Negative terpinen-4-ol modulate potentially malignant and malignant lingual lesions induced by 4-nitroquinoline-1-oxide in rat model

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Abstract
Our aim was to verify the modulative TP-4-ol capacity in 4-nitroquinoline-1-oxide induced oral rat cancer. The stereoisomers of TP-4-ol were used against the human tongue squamous cell line and the negative stereoisomer showed lower IC50. Thirty-one Holtzman rats (120–130 g) were cancer-induced by 4-nitroquinoline-1-oxide (4-NQO/8 weeks/25 ppm) and 32 Holtzman rats (120–130 g) were used to healthy and TP-4-ol toxicity experiments. Six groups were used, healthy, 0.1nL/g of TP-4-ol, 8nL/g of TP-4-ol, 4-NQO, 4-NQO + 0.1nL/g of TP-4-ol, and 4-NQO + 8nL/g of TP-4-ol. We performed the toxicity analysis by biochemical and histopathological analysis. The biochemistry analysis includes alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST), urea, and creatinine and the histopathology analysis includes the liver, kidney, lung, and spleen. Specifically, for malign modulation, we performed a macroscopic and microscopic analysis. The group exposed to 0.1nL/g of TP-4-ol demonstrated a reduced risk of malignancy in dysplasia considering the criteria of architecture and cytology. Similarly, a drop of percentual rats with SCC diagnosis was observed in 4-NQO + 0.1nL/g (41.6%) when compared to 4-NQO (87.5%). Moreover, the 4-NQO group presented a median of 2.62 SCC/rat and the 4-NQO + 0.1nL/g demonstrated a median of 0.75 SCC/rat. For toxicity analysis, 4-NQO + 0.1nL/g showed focal necrosis in the kidney and 4-NQO showed lung hemorrhagic areas. The concentration of 0.1nL/g was more effective in reducing the tongue induction of potentially malignant and malignant lesions by 4-NQO. A kidney toxicity was observed in healthy animals exposed to 0.1nL/g of TP-4-ol. The negative isoform of terpinen-4-ol negatively modulates the development of potentially malignant and malignant lesions in rats (Rattus nonverdixctis albinos, Holtzman) exposed to 4-NQO. (-)-Terpinen-4-ol reduced the mice percentual with squamous cell carcinoma, 87.5 to 41.6%, and decreased the cancer/rat ratio of 2.62 in 4-NQO to 0.75 in 4-NQO + 0.1nL/g. This represents 52.4% by group and 71.3% in the cancer/rat ratio.

Keywords Oral cancer modulation · 4-Nitroquinoline-1-oxide · Terpinen-4-ol · Toxicity

Introduction

Oral cavity cancers (Global Burden of Disease Cancer et al. 2017) are worldwide human cancers associated to high morbidity and mortality (Global Burden of Disease Cancer et al. 2017) and 90% of them are squamous cell carcinomas (SCC) (Pai and Westra 2009). In the oral cavity, the tongue is the most prevalent site accounting for more than 50.4% of oral cancers and males are more affected than females, respectively, 54.5% and 41.8% (Siegel et al. 2018). Cancer-associated behaviors include alcohol ingesting, tobacco smoking, betel quid chewing, and HPV infection (Thomas et al. 2007; Chaturvedi et al. 2013; Lam et al. 2015).

Despite recent advances in the diagnosis and treatment of oral cancer, oral tongue squamous cell carcinoma presents
low prognosis. The 5-year tongue survival rate ranges from 40 to 58% (Bell et al. 2007; Zini et al. 2010). In fact, the 5-year overall survival rate of surgical-treated patients associated or not to adjuvant radiotherapy ranges from 50 to 56% and it drops to 32% with loco-regional metastasis and to 11% with distant metastasis (Prades et al. 2004).

Chemotherapy protocols for SCC have shown controversial results. Sometimes, patients diagnosed with SCC, treated with chemotherapy protocols, demonstrate an increase in the 5-year survival rate; at other times, an improvement in the 5-year survival rate may not be observed (Subramaniam et al. 2018). Monoterpenes is a class of terpenes with two isoprene units and a molecular C_{10}H_{16} and have recently been applied to the human phase II clinical treatment of cancer (Sobral et al. 2014). Monoterpane terpinen-4-ol (TP-4-ol) can be found in tea tree oil (TTO) of Melaleuca alternifolia (de Groot and Schmidt, 2016), orange trees, tangerines, lemons, cedar, and pepper (Pino et al. 2003). TTO was associated with reduced apoptosis induction and cell proliferation inhibition which led to a decrease in 70% of volume and weight of lung and colorectal human cancer xenograft mice tumors (Wu et al. 2012; Shapira et al. 2016). TP-4-ol presents two stereoisomers (mirror images). Enantiomer, also called optical isomer, is one of these stereoisomers (2008). TP-4-ol stereoisomers are the (−)-terpinen-4-ol [(R)-p-Menth-1-en-4-ol, (R)-1-Isopropyl-4-methyl-3-cyclohexen-1-ol] and (+)-terpinen-4-ol [(S)-p-menth-1-en-4-ol, (S)-1-isopropyl-4-methyl-3-cyclohexen-1-ol]. 4-Nitroquinoline oxide (4-NQO) induces oral cancer by genetic oxidation, and it is considered analogous to human oral carcinogenesis (histopathological, molecular, and genetic). This model allows chemotherapy studies in all phases of oral carcinogenesis: initiation, promotion, or progression (Nauta et al. 1995; Herzig and Christofori, 2002; Vered et al. 2005), and it allows biometric (Shiotani et al. 2001; Aloia et al. 2010), biochemical (Viswanadha et al. 2011), macroscopic (Dayan et al. 1997), and histothological (Gautam and Goel, 2014) studies that admit an body/drug interaction analysis (Mohan et al. 2016).

