

Propugnating Effect of Bark of *Rhizophora mucronata* Against Different Toxicants Viz Carbon Tetrachloride, Ethanol and Paracetamol on HepG₂ Cell Lines

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Key Words

cytotoxicity, ethanol: water (3:1) extract, toxicants, viable cells

Abstract

Objective: The aim of the study was to evaluate the hepatoprotective activity of the bark extract (Ethanol: Water) in the ratio of (3:1) of *Rhizophora mucronata* (BERM) by intoxicating the HepG₂ cell lines with different toxicants viz, CCl₄, Ethanol and Paracetamol with different concentrations of the extract were used. The HepG₂ cell lines were subjected to MTT Assay for studying the cytotoxicity.

Methods: HepG₂ cells were plated using 96 well plate in 10% bovine serum, exposed to different toxicants viz, 2% CCl₄, 60% Ethanol and 14 mM Paracetamol respectively. The various test concentrations (18.85, 37.5, 75, 150 and 300 µg/ml) of bark extract of *Rhizophora mucronata* was added and incubated for 24 hours. Medium was removed after incubation period and 0.5 mg/ml MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added and again incubated for 4 hours at 37°C. Then MTT was removed the crystals was dissolved in DMSO and absorbance was measured at 570 nm.

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Results: The result showed that dose dependent increase in percentage of viability at the doses of 18.85, 37.5, 75, 150, 300 µg/ml. The results for the CCl₄ intoxicated, at 300 µg/ml of the concentration of the extract, the % of viable cells was found out to be 99.6%, for Ethanol intoxicated, 97.67%, and Paracetamol induced, 75.37%, IC₅₀ was 21.53 µg/ml, 12.61 µg/ml and 21.42 µg/ml respectively.

Conclusion: Thus, we conclude that, the extract possesses defensive effect against different toxicants and can be used as an alternate drug for hepatotoxicity.

1. Introduction

The liver is considered to be the vital and of paramount importance which is involved in the detoxification of viral infections, chronic alcoholism, drugs and xenobiotics [1]. The damaged liver gives a clear indication that the liver has inherited hepatic injury or damaged parenchyma cells which leads to the imbalance in various metabolic functions [2]. There are various agents responsible for the liver damage and hepatic injuries which includes; chemicals, prolonged drug therapy, paracetamol overdose, viruses, alcoholism, different toxicants, viz, CCl₄ and Ethanol and environmental pollutants [3, 4]

Carbon tetrachloride (CCl₄) is known as toxicant capable of inducing liver damage. It is well known for damaging vital organs of our body viz, kidneys, lungs,

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adrenals, CNS in humans and experimental animals. The possible mechanism of its toxicity has been identified and reviewed to eliminate its toxic capacity and to revive the degenerated cells [5].

Paracetamol overdose may result in hepatic encephalopathy, cardiovascular instability, kidney or renal failure, profound coagulopathy, disturbance in CNS and brain oedema (Prescott, 1996). Its overdose is also attributed to cause side effects viz, vomiting, constipation, insomnia and depression since 1950's [6]. There are other factors may bring into consideration which contribute to an increased risk of hepatic damage in regards to paracetamol administration i.e malnutrition, pre-existing liver disorder, alcohol abuse and reactions with concordant drugs.

In alcoholic fatty liver there are reports stating that there is an increase in lipid accumulation and oxidative stress has been noted [7]. Chronic and excessive ethanol consumption leads to proliferation of cells, fibrosis, cirrhosis and cancer of the liver [8]. Serious damage to hepatocytes occurs as a result of lipid accumulation and oxidative stress due to excess alcohol and chronic exposure. The first stage of alcoholic liver disease (ALD) is fatty liver, followed by oxidative stress then necrosis, fibrosis, cirrhosis and ultimately Hepatic liver failure (HLF).

Thus in this study, the bark extract of *Rhizophora mucronata* (BERM) have been treated with three major toxicants viz, CCl₄, Paracetamol and Ethanol to study their effects and retrieval of regenerative cells on HepG₂ cell lines. This study is an attempt to review the different and potent toxicants and their effects on HepG₂ cell lines.

