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FORMULATION DEVELOPMENT AND EVALUATION OF ANTIDIABETIC ACTIVITY OF AEGLE MARMELOS ROOTS (RUTACEAE)

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ABSTRACT

Diabetes mellitus (DM) is one of the leading metabolic disorders worldwide. Diabetes mellitus is due to a lack of function of β pancreatic cells or insensitivity of target tissue to insulin. This disorder not only affects the metabolic system but also causes various associated problems like neuropathy, nephropathy, retinopathy, and heart-related disease. Treatment of Diabetes mellitus (DM) with allopathic drugs for a long duration has been not safer and may cause several adverse effects along with hypoglycemia. World health organization (WHO) recommended drug researchers emphasize herbal-based research which is safer and more effective for longer regimens in dosing. Hence this work is attempted to exploit natural resources, abundantly available in our surroundings and also evident in the traditional system of medicine. In this research work *Aegle marmolus* roots extract were exploited for anti-diabetic potential by *in-vitro* method. Result were shown α-amylase inhibitory activities IC_{50} 112 µg/ml. EEAMR exhibited highest percentage of glucose uptake *i.e.* 54.84±0.752, which was almost near to the standard *i.e.* 61.74±0.527.

KEYWORDS: Diabetes mellitus, Hypoglycemia, Neuropathy, Retinopathy, α-amylase, *Aegle marmolus.*

INTRODUCTION

Diabetes mellitus is recognized as being a syndrome, a collection of disorders that have hyperglycemias and glucose intolerance as their hallmark, due either to insulin deficiency or to the impaired effectiveness of insulin's action, or to a combination of these.[1] In order to understand diabetes it is necessary to understand the normal physiological process occurring during and after a meal. Food passes through the digestive system, where nutrients, including proteins, fat and carbohydrates are absorbed into the bloodstream.[2]

The human pancreas is basically composed of two types of secretory cells that are both involved in nutrient handling: 98% of the cells- the exocrine type – secrete a food- processing enzyme-bicarbonate mixture into the duodenum, while the remaining 2% - the endocrine type. The islet changes, from a morphological point of view, associated with various types of diabetes can be divided into those with and without severe beta-cell loss. Severe beta-cell loss is found in type I diabetes and some uncommon forms of diabetes such as virus-related diabetes and congenital diabetes. Islets without severe loss of beta-cells are encountered in type II diabetes and in the secondary forms of diabetes.[18]

Unfortunately, there is no cure for diabetes yet but by controlling blood sugar levels through a healthy diet, exercise and medication the risk of long-term diabetes complications can be decreased. Medicinal plants which have showed anti-diabetic activity during earlier investigations include *Panax* species, *Phyllanthus* species, *Acacia arabica, Aloe vera, Aloe barbadensis, Artemisia pallens, Momordica charantia, Alium cepa, Trigonella foenum-graecum* etc.

MATERIALS AND METHODS

Aegle marmelos root obtained from LNCP garden by digging the root & then it was thoroughly washed and dried under shade. Plant prior authenticated by Suman Mishra scientist Vindhya herbal Bhopal. The further drug is cut into small pieces coarsely powdered and pulverized. The coarse powder is then extracted by petroleum ether and then ethyl acetate by the maceration process.Further, it was phytochemically investigated for the presence of various phytoconstituents category.

Preparation of Ethanolic extract of Aegle marmelos.

The powdered plant materials were extracted with 70% v/v ethanol by hot continuous percolation method using soxhlet apparatus (Harborne, 1984). The solvent from the extract was removed under reduced pressure using rotary evaporator and subjected to freeze drying in a lyophilizer until dry powder was obtained. The extract of *Aegle marmelos* was stored in air tight container and utilized.

Qualitative phytochemical analysis[3 16.17]
The extracts obtained by successive solvent extraction were subjected to various qualitative tests to detect the presence of chemical components.

Evaluation of antidiabetic activity by using *in vitro* assays

Alpha-amylase inhibitory assay[5]
The Alfa–amylase inhitory assay of ethanolic extact of Aegle marmolus root was evaluated according to a previously described method by Ranilla et al. (2008) . In brief, 0.5 ml of extract was mixed with 0.5 ml of α-amylase solution (0.5 mg/ml) with 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl). The mixture was incubated at room temperature for 10 min and 0.5 ml of starch solution (1%) in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added. The resulting mixture was incubated at room temperature for 10 min, and the reaction was terminated using 1 ml of dinitrosalicylic acid color reagent. At this time, the test tubes were placed in a water bath (100 °C for 5 min) and cooled until room temperature was attained. The mixture was then diluted with 10 ml of deionized water, and absorbance was determined at 540 nm. The absorbance of blank (buffer instead of extract and amylase solution) and control (buffer instead of extract) samples were also determined. Acarbose was used as standard drug.

