

Determination of dioecy in *Hippophae salicifolia* by evaluating gallic acid content through a validated HPTLC method

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ABSTRACT

Hippophae salicifolia D. Don, belonging to Elaeagnaceae family, exhibits dioecy and is extensively used for multipurpose due to its medicinal and nutraceutical potential. Although several research works have been carried out on the plant, no information is available regarding the impact of its dioecious nature on its phytochemicals and subsequent pharmacological properties. The present study is aimed at development of a high performance thin layer chromatography (HPTLC) method that is specific and reliable for the estimation of a marker compound gallic acid in both female and male counterparts of this dioecious plant, thus evaluating gender impact on chemical profile of the plant. For each sample, an ethyl acetate fraction of the hydrolyzed hydroalcoholic extract of *H. salicifolia* was prepared and applied on pre-coated plates of silica gel G 60F₂₅₄. After developing the plate in the solvent system, toluene:ethyl acetate:formic acid (5:4:1, v/v/v), the identification and estimation was carried out by densitometric scanning at 270 nm. The presence of gallic acid was confirmed by UV overlay of the spot resolving in test tracks with that of reference standard. The results suggested quantitative variation of the marker compound in female and male plants, indicating a clear gender bias. The proposed validated HPTLC method is simple, yet specific and accurate and would contribute to the quality assessment of *H. salicifolia*.

INTRODUCTION

Dioecy is a characteristic feature of species, implying that it has distinct staminate (male) and pistillate (female) flowers on separate plants (Bawa, 1980; Montalvao *et al.*, 2021). It is associated with high diversity rates in morphological and anatomical characteristics of flowering plants (Rabska *et al.*, 2021). Many medicinal plant species are apparently dioecious and quite a few researchers have studied the implications of dioecy (gender impacts) on active phytoconstituents and pharmacological actions of the dioecious medicinal plants, to find out the best gender possessing the optimum therapeutic value (Bajpai *et al.*, 2012; Choudhry *et al.*, 2014; Retuerto *et al.*, 2009). *Hippophae salicifolia* D. Don, belonging to Elaeagnaceae family, is popularly called as “seabuckthorn.” It is a deciduous shrub, occurring in the

icy heights of Himalayan region of India. As per the traditional systems of medicine, the plant shows beneficial effects in asthma, impaired lung conditions, cough, cold, sore throat, stomachache, and also possess wound healing properties (Arimboor *et al.*, 2006a; Garbyal *et al.*, 2005; Man and Dutt, 2008). Seabuckthorn species are extensively used for the treatment of diverse ailments that include gastric ulcers, coronary heart diseases, diarrhea, and also as antioxidant and immunomodulator (Arimboor *et al.*, 2006a; Eccleston *et al.*, 2002; Geetha *et al.*, 2002; Xing *et al.*, 2002). *Hippophae salicifolia* leaf extract is known to enhance the antioxidant defense against reactive oxygen species produced under hyperglycemic conditions, and thus helps in modulation of diabetes mellitus (Middha *et al.*, 2019). *Hippophae salicifolia* is also reported to have anti-inflammatory, anticancer, anti-atherosclerosis, and anti-sterility activities that are attributed to the presence of multivitamins and flavonoids in the aerial parts of this plant (Chakraborty *et al.*, 2015; Dubey *et al.*, 2018; Gupta *et al.*, 2011; Mousmi and Handique, 2013). In addition, its fruits are utilized to improve appetite and as an antidote for food poisoning.

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They are useful in the paralysis of throat muscles and tongue, in lung complaints, influenza and catarrhal issues of airway (Negi *et al.*, 1993; Uniyal, 1990). Seabuckthorn seed oil extracted from the berries possess significant nutritional and antimicrobial properties. It stimulates regenerative processes in the skin epidermis, reduces scars, and also causes healing of wounds. Due to its high content of linolenic acid and vitamin E along with its good antimicrobial property, seed oil is utilized in pharmaceutical, nutraceutical and cosmetic preparations (Moges *et al.*, 2021; Zielinska and Nowak, 2017).

