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ISOLATION AND QUANTIFICATION OF GALLIC ACID FROM *EULOPHIA OCHREATA* LINDL. BY HPTLC

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Keywords:

Eulophia ochreata Lindl. Alcoholic extract, TLC isolate, HPTLC, Gallic acid

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ABSTRACT: In the healthcare system, the development and modernization of phytomedicine in phytochemistry play a central and important role in drug development. Isolation and purification of bioactive compounds from naturally occurring substance are of prime importance. A sensitive and reliable high-performance thin-layer chromatography method has been developed for the estimation of gallic acid in the alcoholic extract of *Eulophia ochreata* Lindl. Alcoholic extract prepared was applied on silica gel G 60 F254 plate. The plate was developed using toluene: ethyl acetate: methanol: formic acid (6:6:0.4:1.6) as a mobile phase detection and quantification were performed by densitometric scanning at 275 nm. The system was found to give well-resolved bands for alcoholic extract having R_f values as 0.8 was matched with the standard R_f values like 0.82. The densitometric chromatogram of HPTLC fingerprint of the alcoholic extract, isolate, and standard gallic acid was obtained. The calibration curve of gallic acid was linear over a concentration range (0.2- 2 microg/ml) with a good correlation coefficient (R^2= 0.9986) and coefficient of variation as CV- 2.4663%. The method was validated for linearity, precision, specificity, and it was found to be precise, reliable, and suitable. The proposed method is simple, rapid, precise, and accurate. The method was found to be suitable for qualitative and quantitative analysis of gallic acid in the alcoholic extract of *Eulophia ochreata* Lindl.

INTRODUCTION: Herbal medicines are believed to have better compatibility with the human body due to their safety, efficacy, cultural acceptability, and lesser side effects [1]. Phenolic acids have been considered as potential therapeutic agents against a wide range of ailments, including neuro-degenerative diseases, cancer, diabetes, cardiovascular dysfunction, inflammatory diseases, and in aging [2].

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The importance of phenolic acids as antioxidant activities and their possible usage have reached a milestone in the health care system. Phenolic acids are diverse group that includes hydroxybenzoic and hydroxycinnamic acids [3]. One such prominent phenolic acid is gallic acid. Gallic acid elicits several interesting and various biological responses, such as antibacterial, anti-fungal, anti-inflammatory, antiviral, anti-cancer, antioxidant, antimutagenic and anti-diabetic activities [4].

Due to these biological activities, gallic acid could be a good lead compound for new drug development [5]. HPTLC is an commonly used technique for qualitative and quantitative analysis of chemical markers in herbal raw materials. HPTLC has advantages of simplicity, sensitivity,

accuracy and is one of the most approached technique. A simple HPTLC method for quantitative estimation of gallic acid is proposed for *Eulophia ochreata* Lindl. [6]

E. ochreata, from the family Orchidaceae, is a ground orchid, commonly known as 'Amarkand'. It is a perennial tuberous herb occurs in rainy seasons in the forest [7]. Ethnobotanical survey of the forest areas of Maharashtra revealed that these tubers are used as specialty food, general tonic, and as rejuvenating herb. It has been used by the tribes for properties like astringent, antifatigue, aphrodisiac, anthelmintic. As a blood purifier [8], [9] tribal's have been using tubers as a general tonic and as rejuvenating since long [10]. On the basis of these prominent uses of *E. ochreata*, tubers were selected for my further work. The lack of well-documented scientific evidence will predominantly impede the progress of an isolated molecule in the avenue. Since, long ago, most of the people used orchids as a source of medicine for millennia to treat different diseases like chest pain, arthritis, inflammations, stomach disorders, menstrual disorder, bone fractures, *etc*. Orchid phytochemicals are generally categorized as alkaloids, flavonoids, carotenoids, anthocyanins, and sterols.

