Abstract. Background/Aim: The leaves of white mulberry (Morus alba L.) contain various polyphenolic compounds possessing strong antioxidant activity and anticancer potential. This study was designed to investigate the chemopreventive effect of aqueous extract of mulberry leaves against N-nitrosodiethylamine (NDEA)-induced liver carcinogenesis. Materials and Methods: Wistar rats were divided into four groups: control, mulberry extract-treated, NDEA-treated, and mulberry extract plus NDEA-treated. Mulberry extract was given in the diet (1,000 mg/kg b.w./day); NDEA was given in drinking water. Results: Mulberry extract reduced the incidence of hepatocellular carcinoma, dysplastic nodules, lipid peroxidation, protein carbonyl formation, and DNA degradation. Treatment with mulberry leaf extract along with NDEA challenge did not affect the activity of antioxidant enzymes and glutathione content. Conclusion: Treatment with mulberry leaf extract partially protected the livers of rats from NDEA-induced hepatocarcinogenesis and a direct antioxidant mechanism appears to contribute to its anticarcinogenic activity.

Since primary liver cancer is the third leading cause of cancer mortality worldwide, chemopreventive strategies aimed at reducing its risk or delaying its onset are highly desirable. The most common type of primary liver cancer is an inflammation-associated cancer developing from hepatocytes, hepatocellular carcinoma (HCC). Hepatocarcinogenesis progresses from chronic intrahepatic inflammation within the state of oxidative stress, which results in continuous cellular injury, necrosis and regeneration along with a genotoxic effect (1, 2). Despite a clear viral etiology, HCC is also mediated through exposure to hepatocarcinogens such as nitrosamines, which cause the generation of radicals or cellular mitochondrial dysfunction (2).

White mulberry (Morus alba) leaves contain abundant varieties of polyphenols, including chlorogenic acid, rutin, isouqueritin, quercetin, astragalin and kaempferol, which are considered strong antioxidants (3). Mulberry leaf extract has been reported to scavenge 1,1-diphenyl-2-picryl-hydrazyl radical and prevent lipid peroxidation in rabbit and human low-density lipoproteins (4). Its antioxidant effect has been also revealed in streptozotocin-induced diabetic rats (5). Anticancer properties of mulberry leaf polyphenols have been demonstrated in numerous assays with various types of human cancer cells of colon (6), liver (7), breast (6), and lung (8), and the underlying mechanisms including antioxidant, antiinflammatory, and proliferative, and cytotoxic activity have been shown. However, to the best of our knowledge, the anticancer effect in an animal model has not yet been investigated.

The present study was designed to evaluate the chemopreventive effect of mulberry leaf extract on N-nitrosodiethylamine (NDEA)-induced liver carcinogenesis in rats. This experimental model of hepatocarcinogenesis, due to histological and biochemical similarities between rodents and human hepatic lesions, is widely used in chemoprevention studies (9).

Materials and Methods

Materials. Dried and crushed M. alba L. (var. wielkolistna zolwinska) leaves were mixed with water (80-90°C) using a counter-flow process (1:10 w/w) and subjected to continuous extraction in a twin-screw extractor (IBPRS, Poznan, Poland). The resulting extract was then concentrated using a vacuum periodic spherical evaporator.
Experimental design. Thirty-two male Wistar rats weighing 250±15 g (12 weeks old) bred at the Department of Toxicology, Poznan University of Medical Sciences, were randomly assigned to four different treatment groups of eight animals each. Experimental groups were treated for 13 weeks as follows: Group 1: Control rats fed the standard diet; group 2: rats given M. alba extract at a concentration of 10 g/kg feed; group 3: rats receiving 0.01% NDEA in drinking water; group 4: rats treated with 10 g/kg feed M. alba extract along with the administration of 0.01% NDEA in drinking water. On the basis of the feed consumption and the nominal dietary concentration of M. alba extract, the calculated mean daily intake of test substance was about 1,000 mg/kg b.w./day. At the end of the 13-week treatment, the animals were fasted overnight and then were anesthetized by ketamine/xylazine (100/7.5 mg/kg b.w., i.p.). The animal experiment was approved by the Local Animal Ethics Committee (permission number 28/2011).

Sample collection. Livers were rapidly removed and processed for histopathological and biochemical examination. For histopathological analysis, a slice of the liver was fixed in 4% paraformaldehyde. Another portion of the liver was homogenized in buffered Tris/sucrose solution (pH 7.55) for differential centrifugation according to the standard procedure to obtain the cytosolic fraction. For glutathione, lipid peroxidation and protein carbonyl determinations, as well as comet assay, other liver tissue sections were homogenized separately. For each fraction, the protein concentration was determined using Folin-Ciocalteu reagent.

Histopathology. After formalin fixation, liver specimens were dehydrated and paraffin impregnated. Paraffin blocks were sectioned at 4 μm then stained with hematoxylin and eosin and then cells and tissues were examined using light microscopy.

