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Original Article

STUDY OF *IN VITRO* ANTI-OXIDANT AND ANTI-DIABETIC ACTIVITY BY *MUSSAENDA MACROPHYLLA* ROOT EXTRACTS

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ABSTRACT

Objective: The systematic study of effective alternative anti-diabetic drugs has great importance to manage diabetes as well as other oxidative stress-related diseases. According to previous research, root and bark of *Mussaenda macrophylla* plant has anti-microbial, anti-coagulant, anti-inflammatory, and hepatoprotective activity. Ethnomedicinal data shows that *Mussaenda macrophylla* is used to treat diabetes as well as oxidative stress. The objective of this research is to investigate *in vitro* anti-diabetic and anti-oxidant activity of root extract of *Mussaenda macrophylla.*

Methods: DPPH free radical scavenging assay was used to detect anti-oxidant potency of ethanol and methanol root extract of the plant and expressed as % of radicle inhibition. Anti-diabetic activity was determined by the glucose diffusion method using a glucose oxidase kit and results were expressed as mean±SD.

Results: The ethanol root extract at the concentration of 50 mg/ml and 100 mg/ml showed better glucose diffusion inhibition than that of methanol extract at the same concentration on increasing time interval. Ethanol extract at the concentration 100 µg/ml displayed better DPPH scavenging activity (89.83±0.19 %) than that of methanol extract (86.61±0.75%).

Conclusion: This study concluded that ethanol and methanol root extract of *Mussenda macrophylla* have potent anti-diabetic as well as anti-oxidant activity but further advance research is necessary in the animal model.

Keywords: *Mussaenda macrophylla*, Anti-diabetic, Anti-oxidant, *In vitro*, DPPH

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INTRODUCTION

Oxidative metabolism is essential for the survival of cells and plays a key role in the normal *in vivo* regulatory system. Accumulation of excess free radicals may result in excess production of some enzymes like superoxide dismutase, catalase, and peroxidase, ultimately leading apoptosis by oxidation of membrane lipids, cellular proteins, DNA, and enzymes, thus shutting down cellular respiration. They can also alter cell signaling pathways [1]. Free radicals play a vital role to increase tissue damage leading to human diseases such as cancer, aging, neurodegenerative disease, arteriosclerosis, and pathological events in living organisms [2]. The imbalance between free radicals production inside the body and its ability to counteract or detoxify their harmful effects through neutralization by antioxidants result in oxidative stress [3]. Antioxidant has the capability to trap free radicals. Secondary metabolites such as flavanoids and flavones are broadly distributed in plants and have anti-oxidant properties [4]. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and butylated hydroquinone are some examples of synthetic antioxidants. Dietary antioxidants like vitamin C and phenolic compounds are abundant in foods to contribute defense against oxidative stress [2]. Due to unwanted effects like carcinogenic effects of synthetic anti-oxidant, interest in plant-based natural antioxidants is growing on [5]. Various parts of plants like bark, leaves, flowers, roots, fruits, seeds, etc. may contain natural antioxidant constituents [4]. In this respect, the genus *Mussaenda* has been a significant source of natural products in the field of pharmacology [6].

Diabetes mellitus is one of the serious metabolic disorders of several etiologies. Chronic hyperglycemia with carbohydrate, fat, and protein metabolism imbalance, etc. is major pathological problems of diabetes which results from abnormal insulin secretion, insulin action, or both. It causes various effects, including long term damage, dysfunction, and failure of various organs. As vast progress has been done regardless of the treatment of diabetes mellitus in the allopathic system, the number of patients with poor glycemic control is still high. A large number of medicines have been developed for the treatment of this disease. New products with enhanced tolerability and better efficacy are in the focus of research and development efforts in today's world. Metformin is extensively used as a first choice of drug for the treatment of type-2 diabetes. Biguanides, Thiazolidinediones, Meglitinides, GLP-agonists, DPP inhibitors, Dopamine agonists, SGLT2 inhibitors are some new class of anti-diabetic drugs approved by FDA since 1990 [7]. Despite all the progress in synthetic chemistry and biotechnology, a number of adverse effects including diarrhea, vomiting, dizziness, headache major problems with these medications so plants are still an indispensable source of medicinal preparations [8].

