EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 8
Issue: 9
471-476
Year: 2021

PHARMACOLOGICAL SCREENING OF EDIBLE UNRIPE FRUIT EXTRACT OF *MUSA BALBISIANA* FOR HEPATOPROTECTIVE ACTIVITY

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Article Received on 06/07/2021 **Article Revised on 26/07/2021** **Article Accepted on 16/08/2021**

ABSTRACT
The objective of the present study was to evaluate the hepatoprotective activity of edible unripe fruit extract of *Musa balbisiana*. The edible unripe fruits were chopped into small pieces and were dried in shade. The powdered drug was extracted with water by cold maceration technique. The extract was subjected to phytochemical analysis. The *In-vitro* hepatoprotective activity was assessed by performing MTT assay in Hep G2 Cell lines using Paracetamol as the toxicant. Wistar albino rats of female sex and Aqueous extract *of Musa balbisiana* (AEMB) at 250 mg/kg and 500 mg/kg and Silymarin (100 mg/kg, p. o.) was used for the study. Paracetamol induced hepatotoxicity was employed to evaluate the hepatoprotective potential of the extract. Biochemical parameters and histopathological examination of liver section were performed. Preliminary phytochemical screening showed alkaloids, flavonoids, tannins, phenolic compounds, terpenoids, saponins, presence carbohydrates, proteins, aminoacids, vitamin C, saponin, starch, and quinones. MTT Assay showed that the extract has a significant protective effect on cell lines against hepatotoxicity and it has increased the cell viability in a dose dependent manner which was further confirmed by morphological assessment. AEMB had shown significant hepatoprotective potential by restoring the biochemical parameters. The same was further confirmed using histopathological studies. The results obtained from the study indicate that unripe fruits of *Musa balbisiana* possess hepatoprotective due to presence of certain constituents like phenols, flavanoids, vitamin C etc.

KEYWORDS: *Musa balbisiana*, MTT assay, Hepatoprotective.

INRODUCTION
The liver is one of the largest organs and the largest gland in the body. It has a weight range of 1.4- 1.8 kg in adult males and 1.2 - 1.4 kg in adult females. It occupies a substantial part of the abdominal cavity. It lies under the diaphragm. It occupies most of the right hypochondrium, part of the epigastrium, and extending into the left hypochondriac region.[1] The etiology of liver diseases varies in different countries and at different times. The prevalence of these etiologies varies according to the changed over the past 10 years. Persistent inflammation of the liver seems to be a risk factor for cirrhosis in spite of the fundamental etiology. Worldwide, cirrhosis of the liver is the 16th leading cause of death and is responsible for hundreds of thousands of deaths each year. Most of the times cirrhosis results in the main cause of Hepatocellular carcinoma. It has the global incidence of over half million, making it the 5th most common cancer in men and seventh most common cancer in the women according to recent findings.[2] According to WHO 3.8% mortality accounts for alcohol consumption every year. Highest mortality was reported in the Europe (0.3%) because of the alcohol, in liver related deaths (Garg et. al., 2012). Also in India alcohol is emerging as the common cause of chronic liver disease. According to WHO 2005 report, 1 billion people are estimated to be overweight and 300 million people are obese.[2,3] Traditional herbal medicines are great gifts to human being. The use of herbal medicines to treat and prevent various diseases has a history of several thousand years. They have been used as the conventional and or complementary therapy in various treatable and incurable diseases. In the prescribed drugs today about 25% drugs are of the plant origin and many are under the clinical exploration. Plants served as precursors for the drug synthesis. The active ingredients of drugs synthesized in the leaf and transported to different parts of the plant are nature's gift to mankind. This showed an importance of the treatment afforded by traditional medicine in comparison to that allopathic drug. Traditional systems like Ayurveda, Siddha and Unani impart knowledge about folklore practices and medicinal importance of drugs of natural origin. The standardization of these drugs is essential since, these drugs are used to treat various ailments of human being.

