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A New Spectroscopic Determination and Stability Indicating Assay for the Estimation of Trimyristin in API and Polyherbal Ayurvedic Formulation

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Abstract: The herbal plant *Myristica fragrans* also called as nutmeg, has many uses in Ayurvedic system of medicine. The principle constituent of the fatty oil of *Myristica fragrans* is a triglyceride named Trimyristin and the principle constituent of essential oil is myristicin.The essential oil part of the nutmeg mostly myristicin was believed to be responsible for the various pharmacological actions of the herb. But to the fact, the triglyceride Trimyristin was reported to have anxiogenic activity which is the principle use of the herbal drug in various polyherbal formulations. So considering the need for the method development a simple and sensitive spectrophotometric method was developed for the determination of Trimyristin in API and in poly herbal Ayurvedic formulations and the method was validated as per the ICH guidelines. Trimyristin was extracted from the seeds of *Myristica fragrans* by reflux method with hexane as a solvent. The UV wavelength was detected at 222 nm by using the co-solvency method. The stability indicating stress degradation studies was also performed as per the ICH guidelines by using a UV spectroscopic method. The method was simple, specific, precise, and accurate. The system obeyes the Beer's law in the concentration range of 10-60 µg/ml. The percentage purity was found to be 99.0% w/v. The percentage recovery studies performed showed a percentage recovery of 98 -101.7%w/v and found to be linear with a correlation coefficient (r^2) of 0.99, method was found to be precise with relative standard deviation of less than 2%, detection limit and quantification limit were estimated to be 0.3µg/ml, 1µg/ml respectively. The stability indicating stress degradation studies showed degradation on subjecting to acidic hydrolysis, basic hydrolysis, oxidative conditions, dry heat and photolytic degradation conditions. This method can be successfully employed for quantitative analysis of Trimyristin in API and in polyherbal formulations.

Key Words: Active pharmaceutical Ingredient, Trimyristin, Stress Degradation, Makaradwaj Vatti, Stability indicating method.

Introduction

The Ayurvedic system of medicine mainly uses herbs, herbal products, in the form of dried powders, extracts, kashayas, asavas, aristas etc. These formulations usually contain more than one type of herb or herbal products. The nutmeg tree is one of several species of trees in genus *Myristica* which is used in herbal medicinal system mainly in Ayurveda[1]. The nutmeg tree biologically termed as *Myristica fragrans* is important for

two spices derived from the fruit they are the seed and mace. This is the only tropical fruit that is the source of two different spices[2]. Several other commercial products are also produced from the trees, including essential oils, extracted oleoresins, and nutmeg butter.

The other major components are fats, 25-40%, and essential oils, 8-15%[3] , the former constitute the major portion of the expressed oil of nutmeg. The expressed oil, also known as nutmeg butter, is composed principally of a triglyceride Trimyristin. The essential oil part of the nutmeg consists of Myristicin, safrole, emelicin, α-pinene and β –pinene[4]. Trimyristin is an example of fundamental type of fat known as a triglyceride. Hydrolysis of one mole of a triglyceride afford one mole of 1, 2, 3-propanetriol (glycerin) and 3 moles of fatty acids, which are carboxylic acids containing the functional group at the end of a long alkyl chain. Chemically Trimyristin is called as 1,3 Di(tetradecanolyloxy) propan-2-yl tetradecanoate[5] as shown in figure 1. The main activity of Trimyristin was reported to be an antidepressant and anxiogenic[6]. Apart from that it has other activities like anti-inflammatory and anti-bacterial.

The literature review reveals no analytical method for the estimation of Trimyristin in polyherbal dosage forms but with few analytical techniques like RP-HPLC[7] method for the estimation of Trimyristin in cooking oil and nutmeg seed extract[8], HPTLC nutmeg seed extract apart from the standardization of unani drugs[9-13] . In view of the need for a suitable method for routine analysis in combined formulations, attempts are made to develop simple, precise and accurate spectroscopic analytical method for estimation of Trimyristin and extend it to their determination in herbal formulation.The herbal formulation includes a compressed tablet Poweromin and a mould Makardhwaj Vatti. So the present work was being validated for various parameters according to the ICH guidelines. The stability of the drug in various environments was also studied by the stress degradation studies as per the ICH guidelines[14,15].

