PROTECTIVE EFFECT OF *Mussaenda frondosa* Linn EXTRACTS ON ISONIAZID INDUCED HEPATOTOXICITY IN WISTAR RATS

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ABSTRACT

The present study was undertaken to investigate the protective effect and possible mechanism of alcoholic (AIE) and aqueous extract (AqE) from *Mussaenda frondosa* Linn leaf (MF) on Isoniazid (INH)-induced hepatic injury in Wistar rat. Hepatotoxic parameters studied in vivo include serum transaminases (AST, and ALT, ALP), bilirubin, protein, lipid profile (Cholesterol, triglyceride, VLDL and HDL) and level of antioxidants together with histopathological examination. Liv 52® was used as a reference hepatoprotective agent (5ml/kg-1.b.w.). AIE and AqE (200 mg/kg-1.b.w.) on oral administration decreased the level of AST, ALP, ALT, bilirubin, cholesterol, triglyceride, VLDL, MDA and increased the level of protein, HDL and antioxidants (SOD, GSH and CAT) in rats being treated with isoniazid (INH). Pentobarbitone-induced sleeping time study was carried out to verify the effect on microsomal enzymes. Histopathological observations confirmed the beneficial roles of MF against INH-induced liver injury in rats. Possible mechanism may involve their antioxidant activity.

Keywords: *Mussaenda frondosa* Linn, hepatoprotective, Isoniazid, Liv 52®, sleeping time.

INTRODUCTION

Liver is a major metabolic organ affected by various chemicals and toxins daily. Identification of successful hepatoprotective agent will provide a useful tool in the treatment of hepatic diseases. In absence of reliable liver protective drugs in modern medicine, a large number of medicinal preparations are recommended for the treatment of liver disorders and quite often claimed to offer significant relief 1. While performing several detoxifications,
liver goes under stress, leads to liver diseases, ending in serious health problems, liver failure and death. Although several synthetic medicines are recommended for liver therapy but most of them are immunosuppressive and symptomatic. To solve this problem, studies on novel therapeutic strategies using plant based drugs are in process of rigorous testing.

Exposure to various organic compounds including a number of environmental pollutants and drugs can cause cellular damages through metabolic activation of those compounds to highly reactive substances such as reactive oxygen species (ROS). Under normal circumstances, reactive oxygen species (ROS) are detoxified by an efficient antioxidant system that includes enzymes such as superoxide dismutase, catalase and glutathione. In case this defense system is inefficient, the cells experiences an oxidative stress which contributes in a variety of chronic inflammatory diseases such as arthritis, atherosclerosis as well as other ailments viz. cancer, diabetes, hepatitis, neuro degeneration and early aging.

Plants and plant products are part of the vegetarian diet and number of them exhibit medicinal properties. Several Indian plants are also being used in Ayurvedic and Siddha medicines. The medicinal properties of several herbal plants have been well documented in ancient Indian literature and the preparations have been found to be effective in the treatment of diseases. The reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases. Hence, search for new synthetic and natural antioxidants is essentially important. Although initial research on antioxidants was mostly on isolated pure compounds, recent focus is more on natural formulations. It has been found that compounds in their natural formulations are more active than their isolated form.

In the absence of reliable modern hepatoprotective drugs, there are a number of traditional medicines recommended for treatment of liver diseases. Traditionally Musaenda frondosa Linn (Rubiaceae) commonly called as Nagavalli reported to possess number of medicinal properties. Traditionally leaves are used in the treatment of jaundice, asthma, hyperacidity, fever, ulcers, leprosy, diuretic, wound, astringent, expectorant, anti-inflammatory, cardiotonic, cough bronchitis, swells, antimicrobial etc.
MATERIALS AND METHODS
Preparation of Mussaenda frondosa Extract
Mussaenda frondosa Linn leaves were collected from open field around the Belgaum city in the month of September and were authenticated by the taxonomist Dr. Harsha Hegde and the herbarium (voucher No.RMRC 484) has been preserved at Regional Medical Research Centre (Belgaum). Shade dried leaves were powdered to moderately coarse grade and subjected for the successive extraction. Alcoholic extract of MF leaves was obtained by soxhlation process and aqueous extract by maceration process kept in an air tight container at 4\(^0\)C for future use. Suspensions of each extract were freshly prepared using 0.1\% Tween 80 for experimental use.

