Original Research Article

Determination of Anti-oxidant activity of Ethanol, Ethyl acetate, Hexane, and Aqueous extracts of Sri Lankan traditional poly-herbal formula*NagaradiPanchakaya*.

ABSTRACT

Natural products are the good source of natural antioxidants. *Nāgarādipanchakaya* is the well-known poly-herbal formula prescribed specially for upper respiratory tract diseases by Sri Lankan Ayurveda medical practitioners. The name implies that it contains five herbal ingredients. This research study was aimed to determine the antioxidant activity of *Nāgarādipanchakaya*, extracted with different solvents (ethanol, ethyl acetate, n-hexane and water). Antioxidant activitywas evaluated by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay method and the Ferric Reducing Antioxidant Power (FRAP) assay. In vitro antioxidant free radicals scavenging capacities of different concentrations of ethanol, ethyl acetate, n-hexane and water extracts, of *Nāgarādipanchakaya* were evaluated. The percentage of inhibition and IC_{50} were calculated. IC_{50} (µg/mL) of water, ethanol, ethyl acetate, n-hexane and Ascorbic Acid ~~was~~ werefound to be179.89±1.25 µg /mL, 248.09±1.74 µg /mL, 169.43± 0.98µg /mL, 189.56±2.23 µg /mL, and 01.18±0.98 µg /mLrespectively in DPPH radical scavenging assay. In FRAP assay the reducing power for the different extracts varied in following order: water > ethanol > ethyl acetate > n-hexane.The total Phenolic content of water, ethanol, ethyl acetate and n-hexane extracts were 1.73±0.51 mg GAE/g, 2.59±0.67 mg GAE/g, 3.67±0.81 mg GAE/g and 1.57±0.09 mg GAE/g respectively**.** The results can be concluded thatEthanol &water extracts exhibit high antioxidant activity possibly due to thepresence of high phenolic content than the other extracts.

Keywords: Antioxidant, DPPH, FRAP, *Nāgarādipanchakaya*~~, respiratory diseases~~.

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1. Introduction

Nāgarādipanchakaya (N5) is a poly-herbal formulation, which is mentioned in traditional medicinal textbooks[1]and in the Ayurveda pharmacopeia of Sri Lanka[2].Since ancient times it has been prescribed by traditional practitioners within the country for treating respiratory tract diseases including cold, cough, sorethroat and fever.As the name implies *Nāgarādipanchakaya* consist of five medicinal plants processed as a hot concentrated water extract known as decoction. In a decoction, the water-soluble active ingredients in plant materials are extracted to water by applying heat and simultaneously concentrating to a specific proportion. The weight of plant materials and the volume of water used are determined according to proportions as mentioned in authentic textbooks[3]. In preparation of decoctions numerous plant materials such

as heartwood, bark, stem, leavesseeds, rhizomesetc. belonging to different genera and families are utilized. N5 also employs different plant partsnamely Rhizome of *Inguru* (*Zingiber officinale)*,Heartwood of *Devadāra* (*Deodar cedar*),seeds of *Kottammalli* (*Coriandrum sativum)*,root and stem of *Ela batu* (*Solanum indicum)* and whole plant except the root of *Katuwelbatu* (*Solanum xanthocarpum)*.

Antioxidants are molecules that neutralize free radicals and thereby prevent the oxidation of other molecules. Imbalance between antioxidant mechanism and free radicals create oxidative stress, which damages cell proteins and adversely affects the biological immune response[4]. Due to long term, oxidative stress the cells of immunity system will be deteriorated consequently leading to a febrile defense system against infections. Therefore, antioxidant agents can bestow a remarkable role in enhancing the immune function within human body. Pathogens such as *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* that target the respiratory system produce redox active toxins and reactive oxygen species respectively[5]. Therefore, decoctions with high antioxidant activity may be a successful treatment for respiratory infections.

