

Original Article

Exploring of Antimicrobial Activity of Triphala *Mashi*—an Ayurvedic Formulation

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Triphala *Mashi* is an ayurvedic formulation that was prepared in our lab. Aqueous and alcoholic extracts of both Triphala and Triphala *Mashi* were used, to evaluate antimicrobial activity. Comparative phytochemical profile of Triphala and Triphala *Mashi* was done by preliminary phytochemical screening, total phenolic content and thin layer chromatography (TLC). Antimicrobial activity includes isolation of pathogens from clinical samples, its characterization, testing its multiple drug resistance against standard antibiotics and antimicrobial activity of aqueous and alcoholic extracts of both Triphala and Triphala *Mashi* against these organisms by using agar gel diffusion method. Triphala *Mashi* containing phenolic compounds, tannins exhibited comparable antimicrobial activity in relation to Triphala against all the microorganisms tested. It inhibits the dose-dependent growth of Gram-positive and Gram-negative bacteria. In conclusion, it appears that Triphala *Mashi* has non-specific antimicrobial activity.

Keywords: antimicrobial activity – clinical sample and Tannin – Triphala – Triphala *Mashi*

Introduction

Current advancements in drug discovery technology and search for novel chemical diversity have intensified the efforts for exploring leads from *Ayurveda* the traditional system of medicine in India. Many plant extracts have been used as a source of medicinal agents to cure urinary tract infections, cervicitis vaginitis, gastrointestinal disorders, respiratory diseases, cutaneous affections, helminthic infections, parasitic protozoan diseases and inflammatory processes (1).

Although extremely effective, antibiotics are able to induce resistance in bacteria. For >50 years, bacterial resistance has been the main factor responsible for the

increase of morbidity, mortality and health care costs of bacterial infections. This bacterial defense mechanism is widely present in bacteria (e.g. *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, *Salmonella*, *Staphylococcus*, *Enterococcus* and *Streptococcus*) and became a world health problem worsened by developments in human, animal and plant transportation (2).

Triphala is a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants *Terminalia chebula*, *Terminalia bellerica* and *Phyllanthus embelica* also known as ‘three myrobalan’. Triphala means ‘three’ (tri) ‘fruits’ (phala) (3).

Triphala is used in Ayurvedic medicine in treating a variety of conditions and also forms part of many other Ayurvedic formulations. Conditions for which Triphala is employed include headache, dyspepsia, constipation, liver conditions, ascites and leucorrhoea. It is also used as a blood purifier that can improve the mental faculties and is possesses anti-inflammatory, analgesic anti-arthritic, hypoglycaemic and anti-aging properties (4–8).

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Phyllanthus embelica contains ascorbic acid (9) astragalín-flavonol (10), gallic acid-benzenoid, emblicol, phyllemblic acid (11), emblicanin A, emblicanin B, pedunculagin, punigluconin-tannin (12), ellagic acid coumarin (13). *Terminalia chebula* contains arjungenin, arjunglucoside I (14), gallic acid-benzenoid (15), chebulic acid, ellagic acid-coumarin, corilagin, punicalagin, terchebulin, terflavin A-tannin (16). β -Sitosterol, gallic acid, ellagic acid, ethyl acetate, galloyl glucose and chebulagic acid have been isolated from fruits of *T. belerica* (3).

Mashī (Black ash) is obtained, when any natural product from vegetable or animal sources is heated slowly, at lower temperature (generally below 450°C). If heating is continued further at higher temperatures (above 450°C) it forms *Bhasma* (White ash). *Mashī* is an intermediate product of *Bhasma* in which unlike *Bhasma*, both organic and inorganic constituents are present.

Mashī is a dosage form in which bulk of raw material is reduced to a greater extent by application of a certain quantum of energy. As a result of this treatment hidden chemical constituents become prominent and/or new chemical moieties are formed which are therapeutically active. Due to thermal degradation or decomposition thermo labile constituent are lost. Therapeutically active organic and inorganic chemical constituents can be prepared by simple heat treatment in a controlled manner. The black color indicates high percentage of carbon and oxides. Non-specific odor and charcoal like taste may be attributed to oxides, inorganic elements and carbon (17).