The aim of our study was to evaluate the modulative potential effect of TP-4-ol in 4-NQO-induced carcinogenesis mice (Sagheer et al. 2021). Oral squamous cell carcinoma cell culture experiments were carried out to verify which stereoisomer would be more effective in further in vivo research. The negative and positive stereoisomers of TP-4-ol were analyzed and we concluded that the negative form was more effective. We used concentrations of 8nL/g and 0.1 nL/g of TP-4-ol (−) to verify its modulating capacity in the tongue induction process of potentially malignant and malignant lesions in rats exposed to 4-NQO (25 ppm) for 8 weeks. Furthermore, we evaluated the general toxicity in healthy and 4-NQO cancer-induced rats (liver, kidney, lung, and spleen).

### Materials and methods

#### Cell cultures

Human tongue squamous cell line (SCC-9) and immortalized keratinocyte cell line (HaCat) were cultured according to the recommendations of the American Type Culture Collection in Dulbecco’s Modified Eagle’s Medium (Sigma Aldrich, St. Louis, MO, EUA) supplemented with 10% (v/v) inactivated bovine serum (Gibco, Life Technologies) and 400 ng/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO, EUA). The cell lineages were donated by Ricardo Della Coleta (Faculdade de Odontologia de Piracicaba, Universidade Estadual Paulista). The cells were routinely cultured in 75-cm² flasks with 15 mL of culture medium (DMEM or DMEM + hydrocortizone and 10% FBS), with 1% (v/v) antibiotics solution (P0781, Sigma-Aldrich), and maintained at 37 °C in a humidified atmosphere 5% of CO₂.

#### Reagent preparation and treatment

(−)-Terpinen-4-ol (Sigma Aldrich, St. Louis, MO, EUA) and (+)-terpinen-4-ol (Sigma Aldrich, St. Louis, MO, EUA) were diluted in complete medium.

#### Dose response curve for cell viability

Cell viability was evaluated by measuring 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide inner salt (MTT, Sigma-Aldrich). To prepare the MTT solution, 25 mg of the methyl tetrazolium salt was added to 5 mL of PBS, reaching a final concentration of 5 mg/mL. A total of 5 × 10³ cells/well were seeded in 96-well plates. SCC-9 and HaCat cells were cultured under adherent conditions for periods of 24 h and treated with TP-4-ol for 2 h. The treatment was carried out in decreasing concentrations of positive and negative terpinen-4-ol. The absorbance was measured at 540 nm in a microplate reader. The drug concentration that reduces 50% of cell viability (IC₅₀) value was calculated (HaCaT SCC-9) in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

The in vitro stereoisomers analysis (TP-4-ol/negative and positive) demonstrated that the IC50 of negative TP-4-ol stereoisomers in SCC-9 (Fig. 1A and C) and HaCAT lineages were lower than positive (Fig. 1B and D). The negative TP-4-ol stereoisomer was chosen for the in vivo mice carcinogenesis 4-NQO induction.
Ethical approval

This project was approved by the Animal Use Ethics Commission (CEUA) at the Araraquara School of Dentistry with protocol n° 8/2015 according to the rules established by the National Council for Animal Experimentation Control (CONCEA) and Normative Instruction No. 04 of June 18, 2014, of the Brazilian National Health Surveillance Agency (ANVISA) on non-clinical studies of toxicology and pharmacological safety necessary for the development and registration of herbal medicines. The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) were used to write this article.

In vivo study

Sixty-four male rats (*Rattus norvegicus albinos*, Holtzman, 4 weeks, 120–130 g) were initiated and maintained to acclimatization for 1 week. The laboratory was housed in a controlled climate environment (24 °C, with light/dark [12/12 h]).

Lingual carcinogenesis 4-NQO induction

Tongue carcinogenesis was carried out by ad libitum water drink (25 ppm of 4-NQO, Sigma-Aldrich®, St. Louis, MO, USA) for 8 weeks. The terpine-4-ol studies started in the 9th week and all animals were sacrificed after 16 weeks when the biochemical and histopathological analyses were performed.

