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Author: A.A.I.S. Menike, V. Paheerathan, R. Piratheepkumar, R. Sivakaneshan

Bandaranayake Ayurveda Research Institute, Eastern University,

University of Peradeniya, Sri Lanka

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EVALUATION OF THE PHYTOCHEMICAL CONSTITUENTS, ANTIOXIDANT CAPACITY, ALPHA AMYLASE INHIBITORY ACTIVITY WITH SPECIAL REFERENCE TO THE HYPOGLYCAEMIC ACTIVITY OF ASPARAGUS RACEMOSUS ON ALLOXAN INDUCED DIABETIC RATS

[1]A.A.I.S. Menike, [2]V. Paheerathan, [3]R. Piratheepkumar, 4R. Sivakaneshan

[1]Intern Medical Officer, Bandaranayake Ayurveda Research Institute, [2,3]Unit of Siddha Medicine, Faculty of Applied Science, Trincomalee Campus, Eastern University, [4]Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Sri Lanka

ABSTRACT

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia due to defects in insulin secretion, insulin action or both. According to the Siddha texts, as per the signs and symptoms, Neerizhivu also known as Mathumegam in Meganeer, can be correlated with Diabetes mellitus in Modern Medicine. In present days, world's focus turns to traditional herbal medicines due to the fewer side effects. Siddha Medicine has better remedies for the treatment and management of diabetes. As per the literature evidences tuberous root of Asparagus racemosus was found to possess anti-diabetic action. Despite the limited scientific validity, the present research was designed to estimate the hypoglycaemic activity of the tuberous root of Asparagus racemosus on the regulation of the blood glucose level of Wistar Albino rats. Further, the study was aimed on the quantitative evaluation of the antioxidant capacity, total phenolic content, flavanoid content and the alpha-amylase inhibitory activity of the leaves, tuberous root and the whole plant of Asparagus racemosus in order to identify the mechanisms involved for its potential hypoglycaemic activity.

Cold and hot water extracts of the leaves, tuberous root and the whole plant with 5 different concentrations were used for the study. The number of phytochemicals and the antioxidant capacity was significantly ($p<0.05$) higher in hot water extracts when compared with that of the cold-water extracts for all the considered plant parts. The plant was found to have a concentration dependent antioxidant capacity of 129.16±7.90µmol Fe2+/g, 95.34 ± 6.08 µmol Fe2+/g and 68.75 ±2.79 µmol Fe2+/g in the hot water extracts of the whole plant, leaves and tuberous root respectively. The flavanoid content was much more when compared with that of the phenolic content. The flavanoid content was 91 ± 6.59 mg TAE/g in the whole plant, 81.66 ± 5.57 mg TAE/g in the leaves and 38.34 ± 2.89 mg TAE/g in tuberous roots. The total phenolic content was in the order of whole plant > leaves> tuberous root with values 39.23±6.40 mg TAE/g, 27.04±1.79 mg TAE/g and 21.18±3.35 mg TAE/g respectively. Lower alpha-amylase inhibitory activity was found in the leaves and the tuberous root ranging from 8%-9%. However, moderate inhibition of 32% was seen in the whole plant extracts. The hypoglycaemic activity of the tuberous

root of the plant was identified via an experimental animal study. Two doses (40mg/150g and 80mg/150g) were administered orally for a period of 14 days to 24 Alloxan induced diabetic rats. Significant blood glucose level lowering effect was seen in the test group which received 80mg/150 with an overall therapeutic effectiveness of 72%. Hypoglycaemic activity of the group which received 40mg/150g was 67%. The mean differences of the blood glucose lowering effect of the groups were in the order of Standard>Test 2>Test1>Control. Hence, the plant exhibits a dose dependent hypoglycaemic activity. Based on the results the hypoglycaemic activity of Asparagus racemosus was scientifically and therapeutically proved as per the traditional Siddha literature and the potential to develop a novel therapy for Diabetes mellitus is being revealed.

Keywords: Asparagus racemosus, Diabetes mellitus, Hypoglycaemic, flavanoids, phenolic content, antioxidant capacity, alpha- amylase.

INTRODUCTION

Diabetes mellitus (DM) is a chronic endocrine disorder caused by an absolute or relative lack of insulin (Type 1 DM) and/or reduced insulin activity (Type 2 DM) that results in hyperglycemia due to abnormalities in carbohydrate, fat and protein metabolism [1]. Type 2 is the most common form of diabetes, accounting for 90% of cases worldwide and many more remain undiagnosed [2]. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 [3]. Sri Lanka is among the countries with the highest diabetes prevalence rates in the world [16]. Diabetes is usually irreversible and its late complications result in reduced life expectancy and major health costs.

In Siddha system of Medicine, diseases are classified into 4448 types. According to sage Yugi in the text Yugi Vaithiya Chinthaamani, Meganoi is classified into 20 types based on the physical characteristics of urine (Neer Perukal Noikal) such as 4 types of Vatham, 6 types of Pitham and 10 types of Kapham [27]. Neerizhivu-Madhumegam or Inippuneer is one among them, which comes under Pitha type called Thithippu Neer. This is explained in the Siddha texts such as Agasthiyar Karukkadai Nool and Sambusivam pillai Dictionary (1936). In Pararajasekaram 5th part (Anonymous 2003) Diabetes mellitus is called as losing water – Neerizhivu or Salakalichchal, water related disease – Salaroham, and sweet urine – Madhumegam. As per Siddha Medicine, Neerizhivu is a disease characterized by the frequent passage of excessive urine resulting in the deterioration of the seven Udal dhathus-fundamental tissues, along with emanciation of the body [24]. In this disease Pitham is affected first, then Vatham and finally Kapham is affected due to internal and external causes. So, all the three Uyir dhathus are imbalanced which in turn affects the seven Udal dhathus (Thirumoolar Vaithya Karukkadai 600-verse 83). Hence according to the clinical features, it can be seen that Neerizhivu in Siddha medicine can be directly correlated with Diabetes mellitus as per modern science.

One of the therapeutic approaches in the management of Type 2 Diabetes mellitus is to decrease the postprandial hyperglycemia. This can be achieved by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes such as alpha-amylases in the digestive tract. Such inhibitors which are presently in the clinical practice for management of Diabetes are known to be associated with various gastrointestinal side-effects [3]. Therefore, it is essential to identify and explore the amylase inhibitors from natural sources having fewer side effects.

