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Evaluation of *in vitro* antioxidant and nephro-protective activities of aqueous extract of *Aragwadadi yogam*

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Abstract
In the present study, we have analyzed the nephro-protective and antioxidant activity of *Aragwadi yogam* using *in vitro* models. The extract of *Aragwadi yogam* showed safety upto 25 µg/ml and also exhibited 72.54% inhibiting of aldose reductase at high concentration of 200 µg/ml. Physico-chemical properties of *Aragwadadi yogam* extract revealed high extract yield (4.53%) and nearly neutral pH of 6.76 (Table 3). Water solubility of the extract is found to be high (78.58%) and also the total phenolic content is 559.68 mg GAE/100 g). Antioxidants activity of *Aragwadadi yogam* extract in terms of radical scavenging activity against DPPH (IC-50 537 µg/ml), superoxide (IC-50 1355 µg/ml), hydrogen peroxide (IC-50 164 µg/ml) and hydroxyl radicals (IC-50 308 µg/ml). Hence *Aragwadadi yogam* could be explored as natural antioxidant and nephro-protective agent.

Keywords: *Aragwadadi yogam*, Nephro-protective, antioxidant activity

1. Introduction
Diabetes mellitus is a chronic metabolic disease which has several complications including diabetic nephropathy, diabetic neuropathy, coronary heart disease and hypertension [1]. The worldwide prevalence of diabetes have increased from around 60 million in 1980 to about 118 million in 1995 and are set to increased to 220 million by the year 2010 [2]. According to the International Diabetes Federation (IDF), Diabetes affects at least 285 million people worldwide and that number will be expected to reach 471 million by the year 2035 [2].
Diabetic nephropathy is a kidney disease caused due to diabetic complication. Uncontrolled diabetes can damage minute blood vessels and the functioning unit of the kidneys and nephrons. This makes the kidneys unable to remove wastes from the body leading to temporary or permanent kidney failure. High levels of blood sugar make the kidneys filter more blood.
Aragwadi yogam consists three herbal ingredients are bark of *Cassia fistula* (*Aragwada*), *Salacia reticulata* (*Ekanayakam*) and *Strychnos potatorum* (*Kataka*). *Aragwada* is a deciduous tree, 6 to 9 m tall with bright yellow flowers in long pendulous racemes, and long cylindrical blackish-brown pods of 25 to 50 cm in length and up to 3 cm in width, found wild and also commonly planted as ornamental tree in most parts of the country up to an altitude of 1200 m. Macroscopically drug occurs in flat or curved thick pieces; outer surface smooth to rough with warty patches; greenish-grey to red; inner surface rough, reddish with parallel striations, fracture, laminate; odour, characteristic taste is astringent. Bark powder have lignified fibres associated with crystal fibres; sieve tubes, many, well-developed, numerous stone cells, thick walled, lumen nearly absent; abundant prismatic crystals of calcium oxalate mostly present singly in a cell and also as numerous crystal fibres; starch grains mostly simple, 2 or 3 in compound grains [3].
Salacia reticulata is a climbing shrub with blackish and prominently lenticellate. Leaves are coriaceous, ovate, reticulate 6-9 x 4–5 cm in size. Small flowers are in each axillary short cymes. Calyx is lobed, short and obtuse. Petals are broad at the base, imbricate, greenish yellow, oblong, 0.5 mm long, apex acute and curved. The roots are acrid, bitter, thermogenic, useful in gonorrhoea rheumatism and skin diseases [5] astringent and anti-inflammatory [6]. They are also useful in vitiated disorders of diabetes, haemorrhoids, gonorrhoea skin diseases and obesity. There are reports indicating that mug made of the wood of this species is used to drink water by diabetic patients [7].

This plant is scientifically investigated for hypoglycemic activity and alpha-glucosidase inhibition activities [8], free radical scavenging activity [9] and anti-bacterial activity [10].

