NOTE / NOTE

Antiglycation activity of *Vaccinium* spp. (Ericaceae) from the Sam Vander Kloet collection for the treatment of type II diabetes[1]

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Abstract: In this report, the inhibition of advanced glycation endproducts (AGEs) by extracts of leaves from a collection of six, mainly tropical, *Vaccinium* L. spp. (Ericaceae) was examined. Indigenous Peoples have used *Vaccinium* species to treat symptoms of type I and II diabetes. Sustained hyperglycaemia, often associated with diabetes, facilitates crosslinking of sugars with proteins, producing AGEs. AGEs are a therapeutic target since they are responsible for many diabetes symptoms and contribute to ageing and the development of atherosclerosis, kidney, vascular, and neurological diseases. *Vaccinium barandanum* S. Vidal, *Vaccinium consanguineum* Klotzsch, *Vaccinium gaultheriifolium* (Griff.) Hook. f. ex C.B. Clarke, *Vaccinium poasanum* Donn. Sm., *Vaccinium tonkinense* Dop, and *Disterigma rimbachii* (A.C. Sm.) Luteyn (outgroup) were collected from Sam Vander Kloet's common garden collection. Ethanolic extracts of leaves of these *Vaccinium* spp. were potent inhibitors of AGEs. *Vaccinium* and outgroup species extracts tested in an AGE inhibition assay demonstrated concentration dependent inhibition, with a half maximal inhibitory concentration (IC_{50}) ranging from 4.2 to 16.2 $\mu g \cdot mL^{-1}$. Phenolic content ranged from 258 to 626 (μg quercetin equivalents·mg $extract^{-1}$). Activity and phenolic content show that these tropical accessions have a higher phenolic content ($p < 0.001$, *t* test) and AGE inhibition ($p < 0.03$, *t* test) than six temperate species from our collections in eastern North America. Significant relationships were found between IC_{50} and latitude of geographic origin.

Key words: diabetes, advanced glycation endproduct, *Vaccinium*, Indigenous Peoples, Ericaceae.

Résumé : Les auteurs ont examiné l'inhibition des produits finaux de la glycation avancée (PFGAs) par des extraits de feuilles provenant de six collections, surtout tropicales, d'espèces de *Vaccinium* L. (Ericaceae). Les peuples indigènes ont utilisé les espèces de *Vaccinium* pour traiter les symptômes des diabètes de type I et II. L'hyperglycémie soutenue, souvent associée aux diabètes, facilite la réticulation des sucres avec des protéines, produisant des PFGAs. Les PFGAs constituent une cible thérapeutique puisqu'ils sont responsables de plusieurs symptômes des diabètes et contribuent au vieillissement, ainsi qu'au développement de l'athérosclérose, et des maladies rénales, vasculaires et neurologiques. Les auteurs ont récolté les *Vaccinium barandanum* S. Vidal, *Vaccinium consanguineum* Klotzsch, *Vaccinium gaultheriifolium* (Griff.) Hook. f. ex C.B. Clarke, *Vaccinium poasanum* Donn. Sm., *Vaccinium tonkinense* Dop, et le *Disterigma rimbachii* (A.C. Sm.) Luteyn (groupe extérieur) provenant de la collection en jardin commun de Sam Vander Kloet. Les extraits éthanoliques des feuilles de ces *Vaccinium* spp. s'avèrent de puissants inhibiteurs des PFGAs. Les extraits des espèces de *Vaccinium* et du groupe ex-

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terne, testés dans un essai d'inhibition des PFGAs, ont démontré une inhibition reliée à la concentration, avec une concentration d'inhibition demi-maximale (IC_{50}) allant de 4,2 à 16,2 μg·mL^{-1}. La teneur en phénols va de 258 à 626 (équivalents mg de quercétine·mg d'extrait^{-1}). L'activité et la teneur montrent que ces accessions tropicales possèdent une teneur en phénol ($p < 0{,}001$, *t* test) et une inhibition des PFGAs ($p < 0{,}03$, *t* test) plus élevées que six espèces de régions tempérées provenant de collections du Nord-est américain. On observe des relations significatives entre les IC_{50} et la latitude géographique de leurs origines.

Mots-clés : diabètes, produits finaux de la glycation avancée, *Vaccinium*, peuples indigènes, Ericaceae.

