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EVALUATION OF ANTIOXIDANT ACTIVITY AND QUANTIFICATION OF α--ASARONE IN RHIZOMES OF *ACORUS CALAMUS* L. USING HPTLC FINGERPRINTING

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Keywords:

Acorus calamus L. Alpha-asarone, Antioxidant, Hydro-alcoholic extract, HPTLC, Maceration

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ABSTRACT: This study aimed to analyze antioxidant activity and quantify alpha-asarone by using High-performance thin-layer chromatography (HPTLC). Alpha-asarone is a secondary metabolite in hydroalcoholic (1:1) rhizome extracts of *Acorus calamus* L. (AC), a medicinal plant being used around the globe traditionally. Sweet flag, AC is a member of the Acoraceae family, and is commonly used unaided as well as in conjunction with other plants in Chinese traditional medicine and the Indian system of medicine. It has sparked a lot of curiosity and has proven to be useful in managing many diseases. *Acorus calamus* and/or its bioactive phytochemical α-asarone, is a well-known medication in a traditional system with various properties. Antioxidants are extremely essential compounds that can defend the body from harm caused by oxidative stress generated by free radicals. HPTLC is a viable option for expanding chromatographic fingerprints in order to identify medicinal plant's key active components. At 100µg/mL, the antioxidant activity of AC demonstrated 52.59 percent and increasing to 56 percent at 500µg/mL. HPTLC fingerprinting profiles revealed the presence of α-asarone is one of phytoconstituent present in hydroalcoholic extract of rhizome extracts of AC. The retardation factor (R_F) was observed at 0.38, and the quantification is done at a wavelength of 260 nm. The amount of α-asarone was found to be 263.6 µg in 1.0 mg of plant extract.

INTRODUCTION: *Acorus calamus* L., (AC), often recognized as "sweet flag" or "calamus," is a flowering plant indigenous to Japan, China, India, Sri Lanka, Southern Russia, Europe, Burma, Mongolia and the United States [1, 2]. It belongs to Acoraceae family, commonly grown in temperate and sub-temperate locations across the globe [3].

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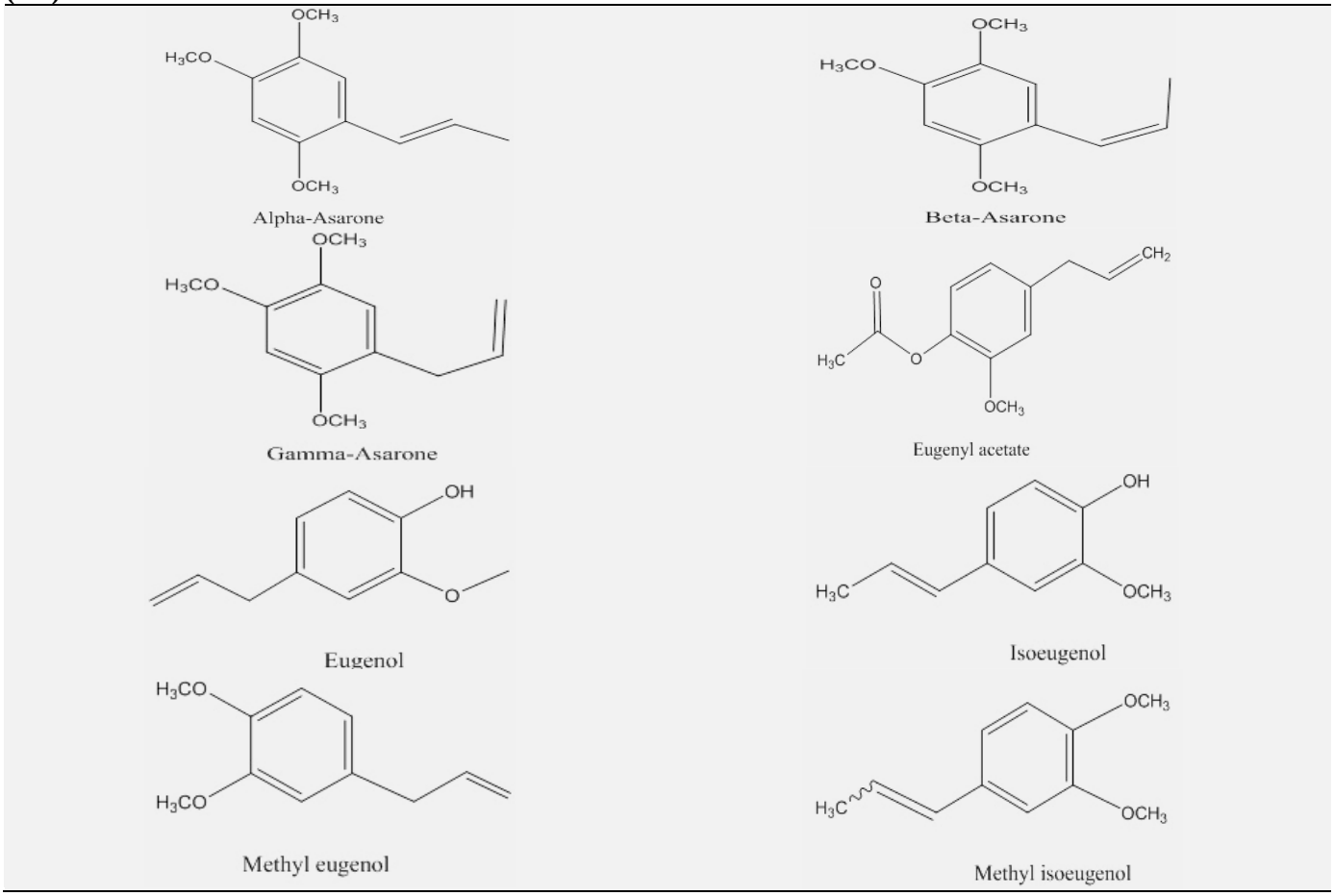
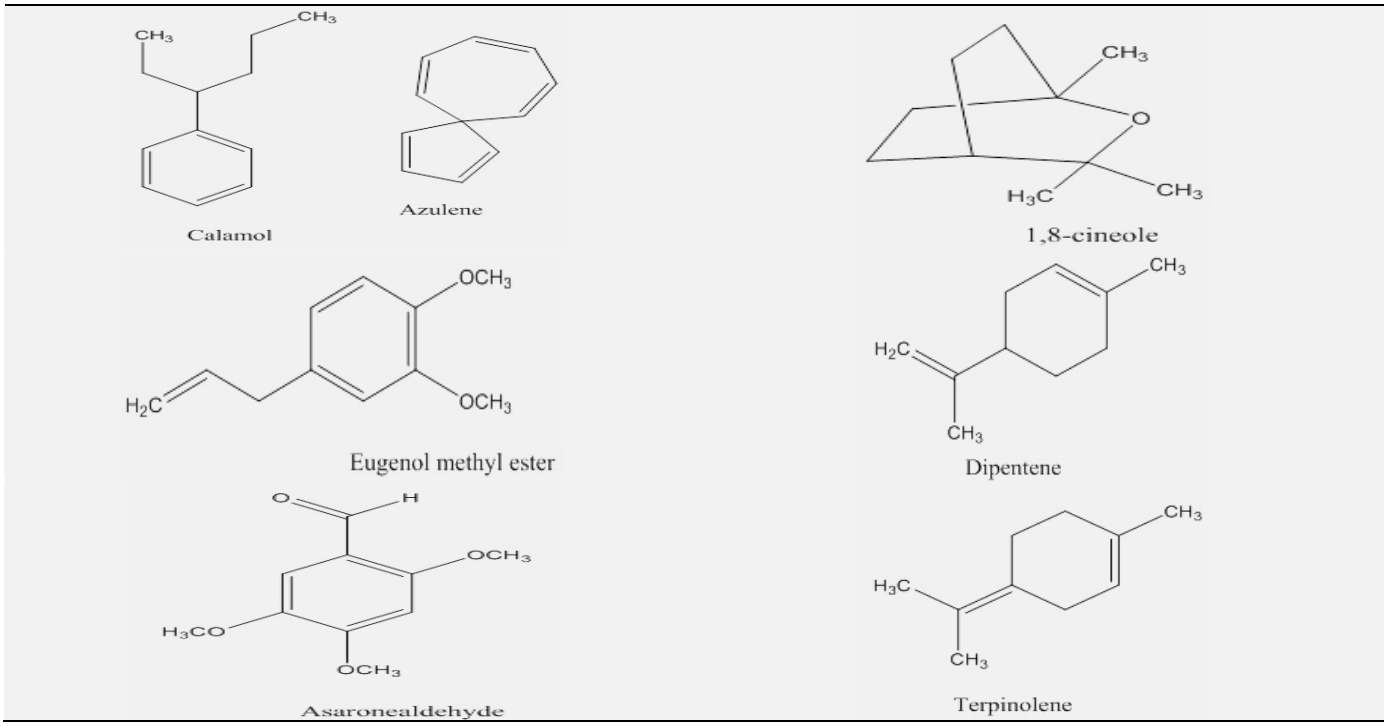
It has been used for medicinal, cultural, and ceremonial purposes for a long time, and its rhizomes are valued in Indian systems of medicine (ISM), Ayurveda and as well as in Traditional Chinese Medicine (TCM) for their possible effects on memory, anti-aging, lipid peroxide content and anti-cholinergic actions [4].

