Anxiety Therapeutic Interventions of \(\beta\)-Caryophyllene: A Laboratory-Based Study

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Abstract
The bicyclic sesquiterpene \(\beta\)-caryophyllene (BCP) has diverse biological activities, including antioxidant, anti-inflammatory, antidiabetic, and analgesic effects. This study evaluates anxiolytic, toxicity, and antioxidant effects of BCP using in vitro and in vivo test models. The anxiolytic effects were tested in Swiss albino mice (\(\text{Mus musculus}\)) by applying the elevated plus-maze, rota-rod, light and dark, and hiding sphere models, while the toxicity was evaluated by brine shrimp (\(\text{Artemia salina}\)) lethality bioassay. Additionally, the antioxidant capacity was tested by using 2,2-diphenyl-1-picrylhydrazyl radical, 2,2ʹ-azinobis-3-ethylbenzothiazoline-6-sulfonic acid hydroxyl radical scavenging, and the \(\text{Saccharomyces cerevisiae}\) test model. The results suggest that BCP exerted a dose-dependent anxiolytic-like effect on the experimental animals. It did not show toxicity in \(\text{A. salina}\) at 24 hours. BCP showed a concentration-dependent free-radical-scavenging capacity, similar to the standard antioxidant Trolox. It also showed protective and repair capacities against hydrogen peroxide-induced damaging effects in isogenic and wild-type \(\text{S. cerevisiae}\) strains. Taken together, BCP exerted antioxidant and protective effects, which can be targeted to treat neurological diseases and disorders such as anxiety.

Keywords
\(\beta\)-caryophyllene, neuropharmacological effect, anxiety, antioxidant

Received: July 24th, 2020; Accepted: September 7th, 2020.
assays report that BCP has an antioxidant capacity as it can act against oxidative damage caused by reactive oxygen species (ROS).\textsuperscript{15,19} The increase in ROS levels that induces oxidative stress results in the development of several diseases, mainly in the central nervous system (CNS), such as epilepsy,\textsuperscript{20} depression,\textsuperscript{21} Parkinson’s disease,\textsuperscript{22} anxiety, and Alzheimer’s disease.\textsuperscript{23,24}

ROS are not only produced as sub-products of the mitochondrial respiratory chain but also can be produced from exogenous sources such as smoke, radiation, and pollution.\textsuperscript{25} ROS overproduction can be highly toxic, causing damage to cellular macromolecules, including deoxyribonucleic acid (DNA), proteins, and lipids.\textsuperscript{26} In this study, we have evaluated the anxiolytic effect of BCP along with its toxicity and antioxidant capacity by applying a number of in vitro and in vivo test models.

Results

Behavioral and Toxic Effects

In the groups treated with BCP (intraperitoneal [i.p.]), there was no change in motor coordination under the parameters evaluated, including the time spent in the rotating bar when compared with the negative control (NC) group at the doses tested, 10 (167.2 ± 6.25), 25 (173.3 ± 4.77), and 50 (172.3 ± 4.85) mg/kg (Figure 1(A)). However, the animals treated with diazepam (DZP) (110 ± 2.14) spent 38.3% less time on the rotating bar when compared with the NC group (178.3 ± 1.16) (P < 0.05), thus demonstrating its muscle relaxant capacity. Similar results were also observed in relation to the number of falls in the BCP-treated groups in comparison to the NC group. The animals treated with DZP 2 mg/kg (i.p.) significantly (P < 0.05) increased the number of falls (2.50 ± 0.22) when compared with the NC group (1.16 ± 0.16) (Figure 1(B)).

In the Artemia salina test, BCP did not show significant toxic effects even at the highest concentration tested (3 mM). The nauplii exposed to BCP at 0.3, 0.7, 1.5, and 3 mM, showed survival by 100 ± 0.0; 90.00 ± 5.77; 83.33 ± 3.33; 73.30 ± 6.66% in the first 24 hours, and 86.67 ± 3.33; 76.67 ± 3.33; 73.33 ± 2.8; 63.00 ± 3.00% after 48 hours of exposure, respectively. However, at 48 hours, the percentage of survival of the shrimp nauplii was reduced by more than 10% for each concentration (Figure 2).

