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DETERMINATION OF HYPOGLYCAEMIC EFFECT OF NATURAL PLANTS AND CALLUS CULTURES OF *MUNRONIA PINNATA* IN TYPE 2 DIABETIC SUBJECTS

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ABSTRACT

Background: *Munronia pinnata* is therapeutically important medicinal plant used for various ailments in traditional medicine in Sri Lanka. *In-vitro* propagation techniques has been practiced due to high demand of this plant. Our previous research has been proved the possible use of callus cultures (calli) as a substitute for whole plant in diabetic animals. **Aim of the study:** The present study was carried out to determine the oral hypoglycemic activity of the aqueous extract of whole plant (MPaq) and calli (MPCaq) of MP in type 2 diabetic patients. **Materials and Methods:** This study was a randomized controlled clinical trial, conducted on 90 newly diagnosed type 2 diabetic patients on diet controlled of either sex for three groups. The two groups were treated with 120.0 mL of MPaq and MPCaq twice a day for three months. The control group was managed with diet control and daily exercises. Glucose challenge, HbA_{1c}, serum insulin levels, liver enzymes and creatinine were tested before and after treatment. **Results:** MPaq significantly lowered (23.30%, $p \leq 0.01$) the serum glucose levels without any adverse effect in diabetic patients compared with the diet control group. MPCaq also reduced the serum glucose levels in diabetic patients, it was not as high as the MPaq (6.60%; $p \leq 0.5$). HbA_{1c} and serum insulin levels were also significantly ($p \leq 0.01$) lowered than the basal level of treatment. **Conclusions:** The MPaq and MPCaq have

hypoglycaemic potential with no adverse effects in Type 2 diabetic patients on diet control and scientifically validated the traditional use of this *M. pinnata* as a hypoglycaemic agent.

KEYWORDS: *Munronia pinnata,* callus culture, aqueous extracts, Type 2 diabetic patients; hypoglycaemic, insulin, HbA_{1c}.

INTRODUCTION

Diabetes mellitus (DM) is one of the most prevalent health problems and the International Diabetes Federation (IDF) estimates that there will be at least 629 million people living with diabetes by 2045.Globally, as of 2011, an estimated 366 million people had DM, with type-2 diabetes (T2DM) making up about 99% of the cases.[1] The number of people with T2DM is increasing in every country with 80% of people with DM living in every population in the world and in all regions, including rural parts of low and middle income countries like Sri Lanka.[2] It is now generally agreed that the most patients of this disease have T2DM, characterized by insulin resistance and β- cell dysfunction, leading to ultimate pancreatic β-cell failure.[3] There is an increasing demand of patients to use natural anti-diabetic agents due to expensive and associated with several complications of synthetic ant diabetic agents in the market. Therefore, herbal medicines are gaining importance as they are cost- effective and also display improved therapeutic effects with lesser side effect.[4]

Due to hypoglycaemic property, many plant species are used for the treatment of diabetes mellitus in traditional or folk medical systems in Sri Lanka. Different parts of the plants or the plant as a whole have been used in the treatment. There is a paucity of published studies on the active ingredients of these herbs. Studies on active ingredients could pave the way to development of new drugs with low cost compared to synthetic drugs.[5] As people are adopted to consumption of locally available herbs either as food or as a remedy, side effects of such newly developed drugs would be minimal. Therefore evaluation of pharmacology of various plants used in traditional systems of medicine is in need.[6] Traditional physicians claim that *M. pinnata* has been used in folk medical practices in Sri Lanka for hundreds of years to treat inflammatory conditions and diabetes mellitus.[7,8,9,10] Further, it has been used as a cure for fever, dysentery, tuberculosis, cough, stomachache, sores, malaria, purification of blood, and skin diseases as a substitute for *Swertia chirata*, (Gentianaceae), which is not a native plant in Sri Lanka.[10]

Investigators reported that the genus *Munronia* is responsible for an array of biological activities such as antibacterial; anti-inflammatory, antipyretic and a number of other pharmacological activities on human being due to the presence of limonoids and triterpenoids.[11]

It has been reported that aqueous and ethanol extracts of *M. pinnata* have the dose dependent hypoglycaemic activity in diabetic induced and healthy Wistar rats.[12, 13, 14] Results of acute and chronic toxicity study indicated that the extract of *M. pinnata* is safe and there are no significant changes in levels of key hepatic enzyme and other haemotological parameters.[15]

Due to high demand, whole plants are uprooted from its natural habitat for medicinal purposes in Sri Lanka and there has been no any practice for commercial cultivation of this plant. As a result of repeated harvesting from its natural resources, this plant has become endangered and if protective measures are not taken, it will face to threat of becoming extinct in Sri Lanka. Further, seed production of *M. pinnata* is low and it is difficult to carry out large scale propagation through seeds.

