

Pharmacological Evaluation of Anti-Diabetic Activity of Myricetin in Different Cell Lines

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Article History	Abstract
Received: 06 June 2023 Revised: 05 Sept 2023 Accepted: 02 Nov 2023	The aim of this research work was to perform pharmacological evaluation of Myricetin by using different cell lines. To check the short-term cytotoxic effect of Myricetin in HepG2 and L6 cells, a colorimetric based MTT assay was performed also the glucose utilization in both HepG2 and L6 cells was estimated in order to measure the in vitro anti-diabetic activity of Myricetin. For Lipid accumulation T3-L1 preadipocytes cells are used. For measurement of nitric oxide (NO) the RAW-264.7 cells are used. The alpha-amylase assay and alpha-glucosidase inhibition assay was performed to check if our drug is having effect in term of inhibiting α-amylase and α-glucosidase respectively. Western blot analysis was performed to check the expressions of iNOS protein in LPS treated RAW 264.7 cells before and after the exposure of Myricetin. The cytotoxicity data suggested that Myricetin displayed low level of cytotoxicity in HepG2 cells and in L6 myoblast cell lines. For glucose utilization experiment, it discovered that glucose uptake level was notably increase in HepG2 cells and in L6 myoblast cell lines with the increasing concentration of Myricetin. Myricetin increase lipid accumulation in 3T3-L1 preadipocytes and also inhibits nitric oxide production in RAW 264.7 cell lines. Research was found that Myricetin had no significant effect in order to alter the expression of alpha-amylase and alpha-glucosidase. Our research concludes that Myricetin can be used as a potent anti-diabetic agent.
CC License CC-BY-NC-SA 4.0	Keywords: Myricetin, anti-diabetic activity, cell lines.

1. Introduction

Diabetes Mellitus is an insistent metabolic disorder characterized by an aberrantly upraised level of blood glucose due to the deficit in insulin secretion by the β -cells of the pancreas and /or resistance toward the exploit of hormone insulin associated with disturbances in the carbohydrates, lipids, and proteins metabolism which leads to long-term complications. International Diabetes Federation conferring 371 million people affected by diabetes and the number likely to elevate 552 million by 2030. Based on the previous experimental and clinical studies recommended that oxidative stress plays a main role in the pathogenesis of diabetes.¹

During DM glucose is abundantly available, which upon oxidation generates excessive free radicals and saturates body's antioxidant defense (catalase, superoxide dismutase, glutathione etc.). These free radicals interact with the biomolecules such as protein, lipids, RNA, DNA etc. and render them non-functional, thereby developing persistent oxidative stress in the body which aids in the development of secondary diabetic complications.² Further diabetes is known to potentiate inflammatory processes within the body via chronically elevating pro-inflammatory cytokines and chemokines and this inflammatory stress further exaggerate diabetic complications.³ Furthermore, DM have been demonstrated to be induce genotoxicity, which is primarily attributed to the interaction of highly reactive free radicals with the DNA, resulting in impaired gene expression. Further, interplay of inflammatory and oxidative stress is known to play an important part in the development of insulin resistance and DM.⁴

Antioxidants are substances able to slow or inhibit the oxidation of other molecules. Recently, the medicinal field focused the antioxidants therapy in the management of numerous diseases, especially

diabetes. Preceding experimental studies and clinical trials have suggested the efficacy of antioxidants in preventing diabetes complication. The therapeutic strategy uses the antioxidants as a substrate, combined drug, synthetic antioxidants, and drug with antioxidants activity. In general, the medicinal plants with antioxidants activity are considered for the treatment of diabetes mellitus. The antioxidants therapy defends the beta-cell against oxidative stress-induced apoptosis and preserves the function of the beta-cell. Data from earlier studies show the antioxidants diminish diabetic-related complication and recover insulin sensitivity. Epidemiological studies revealed a strong association between the dietary antioxidant's intake and protection against diabetes.⁵

