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In vitro validation of anti-diabetic and free radical scavenging property of *Naaval Kottai Mathirai*, a promising Siddha drug for the management of Diabetes mellitus

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The World Health Organization (WHO) reported 422 million total Diabetic population worldwide and about 1.5 million death rates caused due to it. Diabetes Mellitus (*Madhumegam*) is a disease classified under *Neerinai Perkukkal Noi* (diseases causing excessive urination) caused due to increased *kapam humour*. *Naaval Kottai Mathirai* (NKM) is a unique herbal Siddha drug prepared from *Aristolochia bracteolata* which has bitter taste and *Syzygium cumini* which has astringent taste. The herbal formulation NKM neutralizes *kapam* and hence effective in *Madhumegam*. *In vitro* studies like alpha-amylase, alpha-glucosidase inhibitory assays were performed to prove the anti diabetic effect of the drug NKM. DPPH (2,2- Diphenyl 1-2 picrylhydrazyl) assay, hydrogen peroxide scavenging assay (H_2O_2) and nitric oxide radical scavenging assay were done to evaluate its antioxidant potency. The results showed that minimum alpha amylase inhibitory activity ranged from 19.42±5.92 to 60.92±3.98%, with an IC_{50} value 70.9±12.37 µg/mL, when it is compared with standard drug acarbose, which showed inhibition ranged from 39.83±2.18 to 91.08±5.43 with an IC_{50} value 13.29±1.95 µg/mL in various concentration levels of NKM. The IC_{50} value of Butylated Hydroxyanisole (BHA) was noted as 48.61±1.69 µg/mL and NKM as 67.19±7.81 µg/mL for the H_2O_2 scavenging antioxidant activity. In nitrogen scavenging antioxidant activity, it was observed that IC_{50} value of gallic acid was 23.02 µg/mL and NKM was 79.13 µg/mL. In DPPH scavenging activity, for ascorbic acid, the standard showed its IC_{50} value as 13.36 µg/mL and NKM as 53.39 µg/mL. The phytoconstituents present in NKM may enrich the antidiabetic and antioxidant properties of the drug.

Keywords: Anti-diabetic assay, Anti-oxidant assay, *Naval Kottai Mathirai*, Siddha medicine

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WHO reported a rise in the use of traditional medicinal plants upto 80% in developing countries to cure various ailments[1]. International federation report says that 0.42 billion of world population is suffering from Diabetes Mellitus (DM) and the number may increase to 1.55 fold by 2040. The lack of physical exercise, sedentary lifestyle, obesity, and over eating leads to Type II diabetes Mellitus. The estimate of huge increase in prevalence of worldwide DM is expected to increase from 2.8% in 2000 to 4.4% in 2030. DM is a serious health threat to our mankind. The cost of living, treatment cost and the mortality rate are very high in DM as compared to other non communicable diseases. DM affects both carbohydrate and lipid metabolism and hence it is classified under metabolic disorder[2].

Medicinal plants have effective ‘antimicrobial’, ‘antidiabetic’, ‘antioxidant’ activities because of their phytochemicals present in them and hence they are responsible for their therapeutic efficacy in treating diseases[3]. Phenolic compounds present in herbal resources exhibit their hypoglycemic effect by reducing the conversion of starch to sugar, detaining sugar absorption and by decreasing the hydrolysis of starch[4]. Thus, most of the herbs react against the enzymes responsible for hyperglycemia and hence reduce the postprandial sugar level. The herbal medicines assure delaying of the patients from developing secondary complications of DM[5]. Approximately 800 ‘antidiabetic’ plants were explored and these plants were proved for their safety and purity for human consumption[6]. The plant drugs may reduce the plasma glucose level through direct pathways like increasing the glucose uptake activity

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and insulin secretion and through indirect pathways by reducing inflammation and oxidation in the cells[7].

In vitro assays claimed the antidiabetic potential of a drug when it inhibits alpha-amylase and alpha-glucosidase and prevents postprandial hyperglycemia[8].

