Original Research Article

Hepatoprotective activity of 6-Heptadecylcyclohex -3-ene-1 carboxylic acid isolated from the methanol extract of *Dichrostachys cinerea* Wight & Arn. stem bark.

Abstract

Aim of the study :

The phytoconstituent 6-heptadecylcyclohex 3-ene-1 carboxylic acid isolated from the methanol extract of *Dichrotachys cinerea* Wight. stem bark was evaluated for hepatoprotective activity against CCl_4 induced toxicity.

Materials and method :

The constituent 6-heptadecylcyclohex 3-ene-1 carboxylic acid isolated from the methanolic extract of *D. cinerea* and the structure was confirmed by spectroscopic studies. Hepatoprotective property was screened in male Wistar strain rats. The parameters studied were estimation of liver function serum markers such as serum total bilirubin, total protein, alanine transaminase, aspartate transaminase, alkaline phosphatase activities and histological profile of the liver tissue.

Results:

The LD_{50} of methanolic extract and constituent, 6-Heptadecylcyclohex -3-ene-1 carboxylic acid was found to be 500 and 100 mg/kg body weight respectively. The hepatoprotective activity of constituent was more significant as similar to the standard hepatoprotective drug silymarin. The histological profile of the liver tissue showed the presence of normal hepatic cords, absence of necrosis and fatty infiltration as similar to the controls.

Conclusion:

The methanolic extract of *D. cinerea* stem bark and the phytoconstituent 6-heptadecylcyclohex-3-ene-1 carboxylic acid afforded significant protection from CCl_4 induced liver damage.

***Key words*:** *Dichrostachys cineria,* Hepatoprotective, 6-Heptadecylcyclohex -3-ene-1 carboxylic acid.

1. Introduction

Green plants posses the broadest spectrum of synthetic activity and have been the source of many useful compounds. *Dichrostachys cinerea* (Mimosae) is a deciduous medium sized tree commonly distributed in the forests of Africa, Australia, India and parts of South East Asia. It is commonly known as "sickle pod", "acacia Saint Domingue," "aroma," in English and "Vada" among the Kannada speaking people of Karnataka in southern India. As per the traditional claims bark and the leaves were the potential source of drugs for ailments such as jaundice, inflammations rheumatism, fever, asthma, body ache, chest problems, toothache, ulcers, wounds eye diseases and aphrodisiac [1]. Preliminary phytochemical analysis of the plant specially leaves and bark extract contains flavonoids, tannins, triterpens, saponins and steroids [2]. The heartwood of *D. cinerea* contains aliphatics and triterpenoids [3]. Anti bacterial activity of the tannins isolated from the *D. cinerea* [4]. In the present investigation 6-Heptadecylcyclohex -3-ene-1 carboxylic acid was isolated from the methanol extract of *D. cinerea* and its hepatoprotective property was screened against CCl_4 induced hepatic damage in rats.

2. Materials and Methods

2.1 Plant Material

The stem bark of *Dichrostachys cinerea* was collected from the Malebennur reserve forest range of Davanagere District, Karnataka, India. Taxonomic authenticity was confirmed by referring to herbarium specimen at Madras herbarium, Botanical Survey of India, Southern Circle, Coimbatore and a voucher specimen (FDD-53) is deposited at Kuvempu University herbaria, Shankaraghatta.

2.2 Isolation

The fresh stem bark of *D. cinerea* chapped, shade dried, powdered mechanically and was extracted using soxhlet apparatus with methanol for about 48 hrs. (200gm x 5). The extract was filtered and concentrated in vacuum under reduced pressure using rotary flash evaporator (Buchi, Flawil, Switzerland) and allowed it complete evaporation of the solvent. The yield was 2.16% (w/w).

