Journal of
Fundamental and Applied Pharmaceutical Science
https://journal.umy.ac.id/index.php/jfap

Aqueous Leaf Extract of *Chromolaena odorata* Attenuates Methotrexate-Induced Hepatotoxicity in Wistar Rats

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Abstract

Methotrexate (MTX) usage, despite its toxicity in body organs, has increased steadily over the years due to its broad applicability for treating different ailments, including various forms of cancer. Certain plant species have been shown to possess therapeutic properties by offering a protective effect against drug side effects. Thus, the current study was carried out to evaluate the potential of aqueous *Chromolaena odorata* leaf extract (AEOC) to attenuate the effect of MTX-induced hepatotoxicity in Wistar rats. The study divided thirty (30) male Wistar rats into five groups consisting of six each: Group I (control), Group II (AEOC at 250 mg/kg BW), Group III (MTX at 7 mg/kg BW), Group IV (AEOC at 250 mg/kg BW + MTX at 7 mg/kg BW), and Group V (Vitamin C (100 mg/kg BW) + MTX at 7 mg/kg BW). *Chromolaena odorata* and Vitamin C was administered for ten consecutive days, while MTX was administered on day 8 for three consecutive days. Rats were sacrificed 24hrs after the last administration. Serum collected was used for the determination of Aspartate Aminotransferase (AST), Alanine transaminase (ALT), Albumin (ALB), Total Bilirubin (TB), and Total protein (TP), while liver tissue was used for assessment of Superoxide Dismutase (SOD), Malondialdehyde (MDA) and Catalase (CAT) as well as histopathological analysis. The result showed a significant increase in the level of SOD, CAT and a significant reduction in MDA in *Chromolaena odorata* or Vitamin C treated groups compared with MTX. Furthermore, *Chromolaena odorata* or Vitamin C significantly reduced liver function enzymes and Total Bilirubin levels while increasing synthetic molecules compared to the MTX group. *Chromolaena odorata* attenuated the toxic effect of MTX, which was corroborated by histopathological analysis. In conclusion, *Chromolaena odorata* attenuated MTX-induced hepatotoxicity by enhancing antioxidant status; thus, scavenging free radicals and reducing oxidative stress.

Keywords: *Chromolaena odorata*; Hepatotoxicity; Methotrexate; Oxidative Stress; Wistar Rats

Data of article
Received : 29 Jul 2022
Reviewed : 10 Aug 2022
Accepted : 29 Aug 2022

DOI
10.18196/jfaps.v3i1.15652

Type of article:
Research

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INTRODUCTION

Methotrexate (MTX) is an anti-metabolic agent that affects the metabolism of folic acid.[1]. In medical practice, Methotrexate is indicated for a plethora of clinical conditions, including autoimmune rheumatic conditions; rheumatoid arthritis, systemic lupus erythematosus, psoriatic arthritis, juvenile idiopathic arthritis, inflammatory myopathies, sarcoidosis, rheumatic polymyalgia, arthritis related to secondary amyloidosis and others. It is also indicated by other autoimmune conditions, such as Sjögren syndrome, inflammatory bowel disease, vasculitis, and some neoplasms.[2] The importance of MTX as an effective chemotherapy agent for cancer treatment cannot be overemphasized as it has successfully been used in treating breast cancer, leukemia, lung cancer, lymphoma, gestational trophoblastic disease, and osteosarcoma.[3] However, side effects abound, affecting various body systems, including the Gastrointestinal System,[4] Hematopoietic System,[2] Central Nervous System, Respiratory System[5], and Cardiovascular System.[2] MTX also presents renal toxicity and decreases creatinine clearance and glomerular filtration rate.[2] Furthermore, there is evidence that MTX can be oncogenic, notably in lymphomas and leukemias.[5]

