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Carotenoid rodoxanthine obtained from the seeds of *Ricinus communis* act as potential hepatocytes regenerators

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Abstract
The liver is the only visceral organ that possesses remarkable capacity to regenerate. The liver can regenerate after either surgical removal or after chemical injury. It is known that as little as 25% of the original liver mass can regenerate back to its full size. The process of regeneration in mammals is mainly compensatory growth because only the mass of the liver is replaced not the shape. Liver regeneration involves replication of the liver cells, mainly hepatocytes, followed by other cells such as biliary epithelial cells and sinusoidal endothelial cells. Once cell proliferation is completed, the newly divided cells undergo restructuring, angiogenesis and reformation of extracellular matrix to complete the regeneration process. The main objective of present research work is to screen the bio molecules present in EE-CS and isolate the bioactive carotenoid rodoxanthine and evaluate the *in vivo* hepatocytes regenerators' potentiality. Characterization of rodoxanthine was carried out by U.V, IR, ^{1}H-NMR, ^{13}C-NMR, Mass spectra and HPLC etc. The *in vivo* hepatocytes regenerator's potentiality was performed against CCl_4 induced rat hepatocytes. The results obtained from the *in vivo* experimental data had shown that the elevated levels of SGOT, SGPT, ALP and Serum bilirubin due to CCl_4 intoxication were reduced significantly (*P<0.05) in rats, after treatment with EE-CS. Treatment with EE-CS at a both doses of 250 and 500 mg/kg b. w. significantly decreased the SGOT, SGPT, ALP, serum bilirubin levels by 13.05%, 27.98%, 8.82%, and 16.23% (at low dose) and 26.1%, 47.16%, 24.34% and 43.58% (at high dose) respectively. Silymarin used as standard drug showed a reduction of 55.09%, 68.98%, 57.46% and 60.68% receiving CCl_4 alone. So depending upon the recent data it was confirmed that the biochemical parameters of the group treated with EE-CS was significantly lower than the CCl_4-treated group. Histopathological analysis of rat liver sections obtained from CCl_4-intoxicated rats had shown a variety of cavitations and necrosis in hepatocytes, liver tissue section prepared from the 250 mg/kg EE-CS-treated group displayed less cavitation and necrosis, liver tissue section prepared from the 500 mg/kg EE-CS-treated group displayed less cavitation and necrosis, and liver tissue section prepared from the std. drug silymarin-treated group had shown centrilobular regeneration with restoration of central vein, sinusoids and hepatocytes with mild necrosis.

Keywords: Replication, hepatocytes, angiogenesis, sinusoidal endothelial cells, necrosis etc.

Introduction
It is truth that without nature human being life is not possible. The food, clothes and shelter are three basic necessity of human beings and an important one necessity is good health, which provided by plant kingdom. Plant kingdoms are the rich source of organic compounds, many of which have been used for medicinal purposes. In traditional medicine, there are many natural crude drugs that have the potential to treat many disease and disorders one of them is *Ricinus communis*; Family: Euphorbiaceae popularly known as 'castor plant' and commonly known as 'palm of Christ', Jada (Oriya), Veranda (Bengali), Endi (Hindi), Errandi (Marathi), Diveli (Guajarati) [1]. The plant is widespread throughout tropical regions as ornamental plants.

The castor oil plant is a fast-growing, suckering perennial shrub or occasionally a soft wooded small tree up to 6 meter or more, but it is not hardy in nature. This plants was cultivated for leaf and flower colours and for oil production. Leaves are green or reddish in colour and about 30-60 cm in diameter. The leaves contain 5-12 deep lobes with coarsely toothed segments which are alternate and palmate. The stems are varying in pigmentation. The flowers are monoecious and about 30-60 cm. long [2]. The fruit is a three-celled thorny capsule.

The capsule of fruit covered with soft spins like processes and dehiscing in to three 2-valved cocci. The seeds are considerable differences in size and colour. They are oval, somewhat compressed, 8-18 mm long and 4-12 mm broad. The testa is very smooth, thin and brittle. Castor seeds have a warty appendage called the caruncle, which present usually at one end from which runs the raphe to terminate in a slightly raised chalaza at the opposite end of the seed [3]. This plant is common and quite wild in the jungles in India and it is cultivated throughout India, chiefly in the Madras, Bengal and Bombay presidencies. Two varieties of this plant are known A perennial bushy plant with large fruits and large red seeds which yields about 40 P.C of oil. A much smaller annual shrub with small grey (white) seeds having brown spots and yielding 37% of oil.

