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Preparation of Phytol-Loaded Nanoemulsion and Screening for Antioxidant Capacity

ORIGINAL

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Abstract

The study prepared a nanoemulsion with a diterpenoid isoprenoid alcohol called phytol (PYT) and subsequently tested it for antioxidant capacity. For this, PYT-loaded nanoemulsion was prepared by phase inversion method and both PYT-containing nanoemulsion (PNE) and PYT-free nanoemulsion (PFNE) (2-16 µM) were tested for antiradical activity (DPPH•: 1,1-dipheny-picrylhydrazyl radical; ABTS•+: azino-bis-ethylbenzthiazoline-sulfonic acid; •OH: hydroxyl radical scavenging; NO•: nitrite oxide radical), lipid peroxidation (LP), reduction potential (RP), and inhibition of hemolysis (HL) in rat erythrocytes in comparison with an α-tocopherol analogue (Trolox - TRO - positive control). In addition, an in vivo test was performed with wildtype and deficient *Saccharomyces cerevisiae* strains using hydrogen peroxide (H_2O_2) as a stressor. Results suggest that PNE exhibited higher antioxidant than the PFNE. Increasing doses reveled antioxidant capacity in a dose-dependent manner. In the *S. cerevisiae* study, both PFNE- and PNE-treated groups exhibited decreased rates of survival with the highest doses, whichever in the presence of stressor increased the survival rates, which indicates antioxidative defense capacity of PYT. In this occasion, PNE exhibited prominent antioxidative defense in the presence of stressor rather than PFNE. In conclusion, PYT exhibited potential antioxidant activity but at high concentration it was toxic to the yeast cells. The production of PYT-nanoemulsions may be relevant to the pharmaceutical sciences.

Keywords
Antioxidant; Radicals; Nanoemulsion; Phytol; *Saccharomyces Cerevisiae*.

Introduction

Oxidative stress results from the reactive oxygen/nitrogen species (ROS/RNS) and to the damage of essential macromolecules, with apoptosis or necrotic cell death. It is believed that the basic mechanism underlying toxicity may be extracellular reactive species ultimately reaching the nucleus, where it causes DNA damage. Furthermore, DNA damage followed by subsequent cellular proliferation propagates errors and may result in carcinogenesis [1]. Although oxidative DNA damage is repaired by a number of different enzymes, some believe that oxidants and repair enzymes must remain at steady state [2].

Phytol (3, 7, 11, 15-tetrametilhexadec-2-en-1-ol; PYT), along with antioxidant, is already evident to have a number of biological activities, including antimicrobial, antispasmodic, antimutagenic, antibiotic-chemotherapeutic, anticancer, anti-teratogenic, antidiabetic, lipid lowering, anticonvulsant, immunoadjuvancy, antinociceptive, anti-inflammatory, anxiolytic, antidepressant, hair-fall defense and so on [3]. According to Andrade and Fasolo compounds with antioxidant and antimicrobial activities may promote overall health [4]. PYT is still used as a fragrance material in a number of cosmetics and non-cosmetics products [3]. Nowadays extensive research is focusing on the natural products, especially derived from plants, despite of the synthesized medicaments. The use of natural antioxidant compounds as a therapy in diseases related to oxidative stress has gained massive interest for their abilities to quench free radicals and the protection of the body against oxidative stress-induced pathogenesis. It is noteworthy that we have a number of synthetic antioxidants. Having high adverse effects and related other unfavorable factors such as cost, availability, safety and efficacy are the stimulation to search new compounds in this context.

Otherwise, being an essential oil, PYT is a water non-soluble molecule, which limits its application and use in products for humans. So, we prepared PYT-loaded nanoemulsions (PNE), which along with the nanoemulsion-free PYT (PFNE) undergone to investigate the antioxidant capacity in a number of *in vitro*, *ex vivo*, and *in vivo* methods.

Materials and methods

Reagents and chemicals

Tween-80 (0.05%) dissolved in saline solution (0.9% NaCl) served as the vehicle. Trolox (6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid; TRO) was used as the standard for all *in vitro* and *ex vivo* antioxidant assays. PYT, and all the necessary reagents and chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of PNE

PNE was prepared by the emulsion phase inversion method. Briefly, an oil phase composed of medium chain triglycerides (1% w/w) and a surfactant (5% w/w) composed of soy phosphatidylcholine and sodium oleate (1:7 w/w) was slowly titrated with an aqueous solution of 2-methylpyrrolidone (2% w/v) at 70 °C under constant magnetic stirring (1,500 rpm). This was emulsified using a basic Ultra-Turrax T18 equipped with the S 18 N-19 G dispersing tool at 11,000 rpm for one minute followed by four minutes at 7,000 rpm. The surfactant mixture that produced transparent colloidal dispersions was selected in a previous study using a pseudo-ternary phase diagram [5].

Analysis of PYT-nanoparticles size distribution and zeta potential measurement

The mean droplet diameter of the nanoparticles was calculated using the cumulative method of analysis based on the intensity of the light scattered (DLS) in a particle size analyzer (Brookhaven Instruments, USA) at 25 °C using a wavelength of 659 nm and 90° detection angle. The correlation was performed in parallel mode and the data was analyzed using Zeta Plus® Particle Sizing version 3.95

software. Zeta potential (ZP) measurements were performed using the same equipment applying the same field strength (approximately 5.9 V/cm). Five runs for each sample were used to determine the ZP value using the PALS ZP Analyzer software and the electrophoretic mobility according the Helmholtz-Smoluchowski equation. The samples were diluted 1:100 (v/v) with purified water.

Drug loading into the nanoemulsions

The ability of the nanoemulsions to load PYT was tested by adding an excess of the drug into the oil phase before the emulsification step described above. The samples were stored in a thermostatic bath at 25 °C and vortexed for one minute followed by 15 minutes in an ultrasonic bath every 12 h for 72 h. After centrifugation (1000 x g for 15 minutes), the samples were filtered through an acetate membrane (0.45 µm) and loaded PYT was analytically determined by UV-Vis spectrophotometry at 239 nm using the equation from the fitted standard curve plot constructed previously.

Experimental animals

The HL inhibition test was performed in eight week-old Wister albino rats (*Rattus norvegicus*; body weight: 180-220 g) from the Central Animal House of the Federal University of Piaui, Brazil. The animals were allowed free access to food (Purina, Brazil) and tap water *ad libitum* and were kept under controlled lighting (12 h dark/light cycles) at 24 ± 2 °C.

