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IN VITRO EVALUATION OF THE NITRIC OXIDE RADICAL SCAVENGING ACTIVITY OF THIPPILI RASAYANAM: SCIENTIFIC EVIDENCE FOR ITS ANTIOXIDANT POTENTIAL IN CHILDHOOD ASTHMA.

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ABSTRACT

Childhood asthma is a chronic inflammatory airway disease in which oxidative stress contributes significantly to airway inflammation, bronchial hyperresponsiveness, and disease progression through the excessive production of nitric oxide (NO) and other reactive oxygen species. Thippili Rasayanam, a classical Siddha polyherbal formulation traditionally used for the management of respiratory disorders, contains medicinal plants with documented antioxidant and anti-inflammatory properties; however, its nitric oxide radical scavenging activity has not been scientifically validated. The present in vitro experimental study evaluated the antioxidant potential of Thippili Rasayanam using the sodium nitroprusside–Griess assay. The formulation was tested at concentrations of 10–100 µg/mL, with gallic acid serving as the reference standard. Nitric oxide radical scavenging activity was determined by measuring the percentage inhibition of nitrite formation, and antioxidant potency was expressed as the half-maximal inhibitory concentration (IC_{50}). All experiments were performed in triplicate, and the results were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, with $p < 0.05$ considered statistically significant. Thippili Rasayanam exhibited concentration-dependent nitric oxide radical scavenging activity, with percentage inhibition increasing from 9.66 ± 2.59% at 10 µg/mL to 37.68 ± 1.49% at 100 µg/mL, whereas gallic acid showed significantly higher inhibition ranging from 25.57 ± 2.25% to 93.81 ± 2.13%. The IC_{50} values of Thippili Rasayanam and gallic acid were 145.6 ± 4.74 µg/mL and 39.82 ± 3.53 µg/mL, respectively. These findings demonstrate that Thippili Rasayanam possesses significant in vitro nitric oxide radical scavenging activity in a concentration-dependent manner, providing scientific evidence for its traditional use in respiratory disorders. Although its antioxidant potency was lower than that of gallic acid, the formulation exhibited appreciable free radical scavenging activity, supporting its potential as a complementary therapeutic candidate for childhood asthma. Further phytochemical, mechanistic, in vivo, and clinical studies are warranted to validate its therapeutic efficacy.

KEYWORDS: Thippili Rasayanam; Siddha medicine; childhood asthma; antioxidant activity; Nitric oxide radical scavenging; oxidative stress; Polyherbal formulation.

1. INTRODUCTION

Childhood asthma is one of the most common chronic inflammatory respiratory diseases worldwide and remains a major public health concern because of its high prevalence, recurrent exacerbations, impaired quality of life, school absenteeism, and increased healthcare utilization. According to the Global Initiative for Asthma (GINA 2025), asthma is a heterogeneous disease characterized by chronic airway inflammation, variable respiratory symptoms, and reversible airflow limitation. Despite significant advances in pharmacological management, many children continue to experience inadequate asthma control and persistent airway inflammation, emphasizing the need for complementary therapeutic approaches that target the underlying pathogenic mechanisms (1,2).

Oxidative stress has emerged as a critical factor in the pathogenesis and progression of childhood asthma. An imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the endogenous antioxidant defense system leads to oxidative injury of airway epithelial cells, chronic inflammation, bronchial hyperresponsiveness, mucus hypersecretion, and airway remodeling (3–5). Among reactive nitrogen species, nitric oxide (NO) plays an important physiological role in regulating airway tone, vascular homeostasis, and host defense. However, excessive production of NO by inducible nitric oxide synthase (iNOS) during airway inflammation results in the formation of reactive nitrogen intermediates, including peroxynitrite, which induce oxidative damage to lipids, proteins, and nucleic acids and amplify airway inflammation (4–6).

