An Aqueous Extract of the Leaves of *Tridax procumbens* Linn (Asteraceae) Protected Against Carbon Tetrachloride Induced Liver Injury in Wistar Rats.

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ABSTRACT

The potential of an aqueous extract of the leaves of *Tridax procumbens* to protect against carbon tetrachloride-induced liver injury was investigated in Wistar albino rats. The carbon tetrachloride was prepared 1:5 (v:v) in olive oil, and administered subcutaneously at 1 mL/kg body weight. The extract was administered to both normal and carbon tetrachloride treated rats at 100, 200, and 300 mg/kg. On gas chromatographic analysis of the flavonoid fraction of the aqueous crude extract, twenty-three known flavonoids were detected, consisting mainly of apigenin (29.00%), quercetin (21.67%), kaempferol (11.20%), (-)-epicatechin (6.38%), naringenin (4.82%), (+)-catechin (3.28%), biochanin (3.21%), robinetin (3.13%), diadzein (2.57%), and nobiletin (2.07%). Compared to test control, the treatment dose dependently significantly lowered (P<0.05) alkaline phosphatase (54.91-100.52%), aspartate transaminase (37.74-64.79%), and alanine transaminase (32.96-57.82%) activities. The plasma total bilirubin and total protein levels of the treated animals were lower though not significantly. Histopathological studies provided supportive evidence for the biochemical analysis. The results of this study indicated that treatment with the plant extracts protects the liver against carbon tetrachloride induced hepatotoxicity, thus supporting the use of *T. procumbens* in African traditional health care for the treatment of liver problems.

(Keywords: Apigenin, flavonoids, hepatospecific markers, histopathology, quercetin, *Tridax procumbens* Linn)

INTRODUCTION

Tridax procumbens Linn (family Asteraceae) is one of a large number of medicinal plants that have been found to offer some hepatoprotection. It is native to Central and tropical South America, but has spread throughout the tropical and subtropical parts of the world (Jahangir, 2001; United States Department of Agriculture, 2011). It is used as an ornamental or fodder plant, and its leaves are cooked as vegetables (Prajapati *et al*., 2008; Acharya and Srivastava, 2010). Traditionally, it is used for the treatment of bronchial catarrh, malaria, stomachache, diarrhoea, epilepsy, diabetes, hypertension, hemorrhage, liver problems, and to prevent balding (Jahangir, 2001; Salahdeen *et al*., 2004; Hemalatha, 2008).

Earlier, Ikewuchi *et al*. (2009) and Ikewuchi and Ikewuchi (2009a,b) had reported the nutrient potential of the leaves. Their protective effects against cholesterol and salt loading in Wistar albino rats (Ikewuchi and Ikewuchi, 2009c; Ikewuchi *et al*., 2010) as well as their weight reducing (Ikewuchi *et al*., 2011a), hypotensive (Ikewuchi *et al*., 2011b) and analgesic (Prabhu *et al*., 2011) activities have also been reported. In the present study, the hepatoprotective effect of aqueous extract of the leaves of *T. procumbens* against carbon tetrachloride induced liver injury was investigated.

MATERIALS AND METHODS

Preparation of Leaf Extract

Samples of the fresh *Tridax procumbens* plants (Figure 1) were collected from within the Choba and Abuja Campuses of University of Port Harcourt, Nigeria. After due identification at the University of Port Harcourt Herbarium, Port Harcourt, the identity was confirmed/authenticated by Dr. Michael C. Dike of the Taxonomy Unit, Department of Forestry and Environmental Management; Michael Okpara University of Agriculture, Umudike, Abia State,

Nigeria; and Mr. John Ibe, the Herbarium Manager of the Forestry Department, National Root Crops Research Institute (NRCRI), Umuahia, Nigeria. They were cleaned of dirt and the leaves were removed, oven dried at 55 °C and ground into powder. The resultant powder was soaked in hot (about 100 °C), boiled distilled water for 12 h, after which the resultant mixture was filtered and the filtrate was stored in a refrigerator for subsequent use. A known volume of this extract was evaporated to dryness, and the weight of the residue used to determine the concentration of the filtrate, which was in turn used to determine the dose of administration of the extract. The percentage recovery of the crude extract was 4.988%. The resultant residue was use for the phytochemical study, to determine the flavonoid composition.