2. Materials Methods

2.1. Plant material collection and its extraction

The bark of *R. mucronata* were collected from Pichavar-am Mangrove forest, (latitude: 11° 23' to 11° 30' N and longitude: 79° 45' to 79° 50' E) is located between Coleroon and Vellar estuary in the state of Tamil Nadu, identified in the herbarium of C.A.S. in Marine Biology, Annamalai University, Parangipetai, India and authenticated by Prof Jayaraman, Botanist and Director, PARC, West Tambaram, Chennai, Tamil Nadu, India. A voucher specimen was submitted in the department for future reference. The fresh barks were collected from the healthy stem of the plants. These barks were then packed in Poly ethylene plastic and eventually brought to laboratory. The collected bark samples were separated from any undesirable compounds or plant parts. The samples were shade dried for about 15 days to avoid any possible photochemical degradation of bioactive compounds present in them. Then they were coarsely powdered and stored in air tight bottles for further work.

Hot extraction by Soxhlet's apparatus and cold extraction by Maceration process were carried out using various organic solvents starting with Petroleum ether, Diethyl-ether, acetone, ethyl acetate, Iso-propanol, methanol and ethanol:Water (3:1) with increasing polarity. For hot extraction, each solvent 200 ml was taken along with the 50 g of powdered bark of *Rhizophora mucronata* for a period of 8 hours. For cold extraction, a known weight

(200 g) of the extract was taken in the container and 500 ml of above solvents were added starting from petroleum ether, till ethanol: water (3:1) was added one after the other, each solvent was used for 2 weeks, the containers were sealed with occasional stirring and shaking. After 2 weeks, the mixture was filtered through Whatmann filter paper 1. The filtered thus obtained was evaporated in room temperature to obtain the crude extract, which is stored at 4°C for further use.

2.2. Cytotoxicity screening using MTT Assay

The Liver cancer cell line (HepG₂) were plated separately using 96 well plates with the concentration of 1×10⁴ cells/well in DMEM media with 1X Antibiotic Antimycotic Solution and 10% foetal bovine serum (Hi media, India) in CO₂ incubator at 37°C with 5% CO₂. The cells were then exposed to toxicant medium containing 2% CCl₄, Ethanol 60% and paracetamol 14mM. The cells were then washed with 200 µL of 1X PBS, then the cells were treated with various test concentration (18.85, 37.5, 75, 150 and 300 µg/ml) of compound in serum free media and incubated for 24 h. The medium was aspirated from cells at the end of the treatment period. 0.5mg/mL MTT prepared in 1X PBS was added and incubated at 37°C for 4 h using CO₂ incubator. After incubation period, the medium containing MTT was discarded from the cells and washed using 200 µL of PBS. The formed crystals were dissolved with 100 µL of DMSO and thoroughly mixed. The development of color intensity was evaluated at 570nm. The formazan dye turns to purple blue color. The absorbance was measured at 570 nm using micro plate reader. Silymarin was used as the positive control.

3. Results

3.1. CCl₄ induced toxicity

CCl₄ exposed HepG₂ cells showed the percentage viability of 31.03% at the concentration of 2.0% of CCl₄. These exposed cells when treated with different concentration (18.85, 37.5, 75, 150 and 300 µg/ml) of the bark extract of *Rhizophora mucronata* (BERM) showed a dose dependent increase in the percentage of viable cells. The percentage viability ranged between 49.3 to 99.3% at 18.85 to 300 µg/ml (Table 1). The increase in percentage of viable cells at the concentration of 300 µg/ml for the extract has 99.3% whereas for the Silymarin at the same concentration % of viable cells found out to be 92.61% (Fig. 1) and (Fig. 2) respectively. The IC₅₀ value of the treated HepG₂ cells for the sample was noted to be 21.53 µg/ml. Figure-3 depicts the light microscopic images of the Standard and the sample at different concentrations. Thus, from the results it can be concluded that the extract has the same potential bioactivity as compared to the standard Silymarin.

Tested concentration (µg/ml)	CCl ₄ intoxicated	
	Standard (Silymarin) % of Viable cells	BERM Extract % of Viable cells
18.85	59.81 ± 2.98	49.36 ± 1.13
37.5	77.00 ± 2.84	65.50 ± 1.03
75	83.12 ± 1.47	73.94 ± 4.65
150	86.81 ± 0.8	84.28 ± 4.40
300	92.61 ± 1.42	99.36 ± 4.01

Table 1 Illustrates the cyto toxicity (MTT ASSAY) results carried out on the crude bark extract of *Rhizophora mucronata* and the control intoxicated with CCl₄, showing % of viable cells both in extract and standard Silymarin. The results were conducted in triplicates (n=3), Mean ± Standard deviation.

