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Synergistic effect of resveratrol and quercetin on chemically induced hepatocellular carcinoma in rats

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ABSTRACT

Hepatocellular carcinoma is the most common primary cancer of the liver. The objective of this study was to evaluate the effects of resveratrol and quercetin alone or in combination against hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DENA) and phenobarbital (PB) in rats. One hundred and twenty five adult male albino rats were employed in this work which was included in two experiments. In the first, thirty one rats were injected (i.p) with saline solution (control group). The HCC rats group (94 rats) was injected i.p with a single dose of DENA once/week for 3 weeks. After 3 weeks, rats were promoted by i.p injection of PB once a day for 7 days. After the induction period, 10 rats from each of the previous group were taken to compare the alterations in the biochemical and physiological parameters. In the second experiment, four comparisons were made between 21 normal control rats and 84 HCC rats. The HCC rats were divided into the following subgroups; (1) HCC rats left without any further treatment and served as a recovery group. (2) HCC rats received daily i.p resveratrol and served as a resveratrol group. (3) HCC rats treated with i.p injection of quercetin and served as a quercetin group. (4) HCC rats received mixture of resveratrol and quercetin for 2, 4 & 8 weeks and served as a resveratrol and quercetin group.

The obtained data revealed remarkable changes in all studied parameters of hepatocellular carcinoma rats than those in control ones. When HCC rats were treated with combined treatment of resveratrol and quercetin for 2, 4 & 8 weeks a considerable amelioration effects in all studied parameters were pronounced dependent on time of treatment and certain mechanisms which were discussed according to available recent researches.

Keywords: Hepatocellular carcinoma, diethylnitrosamine, resveratrol, quercetin, inflammatory parameters, angiogenesis markers.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major malignancy worldwide ranking 6th among all malignancies due to a high mortality rate (Shankaraiah *et al*., 2019). It is a complex and heterogeneous malignant tumor caused by multiple risk factors. The main origin of the development of HCC is viral hepatitis (HBV & HCV). Other non-viral risk factors include alcoholism, non-alcoholic steatohepatitis (NASH), type II diabetes, obesity and hemochromatosis. Also, environmental and dietary carcinogens such as aflatoxin B1, mycotoxin (soy & peanut), nitrosamines (present in tobacco smoke, cosmetics, gasoline) and various processed foods are known to cause HCC (Zhu *et al*., 2016; Rashed *et al*., 2020).

Diethylnitrosamine (DENA) is one of the chemical family of carcinogenic N-nitroso compounds and is normally used as a carcinogen to induce cancer in animal

models (Qi *et al.*, 2008). It can alkylate DNA molecule with itself being converted to highly reactive molecule by P_{450} dependent oxygenases (Unsal and Belge-Kurutas, 2017). DENA-induced carcinogenesis can be enhanced by promoters such as phenobarbital (PB), which transforms DENA initiated cells to foci and to HCC. PB is a sedative and anticonvulsant, used widely in clinical therapy for long term treatment. It is also well known to be a promoter of rat hepatocarcinogenesis and is an example of a non-genotoxic hepatocarcinogen (Gani *et al*., 2019). Thus, PB does not demonstrate mutagenicity in short-term studies, but long-term feeding results in hepatocarcino-genicity. This may be due to promoting effects on spontaneously initiated hepatocytes (Fornari *et al*., 2019).

Angiogenesis is the process of forming new blood vessels from pre-existing ones. Also, it has an important function in inflammation. (Teleanu *et al*., 2020). Vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF), and matrix metalloproteinases (MMPs) are only a few of substances that promote and mediate the early stages of angiogenesis. Because sprouting angiogenesis is critical for tumor development, invasion, progression and metastasis, thus, inhibiting this process could potentially halt cancer growth and its spread. Anti-angiogenesis targeting strategies are currently being promoted as one of the most important targets in cancer therapy (Haibe *et al*., 2020).

Phytochemicals found in fruits, vegetables, nuts, legumes, spices and traditional medicinal plants could have a tremendous potential to prevent and treat cancer. These agents act *via* different mechanisms to inhibit the angiogenic process by disrupt various components of tumor angiogenesis signaling pathway (Forni *et al*., 2019).

Resveratrol (trans 3,5,4-trihydroxy stilbene) is a non-flavonoid polyphenol found in many plant species including peanuts, blueberries and rhubarb. It has possessed wide range of pharmacological activities including anticancer, antibacterial, anti-oxidative, anti-neurodegenerative, anti-inflammatory and immunomodulatory characteristics (Gianchecchi and Fierabracci, 2020). Indeed, this polyphenol plays a function in preventing undesirable estrogenic situations such as stress, damage or pathogenic attack such as UV irradiation and fungal infection (Koushki *et al.,* 2018).