The use of herbal medicines has continued to gain momentum, particularly in Africa, where 70-80 % of its people depend either totally or partially on it. Certain herbs have been reported to have the potential to alleviate the side effects of most synthetic drugs.[6] *Chromolaena odorata*, a pantropic herb, possess phytochemicals and antioxidant enzymes that activate defense mechanisms and stress-sensing transcription factors to prevent oxidative damage.[7] The dried leaf of *Ch. odorata* contains active phytochemical substances, such as flavonoid aglycones (flavanones, flavonols, flavones). It includes acacetin, chalcones, eupatilin, luteolin, naringenin, kaempferol, quercetin, quercetagetin, sinensetin, terpenes and terpenoids, essential oils, alkaloids (pyrrolizidine, saponins, and tannins), phenolic acids (ferulic acid, protocatechuic acid), and phytoprostane compound including chromomeric acid.[8,9,10] *Ch. odorata* has been reported to be used to treat wounds, burns, and skin infections and possess anticancer, antidiabetic, anti-inflammatory, antimicrobial, anti-diarrheal, astringent, antispasmodic, antihypertensive, anti-inflammatory, diuretic, tonic, antipyretic and heart tonic and also cough remedy agent.[11,12] Therefore, the present study aims to evaluate the potential of *Ch. odorata* to reduce the effect of MTX-induced toxicity in rats.

METHOD

Chemical and Reagent

Methotrexate (liquid) of 50 mg (Zuvius Life Sciences, India) was purchased from MEDVALIK Pharmaceuticals Limited, Lagos, Nigeria. All reagents used were of analytical grade and had the highest purity.

Collection and identification of plant material

Fresh leaves of *Ch. odorata* were collected from within the locality of Iyamho community, Uzairue, in Etsako Local Government Area of Edo State, Nigeria, and taxonomically authenticated at the Department of Plant Biology and Biotechnology Herbarium, Edo State University, Uzairue, Edo State Nigeria with voucher number EUH/00066.

Preparation and Extraction of plant material

The fresh leaves of *Ch. odorata* were thoroughly rinsed and air-dried at room temperature for one month, then pulverized, crushed into a fine powder using an electric blender, and weighed with an electric weighing balance. An aqueous extract of the plant was prepared by soaking 1000g of the dried powdered plant materials in 5 liters of double-distilled water and then kept at room temperature for 48 hours to ensure a thorough extraction process. At the end of the 48 hours, the extracts were filtered first through a Whatman filter paper No. 42 (125mm) and then cotton wool. The resultant filtrate was concentrated using a rotary evaporator set at 40°C to one-tenth of its original volume and then reduced to solid form using a water bath. The solid residue (crude extract) was stored at 4°C. Aliquot portions of the crude plant extract residue were weighed and dissolved in normal saline on each experiment day.

Experimental Animal and Design

Thirty (30) male Wistar rats (180-200g) of the species- *Rattus norvegicus* were purchased from the animal house, Department of Zoology, Ambrose Alli University, Nigeria. The animals were housed in a well-lit, adequately ventilated room using a wood-gauze cage in the Animal house of the Department of Biochemistry, Edo State University Uzairue, Edo State. Standard environmental conditions were used (12 hours light and 12 hours dark) in acclimatizing the animals to the new environment. Animals were fed with standard laboratory pellets and given free access to water. This study was approved by the Ethics committee of the Faculty of Basic Medical Sciences, Edo State University Uzairue, and followed the guidelines for ethical conduct in the care and use of non-human animals in research.[13]

After acclimatization for seven days, the rats were randomly distributed into the following groups: **Group I:** Served as a control and only received normal saline orally once daily. **Group II:** Rats were given aqueous extract of *Ch. odorata* at a dose of 250 mg/kg BW orally once daily for ten days. **Group III**: Rats were given MTX intraperitoneally at a dose of 7 mg/kg BW on day 8 of the experiment for three consecutive days. **Group IV:** Rats were given an aqueous extract of *Ch. odorata* (250 mg/kg BW) orally once daily for ten days and then MTX intraperitoneally (7 mg/kg BW) on day 8 of the experiment for three consecutive days. **Group V:** Rats were given Vitamin C (100 mg/kg BW) orally once daily for ten days and MTX intraperitoneally (7 mg/kg BW) on day 8 for three consecutive days. MTX was dissolved in saline and injected intraperitoneally (i.p.) at 7 mg/kg BW dose.[14] *Ch. odorata* at a 250 mg/kg BW dose was based on another study[15]. Vitamin C, an antioxidant, was chosen as a hepatoprotection; thus, it was a positive control.

