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RESEARCH ARTICLE

6-GINGEROL AND PHYSICOCHEMICAL PROPERTIES AS MONITORING PARAMETER FOR A NATURAL GROWTH PROMOTER NBIOTIC™ PREMIX DURING FEED PELLETIZATION STRESS

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Abstract

Background: Feed is an important input for poultry and swine production, representing 50–70% of the total production costs. Feed processing provides an opportunity to improve poultry and swine performance. Herbal gut function modulators are used in poultry and swine feeds to improve nutrient utilization and efficiencies of biomass production. Nbiotic™ Premix is a phytoformulation of AYURVET LIMITED, INDIA used in poultry and swine as a natural growth promoter (NGP) to effectively replace antibiotic growth promoters (AGP). Pelletization used to prevent wastage of feed, reduce bulk and improve digestibility and palatability. In present work we have assessed the stability of the Nbiotic™ Premix herbal formulation under different pelletization thermal stress conditions.

Methods: Formulation was kept under controlled temperature (90^{o}C) and moisture (2.0 % w/w) for different time intervals (0, 5, 10 minutes). Chromatographic techniques (HPLC and HPTLC) and physicochemical properties were used to monitor the formulations stability. Nbiotic™ Premix was stable at all the experimental investigation of pelletization thermal stress.

Results: No significant change in the 6-gingerol content (250.0 ppm (± 2.0 % at RT 10.05 minutes) and difference between peak areas and the retention factor (RF) values for the control samples and various thermal stress conditioned samples were observed. A comprehensive study of HPLC and HPTLC measurements have been applied for assessing the stability of Nbiotic™ Premix. Developed methods (HPLC & HPTLC) were found to be rapid, sensitive and reliable in determining its stability. As evident, Nbiotic™ Premix can undergo pelletization process without any degradation. In conclusion, present study will help towards developing appropriate stability monitoring parameters for herbal products which is a major challenge in the nutraceutical industry in current scenario.

Introduction:-

In veterinary industry, feeds with antibiotics at sub therapeutic level are used for disease prevention and growth promotion (**Bhaskar 2020).** Immature immune system, hypothermia and immature gastrointestinal system are important challenges for growth in poultry and swine industries. But continuous use of antibiotics has created a threat of drug resistance **(Briggs 1999 & Budino 2005).** As per WHO recommendations, a systematic approach

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towards replacing antibiotic growth promoters with safer alternatives is required for better animal health **(Dozier 2010).**

Nbiotic™ Premix is a proprietary polyherbal formulation developed by AYURVET LIMITED, INDIA which is used as natural growth promoter for pigs and poultry. It is a blend of essential oils and other secondary plant metabolites and act as an effective feed supplement. This formulation is a Premix of herbs such as *Zingiber officinale, Allium sativum* and *Trigonella foenum-graecum* as main constituents. These herbs contain number of secondary plant metabolites such as terpenoids, alkaloids, flavonoids, bitters and tannins. These collectively with a synergistic effect through polyherbal formulation act as a natural growth promoter. The advantage of the natural growth promoter over antibiotic growth promoter is that they do not bear any risk regarding the bacterial resistance or undesirable residue in animal products. Ayurvet limited has previously documented the clinical data reports of the Nbiotic™ Premix as effective growth promoter **(Fairfield 2005, Fairchild 2005 & ICH 2005).**

Feeds with Nbiotic™ Premix processing provide an opportunity to improve poultrys and swine performance and efficiencies of live production. Therefore, an emerging area for research in poultry and animal nutrition is the preparation of feeds prior to ingestion to increase the value of the feed. There are many possible strategies to improve feed processing techniques. The benefits of producing feeds with improved pellet quality have been well documented in previous reports **(Joshua 2020 & Praveen 2015)**.