In present scenario, there is urgent need to utilize ancient knowledge of *Eulophia ochreata* L. to bring its maximum potential in the field of medical and pharmaceutical sciences in novel herbal drug development and its practical application so that a common man in this world will be benefited for improving its health, as *Eulophia ochreata* L. is a valuable and precious gift from nature [11].

MATERIALS AND METHODS:

Chemical: HPLC grade toluene, ethyl acetate, methanol, and formic acid were procured from E. Merck, Mumbai, India. Reference standards of gallic acid were purchased from (Research lab, Mumbai)

Plant Material: The tubers of *Eulophia ochreata* Lindl. was obtained from the Bhimashankar region of Maharashtra, India. Plant material was authenticated by Dr. G. G. Potdar, Department of Botany, Y. C. College of Science, Karad; voucher specimen was deposited at the same college as number AAK1. The tubers were washed with water to remove any dust particles, dried in the shade, powdered, and then sieved through mesh size 85 and stored at 25 °C in an airtight container.

Preparation of Stock Solutions: Stock solutions of gallic acid were prepared in methanol and by appropriate dilution standard solutions were prepared in the concentration of 1.0 mg/ml [12].

Sample Preparation: 60 gm of dried powder was weighed in a round bottom flask. 100 ml of ethanol was added to the flask, and the mixture was extracted by Soxhlet extraction after 12 h. The same procedure was performed twice, and the filtrate obtained was combined together. The extract was then dried.

Isolation and Identification of Bioactive Fraction by Chromatography:

Development of TLC Plate: Thin layer chromate-graphy was performed using silica gel G as adsorbent. A slurry of silica gel was prepared in distilled water. The slurry was applied to get a thin layer of 0.4 mm thickness over a clean and dry glass plate of 10 × 20 cm size by an applicator the plate was activated at 110 °C for one hour.

The Details of TLC as Under:

Adsorbent: Silica gel G (activated)
Thickness: 0.4 mm
Plate size: 10 × 20 cm
Activation Temperature: 110 °C for one hour
Solvent System: Toluene: ethyl acetate: methanol: formic acid (6:6:0.4:1.6).

Different Developing Solvent Systems were: [13]

S1 = Chloroform: methanol: acetic acid (5:1:1)
S2 = Toluene: chloroform: ethanol (2.9:6:1.5)
S3 = Toluene: ethyl acetate: diethylamine (7:2:1)
S4 = Hexane: chloroform: methanol (5:5:0.5)
S5 = Toluene: ethyl acetate: methanol: formic acid (6:6:0.4:1.6):

Isolation of Fractions by Column Chromatography: 150 g of silica for column chromatography was activated in a hot air oven at 110 °C for one hour. The methanol solvent was used to build the silica in the glass column. The activated silica was charged into the column in small portions with gentle tapping after each

addition to ensure uniform packing. The small quantity of solvent system was allowed to remain on the top of the column (about 2 cm). The air bubbles present in the column were removed by gentle tapping to get a uniform bed of adsorbent. The 2 gm of the alcoholic extract was dissolved in ethanol. The activated silica (3 g) was added slowly with continuous stirring to the solution to adsorb on the activated silica. Care was taken for no lumps in the adsorbed silica. Then the adsorbed alumina was charged into the column in a small portion with gentle tapping after each addition in order to ensure uniform packing.

The column was eluted with a mixture of toluene: ethyl acetate: methanol: formic acid (6:6:0.4:1.6). For the selected mobile phase, fractions A, B were collected. Total fractions amounted to 10. TLC of each fraction was carried out to find out homogeneity and detection with various reagents for the separation of active constituents. The chromatographic pattern of each fraction was studied thoroughly, and the fractions belong to the same elute solvent, which gave identical patterns with respect of R_f value and color reaction was mixed. Fraction A was selected for further analysis.

TABLE 1: THE DETAILS OF COLUMN CHROMATOGRAPHY OF ALCOHOLIC EXTRACT OF *EULOPHIA OCHREATA* LINDL.

