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HPLC METHOD DEVELOPMENT FOR ESTIMATION GALLIC ACID AND ELLAGIC ACID IN AN AYURVEDIC ANTI ARTHRITIC FORMULATION SIMHNAD GUGGUL

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ABSTRACT

Present study is a work on results of analytical method development for two important bioactive phyto-constituents of a well-known Ayurvedic formulation Simhnad guggul i.e. gallic acid and ellagic acid. TLC method was used for authentication of these constituents in formulation. Then HPLC methods were developed for quantification of gallic acid and ellagic acid in formulation. The optimized mobile phase for gallic acid was Acetonitrile: water (70:30) and 3% Glacial acetic acid and for ellagic acid it was Acetonitrile: water (80:20) and 1% Glacial acetic acid. The method was applied on market formulations and freshly prepared one. The results indicate that developed methods were found to be precise, sensitive, reliable and accurate.

Keywords: Simhnad guggul, HPLC, Gallic acid, Ellagic acid.

INTRODUCTION

Simhnad guggul (commonly known as *Sihnad guggul* or *Sihnad gugal*) is a very popular and effective ayurvedic anti-arthritis formulation suggested by ayurvedic texts in any type of arthritic patient. According to ayurvedic formulary of India; it is made up of *Commiphora weightii* resin, purified Ganddhak (sulfur), *Terminalia baheda* pericarp, *Terminalia chebula* fruits and *Embelica officinalis* fruits. The formulation needs purification of Guggul resin and raw sulfur by reported traditional methods of shodhana.¹⁻³ Preparation method (Castor oil is used for final rolling). The formulation type is guggul vati (traditionally prepared pills). It is suggested to be taken 4 to 12 in divided dose with warm milk orally.³⁻⁵

Literature review

Sunita, et. al.⁴, reported simhnad guggul to have significant improvement in open label 2 multicenter study of Simhnad guggul and Brihat Saindhavdya Taila in management of Rheumatoid Arthritis on 111 human volunteers for 12 weeks. Singhal H.K., et. al.⁵, studied comparative effect of Singhnad guggulu and Trayodasangaguggulu for treatment of rheumatoid arthritis- Juvenile (amavata) on 10 patients with 2

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mg/Kg dose p.o. 45 days. Mahto R.R., et. al.⁶, reported clinical study results of Rasona Rasonadi ghanvati and Simhnad guggul in 101 amavata patients. Pandey S.A., et. al.⁷ studied comparative clinical effects of Simhnad guggul and Shiva guggulu on Amvata (rheumatoid arthritis) patients. Sumantran V.N., et. al.⁸, They studied chondroprotective effect of Trifala guggul and triphala & Shodhita guggul on the basis of total hyluronidase enzyme content. Debnath Saroj Kumar, et.al.⁹ reported clinical study of 90 Rheumatoid arthritic patients under treatment of Alambushadi Churna and Simh Nadguggul with warm milk, thrice a day, orally for 45 days. Rasool M, et. al.¹⁰, reported anti-arthritic effect of Triphala churna using Adjuvant Induced Arthritis model in mice (1gm/Kg) in comparison with Indomethacin. Shruthi S.D., et. al.¹¹, studied anti-arthritic effect of ellagic acid in formaldehyde induced paw edema for 4 weeks treatment in human at 100 and 250 µg/ml doses in vivo, *in-vitro* and in silico. Allam G., et. al.¹², anti-arthritic effect of ellagic acid was studied in vivo in adjuvant Induced Arthritic mice with 1 week of pre-treatment and 3 weeks of simultaneous treatment with ellagic acid on Adjuvant Induced Arthritis model. Allam G. also did study of mechanism of ellagic acid in Adjuvant Induced Arthritis in mouse at 700 mg/Kg dose (p.o.). Bensaad L.A. et. al.¹³, found potential anti-inflammatory effect of gallic acid, ellagic acid and punicalagin A/B in isolated Frozen RAW264.7 cells at 200 µg/mL doses. Kroes B. H., et. Al.¹⁴, Studied in vitro anti inflammatory and anti oxidant activities of gallic acid in zymosan induced acute food pad swelling in mice. Debnath S.K., et. al.¹⁵, analyzed Simhnad guggul formulation using TLC technique and found 7 phytoconstituents in 254 nm and 8 phytoconstituents in 366 nm.