The role of medicinal plants in traditional system made them back bone of this systems.[3]

MATERIAL AND METHODS

A. *In vitro* pharmacological activity

Hepatoprotective activity

The viability of cells (Hep G2 Cell) were evaluated by direct observation of cells by inverted phase contrast microscope and followed by MTT assay method. After attaining Sufficient growth of cell, Acetaminophen (20mM) was added to induce toxicity and incubated for one hour, prepared extracts in 5% Dulbecco's Modified Eagles Medium (DMEM) were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5g, 6.25ug in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells incubated at 37^0C in a humidified 5% CO_2, incubator.

a) Cytotoxicity Assay by Direct Microscopic observation

Entire plate was observed at an interval of each 24 hours; up to 72 hours in a contrast tissue culture microscope and microscopic observation were recorded. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

b) Cytotoxicity Assay by MTT Method

15mg of MTT was reconstituted in 3 ml PBS until completely dissolved and sterilized between filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilisation Solution (DMSO was added) and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using micro plate reader at a Wavelength of 540 nm.[4,5]

B. *In vivo* pharmacological evaluations

Animals

Rats of Wistar strain (female) were used for pharmacological studies. These animals were kept in animal house of Devaki Amma memorial College of Pharmacy, Chelembra, Malappuram district of Kerala, India. The experimental animals were 22°C (+ 2°C) and the relative humidity was between 50-60%. These animals were fed with standard pellet diet and maintained a drinking water ad libitum. They were kept in 12 h/12 h light/dark cycle d for at least 5 days prior to dosing to allow for acclimatization to the conditions. The animal experimental protocol has been approved by institutional Ethics Committee (DAMCOP/IAEC/037).

a) Hepatoprotective activity (Paracetamol induced hepatotoxicity**)**

The Wistar albino rats (150 -200 g) were randomly divided into five groups, each group comprising of six animals.

Group 1 Normal control receives vehicle of 1 % CMC (1 ml/100g.p.o).

Group 2- Negative control receives Paracetamol (1 g/kg, p.o.) in 1 % CMC

Group 3- Receives Silymarin (100 mg/kg, p. o.) in 1% CMC + Paracetamol (1 g/kg. p. o.)

Group 4- Receives AEMB (250mg/kg, p. o) in 1% CMC + Paracetamol (1 g/kg, p. o.)

Group 5- Receives AEMB (250mg/kg, p. o) in 1% CMC + Paracetamol (1 g/kg, p. o.)

All the animals were intoxicated with paracetamol (1 g/ kg, p. o.) daily for first 7 days except control group. All treatments were given orally from 4 day to 12 day. At the end of the administration, the animals were fasted overnight with water ad libitum. Blood is withdrawn by retro orbital puncture under anaesthesia (Thiopentone sodium 40mg/kg, i. p.) on 13th day. The serum was separated by centrifuge (3000rpm for 15min.) and the serum was used for the estimation of hepatic biochemical markers.[6,7]

Statistical Analysis

The data were expressed as mean ± standard error of the mean (SEM). Different groups were assessed by one way analysis of variance (ANOVA) for multiple comparisons followed by Turkeys' test (Graph pad software Inc, La Jolla, CA. Trial version 6). The criterion for statistical significance was set at $P < 0.05$.

RESULTS

A) Hepatoprotective Activity

MTT Assay

Table 1*: Hepatoprotective activty of AEMB against paracetamol on Hep G2 cell line*.

Sample Concentration (µl/ml)	Sample Concentration (µl/ml)
Control	100±0.0112
Acetaminophen	39.78±0.3118***
AEMB	
6.25	39.78±0.3118***
12.5	56.92 ±0.5656 ***
25	67.42±0.5371 ***
50	76.40±0.3089 ***
100	88.91±0.4116 ***
EC_{50} AEMB = 10.5µl/ml	

The values given are mean +SEM. The extract treated group is compared to standard and the standard and extract group are compared with control by one-way ANOVA (Turkey's method). The data are considered significant if *p<0.05, **p<0.01, ***p<0.001, ns-non significant.

