

EFFECT OF *MURRAYA KOENIGII* LEAVES EXTRACT ON GLUCONEOGENESIS AND GLYCOGENOLYSIS IN ISOLATED RAT HEPATOCYTES CULTURE

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ABSTRACT

Objectives: The present study was aimed to investigate the *in vitro* activity of *Murraya koenigii* extracts through various carbohydrate metabolic pathways in the isolated rat hepatocyte models.

Methods: Different doses of metformin, aqueous and methanol extracts of *M. koenigii* leaves were evaluated in the MTT, glucose, and glycogen content assays in the cultured *in vitro* rat hepatocytes.

Results: The study showed that there was a significant increase in activity with respect to the increased concentration of extracts. Slight effect was observed in the isolated rat hepatocytes culture, *M. koenigii* leaves extract may exert cytoprotective and hypoglycemic action.

Conclusion: It may be needed to determine the effect of *ex vivo* rat hepatocytes isolated from diabetic rats. Effects of the plant or isolated compounds on the genes expression of signaling pathways should be investigated in further studies.

Keywords: *Murraya koenigii*, Non-insulin-dependent diabetes mellitus, Gluconeogenesis, Glycogenolysis.

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INTRODUCTION

Non-insulin-dependent diabetes mellitus (NDDM) or type 2 diabetes mellitus is the most common metabolic syndrome spreading throughout the world [1]. Gluconeogenesis plays a key role to prevent an extreme decline in the blood glucose level during fasting. Augmented hepatic gluconeogenesis is the significant contributing factor to the fasting hyperglycemia which is found in NDDM patients [1,2]. Liver maintains blood glucose levels during fasting through mainly two metabolic pathways by glycogenolysis, i.e., converting its stored glycogen into glucose and by gluconeogenesis, i.e., synthesizing glucose mainly from lactate and amino acids [3]. Failure or lack of insulin secretion or action is the main characteristic of insulin resistance. It involves in both muscle and liver as characteristic features of the glucose intolerance in NDDM individuals. Basically liver holds a major position of insulin resistance, so the overproduction of glucose is reflected despite the presence of both fasting hyperinsulinemia and hyperglycemia [4].

Since there is a shortage of scientific evidence in the review of literature for the effect of *Murraya koenigii* leaves extract on the gluconeogenesis [2], whether it suppresses the generation of glucose. The aim of our study was to test the hypothesis that whether *M. koenigii* leaves extract inhibits the glucose by inhibiting gluconeogenesis and suppressing glycogenolysis in the cultured rat hepatocytes.

METHODS

Drugs and chemicals

Metformin was purchased from Sigma-Aldrich Ltd. Hank's Balanced Salt Solution, Liver digest medium (17701-038), Hepatocyte wash medium (17704-024), Williams E medium (A12176), Fetal Bovine Serum (2614079), Penicillin-streptomycin (15140-122), and Trypan blue stain (15250) were purchased from Gibco, Thermo Scientific Ltd. Dimethyl sulfoxide (DMSO) (D2650) was purchased from Sigma-Aldrich, Ltd. USA, MTT (M6494) from Invitrogen, Thermo Scientific, USA, 6 well plates (CLS3355), 96 well plates (CLS3599), and 5 mL stereopipettes (CLS4487) were purchased from Corning, New York,

USA. All reagents and chemicals used were of analytical grade and stored in a refrigerator at +4°C. The reagents were equilibrated at room temperature for 30 min before the start of the analysis.

Collection and authentication of plant material

Leaves of *M. koenigii* were collected from the local area of Karad in Maharashtra, India (17.2760° N, 74.2003° E), certified and authenticated by the Department of Botany, M. S. Shinde Mahavidyalaya, Tisangi, Kolhapur, India. The plant specimen voucher V03 (Ref: MHST/2016-17/28) of the plant was deposited in the herbarium. Fresh leaves were purchased from the local market of Karad, washed under tap water thoroughly; dried under shade and powdered using a mechanical grinder.

