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Original Article

Identification and Quantification of Four Bioactive Markers in *Sanjivani Vati* Using High-Performance Thin Layer Chromatography

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ABSTRACT

Background: *Sanjivani Vati* (SV) is an extensively used multicomponent Ayurvedic medicine, recommended for fever, indigestion, dysentery, gastroenteritis and more. The pharmaceutical standardization of SV and estimation of its tannin and embelin content have been reported in previous studies. However, the identification and quantification of other bioactive constituents namely, aconitine, gallic acid and β-asarone, etc. remain unaddressed. Also, concerns arise regarding one of its ingredients i.e. *Vatsanabha* (Aconite species), historically recognized as one of the most poisonous plants. It is used after meticulous processing specified in classical texts. Still, it is crucial to identify the amount of aconitine being delivered in the drug to ensure optimal therapeutic benefit while minimizing adverse reactions. In addition, another issue is that *Vatsanabha* is being sold as a mixture of various species in Indian markets. Previous research has highlighted significant qualitative and quantitative variations in the total alkaloid content among different species of *Vatsanabha*. **Materials and Methods:** The present communication deals with identifying and quantifying four bioactive markers viz. Gallic acid, Piperine, β-asarone and Aconitine in SV prepared with two species of *Vatsanabha* namely *Aconitum nepellus* L. (SV -1) and *Aconitum balfourii* Holmes ex Stapf (SV-2) using High-performance Thin Layer Chromatography. **Results:** Gallic acid, piperine and β-asarone were detected in both SV groups. However, aconitine was not detected in any of the samples. Identification was confirmed by matching the R_f and spectrum overlay. The selected solvent system yielded effective separation from the matrix with an R_f value of 0.45±0.01 for gallic acid, 0.27±0.01 for piperine and 0.62±0.01 for β-asarone. The amount of gallic acid was determined to be 1266 µg and 1395 µg in 100 mg of SV1 and SV2 respectively. Piperine was 35.70 µg and 49.95 µg per 100 mg of SV1 and SV2, respectively. β-asarone was 135.3 µg and 236.1 µg in SV1 and SV2 respectively. **Conclusion:** The concentration of all the detected biomarkers is higher in SV-2 than in SV-1. The presence of these marker compounds helps explain the probable mode of action of SV and offers simple, efficient methods for estimating these biomarkers.

Keywords: Ayurvedic medicine, Aconitine, Biomarkers, HPTLC, *Vatsanabha*, *Sanjivani Vati*.

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INTRODUCTION

Ayurveda, an ancient holistic healing system, relies heavily on botanicals to treat various ailments. Phytoconstituents are the bioactive compounds found in plants responsible for their therapeutic benefits. Quantifying these constituents ensures consistency in the formulation, guaranteeing that each dose contains the intended therapeutic compounds in the right proportions. This standardization not only improves results reproducibility but also reduces variability between batches, ensuring predictable therapeutic outcomes. Furthermore, precise quantification enables practitioners to determine the appropriate dosages for optimal therapeutic effects while minimizing the risk of adverse reactions. Therefore, quantifying phytoconstituents is paramount for ensuring the quality, safety and efficacy of Ayurvedic formulations, promoting their acceptance and integration into modern healthcare practices.

Sanjivani Vati (SV) is an extensively used multicomponent Ayurvedic medicine, outlined in the Ayurvedic Formulary of India.[1] Ayurvedic practitioners widely recommend it for addressing various conditions such as fever, indigestion, dysentery, Gastroenteritis, etc. It comprises ten ingredients of herbal origin as enlisted in Table 1. Furthermore, Cow's urine