Free radicals contribute to hundreds of disorders in the human body. The process of oxidation is one of the most important routes in producing free radicals in food, drugs and even in living systems. Antioxidants are important species which possess the ability of protecting organisms from free radical induced oxidative stress [17]. Studies have also shown that the bioactive constituents present in the plants including Phenols and Flavanoids have numerous medicinal effects including anti-diabetic properties through the potential effects of their antioxidant capacities (Ashwini et al, 2018). The antioxidant activity of phenolics is mainly due to the redox properties, which allow them to act as reducing agents, hydrogen donors and metal chelators [17]. Thus, the traditional medical system chosen as an alternative medical source, drugs from medicinal plant sources satisfies this need as they were found to have innumerable benefits for the last three decades [20]. At present globally there is resurgence in the usage of plant-based medicines used in traditional systems such as Siddha because of its holistic approach with maximum therapeutic potency and are considered to be less toxic and with minimum side effects than synthetic ones.

Siddha Medicine serves both therapeutic and prophylactic concepts and hence holistic in nature. Kaya karpam, which is considered as the science of rejuvenation, longevity, and spiritual wellbeing is one of the specific therapeutic formulations in Siddha Medicine advocated for rejuvenation, which is formulated several thousand years back by our great Siddhars for the wellbeing of the human life by power of health securing the body [30]. Kaya kalpa drugs have proven scientific evidences to prevent and manage the Non communicable diseases like Diabetes mellitus, obesity and also chronic deliberating diseases like cancer. This therapy ensures the longevity and elimination of disease-causing factor [20].

Currently research on herbal medicines is encouraged. The need of the hour is to identify and develop innovative plant-based therapies to combat diabetes, and provide evidence – based plant medicine, and prove the advantages of these medicines over the existing therapies. Hence, research is focused to screen the medicinal plants that are used traditionally for diabetes to find a newer leading drug molecule with more potential and less side effects than the existing hypoglycaemic drugs (Chandramohan, 2008). Approximately 126 anti-diabetic plants have been used to treat diabetes in Sri Lanka [8]. However, most of these plants are being used in traditional healing systems without proper scientific validation despite recommendations by World Health Organization for further investigation (Jeyakumaran et al., 2015). Unfortunately, despite the apparent supremacy in terms of multiple therapeutic efficacies of herbal based medicines, well- organized, rigorous clinical trial evidences are not adequately available. Considering these, it is necessary to provide an alternative solution to counter the diabetic plague through indigenous herbal resources used by Siddha practitioners (Ravi, 2015).

Despite the availability of various herbal medicines and traditional treatment methods available in Siddha system of medicine, the plant Asparagus racemosus received the attention for the present study despite of the literal evidences of the use of tuberous root of the plant in Siddha Medicine to cure Neerizhvu- Diabetes mellitus [26]. Additionally, traditional folksier of ancient Tamil literature mentions the use of the plant to prevent its complications which is yet to be scientifically evaluated (Nadkarni 1976, Chadha 1985).

Review of the plant

Asparagus racemosus (A. racemosus) Willd, commonly known as Shatavari

which may be translated as “100 spouses”, also called Thannervittan in Tamil and Hathawariya in Sinhala, is a plant belonging to family Liliaceae [28]. The plant is a scandant, much branched spinous under shrub with woody stems. It possesses a short root stock bearing numerous fusiform tuberous roots tapering at ends. The leaves are reduced to minute chaffy scales and spines. It has white flowers with globose, rugose 1-seeded berries which are red or purplish black in colour [25]. As per Siddha Medicine, Asparagus rcaemosus is known to possess Inippu (sweet) - Suvai (taste), Seetha (cold) -Veeryam (potency) and Inippu (sweet) - Vipakam (effect) and is effective in the treatment of, Neerizhivu (Diabetes mellitus), chronic fever (naatpatta suram), oligospermia and internal heat [26]. It is considered both a general tonic and a female reproductive tonic. The plant is considered as one of the best rejuvenative drugs in Siddha medicine which prevent ageing, increase longevity, impart immunity, improve mental function, vigor and add vitality to the body. It is recommended in traditional medicine for the prevention and treatment of gastric ulcers, dyspepsia, diarrhoea and nervous disorders. The plants also possess antioxidant, immunostimulant, antidyspepsia and antitussive property. Asparagus racemosus constitute of alkaloids, flavonoids, tannins, saponins, phenols, terpenes, polysaccharides and steroids [4]. The root extracts of the plant were found to contain high amounts of flavonoids, polyphenols and Vitamin-C exhibiting a greater antioxidant activity. Shatavarin I to IV, polycyclic alkaloid, Asparagamine A and disaccharide in roots are reported [22]. However, limited research was available to indicate its phytochemical constituents and the antioxidant activity separately in the cold and hot water extracts of the leaves and the whole plant and to determine the pharmacokinetics of the anti-diabetic effect of the tuberous root of this plant. Hence, the present study was carried out in order to determine the hypoglycaemic effect of the tuberous root extract of Asparagus racemosus by identifying its activity on the blood glucose level via an Animal study. Additionally, the plant parts have also been tested for its phytochemical constituents and the alpha amylase inhibitory activity.

Aim and Objectives

To estimate the hypoglycaemic activity of the tuberous root of Asparagus racemosus on the regulation of the blood glucose level of Wistar Albino rats.

Quantitative evaluation of the total antioxidant capacity, total phenolic content, flavanoid content and the alpha-amylase enzyme inhibitory activity of the cold and hot water extracts of the leaves, tuberous root and the whole plant of Asparagus racemosus.

MATERIALS AND METHOD

Study Design

In vitro Phytochemical analysis of the aqueous extracts of Asparagus racemosus to estimate the total antioxidant capacity, flavanoid content and total phenolic content performed as a comparative experimental study. In vitro enzyme assay done to assess the alpha amylase inhibitory activity of the plant parts. The hypoglyvaemic activity of the tuberous root was tested via an experimental animal study.

The study was conducted in the Research Laboratory, Unit of Siddha Medicine, Trincomalee Campus, Eastern University of Sri Lanka during the period from 14th of August 2018 to 19th of April 2019.

