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In Vitro Assessment of Antitumor Effect for *Amphora coffeaeformis* Extract Against HepG2 and HCT116 Cell Cultures

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KEYWORDS

Amphora coffeaeformis, human hepatocellular cancer HepG2 cell line, human colorectal carcinoma HCT116 cell line, human lung normal WI38 cell line, cytotoxicity, apoptosis.

ABSTRACT:

Introduction: Natural products are becoming more widely used as an alternative to conventional cancer treatments. Microalgae can be considered as a potential therapeutic agent in this circumstance due to their rich bioactive content and anticancer properties.

Objectives: This study evaluated the impact of the microalgae *Amphora coffeaeformis* on growth performance of HepG2 and HCT116 cancer cells.

Methods: Gas chromatography-mass spectrophotometry analysis (GC-MS) was used to evaluate the active components of algal extract. The cytotoxicity after treatment with acetone extract of algae was tested against cancer cell lines [human hepatocellular cancer cells (HepG2) and human colorectal carcinoma (HCT116)] as well as normal human lung cells (WI38) by MTT assay. Morphological changes in cells and cell cycle arrest were also assayed. The apoptotic (Annexin V+ /PI+) or necrotic cells (Annexin V− /PI+) were identified by flow cytometry. Gene expression of p53, Bcl-2, Bax and p21 was measured by real-time PCR.

Results: The GC-MS analysis of *Amphora coffeaeformis* extract demonstrated 30 different important compounds. The extract showed inhibitory potentials on cellular proliferation of cancer cells which was obviously along with increasing their concentrations. The extract induced cell growth arrest at G1 phase to HCT116 cells as well as WI38 cells and at pre G1 phase to HepG2 after 24 h exposure. The extract induced death in treated cells, by induction of both apoptosis and necrosis, late apoptosis becomes predominant. Expression of Bax, p53 and p21 genes revealed an increase in treated cancer cell lines compared to untreated control groups. In addition, a decrease in Bcl-2 of treated cancer cells.

Conclusions: Our results revealed the ability of *Amphora coffeaeformis* extract to exert cytotoxic and apoptotic effect on HepG2 and HCT116 cell lines, particularly in the late stage of apoptosis which is an irreversible process. So, *Amphora coffeaeformis* extract exhibited the anticancer activity.

1. Introduction

Cancer is the second serious causes of death all over the world. Nearly 1 out of 6 deaths was due to cancer. Malignant tumors, the uncontrolled cellular proliferations, are related to great pathologic changes. More than 200 various types of malignant tumors are introduce numerous types of cancers that can be metastasized into other tissues resulted in fatal metastatic tumors (1). Attitude of the lifestyle as well as smoking, poor diet, physical inactivity and genital changes raise susceptibility of the body to cancer incidence in less economically developed countries (2).

Most colorectal cancer (CRC) cases are typically diagnosed in adults over 50 years of age. However, mounting evidence from the past decade observed that the incidence of CRC is increasing amongst young adults. CRC is now the third leading cause of cancer death among young adults who are less than 50 years of age (3).

In Egypt, CRC was ranked seventh among the most common malignant tumors with around 3000 cases, representing 4% of totally diagnosed cancers and 53% of gastrointestinal tract (GIT) cancers. The pathogenesis of the CRC is influenced by multiple factors related to

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dietary habits, genetic predisposition, long standing inflammatory bowel disease and presence of colorectal polyps (4).

CRC is mainly treated by surgical resection. Various target drugs have also been developed; however, the cure rate and postoperative survival quality of patients with CRC have not improved significantly (5). Approximately 1.8 million new cases and nearly 900,000 deaths are reported worldwide each year (6).

Moreover, hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide in terms of mortality, and susceptibility is attributed to genetic, lifestyle, and environmental factors. HCC is the sixth most common cancer worldwide, and the fourth most common in Egypt. HCC occurs in several pre-existing conditions, including hepatitis C, hepatitis B, excessive alcohol consumption, diabetes, and non-alcoholic cirrhosis (7, 8).

Despite Chemotherapy, radiotherapy, surgery, hormonal and targeted therapy belong to the modalities for cancer treatment. Side effects of all these types of therapy remain unsolved problems in the clinical treatment of cancer (9, 10, 11). Therefore, in recent years, a great deal of research has been aimed at identifying natural products that can confer protective effects against cytotoxicity and also, natural compounds usually have fewer side effects and cost less. The microalgae and diatoms have attracted major interest as a natural source of natural nutrients (12).

Microalgae were identified as microscopic, unicellular, and photosynthetic organisms and can grow in fresh and saline water (13). Microalgae was focused mainly on its potential as a source of bioactive compounds such as lipopeptides, amino acids, fatty acids, macrolides, and amides, which have long records of human application as a food. Several studies showed that extracts of raw microalgae and its fractions have several therapeutic activities in human and animals like cytotoxic, antitumor, anticancer, antiviral, antibiotics. Microalgae also possesses other biological functions such as antioxidant, antimalarial, antimycotics, antimicrobial, antiepilepsy, anti-inflammatory, and immunomodulatory effects (14, 15, 16).

Amphora coffeaeformis is one of the most abundant species in alkaline fresh, brackish, and marine water. It contains high amount of photosynthetic pigments such as chlorophyll, carotenoids, phenolic, polyphenols, polyunsaturated fatty acids with high levels, and also a series of biologically active substances with antioxidant, antiobesity, antimicrobial, and other properties used in many medical applications (17, 18, 19).

Amphora coffeaeformis algal extract demonstrated scavenging properties against DNA damaged resulted by hydrogen peroxide (20). In addition, *Amphora coffeaeformis* extract exhibited antagonistic effect against the hepatic injury and the deleterious effects induced by paracetamol in rats (21). Till now, few biological studies were conducted on *Amphora coffeaeformis*, besides, there were no studies that assessed the anticancer activity of *Amphora coffeaeformis.* Depending on the proven anticancer effects of microalgae; it can be assumed that *Amphora coffeaeformis* may exert the same anticancer effects against human colorectal carcinoma cells (HCT-116) and human hepatocellular carcinoma cells (HepG2).