Fig 1: Flowering plant, **Fig 2:** Fruit

Pharmacology

1. Antioxidant activity: It is concluded that *R. communis* antioxidant activity by using lipid method and free radical scavenging effect on 2, 2 picrylhydrazyl radical (DPPH) and hydroxyl hydrogen peroxide. The high antioxidant activity of the seed of communis at low concentration shows that it could be very useful for the treatment of disease resulting from oxidative stress. The responsible chemical constituent of antioxidant activity are Methyl ricinoleate, Ricinoleic acid [4] octadecadienoic acid and methyl ester stem and leave extracts also produce antioxidant activity due to the presence of flavonoids in their extracts

2. Antinociceptive activity: The methanolic leaves extract of antinociceptive activity against formalin induced paw licking and The antinociceptive activity showed due to the presence preliminary Phytoconstituents like saponins, steroids and alkaloids -5-en-3-ol, stigmasterol, Y-sitosterol, fucosterol; essential oil using capillary like α-thujone (31.71%) and 1,8- (12.92%) and 30-Norlupan-3β-ol-20-one are bean [5]. seed extracts produced the per oxidation by ferric thiocyanate 2,2-diphenyl-1- radical generated from *R. communis* which produce 12- ester [6]. The *Ricinus communis* extracts [7, 8]. *R. communis* possesses significant acetic acid induced writhing test, tail immersion methods in mice. Alkaloids [9].

3. Antiasthmatic activity: The ethanolic root extract of *R. communis* is effective in treatment of asthma because of its antiallergic and mast cell stabilizing potential effect. Saponins has mast cell stabilizing effect and the flavonoids possess smooth muscle relaxant and bronchodilator activity; the apigenin and luteolin like flavonoids were generally inhibit basophil histamine release and neutrophils beta glucuronidase release, and finally shows in-vivo antiallergic activity. The *R. communis* ethanolic extract decreases milk induced leucocytosis and eosinophilia and possess antiasthmatic activity due to presence of flavonoids or saponins [10].

4. Anti-fertility activity: The methanol extracts of *R. communis* seed possess positive preliminarily Phytochemical tests for both steroids and alkaloids. The pituitary gland releases gonadotropins due to Sex hormones by both positive and negative feedback mechanism and also the pituitary gland block the release of luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) because of the effect of combined oestrogen and progesterone in the luteal phase of the menstrual cycle. Finally it helps the inhibition of maturation of the follicle in the ovary and prevents ovulation. The sex hormone being steroidal compound's (phytosterols) and the presence of steroids in methanol extract of *Ricinus communis* seed produces anti-fertility effects [11, 12].

5. Antihistaminic Activity: The ethanol extract of R. communis root resulted anti histaminic activity at the dose 100, 125, and 150 mg/kg intraperitoneally by using clonidine induced catalepsy in mice [13].

6. *In vitro* immunomodulatory activity: The plant and animal origin immunomodulatory agents generally increase the immune responsiveness of the human body against pathogens by activating the non-specific immune system. The phagocytosis is the engulfment of microorganism by leucocytes. In last the phagocytosis is the intracellular killing of microorganisms by the neutrophils. The presence of tannins in the leaves of *R. communis* significantly increased the phagocytic function of human neutrophils and resulted produces a possible immunomodulatory effect [14].

7. Hepatoprotective activity: *Ricinus communis* leaves ethanolic extract 250/500mg/kg body weight possesses hepatoprotective activity due to their inhibitory activities of an increase in the activities of serum transaminases and the level of liver lipid per oxidation, protein, glycogen and the activities of acid and alkaline phosphatase in liver induced by carbon tetrachloride (CCL_4). The *R. communis* ethanolic extract 250/500mg/kg body weight also treated the depletion of glutathione level and adenosine triphosphatase activity which was observed in the CCl4-induced rat liver. The presence of flavonoids in ethanol extract of *R. communis* produces beneficial effect the flavonoids have the membrane stabilizing and antiperoxidative effects. Hence the *R. communis* increase the regenerative and reparative capacity of the liver due to the presence of flavonoids and tannins. The anticholestatic and hepatoprotective activity was seen against paracetamol-induced hepatic damage due to the presence of N-demethyl ricinine isolated from the leaves of *Ricinus communis* Linn. The whole leaves of *Ricinus communis* showed the protective effect against liver necrosis as well as fatty changes induced by CCL4 while the glycoside and cold aqueous extract provide protection only against liver necrosis and fatty changes respectively, [15, 16, 17].

8. Anti-inflammatory activity: Anti-inflammatory activities of the leaves and root extract were studied in Wistar albino rats in acute and chronic inflammatory models. The study indicated that the paw edema formation due to sub plantar administration of carrageenan, characterizing the cellular events of acute inflammation. The 250 and 500 mg/kg dose of *R. communis* methanolic leaves extract possess protective effect in prevention of cellular

events during edema formation and in allthe stages of acute inflammation. The anti-inflammatory activity of *R. communis* methanolic extract was due to the presence of flavonoids because the flavonoids have the protective effect against carrageenan -induced paw edema in rats [18. 19, 20].

9. Antimicrobial activity: The antimicrobial activities of *Ricinus communis* were good against dermatophytic and pathogenic bacterial strains *Streptococcus progenies, Staphylococcus aureus* as well as *Klebsiella pneumonia, Escherichia coli*. The result showed that the petroleum ether and acetone extracts possess good zone of inhibition where as ethanolic extract having anti-bacterial activity only on higher concentration [21]. The different solvent extracts of roots of *Ricinus communis (*200 mg/ml) possess antimicrobial activity by using well diffusion method against pathogenic microorganisms such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium, Proteus vulgaris, Bacillus subtilis, Candida albicans* and *Aspergillus niger*. The hexane and methanol extracts showed maximum antimicrobial activity where the aqueous extracts has no significant antimicrobial properties [22].

