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Characterization of Polyphenolic compounds from *Bacopa procumbens* and their effects on wound healing process

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Abstract: Wounds represents a medical problem that contribute importantly to patient morbidity and to the healthcare costs in several pathologies. In Hidalgo, Mexico, *Bacopa procumbens* plant has been traditionally used for wound healing care for several generations; *in vitro* and *in vivo* experiments were design to evaluate the effects of bioactive compounds obtained from *B. procumbens* aquoethanolic extract and to determine the key pathways involved in wound regeneration. Bioactive compounds were characterized by HPLC- QTOF-MS and proliferation, migration, adhesion, and differentiation studies were done on NIH/3T3 fibroblasts. Polyphenolic compounds from *Bacopa procumbens (PB)* regulated proliferation and cell adhesion; enhanced migration reducing the artificial scratch area; and modulated cell differentiation. *PB* compounds were included in a hydrogel for topical administration on rat excision wound model. Histological, histochemical and mechanical analysis showed that *PB* treatment accelerates wound closure in at least 48 h; reduce inflammation, increasing cell proliferation and deposition and organization of collagen in earlier times. These changes resulted in the formation of a scar with better tensile properties. Immunohistochemistry and RT-PCR molecular analyses demonstrated that treatment induces: i) overexpression of transforming growth factor beta (TGF-β); and ii) the phosphorylation of Smad 2/3 and ERK1/2, suggesting the central role of some *PB* to enhance wound healing, modulating TGF-β activation.

Keywords: *Bacopa procumbens* HPLC characterization; effect of polyphenolic compounds, *in vitro* and *in vivo* skin wound; collagen organization.

1. Introduction

Wound healing is a highly ordered and synchronized process; tissue repair progresses sequentially through three overlapping phases: inflammatory, proliferative and remodeling; this process requires different cell types, cytokines; growth factors and interaction with the extracellular matrix (ECM) [1–4].

Transforming growth factor beta (TGF-β) is a crucial cytokine during wound healing, it has widespread effects on cell growth, differentiation, migration and deposition of extracellular matrix, inflammation regulation and the promotion of connective tissue regeneration [5–7]. De-regulation of TGF-β produce abnormal wound healing, including scar

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formation. TGF-β stimulate Smad proteins and Smad-independent signal transducers such as mitogen-activated protein kinases (MAPK), implicating it in the pro-abnormal fibrotic effects of this factor [6,8].

New compounds such as pirfenidone (Kitoscell, CellPharma) are commercially available. It is used for the late remodeling phase of wound repair. Activities claim by the pharmaceutic company also includes the reduction of inflammatory response, thereby controlling secondary edema and promoting a better blood supply [9]. Nowadays, no topically effective medication has been developed to accelerate the wound healing process and/or to prevent abnormal cicatrization. New alternatives have been sought for the treatment of wounds, the use of pharmaceutical herbs is in vogue due to the fewer side effects and the effectiveness of certain compounds. In Mexico, different ethnic groups in the state of Hidalgo have used *Bacopa procumbens* (Mill.) Green to treat skin wounds [10]. However, effects on the wound healing process and molecular mechanism involved in it have not been determined. Was evaluate the *B. procumbens* aqueous fraction effect in proliferation, differentiation, and migration of 3T3 fibroblasts as well as in excisional skin lesions produced in rats in which molecular effectors and signaling pathways induced were elucidated.

2. Results

2.1. Phytochemical screening of Bacopa procumbens aqueous fraction

Total aquoethanolic extract (TAE) compounds profile was obtained by HPLC/QTOF-MS. A total of 28 compounds were identified in the extract. Based on the abundance of ions, the most representative compounds are naringenin-C-hexoside, equol 7-O-glucuronide, paeoniflorin, m-hydroxybenzoic acid, 4'-methoxyapigenin rutinoside and methyl ferulate (Table 1).

