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APPLICATION OF PHYTO PIGMENTS IN NOVEL ANALYTICAL METHOD DEVELOPMENT FOR SOME AMIDE GROUP CONTAINING DRUGS

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ABSTRACT

Introduction: The polyphenolic structural features present in synthetic dyes like methyl orange, bromocresol green, thymol blue plays a role in their use as acid base indicators as well as colour complex forming agents in development of spectrophotometric methods. Nature is full of natural colour pigments. Phytochemical investigation of extract of *Delonix regia* flowers shows presence of anthocyanins which have similar polyphenolic nature. Hence as novelty, in present work a successful attempt has been done to develop simple and accurate spectrophotometric method for estimation of glipizide and repaglinide. **Material and Methods:** The estimation of glipizide and repaglinide is done on the basis of formation of colour complex between amide group and polyphenolic structural feature present in extracted pigment of *Delonix regia* flowers. Stock solution of 100µg/ml was prepared and serial dilutions were done to get concentration range of 4 to 20 µg/ml for glipizide and 5 to 40 µg/ml for repaglinide. **Result:** The absorbance of the coloured complex was at λmax of 610 nm for glipizide and at 605 nm for repaglinide against reagent blank. Validation of method was done as per ICH (Q_2) guidelines. Beer's law range was found to be 4 to 20 µg/ml for glipizide and 5 to 40 µg/ml for repaglinide with correlation coefficient 0.9994 and 0.9996 respectively. The LOD and LOQ was found to be 0.2681 and 0.7962 respectively. The existence of Keto-enol tautomerism shown by polyphenolic compounds and presence of loan pair of electron on nitrogen of amide group were found to contribute in formation of colour-complex between amide and the colour pigment which was confirmed by IR. **Conclusion:** It can be concluded that, use of natural colour pigments can be done in development of a simple, accurate,

sensitive and reliable spectrophotometric and can be used in routine quantitative analysis of different API in bulk as well as pharmaceutical formulation.

KEYWORDS: Glipizide; Repaglinide; spectrophotometry; *Delonix regia*; validation.

1. INTRODUCTION

Delonix regia (Fabaceae), an umbrella-shaped tree reaching up to 40 feet height is considered as one of the most attractive tropical trees in the world. It produces outstanding flame like scarlet and yellow flowers in spring before the leaves emerge.[1-4] In Nepal, Pakistan and India it is commonly called as gulmohor. It contains anthrocynines, β-sitoserol, tannins, saponins, flavonoids, steroids, alkaloids, carotene and so on.[2-7]

Repaglinide is a white solid and highly polar compound, chemically 2-ethoxy-4-({[(1S)-3-methyl-1-[2-(piperidin1yl) phenyl] butyl] carbamoyl} methyl) benzoic acid. It was officially in USP which describes liquid chromatography method for quantitation.[8] Glipizide (GZ) is chemically1cyclohexyl3[[p[2(5methylpyrazinecarboxamido)ethyl]phenyl]sulfonyl]urea, used in the treatment of type II diabetes mellitus.[9]

Literature survey revealed that substantial work has been done for quantitative estimation of Repaglinide and Glipizide by HPLC method, spectrophotometric method and HPTLC in pharmaceutical dosage form.[10-15] In present work a novel approach of using a natural color pigment in quantitative estimation API and its formulation has been done. Usually the pigments obtained from plants are only used as acid base indicators. In present work a successful attempt has been done to develop a simple, precise and reproducible colourimetric method for the quantitative estimation of repaglinide and glipizide in bulk and tablet dosage forms by forming a colour complex with pigment extracted from Delonix regia flowers. [16]

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Plant Material

Delonix regia flowers were collected from local area around college campus and Shivaji University, Kolhapur during the blooming season in the month of April to July. Further identification and authentication of plant was done with the help of herbarium sheet (Specimen No.–SUK2604) from Botany Department, Shivaji University, Kolhapur. Further

the herbarium sheet was submitted to the department of Pharmacognosy, Sant Gaganan Maharaj College of Pharmacy, Mahagaon, Gadhinglaj Kolhapur.

2.1.2 Chemical

Glipizide and Repaglinide were procured from Franco-Indian Pharmaceuticals Pvt. Ltd. Mumbai, Sodium hydroxide, hydrochloric acid, chlorbutol, methanol and other chemical used were of analytical grade and were procured from Loba Chemie Pvt. Ltd, Mumbai.

2.2 Method

The fresh flowers were immediately dried in a well-ventilated oven maintained at 40°C overnight. The dried material was packed in plastic bags, sealed under vacuum and preserved in laboratory.

