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EVALUATION OF EFFECT OF ICOFFEE BLACK (SALCITAL-PLUS) ON BIOAVAILABILITY OF METFORMIN HYDROCHLORIDE IN STREPTOZOTOCIN INDUCED RATS BY RP-HPLC METHOD

G. Nagaraju[1*], V. Sirisha[2], Kavati.Ramakrishna[3] and Hareesh Dara[1]

[1*]Department of Pharmaceutical Chemistry, Dhanvanthari Institute of Pharmaceutical Sciences, Sujathanagar, Kothagudem.

[1]Department of Pharmaceutics, Sree College of Pharmacy, Nayakulagudem, Kothagudem, Telangana.

[2]Department of Pharmaceutics, Dhanvanthari Institute of Pharmaceutical Sciences, Sujathanagar, Kothagudem.

[3]Department of Pharmaceutics, Pulipati Prasad College of Pharmacy, Khammam.

***Corresponding Author Email: gdp413@gmail.com**

ABSTRACT

Diabetes Mellitus (DM), DM is a serious metabolic disease that has a significant impact on the health, quality of life and life expectancy of patients, as well as on the health care system Metformin Hydrochloride is a first line drug for DM but has moderate bioavailability (50-60%). It is BCS class III drug, having high solubility and low permeability through intestinal mucosa, so it is necessary to increase the permeability of the drug and thus bioavailability. Enhancing Metformin hydrochloride absorption by oral ingestion is the most convenient and commonly used route of drug delivery. i-Coffee is a poly-herbal product for DM, having instant and high solubility in water. Thus, in continuation to our studies on the enhancement of bioavailability of Metformin hydrochloride using herbal products, herein we felt it worthwhile to study the impact of iCoffee on Metformin hydrochloride as they are widely consumed by the people in various districts of Telangana and other states of India.

KEY WORDS

Diabetes Mellitus, Metformin Hydrochloride, i-Coffee, bioavailability.

INTRODUCTION:

Diabetes Mellitus (DM) is a major chronic life-threatening disorder, in which homeostasis of carbohydrate and lipid metabolism is improperly regulated by the pancreatic hormone, insulin.

In recent years, there has been renewed interest in the DM treatment using herbal drugs as they are generally non-toxic, and World Health Organization (WHO) has also recommended the evaluation of the effectiveness of plants in condition where we lack safe modern drugs [1]

In the year 2000, the total number of people with DM was 151 million and the number is projected to increase by 46% to reach 221 million by the year 2010 and 300 million in 2025. In USA, which ranks third after India and China in the prevalence of diabetes, the growth rate is expected to be much smaller from 13.9 million in 1995 to 21.9 million in 2025[2]

It is an autoimmune disease of the pancreas, characterized by destruction of pancreatic ß-cells (islets of Langerhans) causing insulin deficiency due to the T cell mediated destruction of pancreatic ß-cells. Autoimmune reactions seem to be quite possible for the pancreatic ß-cell destruction, and the activation of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) may play an important role in this process. Approximately 85% of patients have circulating islet cell antibodies and the majority also have detectable anti-insulin antibodies. Most islet cell antibodies are directed

against Glutamic Acid Decarboxylase (GAD) within pancreatic ß-cells [4].

TYPES OF DIABETES MELLITUS:

- Insulin dependent diabetes mellitus (IDDM)-Type 1
- Non-Insulin dependent diabetes mellitus (NIDDM)-Type 2
- Gestational diabetes mellitus (GDM)

ESTIMATION OF BLOOD GLUCOSE LEVELS

There are several methods for the estimation of blood glucose levels. In present study we used the enzymatic, Glucose-Oxidase-Peroxidase (GOD-POD) Method.

Principle:

Glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxidise. In a subsequent peroxidase catalyzed reaction *p*-hydroxybenzoate and 4-amino antipyrine react with hydrogen peroxide to form red colored quinone complex. Absorbance data was measured at 510nm. Intensity of the colour formed is directly proportional to the amount of glucose present in the sample [5].

