Bioorganic & Medicinal Chemistry Letters 26 (2016) 4086–4091

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Antidiabetic effect, antioxidant activity, and toxicity of 3′,4′-Di-*O*-acetyl-*cis*-khellactone in Streptozotocin-induced diabetic rats

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ARTICLE INFO

Article history:
Received 7 June 2016
Accepted 24 June 2016
Available online 25 June 2016

Keywords:
Antidiabetic activity
Antioxidant activity
3′,4′-Di-*O*-acetyl-*cis*-khellactone
Diabetes

ABSTRACT

Pyranocoumarins are compounds with an important pharmacological profile, such as anti-inflammatory, antioxidant, cytotoxic, antiviral, antibacterial, and hypoglycemic effects. These molecules have a widespread presence as secondary metabolites in medicinal plants used to treat Diabetes Mellitus (DM). The aim of this work was to evaluate antidiabetic activity in Streptozotocin (STZ)-induced diabetic rats and the antioxidant effects of 3′,4′-Di-*O*-acetyl-*cis*-khellactone (DOAcK), as well as its toxic potential. We obtained DOAcK with an enantiomeric excess of 70% by chemical synthesis. Our results showed that this compound exerts an important antidiabetic effect: blood glucose decreased in groups treated with DOAcK by 60.9% at dose of 15 mg/kg (p <0.05) compared with the diabetic control group, and demonstrated a statistically significant increase in weight gain (45.7 ± 9.7 in the group treated with DOAcK vs. −23.0 ± 33.1 in the group with diabetes). In a biochemical profile, DOAcK did not modify lipid metabolism and did not cause damage at the renal level. DOAcK administration increased the activities of Catalase (CAT), Glutathione Peroxidase (GPx), and Super Oxide Dismutase (SOD) to levels near those of the healthy group. Histopathological analysis exhibited morphology similar to that of the healthy group and the group treated with DOAcK. DOAcK is not mutagenic by Ames test for *Salmonella typhimurium* strains TA98, TA100, or TA102, and is not genotoxic by Micronucleus assay; median lethal dose (LD_{50}) >2000 mg/kg and, at this dose, no signs of toxicity or death were reported after 14 days of observation. These results indicate that DOAcK can improve glucose metabolism, which may be due to the increased antioxidant activity of CAT, GPx and SOD. In addition, DOAcK is not toxic in the studies tested.

Diabetes Mellitus (DM) is a chronic disease of impaired glucose metabolism characterized by hyperglycemia, which is caused by a deficiency in insulin secretion, insulin resistance, or both.[1] The World Health Organization (WHO) estimates that in 2012, hyperglycemia was the direct cause of 1.5 million deaths. Last year, 9% of adults worldwide had DM, and DM will be the 7th leading cause of death in 2030.[2] Treatment of type 2 DM (DM2) continues to present challenges because many patients have problems achieving adequate glucose levels. Despite the availability of many oral and injected antidiabetic drugs, therapeutic efficacy is regularly accompanied by side effects, such as hypoglycemia, weight increase, and cardiovascular complications, in addition to presenting high costs and, in many cases, drugs are not accessible to the entire population. Therefore, the search for novel drugs with better risk–benefit profiles continues.[3] According to recent reviews, in Mexico there are at least 383 plant species employed for the treatment of Diabetes Mellitus (DM), but only a few of these have been investigated for their preclinical or clinical efficacy.[4] In a previous work, we found that the ethyl acetate extract of *Arracacia tolucensis* carries out significant hypoglycemic activity in Streptozotocin (STZ)-induced diabetic rats,[5] being responsible for the biological activity, two of the major compounds: the chromone (*S*)-(+)-4′-*O*-angeloylvisamminol, and the pyranocoumarin (3′*R*,4′*R*)-(−)-4′-*O*-acetyl-3′-*O*-angeloylkhellactone, also known as Praeruptorin A.[6]

On the other hand, many works report that pyranocoumarin derivatives possess different biological activities, including cytotoxic, anti-inflammatory, antioxidant, antiviral, antibacterial, and hypoglycemic activity.[7] In patients with diabetes, there is a state of Oxidative Stress (OS), which it is due to a persistent production

