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**Hepatoprotective and Antioxidant activity of a Novel active herbal agent against Non-alcoholic fatty liver disease.**

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**Abstract:**

In the present invention we evaluated hepatoprotective and antioxidant activity of the ethanolic leaves extract of Abroma augusta on non-alcoholic fatty liver disease (NAFLD) in SD rats by different screening models. The experimental animals were divided in to five groups (n = 6) and then ethanolic leaves extract of *Abroma augusta (ELEAA)* in dose of 250 and 500 mg/kg body weight was administered. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC) in the serum and high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), free fatty acid (FFA), Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) in liver tissue was evaluated respectively. While hepatoprotective effect were also confirmed by histopathological analysis, the expression level of tumor necrosis factor- α (TNF-α) and cytochrome $P_{450}$ (CYP) 2E1 in Sprague - Dawley (SD) rats liver was determined by immunohistochemistry analysis. A significant decrease were observed in the level of serum ALT (2.85 fold), AST (2.17 fold), and the blood lipid TG (2.44 fold) and TC (1.76 fold) in the dose of 500mg/kg ethanolic leaves extract of *Abroma augusta (ELEAA)* treated Sprague - Dawley (SD) rats ($p < 0.01$), compared to the standard groups.

# Title of Invention

# Hepatoprotective and Antioxidant activity of a Novel active herbal agent against Non-alcoholic fatty liver disease.


**Summary**

This present invention showed that the ethanolic leaves extract of *Abroma augusta* (*ELEAA*) has a protective effect on non-alcoholic fatty liver diseases (NAFLD), down regulate expression of TNF-α, and that CYP2E1 may be one of the action mechanisms of *Abroma augusta*.

Different Sprague Dawley (SD) rat models of non-alcoholic fatty liver disease (NAFLD) were established by feeding Methionine and Choline deficient diet (MCD) for 10 weeks, high fat diet (HFD) for 14 weeks, Cholesterol and Cholate diet (CCD) for 24 weeks and in the chemical model combine with high fat diet (HFD), in this model new born two days after birth Sprague - Dawley (SD) rat were give *streptozotocin* (200µg) and then surviving Sprague - Dawley (SD) rats were started high fat diet (HFD) at four weeks old for 14 weeks. The experimental animals were divided in to five groups (n = 6), Group I - served as normal control and received only as the vehicle (1 mL/kg/day of 1% CMC; p.o.), Group II – served as negative control and not provide any treatment, Group III- received silymarin 100mg/kg as a standard drug, Group IV and Group V received ethanolic leaves extract of *Abroma augusta (ELEAA)* in dose of 250 and 500 mg/kg body weight. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC) in the serum and high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), free fatty acid (FFA), Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) in liver tissue was evaluated respectively. While hepatoprotective effect were also confirmed by histopathological analysis, the expression level of tumor necrosis factor- α (TNF-α) and cytochrome $P_{450}$ (CYP) 2E1 in Sprague - Dawley (SD) rats liver was determined by immunohistochemistry analysis.

A significant decrease were observed in the level of serum ALT (2.85 fold), AST (2.17 fold), and the blood lipid TG (2.44 fold) and TC (1.76 fold) in the dose of 500mg/kg ethanolic leaves extract of *Abroma augusta (ELEAA)* treated Sprague - Dawley (SD) rats ($p < 0.01$), compared to

the standard groups. Pretreatment with silymarin 100mg/kg and 250mg/kg, 500mg/kg ethanolic leaves extract of *Abroma augusta (ELEAA)* significantly raised the levels of antioxidant enzyme SOD in dose dependent manner, to 92.90±8.64, 112.52±12.33, 124.25±10.68, 90.92±9.64, 109.52±11.33, 121.25±10.68, 94.82±7.63, 110.42±13.32, 128.35±11.67 and 93.92±7.64, 113.52±12.33, 125.25±14.68 respectively ($p < 0.05$, $p < 0.01$) as compared to the model groups. Similarly the level of GSH was significantly increased ($p < 0.05$, $p < 0.01$) after the ethanolic leaves extract of *Abroma augusta (ELEAA)* treatment. Meanwhile pretreatment with standard drug 100 mg/kg and 250mg/kg, 500mg/kg of ethanolic leaves extract of *Abroma augusta (ELEAA)* significantly reduced MDA amount by 6.34±1.43, 5.13±1.14, 4.46±1.38, 5.34±1.43, 4.13±1.14, 3.45±1.38, 6.34±1.42, 5.13±1.24, 4.45±1.28 and 5.24±1.43, 4.13±1.14, 3.35±1.38 in the liver homogenates, respectively ($p < 0.01$). The ethanolic leaves extract of *Abroma augusta (ELEAA)* treatment group showed significantly decreased levels of lipid products like low density lipoprotein- cholesterol (LDL-C) ($p < 0.05$, $p < 0.01$), improved high density lipoprotein-cholesterol (HDL-C) level and significantly decrease content of free fatty acid (FFA), compared to the model groups ($p <0.05$, $p < 0.01$). Furthermore, the ethanolic leaves extract of *Abroma augusta (ELEAA)* treated groups also exhibited a down regulation TNF-α and CYP2E1 expression, decreased infiltration of liver fats and reversed histopathological changes, all in a dose dependent manner ($p <0.05$, $p < 0.01$).

**Background**

Non-alcoholic fatty liver diseases (NAFLD) are defined by hepatic fat deposition in the absences of excessive alcohol consumption; it is also associated with the insulin resistance (IR) and metabolic syndrome. Now Non-alcoholic fatty liver diseases (NAFLD) is defined as a concentration of hepatic triglyceride (TG) exceeding 5% liver weight and often show a histological spectrum ranging from simple steatosis to NASH. NASH is characterized by hepatocellular damage, fibrogenesis, and lobular necro-inflammation which may evolve to hepatic cirrhosis and HCC. Although MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD induced Non-alcoholic fatty liver diseases (NAFLD) animals models required a long feeding period and they

are more close to humans Non-alcoholic fatty liver diseases (NAFLD) in pathophysiology, including induced obesity, insulin resistance (IR) and hepatic steatosis in Sprague Dawley - rats. Emotional disorder and poor diet with the key point of blood stasis and phlegm is regarded as the etiology of Non-alcoholic fatty liver diseases (NAFLD) and these etiologies are related to the organs of the liver, spleen and kidney, according to the traditional medicine theory. Promoting blood circulation to remove meridian obstruction, reducing phlegm, removing dampness and liver – kidney – tonifying are an effective approach for the treatment of Non-alcoholic fatty liver diseases (NAFLD). However at present, although tremendous efforts has made in prevention of Non-alcoholic fatty liver diseases (NAFLD) by registered medicinal practitioners (RMPs) and researchers but at the same, there are no approved treatment drugs for the of Non-alcoholic fatty liver diseases (NAFLD). Hence development and exploring a novel agent to detain or reverse the pathogenesis progression in of Non-alcoholic fatty liver diseases (NAFLD) are very important objective.

