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THERAPEUTIC EFFECT OF VISCUM ALBUM AGAINST CCL4 INDUCED HEPATOXICITY IN MALE ALBINO RATS

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ABSTRACT

Viscum album (mistletoe) was used in traditional medicine for the treatment of various disorders, including hepatic disorders. The present study was aimed at evaluating efficacy of viscum album in protecting rat liver against CCL_4-induced liver injury. Biochemical parameters and histological structure were assessed and used as a measure of hepatoprotective potential. The experimental animals (15 male wistar albino rats) weighing between 100-120g were randomly divided into 3 groups. Each group comprised 5 rats and was labeled as group 1, 2 and 3. Group 1 (negative control) animals were administered saline orally daily for 6 weeks (1ml volume per kg body weight) while group 2 (CCL_4group) animals were administered CCL_4 mixed with olive oil as vehicle in 1:1 ratio (3ml/kg body weight). In case of hepatoprotective study (that is post treatment of rats) with extract of the herbal plants (500mg/kg daily for each herb; and 250mg/kg daily for the combination of herbal extracts) administered orally for 6 weeks decreased ($P<0.05$) CCL_4-induced increased in concentrations of total serum protein, albumin total bilirubin, and conjugated bilirubin, and CCL_4 induced increase activities of serum AST, ALT, ALP and GGT. Histological examination of the liver of CCL_4–treated rats also administered the herbal extracts alone showed less destruction of liver architecture in comparison to the group treated with CCL_4 only. The results indicated that the herbal extract investigated (mistletoe) had hepatoprotective effect against CCL_4-induced liver injury when used either singly or in combination and this effect could be due to the phytochemicals present in the herbs.

KEYWORDS: Protective Effects, Viscum album, CCl_4 induced hepatoxicity, male Albino Rats

INTRODUCTION

The liver, as a vital organ in the body, is primarily responsible for the metabolism of endogenous and exogenous agents. It plays an important role in drug elimination and detoxification. Liver damage may be caused by Xenobiotics, alcohol consumption, malnutrition, infection, anemia and medications (Chang & Lee, 1993).

Hepatotoxic agents can react with the basic cellular components and consequently induce almost all types of liver lesions. Toxins and drugs are among the basic etiopathogenetic agents of acute liver failure (Ishak & Irey, 1992). Carbon tetrachloride (CCl_4) is an occupational chemical reagent widely used as a solvent in insecticide industry and is correlated with high incidence of certain types of cancer" (Jeffrey and Allan, 2006).

Studies have shown that regular consumption of fruits, vegetables and seeds can help prevent the risk of many diseases due to their content of bioactive compounds (Peng *et al*., 2012).

The major use of mistletoe, *Viscum album*, is as a palliative cancer therapy. Historically it has been used to treat hypertension, epilepsy, exhaustion, anxiety, arthritis, vertigo, and degenerative inflammation of the joints. *V. album* is the European mistletoe species; it is the species used in the treatment of cancer and it will be the focus of this monograph. Phoradendron, or American mistletoe, is rarely used medicinally. Both species contain lectins, protein toxins, and polysaccharides. The scientific evidence regarding mistletoe's use as a palliative cancer therapy is inconclusive but promising. Mistletoe extracts are usually given parenterally and may cause inflammation at the injection site. Side effects from ingestion of mistletoe include gastrointestinal symptoms such as nausea, vomiting and diarrhea. Poisonings of children after mistletoe ingestion have been reported. There are no data on mistletoe's safety during pregnancy or lactation.

AIM OF STUDY

The aim of this study is to evaluate the protective effects of Mistletoe (*Viscum album)* against CCL4-induced liver injury in Wistar rats.

MATERIALS AND METHOD

Collection and Care of experimental animals

Wistar rats were considered the choicest animals for this experiment because of their availability, cost, genetic makeup, its handling technique and nature of the study.

Fifteen (15) healthy and sexually matured male albino rats of 12 weeks old weighing between 100-120g were used in this study. The rats were obtained from the Experimental Animal Unit of the University. The rats were housed in conventional wire mesh cages under standard laboratory conditions and were allowed free access to water and feed throughout the period of the experiment. The animal feed was gotten from Rumuosi local market Port Harcourt. Constituents of the animal feed are: Maize grains, Wheat brand, Groundnut, Palm kernel, Fish meal. It also contained minerals like sodium and magnesium.