MATERIALS AND METHODS

Raw Materials Procurement and Authentication

The raw materials (dried enrite herb parts) for the formulations were procured in crude forms from Lallubhai VrajLal Gandhi store, Ahmedabad. *Embelica officinalis* fruits procured from same place was authenticated by Morphological, microscopical characters, extractive values and Ash value. *Terminalia bellerica* pericarps were procured from same place was authenticated by Morphological, microscopical characters, extractive values and Ash value. *Terminalia chebula* fruits were also purchased from Lallubhai store and after removal of foreign matter authenticated by Morphological, microscopical characters, extractive values and Ash value. Shuddha Gandhak was procured from Shree Narayan Ayurvedic Pharmacy, Ahmedabad, and was authenticated by its Chemical test, Limit test and sulphated ash value. Shuddha Guggulu was purchased from Lallubhai Vrajlal (LVG) store and was authenticated by Morphological characters, extractive values and Ash value. The study of powdered herbs were done by examining stained and unstained powder slides under 10x and 45x magnification using student microscopes. Guggul Shodhana (Purification), Gandhak Shodhana (purification) and Preparation of SimhNadGuggul^(AFI, API) was done according to Ayurvedic Formulary of India.

Table 1: Simhnadguggul samples details

Parameters	MF1	MF2	MF3	H. F.	L. F.
Name	Sihnadgugul	Sinhanadgugul	Sihnadguggulu	Simhnadguggul	Simhanadguggul
Reference	Bhaishajya Samhita	Bhaishajya Ratnavali (P-444)	Ayurvedic Formulary of India	Ayurvedic Formulary of India	Ayurvedic Formulary of India
Formulation	Coated Tablet	Uncoated Tablet	Coated Tablet	Coated vati	Vati
Indication	Sandhivata, Ashmari, Shool, Prameh,	Amavata, Anaemia, Rectal prolapsed,	Aamvata, vatarakta (arthritis & Gout)	Vata, Shool	Arthritis

	Amlapitta.	Elephantitis.			
Dose	1 to 2 tablet with water.	2 to 4 tablets with warm water / day	1 or 2 tablets twice a day after meal	1 to 2 tablets with warm water.	1 to 2 tablets with warm water.
Quantity	25 gms.	120 tablets	80 tablets	500 gms.	-
Prize	40 Rs.	90 Rs.	145 Rs.	Not for sale	Not for sale
Manuf. license	GA/19	Ayu/157	1800/89	---	---
Shelf life	3 years	Not mentioned	5 years	Not mentioned	3 years
Manufactured by	Shree Narnarayan Ayurvedic Pharmacy, Ahmedabad	Aushadhi Bhavan, Ayurved Seva Sangh, Ganeshwadi, Nashik	Shree Baidyanath Ayurved Bhawan pvt. Ltd., Gwalior Road, Jhansi-284003	Manibai Civil Ayurvedic Hospital, Ahmedabad	Department of Pharmacognosy, L. M. College of Pharmacy

TLC Authentication of Gallic Acid, Ellagic Acid and Catechin in Simhnadguggul

Stationary phase: Silica gel G (ready plates of 0.2 mm thickness).

Spotting solution: Spotting solution: Gallic acid (1mg/5 ml) in methanol. Ellagic acid (1mg/5 ml) in methanol, Catechin 1mg/10 ml in methanol.

Test extract: Lab formulation was defatted with ether and dried. Dried content was then subjected to extraction with methanol and filtered extract was concentrated for spotting.

Band width: 10 mm, Run distance: 8 cm, Chamber saturation time: 15 min.

Temperature: 25°C, Lab room humidity considered 30-35 % w/w (measured by psychrometer)

Mobile Phase: For gallic acid and ellagic acid Toluene: Ethyl acetate: Formic acid (2:2.25:0.5)

Mobile phase: For ellagic acid Toluene: Ethyl acetate: Formic acid (2:2.25:0.5)

Mobile phase: For catechin; Toluene : Ethyl acetate : Glacial acetic acid (5:5:1)

Derivatization: For catechin; 10 % ethanolic Potassium Hydroxide (pink purplish red spot) or UV 365 nm.

