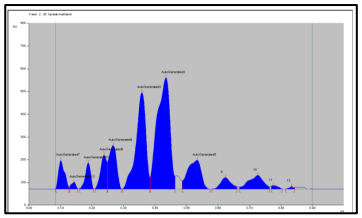
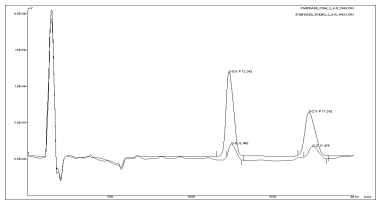
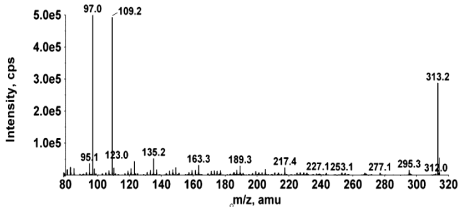
Identification of Biomarker/S from Polyherbal Formulation used in Hyperlipidemia for Qualitative and Quantitative Analysis



Dr. Charmy Kothari

Assistant Professor, Department of Pharmaceutical Analysis

NIRMA UNIVERSITY
INSTITUTE OF PHARMACY

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CONTENTS

Introduction

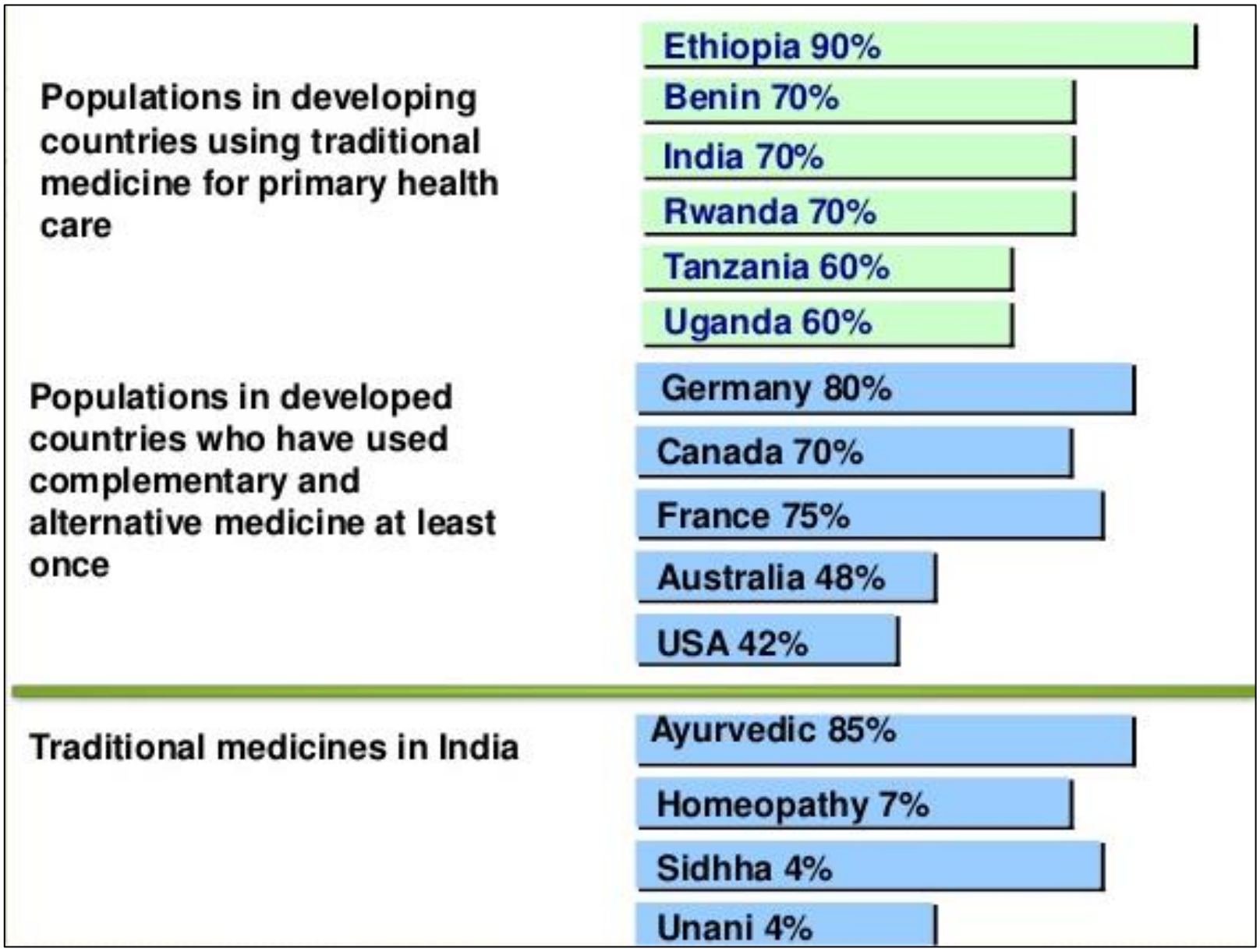
80 %

of the world population still rely on traditional medicine as their primary health care

-WHO estimate, Traditional Medicine, 2013

- **High cost (almost US $ 2000 million), long time (15-20 years) taken in the development of any new synthetic drug.**
- **The several side effects and adverse reactions and poor success are also associated with them.**
- **Inadequate, especially in the management of certain chronic diseases like asthma, cardiac diseases and diabetes.**
- **More than 45,000 plant species found in the ecosystem of the INDIA and only a small percentage of plants has been investigated phyto-pharmacologically.**

Use of Traditional Medicine World Wide



- Herbal oriented pharmaceutical companies are investing crores of rupees on research and development and popularizing OTC remedies at the same time.
- The World Health Organization emphasized the need to ensure quality control of medicinal plants with appropriate modern techniques and suitable standards.
- WHO gives different quality control parameters to standardize the raw material as well as finished products.
- Chemical assay gives the quantitative evaluation of active constituents by using different techniques includes HPTLC method also.

Preparations is challenging for several reasons

- As Analytes, herbs are complex. Even herbal preparations such as extracts contain numerous compounds in concentration that can cover several orders of magnitude.
- The requirements of a fingerprint analysis can be completely different from those for a quantitative determination of marker or key compounds, even after separation of the herbal preparation by fingerprinting. It is necessary to fully separate those compounds from all others.
- Constituents of herbals that belong to very different classes of chemical components can often create difficulties in detection. Keeping the above facts in view, It is required to take initiative for development of advanced quantitation using chromatographic methods.

The products proposed for analytical development of poly Herbal Capsule Formulation containing,

Arjuna (Terminalia Arjuna)	**500 mg**
Pushkarmoola (Inula Racemosa)	**500 mg**
Guggulu (Commiphora Mukul)	**500 mg**

Pharmacology: Cardio-protective, Anti-anginal & Hypolipidemic

Dosage: One capsule twice daily

Packing: 10 capsules in a blister

STORAGE: Store in a cool, dry place. Protect from light

Chemical constituents

ARJUNA [*Terminalia Arjuna*]

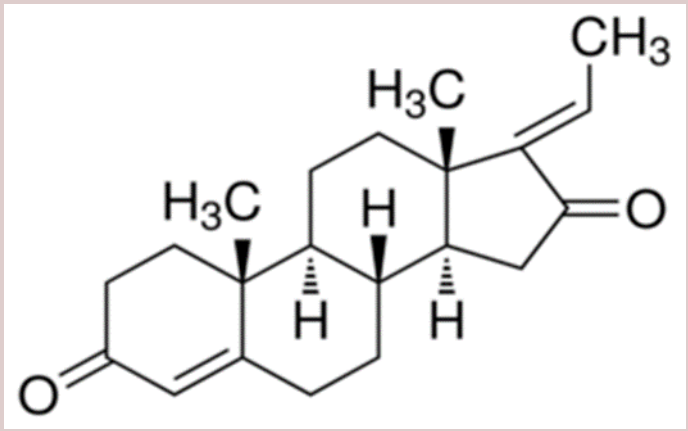
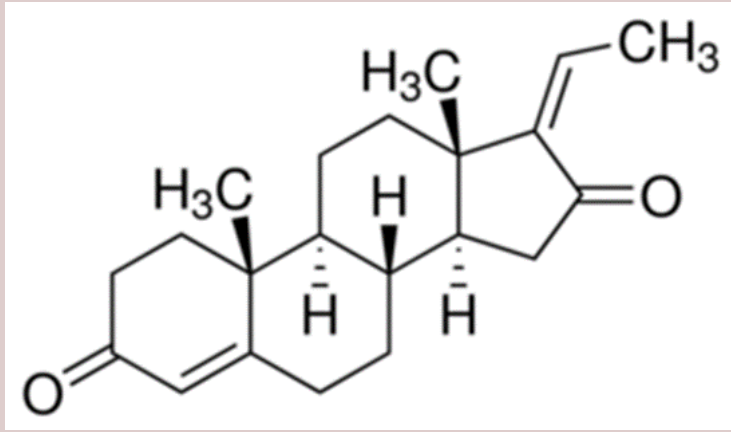
- Triterpene glycoside(Arjunetin, Arjunetoside), Saponin triterpenoid (Arjunic acid, Arjunolic acid, Arjungenin), Flavonoid and Tannins

GUGGUL [*Commiphora Mukul*]

- Gum resin, **Guggulsterone E & Z**, Cambrane-A, Sesanin

PUSHKARMOOLA [*Inula Racemola*]

- Sesquiterpene lactonol (Alantolactone, Isoalantolactone, Dihydroalantolactone, Beta-sitosterol

GUGGULSTERONE-E	GUGGULSTERONE-Z
	
CAS Number: 39025-24-6	CAS Number: 39025-23-5
M. W. : 312.45 gm/mol	M. W. : 312.45 gm/mol
Appearance: White to off White Powder	Appearance: Light Yellow Powder

- It is obtained from gum of plant *Commiphora Mukul* (burseraceae)
- Chemical Formula: $C_{21}H_{28}O_2$
- Index of Refraction: 1.557
- Boiling Point: 463.3 ˚C at 760 mm Hg
- Melting Point: 163.5 ˚C
- Density: 1.1 g/cm^3
- Vapour Pressure: 9.21 E-09 mmHg at 25 ˚C
- Storage Temperature: 2-8 ˚C
- Solubility: Soluble in DMSO, Methanol, Ethanol & DMF. Sparingly soluble in Ethyl Acetate & Pet. Ether.
- Application: Maintain cholesterol level, reduced cholesterol, triglyceride & fatty lipid level.

Aim and Objective

- STANDARDIZATION consists of physicochemical parameter testing, phytochemical tests & Quantitative estimation by suitable analytical techniques.
- Estimation of Guggulsterone E and Z by HPTLC & HPLC Chromatographic method.
- Till date, Arjuna, Guggul & Pushkarmoola has not been standardize in the combined poly-herbal formulation.
- So, that the aim of present work is to Identifiction of biomarker/s from lipistat-polyherbal Formulation for quantitative analysis

Materials

Arjuna, Guggul & Pushkarmoola powder was purchased from LVG Health Care Private Limited(Ahmedabad, India).

Standard Guggulsterone E (>98% Purity) & Guggulsterone Z (>95% Purity) was purchased from Sigma Aldrich (Ahmedabad, India).

Preparation of Powder Extracts

The powder is dried in shade under normal environmental condition and homogenized to coarse powder and stored in opaque screw tight jars until use. Powdered drug was charged into soxhlet apparatus and extraction was carried out with following solvents successively.