10. Antidiabetic activity: The ethanolic extract of roots of *Ricinus communis* (RCRE) was investigated along with its bioassay-guided purification. By Administration of the effective dose (500mg/kg b. w) of RCRE to the diabetic rats for 20 days possess favorable effects not only on fasting blood glucose, but also on total lipid profile and liver and kidney functions. Amongst all fractions the R-18 fraction suggests the significant antihyperglycemic activity. RCRE showed no significant difference in alkaline phosphatase, serum bilirubin, creatinine, serum glutamate oxaloacetate transaminases, serum glutamate pyruvate transaminases and total protein which was observed even after the administration of the extract at a dose of 10 g/kg b.wt. Thus *R. communis* is a potent phytomedicine for diabetes [23].

11. Wound healing activity: The *Ricinus communis* possess wound healing activity due to the active constituent of castor oil which produce antioxidant activity and inhibit lipid per oxidation. Those agents whose inhibits lipid per oxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis. The study of wound healing activity of castor oil was in terms of scar area, % closure of scar area and epithelization in excision wound model. Due to the astringent and antimicrobial property the tannins, flavonoids, triterpenoids and sesquiterpenes promotes the wound healing process, which are responsible for wound contraction and increased rate of epithelialisation. The study resulted that the Castor oil showed wound healing activity by reducing the scar area and also the epithelization time in excision wound model. The comparison study of two different concentrations (5%w/w and 10%w/w) of castor oil was resulted that the 10% w/w Castor oil ointment possesses better wound-healing property [24].

12. Lipolytic activity: The ricin produces the lipolytic activity by using the various substrates: (i) one analogue of triacylglycerol, BAL-TC4; (ii) various chromogenic substrates such as *p*-NP esters of aliphatic short to medium chain acids, and (iii) Monomolecular films of a pure natural diacylglycerol, DC10 in emulsion and in a Membrane-like model. The study concluded that ricin from *R. communis* act as a lipase and has the capability of hydrolyzing different lipid classes. Ricin also hydrolyses phospholipids which are the major components of cellular membranes. The lipolytic activities are maximal at pH 7.0 in the presence of 0.2 M galactose. The action of ricin on membrane phospholipids could occur through a phospholipase A1 activity which is very often a minor activity of lipases [25]

13. Molluscicidal, Insecticidal and Larvicidal activity: The leaf extract of *R. communis* possess molluscicidal activity against *Lymnaea acuminata* and the seed extracts showed better insecticidal and insectistatic activity than the leaf extracts against *S. frugiperda* due to the active ingredients like castor oil and ricinine [26, 27, 28]. The aqueous leaves extracts of *R. communis* possess suitable Larvicidal activity against *Anopheles arabiensis, Callosobruchus chinensis* and *Culex Quinquefasciatus* mosquitoes [29].

14. Antiulcer activity: The castor oil of *R. communis* seed possess significant antiulcer properties at a dose of 500 mg/kg and 1000 mg/kg, but at the dose 1000 mg/kg was more potent against the ulceration caused by pylorus ligation, aspirin and ethanol in rats. The result showed that the antiulcer activity of *R. communis* is due to the cytoprotective action of the drug or strengthening of gastric mucosa and thus enhancing the mucosal defence [30, 31].

R. communis or castor plant is a widely traditionally used and potent medicinal plant amongst all the thousands of medicinal plants. The pharmacological activities reported in the present review confirm that the therapeutic value of *R. communis* is much more. It is an important source of compounds with theirs chemical structures as well as pharmacological properties. The presence of phytochemical constituents and pharmacological activities proved that the plant has a leading capacity for the development of new good efficacy drugs in future.

Materials and Method

Drugs and chemicals: The standard drug silymarin purchased from Local Retail Pharmacy Shop and solvents and other chemicals used for the extraction and phytochemical screening were provided by Institutional Store and were of LR and AR grade.

Instrumentation: The IR spectra were recorded in the solid state as a KBr dispersion medium using the FT-IR (Perkin Elmer, Spectrum 65 & JASCO-FT-IR-430) spectrophotometer. The UV spectrum was recorded on a Shimadzu UV-visible spectrophotometer. The ^{1}H NMR and ^{13}C NMR experiments for rodoxanthine were performed at 400.13 MHz and 100.62 MHz, respectively, on the Bruker Avance 400 MHz FT NMR spectrometer with a multinuclear BBO probe. $CDCl_3$ used as the solvent. HPLC was used for the isolation and purification of rodoxanthine. Mass spectra of rodoxanthine recorded by JEOL GCmate.

Experimental animals: White male albino Wister rats weighing about 200-250gm was used they were obtained from the animal house of C. L. Baid metha College of Pharmacy, chennai. They were kept under observation for about 7 days before onset of experiment to exclude any

intercurrent infection, had free access to normal diet and water. The experimental protocol was approved by IAEC (Institutional Animal Ethics Committee) of CPCSEA: IAEC/XXIX/12/2015.

