"Gene expression and histopathological analysis of the effect of Allium cepa on carbon tetrachloride (CCl4)-induced Anti-Hepatic steatosis in Wister rats"

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) has become the most prevalent chronic liver condition in developed countries, posing a significant public health challenge. Its increasing incidence is attributed mainly to rising rates of obesity and metabolic syndrome, and this highlights the need for effective preventive and management strategies.

Aim: This study investigated onion (*Allium cepa*) as a treatment for carbon tetrachloride (CCl4)induced fatty liver disease.

Materials and Methods: Thirty-five male Wistar rats (150-200g) were divided into seven groups. Group 1 received only water and standard feed; Group 2 received onion extract for 8 weeks; Group 3 received CCl4 (0.5 ml/kg bw, i.p.) twice weekly for 8 weeks; Groups 4 and 5 received CCl4 for 5 weeks followed by 100 mg/kg bw/day and 200 mg/kg bw/day of onion extract, respectively, for 3 weeks; Groups 6 and 7 received CCl4 for 5 weeks followed by raw onion mixed with feed in different ratios for 3 weeks. Liver gene expressions and histology were analyzed.

Results: Exposure to carbon tetrachloride (CCl₄) significantly (P < 0.05) increased levels of alphafetoprotein (AFP), tumor necrosis factor-alpha (TNF- α), and HMG-CoA reductase, indicating liver damage. Histological analysis confirmed structural alterations in all groups except the control. Treatment with onion extract, particularly at 200 mg/kg, reduced AFP, TNF- α , and HMG-CoA reductase levels and improved liver histology.

Conclusion: This study suggests *Allium cepa* may alleviate liver damage in CCl4-induced fatty liver disease, advocating further research on its long-term safety, efficacy, and molecular mechanisms.

Keywords: Allium cepa, Carbon tetrachloride (CCl₄), Therapeutic, Non-alcoholic fatty liver disease, TNF-α, Metabolic syndrome, Obesity, Onion extract, AFP, Liver histology.

1.0 Introduction

Hepatic diseases have been known to cause greater morbidity and mortality globally. Liver disease accounts for approximately 2 million deaths per year worldwide, half of which are due to complications of cirrhosis and the other half as a result of viral hepatitis and hepatocellular carcinoma [1, 2].

Non-alcoholic fatty liver disease (NAFLD) stands out as the leading liver disease in Western countries, which is often related to obesity, metabolic syndrome, or type 2 diabetes, suggesting that obesity and diabetes are predisposing factors for NAFLD and non-alcoholic steatohepatitis

(NASH) [3]. In recent years, this disease has been known to be the most prevalent chronic liver disease in developed nations. The rising prevalence of NAFLD is constantly affecting the developing world as a result of the global obesity epidemic. Moreover, the very close association between NAFLD and metabolic syndrome has been identified [4,5]. NAFLD has now been recognized as a leading cause of chronic liver disease worldwide and is becoming a more common chronic liver disease, particularly in Western industrialized countries [6]. NAFLD has become the leading cause of cirrhosis and hepatocellular carcinoma, affecting more than 25% of the global population, i.e, worldwide prevalence of 25% [7].

Non-alcoholic fatty liver disease (NAFLD), which is characterized by the presence of fat accumulation known as steatosis in >5% of hepatocytes, is of 2 types, namely: Non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), based on histologic findings. Both NAFL and NASH manifest as hepatic steatosis. Still, in addition, NASH is accompanied by inflammation with hepatocyte injury (ballooning) with or without fibrosis, with greater possibilities of progressing to more advanced liver disease such as cirrhosis, hepatocellular carcinoma, and end-stage liver disease [8].

Onion (*Allium cepa*) is a multipurpose food plant used for centuries for its pungency, flavoring value, and medicinal properties. The functional components of onions, which include phenolics and flavonoids, have been reported to possess significant health benefits, some of which include anti-inflammatory, anti-cholesterol, anticancer, antioxidant properties, Immunological effects, and antihypertensive effect. Quercetin, a flavonoid abundantly found in onions, has demonstrated the ability to inhibit the growth of various cancer cells. Studies have shown that quercetin exhibits proapoptotic effects on tumor cells, thereby impeding the progression of numerous human cancers

[9,10]. Allium-derived flavonol-rich extracts have been reported to inhibit adipogenesis and intracellular lipid accumulation in cultured adipocytes and diet-induced obese animal models [11]. The primary aim of this study was to investigate the therapeutic potential of onion (Allium cepa) in mitigating the effects of carbon tetrachloride (CCl4)-induced fatty liver disease in a controlled experimental setting. Carbon tetrachloride is a well-known hepatotoxin that causes liver damage in experimental models, mimicking conditions similar to NAFLD in humans. This study aimed to assess the protective effects of Allium cepa, perform histological analyses, evaluate different dosages, and uncover mechanistic insights. By addressing these objectives, the study aimed to provide comprehensive insights into the efficacy of Allium cepa in protecting against and treating CCl4-induced fatty liver disease, thereby contributing to developing novel therapeutic strategies for liver diseases.