Myricetin is a naturally occurring flavonol with hydroxyl substitutions at the 3, 5, 7, 3', 4' and 5' positions. Its occurrence in nature is widespread among plants including tea, berries, fruits, vegetables and medicinal herbs.⁶ Myricetin's occurrence in berries, vegetables and fruits is mostly in the form of glycosides rather than free aglycones and its content in berries increases considerably as the berries ripen. Myricetin is commonly consumed in our diet in vegetables, fruits and beverages such as tea and wine. Some of the consumed myricetin is absorbed by the gastrointestinal tract, whereas the remainder is metabolized by the gastrointestinal microflora.⁷ The liver is largely responsible for the metabolism of the absorbed myricetin, with the intestinal wall and kidney as the secondary sites. The major metabolite from its metabolism has been identified as 3,5-dihydroxyphenylacetic acid, which is excreted in the urine.⁸ Through the years, a number of studies have been done to investigate its varied therapeutic potential, which includes its use as a potent antioxidant, as an anticarcinogenic agent and in the prevention of platelet aggregation.⁹ The aim of this study was to investigate the anti-diabetic effect of Myricetins in different cell lines.

2. Materials And Methods

Cell Culture and reagent

HepG2 liver cells were cultured in RPMI-1640 supplemented with 10% FBS, 1.5 mM L-glutamine and 1% antibiotics (100 U/ml of penicillin, 10 mg/ml of streptomycin). L6 myoblasts cells were cultured in antibiotic-free growth medium consisting of RPMI 1640 supplemented with 10% FBS. 3T3-L1 cells were cultured in DMEM with 10% fetal bovine serum. RAW 264.7 macrophages cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine supplemented with 10% FBS and 1% antibiotics. All the cell lines were purchased from ATCC, USA and checked for free from mycoplasma contamination. All cell lines were cultured until 90% confluency was reached in a humidified incubator in 5% CO₂ at 37°C and then they were trypsinized and used according to their requirements.¹⁰

MTT Assay

To check the short-term cytotoxic effect of Myricetin in HepG2 and L6 cells, a colorimetric based MTT assay was performed.¹⁰ In brief, cells were dislodged by short exposure to 0.25% Trypsin in phosphate-buffered saline, counted, suspended in new growth medium, and then approximately, 8000 to 10000 HepG2 and L6 cells were seeded in 96-well plates in triplicate and grown to 60-70% confluency. Then, cells were treated with the increasing concentrations of Myricetin for 48 h. After that, media was removed, 20 µL MTT reagent (5mg/mL) was added to each well and incubated at 37°C 2 h for the formation of purple formazan crystals. After removing the MTT solution, purple formazan crystals were dissolved in isopropanol. The color intensity was measured by spectrophotometer at 570 nm using microplate reader (Mithras LB 940, Berthold, Germany).¹¹ The % viability was calculated as mentioned below:

$$\% \text{ Viability} = (\text{Absorbance of test wells} / \text{Absorbance of untreated control}) \times 100$$

Glucose utilization experimental procedure

The glucose utilization in both HepG2 and L6 cells was estimated in order to measure the in vitro anti-diabetic activity of Myricetin.¹¹ Briefly, HepG2 and L6 cells were seeded in 96 wells and then treated with 50, 100, and 200µg/ml concentrations of Myricetin. Two cell-free rows were also included to serve as blanks. After 48 h incubation, the spent culture medium was removed and replaced with a 25 µl incubation buffer (RPMI medium diluted with PBS, 0.1% BSA and 8 mM glucose) and further incubated for an additional 3 h at 37°C. Metformin (0.1 µg/ml) and insulin (6 µg/ml) were used as positive control in HepG2 and L-6 cells, respectively to compare the effectiveness of Myricetin. After incubation, 10 µl of the incubation medium was removed from each well and transferred into a new 96-well plate into which 200 µl of glucose oxidase reagent was added to measure the concentration of glucose in the medium. After 15 min of incubation at 37°C, the absorbance was measured at 492 nm. The amount of glucose utilized was calculated as the difference between the cell-free and cell-

containing wells. The percentage of glucose utilization was calculated in relation to the untreated controls.¹¹

Lipid Accumulation in T3-L1 Preadipocytes

Approximately, 8000 3T3-L1 cells were seeded per well into a 24-well culture plate and allowed to grow until 60-70% confluence was reached. Then, the preadipocytes were treated with the above-mentioned concentrations of Myricetin along with a positive control (Rosiglitazone; 0.4 µg/ml) for 48 h. The cells were then cultured for an additional ten days in normal culture medium (DMEM with 10% FBS) and the medium replaced every two to three days. After ten days, the spent culture medium was removed and gently washed with PBS. The cells were then allowed to fix at room temperature for approximately 1 h by adding 250 µl per well of 10% formaldehyde in PBS. The fix solution was aspirated and later stained by adding 200 µl of pre warmed oil red working solution [6 ml of stock solution (0.5 g oil red dye in 100 ml isopropanol) in 4ml of distilled water] for 15 min at 37°C. After 15 min of incubation, excess dye was extensively washed with water and the plate dried in an oven at 37°C. The dye was further extracted by adding isopropanol (150 µl per well) after which 100 µl was transferred to a 96-well plate and the absorbance measured at 520 nm.¹²

