

Hepatoprotective effects of *Erythrina abyssinica* Lam Ex Dc against Non Alcoholic Fatty Liver Disease in Sprague Dawley Rats

By

1. Felix Kanyi Macharia*

Department of Medical Physiology, School of Medicine, College of Health Sciences,

University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya.

Email Address: hollafelix@gmail.com

2. Peter Waweru Mwangi

Department of Medical Physiology, School of Medicine, College of Health Sciences,

University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya.

Email Address: waweruk2001@gmail.com/peterwaweru@uonbi.ac.ke

3. Abiy Yenesew

Department of Chemistry, College of Physical and Biological Sciences,

University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya.

Email Address: ayenesew@uonbi.ac.ke

4. Frederick Bukachi,

Department of Medical Physiology, School of Medicine, College of Health Sciences,

University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya.

Email Address: fbukachi@uonbi.ac.ke

5. Nelly Murugi Nyaga

Department of Medical Physiology, School of Medicine, College of Health Sciences,

University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya.

Email Address: nellyflashier@gmail.com

6. David Kayaja Wafula

Department of Medical Physiology, School of Medicine, College of Health Sciences,

University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya.

Email Address: dkayaja17@gmail.com

Key: *Corresponding Author

ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome recognized as the most prevalent chronic liver disease across all age groups. NAFLD is strongly associated with obesity, insulin resistance, hypertension and dyslipidemia. Extensive research efforts are geared, through pharmacological approach, towards preventing or reversing this. *Erythrina abyssinica* Lam ex DC is an indigenous tree used widely used in traditional medicine, including for the treatment of liver related diseases, and has been shown to possess hypoglycemic, anti-oxidant, antimicrobial and anti-plasmodia effects. The present study is aimed at establishing the effects of *E. abyssinica* on the development of Non-alcoholic fatty liver disease induced by a high-fat and high-sugar diet in rats, *in-vivo* model.

Methods: Forty rats (40) were randomly divided into five groups: positive control (pioglitazone), Negative control (high fat/high sugar diet), low test dose (200 mg/kg), high test dose (400 mg/kg) and normal group (standard chow pellets and fresh water).

The inhibitory effect of the stem bark extract of *E. abyssinica* on the development of NAFLD was evaluated by chronic administration the herb extracts to rats on a high-fat/high-sugar diet. Biochemical indices of hepatic function including serum lipid profile, serum aspartate transaminase and alanine transaminase levels were then determined. Histological analysis of liver samples was carried out to quantify the degree of steato-hepatitis. Liver weights were taken and used to determine the hepatic index. The data was analyzed using one-way ANOVA, and Tukey's post-hoc tests were done in cases of significance. Histology data was analyzed using Kruskal-Wallis and Dunn's post-hoc test was done in cases of significance. Significance was set at $p < 0.05$.

Results: The freeze dried extract of *E. abyssinica* had significant effects on **fasting blood glucose** [5.43 ± 0.17 (HF/HSD) vs 3.8 ± 0.15 (E 400 mg/kg) vs 4.54 ± 0.09 (E 200 mg/kg) vs 4.16 ± 0.13

(PIOG) vs. 2.91 ± 0.16 (normal control): P value < 0.0001], and **insulin sensitivity** [329.4 ± 13.48 mmol/L · min (HF/HSD) vs. 189.8 ± 12.11 mmol/L · min (E 400 mg/kg) vs. 233.8 ± 6.55 mmol/L · min (E 200 mg/kg) vs. 211.1 ± 7.35 mmol/L · min (PIOG) vs. 142.9 ± 11.94 mmol/L · min: P value < 0.0001],

The extract had significant effects on hepatic indices including, **hepatic triglycerides** (P value < 0.0001), **liver weights** (P value < 0.0001), **liver weight-body weight ratio** (P value < 0.0001), **serum ALT levels** (P value < 0.0001), **serum AST** (P value < 0.0017), **serum total cholesterol** (P value < 0.0001), **serum triglycerides** (P value < 0.0001), and **serum LDL-cholesterol** (P value < 0.0001). The extracts however showed no significant effects on **HDL-cholesterol** (P value = 0.4759).

Histological analysis showed that the extract appears to possess protective effects against steatosis, inflammation and hepatic ballooning, with the high dose (400mg/kg) being more hepato-protective.

Conclusion:

The freeze dried stem bark extract of *Erythina abyssinica* possesses significant inhibitory effects against the development of NAFLD in Sprague Dawley rats.

1.0 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition characterized by excessive accumulation of hepatic fat (primarily triacylglycerols) that occurs in the absence of secondary causes such as alcohol consumption, use of steatogenic medication and steatogenic hereditary disorders[1–4]. This condition which has a prevalence of between 6% and 35% globally may often progress to hepatic failure or hepatocellular carcinoma and is the leading cause of chronic liver disease worldwide [2,3].

Although initially recognized as being primarily as a hepatic disorder, NAFLD is now known to affect multiple organs in the body such as the heart, kidneys and endocrine organs, and is a major risk factor in chronic diseases affecting these organs [5–8]. More pertinently, NAFLD is now recognized as the hepatic manifestation of metabolic syndrome with the majority of NAFLD patients having some, if not all, features of this syndrome [2,9,10]. Indeed, longitudinal studies suggest that NAFLD precedes development of both type 2 diabetes mellitus (T2DM) and metabolic syndrome [6,8,11]. Unfortunately, no pharmacological treatment has been shown to reverse the natural history of NAFLD with current treatment regimens focusing mainly on exercise and dietary modifications [12,13].

This study investigated the effects of the chronic administration freeze dried extracts of *Erythrina abyssinica* Lam ex DC (a plant species which has several medicinal uses in African communities and possesses demonstrated hypoglycemic and cytotoxic effects) in preventing the development of NAFLD in Sprague Dawley rats. The NAFLD was induced by chronically feeding the rats a high fat/high sugar diet containing monosodium glutamate.

The freeze-dried stem bark extracts of *Erythrina abyssinica* Lam Ex DC significantly decreased biochemical and histological markers associated NAFLD namely: fasting blood

glucose, insulin resistance, serum triglycerides, serum total cholesterol, serum ALT, hepatocellular ballooning, micro-vesicular steatosis and lobular inflammation. The freeze-dried extracts of *Erythrina abyssinica* Lam ex. DC therefore significantly inhibited the development of NAFLD in Sprague Dawley rats.

2.0 Materials and Methods

The study was conducted in the Department of Medical Physiology, University of Nairobi.

2.1 Plant Collection and Extract Preparation.

The stem barks of *Erythrina abyssinica* Lam ex DC were harvested from trees growing in Kitui County, Kenya and the identity of the collected plant specimens verified by the staff of the university herbarium located at the School of Biological Sciences, University of Nairobi and a voucher specimen deposited therein (EA2016). The plant parts were air dried for ninety-six hours after which they were milled into a fine powder at the Department of Chemistry, University of Nairobi.

The weight of the resultant powder was then measured using an analytical scale. The powder was then macerated in water in a ratio of 1:10 (weight: volume) and left to stand for one (1) hour. The resulting suspension was then sequentially filtered using cotton wool and Whatmann's® filter paper to obtain the filtrate which was then placed in a standard laboratory freezer overnight. The filtrate was then lyophilized, and the resulting freeze-dried extract weighed and placed in amber colored sample bottles and stored within a laboratory freezer until when required for administration to the experimental animals.

2.2 *In vivo* experiments.

Forty (40) freshly-weaned (4 week old) Sprague-Dawley rats (twenty female and twenty male) weighing between thirty to fifty grams were randomized into the normal control (normal diet and distilled water), negative control (high fat/ high sugar diet only), the positive control (high fat/high sugar diet plus pioglitazone), low dose test (high fat/ high sugar diet plus 200mg/kg freeze

dried extract of *Erythrina abyssinica* and high dose test (high fat/high sugar diet plus 400mg/kg freeze dried extracts of *Erythrina abyssinica*) groups.

The experimental animals were housed at the animal house located within the Department of Medical Physiology University of Nairobi. The following were the conditions maintained within the animal house: at a room temperature of 23.2°C, relative humidity of 60+/- 10 with twelve-hour light/dark cycles. They were habituated for two weeks prior to the start of the study and were fed standard chow pellets ad libitum during this period.

The high fat/ high sugar diet was prepared by the addition of 20% w/w saturated fat (Cowboy™ manufactured by Bidco™ industries) to standard rat chow which contained protein 29.82%, fat 13.43% carbohydrates 56.74%, fiber 5.3%, vitamins and minerals small quantities-ppm (Unga™ Feeds) to ensure that the pellets delivered approximately 30% of calories as fat. Sucrose on the other hand was added to drinking water to make up a 30% sucrose solution. Monosodium glutamate (MSG) was added to the pellets to make up 0.8% of the pellets to increase the palatability of the pellets [14] . The resulting high-fat/high sugar diet contained 30% fat and 30% sucrose in water.

All the animals received diets ad libitum and the extracts/treatments were administered via oral gavage.