2. Objectives

The aim of the present study was to examine the anticancer activity of the *Amphora coffeaeformis* extract that can eradicate cancer cells (HCT-116 and HepG2 cells) without harming normal, healthy cells.

3. Methods

3.1. Algal extract.

Amphora coffeaeformis was obtained from Algal Biotechnology Unit, National Research Centre, Egypt. The fresh material was dried at room temperature then grinded for fine powder. The extraction of algal sample was done using 250gm powder macerated in 500ml acetone and allowed to stand at room temperature for a period of 3 days. For conventional extraction, the extract was placed in a sonicator at 40^{o}C for 60 min. Then this extract was filtered and concentrated under vacuum at 40^{o}C by using Rota vapor to provide crude extract (10gm). The Crude extract was re-dissolved in 250 ml distilled water.

3.2. Gas chromatography–mass spectrometry (GC-MS) analysis.

The chemical composition of samples was performed using Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary

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column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50°C and then increased by 5°C /min to 230°C hold for 2 min then increased to the final temperature 290°C by 30°C /min and hold for 2 min. The injector and MS transfer line temperatures were kept at 250, 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1 µl were injected automatically using Autosampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–1000 in full scan mode. The ion source temperature was set at 200°C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database.

3.3. Cell culture.

Human hepatocellular carcinoma HepG2, human colorectal carcinoma HCT116, and human lung normal WI38 cell lines, were purchased from Cell Culture Research Unit at the Holding Company for Vaccines and Sera (VACSERA), Egypt. Cells were maintained in Roswell Park Memorial Institute (RPMI 1640) culture medium (Gibco, USA) supplemented with 2% fetal bovine serum and 1% antibiotic solution (penicillin-streptomycin), then incubated for 24 h at 37 ℃ in a humidified 5% CO_2 incubator to allow the cells to grow and form a monolayer in the flask. Cells grown to 80-95% confluency were washed twice with phosphate buffer saline and trypsinized with 0.25% trypsin-0.03% EDTA until the cell layer is detached into single cells (usually within 5 to 15 min) then fresh growth medium was used to re-suspend the cells. The stained cells with trypan blue dye were counted using hemocytometer as dead cells however unstained cells were considered viable. Calculation was done according to the following equation:

Number of cells per milli = Average count in the large five squares X 2 (dilution factor) X 10^4

3.4. Cytotoxicity/Viability assessment by MTT assay.

The 96- well tissue culture plate was inoculated with 1 X 10^5 cells / ml (100 µl / well) and incubated for 24 hours in the humidified 5% CO_2 incubator at 37°C and allowed to attach to the substrate. Two-fold dilutions of tested sample made in RPMI maintenance medium were added, and 100µl of each dilution (1000, 500, 250, 125, 62.5, and 31.25 µg/ml) was tested in different wells leaving three wells as control, receiving only maintenance medium. The plate was incubated at 37 °C for 24 h and examined. Cells were checked microscopically for any morphological alterations, such as partial or complete loss of the monolayer, cell rounding and shrinking, or cytoplasm granulation.

To evaluate the cytotoxic effect of the tested sample against human cancer cell lines (HepG2 and HCT116) as well as normal (WI38) cells, MTT [3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution was prepared (5mg/ml PBS). Twenty µl MTT solution were added to each well, and incubate (37ºC, 5% CO_2) for 4 hours to allow the MTT to be metabolized. After the incubation period, MTT solution was discarded and the resulting formazan crystals (MTT metabolic product) were dissolved by adding 200 µl of DMSO / well. Finally, viability was determined by taking optical density (OD) at 560 nm and substrate background at 620 nm by spectrophotometer. The experiments were performed in triplicate (repeated three times for each concentration) and the percentage of viability and toxicity was calculated by using the given equation:

$$\text{Viability (\%)} = \frac{\text{OD of treated cells}}{\text{OD of untreated cells}} \text{X } 100$$

Toxicity (%) = 100 – Viability

The algal extract at 50% inhibitory concentrations (IC50) on HepG2, HCT116 and WI38 cell growth were determined and used for further experiments. The concentration required to cause toxic effects in 50% of cells (IC50), was calculated depending upon the dose-response curve obtained by plotting the percentage of viability versus the concentration of tested extract using excel sheet.

3.5. Apoptosis assessment and cell cycle arrest.

Annexin V- FITC Apoptosis Detection Kit was used to examine the effects of extract on the cell cycle of cells. The kit contains propidium iodide (PI) which can differentiate between apoptosis and necrosis. The cell lines at a density of 1×10^5 cells/well were incubated for 24 h prior to their exposure to IC50 doses of extract (82.42, 137.95 and 166.39 µg/ml) for a time period of

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24 h. Post extract treatment, cells were incubated with 5 µl of Annexin V- FITC and 5 µl of PI and incubate at room temperature for 5 min in the dark. The proportion of cells in G1, S, G2/M and pre-G1 phases were analyzed by examining the intensity of PI fluorescence at 530 nm with a flow cytometer using a FACS flow cytometer (BD FACS Calibur, BioVision, USA). For determination of cell death mode, the quantities of apoptosis/necrosis cells were measured by flow cytometry.

3.6. Quantitative reverse transcriptase PCR analysis.

Total RNA was extracted from 1×10^5 cells cultured for 24 h in the presence of extract (IC50) or from untreated cells utilizing Qiagen RNA extraction kit according to manufacturer's procedure. The concentration of the extracted RNA was evaluated by determining its optical density at 260 nm using spectrophometry. The cDNA high-capacity reverse transcription Kit was used for the reverse transcription of 1µg of total mRNA per sample. The cDNA master mix was prepared according to the kit instructions and was added for each sample. The mix was incubated in programmed thermal cycler for 1 h at 37 °C.

The total reaction volume was 10µl: 1µl cDNA, 5µl SYBR Green Master Mix, 0.5µl of each primer and 3µl Nuclease Free Water. The thermal cycling conditions were as follows:10 min at 95 °C for polymerase activation, then denaturation (95 °C for 15 sec), annealing (55 °C for 30 sec) and extension (72 °C for 30 sec). The expression of tested genes was normalized to the expression of β-actin gene as a housekeeping gene. Primer sequences (5′ to 3′) used for studied genes are listed in Table 1. Analysis of melting curve was carried out to examine the amplification specificity. Difference greater than 1 fold was considered as upregulation while difference less than 1 fold was considered as downregulation.