As per the Siddha text *Kannusamiyam ennum Pathartha Guna Vilakkam - Mooligai Vakuppu*, *Naaval kottai mathirai* (NKM) is a traditional Siddha medicine which is well known for its use in the treatment of *Madhumegam*.

Materials and Methods

In vitro anti-diabetic activity

Alpha-amylase inhibitory assay

The enzyme, alpha-amylase is responsible for destruction of polysaccharides, which further breakdown into glucose and maltose thus increases the postprandial sugar[9]. Thus, when amylase is inhibited, it prevents absorption of starch from the ingested food and hence commonly called as starch blockers[10].

In this *in vitro* assay, 600 µL NKM was dissolved in 30 µL α- amylase enzyme and incubated continuously for 15 min at 37°C. The standard drug used in this assay was Acarbose because it possesses inhibitory action against both the enzymes responsible for postprandial hyperglycemia[11]. 370 µL of 2-chloro-4-Nitrophenyl- α- Maltotrioside was mixed and kept under incubation continuously for 10 min at 37°C. The result was measured through spectrophotometer and percentage of inhibition was calculated[12].

Alpha-glucosidase inhibitory assay

The enzyme alpha-glucosidase degrades the complex carbohydrates into smaller particles and the postprondial sugar level. This enzyme is inhibited by various substances like Acarbose, Miglitol and Voglibose which in turn control postprandial hyperglycemia. Cost of drugs spent by diabetic people worldwide particularly in developing countries is too high. Hence the researchers are working in finding a new drug from plant products to reduce postprandial sugar level by inhibiting this enzyme which may be cost effective[12].

5 µL of NKM was mixed with 20 mM p-nitrophenyl α-D glucopyranoside and 100 mM phosphate buffer (PH-7.0). The above mixture was pre-incubated continuously for 5 min at 37°C. 250 µL of the solution was mixed with 10 mL phosphate buffer (PH-7.0) and it was kept under incubation continuously for 15 min at 37°C. After adding 1000 µL of 200 mM sodium carbonate, the absorbance was determined using UV visible spectrophotometer[13].

In vitro anti-oxidant assays

The phytoconstituents like ascorbic acid, flavonoids and vitamin-C attributes to the protective activity of the plant and enhance the antioxidant property[14]. Studies showed that antioxidant property of certain plants contribute for reducing the complications of chronic illness[15]. Antioxidants play important role as defense factors, fight against free radicals and hence minimizes the oxidative damage. If more free radicals are formed, then the second line of defense mechanism exhibited by vitamins C, E and A destroys the free radicals[16].

2,2-Diphenyl-1-picrylhydrazyl assay

In 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay, the stable free radical DPPH has an affinity for electrons or hydrogen radicals and then transformed into a stable diamagnetic molecule. The ethanol extract of the sample was combined with a stock solution (100 µg/mL or 10 mg/100 mL), and aliquots of 1 mL, 2 mL, 4 mL, 6 mL, 8 mL, and 10 mL were drawn from the stock solution into separate test tubes. Subsequently, serial dilutions were performed using the same solvent, resulting in final volumes of 10 mL per test tube, with concentrations of 10 µg/mL, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, and 100 µg/mL, respectively.

Ascorbic acid was used as standard in this assay. Ascorbic acid in equivalent concentrations was mixed with methanol, by following the same method as sample extract preparation method. In each sample solution of varying concentrations 2.5 mL was taken approximately. A reaction mixture was prepared finally, consisting of 1 mL of 0.3 mM DPPH methanol solution, and allowed to react at room temperature. After 15 min incubation period at 37°C, absorbance readings were taken at different concentrations of the sample (10 µg/mL, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL) using a double-beam UV spectrophotometer, with methanol serving as the blank.

