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Study the effect of purified cow milk glutathione on cancer cell growth (HePG2) in vitro

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***Abstract*---**Glutathione is an important protein in many biological applications as a potential cancer treatment agent .In this study, glutathione was purified from cow milk whey by gelfiltration using Sephadex G-10 column. To know its ability as anticancer agent the study utilized an *in vitro* evaluation for the cytotoxic effect of the purified whey glutathione on *HePG2* cell line at different concentrations and different exposure time of treatment. The purified glutathione concentrations ranging (6.25 to 400) µg/ml for 24,48 and 72 hours. The effect of glutathione was evaluated by employing MTT assay. The results revealed a significant cytotoxic effect at levels ($P<0.05$) for all concentrations and for all exposure time as compared to untreated control cells, The inhibition rate IR% increased with the increased in glutathione concentration and incubation period. The highest inhibitory growth was shown at concentration (400µg/ml) after 72 hrs of exposure time it was 90% .

***Keywords*---**cow milk whey, glutathione, HePG2, MTT assay, anticancer.

Introduction

Cancer is a group of disease characterized by uncontrolled growth and spread of abnormal cancer cells, in which normal cells start multiplying un controllably ignoring signal to stopped accumulating to form a mass that is generally termed a tumor (Skuse, 2015). This disease is the second leading cause of deaths worldwide as it still takes millions of people lives every year around the world. In 2008, almost 12.7 million people were diagnosed with cancer and more than 7.5 million of them were dead (Siegel et. al., 2012) the world health organization (WHO) estimated that the annual global cancer deaths may rise to 19 million by 2020 (Rastogi *et. al.*,2004). Recently in Iraq there is a terrible number of un

published cancer cases beside the published case by Iraqi cancer council in 2016 which were 25.55 thousand and more than 24 thousand of they were dead (IARC,2016). The current conventional cancer treatment options for localized tumors and advanced disease are typically associated with risks and side effects (Shin et. al., 2020.) The discovery of anticancer drugs remains a highly challenging endeavor since cancer a hard to cure disease (Hatzimichael, 2013). The new protocols for cancer therapy include biological natural products. An increasing interest has been reported on the use of biologically active substances from food (Kifah, *etal* .2015 and Comstock et. al.,2014). Whey proteins were received considerable attention in recent years to being one of the biologically role as anticancer specially glutathione (Kifah,*et al*,2015;Zainab,*et al*,2015 and Matés *et.al.,* 2020). glutathione is a tri-amino acid peptide that have a major role in cell biology, The main role of this tri-peptide in cellular activity was maintain as the equilibrium state between oxidation and antioxidant The oxidation state inside the cells generated as a result of the metabolic activity continuously produce what is known the reactive oxygen species (ROS) (Lv *et. al*; 2019). These types of molecules are very harmful if they remain inside the cells for a long period as are self of it high oxidizing capacity, therefore they should be removed from the cells as soon as possible, Here comes the role of glutathione, which reduce all ROS to its ground stat, leaves them harmless to the cell (Wu and Batist 2013). *In vivo* studies showed that oral administration of glutathione reduced tumorigenic in different organs such as breast, liver, lung and colon. Glutathione was found to induce apoptosis in several human cell lines. More over glutathione was effective against breast and liver cancer cells (Kalinina and Gavriliuk, 2020). There is no study about the effect of cow milk glutathione on any cancer cell line in Iraq ,Therefore this project was designed to study the effect of a range of glutathione concentrations at different exposure times on cell viability by using cancer cell line hepatocellular cancer cell line (HePG2) as a model.

Material and Methods

The source of cow's milk

Cow's milk was obtained from the Research Station of the Agricultural Research Directorate - Ministry of Agriculture - Abu Jarib – Baghdad

Preparation of Cheese whey

one liter of fresh cow's skim milk was heated to 40 ° C in a suitable container using a laboratory hot plate. Then the milk was cooled to room temperature to 25 ° C. After that, appropriate amount of rennet solution was added to the milk with stirring and raised the milk to curdle temperature . the curd was cut to formed 1 cm cubes by using a knife. Then the curd was lifted to settle for 30 minutes, and the produced whey was sucked up into a 500 mL beaker (Zainab *et al.*, 2015) .The Cheese whey was skimmed by centrifugation in a Sigma MA3-18 centrifuge at 4000 g/min for 30 min at 4°C. Cheese whey was prepared by precipitation of the casein from skimmed whey in acidic condition with gradual addition from 1N HCl until pH reached to 4.6, the precipitated casein was removed by centrifugation at 12000g/min for 30 min at 4°C. The supernatant (whey) was adjusted to pH 6.8 with 1N NaOH. After adjusting the pH, some turbidity was observed in the solution, so the solution was filtered with 0.22 µM Mlipore membrane filter. The

prepared whey solution was lyophilized and stored at -20°C until used. (Al-Mashakhi and Naqai, 1987).

