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Protective Effects of *Enantia chlorantha* Stem Bark Extracts on Acetaminophen Induced Liver Damage in Rats

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Abstract

The study was designed to evaluate the hepatoprotective activity of different solvent extracts (hexane, chloroform, ethyl acetate and methanol) of *Enantia chlorantha* stem bark in acute experimental liver injury induced by acetaminophen. The effects observed were compared with a known hepatoprotective agent, silymarin (100 mg/kg p.o.). Preliminary phytochemical tests and acute toxicity study were done. The degree of hepatoprotection was measured using serum transaminases (AST and ALT), alkaline phosphatase, bilirubin, albumin, and total protein levels. In the acute liver damage induced by acetaminophen, *E. chlorantha* stem bark extracts (200 mg/kg, p.o.) significantly reduced the elevated serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and bilirubin in acetaminophen induced hepatotoxicity. The total serum protein was significantly increased ($P<0.05$) by all the solvent extracts. Histological examination of the liver tissues supported the hepatoprotection. Our findings suggested that *E. chlorantha* stem bark extracts possessed hepatoprotective activity; the hexane extract of stem bark of *E. chlorantha* plant possesses better hepatoprotective activity compared to other extracts.

Keywords: Hepatoprotection; Acetaminophen; Liver; Stem Bark Extracts; *Enantia chlorantha*

1. Introduction

The liver is the most important organ in the body. The liver plays a pivotal role in regulating various physiological processes (Rajib *et al.,* 2009). It is the centre of metabolism of nutrients such as carbohydrates, proteins and lipids. It is also involved in the metabolism and excretion of waste metabolites, drugs and other xenobiotics from the body thereby providing protection against foreign substances by detoxifying and eliminating them (Mohamed *et al.,* 2010). As a result of this, the liver is exposed to all types of toxic abuse from both endogenous and exogenous substances which may produce liver degeneration.

Liver diseases have become one of the major causes of morbidity and mortality in man and animals and hepatotoxicity due to drugs appears to be the most common contributing factor (Russmann *et al.,* 2009). For instance, drug-induced liver injury accounts for at least 13% of acute liver failure cases in the United States (Au *et al.,* 2011). The manifestations of drug-induced hepatotoxicity are highly variable, ranging from asymptomatic elevation of liver enzymes to fulminant hepatic failure. Acetaminophen also known as paracetamol, taken in overdose can cause severe hepatotoxicity and nephrotoxicity (Yakubu *et al.,* 2008). In spite of the tremendous advances in modern medicine, there is no effective drug available that stimulates liver function, offers protection to the liver from damage or helps to regenerate hepatic cells (Chaudhary, 2010).

Medicinal plants play a key role in human and animal health care. About 80% of the world population relies on the use of traditional medicine, which is predominantly based on plant material (WHO, 1993). Despite the significant popularity of several herbal medicines in general, and for liver diseases in particular, they are still unacceptable treatment modalities for liver diseases due to lack of standardization of the herbal drugs, lack of identification of active ingredient(s)/principles(s), lack of randomized controlled clinical trials (RCTs) and lack of toxicological evaluation (Radha & Yogesh, 2005). Therefore, due importance has been given globally to develop plant-based hepatoprotective drugs effective against a variety of liver disorders.

There are numerous plants and traditional formulations available for the treatment of liver diseases. *Silybum marianum, Orthosiphon stamineus* and *Foeniculum*

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vulgare are amongst natural products whose hepatoprotective effects have been investigated and documented (Hanefi *et al.,* 2004; Pradhan and Girish, 2006; Subramanian *et al.,* 2006).

Enantia chlorantha Oliv (family-*Annonaceae*) common name-African Yellow Wood is widely distributed along the coasts of West and Central Africa and also very common in the forest regions of Nigeria (Adesokan *et al.,* 2007).

Several studies have shown that the stem bark of *E. chlorantha* possesses wide spectrum antimicrobial and antimalarial (Adesokan *et al.,* 2007; Odugbemi *et al.,* 2007) activities. In Cameroon, the stem bark extract is also used to treat jaundice, urinary tract infections (Adjanohoun *et al.,* 1996), hypoglycaemia, typhoid fever (FAO, 2001). The stem bark is also used for treating leprosy spots, as haemostatic agent and uterus stimulant (Gill, 1992). An anti-sickling compound has also been isolated from the ethanolic extract of the plant (Ejele *et al.,* 2012).