Anxiolytic Effects

In the Elevated Plus-Maze (EPM) test, the parameters of total distance moved, distance moved in the open arms versus total distance, number of open arms entries, and anxiety index were evaluated to understand the anxiety levels. There were no significant differences in the total distance moved at doses of 10 (3944 ± 348 cm), 25 (4546 ± 266 cm), and 50 mg/kg (4920 ± 353 cm) of BCP as compared with the NC group. The locomotor activity was also evaluated in the EPM test, where the DZP at 2 mg/kg exhibited a reduced distance moved (2113 ± 219 cm) in the apparatus when compared with the NC group (3714 ± 398 cm). DZP group presented an increase (P < 0.05) in the distance covered in the open arms versus total distance (34.43 ± 1.47) and the number of open arm entries (13.86 ± 1) compared with the NC group (9.00 ± 3.32, 3.42 ± 0.89, respectively) (Figure 3(A)). The anxiolytic effects of BCP were also observed in the distance moved in the open arms versus total distance (Figure 3(B)) and number of open arm entries (Figure 3(C)), when compared with the NC group, especially at doses of 25 (24.99 ± 1.55, 15 ± 0.97) and 50 mg/kg (30 ± 1.04, 16.68 ± 0.69), respectively. BCP at 25 (0.57 ± 0.03) and 50 mg/kg (0.49 ± 0.02), similar to DZP 2 mg/kg (0.45 ± 0.06), significantly lowered the anxiety index as compared with the NC group (0.90 ± 0.03) (Figure 3(D)).

In the light-dark test, the number of crosses and the time spent in the light portion of the box were evaluated. In this case, the animals treated with DZP at 2 mg/kg (256.4 ± 12.00) spent much longer time in the light box than the BCP at 10 (161 ± 10.64) and 25 mg/kg (198.4 ± 9.48) doses. However, BCP at 50 mg/kg exhibited better activity than the DZP group as it increased the light box spent time by 6.90% (274.1 ± 8.04) as compared with the DZP group (Figure 3(E)). When analyzing the number of crosses for the light box, all tested groups, BCP 10 (4.16 ± 0.35), 25 (4 ± 0.42), and 50 mg/kg (6.75 ± 0.36), and DZP (6.37 ± 0.46) showed significantly (P < 0.05) higher number of crosses when compared with the NC group (2.62 ± 0.41) (Figure 3(F)).

In order to confirm the possible anxiolytic effect of BCP, the test of hiding spheres was also performed. There was a reduction of 45.4% of hidden spheres in relation to the NC group (12 ± 0.57) for animals treated with the DZP group (6.55 ± 0.5), while the reduction of hidden spheres was 47.3%, 66.0%, and 79.0% for animals treated with BCP at doses of 10 (6.33 ± 0.42), 25 (4 ± 0.42), and 50 (25.0 ± 0.61) mg/kg,
respectively. It was also observed that BCP at 25 and 50 mg/kg showed a reduction of 38.9% and 61% compared with the DZP group, respectively (Figure 3(G)).

**Antioxidant Effects (In Vitro)**

Percentage of 2,2-diphenyl-1-picylhydrazyl radical (DPPH•) scavenging capacity of BCP at 0.3, 0.7, 1.5, and 3 mM were 39.87 ± 0.43%, 55.23 ± 0.08%, 61.70 ± 0.11%, and 77.37 ± 0.14% radical reduction, respectively. All concentrations significantly (P < 0.05) reduced the DPPH• in comparison to the NC group. The standard antioxidant trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at the same concentrations scavenged DPPH• by 46.33 ± 0.88%, 59.67 ± 1.45%, 62.97 ± 1.96%, and 79.55 ± 0.33%, respectively. The half-maximal effective concentration (EC₅₀) value of BCP was 0.55 nM with a 95% CI (CI 0.50-0.60 mM) (Figure 4(A)).