Animals
The complete course of the experiment was carried out using healthy adult male Wistar rats obtained from registered breeders and was maintained at animal house of the institution. They were fed on commercial laboratory animal feed and tap water ad lib. The rats weighing between 120-150g were housed in laboratory for about a week for acclimatization with natural 12:12 hr light–dark cycle. The animals were starved overnight with tap water ad lib prior to the day of experimentation. Ethical clearance was obtained from Institutional Animal Ethics Committee constituted as per CPCSEA guidelines.

Acute Toxicity Study
Acute toxicity studies were carried out for all the extracts as per OECD guideline 425 in Wistar rats weighing 80 to120g by administering a dose 2000 mg/kg orally. The groups were almost continuously observed for mortality and behavioral changes during first 24hr and then daily for a fortnight. The oral LD\(_{50}\) was found to be more than 2000mg/kg. Therefore 1/10\(^{th}\) of LD\(_{50}\) was used as effective dose in the further study.

Drugs used and their Doses
The rats weighing between 120-150g were divided into five groups (n=6). Group I (normal control) received 0.1\% Tween80, Group II (positive control) received isoniazid 50mg/kg\(^{-1}\), Group III (standard) received Liv52\(^\circ\) 5ml where as Group IV and V received alcoholic & aqueous extract 200mg/kg\(^{-1}\). All the treatments were administered orally for 30 days.
METHODOLOGY

All the treatments were given for a total period of 30 days, on the 31st day all the rats were anaesthetized by halothane; blood was withdrawn by cardiac puncture and animals were sacrificed by over anesthesia to dissect out liver for histopathological studies and oxidative stress markers. Blood was allowed to coagulate for 30 min and serum was separated by centrifugation at 2500 rpm, to estimate alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein and bilirubin (total and direct) content. Lipid profile (Cholesterol, triglyceride, VLDL and HDL). Liver was kept in cold conditions. It was cross chopped with surgical scalpel into fine slices in chilled 0.25M sucrose solution, quickly blotted on a filter paper and used for histopathological study. The part of liver tissue was minced and homogenized in 10mM Tris-HCl buffer, pH 7.4 (10%-w/v) with 25 strokes of tight Teflon pestle of glass homogenizer at a speed of 2500 rpm. The clear supernatant was used for oxidative stress markers assays like lipid peroxidation, reduced Glutathione, Superoxide dismutase and Catalase.

Pentobarbitone induced sleeping time test

The rats were kept on standard diet. Experiments were set as mentioned above for, Isoniazid. Twenty four hours after the last treatment by standard and alcoholic extracts of Mussaenda frondosa, pentobarbitone sodium in water for injection (75mg/kg b.w.) was administered intraperitoneally. Food was withdrawn and water given ad libitum 12hr before, pentobarbitone injection. All the experiments were conducted between 09.00 am to 5.00 pm. in temperature controlled room. The animals were placed on table after loss of righting reflex. The time interval between loss and regain of righting reflex was measured as pentobarbitone induced sleeping time. This functional parameter was used to determine the metabolic activity of the liver.

STATISTICAL ANALYSIS

The results were analysed by ANOVA followed by Tukey’s multiple comparison test and P ≤ 0.05 was considered as significant.

RESULTS

Effect on serum enzymes (AST, ALT and ALP)

The serum activities of AST, ALT and ALP were used as biochemical markers for the early acute hepatic damage The group of animals that received only INH showed significant (P<
0.001) increase in serum AST, ALT and ALP with the mean values of 83.33±1.43, 188.3±1.81, and 118.70 ± 2.06 respectively, as compared to the corresponding values of 45.67±2.89, 35.73±1.39 and 66.5±1.05 in normal control group. The animals treated with Liv52® AlE and AqE showed significant decrease in AST, ALT and ALP compared to the control (Table-1). Response shown by AlE was nearly equal to the Liv52®

**Effect on serum total protein**

The group of animals that received only INH showed significant (P< 0.001) reduction in the level of total protein with the mean value of 5.22±0.15 as compared to 7.72 ± 0.09 of normal control group.

The animals treated with Liv52® showed significant (P< 0.001) increase in serum protein with the mean value of 7.07 ± 0.08. Similarly, animal treated with 200mg/kg-1 of AlE and AqE showed significant (P<0.001) increase in serum protein with the mean values of 6.10 ± 0.02 and 6.04 ± 0.05.

**Effect on serum bilirubin**

The animals that received only INH showed significant (P<0.001) increase in serum bilirubin (total and direct) with the mean values 1.90 ± 0.01 and 0.30 ± 0.01 respectively, as compared to the corresponding values of 0.77 ± 0.03 and 0.12 ± 0.01 in normal control group.