2.3 Ethical approval

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the bio-safety, animal use and ethics committee Department of Veterinary physiology, University of Nairobi (REF: FVM BAUEC/2016/111) In addition, the experimental

design obeyed the 4Rs (reduction, replacement, refinement and rehabilitation) of ethical animal experimental design [15,16] .

2.4 Assay of fasting blood glucose

Assay of fasting blood glucose was performed in the manner described by Lee and Goossens [17]. Briefly blood was sampled from the lateral tail vein after a six hour fast. The tail was warmed by dipping in warm water for five (5) minutes to cause vasodilation of the vein and therefore ease visualization and blood sample collection. The blood glucose was determined using a glucometer (Nova Biomedical) and recorded.

2.5 Insulin tolerance test

Insulin tolerance tests were performed on Days 21, 42 and 56. The insulin tolerance tests were performed according to the protocol by Bowe [18]. Briefly the experimental animals were fasted for six (6) hours. They were then weighed after which soluble insulin (Humulin S™ Eli Lilly USA) was administered by an intraperitoneal injection at a dose of 0.75IU/kg. The blood glucose levels were then assayed at regular time intervals after the administration of the insulin as described in 3.2.2. (at time 0 minutes, 30 minutes, 60 minutes, 90 minutes). A 50% Glucose solution was made available to prevent fatalities due to insulin-induced hypoglycemia (i.e. if blood glucose levels ≤ 1.1 mmol/L). The animals would be withdrawn from the procedure if this happened and the glucose solution administered via oral gavage. The areas under the curve (AUCs) for the respective rats were then calculated and then recorded.

2.6 Assay of serum biochemistry

The procedure for determination of the serum biochemical parameters was as follows: the rats were fasted overnight on the last day of the study (day fifty-six) and then sacrificed under

euthanasia using pentobarbital. The absence of the Pupillary light reflex was used as a confirmative marker of death. Five (5) milliliters of blood were harvested from each using cardiac puncture. The collected blood was then centrifuged to obtain serum which was then assayed for the following biochemical parameters: fasting serum triglycerides, HDL cholesterol, LDL-cholesterol and total cholesterol, aspartate transaminase (AST) and alanine transaminase. The assays were performed at the Clinical Chemistry laboratory, Kenyatta National Hospital.

2.7 Assay of hepatic triglycerides.

A midline abdominal incision was performed after the rats were euthanized and the liver tissues excised. The respective livers were then weighed using an analytical balance and the weights recorded. The respective liver tissues were each divided into two unequal portions with the larger portion weight: smaller portion weight ratios being 2:1. The smaller portions were used in the hepatic triglyceride assay while the larger portions were used in the histological studies.

The smaller portions were stored in a laboratory freezer and then later homogenized with phosphate buffer acting as the liquid medium. The resulting liquid homogenates were then used to determine hepatic triglyceride content using the protocol previously described by Butler et.al [19].

2.8 Liver histology

The larger portions of the harvested liver tissues were preserved in 10 % neutral formaldehyde. Sections (4 mm² in size) were later fixed in PAF and embedded in paraffin. These sections were further sliced to 5 µm and stained with H/E stain for evaluation of hepatic steatosis.

Liver tissues were scored according to the grading system introduced by [20] for NAFLD: Steatosis: Grade 1 < 33 % steatotic hepatocytes, Grade 2 < 33% to 66% steatotic hepatocytes, grade 3 > 66% steatotic hepatocytes in a X 200 microscopic field of view.

Hepatocellular ballooning: Grade 1- minimal zone 3, Grade 2 – present zone 3, Grade 3- marked predominantly zone 3.

Lobular inflammation: 0- absent, grade 1 <2 foci of inflammation, grade 2: 2-4 foci, grade 3 > 4 foci.

Portal inflammation 0 –absent, grade 1 – mild, grade 2- moderate, grade 3-severe.

Stages of fibrosis: stage 1: zone 3 peri-sinusoidal fibrosis, stage 2-focal or extensive periportal fibrosis, stage 3- bridging fibrosis, stage 4-cirrhosis.

2.9 Statistical analysis

The experimental data were expressed as Mean \pm S.E.M. and analyzed using One-way ANOVA and Tukey's post-hoc tests carried out in cases of significance (set at $p < 0.05$) using Graph Pad Prism™ software version 6. The semi-quantitative histological data were analyzed using the Kruskal-Wallis and Dunn's post-hoc tests were carried out in cases of significance which was also set at $p < 0.05$.

3.0 RESULTS

3.1 Extract preparation

The stem barks of *Erythrina abyssinica* Lam ex DC were harvested from trees growing in Kitui county, Kenya. The plant parts were air dried for forty-eight hours. The dried parts were then milled at the Department of Chemistry, University of Nairobi. The weight of the resultant powder was measured using an analytical scale. The powder was mixed in a ratio of 1:10 with water in a flask and swirled severally before being left to settle. The mixture was then filtered using cotton wool and Watmann® filter paper to obtain a crude extract solution which was frozen and lyophilized at ICIPE Nairobi, Kenya.

The percentage yield of the extract was 3.4% (885.3g of plant powder yielded 29.88g of extract)

3.2 Fasting blood glucose results

There were significant differences in blood glucose levels between the experimental groups on day seven (7) [4.89 ± 0.24 mmol/L (negative control) vs 4.01 ± 0.21 mmol/L (test high dose) vs 4.14 ± 0.14 mmol/L (test low dose) vs. 4.13 ± 0.19 mmol/L (positive control) vs 3.56 ± 0.30 mmol/L (normal control): P value = 0.0043]. Post-hoc analysis showed that there were significant differences between the negative control and normal control groups (P value = 0.0017).

There were also significant differences between the experimental groups on day fourteen [5.19 ± 0.24 mmol/L (negative control) vs. 4.03 ± 0.30 mmol/L (test high dose) vs. 4.7 ± 0.20 mmol/L (test low dose) vs. 4.26 ± 0.20 mmol/L (positive control) vs. 3.83 ± 0.40 mmol/L (normal control): P value = 0.01]. Post-hoc analysis showed that there were significant differences between the negative control and the high dose test groups (P value = 0.0402) as well as between the negative control and the normal control groups (P value = 0.0012).

There was also significant difference between the experimental groups on day twenty-one [5.37 ± 0.27 mmol/L (negative control) vs. 4.38 ± 0.15 mmol/L (test high dose) vs. 4.95 ± 0.24 mmol/L (test low dose) vs. 4.8 ± 0.16 mmol/L (positive control) vs. 3.75 ± 0.21 mmol/L (normal control): P value < 0.0001]. Post-hoc analysis showed significant differences between the negative control and high dose test groups (P value = 0.017). Significant differences were also observed between the normal control and low dose test groups (P value = 0.0025) as well between the normal control and the negative control groups (P value < 0.0001).

There were significant differences between the experimental groups on day twenty-eight [5.3 ± 0.21 mmol/L (negative control) vs. 4.39 ± 0.14 mmol/L (test high dose) vs. 4.48 ± 0.29 mmol/L (test low dose) vs. 4.49 ± 0.13 mmol/L (positive control) vs. 3.75 ± 0.13 mmol/L (normal control): P value < 0.0001]. Post-hoc analysis showed that there were significant differences between the negative control and high dose test groups. (P value = 0.0166). In addition, there were significant differences between the two test groups (i.e. low dose and high test) (P value = 0.0407). As previously, there were significant differences between the negative control and normal control groups high-fat/high sugar diet groups (P value < 0.0001).

There were significant differences between the experimental groups on day thirty-five [4.89 ± 0.24 mmol/L (negative control) vs. 4.01 ± 0.21 mmol/L (test high dose) vs. 4.13 ± 0.14 mmol/L (test low dose) vs. 4.13 ± 0.19 mmol/L (Positive control) vs. 3.56 ± 0.31 (normal control): P value < 0.0049]. Post-hoc analysis showed significant differences between the negative control and the high dose test groups (P value = 0.0136) as well as between the negative control and positive control groups (P value = 0.0027).

There were significant differences between the experimental groups on day forty-two [5.26 ± 0.21 mmol/L (negative control) vs. 3.63 ± 0.13 mmol/L (test high dose) vs. 4.06 ± 0.11 mmol/L

(test low dose) vs. 4.23 ± 0.17 mmol/L (positive control) vs. 2.15 ± 0.08 mmol/L (normal control group): P value < 0.0001]. Post-hoc analysis showed significant differences between the negative control and high dose test groups (P < 0.0001) as well as between the negative control and the low dose test groups (P < 0.0001). There were however no significant differences between the low dose test and the high dose test groups (P value = 0.2465) as well as between the high dose and positive control groups (P value = 0.0508).

There were significant differences between the experimental groups on day forty-nine [5.27 ± 0.19 mmol/L (negative control) vs. 3.74 ± 0.08 mmol/L (test high dose) vs. 4.38 ± 0.10 mmol/L (test low dose) vs. 3.73 ± 0.13 mmol (positive control) vs. 2.68 ± 0.15 mmol/L (normal control): P value < 0.0001]. Post-hoc analysis showed there were significant differences between the negative control and high dose test groups (P < 0.0001) as well as between the negative control and the low dose test groups (P < 0.0001). There were also significant differences between the negative control and normal control groups (P < 0.0001).

