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An *In vitro* study on anti-diabetic and antiradical activities of siddha medicine avarai kudiner chooranam

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Abstract
Plants offer us bioactive molecules those may serve as safer therapeutics to combat existing new world ageing related diseases, and diabetic is the major concern among them. Pancreatic lipase inhibitors from plant sources may prove as promising side effects lacking anti-diabetic therapeutics, present study was conceived with the objective of anti-lipase, antiradical activities of Avarai kudiner chooranam decoction. Phytochemical constituents were screened quantifications of total phenolics, tannins, flavonoids, were done by taking tannic acid, quercetin, as reference molecules. Antiradical activities were evaluated by using two different free radicals (ABTS and lipid peroxidation activities). The antiradical activity of Avarai kudiner chooranam decoction was proved to be better than the standard ascorbic acid. Antidiabetic function of Avarai kudiner chooranam decoction was assessed using amylase and glucosidase in an *in vitro* assay system. From the data of the results obtained, maximum percentage of amylase inhibition was shown by Avarai kudiner chooranam decoction (78.32 %) than standard drug. EC_{50} value were calculated using the logarithmic regression of the dose-response curve after subtraction of both blank and inherent sample fluorescence values. In all cases, the coefficients of determination of the regression (R2) were greater than 0.95. EC_{50} are the means ± standard deviations of three determinations.

Keywords: avarai kudiner chooranam, anti-diabetics, antiradical

Introduction
Natural bioactive compounds especially from plant sources, including spices have been investigated for their characteristics and health effects. Plants are potential sources of natural bioactive compounds such as secondary metabolites and antioxidants. They absorb the sun light and produce high levels of oxygen and secondary metabolites by photosynthesis. Medicinal components produced are stored in plant leaves. Most of the secondary metabolites of herbs and spices are commercially important and find use in a number of pharmaceutical compounds. Flavonoids and phenolics acids are the most important groups of secondary metabolites and bioactive compounds in plants (Kim *et al*., 2003). They are also a kind of natural product and antioxidant substance capable of scavenging free superoxide radicals, anti-aging and reducing the risk of cancer (Bodeker, 2000). The Indian sub-continent has an identical amusing diversity of plant species in an extensive range of ecosystems. There are about 18000 species of angiosperm plants, of which roughly 8.000 species, are measured remedial and used by traditional communities, mainly tribal communities, or in traditional medicinal systems, such as the Siddha and Ayurveda. Plant-metabolites are organic compounds which can be divided into primary metabolites and secondary metabolites. Primary metabolites are include glucose, starch, polysaccharide, protein, lipids and nucleic acid are helpful for growth and reproductive activity of plant. Plants secondary metabolites are alkaloids, flavonoids, saponins, terpenoids, steroids, glycosides mainly utilize therapeutic efficacy for curing many diseases (Edeoga *et al.*, 2005) [3].

Materials and Methods
Preparation of Extract
Avarai kudiner chooranam composed of seven plant equal composition (Avarai samoolam Cassia auriculata, Kondrai pattai Cassia fistula, Naval pattai Syzygium cumini, Koraikizhangu Cyperus rotundus, Kostam Saussurea lappa, Marutham pattai Terminalia arjuna, Kadalalingil ver Salacia reticulate). All the dried herbs were finely powdered and triturated in house hold mixer grinder without adding water. Then all the powdered herbs were weighed about 14.28g and mixed evenly. Aqueous decoction made into sterile distilled water in water both 100 ºC for 1h. The extracts were filtered and evaporated to dryness and kept for further studies.

Phytochemical Analysis of Avarai Kudiner Chooranam
The aqueous decoction of Avarai kudiner chooranam were freshly prepared and various chemical constituents were analysed according to methods described by Allen and Harbone. The different chemical constituents tested for included tannins, saponin, glycosides, alkaloids, terpenoids, anthocynin, polyphenol and flavonoids.

Antidiabetic Activity
Glucose uptake in yeast cells
Glucose uptake assay by yeast cells was performed according to Cirillo *et al*. (1963). The yeast cell 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 Avarai kudiner chooranam (25 to 100 µg/ml) were added to 1 ml of glucose solution (20 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. Glycomet was used as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

Increase in glucose uptake%=Abs sample-Abs control/Abs sample×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.

Inhibition of Alpha-Amylase

Inhibition of alpha-amylase method followed by Narkhede *et al.*, (2011) [8]. In this assay, added 390 µl of 0.02 M phosphate buffer (pH 7), positive control (acarbose), different concentrations of Avarai kudiner chooranam and 10 µl of α-amylase enzyme were mixed and incubated at 37°C for 10 min. Added 10 µl of starch to this mixture and again incubated 37°C for 1 h (Megha *et al*., 2013). After incubation, added 0.1 ml 1% iodine solution and 5 ml of distilled water and optical density was measured at 565 nm. Inhibition of enzyme activity was calculated as follows:

Percentage inhibition = (A−C)×100/ (B−C)

Where, A=Absorbance of the sample, B=Absorbance of blank (without α-amylase), and C=Absorbance of control (without starch).