Calculation of fold change:

ΔCTC (of control samples) = TC gene test – HC gene control

ΔCTE (of treated samples) = TE gene test – HE gene control

ΔΔCT = ΔCTE – ΔCTC

Fold change = $2^{-\Delta\Delta CT}$

Table 1: Primers of studied genes utilized for qPCR.

Genes	Primer sequences (5′ to 3′)
p53	F 5'- CCCCTCCTGGCCCCTGTCATCTTC-3' R 5'-GCAGCGCCTCACAACCTCCGTCAT-3'.
Bcl-2	F 5'-CCTGTG GAT GAC TGA GTA CC-3' R 5'-GAGACA GCC AGG AGA AAT CA-3'
Bax	F 5'-GTTTCA TCC AGG ATC GAG CAG-3' R 5'-CATCTT CTT CCA GAT GGT GA-3'
p21	F 5'-GCA CAA AGG TCC TCA TCC AG- -3' R 5'-GAT CAC CAT CTC CGA GGG CT- -3'
β-actin	F 5'-GTGACATCCACACCCAGAGG-3' R 5'-ACAGGATGTCAAAACTGCCC-3'

4. Results

4.1. Chemical composition of *Amphora coffeaeformis*.

The GC-MS analysis of *Amphora coffeaeformis* was presented in Fig. 1 and Table 2. The results showed that there were 30 different components are identified. Thirteen main compounds inclusive Phytol (21.66%), Heptadecane (15.23%), cis-9,cis-12-Octadecadienoic acid (11.93%), n-Hexadecanoic acid (11.06%), 1-Eicosanol (4.58%), 2-Pentadecanone, 6,10, 14-trimethyl, (4.06%), 9,12-Octadecadienoic acid (Z,Z) (3.32%), Palmitoleic acid (3.28%), Neophytadiene (3.22%), 9-Octadecenoic acid (Z) (2.36%), Linolenic acid (2.13%), 3-Hydroxy-à-ionene (2.11%) and 8-Heptadecene (1.84%). In addition, the remains 17 fatty acids founded in minor amount.

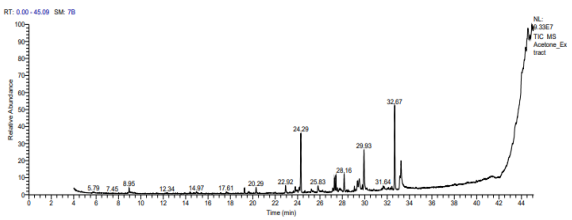

Figure 1: Gas chromatography-mass spectroscopy (GC-MS) spectra of the *Amphora coffeaeformis* active compounds.

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Table 2: Active constituents of *Amphora coffeaeformis* (gas chromatography-mass spectroscopy, GC-MS).

RT	Compound Name	Area %	Formula	MW
8.95	Undecane	1.33	C11H24,	156
19.26	4-(2,6,6-Trimethyl-1-cyclohexenyl)-3-buten-2-one	1.39	C13H20O,	192
20.29	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	1.57	C11H16O2	180
22.92	3-Hydroxy-à-ionene	2.11	C13H20O2	208
23.8	8-Heptadecene	1.84	C17H34	238
23.92	2-Methylhexadecan-1-ol	1.1	C17H36O	256
24.15	3H-3,10a-Methano-1,2-benzodioxocin-3-ol, octahydro-7,7-dimethyl-, (3.alpha,6a.beta,10a.beta)-	1.18	C13H22O3	226
24.29	HEPTADECANE	15.23	C17H36	240
24.66	cis-13-Octadecenoic acid	0.53	C18H34O2,	282
25.24	4-Trifluoroacetoxypentadecane	1.53	C17H31F3O2	324
25.82	9-OCTADECENOIC ACID (Z)-	2.36	C18H34O2	282
27.15	PENTADECANOIC	0.96	C15H30O2	242
27.29	Neophytadiene	3.22	C20H38	278
27.42	2-Pentadecanone, 6,10,14-trimethyl-	4.06	C18H36O	268
27.54	1-HEXADECANOL, 2-METHYL	0.99	C17H36O	256
27.73	Linoleoyl chloride	0.84	C18H31ClO	298
27.8	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.67	C18H32O2	296
28.16	1-Eicosanol	4.58	C20H42O	298
29.08	PENTADECANOIC ACID, 14-METHYL-, METHYL ESTER	1.04	C17H34O2	270
29.34	Linolenic acid	2.13	C18H30O2	278
29.52	Palmitoleic acid	3.28	C16H30O2	254
29.79	Phthalic acid, butyl undecyl ester	0.96	C23H36O4	376
29.93	n-Hexadecanoic acid	11.06	C16H32O2	256
31.65	9,12-OCTADECADIENOIC ACID (Z,Z)-	0.78	C18H31ClO	298
31.73	2-cis-9-Octadecenyloxyethanol	0.56	C20H40O2	312
32.12	cis-13-Eicosenoic acid	0.51	C20H38O2	310
32.42	12-Methyl-E,E-2,13-octadecadien-1-ol	0.78	C19H36O	280
32.67	Phytol	21.66	C20H40O	296
33.13	9,12-OCTADECADIENOIC ACID (Z,Z)-	3.32	C18H32O2	280
33.24	cis-9,cis-12-Octadecadienoic acid	11.93	C18H32O2	280

4.2. Morphological changes.

Inverted light microscope was used at magnification (×200) to investigate the morphological alterations induced by algal extract in both healthy and cancer cell lines. Microscopic examination of HepG2 and HCT116 cancer cell lines revealed cytotoxic features after a 24 h treatment period, where treated cells became rounded, granulated and the cells become detached out of the monolayer along with increasing extract concentrations compared to the normal appearance of untreated cells (Figures 2a&b, 3a&b and 4a&b).