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holds significant importance as one of the primary constituents, utilized for *Bhavana* (impregnation).[2] The pharmaceutical standardization and analytical validation of SV have been performed in our previous study.[3] Additionally, another study attempted to estimate the tannin, embelin and piperine content of the drug.[4] It is important to identify and quantify the maximum possible number of markers in such polyherbal formulations for assessment of Quality, Safety and Efficacy. However, no study has been performed regarding the identification and quantification of the other bioactive constituents viz. Aconitine, Gallic acid and β-asarone in the drug. Moreover, no study has validated the findings of previous research. Despite its established History of Safe Use, concerns arise regarding one of its ingredients i.e. *Vatsanabha*, historically recognized as one of the most poisonous plants. It is crucial to identify the amount of aconitine being delivered in the drug to ensure optimal therapeutic benefit while minimizing adverse reactions. In addition, another issue is that *Vatsanabha* is being sold as a mixture of various species in Indian markets. Previous research has highlighted significant qualitative and quantitative variations in the total alkaloid content among different species of *Vatsanabha*.[5] However, the potential impact of utilizing different species on the quantity of their chief marker compound in various formulations remains unexplored. Therefore, the present communication deals with the identification and quantification of four bioactive markers viz. Gallic acid, Piperine, β-asarone and Aconitine in *Sanjivani Vati* prepared with two different species of *Vatsanabha* namely *Aconitum nepellus* L. and *Aconitum balfourii* Holmes ex Stapf. The selection of these two species was done on the basis of their abundant availability in natural habitat and markets respectively.

MATERIALS AND METHODS

Procurement of Raw Materials

All requisite raw botanicals including a sample of *Vatsanabha* were purchased from a local vendor, Jaipur, in dried form. Another sample of *Vatsanabha* was collected from Chicham Khas, Himachal Pradesh (Elevation-4300 m, Lat. 32.339601^0, Long. 77.997743^0). Fresh *Gomutra* (Cow's urine) was collected from nearby cowsheds in the early morning.

Authentication of Raw material

The procured samples of individual crude drugs were authenticated by Raw Materials Herbarium and Museum, Delhi, under CSIR-National Institute of Science Communication and Policy Research. The authentication numbers for its constituents issued by the institute are as follows: *Vidanga* (4081-82-2), *Nagara* (4081-82-8), *Krishna* (4081-82-9), *Pathya* (4081-82-6), *Amala* (4081-82-5), *Bibhitaka* (4081-82-4), *Vacha* (4081-82-3), *Guduchi* (4081-82-7), *Bhallataka* (4081-82-1). Apart, the fresh sample of *Vatsanabha* was identified and authenticated as *Aconitum nepellus* L. (BSI/BGIR/1/TECH./2021/027/80) by Botanical Survey of India, Botanical Garden of Indian Republic, Noida. The marketed sample of *Vatsanabha* was identified and authenticated as *Aconitum balfourii* Holmes ex Stapf (RRDR/AIIA/129) at Taxonomy and Herbarium laboratory, Regional Raw Drug Repository, All India Institute of Ayurveda, New Delhi. The voucher specimens and herbarium have been preserved in the respective Institutes. Furthermore, the confirmation of the species *Vatsanabha* was also done by DNA fingerprinting.

Preparation of *Sanjivani Vati*

Two groups of *Sanjivani Vati* namely SanjivaniVati-1 (SV-1) and *Sanjivani Vati*-2 (SV-2) were prepared in the Pharmaceutical Laboratory, Department of *Rasashastra* and *Bhaishajya Kalpana*, All India Institute of Ayurveda, New Delhi as per the official reference.[6] SV-1 and SV-2 can be stated as *Sanjivani Vati* prepared from *Aconitum nepellus* L. and *Aconitum balfourii* Holmes ex Stapf respectively. The formulation composition is depicted in Table 1.

Chemicals and Reagents

Standards like Gallic acid (Make: Sigma, Purity: 97%), Piperine (Make: TCI, Purity: 97%), β-asarone (Make: Sigma, Purity: 99%) and Aconitine (Make: Sigma, Purity: 95%) were used for the study. All the solvents used in the experiment such as methanol (Make: Qualigens, Purity: 99.8%), Toluene (Make: Prayog Fine Chem, Purity: 99.8%), Ethyl acetate (Make: EMPARTA Merck life Sciences, Purity: 99.8%), Diethyl ether (Make: Qualigens, Purity: 98%), Formic Acid (Make: Qualigens, Purity: 85%), Diethyl amine (Make: Fisher Scientific, Purity: 99%) Chloroform (Make: EMPLURA, Purity: 99%) were of analytical grade.

