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Original Article

HEPATOPROTECTIVE ACTIVITY OF ETHANOLIC STEM BARK EXTRACT OF *KNEMA ATTENUATA (*HOO K. F. AND THOMSON) WARB

SUPRIYA RAJA H.*

Department of Pharmacology, College of Pharmaceutical Sciences, Govt Medical College, Trivandrum
Email: sraja968@gmail.com

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ABSTRACT

Objective: To investigate the hepatoprotective activity of ethanolic stem bark extract (ESBE) of *Knema attenuata* against carbon tetrachloride (CCl_4) induced hepatotoxicity in Wistar rats using both *in vivo* and *in vitro* models.

Methods: Animals were treated orally with ESBE (250 mg kg^{-1} and 500 mg kg^{-1}) once daily for 6 d and CCl_4 on the 4th d. On the 7th d, animals were sacrificed and the blood samples were collected to measure the serum levels of biochemical parameters, whereas the liver homogenates were utilized for estimating the antioxidant defense. The hepatoprotective efficacy of the extract was further ensured *in vitro* using human liver hepatocellular carcinoma (HepG2) cell line against CCl_4 induced toxicity. The cell line viability was determined using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Results: ESBE effectively reduced ($p<0.001$) the elevated serum levels of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and Alkaline phosphatase (ALP) when compared to the toxicant control group. ESBE 500 mg kg^{-1}significantly raised the antioxidant defense ($p<0.0001$) by reducing the malondialdehyde (MDA) level and enhancing hepatic reduced glutathione (GSH) level in comparison to the CCl_4 control group. The *in vitro* effect was investigated using CCl_4 exposed HepG2 cells. Pretreatment with ESBE showed a dose-dependent increase in percentage cell viability ranged between 44 to 57% at 12.5-100 µg ml^{-1}concentrations ($p<0.001$, when compared to the control cells).

Conclusion: Present study confirms the hepatoprotective activity of the stem bark extract of *K. attenuata* against CCl_4-induced liver damage.

Keywords: *Knema attenuata*, Hepatoprotective, Carbon tetrachloride, MTT assay, Oxidative stress

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INTRODUCTION

The liver, a vital internal organ of the human body, is involved in detoxification and excretion of a majority of xenobiotics, which may cause potential harm to the body. Since it is involved in the biochemical conversions of various endogenous and exogenously administered or ingested substances, highly reactive free radical species may generate [1, 2]. It has an inbuilt protective mechanism (antioxidants) to neutralize them. However, the imbalance in the liver caused by the difference in the production of free radicals and their elimination by protective mechanisms leads to liver toxicity or damage. During liver damage, free radicals can induce the generation of Pro-inflammatory genes, whose over-expression triggers an intracellular signaling cascade that leads to the production of more free radicals. These pathways could result in enhanced oxidative stress and inflammatory lesions, which promote the pathogenesis of liver diseases [3]. Continuous exposures towards environmental toxins, poor drug habits, and alcohol, can exaggerate the condition, which eventually leads to liver diseases such as hepatitis and jaundice. Due to the adverse effects produced by allopathic medicine, the development of effective therapies using herbal preparations is preferred to treat liver ailments [4].

CCl_4 induced hepatotoxicity; a free radical-mediated cytotoxic model is widely employed for the screening of hepatoprotective activity of compounds. The changes associated with CCl_4 induced liver damage to resemble that of acute viral hepatitis [5].

K. attenuata, which belongs to Myristicaceae family, is an endemic tree species native to south India extending from Konkan southwards of Maharashtra, Goa, Karnataka, Tamil Nadu, and Kerala [6]. The species has been used in folk medicine as an ingredient of ayurvedic 'Ashwagandhadi nei', for the treatment of conditions like spleen disorders, breathing disorders, and impaired taste sensation. The stem bark is also being used in the form of decoction for the treatment of jaundice and chronic fever [7]. Studies on the stem bark of *K. attenuata* reported the presence of a lignan attenuol; closely related to those isolated from the plants of Myristicaceae family, which are known to possess antioxidant, liver protection, anti-inflammatory and anti-cancerous properties [8, 9]. Antioxidant, anti-inflammatory, and antimicrobial properties of ethanolic extract of stem bark of *K. attenuata* have already been reported [10-12]. However, there is no scientific work has been performed so far on the hepatoprotective activity of the plant. Therefore, the present study was designed to evaluate the hepatoprotective properties of the ethanolic stem bark of *K. attenuata* using both *in vivo* and *in vitro* screening techniques.

