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Research Article

DETERMINATION OF TOTAL PHENOLIC, FLAVONOID, ALKALOIDAL CONTENTS AND *IN VIVO* SCREENING FOR HEPATOPROTECTIVE ACTIVITY OF *CUSCUTA EPITHYMUM* (L.) L WHOLE PLANT AGAINST CCL_4 INDUCED LIVER DAMAGE ANIMAL MODEL

SERU GANAPATY[1], MADDI RAMAIAH*[2], KANURI YASASWINI[3], CHEPURI RAJESH KUMAR[4]

*[1,2]Department of Pharmacognosy & Phytochemistry, [3]Department of Pharmaceutics, AU College of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530003, [4]Hindu College of Pharmacy, Guntur-522002, A.P., India. Email: rampharma83@gmail.com, ram_pharma83@yahoo.com

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ABSTRACT

Objective: The development of antihepatotoxic drugs being a major thrust area has drawn the attention of workers in the field of natural product research because synthetic drugs may cause serious side effects. The present research was aimed to study the *in vivo* hepatoprotective activity of methanolic extract of *Cuscuta epithymum* (L.) L whole plant, which was used traditionally in Chittoor and Khammam districts of Andhra Pradesh, by carbon tetrachloride (CCl_4) induced hepatotoxicity animal model using albino rats and standard drug silymarin.

Methods: The levels of serum aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), alkaline phosphatase (ALP) and total bilirubin (T. BILI.) were determined to assay hepatotoxicity. CCl_4 administration caused severe hepatic damage in rats as evidenced by elevated serum AST, ALT, ALP and T. BILI. levels. The *C. epithymum* and silymarin administration prevented the toxic effect of CCl_4 on the above serum parameters in both preventive and curative models.

Results and Conclusion: The present study concludes that, methanolic extract of *C. epithymum* has significant hepatoprotective activity against CCl_4 induced hepatotoxicity by suppressing CCl^4 induced cellular oxidative stress, which support folkloric utilization and further confirmed by the histological investigation. The observed activity may be associated with its high bioactive compounds including flavonoids, alkaloids, triterpenoids, glycosides, steroids and carbohydrates.

Keywords: Total phenolic content, Total flavonoid content, Total alkaloid content, Hepatoprotective activity, Histopathology.

INTRODUCTION

Since the introduction of the herbal medicines, many people were impelled to consider the importance of many herbs for treating several forms of disorders. It is no wonder, during the past decade there has been an exponential rise in the application of herbal remedies and such notable increase even continues in these days. However, several herbal products lining in those shelves are not really standardized in terms of its effectiveness and safety [1].

Liver has a pivotal role in the maintenance of normal physiological process through its multiple and diverse functions, such as metabolism, secretion, storage and detoxification of variety of drugs. In the absence of reliable liver protective drugs in modern medicine, in India, a number of medical plants and their formulations are used to cure hepatic disorders in traditional systems of medicine [2]. There are numerous plants and traditional formulations available for the treatment of liver diseases. About 600 commercial herbal formulations with claimed hepatoprotective activity are being sold all over the world [3]. Treating liver diseases with botanical drugs has a long tradition, but evidence for efficacy is sparse. Moreover, synthetic drugs available in the market may cause serious side effects. Keeping this in mind for giving scientific proof, the present work was designed and screened the *C. epithymum*, which was used traditionally for treating liver disorders in Chittoor and Khammam districts of Andhra Pradesh, India [4].

MATERIAL AND METHODS

Materials

The whole plant of *C. epithymum* was collected from Sathupally, Kuppam and surrounding villages of Khammam and Chittoor districts of Andhra Pradesh, India and authenticated by Dr. Madhava Chetty, taxonomist and HOD of Botany, Sri Venkateswara University, Thirupathi, India (Voucher specimen No.SVU-B-13), ascorbic acid (Sigma Aldrich Chemie, Germany), Riboflavin (S.D chemicals, India), silymarin, gallic acid, and catechin (Nature remedies, Bangalore, Karnataka, India). CCl_4 (Poona Chemical Laboratory, Pune, India) and SGOT, SGPT, SALP, BILIRUBIN estimation kits (Span Diagnostics, Surat, India). All other solvents and chemicals used were of analytical grade purchased from local source.