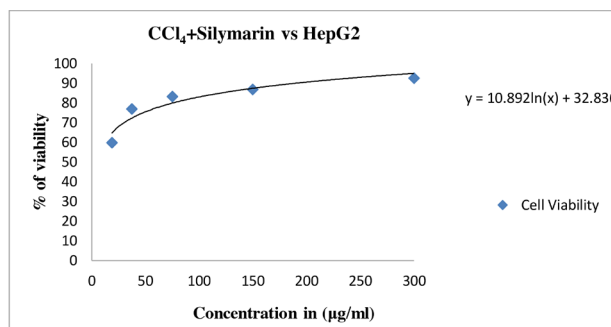


Figure 1 Shows the graph for Standard Silymarin intoxicated with CCl₄, the % percentage of viable cells at different concentrations of standard.

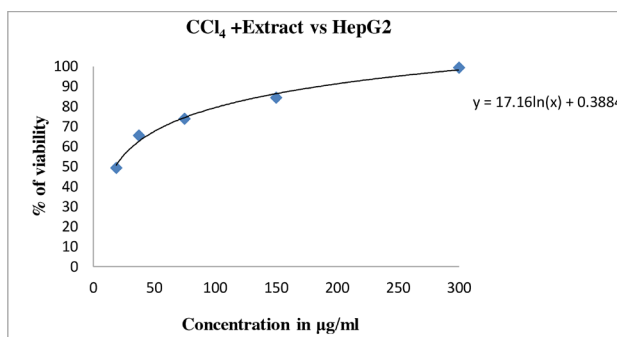


Figure 2 Shows the graph for BERM extract intoxicated with CCl₄, the % percentage of viable cells at different concentrations of extract.

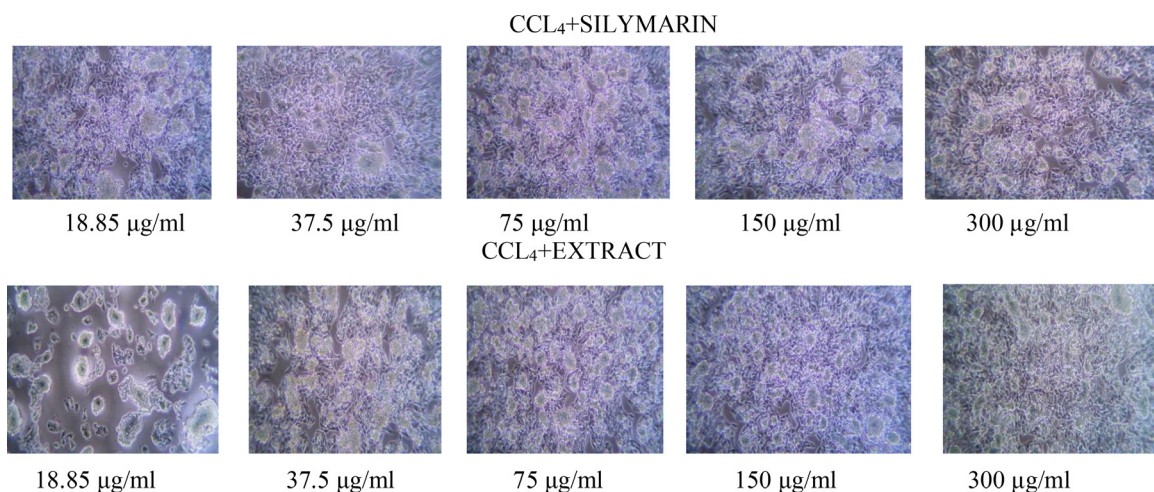


Figure 3 Light microscopic images of Standard Silymarin and the sample BERM at different concentrations when induced with CCl₄ on HepG₂ cell lines

3.2. Ethanol Induced toxicity

Ethanol induced toxicity to HepG₂ Cells showed the percentage viability of 23.17% at the concentration of 60% of Ethanol. These ethanol exposed cells when treated with the different concentrations as stated above of the extract showed a dose dependent increase in the % of viable cells. The percentage viability for the extract was ranged from 52.3% to 97.67% from lower to higher concentration of extract (Table 2). The IC₅₀ values for the extract and the standard silymarin was found to be 12.61 µg/ml and <18.85 µg/ml (Fig. 4) and (Fig. 5) respectively. Fig. 6 depicts the light microscopic images of the Standard Silymarin and the sample at different concentrations. The % of viable cells of the extract was almost similar to as obtained for the standard Silymarin.