Crude methanolic extract of stem bark was subjected for Thin Layer Chromatography (TLC) studies, which showed separation of single spot with the R_f value 0.71. The active constituent was separated by column chromatography (180 g silica gel of 60 -120 mesh, 60x4 cm) and eluted with the solvent system toluene: ethyl acetate: diethyl ether in the ratio of 7:2:1. About 5 ml of collected fractions were monitored by TLC to check further separation and purity of separated constituent, which confirmed the single spot. The compound was re-crystallized in toluene and the yield was 230mg /5gms. The characterization of the compounds was done by IR, MASS, ^{1}HNMR and ^{13}C NMR spectroscopic studies.

2.3 Drug Formulations

Oral suspensions of the methanolic extract (50 mg/ml) and the isolated constituent 6-Heptadecylcyclohex -3-ene-1 carboxylic acid (10 mg/ml) was prepared in gum tragacanth (1 % w/v).

2.4 Animals

Male Wistar albino rats weighing 150-200 g were procured from Central Animal House, National College of Pharmacy, Shivamogga and were maintained at standard housing condition. The animals were fed with commercial diet (Pranav Agro Industries Ltd., Sangli) and water ad- Libitum during the experiment. The Institutional Animal Ethical Committee (Reg.No.144/NCP/IAEC/CLEAR/P.COL.3/2006-07) permitted the study. Acute toxicity study was conducted according to "staircase" method [5]. The LD_{50} of methanolic extract and its constituent, 6-Heptadecylcyclohex -3-ene-1 carboxylic acid were found to be 500 and 100 mg/kg body weight respectively. One tenth of these doses (50 and 10 mg/kg, body weight respectively) was selected as the therapeutic dose for the evaluation of hepatoprotective activity. [6]

2.5 Evaluation of hepatoprotective activity:

The animals were divided into 5 groups of 6 each. The animals of group I (control) received the vehicle gum tragacanth (1 ml/kg/day; 1 % w/v). Carbon tetrachloride with olive oil (1:1) was administered to all the animals of groups II to V in the dose of 0.1 ml/kg/day, ip for 14 days .The group III animals were treated with the standard drug silymarin (Ranbaxy Lab, Dewas;100 mg/kg/day, po). The animals of group IV received methanolic extract (50 mg/kg/day, po) and the animals of group V received the constituent 6-

Heptadecylcyclohex -3-ene-1 carboxylic acid. (10mg/kg/day, po). The drugs were administered concomitantly for 14 days. The animals of all the groups were sacrificed on 14th day under light ether anesthesia. The blood sample of each animal was collected separately by carotid bleeding into sterilized dry centrifuge tubes and allowed to coagulate for 30 min at 37°C. The clear serum was separated by centrifugation at 2500 rpm for 10 min and subjected to biochemical investigation viz., total bilirubin [7]. total protein [8]. serum alanine transaminase, aspartate transaminase,[9], and alkaline phosphatase [10] Results of biochemical estimations were expressed as mean±SE of six animals in each group. The statistical analysis was carried out using one way ANOVA. The difference in values at $P \leq 0.05$ was considered as statistically significant.

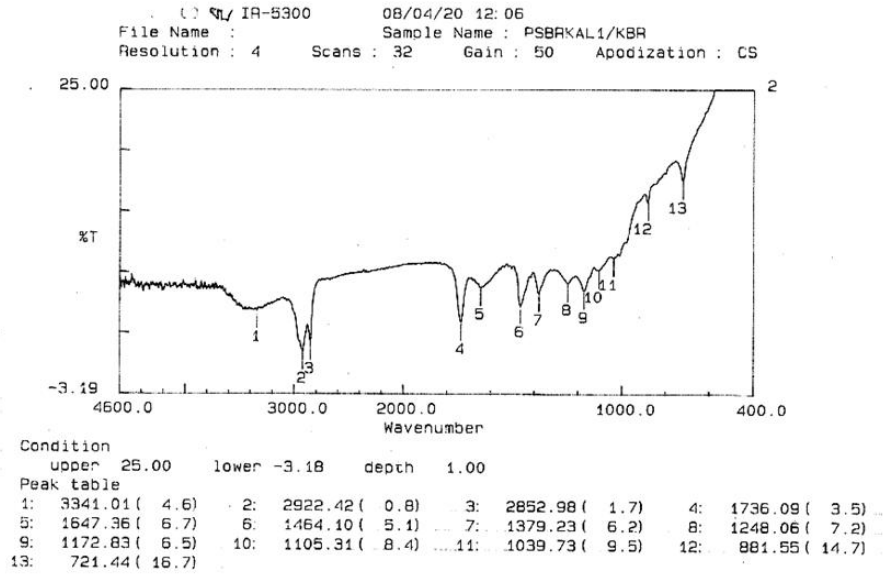
2.6 Study of Histopathology:

The liver samples were excised from the animals of each group after draining the blood and washed with the normal saline. Initially the materials were fixed in 10% buffered neutral formalin for 48 hrs. They were processed for paraffin embedding. The sections were taken at 5 μm thickness, processed in alcohol-xylene series and were stained with alum-haematoxylin and eosin [11][12]. The sections were examined microscopically for the evaluation of histological changes.

3. Results

The compound isolated from stem bark of methanolic extract of *D .cinerea* is a light yellow amorphous powder; Melting point is recorded to be 76 -79 ^{0}C ,

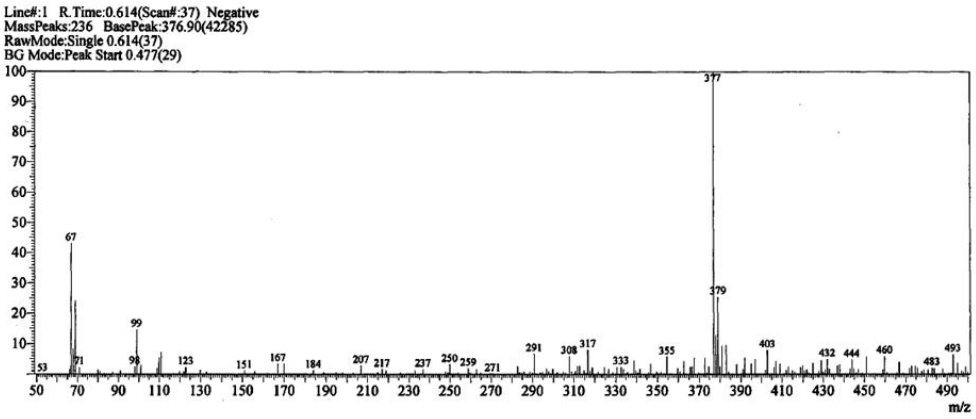
Fig. 1: IR Spectrum- 6-Heptadecylcyclohex -3-ene-1 carboxylic acid



The IR spectrum exhibits bands at 3341 cm-1 for –OH group,1736 cm-1 for carboxylic group, 1647 for C=C **(Fig. 1)**.

