International Journal of Herbal Medicine
Available online at www.florajournal.com

IJHM International Journal of Herbal Medicine

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2015; 3(3): 01-05
Received: 16-05-2015
Accepted: 14-06-2015

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Antioxidant potential of Shivanarvembu Kuzhi Thailam

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Abstract
Shivanar VembuKuzhi Thailam (SVKT) is a poly herbal sidhha drug used for the skin care ailments. The aim of the current study is to evaluate the antioxidant potential of SVKT in the cell free systems. The antioxidant radical scavenging effect of SVKT was evaluated against the free radicals like DPPH radical, superoxide radical, hydroxyl radical, nitric oxide radical, non-enzymatic glycosylation of hemoglobin. The total phenols and the flavonoids were quantified in SVKT. From the results, it can be concluded that, the SVKT exerts the excellent antioxidant potential due to the presence of the polyphenols and flavonoinds.

Keywords: Antioxidant, potential, Shivanarvembu Kuzhi Thailam

1. Introduction
Skin being the outermost organ of the body, is exposed to various pollutants like chemicals and UV radiations. This exposure results in the cellular level insults with the increased production of various types of free radicals. Thus results in the imbalance of free radical to antioxidant ratio in the cell, which induces the the free radical induced damage of the macromolecules such as DNA, Proteins and Lipids [1]. The macromolecular impairs impacts the healthy functioning of the organ. In order to address the free radical induced damage to skin, a poly herbal drug Shivanar Vembu Kuzhi Thailam (SVKT) formulated by siddharthirumoolar has been taken and scientifically validated in this study by evaluating its antioxidant potential.

1.1 Poly Herbal Ingredient Description
It's a polyherbal formulation containing three herbs namely Indigofera aspalathoides (whole herb), Corallocarpus epigeous (tuber) and Celastrus paniculatus (Seeds).

1.1.1 Indigofera aspalathoides
It is popularly known as Shivanarvembu in Ayurveda and siddha system of medicine. It belongs to the family Fabaceae. It is abundantly found in the southern part of India. The scientific literature reveals that the plant is known for the treatments like anti-inflammatory, ant arthritis and carcinogenesis. The phytochemical actives reported in the plant is Indigocarpan, specific to the species [2].

1.1.2 Corallocarpus epigeous
The plant is called as Akashagaruda in Tamil and siddha system of medicine. It belongs to the family Cucurbitaceae. The Rhizome of the plant is known for the ailments like syphilitic rheumatism, veneraldisprders and chonic dysentery. It is also used as an anti-snakedote. It is mainly seen in the deccan and southern regions of India. The pharmacological activities reported for the species are analgesic, anti-pyretic and anti-inflammatory too [3].

1.1.3 Celastrus paniculatus
The plant is called as Valuluvaiarisi in siddha medicine. It belongs to the family Celestraceae. The plant is rich in the sesquiterpene esters namely Celapanin, Celapanagin and Celapagin. The seeds and its oil is bitter, acrid and thermogenic. It is mainly used as a brain tonic, appetizer and treatment for skin disorders. Scientific literature suggests that the plant is an antibacterial, antimalarial, analgesic, antiarthritic agent [4]. SVKT is siddha herbal drug used for most of the skin care ailments. SVKT is prepared with the principle of destructive

distillation process. The drug is used topically on the skin to cure many skin related disorders [5]. This SVKT is prescribed as a carrier matrix for many of the orally taken siddha drugs. In such cases, the role of SVKT is to enhance the bioavailability of the drugs. Thus the current study aims at evaluating the antioxidant potential of the polyherbal drug SVKT.

2. Materials & Methods

The Shivanar Vembu Kuzhi thailam used in the current study was purchased from the Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd (IMPCOPS), Coimbatore.

2.1 Concentration

The concentration of the SVKT sample and the standard solutions (Rutin/ascorbate/Mannitol/Quercetin) used in the final reaction mixture of all the scavenging assays are 1000, 500, 250 and 125µg/ml.