1) Petroleum ether, 2) Benzene 3) Chloroform, 4) Ethyl acetate, 5) Methanol and 6) Water

Preliminary Phytochemical Screening

QUALITATIVE CHEMICAL EVALUATION OF EXTRACTS

Sr. No.	Phyto-Constituents	Extracts					
		Pet. Ether	Benzene	Chloroform	Ethyl Acetate	Methanol	Water
1.	Steroid	-	-	-	+	+	-
2.	Saponin	+	-	+	+	+	+
3.	Alkaloids	-	-	-	+	-	-
4.	Tannin	-	-	-	-	-	-

(+) = Found to be Present and **(-)** = Found to be Absent

Physical Parameters of Herbal Capsules

Parameter	Observation
Average Weight	1095 mg
Length	24 mm
Thickness	1mm
Shape	Oblong
Tensile Strength	702 g/mm^2
Disintegration time	14 min

PHYSICOCHEMICAL PARAMETERS

SR. NO.	TEST	OBSERVATION & VALUES			
		HERBAL FORMULATION	ARJUNA	GUGGUL	PUSHKARMOO -LA
1.	Color	Dark Yellow	Pale Greenish Yellow	Dark Brown	Brown
2.	Odour	Characteristic	Characteristic	Characteristic	Characteristic
3.	Loss on Drying	4.97 %	6.64 %	5.34%	4.63%
4.	Limit test of Arsenic	Passes	Passes	Passes	Passes
5.	Total Ash Value	10.73 %	16.53 %	3.4 %	9.6%
6.	Acid insoluble ash	0.43 %	0.94 %	0.63 %	0.57%
7.	Alcohol soluble extractive	33.12 %	46.38 %	28.93 %	41.63%
8.	Water soluble extractive	41.34 %	43.48%	37.83 %	36.33%

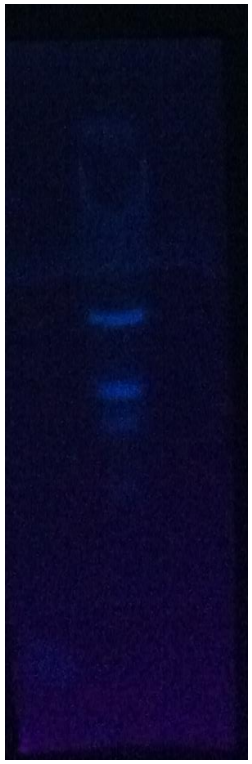
TLC Method

Optimization Of Mobile Phase

SR. NO.	TRIAL	OBSERVATION	REMARK
1.	Toluene : Ethyl acetate (6:4,v/v)	Very high RF Value	Not Satisfactory
2.	Toluene : Ethyl acetate : Formic Acid (6.5:2.5:0.5,v/v/v)	Very high RF Value Tailing	Not Satisfactory
3.	Toluene : Ethyl acetate : Formic Acid (6.8:2.5:0.7,v/v/v)	Variation in RF value	Not Satisfactory
4.	Toluene : Ethyl acetate (6.5:3.5,v/v)	Very high RF Value	Not Satisfactory
5.	Toluene : Ethyl acetate (7:3,v/v)	Variation in RF value	Not Satisfactory
6.	Toluene : Ethyl acetate (7.5:2.5,v/v)	Variation in RF value	Not Satisfactory
7.	Toluene : Ethyl acetate (7.7:2.3,v/v)	High RF Value, Tailing	Not Satisfactory
8.	Toluene : Ethyl acetate (7.9:2.1,v/v)	Tailing	Not Satisfactory
9.	Toluene : Ethyl acetate (8:2,v/v)	Good resolution, Compact spot	Satisfactory

TLC PLATE TRIAL RESULT

[Trial:1]



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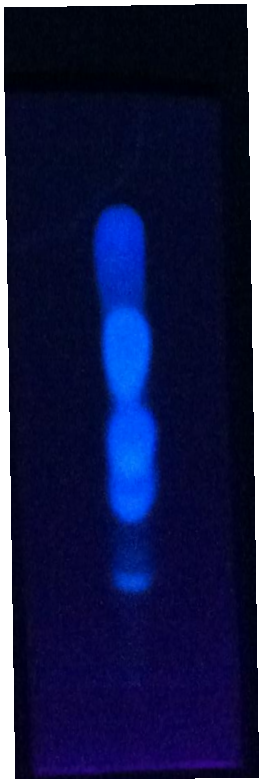
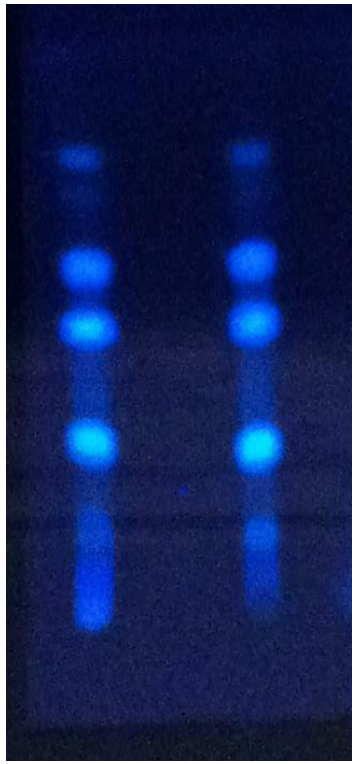


[Trial:7]



[Trial:9]

Optimized Chromatographic Condition

- Stationary phase: Pre-coated silica gel on aluminum plate 60F-254
- Mobile phase: Toluene: Ethyl Acetate (8:2, v/v/v)
- Quantity of mobile phase: 10 ml
- TLC chamber saturation time: 20 min
- Temperature: 25 ˚C

Thin Layer Chromatography - Spot Detection

Test Extract	Solvent System	Numbers of Spot	R_f Value
Petroleum Ether	Toluene: Ethyl Acetate (8:2, v/v)	03	0.62, 0.27, 0.41, 0.54
Chloroform	Toluene: Ethyl Acetate (8:2, v/v)	01	0.39
Ethyl Acetate	Toluene: Ethyl Acetate (8:2, v/v)	05	0.38, 0.41, 0.45, 0.56,0.71
Water	Toluene: Ethyl Acetate (8:2, v/v)	01	0.34
Methanol	**Toluene: Ethyl Acetate (8:2, v/v)**	**05**	**0.2, 0.34, 0.41, 0.61,0.69**

- **Methanolic extract was selected for further analysis**

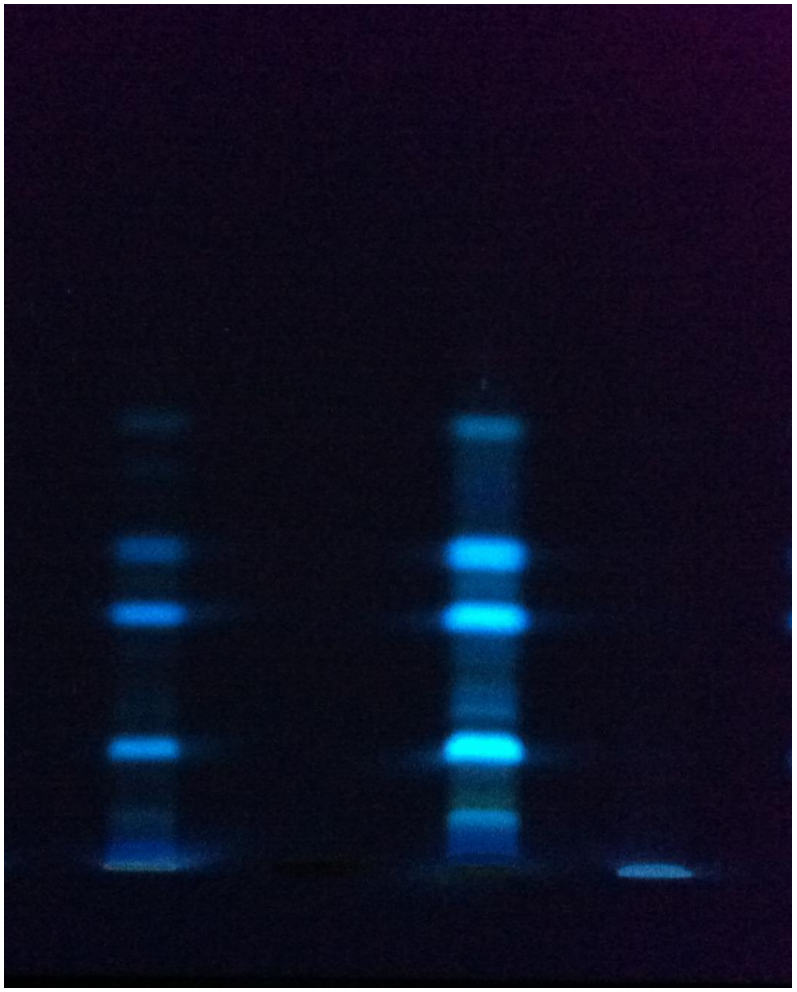
HPTLC Method

Optimized chromatographic conditions

- Stationary phase: Precoated silica gel on aluminum HPTLC plate 60F254
- Mobile phase: Toluene: Ethyl Acetate (8:2, v/v/v)
- Quantity of mobile phase: 10 ml
- TLC chamber saturation time: 20 min
- Run length: 80 mm
- Application rate: 0.1 µl/s
- Scanner band width: 5 mm
- Scanning speed: 20 mm/s
- Detection: UV detector at 251 nm
- Temperature: 25 ˚C

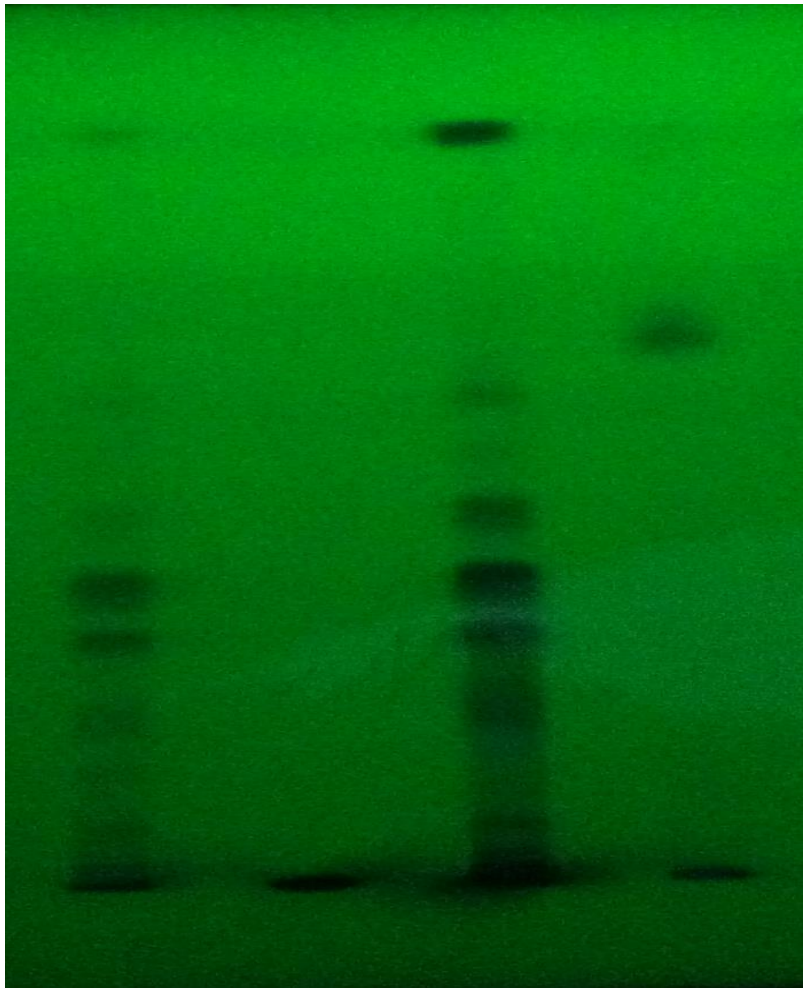
HPTLC Plate of Herbal Formulation & Arjuna, Guggul, Pushkarmoola extract

(Under UV light at 366 nm)



F A G P

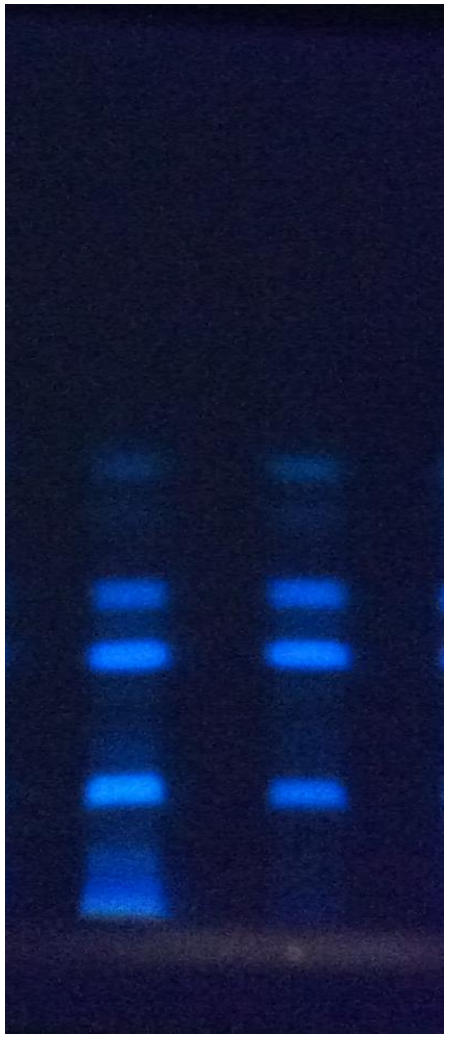
(Under UV light at 254 nm)



