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Infusion of herbal plant extracts for insomnia and anxiety causes a dose-dependent increase of NO and has a protective effect on the renal cellular stress caused by hypoxia and reoxygenation

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Abstract

Background: Herbal plant extracts are a more common alternative to conventional medicine to treat sleep disorders and intermittent hypoxia. Notably, obstructive sleep apnea causes injuries similar to those observed in models of ischemia-reperfusion including the decrease of nitric oxide (NO) availability. Kidney transplantation in end-stage renal disease reverses the sleep apnea. The underlying mechanism linking hypoxia, sleep apnea, and renal protection remains to be defined at the cellular level.

Objective: The aim of this study was to demonstrate the safety and efficacy of herbal plant infusions with a potential for donating NO, to attenuation of damage induced during a hypoxia/reperfusion sequence, on kidney epithelial cells LLC-PK1.

Materials and Methods: Cell death (Lactate Dehydrogenase release assay) and a viability test (MTS assay) after 24 h of incubation with different concentrations of plant infusion were assessed using the LLC-PK1 cell line. Then, measurement of the breakdown product of NO (the $NaNO_2$) and LDH assay were carried out after 24 h of hypoxia, followed by 4 h or 24 h of reperfusion.

Results: The effect of different dilutions of herbal plant infusion on the LLC-PK1 cell viability, after 24 h of incubation, was maximal at a 30% dilution compared to control. After 24 h of hypoxia, there was an increase of $NaNO_2$ and thus of NO, and a concentration-dependent decrease of cell death. Similar results were observed after hypoxia followed by 4 h of reperfusion. These effects were always maximal at 50% dilution of plants infusion.

Conclusion: Safe infusion of plant extracts causes a dose-dependent increase of NO and has a protective effect against the cellular stress caused by hypoxia and reoxygenation. Since it has been demonstrated that there is a NO-dependent mechanism allowing the reduction of injuries induced by ischemia/reoxygenation process, such a mechanism could be responsible for our observations.

Keywords: Herbal plant extracts, Infusion, Nitric oxide, Sleep disorders, Hypoxia/reperfusion, LLC-PK1 cells

Introduction

Herbal plant extracts, alone or in combination, when infused, [1] are common alternatives to conventional medicine to treat sleep disorders and sleep intermittent hypoxia [2-5]. One of the most widespread sleep disorder is apnea, where there is transient cessation of breathing and repetitive hypoxia/reoxygenation; this causes injuries, inflammation, generation of reactive oxygen species (ROS), and the decrease of nitric oxide (NO) availability [6, 7]. Sleep disorders can be considered as the oxidative stress similar to ischemia/reperfusion of many organs [8]. Very recently, sleep disorders have been linked to end-stage renal failure in as many as 80% of dialysis patients [9]. Restoring the renal function in these patients after kidney transplantation reversed the sleep disorders, suggesting underlying mechanisms linking these two conditions of sleep apnea and renal oxidative stress [10].

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Furthermore, renal function should be considered important in the circadian regulation of sleep [11]. NO is a gaseous molecule, produced from the amino acid L-arginine, using proteins belonging to the family of NO synthases (NOS) involved in sleep regulation [12]. In fact, in the mesencephalon, NO is involved in many functions, including these of the awake-sleep cycle. It has been demonstrated in cats that the microinjection of a NO donor, S-nitroso-acetyl-penicillamine, in a sleep control cerebral region, the pedunculopontine tegmentum increased at the same time as slow waves sleep and rapid-eye-movement sleep [13]. Similarly, in rats, the NO precursor, L-arginine, administered during the luminous phase, in this same region, increased the slow wave sleep [14]. Moreover, a specific inhibitor of the neuronal NOS (nNOS) showed that NO controls sleep in rats [15]. All these benefits on sleep from NO addition resulted from local cerebral changes. Endothelial NO is also altered during obstructive sleep apnea at multiple organ levels [16], but activation of NO by plant extract infusion on renal cells following oxidative stress is not known, to our knowledge. The renal production of NO following plant extract infusion could be used to protect kidney cells and consequently to improve sleep physiology. Such ischemia-reperfusion are well characterized for kidney and are successfully used for its preservation during transplantation. Amelioration of hypoxia is one key mechanism, and as kidney transplantation in the end-stage renal disease reverses the sleep apnea, the underlying mechanism linking these conditions remains to be better defined. Pig kidney epithelial cells (LLC-PK1) are well-characterized cells that have been used to study the molecular mechanism of renal injuries [17].

