ORIGINAL ARTICLE

Hepatic Effects of Prolonged Oral Administration of Aqueous Extracts From *Trigonella foenum-graecum* (Fenugreek) Seeds Explored Through Pathology Techniques

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ABSTRACT

Introduction: The escalating global prevalence of complementary and alternative medicine is often linked to the financial challenges of conventional healthcare in developing nations. Trigonella foenum-graecum (TFG), commonly known as Fenugreek, it is rich in proteins, lipids, fatty acids, and minerals, offering numerous health benefits. This study explores the hepatic impact of prolonged oral ingestion of an aqueous extract from Trigonella foenum-graecum seeds in Norvegicus rattus. **Materials and methods:** Thirty-five Norvegicus rattus weighing 150-200g were randomly assigned to five groups, with each subjected to distinct dietary regimens containing 2%, 4%, 6%, and 8% Trigonella foenum-graecum, and a control group. Liver tissues were harvested and processed, and blood samples collected through cardiac puncture for biochemical analysis, comparisons across all groups were performed for all tests using SPSS version 20, with statistical significance determined at $p \leq 0.05$. **Results:** Trigonella foenum-graecum administration resulted in significant increases in serum ALT and AST levels, indicating potential hepatotoxicity. Lower doses exhibited reduced lipid peroxidation (MDA), while higher doses surpassed control group MDA levels, suggesting a dose-dependent response. Antioxidant enzymes (CAT, SOD, GSH, GPX) showed elevation at lower doses but declined at higher doses. Liver histological examinations revealed characteristics indicative of toxicity at higher doses. **Conclusion:** This study establishes the antioxidant and hepatoprotective properties of Trigonella foenum-graecum, highlighting a dose-dependent relationship. While lower doses show beneficial effects, higher doses may lead to adverse effects and liver damage. These findings contribute to understanding the dual nature of Trigonella foenum-graecum in therapeutic contexts.
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INTRODUCTION

The liver, regarded as one of the body's pivotal organs, assumes a critical role in the regulation of numerous physiological processes. Its involvement spans a wide spectrum of biochemical pathways, encompassing growth, immune response, nutrient provision, energy metabolism, and reproduction. Additionally, the liver participates in the intricate processes of carbohydrate, protein, and fat metabolism, as well as the crucial functions of detoxification, bile secretion, and vitamin storage [1]. Furthermore, due to its pivotal role in filtering substances from the portal circulation, the liver is particularly susceptible to initial and sustained exposure to foreign compounds, potentially leading to

liver dysfunction [2].

The global prevalence of complementary and alternative medicine (CAM) is on the rise, with various factors contributing to this trend. One potential driver is the high cost of conventional healthcare, which is often beyond the means of many developing countries. Several medicinal plants and their extracts, derived from various parts such as leaves, seeds, peels, or cores, have shown significant therapeutic potential in animal models for treating induced diseases. During the COVID-19 pandemic, the use of traditional plant-based medicines became particularly prominent, especially in developing nations like Africa. Some of the commonly employed medicinal plants during this period included bitter cola and Mentha spicata (peppermint plant), among others. Nevertheless, it's crucial to acknowledge that this surge in traditional medicine usage also brought to light instances of substance misuse and ethno-medical abuses.

At present, approximately 80% of the global population relies on medications derived from plants. Among these, fenugreek (Trigonella foenum graecum) stands as one of the oldest medicinal plants, thriving as an annual herb across the Mediterranean and Asia. Fenugreek leaves and seeds find diverse applications across various countries, serving medicinal, culinary, and industrial purposes. This versatile plant contributes to the creation of dishes such as rice stews in Iran, cheese flavouring in Switzerland, syrups and bitters in Germany, and mixed seed powder combined with flour for flatbread production in Egypt. It also plays roles in curries, natural dyes, and even as a vegetable when consumed in its youthful form. In some regions, roasted fenugreek grains serve as a coffee substitute, while its extracts find use in controlling insects in grain storage and the perfume industry. Despite the widespread presence of this medicinal plant, Africa harbours a significant portion of its biodiversity. Regrettably, this valuable resource faces continuous threats from ecosystem disruptions brought about by unregulated human activities, including deforestation and industrialization, among other potentially harmful aspects of human development.

Fenugreek seeds exhibit a broad spectrum of beneficial effects, including hypoglycemic (lowering blood sugar), anti-immunological, hypolipidaemic (reducing lipid levels), antifertility (affecting fertility), antiandrogenic (inhibiting male sex hormone activity), antinociceptive (pain-relieving), and anticarcinogenic (cancer-fighting) properties. Additionally, they demonstrate antioxidant activity, stimulate lactation, support wound healing, and serve as a rich source of dietary fibres [3, 4]. Scientific investigations have identified several medicinal applications for fenugreek seeds, including their potential to manage conditions like diabetes and hypercholesterolemia, offer hepatoprotective effects against free radicals, and protect against breast and colon cancer [5].

These protective functions are attributed to the presence of non-nutritive secondary metabolites, commonly referred to as phytochemicals. As mentioned earlier, fenugreek seeds encompass a wide range of components, which can be classified into both non-volatile and volatile constituents (6). Fenugreek holds significant value as a legume crop, particularly in short-term crop rotations, as well as in the production of hay and silage for livestock feed. Its ability to fix nitrogen in the soil contributes to soil fertility enhancement [7]. Additionally, fenugreek serves as a valuable chemurgic crop with wide-ranging industrial applications. Its seeds are of commercial interest due to their steroid diosgenin content, which holds significance in the pharmaceutical industry [8].

