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Identification and quantification of biological active constituents of *Amritarishta*, a herbal formulation

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Herbal formulations have been used by Indian and Chinese traditional systems of medication for a long time. *Amritarishta* is one of the herbal formulations that possess various biological activity viz., antioxidant, anticancer, analgesic, antipyretic, antidiabetic, etc. The active constituents include gallic acid, tannic acid, piperine, and quercetin, etc. Ethanolic extract of the formulation was analysed and quantified. R_f (Retardation factor), functional groups and amount of some of the major chemical constituents were analysed by TLC, FTIR, LC/MS, HPTLC and HPLC, respectively. LC/MS results reveal the presence of quercetin, piperine, tannic acid and gallic acid in the formulation. With the help of HPTLC and HPLC, the quantity of 4 chemical constituents in the formulation was estimated. This type of study is completely new to herbal research.

Keywords: *Amritarishta*, Biological activities, HPTLC, HPLC, LC/MS, Markers

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Ayurveda is a traditional medicine system of India and the name has been derived from two words: Ayur means "life" and Veda means "wisdom" and hence it is the "wisdom of life'. Ayurveda aims to protect from illness and treat the disease[1,2]. Ayurvedic products are manufactured by the different combinations of herbs, minerals, metals, etc. which are nontoxic towards humans as well as animals[3]. Ayurvedic or herbal formulations contain one or more herbs present in definite quantities to produce its effectivness[4,5] and are famous as botanical medicine or phytomedicine[6]. Earlier in twentieth century, the use of herbal medicine increased as it showed a smaller number of side effects. Although fast therapeutic action can be achieved by allopathic medicine and is popular among people but has a major drawback of producing side effects and toxicity. Among the various ayurvedic formulations, Asava (fermented decoction) and Arishta (fermented infusion) are well known for their wide applicability, better quality, good therapeutic properties, and effectiveness of drug delivery in the body[7,8]. In the Indian ayurvedic system, 44 *Arishta* and 45 *Asava* preparations are documented till now concerning their composition and medicinal properties[8-10]. These are biologically active formulation throughout the globe. For example, *Curcuma asava* (the active component of turmeric**)** is used in traditional Chinese Medicine as an antioxidant and anti-inflammatory agent[11-13]. Asava and Arishta of *Withania somnifera* (Ashwagandha) is also widely used to treat inflammation, reduce the blood sugar level, cardiac stimulant, etc[14,15]

Gallic acid possesses anticancer, antifungal, antioxidant, antiviral, antibacterial, antiulcer, and anticholesterol activity[16-19]. Tannic acid is used against CNS disorder, hyperglycemia, obesity disorder (diabetes), hypercholesterolemia or hyperlipidemia[20], Piperine is analgesic, antipyretic and antioxidant and is used for dyspnoea, throat infection, etc[21]. Quercetin is used effectively against diabetes, cardiovascular diseases, asthma, inflammation, viral infections and cancer[22]. Benzoic acid derivatives are used for skin diseases, inflammatory disorders, reducing fevers, wound

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healing, treatment of tumors, affections of eyes, microbial pathologies and respiratory diseases[23]. *Amritarishta* is a polyherbal ayurvedic formulation, used against various diseases such as fever, anemia, typhoid, chronic illness, puerperal, liver disease, and other infectious diseases[24]. The formulation also contains 5% to 10% of alcohol that has health benefits with no side effects[25]. Because of this, we have estimated some of the major chemical constituents present in *Amritarishta* concerning quality and quantity in its marketed formulation.

Its constituents include palmitoside C, palmitoside F, amritoside A, amritoside B, amritoside C, amritoside D[26], gallic acid, ellagic acid[16-19], tannic acid[27], piperine[28], quercetin[29], berberine[30], ascorbic acid [31], caffeic acid[32], benzoic acid, etc. The structure of the chemical constituents is given in (Fig. 1).

Materials and Methods

Derivatizing reagents

Iodine, Anisaldehyde reagent, Vanillin reagent, etc.

