CHAPTER 8

8. Chromatographic Analysis of Active Compositions

Medicinal plants contain various bio-actives that mainly includes the secondary metabolites/ phytochemicals possessing various pharmacological actions. These bio-actives can be used either singly or in combination, where they may potentiate or synergize the effects of other phytochemicals. As mentioned in Chapter 2, various secondary metabolites have been reported to possess PL inhibition activity. Out of all such metabolites, polyphenols are widely reported as PL inhibitors [1]. Extracts containing polyphenols are very well known to lower body weight, plasma FFA levels, and lipid accumulation. Most used polyphenols include various subclasses such as flavonoids and iso-flavonoids that are mostly found in PL inhibitory plants such as citrus, tea etc [2,3]. Apart from polyphenols, saponins and alkaloids are other classes of secondary metabolites that are also found to inhibit PL. Saponins are commonly present in sea cucumber, legumes and plants [4,5]. Amongst saponin Triterpenoids class is commonly reported for PL inhibition [6]. Carbazole, bis-indole and isoquinoline class of alkaloids have been well explored for PL inhibitory potential[7-9].

Taking into consideration the potentiality of these secondary metabolites/phytochemicals, efforts have been made to develop compositions based on extracts rich in such bio-active molecules. However, the previous studies have mainly focused on the inhibition of PL by use of extract either rich in polyphenol/ alkaloid or saponin. The PL inhibitory potential of combinations of either extracts in a single composition has been scarcely explored. Nevertheless, to understand the magnitude and potential of such phytochemicals after their oral intake, it is essential to understand their fate in the gastric environment that in turn may affect the overall enzymatic inhibition process. The activity of the extracts/phytochemicals after the *in vitro* digestion is mainly attributed to a possible effect of the digestion process, temperature, or pH of the media. Thus, these complex factors that are involved during *in vivo* situation play important role while evaluation of the inhibitory activity of extract(s)/fractions/ phytochemicals and their combinations against digestives enzymes [10].

To evaluate the *in vitro* digestion of related phytochemicals in extracts, chromatography based analytical techniques have been commonly used. Various analytical methods have been reported for the quantitative determination of phytochemicals using High-performance Thin-layer chromatography (HPTLC) [11,12], High-performance Liquid chromatography (HPLC) [3,13] and Ultra Performance Liquid chromatography equipped with Mass Spectroscopy (UPLC-

MS/MS) [14,15]. Analytical method development includes optimization of the various stages of sample processing, chromatographic resolution and detection. An elaborative literature survey of the analytical methods on the same or similar analytes is of key importance. Some of the parameters that are frequently evaluated during analytical method development and optimization are selection of detector, column, mobile phase, organic modifier and sample preparation techniques. As per USFDA guidelines an analytical procedure is developed to test a defined characteristic of the drug substance against established acceptance criteria for that characteristic, while method validation is the process of demonstrating that an analytical procedure is suitable for its intended purpose. The typical validation parameters include system suitability; Limit of detection (LOD); Limit of quantification (LOQ); Linearity; Precision; Accuracy; Robustness.

Since, until now no methods have been established for the simultaneous quantitative analysis of the selected bio-active markers of the extracts in all active compositions [16], new methods were developed, optimized and validated as per the USFDA guidelines [17].

8.1 HPLC method development for BARM-GSM

A systematic approach was followed for the method development using suitable chromatographic conditions (Method 1 in Chapter 4). After final optimization, the most suitable chromatographic system for the simultaneous quantification of bio-active markers presents in the composition consisted of the following parameters namely: Hypersil Gold Thermo Scientific C_{18} column (250 × 4.6 mm, 5 μm), mobile phase: A-Acetonitrile B- KH_2PO_4 buffer in gradient mode, 1.5 ml/min flow rate, injection volume of 20 μl and column temperature of 27 °C. This method offered best peak shape and intensities along with the desired selectivity [18].

8.1.1 Analysis of BARM-GSM in SGF media

Using the optimized HPLC method 1, quantification of berberine and palmatine was performed. The % yield of berberine and palmatine in the BARM-GSM were found to be 18.63 and 10.18 % w/w respectively. No peak of gymnemagenin was observed in the composition before SGF treatment.

During the fasted state, the pH of the fluids in stomach ranges between 1 to 7.5. Food intake results in rapid increase of the gastric pH. Depending on the composition of the meal, the fed-state gastric pH increases and is between 4 and 7. Soon after food intake, the gastric pH gradually returns to the fasted-state pH. This constant change in pH of the fluid in stomach in

presence and absence of food decides the fate of the drug molecules. To understand the stability of the bio-active markers at physiological pH, SGF studies were performed [19,20].

Significant changes were observed when the BARM-GSM was exposed to SGF media of pH 1.2. Berberine and palmatine were found to be stable in the media, while a peak at 9.417 min (indicated in red in Figure 57) was detected. Using the previous reported R_t and λ_{max} in literature, the peak was understood to be of gymnemagenin. This peak was further confirmed by comparision with standard gymnemagenin. Presence of gymnemagenin confirmed the digestive instability of gymnemic acid in BARM-GSM to low pH **(Figure 57)**. Earlier reports suggest that gymnemic acid break down into its respective aglycone and sugar residues. The presence of peak of gymnemagenin in the digestion studies confirmed the presence of gymnemic acid I in the extract. Quantification of gymnemic acid has been a difficult task as it is easily hydrolysable to its respective gymnemasaponins **(Figure 58)** [21]. The amount of berberine and palmatine sustained in SGF media were recovered to be 88.08% and 87.14%, respectively. The % yield of gymnemagenin formed after *in vitro* digestion was found to be 0.22% w/w.

