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Evaluation of Anticancer Activities of *Ulva Lactuca* Ethanolic Extract On Colorectal Cancer Cells

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Abstract

The current study aimed to assess the anticancer effects of the ethanolic extract of *Ulva lactuca* (Seaweeds) on Caco-2 cell line. *U. lactuca*, a natural source of poly-unsaturated fatty acids, may be inhibit cancerous cells proliferation through apoptosis and cell death. The MTT experiment revealed that *U. lactuca's* toxicity on Caco-2 cells was concentration-dependent. As the extract concentration reached 0.45 µg, the percentage of cell viability propagated to 100%, and when it reached 250 µg, it decreased to 14.7%. *Ulva lactuca* extract was found to have anti-proliferative activity against Caco-2 cancer cell line, with an IC50 of 17.33 µg/mL. After being exposed to *U. lactuca*, Caco-2 cells underwent cell cycle analysis, which revealed significant DNA accumulation in the S-Phase but not significantly in the G2-M and G0/G1 phases. Apoptosis was confirmed via oxidative activity supported apoptotic potential recording a significantly increased ROS, MDA levels. Here, we used comet assay to examine *U. lactuca's* potential for genotoxicity. When the IC 50 of *U lactuca* was utilized, it induces significant amount of DNA damage to Caco-2 cells. Using the alkaline single cell gel electrophoresis assay, the amount of DNA damage (tail DNA) and the length of DNA migration (comet) were both determined in both control and treated cells.

Keywords: *Anticancer, Cytotoxicity, Apoptosis, Flowcytometry, Caco-2 cells, Comet assay, oxidative stress*

1. Introduction

Colorectal cancer is one of the most serious and common diseases especially in the developed countries. Colorectal cancer is estimated to affect 1.9 million people and kill 900,000 people worldwide **[1].** 3.47% of male cancers and 3% of female cancers are caused by colorectal cancer **[2].** A lack of antioxidant in our regular dietary supply may lead to the development of cancer. Seaweeds, on the other hand, are potential sources of antioxidant agents, and various bioactive compounds contributed with anti-carcinogenic activity against cancer, particularly colon cancer **[3].**

Antioxidant pigments, vitamins such as β-carotene and ascorbic acid, polysaccharides and polyunsaturated fatty acids are found in marine microalgae **[4&5].** All these compounds were subjugated as functional ingredients with antimicrobial **[6- 9],** antioxidant **[10&11]** and anticancer activities **[12].** Oleic acid and olive oil can be found in microalgae have anticancer activity **[13&14].** The anticancer mechanism of oleic and olive acid is due to the influence of available antioxidants on tissue oxidative stress and a change in the apoptosis rate **[15].** Polysaccharide, on the other hand, was isolated from seaweeds and has been demonstrated to have cytotoxic effect against several tumor cell lines and as well as the ability to suppress tumor cell proliferation/ differentiation by production of low cell reactivity **[16].**

One of the marine microalgae (*Ulva lactuca*) is a rich source of marine bioactive compounds with

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numerous application in the biomedical field **[17].** One of the marine microalgae (*Ulva lactuca*) is a rich source of marine bioactive compounds with numerous application in the biomedical field [17]. *Ulva lactuca* contains polysaccharides, polyphenols, plant chemistry and polyunsaturated fatty acids. This green microalgae could be used for treating inflammation, cancer, oxidative stress and others degenerative diseases. Moderate levels of oxidative stress can cause damage to genes involved in cell growth and division which can promote the development of cancer. However, higher levels of oxidative stress trigger apoptosis. Natural anticancer agents whose main mechanism of action is oxidative stress still under investigation. Flow cytometry cell cycle analysis has been recommended for studying single cell properties and proliferation in large cell populations such as cancer cells [18-19]. The current study aims to examine the potential of *Ulva lactuca* for treating colon cancer using Caco-2 as a model, as well as cell-cycle and related oxidative profiles.

