Moringa olifera Attenuated Nitrosodiethylamine-Induced Hepatocarcinogenesis by Modulating the Metabolic Activation and Detoxification Enzymes

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A B S T R A C T

Moringa olifera was shown to exert anti-inflammatory, antioxidant, hepatoprotective properties, and anticancer activity. This study was done to investigate the protective effects of moringa olifera on Diethylnitrosamine (DEN) induced Hepatocellular carcinoma in rats. Fourty five male albino rats were divided into three groups. Group (normal control group): rats administered distilled water only. Group II: rats received diethylnitrosoamine (200 mg/kg b.wt/i.p), two weeks later rats received (2 ml/kg b.wt) Carbon tetrachloride (CCl4) orally at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl4 injections were repeated once again after 1 month from first DEN injection. Group III: rats received DEN then treated with moringa at a dose level of (500 mg/kg b.wt/orally) dissolved in distilled water for 6 weeks. All animals were sacrificed after the end of experiment. DEN induced HCC showed significant increase in hepatic marker enzymes (ALT and ALP), total bilirubin and alpha fetoprotein (AFP) with marked decrease in serum albumin concentration. Also, the results of molecular analysis of liver tissue revealed significant up-regulation in TNF-α gene expression level. Conversely, down-regulation in tumor suppressor gene p53 and Cyp2E1 gene expression compared with control group. Treatment with moringa olifera to DEN induced HCC protects the liver cells from damage by regulating the biochemical parameters. These findings suggest the potential efficacy of moringa as an additional chemopreventive agent in treatment of hepatocellular carcionoma via initiation of tumor suppressor gene (P53) and modulating the metabolic activation of detoxification Enzyme (cytochrome P450 2E1) and anti-inflammatory effect.

Keywords: *Diethylnitrosamine, HCC, Moringa oliefera, P53, cytochrome P450 2E1.*

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1. INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant neoplasm of hepatocytes and constitutes more than 80% of primary malignant liver neoplasms (Satir, 2007). Worldwide, liver cancer is the fifth most common malignancy and the third most common cause of cancer death (Kung *et al.*, 2010).

The major avoidable causes of cancer are smoking, dietary imbalances, chronic infections and hormonal factors which are influenced primarily by lifestyle, other causal factors in human cancer are excessive sun exposure, viruses (such as human papilloma virus and cervical cancer) and pharmaceuticals (such as phenacetin, some

chemotherapy agents, diethylstilbestrol, and estrogen) (Gold et al., 2002). Many hepatocarcinogens such as aflatoxins, acetylaminofluorene3 and diethylnitrosamine have been successfully used to develop hepatocarcinogenesis in animals (Mukherjee et al., 2009). Diethyl nitrosamine (DEN) is a potent hepatocarcinogenic nitrosamine, present in cheddar cheese, cured and fried meals, alcoholic beverages, cosmetics, agricultural chemicals and pharmaceutical agents, ground water having high level of nitrate (Mahmoud and Abdul-Hamid, 2012).

Carbon tetrachloride (CCl4) is classified as a possible human carcinogen based on inadequate evidence of carcinogenicity in humans but sufficient evidence in animals. However, there are major deficiencies in the available cancer studies. Animal studies suggest that the carcinogenicity of carbon tetrachloride is secondary to its hepatotoxic effects, indicating a possible threshold (Provincial, 2010).

A number of modern drugs have been isolated from natural sources and many of these isolations were based on the uses of the agents in traditional medicine. Moringa oleifera leaves have been found to have the same powerful antioxidant agents such as vitamins C, E and A in oranges, pomegranates and carrots, as well as caffeoylquinic acids, carotenoids (i.e., lutein and a- and b-carotene), kaempferol, quercetin and rutin (Smolin and Grosvenor, 2007). Moringa oleifera oil and its micronutrients exhibit antitumor, antioxidant, antiepileptic, anti-diuretic, anti-inflammatory, hepatoprotective and antidiabetic properties (Sreelatha and Padma, 2010). The present study was to investigate the chemopreventive, anti-inflammatory, apoptotic and detoxification effects of moringa olifera on DEN and CCl4 induced- hepatocellular carcinoma in rats through evaluation of some serum liver biomarkers and molecular analysis of CYP 2E1, P53 and TNF-α gene expression in hepatic tissues.