Chemical Reagents

TPTZ – 2, 4, 6 - Tripyridyl-S-triazine solution (10 mM solution in 40mM HCl), 0.4 ml of FeCl3 (20 mmol/L) stock solution was diluted in 50ml of distilled water to make the final concentration 0.16 mmol/L. Acetate buffer (0.3 mol/L, pH = 3.6). $NaNO_2$ solution (5%), AlCl3. $6H_2O$ solution (10%), NaOH (1 M) solution, Folin –Ciocalteu reagent (10%) was prepared by diluting 1mL of the stock solution with 9 mL of distilled water. $NaHCO_3$ (7.5%) solution, Tannic Acid standard solution (1g/L) was used for the Flavanoid assay and Tannic Acid standard solution (0.1g/L) was used for the assessment of the Total Phenolic content. Porcine pancreas α-amylase (3 units/ml), Starch (1% w/v), Phosphate Buffer (pH=6.9), Dinitrosalicylic acid (DNSA) colour reagent. Additionally, Alloxan Monohydrate was used to induce diabetes in rats. All the chemicals were obtained under the proper supervisor authentication and guidance from the Biochemistry Laboratory, Faculty of Medicine, University of Peradeniya, Sri Lanka.

Collection and Preparation of Plant materials

The plants were collected from Trincomalee district, Sri Lanka. The plant species was identified and authorized by the supervisor, Kunapadam laboratory, Trincomalee Campus, Eastern University of Sri Lanka. The collected plants were divided into three groups in order to obtain the required experimental specimens.

Group 1- Extract using fresh plants

Group 2- Extract using leaf powder

Group 3- Extract using tuberous root

Plants were cleaned to remove the contaminated physical impurities and it was further washed with running tap water and distilled water. The whole plant (500 g wet weight) from Group 1, was placed in a stone mortar and chopped into a fine paste and it was further processed through an electrical grinder. Fresh extract was obtained with the help of an extractor and filtered using a Whatman No. 1 filter paper and the samples were freeze dried in a freeze dryer for 2 weeks to obtain the completely dried powders which was properly sealed, labeled and stored at 0°C until further use. The leaves and tuberous root were separated from different plants, obtained from Group 2 and 3 respectively, and was cleaned properly. They were allowed to dry under the shade for approximately one week and were ground using an electrical grinder and fine powders were obtained using a sieving cloth. The prepared powders were weighed separately and were labeled and stored in air tight plastic containers at room temperature until further use.

Figure 1. Authenticated Plant

Preparation of cold and hot water extracts

50 mg of powder of the tuberous roots, leaves and the freeze-dried whole plant were added separately to 10 ml of distilled water and crushed well by using a mortar and pestle. Then it was centrifuged at 10000 rpm for 10 minutes. The supernatant was carefully separated and stored at 25 ° C until further use. The same procedure was adopted to prepare the hot water extracts by heating the plant powders dissolved aqueous solutions in a water bath (100 ° C) for 5 minutes.

The prepared 5mg/ml stock solution was accordingly diluted by adding distilled water to obtain final concentrations 4mg/ml, 3mg/ml, 2mg/ml and 1mg/ml solutions.

Determination of the Total Phenolic Content (TPC)

The Total Phenolic Content (TPC) in cold and hot water extracts was determined according to the method described in Mc Donald et al [31]. 50 µl of extract was mixed with 0.5 ml of 10% Folin – Ciocalteu's reagent and 0.4ml sodium carbonate (7.5%). The tubes were vortexed and allowed to stand for 30 minutes at room temperature. Then the absorbance was measured at 765 nm. Tannic acid (0.1g/L) was used as the standard. The TPC of the extracts were expressed as mg tannic acid equivalent per gram of leaves powder, tuberous root powder and freeze-dried whole plant powder on dry basis. All determinations were performed in duplicates and data was presented as mean ± SD.

The biochemical analysis was separately done for all the different concentrations considered in the study.

Determination of the Total Flavanoid Content

The total flavanoid content was determined by using a colorimetric method described by Enujiugha, 2010 [32]. Briefly, 1.25 ml distilled water and 75 µl 5% $NaNO_2$ was added to 250µl of the extracts. The tubes were vortexed and allowed to stand at room temperature for 6 minutes. Then 150µl of 10% AlCl3 was added to the above mixture and allowed to stand at room temperature for 5 minutes. Finally, the absorbance was measured immediately at 510 nm after adding 0.5 ml of NaOH and 2.5 ml of distilled water. Tannic acid (1g/L) was used as the standard. The flavonoid content was expressed as mg tannic acid equivalent per gram of leaves powder, root powder and freeze-dried whole plant powder on dry basis and all determinations were performed in duplicates and data presented as mean ± SD.

In-vitro antioxidant assay (Ferric Reducing Antioxidant Property)

Total antioxidant capacity was estimated according to the procedure described by Benzie and Strain, 1996 [33] The reagent was prepared by mixing 1ml of 2, 4, 6-Tripyridy-s-triazine (TPTZ) (10mmol/Lin 40mmol/L HCl), 1 ml of FeCl3 (20 mmol/L) and 10 ml acetate buffer (0.3 mol/L, pH=3.6). 20 µl of extract (5mg/ml) was mixed with 1 ml of the mixed reagent and the absorbance was measured spectrophotometrically at 593 nm after incubating at room temperature for exactly 4 minutes, against a reagent blank. The absorbance of 1000 µM (1 mmol/L) $FeSO_4$ standard was also measured following the same procedure as for the samples. The ferric reducing antioxidant power was expressed in µmol/g dry weight (DW).

Alpha- amylase inhibitory assay

Alpha- amylase inhibitory activity was measured in-vitro by the hydrolysis of starch in the presence of alpha-amylase enzyme. The enzyme inhibitory activity was expressed as a decrease in the units of maltose liberated. This process was quantified by using Dinitrosalicylic acid (DNSA), which gives yellow colour with starch [16]. Inhibition of alpha-amylase enzyme by the hot and cold-water extracts of the plant materials were carried out using a method previously described by Geethalakshmi et al [16] according to the following procedure.

