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The vasodilating effect of a *Hintonia latiflora* extract with antidiabetic action

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ABSTRACT

In the present study, it is shown for the first time that an extract of *Hintonia latiflora* (HLE) which is used as an antidiabetic herbal medicine, is not only able to decrease blood glucose concentration but additionally exerts a vasodilating effect. Accordingly, this extract might have a positive influence on diabetes-associated dysfunction of blood vessels.

The vasodilating effect was demonstrated *in vitro* in aortic rings of guinea pigs as well as *in vivo* in rabbits. Aortic rings pre-contracted with noradrenaline (NA) could completely be relaxed by HLE (EC_{50}: 51.98 mg/l). In contrast, potassium-induced contractions were not diminished by HLE. Therefore, it can be suggested that the vasodilating effect of HLE is primarily the result of an inhibition of G protein-induced increase in intracellular calcium and not of a blockade of voltage-operated L-type calcium channels.

The neoflavonoid coutareagenin (COU), a constituent of HLE which in part is responsible for the blood glucose-lowering effect of HLE, also relaxed NA-induced contractions of aortic rings (EC_{50}: 32.55 mg/l) and only weakly inhibited potassium-induced contractions.

Experiments in aortic rat cells revealed that calcium transients evoked by vasopressin were suppressed by 60 mg/l COU supporting the idea of an inhibition of G protein-induced intracellular calcium release by a constituent of HLE.

To study the effect of HLE on vascular tone under *in vivo* conditions, ultrasound measurements were carried out in conscious rabbits which received a single oral dose of HLE. Under the influence of HLE, a vasodilation combined with a lowering of blood flow velocity could be observed in the abdominal aorta and the common carotid artery. Additionally, a decrease in blood glucose concentration in the HLE group occurred.

The combination of a blood glucose-lowering with a vasodilating effect may be helpful for reducing angiopathies, typical long-term complications in patients with diabetes mellitus.

Introduction

Diabetes mellitus (DM) is a widespread disease with an increasing number of patients. The patients generally need medical supervision during lifetime. Type 2 diabetes accounts for almost 90% of all cases of diabetes in adults. Usually, the blood glucose level can more or less be well controlled in these patients and dangerous events such as heavy fluctuation of the blood glucose level and diabetic coma are rare. The main unsolved problems of DM include long term vascular complications which are the leading causes of death in type 2 DM. Chronic hyperglycaemia appears to be one of the central initiating factors responsible for the development of diabetic complications as microangiopathies like retinopathy, neuropathy, nephropathy or macroangiopathies like stroke and myocardial infarction (Brownlee, 2005). For example, glucose can bind to proteins of the body and induce formation of advanced glycosylated end products (AGE products; Brownlee, 2005). Consequences of this formation are dysfunction and destruction of blood vessel walls. Particularly, vascular relaxation will be

Abbreviations: COU, coutareagenin; DM, diabetes mellitus; HLE, *Hintonia latiflora* extract; NO, nitric oxide; NA, noradrenaline; ROS, reactive oxygen species; VP, vasopressin ([Arg^8]vasopressin).

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impaired (Pannirselvam et al., 2003) due to a decrease in endogenous vasodilators and an increase in endogenous vasoconstrictors (Brownlee, 2005). Then, tissues might not be sufficiently supplied with nutrients and oxygen.

On the basis of this knowledge, it was of interest to investigate whether an extract of *Hintonia latiflora* (HLE) has, additionally to its blood glucose-lowering effect, an influence on the tone of blood vessels. An ethanolic-aqueous extract of *H. latiflora* (Copalchi) bark from South America is used as a phytoantidiabetic agent primarily for the treatment of patients with mild to moderate severe type 2 diabetes (Korec et al., 2000; Korecova et al., 2006). The antidiabetic effect of the extract was confirmed in several animal experimental studies (Kaiser and Geyer, 1955; Korec et al., 2000; Pinto et al., 1997; Slijepcevic and Kraus, 1997).

In the present study, aortic rings of guinea pigs were used to study the effects of HLE and COU in arterial blood vessels. Additionally, the action of COU on calcium movements in vascular smooth muscle cells was investigated. Finally, HLE was applied per gavage to rabbits and the influences on abdominal aorta and carotid artery, heart rate, blood pressure and blood glucose level were investigated.

