

Anti-inflammatory and healing action of oral gel containing borneol monoterpene in chemotherapy-induced mucositis in rats (*Rattus norvegicus*)

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The aim of this study was to investigate the effect of gels containing the monoterpene borneol in induced oral mucositis using an animal model. Gels were prepared with borneol at 1.2% and 2.4% (w/w). Oral mucositis was induced by administration of three doses of 5-fluorouracil (30 mg/kg, i.p.) and injury with acetic acid (50%, v/v) soaked in filter paper applied to right cheek mucosa for 60s. Four subgroups comprising 12 animals each were formed. Six animals from each group were sacrificed at days seven and fourteen after oral mucositis induction. Mucous samples were processed and stained with hematoxylin-eosin and Masson's Trichrome. The semiquantitative evaluation involved observation of inflammatory parameters. ImageJ® software was used in the quantitative evaluation. For statistical analyses, Two-way ANOVA, followed by Tukey's post-test ($p < 0.05$), were employed. Borneol 2.4% gel proved effective in the treatment of oral mucositis with statistically significant differences between groups for angiogenesis control, inflammatory cell count reduction and percentage neoformed collagen increase. The confirmation of anti-inflammatory and healing action of borneol in oral mucositis in rats renders it a good marker for predicting this activity for plant extracts rich in this substance.

Keywords: Chemotherapy. Oral gel/Borneol monoterpene/effects. Oral gel/Borneol monoterpene/action anti-inflammatory. Oral gel/Borneol monoterpene/healing action. Borneyl alcohol/effects. Palliative care.

INTRODUCTION

The clinical manifestations most commonly associated with treatment of cancer of the oral cavity are oral mucositis (OM), osteoradionecrosis, dry mouth, radiation caries, trismus, opportunistic infections and dysphagia, among others. OM is an important complication of oncological treatments such as head and neck radiotherapy, chemotherapy and Hematopoietic Stem

Cell Transplantation (HSCT), autologous or allogeneic, having a prevalence of 75%–99%. (Simões, Castro, Cazal, 2011; Patussi *et al.*, 2014). Therefore, the healthcare team should have a broad knowledge of all oral complications of anticancer therapy in order to improve patients' quality of life (Sera *et al.*, 2013).

Among the different oral manifestations, mucositis is the most common and distressing condition that develops as an acute adverse effect of chemotherapy and radiotherapy of the head and neck and is one of the leading causes of disability that limits cancer therapy dose. This complication leads to therapy alteration, dose reduction, delay, interruption or termination and

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can negatively affect the healing process. It is clinically characterized by erythematous and ulcerative lesions that affect the vermilion of the lips and oral mucosa (Smith *et al.*, 2007).

The use of medicinal plants and herbal medicines represents a good alternative treatment approach for this patient group. Products containing *Aloe vera* L., *Leptospermum scoparium* J.R. & G. Forst and *Kunzea ericoides* A. Rich have been used to treat oral mucositis with satisfactory results (Rodríguez-Caballero *et al.*, 2012; Freitas, Rodrigues, Gaspi, 2014.). Chamomile has been used in the treatment of OM with promising results (Dos Reis *et al.*, 2016; Braga *et al.*, 2015; Curra *et al.*, 2013; Pavesi *et al.*, 2011)

Also, *Hypericum perforatum* L. extract, whose phytocomplex contains borneol, has shown anti-inflammatory and healing action in oral mucositis animal models both systemically and locally through oral gel (Tanideh *et al.*, 2014). Borneol (Figure 1) is a monoterpene, bicyclic aromatic alcohol, present in many essential oils of medicinal plants of the Dipterocarpaceae, Lamiaceae (*Rosmarinus officinalis* L., *Thymus vulgaris* L. and *Salvia officinalis* L.) (*Dipterocarpus turbinatus* Gaertn.) Valerianaceae (*Valeriana officinalis* L.) and Asteraceae (*Matricaria chamomilla* L.) families (Horváthová *et al.*, 2009; Borges *et al.*, 2012). In Chinese and Indian folk medicine, borneol is used in the treatment of sore throats, mouth sores, wounds, burns and skin infections (Liu *et al.*, 2009; Kong *et al.*, 2014). Some studies indicate that borneol has analgesic, anti-inflammatory, antioxidant, antibacterial and healing actions (Horváthová *et al.*, 2009; Liu *et al.*, 2009; Barreto, 2013).

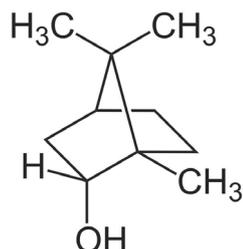


FIGURE 1 - Chemical structure of borneol (monoterpene, bicyclic aromatic alcohol).

Although borneol has been shown to be effective in lesions of the oral cavity, the literature reports no pre-clinical models demonstrating its action in oral mucositis. In order to fill this gap, the present study was conducted investigating the action of gels containing borneol in 5-Fluorouracil-induced oral mucositis using an animal model with Wistar rats.

