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A potential therapeutic impact of Gallic acid in a rat model of hepatocarcinogenesis through inhibition of cell proliferation and oncogenic miRNA-221 and induction of apoptosis by Nrf-2 /Bcl-2/TGF- β1 signaling pathways.

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ABSTRACT

Hepatocellular carcinoma (HCC) is still a main cause of fatality for individuals with chronic liver illnesses. Gallic acid (GA) is among the most probable polyphenols which possess several pharmacological effects as antioxidant, anti-inflammatory, apoptotic, and antitumor activities. The objective of this study was to evaluate the potential therapeutic and anti-cancerous effect of GA on a rat model of HCC. Thirty rats were segregated equally into three groups. G1 (Normal control) were given saline as a vehicle. In G2 (HCC non-treated), HCC was induced via an intraperitoneal injection of diethylnitrosamine (DEN) (200 mg/kg b.wt), then two weeks later the rats were given 3-weekly successive doses of CCl_4 (in corn oil at 1:1 proportion, 3 ml/kg b.wt) orally to boost the carcinogenic impact. DEN and CCl4 administrations were repeated after 5 weeks. In G3 (HCC+GA treated), 15 weeks after HCC induction, treatment with GA (100 mg/kg b. wt.) was given orally and continued for six weeks. The results showed significant upregulations in liver microRNA-221 and TGF-β1, with obvious down-regulation of (Nrf2 and Bcl-2) and insignificant downregulation of caspase 3 gene in HCC-induced rats. GA treatment exhibited a significant decline in ALT, AST, and ALP hepatic enzyme markers with downregulation of TGF-β1, microRNA-221, and upregulation of Nrf2, Bcl-2, and caspase 3 gene expression. In conclusion, GA reduces liver preneoplastic lesions development and has a helpful therapeutic impact against liver cancer, inhibiting growth-promoting oncogene and increasing apoptosis.

1. INTRODUCTION

Cancer of the liver is a highly common fatal cancer worldwide, with over 90% of malignancies being hepatocellular carcinoma (HCC). Nowadays, HCC ranks fourth in terms of cancer-related deaths globally, responsible for over 70,000 mortalities per year (Anwanwan et al., 2020). The greatest risk elements for the growth of HCC are still chronic liver abnormalities and cirrhosis, with viral hepatitis and excessive alcohol intake ranked highest among them (Tunissiolli et al., 2017).

A variety of genetic and epigenetic factors play a role in the complex molecular mechanisms of liver cirrhosis and its progression to HCC (Besheer et al., 2019). Various substances have been identified causing liver damage and are commonly employed to develop models of HCC. They are mainly separated into two categories: (i) genotoxic substances that modify the structure of DNA, and (ii) promoters that can accelerate the growth of malignancies (Zhang et al., 2019). The most popular model for preclinical study among chemically generated liver cancer agents is still diethylnitrosamine (DEN)-induced liver cancer (He et al., 2015). Diethylnitrosamine is mostly directed towards the liver, where centrilobular hepatocyte-resident cytochrome P450 enzymes catalyze its conversion into alkylating compounds that further damage DNA (Connor et al., 2018). Moreover, DEN capacity to induce oxidative stress aids in the emergence of hepatocarcinogenesis (Heindryckx et al., 2009). To recreate the tumor microenvironment of HCC in humans, a single injection of DEN may be combined with frequent doses of CCl_4, serving as a pro-fibrogenic agent. Chronic DEN injections cause fibrosis and inflammation, exactly mimicking the course of human HCC (Jilkova et al., 2019).

