

Research Article



Concentration Dependent Anti-oxidative and Pro-oxidative Effects of Madhumehari in *in-vitro* and in Alloxan Induced Diabetic Mice Model

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Accepted on: 26-09-2013; Finalized on: 30-11-2013.

ABSTRACT

It is believed that oxidative damage is a primary reason for diabetes mellitus. In the present study, aqueous extract of a polyherbal formulation, Madhumehari in different concentrations was evaluated for its antioxidative and anti-hyperglycemic activities. The *in vitro* antioxidative activity of the drug was determined by 1,1-Diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO) and carbon tetrachloride (CCl₄) induced lipid peroxidation (LPO) assays; while, for the study of acute and subacute effects, healthy and alloxan induced diabetic male mice were used, respectively. In all *in vitro* assays the test drug showed significant antioxidative activity in concentration dependent manner. While in subacute drug study, diabetic mice showed increased levels of serum glucose, tissue LPO and water intake (p<0.001 for all) with a decrease in body weight (p<0.001), administration of different doses of the drug extract to diabetic mice showed significant (p<0.001) improvement in all these indices. However, as compared to higher doses, lower dose was found to be more effective. These findings indicated that for the treatment of diabetes mellitus Madhumehari is beneficial at lower concentration and this might be acting through free radical scavenging and/or inhibitory activity. Present findings also suggest that herbal drug may produce dose specific harmful effects. Obviously, one should avoid intake of un-recommended higher doses.

Keywords: Antioxidant, Diabetes mellitus, DPPH, Madhumehari, Oxidative stress, Polyherbal Formulation.

INTRODUCTION

Globally, people are suffering from number of metabolic diseases including diabetes mellitus (DM) and cardiovascular disorders.¹ To cure them man has been using natural as well synthetic drugs from ages. As, a number of adverse effects related to allopathic drugs have been reported,^{2,3} to get safer and effective treatment many people turned towards ayurveda. Recent literature revealed that 80% of the world population avail herbal drugs in different forms and their uses are increasing at a rate of 10-20% every year.² DM is an important human ailment affecting many from various walks of life and proving to be a major health problem. To cure this disorder ayurvedic drugs are believed to be more appropriate and natural for centuries and widely adopted by people.⁴

Over production of free radicals is a prominent factor which is known to initiate and elevate diabetic complications.⁵ Auto-oxidation of glucose due to persisting hyperglycaemia is also one of the major causes of free radical generation, which promotes polyol pathway, prostanoid synthesis, non specific-non enzymatic protein glycation and carbonylation.⁶ Then again, abnormally higher blood glucose enhances lipid peroxidation (LPO) of low density lipoprotein by a superoxide dependent pathway.⁷

Antioxidants are known to protect cells from these free radicals either by inhibiting their generation or by enhancing their removal. Thousands of herbs have been reported to have antioxidative property.^{4,8,9} Similarly, a lot of anti-hyperglycemic herbal drugs are also known to improve enzymatic and/or non-enzymatic antioxidant

potency of the tissues, that protect tissues particularly β -cells which are more vulnerable for oxidative stress.^{7,10} Although, herbal products are thought to be harmless and often used as self medication or over-the-counter drugs,⁹ some reports regarding their harmful effects have also came to light.^{2,11-13} In fact, many of the different polyherbal combinations, used in India as antidiabetic agents are not supported by scientific evidence.^{14,15} Some dose specific undesirable effects of few herbal drugs are also reported.^{3,11} Hence, there is a need to establish scientific evidences regarding safety concern of polyherbal formulation also. Therefore, apart from *in vitro* free radical scavenging assays this study was aimed to find out the effects of graded doses of Madhumehari on blood glucose and LPO on liver and kidney, as these organs play major role in drug metabolism and detoxification.¹⁶ Madhumehari is a commercially available polyherbal mixture of about 16 well known antidiabetic herbs. Though, its antidiabetic activity has been reported,¹⁷ with respect to adverse drug reaction (ADR) no study has been made so far. Particularly, effects of higher doses of this test drug have not been evaluated till now.

MATERIALS AND METHODS

Chemicals and drug

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, St. Louis, MO, USA. Greiss reagent, sodium nitroprusside, phosphoric acid and thio-barbituric acid (TBA), ascorbic acid (vitamin C) were supplied by Hi Media Laboratories Ltd., Mumbai, India. Malondialdehyde (MDA), CCl₄ and all other reagents were purchased from E-Merck Ltd., Mumbai, India. Madhumehari (Baidhyanath



Co., Nagpur - 441107, India. Batch No. 110060) was purchased from authorized medical store of local market, Indore, M.P. and used as aqueous extract.

