ORIGINAL ARTICLE

Gas chromatographic validated method for quantification of ayurvedic polyherbal formulation

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A new gas chromatographic-flame ionization detector (GC-FID) method was developed for quantification of ayurvedic polyherbal formulation. The GC-FID method was found highly accurate, sensitive, simple and precise. This method was validated as per international conference on harmonization (ICH) guidelines. Experimental work was performed by nonpolar capillary column (Zb-5, 5%-Phenyl-95%-dimethylpolysiloxane). Film thickness of capillary column (Zb-5) was (0.25 μm) and length 30 m × 0.25 mm i.d. The temperature of the oven, injector and detector were 200, 210 and 280°C respectively. Data processing system was applied to obtain data. The standards and test samples were prepared in absolute ethanol. The principle constituents t-Anethol, d-Limonene, cuminaldehyde and thymol were found in ayurvedic polyherbal formulation. The ICH validation parameters for the proposed procedure, recovery (limit 98.85–100.76%), precision (<1.00%), limits of detection, limits of quantification and linearity ($r^2 = 0.995 \pm 0.002$) were observed under acceptance limit. Validation results were statistically calculated. The result shows that method is selective and reproducible for quantification of ayurvedic polyherbal formulation. The presented GC method can be applied for the routine analysis of principle constituents as well as ayurvedic polyherbal formulation.

Key words: *Gas chromatography, international conference on harmonization, polyherbal formulation, validation*

INTRODUCTION

The pharmacopeial standard in Ayurvedic system is not adequate enough to ensure the quality of formulations.[1] Analysis of active constituents is necessary to maintain the quality, safety and efficacy of the ayurvedic polyherbal formulation.[2] Herbal formulations have characteristic odor due to volatility of some ingredients. Most of the volatiles ingredients have low molecular weight, typically, monoterpenes, sesquiterpenes and phenylpropenes and their oxygenated derivatives.[3] Volatile oils are plant secondary metabolites that are known for fragrance and flavor. Volatile oils can be presented in different plant organs and materials, and their storage in related to specialized secretary structures. Gas chromatography (GC) is well-established analytical technique for analysis of volatile oils due to the availability of mass spectrometer detector. Once the principle constituents are identified, the interested components can be quantized by the flame ionization detector (FID).[4] No work has been carried out in the estimation of markers compounds in the prepared polyherbal formulation till now.

Gas chromatography is one of the modern sophisticated techniques that can be used for wide diverse applications in essential oil analysis. It is a simple and powerful tool for high-resolution chromatography and trace quantitative analysis. It is one of the most powerful tool for quick and easy determination of purity, quality and authenticity of the crude drugs and ayurvedic formulations by estimation of markers components.[5] The objective of the present work was to develop identification, accurate, specific and reproducible method for the estimation of thymol, cuminaldehyde, t-Anethole and d-Limonene in bulk drug

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as well as polyherbal formulation. The developed method is also utilized to determine the purity and quality of the polyherbal formulation.

MATERIALS AND METHODS

Plant materials

Plant material of following plants *Anethum graveolens*, *Cuminum cyminum*, *Foeniculum vulgare* and *Trachyspermum ammi* were collected from the local market of Pratapgarh, Rajasthan. The materials were authenticated by Prof. S.K. Pandey, Scientist, KNK College of horticulture, Mandsaur, Madhya Pradesh, India. The voucher specimen MIP/P'cognosy/2014/10-13 is submitted in the department of pharmacognosy, MIP, Mandsaur for future reference.

Chemicals and reagents

The thymol, cuminaldehyde and t-Anethole standards were procured from Sigma (Aldrich) and Assigned purity: 98% and 97%. d-Limonene was from Loba Chem. The solvents were from Sigma (Aldrich). All chemicals used were of analytical grade.

