



RESEARCH ARTICLE

Estimation of Gallic Acid in Triphala Using Enzymatic Hydrolysis

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ARTICLE INFO

Article history:

Received 04.09.2021

Revised 11.03.2022

Accepted 25.03.2022

Published 23.05.2022

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[https://doi.org/](https://doi.org/10.18579/jopcr/v21i1.ms21.53)

10.18579/jopcr/v21i1.ms21.53

ABSTRACT

Background: A well-known Ayurvedic formulation, Triphala has numerous health benefits like appetite stimulation, controls diabetes, reduces cholesterol, relieves stress, alleviates inflammation and treats a variety of bacterial and fungal infections along with digestive problems. The phytoconstituents flavonoids, tannins, polyphenols and many chemical compounds are responsible for its claimed health benefits. One of the very significant compounds is gallic acid, which is reported to possess hepatoprotective, anti-inflammatory, antineoplastic and antioxidant properties that are beneficial in treatment of several diseases. **Objective:** In the present work using gallic acid as a marker, an attempt has been made to extract gallic acid from tannins present in Triphala by enzymatic hydrolysis and subsequent estimation of total gallic acid content by high performance thin layer chromatography (HPTLC). **Materials and Methods:** *Rhizopus oryzae* fungus is cultured for the production of enzyme tannase, which converts tannins into gallic acid. The extracted gallic acid is estimated in the biocatalyzed sample at optimal process parameters by high performance thin layer chromatography (HPTLC) method. **Result:** The gallic acid content after treatment with enzyme was found to be doubled 0.296 ± 0.018 mg/ml, Rf value 0.37 at 254nm which was 0.197 ± 0.008 mg/ml and Rf value was 0.37 prior to enzymatic treatment. **Conclusion:** Gallic acid in Triphala is present in free form as well as the constituent of tannins. There is a remarkable yield of gallic acid from tannins using the enzyme Tannase in the conversion process. Application of such advanced technology increases the gallic acid yield from response surfaces in herbal and ayurvedic products containing gallic acid. The extraction condition of phenolic compounds can be optimised using enzyme hydrolysis. The effective factors including the solid to solvent ratio, enzyme concentration, particle size and extraction time can be optimised.

Keywords: Triphala; Gallic acid; Tannin; *Rhizopus oryzae*; Tannase; HPTLC

INTRODUCTION

According to Ayurveda, human body is composed of three doshas, that is, Tridosha which are vata, pitta and kapha.¹ A well-recognized and highly efficacious ayurvedic preparation, Triphala, is useful not only in curing the diseases of Tridosha but also for maintaining health and wellness. Triphala consists of Amla (*Embllica officinalis* Geartn.), Baheda (*Terminalia bellerica* Roxb.) and Harad (*Terminalia chebula* Retz.) in equal ratio as per Ayurvedic Formulary of India (AFI).² Triphala has a unique ability to detoxify and cleanse the system³ and is used to cure wide range of ailments like chronic ulcer, jaundice, asthma, anaemia, diabetes⁴ and improving of digestion, constipation, cleaning gastrointestinal tract⁵ and most importantly in immune system stimulation.⁶

Triphala is known to have many phytoconstituents, polyphenols, tannins and several compounds like ascorbic acid, gallic acid, ellagic acid, chebulagic acid etc. which are responsible for its claimed health benefits.⁷ High performance Liquid Chromatography (HPLC) analysis⁸ and Folin-Ciocalteu and Folin Denis method⁹ showed that Triphala contains $38 \pm 3\%$ polyphenols and $35 \pm 3\%$ tannins weight by weight (w/w). Triphala is reported to contain sufficient gallic acid $0.026\%w/w$ and tannic acid $0.024\%w/w$ whereas each constituent of Triphala, amla contains 0.81% gallic acid, baheda 0.005% gallic acid, 0.004% tannic acid and harad 0.024% gallic acid and 0.011% tannic acid. As the major chemical constituent, Gallic acid (3,4,5-trihydroxybenzoic acid, $C_7H_6O_5$) is a natural phenolic compound known to have several health-promoting effects including antioxidant, antineoplastic, anti-inflammatory,

antimicrobial and also promotes cardiovascular, gastrointestinal, metabolic, neuro psychological health.¹⁰

The HPTLC technique is rapid, comparatively simple, robust, and extremely versatile. HPTLC not only confirm but also establish its identity. It is also an ideal screening tool for adulterations and is highly suitable for evaluation and monitoring of cultivation, harvesting, and extraction processes and testing of stability. A simple and reproducible method using HPTLC was successfully performed for the quantitative analysis of gallic acid in various plants.

