

Chromatographic evaluation of Safranal and Ellagic Acid in Ayurvedic Tea Formulation and its major Ingredients

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Abstract

India is blessed with an extensive variety of plants, trees, shrubs, and herbs that are utilized in the traditional Indian medical system and have been reported to have excellent therapeutic effects in treating ailments. Historically, medicinal plants have been a primary source of compounds used in medicine to treat illnesses. In the indigenous medical system, *Terminalia arjuna* and *Crocus sativus* are the most beneficial and well-recognized medicinal plants for treating a wide range of serious illnesses. The goal of this study is the development of comparative chromatographic profiles of Ayurvedic tea formulation and its major ingredients for identification, and quantitative estimation of concerned PRS/Markers using HPTLC and HPLC. In this study, we have done HPTLC Fingerprint profiling of Ayurvedic tea formulation, *Terminalia arjuna*, *Crocus sativus*, Ellagic acid and Safranal. Safranal and Ellagic acid reference standards were visible in test solution of *Crocus sativus* extract, *Terminalia arjuna* and Ayurvedic tea formulation respectively and found comparable under UV light and after derivatization with derivatizing reagent. The quantification of *Terminalia arjuna* and *Crocus sativus* in formulation was done through HPLC. The quantitative evaluation of Safranal present in Ayurvedic tea formulation and *Crocus sativus* was 0.0330% and 0.1041% respectively. The quantitative evaluation of Ellagic acid present in *Terminalia arjuna*, stem bark and Ayurvedic tea formulation was 0.2814% and 0.1293% respectively.

1. Introduction

Ayurvedic medicine, or traditional Indian medicine, is gaining popularity because it effectively treats a variety of chronic illnesses. Ayurvedic treatments are typically used in conjunction with or subsequent to conventional medical approaches because most patients start taking conventional medications as soon as they are diagnosed. Herbal plants are an important source of therapeutic compounds used to treat human ailments [1]. For instance, Tea and some of its poly-phenolic and flavanol components have been reported to impart beneficial effects such as anti-carcinogenic, anti-mutagenic, preventing from cardiovascular and neuro-degenerative diseases, positive effects on bone metabolism, anti-microbial anti-oxidant and hypocholesterolemic effect [2]. In this regard, the Central Council for Research in Ayurvedic Sciences (CCRAS), New Delhi, India, developed an Ayurvedic tea. Through this study, we have taken two vital ingredients of formulation as *Terminalia Arjuna* and *Crocus sativus*.

Terminalia arjuna has been used extensively as a natural remedy since ancient times. Ancient Indian physicians used the powdered bark of the arjuna tree to treat cardiovascular conditions like angina and "hritshool." *Terminalia arjuna*, a member of the *Combretaceae* family, is known locally as Arjuna [3]. As per Charaka Samhita; (Charaka Samhita - Sutra Sthana – Shadvirechanashatashritiya Adhyaya). Arjuna is mentioned under *Udardprashamana Mahakashaya*. As per Sushruta Samhita; (Sushruta Samhita – Sutra Sthana – Dravyasangrahaniya adhyaya 48–49) Arjuna is mentioned under *Nyagrodhadigana*. The properties and action of Nyagrodhadigana is that it heals the wound, it helps in healing of fractures, it mitigates the Rakta and Pitta dosha, it cures bleeding disorder, burning sensation, obesity and disorder of vagina. The Guna and Karma of Arjuna as mentioned in *Bhavaprakash Nighantu*. Arjuna is a cardiac tonic, it cures the Kshata and Kshayaroga, it acts against the poison, it cures the bleeding disorder,

obesity, and the wound of diabetic patient. It has Sheetal Guna and mitigates the Kapha and Pitta doshas [4]. Ayurvedic and Tibetan medicine doctors both highly regard Saffron as one of those life-saving medications. It is well known in Tibetan medicine to be a potent treatment for all liver illnesses, particularly those brought on by heat [5]. As per Sushruta Samhita; (Sushruta Samhita – Sutra Sthana – Dravyasangrahanīyādhya 24–25). Kesar is mentioned under *Eladigana*. The properties and action of Eladigana is that it mitigates the Vata, Kapha dosha and poison, it enhances the complexion of skin, it cures the itching, eruptions, and rashes. The Guna and Karma of Kesar as mentioned in *Bhava Prakash Nighantu*. Kesar is Katuka, Snigdha and Tikta in nature. It cures the head disorder; it heals the wound and it is anti – helminthic in nature. It is anti – emetic, it enhances the complexion of skin and cures the freckles on skin. Kesar mitigates the tridoshas [6].

