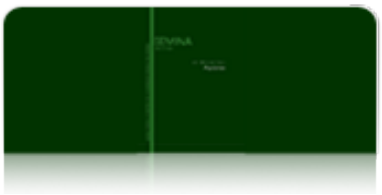

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Triticum aestivum in open skin wounds: cytotoxicity and collagen histopathology

Triticum aestivum em feridas cutâneas abertas: citotoxicidade e histopatologia de colágeno

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Abstract

Phytoterapic compounds have been used in wound healing for many centuries. Nowadays, scientific evidences of phytotherapeutics is a requirement of the legislation. The scientific literature notes the need for healing topics yielding scars that are both aesthetically appealing and resistant. We aimed to evaluate the cytotoxicity of several doses of *T. aestivum* extract (2 mg mL^{-1}, 4 mg mL^{-1}, 6 mg mL^{-1}, 8 mg mL^{-1} and 10 mg mL^{-1}) in a fibroblast cell line and the healing process in an *in vivo* experimental model (New Zealand rabbits). For this, MTT test in 3T6 cells was performed in duplicates using MEM (0 mg ml^{-1}) as negative control. Cell viability was calculated as: absorbance average in treatments/ absorbance average in controls x 100. In vivo test was performed in 78 skin wounds in rabbits that were treated with 2 mg $ml^{-1}$and 10 mg ml^{-1} of *T. aestivum* and non-ionic cream for 21 days. After this period, it was evaluated the histology using picrosorius and Gomori's trichrome staining. Statistical analysis was evaluated using T test (Graphpad) for cytotoxicity assay, Fischer test for the gomori trichrome test (Grahpad) and Kruskal-Wallis (Statistic 9.0) for picrosirius test. The *in vitro* test resulted in cytotoxicity observed at 2mg mL^{-1} whereas cells were viable at higher doses. On the other hand, it was observed that collagen formation of wounds was more uniform with this dose than with 10mg mL^{-1} extract in the *in vivo* study. Thus, we conclude that the 2mg mL^{-1} *T. aestivum* aqueous extract dose was more efficient in the *in vivo* wound healing study, despite its cytotoxic effects *in vitro*.

Key words: Wound healing. Rabbits. Picrosirius. Gomori trichome. Wheat.

Resumo

Os extratos vegetais têm sido utilizados na cicatrização de feridas a muitos séculos. No entanto nos dias atuais a comprovação científica dos fitoterápicos é uma exigência da legislação. Na literatura científica se observa a necessidade de cicatrizantes tópicos que proporcionem uma cicatriz estética e resistente. Devido a isso objetivou-se avaliar a citotoxicidade de diversas doses de *T. aestivum* (2 mg mL^{1}, 4 mg mL^{-1}, 6 mg mL^{-1}, 8 mg mL^{-1} e 10 mg mL^{-1}) em linhagem celular de fibroblasto, e o processo cicatricial em modelo experimental (New Zealand rabbits) *in vivo*. Para isso foi realizado o teste de MTT em linhagem celular 3T. Tests were performed in duplicates, using MEM (0 mg mL^{-1}) as negative control. Cell viability was calculated as: absorbance average in treatments/absorbance average

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in controls x 100. No ensaio *in vivo* foram realizadas 78 feridas experimentais em coelhos que foram tratadas *T. aestivum* 2mg mL^{-1}, *T. aestivum* 10 mg mL^{-1}e creme não iônico por 21 dias, após foi avaliado a histologia do tricrômico de golmori e de picrosirius. Na análise estatística do ensaio de citotoxicidade foi realizado o teste de t (Graphpad), para avaliação do tricomico de golmori foi realizado o teste de fischer (Graphpad) e no picrosirius foi avaliado através de Kruskal - Wallis (Statistic 9.0). O resultado *in vitro* demonstrou que a dose de 2mg mL^{-1} foi citotóxica para as células e que doses maiores a essa apresentavam viabilidade celular. No entanto no estudo *in vivo* foi constatado que as feridas tratadas com essa dose apresentaram a formação de colágeno mais uniforme que as tratadas com 10 mg mL^{-1}. Concluí-se que a dose de 2mg mL^{-1} do extrato aquoso de *T. aestivum* é eficiente no ensaio *in vivo* com as feridas experimentais, o que não foi observado no estudo *in vitro*.
Palavras-chave: Cicatrização. Coelhos. Picro Sirius. Tricrômico de golmori. Trigo.

