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Heme oxygenase – 1 Expression in Liver and Colon of Rats Exposed to Oxidative stress and Dysplasia by a Carcinogen Diethylnitrosamine and the Possible Therapeutic Effects of Probiotic Versus Pyridazine Derivative and Chemotherapy

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Abstract

1. Background: Diethylnitrosamine (DENA) is dietary carcinogen. It is known as cancer initiator in various organs. The present study investigated the destructive changes of DENA in liver and colon and the possible therapeutic effects of doxorubicin (DOX); pyridazine derivative (MDP) and lactobacillus casei (LAB) against DENA induced dysplasia in liver and colon.
2. Methods:
3. *Lactobacillus casei* were tested for their probiotic properties and prepared for rat administration. Sixty adult male albino rats were divided into six groups. A normal control group received the vehicle; DENA group was injected intraperitoneally (ip) with 55mg/kg body weight twice per week for six weeks. DENA+MDP group received MDP at a dose of 10mg/kg (ip) twice per week for the next 4 weeks after DENA administration; DENA+DOX group received DOX at a dose of 10mg/kg (ip) twice per week for the next 4 weeks after DENA administration; DENA+LAB received LAB orally at a dose of (1.5×10^9 CFU) twice per week for the next 4 weeks after DENA administration. DENA+MDP+DOX group received both MDP and DOX as the aforementioned before. Sera, liver and colon were obtained after the end of experiment. Serum aspartate transaminase and alanine transaminase were detected as well as glutathione peroxidase (GSHPX), nitric oxide, tumor necrosis factor (TNF-α), alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA). Histo-pathological studies and immune-histochemical examination of heme oxygenase-1 (HO-1) were done. Morphometric study was performed. All measurements were followed by statistical analysis.
4. Results: DENA induced significant increase in liver enzymes with significant increase in oxidation and inflammation biomarkers and AFP and CEA. Histologically, DENA showed degenerative changes in hepatocytes and dysplastic aberrant crypt foci in colon. Liver and colon displayed increased cytoplasmic and nuclear immune-expression of HO-1. Therapeutic groups showed partial improvement in biochemical parameters and histological structure. However, *Lactobacillus casei* showed the best result in attenuating pathological and biochemical changes in liver and colon.
5. Conclusion: *Lactobacillus casei* displayed a potential anti-tumorigenic activity against DENA in liver and colon. This may be exerted via HO-1 modulation and suppression of oxidation and inflammation.

6. ***Keywords***: Diethylnitrosamine, dysplasia, liver, colon, *lactobacillus casei*

Background:

Diethylnitrosamine (DENA) is a common carcinogenic substance that found in some food specially smoked and fried ones, alcoholic beverages, cosmetics, chemicals and pharmaceutical materials. It is also present in ground water **(1)**. DENA is known to induce different tumors in all animal species by inducing oxidative stress through the metabolism. This may result in cytotoxicity, mutagenicity and carcinogenicity **(2)**.

Pyridazine and pyridazine derivatives showed interesting pharmacological activities, such as

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antiviral, antituberculosis, antimicrobial, and antioxidant (**3, 4)**. Heterocyclic nucleosides have shown interesting effects. So, attention has been paid for synthesizing them. Because many of them revealed toxic effect on the normal cells**,** other newly synthesized *N*-acyclic and *S*-acyclic pyridazine nucleosides were synthesized.

3-Methylsulfanyl-4,6-diphenyl-pyridazine (MDP), one of the pyridazine derivatives, was a modified form from a compound, 4,6-diphenyl-2H-pyridazine-3-thione has anticancer potential against different human cancer cell lines including Lung (A549), Colon (HCT116), Breast (MCF-7), Prostate (PC3) and Liver (HepG2) cancer cell lines with nearly no toxic effect **(5).**

Doxorubicin is an antibiotic extracted from the *Streptomyces peucetius* bacterium. It has used as a chemotherapeutic agent many years ago. Doxorubicin blocks the enzyme topoisomerase II, leading to DNA damage and apoptosis induction. When combining doxorubicin with iron, free radical-mediated oxidative damage to DNA was induced with further DNA synthesis limitation **(6).** Despite its potency as anticancer therapy, it has many hazardous effects on healthy cells of different organs like liver and large intestine **(7).**

Lactic acid bacterial (LAB) strains, such as Lactobacillus strains, have been deliberated as probiotics because of their health benefits **(8)**. These strains have a long history of depletion in old-style provoked foods as natural inhabitants of healthy human gastrointestinal tracts **(9)**. Probiotic bacteria are essential to express great resistance to acid and bile, stick intestinal surfaces, and colonize in the gastrointestinal tract **(10)**. Several studies revealed its protective effect on colon and preventing colorectal cancer. Probiotic bacteria have also an influence on human immunity through regression of carcinogenesis **(11).** So, Probiotic bacteria were considered as a promising approach for cancer therapy and prevention without causing hazardous effects like other therapeutic approaches.

Current study was conducted to investigate the potency of curative effects of MDP, Doxorubicin and *Lactobacillus casei* on DENA-induced oxidative and dysplastic changes in liver and colon in adult male albino rats.

Methods:

Chemicals

1- **N-DiethylNitrosamine (DENA**) was purchased from Sigma Aldrich (St. Louis, MO, USA) (CAS no. 55-18-5). DENA was dissolved in 4 ml normal saline to obtain a final volume of 27.5mg/ml.

2- **Doxorubicin hydrochloride:** Doxorubicin (Adriblastina vials, 50 mg/25 ml) was purchased from Pfizer Company, Egypt.

3- The reagents, indicators, carbon substrates for biochemical tests used in the study were of analytical grade and purchased from Hi Media Chemicals Ltd, Mumbai, India and Oxoid, UK.

4- 3-Methylsulfanyl-4, 6-diphenyl-pyridazine (MDP) synthesis

In a solution of sodium hydroxide (0.04 g, 1 mmol) in ethanol (50 ml), Chloro thiourea derivatives (0.01 mol) was added, and then the reaction mixture was stirred at room temperature for about 1 h. Methyl iodide, 2-chloroethanol, 2-(2-chloroethoxy)-ethanol (0.01 mol) was added and the reaction mixture was stirred at 70°C for 3 h. The reaction mixtures were evaporated under reduced pressure and the residue were crystallized using ethanol to give 3-Methylsulfanyl-4,6-diphenyl-pyridazine synthesis **(5).** For animal injection, MDP was dissolved in normal saline to obtain a final volume of 5 mg/ml.

4- *Lactobacillus casei* preparation

*Lactobacillus case*i were inoculated in De Man, Rogosa, and Sharpe (MRS) broth and grown under aerobic conditions at 37ºC for 24 – 48 h. Bacterial cultures were harvested by centrifugation (20 min, 3500 rpm, 4ºC), and the supernatant was collected **(12).**

Identification of LAB and growth condition:

Twenty-five purified colonies (>100 colonies/each sample) were isolated and identified as described previously **(13)**. Cell morphology and colony characteristics on MRS agar were examined and presumptive lactobacilli, stored at − 80^0 C in 20% glycerol for further identification using VITEK 2 card aerobic (bioMérieux, Marcy l'Etoile, France) in Animal Health Institute, Giza, Egypt.

Lactic acid bacteria properties as probiotic:

1. Antimicrobial susceptibility testing for *Lactobacillus casei* strains:

The disk diffusion method **(14)** was performed to assess the susceptibility of *Lactobacillus casei* strains to the following antimicrobial agents. All antibiotics discs were purchased from Oxide, UK; amikacin (30μg), cefotaxime (30μg), co-amoxicillin (30μg) ceftazidime (30μg), ciprofloxacin (5μg), levofloxacin (5μg), imipenem (10μg), meropenem (10μg), gentamicin (10μg), ampicillin/sulbactam(20μg); piperacillin (10μg), tigecycline (15μg), colistin (10μg), and piperacillin/tazobactam (100 μg/10μg). The susceptibility was classified according to CLSI recommendation (CLSI, 2015)**.** Current quality control testing was done using *Escherichia coli* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853.

Probiotic properties of *Lactobacillus casei*:

Lactobacillus casei isolate was evaluated for its probiotic properties by evaluating its tolerance to temperature, acid, and bile salt as previously described by **(15).**

***Lactobacillus casei* preparation for rat**

Lactobacillus casei, were isolated from yogurt samples as described in our previous study **(13)** and cultured in De Man Rogosa Sharpe (MRS) media at 37 °C for 48 h. After that, the cultures were centrifuged at 2000 rpm for 5 min, washed 3 times with Phosphate buffer (PBS), and then resuspended in PBS. *Lactobacillus casei* suspension was harvested after frequent filtration when its density reached 1.5 x 109 Colony-Forming Units (CFU)/ml and stored at 0–4°C. It was given to the rats via oral gavage twice a week for 4 consecutive weeks. The reagents, indicators, carbon substrates for biochemical tests used in the study were of analytical grade and purchased from Hi Media Chemicals Ltd., Mumbai, India and Oxoid, UK.

Animals and treatments

Sixty adult Sprague–Dawley male albino rats weighting 140±10g were used in this experiment. Care and use of the animals were conducted under supervision of the Animal Ethics Committee of National Organization for Drug Control and Research (NODCAR) and according to international ethics and regulations for animal research in laboratory applications **(16).** After two weeks of acclimatization, rats were randomly divided into six groups of ten animals each, as follows:

1. Group I (Control group): animals were injected intraperitoneally (ip) with saline (2ml/kg).

2. Group II (DENA group): animals were injected intraperitoneally (ip) with diethylnitrosamine (DENA) at a dose of 55mg/kg body weight twice per week **(17)** for 6 weeks, and then received the vehicle that corresponded to other treatments twice a week for the next 4 consecutive weeks; normal saline (2ml/kg, ip) and PBS (1ml/kg, via oral gavage).