Test (T) – 40 µL of aqueous plant extract (1 mg/ml) was mixed with 40 µL of porcine pancreatic alpha-amylase enzyme (3 U/ml) and 2 ml of 80 µL of phosphate buffered saline (pH 6.9).

Test Blank (TB) – Test blanks were conducted in the presence of plant extracts without alpha-amylase enzyme.

Control (C) –Was carried out in the absence of the plant extract or standard inhibitor.

Control Blank (CB) – A blank was prepared without the plant extracts and alpha-amylase enzyme replaced by equal quantities of buffer.

Calculation of the percentage of inhibition of enzyme activity

The inhibition of alpha-amylase enzyme activity is expressed as a percentage calculated by the following equation [8].

$$\% \text{ of Inhibition} = 100 - \left\{ \frac{(\text{Absorbance of Test} - \text{Absorbance of Test Blank}) \times 100}{(\text{Absorbance of Control} - \text{Absorbance of Control Blank})} \right\} \quad (1)$$

Experimental animal study

The hypoglycaemic activity of Asparagus racemosus was identified by testing the cold-water extract of the tuberous root powder on Diabetic rats.

Collection and preparation of animals

Wistar Albino adult male rats (n=24) weighing 150-250g of ages 4-6 weeks, were obtained from the Medical Research Institute, Colombo. The rats were grouped and housed in cages with the standard laboratory conditions in well-ventilated caged and were exposed to 12 hours light and 12 hours dark cycles during the course of the experimental period. The animals were allowed to acclimatize to normal laboratory conditions for one week prior to the study and were provided with standard pellet diet and water [14].

Dose calculation

The dose of the test drug and the standard drug was determined according to the common scale by using the given formula as per the study Shin et al 2010 [7].

$$\text{AED (mg/kg)} = \text{HED (mg/kg)} \times \text{Km ratio} \quad (2)$$

Where, AED – Animal Estimated Dose
HED – Human Estimated Dose

K_m ratio – conversion factor for a rat with average body weight 0.15 kg is 6.17 [7]. The average human body weight was assumed to be, 70kg. The average body weight of the animals selected for the study is 150 g.

Standard drug: Metformin was used as the standard drug. Single dose of Metformin was selected for the present study (850mg/day). The converted animal dose was equivalent to 11 mg/150g per rat.

Test Drug: Normal dosage of the Asparagus racemosus tuberous root chooranam is as 3g – 6g two times a day [34]. Hence these two dosages were selected as the dose to be administered to the animal. The calculated minimum effective dose given to the rats was 40 mg/150 g and the maximum effective dose was 80 mg/150 mg.

Induction of diabetes

The animals were fasted for 12 hours (overnight) prior to the induction of diabetes as described by Joy and Kuttan, 1999 [35]. Then the animals were weighed separately with an electronic weighing scale. Alloxan solution (ALX) - Alloxan monohydrate freshly prepared in distilled water was administered intra-peritoneally

as a single dose of 120 mg/kg body weight [23]. Development of diabetes was confirmed by measuring baseline blood glucose level 2 days after the administration of ALX. Rats with blood glucose level of above >250 mg/dL were considered to be diabetic and were used for the study.

Experimental design

A total of 24 rats were selected for the study and divided into 4 groups consisting of 6 rats in each group according to the following manner;

Group I – Untreated control; fed neither with the test drug nor the standard drug.

Group II – Standard; Diabetic + Metformin 11mg/150g body weight.

Group III - Test 1; Diabetic+ D1 of the test drug (40 mg/ 150g)

Group IV – Test 2; Diabetic + D2 of the test drug (80 mg/ 150g)

(D1 – minimum effective dose, D2 – maximum effective dose)

The animals were allowed to fast overnight before the experimental schedule began but was allowed for free access to water. The base line glucose levels in all the animals were tested 2 days after the induction of diabetes with a glucometer by pricking the tail vein. The test drugs and the standard drugs were administered 7days after the induction of diabetes. Group I was untreated control group which neither received the standard drug nor the test drug. The 2nd group, standard group, received 850 mg/kg (11 mg/150 g) of Metformin dissolved in 1.5 ml distilled water once in the morning. Group III was given the minimum dose of the test drug which is 40 mg/kg and the Group IV rats were given 80 mg/kg of the test drug which is the maximum dose administered twice a day orally dissolved in 1.5 ml of distilled water through a oral gavage tube at 6 hours interval. The treatment procedure was continued for a period of 14 days.

The final blood glucose concentration of all the animals was measured in a similar manner after 14 days with a glucometer by pricking the tail vein of the animals.

Statistical analysis of the animal study

The results were be analyzed statistically. Data is expressed as mean ± S.E.M. In the present study the mean blood glucose level was selected as the independent variable while the tested groups (control, standard, test 1 and test 2), were taken as the dependent variables. The biochemical parameters were analyzed statistically using Paired T- Test in order to compare the mean blood glucose level of the treatment groups before and after treatment and one-way ANOVA (Analysis of variance) was used to identify the relationship of the variables within the groups and among the groups. The test results were used to analyze the maximum therapeutic effectiveness of the test drug with a statistically significant mean difference ($p<0.001$).

RESULTS

Preliminary phytochemical screening

All the plant parts exhibited a statistically significant phytochemical constituents and antioxidant capacity with a p value < 0.05. Hence, the mean values were compared to identify the plant part with maximum effectiveness.

Total Phenolic Content (TPC)

The overall TPC of the hot extracts of 29.15±9.20 mg Tannic Acid Equivalents (TAE)/g was higher than the mean of the cold extracts of 24.92±7.62mg TAE with a significance of p= 0.053.

The TPC of the hot water extracts of the whole plant of Asparagus racemosus were the highest among all the plant extracts considered (39.23 mg ± 6.4 Tannic acid

equivalent/g dry weights) followed by leaves and the tuberous roots.

The TPC of the cold extracts were in the order of was in the order of whole plants >leaves>tuberous root with values 32.79 mg ±2.92, 23.9 mg ±3.34 and 17.79 mg ±1.73 Tannic acid equivalent/g dry weights and respectively.

This indicates a better extraction of phenolic compounds from the all parts at higher temperature.

