Anti-diabetic potentials of stem-bark extracts of *Terminalia avicennioides* on alloxan-induced diabetic rats

SF Yahaya¹*, MM Suleiman¹, A Mohammed² & NDG Ibrahim³

1. Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria
2. Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University Zaria, Nigeria
3. Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria

*Correspondence: Tel.: +234 8032310885; E-mail: sfyahaya22@gmail.com*

Abstract

This study evaluated the extracts of *Terminalia avicennioides* stem-bark for their effect on alloxan-induced diabetes mellitus in male wistar rats. The powdered stem-bark of the plant was extracted with 70% methanol to yield crude methanol extract (CME). The CME was dissolved in distilled water to obtain the aqueous methanol (AME), then partitioned using ethyl acetate and hexane to obtain ethyl acetate (EAE) and hexane (HEX) extracts respectively. Fifty five alloxan induced diabetic rats were randomly divided into 11 groups of five rats each. Rats in groups 1 and 2 received distilled water (DW) and 1% Tween 80 (TW80) at 5 ml/kg, respectively. Rats in group 3 received glibenclamide (GLB) 10 mg/kg. Rats in groups 4, 5, 6 and 7 were administered with 100 mg/kg of CME, AME, EAE and HEX, respectively. Similarly, rats in groups 8, 9, 10 and 11 were given the extracts at 200 mg/kg, respectively. In addition, three normoglycaemic rats were used as non-diabetic non-treated control (group 12). All treatments and diabetic inductions were done intraperitoneally. Treatments started 72 hours after induction of diabetes which served as day 1, then on day 4, 7, 14 and 21. Blood glucose level in all the rats was monitored weekly for three weeks. All animals were sacrificed by jugular venipuncture and serum levels of total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) and triglycerides (TGL) were determined. The extracts significantly (p<0.05) decreased the levels of blood glucose, serum total cholesterol, serum triglyceride and serum low density lipoprotein in diabetic rats when compared with the negative controls. However, HDL level was significantly (p<0.05) increased in the HEX, EAE and CME (100 mg/kg) treated rats. In conclusion, the extracts exhibited an anti-hyperglycaemic and anti-hyper lipidaemic effect which validates the use of the plant in traditional treatment of diabetes mellitus.

Keywords: Alloxan, anti-diabetic, extracts, rats, *Terminalia avicennioides*

Introduction

Diabetes mellitus is a metabolic disease characterized primarily by high blood glucose levels (Baena-Diez *et al.*, 2016), resulting from defects in insulin secretion, insulin action or both (American
Diabetes Association, 2010). It is the most common metabolic disorder. Among adults aged 20-79 years, it had a global prevalence of about 8.8% in the year 2017, and estimated to hit 9.9% by the year 2045 (IDF, 2017). It is a common disease in dogs and cats. The most common form of diabetes in dogs resembles type 1 diabetes in humans whereas the most common form in cats resembles type 2 diabetes in humans (Nelson & Reusch, 2014). It has an estimated incidence as high as 1: 66 (1.52%) for dogs and 1:800 for cats. The disease in dogs occurs most frequently in the mature or older female, and is often associated with estrus. In contrast, male cats appear to be more commonly affected than female (Kaneko et al., 2008).

Although, insulin and oral hypoglycaemic drugs have been the mainstay of the management of diabetes mellitus, there are many proven side effects for these compounds (Cole et al., 2013). Insulin treatment in type 2 diabetics can lead to weight gain (Jansen et al., 2014), pain and hypoglycaemia (Petznick, 2011). Many animals appear to be resistant to insulin, while others, especially cats are very sensitive to its effects and therefore prone to bouts of hypoglycaemia (Wallace & Kirk, 1990). Also, there is an issue of rapid insulin metabolism, whereby insulin wears off quickly in some animals. This leads to the animal requiring a second injection during the day or even additional injections during the day (Brooks, 2010; Ramsey & Maclachlan, 2013).