Ayurvedic System of Medicine has its long history of therapeutic potential. *Ayurveda* is already well accepted and used since thousand years. Now it is time to give it modern scientific proof. Most of the drugs used today are obtained from natural sources or semi synthetic derivatives of natural products as mentioned and used in the Traditional Systems of Medicine. Thus it is a logical approach to drug discovery to screen traditional natural products instead of randomly synthesized chemical moieties. So we undertook this *Triphala Mashī* as a prototype to give it scientific proof. References about *Triphala Mashī* were found in *Bhaisajyaratnavali* and *Bharat Bhaisjya Ratnakar*. The objective of the present investigation was to analyze antimicrobial potential of *Triphala Mashī* and also to assay its toxicities.

Material and Methods

Raw Material

Triphala was procured from local markets, Pune, Maharashtra, India.

Preparation of Triphala Mashī

Triphala *Mashī* was prepared by heating the Triphala in closed silica crucibles in a muffle furnace. Triphala was

heated from 30°C to 450°C higher temperature by continuously increasing temperature to 10°C/min.

Preparation of Triphala and Triphala Mashī Extracts

Aqueous extracts (1:6) of Triphala and Triphala *Mashī* were prepared by hot maceration method and ethanolic extracts (1:6) of both were prepared using soxhlet apparatus. The extracts were filtered and the solvent was removed using rotary evaporator. The extracts were stored in an airtight glass bottle in a refrigerator.

Preliminary Phytochemical Screening (18)

Phytochemical evaluation of aqueous and ethanol extract of Triphala and Triphala *Mashī*: A stock solution was prepared by dissolving 500 mg extract in 20 ml of solvent that was subjected to preliminary phytochemical testing for the detection of major chemical groups.

Antimicrobial Activity using Agar Diffusion Method

We accomplished isolation of pathogens from clinical samples obtained at the hospital, Pune (India) with specific diagnosis of the different human pathologies. They were characterized through microscopic examination, Gram's character, and biochemical test profile. They were analyzed multiple drug resistance against standard antibiotics and antimicrobial activity with Agar Diffusion Method of plant extract against the organisms.

Isolation of Pathogens from Clinical Samples

The organisms were isolated from patients who visited clinical pathology for samples like sputum, wound swab, urine, pus, semen, bronchial lavage, etc. The standard method for isolation and identification based on microscopic examination, cultural characteristics and biochemical properties was followed (19). The samples were collected carefully, avoiding contamination with normal flora of the body and enriched in selective media and subjected to identification and characterization.

Characterization (19)

It was based on the microscopic examination, Gram's character and biochemical test, profile is presented in table 1.

Testing Multiple Drug Resistance against Standard Antibiotics

Isolated cultures were grown overnight in sterile nutrient broth and their optical density (O.D.) adjusted to 0.1. This O.D. adjusted culture was spread on sterile nutrients agar plates. With a sterile forcep the multi-antibiotic disc was placed on the spreaded plate.

Table 1. Microscopic and biochemical profile of clinical isolates

Isolate No.	Name of the organism	Biochemical tests						
		Indole	Urease	Citrate	TSI	Oxidase	Catalase	Coagulase
2443	<i>Escherichia Coli (urine)</i>	+ve	+ve	+ve	A/G	N/A	N/A	N/A
2438	<i>Klebsiella Pneumoniae</i> (extra ETT) secretion	-ve	+ve	+ve	A/G	N/A	N/A	N/A
2379	<i>Pseudomonas Aeruginosa</i> (Wound swab)	-ve	+ve	+ve	A/G	+ve	+ve	N/A
2444	<i>Pseudomonas Aeruginosa</i> (semen)	-ve	-ve	+ve	A/G	-ve	+ve	N/A
2378	<i>Escherichia Coli</i> (urine)	+ve	-ve	-ve	A/G	N/A	N/A	N/A
2401	<i>Staphylococcus aureus</i> (Urine)	-ve	+ve	-ve	A	-ve	+ve	+ve
2384	<i>Klebsiella Pneumoniae</i> (Pus from abdominal wound)	-ve	+ve	-ve	A/G	N/A	N/A	N/A
2381	<i>Staphylococcus aureus</i> (Urine)	-ve	+ve	-ve	A	-ve	+ve	-ve
2445	<i>Staphylococcus aureus</i> (urine)	-ve	+ve	-ve	A	-ve	+ve	-ve
2447	<i>Staphylococcus aureus</i> (urine)	-ve	+ve	-ve	A	-ve	+ve	-ve

N/A, Not Applicable; A/G, Acid and Gas production; A, Acid production; +ve, Positive; -ve, Negative.

