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Phytochemical screening, anti-oxidative activities and anti-microbial studies of an Ayurvedic tonic Triphala along with its components

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Abstract
Triphala has been used in the traditional medicine for the treatment of variety of diseases and therefore it becomes immense to study the phytochemical compounds and antibacterial activities. In the present investigation methanolic extract of triphala and its individuals were subjected to evaluate its antioxidant and antimicrobial properties. Phytochemical studies showed that all the extracts contained alkaloids, glycosides, flavonoids, tannin, saponins, steroids, terpinoids. The results suggest the phytochemical properties of the samples for curing various ailments. Preliminary antioxidative activity of the samples were also determined and among them we found that triphala contained total poly phenols (246.84mg GAE/g sample) as well as DPPH radical scavenging activity (92.32%) and reducing power (OD=1.35). This shows that the product may be potent source of natural antioxidants. However the product showed considerable level of activity against standard strains and clinical isolates of some gram positive and gram negative bacteria. The obtained results provide a support for the use of these plants in traditional medicine and for its further investigation.

Keywords: phytochemical, antioxidative, antimicrobial, triphala

Introduction
Plants are the valuable natural resource of bioactive natural products, which are used to promote human health or to treat diseases. Recently, anti-oxidative and anti-microbial natural products have got much attention. Polyphenols are potential and abundant anti-oxidant in plants. For the prevention and treatment of various diseases in human including radical-induced diseases such as cardiovascular disorders, cancer, aging, inflammation and allergy, plant-based medicines are getting importance in current world due to its multiple beneficial effects in combating diseases with least side effects [1] Modern civilizations now belief to be herbal medicine helps to cure disease without create any problem in the body. Therapeutic uses of plants continued with the progress of civilization and development of human knowledge, scientist endeavored to isolate different chemical constituent from plants, put them to biological and pharmacological tests and thus have been able to identify and isolate therapeutically active compounds. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in-vitro* to have antioxidant and antimicrobial properties [2] Triphala is a botanical preparation comprised of equal parts of three medicinal plants such as Amalaki (*Phyllanthus emblica*), Harritaki (*Terminalia chebula*) and Bohera (*Terminalia bellirica*). The dry fruits of above mentioned three plants and triphala as a whole easily available in the market and is affordable for all socio-economic structure. Triphala used for biliary disorders, diarrhea, dyspepsia, cough, renal problems, and hepatic disorders from ancient time. Triphala has powerful anti-oxidant agents that help in regularizing the metabolism of cells and ease their proper functioning. This reduces the risk of production of free radicals that are the main cause of aging. It also stimulates the functioning of cell organelles like mitochondria, golgi bodies and nucleus that play a vital role in proper functioning of the cell [3] Recent studies conducted that triphala has anti-cancerous activities. It helps in reducing the spindle formations at the mitotic phase there by reducing the risk of growth of metastasis of cancer cells. It also has a strong affinity for the micro-organisms and does not let them to cause any kind of damage in the body [4]. The astringent property of triphala helps in purification of blood and also helps in maintenance of proper density of the blood. Hence it is very helpful in eradicating all blood borne diseases and also skin related problems. The research work presented here deals with an Ayurvedic herbal

formulation triphala and its individual fruit component. The matured fruits of them were used to explore and evaluate antioxidant and antimicrobial activities, which may be utilized in future to peruse a new line of investigation.

Materials and Methods

Collection of the samples: Individual Amlaki, Harritaki, Bohera samples and their combined product triphala were freshly collected from market named Nawa Bazar, Savar, Dhaka and these were identified by registered herbalists.

Preparation of extracts from the plant parts: About 400 gm of powdered materials of each were placed in clean flat bottomed glass containers (4L) and soaked in 1.3 liters of 80% ethanol for the samples separately. The containers were sealed for a period of seven days with occasional shaking and stirring. The whole mixtures were then underwent coarse filtration through a piece of clean, white cotton followed by filtration through Whatman filter paper. The filtrates were concentrated using a rotary evaporator to obtain the crude extracts.

Phytochemical screening: In order to determine the qualitative chemical analysis of different plant species, phytochemical screening was performed. Fresh materials namely *Emblica officinalis*, *Terminalia chebula* and *Terminalia belerica* and triphala were collected. They were dried in the oven at about 40-45 ˚C temperature. The dried materials were then powdered by a grinder machine. About 50g of powdered material was mixed with concentrated ethyl alcohol and was kept for about 24 hours. When the total alcohol was evaporated, different chemical tests were performed by the semi-solid materials (sample).

