

Original article

Antimicrobial activity of Triphala against bacterial isolates from HIV infected patients

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How to cite this article:

Safiullah A, Harish CC, Vijay Anand K, Saira K. Antimicrobial activity of Triphala against bacterial isolates from HIV infected patients. Jundishapur J Microbiol. 2011; 4(Supplement 1): S9-S17.

Received: July 2010

Accepted: October 2010

Abstract

Introduction and objective: The uses of traditional medicinal plants for primary health care have steadily increased worldwide in recent years. 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. Aqueous and alcoholic extracts of both Triphala and its individual components were used, to evaluate antimicrobial activity.

Materials and methods: Phytochemical (phenolic, flavonoid and carotenoid) and antibacterial activities of aqueous ethanolic extracts of Triphala and its individual components (*Terminalia chebula*, *T. belerica* and *Embllica officinalis*) were tested against several bacterial isolates. Isolates were recovered from urethral swabs, seminal fluid, urine, high vaginal swabs, skin swabs, blood, and sputum specimen of HIV infected patients.

Results: *Terminalia chebula* has high phytochemical content followed by *T. belerica* and *E. officinalis*. In anti-bacterial activity, most of the bacterial isolates were inhibited by the ethanolic and aqueous extracts of *T. chebula* followed by *T. belerica* and *E. officinalis* in both disk diffusion and minimum inhibitory concentration (MIC) methods. But as a whole, Triphala did not show antibacterial activity. MIC of aqueous and ethanolic extracts of Triphala and its individual plant components were observed to vary from 0.1-100µg/ml.

Conclusion: In conclusion, this study showed that both ethanolic and aqueous extract of Triphala has potent antibacterial action against the wide variety of bacterial isolates from the HIV infected patients.

Keywords: Triphala; *Terminalia chebula*; *T. belerica*; *Embllica officinalis*; Antibacterial activity

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JJM. (2011); 4(Supplement 1): S9-S17.

Introduction

Recent trends in pharmaceutical industry establishing the technology in medicine which in turn leads to *Ayurvedic* preparations and the plant extracts against certain diseases Triphala is a traditional Ayurvedic herbal formulation [1] consisting of the dried fruits of three medicinal plants *Terminalia chebula*, *T. belerica* and *Embelica officinalis* also known as 'three myrobalan'.

Triphala means 'three' (tri) 'fruits' (phala). Antibiotic resistance has become a global concern [2]. Antibiotics exist in large numbers in today's pharmaceutical market. Despite that, their use is becoming increasingly restricted. The reason behind such a rapid decline is largely attributed to the development of drug resistance among microorganisms. Such a phenomenon is coupled by the toxicity possessed by many antimicrobial. Thus there is need for new antimicrobial agents that can overcome these drawbacks.

Antibiotics were studied and proved in the period of the late 1940s and 1950s, thereby antibiotics chemotherapy came in to full being. These antibiotics were effective against the full array of bacterial pathogens including Gram-positive and Gram-negative bacteria and intracellular parasites. There has been a worldwide move towards the use of traditional medicines due to the concern over the more invasive, expensive and potentially toxic mainstream practices [3].

Earlier study also explains that identified compounds within herbal are effective antibiotics [4]. Traditional healing system around the world that utilizes herbal remedies is an important resource for the discovery of new antibiotics [5]. Some traditional remedies have already produced compounds that are effective against antibiotic-resistant strains of bacteria [6,7]. The effects of fruit extracts on bacteria have

been studied by a number of researchers in different part of the world [7-9].

Triphala contains several compounds that have been proposed to be responsible for its claimed health benefits, including gallic acid, chebulagic acid, and chebulinic acid [10,11]. There is preliminary evidence that Triphala contains compounds with antioxidant properties in isolated cells and rats, however this has not yet been demonstrated in people [10,12-14]. It has been suggested that ethanolic and aqueous extract from the plants are potential source of antimicrobial agents [15]. The WHO estimates that 80% of the people in developing countries of the world rely upon traditional medicine for their primary health care needs and about 85% of traditional medicine involves the use of plant extracts. Medicinal plants provide remedy for all diseases that may afflict human being.

Triphala is a traditional alternative medicine herbal formulation, consisting equal parts of three medicinal plants namely *T. chebula* (Family: *Combretaceae*), *T. belerica* (Family: *Combretaceae*) and *E. officinalis* (*Euphorbiaceae*). The active constituents are unknown. Triphala has been used extensively as a drug against different diseases [16,17]. Formulations of Triphala is claimed to have anti-viral and anti-bacterial effect [18]. Triphala is prescribed for anticaries agent, myocardial injury, cancer etc [19-21].

