Antioxidant and hepatoprotective effects of extract of ginkgo biloba in rats of non-alcoholic steatohepatitis

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ABSTRACT

الأهداف: تقييم الآثار العلاجية لخلاصة أعشاب جينكو بيلوبا (Ginko biloba) وطريقة عملها عند علاج التهاب الكبد الدهني اللاكحولي والذي تم تحفيزه عن طريق الحمية في الجرذان الذين تضمنتهم الدراسة.

الطريقة: أُجريت هذه التجربة في المعمل بقسم الجهاز الهضمي في مستشفى رينمين التابع لجامعة ووهان، ووهان، الصين وذلك خلال الفترة من يونيو 2009م إلى ديسمبر 2009م. ولقد تم تحفيز مرض التهاب الكبد الدهني اللاكحولي عن طريق تقديم حمية عالية الدهون للجرذان الذين تضمنتهم هذه الدراسة. شملت هذه الدراسة 60 جرذياً تم تقسيمهم عشوائياً إلى 3 مجموعات وهي كالتالي: المجموعة العادية وقُدم لها حمية عادية مع شرب الماء، المجموعة الغير مُعالجة وقُدم لها حمية عالية الدهون مع ما يعادل 10 مللتر/كجرام من المياه المقطرة بواسطة التغذية القسرية مرة واحدة يومياً لمدة 12 أسبوعاً، والمجموعة المُعالجة وقُدم لها حمية عالية الدهون مع ما يُعادل 6 مليجرام/كجرام من خلاصة أعشاب جينكو بيلوبا بواسطة التغذية القسرية وذلك مرة واحدة يومياً ولمدة 12 أسبوعاً. وبعد مرور 12 أسبوعاً تم قتل كل الجرذان ومن ثم تم تحليل الكبد وذلك بتحديد مستويات كلاً من: المؤشرات الحيوية الكيميائية في مصل الدم، ومؤشرات تليف الكبد، وإنزيم فوق الأكسيد ديسموتاز (superoxide dismutase)، وألدهايد مالونديادهايد (malondialdehyde)، والتغيرات الأخرى المسببة للمرض، بالإضافة إلى قياس معدلات العامل النووي (KB NF–κB p65 protein).

النتائج: أشارت الدراسة إلى حدوث انخفاض شديد في مستويات كلاً من: ناقلة أمين الألانين، وناقلة أمين الأسبارتات، ومؤشرات تليف الكبد، ونسبة تليف الكبد، ونسبة تركيز المعامل النووي (KB NF–κB p65 protein) في المجموعة المُعالجة من الجرذان بالمقارنة مع المجموعة الغير معالجة. وزاد نشاط إنزيم فوق الأكسيد ديسموتاز، غير أن مستويات ألدهايد مالونديادهايد قد انخفضت في المجموعة المُعالجة بالمقارنة مع المجموعة الغير مُعالجة.

خاتمة: تعد خلاصة أعشاب جينكو بيلوبا من مضادات الأكسدة التي من شأنها وقاية الكبد من الأمراض، كما أن بإمكانها منع إصابة الكبد بالتليف وذلك في الجرذان المُصابين بالتهاب الكبد الدهني اللاكحولي.

Objectives: To evaluate the therapeutic effect and mechanism of extract of ginkgo biloba (EGB) in treatment of diet-induced non-alcoholic steatohepatitis (NASH) in rats.

Methods: The experiment was conducted in the Laboratory, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan, China from June 2009 to December 2009. In this study, the rat model of NASH was produced by feeding high-fat diet. Sixty rats were randomly divided into 3 groups: Normal group: normal diet, drinking water; Model group: high-fat diet, single-distilled water 10 ml/kg gavage once a day for 12 weeks; and Treated group: high-fat diet, EGB 6 mg/kg gavage once a day for 12 weeks. At the end of 12 weeks, all rats were killed. The serum biochemical, fibrosis markers, superoxide dismutase (SOD), malondialdehyde (MDA), the pathological changes, and the expression levels of nuclear factor KB (NF-κB)p65 protein in the liver were observed.

Results: The contents of serum alanine transaminase aspartate aminotransferase, fibrosis markers, and pathological grading of liver fibrosis and the staining intensity of NF-κBp65 protein in the liver of rats in treated group were significantly lower than those in the model group. Activities of superoxide dismutase were elevated, but levels of malondialdehyde were decreased in the treated group as compared with the model group.

