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In vitro antioxidant and antimicrobial efficacy of Triphala constituents: *Emblica officinalis*, *Terminalia belerica* and *Terminalia chebula*

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Abstract
In Indian medicinal preparation, triphala (tri '*three*'; phala '*fruits*') comprises equiproportional herbal fruits: *viz*. *Emblica officinalis* (Syn. *Phyllanthus emblica*), *Terminalia belerica* and *Terminalia chebula*. The '*Tridoshic rasayana*', three constituent elements is characterized with balancing and rejuvenation effects govern the human life. Triphala constituents are rich in anti-oxidants and reported to poses anti-microbial properties. The present study deals with the qualitative and quantitative analysis of triphala components using different extraction techniques. Dry fruit extracts of *E. officinalis*, *T. belerica* and *T. chebula* were screened for the secondary metabolites presence and its antioxidant, antimicrobial activities were determined as well. The sign of biochemical properties opens up an arena to bring its effective use as a single or in combination, against different pathological conditions. Thus results obtained may be helpful rationale to formulate these fruit extract in modern system of health care.

Keywords: Antioxidant, antimicrobial activity, Triphala, phytochemicals

1. Introduction
The traditional system of herbal medicinal practice outcompete the use of synthetic drugs due to the fact of natural origination, cost effectiveness and least side effects. In contrast, synthetic drugs exhibit toxic and mutagenic effects than natural drugs [1]. These attributes brings extensive use of these herbal medicine since thousands of years in developing and developed countries. The most significant character of oriental herbal medicine is that herbal preparations are utilized singly or in composite formulae [2]. Thus the practice of Indian traditional medicine is widely spread and the abundance of plants, encompassing the natural phyto-chemical resources might serve as lead for novel drug development [3].
Researchers have formulated several modes for alienating the health issues and one such option is the facile and cost-effective herbal medicinal practice. A broad spectrum of diseases was treated using herbs since several decades [4]. Almost one fourth of pharmaceutical drugs are derived from the botanical species. For an example, Carbenoxolone, the first effective drug against gastric ulcer was discovered from the use of an indigenous plant (*Glycyrrhiza glabra*), called '*Athimadhuram*' in siddha medicine [5]. Similarly such investigation is the identification of Gefarnate from cabbage [6]. As a result of herbal medicinal practice, several fresh and non-drug substances were innovated. So, it is prime important to formulate novel medicines by eradicating constraints in the herbal medicinal practices.
Triphala is among the most popular herbal medicine in Indian ayurvedic system that could treat several diseases. Triphala churna is the equiproportional powdered formulation of the three myrobalan fruits: *Emblica officinalis* Gaertn. (Syn. *Phyllanthus emblica* L.) (Amla), *Terminalia belerica* Roxb. (Bibhitaki) and *Terminalia chebula* Retz. (Haritaki) that is enriched with bioactive compounds. This formulation has been in extensive use in ayurveda, for treating several disorders such as gastrointestinal, cardiovascular and visual systems [7].
The essential bioactive phyto-chemicals of triphala include alkaloids, essential oils, flavonoids, saponins tannins, terpenoids, and phenolic compounds [8]. The plants that are rich in phenolic compounds serve as resource of antimicrobials and antioxidants [9-12]. Thus ingestion of antioxidant compounds scavenges free radicals such as superoxide, hydrogen peroxide and decreases the levels of oxidative stress by gearing down or prevents complications associated with oxidative stress induced diseases [13]. These constituents have also been reported in ayurvedic medicine for its higher anti-microbial activity against wider spectrum of pathogenic and non-pathogenic bacterial strains [9, 10, 12]. The present study aims to determine the anti-oxidant and anti-bacterial efficacy of the botanical species that constituent triphala all along the phyto-chemical assay as well.

2. Experimental

2.1 Plant material collection: The fruits of *E. officinalis*, *T. belerica* and *T. chebula* were collected from Jaipur (Rajasthan), India and plant specimens were identified and confirmed from the state forest research institute (Jabalpur), India.