2. Experimental

Collection of alga and preparation of extract: *Ulva lactuca* was collected from the coastal area of Red Sea, Hurghada, Egypt, and the collected sample was identified according to Fletcher **[20].** The sample (U. lactuca) was properly washed to eliminate any related debris before being air-dried and crushed into fine powder. The aqueous extract was made by soaking 50 g of alga in 200 mL of aqueous ethanol for 72 h. After soaking, the extract was centrifuged for 20 minutes. The resulting extract was filtered using Whatman filter paper and kept at 4° C for later study.

Cell culture: The colorectal cancer cells (Caco-2 cells) were supplemented from the Egypt's International Center for Training and Advanced Researches (ICTAR). The cells were maintained in accordance with the manufacturer's instructions. For cell culture, the cells were grown in a medium containing 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin. Plates of Caco-2 cells were placed in a humidified environment with 5% CO2 at 37°C

Gas chromatography–mass spectrometry (GC-MS) analysis: The crude extract was analyzed using Gas-Chromatography-Mass spectrum (GC-MS) using a split automatic injector and DB-5 silica capillary column (length: 60 m; ID: 0.32 mm.). The analysis was performed at Al- Azhar University' Regional Center for Mycology and Biotechnology (RCMB).

Cell cytotoxicity assay (MTT): The number of Caco-2 cells was adjusted as 2 × 105/mL/well in the growth medium for 24h at 37°C in an incubator with 5% CO2 environment. In serum-free medium, 100µL/well of two fold serially diluted *Ulva lactoca* mix in the growth medium. Each well received 50µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) solution (of 5 mg/mL) in PBS **[21].** After four hours, the plates formed formazan crystals that were dissolved in 50 µL DMSO and incubated for 30 min at 37°C with shaking. The absorbance of the generated coloring was measured at 570nm using microplate reader, and the percentage (%) of viability was determined.

Cell Cycle Analysis: The DNA content of Caco-2 cells (control and treated) was evaluated at various stages of cell cycle using the nucleic acid stain propidium iodide (PI), followed by flow cytometry analysis. Caco-2 cell line was pelleted by cold centrifugation after treatment with IC50 U. lactoca, washed with 1 mL of cold 1XPBS, centrifuged at 500g for 5 mints, and cell pellets were fixed in 70% cold ethanol at 4 °C for 24 hours. Following that, cells were incubated for 30 minutes at 37 C in the dark with 20X PI (500 µl) + 50 µl 200 X RNase staining solution in combination with FITC conjugated ab139418 according to the manufacturer's procedure. Finally, cell cycle distribution analysis was performed as previously described in **[22].** Flow cytometry was used at 488 nm illumination to calculate the percentages of cell cycle arrest at G1, S, and G2/M phases.

Apoptosis assay (Annexin-V/FITC apoptosis assay): Cell apoptosis was evaluated 24h after treatment with *U. lactoca* using Annexin V-FITC apoptosis/necrosis detection kit. The cells were washed wit PBS and stained with propidium iodide and assessed for apoptosis using flow cytometer.

Comet (DNA fragmentation) Assay: According to **[23],** comet tail lengths were measured from the center of the nucleus to the end of the tail for the count and the size of the comet. Kinetic Imaging, Ltd. (Liverpool, UK) software attached to CCD camera was used to analyze the quantitative and qualitative level of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program determine the value of the tail comet. Each sample, 50 to 100 randomly selected cells were examined.