The therapeutic effects of phytoconstituents in plants have been extensively validated by modern research, which inturns confirms the effectiveness of plant-based treatments in traditional medical systems. Due to qualitative and quantitative variation in biomolecule distribution in medicinal plants,different parts of the same plant display variant therapeutic potentials. Previous researches represented that total phenolic content (TPC) and total flavonoid content (TFC) could be correlated to their antioxidant activities[6,7, 8].

Hence above reasons, there is a possibility for poly-herbalformulations suchas N5 to be more effective rather than the utilization of a single plant. The qualitative analysis of Phytochemicals of different solvent extracts and quantitative analysis of total phenolic and Flavonoid content of N5 was confirmed[9].

The objective of the present study was to assess the medicinal potential of this poly-herbal formulation, especially its antioxidant potential in the form of free radical scavenging activity, using scientific techniques and thus justify its traditional use as a substantial and reliable herbal formulationspecially for respiratory diseases and fever conditions with many other indications.

2. Materials and Methods

2.1Identification and Collection of the Plant Materials

The plant materials such as the rhizomes of *Inguru* (*Zingiber officinale*), stem pieces of *Devadāra* (*Cedrus deodara*), seeds of *Kottamalli* (*Coriandrum sativum*), roots of *Batu* (*Solanum indicum*) and whole plant of *Katuwelbatu* (*Solanum xanthocarpum*) were purchased from local Ayurveda medical shops in Colombo city (6° 55'54.98" N x 79° 50' 52.01" E), Western province, Sri Lanka and were identified by the senior lecturer in the unit of Ayurveda Pharmacology of the Institute of Indigenous Medicine, University of Colombo.

2.2 Preparation of Hot water Extraction

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The aqueous extract was prepared according to the *Kashaya* (Decoction) preparation procedure as mentioned in traditional medicinal textbooks of Sri Lanka[10,11]. The samples were washed under the running tap water to remove soil and other dust particles.Samples were air dried separately under the laboratory condition for 2-3 weeks.Each 24g of five ingredients of *Nāgarādipanchakaya*were weighted and ground to a coarsepowder using grinder(Disk Mill Model FFC-234, China), separately. All the above ingredients were mixed and collected in a clay pot and 1920 mL water was added to the pot. The entire setup was kept on the fire and applied moderate heat to reduce the total volume in to 120 mL (i.e., initial water amount:final water amount = 8:1 ratio). The filtrate was concentrated and converted to powder form using a freeze dryer Telstar LyoBeta, Spain (-40°C to 40°C) and obtained yield of 12.8 g of dry extract. The dried water extract wasstored in freezer at temperature below 0°C for further experiments.

2.3 Preparation of Hexane,Ethyl acetate and Ethanol solvent extracts

To prepare the hexane, ethyl acetate and ethanol extracts, first the materials were sorted out by removing diseased parts, the unnecessary parts and were washed under the running tap water to remove soil and other dust particles. Then the samples were air dried separately under the laboratory condition for 2- 3 weeks. Raw material100g of each of the five ingredients of *Nāgarādipanchakaya* were weighted and ground to a fine powder using grinder(Disk Mill Model FFC-234, China), separately. Grinded 100g of each powder of the above ingredients were mixed and put into a conical flask and solvent was added and kept for 24 hours and sequentially extracted to hexane, ethyl acetate and ethanol. Each extract obtained following successive extraction was filtered using Whatman No. 1 filter papers. The filtrates were then evaporated under reduced pressure and dried using a rotary evaporator at 400C. Finally, the crude was taken into vials, labeled and was stored in a freezer at -40C in the refrigerator, till further use.

2.4 Total Phenolic content

Total phenolic content of the four extracts were determined by the Folin Ciocalteu method[12]with slight modifications. FoilnCiocalteu reagentand plant extract solution were mixed in 1:1 ratio. 0.5mL of 6% w/vNa_2CO_3 solution followed by ~~0~~2 mL distilled water was added to the solution after 05 minutes. The mixture was incubated for 60 minutes in dark at room temperature. Gallic acid which is an abundant phenolic acid in plant kingdom was used as the standard for the calibration curve. The total phenolic content was expressed as mg GAE/g equivalent of the extract.

