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Original Article

Oncology Section

Anticarcinogenic Efficacy of Fucoxanthin on HepG2 Cell Lines

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ABSTRACT

Introduction: Hepatocellular Carcinoma (HCC) is considered one of the deadliest cancers which grow rapidly worldwide. Fucoxanthin can be acquired from edible brown seaweeds, reported that fucoxanthin has numerous physiological functions and biological abilities, and various medicinal properties.

Aim: To evaluate the anticarcinogenic efficiency of fucoxanthin against the Human Hepatoma Cell Line (HepG2).

Materials and Methods: This in-vitro examination and research was carried from June 2018 to August 2018, at Alagappa University, Karaikudi, Tamil Nadu, India. Fucoxanthin at different concentrations was taken to demonstrate its antiproliferative potential and its cytotoxic effect. In this present examination, based on the outcome of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, 80 μM and 100 μM were chosen for further in-vitro studies. It was noted that remarkable leakage of Lactate Dehydrogenase (LDH) and a noteworthy decrease in the Glutathione (GSH) content of 80μ M and 100μM of fucoxanthin treated HepG2 cells. Under light and fluorescence microscopic examination, there was a significant reduction in cell growth and cell proliferation in the fucoxanthin-treated HepG2 cells. Data were presented as the mean±standard deviation. The one-way analysis of variance (ANOVA) and Tukey's multiple comparison method were used to find the level of significance.

Results: The human HepG2 to be studied and analysed were divided into group I: control group; group II: comprising those cells treated with fucoxanthin in concentration of 80 μM; group III: comprising those cells treated with fucoxanthine in concentration of 100 μM. The results of the MTT assay put forward that fucoxanthin has the cytotoxic and antiproliferative potential against HepG2 further fucoxanthin significantly alter the marker enzymes level (p-value <0.05). The therapeutic efficiency of fucoxanthin might be due to the antioxidant effect of the bioactive compound. In this present investigation, the results of in-vitro studies, fucoxanthin strongly put forward that it has potential anticarcinogenic efficacy against the hepatic tumour (p-value <0.05).

Conclusion: The results of this analysis undoubtedly symbolise that fucoxanthin has antioxidant activity and anticarcinogenic efficacy against primary liver cancer HCC. Due to their therapeutic efficacy, may be considered as an excellent candidate against HCC.

Keywords: Antioxidant, Hepatic tumour, Hepatoma cell line, Microscopic examination, Primary liver cancer

INTRODUCTION

HCC, most common primary liver malignancy and it is the most leading deadly tumour in the world. Studies reported that men are mostly affected by HCC when compared with women, especially people over the age of 50 years. Chronic hepatitis B and C virus are the leading causative factors for HCC [1]. Primary HCC, called hepatoma and is one of the common liver malignancies. One of the most effective preventions of HCC is to prevent hepatic viral infection and cirrhosis because these are the important risk factors that are responsible for HCC. The pathogenesis of HCC is comprised of various hereditary/epigenetic variations and changes with many flagging pathways that lead to a known heterogeneity of the sickness biologic and clinical conduct [2].

Fucoxanthin, one of the natural medicines obtained from brown seaweed such as wakame (*Undaria pinnatifida*) and hijiki (*Hijikia fusiformis*) and it is also found in some dietary supplements. Some biological studies reported that fucoxanthin contains different biological benefits and some physiological functions [3]. It has antitumour, antioxidant, anti-inflammatory, antiobesity, and antidiabetic properties. Fucoxanthin's well known metabolites are fucoxanthinol and amarouciaxanthin. Fucoxanthinol was changed over into amaouciaxanthin A which was transcendently appeared in liver microsomes of mice and HepG2 cells [4]. The anticancer impacts of fucoxanthin and fucoxanthinol are conjectured to be brought about by apoptosis enlistment and cell cycle arrest. The objectives of this investigation were to bring out the antioxidant potential, cytotoxic and antioncogenic effect of fucoxanthin. The target of this present study is to scrutiny the antitumourigenic potency of fucoxanthin against HepG2.