## BRIEF DESCRIPTION OF THE DRAWINGS

Other objects, features, and advantages of the embodiment will be apparent from the following description when read with reference to the accompanying drawings. In the drawings, wherein like reference numerals denote corresponding parts throughout the several views.
Preferred embodiments of the present invention are herein further described, by way of non-limiting example only, with reference to the accompanying tables, in which:

Figure 1 Illustrates Appearance of SD-rats liver tissue (A/C/E/G) and Histopathological examination by HE (B/D/F/H, 200 x). I: Control group; II: Model group; III: Standard group; IV: ELEAA 250mg/kg group; V: ELEAA 500mg/kg group.

Figure 2: Representative photographs of immunological histological chemistry examination (200 x). A/C/E/G: TNF-α; B/D/F/H: CYP2E1, I: Control group; II: Model group; III: Standard group; IV: ELEAA 250mg/kg group; V: ELEAA 500mg/kg group; VI: Quantification of TNF-α (A-VI)

and CYP2E1 (B-VI) stained cells. The results are expressed as mean ± SD of 12 rats. $^{b}P < 0.01$ vs control groups; $^{c}P < 0.05$, $^{d}P < 0.01$ vs model groups.

**MODES FOR CARRYING OUT THE PREFERRED EMBODIMENTS**

The embodiments herein and the various features and advantageous details thereof are explained more fully with reference to the non-limiting embodiments that are illustrated in the accompanying drawings and detailed in the following description. Descriptions of well-known components and processing techniques are omitted so as to not unnecessarily obscure the embodiments herein. The examples used herein are intended merely to facilitate an understanding of ways in which the embodiments herein may be practiced and to further enable those of skill in the art to practice the embodiments herein. Accordingly, the examples should not be construed as limiting the scope of the embodiments herein.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

As used herein, the singular forms “a”, “an”, “the” include plural referents unless the context clearly dictates otherwise. Further, the terms “like”, “as such”, “for example”, “including” are meant to introduce examples which further clarify more general subject matter, and should be contemplated for the persons skilled in the art to understand the subject matter. Although this invention has been described in conjunction with the exemplary embodiments’ below, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art. Accordingly, the exemplary embodiments of the invention as set forth above are intended to be illustrative and not limiting. Various changes may be made without departing from the spirit and scope of the invention.

**Experimental design (Methods):**

In the experiment the animals were divided in to five groups (n = 6), Group I - Served as normal control and received only as the vehicle (1 mL/kg/day of 1% CMC; p.o.), Group II – served as negative control and not provide any treatment, Group III- received silymarin 100mg/kg as a

standard drug, Group IV and Group V received ethanolic leaves extract of *Abroma augusta (ELEAA)* in dose of 250 and 500 mg/kg body weight. These were administered orally twice daily at 10:00 and 16:00 hours respectively, for 8 - 24 weeks, as per experimental models for acute hepatoprotective activity against non alcoholic fatty liver disease.

**Experimental Models:**

***MCD Diet (Methionine and Choline deficient diet):***

The male and female Wistar rats were used as 5 weak of age. For the elicitation of non-alcoholic fatty liver disease (NAFLD) in mice. Methionine - choline deficient (MCD) diet model has a high sucrose content and moderate fat content (40% sucrose and 10% fat) but deficient in methionine and choline that are essential nutrients in hepatic β-oxidation and the production of very low density lipoproteins (VLDL). All the diet was γ- irradiated which make the diet safer by reducing the number of harmful parasite and bacteria. Choline deficiency leads to an impaired hepatic very low density lipoprotein (VLDL) secretion, resulting in hepatic lipid deposition, oxidative stress and change in cytokines and adipokines, culminating to liver injury. Increased serum ALT level and steatohepatitis occurred at day 10 in MCD fed diet rats and perisinusoidal fibrosis developed after 8-10 weeks. Development of liver injury was followed by intraperitoneal injection (ip) of 30 mg/kg UDCA-LPE solubilized in 0.5% carboxy-methylcellulose (CMC) two times a week for 3 week on the diet in methionine-choline deficient (MCD) mice. All the animals had free access to diet and drinking water at the duration of study. The mice were anesthetized and killed through cervical dislocation at the end of feeding duration. Livers were collected and a portion of fresh tissue was fixed in 10% buffered formalin. The remaining livers tissues were snap-frozen in liquid nitrogen and stored at -78$^0$C. Blood sample was allowed to clot and serum was separated by centrifuged at 3500 rpm for 15 minutes for carrying out other biochemical investigation. At -78$^0$C the collected serum was stored. In all dietary studies mice were fasted for 4 hours prior to killing. The experiments were approved by animal care and use ethical committee.

**HFD (High Fat Diet):**

The high fat diet (HFD) is widely used to developed Non-alcoholic fatty liver diseases (NAFLD) animal models. In High fat diet (HFD) animal models, diet of groups consist of a variety of regimens with fat content varying between 45 – 75% kcal. The animals total calorie intake is

derived from fat and animals are fed predominantly *ad libitum*. The classic High fat diet (HFD) used rats fed a diet composed of 71% of calories from fat, 11% from carbohydrate and 18% from protein). Similarly to human Non-Alcoholic Fatty Liver Diseases (NAFLD) patients, rats developed IR, as shown by elevated plasma insulin levels, marked pan lobular steatosis, inflammation and fibrogenesis. The mice fed high fat diet (HFD) showing similar result after 16 weeks . Feeding SD-rats with the help of gastrostomy tube with high fat diet (HFD) up to 86% in excess of standard intake for 14 weeks. Obesity, hepatic steatosis, histopathological features similar to NASH in human, as verified by the presences of increased liver triglyceride levels, hepatocyte ballooning, Mallory bodies, higher fasting serum glucose levels and decreased adiponectin levels suggesting hyperglycemia and IR. Their plasma alanine aminotransferase (ALT) levels showed 9 – 10 fold increases.