Acclimatization of animals

After the collection of the animals, they were weighed and identified and kept in a wire gauge cage floored with saw dust to maintain dryness, under favourable condition for two weeks. The animals were feed and handled regularly so as to acclimatize with the handling and environment of human physiology.

Preparation of Aqueous Leaf Extract of Mistletoe (*Viscous album*) (Using Cold Maceration Method)

2kg of powdery form of the **Mistletoe leaves** was taken to the Department of Pharmacognosy laboratory of University of Port Harcourt for extraction.

During the extraction water was used for the maceration. 2kg of the leaf was macerated with water then allowed to stand at room temperature for a period of 3 days with frequent stirring until the soluble matter dissolved. The mixture then was sieved, the damp solid material was pressed, and the solvent was clarified by filtration. The solvent was then placed in the reservoir of soxhlet for extraction. The liquid extract in the reservoir was subjected to heat for several minutes in order to vapourize the moisture. The sample was evaporated over the water bath at a temperature of 45^0C and was constantly monitored until a gelatinous extract was formed. This process was carried out by Mr. Jonah, a laboratory technologist to the Faculty of Pharmaceutical Sciences, University of Port Harcourt River State.

Experimental Design (grouping and treatment of animals)

Fifteen (15) male Wistar albino rats were used for this research and were divided according to their body weight into 9 groups with each group containing five (5) rats.

Group 1 (This was the negative control group; they received 1ml of only distilled water daily for six (6) weeks).

Group 2 (This group was induced with Carbon tetrachloride (CCl_4) and served as a positive Control).

Group 3 (After inducing with CCl_4 this group received 500mg/kg body weight of mistletoe extract only and daily for six (6) weeks).

The summary table is shown below.

Groups	Treatments
Group 1	negative control**
Group 2	positive control**
Group 3	mistletoe+ CCl_4

Negative control = (1ml of distilled water).**

Positive control = (3ml/kg CCl_4 – induced hepatotoxicity)**

(In the study animals, hepatic injury in all groups except standard control was induced by single oral administration of CCl_4 mixed with olive oil as vehicle in 1:1 ratio (3 ml/kg of rat body weight). Animals with hepatic injury were post treated with various single herbal extracts in doses of 500mg/kg and mixed herb extracts in doses of 250 mg/kg orally.)

Procedures for preparation of dose of extracts administered

This was based on the average weight of the experimental animals (rats) in each group; this was done on every week of administration.

Calculation of Doses

Since the dosage of extract was body weight dependent, therefore the formula for calculating dosage was:-

$$\frac{\text{Administrable dose of the extract (mg)}}{1000\text{g}} \times \frac{\text{Average body weight of in the group (g)}}{1}$$

The above formula was used to calculate the dose of extract administered to the rats weekly throughout the period of the experiment.

Procedures for Administration of Extracts

Administration was by oral route. The rat was held at the skin over the head and turned so that the mouth was faced upward and the body lowered towards the holder. The syringe needle knob was then placed into the mouth of the rat a bit laterally to avoid the teeth which are

centrally located. The syringe content was then gradually emptied drop by drop into the mouth of the rat.

Blood Sample Collection and Analysis
The Animals were sacrificed after the fourth week of administration. Blood samples were collected via cardiac puncture for liver enzymes evaluation, this analysis took place at the Research Laboratory of the department of Biochemistry, University of Port Harcourt.

Alkaline phosphatase (ALP) activity was determined using the method of Wright *et al*. (1972). Activities of aspartate transaminase (AST) and alanine transaminase (ALT) were determined based on the method described by Schmidt and Schmidt (1963).

Laboratory procedures for liver enzymes Evaluation
Analytical Techniques Biochemical analysis were carried out to determine the serum concentrations of total protein, albumin, conjugated and total bilirubin, and the activities of liver enzymes such as AST, ALT and ALP using diagnostic kits (Quimica Clinica Applicada, S. A. Spain). Total protein was determined by the Biuret method, albumin by the bromocresol green method, bilirubin was estimated by the method described by Jendrassik and Grof (1938). Alanine and aspartate aminotransferases were determined based on the colourimetric measurement of hydrazone formed with 2, 4 dinitrophenyl hydrazine (Reitman and Frankel, 1957), alkaline phosphatase by the phenolphthalein monophosphate method.