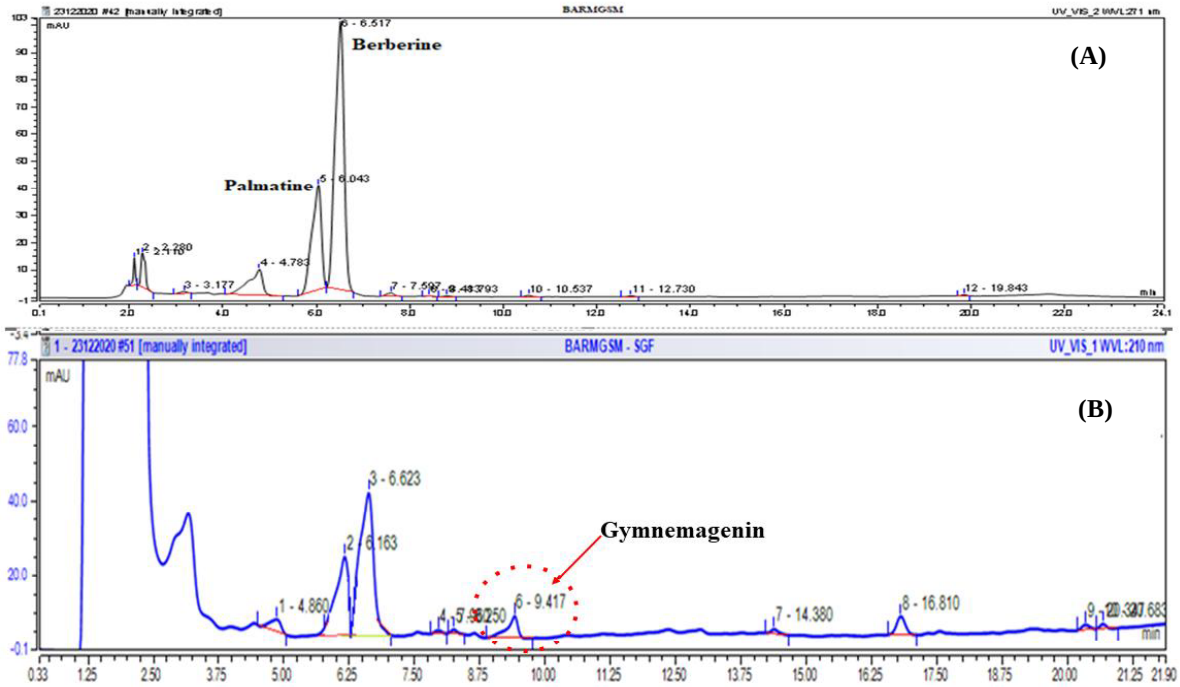

Figure 57: HPLC analysis of BARM-GSM (A) before and (B) after SGF digestion

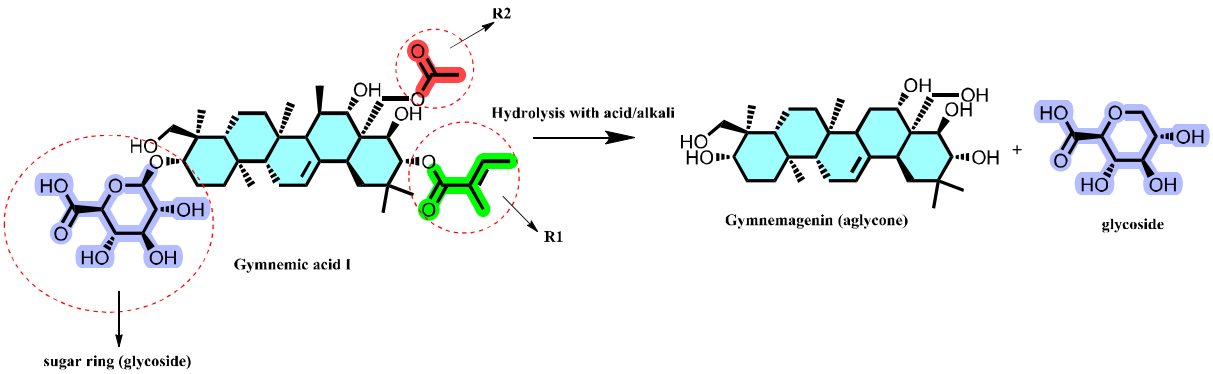

Figure 58: Breakdown of Gymnemic acid I into its aglycone and sugar residues

8.1.2 HPLC method validation

The developed method was validated in terms of specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, intra-day and inter-day precision, accuracy, and robustness for palmatine, berberine and gymnemagenin. Chromatogram of bio-active markers is represented in **Figure 59**.

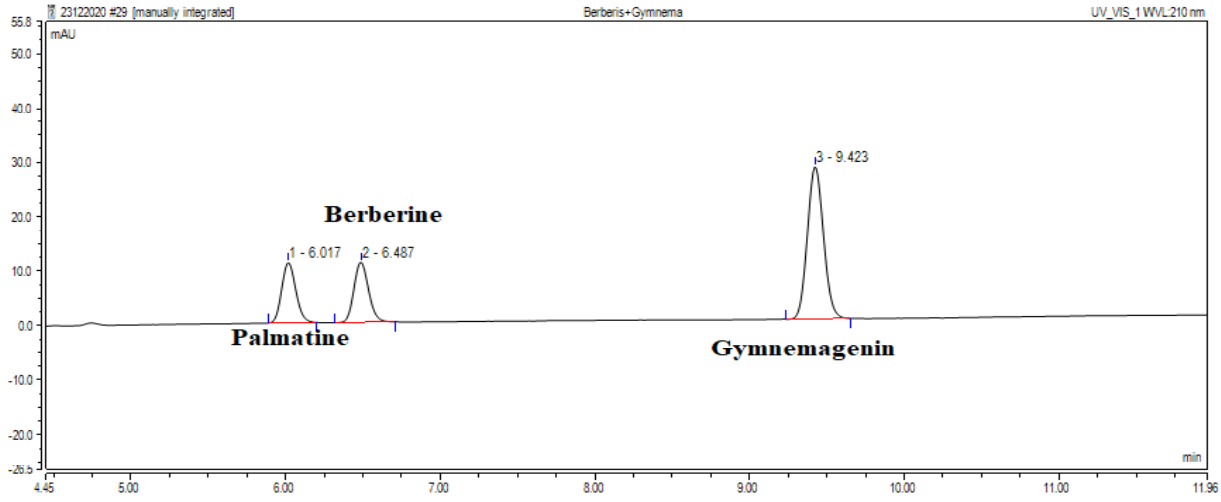

Figure 59: HPLC chromatogram of palmatine, berberine and gymnemagenin

The %RSD of peak area as well as R_t of analytes, was calculated and found to be within 2%, indicating the suitability of the system. The %RSD of the height equivalent to theoretical plates of the column for the seven replicate injections were found to be 20800 ± 1.78% for palmatine 22589 ± 1.12% for berberine and 38669 ± 1.01% for gymnemagenin, respectively. The number of theoretical plates was greater than 2000 and considered to be acceptable for the system suitability test. The retention time (R_t) of all the analytes are tabulated in **Table 10**. The signal-

to-noise ratio of 3.3:1 and 10:1 was obtained for the LOD and LOQ, respectively. The LOD and LOQ were found to be 16.16 ng/ml and 49.46 ng/ml for palmatine, 17.13 ng/ml and 51.41 ng/ml for berberine and 27.01 ng/ml and 82.68 ng/ml for gymnemagenin, respectively.

Linearity was described in terms of the calibration curve. This curve was obtained by plotting the mean peak area of the three analytes against their corresponding concentrations. The results indicated a linear relationship over the concentration range of 49.47-4748.71 ng/ml for palmatine, 51.39-4933.81 ng/ml for berberine and 82.69-7939.17 ng/ml for gymnemagenin, respectively. From the regression data, a linear equation of palmatine, berberine and gymnemagenin were found to be $y = 0.0006x - 0.062$ ($r^2 = 0.9969$); $y = 0.0001x + 0.1043$ ($r^2 = 0.9946$) and $y = 0.0013x - 0.1769$ ($r^2 = 0.9991$), respectively. These results indicated a linear relationship between the mean peak area and concentration of the analytes.