Table 1. Phenolic profile of TAE of *B. procumbens.*

Compound	RT(min)	[M-H]–	Reference mass
Arbutin	0.386	271.0821	272.0896
o-Hydroxybenzoic acid	0.924	137.0242	138.0317
m-Hydroxybenzoic acid	0.959	137.0245	138.0317
Feruloyl glucose	1.161	355.1033	356.1107
Esculetin	1.263	177.0188	178.0266
3-O-Caffeoyl shikimic acid	1.937	335.0771	336.0845
Catalposide	2.88	481.134	482.1424
Homovanillyl alcohol	3.082	167.0711	168.0786
Genistein	3.285	269.0452	270.0528
p-Coumaric acid	3.318	163.0396	164.0473
Z-Astringin	3.486	405.118	406.1264
Naringenin-C-hexoside	3.588	433.1126	434.1213
Astilbin	3.823	449.1092	450.1162
m-Coumaric acid	3.857	163.0398	164.0473
Equol 7-O-glucuronide	4.092	417.1183	418.1264
Syringaresinol-glucoside	4.093	579.2096	580.2156
p-Hydroxybenzoic acid	4.194	137.0243	138.0317
Daidzein 7-O-glucuronide	4.531	429.084	430.09
4'-Methoxyapigenin rutinoside	5.069	591.1709	592.1792
Prunetin	5.071	283.0602	284.0685
Paeoniflorin	5.104	479.1539	480.1632

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Orobol	5.204	285.0409	286.0477
Phloretic acid	5.339	165.0551	166.063
Methyl ferulate	5.34	207.0658	208.0736
Urolithin C	5.745	243.0296	244.0372
Koparin	5.912	299.0551	300.0634
Coumarin	6.115	145.0298	146.0368
Stevenin	6.822	283.0606	284.0685

2.2. In vitro assays

Proliferation. The highest fibroblast proliferation effects were displayed using 10 µg/ml and 100 µg/ml of aqueous and TAE, showing after 48 h of incubation 51% and 35% of growth increment, respectively. In both cases no proliferative effect was observed after 72 h; , hexane (Hx) fraction showed 50% also using 100 µg/ml while in contrast, using the chloroform (Chl) fraction the proliferative effect was only 25% at the same concentration (Figure 1 a-d). Due to the Aqueous fraction showed the highest proliferative effect at lower concentrations, the subsequent analyses were performed using this fraction.

The proliferative effect of the Aqueous fraction at 10 µg/ml was evaluated measuring PCNA expression by Western Blot assays. Results showed that Aqueous fraction induced the expression of PCNA protein at 24 and 48 h increasing 60 and 110 % the basal expression of PCNA from control fibroblast, at 72 h the expression began to diminish (Figure1e).

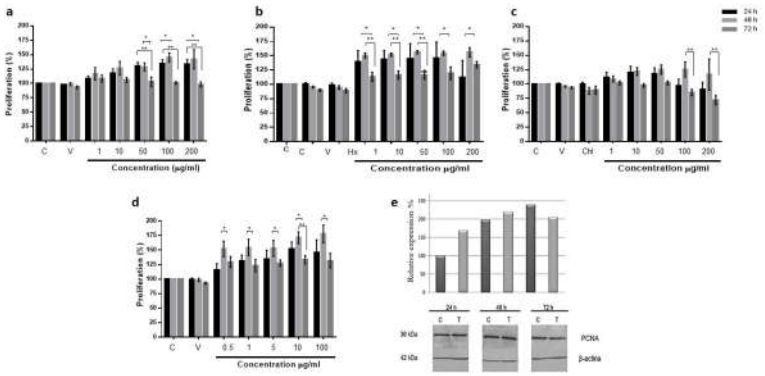

Figure 1. Proliferative effect of *Bacopa procumbens* extracts and fractions on 3T3 fibroblasts. (a) Fibroblasts grown in 2% SFB supplemented media were treated with TAE; (b) Hx; (c) Chl; or (d) aqueous fractions at 0.5, 1, 10, 50, 100 and 200 µg/ml for 24, 48 and 72 h. Cell proliferation was measured by MTT. As solvent controls, fibroblasts were grown with DMSO vehicle (V), 0.25% hexane (Hx), 0.25% chloroform (Chl), or without any solvent as control (C). (e) Total protein extracts from 3T3 fibroblast treated with 10 µg/ml of aqueous fraction were extracted, and WB was carried out to analyze PCNA expression using anti-PCNA antibodies. As for loading control, anti-β actin antibodies were used. The graph shows the densitometric analysis of PCNA expression, protein extracts from 3T3 fibroblasts without treatment (C) and protein extracts from 3T3 fibroblast treated with 10 µg/ml of an aqueous fraction (T). *P<0.05 versus control, **P<0.05 between time. Two Way ANOVA post hoc Tukey´s test.

Migration. - Aqueous fraction at 1, 10 and 50 µg/ml promotes fibroblast migration to an artificially made wound area, reducing the scratch area from 200 µm, at time zero, to 43.65, 73.88, and 56.55% respectively (Figure 2a and 2b). At higher concentrations as in control groups, no significatively migration were observed.

