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STANDARDIZATION OF HERBAL MULTIACTION SKIN FORMULATION NEWCHARM® GEL THROUGH VALIDATED GAS CHROMATOGRAPHY AND ISOCRATIC RP-HPLC

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ABSTRACT

Ensuring the authenticity, safety, and effectiveness of herbal medicines is unquestionably a paramount concern for the industry. Presently, the prevailing quality control framework predominantly centers on chemical markers, primarily focusing on the proper accurate utilization of raw materials and addressing safety concerns. Validated gas chromatography (GC) and reverse-phase high-performance liquid chromatography (HPLC) methods were established with flame ionization (FID) and photo-diode array detection (PDA), respectively, to quantify the levels of 1,8-cineole and *β*-asarone. These compounds are found within a complex natural matrix in the ayurvedic medicine NEWCHARM® GEL. Effective chromatographic separation of 1,8-cineole and *β*-asarone was achieved using a Shimadzu SH-Rxi-5Sil MS 30m x 0.25mm x 0.25µm gas chromatography capillary column and a Phenomenex Luna5µ (RP C18, 25cm × 4.6mm × 100A°) liquid chromatography column. The GC-FID and RP-HPLC-PDA methodologies proposed here underwent thorough statistical validation, encompassing assessments of linearity, range, precision, accuracy, selectivity, and robustness. The calibration curves displayed remarkable linearity within specified ranges: 25.0–125.0µg/mL for 1,8-cineole and 15.0-120.0µg/mL for *β*-asarone, each exhibiting correlation coefficients exceeding 0.998. In the case of the chromatographic analysis approach, its application to the herbal formulation NEWCHARM® GEL resulted in the successful quantification of phytoconstituents, accompanied by excellent recovery values, and notably, no interfering peaks stemming from the excipients were observed. The viability and significance of this methodology for verifying bioactive markers introduce new, exceedingly sensitive, and reliable approaches for standardizing herbal formulations containing 1,8-cineole and *β*-asarone.

KEYWORDS: 1,8-cineole, *β*-asarone, Chromatography, GC-FID, NEWCHARM® GEL, RP-HPLC-PDA.

1.0 INTRODUCTION

The condition of an animal's skin serves as an indicator of its overall health. A healthy, disease-free skin is characterized by a shiny and lustrous body coat that is devoid of any lesions or roughness.[1-2] In companion animals, various factors can affect their skin, including infections (parasitic, bacterial, fungal), exposure to both natural and chemical allergens, as well as trauma and injury. Skin conditions become evident through manifestations such as skin ulcerations, lesions, inflammation, intense itching and irritation in the affected area, edematous swellings, and various types of wounds.[3] Neglecting these conditions can result in the emergence of secondary complications, which, in addition to affecting the skin, have adverse repercussions on the overall health of the pet. Within the realm of pet practice, a significant portion of a clinician's time is dedicated to treating various types of skin ailments, as well as providing care for wounds and injuries.[4-5]

Embracing a natural approach to healthcare, herbal medicine provides a holistic and gentle method for addressing skin issues in pets. This preference for a natural approach is growing among veterinarians and pet owners, as it significantly reduces the risk of adverse reactions in companion animals. It should be understood that Ayurvedic medicines differ from allopathic medicines in that they are not one individual chemical targeted against one individual organism or having one specific action, but a synergistic formulation designed to promote health, in this case, of the skin.[6]

NEWCHARM® GEL is a proprietary polyherbal formulation marketed by AYURVET LIMITED, INDIA which is a multiaction skin preparation for topical use on companion animals, suffering from various skin affections. This gel formulation has been designed to combat common organisms causing skin disease as well as to promote skin healing.