In present work gallic acid, which is found both in free state and as a constituent of tannins, has been chosen as a marker compound and an attempt has been made to estimate the total amount of Gallic acid present in Triphala after enzymatic hydrolysis by high performance thin layer chromatography (HPTLC) method. HPTLC is a simple, sensitive, rapid, reliable and economic high- performance thin-layer chromatography method was developed for the quantification of gallic acid (GA). This study gives an opportunity for standardization of gallic acid in Triphala formulation which was chromatographed on precoated silica gel 60F 254 (Merck Millipore) plates. Toluene, ethyl acetate and formic acid (5:5:1) are used as mobile phase for the development plates, and detection was carried out at 254 nm. Calibration plots established showing the dependence of response on the amount chromatographed. The linear regression analysis data for the calibration plots showed good linear relation with $R^2 = 0.999$. Detection and quantification were performed by densitometer scanning at wavelength $\lambda = 200-400$ nm by using a deuterium lamp. The enzyme, tannin acyl hydrolase (TAH, E.C.3.1.1.20), commonly called Tannase, catalyses the hydrolysis of ester and depside bonds into hydrolysable tannins, releasing glucose and gallic acid.¹¹ It breakdowns hydrolysable tannins such as tannic acid, methyl gallate, ethyl gallate, n-propylgallate, and isoamyl gallate. It hydrolyses the ester bonds of tannic acid completely to gallic acid and glucose through 2,3,4,6,-tetragalloyl glucose and two kinds of mono-galloyl glucose.¹² The products can be detected in the hydrolysate of 1,2,3,4,6,-pentagalloyl glucose and gallic acid, methyl m-di-gallate is liberated as shown in the mechanism of action of Tannase (Figure 1)

Tannic acid $\xrightarrow{\text{Tanninacyl hydrolases}}$ Gallic acid + Glucose

MATERIAL AND METHOD

Preparation of Triphala

The fruits of Amla, Baheda and Harad were identified and authenticated by Botanical Survey of India, Central National Herbarium, Howrah, West Bengal, India. The fruit pulp of Amla, Baheda and Harad were dried, powdered, screened and stored in an air-tight container for future use. Triphala was prepared by mixing the powders in equal proportion

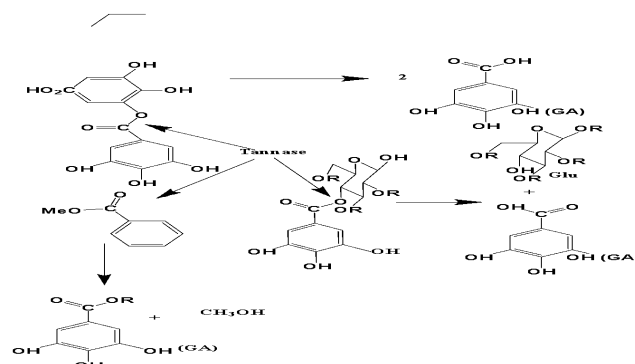


Fig. 1: Mechanism of action of Tannase

(1:1:1) as per Ayurvedic Formulary of India. All the other chemicals used in the experiment were of analytical grade.

Culturing of *Rhizopus oryzae* fungus¹³

Strain of *Rhizopus oryzae* NCIM 879 was collected from National Collection of Industrial Microorganisms NCIM, Pune. The Strain of *Rhizopus oryzae* NCIM 879 was stored at 4°C in refrigerator and subcultured at every 30 days intervals using potato dextrose agar slant, cultivated in potato dextrose agar (PDA) slant for 48 hours, further cultured in modified Zapec dox media broth under liquid condition for production of enzyme. The culture was incubated for 3 days at a temperature of 28°C under shaking condition with the aid of shaker. Then the culture was centrifuged, the biomass was separated and the supernatant containing the crude tannin acyl hydrolase enzyme was dried and used for hydrolysis purpose in citrate buffer pH 5.5.

Analysis of Tannin acyl hydrolase enzyme activity^{14,15}

To 4 ml of 0.35% (w/v) tannic acid in citrate buffer (0.05 M, pH 5.5), 1 ml of the crude tannin acyl hydrolase enzyme sample was added and incubated at 35°C for 45 min. The samples (40 ml) withdrawn at various intervals of time from 0 to 45 min were diluted 100 times with 90% ethanol and absorbance measured at 310 nm.

Conversion of tannin to gallic acid by Tannase enzyme¹⁶

The extraction of total tannin was carried out as described by Scalbert et al. (1989) with modification by adding 1g of sample with 20 ml of solvent mixture (water: methanol:: 20:80) which is centrifuged and the supernatant was collected by filtration. Methanol was evaporated under reduced pressure. Further the aqueous remaining was adjusted to pH 5.5 with citrate buffer to 4 ml of the solution. 1 ml of the enzyme was added and incubated for 45 min at 35°C for conversion of tannin to gallic acid.



Extraction & Estimation of gallic acid¹⁷

Estimation of gallic acid concentration in the bio catalysed sample at optimal parameters is carried out by high performance thin layer chromatography (HPTLC). The bio catalysed was concentrated under reduced pressure. To 10 mg of the sample, 5ml methanol, filtered through 0.2µm filter and applied as 3mm band on pre-coated silica gel 60F 254 (Merck Millipore) plates. Toluene, ethyl acetate and formic acid (5:5:1) are used as mobile phase for the development plates, and detection was carried out at 254 nm.

