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Protective effect of ethanolic extract of C*ucurbita maxima* (PUMPKIN) leaf on acetaminophen-induced acute liver toxicity

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Ethanolic extract of dried leaves of *Cucurbita maxima* (Pumpkin) were screened for their phytochemical composition. The *in vitro* antioxidant property was determined by assessing the free radical (DPPH) scavenging activity. Twenty rats divided into four groups were used for this study with group 4 pre-treated with the extract and later intoxicated with 2 g/kg single dose of acetaminophen. The hepatoprotective effect of the extract was determined by measuring the liver function parameters, liver antioxidant enzyme activities and the rats liver histological micrograph. The ethanolic extract was found to be a rich source of bioactive compounds and showed a direct variation in *in vitro* free radical (DPPH) scavenging property. DPPH scavenging property increases as the concentration of the extract increases from 0.03 to 0.12 mg/l (8.9 - 64.2%) but dropped sharply to 52.2% at a concentration of 0.5 mg/l. A 400 mg/kg daily pre-treatment (for seven days) with ethanolic leaf extract of the plant was able to offer protection to the hepatic cells of the rats. This was evidenced in the significant ($p<0.05$) reduction of the activities of alanine aminotransferase (ALT) from 117.30±57.50 to 31.26±11.22 µ/l and alkaline phosphatase (ALP) from 209.80±67.00 µ/l to 172.00±30.31 µ/l, significant ($p<0.05$) increase of the activities of glutathione peroxidase (GPx) from 115.60±10.03 to 235.45±43.52 µ/mg, superoxide dismutase (SOD) from 0.02±0.01 to 0.09±0.05 U/mg and catalase (CAT) from 2.50±2.60 to 10.23±5.05 U/mg in the test group when compared with the negative control. Also, the lobular architecture of the hepatocytes was well-preserved in the test group. Based on the experimental results obtained here, *C. maxima* has an important role in medicine as it plays a role in scavenging free radicals, stimulating activities of antioxidant enzymes and preserving the liver architecture, thereby protecting the liver against acetaminophen-induced liver toxicity.

Key words: *Cucurbita maxima,* hepatoprotection, oxidative stress, free radical-scavenging, hepatocytes.

INTRODUCTION

The liver is the largest organ of the body which is involved in the metabolism and excretion of unwanted compounds, which may be exogenous (e.g. drugs and poisons) or of endogenous origin (e.g. steroid or

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catecholamine hormones and haem groups). It performs a wider range of biochemical functions than any other organ (Reed, 2009). It is connected with most of the physiological processes, which include growth, immunity, nutrition, energy metabolism and reproduction (Mayuresh et al., 2014).

Paracetamol or acetaminophen is an active metabolite of phenacetin. Acetaminophen (APAP) is an analgesic and antipyretic substance used in the production of the drug paracetamol. It is well tolerated, lacks many of the side effects of aspirin and is available over-the-counter, so it is commonly used for the relief of fever, headache and other minor aches and pain (Vidhya and Bai, 2012). Although, safe at therapeutic doses, APAP had been found to cause severe liver injury (Erica and Emily, 2014). Mitchell et al. (1973) reported that APAP overdose is the predominant cause of acute liver failure in the United States and that toxicity begins with a reactive metabolite that binds to proteins. These findings indicated that acetaminophen was metabolically activated by cytochrome P_{450} (CYP) enzymes to a reactive metabolite that depleted glutathione (GSH) and covalently bonded to protein. It has also been shown by James et al. (2009) that replenishing glutathione (GSH) prevented the toxicity. The mechanism of acetaminophen toxicity is by a complex sequence of events that include but not limited to CYP metabolism to a reactive metabolite which depletes glutathione and covalently binds to proteins, loss of glutathione with an increased formation of reactive oxygen and nitrogen species in hepatocytes undergoing necrotic changes, increased oxidative stress, associated with alterations in calcium homeostasis and initiation of signal transduction responses, causing mitochondrial permeability transition, mitochondrial permeability transition occurring with additional oxidative stress, loss of mitochondrial membrane potential, loss of the ability of the mitochondria to synthesize ATP and loss of ATP which leads to necrosis, (Mitchell et al., 1973; Jack et al., 2009). The reactive metabolite was found to be *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is formed by a direct two-electron oxidation (Dahlin et al., 1984). It was shown that NAPQI is detoxified by glutathione (GSH) to form an acetaminophen-GSH conjugate. After a toxic dose of acetaminophen, total hepatic GSH is depleted by as much as 90%, and as a result, the metabolite covalently binds to cysteine groups on protein, forming acetaminophen-protein adducts (Mitchell et al., 1973). Depletion of GSH which is an intrinsic antioxidant is capable of introducing peroxidation of cell membrane lipids, regeneration of reactive oxygen free radicals and hepatocellular fatty regeneration with centriolobular necrosis of the liver. The cellular damage is due to the failure to eliminate a toxic metabolic intermediate of the drug known as NAPQI (Vidhya and Bai, 2012; Reed, 2009).

