Original research papers

# Evaluation of *in vivo* Synergistic Hypoglycemic & Hypolipidemic Activity of Ethanolic Extract of *Calotropis gigantean* Leaves in Combination to Metformin in Alloxan Induced Rats .


## ABSTRACT:

**Aim:** The present study was designed to investigate the antidiabetic & hypolipidemic activity of *Calotropis gigantean* (Family: Apocynaceae) in alloxan-induced diabetic rat model.

**Study Design:** *In vivo* study was carried out by ethanolic leaf extract was administered in 250mg/kg body weight concentration and then subjected to different rats models to authenticate the antidiabetic and hyperlipidimic properties of the plant.

**Place and Duration of Study:** Department of Pharmacy, Southeast University, Banani, Dhaka-1213,Bangladesh within a period of July 2018 to December, 2018.

**Methodology:** Diabetes was induced in rats by an intraperitoneal injection (i.p) of alloxan (100 mg/kg B.W). Ethanolic leaf extract of *C. gigantean* (250 mg/kg B.W) was administrated orally as a single dose per day to the diabetic rats for 7 days. The negative control group received 0.5 ml of sterile normal saline water orally & positive control group received metformin orally. Synergistic effect of plant was evaluated by combination with 100 mg/kg B.W & 50 mg/kg B.W oral administration of metformin. After 7 days study period, fasting blood glucose, total cholesterol, triglyceride, high-density lipoprotein cholesterol, liver weight & body weight were measured only for diabetic group to observe the effects of diabetes induction.

**Results:** Individual plant extract (250 mg/Kg B.W) & Metformin (100mg/kg B.W) reduced FBG significantly by 52% ($P$<0.001) & 55.3% ($P$<0.001) correspondingly. Metformin (100mg/kg B.W) potentiated reduction (68%) ($P$<0.001) when combined to plant extract (250 mg/Kg B.W). Significant dose dependent manner was followed when metformin (50 mg/kg B.W) was combined to plant extract (250mg/Kg B.W). Our results clearly suggests that *C. gigantean* exhibit hypoglycemic & hypolipidemic activity with an alteration in body-liver weight. The present study also suggested to develop a combination therapy of extract along with metfromin in different doses to minimize the intake of synthetic drug. Significant reduction of TG, TC were noted by extract (250 mg/kg B.W) with 32.42% ($P$<0.001) & 41.32% ($P$<0.001) respectively where standard shown the diminution 43.43% ($P$<0.05) & 47.21% ($P$<0.001) respectively as compare to Untreated diabetic rats. 50.21% ($P$<0.01) & 42.38% ($P$<0.001) reduction of TG & TC were estimated by *C.gigantea* extracts (250 mg/kg B.W) when combined with Metformin (100 mg/kg B.W). 34.53% ($P$<0.05) & 41.54% ($P$<0.001) reduction of TG & TC by *C.gigantea* extracts (250 mg/kg B.W) were confirmed when combined to Metformin (50 mg/kg B.W). Combination therapy also has shown synergistic effect in elevation of plasma HDL-cholesterol.

**Conclusion:** The results of the study concluded that *C. gigantean* have potential antidiabetic and antioxidant properties.



***Keywords:*** *Calotropis gigantean, diabetes mellitus, hypolipidemic activity & antidiabetic activity.*


## 1. BACKGROUND

As Diabetes mellitus is a public health challenge the complications are raising day to day life. According to World Health Organization the diabetic population is likely to increase up to 300 million or more by the year 2025 [1]. Diabetes mellitus (DM) is a severe physiological problem being one of the major causes of death all over the world, and if not treated, it can lead to many complications [2] such as long term damage, dysfunction, and failure of various organs [3]. This disease is caused by the destruction or dysfunction of pancreatic of β-cell and insulin resistance which results in elevating blood glucose level, known as hyperglycemia [4, 5]. Aldose reductases, a key enzyme in the polyol pathway catalyze the glucose to be reduced to sorbitol. Accumulation of sorbitol in the body causes various complications [6]. Over time, diabetic patients with poor glycemic control undergo various life threatening difficulties which include nephropathy, retinopathy, neuropathy, and cardiovascular diseases [7]. Alongside with exercise, modern drugs such as pioglitazone, biguanides, meglitinides, thiazolidinedione, alpha glucosidase inhibitors and sulphonylureas shows considerable benefits with side effects like hypoglycemia, GIT disturbance, , water intoxication, and hyponatremia, obesity when used for long term [8]. Numerous agents that are currently used for the treatment of type 2 diabetes are facing limited efficacy and tolerability [9]. For instance, sulfonylureas induce β-cell death in isolated rodent and human islets while glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors have potential risks for pancreatitis, pancreatic, and thyroid cancers [10]. Alone some synthetic drugs have various side effects due to its high dose, low solubility, low bioavailability [11, 12]. So, it is important to deliver the synthetic drugs along with the natural supplement to overcome their problems. In this scenario, combination therapy is expected to reduce the dosage regimen such that the cost of the treatment and associated adverse events are reduced considerably [13].

