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SHORT COMMUNICATION

In Vitro Antioxidant and Anti-Diabetic Evaluation of a Polyherbal Formulation

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Abstract

Diabetes Mellitus (DM) is a major metabolic disorder causing morbidity and mortality worldwide. The prominent adverse effects of allopathic drugs are the principal reason for an escalating number of people seeking alternative and complementary-based therapies that may have less or no adverse effects. The poly-herbal formulation Vilva Kudineer Choornam (VKC) is a combination of Siddha medicinal plants for the treatment of diabetes. The present study aimed to investigate the antioxidant and antidiabetic efficacy of poly-herbal formulation by invitro assays. The Aqueous Extract of Formulation (AEF) and Ethanol Extract of Formulation (EEF) were used for the assay. In the present research work, the antioxidant potential of polyherbal formulation (VKC) was evaluated using DPPH radical scavenging and Nitric oxide radical scavenging assay. The IC_{50} value of AEF was 333.02 µg/ml and EEF was 86.37 µg/ml in DPPH radical scavenging assay whereas, in nitric oxide scavenging assay, the IC_{50} of AEF and EEF were found to be 8.61 µg/ml and 447.77 µg/ml, respectively. The VKC was also screened for *in-vitro* antidiabetic potential by alpha-amylase inhibition assay and it was shown to have significant percentage inhibition of alpha-amylase. The IC_{50} of EEF and AEF were found to be 868.84 µg/ml and 247.09 µg/ml, respectively. The findings suggest that the polyherbal formulation (VKC) has significant antioxidant and antidiabetic activity. Further *in vitro* studies using cell lines and other enzymes may be carried out to fully establish the anti-diabetic potential of the polyherbal formulation.

Keywords: *Aegle marmelous*, Churna, Diabetes Mellitus, α Amylase

1. Introduction

Diabetes Mellitus (DM) is a major metabolic disorder that is responsible for causing mortality and morbidity worldwide due to elevated plasma glucose levels resulting from insulin resistance, insufficient insulin, or both, along with disturbances in the metabolism of lipids, carbohydrates, and proteins. It is one of the most recurrent metabolic disorders affecting around 2.8 % of the world population, which is expected to traverse to about 5.4 % by the year 2025[1]. The complications associated with DM can be broadly categorized into two types- microvascular complications affecting the blood vessels of the eye (diabetic retinopathy), kidney (diabetic retinopathy), nerves (diabetic neuropathy), and macrovascular complications affecting the heart, brain, and extremities. People with diabetes are at an increased risk (about 2–4 times) of developing coronary heart disease and stroke than non-diabetic people. Besides, poorly controlled DM can also affect the growing fetus in pregnant women leading to birth defects in the newborn baby[2,3]. Incongruity between the production of reactive oxygen species (ROS) and scavenging of these ROS by endogenous antioxidants results in oxidative stress that ultimately culminates in cellular and organ damage in diabetic patients[4]. These effects can be alleviated with increased antioxidant supplementation[5]. A wide variety of treatment

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approaches have been developed for the management of diabetes. Type 1 diabetes can be managed only by the use of insulin injection. The current treatment to combat type 2 diabetes is the usage of oral hypoglycemic drugs such as α-glucosidase inhibitors, sulphonylureas, biguanides, thiazolidinediones, and meglitinide analogs which become important when blood glucose levels cannot be controlled by diet, exercise, weight loss, and oral medications. However, adverse effects associated with the use of such drugs necessitate the development of alternative and complementary therapies having minimal or no adverse effects at all[6]. This has led to the growing interest in phytomedicine, traditional formulations, and the extracts are studied to know about their efficacy, safety, and mechanism of action[7]. In India, various traditional systems of medicines were followed by people for the treatment of diabetes such as Ayurveda, Siddha, Unani, and Homeopathy.

In traditional systems of medicine, there's a consensus about using phytotherapy for the treatment of several systemic disorders. Many of the indigenous/traditional systems of medicine offer superior protective effects than the modern system of medicine. However, the lack of scientifically validated evidence proving their therapeutic applications is one of the prime challenges faced by traditional systems of medicine. The notion of the polyherbal formulation is well documented and has been demonstrated to provide better therapeutic effects than single herb formulations. Hence, in the current study, an attempt was made to formulate a polyherbal formulation consisting of plants with documented antidiabetic properties and subsequently evaluate its antidiabetic potential *in vitro*.