Measurement of nitric oxide (NO)

Nitric oxide has been reported to contribute to the pathogenesis of diabetes and hence inhibition of NO is important to monitor anti-diabetes efficacy as per the protocol described by Chatterjee et al.¹⁰ RAW cells (100000 cells/well) were cultured in 6 well plate and incubated with LPS to induce NO production. After the LPS supplementation, cells were treated with the treatment concentrations of Myricetin for 48 h. After that, cells were lysed with RIPA lysis buffer and protein concentrations were measured. 50 µg protein from different treatment groups were taken in 96 well plate on which 50µl of Griess reagent I and Griess reagent II were added and incubated for 15 min in dark for the pink color formation. After that, absorbance was taken spectrophotometrically at 560 nm and represented graphically.¹³

Alpha-Amylase inhibition assay

The alpha-amylase assay was performed to check if our drug is having effect in term of inhibiting α-amylase.²⁰ Briefly, 15 µl of the Myricetin at different concentrations (50, 100, and 200 µg/ml) was added to 5 µl of enzyme porcine pancreatic solution into 96-well plate. After 10 min of incubation at 37°C, the reaction was initiated by adding 20 µl of starch solution and further incubated for 30 min at 37°C. The reaction was then stopped by adding 10 µl 1M of HCl to each well followed by 75 µl of iodine reagent. A blank containing phosphate buffer (pH 6.9) instead of the extract and a positive control (acarbose, 64 µg/ml) were prepared. No enzyme control and no starch control were included for each test sample.¹⁴ The absorbance was measured at 580 nm and the percentage inhibitory activity was calculated by using the following equation:

$$\% \text{ Inhibition} = [1 - \{ \text{Absorbance of the untreated (Control)} / \text{Absorbance of the test well} \}] \times 100$$

Alpha-Glucosidase inhibition assay

The alpha-glucosidase inhibition assay was to evaluate the Myricetin mediated α-glucosidase inhibition.⁹ In brief, 5 µl of the Myricetin of the above-mentioned treatment concentrations were added to 20 µl of 50 µg/ml alpha-glucosidase solution into a well of a 96- well plate. After that, 60 µl of 67 mM potassium phosphate buffer (pH 6.8) was then added. After 5 min of incubation, 10 µl of 10 mM p-nitrophenyl-α-D-glucoside solution (PNPGLUC) was then added and further incubated for 20 min at 37°C. After incubation, 25 µl of 100 mM Na₂CO₃ solution was added and the absorbance was measured at 405 nm. A blank and sample blank were also prepared by adding 5 µl of deionised water instead of plant extract and 20 µl of deionised water instead of enzyme, respectively. Epigallocatechin gallate (10 µg/ml) was used as a positive control.¹⁵ The percentage inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = [1 - \{ \text{Absorbance of the test well} / \text{Absorbance of the untreated (control)} \}] \times 100$$

Western blot analysis

Western blot analysis was performed to check the expressions of iNOS protein in LPS treated RAW 264.7 cells before and after the exposure of Myricetin as per the protocol.⁹ After the treatment, cells were trypsinized and whole-cell extracts were isolated using RIPA lysis buffer (1% Triton X-100, 20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM sodium pyrophosphate, 1µM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mg/ml PMSF). The extracts were then fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated successively with the primary (1:1000 dilution) and secondary (1:2000 dilution) antibodies and exposed

to the chemiluminescence reagent for signal detection. Densitometric analysis was done by using ImageJ software.^{16,17}

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software, USA. Results represented the mean $\pm$ SD of three separate experiments. Data were analysed using one-way ANOVA followed by Bonferroni's multiple comparison test. Statistical significance of difference in the central tendencies compared to control groups was designated as '*' ($p < 0.05$), '**' ($p < 0.001$) and '***' ($p < 0.0001$).