AC is used in phytotherapy and the alimentary sector for creating foodstuffs and drinks due to its medicinal and fragrant properties [5]. In several herbal traditions, AC has a long history of medical usage. It had been utilized as traditional medicine by many Native American cultures for millennia.

According to Indian Ayurveda, it is a powerful rejuvenator for the brain and nerve system and a treatment for digestive problems, toothaches, and headaches [6]. The primary bioactive component of AC is alpha-asarone (1, 2, 4-Trimethoxy-5-[(E)-prop-1-enyl] benzene), which has a wide spectrum of pharmacological activities such as sedative action, anti-ulcerogenic, anti-atherogenic, neuroleptic, spasmolytic and anti-helminthic. AC is an herbaceous perennial plant that grows in a semi-aquatic and terrestrial environment with creeping rhizomes. Its rhizomes have a bitter flavour, are densely branching, have a pinkish or light green appearance, and have a citrus odour [7]. The dried rhizome of AC is the most often used component, which has anti-inflammatory, anti-ulcer, anti-oxidant, anti-diabetic, anti-microbial and neuron protective qualities. In normal rats, the herb has been shown to reduce stress-induced immuno-logical changes and increase immunity [8]. In the etiology of numerous chronic illnesses, oxidative stress is a considerable risk factor. Oxidative stress and other ROS (reactive oxygen species) are known to be in the pathophysiology of illnesses such as arthritides, diabetes, asthma, Alzheimer's disease, cancer and atherosclerosis. Aging is also considered to be caused by ROS [9, 10]. Chemicals that delay or prevent oxidative stress to a target analyte can be classified as antioxidant [11]. The capacity of antioxidants to capture free ions is its most important characteristic. Anti-oxidant substances, including polyphenols, phenolic acids, and flavonoids, scavenge free radicals, peroxides, and hydrogenperoxides, and inhibit the oxidative pathways that cause neurodegenerative disorders [12]. Since the past times, herbal plants have been thought to be good quality antioxidants. AC had been the subject of a phytochemical inquiry since ancient times [13, 14]. Phenylpropanoids, sesquiter-penoids, monoterpenes, sterols, saponins, triterpene glycosides, triterpenoid and alkaloids have all been identified from AC rhizomes and leave so far. Phenylpropanoids (most notably α, β, γ-asarone, and eugenol) and sesquiterpenoids identified as the most potent chemicals in AC are shown in **Table 1**.

TABLE 1: SHOWS THE DIFFERENT STRUCTURES OF BIOACTIVE COMPOUNDS OF *ACRORUS CALAMUS* (AC)





Due to its reliability, simplicity and quick quantitative quantification of plant biomarkers in herbs, High-performance thin-layer chromatography (HPTLC) is still a powerful analytical approach [15]. The use of HPTLC is to expand chromatographic analysis to perceive the main active components in medicinal plants. The separation and resolution are far superior to thin layer chromatography (TLC) and the results are far more consistent and repeatable. It has the major benefit of *in-situ* qualitative and quantitative assessment by means of scanning densitometry as collective with digital scanning profiling. Additionally, the colorful graphic HPTLC picture gives additional, straightforward visible colors and/or fluorescence characteristics for parallel evaluation on the same plates. It also indicated that individual secondary metabolites may be differentiated better. Establishing a marker profile and the standardization of parameters are critical for preserving the eminence of botanicals as needed by herbal medication producers [16]. There are relatively few proven methods for standardization of α-asarone (in AC) through HPTLC. The purpose of this effort is to develop and verify a method for estimating α-asarone component in AC rhizome using a precise, quick and accurate HPTLC fingerprinting.

Experimentation:

Chemicals and Materials: Standard α-asarone was purchased from TCI Chemicals (India) Pvt. Ltd. Chennai. Ethanol, Ethyl acetate, Toluene, Silica gel, Whatman filter paper, DPPH (2-diphenyl-1-picrylhydrazyl), Ascorbic acid.

Equipment used for Extraction: Maceration Tank, Rotary Evaporator, Lyophilizer, HPTLC, UV Spectrophotometer.

Plant material and Extraction: Dried rhizomes of AC were obtained from a traditional medicine store of India. The dried rhizomes were cut into small pieces, crushed, and placed in a maceration tank of 5 L ethanol: water (1:1) for 3 days.

Collected the menstruum having the active components by using a separating funnel. The rotary evaporator was used to concentrate the filtrated extract below 45°C under reduced pressure. The yield was 14.4 percent w/w after lyophilizing the semi-solid residue to fine powder **Table 2.**

TABLE 2: PERCENTAGE YIELD OF *ACORUS CALAMUS* (AC)

Sample type	Solvent	Sample weight	Solvent used	% yield (w/w)
Dried rhizomes	Hydro-alcoholic (1:1)	1kg	5L	14.4

Quantification Analysis: HPTLC was done by Anchrom Enterprises (I) Pvt. Ltd. Mumbai, India.

Sample Preparation: As per United State Pharmacopoeia Ch. 203- guidelines, Weighed 10 mg of each sample and added 1ml of ethanol in it (10mg/mL). Sonicated the resultant solution for 10 minutes. After that the solution is centrifuged and supernatant collected.

Stationary Phase: TLC silica gel 60 F254 by Merck (Cat. No. 1.05554.0007)

Mobile Phase: Toluene: Ethyl acetate (93:7 v/v)

Developing Distance: 70 mm from the lower edge of the plate.

Sample Application: With the use of Server LABSERVER, version 3.1.21109.3 software, the analysis is done. Seven distinct spots of the standard α-asarone (1, 1, 2, 3, 4, 5, 6 µL) and three replica of the extract (2, 3, 4 µL) were put on HPTLC plates.

Antioxidant Activity: The DPPH technique was used to assess antioxidant activity[17]. The sample was combined with 4.5 mL of 0.1 mM DPPH solution and the different concentrations of extract of 0.5 mL, vortexed for 1 minute, and then incubated in the dark for 30 minutes. At a wavelength of 517nm, the mixture's absorbance was measured. As a control, ethanol was employed. The ability to scavenge free radicals is measured in percent RSA (Radical Scavenging activity).

RESULTS AND DISCUSSION: Fig. 1A showed a development chamber of the plant extract with R_F value 0.38 at 254nm, whereas **Fig. 1B** showed the confirmation of α-asarone in the extract and standard at 366nm. Further, 13 tracks are formed in which the seven tracks are of standard α-asarone (1mg/ml in methanol) of volume (1, 1, 2, 3, 4, 5, 6 µL) and three replicas of the extract (1mg/ml in methanol) of volume (2, 3, 4 µL) are studied under wavelength 254 and 260nm respectively. The wavelength peak at 254nm of the standard are sequenced from **Fig. 2A, 2B, 2C, 2D, 2E, 2F, 2G** and the peak of the extract is started from **Fig. 3A**, **3B, 3C, 3D, 3E** and **3F** respectively. The wavelength peak at 260nm of all the track are started from **Fig. 4A, 4B, 4C, 4D**, **4E, 4F, 4G, 4H, 4I, 4J, 4K, 4L** and **4M,** respectively. The HPTLC Chromatogram of standard α-asarone and the plant extract are shown in **Fig. 5A, 5B, 5C, 5D, 5E, 5F, 5G, 5H** with the R_F at wavelength 260nm. With a correlation value (r) of 0.998563 and calibration curve **Fig. 6** was linear in range of 200–600 ng per spot, indicating satisfactory linear dependency of the peak area on concentration. The calibration's range deviation was found to be 5 percent and coefficient of variation (CV) was 1.59 percent demonstrating the calibration's linearity. The α-asarone in the plant extract was 263.6µg in 1.0mg. The antioxidant activity of A. calamus hydroalcoholic extracts was examined in the search for novel bioactive chemicals from natural resources. Compared to the reference antioxidant, AC showed significant results as shown in **Table 3**. The table below shows AC DPPH scavenging activity. Antioxidants' capacity to scavenge DPPH radicals was assumed to be owing to their hydrogen-donating activity. The radical is scavenged by hydrogen donation as the absorbance of the DPPH radical decreases as the interaction between the antioxidant molecule and the radical develops. A sample of commercial ascorbic acid was used as a control. AC has been proven to have substantial antioxidant activity that is dosage dependent **Fig. 7.**

