Hepatoprotective Activity of Leaf Extract of *Laurus Nobilis* L. against CCL4 Induced Hepatotoxicity in Rats

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Abstract— **The present study was conducted to evaluate the hepatoprotective activity of ethanolic extract of Laurus nobilis against CCL4 induced liver toxic damage in rats. To evaluate the degree of hepatocyte damage in experimental modeling hepatitis the lipid profile and histochemical assay were determined. Introduction of CCl4 in animals was always carried out in fatal to 36 hours. A single injection of the L. nobilis extract simultaneously with CCl4 leads to 100% survivability. L. nobilis leaves extract acts on the liver as a potent scavenger of free radicals to prevent the toxic effects of CCl4 induced hepatotoxicity in rats. Under the influence of CCl4 microscopically normal structure of the liver are violated, the signs of diffuse liver disease with extensive necrosis of hepatocytes, perivascular and pericellular edema are determined. Extract has angioprotective action on the capillary bed of the rat liver, vascular protective effect and prevented the progression of necroinflammation, which can be explained by the presence of antioxidants and antimicrobials both of flavonoids origin, such as quercetin and morin as well as terpens and terpenoids.**

Keywords— **L. nobilis extract, CCl4, hystochemistry, hepatotoxicity, cholesterol, triglycerids.**

I. INTRODUCTION

The problem associated with the liver disorder is still a worldwide health problem. Drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects. Despite the large arsenal used hepatoprotectors, modern medicines are not always helpful to achieve an increase in reparative-regenerative activity, normalization of liver homeostasis and functional activity and prevent the development of fibrosis and cirrhosis. In view of severe undesirable side effects of synthetic agents, there is growing attention to the traditional herbal medicines that are claimed to possess hepatoprotective activity [1]. As one of the candidates for the role of hepatoprotector considered bay laurel, whose medicinal properties have been known for thousands of years and have been widely used in herbal medicine for the various diseases. The leaf of *L. nobilis* used as herbal medicine to treat rheumatism, earaches, indigestion, sprains, migraines, gastrointestinal diseases [2, 3]. Ethanolic extract of *L. nobilis* has antioxidant, antimicrobial and wound-healing effect [4-6]. *L. nobilis* is also used for preventing and treating of Type II-of diabetes, helps in reducing the level of total cholesterol and LDL-cholesterol simultaneously enhancing HDL-cholesterol [7]. There is a lack of scientific reports on the hepatoprotective role of ethanolic extract of *L. nobilis* leaves. One of the classic models of toxic liver damage is the intraperitoneal administration of CCl_4 [8]. The aim of the study was to evaluate the hepatoprotective activities of *L. nobilis* extracts in CCl_4 induced rats.

II. MATERIALS AND METHODS

The leaf of *L. nobilis*, were collected from the Zugdidi region of Georgia. The powdered plant materials were successively extracted with ethanol [5].

The GC-MS analysis of the ethanolic extracts using Hewlett Packard GCD (model 6890) and Hewlett Packard MS (model 5972) with mass selective detector (MSD) was performed by [5]. High-Performance Liquid Chromatography (HPLC) analisys of the samples was performed on an Agilent 1100 chromatograph (Agilent Technologies, USA) with UV - spectrophotometric detector and data processing software ChemStation [6].

Radical scavenging capacity (RSC) of tested extracts, quercetin and rutin solutions was assessed in a chemical model, i.e. DPPH system [5, 9].

The antibacterial activities of plant extracts were investigated by the disc-diffusion method on agar. As an indicator of antibacterial activity the inhibition zone formed after 24 – 48 hours of bacteria *E. coli* K-12 wild type strain development was taken [9]. The square ($pixel^2$) was calculated by special program “Image Repair” [10].

Male Wistar rats (170-200g) were used. Animals experiment was approved by the institutional Animal Ethics Committee. All the animals were divided into 3 groups. Animals of I^{st} group were served as a untreated control. Toxic liver injury was modeled by CCl_4 intraperitoneal injection in a dose of 0.2 ml per 100 g rats mass. III^{rd} group were injected intraperitoneally with 0,2ml on CCl_4 and ethanolic extract of laurel leaves. The animals of the I^{st} and II^{nd} groups were decapitated after 36 hours, while group III^{rd} - were decapitated after 36, 240 and 480 hours, respectively [11].

Cholesterol was determined using a reagents of "kit for cholesterol," the company "Biostart" by [12]. Ttriglyceride was determined by a colorimetric enzymatic method at 546 nm [13].

