



Alpha (α)-amylase Inhibitory Property of *Anthocleista nobilis* Leaf Extract

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Authors' contributions

This work was carried out in collaboration among all authors. Authors KD and FA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BAA and EO managed the analyses of the study. Authors PY managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Alpha (α)-amylase inhibitors from plants are preferable for type 2 diabetes treatment because of their relative potency and safety. This study examined, *in vitro*, the inhibitory effect of *Anthocleista nobilis* leaf extract on starch hydrolysis catalyzed by α -amylase (extracted from moated sorghum).

Place and Duration of Study: Department of Biochemistry (School of Biological Sciences) and Department of Chemistry, School of Physical Sciences, University of Cape Coast, Ghana, between June 2021 and August 2021.

Methodology: Leaves of *A. nobilis* were air-dried, pulverized, and macerated in 70% aqueous ethanol for 72 hrs. The filtrate was concentrated and reconstituted in 0.02M Sodium phosphate buffer (pH 6.9) for further analysis including Phytoconstituents screening. *In vitro* analysis of α -amylase activity as well as inhibitory effect of *A. nobilis* extract on α -amylase was performed. The Lineweaver-Burk plot was employed in the inhibition analysis to determine the inhibition type, maximum initial reaction rate (V_{max}), as well as the Michaelis constant (K_M).

Results: The percentage inhibition of α -amylase ranged from $25.0 \pm 0.46\%$ - $85.7 \pm 2.17\%$ for 0.1mg/mL and 0.9mg/mL of the *A. nobilis* leaf extract respectively. The mode of α -amylase inhibition was found from the Lineweaver-Burk plot as mixed noncompetitive. The K_M and V_{max} were determined as 0.2043 mM and 0.1282 mM/min respectively. In contrast, K_M for the control were 0.1537mM and V_{max} of 0.09750 mM/min. The inhibition property of *A. nobilis* could be attributed to its phytochemicals such as flavonoids, saponins, alkaloids, tannins, and terpenoids that were present.

Conclusion: *Anthocleista Nobilis* leaf extract contains certain naturally occurring anti-diabetic compounds and could be explored for treating type 2 diabetic patients. These findings, however, need further work to validate the exact bioactive constituents responsible for the inhibitory effect.

Keywords: *Anthocleista nobilis*; α -amylase; diabetes mellitus; mixed noncompetitive; moated sorghum.

1. INTRODUCTION

Plants extracts as medicinal resources have been used in the prevention, management, and treatment of a wide range of diseases (Schnell et al., 2016; Wiart, 2007); [1]. Plant species such as *Bligia sapida* and *Vernonia amygdalina* have been explored in managing diabetes due to their relatively low costs, minimal side effects in clinical experience and high potency (Alkhalidy et al., 2018); [2]. Diabetes mellitus is a health problem that occurs due to an increase in the concentration of blood glucose levels as a result of impaired lipid and carbohydrate metabolism which are characterized by defect in insulin secretion and/or insulin action Kazeem et al., [3,4]. Diagnosis of diabetes is based on optimal WHO criteria (a venous plasma glucose concentration of >11.1 mmol/l, 2 hrs after a 75-g glucose tolerance test). Diabetes mellitus is a non-communicable disease whose prevalence continues to increase worldwide [5,6,7]. Explicitly, more than 300 million people are living with diabetes worldwide (Sleddens et al., 2011). The effect of diabetes mellitus includes ketoacidosis, nerve damage, visual disturbances, lack of consciousness, and death [8,9]. Nearly 80 percent of diabetes deaths occur in low- and middle-income countries (Sleddens et al., 2011). It has been estimated that about 366 million of the world population would be diabetic by 2030 [10]. The findings indicated also that the "diabetes epidemic" will continue even if levels of obesity remain constant.

Type 1 (T1D) and type 2 (T2D) are the main types of diabetes mellitus (Piero et al., 2014). T1D, also called Insulin-dependent, is caused by the destruction of the beta cells of the pancreas by the immune system such that no insulin is produced by the body. For the past two decades, T1D represents about 10% of all cases with

diabetes (Sun et al., 2019; Holt, 2004), but it is the most common form of diabetes in children, as it accounts for approximately 80% of pediatric diabetes cases in the United States (Mayer-Davis et al., 2017). The only therapy for the treatment of this metabolic disorder is exogenous insulin injections [11].