The Arjuna can grow up to 100 feet in length. Its branches spread out horizontally and its trunk is buttressed. It is spread across the Deccan, Madhya Pradesh, South Bihar, Uttar Pradesh, and Delhi Indo-sub-Himalayan regions [7]. Saffron is recognized as originating from south Europe and is grown in France, Italy, Turkey, and Australia and India. In India, it is primarily grown in Pampore (Srinagar, Kashmir) at a height 1700 meters and it is world's largest saffron producer [8]. It needs a rich, porous, sandy, or loamy soil that drains well and is devoid of clay and decomposing humus [9]. Arjuna is a rich source of several phytochemicals, including arjunic acid, arjunolic acid, arjungenin, arjunone, arjunolone, luteolin, gallic acid, ellagic acid and oligomeric proanthocyanidins, which have a variety of medicinal uses i.e. antimicrobial, anti-inflammatory, antioxidant and antifeedant [10]. In both experimental and clinical studies, the bioactive components apocarotenoids, monoterpenoids, flavonoids, phenolic acids, and phytosterols are extensively studied for their potential therapeutic effects, particularly on the nervous system. Certain active ingredients in saffron possess high levels of bioavailability, bio accessibility, and blood-brain barrier-crossing ability [11]. *Terminalia arjuna* is beneficial for heart health and for easing the symptoms of anxiety and discomfort. By boosting antioxidative defence mechanisms, it can shield the tissues of the kidney and liver from oxidative stress brought on by CCl₄. It can be taken every day as a tonic for a healthy cardiovascular system because it is among the best heart tonics [12]. Saffron has been utilised in the production of yellow or saffron dyes for painting, fabric dying, and other creative projects. Due to its moisturising qualities and strong antioxidant content, it is frequently used in cosmetic and fragrance products as well as in the prevention of skin cancer. It serves as a UV absorbent ingredient in a variety of sunscreens and lotions, shielding skin from the sun's damaging rays [13].

Through this study, we have been able to confirm the presence of marker compounds Safranal for *Crocussativus* and Ellagic acid for *Terminalia arjuna* by high-performance thin layer chromatography (HPTLC) based analytical method. These findings suggest the presence of Ellagic acid and Safranal in the respective samples, as evident by characteristic bands and matching retention factors. The quantitative evaluation of Ellagic acid present in *Terminalia arjuna* Stem Bark and Safranal for *Crocus sativus* using (HPLC) along with Ayurvedic tea formulation has been done.

2. Materials and Methods

2.1 Chemical constituents

The main chemical constituents of *Terminalia arjuna* include arjunic acid, arjunolic acid, arjungenin, arjunone, arjunolone and luteolin, gallic acid, ellagic acid, oligomeric proanthocyanidins and phytosterols [14, 15]. Saffron includes the glycosides crocin, picrocrocin and other compounds such as lycopene, beta-carotene and together with a crystalline hydrocarbon and zeaxanthin. Ellagic acid is the main bioactive component of *Terminalia arjuna* and safranal is the primary component of *Crocus sativus* which are reported for various biological activities [15].

2.2 Extraction of *Crocus sativus* and *Terminalia Arjuna*

Terminalia arjuna (10.2659 g), Ayurvedic tea formulation (10.3038 g) and *Crocus sativus* (10.2952 g) powders were subjected to 24-hour Soxhlet extraction. 200 ml of methanol was taken as extraction solvent. The resulting extracts underwent evaporation under reduced pressure and the residues having weight 1.1965 g, 1.2939 g and 1.1262 g were obtained respectively [16–26].

