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Research Article

Antihepatotoxic and Free Radical Scavenging Activities of the Methanolic Leaf Extract of *Helianthus annuus*

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ABSTRACT
Helianthus annuus (L.) is commonly used in folkloric medicine for treatment of different diseases including liver disorders especially hepatitis. The methanolic leaf extract was evaluated for its antihepatotoxic and free radical scavenging activities. Hepatic toxicity was induced in the rats by single intraperitoneal injection of 0.5 ml/kg carbon tetrachloride (10% CCL_4 in Olive oil) after overnight fast at the end of treatment period. Three test doses (150, 300 and 600 mg/kg) of *Helianthus annuus* leaf extract (HAE) and a standard reference drug, silymarin (100 mg/kg) were administered to the rats orally for seven days through gastric gavage. Twenty four hours after, blood was obtained from the rats through heart puncture into sample bottles from where serum was obtained for biochemical analysis. Parameters evaluated included: liver function tests, malondiadehyde (MDA) levels and catalase activities. Also the animals were sacrificed by cervical dislocation after light chloroform anaesthesia and the liver harvested for histopathological studies. The effect of the extract was compared with silymarin and distilled water controls. *Helianthus annuus* extract at the doses used caused various levels of significant ($p < 0.05$) reduction of Aspartate aminotransferase (AST), Alanine aminotransferase (AST), Alkaline phosphatase (ALP) and total bilirubin when compared to negative control. The effect of the extract was comparable to silymarin (100 mg/kg). There was no significant difference ($p > 0.05$) in the total protein of both the extract treated and the untreated rats. The free radical scavenging activity was demonstrated by the ability of HAE and silymarin to cause dose-dependent and significant ($p < 0.05$) reduction of MDA and increase in catalase activities of treated rats when compared to the negative control. The protective activity of HAE was confirmed by histopathology in which the extract exhibited various levels of protection of the liver from degenerative changes observed in the untreated rats. In conclusion, the methanolic extract of *Helianthus annuus* demonstrated significant dose-dependent antihepatotoxic and free radical scavenging activities against carbon tetrachloride-induced hepatotoxicity in rats as evidenced by the biochemical, functional and histological parameters

Key words: *Helianthus annuus*, carbon tetrachloride, Aminotransferase, Malondiadehyde, Catalase.

INTRODUCTION

One of the major causes of mortality and morbidity all over the world today is liver diseases (Russman *et al*., 2009)[1]. This results from the fact that liver is the major organ involved in the regulation of many important metabolic functions. Liver is also involved in almost all the biochemical pathways to growth, fighting against diseases, nutrient supply, energy production and reproduction (Ward et al, 1999)[2]. Hepatic injury is directly associated with alterations of these metabolic functions (Mitra *et al*., 1998)[3]. The causes of liver damage ranges from certain xenobiotics, microbial infiltration from ingestion or infection (Sturgill and Lambert, 1997; Jia *et al*., 2011)[4,5] and oxidative damage through free radical generation (Deleve and Kaplowitz, 1995)[6]. The hepatotoxicity manifestations are highly variable and ranges from asymptomatic elevation of liver enzyme markers to serious damage to the integrity of the liver cells and in some cases liver failure (Parmar *et al*, 2010)[7].

In spite of the advances in orthodox medicine, there are hardly any reliable and safe drugs that can completely protect the liver from damage or that can be used for the regeneration of hepatic cells (Bhaskar and Balakrishnan, 2010)[8]. Therefore, the search for effective and safe drugs for the treatment of liver disorders is a continuous process and an area of interest.

In many parts of the world, a number of medicinal plants have been used as alternatives for the treatment and prevention of liver disorders (Charles and Haung, 2009)[9] and various experimental evidences have confirmed the efficacy of some plants used for treatment and prevention of liver disorders (Bhandarkar and Khan, 2004)[10].

Helianthus annuus (Linn) (sunflower), is a coarse, stout and erect annual plant native to the Americans. The common names include: 'Sunflower' (English); 'Tournesol' (France); 'Girassol' (Polland); 'Alizeti' (Sweden) (Atlagic, 2004)[11]. It is also known as 'Ododo-orun' in Nigeria (Odugbemi, 2008)[12]. The stems are straight and rarely branched. The leaves are opposite at the

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Table 1: Effect of *Helianthus annuus* on liver marker enzymes of carbon tetrachloride (CCL_4) included liver toxicity in rats.