There is no practice of using stem cuttings. Previous studies have revealed the possibility of propagating of *M. pinnata* through *in-vitro* hypocotyle callus culture and leaf callus culture.[16, 17] To the best of our knowledge, no work has been reported on clinical study on the biological activities of callus cultures of this valuable medicinal plant.

Therefore, the present study was undertaken to determine the clinical efficacy and safety of the aqueous extract of natural plant (MPaq) and callus cultures (MPCaq) of *M. pinnata* in Type 2 diabetic patients. Such investigations would help to establish more rational use of *M. pinnata* as a drug for long term glucose homeostasis without any adverse effects.

MATERIALS AND METHODS

Study design: Randomized controlled trial.

Study setting

This study was conducted at the Institute of Indigenous Medicine, University of Colombo, Rajagiriya and the National Ayurveda Hospital, Sri Lanka. The protocol of the study was evaluated and approved by the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura (No: 592/11) and registered under the Sri Lanka Clinical Trials Registry (SLCTR/2013/040). Sri Lanka Clinical Trial Registry is linked to the Registry

Network of the International Clinical Trial Registry platform of the world Health Organization (WHO-ICTRP). All subjects (healthy volunteers and type 2 diabetic patients on diet control) provided written informed consent before screening procedures were performed.

Plant material and preparation of extracts

Fresh *M. pinnata* plants were maintained in the greenhouse. Species was taxonomically identified and authenticated by the National Herbarium, where a voucher specimen was deposited (PDA/ MP 01). The aqueous extracts of whole plants and calli (three months old fresh calli) of *M. pinnata* were prepared according to the conventional method used by traditional medical practitioners in Sri Lanka.[8] Air dried coarsely powdered whole plants and *calli* of MP (60.0 g or 12 *kalan)* was mixed with 8 parts (1920.0 mL) of water in an earthen vessel and boiled over moderate heat and reduced to 1/8th part to prepare MPaq and MPCaq extracts. The dose is 120.0 mL twice a day (240.0 ml/day).[8]

Determination of the oral hypoglycaemic activity of MPaq and MPCaq extracts in healthy volunteers

Sixty healthy volunteers (*n* =30/group) were selected for two groups through open advertisement. They were fasted overnight and fasting serum glucose concentrations were assessed using the glucose oxidase reagent kits(Biolabo reagents, France). Baseline values of serum alanine amino transferase (ALT), aspartate amino transfrease (AST), gamma glutamyltransferase (ɤ GT), and creatinine levels were measured by using the reagents kits (Biolabo reagents, France). Serum levels of Alkaline phosphatase (ALP) were measured by using the reagent kits from Stanbio Laboratory, Texas. Creatinine clearance was calculated using Cockroft-Gault equation.[18]

All subjects received distilled water as the control. Thirty minutes later, 75.0 g glucose in 300.0 mL water was administered. Serum glucose levels were measured 2h after glucose load. Two groups of subjects received 120.0 mL of MPaq and MPCaq extracts of MP every morning and evening for two weeks, respectively. At the end of two weeks, following an overnight fast, fasting blood glucose concentrations was accessed. All participants were monitored for 1 month for any adverse effects and at the end of one-month serum levels of ALT, AST, ALP, ɤ GT and creatinine were determined. Creatinine clearance was calculated.

Determination of the oral hypoglycaemic activity of MPaq and MPCaq extracts in Type 2 diabetic patients on diet control

Once diagnosis of diabetes mellitus was made on the basis of Ayurveda signs and symptoms, a detailed proforma was used to record the signs and symptoms, complete history of disease, family history and the history of any other diseases/ illnesses. A total of 90 individuals with type 2 diabetes, (both sex) were randomly divided into three groups (n=30 x3) as Group I and II were allocated for MPaq and MPCaq respectively. Group III for diet control only as a control group.After an overnight fast, fasting serum glucose concentrations and glucose challenge were assessed using the glucose oxidase reagent kits (Biolabo reagents, France). Baseline values of serum alanine amino transferase (ALT), aspartate amino transferase (AST), gamma glutamyltransferase(ɣ GT), and creatinine levels were measured by using the reagents kits (Biolabo reagents, France). Serum levels of Alkaline phosphatase (ALP) were measured by using the reagent kits from Stanbio Laboratory, Texas. Creatinine clearance was calculated.