Table 10: Summary of optimized HPLC method for the analytes of BARM-GSM

Parameter	Analyte	Range
Instrumental precision ThermoScientific Dionex UltiMate 3000	Palmatine (% CV, n=7) Berberine (% CV, n=7) Gymnemagenin (% CV, n=7)	0.71 0.87 0.35
Specificity (n=7)	Palmatine Berberine Gymnemagenin	6.07 ± 0.07 min 6.62 ± 0.10 min 9.39 ± 0.02 min
Limit of Detection (LOD)	Palmatine Berberine Gymnemagenin	16.16 ng/ml 17.13 ng/ml 27.01 ng/ml
Limit of Quantification (LOQ)	Palmatine Berberine Gymnemagenin	49.46 ng/ml 51.41 ng/ml 82.68 ng/ml
Linearity	Palmatine Berberine Gymnemagenin	49.47-4748.71 ng/ml 51.39-4933.81 ng/ml 82.69-7939.17 ng/ml

All the values are expressed as mean± S.E.M(n = 7)

The accuracy was expressed in terms of % biasness (i.e. the proximity to the actual value), while precision was defined in terms of % RSD. The %RSD of peak area for intra-day precision and inter-day precision, % bias at all three QC levels are concised in **Table 11.** These results were found to be within the accepted limits (± 15%, except for LOQ), that verified the applicability of the developed method for routine analysis.

Table 11: Precision (% CV) and accuracy (% bias) of the analytes of BARM-GSM.

Analyte	Nominal Conc (ng/ml)	Intra-day			Inter-day		
		Measured Conc. (Mean ± SD, ng/ml)	Precision (% CV)	Accuracy (% bias)	Measured Conc. (Mean ± SD, ng/ml)	Precision (% CV)	Accuracy (% bias)
Palmatine	100	96.76 ± 1.01	1.04	3.24	97.41 ± 1.20	1.23	2.59
	450	443.66 ± 0.27	0.06	1.41	441.33 ± 0.89	0.20	1.93
	3500	3468.33 ± 1.54	0.04	0.90	3510.12 ± 1.21	0.03	-0.29
Berberine	105	103.67 ± 2.95	2.85	1.27	100.78 ± 1.45	1.44	4.02
	460	459.3 ± 1.93	0.42	0.15	461.6 ± 1.01	0.22	-0.35
	3700	3677.92 ± 2.67	0.07	0.60	3662.07 ± 1.67	0.05	1.03
Gymnema-genin	165	163.66 ± 0.88	0.54	0.81	164.69 ± 2.34	1.42	0.19
	745	745.92 ± 1.02	0.14	-0.12	743.92 ± 1.65	0.22	0.14
	6000	5950.33 ± 0.68	0.01	0.83	5938.23 ± 0.87	0.01	1.03

All the values are expressed as mean± S.E.M(n = 5)

The robustness of the analytical process was evaluated by assessing the effect of small differences in HPLC conditions i.e., change in column oven temperature (25 and 29 °C), flow rate (1.4 ml/min and 1.6 ml/min), and change in pH (4.8 and 5.2) of the solvent system. The results are summarized in **Table 12** and **13.** It was found that a minor change in method condition did not significantly affect the theoretical plates and R_t of the analyte.

Table 12: Method optimization for BARM-GSM: Retention time (R_t) obtained upon making deliberate variations in the chromatographic conditions

Variations in Chromatographic condition	R_t (min)		
	Palmatine	**Berberine**	**Gymnemagenin**
No variation (Optimized)	6.07 ± 0.07	6.62 ± 0.10	9.39 ± 0.02
Flow rate (1.4 ml/min)	6.04 ± 0.23	6.59 ± 0.47	9.33 ± 0.14
Flow rate (1.6 ml/min)	6.15 ± 0.12	6.65 ± 0.34	9.45 ± 0.69
Mobile phase (ACN: KH_2PO_4 buffer; pH=4.8)	6.12 ± 0.06	6.58 ± 0.77	9.44 ± 0.23
Mobile phase (ACN: KH_2PO_4 buffer; pH=5.2)	6.02 ± 0.05	6.49 ± 0.23	9.23 ± 0.33
Column temperature (25 °C)	6.02 ± 0.02	6.52 ± 0.42	9.35 ± 0.01
Column temperature (29 °C)	6.11 ± 0.05	6.62 ± 0.89	9.45 ± 0.12

All the values are expressed as mean± S.E.M (n = 6).

Table 13: Method optimization for BARM-GSM: HETP and number of theoretical plates (N) obtained upon making deliberate variations in the chromatographic conditions

Variations in Chromatographic condition	Effect on chromatographic parameters of analytes (n=6) ± SEM					
	HETP			N		
	Palmatine	**Berberine**	**Gymnemagenin**	**Palmatine**	**Berberine**	**Gymnemagenin**
No variation (Optimized)	20800 ± 1.78	22589 ± 1.12	38669 ± 1.01	28500.94 ± 1965.81	24078.59 ± 926.23	19592.95± 849.29
Flow rate (1.4 ml/min)	20812 ± 1.45	22612 ± 1.01	38623 ± 1.56	26221.11 ± 1080.62	23282.14 ± 3008.07	18677.99 ± 768.23
Flow rate (1.6 ml/min)	20769 ± 1.12	22578 ± 0.95	38679± 0.26	27295.91 ± 860.12	25511.36 ± 566.43	20034.09 ± 616.78
Mobile phase (ACN: KH_2PO_4 buffer pH=4.8)	20754 ± 1.02	22623 ± 1.23	38519 ± 1.31	28790.23 ± 2076.01	24065.23 ± 462.81	19034.67 ± 819.80
Mobile phase (ACN: KH_2PO_4 buffer pH=5.2)	20789 ± 0.64	22487 ± 1.54	38651 ± 0.12	27654.42 ± 978.21	24548.76± 953.49	19786.34 ± 899.48
Column temperature (25 °C)	20921 ± 0.45	22781 ± 1.12	38621 ± 0.98	26543.32 ± 1165.65	25576.98 ± 532.45	20123.56 ± 589.89
Column temperature (29 °C)	20785 ± 0.23	22621 ± 0.99	38667 ± 1.20	28987.23 ± 2000.76	24489.21 ± 943.90	19587.23 ± 856.87

All the values are expressed as mean± S.E.M(n = 6)

8.2 HPLC method development for BARM-TSM

A systematic approach was followed for the method development using suitable chromatographic conditions (Method 2 in Chapter 4), along with an easy and quick sample preparation technique. After the final optimization, method consisting of Hypersil Gold Thermo Scientific C_{18} column (250 × 4.6 mm, 5 µm), mobile phase including A-Acetonitrile B- Water with 0.1% ortho-phosphoric acid (25:75, % *v/v*) in isocratic mode,1 ml/min flow rate, injection volume of 10 µl and column temperature of 35°C was found to be most suitable for the simultaneous quantification of berberine, palmatine, ECG and EGCG. This method offered the best peak shape and intensities along with the desired selectivity.

8.2.1 Analysis of BARM-TSM in SGF media

From the calibration plot, chromatographic evaluation for BARM-TSM was performed. The study revealed that the BARM-TSM contained 2.5% w/w ECG, 5.7% w/w EGCG, 18.3% w/w berberine and 8.2% w/w of palmatine, respectively.

When exposed to SGF medium of pH 1.2, ECG (76.96 %) exhibited some level of digestive instability as compared to EGCG (82.96%), berberine (86.88%) and palmatine (83.66%) (**Figure 60)**.