Figure 1: *Tridax procumbens* Linn

Determination of the Phytochemical Content of the Crude Aqueous Leaf Extract

Calibration, Identification and Quantification: Standard solutions were prepared in methanol. The linearity of the dependence of response on concentration was verified by regression analysis. Identification was based on comparison of retention times and spectral data with standards. Quantification was performed by establishing calibration curves for each compound determined, using the standards. The chromatogram of the extract is shown in Figure 2.

Determination of Flavonoid Composition: The extraction was carried out according to the method of Millogo-Kone *et al*. (2009). The flavonoids were extracted with methanol and the resultant extract was subjected to gas chromatographic analysis. Chromatographic analyses were carried out on an HP 6890 (Hewlett Packard, Wilmington, DE, USA), GC apparatus, fitted with a flame ionization detector (FID), and powered with HP Chemstation Rev. A 09.01 [1206] software, to quantify and identify compounds. The column was a capillary HP INNOWax Column (30 m × 0.25 mm × 0.25 µm film thickness). The inlet and detection temperatures were 250 and 320 °C. Split injection was adopted with a split ratio of 20:1. Nitrogen was used as the carrier gas. The hydrogen and compressed air pressures were 22 psi and 35 psi. The oven was programmed as follows: initial temperature at 50 °C, first ramping at 8 °C/min for 20 min, maintained for 4 min, followed by a second ramping at 12 °C/min for 4 min, maintained for 4 min.

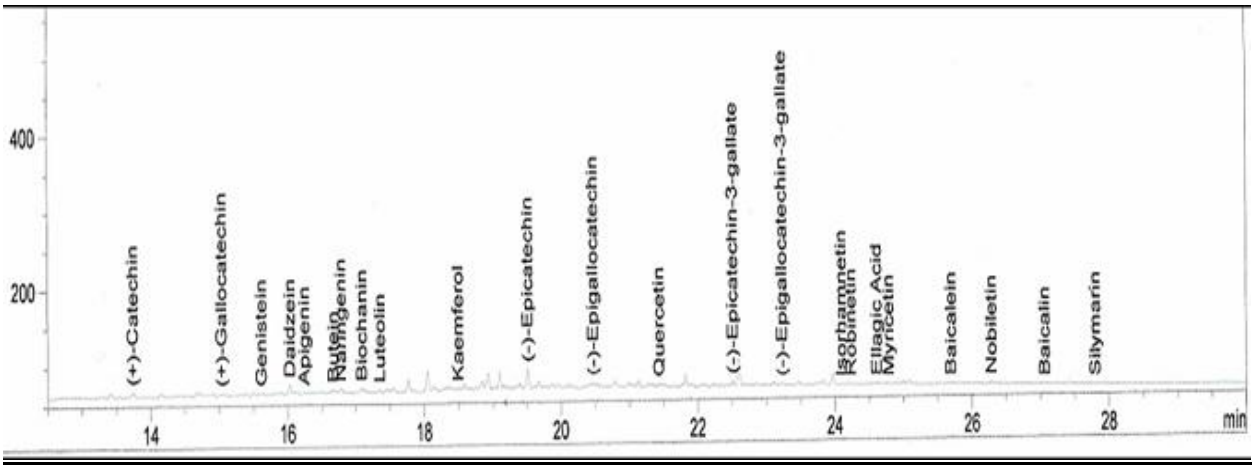

Figure 2: Chromatogram of Flavonoid Composition of Aqueous Extract of *T. procumbens* Leaves.

Experimental Design for the Hepatoprotective Study

Wistar albino rats (185-200 g) were collected from the animal house of the Department of Physiology, University of Nigeria, Enugu Campus. Studies were conducted in compliance with the applicable laws and regulations for handling experimental animals. The rats were weighed and sorted into 8 groups (Table 1) of 5 animals each, so that their average weights were approximately equal. The animals were housed in plastic cages in the animal house of the Department of Biochemistry, University of Port Harcourt. After a 1-week acclimatization period on guinea growers mash (Port Harcourt Flour Mills, Port Harcourt, Nigeria), the treatment commenced.