The mucilaginous properties of fenugreek seeds impart numerous medicinal benefits, making them valuable as a tonic, emollient, carminative, demulcent, diuretic, astringent, emmenagogue, expectorant, restorative, aphrodisiac, and vermifuge. They have historically been employed to treat conditions such as mouth ulcers, chapped lips, and stomach irritation [9]. Furthermore, fenugreek contains furostanolic saponins, which have been associated with potential benefits in promoting the production of the male hormone testosterone. This suggests that fenugreek may have the potential to enhance libido, particularly in older men with reduced testosterone levels.

This environmentally friendly plant exhibits a wide range of potential applications in the food and feed, pharmaceutical, cosmetic, and medical industries, thanks to its rich nutrient and nutraceutical content. Although aqueous extracts of fenugreek seeds have been explored for various therapeutic purposes [9], there is currently no documented evidence regarding their impact on liver function parameters in a dose-dependent manner. Given the tendency for the misuse of substances, especially those associated with aphrodisiac properties, this study was specifically designed to assess the hepatic effects of chronic administration of aqueous fenugreek seed extract on the liver. This research holds significant importance as it contributes to the existing body of knowledge related to the safe administration and consumption of fenugreek seeds. Furthermore, it aims to ascertain the potentially toxic dose of fenugreek and its effects on one of the body's essential organs, the liver, which plays a pivotal role in drug metabolism. The findings from this study will offer valuable insights for individuals seeking to enhance their health by incorporating biogenic elements and healthy fatty acids into their diets.

MATERIALS AND METHODS

Samples and extractions

The Rattus norvegicus used in this study were housed in the central animal facility of the College of Health Sciences at Ebonyi State University. The laboratory testing was carried out in the Histopathology Department at Alex-Ekwueme Federal University Teaching Hospital in Abakaliki. Dried fenugreek seeds (Trigonella foenumgraecum L.) were procured from a local market in Kpiri-kpiri, Abakaliki, Ebonyi State, and subsequently ground into a fine powder. The authenticity of the fenugreek seeds (Trigonella foenumgraecum L.) was verified through botanical authentication conducted at the Department of Biological Sciences, Ebonyi State University, Abakaliki.

Approximately 25 grams of powdered fenugreek seeds were subjected to extraction using 500 mL of boiling distilled water for 5 minutes. The resulting heated decoction was then set aside to cool at room temperature for 30 minutes and subsequently passed through filtration twice. The obtained filtrate was subsequently lyophilized and stored in a refrigerator.

Experimental Design and sample collection

A total of thirty-five (35) Rattus norvegicus, with an average weight falling within the range of 150 to 200 grams, were used for this study. Subsequently, these rats were housed in the Histopathology laboratory and allowed to acclimatize to the standard laboratory conditions (10). The subjects were categorized into five groups, namely G1, G2, G3, G4, and G5. To reduce bias, an Excel random number generator was used to assign the subjects to groups.

All groups were provided with daily dietary supplementation comprising 2%, 4%, 6%, and 8% feeds over 28 days, respectively. It is important to note that Group 1 (G1) served as the control group and did not undergo any dietary intervention. Each of these experimental groups consisted of seven individual subjects, and throughout the study period, the animals were housed in separate cages. This housing arrangement remained constant until the conclusion of the study.

The animals were anaesthetized using ketamine and Xylazine combination which is widely used for rodents and some other small animals. Ketamine provides anaesthesia, while xylazine acts as a sedative and analgesic. The combination offers relatively quick induction and recovery and is commonly used for short procedures. The blood was collected using 5ml syringes through the cardiac puncture in a plain container then, the liver was collected upon dissection into two equal parts and fixed one part in 10% buffered formal saline for histopathological evaluation, and the other half homogenized to demonstrate oxidative stress biomarkers using standard methods described by [10].

Ethical Consideration

Animal procedures were performed under the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals [11].

Histopathological Procedure

Fixed livers were grossed in triplicates and processed using Leica TP1020 Tissue Processor which offers automation of fixation, dehydration, and infiltration of histological tissue samples. Furthermore, tissue embedding was carried out utilizing The HistoCore Arcadia modular tissue embedding system. This system, a product of Leica Biosystems, includes two distinct components: the Arcadia H heated embedding workstation and the Arcadia C cold plate. This setup provides flexibility in organizing the embedding workflow, allowing for adaptation to laboratory requirements and workspace constraints. The two automated machines employed above have working mechanisms based on first principles as discussed under routine manual and rapid processing [10].

Microtomy and Slide Preparation

The tissues embedded with their respective identification numbers were transferred from the cold section of the embedding workstation to the microtome. Microtomy was performed using the HistoCore MULTICUT R - Semi-Automated Rotary Microtome 149MULTIRC1, 14052258221, a Leica Biosystems product. The microtome operates with a horizontal cutting blade, and the tissue-containing block moves up and down through the rotation of a handle attached to the microtome. With each complete 360° rotation of the wheel handle, the block undergoes a downward and then upward movement, resulting in the cutting of a thin ribbon-like section from the tissue (11). Initially, the tissue block is securely placed in the microtome's chuck, and paraffin is trimmed away until the tissue is fully exposed. After this initial trimming, the blocks are allowed to cool for approximately 15–20 minutes. This cooling period helps maintain the consistent properties of both the paraffin and the tissue, ensuring that the cutting process is more manageable.