3. Results and Discussion

Myricetin enhances the glucose utilization in HepG2 liver cell line

In order to reduce the high blood glucose level, potent anti-diabetic substance potentiates the glucose utilization in liver. In our current study when we exposed Myricetin in HepG2 cell line, IC_{50} value was not reached up to the treatment concentration of 200 μ g/ml (Figure 1A). The cytotoxicity data suggested that Myricetin displayed low level of cytotoxicity in HepG2 cells.

Next, when we performed the glucose utilization experiment, it was found that glucose uptake level was significantly increased with the increasing concentrations of Myricetin. Approximately 1.2-, 1.4- and 1.5-fold enhanced glucose utilization was found upon the increasing concentrations (50, 100, and 200 μ g/ml) of Myricetin (Figure 1B). In order to compare the efficacy of our drug, we have used Metformin as a positive control. 0.1 μ g/ml Metformin exposure caused 1.9-fold elevated level of glucose uptake in our treatment procedure and with respect to this, our drug of interest showed comparably lower elevation of glucose utilization (Figure 1B).

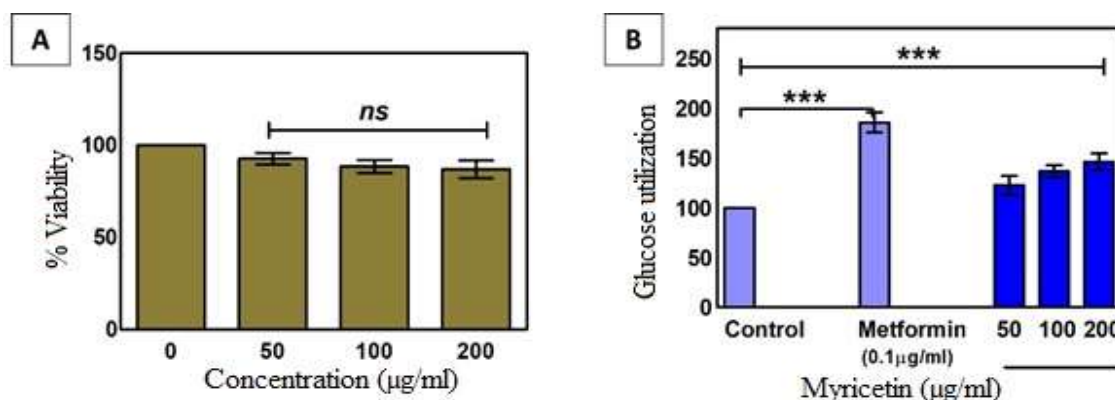


Figure 1: A. MTT cell viability after the exposure of different concentrations of Myricetin in HepG2 liver cells. B. Effect of Myricetin on glucose utilization in HepG2 hepatocytes. Cells were treated for 48 h in the presence or absence of varying concentration of Myricetin. Metformin serves as positive control. Data expressed as mean $\pm$ SD (n = 3). '***' indicates statistical significance ($p < 0.0001$).

Myricetin potentiates the glucose utilization in L6 myoblast cell line

Most of the potent anti-diabetic agents decrease the blood glucose level by increasing the glucose uptake in muscle cells. In our current experiment, in order to evaluate the anti-diabetic efficacy, we have checked the glucose utilization in L6 myoblast cell line. Figure 2A shows the Myricetin mediated decrease in cell viability of myoblast cells. Data suggested approximately 1.06-, 1.1-, and 1.1-fold decrease in cell viability with the increasing treatment concentrations of Myricetin (50, 100, and 200 μ g/ml), which suggest the low level of cell toxicity of it in muscle cells.

Additionally, it was also found that with the increasing concentrations of Myricetin, glucose utilization was also increased in muscle cells also. Upon 200 μ g/ml treatment concentration of Myricetin, 1.4-fold elevated level of glucose uptake was found in myoblast cells (Figure 2B). Insulin (6 μ g/ml) was used as a positive control, which enhanced the glucose utilization 1.5-fold in with compare to this our drug also showed a good potential effect (Figure 2B).