2.3 Preparation of standard solution of Safranal and Ellagic acid

A precisely measured quantity of 1.6 mg of safranal was dissolved in HPLC grade methanol, and the volume was adjusted to 10 ml, resulting in a safranal stock solution with a concentration of 0.16 mg/ml. Simultaneously, 1 mg of Ellagic acid reference standard was meticulously weighed, dissolved in HPLC grade methanol, and the volume was adjusted to 10 ml, yielding a stock solution with a concentration of 0.1 mg/ml for Ellagic acid [16–26].

2.4 Preparation of test solution

50 mg residues of *Crocus sativus*, *Terminalia arjuna* and Ayurvedic tea formulation extracts were dissolved in 10 ml methanol, resulting in concentrations of 5 mg/ml. The solutions were filtered through 0.22 µm membrane filters for HPTLC fingerprint profiling and identification, employing Safranal for *Crocus sativus* and Ellagic acid for *Terminalia arjuna* as phytochemical reference standards [16–26].

2.5 Instrumentation

2.5.1 High-Performance Thin Layer Chromatography (HPTLC) Analysis

HPTLC analysis was performed on a CAMAG HPTLC system (Muttensz, Switzerland) equipped with an automatic TLC sampler Linomat 5, twin trough development chamber, TLC Scanner 3 linked with WINCATS software version 1.4.4 [16–26].

2.5.2 High-Performance Liquid Chromatography (HPLC) Analysis

Ellagic Acid in Ayurvedic tea formulation and *Terminalia arjuna* extract, as well as Safranal in Ayurvedic tea and *Crocus sativus* extract, was performed using High-Performance Liquid Chromatography (HPLC). The quantitative estimation of Safranal, a standard stock solution was prepared by dissolving 2.0 mg of the reference standard in HPLC-grade methanol, resulting in a 0.40 mg/ml stock solution. The test solution involved weighing residues from methanol extracts of Ayurvedic tea formulation and *Crocus sativus* extract, dissolving them in HPLC-grade methanol using volumetric standard flasks, and filtering through 0.22µm (Agilent) membrane filters for HPLC analysis. Chromatographic conditions for Safranal analysis included an Agilent 1260 infinity II instrument with quaternary pump, auto sampler injector, C18 Eclipse, XBD, 4.6mm x 250 mm column, detection at 308 nm by using Diode Array Detector (DAD), a temperature of 27°C ± 0.8° C, a flow rate of 1.2 ml/min, a run time of 5 min., an injection volume of 1µl, and a mobile phase of Acetonitrile: Methanol (80:20) was used for development.

Ellagic Acid, a standard stock solution was prepared by dissolving 1.6 mg of the reference standard in HPLC-grade methanol, resulting in a 0.32 mg/ml stock solution. The test solution involved weighing residues from methanol extracts of Ayurvedic tea formulation and *Terminalia arjuna* extract, dissolving them in HPLC-grade methanol, and filtering through 0.22µm membrane filters for HPLC analysis. Chromatographic conditions for Ellagic Acid analysis included an Agilent 1200 series instrument with a C18 Eclipse, XBD, 4.6mm x150 mm column, detection at 254 nm, a temperature of 25°C, a flow rate of 1 ml/min, a run time of 10 min., an injection volume of 10µl, and a mobile phase of KH₂PO₄ Buffer: Acetonitrile (70:30) was used for development [22–31].

3. Results

3.1 High-Performance Thin Layer Chromatography (HPTLC)

A distinct band at 254 nm (green, R_f 0.44), indicative of Ellagic acid, is observed in both the reference standard and test solutions of *Terminalia arjuna* extract and Ayurvedic tea formulation extract. Additionally, for Safranal, bands at 254 nm (green, R_f 0.88) and 366 nm (brown, R_f 0.88) are visible in both standard and test solution tracks of *Crocus sativus* extract and Ayurvedic tea formulation extract. These findings suggest the presence of Ellagic acid and Safranal in the respective samples, as evidenced by characteristic bands and matching retention factors.

