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PROTECTIVE EFFECT OF AQUEOUS EXTRACT OF NEEM (*AZADIRACHTA INDICA*) LEAVES AGAINST NICOTINE INDUCED OXIDATIVE STRESS IN A MODEL OF HYPERLIPIDEMIA

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ABSTRACT : Nicotine is the most common ingredient in cigarette and is associated with the pathogenesis of lung carcinoma and raise the risk of promoting hepatocellular carcinoma and liver cirrhosis. Prohibition of lung cancer caused by nicotine and liver damage can be achieved by reducing the pathogenic effects of nicotine. The naturalistic nutrition system consists of a variety of components, such as *Azadirachta indica* that shows protective effects against various toxins. Five groups of the male albino rats were used: normal diet control group; high fat diet (HFD) fed group; nicotine-treated group (2.5 mg/kg/day); nicotine with neem extract-treated group (200 mg/kg/day) and nicotine with neem extract-treated group (400 mg/kg/day). Feeding HFD causes significant increase in the levels of total cholesterol, serum triglyceride, serum low density lipoprotein (LDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood glucose, serum protein and Malondialdehyde (MDA) and significant decrease in the level of high density lipoprotein (HDL), catalase (CAT) and superoxide dismutase (SOD). Significant increase in the level of total cholesterol, serum triglyceride, LDL-C, hepatic ALT, AST, ALP, blood glucose, urea, creatinine and (MDA) has been noticed. Whereas, HDL-C, CAT and SOD showed a significant decrease in the nicotine-treated group as compared with control group, while in nicotine+neem groups 200, 400 mg/kg showed significant reduction total cholesterol, serum triglyceride, LDL-C, liver enzymes, urea, creatinine and increment in the HDL-C and antioxidant enzyme in a dose-dependent manner. In conclusion, these findings suggest that aqueous extract of neem leaves extracts have a clear protective effect against nicotine.

Key words : Nicotine, Oxidative stress, *Azadirachta indica*, lipid profile, lipid peroxidation, hypertriglyceridemia.

INTRODUCTION

Tobacco is the most popular drug and is considered as one of the leading causes of illness and premature death in developed as well as developing countries. Epidemiological studies have shown that smoking may accelerate the risks of cardiovascular disorders, lung cancer, and pulmonary diseases. The harmful impacts of smoking have been studied extensively through direct administration studies of nicotine (Mahmoud, 2014).

Nicotine, a principal toxic constituent of cigarette smoking, is known to lead to oxidative stress by stimulating the generation of reactive oxygen species (Sener *et al*, 2007). Nicotine and metabolites increase lipid peroxidation and also affect the activities of antioxidant enzymes, thus, causing oxidative damage. Nicotine increases the results of oxidative stress by generating interactive chemical species called free radicals from several sources and/or from low enzymatic and non-enzymatic antioxidant defenses (Boshra, 2016).

In addition, nicotine is caused the exhaustion of antioxidant defense systems by lowering the level of catalase, phosphide oxide and glutathione peroxidase (Halima *et al*, 2010). Once metabolized, the nicotine is metabolized by the liver to several primary and secondary metabolites (Muthukumaran *et al*, 2008). The main metabolite is cotinine, the primary product of the oxidation pathway of the nicotine transformation that has been used as a marker of nicotine consumption (Yildiz, 2004). Given that the liver is the major site of nicotine metabolism, the liver was expected to be highly sensitive to oxidative stress associated with nicotine toxicity (Wang *et al*, 2005).

In the last years, efforts have been directed to control infectious diseases by the use of herbal medicines, which has fewer side effects and are environmentally safe. Neem (*Azadirachta indica*) is well known for its medicinal features. *A. indica* is a plant in the mahogany family Meliaceae. Neem is the most beneficial traditional medicine as a source of many therapeutic factors in the Indian culture and germinates widely in the tropical and

subtropical countries. Neem leaf extract has been shown to act as a growth promoter (Landy *et al*, 2011), improve performance and hematological parameters (Nayakabr *et al*, 2013) and immune response (Zahid *et al*, 2013).