The extracts were administered orally on daily basis for eight days. The dosages of administration of the extracts were adopted and modified from Ikewuchi *et al*. (2011a,b). The Carbon tetrachloride was prepared 1:5 (v:v) in olive oil, and administered subcutaneously at 1 mL/kg body weight, on days 4 and 8. The dosage and method of administration of Carbon tetrachloride was adopted from Obi and Uneh (2003), with modification. Twenty four hours after the last administration of Carbon tetrachloride, the rats were anaesthetized by exposure to chloroform. While under anesthesia, they were sacrificed and blood was collected from each rat into heparin sample bottles, after which their livers were collected and preserved in 10% formalin, for histological studies. The heparin anti-coagulated blood samples were centrifuged at 1000 g for 10 min, after which their plasma was collected and stored for subsequent analysis.

Assay of Plasma Hepatospecific Markers

The plasma activities of alanine transaminase, aspartate transaminase, and alkaline phosphatase, were determined using Randox test kits (Randox Laboratories Ltd., Crumlin, England, UK). The activities of alanine and aspartate transaminases were respectively measured by monitoring at 546 nm the concentrations of pyruvate and oxaloacetate hydrazones formed with 2,4-dinitrophenylhydrazine. The activity of alkaline phosphatase was determined by monitoring the degradation of p-nitrophenylphosphate to p-nitrophenol, at 405 nm. Plasma total bilirubin and protein concentrations were determined using Randox test kits (Randox Laboratories Ltd., Crumlin, England, UK). The wavelength for the determination of total bilirubin was 578 nm, while that of total protein was 560 nm.

Table 1: Experimental Design for the Hepatoprotective Screening.

S/N	ID	Treatment
1	Normal	Olive oil (1 mL/kg) and water
2	Test control	Carbon tetrachloride (1 mL/kg) and water
3	Treatment control I (TPC1)	Olive oil (1 mL/kg) and extract (100 mg/kg)
4	Treatment control II (TPC2)	Olive oil (1 mL/kg) and extract (200 mg/kg)
5	Treatment control III (TPC3)	Olive oil (1 mL/kg) and extract (300 mg/kg)
6	Treatment I (TP1)	Carbon tetrachloride (1 mL/kg) and extract (100 mg/kg)
7	Treatment II (TP2)	Carbon tetrachloride (1 mL/kg) and extract (200 mg/kg)
8	Treatment III (TP3)	Carbon tetrachloride (1 mL/kg) and extract (300 mg/kg)

Determination of Percentage Protection

The percentage protection provided by the extract against carbon tetrachloride induced liver damage was calculated using the following formula adopted from Al-Qarawi *et al.* (2004)

$$\% \text{ Protection } = \frac{(A - B) \times 100}{A - C} \qquad [Eqn\ 1]$$

Where A = $Parameter_{Test\ control}$; B = $Parameter_{Treatment}$; C = $Parameter_{Control}$

Histopathological Study

The histopathology study was carried out by Professor S.O. Nwosu, of the Department of Anatomical Pathology, University of Port Harcourt Teaching Hospital. Small pieces of liver tissues were collected in 10% formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6 µm in thickness were cut, mounted on slide and stained with hematoxylin and eosin. The sections were then examined via light microscopy (Opticphot-2; Nikon, Tokyo, Japan) at x100 magnification.

Statistical Analysis of Data

Values are reported as the mean ± s.e.m. (standard error in the mean). The values of the variables were analyzed for statistically significant differences using the Student's t-test, with the help of SPSS Statistics 17.0 package (SPSS Inc., Chicago Ill); $p < 0.05$ was assumed to be significant. Graphs were drawn using Microsoft Office Excel, 2010 software.

RESULTS AND DISCUSSION

Table 2 shows the flavonoid composition of an aqueous extract of the leaves of *T. procumbens*. Twenty-three known flavonoids were detected, consisting mainly of 29.00% apigenin, 21.67% quercetin, 11.20% kaempferol, 6.38% (-)-epicatechin, 4.82% naringenin, 3.28% (+)-catechin, 3.21% biochanin, 3.13% robinetin, 2.57% diadzein, 2.07% nobiletin, 1.89% butein, 1.76% baicalein, 1.73% ellagic acid, 1.62% myricetin, and 1.42% (-)-epigallocatechin. All of these compounds have antineoplasmic and anticarcinogenic properties (Dillard and German, 2000; Dewick, 2005).