Strychnos potatorum, family Loganiaceae, also known as clearing nut tree is deciduous tree which has height up to 40 ft. The seeds of the tree are commonly used in traditional medicine for purifying water. Also it is used in Ayurveda, Unani, Siddha and folk medicine for treating several ailments including microbial infections, diarrhoea and diabetes. Kataka (*S. potatorum*) is one of those drug which is given in treatment of Prameha (Diabetes) but not widely used by vaidyas. Nighantu Ratnakar Kayadeva Nighantu and Shaligrama Nighantu have mentioned use of this single dravya for Prameha. *S. potatorum* is a medium-sized, glabrous tree of height 12 - 13 m. It is found in Bengal, Central and plentiful in Southern India. Seeds contain no strychnine but Brucine is present. Seeds are alternative tonic, stomachic and demulcent and they are non-poisonous. Seeds are used to clarify foul and muddy water. According to Ayurveda, seeds are used in various diseases like Prameha, Netravikaram (eye disease) and Mutra vaha strotovikaram (urinary problems).

2. Materials and Methods

2.1 Preparation of extract

Aragwadadi yogam powdered sample (10 g) was taken with 100 ml of distilled water and kept on shaker for 3 h. The extract was then separated by filtration using Whatman No. 1 filter paper and then the filtrate was lyophilized and dry extract was obtained. The extract was then re-dissolved in water at 1 mg / ml ratio and used for further experiments.

2.2 Nephro-protective activity

The kidney cell line (Normal Rat Kidney, NRK-52E) was obtained from American type of cell culture and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing modified to contain 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. The cells were maintained in Minimal Essential Media (MEM) supplemented with 10% Fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100µg/ml) in a humidified atmosphere of 5% CO2 at 37 °C. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week. Cell viability assay was evaluated by the MTT reduction assay [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] [11]. The monolayer cells were detached and single cell suspensions were made using trypsin-ethylene diaminetetraacetic acid (EDTA). A hemocytometer was used to count the viable cells and the cell suspension was diluted with a medium containing 30 mM glucose and 1% FBS in order to obtain final density of 1x105 cells/ml. 96-well plates at plating density of 10,000 cells/well were seeded with one hundred microlitres per well of cell suspension and incubated for cell attachment at 37 °C, 5% CO2, 95% air and 100% relative humidity. Aliquots of 100 µl of different concentrations of plant extract (25, 50, 100 and 200µg/ml) dissolved in DMSO (1%) were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations for 48h at 37 °C, 5% CO2, 95% air and 100% relative humidity. After 48h of incubation, to each well 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) phosphate-buffered saline solution was added and incubated at 37 °C for 4 h. Then, 100µl of 0.1% DMSO is added to each well to dissolve the MTT metabolic product. Then the plate is shaken at 150 rpm for 5 min. Viable cells were determined by the absorbance at 570nm. The medium without samples served as control and triplicate was maintained for all concentrations. The effect of the samples on of NRK-52E was expressed as the % cell viability.

2.3 Determination of aldose reductase activity

For the determination of the aldose reductase inhibitory activity of the different concentration of sample in test tube was taken containing 0.7 mL of phosphate buffer (0.067 M), 0.1 mL of NADPH (25×10−5 M), 0.1 mL of cell suspension, 0.1 mL of DL-glyceraldehyde (substrate) (5×10−4 M) and final volume was made to 1 mL. Absorbance was taken against a reference cuvette containing all components but not DL-glyceraldehyde. The final pH of the reaction mixture was adjusted to the pH 6.2. When substrate was added to the solution mixture, enzymatic reaction started, and absorbance (OD) was recorded at 340 nm for 3 min at 30 sec interval. Aldose reductase activity was calculated and expressed as % of inhibition [9].

2.4 Total phenol content

The total phenolic content of extract was estimated according to the method of Singleton *et al*. [12] Suitably diluted sample (100 µl) was taken with 250 µl of Folin's-Ciocalteu reagent and 1000 µl of 5% of Na2CO3 was added and incubated for 30 min in dark. Then the absorbance was measured at 720 nm using Spectrophotometer. A calibration curve was prepared using standard gallic acid (16 – 100 mg/L; y = 0.0094x – 0.0585; R2 = 0.9939) and used to calculate the total phenolic content of the extract and the results were expressed as gallic acid equivalents (mg GAE / 100 g sample).