Isolation of volatile oils for analysis

The dried materials of selected umbelliferae plants were grinded to get fine powder using a grinder (Voltas-300, Voltas, Mumbai). The grinded powder was then assembled for hydro-distillation to remove volatile oil with the help Clevenger's apparatus. Volatile oils isolation was carried out by hydro-distillation of 500 g of the powdered drug with 1000 ml of tap water for 6 h to obtain the volatile oil of each plant separately. The yield of the extracts was in range 1–1.5% of the total dried material. The same procedure was repeated for other umbelliferous plants. Light yellowish colored oil was obtained having characteristic odor and taste. Moisture from volatile oils was removed by drying over anhydrous sodium sulfate. Volatile extracts stored in a dark glass bottle and kept at 4°C for further analysis.[6-8]

Preparation and standardization of polyherbal formulations for analysis

The present work relates to edible polyherbal compositions that contain large amounts of volatile extracts, are highly palatable when taken orally. Current formulation is particularly useful as carrier for volatile extracts. This polyherbal formulation relates to non-greasy tasting edible compositions, pleasant, preferably in liquid form, containing volatile extracts, lipid soluble flavorant and a highly potent lipid-soluble sweetener. The volatile extracts were mixed by mechanical stirrer, and Saccharin (o-benzoic sulfimide) was pulverized manually with a mortar and pestle, to enhance it dissolution, and was added to the continued agitation. Dissolution of the saccharin appeared complete after about 30 min. The flavor was then added to the oil mixture with agitation that was continued until the mixture, was homogeneous [Table 1].[9]

Sample solution (1% v/v) developed containing polyherbal formulation in methanol was injected into a ZB-5 capillary column. Nitrogen was used as the carrier gas at 1.3 ml/min, and a FID was used. The temperature of the oven, injector and detector were 200°C, 210°C and 280°C respectively. At the same chromatographic condition, standards were spiked in GC injector. GC results were compared with test formulation [Tables 2 and 3]. The chromatogram was recorded with the help of chromatography data station. A fingerprint chromatogram of standards and polyherbal formulation are presented in Figures 1-5.

Method validation

The developed method is validated as per the international conference on harmonization guidelines.[10-13] The validation of the method was developed in terms of precision, accuracy, linearity, recovery, limits of detection (LOD) and limits of quantification (LOQ).

Precision and accuracy

The intra- and inter-day precision, as coefficient of variation (CV, %) and accuracy of the assay determined at thymol, cuminaldehyde, t-Anethole and d-Limonene concentration of 60–300, 120–600, 160–700 and

Table 1: Composition of the polyherbal formulation, as given in the following table, was prepared

Name	Family	Final concentration (v/v)
Trachyspermum ammi Linn.	*Apiaceae* (Umbelliferae)	12 ml (3 ml each)
Cuminum cyminum Linn.	*Apiaceae* (Umbelliferae)	
Anethum graveolens Linn.	*Apiaceae* (Umbelliferae)	
Foeniculum vulgare Mull.	*Apiaceae* (Umbelliferae)	
Saccharin (O-benzoic sulfimide)		0.006 ml
Peppermint concentrate[a]		0.3 ml

[a]A mixture of 4 g of volatile extracts (Moksha Lifestyle Products, 24/157 Shakti Nagar, G.T. Karnal Road, New Delhi, India) and 1 g artificial peppermint flavor (Loba Chemie Pvt. Ltd.)

Table 2: Chemical constituents in polyherbal formulation

Peaks	Time (min)	Component name	Area (μV.s)
1	1.588	Methanol	5767582
3	3.653	Unidentified	758404
4	4.041	Limonene	1779214
6	4.368	Carvon*	1986726
7	4.735	Thymol	136192.4
8	4.910	Unidentified	192385.3
11	5.973	Unidentified	72839.42
12	6.388	Cuminaldehyde	3581044
13	6.509	Unidentified	53068.69
14	6.821	Anethole	3347586