Preparation of Test Solutions

Preparation of Sample Solutions

Both the test drugs viz. SV-1 and SV-2 were accurately weighed (1 g) into a volumetric flask separately. 10 mL of methanol was added to each of them. The mixture was sonicated for 20 min and then filtered through the Whatman filter paper. No. 1. These filtrates were subjected to further analysis for the quantification of Gallic acid, Piperine and β-asarone. However, Chloroform was used as a solvent in place of methanol for the quantification of Aconitine. The concentration of both the sample solutions was 100 mg/mL.

Preparation of Standard Solution of marker compounds

Accurately weighed 1mg quantity of three standards viz. Gallic acid, Piperine and β-asarone were dissolved in HPLC grade methanol in a 10 mL volumetric flask separately, sonicated for 10 min and finally, made to the mark with methanol to obtain standard stock solutions of 100 µg/mL of all marker compounds. For the aconitine standard, the stock solution was prepared in chloroform in a concentration of 1 mg/mL.

High- Performance Thin Layer Chromatography

Instrumentation and Chromatographic Conditions

The test solutions were applied as bands using CAMAG Linomat 5, 100 microlitre Hamilton syringe, a semi-automatic sample applicator on HPTLC silica gel 60 F_{254} (0.2 mm thickness, E. Merck) under a nitrogen stream, with the following parameters: Band length: 8 mm, Dosage speed: 150 nl/s and Pre dosage volume: 0.2 µL. The plate underwent linear ascending development up to 70 mm from the base in an optimized solvent system for different marker compounds within a CAMAG Twin Through Chamber (TTC) equipped with a Stainless Steel lid (20×10cm), previously saturated with the appropriate mobile phase employing saturation pad for 20 min at room temperature. The volume of the solvent system was evenly distributed between the front and rear sections of the chamber. After proper development, the plate was dried at room temperature for 5 min using an air dryer and visualized at λ 254 nm (short UV) and λ 366 nm (long UV) in TLC Visualizer 2. The specific chromatographic conditions for the particular biomarker have been placed in Table 2.

Subsequently, the plates were scanned using CAMAG TLC Scanner 4, with densitometric scanning conducted at a speed of 20 mm/s, using a slit dimension of 6.00×0.45 mm, micron in the absorbance mode at a single wavelength i.e. λ_{max} (corresponding to the absorption maxima of the band) which is selected through generating an absorption spectrum for the respective marker compound. Deuterium lamp, mercury vapor lamp and tungsten lamp were used as radiation sources for wavelengths of 254 nm, 366 nm and white light respectively. The system setup was managed by visionCATS software, version 3.2. The obtained values of Retardation factors on scanning at an optimized wavelength for different bioactive constituents were recorded. Also, the high-resolution images of the HPTLC plate were captured.

Calibration Curve for Bioactive Constituents and their analysis in the test drugs

Calibration curves were plotted to assess the linearity. A solution of 100 µg/mL gallic acid was applied in volumes ranging from 2.0 µL to 10 µL on plates, resulting in amounts ranging from 0.2 µg to 1 µg per band. Likewise, concerned solutions of 100 µg/mL were applied on plates to achieve amounts of 0.2 µg to 0.4 µg piperine and 0.2 µg to 1.4 µg β-asarone per band respectively. Subsequently, all the biomarkers were quantified by plotting a calibration curve following densitometric scanning.

RESULTS

To identify and measure the chosen biomarkers in the test samples, the mobile phase selection was based on polarity. The solvent systems that produced dense and distinct spots with specific R_f values for bioactive components were sought. Among the investigated standards, gallic acid, piperine and β-asarone were detected in both the groups of *Sanjivani Vati*. However, aconitine was not detected in any of the samples. Identification was confirmed by matching the R_f and spectrum overlay. Several solvent systems were assessed in varying proportions. Among these, the selected ones yielded effective separation from the matrix with an R_f value of 0.45±0.01 for gallic acid, 0.27±0.01 for piperine and 0.62±0.01 for β-asarone. The developed HPTLC plate for all the marker compounds can be depicted in Figure 1. While looking over the results obtained on visualization and densitometric scanning, bands of identical color and distinct R_f values corresponding to R_f values of reference standards have been spotted in both the tested samples of *Sanjivani Vati* indicating the

Table 1: Formulation Composition of *Sanjivani Vati*.