Inhibitory Activity of A-Glucosidase

The inhibitory activity of α-glucosidase method was followed by. The first step carried out substrate of starch solution (2% w/v maltose or sucrose, 1 mL) with Tris buffer (0.2 M, pH 8) and various concentrations Avarai kudiner chooranam for 5 min at 37°C. The reaction was initiated by adding α-glucosidase enzyme (1 mL of 1 U/mL yeast α-glucosidase) to the reaction mixture, followed by incubation for 10 min at 37°C. The reaction was terminated by heating the contents in a boiling water bath. 3, 5-dinitrosalicylic acid (1 mL) was added with the product before being incubated for 5 min and added with distilled water (9 mL). The amount of liberated glucose was measured by glucose oxidase peroxidase method.

ABTS (2, 2'-Azino-Bis-3-Ethyl Benzthiazoline-6-Sulphonic Acid) Radical Scavenging Assay

ABTS radical scavenging activity of aqueous decoction of Avarai kudiner chooranam were determined according to Re *et al*. 1999. ABTS radical was freshly prepared by adding 5 ml of a 4.9 mM potassium persulfate solution to 5 ml of a 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to yield an absorbance of 0.70 at 734 nm and the same was used for the antioxidant assay.

The final reaction mixture of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Vit-C. Similarly, in the test group 1 ml reaction mixture comprised 950 µl of ABTS solution and 50 µl of the extract solutions. The reaction mixture was vortexed for 10 s and after 6 min absorbance was recorded at 734 nm against distilled water by using an ELICO (SL150) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

Inhibition of Lipid Peroxidation Activity

Lipid peroxidation induced by Fe_{2+}-ascarbate system in egg yolk by the method of Bishayee and Balasubramaniyam 1971, was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*. 1977. The reaction mixture contained egg yolk 0.1 ml (25% w/v) in Tris-HCl buffer (20mM, pH 7.0); KCl (30mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06mM); and various concentrations of aqueous decoction of Avarai kudiner chooranam in a final volume of 0.5ml.

The reaction mixture was incubated at 37ºC for 1 h. After the incubation period, 0.4ml was removed and treated with 0.2ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100ºC for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed and its absorbance at 532 nm (ELICO (SL150) UV–Vis Spectrophotometer) was measured to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of treatments with that of the control. Ascorbic acid was used as standard. Inhibition of lipid peroxidation (%) by the extract was calculated according to 1- (E/C) X 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample ($Abs_{532+TBA} - Abs_{532\_TBA}$).

Result and Discussion

Phytochemical Screening of Aqueous Decoction of Avarai Kudiner Chooranam

Phytochemical screening provides basic information about medicinal importance of a plant extract. In this study evaluation for qualitative analysis of the chemical constituents of aqueous decoction of Avarai kudiner chooranam decoction showed the presence of various secondary metabolites, alkaloid, saponins, flavonoid, tannins, polyphenols, anthraquinones and triterpenes. Cardiac glycosides were not detected in aqueous decoction (Table-1).

Table 1: Phytochemical screening of aqueous decoction of Avarai kudiner chooranam

S.No.	Phytochemical Constituents	Result indicated	Avarai kudiner chooranam
1.	Alkaloids		
	Dragendroff's reagent	Brown precipitation	+
	Mayer's reagent	Yellow precipitation	+
2.	Flavonoids		
	Alkaline test	Yellow coloration	+
	Lead acetate	Immediate precipitation	+
3.	Polyphenols		
	Ferrozine Test	Blue Coloration	+
4.	Terpenoids		
	Salkowski test	Brown ring	-
5.	Tannins	Dark green blue	+
6.	Glycosides		
	Keller-Killani test	Reddish brown ring	-
	Bronbagers Test	Pink colour in ammonia layer	-
7.	Saponins		
	Froth Test	Foam	+
8.	Anthocynin		
	Ammonia Test	Yellow colour in ammonia layer	+

-- = Negative (absent); + = Positive (present)