At the end of the experiment, after 24hrs of the last administration, the rats were sacrificed, and blood was collected in plain tubes. It was allowed to stand for 45 minutes before being centrifuged at 4000 rpm for 25 min to obtain serum for analysis. The Serum was used for the determination of Aspartate Aminotransferase (AST), Alanine transaminase (ALT), Albumin (ALB), Total Bilirubin (TB), and Total protein (TP).

The liver was immediately excised, washed in ice-cold saline, and weighted. A portion was fixed in 10% phosphate-buffered formalin for histopathological

examination, while the remaining portion was stored at -20°C to determine oxidative stress and endogenous enzymes. 10 % tissue homogenate of the stored liver tissues was prepared using phosphate buffer solution at pH 7.34.

The homogenate was centrifuged at 5000 rpm for 15 minutes, and a clear supernatant obtained used to determine Superoxide Dismutase (SOD), Malondialdehyde (MDA), and Catalase (CAT).

Biochemical Parameter

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) activity were determined using the RANDOX Kit according to the manufacturer's instructions.[16] Total bilirubin was determined using the RANDOX Kit according to the manufacturer's instructions.[17] Total protein was determined by using the RANDOX Kit according to the manufacturer's instructions as described.[18] According to the manufacturer's instructions, albumin was determined using the RANDOX Kit based on the Bromocresol green (BCG) method as described.[19] The reaction of thiobarbituric acid determined malondialdehyde (MDA) as an indicator of lipid peroxidation according to the method of.[20] The level of Superoxide Dismutase (SOD) activity was according to the method of.[21] while the method of[22] was used to determine Catalase (CAT).

Histopathological studies

Rats were sacrificed after, and liver samples were excised and washed with normal saline (0.9% NaCl). The isolated livers were fixed in 10% buffered formalin and were further processed for histopathological investigations. Histopathologically the liver tissues were stained with hematoxylin and eosin (H&E), then sections were examined under a light microscope, Leitz (Biomed), and histopathological changes were captured by a Nikon Camera, EOS700D, 18–55 lens.

Statistical Analysis

All the data in the treatment groups were presented as mean ± Standard error of the mean (SEM), and statistical analysis was carried out using statistical package (SPSS) version 20, Windows 10. Mean values of the different treatment groups were compared using one-way analysis of variance (ANOVA), followed by Duncan multiple range *post hoc* tests. The $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The biomarkers for liver damage in Wistar rats treated with aqueous extract of *Ch. odorata* and administered with MTX are presented in Table 1. Administration of MTX significantly increased ($p > 0.05$) AST (113.67 U/L) and ALT (127.08 U/L) enzymes as well as total bilirubin (17.31 mg/dL), while total protein (3.54 g/dL) and albumin (1.56 g/dL) were reduced significantly when compared with a control group and other groups in this study. However, treatment with *Ch. odorata* or Vitamin C to rats administered MTX significantly ($p > 0.05$) restored the level of AST, ALT, total Bilirubin, total protein, and Albumin towards normalcy.

Table 1. Effects of aqueous leaf extract of *Chromolaena odorata* on Liver Function and Synthetic Molecules in Methotrexate-induced Wistar rats