Glucose oxidase (GOD) and peroxidase (POD) assay

Enzymes are a simple and widely used analytical tool for the study of *in vitro* anti-diabetic effects in the food, biochemical, and pharmaceutical industry. Enzymatic methods are usually specific, reproducible, sensitive, rapid, and, therefore, ideal for analytical purposes. High specificity and sensitivity of enzymes, quantitative assays are conducted on crude materials with little or no sample preparation. This kit is for the quantitative, enzymatic determination of glucose in food and other materials. In this method intensity of color due to the Quinoneimine dye complex is directly proportional to the amount of glucose present in the sample [9].

Principle:-

$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{D-Gluconic Acid} + H_2O_2$$

Peroxidase

H_2O_2+Phenol+4-AAP ⟹ Quinoneimine dye+H_2O

Mussaendamacrophylla is an evergreen shrub belonging to the family Rubiaceae, which is commonly known as the sweet root. It is widely distributed in central and eastern Nepal up to the height of 1800 m. It is also found in northern India, southern China, and Myanmar [8]. This plant consists of a unique feature as it has orange-colored inflorescence and with the beautiful branch. Different parts of *M. macrophylla* can be used to treat persons with sore mouths and sore throats in different parts of Nepal. Root juice is given in fever. It is also used in diabetic patients and good for stomach acidity [10]. Conventionally bark of *M. macrophylla* is used to treat snakebite [8]. Beside traditional medicinal use, *M. macrophylla* has various biological effects such as cytotoxic activity [11], anti-bacterial [12], anti-inflammatory, anti-coagulant, hepatoprotective activity [13] as well as it is effective against oral pathogens [14]. The root bark of this plant consists of iridoid glucoside, sterol, and galactoside [13], flavonoids, and ursane type triterpenoids [14]. In this study, *in vitro* antidiabetic and antioxidant activity of root extract of *Mussaenda macrophylla* was investigated.

MATERIALS AND METHODS

Chemicals and instruments

Ascorbic acid (Merck) DPPH and D-glucose, glucose oxidase kit (Sigma-Aldrich), Nacl (S. D fine chem limited). Digital balance (ATX224, SHIMADZU Corporation, Philippines), Rotary evaporator (R-210/215, BUCHI Labor technok AG, Switzerland), Sonicator (INDOSATI Scientific Lab Equipments), Hot air oven (S. M. Scientific Instruments (P) Ltd., Delhi), UV Spectrophotometer (UV-1800 model; Shimdazu Corporation Pvt. Ltd, China),

Plant materials

The plant was collected from Kaski, Gandaki Zone, and Western Nepal. The selection of the species used in this study was mainly based on their ethnomedicinal evidences (literature) for various diseases such as diabetes, oliguria, Bacterial infection, fever, pain, cancer etc. Scientific name and parts used of collected plant material is given in table 1.

Table 1: Details of plant collection

S. No.	Plant	Local name	Collected parts	Collection site	Collection date
1.	*Mussaenda macrophylla*	Dhobini	Roots	Ghachok, Kaski	October, 2018

Plant identification and herbarium preparation

The plants were identified with the help of Botanist (Homnath Pathak from Prithvi Narayan campus, Nepal) and also compared with the literatures. The voucher specimen was preserved in the Pharmacognosy Laboratory of the Crimson College of Technology (Specimen number: CCT/HRB/2018-121).

Drying and comminution of plants

Collected plant materials were cleaned and divided into small pieces and subjected to shade drying at room temperature in a well-ventilated environment for 30 d. After drying, they were ground to a fine powder using a portable grinding machine and passed through the sieve of mesh size 40 followed by packing into the airtight plastic bottle, sealed to prevent contamination and stored at room temperature in a dark place until use.