MATERIAL AND METHODS

Plant Material

(+)-Borneol 97% was obtained in isolated and powder form (420247 - Sigma Aldrich, USA), Synonym: endo-(1*R*)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol; CAS Number: 464-43-7; Empirical Formula: C₁₀H₁₈O; Molecular Weight 154.25; Lot # BGBB9812V.

Preparation of Aristoflex® gels containing borneol and stability tests

Aristoflex polymer was mixed with methylparaben in water at ambient temperature under stirring at 400 rpm (Heidolph RZR stirrer, Germany) for 30 minutes to complete gelation. The mixture was then incorporated into (+)-borneol powder (Sigma-Aldrich, USA) at concentrations of 1.2% and 2.4%. The gels thus obtained (30 g each, containing borneol at concentrations of 0%, 1.2% and 2.4%) were packaged, labeled and analyzed for organoleptic (appearance, color and odor) and physico-chemical (pH, conductivity and viscosity) parameters and also for both primary and accelerated stability (Oliveira, 2009).

Animals

Male Wistar rats (300-350 g, 12-16 weeks, n=48) were acclimatized for six days prior to experiments and housed during the experimental period at 22±2 °C under a 12/12h light-dark cycle with access to feed and water *ad libitum*. After the onset of oral mucositis, the animals were tagged and randomly distributed into four subgroups of 12 animals each: G1 Group (Borneol 0%); G2 Group (Borneol 1.2%); G3 Group (Borneol 2.4%); and G4 group (Control). All animals were weighed at the beginning and end of the experiment for clinical evaluation. Another 24 animals were used in pilot tests for dose and technique adjustments, but not included in the results of the study. The project was submitted to the Research Ethics Committee on the use of animals (CEUA - UNIVASF) and was approved under permit 0001/140814 on June 2nd, 2015.

Chemotherapy-induced oral mucositis

Oral mucositis was induced by administration of the 5-fluorouracil chemotherapy drug (Eurofarma, São Paulo, Brazil) on days 0, 2 and 4 (30 mg/kg, i.p.) according to Lima *et al.* (2015). On day two, the animals were anesthetized [ketamine hydrochloride 10% (50 mg/

kg) and xylazine hydrochloride 2% (5 mg/kg i.p., 0.4 ml per animal] and right cheek mucous chemically injured by applying 9 mm² filter paper soaked in 10 µL of solution [glacial acetic acid 96% (50%, v/v) in distilled water] for 60 seconds (Figures 2A, 2B and 2C).

Treatment subgroups

The G1, G2 and G3 Groups received a daily application of gels at concentrations of 0%, 1.2% and 2.4%, respectively, using disposable flexible swabs (Figure 2D). The G4 Group (Control) received topical application with distilled water. After the application, all animals were prevented from drinking water or eating for 30 minutes to allow time for the gels to act (Tanideh *et al.*, 2014). The treatments were administered from the third to 13th day after injury with acetic acid.

Mucous removal and histology procedure

Six animals from each group were sacrificed by deep anesthesia [ketamine hydrochloride 10% (100 mg/kg) and xylazine hydrochloride 2% (10 mg/kg) i.p., 0.5 mL per animal] on days seven and fourteen after injury. Subsequently, right cheek mucous was removed from each animal with surgical scissors and scalpel blade number 15 and placed in 10% neutral buffered formalin solution for 48h (Akgullu *et al.*, 2015).

Specimens were dehydrated, diaphonized, embedded in paraffin and cut transversely into 5 µm-thick slices on a microtome (Leica Biosystems, Wetzlar, Germany). Mucous samples were stained with hematoxylin-eosin (HE) and Masson's trichrome. All sections were mounted on glass slides with cover slips using Entellan (Merck, Darmstadt, Germany) for microscopy.



FIGURE 2 - Experimental procedure for testing. A. Placement of filter paper soaked in 50% acetic acid; B. Contact time with cheek mucosa of 60 s; C. Aspect of standardized lesion (9 mm²) after filter paper removal; D. Appearance of gels used in treatments.

Histological evaluation

Fields were scanned using a Leica® DM2500 microscope (Leica Biosystems, Wetzlar, Germany) under x4 and x10 objective lenses, coupled to a CELESTRON® Digital Microscope Imager Model 44421 camera (Torrance, California, United States) using the software supplied with the digital camera. The same light intensity was used in all digitalization; images were acquired in RGB format at a resolution of 2048 X 1536 pixels with JPEG extension. For quantitative analysis, the ImageJ® program was employed using the plugin “Cell Counter” and the number of inflammatory cells and amount of blood vessels stained with HE on the slides were measured. To evaluate percentage collagen, the slides stained with Masson’s trichrome were photographed and then processed using ImageJ® and the Threshold plugin to measure percentage collagen at the injury site (Vilela-Goulart *et al.*, 2008).

