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IN VITRO ANTIOXIDANT PROPERTY OF *SIDDHA* FORMULATION '*NAAGA SANGU PARPAM*'

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ABSTRACT

Free radicals are the natural byproducts of chemical processes, such as metabolism. The human body naturally produces antioxidants to counteract the damaging effects of free radicals. When free radicals accumulate, they may cause a state known as oxidative stress. Chronic oxidative stress can increase the risk of chronic diseases such as heart disease, type 2 diabetes and cancer. To solve this, the method of "*Kaya Karpam*-Antioxidant" is followed in *Siddha* medicine. *Naaga Sangu Parpam* (NSP) is a herbo metal formulation mentioned in *Siddha* literature which is therapeutically used for treating *Moolam* (Hemorrhoids), *Powthiram* (Fistula), *Vellai* (Leucorrhoea), *Vettai* (Venereal disease). *Siddha* medicines not only cure diseases, but it also has rejuvenating property to improve general health and immunity. In this study, the unexplored area of *Naaga Sangu Parpam* was screened for antioxidant activity using DPPH (2,2-diphenyl-1-picrylhydrazyl), Nitric Oxide Radical Scavenging, ABTS Radical Scavenging activity and Hydrogen Peroxide Scavenging activity. NSP exhibited antioxidant activity against DPPH, Nitric Oxide radicals, ABTS and Hydrogen peroxide with IC50 value of 152.4±4.49, 103.5±7.86, 146.5±18.65 and 156.5±21.64 µg/ml respectively. The result of this study shows promising anti-oxidant activity of *Naaga Sangu Parpam* (NSP) by in-vitro methods.

KEYWORDS: Antioxidant, *Naaga Sangu Parpam*, *Siddha* medicine, Traditional medicine.

INTRODUCTION

Siddha is a comprehensive system that places equal emphasis on the body, mind and spirit and strives to restore the innate harmony of an individual. *Kayakarpam* (rejuvenation) is a branch of *Siddha* that deals with prevention or postponing greying of hair, formation of wrinkles and ageing. It emphasizes not only on medicines, but also on the diet and lifestyle along with stress reducing yogic practices. Oxidation is a normal chemical process that takes place in the body every day. It can be accelerated by stress, cigarette smoking and alcohol. When there are disruptions in the natural oxidation process, highly unstable and potentially damaging molecules called free radicals are developed. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA or cell membrane. Normally free radical formation is controlled by various beneficial compounds known as antioxidants. Antioxidants benefit the body by neutralizing and removing the free radicals from the bloodstream. A balance between free radicals and antioxidants is necessary for proper physiological function. Oxidative stress occurs when the production of free radicals goes beyond the protective defences in the body. Oxidative stress and free radical damage to cells may initiate the early stages of cancer[2] and heart disease.[3] Free radicals are also suspect in the development of alzheimer's disease[4], arthritis[5], cataracts[6], diabetes[7], kidney disease[8] and age related diseases.[9] Antioxidant compounds are capable of protecting against oxidative damage by decreasing the number of free radicals which cause chronic diseases. The present study is to evaluate the antioxidant property of *Naaga Sangu Parpam* (NSP) using in-vitro methods.

MATERIALS AND METHODS

Drug selection

The drug *Naaga Sangu Parpam* is a herbo-metal preparation (*Parpam* = one of the 32 types of internal medicine) mentioned in *Siddha* text '*Kannusamy paramparai vaithiyam'*, pg.no: 414, indicated for *Moolam* (Hemorrhoids), *Powthiram* (Fistula in ano), *Vellai* (Leucorrhoea) and *Vettai* (Venereal disease).

Ingredients

1. Purified *Naagam* (Zinc) - 1 *palam* (35 gm)
2. Purified *Sangu* (Conch shell) - 1 *palam* (35 gm)
3. *Uthamani* leaf juice *(Pergularia daemia. Linn)* - 350 ml

Collection of raw drugs

Naagam and *Sangu* were procured from a well reputed country shop in Parrys, Chennai. *Uthamani* was freshly collected from Tambaram Sanatorium. *Naagam* and *Sangu* were purified and the medicine was prepared in *Gunapadam* laboratory of National Institute of Siddha.

Identification and Authentication of the drug

Sangu was authenticated by Dr.Rajkumar Rajan, Scientist D, Marine Biology Regional Centre, Zoological Survey of India, Chennai. Zinc was authenticated by Dr.M.Suresh Gandhi, Department of Geology, University of Madras, Chennai. *Uthamani* was identified and authenticated by Dr.D.Aravind M.D(s), Botanist, National Institute of Siddha, Tambaram Sanatorium, Chennai.