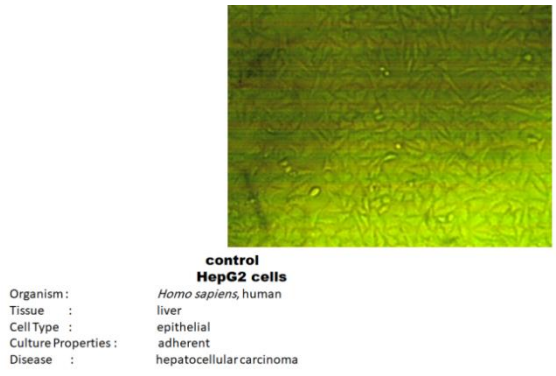

Figure 2 (a): untreated control HepG2 cells. The untreated cells showed a high confluency rate and normal monolayer formation compared with treated cells.

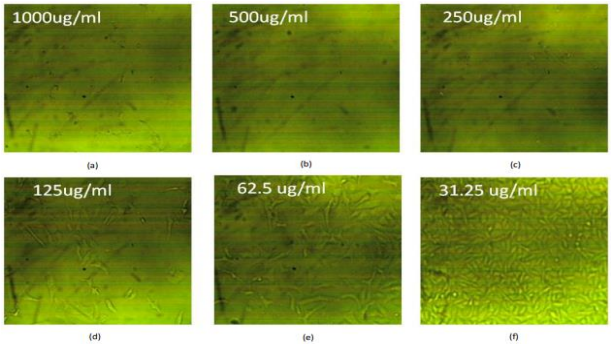

Figure 2 (b): Morphological changes of HepG2 cancer cells 24 h post treatment with algal extract at different concentrations viewed under an inverted light microscope. (a) HepG2 cells treated with 1000 µg/mL; (b): HepG2 cells treated with 500 µg/mL; (c): HepG2 cells treated with 250 µg/mL; (d): HepG2 cells treated with 125 µg/mL; (e): HepG2 cells treated with 62.5 µg/mL; (f): HepG2 cells treated with 31.25 µg/mL respectively. Treated cells exhibited cytotoxicity signs; cells became shrunken and detached out of the monolayer with larger areas devoid of cells at higher extract concentrations (125 to 1000 µg/mL) compared to normal appearance of untreated cells.

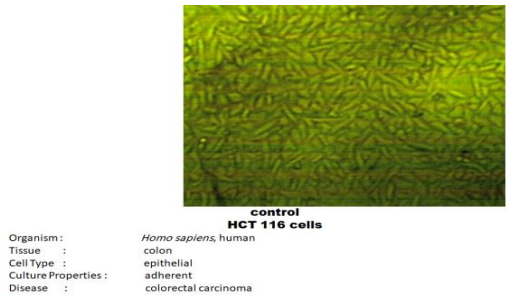

Figure 3 (a): untreated control HCT116 cells. The untreated cells showed a high confluency rate and normal monolayer formation compared with treated cells.

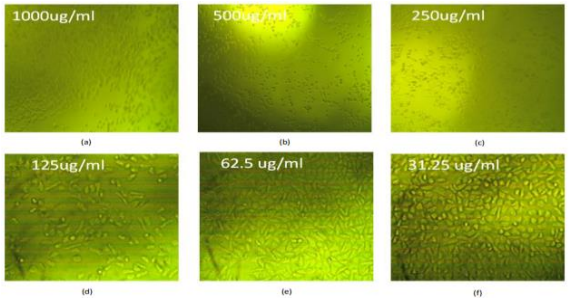

Figure 3 (b): Morphological changes of HCT116 cancer cells 24 h post treatment with algal extract at different

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concentrations viewed under an inverted light microscope. (a): HCT116 cells treated with 1000 µg/mL; (b): HCT116 cells treated with 500 µg/mL; (c): HCT116 cells treated with 250 µg/mL; (d): HCT116 cells treated with 125 µg/mL; (e): HCT116 cells treated with 62.5 µg/mL; (f): HCT116 cells treated with 31.25 µg/mL respectively. Treated cells exhibited cytotoxicity signs; cells became shrunken, rounded in shape, and detached out of the monolayer with larger areas devoid of cells at higher extract concentrations (125 to 1000 µg/mL) compared to normal appearance of untreated cells.

Figure 4 (a): untreated control normal WI38 cells.

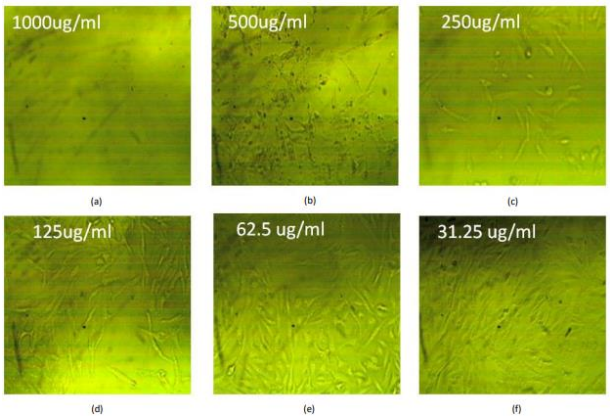

Figure 4 (b): Morphological changes of WI38 control normal cells 24 h post treatment with algal extract at different concentrations viewed under an inverted light microscope. (a): WI38 cells treated with 1000 µg/mL; (b): WI38 cells treated with 500 µg/mL; (c): WI38 cells treated with 250 µg/mL; (d): WI38 cells treated with 125 µg/mL; (e): WI38 cells treated with 62.5 µg/mL; (f): WI38 cells treated with 31.25 µg/mL respectively. No morphological changes were observed in treated cells at 31.25, 62.5 and 125 µg/mL extract concentration, while loss of confluence was observed at 250, 500 and 1000 µg/mL concentration.

4.3. Determination of cytotoxicity on cells.

The degree of cytotoxicity of algal extract to cell lines was determined using MTT assay as shown in Figure 5. The data were presented as viability percentage obtained 24 h post treatment with different concentrations. Cancer cells (HepG2 and HCT116) treated with increasing concentrations of extract (125 µg–1000 µg/mL) exhibited decreased cell viability. The results of the cytotoxic effects of the extract in HepG2 cells are presented in Table 3 while the effect of extract on the viability of HCT116 cells are shown in Table 4. On the other hand, algal extract was toxic to normal WI38 cells at a concentration of 250 - 1000µg/mL (Table 5).