Introduction

Skin wound healing involves dynamic cellular and molecular processes leading to tissue regeneration (MCNES, 2006). Yet, it needs to occur gradually in order to generate a normotrophic and resistant tissue, allowing organized formation of conjunctive tissue and culminating in a maturation phase, with prevalence of type I and oriented collagenous fibres (WOLFRAM et al., 2009; KAHAN, et al., 2009; KIM et. al., 2013).

Moreover, the therapeutic action of phytotherapic compounds has been targeted in studies aiming at producing scars which are both aesthetically appealing and resistant (KRISHANAN, 2006; CESCA et al., 2012). Wheat (*Triticum aestivum*) features among plants with wound-healing properties for its anti-oxidant action and for the presence of fitostimolines which act over fibroblasts, stimulating the synthesis of collagen and glycosaminoglycans (MASTROIANNI et al., 1998; SOLÓRZANO et al., 2001).

In our previous study we found out that treatment with 2mg mL^{-1} wheat extract was effective in yielding resistant scars. Yet, no diferences were observed between treatment and control during the histological analysis of wound healing with haematoxylin and eosin stain (TILLMANN et al., 2014). We thus propose this new study, where we evaluted collagen production as a justification for resistant scar formation.

Our goals were: to evaluate the cytotoxicity of *T. aestivum* extract doses (2mg mL^{-1}, 4mg mL^{-1}, 6mg mL^{-1}, 8mg mL^{-1} and 10 mg mL^{-1}) in a fibroblast cell line; and to assess collagen type and formation during the healing of skin wounds upon treatment with *T. aestivum* doses for 21 days.

Materials and Methods

Extract production

The *T. aestivum* aquous extract used in this study (source: Herbarium PEL, nº PEL 24.600) was obtained through ultrasound sonication. Fifty grams of wheat immersed in distilled water were subjected to sonication for 30 minutes at 30 ºC. The main components of the extract were: carnosic acid (117 mg g^{-1}), kaempferol (163 mg g^{-1}), quercetin (77 mg g^{-1}) e apigenin (38 mg g^{-1}).

In vitro toxicity assay

The 3T6 cell line, from the Universidade do Rio de Janeiro Cell Bank (BCRJ) was used. Cells were cultivated in Minimum Essential Medium (MEM) with 1% antibiotic solution and 8% fetal bovine serum (FBS). After formation of a confluent monolayer, aliquots were collected and sub-cultured in flat bottom 96-well plates where cytotoxicity was assayed after 48 hours using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

Extract doses used on the assay were the following: 2 mg $^{-1}$mL, 4 mg mL^{-1}, 6 mg mL^{-1}, 8 mg mL^{-1} e 10 mg mL^{-1} of *T. aestivum* aqueous extract in MEM. Tests were performed in duplicates, using

MEM (0 mg mL^{-1}) as negative control. Cell viability was calculated as: absorbance average in treatments/ absorbance average in controls x 100, followed by t-test (Graphpad).

In vivo assay

The present study was approved by the animal experimentation ethics commission from the Universidade Federal de Pelotas (CEEA 5104-23110.005104\2009-13). Thirteen male New Zealand rabbits were used, weighting between 2-3kg and kept in individual cages at Biotério Central (UFPel). Seventy-eight experimental wounds were produced via incisions made with punch nº. 8. All the animals received dissociative anaesthetics (xylazine 5 mg kg^{-1} e ketamine 75 mg kg^{-1}) during the procedure, and were treated with analgesics (subcutaneous injections of tramadol chloride 2 mg kg^{-1}, every 12h) for three days after the surgery (SCHANAIDER; SILVA, 2004).

Seventy-eight wounds were randomly divided in three groups, according to the treatments: wounds treated with cream containing 2 mg mL^{-1} *T. aestivum* aqueous extract were called T2; the ones treated with cream containing 10 mg mL^{-1} *T. aestivum* aqueous extract were called T10 and the ones treated with non-ionic cream (control) were called TC. Following the treatments, dressings were made and exchanged every 24 h and the wounds were cleaned with 0.9% physiological saline solution. In addition, wound protection was made with hydrophilic and surgical gauze, which were also exchanged daily.

The three experimental groups were subdivided in groups according the day of euthanasia in seven, 14 and 21 days after the experiment, according to CFMV's, 2012. The areas containing the scars were dissected and placed in flasks with 10% formaldehyde, which were later sent to the Laboratório de Histologia - Instituto de Ciências Biológicas, Universidade Federal de Rio Grande. Samples were processed in automatic tissue processor vacuum (Leica – ASP 200), imbedded and included in Paraplast Xtra (Sigma) and sectioned in an automated rotational microtome (Leica – RM2255), at standard width of 5 µm, with two consecutive cuts made per block. Slides were stained with Gomori trichome and Picrosirius stains.