F A G P

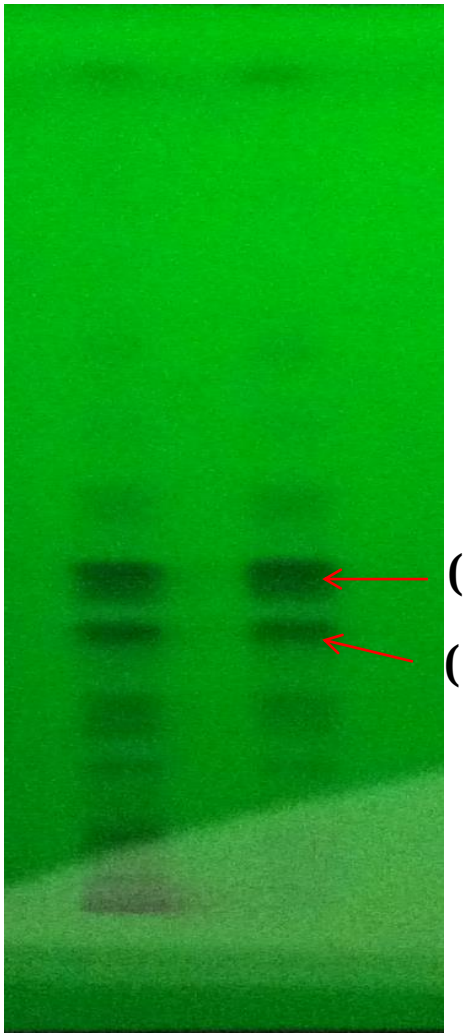
HPTLC Plate of Herbal Formulation & Guggul extract

366 nm



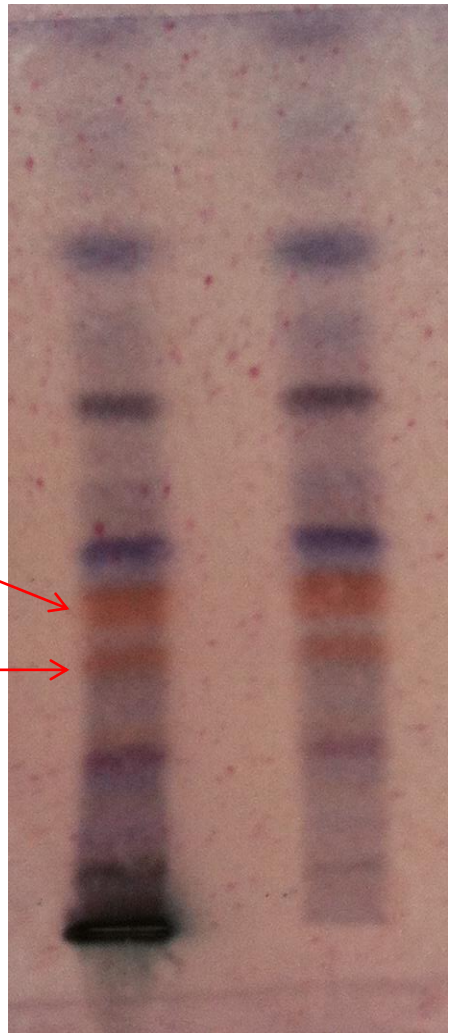
F G

254 nm



F G

(Under day light after spraying with anisaldehyde sulphuric acid reagent)

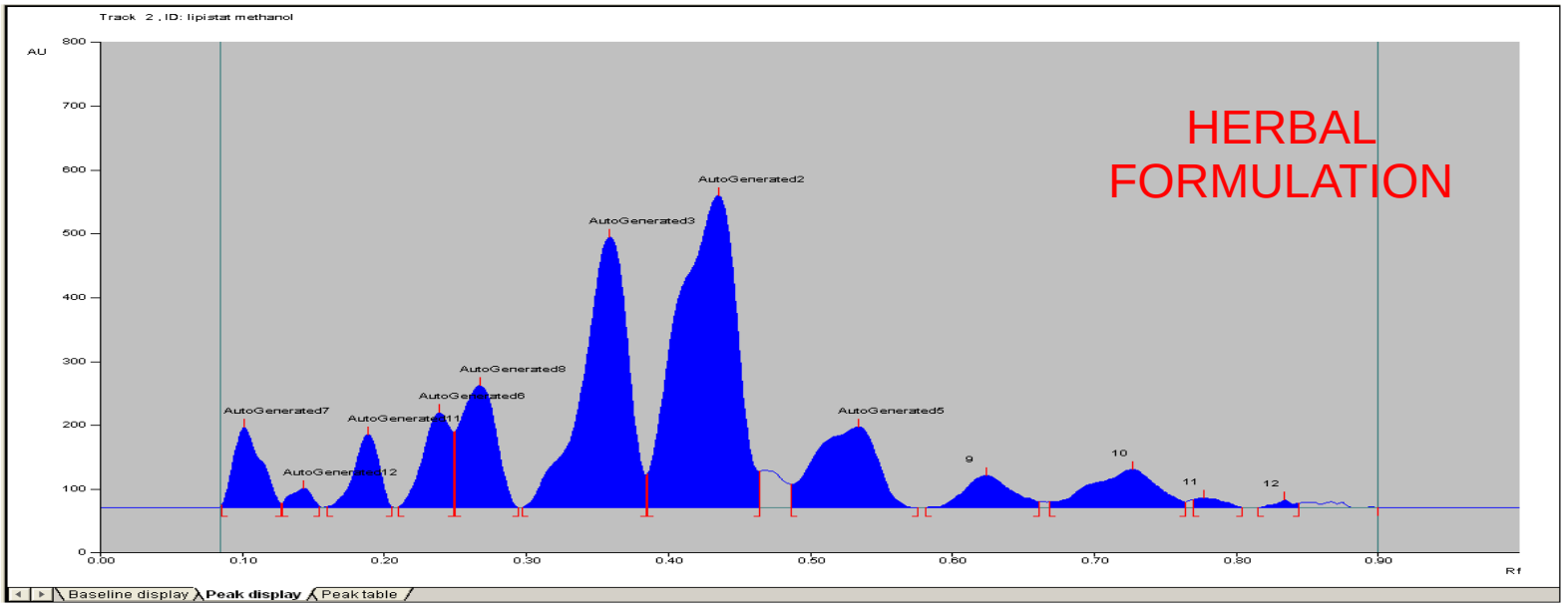


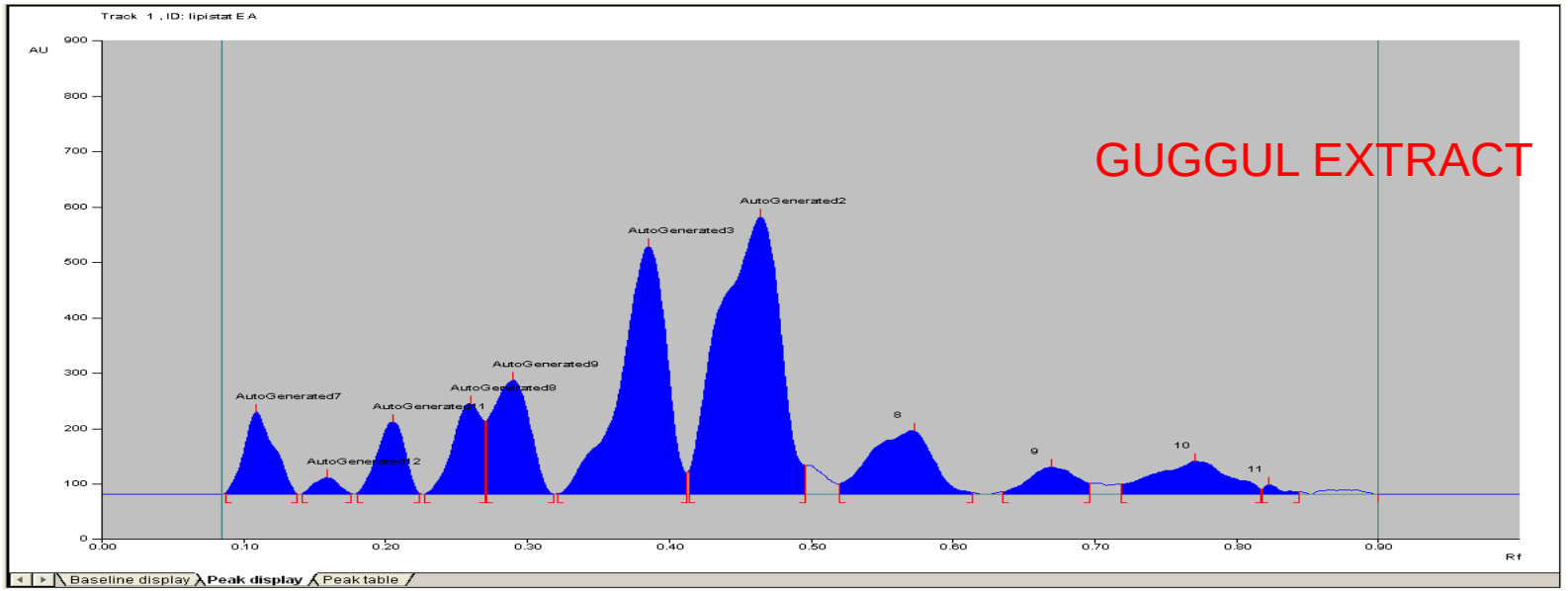
(R_F value: 0.41)

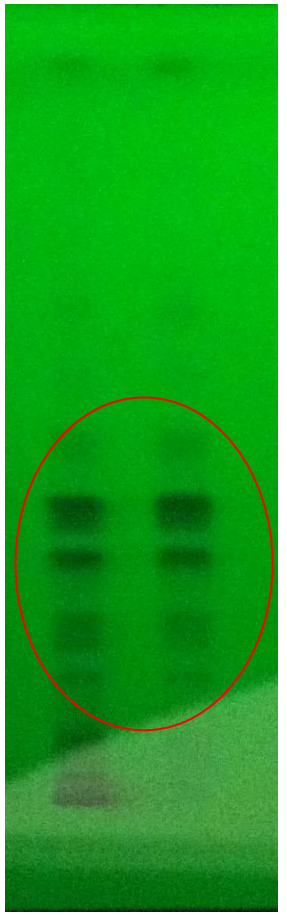
(R_F value: 0.34)

F G

HPTLC Chromatogram of Herbal Formulation & Guggul Extract (R_F value: 0.34 and 0.41) (1.0 µg/spot)