[Traduit par la Rédaction]

Introduction

For millennia, Indigenous Peoples have used all plant parts from numerous species of *Vaccinium* L. (Ericaceae) in traditional medicine (Arnason et al.1981; Lee et al. 2004; Redžić 2006, 2010; Zutter 2009). Leaves of northern *Vaccinium* spp. (blueberries, bilberries, huckleberries, and cranberries) have the following traditional uses: abortifacients, antiemetics, blood and ceremonial medicines, cold remedies, diaphoretics, dietary aids, eye medicines, febrifuges, gastrointestinal and gynecological aids, panacea, and pediatric aids (Moerman 2009; Ferrier et al. 2011). However, the use of leaves from tropical *Vaccinium* specimens is poorly documented or not accessible.

There are a few reports noting Indigenous Peoples use of *Vaccinium* leaves for treating diabetes specifically. Using *Vaccinium myrtillus* L. foliage for infusions was reported in Kelly's (1970) Colorado, USA, woody plants field guide. Ethnobotanical surveys in Lukomir, Bosnia and Herzegovina, report that 4 of 25 traditional healers use the leaves and (or) stems of *V. myrtillus* for a cure-all infusion (Ferrier et al. 2011). Antidiabetic pharmacological studies, guided by anecdotal traditional use reports of the European *V. myrtillus*, evaluated semi-pure and crude leaf extracts in animal and clinical trials with safe and positive antidiabetic effects on depancreatized dogs, insulin reduction and sensitization in diabetic patients, and statistically significant decreases in plasma glucose in streptozotocin-induced diabetic rats (Allen 1927; Cignarella et al. 1996).

The Cree of Eeyou Istchee, Canada, in collaboration with the Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicine undertook analyses of *Vaccinium angustifolium* Aiton. Cree healers recommended *V. angustifolium* and its leaves were shown to have its most potent advanced glycation endproduct (AGE) inhibition activity from leaf material collected in October (Leduc et al. 2006; McIntyre et al. 2009). Harris et al. (2007) demonstrated that *V. angustifolium* contains high levels of the active phytochemicals chlorogenic acid (~100 μg·mg extract^{-1}) combined with many quercetin glycosides at the end of August.

Prolonged hyperglycaemia in diabetes leads to impaired glucose uptake and damages the cells of organs that do not require insulin for glucose uptake (e.g., heart, kidneys, neurons, and small blood vessels). Consequently, these cells have high concentrations of intracellular glucose during elevated hyperglycaemic periods (Ahmed 2005). Cellular T2DM complications begin with formation of "Schiff bases" between glucose and protein, which can progress into an irreversible AGE.

Our goal was to assess the anti-AGE potential of New World and Old World *Vaccinium* spp. that were available for investigation from Sam Vander Kloet's common garden collection. Indigenous Peoples are one of the fastest growing communities on the planet, and with rapidly increasing rates of T2DM they are also some of the most susceptible populations (Helin 2008) that may benefit from diabetic therapies derived from traditional medicines. Because AGEs accumulate with sustained hyperglycaemia causing diabetic complications, AGEs are a therapeutic target. This study examines the relationship of AGE inhibition activity to phenolic content and the latitudinal geographic origin of the species.

Materials and methods

Tropical *Vaccinium* specimens from the Sam Vander Kloet collection

We were very fortunate to have access to the late Sam Vander Kloet's global collection of tropical *Vaccinium* specimens growing in the Harriet Irving Botanical Gardens at Acadia University, Nova Scotia, Canada (ACAD). All common garden leaf samples were collected by J.F. and identified by Sam Vander Kloet (Table 1). *Disterigma rimbachii* (A.C. Sm.) Luteyn was available at ACAD and selected as a common garden Ericaceae outgroup to determine if *Vaccinium* spp. treated herein were more effective than the referenced outgroup family member.

Sam Vander Kloet's specimens were propagated from their wild seeds or cuttings, which grow together under common environmental conditions at the Harriet Irving Botanical Gardens (latitude 45.08686, longitude –64.3681). All common garden specimens grew outdoors during the spring, summer, and autumn; in late autumn plants were moved into a climate controlled greenhouse for the winter period.

