Assessment of anti-oxidant activity and High-Performance Thin-Layer Chromatography characters of different compositions of *Triphala* powder

Silva H.H.S.S., Hapuarachchi S.D.*, Madhushika K.D.C., Perera P.K.

Abstract

Triphala: a polyherbal formulation used in both Ayurveda and Sri Lankan Traditional Medical practices. Aim of the present study is to compare the chemical characteristics of distinct compositions of Triphala powder as mentioned in various Ayurveda Authentic Texts. Dried fruits of *Terminalia chebula* Retz. (TC), *Terminalia bellirica* Gaertn. (TB), and *Phyllanthes emblica* (PE) were purchased and authenticated. Followed by a comprehensive literature review, five different compositions of Triphala dried powdered samples as S_1 (1:1:1), S_2 (1:2:3), S_3 (1:2:4), S_4 (1:2:2) TC: TB: PE respectively and S_5 – 1:2:4 ratio based on the fruits of TC: TB: PE were subjected to ethanol extractions. Anti-oxidant activity and High-Performance Thin-Layer Chromatography (HPTLC) characters of the five samples was evaluated. HPTLC analysis of the samples against the standard solutions of Gallic acid (GA) and Tannic acid (TA) was carried out with Toluene: Ethyl acetate: Formic acid (2:5:1.5) as the mobile phase. Antioxidant activity was evaluated using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay against the standard Ascorbic Acid (AA). The HPTLC analysis of all five samples showed similar patterns with respect to their peaks and intensities while showing peaks corresponding to the peaks of standards. A high level of anti-oxidant activity was found in the samples varying in the decreasing order of S_3 (1:2:4) > S_1 (1:1:1) > S_4 (1:2:2)> S_2 (1:2:3) > S_5 (1:2:4 Fruits). In spite of the high level of antioxidant activity observed with all five samples, the most pronounced level of antioxidant activity was found in S_3 powder sample. Further studies need to be carried out on bioactivity studies to evaluate the therapeutic efficacy of different compositions of *Triphala* powder.

Keywords: *Triphala* powder, Ayurveda authentic texts, HPTLC, Anti-oxidant activity, DPPH assay

Introduction

Triphala constitutes a polyherbal formulation deeply rooted in both Ayurveda and Sri Lankan Traditional Medical practices. *Triphala,* a term derived from the Sanskrit language where "*tri*" signifies "three," and "*Phala*" translates to "fruits" in English, conveys a specific reference to a compound comprising the dried pericarps of three botanical constituents: *Haritaki (Terminalia chebula), Vibhitaki (Terminalia bellirica)* both of which belong to the Combretaceae family and *Amalaki (Phyllanthus emblica)* belong to the Euphorbiaceae family.

This herbal formulation, combining the therapeutic properties of these three fruits, has holistic and numerous health benefits. *Triphala* possesses a myriad of health-enhancing qualities attributable to its various properties, including but not limited to its anti-diabetic, antioxidant, antibacterial, anti-inflammatory, free radical scavenging, immune modulating, appetite stimulation, gastric hyperacidity reduction, prevention of dental caries, antipyretic, analgesic, antibacterial, antimutagenic, wound healing, anticarcinogenic, antistress, adaptogenic, hypo- glycemic, anticancer, hepatoprotective, chemoprotective, radio-

Department of Ayurveda Pharmacology, Pharmaceutics and Community Medicine, Faculty of Indigenous Medicine, University of Colombo, Sri Lanka.