Adhesion. - Concentrations from 1 to 200 µg/ml of Aqueous fraction increase adhesion to fibronectin in all concentrations and incubation times used, having the best results

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using 1, 5 and 10 µg/ml for 30 min, increasing the cellular adhesion 100-125% in comparison to the control group (Figure 2c).

Differentiation. - Expression of α-SMA protein using 10 µg/ml of Aqueous fraction increased significantly after 24 h, displaying a relative increment of about 2 folds in relation to its expression at 0 h. Interestingly, after 48 h expression decrease gradually, while in control without treatment, the α-SMA expression increased continuously to 2.5 folds at 48 and 72 h (Figure 2d).

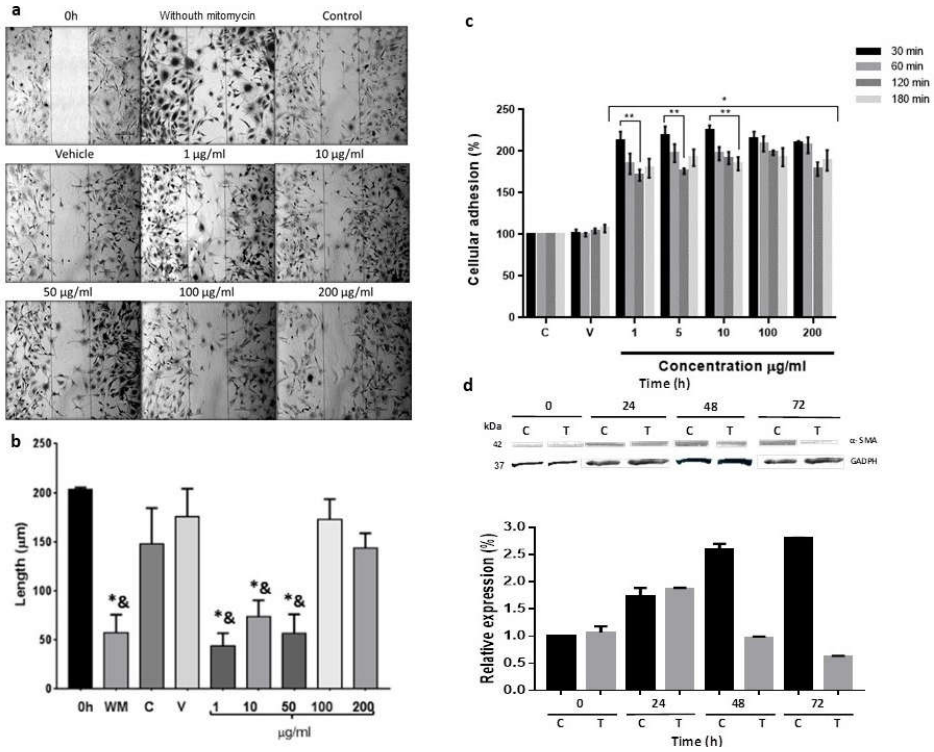

Figure 2. *In vitro* effects on 3T3 fibroblast. (a) Migration, cells were grown at 80% confluence, and scratch was done (0 h). 3T3 fibroblasts were grown with 2% SFB supplemented media without or with 10 µg/ml of mitomycin (control) to inhibit cell proliferation or with 10 µg/ml of mitomycin and 0.2 % DMSO (vehicle); different Aquoeus fraction concentrations (1, 10, 50, 100 and 200 µg/ml) were used for 48 h; image (b) showed the measure of the wound area. (c) Adhesion effect. Culture flasks were covered with fibronectin as described in methods. Cells were grown with 2% SFB supplemented media and treated with 1, 5, 10, 100 and 200 µg/ml of aqueous fraction at 30, 60, 120, and 180 min. Fibroblasts grown with 2% SFB supplemented media (C) or with DMSO vehicle (V) are shown. *p<0.05 versus control, **p<0.05 between time. Two Way ANOVA post hoc Tukey´s test. (d) Differentiation to myofibroblast. Fibroblasts were treated with 2% SFB supplemented media or with 10 µg/ml of aqueous fraction (T) for 24, 48 and 72 h. Total protein extracts were prepared and analyzed by WB using a specific anti- α-SMA antibody for myofibroblast identification. As loading control, anti-β actin antibody was used. The graph shows the densitometric analysis of α-SMA expression.