Treatment of paracetamol overdose is based on replenishment of antioxidant thiols to supplement the role of glutathione (Reed, 2009). Silymarin is a standardized extract obtained from the seeds of *Silybum marianum* containing approximately 70 to 80% of the silymarin flavonolignans and approximately 20 to 30% chemically undefined fraction, comprising mostly polymeric and oxidized polyphenolic compounds. It has been developed into a standard hepatoprotective drug. It is therefore, imperative to identify other plants with potential hepatoprotective effects to help prevent severe damage of the hepatocytes in cases of accidental/intentional over dosage.

Vegetables serve as indispensable constituents of the human diet, supplying the body with minerals, vitamins and certain hormone precursors, in addition to protein and energy (Aja et al., 2010). Leafy vegetables have been found to boost the concentration of red blood cells and significantly increase the serum activity of AST in experimental animals (Ezekwe et al., 2013). Focus on plant research has increased all over the world and a large body of evidence has been collected to show immense potential of medicinal plants for treatment purposes or for the production of drugs (Dahanukar et al., 2001; Olamide and Mathew, 2013; Udochukwu et al., 2015). Their use in ethnomedicine for the management of ailments stem from the presence of phytochemicals (Aja et al., 2010). *Cucurbita maxima* possess some bioactive compounds which make the possibility that the extract of the leaves may have antioxidant and anti-hepatotoxic activities (Shahlah et al., 2013: Alamgir et al, 2016). *Cucurbita* is a genus of herbaceous vines in the gourd family, *Cucurbitaceae* also known as cucurbits (Chakravarthy, 1982). Commonly known as the pumpkin, the plant is called “Ugbogulu” by the Igbo speaking areas of Nigeria. It is broadly grown for consumption as condiment and for therapeutic use (Lindhorst, 2007) and widely used like food and in folk medicine around the world (Perez, 2016). This work was carried out to ascertain the protective effect of ethanol leaf extract of *C. maxima,* a vegetable commonly used in traditional medicine and local diets, on acetaminophen-induced acute liver toxicity in albino rats.

MATERIALS AND METHODS

Pant materials, silymarin and acetaminophen

The plant material is *C. maxima* (pumpkin) leaf. The drug, acetaminophen was a research support from Emzor Pharmaceutical Ltd, Lagos while Silymarin is a branded drug (Sylibon 140) from Micro Laboratory Ltd, India.

Sample collection and preparation

Plant materials were collected in and around Keffi, in Nasarawa state, North Central Zone of Nigeria. The leaves were identified at the University of Ibadan Herbarium, in the Department of Botany and were assigned the voucher number UIH-22682. The leaves were rinsed in water to remove dust and sand particles, and then

dried under room temperature for fourteen (14) days. The dried leaves were then pulverised using Waring laboratory blender. Absolute ethanol (99.9%) from Sigma Chemical Company, London was used to extract the bioactive ingredients from the leaves.

Preparation of extracts

Pulverised plant material was extracted with ethanol by soaking 100 g of the ground samples in 500 ml of absolute ethanol (ratio 1:5 weight to volume) for 48 h. The extract was filtered using muslin cloth and then concentrated by heating in a water bath and stored in airtight containers.

Animal models

Male Wistar albino rats weighing between 120 and 140 g were used for the study. These rats were purchased from the animal house of the National Veterinary Research Institute (NVRI), Vom in Plateau State. They were housed in clean, well ventilated metal cages in the animal house of the Department of Zoology, Nasarawa State University, Keffi. The animals were kept under 24 h light/dark cycling. They were allowed assess to unlimited food and water supply and allowed to acclimatize for two weeks before the commencement of the study. All the animals were marked for identification, and their respective weights recorded. The animals were first fed with the chow (feeds) and intubated with the plant material.