The group treated with Liv52® showed significant (P<0.001) reduction with the mean value of 0.92±0.02 and 0.24 ± 0.01 respectively. The animals treated with 200mg/kg-1 of AlE and AqE showed significant decrease in total and direct bilirubin compared to the control (Table-1).

**Effect on the lipid profile (Cholesterol, triglyceride, VLDL and HDL)**

The group of animals that received only INH showed significantly (P< 0.001) increased cholesterol, triglycerides, VLDL and decreased level of HDL as compared to the corresponding values of normal control group.

The animals treated with Liv52® showed significant (P< 0.001) decrease in cholesterol, triglycerides, VLDL and increase in HDL.
Similarly, animals treated with 200mg/kg-1 of AIE and AqE showed significant decreased in cholesterol, triglycerides VLDL and increased in HDL compared to the control (Table-2). Response shown by AIE was nearly equal to the Liv52.®

**Effect on hepatic antioxidants (GSH, SOD and CAT) in EtOH induced hepatic injury**

The animals that received only INH showed significant (P<0.001) decrease in SOD, GSH and CAT with the mean values of 19.16 ± 0.28, 57.18 ± 0.65 and 92.75 ±1.43 respectively; as compared to the corresponding values of normal control group.

The animals treated with Liv52.® AIE and AqE showed significant increase in GSH, SOD and CAT compared to the corresponding values of normal control group as shown in Table-3.

**Effect on hepatic oxidant MDA**

The animals that received only INH showed significant (P<0.001) increase in MDA level with the mean value of 25.96 ± 0.51 as compared to the corresponding value 5.93 ± 0.28 of normal control group.

The group of animals treated with Liv52,® AIE and AqE showed significantly (P< 0.001) decreased in MDA level with the mean value of 10.47 ± 0.11, 13.82 ± 0.47 and 16.10 ± 0.73 respectively as compared to the the normal. Response shown by AIE was nearly equal to the Liv52®

**Effects on sleeping time**

Effect of hepatotoxicant INH prolonged pentobarbitone induced sleeping time. Sleep duration with pentobarbitone at a dose of 75mg/kg i.p., was 117.20 ± 0.88 min. whereas treatment with INH significantly (P< 0.001) prolonged the pentobarbitone sleeping time with the respective mean duration of 225.90 ± 1.10.

However, prior treatment of animals with Liv52® restored the duration almost to normal with mean duration of 121.40 ± 1.59 min. Similarly, prior treatment of animals with alcoholic extracts of MF significantly (P< 0.001) reduced with mean duration of 124.90 ± 1.09. (Table-4).

**Effects of AIE and AqE on liver histology**

The histological features, as shown in Fig- a indicated a normal liver lobular architecture and cell structure of the livers in the control animals. There were no pathological changes in
healthy control livers which showed normal lobular architecture. Fig.-b shows (INH treated) moderate macrovesicular fatty changes and hepatocellular necrosis Changes were improved in Liv52® AIE and AqE treated rats, which exhibited areas of normal liver architecture (Fig-c, d and e)

**DISCUSSION**

The present study was planned to explore hepatoprotective activity of *Mussaenda frondosa* - leaf extracts, since these plants are widely used by local traditional healers to treat jaundice, which is a major manifestation of liver injury.

The serum marker enzymes (AST, ALT and ALP) are cytoplasmic in nature, but upon liver injury these enzymes enter into the circulatory system due to altered permeability of membrane. Our results showed INH caused a significant elevation of serum levels of ALT, AST and ALP in rats. These effects were markedly reduced if the rats were pre-treated with AIE and AqE of MF. Together these evidences suggest that the hepatoprotective effects of MF might be in part due to its ability to protect biomembrane against free radicals.

Total protein concentration of the INH treated rats was significantly reduced. This suggests a reduction in the protein synthetic function of the liver, which could be as a result of possible damage to the hepatocytes induced by INH. Most protein found in the plasma are synthesized by the hepatocytes and secreted into circulation. Administration of AIE and AqE of MF lead to an increased in the total protein. Bilirubin has been attributed to the damaged structural integrity of the liver; decrease in the level of bilirubin by AIE and AqE of MF indicates its protective effect.

