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RESEARCH ARTICLE

STABILITY MONITORING OF PHYTOGENIC CONSTITUENTS AND PHYSICOCHEMICAL PROPERTIES OF PULMOFARM T HERBAL RESPIRATORY PREMIX DURING FEED PELLETIZATION STRESS

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Abstract

Feed represents a major input in poultry, cattle, and swine production, accounting for approximately 50–70% of total production costs. Feed processing technologies such as pelletization can enhance feed efficiency, reduce wastage, and improve digestibility and palatability. However, the elevated temperature and moisture involved in pelletization may influence the stability of phytogenic feed additives. Livestock species are highly susceptible to respiratory disorders caused by multiple predisposing factors including chronic stress, bacterial or fungal infections, secondary coliform organisms, and environmental allergens. Pulmofarm® T Premix, a phytogenic herbal formulation, is widely used to alleviate symptoms associated with chronic respiratory disease (CRD) such as sniffing, rattling, sneezing, coughing, and respiratory distress. The present study evaluated the phytogenic and physicochemical stability of Pulmofarm® T Premix under simulated pelletization stress conditions.The formulation was exposed to controlled thermal (90 °C) and moisture (2.0% w/w) conditions for 0, 5, and 10 minutes. Stability was assessed using reverse-phase high-performance liquid chromatography with photodiode array detection (RP-HPLC-PDA) and high-performance thin-layer chromatography (HPTLC), along with physicochemical evaluations.The results demonstrated no significant degradation of key phytogenic markers under pelletization conditions. The concentrations of glycyrrhizin (2542.91 ppm ±3.77%, RT 16.82 min) and thymol (281.21 ppm ±4.45%, RT 18.47 min) remained stable, with no notable changes in chromatographic peak areas or retention factor values compared to control samples.

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The analytical methods employed were rapid, sensitive, and reliable for stability assessment.These findings confirm that Pulmofarm® T Premix remains stable during feed pelletization, supporting its suitability for incorporation into pelleted feed systems.Furthermore, the study contributes to the development of robust stability monitoring

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parameters for herbal feed additives, addressing a critical challenge in the nutraceutical and phytogenic feed additive industry.

Introduction:-

Respiratory diseases remain a major health challenge in livestock production systems, particularly in poultry, cattle, and swine, where environmental, infectious, and management-related factors predispose animals to respiratory distress and reduced productivity. In veterinary industry, medicated feeds containing sub-therapeutic levels of pharmacologically active phytogenic substances are frequently utilized for disease prevention and improvement of production performance [1]. Consequently, herbal feed additives have gained increasing attention as natural alternatives for supporting respiratory health and improving livestock resilience [2-3].

Pulmofarm® T Premix, a polyherbal respiratory tonic developed by Zenex Animal Health India Private Limited, is used in poultry, cattle, swine, and other livestock species for the prevention and supportive management of respiratory disorders. Since, they are highly susceptible to respiratory infections caused by pathogens such as Mycoplasma gallisepticum, Pasteurella multocida, and Bordetella bronchiseptica, along with secondary infections by coliform organisms and environmental allergens. In addition to microbial pathogens, several stress-related factors—including transportation, poor ventilation, temperature and humidity fluctuations, high stocking density, and the introduction of new animals into established flocks—contribute significantly to the onset and progression of respiratory disease [4].

Pulmofarm® T Premix has been reported to alleviate clinical manifestations associated with chronic respiratory disease (CRD), including sniffing, sneezing, coughing, rattling, and other signs of respiratory distress [5]. The formulation contains herbs such as Glycyrrhiza glabra and Adhatodavasica, which are widely recognized for their immunomodulatory, expectorant, antimicrobial, demulcent, analgesic, and antispasmodic properties [6-7]. The therapeutic activity of the formulation is largely attributed to bioactive phytoconstituents, including glycyrrhizin from Glycyrrhiza glabra and essential oils such as thymol present in Trachyspermumammi, as well as other phytochemicals present in Adhatodavasica leaves [8]. These compounds exhibit bronchodilatory and mucolytic properties, reducing bronchospasm, and alleviating cough associated with acute and chronic bronchitis. Additionally, Glycyrrhiza glabra demonstrates demulcent and anti-inflammatory activity that helps relieve irritation of bronchial mucosa, sore throat, and asthma-like conditions, making it a widely used herbal remedy for respiratory ailments [9].

Incorporation of herbal premixes such as Pulmofarm® T into feed formulations provides opportunities to enhance livestock productivity and production efficiency. Consequently, optimizing feed processing methods has become an important area of research in animal nutrition. Among these methods, feed pelleting is the most widely used heat-treatment process in poultry and animal feed manufacturing. Pelleting involves the agglomeration of finely ground feed particles into larger pellets, improving feed handling characteristics, increasing feed intake, and enhancing growth performance [10-11].

During pellet production, mash feed is transferred from storage bins to a feeder and conditioner, where it is subjected to heat and moisture before entering the pelleting chamber. The conditioned mash is then forced through a metal die to form pellets, which are subsequently cooled to stabilize their structure [12]. Feed manufacturers frequently adjust processing variables; such as conditioning temperature, moisture content, and residence time—to improve pellet durability and physical quality [13]. Although these modifications enhance pellet quality, the exposure of feed additives to elevated temperature and moisture during pelletization may adversely affect their stability and biological efficacy.