Treatment	AST (U/L)	ALT (IU/L)	ALP (IU/L)	Total protein (g/dl)	Total bilirubin (mg/dl)
Dist.water (10ml/kg)	100.00 ± 5.77	61.30 ± 0.87	659.18 ± 25.24	5.90 ±0.07	5.20 ± 0.01
Sylimarin(100mg/kg)	23.00 ± 4.95*	39.80 ± 2.89*	536.25 ± 28.58*	5.70 ± 0.09	2.18 ± 0.04*
HAE (150 mg/kg)	46.67 ± 12.20*	27.80 ± 3.40*	533.50 ± 27.65*	5.99 ± 0.14	4.99 ± 0.04*
HAE (300 mg/kg)	21.00 ± 6.72*	27.00 ± 7.51*	522.03 ± 10.96*	5.25 ± 0.31	4.17 ± 0.06*
HAE (600 mg/kg)	15.00 ± 2.31*	19.75 ± 0.14*	354.20 ± 26.75*	5.24 ± 0.23	3.17 ± 0.06*

*P < 0.05 when compared to the distilled water treated group (negative group)

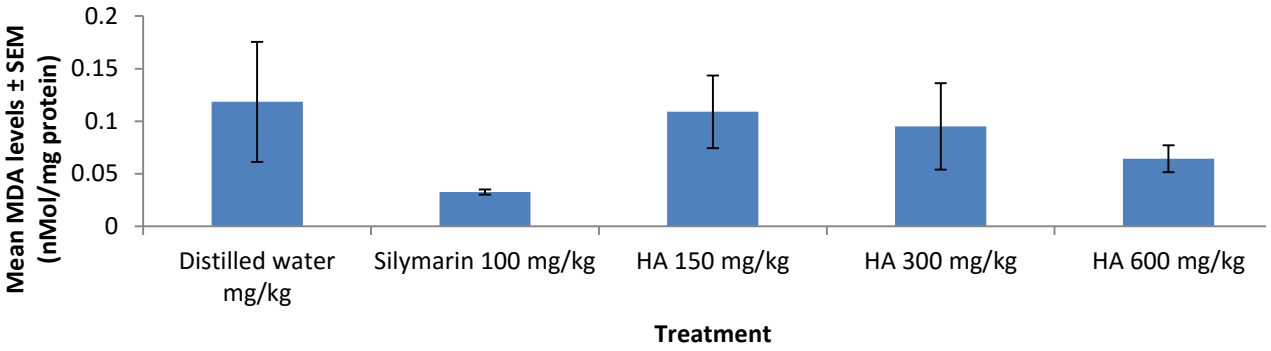

Figure 1: Effect of HAE on the malondiadehyde levels of carbon tetrachloride induced hepatic injured rats

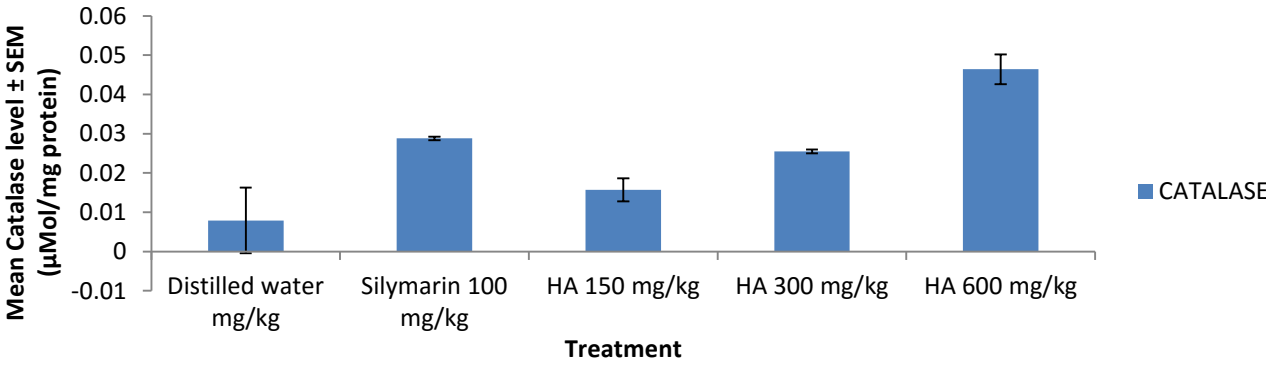

Figure 2: Effect of HAE on the catalase activities of tetrachloride-induced liver injured rats.

lower part of the stem, alternate above, ovate, rough, hairy, with toothed margins, long-stalked, 10-2-cm long; flowers head are solitary or in clusters, up to 40 cm across; disk flowers are yellow and spreading. The involucres bracts are ovate or oblong. *Helianthus annuus* produces grayish-green or black seeds encased in tear-dropped shaped gray or black shell that often times features black and white strips (Heiser, 1976)[13]. The sunflower (*Helianthus annuus*) is a common and wide spread roadside weed. It is common in open site in many different habitats throughout North America, Southern Canada, Mexico and Africa at elevations below 1900 m (Heiser, 2008)[14].