**CCD (Cholesterol and Cholate diet):**

Cholesterol in the diet is an important risk factor for Non-alcoholic steatohepatitis (NASH) because it makes the liver sensitive to tumor necrosis factor- and Fas-induced steatohepatitis. The cholic acid presence promotes the absorption of cholesterol and fat and impedes the conversion of cholesterol to bile acids, hence reducing the removal of cholesterol and increases the cholesterol levels, particularly low-density lipoprotein cholesterol. The cholesterol and Cholate diet containing 1.25% cholesterol and 0.5% Cholate induces progressive formation of steatosis, inflammation, and fibrosis over 6–24 weeks: steatosis and inflammation (after 6 weeks), hepatocellular ballooning and fibrosis (after 24 weeks). In addition, the investigated animals show increased levels of ALT, after 6 weeks. The addition of 60% fat (cocoa butter) to the diet accelerated the development of abovementioned histopathological features. Moreover, the cholesterol and Cholate diet contributes to the presentation of oxidative stress, dyslipidemia, and the activation of stellate cell in the liver. Taken together, it means that atherogenic diet can replicate the pathophysiological findings of human NASH. However, the mice fed this diet did not show systemic IR, only showed the hepatic IR. On the other hand, the mice fed this diet lost 9% of their body weight and showed smaller fat pads and lower triglyceride levels compared with mice fed standard chow. In general, although the cholesterol and Cholate diet replicates the histopathology in human NASH, the metabolic status differs.

**Streptozotocin + HFD:**

Low dose of streptozotocin (STZ) intraperitoneal administration in new born Sprague - Dawley (SD) rats lead to a chemical inflammation and destruction of the pancreatic islets, thus inducing diabetes. This model combine with high fat diet (HFD) can establish a model of non-alcoholic fatty liver diseases (NAFLD). In this model new born two days after birth Sprague - Dawley (SD) rats were give *streptozotocin* (200µg) and then surviving Sprague - Dawley (SD) rats were started high fat diet (HFD) at four weeks old. These rats developed simple steatosis at six weeks, NASH with inflammatory foci and ballooning at eight weeks, progressive pericellular fibrosis at twelve weeks and multiple HCC at twenty weeks of age. The level of transaminase and fasting glycemia are elevated at six weeks of age. This model recapitulates several important histological feature of human non-alcoholic fatty liver disease (NAFLD) and is also relevant to oxidative stress. But streptozotocin recreating beta (β) cell a function rather than a systemic inflammatory insulin resistant milieu, this is different from the human state. However, in similar model where rats were given streptozotocin followed by high fat diet (HFD), an investigator failed to demonstrate concordance between rats and humans with respects to differentially expressed gene.

**Results:**

**Acute toxicity test:**

The ethanolic leaves extract of *Abroma augusta* (*ELEAA*) does not show any sign and symptom of toxicity up to 2000 mg/kg body weight and hence it was considered to be safe and effective.

**Effect of *Abroma augusta* on body weight and liver coefficient:**

After fed with MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD, the body weight of rats in the model group was notably increased compared to that of rats in the control group ($P < 0.01$, Figure 2A). Meantime after ethanolic leaves extract of *Abroma augusta* treatment for six (6) weeks the gain in the body weight for the rats in the 250 mg/kg and 500 mg/kg, ethanolic leaves extract of *Abroma augusta* treated groups was lower than for the rats in the standard and model group ($P < 0.01$, Figure 2A), which indicates ethanolic leaves extract of *Abroma augusta* treatment could inhibit the occurrence of obesity in MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD administrated rats. Furthermore, consistent with these modifications, the liver coefficient was also reduced

markedly in the ethanolic leaves extract of *Abroma augusta* treated rats (*P < 0.05*, *P < 0.01*, Figure 2B), compared to the control group.

**Effect of *Abroma augusta* on serum *ALT* and *AST* levels:**

Serum level of *ALT* and *AST* indirectly reflects the failure of liver function, As shown in Table 1, serum *ALT* and *AST* activates were significantly increased after the administration of MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD, as compared with the standard group, the level of *ALT* and *AST* was significantly deceased in a dose dependent manner after ethanolic leaves extract of *Abroma augusta* treatment 250 mg/kg and 500mg/kg, *(P <0.05, P<0.01,* Table 1).

**Effects of *Abroma augusta* on blood lipid levels:**

MCD (Methionine choline deficient diet), HFD (High fat diet), CCD (Cholate and choline diet) and STZ+HFD (Streptozotocin+High fat diet) induced non-alcoholic fatty liver diseases (NAFLD) produced a marked incremental change in triglycerides (TG) and total cholesterol (TC) levels compared with those in the normal group (*P<0.01,* Table 1), which indicates the successful establishment of the Non-alcoholic fatty liver diseases (NAFLD) models in the rats. However after ethanolic leaves extract of *Abroma augusta (ELEAA)* exposure the concentration of both TG and TC in blood was remarkably decreased in dose dependent manners, as compared to the non-alcoholic fatty liver disease standard groups (*P <0.05, P<0.01,* Table 1). All of these finding indicate that ethanolic leaves extracts of *Abroma augusta (ELEAA)* shows lipid-lowering effects against non-alcoholic fatty liver diseases (NAFLD).