Adsorbent	Silica for column chromatography activated at 110° for 1 h
Length of the column	40 cm
Length of the adsorbent	25 cm
Diameter of the column	Outer 3 cm, inner 2.8 cm
Rate of elution	10-15 drops / min
Volume of each fraction collected	1-5 ml each
Total volume of each mixture elute collected	90-100 ml
Elution	Toluene: ethyl acetate : methanol: formic acid (6:6:0.4:1.6)

TABLE 2: ELUTION SCHEME FOR COLUMN CHROMATOGRAPHY OF ALCOHOLIC EXTRACT OF *EULOPHIA OCHREATA* LINDL.

Parts	Proportion	Fraction
1 to 05	Toluene: ethyl acetate : methanol: formic acid (6:6:0.4:1.6)	Fraction as A
06 to 10		Fraction as B

TABLE 3: TRACK NO SHOWING DESCRIPTION OF STANDARD, SAMPLE AND ISOLATE

Track	Vial ID	Description	Volume	Position	Type
1	1718084-04	Standard GA	0.2 µl	C6	Reference
2	1718084-04	Standard GA	0.4 µl	C6	Reference
3	1718084-04	Standard GA	0.6 µl	C6	Reference
4	1718084-04	Standard GA	0.8 µl	C6	Reference
5	1718084-04	Standard GA	1.0 µl	C6	Reference
6	1718084-04	Standard GA	1.2 µl	C6	Reference
7	1718084-04	Standard GA	1.4 µl	C6	Reference
8	1718084-04	Standard GA	1.6 µl	C6	Reference
9	1718084-04	Standard GA	1.8 µl	C6	Reference
10	1718084	Standard GA	2.0 µl	C6	Reference
11	1718084	Sample alcoholic extract	1.0 µl	C4	Sample
12	1718086	Sample alcoholic Extract	1.0 µl	C4	Sample
13	1718086	Sample isolate 2	1.0 µl	C4	Sample
14	1718086	Sample isolate 2	1.0 µl	C4	Sample
15	1718086	Sample isolate 2	1.0 µl	C4	Sample

Development of HPTLC: Identification and Quantification of Active Constituent: [14, 15] HPTLC was performed on 200 mm × 100 mm aluminum backed plates pre-coated with 0.2 mm layers of silica gel 60 F254 (Merck, Mumbai, India). A CAMAG TLC system comprises of a Linomat-5 applicator and CAMAG TLC III scanner. The stationary phase used was silica gel G 60 F 254, 20 × 10 cm TLC plate. The reference standard gallic acid was obtained from Research Lab, Mumbai, India. Standard solutions of gallic acid and sample solutions were applied to the plates as bands 8.0 mm wide, 7.0 mm apart and 10.0 mm from the bottom edge of the same chromatographic plate by use of a CAMAG (Muttenz, Switzerland) linomat V sample applicator equipped with a 100-ul Hamilton (USA) syringe. Ascending double development to a distance of 80 mm was performed at room temperature (28 ± 2 °C), with toluene: ethyl acetate: methanol: formic acid (6:6:0.4:1.6) v/v as mobile phase, in a CAMAG glass twin trough chamber previously saturated with mobile

phase vapor for 10 min. After development, the plates were dried in the air first and then by keeping on the CAMAG TLC plate heater at 90 °C for 5 min. The developed plate was visualized and photographed at 254 nm, 366 nm in a visualizing chamber and scanned at 254 nm and 366 nm using CAMAG scanner [4]. The separated band appeared for gallic acid in UV, which was scanned at 254 nm and 366 nm with a CAMAG TLC scanner with visioncats-serv, version 2.4.17207.2. The R_f values were calculated.