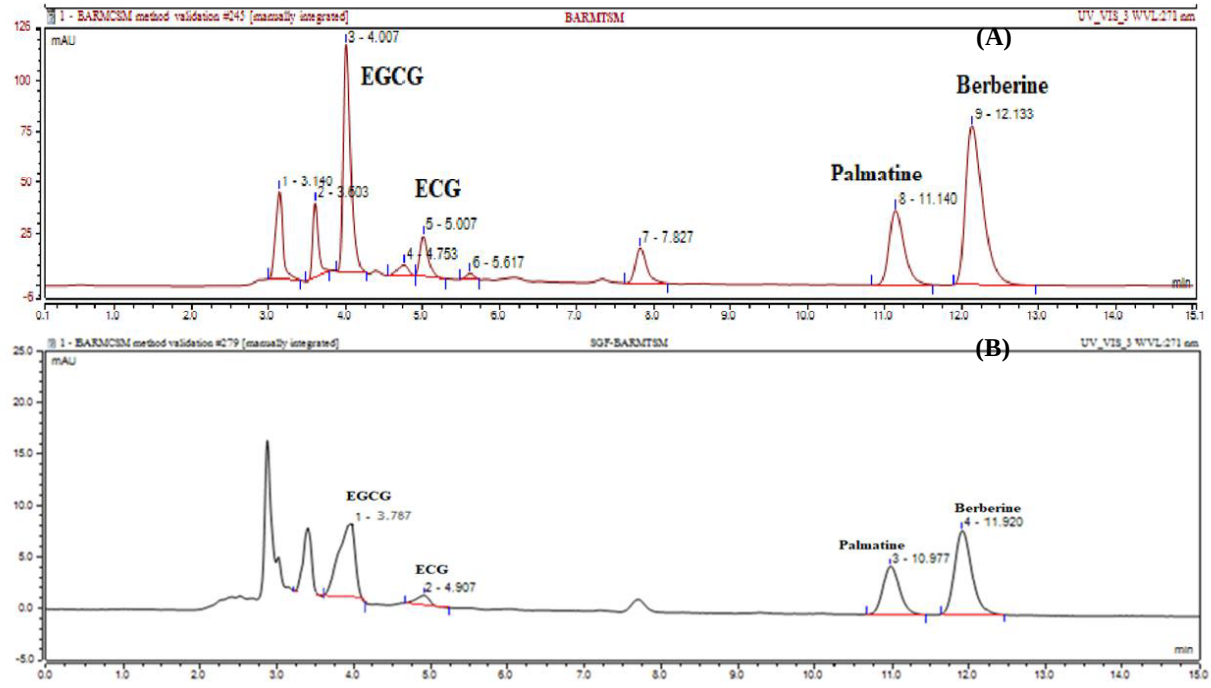

Figure 60: HPLC analysis of BARM-TSM (A) before and (B) after SGF digestion

The above result was supported by the previous reports of Neilson *et al*. (2007) wherein it was observed that EGCG-ECG when digested resulted in the formation of homodimers and presence of residual EGCG and ECG [22].

8.2.2 HPLC method validation

The developed method was validated in terms of specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, intra-day and inter-day precision, accuracy, robustness, and recovery for palmatine, berberine, ECG, and EGCG as per US FDA guidelines **(Figure 61).**

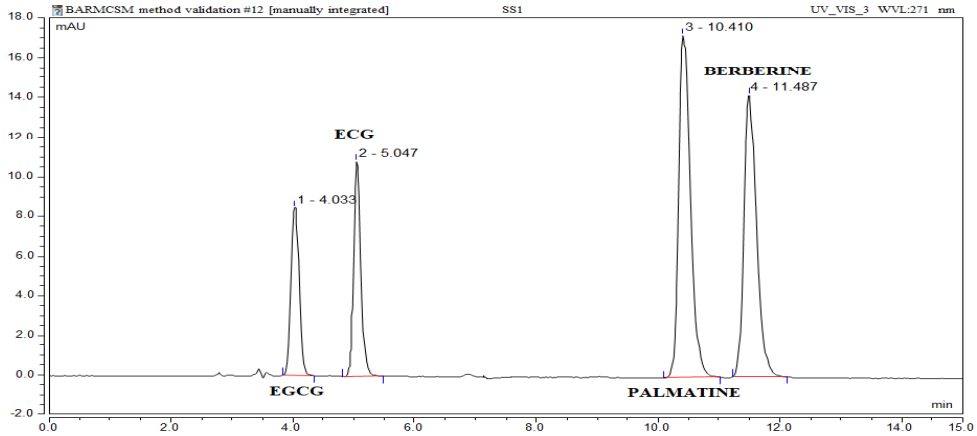

Figure 61: HPLC chromatogram of EGCG, ECG, palmatine and berberine

The %RSD of peak area, as well as R_t of analytes, was calculated and found to be within 2%, indicating the suitability of the system. The %RSD of the height equivalent to theoretical plates of the column for the seven replicate injections were found to be 5235 ± 1.67% for EGCG; 9219 ± 1.98% for ECG; 15098 ± 1.18% for palmatine and 17126 ± 1.23% for berberine. The number of theoretical plates was greater than 2000 and considered to be acceptable for the system suitability test. The retention time (R_t) of all the analytes are presented in **Table 14**. The LOD and LOQ were found to be 47.66 ng/ml and 145.88 ng/ml for EGCG, 13.09 ng/ml and 40.07 ng/ml for ECG, 10.45 ng/ml and 31.99 ng/ml of palmatine, 13.59 ng/ml and 41.61 ng/ml for berberine.

The results obtained from calibration curve provide a linear relationship over the concentration range of 145.0-13920.0 ng/ml for EGCG, 40.0-3840.0 ng/ml for ECG, 31.9-3062.4 ng/ml for palmatine and 41.8-4012.8 ng/ml for berberine. From the regression data, a linear equation of EGCG, ECG, palmatine and berberine were found to be $y = 0.0003x - 0.0118$ ($r^2 = 0.9993$), $y = 0.0002x + 0.0103$ ($r^2 = 0.9992$), $y = 0.0005x + 0.0029$ ($r^2 = 0.9993$) and $y = 0.0006x - 0.0185$ ($r^2 = 0.9980$) respectively.

Table 14: Summary of optimized HPLC method for the analytes of BARM-TSM

PARAMETER	Analyte	RANGE
Instrumental precision ThermoScientific Dionex UltiMate 3000	EGCG (% CV, n=7) ECG (% CV, n=7) Palmatine (% CV, n=7) Berberine (% CV, n=7)	1.42 1.31 0.93 1.48
Specificity (n=7)	EGCG ECG Palmatine Berberine	4.020 ± 0.01 min 5.025 ± 0.03 min 10.354 ± 0.04 min 11.392 ± 0.07 min
Limit of Detection (LOD)	EGCG ECG Palmatine Berberine	47.66 ng/ml 13.09 ng/ml 10.45 ng/ml 13.59 ng/ml
Limit of Quantification (LOQ)	EGCG ECG Palmatine Berberine	145.88 ng/ml 40.07 ng/ml 31.99 ng/ml 41.61 ng/ml
Linearity	EGCG ECG Palmatine Berberine	145.0-13920.0 ng/ml 40.0-3840.0 ng/ml 31.9-3062.4 ng/ml 41.8-4012.8 ng/ml

All the values are expressed as mean± S.E.M (n = 7).

The %RSD of peak area for intra-day precision and inter-day precision, % bias at all three QC levels are summarized in **Table 15.** These results were found to be within the accepted limits (± 15%, except for LOQ), which demonstrated the applicability of the developed method for routine analysis.