Treatment group	AST (U/L)	ALT (U/L)	ALB (g/dl)	TBIL (mg/dl)	TP (g/dl)
Control	$41.33^{a} \pm 3.33$	$36.67^{a} \pm 3.17$	$7.47^{c} \pm 0.99$	$2.39^{a} \pm 0.45$	$9.65^{d} \pm 0.91$
Ch. odorata (250 mg/kg BW)	$43.67^{a} \pm 3.03$	$40.70^{a} \pm 3.31$	$6.04^{c} \pm 0.70$	$1.25^{d} \pm 0.10$	$7.79^{d} \pm 0.93$
MTX (7mg /kg BW)	$113.67^{c} \pm 6.67$	$127.08^{c} \pm 7.55$	$1.56^{a} \pm 0.32$	$17.31^{c} \pm 1.71$	$3.54^{a} \pm 0.11$
MTX (7 mg/kg BW) + *Ch. odorata* (250 mg/kg BW)	$61.33^{b} \pm 4.07$	$62.08^{b} \pm 5.04$	$3.66^{b} \pm 0.72$	$7.23^{b} \pm 0.96$	$5.39^{b} \pm 0.14$
MTX (7 mg/kg BW) + Vit. C (100 mg/kg BW)	$56.33^{b} \pm 4.88$	$73.01^{b} \pm 5.57$	$3.82^{b} \pm 0.60$	$8.87^{b} \pm 0.87$	$6.07^{c} \pm 0.61$

Values are expressed as Mean ± Standard Error of the Mean, n=6. Values with different superscripts down the column differ significantly at ($p<0.05$). AST-Aspartate Aminotransferase; ALT-Alanine Aminotransferase; ALB- Albumin; TBIL- Total Bilirubin; TP- Total protein; Vit. C- Vitamin C; MTX- Methotrexate.

The present study indicated a significant effect of aqueous leaf extract of *Ch. odorata* on liver Function enzymes, AST, and ALT in Methotrexate-induced Wistar rats. The administration of MTX caused significant liver toxicity marked by elevated serum levels of AST and ALT, similar to previous studies.[23,24,25] Uraz *et al.*[26] reported that an increased level of AST indicated damage caused by methotrexate toxicity to the visceral organs. In this present study, MTX at a dose of 7mg/kg BW was recorded to have a higher concentration of AST and ALT in the serum, which vividly indicated hepatotoxicity against other treatment groups with values towards normalcy. However, administration of aqueous leaf extract of *Ch. odorata* or Vitamin C for ten days with an intraperitoneal injection of MTX was found to improve liver functions, evidenced by the reduction in the AST and ALT values similar to previous findings of Patel *et al.*[27] There was no significant difference in the improvement of liver function between Vitamin C and *Ch. odorata* against MTX-induced toxicity. The ability of *Ch. odorata* or Vitamin C treatment groups to reduce the AST and ALT levels may result from the antioxidant activity of vitamin C or bioactive constituents of *Ch. odorata leaves* such as flavonoid.[10] Furthermore, Xu *et al.*[28] reported that ALT is a more specific indicator of liver damage, particularly liver inflammation, than AST. In the present study, MTX elevated ALT more than AST, as observed by Ozogula *et al.*[29]

The present study also showed a significant effect of aqueous leaf extract of *Ch. odorata* on synthetic liver molecules, albumin (ALB), total Bilirubin (TBIL), and total protein (TP) in Methotrexate-induced rats. Although Vitamin C improved in terms of TP level, there was no significant difference in TBIL and ALB levels between Vitamin C and *Ch. odorata* against MTX-induced toxicity. These parameters are used as reliable

checks to indicate liver damage. Based on the results, the low concentration of ALB in blood serum for MTX-administered rats is a clear sign of hepatic impairment compared to the control group. However, administration of aqueous leaf extract of *Ch. odorata* or Vitamin C for ten days with an intraperitoneal injection of MTX recorded much higher values which could be attributed to the protective effect of *Ch. odorata* and vitamin C due to their antioxidant capacity. The findings of this study on the albumin level align with Swayeh *et al.*[30] According to Swayeh *et al.*,[30] these consistent observations are probably due to an indirect effect of Methotrexate on protein synthesis by declining the amount of tetrahydrofolate. This study also recorded a reduction in Total Protein (TP) due to MTX toxicity. Generally, it is expected that the body must constantly produce proteins to fight infections to aid the health and growth of the body's cells and tissues, among others. The level of total proteins shows how well the liver works appropriately to produce these proteins. Conversely, significantly lower values obtained in MTX (7mg/kg BW) group further indicated the extent to which the liver was damaged and could not properly function. The reduction in total protein and albumin could be due to damage to the liver by MTX, increased intestinal protein loss, and protein-losing nephropathy.[31] Similar findings of a decrease in total protein have been previously reported by.[23,32,33,34] However, administration of aqueous leaf extract of *Ch. odorata* or Vitamin C for ten days with an intraperitoneal injection of MTX gradually increased total protein concentration; thus, it demonstrated the protective and antioxidant potential of *Ch. odorata* and Vitamin C.