There were significant differences between experimental groups on day fifty-six [5.43 ± 0.17 mmol/L (negative control) vs. 3.8 ± 0.15 mmol/L (test high dose) vs. 4.54 ± 0.09 mmol/L (test low dose) vs. 4.16 ± 0.13 mmol/L (positive control) vs. 2.91 ± 0.16 (normal control): P < 0.0001]. Post hoc analysis showed there were significant differences between the negative control and the high dose test groups (P < 0.00001) as well between the negative control and the low dose test groups (P < 0.0001). There were significant differences between the two test groups (P value = 0.0070). In addition, there were significant differences between the normal control group and either of the test groups i.e. the low dose test (P < 0.0001) and the high dose test (P = 0.0009) groups. There were however, no significant differences between the positive control and either of the test groups i.e. the high dose test (P = 0.3918) or low dose test (P = 0.3580) groups.

The results of the fasting glucose experiments are graphically represented in figures 1 (A-H).

Figures 1 (A-H) appear here

FIGURE 1 (A-E) Fasting blood glucose in mmol/l among the five groups, on day 7(A), day 14(B), day 21 (C), day 28 (D), day 35 (E), day 42 (F), day 49 (G), and day 56(H). * P VALUE < 0.05, **P VALUE <0.01, ***P VALUE <0.001, **** P VALUE<0.0001.

3.3 Insulin Tolerance Test results

(i) Day 21

There were significant differences in the area under the curves (AUCs) of the insulin tolerance tests between the experimental groups on day twenty-one [237.9 ± 21.39 mmol/L · min (negative control) vs. 179.1 ± 16.55 mmol/L · min (test high dose) vs. 199.3 ± 13.12 mmol/L · min (test low dose) vs. 230.6 ± 17.79 mmol/L · min (positive control) vs. 116.3 ± 12.15 mmol/L · min (normal control): $P < 0.0001$].

Post-hoc analysis showed significant differences in insulin sensitivity between the negative control and normal control (P value < 0.0001) groups as well as between the normal group and the low dose test groups ($P = 0.0092$). There were also significant differences between the normal control and the positive control groups ($P = 0.0239$). A graphical presentation of these experimental data is shown in figure 2.

Figure 2 appears here.

Figure 2. Area under the curve for insulin tolerance tests in mmol/L · min at day 21 *P value < 0.05, **P value < 0.01, ****P value < 0.0001

(ii) Day 42

There were significant differences in the area under the curves (AUCs) of the insulin tolerance tests between the experimental groups on day forty-two [332.8 ± 37.84 mmol/L · min (negative control) vs. 180 ± 7.48 mmol/L · min (test high dose) vs. 258.4 ± 9.29 mmol/L · min (test low dose) vs. 186.6 ± 7.1 mmol/L · min (positive control) vs. 111.4 ± 4.66 mmol/L · min (normal control): $P < 0.0001$].

Post hoc analysis showed significant differences in insulin sensitivity between the negative control and the high dose test ($P < 0.0001$) as well as the low dose test (P value < 0.0471) groups. There were however significant differences between the two test groups (i.e. low dose and high dose) ($P = 0.0399$). There were significant differences between the normal control and low dose test groups ($P < 0.0001$) as well between the normal control and the high dose test groups ($P = 0.0760$). As would be expected, there were significant differences between the negative control and normal control ($P < 0.0001$).

A graphical representation of the experimental data is shown in figure 3

Figure 3 appears here.

Figure 3. Area under the curve for insulin tolerance tests in mmol/L·min at day 42. * $p < 0.05$, ** $p < 0.01$, ** $p < 0.0001$**

There were significant differences in the area under the curves (AUCs) of the insulin tolerance tests between the experimental groups on day fifty-six [329.4 ± 13.48 mmol/L · min (negative control) vs. 189.8 ± 12.11 mmol/L · min (test high dose) vs. 233.8 ± 6.55 mmol/L · min (test low dose) vs. 211.1 ± 7.35 mmol/L · min (positive control) vs. 142.9 ± 11.94 mmol/L · min (normal control): $P < 0.0001$].

Post-hoc analysis showed significant differences in insulin sensitivity between the negative control and the high dose test ($P < 0.0001$) as well as the low dose test ($P < 0.0001$) groups. There were significant differences between the high dose test and low dose test groups ($P = 0.0447$). There were however also significant differences between the normal control and the high dose test ($P \text{ value} = 0.0286$) and the low dose test groups ($P < 0.0001$).

There were no significant differences between the positive control and the low dose test ($P = 0.5635$) or the high dose test ($P = 0.6230$) groups. A graphical representation of the experimental data is shown in figure 4

Figure 4 appears here.

Figure 4. Area under the curve for insulin tolerance tests in mmol/L·min at day 56. *P value < 0.05, **P value < 0.01, **P value < 0.0001.**

3.4 Hepatic Triglycerides

There were significant differences in the mean hepatic triglyceride levels between the experimental groups [6.09 ± 0.56 mg/gram of liver tissue (negative control) vs. 2.06 ± 0.21 mg/gram (test high dose) vs. 3.86 ± 0.55 mg/g (test low dose) vs. 3.92 ± 0.44 mg/ gram (positive control) vs. of 2.05 ± 0.28 mg/gram (normal control): $P < 0.0001$]. Post-hoc analysis showed significant differences in the levels of hepatic triglycerides between the negative control and normal control groups ($P \text{ value} < 0.0001$). There were significant differences between the negative control and both the high dose test ($P < 0.0001$) and low dose test groups ($P = 0.0071$). There were significant differences between the low dose and high dose test groups ($P = 0.0414$).

The experimental data are graphically represented in figure 5.

Figure 5 appears here.

Figure 5: **Hepatic triglycerides in mmol/L . NC (Negative control), PC (Positive control) **P value<0.01****P value < 0.0001**

3.5 Hepatic weights

There were significant differences in liver weights between the five groups [7.02 ± 0.42 g (negative control) vs. 2.68 ± 0.14 g (high dose test) vs. 3.84 ± 0.29 g (low dose test) vs. 5.40 ± 0.29 g (Positive control) vs. 2.65 ± 0.11 g (normal control): P value < 0.0001]. Post-hoc statistical analysis using the Tukey's test showed that there were significant differences between the negative control and the normal control groups (P value < 0.0001). In addition, there were significant differences between the high dose test (P < 0.0001) as well as the low dose test (P = 0.0379) and the negative control groups. There were significant differences in liver weights between the normal control and the low dose test groups (P = 0.031). However, there were no significant differences between the high dose test and the normal control groups (P = 0.99). A graphical representation of the results is shown in figure 6.

Figure 6 appears here

Figure 6. **Mean liver weights in grams. NC (Negative control), PC (Positive control) * P value < 0.05, **P value < 0.01, **** P value < 0.0001.**

3.6 Hepatic Index

There were significant differences in the hepatic index (mean liver-body weight ratio presented as a percentage (%)) between the experimental groups [$6.1 \pm 0.37\%$ (negative control)

vs. $2.4 \pm 0.17\%$ (high dose test) vs. $3.88 \pm 0.37\%$ (low dose test) vs. $4.4 \pm 0.28\%$ (positive control) vs. $1.7 \pm 0.08\%$ (normal control): $P < 0.0001$].

Post-hoc statistical analysis using the Tukey's test showed that there were significant differences between the negative control and the high dose test ($P < 0.0001$) as well as the low dose test groups ($P < 0.0001$). There were significant differences between the high dose and low dose test groups (P value = 0.0022). There were significant differences between the low dose test and the normal control groups ($P < 0.0001$). In contrast, there were no significant differences between the high dose test and the normal control groups (P value = 0.3874).

A graphical representation of the results is shown in figure 7.

Figure 7 appears here.

Figure 7. Hepatic index. NC (Negative control), PC (Positive control) **P value < 0.0001**

3.7 Hepatic Function Tests

3.7.1 Serum ALT

There were significant differences in serum ALT concentration between the five groups [155.6 ± 4.59 IU (negative control) vs. 91 ± 6.85 IU (high dose test) vs. 116.1 ± 3.96 IU (low dose test) vs. 119.9 ± 9.02 IU (Positive control) vs. 76.38 ± 5.01 IU (normal control): P value < 0.0001]. Post-hoc statistical analysis using the Tukey's test showed there were significant differences between the negative control and the normal control groups ($P < 0.0001$). There were also significant differences between the high dose test and negative control groups ($P < 0.0001$) as well as between the Low dose test and negative control groups ($P < 0.006$). There were also significant differences

between the high dose and low dose test groups ($P = 0.0493$). A graphical representation of the results is shown in figure 8.

