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Therapeutic Effects of Resveratrol and Baicalein on Hepatocellular Carcinoma Induced In Rats

Reda M.Fekry[1], Akaber T. Keshta[2], Shimaa R. Nawara[3].

1 Assistant Professor of organic chemistry, Chemistry department, Faculty of Science, Zagazig University, Egypt.
2 Lecture of biochemistry, Chemistry department, Faculty of Science, Zagazig University, Egypt.
3 Biochemistry Division, Biochemistry department, Faculty of Science, Zagazig University, Egypt.

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ABSTRACT

Background: Hepatocellular carcinoma (HCC), one of the most common cancers in the world, is a leading cause of cancer-related mortality. Resveratrol (RSV) and Baicalein (BE) are naturally derived polyphenolic compounds showed promising chemo-preventive effects against cancer. ***Aim:*** The present study aims to investigate the antioxidant activity of RSV and/or BE against induced HCC in rats. ***Methods:*** 48 Swiss male adult albino rats were divided into 6 groups: Group I served as a negative control injected intraperitoneally (I.P) with sterile saline; Group II were injected I.P with (0.25%) Dimethylsulphoxide (DMSO); Group III were injected I.P with Diethylnitrosamine (DEN) (200 mg/kg.bw) once; Group IV injected by DEN then treated with BE (20 mg/kg.bw) I.P for 10 consecutive days; Group V injected by DEN then treated with RSV (2.5 mg/kg.bw) orally for consecutive 10 days; Group VI injected by DEN then treated with BE and RSV. Plasma was collected for some biochemical studies and different antioxidant assays. ***Results:*** DEN-induced HCC that manifested by a significant drop in antioxidants levels, alterations in liver functions, and induced- inflammation. While, treatment with RSV and/or BE ameliorated liver injury by decreasing (alanine transferase (ALT), aspartate transferase (AST) activities, and Galectin-3 (Gal-3) levels. Also, they reversed the oxidative stress by the reduction of malondialdehyde (MDA) and nitric oxide (NO) levels and restoration of superoxide dismutase (SOD), glutathione-S-transferase (GST) activities, and reduced glutathione (GSH) levels. They decreased interlukin-6 (IL-6) levels. These data suggested that RSV or BE exhibited a significant role against HCC, which

Corresponding author:

might be related to their antioxidant and anti-inflammatory activities. ***Conclusions:*** Although combined treatment with RSV and BE significantly exerting a potential therapeutic effects, individual administration was more effective in HCC treatment. These novel findings suggested that RSV and BE have an antagonistic effect suggesting concerted efforts are needed to identify the most optimal combinatorial strategies.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent form of primary liver cancer, it is one of the most common life threatening solid tumors with global annual diagnosis exceeding one million new cases and remains the 3rd leading cause of cancer death **(1)**. Major risk factors for HCC include hepatitis viral infection, alcohol, environmental and industrial toxic chemicals, as well as several dietary carcinogens, such as aflatoxins and nitrosamines, have been shown to be involved in its etiology **(2)**.

Diethylnitrosamine (DEN) is a strong hepatocarcinogenic dialkyl nitrosoamine present in tobacco smoke, water, cheddar cheese, fried meals, agricultural chemicals, cosmetics, and in a number of alcoholic beverages. DEN is metabolized to reactive electrophilic intermediates which interact with DNA causing mutation leading to carcinogenesis. Other mechanisms may be involved through DNA-adduct formation, mutagenicity, and inhibition of many enzymes involved in DNA repair mechanism and tumor initiation **(3)**. Since oxidative stress has been implicated in multistage hepatocarcinogenesis process, dietary antioxidants might be potential protective agents against HCC. Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical-induced oxidative stress **(4)**. Therefore, there is an increasing interest for natural antioxidants, e.g. polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage. In this regard, naturally occurring biologically active polyphenol compounds derived from natural sources are gaining increasing interest as potential cancer therapeutics **(5)**.