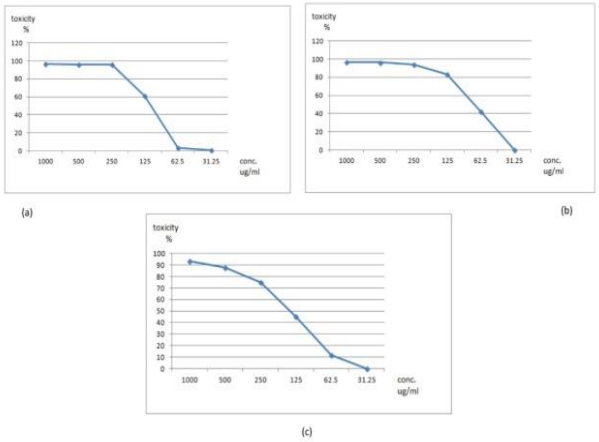

Figure 5: (a) Cytotoxic effect of extract against colon cancer (HCT116); (b) Cytotoxic effect of extract against liver cancer (HepG2); (c) Cytotoxic effect of extract against normal lung (WI38) cell lines using MTT assay. There was a decrease in cellular viability in cancer cells along with increasing extract concentrations with no or little cytotoxic effect to WI38 normal cells at 31.25 to 125 µg /mL.

ID	Conc. ug/ml	O.D			Mean O.D	ST.E	Viability %	Toxicity %	IC50 ug
WI38	1:2	0.326	0.345	0.313	0.328	0.009292	100	0	
Sample 1	1000	0.018	0.024	0.025	0.022333	0.002186	6.808943089	93.19105691	166.39
	500	0.035	0.041	0.046	0.040667	0.00318	12.39837398	87.60162602	
	250	0.095	0.077	0.073	0.081667	0.006766	24.89837398	75.10162602	
	125	0.168	0.172	0.199	0.179667	0.009735	54.77642276	45.22357724	
	62.5	0.278	0.293	0.297	0.289333	0.005783	88.21138211	11.78861789	
	31.25	0.33	0.317	0.337	0.328	0.005859	100	0	

Table 3: The MTT result of the cytotoxic effect of algal extract on human liver carcinoma cells (HepG2)

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following 24 h of exposure. Each experiment was done in triplicate. The dose of algal extract required to produce 50% reduction in the viability of HepG2 cells (IC50) were compound to be 82.42 µg/mL upon 24 h of exposure.

ID	Conc. ug/ml	O.D			Mean O.D	ST.E	Viability %	Toxicity %	IC50 ug
HepG2	1:2	0.491	0.525	0.526	0.514	0.011504	100	0	
Sample 1	1000	0.018	0.018	0.019	0.018333	0.000333	3.566796368	96.43320363	82.42
	500	0.019	0.018	0.019	0.018667	0.000333	3.631647211	96.36835279	
	250	0.034	0.025	0.036	0.031667	0.003383	6.160830091	93.83916991	
	125	0.086	0.094	0.077	0.085667	0.00491	16.66666667	83.33333333	
	62.5	0.295	0.307	0.288	0.296667	0.005548	57.71725032	42.28274968	
	31.25	0.491	0.525	0.526	0.514	0.011504	100	0	

Table 4: The MTT result of the cytotoxic effect of algal extract on human colon carcinoma cells (HCT116) following 24 h of exposure. Each experiment was done in triplicate. The dose of algal extract required to produce 50% reduction in the viability of HCT116 cells (IC50) were compound to be 137.95 µg/mL upon 24 h of exposure.

ID	Conc. ug/ml	O.D			Mean O.D	ST.E	Viability %	Toxicity %	IC50 ug
HCT116	1:2	0.532	0.504	0.527	0.521	0.008622	100	0	
Sample 1	1000	0.017	0.019	0.019	0.018333	0.000667	3.51887396	96.48112604	137.95
	500	0.019	0.02	0.019	0.019333	0.000333	3.71081254	96.28918746	
	250	0.018	0.024	0.019	0.020333	0.001856	3.90275112	96.09724888	
	125	0.214	0.183	0.209	0.202	0.009609	38.77159309	61.22840691	
	62.5	0.483	0.526	0.499	0.502667	0.012548	96.48112604	3.51887396	
	31.25	0.527	0.511	0.513	0.517	0.005033	99.23224568	0.767754319	

Table 5: The MTT result of the cytotoxic effect of algal extract on human lung normal cells (WI38) following 24 h of exposure. Each experiment was done in triplicate. The dose of algal extract that produce 50% reduction in the viability of WI38 cells (IC50) were compound to be 166.39 µg/mL upon 24 h of exposure.

4.4. Cell cycle arrest by flow cytometry.

To evaluate the role of algal extract in the cell death (anticancer/cytotoxic potential) in cells, the cell cycle arrest analysis was conducted. The results of the cell cycle arrest are presented in Fig. 6a, b and Table 6. The results showed that HCT116 and WI38 cells exposed to extract at 137.95 µg/ml and 166.39 µg/ml respectively exhibited cell growth arrest at G1 phase after 24 h exposure. HepG2 cells exposed to extract at 82.42µg/ml, clearly exhibited cell death at preG1 phase.