2,2ʹ-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical (ABTS⁺•) scavenging capacity of BCP at different concentrations has been shown in Figure 4(B). BCP at 0.3, 0.7, 1.5, and 3 mM scavenged ABTS⁺• by 45.93 ± 1.18%, 60.50 ± 0.68%, 69.20 ± 0.05%, and 85.02 ± 0.16%, respectively. Trolox, at the same concentrations, showed radical scavenging capacity by 50.88 ± 0.58%, 64.04 ± 0.91%, 71.72 ± 0.44%, and 83.53 ± 0.31%, respectively. The EC₅₀ value of BCP was 0.39 nM (CI: 0.33-0.45 mM) at 95% CIs.

In hydroxyl radical (•OH) scavenging assay, the radicals were generated from the Fenton reaction. In this case, an antioxidant is able to inhibit the degradation of 2-deoxyribose by the elimination of •OH in relation to the NC group. BCP at 0.3, 0.7, 1.5, and 3 mM reduced the degradation of 2-deoxyribose by 22.63 ± 0.14%, 38.10 ± 0.11%, 44.77 ± 0.08%, and 60.13 ± 0.43%, respectively, while at the same concentration, the standard Trolox between 20.45 ± 0.33% and 53.67 ± 0.88%. The EC₅₀ value of BCP was 0.67 mM (CI 0.59-0.76 mM) at 95% CIs (Figure 4(C)).

**Oxidant/Antioxidant Effect on Saccharomyces cerevisiae (In Vivo)**

BCP exerted preventive effects on hydrogen peroxide (H₂O₂)-induced oxidative damage in proficient and mutated S. cerevisiae strains during pretreatment (Figure 5). It was seen that BCP at the lowest concentration inhibited zones between 7.25 ± 0.47 and 22.25 ± 1.03 mm in all strains, whereas the positive control group by 30.75 ± 2.52 and 36.25 ± 1.37 mm, demonstrating BCP-mediated possible modulatory effects on this test system. Similar response at the highest concentration (3 mM) was observed, even though larger inhibition zones were observed, between 12.5 ± 0.64 and 28.50 ± 0.64 mm. A reduction in H₂O₂-promoted oxidation was observed, indicating that BCP pretreatment presented possible antioxidant activity.

In post-treatment, BCP demonstrated effects on DNA damage repair against H₂O₂ oxidative damage, indicated by decreased zones of inhibition. It was observed that the lower BCP concentration had zones of inhibition between 10.75 ± 0.85 and 29.35 ± 0.47 mm in the evaluated strains, whereas BCP at higher concentrations presented values between 26.44 ± 1.54 and 40.25 ± 0.62 mm, suggesting a repairing capacity of BCP. Similar results were also found for the highest concentration (3 mM), with inhibition zones between 11.50 ± 0.62 and 29.25 ± 0.47 mm (Figure 6).

BCP co-treated with H₂O₂ significantly (P < 0.05) modulated the oxidative damage in the test strains. The lowest BCP concentration (0.1 M) indicated the best antioxidant effect in all S. cerevisiae strains, suggesting the ability to reverse H₂O₂-induced damaging effects in this test system (Figure 7).

**Pearson’s Statistical Correlations Between Anxiolytic and Antioxidant Tests**

The anxiolytic effects of BCP were statistically correlated with the antioxidant activities observed in vitro and in vivo antioxidant assays. Positive statistical correlations were found between anxiety index and ABTS⁺•, DPPH•, and •OH tests. A positive correlation was found for the cytoplasmic superoxide dismutase (Sod1Δ) mutant strain, mitochondrial superoxide dismutase (Sod 2Δ) mutant, as well as for the double mutant Sod1Sod2. The hiding sphere test was positively correlated with antioxidant activity in vitro and in vivo (S. cerevisiae), as well as between the time of distance moved with antioxidant activity of BCP in SOWT and Sod1. However, in vitro tests, correlations of radical capture and antioxidant effects of ABTS⁺•, DPPH•, and •OH radical capture were negative (Table 1).