Extraction procedure

Cold triple maceration technique was applied in which 500 g of the dried powders were soaked with 2500 ml of organic solvents (i.e. ethanol and methanol) with occasional shaking and stirring for 2 d. The mixture was filtered by a piece of clean, white cotton material, marc pressed, and then finally filtered with Whatman filter paper no.1. Using the marc residue, the same process was repeated for two times, and clear infiltrated liquid solutions were mixed and kept in a conical flask; the mouth was covered with aluminum foil and kept in a refrigerator at a temperature of 6 °C until use.

Evaporation of extracts

The filtrate obtained from the extraction process was then evaporated to dryness using a rotary vacuum evaporator. Both methanol and ethanol extracts of root were evaporated at 37 °C–40 °C. The dried extracts were kept in glass vials and the percentage yields of the extract were calculated then extract were covered with aluminum foil and stored in the refrigerator at a temperature of 6 °C until use.

Antioxidant activity

Ethanol and methanol root extracts were evaluated for antioxidant effect by stable DPPH (1,1-diphenyl-2-picrylhydrazyl)) radical scavenging activity assay according to previously established method with slight modification [4, 15]. Four different concentrations of each extract (0.1-100 µg/ml) with 10 fold dilutions were prepared in ethanol and sample solutions containing 2 ml of extract were added to 2 ml 0f 0.1 m mol ethanolic solution of DPPH. The tubes were shaken vigorously and allowed to stand for 30 min at room temperature in a dark place. Changes in absorbance of samples were measured at 517 nm. Ascorbic acid and ethanol were used as the positive and negative control, respectively. All the tests were performed in triplicates.

Free radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Abs control} - \text{Abs sample}) \times 100}{\text{Abs control}}$$

Where,

Abs control is the absorbance of DPPH radical+ethanol

Abs sample is the absorbance of DPPH radical+sample extract/standard.

In vitro anti-diabetic activity

In vitro anti-diabetic activity was measured according to established method with slight modification [16].

Preparation of egg membrane

To investigate the *in vitro* glucose movement, egg membrane was used as semi-permeable membrane. Egg membrane was prepared by dipping the empty egg in a 15% v/v HCl solution. Finally, the white membrane was taken by removing the outer cover of the egg. Thus obtain membrane was kept in distilled water until use.

Preparation of glucose working reagent

Glucose working reagent was prepared by dissolving glucose reagent of concentration 100 mg/dl in a 50 ml buffer solution. Thus prepared reagent was kept in the dark until use.

Preparation of extract solution

For this analysis, 50 mg/ml and 100 mg/ml of two different concentrations of both ethanol and methanol root extract were prepared using 5% tween 80 solutions and used for the study.

Glucose diffusion inhibition [16, 17]

In this study, simple model was employed to understand and determine the *in vitro* effects of *Mussaenda macrophylla* root extract on glucose movement. This model is derived based on the method described by Edwards *et al.,* 1987 [18]. It this method, a sealed egg membrane was prepared instead of a sealed dialysis tube into, which 15 ml of a solution of glucose and 0.15 molar sodium chloride were introduced and the

diffusion of glucose in the external solution was analyzed. The model used in this experiment consisted of an egg membrane into which 1 ml of 50 mg/ml and 100 mg/ml plant extract in 5% tween 80 and 1 ml of 0.15 molar sodium chloride containing 0.22 molar D-glucose was added. The egg membrane was sealed at one end, placed in a 50 ml beaker containing 45 ml of 0.15 molar sodium chloride. The beaker was kept at room temperature. The transfer of glucose into the external solution was measured at set intervals of time. In this experiment, the effects of 50 mg/ml and 100 mg/ml plant extracts on glucose diffusion were compared to a control test which is devoid of plant extract. Determination of glucose concentration was conducted enzymatically with the help of a glucose oxidase kit. All the tests were carried out in triplicate and results were presented as mean±SD.