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Hemorrhagic status of experimental animals liver was assessed by histochemical method using a digital microscope (Intel Play QX3). Liver fresh frozen sections were prepared 150 micrometers thick, which were incubated in the mixture prepared according to method [14].

Experimental results were expressed as means ± SD. All measurements were conducted three to five times. The data were analyzed by a one-way analysis of variance (ANOVA) and the value of $p < 0.05$ was considered as significant.

III. Results and Discussion

Phytochemical composition: The phytochemical composition of *L. nobilis* leaves were also determined by GC-MS analysis (Figure 1, left) has shown that the major compound of extract is an oxygenated monoterpene1.8-cineole (eucalyptol). Other predominant compounds α-thujene, α-pinene, β-pinene, D-limonene, *o*-cymene are. There also are smaller amounts of acyclic monoterpene – myrcene, and cyclic α- and β-phellandrene, camphene, α -terpinene and a terpineol.

HPLC analyses of ethanolic extracts are presented in figure 1 (right). Among the selected phenolic compounds in L. *nobilis* extract were identified: rutin, quercetrin, quercetin, morin, apigenin, kaempferol. As envisioned from the data in the table two major compounds of extract quercetin and morin.

Antiradical activity: There are many reports suggesting that the DPPH scavenging activity of the extracts is directly related with the total phenolic content. The isolated compounds were screened for their *in vitro* antioxidant activity by a DPPH free radical scavenging assay. Results indicate that three isolated compounds contribute importantly to the antioxidant activity of *L.nobilis* leaves, providing a scientific basis for the use of this plant in traditional medicine. The most active compound was found to be quercetin along with two flavonoid compounds, rutin and morin, showed significant scavenging activity with IC_{50} of 10±0.02, 30±0.05 and 54±0.20 μg/ml, respectively.

Antibacterial activity: Flavonoids are efficient antioxidants and plays an important role in augmenting the wound -healing process. Flavonoids and flavons also bind with adhesins that have been termed as the most important determinant of pathogenicity [15, 16]. Terpens and terpenoids are also known to promote the wound -healing process mainly due to their astringent and antimicrobial property[17, 18]. Significant antimicrobial activity of bay extract against *E. coli* strains virulence was observed connected with the adhesion activity [19]. The extracts of *L. nobilis* promote healing activities on induced by *E. coli* purulence wound mode [4]. The results of the antibacterial activity (via the disc diffusion method) showed that square of inhibition under influence of ethanolic extract, quercetin, rutin and morin were 2910 ± 197; 9500 ± 870, 3200 ± 299 and 142000 ± 970 pixel^2, respectively. Since antibacterial activity of morin was more than 15 times greater than the other two components, it can be assumed that the main input in antibacterial activity of extract belongs to morin. The antibacterial activity of bay can be also due compounds non-flavonoid origin, such as *p*-cymene – a precursor of carvacrol [20]. The high contents of eugenol, methyl eugenol and fatty acid methyl esters together with other active components [21] could contribute to its overall antibacterial activity [22].

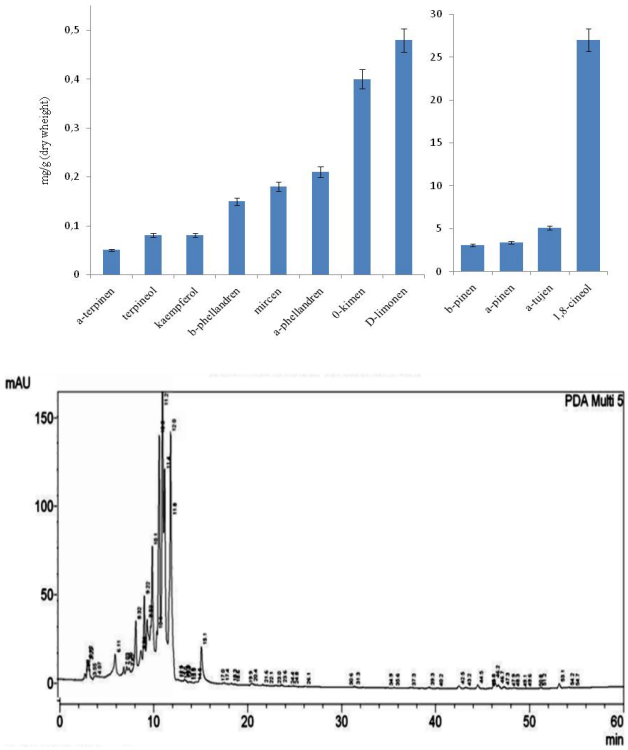

Fig. 1 GC-MS (left) and HPLC (right) analysis of L.nobilis leaf ethanolic extracts

Cholesterol and triglyceride determination: Triglyceride and cholesterol levels in blood are one of the most important determinants of lipid metabolism [23,24]. At liver pathogenesis hepatocytes esterification processes are also inhibited and cholesterol synthesis so accumulated acetic acid is the substrate for its formation. In large quantities acetic acid manifests a cytotoxic effect. Cholesterol is required for the synthesis of the lipid membrane structures, biliary acids [23].