The aim of this study is to demonstrate the efficacy and safety of plant extract infusion using a potential NO donor, during an ischemia/reoxygenation sequence, on kidney epithelial cells LLC-PK1.

Materials and Methods

Plant extract

Standard Dry plant extract (%) from Hawthorn (*Crataegus oxyacantha*) 30%, Melissa (*Melissa officinalis*) 30%, Tila (*Tilia europaea*) 30%, and Cacao (*Theobroma cacao*) 10% were used (Laboratoire Velay, France).

Experimental procedures

Infusions were prepared by the incubation of 2 g of a plant extract with hot water (95°C) for 5 min. Dilutions of plant extract infusions have been used at a range of concentrations from 5%-50%. The 100% dilution was obtained by incubation of 4 g of plant material with hot water (95°C) for 5 min and then diluted by a 2-fold factor in culture media.

These dilutions of plant extract infusions were added to the medium and incubated for 24 h. Cells were washed twice with phosphate buffered saline (PBS) and then covered with Krebs-Henseleit buffer (115 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 1.2 mM $MgCl_2.H_2O$, 1.2 mM $NaH_2PO_4.H2O$, 1.2 mM Na_2SO_4, 20 mM Hepes, 2.5 mM $CaCl_2$, pH 7.4). Cells were stored at 4°C under hypoxic (95% N_2/5% CO_2) conditions. Cold incubation was followed after 4 h or 24 h of rewarming under normoxic (21% O_2/5% CO_2/74% N_2) conditions at 37°C without changing the medium as described previously [18].

Cell culture

The porcine kidney epithelial cell line (LLC-PK1) was obtained from American Type Culture Collection (Rockville, MD, USA), which corresponds to a proximal renal tubule. Cells were grown in T-75 flask (Nunc, Merck-Eurolab, France) using Medium 199 (M199) medium (Gibco, Invitrogen Life Technology, France) supplemented with 10% fetal bovine serum (FBS) (Gibco, France) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Sigma-Aldrich, France) at 37°C in a 5% CO_2/95% air humidified atmosphere. When cell cultures reached about 80% confluence, cells were trypsinized using 0.25% trypsin in EDTA (Gibco, France) and subcultured into 96 well plates [19]. Monolayer's confluent cells were serum-starved for 48 h before experiments.

Viability test

MTS is a colorimetric assay based on the ability of viable cells to convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, France) to formazan; the quantity of formazan product, absorbance, is directly proportional to the number of viable cells in culture. Cells were seeded in 96-well culture plates ($3x10^5$ cells/well) and incubated without or with different dilutions of plant infusion (from 5%-100% dilutions) for 24 h. Cells were then incubated with 20 µl MTS tetrazolium compound for 2 h at 37°C. Absorbance was measured using a multi-plate reader at 490 nm.

LDH release

The release of lactate dehydrogenase (LDH) induced by cell injury was determined by using the TOX-7 assay kit (Sigma-Aldrich, St. Louis, MO, USA). 10^5 cells/ml were cultured in 24-well culture plates. At the end of the experiments, cells were centrifuged at 400xg for 4 min at 20°C). The culture supernatants were collected and incubated with the substrate mixture for 30 min in the dark at room temperature. Absorbance was measured at 490 nm and 690 nm (background absorbance) using a multi-plate reader. LDH release value is the difference between the two absorbance measures.

$NaN0_2$

Production of NO was assessed as the accumulation of nitrite (NO_2^-) in the medium using a colorimetric reaction with the Griess reagent [17]. Briefly, after the treatment period, cell culture supernatants were mixed with an equal (1:1) volume of

Griess reagent (0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4). The absorbance was measured at 540 nm using a 96-well multi-plate reader.