Currently, the use of herbal drugs is being explored in the management of diabetes mellitus (Amraie et al., 2015). Terminalia avicennioides is a yellowish brown, hard and durable wood, commonly found in the savannah region of West Africa (Mann et al., 2011). It is reported to have been used traditionally to treat a variety of diseases in both animals and humans, such as tuberculosis and cough (Mann et al., 2012) and also diabetes mellitus. This study was thus aimed at evaluating the antidiabetic effects of the stem-bark extracts of T. avicennioides in alloxan-induced diabetes mellitus in male Wistar rats.

Materials and Methods

Plant collection

Fresh stem-bark of Terminalia avicennioides was collected from the wilds around Kufena, Zaria, Nigeria, in the month of November. The flower, leaves and seeds of the plant were also collected and sent to the Herbarium, Department of Botany, Ahmadu Bello University, Zaria, Nigeria for identification, where a voucher specimen number 900239 was allocated for reference purpose.

Plant extraction and partitioning

The stem-bark of T. avicennioides was air-dried at room temperature and then pulverized. About 1kg was extracted with three litres of 70% aqueous methanol and evaporated to dryness in a water-bath at 45°C. Fifty grams of the crude methanol extract (CME) were dissolved in 500 ml of distilled water to form an aqueous methanol extract (AME). The solution was transferred to a 1L separating funnel and 600 ml of hexane were added. The funnel was agitated gently, then allowed to stand for some hours after which the lower denser aqueous portion was collected into a conical flask. The remaining upper portion was dispersed into a clean conical flask to obtain the hexane extract (HEX). The procedure was repeated 3 more times and the HEX was pooled together. The remaining aqueous portion was then transferred to a separating funnel and 600 ml of ethyl acetate were added to obtain the ethyl acetate extract (EAE). The procedure was then repeated as described for the hexane fraction.

Phytochemical screening

The crude methanol (CME), aqueous methanol (AME), ethyl acetate (EAE) and hexane (HEX) extracts were all subjected to phytochemical screening using standard procedures (Evans, 2009).

Experimental animals

Adult male wistar rats weighing an average of 170 grams were obtained from the Animal House, Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, and maintained on standard feed. Water was provided ad libitum. The animals were kept in clean iron cages at room temperature throughout the study. The beddings were changed twice every week. Approval for the use of rats was obtained from Ethical Committee on Animal Use and Care, Ahmadu Bello University, Zaria. The approval number MSc/VET MED/ 29391/2012-2013 was allocated.

Acute toxicity studies of the extracts

Acute toxicity studies of the extracts of T. avicennioides were carried out using the OECD fixed dose procedure (OECD, 2001). Sixteen rats were used for the toxicity study. Four rats were selected at random and were individually administered through the intraperitoneal route with 5mg/kg of CME, AME, EAE and HEX. The same procedure was repeated for the extracts using 50, 300 and 2000
mg/kg respectively on other groups of rats. All the rats were observed for any sign of toxicity during the first four hours and thereafter for 24 and 48 hours. All surviving animals were kept under observation for 14 days.

Experimental design
Fifty five adult male Wistar rats were randomly allocated into 11 groups of five rats each. Diabetes was induced in the experimental rats by a single intraperitoneal injection of alloxan monohydrate (150 mg/kg) dissolved in 0.9% cold normal saline solution (Tanko et al., 2014). Seventy two hours after alloxan injection, rats with plasma glucose levels of >140 mg/dl were included in the study (Owoyele et al., 2005; Ravichandra & Paarakh, 2013; Aleem et al., 2014). Rats in groups 1 and 2 received DW (5 ml/kg) and TW80 (5 ml/kg), respectively and they served as negative controls. Rats in group 3 received standard drug glibenclamide (10 mg/kg). Rats in groups 4, 5, 6 and 7 were administered with 100 mg/kg of CME, AME, EAE and HEX respectively. Similarly, rats in groups 8, 9, 10 and 11 received CME, AME, EAE and HEX at 200 mg/kg. Three normoglycaemic rats were also included in the study and they served as group 12. All treatments were administered intraperitoneally. Treatments were done on days 1(72 hours after induction of diabetes), 4, 7, 14 and 21 of the experiment. The weight of each rat was monitored and taken on each treatment day, while body weights, except those in CME 200 and GLB groups when compared with the DW group. From day 4 to 7, the groups experienced a significant (p< 0.05) increase in body weight, except AME 100 and HEX 200 groups which was statistically significant (p< 0.05) when compared to the DW group. Similarly, on day 14 there was significant (p< 0.05) increase in body weights, except those in CME 200 and GLB group that experienced a decrease in body weight by day 21 when compared to the DW group (table 1).