Material and methods

Materials

For the *in vitro* experiments, ±noradrenaline (NA) and milrinone were obtained from Sigma, Germany. Cultivated rat arterial smooth muscle (A10) cells were obtained from DMSZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Germany. The fluorescence dye fura-2/AM was purchased from Microprobes, USA and VP ([Arg^8]vasopressin) from Sigma Aldrich, Germany.

H. latiflora bark native extract and COU were kindly provided by Gehrlicher GmbH, Eurasburg, Germany. *H. latiflora* (Sessé & Moc. ex DC.) Bullock belongs to the family of Rubiaceae and grows in the north of South America and in Central America. The used extract was an ethanolic-aqueous spissum extract (drug-extract relation 3:1; lot no. 7618) with a COU content of 12.9% determined by HPLC. A voucher specimen of the bark and the extract is deposited by Gehrlicher GmbH for future reference.

COU (5-hydroxy-7-methoxy-4-(3,4-dihydroxyphenyl)-2*H*-benzo-1-pyran-2-on) was synthesized by Gehrlicher GmbH, Eurasburg, Germany. For the *in vitro* experiments, *H. latiflora* extract (HLE) and COU were dissolved in water ethanol (1:1) mixture and further diluted with water. For *in vivo* experiments, HLE was dissolved in polyethylene glycol (macrogol 300; Caesar & Lorentz, Germany). NA, milrinone and VP were diluted in water.

Animals and study design

In vitro evaluation in isolated aortic rings of guinea pigs

Guinea pigs were euthanized by concussion (Close et al., 1997). The thoracic aorta was dissected and cut into 2–4 mm ring segments. Isometric force was measured. The bath solution was a modified Krebs–Henseleit buffer (KHB) containing (mM): NaCl, 115; KCl 2.8; $CaCl_2$ 2; $NaHCO_3$, 25; KH_2PO_4 1.2; $MgCl_2$ 1.2; glucose 10. HLE was added in a cumulative manner to the tissue bath. Alternatively, COU, milrinone or ethanol was added cumulatively. Additionally, the effects of HLE and COU on contractions induced by potassium (addition of 30 mM KCl) were observed.

In vitro evaluation of Hintonia latiflora extract in rat aortic (A10) cells

A10 cells were bred on coverslips which were fixed in an experimental chamber (filled with modified KHB with 1.2 mM Ca^{2+}) and put on an inverse microscope. Cells were loaded with 8 to 10 μM fura-2/AM and then superfused to remove extracellular fura-2/AM. For intracellular calcium measurements, cells were illuminated alternately with light of wave-lengths of 340 and 380 nm (AMKO LTI, Germany). The emitted fluorescence at 510 nm was recorded and the ratio between the emitted fluorescence signals was calculated as indicator for the intracellular calcium concentration. Addition of 2 nM VP led to a temporary increase of the intracellular calcium concentration (calcium transient). VP was added after a 45 min rest period and removed after 6 min. After a following rest period of 5 min, COU was added and equilibrated during 40 min. Then addition of VP followed. In control experiments, it was shown that in the absence of COU, the second VP-induced calcium transient did not differ remarkably from the first one.

In vivo evaluation of the effect of Hintonia latiflora by ultrasound

Ultrasound measurements of the abdominal aorta and the common carotid artery were carried out in 8 adult, white New Zealand rabbits (Charles River, Germany) using an ultrasound device (GE Vingmed Ultrasound A/S System VIVID FIVE, N-3191, Norway) fitted to a 10 MHz linear probe. Additionally, mean arterial blood pressure and heart rate were measured by using a special equipment (Data Ohmeda S/5, type F-CM1.00, Finland) connected *via* pressure sensor to Art. auricularis of the animal. Blood glucose was measured in blood of Art. auricularis by using Accu-Check, Roche Diagnostics, Germany. The following parameters were determined: diameter of the abdominal aorta and the common carotid artery, maximum systolic blood flow velocity in these vessels, mean arterial blood pressure, heart rate and blood glucose concentration. After preparing the animals for the experiments, the baseline values were determined. Then, the animals were anesthetized for a short time with 1% propofol (7 mg/kg; Fresenius Kabi GmbH, Germany). During anaesthesia, HLE (200 mg/kg) dissolved in macrogol 300 (1 ml/kg) was administered per gavage to one animal and only macrogol 300 (1 ml/kg) to the other animal. After recovering from anaesthesia, ultrasound measurements were performed at 0.5, 1, 2, 3 and 4 h. All investigations were carried out by a blinded examiner. The animal experiments were performed in concordance with local laws of animal protection and approved by the animal protection committee of the Government of Upper Bavaria, Munich, Germany.