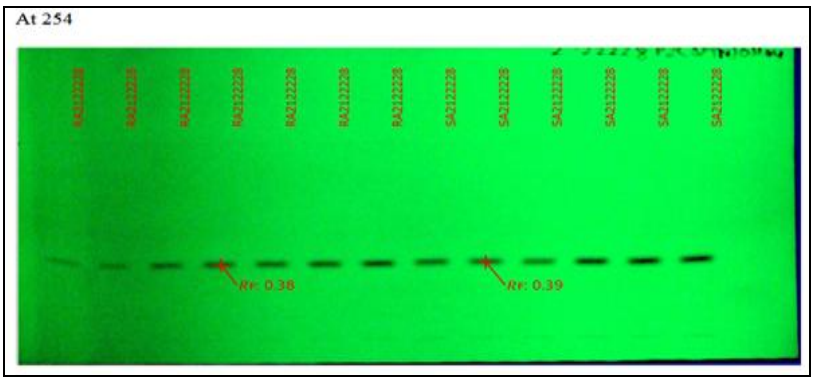
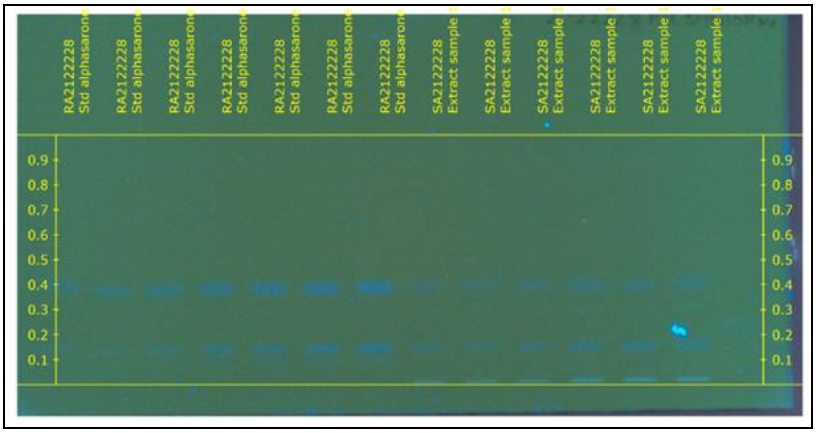