2. Materials and methods

2.1. Experimental animals:

Forty-five white male albino rats of 6- 8 weeks old and weighing 150 - 180 g housed in separated metal cages and kept at constant environmental and nutritional conditions throughout the period of experiment. The animals fed on constant ration and water was supplied ad- labium .

2.2. Chemicals and antioxidant:

All chemicals were of analytical grade and obtained from standard commercial suppliers. The antioxidant and chemicals used in the present study were:

2.2.1. Diethylnitrosamine (DEN) and Carbon tetrachloride (CCl4) were Purchased from SIGMA Chemical Co. (St. Louis, MO, USA) .

Induction of Hepatocarcinogenesis:

Hepatocellular carcinoma was induced in rats by I. P injection of DEN in normal saline (200 mg/kg b.wt), 2 weeks later rats received (2 ml/ kg b.wt) CCl4 orally at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl4 administration were repeated once again after 1 month from the first DEN injection. (Hassan et al.,2014).

2.2.2. Moringa olifera was purchased from National Research Centre, Giza, Egypt. Moringa olifera extract powder was dissolved in distilled water and administered orally at a dose level of 500 mg/kg body weight/day (Bharali et al.,2003).

2.3. Experimental design:

After acclimatization to the laboratory conditions, the animals were randomly divided into three groups (15 rats each) placed in individual cages and classified as follow :

Group I: Control Normal group:

Consisted of 15 male rats fed with ordinary diet only without any treatment during the entire experimental period.

Group II: DEN- induced hepatocarcino-genesis group:

Consisted of 15 male rats received DEN in normal saline (200 mg/ kg b.wt) by I.P injection, 2 weeks later rats received (2 ml/ kg b.wt) CCl4 orally at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl4 injections were repeated once again after 1 month from first DEN injection.

Group III: DEN + moringa treated group

Consisted of 15 male rats received DEN in normal saline (200 mg/ kg b.wt) by I.P injection, 2 weeks later rats received (2 ml/ kg b.wt) CCl4 orally at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl4 injections were repeated once again after 1 month from first DEN injection then treated orally and daily with 500 mg/kg body weight of Moringa oleifera in distilled water.

2.4. Sampling:

2.4.1 .Blood samples:

Twenty-four hours fasting after the last dose of Moringa oleifera administration, rats were anaesthetized under diethyl ether anesthesia. Blood samples were collected by ocular vein puncture in dry, clean tubes and allowed to clot for 30 minutes and serum was separated by centrifugation at 3000 r.p.m for 15 minute. Serum was taken by automatic pipettes and collected in dry sterile tubes, then kept in deep freeze at -20 °C until use for subsequent biochemical analysis. All sera were analyzed for determination of the following parameters: AST, ALP, total bilirubin, Albumin, AFP .

2.4.2 .Liver tissue for molecular analysis

Briefly, liver tissues were cut, weighed and minced into small pieces, about 0.5 g of liver tissues were collected from all animals groups, put in Eppendorf tubes and were immediately kept in liquid nitrogen and stored at -80°C till RNA extraction. The molecular analysis of the relative gene expression in liver tissues evaluated by reverse transcription polymerase chain reaction (RT-PCR) were: (TNF-α, p53 and Cyp2E1).

2.5. Analysis:

2.5.1 Biochemical analysis

Serum ALT and ALP activities, total bilirubin, albumin and AFP concentrations were determined according to the method described by Schumann et al., (2002), EL-Aaser and EL-Merzabani, (1975), Young, (1997) and Doumas et al., (1971) and Engall, (1980), respectively.