Methodology for Soxhlet extraction: First the dried seeds are triturate to make fine powder and the powered material is placed into the thimble made of stout filter paper and the apparatus is fitted up. The flask containing suitable solvent like ethanol is heated on a water bath or on a heating mental. As the solvent boil, its vapors rise through the side tube up into the water condenser. The condensed liquid drops on the solid in the thimble, dissolves the organic substances present in the powdered material and filters out into the space between the thimble and the glass cylinder. As the level of liquid here rises, the solution flows through the siphon back into the boiling flask. The solvent is once again vaporized, leaving behind the extracted substance in the flask. In this way a continuous stream of pure solvent drops on the solid material, extract the soluble substance and returns to the flask. At the end of the operation the solvent in the boiling flask is distilled off, leaving the organic substance behind [32]. Afterwards the ethanolic extract transfer in a clean and dried beaker and is concentrated by placing on a water bath and then cool, keep it in a freeze. From this concentrated extract the preliminary phytochemical screening has to be carried out.

Phytochemical screening and characterization biomolecule rodoxanthine [33-36].
Preliminary phytochemical screening of EE-CS have shown the presence of diverse bioactive molecules such as: carbohydrates, proteins and aminoacids polyphenols, carotenoids, phytosterols and alkaloids which are confirmed by their specific qualitative confirmatory chemical tests. EE-CS have shown the presence carotenoid **rodoxanthine.**

Evaluation of acute oral toxicity [37]. In the present study the acute oral toxicity of the ethanolic extract of castor seeds (EE-CS- *Ricinus communis*) was performed by acute toxic class method. In this method the toxicity of the extract was planned to test using step wise procedure, each step using three Wister rats. The rats were fasted prior to dosing (food but not water should be withheld) for three to four hrs. Following the period of fasting the animals were weighed and the extract was administered orally at a dose of 2000 mg/Kg b.w. Animals were observed individually after dosing at least once during the first 30 min; periodically the surveillance was carried out for the first 24 hrs with special attention given during the first 4 hrs and daily thereafter, for a total of 14 days. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of CPCSEA: IAEC/XXIX/12/2015.

Experimental protocol for Hepatoprotective activity [38].
A total of 30 rats were taken and divided into 5 groups of 6 rats each

(A) Group I: Normal Control Group [NCG - (only the vehicle (1 mL/kg/day of 1% CMC; p.o.)].

(B) Group II: Negative Control Group [Neg.CG – (CCl4 1 mL/kg (1:1 of CCl_4 in olive oil) i.p].

(C) Group III: Positive Control/Standard Group **[SG -** CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) i.p. + Standard Silymarin 100 mg/kg orally (p.o.) for 7 days]

Treatment Groups
(D) Group IV: High Dose Group [HDG - CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) i.p + EE-CS (500 mg/ kg b. w., p.o.)]

(E) Group V: Low Dose Group [LDG - CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) i.p + + EE-CS of BG (250 mg/ kg b. w., p.o.)]. Treatment was given daily for seven days orally.

Collection of blood: On the 8th day, blood was collected by retro orbital puncture, under mild ether anesthesia after 8 hr fasting. Blood samples were centrifuged at 3000 rpm for 20 mins. Serum was separated and stored at – 200^0 C until biochemical estimations.

Biochemical Analysis
The Serum samples were analyzed for
1) Alanine Aminotransferase (ALT) (SGPT)
2) Aspartate Aminotransferase (AST) (SGOT)
3) Alkaline Phosphatase (ALP)
4) Serum Bilirubin

Histopathological Analysis
The liver tissue was dissected out and fixed in 10% formalin solution. It was then dehydrated in ethanol (50%-100%), cleared in xylene and embedded in paraffin wax. Afterwards thick sections (5-6 mm) were made and then stained with hematoxylin and eosin dye for photo microscopic observation. The whole biochemical and histopathological analysis was carried out at V.H.S Hospital in Chennai.

Results and Discussion
Phytochemical screening and spectral data of rodoxanthine
Preliminary phytochemical screening of EE-CS have shown the presence of diverse bioactive molecules such as: carbohydrates, proteins and aminoacids polyphenols, carotenoids, phytosterols and alkaloids which are confirmed by their specific qualitative confirmatory chemical tests. EE-CS have shown the presence carotenoid rodoxanthine characterized by UV, IR, ^{1}H-NMR, ^{13}C-NMR, Mass and HPLC spectral data (Fig: 3-7)

UV spectra: Max (nm): hexane 505 (E 1%1cm = 2350), CHC_{l3} 493s (E 1%1cm = 2140), 511 (2420) and 533s, cyclohexane 472 (E 1%1cm = 2550), 443 (2530), 417 (1605).