Flavanoid content

The concentration of flavanoids in cold and hot water extracts of different parts ranged from 28mg ± 2.45 to 90mg ± 11.46 and from 38mg ± 2.89 to 91mg ± 6.59 Tannic acid equivalent/g dry weights respectively with a significance of p=0.214. The flavonoid concentration of the hot extract of whole plant was 91.28mg ± 6.59 Tannic acid equivalent/g dry weights and that of the cold extract was 89.81mg ± 11.46 Tannic acid equivalent/g dry weights. The lowest flavonoid concentration was found in the cold and hot extracts of the tuberous root with values 28.10 mg ±2.45and 38.35 mg ± 2.89 Tannic acid equivalent/g dry weights respectively. The leaves had values of 56.90 mg± 7.38 and 81.66 mg±5.57 Tannic acid equivalent/g dry weights found in the cold and hot extracts respectively.

According to the study both cold and hot water extracts of whole plant showed highest amount of flavonoid content.

Table 1. Total Phenolic and Flavanoid Content of Asparagus racemosus

Extract	Plant Part	Total Phenolic Content (mg TAE/g)	Total Flavanoid Content (mg TAE/g)
Cold Extract	Leaves	24 ± 3.34	39.84 ± 0.15
	Tuberous root	18 ± 1.73	15.94 ± 0.10
	Whole plant	33 ± 6.53	41.68 ± 0.25
Hot Extract	Leaves	27 ± 1.79	39.03 ± 0.20
	Tuberous root	21 ± 3.35	38.15 ± 0.19
	Whole plant	39 ± 6.41	20.50 ± 0.21

Total Antioxidant capacity (FRAP Assay)

The mean of the cold extract of the whole plant (96.35±4.84 µmol /g) was greater than the mean of the cold extracts of the leaf (77.69±4.74 µmol /g) and tuberous root (48.51±1.88 µmol /g). The mean of the hot extract of the whole plant (129.16±7.9 µmol /g) was greater than the mean of the hot extracts of the leaf (95.340±6.0822 µmol /g) and tuberous root (68.75±2.79 µmol /g). Hence, the means of the molar equivalence of Fe2+ (µmol /g) of the hot extracts were higher than that of the means of the cold-water extracts. This relationship was true and same for all the plant parts considered (leaf, tuberous root and the whole plant) which was statistically significant with $p<0.05$ ($p=0.037$). On overall the hot water extract of the whole plant exhibits the maximum antioxidant capacity of Asparagus racemosus containing the maximum number of Fe3+ being reduced to Fe2+ per gram of the plant product.

Relationship between phytochemical constituents and antioxidant activity

The Antioxidant capacity is positively correlated with flavanoids or total phenolic content. The higher content of flavanoids or total phenols, the stronger the antioxidant capacity [29]. The presence of large quantities of phenolic compounds does not necessarily always correlate with antioxidant effects and according to recent reports, a positive relationship had been found between flavanoids and antioxidant activity appears to be the trend in many plant species in comparison to phenols (Oktay et al., 2003). According to the present study it can be seen that the Total Antioxidant capacity of Asparagus racemosus is mainly due to the presence of higher amounts of flavanoids when compared with that of the phenolic content for all the extracts of the plant parts considered (Figure 2).

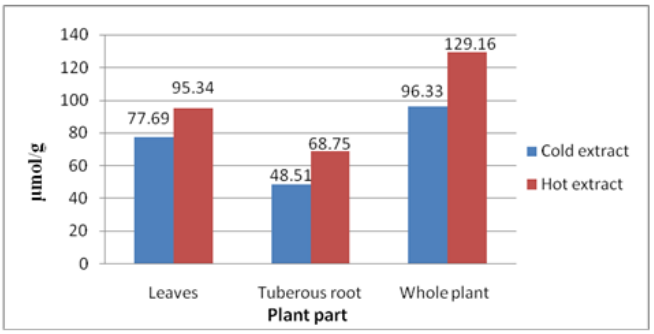

Figure 2. Total anti-oxidant capacity of Asparagus racemosus

Change in the number of phytochemical constituents and the antioxidant capacity with different amounts of dry powder

The number of phytochemical constituents and the antioxidant capacity of the cold and hot extracts of all the plant parts proportionately increases with the increased amount of the dried powder used in the preparation of the extracts with the maximum yield of the biochemicals observed in the hot water extracts (Figure 3, Figure 4 and Figure 5).

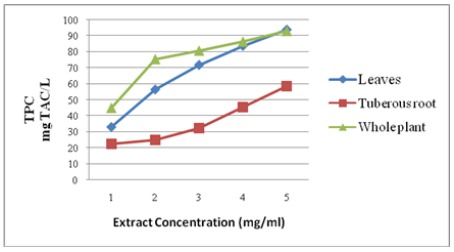

Figure 3. Variation of the TPC of the Hot Extracts with Extract Concentration

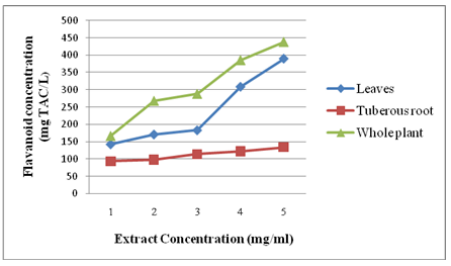

Figure 4. Variation of the Flavanoid content of the Hot Extracts with Extract Concentration

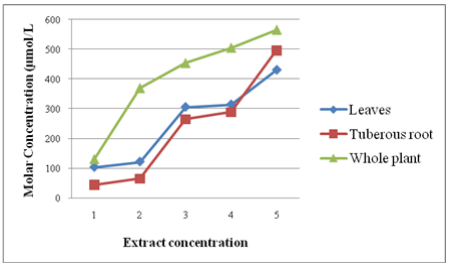

Figure 5. Variation of the Total Antioxidant Capacity of the Hot Extracts with Extract Concentration

Alpha amylase inhibitory activity of Asparagus racemosus

Asparagus racemosus with known anti-diabetic activity was investigated for its potential to inhibit alpha-amylase enzyme and to identify the part with the maximum amylase inhibitory activity. Aqueous extracts of the leaf, tuberous root and the whole plant of Asparagus racemosus were separately analyzed for the enzyme inhibitory activity using a method previously described by Geethalakshmi et al [16].