For Gallic acid and Ellagic acid: 10 % alcoholic Ferric chloride solution (freshly prepared)

HPLC Method for Estimation of Gallic Acid in Simhnad guggul

As per reported method solvents for mobile phase were selected; acetonitrile (ACN), water (demineralized LC grade H2O), methanol (MeOH - AR grade) and Glacial acetic acid (GAA). All formulations (MF1, MF2, MF3, HF, LF) and standard gallic acid in methanol were studied for the chromatographic behavior in following solvent systems.

Different proportions of mobile phases used were; Acetonitrile: water (70:30), Acetonitrile: water (80:20), Acetonitrile: water (20:80), Acetonitrile: water (70:30 + 3% Glacial acetic acid), Acetonitrile: water (55:45), Acetonitrile: water (60:40). Post solvent system development and drying was followed by detection at Short UV or by derivatization using 10% alcoholic Ferric chloride solution.

Instrumentation and Chromatographic Conditions

System: Shimadzu HPLC with UV/PDA detector

Stationary Phase: Column was Phenomax Luna C18 (250 mm × 4.6 mm × 5 μm); LC- 10AS Shimadzu solvent delivery module with isocratic solvent delivery system.

Auto sampler: Shimadzu, (100 μl) injector, syringe 25 μl.

The optimized mobile phase was Acetonitrile: water (70:30) and 3% Glacial acetic acid. The flow rate of mobile phase was kept 1.0 ml/min. Injection volume 10 µL and 10 min total run time initial for checking extra contour later all study was done till 8 min run time.

Detector: SPD- 10A Waters PDA Detector was run by Waters software. Wavelength selected for detection was 255 nm. Peak identity was confirmed by spectrum and retention time comparison with respective reference standards. All the analysis was performed at 25°C temperature.

Preparation of Standard Solutions for Calibration Curve

Stock solution of standard Guggulosterone E in methanol (1 mg/mL) was prepared. From that stock solution 10, 20, 40, 60, 80, 100 µl were diluted up to 1 ml with methanol and injected to have concentrations 10, 20, 40, 60, 80, 100 µg/ml.

Formulation Treatment and Preparation of Extract

All the Lab formulation (LF), Market formulations (SNG MF1, SNG MF2, SNG HF), were accurately weighed 0.5 gm and each were dissolved in methanol 2 ml. The mixtures after sufficient sonication were filtered by cartridge in to HPLC vials and making it to final volume with methanol. Final solution was injected for estimation with injection Volume: 10.00 µl. Run Time: 10 minutes.

Validation

For checking linearity of the method, peak area were measured at concentrations 10,20,40,60,80 and 100 µg/ml concentrations for n=3 and on the bases of graph of AUC plotted against concentration. The regression equation was determined and was used for test extract concentration calculation. Interday and Intraday precision was studied as per ICH guidelines and peak area for 20, 40 and 60 µg/ml concentrations (n=3) were recorded followed by calculation of co-efficient of variance. Repeatability of scanner was studied at 40µg/mL concentration having RSD < 1 % based on seven times measurement of the same peak of single injection. Reproducibility of sample application was studied at 40µg/mL concentration having RSD < 3% based on application of seven injections. Limit of detection was observed at signal to noise ratio of 3:1. Limit of quantification was determined at signal to noise ratio of 10:1.

HPLC Method Development and Validation for Ellagic Estimation in Simhnad guggul

As there was no specific R_f value difference in gallic acid and ellagic acid analyzed by TLC/HPTLC method, so HPLC method was chosen for identification and estimation of ellagic acid. All the marketed, lab formulations were used in filtered extract form to check their behavior in chromatographic instrument under following conditions.

Instrumentation and Chromatographic Conditions

Stationary Phase: Column was Phenomax Luna C18 (250 mm × 4.6 mm × 5 µm); LC- 10AS Shimadzu solvent delivery module with isocratic solvent delivery system.

Auto sampler: Shimadzu, (100 µl) injector, syringe 25 µl. The optimized mobile phase; Acetonitrile: water (80:20) and 1% Glacial acetic acid.

The flow rate: 1.0 ml/min. Injection volume 10 µL and 10 min total run time initial for checking extra contour later all study was done till 8 min run time.

Detector: SPD- 10A PDA Detector was run by Shimadzu software. Wavelength selected for detection was 255 nm. Peak identity was confirmed by spectrum and retention time comparison with respective reference standards. All the analysis was performed at 25°C temperature.