2.3 Macroscopic wound repair effects of Bacopa extract.

Macroscopic analysis of wound healing showed wound area reduction in the animal groups treated by Polyphenolic compounds from *Bacopa procumbens* (*PB)* compared to animals in WOT or with animals treated with KC. At the beginning, the wounds were almost similar. At day 3, wounds WOT and treated with KC were larger in comparison with the wounds treated with *PB* that were smaller, presenting fine scabs (Figure 3a). On days 5 and 7, the area of wound reduction was evident in the *PB* treated groups compared to other groups, showing moderate scabs, while in the KC group the scab was very

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prominent (Figure 3a). Comparison of the wound reduction area of each group (Figure 3b) showed that 50% of wound reduction area was achieved in WOT and KC groups until days 6 and 7, respectively. Instead, the animals treated with 80 mg/ml and 160 mg/ml of *PB* achieved the 50% reduction on day 5 and 4 respectively, suggesting that treatment particularly with 160 mg/ml of *PB* hydrogel accelerates wound closure by at least 48 h (Figure 3b).

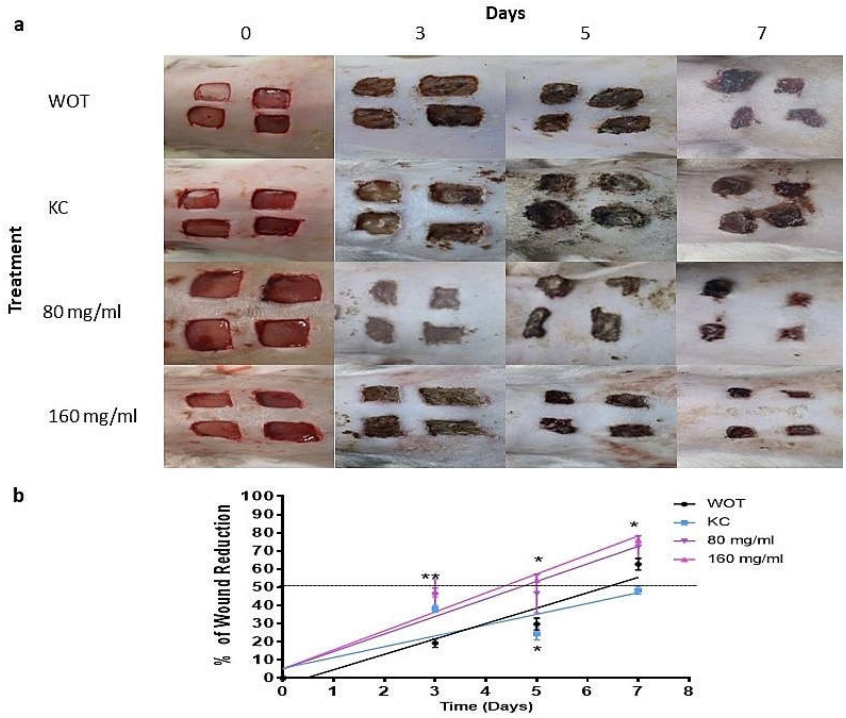

Figure 3. Macroscopic changes induced by *PB* during wound healing. (a) Representative photographs of wounds during the time course of 7 days treatments. (b) Percentage of wound reduction area during the time, continuous lines represent linear regressions of wounds reduction area in WOT, KC and 80 and 160 mg/ml of *PB*. The dashed line represents the 50% of wound reduction. Intersection with linear regressions marks the corresponding times of each treatment to reach 50% wound reduction area. Values are expressed as mean±SEM of at least 6 animals per group. *p< 0.05 versus control as indicated by analysis of variance (ANOVA) and post hoc Tukey's test.

2.4 Histological findings of wound healing from different groups

The lesions at 3, 5 and 7 days after the injury were removed for histopathological analyzes. At day 3, WOT and KC groups showed significant edema and fibrin deposits, infiltration of mononuclear cells and a few amounts of fibroblasts. On the other hand, wounds treated with both *PB* concentrations had less edema and fibrin deposits; infiltration of mononuclear and polymorphonuclear cells and the presence of rounded and disorganized fibroblasts was evident (Figure 4); in group treated with 160 mg/ml of Bacopa extract some elongated fibroblasts were observed. After 5 days, wounds from all groups showed less edema; in the WOT and KC controls, we found some disorganized fibroblasts and an incipient formation of collagen fibers (Figure 5). However, wounds from animals treated with 80 mg/ml *PB*, presented more fibroblast (Figure 4) and an increased collagen content (Figure 4 and 5); wounds from animals treated with 160 mg /ml *PB* showed an important quantity of elongated fibroblasts (Figure 4) and a better distribution and organization of collagen fibers (Figure 4 and Figure 5). On day 7, wounds from all groups showed a remarkable number of fibroblasts and a substantial reduction of inflammatory cells. In the WOT, KC and 80 mg/ml *PB* treated groups, the elongated fibroblasts were still disorganized, few blood vessels were formed, and the collagen fibers were moderately

