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Isolation of Glycosylated Flavonoid from *Ruta graveolens* and study its effect on Hepatocellular Carcinoma

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Abstract

Background: A large number of natural compounds have been demonstrated to have cancer chemopreventive activity and healing due to their strong antioxidant and cytotoxic activities. **Objective:** The present study investigates the effect of encapsulation of rutin with carboxymethyl chitosan against N-Nitrosodiethylamine (Di-ethylnitrosamine) (DENA)-induced and carbon tetrachloride-promoted Hepatocellular carcinoma in male Wistar rats. **Materials and Methods:** Fifty male Wistar rats were divided into two major groups. Group I serves as control; Group II was induced HCC by DENA (200mg/kg b.wt) followed by carbon tetrachloride (3ml/kg subcutaneously) for 6 weeks. Group II was divided into four subgroups. Subgroup I (induced subgroup), which took DENA+CCL4, was only then injected with DMSO. Subgroup II (rutin subgroup) rats induced by DENA+CCL4 then injected with rutinat a dose of 20mg/kg b.w. I.p three times a week. Subgroup III (carboxymethyl chitosan subgroup) rats induced by DENA+CCL4 then injected with carboxymethyl chitosan at a dose of 13.5mg/kg b.w. I.p thee times a week. Subgroup IV (CMC+Rutin subgroup) rats were induced by DENA+CCL4, then injected with the combination at a dose of 16mg/kg b.w. I.P. three times a week. **Results:** Observed that there was a significant increase in liver marker enzymes (ALT $P<0.001$), (AST $P<0.087$), (ALP $P=0.004$) and (Albumin $P=0.003$), tumor marker enzymes (AFP, CEA at $P<0.001$), kidney function (creatinine $P=0.001$) and antioxidant activities have no significant values. **Conclusion:** The results suggested that encapsulation of rutin in carboxymethyl chitosan enhances the effect of rutin against tumor cells.

Keywords: HCC, Rutin *graveolens*, carboxymethyl chitosan, Di-ethylnitrosamine

Introduction

Cancer is a disease characterized by the existence of cells undergoing continuous cell divisions in a quick and uncontrolled manner**[1].** Cancers progress from mutated stem cells that first form mutated clones and then progressively and randomly acquire additional mutations in generations of subclones. Carcinogenesis is the term that discusses the process of cancer development. It is a multistep process that happens over many years. Carcinogenesis starts with the chance mutation of key cancer-controlling genes in adult stem cells, which can divide and propagate these mutations through following cell generations. The first mutating event is called “initiation.” The mutated stem cell then goes through waves of cell division to form a clone of mutated cells. This process is called “promotion” and is driven by factors that enhance mitosis. As the clone expands, daughter cells randomly gain additional mutations, giving rise to subclones with different groups of mutations. This process, known as “progression,” continues throughout the life of the neoplasm, producing

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greater and greater numbers of subclones with unique groups of mutations. The process of progression is the origin of the extraordinary molecular heterogeneity that characterizes most malignant neoplasms[2]. Cancer progresses when there are mutations in the DNA within a cell that allow it to grow out of control. The cancerous cells can then crowd out normal cells or invade body tissues. Radiation, carcinogens, infections, and your genetic makeup can increase your risk of mutations that result in cancer[3].

Hepatocellular carcinoma (HCC) is the fifth common category of cancer and the third leading cause of cancer-related deaths worldwide[4]. Understanding the molecular mechanism convoluted in the development and progression of HCC is imperative for the development of more efficacious therapeutic strategies, given that the worldwide incidence of HCC, a type of cancer with a poor 5-year survival rate (at the late stage) and high rate of recurrence (after surgical resection)[5,6], stands at over one million. The development and progression of HCC is a long-term multistep process, comprising chronic liver injury, necro-inflammation and regeneration, small cell dysplasia, and the appearance of low- and high-grade dysplastic nodules [7,8]. There are many types of cancer treatment. The type of treatment that the patient receives will depend on the type of cancer he has and how advanced it is. But most of the current treatment methods have many side effects that affect the patient's health during the treatment journey and after therapy. So, the world has now turned to nature and natural products, which have vast benefits with minimal side effects. Flavonoids are secondary metabolites that are very plentiful in plants, fruits, and seeds, responsible for the color, scent, and flavor characteristics. In plants, flavonoids play many functions like regulating cell growth, attracting pollinator insects, and defending against biotic and abiotic stresses[9]. For instance, plant flavonoids can function as signal molecules, UV filters, and reactive oxygen species (ROS) scavengers and have many functional roles in drought, heat, and freezing adaptation [10]. In humans, these compounds are associated with a wide range of health benefits arising from their bioactive characteristics, such as anti-inflammatory, anticancer, anti-aging, cardio-protective, neuro-protective, immune-modulatory, anti-diabetic, antibacterial, anti-parasitic, and antiviral characteristics[11]. Flavonoids' chemical construction, principally hydroxy groups, influences humans' bioavailability and biological activity[12]. The present study is designed to investigate the effect of encapsulation of rutin with carboxymethyl chitosan against N-Nitrosodiethylamine (Di-ethylnitrose amine) (DENA)-induced and carbon tetrachloride promoted Hepatocellular carcinoma in male Wister rats *(in-vivo)* and evaluate the effect on the proliferation capacity of a human hepatocellular carcinoma cell line (Hep-G2) (*in-vitro)*.

