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Research Article

"Exploring the Antidiabetic and Antioxidant Potential of Athiyadhi Kashayam: Investigating mechanisms of action in Siddha Medicine"

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ABSTRACT

Diabetes mellitus is a serious condition that affects the majority of the population and leads to mortality, and it may increase tremendously by 2025. The disease is characterized by elevated blood glucose levels resulting from insulin resistance and insufficiency. This leads to various metabolic disorders that affect various organs. The free radicals generated during diabetes conditions are known to elevate the condition much more severely. The antioxidant activity against free radicals and the inhibition of enzymes that are involved in raising blood glucose levels inhibition might prove effective in the treatment of type 2 diabetes. The Siddha formulation Athiyadi Kashayam (AK) was analyzed for its enzyme inhibition study and antioxidant activity in vitro. The results proved the enhanced antioxidant potential. The drug AK also inhibited the enzymes – amylase and glucosidase in a concentration-dependent manner. Thus AK proved to be effective in treating Type 2 Diabetes.

Introduction

Diabetes mellitus (DM) is a prevalent condition affecting adults worldwide, with its incidence rapidly increasing and becoming a leading cause of mortality[1]. According to the International Diabetes Federation, it's projected that by 2030, over 643 million individuals will have diabetes[2]. In conditions of elevated blood glucose levels, various mechanisms, including increased formation of advanced glycated end products, activation of the polyol pathway, mitochondrial dysfunction, impaired electron transport chain, and prolonged inflammation, contribute to heightened generation of reactive oxygen species (ROS) and causes oxidative stress in the body[3,4]. Oxidative stress plays a pivotal role as a triggering factor in the development of Type 2 diabetes, the most common form of the disease, as well as its associated complications such as ischemic heart disease, stroke, neuropathy, nephropathy, and retinopathy[5]. In turn, oxidative stress leads to impaired insulin signalling pathways promoting cell dysfunction, apoptosis, and insulin resistance. The diabetogenic activity of alloxan is also induced by the generation of oxygen-free radicals that damage the pancreas. Thus, the relationship between ROS and Type 2 diabetes is bidirectional. Managing oxidative stress through lifestyle interventions, a balanced diet rich in antioxidants, and pharmacological interventions targeting oxidative stress pathways may offer therapeutic potential in preventing and managing Type 2 diabetes and its complications[3]. Antioxidants play a crucial role in managing diabetes mellitus by mitigating oxidative stress, improving insulin sensitivity, protecting pancreatic β-cells, reducing inflammation, and preventing diabetes-related complications. Plants are abundant sources of phytochemicals that possess antioxidant properties, making plant-based foods valuable components aimed at reducing oxidative stress and promoting the overall health of a diabetic patient[6,7]. Plant extracts and their products have shown potent antioxidant activity [8]. More than 800 plants are reported in the literature for their efficacy in treating T2DM. The active components like alkaloids, phenolics, tannins, and terpenoids are known to exhibit antihyperglycemic activity by inhibiting α-amylase and α-glucosidase enzymes[7]. Currently, traditional medical systems like Siddha and Ayurveda are strongly accepted by everyone as it is safe. In the Siddha system, polyherbal formulations are of clinical importance due to the synergistic effects exhibited by the mixture of herbs as they can act as both scavengers of reactive oxygen species and inhibitors of carbohydrate lysing enzymes[6,9,10].

Many Siddha polyherbal formulations like *Avarai kudineer, Madhumega chooranam, Kabasura kudineer* etc., are already reported for their antidiabetic activity[11–13]. In that context we have developed a formulation called Athiyadhi Kashayam (AK) a mixture of Ficus racemosa, Cassia auriculata, Cassia fistula, Syzygium cumini and Salacia reticulata. The formulation of AK was obtained from the literature of *Mega Nivarana Bodini Ennum Neerizhivu Maruthuvam*[14]. Each ingredient of Athiyadi Kashayam is known for its antioxidant potential and anti-diabetic property[12,15–17]. However, the role of antioxidants in treating diabetes and the potential benefit of the polyherbal formulation has not yet been explored clearly. Hence, in the current study, we determined the antidiabetic and antioxidant activity of Athiyadi Kashayam by in vitro analysis.

