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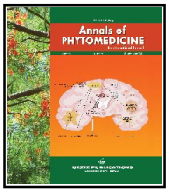

Siddha formution

In vitro free radical scavenging activity of *Nandukkal Parpam*: A herbo-mineral Siddha formulation

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Abstract

Free radical chemistry has considerable attention in recent times. At physiological condition, the imbalance between generation of reactive oxygen species (ROS) and its neutralization by antioxidants creates oxidative stress. As oxidative stress has become a crucial factor in the development of various degenerative diseases such as cancer, diabetes, hyperlipidemia, gastric ulcer, *etc*., there is a raise of interest in discovery of antioxidants from natural sources with minimal side effects. The practice of using Siddha medicine is increasing substantially owing to its excellent antioxidant nature, therapeutic ability, and cost effectiveness compared to modern medicine. *Nandukkal Parpam* (NP) is a herbo-mineral Siddha formulation, prepared from a *Nandukkal*, a fossil stone crab prescribed especially for renal calculi, urinary obstruction, prostatic obstruction of urethra, oliguria/anuria, *etc*. The aim of this study was to investigate the *in vitro* free radical scavenging potential of a herbo-mineral Siddha formulation, *Nandukkal Parpam*. Antioxidant activity assays like DPPH radical scavenging assay, superoxide radical scavenging assay, hydroxyl radical scavenging assay and nitric oxide radical scavenging assay were carried out to determine the free radical scavenging capacity of NP. The results of the above analysis evidenced the antioxidant nature of the NP by exhibiting its IC_{50} value of 120.10 µg/ml, 176.77 µg/ml, and 172.20 µg/ml for DPPH radical scavenging activity, superoxide radical scavenging activity and hydroxyl radical scavenging activity, respectively. From the results, it can be concluded that NP possess worthy scavenging potential of various free radicals. This antioxidant nature of NP elucidates the need of further *in vivo* studies to expose molecular mechanisms behind its therapeutic function.

1. Introduction

Antioxidants are natural or synthetic substances that prevents cell/tissue from oxidative damage caused by various oxidants like reactive oxygen species (ROS), reactive nitrogen species (RNS) and free radicals (Zengin, 2011). Antioxidant deactivates/scavenges, these oxidants before they damage the biological targets at physiological conditions (Nunes *et al*., 2012). Oxidative stress is a pathological condition in which the generation of these oxidants overwhelms the antioxidative defense mechanism which subsequently leads to oxidation of various macromolecules like lipids, proteins and DNA. This in turn leads to tissue injury and accelerated cellular death and paves way for pathogenesis of various diseases (Apak *et al*., 2016). Antioxidant activity/capacity measurement of various natural/synthetic compounds is performed to discover lead molecules for the treatment of various oxidative stress related diseases. Since these antioxidants have diverse biological functions like anti-mutagenic, anticarcinogenic, antiageing, anti-inflammatory, *etc*. (Gulcin, 2012; Gocer and Gulcin, 2011).

Siddha system of medicine is one of the indigenous traditional medical systems of the world. Its origin goes back to BC 5000 to BC 2000. This ancient traditional medical system was developed by a line of 18 *Siddhars* from the land of Tamil like *Agasthiyar, Thirumoolar, etc*. Siddha pharmacology (*Gunapadam*) deals with the study of Siddha drugs (raw drugs). Based on their origin, Siddha raw drugs were classified into 3 broad categories; namely, *Mooligai vaguppu* (plant and its products), *Thaadhu vaguppu* (metals and minerals) and *Jeeva vaguppu* (animals and its products) (Thiagarajan, 1968).

Nandukkal Parpam (NP) is a Siddha formulation mentioned in Siddha classical literature "*Siddha vaidhya thirattu*". It is prepared from 5 ingredients, out of which, *Nandukkal* (Fossil stone crab) is a main ingredient (Kuppusamy Muthaliyar and Uththamarajan, 1977). The other ingredients are: *Kal chunnambu* (lime stone/calcium oxide), *Poo neer* (fullers earth), *Mullangi kilangu charu* (radish juice) and *Sirupeelai chamula charu* (*Aerva lanata* L. whole plant juice). In Siddha literature, NP was indicated for various therapeutic uses like *Neeradaippu* (urinary obstruction), *Kalladaippu* (renal calculi), *Sadhaiyadaippu* (prostatic obstruction of urethra) and *Neerkattu* (oliguria/anuria). This drug was advised (200-400 mg) along with any of the following adjuvants: water

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(*thaneer*), tender coconut (*ilaneer*), *Neermulli kudineer* (decoction of *Asteracantha longifolia* L. Nees) and *Sirupeelai charu* (extract/ juice of *Aerva lanata* L.) (SFI, 2011).