2.2.1 Extraction

The extract was obtained by soaking dried flowers (0.5 kg) overnight in deionized water and methanol (500 mL) separately. The mixture was acidified with 1ml of citric acid (0.05N) and kept at room temperature for 24 hr. The extracts were filtered and subjected to drying process by evaporation on thermostatic water bath.[17-19]

2.2.2 Spectral analysis of extract

Original aqueous and methanolic extracts were having dark pink colour were used for spectral characterization.

2.2.3 Chemical tests for anthocyanins

The extracts obtained were screened for anthocyanins by performing different chemical tests.

2.2.4 Colour change over different pH range

Delonix regia extracts were evaluated at different pH value using buffer solution for their colour change.

2.2.5 Colourimetric method

A Jasco spectrophotometer (model: UV-630) was used to measure the λmax and absorbance of various extracts of Delonix regia flowers solutions at pH values of 5.0-8.0. Sample (0.06 g each) of various extract of Delonix regia flowers was dissolved in 10 ml of appropriate buffer solutions at various pH values. The λmax and absorbance of the solutions were measured in the visible light spectra (400–800 nm).

2.2.6 Determination of pKa

Absorbance of Delonix regia flowers extract (0.12%w/v) was determined at analytical wavelength (AW) in a pH range of 5.0–8.0, because at pH 5.0 the compound exists as a molecular species (unionized form) whereas, at pH 8.0 it occurs in the ionized form. The ionic strength for each buffer used in this study was also calculated. The relationship between the pKa of an indicator (pKin) and pH can be given by Henderson– Hasselbalch equation,

$$pH = pka + [base]/ [acid]$$

The pKa of Delonix regia colour was then determined using the following equation,

$$pKa = pH + \log (di-d)/(d-dm)$$

Where, d is observed colour solution absorbance at the AW, whereas **di** and **dm** are absorbance of the ionized and molecular species at the AW.

2.2.7 Stability of Delonix regia colour

Reversibility of the colour change was also tested for this natural colour. Effect of temperature and pH on the stability of pink colour was studied for 10 days at two different temperatures (23 and 37°C). Delonix regia solutions at two different concentrations (0.1 and 1.0 mg/ml) were prepared in buffer solutions. Samples were kept in screw-capped bottles and shaken continuously (at 80 rpm) in controlled temperature reciprocating shaker cum water bath over a period of 10 days. The absorbance of the solution at 511 nm was monitored and degradation of Delonix regia flowers colour at a particular time was determined.

3. SPECTROPHOTOMETRIC QUANTITATIVE ESTIMATION OF GLIPIZIDE AND REPAGLINIDE IN BULK AND TABLET FORMULATION

3.1 Selection of drug

Phytochemical investigation of extract of Delonix regia flowers shows presence of anthocyanins group of compounds which have polyphenolic nature similar to synthetic dyes like methyl orange, bromocresol Green etc. The synthetic dyes generally show reactivity with amide linkage and hence are responsible for formation of color complex which can be quantitative estimated by spectrometric method. As glipizide and repaglinide in their structure contain amide linkage they were selected as the drug of choice for spectrometric method development.

3.2 Preparation of Standard Stock Solutions

3.2.1 Glipizide

Standard stock solution of glipizide was prepared by dissolving 50 mg of Glipizide in few ml of methanol by sonication and the final volume was made up to 100 ml with using methanol to get stock solution having concentration 500 µg/ml.

3.2.2 Repaglinide

Standard stock solution of repaglinide was prepared by dissolving 20 mg in few ml of methanol by sonication and the final volume was made up to 100 ml with methanol to get stock solution having concentration 200 µg/ml.

3.2.3 Preparation of Standard Working Solutions

Glipizide

4 ml of stock solution was pipette in 100 ml graduated volumetric flask. Volume was made up to 100 ml with methanol to give standard working solution of 20 µg/ml of glipizide.

Repaglinide

10 ml of stock solution was pipette out in 100 ml graduated volumetric flask. Volume was made up to 100 ml with methanol to give standard working solution of 20 µg/ml of repaglinide.

3.2.4 Spectrophotometric analysis

A pink coloured complex of drug and extract of Delonix regia for both drugs was scanned in visible range of 400 to 800 nm and spectra were determined. The complex of drug showed maximum absorbance at 610 nm and 605 nm respectively for glipizide and repaglinide.