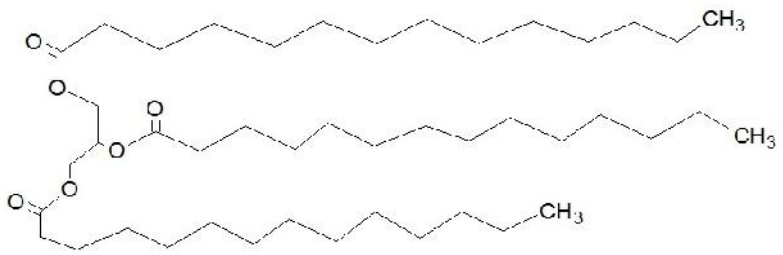

Figure 1: Chemical structures of Trimyristin

Materials and Methods

Apparatus

ELICO-SL 244 UV/ VIS Double beam spectrophotometer, (spectratreats) with PMT detector, was used for the estimation of Trimyristin in the API, extract and in poly herbal Ayurvedic formulations. In addition, an electronic balance (Shimadzu AX200), a ultra sonicator (Spincotech, model2200-MH SOLTECH), a hot air oven (Labhosp), UV chamber (Labhosp) were used in this study.

Chemicals

Trimyristin was obtained from Himedia (RM 1301-1G) (India). Hydrogen peroxide, Sodium Hydroxide was purchased from SDFCL SD Fine Chem Limited and Mio Chem. Pvt. Ltd respectively. Hydrochloric acid was obtained from Merck Chemicals. The polyherbal tablets Poweromin (Apex Laboratories Pvt Ltd) and Makaradhwaj vatti (Godavari Ayurvedic Pharmacy) were obtained from the local ayurvedic store. All other chemicals and reagents were of analytical reagent grade.

Extraction Of Trimyristin

Crushed whole nutmeg seeds of 5gms was weighed and transferred to a 50mL round-bottomed flask. 50ml of hexane was added to the flask which was attached to a water-cooled condenser, the mixture was heated under a gentle reflux for approximately 4 hours at 60^{o}C. The mixture was cooled to room temperature. The hexane layer

was separated out with pipette without disturbing the settled solids. The hexane layer was evaporated by using rotavapour apparatus which on evaporation yields a white crystalline powder. The crude extract was recrystallised by using 95% ethanol to yield pure Trimyristin. The extracted Trimyristin was characterized by IR as shown in figure 2.

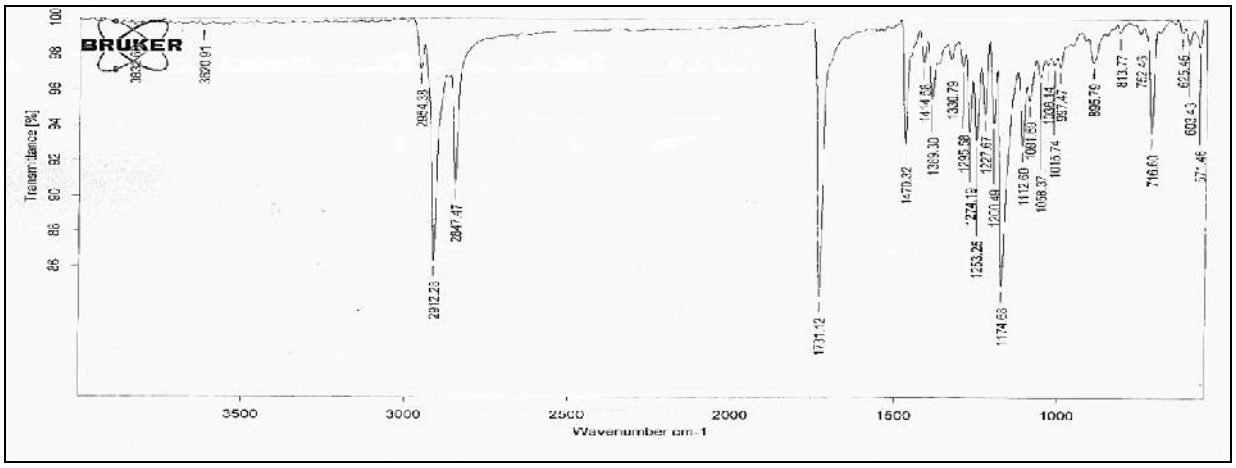

Figure 2: IR Spectrum of extract showing the characteristic peaks.

