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Original Article

EFFECT OF COMBINATION OF TWO PLANT EXTRACTS ON DIABETES MELLITUS

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ABSTRACT

Objective: To investigate the anti-diabetic activity of combined ethanolic extracts (1:1mixture) of dry leaves of *Syzygium cumini* and *Psidium guajava* belonging to the family *Myrtaceae* as well as to compare the anti-diabetic activity of these plants by *in vitro* methods.

Methods: *In vitro* glucose uptake assay was performed on cultured *L6* cell lines (*rat myoblast* cell line) and estimated the glucose uptake using high sensitivity glucose oxidase kit. *In vitro alpha amylase* inhibitory assay was performed on porcine *alpha amylase* and the absorbance was measured at 540 nm using a microplate reader. Acarbose was used as the standard in both the methods.

Results: At a concentration of 100µg/ml the percentage glucose uptake by the combined ethanolic extract (1:1 mixture) of *Syzygium cumini* and *Psidium guajava* leaves was 43.95 while for acarbose the corresponding value was 51.71. At 100 µg/ml the percentage of glucose uptake by *Syzygium cumini* and *Psidium guajava* was 27.62 and 22.17 respectively. The percentage inhibition of *alpha amylase* by the combined ethanolic extract (1:1 mixture) of *Syzygium cumini* and *Psidium guajava* leaves at a concentration of 1000 µg/ml was 36.51 and it was 29.26 for *Syzygium cumini* and 23.43 for *Psidium guajava.* For acarbose the percentage inhibition of *alpha amylase* was 73.82 at the concentration of 1000 µg/ml.

Conclusion: The combined extract of the leaves of the plants selected was found to be more effective than individual plant extracts against diabetes. The percentage glucose uptake of the combined extract was found to be closer to that of the standard drug acarbose. On comparison of two plants *Syzygium cumini* was found to be more active against diabetes than *Psidium guajava.* As the 1:1 mixture of the ethanolic extract is found to be more active, the combination of the two plants can be used to formulate drugs for treating diabetes.

Keywords: Glucose uptake assay, *Alpha amylase* inhibitory assay, *Syzygium cumini* and *Psidium guajava*

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INTRODUCTION

Syzgium cumini (Synonym: *Eugenia jambolan* Linn.) family *Myrtaceae* is a very large evergreen tropical tree [fig. 1]. The different parts of this plants have been used for a wide variety of ailments including cough, diabetes, dysentery, inflammation, pharyngitis, dermopathies, constipation, leucorrhoea and ringworm [1-3]. *Psidium guajava* L. known as Guava is a medicinal plant belonging to the family *Myrtaceae* [fig. 2]. Traditionally it is used for anorexia, cholera, diarrhoea, digestive problems, dysentery, gastric insufficiency, inflamed mucous membranes, laryngitis, skin problems, sore throat, ulcers, aches, bacterial infections, boils, bowel disorders, bronchitis, cold, colic, convulsions, cough, dyspepsia, oedema and epilepsy. *Psidium guajava* leaf extract is a potential reducing agent with biomedical applications [4, 5].

Diabetes mellitus (DM) which is one of the public health issues is a metabolic disorder characterized by the presence of chronic hyperglycaemia accompanied by an impairment of carbohydrate, lipid and protein metabolism that can lead to premature death. According to International Diabetes Federation report by 2030 almost around 552 million will have DM. The 1997 American diabetic association's recommendations for diagnosis of DM focus on fasting plasma glucose (FPG) while WHO focuses on oral glucose tolerance test [6-8]. In 2014, 422 million adults were living with diabetes compared to 108 million in 1980 across the world. Diabetes caused 1.5 million deaths in 2012. An additional 2.2 million deaths were caused by higher than optimal blood glucose by increasing the risks of cardiovascular and other diseases [9].

