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Synergistic, Attenuative and Modulatory activity in Dimethylnitrosamine (DMN)-induced fibrotic rats treated with *Vernonia amygdalina* and *Annona muricata* leaves

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ABSTRACT

This study assessed the effect of *Vernonia amygdalina* and *Annona muricata* leaves on oxidative stress and extracellular matrix induced by dimethylnitrosamine (DMN) - induced liver fibrosis. Control group received physiological saline; Second group received 100mg/kg each of *Vernonia amygdalina* and *Annona muricata* ethanol leaf extract without DMN orally for 14 consecutive days. Third group received 10mg DMN/kg (intraperitoneally) thrice weekly for two weeks, in addition to 100mg/kg each of *Vernonia amygdalina* and *Annona muricata* administered for 14 days consecutively while the last group received only 10mg DMN/kg. At the end of administration, the rats were sacrificed, blood samples collected and sera analysed for hyaluronic acid (HA), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), alanine transaminase (ALT), aspartate transaminase (AST) and total protein (TP) while collagen, catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) levels were assayed in liver tissue homogenate. DMN administration caused significant increases in ALP, AST, HA, ALT, LDH and in liver total collagen content and MDA ($p < 0.05$) and decreased TP, CAT, GSH and SOD. However 100mg/kg *Vernonia amygdalina* and *Annona muricata* leaves attenuated and modulated DMN-induced changes thus giving credence that the leaves possess fibro-suppressant, anti-oxidant and anti-hepatotoxic properties against DMN-induced hepatic fibrosis.

Key words: *Annona muricata*, Dimethylnitrosamine, Fibrosis, Liver, *Vernonia amygdalina*

INTRODUCTION

Liver fibrosis is a pre-pathological cirrhotic state, signalized by collagen and extracellular matrix (ECM) proteins accumulation (produced in damaged liver by stellate cells) in the space of Disse (Shimizu *et al.*, 1999; Poli, 2000; Rojkind *et al.*, 1979). Dimethylnitrosamine (DMN), a liver mutagen and carcinogen has been suggested and proven to be a standard model for studying hepatic fibrosis and cirrhosis associated pathophysiological and biochemical changes (George *et al.*, 2001; Usunobun *et al.*, 2015a, b).

Vernonia amygdalina, a leafy vegetable is used for food and disease treatment including liver and kidney problems, diabetes, malaria, infertility, and gastrointestinal problems (Usunobun *et al.*, 2015a; Farombi and Owoeye, 2011). Nutritional and phytochemical analysis of *Vernonia amygdalina* leaves have revealed levels of crude protein, crude fiber, carbohydrate (Usunobun and Okolie, 2016), phytochemicals (flavonoids, tannins, saponins etc), mineral components (Mg, Ca, K, Na, Mn, Zn, Fe etc) and antioxidant vitamins (A, C, E and riboflavin) (Atangwho *et al.*, 2009; Igile *et al.*, 1994; Usunobun and Okolie, 2015a, b).

Annona muricata, commonly called soursop have its various parts such as bark, roots and leaves being used for treatment of diseases and ailments including diabetes, liver disease, arthritis, bacterial and fungal problems (Adeyemi *et al.*, 2008; Takahashi *et al.*, 2006). Phytochemical screening of *Annona muricata* leaves have shown that it contain alkaloids, flavonoids etc (Usunobun and Okolie, 2015a) as well as mineral elements such as Na, Ca, K, Fe, Zn, Mg etc. Previous studies on *Annona muricata* showed *Annonaceous acetogenins* found in the stem, seed and leaves to be cytotoxic against cancer cells (Chang, 2001; Liaw *et al.*, 2002). This study is aimed at the possible fibro-suppressant and anti-oxidative attenuation of *Vernonia amygdalina* and *Annona muricata* leaves in combine dose on liver fibrosis induced by dimethylnitrosamine (DMN) in wistar rats.