Chemicals

Ruta graveolens was purchased from a local market in Egypt,

DENA (di-ethyl nitrosamine) purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA),

Chitosan was purchased from Chitosan Egypt Co. (Cairo, Egypt).

All solvents were purchased from Sigma-Aldrich Chemical Co.

(St. Louis, MO, USA), with analytical grade.

Procedure

Extraction of Rutin

10 grams of Ruta graveolens was added to 100 ml of 70% ethanol, then sonication was applied by (BRANSON 3200 USA.), sonication for about 15 minutes. The TLC (Thin Layer Chromatography) technique was applied to the extract for identifying the extract by using the BAW (butanol, acetic acid, and water) system. Then HPLC was used to be sure of our extract (HPLC-PDA (SYKM S500 series, Germany; C18 Column,250 x 4.6 mm. the mobile phase was gradients of CH_3CN/H_2O+0.1 Formic Acid at flow rate of 1.0ml min^{-1}.100 grams of Ruta Graveolens was added to 800 ml of 70%ethanol and allowed to stand for extraction.

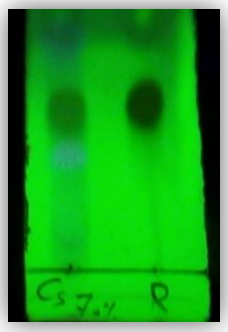

Fig. 1: TLC of the extract Under Where Cs70% our extracted Compound and R are standard rutin.

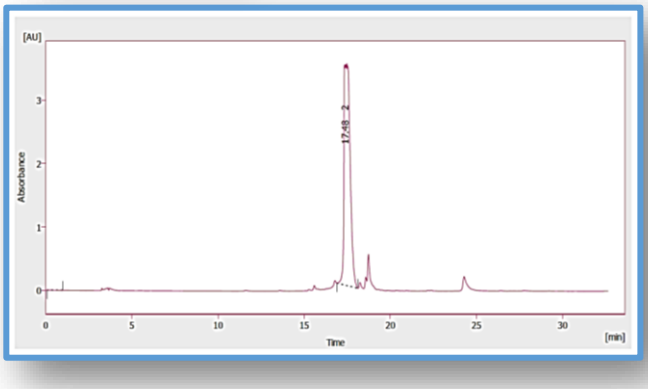

Fig. 2: analysis of the extract (Rutin) by HPLC-PDA (SYKM S500 series, Germany;C18 Column,250 x 4.6 mm. the mobile phase was gradients of CH_3CN/H_2O+0.1 Formic Acid at flow rate of 1.0ml min^{-1} .

Figure 3: The yellow precipitate (rutin) after drying

A yellow precipitate appeared after standing of water extract for 72 hours. In Figure 3, the precipitation was filtered and then washed with petroleum ether. After drying up to 72hrs, the weight of the precipitation was measured.

Preparation of Carboxymethyl chitosan (CMC) and encapsulation of rutin

Rutin has a low oral bioavailability and low water affinity, so encapsulation was applied with carboxymethyl chitosan. Preparation of carboxymethyl chitosan was carried out according to**[13]**. 2 grams of chitosan were dispersed in 20 ml of isopropanol and stirred for 20 min at room temperature. Then 5ml of aqueous 40% NaOH was added with constant stirring at room temperature for 45 min next 12 grams of monochloroacetic acid was added to the suspension at 60 °c with 3hours of stirring.

The encapsulation process was carried out according to**[14].** 2% of carboxymethyl chitosan (w/v) was dissolved in 40 ml of 1% acetic acid using a magnetic stirrer until homogeneous. 0.3 grams of

Tween 80 was added to the solution next, and 11.25 mM of rutin was added to the solution with constant stirring for 30 min. 40 ml of 2% (b/v) TPP (sodium tripolyphosphate $Na_5P_3O_{10}$) solution was added dropwise with constant stirring for 1 hour[15]. The centrifugation was carried out at a speed of 7000 rpm for 10 min. The pellet was then washed and dried.