Histological analyses

The presence or absence of collagen fibres were assessed on Gomori trichome slides, which were quantitatively analysed in optical microscope (bright field) (Olympus® São Paulo: Brazil), according Noronha et al., 2001. Picrusirius slides, on the other hand, were visualized under polarized light microscopy, and images obtained were analysed using the software *Image J®,* where collagen type and pixel percentage per area were assessed, according to Rich and Whittaker (2005) Statistical analysis of the *in vitro* assays were made using t-test (Graphpad). Gomori trichome stains were assessed using Fischer's test (Graphpad), and Picrosirius was evaluated using Kruskal- Wallis (Statistic 9.0).

Results

Results from the *in vitro* assay showed that only the 2mg mL^{-1} *T. aestivum* aqueous extract differed from the control and from the other treatments tested ($P \leq 0.05$), yielding less cellular viability (Figure 1).

Total collagen evaluation at the *in vivo* assay via Gomori trichome staining did not result in significant difference among the groups assessed each day ($P \geq 0.05$) (Figure 2). At day 7, T2 and TC groups displayed higher protein percentage than group T10 (80%, 60% and 40%, respectively), while all groups displayed collagen on 100% of the wounds on the remaining time-points assessed (Figure 2 and 3).

Figure 1. Cell viability percentage of 3T6 cell line 48 hours after treatment with several doses of *T. aestivum* aqueous extract (2 mg ml $^{-1}$, 4 mg ml^{-1}, 6 mg ml^{-1}, 8 mg ml^{-1} and 10 mg ml^{-1}). Different letters indicate statistical significance ($P \leq 0.05$).

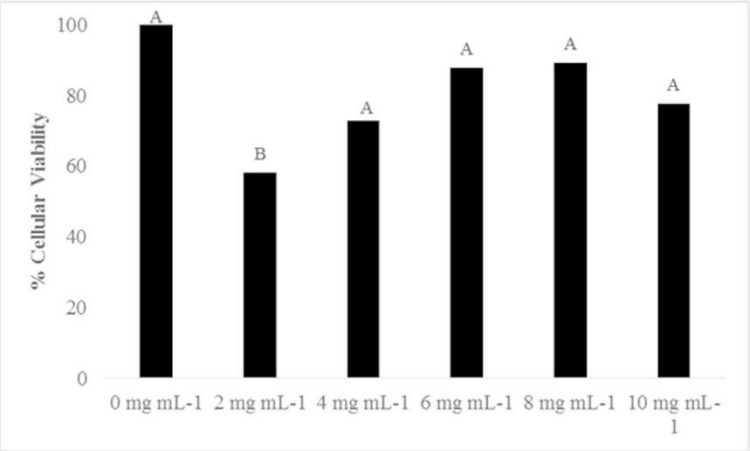


Figure 2. Figure demonstrated the evolution of collagen percentage in the different groups of open skin wounds from rabbits treated with 2 mg ml^{-1} *T. aestivum* aqueous extract (T2- A), 10 mg ml $^{-1}$ *T. aestivum* aqueous extract (T10- B) and non-ionic cream (CT- C) at 7, 14 and 21 day.