Test sample preparation

For the *in vitro* and *ex vivo* assays, PFNE and PNE were emulsified in 0.05% tween-80 dissolved in 0.9% NaCl at final concentrations ranging from 2-16 µM. Standard TRO was also dissolved in the same vehicle to give the same dilutions as PFNE and PNE. For the *S. cerevisiae* assay, PYT (PFNE and PNE) was adjusted to the same dose as above and in the same vehicle H_2O_2 (10 mM) was used as the stressor (STR) in this *in vivo* study.

In vitro antioxidant activity assays

DPPH• scavenging activity

The test for DPPH• scavenging activity was done using a method slightly modified from that described by Manzocco et al. [6]. Briefly, 0.3 mL samples with concentrations from 2-16 µM were added to a 2.7 mL ethanolic solution of DPPH (0.5 mM). After 30 min, the absorbance was measured using a spectrophotometer at 517 nm. A similar concentration of TRO served as the positive control, while only 0.3 mL vehicle was added to the DPPH solution for the negative control (NC). The blank contained no sample. The DPPH radical scavenging potential was calculated using the following equation:

$$\% \text{ inhibition of DPPH• scavenging} = [(A_{br} - A_{ar})/A_{br}]\times 100$$

where A_{br} is the absorbance of DPPH free radicals before the reaction and A_{ar} is the absorbance of DPPH free radicals after the reaction.

ABTS•+ decolorization activity

The ABTS•+ decolorization assay was done as described by Seeram et al. [7] with slight modification. ABTS•+ radicals were produced by adding solid manganese dioxide (80 mg) to a 5 mM aqueous stock solution of ABTS in 75 mM Na+/K+ buffer (pH 7.0). Then, 2.8 mL of the sample was added to 0.2 mL of ABTS•+ solution. The absorbance was read at 750 nm after 5 minutes. The percentage of scavenging capacity was calculated as follows:

$$\% \text{ ABTS•+ scavenged} = [(A_{br} - A_{ar})/A_{br}]\times 100$$

where A_{br} is the absorbance of ABTS•+ free radicals before the reaction and A_{ar} is the absorbance of ABTS•+ free radicals after reaction with the test samples.

•OH scavenging activity

The ability of test samples to scavenge hydrogen peroxide (H_2O_2) was determined according to the method described by Ruch et al. [8]. Briefly, a solution of 40 mM H_2O_2 was prepared in phosphate buffer (50 mM; pH 7.4). The concentration of H_2O_2 was measured spectrophotometrically by determining the absorption at 230 nm. The test samples (2–16 µM) and standards were added to the H_2O_2 and the absorbance at 230 nm was determined after 10 minutes. A solution containing phosphate buffer without H_2O_2 served as the blank. The percentage of H_2O_2 scavenging was calculated using the following equation:

$$\% \ H_2O_2 \text{ scavenged} = [(A_0 - A)/A_0]\times 100$$

where A_0 is the absorbance of the control and A is the absorbance of the test sample.

Nitric oxide (NO•) scavenging activity

To test for NO• scavenging activity, 0.375 mL of the test sample (PNE, FNE, and trolox), 1.5 mL of sodium nitroprusside (10 mM), and 0.375 mL phosphate buffer saline (PBS; pH 7.4) was mixed, and then the absorbance (A_{br}) was read at 546 nm. After incubating the reaction mixture at 37 ˚C for 1 hour, 1 mL of the solution was mixed with 1 mL Griess reagent. The reaction mixture was then incubated at room temperature for 30 minutes and the final absorbance (A_{ar}) was measured at 546 nm. The negative control contained only 0.375 mL of the vehicle [9]. The amount of NO• inhibition was calculated by the following equation:

$$\% \text{ inhibition of NO radical} = [(A_{br} - A_{ar})/Abr]\times 100$$

where A_{br} is the absorbance of NO• free radicals before the reaction and A_{ar} is the absorbance of NO• free radicals after the reaction with the Griess reagent.

Lipid peroxidation inhibition activity

The TBARS (thiobarbituric acid radicals) assay was used to measure the quantity of lipid peroxidation activity. Briefly, 0.1 mL of sample (PFNE/PNE/TRO/NC) was added to 1 mL homogenized egg yolk (1% w/v) in 20 mM phosphate buffer (pH 7.4). Lipid oxidation was induced by adding 0.1 mL of 2,2'-azobis (2-methylpropionamidine) dihydrochloride solution (AAPH; 0.12 M). The reaction mixture was incubated at 37 °C for 15 minutes. After cooling, 0.5 mL of the sample was centrifuged after the addition of 0.5 mL of trichloroacetic acid (15%) at 1,200 × g for 10 minutes. An aliquot of 0.5 mL from the supernatant was mixed with 0.5 mL TBA (0.67%) and heated at 95 °C for 30 minutes. After cooling, the absorbance was measured at 532 nm using a spectrophotometer. The levels of lipid peroxides were expressed as nM of TBARS/mg egg yolk using an extinction coefficient of $1.56x10^5$ mL/cm. Finally, the results were expressed as the percentage of inhibition of lipid peroxidation [10].

Reduction potential (RP) capacity

The RP test was conducted using a method described by Oyaizu [11] with the following modifications. A 0.2 mL sample was added to 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of $K_3Fe(CN)_6$ (1% w/v) and the reaction mixture was heated at 50 ˚C for 20 minutes. Then, 0.5 mL of trichloroacetic acid (10% w/v) was added with constant shaking, followed by the addition of 1.175 mL distilled water and 0.125 mL of $FeCl_3$ (0.1% w/v) after 5 minutes. The absorbance of the sample was taken at 700 nm relative to a blank (without sample) For the NC, only 0.2 mL of the vehicle was added and the samples were treated similarly. The reducing power capacity activity was calculated as follows:

$$\text{Reducing potential } (\%) = [(A_{ts} - A_{bs})/A_{ts}]\times 100$$

where A_{ts} is the absorbance of the test sample and A_{bs} is the absorbance of the blank.