The present study focuses on the antibacterial effect of Triphala, ethanolic and aqueous extract of Triphala against bacterial isolates from HIV infected patients. The phenolics, flavonoids and carotenoids in aqueous and ethanolic extracts of Triphala and its individual fruit component are also correlated with the antimicrobial effect of the Triphala formulation as whole and its different components.

Materials and methods

Collection of plant materials

Fruits belonging to the Triphala formulation were collected and authenticated from The Chief Botanist, Tamil Nadu Medicinal Plants farms and Herbal Medicine Corporation (Tampcol) Ltd., Chennai, India. The plant material was identified and authenticated by plant taxonomist, Dr. RLS. Sikarwar, Deendayal Research Institute, Chittrakoot. The seedless fruits were shade dried and powdered before use. The plant materials used in Triphala formulation include *T. chebula* and *T. belerica* belonging to the family of *Combretaceae* and *E. officinalis* which belongs to *Euphorbiaceae* family.

Preparation of aqueous and ethanolic extracts

Two types of extracts were prepared for this study. For aqueous extract, the powdered form of seedless fruits of *T. chebula*, *T. belerica* and *E. officinalis* and combined (Triphala formulation) maintained at 60°C for 3h in sterile distilled water. The resulting suspensions were filtered and evaporated for dryness at 60°C *in vacuo* for further use. The ethanolic extract was prepared from the powdered seedless fruits of *T. chebula*, *T. belerica* and *E. officinalis* and combined (Triphala formulation). These were extracted using 90% methanol as a solvent in a Soxhlet extraction apparatus 72h at room temperature. The solvent was then evaporated to dryness under reduced pressure in rotary evaporator at 45-70°C. The concentrated methanol extracts was stored in a desiccator for future use.

Microorganisms

Gram-positive and Gram-negative bacterial strains were used for the antibacterial activity of methanolic extract of Triphala. The strains were obtained from NCIM

(National Collection of Industrial Micro-organisms, National Chemical Laboratory), Pune. The Gram-positive strains used were *Bacillus subtilis* (NCIM 2718) and *Staphylococcus aureus* (ATCC 25923). Gram-negative strains used were *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 70063) and *Escherichia coli* (ATCC 25922) [22]. All the strains were maintained on nutrient agar at 4°C and were subcultured every month.

Phytochemical study

A stock solution (aqueous and ethanolic extracts of Triphala and its individual components) was prepared by dissolving 500 mg extract in 20ml of solvent that was subjected to preliminary phytochemical testing for the detection of major chemical groups. Phytochemical study was conducted to estimate phenolics, flavonoids and carotenoids from aqueous and ethanolic extracts of Triphala and its individual fruits combination. Isolation and estimation of phenolics, carotenoids and flavonoids were performed by protocols suggested by standardized procedure.

Extraction and estimation of total carotenoids were applied by standard procedure [23,24]. The quantitative estimation of phenolics, flavonoids and carotenoids done by standardised procedure [25].

Bacterial dilution

The bacterial strains used in this study were clinical isolates from urethral swabs, seminal fluid, urine, high vaginal swabs, skin swabs, blood, and sputum specimen of HIV infected patients. The isolates were identified by standard culture and biochemical methods [22]. The organisms were maintained on agar slope at 4°C and subcultured for 24h before use. Isolated colonies of the bacteria were placed into individual tubes containing 5ml of highly

nutritious general purpose sterile brain-heart infusion broth growth medium (BHIB Himedia, Mumbai, India) and incubated at 37°C, before adjusting the tubes with 0.5 McFarland units using sterile BHIB [26].

The strains were cultivated in chopped-meat medium (CMM) and incubated at 31°C for 18h and colonies were transferred to BHIB. The turbidity of the broth culture was adjusted to a 0.5 McFarland standard (0.048M BaCl₂ (0.5ml) mixed and adjusted volume to 1000ml (pH should be between 6.5-6.7). The dilutions were used within 15mins of their preparation and were vortexed prior to use.