All collections were made from healthy aboveground foliage from plants, older than 10 years. Leaves were collected in paper bags and tobacco offerings were made according to Algonquian custom. Each sample was dried overnight at 37 °C and stored at room temperature prior to a standardized extraction procedure.

For comparison purposes, only six temperate *Vaccinium* spp. (*Vaccinium boreale* I.V. Hall & Aalders, *Vaccinium corymbosum* L., *Vaccinium caespitosum* Michx., *Vaccinium ovalifolium* Sm., *Vaccinium macrocarpon*Aiton, and *Vaccinium uliginosum* L.) were collected in northeastern Canada, processed, and assayed in a similar way. Full details of collection data and activity of individual species will be reported later.

Sample preparation and extraction

All leaves were prepared and extracted using the following

Table 1. A list of species sampled from the common garden with total phenolic (μg quercetin equivalents·mg extract^{-1}) and IC_{50} (μg·mL^{-1}) extract results.

Species	Section	Latitude	Longitude	Alt. (m)	Total phenolics	IC_{50}	Pop. No.	Geographic origin	ACAD Herbarium ID
Vaccinium barandanum S. Vidal	Barandanum	16.583572	120.883592	2455	624.0	16.2	Pop. 1	Mt. Pulog, Luzon, Phillipines	17/XI/92 (AUV.00122)
Vaccinium barandanum S. Vidal	Barandanum	16.583572	120.883593	2455	427.3	6.5	Pop. 2	Mt. Pulog, Luzon, Phillipines	17/XI/92 (AUV.00123)
Vaccinium barandanum S. Vidal	Barandanum	16.583572	120.883594	2455	557.2	11.6	Pop. 3	Mt. Pulog, Luzon, Phillipines	17/XI/92 (AUV.00124)
Vaccinium consanguineum Klotzsch	Pyxothamnus	9.979167	–83.8525	3304	412.6	10.2	Pop. 1	Irazú Volcan, Costa Rica	714686 (AUV00128)
Vaccinium consanguineum Klotzsch	Pyxothamnus	9.979167	–83.8525	3304	460.7	6.0	Pop. 2	Irazú Volcan, Costa Rica	714686 (AUV00128)
Vaccinium consanguineum Klotzsch	Pyxothamnus	9.979167	–83.8525	3304	257.7	6.1	Pop. 3	Irazú Volcan, Costa Rica	714686 (AUV00128)
Vaccinium myrsinites Lam.	Cyanococcus	31.775278	–83.8525	61	437.1	12.1	Pop. 1	Pine Grove,Georgia, USA	422574 (AUV.00295)
Vaccinium gaultheriifolium (Griff.) Hook. f. ex C.B. Clarke	Galeopetalum	22.223	103.7373	622	508.4	11.2	Pop. 3	Phan Xi Păng, Vietnam	1231197 (AUV 00204)
Vaccinium gaultheriifolium (Griff.) Hook. f. ex C.B. Clarke	Galeopetalum	22.223	103.7374	622	585.7	8.9	Pop. 4	Phan Xi Păng, Vietnam	1231197 (AUV 00204)
Vaccinium gaultheriifolium (Griff.) Hook. f. ex C.B. Clarke	Galeopetalum	22.223	103.7375	622	415.1	8.6	Pop. 5	Phan Xi Păng, Vietnam	1231197 (AUV 00204)
Vaccinium poasanum Donn. Sm.	Oreades	10.2	–84.2	2200	431.0	7.6	Pop. 1	Mt. Poas, Costa Rica	20790B (AUV. 00139)
Vaccinium poasanum Donn. Sm.	Oreades	9.509425	–83.711556	2500	625.5	4.2	Pop. 3	Cerro de la Muerte, Costa Rica	113686 (AUV.00124)
Vaccinium tonkinense Dop	NA	21.516667	105.55	483	564.4	6.6	Pop. 3	Tam Dao, Vietnam	127 1197 p. 331 (AUV00142)
Vaccinium tonkinense Dop	NA	21.516667	105.55	483	485.2	8.8	Pop. 4	Tam Dao, Vietnam	127 1197 p. 332 (AUV00142)
Vaccinium tonkinense Dop	NA	21.516667	105.55	483	581.3	7.0	Pop. 5	Tam Dao, Vietnam	127 1197 p. 334 (AUV00142)
Disterigma rimbachii (A.C. Sm.) Luteyn	NA	NA	NA	1995	489.8	13.6	Pop. 1	Ecuador	Outgroup: 8011