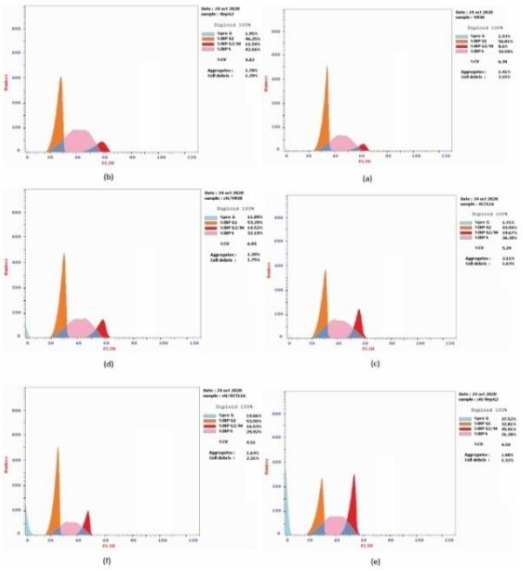

Fig. 6 (a): Flow cytometric analysis was carried out to determine the effects of extract on different phases of cell cycle in cells. [a]: The DNA distribution of WI38 untreated (control) cells; [b]: The DNA distribution of HepG2 untreated (control) cells; [c]: The DNA distribution of HCT116 untreated (control) cells; [d]: The DNA distribution of WI38 cells treated with IC50 concentration of extract for 24 h; [e]: The DNA distribution of HepG2 cells treated with IC50 concentration of extract for 24 h; [f]: The DNA distribution of HCT116 cells treated with IC50 concentration of extract for 24 h.

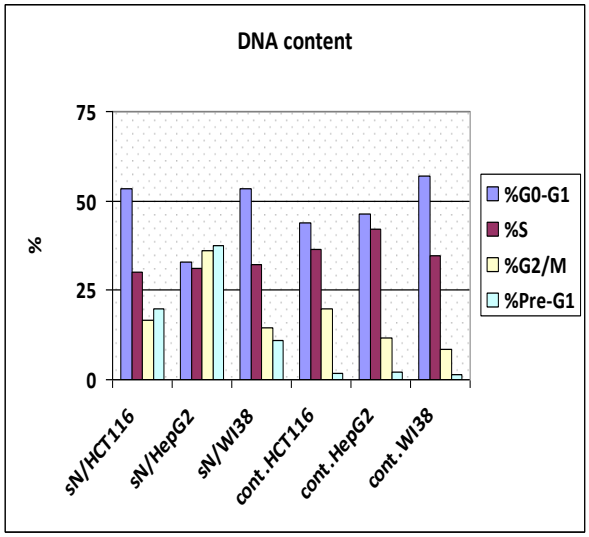

Fig. 6 (b): The distribution of the cell cycle of cells was assessed by flow cytometry after staining with propidium iodide (PI). The bars represent the percentage of cells in different phases of cell cycle. Where sN/HCT116 indicates treated HCT116 cells with

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algal extract, sN/HepG2 indicates treated HepG2 cells with algal extract, sN/WI38 indicates treated WI38 cells with algal extract, cont.HCT116 indicates untreated HCT116 cells, cont.HepG2 indicates untreated HepG2 cells, cont.WI38 indicates untreated WI38 cells.

Table 6: Percentage of cells arrested in different phases of cell cycle after the exposure of extract in HCT116, HepG2 and WI38 for 24 h.

	Sample data		Results DNA content				
	code	IC50 uM	%G1	%S	%G2/M	%Pre-G1	Comment
1	sN/HCT116		53.55	29.92	16.53	19.66	cell growth arrest at G1
2	sN/HepG2		32.81	31.28	35.91	37.52	cell growth arrest at Pre G1
3	sN/WI38		53.29	32.19	14.52	11.09	cell growth arrest at G1
4	cont.HCT116		43.94	36.39	19.67	1.71	No treatment
5	cont.HepG2		46.25	42.16	11.59	1.95	No treatment
6	cont.WI38		56.81	34.59	8.6	1.43	No treatment

4.5. Apoptosis evaluation induced by algal extract in cells.

Determination of phosphatidylserine (PS) externalization is used to determine apoptotic or necrotic cell death by flow cytometry technique. Cells treated with algal extract at IC50 concentration showed the characteristics of apoptosis, i.e., the translocation of phosphatidylserine (PS) to the outer layer of cell membrane demonstrated by Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining at PS and DNA (nucleus), respectively, employing flow cytometry technique as shown in Fig. 7a, b and Table 7. Here, the data are shown in scatter plot, which characterizes the fluorescence generated by FITC and PI staining. The flow cytometry data clearly confirmed that extract primarily initiate cell death by starting early and late apoptotic manners by coincidently convert into the necrotic cell death. Four populations of cells were distinguished from our current results by Annexin-V/PI staining: viable cells (AnnV−/PI−), early apoptotic cells (AnnV+ /PI+), late apoptotic cells (AnnV+ /PI+), and necrotic cells (AnnV- /PI+).

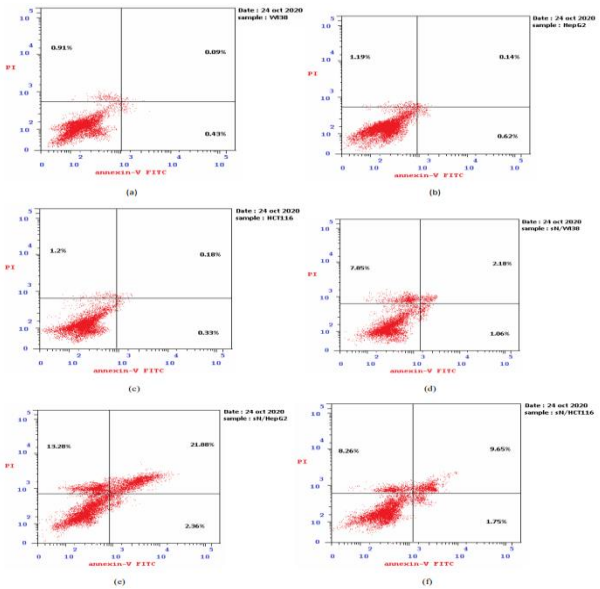

Fig 7 (a): Flow cytometry analysis of apoptosis in cells untreated and treated with IC50 concentration of extract. The scatter plots show total, early apoptosis, late apoptotic and necrotic cells following 24 h exposure. [a]: Control untreated normal WI38; [b]: Hepatocellular carcinoma untreated HepG2; [c]: Colorectal carcinoma untreated HCT116; [d]: Control treated normal WI38; [e]: Hepatocellular carcinoma treated HepG2; [f]: Colorectal carcinoma treated HCT116. The Y-axis represents the PI-labeled population, whereas the X-axis represents the labeled annexin-V FITC-positive cells. The lower left portion of the fluoro-cytogram (An-, PI-) shows viable cells, whereas the lower right portion (An+, PI+) shows early apoptotic cells. The upper right portion (An+, PI+) shows late apoptotic cells, while the upper left portion (An-/PI+) demonstrates the percentage of necrotic cells.