**Table 1: Effect of ethanolic leaves extract of *Abroma augusta* (*ELEAA*) on the serum biochemical levels of SD rats fed with different died models.**

| S.No. | Model | Treatment | Dose mg/kg | ALT (IU/L) | AST (IU/L) | TG nmol/L | TC nmol/L |
|---|---|---|---|---|---|---|---|
| 1. | MCD | Control | - | 17.82±3.72 | 61.63±7.38 | 0.63±0.06 | 0.82±0.07 |
| | | MCD | - | 62.35±14.52 | 165.18± | 1.48±0.31 | 3.06±0.7 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | [b] | 36.43[b] | [b] | 2[b] |
| | | MCD+Standard | 100 | 32.91±4.74[c] | 97.82±12.65[c] | 1.04±0.12[c] | 3.21±0.27[c] |
| | | MCD+ELEAA | 250 | 28.33±3.07[d] | 86.53±8.17[d] | 0.65±0.7[d] | 2.12±0.16[d] |
| | | MCD+ELEAA | 500 | 22.62±4.42[d] | 74.43±7.68[d] | 0.49±0.4[d] | 1.73±0.10[d] |
| **2.** | HFD | Control | - | 16.73±4.74 | 63.53±6.28 | 0.65±0.07 | 0.85±0.08 |
| | | HFD | - | 66.24±12.53[b] | 170.17± 46.42[b] | 1.45±0.32[b] | 3.07±0.71[b] |
| | | HFD+Standard | 100 | 37.62±5.73[c] | 98.72±13.65[c] | 1.03±0.13[c] | 4.22±0.26[c] |
| | | HFD+ELEAA | 250 | 26.42±5.08[d] | 87.63±9.17[d] | 0.63±0.6[d] | 2.14±0.14[d] |
| | | HFD+ELEAA | 500 | 20.53±3.22[d] | 72.42±8.58[d] | 0.48±0.5[d] | 1.63±0.12[d] |
| **3.** | CCD | Control | - | 19.76±4.72 | 63.63±8.37 | 0.62±0.07 | 0.81±0.07 |
| | | CCD | - | 66.34±15.52[b] | 155.17± 35.42[b] | 1.47±0.32[b] | 3.05±0.73[b] |
| | | CCD+Standard | 100 | 34.81±4.73[c] | 98.72±12.64[c] | 1.03±0.13[c] | 4.22±0.26[c] |
| | | CCD+ELEAA | 250 | 29.35±3.06[d] | 84.43±8.17[d] | 0.67±0.6[d] | 2.14±0.16[d] |
| | | CCD+ELEAA | 500 | 23.52±5.42[d] | 73.33±7.68[d] | 0.48±0.5[d] | 1.76±0.12[d] |
| **4.** | STZ+ HFD | Control | - | 17.72±3.62 | 60.64±6.28 | 0.53±0.04 | 0.81±0.06 |
| | | (STZ+HFD) | - | 61.35±13.55 | 162.18± | 1.38±0.21 | 3.04±0.7 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | [b] | 33.53[b] | [b] | 4[b] |
| | | (STZ+HFD) +Standard | 100 | 30.91±3.84[c] | 95.92±13.65[c] | 1.02±0.11[c] | 2.41±0.24[c] |
| | | (STZ+HFD) +ELEAA | 250 | 26.33±2.07[d] | 88.53±9.17[d] | 0.75±0.7[d] | 2.00±0.12[d] |
| | | (STZ+HFD) +ELEAA | 500 | 20.66±3.52[d] | 78.43±9.68[d] | 0.59±0.4[d] | 1.83±0.15[d] |

Data are expressed as mean ± SD ($n$ = 6) for each group, [b]*P < 0.01 vs* control group [c]*P < 0.05*, [d]*P < 0.05*, [d]*P < 0.01 vs* model group. MCD: Methionine choline deficient diet; HFD: High fat diet; CCD: Cholate and choline diet; STZ: Streptozotocin; ELEAA: Ethanolic leaves extract of *Abroma Augusta*; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglyceride; TC: Total cholesterol.

**Effects of *Abroma augusta* on liver tissue *SOD*, *GSH* and *MDA* levels:**

The level of liver antioxidant activity of SOD and GSH was measured due to the oxidative stress manifest in the development of non-alcoholic fatty liver diseases (NAFLD) [38]. The SOD and GSH have the ability of scavenging the lipid hydroperoxides, lipid peroxide radicals and the other products which are toxic metabolite of non-alcoholic fatty liver diseases (NAFLD). Now in this study measured the contents of SOD, GSH and MDA in the liver tissue of the rats. From the table 2, it's clearly seen that the significant difference between the rats fed with MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD model groups and the normal group for the level of SOD and GSH which was mainly decreased (*P<0.01,* Table 2) in the different models treated group compared with that of the normal. Although the pretreatment with 100 mg/kg and 250 mg/kg, 500 mg/kg ethanolic leaves extract of *Abroma augusta (ELEAA)* significantly increase the levels of the antioxidant enzyme SOD in the dose dependent manners 92.90±8.64, 112.52±12.33, 124.25±10.68, 90.92±9.64, 109.52±11.33, 121.25±10.68, 94.82±7.63, 110.42±13.32, 128.35±11.67 and 93.92±7.64, 113.52±12.33, 125.25±14.68 respectively (*P <0.05, P<0.01,* Table 2) as compared with the standard groups. As the same the level of GSH was significantly increased by the treatment with standard and 250 mg/kg, 500 mg/kg ethanolic leaves extracts of *Abroma augusta (ELEAA)* (*P <0.05, P<0.01,* Table 2). MDA is an end product of the

breakdown of the polyunsaturated fatty acid and related esters is an important index of lipid peroxidation in many organ homogenates [34]. The MDA concentration significantly enhanced by the feeding with MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD models as compared with the normal group (*P<0.01,* Table 2). Although the pretreatment with standard and 250 mg/kg, 500 mg/kg ethanolic leaves extract of *Abroma augusta (ELEAA)* significantly reduce the levels of the MDA level by 6.34±1.43, 5.13±1.14, 4.46±1.38, 5.34±1.43, 4.13±1.14, 3.45±1.38, 6.34±1.42, 5.13±1.24, 4.45±1.28 and 5.24±1.43, 4.13±1.14, 3.35±1.38 in the live homogenate respectively (*P<0.01,* Table 2).