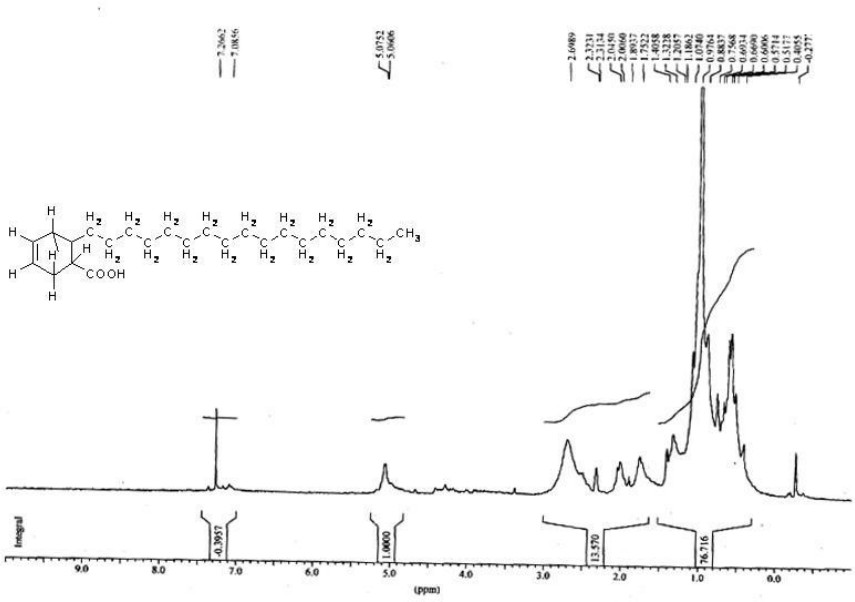
The molecular formula of the compound is $C_{25}H_{46}0_2$ evident from its mass spectrum by exhibiting a pseudomolecular ion $[M-H]^{-}$ at m/z 376.90. The peaks were observed at m/z355,m/z184 m/z170and m/z130**(Fig. 2)**.

Fig.2: MS Spectrum of 6-Heptadecylcyclohex -3-ene-1 carboxylic acid



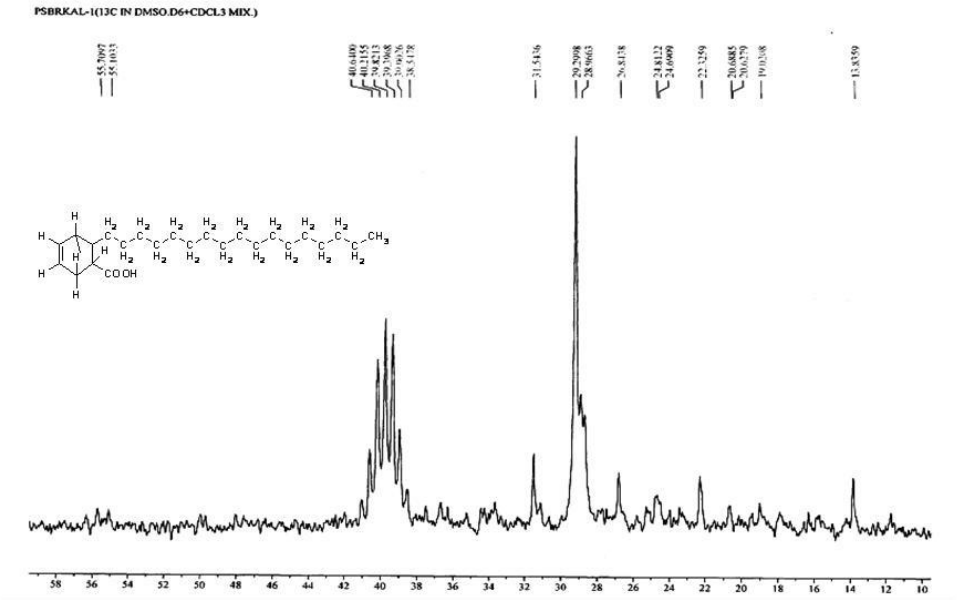
The ^{1}H NMR spectra ($CDCL_3$ +DMSO.D6 MIX.) showed peaks at 5.06(1H,s,OH), 2.0(14H,,J=6.8Hz, CH_2)0.66(3H,t,J=6.80 Hz,CH_3)0.88[28H,s,14xCH_2]. The peaks at δ 0.67 (ppm) for terminal methyl and δ 1.67 (ppm) for a methyl group attached to a double bond. The signal at δ 2.60 is due to a proton attached to a carboxyl group in a ring system. The multiplet at δ 5.54 is due to the presence of an unsaturated proton. In addition, a strong singlet represents the long chain methylene groups **(Fig. 3)**.