Gallic acid (GA), also known as 3,4,5trihydroxybenzoic acid, is a widely recognized polyphenol that's found in various sources such as green tea, pomegranate, grapes, oak bark, different berries, nuts, mango, and red wines. It is also recognized as a key active component in tannins, specifically Gallotannin (Fernandes and Salgado, 2016). Research has indicated that GA possesses a range of pharmaceutical effects such as antioxidant, antitumor, anti-inflammatory, anti-microbial, and anti-viral properties (Locatelli et al., 2009). Treatment with gallic acid provides a versatile strategy for reversing the aggressive nature of HCC in a rat model (Locatelli et al., 2021). Therefore, this study was carried out to evaluate the potential anti-carcinogenic impact of GA on alterations in molecular markers and epigenetic MicroRNA-221 in liver tissues of rats with HCC.

2. MATERIAL AND METHODS

2.1. Experimental Animals:

Thirty male albino rats, aged 4-5 weeks and weighing an average of 150-200 g, were used in this study. The rats were placed in individual metal cages with good ventilation,

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lighting, and humidity levels. The rats had a 15-day adaptation period prior to the start of the study. The rats were given a standard diet pellet and had unlimited access to fresh water. The Animal Care and Use Committee at Benha University approved the experimental protocol (Approval no. BUFVTM 12-02-23).

2.2. Chemicals and drugs:

Diethylnitrosamine (DEN) 1g/1ml vial existing as a transparent yellow liquid form, CAS Number 55-18-5, and Gallic acid (≥98.5% purity) with M.W 188.14 present in white to beige (Off white) powder form, CAS Number 149-91-7 were purchased from Sigma Aldrich Chemical Company (St. Louis, MO., USA).

2.3. HCC Induction:

Hepatocellular carcinoma in rats was generated by administering DEN in normal saline at a dose of 200 mg/kg b. wt. via I.P injection (Singh et al., 2009), then 2 weeks later rats received 3 weekly successive doses of CCl_4 (3 ml/kg b.wt) orally at 1:1 dilution in corn oil as a promoter of a carcinogenic effect (Hassan et al., 2014). A further DEN and CCl4 injection were administered five weeks after the initiation of the DEN injection. About 15 weeks after HCC induction, therapeutic intervention with gallic acid was given and continued for six weeks.

2.4. Experimental design:

Rats were divided equally into 3 groups, (ten each), as follows:

G1 (Normal control): Rats received saline as a vehicle during the entire experimental period of 21 weeks.

G2 (HCC non-treated): HCC was induced in rats by injection of DEN (200 mg/kg b.wt. IP), then 2 weeks later of the DEN injection rats received 3 weekly successive doses of CCl4 (3ml/kg b.wt/ orally) at 1:1 dilution in corn oil as a promoter of a carcinogenic effect. DEN and CCl4 administration were repeated once again after 5 weeks.

G3 (HCC + Gallic acid treated): Rats injected with DEN and CCl_4 injections similarly as G2 then, post-treated orally with GA dissolved in distilled water (100 mg/kg b.wt/day) after 15 weeks from injection of DEN and CCl_4 for 6 weeks (Esmaeilzadeh et al., 2020).

2.5. Sampling:

2.5.1. Blood samples:

Blood samples obtained through ocular vein puncture into tubes with screw caps were subjected to centrifugation at 3000 RPM for 15 minutes. Separated serum was kept in deep freeze at -20 °C until utilized for estimating the liver markers enzyme (Alanine transaminase (ALT), Aspartate transaminase (AST) and Alkaline phosphatase (ALP)).

2.5.2. Liver tissue specimens:

Rats were euthanized at the end of the trial (21 weeks) according to Animal Ethics Committees, the abdomen was then opened, and the liver was removed. About 0.5 g of liver tissue was put in Eppendorf tubes, and immediately placed in liquid nitrogen and kept at -80°C till it used for RNA extraction to determine Nuclear factor erythroid 2-related factor 2 (Nrf2), B-cell lymphoma 2 (Bcl-2), Caspase 3, transforming growth factor-β1 (TGF-β1) and miRNA -221 gene expressions by reverse transcription polymerase chain reaction (RT-PCR).