Recent evidence indicates that dysregulated nitric oxide metabolism and oxidative stress are closely associated with asthma severity, persistent airway inflammation, and poor clinical outcomes in children. Oxidative stress also activates several inflammatory signaling pathways, including nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and nuclear factor erythroid 2-related factor 2 (Nrf2), thereby promoting the production of pro-inflammatory cytokines and perpetuating chronic airway inflammation (5,7,8). Furthermore, fractional exhaled nitric oxide (FeNO) has become a well-established non-invasive biomarker of type 2 airway inflammation and is recommended for evaluating airway inflammation, predicting corticosteroid responsiveness, and monitoring asthma control in children (1,6).

Because oxidative stress plays a pivotal role in asthma pathogenesis, antioxidant therapy has attracted increasing attention as an adjunctive strategy for disease management. Numerous medicinal plants contain phytochemicals such as flavonoids, phenolic acids, alkaloids, tannins, terpenoids, and other polyphenolic compounds that possess potent antioxidant and anti-inflammatory activities. These bioactive constituents scavenge reactive oxygen and nitrogen species, inhibit lipid peroxidation, preserve endogenous antioxidant enzymes, suppress NF-κB-mediated inflammatory signaling, regulate nitric oxide production, and protect airway tissues against oxidative injury (5,7,9).

The Siddha system of medicine has traditionally employed several polyherbal formulations for the treatment of respiratory disorders. Thippili Rasayanam (TR) is a classical Siddha formulation indicated for asthma, chronic cough, and bronchitis. The formulation consists of Piper longum, Piper nigrum, Zingiber officinale, Elettaria cardamomum, Embelia ribes, Syzygium aromaticum, and Terminalia chebula, all of which have been reported to possess antioxidant, anti-inflammatory, bronchodilatory, and immunomodulatory activities. Piperine, the principal alkaloid of Piper longum, together with phenolic compounds, flavonoids, gingerols, shogaols, eugenol, and embelin, has been shown to reduce oxidative stress, modulate inflammatory signaling pathways, and inhibit the production of inflammatory mediators associated with respiratory diseases (9–12).

The nitric oxide radical scavenging assay was selected in the present study because it is a simple, sensitive, reproducible, and widely accepted in vitro method for evaluating the ability of natural products to neutralize nitric oxide-derived free radicals. Sodium nitroprusside spontaneously releases nitric oxide under physiological conditions, which reacts with oxygen to generate nitrite ions that are quantified using the Griess reagent. A reduction in nitrite formation reflects the nitric oxide scavenging capacity of the test compound and provides an important indicator of antioxidant potential. Since excessive nitric oxide production contributes directly to oxidative stress and airway inflammation in childhood asthma, evaluation of nitric oxide radical scavenging activity provides valuable preliminary evidence regarding the antioxidant and potential anti-inflammatory properties of herbal formulations (13–15).

Although several individual constituents of Thippili Rasayanam have been investigated for their pharmacological activities, limited scientific evidence is available regarding the antioxidant activity of the complete Siddha formulation, particularly its nitric oxide radical scavenging potential. Therefore, the present study aimed to evaluate the in vitro nitric oxide radical scavenging activity of Thippili Rasayanam using the sodium nitroprusside–Griess assay and to compare its antioxidant activity with that of gallic acid, thereby providing preliminary scientific evidence supporting its potential therapeutic role in reducing oxidative stress associated with childhood asthma.

2. MATERIALS AND METHODS

2.1 Study Design

This in vitro experimental study was conducted to evaluate the nitric oxide (NO) radical scavenging activity of Thippili Rasayanam (TR), a classical Siddha polyherbal formulation. Antioxidant activity was determined using the sodium nitroprusside–Griess assay, a well-established colorimetric method for assessing nitric oxide radical scavenging activity. The study was performed at Noble Research Solutions Laboratory, Chennai, Tamil Nadu, India. All experiments were carried out in triplicate (n = 3), and the results were expressed as mean ± standard deviation (SD). The experimental procedure was adapted from the method described by Panda et al. with minor modifications (17,19).