Conclusion: Extract of ginkgo biloba has antioxidant and hepatoprotective effects and can inhibit liver fibrosis in rat of NAHS.

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Nonalcoholic fatty liver disease (NAFLD) refers to a wide spectrum of liver disease ranging from simple fatty liver, to nonalcoholic steatohepatitis (NASH) and cirrhosis.[1] At its most severe, nonalcoholic fatty liver disease can progress to liver failure. Unfortunately, the incidence of NASH increases and its pathogenesis is not completely understood. It is reported that oxygen free radicals is involved in the development of NAFLD.[2] Extract of Ginkgo Biloba (EGB) is believed to reduce the free radical induced lipid peroxidation.[3] Studies indicate that EGB on antioxidant activity can contribute to the prevention and treatment of diseases associated with oxidative stress.[4] These features of the EGB are considered to provide many beneficial effects against chronic liver damage and liver fibrosis.[5] Therefore, the purpose of our study was to investigate the protective effect of EGB on chronic liver damage and liver fibrosis caused by diet-induced NASH in rats.

Methods. The experiment was conducted in the Laboratory, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan, China from June 2009 to December 2009. Animal care was in compliance with the guidelines of the Medical Faculty of Wuhan University Research Council Criteria. All experimental protocols were approved by the Wuhan University's Ethics Committee.

Animals and group. In this study, the rat model of NASH was produced by feeding high-fat diet (2% cholesterol, 10% animal oil, and 88% standard rat chow). Sixty clean grade male Wistar rats weighing 180 ± 20 g were randomly divided into 3 groups. Normal group (n=20): normal diet, drinking water; model group (n=20): high-fat diet, single-distilled water 10 ml/kg gavage once a day for 12 weeks; and treated group (n=20 rats): high-fat diet while giving the EGB 6 mg/kg fed once a day for 12 weeks. During the experimental study, animals were raised in the (20±2) clean animal lab, free food and water. After 12 weeks, all rats were sacrificed under anesthesia. Whole blood was centrifuged and the supernatant was stored at -20^{0}C refrigerator. The liver was taken for the same position with 10% neutral formalin fixed, paraffin embedded, sliced. Remaining liver was homogenized in ice-cold phosphate buffered saline (PBS). The homogenates were centrifuged at 4000 rpm for 10 min at 4^{0}C, and the supernatants were stored at -20^{0}C refrigerator.

Histological and biochemical analysis. The sections of the liver were stained with hematoxylin-eosin (HE). Histology was evaluated by a single pathologist who was blinded to the laboratory data. Fibrosis was staged as 0-4 based on Scheuer's scoring system (0=no fibrosis; 1=expansion of portal tract without linkage; 2=portal expansion with portal to portal linkage; 3=extensive portal to portal and focal portal to central linkage; 4=cirrhosis).[6] Automatic biochemical analyzer was employed for determination of serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Serum hyaluronic acid (HA), laminin (LN), procollagen type III (PCIII) and collagen type IV (IV) were detected by radioimmunoassay.[7] The oxidative stress parameters included the activity of superoxide dismutase (SOD) and the levels of malondialdehyde (MDA). The activities of SOD and MDA levels were measured by chemical colorimetry method.[8] The expression of nuclear factor KB (NF-κB)p65 in liver tissue was determined by immunohistochemistry.[9] Immunohistochemical stain was performed on paraffin-embedded sections. The tissue sections were incubated at room temperature with primary antibody of monoclonal mouse antibody against activated NF-κBp65 after antigen retrieval included citrate buffer in an 800-W microwave oven. Antibody binding of the primary antibodies was revealed with anti-mouse immunoglobulin (Ig) G peroxidase kit. It was detected with the Vectastain ABC by horseradish peroxidase-conjugated streptavidin and visualization using diaminobenzidine tetrahydrochloride. Negative control studies were performed in which PBS was used instead of primary antibody. Two independent pathologists who were blinded to the study scored the quantitative analysis of immunohistochemical staining. The number of activated NF-κBp65 was counted (×400) and expressed as percent positive cells.

Statistical analysis. The measurement data were presented as means±SD and compared with analysis of variance. Level count data were analyzed using the rank sum test. A p value less than 0.05 was considered statistically significant. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows version 11 (SPSS Inc., Chicago, Illinois, USA).