MATERIALS AND METHODS

The present study was an in-vitro investigation carried out at Alagappa University Karaikudi, Tamil Nadu, India, from June 2018 to August 2018.

Reagents: Chemical reagents were commercially acquired from Sigma Aldrich, St Louis, covering Dimethyl Sulfoxide (DMSO), Ethidium bromide (EtBr), and essential fucoxanthin in online mode. Culture medium such as RPMI (Roswell Park Memorial Institute)-1640, sodium pyruvate, penicillin, streptomycin, and bovine serum was procured from High Media Chemical Laboratory Pvt Ltd, Bangalore. Trypsin-Ethylene Diamine Tetra-acetic Acid (EDTA) was purchased from the same company at the door of the Chennai branch. In-vitro, culture plates and dishes were collected from Apex biotech ltd., Chennai, Tamil Nadu. Other necessary solvents immaculateness was acquired from Glaxo laboratory. Mumbai, India.

Preparation of drug: Fucoxanthin was diluted in Dimethylsulfoxide (DMSO) to the optimum concentration arranged in order with RPMI medium and placed at 4°C after purification used with syringe filter.

HepG2 cells maintenance and fucoxanthin treatment: Human HepG2 cells were used for this investigation. HepG2 cell lines were procured for this investigations from National Centre for Cell Sciences, Department of Biotechnology, Pune, India. Subsequently [5], deciphered those actions of antineoplastic were represented in monolayer cell culture. By achieved, the enormous quantity of replicate culture techniques has opened up the way and is evident for exploring several compounds [6]. HepG2 is a cell line originating from the liver tissue of humans and grown using the monolayer culture method in the presence of RPMI-1640 medium. A monolayer

culture was established under a controlled environment, humidified atmosphere, used with RPMI-1640 medium, and blended with heat-inactivated Bovine Serum along with few antibiotics such as penicillin 100 IU/mL, streptomycin 10 µg/mL, and 1 mM sodium pyruvate in the ratio of 5% CO_2 at 37°C, Subsequently, after three days' time duration, a reformed medium was observed and incubated, for another one day. The culture was trypsinised well used with 0.05% trypsin and EDTA (0.02%). Among them, 1×10^4 thickness cells were prioritised and chosen for the seeding process in each well plate for the MTT analysis. With a thickness of 0.5×10^6 cells were selected for LDH leakage assay. For subsequent investigations such as the GSH test and others, cells were incubated with or none fucoxanthin for a period of 24 hours.

MTT assay for cell viability: 3 (4, 5 dimethyl thiazol 2 yl) 2, 5 diphenyl tetrazolium bromide (MTT) procedure was executed as defined [7]. The feasibility of the cells was measured by MTT assay, which is based on the lessening of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Using an Enzyme Linked Immunosorbent Assay (ELISA) plate reader, the quantity of formazan was determined and the percentage of viable cells was calculated by measuring the absorbance at 540 nm using an ELISA plate reader.

Experimental setup: The HepG2 cell lines were divided into three groups. Group I was control, Group II & III cell lines were planned to be treated with fucoxanthin based on the results of MTT assay. Cells were placed in a standard humidity and atmosphere of 5% CO_2 and 95% air for a period of 48 hours. Based on the cell viability and cytotoxic assay, 80 and 100 µM were chosen for further enzymatic analysis and microscopic observations. Group II were treated with fucoxanthine in concentration of 80 µM and Group III with concentration of 100 µM.

Assessment of enzyme marker: Estimation of LDH leakage was measured using Grivell and Berry method [7]. The reduction rate of total Glutathione (GSH) was estimated by following the method outlined by Moron MS et al., [8].

Light microscopic study: Morphological alteration and vicissitudes that occurred due to the treatment of fucoxanthin for 24 hours were visualised by an inverted light microscope was attempted [9]. Both control and fucoxanthin-treated HepG2 cells were examined to observe the abnormalities that occurred.