FIG. 1A: SHOWING THE R_F VALUES OF ALPHA ASARONE PRESENT IN THE PLANT EXTRACT AT 254NM



CONFIRMATION OF α-ASARONE IN PLANT EXTRACT AND STANDARD AT 366 NM

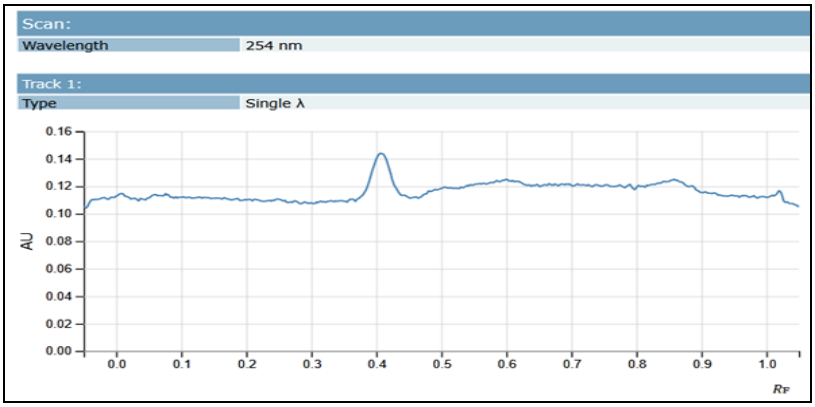


FIG. 2A: R_F VALUE OF STANDARD DRUG (1µL) OF TRACK 1 AT 254 NM

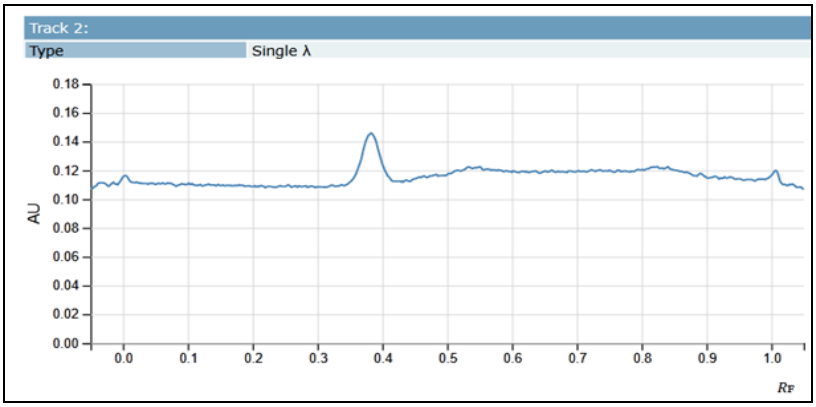


FIG. 2B: R_F VALUE OF STANDARD DRUG (1µL) OF TRACK 1 AT 254 NM

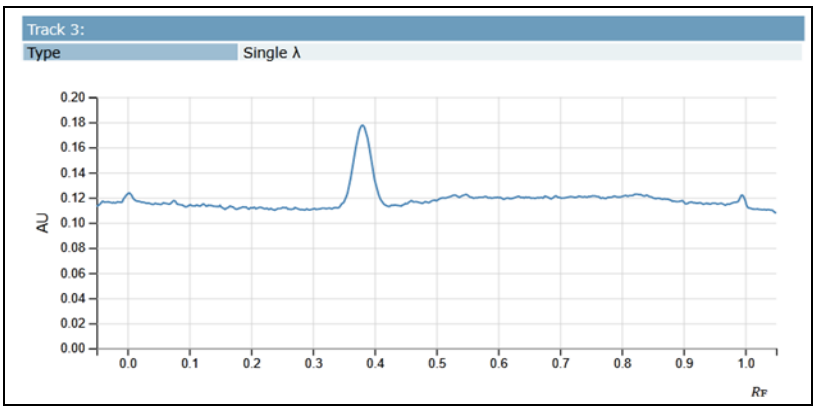


FIG. 2C: R_F VALUE OF STANDARD DRUG (2µL) OF TRACK 3 AT 254 NM

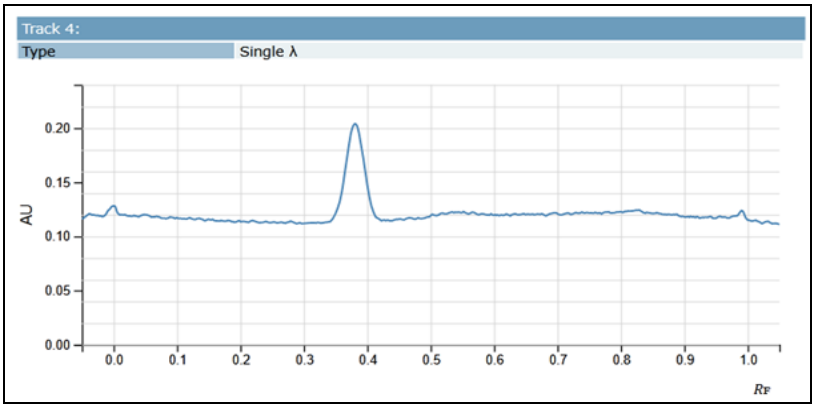


FIG. 2D: R_F VALUE OF STANDARD DRUG (3µL) OF TRACK 4 AT 254 NM

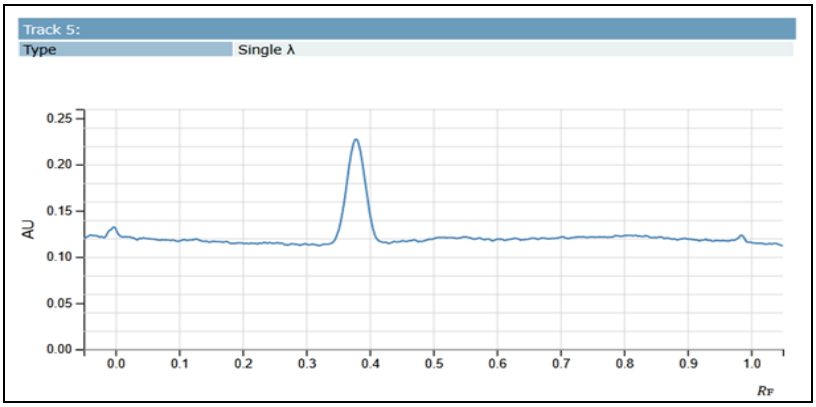


FIG. 2E: R_F VALUE OF STANDARD DRUG (4µL) OF TRACK 5 AT 254 NM

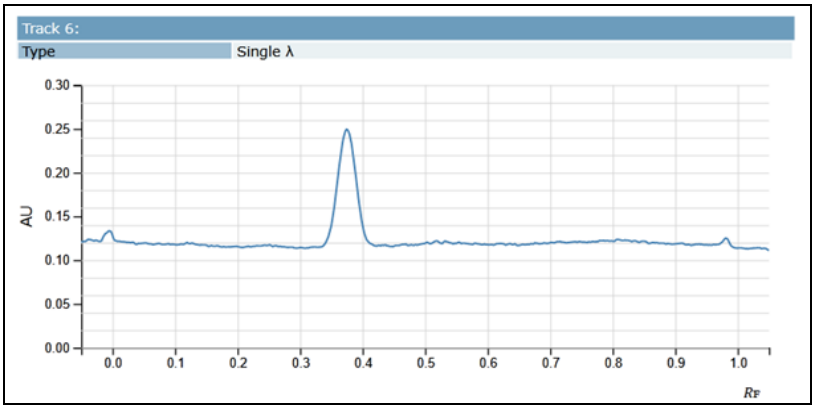


FIG. 2F: R_F VALUE OF STANDARD DRUG (5µL) OF TRACK 6 AT 254 NM

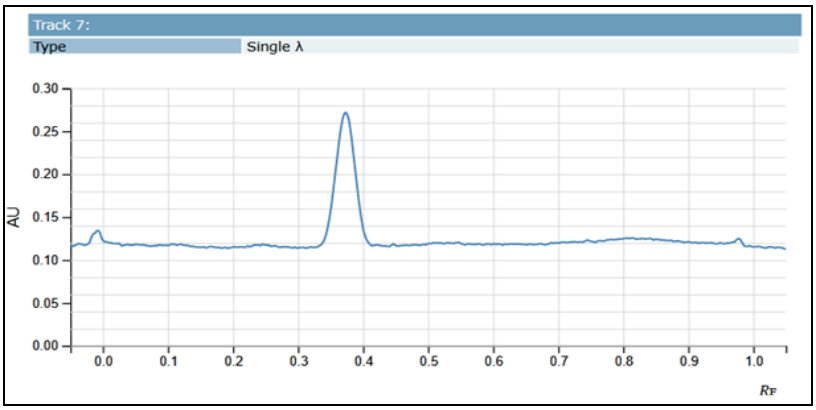