The polyherbal formulation Vilva Kudineer Churna (VKC) consists of *Aegle marmelos* (Rutaceae), *Andrographis paniculata* (Acanthaceae), *Syzygium cumini* (Myrtaceae), *Mangifera indica* (Anacardiaceae), *Azadirachta indica* (Meliaceae), *Adathoda vasica* (Acanthaceae), and *Gymnema sylvestris* (Asclepiadaceae). Literature survey revealed that the ingredients present in the formulation exhibited antioxidant and antidiabetic activity. But no scientific evidence exists for the antioxidant and anti-diabetic potential of polyherbal formulation[8–14]. Considering this and the lack of scientific literature on its antidiabetic potential, the present study were conducted to evaluate the antioxidant and antidiabetic potential of polyherbal formulation (VKC) via the *in vitro* models.

2. Materials and Methods

2.1 Collection of Plant Materials

The plants were collected from the herbal garden of Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, and authenticated by Comparing with Flora by a botanist. Dr. Jayaraman, Plant anatomical Research Centre, Tambaram, Chennai and the voucher specimen number was PARC/2017/2160.

2.2 Preparation of Polyherbal Churna[15]

All the procured and authenticated plant materials (leaves) were shade dried and were subsequently cleaned by hand sorting. The leaves of the medicinal plants were then powdered and passed through mesh no. 80. The formulation was then prepared by weighing the required proportions of each powdered plant material, which were then mixed thoroughly, again sieved and were finally packed in a labeled air-tight container.

2.3 Preparation of Extracts

The prepared formulation (200 g) was macerated by cold maceration process using ethanol and water with occasional shaking. The extract was decanted, filtered, concentrated, and kept in desiccators for complete removal of solvent. The extract was then packed in an airtight container.

2.4 Determination of Physicochemical Parameters

Physicochemical constants such as total ash, acid insoluble ash, water-insoluble ash, water extractive value, ethanol extractive value, loss on drying were determined according to the standard procedures[16].

2.5 Preliminary Phytochemical Analysis

The extracts were subjected to preliminary phytochemical investigations. The extracts were investigated for the presence of different phytochemical constituents such as alkaloids, coumarins, phenols,

glycosides, tannins, steroids, amino acids, etc. according to standard procedures[17].

2.6 DPPH Free Radical Scavenging Assay[18]

A 0.2 mM DPPH solution was freshly prepared by dissolving DPPH in ethanol. The freshly prepared solution was then kept aside (in dark) until further use. About 1 ml of different concentrations of extract (50, 100, 200, 400, 800, 1000 µg) was added to 1 ml DPPH solution. The control was prepared by replacing the extract with ethanol solution. The resulting mixture was then incubated in the dark for about 30 minutes under ambient temperature. After 30 minutes, the resulting absorbance was measured using a UV-Visible spectrophotometer (Perkin-Elmer, USA) at 517 nm. Quercetin was used as a standard. All the determinations were done in triplicate. The percentage radical scavenging activity of the extracts was calculated using the following formula,

$$\% \text{ DPPH Scavevenging Activity} = \left\{\frac{ABS_{CONTROL} - ABS_{SAMPLE}}{ABS_{CONTROL}}\right\} \times 100$$

where, ABS- Absorbance

2.7 Nitric Oxide Radical Scavenging Assay[19]

The nitric oxide scavenging assay was performed based on the Griess assay technique. The sodium nitroprusside (10 mM) in phosphate-buffered solution (2.0 ml) was mixed with 1 ml of various concentrations (50, 100, 200, 400, 800, 1000 µg) of the extracts and incubated for 4 hours at 37° C. After the incubation period, the above solution was added to 0.5 ml Griess reagent. The control solution was prepared without the samples. The absorbance was read at 546 nm in a UV spectrophotometer (Perkin-Elmer, USA). Vitamin C was used as the standard. All the determinations were done in triplicates.

2.8 Alpha-amylase Inhibition Assay[20]

The assay was carried out by utilizing the DNSA reagent. A volume of 500 µl of enzyme solution was mixed with 1ml of various concentrations of extract and was incubated at 37° C for 10 min. Thereafter, 500 µl of the starch solution was added to each test tube and they were incubated for 10 minutes at 37° C. The reaction was terminated by the addition of 1ml of DNSA solution and was incubated for 5 min in a boiling water bath. It was then cooled, diluted with 10ml of water, and measured at 540 nm in UV spectrophotometer (Perkin-Elmer, USA). The control represents 100 % enzyme activity. Absorbance due to the test sample was eliminated by incorporating appropriate control without enzyme and starch. Acarbose was used as standard. All the determinations were performed in triplicates.