Administration of extracts and intoxication of the animals

Twenty albino rats were divided into four groups of five animals each. Group 1 (normal control) received feed and water only, group 2 (the standard control) received feed, water and a pre-treatment with Silymarin (400mg/kg), group 3 (negative control) received feed and water, while group 4 (test group) received feed, water and pre-treatment with ethanol leaf extract of the vegetable, 400 mg/kg for seven days. On the eighth day, the animals in groups 2, 3 and 4 were fasted for up to seven hours, followed by intoxication by oral administration of 2 g/kg acetaminophen and the animals were sacrificed after nine hours.

Animals sacrifice, collection and preparation of samples

At the end of the experimental period, the animals were anaesthetised. Blood samples were collected by cervical decapitation into plain tubes. Serum was collected by centrifuging at 3000 rpm for 10 min.

Preparation of liver homogenate

After bleeding, the livers were carefully removed, trimmed of extraneous tissues and rinsed in ice-cold 1.15% KCl. The livers were then blotted dry, two grams (g) was weighed and homogenized in 8 ml of ice-cold phosphate buffer (100 mM, pH 7.4). The homogenate were then centrifuged first at 6,000 rpm for six 6 min to remove nuclear debris after which the obtained supernatant were centrifuged at 10,000 rpm for twenty min (20 min) to obtain the post-mitochondrial supernatant (PMS), using a refrigerated centrifuge. This was used for the assay of the antioxidant enzymes (super oxide dismutase, catalase and glutathione peroxidase).

Biochemical analysis

Qualitative phytochemical screening of the leaf extract was carried out using standard procedures of the Association of Analytical Chemist (2006) to identify the phytochemicals. The free radical scavenging activity of the plant extracts against DPPH radical was by a slightly modified spectrophotometric method previously described by Afolayan et al. (2014). The serum alkaline phosphatase activities of the experimental animals were estimated using the method of King (1965b). The determination of aspartate aminotransferase and alanine aminotransferase were carried out using the method of King (1965a). The total protein was estimated using the colorimetric method of Lowry et al. (1951). Total bilirubin was determined using the method of Malloy-Evelyn (1937). Superoxide dismutase activity was determined by its ability to inhibit the auto-oxidation of epinephrine and determined by the increase in absorbance at 480 nm as described by Sun and Zigma (1978). The catalase activity was determined according to the method of Beers and Sizer (1952) as described by Usoh et al. (2005) by measuring the decrease in absorbance at 240 nm due to the decomposition of H_2O_2. Determination of glutathione peroxidase (GPx) activity was by the method of Lawrence and Burk (1976). Histopathological study on the liver tissues was carried out using the haematoxylin and eosin stain as described by Bancroft et al. (2013).

Statistical analysis

The data obtained were statistically analysed by analysis of variance (ANOVA). Groups were compared using the least significant difference (LSD) at $P<0.05$.

RESULTS AND DISCUSSION

The ethanol extract of the leaf of *C. maxima* was found to be rich in bioactive constituents as seen in Table 1. Ethanol leaf extract of *C. maxima* was found to exhibit a concentration-dependent free radical scavenging potential from 0.03 to 0.12 mg/l, but sharply decreased at a concentration of 0.5 mg/l (Figure 1). Acetaminophen at a single dose of 2 g/kg caused significant increase ($p<0.05$) in the activities of AST, ALT and ALP in the serum of the rats in the negative control as compared to those in the normal control group. The intoxication decreased the total protein and albumin concentration of the rats in the negative control. However, the pre-treatment with the 400 mg/kg ethanol leaf extract of *C. maxima* for seven day prior to intoxication with acetaminophen led to marked decrease ($p<0.05$) in the activities of ALT and ALP, and increased concentration of the total protein and albumin in the serum as shown in Tables 2 and 3.

A single 2 g/kg oral administration of acetaminophen to the rats caused significant decrease of the activities of SOD and GPx in the negative control as compared to the normal control while the administration of the extract caused significant increase in the activities of the SOD, CAT and GPx of the test animals (Table 4).

DISCUSSION

The ethanol extract of *C. maxima* leaf had a direct variation on the free radical (DPPH) scavenging property

Table 1. The qualitative phytochemical compositions of ethanol leaf extracts of *C. maxima.*

Parameter	Result
Tannin	+
Flavonoids	+
Phenols	+
Cardiac glycosides	+
Triterpenoids	+
Sterol	+
Terpenoids	+
Balsam	+

+: Indicates presence; -: indicates absence.