1. **Alkaloid test:** A sample amount of an alcoholic extract of the plant material was dissolved in water. If the sample did not dissolve in water, a drop of concentrated HCl or H_2SO_4 was added and gentle heat was applied. Different reagents were added in order to observe the precipitation and colour of the sample. If Hager's reagent showed yellow ppt., Mayer's reagent showed off-white ppt., and Wagner's reagent showed brown ppt., then this colour and precipitation indicated the presence of alkaloid.
2. **Tannin test:** To a small amount of alcoholic extract of the sample, 2-3 drops of ferric chloride ($FeCl_3$) solution were added. Black/ blackish brown colour developed indicated the presence of tannin.
3. **Saponin test:** About 0.5 gm of an alcoholic extract of the plant material was vigorously shaken with water. Production of a persistent froth (stable for about 1 minute on heating) indicated the presence of saponin.
4. **Flavonoid test:** A few drops of concentrated HCl were added to a small amount of an alcoholic extract of the plant material. Immediate development of a red colour indicated the presence of flavonoid.
5. **Anthraquinone glycoside test:** About 0.5 gm of powdered material was taken in a dry test tube. 5 ml of chloroform was added and the mixture was shaken at least for 5 minutes. The extract was filtered and the filter was shaken with an equal volume of 10% ammonia solution. Bright pink colour in the aqueous (upper) layer confirmed the presence anthraquinone glycoside.
6. **Cardiac glycoside test:** About 1 gm of the powdered plant material was boiled with 70% alcohol for about 3 minutes and then filtered. To the filtrate, 5 ml of water and 0.5 ml of a strong solution of lead acetate were added. The mixture was shaken well and filtered. The filtrate was extracted with an equal volume of chloroform and was evaporated to dryness. The residue was dissolved in 3 ml of glacial acetic acid and 2 drops of ferric chloride solution were added to it. The mixture was then poured slowly into a test tube containing 2 ml of concentrated H_2SO_4 to form an upper layer. A reddish brown colour was found to be formed at the junction of the low layers and the upper layer turned bluish green on standing. This was an evidence for the presence of cardiac glycoside in the plant material.
7. **Phenols test:** To 1ml of alcoholic solution of sample, 2ml of distilled water followed by a few drops of 10%aqueous ferric chloride solution was added. Formation of blue or green color indicated the presence of phenols
8. **Terpenoid test:** 2ml of chloroform and 1ml of conc. $H_2SO_4$was added to 1mg of extract and observed for reddish brown color that indicated the presence of terpenoid.
9. **Phytosterol test:** The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol. The residue was dissolved in few drops of diluted acetic acid; 3 ml of acetic anhydride was added followed by few drops of Conc. H_2SO_4. Appearance of bluish green color showed the presence of phytosterol.
10. **Steroids test:** One ml of the extracts was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.
11. **Amino acids test:** One ml of the extract was treated with few drops of ninhydrin reagent. Appearance of purple colour shows the presence of amino acids.

Antioxidative activity of the samples

1. **Determination of total phenolic compounds (TPH):** The total concentration of phenolic compounds (TPH) in the fractions was determined according to the Folin-Ciocalteu method with gallic acid (GA) as the standard and expressed (mg) as gallic acid equivalents (GAE)/g of extract. One milliliter of diluted extract was mixed with 1 ml of Folin-Ciocalteu's reagent and vortexed for 5s. Then, 1 ml of a 10% (w/w) sodium carbonate aqueous solution was added to the mixture. The mixture was incubated at room temperature for 1 h, after which colorimetric measurements were made at 700 nm. Each experiment was conducted three times.
2. **DPPH radical scavenging activity:** The reaction mixture (total volume, 3 ml), consisting of 0.5 ml of a 0.5 M acetic acid buffer solution at pH 5.5, 1 ml of 0.2 mm DPPH in ethanol, and 1.5 ml of a 50% (v/v) ethanol aqueous solution with the fractions, was shaken vigorously. After incubation at room temperature for 30 min, the amount of DPPH remaining was determined by measuring absorbance at 517 nm, and the radical-scavenging activity of each sample was expressed using the ratio of the decrease in absorption (%) relative to the control (100%) in the absence of the fraction. If the diluted fraction itself had an absorption value that was more than 1% of the control, it was subtracted from the value for the sample reaction mixture. That is, the radical scavenging activity

(%) = 100 (A – B)/A, where A and B were the absorption of the control and the corrected absorption of the fraction reaction mixture, respectively. Mean values were obtained from triplicate experiments.