2.2 Test Drug

Thippili Rasayanam (TR), a classical Siddha polyherbal formulation indicated for the management of respiratory disorders including asthma, chronic cough, and bronchitis, was selected as the test drug (16). The formulation consists of medicinal plants with reported antioxidant, anti-inflammatory, bronchodilatory, and immunomodulatory properties.

Table 1. Composition of Thippili Rasayanam (16)

S. No.	Botanical name	Part used	Quantity
1	Piper longum	Fruit	70 g
2	Piper longum	Root	8.5 g
3	Zingiber officinale	Dried rhizome	8.5 g
4	Piper nigrum	Dried fruit	8.5 g
5	Elettaria cardamomum	Unripe fruit	8.5 g

6	Embelia ribes	Seed	8.5 g
7	Syzygium aromaticum	Flower bud	8.5 g
8	Terminalia chebula	Fruit rind	8.5 g
9	Honey	–	Quantity sufficient
10	Palm jaggery	–	Quantity sufficient

2.3 Procurement and Preparation of the Formulation

The raw herbal ingredients were procured from Ramasamy Chetty Medical Store, Chennai, Tamil Nadu, India. All botanical ingredients were authenticated by a Medicinal Botanist, National Institute of Siddha, Tambaram Sanatorium, Chennai, India. The formulation was prepared in the Gunapadam Laboratory, National Institute of Siddha, following the purification and preparation procedures described in Sigicha Rathna Deepam and The Siddha Formulary of India (16,18). After purification, the ingredients were gently roasted, powdered individually, blended in the prescribed proportions, and triturated with honey and palm jaggery to obtain a homogeneous semisolid formulation.

2.4 Chemicals and Reagents

All chemicals and reagents were of analytical grade. Sodium nitroprusside was used as the nitric oxide donor, while phosphate-buffered saline (PBS; pH 7.4) served as the reaction medium. The Griess reagent, consisting of sulphanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride dissolved in phosphoric acid, was used for nitrite estimation. Gallic acid was used as the reference antioxidant (17,19).

2.5 Preparation of Test and Standard Solutions

A stock solution of Thippili Rasayanam was prepared in a suitable solvent and serially diluted to obtain concentrations of 10, 20, 40, 60, 80, and 100 µg/mL. Gallic acid was prepared similarly and used as the positive control. Fresh solutions were prepared immediately before each experiment (17,19).

2.6 Nitric Oxide Radical Scavenging Assay

Nitric oxide radical scavenging activity was evaluated using the sodium nitroprusside–Griess assay described by Panda et al. (17). Briefly, 0.5 mL of sodium nitroprusside solution was mixed with 1.0 mL of the test or standard solution at the specified concentrations and incubated at 25°C for 180 min. During incubation, sodium nitroprusside generated nitric oxide, which reacted with atmospheric oxygen to form nitrite ions. After incubation, an equal volume of freshly prepared Griess reagent was added, and the absorbance was measured at 546 nm using a Shimadzu UV-1800 UV–Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Gallic acid served as the positive control, while the reaction mixture without the test sample served as the negative control (19).

2.7 Determination of Nitric Oxide Radical Scavenging Activity

Nitric oxide radical scavenging activity was expressed as the percentage inhibition of nitrite formation using the following equation (19):

percentage nitrite radical scavenging activity:

$$\text{nitric oxide scavenged } (\%) = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100,$$

where A_{control} = absorbance of control sample and A_{test} = absorbance in the presence of the samples extracts or standards.

where $A_{(C)}$ represents the absorbance of the control and $A_{(S)}$ represents the absorbance of the test sample.

2.8 Determination of IC_{50}

The half-maximal inhibitory concentration (IC_{50}) was calculated from the concentration–response curve using nonlinear regression analysis. The IC_{50} value represents the concentration required to inhibit 50% of nitric oxide radicals, with lower IC_{50} values indicating greater antioxidant activity (21).