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http://dx.doi.org/10.1016/j.bmcl.2016.06.071
0960-894X/

of Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), or a decrease in antioxidant enzyme activity.[8] Some coumarins, pyranocoumarins, and their related molecules have been evaluated in animal models, finding that they have hypoglycemic and antioxidant activity by increasing the activity of Catalase (CAT), Glutathione Peroxidase (GPx), and Super Oxide Dismutase (SOD).[9,10] Compounds that inhibit the increase in oxidant species may be drugs of interest for the treatment of DM.[11] Thus, we obtained the pyranocoumarin derivative 3′,4′-Di-*O*-acetyl-*cis*-khellactone (DOAcK) with an enantiomeric excess of 70% by chemical synthesis, and evaluated its antioxidant and antidiabetic activity, as well as its possible toxic effects (Ames test, Micronucleus assay, Acute oral toxicity) in the search for a new drug that provides better quality of life for patients with diabetes.

Synthesis of DOAcK (Fig. 1) was performed in three reactions steps. In the first step, umbelliferone was reacted with 1,1-diethoxy-3-methyl-2-butene in a medium of xylene, obtaining seseline as a yellow solid after purification by column chromatography (80%). Then, seseline was reacted with AD-mix α in water/*t*-butanol to obtain *cis*-khellactone with a yield of 80% and enantiomeric excess of 70% ([α] = +23). We compared this characteristic with those in the literature, and asymmetric dihydroxylation was stereo-selective primarily obtaining the *R*,*R* configuration.[12] In the final step, DOAcK was obtained by the reaction of *cis*-khellactone with acetyl chloride and DMAP in dichloromethane (CH_2Cl_2) to obtain a white solid by column chromatography, for which the yield was 41%.

Administration of DOAcK increased Body Weight (BW) in a significant manner in comparison with untreated groups Table 1. Diabetic and vehicle groups decreased and increased slightly in BW (−23.0 ± 33.1 Standard Error of the Mean [SEM]) and (2.2 ± 18.5), respectively, in contrast with the healthy group (64.3 ± 28.9). The group treated with Glibenclamide and DOAcK increased in 54.8 ± 20.4 and 45.7 ± 9.7, respectively. There was significance between the treated and untreated groups: the group treated with DOAcK exhibited weight close to that of the healthy group.

A survey of the literature illustrated that diabetic rats decreased their BW; however, treatment with extracts, fractions, or compounds from plants with antidiabetic activity can reverse this loss.[5] It has been found that Rutin exhibited an increase in BW up to 12.1%, and *Madhumega chooranam* can increase BW by 42.7%.[13,14]

Insulin comprises the most potent anabolic hormone known and Beta cells are responsible for producing; it is known that the death of these cells could be caused by hyperglycemia, and compounds with pyranocoumarin structure can improve it.[9,15,16] The increase in ROS can lead to death of Beta cells; therefore, an improvement in BW may be due to that DOAcK carries out better control of hyperglycemia by decreasing ROS.[17]

Coumarins and their derivatives have attracted attention due to their anti-inflammatory, antibacterial and antiarthritic activities,[18,19] as well as antidiabetic agents. In some studies it has been reported that different coumarins and pyranocoumarins decreasing blood glucose by up to 42%,[9] and in some cases it has been demonstrated that this improvement is due to changes in the activity of Glucose-6-phosphatase and Fructose-1,6-bisphosphatase.[10] These and many other studies report that coumarins and their derivatives comprise a source of novel molecules of biological interest.