Evaluation of the effect of treatments on serum lipid profile
Serum total cholesterol (TC), serum high density lipoproteins (HDL), serum low density lipoproteins (LDL) and serum triglycerides (TGL) were analysed using an auto-analyser Vitalab Selectra XL, Flexor Company, The Netherlands.

Body weight changes of diabetic rats
There was a decrease in body weight of all alloxan-induced diabetic rats within the first few days of treatment, and it was significant (p< 0.05) on day 4 in all the extract treated groups, TW80, and the GLB groups when compared with the DW group. From day 4 to 7, the groups experienced a significant (p< 0.05) increase in body weight, except AME 100 and HEX 200 groups that experienced a decrease in body weight, when compared with DW control. Similarly, on day 14 there was significant (p< 0.05) increase in body weights, except those in CME 200 and GLB group that experienced a decrease in body weight by day 21 when compared to the DW group (table 1).

Statistical analysis
Data obtained were expressed as mean ± standard error of mean (SEM). Analysis of variance (ANOVA) was used, followed by Tukey’s post-hoc test for multiple comparisons of groups using Graph pad prism version 5. Values of p<0.05 were considered significant.

Results
Phytochemical screening
Carbohydrates, cardiac glycosides, saponins, triterpenes, flavonoids, tannins and alkaloids were detected in the crude methanol extract, aqueous methanol extract, and ethyl acetate extract. However, hexane extract contained only carbohydrates and cardiac glycosides.

Acute toxicity test
The extracts did not cause mortality on rats during the 14 days observation period. The extracts were considered safe up to a dose of 2000 mg/kg bodyweight.

Gross and histopathological examination
At the end of the experiment, each rat was sacrificed and the organs examined for visible gross lesions, which were recorded. The liver and pancreas were dissected out and preserved in 10% buffered formalin. Embedding was carried out a week later and the tissues were prepared for histopathology using the modified method described by Luna (1960). The slides were stained with haematoxylin and eosin and viewed under light microscope at different magnifications for any tissue or cellular morphological changes.
still maintained values below 140 mg/dl, and showed statistically significant (p< 0.05) difference from the non-treated diabetic control group. Normoglycaemic rats maintained normal blood glucose levels throughout the experiment (table 2).

**Serum total cholesterol levels of diabetic rats**
There was a significant decrease (p< 0.05) in total cholesterol levels in rats treated with all the extracts and glibenclamide (10 mg/kg). However, rats treated with CME (100 mg/kg) showed a non-significant decrease when compared to the DW group (figure I).

**Serum high density lipoprotein levels of diabetic rats**
Table 1: Mean body weight of alloxan-induced diabetic rats treated with extracts of *Terminalia avicennioides* for three weeks

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Neutral control</td>
<td>190.40 ± 2.25 179.80 ± 1.07***</td>
</tr>
<tr>
<td>Distilled water (5 ml/kg)</td>
<td>143.00 ± 2.37 106.00 ± 1.58</td>
</tr>
<tr>
<td>Glibenclamide (10)</td>
<td>141.80 ± 1.20 132.80 ± 2.10***</td>
</tr>
<tr>
<td>1% TW80 (5 ml/kg)</td>
<td>157.60 ± 2.66 154.60 ± 2.40***</td>
</tr>
<tr>
<td>CME (100)</td>
<td>147.80 ± 0.97 130.60 ± 0.68***</td>
</tr>
<tr>
<td>CME (200)</td>
<td>143.00 ± 1.79 135.00 ± 1.30***</td>
</tr>
<tr>
<td>AME (100)</td>
<td>141.60 ± 2.80 138.00 ± 1.41***</td>
</tr>
<tr>
<td>AME (200)</td>
<td>146.80 ± 2.08 123.00 ± 1.00***</td>
</tr>
<tr>
<td>EAE (100)</td>
<td>142.20 ± 0.86 134.80 ± 2.18***</td>
</tr>
<tr>
<td>EAE (200)</td>
<td>155.40 ± 2.60 127.60 ± 1.03***</td>
</tr>
<tr>
<td>HEX (100)</td>
<td>148.00 ± 2.02 131.00 ± 0.71***</td>
</tr>
<tr>
<td>HEX (200)</td>
<td>166.40 ± 2.29 134.00 ± 0.54***</td>
</tr>
</tbody>
</table>