Purification process

Naagam

The ghee of south Indian Mahua (*Madhuca longifolia*) – *Illuppai* ghee was taken in a mud pot. Two pieces of Ammonium chloride (*Navaacharam*) were placed in a pot in such a way that half of the portion of the pieces were immersed in the ghee on the opposite direction. The zinc melted in an iron pot was poured twenty-one times to the ghee of south Indian Mahua and washed.

Sangu

Equal quantity of limestone and fuller's earth was taken and mixed with 8 parts of purified water and a clear filtrate was obtained. The Conch shell was allowed to boil in the above said filtrate for 1hr 30 mins.

Uthamani

Before extracting the juice, the leaf of *Pergularia daemia* was washed lightly with cold running water to remove any soil, dust, bugs or other foreign material. The leaf was drained thoroughly on absorbent towel.

Method of Preparation

Purified *Naagam* was taken in an iron pan and subjected to excessive heat, till *Naagam* reached its melting consistency. Conch powder was poured over the melting *Naagam* and the mixture was stirred well till it reached the powder form. The powder was grounded with

Uthamanai leaf juice for 12 hours and made into small cakes and subjected to the calcination process with cow dung cakes. Finally, the *Parpam* was collected and stored in an airtight glass container.

ANTIOXIDANT ACTIVITY

Antioxidant activity was carried out by In-vitro cell line studies using the following assays.

1) DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Assay.
2) Nitric Oxide Scavenging Assay
3) ABTS Radical Scavenging Assay
4) Hydrogen peroxide Radical Scavenging Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Assay

The antioxidant activity of NSP was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. NSP was mixed with 95% methanol to prepare the stock solution in the required concentration (10mg/100ml or 100µg/ml). From this stock solution, 1ml, 2ml, 4ml, 6ml, 8ml and 10ml was taken in six test tubes and by serial dilution with the same solvent, the final volume of each test tube was up to 10 ml whose concentration was then 10 µg/ml, 20 µg/ml, 40µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml respectively. Ascorbic acid was used as a standard which was prepared in the same concentration as that of the sample extract by using methanol as solvent. Final reaction mixture containing 1 ml of 0.3 mm DPPH methanol solution was added to 2.5 ml of sample solution of different concentrations and allowed to react at room temperature. Absorbance in the presence of test sample at different concentration (10 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100µg/ml) was noted after 15 mins incubation period at 37^0C. Absorbance was read out at 517 nm using double-beam U.V Spectrophotometer by using methanol as blank. The effective concentration of test sample required to scavenge DPPH radical by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations.

$$\text{DPPH Scavenging (\%)} = \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} X\ 100$$

Where $A_{control}$ is the absorbance of the control and A_{test} is the absorbance of the test drug.

Nitric Oxide Radical Scavenging Assay

The concentrations of NSP were made into serial dilution from 10−100 µg/ml and standard Gallic acid was also prepared in same concentration as that of NSP. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride immediately before use. A volume of 0.5 ml of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 ml of the different concentrations of the test drug (10−100 µg/ml) and incubated at 25°C for 180 mins. NSP was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the test drug, but with an equal volume of buffer was prepared in a similar manner as it was done for the test samples. The absorbance was measured at 546 nm using a Spectra Max Plus UV-Vis microplate reader (Molecular Devices, GA, USA). Gallic acid was used as the positive control. The percentage inhibition of NSP and the standard was calculated and recorded. The percentage of nitric oxide radical scavenging activity of NSP and Gallic acid was calculated using the following formula,

$$\text{Nitric oxide Scavenging (\%)} = \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100$$

Where $A_{control}$ is the absorbance of the control and A_{test} is the absorbance of the test drug.

ABTS Radical Scavenging Assay

This assay was carried out for evaluating the anti-oxidant potential of NSP against 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS radicals. The ABTS radical cation method was modified to evaluate the free radical-scavenging effect of one hundred pure chemical compounds. The ABTS reagent was prepared by mixing 5 ml of 7 mM ABTS with 88 µl of 140 mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and then diluted with water (1: 44, v/v). To determine the scavenging activity, 100 µl ABTS reagent was mixed with 100 µl of test sample (10-100µg/ml) and was incubated at room temperature for 6 mins. After incubation, the absorbance was measured at 734 nm. 100% methanol was used as a control. Gallic acid with the same concentrations as that of NSP was measured following the same procedures described above and was used as positive control. The ABTS scavenging effect was measured using the following formula,

$$\text{ABTS Scavenging (\%)} = \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100$$

Where $A_{control}$ is the absorbance of the control and A_{test} is the absorbance of the test drug.

