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Protective action of herbal melanin against carbon tetrachloride induced Hepatotoxicity

Protective action of herbal melanin against carbon tetrachloride

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Abstract* – The present study investigated the efficacy of a herbal melanin derived from Nigella sativa L. seed coats (NS melanin) on prevention of carbon tetrachloride (CCl_4) induced acute hepatic injury in Wistar rats. Hepatic damage due to CCl_4 intoxication was assessed by quantifying the markers of hepatic oxidative damage, aspartate transaminase (AST), alanine transaminase (ALT) and hepatic liver peroxide marker malondialdehyde (MDA). Treatment of the rats with CCl_4 increased the levels of AST, ALT in the blood and MDA in liver homogenate. Pre-treatment with herbal melanin solutions resulted in significant reduction in the levels of the hepatic enzymes and peroxides in a dose related manner. Our findings provide evidence to demonstrate that Nigella sativa L. melanin has a potent hepato-protective effect on CCl_4-induced liver injury in rats. It is proposed that the action of melanin is through its antioxidative and immunomodulatory activity via activation of Toll-like receptor 4 and subsequent release of IL-6. *(Abstract)

Keywords— **Protective action; herbal melanin; carbon tetrachloride; hepatotoxicity**

I. *Introduction*

Melanin is a pigment of plants and animals that occurs both externally and internally in tissues and organs (Riley, 1977). Melanin has been extracted from a few plants and recently a herbal melanin (NS melanin) has been extracted from the seed coats of Nigella sativa L. as well as from its cells and tissue cultures (Haseeb and Elhag, 2013, El-Obeid et al., 2006a). Using spectroscopic methods and solubility behavior NS melanin has been characterized to be similar to the well-known eu-melanin of the squid. Melanins are abundant in many human diets and a few authors have suggested a possible immuno-protective role of melanin as a food component in plants (Sava et al., 2003, El-Obeid et al., 2006a, EL-Obeid et al., 2006b, AL-Mufarrej et al., 2006) and as a beneficial immunomodulator (Kerestes et al., 2003). It has also been shown that melanins from various sources exhibit significant antioxidant activities e.g. fungal melanin (Shcherb et al., 2000, Goncalves et al., 2005, Wu et al., 2008, Kumar et al., 2011) skin melanin (Hoogduijn et al., 2003) and tea melanin (Sava et al., 2001, Huang et al., 2002).

Previous studies have shown that both plant and synthetic melanin can modulate cytokines production and enhance several immune parameters (EL-Obeid et al., 2006a, Pugh et al., 2005, Pugh et al., 2008, Mohagheghpour et al., 2000). Melanins isolated from Echinacea and Nigella sativa L seed coats were shown to activate monocytes and induce cytokine production via binding Toll-like receptor (TLR-2) and Toll-like receptor 4 (TLR-4), respectively (Pugh et al., 2005, EL-Obeid et al., 2006b). Toll-like receptors (TLRs) are a group of receptors that sense the invasion of pathogens by recognizing specific molecular patterns present in the microbes. They allow the host to sense the presence of these pathogens and initiate subsequent immune responses. To date, thirteen TLRs (named TLR1 to TLR13) as well as their ligands have been identified in humans (Oldenburg et al., 2012). Stimulation of TLRs by their corresponding T ligands, e.g. TLR-4 by lipopolysaccharides, leads to activation of innate immunity and production of cytokines via activation of the NF-□B signaling pathway (O'Neill and Bowie, 2007). Recently, Oberg et al. (Oberg et al., 2009) and El-Obeid et al. (EL-Obeid et al., 2006b) showed that Nigella sativa L. melanin also activates NF-kB signaling pathway and induces cytokines production.

IL-6 is a cytokine produced by different kinds of cells including macrophages, lymphocytes, fibroblasts, and many types of epithelial cells and it is involved in different pathological and host defenses processes (Akira et al., 1993). It can be produced via activation of the classic membrane bound receptor (mIL-6R) or via Toll like receptors (Gewiese et al., 2010, Oberg et al., 2009). The expression of TLRs (Wei et al. 2008, Peng et al. 2005, Paik et al. 2003) and the production of IL-6 (Panesar et al., 1999, Koleva et al., 2002, Toda et al., 2000) by the liver hepatocytes, Kupffer cells and hepatic stellate cells have been reported. IL-6 in the liver is known to act as a hepatoprotective factor due to its role in liver regeneration (Cressman et al., 1996) repair (Kovalovich et al., 2000) and protection from apoptosis (Kovalovich et al., 2001).