Table 2: *Effect of AEMB on liver enzyme markers against Paracetamol induced hepatotoxicity in rats.*

Sl. no.	Group	SGOT(U/L)	SGPT (U/L)	ALP (U/L)	Total bilirubin
1	Control	49.68±2.02	25.94 ±1.22	123.32 ±1.64	0.46 ±0.02
2	Negative control 1g/kg	75.19±1.45***	45.55±1.15***	192.02±3.25***	1.12±0.072 ***
3	Standrard sylimarin,100 mg/kg	53.57±2.04***	45.55±1.15***	129 ±1.42***	0.50±0.02***
4	AEMB 250mg/kg	67.16±1.09 **	39.30±1.05**	168.46±1.75**	0.76±0.048***
5	AEMB 500mg/kg	58.43±1.37***	32.75±0.68***	141.11±2.32***	0.61±0.01***

The values given are mean +SEM. The extract treated group is compared to standard and the standard and extract group are compared with control by one-way ANOVA (turkey's method). The data are considered significant if *p<0.05, **p<0.01, ***p<0.001, ns-non significant.

Table 3: *Effect of AEMB on oxidative stress enzyme markers against Paracetamol induced hepatotoxicity in rats.*

Sl no.	Group	LPO (nmol/g tissue)	Catalase (U/mg)	Glutathione Peroxidase (U/mg protein
1	Control	8.29±0.26	46.72±0.58	56.96±0.16
2	Negative control 1g/kg	39.15±0.65***	17.95±0.55***	20.85±0.93***
3	Standrard sylimarin,100 mg/kg	10.97±0.41***	42.40±0.21***	48.30±0.30***
4	AEMB 250mg/kg	17.64±0.16***	36.32±0.1 5***	38.93±0.36 ***
5	AEMB 500mg/kg	12.73±0.31***	40.37±0.44 ***	43.23±0.9920***

The values given are mean +SEM. The extract treated group is compared to standard and the standard and extract group are compared with control by one-way ANOVA (Turkey's method). The data are considered significant if *p<0.05, **p<0.01, ***p<0.001,ns-non significant.

Effect on liver enzyme markers

Effect on liver enzyme markers A significant increase in parameters such as SGOT, SGPT, ALP and total bilirubin levels (p<0.001) was observed was observed in rats treated with Paracetamol (1g/kg) in negative control when compared with normal control. There was a significant reduction (p<0.001) in SGOT and SGPT levels in group treated with AEMB at 250 mg/kg and a more significant reduction (p<0.001) in groups treated with two doses of AEMB (250 mg/kg and 500 mg/kg) when compared to negative control and were restored near to normal values in the Silymarin treated groups (p<0.001) as according to the data observed. (Table 2).

Effect on oxidative stress markers

Hepatic intoxication with paracetamol caused a significant increase (p<0.001) in lipid peroxidation in negative groups which reflects hepatocellular damage. Treatment with AEMB at two different doses (250 mg/kg and 500 mg/kg) showed significant reversal (p<0.001) in the toxic effects of Paracetamol as evidenced by a marked decrease in the levels when compared to the negative control. The treatment with Silymarin showed a significant decrease (p<0.001) in levels to normal values. (Table 3).

Catalase and glutathione peroxidase levels were significantly depleted (p<0.001) due to Paracetamol induced liver damage in negative group when compared to the control group. Administration of Silymarin

showed a significant increase ($p<0.001$) in these enzyme levels and was restored when compared to the negative control. Treatment with AEMB (250 mg/kg and 500 mg/kg) also showed a significant increase ($p<0.001$) in the levels when compared to negative control suggesting that it has ability to restore the Cnzyme activity in Paracetamol induced hepatic toxicity.(Table 3).

Histopathological examination of liver of control group was found to have normal morphology. In animals treated with Paracetamol, section of liver showed tissues with partial effacement of architecture. About 50% of cells are enlarged in size, vacuolated cytoplasm and showed disarrangement in normal cells. Section of liver of rats treated with Silymarin showed normal morphology. Liver of rats treated with AEMB at 250 mg/kg possessed 20 % enlarged cells with vacuolated cytoplasm. Kupfer cells and sinusoidal spaces were found to be normal. In animals treated with AEMB at 500 mg/kg, liver sections showed normal morphology. The hepatocytes, portal triads, portal veins and sinusoidal spaces were found to be normal. They showed signs of protection.

