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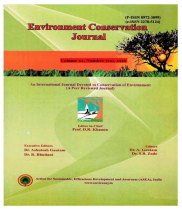

Chemical and morphological variations in *Terminalia bellirica* (Gaertn.) Roxb. – a species of commercial ayurvedic formulation triphla from central India

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ABSTRACT

***Terminalia bellirica* is one of the high traded medicinal plant species, mainly known for its fruits which are bestowed with numerous biological activities and used in treatment of various ailments. The fruits are one of three ingredients of well-known Ayurvedic formulation 'TRIPHLA'. The purpose of this study was to determine the variations for Gallic acid (GA), a chemical marker compound, as well as for key morphological traits (height, girth at breast height, clear bole height, fruit size) in trees from central Indian states viz. Madhya Pradesh, Chhattisgarh and Maharashtra. In this investigation, we also explored the correlation between the chemical marker and morphological features. The study suggested the maximum GA content (0.98±0.42%) in populations of Keregaon range of Dhamtari forest division of Chhattisgarh state which can be considered as superior chemotypes/ populations. The correlation analysis exhibited the positive association between fruit size and GA content.**

Introduction

Terminalia bellirica (Gaertn.) Roxb., commonly known as 'Baheda' or 'Belleric myrobalans' is a prime member of Combretaceae family. It is a widely distributed tree species especially in the Indian subcontinents. There are numerous traditional systems of medicines such as Ayurveda, Siddha, Unani & Chinese medicine that use this plant and its parts. *T. bellirica* itself has been reported to be effective against cancer, diabetes, diarrhoea, microbial infection, wound healing, bronchitis, spasm, fever, and liver disorder (Deb *et al.,* 2016). The pharmaceutical importance of the plant is due to an ample variety of phytochemical constituents in the plant. Some of the important phytoconstituents of the plant are β-sitosterol, gallic acid, chebulagic acid, ethyl gallate, ellagic acid, galloyl glucose (Gupta *et al.,* 2017). The fruit is the most important part of *T. bellerica* which is substantially used in traditional medicines along with fruits of *Terminalia chebula* and *Phyllanthus emblica* for a well-known formulation 'Triphala' (Zhang *et al.,* 2019). This miraculous combination is described to treat a myriad of health disorders. The annual demand of fruits of this species is approximately 2000-5000 metric tonnes (NMPB). The fruit is diarrhoeic, laxative,

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astringent, anthelmintic, antipyretic; used to aid digestion, bronchitis, asthma, dyspepsia, piles, diarrhoea, cough, blisters, eye diseases, scorpion-stings, and as a hair tonic (Mallik *et al.,* 2012).

GA (3,4,5-trihydroxy benzoic acid), one of the subtypes of phenolic acid (Figure 1) is a colourless or slightly yellowish crystalline compound is one of the most profusely found compounds in various parts of *T. bellirica* (Fernandes and Salgado, 2016).

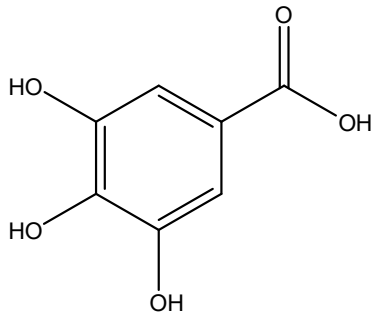

Figure 1: Chemical structure of GA (3, 4, 5-trihydroxybenzoic acid).

This compound for a long has been a keen point of interest for researchers due to its extensive pharmaceutical properties. The anti-microbial property of GA is exhibited due to its ability to disrupt the integrity of bacterial cell membrane, along with inhibition of enzymes in DNA replication, electron transport chain, and cellular respiration (Omojate *et al.,* 2014). The HIV-1 enzymes, attachment, and penetration of HSV-1, HSV-2, and HCV are obstructed by GA (Kahkeshani *et al.,* 2019). GA escalates the activity of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR). Thus, the substance inhibits ROS-induced carcinogenesis and works effectively as an anti-cancerous agent. It is also effective against gastrointestinal (Chatterjee *et al.,* 2012), cardiovascular (Priscilla and Prince, 2009), inflammatory (Bai *et al.,* 2021), metabolic (Gandhi *et al.,* 2014), and neuropsychological diseases (Nagpal *et al.,* 2013).