Despite the growing use of herbal feed additives in livestock nutrition, limited studies have investigated the stability of herbal formulations during feed processing. The stability of herbal feed additives can be influenced by multiple factors, including temperature, moisture, environmental conditions, particle size, and variability in secondary metabolite concentrations [14]. Therefore, monitoring the stability of bioactive constituents during feed processing is essential to ensure product quality and therapeutic efficacy.

The present study was designed to evaluate the stability profile of Pulmofarm® T Premix under pelletization stress conditions, where the formulation is exposed to elevated temperature and moisture for short durations during feed processing. In particular, variations in the concentration of key secondary metabolites were investigated as potential stability markers. Glycyrrhizin and thymol were selected as representative bioactive compounds due to their established pharmacological relevance and their contribution to the respiratory therapeutic activity of the formulation.

Quantitative assessment of these marker compounds, along with evaluation of physicochemical properties, was performed using reverse-phase high-performance liquid chromatography with photodiode array detection (RP-HPLC-PDA) and high-performance thin-layer chromatography (HPTLC). These analytical techniques provide rapid, sensitive, and reliable methods for monitoring phytochemical stability in complex herbal formulations.

The objective of this work was therefore to conduct a comprehensive pelletization stress study to evaluate changes in physicochemical characteristics and the stability of glycyrrhizin and thymol [Figure 1]in Pulmofarm® T Premix using a sequential analytical approach involving RP-HPLC-PDA and HPTLC techniques. The findings of this investigation are expected to contribute to the development of robust stability monitoring parameters for herbal feed additives, addressing a critical challenge in the animal health and feed industry.

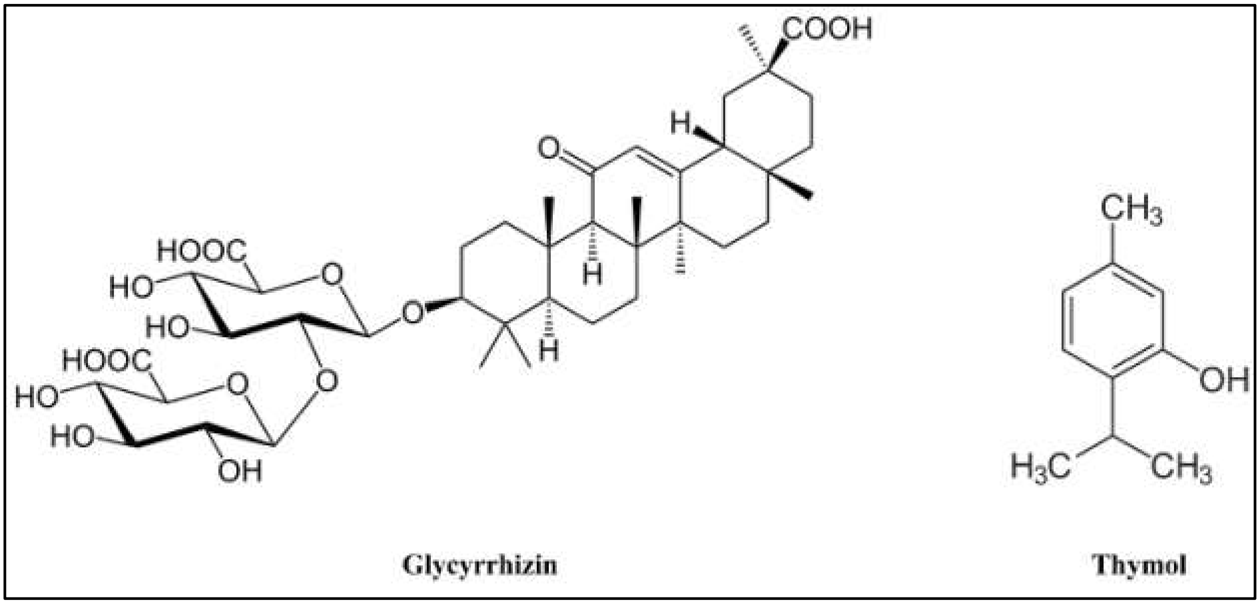

Figure 1: Structure of Glycyrrhizin and Thymol

Material and Methods:-

Chemicals and reagents:

All the reagents and solvents were of AR or HPLC grade as per requirement. The active reference compoundsglycyrrhizin and thymolwere procured from the Sigma Aldrich, while latest controlled sample of Pulmofarm® T Premixwas obtained from the QA/QC department of Zenex Animal Health India Private Limited, Baddi.

Instrumentation:

The RP-HPLC system consisted of WATERS, binary pump 515 with PDA 2996 detector, USA. Separation was obtained on Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 µm). The data were acquired on the Empower 2.0 controlling software (all equipment from Waters, Milford). The HPTLC system consisted of CAMAG-HPTLC system with Scanner III, Linomat V, twin trough chambers and Vision-Cats software.