Degree of substitution

100 mg of CMC was dissolved in 10 ml of 0.12 M NaOH. The mixture was stirred for 30 min at room temperature by a magnetic stirrer. Methyl red was used as an indicator, which was added to the mixture and then titrated with 0.13 M HCl until the mixture became reddish in the recent experiment DS=79%.

$$DS=\frac{MW*M*(B-S)}{1000*W}$$

MW = molecular weight of monomer chitosan (g/mol)

B = volume of HCl blank (mL)

S = volume of HCl sample (mL)

M = molarity of HCl (mol/L)

W = mass of sample (g)

DS = number of substituted hydroxyl groups

Encapsulation efficiency

The encapsulation efficiency was carried out in a direct manner in which the precipitate (5 ml of sample) (CMC+Rutin) was dispersed in 5 ml of deionized water, then centrifuged at 3000 rpm for 5min and filtered by syringe filter Nylon 0.22 Mm. the absorbance was measured by HPLC-PDA (SYKM S500 series, Germany; C18 Column, 250 x 4.6 mm). the mobile phase was a gradient of methanol and water with 0.1 formic acid at a flow rate of 0.5 ml min-1. The PDAs obtained were used to determine the number of encapsulated rutin by using standard curve equations[16]. Encapsulation efficiency (EE) was calculated from Eq. (1),[17].

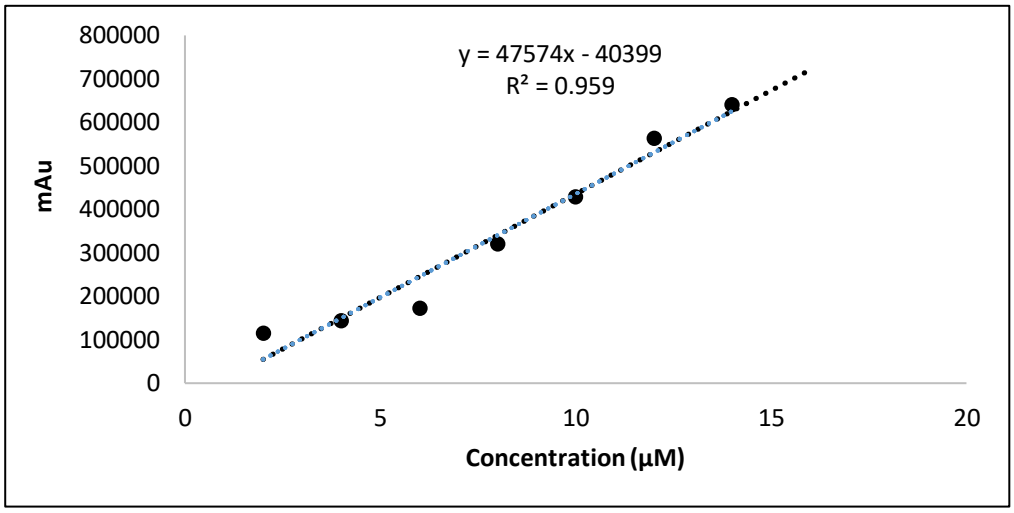

Fig (4. Calibration curve of rutin by HPLC

1) $EE\% = \frac{\text{weight of loaded rutin}}{\text{weight of initial rutin}} *(100\%)$

$$EE\%\frac{Wtr-Wfr}{Wtr} *100$$

Where W_{tr} is the amount of rutin in the encapsulation sample.

W_{fr} amount of free rutin in the supernatant.

$$EE\%=\frac{11.25-1.5}{11.25}*100$$

EE%=86.6 %

In-vitro study

Anti-tumor activity against human hepatocellular carcinoma cell line (Hep-G2):

The MTT Cell Viability Assay:

The method was carried out according to that of **Kumar *et al.* (2018).**

Principle:

Cytotoxicity was measured using the MTT cell viability assay. MTT (3-[4,5-dimethyl thiazole-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form a dark blue insoluble formazan crystals which is largely impermeable to cell membrane, resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT is quantified by measuring the absorbance at 570 nm **[18].**

Animals and experimental design (In-vivo study)

50 male Wistar albino rats weighing 200-230 grams obtained from MASRI (Faculty of Medical Ain Shams Research Institute), the animal house of Ain Shams University, Egypt, were used in this study. Rats were given free access to water and commercial pelleted rodent feed ad libitum. The animals were kept under standard laboratory conditions at a temperature of 22-25 ℃ and 12 hrs artificial light/dark cycle.

To investigate the role of our tested compounds in the protection and enhancement of the treatment of liver cancer, rats were divided into 2 major groups.

Group I (control group) consists of 10 animals, which served as the untreated control group.