3. **GroupIII (DENA+MDP group):** animals received DENA as the aforementioned in group II followed by IP injection of MDP at a dose of 10 mg/kg body weight twice a week for the next 4 consecutive weeks.

4. Group IV **(DENA +DOX)**: animals received DENA as the aforementioned in group II followed by IP injection of DOX at a dose of 10 mg/kg body weight **(18)** twice a week for the next 4 consecutive weeks.

5. Group V (DENA+LAB): animals received DENA as the aforementioned in group II followed by administration of lactobacillus at a dose of (1.5 x 10^9 CFU) orally by gavage **(19)** twice a week for the next 4 consecutive weeks

6. Group VI (DENA+MDP+DOX): animals received DENA as the aforementioned in group II followed by administration of MDP and DOX as mentioned in group III and IV.

Blood sampling

At the end of the experimental period, Blood samples were collected through retro-orbital puncture in centrifuge tubes. After complete coagulation of the blood, tubes were centrifuged at 3000 rpm for 15 minutes**.** Sera were then separated and immediately frozen at -20°C.

Biochemical analysis and estimation of biochemical parameters:

Estimation of serum alpha fetoprotein (AFP), carcinoembryonic antigen (CEA), tumor necrosis alpha (TNF-α) were measured by colorimetric ELISA kit (Abia Ref. DK.045.01.3, Fortress diagnostics Ref. BXE0811A, Sino Gene Clon Ref. SG20127, Diametra Ref DK0015 and Gene II ref A79765 respectively) The serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by colorimetric method using Bio diagnostics kit (AL 1031, AS 1061), while the activity of alkaline phosphatase (ALP) was performed according to the method of Belfield and Goldberg using Reactive Gpl kit Ref. EZ002LQ-SP). Also, the serum activity of gamma glutamyle transferase (GGT) was measured by the method of Persijn and Vander Slik using Vitro Scient Ref. 11714kit. Kits of nitric oxide (NO) and glutathione peroxidase (GSHPX) were purchased from Biodiagnostic Company (Biodiagnostic, Egypt).

Histopathology:

Overnight fasted animals were euthanized by cervical dislocation. Liver and distal colon were removed from rats and fixed in 10% formaldehyde solution. Tissue sections (4µm) mounted on glass slides and stained with haematoxylin & eosin to evaluate normal structure and pathological changes. Immunohistochemical staining was carried out using heme oxygenase -1 (HO-1) (Invitrogen. Thermo Fisher Scientific, USA) Mouse monoclonal antibody (Catalog # MA1-112) where its immunoreactivity was cytoplasmic and nuclear, positive control was human lung tissue. Additional slides of liver and colon specimens were treated with buffer solutions.

Microbiological Analysis

To evaluating the bacterial translocation, blood (5 ml) and tissue samples were immediately collected in ethylene diamine tetraacetic acid-containing (EDTA) sterile tubes and 5 mL of sterile transport medium, respectively. Tissue samples were homogenized in an ultrasonic bath (Ultrasons, USA) for 5 minutes. The counting of total aerobic count was done by inoculating 1 ml of the sample on brain-heart infusion agar (LabM, UK) and incubated at 37^{o}C for 48h. The number of colonies formed on each plate was counted and corrected for the weight of the original tissue.

Quantitative Morphometric Study:

The measurements done by used "Top view" image analyser computer system (China). Mean length of mucosa in the distal colon in millimetres was measured. Number of goblet cells was measured as mean area % in high power fields (x400). The mean area % of HO-1 immunohistochemical stain; using a total magnification 400 was done. Mean number of immunostained nuclei in hepatocytes was counted in high power fields (x400). Digitalized images were captured from 10 randomly selected non-overlapping fields from each section.

Statistical analysis:

Statistical analysis was performed using the arithmetic mean, standard error (SE) after performing Shapiro-Wilk test to check normality of distribution, analysis of variance (one way ANOVA) and comparison between each two groups using post Hoc Tukey test, using the statistical SPSS software for Windows, Version 18 (USA). Significance was P≤ 0.05 **(20).** Statistical analysis of bacteriological data was carried out using GraphPad Prism Statistics software package version 3.00 (GraphPad Prism Software Inc., San Diego, CA, USA). Two-way ANOVA and t-test (two-tailed) were used to determine significant differences in the viability of the Lactobacillus strains. Statistical significance was defined as *P value* less than 0.05

Results

Microbiological analysis

Lactobacillus casei was selected as LAB bacteria strain in our study among twenty-five lactic acid bacteria were collected and identified (**13**). *Lactobacillus casei* was assessed for probiotic properties to fulfill the desirable criteria to be applied **(Table 1).**

Bacterial Translocation. In order to assess the effectiveness of *L. casei* cells in inhibiting cell growth by determining the incidence of oral delivery of *Lactobacillus casei* in blood, liver, colon, and lymph nodes in both control and treated group. *L. casei* cells were increased significantly in the control group (compared with the normal control 24 hours after cancer induction **(Table 2).** No statistical significance showed in the bacterial translocation for both the control and DENA+LAB groups. All animals of both groups showed positive translocation of *L. casei*. However, a significant decrease in the number of colonies translocate to the blood, liver, and colon was observed **(Fig. 1)** that indicated the survival of probiotic bacteria under the different stresses during the 6-week-DENA exposure and reached the target tissues in an optimal number even though they were comparable to the control.

Table (1) Assessment of pH, Temperature and bile tolerance of *Lactobacillus casei* after the allocated incubation period for each.

Parameters	Control	pH tolerance			Temperature resistance (ºC)					Bile salt tolerance (%)			
		2.0	2.5	3.0	25	30	37	40	45	0.2	0.5	1	2
ΔA_{560}	1.58± 0.01	0.003±0.002*	0.07±0.05*†	0.96±0.0*†	0.56±0.04*	0.8±0.0*	1.3±0.0*$	1.17±0.01*	0.5±0.05*	0.8±0.05*#	0.35±0.05*#	0.1±0.02*#	0.09±0.05*
Plate count	S >300	S >50	S 70	S >200	S >30	S >230	S >270	S >120	S >96	S >30	S >88	S >220	NS 0.0

Parameters: ΔA_{56} *:* **Absorbance, (survival) No. of colony (CFU/ml) : Plate count**

* significantly different when compared to control using two-way ANOVA, *P≤0.05,* † significantly different when compared to pH 2 using two-way ANOVA, *P≤0.05,* $ significantly different when compared to temperature 25, 30, 40, 45 oC using two-way ANOVA, *P≤0.05*

significantly different when compared to bile concentration 2% using two-way ANOVA, *P≤0.05*

S: Survival of bacteria at different parameters , NS: not survive

Table 2: Bacterial translocation to blood and organs after cancer induction

Groups	Bacterial counts (log CFUg/tissue)			
	Blood	**Liver**	**Kidney**	**Lymph node**
Control group (*Lactobacillus casei*)	3.7±0.1*	3.8±0.05	2.7±0	3.1±0.2
***Lactobacillus casie* /DENA-treated group**	2.5±0.3†	2.1± 0.2	1.8±0.3	2.4±0.5

* *P* ≤.05 using one-way ANOVA. † *P* ≤.05 compared with control group.

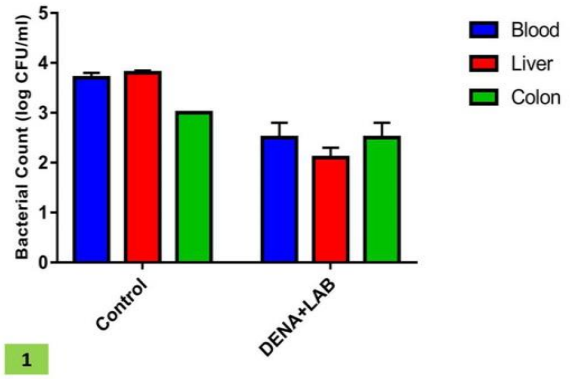


Fig. 1: Translocation of *L. casei* to the blood and testes after twice-weekly treatment for 4 weeks in control animals and after 6-weeks-DENA-exposed group. The difference between groups was statistically examined using one way ANOVA. * DENA-treated group significantly compared with control testes*; P-value* < 0.0001; ** DENA-treated group significantly compared with control blood*; P-valu*e < 0.0001.

Biochemical results:

A significant increment ($p<0.0001$) in the activities of serum AST, ALT, ALP GGT, TNF-α and NO of DENA only treated group when compared with the control group. Otherwise, there was a significant ($p<0.0001$) decrement in the activities of these parameters in therapeutic groups as compared with DENA-treated group. In spite of improvement in these measurements, most of therapeutic treatments did not succeed in reversing them to the control levels. While GSHPX showed significant decrease in DENA and combined therapy group as compared with control, DOX, MDP and LAB treated groups. LAB and MDP treated groups succeeded in reversing glutathione to the control level **(Tables 3, 4).** Regarding AFP, despite the little increase in its levels in all groups as compared with control group but this rise was significant. However, therapeutic groups showed significant improvement when compared with DENA group. CEA showed significant increase in DENA treated rats as compared with control and other therapeutic groups. All therapeutic groups failed to decrease CEA to the control level. DOX and LAB treated groups showed significant ameliorating effect as compared with other therapy groups **(Table 5).**

Table 3: Effect of different treatment on diagnostic markers of liver function in all experimental groups.