3. **Reducing power activity:** The reducing power of the extracts was determined according to the method. Briefly, 2.5 ml of 0.2 M phosphate buffer, pH 6.6, containing different concentrations of the bark fractions were mixed with 2.5 ml of 1% potassium ferricyanide. After incubation at 50 ºC for 20 minutes, the mixtures were mixed with 2.5 ml of 10% trichloroacetic acid followed by centrifugation at 650g for 10 min. The supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Then the absorbance of this solution was measured at 700 nm. Mean values were obtained from triplicate experiments. Ascorbic acid served as positive control.
4. **Determination of total anti-oxidant capacity:** The assay was done according to the method [20]. The tubes containing the fraction and the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 90 ^{0}C for 90 min. The anti-oxidant capacity was expressed as ascorbic acid equivalent (AAE).

Antimicrobial activity of the samples: The agar disc diffusion method was used to evaluate the antimicrobial activity by measuring the inhibition zone against the test organisms [5]. The organisms were inoculated into nutrient agar medium. Sterile filter disc (8mm in diameter) were impregnated with 40µl of sample extract (250mg/ml) and placed on the inoculation plates. The plates were incubated at 35 ºC for 18 hours. Microbial growth inhibition was determined as the diameter of the inhibition zones around the discs. All the tests were performed on triplicate.

Results

Phytochemical Screening of the samples: Preliminary phytochemical investigation of ethanolic extract of the plant materials was carried out for qualitative determination of the groups of organic compounds present in them, by using standard procedure to identify the constituents [6]. Table-1 represents the phytochemical screening of fruit of *Phyllanthus emblica*, *Terminalia bellirica*, and *Terminalia chebula.* All the samples posses carbohydrate, alkaioids, flavonoids, glycosides, phenolic contains etc. *Terminalia belirica* contains anthraquinon glycosides and *Terminalia chebula* contains cardiac glycosides. Saponin is present in *Terminalia belirica* only and steroid is present in *Terminalia chebula* and *Terminalia. belerica*. Triterpinoid is absent in the crude extract of three samples and tannin is present in only *Terminalia chebula* but absent in *Phyllanthus emblica* and *Terminalia belerica*. The extracts of triphala revealed the presence of alkaloids, tannins, cardiac glycosides, reducing sugars, saponins, flavonoids and steroids. Terpenoids and phytosterol were also present in the samples.

Table 1: Result of the samples in phytochemical screening tests.

Name of the Test			Specific Test Name	*Phyllanthus embelica*	*Terminalia chebula*	*Terminalia belerica*	Triphala
Carbohydrates Test		a.	Molish Reagent Test	+	+	+	+
		b.	Fehling's Reagent Test	++	++	++	++
Alkaloids Test		a.	Hager's reagent.	+	++	+	-
		b.	Wagner's reagent.	-	+	+	++
		c.	Mayer's reagent.	-	+	+	+
		d.	Dragendorff's Reagent.	+	-	+	+
		e.	Tannic acid (10%)	+	+	+	+
Flavonoids Test		a.	Ferric Chloride($FeCl_3$)Test	+	-	++	+
		b.	Lead Acetate Test	+	++	+++	+++
		c.	Alkali Test	-	+	+	+
		d.	Con. H_2SO_4 treatment	-	-	+	+
Glycosides test	1.Common test	a.	Ferric Chloride($FeCl_3$)Test	+	++	+	+
	2.Anthraquinon Glycosides test	a.	Borntragers test	-	-	-	-
	3.Cardiac Glycosides test	a.	Keller killiaani test	+	+	+	+
Phenolic compounds & Tannins Test		a.	Ferric Chloride($FeCl_3$)Test	-	+++	++	++
		b.	Lead Acetate Test	+	++	++	+
		c.	HNO_3 test	-	++	-	-
		d.	Amonia (NH_3) test	-	++	-	-
Saponins test		a.	Foam test	-	-	++	+
Terpenoids test	1.Common test	a.	Con. H_2SO_4 test	+	+	-	+
	2.Triterpenoids test	b.	Acetic anhydride test	-	+	-	+
Phytosterol /Steroid test		a.	Salkowski Test	-	+	+	+

"+++" = highly present, "++" = moderately present, "+" = slightly present and "-"= absent

Total phenolic content of the extracts: It has been recognized that the phenolic compounds are class of antioxidant agents which act as free radical terminators.[7] The Folin-Ciocaltue reagent method is actually not an antioxidant test but instead an assay for the quantity of oxidizable substance, that is, phenolic compounds [8] Table 2 shows the content of total phenolic compounds ranged from 68.17 to 246.84 mg GAE/g extract. Triphala with 264.84 mg GAE/g extract of total phenolic content had the highest amount of this substance among the samples in this research. The compounds such as phenolic substances, which contain hydroxyls, are responsible for the radical scavenging effect in the plants [9] According to our study, the high contents of these phytochemicals in triphala explain its high radical scavenging activity. Extract of *Emblica officinalis* contained comparatively lower amount (37.1).