These plates were kept at 10°C to allow diffusion of the drug for half an hour. The plates were then incubated at 37°C for 24 h. Zone of inhibition around the particular drug was observed and recorded.

Testing Antimicrobial Activity of Triphala and Triphala Mashi Extracts (20)

Antimicrobial activity was checked by agar gel diffusion method. The cultures were grown in nutrient broth and incubated at 37°C, for 24 h. After incubation period is finished the O.D. of the culture was adjusted to 0.1 with sterile nutrient broth. The 0.1 ml of the culture was seeded in 25 ml molten nutrient agar butts, mixed and poured into sterile petri plate and allowed to solidify. The wells were bored with 8 mm borer in seeded agar. Then the particular concentration (50–1500 mg/ml) of the Triphala and Triphala *Mashi* extract (0.4 ml each containing 50–1500 mg/ml) was added in each well. Soon afterwards the plates were then kept at 10°C for 30 min. After it normalized to room temperature plates were incubated at 37°C for 24 h. After incubation period was finished the zone of inhibition was measured and recorded.

Acute Oral Toxicity

Acute oral toxicity refers to those adverse effects that occurred following oral administration of a single dose of a substance or multiple doses given within 24 h. OECD Guideline No. 425 (Acute Oral Toxicity–Acute Toxic Class Method) is undertaken as a test procedure to ascertain the acute oral toxicity, that occurred after administration of the test substance. The test procedure described in this OECD guideline is of value in minimizing the number of animals required to estimate

the acute oral toxicity. In addition to the estimation of LD₅₀ and confidence intervals, the test allows observation of signs of toxicity.

Test Report

We worked with six groups of six mice each, of the swiss albino mice species/strain, sourced from National Toxicology Center, Pune, of the female sex, weighing 25–30 g, maintained in standardized environmental conditions (animal house conditions) with free access to food and water and with water used as vehicle. We tested aqueous and alcoholic extracts of Triphala and Triphala *Mashi*, in conditions presented subsequently.

Test Conditions

Individual body weights of rats were determined just before dosing. All extracts were dissolved in water. No information was available on toxicity of the substance to be tested. As per AOT 425, initial dose of 175-mg/kg-body weight was administered. Rats were observed for 48 h. Based on the status (alive or died) of rats during the observation period the next dose of the test drug is decided as per OECD guidelines 425. Stopping criteria was met as three consecutive animals survived at the upper limit (5000 mg/kg) or three consecutive rats died. Oral administration of the drug solution was carried out using a 'gavage'. Drug solution was prepared freshly just before dosing. Dosing volume was 1 ml/100 g of body weight, at 9:30 am. After the period of fasting, performed rats were weighed and then the test substance was administered and after the administration of drug, food was withheld for 1–2 h.

Table 2. Multiple drug resistance pattern against standard antibiotics

Isolate No.	Name of the organism	Amikacin	Bactrim	Ciprofloxacin	Cefotaxime	Cefatazidime	Cefuroxime	Ceftriaxone	Furadentin	Augmetin	Norfloxacin	Netromycin	Cefaparazone	Levofloxacin	Epocinin	Piperacillin	Ofloxacin
2443	<i>Escherichia Coli</i> (urine)	S	S	R	R	R	R	R	S	R	R	R	R	R	R	R	R
2438	<i>Klebsiella Pneumoniae</i> (extra ETT) secretion	R	–	R	I	I	R	R	–	R	–	R	S	–	–	R	–
2379	<i>Pseudomonas Aeruginosa</i> (Wound swab)	S	R	R	R	S	R	R	R	R	R	S	R	R	S	–	R
2444	<i>Pseudomonas Aeruginosa</i> (semen)	S	–	R	R	R	R	R	–	R	–	R	R	–	–	R	–
2378	<i>Escherichia Coli</i> (urine)	S	R	R	S	S	R	R	S	I	R	R	R	I	S	–	R
2401	<i>Staphylococcus aureus</i> (Urine)	S	R	R	S	S	R	R	S	I	R	R	R	I	S	–	R
2384	<i>Klebsiella Pneumoniae</i> (Pus from abdominal wound)	S	R	R	R	R	R	R	R	R	R	R	R	R	I	–	R
2381	<i>Staphylococcus aureus</i> (Urine)	S	–	R	S	S	R	S	–	S	–	S	S	–	S	S	–
2445	<i>Staphylococcus aureus</i> (urine)	R	–	R	R	R	R	R	–	R	–	R	R	–	–	R	–
2447	<i>Staphylococcus aureus</i> (urine)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	–	S

