PhD Synopsis on

Development, Standardization and Evaluation of Herbal Formulation for Obesity

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Introduction:

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. In the obese condition BMI of individual may increases; Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults. It is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2).

Category	**BMI(kg/m2)**
Under weight	< 18.5
Normal range	18.5 - 22.9
Overweight-At risk	23.0 - 24.9
Overweight – Moderately obese	25.0 - 29.9
Overweight – Severely obese	≥ 30.0

Key facts

- ✓ Worldwide obesity has nearly doubled since 1980.
- ✓ In 2008, more than 1.4 billion adults, 20 and older, were overweight. Of these over 200 million men and nearly 300 million women were obese.
- ✓ 35% of adults aged 20 and over were overweight in 2008, and 11% were obese.
- ✓ 65% of the world's population lives in countries where overweight and obesity kills more people than underweight.
- ✓ 42 million children under the age of 5 were overweight or obese in 2013.
- ✓ Obesity is preventable.

Pharmacological remedies for obesity

S. No	Drug Class	Mechanism of Action	Example	Side Effects
1	HMG CoA reductase enzyme inhibitor	Lowering total LDL inhibiting cholesterol biosynthesis	Atrovastatins, Fluvastatin, Lovastatin, Simvastatin	Congestive cardiac failure
2	Fibrates	Enhancing activity of enzyme lipoprotein lipase	Gemfibrozil, Fenofibrate	Upper gastrointestinal disturbance, headache, myalgia
3	Nicotinic acid derivative	Inhibit lipolysis within adipocytes	Niacin	Hyperglycemia, increase uric acid
4	Bile acid sequestrants(Resin)	Bind with bile acid & promote bile acid excretion	Cholestipole, Cholestyramine	Abdominal fullness, constipation
5	Misc.	Inhibit free radicals	Omega 3 fatty acid, Probucol	-

- **Prescription drugs for obesity:**

Drug	Mechanism action	Adverse effects
Orlistat	Reduces fat absorption from the intestine by inhibiting pancreatic lipase and reduces triglyceride hydrolysis. Low fat diet is generally advised.	Steatorrhoea (oily stools).
Sibutramine	Centrally acting sympathomimetic amine that enhances satiety by inhibiting non-selective uptake of nor adrenaline, serotonin and dopamine	Hypertension, serotonin syndrome
Metformin	It activates cAMP-activated protein kinase and suppresses hepatic gluconeogenesis activity.	Lactic acidosis, Gastro-intestinal upset.
Rimonabant	It is an approved but infrequently used drug. It is a canabinoid CB1 receptor antagonist. It selectively acts on CB1 receptor in brain and peripheral organs. reduces lipogenesis in liver. They not only cause weight loss but in addition reverse metabolic effects of obesity.	Severe depression and predisposes to neurodegenerative diseases E.g. Alzheimer's disease, amylotropic sclerosis.

Herbal Remedies for the obesity:

Sr.no	Anti-obesity function	Herbs
1	Inhibiting pancreatic lipase activity	Chitosan,green tea

2	Enhancing thermo genesis	Sea Weed, Bitter Orange, Soybean
3	Preventing adipocyte differentiation	Turmeric, Capsicum, Palm Oil, Banana Leaf, Brown Algae, Garlic, Flaxseed, Black soybean, Kokam fruit
4	Enhancing lipid metabolism	Herb Teas, Cinnamon, Guggul Lipid
5	Decreasing appetite	Pine Nut, Pomegranate Leaf,Ginseng, Hoodia Gordonii, Aghedo, Methi Seeds

Marketed formulation for obesity:

Sr. No.	Name of Formulation	Composition
1	Ayurslim	Garcinia , Indian Bdellium Gymnema, Chebulic Myrobalan, Fenugreek
2	Trim	Garcenia, Pichrorriza Cuprus rotundus, Triphala

Rationale :(Benefit to the patients and health care system)

Obese persons are preferred the use of herbal products for weight management because of following probable reasons:

- ✓ Health benefits of weight loss without any side effects,
- ✓ Less demanding than accepted lifestyle changes, such as exercise and diet,
- ✓ Easily available without a prescription,
- ✓ More easily accepted than a professional consultation with a physician or a nutritionist
- ✓ 100% natural origin and perception that natural means safe

Herbal plants for weight reduction may be effective in the treatment of obesity and associated disorders.

Consistent and safe herbal product for weight reduction is a need of developed and developing countries. In our literature survey, herbal plants showed potential effects on

weight control. However, for the majority of products, more data are needed to assess the suitability as an anti obesity plants**.**

Everyone knows that exercise with a controlled diet is the only way to keep in shape. However, your aim to be slim is obstructed by your urge to eat more and to snack in between meals. It's difficult for many people to resist food or snacks after a long and tiring work day. It's only natural! Tiredness and fatigue can also make people crave sugary food for energy. That's why many are unable to stick to healthy food choices every day.

But now imagine the same situation with a controlled appetite. With your appetite under your control, you can lower the intake of calories.