Pelletization stress stability testing:

The formulated Pulmofarm® T Premixwas assessed for stability under pelletization stress conditions as per the common industrial standards: controlled 90℃ temperature and moisture (2.0 % w/w) exposure for zero, five, and ten minute interval of times. Physicochemical properties, RP-HPLC-PDA, and HPTLC studieswere used to monitor stability.

Physicochemical parameters:
The physical parameters like description,total ash content, extractive values, calcium content, active marker compounds were evaluated for the three samples under study i.e. (1) control, (2) sample exposed to 90°C, 2.0 % w/w moisture for 05 minutes and (3) sample exposed to 90°C, 2.0 % w/w moisture for 10 minutes.

High performance liquid chromatography (RP-HPLC-PDA) study:
Preparation of test solution:
Around 5g of each Pulmofarm® T Premix sample was weighed accurately and transferred into a 250mL round bottom flask. 70mL of methanol was added, and the mixture was refluxed on a water bath using a reflux condenser for one hour. This process was repeated two more times. The solution was then filtered and concentrated to 100mL using a rotavapor, transferred into a 100mL volumetric flask, and made up to volume with methanol. Finally, the solution was filtered through a 0.45 µm filter before being injected into the HPLC system.

Preparation of standard Thymol solutions:
Around 2.5 mg of thymol reference standard was weighed accurately and transferred to a 25 mL volumetric flask. 20 mL of methanol was added, the mixture was sonicated for 5 minutes, and the volume was made up with the same solvent. The solution was filtered through a 0.45 µm filter before being injected into the HPLC system.

Preparation of standard Glycyrrhizin solutions:
Around 5 mg of glycyrrhizin reference standard was weighed accurately and transferred to a 25 mL volumetric flask. 20 mL of methanol was added, the mixture was sonicated for 5 minutes, and the volume was made up with the same solvent. The solution was filtered through a 0.45 µm filter before being injected into the HPLC system.

Chromatographic conditions for Thymol analysis:
Initial trials were carried by a gradient mode of analysis using the mobile phase, which consisted of a gradient solvent system of water (containing 0.2% acetic acid) and acetonitrile (from 50:50 to 100:0 over 20 min). Experiments concluded lack of resolution of a complex mixture of different phytoconstituents and time consuming using the gradient approach of analysis. The simple isocratic mode was opted comprising water and acetonitrile in 50:50 ratio. The elution was clear and well-separated peaks of thymol with a flow rate of 1 mL/min over a runtime of 30 min. The eluent was monitored at 280 nm. The mobile phase was filtered through 0.45 µm Millipore membrane filter and degassed before use. The injection volume was 20 µL and all analysis were performed at ambient temperature.

Chromatographic conditions for Glycyrrhizin analysis:
The simple isocratic mode was opted comprising potassium dihydrogen phosphate buffer solution of 5.3mM and acetonitrile in 65:35 ratio having a pH of 3.5 with acetic acid. The elution was clear and well-separated peaks of glycyrrhizin with a flow rate of 1 mL/min over a runtime of 25 min. The eluent was monitored at 254 nm. The mobile phase was filtered through 0.45 µm Millipore membrane filter and degassed before use. The injection volume was 20 µL and all analysis were performed at ambient temperature.

High performance thin layer chromatography (HPTLC) study:
Preparation of test solution:
(a) Weighed accurately around 5g of each Pulmofarm® T Premix samples and transferred to a 250mL round bottom flask. Added 200 mL of chloroform and refluxed on water bath for 2 hours, cooled, filtered and concentrated up to 100 mL using rotavapor, transferred in to a 100 mLvolumetric flask and made up the volume to 100 mL with chloroform. Filter the solution using 0.45µm syringe filter.Clear resulting solution thus obtained was used for HPTLC analysis.
(b) Dried the marc from above process on water bath and transferred to a 250 mL round bottom flask, added 200 mL methanol, refluxed on water bath for 2 hours, cooled and filtered through filter paper, concentrated up to 100 mL using rotavapor, transferred in to a 100 mL volumetric flask and made up the volume to 100 mL with methanol. Filter the solution using 0.45µm syringe filter.Clear resulting solution thus obtained was used for HPTLC analysis.

Application for chloroform fraction:
Applied 10 µl of solution of each control and exposed sample extracts (control, sample exposed to 90°C, 2.0 % w/w moisture for 05 minutes and sample exposed to 90°C, 2.0 % w/w moisture for 10 minutes) on TLC plate precoated with Silica gel 60F 254 using Linomat applicator. TLC plate was then dipped in saturated twin trough chamber

containing the mobile phase of Toluene: Ethyl acetate 70:30. Eluted TLC plate then scanned in CAMAG-HPTLC Scanner III under Tungsten lamp at 480 nm in absorbance mode. Peaks were integrated and areas were determined. Spectral scan was taken of all peaks to confirm that spot in control and exposed samples track are similar.