Results. ***Hematoxylin-eosin staining of liver tissue.*** In the normal and treated groups, liver sections exhibited normal global histological features (**Figure 1**). Liver sections from rats in the model group revealed that more than one-third of hepatocytes contained macrovesicles of fat, which corresponded to steatosis (**Figure 1**). In addition, necrotic hepatocytes, and collagen deposition were exclusively found in samples from the model group (**Figure 1**). Pathological grading of liver fibrosis in liver tissue was showed in **Table 1**.

Serum alanine aminotransferase and aspartate aminotransferase levels. No significant changes were observed in serum ALT (p=0.001) and AST (p=0.007) between normal group and treated group (**Table 2**). The contents of serum ALT and AST in the normal and

treated group were significantly lower than those in the model group.

Serum fibrotic parameters. Serum HA, LN, PC III, and CIV were significantly higher in the model group than in the normal and treated groups (p<0.05). There were no significant changes in serum HA, LN, PCIII, and CIV between normal group and model group (**Table 3**).

Oxidative stress parameters in liver. Superoxide dismutase activity in the liver was significantly lower in the model group than in the normal and treated groups (p=0.004). Malondialdehyde levels in the liver were significantly higher in the model group than in the normal and treated groups (p=0.005) (**Table 4**).

Immunohistochemistry. Nuclear factor KB p65 positive cell nucleus was stained brown. There were few expressions of NF-kBp65 protein in hepatic sinusoid in the normal rat liver. However, in the model group, positive cells were distributed mainly at the fibro-septa band, the area of necrosis, and inflammatory cell infiltration in hepatic lobules (**Figure 2**). The ratio of positive cells to the total cells in the model group was significantly higher than in the normal and treated groups (p<0.01) (**Figure 3**).

Discussion. Although the pathogenesis of NASH has not yet been fully elucidated, presently "2 hits theory" has been proposed to explain the pathogenesis of NASH.[10] Hepatic fat accumulation in the liver represents the "first hit", which induces a "second hit" including insulin resistance, oxidative stress, and increased endotoxin-mediated cytokine release. These

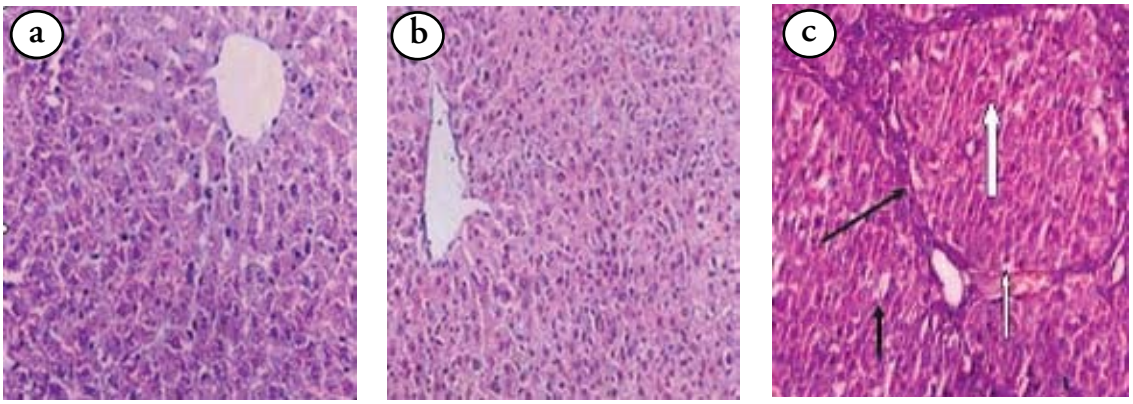

Figure 1 - Liver histopathology of the rat. **a)** The normal group showed normal histology. **b)** The treated group showed normal histology. c) The model group revealed steatosis (long white arrow), hepatocyte ballooning (short white arrow), necrosis (short black arrow), and fibrosis (long black arrow) (hematoxylin-eosin staining, magnification ×100).

Table 1 - Analysis of pathological changes in rats of each group.

Groups	N	Grading of liver fibrosis				
		0	I	II	III	IV
Normal*	20	20	0	0	0	0
Model	20	0	8	9	3	0
Treated*	20	17	2	1	0	0

*p<0.05 versus model group.