Figures 3 and 4 show the effects of an aqueous extract of the leaves of *T. procumbens* on plasma markers of liver integrity/function, in normal and carbon tetrachloride treated Wistar rats. The 100 and 300 mg/kg treatment produced significantly ($P<0.05$) lower alkaline phosphatase, aspartate transaminase and alanine transaminase activities. The plasma total bilirubin and total protein levels of the test animals were lower though not significantly, than the test control. Hepatoprotective activity of an aqueous extract of the leaves of *T. procumbens* in carbon tetrachloride-induced hepatotoxicity in Wistar rats is shown in Figure 5. The protection seemed to be dose dependent, with the 200 and 300 mg/kg doses being more effective. Compared to test control, the treatment dose dependently significantly lowered ($P<0.05$) alkaline phosphatase (54.91-100.52%), aspartate transaminase (37.74-64.79%) and alanine transaminase (32.96-57.82%) activities.

The frequency distribution of the effects of the aqueous extract of the leaves of T. procumbens on the liver histology of normal and carbon tetrachloride treated rats is shown in Figure 6. Sections of the liver samples are shown in Figure 7. The histopathological studies showed that carbon tetrachloride caused fatty degeneration and necrosis of the hepatocytes; a condition that was inhibited by pre-treatment with the extract, thereby, confirming the results of the biochemical studies. This result indicates that treatment with the plant extract protects the liver against carbon tetrachloride-induced hepatotoxicity.

Table 2: Flavonoid Composition of an Aqueous Extract of the Leaves of *Tridax procumbens*

Compounds	Retention Time (min)	Composition (mg/kg)
(+)-Catechin	13.738	497.51
(+)-Gallocatechin	15.039	138.30
Genistein	15.617	147.49
Diadzein	16.034	391.05
Apigenin	16.244	4405.200
Butein	16.667	287.26
Naringenin	16.780	732.73
Biochanin	17.089	487.78
Luteolin	17.357	107.57
Kaempferol	18.491	1701.83
(-)-Epicatechin	19.514	969.34
(-)-Epigallocatechin	20.467	215.13
Quercetin	21.434	3292.48
(-)-Epicatechin-3-gallate	22.512	5.61
(-)-Epigallocatechin-3-gallate	23.226	39.65
Isorhamnetin	24.090	45.57
Robinetin	24.231	475.42
Ellagic acid	24.610	263.30
Myricetin	24.786	245.29
Baicalein	25.690	267.79
Nobiletin	26.281	314.77
Baicalin	27.058	144.34
Silymarin	27.799	17.22
Total	**-**	**15192.61**

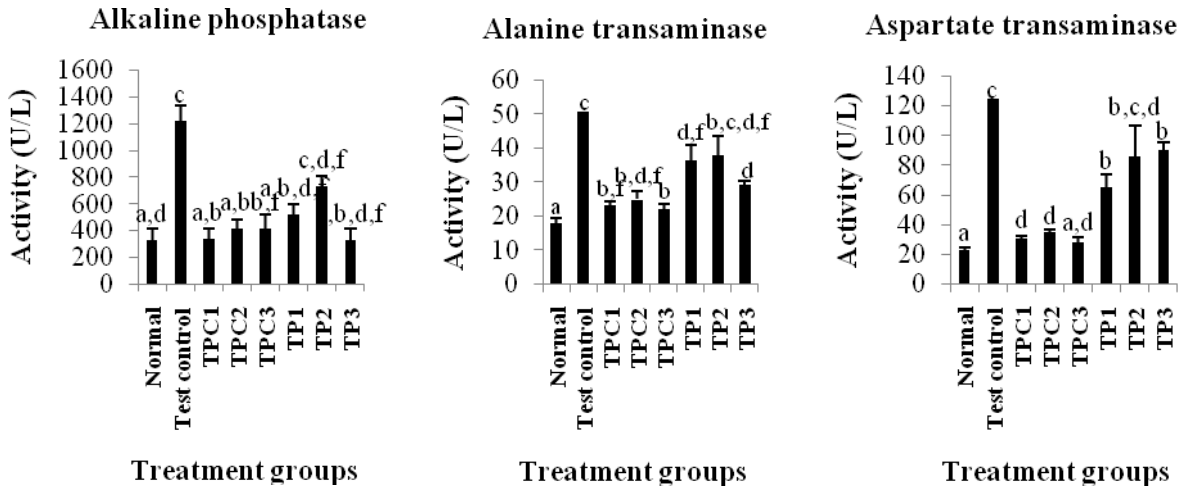

Figure 3: Effect of aqueous extract of the leaves of *Tridax procumbens* on the plasma hepatospecific marker enzymes of normal and carbon tetrachloride treated rats. Values are mean ± s.e.m., n=5, per group. [a,b,c]Values in the same group with different superscripts are significantly different at *P*<0.05.