Instruments

The chromatographic system, to develop the analytical methods for the current investigation was performed on an Agilent Technologies 1200 series, HPLC system (Agilent Technologies, Waldbronn, Germany). It was equipped with a binary pump system (G1312A), an automatic injector (G1329A) and a photodiode array detector (G1315D). Data acquisition was performed using a chromatography software package (EZChrome Elite™). For sample preparation, ultrasonicator (Cleaner 30A), filter assembly, syringe filters and nylon membrane filters (Axiva, Delhi, India) were employed. The samples were accurately weighed using a weighing balance (Citizon, Ambala, India). FT-IR spectra were recorded on Agilent technologies FT-IR (Agilent Technologies, Danbury, USA) (Cary 630). The melting point (°C) of the drugs, was recorded on the melting point apparatus (Sentwin, Ambala, India). A CAMAG (Muttenz, Switzerland) HPTLC system equipped with a Linomat V sample applicator was used to analyze the samples on aluminum backed TLC plates (20×20 cm) precoated with silica gel $60F_{254}$ (Merck, Darmstadt, Germany). For developing the plates, a CAMAG twin trough development chamber was used.

Methods

Procurement of drug

Amritarishta was procured from the local market Solan (Himachal Pradesh), India. The list of ingredients are give in (Table S1).

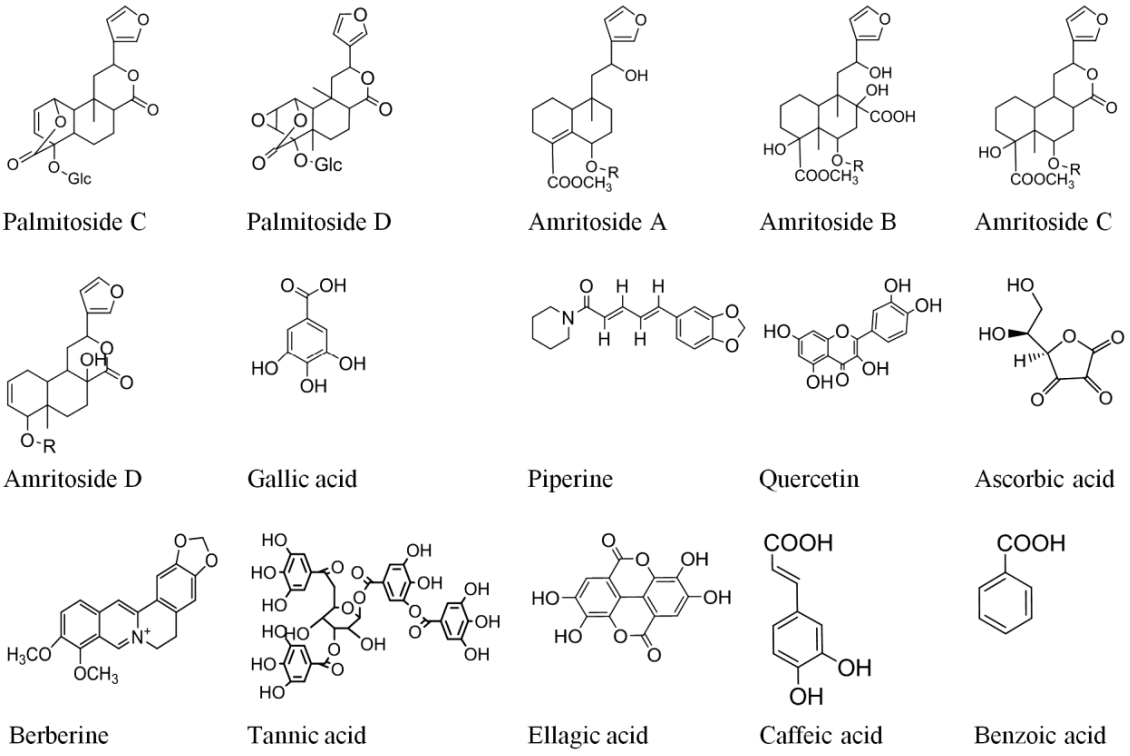

Fig. 1 — Structure of chemical constituent present in *Amritarishta*

Preparation of extract

The formulation was extracted with ethyl acetate and water in a separating funnel, two layers were formed. The organic layer was separated and washed twice with water and dried. The dried material was added to the methanol to form the final extract.

