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INHIBITION OF EXPERIMENTAL CARCINOGENESIS BY THE BIOACTIVE NATURAL PRODUCT BIOBRAN.

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ABSTRACT

To investigate the protective effects of biobran against N-nitrosodiethyamine (NDEA) and carbon tetrachloride CCl_4-induced hepatocarcinogenesis in rats.

Hepatocarcinogenesis was induced in rats by a single intraperitoneal (i.p.) injection of N-nitrosodiethyamine (NDEA) at a dose of 200 mg/kg body weight followed by weekly subcutaneous injections of CCl_4 (3 ml/kg) for 6 weeks, as the promoter of carcinogenic effect. After administration of the carcinogen, 25 mg/kg/day of Biobran were administered i.p., five times a week throughout the study. At the end of 20 weeks, the body weight, liver weight were measured, blood samples were collected for liver function tests, liver biopsies were processed for histopathology examination.

Results demonstrated that biobran has significantly prevented the decrease of the body weight and the increase in the liver weight caused by NDEA. Liver function tests showed significant increase in serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ -glutamyl transpeptidase (γ -GT) of untreated NDEA group, meanwhile treatment with Biobran to rats exposed to carcinogens, significantly minimized the elevation of the liver function enzymes level to be comparable with the normal control values. Histopathological examination of the liver sections of rats subjected to (DENA + CCl4) treatment revealed fibrosis and fatty infiltration of hepatocytes, with inflammatory collection and loss of architecture Biobran treatment showed minimal changes in hepatocyte morphology and histology with no inflammation.

this study showed that Biobran has a protective effect against hepatocarcinogenesis induced by NDEA and CCl4 in rats.

Keywords: *N*-nitrosodiethylamine; Carbon tetrachloride; Carcinogen; Biobran.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer in adult, which account for about 75% of primary liver cancers. It is the 5th most liver common cancer worldwide and represents 83% of all cases (Ferlay *et al* ., 2001) . Liver cancers have different growth patterns; the first type begins as a single tumor that grows larger in hepatic tissue . The second type of is spread through the liver almost from the beginning and is not confined to a single tumor . This is seen most often in people with liver cirrhosis Risk factors for HCC include hepatitis B virus(HBV), hepatitis C virus (HCV) and aflatoxins are assumed to play an important role in high incidence of HCC. HBV vaccination of children and high-risk population must be the priority in reducing the incidence of HCC. Measures to reduce food spoilage by fungi and the associated dietary exposure to aflatoxins are desirable public health goal (Wild and Hall , 2000). Liver carcinogenesis may also develop through progressive accumulation of different mutations (genetic) and/or genetic products (protein), which eventually lead to malignant transformation (Macphee, 1998 and Seufi *et al*., 2009).

N-nitrosodiethylamine, a potent hepatocarcinogenic dialkyl nitrosamine is present in tobacco smoke, water, cheese, cured and fried meats and in a number of beverages (Rajes kumar and kuttan., 2000). A review on NDEA reported that a number of species including mice, rats, guinea pigs, hamsters, rabbits, dogs and monkeys, (Verna *et al*., 1996) developed liver cancer on exposure . It is metabolized to its active ethyl radical(CH_3 CH_2 $^+$) by cytochromes and the reactive product interacts with DNA producing mutation and further oncogenesis .

Biobran is a natural compound made from breaking down rice bran with enzymes from the Shitake mushroom. Previous reports have shown Biobran to be a potent biological response modifier (BRM) that stimulates several different arms of the immune system including natural killer (NK) cells (Ghoneum and Brown., 1999). In addition, MGN-3 is capable of sensitizing human leukemic cell surface CD95 receptors that are involved in the triggering of apoptosis (Ghoneum and Gollapudi., 2003).

MATERIALS AND METHODS

Chemicals & drug:

N-nitrosodiethylamine, was purchased from sigma chemical company, USA. Carbon tetrachloride (CCl4) was obtained from El-Gomhorya company, cairo, Egypt. Biobran was kindly provided by Daiwa Pharmaceuticals Co Ltd., Tokyo Japan.

Animals:

Male albino rats weighing 120-140 g were used. Their age between 8-10 weeks old were procured from the animal house of the Nile Centre for experimental research, Mansura, Egypt. The rats were housed in groups in plastic cages with wood chips for bedding under controlled conditional of temperature (22 ± 3 °C) with a 12 h light/dark cycle respectively for one week before and during the experiment. Animals were allowed to access standard rodent pellets diet and drinking water.