Table 15: Precision (% CV) and accuracy (% bias) of the analytes of BARM-TSM

Analyte	Nominal conc (ng/ml)	Intra-day			Inter-day		
		Measured Conc. (Mean ± SD, ng/ml)	Precision (% CV)	Accuracy (% bias)	Measured Conc. (Mean ± SD, ng/ml)	Precision (% CV)	Accuracy (% bias)
EGCG	290	252.33 ± 6.93	2.75	2.98	257.15 ± 2.06	0.80	2.76
	2610	2526.74 ± 0.57	2.27	3.19	2569.44 ± 0.81	3.14	1.55
	10440	9884.00 ± 1.74	1.76	5.32	10036.80 ± 4.63	0.43	3.86
ECG	80	77.83 ± 0.77	3.71	2.71	79.67 ± 1.26	1.58	0.42
	720	709.50 ± 0.27	3.85	1.45	701.67 ± 7.09	1.01	2.55
	2880	2766.00 ± 0.45	1.61	3.95	2712.67 ± 0.61	2.25	5.81
Palmatine	63.8	60.93 ± 0.95	1.55	4.49	61.87 ± 0.23	0.37	3.03
	574.2	572.40 ± 1.91	0.33	0.31	573.73 ± 1.33	0.23	0.08
	2296.8	2195.01 ± 2.11	0.96	4.43	2181.67 ± 4.96	0.23	5.01
Berberine	83.6	82.94 ± 1.87	2.26	0.78	82.72 ± 0.53	0.65	1.05
	752.4	748.16 ± 4.04	0.54	0.56	750.06 ± 2.34	0.31	0.31
	3009.6	2961.60± 0.71	2.40	1.61	2947.44 ± 0.55	1.85	2.11

All the values are expressed as mean± S.E.M (n =5).

CHAPTER 8

The robustness of the analytical process was evaluated by assessing the effect of small variations in HPLC conditions i.e., change in column oven temperature (30 and 40°C), flow rate (0.9 ml/min and 1.1 ml/min), and change in pH (3.2 and 3.6) of the solvent system. The results are summarized in **Table 16** and **17**. It was found that a minor change in method condition did not significantly affect the theoretical plates and R_t of the analyte. Thus, the proposed method was found to be reliable and robust

Table 16: Method optimization for BARM-TSM: Retention time (R_t) obtained upon making deliberate variations in the chromatographic conditions.

Variations in Chromatographic condition	R_t (min)			
	EGCG	ECG	Palmatine	Berberine
No variation (Optimized)	4.02 ± 0.01	5.02 ± 0.03	10.35 ± 0.04	11.39 ± 0.07
Flow rate (0.9 ml/min)	3.91 ± 0.03	5.01 ± 0.05	10.29 ± 0.06	11.32 ± 0.04
Flow rate (1.1 ml/min)	3.95 ± 0.01	4.98 ± 0.07	10.40 ± 0.02	11.28 ± 0.02
Mobile phase (ACN: Phosphate buffer pH=3.2)	3.94 ± 0.02	4.99 ± 0.03	10.29 ± 0.03	11.18 ± 0.04
Mobile phase (ACN: Phosphate buffer pH=3.6)	4.04 ± 0.03	5.05 ± 0.02	10.38 ± 0.01	11.39 ± 0.09
Column temperature (30 °C)	4.01 ± 0.02	5.01 ± 0.01	10.31 ± 0.06	11.37 ± 0.05
Column temperature (40 °C)	3.96 ± 0.03	4.89 ± 0.03	10.27 ± 0.01	11.30 ± 0.02

All the values are expressed as mean± S.E.M (n =6).

Table 17: Method optimization for BARM-TSM: HETP and number of theoretical plates (N) obtained upon making deliberate variations in the chromatographic conditions.

Variations in Chromatographic condition	Effect on chromatographic parameters of analytes (n=6)							
	HETP				N			
	EGCG	ECG	Palmatine	Berberine	EGCG	ECG	Palmatine	Berberine
No variation (Optimized)	5235 ± 1.67	9219 ± 1.98	15098 ± 1.18	17126 ± 1.23	11501.24 ± 822.46	15588.82 ± 465.61	6254.00± 183.27	5393.93 ± 211.21
Flow rate (0.9 ml/min)	5148 ± 1.09	9390 ± 0.75	15565 ± 0.96	17864 ± 1.76	11528.76± 763.87	15950.73 ± 81.96	6694.15± 81.96	5450.28± 109.04
Flow rate (1.1 ml/min)	5929 ± 1.96	9337 ± 1.56	15709 ± 0.63	17126 ± 0.50	11560.22± 801.98	15841.78 ± 540.01	6730.56± 123.02	5435.81± 107.12
Mobile phase (ACN: Phosphate buffer pH=3.2)	6087 ± 1.65	9708 ± 1.51	16562 ± 2.78	17961 ± 1.56	11552.65± 883.98	15136.79 ± 642.13	6694.15± 147.04	5999.88± 282.01
Mobile phase (ACN: Phosphate buffer pH=3.6)	5123 ± 1.89	8372 ± 0.78	14480 ± 1.90	16821 ± 0.12	11632.19± 854.01	14957.65 ± 152.22	6723.91± 590.00	5075.71± 91.02
Column temperature (30 °C)	5513 ± 0.87	9109 ± 0.88	15610 ± 1.56	17691 ± 0.87	11608.02± 798.43	15848.61 ± 831.54	6700.74±800.03	5868.43± 789.06
Column temperature (40 °C)	5627 ± 1.77	9656 ± 0.97	16054 ± 2.25	16562 ± 2.75	11568.00± 775.67	15740.76 ± 371.33	6687.57± 89.01	5387.04± 234.57

All the values are expressed as mean± S.E.M (n =6).

8.3 HPLC method development for GSM-TSM

A systematic approach was followed for the method development using suitable chromatographic conditions (Method 1 in Chapter 4). After final optimization, Hypersil Gold Thermo Scientific C_{18} column (250 × 4.6 mm, 5 µm), mobile phase including A-Acetonitrile B-KH_2PO_4 buffer in gradient mode, 1.5 ml/min flow rate, injection volume of 20 µl and column temperature of 27°C was found to be the most suitable condition for the estimation of bio-active markers present in the composition, since it offered best peak shape and intensities along with the desired selectivity.

8.3.1 Analysis of GSM-SO & TSM-SO

From the calibration plot, chromatographic evaluation for GSM-TSM revealed that it contained 3.25% w/w of ECG and 4.13 % w/w of EGCG, while no peak of gymnemagenin was found [21].

8.3.2 HPLC method validation

The developed method was validated in terms of specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, intra-day and inter-day precision, accuracy, robustness, and recovery for ECG, EGCG and gymnemagenin as given in US FDA guidelines **(Figure. 62)**.