The current study was designed to investigate the impacts of aqueous extract of neem leaves against nicotine-induced adverse effects on lipid profile, liver function, serum glucose, urea, creatinine, total protein, oxidative parameters, namely MDA and the activities of the antioxidant enzyme superoxide dismutase in serum and liver in rats fed with high fat diet. The importance of this study was explained mainly by the possible application of its results in preventive medicine.

MATERIALS AND METHODS

Chemicals : Nicotine ditartrate was purchased from solarbio life science Company, Tongzhou District, Beijing, China. Deoxycholic acid and Cholesterol has been bought from Hangzhou Sunlong Biotech Co., Ltd. Mainland, China. Neem leaf aqueous extract has been purchased from Marudhar Impex Company, Ahmedabad, Gujarat, India. All other chemicals and reagents used were of analytical grade.

Animals : Male albino Wistar rats, eight weeks of age and weighing about 200- 250 g were purchased by the college of veterinary medicine, Baghdad, Iraq. Animals were kept in regular cages at room temperature of 25 ± 3°C with a 12 h dark/light cycle. At the beginning of the experiment, standard rat pellet and tap water ad libitum have been given to the animals.

Preparation of high-fat diet : Deoxycholic acid (5 g) was mixed with 700 g of powder rat chow diet. In the same time, cholesterol (5 g) was dissolved in 300 g butter. This mix of cholesterol and butter was added into the powder mixture of deoxycholic acid and rat chow to get a soft homogenous cake (Belagali *et al*, 2013a).

Experimental design : Rats in the present study were randomly divided into five groups of eight animals each and treated for two months. Group I (control) rats were given standard chow and tap water. Group II Hyperlipidemic rats were given a high-fat diet and tap water. Group III rats were received nicotine 2.5 mg/kg of body weight orally by gavage and fed with a high-fat diet. Group IV and V rats were given orally nicotine combined with neem leaves aqueous extract 200 and 400 mg/kg respectively, for 60 days of treatment accompanied by nicotine. At the end of the treatments, all the animals were fasted overnight, anesthetized by intra-peritoneal injection with ketamine and xylazine. Blood samples were collected from the heart, sera were separated and used in biochemical assay.

Biochemical assay

Standard Kits for Lipid profile calorimetric, urea, creatinine, total protein, and liver function biomarkers were purchased from Roche Diagnostics Gmbh, Mannheim, Germany. Serum alanine aminotransferase (ALT) (Reitman and Frankel, 1957); Aspartate aminotransferase (AST) (Bablok *et al*, 1988); alkaline phosphatase (ALP) enzyme activities (Greiling and Gressner, 1995); Serum total cholesterol (TC) and triglyceride (TG) (Greiling and Gressner, 1995); High-density lipoprotein cholesterol (HDL-C) (Harris *et al*, 1996); Low-density lipoprotein cholesterol (LDL-C) (Glick *et al*, 1986) were evaluated by previously reported methods.

Antioxidant activity : Superoxide dismutase (SOD), Catalase(CAT) and lipid peroxidation marker malondialdehyde (MDA) have been assessed in serum and liver. The antioxidant enzyme kits were purchased from Solarbiocompany (China). The liver homogenate was prepared by homogenizing 0.1 gm of liver tissue with 1 ml of extraction solution the homogenate was centrifuged by cooler centrifuge at 8000 rpm, 4°C for 10 min in accordance with the manufacturer's instructions, then supernatants were kept at -80°C.

Statistical analysis

Statistical analyses were implemented by the ready statistical program SAS (2002-2010) (Statistical Analysis System). The values are represented by mean ± SE, Duncan's test is used to compare between the groups. P values < 0.01 were considered significant.