Bioactive marker compounds are responsible for the quality and efficacy of medicinal plants and their products which have specific physiological action on the human body (Joshi and Uniyal, 2008; Akinmoladun *et al.,* 2007). WHO and modern herbal pharmacopoeia strongly stressed on the need of quality assurance of medicinal plants with respect to their bioactive ingredients (Kaushik *et al.* 2010; Vasudevan, 2009). GA is reported as a chemical marker compound in fruits of *T. bellirica* (Gupta *et al.,* 2003). The present investigation has been planned for selection and evaluation of natural populations of *T. bellirica* in terms of GA content in its fruits for identification for superior populations in three central Indian states i.e. Madhya Pradesh, Chhattisgarh and Maharashtra. The correlation study between morphological traits of *T. bellirica* with GA content in its fruits was also accomplished.

Material and Methods

Chemicals and reagents

GA standard was purchased from Sigma Aldrich, India. Solvents and chemicals used in the experiments were of AR grade.

Collection of *T. bellirica* fruits and recording of morphological data

Mature fruits of *T. bellirica* were harvested in the last week of February from forest divisions of Madhya Pradesh, Chhattisgarh and Maharashtra states (Figure 2), and brought to the laboratory where they were washed in tap water to remove dust particles, depulped, dried, powdered, and stored in airtight containers for further chemical analysis. Morphological data viz. tree height, GBH, CBH, and fruit size (Figure 3) were also recorded along with GPS coordinates of collection sites.

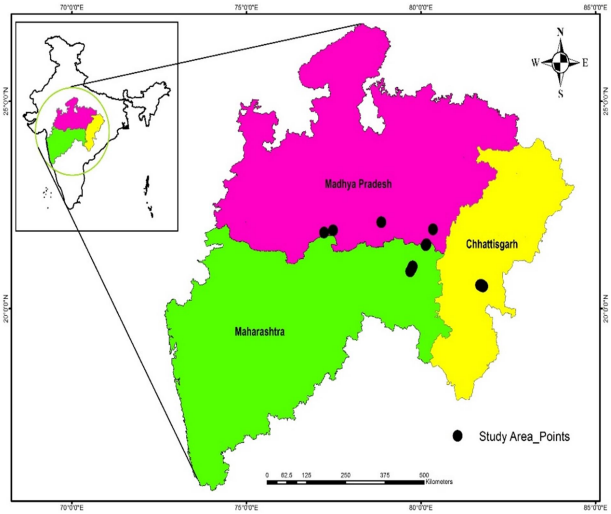

Figure 2: Study sites of *T. bellirica* in central India.

Processing and extraction fruit samples

2.5 gm dried and finely powdered fruit pulp samples were taken in conical flasks containing 50

mL of 2N HCl and heated for 30 minutes over a boiling water bath, cooled and filtered.

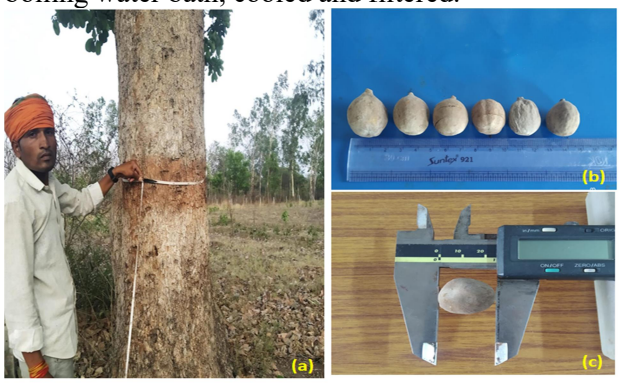

Figure 3: (a) Recording GBH of tree (b) Fruits of *T. bellirica* (c) Recoding size of fruit with vernier caliper

The filtrate was transferred to a separating funnel and extracted twice with 75 mL (50; 25) of diethyl ether. The pooled diethyl ether layers were washed two times with distilled water, dried over anhydrous sodium sulphate and filtered. The filtrate was evaporated and the concentrated extract was dissolved in 10 mL of methanol for analysis (Saxena *et al.,* 2015).