Quercetin (3,3′,4′,5,7-pentahydroxy flavone) is one of the essential components of the polyphenol family of flavonoids. Its biological activity is associated with the presence of five hydroxyl groups on the ring structure (Fernandez-Palanca *et al*., 2019). The beneficial potential of quercetin has been evaluated in a variety of human disorders such as diabetes, cardiovascular disease, neurodegenerative diseases, renal dysfunction and Alzheimer's disease. Also, the protective effects of quercetin against chemically-induced hepatotoxicity are well documented, and have been attributed to its intrinsic antioxidant properties (Khan *et al*., 2020).

The current work was designed to investigate the hepatoprotective potentials of resveratrol or quercetin and their mixture against inflammatory and angiogenesis disturbance of HCC induced by DENA in the presence of PB in rats.

MATERIALS AND METHODS

One hundred and twenty five Wister strain adult male albino rats weighing 140±10.5g were purchased from the animal house of Theodor Bilharz Research Institute, (Imbaba, Giza) and employed in this investigation. Animals were housed in

metallic cages under controlled environmental conditions (25ºC with relative humidity 50-55% and 12 hours light-dark cycle) throughout the experiment. The animals were allowed to adapt to the laboratory conditions one week before the beginning of the experiment and a commercial pellet diet was used during the experiment. The experimental protocols and procedures were approved by Ain Shams University authorities and followed Egyptian rules for animal protection, which was performed according to the U.K. Handling and usage of animals agreed strictly with the regulations and guidelines set by the research Ethics Committee of the Faculty of Science, Ain Shams University.

This study was included two experiments: In the first experiment, thirty one rats were injected intraperitoneally (i.p) with 1 ml normal saline (0.9% NaCl) and served as normal control rats group. The second animals group (94 rats), rats underwent i.p. injected of DENA (Sigma Chem. Co., St Louis, Mo. U.S.A.) with a dose of 100mg/kg body weight to initiate HCC. This was performed three times, once per week. After these 3 weeks, rats received PB (Sigma Chem. Co., St Louis, Mo. U.S.A.) 100mg/kg body weight/day dissolved in normal saline for 7 successive days (Zhang *et al*., 2013 and Elcombe *et al.*, 2014). Ten rats from each previous group were sacrificed to compare the alteration in parameters.

In the second experiment, four comparisons were made between normal control animals group (21 rats) and four HCC subgroups of animals (84 rats); twenty one rats in each one. The first HCC subgroup had no further treatment and served as HCC subgroup. The second HCC rats subgroup was treated intraperitoneally with 50 mg resveratrol (Sigma Chem. Co., St Louis, Mo. U.S.A.) /kg body weight/day as described by Park & Pezzuto (2015). The third HCC rats subgroup was intraperitoneally administered with 50 mg quercetin (Sigma Chem. Co., St Louis, Mo. U.S.A.) /kg body weight/day as described by El-Nekeety *et al.* (2014). Finally, the fourth HCC rats subgroup was injected i.p. with both resveratrol and quercetin daily as above described. All animals in the previous subsets and control group were divided into three periods (2, 4 & 8 weeks).

At the end of the experimental period, rats were overnight fasted, sacrificed by rapid decapitation and their blood samples were collected in a clean, dry test tube to obtain the sera. Sera were separated and kept at -20^{o}C for the determination of physiological and biochemical studied parameters.

The activities of serum transaminases (AST & ALT) and the levels of total protein as well as albumin were determined calorimetrically using commercial kits purchased from BioMed, ARE. The serum levels of alpha-fetoprotein (AFP), cyclooxygenase-2(COX-2) and neutrophil elastase were estimated by the aid of ELISA My BioSource (USA) commercial kits. Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) were measured in serum by sandwich enzyme-linked immunosorbent assay using commercial kits (Boster Biological Technology Co., Ltd. Canada). The level of vascular endothelial growth factor (VEGF) in serum was determined by solid phase sandwich technique (ELISA) using commercial kits R&D Systems, Inc. (USA). In addition, the levels of endostatin were assayed using commercial ELISA (Sandwich Immunoassay Technique) kits using Elabscience (USA). The activity of heparanase was assayed by using commercial ELISA kits purchased from Biomatik, Co., USA according to the manufacturer's instruction.

Statistical analysis

By using a computer program (SPSS, Chicago, IL version 20), data were statistically analyzed by Student "t" test in the first experimental to estimate the difference in parameters tested herein between experimentally hepatocellular carcinoma rats group and normal control rats group. However, ANOVA followed by Duncan's multiple range tests was made in the second experiment to measurement of the differences in the variables dependent on both time and treatment according to Snedecor & Cochran (1982). The criterion for significance was set at $p< 0.05$.

