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A Comparative Analysis for Phytochemical Screening and Antioxidant Potential of Two Different Formulations of *Triphala* Extract

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Abstract

In Ayurveda, *Triphala* is a formulation of three herbs. It is a mixture (equiproportional 1:1:1) of fruits of three plants, *Haritaki* (*Terminalia chebula*), *Bibhitaki* (*Terminalia bellerica*) and *Amalaki* (*Emblica officinalis*). *Triphala* shows antibacterial, antiallergic, antiviral, antifungal, immunomodulatory and antioxidant properties. *Triphala* and its constituents help in the improvement of blood circulation, reduction in level of cholesterol and control of blood pressure. This study focuses on detecting various phytocompounds and their comparative analysis for antioxidant potential in two different formulations of *Triphala* extract. Antioxidant activity was determined by phosphomolybdenum assay and reducing power assay. Our results indicated the presence of various phytocompounds such as phenols, flavonoids, tannins, saponins, alkaloids etc, which may play a role in its biological activities. Both *Triphala* formulations showed significant free radical-scavenging properties as compared to ascorbic acid. Formulation S2 (H:B:A - 1:2:3) showed higher antioxidant activity as compared to formulation S1 (H:B:A- 1:1:1). In conclusion, *Triphala* non-equiproportional mixture may be a more promising Ayurvedic drug in future *Triphala.*

Keywords: Antioxidant, Ayurveda, Phytochemicals, Reducing Power Assay, *Triphala*

1. Introduction

Triphala is a blend of the fruits of three medicinal plants known as the "Three Myrobalans" *Haritaki* (*Terminalia chebula*), *Bibhitaki* (*Terminalia bellerica*) and *Amalaki* (*Emblica officinalis*)[1,2]. *Amalaki* contains many active secondary metabolites such as polyphenols, vitamin C, tannins, flavonoids, terpenoids, glycosides etc., and some individual phytocompounds (e.g. gallic acid, chebulinic acid, phyllemblin, furosin, geranin and quercetin)[3,4]. *Haritaki* contains many bioactive compounds such as tannins, flavonoids, phenolic acids etc. Antioxidant, anti-inflammatory, neuroprotective and cytotoxic effects of *Haritaki* have also been reported[5]. Chebulinic acid, flavonoids, vitamin C, glucosides, tannins, terpenoids, glycosides, gallic acid, saponins, ellagic acid, lignans, terpene acids and bellaric acid are the main bioactive compounds of *Bibhitaki*. It has high antioxidant, anticancer and other biological activities[6].

Triphala demonstrates antifungal, antiviral, antiallergic and antibacterial activities. *Triphala* and its phytocompounds work as a cardiotonic, regulating blood pressure, enhancing blood circulation and lowering cholesterol. It has immunomodulatory effects and strengthens the body's immune system[7-11].

The utilization of natural remedies as medicine is growing in popularity these days. Compared to chemical treatments, several natural formulations that contain plant extracts have been discovered to be safer medications with fewer adverse impacts. *Triphala* formulation is commonly used as an Ayurvedic drug to cure various illnesses such as constipation, jaundice, chronic ulcers, anaemia, fever and asthma[12-15]. It is also been used for the treatment of chronic constipation, digestion problems and colon

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cleansing[14,15]. Recently, many research findings reported that *Triphala* possesses antioxidant, radioprotective and antimutagenic properties[16-23].

Reactive Oxygen Species (ROS), which react with endogenous molecules such as lipoproteins, nucleic acids, lipids and proteins generate oxidative stress, which in turn causes tissue damage or cell injury[24]. ROS-induced cell damage is the root cause of inflammatory, cancerous, cardiovascular and neurological disorders[24,25]. Synthetic antioxidants are not safe to use in humans. Therefore, it is crucial to find new and safe antioxidants from natural sources, such as plants.

Triphala formulations are known to be rich in antioxidant compounds that impart good potential for free radical scavenging. In consideration of this, the current study was designed to determine the antioxidant potential of two different formulations of *Triphala*, one market-purchased formulation (H:B:A -1:1:1) and the other lab-made formulation (H:B:A -1:2:3) with changed proportions of components. Therefore, we tried to explore a new formulation of *Triphala* non-equiproportional mixture (1:2:3) of fruits of three plants, *Haritaki* (*Terminalia chebula*), *Bibhitaki* (*Terminalia bellerica*) and *Amalaki* (*Emblica officinalis*), which was not earlier reported in the literature. For the comparative analysis, phosphomolybdenum assay and reducing power assay were performed in both *Triphala* formulations to determine their antioxidant potential.