The leaves of *Helianthus annuus* have been used as a medicine against diuresis, diarrhea, and several inflammatory diseases (Lewi *et al.*, 2006)[15]. A preparation of the seed is used widely for the treatment of cold and catarrh. The seeds are served as a substitute for quinine in the treatment of malaria and also as a diuretic and expectorant. The leaves have been used to treat kidney dysfunction; a decoction of sunflower root is used to alleviate rheumatism ((Kursheed *et al.*, 2009).)[16]; the infusion of the leaves is used for treatment of asthma; tea made from the sunflower is used in the treatment of malaria and lung ailments. An infusion of the seeds is used for whooping cough; tincture of bark and flower is employed for intermittent of fevers resistant to quinine (Heiser, 1976)[13]. In the Indian and Nigerian traditional folklore medicine, the leaves of *thus annuus* have been employed for management of liver disorders, especially inflammation of the liver or hepatitis (Odugbemi, 2008; Kirtikar and Ahmad, 2012)[12,17].

Hence, the present study was undertaken to evaluate the methanol leaf extract of *Helianthus annuus* for antihepatotoxic and free radical scavenging activities with a view to establishing the pharmacological basis for its folkloric use for management of liver disorders especially hepatitis.

MATERIALS AND METHODS

Collection and identification of plant Material

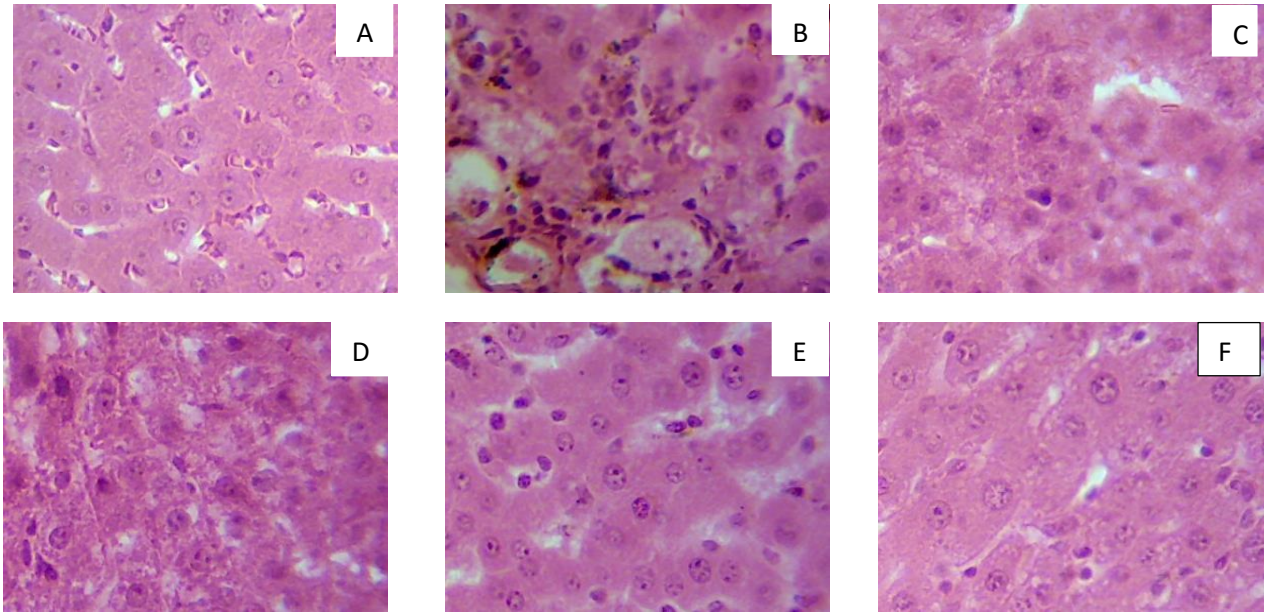

Figure 3: Photomicrographs of liver sections showing A: Normal liver showing normal liver architecture; B (Negative control, CCL4 only): liver showing necrotic and pyknotic hepatocytes, loss of liver architecture and degenerative changes; C (CCl4+ sylimarin 100 mg/kg): liver showing improved liver architecture with cytoplasm containing normal hepatocytes though with a little congestion; D (CCL4+HAE, 150 mg/kg): liver showing some degenerative changes but better than that of B; E (CCL4+HAE, 300 mg/kg): liver showing greatly improved liver architecture; F (CCL4+HAE, 600 mg/kg): liver showing normal liver architecture with normal hepatocytes.