Preparation of extract

Before going to extraction, the collected plant materials i.e., whole plant of *C. epithymum* was subjected to standardization according to the guidelines of WHO for organoleptic, physiochemical, heavy metal, microbiological and pathogen analysis [5]. After collection, the plant materials were shade dried, powdered (40 mesh size) to get a coarse powder and then subjected to Soxhlet extraction continued for 8 cycles (6 hrs) using methanol as a solvent. The extract was filtered and concentrated at reduced temperature on a rotary evaporator. The percentage yield was found to be 27.52 % w/w and then subjected to preliminary qualitative [6-10] and quantitative (for phenolics, flavonoids and alkaloids) phytochemical analysis [Table 1].

Determination of total phenolic content

The total phenolic content was estimated using the modified Folin-Ciocalteu photometric method (11). The appropriate amount of filtered methanol extracts were oxidized with Folin-Ciocalteu's reagents and after 5 minutes was the reaction neutralized with saturated sodium carbonate. The solution was then immediately diluted to the volume of 50 ml with distilled water. The absorbance was measured at 750 nm after 90 minutes of incubation at room temperature against the blank. As the standard was used gallic acid. The total phenolic content is here expressed as g gallic acid equivalents (GAE) per 100 g of dry weight (dw) [Table 1].

Determination of total flavonoid content

The total flavonoid content was measured using a modified colorimetric method [11]. The appropriate amount of extract was added to a test-tube together with distilled water. Then was added 5% $NaNO_2$, after 5 minutes 10% $AlCl_3$ and after another 5 minutes 1 M NaOH followed by the addition of distilled water. The absorbance was measured against the blank at 510 nm after 15 minutes. The standard curve was prepared using different concentration of catechin. The flavonoid content was expressed as g catechin equivalents (CE) per 100 g of dry weight (dw) [Table 1].

Determination of total alkaloid content

The total alkaloid content was determined according to UV-Spectrophotometer method [12]. This method is based on the reaction between alkaloid and bromocresol green. The part of the plant extract was dissolved in 2 N HCl and then filtered. 1 ml of this solution was transferred to separatory funnel and washed with 10 ml chloroform The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. One ml of this solution was transferred to a separating funnel and then 5 ml of bromocresol solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was fractioned with chloroform by vigorous shaking. The fractions were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. All experiments were performed thrice; the results were averaged and reported in the form of mean ± S.E.M. [Table 1].

Acute toxicity study

Acute toxic category method is a method for assessing acute oral toxicity that involves the identification of a dose level that causes mortality. Acute toxicity studies were performed for selected plant methanolic extracts according to the toxic classic method as per guidelines 423 prescribed by OECD [13], 2001 using female albino rats. The selected three extracts showed neither visible sign of toxicity nor mortality. The results clearly indicated non-toxicity of the extracts at a dose of 2000 mg/kg. From this, $1/20^{th}$ $1/10^{th}$, and $1/5^{th}$ and doses were selected for the experimental study. Hence there is no LD_{50} and all the extracts tested are considered safe and nontoxic.