List of Herbal plants utilized for treatment of Obesity

Botanical name	**English name/Common name**	**Parts used**
Acacia arabica	Babbula	Gum, bark, leaf, fruit-pods
Achyranthus aspera	Apamarga	Root, seed, leaf, whole plant
Aconitum heterophyllum	Ativisha	Root, rhizome
Acorus calamus	Vacha	Rhizome
Adathoda vasica	Vasa	Leaf, root, flower
Allium sativum	Garlic	Stem, Fruit
Aloe vera	Kumari	Leaf, root
Betula utilis	Burja	Bark, nodes
Camelia sinensis	Green Tea	Leaves
Catharuths roseus	Barmasi	Whole plant
Commiphora wightii	Guggal	Resin
Coriander sativum	Coriander	Fruits
Cassia tora	Chakramardha	Seed, leaf, root
Cedrus deodara	Devadaru	Hearwood oil
Embelia ribes	Vidanga	Fruit
Emblica officinalis	Amalaki	Fruit
Garcinia indica	Vrikshamla	Fruit, root, bark, oil
Gymnema sylvestre	Meshashringi	Leaf, root, seed
Holarrhena antidysentrica	Kutaja	Seed, bark
Momordica charantia	Karavellaka	Fruit, whole plant, leaf, root
Moringa oleifera	Sigru	Root, bark, seed
Morraya koinigi	Carry Leaves	Leaves
Picrorhiza kurroa	Katuka	Root
Piper longum	Pippali	Fruit, root
Piper nigrum	Maricha	Fruit
Plumbago zeylanica	Chitraka	Root, bark
Punica granatum	Pomegranate	Fruit rind ,leaves

Terminalia arjuna	Arjuna	Bark, root, leaf
Terminalia bellerica	Bibhitaka	fruit
Terminalia chebula	Haritaki	fruit
Terminalia tomentosa	Asana	Bark, heartwood
Thea sinensis	*Oolong tea*	Leaf
Tinospora cordifolia	Guduchi	Stem, root
Trachyspermum ammi	Yavani	Fruit
Tribulus terrestris	Gokshura	Fruit, root, whole plant
Trigonella foenum graceum	Methika	Seed, leaf, whole plant
Valeriana jatamansi	Tagara	Root
Zingiber officinale	Shunti	Rhizome

Formulation and Development:

Selected Herbs: *Achyranthus aspera* ext., *Commiphora wightii* ext., *Garcinia indica* ext., *Morraya koinigi* ext.,

Excipients:

Class	Excipients	Concentration
Direct compressible diluents	MCC	5-15%
	Ethyl Acetate	1-5%
	Dicalcium Phosphate	1-5%
	Lactose	5-20%
	Sucrose	2-25%
	Avicel PH 102	1-20%
Disintegrants	Starch	10-15%
	Sodium Starch Glycocollate	0.5-10%
	Cross carmellose sodium	2-5%
	Crosspovidone	0.5-5%
Lubricants	Talc	1%
	Magnesium stearate	1%
Preservatives	Methyl paraben	1%
	Propyl paraben	0.1%
Adsorbant	Syloid(Sillica)	1 – 5%

Final formulation was prepared by direct compression method with desired result.

Achyranthus aspera ext.	50mg
Commiphora wightii ext	150mg
Morraya koinigi ext.	50mg
Garcinia indica ext	150mg
Avicel PH 102	16.66% (100mg)

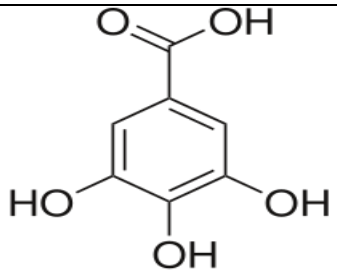
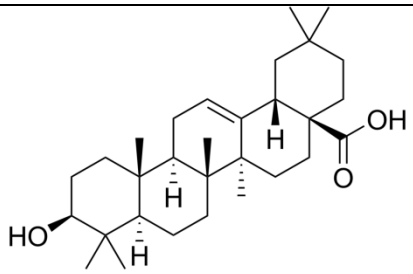
Styloid	2 % (12mg)
SSG	7% (42mg)
Cross carmellose sodium	5% (30mg)
Talc	1% (6mg)
Methyl peraben	1% (6mg)

Evaluation:

Hardness	4.5 kg
Disintegration	9 mins
Friability	>1 %

Analytical method for marker compound:

Here analytical method development of **Gallic acid** and **Oleanolic acid** are developed using Reversed Phase High Performance Liquid Chromatography(RP- HPLC) and validation parameters such as Accuracy, Precision, Linearity, LOQ, LOD and Robustness are performed.

GALLIC ACID(4)	**OLEANOLIC ACID(5)**
	
Solubility: alcohol, ether, glycerol, acetone; negligible in benzene, chloroform, petroleum ether	In Methanol
Molecular weight: 170.12gm/mol	456.7 gm/ mol
Formula: $C_7H_6O_5$	$C_{30}H_{48}O_3$
Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants.	Oleanolic acid or oleanic acid is a naturally occurring pentacyclic triterpenoid related to betulinic acid. It is widely distributed in food and plants where it exists as a free acid or as an aglycone of

	triterpenoid saponins.

General information of Gallic acid and Oleanolic acid

Chemicals and Reagents:

Gallic acid was procured from Sulab (Suvidhinath) Laboratory, Vadodara and **Oleanolic acid** was procured from Sigma- Aldrich , USA.

HPLC Grade Methanol and all other reagents are obtained from Rankem company.

HPLC Grade Water is produced from Double distillation assembly at Laboratory through out the whole study.

Experimental procedure:

HPLC Instrument:

HPLC equipment	**SHIMADZU LC-20AD Prominence**
Column	Hyperchrom 5µ C18 (250 mm x 4.6 mm, 5 µm)
Detector	SHIMADZU SPD-20A Prominence UV/VIS Detector
Injector	Rheodyne 7725 injector valve with fixed loop at 20µl
Software	LC solution
System controller	SBM 20Alite

Preparation of Standard Solution:

Gallic acid stock preparation:

Gallic acid 10 mg dissolved in 10 ml Methanol to prepare 1000 µg/ml solution.