Pelleting is the most prevalent heat treatment process in the production of poultry and animal feed which agglomerates smaller feed particles into larger particles as pellets, enhances the economics of production by increased feed intake, and growth performance. This step involves passing mash feed from the mash bin into the feeder and conditioner. Conditioned mash flows into the pelleting chamber. Pellets are thus formed by passing the hot mash through a metal die followed by cooling **(Thomas 1997 & Van der Fels-Klerx 2011).**

Feed manufactures improve pellet quality through the manipulation of several factors, increasing mash conditioning temperatures and time being well-established methods to improve physical feed quality **(Van Lunen 2003).**

However, there are no study reports monitoring negative aspects of these manipulations (time, temperature and moisture) on the stability and efficacy of feed additives like growth promoters and vitamin supplements for feed pelletization. Monitoring the stability of herbal medicines will add to their quality and efficacy. Temperature, moisture content, secondary metabolite content variations, particle size, environment etc. can all affect the stability of herbal formulations. The purpose of the study was to monitor the stability profile of Nbiotic™ Premix during pelletization stress, where the product is exposed to heat and moisture for a short period of time. The process and the conditions (temperature and moisture) that the pellets are subjected to may have a strong influence on the quality of final product. Selection of variation in the secondary metabolites concentration during process can be a monitoring parameter for the stability of herbal formulation used in the feed pelletization; which has been ignored. Consequently, present study explores the possibility of secondary metabolite (6-gingerol) content along with physicochemical properties by using HPLC and HPTLC as quick, sensitive and reliable methods of monitoring the stability of Nbiotic™ Premix as a nexus for pelletization stress stability profiling of herbal formulations.

Objective of this work was to develop comprehensive pelletization stress study on the physicochemical properties and 6-gingerol stability in Nbiotic™ Premix with sequenced methodology of HPLC and HPTLC techniques. This investigation adds a major contribution towards developing appropriate stability monitoring parameters for herbal products; a major challenge in the animal care industry.

Material and Methods:-

Chemicals and reagents:

All the reagents and solvents were of AR or HPLC grade as per requirement. The active compound 6-gingerol was isolated in R&D Phytochemistry Lab Ayurvet Limited and structure was established by interpreting the 1 H, 13C, and 2D NMR spectra. Latest controlled samples of Nbiotic™ Premix were obtained from the QA/QC department of AYURVET LIMITED, Baddi.

Instrumentation:
The 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 Win Cats software Ver.1.4.1.

Pelletization stress stability testing:
The formulated Nbiotic™ Premix was assessed for stability under pelletization stress conditions: controlled 90^{o}C temperature and moisture (2.0 % w/w) exposure for zero, five and ten minute interval of times. HPLC, HPTLC and physicochemical properties were used to monitor stability.

Physicochemical parameters:
The Physical parameters like description, colour, odour, moisture content, total ash content, extractive values, volatile oil, active marker compound were evaluated for the three samples under study i.e. control, sample exposed to 90^{0}C, 2.0 % w/w moisture for 05 minutes and sample exposed to 90^{0}C, 2.0 % w/w moisture for 10 minutes.

High performance liquid chromatography study:
Preparation of test solution:
Weigh accurately around 5gm of each Nbiotic™ Premix samples and transfer in to 250ml round bottom flask add 70ml methanol and reflux it on water bath using reflux condenser, repeat the process for two more times, filter and concentrate up to 100ml using rotavapor and transfer in to a 100ml volumetric flask, make up the volume with methanol. Filter the solution through 0.45 µm before injecting into HPLC analysis.

Preparation of standard solution:
Weigh accurately around 2.5 mg of 6-gingerol reference standard and transfer to 100ml volumetric flask. Add 70 ml methanol and sonicate for 5 minutes and make up the volume with above solvent. Filter the solution through 0.45µm before injecting into HPLC.

Chromatographic conditions:
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 6-gingerol 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 analyses were performed at ambient temperature.

