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Determination of Anti-oxidant Activity of Ethanol, Ethyl acetate, Hexane, and Aqueous Extracts of Sri Lankan Traditional Poly-herbal Formula *Nāgarādi Panchakaya*

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Authors' contributions

This work was carried out in collaboration among all authors. Author WPR designed the study, wrote the protocol, grant the approval of the research committee, and wrote the first draft of the manuscript and edited the manuscript as per the requirements. Author JAPA managed the analyses of the study edited the manuscript. Author MIM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Natural products are the good source of natural antioxidants. *Nāgarādi panchakaya* is a 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 including *Inguru*

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(*Zingiber officinale), Devadāra* (*Cedrus deodara)*, *Kottammalli* (*Coriandrum sativum), Ela batu* (*Solanum indicum)* and *Katuwelbatu* (*Solanum xanthocarpum)*. This research study was aimed to determine the antioxidant activity of *Nāgarādi panchakaya*, extracted with different solvents (ethanol, ethyl acetate, n-hexane and water). Antioxidant activity was 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 *Nāgarādi panchakaya* 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 were found to be 179.89±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 /mL respectively 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 that Ethanol & water extracts exhibit high antioxidant activity possibly due to the presence of high phenolic content than the other extracts.

Keywords: Antioxidant; DPPH; FRAP; Nāgarādi panchakaya.

1. INTRODUCTION

Nāgarādi panchakaya (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, sore throat and fever. As the name implies *Nāgarādi panchakaya* 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 text books [3]. In preparation of decoctions numerous plant materials such as heartwood, bark, stem, leaves seeds, rhizomes etc. belonging to different genera and families are utilized. N5 also employs different plant parts namely Rhizome of *Inguru* (*Zingiber officinale),* Heartwood of *Devadāra* (*Cedrus deodara)*, 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 in turns 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” [6-8].

Hence above reasons, there is a possibility for poly-herbal formulations such as 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].

“On the other hand, the indications of this poly-herbal formula have mentioned as the respiratory

diseases associated with fever. Oxidative stress is an important feature in the pathogenesis of various respiratory diseases including Chronic Obstructive Pulmonary Diseases (COPD). Targeting oxidative stress with antioxidants or boosting the endogenous levels of antioxidants is likely to be beneficial in the treatment of respiratory diseases. Various research suggest that initiation of fever is associated with oxidative stress; therefore, some antioxidants can reduce pyrexia" [10,11].

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 formulation specially for respiratory diseases and fever conditions with many other indications.

2. MATERIALS AND METHODS

2.1 Identification 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 Faculty of Indigenous Medicine, University of Colombo.

2.2 Preparation of Hot Water Extraction

The aqueous extract was prepared according to the *Kashaya* (Decoction) preparation procedure as mentioned in traditional medicinal textbooks of Sri Lanka [12,13]. 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 coarse powder 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^{o} C to 40^{o} C) and obtained yield of 10.7% of dry extract. The dried water extract was stored in freezer at temperature below 0^{O} 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 plant materials rhizomes of *Zingiber officinale*, stem pieces of *Cedrus deodara*, seeds of *Coriandrum sativum*, roots of *Solanum indicum* and whole plant of *Solanum xanthocarpum* 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. Assays were parallelly conducted for powder extracts of formula used as extraction method in single pure same solvents using rotary evaporator to confirm the avoid in contamination of an extract by the previous solvent. 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 40^{0}C. Finally, the crude was taken into vials, labeled and was stored in a freezer at -4^{0}C 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 [14] with slight modifications. Folin Ciocalteu reagent and plant extract solution were mixed in 1:1 ratio. 0.5mL of 6% w/v Na_2CO_3 solution followed by 02 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 of gallic acid equivalent (GAE) per gram (g) 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 wave length. 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 [15] with slight modifications. Ascorbic acid which is a potent antioxidant was used as the standard. A dilution series was 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.