High Performance Liquid Chromatography (HPLC) analysis for quantification of GA

GA content in fruit pulp was determined by using the reported HPLC method with some modifications (Saxena *et al.,* 2015).

Preparation of standard solution

Standard solution of 1 mg/mL of GA was prepared. Working solutions having concentration range of 100 - 300 µg/ mL was prepared by further diluting with methanol.

Chromatographic analysis

Mobile phase : Methanol: Water: Acetic acid (25: 75:0.1)
Injection volume : 20 μl^{-1}
Flow rate : 1.0 mL min^{-1}
Wavelength : 254 nm
Run time : 10 min

The standard solution and fruit samples were filtered separately using 0.2 µm filters, and 20 µL of each were injected into an HPLC Water 515 series column with a C-18 Xbridge equipped with a Photo Diode Array (PDA) detector, samples were run for 10 minutes. Peak identification was performed by comparison of retention times and diode array spectral characteristics with the standard. The calibration curve was generated by linear regression based on the peak areas. Linearity was obtained over five different concentration range of 2 - 6 µg per injection with $r^2 = 0.997$.

Specificity ascertained by comparing the peak purity of standard and samples through their HPLC chromatograms. Peak corresponding to GA in the sample was completely in agreement with the standard. Quantity of GA in fruit samples was calculated using the obtained linear regression equation.

Morphological traits

Morphological data viz. tree height, girth at breast height (GBH), clear bole height (CBH) and fruit size of *T. bellirica* trees were recorded (Mohammad *et al.,* 2020). A hypsometer (Model - Vertex 5, Make - Haglöf Sweden AB, Sweden) was used to measure tree height and clear bole height. Girth at breast height was measured by looping a measuring tape around the tree trunk at a height of 1.37 m above ground level in a plane perpendicular to the trunk's axis. Size of fruits was measured using vernier caliper.

Statistical analysis

Correlation analysis was conducted between morphometric traits and GA content (Mohammad *et al.,* 2020). For HPLC analysis, the samples were analysed in triplicates and the results are expressed as Mean±SD.

Results and Discussion

Representative HPLC chromatograms of GA standards and samples resolved under the chromatographic conditions is described in Figure 4 and 5. The retention time of GA fruit sample and standard was frame at 5.4±0.01 min.. Chromatographic analysis of extracted fruit samples revealed the presence of GA.

Chemical and morphological variations

Results showing the variations in GA content in fruit samples of *T. bellirica* collected from 07 forest ranges of Madhya Pradesh, Chhattisgarh and Maharashtra states are given in Table 1. GA concentration varies from 0.43±0.09 to 0.98±0.42 per cent among different locations. The highest GA concentration (0.98%) was observed in the fruit sample collected from Keregaon range belonging to Dhamtari forest division of Chhattisgarh state and the lowest GA concentration (0.43%) was found in the fruit sample collected from Chhindwara range

of West Chhindawra forest division of Madhya Pradesh state. The results showed significant variation ($P< 0.05$) in GA content among the 07 locations of different states. Morphological traits showed significant variability among the populations of different locations of three states (Table 1). The tree height ranged from 8.77 m to 19 m, GBH of tree from 0.91 m to 2.43 m, CBH from 3.04 m to 6.75 m and average size of fruit from 323 mm^2 to 725 mm^2.