Application for Methanol soluble fraction:
Applied 10 µl of solution of each control and exposed sample extracts (control, sample exposed to 90°C, 2.0 % w/w moisture for 05 minutes and sample exposed to 90°C, 2.0 % w/w moisture for 10 minutes) on TLC plate precoated with Silica gel 60F 254 using Linomat applicator. TLC plate then dipped in saturated twin trough chamber containing the mobile phase of Chloroform: Methanol in 95:05 ratio. Eluted TLC plate then scanned in CAMAG-HPTLC scanner III under Deuterium lamp at 254 nm in absorbance mode. Peaks were integrated and areas were determined. Spectral scan was taken of all peaks to confirm that spot in control and exposed samples track are similar.

Results and Discussion:-

The impact of heat exposure on the glycyrrhizin and thymol content, physicochemical characteristics, and chromatographic fingerprint profile of Pulmofarm® T Premix was systematically evaluated. The formulation was divided into three groups: a control sample and samples subjected to heat treatment at 90°C in the presence of 2.0% w/w moisture for 0, 5, and 10 minutes.Assessment of key physicochemical parameters—including extractive values, ash content, and calcium content—revealed no significant variation among the treated samples compared with the control [Table 1], indicating that short-term exposure to the applied thermal and moisture conditions did not materially affect the basic physicochemical properties of the formulation.Quantitative analysis of the marker compounds glycyrrhizin and thymol in the treated samples was performed using chromatographic techniques to evaluate the formulation's stability under pelletization-like stress conditions. Analytes quantification were performed via RP-HPLC-PDA utilizing validated methodologies developed in alignment with ICH Q2(R1) statistical guidelines [15]. While the protocol for thymol underwent international standard validation [Tables 2, Table 3, Table 4], the analysis of glycyrrhizin followed previously established and peer-reviewed procedures (Ravikanth K. et al., 2015) [16].The chromatograms demonstrated well-resolved peaks for glycyrrhizin and thymol with retention times of 18.30 minutes and 16.60 minutes, respectively. Spectral scans and minimal differences in peak areas (≤5.0%) across all treated samples provided clear evidence that no significant degradation occurred under the applied heat and moisture treatments [Table 5 & 6, Figure 4 & 5].

Further characterization was performed using High-Performance Thin Layer Chromatography (HPTLC) to assess potential qualitative changes in the formulation profile. Chloroform extracts analyzed at 480 nm and methanolic extracts analyzed at 254 nm provided complementary fingerprint information. The inherent variability of the chloroform extract, presence of more volatile components showed area difference up to 6.28% in HPTLC fingerprint profile. Whereas, overlay peak scans of both solvent extracts from all three treatment conditions [Table 7, Table 8; Figure 2, Figure 3] showed consistent band patterns without the appearance of additional degradation products.
Moreover, variations in the total chromatographic area and active marker compounds in heat-exposed samples relative to the control were within ±10%, which falls within acceptable analytical variability limits. Collectively, these findings indicate that Pulmofarm® T Premix maintains chemical integrity and marker compound stability under the evaluated thermal and moisture conditions, supporting its stability during pelletization-related processing.

Table 1: Physicochemical parameters of Pulmofarm® T Premix.

S. No.	Parameters	Pulmofarm® T PremixControl (00 minutes at 90^{0}C and 2.0% moisture)	Pulmofarm® T Premix: After exposure (05 minutes at 90^{0}C and 2.0% moisture)	Pulmofarm® T Premix: After exposure (10 minutes at 90^{0}C and 2.0% moisture)
1	Description	Greenish yellow color fine powder with herbaceous odour	Greenish yellow color fine powder with herbaceous odour	Greenish yellow color fine powder with herbaceous odour
2	Water soluble extractive value	21.64 %w/w	21.34 %w/w	19.41 %w/w
3	Crude ash	17.95 %w/w	17.47 %w/w	17.37 %w/w

4	Calcium content	6.48 %w/w	6.46 %w/w	6.43 %w/w

Table 2: System Suitability Parameters for the Thymol RP-HPLC-PDA Method.

S. no.	Parameters	Data	RSD
1	Peak area	1196219	0.95
2	Retention time (min)	18.30	0.93
3	Theoretical plates	9954	0.98
4	Tailing factor	0.981	1.04

Table 3: Results of precision, LOD, LOQ, linear regression analysis, and their correlation coefficient for quantitative analysis of thymol by RP-HPLC-PDA.

S. no.	Parameters	Thymol
1	Concentration range for linearity [μg ml^{-1}]	18.0 to 72.0
2	Correlation Coefficient (r2)	0.999
3	Amount of marker compound in Pulmofarm® T Premix [%] (w/w)[a]	0.027
4	Intermediate precision (Reproducibility) RSD [%]	0.50
5	Intraday 1	0.43
6	Interday 3	0.35
7	LOD	0.062 μg ml^{-1}
8	LOQ	0.186 μg ml^{-1}

Table 4: Results from determination of recovery.

S. no.	Parameters	Thymol		
1	Initial concentration in formulation [mg g-1]	0.27	0.27	0.27
2	Concentration added [mg g 1]	**0.0**	**2.0**	**4.0**
3	Total concentration [mg g-1]	0.27	2.27	4.27
4	Concentration found [mg g 1]	0.264	2.07	3.92
5	RSD [%] (n=7)	0.95	0.93	0.92
6	Recovery [%]	97.78	91.19	91.80
7	Mean recovery [%]	93.59		

Table 5: Thymol contents by RP-HPLC-PDA in Pulmofarm® T Premix.