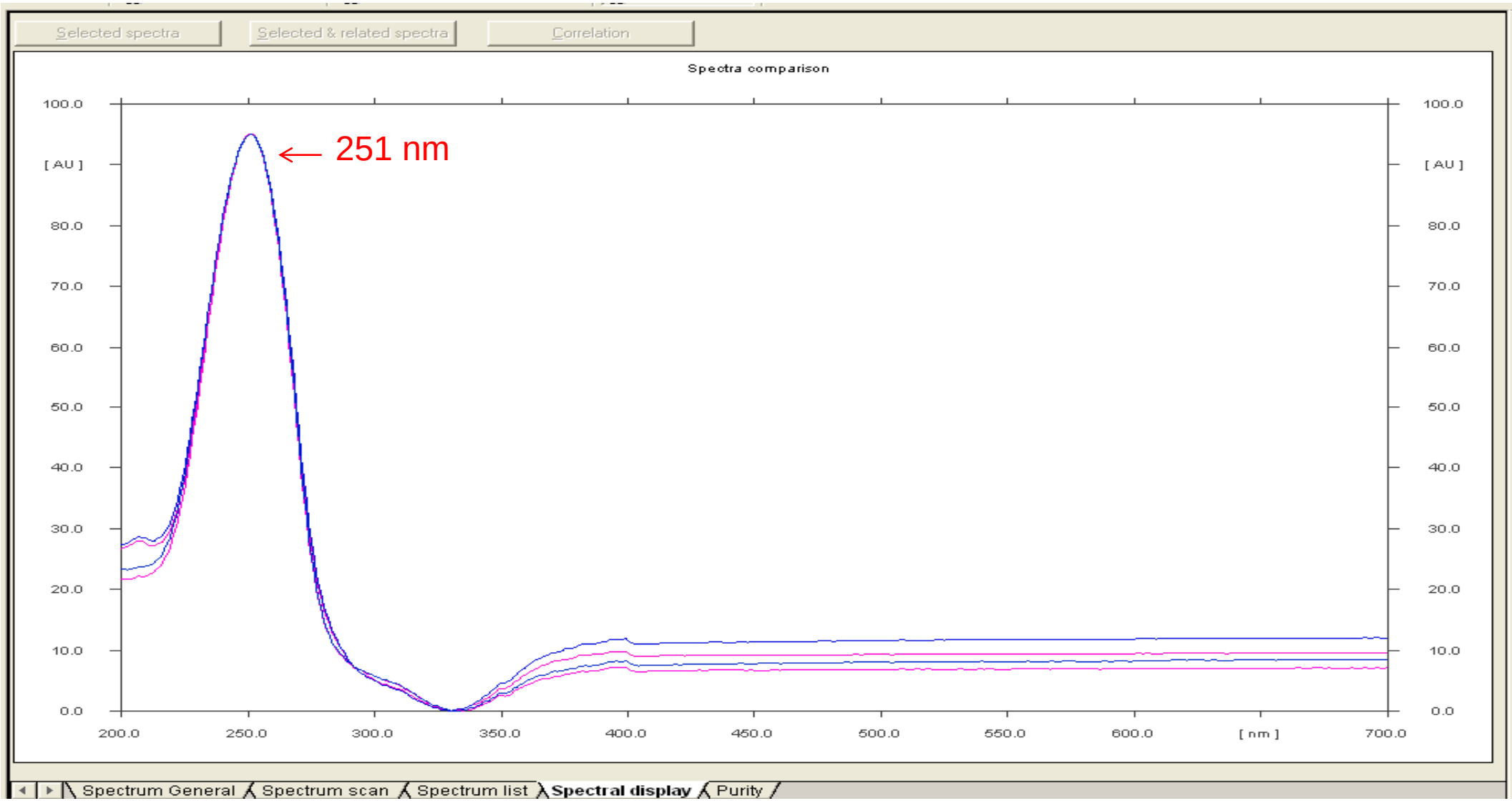






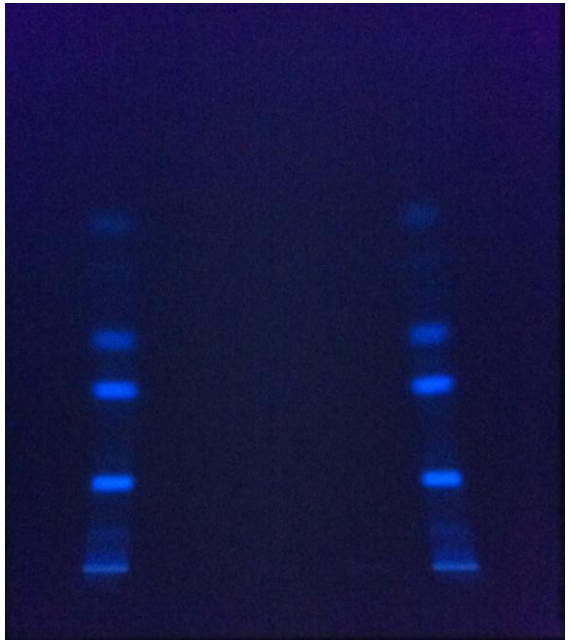
F G

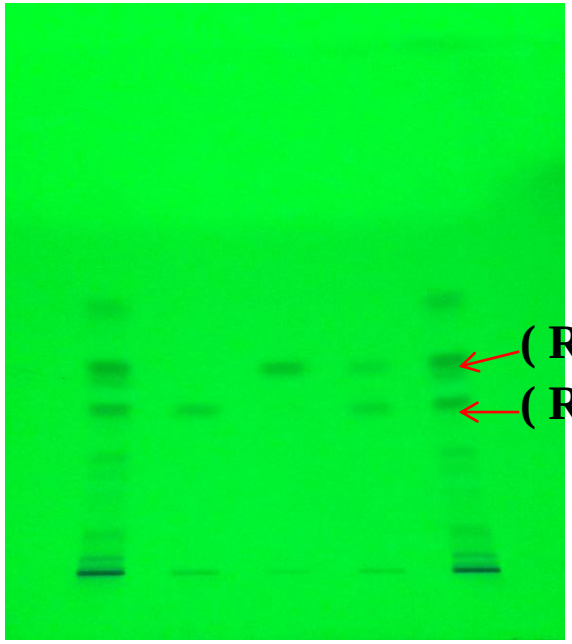
OVERLAY UV SPECTRA OF HERBAL FORMULATION & GUGGUL EXTRACT BY HPTLC

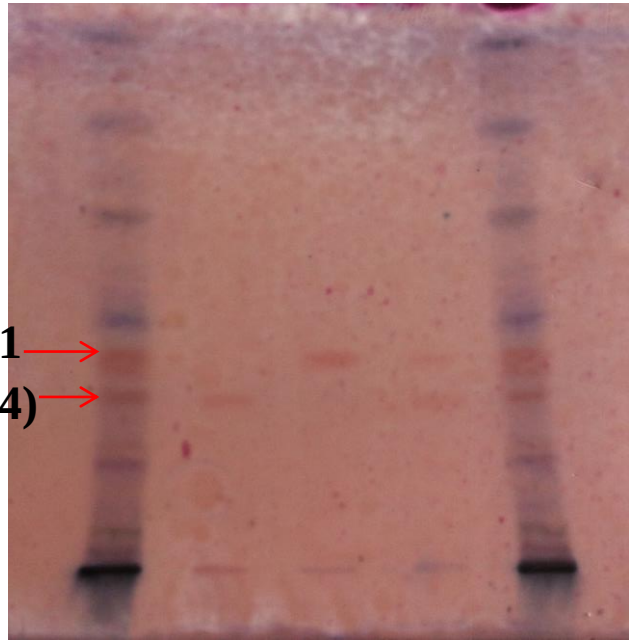


HPTLC results in optimized condition of Herbal Formulation, Guggul extract & Standard Guggulsterone E & Z

366 nm **254 nm** (Under day light after spraying with anisaldehyde sulphuric acid reagent)



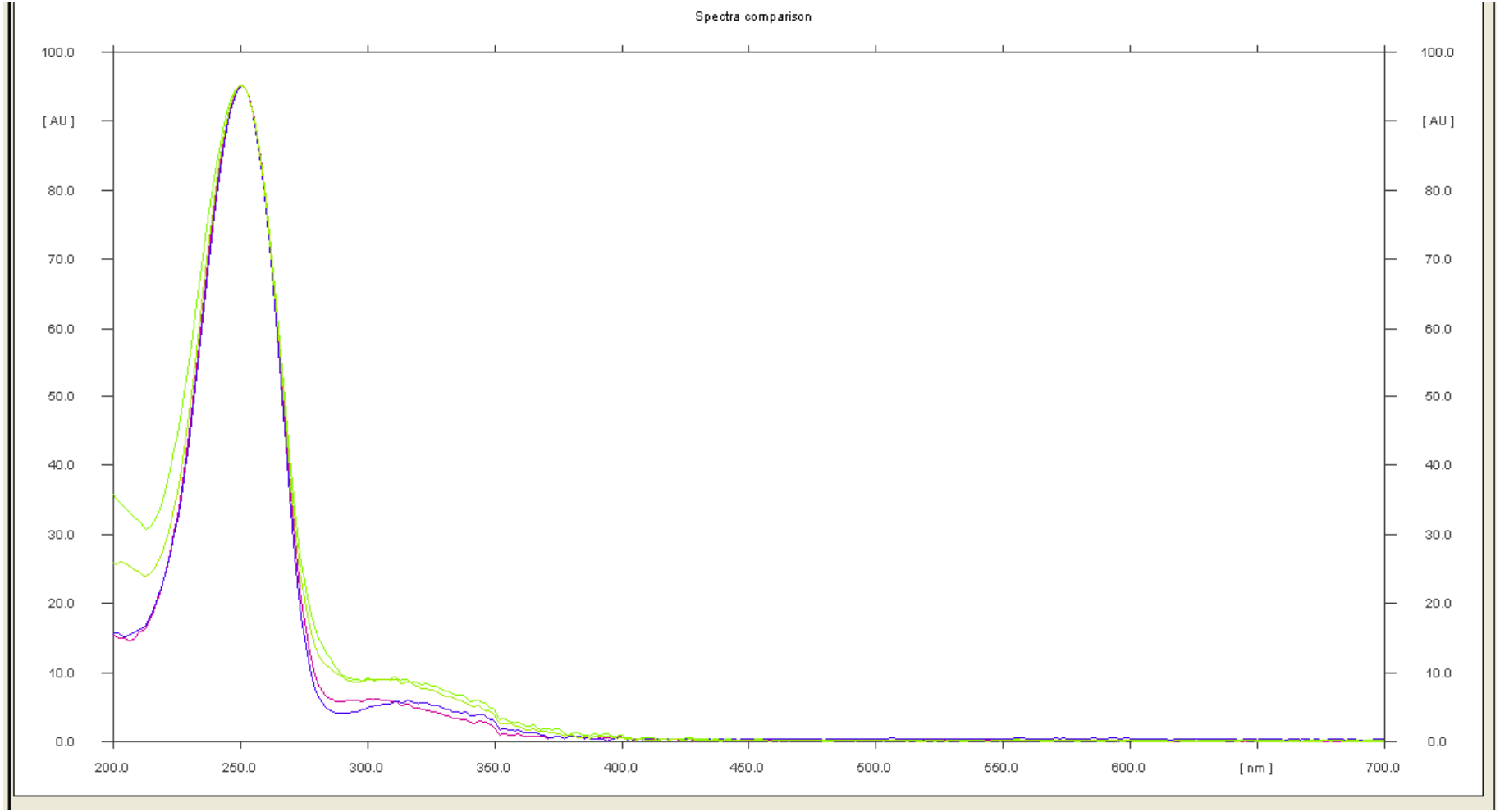


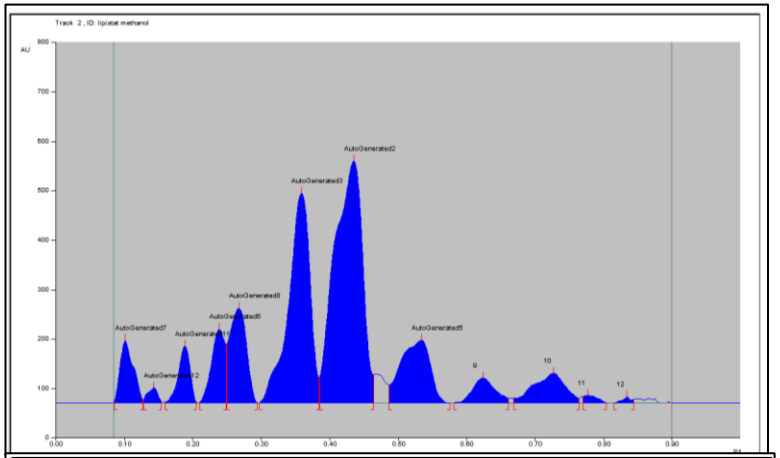


1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

[1: Guggul extract, 2: Guggulsterone E, 3: Guggulsterone Z, 4: Mixture of Guggulsterone E & Z and 5: Herbal Formulation]

