INTRODUCTION
Diabetes mellitus (DM) is a complex chronic disease that requires ongoing medical care and multi-factor strategies to reduce risk beyond glycemic control [1-3]. In DM, a constant increase in glucose levels can lead to chronic micro- and macro-vascular effects [4-7]. A patient with multiple comorbid conditions requires multiple medications to treat each condition, increasing side effects and treatment costs. Combined pharmacotherapies may provide additive benefits that target multiple disease processes [8]. Fixed-dose combination drugs (FDCs) were originally developed to target only one disease. However, CFDCs can also target more than one disease/condition [9, 10]. However, no CDF has been developed for diabetes and its co-morbidities, like hypertension.

Transdermal drug delivery systems offer a promising alternative to oral administration, particularly in preventing difficulties associated with this combination [11, 12]. This is particularly relevant for these products as these two classes of medications are administered at two different times (before and after the diet) when administered by the oral route [13]. Oral administration of GLB causes symptoms like headache or nausea, cold sweats, excessive hunger [14-16]. Atenolol (ATN) is widely used in the management of hypertension as monotherapy or in combination with other classes of antihypertensive agents [17]. The absorption of Atenolol upon oral administration in humans and most laboratory animal species is rapid but incomplete [18]. Thus, the transdermal administration of GLB and ATN would have a better dosage form being that it increases bio-availability.

Therefore, the combination of GLB and ATN through transdermal delivery can be a better therapeutic combination for effective control of diabetes and its coexisting cardiovascular complications (hypertension). The primary objective of this research was to assess the ex vivo and pharmacodynamic behavior of the optimized formulation of GLB and ATN obtained by designing Box Behnken as a proniosomal gel for transdermal administration.

MATERIALS AND METHODS
ATN and GLB were received as gift samples from Sun Pharma Ltd., Mumbai, India. Span 60 and cholesterol were purchased from SD Fine Chemicals, Mumbai, India. Phospholipid (Brand: Phospholipon 90G) was a gift sample supplied by Phospholipid GmbH, Nattermannallee, Koln Germany. The other reagents and chemicals used were of analytical grade and were procured from Merck Limited, Mumbai, India.

Preparation and optimization of proniosomes
Cocreation-phase separation method was followed for the preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were formulation of GLB and ATN obtained by designing Box Behnken as a proniosomal gel for transdermal administration.

RESULTS
The ex vivo permeation behavior through different skins was studied and the findings were also confirmed by the values of the steady-state flux (Jss). The OCPG observed an increase of more than twice in the cumulative amount of impregnated drugs compared to pure drug films. The study on skin irritation revealed the non-irritability of the developed OCPG applied. OCPG significantly showed sustained hypoglycemic activity in rats (p<0.02), when compared to orally treat rats up to 24 h. However, the reduction was slow and sustained in the case of OCPG where a significant response was observed in the performed studies.

Conclusion: All the results show that controlled release GLB and ATN proniosomes offer a useful and promising transdermal delivery system. Henceforth this may be an achievement in treating the diabetic hypertensive patient.
Preparation of rat skin

Wistar albino male rats were obtained from the National center for laboratory animal sciences. All rats were kept under standard laboratory conditions in the 12-h light/dark cycle at 25 °C±2 °C provided by pellet diet (Lipton India Ltd., Bangalore) and water ad libitum. The animals were selected after superficial examination of the skin surface for abnormalities. The hair on the skin of Wistar albino male rat was clipped and subcutaneous tissues were removed, and dermis side was wiped with isopropyl alcohol to remove residual adhering fat. The skin was washed with PBS, wrapped in aluminum foil and stored in a deep freezer at-20 °C till further use (used within 2 w of preparation) [26].

Goat skin

The hair on the skin of slaughtered goat was removed carefully and separated from the underlying cartilage with a scalpel. After separating the full-thickness skin, the fat adhering to the dermis side was removed using a scalpel and isopropyl alcohol [27].