The major ingredient, *Nandukkal* comes under *Uparasam* (other minerals) category of Siddha mineral drugs. The details and therapeutic usage of *Nandukkal* was elaborated in an old Siddha manuscript; namely, *Kal nandu soothiram* (Natarajan, 2015). According to this literature, *Nandukkal* has been used for the treatment of many diseases like urinary disorders, musculo-skeletal disorders, mental disorders, gastrointestinal disorders, ophthalmological diseases, venereal diseases, all types of toxic bites and all types of fever (Arunai Nambiraj *et al*., 2002).

Being such a versatile drug, *Nandukkal* might have many biological functions like antioxidant, antiurolithiatic, anti-inflammatory, *etc*. Hence, the present study was aimed at to evaluate the *in vitro* antioxidant potential of NP, the outcome of which may be first of kind of this report. The results of the study may also throw light on various cellular mechanism in which it elicits its biological/ therapeutic function.

2. Materials and Methods

2.1 Consumables

The study drug, NP was purchased from IMPCOPS, Chennai (Batch No.SII-185). Fine chemicals like curcumin, and nicotinamide adenine dinucleotide hydrogen (NADH) were procured from HiMedia Laboratories Private Limited (Mumbai, India). Ascorbic acid, PMS (*N-Methylphenazonium methosulfate)*, BHT (Butylated hydroxy toluene) were purchased from LOBA Chemie Private Limited (Mumbai, India). NBT (Nitro blue tetrazolium), Gallic acid were of Sisco Research Laboratories Private Limited (Maharashtra, India) make. All other chemicals and reagents used were of analytical grade.

2.2 *In vitro* antioxidant assays

Nandukkal Parpam was dissolved in aqueous solution at a final concentration of 1mg/ml and the following assays were done in dose dependent manner; The DPPH radical scavenging capacity of aqueous solution of NP was estimated according to Suaib Luqman *et al*. (2012) an improved version of Chung *et al*. (2002) method. Superoxide radical scavenging assay of NP was performed as mentioned by Nishi Miki *et al*. (1972) method. The drugs ability to scavenge hydroxyl radicals was assessed using the technique described by Klein *et al*. (1981). Griess reaction method (Jagetia *et al.,* 2004) was adopted for the determination of nitric oxide radical scavenging activity of NP.

2.3 Statistical analysis

All the assays were done in triplicates. The statistical analysis including IC_{50} values and graph for all the assays were generated using GraphPad Prism software (9.3.1).

3. Results

3.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

DPPH radical scavenging activity of NP was tested and compared with standard drug BHT at the concentration ranging from 200-1000 µg/ml. At high concentration (1000 µg/ml), NP showed 72.32% of inhibition. On the other hand, the standard BHT exhibits 94.55% inhibition at 100 µg/ml. The IC_{50} values were calculated from the graph and it was found to be 120.10 µg/ml for NP and 12.24 µg/ml for BHT. The above results were depicted in Figure 1.

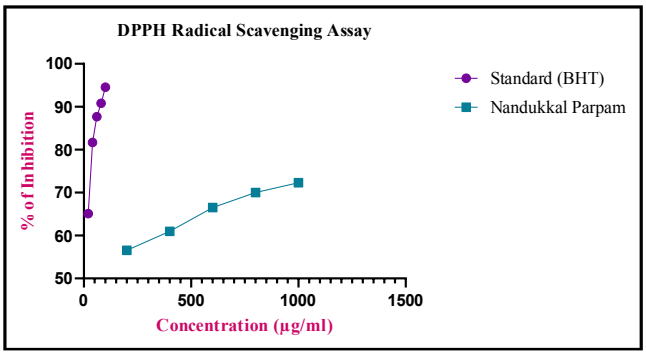

Figure 1: DPPH radical scavenging activity of *Nandukkal Parpam*.

