Hydroalcoholic and Alkaloidal Extracts of Murraya koenigii (L.) Spreng Augments Glucose Uptake Potential against Insulin Resistance Condition in L6 Myotubes and Inhibits Adipogenesis in 3T3L1 Adipocytes

Parameswari Royapuram Parthasarathy¹, Janani Murthy¹, Dinesh Murugan Girija¹, Srivani Telapolu¹, Chamundeeswari Durai-pandian², Thyagarajan Sadas Panatchcharam³

ABSTRACT

Background: Murraya koenigii, commonly known as “curry leaves” is native to India. The highly valued part of the plant is the leaves which possess various biological activities. Objective: The present study aimed to investigate the anti-diabetic effect of Murraya koenigii (MK) leaf extracts, of two different solvent ratios. Materials and methods: 70% hydroalcoholic and alkaloidal extracts of MK leaves were prepared by cold maceration method. Preliminary phytochemical analysis was carried out for both the extracts. In vitro anti-diabetic activity was screened by inhibitory action on α–amylase, α–glucosidase enzymes. Further, the 70% hydroalcoholic and alkaloidal extracts were assessed for glucose uptake potential, anti-adipogenic property, as well as inhibitory activity on diabetes associated complications. HPTLC quantification of major phytoconstituent was carried out. Results: The study showed presence of various phytoconstituents such as, polyphenols, alkaloids, tannins, reducing sugars etc. The 70% hydroalcoholic and alkaloidal extracts of MK leaves exhibited >90% inhibition against carbohydrate metabolising enzymes compared to aqueous and absolute alcohol extracts. Both the extracts showed enhanced glucose uptake in L6 myotubes attenuating the effect of Palmitate induced insulin resistance. Significant inhibition on adipogenesis was exerted by both 70% hydroalcoholic and alkaloidal extracts of MK leaves. Besides, marked inhibition of advanced glycation end products was exhibited by the extracts. HPTLC quantification analysis of the aforementioned extracts showed the presence of major phytoconstituent, Mahanine, in it. Conclusion: The results of the present study showed that MK possesses significant antidiabetic property and also exhibited considerable effect in preventing diabetes associated complications. The potent antidiabetic activity of MK could be attributed to the presence of Mahanine, the major active constituent, which is a carbazole alkaloid.

Key words: Diabetes, Mahanine, α–amylase, glucose uptake, L6 myotubes, complications.

INTRODUCTION

Insulin resistance (IR) is a key contributor for the onset of obesity and type 2 diabetes (T2D). IR is considered as pre-diabetic state, manifested by reduced insulin sensitivity and decreased glucose uptake in peripheral tissues such as skeletal muscle and adipocytes with increased hepatic glucose production, resulting in defective insulin secretion.¹ The imbalanced condition of elevated hepatic glucose production, reduced insulin secretion and compromised insulin action comprehensively lead to oxidative stress which ultimately paves way to long-term complications such as, cardiovascular disease, diabetic retinopathy, diabetic nephropathy and neuropathy.² Therefore drugs that can counteract IR have become the focus of research for the management of T2D. Subsequent to development of unwanted and severe side-effects on long-term usage of various classes of oral hypoglycemic agents and insulin secretagogues, dependence on medicinal plants enriched with bioactive phytoconstituents has geared during recent years to counteract the effects of IR.³

Murraya koenigii (Linn.) Spreng, belonging to Rutaceae family, known commonly as Curry leaves in English is used as an edible spice in Indian kitchens; it possesses significant medicinal values and has been used in traditional medicine either in isolation or in formulations.⁴ The plant is enriched with carbazole alkaloids such as, Mahanine, Mahanimbine and essential oil.⁵ The aerial part of MK has been prescribed for diabetes in Ayurveda.
MATERIALS AND METHODS

Collection of plant material and authentication

The leaves of Murraya koenigii was collected from Tirunelveli district by Dr. V. Chelladurai, Retd Research officer, CCRAS. The leaves were authenticated by Prof. P. Jayaraman, Plant Anatomist Botanists, Plant Anatomy Research Centre, Tambaram (PARC/2015/3167). Further, the leaves were washed thoroughly, shade dried and ground into coarse powder.