**Discussion**

Studies on compounds of plant origin have shown several therapeutic properties and low toxicity. Anxiolytic activities have been reported for BCP in animal models and in the toxicological/behavioral evaluation of this study. BCP did not induce behavioral changes (locomotor) but presented anxiolytic effects at doses of 10, 25, and 50 mg/kg, as observed in the rota-rod, EPM, light-dark, and hiding sphere tests. These tests are widely used in the screening of substances that may have an influence on the CNS. The importance of anxiolytic activity in the doses tested is noteworthy since there are reports that BCP has anxiolytic activity at higher doses (100, 150, and 200 mg/kg) BCP anxiolytic effects were also observed in the 2 highest doses tested (25 and 50 mg/kg), due to increased number of open arm entries in relation to the positive control. Similarly, diazepam also increased the number of entries in the open arms, which demonstrates that BCP has anxiolytic effects analogous to benzodiazepine compounds.

The frequency of mice in the open arms is the highest anxiety index in the EPM test since the open area is extremely aversive to rodents. The anxiety is created by the conflict between the desire to explore and the preference for remaining in the enclosed spaces. As observed in the study, the anxiolytic effects of BCP were also observed in the 2 higher doses...
tested by increased residence time in the light compartment. However, only at the dose of 75 mg/kg were these effects higher than diazepam.

BCP anxiolytic effects, at all doses tested, were evidenced by reducing the number of hidden spheres in a dose-dependent manner, corroborating with the anxiolytic behaviors observed in other tests. The hiding spheres test was also used to confirm the anxiolytic effect observed in the EPM and the light-dark test, since this model is used to infer compulsive and anxious behavior, being widely used as a model of obsessive-compulsive disorder.

Toxicity analysis is of great importance for the selectivity of pharmaceutical activities. As discussed, BCP has anxiolytic effects in several behavioral tests since it did not present toxicological effects due to behavioral changes. In the *A. salina* test, BCP presented toxic effects only at high concentrations (3 mM), corroborating with the previous study, which did not find BCP toxicity extracted from the essential oil of *Croton argyrophylloides*, which contains 10% of BCP in its composition.

The BCP has several pharmacological activities including anti-inflammatory and antianxiety, but its neuroprotective mechanisms of action are not conclusive. Studies have shown that at the dose of 50 mg/kg BCP has shown the endocannabinoid receptor (CB2R) agonist activity. Cell death by necrosis is a marker of ischemia and inflammation and BCP at 0.2, 1, 5, and 2.5 µM, by mechanisms still unknown, is neuroprotective due to its anti-inflammatory activities. Neuroprotective effects were also observed in mice at doses of 8, 24, and 72 mg/kg by mechanisms associated with mixed lineage kinase domain-like protein expression, interaction with protein kinases 1 and kinases 3, and by decreasing levels of toll-like receptor 4, interleukin-1beta, and tumor necrosis factor-alpha.

There are reports that anxiety is associated with oxidative stress, due to the high metabolic demand in the brain related to the amount of oxygen required and reactive species formation. Thus, antioxidant compounds may contribute to the reduction of oxidative stress that is involved in the pathogenesis of various diseases such as epilepsy, Alzheimer’s, depression, and anxiety. Oxidative stress affects cellular functions essential for brain functioning, causing mitochondrial dysfunction, DNA damage, modification of neuronal signaling, and inhibition of neurogenesis.

Regarding BCP antioxidant effects, in vitro tests indicate that BCP exhibits antioxidant effects similar to Trolox. Antioxidant effects were attributed to BCP by free radical capture as well as interference in GSH expression. These effects...
Figure 3. Anxiolytic-like effect of BCP (BCF) on mice. (A-D) Elevated-plus maze test; (E and F) light-dark test; and (G) hidden glass spheres. Values are mean ± SEM (n = 6); *P < 0.05 when compared with the vehicle group; analysis of variance followed by the Neuman-Keuls post hoc test. BCP, BCF, β-caryophyllene; DZP, diazepam.
have also been reported for BCP as the major component of the Marrubium parviflorum subsp. oligodon at concentrations of 0.4, 1.0, and 2.0 mg/mL.55 As shown, the anxiolytic effects were positively correlated (Pearson’s statistical correlation) with the antioxidant test data.