Detecting glutathione (GSH) in the prepared whey sample with the colorimetric method

Glutathione concentration in prepared whey

Glutathione assay kit, Bioassay TM (US Biological, Swampscott, Massachusetts 01907, USA.) was used according to the manufacturer user manual with some modification. Briefly, four 10 mL volume tubes were prepared, the first one contains 1 mg of the lyophilized prepared whey that has been dissolved in 1 mL of phosphate buffer saline pH 7.4 and mixed 600 µL of reagent A of the kit. The second one containing the same measurements for a standard glutathione sample. Tubes were centrifuged for 5 min at 5000 rpm at 4C0. Then 1mL from that supernatant was transferred to another 10 mL tubes and mixed with 1 mL of reagent B of the kit (DNTB reagent), tubes were incubated at RT for 25 min.the development of the colors was observed visually. The third tube containing 1 mL of the kit calibrator (reagentC) and 1 mL of triple distilled water (calibrator tube) and the fourth tube contained 2 mL of the prepared whey (non-fat and non-whey proteins

Isolation and Purification of glutathione

Fágáin *et al.*, (2017) methods were used to separate glutathione from other proteins in Cheese whey The procedure involved gelfltration chromatography by using Sephadex G-10 column**.**

Cell Growth

Hepatocellular cancer cell line (HePG2) and fbroblastic was kindly provided from experimented therapy department cell bank unite Iraqi center of cancer and medical genetic researches, the cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, Streptomycin (0.5 ml/L,(ampicillin (1ml/L), and incubated in 5% CO2 at 37 ºC for 24 h. Cell counts determined using 0.2 ml of trypan blue solution and 1.6 ml PBS , then subculture when monolayer's cells were confluent. Afterwards, 200 µl of cells in growth medium were added to each well of a sterile 96-well microtiter plate. The plates were sealed with a self-adhesive film, lid placed on and incubated in 5% CO2 at 37°C. When the cells are in exponential growth, i.e. after lag phase, the medium was removed and after lag phase, the medium was removed and After that 200 µL of media containing the serial dilutions of glutathaion (100µg/ml- 50µg/ml,25µg/ml and 12.5 were added to the wells. with 3 replicates for each concentration. Beside that RPMI-SF media was added to a separated wells in the same plate to surve as control untreated wells. As a positive control 100 µg/mL of doxorubicin was used to compare the results with it. Afterwards, the plates re-incubated under the same condition for the selected exposure times (24, 48, 72 hrs).

Cytotoxicity assay

Freshney (2005) method was used as a fallow ,two hundred µl of cell suspensions ($1x10^5$ cell/ml) (Confluent monolayer's) of HePG2 wsa seeded into wells of a 96-well plate. After 24 hrs of incubation 200 µl of glutathione extract serial dilutions were added. three replicates were used for each concentration of extract. Afterwards, the plates were re-incubated at 37°C for the selected exposure times (24,48,72) hrs.The cytotoxicity test was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In brief, 100 µl of MTT was added to the wells, the cells were cultured for additional 4 hrs at 37°C. Then 50 µl of DMSO was added to the wells. The solubilized formazan was measured at 570 nm using microplate spectrophotometer (Multiskan, Finland). The % Inhibition were calculated with the following formulae:

Inhibition Rate % = 1-(OD of sample / OD of control) x 100

The Results

Detection of glutathione in the non-fat non-protein cheese whey

The reaction between Ellman's reagent (DNTB) and glutathione was used as a preliminary test to confirm the presence of GSH in the prepared whey. The reaction between this reagent, which is a 5,5-dithiobis (2-nitrobenzoic acid) and the sulfhydryl group of the cysteine in the GSH tripeptide produces a yellow-colored compound, which is the 2-nitro-5-thiobenzoic acid (TNB) have a maximum absorption at 412 nm, this yellow colored compound can easily be recognized visually (Chadda and Robertson, 2016). Fig. 1 shows the visually recognizable reaction between the prepared whey sample and standard GSH sample with DNTB reagent of the used kit. Formation of the yellow color indicating the formation of TNB, which indicates, in turn, the presence of GSH in the prepared whey solution confirmed that GSH present in cheese whey. This result can be compared to the reaction in the blank tube, which hasn't any color and the color of the prepared whey alone. The visually recognizable yellow color intensity indicates the concentration of the GSH in each sample tested (Moser*et al*, 2016). This is, easy, and rapid assay to test the presence of GSH in the produced whey, which helped to continue this study recognizing that the produced whey has glutathione.

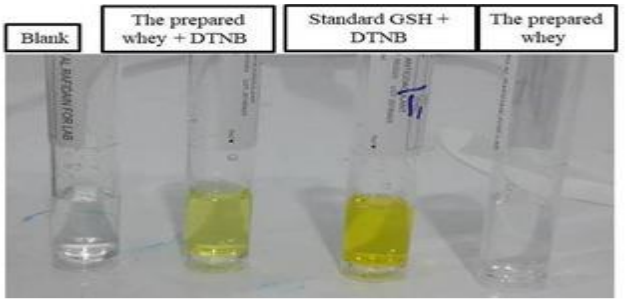

Fig. 1 :Visually recognizable yellow colors were produced via the reaction between Ellman's reagent (DNTB) with the blank sample, a sample of the prepared whey, standard GSH and the prepared whey alone.

Gel filtration chromatography

After preparation of free fat and free protein whey, the prepared whey was lyophilized for application to gel filtration chromatography using a Sephadex G-10 column.

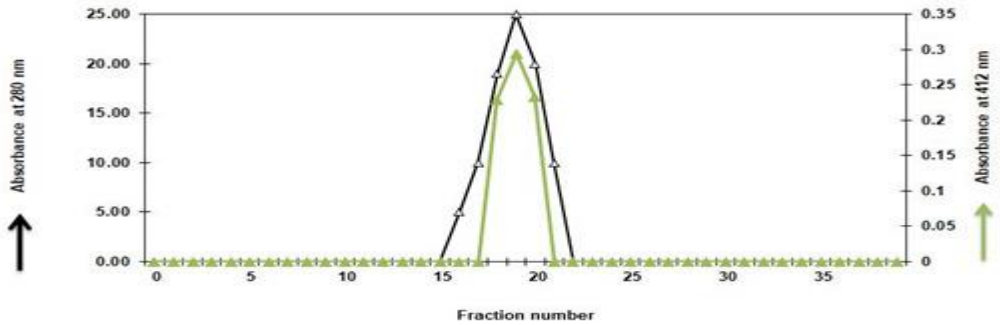

Figure 2 Gel filtration chromatography for purification gsh by using Sephadex G-10 column (1 × 20 cm), equilibrated with Tris-EDTA buffer buffer saline pH 7.4 with a flow rate of 50 ml/hr, 1ml for each fraction.

Fig. 2 represents the result of eluting glutathione through the column of gel filtration on Sephadex G-10 to purify them from previously prepared whey samples .one protein peak with light black color appeared in the eluted fractions (18 to 20) .glutathione protein shown high value when read on the wave length of 412nm Special detects for protein glutathione The presence and the amount of glutathione in the collected fractions were determined using the specific glutathione detection kit which gives the amount of glutathione in the collected fractions by units of micromoles. was 53.55, 81.77 and 55.23 µM, respectively. When using the equations that correlate between molar concentration ratio, molecular weight and mass weight, the amount of glutathione in the 100 mg of the prepared lyophilized whey sample will be equal to 2.915 mg.

