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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(4): 781-786

www.thepharmajournal.com
Received: 04-02-2019
Accepted: 05-03-2019

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Development and characterization of vesicular system containing *aegle marmelos* leaves extract for management of diabetes mellitus

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Abstract
There are many side effects associated with the synthetic drug molecules and in case of diabetes there is drug regimen which means more synthetic drug and more associated side effects. Current work is based on the extraction of leaves of *Aegle marmelos* plant which is of natural origin; this will help to get rid of side effects and after that the problem was dosing frequency which is again a drawback of diabetes treatment. We had selected the vesicular carrier system for the drug release modification. Characterization and performance evaluation of vesicular system loaded with herbal plant extract of the *aegle marmelos* leaves was done. Liposomal dispersion was formulated and evaluated for the vesicle size distribution, percentage drug entrapment and *In vitro* drug release profile along with the stability studies. *In vivo* tissue distribution studies on albino rats suggested the accumulation of formulations in the different organs. These results suggest that the liposomal dispersion are not only effective in rapid attainment of high drug concentration in body and also maintain the same over prolonged period of time.

Keywords: Vesicular system, plant extract, *aegle marmelos*, diabetes mellitus

Introduction
Bael (*Aegle marmelos* Corr.) is another Indian medicinal plant, which has enormous traditional values against various diseases and many bioactive compounds have been isolated from this plant [1]. *Aegle marmelos* commonly known as bael belongs to family Rutaceae [2].

Plant Profile
- Botanical Name: *Aegle marmelos*
- Sanskrit Name: Bilva
- English Name: Bael tree
- Family: Rutaceae
- Parts of Plant used: Leaves, fruit, root and bark[3]

Material and Method
Leaves of Bael (*Aegle marmelos* Corr.) were collected from agriculture college, Indore. Their identification and authentification was confirmed by Department of Botany, Holkar Science College, Indore by correlating their morphological and microscopic characters with those given in literature. The leaves were collected, washed well to remove all the dirt and were shade dried and then powdered transferred into airtight containers with proper labeling for further use.

Preparation of plant extract
Petroleum ether Extract
The coarsely powdered, dried leaves (50 g) were extracted with 300-500 ml petroleum ether by hot extraction process (soxhlet) for 4 hours. After completion of extraction the solvent was removed by distillation and concentrated in vacuo.

Chloroform Extract
The marc left after petroleum ether extraction was dried and extracted with 300 ml -500 ml chloroform by hot extraction process (soxhlet) for 4 hours. After completion of extraction the solvent was removed by distillation and concentrated in vacuo.

Ethanol Extract

The marc left after the methanol extraction was dried and extracted with 300 ml -500 ml ethanol by hot extraction process (soxhlet) for 4 hours. After completion of the extraction the solvent was removed by distillation and concentrated in vacuo.

The above extracts were used for phytochemical studies. The extractive values for each extract were calculated and recorded.

Isolation Method

Preparation of ethanolic extract

The drug powder was taken in the soxhlet extractor and was extracted using ethanol for 72 hours. After the extraction was over the solvent was recovered by distillation and the residue was concentrated in vacuo. The extract obtained was then stored in dessicator.

Fractionation of the ethanolic extract

50gm of ethanolic extract obtained was suspended in distilled water (200ml) in small amounts. It was extracted successively and exhaustively with solvents in increasing order of polarity viz. petroleum ether (60-80° C) (200ml X 5), solvent ether (200 X 5) and ethyl acetate (200X 3). Each fraction was washed with distilled water (5ml), dried over anhydrous sodium sulphate and freed of solvent by distillation. The aqueous remnant was freed of organic solvent by distillation under reduced pressure and then evaporated to dryness on a water bath. The yield of each extract was recorded and subjected to chemical investigation.