RESULTS

Lipid profile

Total cholesterol, serum triglyceride and serum LDL-C have been significantly increased by feeding on HFD as well in nicotine group, while HDL–C was significantly decreased in comparison with the regular diet group (control). Nicotine caused significant increase in cholesterol level in comparison with HFD group. The combination of nicotine +neem extract 200 and 400 mg/kg caused significant decrease in cholesterol level in comparison with nicotine treated group, and showed a significant reduction in serum triglyceride level compared to the nicotine groupand HFD group. There were no significant changes in the LDL-C levelin nicotine + neem extract 200 mg/kg, when compared with nicotine groupand HFD group, whereas significantly decreased in nicotine + neem 400 mg/kgas compared with HFD, nicotine, and nicotine + neem extract 200 mg/kg groups. The level of HDL-C showed a significant rise in both

nicotine + neem extract 200 and 400 mg/kg as compared to HFD group, but only the nicotine + neem 400 mg/kg group showed a significant elevation in HDL-C level (when compared with nicotine group (Table 1).

Hepatic function biomarkers : A significant increase has been found in serum ALT, AST and ALP in both HFD group and nicotine group as compared to the negative control group. While the serum ALT, AST and ALP levels in nicotine + neem 200 mg/kg and nicotine + neem 400 mg/kg groups were significantly reduced when compared to both HFD group and nicotine group (Table 2).

Blood Urea, Creatinine, Glucose and Total protein : Consumption of HFD showed a significant increase in the levels of blood glucose and non-significant changes in urea, creatinine and total protein levels as compared to the control group. Nicotine caused non-significant changes in blood urea, creatinine, glucose and total protein levels as compared to the HFD group. Rats treated by nicotine combined with concentrations of 200 and 400 mg/kg of neem extract showed a significant decrease in the level blood urea, creatinine and glucose in comparison with nicotine group. Nicotine combined with concentrations of 200 and 400 mg/kg of neem extract showed a significant decrease in the levels of total protein when compared to HFD group (Table 3).

Antioxidant activity : Animals fed on HFD showed a significant increase in serum MDA level as compared with the control group. Nicotine group significantly raised the MDA level in comparison with the control, but there was non-significant change when compared with HFD group. Whereas nicotine combined with 200 and 400 mg/kg of neem extract showed a significant decrease in MDA level in comparison with HFD group and nicotine group. The level of SOD and CAT in serum were significantly decreased in both HFD and nicotine groups compared with the control. While the SOD level significantly increased in nicotine + neem extract (in both 200 + 400 mg/kg) as compared with HFD and nicotine group. There was a no significant increase in the level of CAT in nicotine + neem extract (in both 200 + 400 mg/kg)in comparison with HFD and nicotine groups (Table 4).

Rats treated with HFD and nicotine showed a significant increase in MDA level in the liver homogenate in comparison with the control group. Nicotine + neem extract 400 mg/kg significantly lowered the MDA level as compared with the HFD group and nicotine group. Whereas a significant reduction has been found in MDA level innicotine + neem extract (in both 200 + 400 mg/kg) groups when compared with the nicotine group. The levels of SOD and CAT activity in liver homogenate was significantly diminished in both HFD group and nicotine group in comparison with the control group, while SOD

Table 1 : Effect of nicotine and aqueous extract of neem leaves extract on cholesterol, triglyceride, HDL-C, and LDL-C of control and hyperlipidemic male rats.

Parameters / Groups	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-C (mg/dl)	LDL-C(mg/dl)
Control	52.00±1.58^{C}	39.60±1.97^{C}	54.20±2.44^{A}	17.84±0.64^{B}
High Fat Diet (Hyperlipidemia)	71.40±1.60^{B}	73.20±2.04^{A}	43.40±2.16^{B}	32.24±0.88^{A}
Nicotine 2.5mg/kg	81.80±3.26^{A}	70.40±1.364^{A}	44.40±1.36^{B}	29.40±1.36^{A}
Nicotine + Neem extract 200mg/kg	66.80±2.45^{B}	52.40±1.91^{B}	52.00±1.67^{A}	29.00±1.23^{A}
Nicotine + Neem extract 400mg/kg	61.8 ±2. 71^{B}	45.20±2.18^{BC}	55.80±1.59^{A}	22.18±2.79^{B}

Different letters in the same column refer to significant value, while similar letters refer to non-significant value at the 1% level.