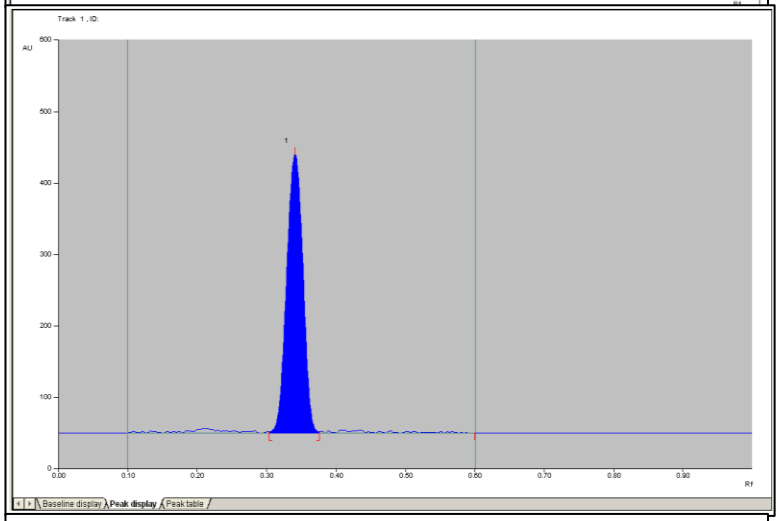
Overlay of standard Guggulsterone E & Z and herbal formulation [Maximum Wavelength : 251 nm]





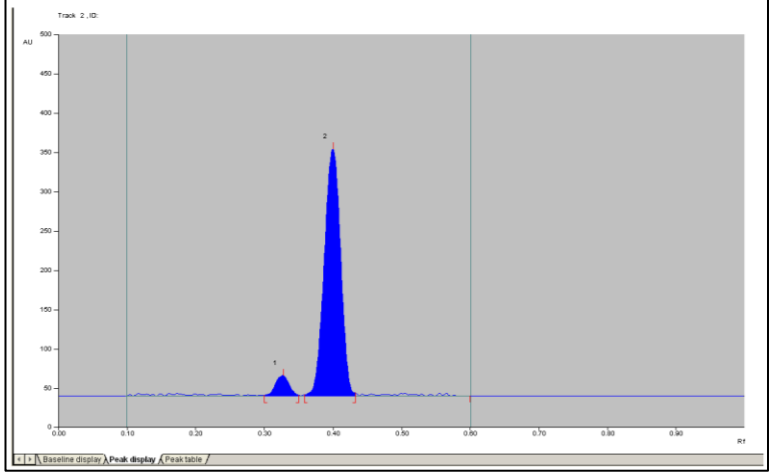
HPTLC chromatogram of Herbal Formulation (1.0 µg/spot)

(R_F value: 0.34 and 0.41)



HPTLC chromatogram of Guggulsterone E standard

(1.0 µg/spot), (R_F value 0.34)

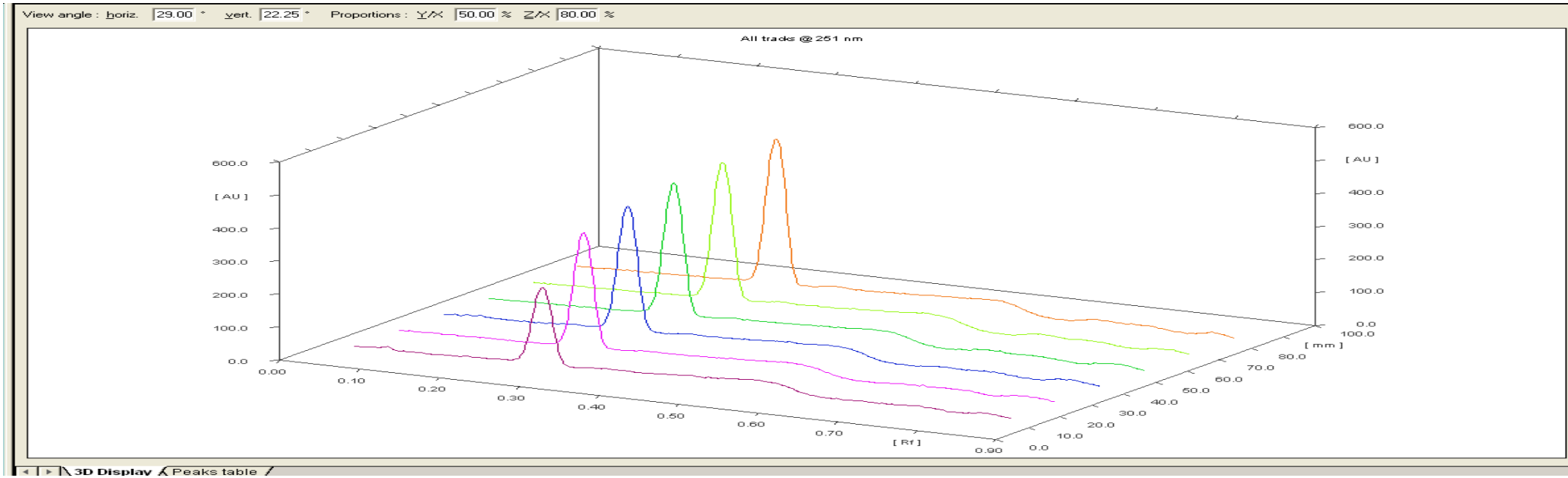


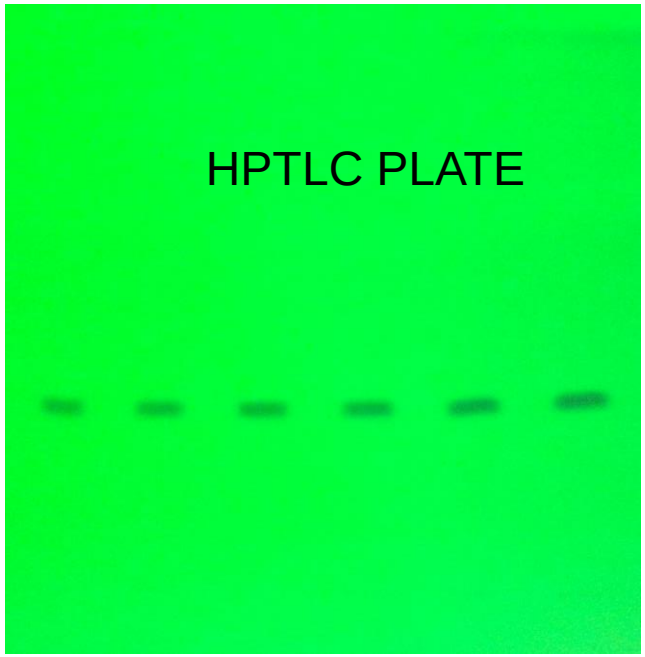
HPTLC chromatogram of Guggulsterone Z standard

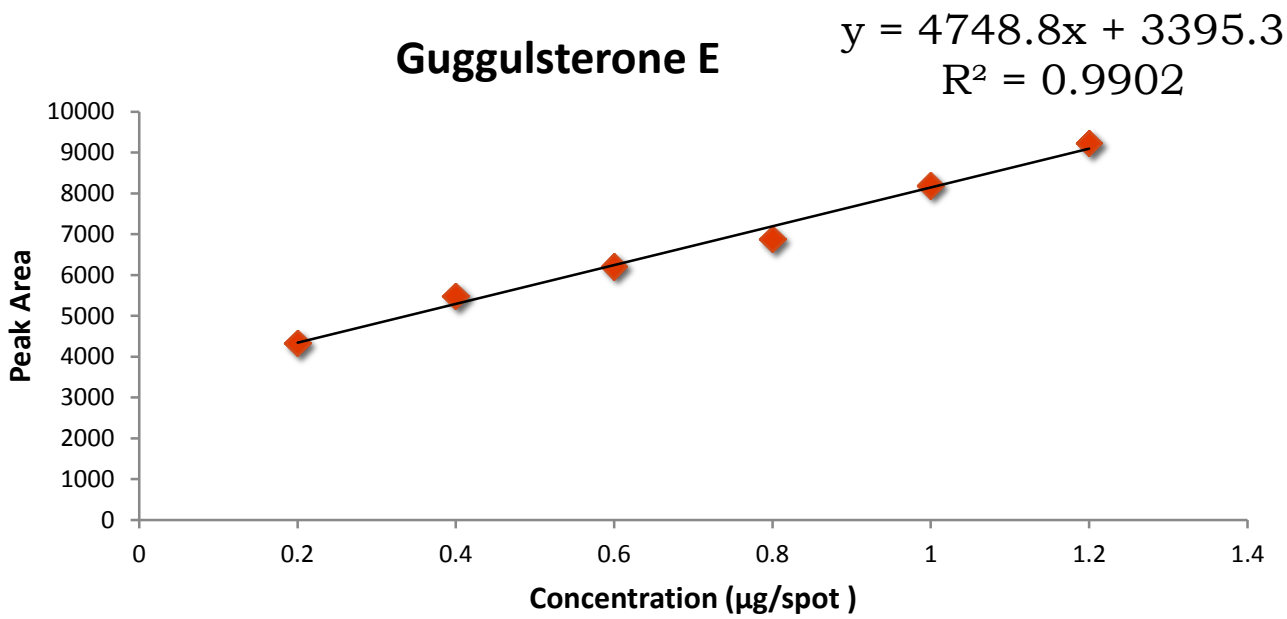
(1.0 µg/spot), (RF value 0.41)

Validation of Developed Method

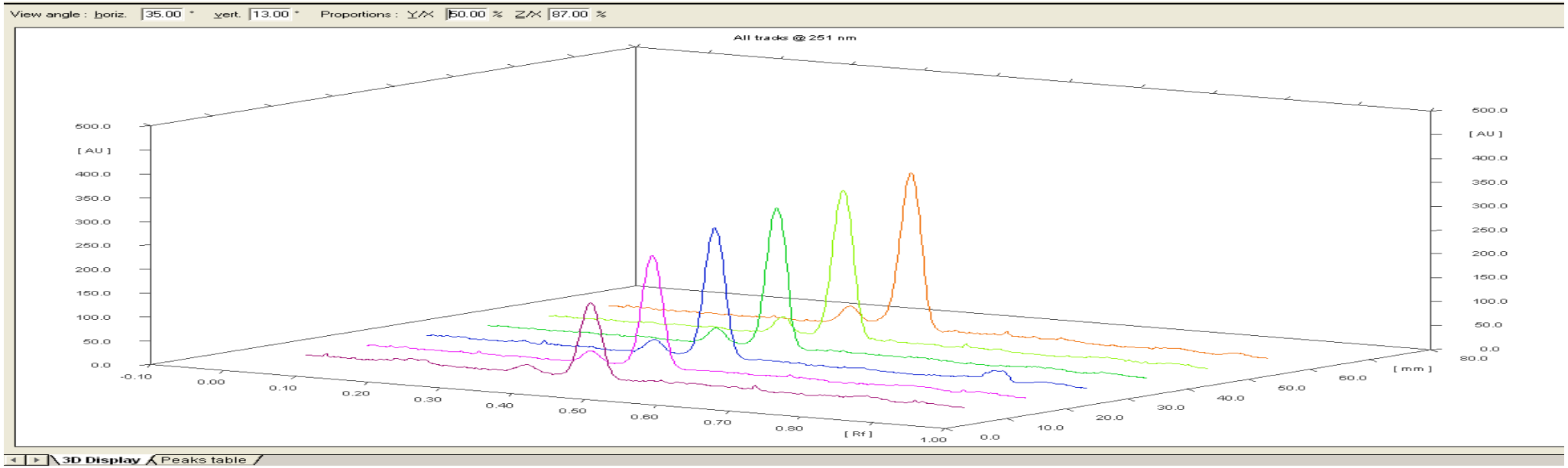
HPTLC Chromatogram (3D view) for linearity of Guggulsterone E