Preparation of Extracts

Extraction of leaf (1g) involved cold maceration with 70% ethanol and 30% chloroform in methanol for 72h and the filtrate was collected. Filtered contents were distilled, evaporated and concentrated in rotary vacuum evaporator at 60°C. The extracts were used for chemo profiling and further in vitro assays.

Phytochemical analysis

Qualitative phytochemical analysis was carried out for 70% hydroalcoholic and alkaloidal extracts of Murraya koenigii as per the method of Harbone, 1984.

α – amylase inhibitory activity

In vitro α-amylase inhibition was studied by the method of Bernfeld, 1955. Briefly, 100µL of different concentrations (1, 3, 10, 30, 100 and 1000µg/ml) 70% hydroalcoholic (HA) and alkaloidal MK extracts could react with 200µL of α-amylase enzyme (Hi media RM 638) and 100µL of 2mM of phosphate buffer (pH-6.9). After 20-min incubation, 100µL of 1% starch solution was added. The same was performed for the controls where 200µl of the enzyme was replaced by buffer. After incubation for 5 min, 500µl of Dinitrosaliclyc acid reagent was added to both control and test. They were kept in boiling water bath for 5 min. The absorbance was recorded at 540nm using spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the formula:

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\text{% inhibition} = \frac{(\text{Control} – \text{Test})}{\text{Control}} \times 100
\]

Suitable reagent blank and inhibitor controls were simultaneously carried out.

α – glucosidase inhibitory activity

The enzyme inhibition activity for α-glucosidase was evaluated according to the method previously reported by Shрути et al. (2011) with minor modifications. The reaction mixture consisted of 50µL of 0.1M phosphate buffer (with pH of 7.0), 25µL of 0.5mM 4-nitrophenol α-D-glucopyranoside (dissolved in 0.1M phosphate buffer, with pH of 7.0), 10µL of different concentrations (1, 3, 10, 30, 100, 300 and 1000µg/ml) 70% hydroalcoholic (HA) and alkaloidal MK extracts and 25µL of α-glucosidase solution (a stock solution of 1mg/mL in 0.01M phosphate buffer, with pH of 7.0 was diluted to 0.1Unit/mL with the same buffer, with pH of 7.0 just before assay). This reaction mixture was then incubated at 37°C for 30 min. Then, the reaction was terminated by the addition of 100µL of 0.2M sodium carbonate solution. The enzymatic hydrolysis of substrate was monitored by the amount of p-nitrophenol released in the reaction mixture at 410nm using microplate reader. Individual blanks were prepared for correcting the background absorbance, where the enzymes were replaced with buffer. Controls were conducted in an identical manner replacing the plant extracts with methanol. Acarbose was used as positive control. All experiments were carried out in triplicates.

Cell Culture Studies

Preparation of cell culture

L6, a monolayer myoblast culture (obtained from NCCS, Pune - Passage no-15) and 3T3L1 pre-adipocytes (obtained from NCCS, Pune - Passage no -19) were cultured in DMEM with 10% foetal bovine serum (FBS) and supplemented with penicillin (120units/ml), streptomycin (75µg/ml), gentamycin (160µg /ml) and amphotericin B (3µg/ml) in a 5% CO2 environment. For differentiation, the L6 cells were transferred to DMEM with 2% FBS for 4 days, post-confluence. The extent of differentiation was established by observing the multinucleate of cells. 3T3L1 pre-adipocytes grown in 48 well plates until 2 days post-confluence and the cells were induced by the differentiation medium (combination of 0.5mM/l of IBMX, 0.25 µM/l of DEX and 1mg/l of insulin in DMEM medium with 10% FBS) to differentiate into adipocytes. Three days after induction, the differentiation medium was replaced with medium containing 1 mg/ml insulin alone. The medium was subsequently replaced again with fresh culture medium (DMEM with 10% FBS) after 2 days the extent of differentiation was measured by monitoring the formation of multi nucleation in cells.