CCL4 intoxicated groups showed increased levels of cholesterol in plasma and tissues attributed to increased β–hydroxyl methyl glutaryl CoA [HMG CoA] reductase activity which is the rate limiting step in cholesterol biosynthesis

[25].The administration of *L. nobilis* ethanolic extract carried out to lowering of total cholesterol and triacylgycerides levels (Table 1).

Table 1 Effect of ethanolic extract of *L. nobilis*on lipid profile in blood plasma of control and CCl_4 administered rats

	control	CCl_4	CCl_4 + *L. nobilis* extract		
			36 h	240 h	480 h
total cholesterol (mM/L)	1,4±0,10	1,8±0,20	1,1±0,05	0,5±0,03	0,7±0,01
triglycerides (mM/L)	1,6±0,06	1,7±0,10	1,2±0,04	0,7±0,03	0,6±0,04

Histochemical assay: Representative images of hematoxylin staining to visualize architecture of the liver and hepatocytes on sections of the liver (40-50 microns) are in the Fig.2.Under the influence of CCl_4 microscopically normal structure of the liver are violated, the signs of diffuse liver disease with extensive necrosis of hepatocytes, perivascular and pericellular edema are determined. It is also noted that diffuse lesion of hepatocytes with more severe changes in pericapillary areas. Often noticeable discomplexation of hepatic beams. Hepatocyte necrosis developed , in the foci of necrosis observed inflammatory infiltration, clearly visible in the central part of the hepatic beams. The single administration of CCl_4 and extract showed improvement in the preservation of hepatocytes both in the 10 and 20 day (Fig. 2). In primary liver tissue the processes of neutralization and reduction in the density of necrotic foci was observed. Much of the tissue did not differ from the normal liver tissue, signs of edema and tissue infiltration are absent. Thus extract has angioprotective action on the capillary bed of the rat liver, vascular protective effect and prevented the progression of inflammation and necrosis, which can be explained by the presence of antioxidants and antimicrobials flavonoid origin, such as quercetin and morin as well as terpens and terpenoids.

However, by the 20th day, there are marked signs lipidation of liver, or steatohepatitis. Steatosis initially causes oxidative stress could be the introduction of CCl_4, which leads to increased formation of free fatty acids. Its can lead to toxicity by stimulation of cellular stresses such as oxidative stress, apoptosis and gut-derived lipopolysaccharide (LPS) trigger an inflammatory response and progressive liver damage [26]. Extract of *L. nobilis* prevented ROS that are generated during free fatty acid metabolism and are responsible for initiating necroinflammation. This lead to increase of triglycerides level and accumulation in liver as a protective mechanism of liver detoxication.

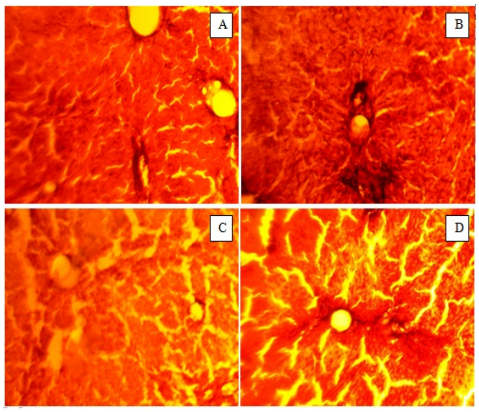

Fig. 2 Photomicrographs of rat liver similar structures sections of the: A - control,- B - CCl4, C -CCl4+ L. nobilis extract (240h), D - (480h) All photomicrographs were taken at a magnification of 200x. The scale bar represents 50μM.