Animals

Healthy in-bred Swiss albino male mice (2-2.5 months old) were housed in polypropylene cages under constant temperature ($27\pm 2^\circ\text{C}$) 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. The approval of departmental ethical committee for handling and maintenance for experimental animals was also obtained before starting the experiments.

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.¹⁸ and gallic acid was used as standard. The results were expressed in mg gallic acid equivalent /100 g dry weight of the drug. The coefficient of determination was $r^2 = 0.979$. Total flavonoid was determined by following the method of Leontowicz et al.¹⁸ as done earlier.^{16,19,20} The coefficient of determination was, $r^2 = 0.967$ and results are expressed as mg of quercetin equivalents/ 100 g dry weight of the extract.

DPPH assay

DPPH free radical scavenging potency was measured by following the protocol of Leontowicz et al.¹⁸ This assay is based on electron transfer, which results change in colour of final reaction mixture. The methanolic stock solutions of different concentrations of drug (5-160 $\mu\text{g/ml}$) were prepared. In reaction mixture 0.5 ml freshly prepared DPPH (0.15 mM) and 1 ml of drug were mixed incubated for 30 min at 20°C . Ascorbic acid was used as standard and percentage (%) scavenging activity was determined using formula, [%RSA = $100 \times (\text{control OD} - \text{sample OD}) / \text{control OD}$].

NO assay

NO free radical scavenging efficacy was estimated by following the method of Marcocci et al.²¹ In this assay, sodium nitroprusside was used as a nitric oxide free radical donor. In assay mixture 0.5 ml sodium nitroprusside (10 mM in 0.2 M PBS, pH 7.4) was added with 0.5 ml of different concentrations (5-160 $\mu\text{g/ml}$) of drugs and incubated for 150 min at 20°C in dark. Then 1 ml Griess reagent was added and OD was taken at 542 nm against blank. Different concentrations of drug were prepared. The NO scavenging (%) activity was measured with respect to control. NaNO_2 was used to prepare standard curve and ascorbic acid was used as standard antioxidant.

LPO assay

LPO was determined by protocol of Ohkawa et al.,²² as routinely followed in our laboratory.^{11,16,19,20} In brief, liver and kidney were excised from healthy mice, washed, chopped and homogenized with Phosphate buffer saline (0.1 M, pH 7.4) to get 10% w/v homogenate. Different concentrations of CCl_4 (25, 50 and 100 μl) in 1 ml (10% w/v) tissue homogenate was used to induce LPO. Finally, considering the most effective concentration of CCl_4 (50 μl) antiperoxidative effect of the test drug was evaluated. As no report was available on *in vitro* study of Madhumehari, wide range of concentrations of the test drug was used in all above assays. LPO was measured in term of nM MDA formed/hr/mg protein. The experiment was repeated with effective concentration of drug to verify the results.

Acute oral toxicity study

Acute oral toxicity study was carried out in young healthy female mice using the 'Limit dose test of up and down procedure' (UDP) according to Organization for Economic Corporation and Development guidelines 425. Dose up to 2000 mg/kg body weight (bw) was given in an increasing dose order and animals were checked for general behavioural, physical and autonomic changes.

Sub acute oral toxicity study

Forty two healthy mice were divided into six groups of seven mice each and acclimatized for one week. Animals of groups 2-6 were rendered diabetic by single intraperitoneal injection of alloxan (150 mg/kg, in normal saline), whereas group 1 (control) animals were injected with normal saline. Hyperglycaemia was confirmed after 72 hours of alloxan treatment (Glucochek glucometer, Aspen Diagnostic, Delhi). Then, animals of group 3rd, 4th, 5th and 6th were treated with Madhumehari at doses of 100, 200, 400, and 600 mg/kg/po respectively; while, group 1st and 2nd were administered with an equivalent amount of distilled water (the vehicle) for next 15 days. Dose was given at a fixed time (10:00-11:00 AM) of the day to avoid circadian variation, if any. Body weight and water intake were measured. On the last day overnight fasted animals were sacrificed by cervical dislocation, blood and tissues were collected and processed for different biochemical estimations.

Biochemical estimations

Serum glucose level was measured by the glucose oxidase/peroxidase method.^{16,19,20} LPO was determined by TBARS method of Ohkawa et al.,²² and expressed as nM MDA formed/h/mg protein. For protein estimation the method of Lowry et al.²³ was followed.