Biochemical analysis Caco-2 cells were washed with cold PBS (by centrifugation), cold centrifuged and pelleted at 1500 RPM for 15 minutes after being treated with U. lactoca IC 50. As described in **[24],** cell lysine was produced by re-suspending the cell pellet in PBS, followed by cold centrifugation at 4,000 ×g for 15 minutes at 4oC. The cell extract was tested for malondialdehyde [MDA] using Lipid Peroxidation Assay Kit (Colorimetric/Fluorometric) (ab118970), and the mixture of MDA free in the samples - TBA (Thiobarbituric Acid) was assessed at 450 nm (ROBONIK P2000 ELISA READER). The level of reactive oxygen species [ROS] activity were measured in treated and untreated cells using readymade kits provided by Bio-diagnostic (Cairo, Egypt) and ROBONIK P2000 ELISA READER at wavelength 450 nm. All previous analysis was determined at Al- Azhar University's ICTAR.

Morphological study: Smears of Caco-2 cells were fixed in methanol, hydrated with ethyl alcohol and stained with Hx and Eosin.

Statistical analysis: Results were expressed as mean ± standard error (SE). The obtained data were analyzed using the one-way analysis of variance (ANOVA) and Tukey's post-hoc test. The difference considered statically significant at $P \leq 0.05$.

3. Results and Discussion

3.1. Phytocomponents identified by GC-MS in the crude ethanolic extract of U. lactuca

Figure (1) and Table (1) revealed the presence of 9 main chemicals in the extract of *U. lactuca*. Octadeconic acid methyl ester (Oleic acid) is the most major unsaturated fatty acid contained in lipid at a high concentration (38.96%) (Fig.1).

GC-MS analysis, on the other hand, revealed that the saturated fatty acids contained in high concentration in the extract of *Ulva lactoca* were palmitic acid (13.30%), followed by Stearic acid (1.83%) and Phenol recorded (4.8%) (Table 1). Other compounds detected in the extract included alcohol (12.3%), aromatic (4.15%), aldehyde (2.2%) and hydrocarbon compounds acid 1.64% (Table 1). The current study concluded that GC-MS analysis of *Ulva lactoca* revealed variances in its fatty acid content, with percentages indicated that the unsaturated fatty acid content was higher than that of the saturated extract.

Polyunsaturated fatty acids (PUFAs) are considered valuable compounds in human nutrition due to their impact on human health **[25].** As a result, higher consumption of unsaturated fatty acids and lower consumption of saturated fatty acids are associated with better health outcomes. Many studies have demonstrated that the colon tumor promoting effect in animals depends not only on the amount of fat but also on its fatty acid composition.

Oleic acid (OA) is an omega-9 monounsaturated fatty acid characterized by potent cytotoxic activity against various disease **[25] [26].** Oleic acid induce apoptosis in several cancer cell lines, and the mechanisms underlying cell death are numerous and include complicated pathways **[27].**