3.2 HPTLC fingerprint Profile

Utilizing a CAMAG Linomat5 sample applicator, 2µl, 4µl, and 6µl of Safranal standard, *Crocus sativus* extract, and Ayurvedic tea Formulation were separately applied onto a silica gel 60 F254 TLC plate (0.2 mm thickness, E. Merck). The plate underwent development in a Toluene: Ethyl acetate: Formic acid (5.5:4.5:0.2; v/v/v) solvent system. Simultaneously, 5µl of Ellagic acid standard solution, *Terminalia arjuna* extract, and Ayurvedic Tea were applied on a similar plate and developed in a *Toluene: Ethyl acetate: Methanol: Formic acid* (4.5:4.5:0.3:0.3; v/v/v/v) solvent system. Both plates were developed until the solvents reached a distance of 8cm.

Both plates were observed through TLC Visualizer under UV at 254 nm and 366 nm and photos were documented. Finally, the plate was dipped in Anisaldehyde sulphuric acid reagent and heated in hot air oven at 105°C until the colour of the spots were appeared and photo was documented under white light and calculated the R_f values given in Figure 1 and Table 1.

3.3 Quantification by HPLC

3.3.1 Calibration curve

Safranal stock solution was diluted to five different concentrations (0.20, 0.10, 0.05, 0.025, 0.0125 mg/ml), and each standard solution was subjected to HPLC analysis, recording the corresponding peak areas. A Standard Calibration curve was established by plotting peak area against Safranal concentration. Similarly, Ellagic Acid stock solution was diluted to five concentrations (0.16, 0.08, 0.04, 0.02, 0.01 mg/ml), stored below 4°C, and each standard solution was analyzed through HPLC, recording peak areas. A Standard Calibration curve was plotted correlating concentration with peak area, demonstrating good linearity for the injected quantities. Both the curves are depicted in Figure 2.

3.3.2 Quantitative analysis

HPLC analysis was used to quantify Safranal in Ayurvedic tea Formulation and *Crocussativus* extract, revealing distinctive peaks that aligned with the reference Safranal 10µl test solution injections were done twice, chromatograms were captured (Figure 2), and the calibration was used to identify the peak region that corresponded to Safranal.

Similar to this, Ellagic Acid was quantitatively analysed using HPLC in Ayurvedic tea Formulation and *Terminalia Arjuna* extracts; the results showed distinctive peaks at the same retention duration as the standard Ellagic Acid. The HPLC system was injected with duplicate 10µl test solutions, and the chromatograms were recorded (Figure 2). Ellagic Acid concentration in the residues of Ayurvedic tea formulation and *Terminalia arjuna* extracts were calculated using the calibration curve's peak region matching to Ellagic Acid.

4. Conclusion

The R_f value of Safranal reference standard 0.88 (Green and Brown) was visible in test solution of *Crocus sativus* extract and Ayurvedic tea formulation and found comparable under UV light at 254 nm and after derivatization respectively. The HPLC chromatogram of *Crocus sativus* extract and Ayurvedic tea corresponding to standard Safranal was shown at a retention time of 4.293 min, at 308 nm wavelength. The quantitative evaluation of Safranal present in Ayurvedic tea formulation and *Crocus sativus* was 0.0330% and 0.1041% respectively.

The R_f value of Ellagic acid reference standard 0.44 (Green) was visible in test solution of *Terminalia arjuna* and Ayurvedic tea formulation and found comparable under UV light at 254 nm. The HPLC

chromatogram of *Terminalia arjuna* and Ayurvedic tea formulation corresponding to standard Ellagic acid was showed at a retention time of 2.245 min, at 254 nm wavelength. The quantitative evaluation of Ellagic acid present in *Terminalia arjuna* stem bark and Ayurvedic tea formulation was 0.2814% and 0.1293% respectively.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Authors give consent for the publication of identifiable details in this manuscript to be published at your Journal.

Availability of data and material: The data that support the findings of this study are available on request from the corresponding author.

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Tables

Table 1. R_f values of Ayurvedic tea formulation, *Crocus sativus*, *Terminalia arjuna*, Safranal and Ellagic Acid.

