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Curcumin-rich extract from Central Brazil *Curcuma longa* protects human umbilical vein endothelial cells under oxidative stress

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Declaration of interests: nothing to declare

ABSTRACT

Introduction: In vascular diseases, the interruption of the local blood flow and the subsequent reperfusion of oxygen can cause deleterious oxidative effects on the cells. Turmeric (*Curcuma longa* L.) presents the capacity to neutralize free radicals along with preventive and therapeutic effects for several diseases. **Objective:** To analyze the bioactive compounds and the antioxidant capacity of the ethanolic extract of Curcuma (EEC), to evaluate its effect on human umbilical vein endothelial cells, and to analyze its effect on cellular signaling pathways. **Methods:** Cells were exposed to different concentrations of EEC for 24, 48, and 72 h. Folin-Ciocalteau test, HPLC-Fluorescence analysis, and DPPH method were used to determine the phenolic compounds, curcumin content, and antioxidant action, respectively; the tetrazolium salt reduction to obtain cell viability, cytotoxicity, and the concentration that inhibits 50% of cell viability; and the immunocytochemistry technique to analyze the expression of caspase3, SIRT1, and mTOR. **Results:** We found the presence of polyphenols in the classes of phenolic acids and curcuminoids in EEC, with 16.7% curcumin content. The number of antioxidants needed to reduce the initial DPPH concentration by 50% was 18.1 µmol/g. The extract mitigated cell damage at a dosage of 100 µg/ml, decreased the immunoexpression of caspase3, and promoted the signaling of the SIRT1 and mTOR survival pathways. **Conclusion:** EEC had a protective effect on human umbilical vein endothelial cells, subjected to oxidative stress, with decreased apoptosis (caspase3) at lower concentrations, cytoprotection by maintaining essential cell functions (mTOR), and signaling of the survival pathway (SIRT1).

Keywords: culture; Curcumin; inhibitory concentration 50; Phytochemicals; Antioxidants.

INTRODUCTION

The interruption of local blood flow, with the subsequent reperfusion of oxygen, can cause deleterious effects and even irreparable damage to the cells of affected tissues. That occurs due to oxidative stress (OS) generated in the cellular environment[1,2].

Turmeric (*Curcuma longa* L.), a spice appreciated in the regional cuisine of Midwest Brazil and Asian countries, has considerable active substances in its rhizomes. In the ethanolic extract of turmeric, phenols such as gallic acid and curcumin (CUR) have been found in significant concentrations, in addition to their high capacity to neutralize free radicals. Besides, the plant has preventive and therapeutic effects on several organic disorders[3-5].

Among the molecular mechanisms involved in the effects of CUR, there is evidence that it can act in the signaling pathways of SIRT1 (Sirtuins type 1), mTOR (mammalian target of rapamycin), and caspase3. Studies with animals submitted to CVA found that curcumin had a neuroprotective effect through the PI3K/Akt/mTOR signaling pathway[6-8].

In another study, endothelial cells under normoxia and OS, when treated with CUR, increased viability via the SIRT1 pathway. In cardiac, brain, and renal ischemic disorders, CUR induced a reduction in cell death, mainly through caspase pathways[8,9].

Therefore, this study aimed to characterize the bioactive compounds and the antioxidant capacity of the ethanolic extract of *Curcuma longa* L. (EEC). In addition, to evaluate the protective effect on human umbilical vein endothelial cells, both under normoxia and oxidative stress, and to analyze the possible activation of the cell signaling pathways of caspase3, SIRT1, and mTOR.

METHODS

The assays were carried out at the Multi-User Laboratory for the Evaluation of Molecules, Cells, and Tissues, at the Veterinary School of the Federal University of Goiás (UFG) and the Laboratory of Pharmaceutical Nanotechnology, at the Center for Research, Technological Development, and Innovation in Drugs, Medicines, and Cosmetics, at the Faculty of Pharmacy at UFG.

Obtaining the ethanolic extract of *Curcuma longa* L.

The rhizomes of *Curcuma longa* L. were acquired from the Mara Rosa Turmeric Products Cooperative (Cooperaçafrão) located in the municipality of Mara Rosa, state of Goiás, Brazil.

All stages of obtaining the crude extract were protected from photodegradation. Processing started with the removal of rhizomes from the soil, followed by washing and drying them in an oven to be processed. Subsequently, 100g of the dry and crushed material was added to 1000 ml of absolute ethanol, which was then cold percolated. The supernatant was filtered, and the solvent evaporated in a rotary evaporator (IKA® RV 05 Basic)[10].

Characterizing the ethanolic extract of curcumin – EEC (*Curcuma longa* L.)

Determination of total phenolics

The phenolic compounds were determined using the Folin-Ciocalteau reagent, following the methodology of Waterhouse[11], with a reading by spectrophotometry at 700 nm. We quantified the extracted sample with the establishment of the gallic acid standard curve. The results were expressed as mg gallic acid equivalent (GAE) per 100 grams of sample.