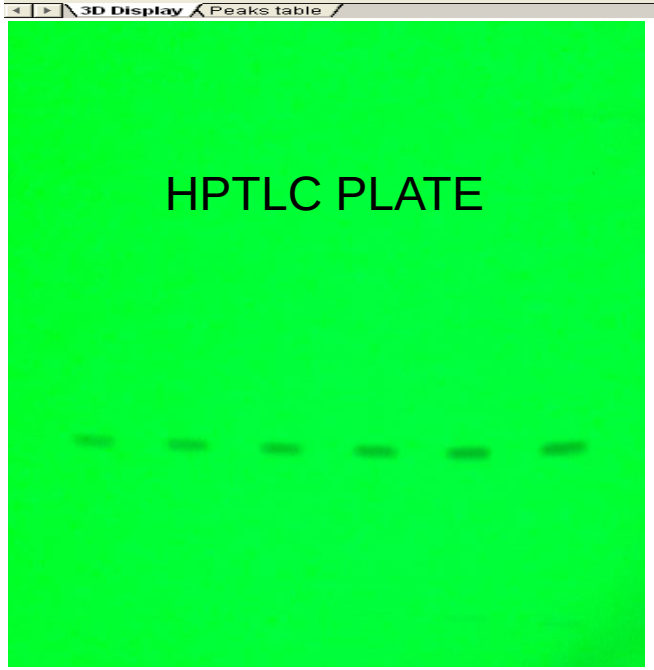


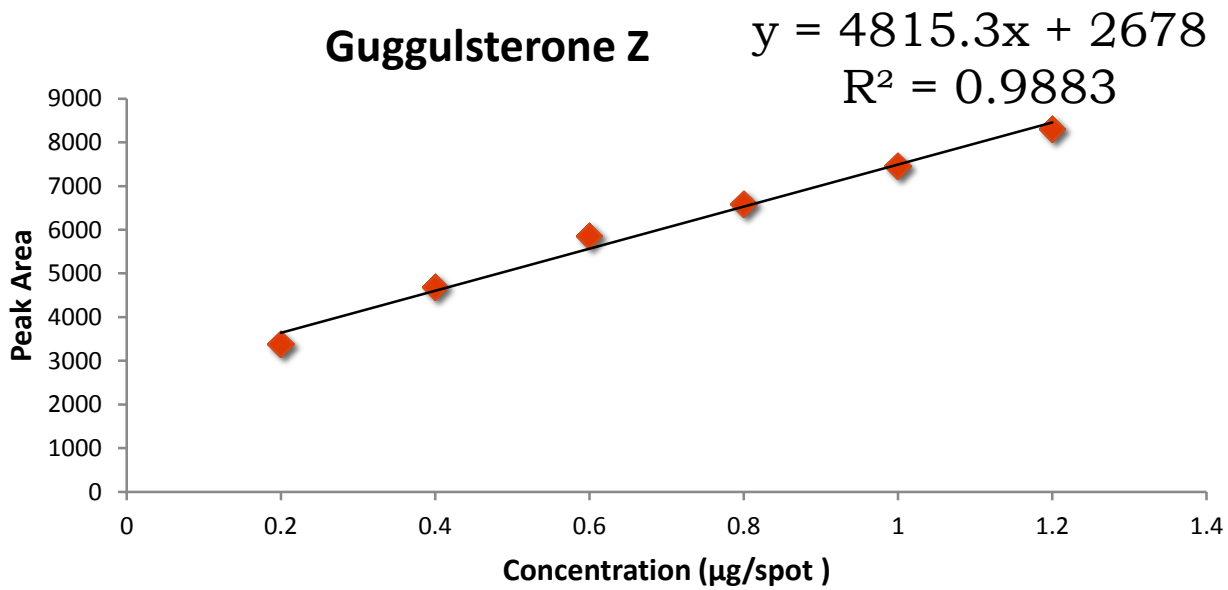




HPTLC Chromatogram (3D view) for linearity of Guggulsterone Z

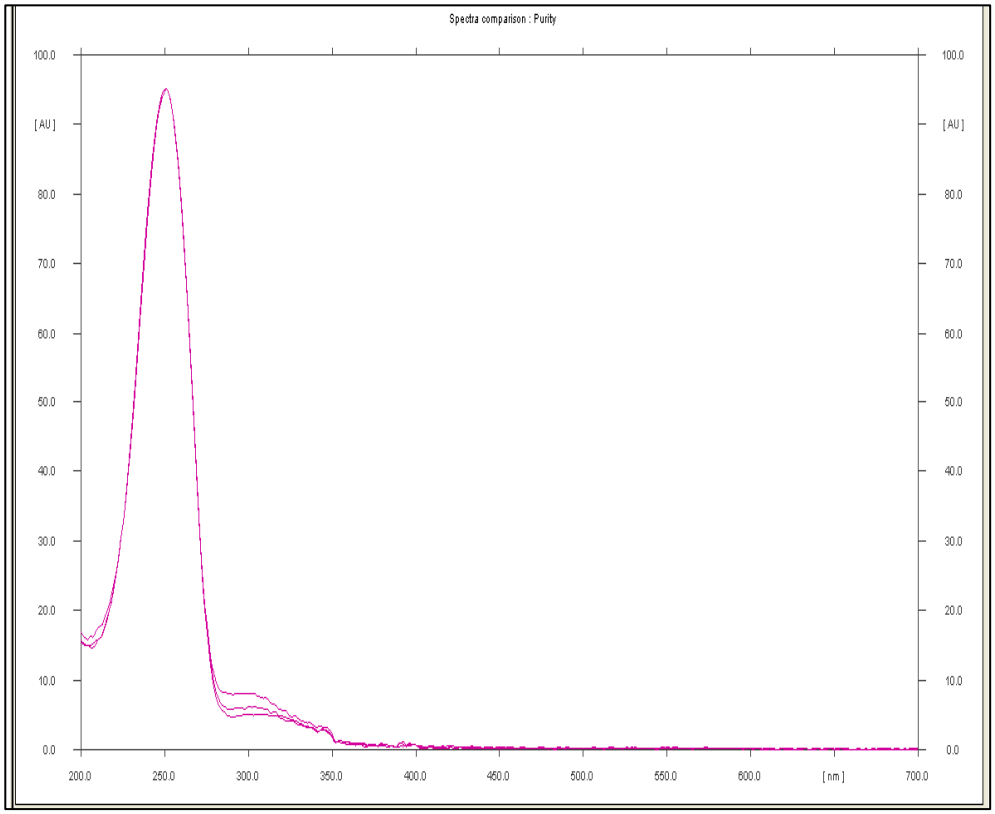


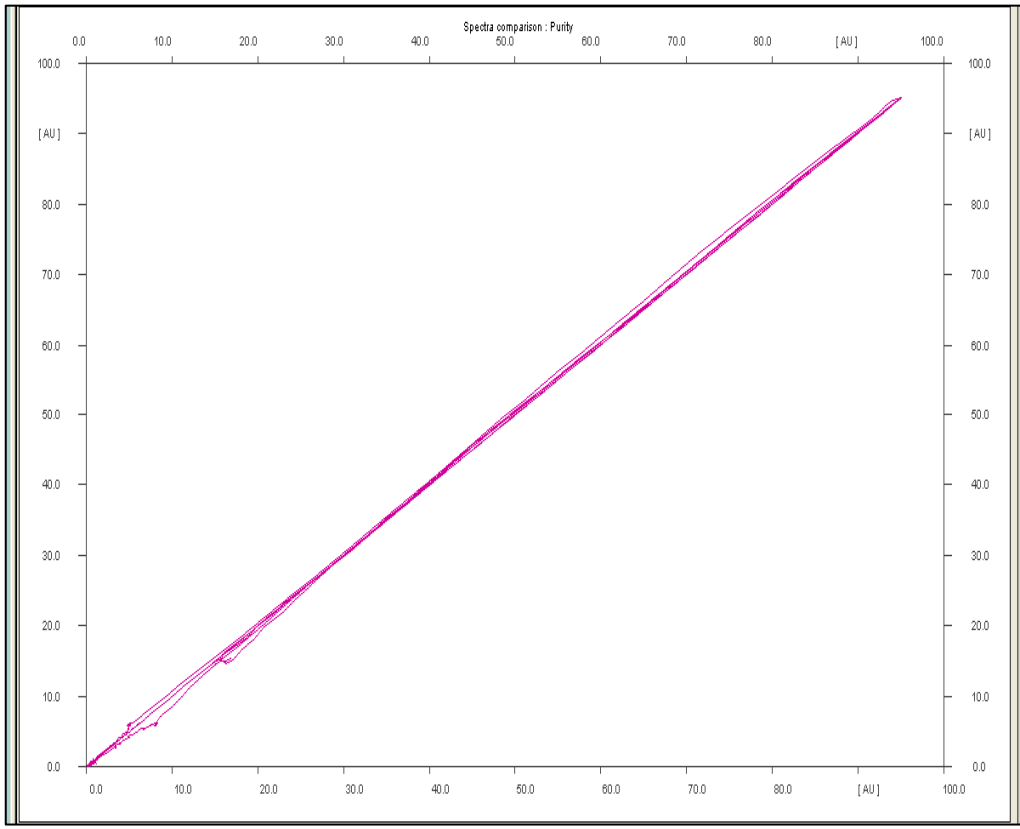




Peak purity spectra of Guggulsterone E

[Maximum Wavelength : 251 nm]



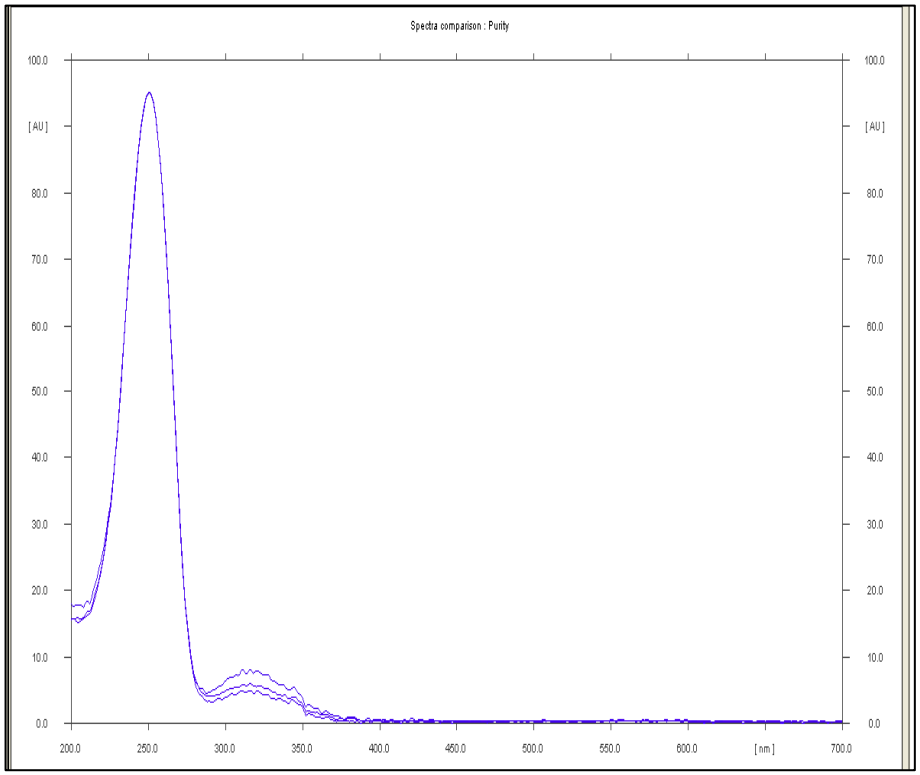


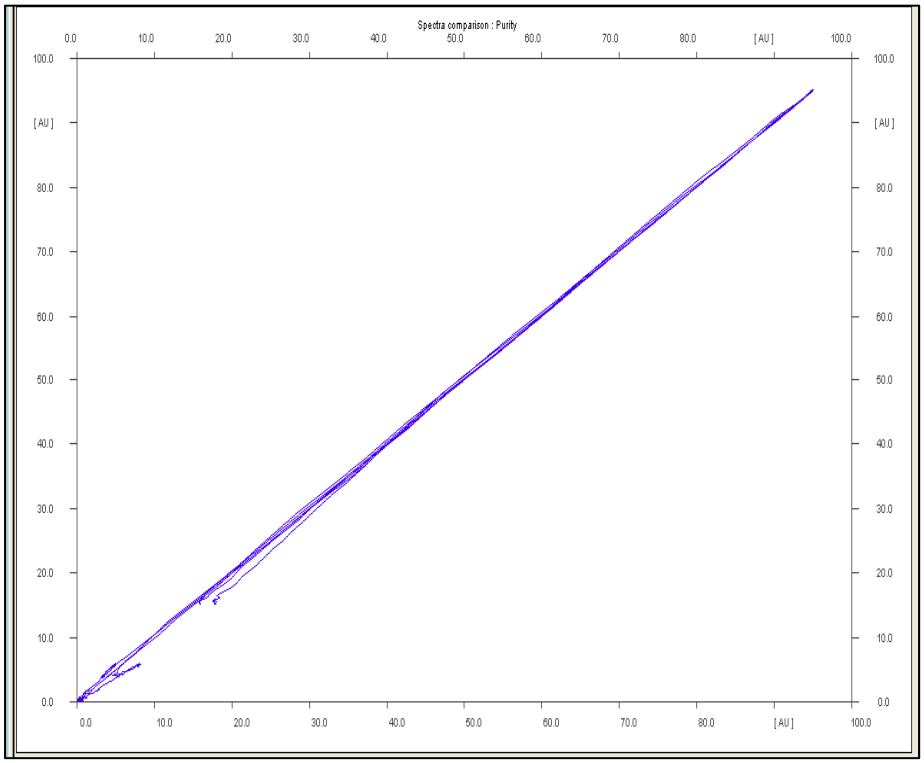
Specificity of Peaks

DRUG	R_F	r (s,m)	r (m,e)
Guggulsterone E	0.34	0.9973	0.9975

Peak purity spectra of Guggulsterone Z

[Maximum Wavelength : 251 nm]