Wavelength	Track 1: Safranal		Track 2: <i>Crocus sativus</i>		Track 3: Ayurvedic tea formulation	
	R _f	Color	R _f	Color	R _f	Color
At UV254 nm	-	-	0.14	Green	0.13	Green
	-	-	0.19	Green	0.20	Green
	-	-	0.30	Green	0.30	Green
	-	-	0.33	Green	0.32	Green
	-	-	0.47	Green	0.46	Green
	-	-	0.51	Green	0.51	Green
	-	-	0.58	Green	0.58	Green
	-	-	0.66	Green	0.65	Green
	-	-	0.76	Green	0.75	Green
	-	-	0.82	Green	0.82	Green
	0.88	Green	0.88	Green	0.88	Green
	-	-	0.94	Green	0.93	Green
At UV366 nm	-	-	0.09	Blue	-	-
	-	-	0.19	Red	0.17	Red
	-	-	0.33	Red	0.41	Red
	-	-	-	-	0.46	Blue
	-	-	-	-	0.52	Red
	-	-	0.55	Blue	0.56	Blue
	-	-	0.60	Blue	0.60	Blue
	-	-	0.76	Red	0.76	Red
	-	-	82	Blue	0.82	Blue
	-	-	0.88	Red	0.88	Red
	-	-	-	-	0.94	Dark Red
	-	-	0.11	Red	0.1	Brown

After Derivatization	-	-	0.15	Red	0.13	Brown
	-	-	0.28	Yellow	0.28	Brown
	-	-	-	-	0.41	Brown
	-	-	0.52	Brown	0.53	Green
	-	-	0.69	Brown	0.67	Brown
	-	-	-	-	0.71	Brown
	-	-	0.84	Brown	0.82	Brown
	0.88	Brown	0.88	Brown	0.88	Brown
	-	-	-	-	0.96	Yellow
Wavelength	Track1: Ellagic Acid		Track2: <i>Terminalia arjuna</i>		Track3: Ayurvedic tea formulation	
At UV 254 nm	R _f	Color	R _f	Color	R _f	Color
	-	-	0.15	Green	0.12	Green
	-	-	-	-	0.20	Green
	-	-	0.29	Green	0.31	Green
	0.44	Green	0.44	Green	0.44	Green
	-	-	0.57	Green	0.58	Green
	-	-	-	-	0.67	Green
	-	-	0.75	Green	0.76	Green
	-	-	-	-	0.84	Green
	-	-	-	-	0.92	Green
	-	-	-	-	0.97	Green
At UV 366 nm	-	-	0.09	Blue	-	-
	-	-	0.15	Blue	-	-
	-	-	0.24	Blue	0.24	Blue
	-	-	-	-	0.31	Red
	-	-	0.44	Blue	0.44	Red
	-	-	-	-	0.50	Blue
	-	-	-	-	0.54	Blue

After Derivatization	-	-	-	-	0.60	Red
	-	-	-	-	0.67	Pink
	-	-	-	-	0.89	Blue
	-	-	-	-	0.97	Pink
	-	-	0.13	Brown	0.13	Brown
	-	-	0.19	Grey	0.18	Grey
	-	-	0.23	Blue	0.23	Brown
	-	-	0.35	Blue	0.34	Brown
	-	-	0.44	Blue	0.44	Brown
	-	-	0.59	Blue	0.60	Grey
	-	-	-	-	0.65	Grey
	-	-	0.84	Brown	0.84	Grey
	-	-	-	-	0.89	Grey
	-	-	0.94	Brown	0.94	Brown

Table 2. Estimation of Ellagic Acid and Safranal bioactive compound in Ayurvedic Tea formulation and its ingredients *Terminalia arjuna* and *Crocus sativus*

S. No.	Sample Name	Weight of Extract used for HPLC (mg/10ml)	Ellagic Acid present (%)	Safranal present (%)
1.	Ayurvedic tea Formulation	10	0.1293	0.0330
2.	<i>Terminalia arjuna</i>	10	0.2814	NA
3.	<i>Crocus sativus</i>	10	NA	0.1041