Image @ 254nm

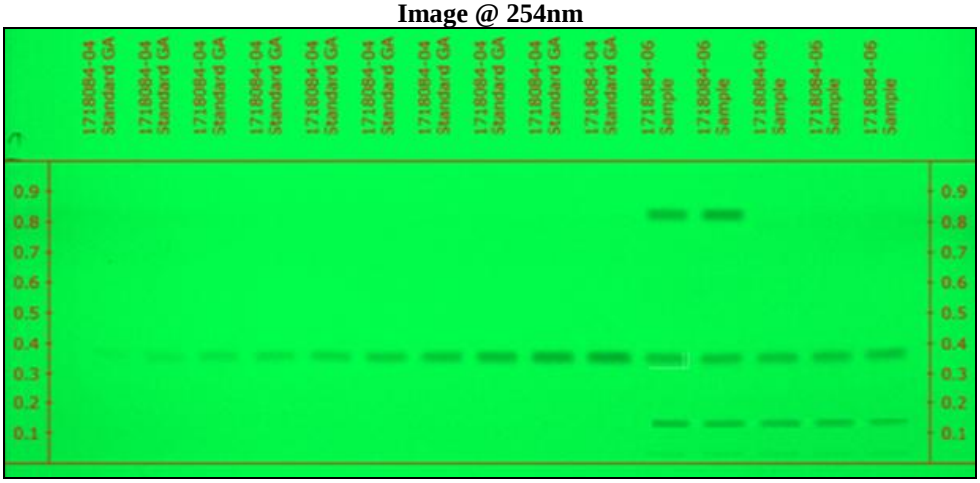


FIG. 1: HPTLC STUDY OF QUANTIFICATION OF ALCOHOLIC EXTRACT AND ISOLATE OF *EULOPHIA OCHREATA* LINDL. @ 254 nm

Image @ 366 nm

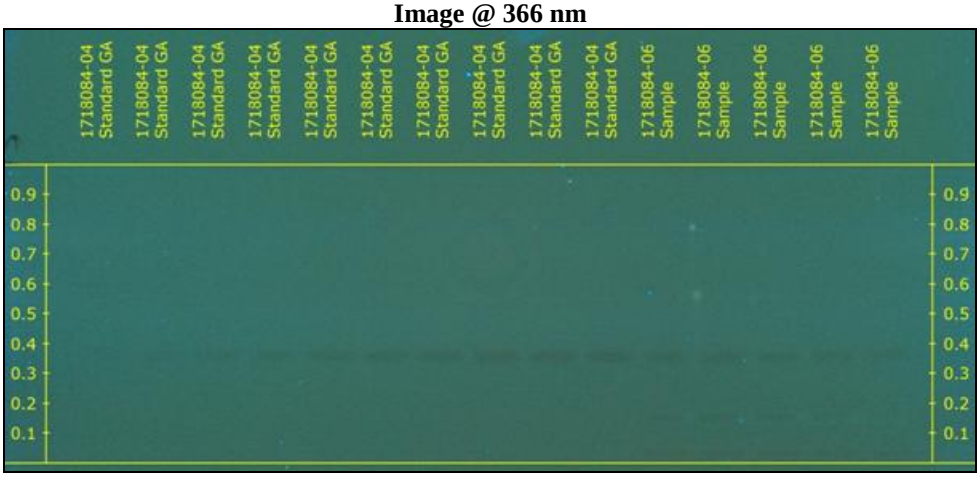


FIG. 2: HPTLC STUDY OF QUANTIFICATION OF ALCOHOLIC EXTRACT AND ISOLATE OF *EULOPHIA OCHREATA* LINDL. @ 366 nm

Track 1-10: Standard gallic acid

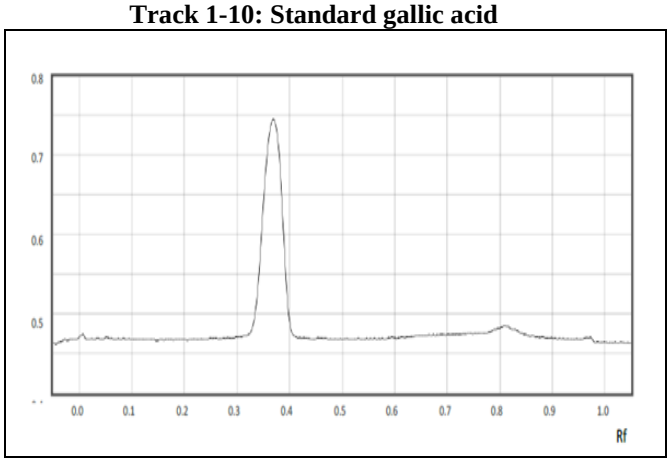


FIG. 3: UV SCANS OF STANDARD GALLIC ACID @ 275 nm

Track 11, 12: Alcoholic extract

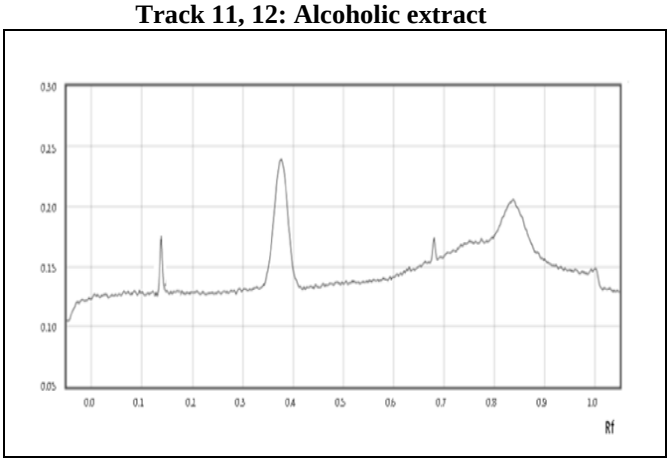


FIG. 4: UV SCANS OF ALCOHOLIC EXTRACT @ 275 nm

Track 13, 14, 15: Sample isolate

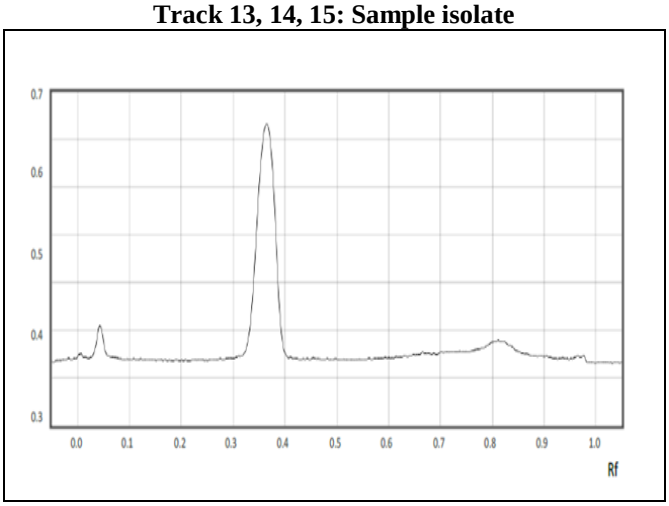


FIG. 5: UV SCANS OF ISOLATE @ 275 nm

Validation of the Method:

Specificity: The R_f values and color of HPTLC bands were compared with those of the standards. And the bands were confirmed. Thus the specificity of the proposed HPTLC method was performed by analyzing standard compounds with samples.

Linearity and Correlation Coefficient: The calibration curve of gallic acid, considering its concentrations and area under curve averages, gave a straight line with the linear regression equation.

Precision:

Repeatability: Repeatability of sample applications and measurement of peak area was carried out using the three replicates of same spot. Repeatability is also termed intra assay precision.

Inter-Day and Intra-day Precision: Intra-day precision that is on the same day and on different days that is inter-day precision variability of the method was studied by analyzing quality control samples of Gallic acid (100 µg/mL).

Stability: Solution stability was evaluated by monitoring the peak area response. Standard solutions were analyzed right after its preparation and after 72 h.