This plant is also found to be rich in vitamin A, B₁, B₁₂, C, and K; flavonoids like isorhamnetin, quercetin, myricetin, and kaempferol and carotenoids like lycopene, β -carotene, δ -carotene, neoxanthin, violaxanthin, cryptoxanthin, and flavoxanthin (Arimboor *et al.*, 2006a, 2006b; Kapoor *et al.*, 1972; Lu, 1992). The vitamin C content of fresh fruits of *H. salicifolia* is reported to be much higher (224 mg/100 g) than the other species under the *Hippophae* genus (Dinca *et al.*, 2018). The phenolic contents identified in other species like *Hippophae rhamnoides* include *p*-hydroxybenzoic acid, gallic acid, protocatechuic acid, *p*-coumaric acid, vanillic acid, cinnamic acid, caffeic acid, and ferulic acid. Among the phenolics, gallic acid was found to be occurring in *H. salicifolia* as a major constituent (Arimboor *et al.*, 2008; Maheshwari *et al.*, 2011; Sharma *et al.*, 2008). Furthermore, gallic acid is a potent antioxidant phytoconstituent and is used as a reference standard in the estimation of phenolics and ascorbic acid. Hence, gallic acid can be considered as a biomarker compound for the qualitative as well as quantitative assessment of *H. salicifolia*.

A comprehensive literature survey revealed that some methods are available for the analysis of gallic in other plant samples, that include liquid chromatography methods, capillary electrophoresis methods, capillary gas chromatography method hyphenated with mass spectrometry and liquid chromatography combined with tandem mass spectrometry (Deodhar *et al.*, 2012; Deshmukh and Prabhu, 2011; Kuskoski *et al.*, 2012; Wang *et al.*, 2013; Yue *et al.*, 2006). But, no simple and validated high performance thin layer chromatography (HPTLC) method was reported for gallic acid estimation in *H. salicifolia*. Moreover, since *H. salicifolia* is an important medicinal dioecious plant, the characterization of gender-specific differences on its active constituents is essential. Unfortunately, no such systematic study has been done on this plant so far. Hence, the objective of the present study was to establish a new, simple, specific and accurate method for gallic acid quantification in the female and male plants of *H. salicifolia*, thereby also emphasizing implications of its dioecious nature on its chemical profile.

MATERIALS AND METHOD

Plant material

As *H. salicifolia* is a dioecious plant, fresh aerial parts of four samples were procured, including both female and male plants from two different provinces of Uttarakhand (India), i.e., Byans valley and Niti valley, in October month. The plant samples were authenticated by Dr. V. P. Bhatt, Taxonomist, Department of Botany, Herbal Research and Development Institute, Chamoli,

Uttarakhand, India. Voucher specimens (PAH/23082015/01-04) have been deposited in Department of Pharmacognosy and Phytochemistry, L. M. College of Pharmacy, Ahmedabad. The fresh aerial parts were cut, dried properly, powdered, and passed through 80# sieve. The powdered samples were stored separately at room temperature in airtight containers.

Chemicals and solvents

Ethyl acetate, petroleum ether (60°C–80°C), 5 N sulphuric acid, toluene, methanol, formic acid, and acetic acid of analytical grade (E. Merck, Mumbai, India) were used. Standard gallic acid (purity 99.9%) was procured from Sigma-Aldrich Chemicals (India).

Extraction and fractionation of plant material

Accurately weighed 200 g of plant powders of the four samples were separately extracted successively with petroleum ether (thrice, 50 ml) and then ethyl acetate (thrice, 50 ml) by hot percolation method. The subsequent ethyl acetate fractions were hydrolyzed with 50 ml of 5 N sulphuric acid and 20 ml of toluene under reflux condition for 5 hours. The hydrolyzed extracts were further fractionated with ethyl acetate (thrice, 20 ml) and the obtained fractions of ethyl acetate were pooled along with the toluene fractions of the hydrolyzed extracts. The pooled fractions were further concentrated under vacuum to dryness to yield semi-solid extracts. 10 mg of these extracts were first dissolved in 5 ml methanol and then further diluted up to 10 ml to yield test extracts of *H. salicifolia*. 15 μ l of test extracts were used for the chromatographic analysis.