$$\textbf{\% Inhibition} = \frac{EC - (ET - TC)}{EC} \times 100$$

3. Results

The polyherbal formulation designated as Vilva Kudineer Churna (VKC) is a combination of medicinal plants that are traditionally used for the treatment of diabetes mellitus in the Siddha system of medicine. The ingredients are *Aegle marmelos* (Rutaceae), *Andrographis paniculata* (Acanthaceae), *Syzygium cumini* (Myrtaceae), *Mangifera indica* (Anacardiaceae), *Azadirachta indica* (Meliaceae), *Adathoda vasica* (Acanthaceae), and *Gymnema sylvestris* (Asclepiadaceae). The formulation was prepared by mixing the ingredients according to Table 1. The physicochemical parameters such as ash values, extractive values, loss on drying of the formulation were determined and the results are tabulated in Table 2. The aqueous and ethanol extracts were prepared, and the results of preliminary phytochemical investigations are tabulated in Table 3. The phytochemical investigation revealed the presence of alkaloids, flavonoids, glycosides, phenolic principles, among others. The Aqueous Extract of Formulation (AEF) and Ethanol Extract of Formulation (EEF) were used for the antioxidant and alpha-amylase inhibition assay.

3.1 DPPH Radical Scavenging Activity

The *in vitro* antioxidant activity of polyherbal formulation was performed using DPPH free radical scavenging assay and the results are tabulated in Table 4 and represented in Figure 1. The ethanol extract and aqueous extract of formulation were subjected to DPPH radical scavenging assay using Quercetin as standard in the concentration of 50–1000 µg/ml. The results showed a dose-dependent scavenging power

Table 1. Ingredients of polyherbal churna (VKC)

S. No	Ingredients	Part used	Required quantity
1	*Aegle marmelos*	LEAVES	16.66 g
2	*Andrographis paniculata*	LEAVES	8.33 g
3	*Syzygium cumini*	LEAVES	16.66 g
4	*Mangifera indica*	LEAVES	16.66 g
5	*Azadirachta indica*	LEAVES	16.66 g
6	*Adathoda vasica*	LEAVES	8.33 g
7	*Gymnema sylvestris*	LEAVES	16.66 g

Table 2. Physico chemical constant of the formulations

S.No	Parameters	% w/w
I	**Ash Values**	
1	Total ash	9
2	Acid insoluble ash	3
3	Water insoluble ash	5.3
II	**Extractive Values**	
1	Water soluble extractive	31.2
2	Ethanol soluble extractive	20.8
III	**Loss on Drying**	3

Table 3. Preliminary phytochemical investigation of various extracts of formulation

S. No	Constituents	Ethanol	Aqueous
1	Alkaloids	+	+
2	Coumarin	-	+
3	Tannins	+	+
4	Flavonoids	+	+
5	Phenol	+	+
6	Saponins	-	+
7	Proteins	-	-
8	Amino acids	-	-
9	Carbohydrates	+	+
10	Glycosides	+	+
11	Steroids	-	-

"+" = Presence **"-"** = Absence

Table 4. Percentage scavenging activity of different extracts of polyherbal formulation (VKC) in DPPH radical scavenging activity

S.No	Concentration (µg/ml)	Percentage scavenging		
		Aqueous Extract	Ethanol Extract	Standard (Quercetin)
1	50	15.12±0.42	38.20±0.57	89.09±0.40
2	100	26.925±0.43	50.04±0.33	90.475±0.41
3	200	32.4±0.42	68.01±0.84	91.28±0.45
4	400	57.355±0.41	81.55±0.35	92.55±0.14
5	800	68.415±0.61	86.97±0.31	93.80±0.24
6	1000	72.07±0.53	88.36±0.40	94.57±0.41

The value represents Mean ± Standard Deviation of the triplicates

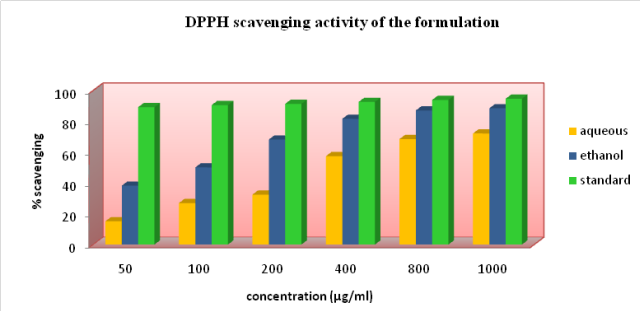

Figure 1. DPPH scavenging activity of the VKC formulation.

of Polyherbal formulation (VKC). The percentage inhibition of ethanol extract was found to be increased from 38.20±0.57 % to 88.36±0.40 % indicating that it has better scavenging ability. With reference to the positive control Quercetin, the results revealed that both extracts have very remarkable antioxidant capacity. The ethanol extract exhibited more activity than aqueous extract. The IC_{50} values of the formulations are tabulated in Table 7. The IC_{50} value of aqueous extract was found to be 333.02 µg/ml and the IC_{50} value of ethanol extract was found to be 86.37 µg/ml.