Experimental design:

Adult male Wister albino rats, 120-140g, the rats were randomly assigned into five experimental groups, group 1& 2 containing 15 rats, groups 3, 4& 5 containing 20 rats.

- Group (1: Control): rats served as controls.
- Group (2:Biobran): rats were given 25 mg/kg/day of Biobran by i.p. injection five times a week throughout the study.

- Group (3:Carcinogen): rats received single intraperitoneal injection of NDEA (200 mg/kg body weight) after one week they are received weekly subcutaneous injections of CCl4 (3ml/kg b.w) for 6 weeks (Sundaresan & Subramanian, 2003).
- Group (4:Biobran +Carcinogen): animals received Biobran as group 2 two weeks before the injection of carcinogens and continued for 20 weeks.
- Group (5:Carcinogen+ Biobran): animals received the carcinogen as in group 3, then treated with Biobran starting from week 10 up to the end of the study.

Body and liver weight changes:

Body weight (BW/g) of the different experimental groups was measured weekly during the experiment time. At the end of experimental study after sacrificing the rats, liver of different groups were excised and weighed.

Histopathological examination:

The liver samples were preserved in phosphate-buffered 10% formalin for 24 hours, cut into small pieces. After fixation, the samples were dehydrated in ascending series of ethyl alcohol 70%, 80%, 90% and 95% for 30 minutes each, then into changes of absolute ethyl alcohol for 30 minutes each. Tissue were cleared in xylene for 20 mintues (two changes), then embedded in paraffin wax. Sections 4 to 5 µm thick were cut using microtome, mounted on glass slide and stained according to the following histological method then examined by light microscope (Weenser, 1968).

Biochemical analysis:

At the end of the experimental period, all the animals were sacrificed. Blood samples were collected in heparinized tubes and centrifuged at (3000 rpm for 20 min) without hemolysis. The levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), were determined using an automatic biochemical analyser (BTS-370, BioSystems S.A., Barcelona, Spain) according to the instructions sup-plied with the commercial assay kits (Roche, Switzerland).

Statistical analysis

Results were expressed as means ± SE. Statistical significance was calculated using one-way analysis of variance (ANOVA) followed by post hoc tests for multiples comparisons. All the statistical analysis carried out with the use of SPSS 18 software. Differences were considered significantly at $P < 0.05$ level.

RESULTS

1. Effect of Biobran on body weight changes induced by NDEA.

Body weight (BW) of the different experimental groups was recorded weekly during the experiment time. Figure 1, shows the BW changes in rats . Initial BW without treatment was comparable between groups. On first week after NDEA treatment, the rats began to show a slow growth and continues gradually through injection of CCl4 for 6 weeks as compared to normal control group. Final body weight of rats showed increased in control group to record (318±7.65 g) and Biobran intake to normal rats recorded (300±6.11g). On the other hand untreated carcinogen group showed highly significant ($p<0.01$) BW loss as compared to the other groups to record (192±3.86 g, -39.54% BW) loss of control group. The body weight in pretreatment group (Biobran+Carcinogen) showed increase as compared to untreated carcinogen group to record (264±5.34 g), -17 % BW, and decreased when compared to the normal control group. Posttreatment animals (Carcinogen+Biobran) significantly recovered the body weight gain of rats (243.5±4.51 g, -23.44% BW) compared to that of carcinogen untreated group.