The patients in group I and II (n=30/group) were treated with the MPaq and MPCaq extracts of *M. pinnata* (120.0 mL) respectively, every morning and evening for three months continuously with diet control. The Group III (control group) was advised for diet control as practiced for three months. All patients were informed on diet as indicated in the national guidelines for the management of Type 2 diabetic subjects (SLMA, 2000). Patients were monitored during the study period for any adverse effects. At the end of three months, fasting blood glucose and glucose challenge were determined. The subjects were monitored for another three months for any adverse effects and serum ALT, AST, Gamma GT and creatinine levels were determined using commercial reagent kits at the end of three months (Biolabo reagents, France). Creatinine clearance was also calculated.

Inclusion criteria

- Type II diabetic patients only on diet control.
- Ability to read and understand Sinhala or English.
- Newly diagnosed people with type 2 diabetes; between 30 to 65 years of age of both sexes.
- Not on insulin therapy.
- Not taking medicine for other health conditions.
- Fasting blood glucose levels between 7.8 and 22.2 mmol/L

Exclusion criteria

- Type II diabetic patients below 30 and above 65 years or pregnant and lactating diabetic patients. Type 1 diabetic.
- Patients on any oral hypoglycaemic medication.
- Macro vascular complications - stroke or myocardial infarction or both.
- Micro vascular complications - confirmed diabetic retinopathy confirmed diabetic nephropathy/ elevated arterial blood pressure/ confirmed kidney failure/ confirmed diabetic neuropathy. Patients on insulin.
- Patients on herbal medications known to have an effect on blood glucose.
- Patients who refuse consent.

Determination of the serum insulin levels of the diabetic patients

Serum insulin levels and glycated haemoglobin levels (HbA_{1c}) were tested in the Type 2 diabetic patients who were recruited in the study (group I, II and control group). Baseline insulin levels and 2 h post glucose load insulin levels were determined. Separated serum was stored in -20^{0}C until use. Insulin levels were determined by an ELIZA based Reagent Kits.[16] Quantitative colorimetric determination of glycated hemoglobin in whole blood was carried out using Diagnostic kits from Stan bio Laboratory.

Statistical analysis

Results were presented as mean ± SEM and significant differences between means of different groups were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD multiple comparisons. Values were considered statistically significant at $p \leq 0.05$.

RESULTS

Hypoglycaemic effect of MPaq and MPCaq extracts in healthy volunteers

Fig.1. shows the results of the oral hypoglycaemic activity of MPaq and MPCaq in healthy volunteers. Fasting serum glucose (FSG) concentration and glucose challenge were analyzed before and after the treatment. The mean FSG of both groups at the before treatment were 5.43±0.11 and 5.31±0.11 mmol/L. After 2 weeks, the mean FSG concentrations were 4.87± 0.09 and 4.99 ± 0.12 mmol/L respectively. The percent inhibition of serum glucose levels (10.26%) of MPaq was highly significant ($p \leq 0.0001$) when compared with the baseline values. Though, the FSG concentration of MPCaq group was reduced (4.99±0.12 mmol/L)

and percent inhibition of blood glucose concentration was 4.99% at the end of 2nd week. This was not significant when comparing with the baseline values of same group (MPCaq). These results indicate that aqueous extracts of *M. pinnata* have ability to control the glucose level in healthy individuals.