Statistical analysis

Results were expressed as mean ± SEM of five to six experiments. Statistical analysis was done using analysis of variance (ANOVA) followed with the Tukey's post-test (GraphPad Prism®, GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Cytotoxicity of infusion

To study the effects of infusion in tissue culture cells, we used a well-established protocol that is known to cause rapid cell growth and reproducible cell toxicity. In order to determine the cytotoxicity of the plants' infusion, cell viability and LDH release under physiological conditions were evaluated. The effect of the infusion, at different dilutions, on the LLC-PK1 cell viability, after 24 h of incubation, was maximal at a 30% dilution of infusion in the medium, as it increases significantly ($p < 0.05$) the number of living cells by 26% compared to control. The increase observed after the treatment with 5% and 10% dilutions were not statistically significant (Fig. 1). A loss of viability was observed at high concentration, about half with 100%, versus control ($p < 0.05$). Thus, the effects of infusion on cell viability were clearly biphasic with an evident dose cytoprotection relationship followed by cell intoxication at the highest infusion concentrations used.

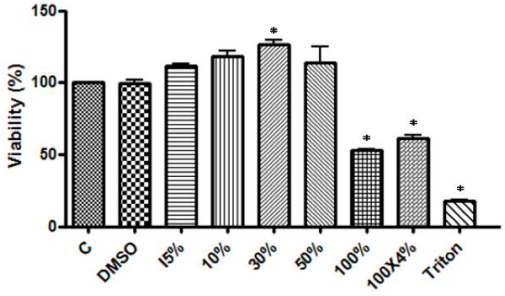

Fig. 1: Effect of the herbal plants extract infusion on cell growth. Cell viability was measured by MTS assay. LLC-PK1 cells were incubated for 24 h without or with plant infusions (5, 10, 30, 50 or 100%). DMSO and Triton were used as negative and positive controls, respectively. C: control. Data are represented as mean ± SEM of six determinations. *p < 0.05 from control.

The effect of plants' infusion on cell LDH release was not found to be significant for any dilution. However, it is interesting that the dose relationship and the maximum cell LDH release occurred at the same dilutions and were maximum at 50% and 100% dilutions (Fig. 2). Thus, the LDH release that is due to cell death in our conditions was less sensitive than the first demonstration found with cell viability.

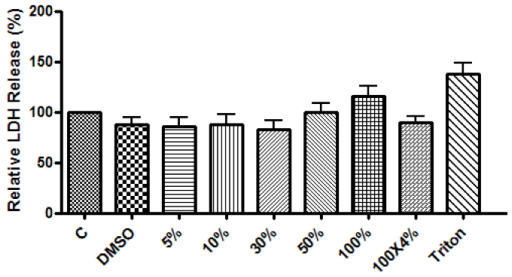

Fig. 2: Effect of the herbal plants extract infusion on cell death. Cell death was measured by assessing the LDH release. LLC-PK1 cells were incubated for 24 h without or with plants infusions (5, 10, 30, 50, or 100%). DMSO and Triton were used as negative and positive controls, respectively. C: control. Data are represented as mean ± SEM of six determinations. *p < 0.05 from control.

Effects of plants infusions on hypoxia and reoxygenation

To determine the potential protective effect of the plant extract infusion, the renal epithelial cells were subjected to hypoxic (24 h) and reoxygenation (4 h and 24 h) conditions. The standard LDH release assay was assessed after periods similar to those in previous reports [17]. Under these conditions that mimic the ischemia-reoxygenation, the LDH release is a well-established and sensitive assay to estimate cell death.

Fig. 3 shows the effect of 24 h of hypoxia on LDH release. A significant decrease of LDH release depending on the range of infusion concentrations used was observed ($p < 0.05$). Fig. 4 shows a similar relationship between infusion dilutions and an increase of $NaNO_2$ that was significant at the two highest concentrations. The two ranges of the dilution dependent effect of infusion appear to be overlapping.

Fig. 5 shows the effect of 24 h of hypoxia followed by 4 h of reperfusion. Fig. 5 shows a significant decrease of LDH release at the two highest concentrations of infusion ($p < 0.05$). Fig. 6 shows a significant increase of $NaNO_2$, only at the highest dose of infusion used ($p < 0.05$). These results appear similar but are smaller than those observed in Fig. 3 and 4. So, only the 50% dilution was an effective dose to prevent damages on cell-associated to LDH release during the cold hypoxia followed by 24 h of warm reoxygenation. Fig. 7 and 8 show the effects of 24 h of hypoxia followed by a 24 h period of warm reoxygenation. The results show trends in affecting cell LDH

release and $NaNO_2$ however, these results obtained at any infusion dilution did not differ from the control values except for the 50% dilution that decreased the LDH cell release.