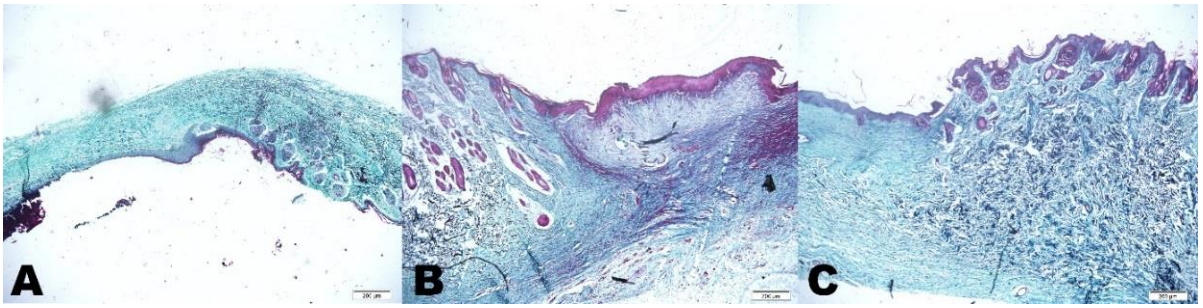
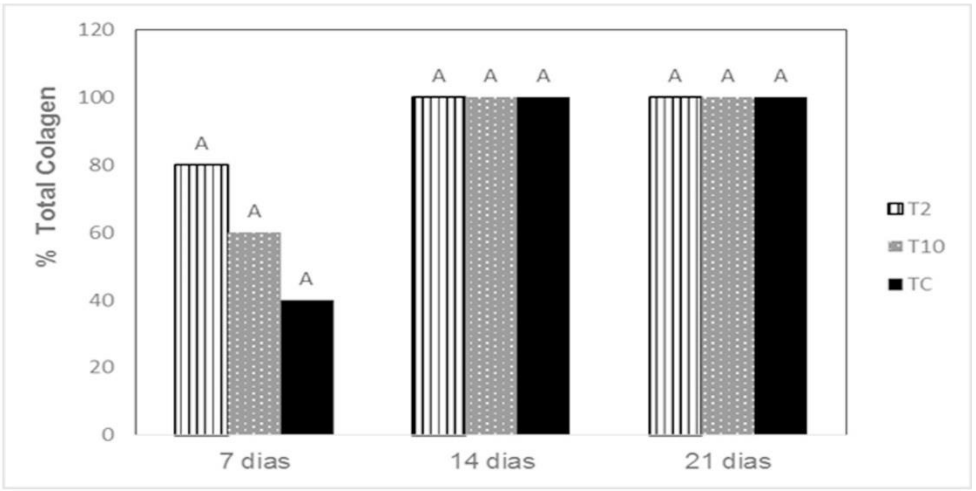


Figure 3. Graphic illustration of the collagen percentage on open skin wounds from rabbits treated with 2 mg ml^{-1} *T. aestivum* aqueous extract (T2), 10 mg ml $^{-1}$ *T. aestivum* aqueous extract (T10) and non-ionic cream (CT), at 7, 14 and 21 days. Different letters indicate statistical significance ($P \leq 0.05$).



Assessment of collagen type through our experimental time-points showed constant formation of type I collagen on all groups tested. In spite of that, when collagen percentage was evaluated, we observed that type I collagen formation was more constant on groups T2 and TC than on T10 (Figure 4 and 5).

Figure 4. Photomicrography of collagen percentage on open skin wounds from rabbits treated with 2 mg ml^{-1} *T. aestivum* aqueous extract (T2- A), 10 mg ml $^{-1}$ *T. aestivum* aqueous extract (T10 B) and non-ionic cream (CT- C), at 7 days. Figure demonstrated that treated wounds with T2 and TC presented presence of collagen (blue coloration), while in the T10 treated wound do not have the protein.

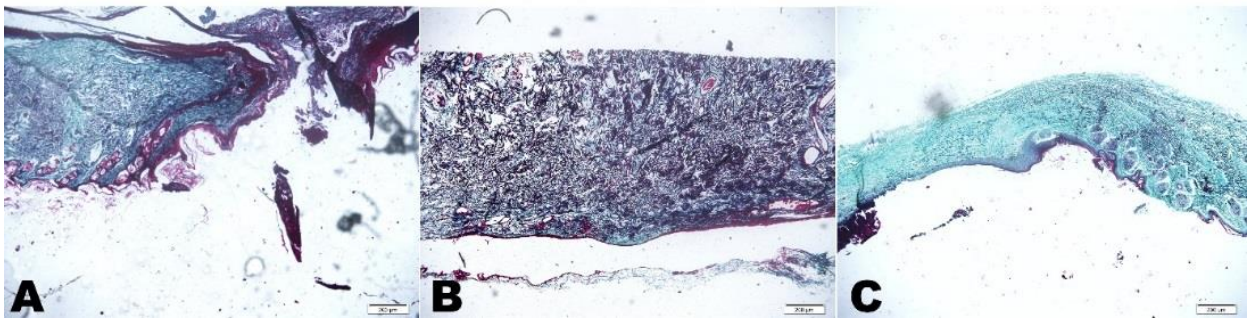
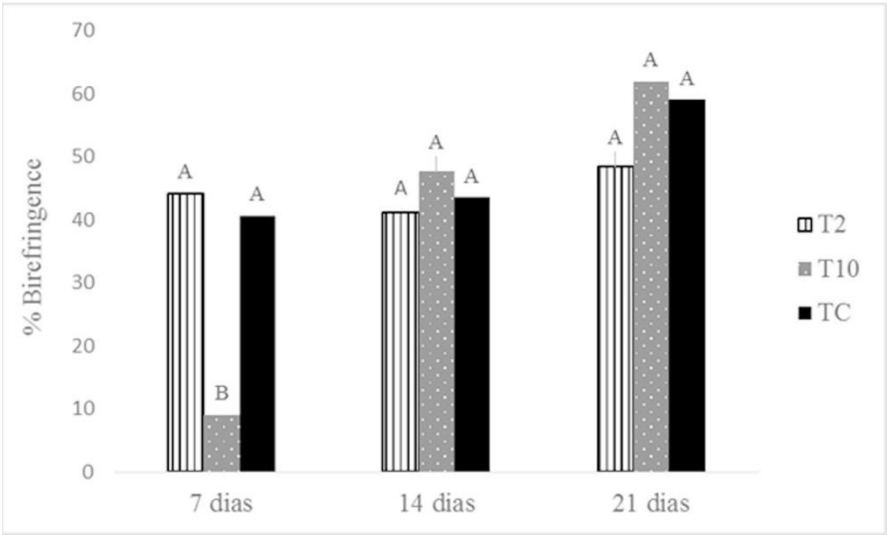