Ex vivo antioxidant activity assay

HL inhibition capacity in rat erythrocytes

To test the inhibition of HL induced by H_2O_2, blood was collected from the retro-orbital plexus of anaesthetized (sodium pentobarbital 35 mg/kg; intraperitoneal) Wistar rats, and the erythrocytes were prepared for 10% suspension in PBS (pH 7.4). Next, 0.15 mL hydrogen peroxide (200 mM in PBS; pH 7.4) and 0.2 mL of the sample solution were added to 0.5 mL of the 10% erythrocytes suspension. The reaction mixture was incubated at 37 °C for 30 minutes and immediately centrifuged at 2,500 rpm for 3 minutes. Next, 0.2 mL of the supernatant was mixed with 2.8 mL PBS (pH 7.4) and the absorbance was measured at 475 nm [12]. For the NC, 0.2 mL of the vehicle was added. The percent rate of hemolysis was calculated using the following formula, taking into account that hemolysis 100% was induced by H_2O_2 (blank):

$$\text{Inhibition of hemolysis (\%)} = [(Abs_{blank} - Abs_{sample}) \times 100] / Abs_{blank}$$

where Abs (blank) is the control absorbance with 100% hemolysis induced by H_2O_2 and reactive Abs (sample) is the absorbance of aliquots containing various concentrations of the sample being studied.

In vivo antioxidant activity

S. cerevisiae assay

Previously sub-cultured yeast strains were linearly swabbed onto sterile YEPD media (0.5% yeast extract, 2% peptone, 2% dextrose, and 2% bacteriological agar). Next, 0.01 mL of either a test sample or control were applied to sterile paper disks and treated accordingly. For sole and co-treated groups, the test samples (PFNE and PNE) and the samples plus STR were added, respectively. NC and STR groups were treated with sterile vehicle (0.05% tween-80 dissolved in 0.9% NaCl) or H_2O_2, respectively. Treatments were done immediately after swabbing the organisms onto the petri dishes. The dishes were then inverted and kept in an incubator at 35 ± 1 °C for 72 h. The inhibition zones (in mm) were then measured. They ranged from 0 mm (full growth) to 40 mm (no growth), based on radius in the petri-dishes. All treatments were performed in duplicate [13].

Statistics

Results are presented as the mean ± standard deviation (SD). For *in vitro* and *ex vivo* antioxidant tests, data were analyzed by means of analysis of variance (one-way ANOVA) followed by *t-Student-Newman-Keuls's post-hoc* test. For *S. cerevisiae*, one-way *Bonferoni's* test and two *Tukey post*-tests were performed by using the GraphPadPrism software (version 6.0), considering $p < 0.05$.

Results and discussion

Preparation and characterization of PNE

Table 1 summarizes the physicochemical characteristics of the transparent oil-in-water nanoemulsions successfully produced in the absence (VNE) or containing PNE. The colloidal dispersions exhibited small and uniformly distributed droplet sizes, with

Table 1. Effect of PYT on droplet size (diameter), zeta potential (ZP) at VNE and drug loading at PNE.

Formulation code, composition (NMP, Mygliol®812, surfactant mixture, Phytol)	Diameter (nm) ± SD	PdI	ZP (mV) ± SD	Drug loading (%)
VNE, 0.2:0.1:0.5:0	130.2 ± 17.17	0.185	-108 ± 3.01	-
PNE, 0.2:0.1:05:0.10	245.5 ± 25.02	0.384	-77.8 ± 2.83	1.55

VNE: vehicle for nanoemulsion; PNE: phytol loaded nanoemulsion; PdI: diameter index for phytol

VNE showing a mean diameter of 130.20 nm and polydispersity index (PdI) of 0.185. Drug loading caused the PNE droplet size to increase to a mean diameter of 245.50 nm (PdI = 0.384). Both VNE and PNE nanoemulsions showed large negative ZP values. The drug loading was calculated using the linear regression equation, A = 0.0045C + 0.005, which was extracted from the standard curve. The measured absorbance was substituted for "C", expressed as µg/mL (PYT). The correlation coefficient of the straight line was 0.9999. The maximum PYT loading on the oil-in-water (O/W) nanoemulsions was 1.55 ± 0.012% (n = 3), which allowed us to produce nanoemulsions containing 1% w/v (10 mg/mL) PNE.

Nanoemulsions are commonly stabilized by relatively large amounts of surfactant in order to produce transparent dispersions [14]. In this study, we have produced extraordinarily transparent nanoemulsions with droplet sizes in the range of 130 to 250 nm using low levels of surfactant (about 5% w/w). This was the result of using a suitable surfactant pair, composed of soy phosphatidylcholine and sodium oleate (1:7 w/w) associated with the co-solvent 2-methylpyrrolidone (2% w/v). The effect of surfactant composition [5] and co-solvent ratio [15] on both droplet size and droplet size distribution has been previously described. In addition, the composition of oil phase/surfactant/co-solvent used in this work produced stable nanoemulsions with high negative ZPs, guaranteeing suitable electrostatic resilience against flocculation.

PYT loading contributed to the increased nanoemulsion droplet size, which probably occurred due to PYT solubilizing in the oil phase. In previous studies with microemulsions containing the antitumor drug doxorubicin, we observed similar results, with drug loading increasing the droplet size of the microemulsions [16]. The same effect was also observed for amphotericin B loaded oil-in-water microemulsions [17]. Thus, PYT is a liquid immiscible with water and, as observed with amphotericin B, its solubilization into oil phase contributes to increased droplet size. The mechanism of the latter case is not clear, but the ZP reduction observed for drug-loaded nanoemulsions confirmed that PYT also disrupts the surfactant film at the oil-water interface. It is possible that the drug is partially located in this region, which is important for PYT's antioxidant effects.

Antioxidant activity assays

Although living systems coexist with free radicals by developing diverse mechanisms for adapting them to advantageous physiological functions, excessive production of ROS and RNS are implicated in various diseases [18].

In vitro antioxidant activity assays

DPPH• scavenging activity

The DPPH• test is the most commonly performed assay for determining antioxidant activity by monitoring the decreased absorbance of the test sample. It assesses radical scavenging activity. Trolox, (+)-catechin, ethyl gallate, ascorbic acid, and a-tocopherol are generally used as standards for screening new compounds [19]. **Table 2** shows that PNE produced greater inhibitory effect when compared to PFNE for all four doses tested, but the activity was lower than the standard (TRO) treated with the same doses. The highest inhibitions were observed for 16 µM of PFNE, PNE, and TRO and were 50.44 ± 0.01, 58.17 ± 0.01, and 76.59 ± 0.01%, respectively. There was a dose-response relationship between the tested doses and inhibition.