Table 1: Successive extractive values of the powdered leaves of *Aegle marmelos*

S. No	Extracts	Yield (% W/W)
1.	Petroleum Ether Extract	2.30
2.	Chloroform Extract	3.15
3.	Ethyl acetate Extract	3.65
4.	Methanol Extract	5.21
5.	Ethanol Extract	4.10

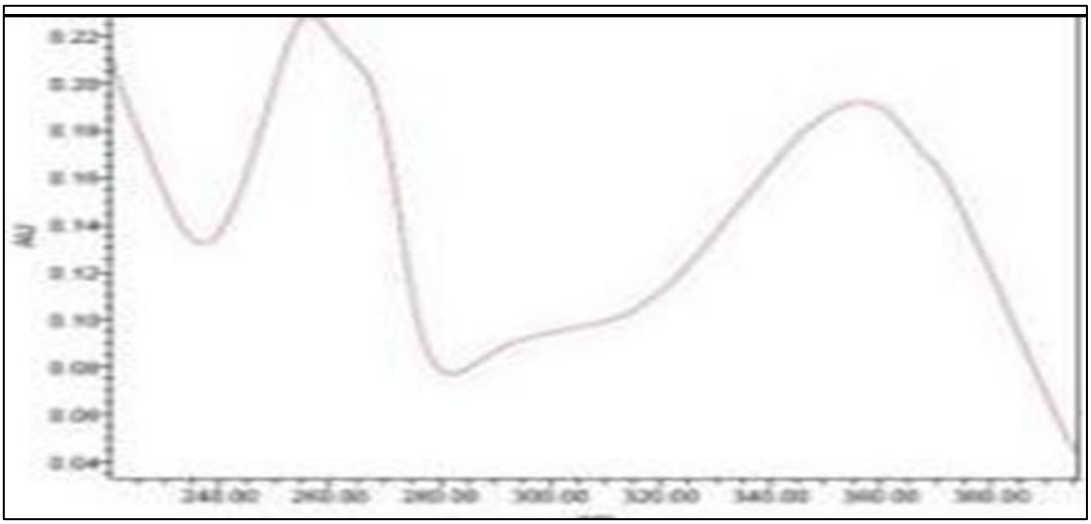

Fig 1: UV Spectra of standard rutin

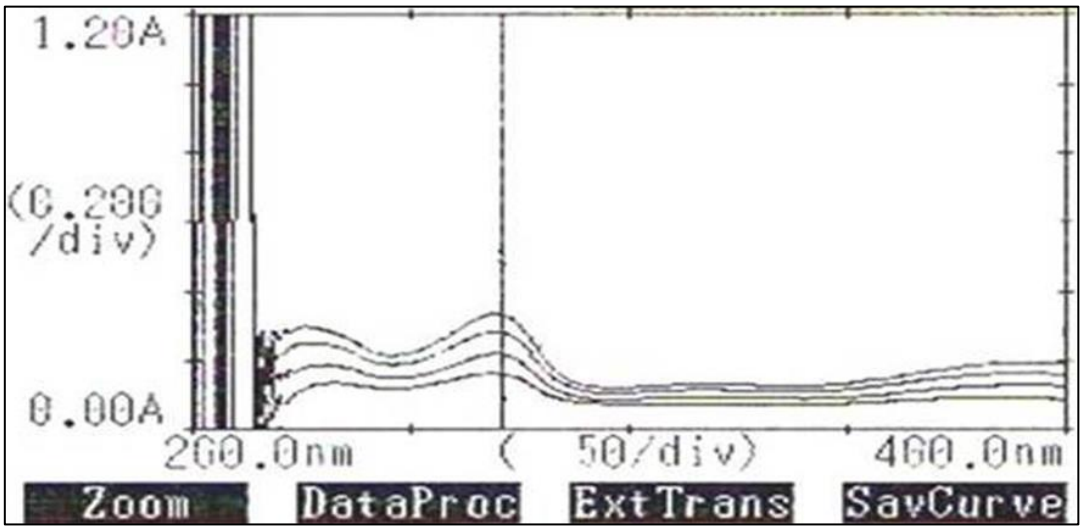