Despite its numerous medicinal uses and importance in the treatment of many illness and diseases in Africa, to our knowledge, no concrete scientific study has been reported to prove the folklore claim of the utility of *E. chlorantha* in the treatment of liver diseases and hence one of the objectives of the present study was to correlate the ethnobotanical evidence with scientific study. Further, the study also attempts to evaluate *in vivo* hepatoprotective and curative effects of stem bark extracts of *E.chlorantha* on acetaminophen induced hepatotoxicity models in rats using solvents of various polarities.

2. Materials and Methods

2.1. Plant Material and Authentication

The plant samples were collected from local region between September and October (rainy season), 2012. The plant was identified and authenticated at the Forestry Research Institute of Nigeria, Ibadan and voucher specimen (FHI. 109950) was preserved at the herbarium.

2.2. Plant Materials Extraction

Stem barks of *E. chlorantha* were dried under shade for 7 days until a constant weight was obtained. This was ground into powder using an electric blender (Blender/Miller III, model MS-223, Taiwan, China). The powder was packed into Soxhlet column and extracted with hexane. The same material was successively extracted with chloroform, ethyl acetate and methanol. The solvents were filtered, squeezed off and evaporated off under reduced pressure in a rotary evaporator to obtain the crude extract. After concentrated preparation, the dried powder extract was stored at 4ºC (Prakash *et al.,* 2008).

2.3. Experimental Animals

Thirty five wistar albino rats (*Rattus norvegicus*) consisting of both male and female with average weight of 150-200 g were obtained from the Animal Holding Unit of the Department of Veterinary Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, University of Ibadan, Nigeria. The animals were allowed free access to feed and fresh water *ad libitum*. All the animals were acclimatized to laboratory conditions for two weeks before commencement of the experiment. The study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Ibadan.

2.4. Drugs and Chemicals

Silymarin (Micro labs, Tamilnadu, India), ethanol, hexane, chloroform, ethyl acetate, methanol and the rest of the chemicals utilized were of analytical grade and were prepared in all glass distilled water. Acetaminophen (Emzor Paracetamol®) was purchased over the counter.

2.5. Phytochemical Screening

The qualitative methods already established to test for classes of compounds in plant extracts by Ciulei (1964) and Chitravadivu *et al.* (2009) were used. The substances that were tested for included: phenolics, alkaloids, steroids, tannins, flavonoids, saponins, glycosides and phlebotanins. The hexane, chloroform, ethyl acetate and methanol extracts of *Enantia chlorantha* stem bark were used to determine the compounds.

2.6. Acute Toxicity Study

This study was conducted according to the Organisation for Economic Cooperation and Development's (OECD) revised up and down procedure for acute toxicity testing (OECD, 2001). Animals were divided into eight groups of five rats each. The control group received distilled water (10 ml/kg) groups II-VIII received 100, 200, 400, 800, 1000, 2000 or 3000 mg/kg of ethanol extract of *Enantia chlorantha* stem bark orally in a single dose. Immediately after dosing, the rats were observed for mortality and clinical signs for the first hour, then hourly for three hours and then periodically for 72hours and then kept for up to 14 days post-treatment in order to observe for any toxic symptoms and mortality.

2.7. Experimental Design

Animals were randomly divided into seven groups (I-VII) of five animals per group. Group I (normal control) received neither the plant extract nor acetaminophen for 8 days. Group II (negative control group) Induction of hepatotoxicity using acetaminophen: The animals received distilled water for 7 days and were administered acetaminophen (500 mg/kg) orally on day 8. Group III (positive control group) – pre-treatment with silymarin (100 mg/kg) for 7 days (p.o) followed by a single dose of acetaminophen on day 8. Groups IV, V, VI and VII – Pre-treatment with hexane, chloroform, ethyl acetate and methanol extract of *Enantia chlorantha* stem bark respectively at 200 mg/kg for 7 days (p.o) followed by a single dose of acetamionophen on day 8.

During the period of drug treatment the rats were fed *ad libitum* with standard pellet diet and had free access to water. The biochemical parameters were estimated after 24 hours following the administration of acetaminophen.

