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PROMISING *INVITRO* ANTIDIABETIC AND ANTIOXIDANT ACTIVITIES OF "AAVARAI KUDINEER" - A POTENT POLYHERBAL SIDDHA FORMULATION

G. Prakash Yoganandam[1]*, V.Gopal[2] and J.Thanka[3]

[1]Assistant Professor, College of Pharmacy, Mother Theresa Post Graduate and Research Institute of Health Sciences, (A Govt. of Puducherry Institution) Puducherry-605 006 and Research Scholar, Sri Ramachandra University, Porur, Chennai-600 116, Tamil Nadu, India.
[2]Professor & Head, College of Pharmacy, Mother Theresa Post Graduate and Research Institute of Health Sciences, (A Govt. of Puducherry Institution), Puducherry-605 006, India.
[3]Professor and Head, Department of Pathology, Sri Ramachandra Medical College & Research Institute, Sri Ramachandra University, Porur, Chennai-600 116, Tamil Nadu, India.

ABSTRACT

Diabetes mellitus is a complex metabolic disorder resulting from either insulin insufficiency or insulin dysfunction. It is a global problem and number of those affected is increasing day by day. Currently used oral hypoglycemic agents use is restricted by their pharmacokinetic properties, secondary failure rates and accompanying side effects. Many Siddha antidiabetic formulations are reputed, popular but scientifically under-explored. One such formulation is Aavarai kudineer which is tailor made, effective, tried and trusted. α-glucosidase and α-amylase inhibitors from plant sources offer an attractive strategy for the control of post prandial hyperglycemia in type –II diabetes mellitus. Thus this research work is a small step towards, scientifically studying the Siddha "Aavarai kudineer" antidiabetic formulation, by *invitro* α-glucosidase and α-amylase inhibitory methods so as to standardize and improve the formulation for the benefit of mankind. From this study, the formulation has been proved to be a significant antidiabetic and antioxidant through *in-vitro* studies.

Key words: Diabetes mellitus, Siddha system of medicine, Aavarai kudineer, *Invitro* antidiabetic, Antioxidant activity.

INTRODUCTION

Diabetes mellitus is a complex metabolic disorder due to defects in carbohydrate metabolism. 346 million people worldwide have diabetes. In 2004, an estimated 3.4 million people died from consequences of high blood sugar. More than 80% of diabetes deaths occur in low- and middle-income countries. WHO projects those diabetes deaths will double between 2005 and 2030. Type I diabetes (insulin dependent) is caused due to insulin insufficiency because of lack of functional β-cells. Patients suffering from this are therefore totally dependent on exogenous source of insulin while patients suffering from Type II diabetes (insulin independent) are unable to respond to insulin and can be treated with dietary changes, exercise and medication (Frier BM and Fisher M, 2006).

Healthy diet, regular physical activity, maintaining a normal body weight and avoiding tobacco use can prevent or delay the onset of type II diabetes. Over time, diabetes can damage the heart, blood vessels, eyes, kidneys, and nerves. Diabetes increases the risk of heart disease and stroke. 50% of people with diabetes die of cardiovascular disease (primarily heart disease and stroke). Combined with reduced blood flow, neuropathy in the foot increases the chance of foot ulcers and eventual limb amputation. Diabetic retinopathy is an important cause of blindness and occurs as a result of long-term accumulated damage to the small blood vessels

Corresponding Author

G.Prakash Yoganandam
Email: gprakashyoga@gmail.com

in the retina. After 15 years of diabetes, approximately 2% of people become blind and about 10% develop severe visual impairment. Diabetes is among the leading causes of kidney failure. 10-20% of people with diabetes die of kidney failure (Davis SN and Granner DK, 2001).
Wide arrays of plant derived active principles representing numerous phytochemicals have demonstrated consistent hypoglycemic activity and their possible use in the treatment of diabetes mellitus. In the traditional system of Indian medicinal plant formulation and several cases, combined extracts of plants are used as drug of choice rather than individual. Many of these have shown promising effect (Mukherjee PK *et al.,* 2006). There are several medicines said in the literatures and practiced successfully by Siddha practitioners. The regulations in food, daily habits etc. are the speciality of most of these medicines. In addition to the prepared medicines there are several herbal combinations said in the texts for the management of this disease. All these medicines are to be used with the prescription of a Siddha medical practitioner and with proper regimen. In which kudineer is one of most important polyherbal formulations equally referred to khashayas in Ayurveda are more useful to prevent the diabetes and their associated complications. Besides, these formulations are only time tested, not scientifically proven and the ingredients are not well established in their scientific terms (Anonymous, 2011). Keeping the above information in view, Aavarai kudineer formulation (AKF) is selected to study the antidiabetic and antioxidant activity so as to create scientific evidence (Anonymous 1979).