**Correspondence: Hapuarachchi S.D., Department of Ayurveda Pharmacology, Pharmaceutics and Community Medicine, Faculty of Indigenous Medicine, University of Colombo, Sri Lanka.*
Email: dr.sdhapuarachchi@fim.cmb.ac.lk

protective, and chemo preventive effects[1]. This polyherbal formulation is proven to enhance proper digestion and absorption of food, improves circulation, increase production of red blood cells and hemoglobin, lowers serum cholesterol levels, relax bile ducts, prevent immune senescence, maintain homeostasis of the endocrine system1. Previous studies have proven the potential of *Triphala* in weight reduction and reduction of body fat. Furthermore, *Triphala* also decreased total cholesterol, triglycerides, and low-density lipoprotein cholesterol[2]. A clinical study carried out on noninsulin-dependent diabetes mellitus patients have clearly shown that supplementation with 5.0 g of *Triphala* powder for 45 days significantly lowered blood glucose levels[3]. Reduction of abdominal pain, hyperacidity, constipation, mucous, and flatulence while improving the frequency, yield, and consistency of stool was observed in a clinical trial that investigated the use of *Triphala* in patients with gastrointestinal disorders[4]. Furthermore, *Triphala churna* has shown to have multiple beneficial effects in diabetic neuropathy which may be attributed to reduced oxidative stress, inhibition of inflammatory cytokines and increased expression of Nerve Growth Factor in rats[5]. Several previous studies proven that *Triphala* exerts an antineoplastic effect on many cancer cell lines, including cancers of the prostate, colon, breast, and pancreas[6-8]. Based on animal studies carried out on stress, researchers have determined that *Triphala* has a potential of protecting against cold-induced stress and reversed stress-induced behavioral alterations and biochemical changes such as increased lipid peroxidation and corticosterone levels[9]. In addition to cold induced stress, previous studies prove that *Triphala* also prevented noise-induced stress[10]. In vitro studies the *Triphala* extract exhibited significant free radical scavenging activity on hydrogen peroxide- induced cell damage and senescence proving that T*riphala* extract exerted highly protective antiaging effects on human skin cells[11].

Antioxidants are crucial for maintaining overall health and preventing oxidative damage. The popularity of the antioxidant activity exhibited by *Triphala* is well-established through various research findings. Antioxidant effects of *Triphala* is considerably more significant to help maintain eye health. *Triphala* serves as a reservoir of vitamin C and a variety of flavonoids. Significant restoration of glutathione levels in eye lenses was observed in a study where *Triphala* was used as a pretreatment in selenite-induced cataracts in mice. Furthermore, *Triphala* increased the activities of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione-S-transferase, and glutathione peroxidase, in the eye lenses[12].

Previous research studies have discovered that tannins, gallic acid, ellagic acid, and chebulinic acid as the major chemical constituents of *Triphala* which are potent antioxidants responsible for the observed immunomodulatory activity of Triphala[13-15]. Along with that, studies have also proven that *Triphala* contains many other bioactive compounds like flavonoids (e.g., quercetin and luteolin), saponins, anthra-quinones, aminoacids, fattyacids, and various carbohydrates[16]. Furthermore, depicting the Anti-oxidant properties, this formulation is proven to contain *Triphala*-derived polyphenols such as chebulinic acid which is transformed by the human gut microbiota into bioactive metabolites, exhibiting its promising in vitro potential for the prevention of oxidative damage[17].

Numerous compositions of this blend have been extensively mentioned within Authentic Ayurveda texts, including Charaka Samhita, Susruta Samhita, Bhavaprakasha, Yogaratnakara, Madanapala Nighantu, Kaiyadeva Nighantu, Chakradatta, Sharangadhara Samhita, and through oral transmission within the Ayurveda tradition. Susruta Samhitha mentions that *Triphala* pacifies *Kapha* and *Pitta dosha*, effective in curing diabetes, skin diseases, promotes vision, enhances digestive fire and cures intermittent fevers[18]. According to Yogarathnakara the combination, *Triphala* is beneficial in edema/ inflammation, diabetes, intermittent fevers, improve appetite, alleviates *Kapha* and *Pitta* and *Kushta* (skin diseases) and has *Rasayana* (rejuvenation) and overcomes eye

diseases[19]. Bhavaprakasha mentions that reducing *Kapha* and *Pitta*, useful in curing urinary diseases and glycosuria, potential laxative, beneficial for eyes, improving appetite, promoting taste sensation and curing Malarial fevers as the properties of Triphala[20]. In Charaka Samhitha, *Triphala* is mentioned under *Rasayana chikitsa* (Rejuvenation Therapy)[21]. According to Acharya Charaka a person undergoing rejuvenation therapy attains long life, good memory, intellect, helps to be free from diseases, maintains youth, promotes excellence of luster, complexion and voice, promote excellent potentiality of the body, senses respect and brilliance [22].

