RESEARCH COMMUNICATIONS

Free radical scavenging reactions and phytochemical analysis of triphala, an ayurvedic formulation

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In order to understand the factors responsible for the potent antioxidant and radioprotecting ability of triphala, it has been evaluated for radical scavenging ability, xanthine oxidase inhibitory activity and phytochemical (phenolics) content. The radical scavenging experiments were carried out using fast reaction kinetic tools and the reactivity of triphala towards different radicals such as hydroxyl radicals, superoxide radicals, DPPH and ABTS* was determined. When triphala was tested for superoxide radical scavenging activity using xanthine and xanthine oxidase assay, it was observed that in addition to reacting with superoxide radical, it also inhibited uric acid formation, indicative of xanthine oxidase enzyme inhibitory activity. Phytochemical analysis showed that triphala is rich in phenols/polyphenols (38 ± 3%) and tannins (35 ± 3%), while flavonoids were found to be absent. HPLC analysis showed that triphala contains 73 ± 5 mg gallic acid per gram of triphala, which was found to increase to 150 ± 5 mg/g upon acid hydrolysis. Relevance of these studies to the antioxidant and radio protection properties of triphala has also been discussed.

Keywords: Gallic acid, free radicals, polyphenols, triphala.

TRIPHALA is an ayurvedic formulation, commonly prescribed by most healthcare practitioners in India. It is an equiproportional mixture of fruits of three medicinal herbs, amalaki (Emblica officinalis), haritaki (Terminalia chebula) and bibhitaki (Terminalia bellercica). It is mild, non-habit forming and rejuvenative, and hence is recommended for all. According to the traditional Indian medicinal system (Ayurveda), triphala strengthens the different tissues of the body, prevents ageing, promotes health and immunity. It corrects constipation, cleanses and tonifies the gastrointestinal tract and also detoxifies the whole body, and improves digestion and assimilation. It exhibits anti-viral, anti-bacterial, anti-fungal and anti-allergic properties. Triphala and its constituents act as cardio- tonic, control blood pressure, improve blood circulation and reduce cholesterol levels. Triphala shows immunomodulatory properties and helps in improving the body’s defence system.

In recent years there are also several reports in the literature which suggest that triphala possesses antimutagenic, radioprotecting and antioxidant activity. Jagetia et al. showed that triphala scavenges free radicals and protects mice from radiation-induced damage when administered intraperitoneally and exposed to 10 Gy of γ- radiation. Kaur et al. studied the antimutagenic activity and showed that acetone extract of triphala exhibits significant cytotoxic effect on cancer cells. Recently, our group has also been actively involved in evaluating a number of herbal extracts, including triphala and its constituents for their potential in vitro antioxidant activity. Our results showed that triphala effectively inhibits radiation-induced lipid peroxidation in rat liver microsomes and strand-breaks in plasmid DNA.

The above reported biochemical studies indicate that triphala is a free-radical scavenger. Therefore, the present work is aimed to evaluate triphala, prepared by aqueous extraction method, for the free radical scavenging ability under different conditions. Attempts have also been made to quantitatively identify important phytochemicals in triphala and correlate its phenolic content with the free radical scavenging reactions.

Dry fruits of the three medicinal plants, viz. E. officinalis, T. chebula and T. bellercica in equal amounts (500 g) were finely powdered and stirred with boiling distilled water for about 90 min. The liquid extract was filtered and the filtrate concentrated up to two parts on a rotary vacuum evaporator under reduced pressure. The concentrated liquid was spray-dried to get the dry powder of triphala (yield ~40%). The powder is readily soluble in water and leaves no undissolved materials. The concentration of triphala is expressed as µg/ml.