Plant secondary metabolites, as well as carotenoids and ascorbic acid, have been shown to exhibit antioxidant activities.56 Studies on protective effects, antioxidant and DNA repair of oxidative damage induced in S. cerevisiae strains indicated that BCP, in concentrations of 0.3; 0.7; 1.5 and 3 mM, showed inhibitory effects of H$_2$O$_2$-induced oxidative damage on proficient and mutated yeast strains. There are reports that BCP at doses of 25 and 50 mg/kg/day inhibits H$_2$O$_2$-induced damage to mouse cells, decreasing the cytokine levels, and, thus, decreasing the oxidative process in cells in the CNS.57 In addition, BCP exhibits anti-inflammatory, as well as protective effects against chemical-induced damage, protecting cellular brain damage.58,59

Natural substances with antioxidant and anxiolytic activity can be an alternative for the treatment of anxiety disorders.60 Through our results, it was possible to verify that BCP leads to a reduction of oxidative stress in vitro, avoiding the production of free radicals that damage all cellular components, including proteins, lipids, and the DNA that is related to the development of several diseases, such as atherosclerosis, Parkinson’s disease, Alzheimer’s disease, and anxiety.61

BCP has antioxidant and antianxiety effects via activation of nuclear factor erythroid 2-related factor 2, which in turn is dependent on the activation of endocannabinoid receptors.62 It reduces the activity of inducible nitric oxide synthase (iNOS), restoring antioxidant enzymes and inhibits lipid peroxidation and glutathione depletion in Wistar rats63 and the production of nitric oxide in BV2 microglial cells.64 Excessive free radical production is known to induce neuronal toxicity in cortical and striatum neurons.65 Moreover, iNOS increases the effect of cyclooxygenase, leading to the formation of arachidonic acid and prostaglandins, which in turn can trigger inflammation66 and anxiety.67

Thus, the function of antioxidants is to neutralize excessive free radical formation, protecting cells against toxic effects, and consequently, preventing pathologies, especially those related to the CNS.68,69 The study indicates BCP to be a promising compound for anxiety therapy, as observed in the animal model, as well as for its correlations with antioxidant activities as observed in S. cerevisiae and in vitro antioxidant tests.
Conclusions

BCP showed an anxiolytic-like effect in mice in a dose-dependent manner. No behavioral changes were observed in mice. BCP did not show toxic effects on *A. salina*. BCP showed antioxidant effects in a concentration-dependent way in vitro studies. The free radical-scavenging capacity of BCP may prevent oxidative damage in *S. cerevisiae*. BCP-mediated anxiolytic-like effects were statistically correlated with its antioxidant effects, suggesting that this compound may act by minimizing the oxidative stress involved in anxiety. However, although BCP is a promising compound for anxiety therapy, additional studies are needed before the use of BCP in pharmaceutical formulations can be recommended.

Materials and Methods

Reagents

The isolated BCP compound (C15H24), purity ≥80%, was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Figure 1). For the in vitro antioxidant tests, Trolox, thiobarbituric acid (TBA), and hydrogen peroxide were used.
trichloroacetic acid (TCA), sodium nitroprusside, 2,2′-azobis-2-amidinopropane dihydrochloride, and 2-deoxyribose were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). In the S. cerevisiae assay, YEL (yeast extract 1%, bactopeptone 2%, dextrose 2%) and YED (yeast extract 1%, bactopeptone 2%, dextrose 2%, agar 2%) were purchased from Microbac (São Paulo, Brazil) for cultivation and sowing, respectively. H$_2$O$_2$ was purchased from Dynamics Química Contemporânea LTDA (São Paulo, Brazil).