NEWCHARM® GEL is a scientifically formulated product with established pharmacological action profile of each of its constituent herbal ingredients.[7-9] This herbal product is formulated in a unique gel base and is prepared from herb's essential oils of proven action profile which is synergy contributes to antimicrobial action against wound-causing organisms, antifungal action against common dermatophytes, antiparasitic and miticidal actions against skin parasites, anti-inflammatory action to treat inflamed skin, vulnerary action for quick healing of wounds, anti-pruritic action for alleviating itching & irritation, and insect repellant action to check cross contamination.[10-15]

NEWCHARM® GEL formulation incorporates essential oils from herbs such as *Eucalyptus globulus, Acorus Calamus, Cedrus deodara, Pongamia pinnata,* and *Azadirachta indica* which contain numerous secondary plant metabolites.[16-21] This variability in ingredient proportions within this polyherbal formulation poses challenges in conducting routine quality analysis. Additionally, the active compounds within these herbs exhibit distinct polarities, leading to different chromatographic behaviors.

Specifically, 1,8-cineole (eucalyptol) is a prominent monoterpene cyclic ether found abundantly in the essential oil extracted from *Eucalyptus globulus* leaves, while *β*-asarone belongs to the phenylpropanoid class of chemical compounds and is present in the essential oil components of plants like *Acorus Calamus* rhizome.

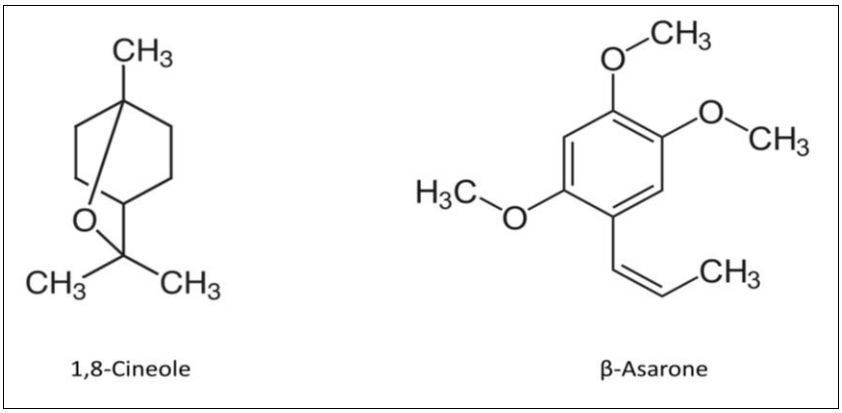

Figure 1: Chemical structure of 1,8-cineole and *β*-asarone.

Hence, the primary objective of our research was to establish chromatographic methods for the quantification of two key analytes, namely, 1,8-cineole and *β*-asarone (Figure 1), which exist in specific concentrations within NEWCHARM® GEL. In pursuit of this goal, we devised two straightforward alternative methodologies for determining these markers within the herbal formulation, employing GC-FID and isocratic RP-HPLC-PDA chromatographic modes, respectively.

Following comprehensive validation of these methods across various parameters, our research successfully demonstrated their quality. Consequently, we applied these validated methods to analyze a commercial product containing these phytoconstituents, sourced from the respective herbs incorporated in NEWCHARM® GEL.

2.0 MATERIALS AND METHODS

2.1 Reagents and materials

All the reagents and solvents were of AR or HPLC grade as per requirement. The active reference marker compounds *β*-asarone (CAS number: 5273-86-9) and 1,8-cineole (CAS number 470-82-6) were procured from Sigma Aldrich. Latest controlled samples of NEWCHARM® GEL were obtained from the QA/QC department of AYURVET LIMITED, Baddi.

2.2 GC–FID analysis conditions

A Shimadzu GC-2014, SPL-2014 (50/60 Hz frequency) equipped with a hydrogen flame ionisation detector (single-FID-2014) (Kyoto, Japan). Chromatographic separation was carried out using a Shimadzu SH-Rxi-5Sil MS column (30m × 0.23mm ID × 0.25μm df, temperature range 320/350°C - USA). The column initial temperature was 50°C, maintained for 2 min, ramped up to 100°C at a rate of 10°C per minute, and maintained for 5 minutes at 70°C and 100°C. Detector temperature was 220°C with sampling rate of 40 msec. Carrier gas was nitrogen at a flow rate of 1.5 mLmin−1, and a split ratio of 10:1. Injection volume was maintained at 2µL for standard and test samples. All data acquired were processed by Shimadzu-LabSolutions software (Kyoto, Japan).