Tested concentration (µg/ml)	Ethanol intoxicated	
	Standard (Silymarin) % of Viable cells	BERM Extract % of Viable cells
18.85	74.47 ± 1.90	52.37 ± 4.14
37.5	77.95 ± 0.60	70.99 ± 2.10
75	84.91 ± 1.04	79.95 ± 4.86
150	89.02 ± 1.19	88.71 ± 3.25
300	95.36 ± 3.25	97.67 ± 2.45

Table 2 Illustrates the cyto toxicity (MTT ASSAY) results carried out on the crude bark extract of *Rhizophora mucronata* and control intoxicated with Ethanol, showing % of viable cells both in extract and standard Silymarin. The results were conducted in triplicates (n=3), Mean ± Standard deviation.

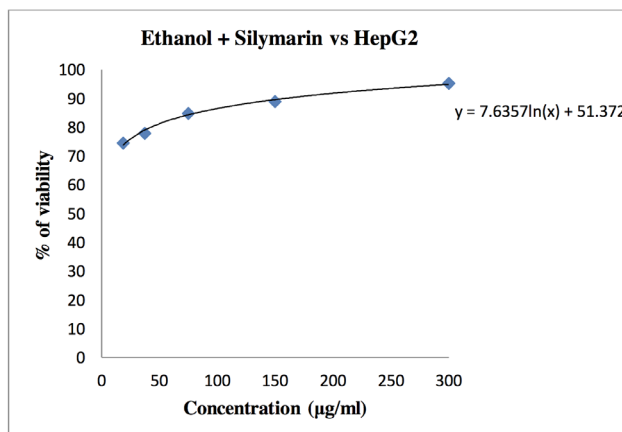


Figure 4 Graph for Standard Silymarin intoxicated with Ethanol, the % percentage of viable cells at different concentrations of standard.

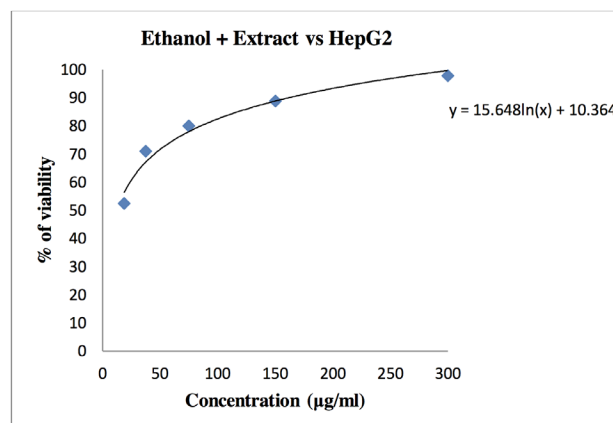


Figure 5 Shows the graph for BERM extract intoxicated with Ethanol, the % percentage of viable cells at different concentrations of extract.

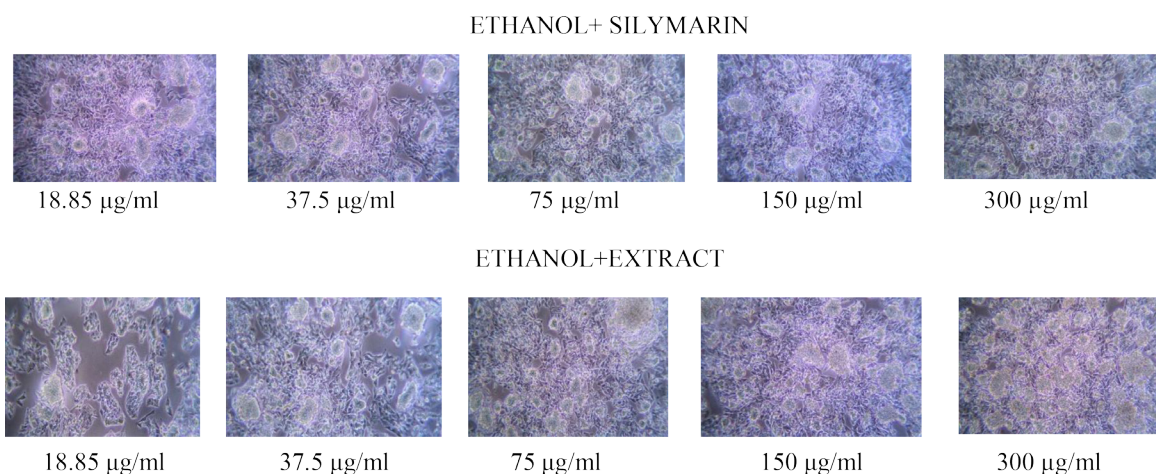


Figure 6 Light microscopic images of Standard Silymarin and the sample BERM at different concentrations when induced with Ethanol on HepG₂ cell lines