2.5 In Vitro DPPH assay for determination of antioxidant activity of aqueous, hexane, ethyl acetate and ethanol extracts.

DPPH [1, 1-di-phenyl-2-picryl hydrazyl] is a stable free radical with purple color, the intensity of which is measured spectrophotometrically at 517 nm wavelength. Antioxidants reduce DPPH to 1, 1-diphenyl-2-picryl hydrazine, a colorless compound.

The antioxidant activity towards the scavenging of DPPH free radical was measured during this assay. Assay was performed according to the previous research[13]with slight modifications. Ascorbic acid which is a potent antioxidant was used as the standard. A dilution series was

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prepared for all the extracts in the range of 1-600 µg /mL.The DPPH solution in ethanol(5mg/100mL) was added extract solutions and was incubated in dark for 15 minutes. The final absorbance was measured at 517 nm range. Obtained absorbance readings were directly utilized to calculate the percentage inhibition according to the following formula.

$$\text{Percentage Inhibition}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \text{x } 100$$

$A_{control}$ – Absorbance of the control

A_{sample}- Absorbance of the sample extracts

The IC_{50} values were calculated using Minitab 19.2software

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2.6In Vitro FRAPassay for determination of antioxidant activity of aqueous, hexane, ethyl acetate and ethanol extracts

The reducing powers of extracts were determined according to the method described in previous research[14]. Substances which have reduction potential react with potassium ferricyanide to form potassium ferrocyanide, which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. The concentration series of extracts was prepared in amounts of15, 20,25,50 and 75 µg/ml.

To 1 mL of the test solution, 2.5 mL of Phosphate buffer (6.6 pH) and 2.5 mL of 1% (w/v) potassium ferricyanide was added. It was incubated at 50 °C for 20 minutes after vortex well. Then 2.5 mL of 10% (w/v) trichloroacetic acid was added and centrifuge for 10 minutes. Next 2.5 mL of distilled water was added to 2.5 mL of centrifuged supernatant solution and 0.5 mL of 0.1% ferric chloride solution was added.It was mixed well and kept 10 minutes. Finally,absorbances were measured at 700 nm. Ascorbic Acid was used as the standard. Higher absorbance indicates the higher reducing power. The assays were carried out in triplicate.

3. Results and Discussion

3.1 Total phenolic content

When considering the four extracts the highest phenolic content, which was 3.67 mg GAE/g, was observed in the ethyl acetate extract. A noticeably low amount was recorded for the hexane extract which was 1.57 mg GAE/g. The total phenolic content of the ethanol and aqueous extracts were 2.59 mg GAE/g and 1.73 mg GAE/g respectively. The total phenolic content of four extracts were varied as Ethyl acetate > Ethanol > Aqueous> Hexane. (Figure No. 1)

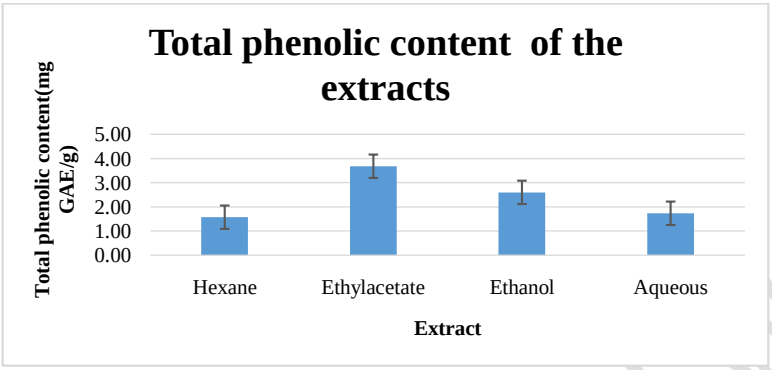

Figure No.1 – Total phenolic content of the extracts

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3.2 Evaluation of DPPH radical scavenging assay