High performance thin layer chromatography study:
Preparation of test solution:
(a) Weighed accurately around 5g of each Nbiotic™ Premix samples and transferred to a 250ml round bottom flask. Added 200 ml of P. Ether (60-80 $^{\circ}$C) and refluxed on water bath for 4 hours, cooled, filtered and concentrated up to 100 ml using rotavapor, transferred in to a 100 ml volumetric flask and made up the volume to 100 ml with P. Ether. 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 4hours, 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 petroleum ether fraction:
Applied 10 µl of solution of each control and exposed sample extracts (control, sample exposed to 90^{0}C, 2.0 % w/w moisture for 05 minutes and sample exposed to 90^{0}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 Toluene: Ethyl acetate 93:07. Eluted TLC plate then scanned in Camag HPTLC Scanner III under Deuterium lamp at 260nm in absorbance mode & at 366nm in fluorescence 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 90^0C, 2.0 % w/w moisture for 05 minutes and sample exposed to 90^0C, 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 Toluene: Ethyl acetate: Formic acid: Water in 3:3:0.80:0.40 ratio. Eluted TLC plate then scanned in Camag HPTLC scanner III under Deuterium lamp at 280nm 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 effect of heat on the 6-gingerol content, physicochemical and fingerprint profile of Nbiotic™ Premix was analyzed. The formulation under study was equally divided as control, to be exposed to heat (90ºC) and moisture (2.0% w/w) for 0, 5 and 10 minutes. It was observed that the physicochemical parameters like extractive values, pH, ash content and volatile oil of three samples under study do not show major variation [**Table 1].**

Quantification of secondary metabolite 6-Gingerol in these treated Nbiotic™ Premix samples with chromatographic technique gives a clear cut data on pelletization stress stability. A validated method in accordance with the statistical method of validation given in ICH Q2R1 **(WHO 1997)** was used for 6-Gingerol quantification by HPLC **[Table 2].** Well separated peaks (RT = 10.05 minutes) in all exposed samples with spectral scans and minuet peak area differences (below 2 %) provide the evidence of no major degradation during the temperature and moisture treatments investigation **[Table 3, Figure 1].**

Strategically HPTLC analysis of control and test Nbiotic™ Premix i.e petroleum ether extracts at 260nm, 366nm and methanolic extracts at 280 nm gives valuable investigation. HPTLC fingerprint profile and overlay peak scan of different solvent treated (Pet ether and methanol) each of three samples under study **[Table 4, Table 5, Table 6** & **Figure 2, Figure 3, Figure 4]** shows no major sign of degradation. The change in the total area & active marker compound of heat exposed samples with respect to control sample is less than ±10 % which is acceptable.

Table 1:- Physicochemical parameters of Nbiotic™ Premix.

Sr. No.	Parameters	Nbiotic™ Premix Control (0 minutes at 90^0C and 2.0%w/w moisture)	Nbiotic™ Premix After exposure (05 minutes at 90^0C and 2.0%w/w moisture)	Nbiotic™ Premix After exposure (10 minute at 90^0C and 2.0%w/w moisture)
1	Description	Fine powder	Fine powder	Fine powder
2	Colour	Greenish colour	Greenish colour	Greenish colour
3	Odour	Aromatic odour	Aromatic odour	Aromatic odour
4	Moisture content	5.0% w/w	4.95%w/w	4.92%w/w
5	Total ash content	8.07%w/w	8.08%w/w	8.04%w/w
6	pH (5% aq.suspension)	5.72	5.70	5.74
7.	Bulk Density (After 50 Taps)	0.643 gm/cc	0.627gm/cc	0.639gm/cc
8.	Water soluble extractive value	17.59%w/w	17.75%w/w	17.70%w/w
9.	P. Ether soluble extractive value	7.12%w/w	7.10%w/w	7.05%w/w
10.	Methanol soluble extractive value	14.54%w/w	14.60%w/w	14.75%w/w
11.	Volatile oil	5.90%v/w	5.85%v/w	5.80%v/w

Table 2:- Results of precision, LOD, LOQ, linear regression analysis, and their correlation coefficient for quantitative analysis of 6-gingerol by HPLC.

Sr. No.	Parameters	6-Gingerol
1	Concentration range (µg/ml)	6 – 48
2	Regression equation	Y = 6462.x + 3050
3	Correlation coefficient (r2)	0.997
4	Amount of marker compound in Nbiotic™ Premix (%) (w/w) (mean, n=3)	0.025 % w/w
5	Method precision (repeatability) n – RSD %	1.7
6	Intermediate precision (reproducibility) – RSD (%) Intraday Interday	 1.21 1.52
7	Recovery (%)	96.57 % w/w
8	LOD LOQ	0.04 µg/ml 0.12 µg/ml

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

Table 3:- 6-gingerol contents by HPLC in Nbiotic™ Premix.