Alanine Aminotransferase (ALT)
Procedure: α-Ketoglutarate reacts with L-alanine in the presence of ALT to form L-glutamate plus pyruvate. The pyruvate is used in the indicator reaction for a kinetic determination of the reduced form of nicotinamide adenine dinucleotide (NADH) consumption. Preincubation of a combined buffer and serum solution to allow side reactions with NADH to occur, 4) substrate start (α-ketoglutarate), and 5) optimal pyridoxal phosphate activation. As a group, the transaminases catalyze the interconversion of amino acids and α-keto acids by transferring the amino groups.

Alkaline Phosphatase (ALP) & Gamma Glutamyl Transferase (GGT)
Procedure

a) Reagent 1 (R1) working solution: Buffer/magnesium (bottles 1 and 1a); 2-Amino-2-methyl-1- propanol D 0.93 mol/l, pH 10.5; magnesium-L-aspartate: 1.24 mmol/l; hydrochloric acid; zinc sulfate hepta-hydrate Using a funnel, transfer 6 tablets of magnesium-L-aspartate (Bottle 1a) into contents of one Bottle 1 (Buffer). Swirl gently to dissolve. Aliquot into clean analyzer bottles and store capped at 2–8°C until the expiration date on the package.
b) Reagent 2 (R2) working solution: 2-Amino-2-methyl-1-propanol D 0.93 mol/l, pH 10.5; pnitrophenyl phosphate: 101 mmol/l; hydrochloric acid; zinc sulfate heptahydrate Dissolve 6 tablets of magnesium from one Bottle 2 (Substrate) by adding R1 Working Solution (Buffer/Magnesium) up to the base of the bottle neck (23 mL). Swirl gently to dissolve. Aliquot into clean analyzer bottles and store capped at 2–8°C until expiration date on package.

Aspartate Aminotransferase (AST)
Procedure
(a) Reagent 1 (R1) working solution: Tris buffer: 100 mmol/l, pH 7,8; L-aspartate: 300 mmol/l; NADH: 0.23 mmol/l (yeast); MDH D 0,53 U/ml (porcine heart); LDH D 0,75 U/ml (microorganisms); preservative Tap the bottom of the granulate bottle (Bottle 1a) before opening. Connect one Bottle 1a (Enzyme/Coenzyme) to Bottle 1 (Buffer) using one of the enclosed adapters. Pour granulate into the buffer and completely dissolve by inverting gently. Aliquot into clean analyzer bottles and store capped at 2–8°C.
(b) Reagent 2 (R2) working solution: ketoglutarate: 75 mmol/l; preservative Use α-ketoglutarate solution, supplied "ready to use." Store capped at 2–8°C until the expiration date on the package.

Albumin
Procedure
Although BCP is structurally similar to the conventional brom cresol green (BCG), its pH color change interval is higher (5.2–6.8) than the color change interval for BCG (3.8–5.4), thus reducing the number of weak electrostatic dye/protein interactions. The BCP system eliminates many of the nonspecific reactions with other serum proteins as a result of the increased pH. In addition, the use of a sample blank eliminates background spectral interferences not completely removed by bichromatic analyses.

Total Bilirubin & Conjugated Bilirubin
Procedure
Reagent 1 (R1) working solution: C2H3NaO2 (sodium acetate buffer): 85 mmol/l; H3NO3S (sulfamic acid): 110 mmol/l; surfactant; solubilizer R2 Use supplied "ready to use." Store at 2–8°C until the expiration date on the package. (b) Reagent 2 (R2) working solution: HCl: 100 mmol/l; diazonium ion: 3 mmol/l Use supplied "ready to use." Store at 2–8°C until the expiration date on the package.

Total Protein
Procedure
(a) Reagent 1 (R1) working solution: Sodium hydroxide: 400 mmol/l; potassium sodium tartrate: 89 mmol/l Use contents of blank, supplied "ready to use." Store at 2–8°C until the expiration date on the package.
(b) Reagent 2 (R2) working solution: Sodium hydroxide: 400 mmol/l; potassium sodium tartrate: 89 mmol/l; potassium iodide: 61 mmol/l; copper sulfate: 24.3 mmol/l. Use supplied "ready to use."

Store at 2–8°C until the expiration date on the package.

Data Analysis

Data were analyzed using appropriate statistical tool known as SPSS version 20. Data were expressed and presented as mean ± SEM (standard error of mean). Post-Hoc test using LSD (Least Significant Difference) was used for multiple comparison among treatment variables.