Hydrogen peroxide Radical Scavenging Assay

The ability of NSP to scavenge hydrogen peroxide was estimated according to the method reported by Ruch et al.[15] with minor modification. A solution of hydrogen peroxide (43 mM) is prepared in phosphate buffer (0.1 M pH 7.4). Different concentration of the sample (10-100µg/ml) was added to a Hydrogen peroxide solution (0.6 ml, 43 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. BHA was used as standard. The free radical scavenging activity was determined by evaluating % inhibition as above. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples using following equation.

$$H_2O_2 \text{ Scavenging } (\%) = \frac{(A_{control} - A_{test})}{A_{control}} X\ 100$$

Where $A_{control}$ is the absorbance of the control and A_{test} is the absorbance of the test drug.

RESULT AND DISCUSSION

Several concentrations ranging from 10 to 100 µg/mL of NSP were tested for their antioxidant activity using different in-vitro models. It was observed that free radicals were scavenged by the test drug NSP in a dose-dependent manner in DPPH, NO radical, ABTS and hydrogen peroxide methods. The results were expressed as Mean ± SD (n=3).

DPPH radical scavenging activity

Naaga Sangu Parpam (NSP) was screened for DPPH radical scavenging activity and the percentage inhibition ranges from 3.44 to 31.87% when compared with standard Ascorbic acid with percentage inhibition ranges from 41.12 to 90.45%. The IC50 value of NSP was found to be 152.4 ± 4.49 µg/ml when compared with standard Ascorbic acid with IC_{50} value 6.234 ± 1.12 µg/ml (Table 1). The DPPH radical scavenging activity of NSP is illustrated in Figure 1.

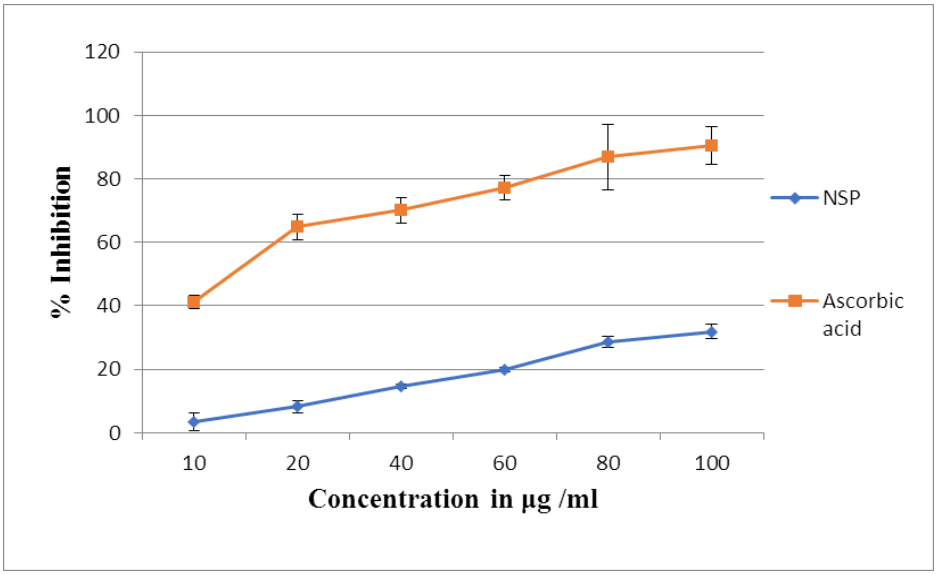

Figure 1: DPPH radical scavenging activity of *Naaga Sangu Parpam* (NSP).

Table 1: IC50 Values for DPPH radical scavenging Assay by NSP and standard.

Test Drug / Standard	IC50 Value DPPH Assay ± SD (µg /ml)
NSP	152.4 ± 4.49
Ascorbic acid	6.234 ± 1.12

NO radical scavenging activity

NO radical scavenging activity of the NSP revealed that the percentage inhibition of the NSP ranges from 6.73 to 46.73% when compared with standard Gallic acid with percentage inhibition ranges from 35.05 to 94%. The corresponding IC50 value of NSP was found to be 103.5 ± 7.86 µg/ml when compared with standard Gallic acid with IC_{50} value 28.93 ± 2.08 µg/ml (Table 2). The NO radical scavenging activity of NSP is illustrated in Figure 2.