To achieve precise sectioning, the block is positioned in the microtome's chuck so that the cutting surface is parallel to the knife. An optimal angle of clearance, typically between 2–5°, is maintained to facilitate high-quality sectioning. The tissue within the block is cut using gentle, smooth, and deliberate strokes, resulting in the production of ribbon-like tissue sections. These sections are then manipulated: the tip of the ribbon is grasped with forceps, and the brush is used to remove the trailing end from the knife edge. The ribbon of the tissue was floated in the water bath, and this made the tissue flat and removed any wrinkling of the tissue. With the help of the forceps, the individual sections were separated from each other. The slide was placed vertically within the water bath in front of the tissue,

and when the tissue was touched, the slides were then withdrawn vertically from the water. The tissue pickup process must be gentle and smooth. To prevent any mix-up, the water bath should be cleaned immediately after cutting each block. The slide containing the picked-up sections was kept in the rack. Then transferred to a hot oven to get dry at a temperature slightly more than the melting point of the paraffin as previously reported by Peachey [11].

Haematoxylin and Eosin Staining Method

The H & E staining technique is based on the chemical affinity between tissues and dyes. Hematoxylin, which is a basic dye, imparts a blue-purple colour to basophilic structures, primarily those containing nucleic acid components such as chromatin, ribosomes, and RNA-rich cytoplasmic regions. On the other hand, an acidic eosin counterstain highlights the acidic elements like red blood cells (RBCs), cytoplasm, muscle, and collagen in varying shades of pink, orange, and red. The process of staining tissue sections involved several sequential steps. First, the sections were dewaxed in xylene, with three changes of xylene for 5 minutes each. Next, the sections were hydrated using descending grades of alcohol, specifically Absolute and 90%, and each hydration step lasted for 5 minutes. After hydration, the sections were brought into contact with water. Subsequently, the sections were immersed in hematoxylin for a 15-minute staining period, followed by rinsing with water. To facilitate differentiation of the sections, 1% acid alcohol was briefly applied, and the sections were then rinsed with water. Scot's water was used for 10 minutes to induce bluing of the sections, followed by rinsing with water. Next, the sections were counterstained with 1% eosin for 4 minutes and rinsed with water.

Dehydration was performed by sequentially placing the sections in ascending grades of alcohol (90%, absolute 1, 2, and 3), with each dehydration step lasting 15 seconds. The sections were then cleared using xylene 1 and 2, with each clearing step lasting 3 minutes. Finally, the stained sections were mounted with DPX mountant, and they were observed under a microscope for examination and photography, following the procedure described by Okorie [10].

Weigert's Iron Haematoxlin Van Gieson

Weigert's hematoxylin, also known as ferric hematoxylin, is a staining technique specifically chosen when an acid solution will be employed in a later step of the staining procedure. This hematoxylin is prepared by combining it with a mordant, typically either potassium aluminium or ammonium aluminium. This mordant addition imparts resistance to acid solutions. In Weigert's hematoxylin staining method, the mordant and the dye are stored separately and are only mixed immediately before the staining process. Consequently, the nuclei exhibit a final colouration ranging from black to dark purple. The staining procedure encompassed several crucial steps: Firstly, the sections were subjected to dewaxing in xylene, involving three changes of xylene, with each change lasting 5 minutes. Subsequently, the sections were hydrated through a series of descending alcohol grades, where each grade was applied for 5 minutes, and this was followed by a thorough water wash. The staining of all the nuclei was executed using Weigert's Iron Hematoxylin solution, with an incubation time of 10 minutes, and then the sections were rinsed under running tap water for an additional 10 minutes. To achieve counterstaining, Van Gieson solution was employed, which is a mixture comprising 100ml of saturated picric acid solution and 10ml of a 1% acid fuchsin solution, and this step was carried out for 5 minutes. Afterwards, there was a further rinsed in distilled water, and subsequently, the sections underwent dehydration, clearing, and mounting using DPX, following the standardized procedure outlined by [10].

Periodic Acid Schiff Technique (control)

The PAS stain is a histochemical reaction where periodic acid is used to oxidize carbon-carbon bonds, resulting in the formation of aldehydes. These aldehydes then react with fuchsin-sulfurous acid, leading to the development of magenta colouration. The staining process consisted of several sequential steps: Firstly, the sections underwent deparaffinization in three changes of xylene, with each change lasting 5 minutes. Following that, the sections were hydrated using descending grades of alcohol, with each grade being applied for 5 minutes, and they were thoroughly washed with water. Next, the sections were oxidized by exposure to periodic acid for 10 minutes, followed by extensive rinsing with tap water. To impart the desired color, the sections were stained with Schiff's reagent for 20 minutes, and afterwards, they were rinsed with distilled water. Counterstaining of the sections was carried out progressively, initially with Harris Hematoxylin, and then they were blued in tap water. Subsequently, the sections were subjected to dehydration, and clearing, and finally, they were mounted in DPX mountant, following the standardized procedure as outlined by Okorie [10].

Pearl's Prussian Blue Staining Technique

In the staining solution, potassium ferrocyanide combines with ferric iron to produce the Prussian blue pigment. The addition of hydrochloric acid enhances the availability of iron within the tissue, facilitating its reaction with potassium ferrocyanide [13]. The chemical reaction for this conversion of iron into Prussian blue [14]:

$4FeCl3 + 3K4Fe(CN)6 \rightarrow Fe4[Fe(CN)6]3 + 12KCl$ (ferric iron) + (potassium ferrocyanide) → (ferric ferrocyanide or Prussian blue) The staining procedure can be broken down into the following steps: The process was initiated by placing the sections in distilled water. Subsequently, the sections were flooded while they were on a rack with a mixture comprising equal parts of ferrocyanide

and hydrochloric acid, and this step was carried out for 10 minutes (or 30 minutes if asbestos bodies were being stained). Afterwards, the sections underwent thorough washing in distilled water, with several changes, and each change was allowed to last for 5 minutes. Following this, the sections were counterstained using a filtered neutral red stain for 1 minute. The sections were then rinsed in distilled water. Rapid dehydration of the sections was accomplished by immersing them in absolute alcohol. Finally, the sections were cleared and mounted per standard laboratory practices.