2.6. Analysis:

2.6.1. Biochemical analysis:

The kinetic method outlined by Schumann et al. (2002) was used to determine the activity of ALT and AST in serum while the method of Tietz et al. (1983) was used for the estimation of ALP activity using a commercial kit supplied by SPINREACT, Santa Coloma, Spain.

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2.6.2. Molecular analysis:

With real-time quantitative PCR analysis, the mRNA transcription levels for Nrf2, Caspase 3, Bcl-2, and TGF-β1 were assessed in the rat livers. Using a complete RNA purification kit and following the manufacturer's guidelines (Thermo Scientific, Fermentas, #K0731), pure RNA was isolated from liver tissues. Each cDNA sample has undergone reverse transcription using Revert Aids TM First Strand CDNA synthesizing kit (#EP0451, Thermo Scientific, Fermentas, USA). Next, real-time PCR with SYBR Green was utilized to evaluate gene expression using gene-specific primers (Table 1) and the manufacturer's protocol (Thermo Scientific, USA, # K0221). The target gene was normalized using glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) using the 2-ΔΔCt technique.

For evaluation of miRNA-221 expression in the liver, Real-time PCR and SYBR green were used with U6 acting as inside management. According to the manufacturer's instructions using (USA, Thermo Scientific, # K0221), the extracted cDNA was amplified, the universal reverse primer integrated with the Quanti-Mir RT kit and a miRNA-specific forward primer (Table 2).

2.7. Statistical analysis:

All data were expressed as means ±SEM. Using SPSS, 18.0 software, 2011, the statistical significance was evaluated by ANOVA (one-way analysis of variance), and DMRT (Duncan's multiple range test) was used to obtain the individual comparisons. A value could be considered significant in statistics when it was $p< 0.05$.

3. RESULTS

The data from table (3) and figure (1) showed that serum AST, ALT, and ALP activities were significantly higher in rats with HCC compared to the control group. However, rats with HCC administered Gallic acid showed a significant decrease in liver marker enzyme activities compared to those not treated (G2).

Table 1 Sequences for forward as well as reverse primers for genes utilized in qPCR:

Gene	Forward primer (′5 ------ ′3)	Reverse primer (′5 ------ ′3)
Nrf2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
Caspase3	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTTCTTTGC
Bcl-2	ATCGCTCTGTGGATGACTGAGTAC	AGAGACAGCCAGGAGAAATCAAAC
TGF-β1	AAGAAGTCACCCGCGTGCTA	TGTGTGATGTCTTTGGTTTTGTCA
GAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA

Table 2 Universal reverse primers sequences for primers used for qPCR:

Gene	Primer sequence (′5 ------ ′3)
MiRNA-221	AGCTACATTGTCTGCTGGGTTTC
U6	TGACACGCAAATTCGTGAAGCGTTC
Universal reverse primer	CCAGTCTCAGGGTCCGAGGTATTC

The qPCR results presented in table (4) and figure (2) indicated a marked downregulation in liver Nrf-2 and Bcl-2 expression, along with an upregulation in TGF- β1 and a non-significant upregulation in Caspase-3 gene expression in HCC-induced rats compared to the control group. Conversely, rats treated with GA showed a significant upregulation in liver Nrf-2, Caspase-3, and Bcl-2 expression, along with a downregulation in TGF- β1 gene expression when compared to the HCC untreated group. The findings presented in table (4) figure (3) indicated that rats exposed to DEN/CCL4 exhibited a significant upregulation in microRNA-221 levels when compared to the control group. However, treatment with GA resulted in a notable downregulation in microRNA-221 levels when compared to the non-treated HCC group.