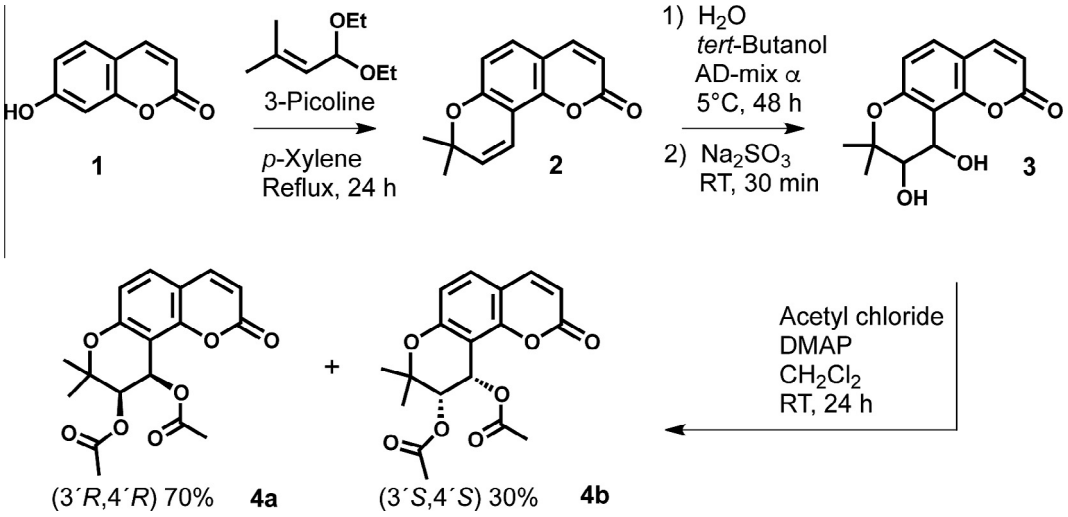

Figure 1. Synthesis of 3′,4′-Di-*O*-acetyl-*cis*-khellactone (DOAcK).

Table 1
Weight after 15-doses of DOAcK (15 mg/kg) in Streptozotocin (STZ)-induced diabetic rats

Group	Day 0 (g)	Day 7 (g)	Day 21 (g)	Weight gain (g)
Healthy	334.5 ± 15.7	345.0 ± 15.8	398.8 ± 24.3	64.3 ± 28.9*
Diabetic	371.6 ± 10.2	370.0 ± 13.0	348.6 ± 31.49	−23.0 ± 33.1#
Diabetic + vehicle	321.5 ± 11.1	323.1 ± 6.4	323.8 ± 14.8	2.2 ± 18.5#
Diabetic + Glibenclamide	330.7 ± 9.2	341.4 ± 13.1	385.5 ± 18.2	54.8 ± 20.4*
Diabetic + DOAcK	319.4 ± 6.9	335.3 ± 10.9	365.1 ± 6.7	45.7 ± 9.7*

* Statistically significant difference versus diabetic group by multiple comparison *Dunnett test*.
\# Statistically significant difference versus healthy group by multiple comparison *Dunnett test*. Vehicle: Water 2:1 DMSO. Glibenclamide was administered at 2.5 mg/kg; Weight gain = Weight on day 21, less weight on day 0 (n = 10; mean ± SEM; p <0.05). Treatment was started on day 7 after STZ-induction; on day 21, 15-doses of DOAcK were completed. (n = 10, mean ± SEM; p <0.05).

Table 2
Fasting blood glucose after 15-doses of DOAcK (15 mg/kg) in Streptozotocin (STZ)-induced diabetic rats

Group	Day 0 (mg/dL)	Day 7 (mg/dL)	Day 14 (mg/dL)	Day 21 (mg/dL)	Day 21 (increased glucose) (mg/dL)	Decrease of blood glucose (mg/dL)
Healthy control	61.25 ± 3.0	106.80 ± 4.8	98.30 ± 3.1	90.50 ± 8.5	29.25 ± 9.1*	–
Diabetic control	54.00 ± 2.6	371.80 ± 81.4	326.50 ± 72.3	327.50 ± 23.9	273.50 ± 21.8	–
Diabetic + vehicle	69.50 ± 8.5	272.00 ± 78.2	368.00 ± 65.3	406.30 ± 38.4	336.80 ± 24.8	–
Diabetic + Glibenclamide	63.75 ± 7.3	211.70 ± 45.2	282.80 ± 75.4	142.00 ± 5.5	78.25 ± 24.8*	185.5 ± 24.5
Diabetic + DOAcK	72.90 ± 5.2	363.20 ± 70.3	280.70 ± 76.4	128.30 ± 6.4	55.35 ± 19.2*	199.2 ± 24.7

* Statistically significant difference versus diabetic group by multiple comparison *Dunnett test*. Vehicle: Water 2:1 DMSO. Glibenclamide was administered at 2.5 mg/kg. Increased glucose = Glucose on day 21, less glucose on day 0. Decrease of blood glucose = Glucose of diabetic group less glucose of treated group. Treatment was started on day 7 after STZ-induction; on day 21, 15-doses of DOAcK were completed. (n = 10, mean ± SEM; p <0.05).