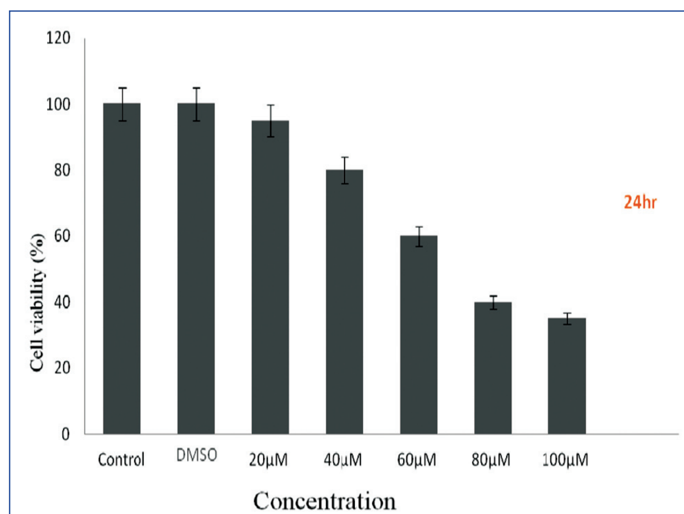
Fluorescent microscopic observation: Cells were stained with the fluorescent dye (Rhodamine Dye). After adequate treatment, cells were recovered carefully, washed well, and suspended in Phosphate buffered saline (PBS). Followed by the Whole Cell Stains (WCS) straining, a buffer solution, 100 µg/mL of phosphate buffer were added to dissolve and cells were tested [10]. Variations in the ingestion of the dye indicated the survival of cells under fluorescent output. Results were analysed. The cytotoxic impacts of different synthetic concoctions and characteristic substances on threatening tumour cells in culture have been broadly considered as an essential screening for antitumour exercises [11].

STATISTICAL ANALYSIS

Data are presented as the mean±standard deviation (SD). The comparison of the means of different groups was achieved with the help of the Statistical Package for Social Sciences (SPSS) student's version 12.5 and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison method was used. Statistically significant values were compared between the control and drug-treated groups. The significance was considered at the level of p-value <0.05.

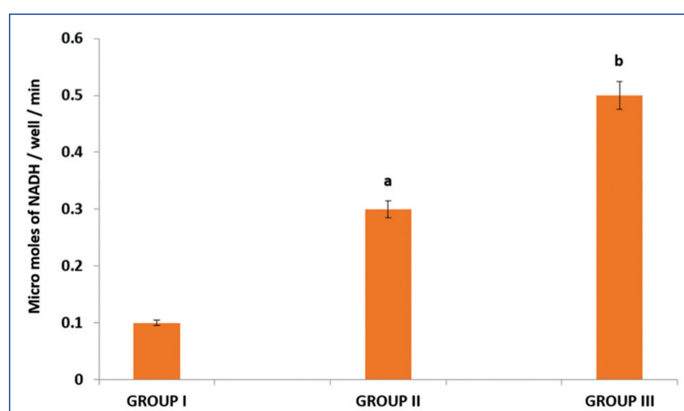
RESULTS

In the present investigation, the chemotherapeutic adequacy of Fucoxanthin against human HepG2 was carried out. In this setting cytotoxicity, LDH enzyme activity, GSH estimation, and microscopic examinations were performed. The antiproliferative impact of fucoxanthin on HepG2 cells was tried by MTT strategy, which is dependable to identify the expansion of cells. [Table/Fig-1] display various concentrations of fucoxanthin (20, 40, 60, 80, and 100 µM) for 24 hours, which brought about diminished cell multiplication in a dose and time-dependent pattern when compared with control cells (p-value <0.05). Hence the responses were analysed by dividing human HepG2 cells into three groups. Group I as control while Group II of those cells treated with fucoxanthine of 80 µM and Group III of those cells treated with concentration of 100 µM.