2.7.1. Serum Biochemical Analyses

Blood was obtained from all animals by puncturing retro-orbital plexus**.** The blood samples were allowed to coagulate and then serum was separated by centrifuging at 3000 rpm for 20 min, collected into sterilized tubes and

stored at -20 °C. Serum biochemical parameters were analyzed: Aspartate aminotransferase (AST) (Reitman and Frankel, 1957), alanine aminotransferase (ALT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Kind and King, 1954), serum bilirubin (Mallay and Evelyn, 1937), total protein, albumin, blood urea nitrogen (BUN) using RANDOX® laboratory reagent kits (RANDOX® Laboratories Ltd., Ardmore, United Kingdom).

2.7.2. Histopathological Examination

After collection of blood samples the rats in different groups were sacrificed. 3-5 mm samples of the liver tissue were collected and placed in 10% formaldehyde solution for histopathological study. The pieces of liver were processed and embedded in paraffin wax and sections were made about 4-6 μm in thickness. After staining with haematoxylin and eosin (H&E), slides were examined under microscope (Olympus, Japan) for histopathological changes and photographed.

2.8. Statistical Analysis

All data were expressed as mean ± standard error of mean (SEM), comparison was by the student t test using Graphpad Prism version 4.00 for Windows, Graphpad Software. Significance was reported at $P < 0.05$.

3. Results

Phytochemical screening of the extracts of *E. chlorantha* stem bark revealed the presence of phenolics, flavonoids, alkaloids, glycosides and saponins (Table 1).

All four extracts of *E. chlorantha* stem bark at a dose of 3000 mg/kg *p.o.*, did not produce any mortality in the rats during the pilot acute toxicity study.

There was a significant ($P < 0.05$) increase in the level of serum total protein (TP) in the test groups when compared with the negative control/ untreated group (distilled water). Rats pre-treated with HX, CH, EE and ME extracts of *E. chlorantha* stem bark showed an insignificant increase ($P > 0.05$) in the serum albumin contents when compared with the untreated group. Groups pretreated with HX, CH, EE and ME extracts of *E. chlorantha* stem bark showed a significant increase in the serum globulin contents when compared with the negative control/ untreated group.

The test groups had a significant decrease ($P < 0.05$) in activities of AST, ALT and ALP when compared with the negative control/ untreated group. The extracts (HX, CH, EE and ME) also decreased significantly the activities of ALT and ALP relative to silymarin (positive control group) (Table 2).

The results reported in table 2 also showed that groups treated with HX, CH, EE and ME extracts of *E. chlorantha* stem bark had significantly decreased ($P < 0.05$) levels of serum bilirubin when compared with the negative control/ untreated group.

Liver sections from acetaminophen treated rats showed vacuolar degeneration and different stages of necrotic alterations in the hepatocytes surrounding the central veins. Focal mononuclear leucocytes inflammatory cells infiltration was observed in between the degenerated and necrotic hepatocytes, as well as in the portal area. There was marked congestion of portal vessels and central vein (Figure 1). Liver sections from rats pre-treated with silymarin showed diffused proliferation of Kupffer cells between the hepatocytes associated with dilatation in the portal vein and inflammatory cells infiltration in the portal area (Figure 2).

Liver of rats pre-treated with hexane extract of *E. chlorantha* prior to acetaminophen administration showed diffused proliferation of the Kupffer cells between the hepatocytes, associated with dilatation in the central vein, and focal inflammatory cells infiltration in the hepatic parenchyma (Figure 3). The liver of rats given chloroform extract of *E. chlorantha* before administration of acetaminophen showed dilatation in the central and portal veins with newly formed bile ductules, oedema and inflammatory cells infiltration in the portal area. Focal haemorrhage was noticed in the hepatic parenchyma (Figure 4). The liver of rats dosed with ethyl acetate and methanol extract of *E. chlorantha* stem bark showed no visible histopathological lesion (Figure 5 and 6, respectively).

Table 1. Phytochemical constituents of hexane, chloroform, ethyl acetate and methanol extracts of *Enantia chlorantha* stem bark.