Group / Parameters	ALT U/L	AST U/L	ALP U/L	GGT U/L
Control	83.5±0.2	35.9±0.5	65±0.5	14±0.4
DENA	91 ± 0.4	56± 0.7*	151±0.5*	32±0.8*
DENA+MDP	87 ± 0.7	43±0.8$^{\#*}$	112±0.4$^{\#*}$	15.6±0.2$^{\#}$
DENA+DOX	88.5±0.2	45±0.2$^{\#*}$	134±0.5$^{\#*}$	19 ± 0.5$^{\#}$
DENA+LAB	87.5±0.5	43±0.5$^{\#*}$	103±0.4$^{\#*}$	12.6±0.2$^{\#}$
DENA+MDP+DOX	88.7±0.2	49±0.3$^{\#*}$	124±0.4$^{\#*}$	28± 0.3$^{\#*}$

Data were expressed as mean values ± SE (n = 6 rats), Means with different superscript letters are significant different at $P \leq 0.05$. Data are analysed with one- way ANOVA followed by Tukey Test *significant difference as compared with control, # significant difference as compared with DENA

Table 4: Effect of different treatment on diagnostic markers of TNF-α, NO and oxidative stress (GSHPX) in all experimental groups.

Group / Parameters	TNF-α ng/ml	NO µmol/l	GSHPX mU/ml
Control	3.4 ± 0.1	4.2±0.3	32 ± 0.4
DENA	7.5 ± 0.1*	9.9±0.5*	11± 0.8*
DENA+MDP	5.1 ± 0.3$^{\#*}$	7.1±0.5$^{\#*}$	31± 0.3$^{\#}$
DENA+DOX	5 ± 0.3$^{\#*}$	9.3±0.4*	21±0.2$^{\#*}$
DENA+LAB	5.3±0.05$^{\#*}$	5.8±0.4$^{\#*}$	31± 0.2$^{\#}$
DENA+MDP+DOX	6 ± 0.1$^{\#*}$	7.5±0.4$^{\#*}$	14±0.5*

Data were expressed as mean values ± SE SE (n = 6 rats), Means with different superscript letters are significant different at $P \leq 0.05$. Data are analysed with one- way ANOVA followed by Tukey Test.
*Significant difference as compared with control, # Significant difference as compared with DENA

Table 5: Effect of different treatment on tumor markers (AFP and CEA) in all groups

Group / Parameters	AFP IU/ml	CEA ng/ml
Control	3.5 ± 0.1	2.08 ± 0.15
DENA	4.5 ± 0.1*	49.8 ± 1.13*
DENA+MDP	4.1 ± 0.2$^{\#*}$	19.01± 1.2$^{\#*@}$

DENA+DOX	3.9±0.14#*	11.1 ± 0.5#*
DENA+LAB	3.9 ± 0.05#*	8.5 ± 0.4#*
DENA+MDP+DOX	3.9 ± 0.1#*	19.16±1.6#*@

Data were expressed as mean values ± SE SE (n = 6 rats), Means with different superscript letters are significant different at P ≤ 0.05. Data are analysed with one- way ANOVA followed by Tukey Test. *Significant difference as compared with control, # Significant difference as compared with DENA, @ Significant difference as compared with DOX and LAB

Histological results

Hematoxylin and eosin results

Liver stained sections of control group showed a central vein & radiating hepatocytes, which have central, rounded, vesicular nuclei and acidophilic cytoplasm. Some cells were binucleated. Blood sinusoids were located between the cords & lined by flat endothelial cells. Presence of some cells with ovoid nuclei, which most probably kupffer cells, was observed **(Fig. 2a, b).** Diethylnitrosamine (DENA) group revealed disturbed architecture of hepatic lobule with bridging necrosis. Ballooned hepatocytes with cytoplasmic foamy vacuolization and hyperchromatic nuclei were observed, other cells were shrunken with small and pyknotic nuclei. Acidophilic Councilman bodies (apoptotic bodies) were noticed **(Fig. 3a, b).** Pyridazine derivative (MDP) group showed partially restored hepatic architecture with dilated and congested central vein and blood sinusoids. Some hepatocytes appeared normal with vesicular nuclei; others showed vacuolated cytoplasm, hyperchromatic nuclei and apoptotic nuclei. Karyorrhexis in nuclei of degenerating cells was observed **(Fig. 4a, b).** Doxorubicin (DOX) group revealed partially restored architecture of hepatic lobule with dilated central vein and marked dilation of blood sinusoids. Most hepatocytes showed cytoplasmic vacuolization with vesicular nuclei. Few hepatocytes with karyorrhexic nuclei were observed **(Fig. 5a, b).** *Lactobacillus casei* (LAB) group showed marked improvement in the form of restored hepatic architecture with minimal dilatation and congestion of central vein and blood sinusoids. Most hepatocytes appeared normal with acidophilic cytoplasm and vesicular nuclei. Some showed hyperchromatic nuclei and apoptotic nuclei **(Fig. 6a, b).** MDP+DOX group revealed disturbed architecture and severe necrotic changes of hepatocytes. Most of them showed vacuolated cytoplasm, hyperchromatic nuclei, karyorrhexis and apoptotic nuclei. Marked dilatation of blood sinusoids and congested central vein were observed **(Fig. 7a, b).** Colon stained sections of control group showed folded mucosa and surface epithelium formed of closely packed tall columnar cells with basal oval nuclei interposed with goblet cells **(Fig. 8).** DENA group showed dysplastic aberrant crypt foci in the form of distorted crypt structure, existence of hyperchromatic nuclei, few goblet cell numbers with apparent short mucosal length as compared with control normal. **(Fig. 9).** MDP group revealed disturbed crypt structure and few goblet cell numbers with apparent short mucosal length as compared with control normal. Most of nuclei appeared hyperchromatic. Necrotic area was also observed **(Fig. 10)**. DOX group showed disturbed crypt structure and few goblet cell numbers as compared with control normal. Most of nuclei were hyperchromatic. Some areas showed existence of nuclear stratification. Ulcerated surface with underlying inflammatory cellular infiltrate was observed **(Fig. 11).** LAB group revealed marked restoration of normal structure of colonic folds with apparent normal goblet cell number. Small areas showed necrotic changes **(Fig. 12).** MDP+DOX group showed severe distortion of colonic crypts with some areas of nuclear stratification. Inflammatory cellular infiltrate and multinucleated giant cells were observed **(Fig. 13a, b).**

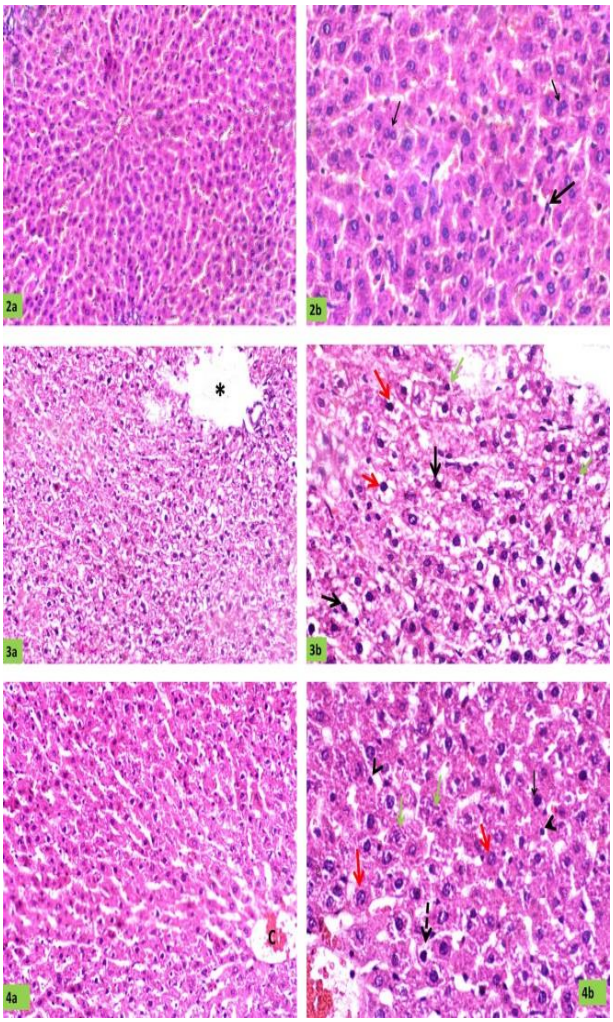
Fig. (2a, b – 4a, b): H&E stained sections of liver from **(2a, b):** control group showing **(a)**: a central vein & radiating hepatocytes, which have central, rounded, vesicular nuclei and acidophilic cytoplasm**. (b):** Some cells are binucleated (thin arrows). Blood sinusoids are located between the cords & lined by flat endothelial cells. Note the presence of some kupffer cells (thick arrow)**. (3a, b):** DENA group showing **(a):** disturbed architecture of hepatic lobule with bridging necrosis (*)**. (b):** Ballooned hepatocytes with cytoplasmic foamy vacuolization and hyperchromatic nuclei (red arrows) are revealed. Other cells are shrunken with small and pyknotic nuclei (green arrows). Acidophilic Councilman bodies (black arrows) are observed**. (4a, b):** MDP group showing **(a):** partially restored hepatic architecture with dilated and congested central vein (C)**. (b):** Some hepatocytes appear normal with vesicular nuclei (red arrows); others show vacuolated cytoplasm (dotted arrow), hyperchromatic nuclei (black arrow) and apoptotic nuclei (arrow heads). Karyorrhexis (green arrows) in nuclei of degenerating cells are observed. Note the dilated blood sinusoids **(Hx & E stain: 2a-4a x200; 2b-4b x400).**

Immunohistochemical results

Liver immunostained sections of control group showed mild cytoplasmic immunoreaction around the central zone area **(Fig. 14a, b).** DENA group showed intense cytoplasmic reaction. Many degenerated hepatocytes showed positive nuclear immunoreaction **(Fig. 15a, b).** MDP group revealed moderated cytoplasmic immunoreaction. Few hepatocytes showed positive nuclear reaction **(Fig. 16a, b).** DOX group showed moderate immunoreaction around the central zone area **(Fig. 17a, b).** . LAB group revealed moderate diffuse cytoplasmic immunoreaction. Few cells showed positive nuclear reaction **(Fig. 18a, b).**