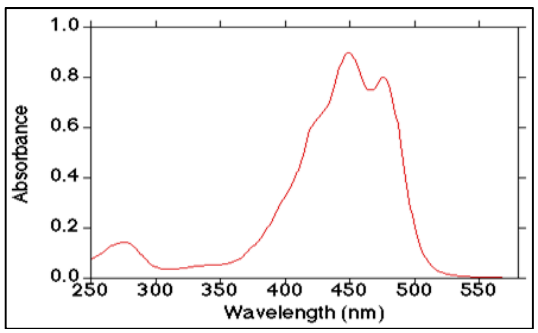

Fig 3: UV spectra of rodoxanthine

IR spectra: Max (KBr)/cm^{-1}: 1659s (conj. CO), 1576m, 1536m (conj. C=C), 992m, 978m (CH=CH, trans).

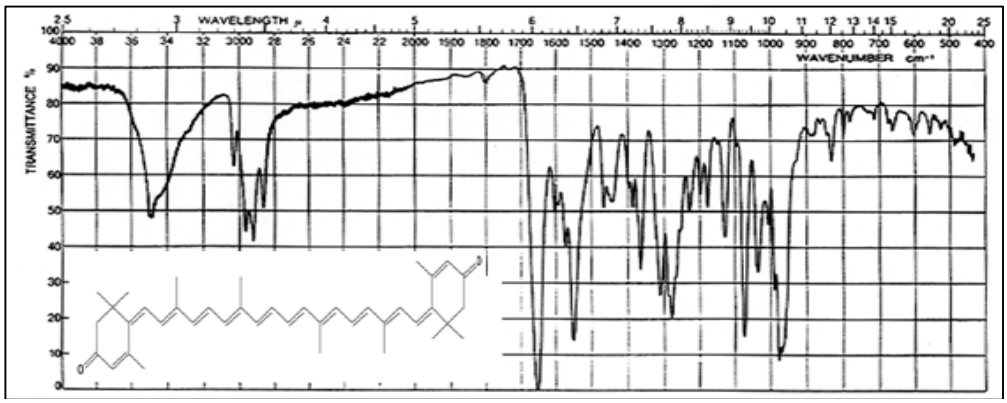

Fig 4: IR spectra of rodoxanthine

NMR spectra

^{1}H-NMR δ(400 MHz, CDCl$_3$) all-trans: 1.39 (s, 16,17,16',17'-Me), 2.00 (s, 20, 20'-Me), 2.03 (s, 19, 19'-Me), 2.16 (d J 1, 18,18'-Me), 2.39 (s, 2,2'-H2), 5.94 (brs, 4,4'-H), 6.26 (d, J 11.5, 12,12'-H), ca 6.43 (m, 14, 14', 15, 15'-H), 6.46 (d, J 15.5, 10,10'-H), 6.79 (dd, J 14.5, J 12, 11,11'-H), 6.80 (d, J 12.5, 8,8'-H), 6.90 (d, J 12.7, 7,7'-H). 6-cis: 1.25 (s, 16,17-Me), 1.39 (s, 16',17'-Me), 1.99 (s, 20'-Me), 1.993 (s, 20'-Me), 2.016 (s, 19-Me), 2.029 (s, 19'-Me), 2.304 (d, J 1.2, 18-Me), 2.16 (d, J 1, 18'-Me), 2.34 (s, 2-H2), 2.39 (s, 2'-H2), 5.94 (brs, 4,4'-H), 6.24 (d, J 11.5, 12-H), 6.26 (d, J 11.5, 12'-H), ca 6.43 (m, 14, 14', 15, 15'-H), 6.40 (d, J 14.5, 10-H), 6.46 (d, J 14.5, 10'-H), 6.74 (dd, J 15, J 11.5, 11,H), 6.78 (dd, 11'-H), 6.55 (d, J 12.2, 8'-H), 6.80 (d, J 12.3, 8'-H), 6.67 (d, J 12.2, 7'-H), 6.90 (d J 12.3, 7'-H).

all-trans ^{13}C-NMR δ(100MHz, CDCl$_3$): 12.41 (19), 12.91 (20), 22.31 (18), 29.87 (16, 17), 38.56 (1), 54.37 (2), 126.05 (4), 129.93 (11), 128.15 (7), 128.32 (8), 129.85 (15), 132.56 (12), 137.51 (13), 137.68 (14), 138.04 (10), 141.32 (9), 142.70 (6), 154.74 (5), 198.95 (3) 6,6'-dicis ^{13}C-NMR δ(100MHz, CDCl3): 12.42 (19), 12.88 (20), 25.42 (18), 28.30 (16, 17), 41.26 (1), 52.50 (2), 125.48 (4), 126.40 (11), 128.79 and 128.83 (4) and (8),129.60 (15), 132.47 (12), 137.87, 137.54 and 137.05 (10), (13) and (14), 139.58 (9), 143.83 (6), 155.46 (5), 198.99 (3).