Measurement of 2-deoxy-D-[1-3H] glucose

L6 myoblasts grown in 24 well plate was subjected to glucose uptake as reported by Anand et al., 2010. In brief, differentiated cells were serum starved for 5h and were incubated with different concentrations (1, 3, 10 and 30µg/ml) of 70% hydroalcoholic and alkaloidal extracts of Murraya for 24h and then cells were either stimulated with 10µM insulin or left untreated for 20min. After experimental incubation, cells were rinsed once with HEPES buffered Krebs Ringer phosphate solution (pH 7.4) and were subsequently incubated for 15 min in HEPES buffered solution containing 0.5µCi/ml 2-deoxy-D-[1-3H] glucose. The uptake was terminated by aspiration of media. Cells were washed thrice with ice cold HEPES buffer solution and lysed in 0.1% sodium dodecyl sulphate (SDS). The lysates were transferred to the plate with glass fibre paper and allowed to air dry. This plate was used to measure the cell-associated radioactivity by liquid scintillation counter. All the assays were performed in duplicates and repeated thrice for concordance. Results have been expressed as % glucose uptake with respective control.

Palmitate induced insulin resistance assay

L6 cells were seeded in 96 well plates. Cells were maintained and differentiated for 9 days. Cells were starved for overnight and pre-treated with different concentrations ranging from 1, 3, 10 and 30µg/ml of aqueous extract of 70% hydroalcoholic and alkaloidal extracts of Murraya for 1h at 37°C. 0.75mM Palmitate was added and incubated for 4h. During the period of incubation L6 cells were stimulated with 100µM insulin and 0.5µCi of 2-deoxy-D-[1-3H] glucose uptake was analyzed following similar protocol of measuring radiolabelled glucose uptake.

Anti - adipogenesis assay

3T3L1 pre-adipocytes were induced by the combination of IBMX, DEX and insulin to differentiate into adipocytes as previous described (day 0). 72 h after induction, the differentiation medium was replaced with 10% FBS–DMEM containing 1mg/l insulin for 48 h (day 5). The medium was replaced again with fresh culture medium for 48 h (day 7). The degree of the differentiation of the cells was investigated by adding different
solvent extracts of 70% hydroalcoholic and alkaloidal extracts of Murraya at doses of 3, 10 and 30µg/ml day 0, a period which covered the entire induction and post induction stages. Before staining with oil red O, cells were washed twice with PBS, fixed with 10% formaldehyde for 15 min at room temperature, washed twice with distilled water and once with 70% isopropanol. Next, cells were stained for 1 h at room temperature with filtered oil red O at a ratio of 60% oil red O stock solution (0.5% w/v in isopropanol) to 40% distilled water. The cells were washed twice with distilled water, twice with PBS, and examined under a microscope. Photomicroscopic evaluation was also carried out for the comparison of triglyceride accumulation using invertoscope (Nikon) was used for imaging at room temperature. For quantitative analysis, oil red O was extracted with isopropanol for 2min, and optical density of each sample was determined at 540 nm.

Advanced Glycation end product (AGE) assay

Bovine Serum Albumin (BSA) (10 mg/ml) was incubated with 1.1 M fructose in 0.1 M phosphate buffered-saline (PBS), pH 7.4 containing 0.02% sodium azide in darkness at 37°C for 7 days. Before incubation, the solution containing different concentrations of 70% hydroalcoholic and alkaloidal extracts of Murraya were added to the mixtures. The glycated BSA formation was determined using fluorescent intensity at an excitation wavelength 355 nm and emission wavelength 460 nm. Amino guanidine was used as a positive control for this study.

HPTLC Standardization of Mahanine

The 70% hydroalcoholic and alkaloidal extracts of MK were prepared at a concentration of 25mg/ml in Methanol. Standard Mahanine was prepared at concentration of 100µg/ml in Methanol. Standard Mahanine was spotted at a range of 200ng – 1000ng and samples at two concentrations of 50µg/ml and 100µg/ml were spotted using CAMAG Linomat 5 applicator. The method was optimized by selecting appropriate mobile phase of n-hexane: ethyl acetate: glacial acetic acid (3:1:0.5) and developed in a twin trough chamber, 10 x 10 cm at 25°C. The plates were dried using hair dryer. The developed plates were scanned at 298nm using CAMAG TLC scanner 3 and photo-documented using CAMAG REPROSTAR 3 at 254nm and 366nm.