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MATERIALS AND METHODS

Plant materials and Extraction

Annona muricata leaves were collected from the tree in Upper Sakponba while *Vernonia amygdalina* leaves were purchased from Oba market in Benin City, Nigeria and thereafter identified by a Botanist in the Department of Basic Sciences, Benson Idahosa University. Washed and air-dried (at room temperature (24°C), *Annona muricata* and *Vernonia amygdalina* leaves were crushed into fine powder and then weighed. Ethanol extracts of the powdered leaves were prepared by weighing and soaking 100g each of the powdered leaves in 1000ml of absolute ethanol for 48 hours (at room temperature). At the end of the 48 hours, the extracts were filtered using Whatmann filter paper and cotton wool. The *Annona muricata* and *Vernonia amygdalina* ethanol extracts were thereafter concentrated using a rotary evaporator (set at 60°C and 40◦C respectively) to 1/10th its original volume followed by freeze drying. The dried crude extract stored at 4ºC was weighed, dissolved (in distilled water) and used for animal experimental study.

Experimental animals

Wistar rats (28 males, 190 - 205g) obtained from Animal Unit facility of the University of Ibadan, Ibadan, Nigeria were housed in wooden cages and used for the study. The rats acclimatized for one week, had free access to drinking water and commercial pelleted rat chow (Bendel Feed & Flour Mill Ltd., Ewu, Nigeria) *ad libitum*. The DMN was synthesized at the Department of Biochemistry, University of Ibadan according to the method of Vogel (1971). The *Annona muricata* and *Vernonia amygdalina* leaf extracts weighed and dissolved in distilled water were administered orally using gavage to rats in second and third groups at a dosage of 100mg/kg for 14 consecutive days (two weeks). Rats in third group were in addition given DMN (via intraperitoneal injection) at a dose of 10mg/kg (dissolved in 0.15M NaCl) in the first three days of each week (for two weeks). Rats in forth group were given same amount of DMN as in third group but without leaf extract, while rats in first group (control group) were given normal saline. The dosage of 100 mg/kg was used based on our previous non-toxic nature of *Vernonia amygdalina* and *Annona muricata* leaves (Usunobun *et al.*, 2015b; Usunobun *et al.*, 2016) By day 15, all the rats were sacrificed (cardiac puncture), blood collected and allowed to stand for 15 minutes before centrifuging at 4000 rpm for 20 min. The serum samples were stored at -20º C until analyzed. Serum was used for determination of hyaluronic acid (HA) level, total protein (TP), aspartate aminotransaminase (AST), Lactate dehydrohenase (LDH), alanine aminotransaminase (ALT), and alkaline phosphatise (ALP). The livers were immediately excised, washed in cold normal saline and blotted individually. A 10% liver tissue homogenate were prepared using normal saline and resulting clear supernatant used for determination of total collagen (ECM component), GSH, CAT, SOD and MDA.

Biochemical assays

Serum LDH, AST, ALT, ALP and TP were determined spectrophotometrically using RANDOX Kit. Serum HA level was determined using ELISA assay kit as described by Chichibu *et al* (1989). Liver total collagen was quantified using QuickZymeR kits. MDA was determined in a colorimetric reaction with thiobarbituric acid (Ohkawa *et al.*, 1979). SOD was determined according to the method of Misra and Fridovich (1972). The catalase assay was by measuring the first order rate constant colorimetrically for H_2O_2 decomposition (Cohen *et al.*, 1970). GSH was determined according to Ellman (1959).

Statistical analysis

Data obtained at the end of this study were expressed as mean ± SD using Statistical Package for Social Sciences (SPSS). A probability level of less than 5% ($p < 0.05$) was considered significant.

RESULTS

The result of liver function enzymes as shown in figure 1 showed that dimethylnitrosamine caused massive elevation in ALT, AST, ALP and LDH after two weeks of administration indicating liver toxicity while simultaneous treatment with a combine dose of 100mg/kg each of *Vernonia amygdalina* and *Annona muricata* significantly reduced the spillage of the enzymes into the blood stream. No significant difference was observed in control rats and rats given *Vernonia amygdalina* and *Annona muricata* alone. The result of the effect of *Vernonia amygdalina* and *Annona muricata* on oxidative stress parameters (figure 2) showed that while DMN caused a significant increase in malondialdehyde (MDA) after two weeks of administration, *Vernonia amygdalina* and *Annona muricata* when combined in usage significantly reduced the MDA level indicating protection. Also, figure 2 results showed significant decline in CAT, GSH and SOD in DMN administered rats whereas *Vernonia amygdalina* and *Annona*

muricata when administered simultaneously significantly increased GSH, SOD and catalase. The result of *Vernonia amygdalina* and *Annona muricata* on ECM proteins in fibrotic rats induced by DMN as shown in figure 3 showed a several folds increase in Hyaluronic acids (HA) and total collagen in rats administered DMN alone whereas in rats simultaneously treated with *Vernonia amygdalina* and *Annona muricata*, HA and total collagen where greatly and significantly reduced. Also figure 3 showed that *Vernonia amygdalina* and *Annona muricata* significantly increased serum total protein (TP) when compared to rats given DMN alone. There was however no significant difference in control rats and rats given *Vernonia amygdalina* and *Annona muricata* alone.