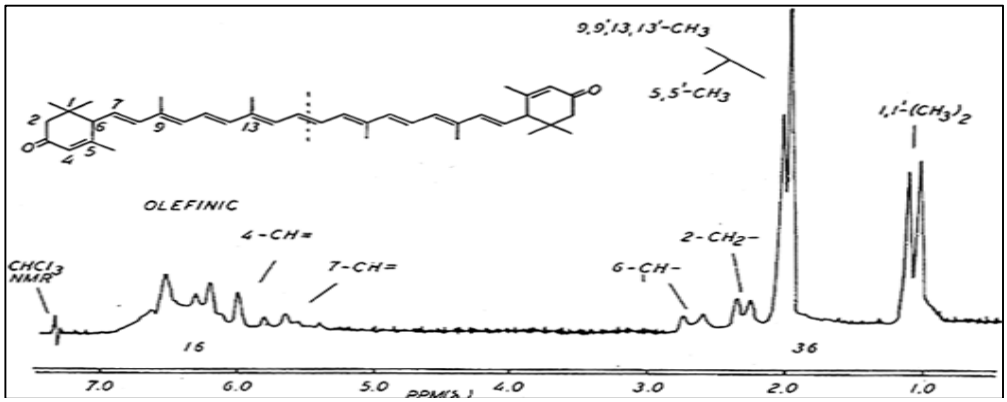

Fig 5: ^{1}H-NMR spectra of rodoxanthine

Mass spectra: m/z: 562 (M), 506 (M-56), 470 (M-92), 456 (M-106).

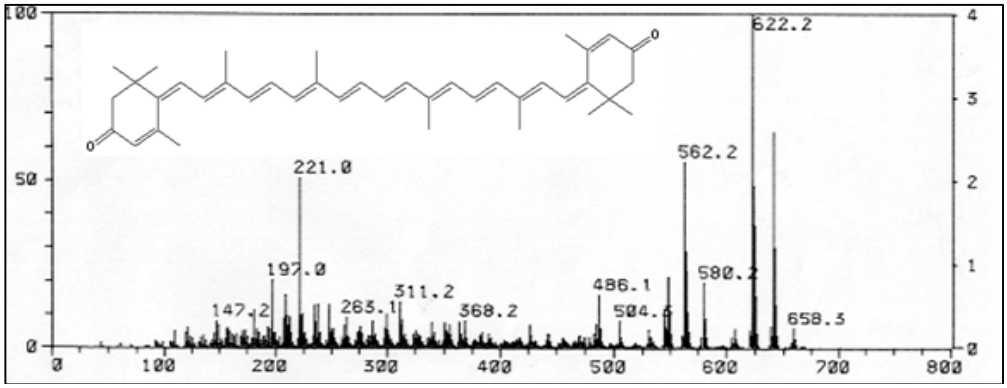

Fig 6: Mass spectra of rodoxanthine

HPLC: (column: Spherisorb S 5-CN), eluent: n-hexane- Isopropyl acetate-acetone (76:17:7), flow rate: 1ml/min.

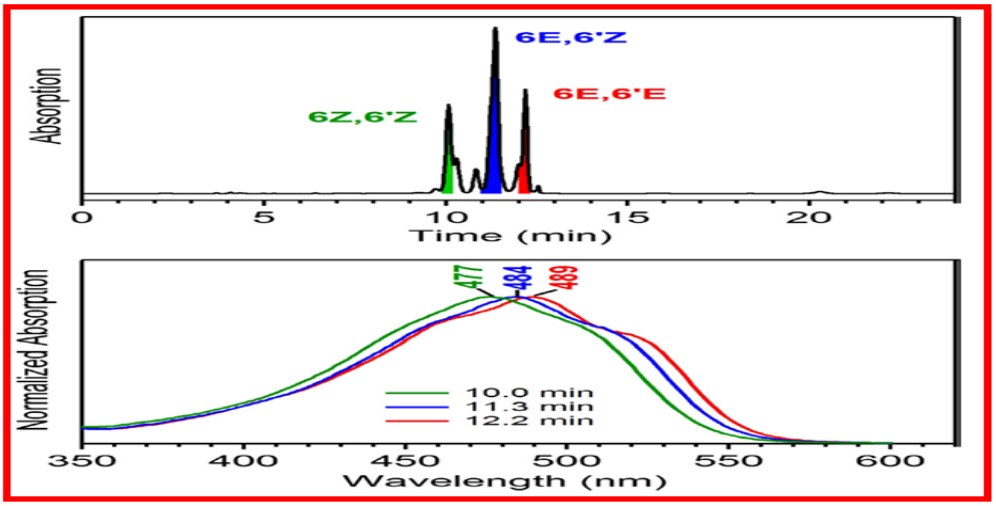

Fig 7: (Top) Normal-phase HPLC chromatogram of EE-CS, with detection at 480 nm. (Bottom) Absorption spectra corresponding to the three major bands colored in the top panel. The absorption of each eluted band was measured while in the mobile phase solvent, which is 12–13% acetone in hexane.

Acute oral toxicity study

1) Acute oral toxicity studies were performed according to the OECD guideline 423 method.
2) This method has been designed to evaluate the substance at the fixed doses and provide information both for hazard assessment and substance to be ranked for hazard classification purposes.
3) The EE-CS was administered initially at a dose of 2000 mg/kg b.w and 1% CMC (p.o) and observed 14 days mortality due to acute toxicity.
4) Careful observation were made at least thrice a day for the effect on CNS, ANS, motor activity, salivation and other general signs of toxicity were also observed and recorded.
5) Since no sign of toxicity observed at 2000 mg/kg b.w. to the group of animals, the LD_{50} value of the EE-CS expected to exceed 2000 mg/kg b. w. and represented as class 5 (2000 mg/kg < LD50 < 2500 mg/kg).
6) From the toxicity studies the data revealed that all the synthesized compounds proved to be non-toxic at tested dose levels and well tolerated by the experimental animals as there LD_{50} cut of values > 2000 mg/kg b. w.