Sample details	6-Gingerol content (ppm)	% Difference of 6-gingerol content with respect to control sample
Nbiotic™ Premix - control	250.0	0.00
Nbiotic™ Premix after exposure to heat For 05 minutes at 90ºC and 2.0% w/w moisture.	246.0	- 1.60
Nbiotic™ Premix after exposure to heat For 10minutes at 90ºC and 2.0% w/w moisture	245.50	-1.80

Table 5:- HPTLC analysis data (366 nm fluorescence) of Petroleum ether (60-80°c) fractions of Nbiotic™ Premix samples.

Sample details	Total area of peaks (HPTLC -366 nm fluorescence)	% Difference in area with respect to control sample
Nbiotic™ Premix - control	14500.50	0.00
Nbiotic™ Premix after exposure to heat for 05 minutes at 90ºC and 2.0% w/w moisture.	14198.90	- 2.07
Nbiotic™ Premix after exposure to heat for 10minutes at 90ºC and 2.0% w/w moisture.	14045.10	-3.14

Table 4:- HPTLC analysis data (260 nm) of Petroleum ether (60-80°c) fractions of Nbiotic™ Premix samples.

Sample details	Total area of peaks (HPTLC - Deuterium 260nm)	% Difference in area with respect to control sample
Nbiotic™ Premix - control	25488.40	0.00
Nbiotic™ Premix after exposure to heat for 05 minutes at 90ºC and 2.0% w/w moisture.	26054.80	+2.22
Nbiotic™ Premix after exposure to heat for 10minutes at 90ºC and 2.0% w/w moisture.	23165.40	-9.11

Table 6:- HPTLC analysis data of methanol fractions of Nbiotic™ Premix samples.

Sample details	Total area of peaks (HPTLC - Deuterium 280nm)	% Difference in area with respect to control sample
Nbiotic™ Premix - control	32621.0	0.00
Nbiotic™ Premix after exposure to heat For 05	31353.50	-3.88

minutes at 90ºC and 2.0% w/w moisture.		
Nbiotic™ Premix after exposure to heat For 10minutes at 90ºC and 2.0% w/w moisture.	31221.50	- 4.29