Now a days medicinal plants show the proof to be used as hypoglycemic agent as most of plants contain glycosides, alkaloids, terpenoids, flavonoids, carotenoids etc [14]. that significantly posses antidiabetic effect. Antihyperglycemic activity of the plants is mainly due to their ability to restore the function of pancreatic tissues by causing an elevation in insulin output or hindering the intestinal absorption of glucose, facilitating of metabolites in insulin dependent or amylase and glucosidase inhibitor as these enzymes are responsible for breaking *α*- 1, 4 bonds in complex carbohydrate to elevate FBGL [15].

*Calotropis gigantean* (Family: Apocynaceae) is a common weed in open waste ground, roadsides, village surroundings and railway lines. *It* is native to continental Asia and South-East Asia and has been introduced in the Pacific Islands, Australia, Central and northern South America and Africa [16]. Also known as crown flower, crown plant, giant milkweed or rubber bush [17]. Different parts of the plant contains stigmasterol, ß-sitosterol [18], mudarine, glycosides (calotropin uscharin, calotoxin), lupeol, calotropin, uscharin, calotoxin, calactin and uscharidin; gigantin, protease such as calotropin DI and DII and calotropin FI and FII [19]. Calotropnaphthalene, calotropises juiterpenol, calotropisesterterpenol and calotropbenzofuranone along with sucrose, have been isolated from *C. gigantean* [20]. Traditionally different parts of the plant are used such as in leprosy, eczema, syphilis, elephantiasis, ulceration, cough [21], purgative, gastrointestinal irritant, abortion inducer [22], paralysis, swellings, intermittent fevers, asthma, anorexia, helmintic infections, inflammations, cutaneous infections, intestinal worms, ascites, bronchitis, dyspepsia (promotes gastric secretions) [23], poisonous snake or rat bites, periodic fever, ulcers, cures dental problems, gonococcal arthritis and other rheumatic complaints[24]. The plant proves to hold some pharmacological effects like proteolytic activity [25][26], antiamoebic [27] wound healing [28], hepatoprotective [29] and anti-oxidant [30] properties. Other reported potentials are analgesic activity [31], antimicrobial [32] and cytotoxic activity [33], anti-diarrhoeal activity, anti-candida activity [34], anti-pyretic activity [35], insecticidal activity [36], CNS activity [37], pregnancy interceptive properties [38] and procoagulant activity [39]. *C. gigantea* is reported to possess major phytochemical groups as alkaloids, cyanogenic, glycosides, phenolics, tannins [40], cardenolides, ester [41,42], flavonoids [43], terpenes [44] (antimosquito larvicidal activity), sterols (campesterol ,stigmasterol, gamma-sitosterol,

desmosterol) with anticervical cancer property), proteinases [45] and nonprotein amino acid [46]. Acetates, benzoates, α-and β-calotropeols, β-amyrin, tetracyclic triterpene compounds, traces of sterols, giganteol acetate and giganteol are also reported from this plant [47].

Therefore, the aim of this study was to find out the scientific basis of the use *C. gigantean* in the management of diabetes & hyperlipidemia used by traditional practitioners using ethanol extracts on alloxan-induced diabetic mice.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Animals

30 Long Evan rats with (gender: male, wg: 80±10g) were obtained from ICDDR, B (International Centre for Diarrhoeal Disease Research, Bangladesh) Mohakhali, Dhaka, Bangladesh. Rats were housed under standard laboratory conditions (22-25$^0$C, humidity 40-60%,12 hr light:12 hr dark cycle) and housed in standard size metallic cages (5 rats/ cages) in properly ventilated room. Through the experiments all rats were fed with standard laboratory diet. Prior to the beginning of the study, animals were allowed for two weeks to acclimatize to laboratory conditions.