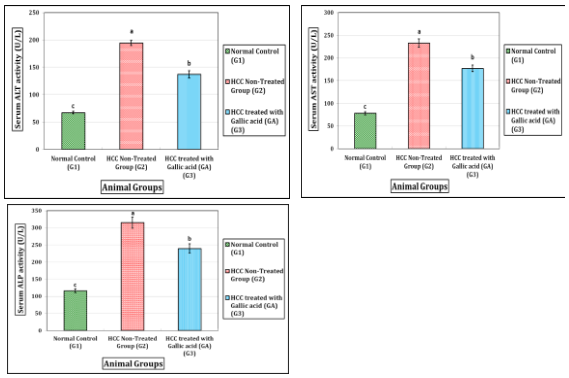

Figure 1: Effect of GA treatment on serum AST, ALT and ALP activities in experimental model of hepatocarcinogenesis in rats.

Table 3 Effects of Gallic acid treatment on serum ALT, AST and ALP activities in DEN/ CCL4-induced HCC in rats

Parameters	Animal groups		
	Control normal. (G1)	HCC non-treated (G2)	HCC+GA treated. (G3)
ALT (U/L)	67.22 ± 2.40^{c}	194.50 ± 4.53^{a}	137.16 ± 6.77^{b}
AST (U/L)	78.26 ± 3.45^{c}	232.66 ± 9.15^{a}	177.01 ± 7.19^{b}
ALP (U/L)	116.28 ± 5.80^{c}	315.61 ± 16.17^{a}	239.74 ± 13.45^{b}

Data are displayed as mean ± SEM. Mean values in the same row with different superscript letters differed significantly at P≤0.05.

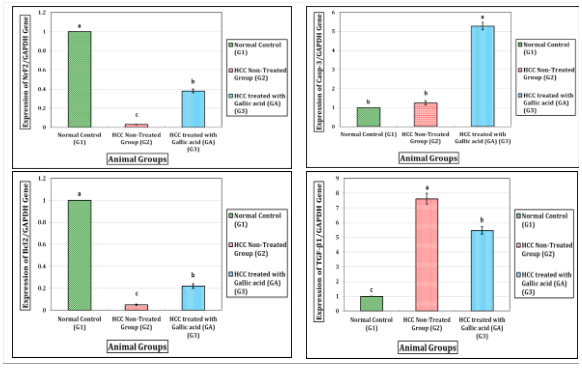

Fig. 2: Effect of GA treatment on Nrf2, Caspase-3, Bcl-2 and FGF-2 of liver tissue gene expressions in experimental model of hepatocarcinogenesis in rats.

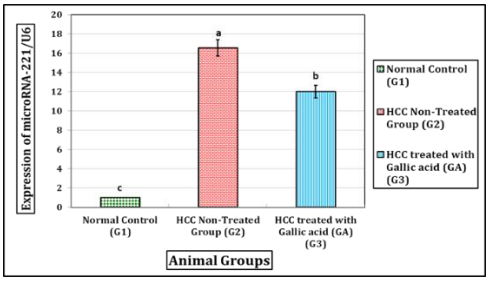

Figure 3: Effect of GA treatment on hepatic tissues microRNA-221 gene expression in experimental model of hepatocarcinogenesis in rats.

Table 4 Effects of GA treatment on liver tissue Nrf2, Caspase-3, Bcl-2, TGF-β1 genes and MicroRNA-221 expressions in DEN/ CCl_4 induced HCC in rats.

Gene (Expression as fold change)	Animal groups		
	Control normal. (G1)	HCC non-treated (G2)	HCC+GA treated. (G3)
Nrf2	1.00 ± 0.00^{a}	0.03 ± 0.002^{c}	0.38 ± 0.02^{b}
Caspase-3	1.00 ± 0.00^{b}	1.24 ± 0.12^{b}	5.28 ± 0.20^{a}
Bcl-2	1.00 ± 0.00^{a}	0.05 ± 0.003^{c}	0.22 ± 0.01^{b}
TGF-β1	1.00 ± 0.00^{c}	7.62 ± 0.36^{a}	5.46 ± 0.25^{b}
MicroRNA-221	1.00 ± 0.00^{c}	16.56 ± 0.85^{a}	12.01 ± 0.64^{b}

Data are displayed as (Mean ± SEM). Mean values in the same row that contain various superscript letters differ significantly at P≤0.05.