Table 2 : Mean± SE of effect of nicotine and aqueous extract of neem leaves on cholesterol, triglyceride, HDL-C, and LDL-C of control and hyperlipidemic male rats.

Parameters / Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Control	43.60±4.43^{B}	125.6±5.50^{B}	110.6±2.46^{E}
High Fat Diet (Hyperlipidemia)	63.60±3.31^{A}	165.6±8.90^{A}	260.2±3.51^{A}
Nicotine 2.5mg/kg	57.60±1.69^{A}	157.6±3.75^{A}	240.2±2.83 ^{B}
Nicotine + Neem extract 200mg/kg	44.00±1.82 ^{B}	113.0±6.33 ^{B}	165.4±2.73 ^{C}
Nicotine + Neem extract 400mg/kg	34.20±1.43 ^{B}	105.8±4.42 ^{B}	151.2±3.02 ^{D}

Different letters in the same column refer to significant values, while similar letters refer to non-significant values at the 1% level.

Table 3: Effects of different treatments on blood glucose; urea, creatinine and total protein in serum of the treated groups.

Parameters / Groups	Blood Urea (mg/dl)	Creatinine(IU/L)	Blood Glucose (mg/ dl)	Total Protein (g/dl)
Control	21.00±0.45^{B}	0.380±0.04^{B}	197.0±13.68^{B}	6.040±0.54^{AB}
High Fat Diet (Hyperlipidemia)	26.40±1.70^{AB}	0.480±0.06^{AB}	256.0±7.27^{A}	5.360±0.43^{B}
Nicotine 2.5mg/kg	31.00±2.21^{A}	0.6200±0.04^{A}	254.0±4.66^{A}	6.480±0.12^{AB}
Nicotine + Neem extract 200mg/kg	23.40±1.12^{B}	0.3800±0.04^{B}	169.8±8.82^{B}	7.020±0.16^{A}
Nicotine + Neem extract 400mg/kg	21.60±1.12^{B}	0.4200±0.04^{B}	185.2±2.75^{B}	6.560±0.29^{AB}

Different letters in the same column refer to significant values, while similar letters refer to non-significant values at the 1% level.

Table 4 : Mean± SE of effect of nicotine and aqueous extract of neem leaves on SOD, MDA and CAT in serumand liver of control and hyperlipidemic rats.

Groups / Parameters		Control	High Fat Diet (Hyperlipidemia)	Nicotine 2.5mg/kg	Nicotine + Neem extract (200 mg/ Kg)	Nicotine + Neem extract (400 mg/Kg)
Serum	MDA(nmol/ml)	2.66±0.07^{B}	4.08±0.20^{A}	4.10±0.19^{A}	2.72±0.29^{B}	2.34±0.36^{B}
	SOD(U/ml)	40.14±3.14^{A}	23.00±4.17^{B}	22.12±3.70^{B}	38.74±3.66^{A}	41.16±4.14^{A}
	CAT(U/ml)	54.12±2.61^{A}	43.28±2.57^{B}	42.71±2.41^{B}	43.68±2.57^{B}	45.90±1.94^{AB}
Liver	MDA(nmol/ml)	17.38±0.43^{C}	25.42±3.59^{AB}	30.46±0.7^{A}	18.68±0.79^{BC}	17.48±1.15^{C}
	SOD(U/ml)	34.64±3.31^{A}	17.42±2.41^{B}	16.84±1.9^{B}	42.16±3.65^{A}	41.70±4.53^{A}
	CAT(U/g)	21.12±0.97^{A}	9.12±0.33^{C}	9.32±0.4^{C}	11.18±0.67^{BC}	12.48±0.69^{B}

Different letters in the same row refer to significant values, while similar letters refer to non-significant values at the 1% level.

and CAT levels in nicotine group was non-significantly decreased in comparison with HFD group. There was a significant raise in SOD activity in liver homogenate in nicotine + neem extract (200 and 400 mg/kg) groups in comparison with HFD group and nicotine group. The levels of CAT in liver homogenate was non-significantly change in nicotine+neem extract in both concentrations 200 and 400mg/kg as compared with both HFD and nicotine groups (Table 4).