The leaves of *Helianthus annuus* were collected from University of Nigeria Nsukka premises in June 2013. The plant material was identified by Mr. A.O. Ozioko of Bioresources Development and Conservation Programme (BDCP Nsukka). A Voucher specimen number MOUAU/CVM/VPP/2013/27 was deposited in department of Physiology, Pharmacology and Biochemistry, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria herbarium for future references.

Animals

Mature adult albino Wistar rats of both sexes weighing between 90-160 g were used for the experiments. They were obtained from the animal house of the faculty of Biological Sciences, University of Nigeria Nsukka. The animals had free access to normal standard chow diet (Vital feed® Nigeria) and clean drinking water. Throughout the experiments, the animals were housed under standard laboratory conditions and maintained at room temperature and relative humidity of 50-60% and under a 12 hour light-dark cycle. The animals were kept in these facilities for two weeks for acclimatization before using them for the experiment. Ethical conditions governing the conduct of experiments with life animals were strictly observed as stipulated by Ward & Elsea (1997) [18] and all animal experiments were conducted in compliance with NIH Guide for Care and Use of Laboratory Animals (pub. No. 85-23, Revised 1985). Also the experimental protocol was approved by the institution's ethical committee.

Preparation of plant material

The leaves of *H. annuus* were cut into pieces, dried under mild sunlight and later pulverized into a coarse powder of about 1mm in diameter. The pulverized plant material was extracted by cold maceration in 80% methanol with intermittent shaking at 2 hr interval for 48 hr. The extract was then filtered using Whatman filter papers (No. 1). The filtrate was concentrated to dryness in an oven (Uniscope SM9023 Laboratory Surgifriend medicals, England.) at 40^{o}C. The percentage yield was 10.04% and was stored as *Helianthus anuus* extract (HAE) in a refrigerator at 4^{0}C until time of use.

Experimental design

Thirty (30) albino Wistar rats of both sexes were used for this experiment using carbon tetrachloride (CCl_4) - induced hepatic injury model (Matsuda *et al*., 1991)[19].

The rats were randomly divided into five (5) groups of six (6) rats per group and were treated as follows:

Group I rats were given distilled water (10 ml/kg) and served as negative control.

Group II rats were given silymarin (100 mg/kg) and served as positive control.

Group II, IV, and V rats received 150 mg/kg, 300 mg/kg and 600 mg/kg of *Helianthus annuus* extract respectively, all by gastric gavage.

The drug and extract were administered for seven (7) days. On day 8, hepatic toxicity was induced in the rat by a single intraperitoneal injection of 0.5 ml/kg CCl_4 (10% CCL_4 in Olive oil) after overnight fast. Twenty four hours after, blood was obtained from the rats through heart puncture into sample bottles from where serum was obtained for biochemical analysis. Also the animals were sacrificed by cervical dislocation after light ether anaesthesia and the liver harvested for histopathological studies.

Biochemical Analysis

Liver function parameters

Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were evaluated using the method of Reitman & Frankel (1957)[20] as described by Randox laboratories, United Kingdom using Randox kits; Alkaline phosphatase (ALP) was assayed based on the methods of Kind & King (1972)[21]; Total protein in serum was assayed using direct biuret method (Gornall *et al*., 1949)[22] while total bilirubin was determined according to the method of Jendrasik & Grof (1938)[23].

Free Radical Scavenging Activity

Malondialdehyde (MDA)

Lipid peroxidation was determined spectrophotometrically by measuring the level of lipid peroxidation product, malondialdehyde (MDA) as described by Wallin *et al.* (1993)[24].

MDA reacts with thiobarbituric acid (TBA) to form a red or pink colored complex which absorbs maximally in acid solution at 532 nm.

Serum sample (0.1ml) was mixed with 0.9 ml of distilled water in a test tube. 0.5 ml of 25% TCA (Trichloroacetic acid) and 0.5 ml of 1% TBA in 0.3% of NaOH were also added to the mixture. The mixture was incubated for 40 min at 95º C. It was then cooled and 0.1ml of 20% sodium dodecyl sulphate (SDS) added.