Statistical analysis

Results are expressed as means ± standard error of the means (SEM). Concentration-effect curves were fitted to a sigmoidal relationship after logarithmic transformation of the concentrations and the EC_{50} values ± SEM determined using GraphPad Prism, USA. The *t*-test was used for the determination of significance in the fura experiments. In the *in vivo* studies, Friedman, Wilcoxon and Mann–Whitney tests were used. Differences were considered as significant at $p \leq 0.05$.

Results

Effects of Hintonia latiflora extract and coutareagenin on noradrenaline-induced contractions

In aortic rings pre-contracted with NA (10 μM), HLE concentration-dependently relaxed the rings (Fig. 1). Relaxation begun at concentrations >45 mg/l and was complete at 60 mg/l (by 99.18 ± 2.35%). The EC_{50} was 51.98 mg/l (51.41 to 52.60 mg/l). Addition of COU also led to a relaxation of the NA-induced contraction. However, relaxation begun at concentrations >15 mg/l and was almost complete at 60 mg/l (by 93.81 ± 5.62%). The EC_{50} was 32.55 mg/l (30.52 to 34.63 mg/l). Accordingly, the potency of the compound is about 1.6 times of that of the extract. Control experiments with ethanol which was used as solubilizers

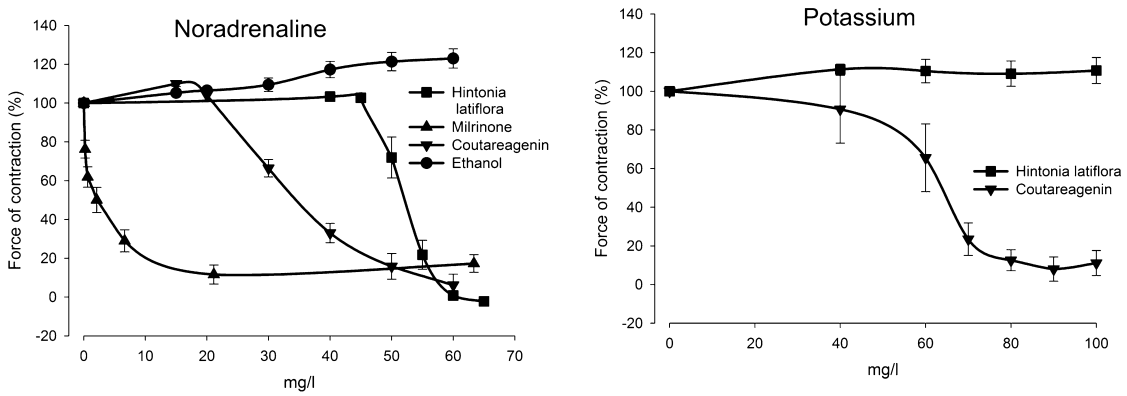

Fig. 1. Effects of *Hintonia latiflora* extract and coutareagenin on NA- and K-induced contractions in aortic rings of guinea pigs. Contractions were elicited by addition of 10 μM NA (left side) or an increase in K^+ concentration by 30 mM (right side). Furthermore, the influences of the solvent (ethanol) and, for comparison, the effect of milrinone on NA-induced contraction are shown. Relative mean values ± SEM of muscles from eight animals for each group are presented.

revealed no relaxing effect. The potency of the reference substance milrinone was rather strong (EC_{50}: 2.43 mg/l; 1.42 to 4.17 mg/l) but the extent was restricted (relaxation by 88.39 ± 4.91%).

Effects of Hintonia latiflora extract and coutareagenin on potassium-induced contractions

In the presence of an elevated potassium concentration, HLE showed no relaxing effect until 100 mg/l. Contrarily, COU showed a relaxing effect. The maximum effect was reached at 90 mg/l COU (by 92.00 ± 6.27%). The EC_{50} was 63.18 mg/l (60.42 to 66.20 mg/l). Regarding the EC_{50} values, the potency of COU in potassium-induced contractions was about half of that in NA-induced contractions.