**Experimental animals**

Swiss mice (*Mus musculus*), male, weighing between 25 and 30 g, aged 6-8 weeks, were obtained from the animal house of the Federal University of Piauí. The animals received water and food (Purina) ad libitum and were kept under controlled lighting (12 hours light/dark cycle) and temperature (26 ± 1°C). Behavioral experiments were performed between 8:00 am to 12:00 am. The animals were monitored for more than 1 hour after behavioral...
tests. The experimental protocols and procedures were approved by the Committee of Ethics in Animal Experimentation of the Federal University of Piauí (No. 008/2015).

**Treatments**

BCP was emulsified with 0.05% Tween 80 (Sigma-USA) and dissolved in 0.9% saline. The animals were treated with the BCP at doses of 10, 25, and 50 mg/kg, intraperitoneally, 30 minutes before the experiments, with 6 animals per group. The NC group received vehicle (0.05% Tween 80 with 0.9% saline) at a constant volume of 10 mL/kg (i.p.). DZP, 2 mg/kg (Sigma Chem Co., St. Louis, MO, USA), used as a positive control, was administered i.p. after being dissolved in 0.9% saline solution.

**Behavioral and Toxicity Studies**

**Rota-Rod Test.** The rota-rod test measures the effect of muscle relaxation and motor coordination after drug administration on animals. For this test, 8 animals per group were treated according to the experimental protocols.
to the experimental protocols. Thirty minutes after treatment, the animals were placed with all 4 legs on a 2.5-cm diameter bar raised 25 cm from the floor in a rotation of 12 rpm for a period of up to 3 minutes to check the residence time in the apparatus and the number of falls.

Artemia salina Test. For general toxicity evaluation, the lethality bioassay with A. salina was used. For that, cysts were incubated in artificial saline water (39.35 mM NaCl, 18.66 mM MgCl2·6 H2O, 28.16 mM Na2SO4, 8.84 mM CaCl2·2H2O, and 9.38 mM of KCl in 1000 mL of water) at 25-30 °C, with pH adjusted (9.0) using sodium bicarbonate. After 48 hours of incubation, 10 live and 10 dead cysts were counted macroscopically. The lethality percentage was determined valid when the number of deaths per concentration. Saltwater was maintained under the same incubation condition for an additional 24-48 hours. Live nauplii were transferred to test tubes containing BCP at the concentrations of 0.3, 0.7, 1.5, and 3 mM.

The final volume of each sample was adjusted to 5 mL with artificial saline and chlorine-free water (1:1, v/v). Samples were maintained under the same incubation condition for an additional 24-48 hours. Live nauplii were counted macroscopically to determine the number of deaths per concentration. Saltwater and potassium dichromate (5 µg/mL) were used as NC and positive control, respectively. The treatments were performed in triplicate, and the results were expressed as a percentage of mortality. We have also determined the median lethal concentration (LC50) values of the BCP at 24 and 48 hours of exposure time.

### Evaluation of Anxiolytic Effect

**EPM test.** The EPM for mice consists of 2 opposing open arms (50 × 10 cm) and 2 closed (50 × 10 × 50 cm), likewise opposing cross-shaped arms. The open and closed arms are connected by a central platform (10 × 10 cm) and 50 cm from the floor. The animals were placed in the center of the apparatus with the head turned toward one of the closed arms, and their behavior was observed for 5 minutes. The behavioral measures were recorded: total distance moved, distance moved in the open arms versus total distance moved, number of entries in the open arms, and length of stay in the open arms.

**Light-Dark Test.** The apparatus used is made of acrylic divided into 2 compartments (light and dark) that communicate through a small door. The dark field (black acrylic, 27 × 18 × 29 cm) is weakly lit. The light box (acrylic, 27 × 18 × 29 cm) is illuminated by ambient light. Thirty minutes after the administration of treatments, the activities of each animal were recorded for 5 minutes. The parameter used was the time spent in the light box in seconds. A compartment entry was considered valid when the animal’s 4 paws were inside that chamber. After each test, the equipment was washed with soap and water, cleaned with ethanol 70%, and dried.