Figures

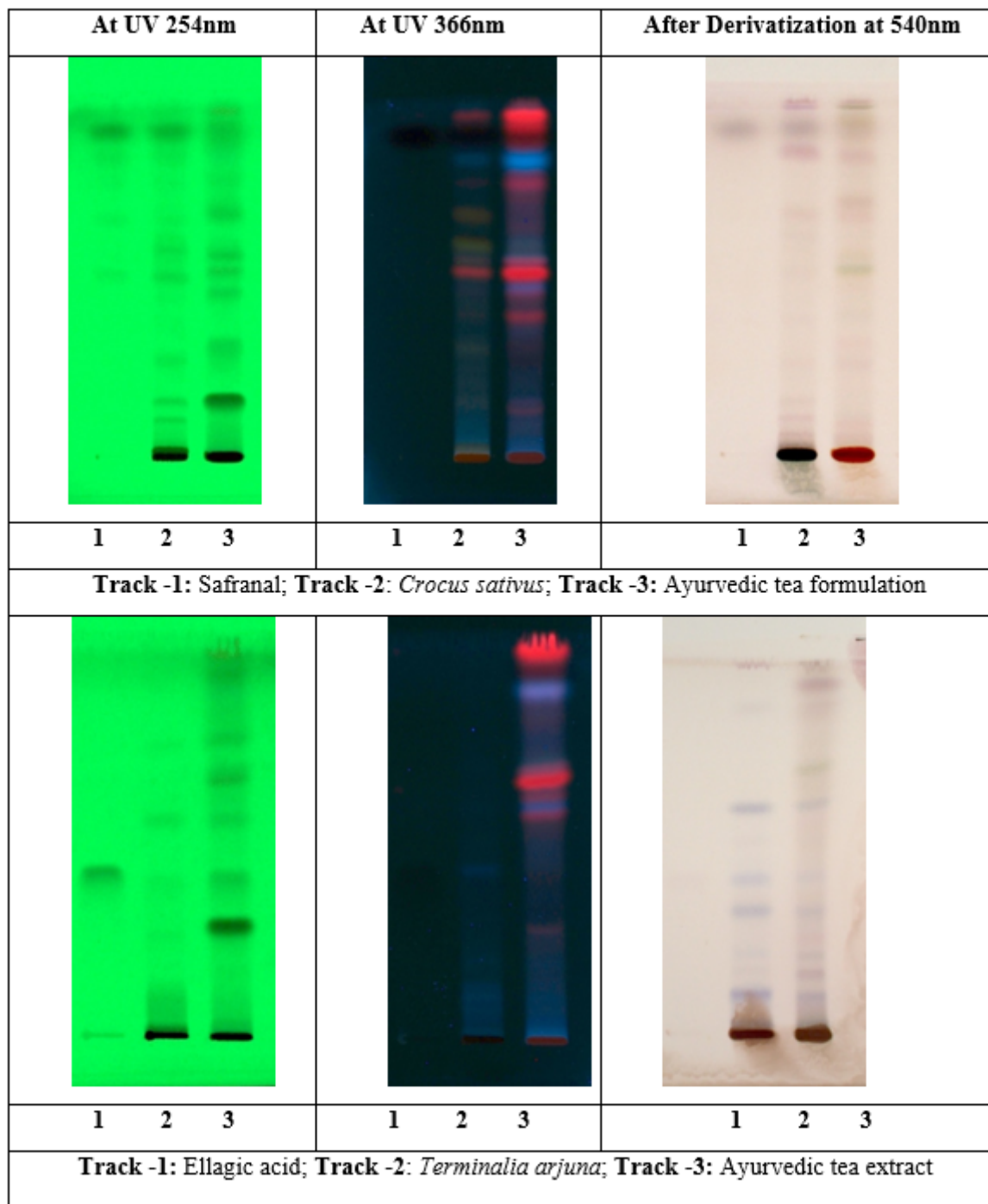


Figure 1

HPTLC fingerprint profile of Ayurvedic tea formulation, *Crocus sativus*, *Terminalia arjuna*, Safranal and Ellagic acid.