The semiquantitative analysis was performed by a blinded evaluator who observed inflammatory parameters including presence of cellular inflammatory infiltrate, vasodilatation, necrosis, ulceration, abscess, hemorrhage and edema. These parameters were attributed scores of 0-3, as described by Lima *et al.* (2005): Score 0 - normal epithelium and connective tissue without vasodilatation; absence of, or mild, cellular infiltration; absence of hemorrhagic areas, ulcerations or abscesses; Score 1 - mild vascular ingurgitation, re-epithelization areas; mild inflammatory infiltration with mononuclear prevalence; absence of hemorrhagic areas, edema, ulcerations or abscesses; Score 2 - moderate vascular ingurgitation, areas of hydropic epithelial degeneration, inflammatory infiltration with neutrophil prevalence, presence of hemorrhagic areas, edema and some ulcerations, absence of abscesses; Score 3 - severe vascular ingurgitation and dilatation, inflammatory infiltration with neutrophil prevalence, presence of hemorrhagic areas, edema and extensive ulceration and abscesses.

Statistical analysis

Comparisons for all variables were performed independent of day, between days, independent of group and between groups. Quantitative and semiquantitative variables were expressed as means. For comparisons, the two-way ANOVA test, followed by Tukey’s post-test, was used for comparisons. In all tests, $p \leq 0.05$ was adopted for the level of rejection of the null hypothesis for significant values.

RESULTS AND DISCUSSION

The formulation developed was stable and transparent with a smooth texture, homogeneous appearance and characteristic aroma. Borneol 0% had pH = 5.0; Conductivity=100.0 mV; Viscosity=58,500 mPa.s; and centrifugation without phase separation. Borneol 1.2% had a pH = 4.7 ± 0.1 ; Conductivity= 120.2 ± 0.5 mV; Viscosity=45,360 mPa.s; and centrifugation without phase separation. Borneol 2.4% had a pH= 4.5 ± 0.2 ; Conductivity= 139.0 ± 1.5 mV; Viscosity=55,560 mPa.s; and centrifugation without phase separation. Acid pH values were expected because the Aristoflex® gel base was formulated with a pH of 5.0. In a previous study, the same gel base had a pH of around 5.0, no phase change and good viscosity (Russo, Guimarães, Cardoso, 2008). Furthermore, borneol is also considered an acid pH substance (Xiao-Fei *et al.*, 2008). Dantas *et al.* (2016) used a topical gel formulation with 5% borneol which had a pH of 3.95.

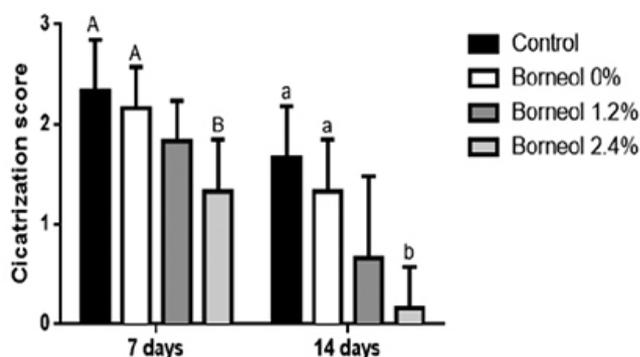


FIGURE 3 - Cicatrization score according to groups and days of experimentation. $p < 0,05$: Control vs. Borneol 2.4% (7 days) ; $p < 0,05$: Borneol 0% vs. Borneol 2.4% (7 days). $p < 0,05$: Control vs. Borneol 2.4% (14 days). $p < 0,05$: Borneol 0% vs. Borneol 2.4% (14 days). (Mann-Whitney test).

At day seven, a statistically significant difference ($p < 0.05$) in scores was found for the G3 group (Borneol 2.4%) compared with both the G1 (Borneol 0%) and G4 (Control) groups. This result indicated better healing for Borneol 2.4% (Figure 3). G1 Group (Borneol 0%) exhibited extensive ulcers and hydropic degeneration, spongiosis and exocytosis. Connective tissue (CT) showed moderate and diffuse presence of polymorphonuclear cells (PMNs) with areas of necrosis and bleeding. The G2 Group (Borneol 1.2%) showed ulcerated areas, hydropic degeneration, spongiosis and exocytosis. CT showed areas of necrosis, presence of some congested blood vessels, as well as moderate and diffuse presence of PMNs. The G3

Group (Borneol 2.4%) had discrete hydropic degeneration, spongiosis, and partial reepithelialization in five animals. CT showed PMNs and the presence of few congested blood vessels. The G4 Group (Negative control) exhibited ulcerated areas, hydropic degeneration, spongiosis and intense exocytosis. CT had edema and necrosis, moderate congested blood vessels, as well as moderate and diffuse presence of PMNs (Figure 4).