Table 2 - Analysis of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in rats of each group (means±SM).

Groups	N	ALT(U/L)	AST(U/L)
Normal*	20	63.6±9.3*	118.7±31.7*
Model	20	259.6±44.5	295.1±47.8
Treated*	20	103.5±31.1*	163.4±36.5*

*p<0.05 versus model group.

Table 3 - Analysis of serum heart rate (HA), laminin (LN), PCIII and CIV in rats of each group (means±SM).

Groups	n	HA (μg/L)	LN (μg/L)	PC III (μg/L)	CIV (μg/L)
Normal	20	75.2±17.6*	122.7±17.2*	110.5±16.4*	33.6±9.5*
Model	20	318.1±51.1	189.7±21.4	297.2±32.5	135.3±22.1
Treated	20	107.3±28.1*	125.9±21.9*	156.3±21.4*	40.9±14.7*

*p<0.05 versus model group.

Table 4 - Analysis of oxidative stress parameters in liver in rats of each group (means±SM).

Groups	n	MDA (μmol/g)	SOD (μmol/L)
Normal	20	0.91±0.31*	27.46±2.91*
Model	20	3.49±0.6	7.88±1.21
Treated	20	1.03±0.34*	24.78±2.84*

*p<0.05 versus model group. MDA - malondialdehyde, SOD - superoxide dismutase

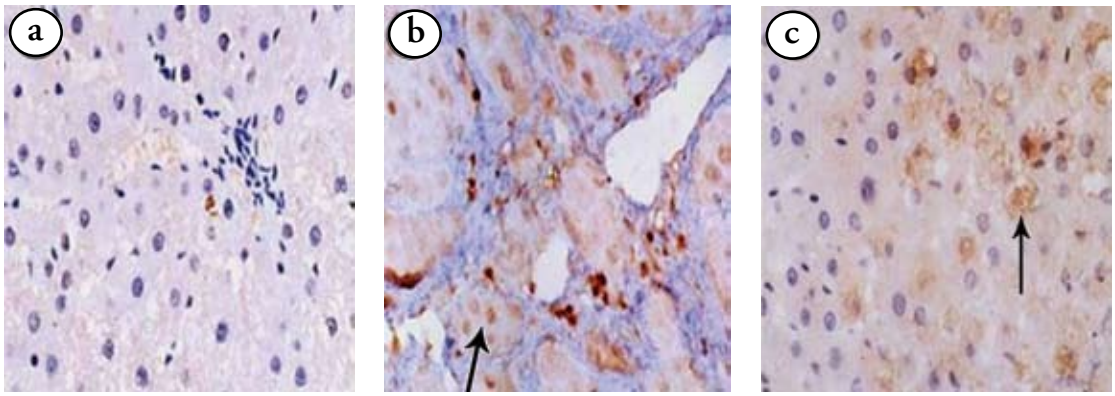

Figure 2 - Immunohistochemical staining for nuclear factor KB (NF-κB)p65 in the rat liver. **a**) There were few expressions of NF-kBp65 protein in hepatic sinusoid in the normal group. **b**) There were partial expressions of NF-kBp65 protein in hepatic lobules (arrow) in the treated group. **c**) Positive NF-kBp65 cells were distributed mainly at the fibro-septa band, the area of necrosis and inflammatory cell infiltration in hepatic lobules (arrow) in the model group (magnification ×400).

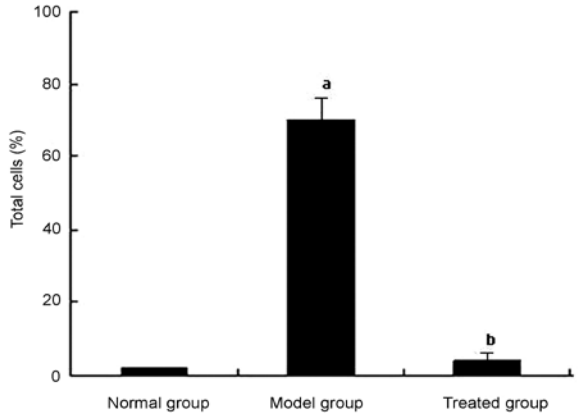

Figure 3 - Comparison of the expression of nuclear factor KB (NF-κB)p65 using immunohistochemical analysis. [a]$p<0.05$ versus normal group; [b]$p<0.05$ versus model group.