Materials and Methods

Animals

48 adult Swiss male albino rats weighed (80-100g) were housed at animal house at the Faculty of Science, Zagazig University. The animals were maintained in controlled environment of temperature, humidity, light, and fed on a commercial standard diet and tap water *ad libitum*.

Chemicals

Diethylnitrosamine (DEN), Resveratrol (RSV), and Baicalein (BE) were purchased from Sigma-Aldrich Chemical Co., (St Louis, MO, USA), Galectin-3 (Gal-3) sandwich enzyme-linked immunosorbent assay (ELISA) Kit method from (BG Medicine, Waltham, MA**)**.

Induction of hepatocellular carcinoma (HCC)

DEN was freshly dissolved in a sterile saline and injected intraperitoneally (I.P) at a dose (200/kg B.W.) once **(12)**.

Experimental design

Animals were divided into 6 groups (8 rats/each one) as following: ***Group I:*** *Negative Control group*: serving as a normal control was injected I.P with a sterile saline, ***Group II:*** *DMSO group:* rats were injected I.P with (0.25%) for 10 days **(13)**. ***Group III:*** *HCC group:* serving as a positive control was injected I.P with DEN (200 mg/kg.BW) once, ***Group IV:*** *Baicalein-treated group (DEN+ Baicalein):* rats were injected with baicalein (20 mg/kg.BW) for 10 days **(14)**

after HCC induction, ***Group V:*** *Resveratrol-treated group* (*DEN+Resveratrol)*: rats were administrated (2.5 mg/kg. BW) of resveratrol orally for 10 days [15] after HCC induction, ***Group (VI):*** *Combination group (DEN + Baicalein + Resveratrol):* Baicalein injected I.P & Resveratrol administrated orally for 10 days after induction of HCC. At the end of the experiment, animals were weighed then anaesthetized under light di-ether and dissected. Blood samples were collected for biochemical analysis.

(A) Biochemical analysis

Malondialdehyde (MDA), nitric oxide (NO), and the antioxidants:superoxide dismutase (SOD), reduced glutathione (GSH), and Glutathione-S- Transferase (GST) were determined using Bio - diagnostic kit method according to **Satoh,** [16], **Montgomery and Dymock,** [17], **Nishikimi *et al.,*** [18], **Beutler *et al.,*** [19], **and Habig *et al.,*** [20] methods; respectively.

Liver enzymes assessed in serum samples:

ALT and AST were measured using Kinetic methods according to the **Schumann and Klauke,** [21] and **Karmen *et al.,*** [22].

Estimation of Galectin-3 (Gal-3):

Gal-3 was determined by using a sandwich enzyme-linked immunosorbent assay (ELISA) Kit method according to the method of **Christenson *et al.,***

***Interleukin-6 (IL-6) measurement*:**

IL-6 antigen was determined by ELISA using a commercially available kit from Promocell (Heidelberg, Germany) according to the method of **Isomura *et al***., [24].

Statistical analysis

Data were evaluated by one-way analysis of variance (ANOVA) by "SPSS" 14.0 for Microsoft Windows, SPSS Inc. [25] and considered statistically significant at a two-sided P < 0.05.Numerical data were expressed as mean ± SD.

Results

Effect of Resveratrol and Baicalein on antioxidants in all studied groups:

The mean levels of MDA and NO in the negative control group were found to be 16.75+1.26 (nmol/ml) and 24.34+3.35 (μmol/L); respectively. HCC group showed a significant increase in MDA levels to be 42.36 ±1.94 (nmol/ml) by 886.25%, and NO levels to be 60.39 ±4.33 (μmol/L) by 148.13%, ($p<0.001$) compared to negative control group. While Resveratrol and/or Baicalein treatment showed a significant decrease in MDA levels to 6.61±0.06, 10.52 ±0.15, and 17.29±0.49 (nmol/ml) by 84.41%, 112.25%, and 632.36%, ($p<0.001$) respectively; compared to the HCC group. Also, NO levels were decreased significantly in Resveratrol, Baicalein, and combination groups to 12.06±1.32, 18.15 ±0.93, and 32.10±2.21 (μmol/L) by 80.03%, 87.75%, and 46.83%, respectively, ($p<0.001$) compared to HCC group, table (I).