3.2.5 Optimization of reagents and reaction condition

3.2.4.1 Glipizide and Repaglinide

For proper development of colour complex there was need to optimize temperature of reaction, quantity and concentration of reagent as well as the sequence of addition of reagents. Solutions having different concentration like 0.5, 1, 1.5, 2 and 2.5 % of extracts were prepared in Phosphate buffer of pH 7. To 1ml of standard working solution of glipizide as well as repaglinide having concentration 20 µg/ml, different volumes of extract having different concentrations were added in different 10 ml graduated volumetric flasks. All the solutions were screened at identified λmax 610 nm for glipizide and 605nm for repaglinide. It

was found that solution of extracted pigment having concentration of 1% and volume of 0.4 ml was found to be optimum for formation of excellent colour complex.

3.3 Procedure for plotting calibration curve

3.3.1 Glipizide

Serial volumes of standard working solution were pipetted out into a series of 10 ml volumetric flasks. To each volumetric flask 0.4 ml of 1% Extract solution of Delonix regia flowers was added and the volume was made up to 10 ml with methanol to get final concentrations of 4 to 20 µg /ml. Flasks were kept aside for 5 to 10 minutes for completion of formation of reaction complex. The absorbance of the red coloured complex was measured at 610 nm against reagent blank.

3.3.2 Repaglinide

Same above procedure was repeated for repaglinide to get final concentration in the range 5 to 40 µg/ml. The absorbance of the pale pink coloured complex was measured at 605 nm against reagent blank.

3.4 FTIR study

FTIR study for plane drugs as well as plane extract as well as for colour complex of both drugs was carried out.

3.5 Method Validation

3.5.1 Analysis of formulation

Twenty tablets of Glipizide and Repaglinide were weighed separately and average weight was calculated. Further the tablets were ground to a fine powder. An accurately weighed tablet powder equivalent to 10 mg of glipizide and 5 mg Repaglinide was dissolved separately in 10 ml of methanol by sonication. The solution was then filtered through whatman filter paper No.41. Appropriate aliquots within the Beer's law limit were analyzed by the proposed method using the procedure described earlier. The concentration of Glipizide and Repaglinide present in the sample solution was calculated by using the formula: Abs = A + B* C where, A = Intercept, B = Slope and C = concentration of drug.

3.5.2 Recovery Studies

To study validity and reproducibility of the proposed method, recovery studies were carried out by adding known amount of drug (glipizide and repaglinide) to pre-analysed sample at four different levels and the percentage recoveries were calculated.

3.5.3 Precision

Precision studies were carried out to determine the reproducibility of the proposed method. Repeatability was determined by preparing six replicates of three different concentrations of the sample and the absorbance was measured.

3.5.4 Limit of quantification (LOQ) and Limit of detection (LOD)

LOD is the lowest amount of analyte in the sample that can be detected. While, LOQ is the lowest amount of analyte in the sample that can be quantitatively determined by suitable precision and accuracy. LOQ and LOD was determined using by using standard deviation of the response and slope of the related calibration curve as defined in International Conference on Harmonization (ICH) guidelines.

3.5.5 Robustness

Robustness is performed by using MeOH: Water (90: 10). Average of nine determinations was used for robustness.

4. RESULTS AND DISCUSSION

Colour Complex Formation

The present work depicts the quantitative reaction of glipizide and repaglinide with solution of Delonix regia flowers extract. Anthocyanins are polyphenolic in nature. Phenol is mostly present in the enol form. The formation of colour-complex is attributed to the existence of Keto-enol tautomerism in phenols. In tautomeric form of phenol in general is going to be acidic, as electrons on oxygen are going to get delocalized in aromatic ring, making it easier for removal of H as H^+ i.e. proton making it acidic. While amide due to presence of lone pair of electron on nitrogen is going to show basic nature which contributes in formation of colour-complex between amide and the colour pigment obtained from Delonix regia.

The reaction based on charge-transfer complex in which glipizide being the electron donor and Delonix regia flowers dried extract solution being the electron acceptor. The formation of charge-transfer complex is shown in Figure 1.