Preparation of Standard Solutions for Calibration Curve

Stock solution of standard ellagic acid in methanol (1 mg/mL) was prepared using methanol. From that stock solution 10,20,40,60,80,100,120,140 µl were diluted up to with 1 ml methanol and injected to have concentrations 10,20,40,60,80,100, 120 and 140 µg/ml.

Formulation treatment and preparation of extract

All the Lab formulation, Market formulations (SNG MF1, SNG MF2, SNG MF3), were accurately weighed 0.5 gm and each were dissolved in methanol 2 ml. The mixtures after sufficient sonication were filtered by cartridge in to HPLC vials. Final solution was injected for estimation with injection Volume: 10.00 μ l. Run Time: 8 minutes.

Validation

For checking linearity of the method, peak area were measured at concentrations 20,40,60,80, 100 and 120 μ g/ml concentrations for n=5 and on the bases of graph of AUC plotted against concentration followed by regression equation determination. Interday and Intraday precision was studied as per ICH guidelines and peak area for 20, 40 and 60 μ g/ml concentrations (n=3) were recorded followed by calculation of coefficient of variance. Repeatability of scanner was studied at 40 μ g/mL concentration having RSD < 1 % based on seven times measurement of the same peak of single injection. Reproducibility of sample application was studied at 40 μ g/mL concentration having RSD < 3% based on application of seven injections. Limit of detection was observed at signal to noise ratio of 3:1. Limit of quantification was determined at signal to noise ratio 10:1 of the instrument.

RESULT AND DISCUSSION

Identification and Authentication of Crude Drugs

Alcohol extractive value (by cold extraction method WHO guideline) for dried amla fruits, baheda pericarps and harde fruits were found 14.8, 12.04 and 17.66 % w/w.

Water extractive value for dried amla fruits, baheda pericarps and harde fruits were found 12.28, 8.75 and 8.22 % w/w. All drugs are found to be authentic in quality. They don't appear aged or rotten or tanned or covered by micro-organisms. Extractive values of individual herbs are within the range prescribed in Indian Pharmacopeia 2008. Triphala powder needed further confirmation for authenticity, so powder microscopy was studied.

TLC authentication Results in Simhnad Guggul

A thin band at 0.65 R_f without tailing, band broadening and merging with no spot reflects that solvent system is most appropriate. TLC results show that catechin and ellagic acid are present Simhnadguggul formulations. Each compounds in formulation show single separated band with slight broadening without interference. Catechin is with no tailing and no band broadening, while ellagic acid band is with little tailing due to excess spotting incorporation. The HPTLC method can't be developed with optimized solvent system as both compounds catechin and ellagic acid come at same R_f thus making them impossible to quantify separately. Retention factor of catechin was found 0.4 and for ellagic acid it as 0.48.

HPLC Method Result for Gallic Acid in Simhnad Guggul

Gallic acid did not show complexity in measurement, except the solutions were older than a day. All the method development, validation and estimations were done using freshly prepared solutions of standard gallic acid and test extracts. HPLC chromatogram of gallic acid in extract in figure reflects retention time 2.776 minute with absolute symmetry and no interference. Tailing factor was found <1 (0.904) with peak purity index 0.9677. UV over laid spectra of test extracts and standard gallic acid have super impossible spectra with same λ_{max} (250 nm \pm 0.5nm)

Validation Results for Gallic Acid

Linearity graph of F.3 shows linearity with 0.996 regressions between 40 to 100 μ g/ml concentration ranges. Method linearity equation is mentioned in graph. Accuracy results of the method are shown in terms of % recovery which indicated that method is accurate with % recovery of gallic acid (in test extract plus standard gallic acid) between 97.643 to 99.7956 and RSD between 0.187 to 0.7078 range.

Repeatability of the method for scanning single peak seven times at 50 µg/ml concentrations was found repeatable with co-efficient of variance 1.2089 (<2). Method was also found of reproducibility for (n=7) sample application with appropriate co-efficient of variance; 1.7597. Method precision study results of Table 3 showed method to be precise for intraday trials with co-efficient of variance less than 1. Method is not only precise intraday but also interday. Interday variations also affect the method to less extent having co-efficient of variance between 0.8884 to 1.3020 (<1.5). Method for gallic acid estimation in methanolic extract of Simhnad guggul formulation was found appropriate by HPLC method. The method had limit of detection 10 µg/ml and limit of quantification 20 µg/ml. Method is sensitive, precise, accurate, repeatable, reproducible and economical. As the peak of gallic acid in HPLC chromatogram was found symmetrical and pure (without interference and tailing) proved that the method is very specific too.