Then the absorbance was taken at wavelength against a blank at 532 and 600nm.

Calculation; $\%MDA=\frac{abs532-abs600\ X\ 100}{0.5271\ X\ 0.1}$

Catalase activity

The method of Aebi (1983) [25] was adopted for this study. Phosphate buffer (2.5 ml), 2 ml of H_2O_2 and 0.5 ml of sample were pipetted into a test tube. To 1 ml portion of the mixture, 2 ml of dichromate acetic acid reagent was added. The absorbance was read at 240 nm at a minute interval into 4 places. Catalase activity was calculated using the following equation:

$$\text{Catalase concentration (IU/L)} = 0.23 \times \frac{\log\frac{Abs\ 1}{Abs\ 2}}{0.00693}$$

Histopathological studies

Samples of liver tissues after sacrifice on day 8 were fixed in 10% formal saline for 24 h. They were washed in ascending grades of ethanol, cleared with xylene, embedded in paraffin wax, sectioned with a microtome and stained with haematoxylin and eosin (H and E) and mounted on Canada balsam. All the sections were examined under a light microscope under different (X100, X200 and X400) magnifications. Photomicrographs of lesions were taken with an Olympus photo microscope for observations and documentations of histopathological lesions (Bancroft and Stevens, 1977)[26].

Statistical analysis

Data obtained were analyzed using one-way analysis of variance (ANOVA) where applicable and the variant mean were separated by least significant difference (LSD) of the different groups. Significance was accepted at the level of $p < 0.05$.

RESULTS

Liver function tests

The result of the effect of methanolic extract of *Helianthus annuus* on the liver function parameters of carbon tetrachloride - induced hepatic toxicity in rats is presented in Table 1.

Aspartate aminotransferase (AST)

The result showed that there was a dose dependent and significant ($p < 0.05$) decrease in the AST values of both silymarin and extract treated groups of rats when compared to the negative control (distilled water treated group).

Alanine aminotransferase (ALT)

The reference drug syimarin (100 mg/kg) and the extract in all doses also caused a significant ($p < 0.05$) decrease in ALT of the treated rats when compared with the untreated group. The reduction was also dose dependent.

Alkaline phosphatase (ALP)

HAE also caused various levels of significant ($p < 0.05$) reduction in the ALP values when compared to the negative control. The effect was also dose-dependent.

Total protein

There was no significant difference ($p > 0.05$) in the total protein of the extract treated rats when compared to the distilled water treated group of rats.

Total bilirubin (TB)

The extract (150, 300 and 600 mg/kg) and the reference drug sylimarin also caused a significant ($p < 0.05$) reduction in the serum bilirubin levels of treated rats when compared to rats given distilled water. The effect of the extract was also dose dependent.

Free Radical Scavenging Activity

Malondiadehyde

The result of the effect of HAE on the malondiadehyde levels of carbon tetrachloride induced hepatic injured rats is presented in Fig. 1. The result showed a dose-dependent and significant ($p < 0.05$) decrease in the serum MDA levels of rats treated with *Helianthus annuus* extract when compared to the negative control rats, reducing the MDA level from 0.12 ± 0.001 mMol/mg in the distilled water treated group to 0.06 ± 0.002 mMol/mg at the dose of 600 mg/kg of the extract.

Catalase

The result of the effect of HAE on the catalase activities of tetrachloride injured rats is presented in Fig. 2. The extract caused a dose-dependent increase in catalase activities of the hepatic injured rats with the extract at the highest dose used (600 mg/kg) increasing the catalase activities from 0.008 ± 0.003 µMol/mg protein in the distilled treated group to 0.046 ± 0.001 µMol/mg protein as compared to 0.29 µMol/mg protein by silymarin (100 mg.kg).

Histopathology

Administration of carbon tetrachloride to untreated rats led to the destruction of the normal liver architecture with congestion, necrotic, pyknotic, disqaumated hepatocytes and degenerative changes. Treatment with sylimarin and the extract at different doses brought about different degrees of amelioration of these changes which was dose dependent, with the extract at the dose of 600 mg/kg maintaining normal liver architecture of treated rats (Fig. 3).