2.Materials

All the reagents used in this study were of laboratory grade.

3. Methodology

3.1 Preparation of Athiyadi Kashayam

Sl.No	Botanical name	Family	Part used	Vernacular name
1	*Ficus racemosa*	Moraceae	Stem Bark	*Athi*
2	*Cassia auriculata*	Caesalpinoidea	Stem Bark	*Avarai*
3	*Cassia fistula*	Caesalpinoidea	Stem Bark	*Sarakontrai*
4	*Syzygium cumini*	Myrtaceae	Stem Bark	*Naval*
5	*Salacia reticulata*	Salvodoraceae	Root	*Kadalazhingil*

3.2 Evaluation of antihyperglycemic efficacy

3.2.1 In-vitro α-Amylase Enzyme Inhibition Study

The enzyme α-amylase (0.5 U/ml) was prepared by mixing 3.24 mg of α-amylase in 100 ml of phosphate buffer (pH 6.9). Test Sample (AK) was serially diluted with double distilled water with a concentration ranging from 100-500 µg/ml. Acarbose 100 µg/ml was used as a reference standard. About 600 µl of test sample were added to 30 µl of α-amylase enzyme solution and incubated at 37°C for 15 min. To this reaction mixture, 370 µl of substrate, 2-Chloro-4-Nitrophenyl-α-Maltotrioside ($CNPG_3$- 0.5 mg/ml) was added, mixed and incubated at 37°C for 10 min. Finally, the absorbance was read at 405 nm against blank in spectrophotometer. A control reaction was carried out without the test sample. Percentage inhibition was calculated by the following formula[18].

$$Percentage\ of\ Inhibition = \frac{AControl - ATest}{AControl} x\ 100$$

$$Percentage\ of\ Inhibition = \frac{AControl - ATest}{AControl} x\ 100 \quad (1)$$

Where, AControl – Absorbance of the control sample, ATest – Absorbance of the test sample

3.2.2 In-vitro α-Glucosidase Enzyme Inhibition Study

The α-glucosidase enzyme solution was prepared by dissolving 0.5 mg of α-glucosidase in 10 ml phosphate buffer (pH 7.0) containing 20 mg bovine serum albumin. About 10µl of the test sample (AK) at varying concentration100-500µg/ml were added to 250 µl of 20 mM p-nitrophenyl-α-D -glucopyranoside and 495µl of 100 mM phosphate buffer (pH 7.0). Acarbose of 100 µg/ml was used as a reference standard. All the solutions were pre-incubated at 37°C for 5 min and the reaction was initiated by the addition of 250µl of the α-glucosidase enzyme solution prepared by 0.5 mg α-glucosidase in 10 ml phosphate buffer (pH 7.0) containing 20 mg bovine serum albumin, after which it was incubated at 37°C for exactly 15 min. 250 µl of phosphate buffer was added instead of enzyme for blank. The reaction was then stopped by addition of 1000µl of 200 mM $Na_2\ CO_3$ solution and the amount of p-nitrophenol released was measured by reading the absorbance of sample against a sample blank (containing PBS with no sample) at 405 nm using UV visible spectrophotometer[19].

$$Percentage\ of\ Inhibition = \frac{AControl - ATest}{AControl} x\ 100$$

$$Percentage\ of\ Inhibition = \frac{AControl - ATest}{AControl} x\ 100 \quad (2)$$

Where, AControl – Absorbance of the control sample, ATest – Absorbance of the test sample

3.3 Evaluation of antioxidant activity

This assay was carried out for the purpose of evaluating the anti-oxidant potential of test drug AK against DPPH (2, 2-Diphenyl 1-2 picrylhydrazyl) assay, Nitric oxide radical scavenging assay, Hydrogen peroxide radical scavenging activity and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS radicals[7,20].