### 2.2 Collection of Plant Material and Preparation of Extracts

*C. gigantea* plant was collected from the natural population growing in the Gazipur, Dhaka, Bagladesh & authenticated by the expert taxonomist from Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh (Accession number: 45130). Leaves were washed and shade dried for several days followed by grinding using mechanical grinder. About 200 gm dried powder were soaked in 800 ml ethanol and kept for a period of about 7 days with occasional shaking and stirring. The whole mixture is then filtered through Whatman No.1 filters paper and concentrated by a rotary evaporate under reduced pressure at 50$^0$C temperature to afford crude extract with gummy or semisolid appearance. The concentrate was stored in an airtight container and kept in a cool, dark and dry place until the next course of action.

**Figure:** *Calotropis gigantea*

### 2.3 Chemicals

Alloxan and metformin were purchased from Sigma-Aldrich and Merck company (Germany) respectively. All other used chemicals were of analytical grade and were obtained from standard commercial suppliers.

## 2.4 Induction of Diabetes

Diabetes was induced in overnight fasted Evan rats by single-dose intraperitoneal injection of freshly prepared alloxan at 140 mg/kg body weight dissolved in 0.5 ml of sterile normal saline water and drink 10% glucose solution to overcome drug induced hypoglycemia. After 72 hours blood glucose level was measured by using tail blood sample. Rats with fasting blood glucose level above 7.0 mmol/L were selected for further study.

## 2.5 Experimental Design

Long Evan rats were randomly assigned into group I, II, III, IV, V, VI (n=5 ) for 7 days treatment due to determination of blood glucose, lipid profile tests.

**Group I:** Non Diabetic Normal Control (Only water & normal diet)

**Group II:** Diabetic Control (Only water & normal diet)

**Group III:** Diabetic Control+ Metformin (100 mg/kg B.W in 0.5 ml 99% DMSO (Dimethyl sulfoxide))

**Group IV:** Metformin(50 mg/kg B.W) + Ethanolic Extract of *C.gigantea* (250mg/kg B.W in 0.5 ml 99% DMSO)

**Group V:** Metformin (100 mg/kg B.W) + Ethanolic Extract of *C.gigantea* (250 mg/kg B.W in 0.5 ml 99% DMSO)

**Grroup VI:** Diabetic Control+ Ethanolic Extract of *C.gigantea* (250 mg/kg B.W in 0.5 ml 99% DMSO)

## 2.6 Collection of blood and determination of Biochemical Parameters

A long term use of alloxan can be toxic and may cause the loss of many animals due to tubular cell necrotic toxicity in kidney. For this reason a 7days study has been carried out for clinical trial on animal [48]

At $0^{th}$, $3^{th}$, $5^{th}$ & $7^{th}$ day, blood samples were collected from tail vein after the administration of metformin & ethanolic extract of *C.gigantea* and blood glucose levels were determined by using by glucose meter. After completing the one week treatment the rats were at first anesthetized with chloroform and 3 ml of blood was directly collected from heart by syringe. Immediately after blood samples collection, serum was isolated by centrifugation at 4000 rpm for 20 min and then analyzed for various biochemical parameters. The serum samples were stored at −80 °C in a freezer until they were analyzed. The concentration of TC, TG, HDL-Cholesterol were measured colorimetrically [49] by blood analyzer using commercially available wet reagent diagnostic kits (HUMAN GmbH, Germany).

## 2.7 Statistical Analysis

The results were expressed as mean ± SD. Data analysis was performed by the SPSS (Statistical Package for the Social Sciences) version 20 using one-way analysis of variance (ANOVA) and Dunnett's test. To assess the individual variations between the control and treatment groups, $P \leq 0.05$ was considered significance level.