Terpinen-4-ol treatment

The TP-4-ol (25 g, molecular weight 154.25 g/mol, 95% purity, Sigma-Aldrich®, St. Louis, MO, USA) was maintained under the manufactured recommended conditions. Both concentrations used in this research were calculated based on the study conducted by Wu et al. (2012). They described the use of TP-4-ol in cancer research and its role in angiogenesis inhibition.

**Fig. 1** Viability of Human tongue squamous cell line (SCC-9) and immortalized keratinocyte cell line (HaCat) exposed to successive fractions of positive and negative terpinen-4-ol stereoisomers. 

A- (+)-Terpinen-4-ol fractions on SCC-9 lineage; B- (-)-Terpinen-4-ol fractions on SCC-9 – lineage; C- (+)-Terpinen-4-ol fractions on HaCAT – lineage; D- (-)-Terpinen-4-ol fractions on HaCAT lineage.
of 11μL of TP-4-ol diluted in stock solution (5.5 mL [0.2%]). The effective intravenous animal application was 100μL. We established 20% of the intravenous dose proposed by Wu et al. (2012). Our daily oral gavage doses were 8nL/g and 0.1nL/g diluted in purified water. These doses represent, respectively, 0.73% and 0.09% of the TP-4-ol Lethal dose (Yang et al. 2011).

**Experimental design**

Sixteen rats were used, separated into two groups of 8 animals. The healthy group was maintained with food and water ad libitum for 16 weeks (healthy) and the 4-NQO group received 25 ppm of 4-NQO in water drink in the first 8 weeks and then were maintained with food and water ad libitum for 8 weeks (4-NQO). The TP-4-ol experimental groups that were initiated used 48 rats, 24 rats received 25 ppm of 4-NQO in water solution for 8 weeks (4-NQO water and food ad libitum), and then 11 received 8nL/g of TP-4ol (4-NQO + 8nL/g) and 12 received 0.1nL/g of TP-4-ol (4-NQO + 0.1nL/g) in dairy oral gavage. The other 24 animals initially received food and water ad libitum and then 8nL/g of TP-4ol (8nL/g) and 12 received 0.1nL/g of TP-4-ol (0.1nL/g) in dairy oral gavage for another 8 weeks (Fig. 2).

**Biometric analysis**

The animals were weighed weekly, and their water and ration consumption were quantified. The data were analyzed to identify weight, water, and/or ration consumption differences.

**Biochemistry**

After euthanasia, the blood from the rats was collected (3.0 mL) in a sodium heparin glass tube. These tubes were centrifuged for 10 min at 2500 rpm. Blood plasmas were collected for biochemical measurements to be performed in the Virtrus® 250 automatic analyzer. Ortho-clinical kits (J&J Company) were used to diagnose alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST), urea, and creatinine. Analyses were performed by dry chemistry and reflectance spectrophotometric system using slides containing 11μL of plasma.

**Macroscopic analysis**

The tongue, liver, kidney, lung, and spleen were collected and immediately fixed in 10% tamponade formaldehyde. All organs and tissues were codified to blind groups. Macroscopic analyses were carried out to describe any tissue/organ macroscopic lesion. Specifically for the tongue, photographs were taken (Canon® Camera-D700 and 105 mm macro lens [Nikon®]) to assist the macroscopic analysis. In the macroscopic analysis, each organ and tissue was measured (length×height×width = mm³) and weighed. The lesions were described by region and position. They were identified on the corresponding elementary lesions described in clinical observation (white spot, plaque, papule, vesicle, petechia, erosion, ulcer). After this, specifically for the tongue, a serial section measuring 3.0 mm was made from the apex to the base of the tongue. The fragments were identified using a sequence of letters and identifying the right, left, and center positions (Fig. 3).

**Histopathology analysis**

After macroscopic analysis, all samples were included in paraffin and 4 μM sections were obtained and stained in hematoxylin and eosin (H&E) stain. The analyses were performed by an experienced pathologist, previously calibrated. The pathologist was blinded to the analysis groups. Analyses were performed at two different times with a minimum interval of 1 week between them. A light microscopy (Carl Zeiss, Primo Star, Germany) was used at 100, 200 and 400 magnifications. The kidney analysis included a cortical region (Bowman’s capsule integrity, urinary space, glomerulus, proximal, and distal ducts and the presence of pigment) and medullar region (duct and type and intensity of inflammatory infiltrate) (Lim et al. 2010; Viswanadha et al. 2011). The liver analysis included portal space, interlobular, and center lobular analysis (congestion, sinusoids, hepatocytes, lipodiosis, pigmentation, and necrosis) (Lim et al. 2010; Viswanadha et al. 2011). The lung analyses included bronchhi, bronchioles, pulmonary alveoli, and supporting tissue.