Sl. No.	Ingredient	Latin name	Part Used	Ratio
1.	*Vidanga*	*Embelia ribes* Burn.	Dried Fruit	1
2.	*Nagara*	*Zingiber officinale* Rosc.	Dried Rhizome	1
3.	*Krishna*	*Piper longum* Linn.	Dried Fruit	1
4.	*Pathya*	*Terminalia chebula* Retz.	Dried Pericarp	1
5.	*Amala*	*Embelica officinalis* Gaertn.	Dried Pericarp	1
6.	*Bibhitaka*	*Termenalia bellirica* Roxb.	Dried Pericarp	1
7.	*Vacha*	*Acorus calamus* Linn.	Dried Rhizome	1
8.	*Guduchi*	*Tinospora cordifolia* Miers ex Hook. and Thoms.	Dried Stem	1
9.	*Shuddha Bhallataka*	Processed *Semecarpus anacardium* Linn.	Dried Fruit	1
10.	*Shuddha Vatsanabha*	Processed *Aconitum nepellus* L. (G-1) /*Aconitum balfourii* Holmes ex Stapf (G-2)	Dried Root tuber	1
11.	*Gomutra*	Cow urine		Q.S. for *Bhavana* (Levigation)

Table 2: **Specific Chromatographic Conditions for Bioactive Constituents.**

Sl. No.	Biomarkers	Mobile Phase (V/V)	Densitometric Scanning @ wavelength
1	Gallic acid	Toluene: Ethyl Acetate: Formic Acid (5:5:1)	280nm
2	Piperine	Toluene: Ethyl Acetate (9:3)	330 nm
3	β-asarone	Toluene: Ethyl Acetate: Diethyl ether (6:3:1)	254nm
4	Aconitine	Toluene: Ethyl acetate: Diethyl amine (7:2:1)	236nm

Table 3: **Analytical Parameters for different Bioactive Constituents.**

Sl. No.	Standard	Calibration function	Coefficient of Variation (CV)	Correlation coefficient (r^2)	LOD (Limit of Detection) (/band)	LOQ (Limit of Quantification) (/band)	Amount per 100.00 mg of Samples	
							SV-1	SV-2
1.	Gallic Acid	$y=1.06\times10^{-8}x + 7.214 \times 10^{-3}$	2.17%	0.99	0.069346	0.21014	1266 µg	1395 µg
2.	Piperine	$y=1.847\times10^{-8}x + 5.575 \times 10^{-3}$	0.81 %	0.99	0.062	0.20	35.70 µg	49.95µg
4.	β-asarone	$y=6.247\times10^{-9}x + 3.101 \times 10^{-4}$	2.35%	0.99	0.066478	0.201447	135.3 µg	236.1µg