RESULTS

Table (1) showed that the induction of HCC in rats group as a result of single intra-peritoneal injection of DENA for 3 weeks followed by intra-peritoneal injection of PB for 7 successive days induced a significant ($p<0.001$) increase in the level of serum tumor marker AFP and the activity of liver enzymes (AST & ALT) as compared to normal control ones. On the other hand, there were significant ($p<0.05$) decreases in the levels of total protein and albumin after the same treatment compared with normal control levels.

Table (1): The mean values of serum α-fetoprotein and liver function tests in control and hepatocellular carcinoma rat groups.

Groups / Parameters	Control	Hepatocellular carcinoma (HCC)	%
AFP (pg/ml)	19.158 ± 0.645	122.128±6.867**	537.48
AST (U/L)	46.614 ± 1.976	118.001 ± 7.901**	153.14
ALT (U/L)	41.585± 2.296	102.057± 4.145**	145.42
Total protein (g/dl)	7.920 ± 0.287	5.201± 0.248*	-34.33
Albumin (g/dl)	4.810 ± 0.231	2.800± 0.161*	-41.79

- Data are expressed as means ± S.E.
- (*) refer to significance ($P<0.05$) and (**) refer to significance ($P<0.001$).

As shown in Table (2), a significant ($P<0.001$) increase in serum VEGF, MMP-2 and MMP-9 activities were reported in HCC rats group as compared to those corresponding control rats group. Moreover, the induction of HCC in rats caused a numerical elevation in the levels of COX-2, neutrophil elastase and heparanase as compared to normal control rats group (Table 2). Whereas, the analysis of data revealed significant ($P<0.05$) decrease in endostatin level as a result of HCC induction compared with the normal control values.

Table (2): The mean values of serum angiogenesis markers in control and hepatocellular carcinoma rat groups.

Groups / Parameters	Control	Hepatocellular carcinoma (HCC)	%
VEGF (pg/mL)	68.020 ± 3.835	145.160 ± 3.139**	113.41
MMP-2 (pg/ml)	240.170 ± 3.062	448.560 ± 3.184**	86.77
MMP-9 (pg/ml)	206.150 ± 3.518	456.280 ± 3.229**	121.33
Endostatin (ng/mL)	4.331± 0.248	2.110 ± 0.168*	-51.28
COX-2 (ng/ml)	4.200 ± 0.173	6.814 ± 0.793**	62.23
Neutrophil elastase (ng/ml)	3.614 ± 0.498	16.340 ± 1.154**	352.13
Heparanase (ng/mL)	2.301± 0.191	6.077 ± 0.508**	164.10

- Data are expressed as means ± S.E.
- (*) refer to significance ($P<0.05$) and (**) refer to significance ($P<0.001$).

It was obvious from data in Table (3) the presence of a significant ($p<0.05$) corrections in serum AFP, total protein and albumin levels as well as the activities of liver enzymes (AST & ALT) after HCC rats group treated by resveratrol or quercetin dependent on time of treatment (2, 4 & 8 weeks) regarding to their corresponding values in the normal control rats groups. Moreover, the maximum amelioration data in these previous parameters were obtained in the hepatocellular carcinoma animals group which treated by mixture of resveratrol and quercetin dependent on the intervals (Table 3).

Table (3): Amelioration effects of resveratrol or/and quercetin supplementation on serum α-fetoprotein and liver function tests in hepatocellular carcinoma rats.