![Fig. 2](Image)
(vessels and connective tissue). The inflammatory infiltrate was classified in intensity (mild, moderate, and severe) and type (acute, mixed, and chronic) and peri-ductal inflammatory infiltrate, specifically analyzed. The circulatory changes were hemorrhage, congestion, thrombosis, or embolism. The emphysema, edema, fibrotic processes, bone metaplasia, ductal squamous dysplasia, hyperplasia, and smooth muscle degeneration, and presence of bacterial colonies and neoplastic tissue (adenoma and adenocarcinomas) were identified and quantified (Kuno et al. 2014). The spleen analysis included capsule (atrophy, hypertrophy, loss of continuity, and normality), white and red pulp rate, marginal region, fibro vascular septum, germinal centers (organized and disorganized), and the presence of granulomas (Gannot et al. 2004; Barcessat et al. 2014).

The tongue histopathology analysis included oral cancer invasion scores (in situ SCC, micro-invasive-SCC, invasive SCC and deep invasive SCC, Table 1). The epithelial dysplasia was identified and classified in low, moderate, and severe dysplasia. Moderate dysplasias were analyzed in a binary system to identify the risk of malignancy. The system used cytological and morphological characteristics that allowed the establishment of a low- or high-risk classification (Kujan et al. 2006; Kwak et al. 2007; Peng et al. 2015). The inflammatory infiltrate was classified in type (acute, chronic, and mixed) and intensity (intense, moderate, and mild). This infiltrate was also analyzed near the SCC. The papilloma, epithelial hyperplasia (acanthosis), and epithelial hyperplasia were also described.

### Statistical analyses

**In vitro** Three independent experiments were performed. The mean and standard deviation or standard error. The differences between two groups were analyzed with the Student’s t test (unpaired, two-sided). All statistical tests were performed on GraphPad Prism 8. All data were included in the analysis. All data were included in the analyses.

**In vivo** The means and standard deviation of the results were established in each experimental group. Multiple comparison of K samples was carried out by ANOVA (one/two-way) followed by the post hoc Tukey test (biometric, macroscopic, and microscopic parameters) and Newman-Keuls (biochemical parameters) using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). A statistical significance was established at 5% (CI 95%; \( p < 0.05 \)). The statistical results were represented by \( P < 0.05(*) \), \( P < 0.01(**) \), \( P < 0.001(***) \), and \( P < 0.0001(****) \).

### Results

One 4-NQO-exposed rat died during the initial 8 weeks. The animal would be part of the 4NQO + 8nL/g TP-4-ol group and it was not replaced. Therefore, all analyses were performed with 11 animals in the 4NQO + 8nL/g TP-4-ol group.

| Classification schemas that histologically categorize SCC* invasiveness |
|-------------------|------------------------------------------------------------------|
| In situ SCC*      | Malignant cells restricted to epithelium surface. Absence of connective tissue invasion |
| Micro-invasive SCC*| Malignant cells that infiltrate the lamina propria (break of basement membrane) |
| Invasive SCC*     | Malignant cells that infiltrate connective tissue (> 3 mm) and infiltrate the striated muscle |
| Deep invasive SCC*| Malignant cells that infiltrate the connective tissue and occupy the lamina propria and infiltrate the striated muscle |

*SCC* squamous cell carcinoma
The mean weight, water, and feed intake are shown in Fig. 4. The 4-NQO showed less weight compared to the healthy group (−32.1% ranged −40.6 to −23.7%, \( P < 0.0001 \)). There was no statistical difference between the groups in the analysis of the average weight of the animals. However, after the 14th week of the experiment, the groups treated with TP4ol showed a detachment from the normal weight curve characterized by mean weight gain (Fig. 4A). The healthy group had a higher mean water consumption when compared to the 4NQO, 4NQO + 8nL/g, and 4NQO + 0.1nL/g groups. On the other hand, the 8nL/g concentration showed lower water consumption than the 0.1 nL/g concentration. In fact, the 0.1nL/g showed more water consumed than its cancer induced group, 4-NQO + 0.1nL/g (74.6% ranged 24.6 to 120%, \( P < 0.002 \)), Fig. 4B. No ration consumption differences were observed (Fig. 4C).

**Biochemistry analysis**

All the study groups had high creatinine levels when compared to the healthy group, except for the 4-NQO + 8nL/g group (Fig. 5A). The 4-NQO showed a higher creatinine level than both TP-4-ol treated groups. For the urea assay, the healthy group and 8nL/g demonstrated similar levels and the other groups showed a higher mean level (Fig. 5B). In the biochemical assays for ALP (Fig. 5C) and ALT (Fig. 5D), the healthy, 8nL/g, and 4NQO + 8nL/g groups...
Fig. 5 Results of plasm biochemical assays by drychemistry technology (spectrophotometry systemanalysis): (A), Dosage of Creatinine; (B), Urea; (C),alkaline phosphatase-ALP; (D), alanineaminotransferase-ALT; (E), aspartate transaminase-AST(* P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001)
Macroscopic lesions demonstrated statistical differences. The 4-NQO rats presented, more frequently, macroscopic lesions than 4-NQO + 8nL/g. The healthy group show no significant to both its TP-4-ol treated groups. 4-NQO and 4-NQO + 8nL/g presented more macroscopic lesions than the healthy group, respectively, 3.5 (1.4 to 5.5 [P<0.001]) and 3.0 (1.1 to 4.9 [P<0.01]). The 4-NQO + 0.1nL/g presented similar results in the healthy group (Fig. 7A). The 4NQO group showed more plaque lesions than healthy and 4-NQO + 0.1nL/g (P<0.0001). The 4-NQO + 8nL/g group also showed more plaque lesions than healthy (P<0.0001) (Fig. 7B).