Phytochemical	Hexane	Chloroform	Ethyl acetate	Methanol
Phenolics	-	+	++	++
Flavonoids	+	+	+	+
Alkaloids	++	++	++	++
Glycosides	±	+	±	+
Saponins	++	++	++	++
Tannins	-	-	-	-
Phlebotanins	-	-	-	-
Steroids	-	-	-	-

++ = Strongly positive, + = positive, ± = weakly positive, and - = not detected

Table 2. Serum biochemical values of rats administered with hexane, chloroform, ethyl acetate, methanol extracts of *E. Chlorantha* stem bark and the control groups

	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
TP (g/dL)	2.75 ± 0.15 [a]	1.28 ± 0.09 [b]	1.88 ± 0.23 [c]	2.65 ± 0.22 [a]	1.84 ± 0.02 [c]	1.46 ± 0.04 [b]	1.98 ± 0.19 [a]
ALB (g/dL)	0.85 ± 0.07 [a]	0.43 ± 0.09 [b]	0.72 ± 0.09 [a]	0.45 ± 0.05 [b]	0.80 ± 0.20 [a]	0.44 ± 0.08 [b]	0.88 ± 0.03 [a]
GLB (g/dL)	1.90 ± 0.18 [a]	0.85 ± 0.05 [b]	1.16 ± 0.09 [c]	2.20 ± 0.17 [a]	1.04 ± 0.02 [c]	1.02 ± 0.10 [c]	1.10 ± 0.08 [c]
AST (U/L)	35.46 ± 2.81 [a]	56.00 ± 1.33 [b]	38.50 ±2.26 [a]	34.50 ±0.63 [a]	43.04 ± 0.60 [c]	35.20 ±4.50 [a]	41.50 ±1.71 [c]
ALT (U/L)	41.39 ± 2.61 [a]	75.25 ±1.80 [b]	59.75 ± 2.40 [c]	42.25 ± 1.03 [a]	55.00± 1.41 [c]	46.00 ± 7.48 [a]	55.25 ± 3.35 [c]
ALP (U/L)	35.61 ± 3.11 [a]	72.25 ± 0.85 [b]	46.25 ± 6.76 [b]	34.00 ± 2.94 [a]	30.00±2.97 [a]	38.40 ± 4.35 [a]	46.75 ± 9.16 [b]
BIL(mg/dl)	6.56 ± 0.34 [a]	9.92 ± 0.17 [b]	8.57 ± 0.06 [b]	6.52 ± 0.36 [a]	9.05 ± 0.15 [b]	6.96 ± 0.94 [a]	7.95 ± 0.20 [c]

Values are expressed as mean ± SEM (n= 5 mice/ group).
TP- total protein ALB- albumin GLB- globulin
AST- aspartate aminotransferase ALT- alanine aminotransferase ALP- alkaline phosphatase
BIL- bilirubin
Means with different superscripts within rows are significantly different at $P < 0.05$

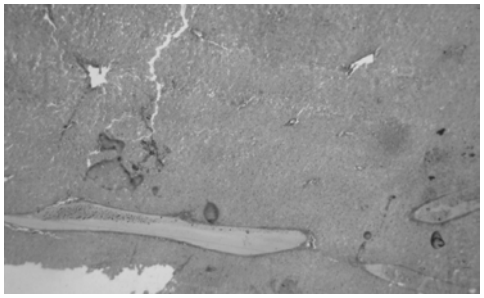

Figure 1. Shows vacuolar degeneration, necrotic hepatocytes, marked congestion of portal vessels and central vein. H & E stain (x100)

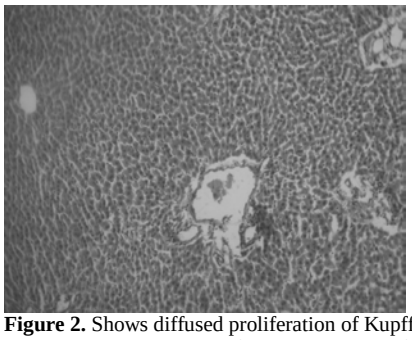

Figure 2. Shows diffused proliferation of Kupffer cells between the hepatocytes inflammatory cells infiltration.H & E stain (x100)

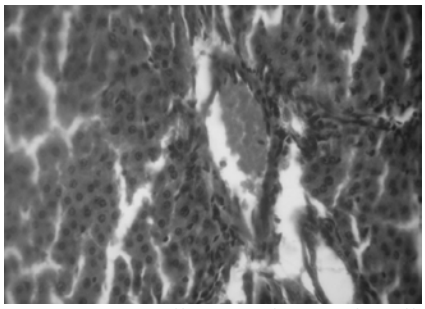

Figure 3. Shows diffused proliferation of Kupffer cells between the hepatocytes dilatation in the central vein and inflammatory cells infiltration in the hepatic parenchyma. H & E stain (x100).