In contrast, type 2 diabetes mellitus is non-insulin dependent, and it occurs as a result of insulin resistant or insulin insensitivity by the peripheral tissues or receptors that mediate glucose utilization in the body [12]. The cascade of events contributing to the development of T2D have been underexplored. However, many genetic factors such as metabolic syndrome (MetS) as well as environmental causes including obesity are attributed to having influence in inducing insulin resistance (Alkhalidy, Wang, and Liu, 2018). Reports show that non-insulin dependent diabetes stands for 90% of diabetic cases [13], (Alkhalidy et al., 2018). Early stages of T2D often results in the development of hyperinsulinemia [14].

The aim of therapeutic management of diabetes is to normalize the blood glucose level. The enzymes such as amylases induce elevated blood glucose levels by hydrolyzing starch molecules (in foods) into its absorbable monomers. The end products of α -amylase action, for instance, are oligosaccharides (maltose, dextrin) and glucose. Postprandial (fed) hyperglycemia contributes substantially to type 2 diabetes development [15] and hence, reducing postprandial hyperglycemia is essential for treating diabetes [16,6,7,17,18]. Synthetic drugs such as metformin, miglitol, alogliptin and acarbose that functions as α -amylase and α -glucosidase inhibitors also aid in diabetes management [19,20]. For instance, acarbose (a complex oligosaccharide) acts as a competitive,

reversible inhibitor of pancreatic α -amylase and membrane-bound intestinal α -glucoside hydrolase. Studies have shown that these α -amylase inhibitory drugs aside reducing blood glucose levels in type 2 diabetic individuals also pose adverse effects such as elevated serum transaminases (Schnell et al., 2016), and GI symptoms, including diarrhea, and abdominal pain (Ziaee et al., 2017); [21]. This makes it ideal to search for alternative inhibitors of α -amylase with minimal side effects, preferably naturally occurring anti-diabetic compounds.

Anthocleista is a genus of tree- and shrub-like tropical plants in the gentian family, (a Potalieae). These species are commonly called Cabbage trees (English) and "Wudifoakete" (Twi) in Ghana. Interestingly, *Anthocleista* species, particularly *A. nobilis*, have been purported to have laxative, anti-plasmodial, antimicrobial, and antioxidant effects that support its uses in traditional medicine [22], and in wound healing [23]. Though there is a dearth of information about its biologically active compounds, health benefits including treatment of T2D obtained from medicinal plants are mostly attributed to the presence of bioactive compounds such as flavonoids, and polyphenols (Sun et al., 2019). It is therefore envisaged that *A. nobilis* will help in reducing blood glucose concentration by retarding the activity of α -amylase. Hence, this study seeks to determine the alpha-amylase inhibition property of the *Anthocleista Nobilis* leaf extract.

2. MATERIALS AND METHODS

2.1 Plant Material

Anthocleista nobilis leaves were collected from the University of Cape Coast botanical garden, located at the northeastern part of the University of Cape Coast. The leaves of *Anthocleista nobilis* were then authenticated by the School of Biological Sciences herbarium, Department of Conservative Biology, University of Cape Coast.

2.2 Chemicals and Equipment

Para-nitrophenyl- α -D-glucopyranoside (PNPG) (Sigma Aldrich, Germany), para-nitrophenol (PNP) (Sigma Aldrich; Germany), Sodium dihydrogen Phosphate, Disodium Hydrogen Phosphate and Sodium carbonate were from Merck Chemical Supplies (Darmstadt, Germany). Starch soluble (extra pure) was obtained from J. T. Baker Inc., USA. Equipment include Labomed

UVD-3200 spectrophotometer, pH meter model NOV-210 (Nova Scientific Ltd., Korea), and Sartorius analytical balance. All other chemicals used, including solvents, were of analytical grade.