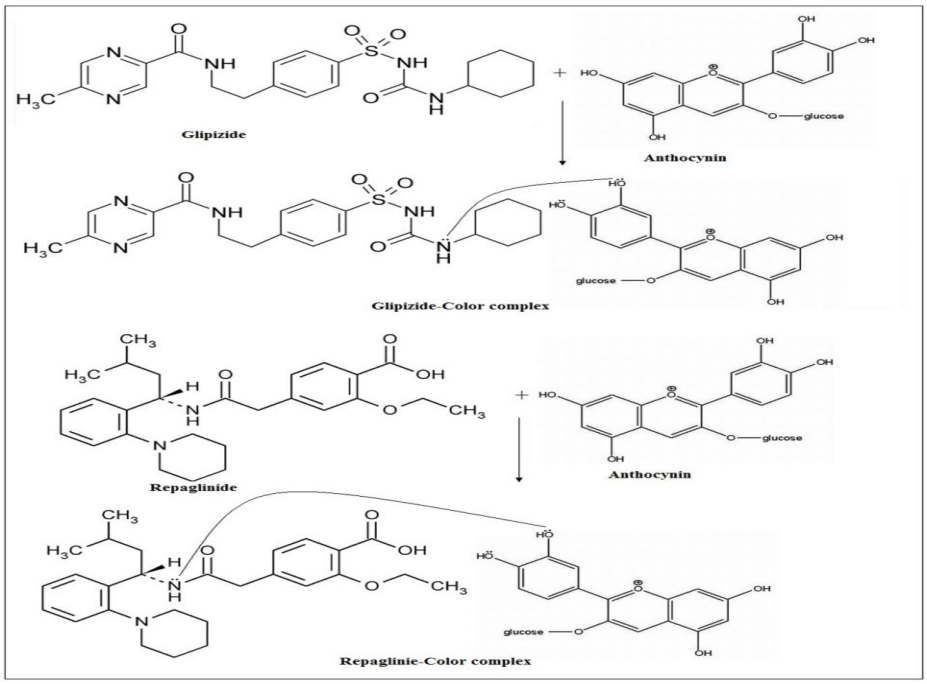

Figure 1: Probable mechanism of formation of charge-transfer complex.

4.1 Spectral analysis of extract

Analytical wavelength (λmax) for water and methanolic extract was found to be at 511nm and 526 nm respectively. Results are shown in figure 2 (A and B).

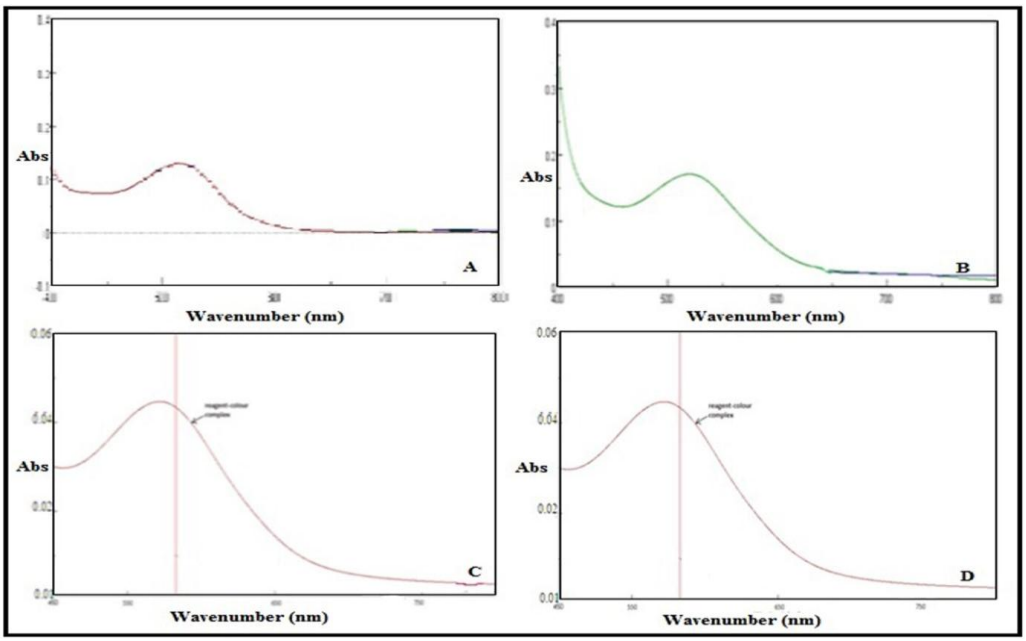

Figure 2: Analytical wavelength (λmax) for water and methanolic extract.

4.1.1 Chemical tests for anthocyanins

Both water and methanolic extracts showed presence of anthocyanins confirmed by the colour reactions. Aqueous and methanolic extract showed Blue to violet colour with aq.

NaOH, yellowish orange with conc. $H2SO_4$ and red with Mg-HCl which are specific test attributed to presence of anthocyanins namely Cyanidin3glucoside and Cyanidin3gentiobioside.