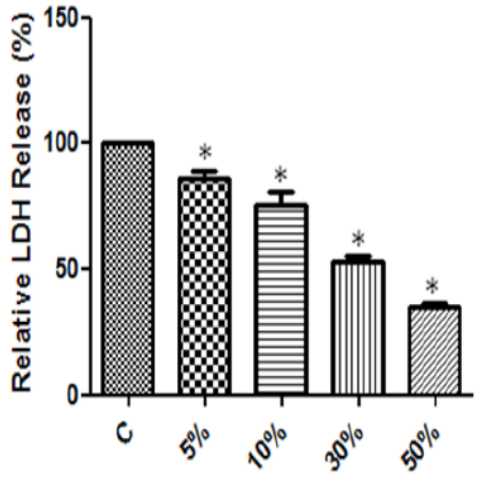

Fig. 3: Protective effect of the herbal plants extract infusion on LLC-PK1 cells injury after 24 h of hypoxia. LDH release of LLC-PK1 cells was assessed after incubation for 24 h in cold (4°C) Krebs-Henseleit buffer (KHB) under hypoxic conditions (95% N2/5% CO2) without or with plant infusions (5, 10, 30, or 50%). C: control. Data are represented as mean ± SEM of five determinations. *p < 0.05 from control.

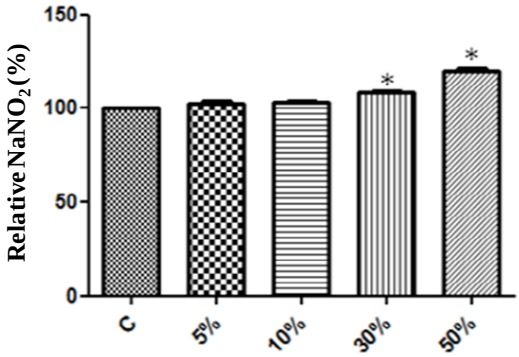

Fig. 4: Effects of herbal plants extract infusion on the production of NO by LLC-PK1 cells after 24 h of hypoxia. The relative $NaNO_2$ release of LLC-PK1 cells was assessed after incubation for 24 h in cold (4°C) Krebs-Henseleit buffer (KHB) under hypoxic conditions (95% N2/5% CO2) without or with plants infusions (5, 10, 30, or 50%). C: control. Data are represented as mean ± SEM of five determinations. *p < 0.05 from control.

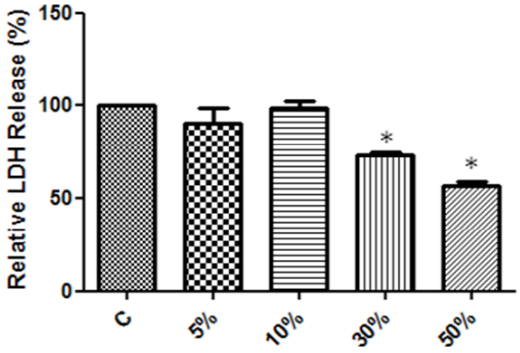

Fig. 5: Protective effect of the herbal plants extract infusion on LLC-PK1 cell injury induced by a sequence of 24 h of hypoxia and 4 h of reoxygenation. LDH release of LLC-PK1 cells was assessed after incubation for 24 h in cold (4°C) Krebs-Henseleit buffer (KHB) under hypoxic conditions (95% N2/5% CO2) followed by 4 h of reoxygenation at 37°C without or with plants infusions (5, 10, 30, or 50%). C: control. Data are represented as mean ± SEM of five determinations. *p < 0.05 from control.