Furthermore, bilirubin analysis is carried out to ascertain the liver's health or monitor the progression of an affected liver. The elevated levels of Total Bilirubin recorded for the MTX (7mg/kg BW) administered group indicated liver damage or disease. It signified that the ability of the liver to clear bilirubin had been impacted; hence, it observed toxicity. This finding aligns with previous studies of.[35,36,37,38] However, administration of aqueous leaf extract of *Ch. odorata* or Vitamin C for ten days with an intraperitoneal injection of MTX reduced total bilirubin concentration to the value recorded by MTX untreated group. This decrease could be attributed to the protection of the liver against oxidative damage caused by MTX.

MDA, an end product of lipid peroxidation, is often used as a marker of lipid peroxidation. In this study, MTX injection for three consecutive days at a dose of 7mg/kg BW significantly increased MDA in the intoxicated group. In contrast, treatment with *Ch. odorata* or Vitamin C significantly reduced MDA; thus, hepatic damage indicated hepatoprotection (Table 2).

Antioxidant enzymes, such as SOD and CAT, were estimated in the present study. There was a significant decrease in CAT content in the liver homogenates of MTX-treated groups compared to the control at $P < 0.05$. The supplementation of *Ch. odorata* with MTX caused a significant increase in CAT compared to the MTX group (Table 2). MTX at a dose of 7mg/kg for three consecutive days also decreased the activity of SOD compared to the control group (Figure 2). However, rats that received *Ch. odorata* or Vitamin C together with Methotrexate experienced a significant increase in SOD activity compared to the MTX-treated group.

Table 2. Effect of aqueous leaf extract of *Chromolaena odorata* on hepatic Oxidative stress parameters in Methotrexate-induced Wistar rats

Treatment group	SOD (U/mg protein)	MDA (µmol/mg protein)	CAT (U/mg protein)
Control	$89.47^{a} \pm 3.13$	$3.29^{a} \pm 1.54$	$2.87^{a} \pm 0.21$
Ch. odorata (250 mg/kg BW)	$93.59^{a} \pm 5.71$	$6.92^{b} \pm 0.85$	$3.50^{a} \pm 0.58$
MTX (7 mg/kg BW)	$29.94^{b} \pm 4.79$	$13.99^{c} \pm 1.23$	$0.93^{b} \pm 0.12$
MTX (7 mg/kg BW) + *Ch. odorata* (250 mg/kg BW)	$73.22^{c} \pm 3.72$	$8.63^{d} \pm 1.37$	$1.17^{c} \pm 0.06$
MTX (7 mg/kg BW) + Vit. C (100 mg/kg BW)	$56.10^{d} \pm 3.87$	$9.22^{d} \pm 0.95$	$1.42^{c} \pm 0.19$

Values are expressed as Mean ± Standard Error of the Mean, n=3. Values with different superscripts down the column differ significantly at ($p<0.05$); SOD- Superoxide Dismutase MDA- Malondialdehyde, CAT- Catalase, MTX- Methotrexate, Vit C- Vitamin C