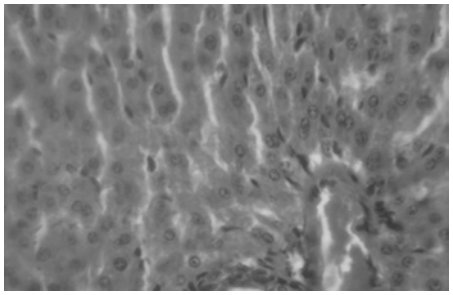

Figure 4. Dilatation in the central and portal veins with newly formed bile ductules, oedema and inflammatory cells infiltration in the portal area. Focal haemorrhage was noticed in the hepatic parenchyma (H & E stain(x100).

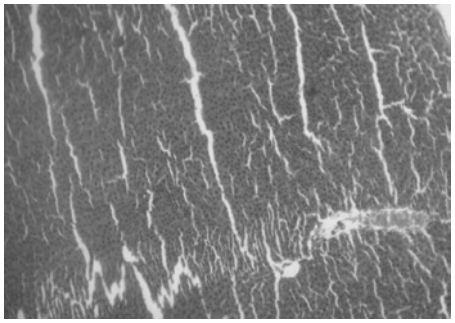

Figure 5. Shows no visible histopathological lesion. (H& E stain) x 100.

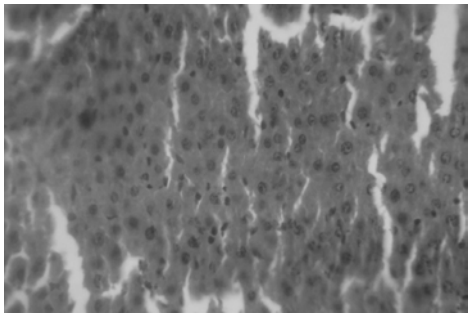

Figure 6. Shows no visible histopathological lesion .H& E stain (100x).

4. Discussion

The stem bark extracts of *E. chlorantha* was found to contain phenolics, flavonoids, alkaloids, glycosides and saponins. These are secondary metabolites which have been reported to cure a lot of diseases (Dongmo *et al*, 2007*;* Suman *et al*, 2011). The fact that methanol exhibited the strongest reactions; thus being able to extract more phytochemicals, could mean that there are more non-polar phytochemicals in the stem bark.

Acetaminophen is a common antipyretic agent, which is safe in therapeutic doses but can produce fatal hepatic necrosis in man, rats and mice with toxic doses (Dash *et al*, 2007). Protection against acetaminophen-induced toxicity has been used as a test for potential hepatoprotective activity by several investigators (Sabir & Rocha, 2008; Parmar *et al*, 2010).

Proteins are important organic constituents of the animal cells playing a vital role in the process of interactions between intra and extra cellular media (Waqar *et al*, 2004). In the present study, the untreated/ negative control group administered with acetaminophen showed a decrease in the level of serum total protein (TP) while pre-treatment with solvent extracts of *E. chlorantha* (500mg/kg) showed an increase in the level of serum protein. Being a part of cell membrane and as an enzyme, protein helps to balance sub cellular fractions. Protein and amino acids are also very important nutrients and they play a major role in the synthesis of microsomal detoxifying enzymes which help detoxify toxicants that enter into the animal's body (Abubakar *et al*, 2010). The reduction in the protein levels in the untreated/ negative control group might thus be as a result of their metabolism to liberate energy during acetaminophen toxicity. The liver is also an important site for the synthesis of many serum proteins (Ahsan *et al*, 2009). The reduction in serum total protein observed in the acetaminophen group may also be associated with decrease in the number of hepatocytes which consequently results in decreased hepatic capacity to synthesize protein. Pre-treatment with HX, CH, EE and ME extracts of *E. chlorantha* stem bark significantly increased TP indicating the hepatoprotective activity of the extracts most probably through hepatic cell regeneration (Olorunnisola *et al*, 2011). These results are in line with the report by Manokaran *et al*, (2008) that oral administration of *Aerva lanata* to acetaminophen treated rats showed increased serum protein level when compared to acetaminophen alone treated rats. Similarly oral administration of hydro-ethanolic extract (70%) of *Calotropis procera* flowers to acetaminophen treated rats showed significantly increased serum protein level (Setty *et al*, 2007). The highest increase in serum total protein content was noticed in the rats treated with the HX extract (this group had more than a two-fold increase) of *E. chlorantha* stem bark and this increase was statistically significant when compared with the acetaminophen treated rats. This also explains the corresponding reduction and increases in albumin in the untreated group and the test groups as about 60% of total serum protein is albumin (Musa *et al*, 2005).

