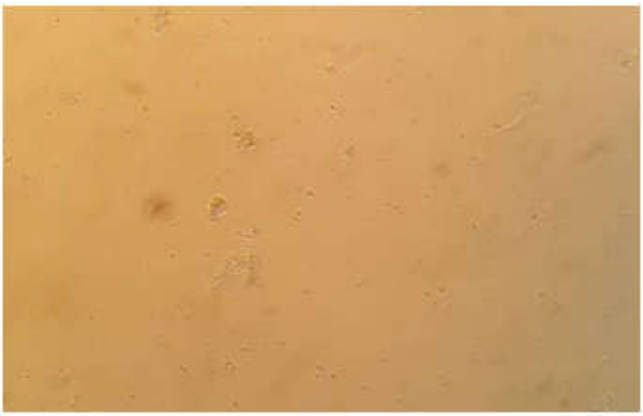


Figure 7: A2 (butanol extract) sample showed completely apoptotic cells. (X:200)

3.3.2. Apoptosis by Annexin V Assay

Table 3: Annexin V assay for Apoptosis:

Sample ID	Cell Line	Sub lethal dose µg/ml	G0-G1 %	% S	G2-M %	Apoptosis %	Comment
A1 (methanolic extract)	HepG2	0.01	62.16	23.42	0.81	13.61	Per-G1 apoptosis
A2 (butanol extract)	HepG2	0.01	64.41	19.21	0.02	16.36	Cell cycle arrest & G1 phase
Flavonoids Standard	HepG2	0.0001	69.23	12.39	0.01	18.37	Cell cycle arrest & G1 phase

Table 3: Apoptosis decreased from A2 and A1 respectively compared to flavonoids standard.

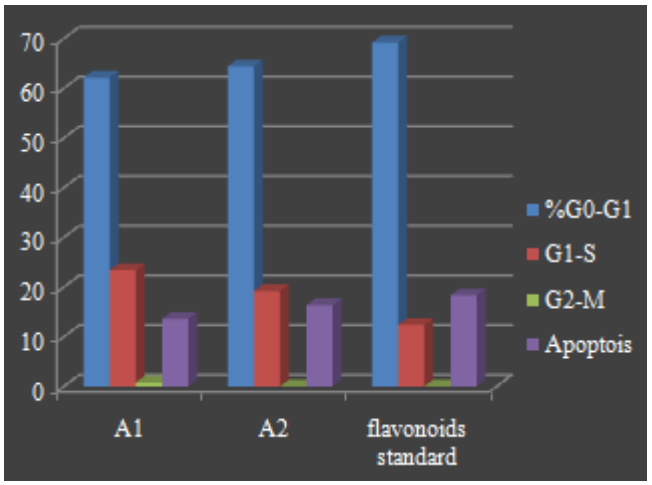


Figure 8: Apoptosis decreased from A2 and A1 respectively compared to flavonoids standard.