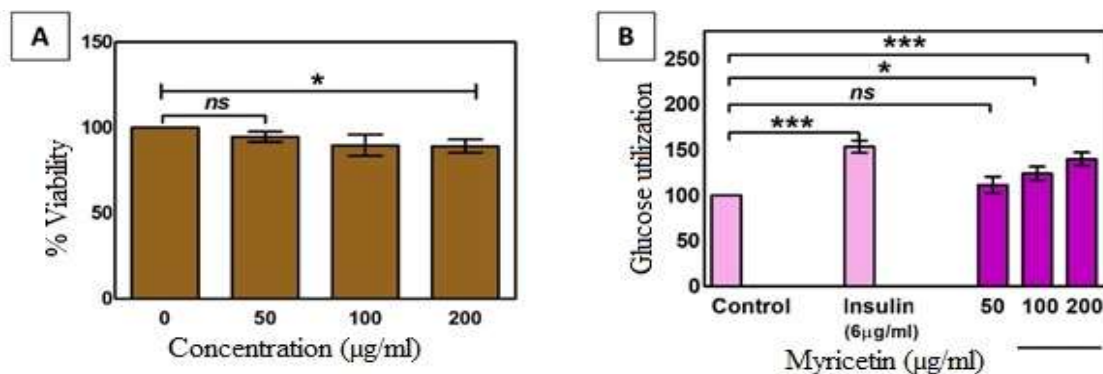


Figure 2: **A.** MTT cell viability after the exposure of different concentrations of Myricetin in L6 myoblast cells. **B.** Effect of Myricetin on glucose utilization in L6 myoblast cells. Cells were treated for 48 h in the presence or absence of varying concentration of Myricetin. Insulin serves as positive control. Data expressed as mean±SD (n=3). 'ns', '*', '***' indicates non-significance (p>0.05) and statistical significance (p<0.05 and p<0.0001, respectively).

Myricetin increases lipid accumulation in 3T3-L1 preadipocytes

Above findings supported the fact that Myricetin shows minimum level of cytotoxicity in normal cells (Figure 1A, 2A). Further to support the anti-diabetic potentiality of Myricetin, we have checked the triglyceride accumulation in 3T3-L1 per adipocytes. Figure 3 displayed an increase in lipid accumulation with the increasing treatment concentrations of Myricetin. At 50, 100, and 200 μg/ml treatment concentrations of Myricetin, approximately 1.2-, 1.4-, and 1.5-fold enhanced lipid accumulation was found which were slightly less than Rosiglitazone (1.6-fold, approximately).

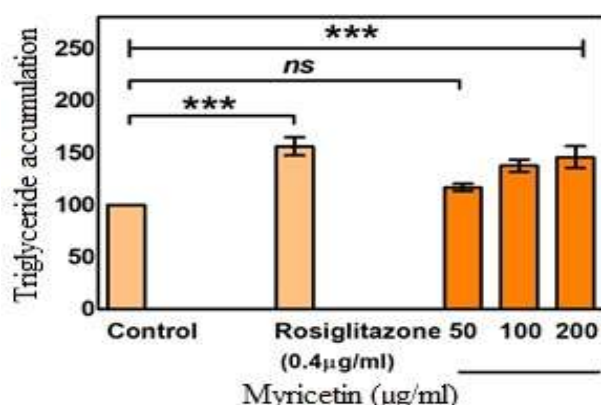


Figure 3: Effect of Myricetin on triglyceride accumulation in 3T3-L1 preadipocytes. Cells were treated for 48 h in the presence or absence of varying concentration of Myricetin. Data were expressed as mean±SD (n=3). 'ns', '***' indicates statistical non-significance (p>0.05) and statistical significance (p < 0.0001). Rosiglitazone serves as positive control.

Myricetin inhibits nitric oxide (NO) production in RAW 264.7 cell line

Inhibition of NO was found to be one of the central mechanisms in many of anti-diabetic substances. In our present work, when we wanted to check the effect of Myricetin in macrophage cell line, it was found that nitric oxide production was significantly affected with the increasing treatment concentrations of Myricetin. Approximately 1.3-, 1.4-, and 2.0-fold reduction in NO production in RAW cell line was found with the 50, 100, and 200 μg/ml treatment concentrations of Myricetin (Figure 4). In order to compare the effectiveness of Myricetin in term of reducing NO production, a positive control, Aminoguanidine was used. 4 μg/ml Aminoguanidine, caused approximately 3.6-fold decrease in NO production, which suggests that our drug of interest is having a great impact in reduction of NO production (Figure 4).