Table 3
Biochemical profile in fasting blood after 15-doses of DOAcK (15 mg/kg) in Streptozotocin (STZ)-induced diabetic rats

Group	Creatinine (mg/dL)	Urea (mg/dL)	Cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
healthy control	0.53 ± 0.02	49.41 ± 3.26	70.54 ± 2.01	30.42 ± 1.57	21.66 ± 2.32
diabetic control	0.58 ± 0.03	60.85 ± 6.89	68.30 ± 1.28	28.26 ± 1.54	12.67 ± 1.53
Diabetic + vehicle	0.56 ± 0.03	62.98 ± 6.21	80.09 ± 9.35	36.31 ± 0.51	23.11 ± 9.82
Diabetic + Glibenclamide	0.62 ± 0.05	58.43 ± 3.89	70.64 ± 2.79	26.60 ± 1.87	21.71 ± 4.68
Diabetic + DOAcK	0.51 ± 0.06	42.04 ± 6.99	70.84 ± 1.51	29.77 ± 1.09	22.38 ± 3.06

DOAck = 3′,4′-Di-*O*-acetyl-*cis*-khellactone. (N = 10; mean ± SEM; p <0.05). Vehicle: Water 2:1 DMSO. (n = 10, mean ± SEM; p <0.05).

Table 4
Catalase (CAT), Glutathione Peroxidase (GPx), and Super Oxide Dismutase (SOD) activity after 15-doses of DOAcK (15 mg/kg) in Streptozotocin (STZ)-induced diabetic rats

	Group	CAT H_2O_2 consumed (μM/min/mg protein)	GPx $NADPH^+$ consumed (μM/min/mg protein)	SOD unit of SOD (U/min/mg protein)
I	Healthy control	132.2 ± 5.9	54.6 ± 5.5	0.83 ± 0.06
II	Diabetic control	47.5 ± 2.6	20.8 ± 3.3	0.44 ± 0.01
III	Diabetic + vehicle	68.9 ± 11.1	10.0 ± 0.6	0.22 ± 0.06
IV	Diabetic + Glibenclamide	93.2 ± 4.9*	48.75 ± 5.8*	0.55 ± 0.02
V	Diabetic + DOAcK	94.8 ± 7.8*	119.0 ± 20.7*	0.73 ± 0.03*

Vehicle: Water 2:1 DMSO. U of SOD: One unit (U) of SOD is defined as the amount in μg of enzyme that causes a 50% decrease of the NBT reduction. (n = 10, mean ± SEM; p <0.05).
* Statistically significant difference versus the diabetic group by the multiple comparison *Dunnett test*.

In Mexico, are at least 383 plant species employed for the treatment of DM.[4] We have evaluated extracts and compounds isolated from *A. tolucensis*[6] with anti-inflammatory and antidiabetic activities in STZ-induced diabetic rats.[5] We now propose the evaluation of the bioisostere of one of the major metabolites of *A. tolucensis*, DOAcK, which is an analogue of Praeruptorin A; we expect that, due to a structural activity relationship, this compound will exhibit the same or better antidiabetic activity.[20] Table 2 shows the values of fasting blood glucose of the experimental groups. DOAcK treatment for 15 days at 15 mg/kg decreased blood glucose values. Blood glucose decreased in the groups treated with Glibenclamide and DOAcK by 56.6 and 60.9% (p <0.05), respectively, when compared with the diabetic control group. These results indicate that DOAcK can improve glucose metabolism. Many works in the literature have evaluated the extracts of plants with antidiabetic activity. *Toddalia asiatica* (L.) Lam. Ethyl Acetate (EtOAc) extract decreasing the blood glucose level from 290.38 ± 1.89 to 108.27 ± 4.38 mg/dL compared with the diabetic group. In addition, this extract increased SOD, CAT, and GPx activity,[21] and the CH_2Cl_2 fraction of *Kalanchoe pinnata* can decrease blood glucose in STZ-induced diabetic rats from 359 ± 11 to 109 ± 10 mg/dL.[22]