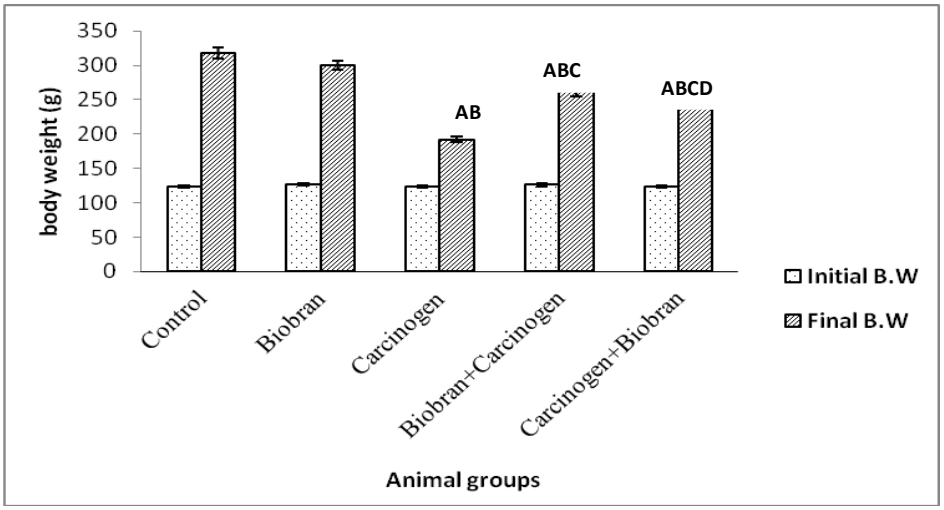

Figure (1): Effect of Biobran intake on rat BW/gm. The data of BW were presented as mean±SE. [A] Significantly different from control group at $p < 0.01$ level. [B] Significantly different from Biobran group at $p < 0.01$ level. [C] Significantly different from Carcinogen group at $p < 0.01$ level. [D] Significantly different from (Biobran+Carcinogen) at $p<0.01$

2. Effect of Biobran on Liver weight

As shown in Figure 2, treatment with Biobran alone to normal animals showed comparable liver weight with the normal control animals and recorded (8.45±0.29 g, 8.56±0.25 g) respectively, liver weight of Carcinogen group animals recorded 10.38±0.34g which represents a marked increase by 24.73%, p<0.01 of untreated normal control group. In the prevention animals by Biobran before induction of tumor (Biobran+Carcinogen) showed a moderate increase in liver weight to record (8.75±0.51g, 3.57%,p<0.01) as compared to normal animals. posttreatment animals (Carcinogen+Biobran) showed slight insignificant increase in liver weight to record 8.66±0.21 g, 2.57% when compared to untreated normal control group.

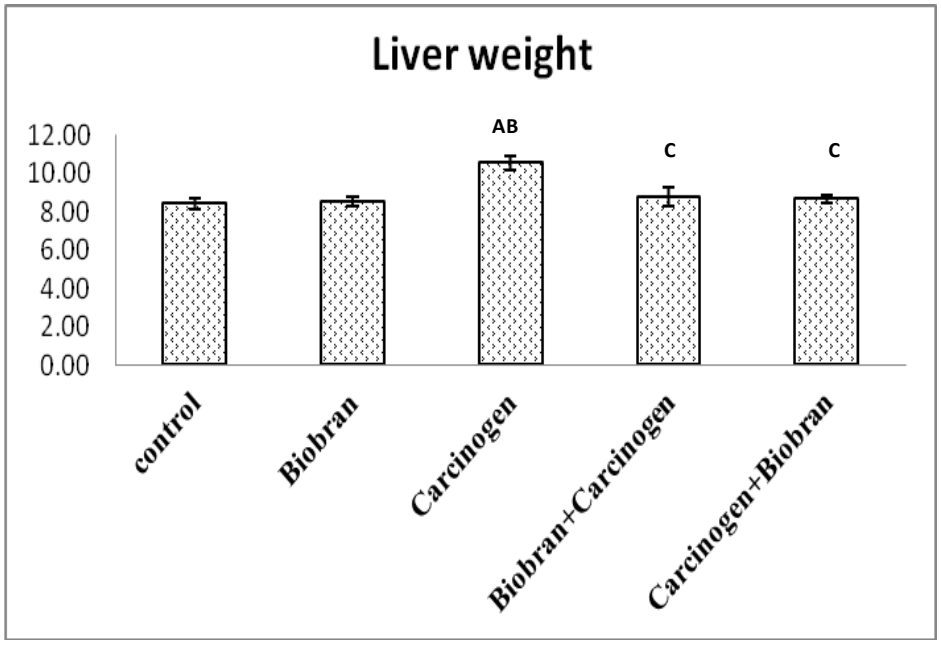

Figure (2): Effect of Biobran on liver weight. Each value represents the mean±SE.

[A] Significantly different from control group at p< 0.01 level. [B] Significantly different from Biobran group at p<0.01 level. [C] Significantly different from Carcinogen group at p< 0.01 level.