The percentage of free radical scavenging property was calculated using the formula:

$$\text{Percentage of scavenging} = \frac{\text{Absorbance of Ascorbic acid} - \text{Absorbance of NKM}}{\text{Absorbance of Ascorbic acid}} \times 100$$

Hydrogen peroxide scavenging assay

Hydrogen peroxide (H_2O_2) generates hydroxide (OH^-) radicals during decomposition, which can trigger lipid peroxidation and causes DNA damage within the body. Assessing the ability of a test drug to scavenge H_2O_2 is crucial in understanding its potential protective effects. A solution containing approximately 40 mM hydrogen peroxide was prepared in a 50 mM phosphate buffer with a pH of 7.4. Using a spectrophotometer at 230 nm, the concentration of hydrogen peroxide was determined through absorption. The sample extract of the drug NKM, prepared at various concentrations, was introduced to the H_2O_2 solution, and absorbance readings were taken after 10 min incubation period against a blank solution (phosphate buffer without hydrogen peroxide) at 230 nm.

Nitric oxide radical scavenging assay

In nitric oxide radical scavenging assay, serial dilutions were performed on the test sample ranging from 10-100 μg/mL, as well as on the standard gallic acid. 1% sulphanilamide and 0.1% naphthylethylene diamine dihydrochloride were taken in equal quantities in 2.5% phosphoric acid and mixed to prepare the Griess reagent. Sodium nitroprusside in phosphate-buffered saline (PBS) was mixed with 1 mL of the sample extract at various concentrations (10-100 μg/mL) and incubated for 180 min at 25°C. Freshly prepared Griess reagent was then added in equal volume to the test sample. Control samples were treated similarly, using an equal volume of buffer. Absorbance results were taken through Spectra Max plus UV-Vis microplate reader at 546 nm. Gallic acid was the positive control. The percentage inhibition of the test drug NKM and the standard was calculated and recorded.

2,2′-Azino-Bis 3-ethylbenzothiazoline-6-sulfonic acid assay

2, 2′-Azino-Bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay was carried out to screen the antioxidant property of NKM. The solution was made with 88 μL of 140 mM Potassium persulfate in 5 mL of 7 mM ABTS. Free radicals generated were diluted in 1:44, v/v of water. 100 μL ABTS reagent was mixed in 100 μL of NKM in various concentrations (10-100 μg/mL) and kept under incubation. In this study, the control was methanol and the positive control was Gallic acid[19].

Results

NKM exhibited 19.42 to 60.92% alpha amylase inhibition effect with IC_{50} value 70.9 μg/mL and the standard drug has 39.83 to 91.08 inhibition and IC_{50} value 13.29 μg/mL (Fig. 1). The alkaloids, phenols, terpenoids or glycosides present in NKM may contribute the medicine as a potential alpha amylase inhibitor. NKM has 54.53% inhibition against α-glucosidase at 100 μg/mL concentration with IC_{50} 87.17 μg/mL and the standard has 85.39% inhibition with IC_{50} 34.48 μg/mL (Fig. 2).

The oxidative stress produced due to various factors causes insulin resistance, enzyme damage and cellular damage[20]. Antioxidants fight against the free radicals which may complicate the diseases like cancer, diabetes. These antioxidants eliminate the free radicals and strengthen the immune system. In DPPH anti oxidant assay, the scavenging effect of Ascorbic acid was 90.07% and study drug was 73.64% (Fig. 3). IC_{50} value was calculated, it is found that, Ascorbic acid has 13.36 μg/mL, while that of the NKM was 53.39 μg/mL. The phenols present in NKM donate electron to H_2O_2, and hence neutralize it to water. The result confirmed the antioxidant activity of NKM by scavenging the BHA as 78.63% and NKM as 69.15% (Fig. 4). BHA has IC_{50} 48.61 μg/mL, and NKM 67.19 μg/mL. The free radical scavenging effect of Gallic acid was 88.9% and NKM 60.4% (Fig. 5). Gallic acid has IC_{50} 23.02 μg/mL and NKM 79.13 μg/mL. ABT s assay also gave well anti oxidant results (Fig. 6).