***In vivo* screening for hepatoprotective activity**

Animals used

Wistar albino rats of either sex weighing between 200-250 g were obtained from Mahaveer Enterprises, Hyderabad. The animals were housed under standard environmental conditions (temperature of 22± 1^0 C with an alternating 12 hrs light- dark cycle and relative humidity of 60±5%), one week before the start and also during the experiment as per the rules and regulations of the Institutional Ethical Committee and by animal regulatory body of the government (Regd: No: 516/01/CPCSEA). They were fed with standard pellet laboratory diet supplied by M/s. Rayans biotechnologies Pvt. Ltd., Hyderabad and water *ad libitum*

Experimental procedure

In this screening [14] albino rats of either sex (200-250 g) were used. The animals were fed with standard diet and water ad libitum two weeks before and during the experimental period. The selected plant methanolic extract was tested at 400 mg/kg dose level. The animals were divided in to 6 groups (I-VI), each group consisting of 6 animals. Group I received 5% gum acacia suspension and acts as a normal control and Group II received CCl4 at a dose of 1 ml/kg orally (p.o.) acts as negative control. Groups III-VI were treated with selected drugs (silymarin and plant extract) for 5 days before the commencement of experiment and on day 6th of the experiment, blood samples were collected (6th day) at 0 hr in all groups and CCl4 was administered to all groups except group I (normal control) one hour after the administration of drugs. On 7th day blood samples were collected from all groups by retro orbital puncture, serum was separated by centrifugation and used for the estimation of blood serum parameters (SGOT, SGPT, SALP and T.BILI.) according to the standard procedures. The liver sections also dissected out subjected to histopathology studies [Table 2 and Figure 1].

Histopathological studies

All the animals were anesthetized with ethyl ether and livers were dissected out quickly by cutting on the ventral side. The isolated liver specimen was trimmed to small pieces and preserved in neutral buffered formalin (10% formaldehyde in phosphate buffered saline) solution for 24 hrs. The liver specimen was subjected to dehydration with acetone of strength 70, 80, 100 % respectively, each for one hour. The infiltration and impregnation was done by treatment with paraffin wax twice each time for one hour. Specimens were cut into sections of 3-5 µm thickness using microtome and were stained with haemotoxylin and eosin and later the microscopic slides of the liver were photographed at 40X magnification [15-16] [Figure 2].

Statistical analysis

The mean±SEM values were calculated for each parameter. Percentage reduction in biochemical parameters with the test samples was calculated by considering the difference between the hepatotoxin treated group and the control group as 100% reduction. For the determination of significant inter group difference, each parameter was analyzed separately using one way analysis of variance (ANOVA) followed by Dunnet's test was carried out to assess the hepatoprotective potency of selected plant extract.

RESULTS AND DISCUSSION

Herbal medicines are free from side effects, adverse effects and they are economical and easily available will be beneficial for the mankind over the centuries.

The selected plant methanolic extract at dose levels of 100 mg/kg b.w, 200 mg/kg b.w and 400 mg/kg b.w, were tested by taking silymarin as a standard. The tested doses exhibited significant hepatoprotective activity against CCl_4-induced liver intoxicated rats by reduction in increased serum levels of SGOT, SGPT, SALP and T.BILI. A slight decrease was found after the treatment with 100 mg/kg b.w dose when compared with the CCl_4 group. However administration of doses at 200 mg/kg b.w and 400 mg/kg b.w produced significant decreasing at serum levels of SGOT, SGPT, SALP and T.BILI. [Table 2 and Figure 1].

Histopathological examination of the liver sections of the control group showed normal architecture of the liver with distinct hepatic cells. The liver section of CCl_4 intoxicated group showed complete disarrangement of normal hepatic cells with intense centrilobular necrosis, vacuolization, fatty changes, sinusoidal haemorrhages and dilatation .The liver sections of silymarin treated rats showed a normal hepatic architecture with normal hepatocytes. Whereas the rats treated with test methanolic extract of *C. epithymum* at doses of 100 mg/kg b.w 200 mg/kg b.w and 400 mg/kg b.w showed recovery from CCl_4 induced liver damage as evident from normal hepatocytes and with higher dose of 400mg/kg b.w showed significant attenuation of inflammatory and necrotic changes and cellular architecture of liver was preserved indicating a marked protective activity similar to that observed in silymarin treated rat liver sections and the effect was found to be dose dependant [Figure 2].