3. Histopathological study

Study of the liver tissue sections from rats in the normal and Biobran control groups revealed a normal hepatic lobular architecture and the presence of normal hepatocytes with granulated cytoplasm and small uniform nuclei and nucleolus, In contrast, the study of sections obtained from rats subjected to (DENA + CCl4) treatment revealed fibrosis and fatty infiltration of hepatocytes, with inflammatory collection and loss of architecture, necrosis and hepatocellular degeneration with frequent mitotic activity. Pretreatment animals with Biobran showed minimal changes in hepatocyte morphology and histology with no inflammation. Animals post-treated with Biobran showed lesser damage of hepatocytes and low index of necrosis, vacuolation of hepatocytes and scanty mitosis.

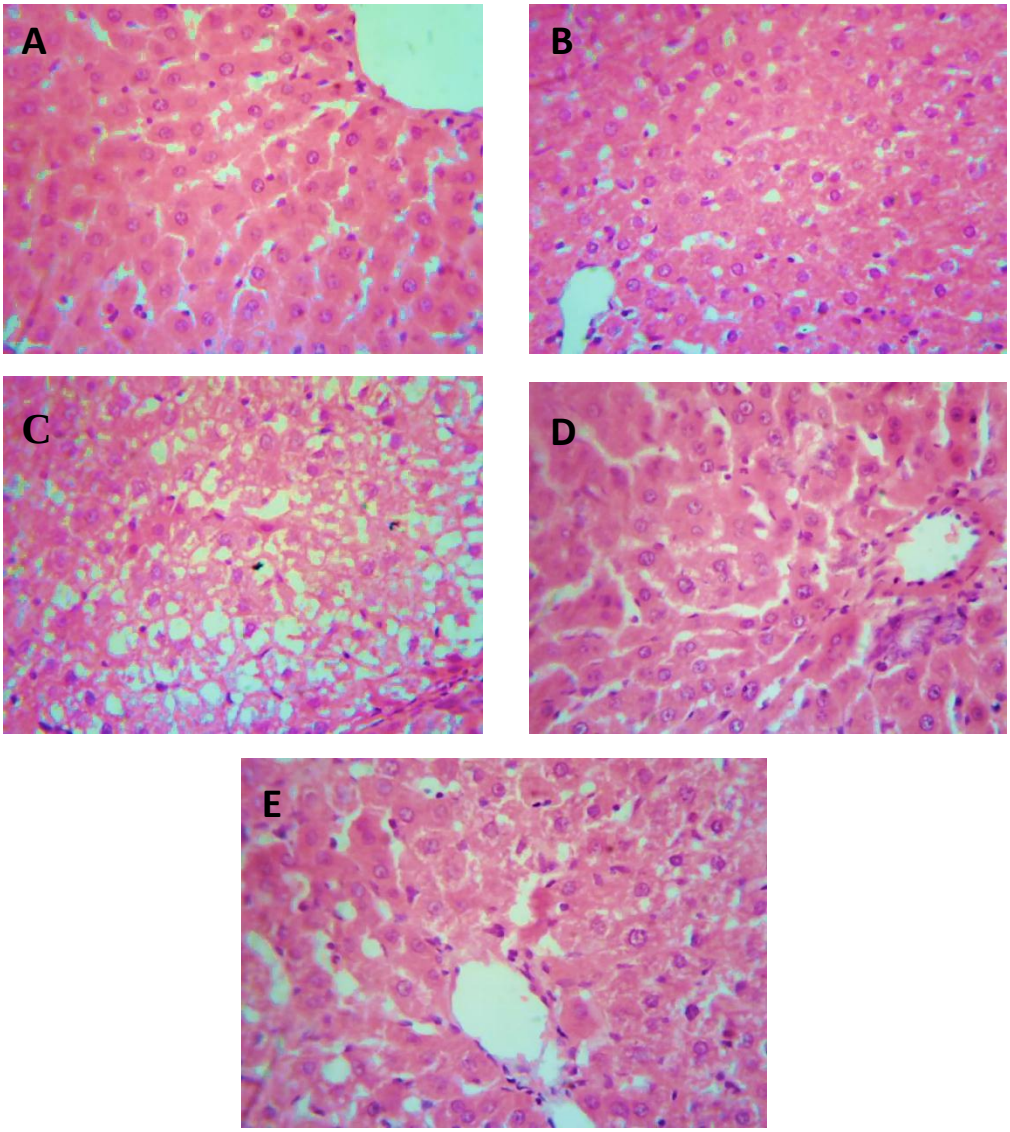

Figure (3): Histopathological effects of biobran treatment against hepatocarcinogenesis in rats. A(untreated),B (Biobran treated): normal control groups showing the normal histological structure of hepatic lobular with granulated cytoplasm and small uniform nucleus and nucleolus. C: (NDEA+CCL_4) showing fatty infiltration of hepatocytes, with inflammatory collection and loss of architecture, necrosis and fibrosis hepatocellular degeneration. D: pre-treatment group (Biobran +Carcinogen) showing preserved hepatic architecture, minimal nuclear changes and vacuolation of hepatocellular cytoplasm and no inflammation. E: animals post-treated (Carcinogen+ Biobran) showed lesser damage of hepatocytes and low index of necrosis, vacuolation of hepatocytes. (H&E x400).

4. Effect of Biobran on liver function tests

Data in Figure 4, represent the activity levels of liver function enzymes AST, ALT, ALP and GGT in serum of rats under different experimental conditions. Animals that administrated of NDEA induced a significant increase ($p<0.01$) in serum levels of AST by 145%, ALT by 224% and 99.23% for ALP as compared with the normal control. Further, serum GGT level showed also a marked high elevation by 1584%, $p<0.01$ of normal values.