Note: Sections follow Vander Kloet et al. (2004), Vander Kloet and Dickinson (2009), and The Plant List (2010). NA, not available. Leaves were collected on 30 September 2009.

standardized method. Dried leaf samples were ground using a Wiley Mill and passed through a 1 mm mesh. Plant material was extracted twice in 95% ethanol (1 g·10 mL^{-1}) at room temperature for 24 h per extraction. The recovered extractions were pooled and dried using a rotary evaporator (Savant Speed-Vac) at room temperature for 24 h to remove EtOH. Extracts were then placed in a freezer for 2 h at –20 °C before lyophilization at –40 °C for 24 h to remove water from the extract. Lyophilization yielded between 0.10 and 0.21 g crude extract·g dried $leaf^{-1}$. Extracts were stored at –20 °C. Stock solutions were prepared in EtOH and distilled H_2O (4:1) for both total phenolic quantification and AGE inhibition assays. All samples were filtered through a 0.2 μm polytetrafluoroethylene (PTFE) nonsterile filter (Chromatographic Specialties Inc., Brockville, Ont., Canada) prior to analysis.

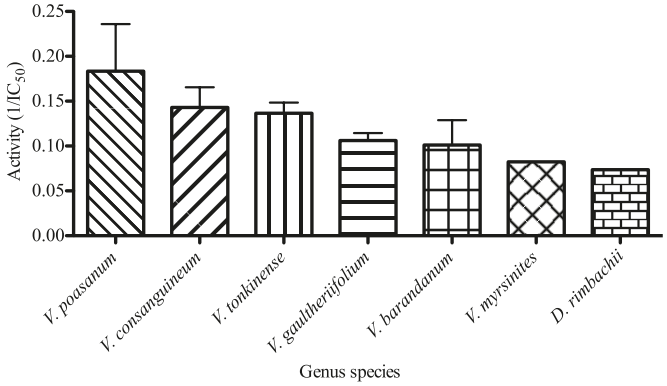
Total phenolics estimation assay

Total phenolic content of *Vaccinium* extracts was estimated colourimetrically with a Folin–Ciocalteu (F–C) reagent based assay (Singleton and Rossi 1965) and modified to reduce volumes as previously described by Farsi et al. (2008), Spoor et al. (2006), and Harbilas et al. (2009). A stock solution of 7.5% $NaHCO_3$ in distilled H_2O was prepared. All extracts were diluted using a 10 mg·mL^{-1} stock solution to optimal assay concentrations of 2 mg·mL^{-1}, 1 mg·mL^{-1}, and 0.5 mg·mL^{-1}. Quantification was based on the standard curve generated at 725 nm of solutions of 0.4, 0.2, 0.1, 0.05, and 0.025 mg·mL^{-1} of quercetin prepared in ethanol/water (4:1). Eighty microlitres of extract or standard solution was added to 400 μL of F–C. The mixture was then vortexed briefly and left to stand at room temperature for 5 min. Two-hundred-seventy microlitres of the $NaHCO_3$ solution was added to the mixture and gently stirred. Samples were transferred to a clear bottom nonsterile 96-well plate (Nalge Nunc International, Rochester, N.Y., USA) in three replicates of 200 μL per well. The plate was covered and incubated at room temperature in the dark for 2 h. Absorbance was read at 725 nm and total phenolic content was calculated in terms of micrograms quercetin equivalents per milligrams of extract.

AGE formation inhibition assay

AGE formation inhibition activity was assessed as described by Farsi et al. (2008) with modifications. Bovine serum albumin (BSA) (1 mg·mL^{-1}) was incubated with 100 mmol·L^{-1} glucose / 100 mmol·L^{-1} fructose in sodium phosphate monobasic monohydrate buffer (pH 7.4) with extract (experimental treatment), ethanol/water (4:1) (negative control), or quercetin, an antioxidant against glycation by way of phenolic hydroxyl groups in the flavonoid structure (24 μg/mL in assay) (Sengupta et al. 2006), which served as a positive control. To control for fluorescence of extracts, a treatment without BSA was included; to control for fluorescence of BSA, a treatment with BSA and vehicle was included. Stock solutions of extracts were serially diluted and tested at five concentrations that were optimized for dissolution and a linear concentration response (40, 20, 10, 5, and 2.5 μg·mL^{-1} in assay). Three replicates were tested in sterile opaque polystyrene 96-well clear bottom plates (Corning Inc., New York, N.Y., USA). Plates were covered, sealed with Parafilm, and incubated for 7 days at 37 °C while shaking. Following incubation, fluorescence was measured using a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, Calif., USA) at excitation and emission wavelengths of 355 and 460 nm. Glucose/fructose and ethanol/water fluorescence was subtracted from all results, and percent inhibition and IC_{50} values were calculated as previously described (Farsi et al. 2008).

