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Original Article

Hepatoprotective and Anti-inflammatory Potential of Crude methanolic extract of *Euphorbia Pilulifera via* NF-KB/Nrf2/Akt/TGF-β1 pathway

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ABSTRACT

Liver fibrosis is a natural process that initiates after liver injury to repair the damaged tissue. The liver has a significant capacity for self-repair of the damaged tissue. To a great extent, the miscellaneous interactions of immune cell subtypes manage these repair procedures like fibrosis and wound healing. **Objectives:** To assess the hepatoprotective and anti-inflammatory potential of *Euphorbia pilulifera* through modulating the NF-KB/Nrf2/AKT/TGF-β1 pathway. **Methods:** *Euphorbia pilulifera* methanolic extract was primarily assessed for its cytotoxic potential against HepG2 cells. Methanolic extract of *E. pilulifera* showed 90% hepatoprotective activity against CCl_4-induced toxicity in HepG2 cells. The methanolic extract downregulated the NF-κB gene by 90%; the AKT, gene by 14%, and the TGF-β1 gene expression by 69% at the concentration of 50µg/ml at the mRNA level. On the other hand, methanolic extract of *E. pilulifera* increased the expression of the Nrf2 gene by 44% at 50µg/ml concentration. Furthermore, the antioxidant activity of leaves extract through DPPH radical scavenging assay was estimated. **Results:** Methanolic extract showed 50.83± 0.39% inhibition against DPPH radicals at 200µg/ml concentration. In addition, the anti-inflammatory potential of the crude extract was assessed. The methanolic extract at 25µg/ml concentration revealed the maximum percentage of hemolysis protection. The methanolic extract was found highly effective against inflammation and hepatotoxicity. **Conclusions:** *Euphorbia pilulifera* leaf extract has the potential to ameliorate hepatic injury and inflammation in HepG2 cells.

INTRODUCTION

Liver fibrosis, chronic fibrosis and hepatic cirrhosis lead to liver cancer[1,2], the third leading cause of cancer-related deaths in the world [3]. Thus, herbal remedies can be substitutional approach against hepatic injury. Medicinal plants serve as a splendid reservoir for drug candidates. From the past few years, herbal medicines, have obtained more scientific awareness and demand against diverse diseases, including neurological malignancies, chronic hepatic diseases, inflammations and many others[4]. The therapeutic relevance of the plants is attributable to their extensive array of phytochemicals called secondary metabolites [5]. These plant-based chemicals provide clinically innocuous, economical and available therapeutic alternative [6]. In this regard, the rich indigenous flora of Pakistan contains a number of plants and their constituents that are mostly effective against malignant diseases such as chronic liver diseases [7]. For instance, *Podophyllum hexandrum* (kakora), *Echinops echinatus* (ont katara), *Glycyrrhiza glabra, Phyllanthus niruri, Picrorrhiza kurroa* and *Silybum marianum* etc. are some of the known local plants having anti-hepatotoxic actions [8]. However, *Euphorbia pilulifera* (snake weed) remains poorly investigated for liver disorders. Thus, therapeutic potential of *E. pilulifera* leaves against liver injury was investigated in

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this study, with the aim to propose new herbal hepatic drugs in near future. To propose a medicinal drug candidate, knowledge of its underlying mechanistic routes is imperative. Among important inflammatory pathways, NF-κB cascade, is a prospective key determinant of hepatic fibrosis which can be targeted to recover injury [9]. NF-κB cascade's role towards apoptosis and oxidative stress stimulation is momentous in all kinds of cellular inflammatory reactions [10] in which Nrf2 activation is also involved. Nrf2 activation reduces the oxidative stress and inflammation by the repression of the NF-KB pathway [11] NF-κB pathway is also repressed by AKT signaling pathway during hepatic fibrosis [12]. Moreover, TGF-β1 actuates Kupffer cells and aggravates hepatic fibrosis [13]. Therefore, the current study is anticipated to halt the NF-κB/Nrf2/AKT/TGF-β1 pathway against liver injury via prospective inhibitory medicinal plant. In the present study, the hepatoprotective and anti-inflammatory potential of *Euphorbia pilulifera* was deliberated to explore. The hepatoprotective activity of the methanolic extract against CCl_4-induced toxicity on HepG2 cells was evaluated via *in vitro* analysis. Furthermore, the antioxidant and anti-inflammatory potential of the methanolic extract was estimated. Hence, it was found that *E. pilulifera* has potential to reverse the liver injury through NF-κB/Nrf2/AKT/TGF-β1 pathway. This study can help to attain the health targets in the near future for feasible development to combat liver-related complications.