TLC (Thin layer chromatography)

TLC studies of extracts were carried out using Silica gel GF-254 as stationary phase and marker compound of gallic acid, tannic acid, piperine, and quercetin was at the baseline along with the formulation. Mobile phase included toluene: ethyl acetate: formic acid (5:4:1) (standard molecules: gallic acid, piperine, quercetin, tannic acid) and Detecting agents used were U.V, Anisaldehyde-sulphuric acid reagent, iodine, etc.

FTIR (Fourier-transform infrared spectroscopy)

FTIR studies of the extract of gallic acid, quercetin, tannic acid, piperine were carried out using the standard solvents to identify fermented FTIR graphs.

LC/MS (Liquid chromatography-mass spectrometry)

The molecular masses of the gallic acid, piperine, quercetin, tannic acid in the formulation were analyzed using LC/MS.

HPTLC (High-performance thin layer chromatography)

HPTLC fingerprint of ethyl acetate extract was recorded at 254 nm. The extracts were subjected to HPTLC studies to develop fingerprints using the same conditions as used for TLC.

HPLC (High-performance liquid chromatography)

Isolated fractions of gallic acid, tannic acid, quercetin, piperine from *Amritarishta* formulation were analysed by HPLC using the following conditions: Column: C18 (25 cm×4.6 mm, i.d.), Mobile phase: methanol: water (60:40) Detection: at 254 nm.

Result and Discussion

In the present study we have identified the major active constituents like gallic acid, quercetin, tannic acid, piperine both qualitatively and quantitatively in the *Amritarishta* formulation. The qualitative study was done by TLC, LC/MS whereas functional group analysis by FTIR and the quantitative estimation was done using HPTLC and HPLC.

The purpose of the TLC was to identify the main chemical constituents present in the formulation. The standard of gallic acid, tannic acid, piperine, quercetin, and methanolic extract of the formulation was spotted on TLC plates simultaneously. The R_f value of the formulation with the standard markers was matched. The R_f of each compound (Gallic acid, Tannic acid, piperine, quercetin as well as the formulation) were calculated after the detection using different detecting reagents. The observation from TLC is given in (Fig. 2) and (Table 1).

FTIR Study

FTIR was used to analyse the functional group present in the formulation (Fig. S1), individual spectroscopy was taken for markers as well as formulation, and the functional group present in the