3.2 Nitric-oxide Radical Scavenging Assay

The Percentage nitric oxide radical scavenging assay was performed for polyherbal formulation and the results obtained are tabulated in Table 5 and represented in Figure 2.

Antioxidant activity of various extracts of the polyherbal formulation was evaluated by the nitric oxide scavenging method and the scavenging ability

Table 5. Percentage nitric oxide radical scavenging of different extracts of polyherbal formulation (VKC)

S. No	Concentration (µg/ml)	Percentage Scavenging Activity		
		Aqueous Extract	Ethanol Extract	Standard (Ascorbic acid)
1	50	64.115±0.36	21.671±0.38	79.505±0.36
2	100	70.94±0.43	24.725±0.24	82.22±0.38
3	200	72.89±0.50	38.455±0.45	85.62±0.52
4	400	82.33±0.41	44.86±0.38	87.415±0.37
5	800	87.01±0.33	75.565±0.55	91.515±0.65
6	1000	87.74±0.28	85.525±0.34	94.145±0.53

The value represents Mean ± Standard Deviation of the triplicates

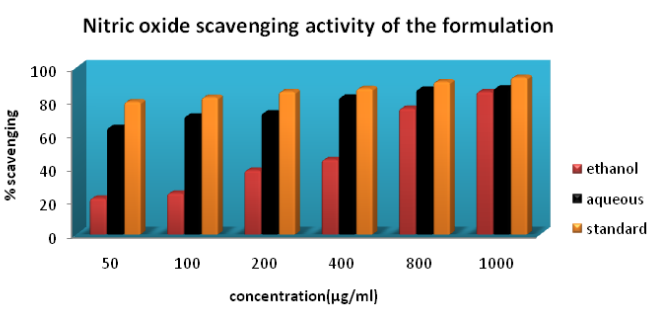

Figure 2. Nitric oxide scavenging activity of the VKC formulation

was shown in Table 3. The aqueous extract exhibited more activity than the ethanol extract. The aqueous extract showed significant inhibition property when compared with the standard ascorbic acid. The IC_{50} values of the formulations are tabulated in Table 7. The IC_{50} of aqueous extract and ethanol extract were found to be 8.61µg/ml and 447.77 µg/ml respectively. The results indicate that aqueous extract has more antioxidant potential than ethanol extract.

3.3 Alpha-amylase Inhibition Assay

In vitro anti-diabetic potential of the polyherbal formulation was evaluated using the α-amylase inhibition method. The alpha-amylase inhibition assay was performed for the formulation extracts viz. ethanol and aqueous and the results are tabulated in Table 6

Table 6. Percentage inhibition of alpha-amylase for different extracts of polyherbal formulation (VKC)

S. No	Concentration (µg/ml)	Ethanol Extract	Aqueous Extract	Standard Acarbose
1	50	2.72±0.36	34.95±0.36	65.68±0.22
2	100	13.73±0.33	42.3±0.56	69.74±0.53
3	200	15.55±0.41	50.39±0.55	70.655±0.33
4	400	27.22±0.16	55.42±0.78	75.795±0.13
5	800	45.57±0.62	58.36±0.37	76.84±0.11
6	1000	56.93±0.65	61.46±0.71	79.67±0.63

The value represents Mean ± Standard Deviation of the triplicates

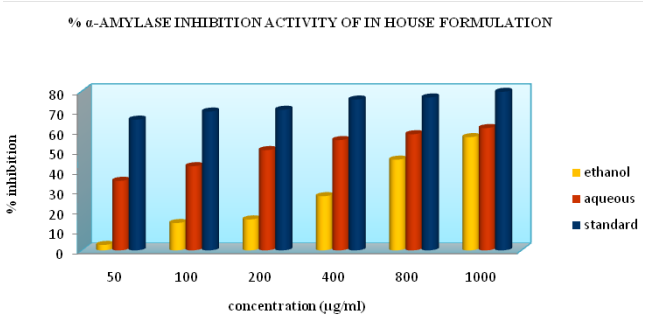

Figure 3. Alpha-amylase inhibition activity of the VKC formulation

Table 7. IC_{50} value of polyherbal formulation (VKC) in *in-vitro* antioxidant and antidiabetic assay