Antioxidant and radioprotection studies indicate that triphala should possess the ability of either inhibiting free radical formation, or itself is a free radical scavenger. During oxidative stress and exposure to radiation, excessive free radicals are produced, which are known to cause damage to biomolecules. Most of the free radicals are reactive and short-lived and in order to monitor their reactions, one has to employ fast kinetic methods. Free radicals have one or more unpaired electrons; therefore, they react with substrates either by electron transfer or by transfer of hydrogen atom. Here employing nanosecond pulse radiolysis, stopped-flow spectrometer and spectrophotometric methods, we studied the reactions of some important free radicals with triphala as discussed below.

Among the several free radicals, hydroxyl radical (‘OH) is the most potent oxidant, produced during radiation exposure as well as Fenton reactions. It is a highly oxidizing radical with standard reduction potential of 1.9 V vs NHE at pH 7. Using nanosecond pulse radiolysis technique, employing 7 MeV electron pulses, with an absorbed dose of 8 Gy, the reactions of hydroxyl radicals with triphala were carried out in microsecond timescale and the transients detected by absorption spectrometry. Rate constant for the reaction of any antioxidant with a free radical indicates its reactivity towards the free radical. However, in the case of plant extracts like triphala, as

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there are mixture of components with unknown concentrations, such estimation of rate constant is difficult. However, it is possible to estimate their relative reactivity in comparison with a standard such as potassium thiocyanate (KSCN) by competition kinetic method. In this method, \( \cdot \)OH is made to react with 2 mM KSCN at pH 7, in the absence and presence of various concentrations of triphala. In the absence of triphala, \( \cdot \)OH reacts completely with SCN\(^-\) to produce (SCN\(^-\))\(^2\). Formation of (SCN\(^-\))\(^2\) was observed by monitoring its absorbance at 500 nm. However in the presence of triphala, decrease in the absorbance of (SCN)\(^2\) was observed. From the extent of decrease, rate of scavenging of hydroxyl radical by triphala (T) was calculated.

The reactions involved in this competition kinetic method are:

\[
\cdot \text{OH} + 2\text{SCN}^- \rightarrow (\text{SCN})_2^+ \quad k_1 = 1.1 \times 10^{10} \text{ M}^{-1} \text{s}^{-1},
\]

\[
\cdot \text{OH} + \text{T} \rightarrow \text{Products} \quad k_2 = ?
\]

The absorbance due to (SCN)\(^2\) at 500 nm in the absence (A\(_0\)) and presence of varying concentrations of triphala (A), is related to the rate constants by the following equation

\[
(A_0/A) - 1 = k_2/k_1 \text{[T]}/[\text{KSCN}].
\]

Slope of the linear plot for \([(A_0/A) - 1] \text{ vs } [\text{T}]/[\text{KSCN}],\) both the concentrations expressed in \(\mu\text{g/ml},\) will give their comparative ability to react with the \(\cdot \text{OH}\) radical with respect to KSCN. Thus, from the slope (inset, Figure 1), the reactivity of triphala towards \(\cdot \text{OH}\) was calculated.

The rate constant\(^{24}\) for the \(\cdot \text{OH}\) reaction with KSCN has been reported to be \(1.1 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}\). Under identical concentration units expressed in \(\mu\text{g/ml},\) the reactivity of triphala was found to be twice that of KSCN.

We also tried to look for the transients formed in such reactions. Thus, the reaction of \(\cdot \text{OH} with triphala,\) produced transient absorbing in the region 350–500 nm (Figure 1a), with a weak shoulder at 350 nm and a major transient absorption peak at 440 nm. The spectrum may correspond to the formation of radicals of phenols/polyphenols present in triphala. To understand this, we compared this spectrum with that obtained for the reaction of gallic acid with \(\cdot \text{OH}\) (Figure 1b). Although some overlap was observed between the two, the spectrum did not completely match with that of the gallic acid radical. This may have been because triphala contains not only gallic acid but also a mixture of other phenols/polyphenols, all of which are reactive towards free radicals.

