Standardization of Selected Indian Medicinal Herbal Raw Materials Containing Polyphenols as Major Phytoconstituents

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The fruits of *Terminalia chebula*, *Terminalia bellerica* and *Emblica officinalis* are important herbal raw materials containing polyphenols. They form the major constituents of widely used Ayurvedic formulations like *Triphala churna*. The extracts of these materials were standardized with respect to their total polyphenol contents as determined by Prussian blue method using gum acacia and phosphoric acid as stabilizers. The antioxidant activities were determined by DPPH (1,1-diphenyl-2-picryl-hydrazyl) method and inhibition of lipid peroxide formation induced by Fe^{2+}-ascorbate system. They were found to strongly correlate with total polyphenol contents. The EC_{50} value (µg/ml) for free radical scavenging activity by DPPH method and IC_{50} value (µg/ml) for lipid peroxidation inhibitory activity along with the total polyphenol contents can be used as quality control parameters for standardization of herbal raw materials containing polyphenols as major phytoconstituents.

Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological activities, higher safety margins and lesser costs. In India, the herbal drug market is about $ one billion and the export of plant based crude drugs is around $ 80 million[1]. But the most important challenges faced by these formulations arise because of their lack of complete standardization. Herbal medicines are prepared from materials of plant origin which are prone to contamination, deterioration and variation in composition. Therefore, quality control of herbal medicines offers a host of problems. To solve this problem, first and foremost task is the selection of the right kind of plant material which is therapeutically efficacious. Fundamentally, a better approach would be the one in which some direct correlation of marker compounds is generated with respect to the biological activity of the extract for a particular therapeutic area or disease pattern[2]. The commonly used analytical methods like chromatography have narrow scope in the analysis of heterogeneous botanical extracts. Most often a desired biological response is due to not one, but a mixture of bioactive plant components and the relative proportions of single bioactive compound can vary from batch to batch, while the bioactivity still remains within tolerable

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limits[3]. New trends are emerging in the standardization of herbal raw materials whereby it is carried out to reflect the total content of phytoconstituents like polyphenols which can be correlated with biological activity like antioxidant activity which many times has a direct or indirect correlation to the pathophysiological disorders like diabetes, cancer, inflammatory and age related disorders. In the present study, we have demonstrated this approach of standardization using fruits of *Terminalia chebula* (Family: Combretaceae), *Terminalia bellerica* (Family: Combretaceae) *and Emblica officinalis* (Family: Euphorbiaceae) which are known to be rich in polyphenols.

The dried and matured fruits of *T. chebula*, *T. bellerica* and *E. officinalis* were collected from three different geographical locations in India that is Maharashtra (MH), Gujarat (GJ) and Uttar Pradesh (UP) in February 2004 and were identified at the Department of Pharmacognosy and Phytochemistry, Prin. K. M. Kundnani College of Pharmacy, Mumbai. The voucher specimens have been retained in the Department of Pharmacognosy and Phytochemistry, Prin. K. M. Kundnani College of Pharmacy. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was purchased from Sigma Aldrich, St. Louis, MO. Gallic acid was purchased from Loba Chemie Pvt. Ltd., Mumbai. All other chemicals and solvents used were of analytical grade.

The fruits of *T. chebula*, *T. bellerica* and *E. officinalis* were powdered separately and 5 g of powder of each was extracted using 100 ml of 70% aqueous acetone by Soxhlet extraction. The concentrated extracts were evaporated to dryness under reduced pressure at 45° and the extractive values were calculated. These extracts were further used for the estimation of total polyphenols and antioxidant evaluation.

The amounts of total polyphenols in the fruit extracts were determined according to the Prussian blue method using 1% gum acacia and 85% phosphoric acid as a color stabilizer[4]. To 0.1 ml of sample solutions, 1 ml of 0.016 M $K_3Fe(CN)_6$ was added followed immediately by 1 ml of 0.02 M $FeCl_3$ in 0.1 N HCl. The contents were mixed well and kept at room temperature for 15 min. This was followed by addition of 5 ml of stabilizer containing water, 85% H_3PO_4 and 1% gum acacia in volume proportions of 3:1:1. The contents were vortexed and the colour density was measured at 700 nm against a reagent blank consisting of all of the reagents except the polyphenols using Shimadzu UV/Vis spectrophotometer-1601. The total polyphenol contents were calculated as % w/w of gallic acid equivalents.

Free radical scavenging activity was assayed spectrophotometrically by DPPH assay[5,6]. The reduction in absorbance of DPPH solution (2 mg/ml) was monitored at 516 nm after addition of various concentrations of test extracts to DPPH reagent and maintaining these solutions at room temperature for 5 min. The EC_{50} values for each test material were calculated from the calibration curves of concentration of extract (µg/ml) verses % reduction in absorbance after subjecting to linear regression between 10-80%. This acitivity was expressed as effective concentration at 50% (EC_{50}) that is the concentration of the test solution required to give a 50% reduction in absorbance of the test solution as compared to that of blank solution. Gallic acid was used as a positive control. The lipid peroxidation inhibitory activity of the fruit extracts was studies by the method Ohkawa *et al.*[7]. The reaction mixture contained mice liver homogenate (0.2 ml, 10% w/v) in 0.15 M KCl, KCl (0.1 ml, 150 µM), Tris buffer (0.4 ml, pH 7.5) and various concentrations of test extracts. *In vitro* lipid peroxidation was initiated by addition of $FeSO_4$. $7H_2O$ (0.1 ml, 10 µM) and ascorbic acid (0.1 ml, 100 µM). The reaction mixture was incubated at 37° for 1 h. After the incubation period, reaction was terminated by addition of thiobarbituric acid (TBA- 2 ml, 0.8%) and by heating the contents for 15 min. for development of coloured complex. The lipid peroxides formed were measured as thiobarbituric acid reacting substances (TBARs) by method of Ohkawa *et al*[7]. The tubes were then centrifuged at 4000 rpm for 10 min. and