2. Materials and Methods

2.1 Plant Materials

Fruits of *Haritaki* (*Terminalia chebula*), *Bibhitaki* (*Terminalia bellerica*), *Amalaki* (*Emblica officinalis*) and commercial *Triphala* were purchased from the local market. In an electric blender, the dried fruits were crushed to make powder after the seeds from the individual fruits were removed. These powders were combined in an unequal ratio (H:B:A -1:2:3) to create a lab-made *Triphala* formulation (S2). These *Triphala* powders were stored in an air-tight container for the experimental studies.

2.2 Chemicals and Reagents

All the chemicals and reagents utilized in the investigation were of the analytical grade. To conduct antioxidant assays, Himedia, SRL and Merck India provided all solvents and chemicals. Ascorbic acid, ammonium molybdate and potassium hexacyanoferrate were procured from Sigma-Aldrich Company.

2.3 Extraction of *Triphala*

For this study two samples of *Triphala* were used, that is market purchased (S1) and lab-made (S2) made by mixing the fine powder of *Haritaki* (*Terminalia chebula*), *Bibhitaki* (*Terminalia bellerica*) and *Amalaki* (*Emblica officinalis*) in proportion of 1:1:1 and 1:2:3 respectively. The extraction was done by the Soxhlet extraction method, where 10gm of the *Triphala* powder was packed in a thimble and extracted with 200ml of ethanol and water separately for 8 cycles at 60°C. The solvent from concentrated extracts was evaporated completely and the dried extract was stored for further analysis.

2.4 Solubility Test of *Triphala* Extracts

The solubility of both *Triphala* formulations (S1 and S2) was checked in different solvents such as chloroform, distilled water, acetone, methanol, and ethanol.

2.5 Phytochemical Screening in *Triphala* Extracts

Different formulations of *Triphala* extract were analysed for the detection of phytochemical compounds including phenols, alkaloids, terpenoids, glycosides, saponins, flavonoids and phytosterols etc. Phytochemicals present in different formulations were confirmed by using standard protocols[26,27] as given below:

2.5.1 Test for Carbohydrates

In 1ml of *Triphala* extracts, 1ml of Molisch's reagent was added and mixed well. 1ml of concentrated sulphuric acid (H_2SO_4) was added to this sample carefully through the side wall of each tube. At the junction of the two layers, a reddish violet ring was seen that confirmed the presence of carbohydrates.

2.5.2 Test for Steroids

1ml of *Triphala* extract was mixed with 5ml of chloroform. 6ml of concentrated sulphuric acid (H_2SO_4) was added drop by drop to the side wall of each tube. The presence of steroids was confirmed by the red colour of H_2SO_4 in

the upper and the yellowish green colour in the bottom layer.

2.5.3 Test for Tannins

In 1ml of *Triphala* extract, 2-4 drops of 5% Ferric Chloride ($FeCl_3$) were added. Blue–black colour appeared confirming the presence of tannin.

2.5.4 Test for Cardiac Glycosides

Keller-killani Test: 1ml of *Triphala* extracts were added in a mixture of 1ml of glacial acetic acid and a few drops of 5% $FeCl_3$. Solutions were then poured into the test tubes and 1ml of concentrated H_2SO_4 was added. A reddish-brown colour was seen at the junction of two liquid layers and the bluish-green colour of the upper layer confirmed the presence of glycosides.

2.5.5 Test for Phenolic Compounds

A few drops of 0.1% v/v $FeCl_3$ were added to 1ml of *Triphala* extract. The appearance of a brownish-green colour confirmed the presence of phenols.

2.5.6 Detection of Coumarins

Triphala extracts were mixed with 2 to 4 drops of 10% Sodium Hydroxide (NaOH) and then Chloroform ($CHCl_3$) was added. The yellow colour confirmed the presence of coumarin.

2.5.7 Detection of Proteins

Xanthoprotein Test: 1ml of *Triphala* extract in a test tube was treated with 2-3 drops of concentrated HNO_3 along the wall of the tube. Yellow-coloured precipitates indicated the presence of protein.

2.5.8 Detection of Alkaloids

1ml of Potassium mercuric iodide solution (Mayer's reagent) was added in a portion of *Triphala* extract. The formation of a light yellow precipitate confirmed the presence of alkaloids.

2.5.9 Detection of Flavonoids

Alkaline reagent was used for the test of flavonoids. 2-3 drops of NaOH were added to the extract of *Triphala*. The appearance of a yellow colour confirmed the presence of flavonoids.

2.5.10 Detection of Saponins

5ml of *Triphala* extract was added to 10ml of distilled H_2O and then mixed vigorously for 2 minutes. A layer of foam was formed. Persistence of froth for 10-15 minutes indicated the presence of saponin.

2.5.11 Detection of Amino Acids

Ninhydrin Test: 1ml of *Triphala* extract was mixed with 2ml of 0.2% ninhydrin solution and boiled. The presence of amino acids and proteins was confirmed by the appearance of violet colour precipitate.