Superoxide radicals are one of the most important reactive oxygen free radicals constantly produced in living cells.\(^{23,25}\) One of the methods of generating superoxide radicals is by xanthine–xanthine oxidase (XO) assay, where XO enzyme catalyses the oxidation of xanthine to uric acid. During this conversion it produces superoxide radical by the reduction of molecular oxygen, which is detected by 2,6-dichlorophenol-indophenol (DCIP), as discussed earlier. Figure 2a shows percentage scavenging of superoxide radicals by triphala as a function of its concentration, from which the concentration of triphala required to inhibit superoxide radicals by 50% (IC\(_{50}\)) was estimated to be 42 \(\mu\text{g/ml}.\) However, this result implies either of the two: (1) inhibition of the enzyme.

Figure 1. Transient spectrum obtained during reactions of hydroxyl radical with (a) triphala and (b) gallic acid. Experimental conditions: \(\text{N}_2\text{O}-\text{saturated aqueous solutions containing 100 \(\mu\text{g/ml,}\) either gallic acid or triphala at pH 7.}\) (Inset) Competition kinetics plot for reaction of hydroxyl radical with varying concentrations of triphala in the presence of 2 mM thiocyanate (KSCN). Dose/pulse = 8 Gy.

Figure 2. (a) Percentage scavenging of superoxide radicals by triphala. Experimental conditions: 16 \(\mu\text{M} \text{xanthine, 0.02 units/ml xanthine oxidase and 10 \(\mu\text{M DCIP were incubated in the presence of varying concentrations (15 to 70 \(\mu\text{g/ml}\) of triphala. (b) Percentage decrease in uric acid formation under similar conditions without any DCIP (triphala concentration varied from 20 to 120 \(\mu\text{g/ml}.\) Uric acid estimated by HPLC analysis, using C18 PCX 500 column and mobile phase of 2.5% acetonitrile, 0.1 M KCl, 0.05 M HCl and detected at 290 nm.)
thereby reducing the formation of superoxide radicals, or (2) direct scavenging of superoxide radicals. In order to resolve this problem, XO inhibitory activity of *triphala* was studied by the estimation of uric acid formation. Generally uric acid concentration is monitored by following its absorbance at 290 nm. However in the present study, due to the interference of absorption by *triphala* at 290 nm, uric acid formed was estimated by HPLC [column, C18 PCX 500; mobile phase, aqueous acetonitrile (2.5%)–HCl (0.05 M)–KCl (0.1 M); detection, absorbance at 290 nm]. In this method, standard sample of uric acid at different concentrations of 2–35 µg/ml was injected and chromatograms at 8.3 min were recorded. By measuring the peak heights at different uric acid concentrations, a calibration plot was obtained. Under similar conditions, uric acid formed from xanthine/XO system was determined both in the absence and presence of *triphala* (20–126 µg/ml). By determining the peak heights and using the calibration plot, the XO inhibitory activity of *triphala* was calculated. Figure 2b shows the percentage inhibition of uric acid formation by different concentrations of *triphala*. Figure 2b also shows that 20 to 126 µg/ml of *triphala* effectively inhibits uric acid formation by 5 to 86%. These studies reveal that *triphala* not only exhibits superoxide radical scavenging ability, but also has XO enzyme inhibitory activity.

After confirming that *triphala* is a scavenger of both hydroxyl radical and superoxide radicals, we further studied the reactions of *triphala* with other radicals like 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS•)). These radicals are stable and are not biologically important. But their reactions can help in understanding the mechanisms and the studies can also be used to estimate the total free radicals scavenging activity of *triphala*.

DPPH is purple in colour (λ<sub>max</sub> 517 nm) and in the presence of a substance (A-H), capable of donating an electron or a hydrogen atom, it loses its radical character and becomes colourless<sup>25</sup>. Initially the absorbance of 125 µM DPPH in methanol at 517 nm, in the absence and presence of different concentrations of *triphala*, was followed, from which the concentration of *triphala* required to scavenge 50% of DPPH (IC<sub>50</sub>) was found to be 7 µg/ml. The kinetics of DPPH radical reaction with *triphala* was studied using stopped-flow kinetic spectrometer in single mixing mode. In the absence of *triphala*, DPPH radical did not show any significant decay (Figure 3a). However, in the presence of *triphala*, it decayed rapidly in seconds (Figure 3b).