The dorsal tongue of rats presented, more frequently, macroscopic lesions, and the distribution of observed lesions was posterior (79.1%) and middle (14.3%) and anterior (6.6%). Only one macroscopic lesion was observed at the ventral tongue (Fig. 8A). Plaque lesions were most frequently detected in 4-NQO and 4-NQO + 8nL/g than in the healthy group and showed distribution on dorsal, posterior, and middle positions. Papule showed similar results but on left side the 4-NQO + 8nL/g presented more papule than healthy group. Ulcer was most frequently detected in 4-NQO and the 4-NQO + 0.1nL/g.
presented statistical reduction of ulcer detection (Fig. 8B). The Fig. 8C shows photo example of rat tongue macroscopic lesions.

**Tongue (microscopic analysis)**

Our results support the efficacy of 4-NQO to induce potentially malignant and malignant lesions on the mouse tongue. The 4-NQO had the highest histopathological lesions compared to both TP-4-ol treatment tested groups. The 4NQO + 8nL/g group had 3.3 times less histopathological lesions than the 4NQO group and the 4NQO + 0.1 nL/g group had 5.2 times less histopathological lesions. Furthermore, the 4NQO + 0.1nL/g had less histopathological lesions than 4-NQO + 8nL/g (Fig. 9A). The analysis of the architecture and cytology criteria for the risk of malignancy showed a statistically significant reduction in the two 4NQO groups treated with terpinen-4-ol when compared to the positive control (4NQO). Furthermore, the 4NQO + 0.1nL/g presented few malignization risks compared to 4-NQO + 8nL/g ($P < 0.05$) (Fig. 9A and B).

Figure 10 presents the patent mean reduction. The acanthosis diagnosis in the 4-NQO + 8nL/g demonstrated −1.5 times (ranging from −0.74 to −2.3) of 4NQO. The dysplasia diagnosis in the 4-NQO + 8nL/g demonstrated −1.0 times (ranging from −0.2 to −1.8) of 4-NQO, and in the 4-NQO + 0.1nL/g, it was −2.0 times (ranged 1.3 to 2.8). Specifically, the moderate dysplasia presented −1.0 times (ranging from −0.2 to −1.7) in the 4-NQO + 8nL/g and it presented −1.2 times (ranged −0.4 to 2.0) in the 4-NQO + 8nL/g (Fig. 10C).

**Malignization risk**

**Tongue (squamous cell carcinoma)**

All 4-NQO-exposed groups presented histopathological oral cancer invasion scores (OCIS). The 4-NQO, 4-NQO + 8nL/g, and 4-NQO + 0.1nL/g groups presented, respectively, 87.5%, 90.9%, and 41.6% of animals with SCC diagnosis that include in situ, micro-invasive, invasive, and deep invasive SCC. The SCC/rat mean revealed statistical differences among 4-NQO (2.62) and 4-NQO + 0.1nL/g (0.75, $P < 0.0001$). The ratio reduction was calculated in 4.0/rat or 71.42% in the SCC. The mean of invasive SCC 4-NQO + 0.1nL/g was lower than 4-NQO + 8nL/g ($P < 0.05$) (Fig. 11A).

On the other hand, when the region, position location, and subtype of SCC were discriminated, we verified differences in the 4-NQO experimental groups that are shown in Fig. 12.

**Discussion**

The TP-4-ol demonstrated the ability to influence the development of potentially malignant and malignant lesions in rats induced to cancer by 4NQO. Our study demonstrated statistical TP-4-ol modulation of lingual carcinogenesis by a decrease in the epithelial dysplasia concomitant to drop malignization risk and squamous cell carcinoma diagnosis. The oral gavage of 0.1nL/g TP-4-ol decreased the upsurge of potential malignant and malignant lesions. Similar results have been described in other studies (Gannot et al. 2004; Lim et al. 2010; Barcessat et al. 2014; Kuno et al. 2014).