2.2 Bacterial strain collection: The clinically isolated pathogenic microbial strains of gram positive and gram negative bacteria comprising *Bacillus subtilis, Enterococcus faecalis, Corynebacterium diphtheria, Staphylococcus aureus, Salmonella typhimurium, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa* were identified and collected from department of microbiology, Nims medical college and hospital, Nims University (Rajasthan), Jaipur, India. All these microbial cultures were incubated on nutrient agar plates and maintained at 4 °C.

2.3 Plant extract preparation: The fruits of the *E. officinalis*, *T. belerica* and *T. chebula* were shade dried and were mechanically crushed. Plant extracts were prepared using different solvents, such as distilled water, methanol and petroleum ether at 65 °C using Soxhlet extractor. The extracts were then filtered and allowed to concentrated in rotary evaporator at 45-50 °C followed by lyophilization of residual semisolid materials was stored at -20 °C till further use [14].

2.4 Phyto-chemical assay: Extracts were tested for the presence of alkaloids, flavonoids, glycosides, phenols, sterols, tannins and terpenoids by standard procedure [15, 16].

2.5 Quantitative estimation of phenolic and flavonoid content

The quantification of phenol and flavonoid contents was determined by standard method [17, 18].

2.6 Anti-oxidant activity by DPPH radical scavenging assay

The antioxidant capacity of the fruit extracts were confirmed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay [19]. Different concentrations (0-100 $\mu g/ml$) of the fruit extracts were mixed with standard trolox in equal volume of ethanol. Latter 50 μl of DPPH solution (1 *mM*) was pipette out into the plant extract-trolox mixture by stirring it thoroughly. The resulting solution was then kept undisturbed for 2 minutes and optical density (OD) was measured at λ = 517 *nm*. After recording six repeated ODs, the percentage of radical scavenging activity was calculated:

% of radical scavenging = $[(A_0 - A_1)/A_0] \times 100$

Here, A_0 is absorbance of control; A_1 denotes the absorbance of the samples and standard. IC_{50} value was calculated to determine the antioxidant potential.

2.7 Antimicrobial assay: The antimicrobial activity was tested against different extracts of *E. officinalis*, *T. belerica* and *T. chebula* fruits by standard protocol [20]. About 20 *ml* of Muller-Hinton agar medium was poured into sterilized petri dishes and allowed to solidify. Bacterial strains were spread over the medium using a glass rod. Wells of 5 *mm* in diameter were punctured in the culture medium using sterile disk borers. The wells were then filled with 1 *ml* of different concentrations *i.e*. 10 *mg/ml*, 20 *mg/ml*, 40 *mg/ml*, 60 *mg/ml*, 80 *mg/ml* of the aqueous, methanolic and petroleum ether extracts of *E. officinalis*, *T. belerica* and *T. chebula* respectively. Plates were incubated at 37 °C for 24 *h*. Antimicrobial activities were evaluated by measuring the diameter (in millimetre) of zone of inhibition. Here, Gentamicin (10 $\mu g/ml$) was used as a standard drug.

2.8 Statistical analysis

Experimental data summarized were presented as Mean ± SD (n=6). Groups were compared together by one way analysis of variance followed by Student Newman-Keuls post hoc test. The acceptance level of significance was $p<0.05$. Here, In Stat (version 3) was used for analysis of experimental data.

3. Results

The preliminary qualitative and quantitative phyto-chemical assays were examined. Presence of bioactive constituents was confirmed with AQ, ME and PE extracts. From the Table (1) it is observed that the presence of alkaloids, flavonoids and phenolic compounds is recorded in all types of extraction techniques. Whereas, the presence of bioactive compounds such as alkaloids, glycosides, sterols, tannins and terpenoids showed distorted pattern pertaining to the solvent type. Presence of flavonoids and phenolic compounds in the extracts bestows the anti-oxidant efficacy [21, 22], Table (2) represents the concentration of flavonoids and phenolic compounds that are determined from *E. officinalis*, *T. belerica* and *T. chebula* using different solvents (AQ, ME and PE). ME extract of *E. officinalis*, *T. belerica* and *T. chebula* were found to poses higher concentration of flavonoids and phenolic compounds. Among these ME extract of *E. officinalis* and *T. chebula* showed highest concentration of phenolic compounds (215.6±0.74) and flavonoids (218.7±0.64) respectively. Whereas lower concentration of flavonoids and phenolic compounds were recorded in AQ extract followed by PE extract.