Preparation of standard and extract stock solution

An accurately weighed quantity of 10mg API and extract was transferred into two separate 10ml volumetric flask and dissolved in dichloromethane to 10ml to give a concentration of 1000µg/ml. The final concentration was brought to 10µg/ml by diluting the stock solution with isopropyl alcohol. This solution was used for further studies. The resulting solution was scanned from 200-800nm against the corresponding blank and the λ_{max} was estimated at 222nm wavelength where the compound showed maximum absorbance.

Assay of marketed Polyherbal Ayurvedic Poweromin tablet

Twenty tablets were weighed and ground to a fine powder using a mortar and a pestle. An accurately weighed portion of the powder, equivalent to 5mg of drug was transferred into a 250ml round bottomed flask and 30ml of petroleum ether was added to it, the above solution was refluxed for 30min at 70℃ . The petroleum ether layer was separated, evaporated completely and diluted to 50ml with dichloromethane. From the above stock solution the dilutions were prepared using isopropyl alcohol such that the final concentration of the solution was 10µg/ml. The resulting solution was scanned at 222nm using UV-Visible double beam spectrophotometer.

Assay of polyherbal Ayurvedic Makaradhwaj Vatti tablet

Twenty tablets were weighed and ground to a fine powder using a mortar and pestle. An accurately weighed portion of the powder, equivalent to 5mg of drug was transferred into 250ml round bottomed flask and added with 10 ml of water, 30ml of dichloromethane respectively. The above solution was sonicated for 20min at 60℃ and centrifuged for 5 minutes. The dichloromethane layer was separated and diluted to 50ml with the same solvent. This solution was further diluted to 10µg/ml with isopropyl alcohol. The resulting solution was scanned at 222nm using UV-Visible double beam spectrophotometer.

Method Validation

The method was validated for linearity, accuracy, precision, LOD, LOQ as per the ICH guidelines[14,15].

Linearity

Five point linearity curves were constructed for both API and extract using the concetrations of 10µg/ml-50µg/ml.

Precision

Precision was assessed by calculating inter and intra-day variability of API and extract samples at low, medium and high concentrations. Inter-day data was obtained by analyzing the samples on two consecutive days of assay, while the intra-day precision data was obtained by analyzing three sets of quality control samples in a single day. Assay precision was calculated using the formula %RSD = (SD/*M*) × 100 where *M* is the mean of the experimentally determined concentrations and SD is the standard deviation of *M*.

Accuracy

The accuracy of the method was established by using analytes at low, medium and high concentrations of 8µg/ml, 10µg/ml, and 12µg/ml for Trimyristin API, extract and polyherbal formulation. The percentage assay was calculated for 2 consecutive days by preparing fresh solutions each day.

Recovery studies

Percentage recovery studies were performed for 80%, 100% and 120% concentrations for both the polyherbal formulations respectively (8 µg/ml, 10 µg/ml and 12 µg/ml). The percentage recovery of Trimyristin from the spiked samples of herbal formulation was calculated.

LOD and LOQ

Standard stock solutions were diluted appropriately to obtain concentrations for the estimation of the limit of detection (LOD) and limit of quantitation (LOQ) according to a signal to noise (S/N) ratio of 3:1 and 10:1, respectively.

Stress Degradation Studies

The stress degradation studies such as hydrolytic (in acidic & alkali medium), photolytic, oxidative and dry heat induced degradations studies were performed for API as per ICH guidelines.