TABLE 4: SUMMARY OF VALIDATION PARAMETERS

Parameter	Gallic acid
Specificity	Specific
Coefficient of variation	2.4663%
Precision (RSD)	≤ 5%
Correlation coefficient	0.9986
Linearity	0.2 to 2 ug/ml
Solution stability after 72 h	Stable, RSD≤ 2%

RESULTS AND DISCUSSION: The aim of our research was to find the better extraction method of active components from *Eulophia ochreata* Lindl. gallic acid was quantified by HPTLC for the first time in *Eulophia ochreata* Lindl. The identity of gallic acid was confirmed by chromatographic technique and comparison with a reference standard when scanned at 275 nm. These active components include isoquinoline alkaloids like phenanthridine and simple phenols like gallic acid, which will be used in further research work. We adopt the phytochemical detection tests, column chromatography, TLC and the HPTLC method to perform the isolation, identification, and characterization of an extract with isolates and biomarkers, and then to identify the best one. Thin-layer chromatography developed the mobile phase as toluene: ethyl acetate: methanol: formic acid (6:6:0.4:1.6), as shown in **Fig. 6**.

FIG. 6: TLC OF ALCOHOLIC EXTRACT OF *EULOPHIA OCHREATA* LINDL.

TLC of alcoholic extract of *Eulophia ochreata* Lindl. showed two distinct spots with R_f Values from below are 0.50 and 0.80. For the selected mobile phase, fractions A, B were collected. TLC of each fraction was carried out to find out homogeneity and detection with various reagents for the separation of active constituents. The chromatographic pattern of each fraction was studied thoroughly, and the fractions belong to the same elute solvent, which gave identical patterns with respect of R_f value and color reaction was mixed. Fraction A was selected for further analysis. Column chromatographic technique utilized for the isolation of fractions. Fractions were collected in sample test tubes and stored for further analysis, as shown in **Fig. 7**. TLC was performed which shows a single spot of R_f value as 0.82 as shown in **Fig. 8**

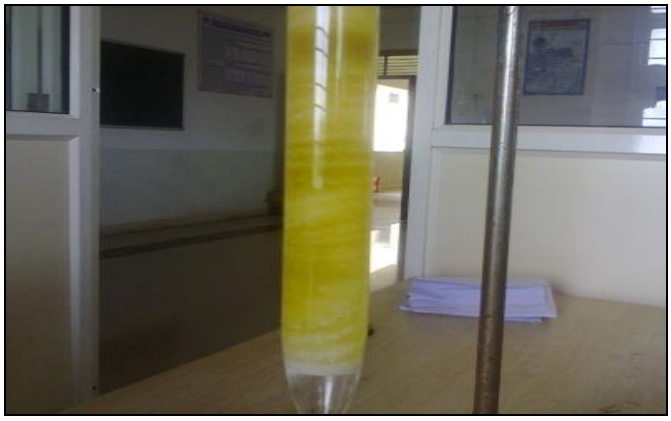
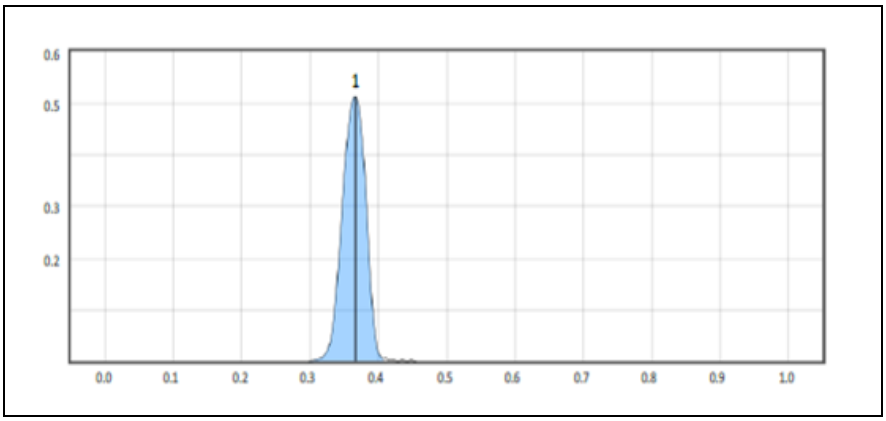