Sample details	**Thymol content (ppm)**	**% Difference in Thymol content with respect to control sample**
Pulmofarm® T Premix - control	281.21	0.00
Pulmofarm® T Premix after exposure to heat for 05 minutes at 90ºC	272.54	-3.18
Pulmofarm® T Premix after exposure to heat for 10 minutes at 90ºC	269.22	-4.45

Table 6: Glycyrrhizin contents by RP-HPLC-PDA in Pulmofarm® T Premix.

Sample details	**Glycyrrhizin content (ppm)**	**% Difference in Glycyrrhizin content with respect to control sample**
Pulmofarm® T Premix - control	2542.91	0.00
Pulmofarm® T Premix after exposure to heat for 05 minutes at 90ºC	2508.85	-1.36
Pulmofarm® T Premix after exposure to heat for 10 minutes at 90ºC	2450.54	-3.77

Table 7: HPTLC analysis data (480 nm) for chloroform fractions of Pulmofarm® T Premix samples.

Sample details	**Total area of peak of (HPTLC chromatogram at Tungsten 480 nm absorbance)**	**% Difference in area with respect to control sample**
Pulmofarm® T Premix - control	0.03517	0.00
Pulmofarm® T Premixafter exposure to heat for 05 minutes at 90ºC	0.03405	-3.29
Pulmofarm® T Premix after exposure to heat for 10 minutes at 90ºC	0.03309	-6.28

Table 8: HPTLC analysis data (254 nm) of methanol fractions of Pulmofarm® T Premix samples.

Sample details	**Total area of peak of (HPTLC chromatogram at Deuterium 254 nm absorbance)**	**% Difference in area with respect to control sample**
Pulmofarm® T Premix - control	0.02216	0.00
Pulmofarm® T Premix after exposure to heat for 05 minutes at 90ºC	0.02197	-0.86
Pulmofarm® T Premix after exposure to heat for 10 minutes at 90ºC	0.02185	-1.42