Hydrolytic degradation under acidic conditions

Hydrolytic degradation studies under acidic conditions were performed by using 2ml (100µg/ml) of stock solution of Trimyristin API, to this 1ml of 1N methanolic HCl was added and volume was made to 10ml with isopropyl alcohol, this solution was kept at room temperature for 90min. 5ml of the above solution was pipetted out into 10ml flask and the volume was adjusted with isopropyl alcohol. Using isopropyl alcohol as a blank, the resulting solution was scanned from 200-400nm.

Hydrolytic degradation under alkaline conditions

Hydrolytic degradation studies under alkaline conditions were performed by taking 2ml (100µg/ml) of stock solution of Trimyristin API, to this 1ml of 1N methanolic NaOH was added and the volume was made up to 10ml with isopropyl alcohol, this solution was kept at room temperature for 90min, from this 5ml of solution was pipetted out into 10ml flask and the volume was adjusted with isopropyl alcohol. Using isopropyl alcohol as a blank, the resulting solution was scanned from 200-400nm.

Dry heat induced degradation

Dry heat induced degradation studies were performed by subjecting 10 mg of Trimyristin API in a standard volumetric flask to a temperature of 80^{o}C for 48 hrs in a hot air oven. After 48 hrs the drug was taken out and diluted with the dichloromethane such that the final concentration was 5µg/ml. Using isopropyl alcohol as a blank, the resulting solution was scanned from 200-400nm.

Oxidative degradation

Oxidative degradation studies were performed by taking 1.5 ml (100µg/ml) of stock solution of Trimyristin API in a 10 ml volumetric flask, to this 1ml of 3% hydrogen peroxide stored over night was added and the volume is made up to 10ml with isopropyl alcohol. Solutions were kept at room temperature for 15 min and then diluted to a concentration of 5 µg/ml. Keeping mixture of 1 ml hydrogen peroxide and isopropyl alcohol as a blank, the resulting solution was scanned from 200-400nm.

Photolytic degradation

Photolytic degradation study was performed by exposing the Trimyristin of API to near UV light for 30 minutes in an UV chamber. After the UV exposure a 10 mg of substance was taken and diluted to get a concentration of 5µg/ml using isopropyl alcohol. Keeping isopropyl alcohol as a blank, the resulting solution was scanned from 200-400nm.

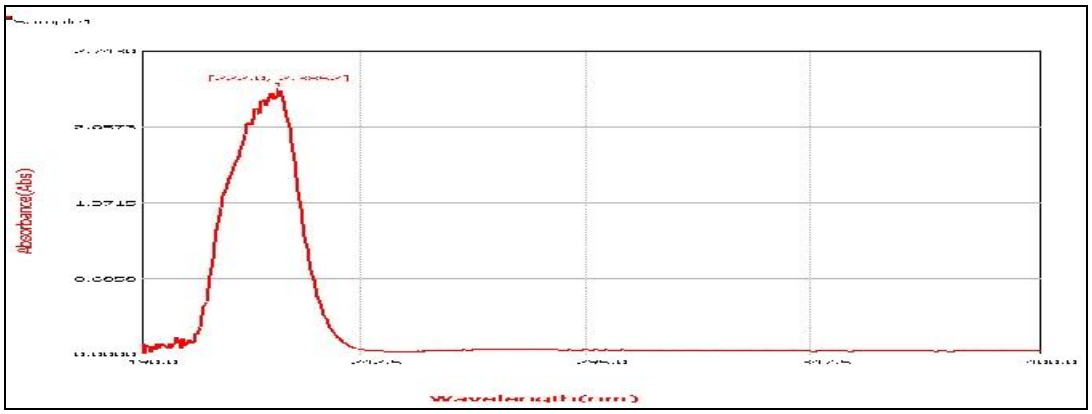

Figure 3: Spectrum of Trimyristin API showing the λ_{max}

Results And Discussion

Assay of Polyherbal formulations

From the spectrum the λmax of Trimyristin API in the given solvent was found to be 222nm as shown in figure 3. Both the herbal formulations Poweromin and Makardhwaj vatti after extraction showed the similar wavelength maxima at the same wavelength. The percentage purity of the assay of both the formulations was found to be 99.23% w/v and 98.65%w/v for Poweromin, Makardhwaj vatti respectively.