There was a progressive increase in radical scavenging ability of the extracts with increasingthe concentration. (Figure No. 2)

IC_{50} (µg/mL) of ethanol, ethyl acetate, n-hexane and water extracts and Ascorbic Acid was found to be248.09±1.74 µg /mL, 169.43± 0.98µg /mL, 189.56±2.23 µg /mL, 179.89±1.25 µg /mL and 01.18±0.98 µg /mL in DPPH radical scavenging assay. As the lowest IC_{50} value was recorded in the ethyl acetate extract depicted the high free radical scavenging capability and the highest IC_{50} value was recorded in the Hexane extract depicted low free radical scavenging capacity when compared with Ascorbic acid.

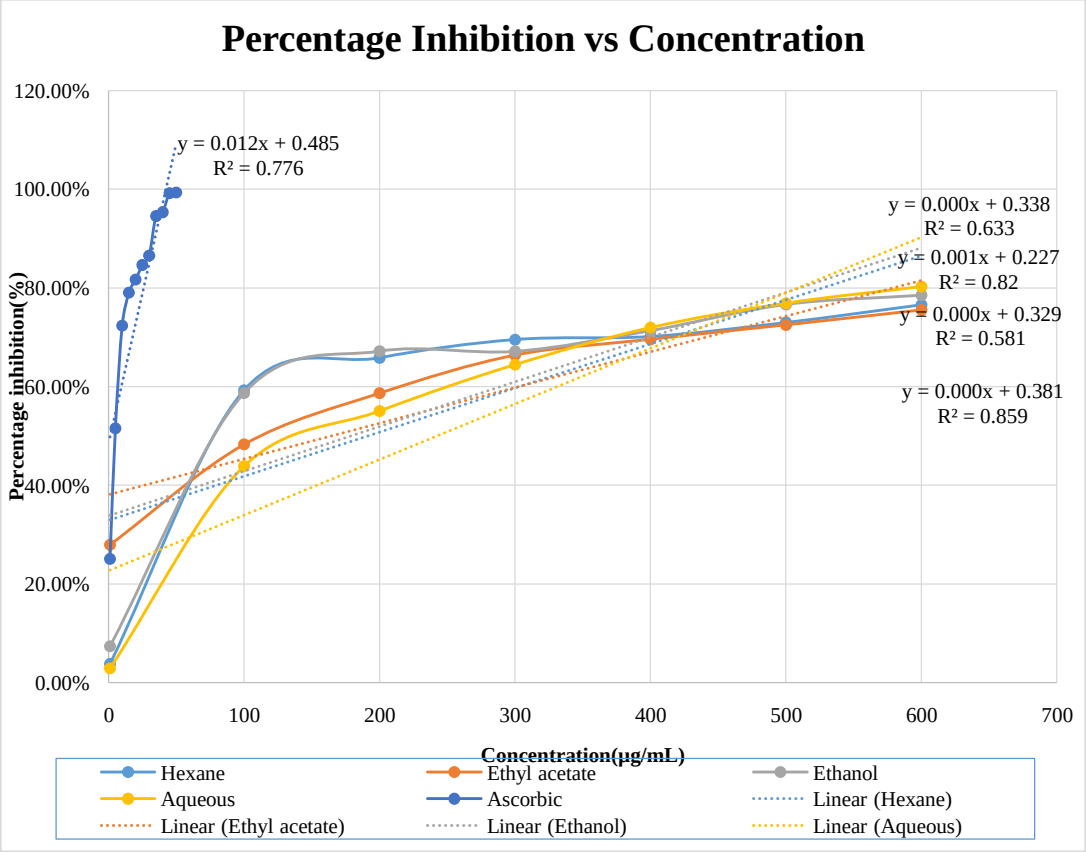

Figure No. 2 - DPPH radical scavenging assay for the extracts

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3.3 Ferric –reducing Antioxidant Power assay