DISCUSSIONS

In biological system, varies free radicals were produced during several metabolic processes. Due to their special chemical characteristics, free radicles can initiate lipid peroxidation, cause DNA strand breaks, and indiscriminately oxidize virtually molecules in biological membranes and tissues, resulting in injury. Substantial evidence has accumulated and indicated key roles for reactive oxygen species and other oxidants in causing liver disorders. Thus antioxidants play major role in treatment of hepatic disorders and symptoms associated with it.[8]

Phytochemical analysis of AEMB indicated the presence of constituents such as flavonoids, phenol, carbohydrate, saponin glycoside, alkaloids, tannins, terpenoids, amino acids, vitamin C, starch, Quinones.[9] In the present study, quantitative estimation of total phenolic content, flavanoid content and vitamin C in AEMB were carried out. Phenols are good sources of potential natural antioxidants because of their abilities to act both as efficient radical Scavengers and metal chelators. Flavonoids are the naturally occurring polyphenolie compounds. Halliwell reported that plants rich in flavonoids are potential sources of natural antioxidants that would add to the overall antioxidant capacity and inhibit lipid peroxidation.[10] the antioxidant potential of flavonoids and phenols has already been established, thus validating the presence of antioxidants in extract.

Ascorbic acid acting as a chain breaking antioxidant impairs with the formation of free radicals. The quantitative determination of vitamin C in AEMB shows that they are good source of ascorbic acid.[11,12]

MTT ASSAY

MIT assay was carried out in Hep G2 cell lines to determine the protective effect on e Viability by the extract against paracetamol induced toxic effects. Tetrazolium salt 4 dimethylthiazole -2 yl)-2, 5 diphenyl tetrazolium bromide) is taken up into CelIs and reduced in a mitochondria dependent reaction to yield a blue coloured formazan product. The product accumulates within the cell, due to the fact that it passes through the plasma membrane. On solubilisation of the cells, the product is liberated can be readily detected and quantified by simple colorimetric method. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which in turn may be interpreted as a measure of viability.[13,14] Determination of their ability to reduce MTT to the formazan derivative after exposure to test compounds the study suggests that AEMB showed a significant increase in percentage of cell viability with increase in concentration (6.25-100 µg/ml) Suggesting that the extract possess besides, the morphological assessment of cells treated with extract showed prominent protective effect when compared to the cells treated with paracetamol. Cells treated with increasing concentrations of extract showed decreased cellular damage confirming that the extract has a significant protective effect against damage confirming hepatotoxicity.[15] (Table no.1)

Hepatoprotective Activity

Paracetamol although safe when used at therapeutic doses, is associated with significant hepatotoxicity when taken in overdose stress, generation of free radicals and by depleting the glutathione levels.[16] SGOT and SGPT are metabolic enzymes found in hepatic cell. Hepatic cell damage, these transaminases leaked out into the systemic circulation due to altered permeability of membrane. The raised levels of cytoplasmic hepatic enzymes: SGOT, SGPT and ALP (due to hepatic-biliary duct damage) are considered as an index of the extent and severity of hepatocellular damage. ALP concentration is related to the normal functioning of hepatocytes. High levels of ALP are related to its increased synthesis by cells lining bile canaliculi usually in response to cholestasis and increased biliary pressure. Serum bilirubin level is another conventional indicator of hepatic cell secretory functions and its elevation is also attributed to hepatic insufficiencies. In the present study too, administration of Paracetamol in Wister albino rats produced a significant increase in SGOT, SGPT, ALP and bilirubin levels, confirming hepatocellular damage.[17] Treatment with AEMB at 250 mg/kg and 500 mg/kg significantly attenuated these changes compared to negative control. Significant reduction ($p<0.001$) in the level of SGPT, SGOT, ALP and total bilirubin towards the normal value upon extract treatment is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damages, Restoration of the levels of these enzymes point towards an Improvement in in the secretory mechanism of hepatic cell. Generation of free radicals and oxidative stress is an important parameter hepatic