Insulin dependent diabetes mellitus (TYPE 1 DM) known as "juvenile diabetes" occurs due to failure of pancreas to secrete enough insulin and is characterized by beta cell destruction caused by an autoimmune process; leading to absolute insulin deficiency. Type 1 DM is characterized by the presence of anti–glutamic acid decarboxylase or insulin antibodies which identify the autoimmune processes that lead to beta cell destruction. Non-insulin dependent diabetes mellitus (Type 11 DM**)** known as adult onset diabetes begins with insulin resistance in which cells fail to respond to insulin properly. Lack of exercise and obesity may lead to this condition. Type 11 DM comprises 80% to 90% of all cases of DM. Diabetes which is triggered by pregnancy is called gestational diabetes as pregnancy may lead to insulin resistance. Around 10% of women with gestational diabetes may develop type II diabetes later [10]. Diabetes due to genetic defects of beta cell function or with defects of insulin action, diseases of the exocrine pancreas such as pancreatitis or cystic fibrosis, dysfunction associated with other endocrinopathies (e. g. acromegaly); and with pancreatic dysfunction caused by drugs, chemicals or infections are grouped together as monogenic or other specific type diabetes [11].

According to world ethno botanical information reports almost 800 plants possess anti diabetic potential. Hypoglycaemic agents from natural products are gaining more importance due to their lower side effects. The use of phytochemicals may delay the development of diabetic complications and may regulate the metabolic abnormalities by different mechanisms. Several plants can be used as potential sources of new drugs to complement existing oral hypoglycaemic agents [12-14].

The aim of the present study was to compare the anti-diabetic activity of ethanolic extract of *Syzygium cumini* and *Psidium guajava* leaves and to investigate the anti-diabetic activity of the combined extract of both the plants. This study was designed with an objective to perform the *in vitro* glucose uptake assay on cultured *L6* cell lines and *alpha amylase* inhibitory assay of the plants under study individually and as a combination of ethanolic leaf extracts of both *Syzygium cumini* and *Psidium guajava.*

Fig. 1: A. *Syzygium cumini* tree, B. *Syzygium cumini* leaves

Source of fig.: Photographs of the tree and leaves were taken from Idukki District of Kerala.

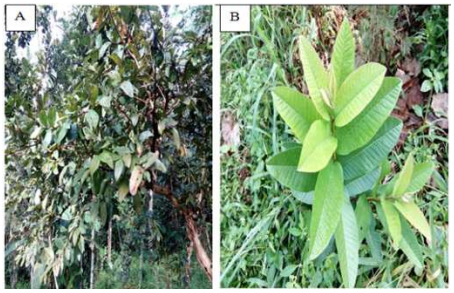

Fig. 2: A. *Psidium guajava* tree, B. *Psidium guajava* leaves

Source of fig.: Photographs of the tree and leaves were taken from Idukki District of Kerala.

MATERIALS AND METHODS

Chemicals and reagents

L6 cell line (*rat myoblast* cell line) was purchased from NCCS Pune and Dulbecco's modified eagle's media (DMEM) from Sigma Aldrich, USA. All other chemicals were procured either from Sigma, Ranbaxy fine Chemicals, New Delhi, Hi Media Mumbai or NICE chemicals Ltd, Cochin, Kerala, India.

Collection and authentication of plant material

The leaves of both plants were collected from the hilly regions of Idukki District, which is a densely forested mountainous region in the south Indian state of Kerala and were authenticated by Dr. Sr. Tessy Joseph, Professor, Department of Botany, Nirmala College, Muvattupuzha. Voucher specimen was deposited in the herbarium of Nirmala college, Muvattupuzha, Kerala, India with a Voucher no: NCBD 3812.

Preparation of extracts

The mature leaves of both *Psidium guajava* and *Syzygium cumini* were shade dried separately powdered in grinder to get coarse powder for extraction. 250 g of the Syzygium cumini leaf powder was extracted in ethanol (2.5 l) in a Soxhlet apparatus and concentrated to yield the crude ethanol extract of *Syzygium cumini* SC EL. Similarly 250 g of the crude powder of *Psidium guajava* leaf was extracted in ethanol (2.5 l) and concentrated to yield the crude ethanol extract of *Psidium guajava* PG EL. The extracts were concentrated using vacuum evaporator.