2.0 Materials and Methods

2.1 Extract preparation and extraction.

Several *Allium cepa* L. were obtained from the local market in Akungba, Ondo state. 1490g of onions were cleaned, chopped into small pieces, mashed using a mortar and pestle, and further shade-dried. They were ground into a powdery form using a mechanical blender and passed through a coarse sieve (0.2mm). The *Allium cepa* L. powder was further macerated with 96% methanol for 72 hours. The extract was passed through the rotary evaporator and freeze-dried. The residue obtained was stored in a refrigerator at -4 °C [12].

2.2 Experimental animal

Thirty-five adult male Wistar albino rats weighing between 150 and 200 g were obtained and maintained at the Animal House of Adekunle Ajasin University. They were acclimatized for two

weeks before the experiment's commencement. They were housed in well-ventilated aluminium cages (0.3 m X 0.3 m X 0.2). Wood shavings were used as non-toxic and absorbent bedding to cover the cages' floors.

The ambient temperature was between 23-25°C, and relative humidity was 40-70%, with a 12hour light/dark cycle. Animals were allowed to have free access to water and standard rodent feed. These animals were maintained under specific pathogen-free conditions to avoid contamination and infection. The total experimental period was for a maximum duration of 8 weeks. The experimental protocols were carried out according to the guidelines for the care and use of laboratory animals approved by the Animal Ethics Committee of Adekunle Ajasin University. The study was carried out according to the policies developed by the Institute for Laboratory Animal Research (ILAR) for animal experiments. The guidelines were followed strictly to ensure the protection of the animals' welfare for the period of the experiment.

2.3 Study design

The animals were randomly allocated into seven groups (5 rats each), n = 5.

Group 1: (Normal control group) received water and rat feed

Group 2: Received onion bulb extract only for 8 weeks

Group 3: Received a single dose of CCl₄ (0.5ml/kg body weight intraperitoneal) twice weekly for 8 weeks.

Group 4: Received a single dose of CCl₄ (0.5ml/kg body weight, intraperitoneal) twice weekly for 5 weeks, after which onion bulb extract (100 mg/kg body weight/day) was administered orally for 3 weeks.

Group 5: Received a single dose of CCl₄ (0.5ml/kg body weight, intraperitoneal) twice weekly for 5 weeks, after which onion bulb extract (200 mg/kg body weight /day) was administered orally for 3 weeks.

Group 6: Received a single dose of CCl₄ (0.5ml/kg body weight, intraperitoneal) twice weekly for 5 weeks, after which raw onion bulb mixed with feed (ratio 30/70) was administered for 3 weeks.

Group 7: Received a single dose of CCl₄ (0.5ml/kg body weight, intraperitoneal) twice weekly for 5 weeks, after which raw onion bulb mixed with feed (ratio 70/30) was administered for 3 weeks.

CCl4 was prepared and administered according to the study of Zhang et al (2019) [27].

2.4 Reverse transcription- polymerase chain reaction

Total RNA was isolated from whole tissues following a method described by Omotuyi *et al.* [13]. Briefly, liver tissues were homogenized in cold (4 °C) TRIzol reagent (Zymo Research, USA) and used for AFP, HMGCoA reductase, marker of proliferation Ki-67 (MKI67), and TNF- α gene expression, with β - β -actin used as the housekeeping gene. DNA contamination was removed from RNA following DNase I treatment (NEB) as specified by the manufacturer. DNA-free RNA was converted to cDNA using the M-MuLV Reverse Transcriptase Kit (NEB). The reaction proceeded at room temperature. Inactivation of M-MuLV Reverse transcriptase was performed at 65°C/20 min.

PCR amplification for the determination of genes was carried out using Ready Mix Taq PCR master mix (One Taq Quick-Load 2x, master mix, NEB), whose primers (Primer3 software) are listed in Table 1 below. PCR amplification was performed in a total of 25 μ l reaction mixture. Initial denaturation at 95 °C for 5 minutes was followed by 20 cycles of amplification (denaturation at 95 °C for 30 seconds, annealing for 30 seconds, and extension at 72 °C for 60 seconds) and

ending with final extension at 72 °C for 10 minutes. The amplicons were resolved on a 1.5% agarose gel (Cleaver Scientific Limited) in Tris (RGT reagent, China) -Borate (JHD chemicals, China) -EDTA buffer (pH 8.4) [14].