At day 14, a statistically significant difference ($p < 0.05$) in scores was found for the G3 group (Borneol 2.4%) compared with both the G1 (Borneol 0%) and G4 (Control) groups. This result indicated better healing for Borneol 2.4% (Figure 3). G1 Group (Borneol 0%) showed hyperkeratosis and full reepithelialization. CT had congested blood vessels, as well as moderate and diffuse presence of Mononuclear (MN) cells. The G2 Group (Borneol 1.2%) showed reepithelialization but with more

blood vessels in G3. CT showed numerous clogged blood vessels and a diffuse mild chronic inflammatory process. The G3 Group (Borneol 2.4%) had a normal aspect and full reepithelialization. CT showed normal features, in addition to granulation tissue with fibroblasts, congested blood vessels and discrete, diffuse presence of MN. The G4 Group (Negative Control) had acanthosis (thickened spinous layer) with loss of integrity of basal membrane and exocytosis. Epithelialization occurred with different morphology in four animals. The CT was found to contain a large amount of granulation tissue with fibroblasts, congested blood vessels and chronic inflammatory infiltrate (Figure 5).

At seven days, the group treated with Borneol 2.4% showed a lower number of vessels than the other groups (G1, G2 and G4). The G3 Group showed statistical significance ($p < 0.05$) when compared to the three other