**Table 2: Effect of ethanolic leaves extract of *Abroma augusta* on the liver antioxidant enzyme specific activity, antioxidant and lipid peroxidation of SD rats fed with different diets models.**

| S.No. | Model | Treatment | Dose mg/kg | SOD (U/mgprot) | GSH mg/gprot | MDA nmol/mgprot |
|---|---|---|---|---|---|---|
| 1. | MCD | Control | - | 132.52±17.28 | 7.86±1.68 | 4.52±1.14 |
| | | MCD | - | 78.72±7.53$^{b}$ | 2.56± 0.83$^{b}$ | 8.57±3.10$^{b}$ |
| | | MCD+Standard | 100 | 92.90±8.64$^{c}$ | 2.72±1.35$^{c}$ | 6.34±1.43$^{c}$ |
| | | MCD+ELEAA | 250 | 112.52±12.33$^{d}$ | 3.76±0.91$^{d}$ | 5.13±1.14$^{d}$ |
| | | MCD+ELEAA | 500 | 124.25±10.68$^{d}$ | 5.59±1.52$^{d}$ | 4.46±1.38$^{d}$ |
| 2. | HFD | Control | - | 131.52±16.28 | 6.86±1.68 | 3.52±1.14 |
| | | HFD | - | 77.72±7.53$^{b}$ | 2.56± 0.83$^{b}$ | 7.57±3.10$^{b}$ |
| | | HFD+Standard | 100 | 90.92±9.64$^{c}$ | 2.72±1.35$^{c}$ | 5.34±1.43$^{c}$ |
| | | HFD+ELEAA | 250 | 109.52±11.33$^{d}$ | 3.76±0.91$^{d}$ | 4.13±1.14$^{d}$ |
| | | HFD+ELEAA | 500 | 121.25±10.68$^{d}$ | 4.59±1.52$^{d}$ | 3.45±1.38$^{d}$ |
| 3. | CCD | Control | - | 133.41±18.38 | 5.86±1.68 | 2.53±1.14 |
| | | CCD | - | 78.62±8.52$^{b}$ | 3.56± 0.83$^{b}$ | 8.57±3.10$^{b}$ |
| | | CCD+Standard | 100 | 94.82±7.63$^{c}$ | 3.72±1.35$^{c}$ | 6.34±1.42$^{c}$ |
| | | CCD+ELEAA | 250 | 110.42±13.32$^{d}$ | 4.76±0.91$^{d}$ | 5.13±1.24$^{d}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | CCD+ELEAA | 500 | 128.35±11.67[d] | 5.59±1.52[d] | 4.45±1.28[d] |
| **4.** | STZ+HFD | Control | - | 138.52±15.28 | 6.86±1.68 | 3.62±1.14 |
| | | STZ+HFD | - | 79.72±7.53[b] | 3.56± 0.83[b] | 7.67±3.10[b] |
| | | (STZ+HFD) +Standard | 100 | 93.92±7.64[c] | 2.72±1.35[c] | 5.24±1.43[c] |
| | | (STZ+HFD) +ELEAA | 250 | 113.52±12.33[d] | 4.76±0.91[d] | 4.13±1.14[d] |
| | | (STZ+HFD) +ELEAA | 500 | 125.25±14.68[d] | 5.59±1.52[d] | 3.35±1.38[d] |

Data are expressed as mean ± SD (n=6) for each group. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group. MCD: Methionine choline deficient diet; HFD: High fat diet; CCD: Cholate and choline diet; STZ: Streptozotocin; ELEAA: Ethanolic leaves extract of *Abroma Augusta*; SOD: Superoxide dismutase; GSH: Glutathione; MDA: Malondialdehyde.

**Effects of *Abroma augusta* on *LDL-C, HDL-C* and *FFA* levels in the liver tissue:**

The volume of lipid products was significantly increased after MCD (Methionine choline deficient diet), HFD (High fat diet), CCD (Cholate and choline diet) and STZ+HFD (Streptozotocin+High fat diet) feeding in the model group as compared to the control group (*P<0.01,* Table 3). Its results shows that the LDL-C was significantly increased in the model groups as compared to the normal group (*P<0.01,* Table 3) and dramatically decreased in the ethanolic leaves extract of *Abroma augusta (ELEAA)* treated groups as compared with that in the standard groups (*P <0.05, P<0.01,* Table 3). In contrast the volume of HDL-C was significantly decreased at the end of the experiments, and the ethanolic leaves extract of *Abroma augusta (ELEAA)* treatment significantly improved the HDL-C volume as compared with that in the standard groups (*P <0.05, P<0.01,* Table 3). Similarly the amount of the free fatty acid (FFA) was notably increased after MCD (Methionine choline deficient diet), HFD (High fat diet), CCD (Cholate and choline diet) and STZ+HFD (Streptozotocin+High fat diet) administration and pretreatment with ethanolic extract of *Abroma augusta (ELEAA)* significantly decreased the content of free fatty acid (FFA) in a dose dependent manner (*P <0.05, P<0.01,* Table 3).

**Table 3: Effects of ethanolic leaves extract of *Abroma augusta* on low density lipoprotein cholesterol, high density lipoprotein cholesterol and free fatty acid level in the liver tissue.**

| S.No. | Model | Treatment | Dose mg/kg | LDL-C mmol/L | HDL-C mmol/L | FFA mmol/L |
|---|---|---|---|---|---|---|
| 1. | MCD | Control | - | 0.33±0.08 | 0.97±0.10 | 0.83±0.13 |
| | | MCD | - | 2.44±0.13[b] | 0.54±0.02[b] | 2.06±0.15[b] |
| | | MCD+Standard | 100 | 1.36±0.10[c] | 0.72±0.04[c] | 1.72±0.12 |
| | | MCD+ELEAA | 250 | 0.92±0.05[d] | 0.76±0.05[d] | 1.54±0.10[c] |
| | | MCD+ELEAA | 500 | 0.58±0.08[d] | 0.82±0.05[d] | 1.32±0.08[d] |
| 2. | HFD | Control | - | 0.34±0.07 | 0.98±0.12 | 0.82±0.14 |
| | | HFD | - | 2.48±0.12[b] | 0.55±0.03[b] | 2.03±0.16[b] |
| | | HFD+Standard | 100 | 1.32±0.11[c] | 0.70±0.05[c] | 1.73±0.14 |
| | | HFD+ELEAA | 250 | 0.95±0.08[d] | 0.80±0.08[d] | 1.56±0.12[c] |
| | | HFD+ELEAA | 500 | 0.62±0.06[d] | 0.87±0.04[d] | 1.34±0.04[d] |
| 3. | CCD | Control | - | 0.36±0.08 | 0.96±0.12 | 0.81±0.12 |
| | | CCD | - | 2.52±0.13[b] | 0.53±0.02[b] | 2.05±0.14[b] |
| | | CCD+Standard | 100 | 1.38±0.10[c] | 0.74±0.04[c] | 1.74±0.13 |
| | | CCD+ELEAA | 250 | 0.97±0.05[d] | 0.85±0.05[d] | 1.51±0.11[c] |
| | | CCD+ELEAA | 500 | 0.60±0.08[d] | 0.92±0.05[d] | 1.30±0.07[d] |
| 4. | STZ+HFD | Control | - | 0.37±0.07 | 0.94±0.10 | 0.82±0.13 |
| | | STZ+HFD | - | 2.40±0.13[b] | 0.55±0.02[b] | 2.07±0.15[b] |
| | | (STZ+HFD) +Standard | 100 | 1.35±0.10[c] | 0.73±0.04[c] | 1.75±0.10 |
| | | (STZ+HFD) +ELEAA | 250 | 0.94±0.05[d] | 0.84±0.05[d] | 1.52±0.12[c] |
| | | (STZ+HFD) +ELEAA | 500 | 0.63±0.08[d] | 0.90±0.05[d] | 1.33±0.07[d] |