Parameters \ Groups		Control	HCC	HCC + Resveratrol	HCC + Quercetin	HCC + Resveratrol + Quercetin
AFP (pg/ml)	2 wks	$19.210\pm0.482^{A}_{a}$	$133.640\pm5.6740^{B}_{a}$	$100.310\pm7.134^{C}_{a}$	$106.900\pm7.224^{C}_{a}$	$95.090\pm6.685^{D}_{a}$
	4wks	$19.160\pm0.836^{A}_{a}$	$148.400\pm4.969^{B}_{b}$	$80.260\pm5.549^{D}_{b}$	$91.700\pm5.674^{C}_{b}$	$75.210\pm6.284^{D}_{b}$
	8 wks	$18.980\pm0.399^{A}_{a}$	$175.420\pm4.679^{B}_{c}$	$40.070\pm3.049^{D}_{c}$	$56.500\pm3.016^{C}_{c}$	$25.030\pm2.720^{E}_{c}$
AST (U/L)	2 wks	$45.020\pm2.090^{A}_{a}$	$140.510\pm7.063^{B}_{a}$	$108.300\pm6.049^{C}_{a}$	$112.200\pm5.083^{C}_{a}$	$98.027\pm5.744^{D}_{a}$
	4wks	$46.20\pm2.029^{A}_{a}$	$189.061\pm4.110^{B}_{b}$	$97.700\pm5.019^{D}_{b}$	$104.400\pm5.949^{C}_{a}$	$81.040\pm5.674^{D}_{b}$
	8 wks	$44.300\pm2.121^{A}_{a}$	$239.022\pm6.156^{B}_{c}$	$77.100\pm5.446^{D}_{c}$	$93.160\pm5.253^{C}_{b}$	$55.410\pm4.545^{E}_{c}$
ALT (U/L)	2 wks	$43.130\pm1.303^{A}_{a}$	$126.320\pm4.857^{B}_{a}$	$90.110\pm5.966^{D}_{a}$	$97.200\pm4.737^{C}_{a}$	$89.101\pm4.195^{D}_{a}$
	4wks	$42.640\pm2.121^{A}_{a}$	$168.450\pm5.186^{B}_{b}$	$81.530\pm4.806^{D}_{b}$	$92.610\pm5.192^{C}_{a}$	$79.200\pm4.726^{D}_{b}$
	8 wks	$42.290\pm2.353^{A}_{a}$	$218.980\pm7.092^{B}_{c}$	$62.001\pm4.123^{D}_{c}$	$73.090\pm5.924^{C}_{b}$	$53.400\pm4.460^{E}_{c}$
T. protein (g/dl)	2 wks	$7.800\pm0.388^{A}_{a}$	$5.040\pm0.386^{B}_{a}$	$5.510\pm0.207^{C}_{a}$	$5.400\pm0.262^{C}_{a}$	$6.011\pm0.141^{D}_{a}$
	4wks	$7.900\pm0.216^{A}_{a}$	$4.600\pm0.209^{B}_{a}$	$5.700\pm0.258^{C}_{a}$	$5.620\pm0.222^{C}_{a}$	$6.301\pm0.173^{D}_{a}$
	8 wks	$8.002\pm0.294^{A}_{a}$	$4.040\pm0.180^{B}_{b}$	$6.120\pm0.165^{C}_{a}$	$6.040\pm0.153^{C}_{a}$	$6.860\pm0.120^{D}_{b}$
Albumin (g/dl)	2 wks	$4.710\pm0.258^{A}_{a}$	$2.720\pm0.193^{B}_{a}$	$3.011\pm0.130^{C}_{a}$	$2.920\pm0.193^{C}_{a}$	$3.520\pm0.188^{D}_{a}$
	4wks	$4.820\pm0.209^{A}_{a}$	$2.650\pm0.147^{B}_{a}$	$3.320\pm0.165^{C}_{a}$	$3.101\pm0.151^{C}_{a}$	$3.711\pm0.130^{D}_{a}$
	8 wks	$4.801\pm0.316^{A}_{a}$	$2.502\pm0.090^{B}_{a}$	$3.700\pm0.158^{C}_{b}$	$3.550\pm0.151^{C}_{b}$	$3.901\pm0.130^{D}_{b}$

- Data are expressed as means ± standard error (SE) for 7 rats/group.
- $^{A, B, C, D, E}$ Means with a common superscript within a row are significantly different ($P<0.05$).
- a, b ,c Means with a common subscript within a column are significantly different ($P<0.05$).

In HCC rats groups which supplemented by resveratrol, a significant ($p<0.05$) correction in the levels of serum angiogenesis markers (VEGF, MMP-2, MMP-9, endostatin, COX-2, neutrophil elastase and heparanase) were recorded regarding to their corresponding animals in the control groups (Table 4). Furthermore, a considerable improvement occurred in the mean values of serum VEGF, MMP-2, MMP-9, endostatin, COX-2, neutrophil elastase and heparanase levels in HCC rats groups which treated with quercetin dependent on time of treatment (2, 4 & 8 weeks). The maximum amelioration were recorded in the levels of serum angiogenesis markers in HCC rats groups which treated with the mixture of resveratrol and quercetin dependent on time of treatment (2, 4 & 8 weeks) as presented in Table (4).

Table (4): Amelioration effects of resveratrol or/and quercetin supplementation on serum angiogenesis markers in hepatocellular carcinoma rats.