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organized. Interestingly, in 160 mg/ml Bacopa treated group, we found elongated and organized fibroblast, considerable blood vessels and the collagen fibers were ticker and perpendicularly oriented (Figure 4 and Figure 5).

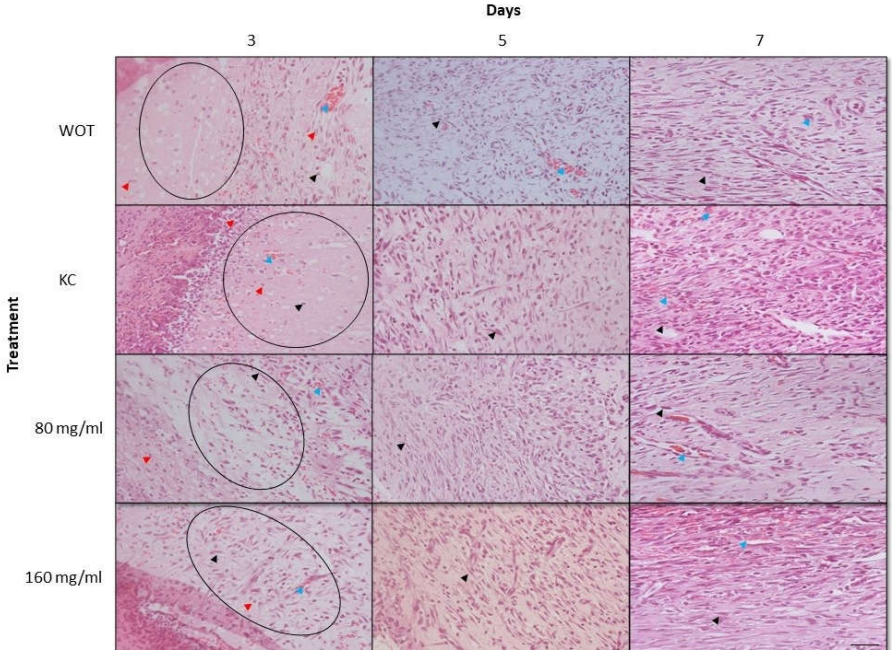

Figure 4. Hematoxyline and eosine (H&E) staining of wounds. Representative microphotographs at 3, 5 and 7 days post-wounding in WOT, KC, and 80 and 160 mg/ml of *PB*. Edema tissue (open circles), inflammatory cells (red arrow), fibroblast (black arrow), blood vessels (blue arrow). Bar = 20 μm