The inhibition of α-amylase was calculated using the following equation.

$$\% \text{ inhibition of } \alpha\text{−Amylase} = \frac{\text{Abs control}-\text{Abs sample}}{\text{Abs control}} \times 100$$

Where Abs control corresponds to the absorbance of the solution without extract (buffer instead of extract) and with α -amylase solution.

Abs sample corresponds to the solution with extract and α -amylase solution

Glucose uptake in yeast cells[6]
Glucose uptake assay by yeast cells was performed according to Cirillo et al. (1963). The yeast, (*Saccharomyces cerevisiae*) suspended in distilled water was subjected to repeated centrifugation (3000 × *g*, 5 min) until clear supernatant fluids were obtained and 10% (v/v) of the suspension was prepared in distilled water. Various concentrations of plant extracts (50to 250 µg/ml) were added to 1 ml of glucose solution (5 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µl of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2500 × *g*,5 min) and amount of glucose was estimated in the supernatant. Acarbose was used as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

$$\text{Increase in glucose uptake \%} = \frac{\text{Abs sample-Abs control}}{\text{Abs sample}} \times 100$$

Where, Abs sample is the absorbance of test sample and Abs control is the absorbance of control reaction (containing all reagents except the test sample). All the experiments were carried out in triplicates.

Capsule Formulation[7.8,9]
EEAMR was taken for the formulation development. The crude extract of *Aegle marmolus* was blended with the various excipients for convenient to formulate as a unit dosage form as capsules and then evaluated. It was carried out with EEAMR-300mg/capsule and cross povidone XL10 as super disintegrant used in the formulation, which is presented in Table 4. The bioactive compounds and excipients were passed through 60 # sieve, weighed and mixed well. Finally, the preservatives were mixed and filled in “0” size hard gelatin capsule using hand filling machine with the average weight of 450 mg per capsule.

EVALUATION OF THE CAPSULE FORMULATION[10, 11]

Uniformity of Weight
An intact capsule was weighed separately and the capsule was opened without any loss or part of the shell and removed the contents as completely as possible. The weight of the content was calculated from the difference between the capsule with content and the shell weight alone.

Moisture Content
Moisture content was determined by using automatic Karl Fischer titration apparatus.

6.9.3. Disintegration Test
The test was carried out on 6 capsules using tablet disintegration tester ED-20 (Electrolab, Mumbai, India) distilled water at 37°C±2°C was used as a disintegration media and the time in second taken for complete disintegration of the capsule with no palable mass remaining in the apparatus was measured in sec.

6.9.4. Microbiological Test
The formulated capsules were subjected to microbiological evaluation, which was carried out as per the procedures of Indian pharmacopoeia, 2007 and WHO Guideline.

I. Total viable count
II. Test for absence of Escherichia coli
III. Test for absence of Salmonella sp.

Bacterial Count
To about 9 – 10cm diameter petridish containing 15ml of liquefied soya bean casein digest agar maintained at not more than 45°C, One ml of pre-treated sample was added. The petridish were incubated at 37°C for 48h and the numbers of colonies were counted.[12]

Test for Absence of E. coli

One ml of pre-treated sample was added to the tube containing 5ml of Mac Conkey Broth. The tube was incubated at 42°C for 48h, and observed for development of pink color.[16, 17]

Test for Absence of Salmonella sp.

Each 10g of sample was taken in 2 different conical flasks. In the first conical flask 100ml of pre sterilized peptone buffer was added. In the second conical flask 100ml of fluid lactose broth was added. From each conical flask 5ml of sample was withdrawn and 100ml of pre-sterilized nutrient broth was added. The nutrient broths with samples were incubated at 35°C for 24h. At this end of the time, one ml of the nutrient broth was added to tetra thionate brilliant green bile broth and the tubes were incubated at 35°C for 48h. After that bile broth was transferred into bismuth sulphate agar plate. In another tube, one ml of bile broth was transferred into brilliant green agar plate. Both the plates were incubated at 35°C for 48h. The colour of bismuth sulphate agar plate changed from green to black metallic color colony. It indicates positive and the brilliant green agar plate turns green to pink color colony.[13, 14]

Analysis of Drug Content

Quantification of Kaempherol-3-O-Glucoside equivalent to Kaempherol by HPTLC The analysis is performed with HPTLC (Camag, Switzerland). The active principle is applied with the linomat IV applicator on the HPTLC silica gel 60 F 254 plates (E-Merck, Germany). The plates are developed with a twin-trough developing chamber. After 70 developments, the plates are scanned with a Camag TLC scanner 3 and the data are processed with Win CATS software. The HPTLC analysis of the active principle is carried out using different solvent systems for individual plant components.[15]

RESULT AND DISCUSSION

Table 1: Phytochemical tests for EEAMR.