DISCUSSION

This study furnishes the first report about the useful application of *Azadirachta indica* leaf aqueous extract versus the worsened toxic impacts that are caused by nicotine in rats under high-fat diet status. The main finding of current study showed that administration of HFD and nicotine caused a significant increase in total cholesterol, serum triglyceride, serum LDL, hepatic enzymes, alkaline phosphatase with enhancing oxidative stress by increasing MDA and diminishing SOD and CAT.

Feeding of rats on HFD has shown a useful model of the supposed effects of dietary fat in humans (López *et al*, 2003). The current results of significant rise levels of plasma TC, triglyceride, LDL-C and a remarkable reduction in plasma HDL-C of the high-fat diet fed male rats, agree with the previous studies. Rat fed with a diet completed with 5 g cholesterol and 5g deoxycholic acid in butter for 60 days were presented as the experimental model (Kumar *et al*, 2008). The action mechanism of deoxycholic acid was to increase the absorption of cholesterol through the property of emulsification and a suppression associated with cholesterol 7á-hydroxylase, which leads to decreased cholesterol excretion (Moghadasian, 2002).

Hyperlipidemia indicates the appearance of abnormalities in lipid metabolism secondary to the manifestation and development of atherosclerosis (Belagali *et al*, 2013b). Hyperlipidemia and oxidative stress have been listed as the essential causative agents for the development of cardiovascular diseases (Madamanchi *et al*, 2005), such as atherosclerosis and its complication, acute myocardial infarction, hypertension and coronary heart disease (Shrivastava *et al*, 2013).

The increase of total cholesterol, serum triglyceride, serum LDL levels, and decrement of HDL-C level in the serum of rats under nicotine treatment clearly showed that nicotine affected the lipid profile severely (Table 1). This result is in consistent with the findings of Chattopadhyay *et al* (2010). Balakrishnan and Menon (2007a) have revealed that nicotine-stimulated catecholamine synthesis, which considers an essential regulator of lipolysis in adipose tissue, that in turn increased the level of cholesterol in the blood .In our study,

the increment of liver function enzymes in HFD group was revealed that high cholesterol diet significantly suppressed hepatic functions as expressed by an increase of serum AST, ALT and ALP as agreed with those reported by Suanarunsawat *et al* (2009). The augmentation in serum ALT, AST and ALP by nicotine stress suggested to causes damage to liver cells by elevating levels of liver enzymes (AST and ALT) in the blood, which indicate liver disease, as in agreement with the previous studies (Balakrishnan and Menon, 2007b; Omar *et al*, 2015; Chattopadhyay *et al*, 2018).

The marked elevation of blood glucose and non-significant changes in urea and creatinine caused by HFD have been agreed with the observations of Garcia *et al* (2018), which stated that mice fed on HFD, the blood glucose levels elevated along with an augment on insulin. Gujjala *et al* (2016)found that HFD caused notable elevation of plasma urea and creatinine by chronic feeding of HFD, which is an indication of defective kidney function in HFD rats. Abnormal renal function is linked with diabetic nephropathy. The pathophysiology includes glucose that attaches irreversibly to proteins in the kidney circulation to produce advanced glycosylation end products. These products can make complexes that contribute to renal damage by stimulation of fibrotic growth factors (Rao and Nammi, 2006). Reduced total protein content with enhanced activities of tissue ALT and AST observed in HFD rats indicates enhanced catabolism of proteins ultimately resulting in enhanced production of urea in these animals (Gujjala *et al*, 2016).