Tab 1. Gene expression analysis by RT-PCR of the Primer sequences of the studied genes.

Name (Gene ID	Sequence for the Forward primer	Sequence for Reverse primer
TNF-α (24835)	Aagtagtggcctggattgcg	Actcaggcatcgacattccg
AFP (24177)	Agtggagcgcatccatttcc	Caacgacaatggtagctacgttaaa
HMG-CoA (24450)	Tgcagagcgatcagtcttgg	Ctgagtcacaagcacgagga
MKI67 (291234)	Ggtttccagacaccagaccat	Gggttctaactggtcttcctgg
β- actin (81822)	Ccaccagttcgccatggat	Cccaccatcacaccctgg

2.5 Histopathological examination

The excised liver tissue was fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections 4 μ m thick were stained with Hematoxylin and Eosin (H and E) and examined using a binocular Olympus CX31 microscope.

2.6 Statistical analysis

All values were presented as means \pm standard error of the means (SEM). Comparisons between different groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. Differences were considered significant when P<0.05. GraphPad prism® software version 8.0 for Windows (USA) was used for these statistical tests.

3.0 Results

3.1 Relative expression pattern of hepatic mRNAs in the Liver tissue

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Fig 1. Relative expressions of AFP mRNA in the Liver of CCl4-induced non- alcoholic Fatty liver disease Wistar rats

Values are expressed as Mean \pm Standard Error of Mean

A statistically significant difference exists between the group labelled a ' and the group labelled

'b'. (P < 0.05). Groups labelled 'c' have no significant difference from groups labelled a.







Values are expressed as Mean \pm Standard Error of Mean.

There is a statistically significant difference between groups labelled a ' and 'c'

(P < 0.05). Group labelled 'b' has no significant difference with group labelled 'a.

African Journa of Gastroenterology 8



Fig 3. Relative expressions of Ki-67 mRNA in the Liver of CCl4-induced non- alcoholic Fatty liver disease Wistar rats. Values are expressed as Mean \pm Standard Error of Mean.

A statistically significant difference exists between the negative control group and the groups

labelled a ' and 'b'. Group labelled 'c' significantly differs from the 'b' group. (P < 0.05) .

African Journa of Gastroenterology 8



Fig 4. Relative expressions of HMG-CoA reductase mRNA in the Liver of CCl4-induced non- alcoholic Fatty liver disease Wistar rats.

Values are expressed as Mean ± Standard Error of Mean.

A statistically significant difference exists between the normal control group and the groups labelled a. There is also a statistically significant difference between groups labelled a and b (P < 0.05). Groups labelled 'c' have no significant difference from a normal control group and the' b' group.

3.2 Histopathological analysis of the Liver





Fig 5. Photomicrograph of liver section, magnified views for Group 1 (Normal control)

Photomicrograph of a liver section stained by Haematoxylin and Eosin showing normal central venules without congestion (white arrow), the morphology of the hepatocytes appears normal (blue arrow), the sinusoids appear normal and not infiltrated (slender arrow), no pathological lesion is seen.



X400

X400

Fig 6. Photomicrograph of liver section, magnified views for Group 2 (Onion extract control)

A photomicrograph of a liver section stained by Haematoxylin and Eosin showing normal central venules without congestion (white arrow), the morphology of the hepatocytes appears normal (blue arrow), the sinusoids appear normal and not infiltrated (slender arrow), no pathological lesion is seen.



Fig 7. Photomicrograph of liver section, magnified views for Group 3 (Carbon tetrachloride control)

A photomicrograph of a liver section stained by Haematoxylin and Eosin shows a dilated venule with moderate congestion(white arrow), the liver parenchyma shows an extended area of severe necrosis (black arrow), and the sinusoids appear mildly infiltrated by inflammatory cells (slender arrow).



Fig 8. Photomicrograph of liver section, magnified views for Group 4 (100mg Onion extract)

Photomicrograph of a liver section stained by Haematoxylin and Eosin showing moderate portal triditis (white arrow), the morphology of the hepatocytes shows microsteatosis and cytoplasmic vacuolation (blue arrow), the sinusoids show moderate infiltration of inflammatory cells (slender arrow).