Determination of oxidative stress biomarker parameters

The determination of oxidative stress parameters through spectrophotometry is a fundamental aspect of biochemical research and clinical diagnostics. Oxidative stress, arising from an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defences, is implicated in various health conditions. Spectrophotometry provides a precise and widely used method for quantifying oxidative stress markers, such as lipid peroxidation, superoxide dismutase activity, glutathione levels, and more. By measuring the absorption of specific wavelengths of light, spectrophotometry enables researchers and clinicians to gain valuable insights into the oxidative status of biological samples, contributing to our understanding of various diseases and potential therapeutic interventions.

Catalase (CAT) activity was assessed following the procedure outlined by (15). The methodology relies on the ultraviolet absorption of hydrogen peroxide, which was quantified at 240 nm. As catalase breaks down hydrogen peroxide (H_2O_2), the absorption decreases over time, allowing for the measurement of catalase activity (15). The procedure involves several steps for assessing catalase (CAT) activity. Firstly, prepare a test tube containing 2.5 ml of phosphate buffer and 2 ml of hydrogen peroxide (H_2O_2). Next, add 0.5 ml of the serum to the same test tube. In a separate test tube, take 1 ml of the reaction mixture, and add 2 ml of dichromate acetic acid reagent to it. Finally, measure the absorbance at 240 nm, recording values at one-minute intervals, while utilizing a blank as a reference, as outlined by (14) in 1983.

The estimation of Super Oxide Dismutase (SOD) activity was conducted per the method outlined by Fridovich and Mc-Cord (16). The SOD assay is based on the ability of superoxide dismutase to hinder the autoxidation of adrenaline. Superoxide, generated by xanthine oxidase, induces the oxidation of adrenaline to adenochrome. The production of adenochrome formed per superoxide added increases with higher pH levels and greater adrenaline concentrations (16). Here is the procedure for determining superoxide dismutase (SOD) activity: To initiate the process, combine 0.2 ml of serum with 2.5 ml of 0.05 phosphate buffer. Maintain the pH at 7.8 and introduce 0.3 ml of freshly prepared adrenaline solution into the reaction mixture. Thoroughly mix the contents by inverting the cuvette. Over 3 minutes, monitor the increase in absorbance at 480 nm at 30-second intervals while using a blank as a reference. The blank, in this case, consists of 0.3 ml of adrenaline and 2.5 ml of buffer, following the procedure outlined by Fridovich and Mc-Cord (16). In summary, SOD activity was assessed by measuring the inhibition of adrenaline autooxidation.

MDA levels were determined through a spectrophotometric method following the procedure described by Wallin et al [17]. This test is based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA), resulting in the formation of a red or pink-coloured complex. This complex, in an acid solution, exhibits maximal absorption at a wavelength of 532 nm (17). To begin, take a test tube and combine precisely 0.1 ml of serum, 0.9 ml of distilled H_2O, 0.5 ml of 25% TCA reagent, and 0.5 ml of 1% TBA reagent in a 0.3% NaOH solution. Next, incubate the test tube at a temperature of 95°C for 40 minutes. After incubation, let the test tube cool in a water bath. Then, add exactly 0.1 ml of 20% SDS (sodium dodecyl sulfate) to the mixture. Measure the sample's absorbance at two wavelengths, 532 nm, and 600 nm, against a blank reagent as outlined by Wallin et al (17).

The concentration of Glutathione (GSH) was determined following the method developed by Ellman (18). This method is based on the formation of a relatively stable yellow colour when Ellman's reagent is introduced to a sulforhydryl compound, specifically 2-nitro-5 thiobenzoic acid. When Ellman's reagent reacts with reduced glutathione (GSH), it produces a chromophoric product that absorbs light at 412 nm. The absorbance at 412 nm is directly proportional to the glutathione content. It's worth noting that reduced glutathione (GSH) constitutes most non-protein sulfhydryl groups (18). Begin by taking milliliter of the sample and adding 4.0% sulfo-salicylic acid to it. Centrifuge the resulting mixture at 3,000 rpm for 15 minutes at a temperature of 2°C. Take the supernatant obtained from the serum and introduce it to 4.5 ml of Ellman reagent. Measure the absorbance of this solution at a wavelength of 412 nm. To prepare the blank, combine 0.5 ml of 4% sulfo-salicylic acid with 4.5 ml of Ellman reagent, and measure its absorbance at 412 nm, following the protocol outlined by Ellman in 1959 (18).

The determination of Glutathione Peroxidase (GPx) activity was conducted following the method developed by Rotruck et al. (19). This analysis is an adaptation of the method originally established by Paglia and Valentine. Glutathione Peroxidase catalyzes the reduction of hydrogen peroxide (H_2O_2), converting reduced glutathione (GSH) into oxidized glutathione (GSSG). Subsequently, GSSG is reduced by the action of glutathione reductase (GR) and β-nicotinamide

adenine dinucleotide phosphate (NADPH), leading to the formation of NADP+ and a corresponding decrease in absorbance at 340 nm. This reduction in absorbance at 340 nm is directly proportional to the concentration of GPx (19). In a test tube, combine 14.0 ml of distilled water, 2.0 ml of a 5% pyrogallol solution, 1.0 ml of a 0.147 M H_2O_2 solution, and 2.0 ml of a 0.1 M phosphate buffer with a pH of 6.0. Allow the mixture to equilibrate at a temperature of 20°C for approximately 5 minutes. Add 1.0 ml of the serum sample to the mixture while ensuring thorough mixing. After precisely 20 seconds, halt the reaction by adding 1.0 ml of 2.0 N H_2SO_4. Measure the optical density of the resulting solution at 420 nm against a blank. The blank should be prepared similarly to the test solution, except that no sample is added to it, and it contains 15 ml of distilled water instead of the 14 ml used in the test solution, following the protocol established by Rotruck et al. in 1973 (19).