Fig. 3: ^{1}HNMR of 6-Heptadecylcyclohex -3-ene-1 carboxylic acid



The ^{13}C-NMR spectra exhibits two signals at δ 127.60 and δ 129.81 were due to the presence of a double bond. The singlet at δ 55.70 and δ 55.10 are due to carbon atoms of long chain methylene groups. The other signals at δ 13.83, δ 22.32, δ 24.69, δ 26.84, δ 28.86, are due to long chain alkyl group **(Fig. 4)**.

Fig. 4: ^{13}C NMR of 6-Heptadecylcyclohex -3-ene-1 carboxylic acid



Based on above facts the structure of the compound written as shown in the Fig 5 and the compound was identified as 6-Heptadecylcyclohex -3-ene-1 carboxylic acid.

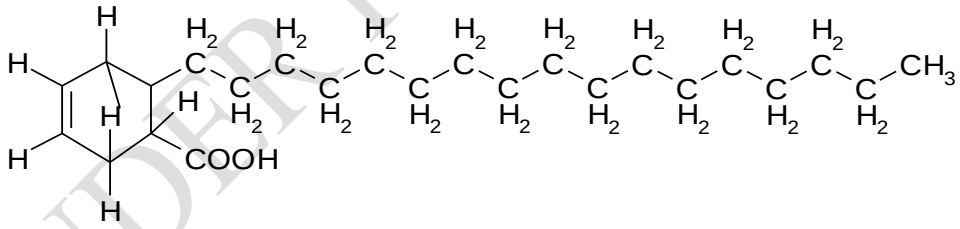


Fig. 5: Structure of 6-Heptadecylcyclohex -3-ene-1 carboxylic acid

After 14 days treatment, biochemical analysis of blood samples of CCl_4 treated animals showed significant increase in the levels of total bilirubin (6.22 fold), aspartate transaminase (5.71 fold), alanine transaminase(4.22 fold) and alkaline phosphatase (3.47 fold) as compared to the controls. In addition, the total protein level (44.86%) was decreased reflecting the liver injury due to the toxic effect of CCl_4. The blood samples of

the animals treated with the methanolic extract and the constituent 6-Heptadecylcyclohex -3-ene-1 carboxylic acid were showed significant reduction in the levels of liver function serum markers. The effect was more pronounced in the animals treated with 6-Heptadecylcyclohex -3-ene-1 carboxylic acid as similar to the effect of the standard drug silymarin **(Table 1).**

Table 1:

Effect of methanolic extract and its constituent 6-heptadecylcyclohex 3-ene-1 carboxylic acid on CCL_4 induced Hepatotoxicity

Groups	T. BIL (mg/dl)	Total protein	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	0.36±0.08	7.40±0.36	209.41±13.57	76.31±9.11	116.86±9.57
CCl4	2.24±0.29	4.08±0.27	1194.08±73.60	321.67±27.93	403.73±8.66
Silymarin+ CCl_4	0.40±0.14	7.34±0.42	217.64±23.8	78.81±8.05	118.21±9.72
Methanol extract + CCl_4	0.58±0.14	6.97±0.38	310.14±36.96	106.50±8.08	144.60±18.68
6-heptadecylcyclohex 3-ene-1 carboxylic acid	0.46±0.12	7.18±0.17	231.72±22.07	82.95±7.17	120.01±9.16
F value	22.4	18.1	114	53.8	113

Values are mean ± SE; n=6 in each group.

P<0.05 when compared to control.

The histological profile of control animal showed normal hepatocytes **(Fig. 6A).** The section of liver of the animals treated with CCl_4 exhibited intense centrilobular necrosis, vacuolization and macro vesicular fatty changes **(Fig. 6B)**. The liver sections of silymarin treated animals showed normal hepatic architecture **(Fig. 7A**). Moderate accumulation of fatty lobules was observed in the liver sections **(Fig. 7B)** of methanolic extract treated animals. The liver sections of the animals treated with the constituent

6-Heptadecylcyclohex -3-ene-1 carboxylic acid exhibited significant liver protection against CCl_4 induced liver damage as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration **(Fig. 7C)**.