Analytical method for quantifying curcumin

The curcumin content in the extract was determined by HPLC-Fluorescence. The analysis was performed by the Agilent HPLC 1260 Infinity system (Agilent, USA), with an Eclipse SDB C18 column (250 X 4.6 mm, 5 µm) at 35 °C. The mobile phase consisted of an acetonitrile mixture: 0.4% acetic acid (45:55, v/v) mixture, at a flow rate of 1 mL/min. The injection volume was 10 µL and the detection of curcumin with excitation at 429 nm and emission at 539 nm. Curcumin standard solutions (Sigma Aldrich, Reference C1386) were prepared in different concentrations, using acetonitrile.

After standardization, approximately 50 mg of the extract, previously homogenized with 10 ml of acetonitrile, was vortexed for three minutes. An aliquot of the extract already solubilized was then diluted (final dilution factor: 1000) in acetonitrile and injected into the HPLC-Fluorescence. The analysis was performed in triplicate.

Antioxidant activity by DPPH

The antioxidant activity of EEC was determined by the DPPH method (2,2 diphenyl-1-picrylhydrazyl), according to the methodology proposed by Rufino et al.[12]. The degree of discoloration of the DPPH radical by the action of antioxidants was measured in triplicate through spectrophotometry, with a concentration of 0.1 $mg.mL^{-1}$, and the results were expressed in % of discoloration. The assay rested for 15 minutes and then read in the spectrophotometer at a wavelength of 520 nm.

Calculations were performed using Equation 1.

$$\% \text{ of discoloration of the DPPH} = (-1 \text{ Abs control} - \text{Abs white} / \text{Abs control}) \times 100 \quad (1)$$

Where:
Abs control = absorbance of the DPPH solution without the sample;
Abs white = Absorbance of the white with the DPPH.

The determination of antioxidant activity was expressed as Trolox equivalent, based on a Trolox calibration curve with concentrations ranging from 0.05 to 0.3 µmol/ml.

Cells and culture

The human umbilical vein endothelial cells - HUVEC (kindly provided by Prof. Dr. Veridiana de Melo Rodrigues Ávila from the Federal University of Uberlândia, state of Minas Gerais, Brazil) originated from the ATCC cell bank (American Type Culture Collection - Manassas, VA, USA). Cultivation was done in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin, and penicillin (10,000 IU/ml - 10 mg/ml), and 1% amphotericin B and L glutamine (Cultilab, Campinas, Brazil). They were later kept in an incubator at 37 °C with a humid atmosphere of 5% CO_2, according to adaptation by Manea[13].

For the assay, three independent experiments were carried out in quintuplicate.

Experimental design

The EEC obtained was diluted in DMEM 1%, for administration to cells in culture, in the different pre-established concentrations: 0 µg/ml, 20 µg/ml, 50 µg/ml, 100µg /ml, and 1000 µg/ml, in the exposure times of 24h, 48h, and 72h.

Evaluation of the effect of ethanolic extract of turmeric on endothelial cells under normoxia

The cultivation of HUVEC occurred in a humidified incubator at 37 °C and an atmosphere of 5% CO2, with a cell concentration of $1x10^4$ cells/well in 96-well culture plates. The treatment with EEC occurred after 24 hours of plating.

Induction assay of oxidative stress

HUVEC were seeded in 96-well culture plates ($1x10^4$ cells/well) and randomly divided into treatment groups with different EEC concentrations. After the treatment period, the cells were subjected to oxidative stress, which was induced with 1% hydrogen peroxide (H_2O_2), adapted from Gong et al[14]., diluted in 1% DMEM, for six consecutive hours.

Tetrazolium salt reduction assay

At the end of the induction of oxidative stress, the liquid content of each well was discarded for the addition of 10 µl of methyl-thiazolyl-tetrazolium (MTT). The plates were incubated for three hours, and then 50 µl of 10% sodium dodecyl sulfate (SDS - Vivantis Biochemical), diluted in HCl (0.01N), was added to each well to stop the MTT reaction. The plates were incubated for 24 hours at room temperature and protected from light.

The absorbance capacity of the converted dye by the viable cells was measured at a wavelength of 540 nm using a spectrophotometer (Awareness Technology Ine®/Stat Fax 2100). The absorbance value for each well was calculated according to the computational formula: absorbance (treatment) / absorbance (control) × 100%, to determine cell viability. From this, the cell cytotoxicity of each well was calculated using the computational formula: 100 - [absorbance (treatment) / absorbance (control) × 100%].

The value of the EEC concentration that inhibited 50% of cell viability (Inhibitory concentration - IC_{50}, in µmol) was calculated using the Graph Pad Prism 5 statistical program (Graph-Pad Software Inc., San Diego, CA, USA). The concentrations of lower cytotoxicities were selected for immunocytochemical analysis.