2.3 RP-HPLC-PDA conditions

β-asarone content were analyzed by High-Performance Liquid Chromatography (WATERS, binary pump-1525, 2707-auto sampler with PDA-2998 detector). The data was acquired on the Empower 3.0 software. Separation was obtained on the Phenomenex luna C-18 column (250 mm x 4.6 mm, 5µm). In the selection and optimization of chromatographic conditions, several mobile phase compositions were tried to optimize the RP-HPLC-PDA parameters. A satisfactory separation and good peak symmetry for *β*-asarone (Figure 1) was obtained by using

methanol : water :: 80 : 20 V/V ratio as a mobile phase in isocratic mode. The mobile phases were filtered through a 0.45µ filter and degassed before use. The flow rate was adjusted to 0.7 mL/minutes with a run time of 25 minutes. The injection volume was adjusted to 20 µL and detection was made with PDA at 245nm.

2.4 Preparation of standard solution of 1,8-cineole

Around 3.0mg of the 1,8-cineole standard was accurately weighed and dissolved in 20mL of n-hexane to obtain stock concentrations of 150µg/mL. The stock solution was further serial diluted to obtain the dilution range of 25-125µg/mL and then injected in GC-FID to prepare the calibration graphs and quantification of bioactive.

2.5 Preparation of standard solution of β-asarone

Around 3.0mg of the *β*-asarone standard was accurately weighed and dissolved in 20mL of methanol to obtain stock concentrations of 150µg/mL. The stock solution was further serial diluted to obtain the dilution range of 15-120µg/mL and then injected in HPLC to prepare the calibration graphs and quantification of bioactive.

2.6 Preparation of test solution (NEWCHARM® GEL) for GC-FID analysis

For the quantification of 1,8-cineole, 1g NEWCHARM® GEL was sonicated with 50mL of n-hexane for 15 minutes and filtered, the process was repeated again. The final volume was made to 100mL with n-hexane and filtered through a 0.45µ membrane filter before injecting into GC-FID.

2.7 Preparation of test solution (NEWCHARM® GEL) for RP-HPLC-PDA analysis

For the quantification of *β*-asarone, 5g NEWCHARM® GEL was sonicated with 60mL of methanol for 15 minutes and filtered, the process was repeated twice. The final volume was made to 200mL with methanol and filtered through a 0.45µ membrane filter before injecting into RP-HPLC-PDA.