The aqueous cold water extracts of the whole plant of A. racemosus showed considerable amount of alpha-amylase inhibitory activity of 32% with 1mg/ml extracts. The percentage alpha-amylase enzyme inhibition of the leaves was 15% in 1mg/ml extracts. However, minimum alpha amylase inhibitory activity was observed in the tuberous root extracts with 6% inhibition at 1mg/ml concentration despite of the evidences for the presence of its anti-diabetic activity

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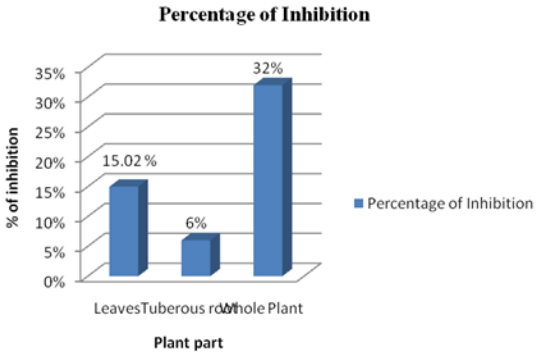

Group	Treatment	Mean	Mean difference	Std. Deviation	Std.Error Mean	Paired "t"	Sig. (2tailed) P
Control	BT_C	513.400	94.20	153.05	68.44	1.376	0.241
	AT_C	419.200					
Standard	BT_S	532.400	409.60	78.72	35.20	11.635	0.000
	AT_S	122.800					
Test 1	BT_T_1	539.800	362.20	112.81	50.45	7.179	0.002
	AT_T_1	177.600					
Test 2	BT_T_2	516.000	369.20	106.38	47.57	7.761	0.001
	AT_T_2	146.800					

Figure 6. Percentage of Alpha Amylase Inhibition

Hypoglycaemic action of aqueous tuberous root extract of Asparagus racemosus on Alloxan induced diabetic rats

The effect of the aqueous tuberous root extract of Asparagus racemosus on the blood glucose levels of normal and diabetic rats is given in table 2. After the intra-peritoneal administration of Alloxan, there was a significant elevation of the plasma glucose levels by 2-3 folds during the experimental time period. The blood glucose levels of the diabetic untreated rats were significantly higher than those of the normal rats.

As shown in Table. 2, the mean blood glucose level was lowest in the standard group with a value of 122.8 mg/dl (±6.87) after 14 days of treatment with Metformin. The second highest blood glucose lowering effect was seen in the Test 2 group which received the maximum dose of the aqueous extract of Asparagus racemosus (80 mg/150g) with a value of 146.8 mg/ml (±12.79). The Test 1 group which received the low dose of the test drug (40 mg/kg) had blood glucose levels of 177.6 mg/ml (±14.86). The lowest blood glucose lowering effect was seen in the control group which was diabetic throughout the experimental period and had mean values of 230.6 mg/ml (±17.68). Hence, according to the results it can be seen that the tuberous root extract of Asparagus racemosus has a significant blood glucose lowering effect towards alleviating diabetes mellitus.

Table 2. Effect of Aqueous Extract of Tuberous Root of Asparagus racemosus on Alloxan Induced Diabetic Rat

BT- Before Treatment, AT-After Treatment, C-Control, S-Standard, T_1-Test 1, T_2-Test-$_2$

Overall therapeutic effectiveness of the test drug

The therapeutic effectiveness of the aqueous extract of the tuberous root of Asparagus racemosus with the highest dose was more when compared with that of the minimum dose of the drug as shown in figure 7. The T2 group treated with the maximum dose of 80mg/150g was shown to have the maximum effectiveness of 72% (71.55%) next to the standard group treated with Metformin (77%). The therapeutic effectiveness of the T1 group which received 40 mg/150g of the test drug showed 67% of overall improvement of the blood glucose level. The lowest blood glucose level lowering effect was seen in the control group which was diabetic throughout the experimental period.

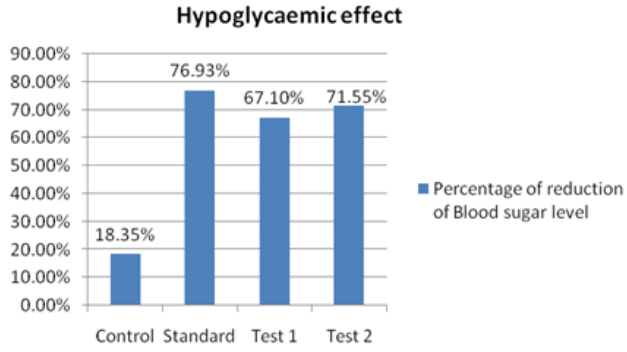

Figure 7. Overall Therapeutic Effectiveness of the Tuberous Root Extract of Asparagus racemosus

DISCUSSION

Diabetes mellitus (DM) is a serious metabolic disorder characterized by chronic hyperglycemia that causes considerable morbidity, mortality and disability primarily due to its micro and macro vascular complications. One of the therapeutic approaches in the management of Type 2 Diabetes is to decrease the post-prandial hyperglycemia. Plants are considered to be the basis for deriving natural or semi-synthetic constituents that can be used against diabetes [36]. Investigations need be carried out to identify the chemical constituent(s) responsible for the anti-diabetic activity of the medicinal plants, and to elucidate their mechanism of action [37]. On the basis of ethno medical/tribal information Asparagus racemosus Willd has been used to treat and prevent diabetes as per the ancient Siddha literature. However, only limited evidence is available for the scientific and medical evaluation of its efficacy. The present study was postulated to evaluate the in-vivo hypoglycaemic activity of the tuberous root of Asparagus racemosus. Further, the study has also assessed the potential inhibitory effect of the plant on one of the major carbohydrate hydrolyzing enzyme; alpha- amylase with special reference to the phytochemical constituents present in the plant.

Preliminary phytochemical screening and Antioxidant capacity

Antioxidants can interfere with the oxidation process by reacting with free radicals [9]. Phenols react with active oxygen radicals such as hydroxyl radical, superoxide anion radical and lipid peroxy radical indicating the antioxidant capacity (Matthias et al., 2015). All the plant parts considered in the comparative study consisted of a comparable amount of phenols present in both the hot and cold water extracts with the highest amount present in the hot extracts. This suggests the increase in yield of extraction of the bioactive components of the plant products at higher temperatures. This principle is effectively used in the preparation of decoctions by the traditional physicians. Hence, presence of the therapeutic effectiveness in the poly herbal decoctions is being scientifically proved by the present study. On the other hand, higher amounts of phenols and flavanoids were found on the whole plant extracts reflecting the contribution of the phytochemical constituents by the flowers and the berries of Asparagus racemosus.