RESULTS

Table 1 shows the results of the qualitative analysis of mistoletoe. The results indicated the presence of alkaloids and tannins in all three herbal extracts. The extracts also contained flavonoids and carbohydrate. The herb was observed to lack the presence of cyanogenic glycosides but they are found to contain cardiac glycosides. Also mistletoe extracts were found to contain anthraquinones.

Table 1: Qualitative Phytochemical Analysis.

	Tests	Qualitative phytochemical analysis mistletoe
1	**Alkaloid Test**	
A	Meyers	Present ++
B	Dragendorffs	Present ++
C	Hagers	Present ++
2	**Tannin Test**	
A	Ferric Chloride	Present ++
B	Bromine Water	Present ++
3	**Flavonoid Test**	
A	Shinoda	Present ++
B	Naoh	Present ++
C	Alkali	Present ++
4	**Carbohydrate**	
A	Fehlings	Present ++
B	Moliseh	Present ++
5	**Saponin Test**	
A	Frothing	Present ++
B	Emulsions	Present ++
6	**Anthraquinnones**	
A	Free Anthraquinnones	Present +
B	Combined	Present v
7	**Cardiac Glycosides**	
A	Keffer Killiani	Present ++
B	Salkawoski	Present ++
C	Liebermann	Present ++
D	Kedde Test	Present ++
8	**Cyanogenic Glycoside**	absent

Note: +ve = present, + + = present in excess.

Table 2 shows the results from the treatment of group 1 with distilled water and induction of liver injury in group 2 with CCL_4. Protein concentration significantly ($p \leq 0.05$) increased from 30.46±5.44g/L in saline group to 81.34±10.08g/L in induced group and albumin, from 25.96±5.01g/L to 78.08±3.94g/L. The pattern is the same for Total and conjugated bilirubin. ALT concentration was similarly found to increase from 7.60±0.89u/L the control group to 19.00±2.12u/L in the CCL_4-induced group ($P<0.05$).

ALP equally was significantly increased ($P \leq 0.05$) from 86.00±1.00u/L in the control group to 387.80±4.82u/L in the CCL_4-induced group while AST showed similar pattern as it increased from 28.40±11.50u/Lin the control to 144.4u/L. The level of GGT significantly increased too from 27.98u/L in saline group to 88.00±4.69u/L in the CCL_4-induced group as shown.

Table 2: Liver function parameters in rats treated with saline & CCL_4.

	Liver function Parameters	Saline-treated (Negative control)	CCl_4-induced (positive control)
1	Protein (g/dl)	30.46± 5.44	81.34±10.08*
2	Albumin (mg/dl)	25.96±5.01	78.08±3.94*
3	Total bilirubin(mmol/L)	10.41±6.09	91.23±1.42*
4	Conjugated. Bilirubin (mmol/L)	6.78±0.64	48.99±1.95*

5	ALT(u/L)	7.60±0.89	19.00±2.12*
6	ALP (u/L)	86.00±1.00	387.80±4.82*
7	AST (u/L)	28.40±11.50	144.40±18.62*
8	GGT (u/L)	27.98±0.78	88.00±4.69*

Values are presented in mean ± SD. n= 5. P ≤ 0.05 * = means values are statistically significant when compared to the Negative control.
Negative control** = (1ml of distilled water).
Positive control** = (3ml/kg CCl_4 –induced hepatotoxicity)

The table 3 below presented results for the treatment of CCL_4-induced hepatotoxicity with 250mg/kg of mistletoe. It was established from the results that the levels of albumin, total bilirubin, and conjugated bilirubin were all decreased upon treatment with mistletoe significantly (P ≤ 0.05) which compared with the positive control values.

CCL4-treated group showed a significant increase (P<0.05) in serum total protein concentration (81.34±10.08g/L), albumin (78.08±3.94g/L), total bilirubin (91.23±1.42mmol/L), and conjugated bilirubin (48.99±1.95mmol/L) as compared to control.

In the mistletoe – treated group, a significant decrease (P≤0.05) was found in albumin (57.04±3.07g/L), total bilirubin (29.52±1.92mmol/L) and conjugated bilirubin concentrations (14.26±0.83mmol/L) as compared to the CCL_4 – treated group.

Table 3: Serum protein and bilirubin concentrations in CCL_4-treated rats following administration of extracts of mistletoe, (*Viscum album*), for 6 weeks.