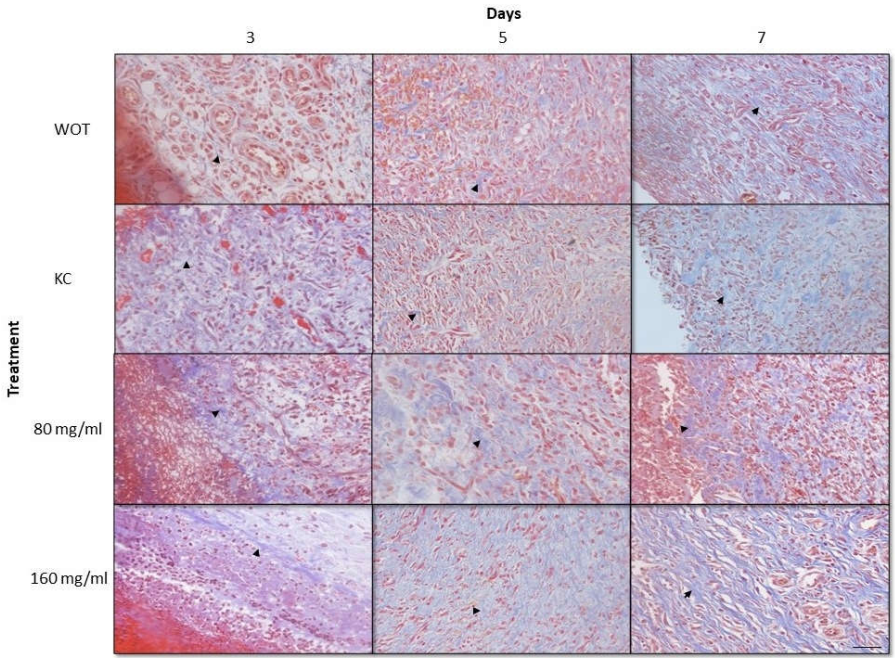

Figure 5. Masson's trichrome staining of wounds. Representative microphotographs at 3, 5 and 7 days post-wounding in WOT, KC and 80 and 160 mg/ml of *PB*. Collagen fibers (black arrow). Bar = 20 μm.

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To determine the collagen types presented in the ECM of the wound repair tissues, Picrosirius Red staining was performed. Under polarized light microscopy, type III collagen, first produced by fibroblasts during wound healing, was detected by greenish birefringence, whereas mature reticular collagen type I is detected in yellow, red [14]. At day 3, both collagens were almost undetectable in the WOT and KC control groups, observing abundant interfibrillar spaces (Figure 6); while in wounds treated with both *PB* concentrations the greenish and yellow-red reticular signals were evident and disorderly arranged (Figure 6). Interestingly, wounds treated with 160 mg/ml *PB* showed thickened collagen fibers. Five days after wounding, the collagen fiber content increased in all groups, in WOT, KC and 80 mg/ml *PB* treated groups the fibers were thin and still disorganized; while in wounds treated with 160 mg/ml *PB*, both types of collagens increased, observing thicker and arranged fibers. After 7 days, collagen fibers tended to be better organized; in WOT the collagen fibers were still thin, whereas, in the KC treatment group, the fibers tend to be arranged in the dermis layer, constituting thicker collagen fibers composed of type I and type III collagens. In wounds treated with 80 mg/ml of *PB*, collagen type I increased but the fibers although oriented, remains thin. Interestingly, in the wounds treated with 160 mg/ml *PB*, the deposition, and orientation of the collagen fibers were highly improved; the arrangement of the collagen fibers tended to be parallel to the surface of the wound and the fibers were mainly yellowish red stained, indicating that the coarse fibers are predominantly compose by type I collagen.

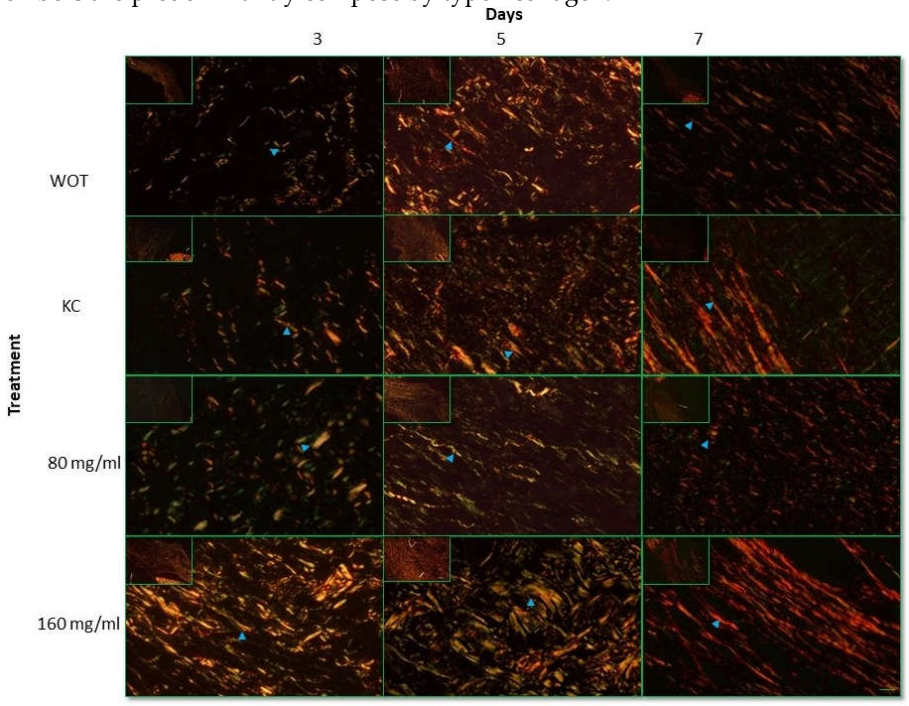

Figure 6. Collagen expression patterns through wound repair process. Representative microphotographs of PicroSiricus-Red staining under polarized light. Greenish fibers (collagen type III) and yellow-red fibers (collagen type I). Collagen fibers (blue arrow). At 3, 5 and 7 days post-wounding in WOT, KC, and 80 and 160 mg/ml of *PB*. Bar = 10 μm