The effects of TP-4-ol and its mechanisms on healthy cells and cancer cells have not yet been clarified. In fact, TP-4-ol has been studied in several studies using cell culture, which demonstrated selective antitumor activities, pro-apoptotic, necrotic cell death, cell arrest, autophagy activities, anti-proliferative, migration, invasion, and interfere the immune response. Banjerdpongchai and Khaw-On studied the effect of TP-4-ol exposition on human leukemic HL-60 cells. TP-4-ol induced ROS generation and autophagic death. Autophagic death was induced by activation of LC3-I/II, Beclin-1, and ATG5 proteins and the increase of Bid expression, cytochrome c release, and caspase-8 activity was related to apoptosis (Banjerdpongchai and Khaw-On, 2013). In fact, TP-4-ol was related to the increase in sensitivity to EGFR antagonists in Ras-mutated tumors (Banjerdpongchai and Khaw-On, 2013; Shapira et al. 2016; Chohan et al. 2018). The extract combination caused necrotic cell death coupled with less activity of apoptosis process in both murine malignant mesothelioma cell line AE17 and murine B16 melanoma cells, and G1 cell cycle arrest (Greay et al. 2010). The TP-4-ol reduced Akt phosphorylation in human meibomian gland epithelial cells (IHMGECs) after 30 min exposition (Chen et al. 2020). The ROCK2 mRNA and protein levels were reduced in PC cells following TP-4-ol treatment in vitro leading to the inhibition of PC cell proliferation and migration (Cao et al. 2022). Additionally, TP-4-ol can interfere with immune response and inhibit the production of inflammatory mediators (IL-1beta, IL-6 and IL-10) (Nogueira et al. 2014) and prevents the production of TNF-α, IL-1β, IL-8, IL-10, and PGE2 via LPS on human macrophages (Hart et al. 2000). The effects of TP-4-ol were mediated by activation of PPAR-γ and subsequent inactivation of the NF-κB signaling pathway (Ning et al. 2018).

TP-4-ol demonstrated dose-dependent growth inhibition in colon, pancreatic, prostatic, and gastric tumor cells. TP-4-ol also demonstrated a synergistic inhibitory effect
on anti-EGFR therapy-resistant malignant cells (mutated KRAS) when combined with cetuximab, resulting in 80 to 90% growth inhibition (Shapira et al. 2016). Sub-toxic concentrations of terpinen-4-ol potentiated growth inhibition of anti-CD24 mAb (90%). A considerable reduction in tumor volume was seen following terpinen-4-ol (0.2%) treatment.

![Architecture and Cytology Criteria](image)

**Fig. 9** Architecture (A) and cytology (B) criteria expressed in all 4-NQO groups (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

![Histopathological Diagnosis](image)

**Fig. 10** (A) graph showing general mean of histopathologic diagnosis in 4-NQO groups; (B) histopathological the yellow arrow shows acantosis and the black arrow shows moderate dysplasia (H&E, 40x); (C) graphic histopathologic diagnosis in 4-NQO groups (**P<0.01; ****P<0.0001)
alone (40%) and with cetuximab (63%). On the other hand, terpinen-4-ol demonstrated an induction of cell cycle arrest in human melanoma cells (Calcabrini et al. 2004).

Moreover, TP-4-ol was shown a dose-dependent cytotoxic response on human lung cancer cells (Wu et al. 2012), low concentrations of TP-4-ol induced early apoptotic death, and the late apoptosis increased at higher concentrations (Shapira et al.). In the same way, TP-4-ol, at democidal concentrations, alters the morphology and inhibits the survival of human meibomian gland epithelial cells (IHMGECs) and, at lower concentrations, also reduces the proliferation and activity of cell survival mediator in IHMGECs (Chen et al. 2020). In fact, Wu et al. (2012) described the lower dose inhibited cell proliferation and higher-dose-induced cell death with marked morphologic changes (shrinkage, rounding, and floating of the cells) in human NSCLC cells. Our in vivo study using 4NQO lingual cancer induction model demonstrated negative modulation at both TP-4-ol concentrations (0.1 nL/g and 8 nL/g). The lower concentration was more effective than the higher one. It was observed that the 4NQO group developed tongue squamous cell carcinomas (SCC) in 87.5% of their animals and that the 4-NQO + TP-4-ol group (0.1 nL/g) reduced this proportion to 41.6% of the animals. In addition, the 4NQO group had, on average, more than two squamous cell carcinomas (SCC) per animal (2.62) while the 4-NQO + 0.1 nL/g group showed less than one tumor per animal (0.75). Therefore, we reduced both the proportion of positive animals and the number of squamous carcinoma, yellow circle, H&E 10x; (B), micro-invasive squamous cell carcinoma, yellow circle, H&E 20x; (D), invasive squamous cell carcinoma, yellow circle, H&E 5x; (E), deep invasive squamous cell carcinoma, yellow circle, H&E 5x.
cell carcinomas (SCC) that each positive animal developed. It represented a decrease of 52.4% by group and 71.3% in the cancer/rat ratio. Other studies also showed better activity in lower doses in natural chemotherapy treatment, including modulation in epithelial dysplasia and, specifically, moderate dysplasia (Ribeiro and Salvadori, 2007; Renugadevi and Prabu, 2010; Helli et al. 2016; Mehdipour et al. 2013; dos Santos-Nascimento et al. 2015; Baracho et al. 2016) and other researchers observed better results in the highest dose, including epithelial acantosis and epithelial dysplasia (Yoshida et al. 2005; Mehdipour et al. 2013).