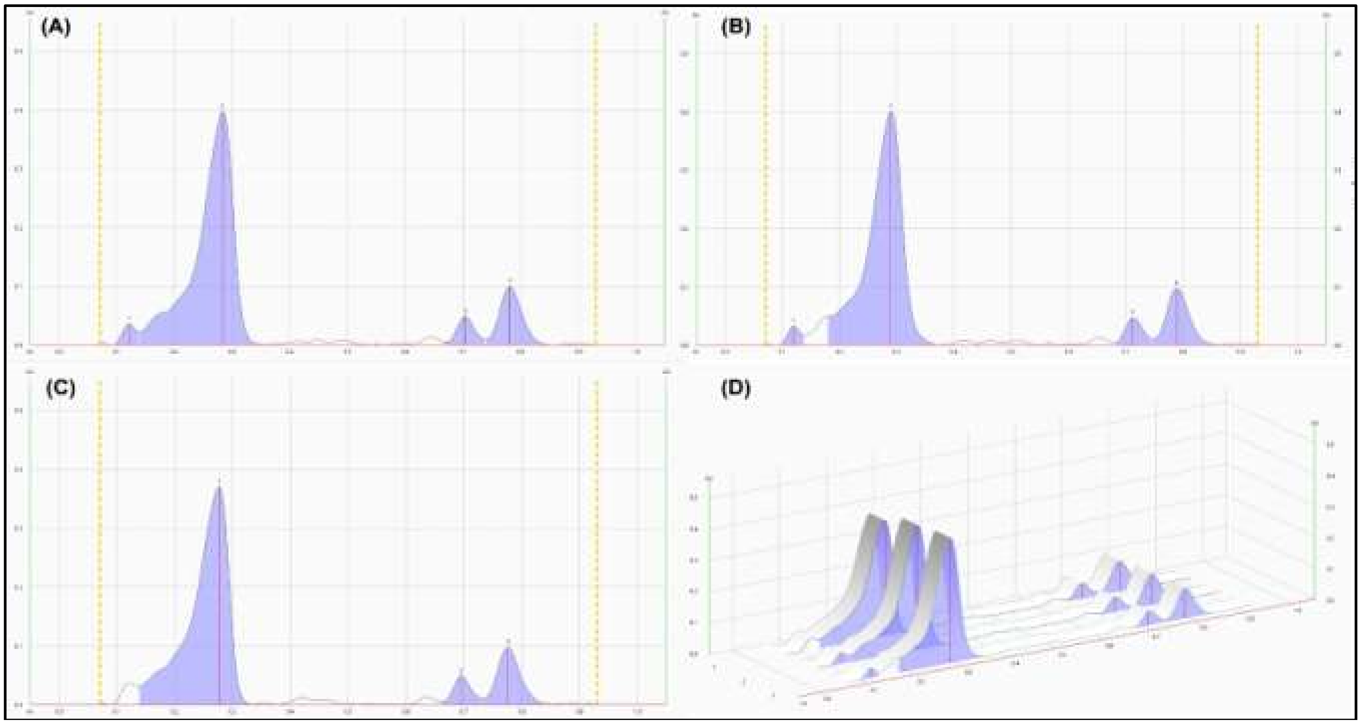

Figure 2: HPTLC analysis data (480 nm) of chloroform fractions of Pulmofarm® T Premix samples. (A) Chromatograms of control Pulmofarm® T Premix sample fraction (zero minute, 90ºC temperature, 2.0% w/w moisture content). (B) Chromatograms of treated Pulmofarm® T Premix sample fraction (5.0 min, 90ºC temperature, 2.0% w/w moisture content). (C) Chromatograms of treated Pulmofarm® T Premix sample fraction (10.0 min, 90ºC temperature, 2.0% w/w moisture content). (D)Three-dimensional overlay chromatogram of (A), (B) and (C).

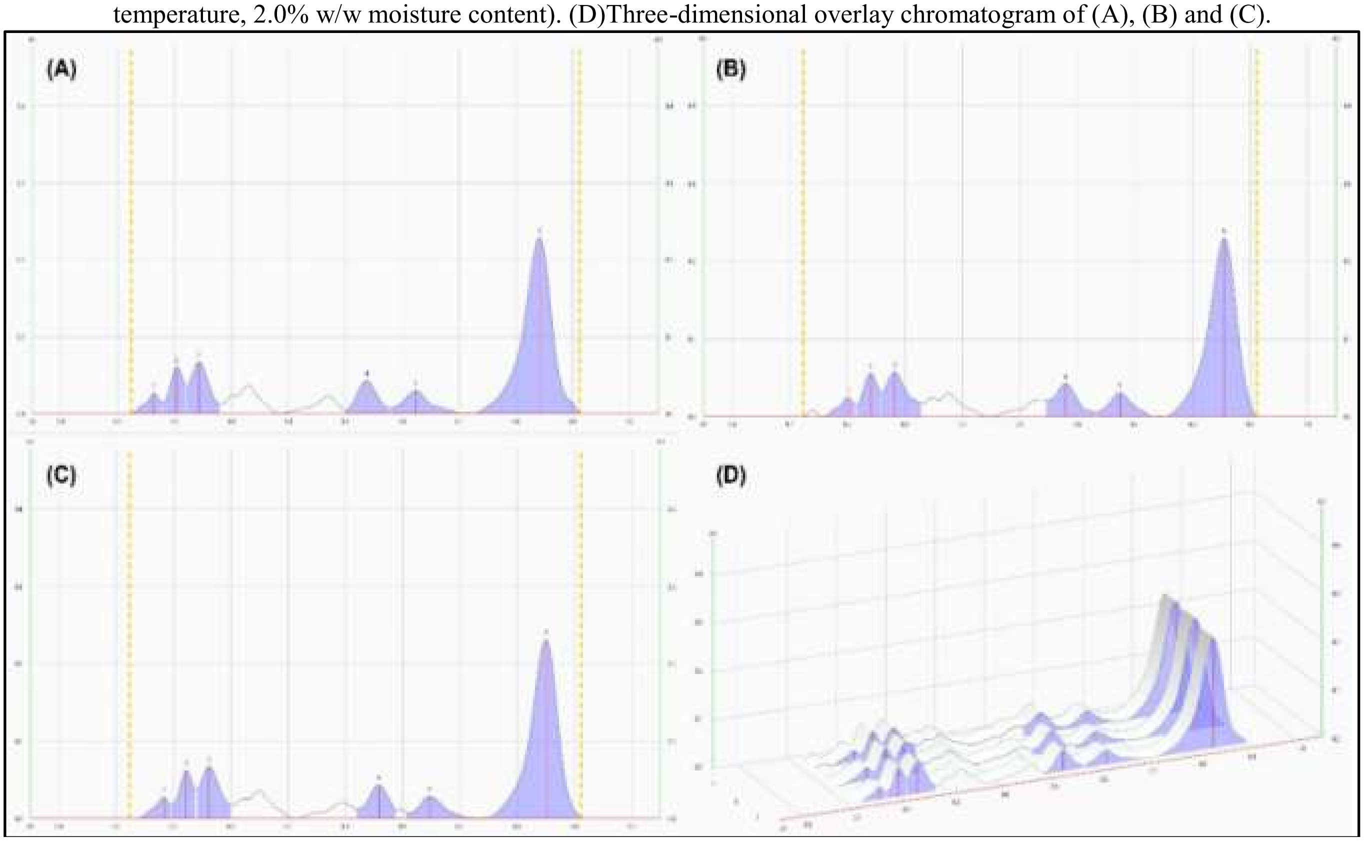

Figure 3: HPTLC analysis data (254 nm) of methanol fractions of Pulmofarm® T Premix samples. (A) Chromatograms of control Pulmofarm® T Premix sample fraction (zero minute, 90ºC temperature, 2.0% w/w moisture content). (B) Chromatograms of treated Pulmofarm® T Premix sample fraction (5.0 min, 90ºC temperature, 2.0% w/w moisture content). (C) Chromatograms of treated Pulmofarm® T Premix sample fraction (10.0 min, 90ºC temperature, 2.0% w/w moisture content). (D) Three-dimensional overlay chromatogram of (A), (B) and (C).