Glucose Uptake in Yeast Cells

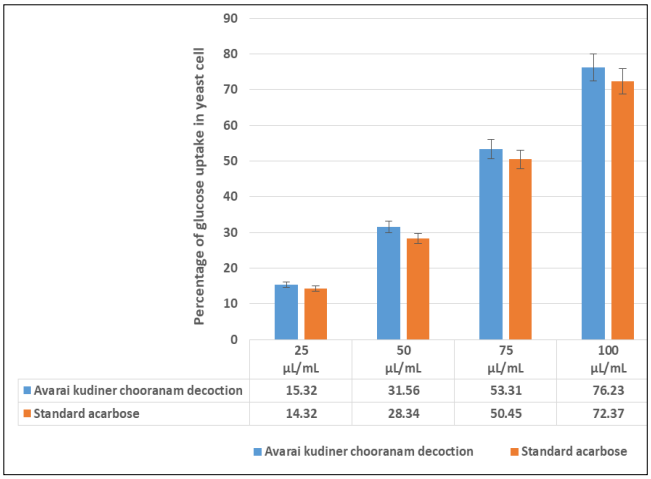

Fig 1. Percentage of Glucose Uptake in Yeast Cells

Different concentrations of Avarai kudiner chooranam decoction were subjected to *in vitro* glucose uptake assay employing yeast as model.

The percentage of glucose uptake in yeast cells by the Avarai kudiner chooranam decoction was compared with standard drug diclofenac sodium (Fig-1).

Avarai kudiner chooranam decoction exhibited highest percentage of glucose uptake 76.23%, which was almost near to the standard 72.37% at 100 µg/ml concentration. Results also indicated that alkaloid rich fraction had nearly same effectiveness in increasing the glucose uptake by yeast cells as compared to standard drug acarbose. Type II diabetes categorized by lack of insulin triggering augmented close in blood glucose level and it be contingent on the uptake of glucose by the cells (Shori, 2015). The increased concentration of Avarai kudiner chooranam decoction similarly increased proportion of glucose uptake in yeast cells.

This result indicated that high concentrations of alkaloid rich fraction displayed high glucose uptake.

Alpha-Amylase Inhibition

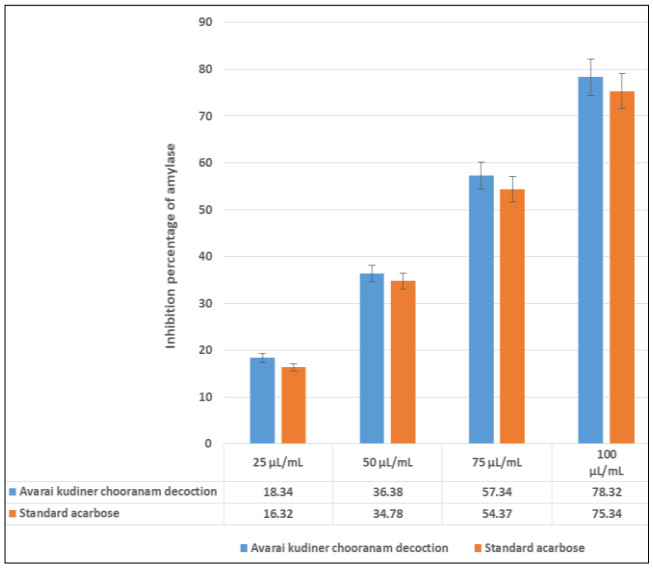

Fig 2: Alpha-Amylase Inhibition Activity of Avarai Kudiner Chooranam Decoction

Inhibitory effects of α-amylase confirmed that Avarai kudiner chooranam decoction at concentrations of 25-100 µg/ml (Fig-2). The maximum inhibition was observed at highest concentration of 100 µg/ml exhibited of 78.23% as compared to standard acarbose which showed significantly lower inhibition of 75.34% at the same concentration. Alpha-amylase is type of the intestinal enzyme which play important role in carbohydrate digestion and glucose absorption (Worthington, 1993). Since Avarai kudiner chooranam decoction, further studies have to conduct on the isolation, and characterization of the compounds in authority for the activity.

Alpha-Glucosidase Inhibition

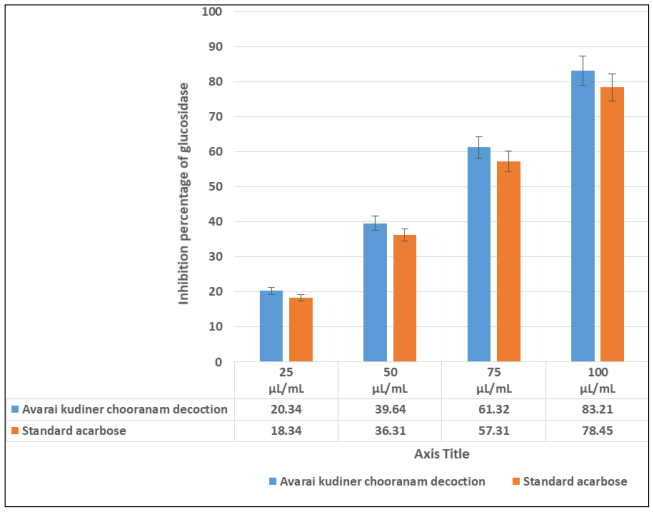

Fig 3: Alpha-Glucosidase Inhibition Activity of Avarai Kudiner Chooranam Decoction