Parameters \ Groups		Control	HCC	HCC + Resveratrol	HCC + Quercetin	HCC + Resveratrol + Quercetin
VEGF (pg/mL)	2 wks	70.310±4.324 $^{A}_{a}$	160.076±3.687 $^{B}_{a}$	135.140±5.176 $^{C}_{a}$	139.022±4.669 $^{C}_{a}$	125.030±3.633 $^{D}_{a}$
	4wks	71.046±3.860 $^{A}_{a}$	189.039±4.183 $^{B}_{b}$	124.340±4.074 $^{C}_{a}$	131.630±4.266 $^{C}_{a}$	110.600±4.658 $^{D}_{b}$
	8 wks	68.610±4.135 $^{A}_{a}$	239.083±4.393 $^{B}_{c}$	99.071±4.347 $^{D}_{b}$	113.100±4.347 $^{C}_{b}$	83.005±5.630 $^{A}_{c}$
MMP-2 (pg/ml)	2 wks	245.011±4.658 $^{A}_{a}$	510.458±6.410 $^{B}_{a}$	410.569±4.289 $^{D}_{a}$	433.640±5.366 $^{C}_{a}$	390.254±4.277 $^{E}_{a}$
	4wks	241.120±5.205 $^{A}_{a}$	609.515±4.785 $^{B}_{b}$	355.626±5.639 $^{D}_{b}$	380.099±4.722 $^{C}_{b}$	340.416±5.504 $^{D}_{b}$
	8 wks	243.390±6.058 $^{A}_{a}$	835.638±7.582 $^{B}_{c}$	315.740±5.319 $^{D}_{c}$	340.990±3.301 $^{C}_{c}$	280.501±3.781 $^{E}_{c}$
MMP-9 (pg/ml)	2 wks	204.100±3.563 $^{A}_{a}$	489.040±9.235 $^{B}_{a}$	414.006±4.919 $^{C}_{a}$	426.008±6.228 $^{C}_{a}$	380.201±7.296 $^{D}_{a}$
	4wks	199.300±2.236 $^{A}_{a}$	529.100±4.690 $^{B}_{b}$	380.030±4.549 $^{D}_{b}$	400.400±6.760 $^{C}_{b}$	341.120±5.431 $^{E}_{b}$
	8 wks	200.020±5.282 $^{A}_{a}$	630.110±4.847 $^{B}_{c}$	340.210±3.847 $^{D}_{c}$	360.130±3.911 $^{C}_{c}$	245.400±3.911 $^{E}_{c}$
Endostatin (ng/mL)	2 wks	4.190±0.325 $^{A}_{a}$	1.800±0.228 $^{B}_{a}$	2.310±0.272 $^{C}_{a}$	2.200±0.173 $^{C}_{a}$	2.420±0.215 $^{C}_{a}$
	4wks	4.300±0.375 $^{A}_{a}$	1.420±0.128 $^{B}_{a}$	2.620±0.070 $^{C}_{a}$	2.400±0.216 $^{C}_{a}$	2.910±0.184 $^{C}_{a}$
	8 wks	4.220±0.193 $^{A}_{a}$	1.001±0.083 $^{B}_{b}$	3.060±0.314 $^{C}_{b}$	2.800±0.273 $^{C}_{b}$	3.320±0.187 $^{C}_{b}$
COX-2 (ng/ml)	2 wks	4.320±0.440 $^{A}_{a}$	7.401±0.615 $^{B}_{a}$	6.310±0.642 $^{C}_{a}$	6.240±0.507 $^{C}_{a}$	5.700±0.535 $^{D}_{a}$
	4wks	4.401±0.412 $^{A}_{a}$	7.903±0.483 $^{B}_{a}$	6.140±0.487 $^{C}_{a}$	5.900±0.425 $^{C}_{a}$	5.220±0.389 $^{D}_{b}$
	8 wks	4.210±0.353 $^{A}_{a}$	9.100±0.789 $^{B}_{b}$	5.101±0.342 $^{C}_{b}$	5.010±0.541 $^{C}_{b}$	4.500±0.240 $^{A}_{c}$
Neutrophil elastase (ng/ml)	2 wks	3.610±0.181 $^{A}_{a}$	18.130±0.907 $^{B}_{a}$	13.500±0.894 $^{C}_{a}$	14.080±0.817 $^{C}_{a}$	12.020±1.068 $^{D}_{a}$
	4wks	3.720±0.164 $^{A}_{a}$	20.021±1.011 $^{B}_{a}$	10.030±1.132 $^{D}_{b}$	11.031±0.925 $^{C}_{b}$	9.400±0.890 $^{D}_{b}$
	8 wks	3.680±0.192 $^{A}_{a}$	26.043±1.224 $^{B}_{b}$	5.502±0.212 $^{D}_{c}$	6.910±0.364 $^{C}_{c}$	4.300±0.192 $^{E}_{c}$
Heparanase (ng/mL)	2 wks	2.200±0.184 $^{A}_{a}$	6.900±0.384 $^{B}_{a}$	5.310±0.391 $^{C}_{a}$	5.510±0.535 $^{C}_{a}$	5.020±0.406 $^{D}_{a}$
	4wks	2.190±0.184 $^{A}_{a}$	7.420±0.452 $^{B}_{a}$	4.810±0.308^{b}	5.010±0.404 $^{C}_{b}$	3.800±0.320 $^{D}_{b}$
	8 wks	2.24±0.249 $^{A}_{a}$	9.050±0.707 $^{B}_{b}$	3.620±0.114 $^{D}_{c}$	4.500±0.230 $^{C}_{c}$	2.800±0.275 $^{E}_{c}$