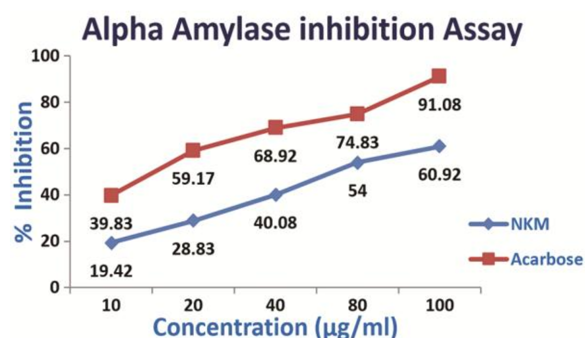

Fig. 1 — Alpha-Amylase inhibitory effect of *Naaval kottai Mathirai*

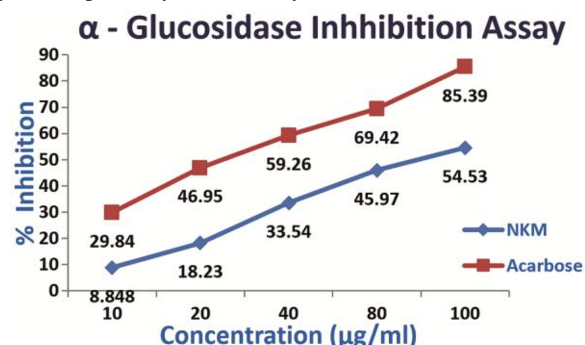

Fig. 2 — α- Glucosidase inhibitory effect of *Naaval kottai Mathirai*

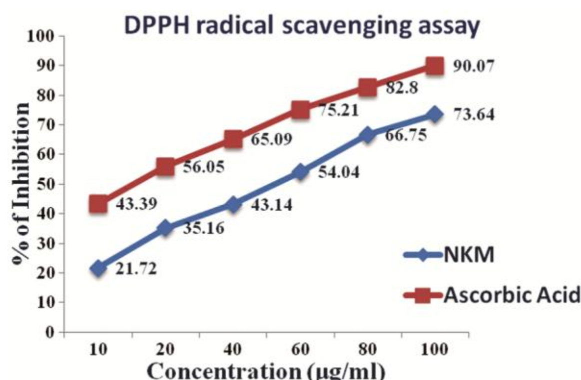

Fig. 3 — DPPH radical scavenging effect of *Naaval kottai Mathirai*

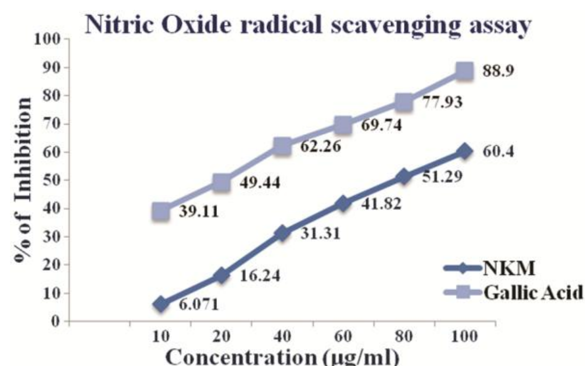

Fig. 5 — Nitric oxide scavenging effect of *Naaval kottai Mathirai*

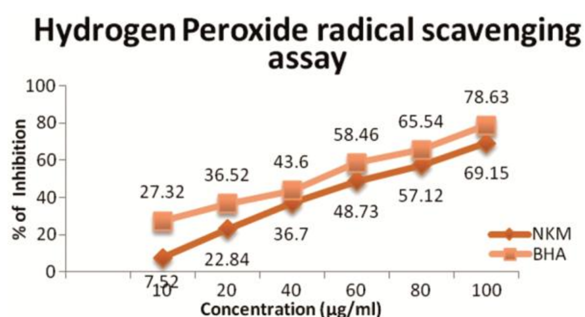

Fig .4 — Hydrogen peroxide radical scavenging effect of *Naaval kottai Mathirai*

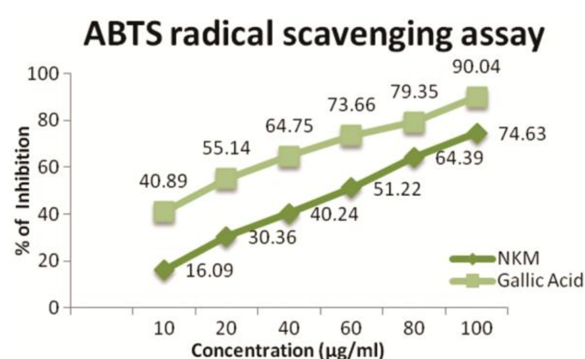

Fig. 6 — ABTS radical scavenging effect of *Naaval kottai Mathirai*

Discussion

Siddha medicines are scientifically validated nowadays to prove the Siddha philosophy, *thirithoda* theory, five elements theory and taste theory in it. WHO realized the vast usage of high quantity of medicinal plants to treat various major ailments like diabetes[21]. Type 2 DM patients relay on the oral hypoglycemic drug which had exhibited many side effects in prolonged use and hence the treatment pattern is shifted to combination therapy[22]. But nowadays amylase inhibition is attained by the phenolic compounds present in many medicinal plants hence they render huge contribution in treating Type 2 diabetes[10]. The aerobic cell metabolism is strongly associated with formation of free radicals. These free radicals are removed by the antioxidant property of phenolic compounds through various mechanisms[10].

In non communicable diseases such as cancer, diabetes, asthma, dementia, parkinsons disease, the free radicals increase the oxidative damage and hence many extracts with the high phenols and flavonoids act as antioxidants[23]. Flavanoids and phenoic acids play a salient role in removing free radicals and perishing of peroxides[24]. Therapeutic efficacy of a plant drug depends on the bioactive compounds like alkaloids, flavanoids, glycosides and steroids[25]. The *in vitro* anti-diabetic effect of NKM was screened through alpha-amylase inhibition method. The