[Table/Fig-1]: Effect of fucoxanthin on HepG2 cells viability-MTT assay.
Each bar represents the mean±SD of control and treated cells; Control Vs Dimethylsulfoxide (DMSO) 20, 40, 60, 80 and 100 µM for 24 hour

The degrees of LDH discharged into the mechanism of control and fucoxanthin-treated HepG2 cells are exhibited in [Table/Fig-2] (p-value <0.05). From this figure, it was seen that marker enzyme LDH leakage was elevated after 24 hours introduction of 80 and 100 µM fucoxanthin. LDH elevation in drug-treated cells when contrast was done with the control confirms the cytotoxic nature of fucoxanthin (p-value <0.05). Each bar represents mean±SD of control and treated cells. It is outstanding that, GSH assumes a significant occupation in securing cells and cell segments against oxidative pressure and in detoxification. It is frequently discovered that GSH levels are expanded in the medication-safe disease cells when contrast was done with the medication induced cells. Hindrance of GSH amalgamation or balance of GSH stockpiles in tumours to lessen anticancer medications opposition may comprise a novel anticancer methodology [12].

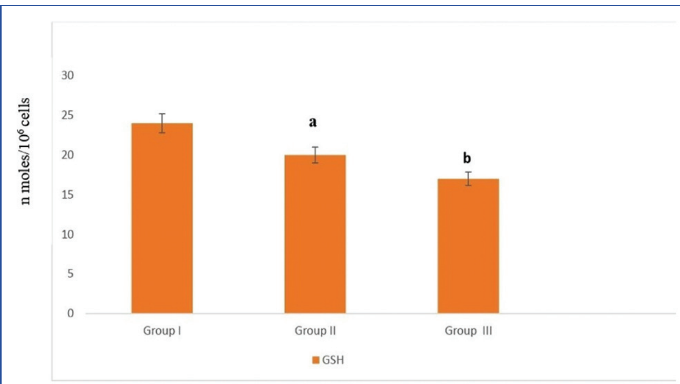


[Table/Fig-2]: Cell viability and LDH leakage in control and fucoxanthin treated HepG2 cells after 24 hour of exposure.
Group I: Control Group; Group II: cells treated with fucoxanthine 80 µM; Group III: cells treated with fucoxanthine 100 µM; Bar a. Group I vs Group II and Group III; Bar b. Group II vs Group III

The levels of GSH content in control and fucoxanthin treated HepG2 cells were exhibited in [Table/Fig-3]. The critical depletion of GSH (p-value <0.05) was seen in fucoxanthin-treated HepG2 cells at 80 and 100 µM concentrations when contrast was done with the control cells.

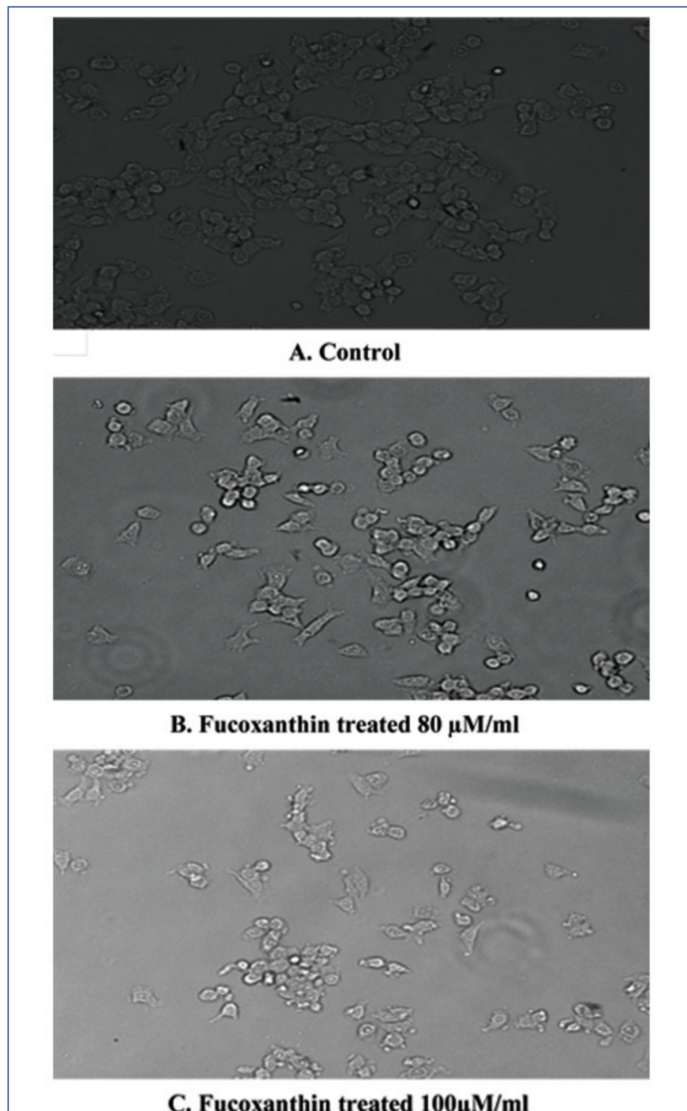
Each bar represents the mean SD of control and treated cells:

a. Group I vs Group II and Group III
b. Group II vs Group III

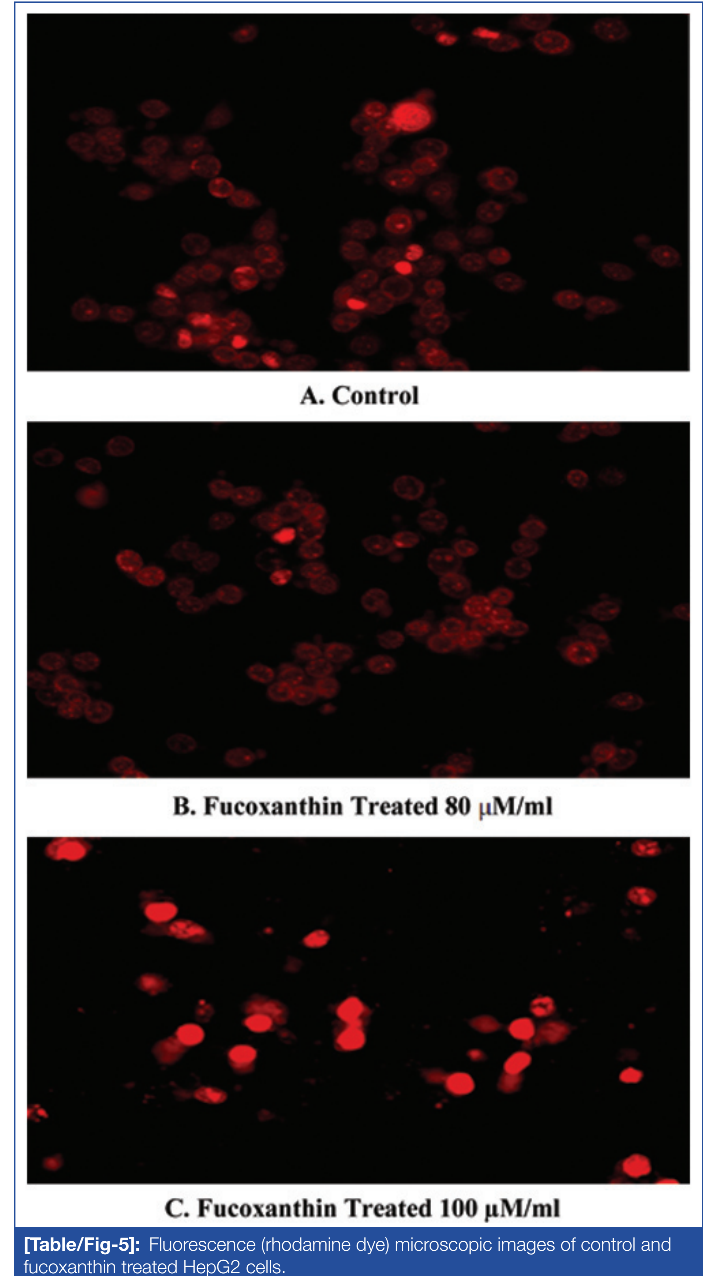


[Table/Fig-3]: Levels of GSH in control and fucoxanthin treated HEPG2 cells.
Group I: Control Group; Group II: cells treated with fucoxanthine 80 μM; Group III: cells treated with fucoxanthine 100 μM; Bar a. Group I vs Group II and Group III; Bar b. Group II vs Group III