4.1.2 Colour change over different pH range

At a low (acidic) pH it has its original red colour, but at a basic pH its colour changes to pale yellow (Figure 3B). Absorbance and λmax of 0.12% (w/v) solutions of extracted pigment from Delonix regia due to colour at different pH values is reported in Table 1.

Table 1: Absorbance and λmax of 0.12% (w/v) solutions of Delonix regia colour at different pH values*

Sr. No.	pH	λ (nm)	Absorbance
1	3	511.3 ± 0.2	0.15 ± 0.03
2	5	522.6 ± 0.3	0.09 ± 0.05
3	7	511 ± 0.3	0.51 ± 0.10
4	8	568 ± 0.4	0.24 ± 0.08

* Indicates ± SD (n=3)

4.1.3 pKa determination

The mean pKa of extract of Delonix regia flowers dye determined by this spectrophotometric method was calculated to be 7.00 ± 0.16 (mean ± S.D.; n=8). Two different anthocyanin have been isolated from Delonix regia flowers colour. Therefore, the pKa reported here will be considered as a macroscopic pKa for a closely related group present in this natural colour. Values of absorbance at different pH, calculated ionic absorbance strength of the buffer (μ) and pKa for extract of Delonix regia flowers solution are shown in Table 2.

Table 2: Absorbance of Delonix regia flowers solution at 511nm.

pH of solution	Calculated ionic absorbance strength of the buffer (μ)	Absorbance (nm)	d (observed at the AW)	Calculated pKa
5	0.0676	1.258	--	--
5.4	0.0703	1.241	1.241	7.09
5.8	0.0763	1.223	1.223	7.16
6	0.0815	1.207	1.207	7.28
6.4	0.0995	0.9256	o.9256	7.57
6.8	0.1289	0.8526	0.8526	7.84
7	0.1448	0.312	0.312	6
7.4	0.1736	0.342	0.342	6.85
7.8	0.1900	0.381	0.381	6.28
8	0.1957	0.4121	--	--

4.1.4 Stability of Delonix regia colour

The solution was red at pH 3.0. Drop wise addition of 0.1N NaOH turned the solution pale yellow around pH 8. When 0.1N HCl was added to this pale yellow colour slowly, the solution changed back to its original colour red around pH 3.

At pH 3, the extract was found to be stable after period of 10 days at 23°C and 37°C. However, at pH 7 and pH 8, % degradation of colour was found to be increased. This indicates the pH dependent degradation of D. regia flower extract solution. Higher concentration of extract also increases the chances of degradation Table 3.

Table 3: Effect of temperature and pH on the degradation of aqueous extract of Delonix regia flowers colour.*

pH	Concentration (mg/ml)	% Degradation of Delonix regia colour over a period of 10 days at different temperature	
		23 ± 1.0°C	37 ± 0.5°C
3	0.1	4 ± 0.1	6.4 ± 0.5
	1	4.5 ± 0.3	10.3 ± 0.3
7	0.1	10 ± 1.6	19.4 ± 1.2
	1	25 ± 0.4	33.2 ± 1.0
8	0.1	35.5 ± 2.5	54.3 ± 0.4
	1	54.6 ± 0.9	71.1 ± 0.3

*Indicates ± SD (n=3); Degradation (%) = (original concentration−determined concentration) ×100

4.2 Use of Delonix regia colour as pH indicator

Chlorobutanol, is a chlorinated alcohol used as a preservative at a concentration of 0.5% (w/v) in pharmaceutical systems. This compound is stable in acidic condition and hydrolyzes in basic or neutral conditions to form hydrochloric acid with a resultant decrease in the pH of the solution. This pH change has also been shown to be dependent of temperature. When exposed to 60 °C, the pH changes in 0.5% (w/v) chlorbutanol solution of was found to be more than 1 pH unit. Therefore, this system was ideal for testing this pH indicator.

The extracted pigment from Delonix regia at acidic pH it has its original red colour, but at a basic pH its colour changes to pale yellow. This colour change is reversible with pH at room temperature. The colour change in a pharmaceutical solution due to a change in the pH of the solution caused due to an in situ degradation can be visually confirmed even at a low concentration (0.015% w/v) of chlorbutol solution of Delonix regia used as a pH indicator. Observed spectral scans at 0 hr and after 12 and 24 h are shown in Figure 3C.