Optimization of the chromatographic method

The test extracts were applied to pre-coated TLC plates at the preliminary stage of method development. Mobile phases, consisting of binary mixtures of solvents, toluene and ethyl acetate, were tested on the basis of polarity. Different proportions of toluene (90% to 50%) and ethyl acetate (10% to 40%) in a mixture were tried. As there was non-satisfactory separation of constituents, different combinations were tried with acetic acid and formic acid which showed that addition of formic acid gave best resolution of the individual constituents as evident from the fingerprint. Then, separation of different samples of *H. salicifolia* was performed on pre-coated HPTLC plates of silica gel G 60F₂₅₄ using the mobile phase, toluene:ethyl acetate:formic acid (5:4:1, v/v/v).

HPTLC analysis

5 mg of standard gallic acid was dissolved in 25 ml of methanol to obtain standard stock solution (200 ng/ μ l). Graded concentrations of the standard solution (1–5 μ l) were applied on pre-coated TLC plates in the form of bands for calibration purpose.

The HPTLC analysis was carried out on 10 \times 10 cm pre-coated HPTLC plates of silica gel G 60F₂₅₄ (E. Merck, Germany). The test extracts were applied to the plate along with the standard solution as 8 mm wide bands (12 mm apart) using CAMAG Linomat-V applicator (Muttentz, Switzerland) with the application speed of 6 seconds/ml. The plate was developed up to 8 cm distance in the mobile phase composed of toluene:ethyl

acetate:formic acid (5:4:1, v/v/v) in pre-saturated CAMAG twin-trough chamber (20 × 10 cm). The plate was dried in air for 5 minutes after development and scanned immediately at 270 nm using a deuterium lamp in CAMAG Scanner-3 (with WinCATS 5 integration software). The photo-documentation of the plate was done using CAMAG Reprostar-3 TLC visualizer. The calibration curve for gallic acid was prepared by plotting the peak area against the concentration of gallic acid and the concentrations of test samples were determined from the calibration curve.

Method validation

Validation of the developed method was carried as per ICH guidelines (ICH, 2005), by determination of linearity, precision (interday and intraday), specificity, accuracy, repeatability, limit of quantification (LOQ), and limit of detection (LOD). The linear response was determined by analyzing gallic acid calibration curve in the range of 200 to 1,000 ng/spot concentration and then applying linear regression analysis. The intraday precision was determined by analyzing standard gallic acid (200, 400, and 800 ng/spot) for three times on the same day, while the interday precision was determined by analyzing the same spot for three consecutive days. Repeatability of peak area measurement was performed by measurement of the same spot for seven times. Seven times application of the same volume of standard solution was done and then peak area was measured for determining the repeatability of sample application. Specificity was determined by analyzing the sample solution for interferences from other constituents present in the *H. salicifolia* extracts. The peak purity of the marker compound was analyzed by comparing the overlay absorption spectra scanned at 270 nm. Accuracy was confirmed at three different levels (50%, 100%, and 150% of working concentration). LOQ and LOD were determined at signal-to-noise ratio of 10:1 and 3:1, respectively.

RESULTS AND DISCUSSION

The literature survey concerning the biological potentiality and phytochemical profile underpin that evaluation of gender-specific variation concerning phytoconstituents and biological efficacy of *H. Salicifolia* has remained unexplored. Hence, this present study is tailored to evaluate gender-specific variation in the phytoconstituents. Hydrolyzed ethyl acetate extract yields of different samples of *H. Salicifolia*, i.e., Male Byans Valley (MB), Female Byans Valley (FB), Male Niti Valley (MN), and Female Niti Valley (FN) were found to be 12.5%, 11.8%, 11.2%, and 12.4% (w/w), respectively. The TLC fingerprint of test extract of each sample, using toluene:ethyl acetate:formic acid (5:4:1, v/v/v) as the mobile phase, showed good separation of bands and revealed the presence of gallic acid as navy blue colored spot in daylight at R_f 0.42, after derivatization by ferric chloride reagent.