Glucose + H_2O → Gluconic acid + H_2O_2

H_2O_2+4-amino antipyrine + *p*-hydroxybenzoate→ Quninone dye

Glucose kit constitutes the following reagents:

1. Glucose reagent → Glucose oxidase peroxidase
2. Glucose diluents → Phosphate buffer, pH 7.4 phenol
3. Glucose standard → Dextrose (100 mg/dL)

Composition of phosphate buffer:

1. Disodium hydrogen phosphate dehydrate [Na_2HPO_4 $2H_2O$]-12.95 g
2. Anhydrous potassium dihydrogen phosphate [KH_2PO_4]- 4.95 g
3. Sodium azide [NaN_3]- 0.5 g

Preparation for working reagent:

Dissolve the contents of glucose reagent with glucose diluents, swirl the mixture gently to dissolve the contents, do not shake vigorously.

Test sample:

After collecting the blood samples into a micro centrifugation tube, it was centrifuged at 3000 rpm for 15-20 min. Then the serum glucose was estimated by making dilutions as shown in Table 1.

Table 1: GOD-POD method

Addition sequence	Blank (mL)	Standard (mL)	Test (mL)
Glucose reagent (L1)	1.0	1.0	1.0
Distilled water	0.01	-	-
Glucose standard (S)	-	0.01	-
Sample (T)	-	-	0.01

Procedure:

Three test tubes were taken and labelled as blank, test and standard as shown in table. To blank 1 mL glucose reagent and 0.01 mL distilled water were added. To the test 1 mL glucose reagent and 0.01 mL sample were added, mixed well and incubated at 37°C for 10 min and the absorbance was measured at 510nm [6].

MATERIALS AND METHODS:

DRUGS AND CHEMICALS: Metformin hydrochloride, iCoffee black, Streptozotocin, Dextrose,
Diethyl ether, Glucose kit, Cholesterol kit, Triglyceride kit,

MAINTENANCE OF EXPERIMENTAL ANALYSIS

Male Wistar rats weighing about 180-250 gm were purchased from Hyderabad, India. Rats were housed in standard polypropylene cages were kept in a room under controlled temperature (20-22°C) and 12hr day-night cycle. Animals were acclimatized for 1 week and then used for study. The animal study protocol was reviewed and approved by the Institutional Animals Ethics Committee (IAEC).

EXPERIMENTAL INDUCTION OF DIABETES MELLITUS

Diabetes was induced by intraperitoneal injection of Streptozotocin dissolved in Normal saline, at a dose of 60 mg/kg. After four days of the development of diabetes, the rats blood glucose range of above 200mg/dL were considered as diabetic and used for the experiment.

EXPERIMENTAL PROCEDURE:

The rats were divided into five groups of three animals in each group (n=3) as follows:

Group I: Normal control rats

Group II: Diabetic control rats

Group III: Administered with *Metformin hydrochloride* (50 mg/kg b. w.) orally suspended in normal saline

Group IV: Administered with i-Coffee black (50 mg/kg b. w.) orally suspended in normal saline

Group V: Administered both *Metformin hydrochloride* and iCoffee black (50 mg/kg b. w.) suspended in normal saline with five min interval.

COLLECTION OF BLOOD SAMPLES

Before the collection of blood samples [7]., animals were fast. Blood samples were collected from retro-orbital vein puncture under diethyl ether anesthesia at 0, 1, 2,4,6,8 and 24 hrs.

EXTRACTION OF SERUM SAMPLES

100 µl of serum was taken in a 2 ml Eppendorf's tube; to it 100 µl of acetonitrile was added to precipitate serum proteins and vortex mixed for 1min, centrifuged at 1300 rpm for 20 min. The supernatant was transferred into a clean, similarly labelled tube and was stored at -20^{0}C until use.

ESTIMATION OF CHOLESTEROL IN SERUM

Principle:

Cholesterol is measured enzymatically [8]. in serum or plasma in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. One of the reactions by products, H_2O_2 is measured quantitatively in a peroxidase catalyzed reaction that produces a colour. Absorbance was measured at 505 nm. The colour intensity is proportional to cholesterol concentration [9]. The reaction sequence is as follows:

Cholesterol ester + H_2O —(Cholesterol ester hydrolase)→ **Cholesterol ester + Fatty acid**

Cholesterol + O_2 —(Cholesterol oxidase)→ **Cholest-4-en-3-one + H_2O_2**

H_2O_2 + 4-aminoantipyrine + phenol —(Peroxidase)→ **Red Quinoneimine complex + H_2O**

Desirable cholesterol levels are those below 200 mg/dL in adults and below 170 mg/dL in children.