2.5 DPPH radical scavenging activity

The DPPH radical scavenging assay was used to analyze the antioxidant property of aqueous extract of the sample by following Sanchez-Moreno *et al*. [13] method. Different concentrations (2000, 1000, 500, 400, 250, 125, 62.50, 31.25, 15.63, 7.81 and 3.91 mg / L) of the extract (100 µl) was added with 0.9 ml of methanolic solution of DPPH (2.5 mg / 100 ml) and incubated at room temperature for 30 min in dark. Different concentrations of Butylated hydroxyanisol (BHA) were used as a standard and the solvent (distilled water) was used instead of extract in control. After 30 min, the absorbance was measured at 515 nm using a spectrophotometer and the radical scavenging activity of the extract was calculated and expressed on percentage basis.

2.6 Superoxide radical scavenging activity

The capacity of extract to scavenge the superoxide anion radical was measured according to the method described by Zhishen *et al*. [14]. The reaction mixture was prepared using 3 x 10^{-6} M riboflavin, 1 x 10^{-2} M methionine, 1 x 10^{-4} M nitroblue tetrozolium chloride and 0.1 mM EDTA in phosphate buffered saline (pH 7.4). For the analysis, 3.0 ml of the reaction mixture was taken with 100 µl of extract in closed tubes and illuminated for 40 min under fluorescent lamp (18 W). The absorbance was then read at 560 nm against the un-illuminated reaction mixture. Results are expressed as superoxide radical scavenging activity on percentage basis.

2.7 Hydrogen peroxide scavenging activity

The effect of extract on hydrogen peroxide was analyzed according to the method proposed by Ruch *et al*. [15] The extract (100 microliter) was mixed with 5 ml of 45 mM

hydrogen peroxide solution in 0.1 M phosphate buffer (pH 7.4). The reaction mixture was vortexed and incubated for 30 min at room temperature and then the absorbency was measured at 230 nm. The extract with phosphate buffer is used as a blank and the level of hydrogen peroxide remaining in the solution was calculated using a calibration curve. The hydrogen peroxide inhibition effect of extract was calculated and expressed on percentage basis.

2.8 Hydroxyl radical scavenging activity

The hydroxyl radical quenching activity of extracts was evaluated according to the method of Hagerman *et al*. [16] The reaction mixture consists of 10 mM phospate buffer (pH 7.4), 2.8 mM Deoxyribose, 2.8 mM H2O2, 0.025 mM FeCl3, 0.1 mM EDTA and 0.1 mM ascorbic acid in a total volume of 3 ml. With the reaction mixture, 100 microliter of extract was added and incubated at 37 ℃ for 15 min. Then the reaction was terminated by the addition of 1 ml of 2.5% ice-cold TCA and 1% TBA. The reactants were mixed well and heated at 90 C for 15 min in a water bath and cooled to room temperature. The chromogen was extracted with 1-butanol and absorbency was measured at 530 nm. Based on absorbency value, the hydroxyl radical scavenging activity of extracts was calculated and expressed on percentage basis.

3. Results and Discussion

Diabetic nephropathy (DN) is a chronic and one of the most serious complications of diabetes. Aldose reductase (AR) pathway is one of the major mechanisms involved in the pathogenesis of DN. Activation of AR has been shown to induce osmotic and oxidative stress in diabetic kidney. Involvement of AR in the activation of endoplasmic reticulum (ER) stress, another pathophysiological alteration observed with DN. Currently, many AR inhibitors (e.g. sorbinil, alrestatin, tolrestat and ponalrestat) have been shown to be effective in the prevention or reversal of DN in animal models, warranting more-focused and dedicated future clinical investigations (Mylari *et al*., 2005).

Cytotoxicity of *Aragwadadi yogam* investigated in normal kidney cell line NRK-52E showed it is safe at low concentration of 25 µg/ml (Table 1). But the extract of *Aragwadadi yogam* is showing high inhibiting of aldose reductase at high concentration of 200 µg/ml (72.54%) (Table 2).