Data are expressed as mean ± SD (n=6) for each group. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group. MCD: Methionine choline deficient diet; HFD: High fat diet; CCD: Cholate and choline diet; STZ: Streptozotocin; ELEAA: Ethanolic leaves extract of *Abroma*

*Augusta*; LDL-C: Low density lipoprotein-cholesterol; HDL-C: High density lipoprotein-cholesterol; FFA: Free fatty acid.

**Table 4: Main features of commonly used SD-rats models of Non alcoholic fatty lived diseases (NAFLD).**

| S.No. | Model | Obesity | IR | Steatosis | SH | Fibrosis | Ballooning | Carcinoma |
|---|---|---|---|---|---|---|---|---|
| **1.** | MCD | Weight Loss | Hepatic IR Only | Yes | Yes | Yes | No | No |
| **2.** | HFD | Yes | Yes | Yes | Yes | Yes (Slight) | No | No |
| **3.** | CCD | Weight Loss | Hepatic IR Only | Yes | Yes | Yes | Yes | No |
| **4.** | (STZ+ HFD) | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

**NAFLD -** Nonalcoholic fatty liver disease; **MCD -** Methionine and choline deficient; **HFD -** High-fat diet; **CCD** - Cholesterol and cholate diet; **STZ** – Streptozotocin; **IR -** Insulin resistance; **SH** – Steatohepatitis.

**Histopathological changes in the liver tissue:**

The histopathological changes in the liver were observed with naked eyes, the liver of the control groups were deep red, moist, glossy and resilient (Figure 1A/C/E/G I) while those of the model groups showed yellow necrotic foci, grey red colour, loss of luster and tumescent (Figure 1A/C/E/G II). But in the ethanolic leaves extract of *Abroma augusta (ELEAA)* treated SD-rats the liver injuries were attenuated dramatically in dose dependent manner as compared with the standard (Figure 1A/C/E/G III-V). In the figure 1B/D/F/H HE- stained section are shown. With the help of the photomicroscope, liver section from the normal control groups showed normal lobular architecture, liver cells are with well preserved cytoplasm and well-defined nucleus (Figure 1B/D/F/H I). For the present the liver sections from the model groups showed full fat vacuoles in the lobule cells, inflammatory cells infiltration, cell swelling and lipid degeneration

in the central region of the lobules (Figure 1B/D/F/H II). But in the liver section of ethanolic leaves extract of *Abroma augusta (ELEAA)* treated Sprague Dawley - rats; inflammatory response and lipid degeneration was remarkably reduced as compared with the standard groups and the liver cell volume became smaller, the droplet numbers of fat was reduced and the hepatic lobules were clearly represent (Figure 1B III-V).

**Effects of ethanolic leaves extract of *Abroma augusta (ELEAA)* on immuno-histochemistry analysis of *TNF – α* and *CYP2E1*:**

Immuno-histochemistry (ICH) analysis of the liver tissue showed that no *TNF – α* expression in the normal groups (Figure 2A/C/E/G I), but increased expression of *TNF – α* in the (MCD, HFD, Cholesterol and Cholate and Streptozotocin+HFD) models groups (Figure 2A/C/E/G II). After pretreatment with standard and ethanolic leaves extract of *Abroma augusta (ELEAA)* [250mg/kg and 500mg/kg], *TNF – α* expression decreased dose dependent manner, still higher than that of the normal groups (Figure 2A/C/E/G III-V). In the figure 3A-VI the quantification of the +ve expression are shown. The results are presented as mean ± SD of (n - 6). $^{b}P< 0.01$ were significantly different from the normal groups; $^{c}P< 0.05$ and $^{d}P< 0.01$ was significantly different from the model groups respectively. In the figure (4B/D/F/H) the normal liver expressed the less amount of CYP2E1 (Figure 2B/D/F/H I). The MCD, HFD, Cholesterol and Cholate and Streptozotocin+HFD model groups showed significantly higher expression of CYP2E1 as compared with the controls ($P< 0.01$ Figure 1B/D/F/H II). But the standard and ethanolic leaves extract of *Abroma augusta* treated groups (250mg/kg and 500mg/kg) showed remarkably decreased CYP2E1 expression (Figure 2B/D/F/H III-V). In the figure 4B-VI the quantification of the +ve expression of CYP2E1 are shown. The results are presented as mean ± SD of (n - 6). $^{b}P< 0.01$ were significantly different from the control group's $^{c}P< 0.05$ and $^{d}P< 0.01$ was significantly different from the model groups respectively.

The ethanolic leaves extract of *Abroma augusta* exhibits the highest and efficient hepatoprotective and antioxidant activity among all the Sprague Dawley - rat models, hence now which have increased their demand in the market of natural and herbal medicine. Lipid

metabolism play an important role in energy dissipation and hence responsible to maintain a steady state in the body. Disruption in the metabolism of lipids may lead to life threating situation like hypercholesterolemia, obesity, atherosclerosis, heart blocked etc. Thus prevention of lipid absorption could be an alternate strategy to treat obesity and NAFLD.