FIG. 2G: R_F VALUE OF STANDARD DRUG (6µL) OF TRACK 7 AT 254 NM

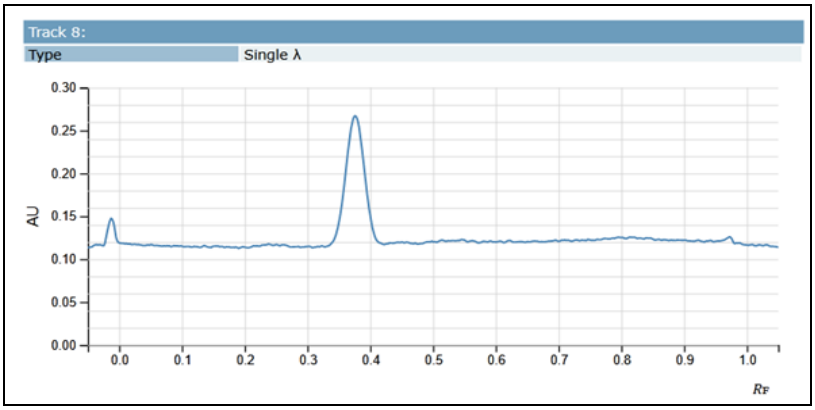


FIG. 3A: R_F VALUE OF PLANT EXTRACT (2µL) OF TRACK 8 AT 254 NM

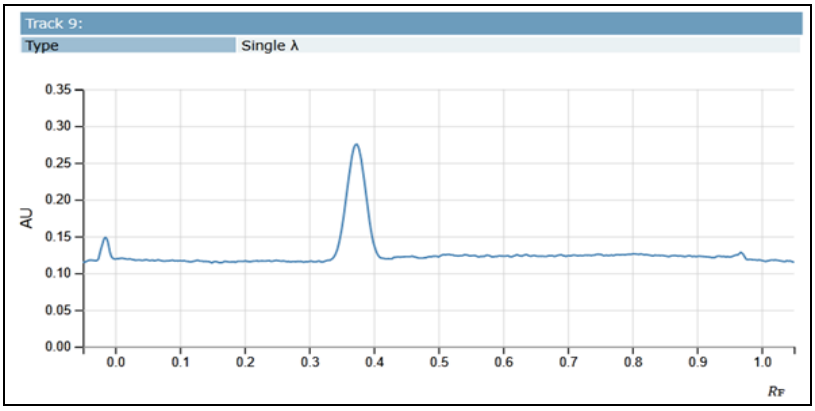


FIG. 3B: R_F VALUE OF PLANT EXTRACT (2µL) OF TRACK 9 AT 254 NM

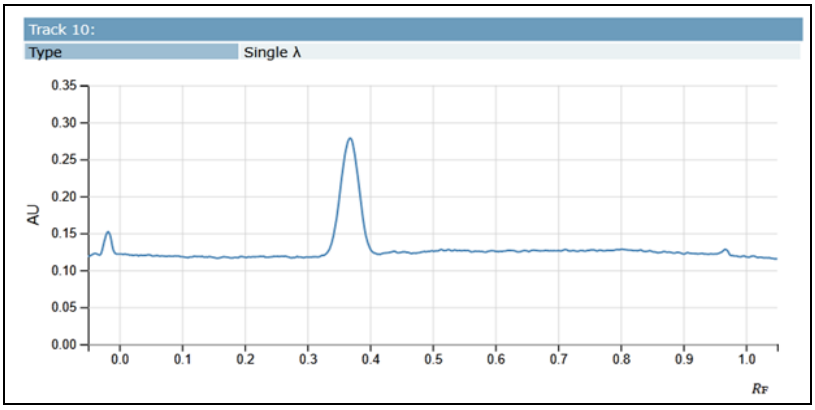


FIG. 3C: R_F VALUE OF PLANT EXTRACT (3µL) OF TRACK 10 AT 254 NM

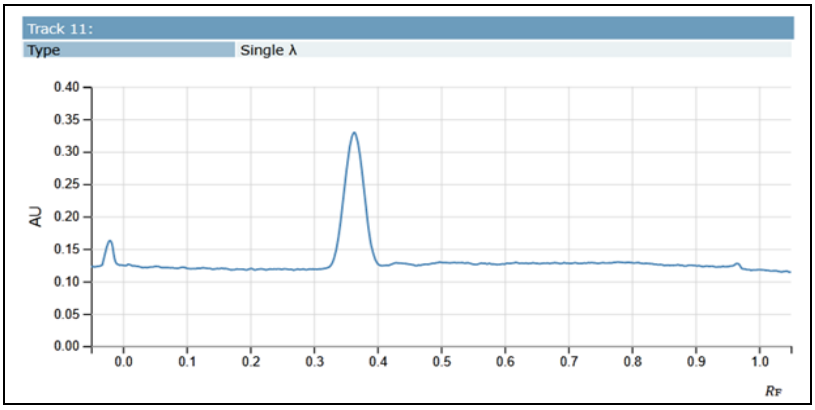


FIG. 3D: R_F VALUE OF PLANT EXTRACT (3µL) OF TRACK 11 AT 254 NM

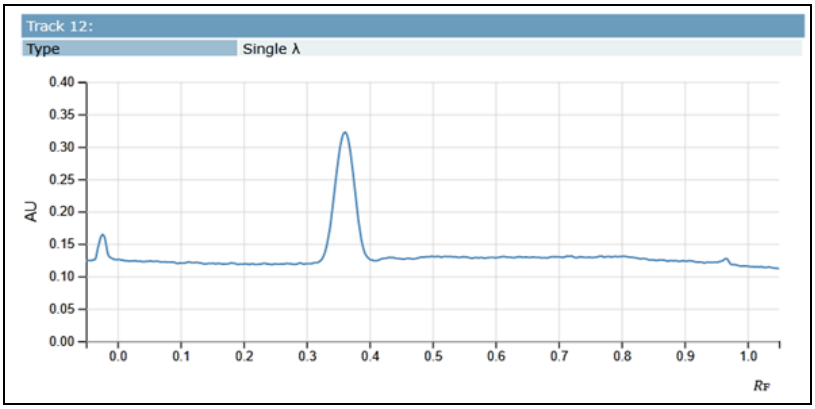


FIG. 3E: R_F VALUE OF PLANT EXTRACT (4µL) OF TRACK 12 AT 254 NM

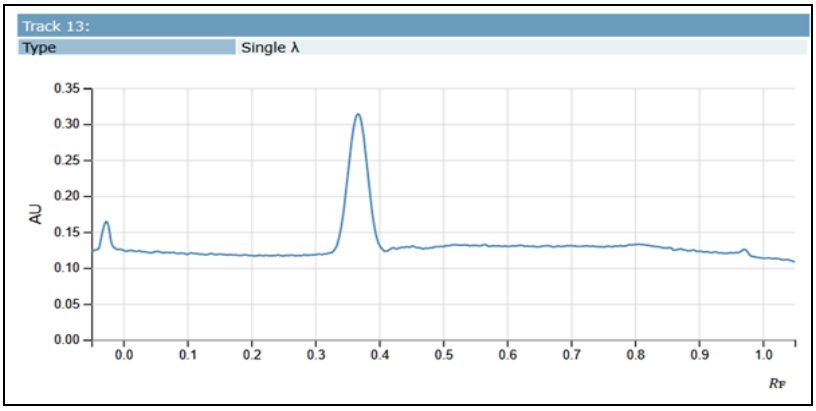


FIG. 3F: R_F VALUE OF PLANT EXTRACT (4µL) OF TRACK 13 AT 254 NM

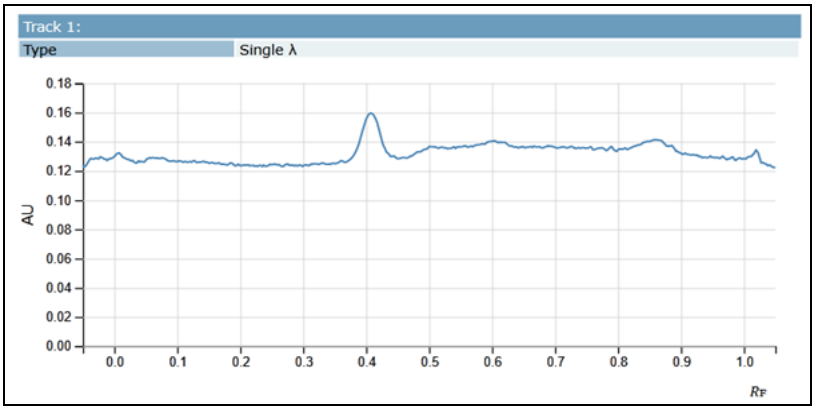


FIG. 4A: R_F VALUE OF STANDARD DRUG (1µL) AT 260 NM

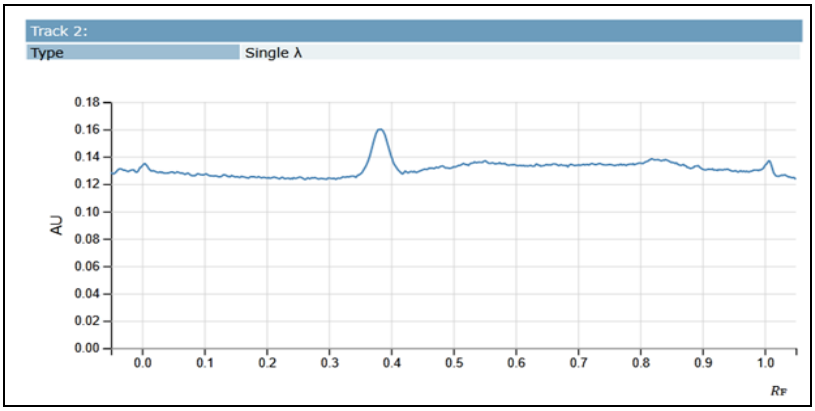


FIG. 4B: R_F VALUE OF STANDARD DRUG (1µL) AT 260 NM