In this assay, the yellow color of the test solution changes to green and blue depending on the reducing power of the test solution. The values of the reducing power (absorbance at 700nm) at different concentration are shown in Table ~~No.~~1 and Figure ~~No.~~ 3. All the extracts exhibited reducing power that increased with concentration. The reducing power for the different extracts was in the following order: aqueous>ethanol>ethyl acetate.>hexane. In this way it was established that phenolic compound contributes directly to the antioxidant activity of the plant extracts.

Table ~~No.~~ 1: Absorbance values of Different concentration of Different test solutions

AQ	ETH	EA	HEX	Ascorbic acid	Concentration(µg/ml)
0.3893	0.3829	0.3802	0.3427	0.8762	75
0.3831	0.3744	0.3608	0.3181	0.6467	50
0.3801	0.3717	0.3785	0.2576	0.5909	25
0.3543	0.3543	0.3633	0.2455	0.5193	20
0.3529	0.3332	0.3257	0.1735	0.4451	15

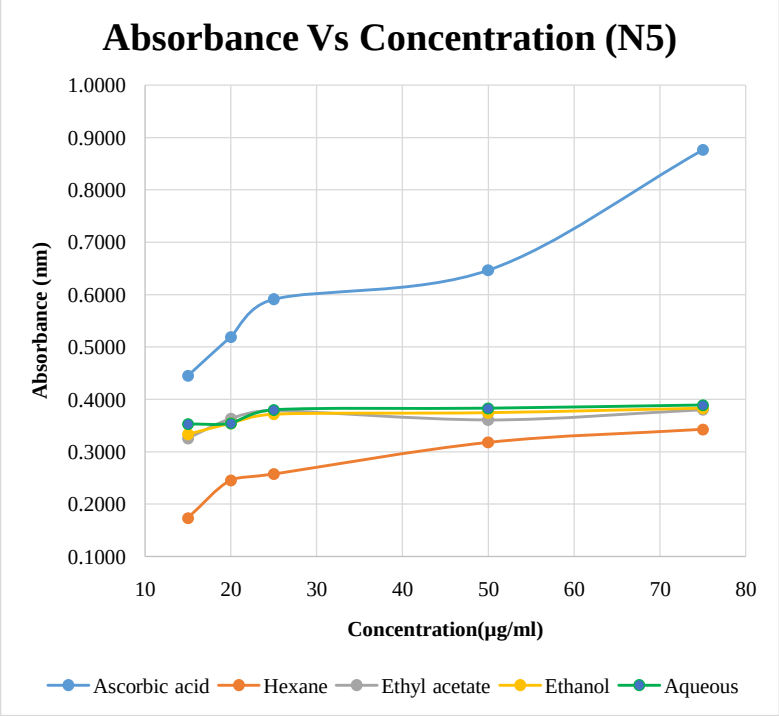

Figure ~~No.~~ 3 - Absorbance Vs Different concentration of Different test solutions

The aqueous extract (decoction)which is mainly used in traditional/ Ayurvedamedicine and the method of preparation of decoction is described in Ayurveda texts. But with the development of

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research culture analysis of various solvent extracts of herbal formulae also latest trend in the world. Antioxidant potential can be increased by the synergistic interactions between different antioxidant compounds present in the mixture of natural products or the mixture of different herbal extracts or plant essential oils. Thesynergistic interactions decrease the requirement of doses of different drugs in combination thus reducing the side effects caused by the high concentrations of a single drug. But very little is known about the mechanism of interactions which are responsible for the synergistic antioxidant activity. Moreover, only one analytical method cannot fully describe the antioxidant activity of the samples, and multiple method approach is necessary for the full evaluation of the antioxidant activity.So, a large number of synergistic combinations are still unexplored. There is also a need for the development of new methods for pharmacological studies and clinical trials evaluating the effects produced by complex mixtures of compounds.