2.5.2 Molecular analysis

Total RNA was isolated from liver tissue of rats using RNeasy Mini Kit (Thermo Qiagen, #74104) according to the manufacturer's protocol. Following determination of RNA concentration and purity by Quawell nanodrop Q5000 (USA), 5 mg of total RNA from each sample was reverse transcribed using Quantiscript reverse transcriptase. The produced cDNA was used as a template to determine the relative expression of Tumor necrosis factor alpha (TNF-α), tumor suppressor (P53) and cytochrome P450 2E1 (Cyp2E1) genes using Step One Plus real time PCR system (Applied Biosystem, USA) and gene specific primers. The reference gene, βactin, was used to calculate fold change in target genes expression. The thermal cycling conditions, melting curves temperatures, and calculation of relative expression was done. For the treated groups, assessment of 2-ΔΔCt determined the fold change in gene expression relative to the control (Livak and Schmittgen, 2001).

Forward and reverse primers sequence for real time PCR.

Gene	Forward primer ('5 ------ '3)	Reverse primer ('5 ------ '3)
TNF-α	GCATGATCCGCGACGTGGAA	AGATCCATGCCGTTGGCCAG
p53	ATGGCTTCCACCTGGGCTTC	TGACCCACAACTGCACAGGGC
CYP2E1	CTCCTCGTCATATCCATCTG	GCAGCCAATCAGAAATGTGG
β-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

2.6. Statistical analysis:

The results were expressed as mean ± SE using SPSS software program version 16 (SPSS© Inc., USA). The data were analyzed using one-way ANOVA to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparison among the groups for testing the inter-grouping homogeneity. Values were considered statistically significant when $p<0.05$.

3. RESULTS

The obtained results demonstrated in table (1) revealed that, serum ALT and ALP activities, total bilirubin and AFP concentrations were significantly elevated and serum albumin level was significantly decreased in DEN – induced liver cancer in rats when compared with the control normal group. Moringa olifera treatment to DEN-induced HCC significantly prevented these changes, resulting in a remarkable protection regarding the same parameters with the ability to restore the value of serum ALT, ALP, total bilirubin, albumin and AFP nearly to the average level of control group when compared with DEN-induced HCC group.

The obtained qPCR results presented in table (2) revealed a significant up-regulation of TNF- α gene expression level in liver tissue of DEN-induced liver cancer in rats. This expression was significantly downregulated after treatment with Moringa olifera. However, a significant downregulation of p53 and Cyp2E1 gene expression levels were observed in liver of DEN induced HCC in rats as compared to the normal control group. This expression was significantly upregulated following treatment by Moringa olifera when compared with DEN group.

Table1. Effect of Moringa olifera treatment on serum ALT and ALP activities, total bilirubin, Albumin and AFP concentrations in DEN-induced HCC in rats.

Parameters Exp. groups	ALT (U/L)	ALP(U/L)	T. bilirubin(mg/dl)	Albumin (g/dl)	AFP (ng/dl)
Group I: Normal control	190.00±11.55^{b,c}	358.00±27.14^{b}	0.58±0.02^{b}	3.23±0.12^{a}	0.73±0.007^{c}
Group II: DEN	365.00±14.43^{a}	490.00±11.55^{a}	1.75±0.03^{a}	1.80±0.17^{b}	4.90±0.47^{a}
Group III: DEN+moringa	218.67±12.13^{b}	402.33±17.32^{b}	0.49±0.09^{b}	3.17±0.12^{a}	3.00±0.06^{b}

Table 2. Effect of Moringa olifera treatment on liver tissue TNF-α, p53 and Cyp2E1 gene expression level in DEN induced HCC in rats.

Parameters Exp. groups	TNF-α Fold change mean± SEM	p53 Fold change mean± SEM	Cyp2E1 Fold change mean± SEM
Group I: Normal control	1.00^{d} ±0.09	1.00^{a} ±0.06	1.00^{a} ±0.06
Group II: DEN group	7.52^{a} ±0.36	0.02^{d} ±0.01	0.05^{c} ±0.005
Group III: DEN +moringa	4.66^{b} ±0.21	0.24^{c} ±0.02	0.23^{b} ±0.02

Data are presented as (Mean ± S.E). S.E = Standard error.