Pretreatment group by Biobran (Biobran +Carcinogen), significantly minimized the elevation of the liver function enzymes level to record 20%, 65.38% & 31.40% for AST, ALT and AL respectively, when compared to the normal control rats. On the other hand, GGT level showed a significant decrease in serum activity ($p<0.01$) and recorded 426% when compared to the normal control. Administration of Biobran to Carcinogen group (Carcinogen+ Biobran) improved the liver function by inducing a remarkable reduction in the elevated AST, ALT & ALP levels in serum to reach 23.89%, 74.85%, 37.74% and 426% respectively, GGT level showed 637% with estimate to normal control values.

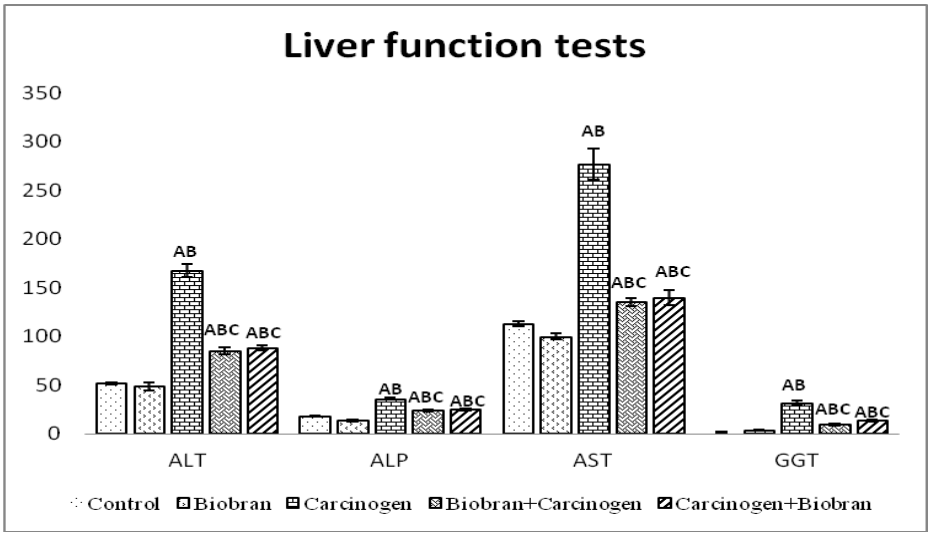

Figure (4): Effect of Biobran on liver function tests. Each value represents the mean±SE .

[A] Significantly different from control group at p < 0.01 level. [B] Significantly different from Biobran group at p <0.01 level. [C] Significantly different from Carcinogen group at p< 0.01 level.

DISCUSSION

N-nitrosodiethylamine (NDEA) is a major environmental carcinogen suggested to increase the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury (Bartsch *et al*., 1989). Since liver is the main site of NDEA metabolism, the production of ROS in the liver may be responsible for its carcinogenic effects (Bansal *et al*., 2005). NDEA is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication (Bhosale *et al*., 2002). Treatment with NDEA and CCl4 has been shown to induce extensive necrosis and inflammatory infiltration, clusters of hepatocyte, necrosis, bile duct proliferation and marked atypia (Sundaresan & Subramanian, 2003, Al-Rejaie *et al*., 2009).