Reagent components

Cholesterol oxidase - 1KU/L
Cholesterol esterase- 0.6KU/L
Peroxidase - 5KU/L
4-aminoantipyrine- 0.5mmol/L
Phenol - 20mmol/L
Phosphate buffer- 50mmol/L
Triton X100- 0.1%

Procedure:

Pipette into 3 test tubes labelled as Blank(B), Standard(S) and Test(T) as shown in Table 2.

Table 2: CHOD/PAP Trinder's method

(Cholesterol Oxidase Phenol 4-aminoantipyrine peroxidase)

Reagent	Blank	Standard	Test
Cholesterol reagent	1.0 mL	1.0 mL	1.0 mL
Cholesterol standard	-	10 µL	-
Serum	-	-	10 µL

The 3 test tubes were incubated for 5min at 37^{o}C, and absorbance was measured at 505nm.

ESTIMATION OF TRIGLYCERIDES IN SERUM

Principle:

Triglycerides are measured enzymatically in serum or plasm using a series of coupled reactions in which triglycerides are hydrolyzed [10]. to produce glycerol. Glycerol is then oxidized using glycerol oxidase and H_2O_2, one of the reaction products, is measured as described above for cholesterol. Absorbance was measured at 546 nm. The reaction sequence is as follows:

Triglycerides + 3 H_2O —(Lipase)→ **Glycerol + Fatty acids**

Glycerol + ATP —(Glycerokinase)→ **Glycerol-3-phosphate + ADP**

Glycerol-3-phosphate + O_2 —(Glycerophosphate oxidase)→ **Dihydroxyacetone phosphate + H_2O_2**

H_2O_2 + 4-aminoantipyrine + ADPS —(Peroxidase)→ **H_2O + HCl + Blue purple complex**

ADPS- (N-ethyl-N-(3-sulfopropyl) m-anisidine

Desirable fasting triglyceride levels are those below 200 mg/dL and are further categorized as Borderline, 200-400 mg/dL; High, 400-1000 mg/dL; and very high (>1000 mg/dL) [72].

Procedure: Pipette into 3 test tubes labelled as Blank(B), Standard(S), and Test(T) as shown in Table 3.

Table 3: GPO-PAP Trinder's method

Reagent	Blank	Standard	Test
Triglycerides reagent	1.0 mL	1.0 mL	1.0 mL
Triglycerides standard	-	10 µL	-
Serum sample	-	-	10 µL

The 3 test tubes were incubated for 5 min at 37°C, and absorbance was measured at 546 nm.

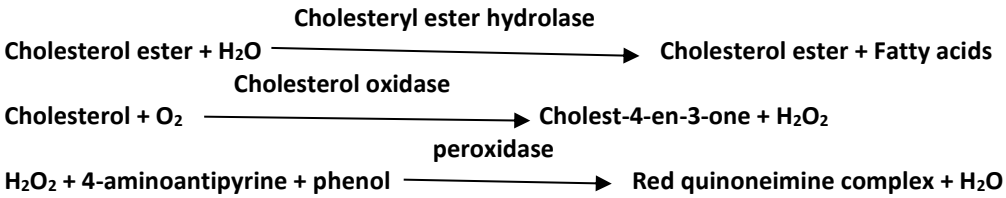
Working reagent components:

Lipase - >5KU/L
Glycerol kinase - >1.25KU/L
Glycerol phosphate oxidase- >5KU/L
Peroxidase - >2KU/L
ATP - >2mmol/L
4-aminoantipyrine - >10mmol/L
ADPS - >0.2mmol/L
Buffer- >20mmol/L
Surfactants and stabilizers

ESTIMATION OF HDL (HIGH DENSITY LIPOPROTIENS) LEVELS IN SERUM

Principle:

The chylomicrons, VLDL (very low density lipoprotiens) and LDL (low density lipoprotiens) are precipitated by addition of phosphotungstic acid and magnesium chloride. HDL fraction remains in the supernatant. After centrifugation the supernatant fluid contains the HDL fraction, and their cholesterol content is determined enzymatically using standard total cholesterol reagents [11].



Procedure:

Step 1: About 0.3 mL of precipitating reagent was added to 0.2 mL of plasma and then centrifuged at 3000 rpm for 10 min to get a clear supernatant.

Step 2: Pipette into 3 test tubes labelled Blank(B), Standard(S), and Test(T) as shown in Table 4.