The present study endeavors to compare the chemical characteristics of distinct compositions of *Triphala* powder as mentioned in various Ayurveda authentic texts. Specifically, this study conducted a comparative analysis of the High-Performance Thin-Layer Chromatography (HPTLC) profiles and evaluated and compared the antioxidant activity of distinct compositions of *Triphala* powder, as specified in diverse Ayurveda authentic texts.

Materials and methods

Identification of different compositions of Triphala powder

The identification of various compositions of *Triphala* powder was achieved following a comprehensive literature review in the Authentic Ayurveda texts: Charaka Samhita, Susruta Samhita, Bhavaprakasha, Yogaratnakara, Madanapala Nighantu, Kaiyadeva Nighantu, Chakradatta, Sharangadhara Samhita, and through oral transmission within the Ayurveda tradition.

Preparation of Triphala powder of different compositions

The fruits of *Terminalia chebula* (TC), *Terminalia bellirica* (TB), and *Phyllanthes emblica* (PE) were purchased from the local market and authenticated from the Department of Ayurveda Pharmacology, Pharmaceutics and Community Medicine, Faculty of Indigenous Medicine, University of Colombo, Rajagiriya. The ingredients were cleaned, washed and dried. The resultant dried materials were finely pulverized (sieve size No. 120), yielding five samples, S_1 (1:1:1), S_2 (1:2:3), S_3 (1:2:4), S_4 (1:2:2) for the powders of TC: TB: PE respectively and S_5 – 1:2:4 for the fruits of TC: TB: PE, in accordance with the specified compositions documented in authentic Ayurveda texts (Table 1).

Preparation of ethanol extracts of Triphala powder of different compositions

Ethanol extracts were prepared from 5.0 g of each sample with 150 mL of ethanol using Soxhlet apparatus. The resultant extract was concentrated and dried at 40^0C using Rotary evaporator to get the Ethanol extracts of *Triphala* powder of different compositions.

Preparation of standards: Gallic acid (GA) and Tannic acid (TA) for HPTLC

Gallic acid (GA) and Tannic acid (TA) were purchased from the local market. A precisely measured quantity of 5 mg of each GA and TA was individually dissolved in 2.5 milliliters of methanol, thereby yielding concentrations of 2 mg/mL for each respective substance.

The High-Performance Thin-Layer Chromatography (HPTLC) analysis

In HPTLC, the Stationary phase was Aluminum plates precoated with Silica gel 60 F 254, size: 10cm x10cm (Merck, Germany). The mobile phase composition was Toluene: Ethyl acetate: Formic acid (2:5:1.5)[27]. Spotted the 5 samples against the standard solutions of Gallic acid (GA) and Tannic acid (TA) at concentrations of 2 mg/mL and allowed to dry at room temperature. After saturation of the twin trough chamber for 30 minutes at room temperature the plate was developed in the solvent system and allowed to dry at room temperature. The comparability of Retention factor (Rf) values among all samples and standards was observed under UV illumination at wavelengths of 254nm and 366nm using win CATS software.