TEST	EEAMR
Alkaloids	
Mayer's test	+ ve
Wagner test	+ ve
Hager's test	+ ve
Carbohydrates And	
Molisch test	+ ve
Legal's test	+ ve
Borntrager's test for anthraquinones	- ve
Glycosides	
Legal test	- ve
Keller Kiliani Test	- ve
Terpenoids & Phytosterols	
Liebermann-Burchard test	+ ve
Salkowski test	+ ve
Flavonoids	
Shinoda test	+ ve
Fluorescence test	+ ve
Tannins & Phenolic compound	
Ferric chloride test	+ ve
Potassium dichromate test	+ ve
Lead acetate test	+ ve
Proteins	
Millon's test	- ve
Biuret Test	- ve
Ninhydrin Test	- ve
Saponins	
Saponification test	- ve
Fats & Lipids	
Spot test	- ve

Alpha-amylase inhibitory assay

The ethanolic extact of *Aegle marmolus* roots were subjected to α-amylase inhibitory assay along with Acarbose as a standard drug. The ethanolic extract showed higher activity among all other extracts tested (Fig-1) which was comparable to standard acarbose. The α-amylase inhibitory activities of differed solvent extracts are recorded in Table 2.

Table 2: Amylase inhibitory activities and IC50 values by Aegle marmolus root extracts.

Sample	Concentration	Inhibition I %	IC_{50}(µg/ml)
Ethanol extract	100 µg/ml	48.264± 0.457	112 µg/ml
Standard (Acarbose)	100 µg/ml	56.752± 0.312	86.22 µg/ml

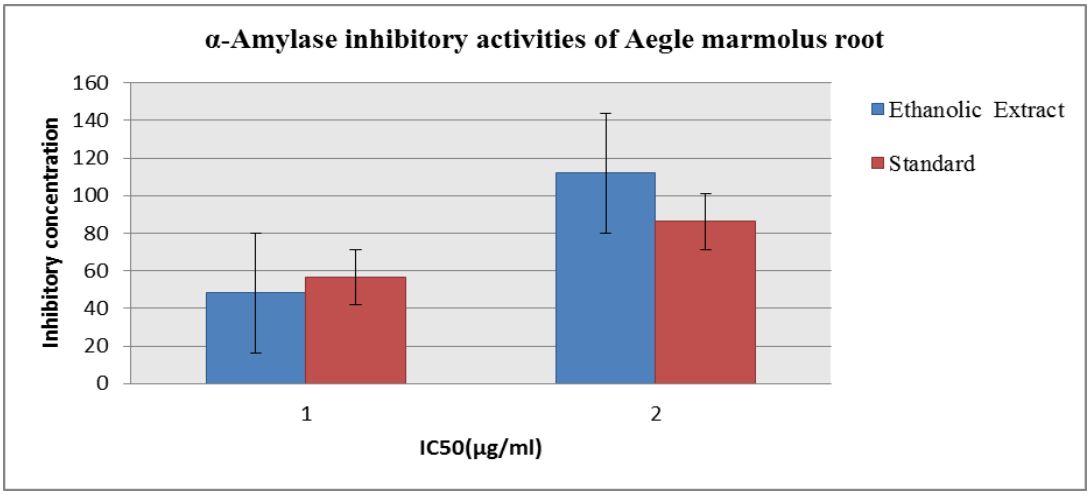

Fig.1. Percentage inhibition of α-amylase by *Aegle marmolus* root extracts.

Glucose uptake in yeast cells

Ethanolic extract of *Aegle marmolus* root extracts are subjected to *in vitro* glucose uptake assay employing yeast as model. The percentage of glucose uptake in yeast cells by the extract was compared with standard drug Acarbose. Aqueous extract exhibited higher activity than the remaining solvent extracts tested (Fig .2). There was concentration dependent increase in percentage of glucose uptake with increasing in concentration of *Aegle marmolus* extract.[5.4] Ethanolic extract exhibited highest percentage of glucose uptake *i.e.* 54.84±0.752, which was almost near to the standard *i.e.* 61.74±0.527 (Fig..2) at 250 µg concentration. Results also indicated that *Aegle marmolus* had almost same efficiency in increasing the glucose uptake by yeast cells as compared to standard drug Acarbose.