Skin permeation studies

The diffusion of GLB and ATN from the prepared gel, either contain pure drugs or OCPG, was carried out utilizing the same conditions as the method previously reported [28, 29]. A two-chamber horizontal Franz diffusion cell (volume of 12.5 ml and surface area for the method previously reported [28, 29]). The study was performed by using healthy Male Newzeland Rabbits (2.5-3.0 kg) which were fed with food and water for 1 w to adapt to the environment before the study. For the skin irritation study, the rabbits were divided into three groups. Group 1 served as control. Group 2 received blank Proniosomal gel and Group 3 received OCPG on the previously shaven (24 h before the study) dorsal side of rabbits. Skin irritation study was performed as per the Draize scoring system (table 2) [31, 32]. The responses scored were noted approximately at 1, 2, 3 and on the 7th day after the removal of the standard irritant and test formulations, rabbits were examined for signs of erythema and edema. The untreated skin of each rabbit was examined, which was reported in our earlier work Anitha et al. [33, 34].

Pharmacodynamic activity of OCPG

Animal experiments were done as per the standards and guidelines set by the institutional animal ethical committee (IAEC No: CBLRC/AEC/13/01-2019). All wistar albino rats (150-180 g) were fed ad libitum and housed in light and dark cycle in an ambient temperature-controlled environment. They were placed into the rat holder for 5–6 d for a period of 10–20 min. By repeating the exercise, the animals taught to remain in the rat holder calmly and they became acquainted with the experimental conditions [36-39].

Antidiabetic activity of OCPG in rats

Studies in normal rats

Male Wistar albino rats (n=6) were fasted overnight only water was given. The hair in the neck region of the animals was removed using...
Levels were measured. GLB was administered orally as a suspension in saline at the interval of 0, 2, 4, 6, 8, 12, and 24 h and blood glucose levels were estimated at intervals mentioned above. In both the cases each animal served as its own control and the hypoglycemic response was calculated by taking the difference in glucose levels at the 0 hour and subsequent hours. For the untreated group (n = 6) of animals, after overnight fasting, the glucose levels were estimated by taking blood samples at 0, 6, 12 and 24 h [40].

Studies in diabetic rats

The rats were fasted for 30 h and later rendered diabetic by an intraperitoneal injection of streptozocin (50 mg/kg body weight) in pH 4.5 citrate buffer. The blood glucose was measured after 24 h and rats with blood glucose levels >250 mg/dL were selected. The experimental protocol used in normal rats was followed in the assessment of hypoglycemic activity. The rats were divided into 3 groups (n = 6). The rats were treated as follows, Group I: oral administration of suspension of the marketed GLB, Group II: GLB (single drug) Proniosomal gel (PNG), and Group III: OCPG (ATN and GLB combination) was applied after application of Dermaroller on the previously shaven dorsal side of rats. An adhesive tape was rolled over the gel formulation to fix it securely to the site of application. The blood glucose level of each rat was measured at the interval of 0, 2, 4, 6, 8, 12, and 24 h using the One Touch glucometer [41].

Antihypertension activity of OCPG in rats

Methyl prednisolone acetate (20 mg/kg/week) for 3 w was administered via the subcutaneous injection to 24 Wistar rats (n = 6) for the induction of hypertension, and the SBP of these rats was measured by tail-cuff method (NIBP system IN125/R; AD Instrument Pvt. Ltd., Australia) and rats with the systolic pressure (SBP) >130 mmHg were then selected for the experiment [42, 43]. Hypertension was successfully induced in all rats. The rats were treated as follows, Group I: oral administration of suspension of the marketed ATN, Group II: ATN (single drug) Proniosomal gel, and Group III: OCPG (both drugs - ATN and GLB) was applied after application of Dermaroller on the previously shaven dorsal side of rats. An adhesive tape was rolled over the gel formulation to fix it securely to the site of application. SBP levels were measured just before and at 0, 2, 4, 6, 8, 12, and 24 h using the tail-cuff system [44, 45].

Data analysis

Data were expressed as the mean±standard deviation of the mean, and statistical analysis was carried out by employing the one-way analysis of variance (ANOVA). A value of p<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

In this study, various formulations were prepared containing GLB and ATN combination given in the design. The optimum formulation of Proniosomal gel was chosen dependent on the criteria of achieving the desirable value of vesicles size, entrapment efficiency of GLB and entrapment efficiency of ATN by applying numerical point prediction method. The OCPG formulation gives the experimentally observed values of vesicles size of 559 nm and entrapment efficiency of GLB is 97.03±1.43% and entrapment efficiency of ATN is 97.30±1.62%. These experimental values of vesicles size, entrapment efficiency of GLB and entrapment efficiency of ATN yielded by the OCPG Formulation were found in agreement with the predicted value of vesicles size [562±17.223 nm], entrapment efficiency of GLB (97.13±1.47%) and entrapment efficiency of ATN (97.42±1.85%) respectively created by design expert software, recommending that the optimized formulation was rational and reliable. The formulation composition with Surfactant (Span 60) (830.272 mg), Cholesterol (191.219 mg) and Phospholipid (900.00 mg) were found to satisfy the essentials of an optimized combination proniosomal gel (OCPG) formulation. This formulation was successful in permitting the transdermal permeation of GLB and ATN.