Group II consists of 40 rats injected with a single intraperitoneal dose of DENA (di-ethylnitrose amine) 200mg/kg b.w., after two weeks of injection, rats received 3ml/kg b.w. CCL4 subcutaneous for 6 weeks. Then, rats were divided into 4 sub-groups, 10 rats in each.

Subgroup I (induced subgroup), which took DENA+CCL4, was only then injected with DMSO.

Subgroup II (rutin subgroup) rats induced by DENA+CCL4 then injected with rutin at a dose of 20mg/kg b.w. I.p three times a week.

Subgroup III (carboxymethyl chitosan (CMC) subgroup) rats induced by DENA+CCL4 then injected with carboxymethyl chitosan at a dose of 13.5mg/kg b.w. I. p. three times a week according to**[19]**.

Subgroup IV (CMC+Rutin subgroup) rats were induced by DENA+CCL4, then injected with the combination at a dose of 16mg/kg b.w. I.P. three times a week.

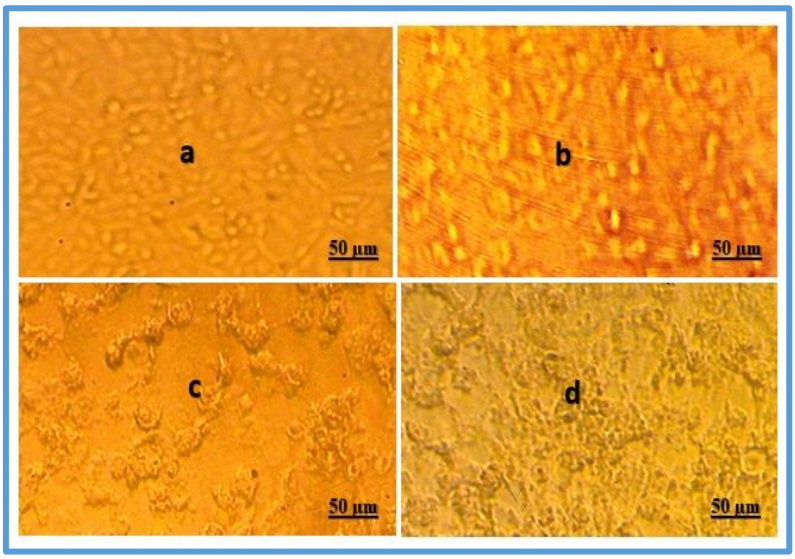

Fig (5): (a)HEPG2 cells without treatment, (b)HEPG2 cells treated with rutin, (c)HEPG2 cells treated with CMC, (d)HEPG2 cells treated with rutin + CMC.

Fig. 6: Some of the experimental rats in the cage.

Results and Discussion:

IRSpectrum

PerkinElmer Spectrum 10.5.4 FTIR spectrophotometer in the range of 4000 - 450 Cm^{-1} was used to identifythe infrared spectroscopy (FTIR) of samples (chitosan and carboxymethyl chitosan), where the KBr technique was used. The infra-red absorption spectra of chitosan and N, O-Carboxymethyl Chitosan areshown in Figures 3 and 4. The peaks that appeared in the infrared spectrum of chitosan and N, O-Carboxymethyl Chitosan are identical. In the infrared spectrum of chitosan, peaks observed at 3437.64 cm-1 and 2922 cm-1 are the stretching vibration of -NH2 and -OH groups. In the infra-red spectrum of N, O-Carboxymethyl Chitosan, carboxymethylation is indicated with the presence of a weak peak at wave numbers 1625.79 cm-1 and a hard peak at wave number 1395 cm-1[20] This wave number 1625.79 cm-1 itself is a characteristic peak of N, O- Carboxymethyl Chitosan that indicates the presence of carboxylic acid salt (the stretching vibration of - COO- antisym)[21]. And to ensure that N, O-Carboxymethyl Chitosan has been formed, it can be done by examining the presence of a peak at wave number 1062 cm-1, which is the C-O-C stretching vibration. In addition, the peak seen at a wavelength of 3238 cm-1, which continues widening and has increasingly small transmittance intensity, also indicates that carboxylation has been formed in both groups, i.e, the amino group and the primary hydroxyl group found in the structure of chitosan[20].