2.3 Preparation of the Plant Extract

The leaves of *A. nobilis* were washed with distilled water and allowed to air dry at room temperature (25°C) for 12 days and then pulverized using an electronic blender. After the pulverization of the sample, 55g of the powdered sample was obtained and transferred into a 1000mL beaker containing 275mL of solvent (70% ethanol and distilled water). The mixture was allowed to stay in the dark for 72 hours [24]. The liquid extract was filtered using a muslin cloth and concentrated to afford the dried leaf extract by continuous evaporation of the extract solution. The dried leaf extract was then kept in a desiccator. The dried extract was re-weighed and dissolved in 0.02M Sodium phosphate buffer (pH 6.9) and the volume was finally made up to 500mL using distilled water.

2.4 Methods for Qualitative Analysis of Phytoconstituents of *A. nobilis* Leaf Extract

The phytochemical compositions were determined by adopting the protocol as described by Prashant et al [25].

2.5 Enzyme (α -amylase) Extraction and Purification

The enzyme was extracted from moated sorghum. Briefly, 15g of the sorghum was weighed into a 250mL beaker containing 45mL of 0.02M sodium phosphate buffer (pH 6.9). It was stirred and left to stand for 5mins. The mixture was transferred into two centrifuge tubes and centrifuged at 1200rpm for 15mins at 4°C. The supernatant was then poured and stored in a fridge at 4°C.

2.6 Determination of α -amylase Activity

The activity of the α -amylase (enzyme) was determined according to the method outlined by Jones and Varner [26] with the following modification: Exactly 0.1mL of the enzyme into six labeled micro-test tubes, then 1.1mL of 0.02M sodium phosphate buffer (pH 6.9) was measured into each micro-test tube and preincubated at room temperature (25°C) for 5 mins. After the

preincubation, varying concentrations of starch 0.1, 0.2, 0.3...1.0 mg/mL were added to the micro-test tubes after which they were incubated at 25°C for 10mins. Then 0.1mL of 0.1M acidified iodine was added to each micro-test tube to stop the reaction. The above procedures were repeated for the standard calibration curve. Absorbance was measured at 620nm.

2.7 Mode of Enzyme (α -amylase) Inhibition

The mode of inhibition of alpha-amylase by *A. nobilis* leaves extract was determined according to the procedure described by [27]. In brief, 0.2mL of 5mg/mL plant extract, 1.1mL of 0.02M sodium phosphate buffer (pH 6.9), and 0.02mL of α -amylase were measured into labeled micro-test tubes respectively. Then these contents in the micro-test tubes were pre-incubated at room temperature (25°C) for 5mins. After the preincubation, 0.2mL of starch was added at increasing concentration 0.1, 0.2, 0.3,0.4,1.0mg/mL in thirty seconds time interval and were incubated again at room temperature (25°C) for 10mins. Next, 0.1mL of previously prepared acidified iodine was added to each micro-test tube to stop the reaction and absorbance was measured at 620nm.

2.8 Alpha (α)-amylase Inhibitory Assay (% Inhibition)

The inhibitory assay was determined by adopting the procedure by Kazeem et al. [28] with slight modifications. The total assay mixture containing 1.0mL of 0.02M sodium phosphate buffer (pH 6.9), the plant extract (increasing concentration from 0.0, 0.1-0.9 mg/mL) and 0.1mL of enzyme (α -amylase) were pre-incubated at room temperature for 5mins. Afterward, 2mg/mL of starch was added to each of the solutions in the test tubes in a time interval of 30 seconds. Then, the solutions in the test tubes were incubated at room temperature for 10mins. 0.1mL of

previously prepared acidified iodine was added to each solution to stop the reaction and absorbance (Abs) was measured at 620nm using a spectrophotometer (Labomed UVD-3200, USA). Positive control was prepared using the above procedure by replacing the extract with distilled water. The percentage inhibition was calculated using the relation below.

$$\%Inhibition = \frac{(Abs_{control} - Abs_{extract})}{Abs_{control}} \times 100$$

2.9 Statistical Analysis

The data were analyzed using GraphPad Prism 9 (GraphPad Software, USA), and MS-Excel (2013 version). The data were analyzed by one way analysis of variance (ANOVA). All the results were expressed as mean \pm SEM for triplicate determinations.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening of *A. nobilis* Leaves Extract