Apoptosis is a hereditarily controlled reaction of a characteristic cell framework that is required for a basic harmony between cell expansion and cell demise in typical improvement and support of homeostasis of a living being. Numerous chemotherapeutic specialists smoother the development of changed or dangerous cells by actuating apoptosis [13]. An enlistment of tumour cell apoptosis is one of the productive focuses for medicating advancement and has become a significant concentration in the investigation of disease treatment. Along these lines, the light and fluorescence minuscule and DNA fracture investigation was concentrated to decide the nearness of apoptosis in fucoxanthin-treated HepG2 cells. [Table/Fig-4] indicated the morphological changes of control and fucoxanthin-treated HepG2 cells at the concentration of 80 and 100 μM for 24 hours of treatment. In fucoxanthin treated HepG2 cells, the obliteration of monolayer was observed. It is not observed in HepG2 cells without fucoxanthin treatment. Control HepG2 demonstrated the growing and adjusted morphology of the cells with dense chromatin and their film. Fucoxanthin-treated cells display condensed chromatin and a shapeless nucleus. Dynamic auxiliary adjustments and a decrease of HepG2 cell populaces were seen in both concentrations of fucoxanthin. [Table/Fig-5] declared that the fluorescent microscopic image of control and 80 and 100 μM fucoxanthin treated HepG2 cells for 24 hours. Normal cores show chromatin with a composed structure, while apoptotic cores show exceptionally condensed chromatin in HepG2 cells. In the present current investigation, typical live cells were showed up in Rhodamine dye shading.



[Table/Fig-4]: Light microscopic images of control and fucoxanthin treated HepG2 cells.



[Table/Fig-5]: Fluorescence (rhodamine dye) microscopic images of control and fucoxanthin treated HepG2 cells.

DISCUSSION

Albeit customarily malignant growth has been battled with the typical armamentarium of chemotherapy and high portions of coordinated radiation, of late there has been more consideration committed to fighting disease through nutritive methods [14]. Specifically, wide scopes of bioactive supplements have been discovered valuable in the physiological fight against malignant growth. HCC is the most widely recognised malignant growth around the world. It is related to poor endurance. HCC is a forceful tumour-related with dreary

anticipation. Careful resection and liver transplantation are the two remedial medicines for HCC, yet are relevant to just a little extent of patients with the beginning phase of tumours [15]. As of now, there is no successful fundamental chemotherapy for HCC, though elective treatment methodologies, for example, transcatheter blood vessel chemoembolisation, percutaneous intratumoural ethanol infusion, and radiofrequency removal are for the most part for vindication and are pertinent just to patients who have tumours confined in the liver. HCC is unmistakably a sickness for which elective remedial modalities are created. An intensive comprehension of the pathogenesis of HCC along these lines holds the guarantee of finding successful chemoprevention and curing malignant growth [16]. Appraisal of the in-vitro cytotoxicity has as of late gotten well known as an essential screening strategy for assessing the antitumour exercises of different synthetics and common substances [17]. To examine the anticancer action of fucoxanthin on cell feasibility, the LDH spillage test and estimation of GSH were acted in the HepG2 cell line. In the present examination, fucoxanthin fundamentally diminished cell suitability in a focus subordinate way. The concealment of cell expansion actuated by fucoxanthin might be because of the acceptance of cell demise. Along these lines, the inhibitory action of fucoxanthin is proof of in-vitro cytotoxicity. Late examinations recommend that the MTT measure is solid to evaluate cell reasonability.

