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Research Article

SPECTROPHOTOMETRIC DETERMINATION OF TOTAL PHENOLIC CONTENT FOR STANDARDIZATION OF VARIOUS *PHYLLANTHUS* SPECIES

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ABSTRACT

Objective: This study aims at the comparison of the total phenolic content of ethanolic extract of various species of *Phyllanthus* available in Gujarat state by spectrophotometric method.

Methods: The total phenolic content was determined quantitatively using the Folin–Ciocalteu reagent, with gallic acid as standard.

Results: The total phenolic content in methanolic extract of various *Phyllanthus* species ranged from 41.801 to 87.542 mg/g of the dry weight of extract, expressed as gallic acid equivalents. *Phyllanthus urinaria* was found to have the highest phenolic content, and *Phyllanthus acidus* was found to have the lowest phenolic content.

Conclusion: The total phenolic content will help develop new drugs and standardize the various *Phyllanthus* species, particularly morphological similar species. The presence of a high entire phenolic content shows that the alcoholic extract of *Phyllanthus* may possess antioxidant properties, which could lead to a new field of research in the future.

Keywords: *Phyllanthus*, Total phenolic content, Gallic acid, Spectrophotometric method.

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INTRODUCTION

The genus "*Phyllanthus*" (Family: Phyllanthaceae, formerly *Euphorbiaceae*) was first described by Linnaeus in 1753. The name *Phyllanthus* means "leaf and flower" because the flower, as well as the fruit, seems to become one with the leaf. It consists of about 833 species of trees, shrubs, and annual or biennial herbs distributed throughout the tropical and subtropical regions. In India, it is represented by 40 species, although Hooker f. has recorded 56 species from the then British India. In total, 12 species of herbaceous *Phyllanthus* have been identified in India [1].

The genus *Phyllanthus* is an essential group of medicinal plants used for various purposes. In *Phyllanthus emblica* Linn., syn: *Emblica officinalis* Gaertn., the fruit is used for diverse applications in healthcare, foo, and cosmetic industry. It has been well studied for immunomodulatory, anticancer, antioxidant, and antiulcer activities [2]. Among the small herbs in this genus, *Phyllanthus amarus* Schum. and Thonn. have been used in Ayurvedic medicine for over 2000 years in problems of stomach, genitourinary system, liver, kidney, and spleen [3].

Unfortunately, a great deal of confusion exists among scientists regarding the identification of *Phyllanthus* species. *P. amarus* has been indexed in the majority of published phytochemical, pharmacological, and ethnobotanical reviews and research articles till date with different names. This species, *P. amarus,* has been confused with *Phyllanthus niruri* Linn. [1]. *P. amarus* is considered by some authors as a variety of *P. niruri*, while in several reports, one name is indicated to be a synonym of the other. Papers are published by the name of *P. niruri* and the same information is given in the monograph of *P. amarus* [4]. Furthermore, *P. niruri* has been indicated as a synonym of *Phyllanthus fraternus* Webster [5]. Thus, the scientists considered these three different species (*P. amarus*, *P. niruri,* and *P. fraternus*) as a single species. However, the fact is that specimens of *P. niruri* have never been confirmed outside the America [6], and indeed, it is a mixture of three distinct species, namely, *P. amarus*, *P. fraternus,* and *Phyllanthus debilis* Klein. ex Willd. in Flora of British India, the true *P. niruri* Linn. being endemic to West Indies [7]. The visually different *Phyllanthus urinaria* was often used interchangeably with those species earlier known as *P. niruri* in the traditional system of India [1].

In Thailand, four species of herbaceous *Phyllanthus* (*P. amarus*, *P. debilis*, *P. urinaria, and Phyllanthus virgatus* Forst.) are collectively called "*Luk-tai-bai*" or "*Ya-tai-bai*" [8]. In India, by the name "*Tamalaki,*" more than one plant (*P. fraternus*, *P. amarus*, and *P. urinaria*) has been taken by the physician due to morphological similarity and multiple therapeutic indices [9].