Immune-histochemical analyses revealed that collagen relative expression after 3 days of wound healing process was similar in the four groups, representing approximately the 50% of the protein expression of normal skin at day 0 (Figure 7a and Figure S1). WOT and KC treated groups kept around 0.5 relative expression levels at 5 and 7 days, while 80 mg/ml *PB* treated group showed 0.6 relative expression at the same days. Interestingly, after 5 days of wound healing process, 160 mg/ml *PB* treatment restored almost normal skin levels of type I collagen protein (Figure 7a). Expression of type I collagen gene showed a differential expression pattern. At day 0 tissues from all groups

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showed basal collagen gene expression. At day 3, in WOT group, collagen mRNA increased its basal expression 600 times, slightly growing up after seven days; in KC treated group, the collagen expression was incipient, increasing slowly, having a maximum level at day 7. Tissues from 80 mg/ml *PB* treated group showed the maximum level at day 5 decreasing progressively to basal expression at day 7. In contrast, the treatment with 160 mg/ml of *PB* presented an initial relative increment of 550 times, having the maximum levels after 5 days, interestingly at day 7, collagen relative expression drops from 1200 to 200 times (Figure 7b).

2.5 Bacopa treatment produced scars with better mechanical properties

Mechanical properties of the scars were evaluated by a tensile test after complete wound healing (24 days) was achieved in WOT, KC and in the *PB* treated group that displayed the best wound healing effect (160 mg/ml). The performance load represents the point of failure that is directly proportional to the physical strength of the healed skin. Mechanical strength force of scar from WOT group was 180.622 N/mm2. Wound healing treated with KC displayed a mechanical strength of 222.82 N/mm2, whereas the scar treated with *PB* had a mechanical force of 229.967 N/mm2, representing an increase of 18.76% and 22.56%, respectively, in comparison to the WOT values (Figure 7c). The higher mechanical properties observed strongly correlate with better collagen deposition and fibers alignment observed in the *PB* treated groups (Figure 7d).

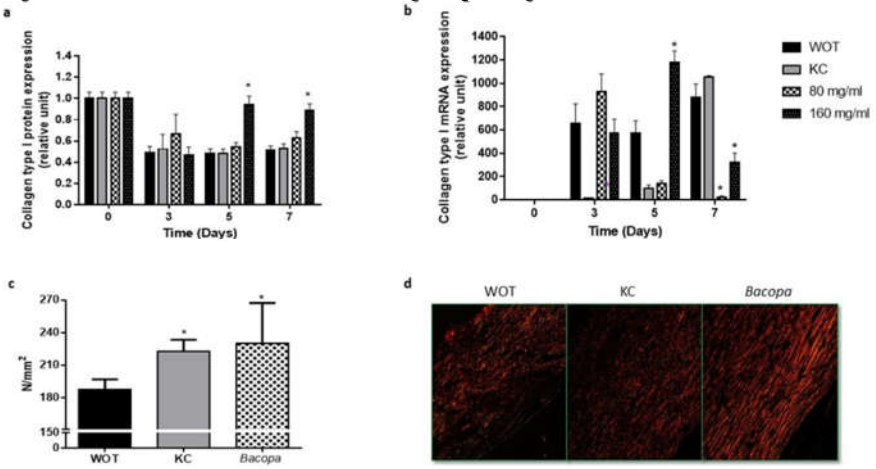

Figure 7. Collagen type I expression and mechanical effect and organization. (a) Collagen type I from wound tissues were analyzed by immunohistochemistry and by (b) qRT-PCR at different periods of time (0, 3, 5 and 7 days). (c) The tensile load applied in wounds were analyzed in scars from WOT, KC, and 160 mg/ml *PB* treated groups after 24 days and (d) representative microphotographs of PicroSirus-Red stain in scars from WOT, KC, and *PB* groups after 24-days of wound healing. Values are expressed as mean±SEM of at least 6 animals per group. *P< 0.05 versus control as indicated by analysis of variance (ANOVA) and post hoc Tukey's test.