Other studies has been demonstrated that administration of Skimmin, a coumarin, decreased blood glucose from 494.4 ± 5.0 to 390.0 ± 10.1 mg/dL; in addition, Skimmin regulated Transforming Growth Factor beta-1 (TGF-β1) protein levels, which improved nephropathy in rats.[23] Clorichromene and XLF-III-43, other coumarins, have been evaluated against diabetic nephropathy; both compounds improved diabetic complications by preserving the blood-retinal barrier[24] and inhibiting advanced glycation end products.[25] The glucose values obtained in this study are similar to the studies discussed previously. DOAcK performed an antidiabetic effect, with a decrease of blood glucose of 199.2 ± 24.7 mg/dL compared with the untreated diabetic group. This result demonstrated that DOAcK possesses interesting antidiabetic activity.

Table 3 depicts the biochemical profile in the experimental groups with and without treatment. No statistically significant differences were observed between all groups compared with healthy and diabetic controls; these results suggest that administration of DOAcK does not modify lipid metabolism and does not cause damage at the renal level. This is consistent with the absence of macroscopic pathologic lesions in the liver and kidney of animals treated with DOAcK.

Oxidative Stress (OS) produced by cellular respiration and environmental factors is the main cause of DM complications (protein oxidation, retinopathies, nephropathies, and neuropathies).[26] Superoxide anion, hydrogen peroxide and radical hydroxyl are the most dangerous radicals known. The cell possesses CAT, GPx, and SOD, enzymes that remove ROS, therefore, the increase of these enzymes provides protection against the harmful effects evoked by ROS.[27]

Extracts of *Stevia rebaudiana* and *Eucalyptus globulus* administered in animal models of diabetic rats increased CAT and GPx activities.[28,29] We performed an evaluation of DOAcK on CAT, GPx and SOD activities. Table 4 illustrates the effect on the antioxidant level in the liver of STZ-induced diabetic rats. Administration of DOAcK and Glibenclamide increased CAT activity to levels near