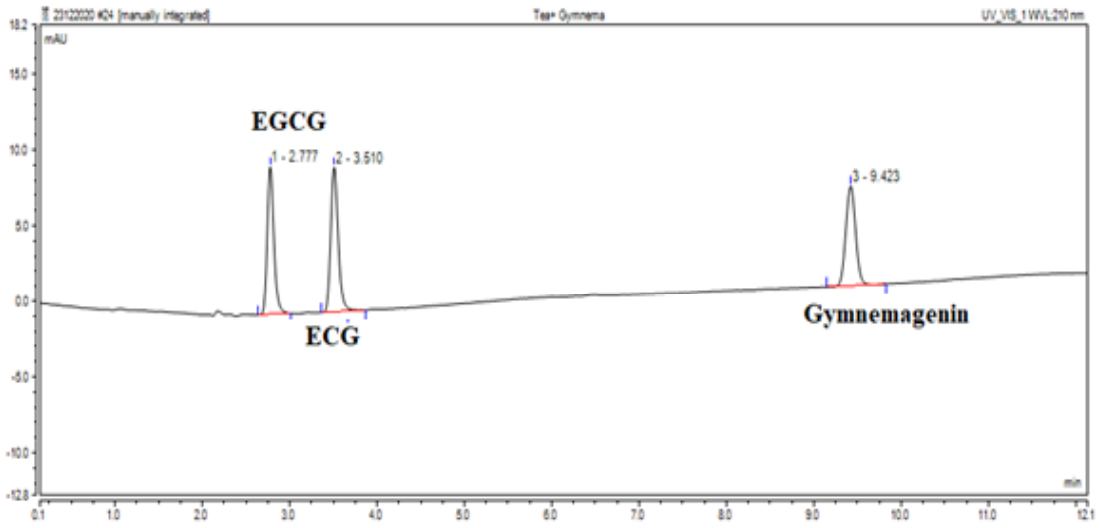

Figure 62: HPLC chromatogram of EGCG, ECG, and gymnemagenin

The %RSD of peak area, as well as R_t of analytes, was calculated and found to be within 2%, indicating the suitability of the system. The %RSD of the height equivalent to theoretical plates of the column for the seven replicate injections were found to be 5861 ± 1.01% for EGCG: 7694 ± 1.14% for ECG and 34346 ± 1.34% for gymnemagenin. The number of theoretical plates was greater than 2000 and considered to be acceptable for the system suitability test. The retention

time (R_t) of all the analytes are tabulated in **Table 18**. The LOD and LOQ were found to be 40.61 ng/ml and 124.37 ng/ml for EGCG, 15.34 ng/ml and 46.96 ng/ml for ECG, 115.00 ng/ml and 351.96 ng/ml for gymnemagenin.

The results obtained from the calibration curve provided a linear relationship over the concentration range of 142.87- 9143.58 ng/ml for EGCG, 46.97- 3005.99 ng/ml for ECG, and 351.96- 22526.06 ng/ml for gymnemagenin. From the regression data, a linear equation of EGCG, ECG and gymnemagenin were found to be $y = 0.0003x - 0.0319$ (r^2 =0.9997), $y = 0.0013x + 0.0594$ ($r^2 = 0.9992$) and $y = 0.0015x - 0.1641$ ($r^2 = 0.9989$), respectively. These results indicated a linear relationship between the mean peak area and concentration of the analytes.

Table 18: Summary of optimized HPLC method for the analytes of GSM-TSM

Parameter	**Analytes**	**Range**
Instrumental precision ThermoScientific Dionex UltiMate 3000	EGCG (% CV, n=7) ECG (% CV, n=7) Gymnemagenin (% CV, n=7)	0.64 1.20 0.52
Specificity (n=7)	EGCG ECG Gymnemagenin	2.78 ± 0.13 min 3.51 ± 0.01 min 9.43 ± 0.05 min
Limit of Detection (LOD)	EGCG ECG Gymnemagenin	40.61 ng/ml 15.34 ng/ml 115.00 ng/ml
Limit of Quantification (LOQ)	EGCG ECG Gymnemagenin	124.37 ng/ml 46.96 ng/ml 351.96 ng/ml
Linearity	EGCG ECG Gymnemagenin	142.87- 9143.58 ng/ml 46.97- 3005.99 ng/ml 351.96- 22526.06 ng/ml

All the values are expressed as mean± S.E.M (n =7).

The %RSD of peak area for inter-day precision and intra-day precision, % bias at all three QC levels are summarized in **Table 19.** These results were found to be within the accepted limits (± 15%, except for LOQ), which demonstrated the applicability of the developed method for routine analysis. The excellent recovery values for accuracy study ascertained that the method was accurate.

Table 19: Precision (% CV) and accuracy (% bias) of the analytes of GSM-TSM.

Analyte	Nominal conc (ng/ml)	Intra-day			Inter-day		
		Measured Conc. (Mean ± SD, ng/ml)	Precision (% CV)	Accuracy (% bias)	Measured Conc. (Mean ± SD, ng/ml)	Precision (% CV)	Accuracy (% bias)
EGCG	285	284.03 ± 5.48	1.93	0.33	278.00 ± 3.08	1.11	2.46
	1285	1293.33 ± 0.13	0.01	-0.65	1275.34 ± 1.09	0.09	0.75
	5000	4904.83 ± 0.89	0.02	1.91	5066.50 ± 0.35	0.01	-1.33
ECG	95	93.44 ± 2.45	2.62	1.63	93.00 ± 2.90	3.12	2.11
	420	422.63 ± 0.71	0.17	0.62	414.7 ± 1.05	0.25	1.26
	2880	1675.44 ± 0.16	0.01	3.95	1693.6 ± 0.76	0.04	0.38
Gymnemagenin	705	709.22 ± 1.34	0.19	-0.60	707.00 ± 1.99	0.28	-0.28
	3200	3211.67 ± 0.67	0.02	-0.36	3218.66 ± 1.33	0.04	-0.56
	12760	12651.33 ± 1.11	0.01	0.38	12452.13 ± 1.11	0.01	1.95

All the values are expressed as mean± S.E.M (n = 5)

The robustness of the analytical process was evaluated by assessing the effect of small variations in HPLC conditions i.e, change in column oven temperature (25 and 29°C), flow rate (1.4 ml/min and 1.6 ml/min), and change in pH (4.8 amd 5.2) of the solvent system. The results are summarized in **Table 20** and **21.** It was found that a minor change in method condition did not significantly affect the theoretical plates and R_t of the analyte. Thus, the proposed method was found to be reliable and robust.

Table 20: Method optimization for GSM-TSM: Retention time (R_t) obtained upon making deliberate variations in the chromatographic conditions

Variations in Chromatographic condition	R_t(min)		
	EGCG	ECG	Gymnemagenin
No variation (Optimized)	2.78 ± 0.13	3.51 ± 0.01	9.43 ± 0.05
Flow rate (1.4 ml/min)	2.76 ± 0.05	3.49 ± 0.11	9.38 ± 0.19
Flow rate (1.6 ml/min)	2.87 ± 0.04	3.57 ± 0.02	9.42 ± 0.03
Mobile phase (ACN: KH_2PO_4 buffer; pH= 4.8)	2.81 ± 0.15	3.48 ± 0.07	9.40 ± 0.17
Mobile phase (ACN: KH_2PO_4 buffer; pH=5.2)	2.77 ± 0.01	3.54 ± 0.15	9.46 ± 0.12
Column temperature (25 °C)	2.71 ± 0.20	3.55 ± 0.11	9.39 ± 0.12
Column temperature (29 °C)	2.76 ± 0.05	3.44 ± 0.02	9.43 ± 0.03

All the values are expressed as mean± S.E.M (n =6).

Table 21: Method optimization for GSM-TSM: HETP and number of theoretical plates (N) obtained upon making deliberate variations in the chromatographic conditions.