study results proved the moderate effect of inhibition 60.9% was observed in NKM against alpha-amylase and the standard as 91.8% which confirmed its effect on diabetes. α-glucosidase inhibitors help in decreasing the disaccharide hydrolysis and hence contribute on controlling of glycemic index in DM patients.

The carbohydrate enriched food results in immediate rise in plasma glucose level due to intestinal absorption of glucose by α-glucosidase and its inhibitors act against this by driving the postprandial glucose and thus maintain the blood glucose level[26]. The NKM was proved to inhibit alpha-glucosidase with moderate effect as 54.53% and 85.39% inhibition was noted in standard. 55 to 82% inhibition was observed in Acarbose in various literatures which coincide with our estimate[11].

Hence NKM is an effective drug for inhibiting α-amylase and α-glucosidase. Anti oxidant activity of NKM was proved through Nitric Oxide, Hydrogen Peroxide, ABTS radical scavenging and DPPH methods.

In previous studies, NKM has been validated for its cytotoxicity and glucose uptake activity in L6 cell

lines[27]. Safety studies of NKM was also done. In acute and 28 days repeated oral toxicity studies, no abnormalities or lesions were reported in the vital organs and so it was claimed as a safe drug for human consumption[28].

Conclusion

NKM is herbal preparation used to treat DM in Siddha system of Medicine. It is a very cost effective drug, it also has many scientific evidences to prove its efficacy in DM. The present study also confirmed its efficacy in diabetes through α-amylase and α-glucosidase inhibiting assays and anti-oxidant assays. Further clinical trials are needed to develop this herbal based anti-diabetic drug.

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Conflict of Interest

Authors declare that there is no conflict of interest.

Author Contributions

SS- Core concept, medicine preparation and the technical work, LJ– Article writing, RMK-Supervision of the work and writing

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