Fig 2: UV Spectra of Isolated rutin

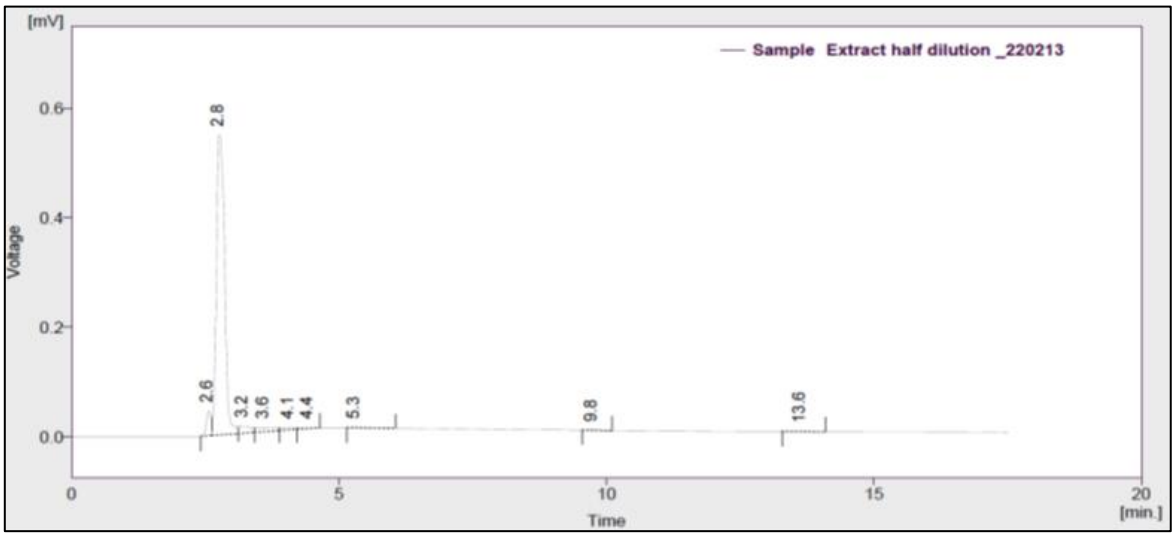

Fig 3: HPLC spectra of isolated Rutin

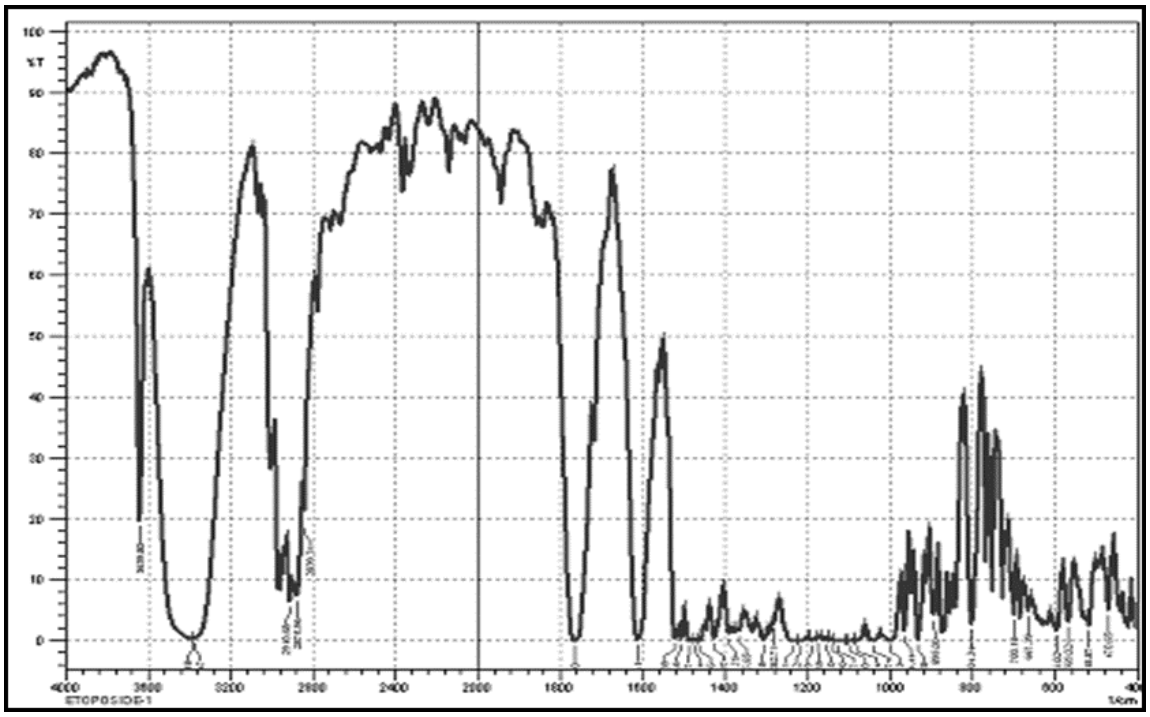

Fig 4: IR Spectra of Isolated rutin

Phytochemical study of *Aegle marmelos*

Isolated rutin from *Aegle marmelos* showed a melting point at 185^{0}C which was in agreement with the standard range of 180-189^{0}C, as reported.