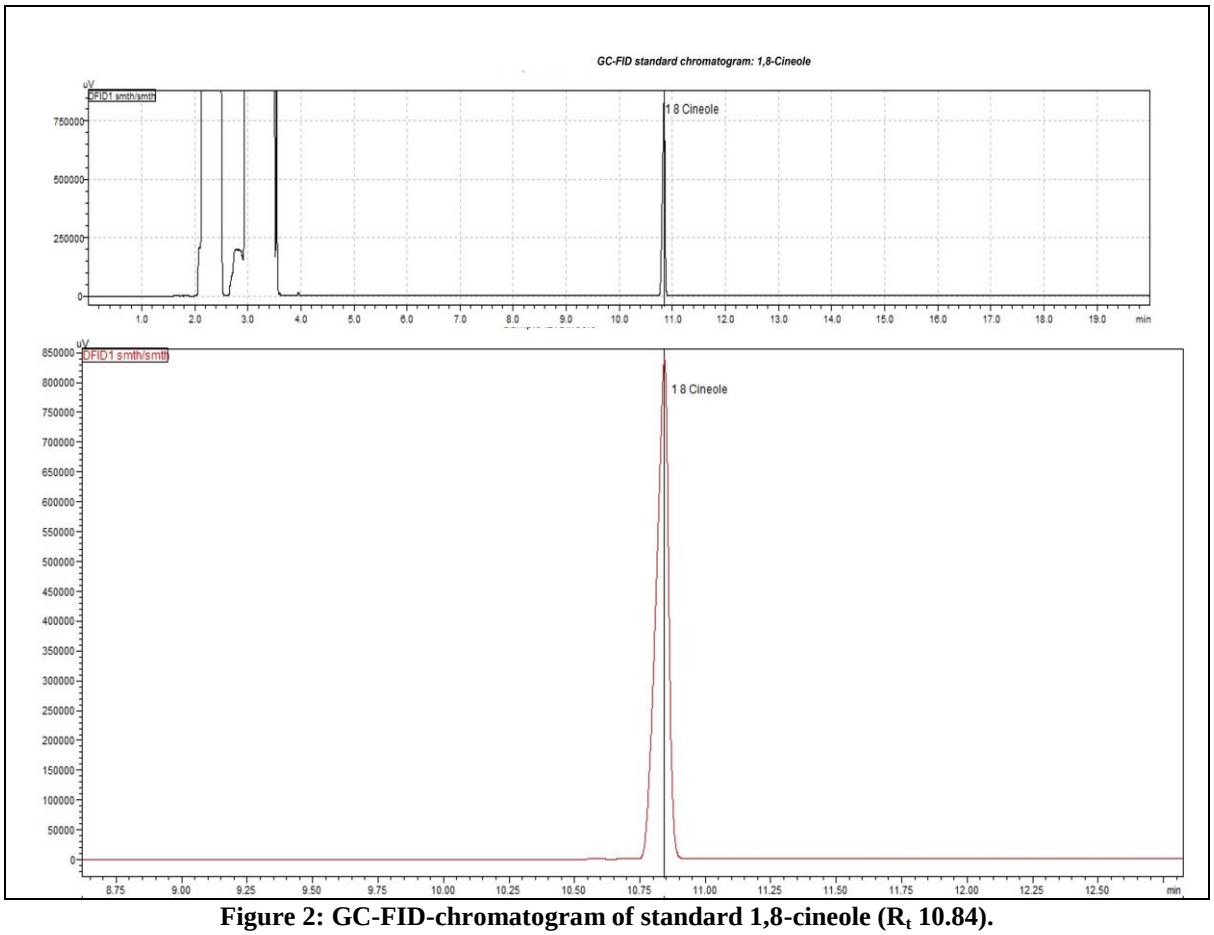

Figure 2: GC-FID-chromatogram of standard 1,8-cineole (R_t 10.84).