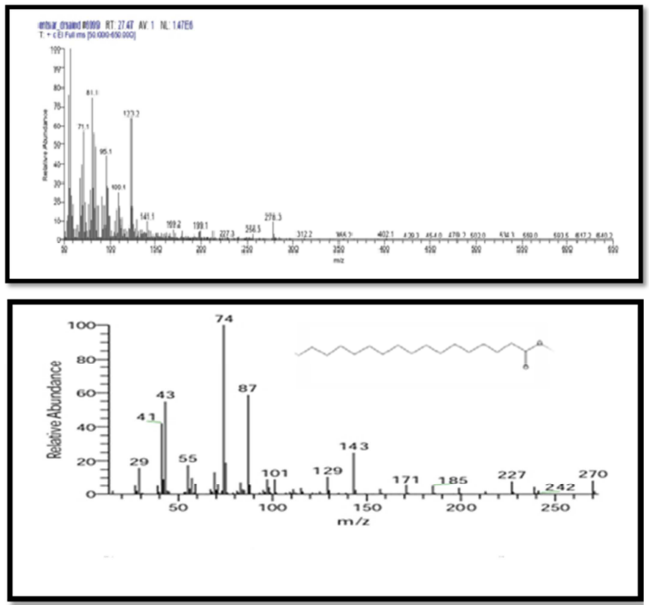


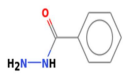
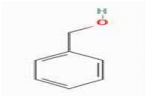
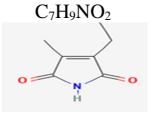
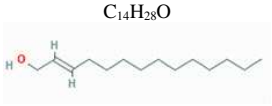
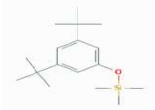
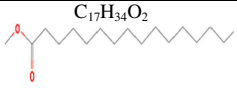
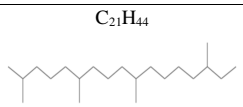
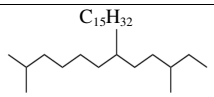
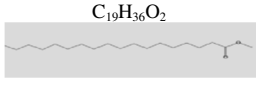
Figure 1: The mass spectrum of octadeconic acid methyl ester (oleic acid) obtained from *Ulva lactuca*

3.2. Cytotoxicity

Caco-2 treated cells with alga extract at concentration 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95, 0.95 and 0.45 µg/ml showed means of cell viability for 24 hours using MTT assay (Fig. 2). The cytotoxic activity of *Ulva lactuca* extract to Caco-2 cancer cells revealed that *U. lactuca* anti-cancer activity was concentration dependent. The percentage of cell viability increased to 100% as the concentration of the extract reached 0.45 µg, and it was decreased to 14.7% at concentration 250 µg of the extract (Fig. 2). The IC50 of the extract was 17. 33 µg/mL for Caca-2 cell line. Similarly in previous study, MTT cytotoxicity assay on *U. lactuca* extracts had significant actions against MCF-7 and Hela cell lines (IC50 of 10.83 1.0 and 12.43 1, 3 g/ml, respectively) [28], *U. fasciata* exhibited significant action against PC3 and HepG2 cell lines (IC50 of 12.99 ± 1.2 and 16.75 ± 1.5 µg/ml, respectively), indicating that the cytotoxicity of *U. lactuca* extract varies depending on the cancer cell type.

Table 1

Phytocomponents identified by GC-MS in the organic extract *U. lactuca*

NO.	. Compound	Molecular Formula	Molecular W.	RT	Peak area %	Chemical group
1	Benzoic acid, hydrazide	$C_7H_8N_2O$ 	136	10.96	2.2	Aldehyde
2	Benzyl alcohol	C_7H_8O 	108.14	9.82	4.15	aromatic
3	3-Ethyl-4-Methyl-1h-Pyrrole-2,5-Dione	$C_7H_9NO_2$ 	139.15	2.34	2.5	Ketone
4	E-2-Tetradecen-1-ol	$C_{14}H_{28}O$ 	212.37	4.56	11.3	Alcohol
5	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$ 	206.32 Used as an antioxidant	18.5	4.8	Phenol
6	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$ 	270	25.78	13.30	Palmitic acid
7	Heptadecane.2.6,10,15-tetramethyl	$C_{21}H_{44}$ 	296.581	20.53	1.83	Stearic acid
8	Dodecan.2.7.10-trimethyl	$C_{15}H_{32}$ 	212.421	14.8	1.640	hydrocarbon
9	9- Octadeconic acid methyl ester	$C_{19}H_{36}O_2$ 	296	20.12	38.96	Fatty acids Oleic acid,

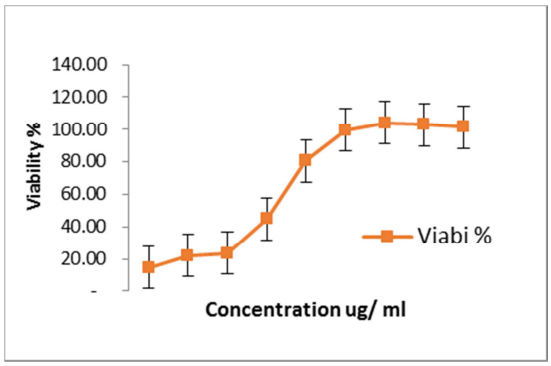


Figure 2: Viability (%) of Caca2 cell line against Caco-2 cell line post 24 h using MTT assay