Fg 9. Photomicrograph of liver section, magnified views for Group 5 (200mg Onion extract)

A photomicrograph of a liver section stained by Haematoxylin and Eosin showing central venules with very mild congestion (black arrow), the morphology of the hepatocytes appears normal (blue arrow), the sinusoids appear mildly infiltrated by inflammatory cells (slender arrow).



Fig 10. Photomicrograph of liver section, magnified views for Group 6 (30/70 Raw onion).

A photomicrograph of a liver section stained by Haematoxylin and Eosin showing moderate portal triditis (white arrow), the morphology of the hepatocytes shows moderate microsteatosis and cytoplasmic vacuolation (blue arrow), the sinusoids appear normal and not infiltrated (slender arrow).



Fig11. Photomicrograph of liver section, magnified views for Group 7 (70/30 Raw onion).

A photomicrograph of a liver section stained by Haematoxylin and Eosin shows normal central venules without congestion (white arrow). The morphology of the hepatocytes shows moderate steatosis due to cytoplasmic infiltration of fat (blue arrow). The sinusoids appear normal and not infiltrated (slender arrow).

4.0 Discussion

4.1 Gene expression analysis

The mRNA expression levels of AFP, TNF- α , and HMG-CoA were significantly (P <0.05) higher in CCl4-only induced rats compared to the normal/ onion control rats.

AFP, a glycoprotein, is usually synthesized by a developing fetus. The circulating AFP level begins to fall before birth and is undetectable or low in healthy adults. AFP serum levels are found to be increased in various forms of chronic hepatic disorders. They are widely used as a serum marker for diagnosing HCC, especially in patients with chronic liver disease, such as severe fatty liver [15,16].

The relative expression of AFP was presented in Fig. 1, obtaining results similar to previous reports. CCl₄ induction resulted in overexpression of AFP in the group treated with CCl4 only, compared to the Normal/ onion control group and CCl4-induced/onion-treated groups, except for the group that was given 70/30 raw onion. Groups treated with 200mg onion extract and 30/70

raw onion showed significant (P < 0.05) downregulation of the AFP gene compared to the CCl4only treated group. Increased serum AFP levels may be due to hepatic inflammation or fibrosis, which was observed to be present [16].

TNF- α plays a central role in the development of hepatic steatosis. It mediates hepatic inflammation, oxidative stress, and necrosis of hepatocytes [17,18]. As shown in Fig. 2, similar to AFP, CCl₄ induction resulted in overexpression of TNF- α in the group treated with CCl4 only, compared to the Normal/onion control group and CCl4-induced/onion-treated groups, except for the group that was given 70/30 raw onion. This confirms previous reports. Quercetin present in onion has antioxidative and anti-inflammatory potential. It has been reported as an effective treatment strategy for patients with liver damage and fibrosis of immune response, due to its ability to modulate and downregulate pro-inflammatory cytokines [10].

HMG-CoA reductase reportedly involves hepatic lipogenesis and lipolytic reactions [19]. In humans, HMG-CoA reductase is the rate-limiting step in cholesterol synthesis and represents the sole primary drug target for contemporary cholesterol-lowering drugs [20]. In Fig. 4, HMG-CoA reductase was significantly (P < 0.05) upregulated in the CCl4-induced and CCl4/100 mg treated groups compared to the normal group; this is in line with a previous report, which stated that NAFLD has been associated with increased HMG-CoA reductase expression [21]. However, rats treated with 30/70 raw onion showed significantly (P < 0.05) down-regulation of HMG CoA reductase, while the group treated with 200 mg showed non-significant (P > 0.05) down-regulation of HMG CoA reductase compared to the group treated with CCl₄ only.

Hepatocellular Proliferation has been found to play a vital role in steatosis and also correlates with inflammatory cells present in non-alcoholic fatty liver disease. The MKI67 gene was used to determine whether steatosis could impact hepatocyte proliferation. In Fig. 3, the gene ki-67 was

least expressed in the negative control group treated with CCl_4 only compared to the other groups. Significant difference (P < 0.05) was observed between the negative control group and other groups, except the 200 mg onion extract group. This result does not agree with previous reports, which support that the MKI67 gene affects hepatocyte proliferation [22,23]. The possible discrepancy in MKI67 gene expression findings compared to earlier reports could be due to differences in experimental conditions, models, methods, onion extract composition, and interplay with other factors.