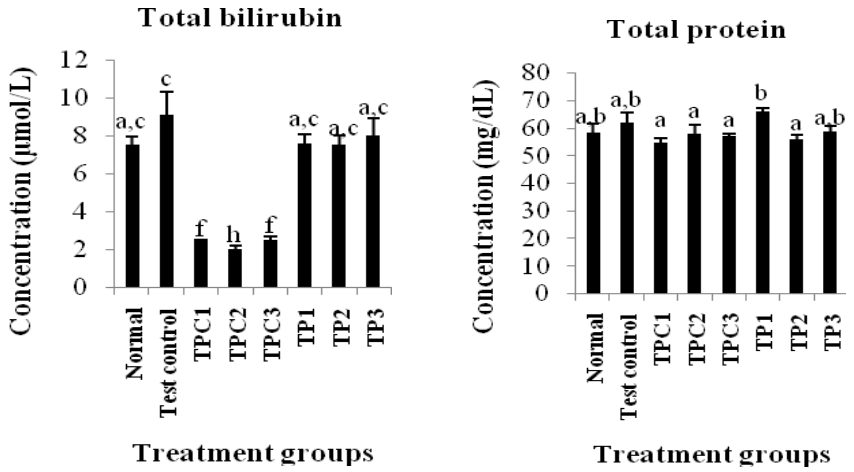

Figure 4: Effect of aqueous extract of *Tridax procumbens* leaves on plasma hepatospecific marker molecules of normal and carbon tetrachloride treated rats. Values are mean ± s.e.m., n=5 per group. [a,b]Values in same group with different superscripts are significantly different at *P*<0.05.

The reduction of carbon tetrachloride-induced elevated plasma activities of aspartate transaminase, alanine transaminase, alkaline phosphatase and total bilirubin level in animals pretreated with the aqueous extract of the leaves of *T. procumbens* shows its ability to protect normal functional integrity of the poisoned liver, and also to protect against subsequent carbon tetrachloride hepatotoxicity. This hepatoprotective activity may have been achieved via any of the following mechanisms.

Reduced metabolic activation of carbon tetrachloride by cytochrome P450 depresses the initial formation of trichloromethyl free radical, resulting in the diminished initiation of lipid peroxidation (Middleton *et al*., 2000), and the consequent toxicity of carbon tetrachloride. It can be suggested that flavonoids in *T. procumbens* leaves (see Table 2, and Ikewuchi *et al.,* 2009), could be responsible for its hepatoprotective ability.