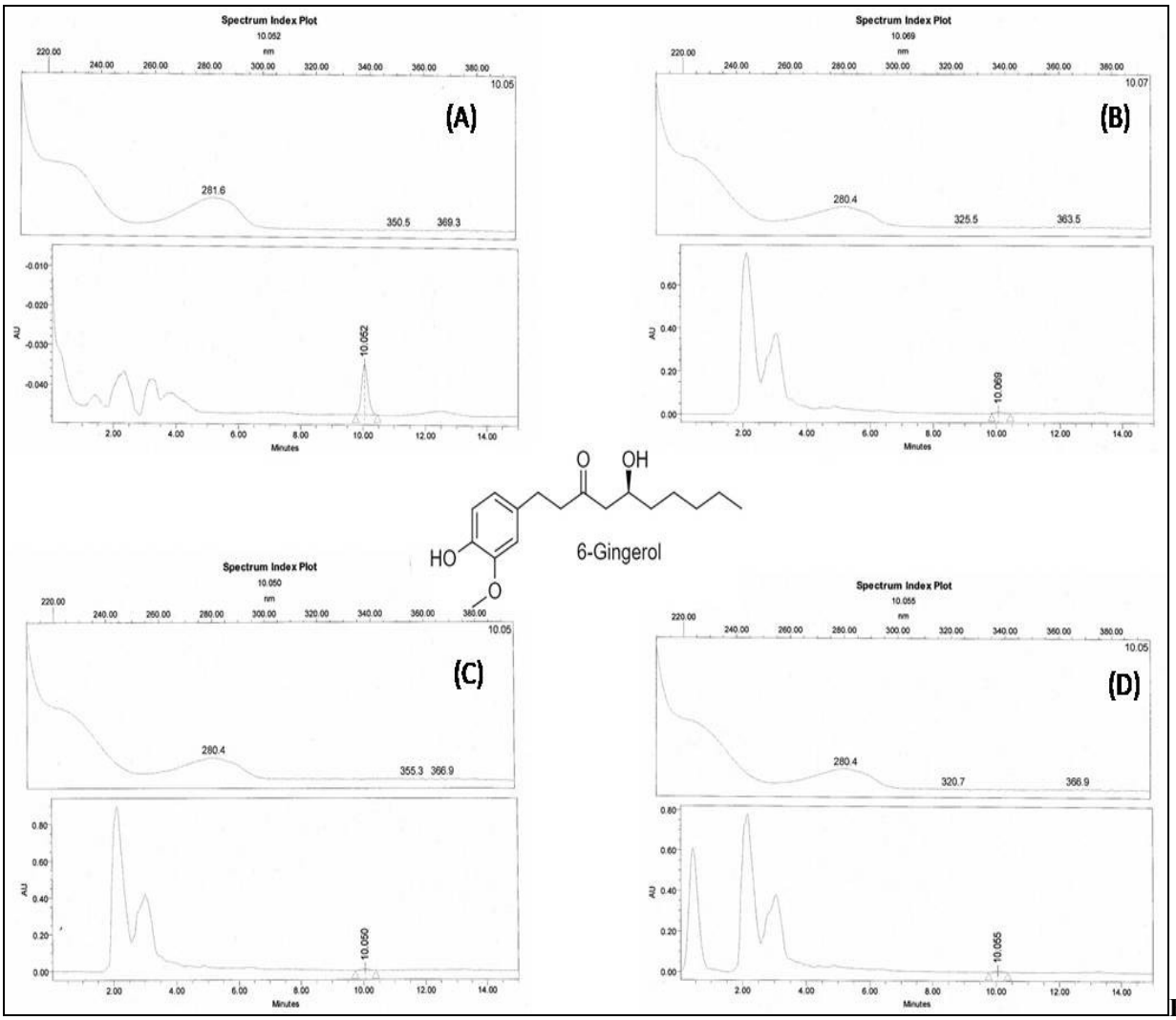

Figure 1:- HPLC-chromatograms (280 nm) and UV spectrum of 6-gingerol in control and treated samples of Nbiotic™ Premix. (A) Standard chromatogram of 6-gingerol (R_t 10.05). (B) Control sample of Nbiotic™ Premix (zero minute, 90ºC temperature, 2.0% w/w moisture content). (C) Treated sample of Nbiotic™ Premix (5.0 minute, 90ºC temperature, 2.0% w/w moisture content). (D) Treated sample of Nbiotic™ Premix (10.0 minute, 90ºC temperature, 2.0% w/w moisture content).