Immunocytochemistry

The immunocytochemistry technique was performed using primary anti-Caspase3 antibodies (polyclonal/rabbit, SC7148, Santa Cruz Biotechnology, USA), anti-SIRT1 (monoclonal/mouse, SC135792, Santa Cruz Biotechnology, USA), and phospho-mTOR (Ser2481, polyclonal/mouse, No. 2974, Cell Signaling Technology, Inc.), in dilutions of 1:500. The negative control was performed by replacing the primary antibodies with 3% BSA. All steps of the technique were performed by the Bond-Max automatic immunocytochemistry processor (Leica).

The slides marked with the antibodies were analyzed (according to Fedchenko & Reifenrath)[15], from the total cell count in each area immunostained by DAB (chromogen 3.3 'diaminobenzidine tetrahydrochloride) and the percentage of positive cells (marked by the antibody). Cells immunostained partially or completely in shades of brown were considered positive, while cells immunostained in shades of blue were considered negative. The ratio of positively stained cells (RCCP) was calculated by the ratio between the number of labeled cells and the total number of cells multiplied by 100:

$$RCCP = \frac{\text{Marked cell numbers}}{\text{Total number of cells}} x100$$

With this value, categories 0 were assigned to 0%, 1 to <1%, 2 for 1-10%, 3 for 11-33%, 4 for 34-66%, and 5 for ≥67%. Semi-quantitative analysis was performed by three researchers, (blind test) to describe the expression intensity, using the scores from 0 to 3. Then, we adopted a combination of the semi-quantitative scoring system (SQSS) and Allred score, which combined the percentage of cells with the staining intensity[16]. The two scores were summed for a final value.

Statistical analysis

To normalize the data obtained in the tetrazolium salt reduction assay and remove possible outliers, a mathematical model was used so that the values above and below these intervals were removed from the quintuplicate data. Then, using the *easyanova* package, the analysis of variance (ANOVA) and the post hoc Tukey test (5% significance) were performed to compare the mean absorbance values among the groups[17].

RESULTS

Characterization of the ethanolic extract of curcumin

In the present ethanolic extract of curcumin (EEC) from Mara Rosa, the standard curve of gallic acid was obtained by the Folin-Ciocalteau test. We obtained the content of total phenolic constituents (TPC) of 691.49 mg of GAE/100 g.

The marking of curcumin in the EEC of the saffron of Mara Rosa was also verified using high-performance liquid chromatography (HPLC or HPLC), the retention times and the respective peaks of the samples of the extract in question were compared with the standard of curcumin acquired commercially and within 15.1 minutes the molecule was identified in the EEC (Figure 1A and 1B).

Using the curcumin analytical curve data, the concentration of this substance in the extract was calculated (Figure 1C), which had an average content of 16.7% (Table 1). Therefore, in our study, the DPPH test was used with comparison by the standard antioxidant: Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and the dispersion curve is represented (Figure 1D). Using the equation obtained, we estimated the antiradical power of EEC by an equivalent amount in Trolox (18.45) and also the IC_{50} (18.10 µg/mL), the index refers to the concentration necessary to inhibit DPPH activity by 50% (Table 1).

Tetrazolium salt reduction assay

The MTT assay results showed no difference ($p<0.05$) between the three different exposure times to EEC of the HUVEC under normoxia (Table 2). In 48 h, we observed a variation between all EEC concentrations used and the control, with the same result for the cell exposure to the compound for 72h.

Besides, when statistically analyzing all concentrations of EEC used (0 µg/ml, 20 µg/ml, 50 µg/ml, 100 µ/ml, and 1000 µg/ml), regardless of exposure time, we found that the mean absorbance of the highest concentration was statistically different ($p<0.05$) from the mean absorbance of the control (0.035; 0.036; 0.035; 0.035; 0.044) (data not shown).

Regarding cytotoxicity, the test demonstrated that, in 24 h, the concentrations of 20, 100, and 1000 µg/ml of EEC did not differ from the control. At the dosage of 50 µg/ml, we found no variation ($p<0.05$), as observed in Table 2. We calculated the inhibitory concentration by 50% of the cell population under the effect of EEC, which showed an IC_{50} index =104.8 µg/ml in 24 h. In 24 hours, the extract inhibited 50% of cell viability with a lower concentration than in other exposure times. There may be a decrease in the action of the extract with increasing exposure time, which may have required a higher concentration to inhibit 50% of cell viability in 48 h and 72 h. However, at 48 and 72 h, these values increased to IC_{50} = 941.4 and IC_{50}=755.8 µg/ml, respectively.