Figure 5. Birefringence percentage (picrosirius), at days 7, 14 and 21, of open skin wounds from rabbits treated with 2 mg ml $^{-1}$ *T. aestivum* aqueous extract (T2), 10 mg ml $^{-1}$ *T. aestivum* aqueous extract (T10) and non-ionic cream (CT). Different letters indicate statistical significance ($P \leq 0.05$).



Discussion

It was observed in our results that wounds from group T2 and the control (TC) had similar responses at the histological analyses, being closer to the physiological scarring process (MANDELBAUM et al., 2003 BALBINO et al., 2005), which was not observed at T10 wounds. This fact justifies wounds displaying higher tension when treated with 2mg ml $^{-1}$ *T. aestivum* aqueous extract, as observed in previous studies (TILLMANN et al, 2014).

The reason for the treatment with the lowest *T. aestivum* concentration (2mg mL^{-1}) having yielded superior results than with the highest concentration is probably justified by the cytotoxic effect of the former over fibroblasts, as shown. While fibroblast are responsible for collagen synthesis, stimulating their production leads to heightened and faster tissue synthesis, preventing proper tissue alignment and maturation (KAHAN, et al., 2009; KIM et al., 2013). The dose being cytotoxic probably leads to cell death resulting in less and slower synthesis, thus allowing adequate tissue formation (SCHREML et al., 2010; SÜNTAR et al., 2012).

Moreover, the reason why the only cytotoxic dose on the cellular assay was 2mg mL^{-1} is probably connected to the hormesis effect (LENZ et al., 2008), where lower doses lead to toxic effects similar to extremely high doses, which does not occur with average doses nor the control. In our experiment, the lowest *T. aestivum* extract dose tested (2mg mL^{-1}) lead to damage in cell metabolism. Nonetheless, as the phytotherapic compound level was low, cells were unable to perceive the damage, thus causing a cytotoxic effect due to the absence of cell protection. On the other hand, average doses (of 4 mg mL^{-1}, 6 mg mL^{-1}, 8 mg mL^{-1}) prompted cells to respond to the metabolic damage caused by the compound, hence its deleterious effect was nulled out and cell toxicity was absent, as observed in the control group (CALABRESE; BRAIN, 2011).

It is possible that *T. aestivum* extract doses higher than 10 mg mL^{-1} lead to cytotoxicity as the 2mg mL^{-1}dose did, although in that case toxicity would be related to the inability of cells in containing damages caused by high extract dosages.

Type III collagen was not observed at wounds from the groups tested. Likely it was formed on the previous time-points, but at day 7 type I collagen was already being formed. During the total and type I collagen assessments it was observed that in wounds from groups T2 and TC, collagen formation occurred according to the physiological scarring process (MANDELBAUM et al., 2003; BALBINO et al., 2005), which was not observed in T10 wounds, since there was intense collagen formation between days 7 and 14. This sudden collagen formation likely prevented proper organization and maturation of the collagen fibres (KAHAN et al., 2009; KIM et al., 2013). As a consequence, the tissue formed in lesions from this group was less mature and displayed higher tension, fact that was not observed at wounds from the other groups tested (TILLMANN et al., 2014). In groups T2 and TC, on the other hand the protein was gradually formed, indicating the occurrence of proper cell signalling and flux to the lesion site, thus leading to formation of a tissue that was more mature tissue and had higher tensiometric strength (MANDELBAUM et al., 2003; BALBINO et al., 2005).

Conclusion

In this study we conclude that the 2mg mL^{-1} *T. aestivum* aqueous extract dose was more efficient in the *in vivo* wound healing study, despite its cytotoxic effects.

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