HPLC Method Result for Ellagic Acid in Simhnad Guggul

Solvent system trial results; Acetonitrile: water (70:30) at 1 ml/min flow rate showed single peak 2.64 minute time with slight tailing. With peak purity index >0.88 and Tailing factor: 1.44

HPLC chromatogram in Figure F.2 show symmetrical peak with slight tailing but with no interference of other constituents in test extract.

Validation Results for Ellagic Acid

The method showed linearity between concentration range of 20 to 100µ/ml of ellagic acid in methanol. Linearity was measured for concentration 10 to 120 µg/ml concentration for n=3. Co-efficient of variance was found lowest 0.5674 and highest 1.5512. Repeatability and reproducibility results of Table 1 have shown co-efficient of variance <2 means tolerable reproducibility. The method was found to be precise for intraday precision study with co-efficient of variance between 0.5273 and 0.7407. The method was found to be precise for interday precision with co-efficient of variance between 1.0853 and 1.4156, ultimately below 2. Method for ellagic acid estimation in Simhnad guggul formulation using HPLC technique was found linear with 0.99 r value and specific. Method was found repeatable, reproducible, precise, accurate and sensitive with limit of quantification 10µg/ml. Method needed solvents, basic apparatus and ideal chromatographic conditions. Recovery study results for this method were found appropriate (Table T.3) that % recovery was found above 98% of actual concentration in test extract of the formulation. Co-efficient of variance for accuracy study have shown results between 0.1926 and 0.5196. Results are calculated as average for n=3 + standard deviation. Ellagic acid being one of the important hydrolysable tannin with great anti-oxidant potential been found with permissible limits in all the formulations. Repeatability of scanner at same wavelength for same peak, seven times have shown co-efficient of variance 0.4045 (less than 1). Reproducibility of the method was studied for sample application seven times keeping scanning wave length and concentration (60µ/ml) same.

Yield of Gallic Acid and Ellagic Acid in Formulations

Gallic acid yield depends on presence of pseudo tannin in formulation, it immediately degrades in presence of oxygen or metal if kept in form of solution for more than a day. So all the extracts were freshly prepared and all the formulations were found to have nearly same content of gallic acid. Ellagic acid being one of the important hydrolysable tannin with great anti-oxidant potential been found with expected limits in all the formulations.

CONCLUSION

Simhnad Guggul formulation is rich in tannins as it contains Triphala, so gallic acid, catechin and ellagic acid being most common of tannins and having profound antioxidant profile, they need to be authenticated by TLC for their presence. Present study has provided TLC authentication method and validated, economical and reliable HPLC methods for estimation of gallic acid and ellagic acid were developed which

found to be sensitive, accurate and precise for their quantification in a complex ayurvedic formulation Simhnad Guggul.