ABTS•+ decolorization activity

The ABTS assay, which monitors the scavenging of ABTS•+ is another common method used to test for antioxidant activity. It is a peroxidase substrate upon oxidation in the presence of H_2O_2 that generates a metastable radical cation [19]. It confirms that there was improvement in the inhibition capability of PNE in comparison to TRO. The 16 µM dose of PNE, or TRO exhibited inhibition of 59.18 ± 0.01% and

Table 2. Antioxidant potential of PFNE/PNE in comparison to controls.

Samples	DPPH (% radical scavenge)			ABTS (% radical scavenge)			OH (% radical scavenge)			NO (% radical scavenge)		
	PFNE	PNE	TRO	PFNE	PNE	TRO	PFNE	PNE	TRO	PFNE	PNE	TRO
16 (μM)	50.44 ± 0.01[a]	58.17 ± 0.01[a]	76.59 ± 0.01[a]	54.59 ± 0.01[a]	59.18 ± 0.01[a]	60.76 ± 0.01[a]	50.79 ± 0.01[a]	62.75 ± 0.02[a]	69.09 ± 0.02[a]	58.36 ± 0.01[a,b]	65.06 ± 0.01[a]	74.69 ± 0.02[a]
8 (μM)	43.63 ± 0.03[a]	34.34 ± 0.02[a]	67.40 ± 0.01[a]	40.03 ± 0.02[a]	39.24 ± 0.01[a]	49.58 ± 0.01[a]	35.93 ± 0.03[a]	36.18 ± 0.01[a]	48.75 ± 0.01[a]	32.81 ± 0.01[a]	37.11 ± 0.02[a]	48.18 ± 0.02[a]
4 (μM)	39.57 ± 0.01[a]	27.16 ± 0.01[a]	45.21 ± 0.01[a]	22.27 ± 0.01[a]	24.41 ± 0.02[a]	27.71 ± 0.02[a]	30.61 ± 0.01[a]	27.41 ± 0.01[a]	41.56 ± 0.01[a]	23.54 ± 0.01[a]	34.23 ± 0.01[a]	39.99 ± 0.01[a]
2 (μM)	31.06 ± 0.01[a,b]	19.49 ± 0.01[a]	26.17 ± 0.02[a]	15.73 ± 0.04[a,b]	16.06 ± 0.01[a,b]	10.36 ± 0.01[a]	21.64 ± 0.01[a]	15.82 ± 0.01[a]	22.66 ± 0.01[a]	12.85 ± 0.02[a]	8.93 ± 0.01[a]	36.56 ± 0.01[a]
NC	2.19 ± 0.05			1.29 ± 0.03			0.89 ± 0.02			1.01 ± 0.01		

Samples	TBARS (% inhibition of lipid peroxidation)			RP (% inhibition oxidation)			HL (% inhibition hemolysis)		
	PFNE	PNE	TRO	PFNE	PNE	TRO	PFNE	PNE	TRO
16 (μM)	66.89 ± 0.01[a]	74.52 ± 0.01[a]	95.02 ± 0.01[a]	64.13 ± 0.01[a]	87.34 ± 0.01[a]	94.09 ± 0.05[a]	58.85 ± 0.01[a,b]	43.82 ± 0.01[a]	65.81 ± 0.01[a,b]
8 (μM)	50.75 ± 0.01[a]	50.75 ± 0.01[a]	81.56 ± 0.01[a]	46.49 ± 0.01[a]	83.88 ± 0.01[a]	87.71 ± 0.02[a]	30.54 ± 0.01[a]	35.19 ± 0.01[a]	34.08 ± 0.01[a,b]
4 (μM)	38.89 ± 0.01[a]	38.89 ± 0.01[a]	65.86 ± 0.01[a]	38.89 ± 0.01[a]	77.82 ± 0.01[a]	83.19 ± 0.03[a]	22.91 ± 0.01[a,b]	19.37 ± 0.01[a,b]	16.76 ± 0.01[a,b]
2 (μM)	31.72 ± 0.01[a,b]	13.91 ± 0.01[a]	16.81 ± 0.01[a]	34.44 ± 0.01[a]	64.47 ± 0.01[a]	80.27 ± 0.05[a]	10.61 ± 0.01[a,b]	13.59 ± 0.01[a,b]	8.01 ± 0.01[a,b]
NC	2.11 ± 0.04			2.03 ± 0.02			1.07 ± 0.02		

[a]$p < 0.05$ compared to NC (0.05% tween 80 in 0.9% NaCl); [b]$p < 0.05$, compared to TRO (ANOVA and t-Student-Newman-Kewls as post hoc test); PFNE: phytol free with nano-emulsion; PNE: phytol in nano-emulsion; TRO: trolox (standard); values are the mean ± SD (n = 5).

Table 3. IC_{50} of antioxidant potential of PYT and standard (trolox).

Parameters	DPPH test			ABTS test			OH test			NO test		
	PFNE	PNE	TRO	PFNE	PNE	TRO	PFNE	PNE	TRO	PFNE	PNE	TRO
IC_{50} (mM/L)	1.39 ± 0.25	4.14 ± 0.53	3.14 ± 0.27	4.23 ± 0.47	4.50 ± 0.47	4.20 ± 0.24	2.76 ± 0.39	4.77 ± 0.55	3.26 ± 0.39	5.17 ± 0.58	4.76 ± 0.70	2.72 ± 0.51
CI	0.63-3.06	1.34-12.82	2.19-4.14	2.18-8.21	2.19-9.26	3.44-5.13	1.07-7.15	1.87-12.16	1.61-6.62	2.12-12.60	1.73-13.09	0.49-15.14
r^2	0.96	0.85	0.98	0.94	0.93	0.99	0.89	0.89	0.93	0.90	0.88	0.75

Parameters	TBARS test			RP test			HL test		
	PFNE	PNE	TRO	PFNE	PNE	TRO	PFNE	PNE	TRO
IC_{50} (mM/L)	2.52 ± 0.38	4.22 ± 0.45	3.21 ± 0.58	2.08 ± 0.43	1.05 ± 0.12	0.41 ± 0.38	5.63 ± 0.66	3.81 ± 0.46	6.72 ± 0.76
CI	0.95-6.71	2.35-7.59	2.30-4.47	0.42-10.27	0.78-1.41	0.01-16.81	2.19-14.49	2.05-7.08	3.52-12.83
r^2	0.89	0.95	0.98	0.82	0.99	0.84	0.89	0.94	0.94

IC_{50}: inhibitory concentration 50% in μM; CI: confidence interval; r^2: coefficient of determination

60.76 ± 0.01%, respectively **(Table 2)**. There was also a dose-response relationship among the test samples.