Mean values with different superscript letters in the same column are significantly different at ($P\leq0.05$).

4. DISCUSSION

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and occurs predominantly in patients with underlying chronic liver disease and cirrhosis (Alison, 2005). *N*-Nitrosodiethylamine (DEN) causes a wide range of tumors in all animal species and such compounds are hazardous to human health. The formation of reactive oxygen species (ROS) is apparent during the metabolic biotransformation of DEN resulting in oxidative stress. Oxidative stress leads to carcinogenesis by several mechanisms including DNA, lipid and protein damage, change in intracellular signaling pathways and even changes in gene expression (Balamurugan and Karthikeyan, 2012). Also, DEN is a potent hepatic carcinogen agent (Mahmoud and Abdul-Hamid, 2012). On the other hand, Carbon tetrachloride (CCl4) produced hepatocellular adenomas and carcinomas in rats, mice and hamsters in oral studies and in rats and mice by inhalation exposure (Manibusan, 2010). DEN and CCl4 is hydroxylated principally by the ethanol inducible CYP2El (cytochrome P450 system) in liver (Verna et al., 1996; Weber et al.,2003).

The obtained results revealed that, serum ALT and ALP activities, total bilirubin and AFP concentrations were significantly elevated and serum albumin level was significantly decreased in DEN/CCl4 – induced liver cancer in rats. DEN+CCl4 administration induce extensive necrosis and inflammatory infiltration, clusters of hepatocytes, bile duct proliferation and marked atypia (Abd EL- Hamid et al., 2013) or caused hepatic damage by those two toxicants which reflects instability of liver cell metabolism that led to leakage of theses enzymes to circulation. (Hassan et al., 2014). Similarity, Hemieda et al. (2016) showed that treatment with DENA/CCl4 elevate the values of serum ALT, AST and ALP activities and total bilirubin level and markedly decreased serum total proteins and albumin concentrations. Also, Hashem et al. (2016) confirmed that administration of DEN/CCl4 significantly increased liver weight, relative liver weight, AST, ALT and ALP while, body weight, total protein, albumin and A/G ratio were markedly decreased. Furthermore, Liver is the main site of DEN metabolism, the generation of ROS in the liver is recognized as an important contributor in DEN-induced damage (Faten et al.,2014). CCl4 is bio-transformed by cytochrome P450 (CYP) enzyme system in the endoplasmic reticulum to produce trichloromethyl free radicals ($CCl3^{\bullet}$). Then $CCl3^{\bullet}$ leads to elicitation of lipid peroxidation (LPO) and destruction of Ca^{2+} homeostasis, resulting in cell death (Talib, 2012). Moreover, Borai et al., (2017) displayed that a significant elevation in serum AST, ALT and ALP enzymes activities were observed in DEN-treated group as compared to control normal rats indicating that DEN could induce a damaged effect on liver tissues. The elevation in enzymes activities is due to the rupture in the architecture of cell membrane and the leakage and liberation of enzymes into the serum as a result of carcinogenesis, necrosis and toxicity. Also, ALP indicates alteration in biliary flow. Therefore, during carcinogenesis, these enzymes could be used as biomarkers for HCC response to therapy according to (Tork et al.,2015). Furthermore, Vandenberghe, (1996) reported that hypoalbuminemia may result from liver disorders, which are accompanied by a reduction in albumin synthesis. Albumin is a key component of serum proteins. Also, liver toxicity resulted in decrease serum albumin level (Adams et al.,2005). The results of the present study are in agreement with this finding and demonstrate the decreased functional ability

of CCl4-injected rat livers (Saravanan et al.,2006).