Method Validation

The method was validated for linearity, accuracy, precision, LOD, LOQ as per the ICH guidelines. The linearity was observed to obey the Beer's law in concentrations ranging from 10-50µg/ml. The linear plot plotted with concentration against absorbance with correlation coefficient (r^2) of 0.993 and 0.9993 for both the API and extract are shown in figure 4a, 4b. The characteristic parameters for regression equation and correlation are given in table 1. The Precision studies were performed for six repeated absorbance of a homogenous solution of concentrations 10, 20, 40µg/ml and the percentage relative standard deviation was calculated for both intraday and interday variation found to be less than 2% which are summarized in table 2. The accuracy of the method was established by using analytes at low, medium and high concentrations of 8µg/ml, 10µg/ml, and 12µg/ml for Trimyristin of API, extract and poly herbal formulation. The percentage assay calculated for 2 consecutive days by preparing fresh solutions each day was found to be in between 98 – 102 % and the results are tabulated in table 3. Percentage recovery studies performed for 80%, 100% and 120% concentrations for both the formulations respectively The percentage recovery of the drug of the spiked sample was found to be in between 98 -101.7%w/v as shown in table 4. Detection limit and quantification limit were found to be 3.035µg/ml and 9.56µg/ml by using the respective S/N ratios of 3 and 10.

Table 1 Linear Ranges and Correlation Coefficients of Calibration Curves

Parameters	**API**	**Extract**
λmax(nm)	222 nm	222 nm
Beer's law limits (mg/ml)	10 -50 µg/ml	10 -50 µg/ml
Correlation Coefficient (r^2)	0.997	0.9993
Regression Equation (Y)*	y = 0.0549x + 0.472	y = 0.0489x + 0.845
Slope (b)	0.0549	0.0489
Intercept(a)	0.472	0.845
LOD	3.035µg/ml	3.035µg/ml
LOQ	9.56µg/ml	9.56µg/ml

Table 2 Precision Studies of Trimyristin

Analyte	**Intraday**		**Interday**	
	Mean±SD	**%RSD**	**Mean±SD**	**%RSD**
API 10µg/ml	9.8± 0.005	0.72	9.02±0.002	0.36
20µg/ml	20.05±0.002	0.19	19.89±0.008	0.72
40µg/ml	41.06 ±0.002	0.13	41.14±0.02	1.16
Extract 10µg/ml	10.07±0.001	0.16	9.42±0.005	0.85
20µg/ml	21.42±0.003	0.25	19.39±0.009	0.72
40µg/ml	40.03±0.0006	0.03	36.39±0.003	1.95

Table 3 Accuracy Studies of Trimyristin

		Day 1			**Day 2**		
Analyte	**Concentr-ation (µg/ml)**	**Amount present (µg/ml)**	**Percentage**	**%RSD n=3**	**Amount present (µg/ml)**	**Percentage**	**%RSD n=3**
API	8µg/ml	7.96	99.5	1.44	7.79	97.29	1.87
	10µg/ml	10.1	101	1.98	9.82	98.3	0.46
	12µg/ml	11.96	99.6	0.94	11.72	97.7	0.85
Extract	8µg/ml	8.10	101.3	1.48	8.07	100.9	0.63
	10µg/ml	9.96	99.6	2.08	10.1	101	1.98
	12µg/ml	11.86	98.8	1.18	11.97	99.8	0.91
Poweromin	8µg/ml	7.94	99.3	0.92	7.64	95.54	1.24
	10µg/ml	10.13	101.3	2.05	9.70	97.06	1.67
	12µg/ml	11.97	99.8	0.91	11.71	97.58	1.93
Makardw-aj Vatti	8µg/ml	8.07	100.9	0.63	7.75	96.9	1.68
	10µg/ml	9.96	97.0	1.67	9.86	98.6	1.17
	12µg/ml	12.10	100.8	1.44	11.73	97.80	1.41