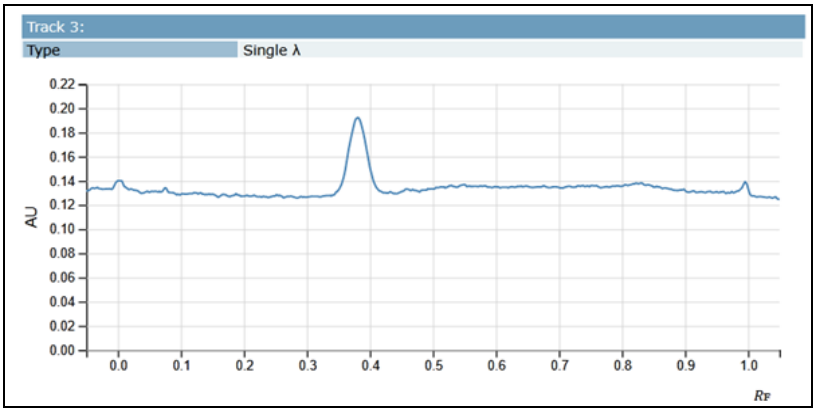


FIG. 4C: R_F VALUE OF STANDARD DRUG (2µL) AT 260 NM

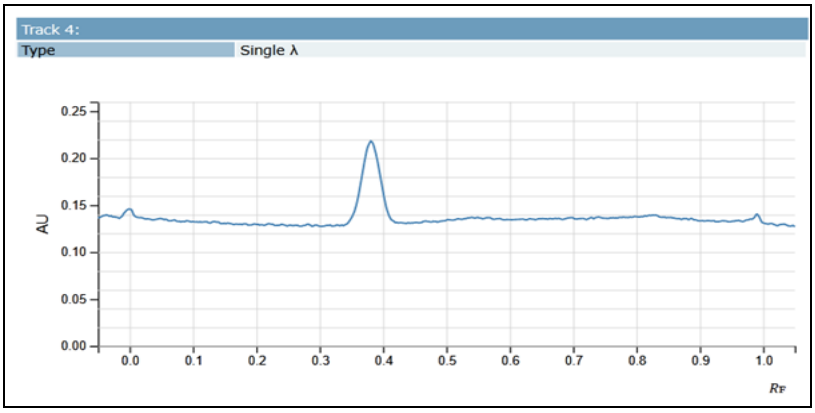


FIG. 4D: R_F VALUE OF STANDARD DRUG (3µL) AT 260 NM

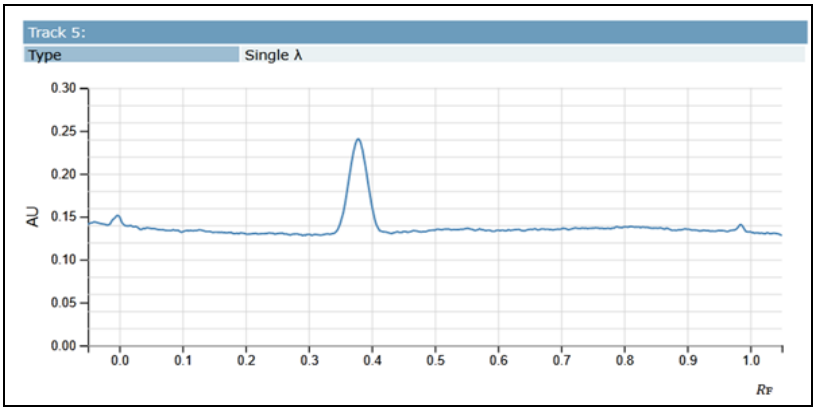


FIG. 4E: R_F VALUE OF STANDARD DRUG (4µL) AT 260 NM

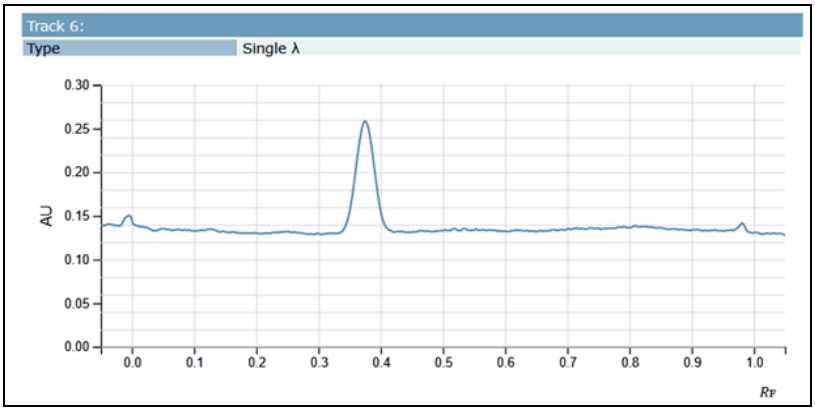


FIG. 4F: R_F VALUE OF STANDARD DRUG (5µL) AT 260 NM

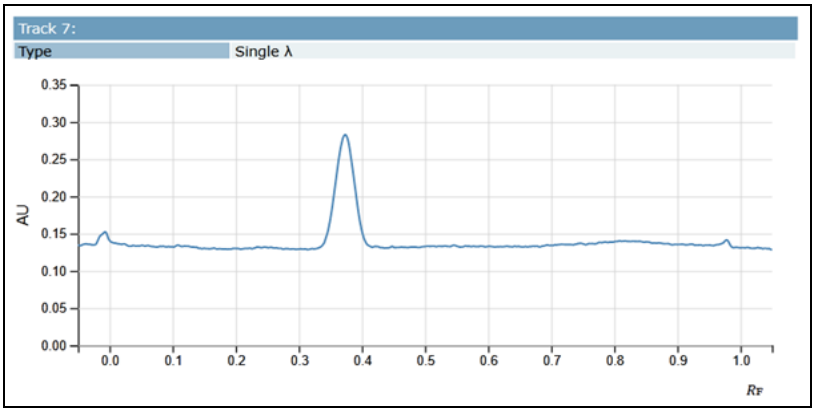


FIG. 4G: R_F VALUE OF STANDARD DRUG (6µL) AT 260 NM

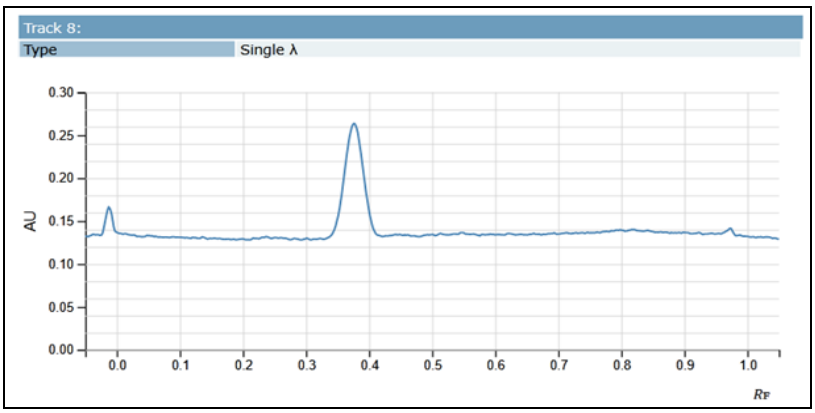


FIG. 4H: R_F VALUE OF PLANT EXTRACT (2µL) AT 260 NM

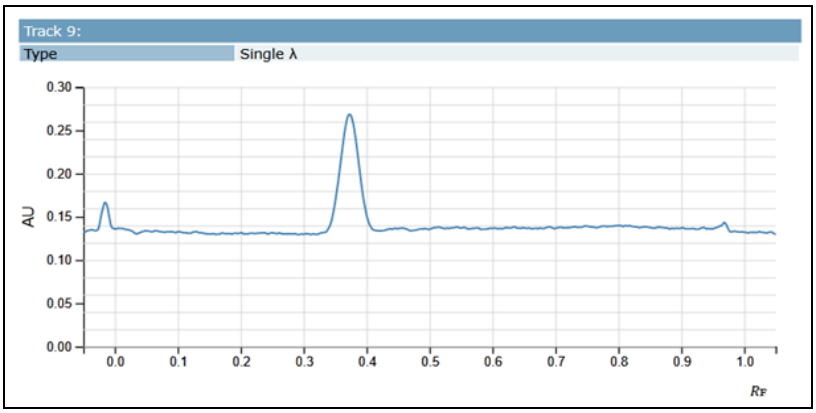


FIG. 4I: R_F VALUE OF PLANT EXTRACT (2µL) AT 260 NM

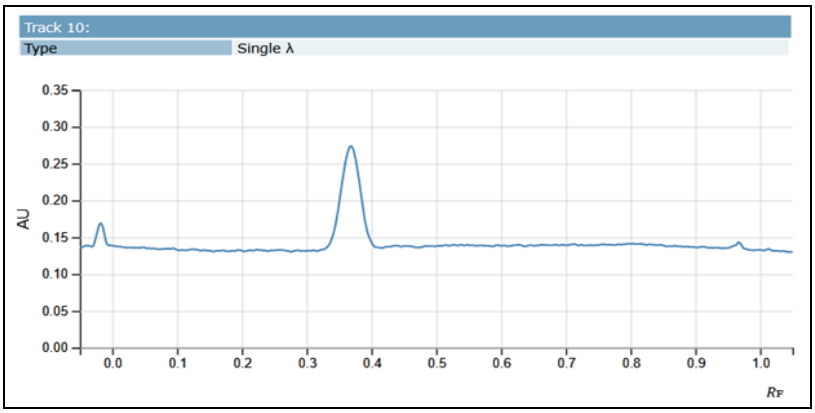


FIG. 4J: R_F VALUE OF PLANT EXTRACT (3µL) AT 260 NM

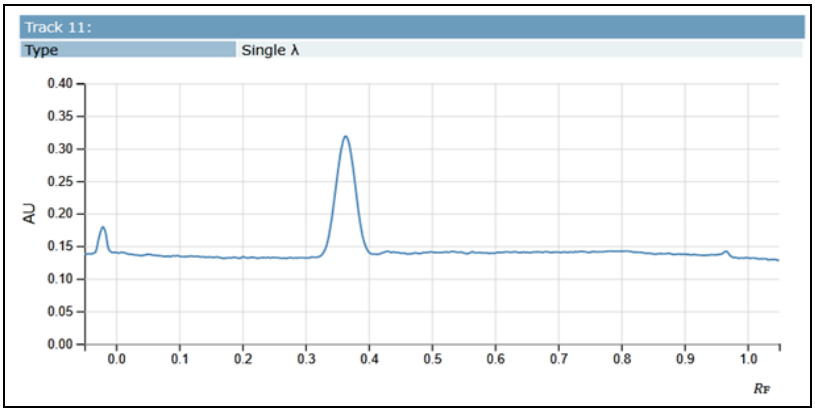


FIG. 4K: R_F VALUE OF PLANT EXTRACT (3µL) AT 260 NM