Another results of antidiabetic activity using α- glucosidase inhibitory assay of the Avarai kudiner chooranam decoction are shown in Fig-3. The alkaloid rich fraction revealed a significant inhibitory action of α-glucosidase enzyme. The percentage inhibition ranges from 20.34% to 83.21% for lowest concentration to highest concentration. Thus the inhibition of the activity of α- glucosidase by Avarai kudiner chooranam decoction desired interruption the degradation of carbohydrate, which would in chance reason a reduction in the absorption of glucose, as a result the decrease of postprandial blood glucose level advancement (Mai *et al*., 2007).

ABTS Radical Activity

Aqueous decoction of Avarai kudiner chooranam exhibited a powerful scavenging activity for ABTS radical cations in a concentration dependent manner (Table-2), showing a direct role in catching free radicals. Maximum discoloration was observed with the aqueous decoction ranges from 23.45 to 75.68% at 25-100 µl/ml than Vitamin-C. This property may be credited to the presence of polyphenolics and flavones in the Avarai kudiner chooranam. Hagerman *et al*. (1998) [4] have stated that the high molecular weight phenolics (tannins) have more abilities to quench free radicals (ABTS) and their effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups. Free radical (ABTS) scavenging activity of Avarai kudiner chooranam decoction might be due to the presence of high molecular weight phenolics such as catechin, and rutin derivatives.

Table 2: ABTS radical activity of Avarai kudiner chooranam decoction

Different concentration of decoction of	[a] Percentage of radical activity	
	Decoction of Avarai kudiner chooranam	Standard Vitamin-C
25µl/ml	23.45±0.89	20.34±0.79
50µl/ml	40.31±2.45**	36.34±2.89
75µl/ml	57.34±1.47	54.34±2.56**
100µl/ml	75.68±1.23	72.34±1.78
EC_{50} Value	66.32 µl /ml	70.34µl /ml

All the observations in different groups showed significant ($P < 0.01$) relationship between the concentration and percentage inhibition (Pearson's correlation analysis). [a] Mean ± SD.

Inhibition of Lipid Peroxidation

Decoction of Avarai kudiner chooranam also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was observed with total aqueous decoction of Avarai kudiner chooranam with inhibition percentage 19.34 to 65.32 at 100µl/ml respectively then standard vitamin-C (Table-3). This inhibition of lipid peroxidation possibly either due to chelation of Fe or by corner of the free radicals. Iron also is playing a major role for the formation of lipid peroxidation in the body. The process of lipid peroxidation has been suggested to proceed via a free radical chain reaction, which has been associated with cell membrane damage. This membranouse damage has been suggested to contribute to various diseases, including diabetes. It is possible that the high level of inhibition on lipid peroxidation displayed by the ethyl acetate fraction is related to the presence of phenolic compounds, which have been correlated with antioxidant activity (Gulcin *et al*., 2002).

Table 3: Inhibition of lipid peroxidation by aqueous decoction of Avarai kudiner chooranam

Different concentration of decoction	[a]Inhibition Percentage of lipid peroxidation	
	Aqueous decoction of Avarai kudiner chooranam	Vitamin-C
25 µl /ml	19.34±1.78	16.34±2.34
50µl /ml	33.64±2.63**	30.32±2.76
750µl /ml	48.37±1.35	45.37±0.86
100 µl /ml	65.32±2.45	63.34±2.78
EC_{50} Value	76.32µl /ml	78.68µl /ml

There was a significant ($^{*}P < 0.01$) relationship between the concentration and percentage inhibition (Pearson's correlation analysis). [a]Mean ± SD

Conclusion

Thus the results of this study indicate that the commonly used aqueous decoction of Avarai kudiner chooranam significantly inhibit the activity of amylase which can be attributed to the presence of polyphenols, flavonoids tannin and saponins which is comparable to Acarbose. According to data achieved from the present study, EC_{50} Value was found to be an effective antioxidant in different *in vitro* assays, including total antioxidant activity determination by ABTS radical and Lipid peroxidation when it is compared to

standard antioxidant compounds used as Vitamin C. These may be used in nutraceuticals and the food industry. However, additional studies are necessary to develop a method for the fractionation and identification of polyphenols and to determine the most active antioxidant compounds in the Avarai kudiner chooranam.

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