Ethanolic Extract of Madhuhari Ameliorates Diabetes–Induced Oxidative Stress in Male Mice

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Abstract: The primary aim of the present investigation was to evaluate the role of an antidiabetic polyherbal formulation, Madhuhari in the alloxan-induced oxidative stress in hepatic and renal tissues. Simultaneously DPPH (1,1-Diphenyl-2-picrylhydrazyl) and in vitro lipid peroxidation (LPO) assays were performed to ascertain the antioxidative potential of the test drug. For in vivo study 5 different doses (100, 200, 400, 800, and 1600 mg/kg) of ethanolic extract of Madhuhari were administered to male mice for 15 days. Alloxan was given on 11th day of drug treatment. On 16th day changes in above mentioned parameters were evaluated. Results revealed that, the lower doses of Madhuhari (100 and 200 mg/kg BW) nearly prevented all alloxan-induced adverse effects, suggesting its antihyperglycemic and antiperoxidative efficacy. However, higher doses were found to be less effective. Results of in vitro study also supported its free radical scavenging efficacy. It appears that the drug primarily acts through its antioxidative properties and pre-treatment with the test drug may ameliorate free radical induced diabetic complications.

Keywords: Madhuhari, lipid peroxidation, antioxidants, diabetes mellitus, liver, in vitro

I. Introduction

Diabetes mellitus (DM), a serious health problem, is increasing day by day and is known to affect almost all the body functions including carbohydrates, lipid and protein metabolism [1]. It is also associated with an enhanced risk for developing premature atherosclerosis, cardiac abnormalities and causes damage to different body organs via enhanced free radical generation [2]. The augmented oxidative stress is also known to increase DM related complications [2,3]. As allopathic drugs, currently in use for treating DM are known to produce side effects, people are gradually turning towards ayurveda, a traditional Indian system of medicine [4].

Though, various new conventional drugs came into market, it is observed that even after chronic treatment one can’t easily get rid from the hitches of DM [5,6]. Further, it is believed that the people with family history of DM or having poorly developed body antioxidant defence system are more vulnerable for this disorder. However, if they are concerned and take preventive measures before onset of clinical symptoms of DM then it may be possible to diminish its severity [4,7].

The involvement of free radicals in DM and in lipid peroxidation (LPO) is well studied [3,5]. Increased blood glucose may also induce more free radical generation which damages membrane lipids and proteins, leading to disturbed cell functions. In fact, in diabetic condition, an increased tissue LPO and/ or decreased antioxidative defence machinery have already been reported earlier [8]. However, it was not known whether the ayurvedic preparation, Madhuhari acts through its antioxidative property or not. It was also not known, if it can prevent diabetes-induced adverse effects in live and kidney.

Many herbs are known to possess medicinal values and reports are available on the use of plant parts and their combinations as source of medicine/ antioxidants [4,9]. From our laboratory also a good number of plants have been reported to be antioxidative in nature [10,11,14-16]. In fact, because of their safer, healthier and economic values they are even preferred and used by healthy individuals. Moreover, some polyphenolic compounds and flavonoids from different plant parts have been known to possess anti-hyperglycemic and free radical scavenging activities [9,14].Madhuhari is a well known multiherbal commercially available antidiabetic drug. It possesses extracts of Gymnema Sylvestre, Ocimum sanctum, Azadirachta indica, Momordica charantia, Eugenia jambolana, Pterocarpous marsupium, Aloe vera, Trigonella foenum gracecum, Tinospora cardifolia etc herbs as its main components. These plants are known to cure DM and related problems [4,5,13,19]. Although, reports reveal the antidiabetic activity of Madhuhari [15,17]; on its mode of action, particularly on the involvement of its antioxidative activity in vital organs such as liver and kidney, nothing was known so far. Therefore, the present investigation attempted to reveal the preventive role of Madhuhari in chemically induced oxidative stress in liver and kidney, that are primarily involved in any drug metabolism and are more susceptible for free radical induced damage [16,17]. A parallel in vitro study was also performed in liver and kidney homogenates [15,18] to affirm its antioxidative potential, if any.
II. Materials And Methods

2.1 Chemicals and drug

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, St. Louis, MO, USA, while thio-barbituric acid (TBA), ascorbic acid were supplied by Hi Media Laboratories Ltd., Mumbai, India, Malondialdehyde (MDA), carbon tetrachloride (CCl₄) and all other reagents were purchased from E-Merck Ltd., Mumbai, India. Madhuhari (Shivayu ayurveda Ltd., Nagpur- 441107, India. Batch No. MHPA/1114) was purchased from authorized medical store of local market, Indore, India and its 70% ethanolic extract was used in the study.