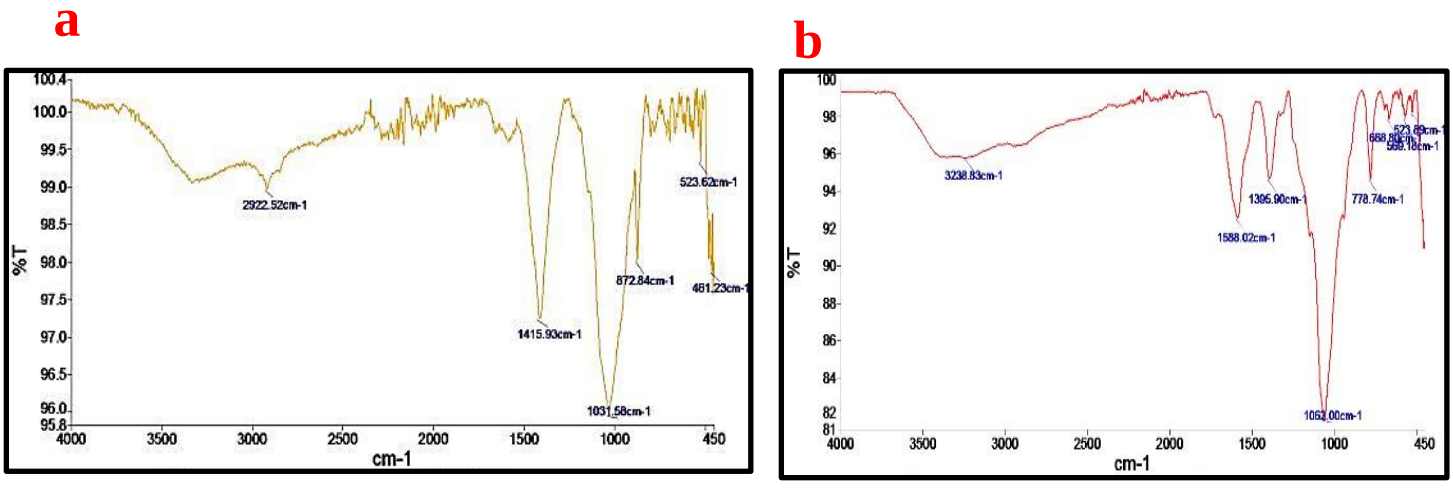

Fig (7): (a) FT-IR of chitosan, (b) FT-IR of carboxymethyl chitosan.

SEM Scanning Electron Microscope analysis

Scanning electron microscope analysis was made using Tescan SEM (Tescan Vega 3 SBU, Czech Republic). Samples were mounted on aluminum microscopy stubs using carbon tape, then coated with gold (Au) for 120 s using Quorum techniques ltd, sputter coater (Q150t, England). In this study, SEM analysis was conducted to determine the surface characteristics of chitosan, N, O-Carboxymethyl Chitosan, and carboxymethyl encapsulated rutin. Based on this SEM analysis, it is revealed that chitosan has a ratherflat and non-porous surface. While the N, O-Carboxymethyl Chitosan has a characteristic of an uneven surface and the surface displays the existence of a new formation that attaches to the surface of chitosan **[22].** The SEM results of carboxymethyl chitosan reveal that after modification, the surface area increases, and the pore diameter results suggest the formation of mesoporous particles. Even though the surface roughness of rutin-encapsulated carboxymethyl chitosan was more than carboxymethylchitosan, the surface area and porosity dropped due to the encapsulation process. Suggests that the surface of carboxymethyl chitosan and rutin carboxymethyl chitosan possesses a different range of pore size, 2.65 micrometers and 0.4 micrometers, respectively**[23].**

X-ray diffraction analysis

In this study, XRD analysis was conducted to test or confirm whether N, O-Carboxymethyl Chitosan had been formed. XRD analysis was conducted using a Cu Kα tube targets with an angle length of 5 to 80 (2θ). Theresults of the XRD diffractogram achieved in this study, both for chitosan and N, O Carboxymethyl Chitosan, show the existence of sharp characteristic peaks at certain angles. In this study, the characteristic peaks of chitosan were observed at an angle of 20.1° with a height of 26. While the characteristic peaks of N, O-Carboxymethyl Chitosan were observed at an angle of 31.681°with a height of 47. Based on the XRD diffractogram data available, it can be said that N, O Carboxymethyl Chitosan has been formed or has been successfully synthesized **[22].**