MTT is a yellow water-soluble tetrazolium salt. Metabolically dynamic cells can change over the salt to water-insoluble dim blue formazan gems [18]. Hence, the measure of formazan created is corresponding to the number of living cells. From the outcomes, it is gathered that the introduction of various convergence of fucoxanthin (20, 40, 60, 80, and 100 μM/ml) for 24 hours came about the decline of cell expansion in a portion subordinate way. Fucoxanthin about represses half-cell populace at the convergence of 80 and 100 μM for 24 hours when contrast was done with control. Along these lines, the inhibitory impact on HepG2 cells by fucoxanthin unequivocally demonstrates the counter expansion property of Fucoxanthin. Ongoing investigations recommend that LDH is an increasingly solid and progressively precise marker to ponder the cytotoxicity. Since harmed cells are divided totally throughout delayed hatching with poisonous substances [19].

In the present investigation, the LDH spillage expanded fundamentally in fucoxanthin treated HepG2 cells when contrast was done and the control cells. This outcome proposes that the hindrance might be because of the antitumour advertisers of the fucoxanthin. In this manner, it is proposed that the LDH spillage in HepG2 cells might be because of the cytotoxic nature of fucoxanthin and affirm its antitumour movement. Various laborers have called attention to those intracellular oxidative metabolites that assume a noteworthy job in the guideline of cell death. In this way, the estimation of GSH in medicating treated HepG2 cells establishes the auxiliary proof for programmed cell death.

Tripeptide GSH comprises a bizarre peptide connection between the amine gathering of cysteine and the carboxyl gathering of the glutamate side-chain. GSH, a cancer prevention agent that shields cells from responsive oxygen species, for example, free radicals and peroxides. GSH is nucleophilic at sulfur and assaults harmful electrophilic conjugate acceptors. GSH is originating only in its decreased structure, subsequently, the protein that returns it from its oxidised structure, GSH reductase, is constitutively dynamic and inducible upon oxidative pressure [20]. The proportion of diminished GSH to oxidised GSH inside cells is utilised deductively as a proportion of cell lethality. Decreased GSH has been estimated to assume a job in the salvage of cells from apoptosis, by buffering an endogenously actuated oxidative pressure. The beginning of apoptosis was related to a fall of intracellular GSH in various cell frameworks. Loss of GSH was demonstrated to be firmly combined with various downstream occasions in apoptosis [21]. It is realised that the lethality of antitumour medications may to a great extent rely upon the intracellular degree of decreased GSH.

GSH is known as a substrate in both conjugation responses and decreases responses, catalysed by GSH S-transferase compounds in the cytosol, microsomes, and mitochondria. Be that as it may, it is likewise fit for taking an interest in non enzymatic conjugation with certain synthetic concoctions. GSH exists in decreased (GSH) and oxidised (GSSG) states. In the decreased express, the thiol gathering of cysteine can give a lessening comparable (H++e-) to other temperamental particles, for example, receptive oxygen species. In giving an electron, GSH itself gets responsive, yet promptly responds with another receptive GSH to frame GSH disulfide (GSSG) and such a response is conceivable because of the generally high convergence of GSH in cells (up to 5 mM in the liver). GSH can be recovered from GSSG by the catalyst GSH reductase. This recommends Reactive Oxygen Species (ROS), particularly H_2O_2 may be associated with the procedure of apoptosis [22]. Concentrates in an assortment of cell types recommend that disease chemotherapeutic medications actuate tumour cell apoptosis to a limited extent by expanding the development of ROS. Since, GSH is known to ensure cells against the danger of various operators.