Various species of *Phyllanthus* are sold in the market in the name of "*Bhuiamlaki*" for the treatment of jaundice. Commercial sample of the same is found to be adulterated with one or more species of *Phyllanthus* such as *Phyllanthus maderaspatensis* Linn., *P. urinaria*, *Phyllanthus virosus* Roxb., and *P. fraternus* [10]. The raw herbal trade samples of *Phyllanthus* comprise 3–5 different species. Most samples contain *P. amarus* along with *P. fraternus* and *P. maderaspatensis* [11]. During market surveillance of herbal drug, it was observed that almost all the commercial samples either comprise of *P. amarus* or *P. maderaspatensis* or mixture of *P. amarus*, *P. fraternus,* and *P. maderaspatensis* [12].

In South India, about 22 herbaceous *Phyllanthus* species are distributed, mostly in moist areas, wastelands, and in agricultural land. All of these species share a common vernacular name, "*Keezhanelli*" (in Tamil) and "*Kirunelli*" (in Kannada), and thus, in the perception of the local collectors, these species are used interchangeably. In Southern India, 76% of the market samples contained *P. amarus* as the predominant species (>95%) and thus were devoid of admixtures. The remaining 24% of the shops had five different species, namely, *P. debilis*, *P. fraternus*, *P. urinaria*, *P. maderaspatensis*, and *Phyllanthus kozhikodianus* Sivar. and Mani [11].

The information available for *P. amarus* is misleading due to incorrect identification of *Phyllanthus* species due to common vernacular name, similarity in gross morphology, close proximity in growth habitat, and lack of legal guidelines to check the authenticity and quality of the medicinal plant sold [13].

According to literature, there is great controversy in the identification of *Phyllanthus* species, especially when the drug is in dried powdered form. To achieve maximum therapeutic efficacy, correct identification of species is necessary. Thus, the current work was undertaken to identify *Phyllanthus* species by total phenolic content determination using spectrophotometric technique species undertaken for the study which are *P. amarus*, *P. debilis*, *Phyllanthus emblica* Linn., *Phyllanthus acidus* (L.) Skeels, *P. fraternus*, *Phyllanthus lawii* J. Graham., *P. maderaspatensis*, *P. urinaria*, *P. virosus*, *P. virgatus,* and *Phyllanthus reticulatus* Poir.

Plant profile of *P. amarus* Schum. and Thonn. [10,14]
Phyllanthus amarus

Phyllanthusamarus

- Common name: Jangliamli, Jaramla, or Bhuiamla
- Source: The drug consists of dried whole plant of *P. amarus*.
- Family: *Euphorbiaceae*
- Habitat: Native of America and India, found commonly as a weed in fallow lands, clearings, and river beds of dry and hotter plains
- Chemical constituents: Phyllanthin, hypophyllanthin, hydrolyzable tannins, phyllanthusiin D, amariin, amarulone, amarinic acid, gallic acid, ellagic acid, niranthin, and phyllnirurin
- Traditional uses: Treatment of dropsy, jaundice, diarrhea, dysentery, intermittent fever, diseases of the urinogenital system, scabies, ulcers, and wounds
- Pharmacological activities: Antifungal, anticancer, antispasmodic, hypoglycemic, antiviral, and hepatoprotective
- Therapeutic claim: Hepatoprotective and antidiabetic
- Adulterants/substitutes: *P. fraternus*, *P. maderaspatensis*, *P. urinaria*, and *P. virgatus.*

METHODS

Chemicals and reagents

Standard compound gallic acid (purity 99%, w/w) was obtained as gift sample from Tetrahedron Ltd., Chennai, India. All chemicals and reagents used were of analytical grade and were purchased from Qualigens Fine Chemicals, Mumbai, India and Spectrochem Pvt. Ltd., Mumbai, India.

Plant collection and authentication

Collection of plant materials

The plant materials required for the experiment were collected from Valsad district in the month of September in monsoon season. Table 1 lists *Phyllanthus* species collected (Fig. 1). The collected plant materials were air-dried and then dried in hot air oven at ≤40°C, stored in air-tight containers at 30°C and powdered to 40 mesh as and when required.

Identification and authentication of the collected species

The collected species were identified referring to standard floras, namely, Flora of Gujarat [15], Flora of Rajasthan [16], and the Flora of the Bombay Presidency [17] and authenticated by a taxonomist.