CCl_4 is a hepatotoxin commonly used for the production of experimental liver toxicity. The carbon tetrachloride mechanism begins with the trichloromethyl radical by the action of the mixed function of cytochrome P-450 oxygenase system. This free radical, which is initially formed as unreactive, reacts very rapidly with oxygen to yield a highly reactive trichloromethyl peroxy radical. Both these radicals are capable of binding with proteins / lipids or abstracting a hydrogen atom from an unsaturated lipid, thus initiating lipid peroxidation. This process of lipid peroxidation can significantly damage hepatic plasma membranes. The increased levels of SGOT, SGPT, SALP and T.BILI. are conventional indicators of liver injury. The ability of hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effect [17].

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscles and kidneys. Elevated levels are found in myocardial infarction, cardiac surgeries, liver disorders, cirrhosis, acute pancreatitis, acute renal diseases and primary muscle diseases. SGOT catalyses the transamination of L - Aspartate and α - Ketoglutarate to form L-Glutamate and Oxaloacetate. In subsequent reaction, malate dehydrogenase (MDH) reduces oxaloacetate to

malate with simultaneous oxidation of nicotinamide adenine dinucleotide [reduced] (NADH) to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm and is directly proportional to SGOT activity in the sample.

SGPT is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. SGPT catalyses the transamination of L-Alanine and α-Ketoglutarate to form pyruvate and L-Glutamate. In subsequent reaction, lactate dehydrogenase (LDH) reduces pyruvate to lactate with simultaneous oxidation to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH to NAD is measured as a decrease in absorbance at 340nm which is proportional to the SGPT activity in the sample.

Serum ALP measured is of particular interest in the hepatobiliary disease and in bone diseases. At the pH 10.3, Alkaline phosphatase (ALP) catalyses the hydrolysis of colourless p-Nitrophenyl phosphate (pNPP) to yellow coloured p-Nitrophenol and Phosphate. Change in absorbance due to yellow color formation is measured kinetically at 405 nm and is proportional to ALP activity in the sample.

Bilirubin is the main bile pigment which is formed form the breakdown of heme of red blood cells by reticuloendothelial system. Total Bilirubin concentration was increased mildly in chronic haemolytic disease, moderately to several in hepatocellular disease and markedly in cholestasis. Total bilirubin reacts in the presence of caffeine with diazotized sulphanilic acid to form azobilirrubin. The color developed is measured at 546 nm and is proportional to the concentration of bilirubin

Phytochemical studies on the selected plant revealed the presence of flavonoids, alkaloids, triterpenoids, glycosides, steroids and carbohydrates. The presence of above constituents in selected plant extract alone or in combination might be responsible for the observed hepatoprotective activity. Further, this was supported by quantitative estimation of phytoconstituents. The total phenolic, flavonoid and alkaloid contents were found to be 4.12±1.14, 3.76±0.68 and 41.74±0.86 respectively [Table 1].

Table 1: Standardization and qualitative-quantitative analysis of whole pant of *C. epithymum*