Specificity of Peaks

DRUG	R_F	r (s,m)	r (m,e)
Guggulsterone Z	0.41	0.9981	0.9998

Validation parameters for HPTLC

Parameters	Guggulsterone E	Guggulsterone Z
Linearity Range (n=6)	0.2-1.2 µg/spot	0.2-1.2 µg/spot
Regression Equation	y = 4748.8x + 3395.3	y = 4815.3x + 2678
Correlation coefficient (r^2)	0.9902	0.9883
Specificity	Specific	Specific
Repeatability (n=6) (%RSD)	0.96	1.65
Intra-day precision (n=3) (%RSD)	1.59	0.78
Inter-day precision (n=3) (%RSD)	1.14	0.79
LOD	0.0437 µg/spot	0.0396 µg/spot
LOQ	0.132 µg/spot	0.120 µg/spot
Recovery (%) ± SD	97.940 ± 0.81	99.053 ± 0.98

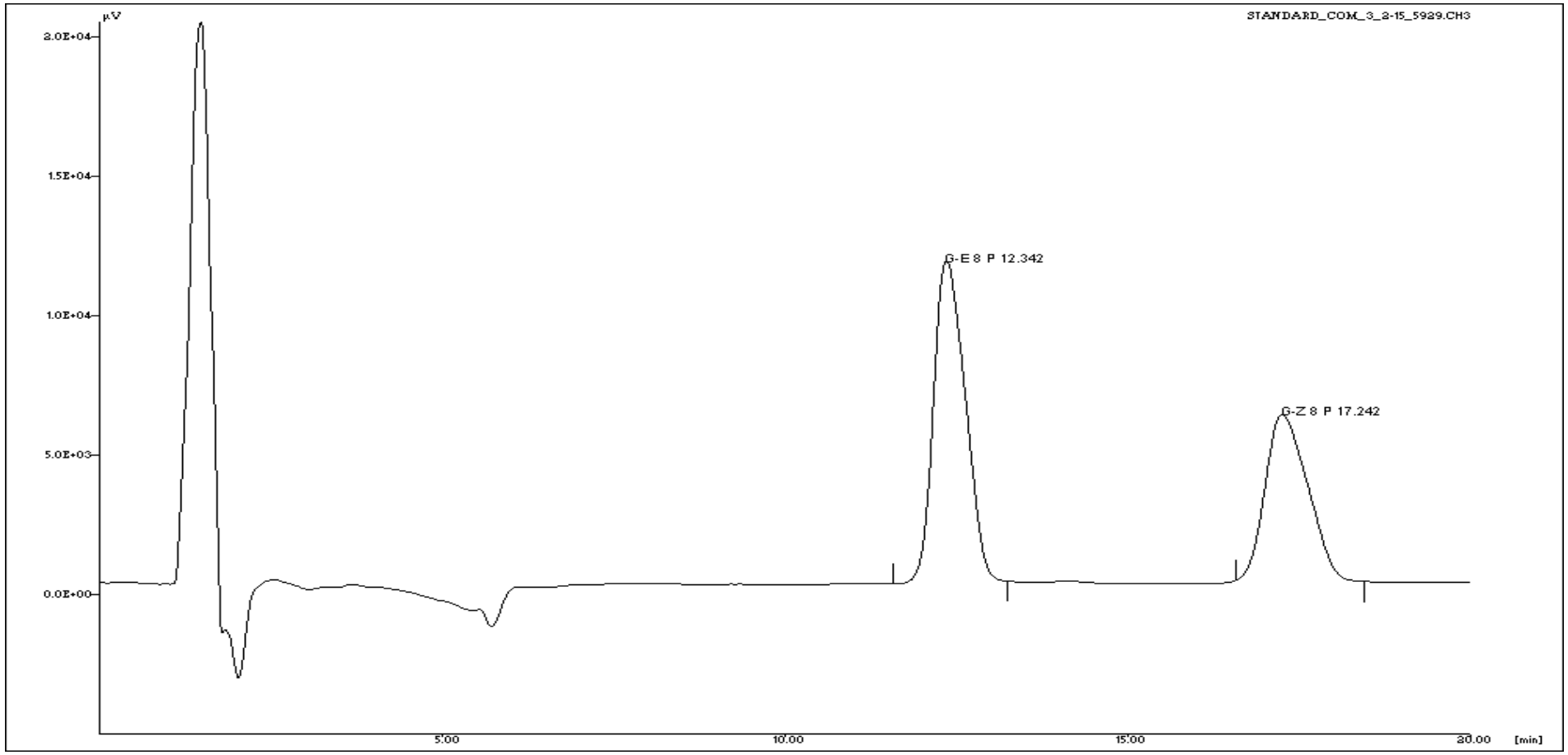
HPLC Method

HPLC METHOD

Optimized condition for HPLC

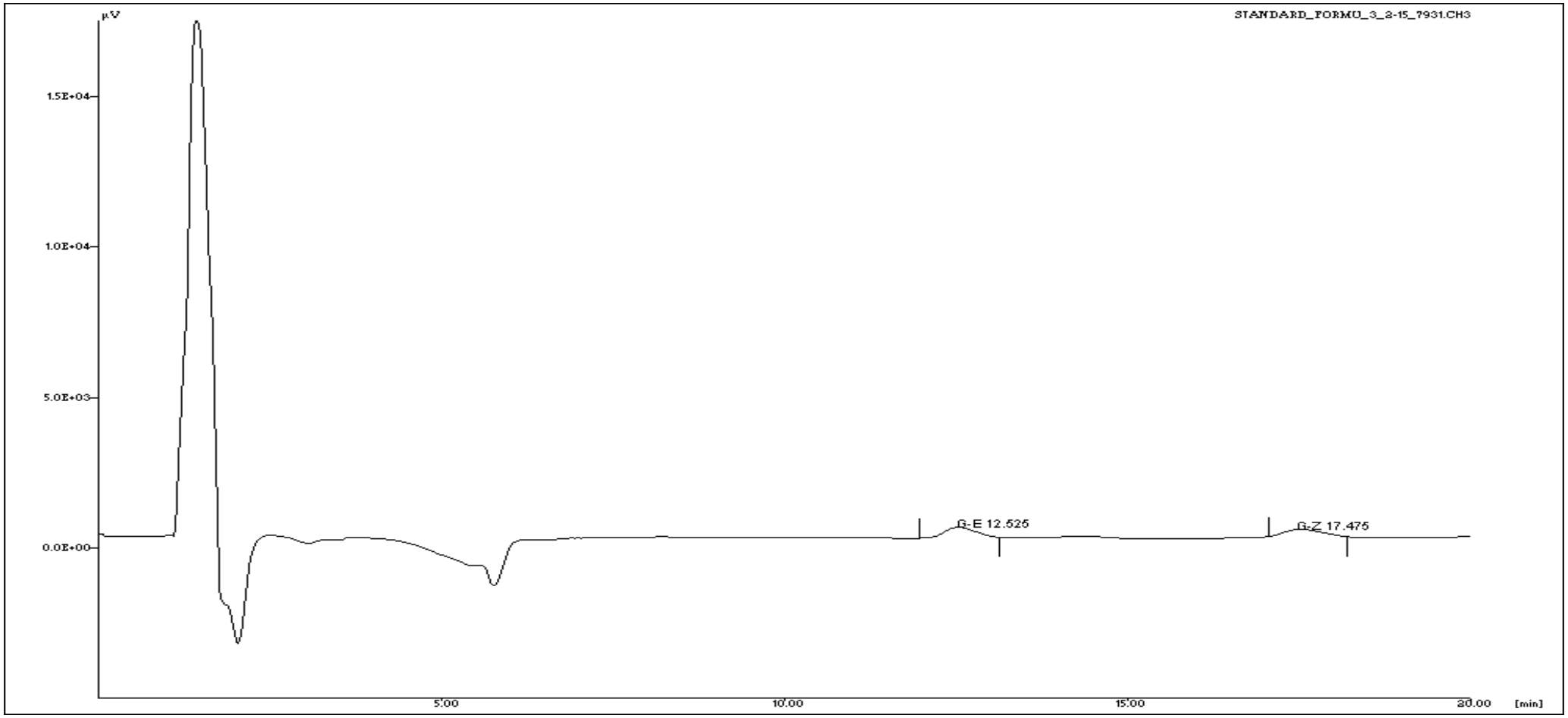
- Stationary Phase: C_{18} (150 mm × 4.6 µm × 5 µm)
- Mobile Phase: Acetonitrile : Water (50:50/v, v)
- Wavelength: 242 nm
- Flow Rate: 1 ml/min
- Run Time: 20 min
- Diluent: Methanol

HPLC Chromatogram of mixture Standard Guggulsterone E & Z (8 PPM) at 242 nm



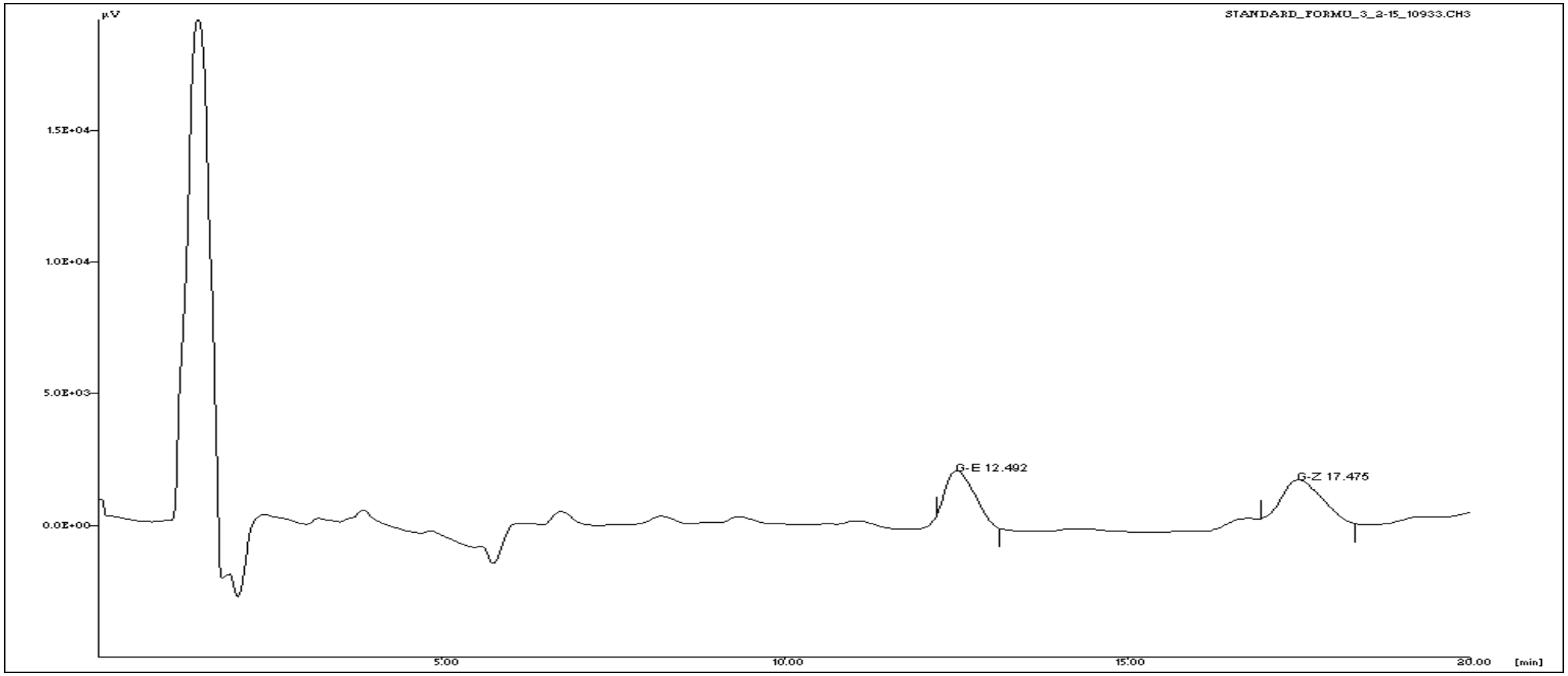
Name	RT	Area [µ V. Sec]	% Area	Asymmetry	Plates	Resolution
Guggulsterone E	12.342	355107	1.23	3447	58.84	0
Guggulsterone Z	17.242	288414	1.26	3603	41.16	4.93