Statistical analysis

The results of statistical analysis for experiments were expressed as mean±SD and all the data were evaluated by the student's t-test, the two-tailed method using Microsoft excel 2018. The results obtained were compared with the controlled group. The $p<0.05$ was considered to be statistically significant.

RESULTS

Extractive yield value

The extractive yield value of root of *Mussaenda macrophylla* in ethanol and methanol are given in the table 2

Table 2: Extraction yield percentage

S. No.	Plants	Ethanol	Methanol
1.	*Mussaenda macrophylla* (Root)	2.6 %	3.5 %

Antioxidant activity

Ethanol and methanol extract of *Mussaenda macrophylla* root and standard ascorbic acid produced dose-dependent free radicle inhibition. Both extracts exhibited potent antioxidant effect at 100 µg/ml concentrations, in which ethanol extract showed significant scavenging activity of 89.83±0.19%. Whereas, 86.61±0.75 % of free DPPH radicle was inhibited by methanol extract at the same concentration. Percentage of DPPH free radicle scavenges by plant extracts and standard ascorbic acid and their half minimum effective concentration (EC_{50}) are described in Table 3 and fig. 1, respectively.

Table 3: DPPH free radical percentage inhibition of ethanolic and methanolic extract of *M. macrophylla* root

S. No.	Concentration(µg/ml)	Percentage inhibition		
		Methanolic extract	Ethanolic extract	Ascorbic acid
1.	0.1	4.8±0.10	5.52±0.38	6.81±0.12
2.	1	20.76±0.38	22.71±0.61	25.43±1.53
3.	10	59.32±0.90	66.1±0.27	88.96±1.13
4.	100	86.61±0.75	89.83±0.19	93.93±0.10

All the data are expressed as mean±SEM.

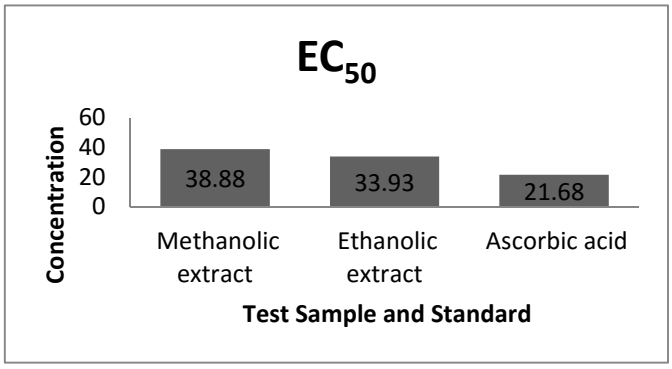

Fig. 1: Graphical representation of EC_{50} value of ascorbic acid, ethanolic, methanolic extract of *M. macrophylla*

Note**:** EC_{50} represents the concentration of test and standard sample in µg/ml at which it shows 50% of DPPH free radical inhibition.

Glucose diffusion inhibition analysis

Data for the extent of glucose diffusion inhibition by plant extract are given at Tables 4 and 5 for 50 mg/ml of ethanol and methanol extract, whereas at Tables 6 and 7 is for 100 mg/ml of extract. The glucose diffusion through the control sample was considered to be 100% for the calculation of relative glucose movement inhibition. Maximum glucose inhibition was exhibited by ethanol extract at both 50 mg/ml (31.00±0.23%) and 100 mg/ml (61.00±0.21%) after 24 h. Similarly, methanol extract also showed an appreciable amount of glucose inhibition with (22.73±0.17%) at 50 mg/ml and (44.43±0.28%) at 100 mg/ml concentration after 24 h.