The characteristic changes observed in the concentrations of plasma lipids, cholesterol and triglycerides and lipoproteins VLDL and HDL in ethanol treated rats. Increase in the level of plasma lipids, cholesterol and triglycerides and lipoproteins VLDL is risk factor for ischemic heart disease. AIE and AqE of MF reduces these levels and increases level of HDL. HDL particles are responsible to remove cholesterol from within artery.

MDA is a major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acid (PUFA). MDA, a secondary product of lipid peroxidation, is a useful indicator of tissue damage involving a series of chain reactions. Recent study also suggested that a reduction in the activity of SOD is associated with the accumulation of
highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes.\textsuperscript{30} GSH, an important protein thiol in living organisms plays a central role in coordinating the body’s antioxidant defense process.\textsuperscript{31} Reducing GSH constitutes the first line of defense against free radicals.\textsuperscript{32} AIE and AqE of MF at tested doses 200mg/kg-1 prevented elevation of liver MDA content, reduction of liver SOD activity, and increase of GSH content resulted from rat liver intoxication with INH challenge. The hepatoprotective ability of MF might be due to its ability to stabilize liver cell membrane. Thus, the activity of SOD was commendably maintained, and the MDA production and the consumption of GSH were decreased.

CAT is a key component of the antioxidant defense system. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage. Excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Homogenated liver CAT activities in AIE and AqE groups were significantly higher than those in INH group. In this study, CAT was increased by administration of AIE, suggesting that it can restore CAT enzyme.

Pentobarbitone -induced sleeping time study was carried out to verify the effect on microsomal enzymes. It was observed; the group received only hepatotoxicant; there was significant increased in the duration of sleep. On treatment of AIE and AqE duration of sleep was significantly reduced.

Liv-52® which contains the various herbal plants mainly \textit{Capparis spinosa}, \textit{Cichorium intybus}, \textit{Solanum nigrum}, \textit{Terminalia arjuna}, \textit{Cassia occidentalis} and \textit{Achillea millefolium} shows hepatoprotective activity by the virtue of their antioxidant property and this is due to the presence of flavanoids, cynogenic glycosides and triterpines. Phytochemical investigation of the \textit{Mussaenda frondosa} showed it contains several types of compounds such of Steroid, Flavonoid, Carbohydrate, Saponin, Resin, and Tannin. Hepatoprotection offered by \textit{Mussaenda frondosa} extracts could be attributed to these constituents, since antioxidants have been reported to posses hepatoprotective activity.\textsuperscript{33} The present study was not aimed to elucidate hepatoprotective mechanisms of \textit{Mussaenda frondosa} extracts. In order to confirm their antioxidant potential and to identify various enzymes involved in generating oxygen free radicals further studies are essential.
Hepatoprotection offered by _Mussaenda frondosa_ extracts could be attributed to these constituents, since antioxidants have been reported to possess hepatoprotective activity.

In order to confirm their antioxidant potential and to identify various enzymes involved in generating oxygen free radicals further studies are essential. These short comings of the present studies open a new arena for the future research. Considering the efficacy of the plants, their phytoconstituents (fractions) need to be isolated in order to explore their hepatoprotective activity. Further activity guided chemical studies of the fractions may help in developing new leads that would be useful for the treatment of presently untreatable hepatotoxocities.

**Table 1: Effect of *Mussaenda frondosa* on isoniazid induced hepatotoxicity**

<table>
<thead>
<tr>
<th>Treatment/Groups</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (IU/L)</th>
<th>Total Protein (g/dl)</th>
<th>Bilirubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>45.67±0.289</td>
<td>35.73±1.39</td>
<td>66.5±1.05</td>
<td>7.72±0.09</td>
<td>0.77±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>INH</td>
<td>83.33#±1.43</td>
<td>188.3#±1.81</td>
<td>118.70#±2.06</td>
<td>5.22#±0.15</td>
<td>1.90#±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.30#±0.01</td>
</tr>
<tr>
<td>Liv52®</td>
<td>56.17***±1.51</td>
<td>80.21***±1.13</td>
<td>82.17***±2.12</td>
<td>7.07***±0.08***</td>
<td>0.92***±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.24***±0.01</td>
</tr>
<tr>
<td>Alcoholic Extract</td>
<td>62.00***±0.81</td>
<td>82.31***±1.27</td>
<td>104.00***±2.62</td>
<td>6.10***±0.02</td>
<td>0.93***±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.24***±0.01</td>
</tr>
<tr>
<td>Aqueous Extract</td>
<td>74.67*±1.14</td>
<td>102.30***±1.27</td>
<td>107.00***±2.36</td>
<td>6.04***±0.05</td>
<td>0.95***±0.01</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25***±0.01</td>
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</tbody>
</table>