The results of the present study seem to provide support for the chemopreventive effects of Biobran against NDEA-induced hepatocarcinogenesis in rats. There is an appreciable reduction in body weight and increase in liver weight observed in carcinogen group rats as compared to control group rats. Decreased appetite and food intake contribute to the weight loss which could be an indication of the declining hepatic function, an increase in the liver weight of the animals. Sreepriya and Bali, 2005 have also reported marked loss of body weight and increase in liver weights. The steadily increase in body weight during the course of the study for the animals pretreated or posttreated with Biobran, might indicate increase in the animal appetite that resulted in prevention of body weight loss. In addition, Biobran treatment maintained normal animal liver weight probably by preventing NDEA and CCl_4 induced hepatotoxicity.

Histopathological examination of the normal control groups showed normal hepatic lobular architecture with granulated cytoplasm and small uniform nucleus and nucleolus. Carcinogen group showed fatty infiltration of hepatocytes with inflammatory collection and loss of architecture, necrosis and hepatocellular degeneration (Ramakrishnan *et al*., 2006). On the other hand, pre-treated group showed preserved hepatic architecture, minimal nuclear changes and vacuolation of hepatocellular cytoplasm with no inflammation. Group post-treated (carcinogen+Biobran) showed lesser damage of hepatocytes and low index of necrosis, vacuolation of hepatocytes.

In the present study, NDEA and CCL4 administration to rats led to marked increase in the levels of serum AST, ALT and ALP compared to the normal group, which indicating that NDEA could induce a liver damage in rats. These results are in agreement with Bansal *et al* (2005) who attributed the elevation of serum transaminases and alkaline phosphatase to the injured structural integrity of the liver as these enzymes released from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage. γ-GT is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain and its liberation into serum indicates damage of the cells and thus injury to liver (Sivaramakrishnan *et al*., 2008). It is important to point out that serum γ-GT activity is considered to be one of the best indicators of liver damage (Jeena *et al*., 1999).These results are also in agreement with Mittal et al (2006) who found that activities of AST, ALT and ALP were increased significantly following nitroso compounds treatment in rats due to substantial liver damage. Pretreatment ~~group~~ and posttreatment with Biobran significantly decreased the elevation in serum liver enzymes levels to a great extent suggesting that Biobran supplementation protects the hepatocytes from injuries and improves the liver functions of tumor- bearing mice due to its antioxidant potency (Noaman *et al*., 2008).

From these observations it can be concluded that Biobran is a potent natural agent that possesses chemopreventive action against NDEA and CCl_4 induced hepacarcinogenesis.

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الدور الوقائي للمادة الطبيعية بيوبران ضد التسرطن التجريبي

ناريمان كمال بدر الدين ، دعاء عبد الحميد على و ريم محمد عثمان
قسم علم الحيوان- كلية العلوم – جامعة المنصورة

يعتبر مرض السرطان من أكثر الأمراض التى تؤدى الى الوفاة فى العالم. و هناك العديد من انواع العلاج منها الجراحى و العلاج الكيميائى و العلاج الأشعاعى. فى هذه الدراسه تم استخدام ماده طبيعيه (بيوبران) مشتقه من قش الأرز المعالج بانزيمات مشتقه من فطر شيتاكى بهدف حماية الكبد من تأثير المسرطنات الكيميائية المتمثلة في مادتي النيتروزداي إيثايل امين ورابع كلوريد الكربون.

في هذه الدراسة تم استخدام إناث الجرذان السويسرية البيضاء وتم حقنها مرة واحدة فقط بمادة النيتروزداي إيثايل امين ٢٠٠ ملجم/كجم من وزن الجسم في التجويف البريتوني وبمادة رابع كلوريد الكربون مرة أسبوعيا لمدة ٦ أسابيع بجرعة ٣ مل/كجم من وزن الجسم تحت الجلد كمواد مسرطنة وتم العلاج بمادة البيوبران ٢٥ ملجم/كجم من وزن الجسم في التجويف البريتوني.