3.3. Paracetamol Induced Toxicity

Paracetamol exposed HepG₂ cells when subjected to a series of known concentrations of extract, there is a dose dependent increase in the % of viable cells were found (Table 3). At 300 µg/ml concentration, the percentage of viable cells for the extract was found to be 75.37% and that for standard to be 78.37% (Fig. 7) and (Fig. 8). The IC₅₀ values for both extract and standard Silymarin was found to be 21.42 µg/ml and 9.07 µg/ml respectively. Fig. 9 depicts the light microscopic images of the Standard and the sample at different concentrations. (Table 4) (Fig. 10) shows an overlay graph of the IC₅₀ values of all the three models both for extract and standard Silymarin.

Tested concentration (µg/ml)	Paracetamol intoxicated	
	Standard (Silymarin) % of Viable cells	BERM Extract % of Viable cells
18.85	55.27 ± 2.58	49.47 ± 0.98
37.5	61.39 ± 0.93	59.91 ± 2.24
75	69.51 ± 0.15	64.76 ± 2.39
150	71.31 ± 0.98	69.30 ± 4.25
300	78.37 ± 4.28	75.37 ± 5.81

Table 3 Illustrates the cyto toxicity (MTT ASSAY) results carried out on the crude bark extract of *Rhizophora mucronata* and control intoxicated with paracetamol, showing % of viable cells both in extract and standard Silymarin. The results were conducted in triplicates (n=3), Mean ± Standard deviation.

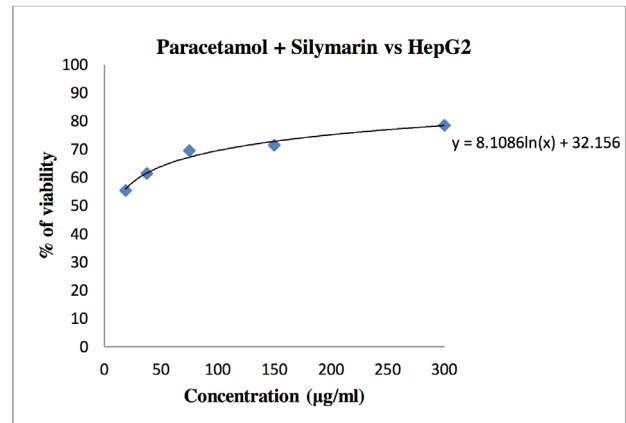


Figure 7 Shows the graph for Standard Silymarin intoxicated with Paracetamol, the % percentage of viable cells at different concentrations of standard.

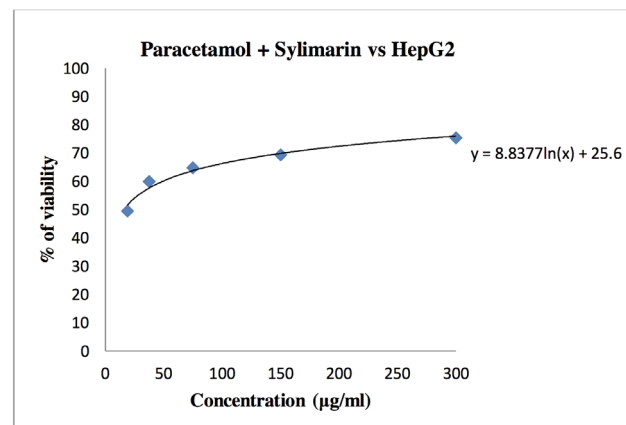


Figure 8 Shows the graph for BERM extract intoxicated with Paracetamol, the % percentage of viable cells at different concentrations of extract.