3.2 Superoxide anion radical scavenging activity

NP was screened for superoxide radical scavenging activity and the result was presented in Figure 2**.** NP aqueous solution instigated a modest dose-dependent inhibition of superoxide anion radical with an IC_{50} of 172.20 µg/ml. Ascorbic acid was used as a reference compound and 45.27 µg/ml ascorbic acid was needed for 50% inhibition. At 100 µg/ml, the percentage inhibition of the NP aqueous solution was 87.24%, whereas that of ascorbic acid was 24.35%. The graph shows dose dependent inhibition of superoxide radicals by the test drug, NP.

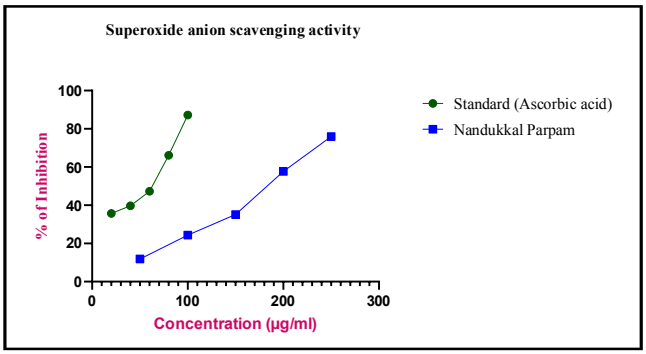

Figure 2: Superoxide anion radical scavenging activity of *Nandukkal Parpam*.

3.3 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of NP were presented in Figure 3. Hydroxyl radical scavenging activity of NP revealed that the scavenging effect varied from the minimum of 56.57% to maximum of 72.32% at the concentration ranging from 200-1000 µg, respectively. The standard gallic acid varied from the minimum and maximum inhibition of 51.78% and 73.80%, respectively at 20-100 µg with IC_{50} value of 19.31µg/ml. Mean while aqueous solution of NP has exhibited appreciable scavenging effect against hydroxyl radicals with the IC_{50} value of 176.77µg/ml.

3.4 Nitric oxide radical scavenging activity

Nitric oxide scavenging activity of NP and the standard curcumin was demonstrated in Figure 4. NP exhibited dose dependent nitric oxide scavenging activity from 0.70% to 33.72% at a very high

concentration ranging from 600-1000 µg. While standard curcumin, exhibited 8.91% to 68.80% inhibition at a very minimal about concentration of 20-100 µg. With an IC_{50} value of 1094 µg/ml, NP has weak inhibition of nitric oxide radical when compared to the standard curcumin since it utilized 66.39 µg/ml for 50% inhibition.

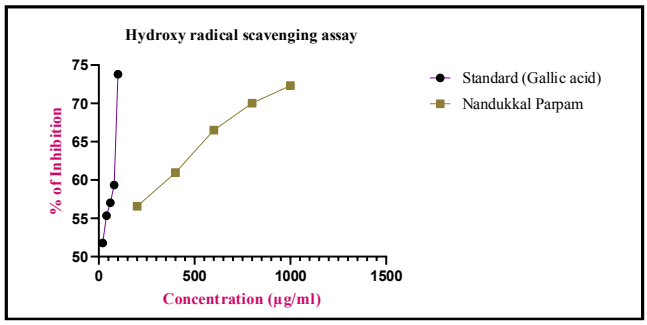

Figure 3: Hydroxyl radical scavenging activity of *Nandukkal Parpam*.

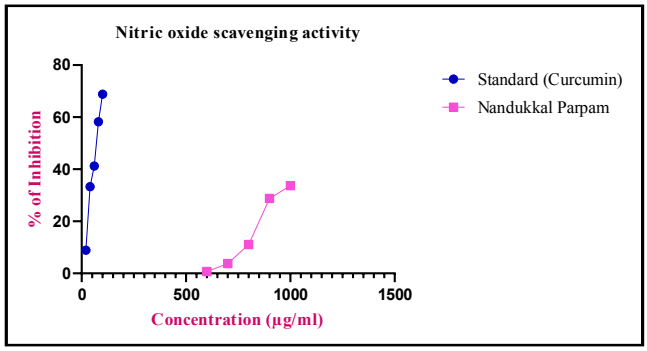

Figure 4: Nitric oxide radical scavenging activity of *Nandukkal Parpam*.