Table- 3. Percentage of glucose uptake in yeast cells treated with *Aegle marmolus* ethanolic root extracts.

Samples	Concentration(µg/ml)	Inhibition (%)	IC50(µg/ml)
Standard	50 µg	42.23±0.241	48.65 µg
	100 µg	46.52±0.513	
	150 µg	49.31±0.362	
	200 µg	55.25±0.421	
	250 µg	61.74±0.527	
Ethanol extracts	50 µg	35.12±0.378	154.87 µg
	100 µg	37.25±0.642	
	150 µg	43.74±0.258	
	200 µg	48.67±0.325	
	250 µg	54.84±0.752	

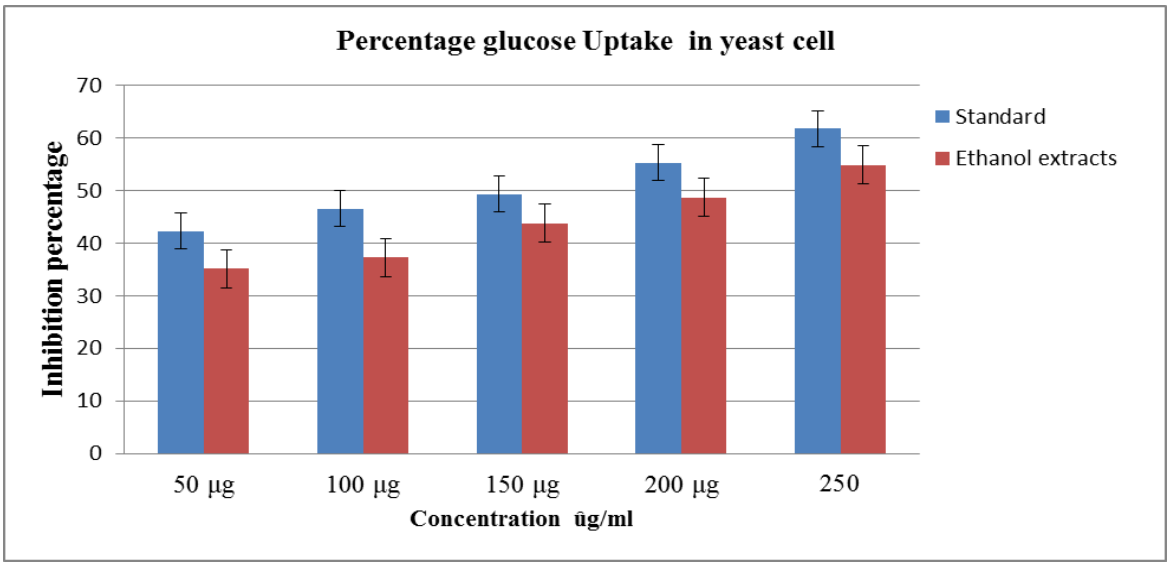

Fig.-2: Percentage of glucose uptake in yeast cells treated with *Aegle mormolus* extracts.

Table- 4: Working Formulas of EEAMR capsules.

S. No	Ingredients	Weight (mg)
1.	EEAMR	300
2.	Dicalcium phosphate anhydrous	105
3.	Sodium lauryl sulphate	10
4.	Cross povidone XL 10	10
5.	Light magnesium stearate	20
6.	Methyl paraben	4
7.	Propyl paraben	1

Table- 5.Physiochemical parameters of capsule.

S. No	Name of the test	Observation
1.	pH	7.52
2.	Moisture Content	3.62%w/w
3.	Weight Variation (Mean ± SEM)	472± 5 mg
4.	Disintegration Time (Mean ± SEM)	5 Min 45 Sec± 0.32

Table 6: Microbiological evaluation of capsule.

S. No	Name of The Test	Limit of Herbal Formulation as Per WHO	Observation
1.	Total microbial count bacterial count (Per gram of sample)	Aerobic bacteriamax10^5/g	874 CFU/g
2.	Fungal count(per gram of sample)	Moulds maximum 10^3/g	360 CFU/g
3.	Presence *of escheria colli*	Should be absent	Absent
4.	Presence of salmonella Sp	Should be absent	Absent

Quantification of Rutin derivative in EEAMR by HPTLC

Quantification of Rutin derivative in EEAMR by HPTLC assay as described in.