MATERIALS AND METHODS

a) *In-vitro* α-Glucosidase Inhibitory Activity

Yeast α-glucosidase was dissolved at a concentration of 0.1 U/ml in 100 mM phosphate buffer, pH 7.0, containing bovine serum albumin 200 mg/ml and sodium azide 200 mg/ml which was used as enzyme source; p-nitrophenyl-α-D-glucopyranoside were used as substrate. Weighed Aavarai Kudineer Formulation (AKF) was made into a concentration of 1 mg/ml and serial dilutions of 500, 250, 125, 62.5, 31.25µg/ml were made with equal volumes of DMSO and distilled water. 10µl of AKF dilutions were incubated for 5 min with 50µl enzyme source. After the incubation, 50µl of substrate should be added and further incubated for 5min at room temperature. The pre-substrate and post-substrate addition absorbance was measured at 405 nm with ELISA microplate reader (Hamdan II and Afifi FU, 2004).
Percent α-glucosidase inhibition was calculated as follows:

$$\% \text{ Inhibiton } = \left(\frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}}\right) \text{x } 100$$

The inhibitory concentration of the AKF required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by graphical method. Experiments were performed in triplicate. Acarbose were dissolved in distilled water and serial dilutions were done and it was used as positive control.

b) *In-vitro* α-amylase Inhibitory Activity

A total of 500 µl of test samples and standard drug (31.25-500µg/ml) were added to 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and were incubated at 25°C for 10 min. After these, 500 µl of a 1% starch solution in 0.20 M phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm (Thalapaneni NR et al 2008). Control represent 100% enzyme activity and were conducted in similar way by replacing AKF with vehicle.

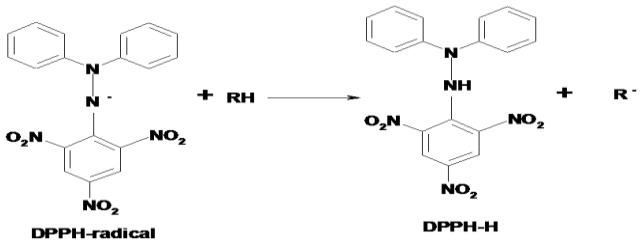
$$\% \text{ Reaction } = \left(\frac{\text{Absorbance of Test}}{\text{Absorbance of Control}}\right) \text{x } 100$$

$$\% \text{ Inhibiton } = 100 - \% \text{ Reaction}$$

For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. The concentration of AKF required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by graphical method. Experiments were performed in triplicate (Heidari R et al 2005).

c) DPPH radical scavenging activity

In the DPPH test, the antioxidants reduce the purple coloured DPPH radical (2, 2- diphenyl-1-picrylhydrazyl hydrate) to a yellow-coloured 2, 2-diphenyl-1-picrylhydrazine and the extent of the reaction was depend on the hydrogen donating ability of the antioxidants. The changes in colour (from deep-violet to light-yellow) was measured at 517 nm wavelength. As DPPH is sensitive to light, it is exposed to minimum possible light (James O and Jacob BO 2010).



Procedure

1 ml each of different concentrations (500, 250, 125, 62.5, 31.25 µg/ml in DMSO and distilled water) of AKF and standard (Ascorbic acid) were added to 2.5ml of a 0.3mM DPPH-methanol solution. The mixture should be shaken vigorously and allowed to stand at room temperature in the dark for 30 mins. The absorbance was measured at 517 nm. The % DPPH radical scavenging activity was calculated using the following formula:

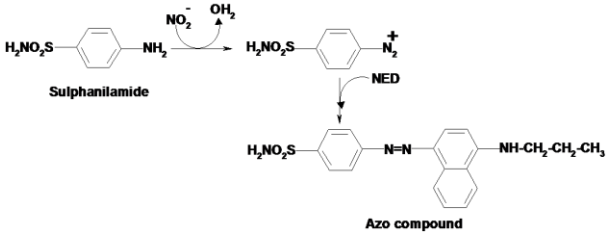
$$\text{DPPH radical Scavenging activity} = \left(\frac{A_{517}^{control} - A_{517}^{test}}{A_{517}^{control}}\right) x\ 100$$

Where, A=Absorbance.