2.2 DPPH radical scavenging Assay

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in color and upon reaction with hydrogen donor changes to yellow color. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490nm [6]. To 200 µl of DPPH solution (100µM), 10 µl of each of the test sample (20mg/ml) or the standard solution rutin (10mg/ml) was added separately. The plates were incubated at 37º C for 30 min and the absorbance was measured at 490 nm, using a microplate reader and the percentage inhibition was calculated as below.

Scavenging activity (%) = (A Control – A sample)/ A Control X 100

2.3 Superoxide Radical Scavenging Assay (Alkaline DMSO Method)

In alkaline DMSO method, superoxide radical is generated by the addition of sodium hydroxide to air saturated DMSO. The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium into formazan dye at room temperature and that can be measured at 560 nm. Superoxide scavenger capable of reacting inhibits the formation of a red dye formazan. The reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5mM NaOH in 0.1ml distilled water) and 0.3 ml of the extracts in DMSO at various concentrations or standards, 0.1 ml of NBT (1 mg/ml) was added to give a final volume of 1.4 ml. The absorbance was measured at 560 nm and the percentage inhibition was calculated [7].

2.4 Nitric Oxide radical scavenging Assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of modified GriessIlosvay reaction. In the present investigation, GriessIlosvay reagent is modified by using Naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Nitrite ions react with Griess reagent, which forms a purple azo dye. In presence of test components, likely to be scavengers, the amount of nitrites will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The absorbance of the chromophore formed was measured at 540 nm [9]. The reaction mixture contains 4ml of sodium nitroprusside (10 mM), 1 ml phosphate buffer saline (pH 7.4) and 1ml extract in DMSO at various concentrations or standard was incubated at 25 ºC for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulphanilic acid reagent (0.33% sulphanilic acid dissolved in 20% glacial acetic acid) was added, mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 ml of NEDD (0.1% Naphthyl ethylene diamine dihydrochloride in 50% glacial acetic acid) was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore was formed. The absorbance of these solutions was measured at 540 nm and the percentage inhibition was calculated.

2.5 Hydroxyl Radical Scavenging Activity

The assay is based on quantification of degradation product of 2-deoxy ribose hydroxyl radical by condensation with TBA [8]. Hydroxyl radical was generated by the Fe^{3+} -Ascorbate – EDTA – H2O2 system (Fenton reaction). The reaction mixture contained 0.1 ml deoxyribose (2.8mM), 0.1 ml EDTA (0.1 mM), 0.1 ml H2 O2 (1mM), 0.1 ml Ferrous ascorbate (0.1mM), 0.1 ml KH2PO4-KOH buffer, PH 7.4 (20mM) and various concentrations of plant extracts in a final volume of 1 ml. The reaction mixture was incubated for 1 hour at 37 °C. Deoxyribose degradation was measured as TBARS and the percentage inhibition was calculated.

2.6 Non-enzymatic haemoglobin glycosylation assay

Antioxidant activity of SVKT was investigated by estimating degree of non-enzymatic hemoglobin glycosylation which was measured calorimetrically at 520nm. Glucose (2%), hemoglobin (0.06%) and Gentamycin (0.02%) solutions were prepared in 0.01M phosphate buffer (pH 7.4). 1ml each of above solution was mixed. 1 ml of different concentrations of the sample (125-1000 µg/ml) was added to above mixture. Mixture was incubated in dark at room temperature for 72 hours. The degree of glycosylation of hemoglobin was measured colorimetrically at 520nm and the percentage inhibition was calculated [12].

2.7 Estimation of total phenols

Sample quantity of 0.25g was added with 2.5 ml of ethanol and centrifuged at 10000rpm at 20 ºC for 10 mins. The supernatant was preserved. Then, the sample was re-extracted with 2.5 ml of 80% ethanol and centrifuged. The pooled supernatant was evaporated to dryness. Then 3 ml of water was added to the dried supernatant. Following 0.5 ml of Folin's phenol reagent and 2 ml of sodium carbonate (20%) was added. The reaction mixture was kept in a boiling water bath for 1 min. The absorbance was measured at 650 nm in a spectrophotometer [11]. A standard was run by using pyrocatechol and the quantity of total phenols present in the SVKT was calculated.