Ex vivo permeation study by using different skins

Following the preparation of proniosomal gel loaded with OCPG, the ex vivo permeation behavior through different skins was studied. The release profile from the transdermal gel loaded with OCPG was superior to normal patches. More than 2-fold increase in the cumulative amount of drug permeated was noticed from the OCPG when compared to pure drug films, which is evident from the values given in table 3. This finding was also confirmed by the values of the steady-state flux (Jss). It is obvious that OCPG formulation demonstrated the greatest flux over the rabbit skin and rat skin (0.221±0.04 mg/cm²/h and 0.583±0.02 mg/cm²/h, 0.229±0.06 mg/cm²/h and 0.588±0.04 mg/cm²/h) of GLB and ATN respectively compared with goat skin (0.186±0.03 mg/cm²/h and 0.516±0.02 mg/cm²/h), which is similar to findings of Imam et al. [46] and Akharr et al. [47]. The permeability coefficient (Ke) valuations for OCPG were 0.0221 cm/min and 0.0233 cm/min, 0.0229 cm/min and 0.02352 cm/min of GLB and ATN respectively from rabbit and rat skin which is greater than goat skin (0.0186 cm/min and 0.0206 cm/min). The permeation profiles of two drugs from different skins were shown in fig. 1 and 2. The permeation of GLB and ATN from the prepared gel either contains a pure drug or OCPG, was in favor of the Higuchi-diffusion model. According to the Korsmeyer-peppas model, the calculated n-values were greater than 0.45 but less than 0.89, indicating non-Fickian or anomalous release, which refers to a combination of both diffusion and erosion controlled-drug release mechanism. These results are in good agreement with previous work for a combination of drugs that have been loaded with a transdermal delivery system [45, 48].

Fig. 1: In vitro skin permeation profile of GLB from OCPG using different skins
Fig. 2: Ex vivo skin permeation profile of ATN from OCPG using different skins

Table 3: Ex vivo permeation data of OCPG for different skin

| S. No. | Ex vivo permeation | Flux (mg/cm²/h) | n-values |
|--------|-------------------|----------------|----------|
|        |                   | GLB            | ATN      | GLB    | ATN    |
| 1      | Rabbit Skin (OCPG) | 0.221±0.04     | 0.583±0.02 | 0.608  | 0.650  |
| 2      | Rat Skin (OCPG)   | 0.229±0.06     | 0.588±0.04 | 0.575  | 0.642  |
| 3      | Goat Skin (OCPG)  | 0.186±0.03     | 0.516±0.02 | 0.595  | 0.493  |
| 4      | Control (Patches) | 0.175±0.03     | 0.399±0.02 | 0.686  | 0.590  |

Data for each response is presented in mean±SD (n=3)

Skin irritation studies

Based on the results, the skin irritation investigation depicted the non-irritancy of the developed OCPG applied. Results showed that the prepared OCPG was safe to be used for transdermal route. No obvious erythema, edema or inflammation was observed on the Male Newzeland Rabbit skin after one week of application of the selected formulation [49].

High-performance liquid chromatography (HPLC) of GLB and ATN

An isocratic LC method, coupled with PDA detection, was developed for the simultaneous determination of ATN and GLB. Chromatogram A and chromatogram B represents the blank mobile phase and an average retention time of 2.322 min for GLB and 3.260 min for ATN, with no interfering peaks, respectively in fig. 3. According to ICH guidelines (International Council for Harmonisation), this method was validated. The validation characteristics were addressed in our earlier work Anitha et al., [35].