Meanwhile, treatment with moringa oliefera to DEN/CCL4 induced HCC rats caused a significant decrease in serum ALT and ALP activities, and total bilirubin and increase in albumin level. These results were agreement with Das et al. (2012) who viewed that moringa oliefera reduced the markers of liver injury evaluated by determining the serum activity of ALT, AST and ALP as these enzymes are released secondarily to liver cell damage. Also, Hamza, (2010) stated that the administration of moringa seed extract decreased the CCl4-induced elevation of serum aminotransferase activities and globulin level. Furthermore, Fakurazi et al. (2008) displayed that reduction of the activity of serum ALT, AST and ALP in groups pretreated with moringa olifera extract compared to those treated with acetaminophen alone. Moreover, Balaraba and Muhammad (2012) confirmed that oral pretreatment and co-administration of aqueous extract of moringa oleifera leaves significantly decreased serum ALT, AST and ALP activities, and total bilirubin concentration compared to the normal control. Moringa leaves have been discovered to contain vitamin E an antioxidant enzyme (Donovan, 2007). Moreover, Moringa contain alkaloids, cinnamates, anthocyanins, quercetin and kaempferol (Siddhuraju and Becker,2003) and proanthocyanidins (Goyal et al.,2007).

Administration of DEN/CCl4 significantly elevated the serum AFP level when compared to normal rats. Similar data was reported by Borai et al. (2017) and Salama et al., (2017) who recognized that AFP concentration was significantly higher in the DEN-treated group as compared to control normal one. It was reported that in DEN induced hepatocarcinogens caused elevation in AFP level (which is widely used as tumor marker for diagnosis of HCC) associated with the increment in tumor growth and progression (Murugan et al.,2015). Similarity, Hashem et al. (2016) reported that a significant increase in AFP level was shown in DEN+CCl4 group. The increase in serum AFP concentration has been used as a clinical marker in the diagnosis and monitoring of HCC (Tork et al.,2015). Moreover, Zaazaa et al., (2018) found that an increase in serum AFP level was observed in DEN-induced HCC in rats when compared to control group. Additionally, alpha fetoprotein (AFP) is the most commonly used tumor markers for the diagnosis of hepatocellular carcinoma (HCC) which is a unique immune-modulatory glycoprotein, and normally made by the immature hepatocytes in the fetus (oncofetal). Detection of AFP during monitoring of liver cancer treatment is well accepted in patients with increased AFP level before therapy. It has been recognized that exposure of animals with DEN increases the circulating AFP level (Sadik et al. et al., 2008).

Treatment with moringa to DEN/CCL4 induced HCC rats caused a significant decrease in serum AFP level when compared with DEN/CCL4 induced HCC group. The results approved by Sadek et al. (2017) who investigated that the decrease in such tumor markers AFP level after moringa oliefera administration, these decline may have been because of abatements in the rate of tumor generation, which phenolic and flavonoid substances are known to be specifically connected to antioxidant activities (Siddhuraju and Becker 2003). The phenolic and flavonoid mixes of moringa oleifera exert their antioxidant activities through scavenging free radicals, restraining enzymatic frameworks and metal chelation (Sadek, 2014).

The obtained qPCR results revealed significant elevation of TNF-α gene expression level in liver tissue of DEN/CCL4

induced HCC in rats as compared to the normal control group. Similarity, Kumar et al., (2016) found that TNF-α level significantly increased in DEN treated animals. Also, Habib et al. (2008) demonstrated that elevation of TNF-α gene expression was observed in hepatic tissue of rats with liver cancer induced by ethionine. Moreover, Song et al. (2013) showed that DEN-induced HCC increased TNF-α, such tumor necrosis factor alpha (TNF-α) is pro-inflammatory cytokines produced by macrophages and it plays an important role under tumor conditions (Lutsiak et al., 2005). It has been reported that TNF-α is an essential factor in tumor promotion (Reuter et al.,2011). Moreover, Hamid et al., (2017) stated that CCl4 elevated proinflammatory cytokines TNF-α, IL-6, COX-2 and NFκB. An increased level of TNF-α was also shown to correlate with hepatic inflammation, necrosis, and hepatic failure (Budhu and Wang, 2006).