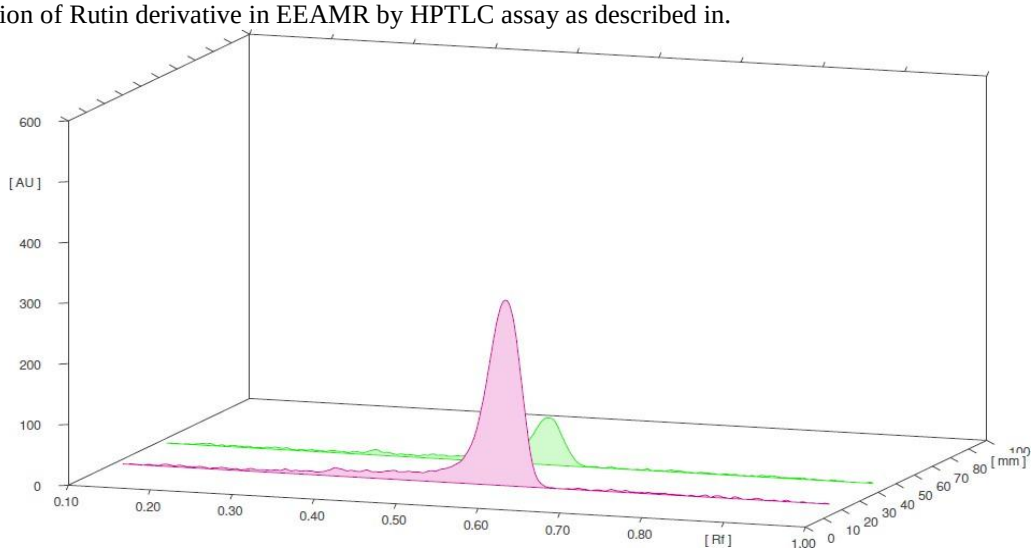

Figure -3: HPTLC peaks of standard Rutin and EEAMR.

Schematic representation of the HPTLC chromatograms of Rutin derivative equivalent to Rutin EEAMR are observed in Figure 3 and Figure 4 and under UV 254nm and UV 366nm the HPTLC plates, which are shown in Figure 5. Rf value and AUC values are shown in Table 6.

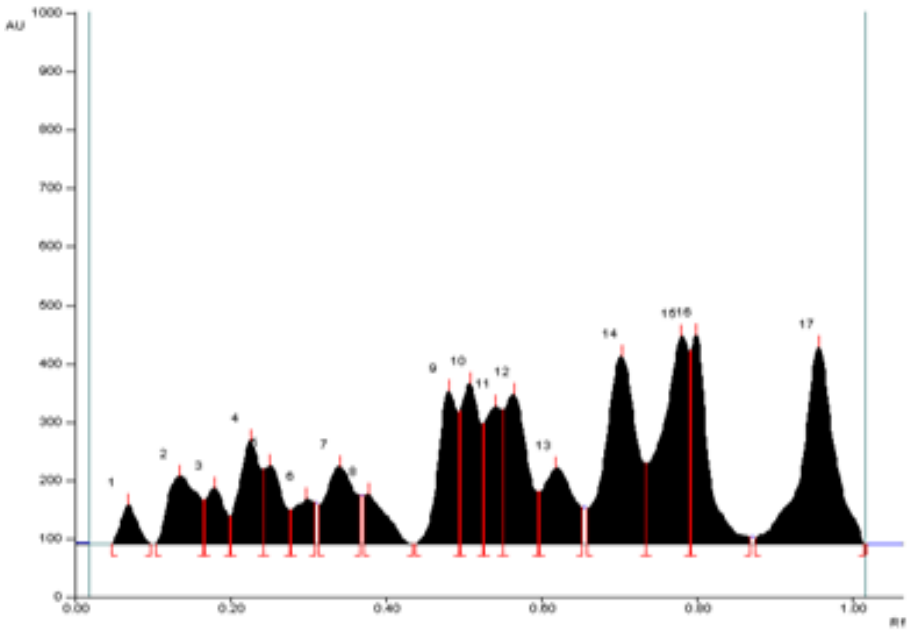

Figure-4: HPTLC peaks of standard Rutin and EEAMR.

CONCLUSION

The antidiabetic effect of plants is attributed to the mixture of phytochemicals or single components of the plant extracts. The phytochemicals responsible for antidiabetic properties mainly are alkaloids, phenolic acids, flavonoids, glycosides, saponins, polysaccharides, stilbenes, and tannins. In the several animal studies reported using different plants, there is a wide variety between the extractions methods. In present study extract of showing excellent antidiabetic potential by in-vitro method. Possible activities due to presence of bioflavonoid & flavonoid constituents present in extract which mimic the insulin activity & glucose uptake by adipose tissue.

CONFLICT OF INTEREST

Author declare that no conflict of interest.

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