3.3.1 DPPH (2, 2-Diphenyl 1-2 picrylhydrazyl) Assay

The antioxidant activity of test drug sample AK was determined using the 2,2-diphenyl 1-2 picrylhydrazyl (DPPH) free radical scavenging assay. Sample AK at the concentration of 10 -100 µg/ml along with standard ascorbic acid was taken for the analysis. Final reaction mixture containing 1 ml of 0.3 mM DPPH methanol solution was added to 2.5 ml of sample solution of different concentrations and allowed to react at room temperature. Absorbance in the presence of test sample AK at different concentration of (10 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100µg/ml) was noted after 15 min incubation period at 37°C. Absorbance was read out at 517 nm using double-beam U.V Spectrophotometer by using methanol as blank.

$$Radical\ Scavenging\ (\%) = \frac{AControl - A\ Sample}{AControl} x\ 100$$

$$Radical\ Scavenging\ (\%) = \frac{AControl - A\ Sample}{AControl} x\ 100 \quad (3)$$

Where, AControl is the absorbance of control sample and ATest is the absorbance of test sample

The effective concentration of test sample AK required to scavenge DPPH radical by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations.

3.3.2 Nitric Oxide Radical Scavenging Assay

A volume of 0.5 ml of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 ml of the different concentrations of the test drug (10–100 µg/mL) and incubated at 25°C for 180 mins. The test drug AK was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the test drug but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. The absorbance was measured at 546 nm using a Spectra Max Plus UV-Vis microplate reader (Molecular Devices, GA, USA). Gallic acid was used as the positive control. The percentage inhibition of the test drug AK and standard was recorded. The percentage nitrite radical scavenging activity of the test drug AK and gallic acid were calculated using the following formula:

$$Nitric\ oxide\ scavenged\ (\%) = \frac{AControl - ATest}{AControl} x\ 100$$

$$Nitric\ oxide\ scavenged\ (\%) = \frac{AControl - ATest}{AControl} x\ 100 \quad (4)$$

Where, AControl is the absorbance of control sample and ATest is the absorbance of test sample

3.3.3 ABTS Assay

The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and was then diluted with water (1 : 44, v/v). To determine the scavenging activity, 100 µL ABTS reagent was mixed with 100 µL of test sample at the concentration of 10-100µg/ml in Double Distilled water and was incubated at room temperature for 6 min. After incubation, the absorbance was measured 734 nm. 100% methanol was used as a control. Gallic acid with same concentrations of test drug AK was measured following the same procedures described above and was used as positive controls. The ABTS scavenging effect was measured using the following formula:

$$Radical\ scavenging\ (\%) = \frac{AControl - ATest}{AControl} x\ 100$$

$$Radical\ scavenging\ (\%) = \frac{AControl - ATest}{AControl} x\ 100 \quad (5)$$

Where, AControl is the absorbance of control sample and ATest is the absorbance of test sample

3.3.4 Hydrogen Peroxide Radical Scavenging Assay

A hydrogen peroxide solution (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Aliquots (0.1 ml) of the test sample AK (different concentration ranging from 10-100µg/ml) were transferred into the test tubes and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). After adding 0.6 ml hydrogen peroxide solution, tubes were vortexed and the absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank. Butylated Hydroxy Anisole (BHA) was used as the positive control. The percentage inhibition of the test drug AK and standard was recorded. The percentage radical scavenging activity of the test drug AK and BHA were calculated using the following formula:

$$Radical\ scavenging\ (\%) = \frac{AControl - ATest}{AControl} x\ 100$$

$$Radical\ scavenging\ (\%) = \frac{AControl - ATest}{AControl} x\ 100 \quad (6)$$

Where, AControl is the absorbance of control sample and ATest is the absorbance of test sample