2.2 Animals

Healthy colony bred healthy Swiss albino male mice, weighing 28±1 g (2-2.5 months old) were housed in polypropylene cages under constant temperature (27±2 °C) and photo schedule (14 h light and 10 h dark). They were provided rodent feed (Golden Feeds, New Delhi, India) ad libitum and had free access to boiled drinking water. Standard ethical guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India were followed. Maintenance of animal was done under the supervision of Institutional Animal Ethical committee (Reg. No. 779).

2.3 Estimation of total Phenolics and Flavonoids

Total polyphenolic contents of the test extract was measured using Folin-Ciocalteu method following the protocol of Leontowicz et al [19], where gallic acid was used as standard. The obtained results were expressed in mg gallic acid equivalent /100 g dry weight of the drug (r² = 0.979). Total flavonoid was determined by following the method of Leontowicz et al [19] using quercetin as standard, as routinely done in our laboratory [13-16]. Results are expressed as mg of quercetin equivalents / 100 g dry weight of the extract (r² =0.965).

2.4 DPPH Assay

DPPH free radical scavenging strength was measured using the protocol of Leontowicz et al [17,19] where ascorbic acid was considered as standard. In this assay change in colour of reaction system is measured which occurs due to electron transfer. In brief, the methanolic stock solutions of different concentrations of drug (2.5-1000 µg/ml) were prepared. In reaction mixture 0.5 ml freshly prepared DPPH (0.15 mM) and 1 ml of drug were mixed and incubated for 30 min at 20°C. Percent (%) scavenging activity was determined using formula, [%RSA = 100 x (control OD – sample OD)/control OD].

2.5 In Vitro Study with CCl₄

In this study CCl₄-induced LPO was measured by following the protocol of Ohkawa et al [20], as done in our laboratory earlier [13-16]. In brief, liver and kidney were excised from healthy male rats, washed, chopped and homogenized with Phosphate buffer saline (PBS, 0.1 M, pH 7.4) to get 10% w/v homogenate. Different concentrations of CCl₄ (25, 50, 75 and 100 µl/ ml) in 1 ml (10% w/v) tissue homogenate were used to induce LPO. Finally, considering the most effective concentration of CCl₄ (50 µl/ ml) antiperoxidative effect of test drug was evaluated. As no report was available on in vitro study of Madhuhari, a wide range of concentrations of the test drug was used. LPO was measured in term of nM MDA formed/ hr/ mg protein. The experiment was repeated with effective concentration of drug to confirm the results.

2.6 In vivo study

An experiment was conducted to see the effects of different doses of the test drug in alloxan induced diabetic mice. For this study doses were taken on the basis of human equivalent dose, and converted to mouse equivalent dose using following formula.

Human equivalent dose in mg/ kg = Animal dose in mg/kg (animal weight in kg/ human weight in kg)

Forty nine healthy male mice were divided into seven groups of seven mice each and acclimatized for one week. Animals of groups 3-7 received 100, 200, 400, 800 and 1600 mg/kg/p.o./day, ethanolic extract of Madhuhari respectively in 0.1 ml distilled water (DW) for 15 consecutive days, while group 1 and 2 received only DW (0.1 ml/ p.o./ day). On 11th day of drug treatment animals of groups 2-7 were given single dose of alloxan after 16 hours fasting (150 mg/kg BW), while those of the group 1 received normal saline and served as control group. Drug was administered at a fixed time (10:00-11:00 AM) of the day to avoid circadian variation, if any. Body weight and water intake was measured routinely. On the last day overnight fasted animals were sacrificed by cervical dislocation, blood was collected and serum was separated for glucose estimation. Liver and kidney were removed quickly, cleaned and washed twice with chilled PBS (0.1 M, pH 7.4), homogenized and processed for different biochemical estimation [14,16,17].

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Serum glucose level was measured by the glucose oxidase/peroxidase method. LPO was determined by TBARS method of Okhawa et al.[20], and expressed as nM MDA formed/h/mg protein. For protein estimation and activity of SOD, the method of Lowry et al.[19] and Markland and Markland[22] were followed respectively, while CAT and GSH were measured by the methods of Aebi[23] and Ellman[24] respectively, as routinely done in our laboratory.[24,34].

2.8 Statistical Analysis

Data are expressed as mean ± SE. Statistical analysis was done by using one-way ANOVA followed by unpaired student’s t-test and P-value of 5% and less were considered as significant. Polyphenolic and flavonoid compounds values were calculated from the linear regression equation.