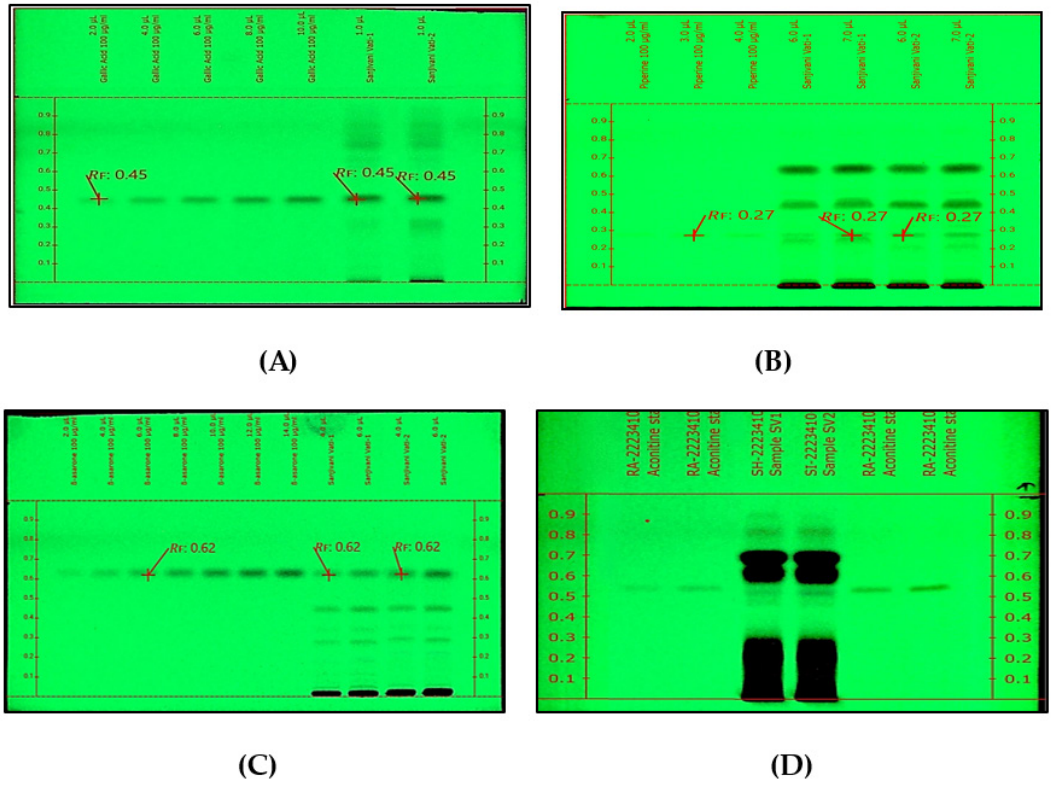

Figure 1: Developed HPTLC plates for all marker compounds. A. Gallic acid, B. Piperine, C. β-asarone, D. Aconitine).

presence of all the examined markers except aconitine in both SV-1 and SV-2.

The chromatograms for the identified standard as well as both samples have been produced and shown in Figure 2. Further, a strong linear relationship between peak area and quantity of the analyte was noted. The collated presentation of the Calibration plot for all the bioactive constituents can be seen in Figure 3. The Linearity of an analytical procedure is defined as its capacity (within a specified range), to obtain test results directly, or through a mathematical transformation, proportional to the analyte concentration in the sample.[7] Linear-2 regression mode with a range deviation of 5% was employed for the quantification of the targeted compounds. The Calibration function, Coefficient

of Variation (CV) and Correlation coefficient (R2) along with the detected quantity of biomarkers in both samples have been tabulated in Table 3. Also, the Limit of Detection (LOD) and the Limit of Quantification (LOQ) under the chromatographic conditions used were separately determined at Signal-to-Noise ratios (S/N) of 3 and 10, respectively.

DISCUSSION

Through Table 3, it is evident that the concentration of all the detected biomarkers is higher in SV-2 compared to SV-1. The only difference between the two groups is the species of *Vatsanabha* used, with all other ingredients remaining constant. Despite this, a significant variation in the quantity of bioactive constituents was observed between the two groups of *Sanjivani Vati*. None of the tested marker compounds were found in any of the investigated aconitine species, indicating that the species of *Vatsanabha* alone cannot explain these findings. Interactions between the drugs might lead to differences in the synthesis and accumulation of bioactive compounds. The primary reason for these variations could be batch-to-batch differences in the formulation and the amount of bioactives extracted in the test samples. Regarding the source of these bioactives, it is inferred that gallic acid in the test drugs likely originates mainly from *Embelica officinalis* Gaertn., *Terminalia chebula* Retz. and *Terminalia bellirica* Roxb.[8] Other compounds, such as piperine and β-asarone, likely come from *Piper longum* Linn. and *Acorus calamus* Linn., respectively, as these are active ingredients in these herbs.[9,10] However, Aconitine, a diterpenoid alkaloid and key phytoconstituent of *Vatsanabha* was not identified in either group of *Sanjivani Vati*. The underlying cause may be its specific traditional treatment with Cow urine (*Shodhana*) done before preparing the test drugs. Such specific treatments are known to enhance efficacy and reduce