4. DISCUSSION

Hepatocellular Carcinoma is recognized as one of the most common causes of death worldwide yearly (Elkenawy et al., 2022). The interesting features of natural products in combating cancer have garnered a lot of interest in the last three decades. Consequently, they are excellent choices for the creation of new anticancer and chemo-preventive treatments. The current data indicated that the activity of AST, ALT, and ALP in the serum was significantly elevated in rats with liver cancer. This result was consistent with Reyes-Esparza et al. (2019), who observed that there was an increase in serum ALT, AST, and ALP activities in rats with pre-neoplastic lesions after receiving a single dose of DEN 200 mg/kg i.p., followed by a single dose of CCl_4 2 mL/kg i.p. two weeks later, in comparison to the control group. Also, Lyngdoh et al. (2023) reported that a significant increase in serum ALT, AST, and ALP activities was observed in DEN-induced HCC in mice. Moreover, the delivery of DEN led to an increase in the serum stress-specific enzymes (ALT, AST, and ALP), suggesting that DEN has a toxic effect on liver tissue, causing membrane permeability and subsequent enzyme leakage into the circulation (Pradeep et al., 2010). Nevertheless, rats with liver cancer treated with Gallic acid showed a marked reduction in serum ALT, AST, and ALP activities.

Nrf-2 deficiency has been linked to several diseases because of its vital role in regulating oxidative stress, involving diabetes, hyperglycemia, acute kidney damage, ischemia, atherosclerosis, and liver diseases. Concerning the liver, Nrf-2 also plays a significant role in the initiation of transporters and detoxification enzymes which help to eliminate harmful substances (Fuertes-Agudo et al., 2023). In the current study, a significant downregulation in liver Nrf-2 gene expression was observed in HCC-induced rats Similarly, Mansour et al. (2019) found that rats injected with a single dose of diethylnitrosamine (200 mg/kg b.wt) had significantly reduced levels of Nrf-2 in their liver, colon, and stomach compared to normal rats. Conversely, liver cancer rats treated with GA showed a marked upregulation of Nrf-2 gene expression compared to HCC untreated group. In a study conducted by Ma et al. (2014), it was found that GA increased Nrf-2 expression levels in a mouse model of hepatotoxicity induced by diethylnitrosamine. This, in turn, led to the activation of redox stabilizers through bonding with DNA sequences. Similarly, a study conducted by Sanjay et al. (2021) demonstrated that Gallic acid at a dosage of 150 mg/kg significantly elevated the level of Nrf2 expression compared to a group that was given isoniazid and rifampicin to induce hepatotoxicity. Elmileegy et al. (2023) also demonstrated that treatment with GA up-regulated the immune expression of Nrf-2 in liver tissues that were exposed to uranyl acetate-induced hepatic dysfunction.

Caspase-3 is the basic element of the apoptotic process, apoptosis doesn't initiate unless a cell is activated, damaged, or given the order to die. A previous study done by Ding et al. (2010) revealed that, positive Caspase-3 expression was substantially less in HCC than that in paracancerous tissues