* - Statistically significant (0.01<P<0.05)
** - Highly significant (0.001<P<0.01)
*** - Very highly significant (P<0.001)

**Table 2: Mean blood glucose levels of alloxan-induced diabetic rats treated with extracts of *Terminalia avicennioides* for three weeks**

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Neutral control</td>
<td>112.00 ± 6.53</td>
</tr>
<tr>
<td>Distilled water (5 ml/kg)</td>
<td>542.60 ± 23.83</td>
</tr>
<tr>
<td>Glibenclamide (10)</td>
<td>454.60 ± 44.24</td>
</tr>
<tr>
<td>1% TW80 (5 ml/kg)</td>
<td>528.40 ± 25.97</td>
</tr>
<tr>
<td>CME (100)</td>
<td>473.60 ± 36.14</td>
</tr>
<tr>
<td>CME (200)</td>
<td>500.00 ± 25.82</td>
</tr>
<tr>
<td>AME (100)</td>
<td>545.40 ± 23.32</td>
</tr>
<tr>
<td>AME (200)</td>
<td>517.80 ± 21.00</td>
</tr>
<tr>
<td>EAE (100)</td>
<td>472.80 ± 24.15</td>
</tr>
<tr>
<td>EAE (200)</td>
<td>537.00 ± 9.21</td>
</tr>
<tr>
<td>HEX (100)</td>
<td>551.40 ± 36.80</td>
</tr>
<tr>
<td>HEX (200)</td>
<td>510.00 ± 9.42</td>
</tr>
</tbody>
</table>

* - Statistically significant (0.01<P<0.05)
** - Highly significant (0.001<P<0.01)
*** - Very highly significant (P<0.001)
Serum triglyceride levels of diabetic rats
There was a significant (p< 0.05) decrease in all the extract treated groups when compared to the DW group except HEX (100 mg/kg) where there was an increase (figure IV).

Histopathology
In the CME treated rats, photomicrographs of the liver showed vascular congestion at (100 mg/kg) and congested sinusoids at 200 mg/kg (plate I). The pancreas showed partial regeneration of the islet cells (plate IV) at 100 mg/kg and no lesions at 200 mg/kg. In the AME treated rats, photomicrographs of the liver showed congested central vein (plate II), while the pancreas showed no observable lesions. Photomicrographs of the liver and pancreas of the rats treated with EAE (100 and 200 mg/kg) showed no observable lesions. In the HEX treated rats, photomicrographs of the liver showed slight focal hepatocyte necrosis at 100 mg/kg (plate II) and congested central vein at 200 mg/kg. The pancreas showed no observable lesions.

Discussion
The extracts of *T. avicennioides* stem bark showed no toxicity up to 2000 mg/kg. This result did not agree with the work of Abdullahi *et al.* (2001) where the intra peritoneal LD<sub>50</sub> was found to be between 871 – 917 mg/kg. A number of factors could be responsible for this variation. These include age of the plant, altitude and ecological factors. Age (Riet-Correa *et al.*, 2011) and altitude (Ganzera *et al.*, 2008) could affect the contents of active
ingredients in the plant. Liu et al. (2015), in a study confirmed that ecological factors such as annual precipitation, annual sunshine duration, soil pH, organic matter and also readily available potassium which vary based on geographical location significantly affect active ingredient contents of a plant. Similarly, Stefanucci et al. (2018), in a study using Capparis spinosa showed that geographical, climatic variations and also extraction techniques could have effect on the phytochemical composition of a plant.