CCl4 is one of the chlorinated hydrocarbons that have a widely spread use as a chemical inducer of experimental liver injury (Kamalakkannan et al., 2005). Administration of CCl4 increases the concentration of serum hepatic enzymes and leads to fibrosis, cirrhosis and hepatic carcinoma (Perez, 1983). Immunologically, it has been shown that CCl4 activates the immune system and induces the secretion of inflammatory mediators such as cyclooxygenase-2 (COX-2) in response to oxidative stress (Uzma et al., 2011). Additionally, Aisaka et al.(Shi et al., 1998) has reported evidence of hepatocytes apoptosis in rat liver after the administration of carbon tetrachloride. However, the effect of Nigella sativa L. melanin, as an antioxidant and inducer of IL-6, on carbon tetrachloride (CCl4) induced liver injury is unknown. The aim of this study is to use CCl4 to induce liver intoxication in a rat

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MATERIALS AND METHODS

Preparation of Nigella sativa L. melanin:

Melanin has been extracted from the seed coats of the Nigella sativa L. herb via alkali solubilization and acid aggregation method. The melanin nature of the extract has been verified via ESR, infrared (IR), ultraviolet-visible (UV-VIS), XRD, Fluorescence, solubility studies and elemental analysis. The melanin working solution was prepared as described before [4]. Briefly, the powder of Nigella sativa L. seed coats was solubilized in an alkaline solution of NaOH (pH 12.5) for 3 hours. The melanin solution was then centrifuged, filtered and melanin was precipitated from it at pH 2 using conc. HCl. This alkali–acid treatment was repeated 2–3 times to ensure a higher purity of melanin. The precipitate was thoroughly washed with distilled water, filtered out and dried at 80^{o}C. The dry powder was later dissolved in 10% aqueous NaOH at 1 g/l. The solution was neutralized with 36% HCl and each ml of the solution was adjusted to contain 40 mg/ml.

Preparation of the Carbontetrachloride solution (CCl_4):

CCl_4 was dissolved in light liquid paraffin in the ratio of 1:1 (v/v).

Treatment of animals:

Male Wistar rats (250 g body weight) were randomly divided into 4 groups (N= 8 animals per group). Rats were maintained under defined conditions of temperature (22+/- 2^{o}C), humidity (55+/- 5%) and light-dark conditions (12/12 h light/dark). The conduct of the experiments was approved by the ethics committee of the Experimental Animal Care Centre , College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. All animals were fasted for 12 hours before start of the experiments. Group 1 was administered melanin intraperitoneally (i.p.) and CCl_4 solvents. Group 2 was injected (i.p.) with CCl_4 (2.5 ml/kg) plus melanin solvent. Group 3 and 4 were injected (i.p.) with melanin 100 and 200 mg/kg, respectively, plus CCl_4 solvent. All animals were then provided with food and water ad libitum. Twenty four hours later, the animals were anesthetized with ether and 5 ml of blood were obtained from each animal using cardiac puncture. The blood was placed in centrifuge tubes containing 3.6% trisodium citrate in water to give a ratio of 9:1(Blood: citrate) and centrifuged at 2000 rpm for 10 minutes. The separated plasma was collected and immediately used for the determination of the two liver enzymes Glutamic oxaloacetic acid transaminase (GOT) and Alanine transaminases (ALT).

Determination of MDA:

Following removal of blood, the animals were killed using ether overdose. The abdomens were opened, the livers removed, washed in ice-cold 0.9% NaCl, blotted dry and weighed. Each gram was homogenized in 9 ml of 1.15% KCL in water to obtain liver total homogenate as outlined by Ohkawa et al (Ohkawa et al., 1979). To determine the level of hepatic peroxides each 0.1 ml of liver homogenate was mixed with 0.2 ml of 8.1% of aqueous sodium dodecyl sulphate, 1.5 ml of 0.8% aqueous thiobarbituric acid. The volume was completed to 4 ml using distilled water and the mixture was heated at 95^{0}C for 60 minutes using a glass ball as a condenser. One ml of distilled water was added to the cool mixture together with 5ml of n-butunol/pyridine mixture (15:1 v/v), vigorously shaken, and centrifuged at 3000 rpm for 10 minutes. The upper red layer was aspirated and its absorbance was determined at 532 nm. Standard curve was constructed for calculating the lipid peroxides contents using 1, 1, 3, 3,-tetramethoxy propane (TMP) that was pre-heated at 95 ^{0}C for 60 minutes. Concentrations of TMP used ranged from 2 to 16 nmoles. The levels of lipid peroxides were expressed in nmoles malondialdehyde MDA per 100mg protein (Ohkawa et al., 1979). The protein content in the homogenates was determined using Randox diagnostic kit (Randox Laboratories Ltd., UK) based on Biuret method (Gornall et al., 1949).