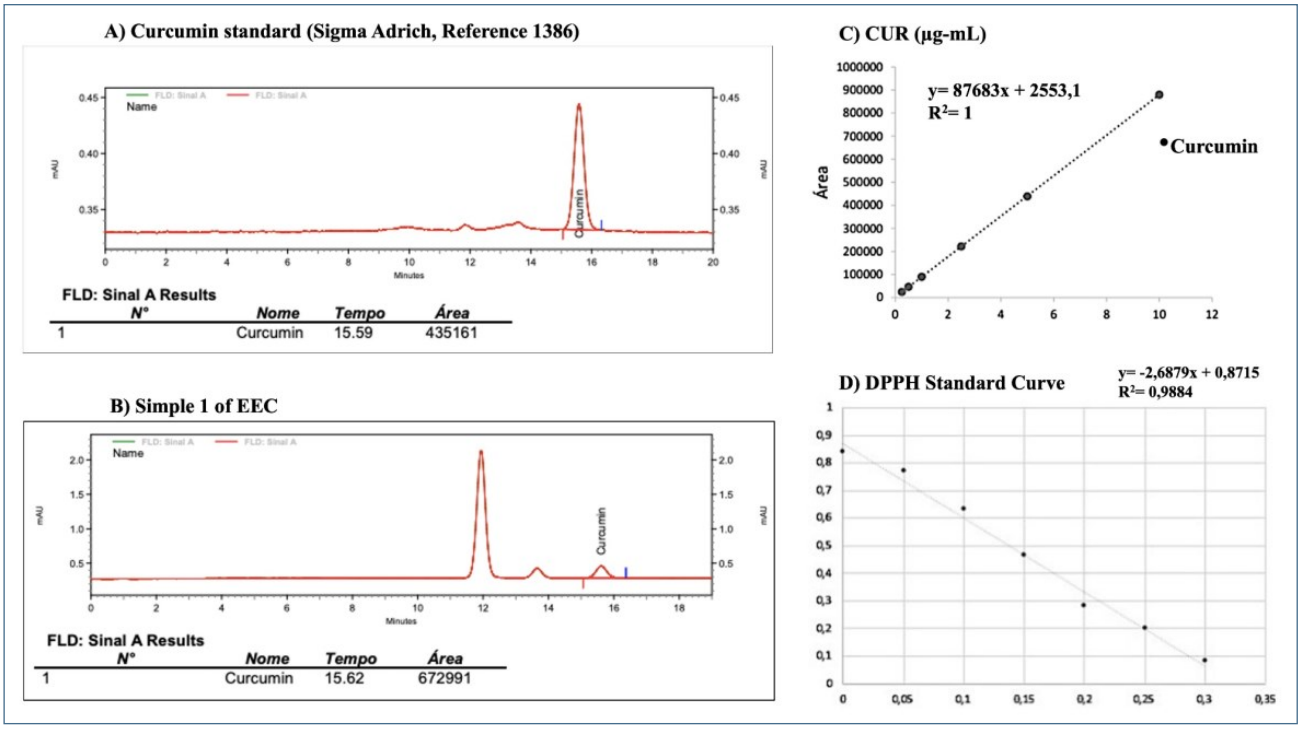

Figure 1: Chromatogram of the curcumin molecule. A) Standard curcumin retention time and area; B) Graphs of the sample of the ethanolic extract of Curcuma longa L. (EEC) from Mara Rosa, Goiás, showing the curcumin peaks found. C) Representation of the calibration curve, equation, and coefficient of determination of the curcumin standard. D) Dispersion graph between absorbance (y-axis) and concentration of standard Trolox (x-axis).

We also analyzed the effect of EEC on HUVEC cells under oxidative stress (OS). Thus, the statistical analysis referring to the set of variables time and concentration showed no difference ($p<0.05$) neither between the three different exposure times nor between the concentrations used within the referred periods of exposure (Table 3).

Table 1: Results of curcumin purity and Standard Trolox-equivalent antioxidant capacity (mol Trolox/mL) of the ethanolic extract of turmeric from Mara Rosa.

HPLC					
Sample	**Area**	**[CUR] (µl/ml)**	**[ ] Dilution factor (CUR mg final)**	**Extract weight (mg)**	**CUR content (%)**
EEC 1	672991	7.65	7.65	43.3	17.7
EEC 2	707384	8.04	8.04	49	16.4
EEC 3	837899	9.53	9.53	59.8	15.9
					Mean 16.7

DPPH	
Sample	**Ethanolic Extract of Curcumin**
Abs 1	0.5
Abs 2	0.5
Abs 3	0.51
Mean	0.502
Standard Deviation	0.0035
White sample	0.126
White solution	0.839
Abs white	0.376
Extract concentration (mg)	0.01
Antioxidant activity (µM TROLOX/g sample)	18.45
% Discoloration of DPPH	55
IC50 (µg/mL)	18.1

Table 2: Cell viability and cytotoxicity values obtained from absorbance by spectrophotometry for HUVEC samples under normoxia, after continuous exposure to EEC for 24, 48, or 72 hours.