2.8 Estimation of flavonoids

0.2g of SVKT extract was mixed with ethanol-water in two different ratios namely 9:1 and 1:1 respectively. The homogenate was filtered and the filtrate of the two ratios was combined. This was evaporated to dryness until most of the ethanol was removed. The resultant aqueous extract was extracted in a separating funnel with hexane or chloroform. The solvent extracted aqueous layer was concentrated. 0.5 ml of the concentrated extract was pipetted-out in a test tube; 4 ml of the vanillin reagent (1% vanillin in 70% conc. H_2SO_4) was added and kept in a boiling water bath for 15 mins. The absorbance was read at 360 nm [10]. A standard was run by

using catechin and the quantity of flavonoids present in the SVKT was calculated.

3. Results & Discussion

Reactive Oxygen species (ROS) and Reactive Nitrogen species (RNS) are the two major types of free radicals. These free radicals initiates the chain reaction and propogates to the essential macromolecules in the cells, thereby induces the damage to protein, DNA and lipids. Thereby haulting this chain reaction with a reactive molecules (Antioxidants) to yield a non-reactive end product becomes very essential. In order to escort the cells from free radical damage, scouting for the potent antioxidant becomes must. The SVKT was evaluated against various types of radicals for its scavenging potential and the results are discussed in this section in comparison to standard antioxidant bench marks.

3.1 DPPH radical scavenging results

DPPH radical scavenging assay is high throughput cum widely used method for evaluating the antioxidant activity of any molecule or extract. The antioxidant molecules preset in the SVKT donates hydrogen ions to the stable free radical DPPH· there by forms a non-reactive end product DPPH-H, thus decreases the absorbance, which is interpreted as antioxidant activity [13]. The results depicted in the Figure 1 reveals that the SVKT has the excellent antioxidant potential in scavenging the high molecular weight free radicals like DPPH. From the study we can also infer that the potential of SVKT is way better than the known standard antioxidant rutin in all the tested concentrations. This is confirmed from the IC 50 values, i.e., Rutin IC50 is 558µg/ml whereas SVKT IC50 is only 207µg/ml. of rutin.

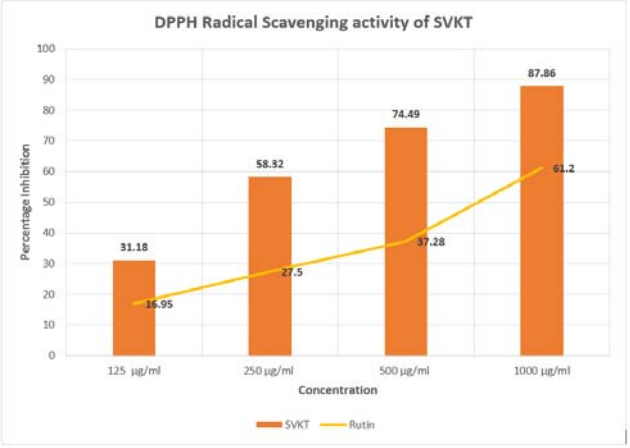

Fig 1: DPPH radical scavenging activity of Shivanar Vembu Kuzhi thailam Vs standard Rutin

3.2 Superoxide Radical scavenging results

Superoxide free radicals are the inevitable bye products of the cellular respiration. The superoxide radicals generated by the mitochondrial metabolism induces the severe damage to various organelles of the cells there by impairs the function [14]. In the current study, the generated superoxide radicals reduces the NBT there by resulting in a formazon which absorbs at 560 nm [15]. In presence of the potent antioxidants like SVKT, there will be a decrease in the absorbance. The polyherbal siddha preparation SVKT has an excellent Superoxide radical scavenging potential compared to standard compound quercetin. This can be inferred from the Figure 2. The IC50value of the SVKT against superoxie radical is 339.45µg/ml, whereas the standard flavonoid quercetin has the IC 50 concentration is double of the SVKT i.e., 645µg/ml.