Table 1: Morphological traits and gallic acid (%) of *T. bellirica*

States	Forest Divisions	Forest Ranges	Height (m)	Girth at Breast Height (m)	Clear Bole Height (m)	Avg. size of fruit (length x width) in mm^2	Gallic acid (%) Mean ± SD
Maharashtra	Bhandara	Bhandara	14.87±6.10	1.59±0.82	4.10±2.48	537.67±175.29	0.84±0.30
	Gondia	Gondia	11.00±1.73	1.07±0.38	3.87±1.86	725.00±147.10	0.60±0.04
Chhattisgarh	Dhamtari	Keregaon	9.53±2.40	0.91±0.13	4.95±3.23	652.75±105.90	0.98±0.42
		Singpur	8.77±2.29	0.95±0.23	3.04±1.59	640.25±85.15	0.71±0.19
Madhya Pradesh	West Chhindwara	Chhindwara	19.00±3.56	1.68±0.19	6.75±0.50	494.00±101.60	0.43±0.09
	South Balaghat	Lougur	18.50±3.54	2.43±0.30	5.50±2.12	423.00±89.10	0.65±0.02
	Betul Forest	Mohada	11.00±3.0	1.80±0.20	4.00±1.00	323.00±23.0	0.60±0.30

Table 2: Inter-character correlation between different traits of *T. bellirica*

	Avg. size of fruit (length x width) in mm^2	Girth of tree (m)	Height of tree (m)	Clear bole (m)	Gallic acid (%) in fruits
Avg. size of fruit (LxW) in mm)	1	-0.82	-0.47	-0.31	0.36
Girth of tree (m)	-0.82	1	0.79	0.48	-0.41
Height of tree (m)	-0.47	0.79	1	0.80	-0.50
Clear bole (m)	-0.31	0.48	0.80	1	-0.37
Gallic acid (%) in fruits	0.36	-0.41	-0.50	-0.37	1