Table 1: Effect of varying concentrations of *Aragwadadi yogam* on cell viability of NRK-52E (Normal rat kidney-NRK) cell lines as determined by MTT assay

S. No.	Concentrations (µg/ml)	Cell Viability (%)
	Control	100
1.	25	92.50
2.	50	86.54
3.	100	80.45
4.	200	74.85

Table 2: Effect of varying concentrations of *Aragwadadi yogam* on aldose reductase activity in NRK-52E cell lines

S. No.	Concentrations (µg/ml)	% of inhibition
1.	25	18.25
2.	50	35.20
3.	100	54.87
4.	200	72.54

Physico-chemical properties of *Aragwadadi yogam* extract revealed high extract yield (4.53%) and nearly neutral pH of 6.76 (Table 3). Water solubility of the extract is found to be high (78.58%) and also the total phenolic content is 559.68 mg GAE/100 g).

Table 3: Physico-chemical properties of aqueous extract of herbal formulation.

S. No.	Physico-chemical properties	Solvent extract
1	Colour	Light Brown
2	Odour	Characteristic
3	Extract yield (%)	4.53 ± 0.26
4	pH	6.76 ± 0.39
5	Water solubility (%)	78.58 ± 2.45
6	Total phenolic content (mg GAE/100 g)	559.68 ± 68.93

Antioxidants can reduce DPPH through hydrogen transfer into its non-radical form (DPPH-H) and hence the absorption disappears at 515 nm. The decrease in absorbency at 515 nm may be due to the reaction between phytochemicals and DPPH, which indicates the antioxidant power. The *Aragwadadi yogam* extract showed high level of DPPH inhibition potential with a IC-50 value of 537 µg/ml) (Figure 1A).

The superoxide radical scavenging activity of samples was investigated by generating superoxide through photo-induced reduction of riboflavin, which can generate superoxide radical in the presence of methionine. The generated superoxide radical reduce the NBT into purple colour formazan, which was measured at 560 nm. In presence of antioxidant, the generated superoxide radicals were scavenged and hence, formation of purple colour formazan is minimum or nil. The superoxide inhibition capacity of *Aragwadadi yogam* extract was 45% (IC-50 1355 µg/ml) (Figure 1B).

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. Thus, removing hydrogen peroxide is very important for protection of cellular system. The hydrogen peroxide can decompose into water by accepting two electrons and protons. The level of hydrogen peroxide in buffer solution can be detected spectrometrically at 230 nm. If antioxidants (electron donors) are added to the reaction mixer, they can accelerate the conversion of hydrogen peroxide into water. *Aragwadadi yogam* extract revealed 73% of inhibition of hydrogen peroxide (Figure 1C).