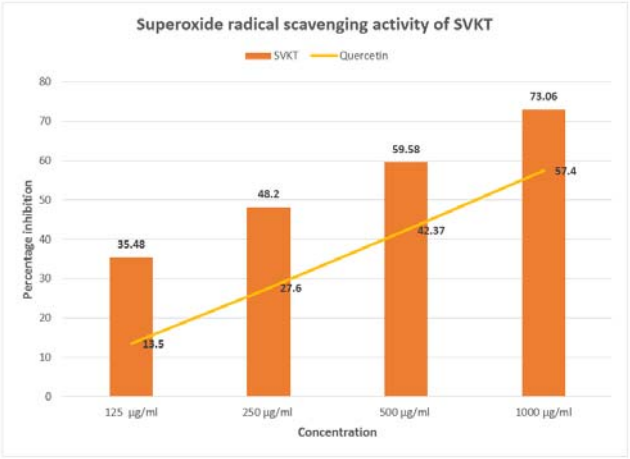

Fig 2: Superoxide Radical Scavenging activity of Shivanar Vembu Kuzhi thailam Vs Standard Quercetin

3.3 Nitric oxide radical scavenging Results

In the Physiological process such as smooth muscle relaxation, neuronal signaling and platelet aggregation inhibition, and nitric oxide plays a crucial role [18]. But imbalance in the concentration of the Nitric oxide to the cellular antioxidant defence results in the diseased condition [19]. In order to combat the nitric oxide radicals and to inhibit the nitrite ion formation, the extracts or molecules were screened. The SVKT may not be a right choice for encountering the nitric oxide radical's damage in the cells. This can be inferred from the results shown in the Figure 3. The IC50 value of the SVKT is 2000µg/ml approximately, which is very high value compared to the standard nitric oxide scavenger mannitol, whose IC50 value is 650µg/ml.

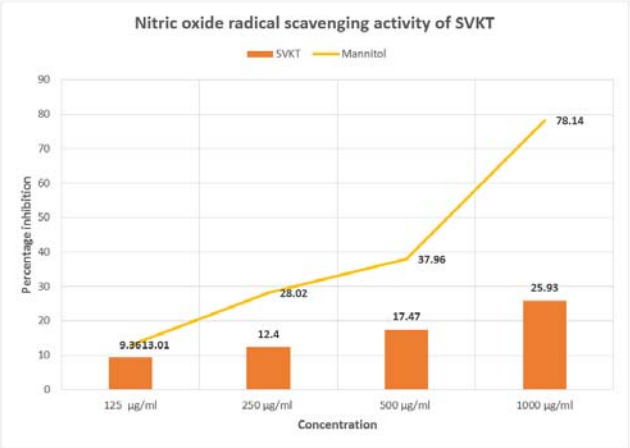

Fig 3: Nitric oxide radical scavenging activity of Shivanar Vembu Kuzhi Thailam Vs Mannitol

3.4 Hydroxy Radical scavenging results

Hydroxy radicals are the short living, but highly reactive potent radical moiety generated from the normal cellular metabolism [16]. It can induce the irreversible damage to the DNA and proteins [17]. In order to combat the hydroxyl radicals, SVKT was evaluated for the inhibition of the formation of TBARS. The hydroxyl radical scavenging potential of SVKT is not be much pronounced as the IC50 value of the standard potent antioxidant Ascorbate is

317.7µg/ml, whereas the SVKT IC50value is 735µg/ml, which is double concentration of ascorbate. From this we can infer that SVKT is not potent against the hydroxyl radicals. This is depicted in the below figure 4.