Figure 8 appears here

Figure 8. serum ALT in IU/L NC (Negative control), PC (Positive control). *P value < 0.05, * P value < 0.001, **** P value < 0.0001.**

3.7.2 Serum AST levels

There were significant differences in serum AST levels between the five groups [133 ± 8.27 IU/L (low dose test) vs. 123.6 ± 11.72 IU/L (high dose test) vs. 150.9 ± 8.39 IU/L (negative control) vs. 132.8 ± 11.89 IU/L (positive control) vs. 92.7 ± 4.05 IU/L (normal control): $P < 0.0017$]. Post-hoc statistical analysis using the Tukey's test showed significant differences between the normal control and low dose test groups ($P = 0.0294$). There were significant differences between the high dose test and the normal control groups ($P = 0.308$).

There were however no significant differences between the negative control and the low dose test groups ($P = 0.648$) as well between the negative control and the high dose test groups ($P = 0.243$). A graphical representation of these results is shown in figure 9.

Figure 9 appears here

Figure 9 serum AST in IU/L. NC (Negative control), PC (Positive control). *p < 0.05, *P < 0.00001.**

3.8 Lipid Profiles

3.8.1 Total Cholesterol

There were significant differences in serum total cholesterol levels between the five groups [3.39 \pm 0.21 mmol/L (negative control) vs. 1.91 \pm 0.16 mmol/L (high dose test) vs. 2.58 \pm 0.15 mmol/L (low dose test) vs. 2.59 \pm 0.16 mmol/L (positive control) vs. 1.62 \pm 0.15 mmol/L (normal control): P < 0.0001].

Post-hoc statistical analysis using the Tukey's test showed that there were significant differences between the negative control and the normal control groups (P < 0.0001). There were also significant differences between the negative control and the high dose test groups (P < 0.0001) as well as between the negative control and the low dose test groups (P = 0.0071). There were also significant differences between the high dose and low dose test groups (P = 0.0419).

A graphical representation of the results is shown in Fig. 10.

Figure 10 appears here

Figure 10. of serum total cholesterol in mmol/l. NC (Negative control), PC (Positive control)

P value < 0.05, **P value < 0.01, ***P value < 0.001, * P value < 0.0001.**

3.8.2 Serum triglycerides

There were significant differences in the serum triglyceride levels among the five groups [2.20 \pm 0.16 mmol/L (negative control) vs. 0.74 \pm 0.12 mmol/L (high dose test) vs. 1.26 \pm 0.16 mmol/L (low dose test) vs. 1.99 \pm 0.07 mmol/L (positive control) vs. 0.5 \pm 0.06 mmol/L (normal control): P < 0.0001].

Post-hoc statistical analysis using the Tukey's test showed that there were significant differences between the negative control and the high dose test groups (P < 0.0001) as well as between the

low dose test and negative control groups ($P < 0.0001$). There were also significant differences between the high dose and low dose test groups ($P = 0.0345$).

A graphical representation of these results is shown Figure 11.

Figure 11 appears here

Figure 11. Serum triglyceride levels in mmol/L. NC (Negative control), PC (Positive control).

P value < 0.05, *P value < 0.0001.**

3.8.3 Serum LDL cholesterol

There were significant differences in serum LDL cholesterol concentration among the five groups [2.02 ± 0.27 mmol/L (negative control) vs. 0.56 ± 0.09 mmol/L (high dose test) vs. 1.30 ± 0.11 (low dose test) vs. 1.16 ± 0.14 mmol/L (positive control) vs. 0.05 ± 0.08 mmol/L (normal control): $P < 0.0001$].

Post-hoc statistical analysis using the Tukey's test showed that there were significant differences between the negative control and high dose test groups ($P < 0.0001$) as well as between the low dose test and negative control groups ($P = 0.0172$). In addition, there were significant differences between the low dose test and high dose test groups ($P = 0.0149$).

A graphical representation of these results is represented in Fig. 12.

Figure 12 appears here.

Figure 12. Serum LDL-cholesterol in mmol/L. NC (Negative control), PC (Positive control)

*** P value < 0.05, **P value <0.01, ****P value < 0.0001.**

There were no statistically significant differences in HDL cholesterol concentration among the five groups (P value = 0.4759). However, the negative control group had the lowest HDL concentration among the groups

3.9. Histological evaluation of the liver

There were significant differences in hepatocellular ballooning among the five groups (P < 0.0001). Post-hoc analysis statistical demonstrated that the negative control group had significantly higher hepatocellular ballooning than the normal control group (P < 0.0001). There were significant differences between the negative control and the high dose test group (P = 0.0002 as well as between the low dose test and negative control groups (P = 0.0055). These results are represented in table 2 and table 3.

There were significant differences in occurrence of micro vesicular steatosis among the five groups (P < 0.0001). Post hoc statistical analysis showed that the negative control group had significantly more steatosis compared to: the high dose test (P = 0.0009), low dose test (P = 0.0009) and the normal control (P < 0.0001) groups. These results are represented in table 1 and table 3

There were significant differences in lobular inflammation among the 5 groups (P < 0.0001). Post-hoc statistical analysis showed that there were significant differences in lobular inflammation in the negative control group compared to: high dose test (P = 0.0001), low dose test (P = 0.0060) and normal control groups (P = 0.0001). These results are represented in table 1 and table 3.

There were no significant differences between the high dose test and the low dose test groups with respect to hepatocellular ballooning (P = 0.99), micro vesicular steatosis (P = 0.99) and lobular inflammation (P = 0.99).

There were significant differences in portal inflammation between the five groups (P value = 0.0460) but no significant differences were observed on post-hoc statistical analysis (P>0.05). The negative control group however had the highest incidence rates of grade 2 (50%) and grade 1 (50%) portal inflammation. These results are represented in table 2 and table 3.

There was no fibrosis or macro vesicular steatosis was observed in any of the experimental groups.

Table 1 .Table showing incidence rates (%) of steatosis and lobular inflammation among the different groups.

GRADE	STEATOSIS				LOBULAR INFLAMMATION			
	0	1	2	3	0	1	2	3
200mg/kg	62.5%	25%	12.5%	–	75%	25%	–	–
400mg/kg	87.5%	12.5%	–	–	100%	–	–	–
Negative control	–	25%	75%	–	–	62.5%	37.5%	–
Positive control	75%	25%	–	–	75%	25%	–	–
Normal control	100%	–	–	–	100%	–	–	–

Table 2. Table showing incidence rates (%) of hepatocellular ballooning and portal inflammation.

GRADE	HEPATOCELLULAR BALLOONING				PORTAL INFLAMMATION			
	0	1	2	3	0	1	2	3
200mg/kg	50%	37.5%	–	12.5%	75%	25%	–	–
400mg/kg	100%	–	–	–	100%	–	–	–
Negative control	–	12.5%	37.5%	50%	–	50%	50%	–
Positive control	87.5%	12.5%	–	–	62.5%	37.5%	–	–
Normal control	100%	–	–	–	100%			

Table 3. Histology data presented as median (min.-max.) *significantly different from the Negative control group (p value< 0.05).

HISTOLOGICAL FEATURE	200mg/kg	400mg/kg	Negative control	Positive control	Normal control
Macro-vesicular steatosis	–	–	–	–	–
Micro-vesicular steatosis	0(0-1)*	0(0-1)***	1.5(1-2)	0(0-1)**	0(0-0)***

Hepatocellular ballooning	0.5(0-1)**	0(0-1)***	2.5(2-3)	0.5(0-2)**	0(0-0)****
Lobular inflammation	0(0-1)**	0(0-1)***	0(1-2)	0(0-1)**	0(0-0)***
Portal inflammation	0(0-0)	0(0-0)	0.5(0-2)	0(0-1)	0(0-0)

4.0 Discussion

Nonalcoholic fatty liver disease (NAFLD) covers a wide range of hepatic changes, ranging from simple steatosis to non-alcoholic steato-hepatitis (NASH), liver cirrhosis and hepatocellular carcinoma and is one of the most common chronic liver disorders worldwide [1,3,5]. It is associated with diverse clinical conditions including but not limited to obesity, renal failure, osteoporosis, type 2 diabetes, myocardial infarction, hypothyroidism, male infertility among others [5,6,8,21–25]. Current therapies for this condition however remain unsatisfactory underscoring the need for discovering new efficacious treatments for this condition [12,13,26].

Various animal models that aim to mirror both the histopathology and the pathophysiology of each stage of human NAFLD have been developed [27,28]. Indeed, selection of the appropriate animal model remains an important consideration in experimental design. We used a modified high fat/high sugar diet which was prepared by the addition of 0.8% monosodium-glutamate to a standard high-fat/high sugar diet. High-fat/high-sugar diets (“cafeteria diets”) are a popular animal model for the induction of NAFLD and are normally administered chronically over a period of six to sixteen weeks [29]. However, high-fat/high-sugar diets administered ad-libitum normally have the limitation of reduced food consumption compared to normal rat chow and this has been attributed to reduced palatability and satiety induction in the cafeteria diets [30]. Monosodium glutamate is a dietary tastant that confers the umami flavor to food and has been previously shown to increase food consumption was therefore added to the diet.

The NAFLD model used in this study reproduced the serum biochemical and histological (steatosis, inflammation and hepatocellular injury) features of NAFLD [27] showing that the model possessed both construct and ecological validity and can therefore be used experimentally in the evaluation of anti-NAFLD therapies.