METHODS

***E. pilulifera* crude extract preparation:** The leaves of *Euphorbia pilulifera* were collected from CEMB (Centre of Excellence in Molecular Biology) and identified by Dr. Zahoor of the Botany Department, University of the Punjab Lahore, Pakistan. The leaves of the plant were washed with tap water, air-dried, and then grounded into a very fine powder in an electric grinder. Approximately 100g of powder was weighed and soaked in 500ml of 70% methanol and left overnight at room temperature. The next day, the mixture was filtered through a Whatman No.1 filter paper. The remnant was again soaked overnight in 50% methanol and repeated the procedure two more times. The filtrate obtained after three times extraction was combined and dried. The dried crude extract was weighed, and stored for further use.

MTT cytotoxicity assay on HepG2 cells: MTT assay was used to evaluate the cytotoxic potential of *E. pilulifera* methanolic extract on HepG2 cells. For this purpose, HepG2 cells at the density of 2×10^4 cells/well were plated in a 96-well plate and incubated in 5% CO_2 atmosphere at 37°C. The next day cells were analyzed and upon 70–80% confluency, treated with several concentrations of extract starting from 200µg/ml-3.12µg/ml in triplicate in corresponding wells. Plain cells without treatment were considered as control. After 24 hours of treatment, media from the plate was removed, and 20µl of freshly prepared MTT solution was added. After 3 hours, purple-colored formazon crystals were developed, which were dissolved by adding 100µl of Dimethyl sulfoxide for 20 minutes. The developed color was quantified at 570nm and 650nm using ELISA (enzyme-linked immunosorbent assay) spectrophotometer. The % of cell viability was determined using the formula below:

$$\%\ Cell\ viability = \frac{OD\ test\ (570\text{-}650)}{OD\ Control\ (570mm\text{-}650mm)} \times 100$$

Estimation of hepatoprotective activity of *Euphorbia pilulifera*: HepG2 cells were cultured in DMEM media in 96-well plate. The next day, the media was aspirated, and cells were washed with IX PBS. After washing, the cells were treated with different concentrations of *E. pilulifera* methanolic extract, serially diluted from 200µg/ml to 3.12µg/ml in DMEM. After 24 hours, media was discarded and cells were incubated in 0.1% CCl_4 serum-free media for 2 hours. The next day, MTT assay was performed to analyze the hepatoprotective activity of the methanolic extract.

Expression analysis of genes by Real-Time PCR: The total RNA was isolated using TRIzol reagent and cDNA was synthesized using cDNA synthesis kit in accordance with the manufacturer's directions. To evaluate the gene markers (Table 1) and pathway in samples, a real-time polymerase chain reaction (PCR) was conducted using SYBR Green master mix. Moreover, by using the RT-PCR comparative CT method (ΔΔCT), fold change was analyzed.

Gene	Forward primer	Reverse primer	Product size
AKT	GGACCTCAAGCTGGAGAACC	CGACCGCACATCATCTCGTA	199bp
Nrf2	TTAGTCAGCGACAGAAGGAC	TCCACTGGTGTCTGTCTGGAT	179bp
GAPDH	CGGATTTGGTCGTATTGG	AGATGGTGATGGGATTTC	473bp
TGF-β1	ACTGCGCCCTTCTCCCTG	CTTCACCAGCTCCATGTCGATAG	188bp
NF-KB	GTTTGTCCAGCTTCGGAGGA	GACCTGTACTTCCAGTGCCC	153bp

Table 1: Gene's primer sequences and product size used in this study

DPPH radical scavenging activity: To evaluate the anti-oxidative properties of the methanolic extract, DPPH free radical scavenging activity assay was conducted following the reported protocol. Briefly, different concentrations (3.125µg/ml to 200µg/ml) of methanolic extract and standard (Gallic acid) were added, and then 0.1% DPPH solution was added to it. The mixture was incubated in the dark for 30 minutes. The absorbance was read at 517 nm. The % inhibition of DPPH was determined from the equation below:

$$\%\ Hemolysis = \frac{Absorbance\ of\ test\ sample}{Absorbance\ of\ control} \times 100$$

Membrane stabilization percentage was also determined

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$$\% \ Protection = \left(100 - \left[\frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \right] \right) \times 100$$

Statistical analysis: The scientific data was presented in the form of means and standard error and calculated via Microsoft Excel. Data was evaluated by GraphPad Prism software (version 8). Statistical significance was analyzed by *two-way ANOVA*.