TABLE 1: EXTRACTIVE VALUES OF *T. CHEBULA, T. BELLERICA* AND *E. OFFICINALIS* FROM DIFFERENT GEOGRAPHICAL LOCATIONS

Samples	Extractive value % W/W* ± SD		
	MH	GJ	UP
T. chebula	55.57±0.35	57.35±0.05	60.28±0.15
T. bellerica	39.83±0.67	41.64±0.18	42.61±0.37
E. officinalis	38.63±0.87	40.78±0.27	44.78±0.54

*Mean of three determinations, MH- Maharashtra, GJ – Gujrat, UP- Uttar Pradesh

TABLE 2: TOTAL POLYPHENOL CONTENTS OF EXTRACTS OF *T. CHEBULA, T. BELLERICA* AND *E. OFFICINALIS*

Samples	% W/W* ± CV		
	MH	GJ	UP
T. chebula	60.44±1.32	63.37±0.92	67.33±0.45
T. bellerica	55.07±1.80	57.85±1.42	58.41±0.59
E. officinalis	38.80±0.40	41.29±0.71	45.76±1.08

*Mean of six determinations, expressed as gallic acid equivalents (GAE), CV- Coefficients of variation, MH- Maharashtra, GJ- Gujarat, UP- Uttar Pradesh.

TABLE 3: FREE RADICAL SCAVENGING ACTIVITY OF EXTRACTS OF *T. CHEBULA*, *T. BELLERICA* AND *E. OFFICINALIS* BY DPPH ASSAY

Samples	EC_{50} (µg/ml) ± SD					
	MH	r*	GJ	r*	UP	r*
T. chebula	3.56±0.04	0.96	3.53±0.08	0.96	3.29±0.04	0.95
T. bellerica	4.68±0.02	0.96	4.57±0.01	0.97	4.52±0.08	0.97
E. officinalis	5.15±0.06	0.96	4.90±0.02	0.97	4.85±0.03	0.97
Gallic acid	1.12±0.01	0.98	-	-	-	-

Values are mean±SD; n =3, r* Correlation coefficient, MH- Maharashtra, GJ – Gujarat, UP- Uttar Pradesh

cooled. The % inhibition of lipid peroxidation was determined by comparing the results of test compound with those of controls not treated with extracts by monitoring the colour intensity at 532 nm. Gallic acid was used as a positive control. The results were expressed as IC_{50} values that is the concentration of extracts required for 50% inhibition of production of lipid peroxides.

The phenolics, particularly polyphenols exhibit a wide variety of beneficial biological activities in mammals, including antiviral, antibacterial, immunostimulant, antiallergic, antihypertensive, antiischemic, antiarrhythmic, antithrombotic, hypocholesterolemic, hepatoprotective, antiinflammatory and anticarcinogenic[8]. A positive role of antioxidants is established in many of the disease states like liver toxicity [9], diabetes[10], cancer[11], neurodegenerative and several cardiovascular diseases[8,12]. The fruits of *T. chebula*, *T. bellerica* and *E. officinalis* are native plants of India and South East Asia and occupy important place in indigenous medicines, which when admixed in equal proportions form Ayurvedic formulation *Triphala churna*[13]. They exhibit wide varieties of activities like antimutagenic[11,14], antidiabetic[10], hepatoprotective[15]. Hence it is important to determine their total polyphenol contents which can be used as one of the quality control parameters for their standardization.

The fruits of *T. chebula*, *T. bellerica* and *E. officinalis* exhibited a geographical variation in their contents of phytoconstituents as revealed from their extractive values (Table 1) and total polyphenol contents (Table 2). The UP variety was found to be the most superior in this respect. Out of the three plant materials highest amount of total polyphenols were found in *T. chebula* followed by *T. bellerica* and *E. officinalis* as shown in Table 2.

The free radical scavenging activity as measured by DPPH assay reveals the hydrogen donating capacity of the constituents present in the extracts. Polyphenolic phytoconstituents like flavonoids, hydrolysable and condensed tannins usually are good antioxidants because of their proton donating properties. In the present investigations the extract of *T. chebula* showed highest activity with the lowest EC_{50} value followed by the extracts of *T. bellerica* and *E. officinalis* (Table 3).

A dose dependent relationship was observed when the extracts of *T. chebula*, *T. bellerica* and *E. officinalis* were tested for their ability to reduce lipid peroxides generated by Fe^{2+} -ascorbate system. In this evaluation also the highest activity was shown by *T. chebula* extract (Table 4).

Our investigations have revealed a strong correlation between the contents of polyphenols in the fruits of *T. chebula*, *T. bellerica* and *E. officinalis* and their respective antioxidant activities. Hence the EC_{50} values (µg/ml) and the IC_{50} values (µg/ml) along with total polyphenol contents of the extracts of these fruits can be used as quality control parameters for their standardization.

TABLE 4: EFFECT OF *IN VITRO* ANTIOXIDANT ACTIVITY OF EXTRACTS OF *T. CHEBULA*, *T. BELLERICA* AND *E. OFFICINALIS* BY INHIBITION OF LIPID PEROXIDATION

Samples	IC_{50} (µg/ml) ± SD		
	MH	GJ	UP
T. chebula	13.78±0.62	10.34±0.57	8.82±0.05
T. bellerica	20.45±0.68	18.89±0.65	17.62±0.12
E. officinalis	37.42±0.74	35.01±0.71	32.93±0.17
Gallic acid	5.39±0.25		

Values are mean ± SD; n =3, MH- Maharashtra, GJ – Gujarat, UP- Uttar Pradesh

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