Table 4: Effect of *Mussaenda macrophylla* root extracts (50 mg/ml) on the movement of glucose out of Egg membrane over 24 h incubation periods

Time	1 h	3 h	5 h	15 h	24 h
Control (without extract)	209.37±0.76	302.83±0.44	316.33±0.82	344.83±0.26	365.3±0.89
Ethanol Extract	185.4±0.77	236.53±0.94	240.43±0.61	256.93±0.54	252.03±0.87
Methanol Extract)	188.66±0.44	272.1±0.28	275.96±0.09	276.33±0.86	282.26±0.65

Concentration of glucose was measured in mg/dl. The values are expressed as mean±standard deviation for triplicate. All values are significant at $P<0.005$

Table 5: Relative inhibition of glucose movement (in percentage) by *Mussaenda macrophylla* root extracts (50 mg/ml) out of egg membrane over 24 h incubation periods

Time	1 h	3 h	5 h	15 h	24 h
Ethanol Extract	11.45±0.37	21.89±0.31	23.99±0.19	25.49±0.15	31.00±0.23
Methanol Extract	9.88±0.21	10.14±0.09	12.76±0.02	19.86±0.25	22.73±0.17

The values are expressed as mean±standard deviation for triplicate.

Table 6: Effect of *Mussaenda macrophylla* root extracts (100 mg/ml) on the movement of glucose out of egg membrane over 24 h incubation periods

Time	1 h	3 h	5 h	15 h	24 h
Control (without extract)	209.37±0.76	302.83±0.44	316.33±0.82	344.83±0.26	365.3±0.89
Ethanol extract	168.53±0.54	207.66±1.18	196.73±0.49	177.26±0.49	142.46±0.77
Methanol extracts)	172.43±0.57	240.2±0.86	244.8±0.57	216.26±0.82	203.16±1.18

Concentration of glucose was measured in mg/dl. The values are expressed as mean±standard deviation for triplicate. All values are significant at P<0.005

Table 7: Relative inhibition of glucose movement (in percentage) by *Mussaenda macrophylla* root extracts (100 mg/ml) out of egg membrane over 24 h incubation periods

Time	1 h	3 h	5 h	15 h	24 h
Ethanol Extract	19.5±0.26	31.42±0.39	37.80±0.15	48.59±0.14	61.00±0.21
Methanol extracts	17.64±0.27	20.68±0.28	22.61±0.18	37.28±0.23	44.43±0.28

The values are expressed as mean±standard deviation for triplicate.

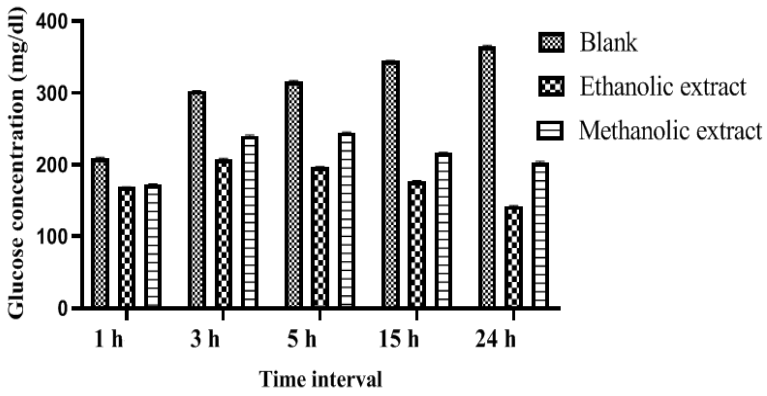

Fig. 2: Bar diagram showing the effect of *Mussaenda macrophylla* root extract (100 mg/ml) on the movement of glucose out of egg membrane over 24 h incubation periods