In this current study, it was seen that the degrees of GSH were essentially diminished in fucoxanthin-treated HepG2 cells at the grouping of 80 and 100 μM. This demonstrates the lessening in GSH might be engaged with the restraint of HepG2 cell development and the basis of apoptosis. Consumption of GSH has been depicted for a few operators, for example, oxidative and alkylating specialists in different cell types. The present investigation demonstrates that the fucoxanthin may quickly initiate intracellular oxidation in HepG2 cells and leads to apoptosis which causes cell passing. It is theorised that fucoxanthin may incite ROS generation in HepG2 cells and in this way induce apoptosis. Not withstanding, ROS was not the immediate factor to cause apoptosis incited by the medication, however intracellular ROS may regulate the qualities associated with cell death. In the present examination, fucoxanthin prompted oxidative anxieties upstream of flagging occasions that may modify the professional and antiapoptotic balance in the HepG2 cells.

The phenolic mixes are having their cancer prevention agent impacts as well as expert oxidant activities under the in-vitro conditions [23]. In this examination, it appeared to be conceivable that treatment with fucoxanthin in HepG2 cells exhaust the GSH levels and potentiates somewhat of oxidation acceptance, which exchanging the method of death employing apoptosis. Along these lines, the cytotoxic activity of this medication might be credited to its genius oxidant activity on the cells. This might have the option to represent the inconsistency in-vitro cytotoxicity and in-vivo antitumour exercises of fucoxanthin. Apoptosis is vitality subordinate, firmly managed and specific physiological procedure that oversees the expulsion of excessive or faulty cells. It happens in an ordinary biological manner. It can likewise be activated by various pathologies. In sound tissues, the fundamental job of cell death is to keep up the ideal quantity of cells in tissues and organs by expelling the excess, harmed, or practically anomalous cells [24].

The most transcendent morphologic highlights of apoptosis are film blebbing, cell shrinkage, and chromatin build-up. Apoptosis can be initiated by various boosts including some cell harming specialists and malignant growth treatment. In this association, light, fluorescence infinitesimal perception, DNA fracture and protein articulation in fucoxanthin treated HepG2 cells were contemplated. In the present examination, light tiny perception of fucoxanthin examined HepG2 cells at groupings of 80 and 100 μM after 24 hours of introduction demonstrated the run-of-the-mill morphological highlights of apoptosis in HepG2 cells. The structural variations watched were the decrease in cell volume, cell shrinkage, decrease in chromatin build-up, and development of cytoplasmic blebs. Be that as it may, the control HepG2 cells were observed with the higher intersection of the monolayer

with no obliteration. It was seen that the fluorescence infinitesimal examination indicated apoptosis in fucoxanthin-treated HepG2 cells at convergences of 80 and 100 μM. In the present examination, control HepG2 cells and fucoxanthin-treated cells were recolored green in shading. When all is said in done, cytotoxic medications actuate an enormous breakage of DNA into oligonucleosomal parts is a late occasion of apoptosis. In this way, the fucoxanthin prompts DNA harm in HepG2 cells and along with these lines' causes' cell death. From this perception, it is deduced that fucoxanthin may apply an anticancer impact through DNA harm in HepG2 cells and advancing apoptosis. Hence, the likely utilisation of fucoxanthin and its derivatives, as co-adjuvant specialists in the therapy of disease should be added in-vivo and in-silico researched.

Limitation(s)

Combined therapies of fucoxanthin or fucoxanthinol with normal anticancer medicines can uphold regular restorative procedures by lessening drug opposition. Undoubtedly, as an anticancer atom, fucoxanthinol seems, by all accounts, to be a more viable bioactive compound than fucoxanthin. Hence, the likely utilisation of fucoxanthinol and other fucoxanthin subordinates, as co-adjuvant specialists in the therapy of disease ought to be additionally researched.

CONCLUSION(S)

From the present analysis, it was concluded that fucoxanthin has notable cytotoxic and antiproliferative efficacy by damaging carcinogenic cells through realtering the cancer cell growth factors. It has apoptotic efficiency which was confirmed in microscopic investigated HepG2 cells. Previously, it has been reported that bioactive compounds flavonoids, alkaloids phenolics, and terpenoids exhibited antineoplastic activity in various in-vitro studies which supports these conclusions. Thus, it was powerfully recommended that fucoxanthin is a spectacular contender against human hepatoma cells HepG2.

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