Table 1: *Phyllanthus* species collected from Valsad district

S. No.	Biological name	Habit	Parts collected	Region of collection
1	*P. amarus*	Herb	Whole plant	Killa Pardi
2	*P. acidus*	Tree	Aerial parts	Killa Pardi
3	*P. emblica*	Tree	Aerial parts	Dharampur
4	*P. fraternus*	Herb	Whole plant	Killa Pardi
5	*P.maderaspatensis*	Herb	Whole plant	Dharampur
6	*P. reticulatus*	Shrub	Aerial parts	Killa Pardi
7	*P. urinaria*	Herb	Whole plant	Dharampur
8	*P. virgatus*	Herb	Whole plant	Dharampur
9	*P. virosus*	Shrub	Aerial parts	Killa Pardi

P. urinaria: Phyllanthus urinaria, P. acidus: Phyllanthus acidus, P. emblica: Phyllanthus emblica, P. fraternus: Phyllanthus fraternus, P. virgatus: Phyllanthus virgatus, P. maderaspatensis: Phyllanthus maderaspatensis, P. reticulatus: Phyllanthus reticulatus, P. virosus: Phyllanthus virosus, P. amarus: Phyllanthus amarus

Table 2: The yields of solid residue after extraction and evaporation from 1 g dried plant parts methanolic extract of various *Phyllanthus* species

S. No.	Methanolic extract of various *Phyllanthus* species	Yield (gm)[1]
1	*P. acidus*	0.15±0.815
2	*P. urinaria*	0.28±0.210
3	*P. emblica*	0.34±0.315
4	*P. amarus*	0.23±0.687
5	*P. virgatus*	0.24±0.555
6	*P. virosus*	0.27±0.234
7	*P. reticulatus*	0.21±0.123
8	*P. fraternus*	0.30±0.235
9	*P. maderaspatensis*	0.18±0.675

[1]Each value is the average of three measurements±standard deviation, *P. urinaria: Phyllanthus urinaria, P. acidus: Phyllanthus acidus, P. emblica: Phyllanthus emblica, P. fraternus: Phyllanthus fraternus, P. virgatus: Phyllanthus virgatus, P. maderaspatensis: Phyllanthus maderaspatensis, P. reticulatus: Phyllanthus reticulatus, P. virosus: Phyllanthus virosus, P. amarus: Phyllanthus amarus*

Table 3: Total phenolic contents in methanolic extracts of various *Phyllanthus* species expressed in terms of gallic acid equivalent (mg of GA/g of extract)

S. No.	Methanolic extract of various *Phyllanthus* species	Total phenolic content (mg GAE gm)[1]
1	*P. acidus*	41.801±0.815
2	*P. urinaria*	87.542±0.312
3	*P. emblica*	66.469±0.245
4	*P. amarus*	73.969±0.341
5	*P. virgatus*	58.969±0.654
6	*P. virosus*	73.879±0.436
7	*P. reticulatus*	41.981±0.254
8	*P. fraternus*	52.192±0.616
9	*P. maderaspatensis*	80.433±0.556

[1]Each value is the average of three analyses±standard deviation, *P. urinaria: Phyllanthus urinaria, P. acidus: Phyllanthus acidus, P. emblica: Phyllanthus emblica, P. fraternus: Phyllanthus fraternus, P. virgatus: Phyllanthus virgatus, P. maderaspatensis: Phyllanthus maderaspatensis, P. virosus: Phyllanthus virosus, P. amarus: Phyllanthus amarus, P. reticulatus: Phyllanthus reticulatus*