In the present study, diabetes mellitus was induced using alloxan at a dose rate of 150 mg/kg as animals in the study had a blood glucose level of above 140 mg/dl. This is in agreement with the works of Owoyele et al. (2005); Aziz (2009); Ravichandra & Paarakh (2013). Loss of body weight is a major consequence of diabetes in rats (Ramachandran et al., 2012). The decrease in body weight of experimental rats in the present study is in conformity with the findings of Aziz (2009) in rats. The reduction of body weight in diabetic rats is due to dehydration and catabolism of fats and proteins (Hakim et al., 1997); increased catabolic reaction leading to muscle wasting can be the cause of the reduced body weight gain in diabetic rats.

Figure III: Effect of crude methanol (CME), aqueous methanol (AME), ethyl acetate (EAE) and hexane (HEX) extracts of Terminalia avicennioides on concentration of serum low density lipoprotein level of diabetic rats. Glibenclamide (GLB) was used as treated control, while distilled water (DW) and tween-80 (1 %) were used as untreated control. Non-diabetic and non-treated group (NT) was used as neutral. Means having different superscript letters (a, b) are significantly different (p< 0.05).

Figure IV: Effect of crude methanol (CME), aqueous methanol (AME), ethyl acetate (EAE) and hexane (HEX) extracts of Terminalia avicennioides on concentration of serum triglyceride level of diabetic rats. Glibenclamide (GLB) was used as treated control, while distilled water (DW) and tween-80 (1 %) were used as untreated control. Non-diabetic and non-treated group (NT) was used as neutral. Means having different superscript letters (a, b) are significantly different (p< 0.05).
Plate I: Photomicrograph of liver of rats induced with diabetes mellitus using alloxan (150 mg/kg) and treated with CME (100 mg/kg) showing vascular congestion (arrow A) liver of rat treated with CME (200 mg/kg) showing congested sinusoids (arrow B), liver of rat treated with DW (5 ml/kg) showing lymphocytes (black arrow C) and kupfer cells hyperplasia (white arrow C), liver of rat in neutral group showing normal histology (D) (H and E × 400)

Plate II: Photomicrograph of liver of rats induced with diabetes mellitus using alloxan (150 mg/kg) and treated with AME (100 mg/kg) showing vascular congestion (arrow A), b. diabetic rat liver treated with HEX (100 mg/kg) showing focal hepatocyte necrosis (arrow B), diabetic rat liver treated with 1% TW80 (5 ml/kg) showing lymphocytes (big arrow C) and kupfer cell (small arrow C) hyperplasia, liver of rat in neutral group showing normal histology (D) (H and E × 400)

Plate III: Photomicrograph of pancreas of rats induced with diabetes mellitus using alloxan (150 mg/kg) and treated with CME (100 mg/kg) showing partial regeneration of islet cells (arrow A), DW (5 ml/kg) showing severe necrosis of islet cells (arrow B), pancreas of rat in neutral group showing normal histology (C) (H and E × 200)
flavonoids have been isolated as part of the phytoconstituents of *T. avicennioides* stem-bark extracts. Hyperglycaemia generates glucose auto-oxidation and auto-oxidative glycosylation of proteins which leads to increased oxidative stress by increasing reactive oxygen species (Shabeer *et al.*, 2009). The increased oxidative stress results to depletion of majority of plasma antioxidants (Price *et al.*, 2001; Valabhji *et al.*, 2001; Mooradian, 2006). Expression of antioxidant enzymes is known to be very low in islet cells compared with other tissues and cells (Tiedgar *et al.*, 1997), therefore, once beta cells are exposed to oxidative stress, they are rather sensitive to its damaging effects suggesting that oxidative stress, may in part mediate toxicity of hyperglycaemia. Flavonoids, saponins and alkaloids have potent antioxidant activities. Flavonoids can exert their antioxidant activity by various mechanisms, e.g., by scavenging or quenching free radicals, by chelating metal ions, or by inhibiting enzymatic systems responsible for free radical generation (Bláha *et al.*, 2004; Dias *et al.*, 2005). Flavonoids are also known to regenerate the damaged beta cells in the alloxan induced diabetic rats and act as insulin secretagogues (Alagammal *et al.*, 2012). In another study, ellagic acid which is also found in *T. avicennioides* (Shuaibu *et al.*, 2007) exhibited anti hyperglycaemic activities. Its activity has been demonstrated in rats and the proposed mechanism of action was by increasing the peripheral utilisation of glucose and inhibiting glucose transporter activity from the intestine (Jadhav & Pushchakayala, 2012).