Statistical analysis

All the experiments were performed in triplicate and the results were expressed as Mean ± SEM. One-way analysis of variance (ANOVA) was used to calculate the statistical significance of differences between groups for cell line based studies, followed by Tukey’s test for multiple comparisons among groups using Graph Pad Prism v.5.0. P<0.05 were considered as statistically significant. Linear regression analysis was done for in vitro extract based assays.

RESULTS

Preliminary phytochemical analysis

Preliminary phytochemical analysis showed the presence of phenolic compounds, flavones, and tannins, alkaloids, reducing sugars, proteins and carbohydrates in both the extracts (Table 1).

In – vitro α – amylase and α – glucosidase inhibitory activities

The, 70% hydroalcoholic and alkaloidal extracts of MK were evaluated for α – amylase and α – glucosidase inhibitory activity (Figure 1). The 70% hydroalcoholic extract of MK showed potent inhibitory activity against α - amylase enzyme with a maximum inhibition percentage of 92.23% at 1000µg/ml which compared with standard Acarbose. The IC50 of 70% hydroalcoholic extract of MK was found to be 37.09µg/ml which was found to be close enough to standard Acarbose whose IC50 is 34.83µg/ml. The alkaloidal extract showed its maximum inhibition percentage of 91.79% at 1000µg/ml and its IC50 value was found to be 48µg/ml. A similar inhibitory pattern was observed for MK extracts in case of α – glucosidase. The maximum inhibition of α – glucosidase of 70% HA and alkaloidal extracts of MK was found to be 95.57% and 99% respectively whereas for Acarbose it was 97.29%. The corresponding IC50 was found to be 21.56µg/ml, 40.22µg/ml and 42.11µg/ml for 70% HA MK extract, alkaloidal extract and Acarbose respectively.

[3H]-2-Deoxy glucose (Radiolabelled) uptake assay

Glucose uptake assay was carried out in differentiated L6 myotubes using radio – labelled glucose [3H]-2-Deoxy glucose. Incubation of L6 myotubes with different concentrations (1, 3, 10 and 30µg/ml) of 70% HA and alkaloidal extracts of MK exhibited a dose – dependent increase in the glucose uptake activity which was comparable with that of Insulin (Figure 2). In the absence of insulin, 70% HA extract of MK showed maximum glucose uptake of 65.80% at 30µg/ml concentration, while the alkaloidal extract MK exhibited 55.99% glucose uptake at the same concentration. The glucose uptake activity in both 70% HA and alkaloidal groups in presence of insulin did not vary significantly compared to treatment without insulin. Hence it was revealed from the results that no additive or synergistic effect was exhibited by the MK extracts and insulin.

Palmitate induced insulin resistance assay

The effect of 70% HA and alkaloidal extracts of MK was evaluated against Palmitate induced insulin resistance (Figure 3). Significant attenuation (p<0.001) of glucose uptake in L6 skeletal muscle cells was observed in Palmitate induced group due to insulin resistant condition. The 70% HA and alkaloidal extracts of MK at different concentrations (1, 3, 10 and 30µg/ml) exhibited dose – dependent increase in uptake of glucose by attenuating the effect of Palmitate. While insulin (10µM) exhibited 85.43% uptake of glucose, 70% HA and alkaloidal extracts of MK showed maximum uptake of 67.23% and 61.12% of glucose at 30µg/ml.

Oil O red adipogenesis assay

The effect of 70% HA and alkaloidal extracts of MK on lipid accumulation was investigated using 3T3L1 adipocytes at various doses of 1, 3, 10 and 22
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Following eight days of differentiation, intracellular accumulation of lipid content was analysed by Oil O red staining. Increased lipid accumulation was visualized in control differentiated adipocytes indicated by increase in staining intensity. The 70% HA and alkaloidal extracts of MK treated cells lowered triglyceride accumulation which was exhibited by decrease in staining pattern, suggestive of anti-adipogenic property of the extracts.

Diabetes associated complications assay – AGE product assay

The potential of 70% HA and alkaloidal extracts of MK were studied against AGE product inhibitory activity (Figure 5). Different doses of the extracts (10, 30, 100, 300 and 1000µg/ml) were examined against AGE formation. The alkaloidal Murraya extract showed a considerable inhibition of 84% at a concentration of 1000µg/ml while with 70% HA extract of Murraya, inhibition was about 82% at the same concentration level. Gallic acid was used as positive control and it showed an inhibitory potential of about 73%.