This study investigated the effects of the chronic administration of the freeze-dried stem bark extracts of *Erythrina abyssinica* on the development of NAFLD. This plant species finds extensive application in traditional medicine and is used in the preparation of health tonics by the Maasai community of East Africa [31,32]. In addition, this plant is shown to be rich in flavonoids, a group of natural products known to be efficacious in the management of NAFLD and other metabolic syndrome conditions [33–36].

The freeze-dried stem bark extracts of *Erythrina abyssinica* displayed potent dose-dependent antihyperglycemic effects in rats chronically fed a high-fat/high-sugar diet. Previous studies have shown that plant species belonging to this genus including the species that is the subject of this study possess antihyperglycemic activity [37,38]. The freeze-dried extracts of *Erythrina abyssinica* also possessed significant effects on insulin sensitivity. This was a significant finding in our opinion since insulin resistance is regarded as the most important pathophysiological mechanism of NAFLD [10,39]. In addition, it is also known that insulin resistance is one of the multiple hits determining the progression from NAFLD to non-alcoholic steato-hepatitis (NASH) [10,20,40]. It can therefore be seen that the extract can potentially prevent the establishment of, as well as slow the progression of NAFLD via the positive effects on insulin resistance.

There several putative mechanistic explanations for the observed effects on blood glucose levels as well as the insulin resistance. Benzo furans and Coumestan which possess agonist activities on AMP Kinase (AMPK) have been isolated from this plant species [41]. Activation of this enzyme is an attractive pharmacological target for the treatment of type 2 diabetes and other metabolic disorders [42]. Indeed leading diabetic drugs such as metformin and rosiglitazone mediate their metabolic effects partially through AMPK activation [42–44].

In addition, fifteen pterocarpan derivatives which possess inhibitory effects on protein tyrosine phosphatase 1B (PTP1B) have been isolated from the stem bark of this plant species [33,38]. Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of the leptin and insulin signaling pathways and plays important roles in the pathogenesis of obesity and diabetes [45]. The inhibition of this enzyme can therefore be expected to have beneficial effects in obesity, diabetes as well as NAFLD. This observation is supported by the fact that mice with the whole body deletion of PTP1B were protected against the development of obesity and diabetes [45,46].

The freeze-dried extracts possessed significant anti-dyslipidemic effects i.e. lowering plasma total-cholesterol, LDL-cholesterol and serum triglycerides. This was an important finding in our opinion. This is because NAFLD is characterized by atherogenic dyslipidemia, postprandial lipemia and high-density lipoprotein (HDL) dysfunction which are established risk factors for both cardiovascular and liver disease [47,48]. A possible mechanistic explanation for the observed anti-dyslipidemic effects is the AMP kinase inhibition described previously. It is known that AMP-kinase phosphorylates and inactivates the Sterol Regulatory Element Binding Protein (SREBP1) which has been implicated in the hepatic accumulation seen in NAFLD via its effects on lipid biosynthesis, LDL receptor biosynthesis and de novo cholesterol biosynthesis [49].

The freeze-dried stem bark extracts of *Erythrina abyssinica* significantly reduced hepatic lipid deposition as shown by the reduced hepatic triglyceride concentrations, liver weights and liver weight-body. These hepatic indices have been shown to be increased in NAFLD as well as in insulin resistance [50]. This was also a key finding in our opinion since according to the “two-hit” hypothesis on the pathophysiology of NAFLD, the hepatic triglyceride deposition is the first hit while the lipotoxicity that accompanies this deposition leads to hepatic injury and inflammation marks the second hit [51].

The extract of *Erythrina abyssinica* had significant effects on the serum alanine amino-transferase levels (ALT) which are a recognized marker of hepatocellular injury. Indeed, several epidemiologic association studies have shown that this enzyme is elevated in, and consequently can be used as a biomarker of NAFLD [52–54].

The extract significantly prevented the development of Histopathological evaluation of the liver showed prevention of steatosis, inflammation and hepatocellular injury. These effects were especially marked in the higher dose test group (400 mg/kg). It can therefore be seen that the results of the histopathological examination agree with those of the hepatic enzymes assay: i.e. that the extracts prevent the development of hepatic injury despite the chronic administration of a high fat/high sugar diet for eight (8) weeks.

Conclusions and future directions

The freeze-dried extracts of *Erythrina abyssinica* had significant protective effects against the development of NAFLD in this study and show potential as a prophylactic against the development of this condition. These beneficial actions can be attributed to the rich variety of chemical compounds found in this plant species which have been shown to have positive effects in metabolic syndromes, cardiovascular disease and other conditions associated with aging [33,35,38,41,55]. The results of this study also validate the traditional use of this plant species as a tonic for the maintenance of good health.