The TP-4-ol interfered with migration and invasion process of drug-sensitive (Bozzuto et al. 2011). In addition, reduction of squamous cell carcinoma in situ, micro-invasive, and invasive have also been described (Fong et al. 2006; Abbasi et al. 2014). Our study presented reduction of SCC diagnosis 4-NQO + 0.1nL/g group (41.6%) compared to 4-NQO (87.5%). The diagnosis included in situ, micro-invasive, invasive, and deep invasive SCC and the SCC/rat mean revealed reduction of 2.62 (4-NQO) to 0.75 (4-NQO + 0.1nL/g) that represents a reduction of 4.0/rat or 71.42% in the SCC. Our lower dose also presents less mean of invasive SCC than the higher dose. The TP-4-ol interfered with in vitro cell migration and invasion of adriamycin-sensitive and resistant M14 cells by inhibiting the intracellular pathway induced by the multidrug transporter p170 glycoprotein (Bozzuto et al. 2011). The synergistic effect of terpinen-4-ol might be due to its lipophilic character and ability to interact with phospholipids of plasma membranes, which might result in reorganization of their lipid architecture and easier entry of the drug into the cell (Batista et al. 2020).

Nonetheless, it is important to note the results of our toxicity analysis. Previous studies describe the absence or low toxicity of TP-4-ol (Fong et al. 2006; Banjerdpornchai and Khaw-On, 2013; Abbasi et al. 2014; Shapira et al. 2016; Chohan et al. 2018). The oral gavage of TP-4-ol in healthy animals demonstrated relative toxicity in the kidney analysis. In the biochemical analysis of creatinine levels, the 4NQO + 8nL/g group showed concentrations similar to the healthy group. Furthermore, the 4NQO group showed higher concentrations than its two experimental groups 4NQO + TP-4-ol (0.1nL/g and 8nL/g). For the urea analysis, the 8nL/g TP-4-ol shows similar results to the healthy group and the other groups had the highest levels in this biochemical parameter. Serum creatinine and urea levels are important biochemical markers of glomerular function. An increase in these markers is associated with progression to chronic kidney disease. However, it was not possible to find specific serum levels in the literature (Silva et al. 2008; Baracho et al. 2016).

These lower toxicities disappear in both carcinogenic TP-4-ol treated groups. In the kidney histopathology analysis, the 0.1nL/g TP-4-ol group showed yellowish pigment and ductal and distal necrosis. On the other hand, the 4-NQO + 8nL/g TP-4-ol group exhibited distal duct edema. These findings demonstrated low kidney toxicity in healthy TP-4-ol oral gavage. Shapira et al. (2016) described mild toxicity of TP-4-ol that was associated with modulation of ion channels and cellular excitability that possibly affects the organ. A blockade of K+ channels with membrane depolarization was described and its control was critical for renal function. Shuvy et al. (2011) showed similar kidney lesions describing ductal edema and pigment.

The spleen is a secondary lymphoid organ and its white pulp/red pulp ratio is associated with greater or lesser immunological activity to blood antigens (Gannot et al. 2004; Peng et al. 2015). Our study demonstrated a reduction in white pulp in the 4NQO group and both TP-4-ol treatment groups (4NQO + TP-4-ol) showed similar results to the healthy group. To the lung, the toxicity of 4-NQO described proliferative lung lesions, adenomas, and adenocarcinomas (Barcessat et al. 2014). Our results demonstrated the presence of hemorrhagic processes in the 4NQO group. Furthermore, we observed a reduction in these bleeding processes in the 4NQO + 8nL/g group and they disappeared in the 4NQO + 0.1nL/g group. No other study with 4NQO has described pulmonary hemorrhagic processes in rats. Recently, TP-4-ol was described as having antihypertensive capacity and this could explain our results with reduction of pulmonary hemorrhagic processes in animals treated with TP-4-ol. Again, the lowest concentration had the best effects.