Sample collection and biochemical evaluation of the liver

Blood samples were obtained through cardiac puncture and placed into plain vacutainer tubes, with careful attention to preventing haemolysis. Subsequently, the samples were allowed to clot and undergo clot retraction. The samples were then separated by centrifugation at 2500 rpm for 15 minutes, and the resulting serum was archived in cryovials prelabeled for each sample group. These serum samples were subsequently analyzed for various hepatic evaluation parameters, including alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST). The analysis of the serum samples was performed using the Selectra ProS™ benchtop system, a fully automated robotic clinical chemistry analyzer. Before running the samples, the machine underwent rigorous quality control procedures to ensure the proper functioning of reagents and samples. Results were obtained from the machine's monitor compartment, and strict adherence to the manufacturer's instructions for loading samples and reagents was maintained to ensure the accuracy and reliability of the results.

Statistical analysis

Descriptive analysis was conducted for each variable, and comparisons across all groups were executed for all tests, with statistical significance set at a threshold of $p \leq 0.05$. The data underwent analysis using SPSS Version 20, and measurements were expressed as mean ± SD.

RESULTS

Histopathological evaluation of the Liver

The histopathologic studies of the liver section of Norvergicus rattus revealed different characteristics among the groups studied. The liver section of the normal control group, which received diet and distilled water, showed clear sinusoidal space, central vein and hepatocytes (see Plate A). Similarly, the liver section of the group administered diet supplemented with 2% TFG/bw showed clear sinusoidal spaces and normal hepatocytes (see Plate B). In the group fed diet supplemented with 4% TFG/bw, the liver section showed clear sinusoidal spaces, normal hepatocytes, and few reactive changes (see Plate C). The liver section of Norvergicus rattus on a diet supplemented with 6% TFG per body weight/bw showed relatively poor cellular architecture and conspicuous positive Pearls Prussian Blue (see Plate D). Finally, the liver section of Norvergicus rattus fed a diet supplemented with 8% TFG/bw showed relatively poor cellular architecture, fibrotic connective tissues and marked necrotic cells (see Plates E and F).

Serum biochemical evaluation of the liver

There were no significant differences in ALP values among all the study groups ($p = 0.224$). However, when it comes to serum ALT levels, the groups that received 6% (78.33 ± 2.78 U/L) and 8% (91.41 ± 4.16 U/L) TFG showed significantly higher levels compared to the control group (64.37 ± 1.00 U/L). Notably, the increase in ALT was more pronounced in the group that received 8% TFG than in the group that received 6% TFG. Conversely, the groups that received 2% (64.21 ± 1.44 U/L) and 4% (64.53 ± 1.53 U/L) TFG did not exhibit any significant differences from the control group in terms of ALT levels. In the case of serum AST levels, the Treatment group that received 6% TFG (68.67 ± 1.69 U/L) showed significantly higher levels than the control group (63.84 ± 0.96 U/L). However, there were no significant differences between the groups that received 2% (63.31 ± 0.76 U/L), 4% (64.43 ± 1.60 U/L), and 8% (65.14 ± 3.04 U/L) TFG and the control group concerning AST levels.

Serum MDA levels exhibited a significant reduction in the groups administered 2% (3.30 ± 0.04 mmol/mg), 4% (3.29 ± 0.03 mmol/mg), and 6% (3.84 ± 0.21 mmol/mg) TFG when compared to the normal control group (4.37 ± 0.01 mmol/mg). However, in the group administered 8% TFG, the MDA level increased significantly compared to the control group (4.98 ± 0.10 mmol/mg versus 4.37 ± 0.01 mmol/mg).

Regarding serum CAT levels, the groups that received 6% (12.11 ± 0.02 u/mg) and 8% (11.00 ± 0.50 u/mg) doses of TFG showed significantly lower levels compared to the control group (12.38 ± 0.04 u/mg). Notably, the decrease in CAT levels was more pronounced in the group that received 8% TFG when compared to the group that received 6% TFG.

Serum SOD levels were significantly elevated in the groups that received 2% (33.78 ± 0.04 u/mg) and 4% (33.95 ± 0.02 u/mg) doses of TFG when compared to the control group (33.31 ± 0.05 u/mg). However, these levels decreased with the administration of 6% and 8% TFG. Notably, the group that received 8% TFG exhibited a significant reduction in SOD levels (32.06 ± 0.25 u/mg) compared to the control group. Serum GPX levels

were significantly lower in the treatment groups that received 6% (25.06 ± 0.40 u/mg) and 8% (24.09 ± 0.10 u/mg) TFG compared to the control group (26.16 ± 0.02 u/mg). The group that received 4% TFG (27.05 ± 0.73 u/mg) had the highest GPX level, although this value was not significantly different from the control group.

In contrast, serum GSH levels in the group that received 4% TFG (22.48 ± 0.21 u/mg) were significantly higher than those in the control group. However, the values of GSH in the groups administered 2% (21.64 ± 0.14 u/mg) and 8% (20.40 ± 0.54 u/mg) TFG were significantly lower than in the normal control group (21.89 ± 0.05 u/mg).