Form this solution 1 ml was taken and diluted up to 10 ml with methanol to prepare 100 µg/ml.

Oleanolic acid stock preparation:

Oleanolic acid 10 mg dissolved in 10 ml Methanol to prepare 1000 µg/ml solution.

Mixture:

From **Gallic acid stock solution**, 0.4 ml taken and 4 µg/ml and from **Oleanolic acid stock solution** 0.8 ml taken and diluted to 10 ml with methanol to make **4, 80** µg/ml for injection in HPLC.

Preparation of Sample Solution:

Polyherbal Tablet A and B were formulated in Laboratory using herbal extracts in which these phytoconstituents GA and OA were present. Approximately five tablets were crushed

and 500 mg tablet powders dissolved in 50 ml of methanol. From this solution, 1 ml was to be diluted up to 10 ml with methanol and injected in HPLC after filtered through 0.22 micron syringe filter.

Selection of wavelength (Iso-absorptive point)

Selection of wavelength of both makers was done by using UV spectrophotometer. Standard solutions of Gallic acid (100µg/ml) and Oleanolic acid (1000µg/ml) were scanned between 200-400nm under UV-Vis spectrophotometer and intercept at 222nm as shown in figure , which was selected as detecting wavelength.

Optimization of Mobile Phase:

Based on sample solubility and suitability various chromatographic condition such as mobile phase, pH, wavelength were tried to get good resolution and sharp peaks.

Mobile phase	**Ratio**	**Gallic acid**		**Oleanolic acid**	
		RT(min)	**Tailing factor**	**RT(min)**	**Tailing factor**
Water : Acetonitrile	50:50	3.6	2.5	-	-
Water(0.3 %OPA) : Acetonitrile	15:85	5.3	1.2	-	-
Water : ACN : Methanol	45:10:45	2.9	1.3	-	-
Water: Methanol	95:5	2.9	1.2	11.7	1.05
OPA (0.1%) : Methanol	10:90 (0.8ml/min)	2.8	1.0	17.2	1.0
OPA (0.1%) : Methanol	2:98 (0.8ml/min)	3.5	1.9	9.5	1.0
OPA (0.1%) : Methanol	2:98 (1 ml/min)	2.8	1.5	7.5	1.1
OPA (0.1%) : Methanol	3:97 (1 ml/min)	2.8 (Shape was not	1.5	7.9	1.1

		good)			
OPA (0.1%) : Methanol	5:95 (1 ml/min)	2.8	1.2	9.5	1.0

(ACN = Acetonitrile, OPA= Ortho phosphoric acid)

Chromatographic condition After the all these trial performed mobile phase 0.1% Ortho Phosphoric acid: Methanol (5:95) was selected for HPLC method which gives sharp and symmetric peaks for both the markers with good resolution.

Column	Hyperchrom ODS BP C18 (Size: 250*4.6 mm, 5µ)
Flow rate	1.0 ml/min
Detection wavelength	222 nm
Mobile Phase	Ortho Phosphoric acid 0.1 % in Water : Methanol (5:95) It was filtered through 0.45 µm Nylon filter and sonicated for 5 min.
Injection Volume	20 µl through rheodyne manual injector.
Temperature	Ambient
Retention Time	2.8 min for Gallic acid and 9.9 min for Oleanolic acid

Method Validation for HPLC Fingerprinting(6):

The method was validated according to ICH guidelines for Linearity, Precision, Accuracy, Limit of Detection and Limit of Quantification.

Linearity:

Linearity of the method was performed by analyzing both the markers in combination as following concentration range.

Linearity Solution	Concentration of GA (µg/ml)	Concentration of OA (µg/ml)
1	1	50
2	2	60
3	3	70

4	4	80
5	5	90
6	6	100

Concentration of Gallic acid(GA) and oleanolic acid(OA)

Now calibration curve was plotted against Area of peak verses Concentration of injected linearity standards. From the graph, correlation co-efficient and regression line equation were to be determined.

Accuracy

The accuracy was determined by calculating % recoveries of GA and OA(Spiking method). It was carried out by adding known amounts of each analyte corresponding to three concentration levels (80, 100, and 120%) of the labeled claim to the excipients. At each level, two determinations were performed, and the accuracy results were expressed as percent analyte recovered by the proposed method.

Precision

Precision of an analytical method is usually expressed as the standard deviation. The repeatability studies were conducted by estimating the response of GA and OA in six times.

Reproducibility of methods was checked by performing intra-day precision (three times a day) and inter-day precision (repeated triplicates for three consecutive days). Results are expressed in terms of standard deviation and % Relative standard Deviation (RSD).

Intraday precision was determined by estimation of mixture of standard markers solution in lower, middle and higher concentration in triplicates on the same day.

Interday precision was determined by estimation of mixture of standard markers solution in lower, middle and higher concentration on three different days.

Robustness

Robustness of the method was investigated under a variety of conditions including changes of composition of buffer in the mobile phase, flow rate, and temperature. This deliberate change

in the method has no effect on the peak tailing, peak area, and theoretical plates and finally, the method was found to be robust.

Limit of Detection (LOD):

The LOD can be defined as the lowest amount of analyte that can be detected but not quantitated.