Preparation of *M. koenigii* leaves extracts

Methanolic (MEMK) and aqueous (AEMK) extracts of *M. koenigii* leaves were prepared by soxhletation method. Extracts were concentrated in a vacuum rotary evaporator and air evaporated.

Experimental animals

Healthy Wistar rats of either sex, weighing between 170 and 200 g were used in the study. Experiment study was conducted with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) for animal experimentation of laboratory and Institutional Animal Ethics Committee (Reg. No. 255/PO/2000/bc/CPCSEA), Krishna Institute of Medical Sciences, Karad, has approved the study.

Rat hepatocyte culture preparation

The method of rat hepatocytes culture was modified and prepared in accordance with Shen *et al.* [5].

Rat dissection and isolation of liver

Rats of either sex 200 g body weight were anesthetized in diethyl ether chamber. Depth of anesthesia was monitored by toe pinch. Hepatic portal vein was exposed by carefully moving the viscera to the right outside of

the abdominal cavity. After saline perfusion, the liver was dissected and placed in a pre-chilled sterile beaker with 20 mL HBSS medium.

Hepatocyte isolation

In the biosafety cabinet Type II, the liver tissue was washed thoroughly with HBSS containing penicillin/streptomycin to remove blood stains. The tissue was dissected into small pieces with a surgical blade and scraped to release cells into the buffer. Tissue pieces were transferred into sterile 15 mL centrifuge tube along with HBSS buffer containing penicillin-streptomycin (100 units/mL/100 µg/mL) antibiotics and washed 2-3 times by centrifugation at 1000 g for 5 min. Then, liver tissue pieces were digested with an appropriate volume of liver digest medium containing collagenase - I and dispase - II for 2 h. The dispersed hepatocytes into single cell suspension were filtered through a 100 µm pore size cell strainer into a 50 mL conical tube to remove connective tissues and undigested tissue fragments. The single cell suspension was washed with ice-cold hepatocyte wash medium for 2-3 times. The purified hepatocytes in cell pellets were re-suspended in 40 mL William's E Medium supplemented with 1 mL of penicillin-streptomycin and determined viable cell density by cell viability by Trypan blue staining.

Hepatocyte culture

The hepatocytes with 95-98% cell viability were grown in Williams E medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 units/mL/100 µg/mL) antibiotics. It was diluted cells with William's E medium to the required concentration. In 25 mL capacity Nunc cell culture flask containing 5 mL of William's E medium supplemented with 10% fetal bovine serum (FBS) and 100 µL of penicillin and streptomycin, 1×10^8 cells were seeded and maintained in a CO₂ incubator at 37°C in 95% humidity and 5% CO₂. After 24 h culture incubation, the cells were maintained in the medium with serum-free medium. The serum-free medium helped to maintain cell morphology with no adverse effects from hormones when the serum-free medium was used.

Cell viability in isolated rat hepatocytes by MTT method

Cell viability assay was performed according to the method described by Mosmann T [6]. *In vitro* cytotoxicity effects of the metformin, AEMK and MEMK on hepatocytes were determined by MTT assay. The cells were maintained in Williams E medium supplemented with 10% FBS, penicillin-streptomycin at 100 U/mL/100 µg/mL in a humidified atmosphere of 5% CO₂ at 37°C. 10,000 cells were seeded in a 96 well plate and incubated at 37°C, 5% CO₂. After 24 h of incubation, the cells were exposed to the treatment of AEMK and MEMK at concentrations of 1, 2.5, 5, and 10 mg/mL in culture medium without FBS and incubated for further 24 h at 37°C and 5% CO₂. After 24 h completion of treatment, cells were washed with sterile HBSS medium; thereafter, cell viability was determined using MTT dye. In 10 µL/well, MTT (5 mg/mL) was added to the cells, and the plates were further incubated at 37°C, and 5% CO₂ atmosphere. After 4 h incubation, the MTT solution was carefully removed, and 200 µL of DMSO was added to each well. The absorbance of purple color developed was measured at 560 nm wavelength using UV-visible 1800 spectrophotometer (Shimadzu). The results were expressed as a percentage of cell survival as compared to the control. All experiments were performed in triplicates.