The phytochemical analyses of *Anthocleista Nobilis* leaf revealed that the plant extract is rich in saponins, tannins, terpenoids, flavonoids, and alkaloids (Table 1). The inhibitory effects of the ethanol extracts of *Anthocleista Nobilis* on α -amylase activity may be as a result of the presence of phytochemicals such as flavonoids tannins, alkaloids, terpenoids, and saponins. This is because some of these secondary metabolites inhibit the enzyme by blocking the active sites [29]. Studies have also shown that flavonoids help in reducing the blood glucose level by inhibiting α -amylase activity [30,31,32]. Alternatively, saponins, terpenoids, tannins, and alkaloids are known to contain some biological properties that aid in controlling postprandial hyperglycemia in diabetic (non-insulin dependent) individuals [3,33,34,35] due to their synergistic effect.

Table 1. Qualitative screening of *A. Nobilis* leaves extract

Phytochemicals	Test	Status
Saponin	Foam test	+
Tannins	Ferric chloride test	+
Steroids	Lieberman Burchardt	-
Alkaloids	Wagner test	+
Flavonoids	Lead acetate test	+
Terpenoids	Salkowski test	+

* +: (present); - (not detected)

3.2 Quantitative Analysis

3.2.1 Enzyme (α – amylase) activity

In this study, the activity of the α -amylase was determined (Fig 1a and 1b) and it was observed that as the concentration of the substrate (starch) increases, the activity of the enzyme (α -amylase) also increased. This implies that the higher the amount of substrate available, the higher it binds the active site of the enzyme thus enhancing the catalytic activity of α -amylase. Using GraphPad prism, the K_M (substrate concentration at half V_{max}) was determined as 0.1537mM on the Michaelis-Menten graph (Fig. 1a) showing the activity of alpha-amylase with V_{max} (maximum rate of reaction) of 0.09750 mM/min. The observed increase in the activity of the enzyme could be because factors (such as pH,

temperature, concentration, inhibitors or activators) that affect the activity of enzymes were kept constant with negligible fluctuations while increasing the concentration of substrate [12]. Also, it is known that the activity of an enzyme increases as the substrate concentration increases to a point where further increase in substrate concentration shows no substantial change in the activity of the enzyme (Valithan, 1998; Adinortey et al., 2018). This is because as the substrate concentration increases, the enzyme's active sites allow complementary substrates to bind forming an enzyme-substrate complex. When the active sites of the enzymes become saturated with substrates, a further increase in the concentration of the substrate will have no substantial effect on the rate of reaction which is seen in the Michalis-Menten graph as asymptote (Fig. 1a).