4.2 Histopathological findings

Signs of fatty liver were further confirmed by liver histopathological examination in this study. The histological analysis of liver tissue of the rats in the normal group shows that the size and shape of the hepatocytes appear normal, showing normal venules without congestion. The sinusoids also appear normal and not infiltrated; no pathological lesion was observed. This same morphology, seen in the normal group, was also observed in the onion control group, as no pathological lesion was seen. A different morphology was observed in the CCl4-induced group. The structural design was distorted, as evident by the appearance of enlarged venules with moderate congestion compared to the standard control. The liver parenchyma shows an extended area of severe necrosis, while the sinusoids appear mildly infiltrated by inflammatory cells. These structural characteristics have been observed in previous studies [24,25].

These structural changes observed in the CCl4-induced group were significantly reduced in the CCl4-induced/onion-treated groups. The morphology of the hepatocytes of the groups treated with raw onions shows moderate steatosis due to cytoplasmic infiltration of fat. Normal morphology was observed in the group treated with 200 mg onion extract, but the sinusoids appeared mildly infiltrated by inflammatory cells. The group treated with 100 mg onion extract shows moderate

portal triditis, the morphology of the hepatocytes shows microsteatosis and cytoplasmic vacuolation, and mild infiltration of inflammatory cells is observed in the sinusoids. Pathological lesions, such as steatosis and necrosis, are seen in some groups as part of the chain reactions that occur due to the production of free radicals because of oxidative stress caused by CCl₄ [26].

4.3 Limitations

While this study presents promising findings regarding the therapeutic potential of *Allium cepa* on CCl4-induced liver damage, several limitations must be addressed. Firstly, the study was conducted on a small sample size of Wistar rats, which may limit the generalizability of the results to humans. The study did not explore prolonged onion extract administration's long-term effects and safety. The mechanisms underlying the hepatoprotective effects of onion were not fully elucidated, warranting further molecular investigations. Furthermore, the study focused solely on male rats, and it remains unclear whether similar results would be observed in females or other animal models. Finally, while the down-regulation of specific markers was observed, the functional outcomes related to liver function and overall health improvement were not comprehensively evaluated.

4.4 Further Research

Future research should aim to validate these findings in larger, more diverse animal models and, ultimately, in clinical trials involving human participants. Long-term studies are necessary to assess the safety and efficacy of onion extract over extended periods. Investigating the detailed molecular pathways involved in the hepatoprotective effects of *Allium cepa* will provide a deeper understanding of its therapeutic potential. Additionally, exploring the impact of onion extract in

combination with other treatments for NAFLD could offer insights into more comprehensive management strategies. Gender differences and the potential benefits in different stages of liver disease should also be explored to enhance a better understanding of the pharmacokinetics of *Allium cepa*.

5.0 Conclusion

The result showed that Allium cepa exhibited ameliorative potential on the hepatocellular damage induced by CCl4 through maintenance of hepatic membrane integrity as observed from the down-regulation of the genes expressed and the reversal of liver micro-morphological alterations. Although groups administered onion extract, particularly 200 mg, produced a better result compared to the groups given raw onion. The action of onion may be attributed to its antioxidative and anti-inflammatory potential and ability to modulate pro-inflammatory cytokines. The results revealed that onion (*Allium cepa*) might be a potential therapeutic agent for treating and managing Non-alcoholic fatty liver disease. Further research is essential to confirm its long-term safety and efficacy, uncover the precise mechanistic insights underlying its hepatoprotective effects, and explore potential interactions with other drugs.

Footnotes.

Ahmed Fathy (Professor of internal medicine, gastroenterology, and hepatology unit), Hayam Rashed (Professor of pathology), Samia Hussien (Professor of Medical Biochemistry and Molecular Biology), and Amany Mohamed (Professor of family medicine, biostatistician) were the peer reviewers.

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Ethics approval

The experimental protocol used in this study was carried out according to the guidelines for the care and use of laboratory animals approved by Adekunle Ajasin University's Animal Ethics Committee and the European regulations. All procedures that can give welfare and minimize pain or discomfort to the animals were done. The animals were acclimatized to laboratory conditions and had ad libitum access to food and water before experimentation.

All animals were euthanized for tissue collection as indicated in the AVMA guidelines for the Euthanasia of Animals, edition 2013.

Study protocol:

Data and materials availability: The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests: The authors declare that they have no competing interests. **Funding**: This study had no funding from any source.

This work was done according to the guidelines.

Authors' contributions:

OYA designed the research and drafted the manuscript. BPF prepared the extract and participated in animal care, conducted statistical data analysis and gene expression analysis, and drafted the manuscript. DSM designed the research and carried out histopathological analysis. AOA prepared the extract and participated in animal care. OOA prepared the extract and participated in animal care. ABR made some contributions and edited and proofread the manuscript. All authors read and approved the final manuscript.

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