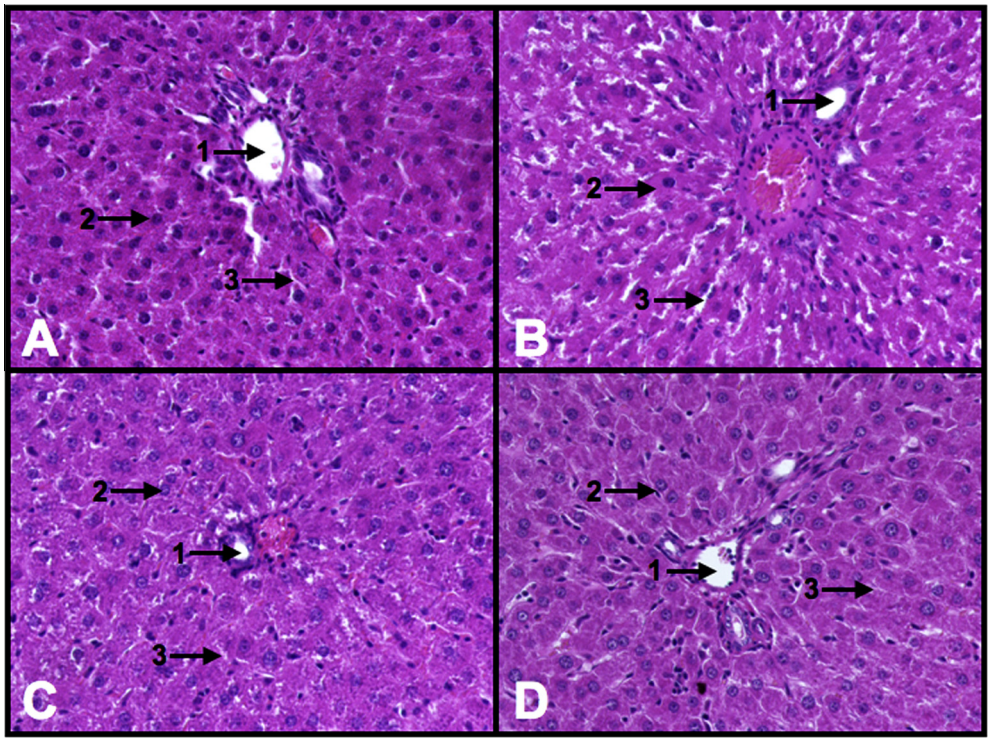

Figure 2. Histopathological analyses of liver in Streptozotocin (STZ)-induced diabetic rats. (A) Healthy control, (B) Diabetic control, (C) Diabetic + Glibenclamide, (D) Diabetic + DOAcK. (1) Central vein, (2) Hepatocyte, (3) Sinusoids. Images are representative of each experimental group (200×).

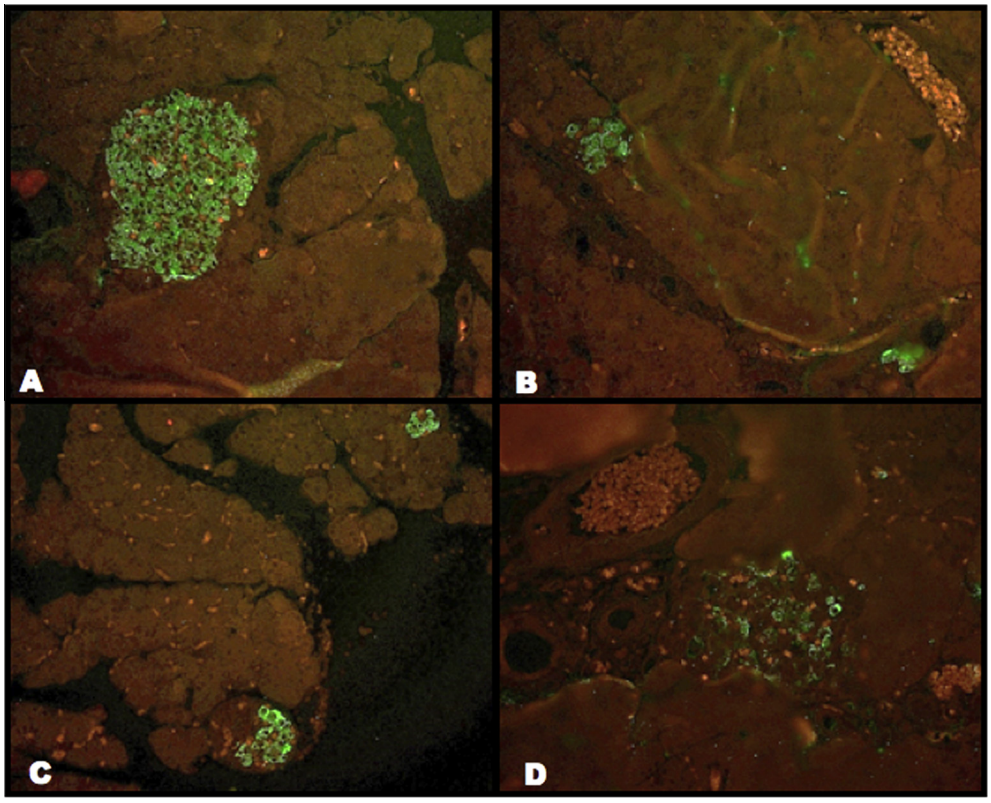

Figure 3. Fluorescence microphotographs of the Islets of Langerhans of Streptozotocin (STZ)-induced diabetic rats. Insulin is displayed in green. (A) Healthy control, (B) Diabetic control, (C) Diabetic + Glibenclamide, (D) Diabetic + DOAcK. Images are representative of each experimental group (200×).