Paracetamol induced toxicity. The body has an effective defence mechanism to prevent and neutralize free radicals-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as catalase, superoxide dismutase, Glutathione peroxidase etc. Catalase is an antioxidant and results in decomposition of hydrogen peroxide. Glutathione peroxidase is a seleno enzyme and it catalyses. Reaction ion of Hydroperoxides with reduced glutathione to form glutathione disulphide Discussions (GSSG) and the reduction product of the hydroperoxide. It plays an essential role in liver by eliminating toxic compounds by conjugating them with glutathione. In induced hepatotoxicity, the balance between ROS production and Paracetamol antioxidant defences may be lost hence Oxidative stress results which, through a series of events deregulate cellular functions leading to hepatic necrosis. The reactive intermediate formed is believed to cause lipid peroxidation and breakdown of cellular membrane.[18] in the present study, Paracetamol treatment produced a significant depletion of antioxidant enzymes like Catalase and Glutathione peroxidase and showed a marked increase in lipid peroxidation indicating oxidative stress. Treatment with AEMB at 250 mg/kg and 500 mg/kg showed a significant elevation of depleted catalase and glutathione levels. AEMB at 500 mg/kg produced more significant effect ($p<0.001$) and was comparable to the effect produced by Silymarin. The level of lipid peroxidation was increased significantly ($p<0.001$) in Paracetamol treated groups when compared to the control groups. However, treatment with AEMB at 250 me/kg d 500 mg kg showed an anti-lipid per oxidative effect. AEMB at 500 mg/kg showed a marked decrease ($p<0.001$) in lipid peroxidation and was restored near to normal values. The findings suggest that the extract has a potential to counteract with the Idative stress produced by Paracetamol intoxication. Thus, AEMB has greatest ability to reduce oxidative stress by increasing the depleted glutathione and catalase Levels and by reducing lipid peroxidation. Effect of AEMB was further confirmed by histopathological studies. Histological imaging of the negative control group showed tissues with partial architecture. Treatment with AEMB at 250 mg/kg and 500 mg/kg attenuated these toxic changes when compared to the negative control. Silymarin also produced significant hepatoprotective effect.[19] (Table no.2, 3)

The findings from the study suggest. AEMB at 500 mg/kg showed more protective effect against paracetamol induced hepatic damage. Because of the antioxidant potential, the extract restored glutathione levels so that the glutathione could undergo rapid conjugation with the toxic intermediate (NAPQ) and thereby forming non-toxic mercaptate and cysteine compounds scavenging free radicals, possible mechanism responsible for the protection of the Paracetamol induced liver damage by AEMB may be as a result of the extract acting as a free radical scavenger by intercepting certain in phytochemicals because a number of scientific reports indicates the role of certain flavonoids, phenols, vitamin C in hepatoprotection against hepatotoxins. Acting as antioxidants, these constituents offers protection against liver damage by reducing the oxidative stress developed in the system. Cysteine thus compounds and thus decreasing the toxic effects, besides the free radicle scavenging ability radicals, of AEMB offers production against the destructive effect of free radicals hydrogen peroxide formed due to hepatotoxicity. possible mechanism responsible for the protection of the Paracetamol induced liver damage by AEMB may be as a result of the extract acting as a free radical scavenger by intercepting the radicals involved in paracetamol metabolism or the presence of certain in phytochemicals because a number of scientific reports indicates the role of certain flavonoids, phenols, vitamin C in hepatoprotection against hepatotoxins. Acting as antioxidants, these constituents offers protection against liver damage by reducing the oxidative stress developed in the system.[20]

CONCLUSION

The finding from the present study suggests that unripe fruits of *Musa balbisiana* possess hepatoprotective. It might be due to its antioxidant potential and presence of certain constituents like flavonoids, phenolic compounds, vitamin C, and minerals like Iron, Zinc, and Copper etc. Thus, it could be a better choice for liver diseases. This study revealed the efficacy of the extract and it would definitely have wide scope in future. There is further scope for research in isolating the phytoconstituents responsible for the pharmacological activities.

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