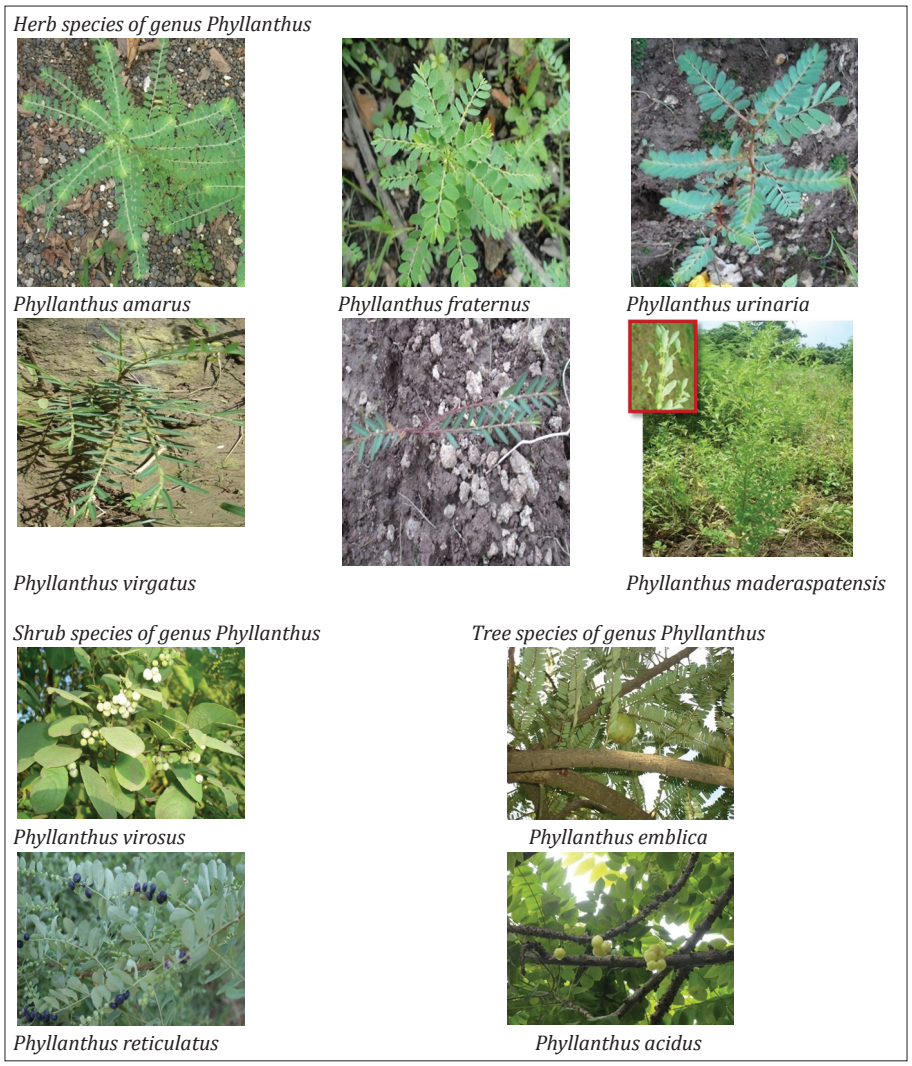

Fig. 1: *Phyllanthus* species collected from Valsad district

Voucher specimens of all the species collected were preserved at School of Pharmacy, Devi Ahilya Vishwavidyalaya for future reference.

Preparation of plant extracts

Accurately weighed (1 g) dried powdered drug of the collected species was extracted exhaustively with methanol (4 times × 10 ml) on a water bath. The extracts were cooled, filtered through Whatman I filter paper. Combined supernatants were evaporated to dryness under vacuum at 40°C using rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C. Stock solutions were prepared at a concentration of 1 mg/ml and subjected to spectrophotometric measurements to determine the total phenolic content.

Preparation of standard solution of gallic acid

Stock solution of 1 mg/ml of gallic acid was prepared by dissolving 50 mg of accurately weighed gallic acid in methanol and volume was made up to 50 ml with methanol in a volumetric flask. The aliquots (0.1–0.6 ml) of stock solutions were transferred to 10 ml volumetric flasks and volume of each was adjusted to 10 ml with methanol, to obtain standard solutions containing 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml of gallic acid.

Plotting of calibration curve of gallic acid

The calibration curve (Fig. 2) was plotted by mixing 1.0 ml aliquots of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml of gallic acid with 5 ml of 10% Folin–Ciocalteu's reagent dissolved in water and 4 ml of 7.5% $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45°C for 45 min. The absorbance was noted using spectrophotometer at λ_{max}= 765 nm. The absorbances were recorded and calibration curve was prepared by plotting absorbance versus concentration. The calibration curve was obtained by linear regression analysis.