4. Discussion

Antioxidants are generally divided into two categories, *viz.,* primary or chain breaking antioxidants and secondary or preventative antioxidants. Chain breaking antioxidants act mainly by scavenging ROS and RNS. Whereas, preventative antioxidants act by transition metal ion chelation (Madhavi, 2014). Whatever may be the type, antioxidants may act directly by scavenging ROS/RNS or by inhibiting their production. It may also have indirect action through up regulation of various endogenous antioxidant defense proteins. The present study was carried out to analyze the capacity of a Siddha formulation, NP in scavenging various free radicals that are generated in *in vitro*. DPPH radical scavenging assay is a mixed mode assay in which hydrogen atom transfer (HAT), electron transfer and proton coupled electron transfer (PCET) mechanisms play different roles based on pH, solvent and other reaction conditions (Apak *et al*., 2016). The other assays of the study; namely, superoxide radical scavenging assay hydroxyl radical scavenging assay and nitric oxide radical scavenging assay falls under ROS/RNS scavenging methods of antioxidant assays. In all these assays, free radical/reactive species are generated by redox active chemical reagent and subsequent conversion of probe was measured either by spectroscopically or by electrochemically. The results of these assays was a direct measure of ROS/RNS concentration and its depletion demonstrates the scavenging activity of the test drug, NP.

Various concentrations of the NP solution were tested for these antioxidant assays and the results obtained are in dose dependent manner. As observed from the results, the IC_{50} of NP for DPPH assay was found to be 120.10 µg/ml. Whereas, IC_{50} of its reference compound, BHT was 12.24 µg/ml. This shows that NP has moderate scavenging action *via* hydrogen atom transfer mechanisms. From Figure 2, it was observed that ascorbic acid (reference compound) IC_{50} value was 45.27 µg/ml, whereas NP has IC_{50} value of about 172.20 µg/ml. It was approximately four fold greater than that of ascorbic acid, and hence evidenced that it has appreciable scavenging effects towards superoxide radicals. For hydroxyl radical scavenging assay, gallic acid was used as reference compound and it exhibited 19.31 µg/ml as IC_{50} value. NP could scavenge 50% of hydroxyl radical at a concentration of about 176.77 µg/ml. Both the superoxide radicals and hydroxyl radicals are reactive oxygen species. Superoxide anions are very harmful to cellular components (Korycka-Dahl *et al*., 1978). While hydroxyl radicals form the major ROS that causes lipid peroxidation and enormous biological damage (Aurand *et al*., 1977). The results of the present study suggest that NP was a potent scavenger of both the reactive oxygen species.

Nitric oxide (NO) plays a key role in various inflammatory processes. Increased levels of the NO production were toxic to tissues directly and contributes to vascular collapse associated with septic shock. Prolonged increase in nitric oxide levels leads to various cancers and inflammatory conditions like diabetes, multiple sclerosis, arthritis, ulcerative colitis, *etc*. (Taylor *et al*., 1997). The effect of NO amplifies in the presence of superoxide radicals as it reacts to form peroxynitrate anion (Huie and Padmaja, 1993). In the current study, NO scavenging activity of NP was studied and it could scavenge NO at an IC_{50} value of 1094 µg/ml. The reference compound, curcumin, IC_{50} value was found to be 66.39 µg/ml. This shows that NP was a weak NO scavenger. Though, a much greater dose was required to scavenge NO, NP was used for the treatment of many inflammations associated diseases. Being a *parpam* (oxide) form of drug, which was prepared by incineration process, only oxidized form of the ingredients would be available in the finished product. Hence, the biological function of this Siddha drug could be because of its main ingredient *Nandukkal*, a fossil crab stone.

As already mentioned earlier, *Nandukkal*, a fossil crab stone has diverse therapeutic uses, *viz.*, urinary disorders, mental disorders, ophthalmological disorders, veneral diseases, musculoskeletal disorder, *etc*. As many of these diseases involve oxidative stress, this significantly contributes to the high mortality rates associated with immune system dysregulation and other diseases (Dolly Verma *et al.,* 2022), the antioxidant nature of NP may be claimed for its therapeutic potential. Besides, elaborate studies on individual biochemical/metabolic pathways may lead to identification of cellular/ molecular mechanisms involved.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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