RESULTS

Dose-dependent non-toxic potential of *Euphorbia pilulifera* methanolic extract: MTT assay of the crude methanolic extract showed more than 90% cell viability up to 200µg/ml concentration (Figure 1). So, the methanolic extract was considered as non-toxic as their CC_{50} was more than 200µg/ml.

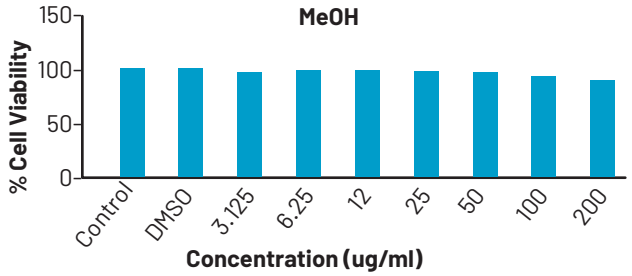

Figure 1: The dose-dependent non-toxic potential of E. Pilulifera methanolic extract in HepG2 cells

Data are expressed as a percentage of control. Results are presented as means ± SEM from 3 experiments conducted in triplicates.

Cytoprotective effect of *E. pilulifera* crude extract against CCl_4 & H_2O_2-induced toxicity: MTT cytotoxic assay of the methanolic crude extract showed cell viability up to 90.04± 0.90% at 200µg/ml against CCl_4 and 86.54± 1.24% at 200µg/ml against H_2O_2 toxicity (Figure 2). So, the *E. pilulifera* methanolic extract is considered as hepatoprotective.

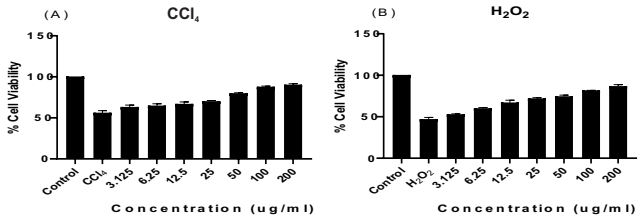

Figure 2: The dose-dependent cytoprotective activity of E. pilulifera methanolic extract in HepG2 cells

(A) Cytoprotective activity against CCl4. **(B)** Cytoprotective activity H2O2-induced toxicity. Results are presented as means± SEM from 3 experiments conducted in triplicates.

DPPH radical scavenging activity: The methanolic extract of E. pilulifera showed a dose-dependent antioxidant activity against DPPH free radicals (Figure 3). Methanolic extract showed a significant DPPH-radical scavenging effect with an IC50 of 165.70 µg/ml.

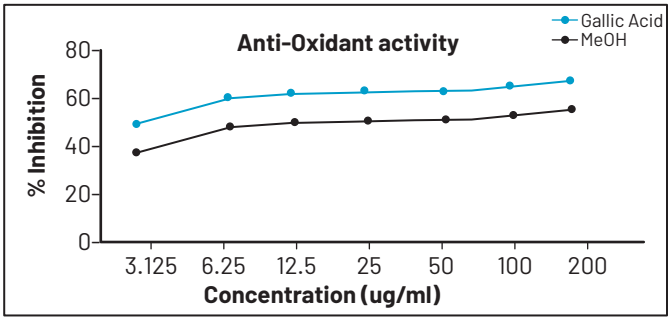

Figure 3: DPPH radical scavenging activity of the methanolic extract and the standard compound gallic acid

The values are means± SEM of three independent experiments performed in triplicates.

Anti-inflammatory activity of Euphorbia pilulifera methanolic extract: E. pilulifera methanolic extract at 25µg/ml concentration revealed the maximum percentage of hemolysis protection (Figure 4A). In the heat-induced hemolysis method, the E. pilulifera methanolic extract at 25µg/ml concentration indicated the lowest hemolysis (Figure 4B).