The Rf values of isolated and standard rutin in several mobile phases are shown in table below.

Table 2: Comparison between the RF Values of Isolated and Standard Rutin in Different Mobile Phase (TLC)

Solvent system in TLC	Rf value of isolated rutin	Rf value of standard rutin
Ethyl acetate : formic acid : acetic acid : water	0.39	0.34

Table 3: Comparison between the RF Values of Isolated and Standard Rutin in Different Mobile Phase (Paper Chromatography)

Solvent system in Paper Chromatography	Rf value of isolated rutin	Rf value of standard rutin
Isopropyl alcohol : water	0.55	0.59

Pharmaceutical screening of active constituent

Acute Toxicity study: The acute toxicity study is use to establish the therapeutic index, i.e. the ratio between the pharmacologically effective dose and lethal dose on the same strain and species (LD_{50}/ED_{50}). The animals were divided into four groups and each group consisted of five mice. The defined or fixed dose level of aqueous and ethanolic extracts (2000 mg/kg) were given orally to identify a dose producing evident toxicity. The animals were observed continuously for 2 hours for behavioral, neurological and autonomic profiles. The toxicity signs were observed after 24 hours till fourteen days for any lethality or death.

Table 4: Result of acute toxicity study of Rutin

S No.	Group	No. of animal used	Treatment Dose (mg/kg) body wt.	No. of animals recovered after study		
				24 hrs.	72 hrs.	14 days
1	Group A (Alcoholic Extract)	5	2000	5	4	4
2	Group B (Aqueous Extract)	5	2000	5	5	4

Oral Glucose Tolerance Test: Animals were divided in nine groups and each group consisted of six rats. Overnight fasted rats were used for study.

Group I: Normal control rats administered saline (0.9% w/v);

Group II: Diabetic rats administered standard drug Glibenclamide (2.5 mg / kg) daily

Group III: Diabetic rats administered test sample (50 mg/kg);

Group IV: Diabetic rats administered test sample (100 mg/kg);

Table 5: Effect of Rutin from *Aegle marmelos* on oral glucose tolerance test in rats

S. No.	Treatment n=6	Fasting blood glucose level (mg / dl)			
		0 min	30 min	60 min	120 min
1	Normal	91.42± 0.92	132.33± 1.12	117.29± 1.11	111.03± 1.17
2	Standard (Glibenclamide, 2.5mg/kg)	94.01± 0.73	110.33±0.56*	83.09 ± 0.97*	79.39± 0.05*
3	Rutin (50mg/kg)	95.01± 1.32	123.33±1.48*	104.67±0.92*	92.01± 0.37*
4	Rutin (100mg/kg)	103.09±1.67	129.04±1.46	108.31±1.87*	94.83± 2.11*

Normal Control- Vehicle 10 ml/kg, Reading are values ± S.E.M, n = Numbers of animals in each group* P < 0.05 v/s Normal control; One-way ANOVA followed by Dunnett test

3. FBS (Fasting blood glucose level): Fasting blood sugar level was determined by using glucose oxidase peroxidase reactive strips.

Table 6: Effect of Rutin on fasting blood glucose levels in rats.

Sl No.	Treatment n=6	Fasting blood glucose level (mg / dl)			
		Day 0	Day 5	Day 10	Day 15
1	Normal	97.14±1.53*	94.17±1.25 *	91.83± 1.01*	88.67±1.15 *
2	Diabetic control	181.67±1.12	189.11± 0.88	196.83± 1.08	199.18± 1.31
3	Standard (Glibenclamide, 2.5mg/kg)	184.33±1.45	127.55±0.76*	116.51±1.01*	107.67±1.14*
4	Rutin (50mg/kg)	180.11±1.83	137.33±1.33	125.83±1.34*	119.18±0.97*
5	Rutin (100mg/kg)	179.67±0.65	135.65±1.50*	126.33±1.03*	116.52±1.08*

Values expressed as mean ±S. E. M.; n = no. of animals in each group. *p < 0.05 significant Vs diabetic control. One-way ANOVA followed by Dunnett test

