

# In Vitro Activation of eNOS by *Mangifera indica* (Careless™) and Determination of an Effective Dosage in a Randomized, Double-Blind, Human Pilot Study on Microcirculation

## Authors

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## Key words

- ◉ *Mangifera indica*
- ◉ Anacardiaceae
- ◉ Careless™
- ◉ microcirculation
- ◉ endothelial function
- ◉ human study
- ◉ eNOS
- ◉ HUVEC

## Abstract

▼ *Mangifera indica* fruit preparation (Careless™) activates the evolutionary conserved metabolic sensors sirtuin 1 and adenosine monophosphate-activated protein kinase, which have been identified as playing a key role in microcirculation and endothelial function. Here, an acute effect of a single dose of 100 mg or 300 mg Careless™ on microcirculation was investigated in a randomized, double-blind, crossover pilot study in ten healthy women to determine the effective dosage. Microcirculation and endothelial function were assessed by the Oxygen-to-see system and pulse amplitude tonometry (EndoPAT™), respectively. Cutaneous blood flow was increased over time by 100 mg (54% over pre-values,  $p = 0.0157$ ) and

300 mg (35% over pre-value,  $p = 0.209$ ) Careless™. The EndoPAT™ reactive hyperemia response was slightly improved 3 h after intake compared to pretesting with 300 mg Careless™. Furthermore, activation of endothelial nitric oxide synthase, as an important regulator for endothelial function, was tested *in vitro* in primary human umbilical vein endothelial cells. Careless™, after simulation of digestion, increased the activated form of endothelial nitric oxide synthase dose-dependently by 23% (300 µg/mL), 42% (1500 µg/mL), and 60% (3000 µg/mL) compared to the untreated control. In conclusion, the study suggests moderate beneficial effects of Careless™ on microcirculation, which is at least partly mediated by endothelial nitric oxide synthase activation.

## Introduction

▼ The fruits of *Mangifera indica* L. (Anacardiaceae) are known to contain high levels of bioactive compounds such as vitamins, carotenoids, and polyphenols. The occurrence of high levels of polyphenols, such as the xanthonoid mangiferin, flavonoids, phenolic acids, and different derivatives, provide a good basis for *M. indica* fruits as a healthy food [1].

Recently, it could be demonstrated that Careless™ activates the evolutionary conserved metabolic sensors sirtuin 1 (Sirt1) and AMP-activated protein kinase (AMPK) and, furthermore, stimulates mitochondrial biogenesis and has antioxidative effects based on an increase in superoxide dismutase activity [2]. Sirt1 and AMPK are key players in regulating energy metabolism [3,4]. They are activated in response to a variety of stimuli, including cellular stress, exercise, calorie restriction, and a wide range of hormones and agents that exert impacts on cellular metabolism [3,4]. Once activated, Sirt1 or AMPK stimulate a cascade of

metabolic processes, e.g., increased fatty acid oxidation, inhibition of cholesterol, fatty acid as well as triglyceride synthesis, enhanced muscle glucose uptake, and modulation of insulin secretion [3,5].

AMPK in endothelial cells has been implicated in the regulation of fatty acid oxidation, small G protein activity, inflammation, and angiogenesis [6]. Endothelium-dependent vascular relaxation via the activation of endothelial nitric oxide synthase (eNOS) and nitric oxide production has been shown to be stimulated by AMPK [6]. Moreover, there is evidence indicating that the activation of the AMPK pathway may help to prevent the vascular complications associated with insulin sensitivity [7].

The prevalence of metabolic syndrome, type 2 diabetes, and being overweight has reached epidemic proportions in Western society [8]. Being overweight is associated with impaired microcirculation through alterations in endocrine and vasocrine signals that cause alterations in microvascular endothelial and skeletal muscle signal-