RESULTS

The amount of gallic acid present in Triphala was computed from the calibration curves shown in Figure 2 from the data obtained which is presented in Table 1. The linear regression analysis data for the calibration plots (Figure 2) shows a good linear relation $y = 13.24x + 1411$ with $R^2 = 0.999$ peak area of gallic acid, concentration range of gallic acid was 200 -900ng/band. The HPTLC profile of gallic acid before and after treatment with the enzyme are shown in Figure 3 and Figure 4 respectively. As seen from chromatogram the clear peaks indicate the concentration of gallic acid before treatment with enzyme was 0.197 ± 0.008 mg/ml with retention factor (Rf) value: 0.37 (figure3) and concentration of gallic acid after treatment of enzyme was found to be 0.296 ± 0.018 mg/ml with Rf value: 0.37(Figure 4).

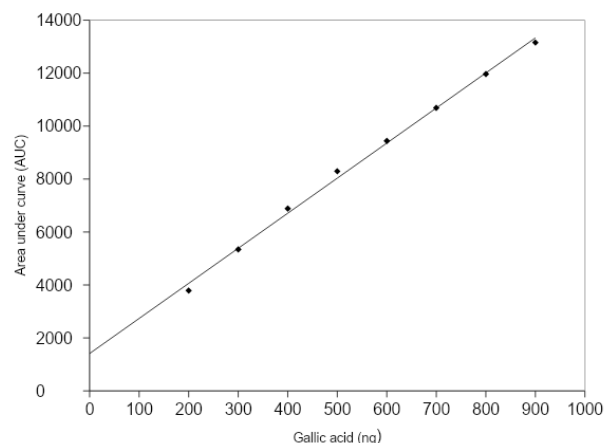


Fig. 2: Standard plot of gallic acid

Table 1: Data for Standard plot of the Gallic acid

Concentration	AUC
200	3787.22
300	5344.09
400	6883.92
500	8294.05
600	9441.31
700	10687.67
800	11965.95
900	13155.14

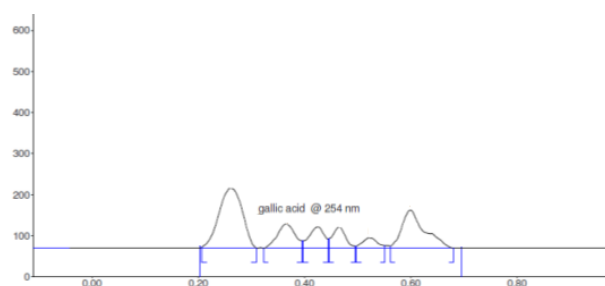


Fig. 3: Chromatogram of Gallic acid (concentration before treatment with enzyme 0.197 ± 0.008 mg/ml; Rf value: 0.37)

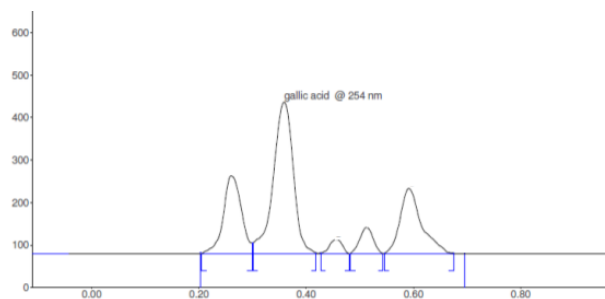


Fig. 4: Chromatogram of Gallic acid (concentration after treatment with enzyme 0.296 ± 0.018 mg/ml; Rf value: 0.37)

DISCUSSION

From the result obtained, estimation of gallic acid in Triphala using the novel method of enzymatic hydrolysis seems to be very efficient. It was observed that on the treatment of triphala with tannase enzyme, there was a marked increase in gallic acid content to almost double. The chromatograms obtained show that without treatment of enzyme tannase concentration of gallic acid was found to be 0.197 ± 0.008 mg/ml (figure3) and after treatment of enzyme it was found to be 0.296 ± 0.018 mg/ml (figure 4) which is 1.5 times the yield obtained without treatment with enzyme. The microbial enzyme, tannase catalyses the breakdown of the ester bonds of tannic acid to gallic acid and glucose thereby

showing remarkable rise in the gallic acid content. Thus, this method can be utilised to increase the gallic acid yield in other herbal and ayurvedic products containing gallic acid.

CONCLUSION

In earlier works reported on estimation of gallic acid in Triphala, only the free gallic acid has been estimated. However, by using novel enzymatic hydrolysis technique

enormous amount of tannins present in Triphala can be converted to the gallic acid to obtain a better yield. Thus, there is a clear indication from the experiment that the content of gallic acid in Triphala gives better yield after treatment with the microbial enzyme Tannase. It is also used to mask the astringent and bitter taste of the herbal drugs. Use of such cutting-edge innovation increases the gallic acid yield from response surfaces in herbal and ayurvedic products containing gallic acid.

FINANCIAL SUPPORT AND SPONSORSHIP

Nil

CONFLICTS OF INTEREST

There are no conflicts of interest

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