## 3. RESULTS

### 3.1 Antidiabetic Activity:

**Table 01: Effect of *C. gigentea* on fasting blood glucose levels in alloxan induced diabetic rats.**

| Fasting Blood Glucose Level (FBGL) (mmol/l) | | | | |
|---|---|---|---|---|
| **Animal Grouping** | **Day 1** | **Day 3** | **Day 5** | **Day 7** |
| **Control** | 4.3±0.29 | 3.9±0.37 | 3.7±0.20 | 4.02±0.26 |
| **Untreated diabetic** | 8.02±0.53 | 9.05±1.02 (12.84) | 13.6±1.02 (69.58) | 15.00 ±3.15 (87.03) |
| **Diabetic+Metformin (100 mg/kg B.W)** | 9.6±0.98*** | 6.07±0.37*** (36.77) | 5.62±0.07*** (41.46) | 4.30±0.07*** (55.21) |
| **Metformin (100 mg/kg B.W)+Extract (250 mg/kg B.W)** | 8.6±0.37*** | 6.2±0.12*** (27.91) | 5.44±0.17*** (36.74) | 2.75±0.35*** (68.02) |
| **Metformin (50 mg/kg)+Extract (250 mg/kg B.W)** | 8.62±0.28*** | 7.2±0.12*** (16.47) | 5.92±0.09*** (31.32) | 3.48±0.37*** (59.63) |
| **Extract (250 mg/kg B.W)** | 8.74±0.46*** | 6.98±0.24*** (20.14) | 5.76±0.29*** (34.1) | 4.18±0.24*** (52.17) |

Values are expressed as mean ± SD ($n$ = 5 rats). Significance level among different groups at $P \leq 0.05$. ($^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$); Diabetic rats were compared with normal rats. **Metformin** and ***C.gigantea*** treated diabetic rats were compared with diabetic rats.

At all-time points, blood glucose concentration remain unchanged in normal rats treated with distilled water. Table 01 indicates gradual decrease of FBGL for each group at 1st, 3rd, 5th & 7th day. The FBGL of all groups were compared to untreated diabetic group. At 7th day, oral administration of *C.gigantea* extracts (250 mg/kg B.W) significantly decreased the blood glucose level 52.17% ($P<0.001$). Combination therapy was performed to establish synergistic effect with two doses. Metformin (100 mg/kg B.W) has shown significant FBGL reduction by 55.21% ($P<0.001$) individually but potentiated reduction (68.02%) ($P<0.001$) when combined to plant extract (250 mg/Kg B.W). Dose dependent manner (59.63%) ($P<0.001$) was followed when metformin (50 mg/kg B.W) was combined to plant extract (250 mg/Kg B.W) with reduced dose. The possible mechanism by which *C.gigantea* brings about its hypoglycemic action may be stimulating the insulin effect of serum by increasing either the pancreatic secretion of insulin from the beta - cells of islets of langerhans or its release from bound insulin. Thus, the significant antidiabetic effect of the extracts could be due to the presence of the flavonoids, tannin and alkaloid in the extracts, which could act synergistically and/or independently to enhance the activity of glycolytic enzymes.

### 3.2 Hypolipidemic Activity:

**Table 02: Effect of *C.gigentea* on lipid profile in alloxan induced diabetic rats.**

| | Liver Weight (mg/gm) | Lipid profile (mmol/l) | | |
|---|---|---|---|---|
| **Animal Grouping** | | **TG** | **TC** | **HDL-C** |
| **Control** | 4.73 | 3.75±0.95 | 8.86±0.95* | 8.12±0.41 |
| **Untreated diabetic** | 4.15*** (12.26) | 4.72±0.64*** 25.87 | 9.51±0.76*** 7.34 | 5.43±0.20*** 33.13 |
| **Diabetic+ Metformin (100 mg/kg B.W)** | 4.49* (8.19) | 2.67±0.29*** (43.43) | 5.02±0.12*** (47.21) | 6.78±0.415** (24.86) |
| **Metformin (100 mg/kg)+Extract (250 mg/kg B.W)** | 4.61** (11.08) | 2.35±0.37** (50.21) | 5.48±0.46*** (42.38) | 6.81±0.26** (25.23) |
| **Metformin (50 mg/kg B.W)+Extract (250 mg/kg B.W)** | 4.31** (3.86) | 3.09±0.49 (34.53)* | 5.56±0.62*** (41.54) | 6.64±0.98** (22.28) |
| **Extract (250 mg/kg B.W)** | 4.25*** (2.41) | 3.19±0.40 (32.42)* | 5.58±0.35*** (41.32) | 6.60±0.415** (21.55) |

Values are expressed as mean ± SD (*n* = 5 rats). Significance level among different groups at $P \leq 0.05$. ($^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$); Diabetic rats were compared with normal rats. **Metformin** and ***C.gigantea*** treated diabetic rats were compared with diabetic rats.