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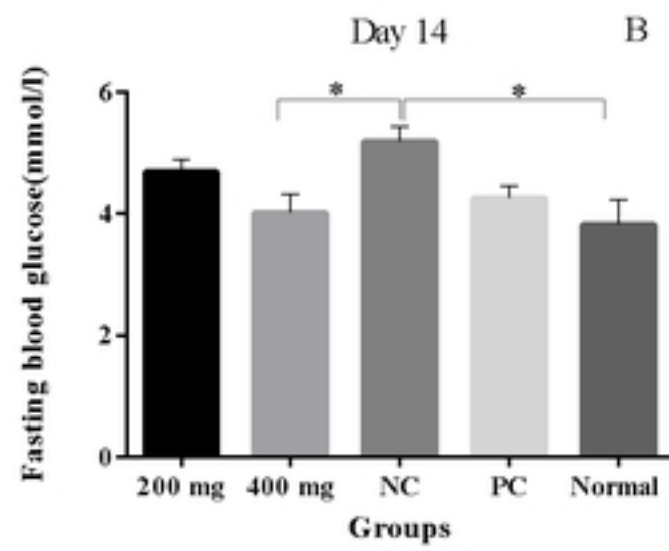
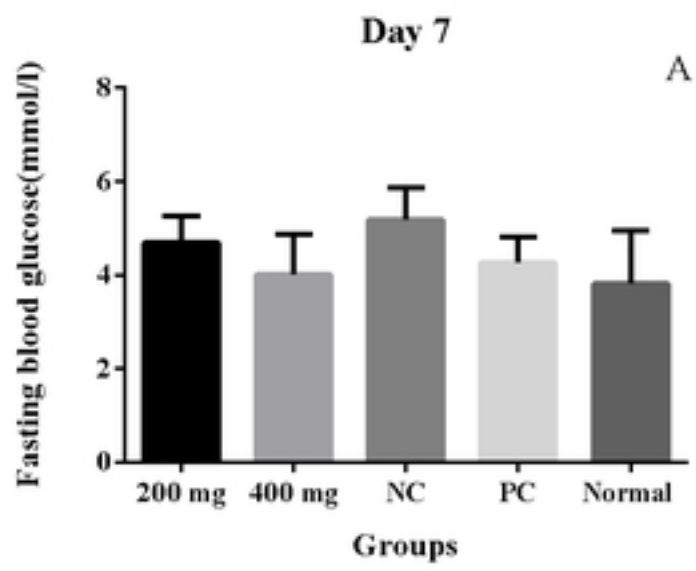
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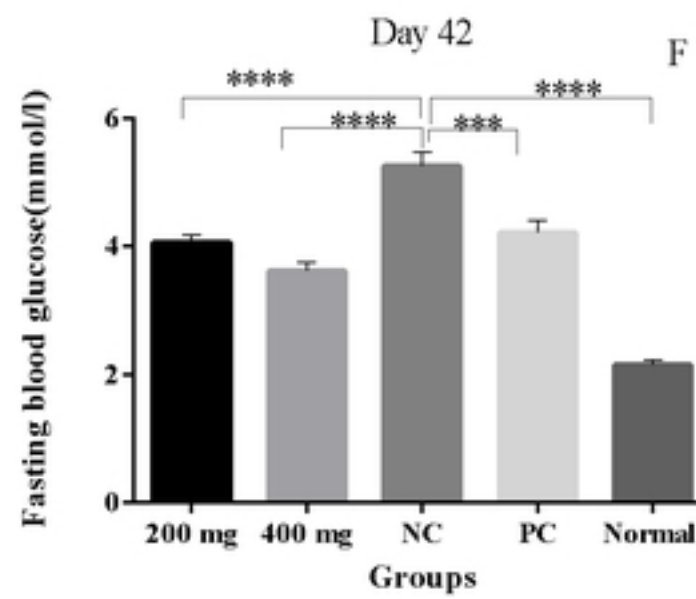
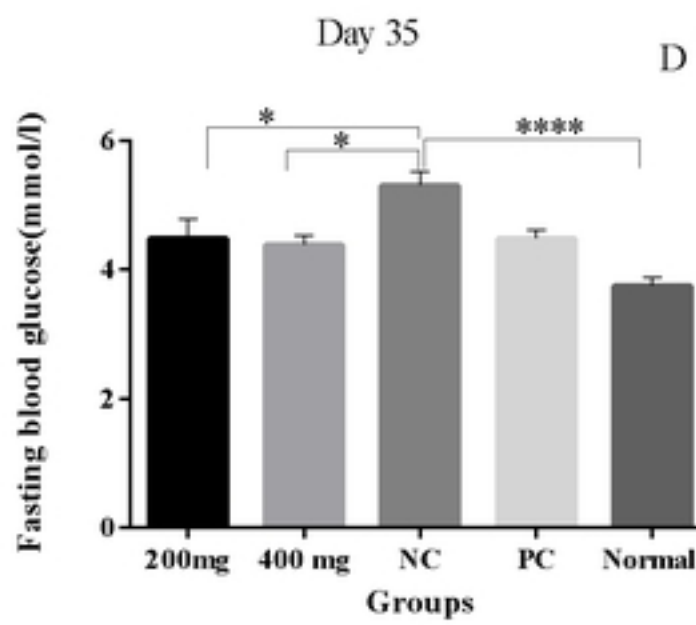
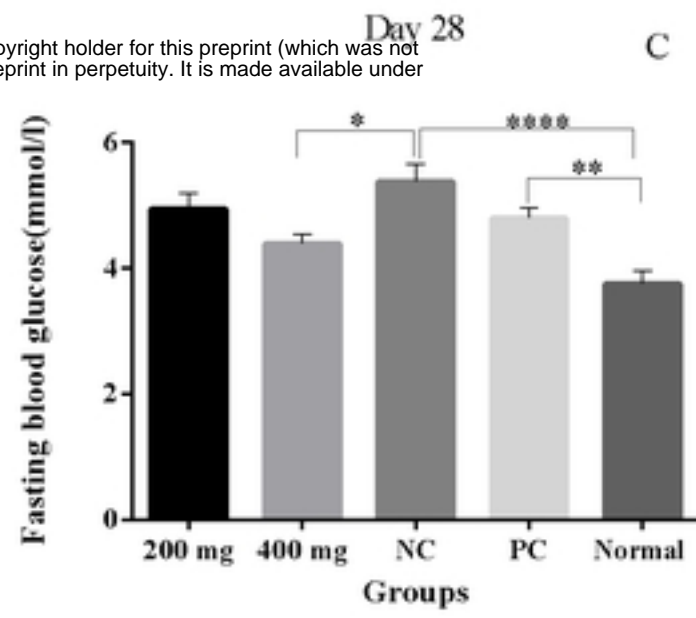
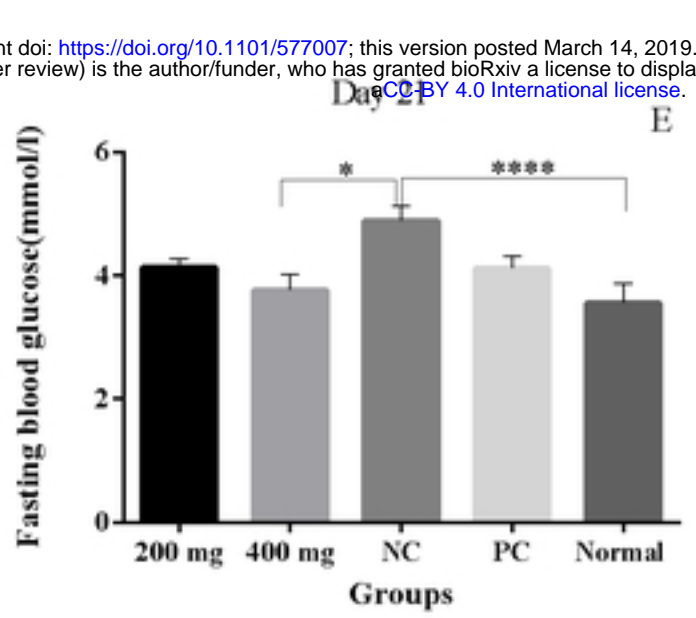
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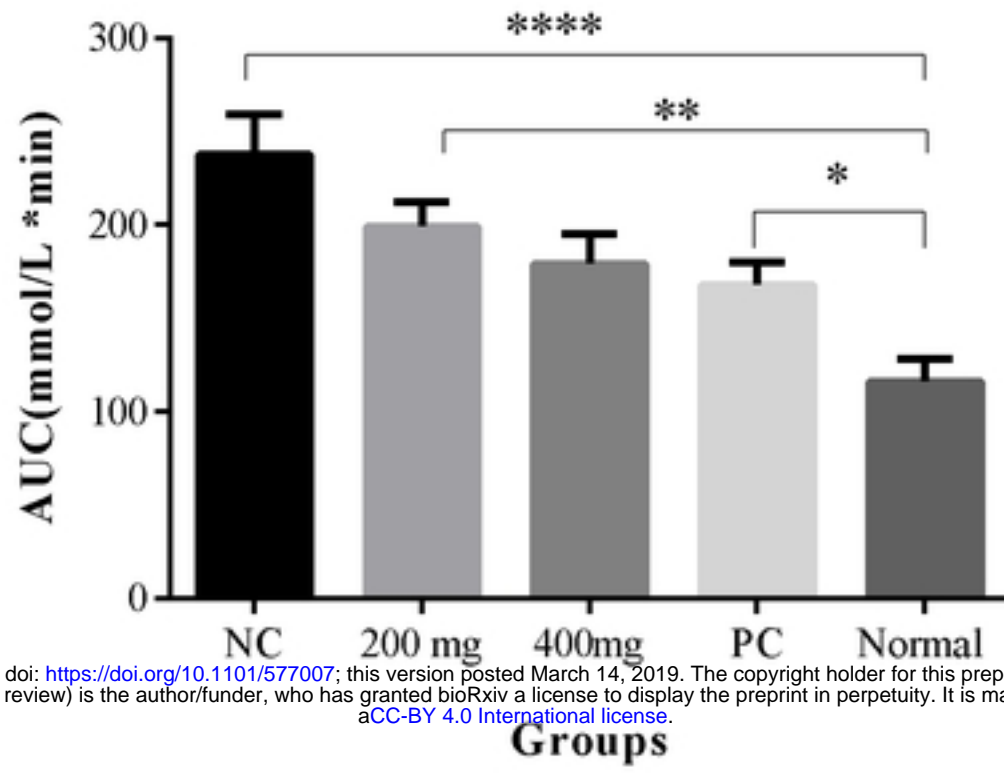
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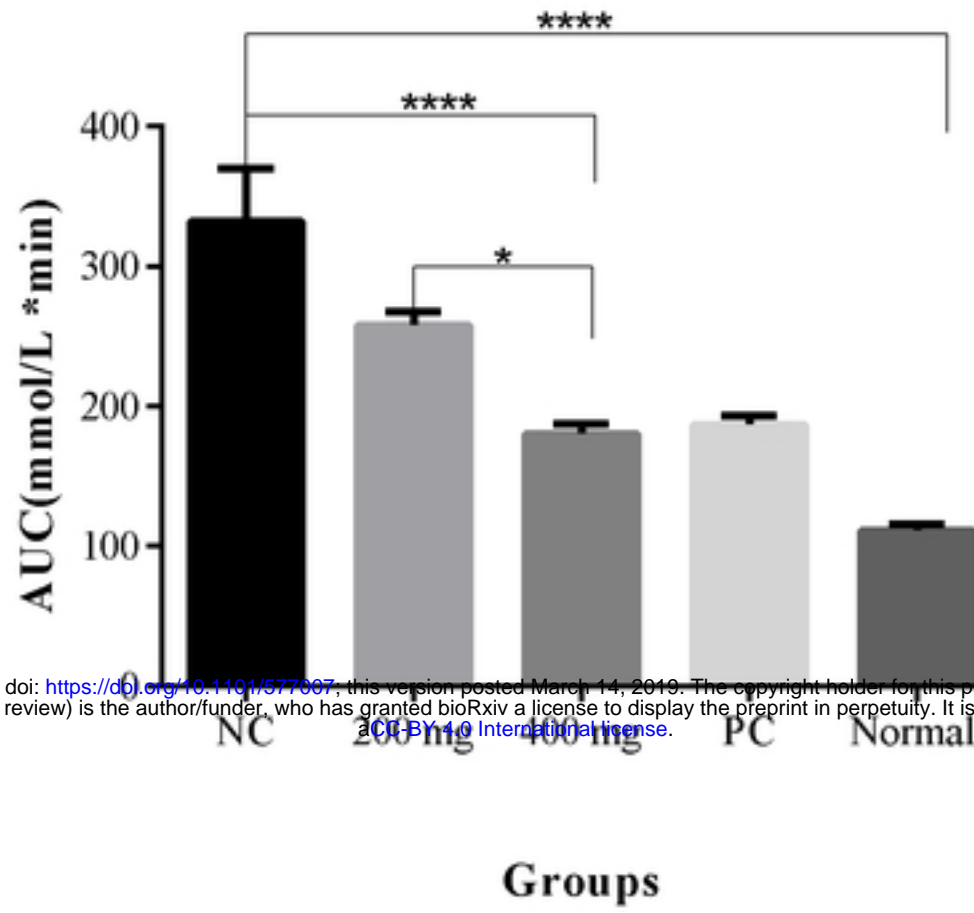