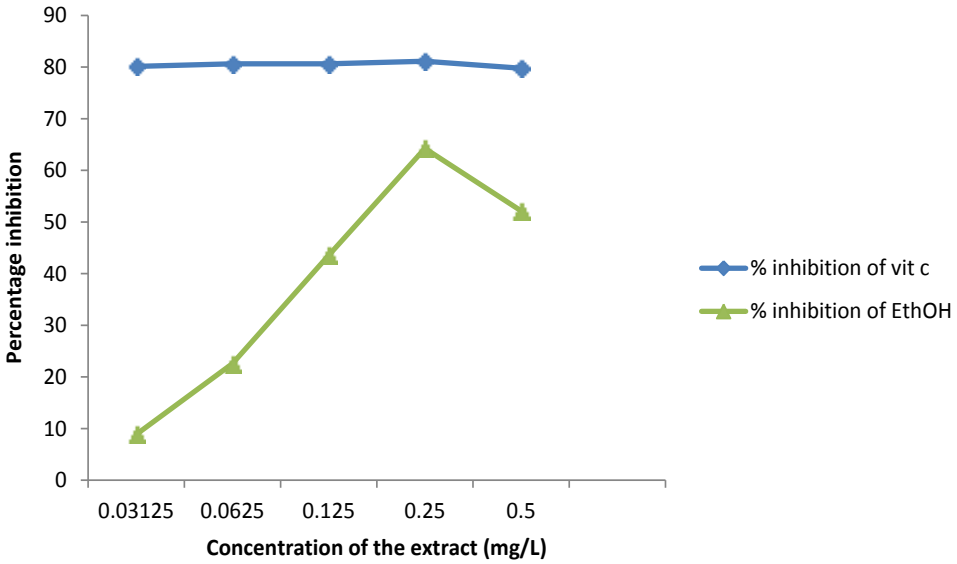

Figure 1. The free radical scavenging activity of ethanol leaf extract of *C. maxima* using ascorbic acid as standard.

with increase in concentration of the extract from 0.03 to 0.12 mg/l (8.9 to 64.2%) and dropped sharply to 52.2% at a concentration of 0.5 mg/l. This free radical scavenging property may be due to the presence of flavonoids and phenols (Table 1) which are good antioxidants. The methanol extract of the plant has been reported to have reasonable *in vitro* antioxidant potentials (Alamgir et al., 2016). However, the ascorbic acid had more DPPH-scavenging (*in vitro* antioxidant) potential than the ethanol leaf extract of *C. maxima* at the concentrations stated above.

The aminotransferase are abundant in the liver and are released into the blood stream following hepatocellular damage, making them sensitive markers of liver damage (Al-Mamary, 2002; Sarvesh 2012). 2 g/kg single dose acetaminophen caused the perturbation of the liver as evidenced in the significantly ($p<0.05$) raised activities of ALT, AST and ALP. This is in consonance with the work of Prabu et al. (2011) and Ekor et al. (2006), which reported liver damage as a result of the administration of 2 g/kg of acetaminophen in albino rats. Therefore, a marked increase in the serum ALT and AST activities is indicative of liver damage. Serum levels of aminotransferase are used as an indicator of damage to the liver structural integrity because these enzymes are cytoplasmic in location and are released into the circulating blood only after structural damage (Okediran et al., 2014). The present study provides evidence that the pre-treatment of rats, with a 400 mg/kg per day with ethanol leaf extract of *C. maxima*, for seven days, was able to offer protection to the hepatic cells of the rats against toxicity and oxidative stress arising from a 2 g/kg oral intoxication with acetaminophen over nine hours (9 h). The pre-treatment with the leaf extract led to

Table 2. The effect of pre-treatment with 400 g/kgbw ethanol leaf extract of *C. maxima* on the serum activities of AST, ALT and ALP of rats intoxicated with 2 g/kg single dose of acetaminophen.

Group	AST (u/l)	ALT (u/l)	ALP (u/l)
Normal control	41.90±3.30	27.90±8.20	86.30±58.80
Standard control	42.80±10.80	17.90±3.10	167.30±107.70
Negative control	67.40±26.40^{++}	117.30±57.50^{++}	209.80±67.00^{++}
Cucurbita maxima (400 mg/kg)	72.18±42.92^{a}	31.26±11.22**^{a}	172.00±30.31**

Values are mean ± SD of five (5) results, * and ** show values with significant increase and decrease respectively, compared to the negative control while $^{++}$ and $^{--}$ indicate values with significant increase and decreases respectively as compared to the normal control. ^{a}Significant difference from standard control. AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phosphatase.