Statistical analyses

Data are expressed as mean \pm SE. Statistical analysis was done by using one-way ANOVA followed by unpaired student's t-test and p-values of 5% and less were considered as significant. Values of polyphenolic and

flavonoid compounds were calculated from the linear regression curve equation.

RESULTS AND DISCUSSION

The amount of total polyphenols and flavonoids in the test drug were found to be 50.42 ± 2.96 mg gallic acid equivalent/100 g dry weight and 71.21 ± 12.39 mg quercetin equivalents/100 g dry weight of the drug, respectively. In DPPH assay Madhumehari showed significant antioxidant activity in concentration

dependent manner and the highest scavenging activity was found to be 85.89% at 80 $\mu\text{g/ml}$ of the drug that is comparable to the standard antioxidant. Similar pattern was observed in NO scavenging assay where highest inhibition (88.67%) was obtained at 40 $\mu\text{g/ml}$ of the drug. Unpredictably in both assays, test drug at higher concentration showed decreased % free radical scavenging activity ($p < 0.005$) than lower concentration (Table 1).

Table 1: 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) free radical scavenging activities (in %) of test drug at different drug concentrations as compared to a standard, ascorbic acids.

Drugs ($\mu\text{g/ml}$)	5	10	20	40	80	160
DPPH assay						
Madhumehari	27.42 ± 0.31	40.58 ± 0.44	55.30 ± 0.61	72.68 ± 0.79	85.89 ± 0.46	76.02 ± 0.83^a
Ascorbic acid	29.70 ± 0.35	49.96 ± 0.43	80.34 ± 0.73	87.56 ± 0.95	95.55 ± 0.98	96.98 ± 1.06
NO assay						
Madhumehari	71.45 ± 0.64	76.89 ± 0.78	83.67 ± 1.04	88.67 ± 0.89	81.47 ± 0.47^a	67.96 ± 0.55^b
Ascorbic acid	78.74 ± 0.53	84.55 ± 0.52	86.89 ± 0.94	92.89 ± 0.77	94.91 ± 0.93	94.71 ± 0.86

Values are given as mean \pm SEM (n=3); ^a $p < 0.001$ less effective than the highest inhibitory concentration of Madhumehari.

Table 2: Induction of LPO (in nM MDA/h/mg protein) in tissue homogenates by CCl_4

Tissue	Control	25 μl	50 μl	100 μl
Liver	1.84 ± 0.046	$2.97 \pm 0.088^{**}$ (61.41%) \uparrow	$3.45 \pm 0.068^{**}$ (87.50%) \uparrow	$2.56 \pm 0.149^*$ (39.13%) \uparrow
Kidney	2.42 ± 0.039	$3.14 \pm 0.076^{**}$ (29.75%) \uparrow	$3.89 \pm 0.089^{**}$ (60.74%) \uparrow	$3.42 \pm 0.269^*$ (41.32%) \uparrow

Values are given as mean \pm SEM (n= 3). * $p < 0.05$ and ** $p < 0.01$ as compared to the respective control values.

Table 3: Inhibition of LPO (nM MDA/h/mg protein) by different concentrations of Madhumehari (mg/ml), induced by CCl_4 (% inhibition)

Tissue	Cont.	CCl_4	$\text{CCl}_4+0.30$	$\text{CCl}_4+0.60$	$\text{CCl}_4+1.25$	$\text{CCl}_4+2.5$	$\text{CCl}_4+5.0$	CCl_4+10
Liver	2.01 ± 0.12	4.98 ± 0.25^p (146.65%) \uparrow	2.73 ± 0.02^c (45.18%) \downarrow	1.93 ± 0.04^c (61.24%) \downarrow	1.62 ± 0.04^c (69.47%) \downarrow	1.83 ± 0.06^c (63.25%) \downarrow	$2.34 \pm 0.15^{c,p}$ (53.01%) \downarrow	$3.86 \pm 0.27^{a,p}$ (22.48%) \downarrow
Kidney	2.53 ± 0.075	3.96 ± 0.073^p (56.52%) \uparrow	2.57 ± 0.06^d (35.10%) \downarrow	2.22 ± 0.05^d (43.93%) \downarrow	1.79 ± 0.04^d (54.79%) \downarrow	1.03 ± 0.07^d (73.88%) \downarrow	1.65 ± 0.176^d (58.33%) \downarrow	$2.81 \pm 0.08^{b,p}$ (22.97%) \downarrow

Values are given as mean \pm SEM (n= 3). ^p $p < 0.001$ as compared to the respective control values. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ and ^d $p < 0.0001$ compared to respective CCl_4 treated values.