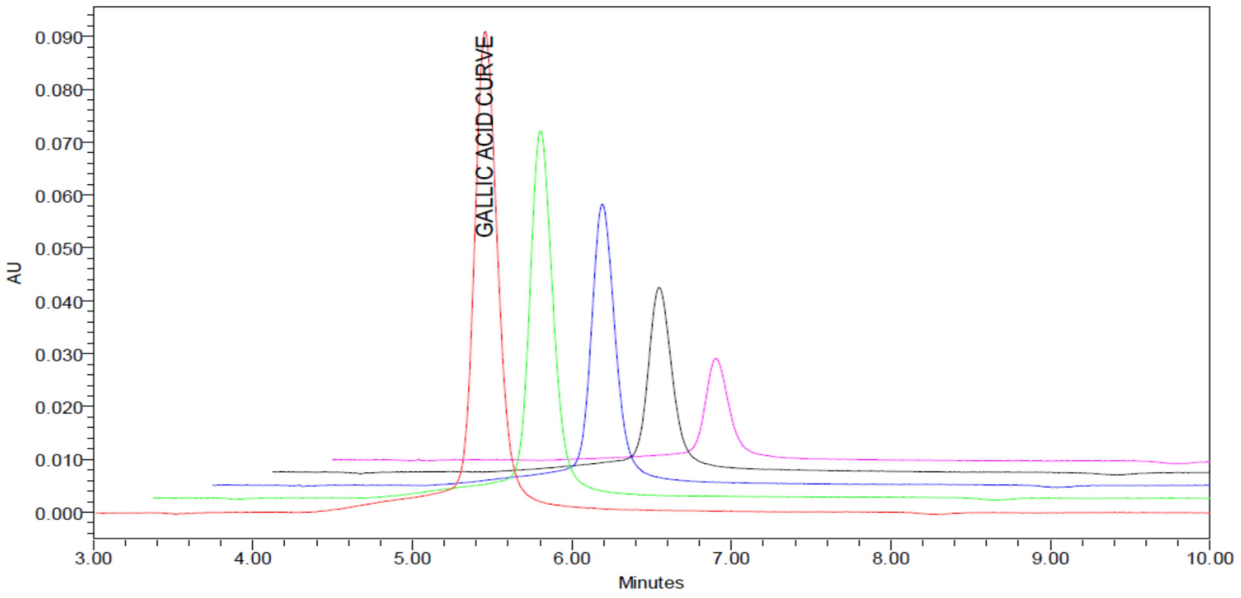

Figure 4: HPLC chromatogram of gallic acid standard

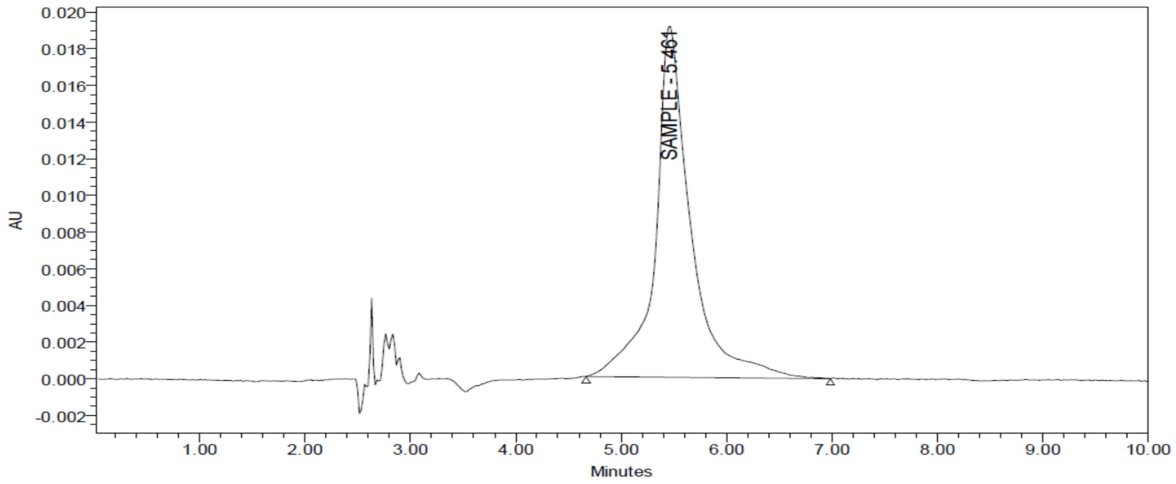

Figure 5: HPLC chromatogram of fruit sample

	Avg. size of fruit (length x width) in mm^2	Girth of tree (m)	Height of tree (m)	Clear bole (m)	Gallic acid (%) in fruits
Avg. size of fruit (length x width) in mm^2	1	-0.82	-0.47	-0.31	0.36
Girth of tree(m)	-0.82	1	0.7864	0.48	-0.41
Height of tree (m.)	-0.47	0.79	1	0.80	-0.5
Clear bole (m.)	-0.31	0.48	0.80	1	-0.37
Gallic acid (%) in fruits	0.36	-0.41	-0.5	-0.37	1

Figure 6: Shaded correlation matrix depicting relationship between different morphometric traits with GA (%) in fruits of *T. bellirica.*

Correlation study

Phenotypic inter-character correlation in all the possible combinations were computed and showed as shaded correlation matrix that depicting relationship between different morphometric traits and GA content (Figure 6). A close examination of the correlation analysis between morphological and chemical parameters revealed that GA content showed positive and significant correlation only with fruit size. With rest of characters, GA content showed a non-significant association (Table 2).

The positive correlation between fruit size and GA content may be explained by the hypothesis that the fruit colour modifies the chemical composition of phytochemicals, bioactive (phenolics) compounds etc. In case of *T. bellirica*, with the maturity, the fruit colour and size modify which may be the reason for increased GA content in bigger fruits. Our notion is corroborated by previous research revealing more coloured and larger fruits with improved pomological features and altered nutritional components and phenolics in the 'Kordia' sweet cherry (*Prunus avium* L.) (Usenik *et al.,* 2014). Moreover, another study reported the inconstant change in phytochemicals with respect to the fruit size (Kesta, 1988). Thus, it can be concluded that there is no specific trend of correlation of chemical content with the fruit size and it may vary according to the species, locations and environmental conditions.

Conclusion

Present work is the first comprehensive investigation revealing the high level of variations in chemical and morphological traits of *T. bellirica*. The study showed the populations of Keregaon range belonging to Dhamtari forest division of Chhattisgarh state contained maximum GA content, hence the superior chemotypes. Further, since the fruit size was found to have positive association with GA content, it should be given high weightage during the selection of superior chemotypes of *T. bellirica*. The work carried out in this study will be

of great help in management and conservation of this valuable species.

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Conflict of interest

The authors declare that they have no conflict of interest.

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