Table 4: Phosphotung state precipitation method

Reagent	Blank	Standard	Test
Cholesterol reagent	1.0 mL	1.0 mL	1.0 mL
HDL Cholesterol standard (50 mg/dL)	-	100 µL	-
Serum sample (supernatant)	-	-	100 µL
Distilled water	100 µL	-	-

The 3 test tubes were incubated for 5 min at 37°C, and absorbance was measured at 505 nm.

RESULTS:

MEAN GLUCOSE LEVELS OF DIABETIC RATS

The mean serum glucose of diabetic rats is shown in Table 5:

Table 5: Mean glucose levels in diabetic rats

Group	0 day	7 days	14 days
Group I	81.33±7.0	82.66±7.5	81.66±12.5
Group II	234.66±11.5	232.33±11.5	227.33±9.5
Group III	207.33±15.9	204.66±16.5	203±16.3
Group IV	223.66±11.1	219.33±10.9	216.66±11.1
Group V	213.66±7.76	208.66±7.7	205.66±6.8

All values expressed as Mean±SD., n=3

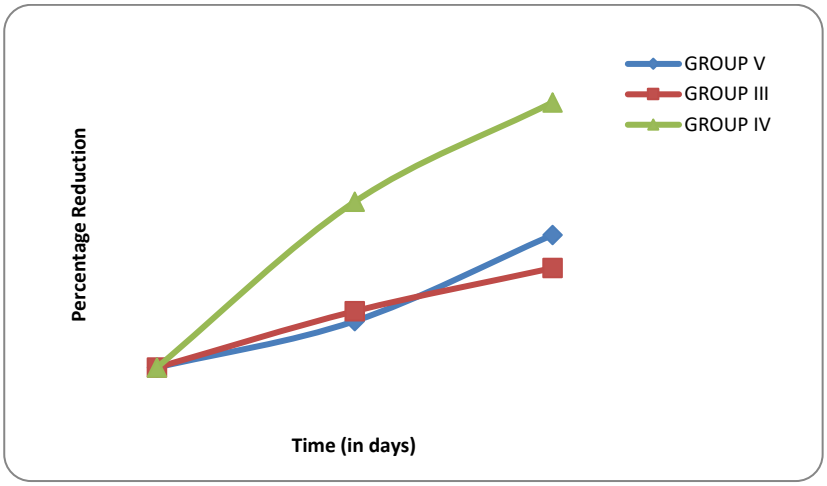


Fig 1: Percentage reduction of glucose levels on different days

PHARMACODYNAMIC STUDIES

Table 6: Mean serum glucose levels in diabetic rats at different time intervals

Time (hr)	Group I	Group II	Group III	Group IV	Group V
0	81.66±12.5	227.33±9.5	203±16.3	216.66±11.1	205.66±6.8
1	82.66±12.8	210.66±8.0	208.66±7.7	206.66±7.7	204.33±6.4
2	84.33±12.6	208.66±7.0	205.66±7.0	203.66±7.0	202.66±5.8
4	83.66±13.0	206.66±6.5	204±6.5	201.66±7.0	200.33±6.42
6	83±13.4	203.33±8.2	201.33±3.5	198±7.2	96.33±5.6
8	84±12.4	201.33±9.0	197.33±2.5	196±7.2	194.66±6.8
24	83.66±13.3	203±7.2	202.66±2.0	199.66±6.1	196±4.5

All values are expressed as Mean±SD.

Body weight:

The rats in group III [12]. showed a gradual and significant increase in body weight as shown in Table 8 and Fig 21. The effect of iCoffee black on the body weight of rats belonging to Group IV showed a significant decrease in the body weights compared to Group V.

Table 7: Comparison of body weights

Group	Initial (gms)	Final (gms)
Group III	233.33±30.5	243.33±23.0
Group IV	260±45.8	240±30.4
Group V	250±43.5	241.66±14.4

All values are expressed as Mean±SD.,

Lipid profile

Table 8: Comparison of Cholesterol, Triglycerides and HDL levels

	Group III	Group IV	Group V
Cholesterol (mg/dL)	220±2.2	180±4.8	175±3.7
Triglycerides (mg/dL)	132.4±3.2	121.2±2.8	118.6±4.1
HDL levels (mg/dL)	42±2.5	58±2.5	72±2.5

All values are expressed as Mean±SD.