In the HPTLC analysis, the peaks resolving at R_f 0.42 in test solutions superimposed with that of gallic acid standard. The HPTLC fingerprint and the densitometric chromatogram of test extracts of all four samples is shown in Figure 1. The study of UV overlay absorption spectra obtained from the reference

standard and the respective peaks from test indicated peak purity and specificity of the developed method (Fig. 1). The method was also accurate as indicated by the results of recovery studies. The calibration plot indicated that the peak area response was in linear association with standard gallic acid concentration in the range of 200 to 1,000 ng. The data of linearity, precision, repeatability, LOQ, LOD, specificity, and accuracy are given in Table 1. The amount of gallic acid in different test samples was found to be 0.426%, 0.718%, 0.143%, and 0.566% (w/w) in MB, FB, MN, and FN samples of *H. salicifolia*, respectively.

In the present investigation, gallic acid was estimated as a marker compound by HPTLC method for the first time in both female and male plants of *H. salicifolia* procured from two different provinces of Uttarakhand, namely, Byans valley and Niti valley. The content of gallic acid was found to vary from 0.143% to 0.718% w/w in different samples, the highest amount being found in the aerial parts of female plants procured from Byans valley (FB) and the lowest in male plants of Niti valley (MN). It was also observed that the amount of gallic acid was marginally higher in the female plants than their respective male plants. Thus, in line with the earlier observations (Cornago *et al.*, 2011; Simpson, 2013), our experimental results also showed a significant correlation between the phenolic content and dioecy which is lacking in earlier findings. The HPTLC fingerprints of all the samples also revealed more number of bands in female plant extracts showing abundance of phytoconstituents as compared to

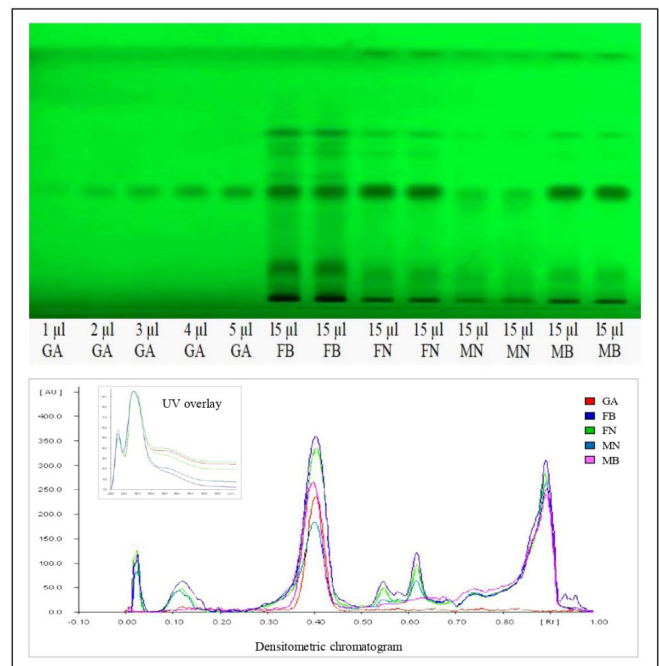


Figure 1. HPTLC plate and densitometric chromatogram of standard gallic acid with test extracts. GA, gallic acid standard; FB, female byans valley; FN, female niti valley; MN, male niti valley; MB, male byans valley.

Table 1. Results of linearity ($n = 6$) and method validation parameters.

Parameter	Result
Linearity range	200–1,000 ng/spot
Regression equation	$y = 11.49x - 999.7$
Correlation coefficient (R^2)	0.991
Intraday precision	0.31%–0.72% RSD
Interday precision	0.32%–0.88% RSD
Repeatability of peak area measurement	0.072% RSD
Repeatability of sample application	0.213% RSD
LOD	1.891 ng/spot
LOQ	5.731 ng/spot
Accuracy	97.56%–99.40%
Specificity	Specific

their male counterparts. Hence, the study indicated clear gender bias as well as influence of geographical differences on this plant.

CONCLUSION

The validated analytical method was developed with optimized chromatographic conditions for estimation of gallic acid as a marker compound in aerial parts of both female and male plants of *H. salicifolia*. The developed method is sensitive, precise, reliable, and specific as indicated by the results of validation parameters and has significant potential to indicate gender impact on this plant based on their chemical profile. Thus, the proposed validated HPTLC method would enable easy, yet accurate and specific analysis of gallic acid in the aerial parts of both genders of this plant and can be applied for the routine qualitative and quantitative assessment of *H. salicifolia* plants.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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