Table 2: Total phenolic content of the samples (100 times dilution)

Sample	Absorbance at 700 nm			Average	Total phenolic content(mgGAE/g)
	R-1	R-2	R-3		
Amlaki	0.487	0.495	0.508	0.496667	68.1733
Harritaki	0.584	0.579	0.573	0.578667	79.42875
Bohera	0.629	0.63	0.632	0.630333	86.52061
Triphala	1.792	1.815	1.788	1.798333	246.8422
GA100µM	0.681	0.688	0.687	0.685333	
GA200µM	1.373	1.392	1.396	1.387	

Antioxidant assay

DPPH radical-scavenging activity: DPPH is a useful reagent for investigating the free radical-scavenging activities of compounds. In the DPPH test, the extracts were able to reduce the stable radical DPPH to the yellow-colored diphenylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction [10] As seen in Table 3, the methanolic extract of all samples exhibited considerable DPPH radical scavenging activity. At 100 µg/ml concentration, extract of triphala showed the highest DPPH radical scavenging activity (92.32%) followed by, *Emblica officinalis* (88.16%), *Terminalia chebula* (83.62%), and *Terminalia belerica* (72.64%). Since triphala exhibited strongest activity it could be used as antioxidative substrate.

Table 3: DPPH radical scavenging activity of the samples.

Samples	Absorbance at 517 nm			Average	Activity (%)
	R-1	R-2	R-3		
Amlaki	0.449	0.447	0.44	0.445333	88.16
Harritaki	0.411	0.407	0.414	0.410667	83.62
Bohera	0.526	0.558	0.531	0.538333	72.64
Triphala	0.344	0.353	0.356	0.351	92.32
Control	1.926	1.925	1.921	1.924	

Reducing power: The reducing capability of a compound may serve as a significant indicator of its potential antioxidant activity. Table 4 shows the reductive activity of the extracts at a concentration of 400 µg/mL in phosphate buffer. The most reducing activity comes from the sample triphala (O.D. 1.781) followed by *Terminalia bellirica*, *Terminalia chebula* and *Emblica officinalis* also has some activity.

Table 4: Reducing power of the samples

Samples	OD at 700 nm			Average absorbance
	R-1	R-2	R-3	
Amlaki	1.2	1.184	1.221	1.20
Harritaki	1.176	1.22	1.221	1.20
Bohera	1.18	1.184	1.187	1.18
Triphala	1.358	1.347	1.345	1.35
Ascorbic acid100µg/ml	1.46	1.421	1.379	1.42
Ascorbic acid200µg/ml	2.773	2.798	2.786	2.786

Antimicrobial activity of the samples: Methanolic extracts (80%) of the samples triphala was tested for their antimicrobial activity by using disc diffusion method at 250mg/ml concentration, shown in the table 5. The extract displayed the height level of activity against *Bacillus subtilis* and *Salmonella typhi.* The inhibition zone was 13.08 mm and 11.44 mm. Activity was also detected against *Bacillus cereus, Staphylococcus aureus, E. coli* and *Salmonella typhi.* Gram negative bacteria, *Shigella flexneri* was showing no activity.The present findings support the applicability of the samples in traditional system for its claimed uses and can be recommended by the scientific community accessible alternative to synthetic antibiotics. The high degree of antimicrobial activity seems to support the folk therapy for infectious and traditional therapeutic claims of this plant.

Table 5: Antibacterial activity (inhibition zone) of Triphala using disc diffusion method.