ESTIMATION OF METFORMIN HYDROCHLORIDE BY RP-HPLC METHOD

The concentration of *Metformin hydrochloride* present in the acetonitrile extracts of serum samples were estimated by a modified RP-HPLC method. The SHIMADZU HPLC system used in the study.

METHOD:

PREPARATION OF THE STANDARD SOLUTIONS:

Stock and working standard solutions:

Standard solution of *Metformin hydrochloride* (1mg/ml) was prepared [13]. in water. The serial dilutions of stock solution were made to obtain working standard solutions of 1, 3, 5,7,10,15,20,25 and 50 µg/ml strength. These solutions were freshly prepared every week and stored at 4-8^{0}C.

Sample preparation:

Serum samples were stored at -20^{0}C and allowed to thaw at room temperature before processing. A standard graph was prepared by adding1[4]. known concentration of *Metformin hydrochloride* free rat serum 100µl and 100µl aliquot of working standard solution of *Metformin hydrochloride* was added in a polypropylene centrifuge tube.

To this 100µl of acetonitrile was added for precipitation and the tubes were vortexed for 1min. Then all the tubes were centrifuged for 20 min at 3000rpm. Clear supernatant was collected in another centrifuge tube and 20µl aliquot was injected into the analytical column.

DATA ANALYSIS

Linearity and limit of quantification:

Lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method is limit of quantification.

Calibration curve was established by spiking rat serum (500µl) with known amount of *Metformin hydrochloride*. The lowest concentration of the analyte that gives atleast five times as the response as compared with blank was considered as the limit of quantification (LOQ). Quantification of *Metformin hydrochloride* in rat serum was done by reading the analyte response against the calibration curve.

Accuracy and Precision:

In order to determine the intra-day and inter-day accuracy and precision, the concentration of *Metformin hydrochloride* is present in five replicates [15]. of plasma spiked with 5, 10, 20µg/ml. *Metformin hydrochloride* was estimated by HPLC within a day or on three consequent days. Accuracy with 98% and coefficient variation (CV) values <15% except at LLOQ (accuracy 80-120% and CV ≤20%) were considered acceptable.

Recovery:

Recovery of *Metformin hydrochloride* from serum was estimated at 8, 16, 24µg/ml concentrations by comparing peak area of spiked serum standards with those of corresponding plain standards containing the corresponding concentration in mobile phase that represent 100% recovery.

Stability:

Analyte stability in serum was tested at 8, 16, 24µg/ml concentrations in replicates of three for one freeze-thaw cycle, long term (30 days), and short term (24 hr) stabilities. Initially, a serum standard containing the above concentrations was prepared and divided into four fractions. One fraction was analyzed immediately, and its area was noted. The other three fractions were stored, two at -20^{0}C, and remaining at room temperature. The sample kept at room temperature was analysed after 24hrs. One of the samples kept at -20^{0}C was analyzed after one freeze-thaw cycle and the other was analyzed after one month storage. The peak area at the end of the study was compared with that of the standard and stability was calculated.

Deproteinization of serum and analyte extraction:

Blood samples (1ml) were collected in 2ml heparinized Eppendorf's tube and centrifuged at 3000rpm for 15min to separate the serum. To deproteinize serum and *Metformin hydrochloride*, acetonitrile (1ml) was added to serum (0.5ml) then the mixture was vortexed for 30sec. After standing for 10min, the mixture was centrifuged at 5000rpm for 10min. The upper layer (about 100µl) was separated and filtered through nylon membrane filters (0.22µ, 13mm) and stored at 20^{0}C until analysis. About 20µl of filtrate was used for estimating *Metformin hydrochloride* by HPLC method.

In the present method, acetonitrile was used to deproteinize serum and *Metformin hydrochloride*. The recovery studies indicate that this solvent was effective to *Metformin hydrochloride* from the spiked serum. In fact, acetonitrile is a polar solvent, and the present studies indicated that it has effectively deproteinized the serum. Hence, there is no necessity of separately using acid for this purpose. Since acetonitrile could also extract *Metformin hydrochloride*, the present method appears comparatively simple for serum preparation process.