2.9 Statistical Analysis

All experiments were performed in triplicate (n = 3), and the results are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism version 10.0 (GraphPad Software Inc., San Diego, CA, USA) (20). Differences between Thippili Rasayanam and gallic acid at different concentrations were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A p-value < 0.05 was considered statistically significant.

3.RESULTS

3.1 Nitric Oxide Radical Scavenging Activity

The nitric oxide radical scavenging activity of Thippili Rasayanam (TR) was evaluated at concentrations ranging from 10–100 µg/mL and compared with the reference antioxidant, gallic acid. TR demonstrated a concentration-dependent increase in nitric oxide radical scavenging activity, with percentage inhibition increasing from 9.66 ± 2.59% at 10 µg/mL to 37.68 ± 1.49% at 100 µg/mL (Table 1; Figure 1). Gallic acid exhibited significantly higher scavenging activity at all tested concentrations, with percentage inhibition ranging from 25.57 ± 2.25% to 93.81 ± 2.13%. Statistical analysis using one-way ANOVA followed by Dunnett's multiple comparison test demonstrated that the nitric oxide scavenging activity of gallic acid was significantly greater than that of TR at each tested concentration ($p < 0.05$).

Table 1. Nitric oxide radical scavenging activity of Thippili Rasayanam and gallic acid.

Concentration (µg/mL)	Thippili Rasayanam (% inhibition, Mean ± SD)	Gallic acid (% inhibition, Mean ± SD)
10	9.66 ± 2.59	25.57 ± 2.25
20	15.96 ± 2.25	41.25 ± 3.60
40	19.49 ± 1.48	49.42 ± 6.36
60	26.14 ± 1.53	58.71 ± 1.68
80	29.62 ± 1.93	82.50 ± 2.68
100	37.68 ± 1.49	93.81 ± 2.13

3.2 Comparative Antioxidant Activity

A progressive increase in nitric oxide radical scavenging activity was observed for both TR and gallic acid with increasing concentration. However, gallic acid consistently demonstrated significantly greater antioxidant activity than TR at all concentrations evaluated ($p < 0.05$). At the highest concentration tested (100 µg/mL), gallic acid produced 93.81 ± 2.13% inhibition, whereas TR produced 37.68 ± 1.49% inhibition, indicating comparatively lower antioxidant potency of the Siddha formulation.

3.3 IC_{50} Determination

The antioxidant potency of the test formulation and reference standard was assessed by calculating the half-maximal inhibitory concentration (IC_{50}) from the concentration-response curves (Table 2; Figure 2). Thippili Rasayanam exhibited an IC_{50} value of 145.6 ± 4.74 µg/mL, whereas gallic acid exhibited a significantly lower IC_{50} value of 39.82 ± 3.53 µg/mL, indicating stronger nitric oxide radical scavenging activity of the reference antioxidant.

Table 2. IC_{50} values of Thippili Rasayanam and gallic acid.

Sample	IC_{50} (µg/mL, Mean ± SD)
Thippili Rasayanam	145.6 ± 4.74
Gallic acid	39.82 ± 3.53