MDP+DOX group showed intense diffuse cytoplasmic reaction. Many hepatocytes revealed positive nuclear reaction **(Fig. 19a, b).**

Colon immunostained sections of control group showed positive cytoplasmic and nuclear immunoreaction in lining epithelium of colonic mucosa and cells in lamina propria **(Fig. 20).** DENA group revealed intense cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria **(Fig. 21).** MDP group showed moderate cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria **(Fig. 22).** DOX group showed moderate cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria **(Fig. 23).** LAB group revealed positive cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria **(Fig. 24).** MDP+DOX group showed moderate cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria **(Fig. 25).**



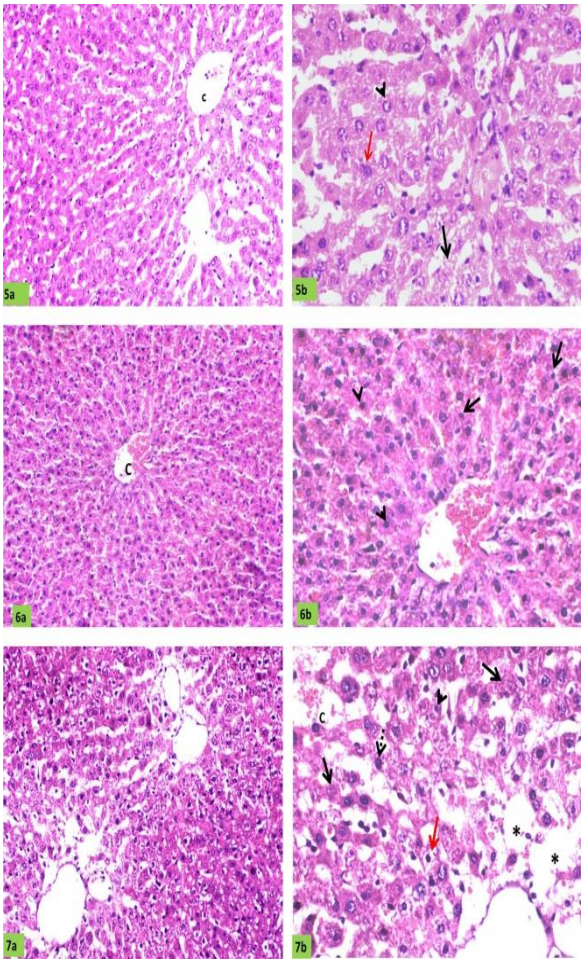


Fig. (5a, b – 7a, b): H&E stained sections of liver from **(5a, b):** DOX group showing **(a):** partially restored architecture of hepatic lobule with dilated central vein (C)**. (b):** Most hepatocytes show cytoplasmic vacuolization (black arrow) and vesicular nuclei (arrowhead). Few hepatocytes reveal karyorrhexic nuclei (red arrow). Marked dilation of blood sinusoids is observed. **(6a, b):** LAB group showing **(a):** Marked restoration of hepatic architecture with minimal dilatation and congestion of central vein (C)**. (b):** Most hepatocytes appear normal with acidophilic cytoplasm and vesicular nuclei. Some cells show hyperchromatic nuclei (arrowheads). Apoptotic nuclei (arrows) are noticed. Minimally dilated blood sinusoids are detected. **(7a, b):** MDP+DOX group showing **(a):** disturbed architecture and severe necrotic changes of hepatocytes**. (b):** Most cells show vacuolated cytoplasm (red arrow), hyperchromatic nuclei (dotted arrow), karyorrhexis (black arrows) and apoptotic nuclei (arrowhead). Marked dilatation of blood sinusoids (*) and congested central vein (C) are observed **(Hx & E stain: 5a-7a x200; 5b-7b x400).**

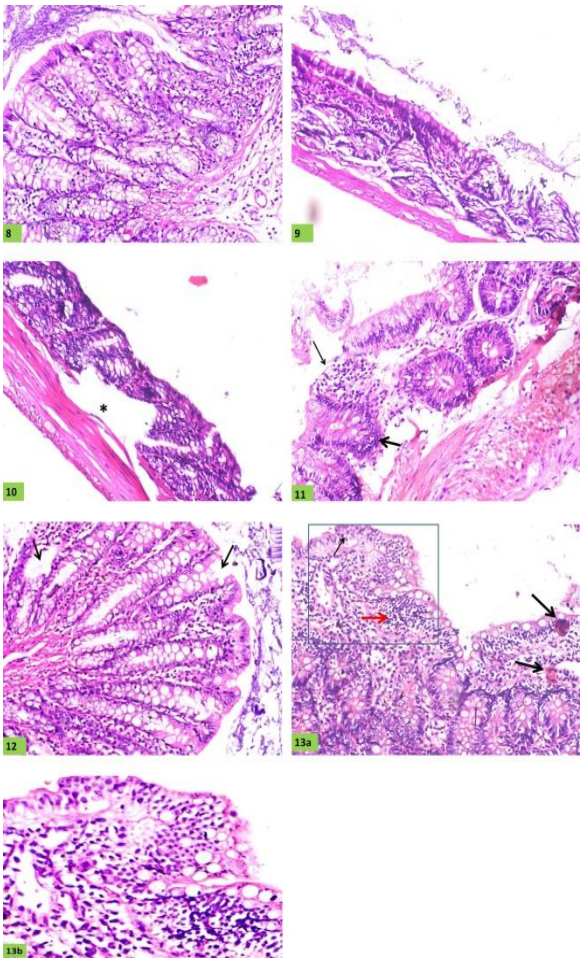


Fig. (8 – 13): H&E stained sections of colon from **(8):** control group showing folded mucosa and surface epithelium formed of closely packed tall columnar cells with basal oval nuclei interposed with goblet cells. **(9):** DENA group showing dysplastic aberrant crypt foci in the form of distorted crypt structure and existence of hyperchromatic nuclei. Few goblet cell numbers with apparent short mucosal length as compared with control normal are noticed. **(10):** MDP group showing disturbed crypt structure and few goblet cell numbers with apparent short mucosal length as compared with control normal. Most of nuclei appear hyperchromatic. Necrotic area is observed (*)**. (11):** DOX group showing disturbed crypt structure and few goblet cell numbers as compared with control normal. Most of nuclei are hyperchromatic. Some areas show existence of nuclear stratification (thick arrow). Note the ulcerated surface with underlying inflammatory cellular infiltrate (thin arrow). **(12):** LAB group showing marked restoration of normal structure of colonic folds with apparent normal goblet cell number. Small areas show necrotic changes (arrows). **(13a, b):** A photomicrograph of H&E stained sections of colon from MDP+DOX **group** showing **(a):** severe distortion of colonic crypts. Some areas show nuclear stratification (thin arrow). Inflammatory cellular infiltrate (red arrow) and multinucleated giant cells are observed (thick arrows)**. (b):** a higher magnification of the boxed area **(x400). (Hx & E stain: 8-13a x 200).**

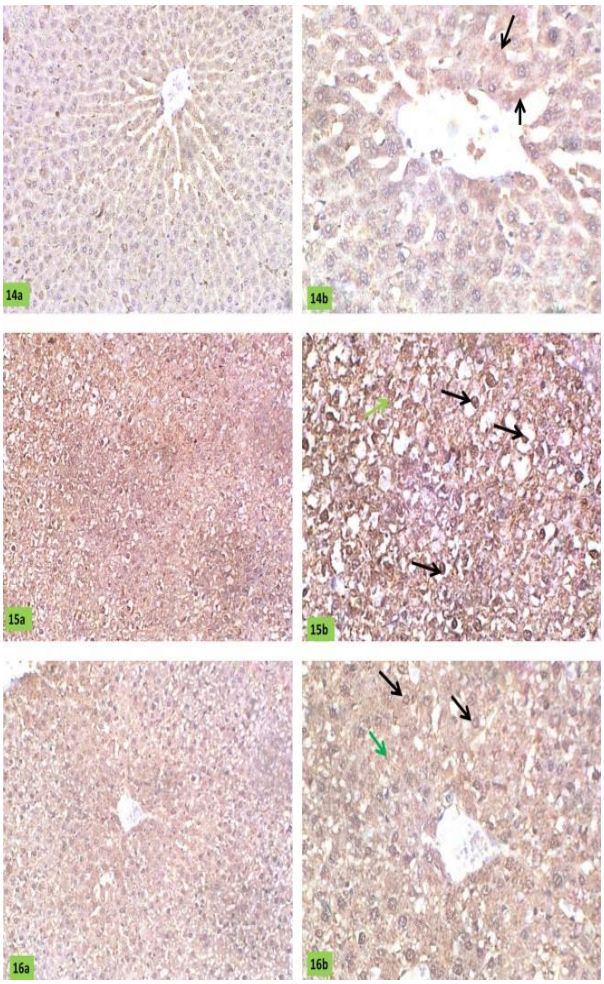


Fig. (14a, b – 16a, b): HO-1 immunostained sections of liver from **(14a, b):** control group showing mild cytoplasmic immunoreaction (arrow) around the central zone area**.. (15a, b):** DENA group showing intense cytoplasmic reaction (green arrow). Many hepatocytes show positive nuclear immunoreaction (black arrows). **. (16a, b):** MDP group showing moderate cytoplasmic immunoreaction (green arrow). Few hepatocytes show positive nuclear reaction (black arrows). **(HO-1 immunostain: 14a-16a x200; 14b-16b x400).**