Bacterial strains	Type	Inhibition zone (mm) (120µl/ml)			Average inhibition zone	Standard
		R-1	R-2	R-3		
Bacillus subtilis	+	13	12	14.25	13.08	22.3
Bacillus cereus	+	11.5	12.5	10	11.33	25.3
Salmonella paratyhpi	-	11	11.33	12	11.44	23.2
Vibrio colerae	-	6.1	8.7	10	8.27	22
Escherichia coli	-	8	8.3	8.3	8.3	22.4
Pseudomonusaerus	-	12.5	11	13.75	12.42	18.3

Discussion

Triphala is a traditional Ayurvedic herbal formulation, consisting equal parts of three medicinal plants namely *Emblica officinalis*, *Terminalia chebula,* and *Terminalia belerica.* Triphala is considered as a 'rasayan' having balancing and rejuvenating effects on the three constitutional elements that govern human life *i.e.* vata, pitta and kapha [11]. Triphala has been reported to possess antioxidant rich herbal formulation [12], improves the mental faculties and also assists in the weight loss [13]. Triphala contains about 20% tannins of both condensed and hydrolysable type. Other constituents identified in the fruit include lipids, sitosterol, saponins, gallic and ellagic acids, cardiac glycoside and various carbohydrates. Fruits of triphala are claimed to have various biological activities such as, exerts a marked heart-protective and cardio-tonic effect, improves digestion and improves liver function [14]. Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases. For example, phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids have anti-inflammatory effects [15-19] Glycosides, flavonoids, tannins and alkaloids have hypoglycemic activities [20, 21] Past study reported that saponins possess hypocholesterolemic and antidiabetic properties [22]. The terpenoids have also been shown to decrease blood sugar level in animal studies [23] Steroids and triterpenoids showed the analgesic properties [24] The steroids and saponins are responsible for central nervous system activities [25] In the present study, we have found that most of the biologically active phytochemicals were present in the ethanol extracts of plant samples. The medicinal properties of the plant extracts may be due to the presence of above mentioned phytochemicals. The various phytochemical compounds detected are known to have beneficial importance in medicinal sciences. It has long recognized that plants contain many secondary metabolites such as alkaloid, flavonoid, tannin, saponin, glycosides, phenolic etc. In the present study it was found that phytochemical screening of all the samples (*Emblica officinalis, Terminalia chebula and*

Terminalia belerica and their equal-proportional mixture, triphala) were revealed the presence of various phytochemicals of therapeutic significance. The qualitative chemical examination of methanolic crude extracts was found to contain carbohydrates, alkaloids, tannin, flavonoids, glycosides, terpenoids, steroids and saponins. These phytoconstituents present in the extracts may account for their various pharmacological activities [26]. The present study investigated the antioxidant effect of methanolic extracts of triphala and its individual plant component. The evaluation of the in vitro antioxidant activity of aqueous extract of the fruits of *Emblica officinalis*, *Terminalia chebula and Terminalia belerica* and their equi-proportional mixture, triphala, has indicated their strong ability to scavenge free radicals such as DPPH and super oxide. Results of DPPH reduction have shown that triphala had a synergistic effect, compared to each individual constituent, and it may be useful for free radical induced disorders such as paracetamol toxicity, heavy metal and radiation. As the phenolic compound present in these extracts are mostly responsible for their radical scavenging activity, the total phenolic content present in these extracts has been determined and found to vary from 33% to 44% in terms of Gallic acid equivalents. These studies revealed that all three constituents of Triphala are active and they exhibit slightly different activities under different conditions. *Emblica officinalis* shows greater efficiency in lipid peroxidation and plasmid DNA assay, while *Terminalia chebula* has greater radical scavenging activity. Crude methanolic extracts of triphala showed antibacterial against *Bacillus subtillis*, *Escherichia coli* and *Vibrio cholera*, on the other hand the extract did not show any antibacterial activity against *Serratia* sp. The result of antibacterial activity measured in term of diameter of zone of inhibition in mm. Standard antibiotic discs of Ciprofloxacin was used as standard comparison purpose. The samples extract showed antibacterial activity against both gram positive and gram negative bacteria. But the gram positive bacteria are more susceptible than gram negative bacteria. The antibacterial activity may be due to the presence of phenolics, flavonoids and carotenoids etc. and this activity can be corroborated by the usage of this sample in folk medicine.

Conclusion

The results of this study show that methanolic extract of triphala (*Emblica officinalis*, *Terminalia chebula*, and *Terminalia belerica*) has antioxidant and antibacterial effect and hence triphala can be used for the prevention and treatment of various diseases in human. Further detail analysis is required in order to confirm the prediction. So, further fractionating and studying antimicrobial effect is necessary to possibly reveal molecules of triphala and its formulation mixtures containing *Emblica officinalis*, *Terminalia chebula*, and *Terminalia belerica*. There is a need to pursue the characterization of active principles, to optimize the observed activity.

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