INSULIN TOLERANCE DAY 21



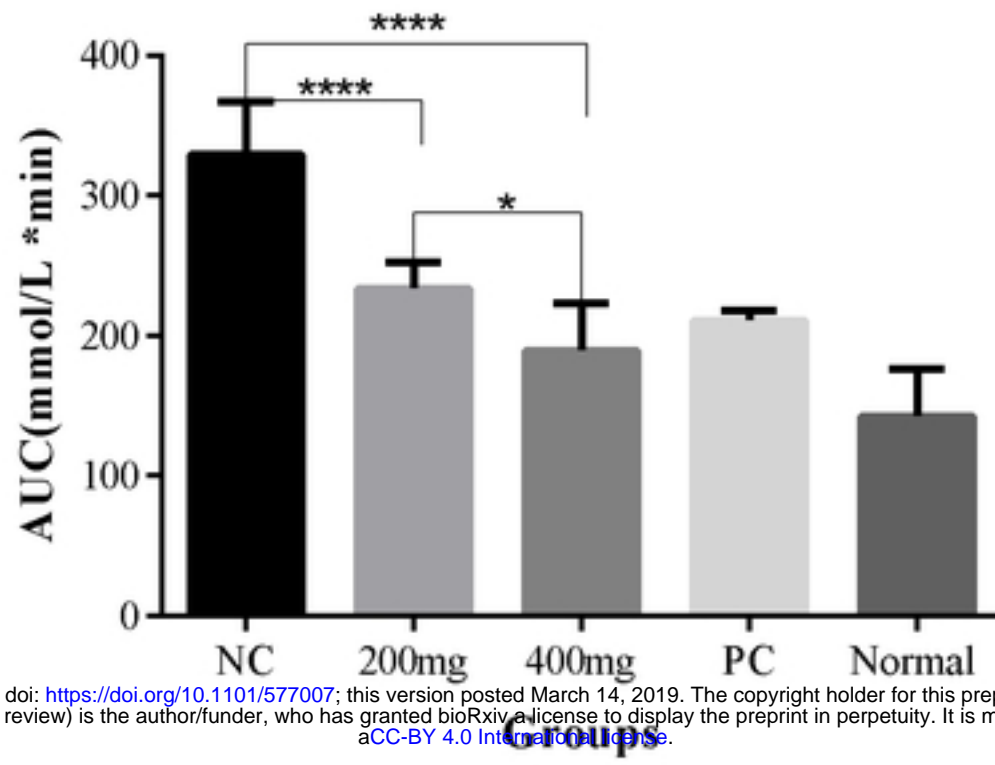
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INSULIN TOLERANCE DAY 42

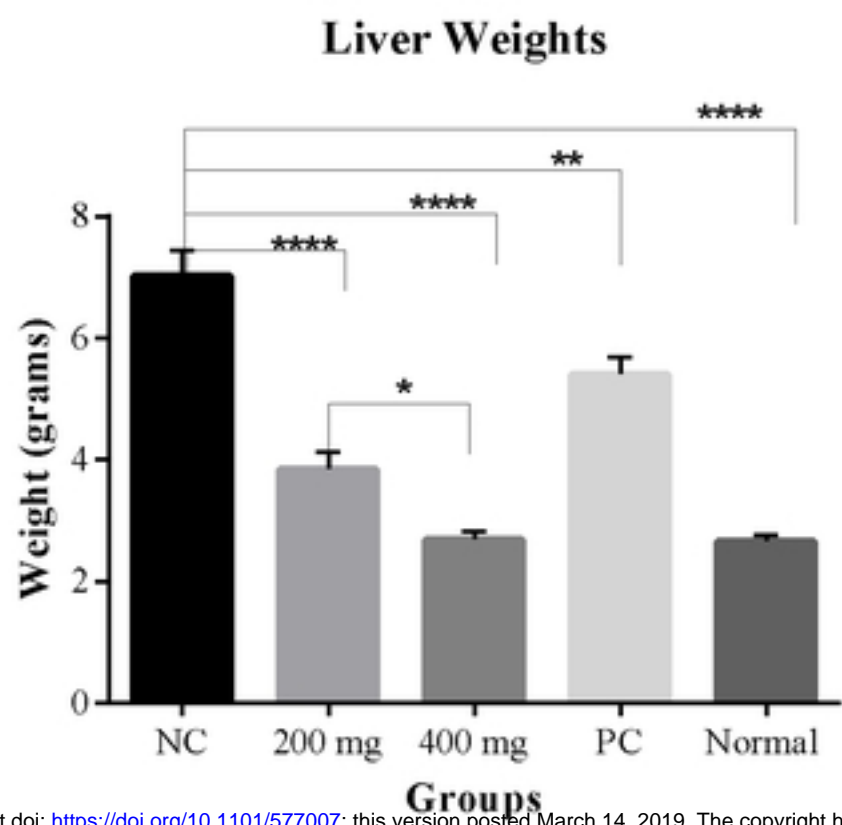


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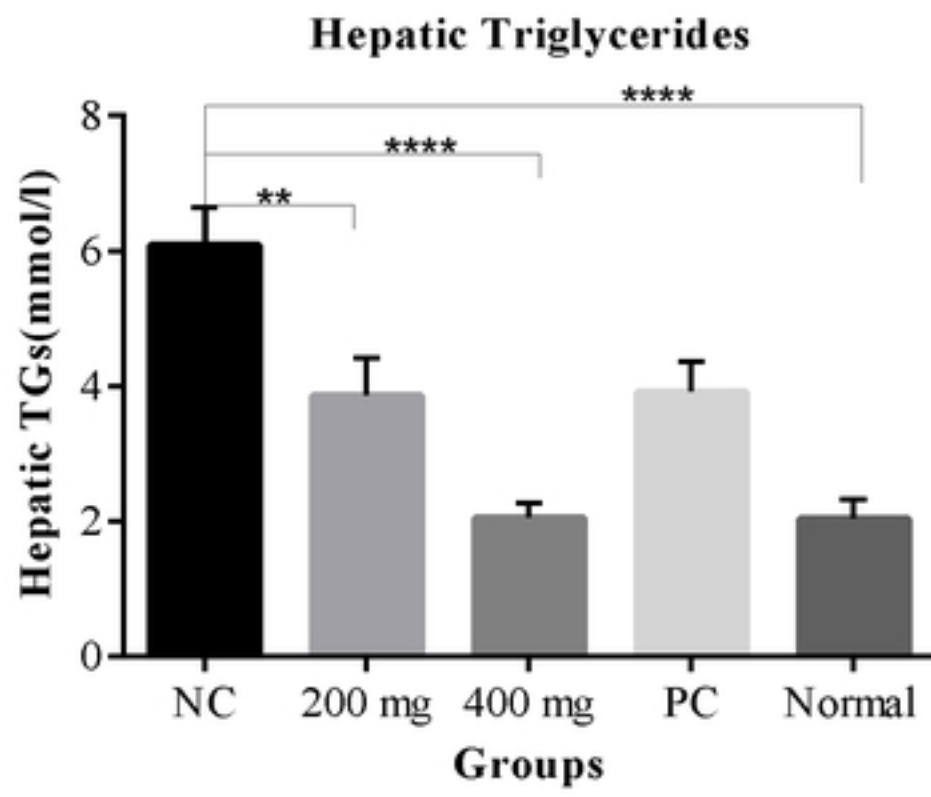
INSULIN TOLERANCE DAY 56