Morphometrical and statistical results

Regarding length of colonic mucosa, there was a significant decrease in DENA group as compared with negative control and LAB treated group. Other therapeutic groups didn't show any significant improvement. Number of goblet cells showed significant decrease in DENA group as compared with negative control and LAB group. It worthy to mention that MDP and DOX treated groups showed more decrease in number than DENA while LAB treated group succeeded in restoring number of goblet cells to the control level **(Table 6).**

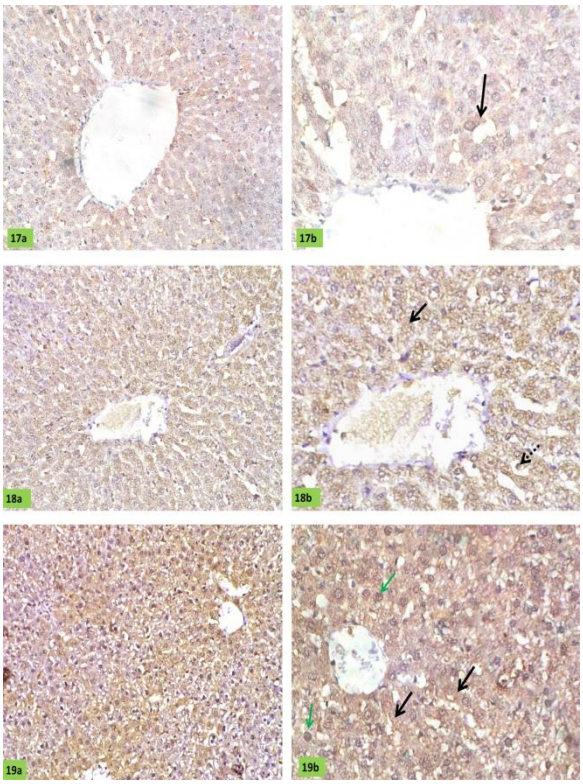


Fig. (17a, b – 19a, b): HO-1 immunostained sections of liver from **(17a, b):** DOX group showing moderate cytoplasmic immunoreaction (arrow) around the central zone area.**. (18a, b):** LAB group showing moderate diffuse cytoplasmic immunoreaction (arrow). Few cells show positive nuclear reaction (dotted arrow)**.. (19a, b):** MDP+DOX group showing intense diffuse cytoplasmic reaction (black arrows). Many hepatocytes show positive nuclear reaction (green arrows). **(HO-1 immunostain: 17a-19a x200; 17b-19b x400).**

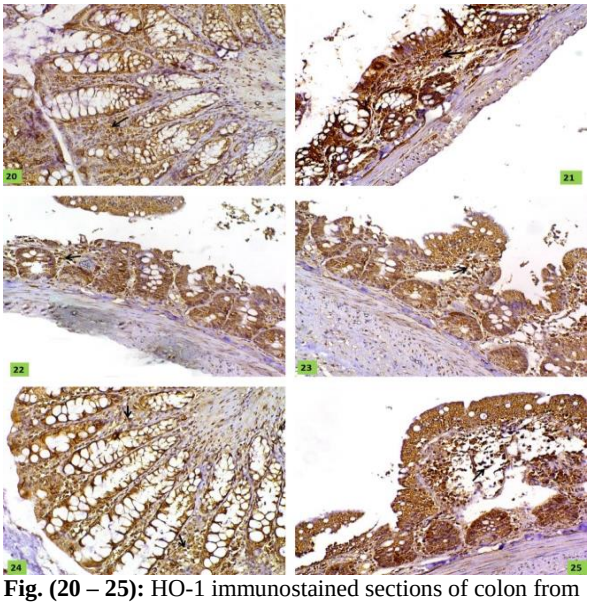


Fig. (20 – 25): HO-1 immunostained sections of colon from **(20):** control group showing positive cytoplasmic and nuclear immunoreaction in lining epithelium of colonic mucosa and cells in lamina propria (arrow). **(21):** DENA group showing intense cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria (arrow). **(22):** MDP group showing moderate cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria (arrow). **(23):** DOX group showing moderate cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria (arrow). **(24):** LAB group showing positive cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria (arrow). **(25):** MDP+DOX group showing moderate cytoplasmic and nuclear immunoreaction in epithelium of colonic mucosa and cells in lamina propria (arrow). **(HO-1 immunostain: 20-25 x 200)**.

Table 6

Group / Parameters	Length of of colonic mucosa (mm)	Area% of goblet cells in mucosa
Control	0.44± 0.02	28.3±0.21
DENA	0.03 ± 0.001*	12.08±0.12*
DENA+MDP	0.04 ± 0.001*	10.6±0.18*#
DENA+DOX	0.05 ± 0.002*	8.5±0.17*#
DENA+LAB	0.42 ± 0.02#	28.5±0.4#
DENA+MDP+DOX	0.09± 0.001*	11.3±0.13*

Data were expressed as mean values ± SE with significant difference when P value ≤0.05.
*Significant difference as compared with control
Significant difference as compared with DENA

Regarding HO-1 immunoreactivity in colon, there was a significant increase in DENA group as compared with negative control and other therapeutic groups. However, LAB and negative control groups showed no significant difference; while, other therapeutic groups were significantly different when compared with the control group.

Liver tissues showed significant high cytoplasmic and nuclear immunoreactivity in DENA treated group as compared with control and other therapeutic groups. However, DOX and LAB treated groups showed significant difference in cytoplasmic reaction when compared with control group; while, they showed no significant difference as regard nuclear immunoreactivity. MDP and combined groups were significantly different than control group as regard both cytoplasmic and nuclear immunoreaction **(Table 7).**

Table 7: Mean ± SE of area% of HO-1 immunoreactivity, number of HO-1 immunostained hepatocyte nuclei, in different studied groups.

Group / Parameters	Area% of HO-1immunoreactivity in mucosa of colon	Area% of HO-1immunoreactivity in liver	Number of HO-1 immunostained hepatocyte nuclei
Control	27.2 ± 0.3	7.4 ± 0.8	1.3±0.21
DENA	48.1 ± 0.5*	27.2 ± 1.1*@	31.16±0.4*
DENA+MDP	36.7 ± 0.19#*	23.6 ± 0.3#*@	10±0.25*#
DENA+DOX	40.7± 0.4#*	14.4 ± 0.2#*	1.3±0.4#
DENA+LAB	28.5 ± 0.7#	18.2 ± 0.3#*@	1.6±.3#
DENA+MDP+DOX	45.4 ± 0.61#*	20.06± 0.5#*@	10.3±2.4*#

Data were expressed as mean values ± SE with significant difference when P value ≤0.05.
*Significant difference as compared with control
Significant difference as compared with DENA
@ Significant difference as compared with DOX

Discussion

Nitrosamines are dietary carcinogens which are considered one of the common causes of hepatocellular and intestinal carcinogenesis. They also contribute to the development of oxidative stress, chronic inflammation, and dyplastic or preneoplatic changes in response to tissue injury **(21, 22, 23).** Diethylnitrosamine **(**DENA), one of the common nitrosamines, is used to investigate its cytotoxic mechanisms on different tissues and organs. Moreover, DENA leads to changes in serum and tissue enzyme markers **(24).**

DENA cause agitations in the nuclear enzymes involved in DNA repair/replication It is usually used as a carcinogen for induction of liver and colon cancer in experimental animals **(2)**. DENA has been metabolized to its active form named ethyl radical metabolite. this reactive product interacts with DNA leading to mutation, with subsequent carcinogenesis **(25)**.

Probiotic have been known for several years due to its health benefits exert when applied in treatment especially in cancer treatments. Cancer caused a compromised immunity of patients that in turn provide the desirable effect of probiotic in regulating proliferation and apoptosis of cancer cells **(26)**

In the present study, we have investigated the therapeutic effects of 3-Methylsulfanyl-4,6-diphenyl-pyridazine, Lactobacillus and Doxorubicin on different biochemical studies and histological structures of rat livers and colon in DENA-induced early oxidative and preneoplastic changes.

The results of this study revealed that DENA group showed a significant elevation in serum liver enzymes, tumor markers AFP and CEA as compared to the negative control group. This finding was in agreement with **(25)** and **(27)**. These results could be as a result of hepatocellular damage and impairment of liver function by DENA. These findings were similar to other published data which revealed incidence of HCC in rats by DENA **(28)**. AST and ALT are sensitive markers of liver damage. ALT is mainly present in the cytoplasm of hepatocytes while AST is mainly present in the cytoplasm and mitochondria of hepatocytes. So, the increased level of ALT and AST in the serum may be an indicator of damaged liver cell membranes and mitochondria **(29).**

The AFP is an onco-fetal protein that is abundantly synthesized in the fetal and new-born rat liver and it is absent in adult animals. The elevation of AFP in cancer may be due to either increased transcription of AFP gene affecting AFP production. The DEN-treated group rats exhibited the presence of this biomarker **(30).** So, increase its level in this study may be an indicator of preneoplastic alteration.