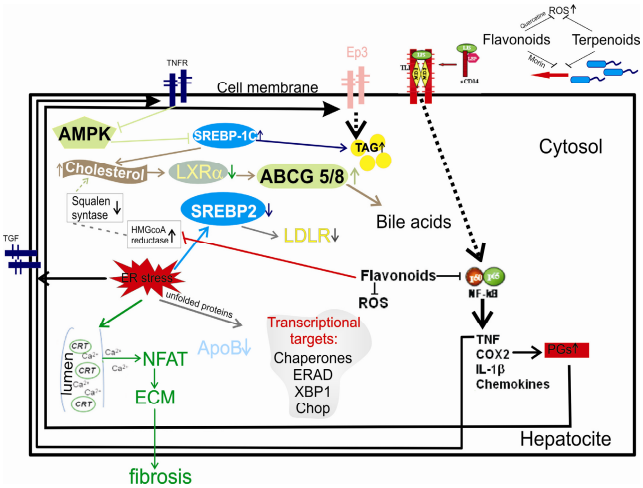

Fig. 3 Hypotetic scheme of multiple pathophysiological ways of hepatosteatosis induced by CCl4 and L.nobilis extract compounds action

A central role in cell metabolism by controlling synthesis of fatty acids, triglycerides, and cholesterol play transcription factors called sterol regulatory element-binding proteins (SREBPs) [23], that integrate multiple cell signals to control lipogenesis. SREBP-1a activates fatty acid and cholesterol synthesis, SREBP-1c fatty acid synthesis and SREBP-2 cholesterol synthesis and uptake. Under normal conditions, these pathways interact with each other and act as regulators of hepatic cholesterol levels and activate genes involved in the synthesis of cholesterol and free fatty acids carried out on the basis of feedback system regulation [27]. However during liver pathogenesis these systems are highly

disorganized and in the context of liver damage the regulatory loop of SREBP is disturbed, regardless of the intracellular levels of cholesterol and/or fatty acids [28] and despite excess cholesterol accumulation in hepatocytes, de novo cholesterol synthesis remained greatly enhanced even though SREBP-2 expression while it uptake suppressed because of markedly downregulated expression of LDL receptor [27].

Toxic lipids such as free fatty acids, diacylglyceride, phospholipids and free cholesterol activate several cellular stress pathways [29. One epicenter for these stress responses is the endoplasmic reticulum (ER) stress as an emerging factor in fibrotic disease, although precise mechanisms are not clear [30]. Free cholesterol accumulation triggers ER stress by altering the critical free cholesterol-to-phospholipid ratio of the ER membrane. An important element of the fibrogenesis is endotoxin exposure, leading to activation of cytokines, including transforming growth factor (TGFβ), under which influence the transformation of fat accumulating cells in fibroblasts.

ER stress and fibrous tissues activated chaperone calreticulin (CRT), which regulates extracellular matrix by stimulation of TGFβ, with controlling the level of intracellular Ca^2 and NFAT signaling. This leads to the activation of calcineurin, which activates NFAT nuclear translocation and promotes that can directly or indirectly stimulate transcription of extracellular matrix proteins (ECM), such as type I collagen involved in the formation of fibrosis. Prolonged / or strong ER stress can also lead to misfolding of many secretory proteins in particular, ApoB100 due to failure ER chaperones, such as GRP 78, GRP94, protein-72 ER, calreticulin and calnexin.

Gut microbiota play many roles in the hepatosteatosis, hepatocellular carcinoma, cardiac functions, vascular atherosclerosis, diabetes and other conditions. Endotoxin or LPS produced by gut microbiota could be delivered to the liver via the portal vein through the intestinal barrier. LPS – compound of outer membrane of Gram- bacteria binds with LBP and transports to liver, where interact with $CD1_4$ and TLRs with following activation of nF-κB and its transcriptional targets –proinflammatory TNF, IL-6,- 1β, NOS, COX1/2, ROS, free fatty acids, etc. COX-1 is constitutively expressed in many tissues and is responsible for a variety of physiological functions, and does not change with the liver lipidation. COX-2 in the normal liver is not detected but induced *de novo* in fatty liver [31]. This increases prostaglandin synthesis that interacting with receptors Ep3, help to increase the synthesis of triglyceride and their accumulation in the liver by preventing lipolysis.

We have proposed a scheme (Fig.3), taking into account the multiple parallel pathophysiological ways of hepatic steatosis including microbiota LPS, oxidative stress and pro-inflammatory cytokines. A single administration of the extract L.nobilis led to a reduction of necroinflammation of the liver tissue possibly due lowering of ROS level, and the binding of endotoxin to the touch receptors, reducing induction of HMG-CoA reductase and can be one of the possible causes of lowering blood cholesterol levels.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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