MATERIALS AND METHODS

Collection of plant material

The stem bark of *K. attenuata* for the proposed study was collected from Kerala Forest Research Institute, Peechi, Thrissur in January 2015. The identification and authentication of species were carried out by Dr. V. B Sreekumar, Scientist (Botany Department), Kerala Forest Research Institute, Peechi, Thrissur. The fresh stem barks were collected, thoroughly washed, spread in trays, and air-dried for three w. The cleaned and air-dried barks were subjected to coarse powdering and sieved (sieve no: 44) to obtain a uniform size for extraction [13].

Preparation of extract

A 50 g of dried stem bark powder of *K. attenuata* was packed in a thimble and loaded in a soxhlet apparatus. After defatting stem bark powder with petroleum ether (60-80 °C), it was subjected to continuous extraction with 300 ml of 95 % ethanol to get an ethanolic fraction. The filtrate was evaporated to vacuum (at 40 °C) to obtain the dried ESBE with a percentage yield of 17.8% w/w. The extract was solubilized using water to feed rats in subsequent studies [14].

Drugs and chemicals

HepG2 cell lines were purchased from the National Centre for Cell Sciences, Pune. Silymarin and Thiobarbituric acid (TBA) were

purchased from Sigma chemicals, USA. Dulbecco's modified eagle's medium and Trypsin-EDTA solution were procured from Himedia laboratories, Mumbai. Commercial kits used for determining biochemical parameters were obtained from Agappe Diagnostics Ltd, Kerala. All chemicals and reagents used were of analytical grade.

Animals

In this study, Wistar rats of either sex (150-250 g), were procured from the animal house (Reg No: 752/02/a/CPCSEA) of Govt. Medical College, Trivandrum. The animals were housed in propylene cages (3 per cage) with dust-free husk as bedding material under standard condition of temperature (25±2 °C) and relative humidity (30-70 %) with a 12:12 light-dark cycle and fed with standard rodent pellet and water except during experimentation. Institutional Animal Ethics Committee, Govt. Medical College, Thiruvananthapuram, approved the experimental protocols on 17/12/2014 (Approval no: 03/13/2014/MCT).

Cell culture

HepG2 hepatic carcinoma cell lines were maintained in Dulbecco's modified eagle's media supplemented with 10% Fetal bovine serum (FBS) and grown to confluency at 37 °C in 5 % CO_2 in a humidified atmosphere in a CO_2 incubator.

Acute toxicity studies

Acute oral toxicity test was carried out as per the Organization for Economic Co-operation and Development (OECD) Guidelines for the testing of Chemicals number 425. Since there was little information about the toxicity of extract, the main test was performed. All the animals were observed 14 d for any sign of toxicity and mortality [15].

Hepatoprotective activity

Wistar rats of either sex (150-250 g) were randomly divided into 5 groups with each group consisting of 6 animals. Animals of group1 served as control and were given only vehicle (water) for 6 d, animals of group 2 served as toxin control and were administrated with CCl_4 (in liquid paraffin, 1 ml kg^{-1} orally) on 4th d and with the vehicle on rest of the d. Animals of group 3 received standard drug of silymarin (100 mg kg^{-1}, orally) for 6 d orally as well as CCl_4 (1 ml kg^{-1}) on 4th d. Animals of group 4 and group 5 received ESBE 250 mg kg^{-1}, ESBE 500 mg $kg^{-1}$for 6 d orally, and CCl_4 (1 ml kg^{-1}) on 4th d.