The body's antioxidant defense system is the primary line of defense that counteracts the deleterious effects of free radicals, oxidative damage, and oxidative stress. Data from this study indicated a remarkable decrease in the activity of CAT and SOD in the group administered with MTX (7 mg/kg BW) compared to the control group (Table 2). According to Sener *et al.*,[39] oxidative stress leads to pathological and cellular damage to liver tissues. However, administration of aqueous leaf extract of *Ch. odorata* or Vitamin C for ten days with an intraperitoneal injection of MTX showed a significant increase in the activity of CAT and SOD in the liver. This result conforms to a previously reported study of.[40-41] Unlike SOD and CAT, an increase in MDA values was observed for the group administered with MTX. It occurred because lipid peroxidation decreased membrane fluidity[42] and could compromise the integrity and function of the plasma membrane, thereby leading to leakages of materials from hepatocytes into the blood. However, administration of aqueous leaf extract of *Ch. odorata* or Vitamin C for ten days with an intraperitoneal injection of MTX had recorded a much-reduced MDA compared to the group administered with MTX (7 mg/kg BW). It is similar to previous studies of.[43,44,45,40] Although *Ch. odorata* had better improvement in increased SOD levels, there was no significant difference in MDA and CAT levels between Vitamin C and *Ch. odorata* against MTX-induced toxicity.

Furthermore, the histopathological analysis showed that MTX (7 mg/kg BW) caused distortion necrosis, congestion, cell infiltration, irregular loss of hepatocytes architecture with dilated central veins and hepatic sinusoid vacuolar degeneration (Figure 2). However, administration of aqueous leaf extract of *Ch. odorata* or Vitamin C for ten days with an intraperitoneal injection of MTX showed a reduction in hepatic lesions with moderately spaced central veins surrounded by uniform hepatocytes distribution (Figures 4 and 5).

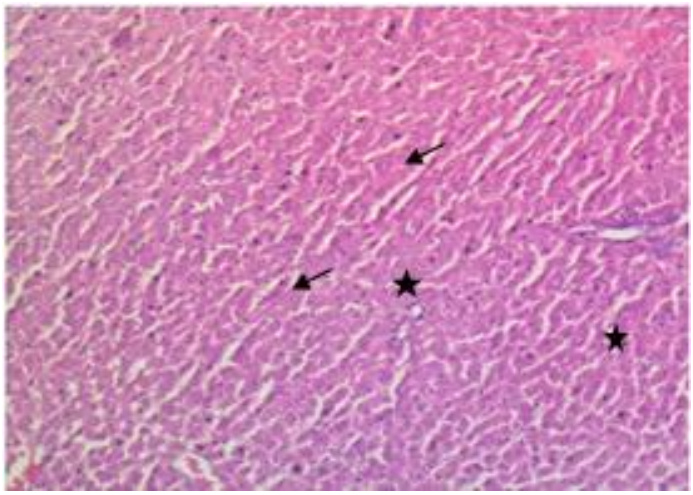

Figure 1. Photomicrograph of liver of Control rats that received normal saline showing normal liver architecture

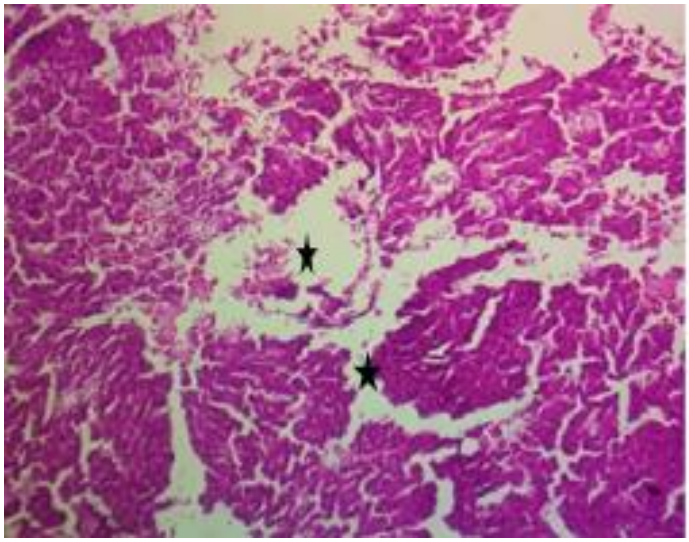

Figure 2. Photomicrograph of liver given MTX at a dose of 7 mg/kg BW for three consecutive days showing necrosis, cell infiltration dilation, and congestion