**Table 03: Effect of *C.gigentea* on mean weight of liver in alloxan induced diabetic rats.**

| Groups | Mean weight of animals at sacrificial time (Grams) | Mean weight of Liver at sacrificial time (Grams) | Weight of liver in grams/kg body weight |
|---|---|---|---|
| Control | 110 | 4.73 | 43 gm/kg |
| Untreated diabetic | 95 | 4.15 | 43.68 gm/kg |

After alloxan induced, the result showed that TG, TC increased while HDL decreased compare to Untreated diabetic rats **(Table: 02).** Highest reduction of TG, TC were shown by Metformin (100 mg/kg B.W) like 43.43% ($P<0.05$) & 47.21% ($P<0.001$) respectively where extracts shown significant diminution by 32.42% ($P<0.001$) & 41.32% ($P<0.001$) respectively. Combination study of Extract to Meformin was performed to develop the synergistic effect with different doses in dose dependent activity. Metformin (100 mg/kg B.W) with *C.gigantea* extracts (250mg/kg B.W) reduced TG & TC by 50.21% ($P<0.01$) &

42.38% (*P*<0.001) respectively & metformin (50 mg/kg B.W) with *C.gigantea* extracts (250mg/kg) lessened TC & TC level by 34.53% (*P*<0.05) & 41.54% (*P*<0.001) The administration of the extract of *C.gigantea* produced a significant increase in the level of High-density lipoprotein-cholesterol (HDL-C) in individual & combination groups. Individual extract showed elevation of HDL-C by 21.55%. Metformin (100 mg/kg B.W) with *C.gigantea* extracts (250mg/kg B.W) increased 25.23% & metformin (50 mg/kg B.W) with *C.gigantea* extracts (250mg/kg B.W) increased 22.28% of HDL-C.

Significant decrease of liver weight was revealed in diabetic rat (12.26%) (*P*<0.001) as compared to control **(Table: 03).** Liver weight was slightly increased by1.58% (*P*<0.001) with 43.68 gm/kg B.W in diabetic rats when compared with non-diabetic rats (43.00 gm/kg B.W). The observed significant reduction in serum total lipids, total cholesterol and LDL cholesterol by the extract which can attribute the presence of phytochemical constituents like flavonoid [50] which is a active biological principle of most medicinal plants with hypoglycemic and antidiabetic activities.that propose the cardioprotective features with prevention of cardiovascular complications arising from hyperlipidemia [51].

## Discussion

New antidiabetic drugs from natural plants are already in search that contain phytochemical compounds with high efficacy with minimum toxicity. As most of plants contain glycosides, alkaloids, terpenoids, flavonoids, carotenoids, *etc.*, that are significantly posses antidiabetic effect [12] Plant extracts are evaluated to balance the liberation and absorption of glucose is becoming a striking therapeutic choice in the treatment of diabetes mellitus.

Alloxan, a beta cytotoxic agent, rapidly and selectively accumulates in pancreatic beta cells] and causes beta cell death and apoptosis by generation of reactive oxygen species (ROS), super oxide radicals and hydrogen peroxide [52]. Sequential injection of alloxan caused a significant increase in blood glucose concentration for 7 days in all group of rats compared with their respective baseline blood glucose and to control values. Single & combination therapy was performed to establish synergistic effect with two doses of metformin for 7 days. The estimated results were taken after $7^{th}$ days. Individual plant extract & standard reduced FBG significantly by 52% (*P*<0.001) & 55.3% (*P*<0.001) correspondingly. Metformin (100mg/kg B.W) potentiated reduction (68%) (*P*<0.001) when combined to plant extract (250 mg/Kg B.W). Significant dose dependent manner was followed when metformin (50 mg/kg B.W) was combined to plant extract (250mg/Kg B.W) with reduced dose. This results can led to a development of new drug design with reduced dose of standard when taken with leaf extract of *C.gigantea*. It can be due to probable reduced absorption of glucose from the small intestine as glucose liberation from disaccharides is reduced. In our study, it is found that extract have hypoglycemic effect in glucose induced hyperglycemic rats.