Glucose assay in isolated rat hepatocytes

Glucose assay was assessed by the method of Yan *et al.* [7]. Cells were cultured in collagen M199 medium supplemented with 5% FBS, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin in a 12-well plate and treated with metformin (0.1-1 mg/mL) and extracts (10-1 mg/mL) for 24 h. After centrifugation, the supernatant medium was collected, and glucose concentration was measured with a DNS reagent. Absorbance was determined at 540 nm [7].

Glycogen content assay in isolated rat hepatocytes

Glycogen content assay was assessed by the method of Yan *et al.* [7]. Cells were seeded in a 6-well plate and treated with metformin

(0.1-1 mg/mL) and extracts (10-1 mg/mL) for 24 h. Cells were collected and adjusted to the same concentration. The cells were then homogenized using 0.5 mL of 30% (0.3 g/mL) potassium hydroxide and boiled for 15 min to destroy glucose, followed by centrifugation at 15,000 g for 15 min after adding 1.5 mL ethyl alcohol. The precipitates were dissolved in 0.5 mL of distilled water and boiled for 20 min after adding 0.2% anthrone diluted with 98% H₂SO₄. The glycogen content was determined using the anthrone reagent. The absorbance was detected at 620 nm [7].

Statistical analysis

Data were expressed as the mean ± standard deviation. Data analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). All data were analyzed using two-way repeated analysis of variance, and Tukey's test was applied for *post hoc* analysis. The value of $p < 0.05$ was considered to be statistically significant.

RESULTS

It showed that the findings increase with respect to the increased concentration of extracts. Since there was a slight effect observed in the isolated rat hepatocytes culture, it may be needed to determine the effect of *ex vivo* rat hepatocytes isolated from diabetic rats. It was observed extremely significant ($p=0.0054$) in MTT assay as shown in Fig. 1 while was found to be significant in glucose ($p=0.0196$) and glycogen ($p=0.0218$) assays as shown in Tables 1 and 2.

DISCUSSION

Glucose is the fundamental source of energy for the vital organs such as brain, heart, and red blood cells. It is stored in the form of glycogen in the liver and skeletal muscle. In NDDM, the failure of suppressing gluconeogenesis results in decreased liver glycogen storage in the postprandial state [4] along with reduced glucose deposition in skeletal muscle, heart, and adipose tissue [1].

Pyruvate-dependent gluconeogenesis involves in converting pyruvate to phosphoenol pyruvate by the enzyme phosphoenolpyruvate carboxykinase (PEPCK) present in the cytosol, and mitochondrial dicarboxylic acids are also involved [4]. Insulin inhibits gluconeogenesis by suppressing two rate-limiting gene expressions of PEPCK and glucose-6-phosphatase (G6Pase) while glucagon stimulates both genes, PEPCK, and G6Pase [1]. The enzyme G6Pase converts G6Pase to glucose as a final step in the metabolic pathways of glycogenolysis and gluconeogenesis. Thus, inhibiting this enzyme reduces hepatic glucose output [3].

Gluconeogenesis is involved in the conversion of carbohydrate as well as non-carbohydrate precursors to glucose or glycogen. G6Pase and

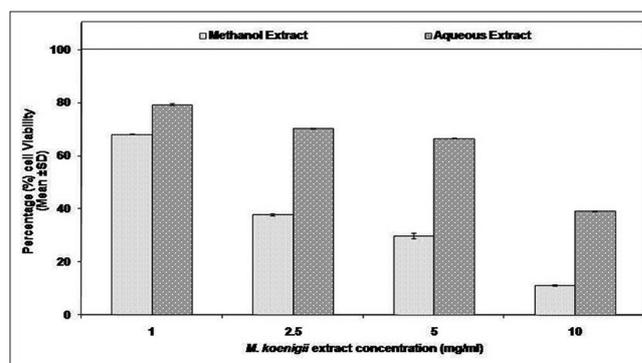


Fig. 1: Representative histogram showing the percentage cell viability of rat hepatocytes exposed to different concentrations (1, 2.5, 5, and 10 mg/mL) of methanol and aqueous extract of *Murraya koenigii* leaves measured by MTT assay. The results represent the means of three independent experiments and error bars represent the standard deviation of the means