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

$A_{control}$ – Absorbance of the control
A_{sample}- Absorbance of the sample extracts

The concentration of the extracts needed to produce a 50% inhibition (IC_{50}) was calculated using Probit analysis (MINITAB Release 14.2 Minitab Inc. 2003 statistical software) and data are shown in Fig. 2.

2.6 *In vitro* FRAP Assay 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 [16]. 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 of 15, 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 (Fig. 1).

3.2 Evaluation of DPPH Radical Scavenging Assay

There was a progressive increase in radical scavenging ability of the extracts with increasing the concentration (Fig. 2).

IC_{50} (µg/mL) of ethanol, ethyl acetate, n-hexane and water extracts and Ascorbic Acid was found to be 248.09±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.

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 1. 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.

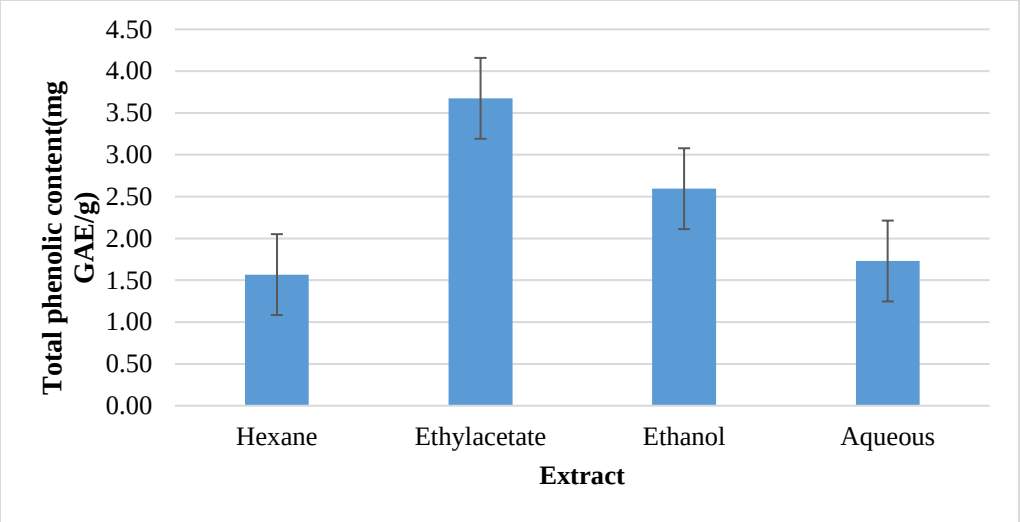

Fig. 1. Total phenolic content of the different extracts of the *Nāgarādi panchakaya*