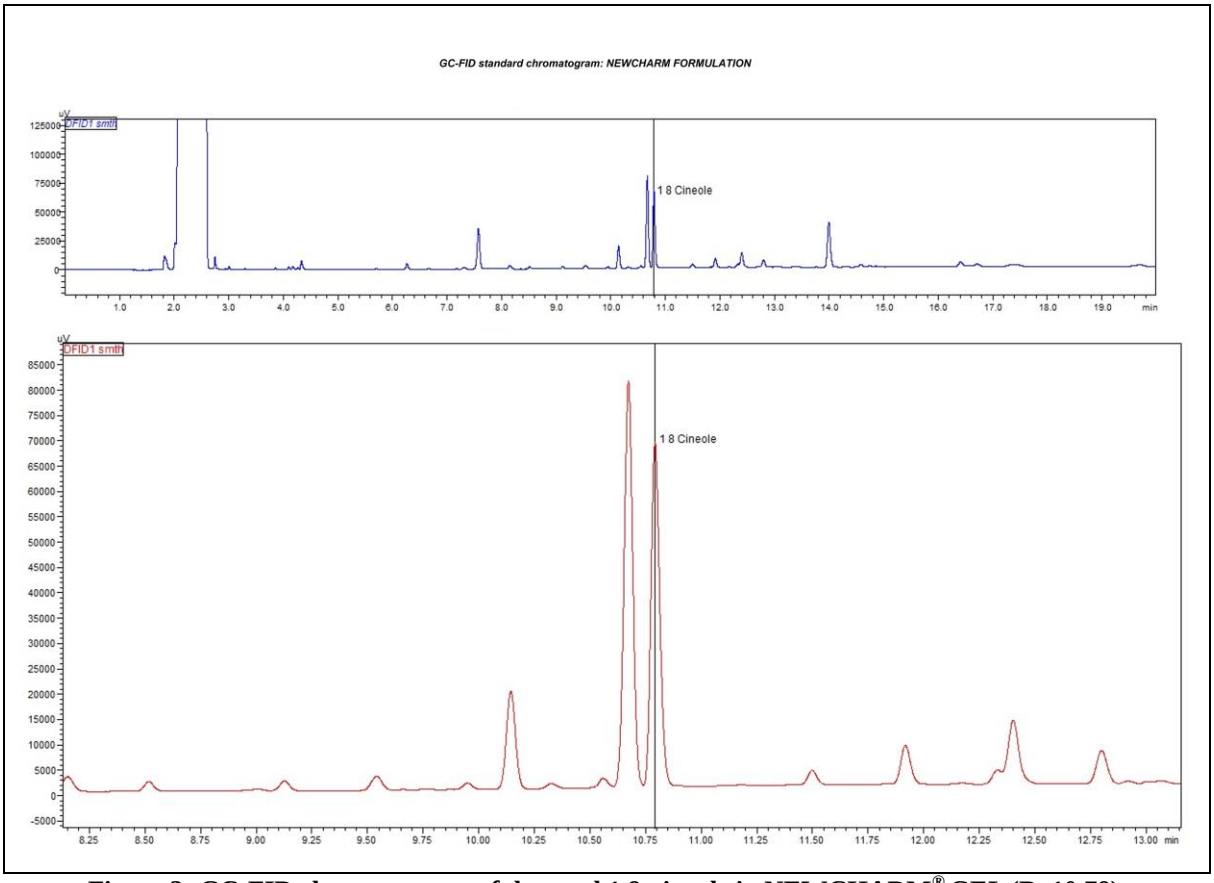

Figure 3: GC-FID chromatogram of detected 1,8-cineole in NEWCHARM® GEL (R_t 10.79).