3.3. Cell cycle analysis

Flow cytometry was used in the current to identify the number of cells in each phase of its cell cycle. Caco-2 cells displayed normal cell cycle peaks in G1, S and G2/M phases (Fig. 3A). Flow cytometry examination, however, revealed changes in the distribution of G1, S, and G2/M peaks in Caco-2 cells treated with *Ulva lactuca* (Fig. 3B). In addition, the percentage of DNA content in Caco-2 cells after treatment with *Ulva lactuca* recording (42.9 %), was significantly higher [P < 0.05] at the S phase compared to the value in the cell control (29.82%) (Fig. 3C). While, G0-G1 phase demonstrated a significant lower percentage of arrest recording (43.8%) of DNA content after treatment than the control cells (55.72%). At the same time, G2/M phase showed a negligible change [p > 0.05] of DNA content compared with that of the un-treated cell control (Fig 3C).

Annexin V-FITC apoptotic/necrotic analysis with flow cytometry revealed an increase in the number of apoptotic cells (S phase) treated with *Ulva lactuca* relative to non-treated cells after 24 h (Fig. 4 A, B).

In addition, there was clearly elevated total apoptosis [P< 0.05] compared to apoptotic values detected in non-treated cell control (Fig. 4), and the apoptosis percentage in CaCo-2 cell line was significantly [P< 0.05] higher than in untreated control cells (41.39%).

At the same time, *Ulva lactuca*-treated cells had a significantly higher level of early and late apoptosis when compared to control values. CaCo-2 treated

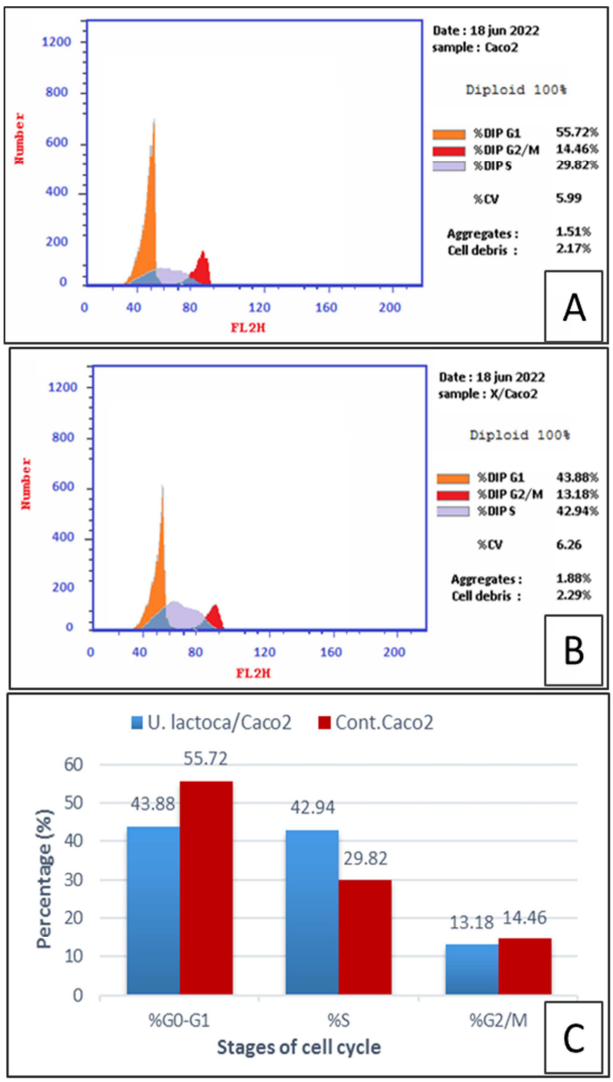


Figure 3: Evaluation of cell cycle profile induced in Caco-2 cancer cell lines under the effect of *Ulva lactuca* using flow cytometry. A. Caco-2 Control, B Caco-2 treated with *Ulva lactuca*. C. DNA content in different stages of cell cycle analysis

cells had 4.39 %, total early apoptotic cells, while control had 0.54%. Total late apoptotic treated cells were 16.01%, while control cells were 0.16 %. Finally, necrotic activity was significantly increased (P<0.05) following Ulva lactuca cell treatment (Fig. 4C).