4. Results and Discussion

4.1 Percentage inhibition of test drug AK on α-Amylase and α-Glucosidaseenzymes

The percentage of inhibition of AK against α-Amylase and α-Glucosidase was determined at different concentrations ranging from 100 - 500µg/ml with acarbose 100µg/ml as the standard and represented in fig 1a and 1b respectively. It was observed from the results of the present investigation that the formulation AK shown promising α- amylase and α-glucosidase enzyme inhibition potential with the maximum inhibition of about 39.95 ± 0.5029 %, and 37.09 ± 8.128 % with the corresponding IC_{50} value of 657.1 ± 28.89 µg/ml and 682.9 ± 164.5 µg/ml respectively. Standard acarbose exhibited significant inhibition in α-amylase and α-glucosidase enzymes with the maximum inhibition of about 99.16 ± 0.375 % and 98.52 ± 1.812 % with the corresponding IC_{50} value of 15.23 ± 6.542 µg /ml and 27.25 ± 6.738 µg /ml.

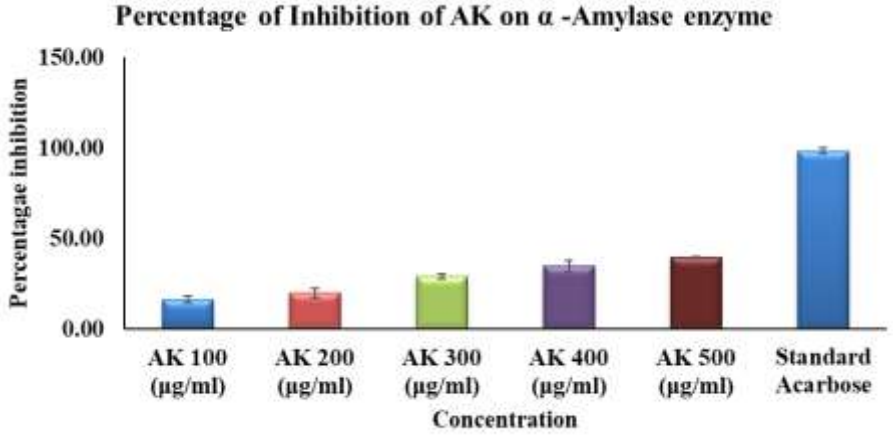

Fig.1 represents the percentage of inhibition of the drug AK against α-amylase at 5 different concentrations with acarbose 100µg /ml as standard

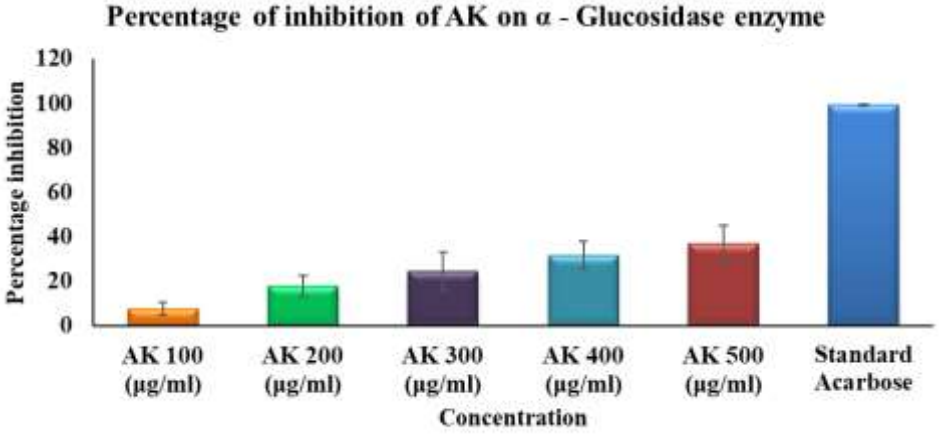

Fig.1b represents the percentage of inhibition of the drug AK against α-glucosidase activity at 5 different concentrations with acarbose 100µg /ml as standard

4.2 Antioxidant potential of AK

The in vitro antioxidant activity of the AK drug was analysed using DPPH, NO, ABTS and Hydrogen peroxide radical scavenging activity.