HPTLC quantification of Mahanine in 70% hydroalcoholic and alkaloidal extracts of MK

The HPTLC chromatograms of Mahanine, carbazole alkaloid are depicted in Figure 6. The retention factor (Rf) of Mahanine in 70% hydroalcoholic and alkaloidal extracts of MK was found to be 0.73. The quantification of Mahanine at scanning wavelength of 298nm in the solvent system of n-hexane: ethylacetate: glacial acetic acid (3:1:0.5) demonstrated the presence of Mahanine in both the extracts. The percentage content of Mahanine in 70% hydroalcoholic and alkaloidal extracts of MK was found to be 19.68% and 26.59% respectively.

DISCUSSION

Diabetes is a chronic long-term metabolic disorder which is associated with persistent complications. Review of glycaemic control at regular time points with proper therapeutic treatment can develop control over the disorder. To avoid the adverse effects caused by modern medicines, options for complementary and alternative medicines have emerged. In this background, the results of the present study exemplify the effect
of Murraya extracts on carbohydrate metabolizing enzymes, glucose uptake potential, insulin resistance, and adipogenesis as well as on diabetes-associated complications.

Hyperglycemia is one of the hallmark features of T2D; hence regulating plasma glucose plays a crucial role in preventing T2D. α – amylase and α – glucosidase are the two key enzymes that are responsible for postprandial hyperglycemia. Hence inhibiting the activity of these enzymes would be one of the best approaches in the management of diabetes, as it delays the absorption of ingested carbohydrates.25 The results of our present study exhibited that aqueous, absolute alcohol, 70% hydroalcohol and alkaloidal extract of MK have effectively inhibited these enzymes which suggests that Murraya koenigii possess carbohydrate metabolism regulating property, thereby beneficial in reducing postprandial glucose. Skeletal muscle is the predominant regulatory site for insulin stimulated glucose uptake which helps in maintenance of glucose homeostasis. Major portion of glucose is stored in muscle cells as glycogen; it is glucose uptake which helps in maintenance of glucose homeostasis.

Metabolic imbalance in T2D results in generation of reactive oxygen species (ROS) which decreases the antioxidant levels. This leads to increased oxidative stress which in turn, activates the polyol pathway. Activation of polyol pathway leads to increased production of intracellular sorbitol and fructose, thereby increasing the production of AGEs.33 Hence, any compound which possesses the property of inhibiting both these activities may be beneficial. Studies have demonstrated that plants rich in flavonoids possess aldolase inhibitory property,34,35 whereas plants enriched with alkaloids have shown potent AGE inhibitory property.36,37 70% hydroalcoholic and alkaloidal extracts of MK effectively inhibited the formation of AGE, which may be due to the presence of carbazole alkaloids.

CONCLUSION

Results of our study demonstrate multiple effects of Murraya koenigii in the management of diabetes as well as its associated complications,
thereby suggesting that the effective bioactive ingredients present in MK can be used for future research, both in vitro and in vivo and evaluate its antidiabetic activity by exploring the underlying mechanisms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

IR: Insulin resistance; T2D: Type 2 diabetes; MK: Murraya koenigii; HA: hydroalcoholic; CCRAS: Central Council for Research in Ayurveda and Siddha; NCCS: National Centre for Cell Science; DMEM: Dulbecco’s Modified Eagle’s Medium; PBS: Fetal bovine serum; IBMX: 3-isobutyl-1-methylxanthine; DEX: Dexamethasone; PBS: Phosphate buffered Saline; AGE: Advanced Glycation End Product; HPTLC: High Performance Thin Layer Chromatography; ROS: Reactive oxygen species.

REFERENCES

Parameswari, et al.: Anti-Diabetic Effect of *Murraya koenigii*

**SUMMARY**

- The 70% hydroalcoholic and alkaloidal extract exhibited beneficial effect against insulin resistance and adipogenesis.
- The extracts were found to be effective not only on obesity and type 2 diabetes but were also potent enough in prevention of diabetes associated complications.
- The anti-diabetic effect of *Murraya koenigii* might be attributed to the presence of major phyto constituent Mahanine, carbazole alkaloid present.