On 7th d, animals were sacrificed by cervical dislocation. The blood was collected by cardiac puncture of each animal and serum was separated, which was analyzed for assessment of enzyme activity. After collecting blood from each animal, the liver was separated, washed with ice-cold PBS and soaked in filter paper. Liver tissue was minced; added 5 ml ice-cold PBS and was homogenized in tissue homogenizer at 4000 rpm for 5 min and then centrifuged at 5000 rpm for 5 min. The clear supernatant was stored at 4 °C until use [16, 17].

Estimation of serum biochemical parameters

Serum biochemical parameters were estimated according to standard methods. The activity of the enzymes ALT, AST, and ALP was measured using commercial enzymatic biochemical diagnostic kits.

Estimation of tissue biochemical parameters

a) Lipid peroxidation

The extent of lipid peroxidation in the liver was determined in supernatant of liver tissue homogenate following standard methodology [18]. The amount of MDA was measured by reaction with TBA at 532 nm and the MDA level was expressed as nmol mg^{-1} protein.

b) Estimation of GSH levels

GSH level was estimated according to the method described by Ellman GL [19]. The sulfhydryl group of GSH reacts with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB) with a maximum absorbance at 412 nm. The results were expressed as nmol mg^{-1} protein.

***In vitro* hepatoprotective activity against CCl_4 induced HepG2 cell toxicity**

HepG2 hepatic carcinoma cells were trypsinized (500 µl of 0.025% Trypsin in PBS/0.5 mmol EDTA solution) for 2 min and transferred to T flasks in complete aseptic conditions. 200 µl of the cell suspension was plated into a 96-well culture plate and incubated for 24 h. Post incubation, the cells were treated with different fractions of ESBE at a concentration ranging from (12.5–100 µg ml^{-1}) for 24 h, to investigate the possible toxic effect. MTT assay was employed to measure cell viability. Later, for studying the hepatoprotective activity, cells were pretreated with different fractions of ESBE and incubated for 2 h. Then CCl_4 (0.1%) was added to the wells and kept for 24 h. Untreated cells were kept as control. Thereafter, the viability of HepG2 cells was estimated by MTT reduction assay [20].

MTT assay

The cells were washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT-5 mg ml^{-1} dissolved in PBS). It was then incubated at 37 °C for 3 h. MTT was removed by washing with 1x PBS and 200 µl of DMSO was added to the culture. Incubation was done at room temperature for 30 min until the cell got lysed and the color was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 min to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a microplate reader [17, 21].

$$V\% = \left[\frac{(OD_t - OD_b)}{(OD_c - OD_b)}\right] \times 100$$

Where, V% is percentage cell viability; OD_t is the optical density of treated cells; OD_b is optical density of blank cells; and OD_c is optical density of control cells.

Statistical analysis

The obtained data were analyzed by one-way ANOVA followed by Dunnet's t-test and $p<0.05$ was considered statistically significant.

RESULTS

Acute toxicity studies

Neither toxic symptoms nor mortality was observed throughout the observation period even at the highest dose (5000 mg kg^{-1}) indicating low toxicity of extract. There were also no significant changes in food consumption, water uptake, and body weight observed in animals after treatment with the stem bark extract of *K. attenuata*. Additionally, the histopathological analysis of vital organs (data not shown) also didn't show statistically significant changes upon the treatment. The results imply that (supported by results from AOT425 stat program version 1.0) that the oral lethal dose (LD_{50}) of *K. attenuata* is greater than 5000 mg ml^{-1}.

Hepatoprotective effect of ESBE in CCl_4-induced liver damage

Effect of ESBE on liver function parameters

Hepatic injury by CCl_4 was confirmed by measuring the activity of hepatic marker enzymes ALT, AST, and ALP of rats intoxicated by CCl_4. A significant increase in the levels of ALT, AST, and ALP was observed in CCl_4-treated rats from those of the control group (fig. 1). Administration of ESBE (250 mg kg^{-1} and 500 mg kg^{-1}) significantly attenuated the elevation of these parameters in a dose-dependent manner. The hepatoprotective effect shown by ESBE was almost comparable to that of silymarin.