Figure 6

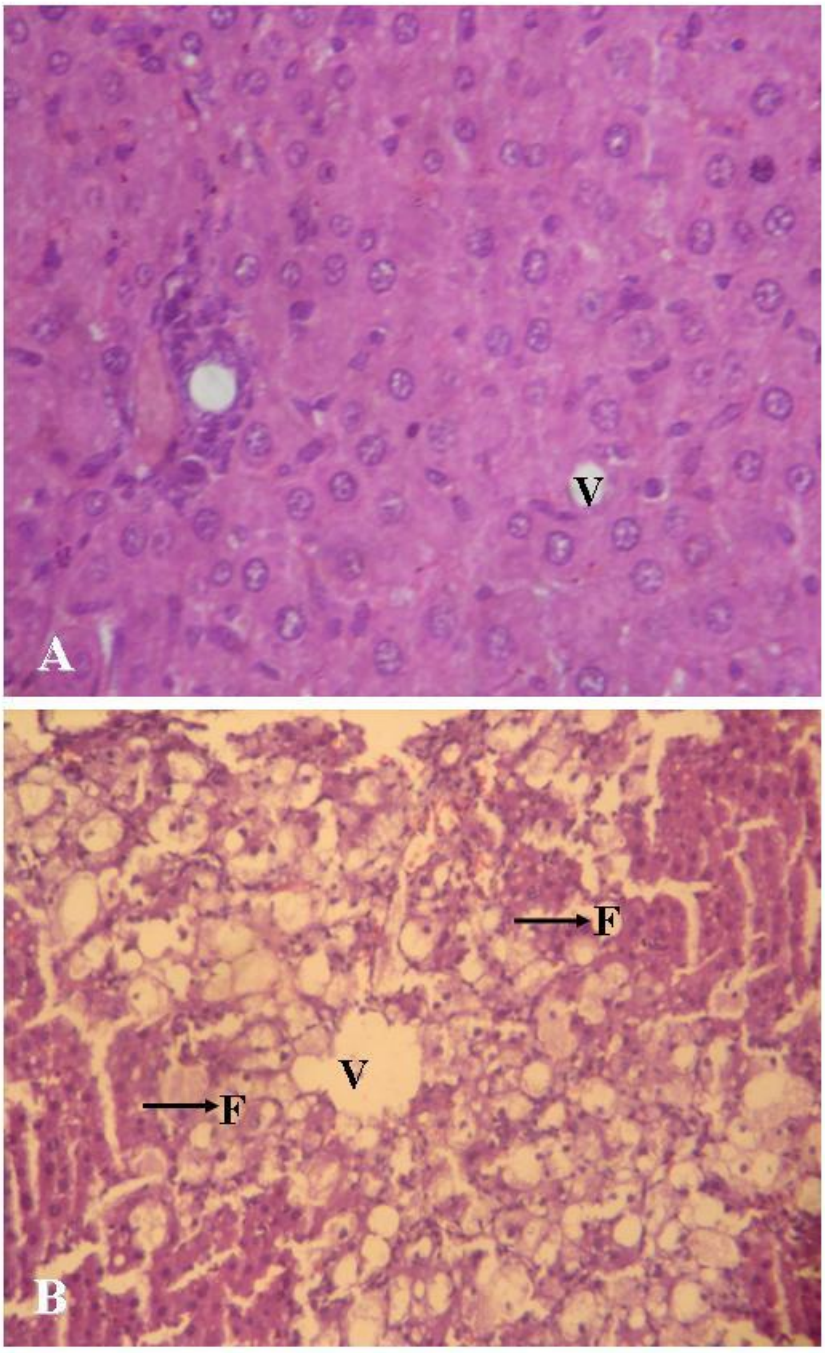


Figure 7

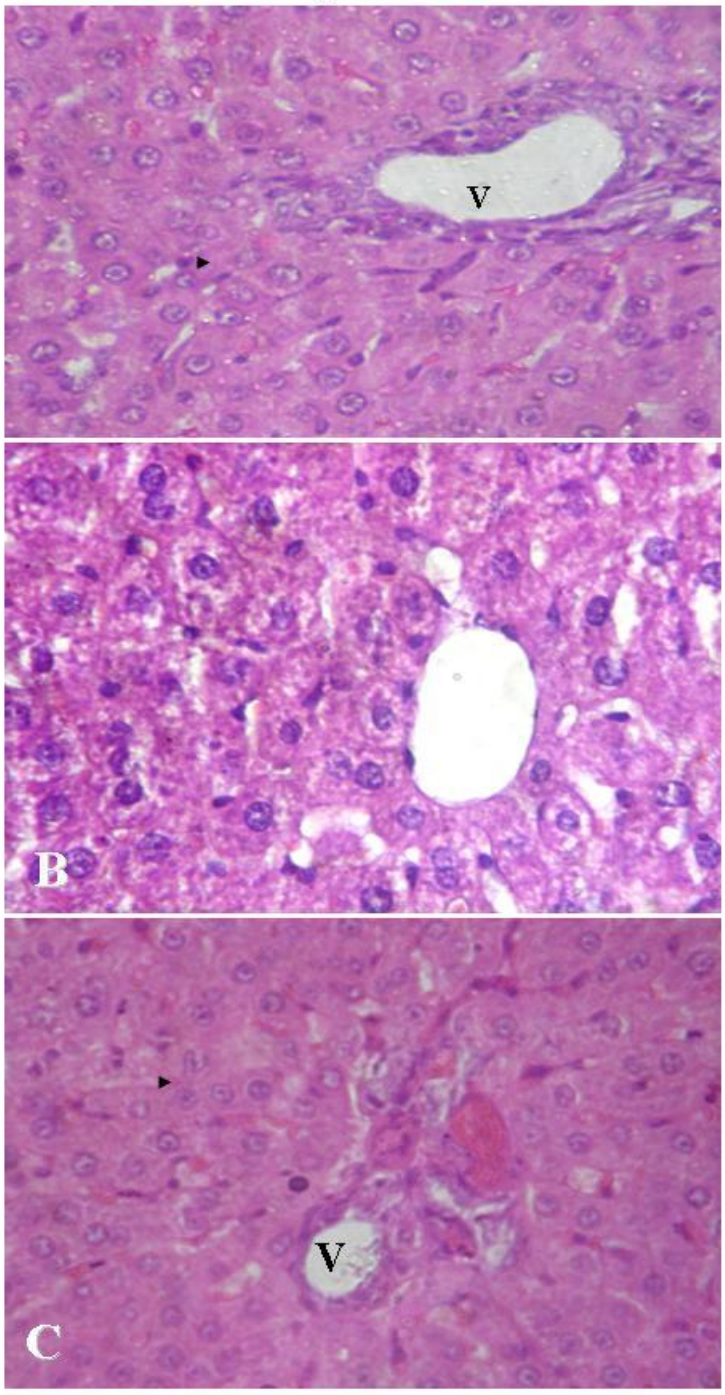


Fig. 6, 7. Histological slides

4. Discussion

Medicinal plants are an integral component of research developments in the pharmaceutical industry. Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs from plants as well as from traditionally used rural herbal remedies. The constituent 6-Heptadecylcyclohex -3-ene-1 carboxylic acid isolated from the stem bark was showed significant hepatoprotective activity against CCl_4 induced hepatotoxicity. To the best of our knowledge the 6-Heptadecylcyclohex -3-ene-1 carboxylic acid is a novel phytoconstituent isolated for the first time from *D. cinerea*. Similar fatty acid derivatives such as 5-hexyl-2-(8-oxooctyl)cyclohexane 1-carboxilicacid(Pubchem CID23345599) with **MW**: 354.524020 g/mol | MF: $C_{21}H_{38}O_4$ 3-Heptylcyclohexane-1-caboxylic acid (PubchemCID 20320740) with MW 226 355040 g/mol | MF**:** $C_{14}H_{26}O_2$. 2-butylcyclohexane-1-caboxylic acid (PubchemSID53962009) has MW**:** 184g/mol g/mol |MF:$C_{11}H_{20}O_2$ and 2-butylcyclohexane-1-carboxylic acid MW: 184.275300 g/mol | MF: $C_{11}H_{20}O_2$ (PubchemCID 12933617) have been reported. Reports also indicated that ω-3 and ω-6 fatty acids exhibited significant hepatoprotective property in rats [13] and supplementation of omega-3 fatty acid would reduce hepatocellular damage and cell death in a model of murine common bile duct ligation [14][15].

It is well established that CCl_4 induces hepatotoxicity by metabolic activation, therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function [16][17]. CCl_4 is bio-transformed by the cytochrome P450 system in the