Disk diffusion analysis

Sterile blank diffusion disks were placed into labeled trays for each extracts. A 10% of extract was prepared with both distilled water and ethanol. Then, this preparation was processed to prepare disks with various concentrations by saturating with 5µl of the individual aqueous or ethanol extracts. The positive control disks were prepared by saturating blank discs in either ethanol or sterile distilled water and allowing all the solvent to evaporate. The filter paper discs (6mm in diameter) were loaded with methanol extract (3mg/disc) and were allowed to dry completely. Discs with 10µl DMSO and gentamicin (10µg/disc) were placed as controls. The standardized inoculum 1-2×10⁷CFU/ml (0.5 McFarland standards) was lawn cultured on the surface of sterile Muller-Hinton agar (Himedia, Mumbai, India) pH 7.2-7.4 plates using sterile swab.

The inoculations were done along three axes in rolling motion to ensure uniform bacterial distribution and plates were incubated overnight at 37°C. The antibacterial activity against each test organism was quantified by determining average diameter of the zone of inhibition around the paper discs in millimetres. The

tests were performed twice and average diameters of zones were calculated

Minimum inhibitory concentration (MIC)

MIC values were determined using broth-dilution method. The extracts, sterilized by 0.45mm Millipore filters and inoculums were added to MH broth medium. Serial 10-fold dilutions were made that furnished a concentration range from 0.01-1000µg/ml for each extracts and the tubes incubated aerobically at 37°C for 18-24h. Two control tubes include antibiotic control (containing the growth medium without extract) and organism control (containing growth medium, physiological saline and the inoculum). The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes were regarded as MIC.

MIC of ethanolic extract was determined by serial dilution method. A total of 500µl of MHB was added to tubes. Stock solution of 50mg/ml of ethanolic extract was subjected to two fold dilutions, these concentration ranged between 50mg/ml and 0.0156 mg/ml. Again, 10µl of 10⁶CFU bacterial suspensions were added to the tubes. The tubes were incubated at 37°C for 24h. MIC was taken as the highest dilution of the extract that inhibited the growth of the bacteria. Lowest concentrations of the ethanolic extract, which inhibited the bacterial growth after a period of 24h of incubation at 37°C, were recorded as MIC. Minimum bactericidal concentration (MBC) was determined by subculturing 10µl of the MIC tube solution (showing no visible growth) on a fresh drug free MHA plate and incubating for 24h at 37°C. The highest dilution that yielded no bacterial growth was taken as MBC [27].

Results

The present study investigated the antibacterial effect of aqueous and ethanolic extracts of Triphala and its individual plant component against common bacterial isolates from HIV infected patients. The percentage of individual and combined formulation of Triphala extract yields of aqueous and ethanolic (%w/w) are given in table 1. The percentage of yields in ethanolic extracts of all plant materials such as *T. chebulla*, *T. belerica* and *E. officinalis* and Triphala equal mixtures (*T. chebulla*, *T. belerica* and *E. officinalis*) were found to be high compared with aqueous extracts of the same plant materials.

Table 1: The aqueous and ethanolic extracts yield of Triphala and its individual components

Plant materials	Aqueous (%)	Ethanolic (%)
Triphala (equal mixtures of ^{a, b} and ^c)	12	14
<i>T. chebulla</i> ^a	12	16
<i>T. belerica</i> ^b	13	15
<i>E. officinalis</i> ^c	12	18

In Kirby-Bauer's disk diffusion method aqueous and ethanolic extract of *T. chebulla* showed zone of clearance to most of the bacterial isolates. In aqueous extract, only *T. chebulla* showed zone of clearance against *Shigella sonnei*, *S. flexneri*, *Vibrio cholerae*, *E. coli* and *Enterococcus faecalis*, and the rest of aqueous extracts showed no zone of clearance (Table 2). The aqueous extract of *T. chebulla* (ATc) showed maximum inhibition zone (16mm ± 0.2) in *S. sonnei*, *E. coli*, and *S. typhi*-, 16mm ± 0.1

in *S. aureus* and *E. faecalis* and 15±0.00mm in *S. flexneri* and *V. cholera*. But no zone of clearance was observed in other aqueous extract of Triphala (AT), *T. belerica* (ATb) and *T. officinalis* (ATo).

The ethanolic extract of *T. chebulla* (ETc) and *T. belerica* (ETb) showed the maximum zone of inhibition rather than ethanolic extract of Triphala (ET) and *E. officinalis* (ETo) against *S. sonnei*, *S. flexneri*, *S. aureus*, *V. cholera*, *E. coli* and *E. faecalis*. The bacterial isolates namely *P. aeroginos* *Salmonella paratyphi* B and *S. typhi* were resistant to both aqueous and ethanolic extracts, particularly more sensitive to ethanolic than aqueous extract.