LOD can be calculated as per following equation:

$$LOD = 3.3\ \sigma/S$$

Where σ is standard deviation of regression line and S is slope of calibration curve

Limit of Quantification:

Quantification limit of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precesion and accuracy.

$$LOQ = 10\ \sigma/S$$

Where σ is standard deviation of regression line and S is slope of calibration curve

Quantification of GA and OA in polyherbal tablet:

Applicability of proposed method for the laboratory based formulation tablet was quantified for the marker components – Gallic acid and Oleanolic acid. The content of all two markers were determined by injecting the prepared laboratory sample as per proposed chromatographic condition. The concentrations of markers were determined by following equation.

$$\% \text{ Assay} = \frac{Area\ of\ sample\ x\ Std\ wt\ taken\ x\ Sample\ dilution}{Area\ of\ std\ x\ Std\ dilution\ x\ Sample\ wt\ taken} \text{ x } 100$$

RESULTS AND DISCUSSION:

Isoabsorptive point (Wavelength selection) :

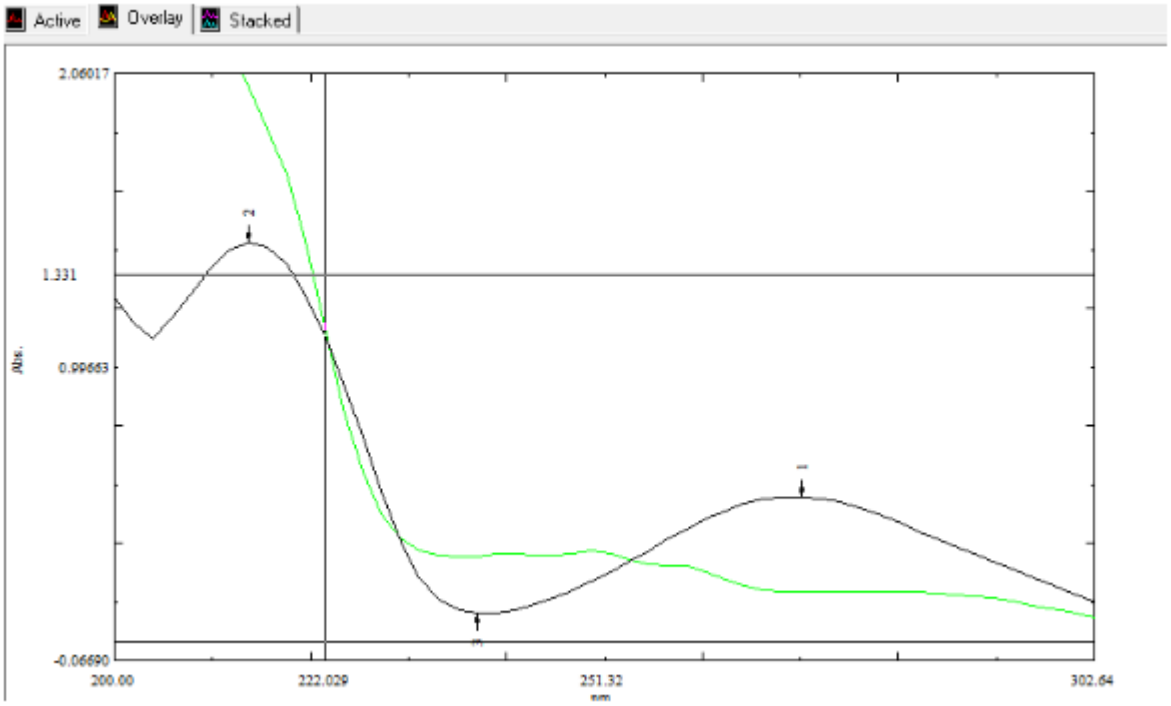
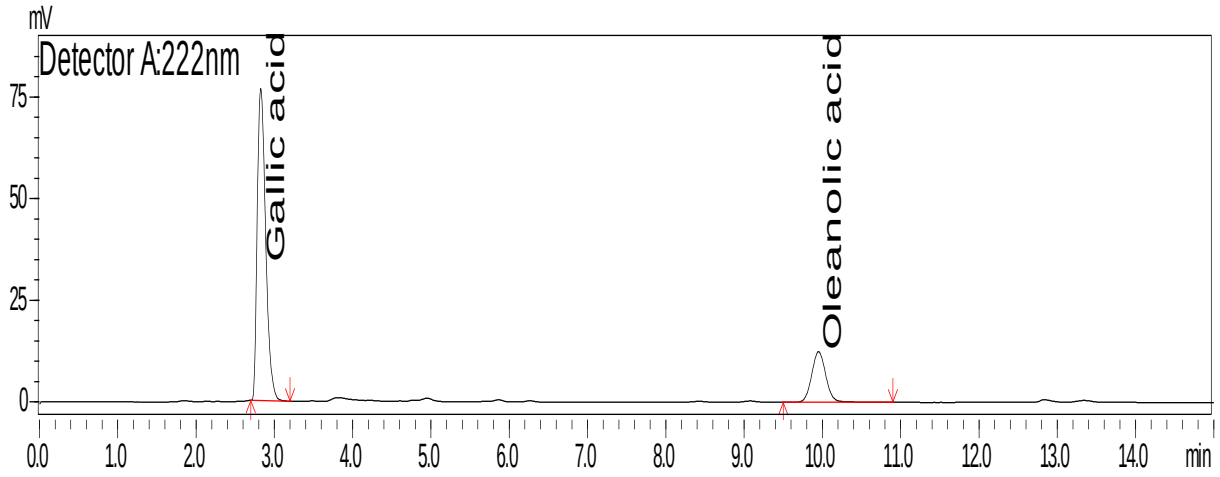


Figure 1: Overlay spectra for both markers GA and OA.