those of the healthy group, but interestingly, DOAcK increased GPx activity more than Glibenclamide treatment. On the other hand, administration of DOAcK increased SOD activity of statistically significant manner. CAT, GPx, and SOD are three of the major antioxidative enzymes. It is known that there are compounds that are able to increase the expression of these enzymes. The

Table 5
Mutagenicity index of DOAcK in *Salmonella typhimurium* strains TA98, TA100, and TA102

Substance	Concentration μM/plate	TA98		TA100		TA102	
		With S9	Without S9	With S9	Without S9	With S9	Without S9
DOAcK	8.65	1.1	0.7	0.9	1.3	0.8	0.7
DOAcK	17.30	1.2	1.0	0.9	1.0	1.2	0.7
DOAcK	34.60	1.2	0.8	0.8	1.0	1.4	1.0
DOAcK	50.00	1.1	1.1	0.9	0.9	1.2	1.1
DOAcK	100.00	1.4	0.4	1.0	1.4	0.9	0.8
DMSO 0.1%	141.00	1.1	0.4	0.9	1.0	1.1	1.0
Picrolonic acid	0.19		27.3				
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine	0.007				22.9		
4-Nitroquinoline-1-oxide	0.003						3.1
2-Aminoanthracene	0.005	54.1		19.1		3.5	

DMSO = Dimethyl sulfoxide.

thiazolidinediones comprise a family of compounds that possess this activity; however, their toxic effects have removed some of these compounds.[30] Our results provide evidence that DOAcK can increase the activity of CAT, GPx and SOD, thus increasing antioxidant activity in order to attenuate damage generated by ROS. These results are in agreement with the results obtained in the Ames assay: DOAcK are not mutagenic on the TA102 strain, which is able to detect chemical compounds with the ability to generate ROS.

Histopathological analysis of the healthy rats group demonstrated normal architecture with a central vein and hepatocytes surrounding this vein, while the diabetic group exhibited more sinusoidal spaces due to hepatocyte inflammation and even death. Groups treated with Glibenclamide and DOAcK showed a morphology similar to that of the healthy group (Fig. 2); this could be due to improved glucose control. In other studies it has been reported that *Cyclocarya paliurus* and mangiferin in STZ-induced diabetic rats, can decrease the damage observed in different tissues, in contrast with the untreated groups.[31,32]

Figure 3 depicts the Islets of Langerhans of the experimental groups. The diabetic untreated group exhibits a constriction of these Islets, as well as a decrease of β-cells versus the healthy group. In contrast, the DOAcK-treated group demonstrated morphology close to that of the healthy group. Patients with diabetes exhibited a decrease of β-cells after having been diagnosed with DM, due to progressive damage by OS evoked by ROS and hyperglycemia.[33] The morphology observed in the group treated with DOAcK is probably due to attenuation in ROS levels.

None of the five DOAcK concentrations tested were mutagenic for *S. typhimurium* strains TA98, TA100, or TA102 in the absence or presence of the S9 fraction. There are natural products that possess potential mutagenic activity, such as flavonoids and alkenylbenzenes;[34] therefore, in order to avoid potential health risks, we evaluated the mutagenic potential of DOAcK by means of the Ames test. Only positive controls increased the revertants number under all conditions. Table 5 presents the mutagenic index of the doses used, which were determined as follows: the average number of revertants per plate test divided by the average number of revertants per baseline control plate. Since 1983, Ohta found that coumarin and 7-hydroxy-coumarin have antimutagenic effects;[35] furthermore, in 2004, Marques and Lin found that 4-hydroxy-coumarin can be an antimutagen agent. This compound might form hydrogen-bonds between its carbonyl group and amino group of mutagenic compounds to produce stable complexes.[36] Although it is necessary to conduct other studies, we think that DOAcK and other coumarins would possess a mechanism similar to that demonstrated by 4-hydroxy-coumarin.