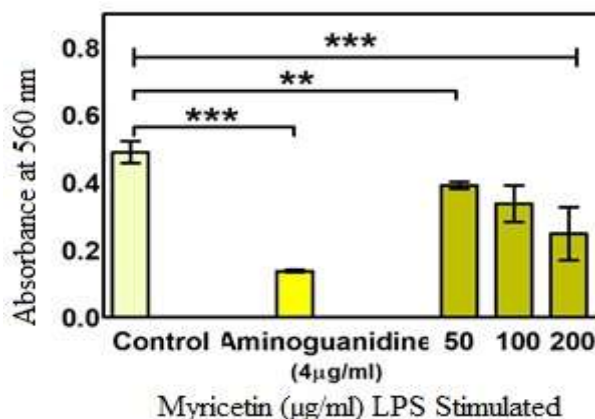


Figure 4: The effect of Myricetin on NO production by LPS-stimulated RAW macrophage cells. Data are expressed as mean $\pm$ SD (n=3). ‘***’, ‘****’ indicates statistical significance (p<0.001 and p<0.0001, respectively). Aminoguanidine serves as a positive control.

Effect of Myricetin in α -amylase and α -glucosidase

Research suggests that some of the anti-diabetic drugs lower the blood glucose level by downregulating the expression of two enzymes, α -amylase and α -glucosidase. In this study, when we wanted to illustrate the molecular mechanism behind the way of action of Myricetin by measuring the level of α -amylase and α -glucosidase, it was found that Myricetin had no significant effect in order to alter the expression of these two enzymes (Figure 5A, 5B).

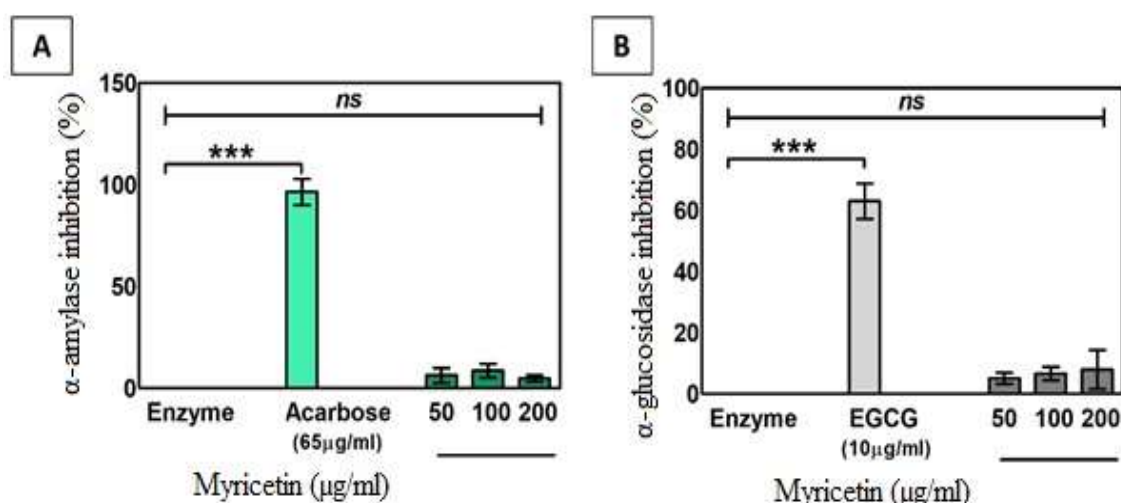


Figure 5: The effect of Myricetin on α -amylase (A) and α -glucosidase (B) activity. Data expressed as mean $\pm$ SD (n=3). ‘ns’, ‘***’ indicates statistical non-significance (p>0.05) and statistical significance (p<0.0001). Acarbose and Epigallocatechin gallate (EGCG) act as positive controls, respectively.

Molecular mechanism behind Myricetin mediated anti-diabetic activity

Above data give us the idea that Myricetin shows anti-diabetic efficacy and it is independent of modulating α -amylase and α -glucosidase (Figure 5). However, our above observation appears Myricetin mediated inhibition of NO level (Figure 4). Hence, in order to evaluate the molecular mechanism behind the Myricetin mediated anti-diabetic potentiality, we have checked the iNOS expression in LPS stimulated RAW macrophage cells. Figure 6 shows the expression of iNOS. Upon the exposure of Myricetin, iNOS level were found to be downregulated. At 200µg/ml Myricetin treatment concentration, the expression of iNOS was found to be decreased by -fold.