T	Absorbance		Cell viability			Cytotoxicity		
µg/ml	MEAN	SD	Time	%	TT	Time	%	TT
0	0.035	0.001		100.00	**a**		0.00	**a**
20	0.036	0.002		98.65	**a**		1.35	**a**
50	0.035	0.001	24h **A**	96.80	**a**	24h **A**	3.20	**b**
100	0.035	0.001		101.22	**a**		-1.22	**a**
1000	0.034	0.007		107.72	**a**		-7.72	**a**
0	0.035	0.002		100.00	**a**		0.00	**a**
20	0.035	0.002		99.51	**b**		0.49	**a**
50	0.036	0.003	48h **A**	102.60	**b**	48h **B**	-2.60	**a**
100	0.035	0.002		98.85	**b**		1.15	**a**
1000	0.049	0.008		140.67	**b**		-40.67	**a**
0	0.035	0.001		100.00	**a**		0.00	**a**
20	0.037	0.002		103.90	**b**		-3.90	**a**
50	0.035	0.002	72h **A**	98.46	**b**	72h **B**	1.54	**a**
100	0.036	0.002		101.04	**b**		-1.04	**a**
1000	0.049	0.018		136.61	**b**		-36.61	**a**

HUVEC: Human umbilical vein endothelial cells; EEC (ethanolic extract of *Curcuma longa* L.); % (percentage of cell viability); uppercase letters indicate the statistical difference between the time of exposure and EEC, and the lowercase letters among the concentrations used ($p<0.05$). T: Treatment; SD: Standard Deviation; TT: Tukey Test.

Table 3: Cell viability and cytotoxicity assay by the tetrazolium salt reduction method, in endothelial cells, submitted to oxidative stress (H_2O_2 at 1%). Analysis of mean absorbance values concerning the EEC concentration used and the exposure times (24, 48, and 72 h).

T	Absorbance		Cell viability			Cytotoxicity		
µg/ml	MEAN	SD	TIME	%	TT	TIME	%	TT
0	0.032	0.000		100.00	**a**		0	**a**
20	0.033	0.000		103.45	**a**		-3.45	**a**
50	0.031	0.000	24h **A**	99.63	**a**	24h **A**	0.37	**a**
100	0.033	0.001		104.43	**a**		-4.43	**a**
1000	0.032	0.000		101.59	**a**		-1.59	**a**
0	0.032	0.000		100.00	**a**		0	**a**
20	0.033	0.000		101.97	**a**		-1.97	**a**
50	0.032	0.000	48h **A**	99.65	**a**	48h **B**	0.35	**a**
100	0.034	0.001		99.67	**a**		0.33	**a**
1000	0.033	0.001		102.99	**a**		-2.99	**a**
0	0.034	0.001		100.00	**a**		0	**b**
20	0.034	0.001		99.58	**a**		0.42	**a**
50	0.034	0.003	72h **A**	101.31	**a**	72h **B**	-1.31	**ab**
100	0.036	0.002		104.49	**a**		-4.49	**ab**
1000	0.035	0.003		103.37	**a**		-3.37	**ab**

HUVEC: Human umbilical vein endothelial cells; EEC (ethanolic extract of *Curcuma longa* L.); % (percentage of cell viability); uppercase letters indicate the statistical difference between the time of exposure and EEC, and the lowercase letters among the concentrations used ($p<0.05$). T: Treatment; SD: Standard Deviation; TT: Tukey Test.

However, when statistically analyzing only the mean absorbance of the concentrations (0 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml, 1000 µg/ml), we found that, at all times of exposure, the dosage of 100 µg/ml (0,034) was significantly different ($p<0.05$) from the control (0,032). From this, we can infer that this dose caused a protective reaction in the cells against OS damage.

In the cytotoxicity analysis of the compound, we found no likely cytotoxicity in 24 and 48 h due to the lack of statistical difference, neither between the dosages used nor between the times of exposure to EEC. At 72 h, the group of cells exposed to 20 µg/ml of extract varied statistically from the control (Table 3).

Immunocytochemistry

From the data found, we selected the concentrations of 20, 50, and 100 µg/ml and the exposure time of 24 h to investigate, through the immunocytochemistry technique, the possible signaling pathways for cell survival (SIRT1) and death (Caspase3, mTOR) in HUVEC, both under normoxia and OS. The results demonstrated the presence of the mentioned routes, with down-regulated by Caspase3 and up-regulated by SIRT1 and mTOR (Figure 2).

Morphological analysis showed changes between control cells and those treated with EEC. HUVEC exposed to low dosages of EEC showed an increase in the number of cells in culture (mitosis figures), and the original characteristics were maintained as monolayer growth, a polygonal shape, a centralized nucleus, and preserved edges. In higher concentrations, both under normoxia and OS, some dysmorphia, such as irregular cytoplasmic projections and coarse chromatin, could be seen, in addition to the loss of cell adhesion and decrease in the number of cells, data not shown in the image.