It has been reported in several studies that liver enzymes are liberated into the blood whenever liver cells are damaged and enzyme activity in the plasma is increased (Chang, 2009). Thus ALT, AST and ALP activity and serum bilirubin level are largely used as most common biochemical markers to evaluate liver injury (Ajayi *et al*, 2009). Elevation of these liver enzymes is also associated with cell necrosis of many tissues especially the liver (Adedapo *et al*, 2004). The current study also confirmed these effects of acetaminophen overdose toxicity, as indicated by marked increases in serum hepatic enzymes in the control/ untreated group. This is in consonance with the findings of Vadivu *et al*, (2008) who stated that acetaminophen causes liver damage in rats and significantly ($P < 0.05$) increased the AST and ALT levels in serum when compared with silymarin which has a remarkable protection of serum AST and ALT levels towards acetaminophen induced hepatotoxicity. The significant ($P<0.05$) decrease in activities of these enzymes by the extracts may indicate that the plant extracts did not have necrotic effect on the liver. This may be due to the fact that the extracts offer protection and maintain the functional integrity of hepatic cells. The protective effect may be the result of stabilization of plasma membrane thereby preserving the structural integrity of cell as well as the repair of hepatic tissue damage caused by acetaminophen (Murugaian *et al*, 2008). The increased ALP concentration following acetaminophen administration is in line with existing literature that ALP synthesis is increased by cells lining bile canaliculi usually in response to cholestasis and increased biliary pressure (Gaw *et al.*, 1999). Increased level was obtained after acetaminophen administration and it was brought to near normal level by *E. chlorantha* treatment. This further signifies the curative nature of the extract against acetaminophen toxicity.

Serum bilirubin is considered an index for the assessment of hepatic function and any abnormal increase indicates hepatobiliary disease and severe disturbance of hepatocellular architecture (Martin and Friedman, 1992). Acetaminophen administration resulted in increased serum bilirubin level, (Table 2) thereby suggesting severe hepatic injury and confirming the hepatotoxic nature of acetaminophen. Treatment with *E. chlorantha* stem bark extracts significantly decreased the elevated level of total bilirubin in serum towards normalcy indicating its hepatoprotective efficacy. The hexane extracts demonstrated the highest potency in this regard.

Liver of rats administered with acetaminophen showed severe necrosis, with disappearance of nuclei. This could be due to the formation of highly reactive radicals because of oxidative threat caused by acetaminophen (Shardul, 2010). Histopathological changes of the group pre-treated with the extracts showed significant improvement in architecture. Pretreatment with the ethyl acetate and methanol extracts restored the hepatic architecture and protected the liver tissue from fatty and degenerative changes, by preventing the toxic chemical reaction. Although, necrotic changes were still evident in the liver of rats

pre-treated with the hexane and chloroform extracts, the severity of the damage was less intense significantly. The various phytoconstituents of the stem bark extracts of *E. chlorantha* might be helpful in the changes in the membrane, in the mitochondria or at the ionic level like calcium (Rang *et al*., 2003*)*.The extracts of *E. chlorantha* stem bark may have a role in the process of regeneration and prevention of fibrosis. However, our study has shown the centrizonal necrosis by acetaminophen and prevention of such changes and restoration to normalcy in the centrizonal area by extracts of *E. chlorantha* stem bark.

The possible mechanism responsible for the protection of the acetaminophen induced liver damage by the extract of *E. chlorantha* maybe a result of the extract acting as a free radical scavenger by intercepting the radicals involved in acetaminophen metabolism by microsomal enzymes or the phytochemicals constituents of the plant because a number of scientific reports indicate the role of certain flavonoids and steroids in hepatoprotection against hepatotoxins. The presence of these compounds in *E. chlorantha* may be responsible for the protective effect on acetaminophen induced liver damage in rats.

5. Conclusion

Based on the above results, it could be concluded that hexane, chloroform, ethyl acetate and methanol extracts of *Enantia chlorantha* stem bark exert significant hepatoprotection against acetaminophen-induced toxicity.

The hexane extract of *E. chlorantha* showed better hepatoprotective activity in acetaminophen induced liver damage compared to chloroform, ethyl acetate and methanolic extract(s) as indicated by maximum prevention of increased serum biochemical parameters.

The stem bark extract of *E. chlorantha* extract may be hepatoprotective.

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