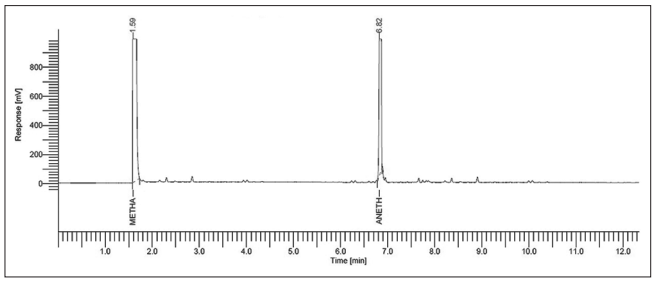

Figure 1: Gas chromatogram of standard t-Anethole

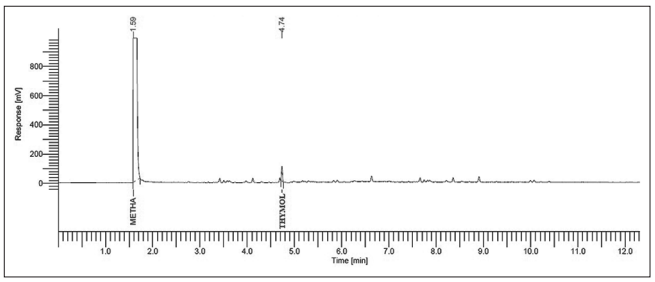

Figure 2: Gas chromatogram of standard thymol

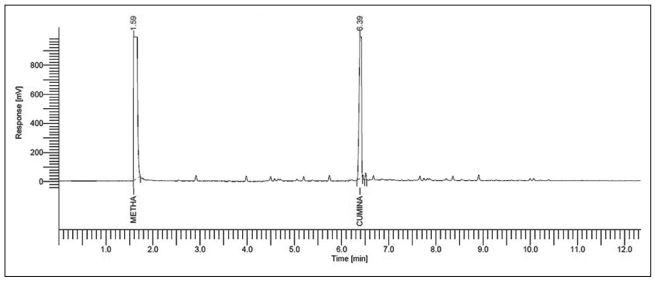

Figure 3: Gas chromatogram of standard cuminaldehyde

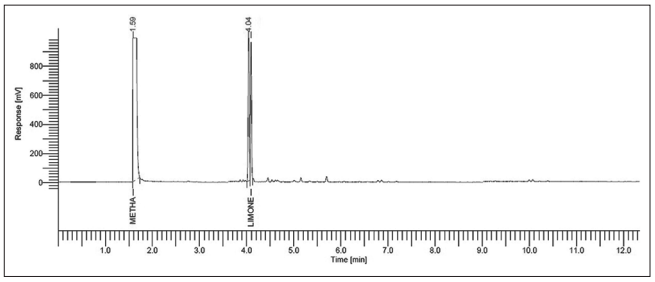

Figure 4: Gas chromatogram of standard d-Limonene

Table 3: Optimum GC conditions

Parameters	Optimum condition
Injection volume	10 µl
Injector temperature (split with split ratio of 5)	210°C
Detector temperature (FID)	280°C
Column	Zb-5, 30 m×0.25 mm×0.25 µm (0.25 µm film thickness)
Oven temperature programming	90°C-190°C (ramp of 10°C)
Detector	FID
Carrier gas N, H and Air	1.3, 10 and 20 ml/min respectively

FID: Flame ionization detector, GC: Gas chromatographic

200–1000 µl/ml has been summarized. The intra-day precision (n = 5) was <1.00%. The inter-day precision over three different days was ≤0.5%. The intra-day and inter-day accuracy were in the range of 98.85–100.43% and 98.42–100.98%, respectively. The repeatability of the method was studied on five samples of thymol, cuminaldehyde, t-Anethole and d-Limonene at same concentration under the same experimental conditions. The results were observed under the acceptable range, and so I conclude that the presented method was reproducible, accurate and reliable in the day to day routine analysis.

Sensitivity and linearity

In order to estimate detection (LOD) and quantification (LOQ) limits, blank methanol (n = 6) spiked in GC column, followed by the same method as explained under the section of chromatographic conditions and the standard deviation (SD) (σ) of the magnitude of analytical response was determined. The LOD was expressed as (LOD = 3.3 σ/slope of thymol, cuminaldehyde, t-Anethole and d-Limonene calibration curve), whereas LOQ was expressed as (LOQ = 10 σ/slope of thymol, cuminaldehyde, t-Anethole and d-Limonene calibration curve).

Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The GC graph for thymol, cuminaldehyde, t-Anethole and d-Limonene in the sample were confirmed by comparing R_t and area under the curve of GC graph with that of the standard.

RESULTS AND DISCUSSION

Selection and optimization of gas chromatographic conditions

On the basis of literature reviews and experiments, some conditions were decided. Finally, optimum conditions for GC are following

Calibration curve

Calibration was linear in the concentration range 240–1200 µl/ml. The linear regression equation was $Y = 737.9x + 13435$, $Y = 1581x + 50572$, $Y = 618.2x + 17785$ and $Y = 1559x + 12978$, for thymol, cuminaldehyde, t-Anethole and d-Limonene respectively, while the correlation coefficient (r^2) was 0.998, 0.995, 0.995 and 0.993 with high reproducibility and accuracy [Table 4].