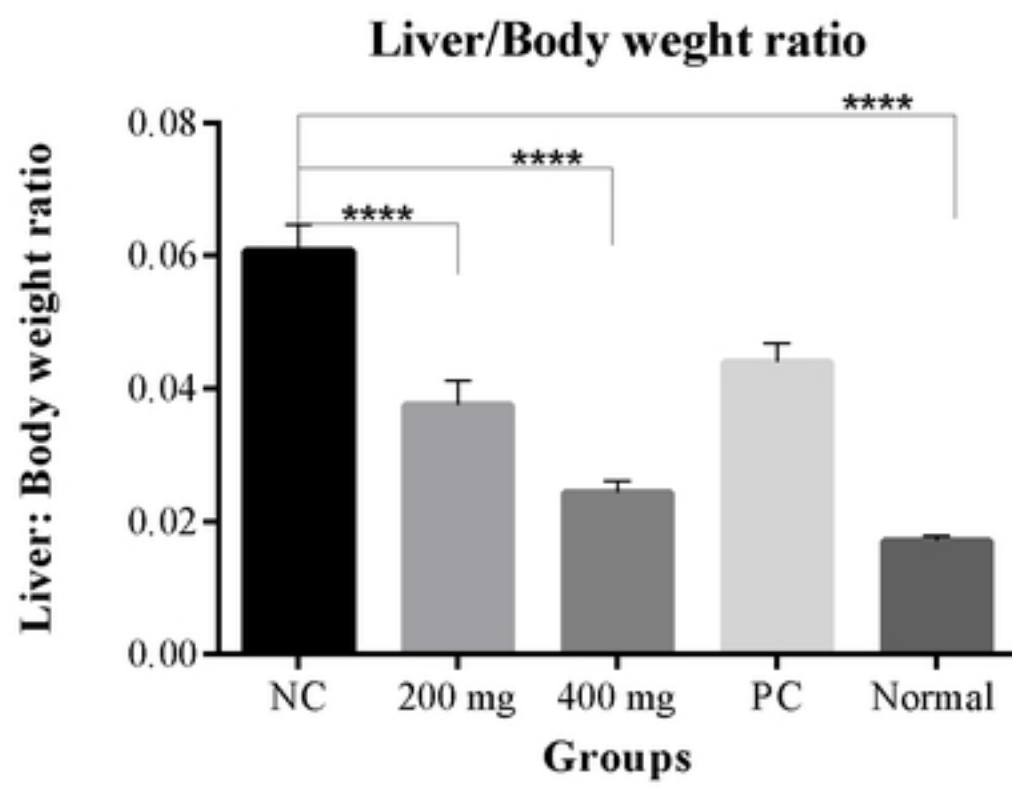
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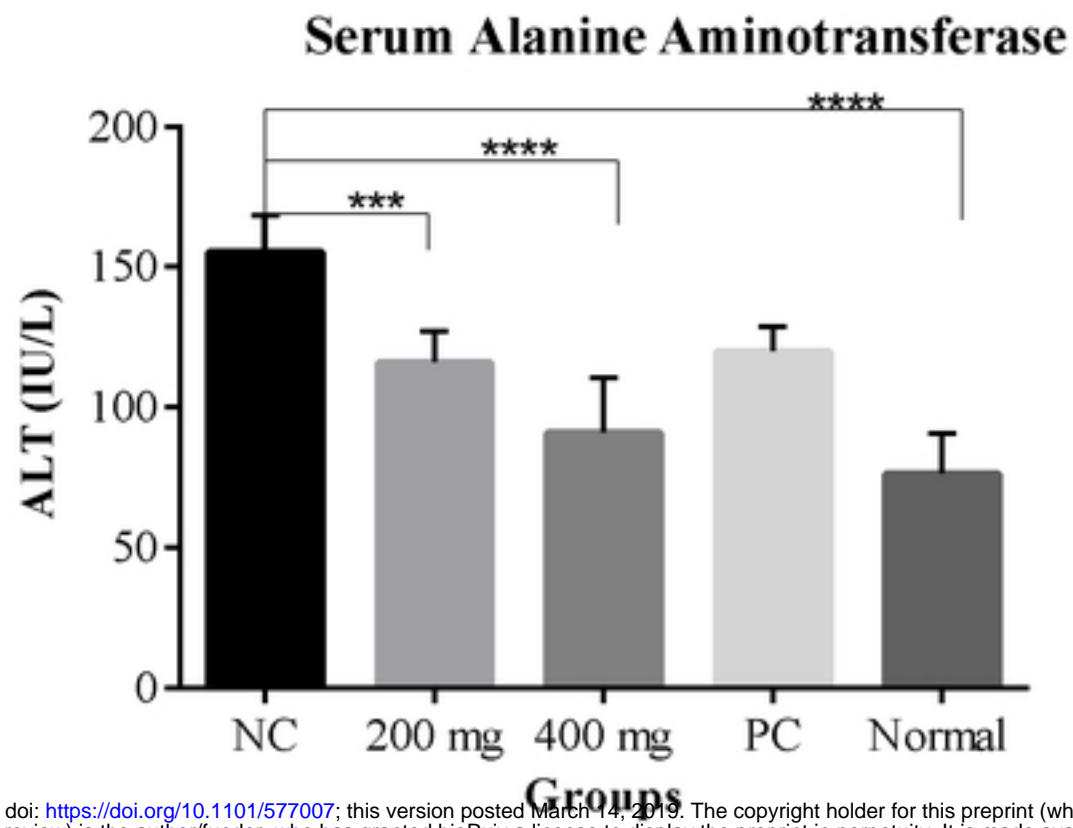
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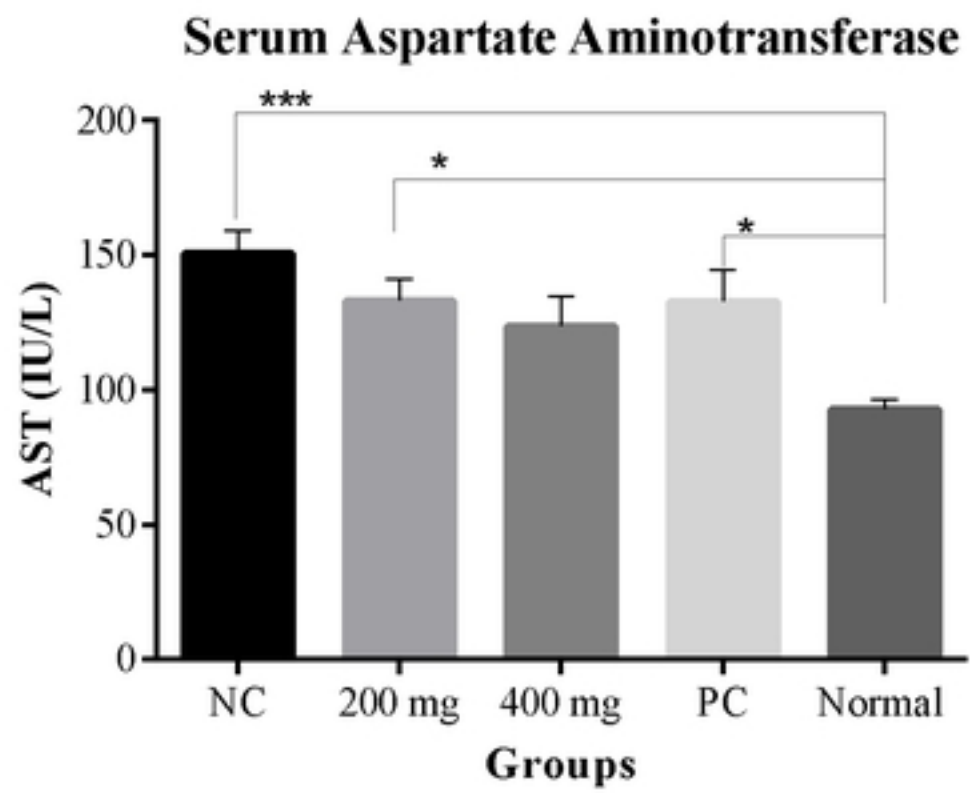
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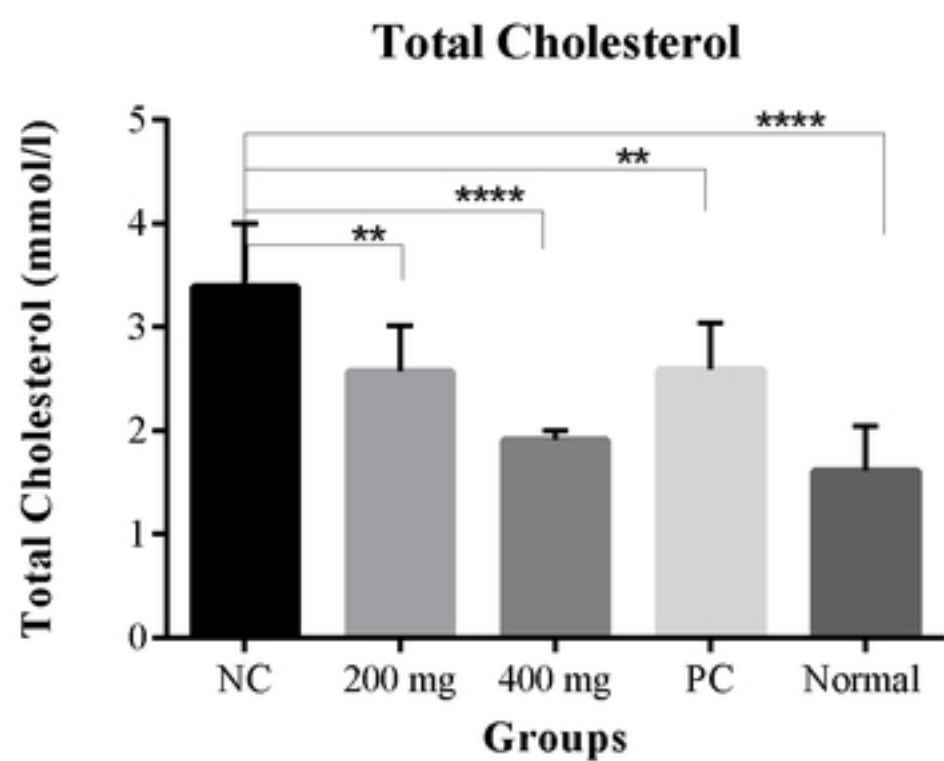
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Serum Triglycerides