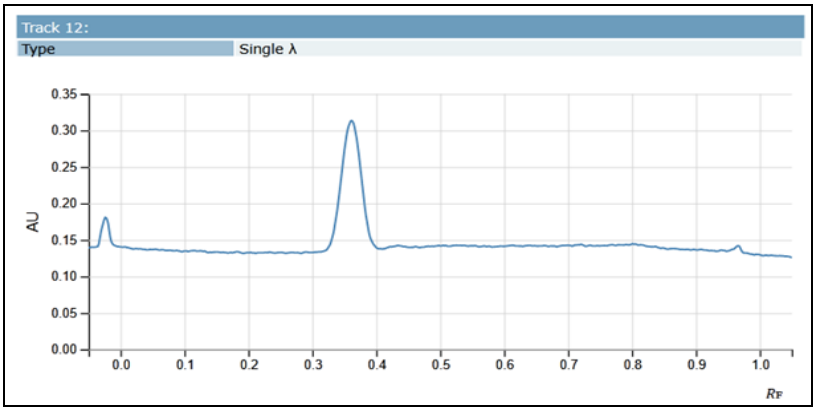


FIG. 4L: R_F VALUE OF PLANT EXTRACT (4µL) AT 260 NM

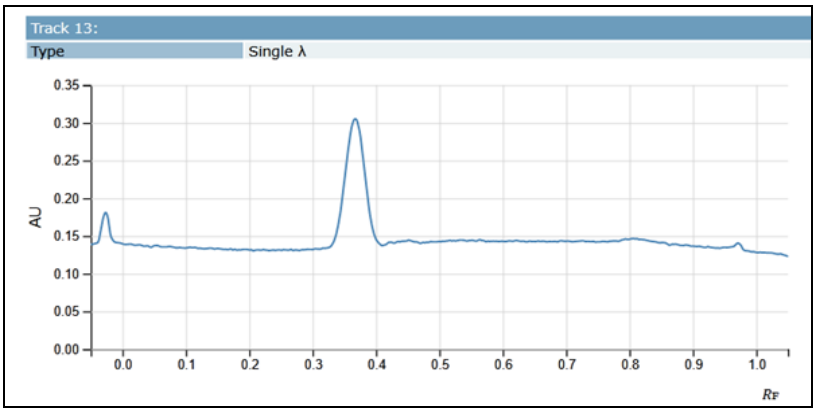


FIG. 4M: R_F VALUE OF PLANT EXTRACT (4µL) AT 260 NM

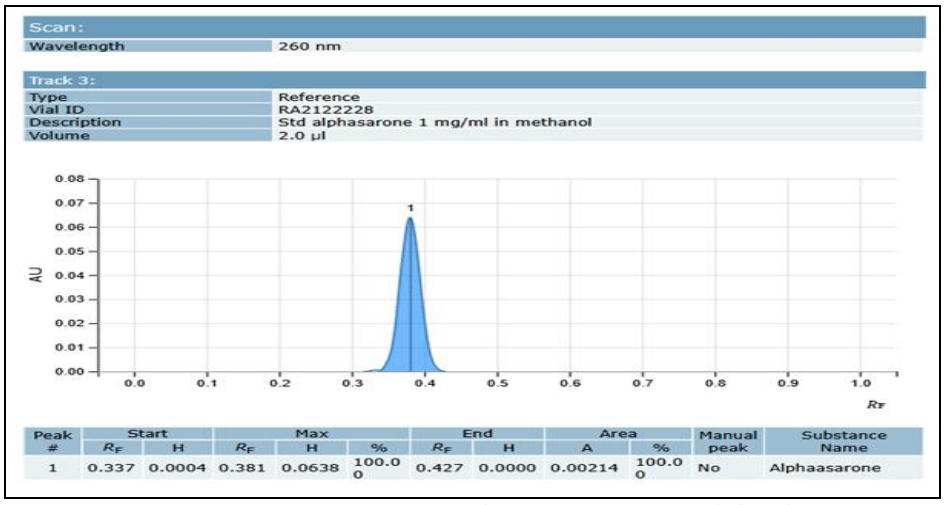
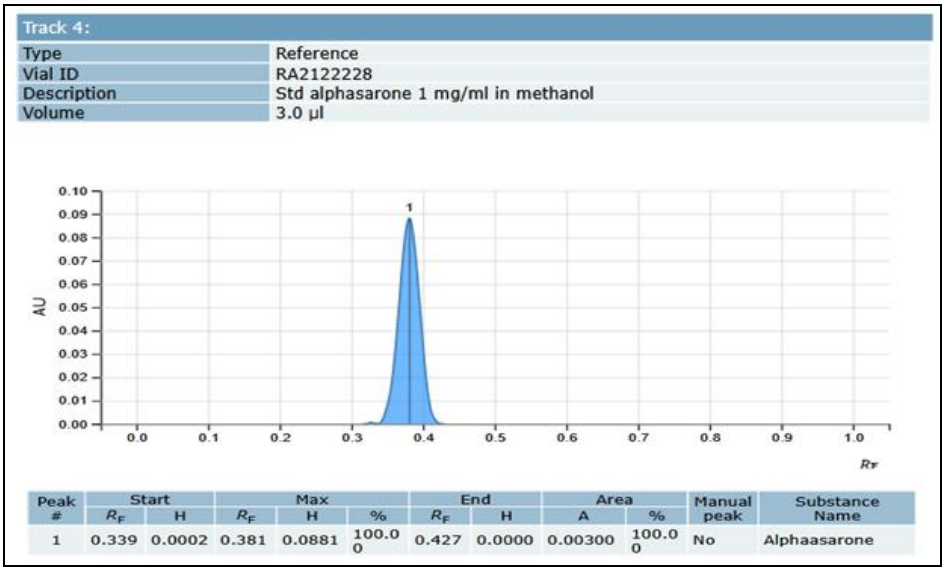
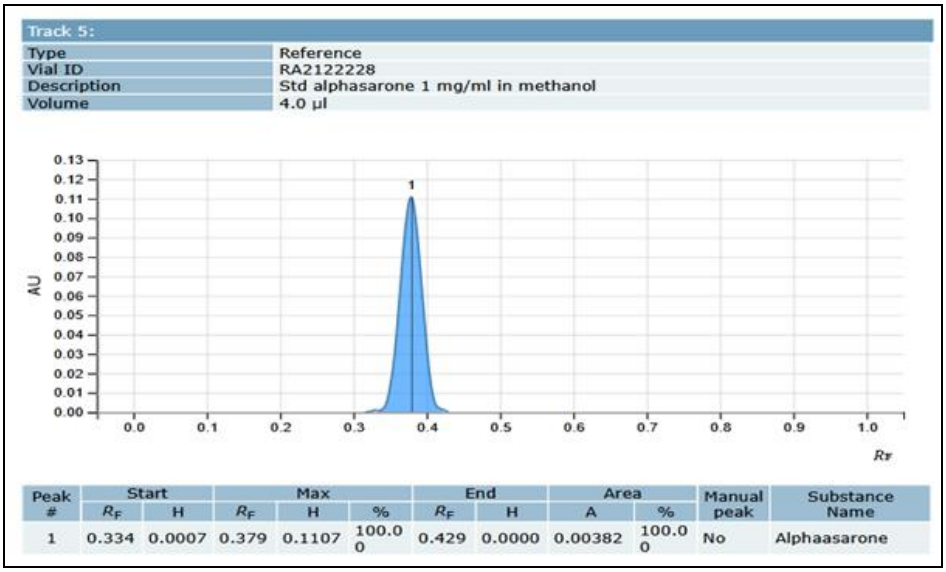


FIG. 5A: PEAK OF STANDARD DRUG (ALPHA ASARONE) (2µL) AT 260 NM



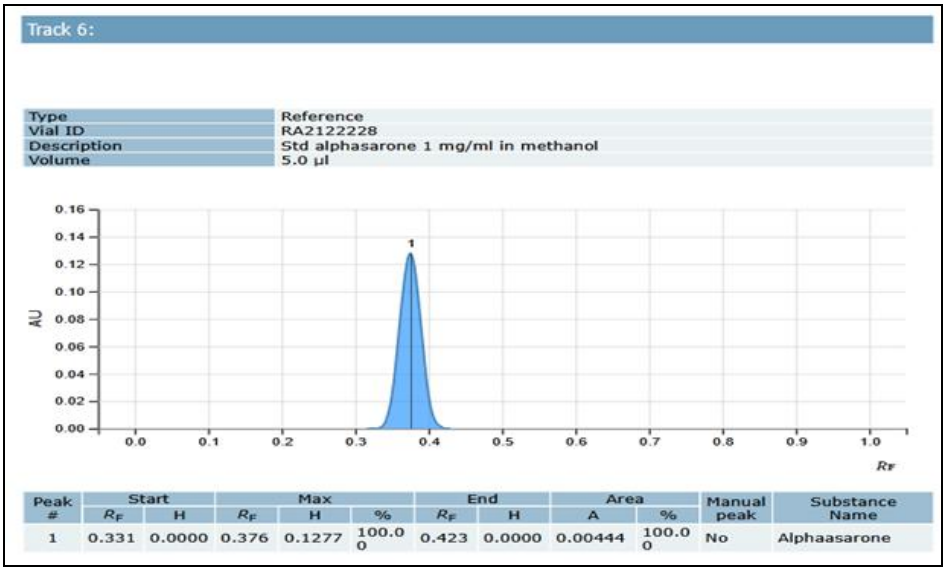
Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %	Manual peak	Substance Name
1	0.339	0.0002	0.381	0.0881	100.00	0.427	0.0000	0.00300	100.00	No	Alphaasarone

FIG. 5B: PEAK OF STANDARD DRUG (3µL) AT 260 NM



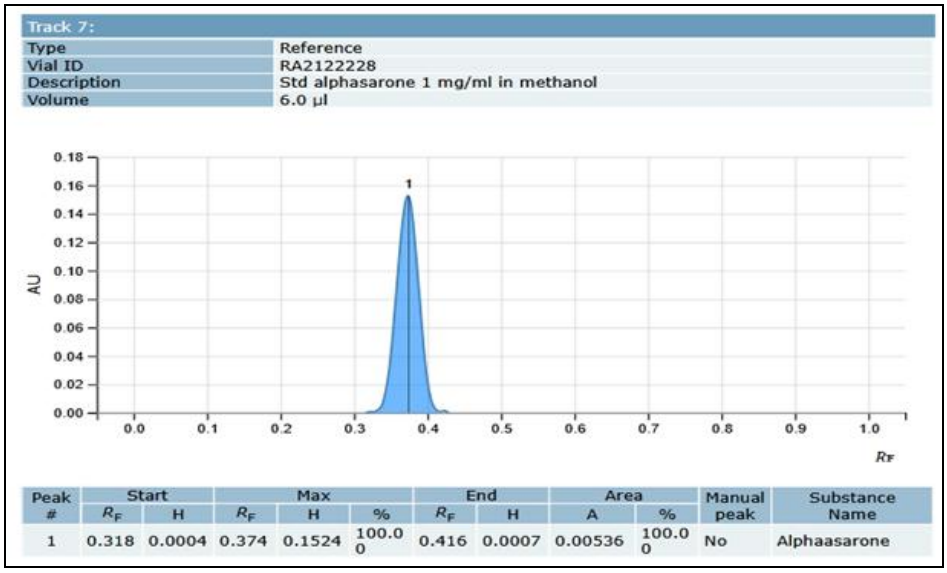
Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %	Manual peak	Substance Name
1	0.334	0.0007	0.379	0.1107	100.00	0.429	0.0000	0.00382	100.00	No	Alphaasarone