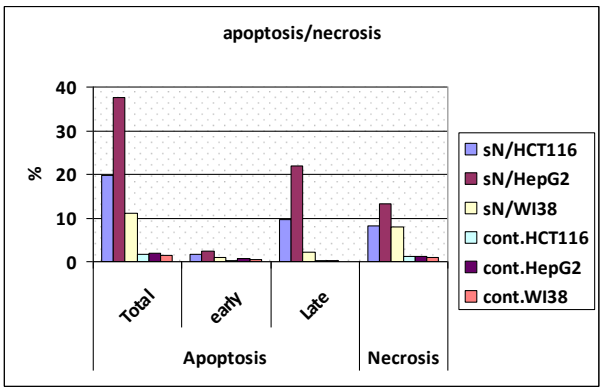

Fig 7 (b): Bar graphs of the percentage of cell populations by annexin-V FITC flow cytometry

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analysis in both untreated and extract treated cells that demonstrate a highly percent increase in the late apoptosis of extract/cells at 24 h as compared to the untreated group.

Table 7: Percent early apoptosis, late apoptotic and necrotic cells following 24 h exposure to extract.

	code	conc	Apoptosis			Necrosis
			Total	Early	Late	
1	sN/HCT116		19.66	1.75	9.65	8.26
2	sN/HepG2		37.52	2.36	21.88	13.28
3	sN/WI38		11.09	1.06	2.18	7.85
4	cont.HCT116		1.71	0.33	0.18	1.2
5	cont.HepG2		1.95	0.62	0.14	1.19
6	cont.WI38		1.43	0.43	0.09	0.91

4.6. Effect of extract on expression levels of apoptosis related genes.

The expression of pro-apoptotic proteins (Bax, p53 and p21) were increased while that of anti-apoptotic protein (bcl2) was decreased in extract-treated cancer cells (sN/HCT116 and sN/HepG2) as well as control treated cells (sN/WI38) compared to control untreated cells (cont.HCT116, cont.HepG2 and cont.WI38) at IC50 concentrations. After 24 h of exposure of HCT116 to extract (137.95 µg) the expression of Bax (4.27-fold), p53 (3.077-fold), and p21 (3.357-fold) were up-regulated. Exposure of HepG2 to extract (82.42 µg) the expression of Bax (12.84-fold), p53 (5.153-fold), and p21 (6.607-fold) were up-regulated. In addition, treatment of normal WI38 cell line to extract (166.39 µg) the expression of Bax (1.38-fold), p53 (1.290-fold), and p21 (1.424-fold) were also up-regulated compared to untreated controls. On the other hand, the expression of bcl2 was down-regulated to 0.396-fold in treated HCT116, 0.514-fold in treated HepG2 and to 0.939-fold in treated WI38 compared to untreated controls. Relative quantities of mRNA of the selected genes in treated cells compared to the untreated controls are presented in Table 8 and Figure 8.

Table 8: Fold changes in gene expression of studied genes in control and treated cell lines to the extract post 24 h of treatment.

	Sample		Results Fold Change				
	code	conc	Bax	bcl2	p53	p21	
1	sN/HCT116		4.27	0.396	3.077	3.357	
2	sN/HepG2		12.84	0.514	5.153	6.607	
3	sN/WI38		1.38	0.939	1.290	1.424	
4	cont.HCT116		1	1	1	1	
5	cont.HepG2		1	1	1	1	
6	cont.WI38		1	1	1	1	

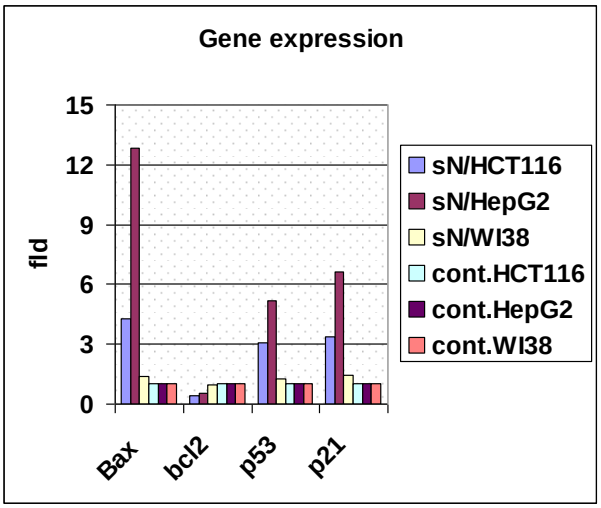

Fig. 8: Gene expression of Bax, bcl2, p53 and p21occurred in studied untreated and treated cell lines after 24 of treatment.

5. Discussion

Carcinoma refers to a wide range of illnesses characterized by unregulated cell growth in the body. There are over 200 distinct forms of cancer, and certain tumors may gradually extend into other organs, creating fatal metastasis. During century, surgery, chemotherapy, and radiation were the only options (22). New therapeutic compounds must be developed which are anticipated to be safer, more chemopreventive and successful at therapeutic strategies of cancer treatment, and less expensive than currently available chemical-based treatments. In this context, natural compounds with less toxicity and side effects should be preferred in the treatment of carcinogenesis (23). More recently, there is great interest in exploring the biotechnological potential of microorganisms such as microalgae since they are easier to cultivate, have short generation times and represent a renewable and still poorly explored resource for drug discovery (24).

Due to being rich in bioactive substances, microalgae play a crucial role in the prevention and treatment of cancer. Microalgae are photosynthetic microorganisms that produce abundant bioactive compounds such as proteins, polysaccharides, lipids, polyunsaturated fatty acids, vitamins, pigments and enzymes (25). These bioactive compounds show anti-microbial, antioxidant, anti-inflammatory, neuroprotective, and anti-cancerous effects (26).