S. No.	Parameter	*Cuscuta epithymum*	
1.	**Organoleptic characters**		
	Colour	Pale pinkish red	
	Odour	Characteristic	
	Taste	Characteristic	
	Physical appearance	Free flowing powder	
2.	**Physiochemical characters**		
	Water soluble extractive	61.11%	
	Alcohol soluble extractive	82.67%	
	PH 1% w/v solution	4.28	
	Loss on drying	5.56%	
	Ash content	7.02%	
	Acid insoluble ash	2.13%	
	Moisture content by K.F	2.86%	
	Foreign organic matter	1.92%	
3.	**Heavy metals**		
	Lead	6.04 ppm	
	Arsenic	1 ppm	
	Cadmium	0.3 ppm	
	Mercury	1 ppm	
4.	**Microbiological analysis**		
	Total aerobic count	327 CFU/g	
	Yeast & mould	42 CFU/g	
5.	**Pathogen analysis**		
	E. Coli	Absent	
	Salmonella	Absent	
	Pseudomonas aeruginosa	Absent	
	Staphylococcus aureus		
6.	**Qualitative preliminary phytochemical analysis**		
	Alkaloids	**+**	
	Carbohydrates	**+**	
	Flavonoids	**+**	
	Glycosides	**+**	
	Phytosterols	**+**	
	Proteins & amino acids	**-**	
	Saponins	**-**	
	Tannins	**-**	
	Triterpenoids	**+**	
7.	**Quantitative phytochemical analysis**		
	Phenolic content (g GAE/100 g dw)	Flavonoid content (g CE/100 g dw)	Alkaloid content (mg/100 g plant material)
	4.12±1.14*	3.76±0.68*	41.74±0.86*

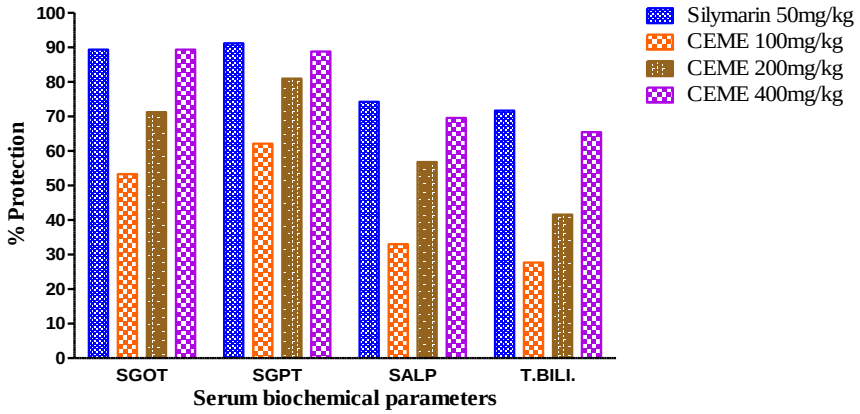
'+'Present, '-' Absent

*Values are means of triplicate determination ± Standard deviation

Table 2: Effect of methanolic extract of whole plant of *Cuscuta epithymum* against CCl_4 - induced hepatotoxicity in albino rats

S. No.	Treatment group	Serum biochemical parameters			
		SGOT (IU/L)	SGPT (IU/L)	SALP (IU/L)	T.BILI. (mg/dl)
1	Control (5% gum acacia 1ml/kg p.o.)	83.83±0.57	73.25±0.38	140.85±0.39	0.66±0.05
2	Hepatotoxin - CCl_4 (1ml/kg p.o.)	486.67±0.44***	397.80±0.55***	812.95±0.69***	4.05±0.07***
3	Standard- Silymarin (50 mg/kg p.o.)	124.79±0.46***	101.80±0.55***	313.90±0.65***	1.62±0.01***
4	Methanolic extract of *Cuscuta epithymum* (100mg/kg p.o.)	270.95±0.73***	196.10±0.46***	590.81±0.45***	3.11±0.02***
5	Methanolic extract of *Cuscuta epithymum* (200mg/kg p.o.)	198.28±0.63***	135.00±0.64***	430.97±0.61***	2.64±0.04***
6	Methanolic extract of *Cuscuta epithymum* (400mg/kg p.o.)	138.79±0.54***	109.95±0.30***	345.25±0.31***	1.83±0.02*

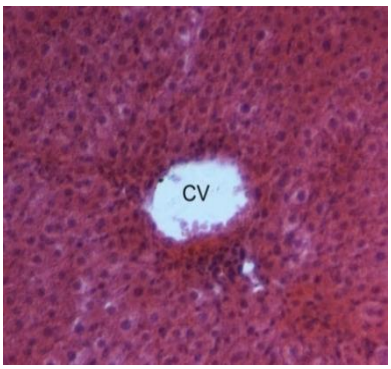
Values are mean ± SEM, n=6, Significance: *P<0.05, **P<0.01, ***P<0.001