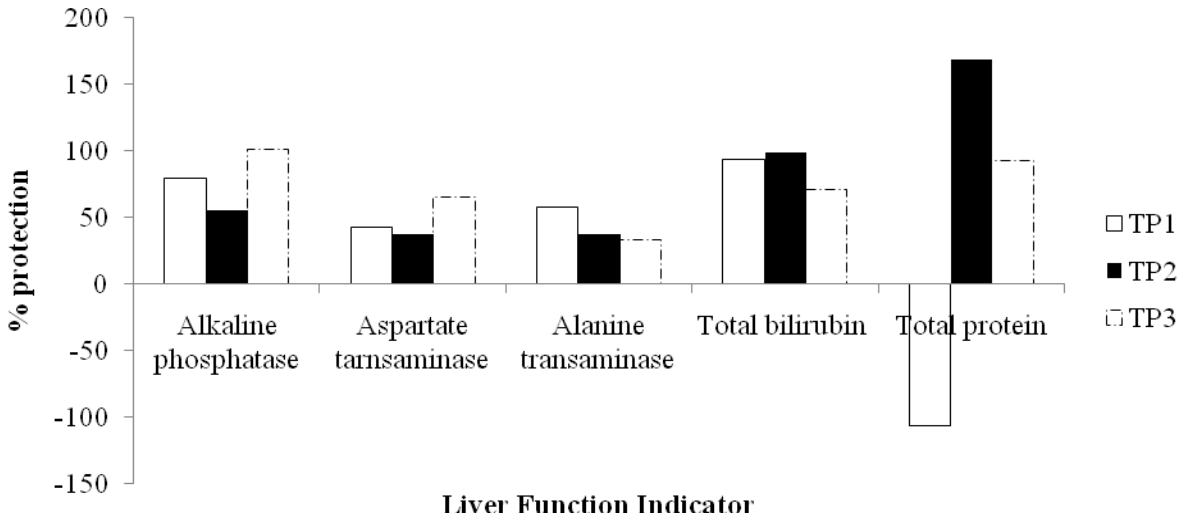

Figure 5: Hepatoprotective activity of aqueous extract of *T. procumbens* leaves in CCl_4-induced hepatotoxicity in Wistar rats.

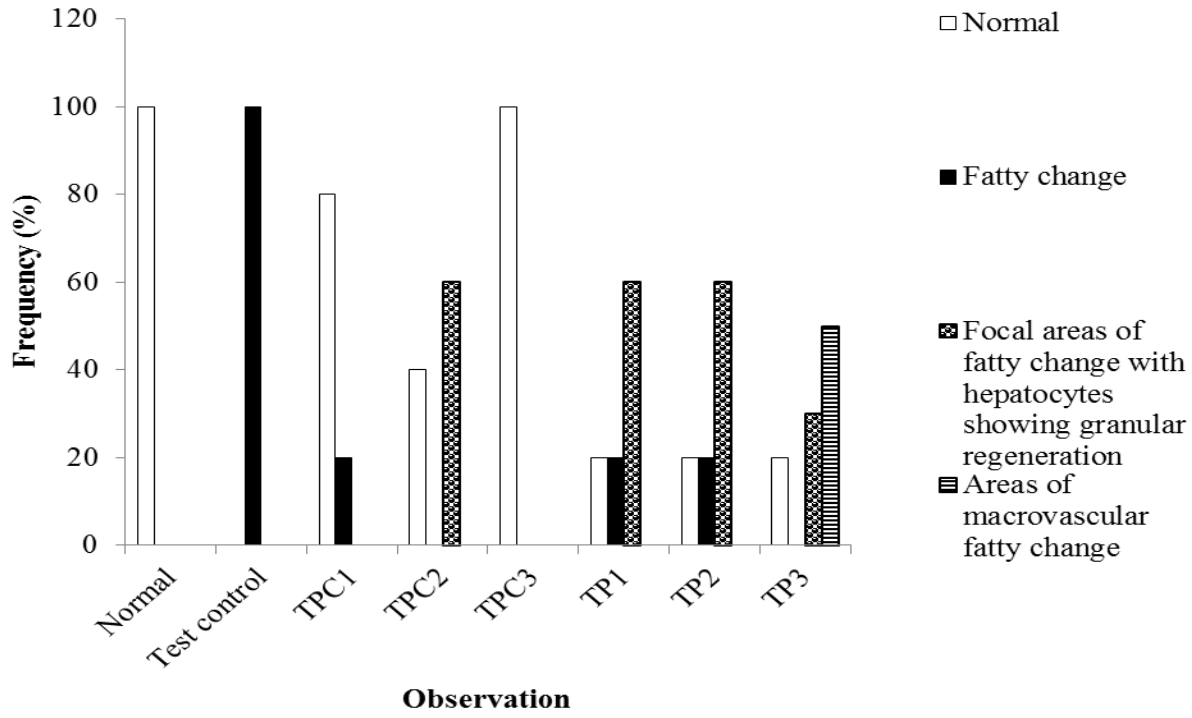

Figure 6: The frequency distribution of the effects of aqueous extract of the leaves of *T. procumbens* leaves on the liver histology of normal and carbon tetrachloride treated rats