S, Sensitive; R, Resistance; I, Insensitive. (i) Aminoglycosides (1. Amikacin, 2. Netromycin.) (ii) Cephalosporins (1. Cefotaxime, 2. Cefatazidime, 3. Cefuroxime, 4. Ceftriaxone, 5. Cefaparazone.) (iii) Quinolones (1. Ciprofloxacin, 2. Norfloxacin, 3. Ofloxacin, 4. Levofloxacin) (iv) Sulphamethoxazole with trimetropim (Bactrim) (v) Clavulanic acid with amoxicillin (Augmetin) (vi) Penicillin (Piperacillin) (vii) Furadentin (Nitrofurantoin).

Results

Antimicrobial activity

Effectiveness of Extracts

In general, the aqueous and ethanolic extracts of Triphala and Triphala *Mashi* exhibited a broad-spectrum antimicrobial activity against all the microorganisms from human secretions and from pathology lab with prior diagnosis. It inhibited the growth of all Gram-positive and Gram-negative bacteria. In agar diffusion method, with the broad range of concentrations of 50–1500 mg/ml of the extract, the growth of all microorganisms was inhibited. Aqueous extracts shows better activity than ethanolic extract for all strains. More inhibitory zone is observed for the strains *E. coli*, *S. aureus*. All extracts show dose-dependant activity (Fig. 1). All the microorganisms that presented resistance to certain tested antibiotics, showed good susceptibility to the extracts of Triphala and Triphala *Mashi*. Variations of susceptibilities and resistance existed among same microorganisms to the same antibiotics.

Toxicity Study

No signs of toxicity were observed in short-term analysis but mortality was seen in long-term study at the dose of 5000 mg/kg. The data collected for individual animals is presented in Table 4. Triphala aqueous and alcohol extract (50–1500 mg/ml)/Triphala *Mashi* aqueous and alcohol extract (50–1500 mg/ml). *Escherichia coli* (2443/2378/2381)/*Klebsiella* (2438/2384)/*Pseudomas* (2379/2444)/*Staphylococcus aureus* (2401/2445/2447).

Discussion

The antimicrobial mechanisms of tannins can be summarized as follows (21). (i) The astringent property of the

tannin may induce complexing with enzymes or substrates (22). Many microbial enzymes in raw culture filtrates or in purified forms are inhibited when mixed with tannins (23). (ii) Tannins toxicity may be related to their action on membranes of the microorganisms. (iii) Complexation of metal ions by tannins may account for tannin toxicity (24). The ester linkage between gallic acid and glucose was important to the antimicrobial potential of these compounds.

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two 2OH groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on and the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. In addition, some authors have found that more highly oxidized phenols are more inhibitory. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with the proteins (25).

Several studies have shown that purified catechin fractions from green and black tea, and Epicatechin gallate (ECG) and Epigallocatechin gallate (EGCG) in particular, inhibit the growth of many bacterial species and possess anticariogenic properties (26–29). ECG and EGCG, but not Epicatechin (EC) or EGC, have been reported to be powerful antagonists of human immunodeficiency virus reverse transcriptase, causing 50% inhibition at concentrations of 10–20 ng/ml (30). Ikigai *et al.* (31) showed that EC was much less active than EGCG. *Staphylococcus aureus* was more susceptible than *E. coli*, consistent with a much greater binding of EGCG to staphylococci. The MICs of EGCG and EC were 73 and 573 mg/ml, for *S. aureus* and 183 and 1140 mg/ml, for *E. coli*, respectively. The bactericidal effect of EGCG was attributed to membrane perturbation.

Table 3. Preliminary phytochemical screening

Chemical test	Triphala aqueous	Triphala <i>Mashi</i> aqueous	Triphala alcoholic	Triphala <i>Mashi</i> alcoholic
Alkloids	–	–	–	–
Anthraquinone glycosides	+	–	+	–
Saponins	++	+	–	–
Steroids and triterpenoids	–	–	–	–
Phenolic compounds	++	++	++	++
Tannins				
Proteins	–	–	–	–
Ascorbic acid	+	+	+	+
Sugars	–	–	–	–

–, Negative; +, Positive; ++, Positive.