CHROMATOGRAMS:

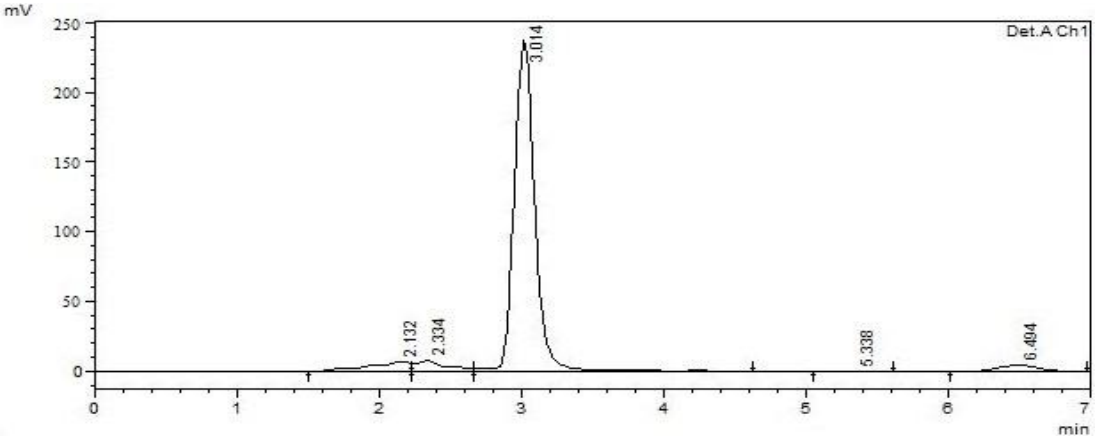


Fig 2: Chromatogram of Metformin hydrochloride, showing drug peak at 3.0 min

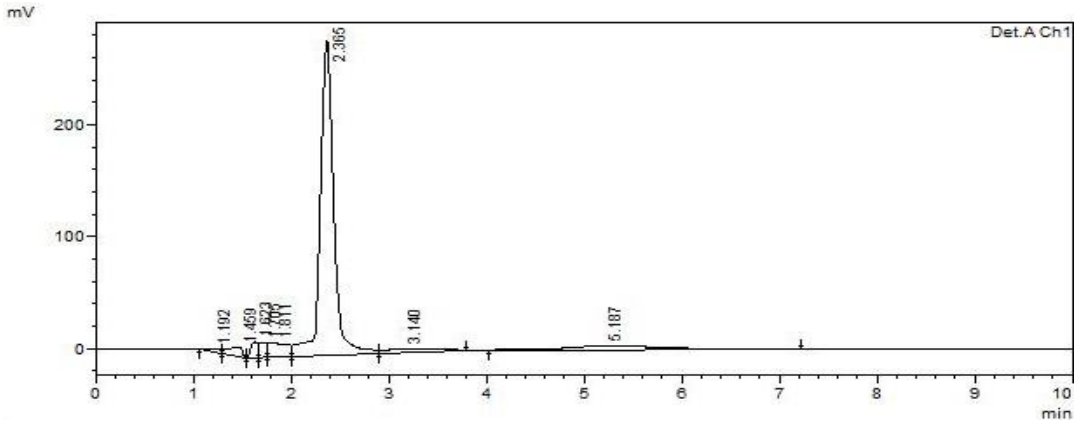


Fig 3: Chromatogram of Blank serum, showing peak at 2.3 min

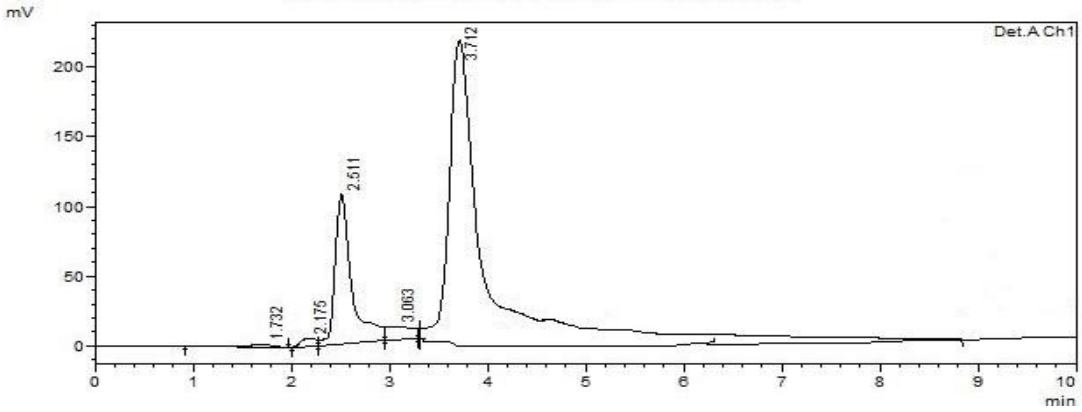


Fig 4: Chromatogram of Metformin hydrochloride in diabetic rats (2hr sample), showing peak at 3.7 min