SOD and GST activities and GSH concentration were decreased from 293.08±3.03 (U/ml), , 267.20±21.05, (U/L) 9.08±0.67 (nmol/ml) in negative control group to 99.11 ±5.08 (U/ml), , 126.39±16.19 (U/L), 4.16±0.49 (nmol/ml) in HCC group by 66.18%, 54.15% and 52.70%; respectively, ($p<0.001$). While, their activities were significantly increased by 505.21%, 215.71%, and 305.57% in Resveratrol group, by 576.29%, 132.2%, and 973.72% in Baicalein group, and by 3359.7%, 128.13%, and 149.87% in combination group; respectively, ($p<0.001$) compared to HCC group, as shown in table (I).

Effect of Resveratrol and Baicalein on liver functions in all studied groups:

ALT and AST activities were found to be increased from 55.7+0.76 (U/L) and 118.5+0.34 (U/L) in negative control group to 79.84+0.87 (U/L) and 176.45+12.52 (U/L) in HCC group by 105.48% and 48.90%; respectively, ($p<0.001$). These high activities of liver

enzymes were significantly reduced respectively in Resveratrol group by 67.647% and 39.683%, and in Baicalein group by 60.598% and 45.39%, and also in combination group by 48.581% and 36.322%; respectively, ($p<0.001$) compared to HCC group, as shown in table (II).

Effect of Resveratrol and Baicalein on Galectin-3 concentration in all studied groups:

Gal-3 concentration was significantly elevated from 3.76+0.08 (ng/ml) in negative control group to 22.15 ±1.74 (ng/ml) in HCC group by 490.67%, ($p<0.001$). While, Gal-3 was significantly decreased to 3.74±0.53 (ng/ml) by 83.12% in Resveratrol group, and 7.18±0.46 (ng/ml) by 98.54% in Baicalein group, and 9.17±0.81 (ng/ml) by 58.60% in combination group; respectively, ($p<0.001$) compared to HCC group, as shown in table (III).

Effect of Resveratrol and Baicalein on Interleukin- 6 levels in all studied groups:

IL-6 was found to be 3.18+0.32 (pg/ml) in negative control group, while in HCC group there was a high significant increase in serum IL-6 by 10.58±1.22 (pg/ml) by 233.07% compared to normal control group, Table (IV). Groups treated with Resveratrol and/or Baicalein showed a high significant decrease in IL-6 level by 1.96±0.21, 5.18±0.41, and 5.18±0.41 (pg/ml) by 81.41%, 97.18%, and 50.99%; respectively as compared with HCC control group.

Hepatocellular carcinoma (HCC) is the most frequent primary hepatic malignancy, and it represents an important health issue due to its increasing incidence and poor survival, where in many years, natural products have considerable attention in scientific community for their therapeutic efficacy against various ailments such as cancer [26].

Our results showed that, DEN caused significant elevation in MDA & NO levels, and significant reduction in the activity of SOD and GST and the concentration of GSH in HCC group, compared to negative group. While treatment with resveratrol and/or baicalein revealed a significant decrease by in MDA & NO levels and a significant increase in SOD, GSH & GST activities, compared to the HCC group. There were some alteration between DMSO group and negative control group in all studied parameters. DMSO was only used as solvent for natural products (RSV & BE); also it used as an alternative treatment for cancer [27].

DEN confers its hepato-carcinogenicity through its metabolic activation in the hepatic microsomes, resulting in the release of ethyl-carbonium ions that bind to the DNA, producing adducts and generating superoxide radicals through lipid peroxidation (LPO) of phospholipids membrane fatty acids ultimately leading to oxidative stress and carcinogenesis [28]. The reduction in GSH level may be due to direct conjugation of GSH with electrophiles species since DEN is an electrophilic carcinogen, this depletion of GSH, may be responsible for the increased LPO [29].