Table 3. The effect of pre-treatment with 400 m/kg ethanol leaf extract of *C. maxima* on the serum concentrations of total protein, albumin, and bilirubin of rats intoxicated with 2 g/kg single dose of acetaminophen.

Groups	T. Protein (g/dl)	Albumin (g/dL)	T. Bilirubin (mg/dl)	D. Bilirubin (mg/dl)	Ind. Bilirubin (mg/dl)
Normal control	75.10±5.70	18.67±5.00	12.50±2.70	5.30±1.40	5.90±0.70
Standard control	76.80±10.50	4.70±2.30	9.00±5.20	6.50±2.90	4.00±3.30
Negative control	62.80±1.20	4.00±1.42^{--}	16.40±4.20	4.30±2.50	7.20±1.90
*C. maxima (*400mg/kg*)*	64.334±4.65	6.78±1.67*	11.92±7.08	3.40±2.05	8.52±6.65

Values are mean ± SD of five (5) results, *show values with significant increase as compared to the negative control. $^{--}$ ndicates values with significant decreases as compared to the normal control.

significant ($p<0.05$) decrease of the serum activities of ALT and ALP of the animals. However, the decrease in the serum activities of AST and ALT of the rats pre-treated with these extracts was significantly lower ($p<0.05$) than that of Silymarin treated group. The activities of the liver antioxidant enzymes, SOD and GPx were significantly reduced ($p<0.05$) in negative control group (Table 3). The activity of CAT was also reduced, although the reduction was not statistically significant ($p>0.05$). This is an indication of oxidative stress in the liver. Disrupted hepatic lobular architecture of the rats was also observed (Plate 1C). All these alterations were seen in the negative control (Group 3) as compared to the normal control, group 1. The toxicant also altered the concentration of protein (total protein and albumin) in the serum of the rats, which could be as a result of the binding of NAPQI to proteins or the effect of NAPQI on the protein synthesizing/metabolizing ability of the liver. A marked rise in the serum activity of ALT, reduction in total serum protein and abnormal increase in serum bilirubin had been reported in hepatotoxicity (Olamide and Mattew, 2013; Olorunnisola et al., 2011; Martin and Friedman, 1992). A decrease in total protein and album shows that the liver's ability to synthesis protein (example albumin) has been impaired, hence indicative of liver damage. NAPQI is an oxidative product of acetaminophen metabolism that binds covalently to the sulphydryl groups of proteins, resulting in cell necrosis and lipid peroxidation induced by decrease in glutathione in the liver causing hepatotoxicity (Kanchana and Mohammed Sadiq, 2011). Results from the present study provide evidence of the induction of oxidative stress nine hours following acute acetaminophen intoxication. The induced oxidative stress as found in this study is evident in the significantly ($p<0.05$) decreased activities of the SOD, CAT and the GPx of the animals in the negative control group as compared to the normal control group. Ekor et al. *(*2006) reported that after seven hours, following paracetamol (PCM) intoxication, there was a rise in GST activity, indicating increased GST-catalysed conjugation of PCM toxic metabolite NAPQI with GST leading to the depletion of cellular GSH level. Histological profile of the livers of the rats in the negative control group showed a poorly preserved hepatic lobular architecture, sharply demarcated hepatocyte, necrosis and exhibited peri-portal sinusoidal congestion (Figure 2) which is a confirmation of liver injury.

The activities of CAT, SOD and GPx increased significantly at 95% confidence level by the actions of the ethanol extract of *C. maxima* leaf. Jain and Pathak (2012) also reported the hepatoprotective activity of methanol extracts of *C maxima* seeds against paracetamol-induced hepatotoxicity. Catalase, superoxide dismutase and glutathione peroxidase are the primary intracellular defence mechanism to cope with increased oxidative stress, eliminating superoxide anion and hydrogen peroxide that may oxidise cellular substrates thereby preventing free radical chain reactions (Ekor, et al., 2006).

Table 4. The effect of pre-treatment with 400 g/kg ethanol leaf extract of *C. maxima* on the activities of the liver antioxidant enzymes of rats intoxicated with a 2g/kg single dose of acetaminophen.

Samples	SOD (U/mg)	CAT (U/mg)	GPx (µ/mg)
Normal control	0.05±0.01	3.00±2.30	397.00±100.00
Standard control	0.20±0.40	20.90±14.90	184.60±23.60
Negative control	0.02±0.01¨	2.50±2.60	115.60±10.03¨
Cucurbita maxima	0.09±0.05*	10.23±5.05*	235.45±43.52*

Values are mean ± SD of six (5) results, *values with significant increase as compared to the negative control, while ¨indicates values with significant decreases respectively as compared to the normal control.