Elevated serum CEA levels with DENA administration was most probably linked to production rates of tumor besides its location, stage, size, differentiation and vascularity. The tumor could release CEA from damaged liver cells or colon. Other studies discovered that CEA is normally eliminated from circulating plasma by the liver. Accordingly, the increased serum levels of CEA might consequently result from compromised hepatic uptake of CEA or CEA-like glycoproteins **(31).**

DENA prompts hepatic and colon injury through disturbances in antioxidant defense systems, rises in the reactive oxygen species (ROS) and membrane lipid peroxidation with subsequent bio-membranes damage **(32).** ROS can harmfully affect different cellular biomolecules like protein, RNA and DNA causing severe destruction to tissues and organs resulting in chronic disease such as cancer, heart disease, diabetes mellitus, arthritis and neurodegenerative disease **(33, 34)**. Non-enzymatic (mainly GSH) and enzymatic antioxidant such as GPx are the crucial enzymes in removing free radicals. Our present study revealed oxidative stress by increased production of NO and TNF-α accompanied with decreased activities of antioxidants including GSH level in rats treated with DENA as compared with control group which lead to liver and colon damage as confirmed by histopathological studies. Our data are in agreement with previous findings of **(35) and (36).**

The current data indicated that different therapeutic approaches used in this study caused partial improvement in different biochemical parameters as compared with DENA treated group but none of them succeeded in reversing any parameter to normal. This might be attributed to anti-inflammatory and anti-oxidant activity of pyridazine derivatives **(37),** and lactobacillus **(38).** *L. casei* had significantly lower serum enzyme levels, less inflammation and attenuates liver fibrosis development as proved by **(39).**

It is worth mentioning that despite the pro-inflammatory and oxidative effect of doxorubicin, but it also has partial ameliorating effect on biochemical parameters. This might be due to its anti-proliferative effect **(40).** Moreover, previous study revealed that doxorubicin significantly elevated glutathione-S-transferase (GST) activity in a time-dependent fashion (**41).**

A histological and immunohistochemical study was done to confirm the biochemical results. Hematoxylin and eosin stained sections of liver and colon were investigated. DENA treated groups showed disturbed architecture and degenerative changes in the form of cellular destruction that was correlated with oxidative stress as revealed by the measured inflammatory markers. This was in agreement with **Bingül et al. (42) and Mansour et al. (43)** who revealed degenerative and preneoplastic changes in liver with DENA administration.

It worthy to mention that there was no neoplastic foci detected despite increased level of serum AFP. However, this increase may be an indicator of preneoplastic or dysplastic changes.

As regard colon, stained sections in DENA group revealed significant decrease in length of mucosal folds and significant decrease in goblet cells with evident dysplasia in the form of dysplastic aberrant crypt foci. This result was consistent with **Perše & Cerar, (44) and Suzui et al. (45)** who revealed similar changes with carcinogen substances. This result was also correlated with the increased CEA tumor marker. Treatment with MDP and DOX showed partially restoring the histological structure of liver tissue. This partial improvement may be attributed to their side effects as anticancer drugs. LAB treated group showed the best result in ameliorating liver tissue structure. The efficacy of lactobacillus on liver was confirmed by a study of **Zhao et al. (46)** who revealed that dietary *L. casei* alleviates LPS-induced liver injury via reducing pro-inflammatory cytokines and increasing anti-oxidative capacity.

Regarding effect of MDP and DOX treatment on colon, they minimally improve the histological structure of colon while lactobacillus had an amazing effect in restoring normal structure of colon. However, it failed to normalize serum level of CEA tumor marker to normal level. This was in agreement with **Sivamaruthi et al. (47)** who stated that probiotics may help to prevent colon cancer and the adverse effects caused by related therapies and with **Zackular et al. (48)** who revealed that gut microbiome could modulate colon tumorigenesis.

Lactobacillus strains have many anticancer properties that proved in several studies. LAB has shown preventive effects on cancer colon, bladder, liver, breast, and gastric cancers. This was achieved by alteration in gastrointestinal microflora, increasing the host's immune response, and antioxidative and antiproliferative activities of LAB **(49, 50)**. Moreover, degradation and inhibition of enzymes that produce carcinogenic compounds, improving chronic inflammation by immunomodulation, decrease intestinal pH were also evident (51, 52). Probiotic has also produced short-chain fatty acids (SCFAs), and lactic acid, butyrate and pyridoxine. SCFAs are the energy source of colon cells, preserving the acidic environment of the intestine. It also inhibits the formation of high levels of secondary bile acids with subsequent promotion of acidosis and apoptosis of cancer cells (53).

It was surprising that combined therapy group showed evident multinucleated giant cells. This finding was consistent with that of Kambham et al. (54) who revealed infrequently peculiar multinucleated giant cell within the crypts of inflamed colon polyps with ragged architecture. This could be explained as dysplasia or viral infection. Their Immunohistochemical and ultrastructural studies did not succeed in detection a viral etiology. Follow-up also did not reveal clinically hostile disease. These changes may represent a nonspecific, degenerative response to inflammation and injury.

Heme oxygenase is one of the heat shock proteins (HSPs). It is named as HSP32. It is present in different organelles like endoplasmic reticulum, mitochondria, and plasma membrane **(55, 56).** HO-1 is detected in all tissues at low levels. It is up-regulated under stressful circumstances. It is highly detected in spleen macrophages and Kupffer cells in the liver. This is due to its role in old red blood cells clearance. It is also highly expressed in hematopoietic stem cells in the bone marrow to control the levels of heme that is an erythroid differentiation factor (**57)**.

Under stressful environments, HO-1 could translocate to the nucleus. It functions non-enzymatically to regulate its own expression. It also controls transcriptional factors that are linked to oxidative stress responses, which may be related to progression of cancer **(58, 59).**

HO-1 protects cells by reducing superoxide and other reactive oxygen species. Heme oxygenase expression is induced by oxidative stress. Increasing its expression seems to be protective through antioxidant, anti-inflammatory and cyto-protective effects by its by-products including carbon monoxide (CO), biliverdin/bilirubin and free iron **(60, 61).**

Apart from the spleen and liver, HO-1 levels also seem to be constitutively expressed at the gastrointestinal system **(55).** Since the digestive system is continuously exposed to a wide variety of stress conditions, the HO system seems to play an important part in gastrointestinal tract health and disease.

Regarding heme oxygenase-1 (HO-1) immunoreaction in liver and colon, a significant increase (represented by area %) was detected in DENA group as compared with the control and other treated groups. This increased expression may be due to its stimulation by released nitric oxide as revealed by several studies **(62, 63).** This was correlated with biochemical results of the current study regarding nitric oxide levels. This was accompanied by significant increase in nuclear immunostaining of degenerated hepatocytes. In spite of the cytoprotective actions of HO-1, HO-1 and its by-products may be cytotoxic in a tumorigenic environment **(64).** So, its marked increase in DENA group may be an indicator of worsening of tissue. The nuclear translocation besides high intensity of cytoplasmic reaction may also indicate early dysplastic changes in liver and colon. This was in agreement with **Namba et al. (65)** who reported that enhanced lung nuclear HO-1 levels impaired recovery from hyperoxic lung injury by disabling PAR-dependent regulation of DNA repair. They mentioned also that both high cytoplasmic and nuclear expression of HO-1 may predispose to long-term abnormal lung cellular proliferation. Many previous researches have shown opposite roles (protumor or antitumor) of HO-1 in cancer progression. However, the underlying basis for this dual behavior remains mostly elusive. Cytoplasmic-nuclear shuttling of HO-1 was revealed in several malignant and non-malignant conditions. This may explain partially the dual role of HO-1 in cancer. However, nuclear HO-1 has been associated with a protumor role in the majority of tumor types studied so far. This protumor behavior of nuclear HO-1 may be due to the induction of some cellular processes such as cell proliferation, migration, invasion, metastasis, and chemoresistance **(66).**

Regarding therapeutic groups, all showed significant decrease in HO-1 immunoreaction in liver and colon as compared with DENA group. However, the immunoreactivity was still higher than the control. This increased expression may exert some cytoprotective mechanisms that noticed as partial restoration of histological structure. This was in agreement with **Yang et al. (67)** who revealed that increased HO-1 attenuated the severity of liver injury and with **Onyiah et al. (68)** who reported that HO-1 induction inhibited epithelial chemokine expression in inflammatory condition of intestine. It worth mentioning, that LAB and DOX groups showed no significant nuclear immune-staining in hepatocytes as compared with control. This may indicate that they may have powerful anti-tumorigenic effects. LAB group showed also no significant difference in immunoreaction in colon as compared with control group. The combined therapy group was revealed as the worst group in ameliorating liver and colon. This might be attributed to the side effect of both drugs as anticancer therapy. However, *Lactobacillus casei* showed the best therapeutic effect especially its effect on colon.

Conclusion:

In conclusion, Administration of DENA twice per week for six weeks has induced degenerative and dysplastic changes in liver and colon. The liver changes could be reversed partially by *L. casei* and DOX and MDP treatment. In addition, dysplastic changes in colon were markedly reversed by *L. casei.* The result of the current study revealed that *L. casei* had also very surprising in vitro probiotic properties. So, *L. casei* displayed a very promising anti-tumorigenic effect without the side effect of other anticancer therapy. This may be exerted via HO-1 modulation and suppression of oxidation and inflammation. The present study also shed the light on the dual action of HO-1 as its increased expression and/or its nuclear localization could be protective or protumor.

List of Abbreviations

DENA: diethylnitrosamine
MDP: pyridazine derivative
DOX: doxorubicin
LAB: *Lactobacillus casei*
HO-1: heme oxygenase -1
AFP : alpha fetoprotein
CEA : carcinoembryonic antigen
TNF-α: tumor necrosis alpha

Declarations

Ethical approval and consent to participate

Departmental approval was granted to the protocol of the study project on 2/1/2020. All ethical issues and research settings regarding animal biosafety, animal handling, and reducing animal's suffering, distress and pain were followed according to the guidelines of NODCAR animal house which conforms with the recommendations of the National Institute of Health

Guide for Care and Use of Laboratory Animals [National Research Council. (2010). Guide for the care and use of laboratory animals.

Consent for publication

I and my co-authors agreed the current version of the manuscript for publication

Availability of supporting data

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no conflict or competing interest to declare.

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Authors' contribution

AMM: contributed to the conceptualization of the idea of the research, carried out the experiment and contributed to the interpretation of the results, reviewing of the manuscript.