In the present study, compared to normal control groups, it was demonstrated that the liver coefficient and the levels of serum *ALT*, *AST*, *TG* and *TC* were significantly increased, the levels of *LDL-C* and *FFA* in liver was markedly increased, and *HDL-C* were markedly reduced in the MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD -induced Non-alcoholic fatty liver diseases (NAFLD). Pretreatment with ethanolic leaves extract of *Abroma augusta (ELEAA)* showed that *Abroma augusta* is able to inhibit the incremental changes in *ALT* and *AST*, to decrease the *TG*, *TC*, *LDL-C* and *FFA* levels, and to increase *HDL-C* level. In addition, the histopathological changes from the microscopy observation correlated with the examination of the liver function. The centrolobular hepatic necrosis, ballooning degeneration, fatty changes and infiltrating lymphocytes was observed in Non-alcoholic fatty liver diseases (NAFLD) models groups. The treatment with ethanolic leaves extract of *Abroma augusta* (*ELEAA*) prevents these histopathological changes in the Sprague Dawley - rats with MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD induced NAFLD. Thus, these results suggested that the inhibition of the elevation of liver function markers, obvious lipid-lowering and liver damage may related to the protective effects of ethanolic leaves extract of *Abroma augusta (ELEAA)* against MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD - induced Non-alcoholic fatty liver diseases (NAFLD). Ethanolic leaves extract of *Abroma augusta* enhanced the activity of SOD, increased GSH and decrease MDA against the MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD - induced Non-alcoholic fatty liver diseases (NAFLD) in the Sprague Dawley - rats, suggesting that the activity of antioxidants may play a role in the mechanism of its hepatoprotective activity.

The central proinflammatory cytokine TNF – α is associated with the variety of physiological and pathological conditions, including Cytotoxicity, growth stimulation, immune modulation and

proinflammatory activity. The TNF – α is predominantly produced by the monocyte's macrophage lineage in the liver, and the main population of this lineage is Kupffer cells. Thus, increased TNF – α production by activation of Kupffer cells may be responsible for Non-alcoholic fatty liver diseases (NAFLD). Furthermore, the most current studies have indicated that inhibition of TNF – α could decrease the content of hepatic fatty storage in the MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD – induced Non-alcoholic fatty liver diseases (NAFLD) models. In the present study the effects of TNF – α in damaged liver was evaluated by IHC. Compared to the normal groups, Sprague Dawley - rats treated with MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD showed up – regulated expression of TNF – α, while pretreatment with ethanolic leaves extract of *Abroma augusta* led to down – regulated expression of TNF – α compared to the MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD – models groups. The isoforms 2E1 of CYP is one of the most potent microsome cytochrome to generate ROS, and it is involved in the metabolism of Isoniozid and the mediation of its Hepatotoxicity [48], which has been exhibited to be invariably increased in the livers of Non-alcoholic fatty liver diseases (NAFLD) patients. In the present study, the expression of CYP2E1 in MCD Diet (Methionine and Choline deficient diet), HFD (High Fat Diet), CCD (Cholesterol and Cholate diet) and Streptozotocin + HFD models groups was observed to be increased, while the ethanolic leaves extract of *Abroma augusta* treated groups showed a significant down regulation of its expression, especially in the high dose of ethanolic leaves extract of *Abroma augusta* treated groups.

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**Claims:**

1) Herein we claim Hepatoprotective and Antioxidant activity of a Novel active herbal agent against Non-alcoholic fatty liver disease.
2) We also claim the method to develop the formulation claimed in 1.

3) For increasing absorption of formulation claimed in 1 the ethanolic extract is most suitable method.
4) We also claim that ethanolic leaves extract of Abroma augusta (ELEAA) has a protective effect on nonalcoholic fatty liver diseases.
5) We also claim that active herbal agent down regulate expression of TNF-α, and that CYP2E1 is one of the action mechanisms of Abroma augusta.