Table 4 Recovery Studies of Trimyristin

Tablet	Concentration		Amount added (µg/ml)	Amount recovered (µg/ml)	Percentage	%RSD n=3
Poweromin	Low	8µg/ml	10 µg/ml	17.93	99.66%	0.98
	Medium	10µg/ml	10 µg/ml	19.95	99.79%	1.02
	High	12µg/ml	10 µg/ml	22.43	101.97%	1.38
Makardhwaj Vatti	Low	8µg/ml	10 µg/ml	17.97	99.83%	1.70
	Medium	10µg/ml	10 µg/ml	19.75	98.75%	1.29
	High	12µg/ml	10 µg/ml	22.06	100.2%	1.60

Stress Degradation Studies

The stress degradation studies were performed for the standard Trimyristin (API) as per the ICH guidelines by subjecting the drug for different conditions that stimulate the drug degradation.The spectrum of the drug was analysed from 200-450 nm for the degradation changes figure 3-6. The drug showed degradation on subjecting to acidic hydrolysis, basic hydrolysis, oxidative conditions, dry heat and photolytic degradation conditions. The shift of wavelength indicated the degradation changes. The observations are shown in table 5.

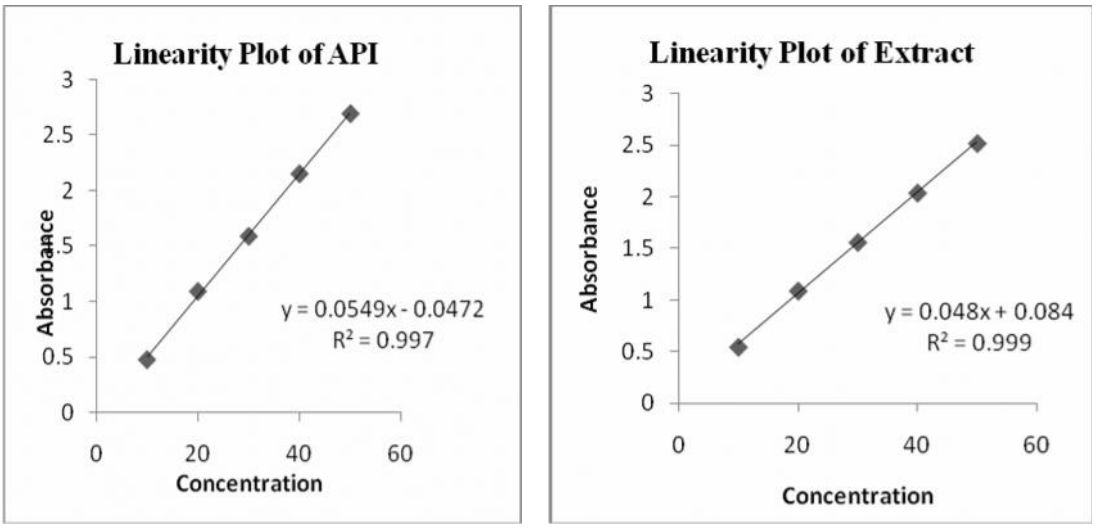

Figure 4a, 4b: Linearity plot of Trimyristin API and extract

Figure 5: Spectrum of Trimyristin under acidic conditions showing the shift of wavelength