Lower absorbance of the reaction mixture indicates higher radical scavenging activity. All the tests were performed in triplicate and the graph were plotted with the mean values. The concentration of sample required to scavenge 50% of DPPH (IC_{50}) was determined by graphical method (Badami S et al 2003).

d) Nitric oxide scavenging method

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess illosvoy reaction. In the present investigation, Griess illosvoy reagent is modified by using naphthyl ethylenediamine-di-hydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). Scavengers of nitric oxide compete with oxygen leading to reduce the production of nitric oxide (Ardestani A and Yazdanparast R, 2007).



Procedure

The reaction mixture (3ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and AKF and standard solution (0.5 ml) were incubated at 25 ^{0}C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite were pipetted and mixed with 1ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5min for completing diazotization. Then, 1ml of naphthyl ethylenediamine-di-hydrochloride (0.1%) was added, mixed and allowed to stand for 30 min. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions (Jie Yin et al 2008). The amount of nitric oxide radical inhibited by the extract was calculated using the following equation:

$$\%\ \text{of Scavenging activity} = \left(\frac{A_{540}^{control} - A_{540}^{test}}{A_{540}^{control}}\right) x\ 100$$

Where A=Absorbance

RESULTS

Table 1. α -Glucosidase Inhibitory Activity of aqueous extract of AKF

S.NO	Concentration (µg/ml)	Log conc.	Standard (Acarbose)		AKF extract	
			% of Inhibition (Mean ± SEM)	IC_{50} (µg/ml)	% of Inhibition (Mean ± SEM)	IC_{50} (µg/ml)
1	31.25	1.4948	31.56± 0.460	56.92±0.20	25.06± 0.045	64.0±0.23
2	62.5	1.7958	53.20± 0.400		49.41± 0.005	
3	125	2.0969	62.03± 0.015		58.72± 0.030	
4	250	2.3979	73.07± 0.050		69.52± 0.020	
5	500	2.6989	81.87± 0.050		74.72± 0.015	

Data were expressed as Mean ± SEM, (n=3)

Table 2. α -Amylase Inhibitory Activity of aqueous extract of AKF

S.NO	Concentration (µg/ml)	Log conc.	Standard(Acarbose)		AKF extract	
			% of Inhibition (Mean ± SEM)	IC_{50} (µg/ml)	% of Inhibition (Mean ± SEM)	IC_{50} (µg/ml)
1	31.25	1.49485	43.75 ± 0.225	41.82±0.28	33.81± 0.225	64.0± 0.21
2	62.5	1.79588	59.22 ± 0.370		49.35± 0.015	
3	125	2.09691	69.20 ± 0. 33		58.72± 0.030	
4	250	2.39794	77.22 ± 0.133		69.02± 0.452	
5	500	2.69897	93.97 ± 0.356		82.92± 0.371	

Data were expressed as Mean ± SEM, (n=3)

Table 3. DPPH scavenging activity of aqueous extract of AKF

S.NO	Concentration (µg/ml)	Log conc.	Standard (Ascorbic acid)		AKF extract	
			% Scavenging (Mean ± SEM)	IC_{50} (µg/ml)	% Scavenging (Mean ± SEM)	IC_{50} (µg/ml)
1	31.25	1.49485	39.06±0.370	**47.37±0.22**	28.81±0.160	**61.30±0.12**
2	62.5	1.79588	58.74±0.130		49.53±0.030	
3	125	2.09691	64.35±0.375		53.87±0.015	
4	250	2.39794	72.03±0.050		61.65±0.410	
5	500	2.69897	89.87±0.210		76.48±0.005	

Data were expressed as Mean ± SEM, (n=3)

Table 4. Nitric oxide scavenging activity of aqueous extract of *AKF*

S.NO	Concentration (µg/ml)	Log conc.	Standard (Ascorbic acid)		AKF extract	
			% Scavenging (Mean ± SEM)	IC_{50} (µg/ml)	% Scavenging (Mean ± SEM)	IC_{50} (µg/ml)
1	31.25	1.49485	41.83 ± 0.19	47.87±0.15	29.88± 0.25	70.05±0.28
2	62.5	1.79588	57.07 ± 0.25		46.91 ± 0.30	
3	125	2.09691	69.98± 0.13		59.17 ± 0.32	
4	250	2.39794	73.54 ± 0.01		64.99 ± 0.15	
5	500	2.69897	82.21 ± 0.21		76.23 ± 0.39	

Data were expressed as Mean ± SEM, (n=3).