Detection limit of thymol, cuminaldehyde, t-Anethole and d-Limonene was determined by plotting a series of concentrations. The lowest amount of thymol, cuminaldehyde, t-Anethole and d-Limonene which could be detected (LOD), were 5.34 µl/ml, 30.19 µl/ml, 2.78 µl/ml and 0.99 µl/ml respectively. The lowest amount of thymol, cuminaldehyde,

t-Anethole and d-Limonene which could be quantified (LOQ), were found to be 16.18 µl/ml, 91.17 µl/ml, 8.42 µl/ml and 2.99 µl/ml respectively.

Validation of method

Recovery studies

The proposed method, when used for estimation of thymol, cuminaldehyde, t-Anethole and d-Limonene after spiking with 0%, 25%, 50% and 75% of additional drug, afforded recovery ranging from 99.69%, 99.78%, 101.66% and 100% and relative standard deviation (RSD) was 0.910%, 1.139%, 0.676% and 1.284% for thymol, cuminaldehyde, t-Anethole and d-Limonene were obtained respectively as listed in Table 5.

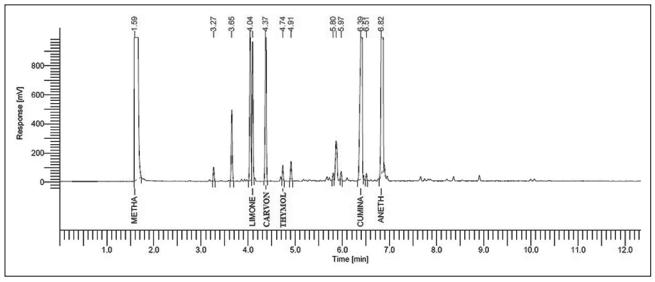

Figure 5: Gas chromatogram of polyherbal formulation

Table 4: Linear regression data for the calibration curves

Ingredients	Linearity range (µl/ml)	r^2	Slope	Intercept
Thymol	240-1200	0.998	737.9	13435
Cuminaldehyde	240-1200	0.995	1581	50572
t-Anethole	240-1200	0.995	1559	12978
d-Limonene	240-1200	0.993	618.2	17785

Precision and accuracy

The intra- and inter-day precision, as CV, % and accuracy of the assay determined at thymol, cuminaldehyde, t-Anethole and d-Limonene concentration of 60–300, 120–600, 160–700 and 200–1000 µl/ml has been summarized in Table 6. The intra-day precision ($n = 5$) was <1.00%. The inter-day precision over three different days was ≤0.5%. The intra-day and inter-day accuracy were in the range of 98.85–100.43% and 98.42–100.98%, respectively. The repeatability of the method was studied on five samples of thymol, cuminaldehyde, t-Anethole and d-Limonene at same concentration under the same experimental conditions. The results were identified within the acceptable range, so we can conclude that the method was reliable, reproducible and accurate.

Robustness of the method

The SD and % RSD of peak areas were calculated for each parameter. Results were identified in the acceptable range. The low values of SD (<2.0) and % RSD (<1.00) obtained after introducing small deliberate changes in the developed GC-flame ionization detector (GC-FID) method indicated the robustness of the method [Table 7].

Limit of detection and limit of quantification

The calibration curve in this study was plotted between amount of analyte versus average response (peak area) and the regression equation was obtained $Y = 737.9x + 13435$, $Y = 1581x + 50572$, $Y = 618.2x + 17785$ and $Y = 1559x + 12978$ over the concentration range 240–1200 µl/ml with respect to the peak area with a regression coefficient of 0.998, 0.995, 0.995 and 0.993 respectively. LOD and LOQ were calculated by the method as described in

Table 5: Recovery studies (*n*=4)

Mixture	Concentration (µl/ml)	Standard added (µl/ml)	Response (µV*s)	Amount found (µl/ml)	Recovery %	Mean recovery±SD	CV %
1	240	0	190286	242	100.83	Thymol	Thymol
	480	0	795397.03	471.11	98.15	99.69±0.907	0.910
	640	0	422533	654.72	102.30		
	800	0	1275585	809.88	101.24		
2	240	60	231733.65	295.83	98.61	Cuminaldehyde	Cuminaldehyde
	480	120	998133.8	599.34	99.89	99.78±1.137	1.139
	640	160	523166.3	817.5	102.19		
	800	200	1601481	1018.92	101.89		
3	240	120	278083	358.65	99.63	d-Limonene	d-Limonene
	480	240	1194483	723.53	100.49	101.66±0.687	0.676
	640	320	618799	972.19	101.27		
	800	400	1863378	1186.91	98.91		
4	240	180	322427	418.74	99.70	t-Anethole	t-Anethole
	480	360	1386787	845.17	100.62	100±1.284	1.284
	640	480	716433	1130.13	100.90		
	800	600	2207274	1407.5	100.54		