Table 1: for the dose selection by acute toxicity class method (OECD) guide lines 423 of EE-CS

Sl. No.	Treatment group	Dose mg/kg	Sign of toxicity	Onset of toxicity	Duration
1	EE-CS	250	No	No	14 days
2	EE-CS	500	No	No	14 days

Hepatoprotective activity

Statistical analysis: The data were expressed as mean ± SD. Statistical differences at *P<0.05 between the groups were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison Test using Graph Pad Prism 5.04 In state software package. The data's were compared with group 2 i.e. Negative Control group.

Biochemical analysis: The effects of EE-CS on liver marker enzymes and serum bilirubin levelsare displayed in table 2 and fig: 8-12. The data exhibited that Normal Control Group (NeCG) demonstrated a normal range of AST, ALT, and bilirubin levels while the CCl_4-treated group showed elevated levels of AST, ALT, and bilirubin, thus confirming that CCl_4 causes hepatocellular degeneration at higher doses. The elevation of cytoplasmic AST and ALT is considered an indicator for the release of enzymes from disrupted liver cells. Bilirubin concentration has been used to evaluate chemically induced hepatic injury. The Results displayed in table 2 and fig 8-12: were indicated that the elevated levels of SGOT, SGPT, ALP and Serum bilirubin due to CCl_4 intoxication were reduced significantly (*P<0.05) in rats, after treatment with EE-CS. Treatment with EE-CS at a both doses of 250 and 500 mg/kg b.w. significantly decreased the SGOT, SGPT, ALP, Serum Bilirubin levels by 13.05%, 27.98%, 8.82%, and 16.23% (at low dose) and 26.1%, 47.16%, 24.34% and 43.58% (at high dose) respectively. Silymarin used as standard drug showed a reduction of 55.09%, 68.98%, 57.46% and 60.68% receiving CCl_4 alone. So depending upon the data of table 2 it was confirmed that the biochemical parameters of the group treated with EE-CS was significantly lower than the CCl_4-treated group. Moreover the treatment with the EE-CS significantly reduced the previously raised levels of AST, ALT, ALP and bilirubin in hepatotoxic rats.

Histopathological Analysis: The results of light microscopy examination of the transverse section of control, CCl_4-treated and treated with EE-CS rat livers were represented in fig 5. It was revealed that the liver section of animals treated with CCl_4 showed a high degree of damage characterized by cell vacuolation, pyknotic and degenerated nuclei and wall of bile capillaries. The normal architecture of the liver was lost. The intralobular vein was badly damaged with wide spaces at some sinusoids. Liver sections of these rats indicated necrosis, ballooning and degeneration in hepatic plates and loss of cellular boundaries. There was also a heavy accumulation of neutrophils surrounding the portal vein. These neutrophils act as an indicator of the occurrence of cell damage as they are absent in normal healthy tissues. The hepatocytes are disrupted and sinusoids are damaged as well.

Table 2: for the assessment of Biochemical parameters

Sl. No.	Groups	AST(SGOT) IU/L	ALT(SGPT) IU/L	ALP(SALP) IU/L	Sr. bilirubin mg/dL
1.	NCG	53.00±8.672	46.60±11.95	139.2±6.914	0.58±0.08
2.	NeCG	202.2±30.45	204.4±47.74	399.2±16.18	1.17±0.16
3.	LDG	175.8±14.48**	147.2±39.79**	364.0±17.52**	0.98 ± 0.88**
4.	HDG	149.6±12.52***	108.0±18.48***	302.0±36.76***	0.66±0.10***
5.	SG	90.80±17.61***	63.40±15.73***	169.8±8.55***	0.46±0.14**

Data are expressed as mean±SD (n = 6). One-way ANOVA followed by Dunnett's Multiple Comparison Test (* P<0.05) compared with group 2 ;(ns=non-significant).

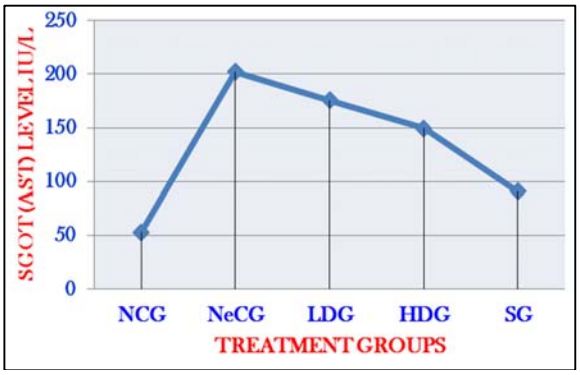

Fig 8: Comparison of AST (SGOT) Level.