The absorption–time plot was fitted to a single exponential function to get an observed rate constant (k<sub>obs</sub>), which was found to increase linearly with increasing *triphala* concentration from 10 to 40 µg/ml (inset, Figure 3), suggesting that some of the constituents present in *triphala* are capable of efficiently reacting with DPPH. Most of the plant phenols like ascorbic acid and gallic acid show high rate constants<sup>3</sup> with DPPH and the above reaction taking place in seconds confirms the presence of such phenols.

ABTS• radical is also stable like DPPH, and shows absorption in the wavelength region 400 to 750 nm, with extinction coefficient at 417 nm (ε = 3.47 × 10<sup>5</sup> M<sup>-1</sup> cm<sup>-1</sup>), 645 nm (ε = 1.35 × 10<sup>6</sup> M<sup>-1</sup> cm<sup>-1</sup>) and at 728 nm (ε = 1.5 × 10<sup>5</sup> M<sup>-1</sup> cm<sup>-1</sup>)<sup>29</sup>. The ABTS• radicals reactions involve electron transfer and take place at a much faster rate compared to DPPH radicals. Hence we employed pulse radiolysis technique to study ABTS• radical reactions. Details of generation of ABTS• radicals are given elsewhere<sup>10</sup>. In the absence of *triphala*, ABTS• radical did not show any decay (Figure 4b); however, in the pres-

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**Figure 3.** Absorption–time plot showing decay of 25 µM DPPH radical in methanol at 517 nm in the absence of *triphala* (a) and in the presence of 10 µg/ml of *triphala* (b). (Inset) Linear variation of k<sub>obs</sub> for decay of DPPH radical as a function of concentration of *triphala*.

**Figure 4.** (a) Linear plot showing variation of k<sub>obs</sub> for decay ABTS• radical as a function of ascorbic acid concentration. (Inset) Absorption–time profiles at 645 nm for decay of ABTS• radical in the absence (b) and presence of 45 µg/ml of *triphala* (c) and 45 µg/ml ascorbic acid (d).
ence of triphala it decayed in milliseconds (Figure 4c). The observed decay constant (k_{obs}) was monitored under pseudo first-order condition, where concentration of the radical was less than that of triphala. It was found that k_{obs} increased linearly with increase in concentration of triphala. It is possible to use such a reactivity parameter to estimate antioxidant equivalents in terms of a standard like ascorbic acid or gallic acid. In these studies we tried to estimate the antioxidant equivalents for triphala in terms of ascorbic acid as reference. A typical absorption trace showing the decay of ABTS^{•−} radical in the presence of ascorbic acid is given in Figure 4d. For the estimation of ascorbate equivalents, k_{obs} obtained from the decay of ABTS^{•−} radical in the presence of different concentrations of ascorbic acid (4–68 μg/ml) was plotted as a function of ascorbic acid concentration, to obtain a linear calibration plot (Figure 4a). Using this plot and the estimated reactivity parameter obtained from the decay of ABTS^{•−} radical in the presence of known amount of triphala, the ascorbate equivalents for triphala were calculated to be 33 ± 5%. These studies confirm that triphala possesses high amount of phenols/polyphenols, accounting for its activity towards several free radicals. Such antioxidants can take part in electron transfer and hydrogen atom transfer reactions with many oxidizing free radicals.

Since many other methods are available in the literature for the estimation of phenolic content of plant extracts, we tried to use some of them to find out the phenolic content of triphala.