III. Results

Total Phenolics And Flavonoids - The amount of total polyphenols and flavonoids in the test drug was calculated out to be 92.66 ±6.94 mg gallic acid equivalent/100 g dry weight and 124.61±8.46 mg quercetin equivalents/100 g dry weight of the drug.

In Vitro Assays - In DPPH assay the drug showed concentration dependent free radical inhibition, the highest inhibition was found at 80 µg/ ml (85.63%) which is comparable to standard ascorbic acid (Table 1).

Table 1 - DPPH free radical scavenging activity of test drug at different drug concentrations in comparison to standard, ascorbic acid. Data are in mean ± SEM (n=3). Less scavenging activity at higher concentrations of the drug may be noted.

<table>
<thead>
<tr>
<th>Conc. (µg/ ml)</th>
<th>Madhuhari (µg/ mg protein)</th>
<th>Ascorbic acid (µg/ mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>26.96± 0.037</td>
<td>80.34± 0.017</td>
</tr>
<tr>
<td>20</td>
<td>73.31± 0.042</td>
<td>87.56± 0.023</td>
</tr>
<tr>
<td>40</td>
<td>79.55± 0.032</td>
<td>92.45± 0.021</td>
</tr>
<tr>
<td>80</td>
<td>85.63± 0.013*</td>
<td>96.98± 0.015</td>
</tr>
<tr>
<td>160</td>
<td>75.01± 0.023</td>
<td>97.53± 0.012</td>
</tr>
<tr>
<td>320</td>
<td>41.56± 0.031</td>
<td>97.68± 0.022</td>
</tr>
<tr>
<td>640</td>
<td>34.67± 0.029</td>
<td>96.96± 0.018</td>
</tr>
</tbody>
</table>

Data are mean ±SE (n=3); * P<0.0001 as compared to the respective values.

With respect to the inhibition of CCl4-induced LPO in the liver and kidney homogenates, while a significant (P<0.001) increase in LPO was observed following CCl4 administration, an inhibition in LPO occurred in tubes in which both drug and CCl4 were added. However, the highest inhibition was found at 0.31 gm/ml of the drug with 75.80% and 88.82% reduction in hepatic and renal tissues respectively (Fig. 1 & 2). The inhibition was less below and above this concentration.

In Vivo Study – While alloxan administration enhanced the serum glucose and tissue LPO significantly (P<0.0001 for both), a marked decrease in the same was observed when 100 and 200 mg/Kg of the test drug were administered (P<0.001 for both, Fig 2-4). There was also a significant decrease in body weight (P<0.001)
with concomitant increase in water intake ($P<0.001$) in the alloxan treated animals (Table 4), that were nearly reversed by drug administration. Also in these drug treated groups a significant increase in the activities of SOD and CAT and in GSH content ($P<0.0001$ in all) were noticed. However, at higher doses (800 and 1600 mg/kg BW), no significant changes were observed in most of the above studied parameters, as compared to alloxan treated groups (Table 2 & 3).

**Table 2-** Effects of different doses of Madhuhari extract for 15 days on liver superoxide dismutase (SOD, in IU/mg protein), catalase (CAT, in IU/mg protein) and reduced glutathion (GSH, in M GSH/mg protein) in male mice. Cont (Control), Allox. (Diabetic), D1 (100 mg/kg), D2 (200 mg/kg), D3 (400 mg/kg), D4 (800 mg/kg) and D5 (1600 mg/kg) body weight.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD</th>
<th>CAT</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont.</td>
<td>6.79± 1.03</td>
<td>41.33± 4.31</td>
<td>5.54± 0.783</td>
</tr>
<tr>
<td>Allox.</td>
<td>3.23± 0.973</td>
<td>26.89± 3.28</td>
<td>3.41± 0.498</td>
</tr>
<tr>
<td>Allox.+ D1</td>
<td>5.89± 1.23*</td>
<td>45.67± 5.34*</td>
<td>5.72± 0.878*</td>
</tr>
<tr>
<td>Allox.+ D2</td>
<td>5.71± 1.10*</td>
<td>43.81± 4.89*</td>
<td>5.69± 0.745*</td>
</tr>
<tr>
<td>Allox.+ D3</td>
<td>4.19± 1.78*</td>
<td>33.56± 4.54*</td>
<td>4.45± 0.534*</td>
</tr>
<tr>
<td>Allox.+ D4</td>
<td>3.08± 1.52</td>
<td>34.67± 4.86</td>
<td>4.37± 0.822*</td>
</tr>
<tr>
<td>Allox.+ D5</td>
<td>2.67 ± 1.05</td>
<td>28.98± 3.52</td>
<td>3.22± 0.921</td>
</tr>
</tbody>
</table>

Data are mean ±SE (n=7); * p<0.001 as compared to the respective control values. $^b$ p<0.01 and $^a$ p<0.001 compared to respective value of the diabetic group.