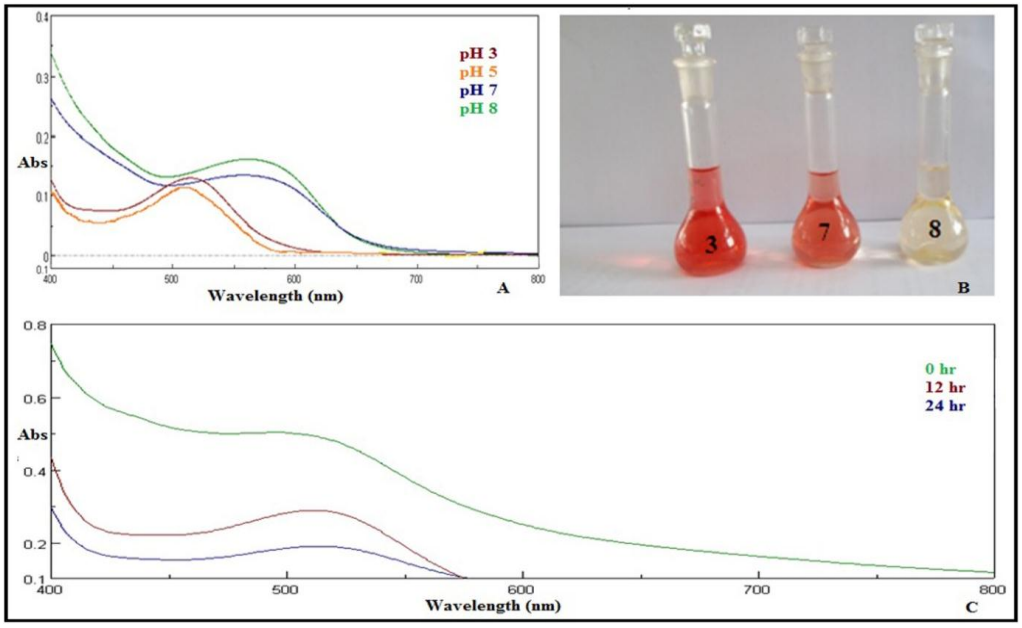

Figure 3: Effect of temperature and pH on the degradation of aqueous extract of Delonix regia flowers colour.

4.3 Spectrophotometric analysis of glipizide and repaglinide

UV-Visible spectra of D. regia colour, glipizide and glipizide-colour complex has been reported in Figure 2C. While UV spectra of D. regia colour, repaglinide and repaglinide-colour complex has been reported in Figure 2D. UV- visible overlain spectra of glipizide-colour complex and repaglinide-colour complex has been shown in Figure 4(A) and 4(B) respectively.

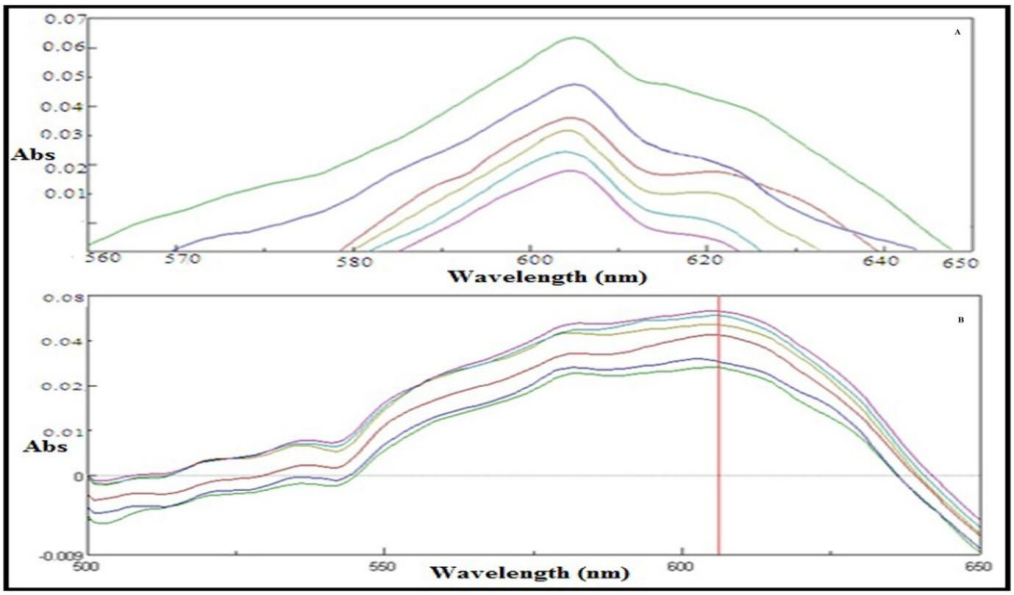

Figure 4: UV- visible overlain spectra of glipizide-colour complex and repaglinide-colour complex.