Table 5. Comparison of IC_{50} value of *In-vitro* antidiabetic activity:

Methods used	IC_{50} value (µg/ml)	
	Standard(Acarbose)	Test drug(AKF)
α-glucosidase inhibitory method	41.82±0.28	64.0±0.21
α-amylase inhibitory method	56.92±0.20	64.0±0.23

Table 6. Comparison of IC_{50} value of *In-vitro* antioxidant activity:

Methods used	IC_{50} value (µg/ml)	
	Standard(Ascorbic acid)	Test drug(AKF)
DPPH	47.87±0.15	70.05±0.28
Nitric oxide	47.37±0.22	61.30±0.12

Figure 1. Comparision α -Glucosidase Inhibitory activity of aqueous extract of *AKF* with standard Acarbose

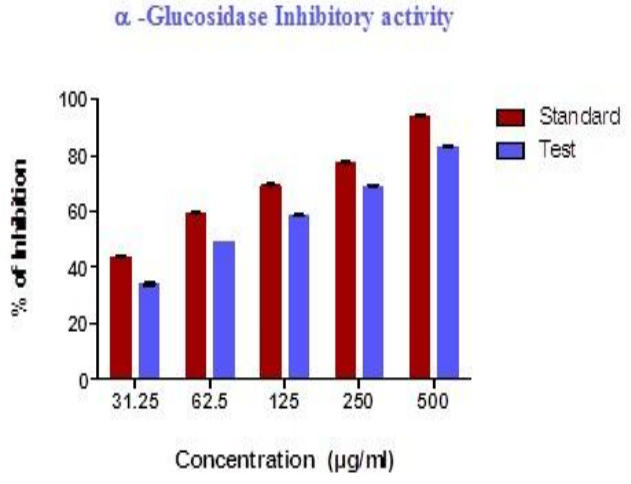


Figure 2. Comparision α–Amylase inhibition activity of aqueous extract of AKF with standard Acarbose

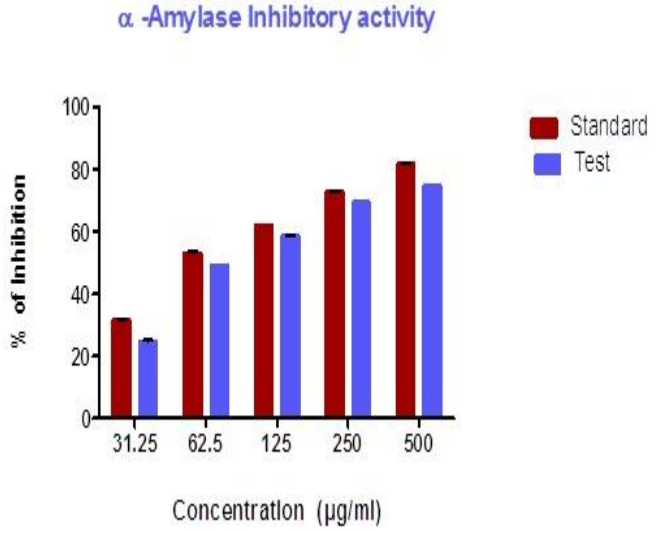


Figure 3. Comparison of DPPH scavenging activity of aqueous extract of *AKF* with standard Ascorbic acid.

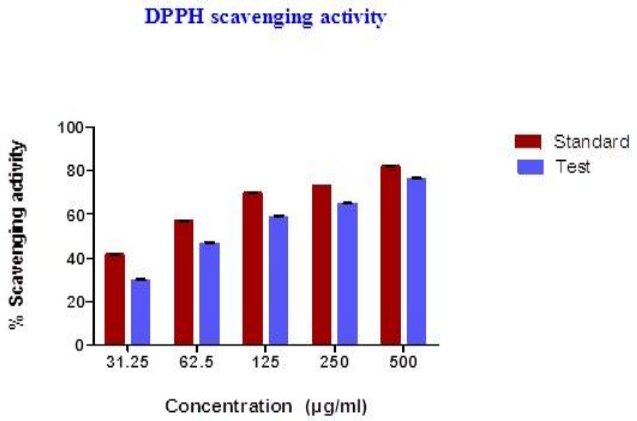
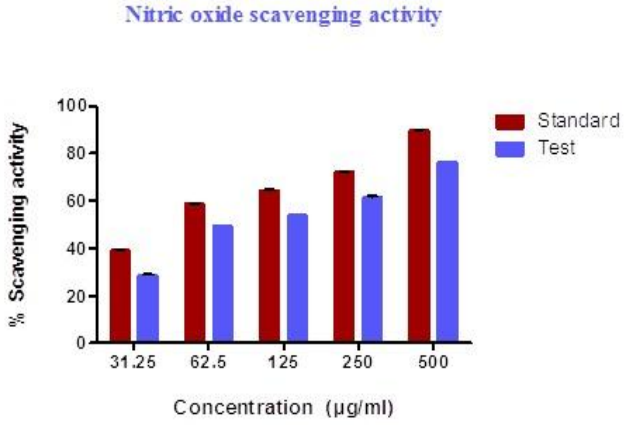