NAS performed all microbiological methods regarding the preparation, testing of bacteria, analysis and interpretation of the bacteriological data.

RAA, HMA, TEM: implemented the experiment, collect data, and performed data analysis and interpretation, contributed in writing the manuscript.

NAI and WMR: contributed to the design of animal grouping, image analysis of the captured photomicrographs, data curation, writing of the photos' legends, writing the initial draft of the manuscript.

DIE: contributed to the histological and immunohistochemical preparations, capturing of photomicrographs, interpretation of the photomicrographs, writing the initial draft of the manuscript.

All authors designed the model and the computational framework and analyzed the data and performed the calculations, discussed the results and contributed to the final manuscript, and agreed to its published version.

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References

1- Amin, H. A. M., Arihan, O., & Ragbetli, M. C. (2017). Effect of thymoquinone administration on erythrocyte fragility in diethylnitrosamine administered rats. *Journal of Cellular Biotechnology*, *3*(1), 1-7.

2- Elguindy, N. M., Yacout, G. A., & El Azab, E. F. (2018). Amelioration of DENA-induced oxidative stress in rat kidney and brain by the essential oil of Elettaria cardamomum. *Beni-Suef University Journal of Basic and Applied Sciences*, *7*(3), 299-305.

3- Flefel, E. M., Tantawy, W. A., El-Sofany, W. I., El-Shahat, M., El-Sayed, A. A., & Abd-Elshafy, D. N. (2017). Synthesis of some new pyridazine derivatives for anti-HAV evaluation. *Molecules*, *22*(1), 148.

4- Popovici, L., Amarandi, R. M., Mangalagiu, I. I., Mangalagiu, V., & Danac, R. (2019). Synthesis, molecular modelling and anticancer evaluation of new pyrrolo [1, 2-b] pyridazine and pyrrolo [2, 1-a] phthalazine derivatives. *Journal of enzyme inhibition and medicinal chemistry*, *34*(1), 230-243.

5- El Malah, T., E Rashad, A.E., Shamroukh, A.H., Ali, M.M., Mahmoud A.E. (2017). Synthesis and *In Vitro* Anticancer Evaluation of some Acyclic *N*- and *S*-nucleosides of Pyridazine Derivatives. Der Pharma Chemica, 9(19):79-84

6- Gorini, S., De Angelis, A., Berrino, L., Malara, N., Rosano, G., & Ferraro, E. (2018). Chemotherapeutic drugs and mitochondrial dysfunction: focus on doxorubicin, trastuzumab, and sunitinib. *Oxidative medicine and cellular longevity*, *2018*.

7- Ajaykumar, C. (2020). Overview on the Side Effects of Doxorubicin. In *Overview of Doxorubicin-Clinical Use, Resistance, Side Effects, and Palliative Care*. IntechOpen.

8- Evivie, S. E., Huo, G. C., Igene, J. O., & Bian, X. (2017). Some current applications, limitations and future perspectives of lactic acid bacteria as probiotics. *Food & nutrition research*, *61*(1), 1318034.

9- Behera, S. S., Ray, R. C., & Zdolec, N. (2018). Lactobacillus plantarum with functional properties: an approach to increase safety and shelf-life of fermented foods. *BioMed Research International*, *2018*.

10- Nemska, V., Logar, P., Rasheva, T., Sholeva, Z., Georgieva, N., & Danova, S. (2019). Functional characteristics of lactobacilli from traditional Bulgarian fermented milk products. *Turkish Journal of Biology*, *43*(2), 148-153.

11- Górska, A., Przystupski, D., Niemczura, M. J., & Kulbacka, J. (2019). Probiotic bacteria: a promising tool in cancer prevention and therapy. *Current microbiology*, *76*(8), 939-949.

12- Escamilla, J., Lane, M. A., & Maitin, V. (2012). Cell-free supernatants from probiotic Lactobacillus casei and Lactobacillus rhamnosus GG decrease colon cancer cell invasion in vitro. *Nutrition and cancer*, *64*(6), 871-878.

13- Motawee, M. M., & Saleh, N. M. (2016). MICROBIOLOGICAL QUALITY AND SAFETY OF COMMERCIAL MARKET YOGURT IN GIZA, EGYPT. *Journal of Food and Dairy Sciences*, *7*(1), 11-17.

14- Bauer, A. T. (1966). Antibiotic susceptibility testing by a standardized single disc method. *Am J clin pathol*, *45*, 149-158.

15- Reddy, K. B. P. K., Madhu, A. N., & Prapulla, S. G. (2009). Comparative survival and evaluation of functional probiotic properties of spray-dried lactic acid bacteria. *International Journal of Dairy Technology*, *62*(2), 240-248.

16- Gluck, J. P., DiPasquale, T., & Orlans, F. B. (Eds.). (2002). Applied ethics in animal research: philosophy, regulation, and laboratory applications. Purdue University Press.

17- Li, Y., Zhang, Y., Yu, Q., & Meng, F. (2017). Effect of ligustrazine on a rat model with hepatic preneoplasia. *microbiology*, *5*(13), 26.

18- Pieniążek, A., Czepas, J., Piasecka-Zelga, J., Gwoździński, K., & Koceva-Chyła, A. (2013). Oxidative stress induced in rat liver by anticancer drugs doxorubicin, paclitaxel and docetaxel. *Advances in medical sciences*, *58*(1), 104-111.

19- Matuskova, Z., Anzenbacher, P., Vecera, R., Siller, M., Tlaskalova-Hogenova, H., Strojil, J., & Anzenbacherova, E. (2017). Effect of Lactobacillus casei on the pharmacokinetics of amiodarone in male Wistar rats. *European journal of drug metabolism and pharmacokinetics*, *42*(1), 29-36.

20- Emsley, R., Dunn, G., & White, I. R. (2010). Mediation and moderation of treatment effects in randomised controlled trials of complex interventions. *Statistical methods in medical research*, *19*(3), 237-270.

21- Darvesh, A. S., & Bishayee, A. (2013). Chemopreventive and therapeutic potential of tea polyphenols in hepatocellular cancer. *Nutrition and cancer*, *65*(3), 329-344.

22- Zhu, Y., Wang, P. P., Zhao, J., Green, R., Sun, Z., Roebothan, B., ... & Mclaughlin, J. R. (2014). Dietary N-nitroso compounds and risk of colorectal cancer: a case–control study in Newfoundland and Labrador and Ontario, Canada. *British journal of nutrition*, *111*(6), 1109-1117.

23- Carrasco-Torres, G., Monroy-Ramírez, H. C., Martínez-Guerra, A. A., Baltiérrez-Hoyos, R., Romero-Tlalolini, M. D. L. Á., Villa-Treviño, S., & Vásquez-Garzón, V. R. (2017). Quercetin reverses rat liver preneoplastic lesions induced by chemical carcinogenesis. *Oxidative Medicine and Cellular Longevity*, *2017*.

24- Atakisi, O., Atakisi, E., Ozcan, A., Karapehlivan, M., & Kart, A. (2013). Protective effect of omega-3 fatty acids on diethylnitrosamine toxicity in rats. *Eur Rev Med Pharmacol Sci*, *17*(4), 467-71.

25- Anwar, H. M., Moghazy, A. M., Osman, A. A. E., & Abdel Rahman, A. A. (2021). The Therapeutic Effect of Myrrh (Commiphora molmol) and Doxorubicin on Diethylnitrosamine Induced Hepatocarcinogenesis in Male Albino Rats. *Asian Pacific Journal of Cancer Prevention*, *22*(7), 2153-2163.

26- Śliżewska, K., Markowiak-Kopeć, P., & Śliżewska, W. (2021). The Role of Probiotics in Cancer Prevention. *Cancers*, *13*(1), 20.

27- Dar, K. K., Ali, S., Ejaz, M., Nasreen, S., Ashraf, N., Gillani, S. F., ... & Mughal, T. A. (2019). In vivo induction of hepatocellular carcinoma by diethylnitrosoamine and pharmacological intervention in Balb C mice using Bergenia ciliata extracts. *Brazilian Journal of Biology*, (AHEAD).

28- Singh, D., Singh, M., Yadav, E., Falls, N., Dangi, D. S., Kumar, V., ... & Verma, A. (2018). Attenuation of diethylnitrosamine (DEN)–Induced hepatic cancer in experimental model of Wistar rats by Carissa carandas embedded silver nanoparticles. *Biomedicine & Pharmacotherapy*, *108*, 757-765.

29- Contreras-Zentella, M. L., & Hernández-Muñoz, R. (2016). Is liver enzyme release really associated with cell necrosis induced by oxidant stress?. *Oxidative Medicine and Cellular Longevity*, *2016*.

30- Wang, X., & Wang, Q. (2018). Alpha-fetoprotein and hepatocellular carcinoma immunity. *Canadian Journal of Gastroenterology and Hepatology*, *2018*.

31- Ahmed, H. H., Shousha, W. G., El-Mezayen, H. A., El-Toumy, S. A., Sayed, A. H., & Ramadan, A. R. (2017). Biochemical and molecular evidences for the antitumor potential of Ginkgo biloba leaves extract in rodents. *Acta Biochimica Polonica*, *64*(1), 25-33.

32- Roy, S. R., & Gadad, P. C. (2016). Effect of β-asarone on diethylnitrosamine-induced hepatocellular carcinoma in rats. *Indian Journal of Health Sciences and Biomedical Research (KLEU)*, *9*(1), 82.

33- Glory, M. D., & Thiruvengadam, D. (2012). Potential chemopreventive role of chrysin against N-nitrosodiethylamine-induced hepatocellular carcinoma in rats. *Biomedicine & Preventive Nutrition*, *2*(2), 106-112.