Phenolic acids, flavonoids and tannins are the most commonly found polyphenolic compounds in plant extracts^{28,30}. In the present study we have estimated the total phenolic content in triphala using spectrophotometric methods^{30–32}. The Folin–Ciocalteau method, which is based on the principle of reduction of phosphomolybdic acid by phenols in the presence of aqueous alkali, was employed to determine the total phenolic content^{30}. Gallic acid was used as a standard, and the results expressed as % gallic acid equivalents, gave a mean value of 38 ± 3%. Tannins are naturally occurring, high molecular weight plant polyphenols. They are usually subdivided into two groups, hydrolyzable tannins and condensed tannins. The total tannin content present in triphala was measured using a colorimetric Folin–Denis method^{31}. The measurements were compared with standard tannic acid sample and results expressed in terms of % tannic acid equivalents. The tannin content in triphala was found to be 35 ± 3%. Analysis of total flavonoid content in triphala has been done using colorimetric method and quercetin as standard flavonoid^{32}. The results showed that triphala did not contain any significant amount of flavonoids.

Recent reports indicate the presence of gallic acid in the fruits of E. officinalis, T. chebula and T. bellierica^{11,21,33}. In these fruits, gallic acid is present either in free form or bound tannin form (gallotannins and ellagotannins). The amount of free gallic acid present in triphala formulations varies with the extraction process. During the extraction process, tannins present in the fruit samples may get hydrolysed to give free gallic acid or they may remain in bound form. Thus the exact quantification of free gallic acid in triphala is necessary. Therefore, HPLC method was used for the quantification of gallic acid in triphala [column, C18 PCX 500; mobile phase, aqueous acetonitrile (10%)-HCl (0.05 M)–KCl (0.1 M); detection, absorbance at 260 nm]. The chromatogram of triphala showed (Figure 5a) several prominent peaks having retention time of 8.30, 9.44, 10.88 and 14.33 min. The peak at 14.33 min corresponded to gallic acid as determined by comparison with standard (Figure 5b). The amount of gallic acid in triphala was quantified by a calibration curve (inset, Figure 5), where peak areas were plotted as a function of different standard gallic acid concentrations (1–8 μg/ml). By comparing the peak areas, the amount of gallic acid in triphala was calculated and found to be 73 mg/g. This accounts for only ~7% of the total phenolics estimated by other methods. The peaks at 8.30, 9.44, 10.88 min did not match with any of the standards in hand, namely acorbic acid, caffeic acid, ferulic acid, vanillin or some of the other carboxylic acids. It is possible that some of the polyphe-
nols in the extract are present in either glycosylated/esterified form or in another bound form that does not allow estimation as free acids/phenolics. In such case, even though *triphala* exhibits high reactivity with free radicals, the gallic acid estimation may not match and can be misleading. Therefore, we subjected the aqueous solution of *triphala* to acid hydrolysis by boiling with 0.1 N HCl for 2 h. The hydrolyzate, on examination by HPLC, showed significantly increased amount of gallic acid (150 mg/g), which is ~15%, but still less than the total percentage of phenolics present in *triphala*. This clearly showed that other unidentified phenols and polyphenols significantly contribute to the overall free radical activity of *triphala*, and most of them may be in bound form. Although there is a mismatch in their estimation, this simple and easy method of estimation of gallic acid in *triphala* is of significant importance, as it can be used as a ‘marker’ (the guide) for standardization of different methods of preparations of *triphala* extracts.

*Triphala*, a well-known ayurvedic formulation, having potent antioxidant and radioprotective ability, has been found to be an excellent scavenger of hydroxyl radicals and superoxide radicals, whose excessive formation is implicated in oxidative stress and exposure to radiation. Employing other non-biological and stable free radicals, DPPH and ABTS, the total free-radical scavenging ability of *triphala* in terms of ascorbic acid equivalents was estimated. The total polyphenolic content was estimated by several known spectrophotometric methods, which confirmed that *triphala* contains high level of polyphenols. HPLC analysis of *triphala* showed a number of components, one of the prominent products being gallic acid. The above-studied free radical reactions and estimated polyphenolic content in *triphala* confirm that the antioxidant and radioprotective ability of *triphala* arise from the polyphenols, which reduce oxidative stress by converting the reactive oxygen free radicals to non-reactive products. The studies are of great significance as the demand for herbal products as antioxidants and radio protectors is increasing constantly.