تم تقسيم ٩٠ جرذ الى خمس مجموعات كالاتي:

- المجموعة الاولى (المجموعة الضابطة): ١٥ جرذ لم يتم حقنها بالمواد المسرطنة ولم تتلقى اي مادة علاجية
- المجموعة الثانية (مجموعة البيوبران): ١٥ جرذ تم معاملتهم بمادة البيوبران بجرعة ٢٥ملجم/كجم من وزن الجسم في التجويف البريتوني ٥ أيام في الاسبوع ابتداء من اليوم ٠ للتجربة حتى نهاية التجربة (٢٢ أسبوع).
- المجموعة الثالثة (المجموعة المسرطنة): ٢٠ جرذ تم حقنهم بمادة النيتروز امين ٢٠٠ ملجم/كجم من وزن الجسم في التجويف البريتوني وبعد أسبوع تم حقنهم بمادة رابع كلوريد الكربون ٣ ملجم/كجم من وزن الجسم وذلك تحت الجلد مرة في الاسبوع لمدة ٦ أسابيع.
- المجموعة الرابعة (المجموعة المعاملة بالبيوبران اولا ثم تم حقنها بالمسرطنات): لقد تم معاملة هذه المجموعة والتي تحتوي على ٢٠ جرذ بمادة البيوبران لمدة اسبوعين قبل حقنها بالمواد المسرطنة والاستمرار بعد ذلك حتى نهاية التجربة.
- المجموعة الخامسة (المجموعة المسرطنة ثم المعاملة بالبيوبران): ٢٠ جرذ تم حقنهم بمادة البيوبران ابتداء من الاسبوع العاشر من التجربة ٢٥ ملجم/كجم من وزن الجسم حتى نهاية التجربة.

ويمكن تلخيص النتائج التي حصلنا عليها كالاتي:

أظهرت نتائج الدراسة بأن معالجة الجرذان بمادة البيوبران قد منع الى حد كبير فقدان وزن الجسم بالمقارنة مع مجموعة الجرذان المسرطنة. وكذلك وجد أن المجموعة المسرطنة أظهرت ازدياد ملحوظ في وزن الكبد بنسبة (٢٤.٧٣%) مقارنة مع المجموعة الضابطة، بينما ادى العلاج بمادة البيوبران الى حد ما إلى الحفاظ على الوزن الطبيعي بالمقارنة مع المجموعة الضابطة.

كما تم قياس مستوى إنزيمات وظائف الكبد فوجد أن المجموعة التى تم حقنهابالمواد المسرطنة غير المعالجة أظهرت أرتفاعا ملحوظا في مستوى إنزيمات الكبد ALTبنسبة(٢٢٤%) وALP بنسبة (٩٩.٢٣ %) وAST بنسبة (١٤٥%) وGGT بنسبة (١٥٨٤ %) بينما نجد في المجموعة التى تم حقنهابالمواد المسرطنة والمعالجة بمادة البيوبران تراجع لحد كبير في مستوى إنزيمات وظائف الكبد ALT بنسبة (٧٤.٨٥%) و ALP بنسبة (٣٧.٧٤%) وAST بنسبة (٢٣.٨٩%) و GGT بنسبة ٦٣٧%. و من ناحيه اخرى لوحظ تراجع وانخفاضا اكبر في مستوى هذه الأنزيمات بالنسبه للمجموعة المعاملة بالبيوبران اولا ثم المواد المسرطنة وكان ALT بنسبة (٦٥.٣٨ %)و ALP بنسبة (٣١.٤٠ %)و AST بنسبة ٢٠% و GGT بنسبة ٤٢٦% بالمقارنة مع المجموعة الضابطة.

اظهر ايضا الفحص المجهرى باستخدام الميكروسكوب الضوئى light microscopy لانسجه الكبد فى الجرذان الغير معالجه وجود الخلايا السرطانيه المتخلله للنسيج الكبدي ووجود تجمعات ليفية ودهنية. اما أنسجه الكبد التالفة فى الجرذان المعالجه بمادة البيوبران فقد ظهر التقلص الواضح فى عدد الخلايا الكبدية السرطانية ووجود عدد كبير من الفجوات السيتوبلازميه مع ارتفاع نسبه الموت الخلوى مع عدم وجود اي التهاب بعد العلاج.

نستخلص من هذه الدراسة أن تناول بيوبران الطبيعية ادى الى حمايه خلايا الكبد من تأثير المواد المسرطنه بدرجه عاليه كما منع الى حد كبير فقدان وزن الجسم و حافظ على المستوى الطبيعى لإنزيمات وظائف الكبد. مما سبق نتوقع ان تؤدي نتائج هذه الدراسة الى إمكانية إستخدام مادة بيوبران الطبيعية الأمنة كماده واقيه ضد الأصابه بسرطان الكبد.