- Data are expressed as means ± standard error (SE) for 7 rats/group.
- $^{A, B, C, D, E}$ Means with a common superscript within a row are significantly different (P<0.05).
- a, b, c Means with a common subscript within a column are significantly different (P<0.05).

DISCUSSION

Hepatocellular carcinoma is one of the most common malignant neoplasms and a major cause of morbidity and mortality worldwide. In the current investigation, the significant elevation of AFP levels confirmed the diagnosis of DENA/PB induced HCC in rats. AFP, a tumor-associated fetal protein, has long been employed as a serum fetal tumor marker to monitor disease progression. It is detectable only in minute amounts in the serum of normal adults, while the level is increased in conditions like hepatocellular and germ cell carcinoma. Moreover, elevated serum concentrations of AFP can be achieved in the adult by exposure to hepatotoxic agents or hepatocarcinogens (Liu *et al.*, 2019). Because of its relative small molecular size, AFP can pass through the glomerular basement membrane and so can be detected in urine (Jahan *et al.*, 2011). Aminotransferases AST and ALT reflect the physiological state of the liver function. These enzymes are changed according to the distortion of liver, resulting from cellular injury caused by toxic metabolites and diseases (Liu *et al.*, 2019). In the current

work, DENA/PB-induced group rats recorded hepatic injury which was evident by significant elevation in the serum activities of ALT and AST associated with a significant decline in the levels of total protein and albumin as compared to their corresponding normal rats group. The rise in the enzyme activity of AST is usually accompanied by an elevation in the activity of ALT, which plays a vital role in the conversion of amino acids to keto acids. The leakage of large quantities of enzymes into the blood stream was associated with centrilobular necrosis and ballooning degeneration of the liver (Heibashy & Mazen, 2011). The authors attributed these results to the dissociation and destruction of endoplasmic reticulum polysomes that play an important role in protein biosynthesis. The decrease in total protein and albumin levels is used as an indicator of decreased protein biosynthesis, induced by DENA/PB poisons (Gani *et al.,* 2019). These results backed the hepatocarcinogenic effect of DENA/PB.

After HCC groups treated with resveratrol or/and quercetin, the activities of ALT, AST and AFP levels were significantly decreased while total protein and albumin were significantly increased in all intervals compared with corresponding control groups. These results may be attributed to the anticancer effect of resveratrol and quercetin (Mrkus *et al*., 2019). AFP is an indicator of HCC; a decrease in its level indicates that the development of HCC is inhibited, which is likewise supported by the improved liver function enzyme activity compared with HCC rats (Serra *et al*., 2020). In this study, treatment with resveratrol and quercetin reversed DENA/PB-induced HCC in the contents of ALT and AST. These results may be due to reduced cell turnover leading to minimization in the release of the enzyme into the circulation (El-Nekeety *et al.,* 2014 and Su *et al.,* 2019). This indicates that resveratrol and quercetin protect the structural integrity of liver cell membranes and ultimately inhibit the leakage of these enzymes into the circulatory system (Hussein *et al*., 2017 and Abdu & Al-Bogami, 2019). Furthermore, the levels of total protein and albumin increased significantly after treatment by resveratrol or/and quercetin. This correction may be reflecting to the ability of resveratrol and quercetin to repair liver damage caused by DENA/PB. Also, due to the reduction of oxidative stress caused by DENA/PB, the plasma membrane maintains its strength. This amelioration may be the main reason for the anti-cancer properties of resveratrol and quercetin and their ability to regulate the uncontrolled proliferation of cancer cells, thereby improving the cell damage caused by DENA/PB (El-Nekeety *et al.,* 2014; Su *et al.,* 2019).