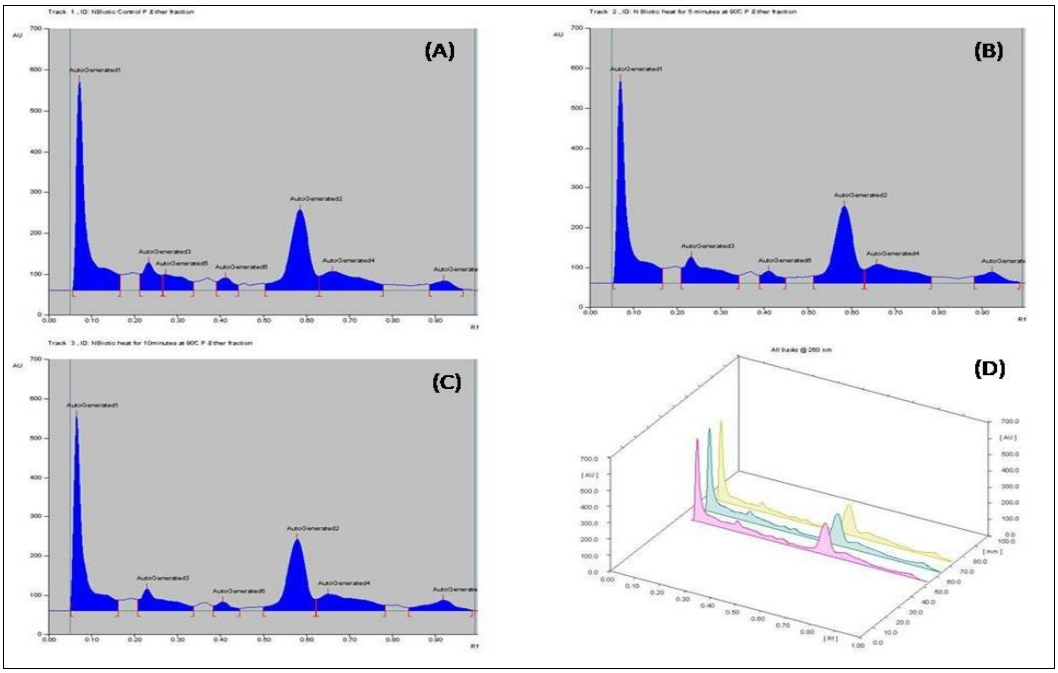

Figure 2:- HPTLC analysis data (260 nm) of petroleum ether (60-80°c) fractions of Nbiotic™ Premix samples. (A) Chromatograms of control Nbiotic™ Premix sample fraction (zero minute, 90ºC temperature, 2.0% w/w moisture content). (B) Chromatograms of treated Nbiotic™ Premix sample fraction (5.0 minute, 90ºC temperature, 2.0% w/w moisture content). (C) Chromatograms of treated Nbiotic™ Premix sample fraction (10.0 minute, 90ºC temperature, 2.0% w/w moisture content). (D) Three dimensional overlay chromatogram of (A), (B) and (C).

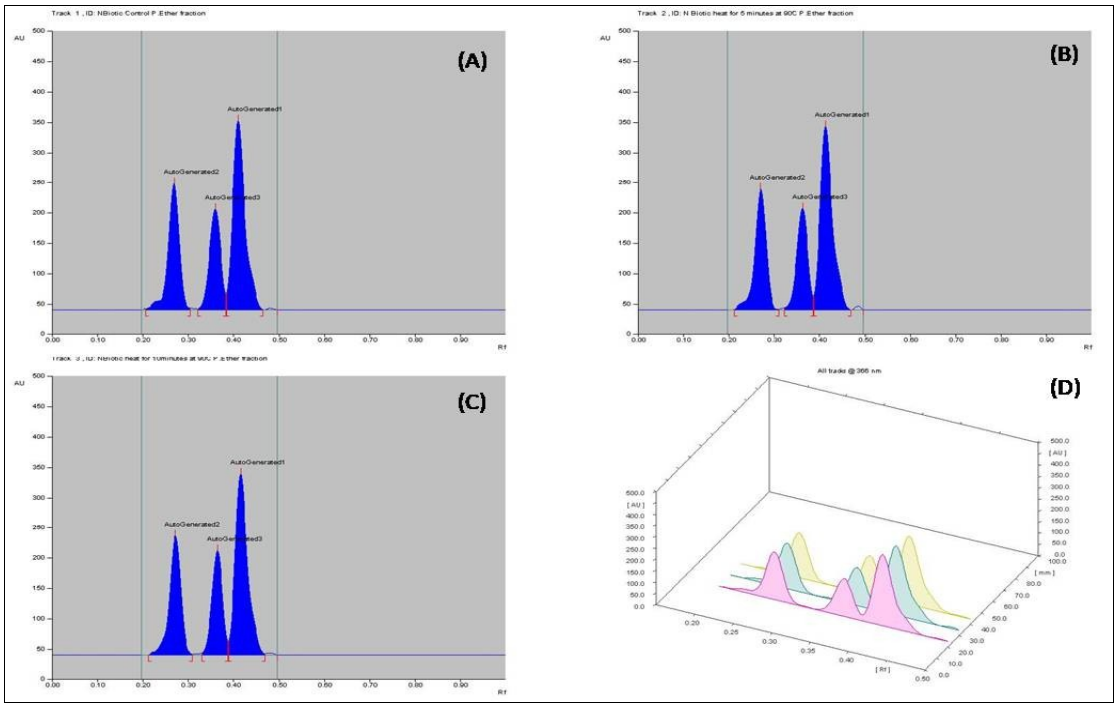

Figure 3:- HPTLC analysis data (366 nm-Fluorescence) of petroleum ether (60-80°c) fractions of Nbiotic™ Premix samples. (A) Chromatograms of control Nbiotic™ Premix sample fraction (zero minute, 90ºC temperature, 2.0% w/w moisture content). (B) Chromatograms of treated Nbiotic™ Premix sample fraction (5.0 min, 90ºC temperature, 2.0% w/w moisture content). (C) Chromatograms of treated Nbiotic™ Premix sample fraction (10.0 min, 90ºC temperature, 2.0% w/w moisture content). (D) Three dimensional overlay chromatogram of (A), (B) and (C).