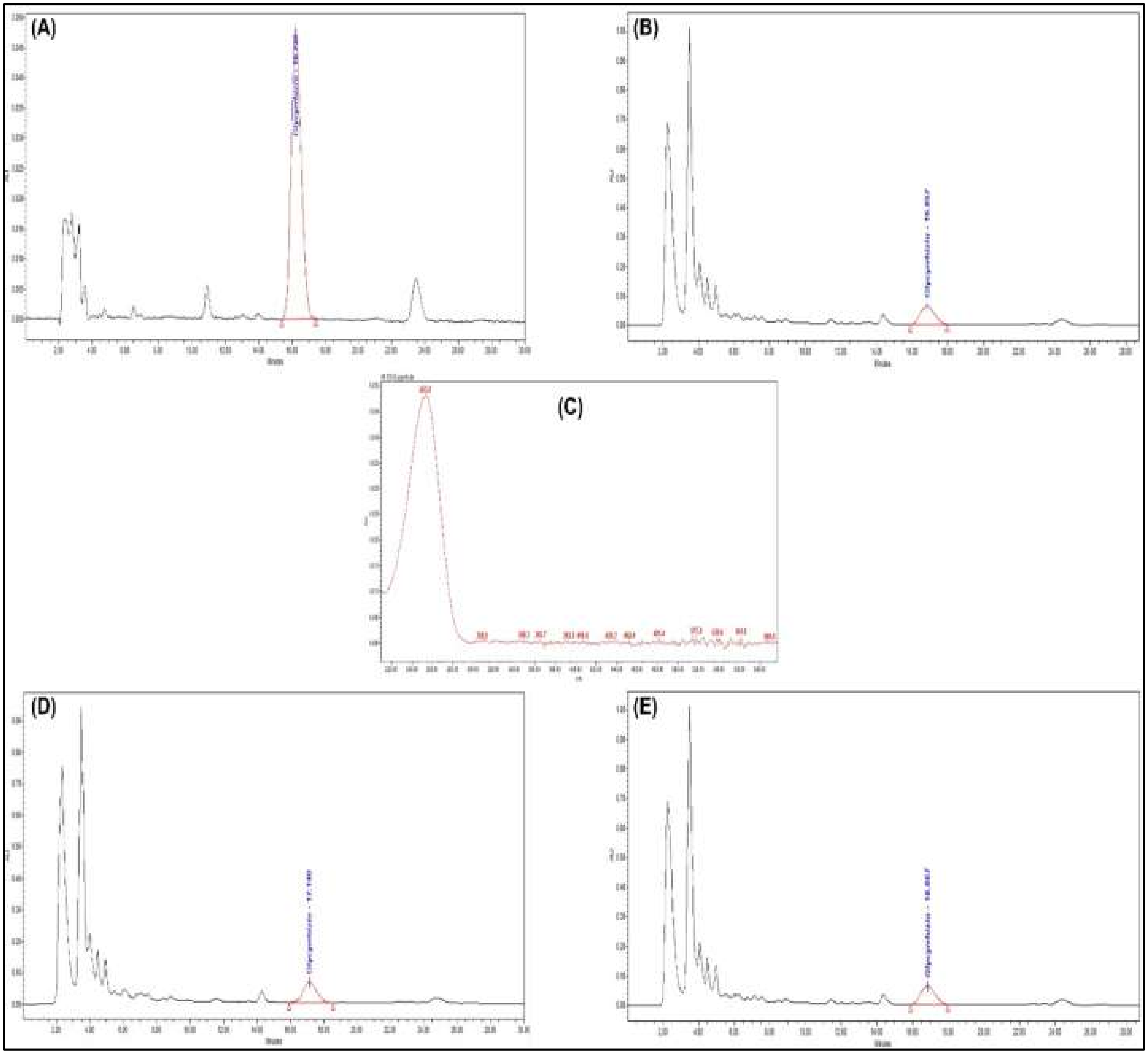

Figure 4: RP-HPLC-PDA chromatograms (254 nm) and UV spectrum of glycyrrhizin in control and treated samples of Pulmofarm® T Premix. (A) Standard chromatogram of glycyrrhizin (Rt 16.82). (B) Control sample of Pulmofarm® T Premix (zero minute, 90ºC temperature, 2.0% w/w moisture content). (C) Obtained UV-spectral scan for standard and samples. (D) Treated sample of Pulmofarm® T Premix (5.0 minute, 90ºC temperature, 2.0% w/w moisture content). (E) Treated sample of Pulmofarm® T Premix (10.0 minute, 90ºC temperature, 2.0% w/w moisture content).