modifications enhance lipid peroxidation, hepatocyte injury, and release of toxic byproducts, resulting in inflammation, necrosis, and fibrosis.[11] Nonalcoholic steatohepatitis may develop into liver fibrosis and cirrhosis.[12] Therefore, it is very important for patients with NASH to slow down the progression of fibrosis. The liver in metabolism and other physiological activities can produce a small amount of free radicals, because there is a normal antioxidant system usually does not cause liver cell injury. However, in some pathological conditions, such as liver ischemia and hypoxia, free radicals increased, hepatic antioxidant system was restrained, resulted in lipid peroxidation.[13] Oxide content increased and antioxidant levels decreased in liver tissue can lead to free radicals and MDA increased, leading to cell dysfunction and even death, and lipid peroxidation may lead to lobular inflammatory cell infiltration and fibrosis.[14] Decreased lipid peroxidation can slow down the progression of fibrosis.

Ginkgo originated in China and is one of the oldest living tree species in the world. Now its leaves are more widely used medicinal plants. Ginkgo leaves contain 2 types of chemical composition (flavonoids and terpenoids) are effective antioxidants.[15] Its purported biological effects include: scavenging free radical; lowering oxidative stress; anti-inflammation; anti-tumor activities; and anti-aging.[16] Thus, EGB has a wide range of antioxidant for the treatment of many diseases. Recent studies have showed that oxidative stress is concerned with the formation of fibrosis in most chronic diseases.[17] Prevalence of NASH is increasing in global populations.[18] At present, no effective drugs can be used to treat NASH. Under the circumstances, we consider that antioxidant therapy could protect hepatic cells from lipid peroxidation, and prevent or relieve chronic liver damage processes. Therefore, we designed the experiment to investigate the effect of EGB on NASH in rats. This study showed that the levels of hepatic enzyme indicator MDA and the serum levels of ALT and AST were decreased while the activity of SOD in the treated group increased when compared with model group. Malondialdehyde is the direct product of lipid peroxidation.[19] Therefore, the extent of lipid peroxidation can be assessed by measuring MDA levels in tissues. Superoxide dismutase is the major enzyme for scavenging oxygen free radicals, and its activity can reflect its functional status.[20] These findings are fully confirmed that EGB can inhibit lipid peroxidation in plasma and liver tissue and has protective effect on NASH. The grading of histological hepatic fibrosis in the treated group showed significantly lower than that in the model group ($p<0.05$). Compared with model

group, fibrosis markers in treated group, such as serum HA, LN, PCIII and CIV levels reduced significantly (p<0.05). Co-detections of serum HA, LN, PCIII and CIV can be useful for diagnosing in hepatic fibrosis.[21] The results show that EGB could effectively prevent the progression of liver fibrosis. Nuclear factor KB plays a key role in immune and inflammatory responses.[22] Because NF-κB controls many genes involved in inflammation, it is not surprising that NF-κB is found to be chronically active in many inflammatory diseases.[23] To investigate the antioxidant mechanism of EGB, we examined the expression of NF-κBp65 using immunohistochemical methods, as it may be involved in the regulation of oxidative stress. This study indicated that the expression of NF-κBp65 significantly decreased in T group. Inhibition of oxidative stress by EGB in liver cirrhosis may be due to suppression of the NF-κBp65 signaling pathway. Therefore, a potential approach to block oxidative stress in liver cirrhosis is to target towards NF-κB signaling pathway.

In conclusion, our study clearly demonstrates that EGB is effective against oxidative liver damage and liver fibrosis caused by diet-induced NASH. The protective effect may be due to its radical scavenging action or antioxidant activity. Thus, we propose that this novel therapeutic approach for NASH be tested in future clinical studies.