	Groups	Protein Carbonyl (g/L±SD)	Albumin (g/L±SD)	Total bilirubin (mmol/L±SD	Conj. Bilirubin (mmol/L±SD)
Group 1	Negative control**	30.46± 5.44	25.96±5.01	10.41±6.09	6.78±0.64
Group 2	positive control**	81.34±10.08	78.08±3.94	91.23±1.42	48.99±1.95
Group 3	miistletoe+ccl4	79.44±5.14*	57.04±3.07*¥	29.52±1.92*¥	14.26±0.83*¥

Values are presented in mean ± SD. n= 5. P ≤ 0.05 * = means values are statistically significant when compared to the Negative control and ¥ = means values are statistically significant when compared to the Positive control.
Negative control** = (1ml of distilled water).
Positive control** = (3ml/kg CCl_4 –induced hepatotoxicity)

Assessment of Liver Histology in the Negative Control Group (Not Induced With Ccl_4), Positive Control Group (Ccl_4-Induced Hepatotoxicity), And In Treatment Groups With Various Herbs

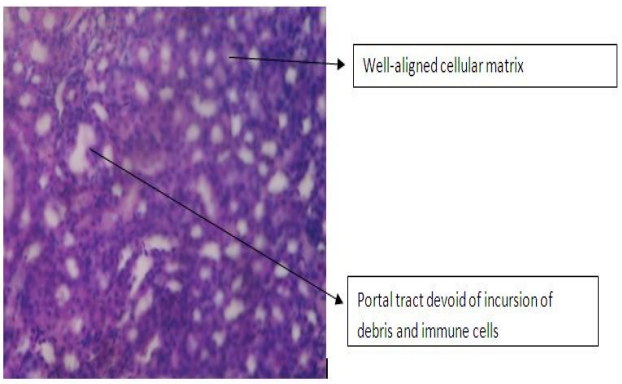

Plate 1: Photo micrographic slide of liver organ of group (control saline) H & E X400.

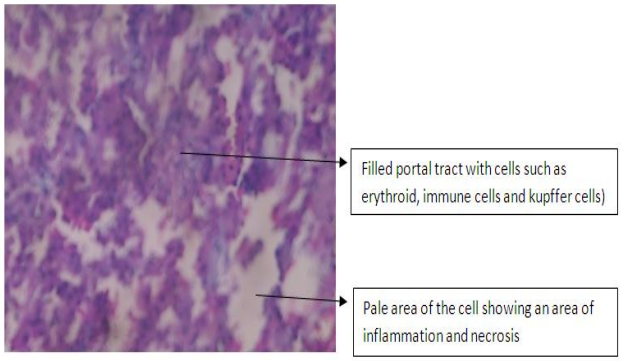

Plate 2: Photo micrographic slide of liver organ of group 2 (*positive control CCL_4-induced hepatotoxicity*) H & E X400.

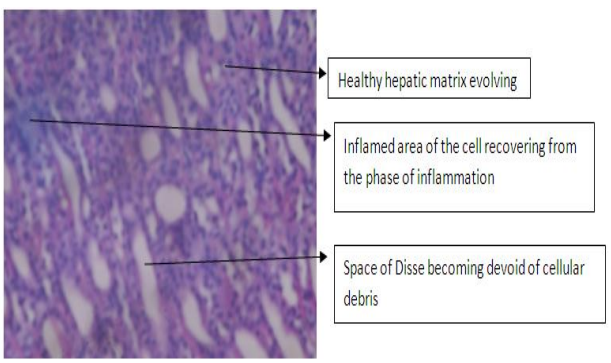

Plate 3: Photo micrographic slide of treated liver organ of group 3 using aqueous extract of *mistletoe* only. (500mg/kg). H & E X400.

DISCUSSION

Assessment of the activities of marker enzymes, like AST and ALT can be used in the analysis of liver function (Daniel *et al*, 1999). Aspartate and alanine aminotransferases (AST and ALT) are normally localized within the cells of the liver, heart, gill, kidney, muscles and other organs. The enzymes are of major importance in assessing and monitoring liver cytolysis (Rosen and Keefe, 2000). Their presence in the serum may give information on organ dysfunction (Sherlock, 1997). The general increase in the activity of liver AST and ALT following the administration of CCl4 could be due to a possible destruction of liver cells, leading to hepatic dysfunction.