Table 1: Different compositions of *Triphala* powder in various Ayurveda authentic texts

Sample	Form	Composition of three ingredients			Original Source
		TC	TB	PE	
S_1	Powder	1	1	1	1) Susruta Samhitha, *Sutrasthana*[18] 2) Bhavaprakahsa, *Purva Khanda*[20]
S_2	Powder	1	2	3	Oral Tradition
S_3	Powder	1	2	4	1) Yogarathnakara, Basic concepts of Ayurveda[19] 2) Madanaphala Nighantu, *Abhayadi varga*[23] 3) Kaiyadeva Nighantu, *Oshagivarga*[24] 4) Chakradatta, *Rasayanadhikara*[25]
S_4	Powder	1	2	2	Oral Tradition
S_5	Fruits	1	2	4	1) Charaka Samhitha, *Chikithsastana*[21] 2) Sharangadhara Samhitha, *Madhyama Khanda*[26]

Determination of antioxidant activity of the different compositions of Triphala

Preparation of concentration series for samples and the standard: Ascorbic Acid (AA) to analyze the antioxidant activity

A concentration series for the five distinct samples and the standard Ascorbic Acid (AA) was precisely made over a range spanning from 5 parts per million (ppm) down to 0.6 ppm. This was obtained through the dissolution of both the five samples and the standard Ascorbic Acid (AA) in methanol.

To ensure reliability, this concentration series was triplicated, reinforcing the consistency and accuracy of the analytical measurements and assessments.

Analysis of the Antioxidant Activity- 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

Antioxidant capacity of all samples S_1, S_2, S_3, S_4, S_5 and the standard AA were determined through the evaluation of free radical scavenging effect on the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical[28]. A freshly prepared DPPH solution was made up to 0.5mg/mL concentration with methanol and the solution was mixed with sample series /AA solution series in methanol in 96 well plate. The entire experimental setup was then incubated in dark conditions at room temperature for a duration of 10 minutes. Upon the completion of the incubation period, the absorbance of the reaction mixtures was measured at a wavelength of 517 nanometers (nm). This quantification was performed using a UV visible spectrophotometer (Multiskan SkyHigh UV/Vis), equipped with specialized software Skanlt TM. Radical scavenging activity which was expressed as the inhibition percentage was calculated using the following formula[29];

$$\text{Percentage Inhibition} = \frac{Ac - As}{Ac} \times 100\%$$

Ac -Absorbance of the control, As - Absorbance of the sample.

Results

HPTLC analysis

The HPTLC profiles revealed the presence of nine distinct peaks exhibiting similar patterns in terms of peak profiles and their respective intensities in all samples, characterized by a range of Retention factor (Rf) values spanning from 0.01 to 0.88. The Rf values corresponding to the standards, GA and TA were identified as 0.88 and 0.73 respectively. In each of these samples, similar peaks were observed, aligning closely with the characteristic peaks demonstrated by the standards of GA and TA.

Figure 1 to 5 shows the developed HPTLC and TLC Profiles with peak densitogram of ethanol extracts of S_1, S_2, S_3, S_4, S_5 respectively. Figure 6 and 7 shows the TLC chromatogram under 366 and 254nm respectively.