In vitro glucose uptake assay on cultured *L6* cell lines

The *L6* cell line (*rat myoblast* cell) was maintained in Dulbecco's modified eagles media supplemented with 10% *foetal bovine serum* (FBS). The cells were grown to confluency at 37 °C in 5 % CO_2 in a humidified atmosphere in a CO_2 incubator (NBS, Eppendorf, Germany). The cells were trypsinized with 500 µl of 0.025% trypsin in phosphate buffered saline (PBS)/0.5 mmol ethylene diamine tetra acetic acid (EDTA) solution (In vitrogen) for 2 min and passaged to T flasks in complete aseptic conditions. The cells were subcultured to a 24 well plate. The cells were kept in DMEM without glucose for 24 h after attaining 80% confluency. To the grown cells added the extracts at a final concentration of 25 µg, 50 µg and 100 µg from a stock of 1 mg/ml and incubated in DMEM containing 300 mmol glucose for 24 h. An untreated control with high glucose was also maintained. The cells were isolated after incubation by spinning at 6000 rpm for 10 min. The supernatant was discarded and added 200 µl of cell lysis buffer (1MTrisHcl, 0.25M EDTA, 2M Nacl, 0.5% Triton). The mixture was incubated for 30 min at 4 °C and estimated the glucose uptake using high sensitivity glucose oxidase kit. All experiments were repeated in triplicates and mean average was used for calculations [15].

$$\% \text{ Glucose uptake } = \text{OD of test} - \text{OD of Control} \div \text{OD of test} \times 100$$

In vitro alpha amylase inhibitory assay

Different concentrations of samples (125µg/ml–1000µg/ml) were prepared from a stock concentration of 10 mg/ml and made up to 1000 µl using 25 mmol phosphate buffer pH 6.9, containing 25 µl of porcine *α amylase* at a concentration of 0.5 mg/ml. The mixtures were incubated at 25 °C for 10 min. After pre incubation, 25 µl of 0.5% starch solution in 25 mmol phosphate buffer pH 6.9 was added to the mixtures. The reaction mixtures were then incubated at 25 °c for 10 min. The reaction was stopped with 50 µl of 96 mmol 3, 5-dinitro salicylic acid colour reagent. The micro plate was then incubated in a boiling water bath for 5 min and cooled to room temperature. Absorbance was measured at 540 nm using a microplate reader (Erba, Lisascan) [16].

CALCULATION

$$\% \text{ inhibition} = \frac{\text{control} - \text{test}}{\text{control}} \times 100$$

Table 1: Effect of different concentrations of acarbose and the plant extracts on glucose uptake by the *L6* cell lines

S. No.	Sample	Concentration	Absorbance	Percentage glucose uptake (µg/ml)	Bias
1	Control	-	0.1702±0.96	-	
2	Acarbose	25	0.2900±1.02	41.31	
	(Standard)	50	0.3189±1.34	46.63	
		100	0.3525±1.22	51.71	
3	Ethanolic extract of	25	0.2007±0.98	15.23	0.0893
	Syzygium cumini	50	0.2148±1.65	20.79	0.1041
	(SC EL)	100	0.2351±0.94	27.62	0.1174
4	Ethanolic extract of	25	0.1836±1.79	7.3	0.1064
	Psidium guajava	50	0.2035±0.98	16.4	0.1154
	(PG EL)	100	0.2186±0.86	22.17	0.1339
5	1:1 mixture of	25	0.2273±1.54	25.15	0.0627
	SC EL and PG EL	50	0.2654±1.12	35.87	0.0535
		100	0.3036±1.42	43.95	0.0489