Influence of coutareagenin on intracellular calcium

The temporary intracellular rise in calcium concentration in single aortic smooth muscle cells evoked by 2 nM vasopressin was remarkably suppressed in the presence of COU (Fig. 2). Superfusion of the cell with 60 mg/l COU before addition of vasopressin led to complete suppression of the rapid initial increase of the calcium signal. The maximum increases of the ratio values of calcium transients were reduced under the influence of COU from 0.664 ± 0.083 to 0.184 ± 0.030 or by 72.3% ($p < 0.05$).

Effect of Hintonia latiflora on diameter and maximum systolic blood flow velocity in blood vessels of rabbits

Abdominal aorta

Following application of HLE, a slight increase in the **diameter of the abdominal aorta** was observed (Fig. 3) which was more pronounced after 0.5 h (from 100% to 105.28 ± 1.53%) and after 3 and 4 h (increases to 105.80 ± 1.66 and 107.03 ± 1.99%). In contrast, in the control group, there was a slight decrease in the diameter (to 95.86 ± 2.69% at 0.5 h and to 97.88 ± 3.07% at 3 h). The values in the treatment group with exception of the value after 1 h were significantly different ($p < 0.05$) from the initial value. The control group did not show significant differences to the initial values. The difference between the treatment group and the control group was significant ($p = 0.016$) after 0.5 h and there was a tendency to significance at 3 and 4 h ($p = 0.059$).

The application of HLE induced a transient increase of the **maximum systolic blood flow velocity** after 0.5 h (from 100 to 110.86 ± 5.76%) which was followed by a pronounced decrease of the velocity after 3 to 4 h (from 100% to 92.77 ± 5.75% and to 93.13 ± 7.7%, respectively; Fig. 3). The values in the control group increased somewhat. All values were not significantly different from the initial values and also the differences between the treatment and the control group did not reach significance although the difference at 3 h showed a tendency to significance ($p = 0.093$).

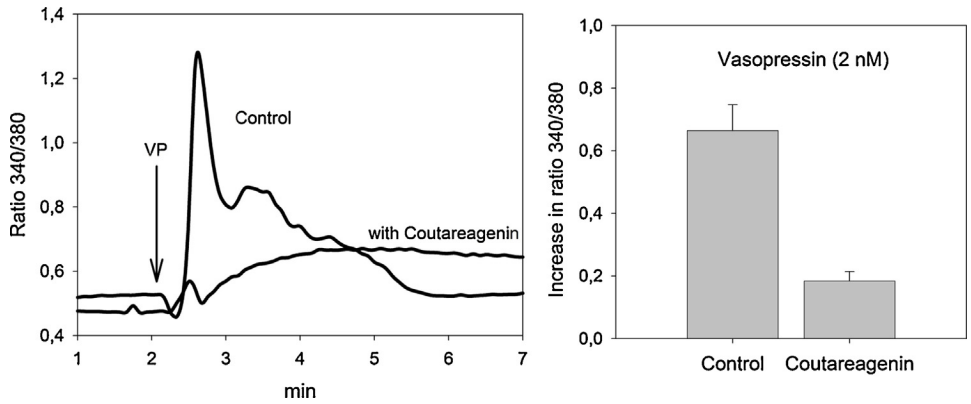

Fig. 2. Suppression of vasopressin-induced calcium transients in A10 cells by coutareagenin. A10 cells were loaded with calcium-sensitive dye fura-2. Ratios of light emitted at wavelength of 510 nm after excitation of dye at 340 and 380 nm were determined as indicator of intracellular calcium concentrations. Calcium transients were elicited by adding vasopressin (VP; 2 nM) in absence or presence of coutareagenin (60 mg/l). Left side: original recordings of the transients. Right side: Mean values (%) ± SEM of increases in ratios after VP addition recorded in eight cells.