which indicated that the growing phase in HCC cells may be present but with low apoptosis. This may consistence with our investigation that demonstrated a non-significant upregulation of caspase-3 in HCC-induced rats. Conversely, rats treated with GA significantly upregulate caspase-3 gene expression. Consequently, the existing results were consistent with results of Huang et al. (2021), who proved that, treatment of HCC cells with methyl gallate, a compound produced from gallic acid, led to the overexpression of caspase3. The Bcl-2 family that modulates the intrinsic apoptotic pathway consists of proapoptotic proteins such as Bak, Bad, and Bax plus anti-apoptotic proteins like Bcl2 and Bcl-XL (Brentnall et al., 2013). The obtained results show significant downregulation in the expression of Bcl-2 level in the liver of DEN/CCL_4 treated rats. Similarly, HepG2 cells subjected to oxidative stress caused by cadmium telluride quantum dots show lower levels of Bcl-2 (Nguyen et al., 2013). Chiu et al. (2003) reported that the difference in Bcl-2 expression levels possibly connected to p53 state, Bcl-2 is noticeably up-regulated in p53-positive HCC tissues but down-regulated in p53-negative ones. Also, earlier studies established that Bcl-2 expression prevents and postpones DEN-induced hepatocarcinogenesis (Pierce et al., 2002). Therefore, our study suggests that in hepatocellular carcinoma induced by DEN and CCL4, the progression of hepatocarcinogenesis is associated with a decrease in Bcl-2 expression. However, treating HCC with GA leads to a significant increase in Bcl-2 gene expression level when compared to the HCC non-treated group. TGF-β1 is considered as an inflammatory cytokine, immune-suppressive substance, pro-fibrogenic indicator, and procarcinogenic growth element which exhibits increasing levels along the stages of liver failure, from hepatitis to HCC. Bad prognosis and survival rate are linked to aberrant TGF-β1 activity in HCC (Devan et al., 2022). In both cancerous and normal hepatic cells, TGF-β could lower the expression level of Bcl-2 and hence induce apoptosis (Zhang et al., 2020). The collected result proved that the untreated group's TGF-β1 expression level is highly upregulated when compared to normal. These results were consistent with Jin et al. (2022), who revealed that molecular and cell-based information showed higher TGF-β1 mRNA expressions in both human cell lines with HCC (Huh7 and HepG2) compared to the normal hepatic one. Also, the induction of liver fibrosis by Diethylnitrosamine therapy resulted in a significant 2-fold rise in TGF-β1 levels (Chen et al., 2018). However, treatment of the HCC group with GA exhibits a significant downregulation of TGF-β1 expression level. This came in agreement with Chen et al. (2018), who claimed that TGF-β1 levels were considerably decreased after administration of a medium and high dose of gallic acid than in the simulation group. Additionally, Hussein et al. (2020) found a significant decrease in TGF-β1 protein levels in animals with liver fibrosis induced by thioacetamide and treated with GA.

The obtained results indicated that rats exposed to DEN/CCL4 exhibited a significant upregulation in microRNA-221 levels compared to the control group. Similarly, Yun et al. (2022), showed higher levels of miR-18a and miR-221 expressions in HCC tissue compared to neighboring non-cancerous liver tissue. The study also suggested a correlation between miR-221 expression and greater tumor growth. Callegari et al. (2012) also mentioned that in a liver-specific transgenic mouse model (TG221) for miR-221, the administration of DEN accelerated the progression of liver cancer. It is worth mentioning that the extent of miR-221 overexpression was directly related to the degree of miR-221 dysregulation in human HCC, with levels increasing by 2 to 3 times when comparing hepatocellular carcinoma to surrounding tissue. Patients with hepatocellular carcinoma (HCC) have been found to exhibit higher levels of miRNAs (21, 221, and 222) expression in comparison to those with healthy livers. Additionally, these miRNAs can promote the development of liver fibrosis by increasing TGF-β signaling, as demonstrated in former studies (Karakatsanis et al., 2013; Gupta et al., 2019). Conversely, treatment with GA resulted in a significant downregulation in oncogenic microRNA-221 levels when compared to the non-treated HCC group. Also, Hussein et al. (2020) showed a significant decrease in the expression of miR-21 in the rats treated with thioacetamide and GA when compared to the untreated group.

5. CONCLUSIONS

It could be concluded that, Gallic acid has the potential effect to inhibit the progression of HCC by controlling various genes associated with HCC such as Nrf2 which regulates oxidative stress, Caspase-3 and Bcl-2 affect apoptotic mechanisms, and TGF-β1 which plays a role in cell proliferation and tumor growth. Furthermore, Gallic acid's ability to regulate the oncogenic miR-221 could be beneficial and have a strong therapeutic impact on HCC.

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