Through the Allred score system, we observed that, under normoxia, the magnitude of caspase3 immunostaining, a marker of cell death, gradually decreased with the increase in the concentration of EEC (Table 4). In the studied HUVECs under oxidative stress, the lowest degree of labeling of the caspase3 was at the concentration of 50 µg/ml of EEC (Table 4).

DISCUSSION

The choice of ethanol as the solvent for the extraction of curcumin from rhizomes was made according to the criteria listed in the literature, which demonstrates the best substances to solubilize curcuminoids. These compounds are characterized as hydrophobic, i.e., using water as a solvent decreases the efficiency in the extraction of CUR. Carvalho and colleagues demonstrated that the solubility of this molecule in water was 1.3 $mg.L^{-1}$, while in ethanol (99.5%), this value increased to 8895.9 $mg.L^{-1}$. Solvents, such as acetone and methanol, are also capable of extracting the molecule. However, the pharmaceutical and food industries value the use of ethanol to reduce the risk to consumers' health due to its high bioavailability of curcuminoid[18].

When comparing the value of the content of total phenolic constituents (TPC) found in this experiment, 691.49 mg of GAE/100 g, with the value of pure curcumin, commercially acquired (Sigma Aldrich), we noticed that its value was lower

(495±8 mg of GAE/100 g), which can be attributed to the other extract components. Twenty-three *Curcuma species* were analyzed and the total phenolic content ranged from 0.4 ± 0.1 to 22,3 ± 2,4 mg GAE/g. The highest phenolic contents were observed in the rhizomes of *C. longa* (22,3±2,4 mg GAE/g), followed by *C. parfivlora* (15,3±1,2 mg GA /g) and *C. latifolia* (12,9±0,3 mg GAE/g). The lowest phenolic contents were observed in the rhizomes of *C.* Wan Rang-Jud (0,4±0,1 mg GAE/g) followed by *C.* Wan Ma-Hor (0,5±0,1 mg GAE/g) and *Curcumamangga* (0,6±0,3 mg GAE/g)[19].

Gallic acid is a phenolic compound recognized as a potent neutralizer of reactive oxygen species. It is commonly used in pharmaceutical, cosmetic, and food industries, and its therapeutic effect, as well as of CUR, has shown antibacterial, antiallergic, anti-inflammatory, and antioxidant action. As a result, the TPC value can be determined as the equivalent of gallic acid since it is a highly recommended reference compound[20].

We found an average content of 16.7% curcumin in the EEC, it is known that the curcumin content in the plant can vary due to factors such as soil type, geographic location, climate, spacing