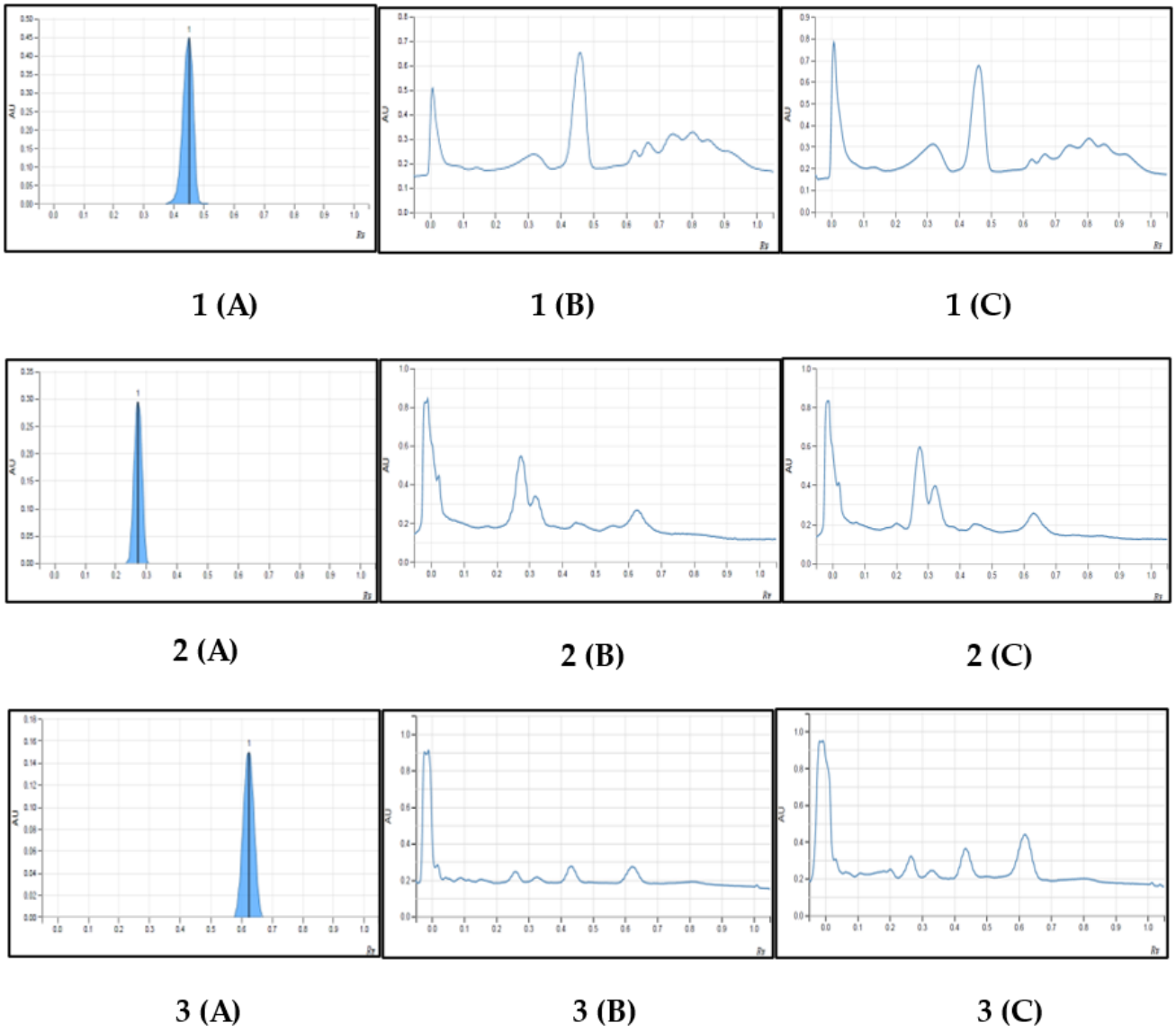

Figure 2: Chromatograms of Standards and Samples. {2.1 - Chromatogram of Gallic acid (A: Standard, B: SV-1, C: SV-2), 2.2 - Chromatogram of Piperine (A: Standard, B: SV-1, C: SV-2), 2.3 - Chromatogram of β-asarone (A: Standard, B: SV-1, C: SV-2)}.