Effect of ESBE on liver oxidative status

As illustrated in table 1, the MDA level was significantly increased in the CCl_4 group as compared to the control group. Silymarin 100 mg kg^{-1} showed significant protective activity against CCl_4-mediated lipid peroxidation. Pretreatment of rats with ESBE at doses of 250 and 500 mg kg^{-1} also caused a significant diminution of elevated MDA level, indicating that ESBE could effectively inhibit the lipid peroxidation induced by CCl_4. Besides, the decrease in the content of GSH caused by CCl_4 was effectively reversed by ESBE in a dose-dependent manner.

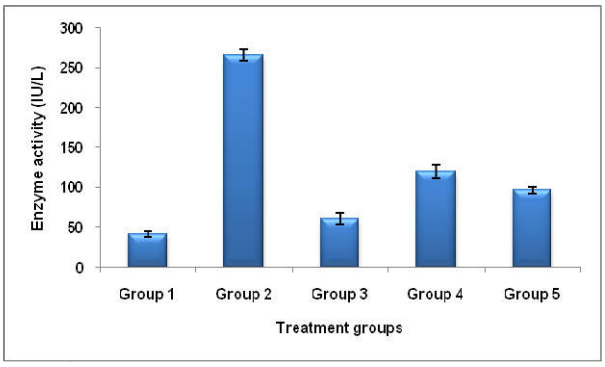

Fig. 1: **Effects of ESBE of *K. attenuata* on serum levels of ALT in CCl_4-treated wister rats. n=6; Values were expressed in mean±standard error of the mean. Silymarin 100 mg kg^{-1}, ESBE 250 mg kg^{-1}, and 500 mg kg^{-1} were compared to toxicant control; one-way ANOVA followed by Dunnett's t-test. p<0.001 as compared to the toxicant control group**

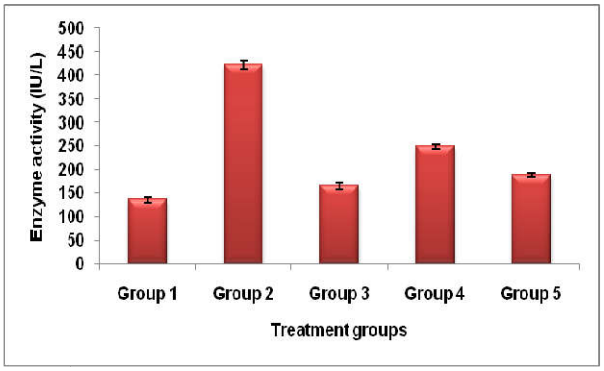

Fig. 2: **Effects of ESBE of *K. attenuata* on serum levels of AST in CCl_4-treated wister rats. n=6; Values were expressed in mean±standard error of the mean. Silymarin 100 mg kg^{-1}, ESBE 250 mg kg^{-1}, and 500 mg kg^{-1} were compared to toxicant control; one-way ANOVA followed by Dunnett's t-test. Silymarin 100 mg kg^{-1}, ESBE 250 mg kg^{-1}; p<0.01 and ESBE 500 mg kg^{-1} p<0.001 as compared to the toxicant control group**

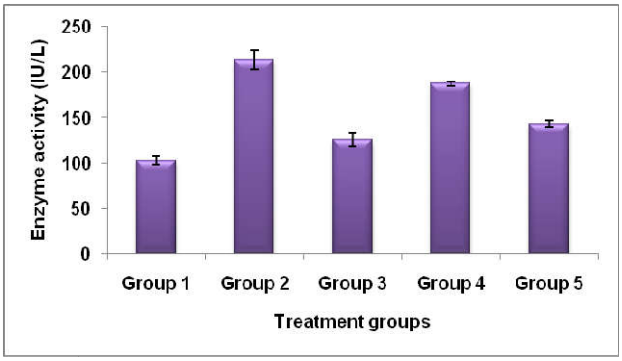

Fig. 3: **Effects of ESBE of *K. attenuata* on serum levels of ALP in CCl_4-treated wister rats. n=6; Values were expressed in mean±standard error of the mean. Silymarin 100 mg kg^{-1}, ESBE 250 mg kg^{-1}, and 500 mg kg^{-1} were compared to toxicant control; one-way ANOVA followed by Dunnett's t-test. p<0.001 as compared to the toxicant control group**