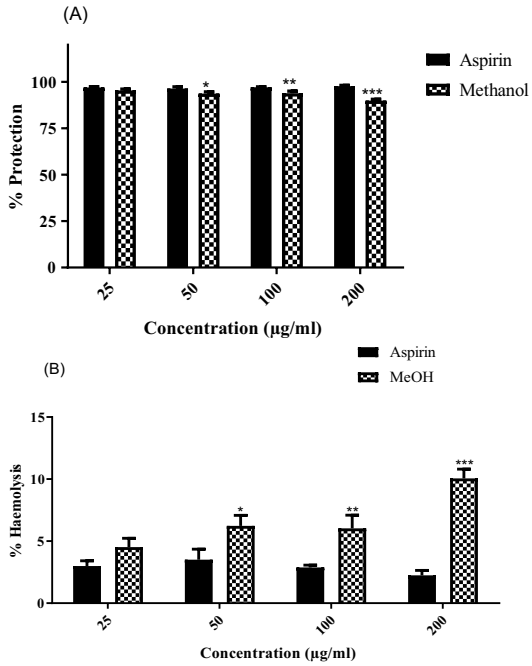

Figure 4: Anti-inflammatory activity of Euphorbia pilulifera methanolic extract

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(A) The bar plot represents the percentage protection of human red blood cells by methanolic extract of Euphorbia pilulifera as compared to aspirin (standard drug) as well as their corresponding percentage error. **(B)** The bar plot represents %age inhibition of hemolysis of human red blood cells by methanolic extract of Euphorbia pilulifera as compared to aspirin as well as their corresponding percentage error. *p<0.01, ** p<0.001, ***p<0.0001 vs. control(Aspirin).

Euphorbia pilulifera methanolic extract ameliorates CCl4-induced injury through NF-κB/Nrf2/AKT/TGF-β1 pathway: E. pilulifera methanolic extract alleviated CCl4-induced damage in HepG2 cells via regulation of NF-κB/Nrf2/AKT/TGF-β1 pathway. The results showed that E. pilulifera methanolic extract downregulated NF-κB gene by 90% and TGF-β1 gene expression by 69% at the concentration of 50µg/ml and AKT gene by 30% at 100µg/ml concentration at mRNA level. While E. pilulifera methanolic extract was found to increase Nrf2 expression by 44% at 50µg/ml concentration as compared to control cells(Figure 5).

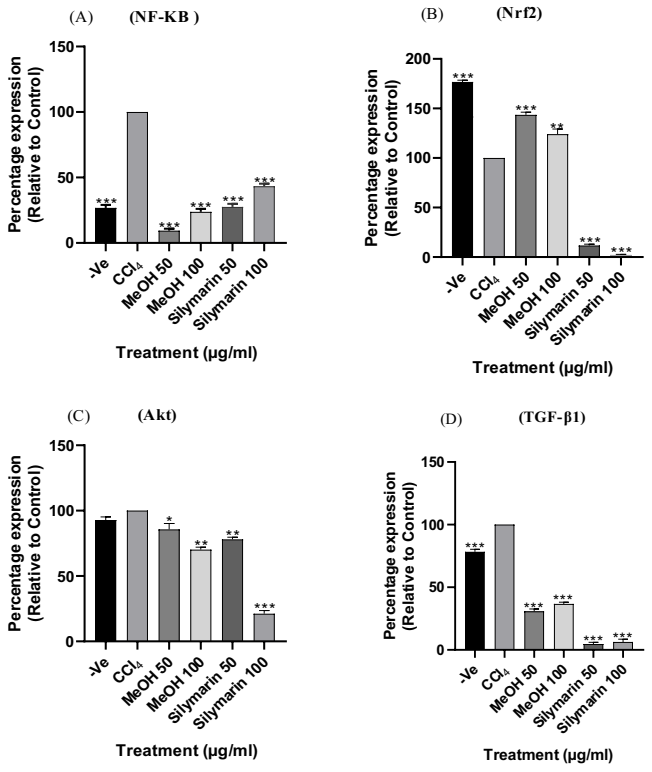

Figure 5: Effect of E. pilulifera methanolic extract on NF-κB/Nrf2/AKT/TGF-β1 expression

(A) Downregulation of NF-κB **(B)** Upregulation of Nrf2 gene. (C) AKT gene mRNA expression. (D) Modulation of TGF-β1 mRNA expression. The results are denoted as means ± SEM acquired from three independent experiments and are demonstrated as the relative percentage of the control. -Ve: Negative cells without any treatment, CCl4:CCl4 treated cells (control). *p <0.01, **p < 0.001, ***p<0.0001 vs. control.