Figure 4. Comparison of Nitric oxide scavenging activity of aqueous extract of *AKF* with standard Ascorbic acid



DISCUSSION

In-vitro antidiabetic screening of AKF by α-glucosidase & α-amylase inhibitory methods

Lack of insulin affects the metabolism of carbohydrates, proteins, fat and causes significance disturbance in metabolic function (Neelesh M *et al.*, 2010). Recent advances in understanding the activity of intestinal enzymes (α-amylase and α-glucosidase both are important in carbohydrate digestion and glucose absorption) have lead to the development of newer pharmacological agents. A high postprandial blood glucose response is associated with micro- and macro-vascular complications in diabetes and is more strongly associated with the risk for cardiovascular diseases than are fasting blood glucose. α-glucosidase enzymes in the intestinal lumen and in the brush border membrane play main roles in carbohydrate digestion to degrade starch and oligosaccharides to monosaccharides before they can be absorbed. It was proposed that suppression of the activity of such digestive enzymes would delay the degradation of starch and oligosaccharides, which would in turn cause a decrease in the absorption of glucose and consequently the reduction of postprandial blood glucose level elevation (Puls W *et al.*, 1997). α-glucosidase inhibitor retards the digestion of carbohydrates and slows down the absorption. Acarbose are competitive inhibitor of α-glucosidases and reduces absorption of starch and disaccharides (Mukherjee PK *et al.*, 2006).

The α-amylase inhibitors act as an anti-nutrient that obstructs the digestion and absorption of carbohydrates. Acarbose is complex oligosaccharides that delay the digestion of carbohydrates. It inhibits the action of pancreatic amylase in breakdown of starch. Synthetic inhibitor causes side effect such as abdominal pain, diarrhoea and soft faeces in the colon. This finding reveals that aqueous extract of AKF effectively inhibits α-glucosidase and α-amylase enzyme by in vitro method when compared with standard (Acarbose) at different concentrations in serial dilution 500, 250, 125, 62.5, 31.25µg/ml showed significant($P<0.05$) antidiabetic activity (Table 5).

In-vitro antioxidant activity by DPPH and Nitric oxide free radical scavenging methods

DPPH is usually used as a substrate to evaluate antioxidant activity. DPPH assay is based on the measurement of the scavenging ability of antioxidant towards the stable DPPH radical. This method is based on the reduction of purple colored methanol solution of DPPH in the presence of hydrogen donating antioxidants, by the formation of yellow colored non radical form of DPPH. Lower the absorbance higher the free radical scavenging activity. Aqueous extract of AKF were able to reduce purple colored DPPH to yellow colored picryl hydrazine at different concentrations in serial dilution 500, 250, 125, 62.5, 31.25µg/ml showed significant ($P<0.05$) antioxidant activity.

Nitric oxide is implicated in diseases such as cancer and inflammation. It also mediates smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated cytotoxicity. Sodium nitroprusside spontaneously generates nitric oxide at physiological pH, in aqueous solutions. The nitric oxide, generated is converted into nitric and nitrous acids on contact with dissolved oxygen and water. The liberated nitrous acid was estimated using a modified Griess-Illosvoy method. Nitrous acid reacts with Griess reagent, to form a purple azo dye. In presence of antioxidants, the amount of nitrous acid will decrease and the degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. Aqueous extract of AKF when compared with standard (Acarbose) at different concentrations in serial dilution 500, 250, 125, 62.5, 31.25µg/ml showed significant ($P<0.05$) antioxidant activity (Table 6).

CONCLUSION

The Siddha system of medicine is a traditional Indian system of medicine. Among the various Siddha formulations useful in the treatment of diabetes mellitus, "Aavarai Kudineer" (AKF) is a reputed formulation. AKF is, "make your own medicine" type, formulation. It is freshly prepared and consumed. Lot of variation has been observed in the preparation of AKF; hence to avoid the variation and to have reproducible results a small step has been taken through the raw materials and finished product of this formulation have been standardized. This study will be helpful in maintaining the quality of AKF. Further the formulation has been proved to be an effective antidiabetic through *in-vitro* studies. Also the selected formulation was found to possess excellent antioxidant properties. These studies will help the formulation attain the quality standards at par with their modern medicine counterparts.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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