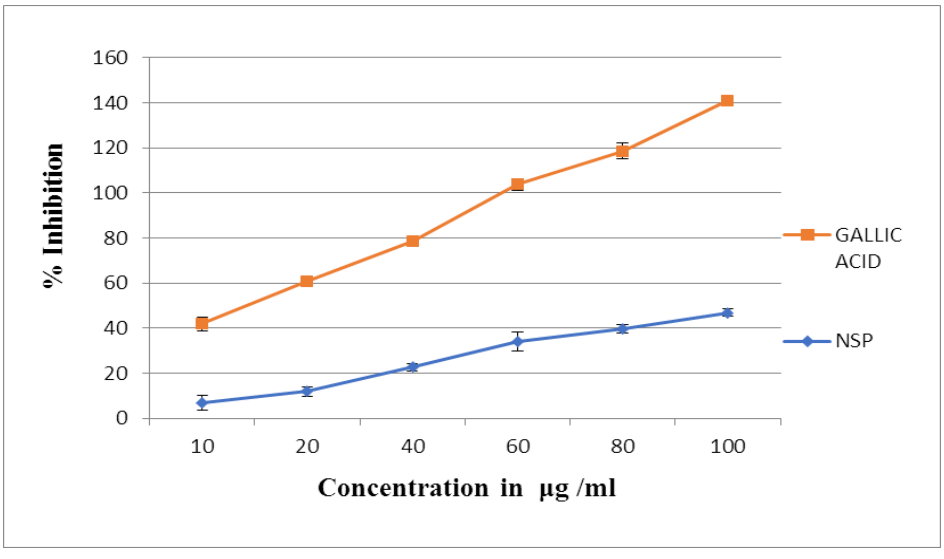

Figure 2: Nitric oxide radical scavenging activity of *Naaga Sangu Parpam* (NSP).

Table 2: IC50 Values for Nitric Oxide radical scavenging assay by NSP and standard.

Test Drug / Standard	IC50 Value NO Assay ± SD (µg /ml)
NSP	103.5 ± 7.86
Gallic acid	28.93 ± 2.08

ABTS radical scavenging activity

NSP was screened for hydrogen peroxide radical scavenging activity and the percentage inhibition ranges from 12.41 to 37.33% when compared with standard Gallic acid with percentage inhibition ranges from 47.86 to 84.97%. The corresponding IC50 value of the NSP was found to be 146.5 ± 18.65 µg/ml when compared with standard Gallic acid with IC_{50} value 7.012 ± 6.095 µg/ml (Table 3). The ABTS radical scavenging activity of NSP is illustrated in Figure 3.

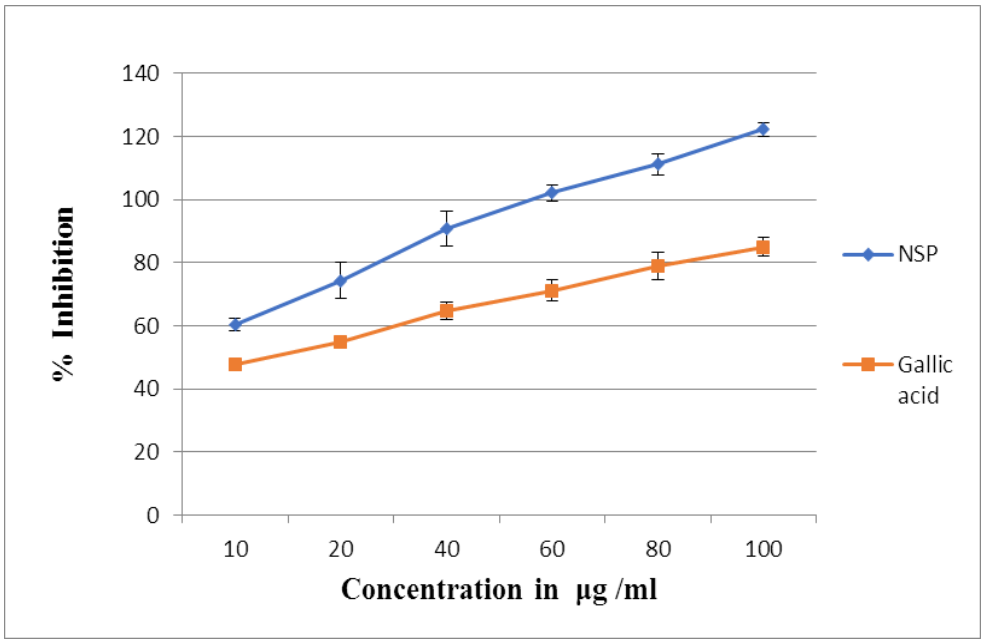

Figure 3: ABTS radical scavenging activity of *Naaga Sangu Parpam* (NSP).