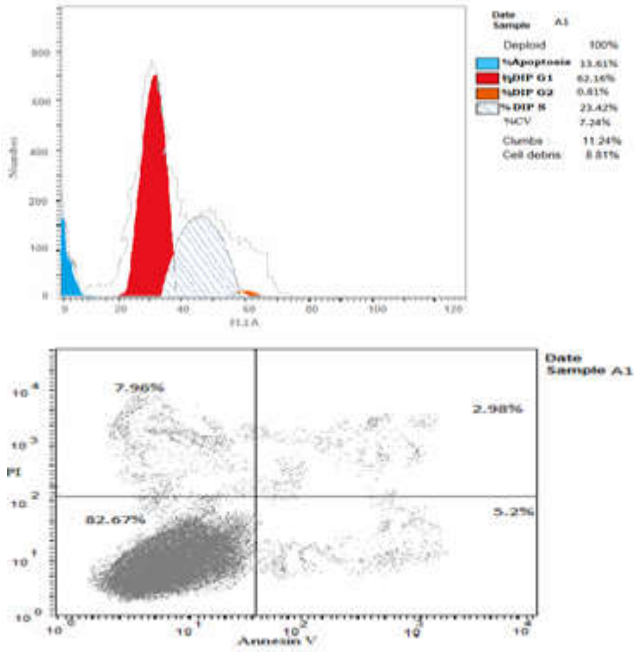


Figure 9: A1 apoptosis

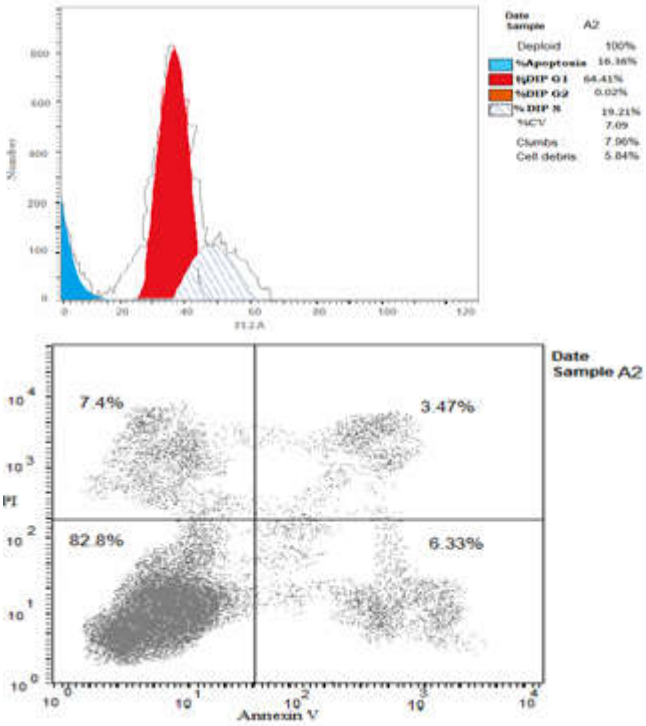
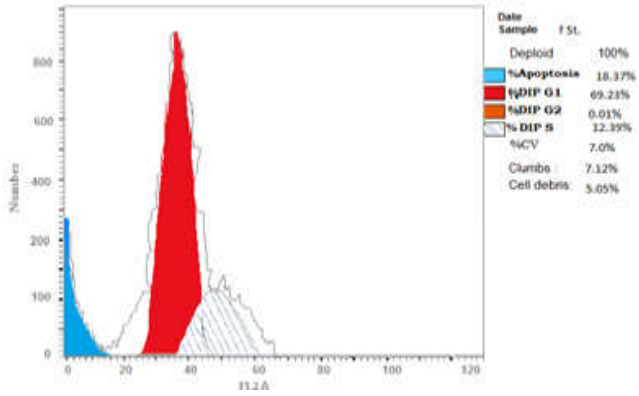


Figure 10: A2 apoptosis



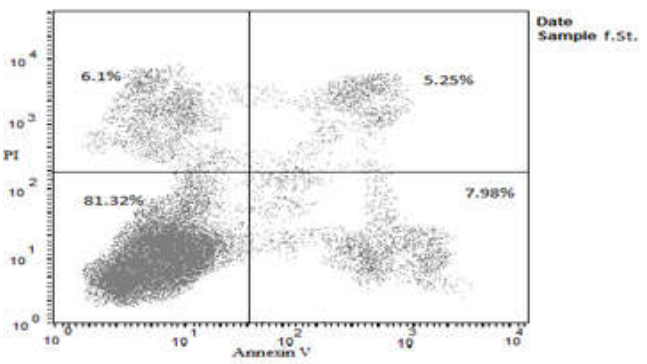


Figure 11: Flavonoids standard

3.3.3. Quantitative RT-PCR of BCL2 and P53 gene expression:

Table 4:

Sample	Cell line	Sub lethal dose	BCL2 IU/ml		P53 IU/ml	
Control	HepG2	----	CT	Conc.	CT	Conc.
A1 (methanolic extract)	HepG2	0.01	24.58	195,489	28.72	11,158
A2 (butanol extract)	HepG2	0.01	27.13	91,367	26.28	115,820
Flavonoids Standard	HepG2	0.0001	31.28	43.452	25.20	180,225

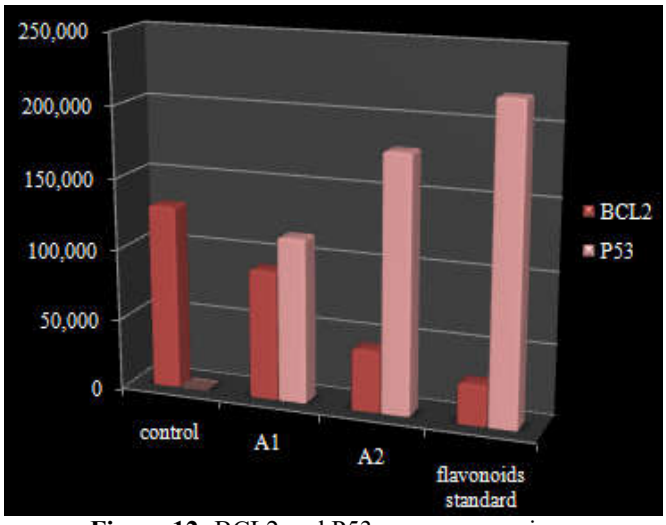


Figure 12: BCL2 and P53 gene exepression

Data showed detected level of P53gene expression decreased from A2, A1 Data level of BCL2 gene expression decreased from A1 and A2 respectively, Compared to flavonoids standards.