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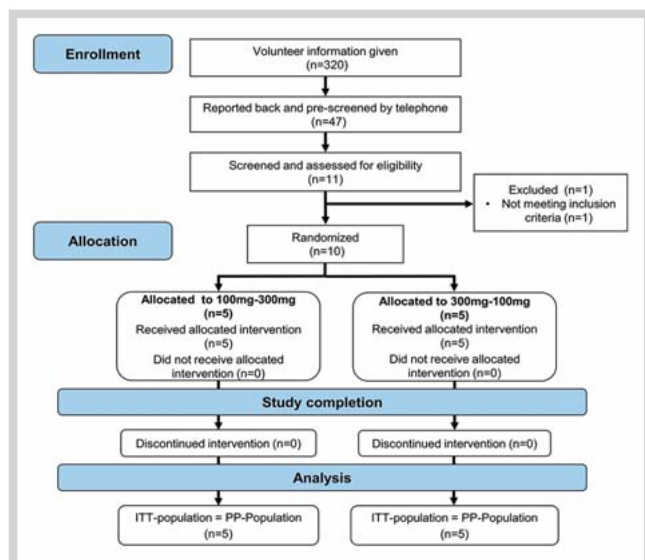


Fig. 1 Disposition of volunteers. (Color figure available online only.)

ing. Impaired microcirculation, in turn, compromises the timely access of glucose and insulin to their target tissues, and is therefore a contributor to insulin resistance. Emerging research demonstrates that impaired microcirculation may be a key feature in the development of overweight-related insulin resistance [9]. Interventions improving microcirculation can be beneficial to maintaining a healthy metabolism and energy balance and in reducing the risk of becoming overweight as well as developing insulin resistance and, consequently, type 2 diabetes and CVD [7,9]. Therefore, the aim of the current pilot study was a first plausibility and dosage finding test to investigate the acute effects of Careless™ on microcirculation measured by pulse amplitude tonometry (EndoPAT™) and the Oxygen-to-see (O2 C) system in a monocenter, randomized, double-blind, crossover pilot study in healthy women. Additionally, eNOS activation was investigated *in vitro* using primary human umbilical vein endothelial cells (HUVEC). In this model, the effects of Careless™ after an *in vitro* simulation of digestion in the human stomach and small intestine was compared to undigested Careless™.

## Results

In the presented human pilot study, 11 healthy women were screened for eligibility, 10 were included, and all 10 volunteers completed the study according to the protocol (○ Fig. 1).

Volunteers included in the current study were, on average,  $55.0 \pm 9.6$  years old, with a BMI of  $25.0 \pm 2.8$  kg/m<sup>2</sup>. They were characterized by a normal balanced nutrition. Vital signs and blood routine parameters measured during a screening visit were within the normal range (○ Table 1).

After a pre-measurement of cutaneous flow and endothelial function, a single dose of either 100 mg or 300 mg Careless™ was administered. An increase of cutaneous blood was observed for both dosages reaching maximal values after 6 h (○ Fig. 2) when compared to the pre-measurement before the intake of Careless™. Concomitantly, oxygen saturation of hemoglobin increased from  $51.6 \pm 12.5\%$  before the intake of Careless™ to  $62.9 \pm 13.2\%$  at 6 h for the 100 mg Careless™ group and from

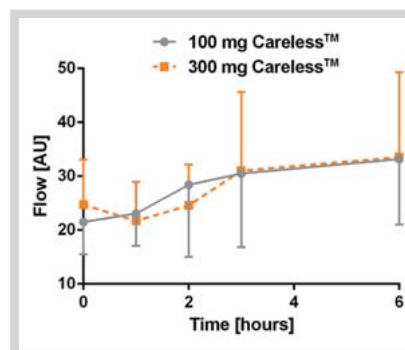


Fig. 2 Effects of 100 mg and 300 mg Careless™ on cutaneous blood flow [AU] over time measured by O2 C-System (100 mg:  $p = 0.0157$ ; 300 mg:  $p = 0.209$ ). Mean  $\pm$  95% CI is expressed. Effect size 0 h to 6 h: 100 mg,  $d = 0.79$ ; 300 mg,  $d = 0.46$ . (Color figure available online only.)

Table 1 Baseline characteristics. Values are means  $\pm$  SD.