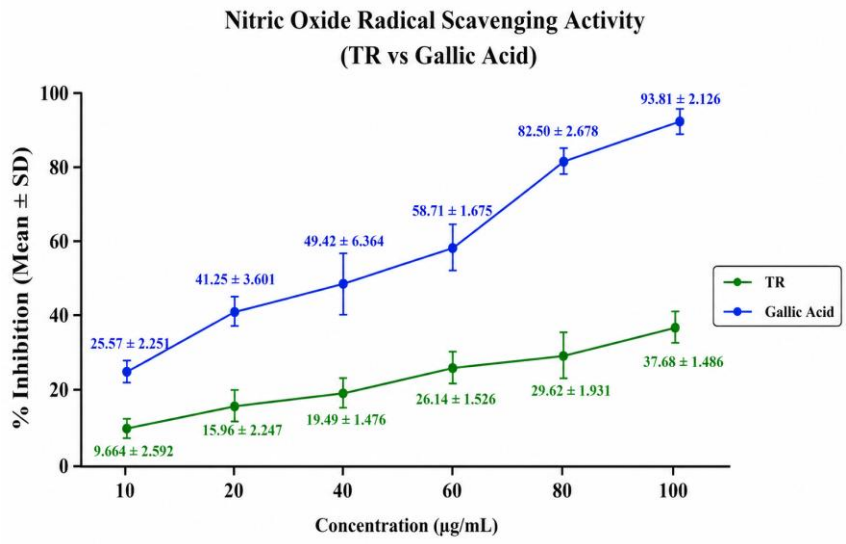

Figure 1. Concentration-dependent nitric oxide radical scavenging activity of Thippili Rasayanam and gallic acid. Values are expressed as mean ± SD (n = 3).

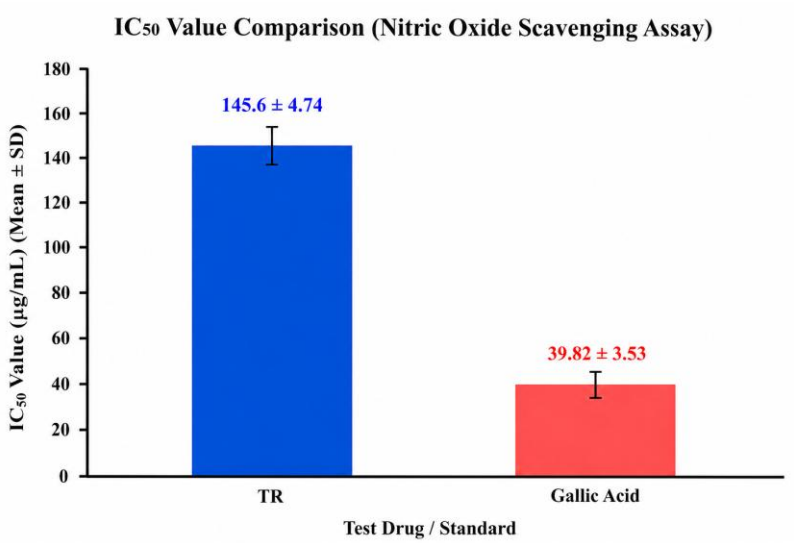

Figure 2. IC_{50} values of Thippili Rasayanam and gallic acid obtained from concentration-response curves.

4. DISCUSSION

Oxidative stress is recognized as a key pathogenic factor in childhood asthma, resulting from an imbalance between the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the endogenous antioxidant defence system. Excessive production of reactive species, particularly nitric oxide (NO), contributes to airway epithelial injury, chronic inflammation, bronchial hyperresponsiveness, mucus hypersecretion, and airway remodelling. During airway inflammation, inducible nitric oxide synthase (iNOS) generates large amounts of NO, which reacts with superoxide radicals to form peroxynitrite, a highly reactive oxidant capable of damaging cellular lipids, proteins, and nucleic acids. Consequently, antioxidants capable of scavenging nitric oxide radicals may help attenuate oxidative stress and reduce inflammatory damage associated with childhood asthma (22–24).

In the present study, Thippili Rasayanam (TR) exhibited concentration-dependent nitric oxide radical scavenging activity, with percentage inhibition increasing from 9.66 ± 2.59% at 10 µg/mL to 37.68 ± 1.49% at 100 µg/mL. In comparison, the reference antioxidant gallic acid demonstrated significantly greater scavenging activity across all tested concentrations. The comparatively lower antioxidant activity of TR was further reflected by its higher IC_{50} value (145.6 ± 4.74 µg/mL) compared with gallic acid (39.82 ± 3.53 µg/mL). Nevertheless, the concentration-dependent response observed in the present investigation indicates that the formulation possesses measurable nitric oxide scavenging activity and moderate antioxidant potential (22,25).