4. Discussion

Screening of phytochemicals for anticancer activity is extremely important today's context of a variety of tumors getting resistant to conventional chemical anticancer drugs (Avni et al., 2008).

The present study aims to proliferation inhibition and apoptosis induction in cell line.

For the confirmation of anti-tumor effect 3 methods were used in this study morphological study, apoptosis study and detection of gene expression of BCL2 and P53.

Morphological study allows us to detect morphological changes in HepG2 monolayer cells after treatment; cell shrinkage, leading to the formation of apoptotic bodies got detached from the growth surface shown cells flagging and clump formation.
High level apoptosis detected from Chenopodium album leaves flavonoids component (rutin hydrate, cinnamic acid, quercetin, and Kaempferol)

The tumor suppressor gene p53 high expressed by Chenopodium album leaves flavonoids component (rutin hydrate, cinnamic acid, quercetin, and Kaempferol)

Anti-apoptotic gene Bcl-2 low expressed by Chenopodium album leaves flavonoids component (rutin hydrate, cinnamic acid, quercetin, and Kaempferol)

C. album leaves Flavonoids component (rutin hydrate, cinnamic acid, quercetin, and Kaempferol) have anti-tumor activity.
Data obtained from the present study suggest that detailed studies in-vivo.

5. Conclusions

The extracts of Chenopodium album contained significant amounts of flavonoids, which play a major regulatory role as antitumor.

The findings of this study revealed that the Chenopodium album flavonoids could be used as a readily accessible source of natural antitumor. Further research to purification and Identification of active components is required to widen the knowledge about the antitumor mechanisms and possible future applications.

References

[1] Avni GD, Ghulam NQ, Ramesh KG, Mahmoud E-T, Jaswant S, et al. Medicinal Plants and Cancer Chemoprevention. Current Drug Metabolism, (2008); 9(7): 581-591.
[2] Bahgat A. EL-Fiky.,ShadenMuawia H., TarekA. Salem, AmalA. AbdEl-Aziz1 and M. Shokair(2011).Influence of anticancer drugs on DNA methylation in the liver of female mice. American Journal of Molecular Biology,doi:10.4236/ajmb.2011.12008.
[3] Bustin SA, Benes V, Nolan T. and Pfaffl MW.(2005): Quantitative real-time RT-PCR a perspective. Journal of molecular endocrinology; 34: 597-601 .
[4] Cutillo F, DellaGreca M, Gionti M, Previtera L. and Zarrelli, A.(2006): Phenols and lignans from Chenopodium album. Phytochem Anal. 1 7(5): 344-349.
[5] Hertog M L , Hollman P H. and van de Putte B. (1993): Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. Journal of Agricultural and Food Chemistry 41: 1242-1246.
[6] Jiang JB, Grieb A,Thyagarajan, and D.sliva.(2008).Ganoderic acids suppress growth and invasive behavior of breast cancer cells by Modulating AP-1 and NF-KappaB signaling.Int J Mol Med 21:577-84.
[7] Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A. & Zoric N. The real-time polymerase chain reaction. Molecular Aspects of Medicine. 27: 95-125 (2006).
[8] Lamaison JC. and Carret A.(1990): Teneurs en principaux flavonoids des fleurs de Crataegus monogyna Jacq et de Crataegus laevigata (Piret DC) en fonction de la vegetation," Plantes Medicinales et Phytotherapie, 25; 12-16.
[9] Matthaus, B. (2002):Antioxidant activity of extracts obtained from residues from different oilseeds. Journal of Agriculture and Food Chemistry; 50: 3444-3452.
[10] Penarrieta JM, Alvarado JA,AkessonB. and Bergenstahl B. (2008):Total antioxidant capacity and content of flavonoids and other phenolic compounds in Canihua (Chenopodium pallidicaule): an Andean pseudocereal. Mol Nutr Food Res. 52(6): 708-717.
[11] Siddhuraju, P. and Becker K. (2003): Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera Lam). Journal of Agriculture and Food Chemistry 15: 2144-2155.