4. Effect of rutin on serum lipid profile

Table 7: Effect of rutin on serum lipid profile in rats

Sl. No.	Treatment n=6	TG	TC	HDL	LDL	VLDL
1	Normal	88.86±1.04*	55.52±0.94*	23.88±0.73	13.45±0.87*	18.72±0.20
2	Diabetic control	136.52±1.54	96.56± 1.03	15.55±0.83	53.73± 0.41	27.29± 0.33
3	Standard (Glibenclamide, 2.5mg/kg)	101.56±1.07*	57.21±1.08*	19.20±0.52	17.35±0.44*	20.85±0.21
4	Rutin (50mg/kg)	115.87±1.14*	75.27±1.20*	14.26± 0.39	37.84±0.86*	23.17±0.23
5	Rutin (100mg/kg)	107.06±1.04*	65.19±1.24*	18.59±0.38	25.23±1.40*	21.37±0.30

Values expressed as mean ± S. E. M.; n = no. of animals in each group. * p < 0.05 significant Vs diabetic control. One-way ANOVA followed by Dunnett test

Preparation of Vesicular system

Vesicles were prepared and optimized on the basis of % entrapment, drug content and no. of vesicles formed. Optimized formula used for further work. Hand shaken method has been used in which Lecithin and cholesterol was dissolved in minor amount of DMSO. The ratio of lecithin and cholesterol kept constant (7:3), while rutin content was varied in different preparations for determining optimum rutin content. The organic solvent mixture was removed using a rotary flash evaporator (Stereoglass Rotavap, Italy) under reduced pressure. The dried film was hydrated with 10 ml of PBS (pH 7.4) followed by continuous vortexing of the flask for about an hour to get multi lamellar liposomes. Liposomal suspension was allowed to stand for further 3 to 4 hours in dark at room temperature to allow complete swelling of the vesicles. The suspension was then centrifuged at 2000 rpm for 4 h, and the pellet was resuspended in PBS (pH 7.4).

The liposomal formulation were centrifuged through sephadex G-50 mini-column at 2000 rpm for 3 min to remove the free drug and stored in dark at low temperature. The liposomal fraction was added with minimum amount of Triton X-100 (0.1%, v/v), drug content was determined spectrophotometrically at 268 nm and percent drug entrapment was calculated.

Table 8: Optimization of Drug: polymer ratio

Formulation code	Ratio (Drug:Polymer)	Average size(µm)	No. of particles per mm^3x1000	% Entrapment
PL-1	9:1	2.23±0.35	27±2.5	64.4±1.2
PL-2	8:2	2.34±0.54	28±2.2	68.8±0.98
PL-3*	7:3	2.69±0.57	36±1.9	70.5±1.10
PL-4	6:4	2.75±0.15	28±1.6	65.4±1.43
PL-5	5:5	2.77±0.24	23±1.5	64.8±0.85

*Data are shown as mean ± SD (n= 3)

Characterization of Liposomes

Vesicle Shape: Microparticles were visualized under Philips Morgani 268 Transmission Electron Microscope. A drop of the different formulations was placed on different carbon coated copper grids to leave a thin film on the grids. Then, the film was negatively stained with 1% phosphotungastic acid (PTA) by placing a drop of the staining solution on to the film and the excess of the solution was drained off with a filter paper. The grid was allowed to dry thoroughly and formulations were viewed under a transmission electron microscope and photographs were taken at suitable magnification. (Fig No.: 5)

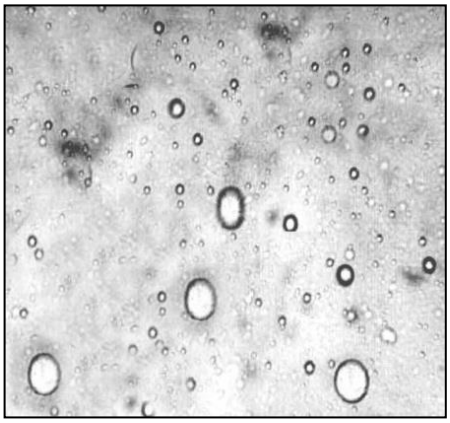

Fig 5: Photomicrograph of Vesicles

Particle size and distribution: The size and size distribution of vesicles was determined using laser diffraction particle size analyzer (Cilas, 1064 L, France). The liposomal suspension was dispersed in distilled water and then it was put into the sample chamber of particle size analyzer and measurement of vesicular size was carried out (Table No. 8).