DISCUSSION

Chronic liver diseases are a severe healthcare burden globally due to increase mortality and morbidity rate. Due to the prevalence of hepatic ailments globally and the limitations of existing remedies, the progress of safe, innovative, and inexpensive drugs with enhanced efficacy is essential. Medicinal plants are an appropriate source of dormant pharmacological agents that need to be investigated . Therefore, the current study focuses on identifying plant-based natural compounds for the treatment of liver-related diseases, aiming to find out drugs averting the development of liver diseases. For this purpose, Euphorbia pilulifera was selected because of its well-known therapeutic potential against different diseases. Euphorbia pilulifera is a weed belonging to the genus Euphorbiaceae reported to have many biological activities such as anti-inflammatory, analgesic, wound healing, anti-asthmatic, anti-diabetic, etc. Many plants of the genus Euphorbiaceae are reported to possess protective activity against chronic liver injuries . The methanolic extract with different concentrations (3.125µg/ml -200µg/ml) of E. pilulifera samples was considered non-toxic in HepG2 cells even at higher doses with more than 90% cell viability, so, they were further studied for their potential therapeutic activity against liver injury. A previous study revealed that Euphorbia pilulifera extracts were non-toxic to the Vero cell line at a higher concentration of 100µg/ml and its IC50 was greater than 100µg/ml . Further, the in vitro hepatoprotective activity of the methanolic extract against the CCl4 and H2O2-induced cytotoxicity was investigated. The extract of E. pilulifera displayed significant cytoprotective potential by averting the HepG2 cells from cell death caused by CCl4. A previous study on the in vitro hepatoprotective effect of crude methanolic extract and sub-fractions of Inula crithmoides ethyl acetate fraction showed the highest hepatoprotective effect against CCl4-induced toxicity . Oxidative stress, which results from the production of free radicals in the body, is one of the major mechanisms underlying the progression of several pathological conditions . Medicinal plants and their constituents can alleviate these free radicals. DPPH radical scavenging assay showed that E. pilulifera methanolic extract showed significant DPPH-radical scavenging activity with IC50

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15.39± 0.94µg/ml. Likewise, the antioxidant potential of Albizia odoratissima was evaluated through DPPH . Inflammation is reflected as an advantageous process under normal circumstances. E. Pilulifera methanolic extract also exhibit anti-inflammatory potential. E. Pilulifera methanolic extract at 25µg/ml concentration revealed the maximum percentage protection from hemolysis. Rehman et al., likely described the anti-inflammatory potential of Beta vulgaris L. extracts by executing a dose-dependent HRBC membrane stabilization assay. Further, the downstream pathway involved in the hepatoprotective activity of the E. pilulifera was investigated. Herein, NF-κB pathway activation contributes to oxidative stress and is considered in almost all inflammatory reactions in the cell. Deregulation of the NF-κB pathway can cause a reduction in oxidative stress, therefore making it an effective therapeutic target . Some other studies specified that Nrf2 activation diminished oxidative stress and inflammation via repression of the NF-κB pathway . In the current study, the results indicated that E. pilulifera methanolic extract downregulated the NF-KB gene by 90% at 50 µg/ml concentration. Furthermore, the Nrf2 gene expression was increased by 44% at 50 µg/ml concentration compared to control cells (CCl4-treated cells). The results demonstrated that E. pilulifera methanolic extract could avert liver fibrosis through modulation of NF-κB and Nrf2 expression. Likewise, TGF-β is an essential cytokine that plays an important role in liver physiology and disease progression . E. Pilulifera methanol extract downregulated AKT and TGF-β1 genes by 14 % and 69 % respectively at 50µg/ml concentration as compared to control cells (CCl4-treated cells). AKT signaling pathway also has a vital role in tumor oncogenesis, as it regulates cell propagation and apoptosis.

CONCLUSION

The results demonstrated that E. pilulifera methanolic extract can treat liver fibrosis by targeting TGF- β1 and AKT genes. In conclusion, E. Pilulifera methanolic extract is found highly effective in reversing the effect of CCl4 damage. The methanolic extract of E. pilulifera was also found to modulate NF-KB/Nrf2/AKT/TGF-β pathway to reverse the liver damage. In the future, the E. pilulifera leaf extract can be explored for active constituent which may prove helpful in obtaining cost-effective and potent drugs against liver diseases.

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