34- Kruk, J., Aboul-Enein, H. Y., Kładna, A., & Bowser, J. E. (2019). Oxidative stress in biological systems and its relation with pathophysiological functions: the effect of physical activity on cellular redox homeostasis. *Free radical research*, *53*(5), 497-521.

35- Chen, B., Ning, M., & Yang, G. (2012). Effect of paeonol on antioxidant and immune regulatory activity in hepatocellular carcinoma rats. *Molecules*, *17*(4), 4672-4683.
36- Ghosh, D., Choudhury, S. T., Ghosh, S., Mandal, A. K., Sarkar, S., Ghosh, A., & Das, N. (2012). Nanocapsulated curcumin: oral chemopreventive formulation against diethylnitrosamine induced hepatocellular carcinoma in rat. *Chemico-biological interactions*, *195*(3), 206-214.
37- Abdelbaset, M. S., Abdel-Aziz, M., Abuo-Rahma, G. E. D. A., Ramadan, M., & Abdelrahman, M. H. (2019). Pyridazinones and pyrrolones as promising Scaffolds in Medicinal Chemistry. *Journal of advanced Biomedical and Pharmaceutical Sciences*, *2*(1), 19-28.
38- Chen, X., Zhang, J., Yi, R., Mu, J., Zhao, X., & Yang, Z. (2018). Hepatoprotective effects of Lactobacillus on carbon tetrachloride-induced acute liver injury in mice. *International journal of molecular sciences*, *19*(8), 2212.
39- Jantararussamee, C., Rodniem, S., Taweechotipatr, M., Showpittapornchai, U., & Pradidarcheep, W. (2020). Hepatoprotective Effect of Probiotic Lactic Acid Bacteria on Thioacetamide-Induced Liver Fibrosis in Rats. *Probiotics and Antimicrobial Proteins*, 1-11.
40- Doroshow, J. H., Esworthy, R. S., & Chu, F. F. (2020). Control of doxorubicin-induced, reactive oxygen-related apoptosis by glutathione peroxidase 1 in cardiac fibroblasts. *Biochemistry and biophysics reports*, *21*, 100709.
41- Paranka, N. S., & Dorr, R. T. (1994). Effect of doxorubicin on glutathione and glutathione-dependent enzymes in cultured rat heart cells. *Anticancer research*, *14*(5A), 2047-2052.
42- Bingül, İ., Başaran-Küçükgergin, C., Aydın, A. F., Soluk-Tekkeşin, M., Olgaç, V., Doğru-Abbasoğlu, S., & Uysal, M. (2016). Blueberry treatment attenuated cirrhotic and preneoplastic lesions and oxidative stress in the liver of diethylnitrosamine - treated rats. *International journal of immunopathology and pharmacology*, *29*(3), 426-437.
43- Mansour, D. F., Abdallah, H. M., Ibrahim, B. M., Hegazy, R. R., Esmail, R. S., & Abdel-Salam, L. O. (2019). The carcinogenic agent diethylnitrosamine induces early oxidative stress, inflammation and proliferation in rat liver, stomach and colon: protective effect of ginger extract. *Asian Pacific journal of cancer prevention: APJCP*, *20*(8), 2551.
44- Perše, M., & Cerar, A. (2010). Morphological and molecular alterations in 1, 2 dimethylhydrazine and azoxymethane induced colon carcinogenesis in rats. *Journal of Biomedicine and Biotechnology*, *2011*.
45- Suzui, M., *Morioka*, T., & Yoshimi, N. (2013). Colon preneoplastic lesions in animal models. *Journal of toxicologic pathology*, *26*(4), 335-341.
46- Zhao, D., Wu, T., Yi, D., Wang, L., Li, P., Zhang, J., ... & Wu, G. (2017). Dietary supplementation with Lactobacillus casei alleviates lipopolysaccharide-induced liver injury in a porcine model. *International journal of molecular sciences*, *18*(12), 2535.
47- Sivamaruthi, B. S., Kesika, P., & Chaiyasut, C. (2020). The role of probiotics in colorectal cancer management. Evidence-Based Complementary and Alternative Medicine, 2020.
48- Zackular, J. P., Baxter, N. T., Iverson, K. D., Sadler, W. D., Petrosino, J. F., Chen, G. Y., & Schloss, P. D. (2013). The gut microbiome modulates colon tumorigenesis. *MBio*, *4*(6).
49- Delesa, D. A. (2017). Overview of Anticancer activity of Lactic Acid bacteria. *Int. J. Adv. Res. Biol. Sci*, *4*(12), 166-177.
50- Nozari, S., Faridvand, Y., Etesami, A., Ahmad Khan Beiki, M., Miresmaeili Mazrakhondi, S. A., & Abdolalizadeh, J. (2019). Potential anticancer effects of cell wall protein fractions from Lactobacillus paracasei on human intestinal Caco-2 cell line. *Letters in applied microbiology*, *69*(3), 148-154.
51- Dos Reis, S. A., da Conceição, L. L., & Peluzio, M. D. C. G. (2019). Intestinal microbiota and colorectal cancer: changes in the intestinal microenvironment and their relation to the disease. *Journal of medical microbiology*, *68*(10), 1391-1407.
52- Molska, M., & Reguła, J. (2019). Potential mechanisms of probiotics action in the prevention and treatment of colorectal cancer. *Nutrients*, *11*(10), 2453.
53- Kahouli, I., Tomaro-Duchesneau, C., & Prakash, S. (2013). Probiotics in colorectal cancer (CRC) with emphasis on mechanisms of action and current perspectives. *Journal of Medical Microbiology*, *62*(8), 1107-1123.
54- Kambham, N., Troxell, M., & Longacre, T. A. (2005). Multinucleated epithelial giant cells in colorectal polyps: a potential mimic of viropathic and/or dysplastic changes. *The American journal of surgical pathology*, *29*(7), 912-919.
55- Barton, S. G. R. G., Rampton, D. S., Winrow, V. R., Domizio, P., & Feakins, R. M. (2003). Expression of heat shock protein 32 (hemoxygenase-1) in the normal and inflamed human stomach and colon: an immunohistochemical study. *Cell stress & chaperones*, *8*(4), 329.

56- Hopper, C. P., Meinel, L., Steiger, C., & Otterbein, L. E. (2018). Where is the clinical breakthrough of heme oxygenase-1/carbon monoxide therapeutics?. *Current pharmaceutical design*, *24*(20), 2264-2282.

57- Puentes-Pardo, J. D., Moreno-SanJuan, S., Carazo, Á., & León, J. (2020). Heme Oxygenase-1 in Gastrointestinal Tract Health and Disease. *Antioxidants*, *9*(12), 1214.

58- Chau, L. Y. (2015). Heme oxygenase-1: emerging target of cancer therapy. *Journal of biomedical science*, *22*(1), 1-7.

59- Nitti, M., Piras, S., Marinari, U. M., Moretta, L., Pronzato, M. A., & Furfaro, A. L. (2017). HO-1 induction in cancer progression: a matter of cell adaptation. *Antioxidants*, *6*(2), 29.

60- Chang, M., Xue, J., Sharma, V., & Habtezion, A. (2015). Protective role of hemeoxygenase-1 in gastrointestinal diseases. *Cellular and molecular life sciences*, *72*(6), 1161-1173.

61- Zhang, L., Zhang, Z., Liu, B., Jin, Y., Tian, Y., Xin, Y., & Duan, Z. (2017). The protective effect of heme oxygenase-1 against intestinal barrier dysfunction in cholestatic liver injury is associated with NF-κB inhibition. *Molecular Medicine*, *23*(1), 215-224.

62- Zuckerbraun, B. S., Billiar, T. R., Otterbein, S. L., Kim, P. K., Liu, F., Choi, A. M., ... & Otterbein, L. E. (2003). Carbon monoxide protects against liver failure through nitric oxide–induced heme oxygenase 1. *The Journal of experimental medicine*, *198*(11), 1707-1716.

63- Liu, X. M., Peyton, K. J., Ensenat, D., Wang, H., Hannink, M., Alam, J., & Durante, W. (2007). Nitric oxide stimulates heme oxygenase-1 gene transcription via the Nrf2/ARE complex to promote vascular smooth muscle cell survival. *Cardiovascular research*, *75*(2), 381-389.

64- Yin, H., Fang, J., Liao, L., Maeda, H., & Su, Q. (2014). Upregulation of heme oxygenase-1 in colorectal cancer patients with increased circulation carbon monoxide levels, potentially affects chemotherapeutic sensitivity. *Bmc Cancer*, *14*(1), 1-14.

65- Namba, F., Go, H., Murphy, J. A., La, P., Yang, G., Sengupta, S., ... & Dennery, P. A. (2014). Expression level and subcellular localization of heme oxygenase-1 modulates its cytoprotective properties in response to lung injury: a mouse model. *PloS one*, *9*(3), e90936.

66- Mascaró, M., Alonso, E. N., Alonso, E. G., Lacunza, E., Curino, A. C., & Facchinetti, M. M. (2021). Nuclear Localization of Heme Oxygenase-1 in Pathophysiological Conditions: Does It Explain the Dual Role in Cancer?. *Antioxidants*, *10*(1), 87.

67- Yang, H., Zhao, L. F., Zhao, Z. F., Wang, Y., Zhao, J. J., & Zhang, L. (2012). Heme oxygenase-1 prevents liver fibrosis in rats by regulating the expression of PPARγ and NF-κB. *World journal of gastroenterology: WJG*, *18*(14), 1680.

68- Onyiah, J. C., Schaefer, R. E., & Colgan, S. P. (2018). A central role for heme oxygenase-1 in the control of intestinal epithelial chemokine expression. *Journal of innate immunity*, *10*(3), 228-238.