RESULTS AND DISCUSSION

The 1:1 mixture of ethanolic extract of *Psidium guajava* and *Syzygium cumini* leaves was found to possess good glucose uptake activity than either of the individual extracts. The percentage of glucose uptake by ethanolic extract of the leaves of *Psidium guajava, Syzygium cumini*, 1:1 mixture of both and acarbose, the standard drug are tabulated in table 1. The result showed that the combined extract has good activity comparable with that of acarbose. Results are expressed as mean±SD (n=3). The glucose uptake assay on *L6 rat myoblast* cells was performed at three concentrations 25, 50 and 100 µg/ml. The results revealed that the combined ethanolic extract (1:1 mixture) of *Psidium guajava* and *Syzygium cumini* at 100 µg/ml increased the glucose uptake by 43.95% compared to control. The percentage increase in glucose uptake by acarbose the standard drug at 100 µg/ml was 51.71. At 100 µg/ml the percentage increase in glucose uptake by *Syzygium cumini* and *Psidium guajava* was 27.62 and 22.17 respectively on comparison with control. All the extracts showed a dose dependant increase in glucose uptake by *L6 rat myoblast* cells.

Table 1: The percentage increase of glucose uptake of standard drug acarbose, ethanolic extract of *Syzygium cumini* (SC EL), *Psidium guajava* (PG EL) and the 1:1mixture of ethanolic extracts of *Syzygium cumini* and *Psidium guajava* (SC EL+PG EL) at 25 µg/ml, 50µg/ml and 100µg/ml with respect to control. Results are expressed as mean±SD (n=3) and statistical significance was evaluated by one-way analysis of variance (ANOVA). The percentage of glucose uptake by SC EL, PG EL and the 1:1 mixture of SC EL+PG EL was compared with standard acarbose and the P values are 0.827, 0.856 and 0.359 respectively. The mixture SCEL+PG EL has least difference from the standard acarbose when compared with others. The bias of absorbance values calculated with respect to the standard drug acarbose is least for 1:1 mixture SC EL+PG EL when compared to the other two test groups.

The *In vitro α-amylase* inhibitory studies demonstrated that the combined ethanolic extract (1:1 mixture) of *Psidium guajava* and *Syzygium cumini* has more inhibitory activity on *α-amylase* than individual ethanolic extracts of *Psidium guajava* and *Syzygium cumini*. The extracts were tested and compared with the standard drug acarbose for *α-amylase* inhibitory activity at four concentrations 125, 250, 500 and 1000 µg/ml. The percentage inhibition of *α-amylase* activity by ethanolic extracts of the leaves of *Psidium guajava, Syzygium cumini,* 1:1 mixture of both and acarbose are tabulated in table 2. Results are expressed as mean±SD (n=3). All the extracts showed a dose dependant increase in the inhibitory activity. At a concentration of 1000µg/ml the combined ethanolic extract (1:1 mixture) of *Psidium guajava* and *Syzygium cumini* showed an inhibition of 36.51 % while it was 29.26% for *Syzygium cumini* and 23.43 % for *Psidium guajava*. For standard drug acarbose the percentage inhibition of *alpha amylase* at 1000µg/ml was 73.82.

Table 2: *Alpha amylase* inhibitory activity of different concentrations of acarbose and the plant extracts on porcine *alpha amylase*

S. No.	Sample	Concentration (µg/ml)	Absorbance at 540 nm	Percentage of inhibition	Bias
	Acarbose	125	0.0543±0.86	55.85	
1	(Standard)	250	0.0539±0.57	58.61	
		500	0.0492±0.64	60.00	
		1000	0.0322±0.75	73.82	
		Control	0.1230±0.45	-	
2	Ethanolic extract of	125	0.2113±0.67	12.54	0.1570
	Syzygium cumini	250	0.1941±0.34	19.66	0.1402
	(SC EL)	500	0.1805±0.53	25.29	0.1313
		1000	0.1709±0.66	29.26	0.1387
		Control	0.2416±0.47	-	0.1186
3	Ethanolic extract of	125	0.2132±0.66	11.75	0.1589
	Psidium guajava	250	0.2016±0.57	16.56	0.1477
	(PG EL)	500	0.1977±0.76	18.17	0.1485
		1000	0.1850±0.87	23.43	0.1528
		Control	0.2416±0.42	-	0.1186
4	1:1 mixture of	125	0.1905±0.43	21.15	0.1362
	SC EL and PG EL	250	0.1767±0.42	26.86	0.1228
		500	0.1739±0.75	28.02	0.1247
		1000	0.1534±0.68	36.51	0.1212
		Control	0.2416±0.74	-	0.1186