One way ANOVA followed by Turkey’s multiple comparison tests.
# P<0.001 when compared with Normal control group. *** P<0.001, ** P<0.01 , *p<0.05

**Table 2: Effect of *Mussaenda frondosa* on isoniazid on lipid profile**

<table>
<thead>
<tr>
<th>Treatment/Group</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>VLDL (mg/dl)</th>
<th>HDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>101.80±1.30</td>
<td>67.00±0.68</td>
<td>13.37±0.13</td>
<td>26.83±0.70</td>
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<tr>
<td>INH</td>
<td>159.30#±1.70</td>
<td>102.30#±2.86</td>
<td>20.47#±0.57</td>
<td>9.83#±0.60</td>
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<tr>
<td>Liv 52®</td>
<td>111.20***±2.16</td>
<td>69.17***±1.68</td>
<td>13.83***±0.32</td>
<td>25.17***±0.70</td>
</tr>
<tr>
<td>Alcoholic Extract</td>
<td>122.50***±1.94</td>
<td>81.17***±0.94</td>
<td>16.23***±0.18</td>
<td>21.83***±0.79</td>
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<tr>
<td>Aqueous Extract</td>
<td>147.70*±1.47</td>
<td>86.00***±0.73</td>
<td>17.20***±0.14</td>
<td>14.33**±0.91</td>
</tr>
</tbody>
</table>

One way ANOVA followed by Turkey’s multiple comparison tests.
#P<0.001 when compared with Normal control group. ***P<0.001, **P<0.01, *p<0.05

Table 3: Effect of *Mussaenda frondosa* on isoniazid induced changes on antioxidants and oxidant

<table>
<thead>
<tr>
<th>Treatment/ Group</th>
<th>GSH (µg/ mg protein)</th>
<th>SOD (U/ mg protein)</th>
<th>CAT (U/ mg protein)</th>
<th>MDA (nm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>101.4±1.31</td>
<td>45.34±1.21</td>
<td>280.9±1.50</td>
<td>5.93±0.28</td>
</tr>
<tr>
<td>INH</td>
<td>57.18±0.65</td>
<td>19.16±0.28</td>
<td>92.75±1.43</td>
<td>25.96±0.51</td>
</tr>
<tr>
<td>Liv 52®</td>
<td>84.94***±0.58</td>
<td>32.99***±0.61</td>
<td>254.50***±1.85</td>
<td>10.47***±0.11</td>
</tr>
<tr>
<td>Alcoholic Extract</td>
<td>77.01***±0.90</td>
<td>31.50***±0.58</td>
<td>248.30***±3.28</td>
<td>13.82***±0.47</td>
</tr>
<tr>
<td>Aqueous Extract</td>
<td>70.25*±0.66</td>
<td>27.56***±0.60</td>
<td>228.80***±4.34</td>
<td>16.10***±0.73</td>
</tr>
</tbody>
</table>

One way ANOVA followed by Turkey’s multiple comparison tests.

#P <0.001 when compared with Normal control group. ***P<0.001, *p<0.05

Table 4: Effect of Alcoholic extract of *Mussaenda frondosa* on pentobarbitone induced sleeping time (in minutes)

<table>
<thead>
<tr>
<th>Treatment - DOSE/kg⁻¹</th>
<th>MEAN ± SEM (in minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control /Pentobarbitone 75mg (i.p.)</td>
<td>117.20 ±0.88</td>
</tr>
<tr>
<td>INH 50mg + Pentobarbitone 75 mg (i.p.)</td>
<td>225.90 ± 1.10</td>
</tr>
<tr>
<td>Liv52® 5ml + INH 50mg + Pentobarbitone 75mg (i.p.)</td>
<td>121.40* ± 1.59</td>
</tr>
<tr>
<td>MF- AIE 200mg.+ INH 50mg + Pentobarbitone 75mg (i.p.)</td>
<td>124.90* ± 1.09</td>
</tr>
</tbody>
</table>

*P<0.001
HISTOPATHOLOGY

Fig-a: Normal
Fig-b: INH
Fig-c: INH + Liv 52®
Fig-d: INH + Alcoholic extract
Fig-e: INH + Aqueous extract

INH treated group showing portal triaditis inflammatory cells and moderate necrosis. Liv52® & Alcoholic extract treated groups showing marked improvement towards normal hepatic architecture compared to aqueous extract treated group.

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