Table 4. Effect of Triphala and Triphala *Mashi* on mortality in *Swiss albino mice*

Sr.No.	Animal ID (n=6)	Dose (mg/kg)	Short-term result	Long-term result
1	H	175	O	O
2	B	550	O	O
3	T	1750	O	O
4	HB	5000	O	X
5	BT	5000	O	X
6	HBT	5000	O	X

X, Died; O, Survived.

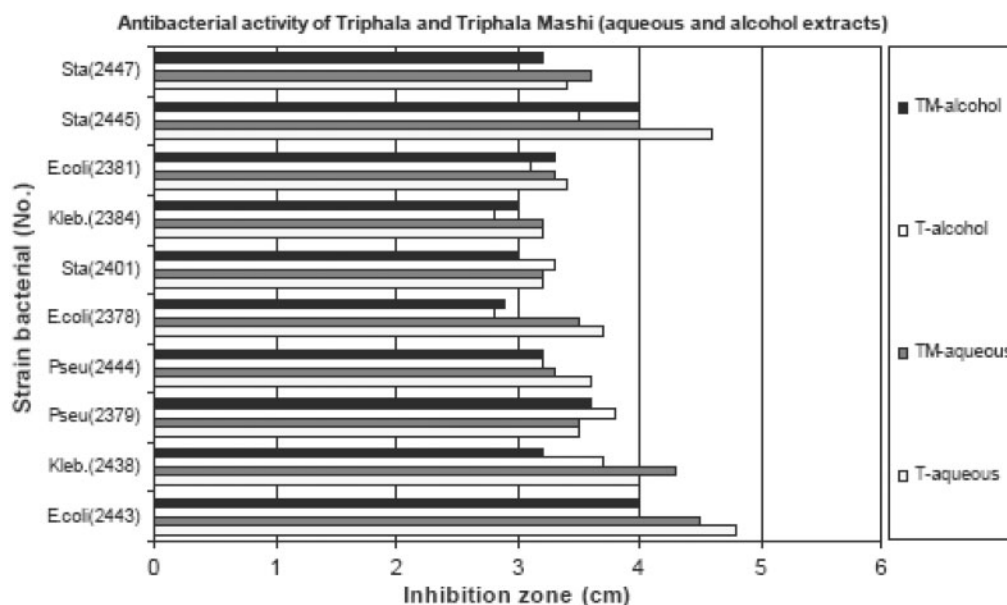


Figure 1. Antibacterial activity of Triphala and Triphala *Mashī*.

In Ayurvedic texts *Triphala Mashī* has been mentioned for its antimicrobial effect. To justify the claims made in *Ayurveda*, its antimicrobial activity was checked and results were expressed in terms of zone of inhibition (in cm). Bacterial strains were multiple drug resistance strains of both Gram-positive and Gram-negative (Table 2). Both aqueous and alcoholic extracts of Triphala and Triphala *Mashī* were used for antimicrobial activity. Different concentrations of samples from 50 mg/ml to 1500 mg/ml showed linear activity with increase in concentration. Total organic carbon content was determined to analyse the organic constituent's degradation in Triphala *Mashī*. Preliminary phytochemical screening of both Triphala and Triphala *Mashī* indicates the presence of tannins and ascorbic acid. Anthraquinone glycosides were present only in Triphala extract but these are absent in Triphala *Mashī* (Table 3). This shows that the degradation of anthraquinones takes place as temperature increases.

Estimation of total phenolic content and total tannins give reproducible quantity of tannins and phenolics in both Triphala and Triphala *Mashī*. TLC study also reveals that Triphala *Mashī* contains comparable gallic acid, ellagic acid and other tannins as that of Triphala. In the present circumstances antimicrobial effect may be attributed to these constituents. Further detail analysis is required in order to confirm the prediction. Also during TLC research there are new bands formed in Triphala *Mashī* which are not present in Triphala that may be due to heat treatment. So further fractionating and studying antimicrobial effect is necessary to possibly reveal molecules from Triphala *Mashī*. There is need to pursue the characterization of active principles, to optimize the observed activity.

Such results are interesting, because bacteria was isolated from a hospital environment and its control is difficult by the usual therapeutic means. Studies regarding the mode of action for these compounds in the bacterial cell should be done.

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