•OH scavenging activity

The free hydroxyl radical (•OH) generally reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes cellular destruction [20]. Measuring the hydroxyl anionic radical (-•OH) is done similarly to the DPPH and ABTS assays, where •OH produced from the H_2O_2 is scavenged by the sample tested. We found that PNE, rather than PFNE, showed higher inhibition. The greatest inhibition was observed for the highest dose (16 µM), resulting in 50.79 ± 0.01%, 62.75 ± 0.02%, and 69.09 ± 0.02% by PFNE, PNE, and TRO, respectively. The CI revealed that both PFNE (CI = 1.07-7.15 µM; r^2 = 0.89) and PNE (CI = 1.87-12.16 µM; r^2 = 0.89) had improved activity in comparison to TRO (CI = 1.61-6.62 µM; r^2 = 0.93; **Table 3**). There was also a dose-response relationship for the tested sample doses.

Nitric oxide (NO•) scavenging activity

The sodium nitroprusside in the NO• scavenging test was used as the source of nitric oxide (NO) radical. Under aerobic conditions, NO reacts with oxygen to produce a stable nitrate (NO_3•–) and nitrite (NO_2•–) radicals, the quantities of which can be determined using the Griess reagent. In this test, PNE, rather than PFNE, had strong NO• scavenging activity, with an inhibition of 65.06 ± 0.01% by the 16 µM dose, while TRO by 74.69 ± 0.02%. However, increasing doses exhibited greater inhibition, thus indicating a dose-response relationship. The CIs for PFNE, PNE, and TRO were 2.12-12.60 µM (r^2 = 0.90), 1.73-13.09 µM (r^2 = 0.88), and 0.49-15.14 µM (r^2 = 0.75), respectively **(Table 3)**. Since PNE showed strong inhibition of NO• production, it could have physiologically important roles and could potentially prevent nitrosative stress [21].

Lipid per-oxidation inhibition activity

In the TBARS assay, lipid peroxidation was detected by measuring the amount of malonyl aldehyde (MDA) formed from unsaturated fatty acids. The more MDA that reacted with thiobarbituric acid (TBA), the stronger the pink coloration of the sample and the greater the absorbance [19]. Lipid peroxidation is usually completed by AAPH (*in vitro*), but it was strongly inhibited by TRO, followed by PNE and PFNE. The CI parameter demonstrates the prominence activity of PNE with a coefficient of 0.95 (r^2) **(Table 3)**. However, in this study, 16 µM of PNE and PFNE exhibited inhibition of LP by 74.52% and 66.89% respectively. The oxidation/peroxidation of lipids by foreign chemicals is a major cause of liver injury [22]. In this *in vitro* test, the dose-response relationship and the significant ($p < 0.05$) inhibition of LP suggests PYT has potent antioxidant capacity.

Reduction potential (RP) capacity

The RP assay is a rapid and sensitive antioxidant test, in which absorbance increases upon formation of a color complex with potassium ferricyanide, trichloro acetic acid, and ferric chloride. An increase in the absorbance indicates antioxidant activity of the test sample [23]. Antioxidants are the reducing agents. According to the **Table 2**, PNE improved the reduction activity more than PFNE for all of the doses tested. PFNE (16 µM) showed a similar reduction capability as the lowest dose of PNE (2 µM). The CI calculated for PFNE, PNE, and TRO were 0.42-10.27 µM (r^2 = 0.82), 0.78-1.41 µM (r^2 = 0. 99), and 0.01-16.81 µM (r^2 = 0.84), respectively **(Table 3)**. Even though the activity was lower than the TRO, there was a dose-response relationship in all cases. A possible simplified mechanism for the antioxidant activity of PYT is shown in **Figure 1**.

Ex vivo antioxidant activity assay

HL inhibition capacity in rat erythrocytes

Ex vivo HL was carried out by first treating rat erythrocytes with a lysis agent, H_2O_2. Then, HL in-