DISCUSSION

The objective of this research was to assess the impact on the liver resulting from extended oral exposure to TFG, as illustrated in Figure 1. Plate A (Magnification ×200, Haematoxylin and Eosin Staining) presents a liver section from a Norvergicus rattus specimen exposed to a diet supplemented with distilled water, serving as the normal control group. Noteworthy features include well-defined sinusoidal spaces (indicated by the black arrow), a discernible central vein, and healthy hepatocytes (highlighted by the black). These observations collectively indicate a state of normalcy in the liver's histological structure. Plate B (Magnification ×200, Haematoxylins and Eosin Staining): Presented here is a liver section from a Norvergicus rattus specimen, part of a group fed a diet enriched with 2% TFG per body weight (bw). This image highlights well-defined sinusoidal spaces and the presence of healthy hepatocytes (indicated by the black arrow). Overall, the liver's histological structure appears normal, reflecting a state of typicalcy. Plate C (Magnification ×200, Haematoxylins, Van Gieson Staining): This image displays a liver section from a Norvergicus rattus subjected to a diet supplemented with 4% TFG per body weight (bw). In the image, you can observe well-defined sinusoidal spaces, normal hepatocytes (highlighted by the red arrow), and a few hepatocytes with reactive changes (deep brown colour at the centre). These reactive changes are noticeable within the liver section, indicating some alterations in its histology. Plate D (Magnification ×200, Perls Prussian Blue Staining): This image illustrates a liver section from a Norvergicus rattus that was fed a diet supplemented with 6% TFG per body weight (bw). In the image, you can observe a relatively poor cellular architecture and the presence of noticeable positive Pearls Prussian Blue staining (indicated by the black arrow). This staining suggests the deposition or accumulation of iron within the liver tissue. Further assessment on the impact of prolonged oral administration of an aqueous extract of TFG (TFG) seeds on liver function parameters (ALT, AST, and ALP) and investigation of the antioxidant potential of TFG seeds using oxidative stress biomarkers (MDA, GSH, SOD, CAT, GPX) was reported in Figure 3 and Figure 4 respectively which also reminiscence the dose-dependent hepatic effect observed in histopathology results of Figure 1 and Figure 2. It has been established that under conditions of oxidative stress, reactive oxygen, and nitrogen species (ROS/RNS) are known to cause damage to various cellular biomolecules, including lipids, sugars, proteins, and polynucleotides (20). Consequently, cells have evolved multiple defense mechanisms to counteract the excessive increase in ROS. These defense systems comprise non-enzymatic molecules such as glutathione, vitamins A, C, and E, as well as various antioxidants found in foods. Additionally, there are enzymatic scavengers of ROS, with superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) being prominent components of these defense systems (15).

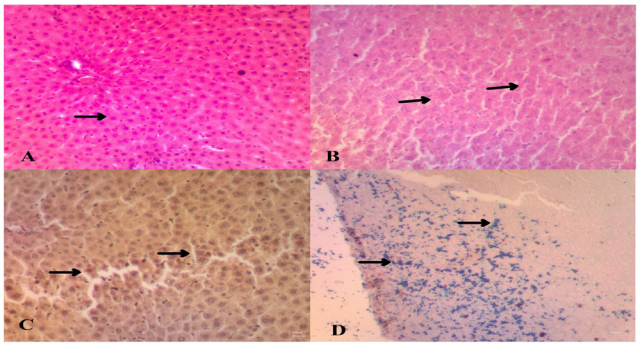

Figure 1: The assessment on the liver resulting from extended oral exposure to TFG.

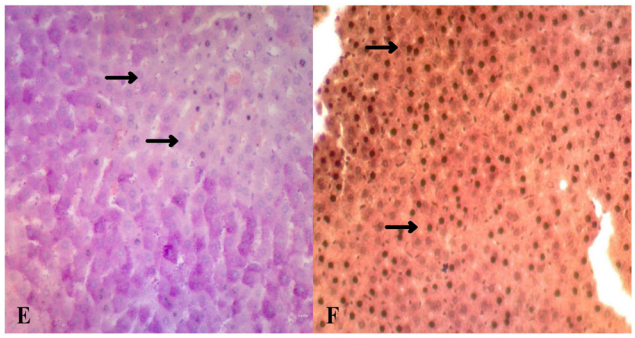

Figure 2: Plate E (Magnification ×200, PAS Staining): This image displays a liver section obtained from a Norvergicus rattus that was fed a diet supplemented with 8% TFG per body weight (bw). The liver section showed moderate increase of connective tissue and alteration of liver architecture (see black arrows in plate E). Plate F (Magnification ×200, HVG Staining): This image depicts a liver section from a Norvergicus rattus that received a diet supplemented with 8% TFG per body weight (bw). The slide revealed conspicuous reactive changes with brown discolouration of the hepatocytes. This shown that, at higher dose of the TFG, liver may not be safe.

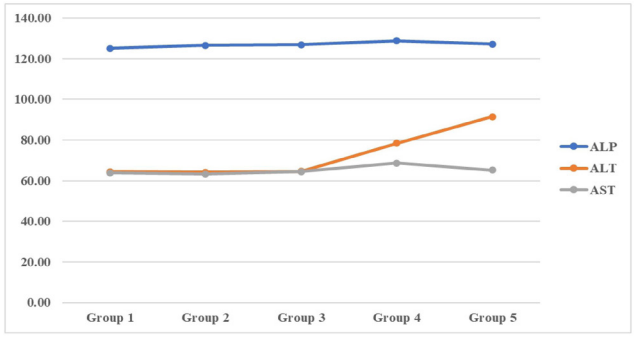

Figure 3: Experimental group description: Group 1 (normal control) received only food and water, Group 2 received a diet supplemented with 2% TFG, Group 3 received a diet supplemented with 4% TFG, Group 4 received a diet supplemented with 6% TFG, Group 5 received a diet supplemented with 8% TFG and its effects on liver function biomarkers.