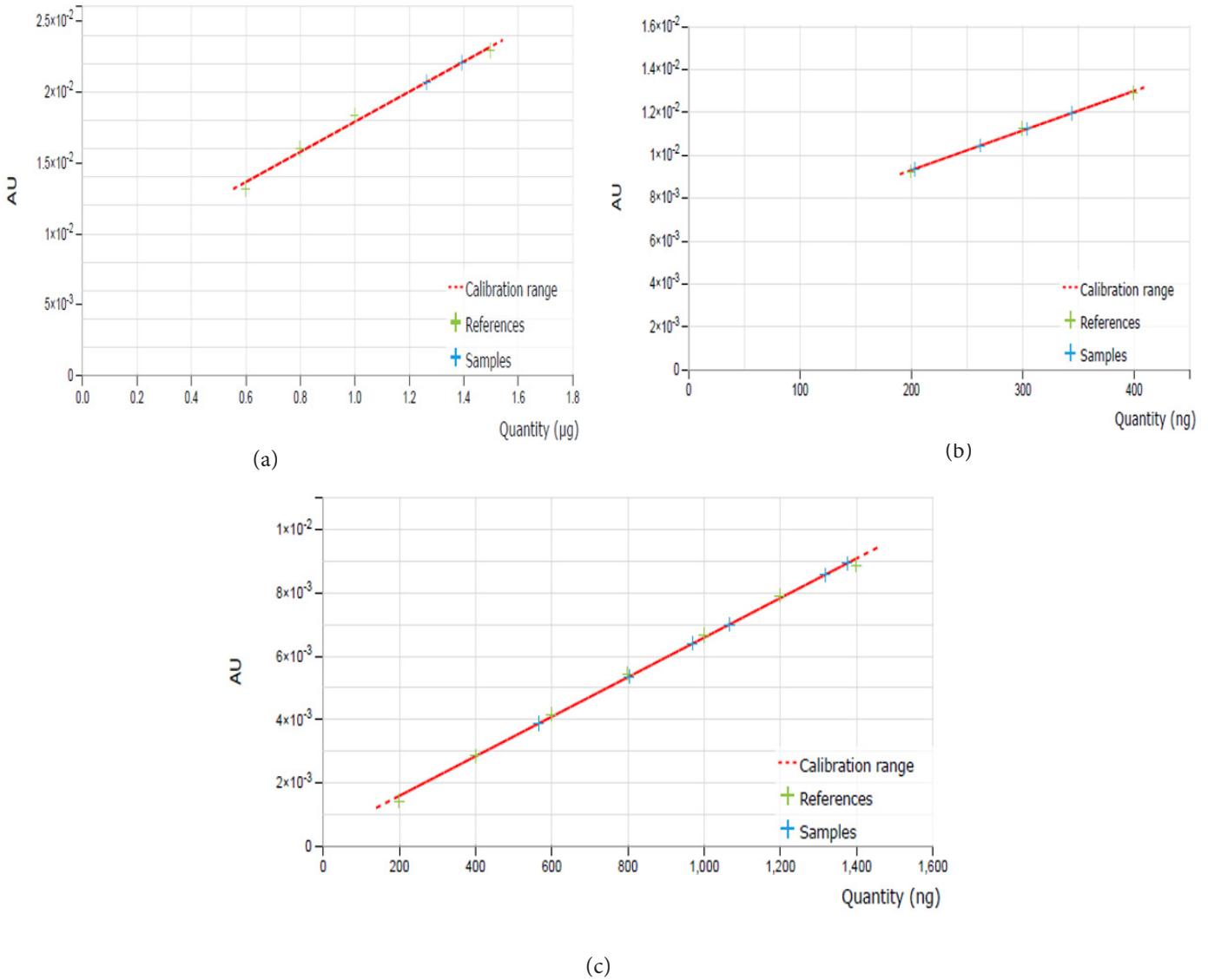

Figure 3: Calibration plot (a) Gallic acid @280nm (b) Piperine @330 nm (c) β-asaron @ 254nm.

the toxicity of crude drugs by altering their pharmacodynamic properties. For Aconite roots, changes in pharmacodynamics and toxicity levels occur due to the modification in the structure of Diester Diterpenoid Alkaloids (DDAs) during processing, leading to the formation of Monoester Diterpenoid Alkaloids (MDAs).[11,12] Examining the composition of *Sanjivani Vati* reveals that *Vatsanabha* is one of its ten ingredients, comprising approximately 1/10th of the entire formulation. This suggests a meager amount of aconitine in the test drug. Additionally, the processing of *Vatsanabha* likely modified the alkaloid structure, resulting in its absence in the analytical study.