Biochemical assays. The level of microsomal lipid peroxidation was assayed by measuring thiobarbituric acid reactive substances (TBARS) (11). Protein carbonyl concentration was assessed using a commercial enzyme-linked immunosorbent assay kit from BioCell Corporation, Auckland, New Zealand.

An alkaline comet assay was conducted according to the method of Hartmann et al. (12). After cell lysis, DNA unwinding, electrophoresis and neutralization, the slides were stained with ethidium bromide. Images of comets from a Zeiss fluorescence microscope (magnification ×400) were captured with a digital camera and scored into 5 groups according to the degree of DNA damage (13). Reduced glutathione content was assessed by its reaction with Ellman’s reagent (14).

Antioxidant enzyme activities were determined in the liver cytosol using spectrophotometric methods. Superoxide dismutase assay was based on its ability to inhibit spontaneous epinephrine oxidation (15). Catalase activity was assessed by the measurement of the rate of H2O2 decomposition (15). Glutathione peroxidase activity was determined according to Mohandas et al. (16), with hydrogen peroxide as a substrate; the rate of the NADPH disappearance at 340 nm was a measure of the enzyme activity. Glutathione reductase activity was assayed by measuring NADPH oxidation at 340 nm in the presence of oxidized glutathione (16).

Glutathione-S-transferase activity measurement was based on the determination of 1-chloro-2,4-dinitrobenzene conjugate formed in a glutathione (GSH)-coupled reaction (16). Paraoxonase-1 activity was measured with phenylacetate as a substrate; the rate of phenol generation was a measure of the enzyme activity (17). The data are expressed as the mean±SD. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test was used. A value of p<0.05 was considered statistically significant.

Results

Microscopic evaluation. Histopathological examination of liver sections from controls and rats treated with the mulberry extract revealed normal architecture (Figure 1A and B). Liver tissue of NDEA-administered animals showed the presence of hepatocellular carcinoma, dysplastic nodules and cirrhosis (Figure 1C). Administration of the M. alba extract resulted in a modest improvement of histological architecture of the liver tissue (Figure 1D) and reduced the incidence of hepatocellular carcinoma and dysplastic nodules (Table II).

Oxidative stress evaluation. The effects of M. alba leaf extract on the levels of oxidative modification of lipids, proteins and DNA are illustrated by the data in Table III. In rats exposed to NDEA, a significant increase in protein oxidation, of more than six-fold, as well as in lipid peroxidation and DNA degradation by 142% and 64%, respectively, was observed. Mulberry extract administration to NDEA-treated rats caused a significant decline in levels of protein carbonyls and TBARS, as well as a decrease in comet size by 75%, 66%, and 6%, respectively, as compared to those in rats treated with NDEA alone. In rats treated with NDEA alone, the hepatic GSH level was increased by 28% as compared to that in the controls (Table III). Administration

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\begin{array}{|c|c|}
\hline
\text{Compound} & \text{Content (g/100 g)} \\
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\text{Gallic acid} & 0.28 \\
\text{Picrotocatechic acid} & 0.08 \\
\text{p-Hydroxybenzoic acid} & 0.11 \\
\text{Vanillic acid} & 0.42 \\
\text{Chlorogenic acid} & 2.33 \\
\text{Caffeic acid} & 0.66 \\
\text{p-Coumaric acid} & 0.12 \\
\text{Ferulic acid} & 0.09 \\
\text{Sinapic acid} & 0.11 \\
\text{Total phenolic acids} & 4.27 \\
\text{Rutin} & 0.90 \\
\text{Quercetin 3-β-D-glucoside} & 0.47 \\
\text{Kaempferol 3-β-D-glucopyranoside} & 4.00 \\
\text{Total flavonoids} & 1.56 \\
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of mulberry leaf extract to NDEA-challenged rats slightly reduced the level of GSH; however, the difference was not statistically significant. The response of hepatic antioxidant enzymes to NDEA administration was diverse (Table IV). The superoxide dismutase and catalase activities remained unchanged. The activities of glutathione-S-transferase and glutathione reductase were increased by 49% and 82%, respectively, whereas the activity of glutathione peroxidase was inhibited by 68%, compared with those in the control rats. Nevertheless, supplementation with M. alba leaf extract did not have a significant effect on NDEA-induced changes in activity of these enzymes. NDEA also caused an increase in the hepatic paraoxonase-1 activity, by 38%, which remained unchanged after co-treatment with mulberry extract.