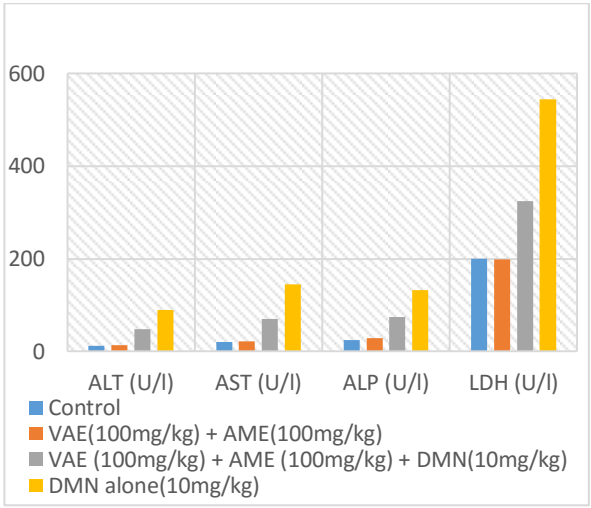

Figure 1: Effect of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) on serum liver function enzymes in Dimethylnitrosamnie (DMN)-induced hepatic fibrotic rats.

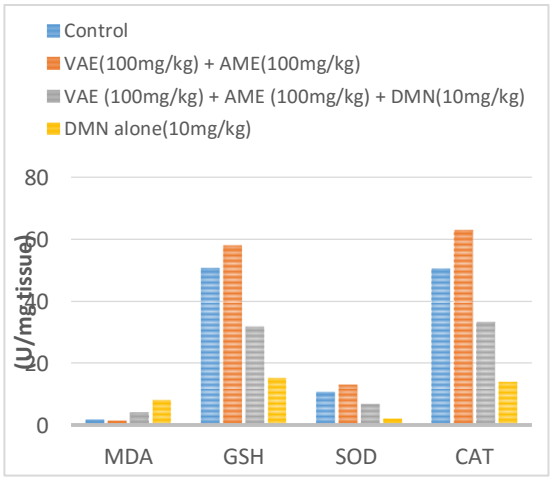

Figure 2: Effect of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) on oxidative stress parameters in Dimethylnitosamine (DMN)-induced hepatic fibrotic rats

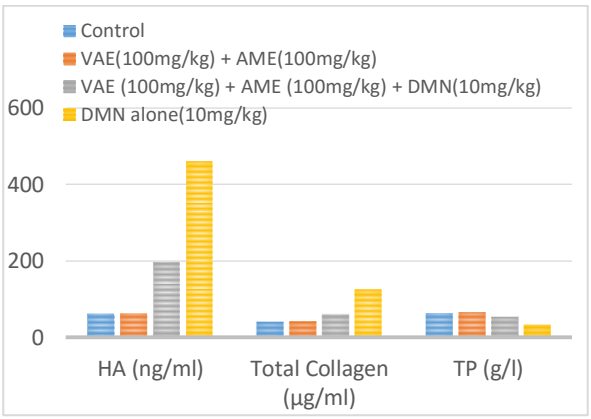

Figure 3: Effect of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) on hyaluronic acid (HA), total collagen and total protein (TP) in DMN-induced hepatic fibrotic rats