Sanjivani Vati has traditionally been used to treat gastrointestinal disorders and pyrexia. A literature review reveals that gallic acid plays a significant role in protecting the mucosal layer of the gastrointestinal tract from ulcers through various mechanisms. These include reducing acid secretion, promoting the release of endogenous antioxidants and defensive factors and decreasing oxidative stress and lipid peroxidation.[13,14]Additionally, it interferes with intracellular inflammatory pathways that lead to ulcerative colitis by inhibiting nuclear transcription factors, such as nuclear factor (NF)-κB and signal transducer and activator of transcription 3 (STAT3) and down-regulating their inflammatory downstream targets.[15,16] It also mitigates inflammation by reducing the expression and release of pro-inflammatory and inflammatory mediators like bradykinin, substance P, COX-2, NF-κB, IL-2, IL-4, IL-5, IFN-γ and TNF-α.[17] Similarly, Piperine, an amide alkaloid, is known for its anti-inflammatory properties[18] and its ability to enhance the bioavailability of various compounds.[19,20] Likewise, β-asarone exhibits antioxidant as well as anti-inflammatory effects.[21] Thus, the presence of these marker compounds helps in explaining the probable mode of action of *Sanjivani Vati*. Thus, the study offers simple and efficient techniques to estimate the investigated biomarkers in the tested drug. Nevertheless, it is essential to validate all these methods in terms of accuracy, precision, repeatability, robustness

and specificity in accordance with the guidelines outlined by the International Conference of Harmonization (ICH Q2 R2).[22] Furthermore, efforts should be made to identify and quantify the other bioactive constituents such as embelin, chebulagic acid, 6-gingerol, quercetin, bhilawinol and pseudoaconitine etc., as per the constituents of the drug. Then, the findings must be further verified by employing advanced instrumentations to develop international standards.

CONCLUSION

Gallic acid, piperine and β-asarone were detected in both groups of SV. However, aconitine was not detected in any of the samples. Identification was confirmed by matching the R_f and spectrum overlay. The selected solvent system yielded effective separation from the matrix with an R_f value of 0.45±0.01 for gallic acid, 0.27±0.01 for piperine and 0.62±0.01 for β-asarone. The amount of gallic acid was determined to be 1266 µg and 1395 µg in 100 mg of SV1 and SV2 respectively. Piperine was 35.70 µg and 49.95µg per 100 mg of SV1 and SV2, respectively. β-asarone was 135.3 µg and 236.1µg in SV1 and SV2 respectively. The concentration of all the detected biomarkers is higher in SV-2 (*Aconitum balfourii* Holmes ex Stapf) than in SV-1 (*Aconitum nepellus* L.). The presence of these marker compounds helps explain the probable mode of action of SV and offers simple, efficient methods for estimating these biomarkers.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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ABBREVIATIONS

SV: *Sanjivani Vati;* **HPTLC:** High-Performance Thin Layer Chromatography; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification **R_f:** Retardation factor; **CV:** Coefficient of Variation.

SUMMARY

This study focuses on the identification and quantification of four bioactive markers-gallic acid, piperine, β-asarone and aconitine-in *Sanjivani Vati* (SV) prepared using two species of *Vatsanabha*: *Aconitum nepellus* L. (SV-1) and *Aconitum balfourii* Holmes ex Stapf (SV-2), utilizing High-Performance Thin Layer Chromatography (HPTLC). Among them, gallic acid, piperine and β-asarone were detected in both the formulations, while aconitine was not detected. The solvent system effectively separated the markers, with R_f values of 0.45±0.01 (gallic acid), 0.27±0.01 (piperine) and 0.62±0.01 (β-asarone). Quantification revealed higher concentrations of all detected markers in SV-2 compared to SV-1, indicating species-based variation. The results underscore the need for stringent standardization of SV, particularly concerning the aconite species used. This study not only ensures the safety and efficacy of SV but also highlights a reliable approach for marker-based quality assessment, contributing to the pharmaceutical standardization of Ayurvedic formulations.

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