FIG. 5C: PEAK OF STANDARD DRUG (4µL) AT 260 NM



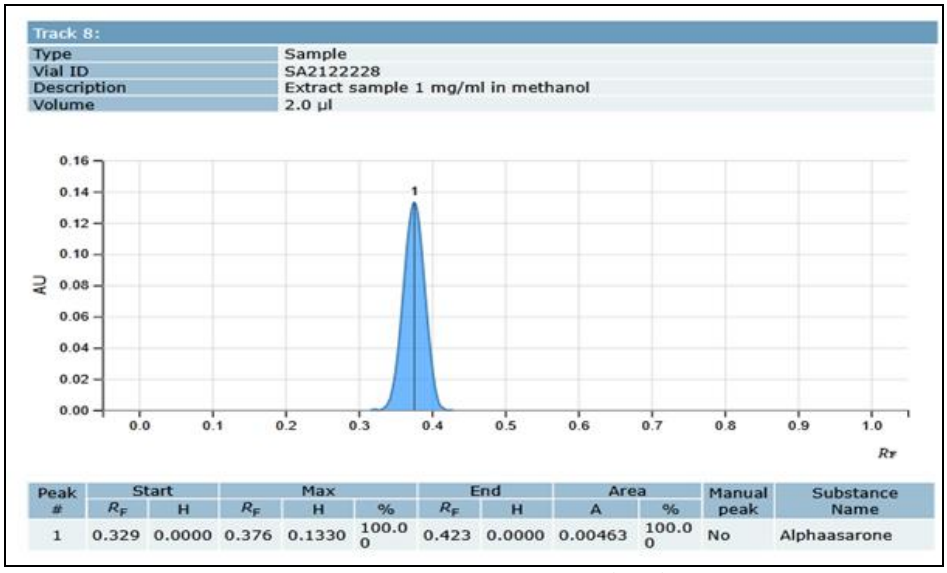
Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %	Manual peak	Substance Name
1	0.331	0.0000	0.376	0.1277	100.00	0.423	0.0000	0.00444	100.00	No	Alphaasarone

FIG. 5D: PEAK OF STANDARD DRUG (5µL) AT 260 NM



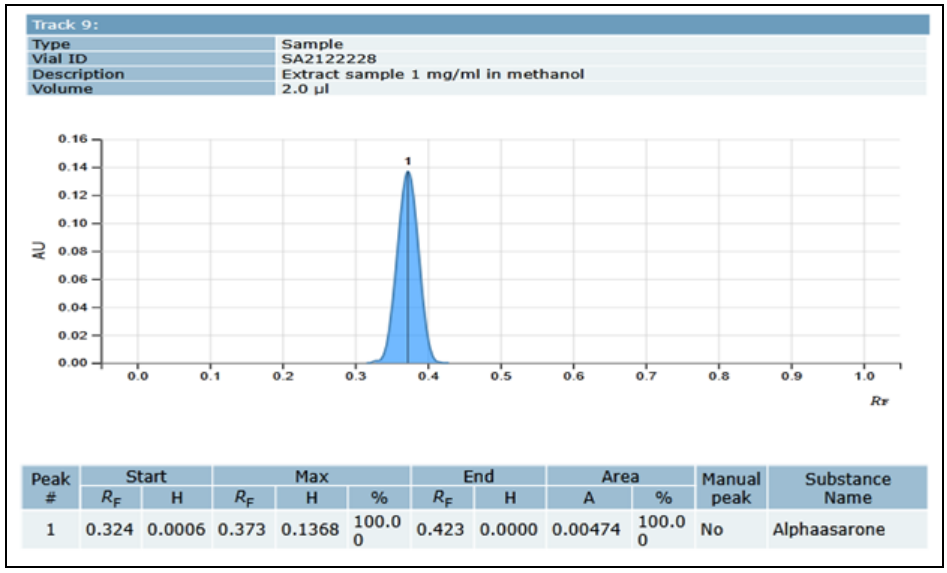
Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %	Manual peak	Substance Name
1	0.318	0.0004	0.374	0.1524	100.00	0.416	0.0007	0.00536	100.00	No	Alphaasarone

FIG. 5E: PEAK OF STANDARD DRUG (6µL) AT 260 NM



Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %	Manual peak	Substance Name
1	0.329	0.0000	0.376	0.1330	100.00	0.423	0.0000	0.00463	100.00	No	Alphaasarone

FIG. 5F: PEAK OF PLANT EXTRACT (2µL) AT 260NM



Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %	Manual peak	Substance Name
1	0.324	0.0006	0.373	0.1368	100.00	0.423	0.0000	0.00474	100.00	No	Alphaasarone

FIG. 5G: PEAK OF PLANT EXTRACT (2µL) AT 260 NM

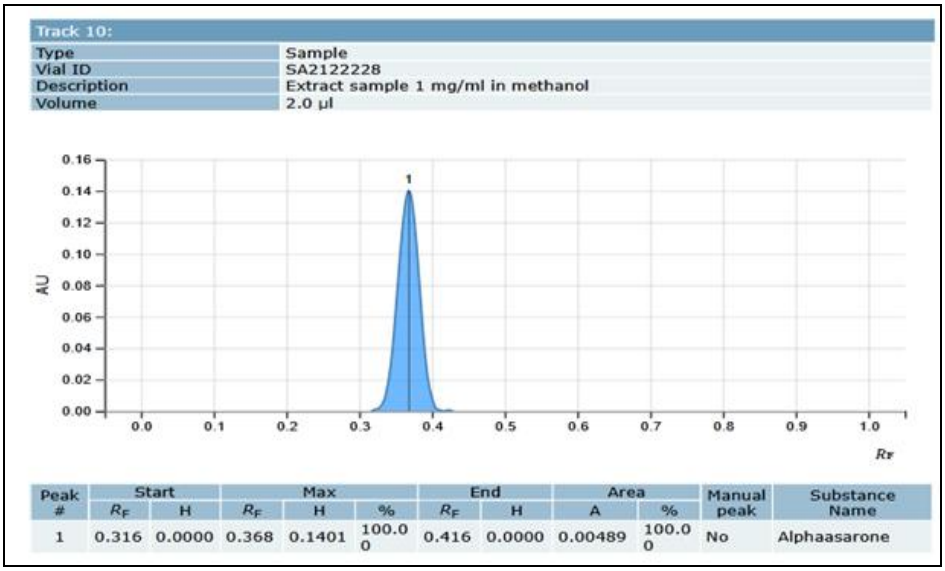


FIG. 5H: PEAK OF PLANT EXTRACT (2µL) AT 260 NM

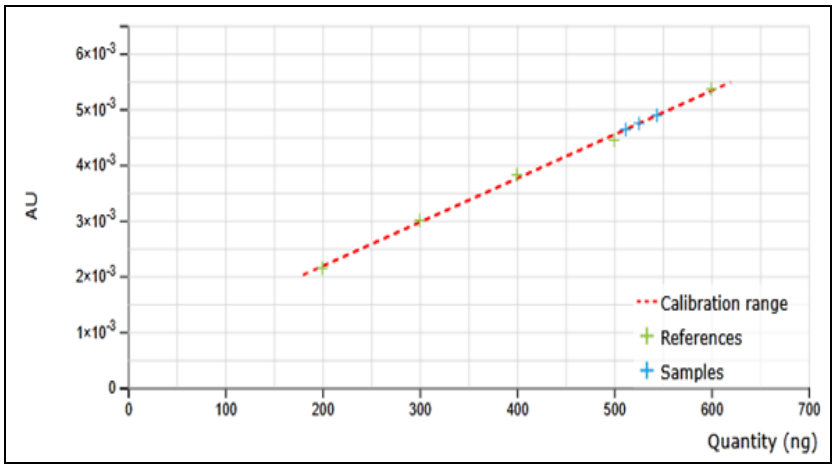


FIG. 6: CALIBRATION RANGE OF THE STANDARD DRUG (α-ASARONE) AND THE PLANT EXTRACT

TABLE 3: DPPH SCAVENGING ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *ACORUS CALAMUS* LINN. (HAEAC)

S. no.	Conc. (µg/mL)	% DPPH free radical scavenging activity of ascorbic acid	% DPPH free Radical scavenging activity of HAEAC
1.	100	64.13%	52.59%
2.	200	66.32%	53.95%
3.	300	67.46%	55.19%
4.	400	68.81%	55.30%
5.	500	70%	56%

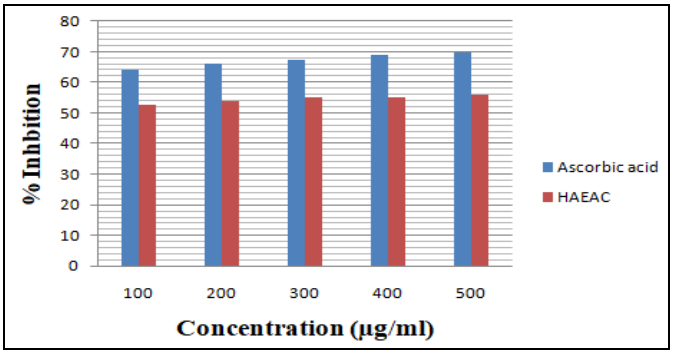


FIG. 7: DPPH SCAVENGING ACTIVITY OF THE PLANT EXTRACT

CONCLUSION: The current procedure is straightforward, exact, and repeatable. It offers a lot of potential for developing herbal drugs, antioxidant studies, quality control, and standardization. In AC, α-asarone is an essential marker chemical and has antioxidant properties. This substance has been quantified in the plant's rhizome using a simple and quick HPTLC densitometric approach devised and verified. This approach might effectively create quality control and profile analysis of this medicinal plant.

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