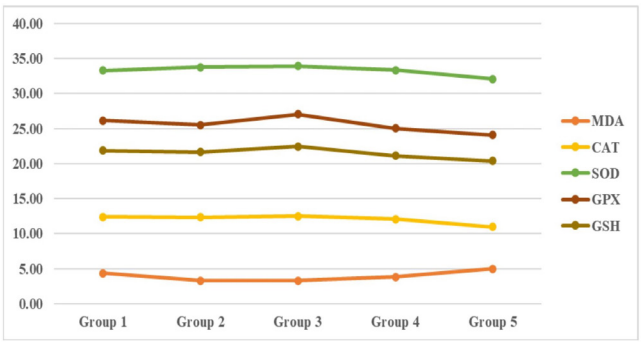

Figure 4: The experimental group and the parentage effects of dietary on the oxidative stress biomarkers.

The analysis of liver function biochemical parameters Table I and II revealed an increase in the levels of ALP, ALT, and AST in the groups administered TFG when compared to the control group. Although the increase in ALP was not statistically significant, significant elevations in AST levels were observed in the groups administered 6% TFG, and significant increases in ALT levels were observed in the groups administered 6% and 8% TFG. This elevation in serum AST and ALT levels can be attributed to the compromised structural integrity of the liver, as these enzymes are typically located in the cytoplasm and are released into the bloodstream following cellular damage, which is consistent with the degree of liver damage [21, 22].

Table I: Effect of TFG on liver function parameters

Groups	N	Liver Function Parameters					
		ALP (U/L)		ALT (U/L)		AST (U/L)	
		Mean	SD	Mean	SD	Mean	SD
Group 1	7	125.00	1.53	64.37	1.00	63.84	0.96
Group 2	7	126.57	2.70	64.21	1.44	63.31	0.76
Group 3	7	126.86	3.98	64.53	1.53	64.43	1.60
Group 4	7	128.86	4.26	78.33	2.78	68.67	1.69

CONTINUE

Table I: Effect of TFG on liver function parameters. (CONT.)

Groups	N	Liver Function Parameters					
		ALP (U/L)		ALT (U/L)		AST (U/L)	
		Mean	SD	Mean	SD	Mean	SD
Group 5	7	127.14	3.81	91.41	4.16	65.14	3.04

*Abbreviations: ALP; Alanine Phosphatase, ALT; Alanine aminotransferase, AST; Aspartate aminotransferase, N; Number of observations, SD; Standard Deviation. TFG; Trigonella foenum-graecum
*Experimental group description: Group 1 (normal control) received only food and water, Group 2 received diet supplemented with 2% TFG, Group 3 received diet supplemented with 4% TFG, Group 4 received diet supplemented with 6% TFG, Group 5 received diet supplemented with 8% TFG

Table II: Differences in the effect of TFG on liver function parameters

Parameters	Group 1 Mean ± SD. (n = 7)	Group 2 Mean ± SD. (n = 7)	Group 3 Mean ± SD. (n = 7)	Group 4 Mean ± SD. (n = 7)	Group 5 Mean ± SD. (n = 7)	p value
ALP (U/L)	125.00 ± 1.53	126.57 ± 2.70	126.86 ± 3.98	128.86 ± 4.26	127.14 ± 3.81	0.224
ALT (U/L)	64.37 ± 1.00	64.21 ± 1.44	64.53 ± 1.53	78.33 ± 2.78[a]	91.41 ± 4.16[a]	0.000
AST (U/L)	63.84 ± 0.96	63.31 ± 0.76	64.43 ± 1.60	68.67 ± 1.69[a]	65.14 ± 3.04	0.000

P < 0.05 is considered statistically significant, values bearing superscript 'a' are significantly different from the normal control group in that same row (p < 0.05).
Abbreviation: ALP; Alanine Phosphatase, ALT; Alanine aminotransferase, AST; Aspartate aminotransferase, n; Number of observations, SD; Standard Deviation.
Experimental group description: Group 1 (normal control) received only food and water, Group 2 received a diet supplemented with 2% TFG, Group 3 received a diet supplemented with 4% TFG, Group 4 received a diet supplemented with 6% TFG, Group 5 received a diet supplemented with 8% TFG.

ALP, being a membrane-bound enzyme, is released unequally depending on the underlying pathological processes [23]. The findings suggest that higher doses of TFG may potentially induce liver damage. These results contrast with the findings [24], who reported a significant reduction in serum AST and ALT following the administration of an aqueous extract of TFG. They also differ from the results of other studies [25]. However, they align with a case report presented by Bernard Reye et al [26], where acute liver injury, accompanied by rising AST and ALT levels, was diagnosed in a previously healthy 34-year-old female who had been using TFG to enhance lactation. Interestingly, upon discontinuation of TFG, liver enzyme levels gradually returned to normal without further medical intervention.

On a different note, the lipid peroxidation index, represented by MDA levels, exhibited significant reductions in groups treated with 2%, 4%, and 6% TFG Tables III and IV referred. This decrease in MDA levels is consistent with the findings of [27], who reported a dose-dependent reduction in MDA levels induced by TFG. These results also align with the outcomes of other studies, [24 and 28]. However, in contrast to these studies, the administration of 8% TFG resulted in a significant increase in MDA levels beyond those

of the control group. This observation suggests that the mitigating effect of TFG on lipid peroxidation may be dependent on a threshold, where higher doses could potentially become toxic.