Resveratrol has been reported to prevent oxidative stress and LPO processes, which might be due to its free radical scavenging effect; owing to the phenolic moiety present in its structure, these effects could be also related to its antioxidant properties in the liver [30]. Baicalein functioned as an anti-lipid propagating agent against oxidative damage through termination of peroxyl radical-mediated reaction; depending on its lipophilicity and its ability to bind to membranes phospholipids, as it is able to restore nuclear Nrf2 protein expression and its antioxidant response element (ARE) binding activity, which is a critical regulator of SOD and many of phase II genes such as GSTs [31]. Our findings were in accordance to many authors; **Lee *et al.,*** [32] showed that resveratrol treatment intensively lowered MDA level than ischemia rats. **Gao *et al.,*** [33] confirmed that baicalein showed a significant reduction in LPO along with a significant decrease in MDA levels. Also, **Sharma *et al.,*** [34] reported the potential protective role of resveratrol increasing hepatic SOD and GSH activities as well as activity of GST. **Naveenkumar *et al.,*** [35]

reported that baicalein treatment recovered the decreased hepatic GSH, SOD, and GST levels towards normal. Both of resveratrol and baicalein have been reported to possess a significant anti-inflammatory activity in various cells and tissues; since it decrease DEN-induced translocation of NF-kB to the nucleus; as a result reversed the elevated inducible NO synthase (iNOS) expression and thereby reduced the subsequent NO production [36]. Our results were in agreement with **Xin *et al.*,** [37] who reported that baicalein is able to inhibit iNOS gene expression and down-regulate NO production induced by various inflammatory stimuli *in vitro* and *in vivo* in animal models.

Serum AST and ALT are reliable marker enzymes of liver and they are the first enzymes to be used in diagnostic enzymology as an indicator for the extent of liver damage. The elevation of these enzyme activities was indicative of the toxic effect of DEN on the liver tissue; due to over production of these enzymes in tumor cells, which might have caused increase in the permeability of cell membrane resulting in liberation of these enzymes into serum [38]. Resveratrol treatment significantly attenuated the increased activities of these enzymes; which can be attributed to its capability to conserve the membrane integrity of cellular organelles [39].
While baicalein is able to uphold parenchymal cell regeneration in liver, by repairing hepatic tissue damage caused by tumor induction, thus protecting membrane integrity and thereby decreasing enzymes leakage. In addition, it is suggested that baicalein showed significant antioxidant activity, which might be in turn responsible for its hepato-protective activity [40]. Galectin-3 (Gal-3) is an intracellular and extracellular lectin which is presumed to interact with glycoproteins of the cell surface matrix, Gal-3 is over expressed in HCC tissues and is correlated with the tumor differentiation, suggesting that Gal-3 may be associated with the carcinogenesis and development of HCC. Thus, Gal-3 could be a novel serum tumor marker for HCC [41]. In the present study, we have observed an elevation in Gal-3
levels in HCC group compared to control group, Gal-3 expression levels increased in HCC tumor tissues relative to normal tissues, and might be associated with metastasis and cell growth
during the progression of HCC. Hence, Gal-3 might serve as a prognostic factor for HCC and have an important role in the diagnosis and treatment of this disease [42]. Both of resveratrol and
baicalein exert their anti-inflammatory potential through inhibition of NF-kB; thus attenuated the induction of Gal-3 in HCC cells expression at both the protein and mRNA levels [43].

Interleukin (IL-6) levels were found to be elevated in the HCC group compared to negative control group. Meanwhile, its levels were significantly decreased after treatment with resveratrol and/or baicalein. It has been demonstrated that DEN-induced inflammation triggers ROS production, which activates NF-κB followed by release of pro-inflammatory cytokine (IL-6), chemokines and iNOS- all these events leads to hepatocarcinogenesis [44]. Our results are consistent with the research results of **Jelic *et al.*,** [45] confirming the anti-inflammatory potential effect of resveratrol decreasing IL-6 levels.

Conclusion:

Resveratrol or Baicalein exert a potential chemotherapeutic activity against HCC induced in rats, while co-administration of both may enhance toxic effects; as the alternation in the pharmacokinetics of both natural compounds have very serious toxicological consequences.