Scanning of Gallic acid standard and Oleanolic acid standard were run by UV Visible spectroscopy and both the markers were intercept at 222 nm. Therefore 222nm was selected as detection wavelength for further study.

System Suitability Parameters:

After various trials the mobile phase 0.1 % Orthophosphoric acid and methanol with the ratio of 5:95 would give a good resolution and sharp peak. The below mentioned chromatogram passed the system suitability parameters such as tailing factor, theoretical plates and resolution.



HPLC Chromatogram of Simultaneous estimation of Gallic acid and Oleanolic acid.

Name	Retention time	Peak start	Peak End	Height	Area	Area %	Tailing factor	Theoritical plate	Resolution
Gallic acid	2.844	2.700	3.200	61108	575350	78.65	1.218	3088.996	-
Oleanolic acid	9.949	9.500	10.90	11714	156109	21.34	1.076	14402.220	26.501

Table 6 : peak symmetry for Gallic acid and Oleanolic acid

Method Validation parameters for HPLC fingerprinting:

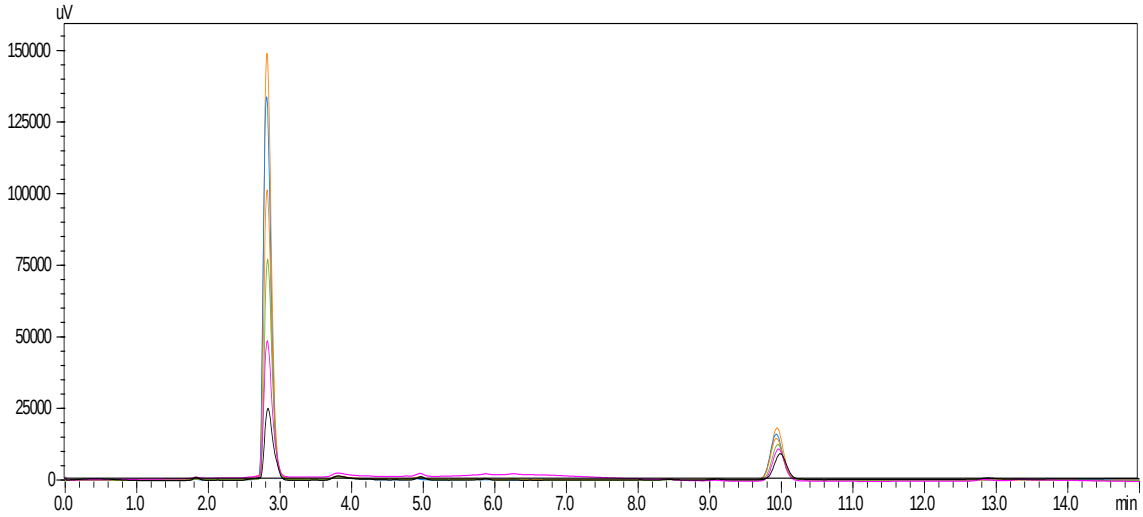
Linearity Parameter:

Concentration of GA in µg/ml	Avg. Area of Gallic acid
1	204339
2	379961
3	597321
4	775443
5	984878
6	1128298

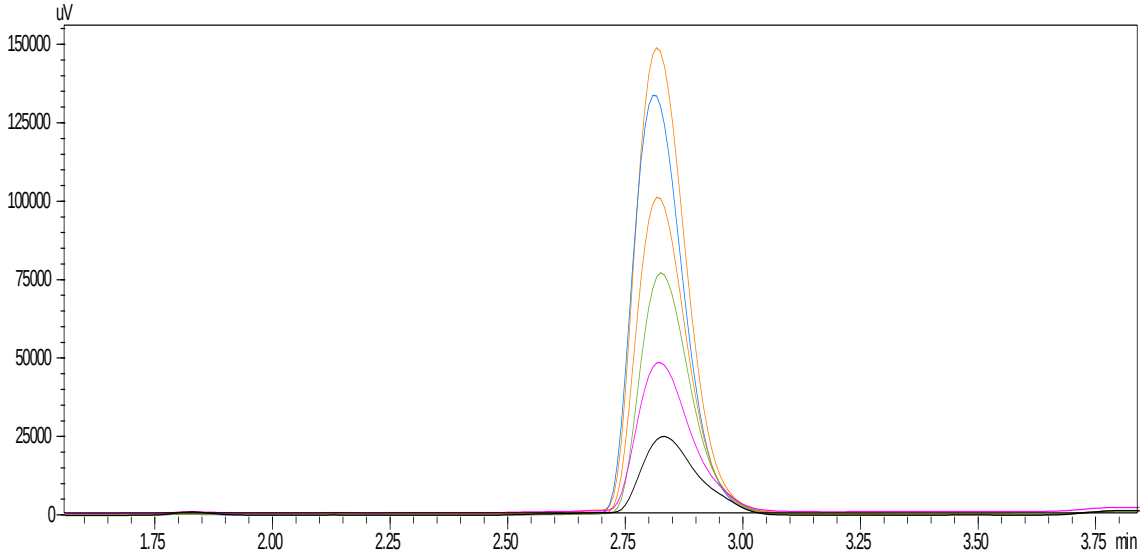
Peak area of GA

Concentration of OA in µg/ml	Avg. Area of Oleanolic acid
50	118299
60	140246
70	163724
80	189978
90	209210
100	229411