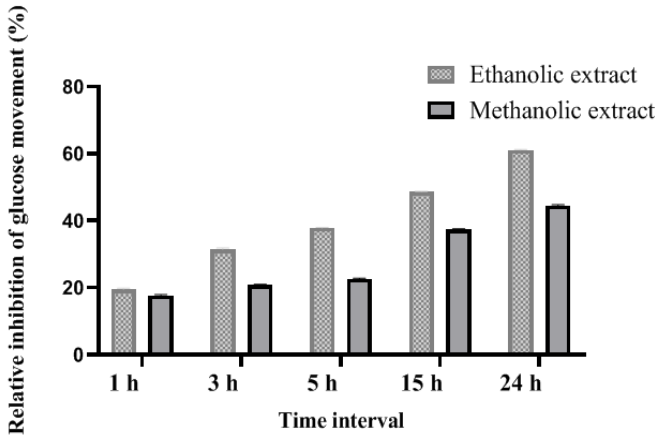

Fig. 3: Bar diagram showing the percentage of relative glucose movement by *Mussaenda macrophylla* root extracts (100 mg/ml) out of egg membrane over 24 h incubation periods

DISCUSSION

The present study was designed to investigate the anti-oxidant and anti-diabetic properties of the plant *Mussaenda macrophylla* which have significant ethno medicinal value in different parts of Nepal. In the anti-oxidant activity assay, table 3 revealed that both extracts inhibited free radical potently in dose dependent manner. Ethanol extract of root was found to be more effective to inhibit DPPH free radical compared to methanol extract in all tested concentrations and its antioxidant potency is almost similar to ascorbic acid, which is also supported by fig. 2. The reason for more antioxidant potency of ethanol extract may be due to the synergistic action of chemical constituents extracted by ethanol. In the present study, the minimum effective concentration value of ethanol and methanol extract is 33.93µg/ml and 38.88 µg/ml respectively, whereas ascorbic acid has EC_{50} of 21.68 µg/ml, which signify that both extracts even in low concentration showed their antioxidant potency comparable to the standard solution. According to an earlier study, the minimum effective concentration of whole plant extract was reported to be 63 µg/ml [19].

Diabetes mellitus is a devastating and often life-threatening metabolic disorder with increasing incidence throughout the world. With the rapid rise in the rate of incidence of diabetes mellitus it has been estimated that 1 in 5 may have diabetes by 2025 [20]. Anti-hyperglycemic phytochemicals of plants can increase glucose transport and metabolism in muscle or to stimulate insulin secretion [18]. In the present study, a study was conducted to determine the extent of glucose diffusion inhibition by ethanol and methanol extract through a semipermeable membrane that can correlate their ability to slow down the diffusion and movement of glucose in the intestinal tract [17].

From the table 4, in the case of control sample (without plant extract), after 1h of incubation, 209.37 mg/dl glucose was diffused outside the membrane which reached a plateau up to 365.3 mg/dl mean concentration in 24 h. But in the sample containing 1 ml ethanol and methanol extract of both concentrations showed a significant effect on glucose diffusion inhibition after 24 h as compared to control. Ethanol extract has prominent glucose movement inhibition potency in comparison to methanol extract. Thus observed the hypoglycemic effect of both ethanol and methanol extract of the root of this plant is may be due to the presence of diverse chemical compounds like iridoids, triterpenoid glycosides, flavonoids, sterols [14]. This effect was reported for the first time in this study.

CONCLUSION

The result in this study revealed that both ethanol and methanol root extract of *Mussaenda macrophylla* has anti-oxidant as well as anti-diabetic potency. Ethanol extract in comparison to methanol extract was found more potential in scavenging the free radicals thus may be capable of reducing oxidative stress inside our body. Similarly, inhibition of the movement of glucose was found to be better by the ethanol extract in comparison to methanol extract, from which we can conclude that the ethanol extract of this plant root has potent glucose movement inhibition capacity from the intestine for the prevention of diabetes. Further advance research should be done to confirm its anti-diabetic and anti-oxidant potency.

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Nil

AUTHORS CONTRIBUTIONS

Jitendra Pandey and Deepak Shrestha act as First author. Ravin Bhandari act as Corresponding author.

CONFLICT OF INTERESTS

We declare that we have no conflict of interest.

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