Table 1: Effect of *M. koenigii* on glucose content in isolated rat hepatocytes

Concentration (mg/mL)	MEMK	AEMK	Concentration (mg/mL)	MET
10	0.67±0.09	0.71±0.007	1	0.037±0.001
5	0.58±0.04	0.45±0.008	0.5	0.047±0.005
2.5	0.37±0.07	0.26±0.007	0.25	0.049±0.001
1	0.26±0.03	0.14±0.001	0.1	0.048±0.003

Values are expressed in mean±SD, MET: Metformin, AEMK: Aqueous extract of *M. koenigii* leaves, MEMK: Methanolic extract of *M. koenigii* leaves, p=0.0196, *M. koenigii*: *Murraya koenigii*

Table 2: Effect of *M. koenigii* on glycogen content in isolated rat hepatocytes

Concentration (mg/mL)	MEMK	AEMK	Concentration (mg/mL)	MET
10	0.053±0.001	0.141±0.001	1	0.018±0.001
5	0.072±0.001	0.100±0.001	0.5	0.015±0.001
2.5	0.029±0.001	0.052±0.001	0.25	0.014±0.002
1	0.026±0.001	0.049±0.0005	0.1	0.019±0.002

Values are expressed in mean±SD, MET: Metformin, AEMK: Aqueous extract of *M. koenigii* leaves, MEMK: Methanolic extract of *M. koenigii* leaves, p=0.0218, *M. koenigii*: *Murraya koenigii*

fructose-1, 6-diphosphatase (F1-6DPase) are the important regulatory enzymes in the gluconeogenic pathway. Both get altered in uncontrolled diabetes. G6Pase catalyzes the conversion of G6Pase splitting to glucose and hydrogen, which binds with NADP+ in the form of NADPH and enhances the synthesis of fats from carbohydrates, i.e., lipogenesis. The generation of fructose-6-phosphate from fructose-1, 6-diphosphate is catalyzed by F1-6DPase. The activities of these two enzymes are increased in the diabetic control rats compared with the normal rats [8].

Decreased glycolysis, impeded glycogenesis and increased gluconeogenesis are some of the changes in glucose metabolism in the diabetic liver. In the present study, *M. koenigii* could be proven to inhibit glucose inhibition which is in accordance with the study by Cheng *et al.*, [9]. Cinnamon aqueous extract and its polyphenolics demonstrated dose-dependent inhibition of hepatic glucose production. Metformin decreased glycogen synthesis in a dose-dependent manner [10] in cultured rat hepatocytes which are in agreement with the present study. *Rhodiola crenulata* root extract exert the glucose-lowering effect partly by inhibiting hepatic gluconeogenesis through activating the AMPK signaling pathway in the report of Lee *et al.* [11] which is in concurrence with the results of our study. In future plans, various spices [12] and medicinal plant like *Kalanchoe pinnata* [13] will be utilized to study their effects on *in vitro* isolated rat hepatocytes culture.

CONCLUSION

M. koenigii leaves may exert a cytoprotective effect on the normal rat hepatocytes in the *ex vivo* assay. Effects of the plant or isolated compounds on the genes expression of G6PAS, PEPCK, and AMPK signaling pathways should be investigated in the further study.

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AUTHORS' CONTRIBUTION

Rohan S. Phatak, Chitra C. Khanwelkar, Kailas D. Datkhile: Concept and Design, Rohan S. Phatak, Kailas D. Datkhile, Pratik Durgawale: Laboratory work. Rohan S. Phatak, Kailas D. Datkhile, Pratik Durgawale: Collection, Analysis and Interpretation of data. Rohan S. Phatak, Chitra C. Khanwelkar, Kailas D. Datkhile: Drafting of the Article. Rohan S. Phatak, Chitra C. Khanwelkar, Kailas D. Datkhile: Critical revision of intellectual contents and Final Approval of the article.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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