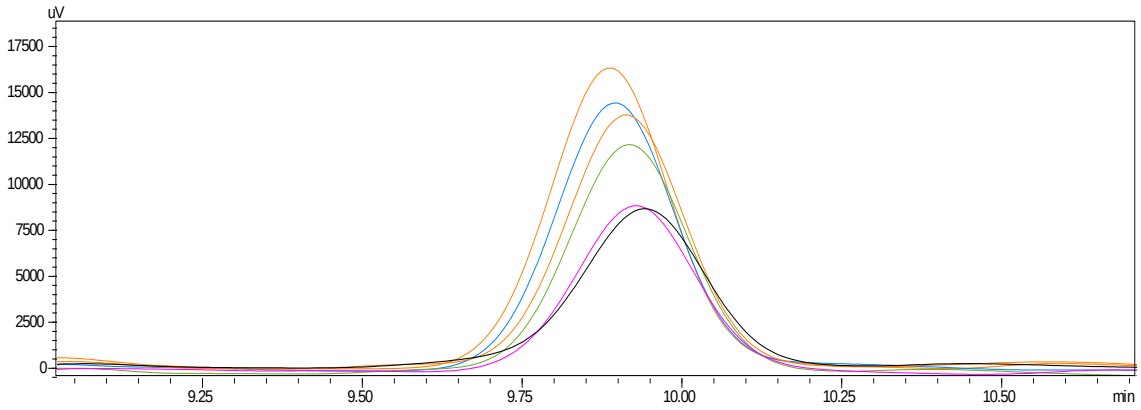
Peak area of OA



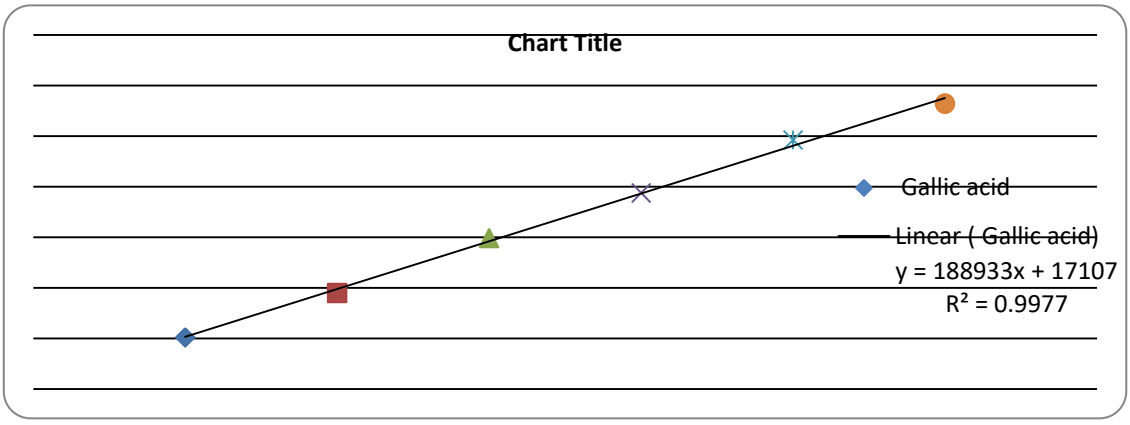
Overlay HPLC Chromatogram for different linearity concentration for both markers.



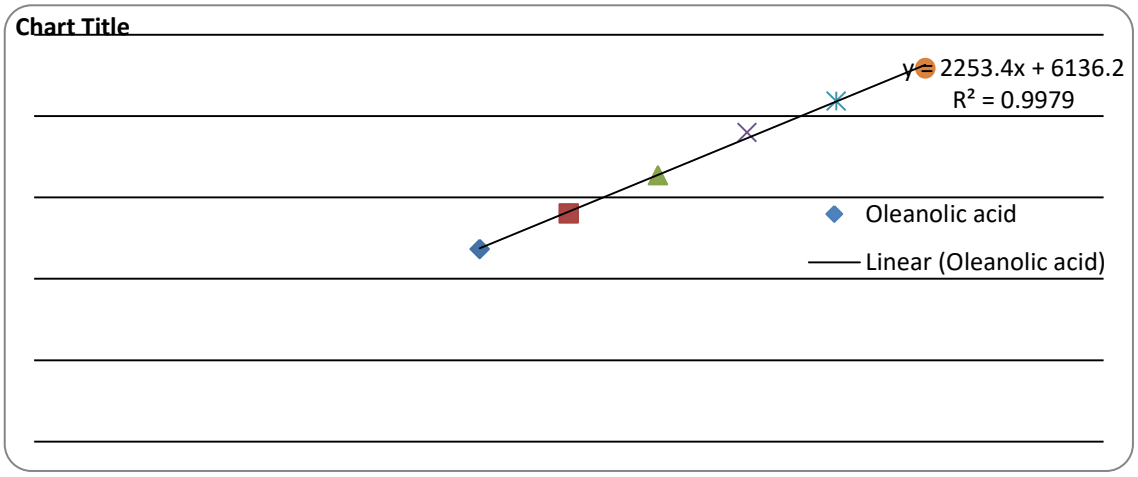
Overlay HPLC Chromatogram for different linearity concentration for Gallic Acid



Overlay HPLC Chromatogram for different linearity concentration for Oleanolic Acid



Calibration curve between Area of peak GA verses its Concentration .