Flavonoids play an important role in antioxidant system in plants. The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper and inhibition of enzymes responsible for free radical generation (Benavente-Garcia et al., 1997). Depending on their structure, flavonoids are able to scavenge practically all known Reactive Oxygen Species resulting in higher Antioxidant Properties. According to the present study, the high contents of flavonoids in Asparagus racemosus extracts when compared with that of the phenolic content can explain its high radical scavenging activity and it has been scientifically proved that the high Antioxidant capacities of the plant is mainly by the contribution from the flavanoids rather than from the phenols. Similarly, the present study also has justified that the Antioxidant capacity of the plant extracts proportionately increase with increase in the amount of the plant material in the extracts which has been presented by the previous study Karuna et al [6].

Asparagus raemosus is considered as one of the rejuvenating herbs in Siddha medicine (Moolikai karpam). According to the modern concept Kaaya karpam can be correlated with the Antioxidant concept [30]. Hence, from the present study the use of Asparagus racemosus as a rejuvenating

herb is also scientifically proved with its high Antioxidant capacity.

Comparison of the total phenolic and flavanoid content with that of the antioxidant capacity

Antioxidant capacity is positively correlated with flavonoids or total phenolic content. Higher the content of flavonoids or total phenols, stronger is the antioxidant capacity [29]. Studies had revealed that there is high correlation between antioxidant capacity of the plant materials and the phenolic content [30]. The presence of large quantities of phenolic compounds does not necessarily always correlate with antioxidant effects and according to recent reports, a positive relationship between flavanoids and antioxidant activity appears to be the trend in many plant species in comparison to phenols (Oktay et al., 2003).The statement has been justified in the current study where the total antioxidant capacity showed a positive relationship with a high flavanoid content present in the plant.

Alpha-amylase inhibitory activity of Asparagus racemosus

One of the therapeutic approaches is to decrease the postprandial hyperglycemia, by retarding the absorption of glucose by inhibition of carbohydrate-hydrolyzing enzymes, such as alpha-amylase and alpha-glucosidase, thus being an interesting and novel therapeutic target for diabetes mellitus treatment [21]. From this point of view, many efforts have been made to search for more effective and safe inhibitors of alpha-glucosidase and alpha-amylase from natural materials to develop functional foods to treat diabetes. Literature survey showed that active phytochemical components of plants such as vitamins, carotenoids, flavonoids, anthocynins and other phenolic compounds can reduce blood glucose in diabetic patients, inhibiting alpha-amylase (Gomina et al., 2014).

The leaves and the tuberous extract of the plant did not show considerable alpha-amylase inhibitory activity despite its hypoglcaemic effect. The percentage of inhibition seen in the leaves was 15 % (15.02%) and that of the tuberous roots were 6% (6.33%). The whole plant extract showed a considerable inhibitory activity with 32% of inhibition at 1mg/ml extract concentration. Hence, the hypoglycaemic effects known to exist within the tuberous root of Asparagus racemosus according to ancient literature may be exerted by mechanisms of other enzyme inhibotry effects such as alpha-glucosidases rather than alpha amylase enzyme. Hence, further studies are necessary to identify these mechanisms. The results in the present study are not in consistent with the study carried out by Vadivelan et al., (2009) which showed dose dependent significant alpha amylase inhibitory activity in aqueous extracts of the leaves of A. racemosus than that of the methanol, ethyl acetate extracts and the Arcabose. This effect may be due to factors such as different geographical locations and the native habitats of the plant. On the other hand, it would be due to the effectiveness of the method of extract preparation adopted in the study Vadivelan et al., (2009) where the extracts ad been prepared by the method of maceration of the dried leaf powders. Prolong maceration involves the soaking of the dried leaf powders which would result in the increased osmotic pressure of the cells and rupture of the cells. This process would enhance the maximum yield of the active compounds to the medium. However, no study was available to illicit the enzyme inhibitory effects of the tuberous root in the aqueous medium despite its anti-diabetic action.

Anti-diabetic action of the tuberous root extracts of Asparagus racemosus

According to the literature evidence given in the standard Siddha text Kunapadam Porutpanpunool (Part I) written by K.S.Murugesamuthaliyar [26] has mentioned that the tuberous root of Asparagus racemosus is used to treat Neerizhivu. Hence the present research has scientifically proved the hypoglycaemic activity of Asparagus racemosus by and experimental animal study, in consistent with the presence of the bioactive phytochemicals and ezymes to understand the pharmacokinetics of its Anti-diabetc action.

The groups treated with the minimum and the maximum doses of the test drug showed a significant Hypoglycaemic effect ($p<0.05$) when compared with that of the control group. Hypoglycaemic effect of tuberous extract of Asparagus racemosus is consistent with the study of Kannan et al, (2014). The maximum effect was seen in the high dose of the test drug where the rats were treated with 80mg/150g of root extract. The mean blood glucose level was reduced from 516mg/ml to 146.8 mg/ml and this was a 369mg/ml reduction in the total blood glucose level. The low dose of the test drug also showed beneficial results where the blood glucose level was reduced from 540 mg/ml to 178 mg/ml within a period of 14 days of treatment of dose 40 mg/150g body weight of the animals. A dose dependent hypoglcaemic activity of the tuberous root of Asparagus rcaemosus was observed. The above results of the present study are in consistent with the study Dheeba et al, 2012 [14]. which had shown an anti-diabetic action by the ethanolic extracts of the root of Asparagus racemosus. Most of the medicinal preparations used in ancient traditional systems of Medicine are effectively administered in the form of decoctions where the active components of the medicinal herbs are being extracted to the aqueous medium. However, no study was available to illicit to anti-diabetic effect of the tuberous root of Asparagus racemosus in aqueous cold water extracts, Hence, the present study is an approach to provide an evidence based scientific evaluation for the success of the treatment procedures adopted by our ancient traditional healers.