2.5.12 Detection of Terpenoids

Salkowiski Test: 1ml of *Triphala* extract was mixed with 0.4ml of chloroform ($CHCl_3$) and 0.6ml of concentrated H_2SO_4 was added in solution to form a layer. On the surface of the solution, a reddish-brown colour indicated the presence of terpenoids.

2.6 Determination of Antioxidant Activity

2.6.1 Phosphomolybdenum Assay

According to Prieto's *et al.*,[28] protocol, the phosphomolybdenum technique was used to assess the antioxidant activity of *Triphala* extracts. A tube containing an aliquot of 0.1ml of each extract was combined with 1ml of the reagent solution (28mM sodium phosphate, 0.6 M sulfuric acid, and 4mM ammonium molybdate) and incubated in a water bath at 95°C for 90 minutes. Samples were cooled to room temperature after incubation, and the absorbance of the mixture was measured at 765nm against a blank. Ascorbic acid was used as standard.

2.6.2 Reducing Power Assay

The Fe^{3+} reducing power of *Triphala* extracts was determined by the method of Oyaizu[29]. Different concentrations of *Triphala* extracts (0.75ml) were added in 0.75ml of $K_3Fe(CN)_6$ (potassium hexacyanoferrate) (1%, w/v) and 0.75ml of phosphate buffer (0.2M, pH 6.6). These sample mixtures were incubated in a water bath for 20 minutes at 50°C. After adding 0.75 ml of 10% Trichloroacetic Acid (TCA) solution to halt the reaction, samples were centrifuged at 3000 rpm for 10 minutes. Ferric Chloride ($FeCl_3$) solution (0.1%, w/v), 1.5ml of distilled water and 0.1ml of the supernatant were combined for 10 minutes. The reducing power was calculated using the absorbance at 700nm. The reaction mixture's higher

absorbance revealed more reducing power. Ascorbic acid was used as a standard for the reference value.

2.7 Statistical Analysis

Each experiment was performed in triplicates (n = 3). All statistical analysis, including calculation of mean + SD (mean and standard deviation) and creation of graphs, were done using Graphpad Prism 6.0.

3. Results and Discussion

The results of increasing the order of solubility test in different solvents are shown in Figures 1 and 2. Our results show that maximum solubility was observed in ethanol. Therefore, the phytochemical screening and antioxidant activities were done in ethanolic extracts of both *Triphala* formulations. The phytochemical analysis indicated the presence of phenol, flavonoids, tannins, saponin, terpenoids, alkaloids and steroids in both the formulations of *Triphala* extracts (Tables 1 and 2). Therefore, both formulations have their own nutritional and pharmaceutical values. World Health Organization reported that naturally occurring phytochemicals are a very good option for the discovery of future medicines[30].

Steroid compounds have very good anti-inflammatory and antibacterial properties[31]. Therefore, steroid compounds can be used for the treatment of many dangerous diseases like allergies, fatigue, arthritis and hormonal imbalance[32]. Testing of *Triphala* extract using ethanol solvents indicated positive results for alkaloids. Alkaloid compounds have many beneficial health effects[33].

Our results showed that both the formulations of *Triphala* extracts contain tannins, which is well known for their antioxidant property. It can protect our skin from ultraviolet radiations[34]. In a few plants, tannin compounds are reported for their anti-inflammatory and anti-diarrhea properties[35].

Tests for the flavonoid compounds were positive in our ethanolic *Triphala* extracts. Flavonoid compounds belong to a class of polyphenolic compounds, which have free radical scavenging and anti-inflammatory properties and well known inhibitors of oxidative and hydrolytic enzymes[36]. They are also useful for enhancing the effectiveness of ascorbic acid, preventing bone loss and protecting cell structures[37]. Saponins also have anti-inflammatory, anti-fungal and anti-microbial activities. They can be used for the treatment of ulcers, dysentery and leucorrhoea[38,39]. In many medicinal plants, terpenoid compounds act as antibacterials, anti-inflammatory, cholesterol synthesis, cancer cell inhibitors, snake bites, menstrual and skin disorders[40].

The antioxidant activity was determined through the phosphomolybdenum assay which reduces Mo (VI) to Mo (V) by the *Triphala* extract containing antioxidant phytochemicals. In our study, S2 *Triphala* formulation was more effective in reducing Mo (VI) to Mo (V) than the S1 formulation of the *Triphala* extract. The reduction of Mo (VI) to Mo (V) by ascorbic acid which was used as a standard (Figure 3) indicated that effective antioxidants were present in both the formulations.