Determination of plasma AST (GOT):

For the determination of AST in the plasma, Deneke et al. (Deneke et al., 1985) method was used; this involved the use of the Reflotron Instrument and the provided kits (Roche Diagnostics, Germany). Briefly, the principle depends upon the ability of the enzyme AST to act on ketoglutrate and alanine sulfinate to produce glutamate and pyruvate**.** The latter, in presence of molecular oxygen, phosphate ions and water is acted on by the enzyme pyruvate oxidase to produce CO_2, acetyl phosphate and H_2O_2. The released H_2O_2 in the presence of the peroxidase enzyme and the indicator 4-(4-dimethylaminophenyl)-5-19methyl-2-(3,5-di-t-butyl-4-hydroxy phenyl)-imidazole dihydrochloride gives a blue color whose intensity can be measured.

Determination of blood ALT:

In order to determine levels of ALT in the plasma, the method used by Deneke et al. (1986) was employed using a Reflotron Instrument and the provided kits (Roche Diagnostics - Germany). The method is based on the ability of ALT to convert ketoglutarate and alanine to glutamate and pyruvate. In presence of water, phosphate, molecular oxygen and the enzyme pyruvate oxidase pyruvate is converted to acetyl phosphate, CO2 and H2O2. The released H2O2 in the presence of peroxidase enzyme and the indicator 4-(4-dimethylaminophenyl)-5-methyl-2-(3,5-di-t-butyl-4-hydroxyphenyl)-imidazole ihydrochloride gives a blue color whose intensity can be measured.

Statistical analysis:

All values were reported as mean +/- s.e.m. with N = number of animals used Significant differences were calculated using ANOVA.

RESULTS AND DISCUSSION

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In this study, the effect of Nigella sativa L melanin was tested on CCl_4- induced hepatotoxicity. CCl_4 intoxication induced liver damage, as evident from higher serum glutamic oxaloacetic transaminase (AST), serum glutamic-pyruvic transaminase (ALT) and liver peroxide malondialdehyde (MDA) levels. Our results show that treatment of the rats with the solubilized CCl_4 in a dose of 2.5 ml/kg (i.p) resulted in 5-fold increase in the levels of the hepatic enzymes AST and ALT in the blood and about 3-fold increase in the level of hepatic lipid peroxides (Tables 1 and 2, respectively). Treatment of the animals with melanin in doses of 100 and 200 mg/kg concurrently with CCl_4 resulted in significant reductions in the levels of the hepatic enzymes and peroxides in a dose related manner. The reductions in the AST were 35 and 64.7%, those in ALT were 22.2 and 59.7%, and those in the peroxide were 41.1 and 70%, following treatment with the two doses of melanin, respectively. Our results clearly demonstrate that pretreatment of the rats with NS melanin has significantly decreased the serum markers of hepatic injury and lipid peroxidation during CCl_4-induced liver injury in rats. Previously Shim et al. (Shim et al., 2009) has attributed the observed increase in blood hepatic enzymes (AST and ALT), following CCl_4 administration, to the release of these enzymes into the circulation following the damage inflicted by the peroxides. CCl_4 causes direct liver injury as it leads to the generation of reactive oxygen species (ROS) and oxidative stress. ROS-mediated oxidative stress is known to attack DNA and cause DNA lesions, such as base modifications, single-strand breaks and double strand breaks and, in turn, leads to mutations, genomic instability and cell death (Yang et al., 2011). In the liver CCl_4 is transformed to trichloromethyl free radicals CCl3* and CL3COO* by the enzyme cytochrome P450. These active free radicals react with proteins, lipids and nucleic acids causing liver damage and the consequent increase in concentration of AST, ALT and MDA (Weber et al., 2003, Campo et al., 2004, Hsu et al., 2010, Morel and Barouki, 1999).

Halim and co-workers (Halim et al., 1997) reported that the antioxidant action plays an important role in protection against CCl_4-induced liver injury. Other investigators have shown that the depletion of the antioxidant glutathione (GSH) by CCl_4 can lead to liver damage by reactive oxygen species such as free radicals and peroxides (Pompella et al., 2003, Williams et al., 1990). The protective effects of various natural and synthetic antioxidants, such as vitamin C, vitamin E and silymarin against hepatotoxicity have been reported (Carbonari et al., 2006, Lin et al., 2008). Similarly, Shim et al.(Shim et al., 2009) reported that ginsan isolated from Pantax ginseng has effectively prevented liver injury and they attributed the hepatoprotective effect of ginsan to the induction of antioxidant protein contents, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) as well as the restoration of the hepatic glutathione (GSH) concentration.

Melanins extracted from different resources had displayed high antioxidant activity (Shcherb et al., 2000, Goncalves et al., 2005, Kumar et al., 2011). Using a melanin-like pigment (MLP), derived from tea, Sava et al.(Sava et al., 2003) showed that pretreatment of animals with MLP prevented the rise of serum alanine transferase activity and the decrease of glutathione level in the liver challenged by hydrazine. Similar results were reported by Hung et al.(Hung et al., 2003) who used melanin derived from tea (MDFT) to show that MDFT can prevent the formation 8-hydroxy-deoxyguanosine (8-OH-dG) DNA adducts.