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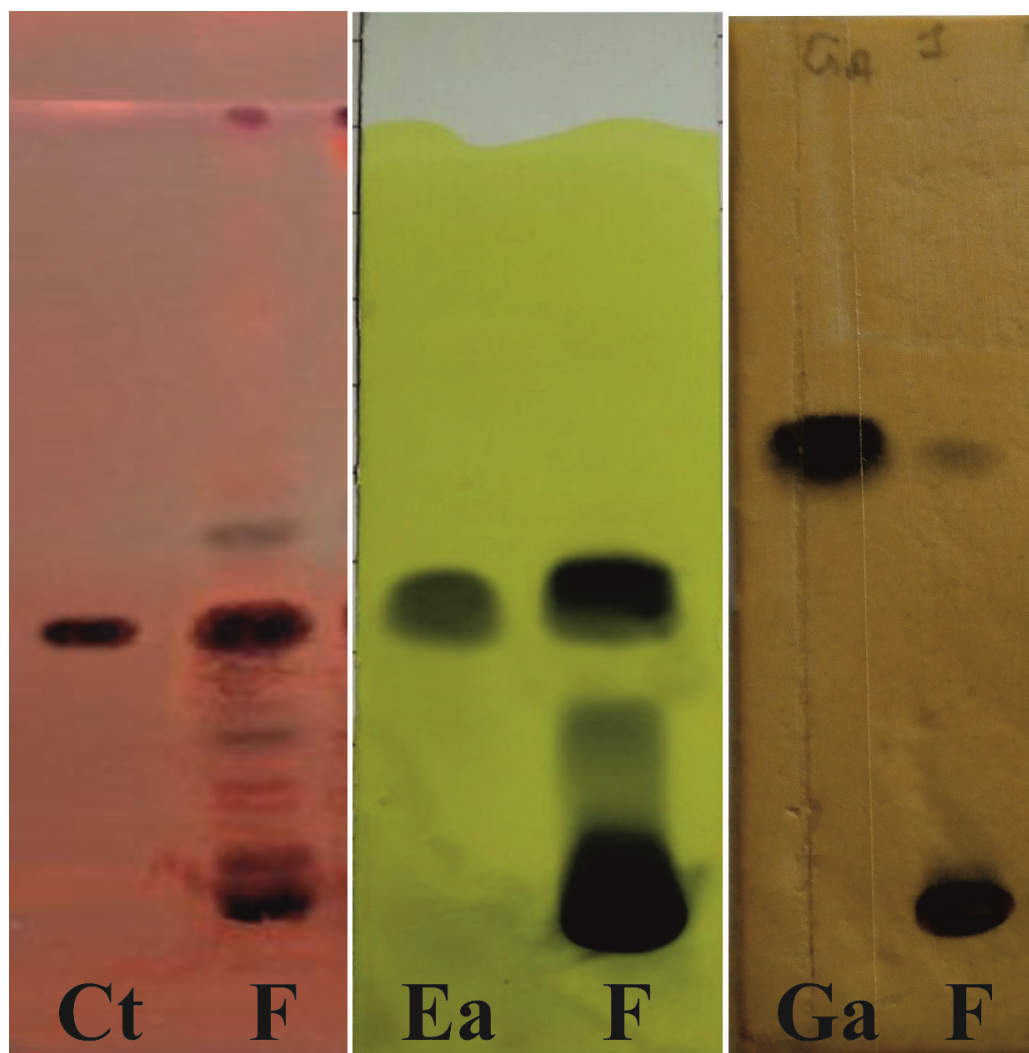


Figure 1: TLC result of Std. Gallic Acid [GA] Catechin [CT] and Ellagic Acid [EA] in alc. extract of Simhnad Guggul formulation [F]