DISCUSSION

Connective tissue proteins accumulation such as collagen and hyaluronic acid (HA) has been reported and its measurement can serve in quantifying fibrosis (Yuan *et al.*, 2004). In this study by day 15, total collagen was increased linearly following administration of DMN (figure 3), an indication of hepatic fibrosis similar to previous reports (George *et al.*, 2001; George and Chandrakasan, 2000; Limuro and Fujimoto, 2003; Mu *et al.*, 2006). The hyaluronic acid increase in this study could be explained by its increased synthesis by activated hepatic stellate cells (HSCs) resulting in its simultaneous leakage into the blood stream along with the liver function enzymes. The high rise in hyaluronic acid may also be as a result of its degradation by hyaluronidases enzymes (Fraser *et al.*, 1997). However, the combination of *Vernonia amygdalina* and *Annona muricata* leaves showed anti-fibrogenic activity expressed in decrease of total collagen and hyaluronic acid content most probably due to the antioxidants and phytochemicals such as flavonoids, saponins and tannins present in the leaves as reported in previous study (Usunobun and Okolie, 2015a, b; Usunobun and Okolie, 2016). The decrease of HA activity by *Vernonia amygdalina* and *Annona muricata* leaves may also be due to HA degrading enzymes activation caused by the bioactive agents such as flavonoids, saponins and tannins present in the leaves (Usunobun and Okolie, 2015a, b; Usunobun and Okolie, 2016). The protective effect of

Vernonia amygdalina and *Annona muricata* leaves is in tandem with similar studies by Shin and Moon (2010); Yan *et al* (2010); George *et al* (2006). In this study, DMN administration caused a marked rise in serum AST, LDH, ALT and ALP indicating damage of the liver and consequent cytolysis of hepatic cells similar to previous reports (George *et al*., 2001; Bansal *et al*., 2005; Vimal and Devaki, 2004). The release of the liver function enzymes from cytoplasm into blood can be said to be due to rupture of plasma membrane and cell damage. The high rise in AST compared to ALT may be due to mitochondrial AST release into the blood stream, a consequence of severe hepatocyte damage. The results of Qing-Wei and Geng-Tao (2006) were similar to our findings which reported significant increase in liver function enzymes in mice/rats treated with DMN intraperitoneally. Consequently, a combined dose of *Vernonia amygdalina* and *Annona muricata* leaves against DMN-induced fibrosis significantly reduced the levels of liver function enzymes compared to rats given only DMN, an indication that the leaves offered protection against liver cell damage. The protection offered by *Vernonia amygdalina* and *Annona muricata* in this study is similar to previous studies of Shin and Moon (2010). Lipid peroxidation (with Malondialdehyde, MDA considered its most significant indicator) is the most recognized mechanism in studying liver injury pathogenesis by several toxic agents (Vendemiale *et al*., 2001; George, 2003; Halliwell and Gutteridge, 1995). In this study, the massive rise in MDA is an indication that DMN-induced damage of liver cell membrane results in free radical production thereby enhancing oxidative stress. However, simultaneous supplementation with combined dose of *Vernonia amygdalina* and *Annona muricata* leaves significantly reduced MDA levels, an indication that the plant leaves contain bioactive agents that can scavenge and detoxify free radicals similar to previous findings of Shin and Moon (2010). Also in this study, antioxidant component of self-defense system (GSH, CAT, and SOD) were reduced in rats given DMN, a finding similar to previous investigations (Priya *et al*., 2011a, b; Hong *et al.*, 2010, Wang *et al.*, 2010). The continuous mitochondria production of superoxide radical might be responsible for the reduction in liver SOD and CAT in rats given DMN. The drop in CAT, SOD and GSH biosynthesis during liver cell damage might be responsible for the depletion in the non-enzymatic and enzymatic self-defense component. However, treatment with combined dose of *Vernonia amygdalina* and *Annona muricata* leaves simultaneously with DMN significantly raised the antioxidants (SOD, CAT and GST) compared to rats given only DMN. *Vernonia amygdalina* and *Annona muricata* leaves contain phytochemical compounds such as flavonoids with strong free radical scavenging potentials. These protection offered by combined dose of both leaves is similar to previous work of Sharma and Singh (2014). In conclusion, *Vernonia amygdalina* and *Annona muricata* leaves possess synergistic, anti-fibrotic, free radical scavenging and hepatoprotective potentials against DMN-induced liver disease and the bioactive agents including flavonoids in both leaves may be responsible.

Conflict of Interest

We the authors hereby declare no conflict of interest.