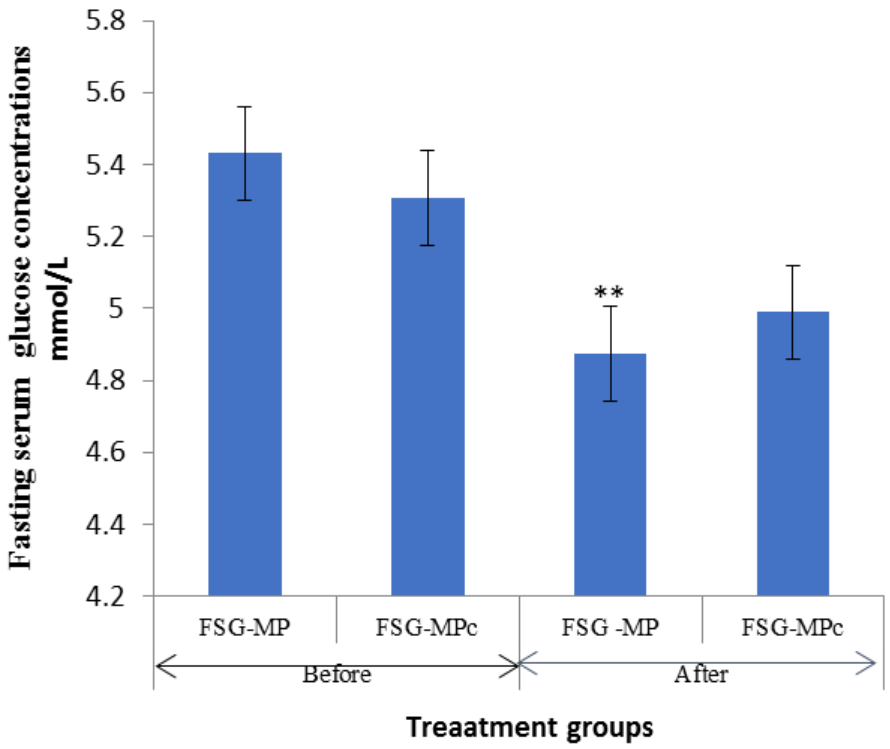

Fig. 1: Effect of oral hypoglycaemic of natural plants (MP) and callus cultures (MPc) of *M. pinnata* in healthy volunteers. (B/T) - before treatment; (A/T) - after treatment, FSG-fasting serum glucose level of conrol group; $^{**}p \leq 0.001$ compared with control.

Hypoglycaemic effect of MPaq and MPCaq extracst in Type 2 diabetic patients on diet control

Following three months of treatment, the average FSG concentration in MPaq and MPCaq groups at the beginning of the study were 7.8 ± 0.1 and 8.0 ± 0.1 mmol/L, which decreased significantly ($p < 0.01$) to 4.98± 0.08 (MPaq) and 7.78 ± 0.18 (MPCaq) compared with the control group (Fig.2). Type 2 diabetic subjects showed a significant reduction (23.30%, $p< 0.01$) in the blood glucose concentrations at2h post glucose load when administered with MPaq. Though the MPCaq reduces the serum glucose concentration in Type 2 diabetic patients, it was not as high as the MPaq extract (6.60%, $p< 0.05$). The average concentration of HBA1c was significantly ($p < 0.01$) lower than the initial level when compared at the end of drug treatment of MPaq and MPCaq (6.38± 0.4 and 7.13 ± 0.3) respectively (Fig. 3).

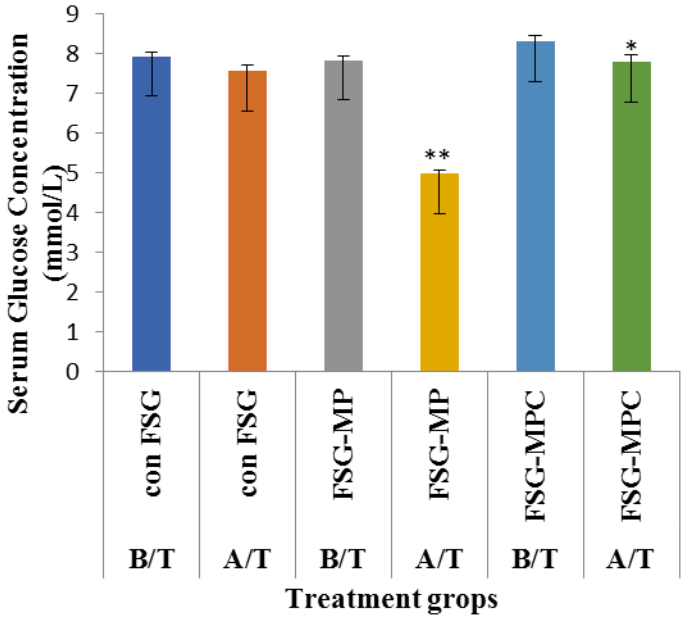

Fig. 2: Serum glucose levels of Type 2 diabetic patients after three months treatment of *M. pinnata* extracts.