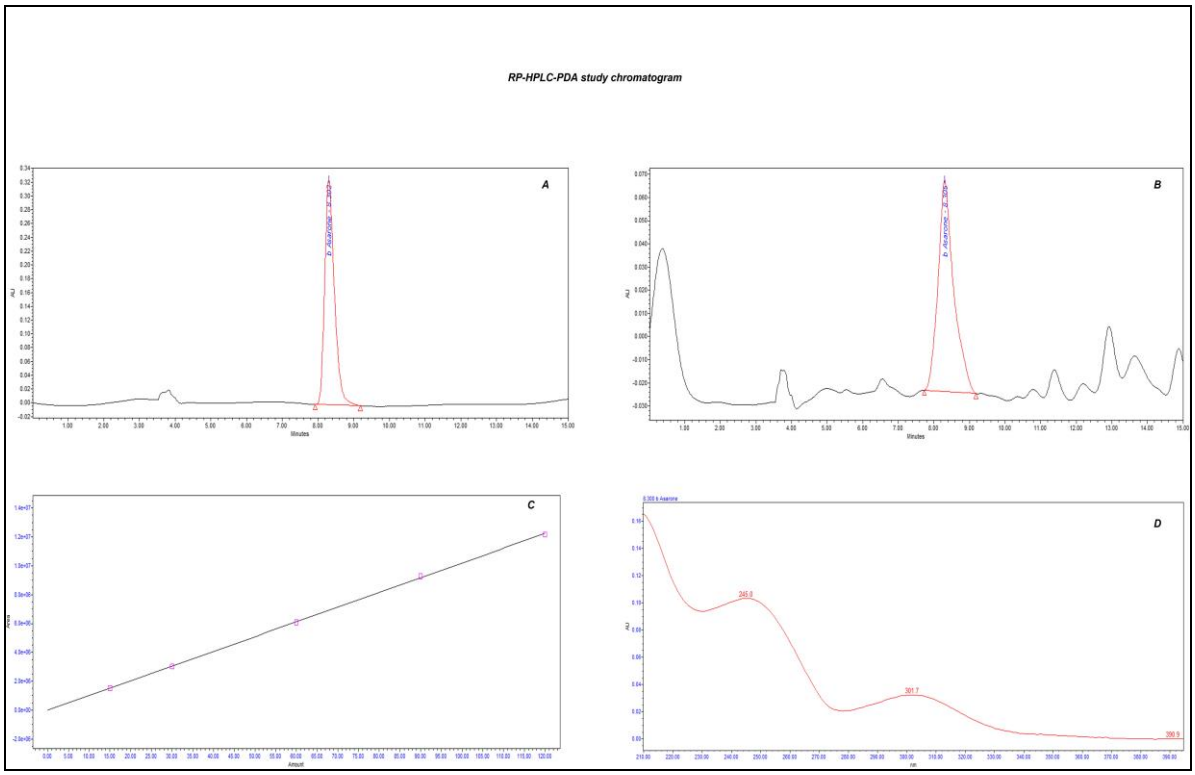

Figure 4: RP-HPLC-PDA-chromatograms (245 nm). (A) Standard chromatogram of *β*-asarone (R_t 8.30). (B) Chromatogram of detected *β*-asarone in NEWCHARM® GEL sample (R_t 8.30). (C) Linearity curve for the developed method of *β*-asarone. (D) UV spectrum of *β*-asarone in standard and samples of NEWCHARM® GEL.

3.0 RESULTS AND DISCUSSION

The development of the GC-FID and RP-HPLC-PDA methods was informed by the experience gained from previously established chromatographic methods for the analysis of polyherbal formulations. It is crucial to note that the analytical results obtained through these methods hold validity only when the defined system suitability criteria are met.

Throughout this investigation, the experimental findings strongly indicate that the chromatographic systems were indeed suitable for the intended analysis. To assess reproducibility, standard solutions containing known concentrations of 1,8-cineole and *β*-asarone were each injected seven times in GC-FID and RP-HPLC-PDA, respectively. The relative standard deviation (RSD) values for peak area and retention time of these standards confirmed the reproducibility of these parameters.[22]

The proposed methods were then subjected to validation for the determination of 1,8-cineole and *β*-asarone, following the parameters outlined in accordance with the guidelines established by the International Council for Harmonization (ICH).[23]

Table 1: Results of precision, LOD, LOQ, linear regression analysis, and their correlation coefficient for quantitative analysis of 1,8-cineole by GC-FID.

S. no.	Parameters	1,8-cineole
1	Concentration range (µg/mL)	25–125
2	Regression equation	Y = 4374x + 1754
3	Correlation coefficient (r^2)	0.998
4	Amount of marker compound in NEWCHARM® GEL (%) (w/w) (mean, n=4)	0.55%w/w
5	Method precision (repeatability n=7) – RSD %	1.8%
6	Intermediate precision (reproducibility) – RSD (%) Intraday Interday	 1.67% 1.74%
7	Mean recovery (n=3) (%)	94.45%w/w
8	LOD LOQ	0.09µg/mL 0.27µg/mL

y=Peak area response, x=Amount of marker compound. LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation.

Table 2: Results of precision, LOD, LOQ, linear regression analysis, and their correlation coefficient for quantitative analysis of *β*-asarone by RP-HPLC-PDA.