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References

1. Mensink RP, Plat J, Schrauwen P. Diet and nonalcoholic fatty liver disease. ***Curr Opin Lipidol*** 2008; 19: 25-29.
2. Patrick-Melin AJ, Kalinski MI, Kelly KR, Haus JM, Solomon TP, Kirwan JP. Nonalcoholic fatty liver disease: biochemical and therapeutic considerations. ***Ukr Biokhim Zh*** 2009; 81: 16-25.
3. Sener G, Kabasakal L, Atasoy BM, Erzik C, Velioglu-Ogunc A, Cetinel S, et al. Ginkgo biloba extract protects against ionizing radiation-induced oxidative organ damage in rats. ***Pharmacol Res*** 2006; 53: 241-252.
4. Bridi R, Crossetti FP, Steffen VM, Henriques AT. The antioxidant activity of standardized extract of Ginkgo biloba (EGb 761) in rats. ***Phytother Res*** 2001; 15: 449-451.
5. Yao P, Li K, Song F, Zhou S, Sun X, Zhang X, et al. Heme oxygenase-1 upregulated by Ginkgo biloba extract: potential protection against ethanol-induced oxidative liver damage. ***Food Chem Toxicol*** 2007; 45: 1333-1342.
6. Goodman ZD. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. ***J Hepatol*** 2007; 47: 598-607.
7. Xu GG, Luo CY, Wu SM, Wang CL. The relationship between staging of hepatic fibrosis and the levels of serum biochemistry. ***Hepatobiliary Pancreat Dis Int*** 2002; 1: 246-248.
8. Yao J, Wang JY, Liu L, Li YX, Xun AY, Zeng WS, et al. Anti-oxidant effects of resveratrol on mice with DSS-induced ulcerative colitis. ***Arch Med Res*** 2010; 41: 288-294.
9. Gao L, Wang F, Wang B, Gong B, Zhang J, Zhang X, et al. Cilostazol protects diabetic rats from vascular inflammation via nuclear factor-kappa B-dependent down-regulation of vascular cell adhesion molecule-1 expression. ***J Pharmacol Exp Ther*** 2006; 318: 53-58.
10. Younossi ZM. Review article: current management of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. ***Aliment Pharmacol Ther*** 2008; 28: 2-12.
11. Wu D, Cederbaum AI. Oxidative stress and alcoholic liver disease. ***Semin Liver Dis*** 2009; 29: 141-154.
12. Schuppan D, Gorrell MD, Klein T, Mark M, Afdhal NH. The challenge of developing novel pharmacological therapies for non-alcoholic steatohepatitis. ***Liver Int*** 2010; 30: 795-808.
13. Fouad AA, El-Rehany MA, Maghraby HK. The hepatoprotective effect of carnosine against ischemia/reperfusion liver injury in rats. ***Eur J Pharmacol*** 2007; 572: 61-68.
14. Albano E, Mottaran E, Vidali M, Reale E, Saksena S, Occhino G, et al. Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis. ***Gut*** 2005; 54: 987-993.
15. He SX, Luo JY, Wang YP, Wang YL, Fu H, Xu JL, et al. Effects of extract from Ginkgo biloba on carbon tetrachloride-induced liver injury in rats. ***World J Gastroenterol*** 2006; 12: 3924-3928.
16. Chan PC, Xia Q, Fu PP. Ginkgo biloba leave extract: biological, medicinal, and toxicological effects. ***J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*** 2007; 25: 211-244.
17. Cottone S, Lorito MC, Riccobene R, Nardi E, Mulè G, Buscemi S, et al. Oxidative stress, inflammation and cardiovascular disease in chronic renal failure. ***J Nephrol*** 2008; 21: 175-179.
18. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. ***Dig Dis*** 2010; 28: 155-161.
19. Saleh NK, Saleh HA. Protective effects of vitamin E against myocardial ischemia/reperfusion injury in rats. ***Saudi Med J*** 2010; 31: 142-147.
20. Ceylan BG, Yilmaz F, Eroglu F, Yavuz L, Gulmen S, Vural H. Oxidant and antioxidant activities of different anesthetic techniques. Propofol versus desflurane. ***Saudi Med J*** 2009; 30: 371-376.
21. Wang L, Cheng D, Wang H, Di L, Zhou X, Xu T, et al. The hepatoprotective and antifibrotic effects of Saururus chinensis against carbon tetrachloride induced hepatic fibrosis in rats. ***J Ethnopharmacol*** 2009; 126: 487-491.
22. Wullaert A. Role of NF-kappaB activation in intestinal immune homeostasis. ***Int J Med Microbiol*** 2010; 300: 49-56.
23. Marcu KB, Otero M, Olivotto E, Borzi RM, Goldring MB. NF-kappaB signaling: multiple angles to target OA. ***Curr Drug Targets*** 2010; 11: 599-613.