Entrapment Efficiency: 1 g sephadex G-75 was allowed to swell in 10 ml of 0.9% NaCl solution in distilled water in a glass screw capped bottle for 5 hours at room temperature. The hydrated gel was filled to the top in the barrel of 1ml disposable syringe plugged with whatman filter pad. The barrel was then placed in the centrifuge tubes. The tubes were centrifuged at 2000 rpm for 3 minutes to remove excess saline solution. Eluted saline was removed from the centrifuge tubes and exactly 0.2 ml of suspension (undiluted) was applied dropwise on the top of the gel bed in the center. Columns were again centrifuged at 2000 rpm for 3 minutes to expel and remove void volume containing liposomes in to the centrifuge tubes. Elute was removed and 0.25 ml saline was applied to each column, and centrifuged as previously. The amount of drug entrapped in the vesicles was then determined by disrupting the vesicles followed by filtration and subsequent determination of the drug content using spectrophotometric method (Table No. 8).

***In vitro* drug release:** 1 ml of pure suspension was placed in dialysis tube, which in turn was placed in a beaker containing 20 ml of PBS (7.4 pH). The solution containing the dialysis tube was stirred on a magnetic stirrer while keeping the temperature constant at 37±1°C throughout the study. Samples were withdrawn at different time intervals with subsequent analyzed for drug using Shimadzu 1601 UV spectrophotometer at 268nm.

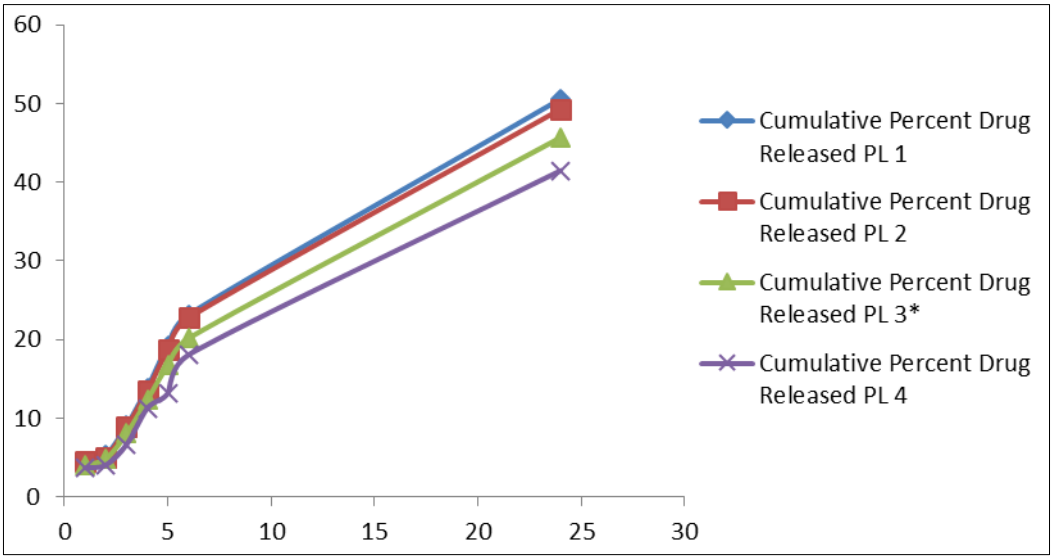

Fig 6: *In vitro* drug release profile

Vesicle count

To characterize liposomal formulation for vesicular count, the liposomal formulation was diluted (5 times) with PBS (pH 7.4) and liposomes /mm [3] were counted by optical microscopy. The liposomes in 80 small squares were counted and calculated using the following formula: (Table 8).

Total numbers of liposomes/mm [3]:

Total number of liposomes counted x Dilution x 4000

Results and Discussion

Isolated rutin shows effective decrease in blood glucose level as compared the standard glibenclamide drug which is tested by animal studies. Drug release pattern also altered with the vesicles prepared by hand shaken method shows improved the drug release profile. *In vitro* drug release studies shows that drug release controlled over prolong period of time i.e. after 24hrs. 45.7% drug was released, this will also decrease the dosing frequency of active constituent.

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