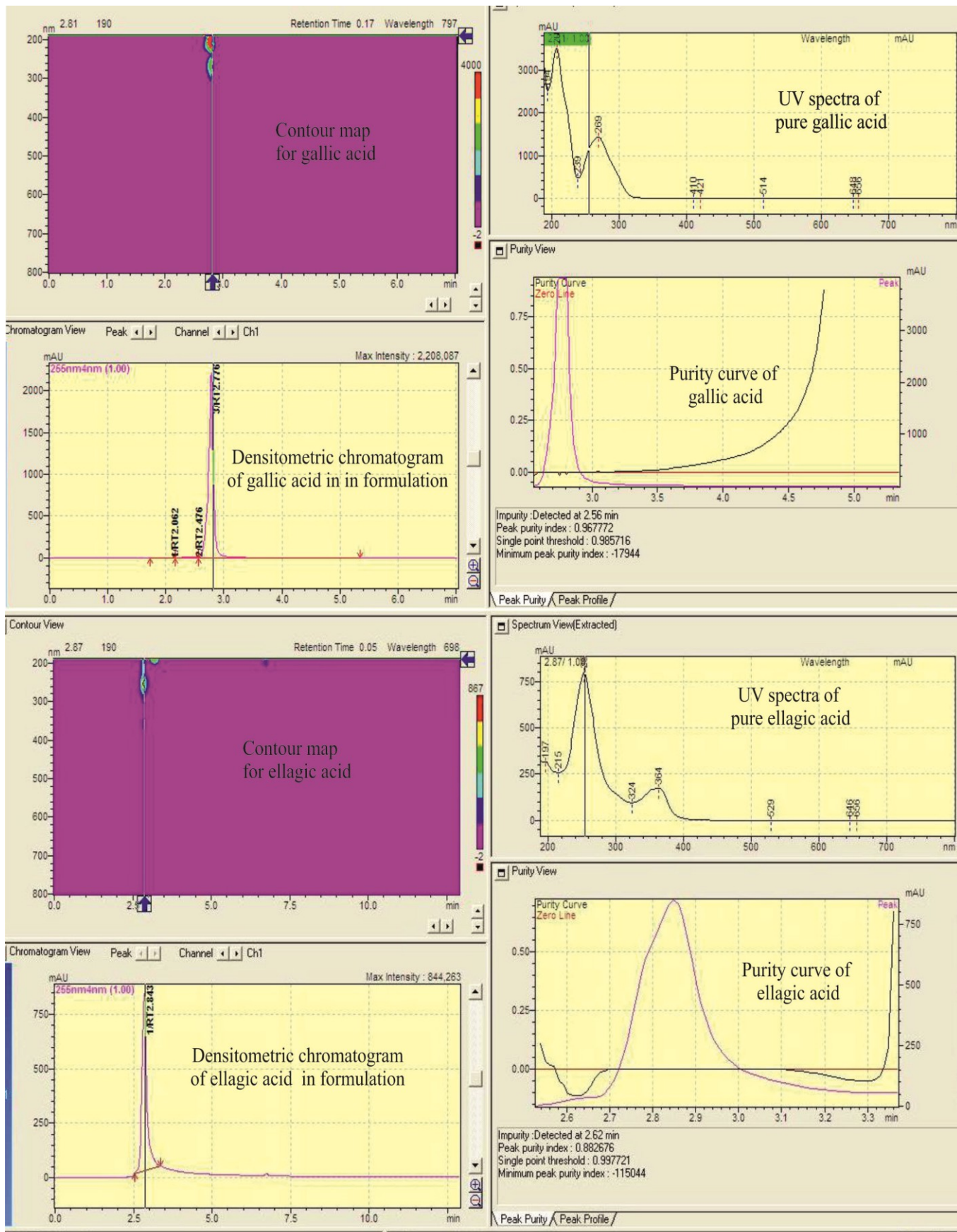


Figure 2: HPLC study results for Gallic Acid and Ellagic Acid

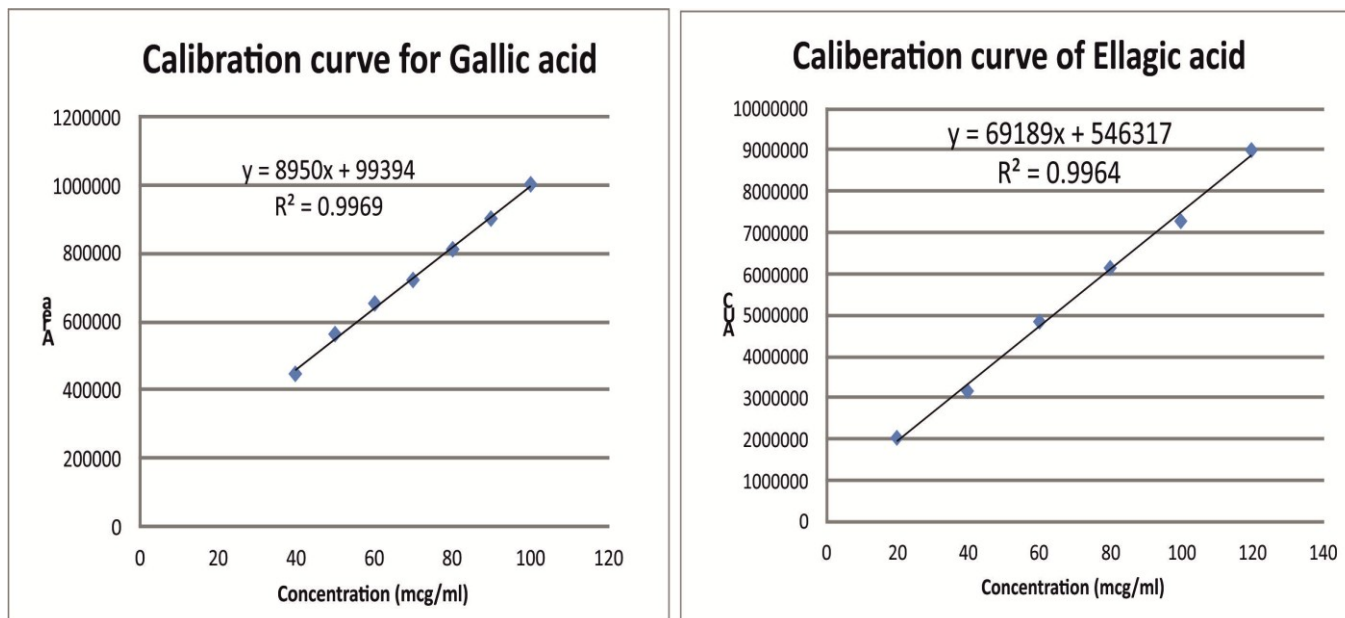


Figure 3: Linearity graph for Gallic Acid and Ellagic Acid

Table 2: Repeatability and Reproducibility Results

	Ellagic acid		Gallic acid	
	Repeatability	Reproducibility	Repeatability	Reproducibility
	4814318	4787482	559416	554416
	4789245	4813375	545723	539411
	4780465	4842096	561023	542767
	4801668	4776158	557869	530189
	4770465	4630125	547162	549701
	4810086	4782560	560152	530888
	4764523	4597852	549138	532746
Average	4790110	4747093	554354.71	540016.86
Standard deviation	19378.04	94080.83	6701.9538	9502.9509
co-var	0.4045	1.9818	1.2089	1.7597

Table 3: Intraday and Interday Precision Study Results

		Conc. µg/ml	1	2	3	Avg.	std. dev.	co-var.
Gallic acid	Intra- day precision	50	564263	560751	567621	564211.7	3435.288	0.6088
		60	649126	652459	659924	653836.3	5529.194	0.8456
		70	718862	721428	731426	723905.3	6638.253	0.9170
	Inter- day precision	50	560456	562246	571726	564809.3	6056.503	1.0723
		60	648236	652459	659715	653470	5805.898	0.8884
		70	716256	720456	734266	723659.3	9422.634	1.3020
Ellagic acid	Intra- day precision	40	3126692	3145986	3163407	3145362	18365.46	0.5838
		60	4792482	4813375	4843026	4816294	25398.15	0.5273
		80	6060014	6104820	6150456	6105097	45221.63	0.7407
	Inter- day precision	40	3110782	3145986	3182045	3146271	35632.35	1.1325
		60	4762753	4813375	4867241	4814456	52252.39	1.0853