Values are expressed as mean ± SEM (n=30 in each group).* $p \leq 0.01$, ** $p \leq 0.001$compared with control.

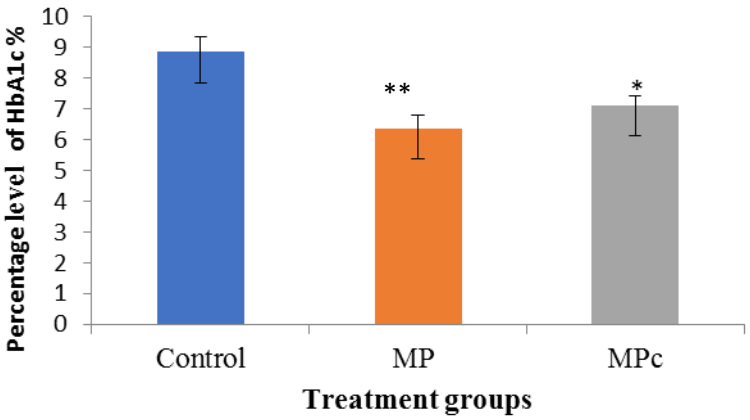

Fig. 3: Glycosylated haemoglobin (HbA_{1c}) concentration after three months of *M. pinnata* treatment in type 2 diabetic patient.

Values are expressed as mean ± SEM (n=30 in each group). * $p \leq 0.01$, ** $p \leq 0.001$ compared with control.

Possible hypoglycaemic mechanisms in Type 2 diabetic patients

Insulin plays a major biochemical role in stimulating the uptake of glucose by different cells of the body for the production of energy.[19] In this study, after three months treatment of both aqeous extracts (MPaq and MPCaq) were able to reduce the serum insulin levels "Fig.4".

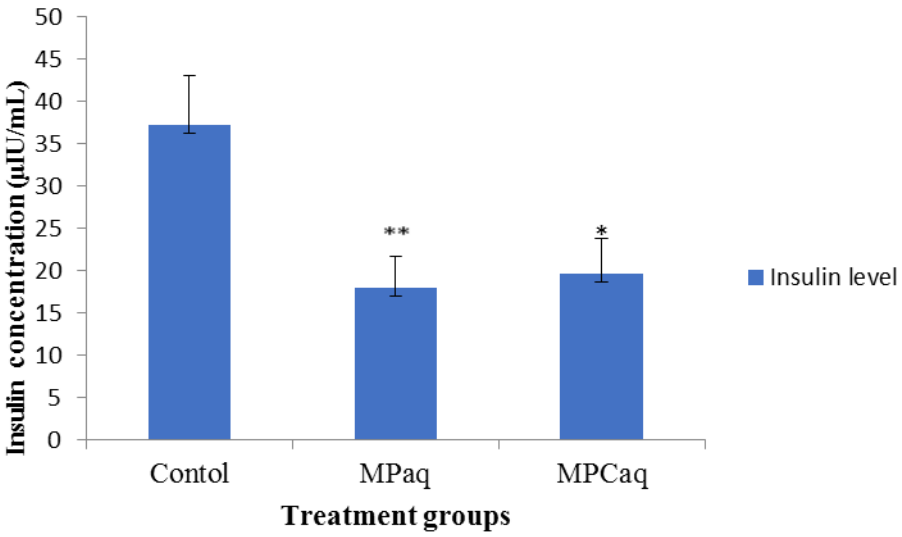

Fig. 4: Insulin concntration of after three months treatment of aqueous extracts ofnatural plants and calli of *M. pinnata* (MPaq and MPCaq) in diabetic pateints.

*p < 0.05 and **p < 0.01 are compared to Control

Effect of multiple doses of MPaq and MPCaq extracts on serum levels of key hepatic enzymes (ALT, AST, ALP) and creatinine

There were no clinically significant adverse reactions reported by either patients or observed by the investigators during the period of treatment. Table 1 shows the serum levels of key hepatic enzymes, creatinine and calculated creatinine clearance following the intervention. No clinically significant changes were observed in serum levels of ALT, AST, ALP, ɤ-GT and creatinine as well as calculated creatinine clearance compared with the baseline values of the test and control groups after 3 months of the treatment (p> 0.05).

Table 1: Effect of multiple doses of MPaq and MPCaq extracts on serum levels of key hepatic enzymes (ALT, AST, ALP) and creatinine.