Meanwhile, treatment with moringa to DEN/CCL4 induced HCC rats caused a significant downregulated in TNF-α gene expression and NF-κB gene expression. The obtained results are noted by Kooltheat et al., (2014) who investigated that moringa depress the expression of *RelA*, a gene important in NF-κB signaling inflammatory reaction. Also, Tan et al. (2015) displayed that moringa downregulated the expression of inflammatory mediators (NF-κB, iNOS, and COX-2) and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and PGE_2). Also, Tan et al. (2015) reported that moringa leaves are enriched with flavonoids such as kaempferol and quercetin (Karthivashan et al.,2013), and also reported the presence of high flavonol contents in Moringa oleifera flowers grown at South Africa (Pakade et al.,2013).

A significant down regulation of p53 gene expression level was observed in liver tissue of DEN/CCL4 induced HCC in rats as compared to the normal control group. Similarity, Loyden et al., (2017) demonstrated that a significant reduction in p53 gene expression was showed in DEN group administration. The p53 protein acts as a central response to cellular stress or DNA damage by inducing cell cycle arrest, apoptosis, senescence, and other tumor-suppressive actions (Bisteau et al.,2014). Moreover, Khan et al. (2016) stated that decrease in p53 expression induced with CCl4, which explained that CCl4 acts as a tumor promoter through increasing the intracellular concentration of ROS necrosis/regeneration and cell proliferation and/or may be due to mutation of p53 led to regarding p53 (Farazi et al., 2006).

Treatment with moringa to DEN/CCL4 induced HCC rats caused a significant upregulation in p53 gene expression level when compared with DEN/CCL4 induced HCC group. Similarity, Madi et al. (2016) found that moringa olifera extract treatment resulted in a significant increase in p53 gene expression. Also, Madi et al. (2016) explained that moringa olifera extract treatment resulted in a significant decrease in mitochondrial membrane potential, followed by an increase in ROS, caspase activation, proapoptotic proteins expression (p53, SMAC/Diablo, AIF), and PARP-1 cleavage.

The obtained results revealed significant dysregulation of Cyp2E1 gene expression level in liver tissue of DEN/CCL4 induced HCC in rats as compared to the normal control group. Similarity, Zhang et al., (2013) found that DEN treatment resulted in significant decreases of the activities of CYP2E1, CYP1A2. It has been well documented that DEN-induced hepatocarcinogenesis requires metabolic activation by some forms of CYP450s, especially CYP2E1. Furthermore, Khan et al., (2016) displayed that significant down

regulation in CYP 2E1 expression was observed in CCl4-induced hepatotoxicity. Who confirmed that reactive oxygen species (ROS) formed during the biotransformation process of CCl4 are more reactive and toxic than the parental compound. Biotransformation of CCl4 occurs in the endoplasmic reticulum and the isoenzyme implicated in this process is CYP2E1 (Knockaert et al., 2012). Treatment with moringa to DEN/CCL4 induced HCC rats caused a significant up regulation in Cyp2E1 gene expression when compared with DEN/CCL4 induced HCC group. Various medicinal phytochemical plants caused up regulation in Cyp2E1 gene expression. Resembling Khan et al., (2012) who showed that the active free radical/intermediate of CCl4 caused a reduction in CYP2E1, which was markedly restored by routine treatment.

5. Conclusion

The present findings exhibited that moringa olifera improve liver cells damage which caused marked decrease in serum liver marker enzymes (ALT and ALP), total bilirubin and AFP and significantly increased serum albumin concentration. Also, the results of molecular analysis showed significant up-regulation of TNF-α and significant down-regulation in p53 and CYP2E1 gene expression level in hepatic tissues. These findings suggest the potential ameliorating effect of moringa olifera as an additional powerful natural chemopreventive agent in treatment of hepatocarcinogenesis via initiation of tumor suppressor gene P53 and modifying the metabolic activation of detoxification Enzyme (cytochrome P450 2E1) and palpable anti-inflammatory effect.

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