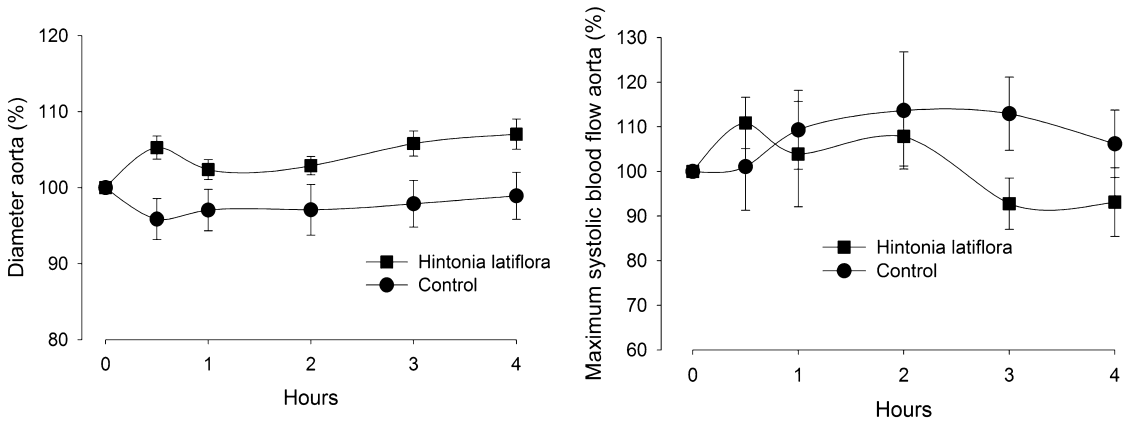

Fig. 3. Effect of *Hintonia latiflora* extract on the diameter and the maximum systolic blood flow velocity of the abdominal aorta of rabbits. Blood vessel diameter (left side) and blood flow velocity (right side) were determined by sonography. *Hintonia latiflora* (200 mg/kg in macrogol 300) or only macrogol 300 were administered after measurement of the baseline values. Mean values (%) ± SEM from eight animals in each group.

Common carotid artery

Measurements in the common carotid artery revealed similar effects as in the aorta, *i.e.* some vasodilation and reduction of the blood flow under the influence of HLE (results not shown). The differences to control values and between the treatment and control group did not reach significance.

Effects of Hintonia latiflora on heart rate and blood pressure

Under the influence of HLE there appeared no remarkable differences between the treatment and the control group regarding heart rate and blood pressure of the rabbits (results not shown). Additional experiments in isolated heart preparations did not show any effect on heart muscle contractions.

Effect of Hintonia latiflora extract on blood glucose concentration

HLE induced a decrease in blood glucose concentration. The concentration decreased under HLE from 100.50 ± 3.55 to 89.14 ± 1.86 mg/dl after 2 h or by 10.87 ± 2.33% (Fig. 4). A minimum value of 89.13 (mg/dl) was achieved after 2 h in the HLE group. The decreases were significant after 0.5, 1 and 2 h. Comparing both groups, the differences after 1 and 2 h were significant.

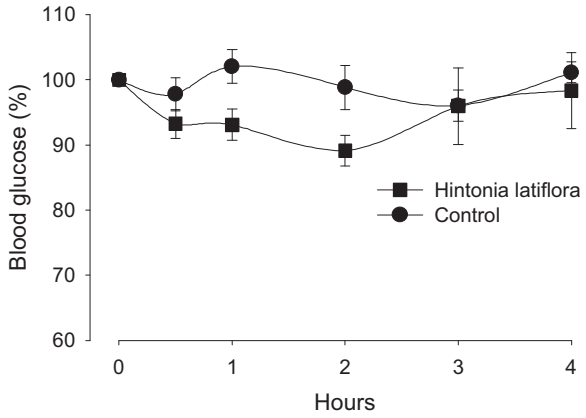

Fig. 4. Effect of *Hintonia latiflora* extract on blood glucose concentration of rabbits. Same experiment as described in Fig. 3. Mean values (%) ± SEM from eight animals in each group are shown.

Discussion

The purpose of the present study was to investigate whether HLE which is used for the treatment of mild to moderately severe type 2 DM, has, additionally to its blood glucose-lowering effect, an influence on the cardiovascular system. A vasodilating effect of HLE could be helpful to avoid long-term complications in DM patients. In *in vitro* experiments, it was demonstrated that NA-induced vasoconstriction could be completely abolished by addition of HLE. Furthermore, in *in vivo* experiments, it was shown that administration of a single dose of HLE led to some dilation of the aorta together with a reduction of blood flow. Similar effects were seen in the common carotid artery. These effects were combined with a decrease in blood glucose concentration pointing to the antidiabetic property of the extract.