Determination of total phenolic contents in the plant extracts

The total phenolic contents of the methanolic extracts of nine species of *Phyllanthus* were determined using Folin–Ciocalteu reagent (Singleton *et al*., 1999) [18]. Stock solutions were prepared at a concentration of 1 mg/ml for the spectrophotometric analysis. The reaction mixture was prepared by mixing 1 ml of methanolic solution of extract, 5 ml of 10% Folin–Ciocalteu's reagent dissolved in water, and 4 ml of 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 1 ml methanol, 5 ml of 10% Folin–Ciocalteu's reagent dissolved in water, and 4 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45°C for 45 min. The absorbance was noted using spectrophotometer at λ_{max}=765 nm to determine the total phenolic contents in methanolic extracts of nine *Phyllanthus* species. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then, the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract) using the formula:

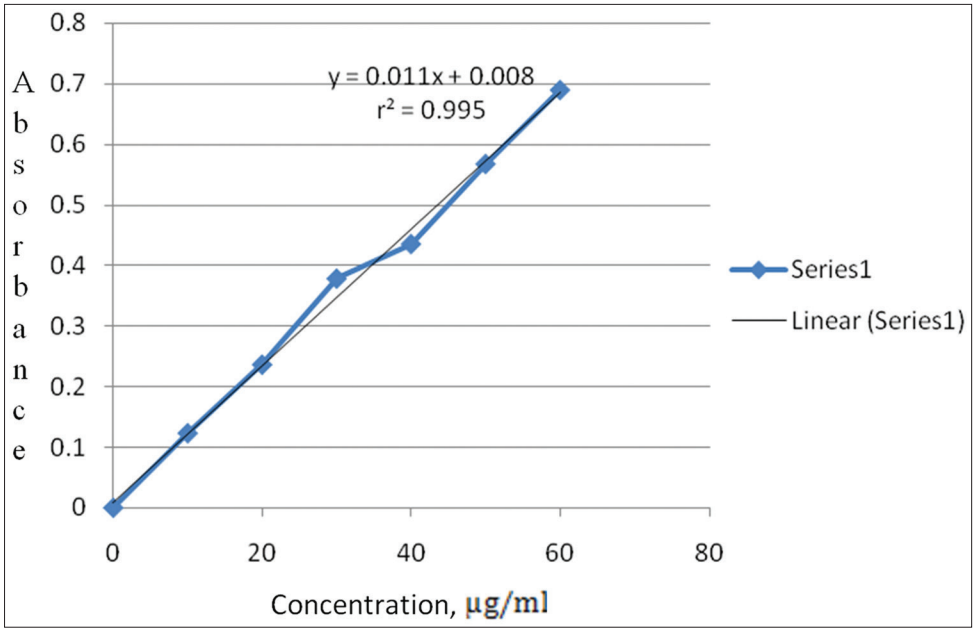

Fig. 2: Calibration curve of gallic acid in methanol

Total phenolic content in mg/g gallic acid equivalent = CV/M

Where, C=Concentration of gallic acid in the extract as obtained from calibration curve in mg/ml
V=Volume of extract used in ml
M=Weight of extract used in g.

RESULTS AND DISCUSSION

Methanolic extracts were prepared to examine the total phenolic content in nine species of *Phyllanthus.* The yield of extracts obtained from 1 g of dry plant material was calculated (Table 2). The highest yield of solid residue was obtained in methanolic extract of *P. emblica.*

The total phenolic contents in the methanolic extract of various *Phyllanthus* species using the Folin–Ciocalteu's reagent are expressed in terms of gallic acid equivalent (the standard curve equation: y=0.011x+0.008, r^2=0.995). The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract (Table 3).

The total phenolic content in methanolic extract of various *Phyllanthus* species ranged from 41.801 to 87.542 mg/g of dry weight of extract, expressed as gallic acid equivalents. *P. urinaria* was found to have highest phenolic content and *P. acidus* was found to have lowest phenolic content.

CONCLUSION

The total phenolic content in methanolic extract of various *Phyllanthus* species was determined and can serve as tool for standardization and authentication of various *Phyllanthus* species. The presence of a high total phenolic content shows that the alcoholic extract of *Phyllanthus* may possess antioxidant properties, which could lead to a new field of research in the future. Hence, in further studies total flavonoid content, antioxidant activity of the methanolic extract of various *Phyllanthus* species can be evaluated. Numerous investigations concluded high linear correlation between phenolic content and antioxidant activity. Phenols have free radical scavenging ability; hence, the phenolic content of plants may contribute directly to their antioxidant action.

AUTHORS' CONTRIBUTIONS

The literature review, design of the study, experiments, interpretation of the data, writing of the paper, and all correspondences and revisions were performed by Rakhi Khabiya under the guidance and supervision of Dr. Gajendra Choudhary and Dr. G. N. Darwhekar.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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