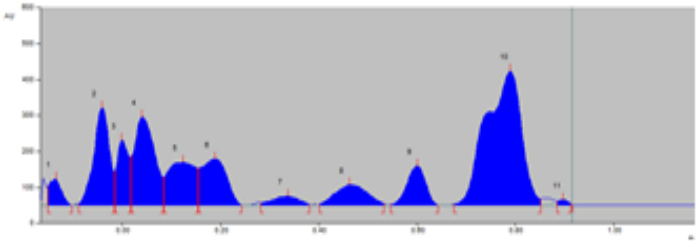

Fig. 1: HPTLC and TLC Profile with peak densitogram of ethanol extracts of S_1

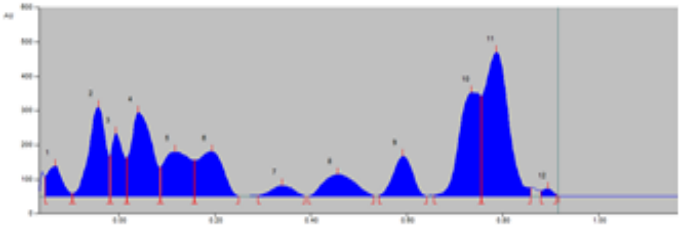

Fig. 2: HPTLC and TLC Profile with peak densitogram of ethanol extracts of S_2

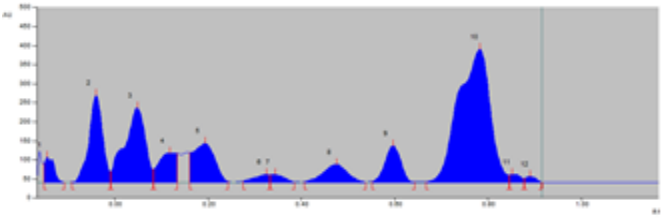

Fig. 3: HPTLC and TLC Profile with peak densitogram of ethanol extracts of S_3

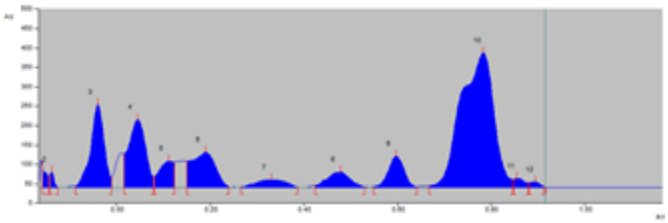

Fig. 4: HPTLC and TLC Profile with peak densitogram of ethanol extracts of S_4

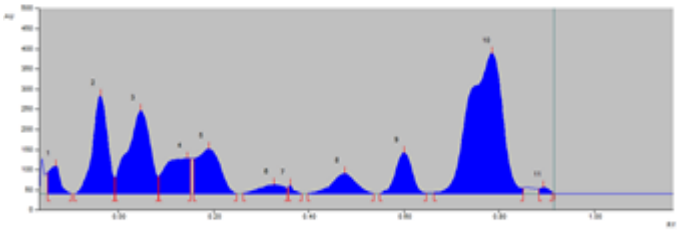

Fig. 5: HPTLC and TLC Profile with peak densitogram of ethanol extracts of S_5

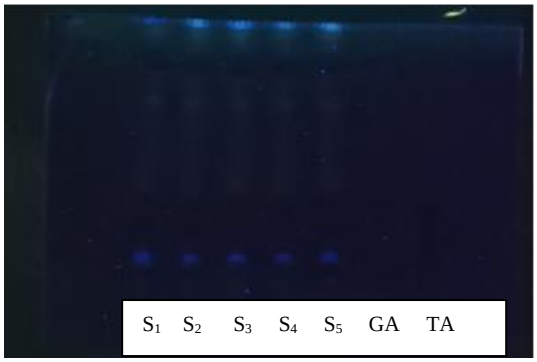

Fig. 6: TLC chromatogram under 366nm

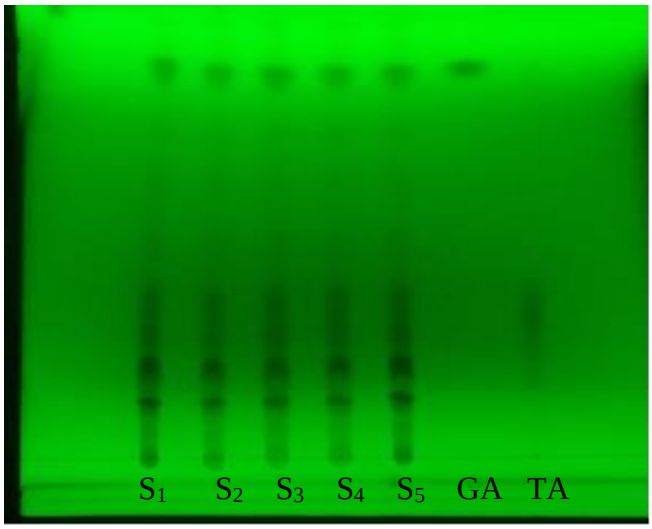

Fig. 7: TLC chromatogram under 254nm

Table 2 shows the Rf value (Retardation Factor), AU value (Area Under curve) of the 5 samples. The highest area distribution is shown a particular substance referred to as the 10th peak of S_1 (1:1:1), S_3 (1:2:4), S_4 (1:2:2) powders respectively and S_5 (1:2:4 for the fruits) and the 11th peak of S_2 (1:2:3) for the powders of *Triphala.*