The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in plasma were significantly higher in CCl4-treated rats than in the saline control group, indicating the severity of hepatic injury and cholestasis caused by CCl4. *Viscum album* (Mistletoe) administered at 250mg/kg significantly reduced the elevated plasma ALT, AST, ALP and GGT in the CCL4-induced group.

The livers of saline control rats revealed the characteristic hepatic architecture (plate 1). The liver of rats subjected to CCl4 showed loss of liver tissue architecture, severe dilatation and congestion of blood vessels (either central veins or portal tract vessels), marked lymphocytic infiltration, and fibrosis extending between the portal areas (plate 2).

Sections of liver tissue from rats treated with Viscum album at 250mg/kg showed slight distortion of the normal architecture of liver tissue with marked dilatation and congestion of the portal veins in portal areas (plate 3).

Specifically, in the present study, acute hepatic injury was induced by injection of CCl4 in rats, leading to high levels of serum aminotransferases and considerable perivenular necrosis. Hepatic injury was reduced significantly by the administration of *Viscum album* with a reduction in plasma levels of hepatocellular enzymes ALT and AST as well as the cell membrane enzyme ALP and with marked improvement in liver morphology. These results pointed to a hepatic protective effect of *Viscum album*. The study also indicated the usefulness of combining both *Viscum album* and other herbs. Little information is available in the literature on the effect of *Viscum album* on hepatic injury. The plant, however, has been widely investigated for its anticancer properties (Osadebe and Ukwueze, 2004).

Mistletoe (*Viscum album*) extract was proven to be potent at reversing the deleterious effect of hepatic injury caused by CCl4 induction. In fact, two recent studies done in patients with chronic hepatitis C, treated with a mistletoe preparation as monotherapy for 1 year, reported a significant improvement in elevated transaminases (Rosalki and Mcintyre, 1999). The mechanism (s) by which *Viscum album* modulates hepatic inflammation remains, however, unclear. Viscum album extracts contain mistletoe lectins, cytotoxic glycoproteins also known as viscumin or agglutinin, which are members of the family of type 2 ribosome-inactivating proteins, and viscotoxin, which is a 46-amino acid peptide that damages cell membranes (Green and Flamm, 2002). Other constituents include polysaccharides (galacturonan and arabinogalactan) and alkaloids. Viscum album extracts have both immunomodulatory (induces TNF-α and IL-12) and apoptosis-inducing properties, which are likely to be dose-dependent (Boyde and Latner 1967).

The perioperative administration of Viscum album attenuated the immuno-suppressive effects of surgery, increasing the number of NK cells, the T and B cells, complement, and IgA, IgG, and IgM values (Nalpus et al., 1996). Studies also suggested that European mistletoe possesses insulin-secreting (Jendrassik and Grof, 1985), antihyperglycemic, antioxidant activity (Kafaru, 1993), and cholinomimetic activities (Obatomi *et al*, 1996). Findings in the present study suggest that the use of other herbs with Viscum album can have an additive beneficial effect in lessening liver inflammation and necrosis caused by CCL_4.

The release of aminotransferases into the plasma was markedly reduced, indicating a reduction in the severity of liver damage by the combination. Aminotransferases are sensitive indicators of liver-cell injury and are released into the blood in increasing amounts whenever the liver cell membrane is damaged (Oyetayo and Osho, 2004).

Mistletoe preparations are among the most widely used unconventional cancer therapies in many countries (Pandit et al., 2004).

The results of the present study suggest that mistletoe preparations may be a useful therapeutic intervention for patients with chronic liver disease. Clearly, further studies are required to elucidate the mechanism(s) by which mistletoe preparations exert their hepatoprotective effects seen in the present study.

CONCLUSION

Evidently, the use of herbs as shown in this study is efficacious in all ramifications especially in reversing the lopsided concentrations of liver markers upon treatment during the hepatic injury induced by CCL_4.

In the present study, CCL_4 –induced liver damage is manifested by increases in serum ALP, GGT, and bilirubin levels. From our observation on the preventive effects of herbs on liver damage, the differences between the activities of the liver enzymes in those untreated and those treated with mistletoe extracts were all significant.

The herbal plant investigated was able to exert a possible hepatoprotective potential because of their phytochemical constituents. From the results of the histological findings it can be further inferred that extracts of mistletoe possesses natural antioxidants necessary for protection against the possible free radical damage induced by CCL_4 in rat liver.

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