Hyperlipidemia is a recognized outcome of Diabetes mellitus [53]. Abnormal high concentration of serum lipids result from increase in the mobilization of free fatty acids from the peripheral storehouse. The marked hyperlipidaemia that characterizes the diabetic state is the consequence of the dysfunction of lipolytic hormones on the fat depots [54]. Hyperlipidemia associated with diabetes mellitus is reduced by limited absorption of free fatty acids and free cholesterol following inhibition of pancreatic lipase and pancreatic cholesterol esterase [55]. When compare to untreated diabetic rats significant reduction of TG & TC were noted by extract (250 mg/kg B.W) with 32.42% (*P*<0.001) & 41.32% (*P*<0.001) respectively where Standard shown the diminution by 43.43% (*P*<0.05) & 47.21% (*P*<0.001). 50.21% (*P*<0.01) & 42.38% (*P*<0.001) reduction of TG & TC were studied by *C.gigantea* extracts (250 mg/kg B.W) when combined to Metformin (100 mg/kg B.W). Dose dependent manner was followed by the extract (250

mg/kg B.W) when combined with metformin at lower dose (50 mg/kg B.W) where TG & TYC were lessened by (34.53%) ($P$<0.05) & (41.54%) ($P$<0.001) reppectively. Highest reduction of TG & TC were shown by Metformin (100 mg/kg B.W) like 43.43% ($P$<0.05) & 47.21% ($P$<0.001) respectively where extracts shown significant diminution by 32.42% ($P$<0.001) & 41.32% ($P$<0.001) respectively. Combination study of Extract to Meformin was performed to develop the synergistic effect with different doses in dose dependent activity. Metformin (100 mg/kg B.W) with *C.gigantea* extracts (250mg/kg) reduced TG & TC by 50.21% ($P$<0.01) & 42.38% ($P$<0.001) respectively & metformin (50 mg/kg B.W) with *C.gigantea* extracts (250mg/kg) lessened TC & TC level by 34.53% ($P$<0.05) & 41.54% ($P$<0.001) The administration of the extract of *C.gigantea* produced a significant increase in the level of High-density lipoprotein-cholesterol (HDL-C). The plant demonstrated a cardioprotective effect via an increase in HDL-cholesterol levels. Combination therapy also shown synergistic effect in elevation of plasma HDL-cholesterol that prevent risk of developing cardiovascular disease.

The present study has shown related reduction of liver weight according to the dose of studied sample & standard in individual & combination design. The liver is an insulin-sensitive organ that undergoes functional abnormalities in individuals with untreated diabetes [56]. In this study, the liver of diabetic animals & control animals were compared. An increase (hypertrophy) in the weight of liver in proportion to the body weight was observed despite the reduction of the mean weight of all the animals in Alloxan induced group. It could be ascribed to increased triglyceride accumulation that can lead to liver enlargement by reason of increased entry of fatty acids into the liver induced by hypoinsulinemia [57] and the less elimination of lipoprotein from liver. Previous research articles also present the same agreement with the present findings [58].

## 4. Conclusion

In the present study, reduction in the concentration of glucose, TG (total triglyceride), TC (total cholesterol) and increase in HDL cholesterol were observed for ethanolic extract of *C.gigantea* leaves. Synergistic effect was estimated in combination with metformin. The results propose the probability of dose reduction of synthetic drug with required pharmacological activity when taken with *C.gigantea* leaves. The antidiabetic and hypolipidemic activity of the plant source is due to the phyto chemical constituents present in the plant. This study justifies ethnomedicinal use of the plant and can be exploited in the management of diabetes induced hyperlipidemia. Further studies are in progress for isolation and identification of lead compound to design a combination therapy in conjunction with synthetic drug.

## Ethical Considerations

This was carried out in strict compliance with the National Research council guidelines on the care and use of laboratory animals to minimize research animal pain and suffering [59].

**Consent:** NA

## Conflict of Interests

The authors declare that they have no conflicts of interest.

## Data Availability

The data used to support the findings of this study are included within the article.

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