Oleic acid (OA) is an omega-9 monounsaturated fatty acid characterized by potent cytotoxic activity [25&27]. Oleic acid induces apoptosis in several cell lines and the mechanisms behind cell death are numerous and involve a complex set of pathways.

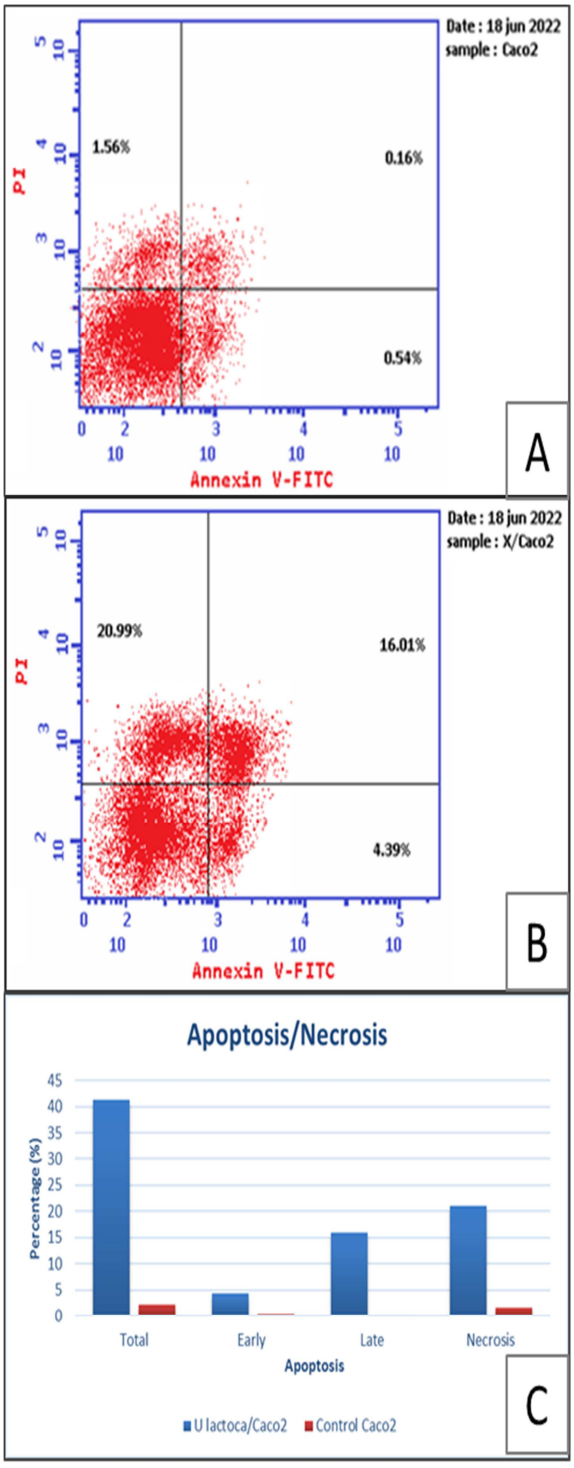


Figure 4: Apoptotic/ necrotic analysis in Caco-2 cancer cell line and under the effect of *Ulva lactuca* (Annexin V-FITC and P1 staining). A. Control Caco-2, B. Caco-2 treated with *U. lacoca*, C. Evaluation of DNA content and the % of apoptotic (early and late) / necrotic cells of Caco-2 cell lines treated with *Ulva lactuca* compared with untreated cell control

The cytotoxic activity in tumor cells is most likely due to proteasome inhibition [29], resulting in an increase in the amount of ubiquitin proteins within the cells, which in turn triggers activation of autophagy and apoptosis processes, resulting in cell death.