### Table 2. Saccharomyces cerevisiae Strains and Genotypes.

| Strains | Genotype | Origin |
|---------|----------|--------|
| EG103 (Sodwt) | MATa leu2-3,112 trp1-289 ura3-52 GAL+ | Edith Gralla, L Angeles |
| EG118 (Sod1Δ) | sod1: URA3 all other markers as EG103 | |
| EG110 (Sod2Δ) | sod2: TRP1 all other markers as EG103 | |
| EG133 (Sod1ΔSod2Δ) | sod1: URA3 sod2::TRP1 double mutant all other markers as EG103 | |
| EG223 (Cir1Δ) | EG103, except car1: TRP1 | |
| EG (Sod1ΔCir1Δ) | EG103, except sod1: URA3 and cir1:: TRP1 | |

Adapted from Oliveira et al.35
Table 3. *Saccharomyces cerevisiae* Experimental Procedure.

| Treatments       | Procedure                                                                 |
|------------------|---------------------------------------------------------------------------|
| Pretreatment     | Addition of 10 µL of BCP in different concentrations, on the central paper disk in Petri dishes. After 2 hours, 10 µL of H₂O₂ were added and incubated at 30 °C for 48 hours. |
| Co-treatment     | Addition of 10 µL H₂O₂ + 10 µL of the BCP in different concentrations, on the central paper disk in Petri dishes and incubation at 30 °C for 48 hours. |
| Post-treatment   | Addition of 10 µL H₂O₂ on the central paper disk in Petri dishes. After 2 hours, 10 µL of BCP was added at different concentrations and incubation at 30 °C for 48 hours. |
| Positive control | Addition of 10 µL of H₂O₂ on the central paper disk in Petri dishes and incubation at 30 °C for 48 hours. |
| Negative control | Addition of 10 µL of saline solution (0.9%) as a negative control in order to evaluate spontaneous oxidative damage to yeast strains and incubation at 30 °C for 48 hours. |

Abbreviations: BCP, β-caryophyllene; H₂O₂, hydrogen peroxide.

**Hiding Spheres Test.** The procedure of hiding spheres has been proposed since rodents exhibit the behavior of hiding (burying) aversive objects, including sources of shock, harmful foods, or inanimate objects such as glass spheres. Administration of substances with a possible anxiolytic effect in rodents tends to reduce the number of hidden spheres (NHSs) in this test. The groups were divided into the vehicle (0.05% Tween 80 dissolved in 0.9% saline, i.p.), DZP (2 mg/kg, i.p.), and the other groups were treated with different doses of BCP (10, 25, and 50 mg/kg, i.p.). After 30 minutes of treatment, the animals were individually placed in polycarbonate boxes (27 × 16 × 13 cm), which were lined with a 5-cm layer of shavings, which were uniformly distributed 25 glass spheres with 1.5 cm in diameter. The NHSs were recorded. The spheres that were completely covered by the shavings were considered hidden. After each individual test, the beads were cleaned with 70% alcohol, and the shavings were removed to leave no traces of the previous animal.

**In Vitro Antioxidant Tests**

**DPPH radical scavenging assay.** This method is based on the elimination of the stable free radical DPPH•. The evaluation of the antioxidant capacity against the radical DPPH• was performed according to Silva and Eleutherio. For that, the association of BCP at various concentrations (0.3, 0.7, 1.5, and 3.0 mM) with the DPPH radical was vigorously agitated and incubated at room temperature in the absence of light for 30 minutes. The antioxidant evaluation was performed in triplicate and the absorbance values (517 nm) were expressed as percent inhibition of the DPPH radical in relation to the System (100% DPPH• radical). The standard antioxidant Trolox was tested at 0.1-3 mM, and the EC₅₀ values for BCP and Trolox were also determined.

**ABTS⁺ Radical-Scavenging Assay.** To evaluate the antioxidant capacity with the ABTS⁺• radical, the radical cation ABTS⁺• was initially formed according to the procedure described by Re et al. The initial solution was then diluted in ethanol to reach a solution with the absorbance of 1.00 (±0.05) at 734 nm. In the dark environment and at room temperature, a reaction mixture of BCP (0.1-3 mM) was made with the ABTS⁺• solution. The experiment was performed in triplicate, and the absorbance readings were performed after 6 minutes in a spectrophotometer (734 nm). The results were expressed as percent inhibition of the ABTS⁺• radical relative to the System (100% ABTS⁺• radical). Trolox was tested at the same concentration as described in the DPPH• assay, and the EC₅₀ values for BCP and Trolox were also determined.