		80	6024347	6104820	6197164	6108777	86476.43	1.4156

Table 4: Recovery Study Results

	Test µg/ml	STD µg/ml	Actual µg/ml	Average AUC (n=3)	Std. dev.	Co- var.	Conc. (µg/ml)	% Recovery
Ellagic acid	44.6335	20	64.6335	4478928	16705.03	0.3729	63.9450	98.9349
	44.6335	40	84.6335	5908776	11380.29	0.1926	84.6109	99.9733
	44.6335	60	104.6335	7166839	37241.72	0.5196	102.7939	98.2418
Gallic acid	Test Conc. µg/ml	Std	Total Conc.	Avg AUC (n=3)	std. dev.	Co-var	Practical Conc.	% Recovery
	36.33	16	52.33	556708.6	3940.663	0.7078	51.0966	97.6430
	36.33	32	68.33	709698	1329.383	0.1873	68.1903	99.7956
	36.33	48	84.33	851937.3	1665.471	0.1954	84.083	99.7071

Table 5: Validation result summary

Parameters	For Gallic acid	For Ellagic acid
Linearity range	40 to 100 µg/ml	20 to 140 µg/ml
Correlation co-efficient	0.996	0.996
Repeatability	1.2089	0.4045
Reproducibility	1.7597	1.9818
Interday	0.8884-1.3020	1.0853-1.4156
Intraday	0.6088-0.9170	0.5273-0.7407
Accuracy (% recovery)	97.6430-99.7956	98.2418-99.9733
Limit of detection	10 µg/ml	5 µg/ml
Limit of quantification	20 µg/ml	10 µg/ml
Limit of Linearity	100 µg/ml	140 µg/ml
Specificity	specific	specific

Table 6: Yield of Gallic Acid and Ellagic Acid

	Total Gallic acid (% w/w)	Total Ellagic acid (% w/w)
MF1	0.9836 ± 0.0108	0.4009 ± 0.0009
MF2	0.8525 ± 0.0034	0.3803 ± 0.0041
MF3	0.8215 ± 0.0028	0.4304 ± 0.0049
HF	0.9012 ± 0.0040	0.4157 ± 0.0004
LF	1.0126 ± 0.0114	0.4387 ± 0.0011
Triphala	3.824±0.011	1.0251 ± 0.0058

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