DPPH scavenging activity and IC_{50} value of different fruit extracts of *E. officinalis*, *T. belerica* and *T. chebula* were keyed in. Here ascorbic acid is taken as the reference antioxidants. DPPH scavenging activity is that it determines the ability of stable free radicals to accept an electron or hydrogen radical, so as to exhibit stable diamagnetic molecular property [23]. Reduction in the potential of DPPH radicals was determined by decrease in absorbance induced by the anti-oxidants. Minimum is the IC_{50} represents the maximal anti-oxidant efficacy of fruit extracts. IC_{50} is defined as the total antioxidant necessary to decrease the initial DPPH radical by 50% and was calculated for all the extracts based on the percentage of DPPH radicals scavenged. From present study, it is observed that ME fruit extracts exhibited competent IC_{50} than AQ and PE fruit extracts. Figure (1) clearly shows that ME fruit extract of *E. officinalis* (10 $\mu g/ml$) was found to pose efficient microbial inhibition, whereas ME fruit extract of *T. belerica* and *T. chebula* had exhibited maximal anti-microbial activity at 20 $\mu g/ml$. Thus the ME fruit extract of *E. officinalis* could significantly work more as efficient anti-oxidants and antimicrobials as compare to the other components among triphala.

Antimicrobial assay is to determine in terms of zone of inhibition (in *mm*), where maximum the zone of inhibition indicates the maximum antimicrobial activity. In the present study, growth of microbial inhibition was found to be induced by AQ, ME and PE fruit extracts of *E. officinalis*, *T. belerica* and *T. chebula* against the pathogenic bacterial strains. Here the ME fruit extract of *E. officinalis*, *T. belerica* and *T. chebula* was found to poses significant inhibitory zone as

compared to the standard gentamicin antibiotic. From the Table 4, it is revealed that maximal zone of inhibition is found in ME fruit extracts towards the examined bacterial strains. Among these, highest microbial inhibition was recorded against *S. typhi* (24.0±0.6) and *E. faecalis* (23.5±0.7) with *T. chebula* and *E. officinalis* fruit extracts respectively. Here, Gentamycin serves as control antibiotic throughout the study.

Form the gram +ve bacterial strains studied, efficient microbial inhibition (23.5±0.7, 16.0±0.5 and 14.7±0.5) was exhibited by ME fruit extract of *E. officinalis* against *E. faecalis*, *C. diphtheria*, *B. subtilis* respectively. Whereas, similar inhibition zone (13.9±0.5) was observed with *E. officinalis* and *T. chebula* against *S. aureus*. Among the examined gram –ve bacterial strains, the fruit ME extract of *E. officinalis* exerted the maximum (23.9±0.6 and 15.8±0.5) restrained growth of *S. typhi* and *K. pneumoniae*. Alongside ME fruit extract of *T. chebula* depreciated the incubation of *P. aeruginosa* (22.4±0.8) followed by *E. coli* (19.4±0.7).

4. Discussion

Traditional systems of Indian medicine comprise the botanical species that are rich in bioactive compounds exhibiting anti-oxidant [24], anti-microbial [25] and free radical scavenging activities [26]. The primary phyto-chemical identification revealed the presence of aforementioned bioactive compounds (Table 1) that are characterized with DPPH antioxidant and antibiotic activities. It has been reported that alkaloids, flavonoids, glycosides, phenols, sterols, tannins and terpenoids are found in aqueous fruit extract of *E. officinalis* [27]. Similarly, methanolic extract of *T. belerica* fruit found to have alkaloids, flavonoids, glycosides, phenols, sterols, tannins and terpenoids [25]. The plant extracts that constitutes alkaloids, flavonoids and phenolic compounds are responsible for their free radical scavenging and antimicrobial potential [9, 18, 28].