indicating the degradation of the drug

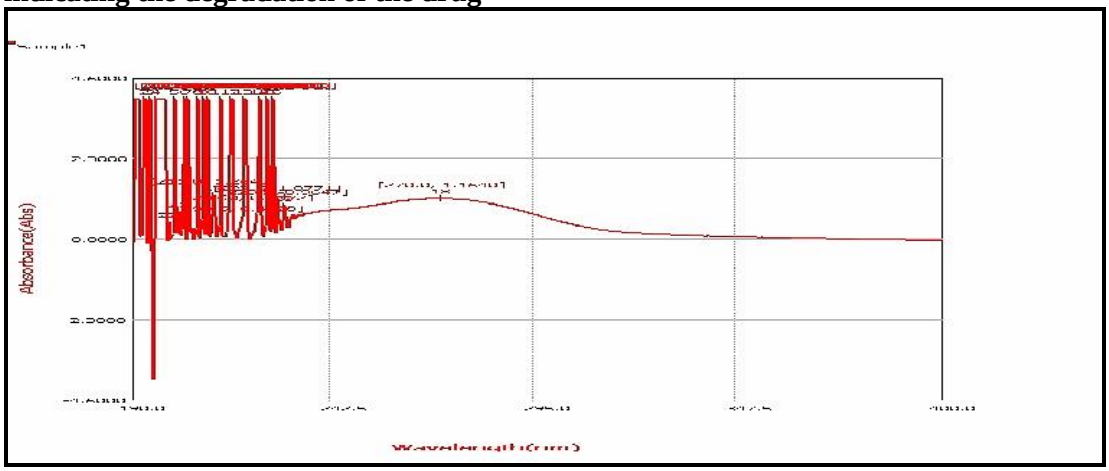

Figure 6: Spectrum of Trimyristin under alkali conditions showing the shift of wavelength indicating the degradation of the drug

Table 5 Showing the Stress Degradation Studies of Trimyristin Api

S.No.	Stress Degradation Studies	Time Period	Standard Observation
1.	Hydrolytic Degradation Under Acidic Conditions	90 min.	Shift of λmax towards higher wavelength indicating degradation
2.	Hydrolytic Degradation Under Alkali Conditions	90 min.	Shift of λmax towards higher wavelength indicating degradation
3.	Dry Heat Induced Degradation	48 hrs	Shift of λmax towards higher wavelength indicating degradation
4.	Oxidative Degradation	15 min.	Shift of λmax towards lower wavelength indicating degradation
5.	Photolytic Degradation	30 min	Shift of λmax towards lower wavelength indicating degradation

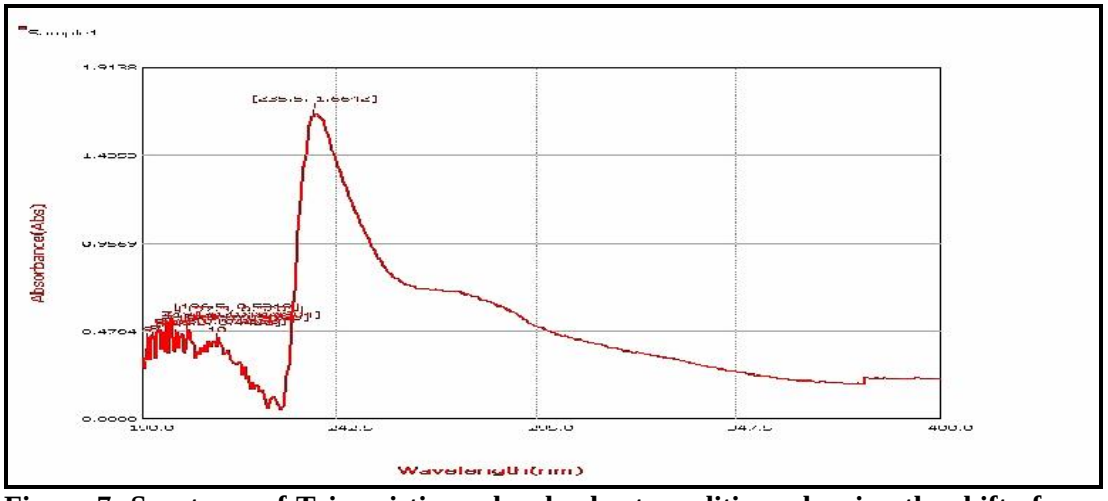

Figure 7: Spectrum of Trimyristin under dry heat conditions showing the shift of wavelength indicating the degradation of the drug