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Table (I): Changes in levels of antioxidants in all studied groups:

Variable	Negative control group		DMSO Control		HCC Control Group		Resveratrol Group (DEN+ RSV)		Baicalein Group (DEN + BE)		Combination Group (DEN+RSV+BE)	
	Mean± SD.	% Change	Mean±SD.	% Change	Mean± SD.	% Change	Mean±SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change
MDA (nmol/ml)	16.75+1.26	------	14.73+1.94	-12.09	42.36+1.94a	+886.25	6.61+0.06b	-84.41	10.52±0.15b	-112.25	17.29±0.49b	+632.63
NO (nmol/ml)	24.34+3.35	-------	19.75+1.17a	-18.85	60.39+4.33a	+148.13	12.06+1.32b	-80.03	18.15±0.93b	-87.751	32.10±2.21b	-46.83
SOD (U/L)	293.08+3.03	-------	251.38+2.55a	-14.23	99.11+5.08a	-66.18	599.84+45.39b	+505.21	461.48±30.36b	-576.29	315.18±1.21b	+3359.7
GSH (nmol/ ml)	9.08+0.67	-------	8.14+0.05a	-10.29	4.16+0.49a	-54.15	13.14+1.12b	+215.71	17.43±1.13b	-132.2	9.49±0.04b	+128.13
GST (nmol/ ml)	267.2+21.05	-------	324.28+1.81a	+21.36	126.39+16.19a	-52.70	512.59+7.97b	+305.57	460.47±41.51b	-973.72	315.78±9.36b	+149.87

Significant difference from control value at $P < 0.001$,
(a) Compared with negative control
(b) Compared with HCC group

Table (II): Changes in liver enzymes activities in all studied groups:

Variable	Negative control group		DMSO Control		HCC Control Group		Resveratrol Group (DEN+ RSV)		Baicalein Group (DEN+ BE)		Combination Group (DEN+RSV+BE)	
	Mean±SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change
ALT (U/L)	55.7+0.76	----	79.84+0.87a	+43.33	114.45+1.93a	+105.48	37.06±.077b	-67.647	41.63±0.48b	-60.598	58.90±1.13b	-48.581
AST (U/L)	118.5+0.34	----	95.06+0.51a	-19.78	176.45+12.52a	+48.90	106.43±1.18b	-39.683	71.10±4.98b	45.39	112.36±0.52b	-36.322

Significant difference from control value at $P < 0.001$
(a) Compared with negative control
(b) Compared with HCC group

Table (III): Changes in Galectin-3 concentration in all studied groups:

Variable	Negative control group		DMSO Control		HCC Control Group		Resveratrol Group (DEN+ RSV)		Baicalein Group (DEN+ BE)		Combination Group (DEN+RSV+BE)	
	Mean±SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change
Gal-3 (ng/ml)	3.76+0.08	----	4.00+0.33a	+6.56	22.15±1.74a	490.67	3.74±0.53b	-83.12	7.18±0.46b	-98.54	9.17±0.81b	-58.60

Significant difference from control value at $P < 0.001$
(a) Compared with negative control
(b) Compared with HCC group

Table (IV): Changes in levels of IL-6 in all studied groups:

Variable	Negative control group		DMSO Control		HCC Control Group		Resveratrol Group (DEN+ RSV)		Baicalein Group (DEN+ BE)		Combination Group (DEN+RSV+BE)	
	Mean± SD.	% Change	Mean ± SD.	% Change	Mean± SD.	% Change	Mean ± SD.	% Change	Mean± SD.	% Change	Mean± SD.	% Change
IL-6 (pg/ml)	3.18+0.32	---	3.49+0.67a	1.97+0.21	10.57±1.22a	233.07	1.96±.21b	-81.41	6.58±0.62b	-97.18	5.18±0.41b	-50.99

Significant difference from control value at $P < 0.001$, (a) Compared with negative control , (b) Compared with HCC group