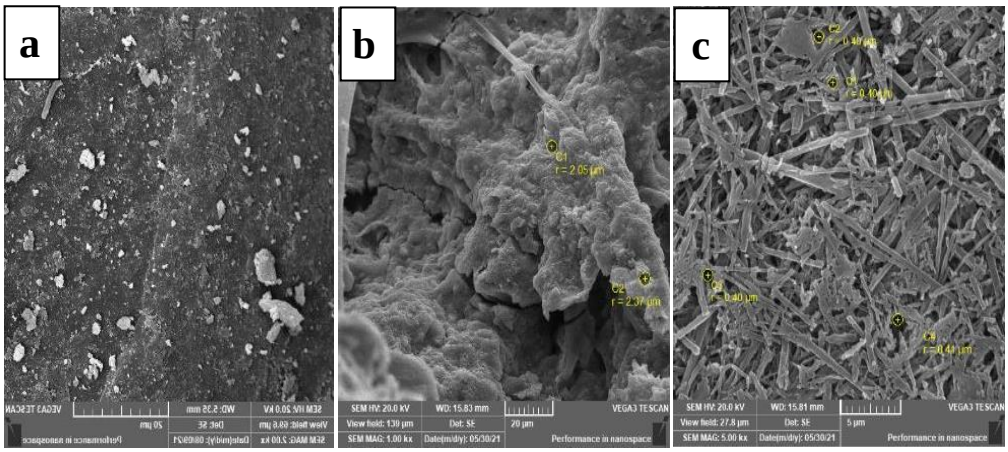

Fig (8): SEM of (a) chitosan, (b) carboxymethyl chitosan, and (c) rutin encapsulated with carboxymethyl chitosan.

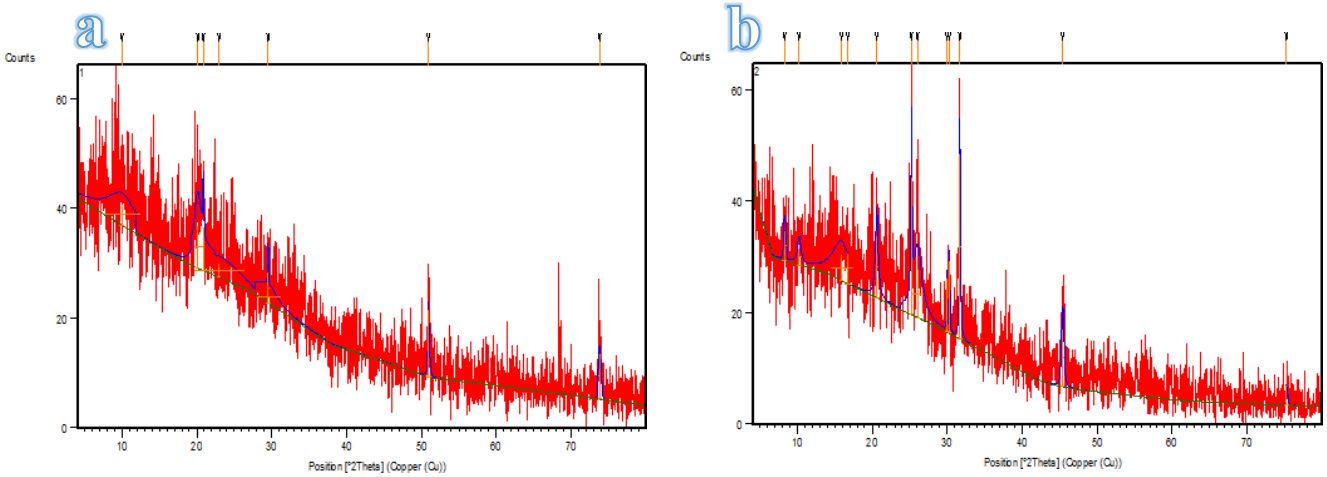

Fig (9): (a) X-ray diffraction of chitosan, (b) X-ray diffraction of carboxymethyl chitosan.

Biochemical parameters

This study shows the effect of our tested compounds on ten different parameters (AST, ALT, ALP, Albumin, Creatinine, CEA, AFP, GSH content, GSH peroxidase, and lipid peroxidation). Data was analyzed using one-way analysis of variance (ANOVA) using the statistical package for social science (SPSS) program (Norusis, 1993). Numerical data were expressed as mean ± S.D., minimum, and maximum. Qualitative data were expressed as frequency and percentage. The degree of association between thedifferent measurements was measured by Pearson´s correlation coefficient (r).

Histopathological results

Liver specimen from rats was fixed in 10% neutral buffered formalin, sectioned at 3 µm, and stained with Haematoxylin and Eosin (H & E) stain for light microscopic examination and examined microscopically as shown in Figure 14. the control rats showing normal liver architecture; DENA induced liver fibrosis (positive control)showing congested central vein and blood sinusoids, focal lymphoplasmacytic infiltrates, hepatocytes ballooning, and fibrosis; CMC treated group, rutin treated group showed mild small localized vascular congestion and extravasation, few intra-acinar infiltration of lymphocytes associated with regenerativehepatocytes; CMC+Rutin treated group showed maintained hepatic architecture, with minimal damage.