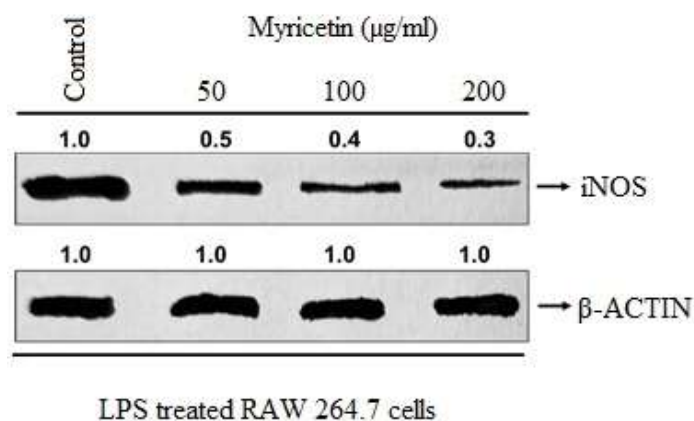


Figure 6: The effect of Myricetin on iNOS expression. β -ACTIN served as a loading control.

Diabetes is a disease where enough insulin is not produced or body is unable to use the insulin effectively, as a result, the blood glucose level raised excessive high. Untreated this disease caused severe health related complications. Here acute complications can include diabetic ketoacidosis, nonketotic hyperosmolar coma, or death. Serious long-term complications include heart disease, stroke, chronic kidney failure, foot ulcers and damage to the eyes.

In this study, we have systematically studied the effect of Myricetin in order to establish it a potent anti-diabetic substance. Myricetin is a natural anti-oxidant having many commercial values. Being a carotenoid and an antioxidant, it was thought to be non-toxic or less toxic in nature. In this present study, when we exposed Myricetin to liver cells and muscle cells, very less Myricetin mediated cytotoxicity was noted (Figure 1A, 2A). Moreover, up to the treatment concentration of 200 μ g/ml, IC₅₀ value of Myricetin was not found. Hence, relatively low level of toxicity exhibited by Myricetin raises prospects that Myricetin could be potentially safe for the users.

Further, various biochemical assays were used to evaluate probable antidiabetic action of Myricetin. Glucose utilization experiments suggested that, Myricetin enhances the glucose uptake in liver and muscle cells (Figure 1B, 2B). Metformin and Insulin were used to compare the effectiveness of Myricetin in order to enhance the glucose uptake in liver and muscle cells, respectively. Although, Myricetin was not found to be that much effective than Metformin and Insulin, it showed a significant impact in enhancing the glucose uptake.

4. Conclusion

Lipid accumulation data further showed that Myricetin has some role in enhancing the triglyceride accumulation. The potentiality of adipose tissue to lodge excess lipid can be exceeded in obese patients, which results in the abnormal accumulation of lipid in muscle, liver, and pancreatic islet leading to lipotoxicity. Higher level of lipid accumulation in 3T3-L1 adipocytes suggesting that Myricetin might be a good therapeutic agent in order to lower the lipid profiles (Figure 3).

Above observations suggested the anti-diabetic potentiality of Myricetin. However, to evaluate the molecular mechanism of Myricetin mediated anti-diabetic action, some experiments were carried out. Nitric oxide plays an important role in the onset of diabetes. So, we wanted to check if Myricetin has some effect in lowering the NO production in RAW cells or not. Our study revealed that Myricetin has a significant role in order to reduce the NO level which could be the potential way of action of Myricetin mediated anti-diabetic activity (Figure 4).

At present, several antidiabetic drugs that are used to manage diabetes exhibit the inhibition of alpha-amylase and/or alpha-glucosidase. So, here also, we wanted to check if Myricetin has any role in order to uplift the level of these two enzymes or not. Our findings suggested that Myricetin doesn't play a significant role in inhibition of alpha-amylase and alpha-glucosidase (Figure 5A, 5B).

Taken together, our findings suggest that Myricetin has no effect in altering the level of alpha-amylase and alpha-glucosidase, it has a great role in reducing the nitric oxide production. Further, this Myricetin mediated inhibition of nitric oxide was found to be due to downregulation iNOS. Collectively. Our current piece of work seems to be an excellent study and Myricetin can be used as a potent anti-diabetic agent.

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