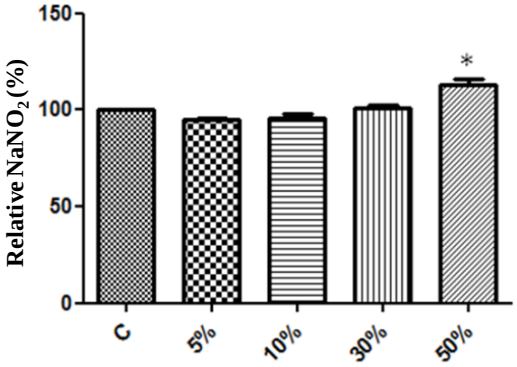

Fig. 6: Effects of herbal plants extract infusion on the production of NO by LLC-PK1 cells induced by a sequence of 24 h of hypoxia and 4 h of reoxygenation. The relative $NaNO_2$ release from LLC-PK1 cells was assessed after incubation for 24 h in cold (4°C) Krebs-Henseleit buffer (KHB) under hypoxic conditions (95% N2/5% CO2) followed by 4 h of reoxygenation at 37°C without or with plants infusions (5, 10, 30, or 50%). C: control. Data are represented as mean ± SEM of five determinations. *p < 0.05 from control.

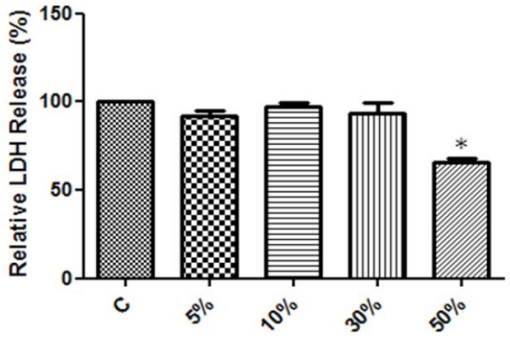

Fig. 7: Protective effect on LLC-PK1 cells injury induced by a sequence of 24 h of hypoxia and 24 h of reoxygenation. LDH release from LLC-PK1 cells was assessed after incubation for 24 h in cold (4°C) Krebs-Henseleit buffer (KHB) under hypoxic conditions (95% N2/5% CO2) followed by 24 h of reoxygenation at 37°C without or with plants infusions (5, 10, 30, or 50%). C: control. Data are represented as mean ± SEM of five determinations. *p < 0.05 from control.

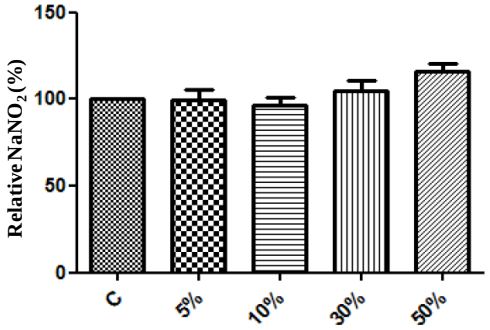

Fig. 8: Production of NO by LLC-PK1 cells induced by a sequence of 24 h of hypoxia and 24 h of reoxygenation. The relative $NaNO_2$ release of LLC-PK1 cells was assessed after incubation for 24 h in cold (4°C) Krebs-Henseleit buffer (KHB) under hypoxic conditions (95% N2/5% CO2) followed by 24 h of reoxygenation at 37°C without or with plants infusions (5, 10, 30, or 50%). C: control. Data are represented as mean ± SEM of five determinations. *p < 0.05 from control.

Discussion

In this study, we first documented the safety and secondly, the efficacy of plant extract infusion having NO donor potential to attenuate the damages induced during an ischemia/reoxygenation sequence on kidney epithelial cells, LLC-PK1, derived from the proximal tubule.

Cellular injuries, due to oxidative stress induced by ischemia/reoxygenation sequences in LLC-PK1 cells, were assessed by the LDH release evaluation. The plant extract infusions were added to the cell culture for 24 h as a pretreatment.

This study was aimed to demonstrate the safety of infusion over a wide range of concentrations and its efficacy on hypoxic and reoxygenation-induced stress. This last effect was extrapolated to NO increase in the cell culture, meaning that infusion increases NO, either via a NO donor or by inducing the cellular production of NO.

The initial experiments in this work were performed under physiological conditions, and they showed that the plant extract infusion is safe except at the concentration corresponding to a 100% dilution. This apparent toxic effect to the cells could be the real toxicity, but in our conditions could also be due to a weakened effect of the media altering the optimal cell condition culture. Thus, we avoided the use of this dilution in our subsequent experiment where cell cultures were affected during hypoxia and reoxygenation. Indeed, the study of cell death via the LDH release measurement showed no significant difference in a dilution ranging effect. Moreover, experiments studying cell viability at the 30% dilution suggests an improvement in cell culture rather than the dysfunction.