The antioxidant activity of Thippili Rasayanam is likely attributable to the synergistic action of its diverse phytochemical constituents. The formulation contains medicinal plants such as Piper longum, Piper nigrum, Zingiber officinale, Elettaria cardamomum, Embelia ribes, Syzygium aromaticum, and Terminalia chebula, which are rich sources of bioactive compounds including piperine, gingerols, shogaols, eugenol, embelin, flavonoids, tannins, and phenolic compounds. These phytochemicals have been reported to neutralize reactive oxygen and nitrogen species, inhibit lipid peroxidation, preserve endogenous antioxidant enzymes, suppress NF-κB-mediated inflammatory signaling, and regulate nitric oxide production, thereby protecting airway tissues from oxidative injury and inflammation (23,24,26).

Nitric oxide radical scavenging assays provide an important preliminary approach for evaluating the antioxidant potential of herbal formulations because nitric oxide is a major mediator of inflammatory responses in asthma. Although physiological concentrations of nitric oxide contribute to normal airway function, excessive nitric oxide production during chronic airway inflammation amplifies oxidative stress and inflammatory signaling. Therefore, compounds capable of reducing nitric oxide-derived free radicals may contribute to improved airway protection and reduced inflammatory responses. The concentration-dependent nitric oxide scavenging activity observed for Thippili Rasayanam suggests that its antioxidant effect may contribute, at least in part, to its traditional therapeutic use in respiratory disorders (22–24).

The observed antioxidant activity is consistent with previous reports demonstrating that polyherbal formulations exert pharmacological effects through the combined action of multiple phytochemicals rather than a single active constituent. Synergistic interactions among antioxidant and anti-inflammatory compounds may enhance free radical scavenging capacity while simultaneously modulating inflammatory pathways involved in asthma pathogenesis. Such multitarget activity is particularly relevant in childhood asthma, where oxidative stress, immune dysregulation, and chronic inflammation coexist and contribute to disease progression (23,24,26).

The present study has certain limitations. The antioxidant activity of Thippili Rasayanam was evaluated only under in vitro conditions using the nitric oxide radical scavenging assay. Although this assay provides a reliable preliminary assessment of antioxidant potential, it does not fully represent the complex biological processes occurring in vivo. Furthermore, only one antioxidant assay was employed; therefore, additional investigations using complementary antioxidant assays, detailed phytochemical characterization, cellular and animal models,

and well-designed clinical studies are required to confirm the antioxidant efficacy and therapeutic mechanisms of Thippili Rasayanam in childhood asthma (25,27).

5. CONCLUSION

The present study demonstrated that Thippili Rasayanam possesses concentration-dependent nitric oxide radical scavenging activity, indicating its antioxidant potential. Although its antioxidant activity was lower than that of the standard antioxidant, the formulation exhibited appreciable free radical scavenging capacity. The observed antioxidant effect may be attributed to the synergistic action of its phytoconstituents, including phenolics, flavonoids, tannins, and piperine, which are known to modulate oxidative stress and inflammatory pathways. These findings provide scientific support for the traditional Siddha use of Thippili Rasayanam in the management of childhood asthma and other respiratory disorders. However, further phytochemical investigations, mechanistic studies, in vivo experiments, and well-designed clinical trials are required to confirm its therapeutic efficacy and elucidate its mechanism of action.