The raised serum levels of urea and creatinine are universal markers for detecting nephrotoxicity in conventional clinical pathology (Bonventre *et al*, 2010), which is consistent with the observed elevation in serum urea and creatinine in nicotine treated rats in this study.

Increased MDA levels and decreased SOD activities in the HFD group may already indicate an increased amount of oxidative stress in rats. Significant changes were observed in the antioxidant systems, the comparing between HFD and normal diet groups, indicating that ROS generation was enhanced by high-fat diet intake. Indeed, there is a rising awareness that the use of oxygen is essential for oxidative phosphorylation by the electron transfer system and variation in food constituents, as occur in high-fat diet intake, resulted in higher ROS production, thus inducing oxidative stress and lipid hydroperoxide formation. This result comes in agreement with the outcome of previous studies (Novelli *et al*, 2007; Rocha *et al*, 2009). We also observed an increased level of MDA and decreased SOD level in plasma and liver of nicotine-treated rats. This result is in accordance with the data of an earlier study (Ashakumary and Vijayammal, 1996). Minamisawa *et al* (1990) showed that the activity of rabbit erythrocyte antioxidant enzymes such as catalase and SOD had been suppressed by cigarette smoking. The SOD enzyme is reported to involve in the defense mechanisms versus the harmful effect of oxygen free radicals in the biological system (Kumari and Menon, 1987). The decreasing activity of this enzyme leads to accumulation of O_2^- and H_2O_2, which in turn forms the hydroxyl radical OH•, which can involve in several toxic reactions (Sacks *et al*, 1978). In this study, *Azadirachta indica* leaf aqueous extract in two concentrations accompanied by nicotine succeeded in alleviating the adverse effects induced by nicotine. This ameliorative effect was also depended on the concentration of *A. indica* extracts; the higher dose of the extract had the best-improved effects.

It had been found that neem extract reduces the concentrations of blood glucose, urea, total cholesterol and creatinine. The reduction in the creatinine and urea levels in the serum samples was due to the action of neem extract on blood vessels. This result agrees with that reported earlier by Khosla *et al* (2000). Chemical analysis disclosed that *A. indica* leaf extract contains the following six compounds: Quercetin-3-O-B-D-glucoside, Myricetin-3-Orutinoside, Quercetin-3-O-rutinoside, Kaempferol3-O-rutinoside, Kaempferol-3-O-B-D-glucoside and Quercetin-3-O-L-rhamnoside (Chattopadhyay, 1999). It is assumed that these compounds either in whole or in part may be responsible for the antihyperlipidemic activity. Thereby according to our findings it can be concluded that the levels of total serum cholesterol, triglycerides and LDL-cholesterol, which are actually had been raised in HFD group can be lowered with *A. indica* leaf extract.

Furthermore, its antihyperlipidemic effect could represent a protective mechanism against the progression of atherosclerosis. Biswas *et al* (2002) revealed that the elevated levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyltranspeptidase (GGT) are indicatives of liver damage, can be significantly reduced by administration of the neem leaf aqueous extract *Azadirachta indica* has an antioxidant property that inhibits the *in vitro* generation of O_2^- and OH• free radical in the enzymatic system.

As could be concluded from our data, neem extract significantly reduces total cholesterol, triglyceride, serum LDL, glucose, urea, creatinine, total protein levels, liver enzymes, lipid peroxidation marker (MDA), while increases HDL-C, antioxidant enzyme (SOD) and brought

them near the normal value. Nicotine intakes cancreate oxidative stress leading to hepatic injuries. *Azadirachta indica* leaf aqueous extract co-treatment led to the prevention of damage caused by nicotine. The protective impacts of neem leaf aqueous extract were due to the radical scavenging of their components. Thus, neem extract is useful in the prophylactic treatment of nicotine toxicity.

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