Coming along, DNA damage, our study found that treated cells had significantly more DNA damage, indicating structural integrity defects, similar to the results of [30], indicating variable ROS-induced genotoxicity changes such as oxidized single-strand breaks, base and sugar damage, as well as DNA–DNA and DNA–protein cross-links [31]. *Ulva lactuca* is an effective anti-cancer agent induced DNA damage which is consistent with our findings [32]. Many studies have shown that the colon tumor promoting effect in animals depends not only on the amount of fat but also on its fatty acid composition.

3.4. Comet assay (Single cell gel electrophoresis)

DNA damage was assessed using alkaline comet assay (Fig.5). There was a significant damage of DNA in the Caco-2 treated with *U lactuca* represented by decrease in the percentage of intact nucleus and increase of tailed DNA, increase of tail length and tail moment (Fig. 5). Tail DNA (%) following treating cells with a concentration of IC50 17.3 µg/mL of *U. lactuca* were compared to untreated Caco-2 cells..

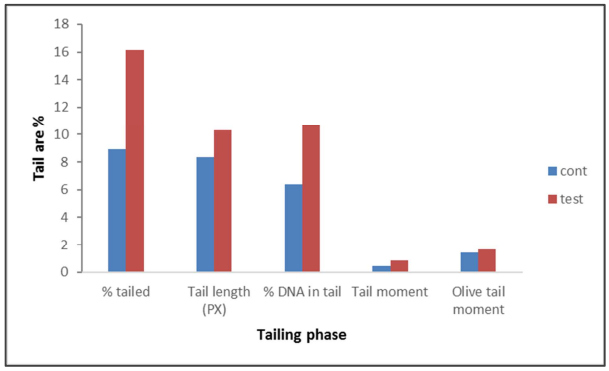


Figure 5: Comet (alkaline) assay. Determination of DNA damage (double-strand breaks) induced by IC50 concentrations of *ulva lactoca* in cancer cell line Caco-2 after 24 h incubation of cells with tested compounds

3.5. Hx. & Eosin examination

Conventionally, Hx & Eosin results showed that the control cells have regular hyperchromatic nuclei and pleomorphic cells.

However, the treated cells showed many shrunk apoptotic cells or necrotic cells with eosinophilic cytoplasm (fig. 6).

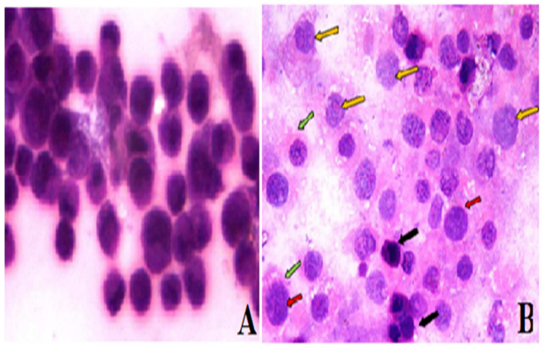


Figure 6: A photomicrograph showing Caco-2 cell line A: Control cells and B: Treated cells with Ulva lactuca IC50.Apoptotic cells with condensed chromatin (Black arrows), and irregular cell and nuclear membranes (Green arrows), necrotic cells with swilling (Orange arrows) (H and E, Original magnification 100X, Oil)

3.6. Biochemical analysis

Figure 7 shows that *U. lactoca* induced oxidative damage to Caco-2 cells. Administration of alga extract induced significant (P<0.05) elevated level of MDA, a biomarker of oxidative stress and indicator for the degree of lipid peroxide (LPO) in comparison to the control Caco-2 cells. In the same consequence significant (P<0.05) increase in ROS concentration (Fig. 7 A&B). It was noticed that *Ulva lactuca* showed a significant (P<0.05) elevated MDA level recording (12.27±0.08 n mol) compared with its level of untreated cell control (7.869±0.03 n mol) (Fig. 7 A). In the mean times, reactive oxygen species (ROS) level showed significant elevated values (433.5pg/ml) compared with untreated cell control value (308.0pg/ml) (Fig.7B).