The bacterial isolates showed sensitivity to both aqueous and ethanolic extracts of Triphala and its individual components were selected for MIC determination. The *S. sonnei*, *S. flexneri* and *S. aureus* were sensitive to low concentration (0.01µg/ml) of ethanolic extracts of *T. chebulla*. Even aqueous extract showed antibacterial effect on the most of the bacterial isolates except *K. pneuminiae* and *S. typhi* (Table 3).

The ethanolic extract yield was more in both Triphala and its individual plant components than aqueous, followed by Triphala, *T. belerica* and *E. officinalis* respectively (Table 4). Such results are interesting because bacteria were 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.

Table 2: Antibacterial activity of aqueous and ethanolic extracts of Triphala and its individual plant components by Kirby-Bauer's disk diffusion

Tested organisms	Zone of inhibition (mm)							
	ATc	ATb	AEo	AT	ETc	ETb	EEo	ET
<i>P. aeruginosa</i>	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	16±0.2	≤ 5	15±0.0
<i>K. pneumoniae</i>	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5
<i>S. sonnei</i>	16±0.2	≤ 5	≤ 5	≤ 5	19±0.4	18±0.4	15±0.0	16±0.2
<i>S. flexneri</i>	15±0.0	≤ 5	≤ 5	≤ 5	18±0.4	16±0.1	16±0.1	16±0.2
<i>S. aureus</i>	16±0.1	≤ 5	≤ 5	≤ 5	18±0.4	16±0.1	16±0.1	15±0.0
<i>V. cholera</i>	15±0.0	≤ 5	≤ 5	≤ 5	15±0.0	17±0.3	16±0.2	16±0.2
<i>S. para typhi-B</i>	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5
<i>E. coli</i>	16±0.2	≤ 5	≤ 5	≤ 5	16±0.2	16±0.1	15±0.0	16±0.2
<i>E. faecalis.</i>	16±0.1	≤ 5	≤ 5	≤ 5	16±0.2	15±0.0	16±0.1	16±0.1
<i>S. typhi</i>	16±0.2	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5	≤ 5

ATc: Aqueous extract of *T. chebula*, EEo: Ethanolic extract of *T. officinalis*, ATb: Aqueous extract of *T. belerica*, ETb: Ethanolic extract of *T. belerica*, AEo: Aqueous extract of *T. officinalis*, x: No bacterial activity, AT: Aqueous extract of Triphala, ETc: Ethanolic extract of *T. chebula*, ET: Ethanolic extract of Triphala

Table 3: Minimum inhibitory concentration of aqueous and ethanolic extracts (µg/ml) of Triphala and its individual plant components

Tested organisms	AT	ATc	ATb	AEm	ET	ETc	ETb	EEm
<i>P. aeruginosa</i>	100	10	100	R	0.1	0.1	0.1	10
<i>K. pneumoniae</i>	R	100	R	R	10	10	10	10
<i>S. sonnei</i>	10	0.1	100	100	0.1	0.01	0.1	0.1
<i>S. flexneri</i>	10	0.1	10	100	0.1	0.01	0.1	0.1
<i>S. aureus</i>	10	0.1	10	100	0.1	0.01	0.1	0.1
<i>V. cholera</i>	10	0.1	10	100	0.1	0.1	0.1	0.1
<i>S. paratyphi-B</i>	10	0.1	10	100	0.1	10	10	100
<i>E. coli</i>	10	0.1	10	100	0.1	0.1	0.1	0.1
<i>E. faecalis.</i>	10	0.1	10	100	0.1	0.1	0.1	0.1
<i>S. typhi</i>	100	0.1	R	R	10	10	10	100

MIC: Values are expressed as µg/ml, R: No inhibition even at the lowest tested concentration, AT: Aqueous extract of Triphala, ET: Ethanolic extract of Triphala, ATc: Aqueous extract of *T. chebula*, ETc: Ethanolic extract of *T. chebula*, ATb: Aqueous extract of *T. belerica*, ETb: Ethanolic extract of *T. belerica*, AEm: Aqueous extract of *E. officinalis*, EEm: Ethanolic extract of *E. officinalis*

The phytochemical analysis of the ethanolic extracts performed showed the presence of phenolics, carotenoids, and flavonoids (Table 4). The concentrations (µg/ml) of Phenolics and flavonoids are found to be high in both aqueous and ethanolic extracts. But high content (µg/ml) of carotenoids observed in ethanolic extract rather than in aqueous extract.