Table 3: IC50 Values for ABTS radical scavenging assay by NSP and standard.

Test Drug / Standard	IC50 Value ABTS Assay ± SD (µg /ml)
NSP	146.5 ± 18.65
Gallic acid	7.012 ± 6.095

Hydrogen peroxide radical scavenging activity

NSP was screened for hydrogen peroxide radical scavenging activity and the percentage inhibition ranges from 5.38 to 33.11% when compared with standard BHA with percentage inhibition ranges from 37.01 to 94.5 %. The corresponding IC50 value of NSP was found to be 156.5 ± 21.64 µg/ml when compared with standard BHA with IC_{50} value 16.12 ± 10.99

µg/ml (Table 4). The Hydrogen peroxide radical scavenging activity of NSP is illustrated in Figure 4.

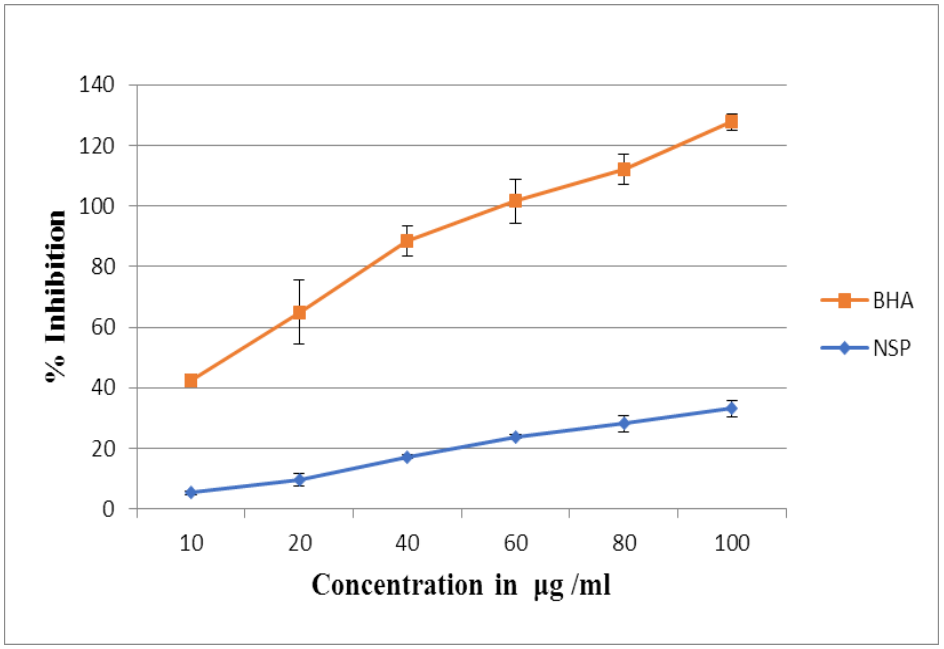

Figure 4: Hydrogen peroxide radical scavenging activity of *Naaga Sangu Parpam* (NSP).

Table 4: IC50 Values for Hydrogen peroxide radical scavenging by NSP and standard.

Test Drug / Standard	IC50 Value Hydrogen peroxide radical scavenging Assay ± SD (µg /ml)
NSP	156.5 ± 21.64
BHA	16.12 ± 10.99

CONCLUSION

Antioxidant properties of "*Naaga Sangu Parpam*" was studied using DPPH, Nitric Oxide Scavenging, ABTS and Hydrogen Peroxide Scavenging activity. The results indicate that the *Siddha* formulation NSP decreased oxidative stress via its antioxidant properties and was found to be an effective scavenger of DPPH, ABTS, H_2O_2 & NO. Thus, it is concluded that *Naaga Sangu Parpam* has promising anti-oxidant activity and may act therapeutically in treating several oxidative stress related disorders.

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