In pharmacognosy, presence of bioactive compounds as secondary metabolite is recorded in almost all plant species whereas its concentration varies with the plant part, environmental factors and solvents used for extract preparation. In the present study it is observed that on varying the solvents used for extract preparation resulted in diverse distribution of phytochemicals (Table 1) and higher concentration of bioactive compounds were recorded with ME fruit extract (Table 2). The presence and concentration of bioactive compounds are determined by the compatibility with the solvents polarity.

The present study demonstrates that ME fruit extract of *E. officinalis*, *T. belerica* and *T. chebula* were found to have significant DPPH radical scavenging and antimicrobial activity. As mentioned earlier, that IC_{50} of ME fruit extracts exhibited competent anti-oxidant activity than AQ and PE fruit extracts (Figure 1).

Antibacterial activity was estimated by the zone of inhibition. Maximum the zone of inhibition indicate greater the antimicrobial activity. The ME fruit extract of *E. officinalis*, *T. belerica* and *T. chebula* showed significant inhibition of examined bacterial strains (Table 3). Presence of higher concentration of flavonoids [21] and phenolic compounds [22] were in line with the maximal microbial inhibition [29] as was recorded against *S. typhi* (24.0±0.6) and *E. faecalis* (23.5±0.7) with *T. chebula* and *E. officinalis* fruit extracts respectively.

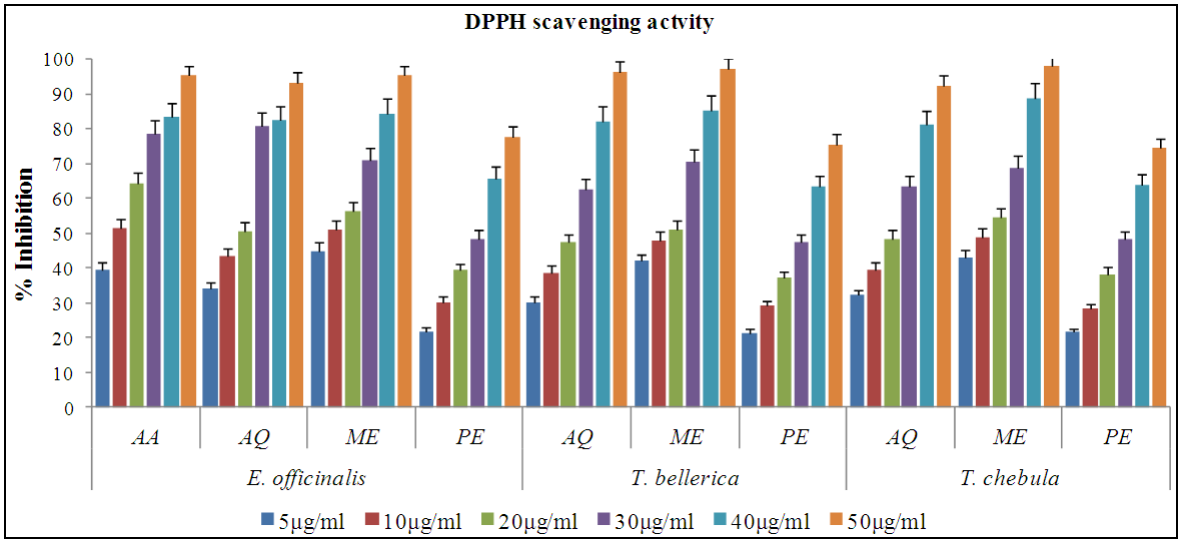

Fig 1: DPPH scavenging activity (%) presented at different concentration of aqueous extract (AQ), methanolic extract (ME) and petroleum ether extract (PE) of *E. officinalis, T. belerica and T. chebula.*

Table 1: Qualitative estimation of phyto-chemicals in the fruits of *E. officinalis*, *T. belerica* and *T. chebula* using aqueous extract (AQ), methanolic extract (ME) and petroleum ether extract (PE).