REFERENCES

Adeyemi D, Komolafe O, Adewole S, Obuotor EM, Adenowo T (2008). Antihyperglycemic activities of *Annona muricata*. *Afr. J. Tradit. Complement. Altern. Med* 6:62–69

Atangwho IJ, Ebong PE, Eyong EU, Williams IO, Eteng MU, Egbung GE (2009). Comparative chemical composition of leaves of some antidiabetic medicinal plants: *Azadirachta indica, Vernonia amygdalina and Gongronema latifolium*. *Afr. J. Biotechnology* 8(18):4685–4689.

Bansal AK, Bansal M, Soni G, Bhatnagar D (2005). Protective role of Vitamin E pre-treatment on N-nitrosodiethylamine induced oxidative stress in rat liver. *Chem. Biol. Interact* 156(2-3):101-111.

Chang FR (2001). Novel cytotoxic annonaceous acetogenins from *Annona muricata*. *J. Nat. Productivity* 64:925–931.

Chichibu K, Matsuura T, Shichijo S (1989). Assay of serum hyaluronic acid in clinical application. *Clinica Chimica Acta*, 181: 317-324.

Cohen G, Dembiec D, Marcus J (1970). Measurement of catalase activity in tissues. *Analyt Biochem.* 34:30-38.

Ellman GL (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys* 82:70–77.

Farombi EO, Owoeye O (2011). Antioxidant and chemopreventive properties of *Vernonia amygdalina* and Garcinia biflavonoid. *Int. J. Env. Res. Pub. Health* 8: 2533–2555.

Fraser JRE, Laurent TC, Laurent UBG (1997). Hyaluronan: its nature, distribution, functions and turnover. *J. Internal Med.* 242(1) : 27-33.

George J, Chandrakasan G. (2000). Biochemical abnormalities during the progression of hepatic fibrosis induced by dimethylnitrosamine. *Clin. Biochem.* 33(7):563-570.

George J, Rao KR, Stern R, Chandrakasan G (2001). Dimethyl-nitrosamine-induced liver injury in rats: the early deposition of collagen. *Toxicology* 156(2-3): 129-138.

George J (2006). Mineral metabolism in dimethylnitrosamine-induced hepatic fibrosis. *Clin. Biochem* 39: 984–991.

George J (2003). Ascorbic acid concentrations in dimethylnitrosamine-induced hepatic fibrosis in rats, *Clin. Chim. Acta* 335: 39–47.

Halliwell B, Gutteridge JMC (1995). The definition and measurement of antioxidants in biological systems. *Free Radic. Biol. Med* 18: 125-126.

Hong SW, Jung KH, Zheng HM, Lee HS, Suh JK, Park IS, Lee DH, Hong SS (2010). The protective effect of resveratrol on dimethylnitrosamine-induced liver fibrosis in rats. *Arch. Pharm. Res* 33(4):601–609.

Igile GO, Oleszek W, Jurzysta M, Burda S, Fanfunso M, Fasanmade AA (1994). Flavonoids from *Vernonia amygdalina* and their antioxidant activities. *J. Agric. Food Chem.* 42: 2445–2448.

Liaw CC, Chang FR, Lin CY, Chou CJ, Chiu HF, Wu MJ, Wu YC. (2002). New cytotoxic monotetrahydrofuran annonaceous acetogenins from *Annona muricata*. *J. Nat. Productivity* 65: 470–475.

Iimuro Y, Fujimoto J. (2003). Strategy of gene therapy for liver cirrhosis and hepatocellular carcinoma. *J. Hepatobiliary Pancreat. Surg.* 10(1): 45-47.

Misra HP, Fridovich I. (1972). The role of superoxide ion in the autooxidation of epinephrine and a simple assay of superoxide dismutase. *J. Biol. Chem* 247: 3170– 3175.

Mu YP, Liu P, Liu Y, Li FH, Chen GF, Gu HT, Du GL, Wang L, Wang CS (2006). Inhibiting action of Xiayuxue decoction on hepatic fibrosis at progressive stage in rats and study on the prescription and syndrome. *J. Tradit. Chin. Med*. 47: 215-218

Ohkawa H, Ohishi N, Yogi K (1979). Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Annals Biochem* 95: 351–358.

Poli G (2000). Pathogenesis of liver fibrosis: role of oxidative stress. *Mol. Aspects Med.* 21: 49–98.

Priya S, Vijayalakshmi P, Vivekanandan P, Karthikeyan S (2011a). *N*-Acetylcysteine attenuates dimethylnitrosoamine induced oxidative stress in rats. *European Journal of Pharmacology* 654: 181–186.