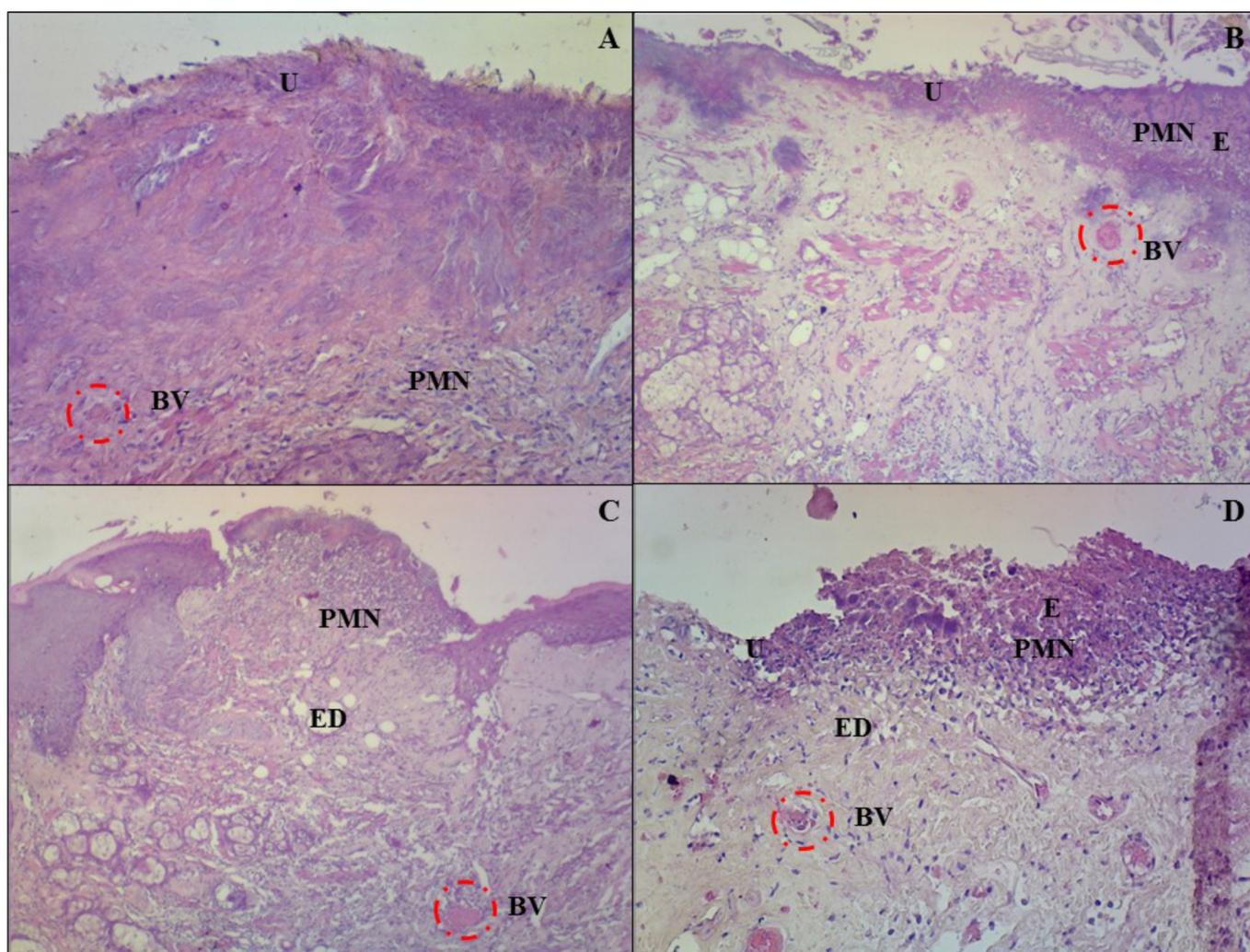


FIGURE 4 - Microscopic aspects of groups at seven days of experimentation. A Group treated with gel base (Borneol 0%); B Group treated with gel (Borneol 1.2%); C Group treated with gel (Borneol 2.4%); and D Group (Negative control). PMN=Polymorphonuclear cells; BV=Blood vessel; U=Ulcerated area; E=Exocytosis; ED=edema. Magnification x10. HE staining.

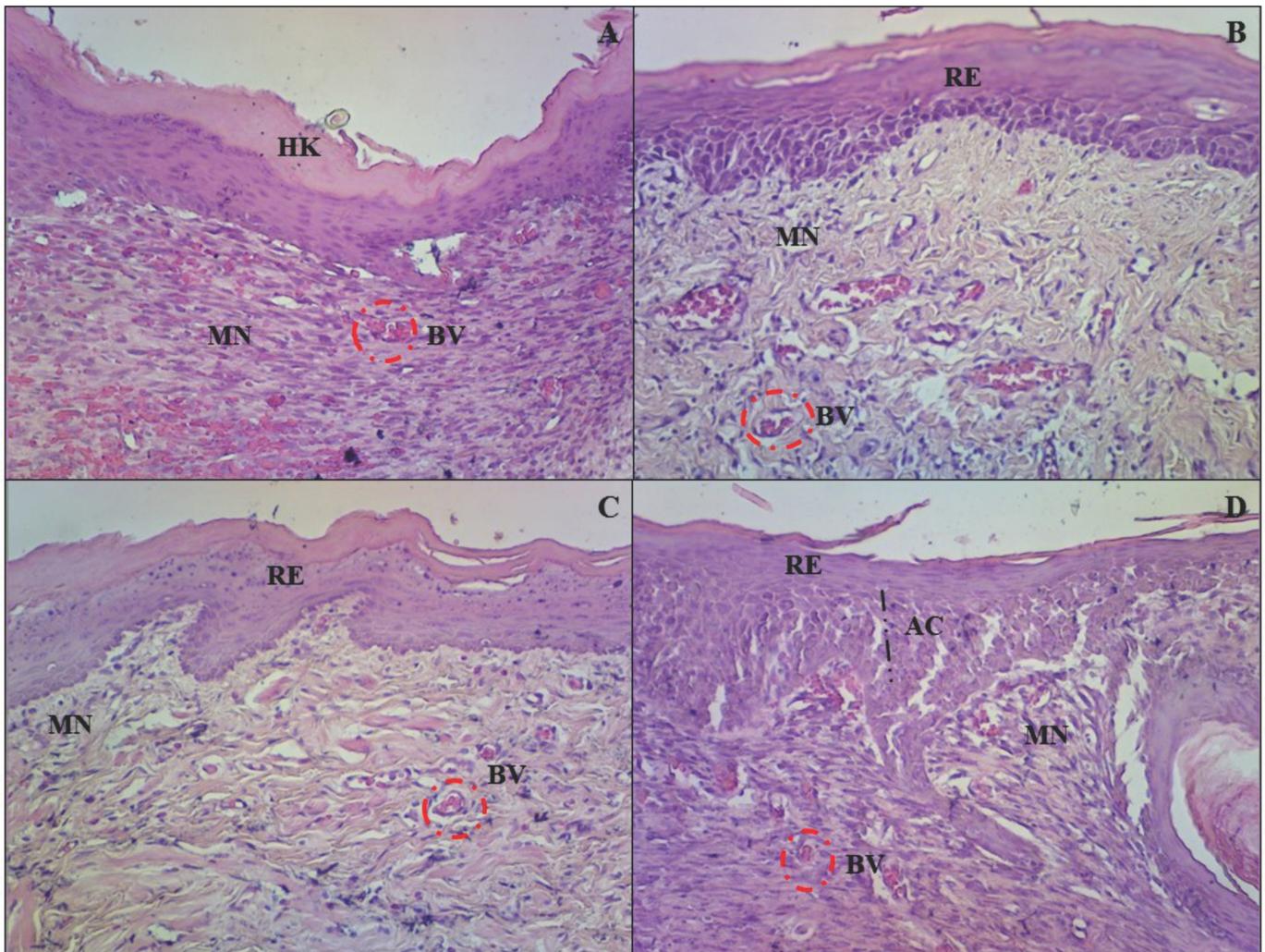


FIGURE 5 - Microscopic aspects of groups at fourteen days of experimentation. A Group treated with gel base (Borneol 0%); B Group treated with gel (Borneol 1.2%); C Group treated with gel (Borneol 2.4%); and D Group (Control). RE=reepithelialization; HK=Hyperkeratinization; BV=Blood Vessel; MN=Mononuclear; AC=acanthosis. Magnification x10. HE staining.

groups. The G2 Group (Borneol 1.2%) showed statistically significant differences when compared to both the G1 Group (Borneol 0%) and Control Groups. There were no differences between the G1 and Control Groups. At 14 days, there was statistical significance between all cross comparisons except the G1 Group and Control Group (Figure 6).