For thymol 240 µl/ml; cuminaldehyde 480 µl/ml; d-Limonene 640 µl/ml and t-Anethole 800 µl/ml respectively. SD: Standard deviation, CV: Coefficient of variation

Table 6: Intra- and inter-day precision of GC-FID method (*n*=5)

A. Intra-day analysis of GC-FID method (*n*=5)

Mixture	Concentration µl/ml	Area (µV*s)			Concentration found (µl/ml)			Mean	Standard	CV %
		A	B	C	A	B	C			
1	60	57386.9	56879	57434	59.56	58.88	59.63	59.36	0.417	0.703
	120	235397	237698	236578	116.90	118.36	117.65	117.64	0.728	0.619
	160	119533	119786	120675	164.59	165.00	166.43	165.34	0.970	0.587
	200	340585	339677	338769	210.14	209.56	208.97	209.56	0.582	0.278
2	120	101380.8	102965	102354	119.18	121.33	120.50	120.34	1.083	0.900
	240	441325.1	438978	442345	247.16	245.67	247.80	246.88	1.092	0.442
	320	215623	214685	216534	320.02	318.51	321.50	320.01	1.496	0.467
	400	629690	628677	626787	395.58	394.93	393.72	394.74	0.945	0.239
3	180	149246.2	148769	148875	184.05	183.40	183.55	183.67	0.340	0.185
	360	629397.1	628679	628754	366.11	365.66	365.71	365.83	0.250	0.068
	480	314621	316742	315775	480.16	483.59	482.03	481.93	1.718	0.356
	600	939585	942564	938948	594.36	596.27	593.95	594.86	1.238	0.208
4	240	191380.3	189987	190465	241.15	239.26	239.91	240.11	0.959	0.400
	480	815462	819564	818546	483.80	486.40	485.75	485.32	1.351	0.278
	640	428526	426759	428674	664.41	661.56	664.65	663.54	1.724	0.260
	800	1271585	1266498	1268475	807.32	804.05	805.32	805.56	1.645	0.204
	300	241273.2	241796	242324	308.77	309.47	310.19	309.48	0.712	0.230
	600	999297	997987	998975	600.08	599.25	599.88	599.73	0.432	0.072
	700	451429	452286	451978	701.46	702.85	702.35	702.22	0.702	0.100
	1000	1591481	1589453	1593456	1012.51	1011.21	1013.78	1012.50	1.284	0.127
Mean CV %	Thymol									0.483
	Cuminaldehyde									0.296
	d-Limonene									0.354
	t-Anathole									0.211

B. Inter-day analysis of GC-FID method (*n*=5)

Mixture	Concentration µl/ml	Area (µl/ml)			Concentration found µg/ml)			Mean	Standard	CV %
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3			
1	60	57376	56878	57464	59.55	58.87	59.67	59.36	0.428	0.721
	120	237397	237868	236878	118.17	118.47	117.84	118.16	0.313	0.265
	160	119833	119767	120975	165.07	164.97	166.92	165.65	1.099	0.663
	200	338585	339687	338769	208.86	209.56	208.97	209.13	0.379	0.181
2	120	101480	102865	102634	119.32	121.20	120.88	120.47	1.006	0.835
	240	441325.1	439778	439745	247.16	246.18	246.16	246.50	0.571	0.232
	320	215326	214387	215434	319.54	318.02	319.72	319.09	0.931	0.292
	400	629878	628718	626787	395.70	394.96	393.72	394.79	1.002	0.254
3	180	149246.2	148839	147875	184.05	183.50	182.19	183.25	0.954	0.521
	360	629495	627269	628457	366.18	364.77	365.52	365.49	0.705	0.193
	480	315421	316742	316585	481.46	483.59	483.34	482.80	1.167	0.242
	600	938985	939464	935448	593.97	594.28	591.71	593.32	1.407	0.237
4	240	189880.3	189987	190564	239.12	239.26	240.04	239.48	0.498	0.208
	480	817662	819564	818365	485.19	486.40	485.64	485.74	0.608	0.125
	640	427296	426759	427674	662.42	661.56	663.04	662.34	0.744	0.112
	800	1269485	1266498	1265275	805.97	804.05	803.27	804.43	1.389	0.173
5	300	241543.2	241693	242524	309.13	309.33	310.46	309.64	0.716	0.231
	600	998877	997987	997675	599.81	599.25	599.05	599.37	0.395	0.066
	700	451878	452676	452180	702.19	703.48	702.68	702.78	0.652	0.093
	1000	1592541	1590553	1592756	1013.19	1011.91	1013.33	1012.81	0.779	0.077
Mean CV %	Thymol									0.503
	Cuminaldehyde									0.176
	d-limonene									0.280
	t-Anathole									0.184