4.4 FTIR study

FTIR study of pure glipizide and repaglinide, extract of Delonix regia and complex of extracted pigment with drug indicates formation of complex. Delonix regia extract showed major peak at 3376.9, 2946.83, 1451.28 and 666.92 cm^{-1} attributed to phenols -OH stretching, aromatic C-H stretching, aromatic -C=C- stretching and aromatic substitution respectively. The Glipizide-colour complex and Repaglinide-colour complex shows peak at which are different from original pure extract. Hence it can be concluded that positive interaction between drug and extract is seen(Figure 5).

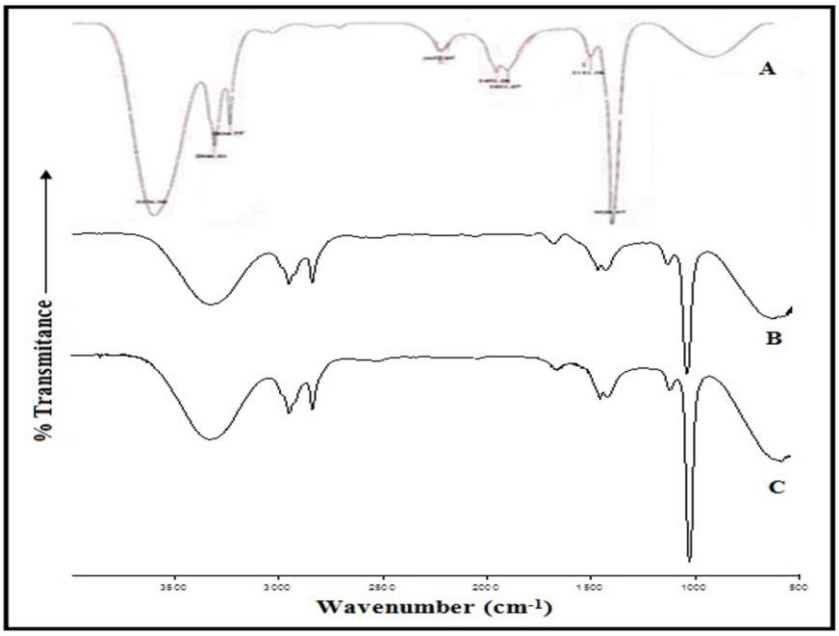

Figure 5: FTIR spectra of (A) *Delonix regia* flowers extract (B) Glipizide–colour complex (C) Repaglinide–colour complex.

4.6 Method validation

4.6.1 Analysis of tablet formulation

Result of analysis of tablet formulation showed % SD values in the range of 98.34 to 100.58% which indicates high precision of the method (Table 4).

Table 4: Results of tablet analysis.

Analyte	Label claim (mg/tab)	% Label claim estimated* (Mean ± SD)	RSD
Glipizide	5	98.61 ± 0.03	0.563
Lab sample	5	99.58 ± 1.16	--
Repaglinide	5	99.46 ± 1.0073	1.57
Lab sample	5	99.35 ± 0.65	--

* Indicates ± SD (n=3)

4.6.2 Linearity study

4.6.2.1 Glipizide

A calibration curve was constructed at optimum experimental conditions using absorbance values at 610 nm versus concentration in the range of 4 to 20 µg /ml (Table 5; Figure 6A).

Table 5: Absorbance values for calibration curve of Glipizide colour complex and Repaglinide colour complex.

Sr. No.	Glipizide colour complex		Repaglinide colour complex	
	Concentration (µg/ml)	Absorbance	Concentration (µg/ml)	Absorbance
1	4	0.00814	5	0.0114
2	8	0.0212	10	0.236
3	12	0.0333	20	0.0413
4	16	0.0462	30	0.0610
5	18	0.0538	35	0.0722
6	20	0.0611	40	0.0834

It has shown linear data. High value of the correlation coefficient (r=0.9994) indicates a good linearity and adherence of the method to Beer's law.

4.6.2.2 Repaglinide

A calibration curve was constructed at optimum experimental conditions using absorbance values at 605 nm versus concentration in the range of 5 to 40 µg/ml (Table 5; Figure 6B).