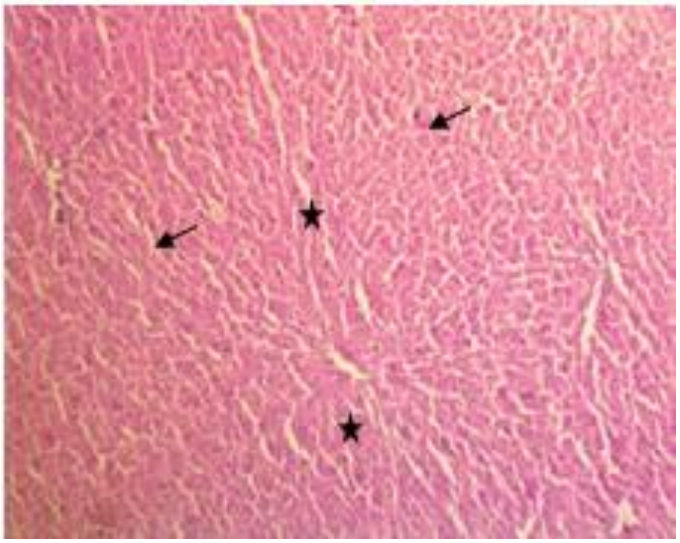

Figure 3. Photomicrograph of liver of rats that received 250 mg/kg BW *Ch. odorata* for ten consecutive days showing normal liver architecture

Figure 4. Photomicrograph of liver of rats given 100 mg/kg BW Vitamin C for ten consecutive days and 7 mg/kg BW MTX for three consecutive days showing a reduction in hepatic lesions

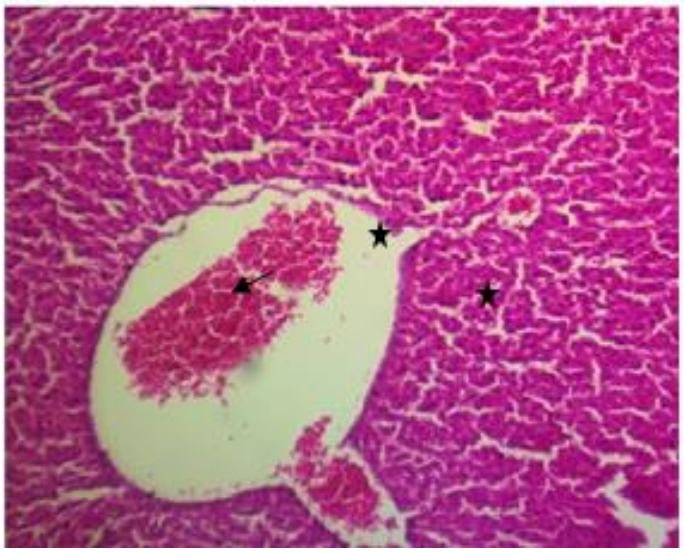

Figure 5. Photomicrograph of liver of rats given 250 mg/kg BW *Ch. odorata* for ten consecutive days and 7 mg/kg BW MTX for three consecutive days showing a reduction in the histopathological lesions

Histopathological analysis showed that MTX (7 mg/kg BW) had significant distortion on the hepatocytes as seen with observed necrosis, congestion, cell infiltration, irregular loss of hepatocytes architecture with dilated central veins, dilated hepatic sinusoid as well as vacuolar degeneration similar to findings of.[46] However, administration of aqueous leaf extract of *Ch.* or Vitamin C for ten days with an intraperitoneal injection of MTX showed a reduction in hepatic lesions with moderately spaced central veins surrounded by uniform hepatocytes distribution.

CONCLUSION

Based on the result of this study, it can be concluded that *Ch. odorata* attenuated MTX-induced hepatotoxicity by

scavenging free radicals, reducing oxidative damage and oxidative stress, thus enhancing antioxidant status.

ACKNOWLEDGMENT

The Authors would like to thank the animal house staff, Department of Biochemistry, Edo State University Uzairue, for all their support in this research.

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