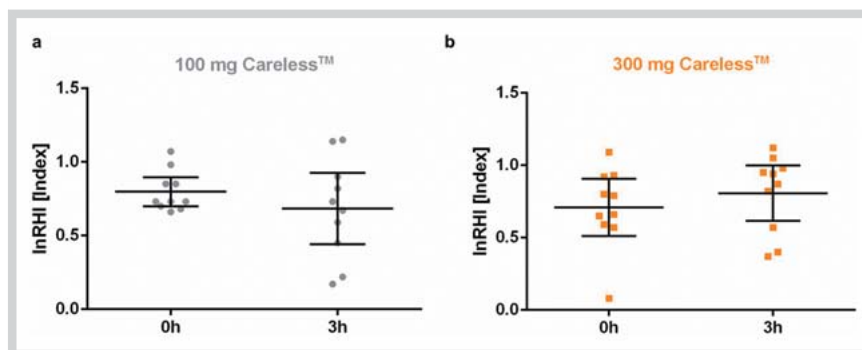
Characteristics	Value
Age (years)	55.0 $\pm$ 9.6
Body mass index (BMI) (kg/m <sup>2</sup> )	25.0 $\pm$ 2.8
Systolic blood pressure (mmHg)	125.3 $\pm$ 29.0
Diastolic blood pressure (mmHg)	79.6 $\pm$ 11.8
Heart rate (bpm)	68.4 $\pm$ 8.9
Fasting glucose (mg/dL)	87.3 $\pm$ 11.4

$59.2 \pm 11.5\%$  before the intake of Careless™ to  $63.6 \pm 14.0\%$  for the 300 mg Careless™ group. The increase in flow in the 100 mg dosage group reached significance ( $p = 0.0157$ ) over time after 6 h compared to pre-values measured before the intake of the study product. The characteristics of curve progression were very similar for both dosages. However, due to slightly higher baseline values in the 300 mg group as a result of individual variation, there was a slight descent between baseline and 1 h in this group and the relative increase was less pronounced than in the 100 mg group. The increase of cutaneous blood flow was 54% over baseline for 100 mg and 35% for 300 mg Careless™. The effects were not significantly different between the two dosages.

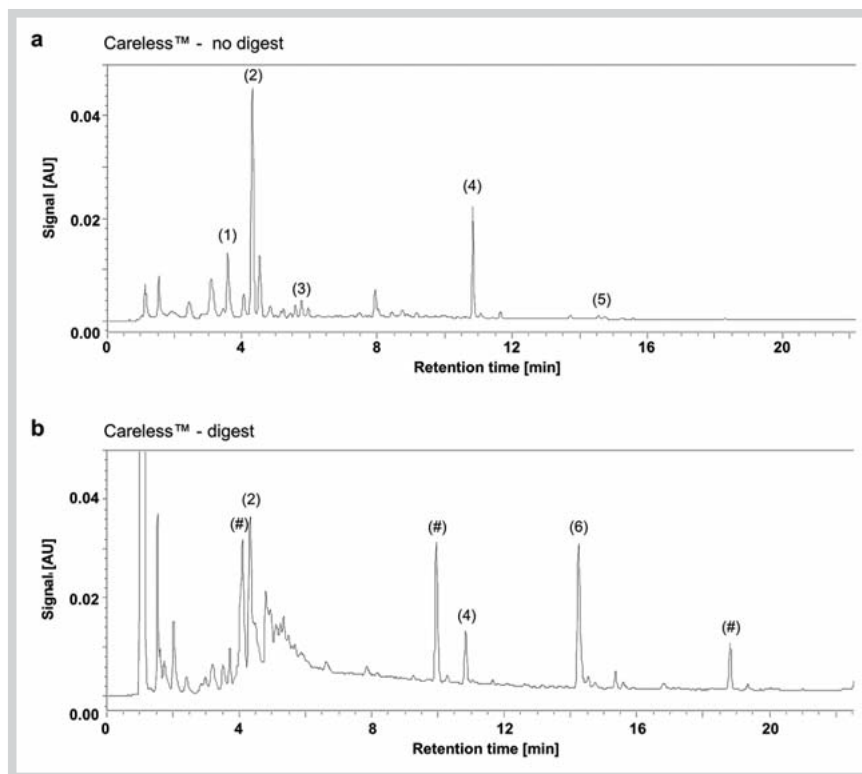
The endothelial function, measured as lnRHI (reactive hyperemia index) with the EndoPat™ method, showed a slight decrease compared to baseline 3 h after the intake of the 100 mg dosage (○ Fig. 3 a). In contrast, the lnRHI in the 300 mg dosage group slightly increased (○ Fig. 3 b). However, there were no significant differences, neither within dosages nor between dosages. Similar results were obtained measuring the RHI (data not shown).