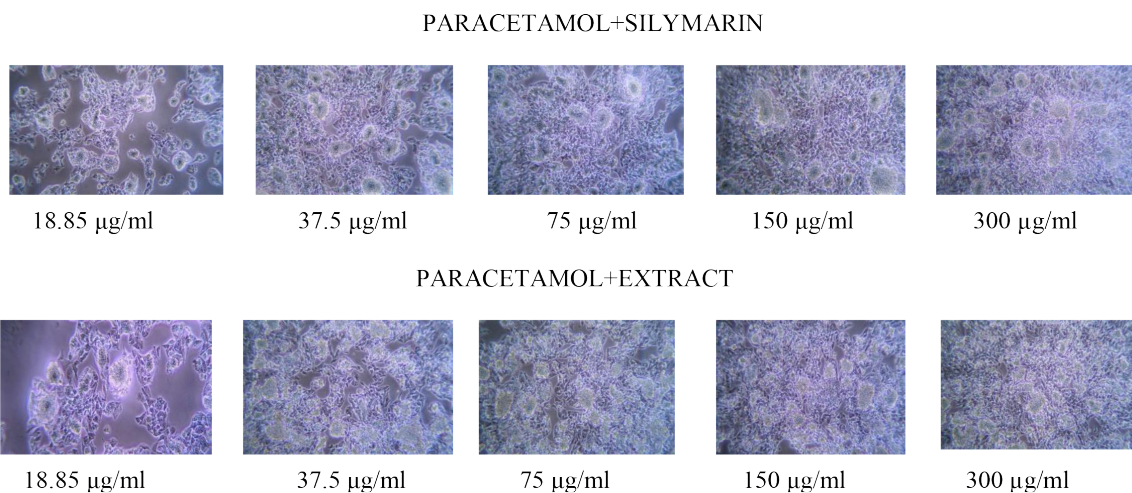


Figure 9 Light microscopic images of Standard Silymarin and the Sample BERM at different concentrations when induced with Paracetamol on HepG₂ cell lines.

Model	Sample and Standard	IC50 values (µg/ml)
CCl ₄ model	CCl ₄ + Silymarin	4.806 (< 18.85 µg/ml)
	CCl ₄ + Extract	21.53
Ethanol model	Ethanol + Silymarin	1.19 (< 18.85 µg/ml)
	Ethanol + Extract	12.61
Paracetamol model	Paracetamol + Silymarin	9.07 (< 18.85 µg/ml)
	Paracetamol + Extract	21.42

Table 4 Illustrates the overlay data of three models (CCl₄, Ethanol and Paracetamol intoxicated) on HepG₂ cell lines, IC50 values (µg/ml) for standard and extract respectively.

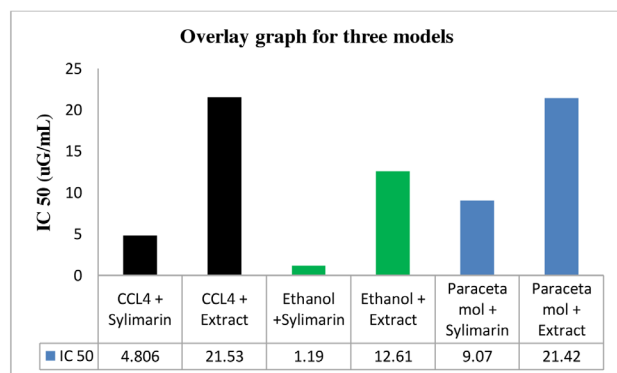


Figure 10 Illustrates the overlay data of IC50 values for all the three models for the extract and standard Silymarin respectively.

4. Discussion

The most commonly used model for screening of anti-hepato toxic or hepatoprotective activity of drugs are CCl₄ induced toxicity in cell lines [9, 10]. The xenobiotic hepatotoxicity is characterized by liver injuries caused due to CCl₄. Since, viral acute hepatitis is similar to that of CCl₄ induced damage to liver [11], in this study CCl₄ toxicity has been chosen as one of the model. It is well established that CCl₄ gets accumulated at parenchymal and other cells in liver, and gets activated by cytochrome-450 monooxygenase to form an unstable compound called trichloromethyl radical (CCl₃). This CCl₃ molecule produces lipid peroxides by alkylating the cellular proteins, PUFA and other macromolecules in the presence of oxygen leading to the damage of liver cells [12]. In the present study, the extract is capable of neutralize the CCl₄ toxicity as per the results obtained. The maximum concentration at 300 µg/ml, the viable cells found to be approximately 90% which indicates that the extract has the potency to regenerate the CCl₄ toxicated cells. Similar results were obtained in

the study by [13] wherein the total alkaloid fraction of *Hypophylla auriculata* at the concentration of 80 µg/ml obtained 92% of viable cells intoxicated with CCl₄.