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REFERENCES

1. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention: 2025 Update. Fontana (WI): Global Initiative for Asthma; 2025. Available from:
2. Papadopoulos NG, Custovic A, Cabana MD, et al. Childhood asthma: recent advances in pathogenesis, diagnosis, and management. Lancet Respir Med. 2024.
3. Barnes PJ. Oxidative stress-based therapeutics in asthma and chronic obstructive pulmonary disease. Antioxid Redox Signal. 2020;33(3):181-199.
4. Holguin F. Oxidative stress in airway diseases. Ann Am Thorac Soc. 2013;10(Suppl):S150-S157.
5. Aldakheel FM, Thomas PS, Bourke JE, Matheson MC. Oxidative stress and asthma: molecular mechanisms and therapeutic strategies. Antioxidants (Basel). 2022;11:975.
6. Dweik RA, Boggs PB, Erzurum SC, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FeNO) for clinical applications. Am J Respir Crit Care Med. 2011;184(5):602-615.
7. Ahmad A, Rehman MU, Ahmad P, et al. Phytochemicals as modulators of oxidative stress and inflammation in respiratory diseases. Biomed Pharmacother. 2022;152:113248.
8. Bezerra Filho CSM, et al. Piperine: pharmacological activities and therapeutic potential. Phytother Res. 2023;37:2251-2274.
9. Gorgani L, Mohammadi M, Najafpour GD, Nikzad M. Piperine—the bioactive compound of black pepper: from isolation to medicinal formulations. Compr Rev Food Sci Food Saf. 2017;16(1):124-140.
10. Kannusami Pillai. Sigicha Rathna Deepam. Part I. Chennai: Department of Indian Medicine and Homeopathy; 2007.
11. Serreli G, Deiana M. Role of dietary polyphenols in nitric oxide regulation and antioxidant activity. Antioxidants (Basel). 2023;12(1):147.
12. Subedi L, Gaire BP, Parveen A, Kim SY. Nitric oxide as a therapeutic target for phytochemicals. Int J Mol Sci. 2021;22(9):4771.
13. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem. 1982;126(1):131-138.
14. Panda BN, Raj AB, Shrivastava NR, Prathani AR. Evaluation of nitric oxide scavenging activity of Acalypha indica Linn. root. Asian J Res Chem. 2009;2(2):148-150.
15. Sebaugh JL. Guidelines for accurate EC50/IC50 estimation. Pharm Stat. 2011;10(2):128-134.
16. Kannusami Pillai. Sigicha Rathna Deepam. Part I. Chennai: Department of Indian Medicine and Homeopathy, Government of Tamil Nadu; 2007.
17. Panda BN, Raj AB, Shrivastava NR, Prathani AR. Evaluation of nitric oxide scavenging activity of Acalypha indica Linn. root. Asian J Res Chem. 2009;2(2):148–150.
18. Anonymous. The Siddha Formulary of India. Part I. 1st ed. New Delhi: Ministry of Health and Family Welfare, Government of India; 1992.
19. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem. 1982;126(1):131–138.

20. GraphPad Software LLC. GraphPad Prism User Guide. Version 10.0. San Diego, CA: GraphPad Software LLC; 2023.
21. Sebaugh JL. Guidelines for accurate EC50/IC50 estimation. Pharm Stat. 2011;10(2):128–134.
22. Serreli G, Deiana M. Role of dietary polyphenols in nitric oxide regulation and antioxidant activity. Antioxidants (Basel). 2023;12(1):147.
23. Subedi L, Gaire BP, Parveen A, Kim SY. Nitric oxide as a therapeutic target for phytochemicals. Int J Mol Sci. 2021;22(9):4771.
24. Barnes PJ. Oxidative stress-based therapeutics in asthma and chronic obstructive pulmonary disease. Antioxid Redox Signal. 2020;33(3):181-199.
25. Sebaugh JL. Guidelines for accurate EC50/IC50 estimation. Pharm Stat. 2011;10(2):128-134.
26. Ahmad A, Rehman MU, Ahmad P, et al. Phytochemicals as modulators of oxidative stress and inflammation in respiratory diseases. Biomed Pharmacother. 2022; 152:113248.
27. Organisation for Economic Co-operation and Development (OECD). Guidance Document on Good In Vitro Method Practices (GIVIMP). OECD Series on Testing and Assessment No. 286. Paris: OECD Publishing; 2018.