Rapid and specific detection of luminous and non-luminous *Vibrio harveyi* isolates by PCR amplification

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*Vibrio harveyi* is the major causal organism of luminous vibriosis, which causes potential devastation to diverse ranges of marine invertebrates over a wide geographical area. These microorganisms, however, are extremely difficult to identify because they are phenotypically diverse. Biochemical identification techniques involve many tests which may be time-consuming and expensive. The development and sustainability of shrimp aquaculture industry requires a simple, fast and reliable technique for species-specific identification of *V. harveyi* in order to control it effectively. The present communication describes a simple, cost-effective and rapid PCR-based and species-specific detection technique to facilitate early detection and identification of luminous and non-luminous *V. harveyi* isolates. Many of these isolates are also resistant to multiple antibiotics such as ampicillin, chloramphenicol, nalidixic acid, rifampin, polymyxin-B, trimethoprim and penicillin.

**Keywords:** Luminous *V. harveyi*, PCR amplification, shrimps, *Vibrio harveyi*.

*Vibrio harveyi*, a marine bacterium, is not only ubiquitous in the marine environment but is also considered one of the main bacterial species which is part of normal microflora of healthy shrimps. However, over the past few decades, bacterial strains of this species have been recognized as significant pathogenic agents and a cause of high rates of shrimp mortality in the shrimp culture industry worldwide. In various parts across the globe, *V. harveyi* drastically affects the production of *Litopenaeus vannamei*, the most extensively cultured penaeid in all the zones. Vibriosis, especially luminous disease has caused serious loss in prawn hatcheries. *V. harveyi* was reported as the causative bacterium of vibriosis in pearl oyster (*Pinctada maxima*), black tiger prawn (*P. monodon*) and kuruma prawn (*P. japonicus*). Larval prawns are particularly susceptible to *V. harveyi*, succumbing to what has been termed as luminescent bacterial disease. This disease has been identified as a major problem in the Philippines, Japan, Southeast Asia and European countries, causing severe losses of juvenile prawns in several hatcheries.

Among the common technologies used for diagnosis and detection of *V. harveyi* in shrimp farms are biochemical tests. Although these phenotype-based identifications of marine bacteria are useful, they are time-consuming and can generate false-positive results. The development and sustainability of shrimp aquaculture industry urgently requires a simple reliable and fast method for species-specific identification of *V. harveyi* for its adequate control. Likewise several highly powerful molecular techniques, e.g. ELISA, amplified fragment length polymorphism (AFLP) and repetitive extragenic palindromic elements polymerase chain reaction (REP-PCR), FAFLP, and IGSPCR have become readily available for the identification of bacteria, including *Vibrios*. Keeping in view these important facts, we have developed a simple, cost-effective reliable and fast PCR-based technique to identify *V. harveyi* isolates. This genomic approach used for the identification and typing of *Vibrio* strains is useful for taxonomic studies, including identification up to the subspecies level. The genus *Vibrio* contains a large number of closely related bacterial species with 16S rRNAs differing in nucleotide sequence from less than 1 up to 6%. Our results have clearly demonstrated the rapid detection of marine luminous and non-luminous *V. harveyi* isolates for molecular epidemiology purpose. This approach may also be applied to the detection of other marine *Vibrio* species involved in aquaculture diseases. These *V. harveyi* isolates were also screened for antibiotic sensitivities. Interestingly, several isolates were found to be resistant to ampicillin, chloramphenicol, nalidixic acid, rifampin, polymyxin-B, trimethoprim, and penicillin; few isolates were sensitive to tetracycline, streptomycin and novobiocin.

Environmental isolates of luminous and non-luminous *V. harveyi* screened were obtained from sea water samples collected from various sampling sites along the west coast of Goa, i.e. Goa Shipyard Limited, Western India Shipping Limited and beaches such as Majorda, Benaulim, Miramar, Donapaula, Anjuna, Tirakol, Colva and Kakra.