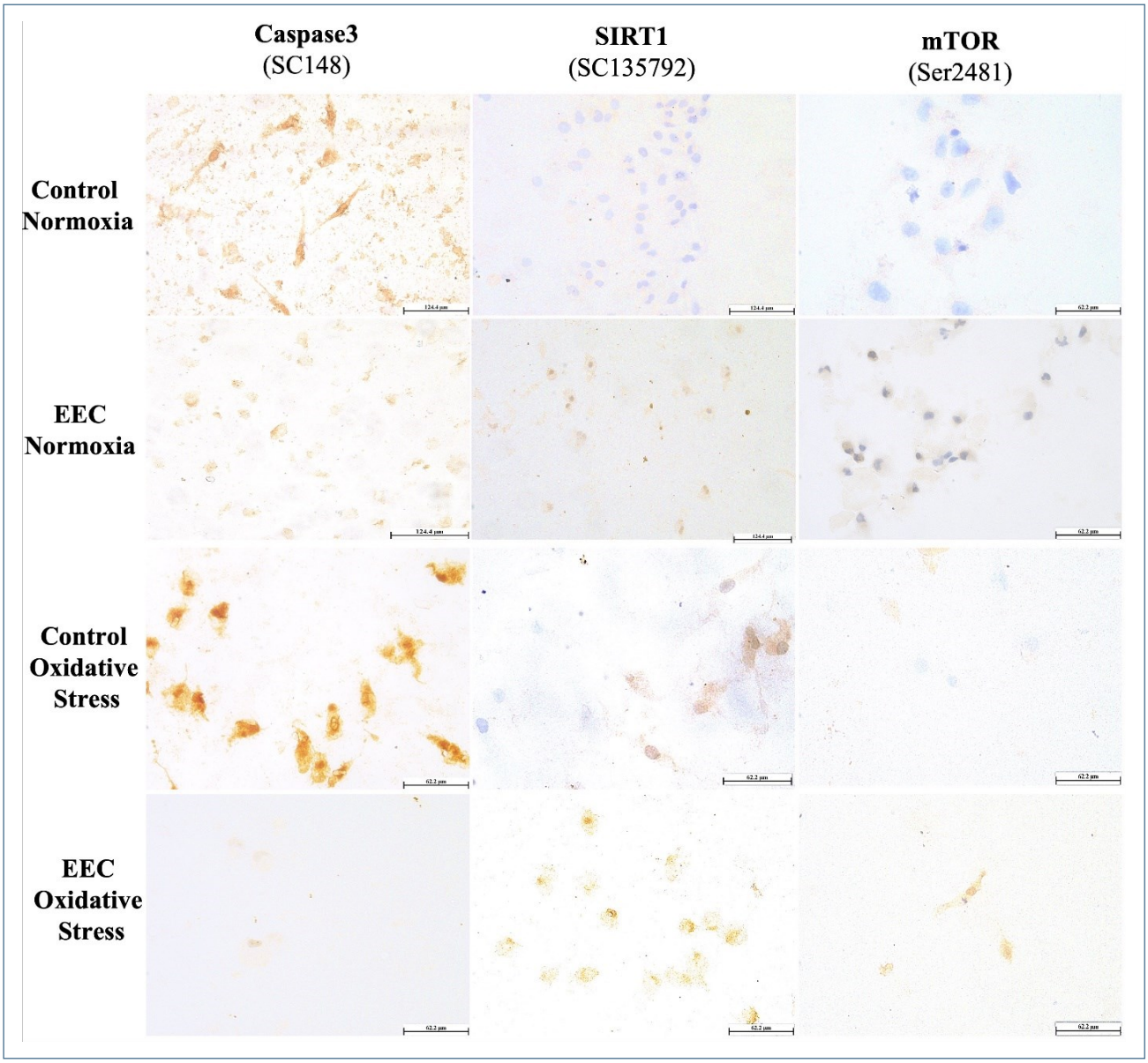

Figure 2: Photomicrographs of slides containing human umbilical vein endothelial cells under normoxia and oxidative stress, after the immunocytochemistry assay by the automatic Bond-Max processor (Leica). In normoxia, the intensity of immunostaining by the caspase3 antibody was lower in cells treated with the ethanolic extract of *Curcuma longa* L; with the SIRT1 and mTOR antibody, we noticed more labeling in the exposed cells. With oxidative stress, the caspase3 immunostaining reaction decreased markedly in cells exposed to the ethanol extract of Curcuma longa L. compared to the control; the opposite was observed with the use of antibodies SIRT1 and mTOR, in which the immunostaining in the control groups was lower than the treatment with the extract. Staining: DAB, counterstaining with hematoxylin. 100 µg/ml in 24 h.

Table 4: Allred scores for the evaluation of immunostaining in human umbilical vein endothelial cells under normoxia conditions and oxidative stress.

Antibody	T µg/ml	Normoxia			Oxidative stress		
		C	SC	SQSS	C	SC	SQSS
Caspase3	0	5	2	7	5	2	7
	20	5	3	8	5	2	7
	50	5	2	7	2	2	4
	100	5	1	6	5	3	8
SIRT1	0	0	0	0	4	3	7
	20	5	1	6	5	2	7
	50	5	1	6	5	2	7
	100	5	1	6	5	3	8
mTOR	0	3	1	4	0	0	0
	20	5	1	6	3	1	4
	50	5	1	6	5	2	7
	100	5	1	6	5	2	7

T: Treatment; C: Category; SC: Score; SQSS: Combined Semiquantitative Scoring System.

between plants, type of mineral fertilizer, rhizome storage period, exposure to light and others[21-23]. Ferreira[21] and collaborators reported a 14.83% curcumin content in the rhizome powder obtained from Cooperaçafrão, through extraction by ethanol and analysis by HPLC. Akbar et al.[22] found outliers in different regions of India (0.4% to 7.2%), realizing that the curcumin content decreased progressively at lower altitudes[21,23].

The IC_{50} value found in the searched extract (18.10 µg/mL) corroborates the value described by Tanvir et al.[24] (16.55 µg/mL), which analyzed the hydroalcoholic extract, obtained from saffron rhizomes grown in the Chittagong region of Bangladesh. These results are in line with the value already demonstrated in ascorbic acid (16.48 ± 0.11 µg/mL), whose antioxidant activity is well established in the literature[24]. Thus, it can be inferred that the EEC has an effective antioxidative action[24].

Carvalho et al.[25] also evaluated the antioxidant potential of curcumin. However, they used the pure molecule, commercially acquired (Sigma Aldrich), and diluted in methanol. Their result (14.09±0.60 µg/mL) was a little more effective than our result using the EEC from Mara Rosa. Thus, we can infer that the use of methanol as a solvent and the molecule purity influenced the antiradical reaction.

These results show the importance of characterizing the components of the EEC, given that the qualitative and quantitative evidence of antioxidants in the Mara Rosa's turmeric validates the perspective of its use as a protector.