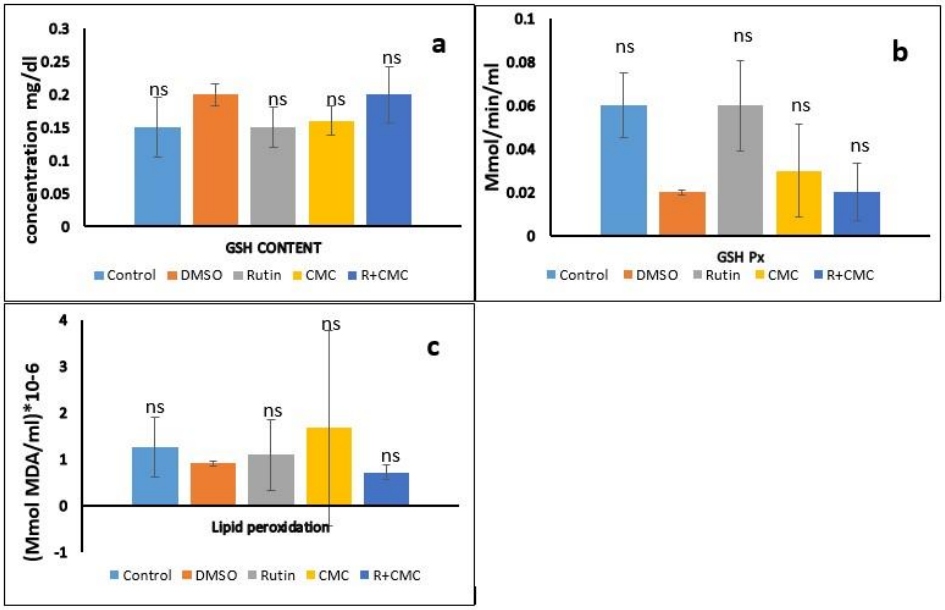

Fig (10): shows the effect of our tested compounds on antioxidant activities (a) glutathione (gsh) content, (b) GSH peroxidase (Px) and (c) lipid peroxidation Results were expressed as Mean ± SD, Comparisons were made as *** statistical significant at P< 0.001, ** Statistical significant at P< 0.01, * statistical significant <0.05 and ns at non statistical significance compared with DMSO group.

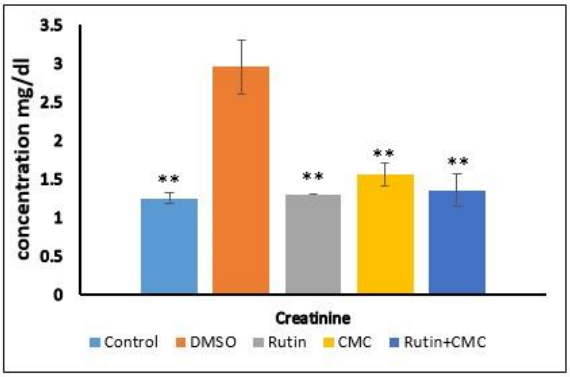

Figure 11 illustrates the effect of the tested compoundson one of the kidney functions (creatinine). Results were expressed as Mean ± SD Comparisons were made as *** statistically significant at P< 0.001, ** statistically significant at P< 0.01, * statistically significant <0.05, and ns at non-statistical significance compared with the DMSO group.

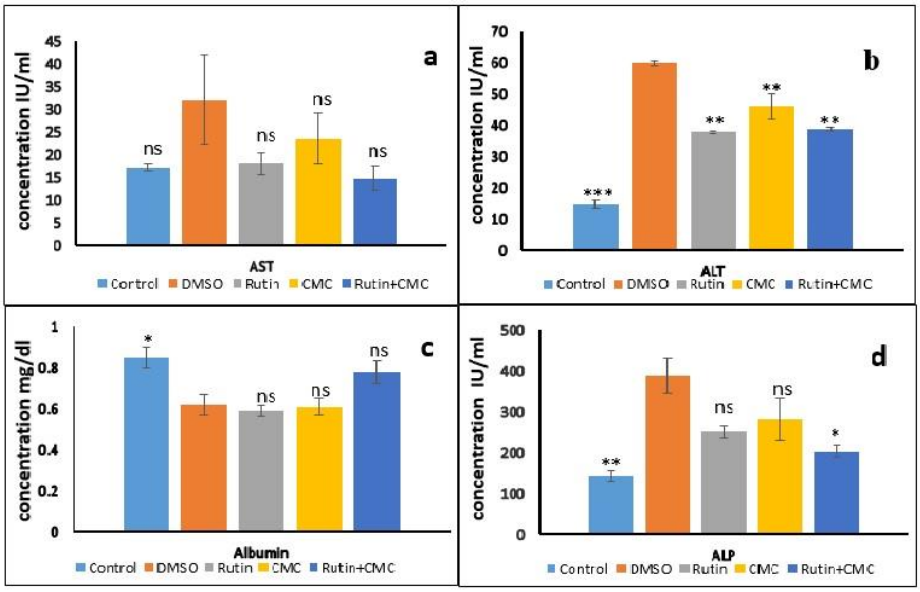

Figure 12 illustrates the effect of tested compounds on liver enzymes (a) change in AST level, (b) change in ALT level, (c) change in Albumin level, and (d) change in ALP level. Results were expressed as Mean ± SD Comparisons were made as *** statistically significant at P< 0.001, ** statistically significant at P< 0.01, * statistically significant <0.05, and ns at non-statistical significance compared with the DMSO group.