*Phytochemicals**	*E. officinalis*			*T. belerica*			*T. chebula*		
	AQ	ME	PE	AQ	ME	PE	AQ	ME	PE
Alkaloids	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+
Glycosides	+	+	+	+	+	-	+	-	-
Phenolics	+	+	+	+	+	+	+	+	+
Sterols	-	+	+	+	+	+	-	-	-
Tannins	+	+	+	-	+	-	+	-	+
Terpenoids	+	-	-	+	+	+	+	+	-

*The designated phytochemicals represented as presence (+) and absence (-)

Table 2: Quantitative estimation of phenolic and flavonoids contents in fruits of *E. officinalis*, *T. belerica* and *T. chebula* using aqueous extract (AQ), methanolic extract (ME) and petroleum ether extract (PE).

Phytochemicals	*E. officinalis*		*T. belerica*		*T. chebula*	
	Total phenolics	*Total flavonoids*	*Total phenolics*	*Total flavonoids*	*Total phenolics*	*Total flavonoids*
AQ	195.6±0.25	169.5±0.70	104.3±0.45	128.4±0.58	97.3±0.11	210.6±0.58
ME	215.6±0.74	176.5±0.20	133.3±0.80	137.4±0.93	127.4±0.00	218.7±0.64
PE	098.3±0.06	097.3±0.08	078.2±0.67	090.2±0.71	043.1±0.43	078.2±0.91

Data are represented as mean ± SEM

Table 3: Summary of antimicrobial efficacy recorded from *E. officinalis*, *T. belerica* and *T. chebula* fruit (AQ, ME, PE) extracts.

Bacterial strain		*Standard Antibiotic*	*Emblica officinalis*			*Terminalia belerica*			*Terminalia chebula*		
		GE	AQ	ME	PE	AQ	ME	PE	AQ	ME	PE
Gram +ve bacteria	*B. subtilis*	19.0±0.6	13.0±0.4	14.7±0.5	06.4±0.2	13.0±0.4	13.9±0.4	07.0±0.3	12.6±0.4	14.0±0.5	07.8±0.4
	E. faecalis	26.0±0.8	12.9±0.4	23.5±0.7	08.5±0.3	12.5±0.4	21.8±0.8	09.7±0.3	13.5±0.4	22.1±0.7	09.4±0.3
	C. diphtheria	19.8±0.6	12.1±0.4	16.0±0.5	08.5±0.3	12.1±0.4	15.0±0.5	09.0±0.3	14.4±0.5	14.9±0.5	10.0±0.4
	S. aureus	14.9±0.5	11.6±0.4	13.9±0.4	09.5±0.3	12.7±0.4	13.3±0.4	09.6±0.3	12.5±0.4	13.9±0.5	10.5±0.3
Gram -ve bacteria	*S. typhi*	27.1±0.6	16.4±0.6	23.9±0.6	09.9±0.4	14.4±0.5	22.4±0.7	09.3±0.4	15.6±0.5	24.0±0.6	08.1±0.2
	K. pneumoniae	19.0±0.6	14.2±0.5	15.8±0.5	07.7±0.4	13.9±0.6	14.0±0.8	08.6±0.3	14.8±0.5	15.5±0.6	08.6±0.5
	E. coli	21.0±0.6	14.0±0.6	18.8±0.6	07.0±0.4	12.5±0.5	19.0±0.5	06.1±0.3	13.0±0.4	19.4±0.7	06.0±0.3
	P. aeruginosa	27.1±0.9	11.7±0.5	21.4±0.5	07.0±0.2	13.8±0.5	20.8±0.5	07.4±0.3	12.8±0.4	22.4±0.8	08.0±0.4

5. Conclusion

The present study reveals that presence of significant bioactive compounds that includes alkaloids, flavonoids and phenolic compounds as responsible entities for anti-oxidant and anti-bacterial efficacy against the examined pathogenic strains. From the study it was observed that methanolic fruit extracts, obviates further incubation of tested pathogens. These attributes supports that methanolic fruit extracts of *E. officinalis, T. chebula* and *T. belerica* could serve as a potential antioxidant and antibacterial constituents. The sign of biochemical properties opens up an arena to bring its effective use as a single or in combination, against different pathological conditions. Thus results obtained may be helpful rationale to formulate these fruit extract in modern system of health care.

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