endoplasmic reticulum to produce trichloromethyl free radical (•CCl_3). Trichloromethyl free radical when combined with cellular lipids and proteins in the presence of oxygen form trichloromethyl peroxyl radical, which may degrade lipids on the membrane of endoplasmic reticulum, thus leads to elicit lipidperoxidation and finally results in cell death [14]. In this present study it was noted that the administration of CCl_4 decreased the levels of total protein and increased levels of marker enzymes. The estimation of total bilirubin depicts the depth of jaundice and the elevated level of marker enzymes, ALT, AST and ALP indicates the intensity of liver damage. Marked elevation in the levels of total bilirubin and marker enzymes in the serum of CCl_4 intoxicated rats (1194.08±73.60, 321.67±27.93 and 403.73±8.66 respectively) were significantly decreased in the serum of the animals treated with methanol extract (310.14±36.96,106.50±8.08 and 144.60±18.68 respectvely) and 6-Heptadecylcyclohex-3-ene-1 carboxylic acid (231.72±22.07,82.95±7.17and 120.01±9.16 respectively). The protection against the injurious effects of carbon tetrachloride that may result from the interference with cytochrome P450, resulting in the hindrance of the formation of hepatotoxic free radicals. The site-specific oxidative damage in some susceptible amino acids of proteins may regarded as the major cause of metabolic dysfunction of liver during pathogenesis [18]. Bilirubin is the conventional indicator of liver diseases [19]. The