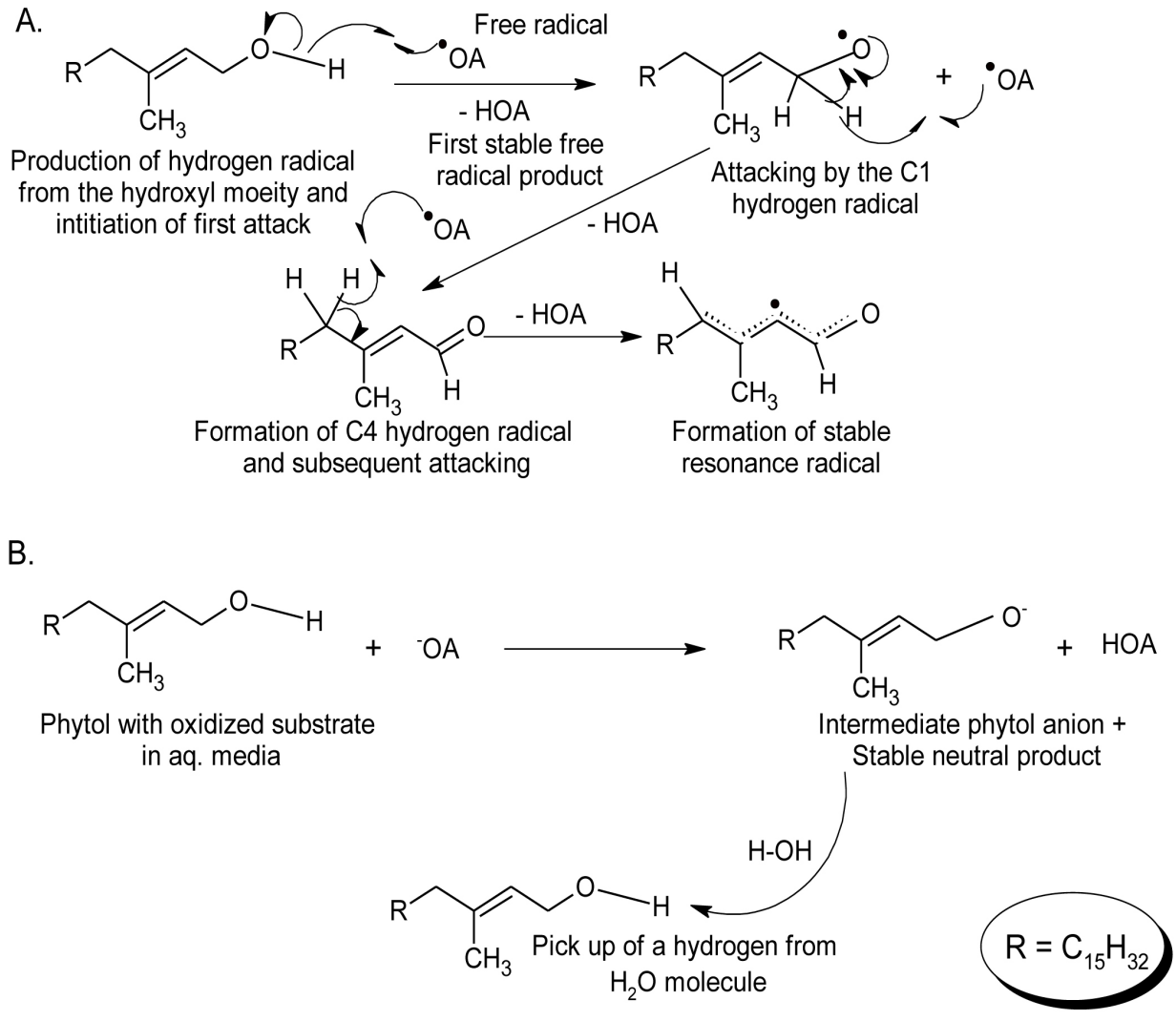
hibition was assessed by measuring the hemoglobin concentration in the reaction [12]. In this study, PFNE proficiently up-regulated erythrocyte membrane stability and, thus, reduced HL. Erythrocyte lysis was decreased by 58.85 ± 0.01%, 43.82 ± 0.01%, and 65.81 ± 0.01% after treatment with 16 µM of PFNE, PNE, and TRO, respectively. Oxidative radicals, especially NO•, can bind to Fe^{2+}-bound heme, which alters its physiological functions [24], since free hemoglobin (Hb) is crucial for dissolved oxygen (O_2) and carbon-di-oxide (CO_2) respiration during normal metabolic processes. Since PYT exhibited strong NO• scavenging activity in this study, it may alter the detrimental *in vivo* effects of this radical. However, the lower inhibition of HL with PNE may be due to its greater hydrophobicity at high concentration and antioxidant-induced pro-oxidative effects [25].

In vivo antioxidant activity

S. cerevisiae assay

Currently, there is evidence that the body prevents free radical damage [26] using some enzymes and substrates [27]. For example, the dismutation enzyme, superoxide dismutase (SOD), can convert

Figure 1: Simplified possible reducing mechanisms of phytol. (A: Free radical (neutralization) pathways. B: Ionization pathway).



O_2• into ordinary oxygen (O_2) or H_2O_2. On the other hand, the catalase (CAT) enzyme, which is ubiquitous in most living organisms, is responsible for converting H_2O_2 to water (H_2O) and oxygen (O_2) [28]. These two enzymes are essential for the neutralization of ROS and keeping cells free from oxidative stress-mediated damage [29]. *S. cerevisiae* have antioxidant compounds in the cytoplasm, mitochondria, and on the entire surface of the cell [30]. In addition, one of its cell wall structural polymers (1-3), (1-6)-b-D-glucan) has antioxidant [31] and immunomodulatory activities [32]. Therefore, this organism can be used to measure the antioxidant action of a wide variety of organic and inorganic substances. In addition, this eukaryotic model allows for the investigation of the effects of antioxidants at the cellular level and is, thus, an attractive alternative to mammalian cell lines. Based on colony formation tests performed by Bakkali et al. [25], certain EOs are cytotoxic to *S. cerevisiae*, mainly in the stationary growth phase. However, it depends on the state of cell growth as the cells in vegetative stage are much more sensitive due to high penetration power of EOs more efficiently at the budding sites. EOs containing phenolic, aldehyde, and alcoholic OH are cytotoxic in nature [33] and they may act against fungi [34].

Study-based conclusive talks

The use of PYT in aqueous dispersions requires a powerful solubilizing strategy, such as O/W nanoemulsion. Previous studies have reported the efficacy of loading insoluble terpenes into nanoemulsions and the increased stability of these mixtures [35]. In addition, an antioxidant synergy formulation (ASF) containing delta, alpha, and gamma tocopherol loaded into nanoemulsions decreased inflammation in *CD-1* mice, which was attributed to the increased bioavailability of gamma (2.2 folds) and delta (2.4 folds) tocopherol when compared to pure compounds [36]. The antioxidant activity of the anticancer compound quercetin was also increased when loaded into nanoemulsions, as measured as a function of prophylactic antitumor efficacy against DMBA-induced breast tumors [37]. It was also shown to combat doxorubicin- and cyclosporin A-induced cardiotoxicity and nephrotoxicity [38].

EOs are known for their antioxidant and cytotoxic, rather than mutagenic, activities. At low concentrations, antioxidants cannot penetrate the mitochondrial membrane and, thus, may retain antioxidant, rather than cytotoxic or prooxidant, activities. Low concentrations of retinol and tocopherol exhibit antioxidant and antimutagenic activities, whereas, at high concentration, they are genotoxic [39]. Therefore, both the concentration of the antioxidant and the integrity of the mitochondrial membrane are keys for the antioxidant and cytotoxic activities of the EOs.

Based on the results shown in **Table 4**, it is clear that PFNE had oxidative activities in Sod1ΔSod2Δ at all tested doses, but the inhibition zones (7.00-11.00 mm) were lower than those seen for the STR (25.25 mm). The PFNE also exhibited an oxidative effect in the SOD-WT and Sod1Δ strains at 16 µM. Survival of the rest of the strains was significantly ($p < 0.05$) increased with the treated doses, where PFNE produced a statistical difference ($p < 0.05$) of 2 µM with 16 µM in SOD-WT, 2 µM and 4 µM with 16 µM in Sod1Δ, 2 µM with 4 µM, 8 µM and 16 µM in Sod2Δ and 2 µM with 16 µM in Cat1Δ strains, respectively.