In 24 hours, the extract inhibited 50% of cell viability with a lower concentration than in other exposure times. There may be a decrease in the action of the extract with increasing exposure time, which may have required a higher concentration to inhibit 50% of cell viability in 48h and 72h. Hosseini and colleagues[26] did not find, through survival tests, a decrease in HUVEC viability exposed to pure curcumin until 72 h (1, 5, 25, 50,100 µM). Our results corroborate those findings, given that the highest percentages of cell viability found here were in the dosages of 1000 µg/ml for 48 h (140.67%) and 72 h (136.71%). In addition, at the 72-hour exposure time, the 20ug / ml dose varied in toxicity compared to the control group and the other higher doses did not. This is because the higher dosages showed negative cytotoxicity values, which means that there was no cytotoxicity. On the contrary, such dosages have led to greater cell survival, which may indicate a greater cell protection action.

Regarding cytotoxicity, Mohammad et al.[27] found that the cytotoxicity of the hexanic extract of Curcuma longa L. was dose-dependent (0.28, 0.27, and 0.23 mg/ml, at 24, 48, and 72 h). In addition, they also observed that in studies using pure curcumin, the IC_{50} value was significantly lower compared to the rhizome extract. Thus, it is believed that other substances contained in EEC, in addition to curcumin, could interfere with its mechanism of action.

Through the immunocytochemistry technique, we demonstrated the signaling pathways of cell survival (SIRT1) and death (Caspase3, mTOR) in HUVEC, both in normoxia and in OS. SIRT1 is a protein-related to metabolism and cell survival, and its relevance in the vasculature is due to its expression being associated with the endothelium protection against senescence and dysfunction, in addition to promoting DNA repair[28,29].

The activation of MAPK signaling cascades occurs in cells under oxidative stress, which result in stimulation of caspase3 and, consequently, apoptosis. In the studied HUVECs under oxidative stress, the lowest degree of labeling of this protein was at the concentration of 50 µg/ml of EEC. The study by Han and colleagues showed a reduction in the activity of caspase3 and caspase-9 in endothelial cells treated with pure curcumin at a concentration of 10 µg/ml.[7] These findings corroborate the literature showing that

curcumin can mitigate cell death at lower concentrations since high dosages have been suggested to induce apoptosis of neoplastic cells: Curcumin at a dosage of 20 µM decreased cell viability and induced apoptosis in osteosarcoma, breast, and melanoma cells better than with 10 µM[30].

Regarding the cell survival protein, studies have shown that phenolic compounds, such as resveratrol and curcuminoids, attenuate cell damage caused by OS by increasing the expression of SIRT1. Thus, our results showed that pre-treatment with EEC increased the expression of SIRT1 in HUVEC exposed to H_2O_2, probably in a dose-dependent way, since immunostaining was higher in 100 µg/ml than in the other dosages. There was an increase in the scores for caspase-3, however, despite this event, there was an increase in SIRT1, a protein related to cell survival, and an increase in mTOR, related to cytoprotection and maintenance of essential cell functions. For this reason, we conclude the possibility of the protective action is greater than the stimulation of caspase-3.

This result corroborates the findings of Sun and colleagues, who investigated the senescence of HUVEC through the exposure of H_2O_2, observing immunostaining of SIRT1 when treating cells with pure curcumin. Xiao and colleagues also observed this protein increase in myocardial cells subjected to oxidative stress, and they explained that the protective effects of curcumin were attributed to SIRT1 activation, suggesting the agent as a future therapy[8,29].

The mTOR (mammalian target of rapamycin) activation is associated with events of cell damage and carcinogenesis. Some studies have suggested that curcumin acts on the regulation of mTOR, mainly via the AMPK/mTOR or PI3K/AKT/mTOR pathways, providing cytoprotection and maintaining essential cellular functions[31,32].

In the present study, this protein labeling was more intense in cells treated with EEC in a dose-dependent way, both in HUVEC under normoxia and OS, suggesting cytoprotection through this pathway. This finding corroborates studies that found higher cell viability by increasing mTOR expression in endothelial cells exposed to H_2O_2 and pretreated with pure curcumin (5 and 20 µg/ml)[27,32].

It is noteworthy that the results found in the literature are from experiments using pure curcumin acquired commercially. In addition, the results obtained with EEC are superior to those obtained with the standard solution of Curcumin, in another research carried out in the same laboratory (data not shown). Therefore, it is necessary to distinguish and analyze more constituents of EEC to elucidate the presence of other compounds and possible activation pathways of signaling proteins. We expect that EEC may represent an economically viable alternative as a preventive agent for injuries caused by oxidative stress.

Conclusion

The ethanolic extract of Curcuma longa L., produced from the turmeric rhizomes of Mara Rosa, contains 16.7% curcumin and antioxidant activity. In addition, in human umbilical vein endothelial cells, subjected to oxidative stress, at a dosage of 50µg/ml, the immunoexpression of caspase3 decreased and mTOR increased. At the dosage of 100 µg/ml, there was an increase in the immunoexpression of SIRT1 and mTOR. Thus, there was a decrease in apoptosis at lower concentrations and cytoprotection due to the maintenance of essential cellular functions and signaling of the survival pathway.

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