While healing depends on the neoformation of blood vessels, persistent or excessive vessels may denote the existence of chronic inflammation or fibrosis (Abbas *et al.*, 2010). On histological analysis of the blood vessels, the Control Group showed a greater number of blood vessels in the final process of wound healing compared to the other groups (G2 and G3). This indicates that the healing process in the Control Group required a greater angiogenesis for the formation of granulation tissue (Abruceze, 2013).

The proliferation of blood vessels is stimulated by

vascular endothelial growth factor (VEGF). Some factors stimulate the expression of VEGF in angiogenesis and ischemia, while increases in certain growth factors are responsible for the elevated VEGF expression in chronic inflammation and wound healing. The inflamed tissue is generally in hypoxia, where inflammation may promote angiogenesis by inducing the release of angiogenic factors leading to fibrosis (Nagy, Dvorak, Devorak, 2007). According to a study by Dai *et al.* (2009), borneol showed anti-fibrotic action.

Regarding inflammatory cell count, the gels containing borneol at concentrations of 1.2% and 2.4% exhibited anti-inflammatory action at seven and 14 days, reducing the number of these cells at the injury site. The Control Group and Borneol 0% groups, at seven days, showed a much more intense inflammatory process than the treated groups (Borneol 1.2% and Borneol 2.4%), but

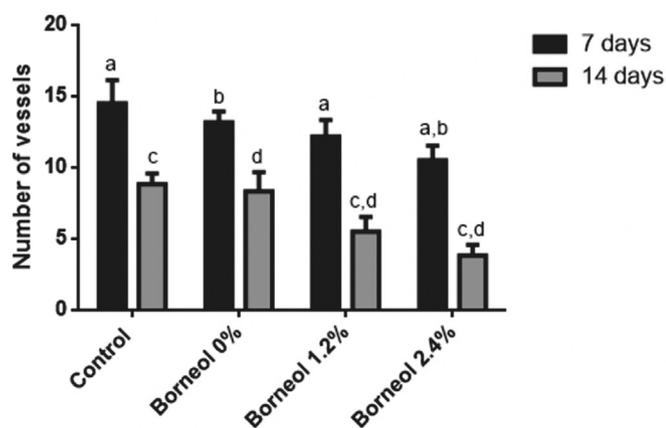


FIGURE 6 - Vessel count according to group and day of experimentation on anti-inflammatory activity test of oral gel containing different concentrations of borneol. ^a $p < 0.05$: Control vs. Borneol 1.2% and Borneol 2.4% (7 days); ^b $p < 0.05$: Borneol 0% vs. Borneol 1.2% and Borneol 2.4% (7 days). ^c $p < 0.05$: Control vs. Borneol 1.2% and Borneol 2.4% (14 days). ^d $p < 0.05$: Borneol 0% vs. Borneol 1.2% and Borneol 2.4% (14 days) (Two-Way Analysis of Variance ANOVA, followed by Tukey's post-test).

no differences were observed between the two borneol concentrations. Cell infiltrates were less intense in all groups at 14 days. Differences were observed between the G1 Group and G3 Group and also between the two gels (Borneol 1.2% and Borneol 2.4%) compared to the Control Group, but no differences between the two concentrations were evident (Figure 7).

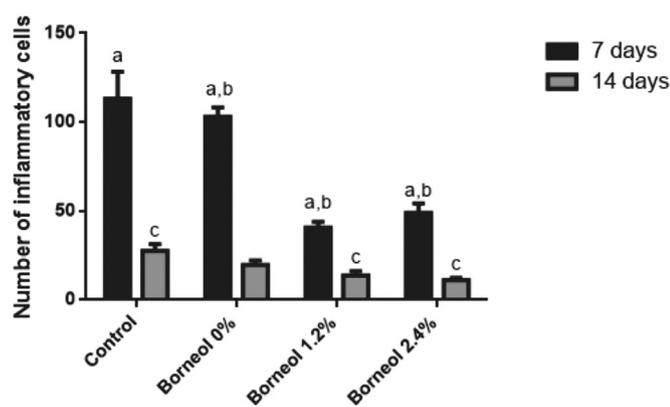


FIGURE 7 - Inflammatory cell count according to group and day of experimentation. ^a $p < 0.05$: Control vs. Borneol 0%, Borneol 1.2% and Borneol 2.4% (7 days); ^b $p < 0.05$: Borneol 0% vs. Borneol 1.2% and Borneol 2.4% (7 days). ^c $p < 0.05$: Control vs. Borneol 1.2% and Borneol 2.4% (14 days) (Two-Way Analysis of Variance ANOVA, followed by Tukey's post-test).