Table 5 shows that 2-16 µM PNE had an oxidative effect on the Sod1ΔSod2Δ and Sod1ΔCat1Δ strains, and 4-16 µM PNE had an effect in the Cat1Δ strain, but the inhibition zones were smaller when compared to STR. Only the 2 µM dose had an antioxidative effect in Cat1Δ when compared to STR. In this case, 16 µM PNE also had oxidative activity in the SOD-WT strain. However, there was increased survival rates in the rest of the strains tested. The data presented here suggest no statistical difference between the doses of PNE tested. On the other hand, there was a difference in the results for PFNE and PNE treatments for the Cat1Δ and Sod1ΔCat1Δ strains. PFNE increased survival rates significantly (p

Table 4. IC_{50} of antioxidant potential of PYT and standard (trolox).

Parameters	Inhibition zones in millimeters in test strains					
	SOD-WT	Sod1Δ	Sod2Δ	Cat1Δ	Sod1ΔSod2Δ	Sod1ΔCat1Δ
NC (10 µL)	5.00 ± 1.24	5.50 ± 1.04	6.25 ± 0.73	6.75 ± 0.85	0.50 ± 0.33	0.25 ± 0.29
STR (10 mM)	20.25 ± 0.29[a]	24.50 ± 1.94[a]	24.00 ± 0.67[a]	24.00 ± 2.73[a]	25.25 ± 3.57[a]	12.75 ± 0.73[a]
Nullified STR						
16 (µM)	7.75 ± 1.52a	10.75 ± 0.48[a]	10.25 ± 0.55	10.75 ± 0.86	11.00 ± 0.47[a]	8.75 ± 0.99
8 (µM)	6.50 ± 2.03	9.75 ± 0.48	9.75 ± 0.87	9.25 ± 0.76	8.75 ± 1.37[a]	7.75 ± 1.19
4 (µM)	7.50 ± 0.58	9.25 ± 0.63	9.25 ± 1.09	8.00 ± 0.56	8.75 ± 1.37[a]	7.00 ± 0.82
2 (µM)	5.00 ± 0.67	8.50 ± 0.29	8.50 ± 0.75	7.50 ± 0.74	8.50 ± 1.00[a]	5.50 ± 0.75
PFNE+STR						
16 (µM)	10.25 ± 0.29[a]	13.25 ± 1.89[a]	7.75 ± 1.73[b]	6.50 ± 1.89[b]	8.50 ± 1.00[a]	8.75 ± 0.99[b]
8 (µM)	9.50 ± 0.33[b]	7.00 ± 1.68[b]	8.25 ± 0.85[b]	5.00 ± 1.58[b]	7.00 ± 0.82[a]	7.75 ± 1.19[b]
4 (µM)	9.50 ± 1.00[b]	5.50 ± 0.87[b,c]	7.50 ± 1.38[b]	7.50 ± 1.29[b]	9.00 ± 1.25[a]	7.00 ± 0.82[b]
2 (µM)	8.25 ± 0.73[b,c]	5.25 ± 0.75[b,c]	4.25 ± 0.73[b,c,d,e]	3.75 ± 0.46[b,c]	8.75 ± 1.28[a]	5.50 ± 0.75[b]

[a]$p < 0.05$ compared to NC (0.05% tween 80 in 0.9% NaCl); [b]$p < 0.05$ compared to STR (H2O2); [c]$p < 0.05$ compared to PFNE 14.16 µM; [d]$p < 0.05$ compared to PFNE 7.08 µM; [e]$p < 0.05$ compared to PFNE 4.72 µM (one-way ANOVA followed by Bonferoni's test within 95% confidence interval); values are the mean ± SD (n = 4).

Table 5. Inhibition zones of PNE, NC and STR in proficient and mutant *S. cerevisiae* strains.

Parameters	Inhibition zones in millimeters in test strains					
	SOD-WT	Sod1Δ	Sod2Δ	Cat1Δ	Sod1ΔSod2Δ	Sod1ΔCat1Δ
NC (10 µL)	5.00 ± 1.24	5.50 ± 1.04	6.25 ± 0.73	6.75 ± 0.85	0.50 ± 0.33	0.25 ± 0.29
STR (10 mM)	20.25 ± 0.29[a]	24.50 ± 1.94a	24.00 ± 0.67[a]	24.00 ± 2.73[a]	25.25 ± 3.57[a]	12.75 ± 0.73[a]
Nullified STR						
16 (µM)	9.25 ± 0.87[a]	10.00 ± 0.82	8.75 ± 1.09	8.25 ± 1.19[a]	10.25 ± 0.67[a]	9.50 ± 1.11[a]
8 (µM)	8.75 ± 0.87	8.50 ± 1.11	9.25 ± 0.55	8.75 ± 0.55[a]	9.50 ± 0.75[a]	9.25 ± 0.87[a]
4 (µM)	8.25 ± 0.55	7.00 ± 0.82	7.50 ± 1.00	8.25 ± 0.55[a]	8.00 ± 0.47[a]	8.25 ± 0.87[a]
2 (µM)	6.50 ± 0.75	6.25 ± 0.55	6.25 ± 0.55	6.50 ± 0.58[a]	6.25 ± 0.55[a]	7.00 ± 0.82[a]
PNE+STR						
16 (µM)	9.75 ± 0.85[a]	6.25 ± 0.55[b]	7.75 ± 1.52[b]	6.75 ± 1.79[a]	8.25 ± 0.99[a]	7.75 ± 1.78[a]
8 (µM)	7.00 ± 1.70[b]	8.25 ± 1.28[b]	8.00 ± 1.25[b]	6.00 ± 1.83[a]	9.00 ± 0.94[a]	8.50 ± 1.38[a]
4 (µM)	8.00 ± 0.47[b]	7.50 ± 1.53[b]	8.25 ± 1.59[b]	8.25 ± 0.98[a]	6.25 ± 0.29[a]	7.75 ± 0.73[a]
2 (µM)	5.50 ± 0.33[b]	6.00 ± 1.06[b]	5.50 ± 0.75[b]	5.00 ± 0.82[b]	6.00 ± 0.67[a]	6.00 ± 1.06[a]

[a]$p < 0.05$ compared to NC (0.05% tween 80 in 0.9% NaCl); [b]$p < 0.05$ compared to STR (H_2O_2) (one-way ANOVA followed by Bonferoni's test within 95% confidence interval); values are the mean ± SD (n = 4).