Similar findings **[33]** found that olive oil and sesame extract significantly reduced congestion, mitotic index and inflammation in cancer cells. They are also used in cancer treatment since they are less toxic and presents less risk to human health than chemotherapy. The pro-oxidant activity of polyphenol may play an important role in the regulation of cancer cells **[34]**.

Oxidative stress has an impact in cancer initiation and proliferation. However, high level of reactive oxygen species (ROS) can cause DNA damage and mutation because ROS can oxidize nucleobases and producing unique forms of oxidative harm products with mutational properties **[35].** The mechanisms behind the apoptotic event induced by oleic acid could be related to an increase in intracellular ROS production or MDA level that leads to caspase-3 activity and apoptosis. In addition, fatty acid may disturb the redox state of the cells not only owing to an increase in ROS generation as previously shown, but also due to a reduction in antioxidant enzyme activities. Thus, some researchers demonstrated that OA reduced catalase activity in human leukemia cell lines **[36].**

The high accumulation of ROS can inhibit tumor cell proliferation. The massive accumulation of ROS lead to cancer cell death by the activation of ER stress, mitochondrial, P53 apoptotic pathways in cancer cells **[37].**

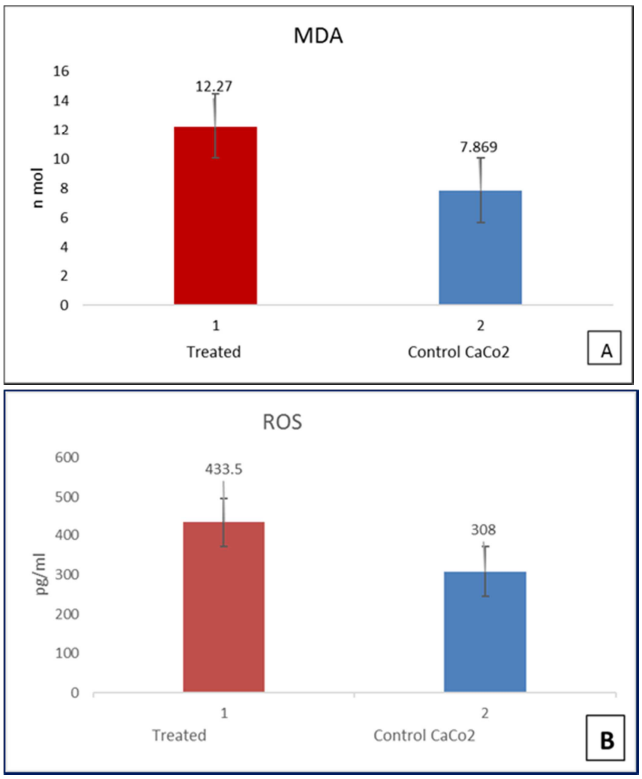


Figure 7: MDA levels and ROS (Reactive oxygen species) levels in control and U. lactoca treated Caco-2 cells

4. Conclusions

Ulva lactuca is a vital source of many essential bioactive compounds, from antioxidants and essential minerals to a highly vital and complete profile of amino acid and fatty acid. This study shows that the ethanol extract of *Ulva lactuca* acts as apoptotic

agent, and participates in the inhibition of Caco-2 cell development by increasing its oxidative activity. This role could be further explored in future clinical trials.

5. Conflicts of interest

The authors declare that there are no conflicts of interest.

6. Formatting of funding sources

This study didn't receive any funding.

7. Acknowledgments

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