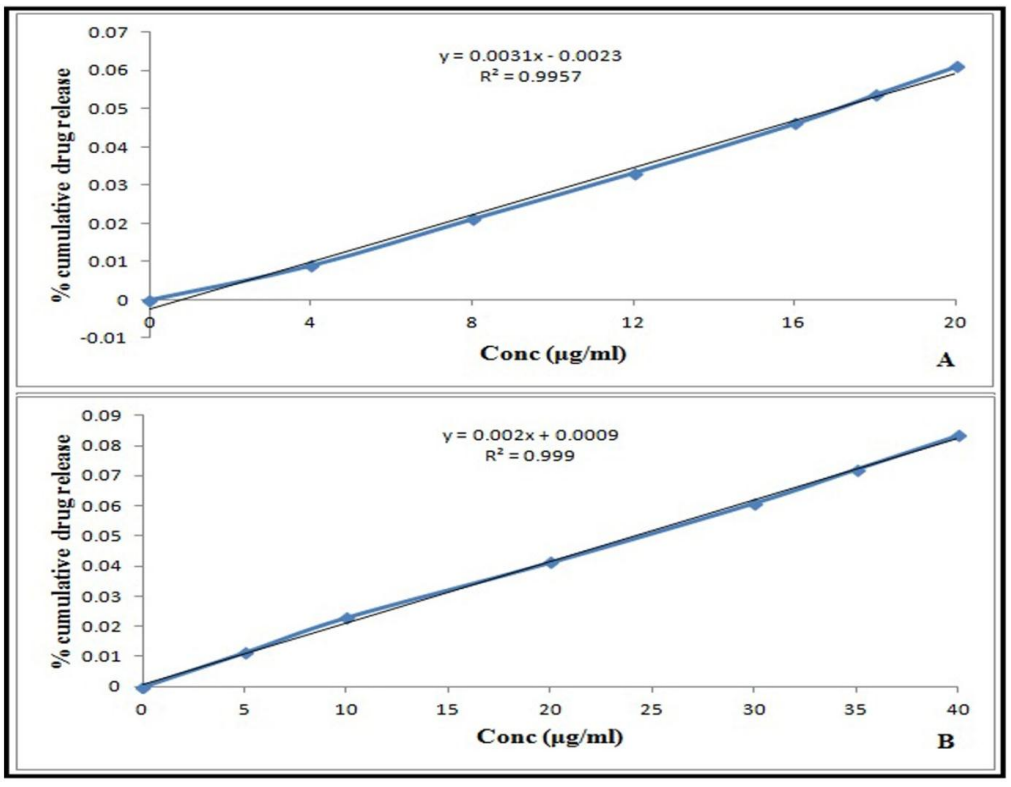

Figure 6: A calibration curve for Glipizide(6A) and Repaglinide(6B).

It has shown linear data. High value of the correlation coefficient (r=0.9996) indicates a good linearity and adherence of the method to Beer's law.

4.7 Recovery studies and Repeatability

The results indicated excellent recoveries for both ranging from 98.65 to 100.43 %. Recoveries obtained for the drug do not differ significantly from 100% showing that there was no interference from common excipients used in the formulation and thus indicates accuracy and reliability of the method. Result of recovery studies and Repeatability for both drug are shown in Table 6.

Table 6: Result of recovery study and repeatability.*

Analyte	Label claim (mg/tab)	% Recovery estimated* (Mean ± SD)	RSD	Repeatability % Label claim estimated* (Mean ± RSD)
Glipizide	5	99.22 ± 0.04	0.79	99.34 ± 1.18
Repaglinide	5	99.54 ± 0.89	0.88	99.65 ± 1.06

* Indicates ± SD (n=3)

4.8 Robustness

Robustness value for Glipizide and Repaglinide was found to be 99.87 ± 0.35 and 99.25 ± 0.65 respectively (Table 7). This showed ability of method to remain unaffected by small but deliberate change in reaction conditions.

5.9 LOD and LOQ

LOD and LOQ values are found to be 0.2681 and 0.7962 respectively (Table 7).

Table 7: Results of robustness, LOD and LOQ.

Parameters	Glipizide	Repaglinide
Robustness		
Label claim (mg/tab)	5	5
% Label claim estimated* (Mean ± % SD)	99.87 ± 0.35	99.25 ± 0.65
RSD	0.21	0.66
LOD (µg/ml)	0.2681	0.2681
LOQ (µg/ml)	0.7962	0.7962

* Indicates ± (n=9); RSD- Relative Standard Deviation

5. CONCLUSION

The proposed method for colourimetric quantitative estimation of Glipizide and Repaglinide in bulk and tablet dosage form using Delonix regia flowers extract as colour complex forming

agent was found to be accurate, precise, yield reproducible result and rugged. Moreover the method is economic, simple and rapid, hence can be employed for routine analysis in quality control laboratories.

Conflict of interest

Authors declare that they have no conflict of interest.

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