Table 1: Determination of oxidative stress in liver homogenate, n=6; Values were expressed in mean±standard error of the mean. ****p<0.0001,***p<0.001 compared to CCl_4 control group

Group	GSH (nmol mg^{-1} liver)	MDA(nmol mg^{-1} liver)
Group 1	147.93±23.71	3.08±0.06
Group 2	74.76±7.15	6.34±0.04
Group 3	138.89±6.07***	3.67±0.11****
Group 4	113.17±2.52****	4.96±0.05****
Group 5	129.61±3.14****	4.12±0.15****

***In vitro* hepatoprotective activity using HepG2 cell lines**

The exposure of HepG2 cells to varying concentrations of ESBE of *K. attenuata* alone for 24 h did not alter the viability (fig. 4). Upon toxicant administration, the CCl_4 exposed cells showed the percentage viability of 22.49 %. These exposed cells, when treated with different concentrations of extract, a dose-dependent increase in percentage viability that ranged between 44.33-57.41% was observed, indicating that pre-treatment with ESBE has considerably prevented cell death in a concentration-dependent manner. The results of cell viability are depicted in table 2.

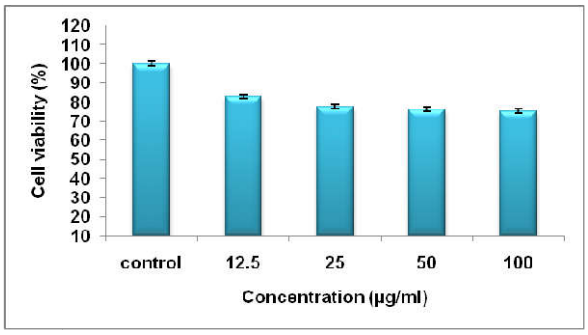

Fig. 4: Effects of ESBE of *K. attenuata* on HepG2 cell viability. n=3; values were expressed in mean±standard error of the mean

Table 2: The percentage cell viability after drug administration, an average of 3 determinations, carried out in triplicates, values were expressed in mean±standard error of the mean, $^{***}p<0.001$ compared to control group

Groups	Treatment	Percentage cell viability (%)
Control	Untreated cells	100
Toxicant control	CCl_4(0.1%)	22.49±0.871
Cells treated with toxicant and Standard	CCl_4 with Silymarin 100 µg ml^{-1}	80.35±2.05
Cells treated with toxicant and ESBE	CCl_4 with ESBE 12.5 µg ml^{-1}	44.33±1.21***
	CCl_4 with ESBE 25 µg ml^{-1}	45.25±2.61***
	CCl_4 with ESBE 50 µg ml^{-1}	52.08±1.77***
	CCl_4 with ESBE 100 µg ml^{-1}	57.41±2.33***

DISCUSSION

Natural products isolated from medicinal plants have gained significance and immense popularity in the modern health care system. Herbal drugs, which offer protection from hepatic disturbances or help in the regeneration of hepatic cells, come under hepatoprotective agents. These drugs contain a variety of chemical constituents like phenols, coumarins, curcuminoids, lignans, essential oils, and terpenoids [22]. The present study demonstrates the effect of ESBE as a possible hepatoprotective agent.

According to the guidelines recommended by OECD, acute oral toxicity testing of crude ESBE was carried out to find the safety profile and the LD_{50} value for the extract based on which dose for further studies can be selected. As per the study, LD_{50} value was obtained as greater than 5000 mg kg^{-1}, indicating that the extract was relatively non-toxic [15]. Two doses 250 mg kg^{-1} and 500 mg kg^{-1} were selected for carrying out the screening of hepatoprotective activity.