Table 4: Effects of different concentrations of Madhumehari on body weight (% change) and water intake (ml/day)

Parameters	Cont.	Diab	LD	MD	HD
Body weight					
On 8 th day	$+1.16 \pm 0.16$	-6.14 ± 0.20	-4.12 ± 0.13	-7.18 ± 0.11	-6.81 ± 0.19
On 16 th day	$+3.07 \pm 0.67$	-12.28 ± 0.85^a	$+1.27 \pm 0.039$	$+0.58 \pm 0.12$	-10.73 ± 0.56^a
Water intake					
On 8 th day	3.17 ± 0.41	6.89 ± 0.78	$5.49 \pm 0.56^*$	6.46 ± 0.39	6.83 ± 0.62
On 16 th day	3.23 ± 0.36	$8.11 \pm 0.88^{**}$	$4.56 \pm 0.74^{**}$	$6.73 \pm 0.65^*$	8.24 ± 0.87

Values are given as mean \pm SEM (n=7). Cont= Normal cont, Diab= diabetic control, LD= low dose (100 mg/kg), MD= medium dose (200 mg/kg) and HD= high dose (400 mg/kg). ^a $p < 0.001$, as compared to respective initial body weight. * $p < 0.01$ and ** $p < 0.001$ as compared to normal control value.

Upon treatment with CCl_4 a significant increase in LPO ($p < 0.001$) was seen with all its concentrations, but maximum increase was found at 50 μl of CCl_4 in both liver ($p < 0.001$) and kidney ($p < 0.001$) homogenates (Table 2). Therefore, this concentration was used for further experiments. Here again, concentration dependent

inhibition was observed. However, the highest inhibition (69.47% and 73.88%) was found at 1.25 mg/ml ($p < 0.001$) and at 2.5 gm/ml ($p < 0.001$) in liver and kidney respectively, as compared to the values of respective CCl_4 treated homogenates. Moreover, a significant ($p < 0.05$) decreased anti-peroxidative activity was seen at higher

concentrations of test drugs in both hepatic and renal tissues (Table 3).

In acute oral toxicity study, no mortality was found up to the dose of 2000 mg/kg body weight, while with subacute dose a significant increase in serum glucose, tissue LPO in both the studied organs of diabetic animals was found as compared to that of normal animals. Unpredictably, 100% mortality was seen in the animals of 6th group at different time intervals. However, significant antidiabetic effects were found at all other doses but the lowest dose (100 mg/kg bw) which was observed to be the most effective and safe ($p < 0.05$) one (Figure 1 & 2). Daily water intake and the bw were also nearly normalised in drug treated mice (Table 4).

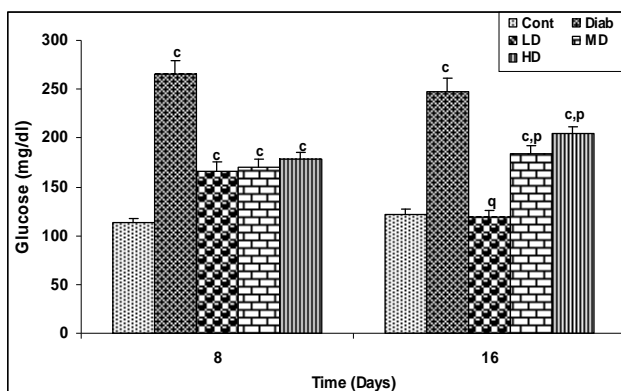


Figure 1: Effects of different drug doses on fasting blood glucose on 8th and 16th day. Values are given as mean \pm SEM (n=7). Cont= Normal cont, Diab= diabetic control, LD= low dose (100 mg/kg), MD= medium dose (200 mg/kg) and HD= high dose (400 mg/kg). ^a $p < 0.01$ ^b $p < 0.001$ and ^c $p < 0.001$, increase in fasting blood glucose as compared to the respective control group, whereas ^p $p < 0.05$ and ^q $p < 0.001$ decrease in fasting blood glucose as compared to the respective values of diabetic group.