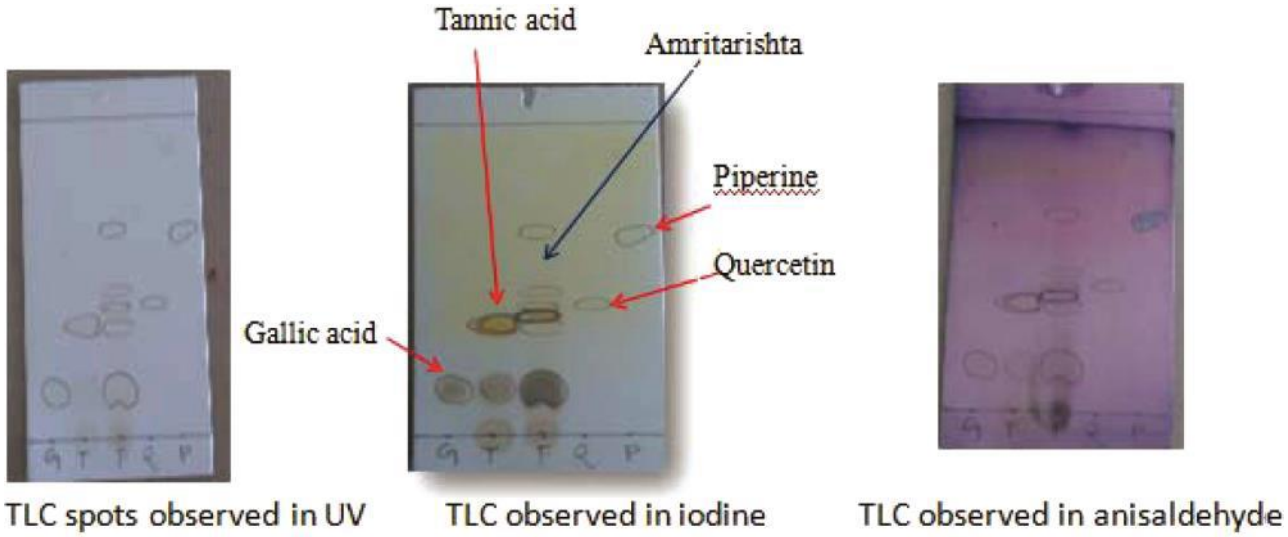

Fig. 2 — Thin layer chromatographic analysis of some major ingredients present in the *Amritarishta* formulation.

formulation and are represented in (Table 2). Analysis of IR frequencies from "Table 2" shows the (C-O) bond was present in all the members as well as in the formulation. (C=C) bond in 1400-1600 was formed in all (medium-weak bands). The FTIR analysis gave a rough idea about the presence of these markers in the formulation. IR of gallic acid, quercetin, tannic acid are given supplementary file (Fig. S2-S4).

LC/MS Study

After the FTIR analysis we have performed LC/MS analysis of the formulation to analyse the presence of major chemical constituents present. The mass chromatogram for *Amritarishta* formulation showed peculiar molecular weight for some of the molecules present. The result signifies the presence of gallic acid (170.13), piperine (285.15), quercetin (302.17) present in the formulation (Fig. S5). Some other peaks (Molecular mass peaks) were also present which was not taken into consideration. Unfortunately, identification of tannic acid was not possible due to its heavy molecular weight (1701.19 g/mol). For the chromatography /mass spectroscopy in (Table 3).

After the qualitative analyses of the *Amritarishta* formulation via., TLC, FTIR and LC/MS. We ascertained the presence of four markers in our formulation. Then we proceed for the quantification and estimation of the (% amount) of the individual major constituent present. For this purpose, we have performed HPTLC (High-performance thin-layer chromatography) and HPLC (High-performance liquid chromatography).

HPTLC

The R_f value of standard gallic acid was found to be 0.46 and peak area 18470.0. Aqueous extract of *Amritarishta* peak having R_f value 0.44 coincides with the standard R_f value and its peak area was found to be 38325.2. The percentage amount of gallic acid in the formulation was found to be 0.18% w/v (Table 4). The R_f value of standard piperine was found to be 0.00 and its peak area was 19837.0. The formulation peak R_f value 0.00 coincides with the standard R_f value and its peak area was 22900.0. The amount of piperine was found to be 0.03% w/v (Table 4). The R_f value of standard quercetin was found to be 0.69 and peak area 40889.7. The aqueous extract of *Amritarishta* peak Rf value 0.69 was coinciding with the standard Rf value and its peak area was 66330.0. the amount of quercetin was found to be 0.053% w/v. The Rf value of standard tannic acid was found to be 0.43 and its peak area was 22054.8. The peak of R_f

Table 1 — Observation of TLC

Standard	UV[a]	Iodine[a]	Anisaldehyde[a]	Characteristics[b]	R_f[c]	Remarks in formulation[d]
Gallic acid	✓	X	✓	Most intensity	0.2	Less amount
Piperine	✓	✓	X	Less intensity	0.64	More amount
Quercetin	✓	✓	X	Medium intensity	0.44	Less amount
Tannic acid	✓	✓	✓	Most intensity	0.4	Less amount

[a]Detecting agent [b] Characterisation of the chemical constituent observed on TLC plate [c]Retardation factor [d]Area calculation