Fig. 1. *Vaccinium* spp. and outgroup mean advanced glycation endproduct (AGE) inhibition activity from common garden populations (n = 3, except for *Vaccinium poasanum* (n = 2), *Vaccinium myrsinites* (n = 1), and *Disterigma rimbachii* (n = 1)).



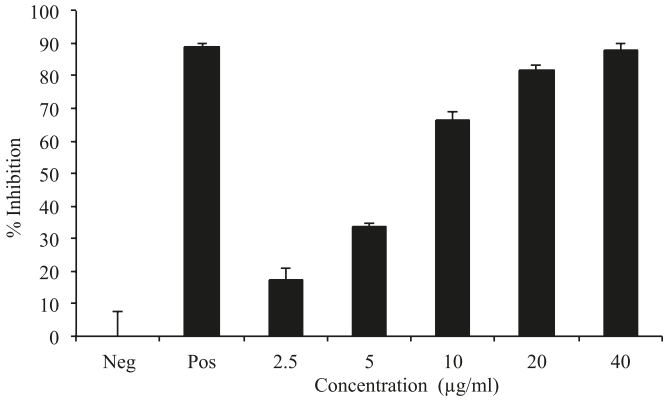
Statistics and geographic information system analysis

Statistical analysis was conducted using Prism 5.0d trial software. All specimen localities were imported into ArcGIS version 9.3 and projected to an Albers projection with a World Geodetic System 1984 datum. Utilizing the altitude variable from the Worldclim dataset at 30 arc seconds resolution (Hijmans et al. 2005), values were extracted for each unique specimen locality using the "Extract Values to Points" tool in the Spatial Analyst extension of ArcGIS version 9.3. These altitude values were compared between the latitudinal geographic origins of wild and garden groups using the nonparametric Kolmogorov–Smirnov z test, because assumptions of data normality and homogeneity of the variance were not met (data not shown).

Results and discussion

All *Vaccinium* spp. showed an inhibition of AGE formation (Table 1; Fig. 1). To illustrate the results on an activity basis, $1/IC_{50}$ is plotted (Fig. 1). The highest mean activity ($1/IC_{50}$) from the common garden group was 0.169 from *V. poasanum* (Costa Rica) (Fig. 1). The mean ± SE of the IC_{50} for all tropical common garden species (six species) was 8.77 ± 0.79 μg·mL^{-1} with a range from 4.2 to 16.2 μg·mL^{-1}. The common garden reference outgroup (*D. rimbachii*) was the least effective extract in terms of AGE inhibition activity (Fig. 1). All *Vaccinium* sp. extracts tested in the AGE inhibition assay displayed highly linear concentration dependant inhibition of AGE formation with activity higher or comparable to the positive control, quercetin. The data for the most active species, *V. poasanum*, is shown (Fig. 2). *Vaccinium poasanum* was reported to contain quercetin-3-galactoside, chlorogenic acid, quercetin-3-glucoside, quercetin-3-arabinoside, quercetin-3-rhamnoside, quercetin glycoside, myricetin, myricetin glycoside 4, and

Fig. 2. *Vaccinium poasanum*: percent inhibition of advanced glycation endproducts (AGEs) with increasing concentration of crude leaf extract. *Vaccinium poasanum* (VPO 11) exhibits an IC_{50} = 7.62 μg·mL^{-1} (R^2 = 0.953). The positive control, quercetin, displayed a 90% inhibition at 24 μg·mL^{-1} concentration.



procyanidin b2 biomarkers in a crude leaf extract (Saleem et al. 2010).