Table 2: *Alpha amylase* inhibitory activity of standard drug acarbose, ethanolic extract of *Syzygium cumini* (SC EL), *Psidium guajava* (PG EL) and the 1:1mixture of ethanolic extracts of *Syzygium cumini* and *Psidium guajava* (SC EL+PG EL) at 125 µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml with respect to control. Results are expressed as mean±SD (n=3) and statistical significance was evaluated by one-way analysis of variance (ANOVA). The percentage inhibition of *alpha amylase* by SC EL, PG EL and SCEL+PG EL was compared with standard acarbose and the P values are 0.937, 0.968 and 0.908 respectively. The mixture SCEL+PG EL has least difference from the standard acarbose, when compared with others. The bias of absorbance values calculated with respect to the standard drug acarbose is least for 1:1 mixture SC EL+PG EL when compared to the other two test groups.

Insulin resistance, which is the reduced response of target tissues such as the skeletal muscle, liver, and adipocytes to insulin, plays a major role in the pathogenesis of Type II Diabetes. Skeletal muscle is the predominant site of insulin-mediated glucose uptake in the postprandial state. Normal glucose homeostasis depends on a well-balanced interaction between tissue (muscle, liver and fat) sensitivity to insulin and insulin secretion [17]. The present study showed that the ability of the combined ethanolic extract of *Syzygium cumini* and *Psidium guajava* to increase the percentage glucose uptake is comparable to that of the standard drug acarbose showing its ability to reduce post prandial blood sugar level.

Type II Diabetes mellitus can be treated by reducing post prandial hyperglycaemia. The intestinal digestive enzyme *alpha amylase* is a carbohydrate hydrolyzing enzyme. *Alpha amylase* inhibitors prevent break down of polysaccharide in to mono and disaccharide. Thus *alpha amylase* inhibitors can prevent the postprandial hyperglycaemia by preventing glucose release from starch and delaying carbohydrate metabolism [18]. This research demonstrated the better anti diabetic potential of the 1:1 mixture of ethanolic extract of *Syzygium cumini* and *Psidium guajava* leaves by its ability to inhibit *alpha amylase* more effectively than either of the individual plant extracts of *Syzygium cumini* and *Psidium guajava leaves*.

The results of the glucose uptake assay and *alpha amylase* inhibitory assay demonstrated that even though the individual ethanolic plant extracts of *Syzygium cumini* and *Psidium guajava* could increase the glucose uptake and inhibit the *alpha amylase* activity, the combined ethanolic extracts of both the plants increased the glucose uptake

and inhibited the *alpha amylase* more efficiently than individual plants. So the combined ethanolic extract (1:1 mixture) of *Psidium guajava* and *Syzygium cumini* can act as a better anti diabetic agent. Plants containing various phytochemicals exhibit additive and synergistic interaction in exerting anti diabetic properties which is a beneficial effect to formulate more potent anti-diabetic drugs in combination [19].

CONCLUSION

In the present study the ethanolic extract of leaves of *Psidium guajava, Syzygium cumini* and 1:1 mixture of ethanolic extract of both *Psidium guajava* and *Syzygium cumini* leaves increased the glucose uptake by *L6 rat myoblast* cells and exhibited *alpha amylase* inhibition properties. This indicates the ability of the plants under study to act as anti-diabetic agents. Among the three extracts studied the combined extract of *Psidium guajava* and *Syzygium cumini* was found to be more active than either of the plants alone. Further studies are recommended to find the mechanism behind this synergistic or additive effect. This study also suggests that the active compounds isolated from these plants can be used as lead compound for designing potent anti-diabetic drugs. Based on the future investigations the plants can be utilised as the components of a polyherbal formulation for treating diabetes.

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AUTHOR CONTRIBUTIONS

Jose Deepa conducted the experiment and prepared the manuscript. Dr. N A Aleykutty designed the experiment and Dr. Harindran Jyoti contributed in experimental part of the work.

CONFLICT OF INTERESTS

The authors confirm that this article content has no conflict of interest.

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