Cytotoxic activity of glutathione

The result of the cytotoxic activity of glutathione against hepatocellular cancer cell line (HePG2) determined by MTT assay and percentage of inhibition calculated by microplate reader at 570nm , were shown in figure 3

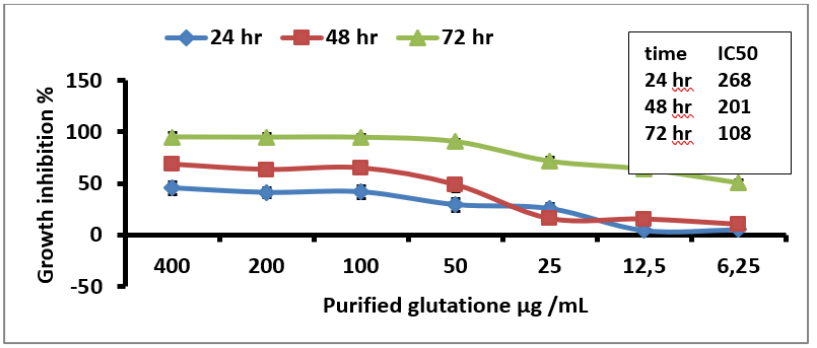

Figure 3: : Anti-cancer activity of the purified glutathione against HepG2 cell line with IC50 of 268, 201, and 108 microgram / mL after 24, 48,and 72 hr respectively.

From the figure (3) it's could be indicated that the least growth inhibition percentage after 24, 48, and 72 hours was 6.25 µg /mL which was 10, 20, 70% respectively. and the growth inhibition percentage increased after 24, 48, and 72 hours by increasing the glutathione concentration to reach 40, 65, and 85% in 100 µg / mL of glutathione respectively. The highest growth inhibition percentage was elevated to higher levels when 400 µg / mL of glutathione was used and it was 50, 70, and 90% respectively The growth inhibition 50% (IC50) for the cancer cells varied according to the incubation time as can be seen in the figure3. It was 268, 201, and 108 microgram / mL of the glutathione for HepG2 cell line at 24, 48, 72 hr of incubation time. This concentration reflects the effectiveness of the glutathione in reduce cancer cells viability, the more the IC50 increased the less the sensitivity of the cells to the glutathione. In order to compare this growth inhibition that used the purified glutathione with that of the standard glutathione, the same cell lines were exposed to the same range of standard glutathione concentration and the same incubation times. Figure 4 show the results.

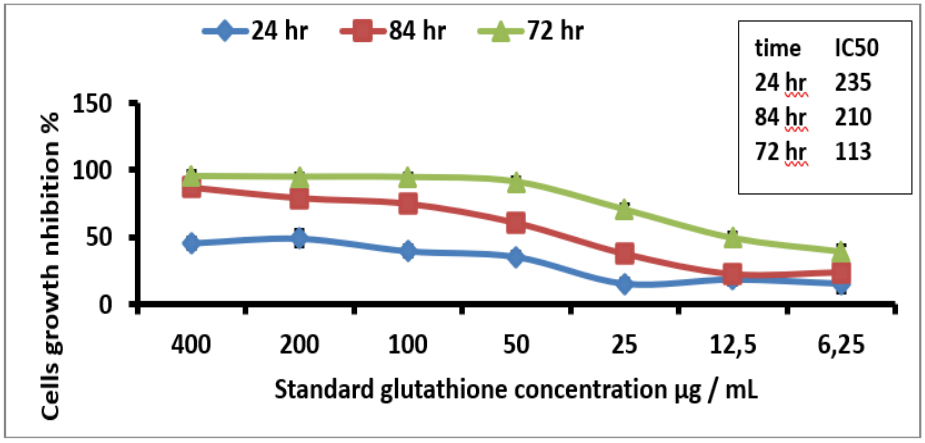

Figure 4: Anti-cancer activity of the standard glutathione against HepG2 cell line with IC50 of 235, 210, and 113 microgram / mL after 24, 48,and 72 hr respectively

The results in figure (4) showed that the lowest growth inhibition percentage after 24, 48, and 72 hours of incubation at 6.25 µg /mL concentration of glutathione was 8, 15, 70% respectively. The growth inhibition percentage increased by increasing the glutathione concentration to reach 40, 62, and 98% in 100 µg / mL of glutathione respectively. The highest growth inhibition percentage was elevated to higher levels when 100 µg / mL of glutathione was used and it was 55, 68, and 99% respectively.

For HepG2 cell line the growth inhibition patron was similar to that for purified glutathione, however the IC 50 for the three incubation times were higher in this experiments for the three incubation times as can be seen in figure 4. With the standard glutathione the growth inhibition of the cancer cell line were evident the effect was started with the use of lowest glutathione concentration and it was escalated with the increment of glutathione concentration. Its noticeable that the standard glutathione was having a little bit lower IC50 which indicate some kind of efficiency for the standard glutathione over the purified one .