The inhibition of AGE formation by the Sam Vander Kloet tropical *Vaccinium* collection is comparable or higher than northern wild *Vaccinium* collections. A wild collection of six *Vaccinium* spp. from northeastern North America were assessed under comparable methods and had a mean ± SE IC_{50} of 28.9 ± 4.7 μg·mL^{-1}, with a range of 3.6–78.4 μg·mL^{-1}. Data for individual species will be published in a later publication. Clearly, the mean activity of the tropical collection is higher (~3×) than the northern wild *Vaccinium* collection ($p < 0.03$, *t* test), although there is some overlap of the range for individual species.

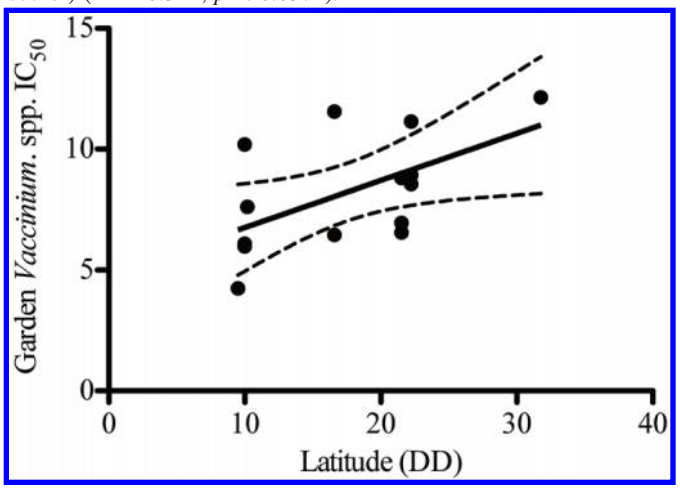
Tropical species had a high phenolic content and the antioxidant properties of phenolics are known to inhibit the formation of AGEs (Farsi et al. 2008). Mean ± SE phenolic concentration for the six tropical species was 492 ± 25 μg quercetin equivalents·mg extract^{-1} with a range of 271–675 μg quercetin equivalents·mg extract^{-1}. Tropical specimens had ~3× greater phenolic content compared with a collection of northern *Vaccinium* analyzed with the same methodology (data not shown) (mean ± SE = 142 ± 8 μg quercetin equivalents·mg extract^{-1}, range = 80–248 μg quercetin equivalents·mg extract^{-1})($p < 0.001$, *t* test).

In the Geographic Information System analysis, the altitude values extracted for each of the specimen localities show clear differences between the wild and the garden *Vaccinium* spp. groups. The Kolmogorov–Smirnov *z* test found significant differences in altitude for the locations of wild and garden species ($D(35) = 1.593$, $p < 0.05$).

Tropical *Vaccinium* spp. normally grow at high altitudes with high UV irradiances. As a genetic adaptation to these environments, these species appear to have high phenolic production, possibly to avoid photo-oxidant damage by the antioxidant properties of phenolics. For example, in other Ericaceae, Rapinski et al. (2011) found a relationship between phenolic concentrations and mean July solar radiation.

A linear regression of IC_{50} with respect to latitudinal geographic origin (R^2 = 0.314, n = 13, $p < 0.037$ (Fig. 3)) for the tropical species indicated that latitudinal geographic origin explains 31% of variation in common garden IC_{50} values.

Fig. 3. A linear regression (with 95% confidence intervals) showing the relationship between latitudinal geographic origins (latitude) and IC_{50} of garden *Vaccinium* spp. (minus one *Vaccinium barandanum* outlier) (R^2 = 0.314; $p < 0.0371$).



Latitude is not a parameter affecting plant physiology or genetic adaptation directly, but latitudinal variation in climatic parameters affecting plant growth are likely primarily responsible for this correlation.

Vaccinium species have a long history of use among Indigenous Peoples (Moerman 2009). Traditional use suggests they are also very safe for consumption on a day-to-day basis. All *Vaccinium* spp. grown in the Harriet Irving Botanical Garden showed potent AGE activity. Our results confirm that all species tested in the genus *Vaccinium* are a significant source of antiglycation agents. This report shows that *Vaccinium* of the Sam Vander Kloet collection have the potential to reduce complications of T2DM. The genus clearly merits further in vivo pharmacological and phytochemical study.

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