The results of the safety analysis did not hint at any safety issues of Careless™ at dosages of 100 mg and 300 mg. After a single dose intake of the study product, no adverse supplement reactions occurred. During the study, only some volunteers reported a slight headache, probably caused by caffeine withdrawal or weather influences but not by the study product. All volunteers rated the tolerability of Careless™ with the best category ("well tolerated") for both dosages.

To elucidate the mechanism behind the effect of Careless™ on microcirculation and endothelial function, the effect of Careless™ on eNOS was studied *in vitro* in HUVEC. Based on literature data, it was hypothesized that potential active molecules such as flavonoids in Careless™ need to be converted to the active form by digestion [10]. Therefore, the effects of Careless™ on eNOS activity was investigated after an *in vitro* simulation of digestion in the human stomach and small intestine compared to the effects of the non-digested Careless™ preparation. A comparison of the high-pressure liquid chromatography (HPLC) fingerprint before



**Fig. 3** Effects of **a** 100 mg and **b** 300 mg Careless™ on endothelial function measured as lnRHI [index] at baseline and after 3 h. Mean ± 95% CI is expressed. Effect size and significance 0 h to 3 h: 100 mg,  $d = 0.41$ ,  $p = 0.625$ ; 300 mg,  $d = 0.36$ ,  $p = 0.695$ . Effect size and significance 100 mg vs. 300 mg at 3 h:  $d = 0.93$ ,  $p = 0.1815$ . (Color figure available online only.)



**Fig. 4** HPLC chromatogram at 260 nm of Careless™ **a** before and **b** after *in vitro* simulation of the digestion process. (1) Gallic acid, (2) not identified, (3) protocatechuic acid, (4) mangiferin, (5) flavonol structure, and (6) rutin; (#) peak due to digestion solution.

and after the simulation of digestion of Careless™ is shown in **Fig. 4**.

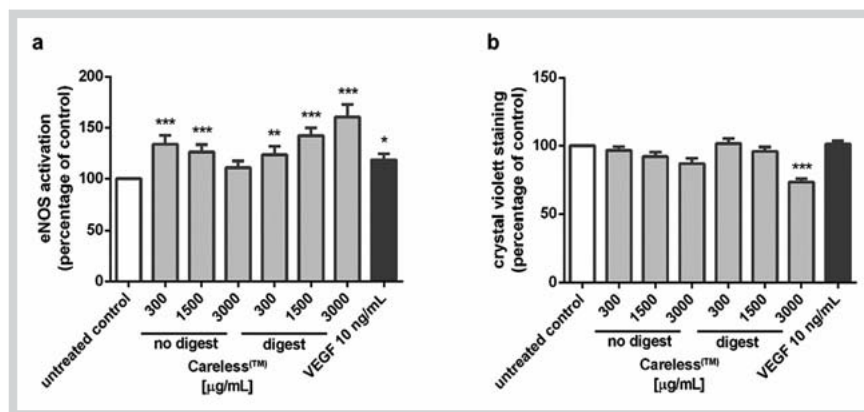
Digested Careless™ showed a dose-related significant activation of eNOS after 5 min of treatment dependently by 23% (300 µg/mL), 42% (1500 µg/mL), and 60% (3000 µg/mL) compared to the untreated control. The rate of activation was even higher than that of the positive control vascular endothelial growth factor (VEGF). Undigested Careless™ showed an inverse dose dependency with significant activation in the two lower concentrations (1500 and 300 µg/mL;  $p < 0.05$ ) (**Fig. 5a**).

To ensure that no cytotoxic effects influenced the outcome of the experiment, a crystal violet staining was performed. Only the highest concentration (3000 µg/mL) of the digested Careless™ showed a significant reduction in cell number compared to untreated cells (**Fig. 5b**). However, the results of the eNOS activity assay were corrected for differences in cell number measured by crystal violet staining.