Serum transaminases (ALT, AST) and ALP have been useful to indicate liver and hepatobiliary system damage in the liver diseases (Renugadevi and Prabu, 2010) (Karthikeyan, 2004). High levels of aspartate transaminase and alanine transaminase detect liver cellular parenchyma damage (1997). Renugadevi and Prabu described the increased activities of AST, ALT, and ALP, in the serum of Cd-treated rats which are related to the status and function of hepatic and hepatobiliary cells (Renugadevi and Prabu, 2010). Our results demonstrated healthy 0.1nL/g with higher levels of ALP and ALT. Serum ALT levels in animals exposed exclusively to 0.1 nL/g of TP-4-ol were similar to those found in the positive control (4NQO). However, animals exposed to 0.1 nL/g of TP-4-ol had higher serum levels of ALT and ALP than the healthy group. Grando et al. described no toxic effect to the liver and kidney using 200nL/g (0.2 mL/kg) and 500nL/g (0.5 mL/kg) and both doses were able to prevent AST and ALT increase in Haemonchus contortus-infected animals (Grando et al. 2016). Considering the lethal dose of 1.4144 g/kg (Yang et al. 2011), the density mass of 0.929, and 95% purity of terpinen-4-ol (Sigma-Aldrich®), the described dose corresponded to 18.25% and 45.62% of lethal dose, respectively. Our dose was 0.73% and 0.09% of the lethal dose.

Body weight is useful in identifying cancer progression in mice induced by 4NQO. Our studies demonstrated
weight loss in the 4NQO group compared to the healthy group (Kujan et al. 2006). The other groups demonstrated weight gain. Mehdipour et al. (2013) show loss of body weight in all 4-NQO studied groups. Araki and Salvadori (2007) described an expressive reduction of body weight in animals exposed to 4-NQO. On the other hand, Makita et al. described weight gain in the 4-NQO group compared to its experimental groups. Water and feed consumption was described by some authors as not affected by exposure to 4NQO; however, other studies described results similar to ours (Shuvy et al. 2011; Mehdipour et al. 2013).

Finally, the free TP-4-ol concentrations in dermal tissue are lower than plasma levels. The plasma levels do not provide information of TP-4-ol dermal tissue concentrations (Chooluck et al. 2013). Wahyuni et al. (2009) studied 29 healthy human and use three groups of dose regimens of Melaleuca alterinifolia (60% TP-4-ol), single oral dose of 300 mg and 600 mg, and multiple oral doses of 300 mg. They described serum concentrations of 2.84 ng/mL (300 mg) and 5.15 ng/mL (600 mg) after 1–2 h of ingestion. They described TP-4-ol widely distributed in the body with a slow elimination rate (half-lives vary from 28.5 to 42.6 h). They also described that TP-4ol was slowly excreted into urine with average excretion rates of 177.78 ng/h to single oral dose 300 mg and 118.49 ng/h to single-dose 600 mg (WAHYUNI et al. 2009). Our in vivo results described kidney toxicity and we consider that it will be necessary to further research to verify the potential toxicity of TP-4ol on healthy people.

**Conclusion**

Our results demonstrated that 0.1nL/g of TP-4-ol is more effective than 8nL/g of TP-4-ol to modulate potentially malignant and malignant tongue lesions induced by 4-NQO (25 ppm) in rats (Rattus nervigeric us albinos, Holtzman). Kidney toxicity was observed in animals exposed exclusively to 0.1nL/g of TP-4-ol. However, this toxicity was not observed in animals previously induced by 4NQO.

**Author contribution** The authors declare that all data were generated in-house and that no paper mill was used. JNCN and CLRA conceived research, designed research, acquired data, analyzed data, interpreted data, drafting the article, revised the article, and approved the final version. AM and BB conducted experiments. GR contributed new reagents or analytical tools. CAAF conceived research, acquired data, analyzed data, and approved the final version. TFMS conceived research, designed research, acquired data, revised the article, and approved the final version. DAR conceived research, designed research, interpreted data, revised the article, and approved the final version. ILB conceived research, designed research, acquired data, analyzed data, revised the article, and approved the final version. CRLA wrote the manuscript.

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**Data availability** Data archiving is not mandated but data will be made available on reasonable request.

**Declarations**

**Ethical approval** This project was approved by the Animal Use Ethics Commission (CEUA) at the Araquara School of Dentistry with protocol n° 8/2015 according to the rules established by the National Council for Animal Experimentation Control (CONCEA) and Normative Instruction No. 04 of June 18, 2014, of the Brazilian National Health Surveillance Agency (ANVISA) on non-clinical studies of toxicology and pharmacological safety necessary for the development and registration of herbal medicines. The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) were used to write this article.

**Consent to participate** Not applicable.

**Consent for publication** Author agreement form

Journal Name: Naunyn–Schmiedeberg’s Archives of Pharmacology, the manuscript entitled: “Negative Terpinen-4-ol modulate potentially malignant and malignant lingual lesions induced by 4-nitroquinoline-1-oxide in rat mode” within author and the co-authors: José Nunes Carneiro Neto, Juliana Maria Sorbo, Carlos Alberto Arcaro Filho, Thais Fernanda Moreira Sabino, Daniel Araki Ribeiro, Iguatemy Lourenço Brunetti, and Cleverton Roberto de Andrade in submitting the aforementioned manuscript to the Journal. The corresponding author of the above specified manuscript, Cleverton Roberto de Andrade, certifies that he is authorized by all co-authors to enter into the agreement given below. On behalf of myself and my co-authors, I certify that:

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**Conflict of interest** The authors declare no competing interests.

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