HCC is type of hypervascular tumors, generally due to the neo-angiogenesis forms that determine primary node growth, metastasis development and disease prognosis (Fodor *et al*., 2019). However, angiogenesis and blood supply of tumor tissues are the considerable importance for hepatic carcinogenesis (Moawad *et al.,* 2020). The mechanisms underlying angiogenesis HCC are the secretion of angiogenic factors and the activation, proliferation and migration of endothelial cells (Feng *et al.,* 2017). Disturbances of the balance between endogenous pro and antiangiogenic factor levels lead to the uncontrolled growth of blood vessels mainly *via* stimulation of VEGF, the master regulators of vascular growth (Lee *et al.,* 2015).

Considering the present data, VEGF, MMP-2, MMP-9 and COX-2 were highly significant in the 2, 4 & 8 weeks in HCC group while groups treated with resveratrol or/and quercetin, the values of them were

significantly decreased in all intervals compared with corresponding control groups. The anticancer effects of resveratrol and quercetin may be due to the inhibition of DNA synthesis or the down-regulation of VEGF, MMP-2, MMP-9 and COX-2, which are involved in angiogenesis, apoptosis and cytotoxicity effects (Pratheeshkumar *et al*., 2012). Wu *et al*. (2019) found that resveratrol and quercetin can inhibit the VEGF signal pathway, due to the direct effect on VEGF/VEGFR2 or by regulating the downstream signal mediated by VEGFR2. Most MMPs are related to the tumorigenesis of various human malignancies. Among these proteases, MMP-9 is reported to have important significance in the occurrence of cancer invasion and metastasis. Studies have shown that inhibiting the expression of MMP-9 can inhibit metastasis in cancer progression (Bai *et al*., 2017; Wu *et al*., 2019). In the present work, resveratrol and quercetin decreased secretion of MMP-2 and MMP-9. The capability of resveratrol and quercetin to reduce angiogenesis stimulated with phorbol myristate acetate. The inhibitory impact associated with down regulation of COX-2 expression as well as inhibition of MMP-9 protein release and gelatinolytic activity. In addition, resveratrol and quercetin can block MMP-2 and MMP-9 signaling through inhibition of the MAPK and PI3K/AKT signaling pathways (Pilatova *et al*., 2010; Wolfe *et al*., 2015). The neutrophil depletion results in lowers levels of VEGF/ VEGFR signaling and a delay of the angiogenic switch (Nozawa *et al.,* 2006). Resveratrol and quercetin decreased neutrophil percentage in HCC rat groups. These results are in parallel with that obtained by Pelus *et al.* (2004) who revealed that MMP-9 involved in regulation of leukocytosis by the release of hematopoietic progenitor cells from the bone marrow in HCC group induced by DENA/PB.

Endostatin has been shown to inhibit angiogenesis under various pathological conditions characterized by increased angiogenesis, such as tumors (O'Reilly *et al*., 1997). Numerous studies have demonstrated that endostatin interferes with VEGF/VEGFR signaling and improves peritoneal sclerosis by reducing expression of transforming growth factor-beta 1 (TGF-β1) which is the most important profibrotic growth factor. Based on these reports, endostatin has protective effects by repress the production of pro-inflammatory cytokines and by inhibiting angiogenesis (Hajitou et al. 2002; Kim *et al.,* 2002). As shown in current results the level of endostatin was significantly decreased in DENA/PB group as compared with the normal control group, and highly significant depletion in the second, four and eight weeks in HCC groups while HCC groups treated with resveratrol or/and quercetin, the values of them were significantly increases in all intervals compared with HCC group. The antiangiogenic properties of resveratrol and quercetin have been shown to suppress both the nuclear factor-kappa-B (NF-κB) and protein kinase B (AKT) pathways. Inhibition of endothelial cell proliferation is primarily associated with induction of apoptosis and reduced secretion of VEGF. Interference at several points in the angiogenesis process, including inhibition of endothelial cell migration and invasion is considered to be the essential step of angiogenesis (Sun *et al*., 2015).

Tumor metastasis is a process that involves the release of single tumor cells, migration of these cells to blood vessel, penetration into the blood stream or lymph stream and finally adhesion to vessel endothelium and extravasation into the tissue at the metastatic location (Zijl *et al*., 2011). Heparanase modulates two critical systems involved in tumor progression, VEGF expression and EGFR activation.