Thus, the plant extract of the Asparagus racemosus may be used in the management of type 2 diabetes mellitus with few or no side effects. However, further studies of the plant extract of the Asparagus racemosus using in vitro and in vivo models need to be performed to elucidate its insulin mimetic activity and reduction of insulin resistance and also to develop medicinal preparations, nutraceuticals or functional foods for diabetes and related symptoms.

Discussion of the anti-diabetic action of Asparagus racemosus on Siddha aspect

According to the Mukkutra Verupadugal (etio-pathogenesis) of Neerizhivu explained under the literature Pitta and Vatham are the main doshas vitiated at the initial stages of the disease as mentioned in the quotation of the Siddha text Pathinen Siddhar nadi Nool. Finally Iyam is affected with the vitiated Vatham. So, all the three Uyir Thathus and seven Udal thathus are affected. Gradually body becomes emaciated and these are excreted through urine.

As per the Siddha text Kunapadam Porutpanpunool (Part I) by K.S.Murugesamuthaliyar [26] Asparagus racemosus possess Inippu (sweet) Suwai (taste), Seetha (cold) veeriyam/Thanmai (potency), Inippu (sweet) Vipakam/Pirivu (action) and Singdha (unctuous) and Mandha (dullness) in Gunam (character).

Inippu (sweet) taste is formed by the Panchabhoothic composition of the Mann (Earth) + Neer (water). According to Siddha medicine, drugs and diet having sweet taste enhance the growth of Saram

(plasma), Seneer (blood), Uoon (muscles), Kozhuppu (fat), Enbu (bones), Moolai (bone marrow) and Sukkilam (sperm) or Suronitham (Ova). On the other hand, it also alleviates Pitham and Vatham. Hence accordingly, it can be seen that that the Suvai of the plant is effective in the management of the Neerizhivu/Mathumegam noi by balancing the imbalanced Pittam and Vatham aggravated at the initial stage of the disease condition. On the other hand the deteorated sapthdhathus would also be enhanced by the action of the sweet taste. Similarly general hyperglycaemic symptoms seen in Neerizhivu such as increased thirst and emaciation due to the loss of excess "rasa" (chyme) can also be treated with the Inippu taste herbal preparation of Asparagus racemosus. This has been further proved by the study Susila et al, (2017) which has concluded that tuberous roots of Asparagus racemosus of Suvai Inippu is suitable for the treatment of Diabetes in reference to the vitiation of Pitta dosha at the initial stage of the disease. However, in the chronic stage of the disease condition the Vatham would combine with Iyam and would further manifest the pathogenesis. Hence, in chronic diabetes conditions the Iya dosha consisting of Mann and Neer Panchabhothic compositions would be aggravated. Since the suvai of Sathawariya is also Inippu consisting of same panchabhoothic compositions administration of the drug in chronic disease would further aggravate the condition. So, further research is needed to identify the mechanism of treatment of chronic Neerizhivu conditions with respect to the doshas involved as per the pathogenesis of the disease condition.

The panchabhoothic composition of Seetha Veeriyam is Pirithu+ Neer (Earth and water) and it alleviates Pittam (Pitha samani). Hence according to the veeriyam of the plant also it is suitable to balance the imbalance doshas and to alleviate the immediate as well as the long term effects of Nerizhivu-Mathumegam.

The Panchabhutha composition of Mantham is Pruthiri + Punal (Earth and water). It possesses – Sweet – Astringent – bitter Taste and result in Sweet Vipakam as the end products of digestion. Seetha Veeriyam can be seen as the potency of Mantha gunam. The property which is responsible for the slow activity or delayed response of a substance is known as mandhagunam. It is otherwise defined as the quality which will subscide the vitiated doshas or which is helpful in the palliative treatment of diseases .This activity makes the drug to travel through the body for a longtime. It aggravates kapha and subscides vatham. It strengthen the tissues, does not help in the expulsion of excreta. Hence, it is seemed to be suitable to treat the condition by balancing the vitiated Pitham.

According to the Gunam of the drug also it can be seen that Asparagus racemosus is effective in the management of Neerizhivu by balancing the Doshas and enhancing the growth of the detiorated tissues.

Hence, accordingly by the present study it has been proved that the Asparagus racemosus (Thanneer vittan/Sathawari) is suitable for the management of Neerizhivu (Type 2 Diabetes Mellitus) as per the basic concepts of Siddha medicine.

So, the quotation mentioned in the text Kunapadam Porutpanpunool (Part I) by K.S.Murugesamuthaliyar [26] related to the Anti-diabetic action of tuberous root of Asparagus racemosus is scientifically and theoretically proved by the present study.

CONCLUSION

On the basis of the results and findings of the present study it can be concluded that Asparagus racemosus has potential hypoglycemic action.

The effectiveness of the hypoglycemic activity of the tuberous root of Asparagus racemosus was proved in the Animal study. The maximum hypoglycaemic activity was seen in the animals treated with the maximum dose of the test drug (80mg/150g). The plant also possess antioxidant capacity which was seen to be more in the hot water extracts when compared with that of the cold water extracts suggesting that the process of boiling the extracts increases the yield of the phytochemical constituents of the plant.

The study also has shown the presence of falvanoids and phenols in the plant extracts. The major phytochemical constituents contributing to the total antioxidant capacity of the plant was seen to be provided by the flavanoids. The tuberous root and the leaf had minimum Alpha amylase inhibitory activity. However, the whole plant exhibited a 32% of the inhibitory action. This suggests that other mechanisms would be involved with the potent hypoglycaemic action of the plant. As per the Suvai, Veeriyam, Vipakam and Gunam of Asparagus racemosus it can be seen that the plant illicit its hypoglycaemic action by balancing the vitiated Pitham and the Vatham aggravated in the initial stage of Neerizhivu. On the other hand the vipakam of the plant helps in the rejuvenation of the body. Thus, the hypoglycaemic action of Asparagus racemosus as per the Siddha literature is scientifically confirmed and proved.

Further it would be suggested that future research needed to be carried out to analyze the anti diabetic action of the plant by a clinical trial with hot water extracts. It is also suggestive to identify the other enzymatic mechanisms involved with the hypoglycaemic activity of the plant.

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