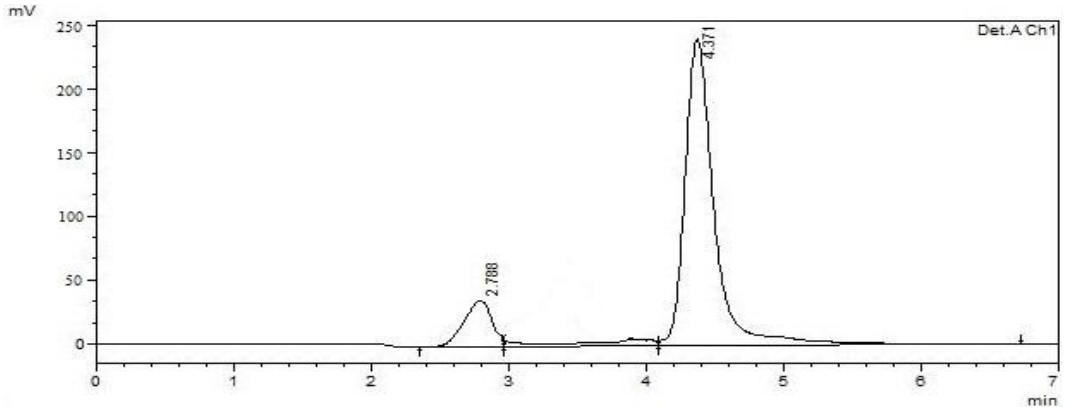


Fig 5: Chromatogram of iCoffee black in diabetic rats (2hr sample), showing peak at 4.3 min

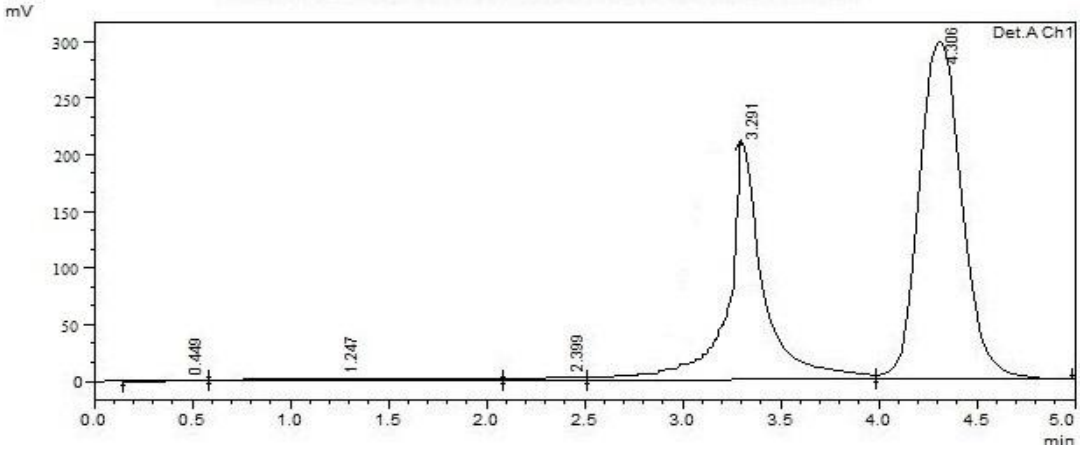


Fig 6: Chromatogram of Metformin hydrochloride in diabetic rats pretreated with iCoffee black (2hr sample), showing peaks at 3.2 & 4.3 min

PHARMACOKINETIC STUDIES:

The pharmacokinetic parameters were [15]. calculated from the serum concentration time profiles of Group III, IV and V diabetic rats (Fig 28).