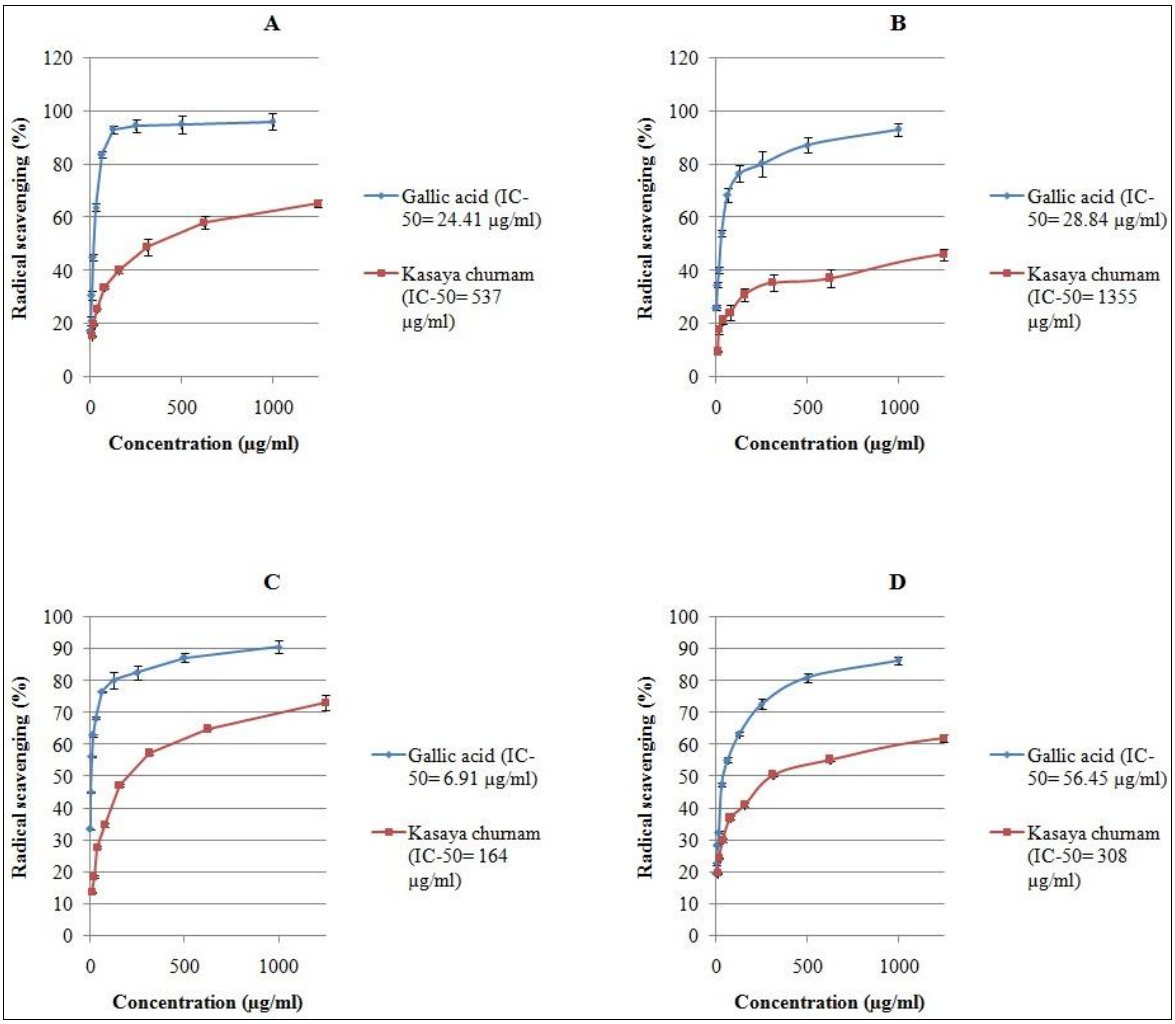

Fig 1: Antioxidant activity of *Aragwadadi yogam*: (A) DPPH radical scavenging activity, (B) Superoxide radical scavenging activity, (C) Hydrogen peroxide scavenging activity and (D) Hydroxyl radical scavenging activity.

Hydroxyl radicals are produced by the Fenton reaction between Fe(II)-EDTA and hydrogen peroxide. The hydroxyl radicals (OH.) degrade Deoxyribose and produce MDA, which can be measured by TBARS reaction. The TBA can react with MDA in acidic medium to form pink colour chromogen, which could be extracted with 1-butanol and read at 530 nm. OH radicals may attack various biomolecules including proteins, lipids, and DNA and cause oxidative damage to the cellular components and hence it is considered to be biologically dangerous free radical. The hydroxyl radical inhibition activity of *Aragwadadi yogam* extract was found to be 61% (IC-50 308 µg/ml) (Figure 1D).

4. Conclusions

NRK cells exposed to high glucose (30 mM and 1% FBS) for 48 h showed the decreased in viability with progressive decline after 48 hours and the inhibition of aldose reductase activity exhibited by the extract is beneficial to Diabetic nephropathy patients. The present study revealed the high antioxidant potential of *Aragwadadi yogam* extract in all the radical scavenging assays, which will be useful in using this extract in the management of diabetic nephropathy.

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