In vivo Micronucleus assay can detect genotoxic agents that cause damage to the chromosomes or to the mitotic spindle.[37] We evaluated the potential of DOAcK to produce micronucleus

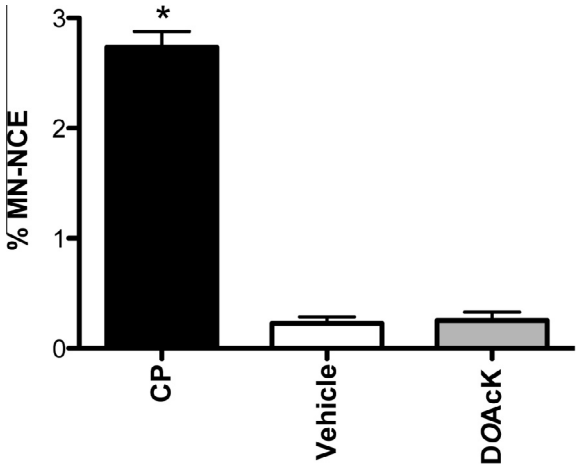

Figure 4. Percentage of erythrocytes with micronucleus in the different groups. Data are expressed as mean ± Standard Error of the Mean (SEM). *Significant statistical differences (p <0.05) against the negative control. CP: Cyclophosphamide; vehicle (water 2:1 DMSO).

48 h after treatment was administered. Figure 4 depicts the percentage of micronuclei of treated groups. The positive control (Cyclophosphamide) was significantly increased compared with the vehicle control (water 2:1 DMSO), whereas administration of DOAcK did not affect the percentage of micronuclei. When a new drug is proposed for use in humans, it is necessary to analyze its toxic activity in order to avoid partial or mortal complications. Glibenclamide has been reported as not evocating any significant increase in the frequencies of micronucleated cells.[38] However, recently it was reported that Metformin and Glimepiride increased the frequency of micronucleated cells.[39] The presence of genotoxicity in drugs currently employed to treat DM is evident; in contrast, our results show that DOAcK are not aneuploid or clastogenic agent.

The OECD-423 test was conducted to evaluate the acute toxicity of DOAcK at the initial dose of 2,000 mg/kg.[40] At this dose, no signs of toxicity or death were reported after 14 days of observation. Animals treated with DOAcK increased BW in the same manner as the untreated control group (Table 6). Hence, according to the Globally Harmnonized System (GSH) system, the LD_{50} of DOAcK is >2000 mg/kg. Although DOAcK LD_{50} has not been previously determined, its precursor, the coumarin, has an LD_{50} >700 mg/kg,[41] both toxicity values suggesting that the acute toxicity of this family of compounds is relatively elevated. The biochemical profile in diabetic rats showed similar values to healthy group, indicating no toxic activity at the dose tested (Table 3).

Table 6
Body weight (BW) of animals after 14 days of treatment with 2000 mg/kg of DOAcK

	Day 1		Day 14		Weight gain 14	
	Control	DOAcK	Control	DOAcK	Control	DOAcK
Weight	22.3 ± 1.8	21.0 ± 1.0	35.7 ± 1.5	34.0 ± 0.0	13.3 ± 0.3	13.0 ± 1.0

The search for new drugs to treat DM is essential, despite that there are many medicaments available to the public and many others under investigation; these drugs have different degrees of effectiveness and side effects. The pyranocoumarins are compounds that possess an important pharmacological profile; we have provided evidence regarding how DOAcK can improve glucose metabolism, which may be due to the increased antioxidant activity of CAT, GPx, and SOD, in addition to not presenting high toxicological potential. Therefore, DOAcK could be an alternative in treating DM. However, more studies are necessary to warrant this: it is important to evaluate DOAcK in a different animal model of diabetes and to determine its sub-chronic and chronic oral toxicity. In this moment, we are evaluating DOAcK in an animal model of diet-induced obese mice to evaluate its potential for treating metabolic syndrome. The antidiabetic activity of DOAcK is the subject of a patent.

Acknowledgments

We thank Ricardo Gaxiola-Centeno, MVZ, for his excellent technical support in the model of Streptozotocin-induced diabetic rats. The authors are very grateful for the financial support from SIP-IPN – México (20140974 and 20150719). Our thanks also go to CONACYT – México for the fellowship granted to EAD-M (240114), he studied in the program of 'Doctorate of Science in Biotechnology on Network of the National Polytechnic Institute'.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.071.

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