Biochemical parameter	Group	At base line	At the end of study	*p* value
AST (IU/L)	MPaq	22± 0.5	22.3±1.2	0.4
	MPCaq	23± 0.4	24.0± 0.2	0.2
	Control group	20 ± 0.4	24.5± 3.7	
ALT (IU/L)	MPaq	19± 0.9	18.6± 3.6	0.1
	MPCaq	20± 0.5	21.3± 5.2	0.3
	Control group	20± 0.4	25.1± 4.6	
GGT (mmol /L)	MPaq	41.8±7.8	42.0± 4.0	0.4
	MPCaq	40.3±1.5	41.0± 0.6	0.3
	Control group	40.5±4.2	42.4± 7.1	
Creatinine (mmol/L)	MPaq	88.5±3.1	90.8±4.1	0.3
	MPCaq	87.0±5.7	89.7±3.3	0.2
	Control group	88.9±0.3	93.6±5.3	
ALP (IU/L)	MPaq	62.4±3.2	63.1±6.1	0.2
	MPCaq	65.1±5.3	67.4±4.2	0.1
	Control group	65.2±4.1	68.5±3.4	

Values are expressed as mean ± SEM (n=60). $p < 0.05$ is compared to Control.

MPaq- decoction of the natural plants of *M. pinnata*;

MPCaq - decoction of the calli of *M. pinnata.*

DISCUSSION

In the present study, an attempt has been made to evaluate the clinical efficacy and safety of the MPaq in Type 2 diabetic patients. A significant reduction in the blood sugar level and glycosylated haemoglobin levels in the newly diagnosed Type 2 diabetic patients was observed in this study. Similarly, there was a significant improvement in general symptoms in test group after three months treatment of the MPaq. Further, MPaq was able to reduce the serum insulin levels (Fig.4) in diabetic patients compared to the control group. This observation implies that this herb may potentiate the sensitivity of the insulin receptors for insulin and increase the receptor binding. In general, type 2 diabetics have an elevated level of serum insulin due to resistance of the receptors.[19] Insulin plays a major biochemical role in stimulating the uptake of glucose by different cells of the body for the production of energy.[20] The majority of the herbs act as anti diabetic by increasing insulin secretion and exerting pancreatic mechanisms; such as enhancing glucose uptake by adipose and skeletal muscle tissues, inhibiting intestinal glucose absorption and inhibiting hepatic glucose production.[21] Plants such as *Allium cepa, Clerodendrum phlomoides, Cinnamomum tamala, Coccinia indica, Enicostemma littorale, Ficus bengalensis, Gymnema sylvestre leaves,*

Momordica charantia, *Pterocarpus marsupium* and *Syzygium cumini* exhibit a great anti diabetic potential through various mechanisms.[22,23]

M. pinnata and *Andrographis paniculata* are used for the drug preparations in the traditional and ayurvedic medicine as substitutes for *Swertia chirata* (Family- Gentianaceae), which is not available in Sri Lanka. The hypoglycaemic activities of *S. chirata* and *A. paniculata* have been studied extensively in diabetic and healthy rats. [24, 25] As per our previous animal studies using healthy and diabetic Wistar rats,[12-14] significant hypglycaemic activity of aqueous and ethanol extracts of *M. pinnata* also exhibited without any toxic effect. This is the first time that the clinical efficacy of *M. pinnata* in the management of Type 2 diabetes is reported. The results of key hepatic enzyme assays at the end of three months emphasized that regular treatment with *M. pinnata*is a clinically safe drug for long term treatment for diabetic patients. This is important in the case of hypoglycaemic drugs, which have to be administered over a relatively long period of time in the therapeutic practice in Ayurveda as well as traditional and allopathic medical systems.

In conclusion, both aqueous extracts of MPaq and MPCaq exhibit a significant hypoglycaemic effect in healthy volunteers and type 2 diabetic patients when subjected to glucose challenge. Further, this study demonstrated that the hypoglycaemic potential of *M. pinnata* in healthy and diabetic individuals with no possible adverse effects, and its use in traditional medicine has been scientifically validated. Moreover, these findings suggest that the calli of *M. pinnata* also has good prospects for futher study to apply *in-vitro* propagation techniques to meet the increasing demand of this plant.

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Author Disclosure Statement

The authors have declared that there is no conflict of interest.

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