CCl_4 is a hepatotoxic compound that causes severe hepatic injury. The compound undergoes metabolism by the action of the enzyme cytochrome p 450 forming unstable and highly reactive free radicals such as tri-chloro-methyl (CCl_3-) and tri-chloro-methyl peroxy radical (CCl_3OO–). Hepatotoxicity induced by CCl_4 involves the action of these free radicals, which alters the permeability of hepatic cell membrane, causing leakage of liver enzymes such as ALT, AST, and ALP into the blood circulation. Therefore, elevated levels of these liver enzymes are important biomarkers that indicate hepatotoxicity or injury to the liver [23, 24]. Pre-treatment with ESBE before the introduction of CCl_4 has shown a promising dose-dependent potential to restrain the elevated level of liver enzymes in the serum.

Free radicals generated by CCl_4 induce substantial oxidative stress and attack polyunsaturated hepatic membrane lipids causing lipid peroxidation. The end products of this oxidation are MDA, 4-hydroxynonenal, and 4-hydroxy-2, 3-alkenals [25]. The results obtained indicated elevated MDA levels in the liver as a response to CCl_4 treatment, implying enhanced oxidative damage to the liver. However, the results also suggested that the treatment of CCl_4 intoxicated rats with ESBE caused a significant reduction of MDA back to their control levels in the serum.

Glutathione is an antioxidant capable of scavenging free radicals and thereby inhibiting the chain reaction of lipid peroxidation. Glutathione can exist in both reduced (GSH) and oxidized (GSSG) states. The sulfhydryl residues of GSH molecule get oxidized to GSSG, which in turn, can be converted back to GSH using glutathione reductase (GR). The redox ratio of GSH/GSSG within cells is a measure of cellular oxidative stress. Therefore, the depletion in the hepatic GSH level indicates the extent of oxidative injury to the liver [26]. The intoxication with CCl_4 causes a reduction in the synthesis and functioning of GSH; however, the administration of ESBE reverses this, suggesting a protective effect. Hence, ESBE exhibits antioxidant activity by decreasing the extent of lipid peroxidation and maintaining the glutathione level and thereby relieving oxidative stress.

HepG2 cell line is considered as an effective *in vitro* model to study the hepatoprotective potential of phyto compounds as they possess functional similarity to normal human hepatocytes [27]. The investigation carried out on HepG2 cells against CCl_4-induced damage also confirmed the hepatoprotective nature of ESBE indicating a good correlation between *in vivo* and *in vitro* studies.

Phytoconstituents like the phenolic compounds and flavonoids are reported to be present in ESBE, which showed promising antioxidant activity [10]. The mechanism of hepatoprotection exhibited by the ethanolic extract of stem bark of *K. attenuata* can be attributed to their antioxidant potential. ESBE can reduce free radicals that may lessen the oxidative damage to the hepatocytes and improve the activities of the liver antioxidant enzymes, thus protecting the liver from CCl_4 induced damage. Its hepatoprotective action can be attributed to the presence of flavonoids and polyphenolic compounds identified in the preliminary phytochemical analysis of extract.

CONCLUSION

The stem bark of *K. attenuata* has been used in folk medicine for the treatment of liver disorders. The present study on ESBE of *K. attenuata* shows promising hepatoprotective activity in both *in vitro* and *in vivo* studies and thereby provides proper scientific evidence for its traditional use. The study also reaffirms its antioxidant activity, which is due to the presence of flavonoids and phenolic compounds. Therefore, it can also be useful in several diseases that cause oxidative stress. Further work is in progress to isolate and characterize the active constituents responsible for the various pharmacological activities of stem bark of *K. attenuata*.

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Nil

AUTHORS CONTRIBUTIONS

Conception and design, acquisition, analysis and interpretation of data and writing of the manuscript was carried out by the corresponding author.

CONFLICTS OF INTERESTS

The author declares no conflicts of interest in preparing this article.

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