## Discussion

Measurement of microcirculation and endothelial function provides information on physiology and tissue nutrition [11], metabolism [9], and vascular reactivity [12]. Changes in microcirculation have been found to occur years prior to the manifestation of symptoms [11]. Microcirculation is an indicator for age and the risk to develop circulation- and metabolism-related disorders, e.g., lack of energy and muscle power, diabetes type II, or cardiovascular disease [13, 14]. Emerging research revealed that impaired microcirculation contributes to insulin resistance and thereby may be an important feature in the development of metabolic syndrome and type 2 diabetes [9]. Therapeutic interventions that improve endothelial function often simultaneously ameliorate insulin resistance and vice versa [7].

In our pilot dosage finding study, we demonstrated moderate effects of Careless™ on microcirculation. Following consumption of 100 mg Careless™, a significant increase of cutaneous blood flow, measured by the O<sub>2</sub>C system, was detected compared to the pre-measurement before intake of the study product. Ghazanfari et



**Fig. 5** **a** eNOS activation and **b** number of living cells measured by crystal violet staining after removal of dead cells after treating HUVEC cells for 5 min with digested or undigested Careless<sup>TM</sup> in the concentrations 3000 µg/mL, 1500 µg/mL, and 300 µg/mL. Untreated control is set to 100%. \**P* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

al. reported that an increase of flow above 10% could be interpreted as an interventional threshold. A change of flow below this threshold may be based on physiologic variability (temperature, parasympathical influence, etc.) rather than on the intervention itself [15]. The effects documented in the presented study are clearly above this interventional threshold (54% over pre-measurement in the 100 mg group and 35% over pre-measurement in the 300 mg group) and therefore can be interpreted as biologically relevant [15].

The increase in cutaneous blood flow is consistent with previous work with other polyphenol-rich plant sources, which also demonstrated the vasodilator properties of polyphenols in humans [16,17]. Upon ingestion of a green tea extract, a transient early effect on blood flow was observed, but no dose dependency occurred after a single dose of 0.5 g, 1 g, or 2 g [16], which is similar to our findings. In a study with cocoa-flavanols, a dose-dependent influence of blood flow was observed after treatment of a high and low dosage of cocoa-flavanols [17].

The specific active compounds in Careless<sup>TM</sup> responsible for the beneficial effects on microcirculation have not been fully elucidated yet. However, several compounds in Careless<sup>TM</sup> are described for their vasoactive function. For example, mangiferin, which is found in Careless<sup>TM</sup>, belongs to the substance class of xanthones. Vasorelaxant activities of xanthones have been summarized by Jiang et al. [18] and the mangiferin aglycon norathyriol has been found to relax rat thoracic aortas in an *ex vivo* model [19]. Furthermore, rutin another substance found in Careless<sup>TM</sup>, induced eNOS activity [20] and led to relaxation of rat aorta rings [21].

In previous studies on microcirculation with natural products, flavanols from cocoa or green tea showed the highest increase of blood flow after 2 h (cocoa flavanols) or approximately 15–30 min (green tea) [16,17]. In contrast, the present study indicates that the maximum flow occurred at the last time point (6 hours after the intake of Careless<sup>TM</sup>), indicating a different pharmacokinetic of flavanols and the compound relevant for the effect of Careless<sup>TM</sup>.

Mangiferin is described as needing several digestive steps to be transformed into its active metabolite [22]. Liu et al. reported that mangiferin undergoes extensive metabolism, and up to 48 different metabolites (norathyriol being one of them) were detected in urine, plasma, and feces after oral administration of mangiferin in rats [10].

However, a relative change of flow after 6 h in the current study with a 54% increase for 100 mg and a 35% increase for 300 mg Careless<sup>TM</sup> was comparable to the 70% increase of blood flow by

the acute intake of cocoa with a high (329 mg) content of flavanols after 2 h [17].

Besides microcirculation, endothelial reactivity measured by EndoPat<sup>TM</sup> was also assessed in the present study. Due to the study protocol, the volunteers had a delayed intake of breakfast resulting in a prolonged fasting, since a first light meal was served after the 3 h EndoPat<sup>TM</sup> measurement and after overnight fasting. Such prolonged fasting states are known to lead to a progressive decrease of endothelial function [23]. We can only speculate that a control group receiving only water would have had a reduced endothelial function and that the slight improvement of endothelial function observed in the group receiving 300 mg Careless<sup>TM</sup> was buffering this effect. The difference between the 100 mg Careless<sup>TM</sup> group and the 300 mg Careless<sup>TM</sup> group did not reach significance, probably due to a high variability and low sample size, but the calculated *cohens d* indicated a strong effect. Also, a continuous increase in flow over time with the highest levels at 6 h was observed, suggesting that the effects on endothelial function would have been higher at later time points. Taken together, further studies, including a placebo control group and measurements at later time points, are necessary to elucidate effects of Careless<sup>TM</sup> on endothelial function.