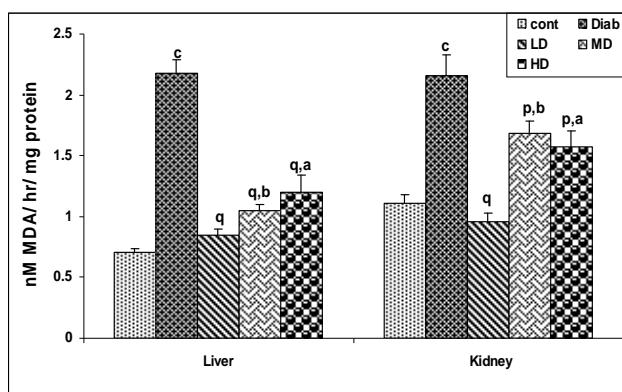


Figure 2: Effects of different drug doses on lipid peroxidation (LPO). Values are given as mean \pm SEM (n=7). Cont= Normal control, Diab= diabetic control, LD= low dose (100 mg/kg), MD= medium dose (200 mg/kg) and HD= high dose (400 mg/kg). ^a $p < 0.01$ ^b $p < 0.001$ and ^c $p < 0.0001$, increase in LPO as compared to the respective control group, whereas ^p $p < 0.05$ and ^q $p < 0.001$ decrease in LPO as compared to the respective values of diabetic group.

Our finding clearly revealed the concentration dependent antioxidative potential of test drug, which could be a reason for its anti-hyperglycemic activity. Interestingly, the lowest dose of drug was found to be more effective and safe than the higher doses. In addition, *in vitro* results also revealed its concentration specific antioxidative effects.

Both DPPH and NO are synthetic free radicals and are commonly used to evaluate antioxidative potential of drugs.¹⁹ As NO at higher concentration is known to severely damage β -cells of pancreas and enhances the probability of DM,^{4,10,24} drugs having better NO inhibition efficacy may be considered as more effective *in vivo* antioxidant.²⁵⁻²⁷ Interestingly, in both assays test drugs showed noticeable free radicals scavenging activity.

In microsomal system, CCl₄ is rapidly converted to trichloromethyl peroxy free radical (CCl₃OO \cdot), that interacts with membrane lipids and causes their disintegration and peroxidation.^{20,28,29} Therefore, it is very often used to induce LPO.²⁰ In our study also in CCl₄ treated tubes increased LPO was observed in both tissues, which was found to be decreased upon drug treatment. Since, the LPO inhibitory efficacy was observed to be concentration dependent, here again higher concentrations were found to be less effective.

Alloxan induced increase in serum glucose, tissue LPO and in body weight are consistent with the earlier reports.^{16,19,28} In drug treated groups with respect to glucose, hepatic and renal LPO, a significant decrease was noticed at all test doses indicating its diabetes ameliorating potential. In fact, both *in vitro* and *in vivo* studies revealed concentration specific protective effects. Higher doses were less effective, rather high concentration appeared to be toxic and exerted undesirable effects on the tested tissues after chronic treatment. Such observations can be explained by earlier studies, which proved that the antidiabetic and antioxidative properties of herbs are due to the presence of different phytochemicals such as polyphenols, flavonoids, terpenoids etc.^{11,19,30} In this study also results can be supported by high content of these phytochemicals.^{31,32} Thus, from the present findings it can be predicted that in physiological systems the test drug either up-regulates the synthesis of antioxidants, as a self-protective response against oxidative stress or might be playing a role in direct free radical scavenging. Although, antidiabetic activity of the test drug has been reported by,¹⁷ to the best of our knowledge it is the first report which revealed *in vitro* and *in vivo* antioxidative and antiperoxidative activities as well as its dose dependent effects on tissue LPO.

Here, lower concentration of Madhumehari observed to be more beneficial and decreased efficacy of test drug at higher dose can be compared with earlier reports with other herbal drugs^{31,33} where higher concentration of polyphenolic and flavonoid compounds proved to be toxic. This possibly happened due to herb-herb

interaction within pharmacological combination; which ultimately alter the bioavailability and therapeutic activity of active component of drugs.³⁴

CONCLUSION

From this study, it can be concluded that Madhumehari is a potent antidiabetic herbal formulation and proved to be effective and safe against hyperglycaemia and oxidative stress particularly, at lower dosage. We suggest that its higher dose should be avoided, in which it causes adverse effects. Therefore, to improve the safety and consistency of herbs and to define the pharmacology, stability and safety of the test drug, additional research is needed.

Acknowledgement: Financial support from University Grant Commission (UGC), New Delhi, India (NET – JRF, Reference No. 2120930513/ 20-12-2009 EU IV), is gratefully acknowledged.

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Source of Support: Nil, **Conflict of Interest:** None.