< 0.05), but PNE exhibited oxidative effects at all of the doses tested, except 2 µM. There may be an effect of the vehicle in which the PYT nanoparticles were emulsified. However, the data presented in both **Tables 4** and **5** suggest lower zones of inhibition for both formulations, which were lower than the STR-treated group. The data shown in Tables 3 and 4 reveal that both PYT formulations effectively protected all *S. cerevisiae* strains tested, thus demonstrating the antioxidant defense capacities of PFNE and PNE.

However, in the without-STR (nullified) treated groups and groups co-treated with 16 µM of PFNE/PNE, the zones of inhibitions were slightly larger than for the other co-treated doses. There may be a hyperoxy effect; which was eventually minimized

after the application of STR encountered activity of both the PFNE and PNE. In the *in vitro* •OH scavenging test **(Table 2)**, PYT significantly ($p < 0.05$) augmented •OH scavenging potential, thus the STR in this case produces OH radicals that may encounter PYT (PFNE/PNE). While the defense increased with decreasing doses of PFNE and PNE, the activity was significant ($p < 0.05$) when compared to the NC and STR-treated groups.

It is clear that, like other aerobes, *S. cerevisiae* has a number of antioxidant defenses, such as: (i) a cytosolic copper-zinc superoxide dismutase (CuZn-SOD; Sod1) encoded by the *SOD1* gene; (ii) a mitochondrial manganese superoxide dismutase (Mn-SOD; Sod2) encoded by the *SOD2* gene; (iii) a cytosolic catalase encoded by the *CTT1* gene; and (iv) a peroxisomal catalase encoded by the *CTA1* gene. However, *S. cerevisiae* can perform both aerobic and anaerobic metabolism depending on the nature of the carbon source. Under aerobic conditions, *S. cerevisiae* produces energy mainly within its mitochondria using a process similar to that of human cells. According to Costa et al. [40], when wildtype *S. cerevisiae* is exposed to ethanol-generating superoxide (O_2•-) and H_2O_2 during both diauxic and post-diauxic growth and the mitochondrial Sod mutant (Sod2Δ) produces essential protection against oxidative stress. A similar study was suggested while working on L-ascorbic acid by Saffi et al. [41]. In this study, both formulations of PYT protected the cytosolic Sod1Δ, mitochondrial Sod2Δ, and liver Cat1Δ mutants, which may confirm the potential protection against oxidative damage by PYT.

Previous *in vivo* studies showed that 6, 12, 24, and 48 µM PYT had antioxidant activity in mice. It was also postulated to have significant •OH, O_2•, methoxy radical (root CH_2O•-), carbon-dioxide anion radical (CO_2•), NO•, and DPPH• scavenging activities. There is also *ex vivo* evidence for the antioxidant effects of PYT at doses of 2, 6, 12, and 18 µM in *Swiss* mice [3].

There are compounds [42] (Nunes et al., 2014), including diterpene and carnosic acid ($C_20H_{28}O_4$), that have antioxidant activity due to the presence of active -OH groups [43]. There are a number of commercially used natural antioxidants, including some diterpenes- kahweol, cafestol [44], carnosic acid, and carnosol [19], and oxidation products of carnosic acid (rosmanol and isorosmanol) [45]. On the other hand, there are also numerous natural antioxidants with reactive hydroxyl (–OH) groups, including carvacrol, thymol, euginol, isoeuginol, gereniol, caffeic acid, p-cumaric acid, terpein-4-ol, alpha-terpenol, limonen, and p-cymene [46].

PYT is a phenolic EO containing an OH group at its C1 position, which is of interest for its antioxidant activity. In addition, an increased number of OH groups may increase the polarity of diterpenes. Recently, aldehyde, oxime, and ethyl bromocrotonyl derivatives of PYT have been shown to have 2, 4, and 6.5 folds more potent antitubercular activities, respectively [47], thus featuring a better pharmacological profile with synthetic products of PYT.

Plant-based antioxidants (natural) have recently gained popularity due to their role as dietary supplements with minimal side effects. There is no doubt that earlier researches done on PYT are demonstrating its potential antioxidant capacity. Our study is also connecting the earlier reports on this diterpene. Both pure phytol (PFNE) and phytol-loaded nanoemulsions (PNE) in this study exhibited potent antioxidant activities in *in vitro*, *ex vivo*, and *in vivo* assays. In addition, the application of PNE as an innovative nanocarrier with a considerable soluble fraction of the studied terpene in chemical, food, and pharmaceutical industries have been well demonstrated. Nevertheless, earlier information about antioxidant potentials of PYT regarding its antiradical capacity is available for researchers. Results emerging from this study will substantially improve the advances in further non-clinical and clinical settings for a wide range of illnesses related to oxidative stress. Furthermore, PYT in nanopreparation

(PNE) exhibited better antioxidant activities than its free form (PFNE), which may suggest that emulsion preparation is possible by taking into pharmaceuticals consideration.

Abbreviations

AAPH: 2,2'-azobis(2-methylpropionamidine) dihydrochloride; ABTS: azino-bis-ethylbenzthiazoline-sulfonic acid; CAT/Cat: catalase; DPPH: 1,1-diphenyl-2-picryl-hydrazyl; HL: hemolysis inhibition; LP: lipid peroxidation; MDA: malonyl aldehyde; NO: nitric oxide; RNS: reactive nitrogen species; OH: hydroxyl; OS: organoselenium; PBS: phosphate buffer saline; PFNE: phytol free with nano-emulsion; PNE: phytol in nano-emulsion; PYT: phytol; RBC: red blood corpuscle; ROS: reactive oxygen species; RP: reduction potential; SOD/Sod: superoxide dismutase; SOD-WT: superoxide dismutase-wild type; STR: stressor; TBA: thiobarbituric acid; TBARS: thiobarbituric acid; TRO: 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid; YEPD: yeast extract-peptone-dextrose media.

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Ethical statement

This study is conducted under a doctoral project approved by the Ethics Committee of Federal University of Piaui (UFPI), Brazil (#109/14)..

Conflict of interest

We do declare that we have no conflict of interest.

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