**Fig. 3 -** Effects of different doses of Madhuhari for 15 days on fasting serum glucose (FSG in mg/dl).

**Fig. 4 -** Effects of different doses of Madhuhari for 15 days on liver tissue lipid peroxidation (nM MDA/ h/mg protein).

**Fig. 5 -** Effects of different doses of Madhuhari for 15 days on kidney tissue lipid peroxidation (nM MDA/ h/mg protein).

In Fig. 3, 4 and 5, each bar represents the mean± SEM (n=7). Cont (Control), Diab. (Diabetic), D1 (100 mg/kg), D2 (200 mg/kg), D3(400 mg/ kg), D4 (800 mg/ kg) and D5 (1600 mg/kg) body weight. * P<0.0001 compared to control value. $^a$ P<0.0001 and $^b$P<0.001 compared to diabetic group.
IV. Discussion

Results of the present investigation revealed the potential of Madhuhari extract to inhibit alloxan induced hyperglycaemia. However, the effects were concentration specific, as out of five, only two lower doses (100 and 200 mg/ kg/ day) were found to be effective and safe. Although, animals treated with three higher doses also showed some short of antihyperglycemic activity, only 400 mg/ kg exhibited significant effect. With respect to tissue LPO and activity of antioxidants, such as SOD, CAT and GSH; no significant changes were observed suggesting their ineffectiveness, at higher doses. Similarly, results from DPPH and in vitro anti-lipid peroxidation assays indicated an inhibition/ scavenging of free radicals by test drug at different concentrations, but exhibited better inhibition at lower concentrations, again demonstrating and supporting their dose dependent antiperoxidative nature. Such type of concentration dependent inhibition of the drug can be compared with the earlier reports on some other plant extracts [13,15-17].

Carbon tetrachloride (CCl₄) is rapidly converted to trichloromethyl (CCl₂O) by P450-2EL (CY2E1) in microsomes [10], which readily reacts with free oxygen and gets transformed to trichloromethyl peroxyl free radical (CCl₃OO⁻), that in turn interacts with membrane lipids and causes their disintegration and peroxidation [10,15] leading to impairment of membrane fluidity [10]. Therefore, CCl₄ is very often used to induce LPO [15]. In our study also, while, hepatic and renal LPO significantly increased with addition of CCl₄, when incubated along with test drug a decrease in the same was observed, indicating its protective efficacy.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD</th>
<th>CAT</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>4.44±k 0.783</td>
<td>53.41±6.31</td>
<td>5.77±0.863</td>
</tr>
<tr>
<td>Allox.</td>
<td>1.94±k 0.387*</td>
<td>30.00±4.48*</td>
<td>3.11±0.281*</td>
</tr>
<tr>
<td>Allox+D1</td>
<td>5.19±k 0.823*</td>
<td>51.34±5.14*</td>
<td>4.88±0.84k*</td>
</tr>
<tr>
<td>Allox+D2</td>
<td>4.81±k 0.912*</td>
<td>48.88±5.80k</td>
<td>4.69±0.84k*</td>
</tr>
<tr>
<td>Allox+D3</td>
<td>3.39±k 0.748*</td>
<td>37.76±4.74*</td>
<td>3.56±0.78k</td>
</tr>
<tr>
<td>Allox+D4</td>
<td>3.01±k 0.522*</td>
<td>38.59±4.83k</td>
<td>3.37±0.62k</td>
</tr>
<tr>
<td>Allox+D5</td>
<td>2.11±k 0.488</td>
<td>26.78±3.32</td>
<td>2.62±0.52k</td>
</tr>
</tbody>
</table>

Data are mean ±SE (n=7); * p<0.001 as compared to the respective control values. ** p<0.01, # p<0.001 and * p<0.0001 compared to respective value of the diabetic group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (gm)</th>
<th>Water intake (ml/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27.42±k 1.616</td>
<td>13.67±k 1.47</td>
</tr>
<tr>
<td>Allox.</td>
<td>28.14±k 0.799</td>
<td>28.72±k 1.49*</td>
</tr>
<tr>
<td>Allox+D1</td>
<td>28.28±k 1.016</td>
<td>24.66±k 0.360</td>
</tr>
<tr>
<td>Allox+D2</td>
<td>27.72±k 0.300</td>
<td>24.75±k 0.448</td>
</tr>
<tr>
<td>Allox+D3</td>
<td>28.80±k 0.828</td>
<td>27.00±k 0.448</td>
</tr>
<tr>
<td>Allox+D4</td>
<td>29.55±k 0.431</td>
<td>29.15±k 0.723</td>
</tr>
<tr>
<td>Allox+D5</td>
<td>29.57±k 0.366</td>
<td>26.38±k 0.495</td>
</tr>
</tbody>
</table>