Considering previous studies on the role of different melanins as antioxidants, we therefore suggest here a similar protective antioxidant role for melanins in protecting the liver from CCl_4 induced damage. The important roles of IL-6 and TNF-α as key regulators in liver regeneration and liver protection against damage has previously been studied. Liver regeneration is impaired in their absence (Gewiese-Rabsch et al., 2010, Michalopoulos and DeFrances et al., 1997, Taub, 2004). Camergo et al.(Camergo et al., 1997) had noted an inverse correlation between IL-6 and subsequent TNF-a levels and they suggested a possible hepatoprotective effect of IL-6 as a result of TNF-a down-regulation. The role of IL-6 in the protection of CCl_4 induced-liver damage was shown by Kovalovich et al.(Kovalovich et al., 2000) who reported that IL-6 deficient mice were more sensitive to CCl_4 induced liver injury than wild type. They showed that pretreatment with IL-6 before CCl_4, reduced acute CCl_4 injury and apoptosis and accelerated liver regeneration and they indicated the importance of IL-6 in reducing CCl_4-induced liver injury. Later on, they reported that IL-6 acted at several points in the apoptotic pathway to protect the liver against cell death by establishing and maintaining an adequate level of anti-apoptotic hepatic proteins (Kovalovich, 2001). Similarly, Bansal et al. (Bansal et al., 2005) reported that IL-6 had protected hepatocytes from CCl_4-mediated necrosis and apoptosis in mice. They attributed the protective role of IL-6 to the down-regulation of MMP-2 expression and suggested a role for MMP-2 in amplifying liver injury in vivo. The protective role of IL-6 to liver was further indicated by Fukumura et al.(Fukumura et al., 2007) who used IL-6 inducer ME3738 to prevent the development of fatty liver caused by chronic ethanol consumption.

In a former study, we have shown that NS melanin can induce IL-6 and TNF-□ production (EL-Obeid et al., 2006a). We have also shown that NS melanin can activate TLR4 and induce cytokine production via activation of NF-kB signaling pathway (EL-Obeid et al., 2006b, Oberg et al., 2009). It has been shown that TLR4 is expressed in liver hepatocytes, Kupffer cells and hepatic stellate cells (Wei et al., 2008, Peng et al., 2005,

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Paik et al., 2003). In accordance with results obtained here and those reported earlier we therefore assume that the intraperitoneal injection of NS melanin has activated TLR4 expression on the different liver cells and lead to IL-6 production. This assumption can clearly explain the results obtained previously by Su et al.(GL et al., 2004) who studied the role of toll-like receptor 4 in acute liver injury by carbon tetrachloride utilizing a naturally occurring TLR- 4 mutant and wild-type mice strains. Their results suggest that toll-like receptor 4 is important in the hepatic regenerative response to CCl_4 liver injury and they concluded that their data support the hypothesis that TLR4 mediates liver regeneration through its modulation of IL-6 production**.**

The combined effects of the antioxidant action of melanin and its immunomodulatory role are suggested here as efficient hepatoprotective mechanisms against CCl_4 injury. Application of NS melanin is proposed as a promising clinical alternative in hepatoprotective treatments. Further investigation on the detailed mechanism of TLR4 versus CCl_4-induced liver injury is underway.

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Authors' contributions

Authors have contributed equally to the manuscript.

Conflict of interest

Authors declare no conflict of interest.

TABLE 1. EFFECT OF MELANIN ON CCL_4-INDUCED ELEVATION OF LIVER ENZYMES.

Treatment	**SGOT(AST)** units/liter	**ALT** units/liter
Control+solvent[a]	43^{+}/-5	30^{+}/-4
CCl_4+solvent[b]	222^{+}/-6	148^{+}/-4
CCl_4+melanin1 (100 mg/kg)	44.3^{+}/-3.9*	115^{+}/-3.1*
CCl_4+melanin2 (200 mg/kg)	78.2^{+}/-6.3*	59^{+}/-6.6*

a: Solvent was neutralized aqueous NaOH and paraffin oil
b: Solvent was neutralized aqueous NaOH solution.
** $p < 0.01$, N=6.

TABLE 2. EFFECT OF MELANIN ON CCL4-INDUCED ELEVATION OF HEPATIC PEROXIDES IN THE RAT LIVER.

Control+solvent[a]	122^{+}/-5.6
CCl_4+solvent[b]	367^{+}/-11.8
CCl_4+melanin1 (100mg/kg)	216^{+}/-7.9**
CCl_4+melanin2 (200mg/kg)	110^{+}/-9.3**

a: Solvent was composed of two solutions: neutralized aqueous NaOH and paraffin oil.
b: Solvent was neutralized aqueous NaOH solution.
** $p < 0.01$, N=6.

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