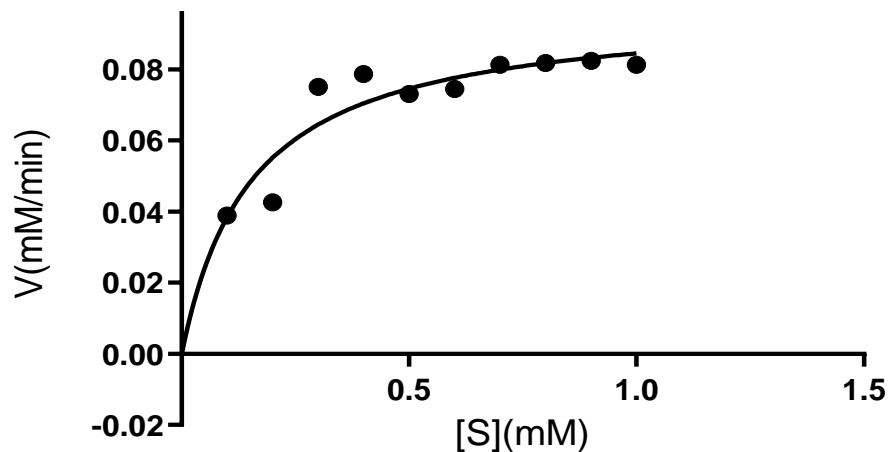


Fig. 1a. Michaelis- Menten plot of the activity of alpha (α) amylase

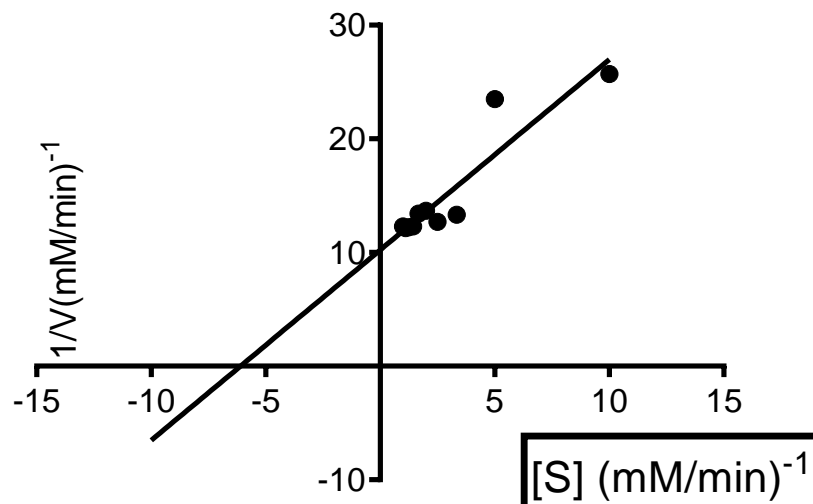


Fig. 1b. Lineweaver-Burk representation of the activity of α -amylase

3.2.2 Percentage inhibition of α – amylase by *A. nobilis* leaf extract

According to Valiathan [36], Plants which are inhibitors of α -amylase may serve as an effective therapy for postprandial hyperglycemia with no significant side effects, thus the bioactive agent in this plant extract could be isolated for therapeutic purpose. In the vitro analysis of α -amylase inhibition by aqueous *Anthocleista nobilis* leaf extracts was evaluated and the percentage inhibition of the leaf extract increased with increasing concentrations of both the substrate and leaf extract. From Fig. 2, the lowest percentage inhibition was 25% and the highest was 85.7% which were 0.1 mg/mL and 0.9 mg/mL of the leaf extract. This revealed that *Anthocleista nobilis* leaf extracts could be a powerful inhibitor of α -amylase. On one hand, the observed *Anthocleista nobilis* leaf percentage inhibition of α -amylase is higher than 49% of lichens the methanolic extract of *F. caperata* that was reported by [37]. On the other hand, the present data is comparable to 79% inhibition of *Strychnos Spinosa* leaves of α -glucosidase reported by [30]. However, the observed findings do not the claim that *Anthocleista nobilis* leaves could not significantly reduce fasting blood glucose concentration [38].

3.2.3 Mode of Enzyme Inhibition

The Lineweaver-Burk plot (Fig 3b), a double-reciprocal plot of the Michaelis-Menten equation, is generally employed in inhibition analysis to

determine important factors such as the inhibition type, maximum initial reaction velocity (V_{max}), as well as the Michaelis constant (K_m) (Sun, L., Warren, and Gidley, 2019; Yang, He, and Lu, 2014). From Fig 3a, the K_M and V_{max} for α -amylase activity in the presence of *Anthocleista nobilis* leaf extract were determined as 0.1282 mM/min and 0.2043 mM respectively. However, the K_M for the enzyme activity (without the sample extract) (Fig 1a) was greater than the K_M value of the mode of α -amylase inhibition (Fig 3a), but the V_{max} of the enzyme's activity was lower than the V_{max} of the mode of inhibition. This might be a result of a large binding affinity of α -amylase for its substrate due to the presence of some active components sample extract. However, these results also conform to the findings of Kazeem et al. [28] on the mode of inhibition of α -glucosidase by aqueous extracts of *Morinda Lucida Benth* leaf. Further studies revealed that the mode of inhibition was mixed non-competitive inhibition (Fig 3b). This connotes that the active components aqueous extract of *A. nobilis* active component bind to the allosteric site of the enzyme rather than the substrate-binding site. This kind of binding leads to deformation in the protein structure of α -amylase; thereby reducing its catalytic activity of the enzyme [3,4,[39],[40]. This signifies that this plant extract contains some active ingredients that have resulted in a decrease in the catalytic efficiency of the enzyme. However, this type of inhibition is not contrary to the mode of inhibition of α -amylase by *Blighia Sapida* leaf extract [3].