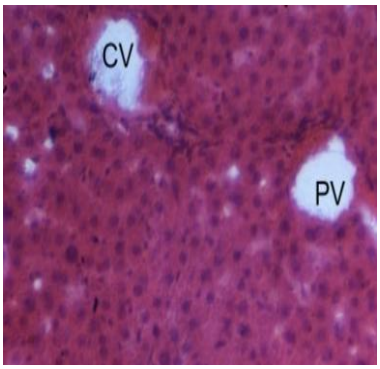
CEME: Methanolic extract of *Cuscuta epithymum*

Fig. 1: Percentage reduction of various serum biochemical parameters due to treatment with methanolic extract of whole plant of *Cuscuta epithymum* against CCl_4 - induced hepatotoxicity in albino rats

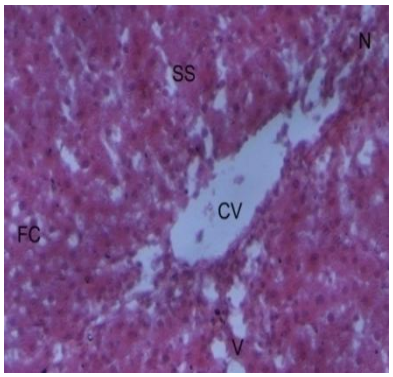
Histopathology: Photomicrographs of liver sections



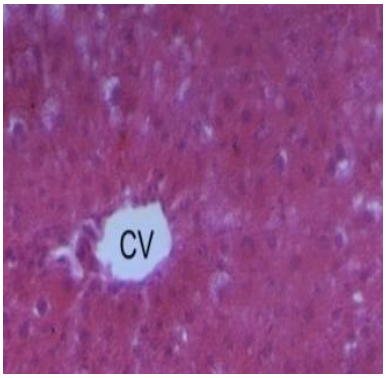
Normal control



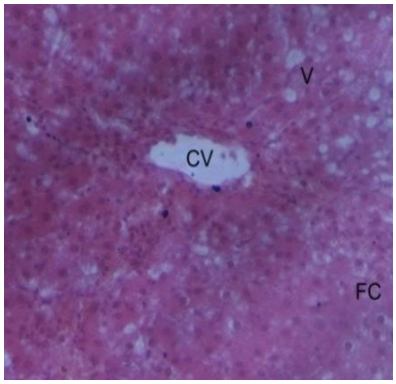
CEME 100 mg/kg b.w

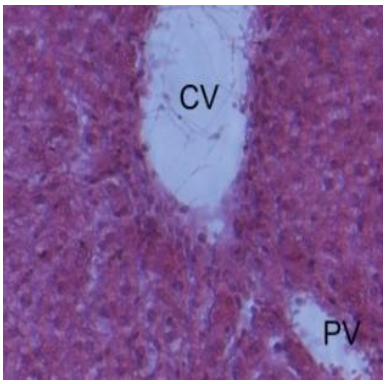


Negative control (CCl_4 treated)



CEME 200 mg/kg b.w





Positive control (silymarin treated) **CEME 400 mg/kg b.w**

CV-Central Vein; PV-Portal Vein; V-Vacuole; N-Necrosis; SS –Sinusoidal Spaces; FC- Fatty Changes

CEME: Methanolic extract of *Cuscuta epithymum*

Fig. 2: Effect of methanolic extract of *Cuscuta epithymum* against CCl_4 - induced hepatotoxicity in albino rats

CONCLUSION

All these scientific observations support the traditional use of *Cuscuta epithymum* (L.) L for treating liver disorders could be due to generation of free radicals. The free radical scavenging and antioxidant properties of phytoconstituents may be the possible mechanism.

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