One possible mechanism by which Careless<sup>TM</sup> influences microcirculation is via Sirt1 and AMPK activation [2], which are involved in endothelial homeostasis [6]. Another possible mechanism known to be involved in microcirculation is the activation of eNOS, which has been described for many plant-derived products such as grapes, black and green tea, and cocoa amongst others [24]. Therefore, the effect of Careless<sup>TM</sup> on eNOS *in vitro* was investigated using primary human endothelial cells. Data from the literature show that at least one of the potentially active molecules, mangiferin, in Careless<sup>TM</sup> needs to be converted to the active metabolite [10]. Hence, the effects of Careless<sup>TM</sup> were investigated after an *in vitro* simulation of digestive processes in the human stomach and small intestine. A comparison of the HPLC chromatogram before and after the simulation of the digestion revealed that potential substances relevant for microcirculation, such as rutin, are increased by the procedure (● Fig. 4). Digested Careless<sup>TM</sup> showed a dose-related significant activation of eNOS after 5 min of treatment. Undigested Careless<sup>TM</sup> showed an inverse dose dependency with significant activation with the two lower concentrations. It is therefore concluded that one reason for the observed beneficial effects of Careless<sup>TM</sup> on microcirculation *in vivo* might be its activating effect on eNOS.

However, our model is limited to the digestion processes occurring in the stomach and the small intestine. For mangiferin, it



has been shown that the degradation occurring by intestinal flora leads to the formation of different metabolites [22]. Therefore, there might be relevant degradation of Careless™ by gut bacteria. Taken together, the present pilot findings indicate moderate beneficial effects of Careless™ on microcirculation in healthy women and could successfully determine an active dosage of Careless™. This pilot study demonstrated that Careless™ is a safe and well-tolerated ingredient for herbal supplementation, which might be of interest for subjects with alterations of microcirculation and endothelial function.

## Materials and Methods

### Plant material

The investigated Careless™ (batch number VSMI2013003) is a 100% pure fruit powder obtained from the *M. indica* variety Kili-Mooku cultivated in the Tamil Nadu region of India. The fruits for Careless™ are harvested when they have reached their full size (> 12 cm), but are still green and hard without yellow spots, which corresponds to an unripe stage. The harvest period is from April to May. The fruits are then sliced, dried, and subsequently ground to a fine powder. The final water content is below 5%. Careless™ is available from Vital Solutions GmbH. A voucher specimen (CoA\_BID\_13\_02) is deposited at the Herbarium of Vital Solutions in Germany.

### Human study

The acute effects of a special Careless™ on microcirculation and endothelial function were investigated in a monocenter, randomized, double-blind, crossover study in healthy women. The trial was registered in the Clinical Trials Registry (clinical trial.gov NCT02511899). The study was a nutritional study and was conducted in accordance with ICH-GCP guidelines and in compliance with the declaration of Helsinki. The study was reviewed and approved by the Institutional Review Board (IRB) "Landesärztekammer Baden-Württemberg" and all subjects signed the IRB-approved informed consent prior to any procedures being conducted (F-2015-017).

### Study population

Apparently healthy women aged 40–70 years with a body mass index of 19–30 kg/m<sup>2</sup> were recruited. Before study entrance, the volunteers were medically checked for their physical condition. Blood samples were collected at screening, after overnight fasting for at least 10 h, and differentiated hemogram, clinical laboratory, blood lipid status, creatinine, uric acid, and fasting glucose were determined. Smokers and volunteers with an intake of more than 275 mg caffeine per day, a diet high in vegetables and fruits (≥ 5 portions per day), or regular medication supplements that would potentially interfere with the study (e.g., polyphenols, L-Arginine, Niacin, medication of hemodilution, blood flow stimulating products like Aspirin®) were excluded.