The reducing power of *Triphala* extracts may act as an indicator of its antioxidant potential. Figure 4 depicts the reducing power of both formulations of *Triphala* extracts. In our study, we found that the reducing power of the S2 formulation was more than the S1 formulation with increasing concentrations. The result of this study demonstrated that *Triphala* formulations consist of polyphenolic compounds that may be responsible for their greater reducing power.

Figure 1. Increasing order of solubility of S1 formulation of *Triphala* extract in different solvents.

Figure 2. Increasing order of solubility of S2 formulation of *Triphala* extract in different solvents.

Table 1. Phytochemical screening of S1 formulation of *Triphala* extract

Test Performed	Solvent used				
	Chloroform	Distilled Water	Acetone	Methanol	Ethanol
Detection of carbohydrates (Molisch's Test)	–	+	+	–	–
Test for steroids	+	+	+	+	+
Test for tannins	–	+	+	+	+
Test for cardiac glycosides (Keller- Killani Test)	–	–	+	+	–
Test for phenolic compounds ($FeCl_3$ Test)	–	+	+	+	+
Detection of coumarins	+	+	+	+	+
Test for proteins (Xanthoprotein Test)	+	–	+	–	+
Detection of alkaloids	–	+	+	–	+
Detection of flavonoids	–	+	+	–	+
Test for saponins (Foam Test)	–	+	+	+	+
Test for amino acids (Ninhydrin Test)	–	–	–	–	–
Test for terpenoids (Salkowiski Test)	+	+	+	+	+

Table 2. Phytochemical screening of S2 formulation of *Triphala* extract

Test Performed	Solvent Used				
	Chloroform	Distilled Water	Acetone	Methanol	Ethanol
Detection of carbohydrates (Molisch's Test)	–	+	–	–	–
Test for steroids	+	+	+	+	+
Test for tannins	–	+	+	+	+
Test for cardiac glycosides (Keller-Killani Test)	–	–	+	+	–
Test for phenolic compounds ($FeCl_3$ Test)	–	+	+	+	+
Detection of coumarins	+	+	+	+	+
Test for proteins (Xanthoprotein Test)	–	+	+	–	+
Detection of alkaloids	–	+	+	+	+
Detection of flavonoids	+	+	+	+	+
Test for saponins (Foam Test)	–	–	+	+	+
Test for amino acids (Ninhydrin Test)	–	–	–	–	–
Test for terpenoids (Salkowiski Test)	+	+	+	+	+

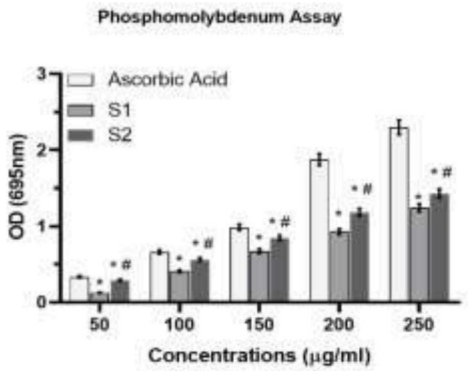

Figure 3. Phosphomolybdenum assay**:** The bar plot indicates the concentration-dependent differential change in the OD among the indicated groups. Data values are representative of the mean ± SD of triplicate measurements (n = 3), and statistical significance was calculated using Student's paired t-tests where p-value presented as *p ≤ 0.05 (Control versus S1 and S2), #p ≤ 0.05 (S1 versus S2).

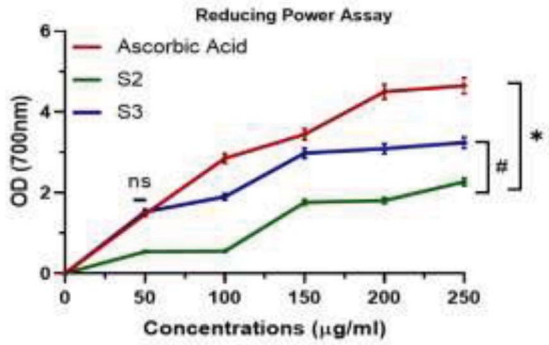

Figure 4. Reducing power assay**:** The line graph indicates the concentration-dependent increase in the reducing power of the mentioned treatment groups. Data values are representative of the mean ± SD of triplicate measurements (n = 3), and statistical significance was calculated using paired t-tests of students where p-value presented as *p ≤ 0.05 (Control versus S1 and S2), #p ≤ 0.05 (S1 versus S2).

4. Conclusion

This study demonstrated the presence of significant bioactive phytochemicals such as phenols, flavonoids, tannins, saponins, alkaloids etc which might play a role in its biological activities. Further, we also found that formulation two (1:2:3) of *Triphala* extract (S2) revealed higher antioxidant activity as compared to formulation one (1:1:1) (S1). In conclusion, the *Triphala* non-equiproportional mixture may be a more promising ayurvedic drug in future pharmaceuticals.

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