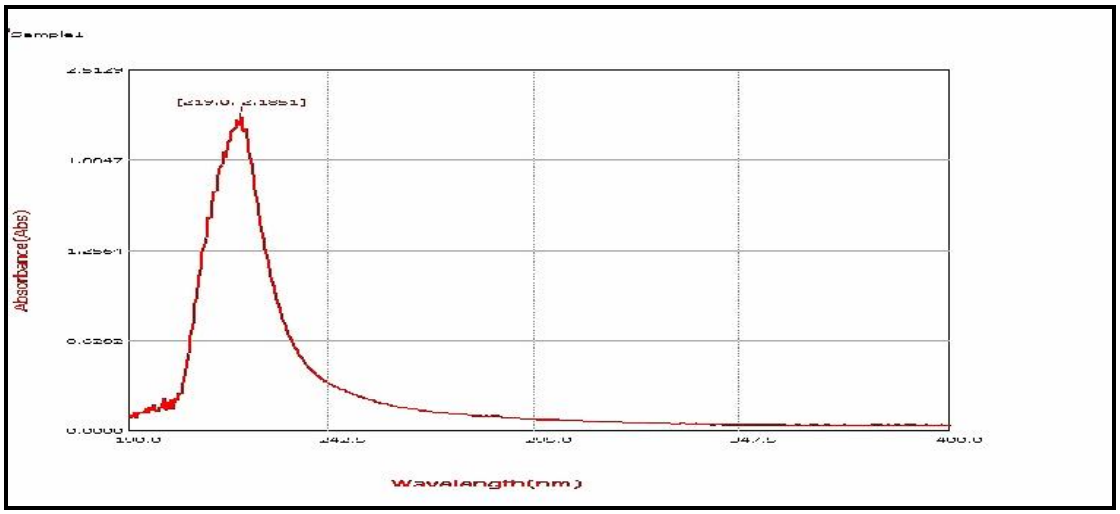

Figure 8: Spectrum of Trimyristin under oxidative conditions showing the shift of wavelength indicating the degradation of the drug

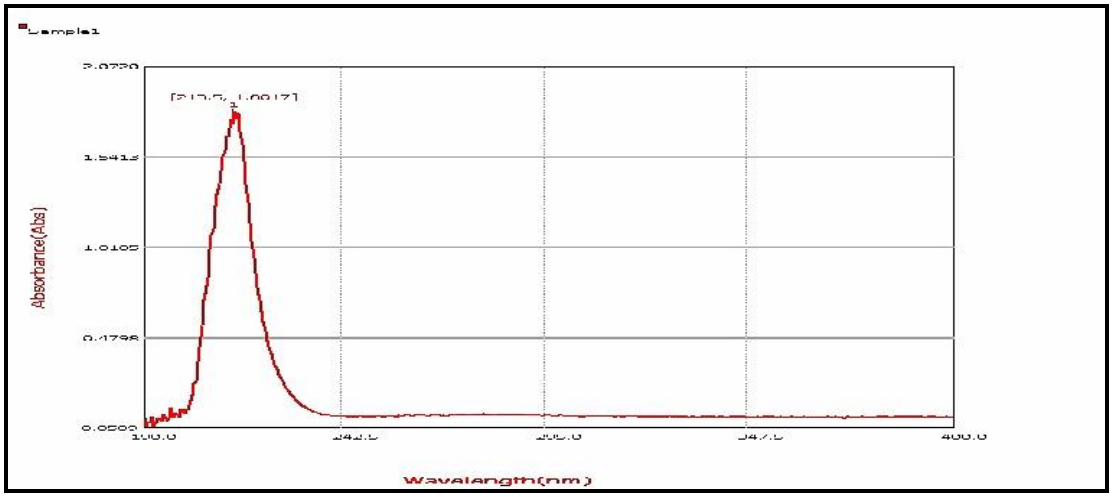

Figure 9: Spectrum of Trimyristin under photolytic exposure conditions showing the shift of wavelength indicating the degradation of the drug

Conclusion

Previously published methods were only confined to the estimation of various components of the herbal plant *Myristica fragna*ns. The present method was used for the estimation of Trimyristin by extracting from the poly herbal formulation. Recent investigations have confirmed that Trimyristin have vulnerable and wide-ranging pharmacological properties such as anxiogenic, anti-inflammatory properties. So the present cost-effective method can be used for the market analysis of Trimyristin in polyherbal formulations and this particular stability indicated spectroscopic method was found to be simple, accurate and precise. Hence the method can be recommended for routine analysis of Poweromin tablet and Makardhwaj vatti polyherbal formulation containing the Trimyristin. The stability indicating stress degradation studies crams various physical stability properties of the drug in the formulation which helps for the better formulation development.

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