restoration bilirubin levels may be due to the inhibitory effects on cytochrome P450 or/and promotion of its glucuronidation [20]. The attainment of these marker enzymes towards a near- normalcy in the animals treated with methanol extract and its isolated phytoconstituent 6-heptadecylcyclohex-3-ene-1 carboxylic acid confirms the hepatoprotective effect. The results were found comparable to a commercial hepatoprotective drug silymarin which is

a composite name of three flavonoids isolated from milk thistle Silybum marinum. Among all the treated groups the hepatoprotective activity was more in the animals treated with phytoconstituent 6-heptadecylcyclohex-3-ene-1 carboxylic acid. Similar type of investigations on the effect of phytoconstituents isolated from the medicinal plants in restoring the levels of liver function markers was reported [21,22, 23 & 24].

5. Conclusion.

In conclusion, the constituent 6-heptadecylcyclohex-3-ene-1 carboxylic acid isolated from the methanolic extract of *D .cinerea* is a novel phytoconstituent afforded protection from CCl_4 induced liver damage. The possible mechanism that it may protect the liver by CCl_4 toxicity or may act as a free radical scavenger intercepting those radicals involved in CCl_4 metabolism by microsomal enzymes. By trapping oxygen related free radicals the compound could hinder their interaction with polyunsaturated fatty acids and would abolish the enhancement of lipid peroxidative processes. The present investigation also supports the ethnomedical uses of *D .cinerea.*

6. References

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