Priya S, Vijayalakshmi P, Vivekanandan P, Karthikeyan S (2011b). Influence of N-acetylcysteine against dimethylnitrosoamine induced hepatotoxicity in rats. *Toxicol. Ind. Health* 27(10): 914–922.

Qing-Wei H, Geng-Tao L (2006). Effects of bicyclol on dimethylnitrosamine-induced liver fibrosis in mice and its mechanism of action. *Life Sci*ence 79: 606–612.

Rojkind M, Giambrone MA, Biempica L (1979). Collagen types in normal and cirrhotic liver. *Gastroenterology* 76:710–719.

Sharma V, Singh, M (2014). Attenuation of N-nitrosodimethylamine induced hepatotoxicity by *Operculina turpethum* in Swiss Albino mice. *Iranian J. Basic Med. Science* 17:73-80.

Shin M, Moon J (2010). Effect of dietary supplementation of grape skin and seeds on liver fibrosis induced by dimethylnitrosamine in rats. *Nutri. Res. Pract.* 4(5):369- 374.

Shimizu I, Ma YR, Mizobuchi Y (1999). Effects of Sho-saikoto, a Japanese herbal medicine, on hepatic fibrosis in rats. *Hepatology* 29: 149–160.

Takahashi JA, Pereira CR, Pimenta LP, Boaventura MA, Silva LG (2006). Antibacterial activity of eight Brazilian Annonaceae plants. *Nat. Prod. Res* 20(1): 21-26.

Usunobun U, Okolie NP (2015a). Phytochemical analysis and mineral composition of *Annona muricata* leaves. *Int. J. Res. Curr. Development* 1(1): 38-42.

Usunobun U, Okolie NP (2015b). Phytochemical, trace and mineral composition of *Vernonia amygdalina* leaves. *Int. J. Biol. Pharmaceut. Res.* 6(5): 393-399.

Usunobun U, Okolie PN (2016). Phytochemical analysis and proximate composition of *Vernonia amygdalina. Int. J. Sci. World* 4(1):11 -14.

Usunobun U, Okolie NP, Eze IG. (2015a). Attenuation of N, N-Dimethylnitrosamine -induced liver Fibrosis in rats by ethanolic leaf extract of *Annona muricata*. *Saudi J. Med. Pharmaceut. Science* 1(2): 62-69.

Usunomena U, Okolie NP, Eze IG (2015b) Inhibitory Effect of *Vernonia amygdalina* on Dimethylnitrosamine (DMN)- induced Liver Fibrosis

in Rats. *Int. J. Clin. Pharmacol. Toxicol.,* 4(4) : 179-184.

Usunobun U, Anyanwu OG, Okolie PN (2016). Anti-anemic Effect of *Annona muricata* Ethanolic Leaf Extract in Dimethyl Nitrosamine (DMN)-Induced Hepatic Fibrosis in Rats. *EC Pharmacology and Toxicology* 2.2 : 91-95.

Vimal V, Devaki T (2004). Hepatoprotective effect of allicin on tissue defense system in galactosamine/endotoxin challenged rats. *J. Ethnopharmacology* 90: 151–154

Vendemiale G, Grattagliano I, Caruso ML, Serviddio G, Valentini AM, Pirrelli M, Altomare E (2001). Increased oxidative stress in dimethylnitrosamine-induced liver fibrosis in the rat: effect of N-acetylcysteine and interferon-alpha. *Toxicol. Appl. Pharmacology* 175: 130-9.

Vogel AI (1971). A textbook of practical organic Chemistry including qualitative organic analysis. Longman group limited, London. p426.

Wang JH, Shin JW, Son JY, Cho JH, Son CG (2010). Antifibrotic effects of CGX, a traditional herbal formula, and its mechanisms in rats. *J. Ethnopharmacol* 127: 534–542.

Yan W, Juncha G, Di Z, Jian Z, Junji M, Huiqing J (2010). New insights into the antifibrotic effects of sorafenib on hepatic stellate cells and liver fibrosis. *J. Hepatology* 53: 132–144

Yuan GJ, Zhang ML, Gong ZJ (2004). Effect of PPAR agonist pioglitazone on rat hepatic fibrosis. *World J. Gastroenterol*ogy 10: 1047–1051.