Thus, providing a strong clinical support for the pro-metastatic and proangiogenic functions of the enzyme and positioning heparanase as an attractive target for the development of anticancer drugs (McKenzie, 2007; Cohen-Kaplan, *et al.,* 2008). In addition, neutrophil elastase is another broad-spectrum proteolytic enzyme believed to be a tumor-stimulator involved in the increased invasion of cancer cells (Kistowski *et al.,* 2017). Furthermore, neutrophil elastase is a protease that can degrade insoluble elastin, a structural component of elastic tissues such as blood vessels, skin, lung, liver, and breast tissue (Akizuki *et al.,* 2007). As shown in current results the activities of neutrophil elastase and heparanase enzymes were significantly increased in DENA group as compared to control group, while after treatment of resveratrol or/and quercetin, the values of them were significantly decreased in 2,4 weeks and highly significant decreases in 8 weeks compared with HCC groups. The results showed that the activities of neutrophil elastase and heparanase enzymes were significantly decreased with resveratrol or quercetin which indicated that resveratrol or quercetin inhibit metastasis (Singh *et al*., 2020). Expression of heparanase correlates with the metastatic potential of cancer cells and treatment with heparanase inhibitors markedly reduces the incidence of metastases in experimental animals (Mohan *et al.,* 2019). Furthermore, elevation neutrophil elastase destroys the barrier between the tumor and the local circulation, either lymphatic or hematological, and leads to at least localized regional metastases (Mejías *et al*., 2019).

The obtained data in the current study is strong enough to advocate and support further studies on the effect of resveratrol or quercetin in HCC in animal model. Also, supplementation of resveratrol or quercetin to carcinogenic patients may be hereby highly recommended to improve the odds of stimulated lesions and changes in liver, stomach and intestine tissues. This 'anti-carcinogen' effect becomes more pronounced after mixing resveratrol and quercetin after the DENA treatment. This investigation can practically help to encourage the clinical use of these compounds as a treatment for hepatocellular carcinoma.

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التأثير التآزري لريسفيراترول وكيرسيتين على سرطان الخلايا الكبدية المستحث كيميائياً في الجرذان

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المستخلص

سرطان الخلايا الكبدية هو أكثر أنواع السرطانات الأولية شيوعًا في الكبد . والهدف من هذه الدراسة هو تقييم آثار ريسفيراترول وكيرسيتين بمفردهما أو معًا ضد سرطان الخلايا الكبدية المحدث بواسطة ثنائي إيثيل نيتروزامين والفينوباربيتال في الجرذان. تم استخدام١٢٥جرذ ا بالغًا من ذكور الجرذان البيضاء في هذة الدراسة التى تتضمن تجربتين.في التجربة الأولى ، تم حقن ٣١جرذ بمحلول ملحي (المجموعة الضابطة) وحقن مجموعة الجرذان المصابة بسرطان الخلايا الكبدية (٩٤ جرذاً) بجرعة واحدة من ثنائي إيثيل نيتروزامين مرة واحدة في الأسبوع لمدة 3 أسابيع و فى نهاية هذه المدة تم حقن الجرذان بالفينوباربيتال مرة واحدة يوميًا لمدة ٧ أيام. ثم بعد مرور٧ أيام تم أخذ ١٠جرذان من كل مجموعة سابقة لمقارنة التغييرات في المعايير البيوكيميائية والفسيولوجية.

في التجربة الثانية ، أجريت أربع مقارنات بين ٢١ جرذًا من المجموعة الضابطة و ٨٤ جرذًا من مجموعة الجرذان المصابة بسرطان الخلايا الكبدية وتم تقسيم الجرذان المصابة بسرطان الخلايا الكبدية إلى المجموعات الفرعية التالية ؛(١) تركت الجرذان المصابة بسرطان الخلايا الكبدية دون أي علاج إضافي وعملت كمجموعة تعافي. (٢) اعطاءالجرذان المصابة بسرطان الخلايا الكبدية يوميًا ريسفيراتر ولبريتونيا وعملت كمجموعة ريسفيراترول .(٣) الجرذان المصابة بسرطان الخلايا الكبدية تم عالاجها بالحقن بريتونيا بالكيرسيتين وعملت كمجموعة كيرسيتين . (٤) اعطاء الجرذان المصابة بسرطان الخلايا الكبدية خليط من ريسفيراترول وكيرسيتين لمدة ٢ و ٤ و ٨ أسابيع وعملت كمجموعة ريسفيراترول وكيرسيتين.

أوضحت النتائج التي تم الحصول عليها عن تغيرات ملحوظة في جميع المتغيرات المدروسة لجرذان سرطان الخلايا الكبدية مقارنة بتلك الموجودة في المجموعة الضابطة .عندما عولجت جرذان سرطان الكبد بالعلاج المشترك من ريسفيراترول وكيرسيتين لمدة ٢ و ٤ و ٨ أسابيع ، اظهرت تحسن ملحوظ في جميع المعايير المدروسة اعتماداً على وقت العلاج وآليات معينة تمت مناقشتها وفقًا للأبحاث الحديثة المتاحة.