Discussion

Isolation of microbial agents less susceptible to regular antibiotics and increasingly resistant isolates during antibacterial therapy is raising throughout the world, which highlights the need for new principles. Triphala and its individual components showed antibacterial effect on both Gram-positive as well as Gram-

negative bacteria suggesting the passage of the active chemical through both type of the bacterial cell walls.

Table 4: The phytochemical (phenolics, flavonoids and carotenoids), in aqueous and ethanolic extracts of Triphala and its individual fruit component

Drug (Triphala formulation)	Phenolics (µg/ml)	Flavonoids (µg/ml)	Carotenoids (µg/ml)
Aqueous extract			
<i>T. chebula</i>	9084	712	196
<i>T. belerica</i>	8256	624	166
<i>E. officinalis</i>	9520	682	194
Triphala	8026	680	184
Ethanolic extract			
<i>T. chebula</i>	11260	806	322
<i>T. belerica</i>	10860	740	286
<i>E. officinalis</i>	8620	710	280
Triphala	10600	724	300

In the present study, ethanolic extracts showed higher antibacterial effect than aqueous extracts. This might be due to the less solubility of the active constituents in aqueous solutions, which results in less or no antibacterial effect on the bacterial isolates at lower concentration. Moreover, the methanol fraction may facilitate the solubility of a mixture of active phyto components due to its high polarity. As many of the antibiotic compounds already identified in herbs are aromatic or saturated organic molecules, ethanol is an ideal solvent [28].

Even though the ethanolic extract of *E. officinalis* showed 18% yield but it showed poor antibacterial activity, perhaps due to increased temperature during extract procedures, which denature the active constituents. The ethanolic extract of *T. chebulla* (ETc), *T. bellerica* (ETb), Triphala (ET) and *E. officinalis* (ETo) showed maximum zone of inhibition in isolates of *S. sonnei*, *S. flexneri*, *S. aureus*, *V. cholera*, *E. coli* and *E. faecalis*. This might be due to the presence of more active phytochemical elements in the ethanolic extraction. But in

aqueous extract, only *T. chebula* showed zone of clearance against *S. sonnei*, *S. flexneri*, *V. cholerae*, *E. coli* and *E. faecalis*. The results obtained in this study are similar to that of the earlier study where methanol extract of *Evolvulus nummularius* has showed antibacterial activity [29]. The phytochemicals (Phenolics, flavonoids and carotenoids) were reported to have antibacterial activity [30] which support the present study.

Some of the simplest bioactive phyto chemicals consist of phenolic ring, shown to be toxic to microorganisms. The site(s) and number of hydroxyl and the phenol group are thought to be related to their relative toxicity to microorganisms on the 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 [28].

The present study indicates that most of bacterial strains are more sensitive to *T. chebula* than *T. belerica* and *E. officinalis* and this is in contrast to the earlier study in which methanol extract of *E. nummularius* (L) showed susceptibility to only *E. coli* and *B. subtilis* [29]. This may be due to the presence of high phytochemicals content. The mechanisms of antibacterial activity are complex and could be attributed to either inhibiting cell division or by damaging the cell walls of the bacterium. In the present circumstances, antimicrobial effect may be attributed to these constituents.

Chemical spectral and biochemical composition studies support this study. Further fractionation of ethanolic preparation is necessary to study antimicrobial activity. Such results are interesting because the bacteria were isolated from a hospital environment and their control is difficult by the usual therapeutic means. Studies regarding the mode of action for these compounds in the bacterial cell should be done. 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 *T. chebula* than *T. belerica* and *E. officinalis*. There is a need to pursue the characterization of active principles, to optimize the observed activity. The bacterial isolates namely *P. aeruginosa*, *S. paratyphi* B and *S. typhi* were resistant to both aqueous and ethanolic extracts but in the case of methanolic extract of *E. nummularius* the resistant strains were *S. aureus*, *K. pneumoniae* and *P. aeruginosa* [29].

Conclusion

The results of this study show that aqueous and ethanolic extract of Triphala (*T. chebula*, *T. belerica* and *E. officinalis*) has antibacterial effect and hence Triphala can

be used as an antibacterial drug. The antibacterial activity may be due to the presence of phenolics, flavonoids and carotenoids etc. The antibacterial activity exhibited by the ethanolic extract can be corroborated by the usage of this plant in Indian folk medicine.

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