S. No	Extracts	IC_{50} (µg/ml)		
		DPPH radical scavenging assay	Nitric oxide radical scavenging assay	Alpha-amylase inhibition assay
1	Aqueous Extract	333.02	8.61	247.09
2	Ethanol Extract	86.37	447.77	868.84

and represented in Figure 3. The percentage inhibition of alpha-amylase by aqueous extract of the formulation was ranging from 34.95±0.36 % to 61.46±0.71 %. The aqueous extract exhibited more activity than the ethanol extract of the formulation. IC_{50} values of the formulation are tabulated in Table 7. The IC_{50} of

ethanol extract and aqueous extract of Polyherbal formulation were found to be 868.84 µg/ml and 247.09 µg/ml, respectively. The lower IC_{50} value indicates the significant antidiabetic potential of aqueous extract of the polyherbal formulation.

4. Discussion

It is imperative to discover and develop novel drugs and therapeutic strategies for the management of DM. There has been a positive trend in the number of medicinal plants being screened for their antidiabetic activity. Considering the recommendations proposed by WHO (that consists of regular exercise along with intake of healthy food) for the effective management of type II DM, urging the urban population to follow a healthy lifestyle along with the use of herbal remedies possessed with antidiabetic properties will be one of the most economical ways of managing the disease burden[21].

It has been demonstrated that complications of DM are associated with free radical-induced oxidative stress generated from the oxidation of glucose and degradation of glycosylated proteins[22]. Hence, antidiabetic medications are usually recommended along with antioxidants to avoid such complications. In the present study, the antioxidant potential of the formulation was measured by DPPH scavenging and Nitric oxide scavenging assay. DPPH scavenging study revealed that the ethanol extract of formulation exhibited more activity than the aqueous extract. IC_{50} values of the aqueous and ethanol extracts of formulation were found to be 333.02 µg/ml and 86.37 µg/ml respectively (Table 7). Furthermore, the activity of formulation was comparable to that of standard quercetin.

The antioxidant efficacies of the formulations were further established by performing nitric- oxide radical scavenging assay. The study revealed that the aqueous extract of formulation exhibited more activity than the ethanol extract. IC_{50} values of the aqueous extract and ethanol extracts of formulation were found to be 8.61 µg/ml and 447.77 µg/ml respectively (Table 7). The formulation exhibited similar activity as that of the standard ascorbic acid.

The *in vitro* anti-diabetic efficacies of the formulations were established by performing alpha-amylase inhibition assay. The study revealed that the aqueous extract of the formulation exhibited more activity than the ethanol extract. The IC_{50} values of the ethanol extract and aqueous extracts of formulation were found to be 868.84 µg/ml and 247.09 µg/ml respectively (Table 7). The formulation exhibited alpha-amylase inhibition similar to standard drug acarbose. These α- amylase inhibitors act by inhibiting the breakdown of 1,4-glycosidic linkages of starch and other oligosaccharides into simple sugars like maltose and triose[23].

In the present research work, the antioxidant potential of polyherbal formulation (VKC) was evaluated using DPPH radical scavenging assay and Nitric-oxide radical scavenging assay. The results revealed that the polyherbal formulation (VKC) possesses antioxidant potential. The VKC was also screened for *in-vitro* antidiabetic potential by alpha-amylase inhibition assay and it was shown to have significant percentage inhibition of alpha-amylase. The findings of the research work suggest that the polyherbal formulation (VKC) has significant antioxidant and antidiabetic activity. This may be due to the synergistic effect of herbs in the formulation and the phytoconstituents present such as alkaloids, tannins, saponins, glycosides, flavonoids, carbohydrates, etc. Hence the above results suggest that the polyherbal formulation could be beneficial in reducing starch absorption and thus can be effectively used in the management of diabetes.

5. Conclusion

The present study provides scientific evidence for the polyherbal formulation (VKC) possessing antioxidant and alpha-amylase inhibition activity *in vitro*, therefore helping in reducing Post-Prandial Hyperglycemia (PPHG) which is very useful in diabetes. Further *in vitro* studies using cell lines and other enzymes may be carried out to fully establish the anti-diabetic potential of the polyherbal formulation.

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