Hyperlipidaemia, a common complication of both clinical and experimental diabetes mellitus, is characterised by increase in serum total cholesterol, triglycerides, low density lipoprotein and a decreased high density lipoprotein (Balamurugan *et al.*, 2014). This marked hyperlipidaemia may be regarded as uninhibited actions of lipolytic hormones on fat depots (Patel *et al.*, 2012). In the present study, the stem bark extracts of *T. avicennioides* exhibited anti hyperlipidaemic effects. Its activity has been demonstrated in rats and the proposed mechanism of action was by increasing the peripheral utilisation of glucose and inhibiting glucose transporter activity from the intestine (Jadhav & Pushchakayala, 2012).

Hyperlipidaemia, a common complication of both clinical and experimental diabetes mellitus, is characterised by increase in serum total cholesterol, triglycerides, low density lipoprotein and a decreased high density lipoprotein (Balamurugan *et al.*, 2014). This marked hyperlipidaemia may be regarded as uninhibited actions of lipolytic hormones on fat depots (Patel *et al.*, 2012). In the present study, the stem bark extracts of *T. avicennioides* exhibited anti hyperlipidaemic effects. Similar anti hyperlipidaemic effects to that of *T. avicennioides* have been reported in studies using other members of the combretaceae family such as *Terminalia arjuna* (Morshed *et al.*, 2011) *Terminalia catappa* (Ahmed *et al.*, 2005), *Terminalia paniculata*, (Ramachandran *et al.*, 2013), *Terminalia superba* (Desire *et al.*, 2014). These effects could be attributed to the action of phytochemicals of plants. Many studies have demonstrated that saponins exert anti hyperlipidaemic effects such as the works of Han *et al.* (2000) and Patel *et al.* (2012), and the proposed mechanism of action of saponins is by inhibition of pancreatic lipase enzyme (Han *et al.*, 2000). Saponins have also been reported to alleviate alloxan-induced diabetes by decreasing the level of lipid peroxidation and increasing the antioxidant defence system in the serum, liver and pancreas (Elekofehinti *et al.*, 2013). Triterpenes have also been reported to have anti hyperlipidaemic activities. Gutierrez (2013) and Machaba *et al.* (2014) demonstrated this and proposed that the mechanism of action was by decreased degradation of glycogen and decreased rate of gluconeogenesis. Saponin and flavonoids isolated from plants exhibit hypoglycaemic effect by increasing insulin release from pancreatic beta cells, increasing peripheral glucose uptake and by reducing glucose absorption (Luo *et al.*, 2005; Saravanan & Pari, 2008; Zambare *et al.*, 2011). Tannins are insulin – like substances (Mota *et al.*, 1985; Tullo, 2008) and mimic the effect of insulin on glucose metabolism and enhanced insulin secretion. These substances have been isolated from *T. avicennioides* stem bark in this study. *T. avicennioides* stem bark could also possibly...
have exerted its anti hyperglycaemic effect by attenuating the body antioxidant system.

Photomicrographs of the liver showed congested central vein in some of the extract treated groups as well as the GLB group which is similar to the result in an anti-diabetic study by Oyebadejo et al. (2014). Photomicrographs of the pancreas revealed necrosis of islet cells in diabetic control and partial or complete restoration of islet cells in extract treated groups, which is similar to results obtained by Natarajan et al. (2012). Similar results were also obtained by Ahmed et al. (2005) using Terminalia catappa.

In conclusion, the results of this study showed that the stem-bark extracts of T. Avicennioides possess anti-diabetic effects in rats, the crude methanol and hexane extracts at 100 mg/kg were able to reduce blood glucose levels below 140 mg/dl. They also caused significant increase in serum high density lipoprotein, significant decrease in serum low density lipoprotein levels and also serum triglyceride levels. This validates the use of the plant traditionally for the management of diabetes mellitus.

Conflicts of Interest
The authors declare no conflicts of interest.

References