During DPPH inhibition study, the IC_{50} value of the AK sample was found to be 120±38.31µg /ml when compared with standard ascorbic acid with IC_{50} value of 49.37±3.299 µg/ml. DPPH radical scavenging activity and the percentage of inhibition of AK ranged from 6.449±3.285, 14.61±4.742, 25.58±11.91, 29.81±9.642, 36.98±12.1, 41.73±11.57% which was less when compared with standard ascorbic acid inhibition ranging from 14.74± 2.313, 28.25±2.62, 50.74±5.247, 60.18±4.249, 72.26±1.661, 88.43±1.967 %.

Nitric Oxide radical scavenging activity of the AK sample revealed that the percentage inhibition ranging from 6.759±5.657, 12.18±7.161, 16.14±8.826, 21.57±10.11, 25.99±7.795,31.43± 8.68 % when compared with standard gallic acid with percentage inhibition ranging from 26.1±4.802, 40.59±5.316, 51.79±4.829, 59.13±5.66,82.97±3.437,93.59±4.213%. The corresponding IC_{50} value of AK was found to be 174.5±45.58 (µg /ml) when compared with standard gallic acid with (IC_{50} value 39.1±6.586 µg/ml)
The hydrogen peroxide radical scavenging activity and the percentage inhibition of AK ranged from 7.691±6.896, 10.65±7.119, 17.77±7.675, 23±7.066, 26.26±7.777 and27.35±7.142 % when compared with standard gallic acid with percentage inhibition ranging from 25.86±6.812, 48.61±4.418, 61.62±5.115, 76.34±5.431, 83.33±3.64,94.48±4.074%. The corresponding IC_{50} value of AK was high with a value of 162.1±36.3 (µg /ml) when compared with standard Gallic acid with of 29.87±7.46 µg/ml.
The hydrogen peroxide radical scavenging activity and the percentage inhibition of AK ranged from 13.4±7.25, 20.81± 6.611, 23.64±7.599, 30.19±9.326, 33.93±9.383 % when compared with standard BHA with percentage inhibition ranging from 23.21±3.198, 35.52±5.166, 46.31± 2.559, 54.57±7.22, 88.5±0.9255 %.The corresponding IC_{50} value of AKwas found to be 221.1 ± 47.02 (µg /ml) when compared with standard BHA with (IC_{50} value 46.6 ± 5.204µg/ml)
Similar observations of higher IC_{50} when compared to the standard was reported by Savych et al[4]. The decrease in DPPH radical scavenging activity of the AK sample was due to the high phenolic contents and antioxidant scavenging potential of the mixtures of AK.

5. Discussion

The anti-diabetic and antioxidant efficacy of the components of the AK drug, the *Ficus racemosa, Cassia auriculata, Cassia fistula, Syzygium cumini and Salacia reticulata* are already individually reported[21–25]. The antioxidant potential of a drug is recorded to reduce the oxidative stress that triggers the insulin resistance and diabetic complications by quenching the free radicals. The antioxidant activity of AK analysed in this study using the DPPH, ABTS, NO and Hydrogen peroxide confirmed AK to possess potent radical scavenging ability. The activity of α-amylase and α-glucosidase was also inhibited by AK. Further, the individual components used to prepare AK also had the enzyme inhibiting efficacy and potent antioxidant property. Thus, the herbal mixture AK proves to be effective in the treatment of Type 2 diabetes as it can reduce the ROS formation by inhibiting enzymes or by chelating trace elements. The synergistic effect of antioxidant and antihyperglycemic effect of the drug AK proved as an effective treatment for Type 2 diabetes.

6. Conclusion

The in vitro antioxidant study and the enzyme inhibition study showed that the Siddha formulation of AK can reduce the actions of oxidative stress as a triggering effect of diabetes and its allied complications. The drug quenches the free radicals and inhibits the enzyme that raises the blood sugar level. The results of this study prove the formulation of AK to be effective in treating Type 2 diabetes. Based on the results obtained, the Siddha formulation AK has promising anti-diabetic and anti-oxidant activity. With further analysis it can be recommended for diabetic patients.

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Authors Contribution

Dr. Bharathkumar designed the work, Dr. Meenakshi carried out the bench work, and both authors wrote and edited the manuscript.

Conflict of Interest

The authors declare that there are no conflicts of interest

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