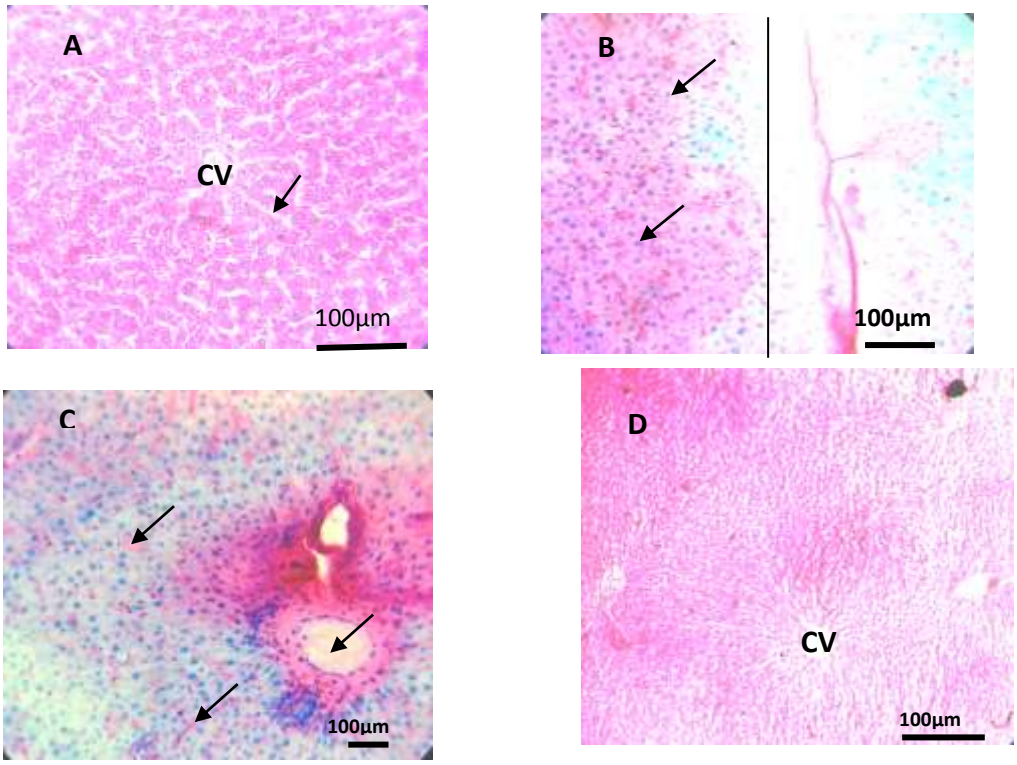

Figure 2. The histological micrograph of the rats' hepatocytes. A: The normal control, the section shows a well-preserved hepatic lobular architecture, with normal appearing cords hepatocytes interspersed by hepatic sinusoids (arrow). A normal appearing central vein (CV) is also seen. B: The negative control, section show a poorly preserved hepatic lobular architecture exhibiting peri-portal sinusoidal congestion and sharply demarcated hepatocyte necrosis as seen on plate B (arrows). C: The standard control, the liver section showed a well-preserved hepatic lobular architecture with sharply demarcated patchy areas of hepatocyte necrosis (right of image) and sinusoidal congestion (arrows). D: The test animal, section shows a well-preserved hepatic lobular architecture, with normal appearing hepatocytes and a minimal diffuse chronic inflammatory infiltrate. CV = central vein.

The induction of higher activities of these antioxidant enzymes is suggested for the protection of the livers by reducing oxidative stress on the organ. All these protections may be due to the antioxidant properties of the plants, which stem from its phytochemical components. Prerona et al. (2011) posited that the potent hepatoprotective activity of *C. maxima* aerial parts against CCl_4 induced hepatic damage may be due to its antioxidant activity and free radical scavenging property. However, it is not known whether the health benefits are the result of individual phytochemicals, the interaction of various phytochemicals, the fibre content of plant foods or the interaction of phytochemicals and the vitamins and minerals found in the same foods.

Conclusion

The vegetable was found to be a potential antioxidant and offered protection to the hepatic cells. Therefore, it can be a good source of raw materials for the production of medicine/drugs for the prevention and treatment of liver and associated diseases. The vegetable is therefore recommended in diets.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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