Antioxidant activity of the different compositions of Triphala

DPPH Assay

Table 3 shows the ability of the 5 samples to scavenge the DPPH free radical from the obtained respective percentage inhibition values at different concentrations.

Table 4 shows the shows ability of Ascorbic acid to scavenge the DPPH free radical at different concentrations.

Table 5 shows the IC_{50} values of the *Triphala* samples and Ascorbic acid. Among the 5 samples, the lowest IC_{50} (1.32 ± 0.29 ppm) showed from S_3 sample and it varied in the ascending order ($S_3 < S_1 < S_4 < S_2 < S_5$). IC_{50} value of Sample S_3 is very much close to the standard IC50 value.

Table 2: Peak distribution and area distribution of samples in HPTLC

Peak	S_1 (1:1:1)	S_2 (1:2:3)	S_3 (1:2:4)	S_4 (1:2:2)	S_5 (1:2:4 Fruits)
4	0.02-0.08 Rf 6964.1AU	0.02-0.09 Rf 7264.4 AU	0.08 - 0.13 Rf 2036.5 AU	0.01 - 0.08Rf 4439.0 AU	0.09 - 0.15 Rf 3173.2 AU
5	0.09-0.15 Rf 4573.0AU	0.09-0.16 Rf 4967.0 AU	0.16 - 0.24 Rf 3375.1 AU	0.08 - 0.12Rf 1474.6 AU	0.16 - 0.25 Rf 3762.2 AU
6	0.16-0.24 Rf 4571.6AU	0.16-0.25 Rf 4692.0 AU	0.28 - 0.33 Rf 480.0 AU	0.15 - 0.24Rf 3232.5 AU	0.26 - 0.35 Rf 803.3 AU
7	0.28-0.38 Rf 926.4AU	0.29-0.39 Rf 971.4 AU	0.33 - 0.38 Rf 426.1 AU	0.26 - 0.39Rf 898.5 AU	0.36 - 0.38 Rf 158.1 AU
8	0.40-0.53 Rf 2556.0AU	0.40-0.53 Rf 2881.9 AU	0.41 - 0.54 Rf 1749.3 AU	0.42 - 0.53Rf 1370.2 AU	0.40 - 0.54 Rf 1745.4 AU
9	0.55-0.64 Rf 2589.7AU	0.54-0.64 Rf 3210.8 AU	0.55 - 0.64 Rf 2290.9 AU	0.55 - 0.64Rf 1910.0 AU	0.55 - 0.65 Rf 2416.6 AU
10	0.68-0.85 Rf 18716.9AU	0.66-0.75 Rf 9051.5 AU	0.67 - 0.85 Rf 16620.0 AU	0.67 - 0.85Rf 16960.8 AU	0.66 - 0.85 Rf 16736.0 AU
11	0.88-0.95 Rf 207.7AU	0.76-0.86 Rf 14141.9 AU	0.85 - 0.88 Rf 345.5 AU	0.85 - 0.88Rf 373.0 AU	0.88 - 0.91 Rf 212.8 AU
12		0.88 - 0.91 Rf 333.0 AU	0.88 - 0.92 Rf 247.4 AU	0.88 - 0.91Rf 214.6 AU	

Table 3: Percentage of Inhibition of different samples (S_1, S_2, S_3, S_4, S_5)