FIG. 7: COLUMN CHROMATOGRAPHY

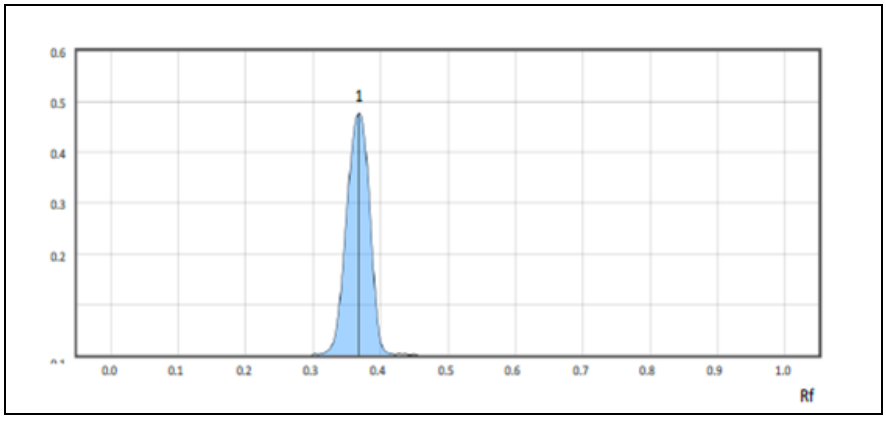
FIG. 8: TLC OF ISOLATED FRACTION

Peak purity was studied by UV spectra obtained from the standards and from the corresponding peaks from test as shown in **Fig. 3, 4** and **5**. The densitometric chromatogram of HPTLC fingerprint of the alcoholic extract, isolate, and standard gallic acid is shown in **Fig. 9, 10, 11,** respectively.



Peak	Start		Max			End		Area		Manual peak	Substance R_f name
1	RT	H	RT	H	%	RT	H	A	%		Alcoholic extract
	0.298	0.0000	0.366	0.5126	100.00	0.408	0.0042	0.02074		No	containing gallic acid

FIG. 9: Ddensitometric chromatogram of HPTLC fingerprint of the alcoholic extract

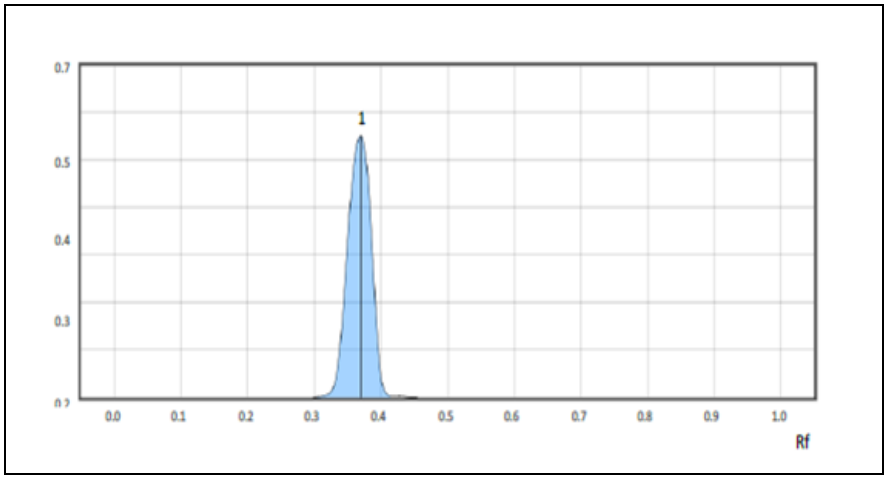


Peak	Start		Max			End		Area		Manual peak	Substance name
1	RT	H	RT	H	%	RT	H	A	%		Isolate 2 gallic
	0.300	0.0006	0.368	0.4777	100.00	0.421	0.0006	0.01914	100.00	No	acid