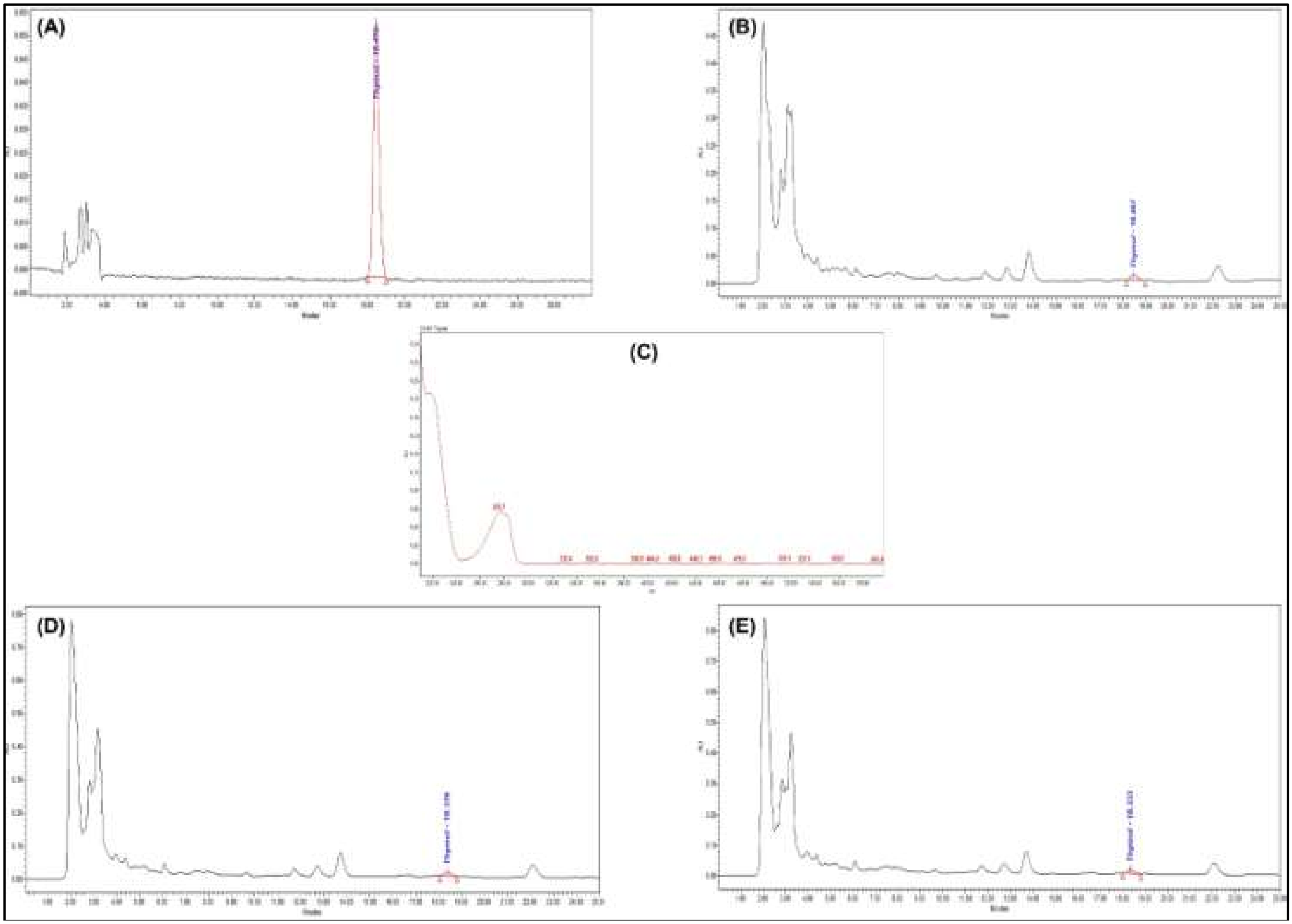

Figure 5: RP-HPLC-PDA chromatograms (280 nm) and UV spectrum of thymol in control and treated samples of Pulmofarm® T Premix. (A) Standard chromatogram of thymol (Rt 18.47). (B) Control sample of Pulmofarm® T Premix (zero minute, 90ºC temperature, 2.0% w/w moisture content).(C) Obtained UV-spectral scan for standard and samples.(D) Treated sample of Pulmofarm® T Premix (5.0 minute, 90ºC temperature, 2.0% w/w moisture content). (E) Treated sample of Pulmofarm® T Premix (10.0 minute, 90ºC temperature, 2.0% w/w moisture content).

Conclusions:-

In conclusion, Pulmofarm® T Premix demonstrated stability under pelletization stress conditions, including elevated temperature and increased moisture levels. The results indicate that the formulation can be incorporated as a stable feed supplement during the pelletization process without significant degradation of its key constituents.The present investigation advances to the development of appropriate stability monitoring parameters for herbal formulations. The study provides supporting evidence that chromatographic techniques such as RP-HPLC-PDA and HPTLC, when used in conjunction with established physicochemical evaluations, represent robust analytical approaches for assessing the stability of complex herbal preparations. These techniques can therefore be effectively employed to generate scientifically reliable stability data suitable for regulatory submissions to relevant authorities.Furthermore, the application of these analytical methodologies facilitates the prediction of shelf life and appropriate storage conditions for herbal products. Such assessments are particularly critical for herbal formulations due to their multicomponent composition and inherent chemical complexity, which can influence stability during processing and storage.

Conflict of Interest:-

Author has no conflict of interest.

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