Concentration (ppm)	S_1 Inhibition %	S_2 Inhibition %	S_3 Inhibition %	S_4 Inhibition %	S_5 Inhibition %
5	61.89	63.93	74.35	63.53	57.29
4	59.95	54.11	70.49	62.73	53.36
3	54.51	57.04	63.11	55.44	49.24
2	52.07	47.83	58.84	52.27	42.28
1	47.74	46.33	50.32	48.77	37.02
0.8	46.37	41.54	45.73	44.13	36.5
0.6	45.50	42.12	39.45	35.77	38.25

Table 4: Percentage Inhibition of Ascorbic acid

Concentration(ppm)	Percentage Inhibition	Average
4	72.10784713	72.11
3	62.83760059	62.84
2.5	57.55104515	57.55
2	55.32973414	55.33
1.5	51.57644045	51.58
1	47.01771475	47.02
0.5	44.72366102	44.72
0.1	45.84138189	45.84

Table 5: IC_{50} Values of samples *Triphala* and standard

Sample /Standard	IC $_{50}$ value
Ascorbic Acid	1.17 ± 0.05 ppm
S1 (1:1:1 powder)	1.67 ± 0.16 ppm
S2 (1:2:3 powder)	2.25 ± 0.47 ppm
S3 (1:2:4 powder)	1.32 ± 0.29 ppm
S4 (1:2:2 powder)	2.01 ± 0.64 ppm
S5 (1:2:4 Fruits)	3.40 ± 0.22ppm

Discussion

HPTLC analysis showing presence of nine distinct peaks exhibiting similar patterns and respective intensities in S_1, S_2, S_3, S_4, and S_5 reveal that all the samples consist of similar chemical substances.

Antioxidant activity refers to the ability of certain substances, known as antioxidants, to neutralize or counteract the damaging effects of molecules called free radicals within the body. Based on the values obtained from Percentage Inhibition to scavenge the DPPH free radical and Half Maximal Inhibitory Concentration (IC_{50}) which determines the concentration at which a substance exerts half of its maximal inhibitory effect, it is clear that all five samples: S_1, S_2, S_3, S_4, and S_5, exhibited pronounced and high levels of antioxidant activity in DPPH Assay. Specifically, it is observed that the levels of antioxidant activity exhibited a descending order as follows: S_3 (1:2:4 powder of TC: TB:PE) > S_1 (1:1:1 powder of TC: TB:PE) > S_4 (1:2:2 powder of TC: TB:PE) > S_2 (1:2:3 powder of TC: TB:PE) > S_5 (1:2:4 Fruits of TC: TB:PE). The highest antioxidant potential was identified in sample 3 (IC_{50}=1.32 ± 0.29 ppm) with a level that closely approaches the antioxidant activity observed in the standard ascorbic acid (IC_{50}=1.17 ± 0.05 ppm). This heightened antioxidant potency compared to other samples can be attributed to the fact that S_3 (1:2:4 powder of TC: TB: PE) possesses the highest concentration of *Amalaki*, one of constituent fruits in *Triphala*. Charaka Samhitha, Sutrasthana mentions *Amalaki-Phyllanthus emblica* as one ingredient among the *Vayasthapana dashaka* (10 longevity promoters).[30] S_1 (1:1:1) has the potential of Kapha and Pitta pacifying properties and S_3 (1:2:4) is rich with *Rasayana* (Rejuvenation) action which destroys senility, diseases and promote longevity due to the highest concentration of *Amalaki*.

It is recommended that future research could be done on bioactivity studies to assess the therapeutic efficacy of different compositions of *Triphala* powder based on Ayurveda Authentic Texts.

Conclusion

From the present study it can be concluded that, even though the chemical characteristics of various formulations of *Triphala* powder, as specified in diverse Ayurveda authentic texts exhibited notable similarity, the highest antioxidant activity was found in Sample 3 (1:2:4 powder of TC: TB:PE).

Conflict of Interest

The authors have declared that there is no conflict of interest.

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