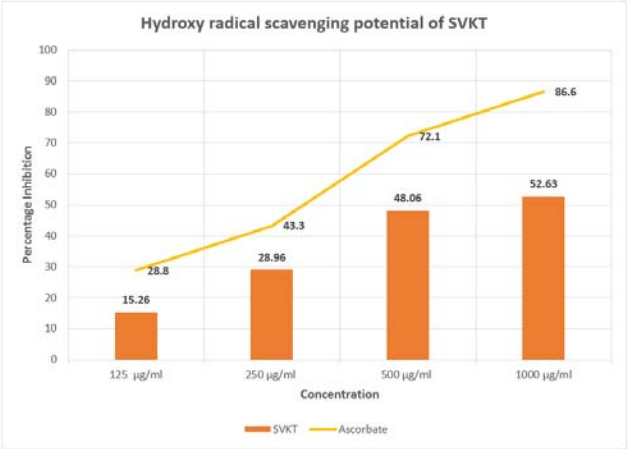

Fig 4: Hydroxy radical scavenging actitiy of ShivanarVembuKuzhi thailam Vs Ascorbate

3.5 Non-enzymatic hemoglobin glycosylation Results

Non enzymatic glycosylation of Hemoglobin is a well-known oxidation reaction. The degree of hemoglobin glycosylation is inhibited with the increase in the concentration of the potent herbal extracts. This change is monitored spectrophotometrically. The results of Non-enzymatic hemoglobin glycosylation assay results reveals that the SVKT has the potential to inhibit the hemoglobin glycosylation compared to the standard benchmark ascorbate. The results are depicted in the Figure 5. This can be inferred from the IC 50 values of Ascorbate (528µg/ml) and the SVKT (444µg/ml).

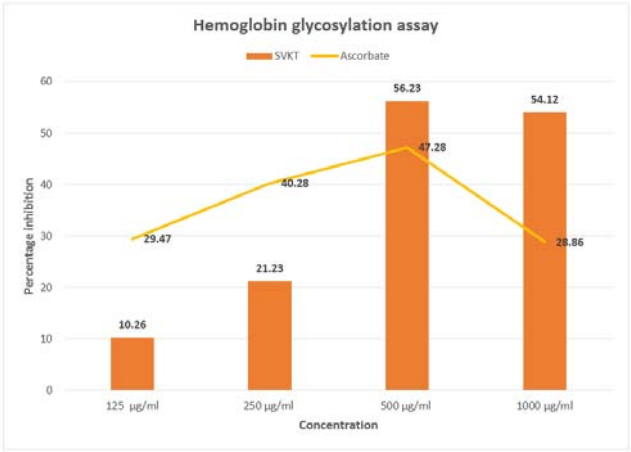

Fig 5: Non-enzymatic glycosylation inhibition potential of Shivnar Vembu Kuzhi Thailam Vs Ascorbate

3.5 Total Phenols & Flavonoids

Polyphenols and flavonoids are the potent secondary metabolites play a significant role in the defence mechanisms in plants [20]. These molecules have the high redox potential. They can donate hydrogen radicals, thereby attenuates the chain reaction of harmful free radicals [21]. These secondary metabolites, upon uptake enhances the expression of many genes in the cells and thereby maintains the healthy living of the cells as well as the individuals [22].

Table 1: Total Polyphenol content & Flavonoid content of Shivanar Vembu Kuzhi Thailam.

S.No	Parameters	Results
1	Total Phenols(mg of Pyrocatechol equivalents / g of the sample)	13.56±0.4 mg
2	Flavonoids (mg of Catechin equivalents /g of sample)	1.63±0.02mg

The total polyphenol content of the SVKT thailam was expressed as the pyrocatechol equivalents and the flavonoid content as Catechin equivalents in the above table1.

4. Conclusion

In continuation to the results mentioned in the above section, it can be concluded that the SVKT is a potent antioxidant due to the presence of polyphenols and flavonoids. Its antioxidant capacity is potent enough to encounter the high molecular weight free radicals like DPPH and also the superoxide radical. It also inhibits the glycosylation of proteins due to prolonged exposure of proteins to sugars in the diseases conditions like diabetes or free radical induced damage. Eventhough the SVKT exerts an antioxidant activity against the highly sensitive free radicals like hydroxy and nitric oxide radical, but the intensity of the activity is not better than the benchmark standards. As the results are from the in-vitro cell free systems, the activity can be studied in the cell lines and animal models to confirm the antioxidant potential of SVKT.

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