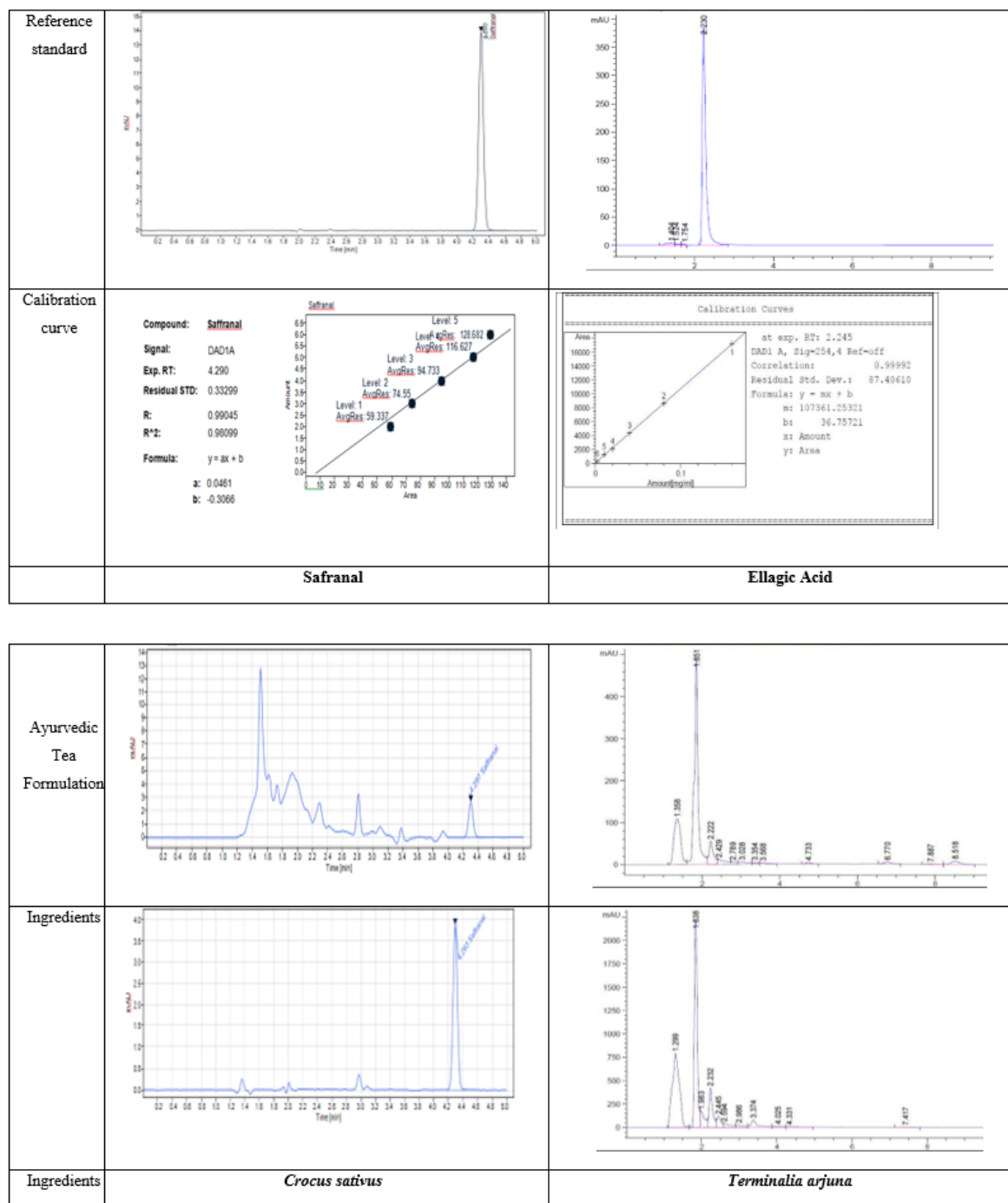


Figure 2

HPLC Calibration curve and Chromatogram of Safranal, Ellagic Acid bioactive compounds in Ayurvedic Tea formulation and its ingredients *Crocus sativus*, *Terminalia arjuna*