Calibration curve between Area of peak OA verses Concentration .

Precision data:

Markers	Precision	% RSD of Retention time	% RSD of Area
Gallic acid	**Interday**	0.58	1.94
	Intraday	0.10	1.02
Oleanolic acid	**Interday**	0.25	1.41
	Intraday	0.18	0.72

Interday and Intraday precision data.

Limit: % RSD of RT should be less than 2.0 and for area NMT 5.0

Here both the markers in combination mixture at lower, middle and higher concentration range showed %RSD of Retention time and Peak area in limit specified in ICH guideline.

Accuracy

Accuracy was performed by recovery study where known concentrations of markers were to be added and calculated the amount to be recovered which shown in following table.

Markers	Initial Amount(A)	Addition of known quantity(B)		A+B	Amount recovered (mg)	% Recovery	Accepted % Limit for Recovery
Gallic acid	0.031	80%	0.025	0.0558	0.0561	100.54	98-102%
		100%	0.031	0.062	0.0619	99.84	
		120%	0.0372	0.0682	0.0689	101.03	
Oleanolic acid	0.01	80%	0.008	0.018	0.0182	101.1	
		100%	0.01	0.02	0.0198	99	
		120%	0.012	0.022	0.0219	99.54	

Table 10: Recovery study of HPLC method

Robustness data:

Parameters	Changes	Concentration in	Retention time(RT) in	RSD of RT	Area Under Peak	RSD of Area

		µg/ml		minute							
		GA	**OA**	**GA**	**OA**	**GA**	**OA**	**GA**	**OA**	**GA**	**OA**
Flow rate	0.9 ml	4	80	3.136	10.987	0.08	0.05	1007007	234933	0.15	1.40
	1 ml			2.827	9.93	0.10	0.41	769777	191644	1.11	0.62
	1.1 ml			2.56	9.020	0.11	0.07	828029	193256	0.23	0.6
Detection wavelength	221 nm			2.827	9.805	0.089	0.76	839239	230555	2.285	0.76
	222 nm			2.835	9.687	0.058	0.26	775443	189978	1.154	0.12
	223 nm			2.817	9.751	0.23	0.45	725557	156890	1.852	0.25
Mobile phase composition	90: 10			2.829	19.3	0.35	0.21	243330	137715	2.012	1.87
	98 : 2			2.804	7.5	0.21	0.14	238514	129056	1.478	2.45
	97 : 3			2.826	7.916	0.41	0.45	256412	156256	0.75	1.89

Robustness data for method validation

For changes in mobile phase combination, flow rate and detection wavelength, the results showed that the % Relative Standard Deviation of RT and Peak area passed the specified limit as per ICH Guideline. Therefore, method should be robust.

LOD and LOQ:

Parameters	Gallic acid	Oleanolic acid
LOD	0.012	1.2116
LOQ	0.039	3.6723

Sensitivity of method

Quantification of Markers in developed polyherbal tablet:

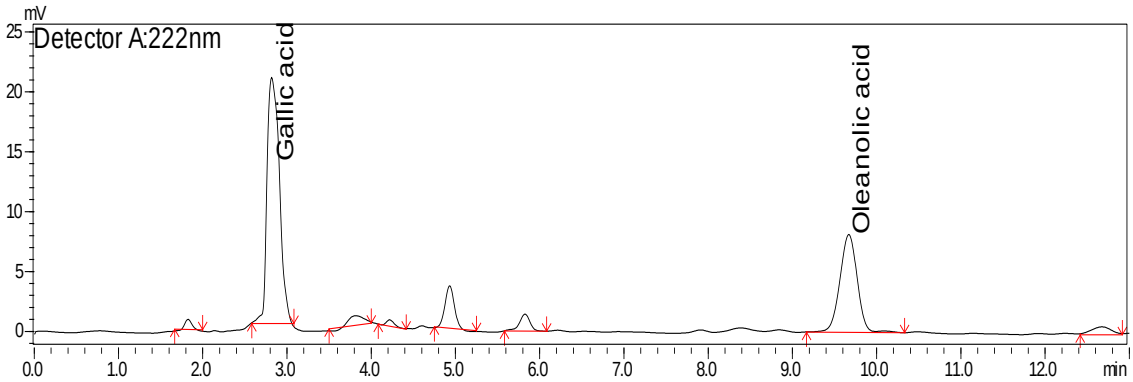


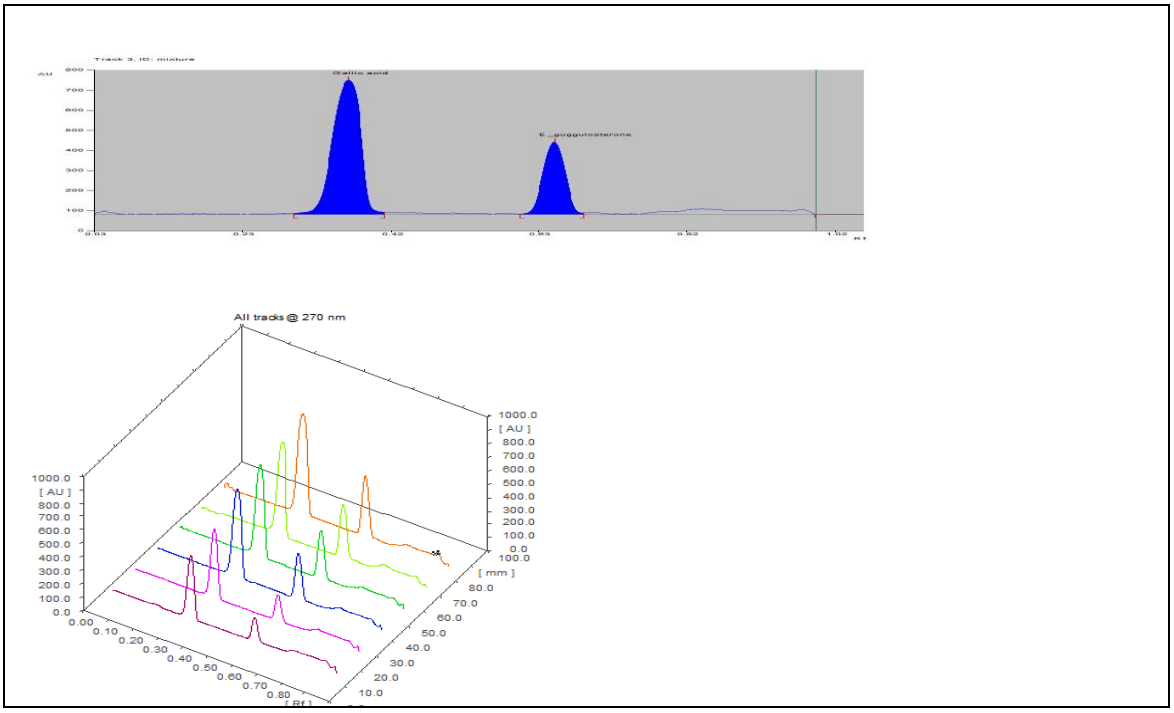
Figure 5: HPLC Chromatogram for developed polyherbal tablet.

Sample	Amount	
	Gallic acid %	Oleanolic acid %
Polyherbal tablet	0.031	0.01

Quantification of markers in laboratory formulated tablet.

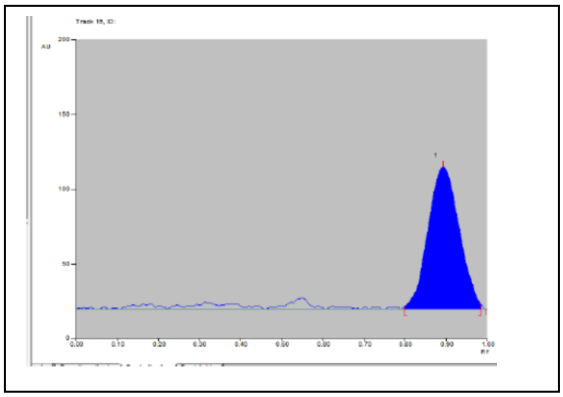
HPTLC Method Development and Validation of simultaneous estimation of Gallic acid, Oleanolic acid and E-Guggulosterone

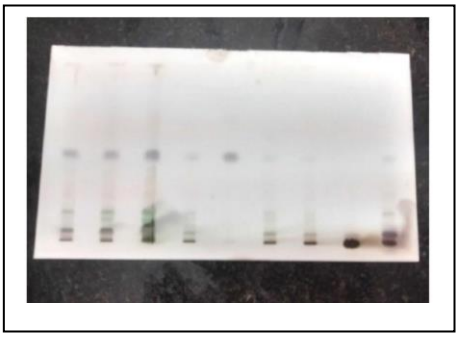
Parameters	Gallic acid	Oleanolic acid	E_Guggulosterone
Retardation factor	0.32	0.68	0.8
Deection wavelength	270 nm		368 nm
Linearity(Corelation coefficient)	0.9987	0.9981	0.9983
Beer's range(µg/ml)	5-30 ug/spot		
Regression equation	y= 661.08x + 8963.6	y = 348.26x + 2240.3	y = 162.37x + 633.33
Precision	0.21-1.58	0.14-1.87	0.56-1.54
LOD	0.243	0.512	0.846
LOQ	0.737	1.554	2.563
Accuracy	99.84-100.82	99.14-101.2	98.5-101.2
Robustness	Robust	Robust	Robust



HPTLC Method for Mahanimbine in *Morraya koinigi* ext.

Stationary phase Aluminum oxide 150 F254, neutral
Mobile phase n- hexane: ethyl acetate (9:1)
Calibration range 2- 15 µL
Detection Scanned under UV at 254 nm
Derivatization 5 or 10% H2SO4 : violet purple spots
Regression equation (area vise) Y= 610.4x + 164.6
R2 value (area vise) 0.999





Sr.No	Parameter	Values
1	Linearity	Y= 610.4x + 164.6
2	R_2 value	0.999
3	Interday precision	(RSD) 0.119451
4	Intraday precision	(RSD) 0.11744
5	Assay	0.66 µg/ml
6	Limit of detection (LOD)	0.0140
7	Limit of quantification (LOQ)	0.0426

8	Recovery	
	80%	100.81%
	100%	98.95%
	120%	99.44%

In Silico Method for screening of marker compounds for obesity

The OB-receptor or leptin receptor (LR) is crucial for energy homeostasis and regulation of food uptake. Leptin is a 16 kDa hormone that is mainly secreted by fat cells into the bloodstream. Under normal circumstances, circulating leptin levels are proportionate to the fat body mass. Sensing of elevated leptin levels by the hypothalamic neuro-circuitry activates a negative feedback loop resulting in reduced food intake and increased energy expenditure.

Work is under progress with other marker compound and other related receptor for obesity.

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