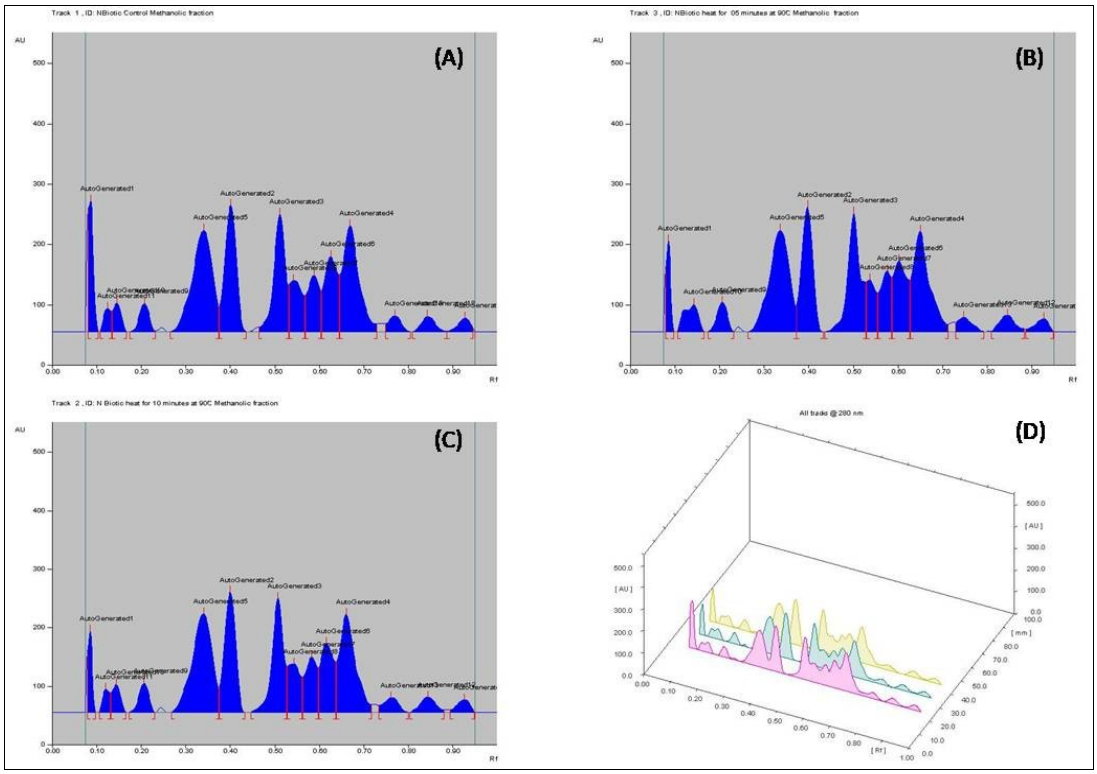

Figure 4:- HPTLC analysis data (280 nm) of methanolic fractions of Nbiotic™ Premix samples. (A) Chromatograms of control Nbiotic™ Premix sample fraction (zero minute, 90ºC temperature, 2.0% w/w moisture content). (B) Chromatograms of treated Nbiotic™ Premix sample fraction (5.0 minute, 90ºC temperature, 2.0% w/w moisture content). (C) Chromatograms of treated Nbiotic™ Premix sample fraction (10.0 minute, 90ºC temperature, 2.0% w/w moisture content). (D) Three dimensional overlay chromatogram of (A), (B) and (C).

Conclusions:-

In conclusion, Nbiotic™ Premix is stable to pelletization stress conditions like high temperature and elevated moisture and can be used as stable feed supplement through pelletization process without degradation. This study is a major contribution towards developing appropriate stability monitoring parameters for herbal products. We hereby provide evidence that HPLC and HPTLC along with physicochemical properties are powerful analytical tools that can be used to predict or assess the stability of herbal preparations in order to generate stability data for submission to the relevant authorities. In addition, they assist in predicting shelf life or storage conditions. This is very important in the case of herbal preparations due to their complex nature.

Conflict Of Interest:

Author has no conflict of interest.

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