CV: Coefficient of variation, GC-FID: Gas chromatographic-flame ionization detector

validation section and was found to be 5.34 µl/ml, 30.19 µl/ml, 2.78 µl/ml and 0.99 µl/ml and 16.18 µl/ml, 91.17 µl/ml, 8.42 µl/ml and 2.99 µl/ml respectively, which indicates the sample sensitivity of the method.

Specificity

The specificity of the proposed method was determined by comparing the sample and standard peak for its R_t and GC-FID graph. Three point peak purity that is, peak start, peak apex, and peak end, were compared and found superimposed. This indicated that standard thymol, cuminaldehyde, t-Anethol and d-Limonene sample peaks were not merging with any other components or impurities. The peak purity of thymol, cuminaldehyde, t-Anethol and d-Limonene were assessed by comparing the spectra at three different levels that is, peak start, peak apex and peak end positions [Figure 3]. A well resolved t-Anethole, thymol, cuminaldehyde and d-Limonene were observed at R_t value 0.72 ± 0.02 in the chromatogram of the samples extracted from Umbelliferae seeds and formulations. The thymol, cuminaldehyde, t-Anethol and d-Limonene contents were observed and calculated [Table 8].

A validated GC-FID method has been developed for the determination of thymol, cuminaldehyde, t-Anethol and d-Limonene in bulk drug and its formulation. The proposed method is reliable, simple, precise, accurate, specific, less time consuming and cost effective. Statistical analysis proved that the method is evitable for the analysis of thymol, cuminaldehyde, t-Anethol and d-Limonene respectively. The developed GC-FID method will help the manufacturer for quality control and standardization of herbal formulations. In this experiment, the contents of thymol, cuminaldehyde, t-Anethol and d-Limonene were found. The method established in this study could be used for the quality control of herbal medicines derived from different species thymol, cuminaldehyde, t-Anethol and d-Limonene containing plant.

Table 7: Robustness testing (*n*=4)

Parameters	Ingredients	SD* of peak area	RSD %
Flow rate	Thymol	0.14	0.02
	Cuminaldehyde	0.02	0.00
	t-Anethol	0.02	0.00
	d-Limonene	0.02	0.00
pH	Thymol	0.05	0.00
	Cuminaldehyde	0.09	0.01
	t-Anethol	0.19	0.04
	d-Limonene	0.06	0.00

*Average of three concentrations 240, 480, 640 µl/ml, 480, 640, 800 µl/ml, 640, 800, 1200 µl/ml and 800, 1000, 1200 µl/ml of thymol, cuminaldehyde, t-Anethole and d-Limonene respectively. RSD: Relative standard deviation

Table 8: The content of thymol, cuminaldehyde, t-Anethole and d-Limonene in bulk drug and formulations

Ingredients	Bulk drugs (%), (*n*=4)	Formulation (% w/w), (*n*=4)
Thymol	16	3
Cuminaldehyde	18	3
t-Anethole	51	3
d-Limonene	5	3

CONCLUSION

In this research, an antispasmodic polyherbal formulation in Ayurvedic system of medicine was identified as thymol, cuminaldehyde, t-Anethol and d-Limonene and confirmed by GC-FID. The GC method developed to quantify the thymol, cuminaldehyde, t-Anethol and d-Limonene in Bulk drug, and polyherbal formulation were shown to be rapid, reliable and accurate. In the present study, the method was found to be useful in detecting the geniuses of the formulation.

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