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In the current study, anticancer properties of *Amphora coffeaeformis* were evaluated by testing the antiproliferative activity against human HepG2 and HCT116 cancer cells. The present study reported that the GC-MS analysis of fatty acids in the *Amphora coffeaeformis* showed isolation of 30 different fatty acids. Thirteen main compounds inclusive Phytol (21.66%), Heptadecane (15.23%), cis-9,cis-12-Octadecadienoic acid (11.93%), n-Hexadecanoic acid (11.06%), 1-Eicosanol (4.58%), 2-Pentadecanone, 6,10, 14-trimethyl, (4.06%), 9,12-Octadecadienoic acid (Z,Z) (3.32%), Palmitoleic acid (3.28%), Neophytadiene (3.22%), 9-Octadecenoic acid (Z) (2.36%), Linolenic acid (2.13%), 3-Hydroxy-à-ionene (2.11%) and 8-Heptadecene (1.84%). In addition, the remains 17 fatty acids founded in minor amount. These results are in similar to the previous study that reveals *Amphora coffeaeformis* composed of substantial amounts of lipids, proteins, minerals, sugars, carotenoids, chlorophylls, fatty acids, and polyphenols (27). Moreover, *Amphora coffeaformis* contained polyunsaturated fatty acids as linoleic acid (7.28 %, omega 6 FA) and linolenic acid (1.84 %, omega 3 FA). Besides, *Amphora coffeaformis* contains monounsaturated fatty acids as oleic acid (44.30%), stearic acid (9.24 %), and vaccenic acid (2.28%). It also contains saturated fatty acids as palmitic acid (30.18%), palmitoleic acid (3.86%), and undecanoic acid (1.01%) (17, 18, 28).

In our study, microscopic examination of HepG2 and HCT116 cancer cell lines revealed cytotoxic features after a 24 h treatment period, where treated cells with *Amphora coffeaeformis* became rounded, granulated and the cells become shrunken and detached out of the monolayer along with increasing extract concentrations (125 to 1000 µg/mL) compared to the normal appearance of untreated cells. These results may be due to different bioactive components in *Amphora coffeaeformis* extract that may modulate immune system and decrease growth of cancer cells. Our results are in agree with (29), who demonstrated that *Amphora coffeaeformis* supplementation has been shown to improve fish immunity by reducing pathogenic bacteria and increasing the innate immune system response of fish.

In the present investigation the degree of cytotoxicity of algal extract to cell lines was determined using MTT assay. Cancer cells (HepG2 and HCT116) treated with increasing concentrations of extract (125 µg–1000 µg/mL) exhibited decreased cell viability. However, the results were better in HepG2 where the extract displays toxicity to 50% of cells at low IC50, 82.42µg/ml compared to IC50 (137.95 µg/ml) of extract with HCT116. The extract exhibits also toxicity to normal cells (WI38) but it could induce death to 50% of cells at high IC50, 166.39µg/ml. These data showed that *Amphora coffeaeformis* have ability to limit and decrease cell cancer viability in a dose-dependent manner. This may be due to different important fatty acids in *Amphora coffeaeformis* such as phytol, heptadecane, palmitoleic acid, linolenic acid and so on. These results are in agreement with (17) who demonstrated that *Amphora coffeaeformis* contain bioactive constituents such as polyunsaturated fatty acids, sulfated polysaccharides, carotenoids, astaxanthin, canthaxanthin, b-glucans, and vitamins C and E which have antioxidant properties against oxidative stress. In addition, oleic acid significantly reduced viability of hepatocellular carcinoma cell lines at 300µM through Alamar Blue staining evaluation (30).

Furthermore, the results of the cell cycle arrest showed that HepG2 cells treated with Amphora extract exhibit proliferative arrest at preG1 stage. While HCT116 cells and WI38 cells exhibited cell growth arrest at G1 phase after 24 h exposure. We next investigated whether apoptosis could be the cause of the extract-induced cell anti-proliferation; thus, an Annexin V-FITC/PI double staining assay was performed. Results showed that algal extract induced death in cells, by induction of both apoptosis and necrosis but mainly through late apoptosis. We suggest that *Amphora coffeaeformis* has a negative regulator role of proliferation in cell lines. So, *Amphora coffeaeformis* extract reduces cancer cells viability and increase cell death. Previous studies reported that oleic acid has cell proliferation inhibition and apoptosis induction (30). In addition, *Amphora coffeaeformis* has antioxidant and radical scavenging activity through their strong hydrogen donating activity in vitro (28).

Furthermore, the expression of pro-apoptotic proteins (Bax, p53 and p21) were increased in all extract-treated cells (sN/HCT116, sN/HepG2 and sN/WI38) but it was highly upregulated in HepG2 while that of anti-apoptotic protein (bcl2) was decreased in all extract-treated cells specially HCT116 compared to control

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untreated cells (cont.HCT116, cont.HepG2 and cont.WI38) at IC50 concentrations. These data represented that *Amphora coffeaeformis* as a possible inducer of cell death processes in human hepatocellular carcinoma HepG2 and human colorectal carcinoma HCT116 cell lines by modulating cell death regulators. Our results are in agreement with previous results of (31) who reported the various species of microalgae have anti-tumor activity, the tumor cell lines altered through using microalgal extracts along with the levels of such extracts that reported to its inhibitor effect against cell cycle and proliferation.

DNA damage is a common event in life following which, repair mechanisms and apoptosis will be activated to maintain genome integrity. The tumor-suppressor genes Bax, p53, and p21 provide an important link between DNA damage and apoptosis. DNA damage results in cell cycle arrest at checkpoints or at G1 or G2 stage to inhibit cell cycle progression and to induce apoptosis, thus protecting cells against further damage (32, 33).

6. Conclusion

The active molecules from algal extract likely interact with special cancer-associated receptors or cancer cell special molecules, thus triggering specific mechanisms (such as apoptosis of tumor cells) that caused cancer cell death. The extract increased the expression of P53, Bax and p21 tumor suppressor genes and inducing a reduction of Bcl-2 apoptosis suppressor gene, which subsequently can either enforce cell cycle arrest or induce apoptosis against the damaged cell to protect the organism.

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