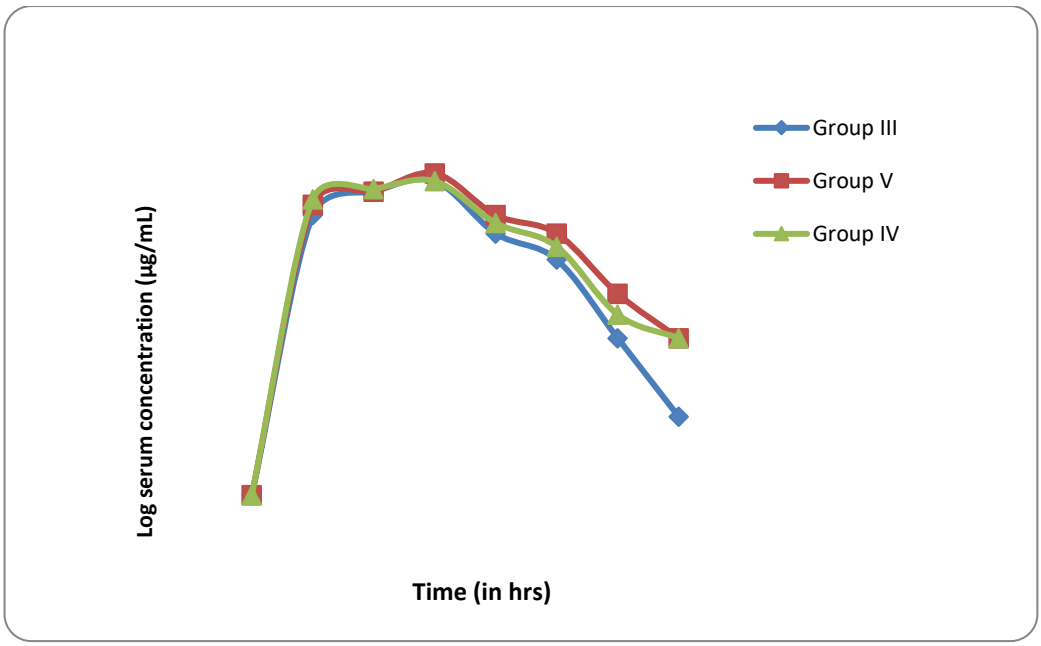


Fig 7: Log serum concentration-time profile of Group III, IV and V diabetic rats

Table 9: Pharmacokinetic parameters of Group III, IV and V Steptozotocin diabetic rats

Parameters	Group III	Group IV	Group V
AUC (µg.hr/ml)	164.514	185.9	183.102
Cmax(µg/ml)	16	17	16
Tmax(hr)	6	6	6
Elimination rate constant (hr^{-1})	0.1533963	0.10446	0.11515
Clearance (L/hr)	0.2896861	0.231	0.2464

C_{max}: Peak plasma concentration; T_{max} : Time to reach peak plasma concentration; AUC : Area under serum concentration/time.

The pharmacokinetic data in Table 12 reveals that the pretreatment of diabetic rats with iCoffee black produced noticeable changes in pharmacokinetic parameters of *Metformin hydrochloride*. Group IV, which is pretreated with iCoffee black had an increased AUC, C_{max} and all three groups have the same T_{max}. The constant elimination rate and clearance was decreased in group IV when compared with group V and III.

CONCLUSION:

The iCoffee black administration has shown a significant impact on blood glucose levels and body weight on Streptozotocin induced diabetic rats.

According to this study, the glucose levels in diabetic rats was decreased in Group IV(i.e., administered with iCoffee black) with 8% when compared with Group V (i.e., administered with both iCoffee and *Metformin hydrochloride*) with 4% and Group III (i.e., administered with *Metformin hydrochloride*) with 3% and also the mean serum glucose levels at different time intervals significantly decreased in Group IV with 7.8% as compared to Group V with 4.3% and Group III with 0.4%. This research has a great impact on obesity, as rats gradually decreases their body weight in Group IV when compared with Group V. Whereas, in Group III the body weight of rats was increased.

The serum Cholesterol, Triglycerides and HDL levels were found to be normal range in all the animals.

As the *Metformin hydrochloride* showing low peak height compared with iCoffee black, it is considered as having a good bioavailability.

The pharmacokinetic data indicates that iCoffee black improved the anti-diabetic effect of *Metformin hydrochloride*. Hence, this suggests iCoffee black as a bioenhancer for *Metformin hydrochloride*. Undoubtedly, it can be recommended in patients with type-2 diabetes who are administering *Metformin hydrochloride*.

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***Corresponding Author:**
G. Nagaraju*
Email: **gdp413@gmail.com**