The findings for number of inflammatory cells corroborate the results of the study by Almeida *et al.*

(2013), in which the authors confirmed the action of monoterpene and suggested that the substance has therapeutic potential in painful and inflammatory diseases. Tanideh *et al.* (2014) tested the action of *Hypericum perforatum* L. extract for treating induced oral mucositis in animals. The extract of this plant contains Borneol (CIV, 2012) and was administered by gavage (systemically) and locally via an oral gel. The extract showed anti-inflammatory and healing action, especially systemically.

In the present study, percentage collagen at seven days showed statistical significance on comparison of the G1 Group (Borneol 0%) with the G3 Group (Borneol 2.4%) and also on comparisons of the G2 Group and G3 Group with the G4 Group (Control). Comparison of the G2 and G3 Groups with the G4 Group also proved significant at 14 days (Figure 8).

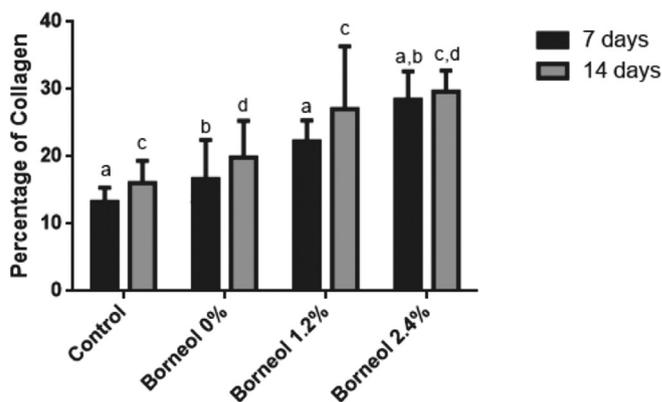


FIGURE 8 - Percentage collagen according to group. ^a $p < 0.05$: Control vs. Borneol 1.2% and Borneol 2.4% (7 days); ^b $p < 0.05$: Borneol 0% vs. Borneol 2.4% (7 days). ^c $p < 0.05$: Control vs. Borneol 1.2% and Borneol 2.4% (14 days). ^d $p < 0.05$: Borneol 0% vs. Borneol 2.4% (14 days) (Two-Way Analysis of Variance ANOVA, followed by Tukey's post-test).

Percentage collagen in the G2 and G3 Groups on slides stained with Masson's trichrome was higher than in both G1 and G4 Groups at 14 days. This was due to the healing effect of borneol which promoted greater formation of fibroblasts with better collagen and fibronectin production in local lesions (Figure 9). Tanideh *et al.* (2014) also observed increased stimulation of collagen production by topical gel *Hypericum perforatum* L., which helped repair the wounds, closing areas of damaged oral mucosa in the animals. This effect may be associated with the bactericidal action of borneol, which prevents secondary infection by fungi and bacteria, promoting reepithelialization and standardization of collagen fiber content (Tabanca *et al.*, 2001; Wenqiang *et al.*, 2006).