### Study procedure

After having provided written informed consent, and after completion of the screening procedures, volunteers were invited for study visits. At visit 1, the subjects were randomly assigned to one of the two sequence groups (first 100 mg, afterwards 300 mg or first 300 mg, afterwards 100 mg of Careless™ as a single dose). To avoid differences in color and taste between the two dosages, Careless™ was provided as a standardized drink formu-

lation, e.g., mango aroma and colorants, additives that had no effect on the investigated activities. Prior to consumption, the study preparation was dissolved in 250 mL of still water. Study products were ingested after overnight fasting and under supervision of the study staff. Microcirculation was determined using a micro-lightguide spectrophotometer (O2 C). After pre-measurement, the volunteers ingested the study products, and at 1 h, 2 h, 3 h, and 6 h post-intake, repeated measurements of microcirculation were performed. Furthermore, endothelial function was determined using EndoPAT™ pre- and 3 h post-intake of Careless™. A standardized breakfast was served after the 3 h post-measurements. Between visits 1 and 2, there was a wash-out phase of 2 weeks. During this time period, no study products were taken.

### Oxygen-to-see measurement

Blood flow in the skin and oxygen saturation of hemoglobin was determined with the O2 C system (LEA Medizintechnik). This technique combines the laser doppler technique with tissue spectroscopy and thus allows to record simultaneously the post-capillary oxygen saturation of hemoglobin (SO<sub>2</sub>), the regional amount of hemoglobin (rHb), and the local blood flow (Flow) in the microcirculation. For measurement in the 1–2 mm depth, the glass fiber probe LFX29 was used. The measurement was performed in the lying position at the inner forearm of the dominant arm. Microcirculation was measured under standardized conditions with at least 30 min of acclimatization in the morning prior to the pre-measurements with at least 8 min of calm relaxing rest in the lying position prior to recording of the parameters of microcirculation over a period of 40 s. After 20 s of monitoring baseline measurements, a mark was set and an additional 20 s were recorded. The standard procedure with average values of 10 s time intervals starting from the mark was used for analysis of the data. For data analysis, the software O2CevaTime version 23.2.0 was used.

### EndoPAT™ measurement

EndoPAT™ was determined using the Endo-PAT2000 device (Itamar). After assessing the baseline signal, blood flow was occluded for 5 min, and after deflating, the endothelial-dependent dilatation of the blood vessels was measured. Data prior to and after ischemia were analyzed automatically by integrated EndoPAT™ Software. The reactive hyperemia index (RHI and lnRHI) was calculated by the increase of the peripheral artery tone signal (PAT) in the fingertip after 5 min of occlusion of the arm in comparison to a 5-min baseline signal. The non-occluded arm was used as a control arm. EndoPAT™ measurements were performed in the lying position after at least 10 min of resting time and at the same time during visits 1 and 2 to avoid circadian influences. lnRHI is said to be the more robust measure. Therefore, this parameter is presented. Values below 0.51 indicate endothelial dysfunction.

### Safety parameters

For monitoring of safety parameters, the subjects were asked about tolerability and adverse events at the end of each visit. Additionally, adverse events in the period between the two study days and concomitant medication were recorded in volunteer diaries. Adverse events were documented in accordance with ICH/GCP guidelines.

### *In vitro* study

The effect of Careless™ on eNOS activity was investigated in primary endothelial cells (HUVEC) after an *in vitro* simulation of digestion in the human stomach and small intestine and compared to the effect of an undigested Careless™ preparation.

The *in vitro* simulation of the digestive tract has been described elsewhere and was adapted for this study [25,26]. Briefly, to simulate first the human stomach condition, 360 mg of Careless™ were added to 105 mL of digestion solution [adjusted to pH 2 with 0.5 mol/L HCl solution, including mucin (2.9 mg/mL) and pepsin (0.08 mg/mL)] and incubated on a shaker at 37 °C for 2 h. Subsequently, the solution was filled up to a volume of 120 mL with 2.8% NaCl solution and the pH was adjusted to 7.5 with NaCO<sub>3</sub>. Pancreatin (2.9 mg/mL), trypsin (0.08 mg/mL), and lyophilized bile (2.9 mg/mL) were added to simulate intestinal digestion for 4 h [26]. After the incubation, enzymes were removed by filtration through an ultrafiltration device with a molecular cutoff of 10000 (Vivaspin 20, Sartorius AS) prior to the cell culture investigations. To obtain undigested control samples, 30 mg of Careless™ were suspended in 10 mL of Emsure water (Merck Millipore) and filtered through the same ultrafiltration device as the digested control to remove the insolvent fibers. To adjust the different test concentrations, the digested and undigested solutions were diluted in endothelial cell growth medium (Promocell). All chemicals for digestion were purchased from Sigma-Aldrich. HUVEC cells were obtained from Promocell and were maintained in 75 cm<sup>2</sup> flasks at 37 °C in an incubator with a 5% CO<sub>2</sub>/95% air atmosphere at constant humidity.