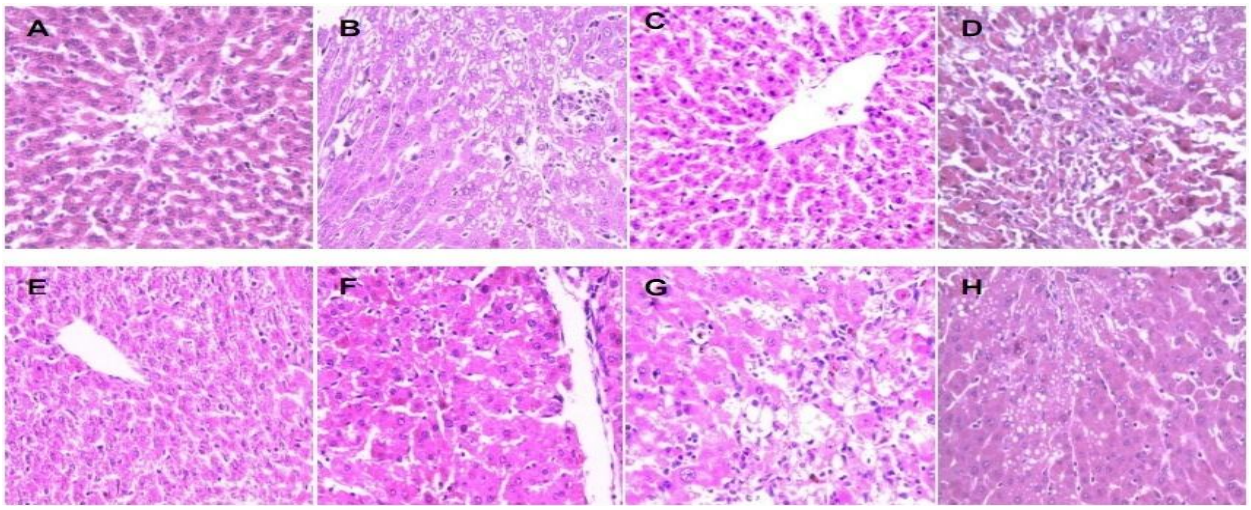

Figure 7: Sections (x20) of the liver samples showing the effect of an aqueous extract of the leaves of *Tridax procumbens* on the liver histology of normal and carbon tetrachloride treated rats. A: Section of the liver of rats administered olive oil (1 mL/kg) and treated with water, showing normal cells. B: Section of the liver tissue of rats administered carbon tetrachloride (1 mL/kg) and treated with water, showing fatty change. C: Section of the liver of rats administered olive oil (1 mL/kg) and treated with 100 mg/kg extract, showing normal cells. D: Section of the liver of rats administered olive oil (1 mL/kg) and treated with 200 mg/kg extract, showing microvesicular fatty change. E: Section of the liver of rats administered olive oil (1 mL/kg) and treated with 300 mg/kg extract, showing normal cells. F: Section of the liver of rats administered carbon tetrachloride (1 mL/kg) and treated with 100 mg/kg extract, with very few cells showing fatty change. G: Section of the liver of rats administered carbon tetrachloride (1 mL/kg) and treated with 200 mg/kg extract, showing focal areas of fatty change, with hepatocytes showing granular regeneration. H: Section of the liver of rats administered carbon tetrachloride (1 mL/kg) and treated with 300 mg/kg extract, showing areas of macrovesicular fatty change.

Flavonoids have been reported to inhibit lipid peroxidation by either inhibiting cytochrome P450 aromatase and/or exerting a membrane-stabilizing action (Kowalska *et al*., 1990; Middleton *et al*., 2000). Of the many flavonoids detected in the extract (Table 2), apigenin (Zheng *et al*., 2005; El Alfy *et al*., 2010), quercetin (Pavanato *et al*., 2003; Mandal and Das, 2005), kaempferol (Oh *et al*., 2004) and naringenin (Pari and Gnanasoundari, 2006) have potent hepatoprotective properties.

Another component of *T. procumbens* leaves that may also have contributed to its hepatoprotective activity is vitamin C, a compound that has been reported to be present in *T. procumbens* (Ikewuchi and Ikewuchi, 2009a). In vivo studies have shown that hepatic microsomal drug metabolism decreases in ascorbic acid deficiency, due to reduction in cytochrome P450, and is augmented with vitamin C supplementation (Sato and Zannoni, 1976; Rikans *et al*., 1978).

CONCLUSION

This study clearly demonstrates that aqueous extract of the leaves of *T. procumbens* is an effective agent in the treatment and prevention of carbon tetrachloride-induced hepatic cytotoxicity. The data suggest that the daily oral consumption of the extract was prophylactic to carbon tetrachloride poisoning. This confirms the use of *T. procumbens* in traditional health care for the treatment of liver problems.

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