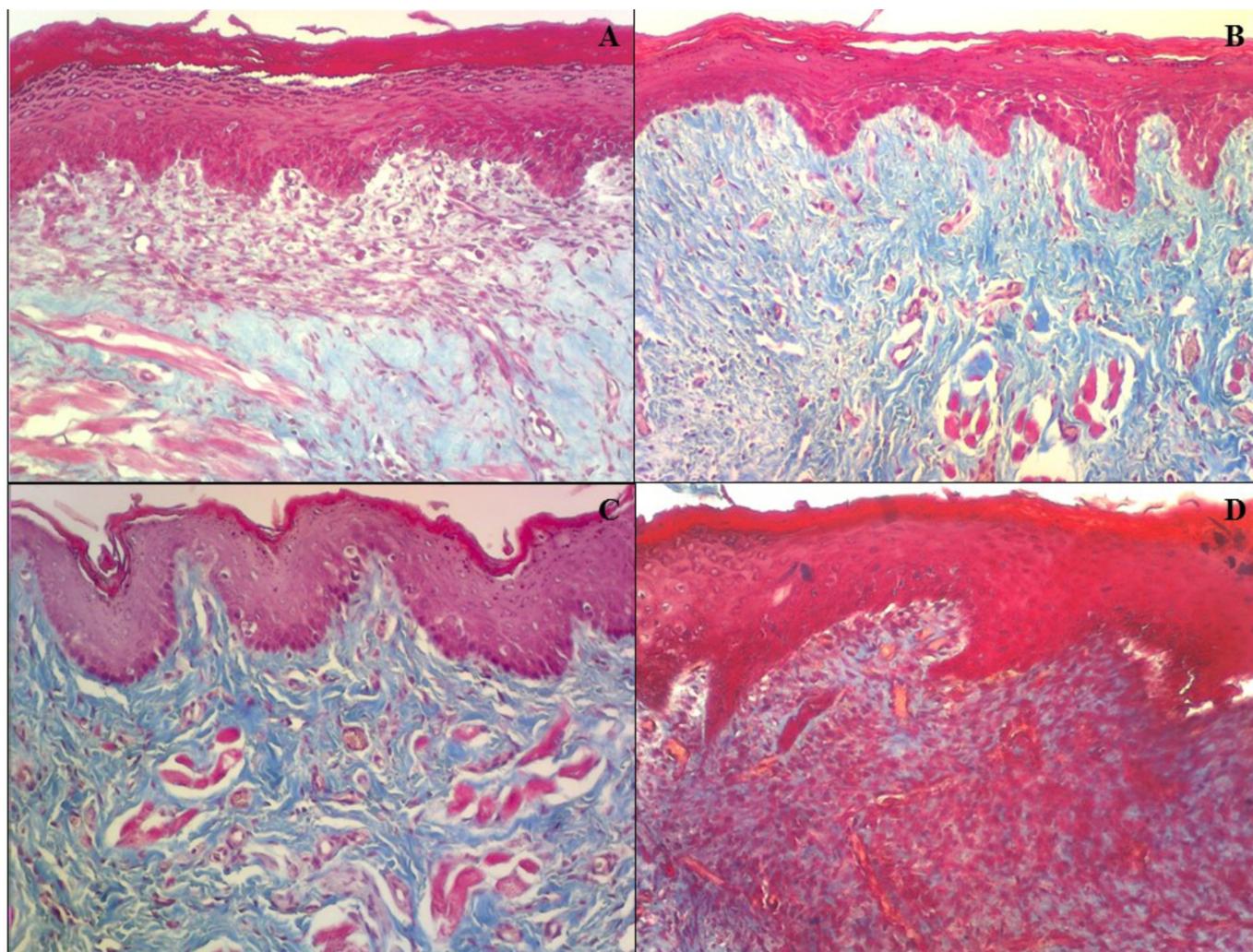


FIGURE 9 - Microscopic aspects of groups at fourteen days of experimentation. G1 Group treated with gel base (Borneol 0%); G2 Group treated with gel (Borneol 1.2%); G3 Group treated with gel (Borneol 2.4%); and G4 Group (Control). Note the greater quantity of newly formed collagen in treated groups (G2 and G3). Magnification x10. Masson's trichrome.

All animals lost weight throughout the experiment due to the side effects of 5-fluorouracil, oral mucositis and reduction in water and feed intake. At 14 days of experimentation, the Borneol 2.4% Group had a lower percentage of weight loss relative to the other three groups (Borneol 0%, Borneol 1.2% and Control), probably due to the greater action of Borneol 2.4% in reducing inflammation and promoting wound healing (Figure 10).

CONCLUSIONS

The Borneol 2.4% Group was statistically superior to the Borneol 1.2%, Borneol 0% and Control Groups in the treatment of induced oral mucositis in rats, promoting angiogenesis control, inflammatory cell count reduction at the injury site and percentage neofomed collagen increase in reepithelialization. The G3 group (Borneol 2.4%) was

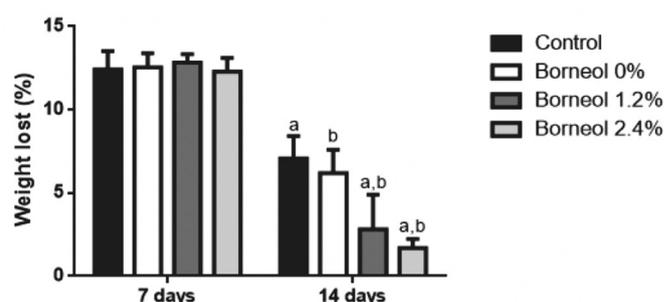


FIGURE 10 - Percentage weight loss according to group and day of experimentation. ^a $p < 0.05$: Control vs. Borneol 1.2% and Borneol 2.4% (14 days). ^b $p < 0.05$: Borneol 0% vs. Borneol 1.2% and Borneol 2.4% (14 days) (Two-Way Analysis of Variance ANOVA, followed by Tukey's post-test).

statistically superior to the other three groups on the semiquantitative evaluation, promoting improvement in

reepithelialization and healing of chemotherapy-induced oral mucositis.

The confirmation of anti-inflammatory and healing action of borneol (especially Borneol 2.4%) in oral mucositis in rats renders it a good marker for predicting this activity for plant extracts rich in this substance. The study results showed that borneol has anti-inflammatory and healing action in induced oral mucositis in cheek mucosa of Wistar rats.

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