In another study, during the evaluation of hepatoprotective and antioxidant potential of methanolic extract of *Polyalthiya longifolia* fruits, intoxicated with CCl₄, the fruit extract at 500 µg/mL, able to retrieve 84.5% of viable cells [14].

In worldwide, paracetamol is used either as antipyretic or analgesic drugs since 19th century. It is similar to non-steroidal anti-inflammatory drugs (NSAIDs) in its mechanism of action. However, it is considered safe when it has been taken within the therapeutic dosage limits. Paracetamol overdose may give rise to a wide range of disorders in almost different vital organs viz, liver, kidneys, adrenals, brain, etc. The hepatotoxic effects of Paracetamol are attributed to yield toxic trichloroethylene radicals that can act as free radical initiators. These free radicals react with complex macromolecules within the tissues and aids lipid per oxidation which further leads to the damage and chronic injury to the cells. The symptoms which include nausea with or without vomiting, malaise and abdominal pains due to paracetamol overdose may often mistake for viral prodrome.

In a similar study, wherein in-vitro hepatoprotective activity of *Albizia lebbek*, *Cassia occidentalis* and *Swertia chirata* on HepG₂ cells [15] on comparing with the reference standard where Silymarin showed 156.3 ± 1.69% cell viability in seed extracts of *A. lebbek*, 154 ± 1.63% cell viability in seed extracts of *C. occidentalis* and 156.6 ± 2.49% cell viability in leaves extract of *S. chirata* against Paracetamol induced hepatotoxicity on HepG₂ cell lines. Studies have promisingly shown hepato protection and immune modulation by methanolic and ethanolic extract of *A. lebbek* and *S. chirata* [16-18]. Promising results have also been shown in the model of Wistar rats proving histologically the beneficial effect of the ethanolic extract of *A. lebbek* in hepato protection [19]. Our study also showed the hepato protection of the bark extract (3:1) of *Rhizophora mucronata* against Paracetamol induced intoxication.

Alcoholism, intake of alcohol may one of the major causes of hepatic injury or liver damage. Although the mechanism of alcohol induced hepatotoxicity is very complex and in depth knowledge of different pathways required to understand for its toxicity. However oxidative stress and lipid per oxidation may be attributed to its toxicological nature. Due to excessive alcohol consumption one can inherit alcoholic disorder which in turn leads to chronic liver disorders. Excessive alcohol consumption leads to alcohol hepatitis, then fibrosis, liver cirrhosis, then ultimately hepatic liver failure (HLF). In the present study, ethanol induced hepatotoxicity on HepG₂ cells when treated with the extract neutralized by the extract at the concentration of 300 µg/mL, able to retrieve more than 75% of cells. This is the indication that the extract is capable of regenerating the damaged cells caused due to the toxicant ethanol.

Thus, our study document the potential influence of the bark extract of *Rhizophora mucronata* extract against HepG₂ cell lines using MTT assay. The intensity of HepG₂ cell density was increased while increasing the concentration of *Rhizophora mucronata* extract from 18.85 µg/

ml to 300 µg/ml when treated with different toxicants viz, CCl₄, ethanol and paracetamol. This infers the existence of dose dependent properties of *Rhizophora mucronata* extract against different toxicants on cell lines which was found effective. Study reveals that the ethanol: water (3:1) fraction of *Rhizophora mucronata* evidence the existence of cytotoxic effect and also document the evidence of bioactive compounds which profound to be the influencing factor for the anti-hepato toxic effect. Thus, from the study it can be concluded that the extract of *Rhizophora mucronata* contains potent bioactives capable of inducing the hepatoprotective activity.

5. Conclusion

The Several studies have been carried on *Rhizophora mucronata* and its parts for its therapeutic effects, however very little has been documented. The results obtained from the current study, clearly suggested that the bark extract of *Rhizophora mucronata* at different concentrations capable of neutralizing a wide range of toxicants viz, CCl₄, paracetamol and alcohol overdose. Further, the study also proved that the extract contained potent bioactives with high clinical value which reverted the toxic cells to the normal ones. The study can be further taken to next level, the extract can be tested in animals to support or confirm the findings in our present study. The extract can be used as an alternate source of hepatoprotectant w.r.t the conventional liver savers.

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