The optimal effect was obtained with the 50% dilution. It has been shown that hypoxic conditions, but also cold storage, are responsible for damaging kidney cells. The damage is mediated by ROS. This production was observed both during hypoxia and hypothermia [20-22]. Additional damage to tissues is detected after reoxygenation and rewarming since several studies have shown the implication of ROS. Thus, injuries of renal cells may occur on renal cells at both of the two phases of cold hypoxia and warm reoxygenation by involving different mechanisms [22, 23]. However, many studies have shown altered NO levels using the induction of endothelial NO synthase (eNOS), the addition of NO donors in the cold storage liquid or the use of NO to mimic ischemic preconditioning [24-26]. It was seen that some sleep disorders cause the same damage as ischemia/reoxygenation, thus the use of NO or NO donors may be useful to counter the adverse effects associated with these sleep disorders.

The NO production is dependent on dilution and on condition. In fact, at the same dilution, or in control condition, the NO production was lower without reperfusion and increased with the duration of reperfusion. This is in agreement with previous studies analyzing the endogenous production of NO. In fact, it has been shown experimentally that the renal tubule and glomerulus have the ability to produce NO in response to injury [27, 28] and NO production in proximal tubules could be substantially and rapidly enhanced under situations such as hypoxia/reoxygenation [29]. The overproduction of NO in

kidneys persists at 24 h after ischemia-reperfusion injury [30, 31].

Evidence for the hypoxic alterations associated with the LDH release occurs in the reoxygenation step. This is in contrast with a previous study [18] that showed a continuous increase of LDH release during the first four hours of rewarming. We can speculate that LDH release during rewarming is the consequence of two effects: a reoxygenation or a combination of rewarming and reoxygenation. It is possible that the temperature transition between the two phases takes more than 4 h to show the effects of warm reoxygenation on cell injuries.

Thus, the physiological relevance of the use of plant extract infusion is the prevention of sleep disorders. One of the most widespread sleep disorder is obstructive sleep apnea (OSA). Repetitive hypoxia/reoxygenation occurring, during transient cessation of breathing, in OSA is similar to ischemia/reoxygenation injury. Although perfusion remains intact during obstructive events in OSA, the alterations, inflammation, generation of ROS, and the decrease of NO availability are related to those observed in models of ischemia-reperfusion [6, 8].

The prevention by herbal plant extract infusion of deleterious hypoxia and reoxygenation demonstrated using cultured renal cells should be corroborated by clinical studies focusing on renal function in patients with sleep disorders.

Conclusion

In conclusion, the results of this study demonstrate the safety of the herbal plant extract infusion containing Hawthorn (*Crataegus oxyacantha*), Melissa (*Melissa officinalis*), and Tila (*Tilia europaea*) in similar ratios as those traditionally used for insomnia and anxiety treatments. This suggests a protective effect of the herbal plant extract when used as a preventive method to protect kidney epithelial cells (LLC-PK1) against hypoxia/reoxygenation injuries. This effect could be a result of the increase of NO induced by the infusion. NO donors provide significant protection in ischemia-reperfusion in several organs [32, 33]. In addition, inhibition of NO can induce or aggravate most of the alterations elicited by ischemic damage [34, 35]. However, the cellular response to NO depends on the reducing potential produced by the internal compounds and free thiols, and its statement oxygenation, especially in the presence of species derived from oxygen. Thus, NO can also lead to the development of nitrosative stress by acting on antioxidants and cell signaling pathways. NO is a molecule whose effects are complex and can be either positive or deleterious. Thus, its effect on the stress injuries induced by the oxidative stress should be further explored as we have previously done with other herbal remedies (Gingko Biloba, Desmodium adscendens) or tea infusions [17, 36-38].

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

The authors' responsibilities were as follows: CF, JMM - study design and writing the manuscript; CF and MF - conduct cell culture studies; CF, MF - review of the manuscript. All authors participated in the study and take responsibility for the content of this report. None of the authors had a personal or financial conflict of interest.

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