HPLC Chromatogram of Herbal Formulation (10 PPM) at 242 nm



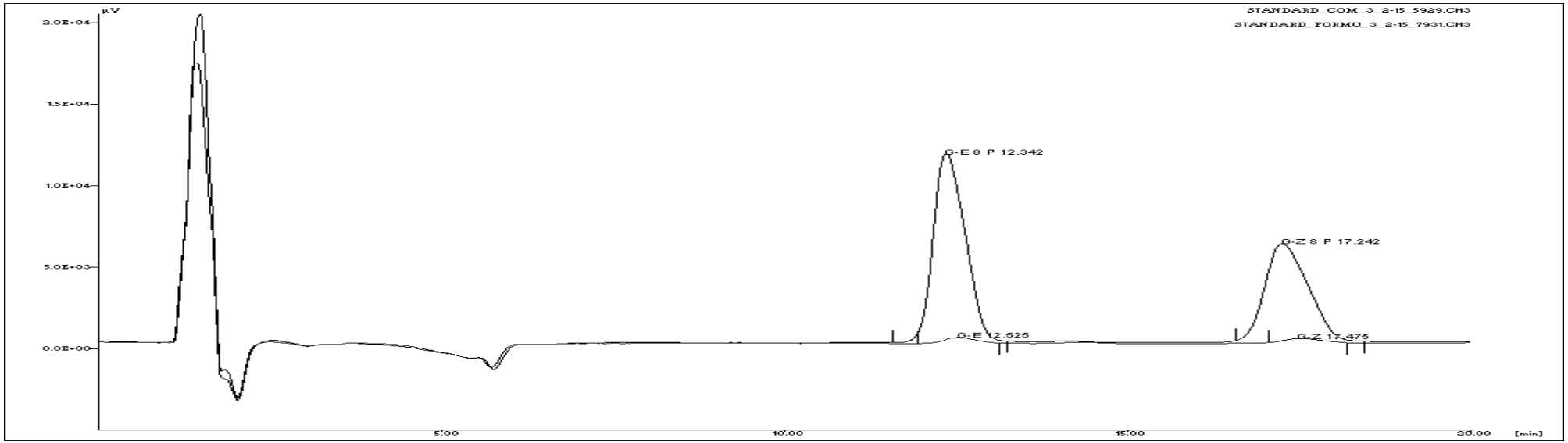
Name	RT	Area [µ V. Sec]	% Area	Asymmetry	Plates	Resolution
Herbal Formula-tion	12.525	10555	54.46	1.09	3636	-
Herbal Formulation	17.475	8826	45.54	1.37	4672	5.35

HPLC Chromatogram of Herbal Formulation (300 PPM) at 242 nm

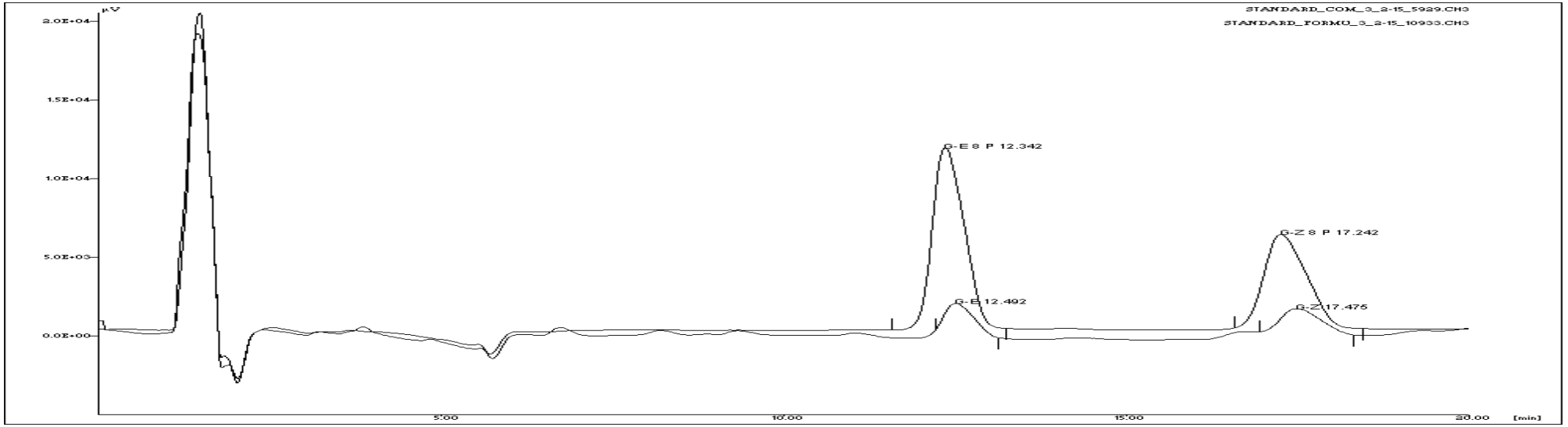


Name	RT	Area [µ V. Sec]	% Area	Asymmetry	Plates	Resolution
Herbal Formulation	12.492	50200	45.75	1.56	4048	-
Herbal Formulation	17.475	59519	54.25	1.29	4257	5.37

Overlay HPLC Chromatogram Of Herbal Formulation (10 ppm) & Guggulsterone E & Z (8 ppm) at 242 nm

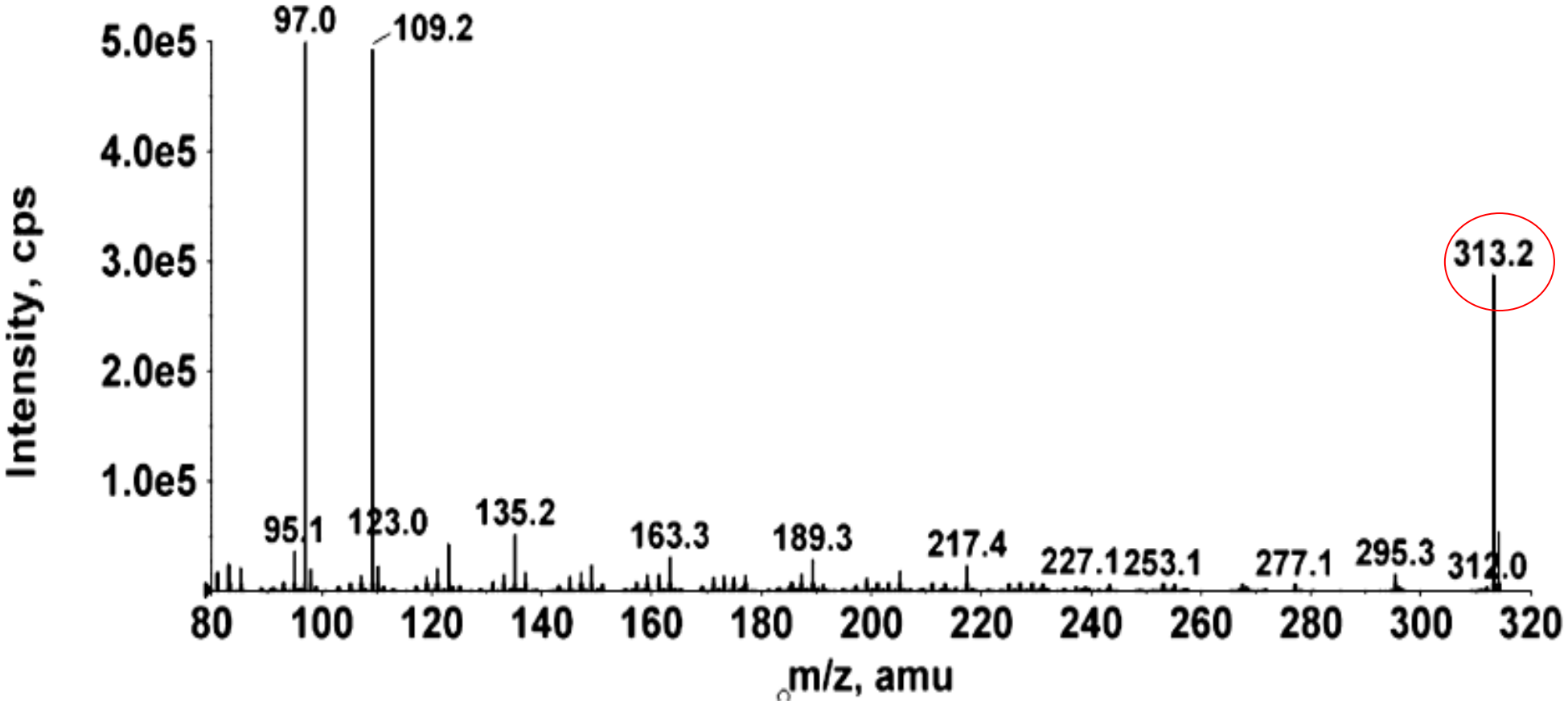


Overlay HPLC Chromatogram Of Herbal Formulation (300 ppm) & Guggulsterone E & Z (8 ppm) at 242 nm



Mass Analysis

Mass Spectrum of isolated Guggulsterone from Herbal Formulation



Spectrum RT 0.55 - 0.69 {5 scans} - Background Subtracted 0.13 - 0.48
T 2015.05.16 14:16:57 ; ZRC. ESI + Max: 1.2E7

Estimation of Guggulsterone in Herbal Formulation done by HPTLC method

- Two spots at Rf 0.34 and 0.41 was observed in the chromatogram herbal formulation. There was no interference in analysis of isomers from the other components present in the capsules.
- The total Guggulsterone content was found to be 0.87% (w/w) with a R.S.D.% of 1.57 out of which E and Z contributes 33.21 and 66.79% (w/w), respectively, of total Guggulsterone content in capsule .

CONCLUSION

- Polyherbal formulation were standardize by evaluating physicochemical parameters, physical parameters of polyherbal formulation and quantification done by two analytical techniques HPTLC and HPLC.
- All evaluated parameters were within range and developed methods for quantification are simple, rapid, accurate, precise, sensitive for determination of Guggulsterone E and Z in polyherbal formulation. Excipient present in formulation doesn't interfere with quantification of Guggulsterone E and Z, indicates that method is specific.

- The HPTLC profile of methanol extract of poly herbal formulation could be used as a valuable analytical tool in the routine standardization of poly herbal formulation to check the batch to bath variation. The study reveals that sufficient quality control parameters were followed during the manufacturing process. Organoleptic characteristics, Physicochemical parameters, physical parameters and HPTLC analysis indicates genuineness of finished product.

- Identified E and Z Guggulsterone can be used as a biomarker controlling quality of polyherbal formulation containing Arjuna, Guggul & Pushkarmoola.

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Thank You

Contact Detail
Dr. Charmy Kothari
charmy.kothari@nirmauni.ac.in