**Hydroxyl Radical Scavenging Assay.** To evaluate the antioxidant capacity of the hydroxyl radical generated by the Fenton system, aliquots of BCP (0.1-3 mM) were added to the reaction medium containing 2-deoxyribose, 3.2 mM ferric chloride (50 mM), H₂O₂ (100 mM), and phosphate buffer (20 mM, pH 7.4). The reaction mixture was incubated at 50 °C for 40 minutes. After this procedure, 10% TCA and 1% TAB (50 mM sodium hydroxide) were added. The mixture was then heated for 15 minutes at 95 °C, cooled, and the absorbances were measured at 532 nm. The results were expressed as percent inhibition of 2-deoxyribose degradation relative to the System (100% hydroxyl radical). Trolox was tested at the same concentration as described above, and the EC₅₀ values for BCP and Trolox were also determined.

**Oxidant/ Antioxidant Assay**

*S. cerevisiae test.* *Saccharomyces cerevisiae* is 1 of the major eukaryotic model organisms used in emerging studies to understand the cellular response to oxidative stress and antioxidant defense systems in natural products. Six yeast strains of *S. cerevisiae* species proficient and mutated in their antioxidant defenses were used for this study. The EG118 line is defective in the enzymatic system involving the enzyme cytoplasmic superoxide dismutase (CuZnSOD-Sod1Δ gene product), EG110 have a mutation in the mitochondrial Sod (MnSOD- Sod2Δ gene product), EG133 is the double mutant defective in the enzymatic system involving the enzyme cytoplastic superoxide dismutase (CuZnSOD- Sod1Δ gene product), EG103 corresponds to the wild strain (Table 2).

The strains were cultured in YEL medium (0.5% yeast extract, 2% bacto-peptone, 2% glucose) at 28 °C on an orbital shaker until they reached a stationary growth stage. Suspension cells were seeded from the center to the margin of Petri dishes in a continuous movement on both sides of the dish containing a sterile filter paper disc in the center of the plate, in which 10 µL of the BCP concentrations (0.3, 0.7, 1.5, and 3 mM) was added. H₂O₂ was used as a positive control (10 mM) and the saline solution (0.9%) as a negative control. After 48 hours of incubation at 30 °C,
inhibition halos (in mm) were measured from the filter paper disc margin to the yeast growth (Table 3).

The percentage values of BCP-modulation on oxidative damage caused by H_{2}O_{2} were calculated based on the absence of yeast growth from the central paper disc to the margin of the Petri dish (40 mm). After obtaining the oxidation values, the percentage of modulation was calculated using the following formula: \% M = [PC - (|BCP + PC|)/PC] \times 100, where \% M represents the percentage of BCP modulation; PC is the percentage of oxidative damage induced by H_{2}O_{2}; BCP + PC is the percentage of oxidative damage induced by BCP associated with H_{2}O_{2}.

\textbf{Statistical Analysis}

The results were presented as mean ± SD. The data were analyzed using ANOVA, followed by Tukey’s and Dunnett’s Bonferroni tests using GraphPad Prism (version 6.0), considering \( P < 0.05 \) with a confidence level of 95\%. Pearson’s statistical correlations were made to evaluate associations of the anxiolytic effects of BCP with antioxidant activities.

\textbf{Statement of Human and Animal Rights}

All of the experimental procedures involving animals were conducted in accordance with the \textit{Guide for the Care and Use of Laboratory Animals}, Eighth Edition, which was approved by the Committee of Ethics in Animal Experimentation of the Federal University of Piauí (nº 008/2015).

\textbf{Declaration of Conflicting Interests}

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

\textbf{Funding}

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: We are grateful to the National Council for Scientific and Technological Development (CNPq) and UFPI for funding and hosting of this project, respectively.

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