Table III: Effect of TFG on oxidative stress biomarkers

Groups	N	Oxidative Stress Markers									
		MDA (mmol/mg)		CAT (u/mg)		SOD (u/mg)		GPX (u/mg)		GSH (u/mg)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Group 1	7	4.37	0.01	12.38	0.04	33.31	0.05	26.16	0.02	21.89	0.05
Group 2	7	3.30	0.04	12.37	0.09	33.78	0.04	25.51	0.61.0	21.64	0.14
Group 3	7	3.29	0.03	12.49	0.08	33.95	0.02	27.05	0.73	22.48	0.21
Group 4	7	3.84	0.21	12.11	0.02	33.34	0.08	25.06	0.40	21.15	0.57
Group 5	7	4.98	0.10	11.00	0.50	32.06	0.25	24.09	0.10	20.40	0.54

Abbreviations: MDA; Malondialdehyde, CAT; Catalase, SOD; Superoxide dismutase, GPX = Glutathione peroxidase, GSH; Glutathione, N; Number of observations, SD = Standard Deviation. Experimental group description: Group 1 (normal control) received only food and water, Group 2 received diet supplemented with 2% TFG, Group 3 received diet supplemented with 4% TFG, Group 4 received diet supplemented with 6% TFG, Group 5 received diet supplemented with 8% TFG.

Table IV: Differences in the effect of TFG on oxidative stress biomarkers

Parameters	Group 1 Mean ± SD. (n = 7)	Group 2 Mean ± SD. (n = 7)	Group 3 Mean ± SD. (n = 7)	Group 4 Mean ± SD. (n = 7)	Group 5 Mean ± SD. (n = 7)	p value
MDA (mmol/mg)	4.37 ± 0.01	3.30 ± 0.04[a]	3.29 ± 0.03[a]	3.84 ± 0.21[a]	4.98 ± 0.10[a]	0.000
CAT (u/mg)	12.38 ± 0.04	12.37 ± 0.09	12.49 ± 0.08	12.11 ± 0.02[a]	11.00 ± 0.50[a]	0.000
SOD (u/mg)	33.31 ± 0.05	33.78 ± 0.04[a]	33.95 ± 0.02[a]	33.34 ± 0.08	32.06 ± 0.25[a]	0.000
GPX (u/mg)	26.16 ± 0.02	25.51 ± 0.61	27.05 ± 0.73	25.06 ± 0.40[a]	24.09 ± 0.10[a]	0.000
GSH (u/mg)	21.89 ± 0.05	21.64 ± 0.14[a]	22.48 ± 0.21[a]	21.15 ± 0.57	20.40 ± 0.54[a]	0.000

P < 0.05 is considered statistically significant, values bearing superscript 'a' are significantly different from the normal control group in that same row (p < 0.05)
Abbreviation: MDA; Malondialdehyde, CAT; Catalase, SOD; Superoxide dismutase, GPX; Glutathione peroxidase, GSH; Glutathione, n; Number of observations, SD; Standard Deviation. Experimental group description: Group 1 (normal control) received only food and water, Group 2 received a diet supplemented with 2% TFG, Group 3 received a diet supplemented with 4% TFG, Group 4 received a diet supplemented with 6% TFG, Group 5 received a diet supplemented with 8% TFG and the differential effects on the oxidative stress biomarkers.

Most studies have primarily examined the beneficial properties of TFG, with fewer investigations into its potential side effects. Toxicity assessments of TFG have generally indicated a low risk of toxicity. For example, Yadav and Baquer [29] conducted a comprehensive review of TFG's pharmacological effects on both health and disease, concluding that TFG tends to have minimal or negligible side effects. Furthermore, Zargar [30] reported that hydroalcoholic extracts derived from TFG partially mitigate liver cirrhosis. An evaluation of acute liver toxicity associated with TFG seed aqueous extract was conducted by Alfarisi et al. [31]. Their results demonstrated that the administration of TFG at a dose of 9g/kg body weight yielded a 66.7% survival rate, while lower doses (3g/kg and 6g/kg body weight) resulted in 100% survival. Importantly, none of the administered doses induced any signs of acute toxicity.

Regarding histological examinations, the liver sections of Norvergicus rattus administered 2% TFG exhibited normal liver histology, comparable to the normal control group. In the group administered 4% TFG, normal hepatocytes were observed, along with minor reactive changes in the liver section. However, the liver section of Norvergicus rattus in the 6% TFG group exhibited features suggestive of iron deposition or accumulation. Administration of 8% TFG led to features indicative of liver toxicity and damage. These histological findings, when considered alongside the results related to liver enzymes and oxidative biomarkers, suggest a potential for hepatotoxicity at higher levels of TFG.

It's worth noting that histological studies are scarce and specifically focused on TFG toxicity. Alfarisi et al. [31] conducted a histological study that revealed mild portal inflammation and mild mononuclear cell infiltration in hepatic parenchyma, in addition to mild bile stasis, primarily in the group that received 9g/kg. This finding indicates that TGF has a minimum lethal oral dose and may induce mild liver histopathological inflammatory changes.

CONCLUSION

his study revealed antioxidant and hepatoprotective properties of TFG and further established adverse effects and, liver damage in higher doses response. Further research aimed at isolating the bioactive compounds present in TFG and elucidating their mechanisms of action will facilitate the harnessing of the most beneficial bioactive compounds for potential therapeutic use.

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