Table 2 — FTIR analysis of the *Amritaristha* formulation

Functional groups	Characteristic absorption (cm^{-1})	Formulation	Gallic acid	Piperin	Quercetin	Tannic acid
C-H (strong)	2850-3200	2936		2948		
O-H (strong)	3200-3600	3443,3350, 3402	3316	3290		3342, 3365
-C=C- (variable, not present in symmetrical alkynes)	2100-2260	2125				
C=O (strong)	1670-1820	1711				1704
N-H (strong)	1550-1640	1611				
C=C (medium – weak, multiple bands)	1400-1600	1518,1454, 1406	1421	1451		1454
C-F (strong)	1000-1400	1127,1231				
C-O (strong)	1000-1300	1048	1026	1022	1022	1041,1212, 1032
C-Cl (strong)	600-800	717 ,747, 780				

Table 3 — LC/MS analysis of the *Amritarishta* formulation

Standard	Actual molecular mass (g/mol)	Formulation compound molecular weight (g/mol)
Gallic acid	170.12	170.13
Piperine	285.343	285.15
Quercetin	302.236	302.17

Table 4 — HPTLC analysis of *Amritarishta* formulation

Sample	Wavelength (nm)	Max. R_f	Area	Vol. of sample (µL)	Concentration in the formulation (%w/v)
Gallic acid	254	0.46	18470.4	5.0	0.18
Formulation	254	0.44	38325.2	15.0	
Piperine	254	0.00	19837.0	4.0	0.03
Formulation	254	0.00	22900.0	4.0	
Quercetin	254	0.69	40889.7	5.0	0.053
Formulation	254	0.69	66330.0	4.0	
Tannic acid	254	0.43	22054.8	1.0	0.047
Formulation	254	0.00	59747.3	15.0	

value 0.00 coincides with the standard R_f value and its peak area was 59747.3 and the amount of tannic acid was found to be 0.047% w/v (Table 4). For the detailed calculation and associated information regarding HPTLC (see the supplementary file Fig. S6)

HPLC Analysis

Along with the HPTLC study, we have done an HPLC analysis of the formulation. Though alone the HPTLC study is not enough to estimate the chemical constituent accurately. The HPLC chromatogram of gallic acid, tannic acid, piperine, quercetin was obtained at a wavelength of 254 nm. The HPLC chromatogram of a methanolic extract of the formulation was compared with the UV spectrum of individuals in the test sample and presented. Gallic acid and tannic acid in the formulation are being reported for the first time and it was found to be 0.16% w/v and gallic acid having a retention time of 4.40 min, was found to be 0.052% w/v. The tannic acid in the methanolic extract having a retention time of 13.00 min (see supporting Fig. S7). The results observed in HPTLC and HPLC were similar but not the same. It may be due to some manual or instrumental errors during the analysis.

Conclusion

The marketed *Amritarishta* formulation was analyzed using TLC, FTIR, LC/MS, HPTLC and HPLC. In the TLC analysis, the R_f values of standards were compared with the R_f values of gallic acid (0.2 cm), piperine (0.64 cm), quercetin (0.44 cm) and tannic acid (0.4 cm) present in the formulation. Which gave us a rough idea of their presence in the formulation. FTIR was done and the functional groups were also matched. LC/MS analysis proved the molecular weight of gallic acid (170.13 g/mol, piperine (285.15 g/mol), quercetin (302.17 g/mol) and approved the presence of these four chemical constituents present in it. HPTLC and HPLC gave us the percentage amount of the chemical constituents present in it. The percentage amount of gallic acid, piperine, quercetin and tannic acid were 0.18%w/v, 0.03% w/v, 0.053% w/v 0.047% w/v respectively. These markers were present in the *Amritarishta* formulation. This research has never been reported earlier. This will provide an analysis technique to evaluate different formulations and validatation.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at http://nopr.niscpr.res.in/jinfo/ijtk/IJTK_21(04)750-755(2022)_SupplData.pdf

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Ethical statement

The article does not contain any studies with animal/human performed by any of the authors.

Conflict of Interest

Authors declare no conflict of interests.

Authors' Contributions

BPD, AKD, DK: Conceptualization, Resources, Visualization; PS, DB: Methodology, Visualization, Writing -original drafts; SL: Writing -review & editing.

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