Data are mean ±SE (n=7); ** P< 0.005, *** P< 0.001 and * P< 0.0001 as compared to control group. "MDA is a well known terminal product of lipid peroxidation, which is predominantly used to estimate the extent of LPO. In this study, a significant increase in tissue LPO following alloxan treatment suggested the induction of tissue damage [27]. These results are consistent with the results reported by others [15,16,20,26]. Decrease in final BW and an increase in water intake were also in accordance with the earlier findings [10,20,22] that also confirmed the induction of diabetes in the experimental animals.

Alloxan is an oxygenated pyrimidine derivative beta-cytotoxin, also well known for its toxic effects on other body organs as well as for its diabetogenic activity [7,8,27]. In our study also, alloxan induced the tissue LPO. But in drug treated groups (100 and 200 mg/ kg/ day) a significant decrease in serum glucose, hepatic and...
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renal LPO, and water intake was observed as compared to the respective values of control animals, suggesting their potential to ameliorate alloxan-induced tissue peroxidation and hyperglycemia. The higher doses were not found to be effective. With respect to alterations in different antioxidative enzymes, alloxan administration decreased the level of SOD and CAT, and in non-enzymatic antioxidant pool of GSH as observed by earlier workers [14,18,19,29]. On the other hand, simultaneous administration of alloxan and Madhuhari extract, particularly at 100 and 200 mg/kg showed most of these changes. But, Madhuhari at higher doses (800 and 1600 mg/ kg/ day) showed a diminished SOD, CAT activities with lesser GSH content as well as no change in BW as compared to that of alloxan treated animals, again revealing their ineffectiveness at relatively higher concentrations.

An increase in main antioxidative enzymes such as SOD, CAT and GSH following the administration of lower doses of test drug indicate the enhancement of anti-oxidative process that might have helped in scavenging excess superoxide and peroxide anions as suggested earlier [21,30,31]. From these findings it can be hypothesized that the alloxan-induced free radical overproduction might have been counteracted by the drug-induced increase in biosynthesis of antioxidant enzymes [10,16,31].

Glutathione is a tripeptide, γ-L-glutamyl-L-cysteinyl-glycine, found in all mammalian tissues [28]. It serves as a cofactor in the GPx mediated destruction of hydroperoxides, which protects membrane against oxidative damage. In this study, we noted marked reduction in GSH levels in alloxan treated mice, which reflects its higher consumption in the oxidative stress [18,29,30]. However, lower two doses of the test drug triggered the GSH synthesis and turnover rate, as indicated by increased GSH content in both liver and kidney. Thus, in the present investigation test drugs might have up-regulated the synthesis of antioxidants, as a self-protective response against oxidative stress [32].

It appears that the drug might be acting through multiple mechanism(s) on different body organs. Because, it normalized alloxan induced serum glucose level, it seems that the test drug may have β-cells protective and/or β-cells proliferative activities which enhance insulin secretion. In fact, its individual herbal components were also also reported earlier to posses similar properties [28,33]. Some reports indicated that its components inhibit gluconeogenesis or glycogenolysis, which exert their antidiabetic actions as suggested earlier [18,32]. We also clearly observed positive changes in the antioxidative activities. As diabetes is mainly coupled with the free radical induced β-cell destruction [32] it seems that the test drug acts through scavenging free radicals. This possibility is further consolidated by the presence of rich amounts of polyphenols and flavonoids in test drugs, which are antioxidative in nature [33]. Since it has been found that antidiabetic and antioxidative properties of herbs and many herbal mixtures are associated with the presence of different phytochemical such as polyphenolic compounds, flavonoids, terpinoids etc [10,16,34-36], the findings can be supported by high content of these phytochemicals [35,36]. Interestingly, reduced efficacy of the test drug was seen at higher concentration. This can be explained by the fact that various polymerization reactions take place in phytochemicals present in the poly herbal formulation that in turn result in the alterations in their chemical structure, spatial arrangements and finally their action as therapeutic agent. In fact, flavonoids also show dose dependency in their action [6,35,36].

Reviewing all these findings we conclude that the test drug, Madhuhari not only ameliorates diabetes mellitus, but also its associated lipidperoxidation in liver and kidney tissues,primarily through a reduction in the tissue oxidative stress. However, its higher concentrations should be avoided as they were found to be less effective and were not protective from alloxan-induced peroxidation of tissues.

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