DISCUSSION

This study evaluated the effect of *Helianthus annuus* (L.) on hepatotoxicity in rats induced by carbon tetrachloride. Carbon tetrachloride-induced hepatotoxicity is one of the most common experimental models in rats used for the study of the effects of drugs or extracts on the liver (Johnson and Kroening, 1998)[27]. The hepatotoxic effect of carbon tetrachloride is attributed to its active metabolite, trichloromethyl radical, which binds covalently to the macromolecules to induce lipid peroxidative degradation of membranes and consequent liver damage (Bhaskar and Balakrishnan, 2010)[8].

The levels of serum liver marker enzymes (AST, ALT, ALP) including protein and bilirubin are used indirectly to assess the toxic effects of substances on the liver (Mukinda and Syce, 2007)[28].

Administration of carbon tetrachloride to rats caused marked increases in these biochemical markers of liver injury as seen in the negative control group of rats indicating damage to the liver (Table 1). These enzymes are cytoplasmic in location and are only released into circulation following liver cellular damage indicating hepatotoxicity (Gutierrezl and Solis, 2009)[29].

These increases in the levels of serum AST, ALT, ALP were significantly reduced by pretreatment with *Helianthus annuus* extract which was dose-dependent. This finding evidently suggests hepatoprotective effect of the leaf extract of the plant. The low levels of the serum liver marker enzymes of the pretreated rats is an indication of plasma membrane stabilization by HAE which prevented damage to the liver cells by carbon tetrachloride as suggested by Mulla *et al*. (2009)[30].

Bilirubin is a breakdown product of hemoglobin and its increased level in the serum as seen in the distilled water treated group of rats in this study, is associated with hepatotoxicity such as hepatobilliary diseases (Thapa and Walia, 2007) [31]. Pretreatment with HAE also significantly reduced the serum levels of bilirubin in rats when compared with the distilled water treated group. This further indicates the ability of the extract to protect the liver against intoxicants.

In this study, there was no significant difference in the protein levels of both treated and negative control rats and this may be attributed to the duration of the experiment.

One of the major mechanisms of hepatotoxicity is lipid peroxidation resulting from oxidative stress due to the activities of free radicals (Winrow *et al*., 1993)[32]. The effect of HAE on lipid peroxidation was evaluated using malondiadehyde level and catalase activities.

In this study, there was a marked elevation of the MDA levels of the untreated rats by CCL_4 which suggested enhanced lipid peroxidation and failure of antioxidant defense mechanisms to prevent formation of excess free radicals leading to liver tissue damage (Pajero, 2002)[33].

In the *Helianthus annuus* extract pretreated rats, the MDA levels were dose-dependently reduced, which further reinforces the antihepatotoxic property of the plant. This decrease in the MDA levels may have resulted from increase in the activities of the glutathione peroxidase or may be as a result of direct inactivation of lipid peroxidation thereby resulting in the reduction of oxidative stress (Afshari et al., 2007)[34].

Catalase converts harmful hydrogen peroxide into water and oxygen and protects the tissues from highly reactive hydroxyl radicals and the reduction in the activity may lead to a number of deleterious effects due to accumulation of highly toxic metabolites (Chance and Greenstein, 1992)[35]. Reduced catalase activities observed in the distilled water treated group when compared to the extract pretreated rats, is an indication of generation of free radicals and also a feature of hepatic damage (Friday, *et al*., 2010)[36]. The reduction in the catalase activities may be due to exhaustion of the enzyme as a result of the oxidative stress caused by carbon tetrachloride (Jia *et al*., 2011)[5].

The free radical scavenging enzyme, catalase, activities was significantly increased in the treated rats, suggesting that free radical scavenging may be a possible mechanism of the antihepatotoxic activity of *H. annuus*.

Histological examination of the liver sections revealed that the normal liver architecture was disrupted by carbon tetrachloride intoxication showing necrotic, pyknotic and disqaumated hepatocytes among other degenerative changes in the distilled water treated rats (Fig. 3, slide B). Pretreatment with HAE protected the rat liver from such degenerative changes as was evident in the retention of normal liver architecture which was more prominent at the dose of 600 mg/kg (Fig.3 slide A). This further strengthens the antihepatotoxic property of *H. annuus* extract which may have resulted from promotional activation of antioxidative enzymes and protection of the functional and structural integrity of the liver cells.

In conclusion, the methanolic extract of *Helianthus annuus* in this study exhibited a significant dose-dependent antihepatotoxic and free radical scavenging activities against carbon tetrachloride-induced hepatotoxicity in rats as evidenced by the biochemical, functional and histological parameters. However, further work is required to isolate and characterize the active compound responsible for the antihepatotoxic action.

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