FIG. 10: DENSITOMETRIC CHROMATOGRAM OF HPTLC FINGERPRINT OF THE ISOLATE

The peaks resolving at R_f 0.298 in alcoholic extract and 0.300 in isolates were found to be super-imposed with those of respective standards of gallic acid having an R_f value of 0.302. The content of

gallic acid in the plant was calculated on the basis of peak area and was found to be 0.13% (w/w), respectively. The calibration plots indicate that the peak-area response was a polynomial function of the amount of standards gallic acid. The calibration curve of gallic acid was linear **Fig. 12** over a concentration range 0.2-2 microg/ml with a good correlation coefficient (R^2 = 0.9986) and coefficient of variation as CV-2.4663%.



Peak	Start		Max			End		Area		Manual peak	Substance name
1	RT	H	RT	H	%	RT	H	A	%	No	Gallic acid
	0.302	0.0005	0.369	0.5525	100.00	0.421	0.0020	0.02298	100.00		

FIG. 11: DENSITOMETRIC CHROMATOGRAM OF HPTLC FINGERPRINT OF THE STANDARD GALLIC ACID

Sample 1/18084-06 and isolate 2	1.383 mg/ml	CV = 3.780%
Volume: 1.0 µ	1.383 mg/ml	CV = 3.780%
Tract 11 alcoholic extract	1.470 mg/ml	1.470 µg
Tract 12 alcoholic extract	1.391 mg/ml	1.391 µg
Tract 13 isolate 2	1.349 mg/ml	1.349 µg
Tract 14 isolate 2	1.366 mg/ml	1.366 µg
Tract 15 isolate 2	1.340 mg/ml	1.340 µg

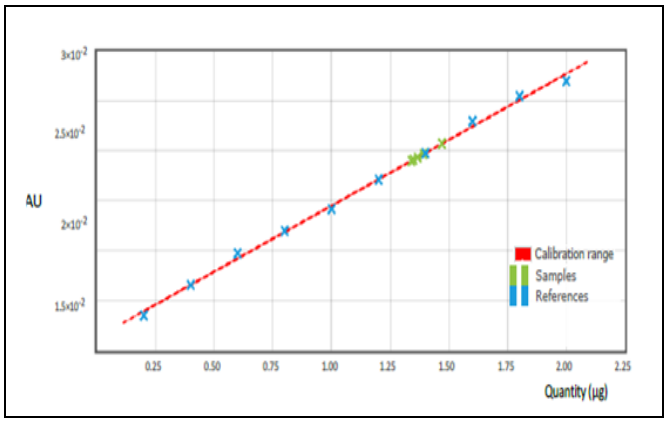


FIG. 12: CALIBRATION CURVE OF GALLIC ACID OF *EULOPHIA OCHREATA* LINDL.

Regression mode	Linear-2
Range deviation	5.00%
Related substance	Default
Number of references	10
Calibration function	$Y = 1.322 \times 10^{-8} x + 1.311 \times 10^{-3}$
Coefficient of variation	CV 2.4663%
Correlation	R = 99.867727%

Intraday and inter-day precision were calculated for the developed method by performing the analysis at three different levels on the same day as well as on different days, respectively. The results were expressed as ≤ 5% RSD. From all validation parameters, which indicated that the developed method was accurate and satisfactory.

Solution stability was evaluated by monitoring the peak area response. Standard solutions were analyzed right after its preparation and after 72 h. There was no significant change (% RSD ≤ 2%) in the R_f and area values of the standard peak.

CONCLUSION: The method was validated for linearity, precision, specificity, and it was found to be precise, reliable, and suitable because of the good recovery data.

The proposed method is simple, rapid, precise, and accurate. The method was found to be suitable for qualitative and quantitative analysis of gallic acid in the alcoholic extract of *Eulophia ochreata* Lindl.

The method established in this work can, therefore, be used as a quality control method for other market formulations or dietary supplements containing gallic acid as one of the components.

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CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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