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4. Conclusion

When analyzing the results of this study N5 decoction clearly shows significant concentration dependent antioxidant activity for DPPH and FRAPS assays. Presence of remarkable antioxidant activity in a decoction plays a vital role in protection against infections and maintaining overall health of human beings. As antioxidants maintain the proper functioning of immune cells, N5 decoction with significant antioxidant activity can act as a potent immune booster thus preventing the respiratory diseases.Therefore, this study scientifically validates the traditional use of N5 decoction for respiratory diseases.

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NOTE:

The study highlights the efficacy of "Ayurved" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

5. References

01. Kumarasighe, A., Sri Lanka Deshiya ChikitsaSangrahaya (Compendium of Traditional preparations), 1st print, Vol. 1, Department of Ayurveda, Colombo, (Sathosa print) 1981, pg. 173.

02. Ayurveda Pharmacopoeia Committee (APC), Ayurveda Pharmacopoeia, Vol. 1, Part One, Department of Ayurveda, Colombo, 1961, pg. 138.

03. Reddy, K.R.C., 1998.Bhaisajya Kalpana Vignana,ChaukhambhaSansskrit Bhawan, Varanasi, India, 1998. pg.159

04. Kaur, J., Kaur, R. and Kaur, A., 2018. Dietary Antioxidants and Infectious Diseases. *Infectious Diseases and Your Health*, pp.307-316.

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05. Chaplin, D., 2010. Overview of the immune response. *Journal of Allergy and Clinical Immunology*, 125(2), pp.S3-S23.

06. Mongkolsilp S, Pongbupakit I, Sae-Lee N, Sitthithaworn W. Radical scavenging activity and total phenolic content of medicinal plants used in primary health care. SWU J Pharm Sci. 2004;9(1):32-5.

07. Xu BJ, Chang SK. Total phenolic content and antioxidant properties of eclipse black beans (*Phaseolus vulgaris* L.) As affected by processing methods. J Food Sci. 2008;73(2):H19-27.

08. Mashkor IM. Phenolic content and antioxidant activity of fenugreek seeds extract. Int J PharmacognPhytochem Res 2014;6(4):841-4.

09. P. R. Waratenne, A. P. A. Jayasiri, I. M. Manuha and P. M. H. K. Maduratharangi (**2020).** Phytochemical Analysis of Different Extracts of *Nagaradipanchakaya*. South Asian Research Journal of Natural Products - 3(4): 41-49.https://journalsarjnp.com/index.php/SARJNP/article/view/47

10. Kumarasighe, A., Sri Lanka Deshiya ChikitsaSangrahaya (Compendium of Traditional preparation), 1st print, Vol. 1, Department of Ayurveda, Colombo, (Sathosa print) 1981, pg. 90.

11. Ayurveda Pharmacopoeia Committee (APC), Ayurveda Pharmacopoeia, Vol. 1, Part one,Department of Ayurveda, Colombo, 1976, pg. 30.

12. Oyalinka, A.A., and Anthony, I. O. (2009) phytochemical screening and polyphenolic antioxidant activity of aqueous extract of *Helichrysum pedunculatum*. *Int. J. Mol. Sci.10*, 4990-5001.

13. Fejes S, Blazovics A, Lugasi A, Lemberkovics E, Petri G, Kery A, In vitro antioxidant activity of Anthriscus cerefolium L. (Hoffm.) extracts. J Ethnopharmacol, 2000; 69: 259-265.

14. Ujwal K, Tanvir H, Zahidul I. Phytochemical investigation, Antioxidant activity and Antihelminthic activity of Micania micrantha leaves. World Journal of Pharmaceutical Research, 2015;Vol. 4, Issue 5, 121-133