The presence of phosphorylated and unphosphorylated eNOS was assessed with an ELISA assay [CytoGlow eNOS (Phospho-Ser1176) #CBP1542 Assay Biotechnology Company, Inc. Sunnyvale] according to the user manual. Briefly, cells at passage 3 were grown in 96-well plates to confluence and then stimulated with either 300 µg/mL, 1500 µg/mL, or 3000 µg/mL Careless™ or VEGF (10 ng/mL, Promocell) as a positive control [27] for 5 min. Subsequently, cells were fixated and stained either with anti-eNOS antibody for the phosphorylated or the unphosphorylated form. Cytotoxic effects were expected to be low due to the short time of incubation. However, to control for cytotoxic effects, crystal violet staining was included in the experiment after removing dead cells by washing steps during the assay performance and results were corrected for crystal violet staining. eNOS activation was calculated as the ratio of phosphorylated eNOS corrected for crystal violet staining to total eNOS corrected for crystal violet staining.

### High-pressure liquid chromatography analyses

Careless™ samples before and after *in vitro* simulation of the digestive process were characterized by a chromatographic fingerprint obtained using the following HPLC method: before HPLC analyses, 40% (v/v) methanol was added to all samples followed by filtration through a 0.22-µm centrifugal filter device (Merck-Millipore).

HPLC was performed using a Waters Alliance apparatus equipped with a Waters 2996 Photodiode Array Detector and a 100 × 4.6 mm, 2.6 µm C18 100 A column (Phenomenex Kinetex™). Separation was achieved with an increasing amount of 0.1% trifluoroacetic acid in acetonitril (B) and 0.1% trifluoroacetic acid in water (A): 0–20 min, 1–30% B, linear gradient; 20–25 min, 30–60% B, linear gradient; 25–30 min, 60% B, isocratic; 30–30.5 min, 90–1% B, linear gradient; and 30.5–35 min, 1% B, isocratic at a flow rate of 1.0 mL/min. The temperature of the samples and the column oven

was 25 °C. The fingerprint is presented at the wavelength of 260 nm.

### Statistical analyses

Statistical evaluation for the human pilot study was defined prior to the start of the study and described in the protocol. No subgroup analysis or interim analysis was performed. The distribution of parameters was tested for normality for each single parameter, which was the basis for selecting a parametric or non-parametric approach. For comparison of the two dosage groups, the pre-post differences were compared with each other using the crossover analysis by strict separation of the treatment effects from the period effects and investigation of the sequence groups using Student's *t*-test for independent samples. This was performed for each time point. Multiplicity in hypothesis testing was not controlled during this hypothesis-generating approach. The existence of carryover effects was ruled out for this method. Repeated measures ANOVA with Dunnett's multiple comparison test was used to test the increase over time in comparison to baseline values within groups for parameters of microcirculation. The Wilcoxon matched-pairs signed-rank test was used to test the effects within groups for EndoPAT™. All statistical tests were performed two-sided. The significance level was set to 5%. For the *in vitro* analysis, one-way ANOVA with a post-test (Dunnett's multiple comparison test) was used. Results with *p* values < 0.05 were considered significant.

### Acknowledgements

▼ The authors would like to thank all of the subjects who took part in this trial.

### Conflict of Interest

▼ This study was sponsored by Vital Solutions Swiss AG. The sponsors contributed to discussions about study design and selection of outcome measures prior to the start of the study. During study realization and data analysis, all data were blinded. Study realization, data analysis, and reporting were undertaken independently from the sponsors. Vital Solutions own the proprietary ingredient used in the study.

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