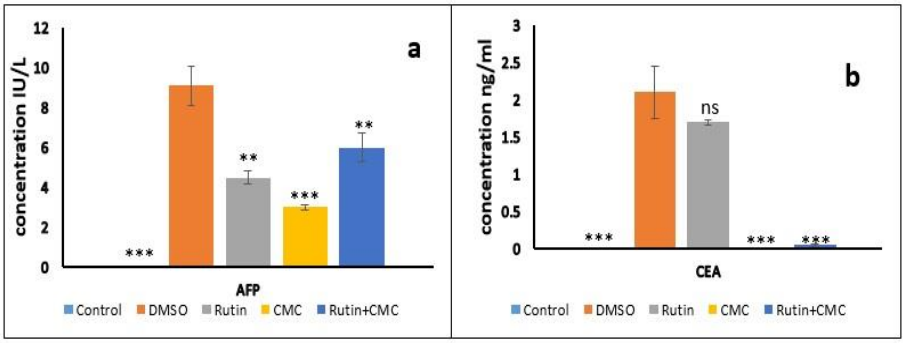

Fig 13 illustrates the effect of tested compounds on tumor markers (a) alpha-fetoprotein (AFP) and (b) carcinoembryonic antigen (CEA). Results were expressed as Mean ± SD Comparisons were made as *** statistically significant at P< 0.001, ** statistically significant at P< 0.01, * statistically significant <0.05, and ns at non-statistical significance compared with the DMSO group.

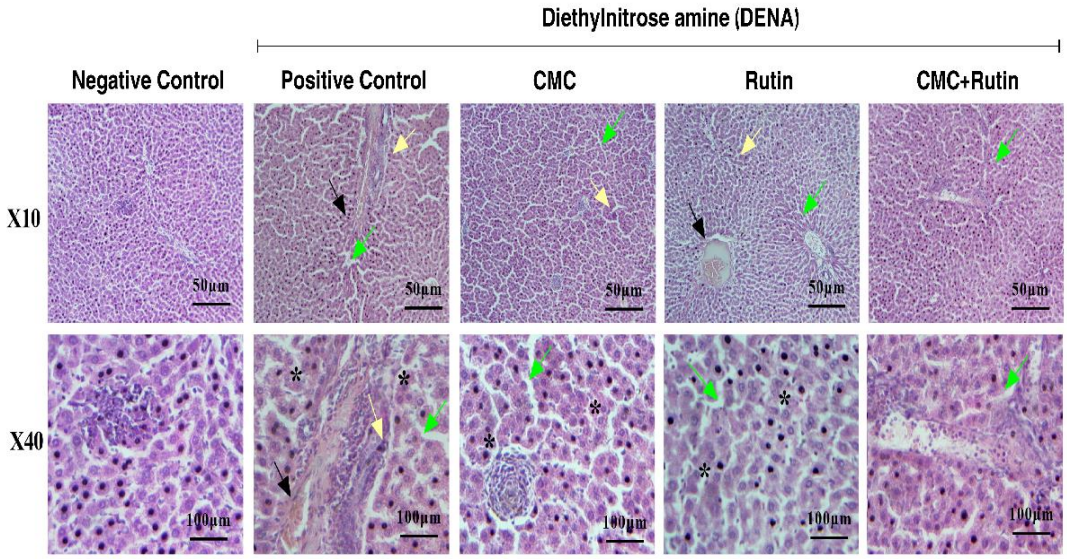

Fig (14): photomicrographs of liver sections stained with haematoxylin and eosin (HE, x100, x400). Blood sinusoids (black arrows);*: hepatocyte ballooning, focal lymphoplasmacytic infiltrates (yellow arrows); fibrosis (green arrows).

Conclusion

The goal of this study was to find a natural treatment for HCC that would be less toxic for normal cells. Rutin and CMC were chosen due to their biodegradability and bioavailability, where rutin was encapsulated with CMC and utilized to treat HCC in vitro and in vivo. The results confirmed the antitumor activity of rutin, which was significantly enhanced by encapsulating it with a hydrophilic chemical (CMC) in both in vitro and in vivo tests.

Conflict of interest: NIL

Funding: NIL

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