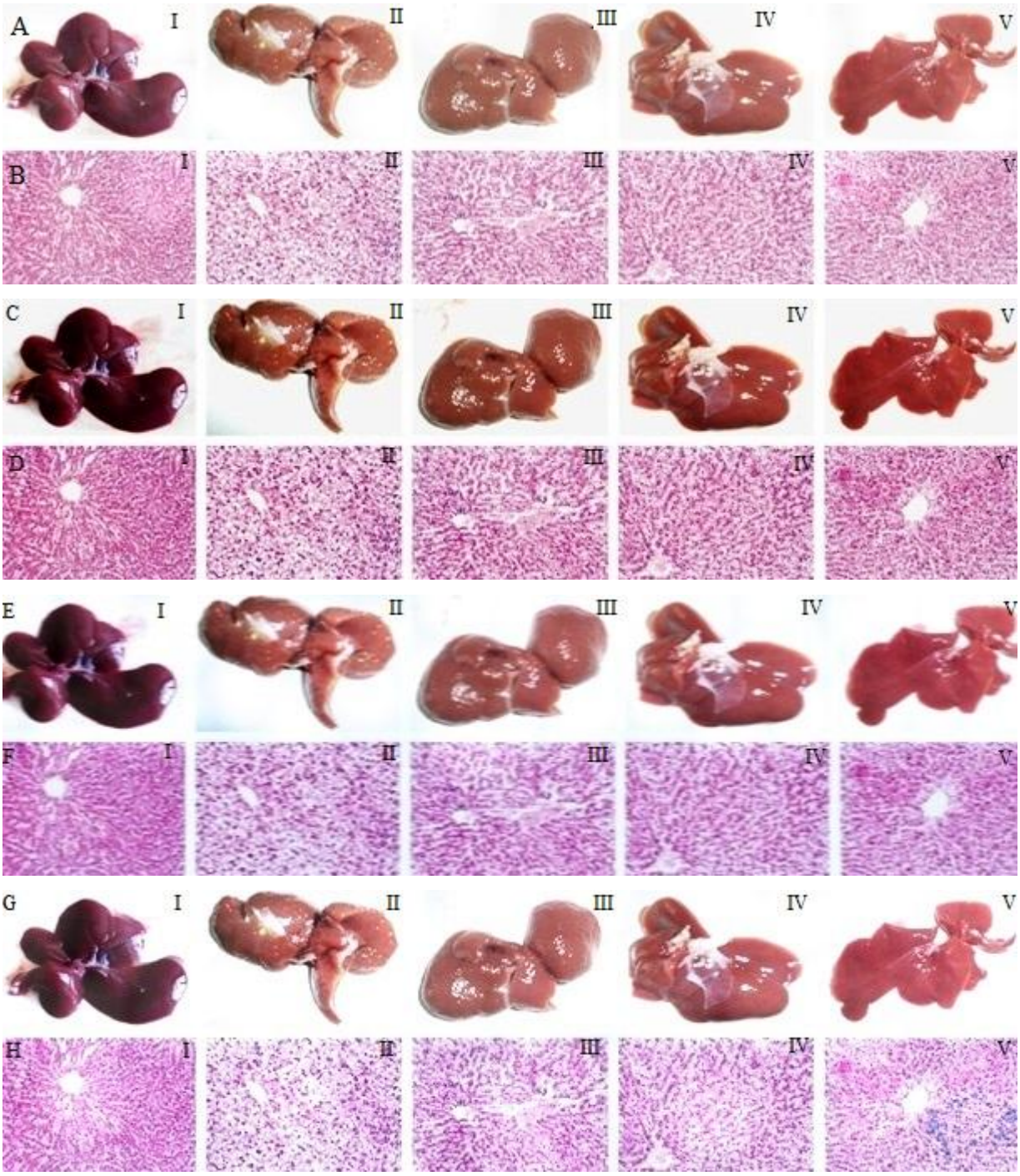


**Figure 1:** Appearance of SD-rats liver tissue (A/C/E/G) and Histopathological examination by HE (B/D/F/H, 200 x). I: Control group; II: Model group; III: Standard group; IV: ELEAA 250mg/kg group; V: ELEAA 500mg/kg group.

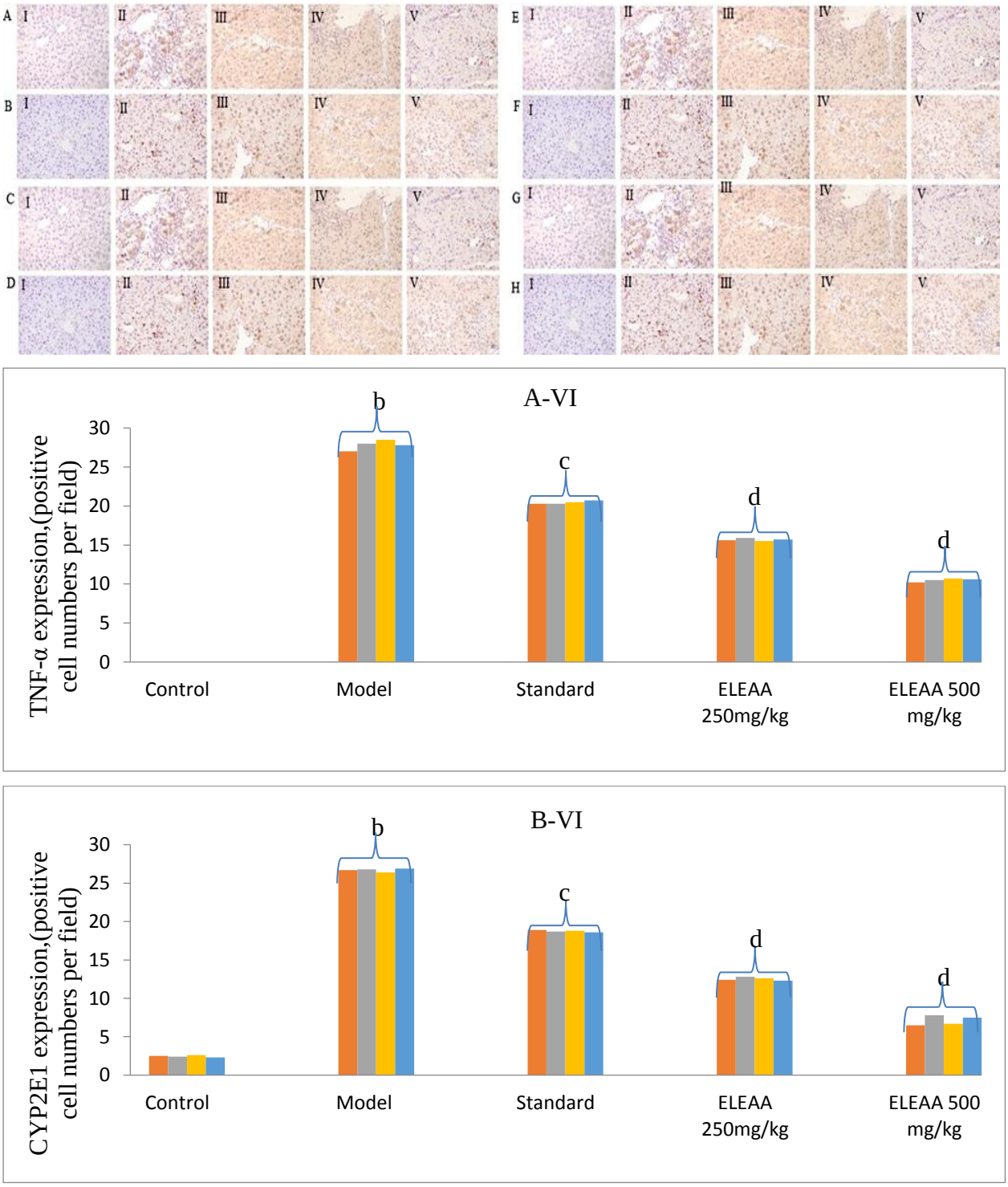


**Figure 2:** Representative photographs of immunological histological chemistry examination (200 x). A/C/E/G: TNF-α; B/D/F/H: CYP2E1, I: Control group; II: Model group; III: Standard group; IV: ELEAA 250mg/kg group; V: ELEAA 500mg/kg group; VI: Quantification of TNF-α (A-VI) and CYP2E1 (B-VI) stained cells. The results are expressed as mean ± SD of 12 rats. $^{b}P < 0.01$ vs control groups; $^{c}P < 0.05$, $^{d}P < 0.01$ vs model groups.