S. no.	Parameters	*β*-asarone
1	Concentration range (µg/mL)	15 – 120
2	Regression equation	Y = 1.02x + 2.05
3	Correlation coefficient (r^2)	0.999
4	Amount of marker compound in NEWCHARM® GEL (%) (w/w) (mean, n=4)	0.66%w/w
5	Method precision (repeatability n=7) – RSD %	1.0%
6	Intermediate precision (reproducibility) – RSD (%) Intraday Interday	 1.30% 1.21%
7	Mean recovery (n=3) (%)	97.79%w/w
8	LOD LOQ	0.06µg/mL 0.18µg/mL

y=Peak area response, x=Amount of marker compound. LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation.

3.1 Validation of the proposed method

The proposed methods were validated for the determination of 1,8-cineole and *β*-asarone by GC-FID and RP-HPLC-PDA respectively using the following parameters as per ICH guidelines.

- Calibration: The marker compounds in the formulation were quantified using a calibration curve established with five dilutions of the standard. The corresponding peak area in the formulation was plotted against the concentrations of the standard injected in GC-FID and RP-HPLC-PDA. Peak identification was achieved by comparison of the retention time (Rt) and UV absorption spectrum (RP-HPLC-PDA study) with those obtained for standard.
- Linearity: Linear regression analysis was used to calculate the slope, intercept, and regression coefficient (r^2) for the calibration plot. Linearity was determined by using five concentrations of the standard solution. The response was found to be linear in the concentration ranges investigated (Table 1 and 2).
- Range: Range is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical method has a suitable level of precision, accuracy, and linearity. The

linear response was observed over a range of 25-125 µg/mL for 1,8-cineole and 15-120 µg/mL for *β*-asarone (Table 1 and 2).

- Precision: Three different concentrations of marker compound solution in triplicates were injected at three different times within the same day and repeated the same on three different days to record intra-day and inter-day variations in the results. The low % RSD values of intraday and interday (Table 1 and 2) for the marker compounds 1,8-cineole and *β*-asarone reveal that the proposed methods by GC-FID and RP-HPLC-PDA are precise.
- Limit of Detection (LOD) and Limit of Quantification (LOQ): For determination of limits of detection and quantification, different dilutions of the markers were injected with mobile phase as blank and determined based on signal-to-noise ratio 3:1 and 10:1 respectively. The LOD and LOQ for the standard compounds were calculated and tabulated (Table 1 and 2).
- Selectivity: The retention time of 1,8-cineole was 10.81±0.02 minutes and 8.30±0.05 minutes retention time was observed for *β*-asarone in the formulation respectively. The UV-Vis spectrum of *β*-asarone marker compound was compared with their counterpart in the formulation at three different positions, the peak start, peak center, and peak end. There was a good correlation between spectra obtained at each of the three positions. The 1,8-cineole and *β*-asarone peaks were, therefore, not masked by any peak of another compound present in the formulation (Figures 2-4) which was indicative of peak purity.
- Accuracy: Recovery experiments were conducted to check for the presence of positive or negative interferences from other ingredients/excipients present in the formulation and to study the accuracy of the method. Recovery was determined by the standard addition method. 1,8-cineole and *β*-asarone standard were added to the formulation at two different concentrations, extraction and analysis (respectively on GC-FID and RP-HPLC-PDA instrumentations) were performed as described above. Recovery was calculated for each standard at each concentration (Table 1 and 2).

4.0 CONCLUSION

New analytical techniques employing both GC-FID and RP-HPLC were devised to achieve enhanced separation and quantification of two key phytoconstituents within NEWCHARM® GEL, an exclusive herbal medicine developed by Ayurvet Limited. The establishment of standardized analysis methods for the phytotherapeutic components, 1,8-cineole and *β*-asarone, plays a pivotal role in ensuring consistent quality and efficacy of the product across various production batches on a commercial scale. Moreover, the methodologies presented in this study are characterized by their simplicity, precision, accuracy, making them well-suited for routine analysis and quantification of the active constituents within formulations that incorporate these compounds.

5.0 CONFLICT OF INTEREST

Authors have no conflict of interest.

6.0 ACKNOWLEDGMENTS

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