To study the effect of GSH whey as an anticancer against one cell line (HepG2 cell line), an assay for chemotherapy doxorubicin was carried out for the one cell line under this study. As can be shown in figure 5, the doxorubicin can inhibits the growth of cell line under the study in concentrations far lower than the glutathione during 24hr time of incubation. The IC50 for doxorubicin were 0.103, microgram/ mL for the cell HepG2 after 24 hr incubation . These IC50 are more than 1000 times lower than the IC50 achieved using the purified glutathione or the standard glutathione. These results are logical since glutathione is originally nontoxic material and its considered safe for human consumption. In some studies recommendations were suggested to use glutathione for its cosmetic properties, and studies showed that supplementation of reduced form of glutathione (GSH, 500 mg/d) has a skin-lightening efficacy in humans. (Weschawalit *et al.*, 2017) Whereas the doxorubicin can't be used in dosages like those used with glutathione since its doses used in cancer patients ranged between 2 to 60 mg/M2 of body mass (Vaitiekus etal., 2020). Whoever for overall anticancer activity caparisoning purposes the assay for doxorubicin against the studded cell lines was jest carried out**.**

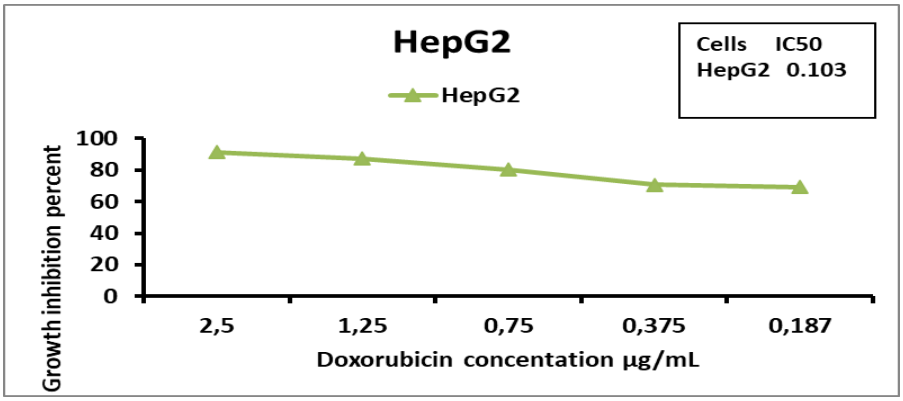

Figure (5): Anti-cancer activity of the chemotherapy doxorubicin against HepG2 cell line with IC50 of 0.103 microgram / mL after 24hrt

The results of this study were consistent with the results of other concern on the role of glutathione or glutathione reductase in the prevention of cancer or even treat cancer when used with some chemotherapies. The modulation of the GSH-antioxidant system has provided promising preclinical results and GSH-based medication has also been successfully employed to protect against cisplatin induced nephrotoxicity (Dong etal., 2018) .Other researches reviewed that the multifaceted roles of glutathione and glutathione-based systems in carcinogenesis, anticancer drug resistance, and clinical applications (Hatem, *etal.*, 2017) . Chronic infection, injury and inflammation have long been recognized as risk factors for the development and progression of various human cancers (Furman etal., 2019).

There is ample evidence to indicate that factors present in the tumor microenvironment (TME), such as products of inflammatory cells reactive nitrogen and oxygen species may cause DNA damage to the neighboring cells, thereby contributing to tumor progression (Greten etal., 2019). A detailed review of many roles of the inflammation-driven changes in tumorigenesis has been recently provided (Greten etal., 2019).

It has been established that GSH deficiency or a change in GSH/GSSG ratio increases the vulnerability of cells to oxidative stress, inflammation, and tumor progression. However, elevated GSH levels increase antioxidant capacity and resistance to oxidative stress as is evident in many tumors. It has been demonstrated that exogenously added GSH inhibits the inflammatory response through regulation of ROS while endogenous GSH has been recently shown to have a role in fine-tuning the innate immune response to infection thereby regulating inflammation (Diotallevi etal., 2017). GSH therefore has a dual role in the inflammatory response as an antioxidant ROS scavenger in the oxidative stress as well as signaling molecule that regulates protein function through thiol–disulfide exchange reactions such as protein glutathionylation with many examples of regulation

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