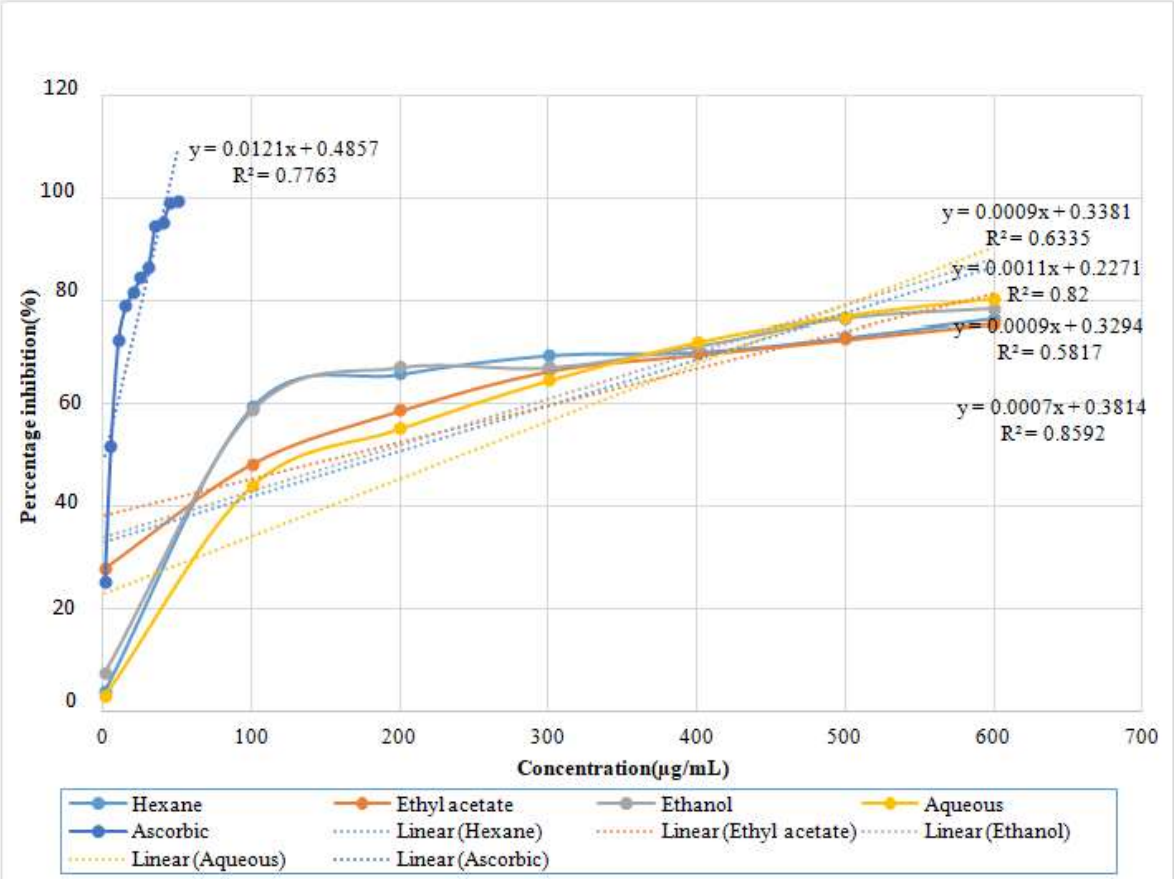

Fig. 2. DPPH free radical scavenging activity for standard and different extracts of *Nāgarādi panchakaya*)

Table 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

AQ – Aqueous extract; ETH – Ethanol extract; EA – Ethyl acetate extract; HEX – Hexane extract

The *Kashāya kalpanā* (hot water extracts /decoction) which is mainly use dosage form in Traditional/ Ayurveda medicine. The method of preparation of hot water extracts/decoction also described in Ayurveda texts.

Natural antioxidants increase the strength of the antioxidants in the body and as a result, reduce the incidence of certain diseases, such as cancers, heart disease, and stroke. Also, Natural antioxidant compounds are significant as important drug compounds and have a high ability to cope with many diseases [17]. Research works on the antioxidant activity of such poly-herbal decoction (hot water extract) were very few. Among them also the method preparation of hot water extract was not followed the way which has mentioned in authentic texts [18]. In this study it is also revealed that the extracts prepared by the traditional methods (hot water extract) has shown the highest antioxidant activity. But the antioxidant studies on single herbs used in traditional medicine in various countries were easily available [19-21].

With the development of research culture, the analysis of various solvent extracts of herbal formulae also latest trend in the world. One study was conducted in Korea, by using the hexane, ethyl acetate, n-butanol, and water extracts of 10 Korean herbal medicines. They were screened and compared for their antioxidant activities. Among the four solvent extracts, the antioxidant activities of more-polar solvent extracts (n-butanol and water extracts) were relatively higher than that of non-polar solvent extracts (hexane and ethyl acetate extracts) [22]. In this study also it is revealed that antioxidant activity was more dominant in aqueous extract and Ethanol extracts comparing with other two solvents.

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. The synergistic 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.

4. CONCLUSION

When analyzing the results of this study N5 decoction clearly shows significant concentration dependent antioxidant activity for DPPH and FRAP 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 and these results will be useful to further analysis of the herbal medicines that contain the highest antioxidant activity in order to identify the active principles that can be used for their clinical application.

5. LIMITATION OF THE STUDY

Analysis of Anti-oxidant activity of the research formula was limited for DPPH and FRAP assays.

NOTE

The study highlights the efficacy of "Ayurveda" 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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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