Variations in Chromatographic condition	Effect on chromatographic parameters of analytes (n=6)					
	HETP			N		
	EGCG	ECG	Gymnemagenin	EGCG	ECG	Gymnemagenin
No variation (Optimized)	5861 ± 1.01	7694 ± 1.14	34346 ± 1.34	34937.30 ± 1226.93	29429.07 ± 626.82	19142.77 ± 927.03
Flow rate (1.4 ml/min)	5859 ± 0.77	7692 ± 1.21	34343 ± 1.15	34686.99 ± 1640.64	29345.78 ± 898.34	19145.98 ± 811.76
Flow rate (1.6 ml/min)	5852 ± 0.69	7697 ± 1.12	34356 ± 1.57	35550.18 ± 2494.23	29543.55 ± 765.98	19630.78 ± 777.09
Mobile phase (ACN: KH_2PO_4 buffer; pH= 4.8)	5859 ± 0.91	7693 ± 1.66	34350 ± 2.45	34897.45 ± 1842.19	29577.65 ± 845.56	19032.85 ± 990.00
Mobile phase (ACN: KH_2PO_4 buffer; pH=5.2)	5863 ± 1.99	7688 ± 0.74	34351 ± 1.99	34798.13 ± 1598.45	29445.78 ± 634.43	19243.54 ± 845.29
Column temperature (25 °C)	5868 ± 0.37	7696 ± 0.53	34342 ± 1.23	34956.37 ± 1265.89	29648.28 ± 678.35	19135.62 ± 634.76
Column temperature (29 °C)	5865 ± 1.35	7690 ± 1.78	34348 ± 2.67	34877.77 ± 1877.09	29789.21 ± 771.89	19187.57 ± 881.79

All the values are expressed as mean± S.E.M (n =6)

CHAPTER 8

8.4 HPLC method development for AMM-PLM

A systematic approach was followed for the method development by using suitable chromatographic conditions (Method 3 of Chapter 4). After final optimization, reverse phase C_{18} column (Waters) of dimension 250 × 4 mm with 4µm particle size were used. The wavelength was set at 342 nm and 271 nm. Total run time was set as 20 min using a gradient mobile phase as 50:50% *v/v* (0-5 min), 60:40% *v/v* (5-10 min), 70:30 % *v/v* (10-15 min) and 50:50% *v/v* (15-20 min) of Methanol: Water. The total injection volume for analysis was 20 µl and the flow rate was 1.0 ml/min. The column temperature was set at 30°C.

8.4.1 Analysis of AMM-PLM

From the HPLC chromatogram, identification of alloimperatorin from AMM-SO, while piperine and pellitorine from PLM-SO was done. The calibration plot quantified the amount of these bio-active markers in the AMM-PLM. It was found that 5.65 % w/w of alloimperatorin, 6.73% w/w of piperine and 4.34% w/w of pellitorine was present in the composition.

8.4.2 HPLC method validation

The developed method was validated in terms of specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, intra-day and inter-day precision, accuracy, robustness, and recovery for alloimperatorin, piperine and pellitorine as given in US FDA guidelines **(Figure 63)**

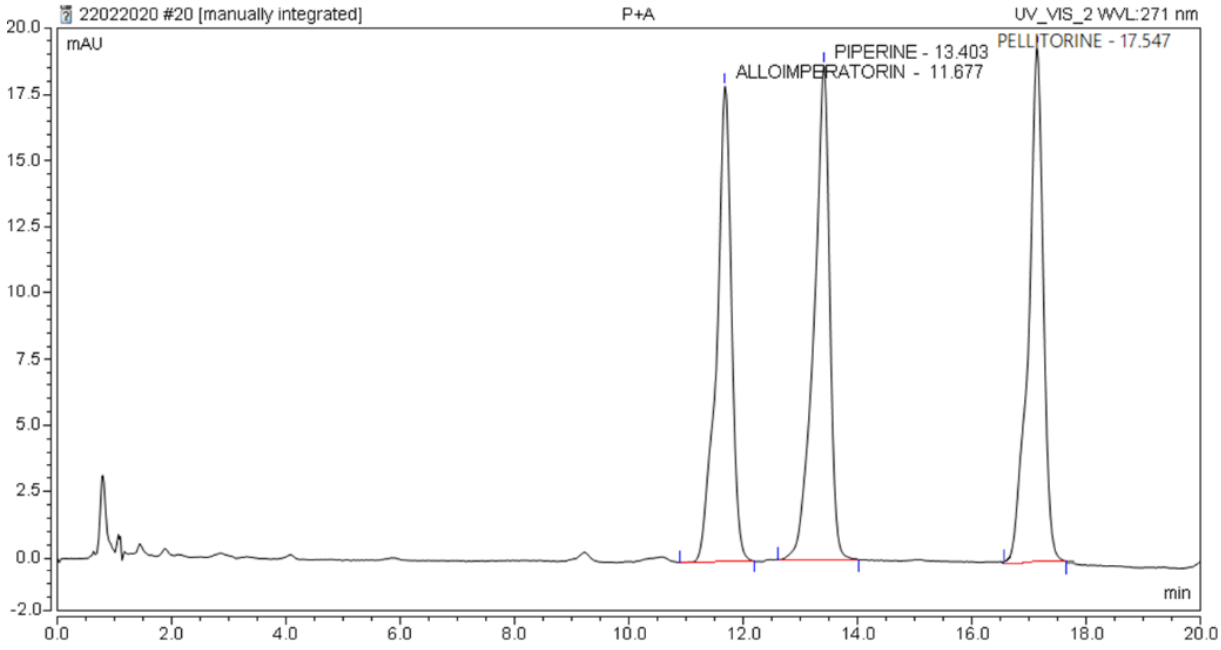

Figure 63: HPLC chromatogram of alloimperatorin, piperine and pellitorine

The %RSD of peak area, as well as R_t of analytes, was calculated and found to be within 2%, indicating the suitability of the system. The %RSD of height equivalent to theoretical plates of the column for the seven replicate injections were found to be 9495 ± 1.00% for alloimperatorin, 8890 ± 1.32% for piperine and 9120 ± 1.04% for pellitorine. The number of theoretical plates was greater than 2000 and considered to be acceptable for the system suitability test. The LOD and LOQ were found to be 36.26 ng/ml and 108.89 ng/ml for alloimperatorin, 12.27 ng/ml and 36.36 ng/ml for piperine, 11.62 ng/ml and 34.88 ng/ml for pellitorine (**Table 22**).

The results provide a linear relationship over the concentration range of 0.11-64 ng/ml for alloimperatorin, 0.36-24 ng/ml for piperine, and 0.34-20 ng/ml for pellitorine. From the regression data, a linear equation of alloimperatorin, piperine and pellitorine were found to be $y = 0.1499x + 0.0344$ ($r^2 = 0.9994$); $y = 0.6357x + 0.2357$ ($r^2 = 0.9984$) and $y = 0.9439x – 0.7895$ ($r^2 = 0.9942$), respectively. These results indicated a linear relationship between the mean peak area and concentration of the analytes.