Pharmacodynamic activity

Antidiabetic activity of OCPG in rats

Studies in normal rats

Hypoglycemic effect was significant in oral and OCPG applied group when compared with the untreated group (fig. 4). The untreated group of animals did not show any noticeable hypoglycemia. Oral GLB produced a decrease of 40.7±5.4% in blood glucose levels after 2 h. In the case of OCPG, the maximum response was observed after 8 h (30.4±5.4%) and thereafter remained stable up to 24 h (42.4±5.6%) and the hypoglycemic response was gradual. The blood glucose levels declined after 8 h and were only 5.5±4.3% after 24 h in an orally treated group, which is similar to findings mentioned in Sridevi et al., [39].

Fig. 3: HPLC chromatograms of mobile phase containing 10 µg/ml GLB and 25 µg/ml ATN (chromatogram B)
Studies in diabetic rats

Results obtained from the diabetic rats after application of OCPG, GLB PNG and GLB oral administration are shown in (fig. 5). Blood Glucose levels obtained by from diabetic rats showed significant and almost similar hypoglycemic activity up to 24 h. The hypoglycemic effect produced by OCPG and GLB PNG in the rats is significantly less when compared to oral administration in the initial 4 h. The observed effect was found to be significant in case of OCPG treated animals (P<0.001), when compared to orally treat animals up to 24 h. The GLB (oral) produced a decrease in blood glucose level up to 90.12±9.23 mg/dL (P<0.05) for 4 h. In case of OCPG and GLB PNG, the hypoglycemic response was gradual. A maximum hypoglycemic response was observed after 8 h and remained stable up to 24 h (fig. 5). The plasma insulin level was elevated to the maximum in oral, transdermal proniosomes (OCPG and GLB PNG) treated groups at 4 h and 12 h, respectively as similar to the findings mentioned in Vijayan et al., [50].

Antihypertension activity of OCPG in rats

During antihypertensive study, hypertension was induced in all rats in a successful manner after subcutaneous injection of methyl prednisolone acetate (20 mg/kg/week for 3 w). There is a significant difference in SBP values in pre-and post-treatment with methylprednisolone acetate in Wistar albino rats. The changes in systolic blood pressure (SBP) (mm Hg) after oral administration of marketed ATN, ATN PNG and OCPG in methylprednisolone acetate-induced hypertensive wistar rats are presented in fig. 6. An early drug action was observed in Group 1 after oral administration of ATN; hypertension was significantly reduced to normal value with the maximum reduction in SBP (110.67±3.35 mm Hg) observed at 2 h. After 6 h of oral administration, SBP progressively started to rise and reached up to 125.50±4.62 mm Hg at 12 h and reached 140.83±3.12 mm Hg at 24 h (P<0.05) after oral administration. The observed effect was found to be significant in the case of OCPG treated rats (P<0.02), when compared to orally treat rats up to 24 h. On the other hand, ATN PNG and OCPG steadily decrease the SBP and maximum action of the drug observed after 4 h after transdermal application (SBP 115.50±4.26 mm Hg) in hypertensive rats, which is similar to findings in Abdul Ahad et al., [51]. The SBP lowering effect of proniosomal gel formulation was sustained and maintained up to the complete duration of study i.e. 24 h. It is concluded that the developed OCPG formulation released the drug gradually and efficaciously controlled the SBP in rats up to 24 h.
efficient than oral administration in rats. However, ex vivo addition to reversing complications of diabetes mellitus more showed better control of high blood sugar and hypertension, in contrast to the oral group but also an effective approach for Transdermal route not only effectively maintained normoglycemic hypertensive patients. This study showed that proniosomal transdermal administration of a combination of GLB and ATN showed better control of high blood sugar and hypertension, in addition to reversing complications of diabetes mellitus more efficiently than oral administration in rats. However, ex vivo assessment, pharmacokinetics and pharmacodynamics of these systems in human volunteers is required to confirm these results.

CONCLUSION
Transdermal route not only effectively maintained normoglycemic levels in contrast to the oral group but also an effective approach for treating its Comorbidities i.e., hypertension in combination, which produced remarkable hypoglycemia and controlled SBP, which is an indication that a similar episode might be prevented in diabetic hypertensive patients. This study showed that proniosomal transdermal administration of a combination of GLB and ATN showed better control of high blood sugar and hypertension, in addition to reversing complications of diabetes mellitus more efficiently than oral administration in rats. However, ex vivo assessment, pharmacokinetics and pharmacodynamics of these systems in human volunteers is required to confirm these results.

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AUTHORS CONTRIBUTIONS
All the author has contributed equally.

CONFLICTS OF INTERESTS
Declared none

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