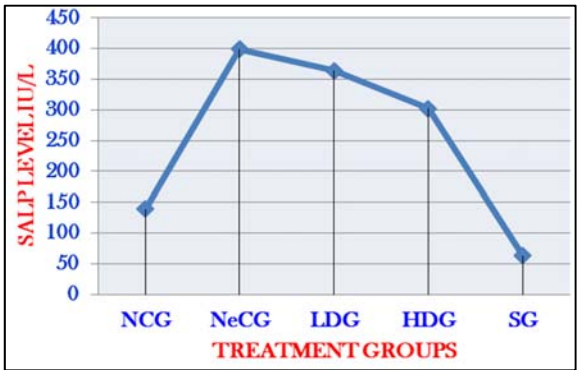

Fig 10: Comparison of SALP evel.

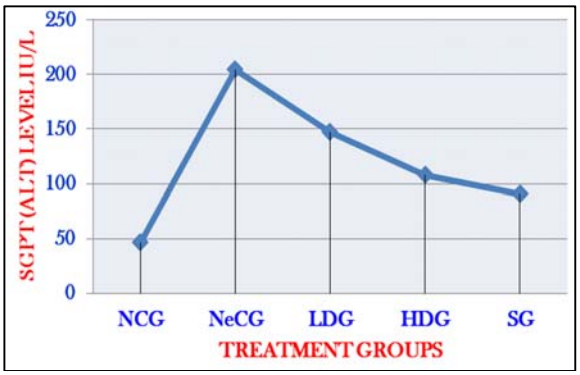

Fig 9: Comparison of ALT (SGOT) Level.

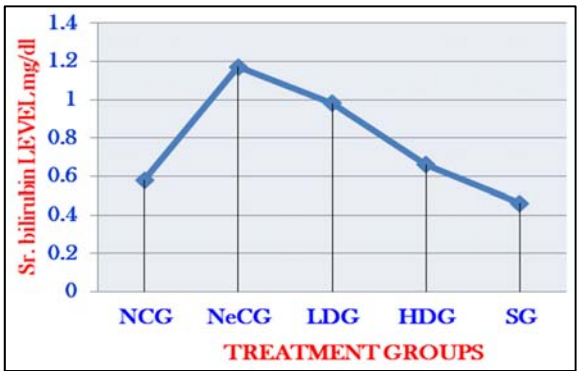

Fig 11: Comparison of Sr. bilirubin Level.

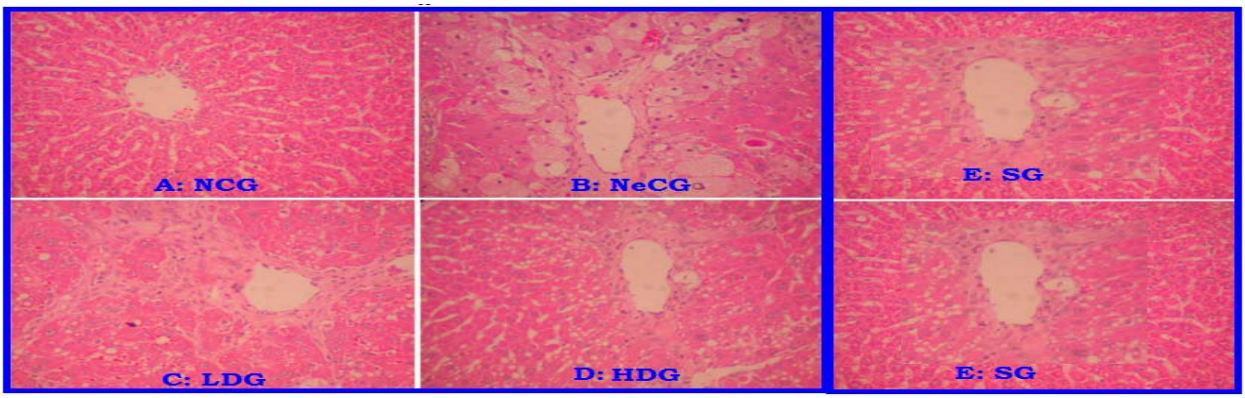

Fig 12: Histopathological analysis of rat liver sections using H&E staining. A: Section from a normal control rat liver. B: The liver section obtained from CCl_4-intoxicated rats shows a variety of cavitations and necrosis in hepatocytes. C: Liver tissue section prepared from the 250 mg/kg EE-CS-treated group shows less cavitation and necrosis compared to B. D: Liver tissue section prepared from the 500 mg/kg EECS-treated group shows less cavitation and necrosis than C. E: Liver tissue section prepared from the std. drug silymarin-treated group shows centrilobular regeneration with restoration of c.v, ss and hepatocytes with mild necrosis.

Conclusion

From the above experimental data, here I concluded that the EE-CS contained various bioactive molecules which were identified by their specific qualitative tests and executed moderate to good hepatoprotective activity. When compared with standard drug silymarin it displayed that EE-CS had the ability to restore and regenerate the CCl_4 induced hepatocytes due to the presence of bioactive molecule rodoxanthine.

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