Considering the mechanism of action of the vasorelaxant effect, the fact that in isolated preparations a complete relaxation was observed under the influence of NA but no relaxation in K^+-induced contractions argues against a blocking effect of the extract on voltage-dependent L-type Ca^{2+} channels. Blockers of these channels like nifedipine preferentially inhibit K^+-induced contractions of aortic muscle (Godfraind, 1983). A strong further argument against a Ca^{2+} channel-blocking effect of HLE comes from experiments in heart muscle preparations (not shown): the extract exerted no negative inotropic effect and induced no change in the shape of the contraction curve typical for blockers of L-type Ca^{2+} channels (Sensch et al., 2000). The fact that the K^+-induced increase in vascular tone was not diminished by HLE argues against a nitric oxide- (NO—) mediated effect or a phosphodiesterase inhibiting action. Also relaxation by activation of membrane K^+ channels is not probable. Hypothetically, a direct influence of the extract on the inositol trisphosphate (IP_3) pathway of the smooth muscle cell can be assumed.

COU, a lead compound of HLE, showed a stronger relaxing effect than HLE. Regarding EC_{50} values, the effect of COU was about 1.5 times stronger than that of HLE. However, the determined content of COU was 12.9%. This means, if COU would be the only active compound of HLE, the potency of COU should be about 7.7 times of that of HLE. Therefore, other compounds beside of COU should contribute to the relaxing effect of COU. Indeed other neoflavonoids and oxidocoumarins as well as cucurbitacins were isolated from stem bark extracts of *H. latiflora* (Guerrero-Analco et al., 2007; Reguero et al., 1987) which may contribute to the vasodilating effect of HLE.

As shown, COU was able to suppress the intracellular calcium release induced by the vasoconstringent peptide VP in aorta

cells. Because VP also induces Ca^{2+} release *via* activation of the phosphatidylinositol cascade (Doyle and Ruegg, 1985; Simpson and Ashley, 1989), the result supports the hypothesis that COU and also HLE inhibit a step in the phosphatidylinositol cascade. It also indicates that, in view of the absence of endothelial cells, release of nitric oxide (NO) from the endothelium is not essential for vasodilation.

The combination of a blood glucose-lowering with a vasodilating effect is a very interesting principle, because elevated blood glucose and vasoconstriction certainly play a decisive role for the development of long-term complications of diabetes like microangiopathies and macroangiopathies. Blood glucose or metabolites of it can, in continuously increased concentrations, bind to certain molecules, *e.g.* proteins, and disturb their function mainly in endothelial and smooth muscle cells of blood vessels (Brownlee, 2005). A marker of this interaction is an increase in glycated haemoglobin (HbA1c). Moreover, increased formation of reactive oxygen (ROS) due to an augmented glucose metabolism leads to a secondary damage of certain cells. Additionally, in diabetes, an increase in vasoconstrictive factors like endothelin and a decrease in vasodilating compounds like nitric oxide (NO) were observed. This combination leads to blood vessel damage and vasoconstriction resulting in impaired support of tissues with oxygen and nutrients.

HLE can reduce the burden of hyperglycaemia and concomitantly the glycation of target molecules (reduction of HbA1c) as shown in a study in patients with type 2 diabetes (Korecova et al., 2006). Therefore, a reduction of vascular complication in these patients can be expected. As a result of the present study, a vasodilating effect of HLE may help to improve the perfusion and nutrition of critical tissues. It is interesting that the observed dilation of the blood vessels was not combined with an increase in heart rate and therefore a heart rate-induced increase in oxygen consumption can be avoided.

As shown by Reguero et al. (1987) and Guerrero-Analco et al. (2007), their *H. latiflora* extract contained beside neoflavonoids also cucurbitacins as active blood-glucose lowering compounds. Cucurbitacin glucosides exert a strong antioxidant and free radical scavenging effect (Tannin-Spitz et al., 2007). Probably, also COU and other neoflavonoids have some antioxidant effects (Lee et al., 2001). If we consider that the formation of ROS substantially contributes to the damaging effect of high glucose levels on blood vessels, then a further favourable effect of the extract regarding long-term complications of diabetes can be expected.

The combination of a mild hypoglycaemic effect with vasodilating and antioxidant properties of HLE may not only be helpful for the treatment of patients with manifest but also with prediabetes if dietetic interventions and physical activities are not sufficient.

Conflicts of interest

The authors declare that there is no conflict of interest.

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