**Discussion**

Several reports have demonstrated that oxidative stress may be linked to carcinogenesis in the case of NDEA-induced HCC (9,18). Previously, we demonstrated antioxidant activity of M. alba leaf extract in assays based on the scavenging of stable 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2’-azinobis-3-ethylbenzo thiazoline-6-sulfonate (ABTS) radicals, as well as the ability to chelate iron (II) (10). In this study, we investigated whether the extract tested can provide some protection against NDEA-induced hepatocarcinogenesis in rats. Microscopic studies of livers stained with hematoxylin and eosin revealed extensive tissue damage as evidenced by cirrhosis, dilatation of bile ducts and ballooning degeneration,
as well as the presence of HCC and dysplastic nodules in rats treated with NDEA. These histological findings corroborate those from other studies (19, 20). Shen et al. reported that in rats exposed to NDEA, small pre-neoplastic focal lesions undergo malignant transformation, with the formation of neoplastic nodules and ultimately HCC (21). The mulberry extract inhibited the malignant transformation process since the incidence of HCC and dysplastic nodules was reduced in the livers of rats receiving the extract and NDEA. Additionally, the extract partially improved the hepatic cellular architecture. It has been reported by others that polyphenols such as: morin (22), apigenin (19), green tea polyphenols (21), barks (23), moderately improved the hepatocellular structure and architecture. It has been reported by others that polyphenols such as: morin (22), apigenin (19), green tea polyphenols (21), barks (23), moderately improved the hepatocellular structure and architecture. It has been reported by others that polyphenols such as: morin (22), apigenin (19), green tea polyphenols (21), barks (23), moderately improved the hepatocellular structure and architecture.
damaged by NDEA treatment. It is widely accepted that NDEA undergoes metabolic activation by cytochrome P450 2E1 (CYP2E1) and consequently oxygen radical by-products and the active ethyl radical metabolite (CH$_3$CH$_2$) are formed. NDEA–generated reactive oxygen species cause oxidative modifications of macromolecules which, together with DNA alkylation can initiate hepatocarcinogenesis (20). Since hepatic oxidative stress in rats following NDEA administration can promote hepatocarcinogenesis, the suppressive effects of mulberry leaf extract on the development of HCC may be due to its ability to scavenge free radicals or to the induction of hepatic antioxidant defense.

The current study confirmed that oxidative stress contributed to NDEA-induced hepatocarcinogenesis, as was evidenced by the increase in the content of protein carbonyls and lipid peroxidation products and damage to DNA. Aparicio-Bautista et al. demonstrated that NDEA-induced thiol protein oxidation was involved in the early stages of hepatocarcinogenesis (24). The increase of the hepatic level of protein carbonyls in NDEA-treated animals was also corroborated by Bishayee et al. (25). NDEA-induced products of oxidative damage to lipids can attack DNA, among other cellular targets, thereby promoting mutagenicity and carcinogenicity (20). The electrophilic by-product generated during NDEA biotransformation has been reported to alkylate DNA to form pro-mutagenic oxidized bases and to initiate DNA fragmentation (26). In the current experiment, NDEA increased DNA damage, as was evidenced by the increase in comet tail length. The administration of mulberry leaf extract markedly inhibited protein and lipid oxidation and ameliorated DNA damage, which can be interpreted as a suppression of NDEA-induced oxidative stress. These findings fully corroborate our earlier in vitro study in which we found a high capacity of the extract to reduce ABTS cation and DPPH: 41.36 μMol Trolox g$^{-1}$ dry weight and 137.60 μMol Trolox g$^{-1}$ dry weight, respectively (10). Similar results regarding protective effects of mulberry leaf extracts against oxidative damage of macromolecules have been reported in the liver of high fat diet-induced obese mice (27).

Glutathione serves numerous important functions including neutralization of free radicals, detoxification of toxic electrophiles and peroxides (or other oxidizing reagents), as well as mediation in S-glutathionylation (28). Marinho et al. reported that chronic exposure to NDEA caused the induction of gamma-glutamyl transferase and gamma-glutamylcysteine synthetase activity, resulting in an increase in the content of GSH in the liver (29). In the present study, the enhancement of hepatic GSH level in NDEA-treated rats was accompanied by an increase in activities of glutathione S-transferase and glutathione reductase, and these findings are consistent with those of Santos et al. (18). Furthermore, we noticed an increase in the activity of paraoxonase 1, an enzyme involved in protection against lipid peroxidation (21). Overall, these results can be interpreted as a cytoprotective response against electrophiles and oxidants (30). Treatment with the mulberry leaf extract along to NDEA challenge did not affect the activity of antioxidant enzymes or glutathione content.

Taken together, the histological and biochemical findings of the present study demonstrate that treatment with mulberry leaf extract partially suppressed NDEA-initiated hepatocarcinogenesis and the appearance of pre-neoplastic lesions. Since the extract tested has been reported to possess strong free radical-scavenging properties in vitro and we showed its ability to prevent oxidative damage of macromolecules in rats, it could be suggested that a direct antioxidant mechanism contributes to its anticarcinogenic activity. However, further research should be undertaken to examine whether the anti-inflammatory mechanism is also responsible for the chemopreventive effect. In conclusion, M. alba leaf extract appears to be an attractive candidate for chemoprevention of liver cancer.

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