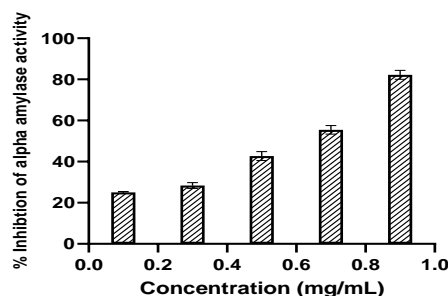


Fig. 2. Effect of different doses of aqueous extract of *Anthocleista nobilis* (mg/mL) on Alpha(α)-amylase activity at the same time intervals (10 mins)

Test extracts were significant ($P < 0.05$) from normal control, The values expressed as Mean \pm S.E.M for $n = 3$ experiments. The percentage inhibition of alpha-amylase activity by *Anthocleista nobilis* leaf extract increased with increasing concentration of the extract

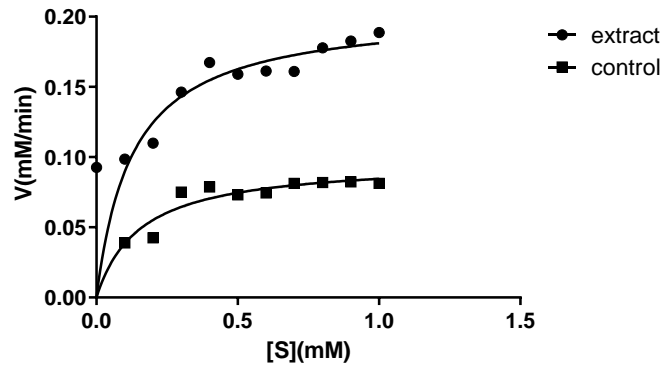


Fig. 3a. Mode of alpha amylase inhibition by aqueous *Anthocleista nobilis* leaf extracts. This is the Michaelis-Menten plot of how the leaf extract inhibits the activity of α -amylase. The control is a substrate (i.e. starch) for α -amylase without any extract.

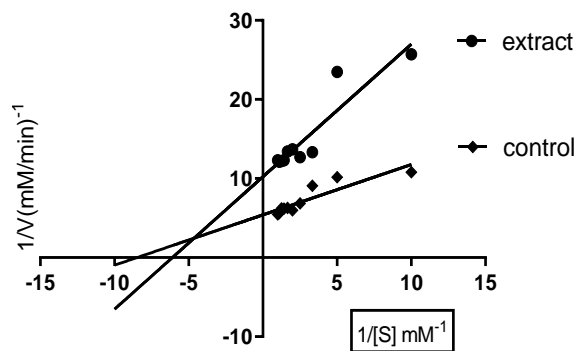


Fig. 3b. The Lineweaver-Burk plot (Double reciprocal plot) was used to determine the mode of inhibition of the aqueous extract of *Anthocleista nobilis* leaf on α - amylase, which displayed a mixed inhibition of the enzyme activity where the inhibitor binds both the enzymes active sites and enzyme-substrate complex decreasing or inhibiting the activity of α -amylase. The K_M (substrate concentration at half V_{max}) and V_{max} (maximum reaction rate) were determined as 0.1282 mM/min and 0.2043 mM respectively. In contrast, K_M and V_{max} for the control (i.e. for the enzyme without the inhibitor extract) were 0.1537mM and V_{max} of 0.09750 mM/min respectively. This implies that the activity of α -amylase increases when the concentration of the substrate increases in the absence of the extract but decreases when the extract was added. For this mixed inhibition consisted of lines with a slope $\alpha K_M / V_{max}$, a $1/V_o$ intercept of α / V_{max} , and a $1/[S]$ intercept of $-\alpha / \alpha K_M$

4. CONCLUSION

From the results of this study, *Anthocleista Nobilis* leaf extracts have inhibitory effects on the activity of α -amylase and the type of inhibition is mixed non-competitive inhibition. Therefore, *Anthocleista Nobilis* leaf extract has medicinal properties that make it a potent inhibitor of the enzyme (α -amylase) whose hydrolytic activity could result in postprandial hyperglycemia. However, further study should be carried out to isolate, characterize and therapeutically exploit the active principle(s) in

this plant that causes this alpha-amylase inhibitory effect.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. there is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. also, the research was not funded

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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