Table 22: Summary of optimized HPLC method for the analytes of AMM-PLM

PARAMETER	**ANALYTES**	**RANGE**
Instrumental precision ThermoScientific Dionex UltiMate 3000	Alloimperatorin (% CV, n=7) Piperine (% CV, n=7) Pellitorine (% CV, n=7)	1.18 0.77 0.66
Specificity (n=7)	Alloimperatorin Piperine Pellitorine	11.56 ± 0.88 min 13.38 ± 0.31 min 17.64 ± 0.11 min
Limit of Detection (LOD)	Alloimperatorin Piperine Pellitorine	36.26 ng/ml 12.27 ng/ml 11.62 ng/ml
Limit of Quantification (LOQ)	Alloimperatorin Piperine Pellitorine	108.89 ng/ml 36.36 ng/ml 34.88 ng/ml
Linearity	Alloimperatorin Piperine Pellitorine	0.11-64 ng/ml 0.36-24 ng/ml 0.34-20 ng/ml

All the values are expressed as mean± S.E.M (n = 7)

The %RSD of peak area for intra-day precision and inter-day precision, % bias at all three QC levels are précised in **Table 23.** These results were found to be within the accepted limits (± 15%, except for LOQ), which demonstrated the applicability of the developed method for routine analysis. The excellent recovery values for accuracy study ascertained that the method was accurate.

Table 23: Precision (% CV) and accuracy (% bias) of the analytes of AMM-PLM

Analyte	Nominal conc (ng/ml)	Intra-day			Inter-day		
		Measured Conc. (Mean ± SD, ng/ml)	Precision (% CV)	Accuracy (% bias)	Measured Conc. (Mean ± SD, ng/ml)	Precision (% CV)	Accuracy (% bias)
Alloimperatorin	350	356.09 ± 0.66	2.13	-1.74	347.30 ± 0.01	1.76	0.77
	6000	5829.652 ± 0.03	0.54	2.84	6160.64 ± 0.17	2.84	-2.68
	35000	36817.6 ± 0.27	0.04	-5.19	35424.36 ± 0.92	2.59	-1.21
Piperine	200	198.05 ± 0.01	6.96	0.98	198.93 ± 0.01	5.65	0.50
	4000	3957.73 ± 0.06	1.59	1.06	4257.93 ± 0.08	1.81	-6.45
	10000	9654.50 ± 0.45	0.21	3.45	9740.60 ± 0.12	1.22	2.59
Pellitorine	200	196.08 ± 0.02	3.52	0.70	196.96 ± 0.01	4.77	1.52
	4000	4001.02 ± 0.04	1.07	0.03	3996.87 ± 0.03	0.80	0.08
	10000	9945 ± 0.06	0.56	0.55	9876.56 ± 0.22	0.22	1.24

All the values are expressed as mean± S.E.M (n = 5).

The robustness of the analytical process was evaluated by assessing the effect of small variations in HPLC conditions i.e., change in column oven temperature (25 and 35°C), flow rate (0.9 ml/min and 1.1 ml/min), and change in pH (6.4 and 6.8) of the solvent system. The results are summarized in **Table 24** and **25** and found that a minor change in method condition did not significantly affect the theoretical plates and R_t of the analyte. Thus, the proposed method was found to be reliable and robust.

Table 24: Method optimization for AMM-PLM: Retention time obtained upon making deliberate variations in the chromatographic conditions

Variations in Chromatographic condition	R_t(min)		
	Alloimperatorin	Piperine	Pellitorine
No variation (Optimized)	11.60 ± 0.88	13.38 ± 0.31	17.64 ± 0.11
Flow rate (0.9 ml/min)	12.21 ± 0.09	14.10 ± 0.08	18.25 ± 0.24
Flow rate (1.1 ml/min)	10.23 ± 0.04	12.10 ± 0.08	16.96 ± 0.44
Mobile phase (MeOH: H_2O pH=6.4)	11.51 ± 0.04	13.32 ± 0.03	17.59 ± 1.09
Mobile phase (MeOH: H_2O pH=6.8)	11.40± 0.01	13.28 ± 0.01	17.45 ± 0.32
Column temperature (25 °C)	11.52 ± 0.08	13.33 ± 0.05	17.49 ± 0.78
Column temperature (35 °C)	11.48 ± 0.09	13.66 ± 0.34	17.36 ± 0.61

All the values are expressed as mean± S.E.M (n = 6)

Table 25: Method optimization for AMM-PLM: HETP and number of theoretical plates (N) obtained upon making deliberate variations in the

Variations in Chromatographic condition	Effect on chromatographic parameters of analytes (n=6)					
	HETP			N		
	Alloimperatorin	Piperine	Pellitorine	Alloimperatorin	Piperine	Pellitorine
No variation (Optimized)	9495 ± 1.00	8890 ± 1.32	9120 ± 1.04	9131.59 ± 323.46	7624.78 ± 274.07	9343.21 ± 343.89
Flow rate (0.9 ml/min)	9487 ± 0.50	8888 ± 1.14	9116 ± 0.67	8299.45 ± 306.14	6034.30 ± 100.73	9143.56 ± 313.98
Flow rate (1.1 ml/min)	9499 ± 1.41	8892 ± 1.07	9121 ± 0.76	11317.16 ± 281.90	10859.55 ± 250.21	11234.89 ± 276.09
Mobile phase (MeOH: H_2O pH=6.4)	9488 ± 1.50	8887 ± 1.05	9117 ± 0.34	9393.18 ± 112.40	7195.64 ± 70.55	9541.12 ± 117.67
Mobile phase (MeOH: H_2O pH=6.8)	9503 ± 2.07	8890 ± 1.54	9125 ± 0.12	9613.81 ± 171.23	7214.90 ± 106.02	9865.23 ± 123.11
Column temperature (25 °C)	9491 ± 1.42	8891 ± 1.54	9124 ± 1.09	9621.37 ± 334.85	7609.44 ± 348.23	9903.43 ± 356.90
Column temperature (35 °C)	9484 ± 1.04	8880 ± 1.61	9118 ± 0.98	9363.33 ± 327.40	7528.61 ± 273.80	9756.34 ± 341.97

All the values are expressed as mean± S.E.M (n = 6).

CHAPTER 8

In conclusion, the bio-active markers in the compositions were estimated using newly developed HPLC methods (1-3). The new HPLC methods were validated as per USFDA guidelines. From the *in vitro* results for BARM-GSM and BARM-TSM in SGF media, the stability of ECG, EGCG, berberine and palmatine was estimated, while the formation of a hydrolysis product gymnemagenin was observed. The amount of berberine and palmatine in BARM-GSM was found to be 18.63 and 10.18 % w/w. After SGF digestion of BARM-GSM composition, berberine (88.08%) and palmatine (87.14%) in BARM-GSM were found to be stable in SGF media, while the amount of gymnemagenin formed was 0.22% w/w. The amount of ECG, EGCG, berberine and palmatine in BARM-TSM were found to be 2.5, 5.7, 18.3 and 8.2 % w/w, respectively. ECG (76.96 %), EGCG (82.96 %), berberine (86.88 %) and palmatine (83.66 %) in BARM-TSM were found to be stable in SGF media. In GSM-TSM, the amount of ECG and EGCG were found to be 3.25% and 4.13 %, respectively. Using HPLC method 3, alloimperatorin (5.65 % w/w), piperine (6.73% w/w) and pellitorine (4.34% w/w) were identified in the AMM-PLM.

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