2.6 Key molecular effectors of wound repair induced by Bacopa

The proliferative effect of *PB* extract on wound tissue was evaluated by measuring the expression of proliferating cell nuclear antigen (PCNA) (Figure 8a and Figure S2). Results showed that PCNA expression slowly increases in WOT group, having the maximal expression 7 days after injury. In the KC group, the PCNA expression also increased having the expression pick at day 5, decreasing slightly at day 7. In both *PB* treated groups, the PCNA expression was enhanced very early (day 3). Interestingly, after five days, PCNA expression declined, almost reaching the basal expression level at day 7 (Fig.8a and Figure S2).

Immunohistochemistry and RT- qPCR analyses for key molecular effectors TGFβ1, smad 2/3-p and ERK 1/2 -p were done. TGFβ1 protein expression (Figure 8b and Figure S3) reached the maximum expression levels at 7, 5 and 3 days in WOT, KC and *PB*

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treatments, respectively. At day 7 the TGFβ1 expression was down-regulated in KC and 160 mg/ml *PB* treated groups. On the other hand, analysis of TGFβ1 mRNA expression showed (Figure 8c) the highest values in WOT at day 5 decreasing suddenly at day 7; while in KC group, TGFβ1 gene was not expressed at day 3, increasing slowly at day 5, displaying 4 times increment of basal expression at day 7. In contrast, in 80 mg/ml *PB* treated group, TGFβ1 mRNA increase 1.7 and 1,6 folds at day 3 and 5, respectively and decrease at day 7; in 160 mg/ml *PB* a treated group, TGFβ1 mRNA expression augment 2 folds at day 3, having its maximal expression (4.5-fold) at day 5; and suddenly it was almost turned off at day 7 (Figure 8c).

TGFβ could act through a canonical pathway mediated by the phosphorylation of Smad 2/3 [2,8,15] or by an alternative pathway that involves the MAPK, which provokes ERK1/2 phosphorylation [16]. Our results showed that smad 2/3 phosphorylated is slightly overexpressed at day 3 in KC and in both *PB* treated groups. At day 5, its expression increased in all groups and finally at day 7 its expression almost returned to the basal levels (Figure 8d and Figure S4). In contrast, ERK 1/2 phosphorylated remained at basal levels in WOT and KC groups at day 3, while at days 5 and 7, its expression was slightly down-regulated. Interestingly, *PB* treatment increase the ERK 1/2 phosphorylated 1.7 folds at day 3 in both concentrations, and its expression gradually goes down at day 5 (1.4-fold), returning almost to basal levels in wounds treated with 160 mg/ml of *PB* t at day 7, while in 80 mg/ml Bacopa the ERK 1/2 phosphorylated protein was down-regulated similar to WOT and KC groups (Figure 8e and Figure S5).

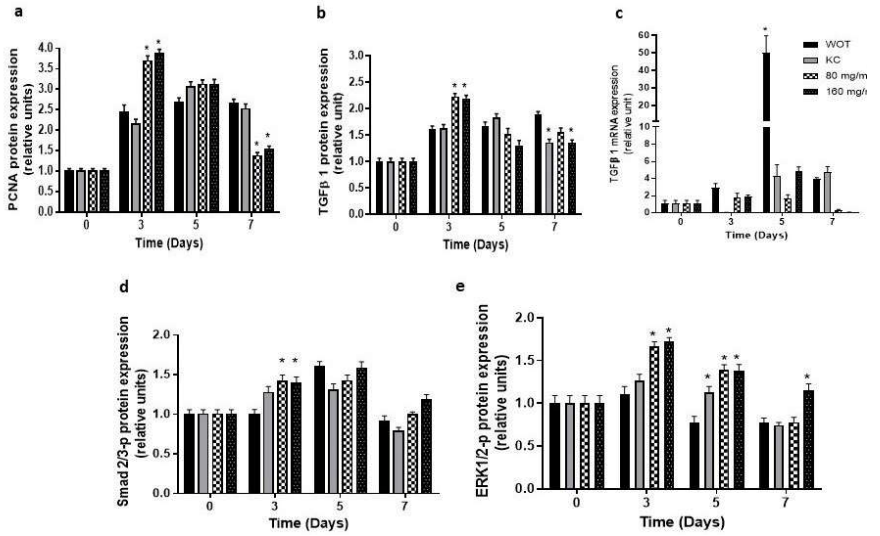

Figure 8. Expression of key regulators of skin wound healing from canonical and non-canonical pathways. (a) Proliferating cell nuclear antigen (PCNA) in wound tissue was analyzed by immunohistochemistry; Transforming growth factor beta (TFGβ1) analyzed by (b) immunohistochemistry and (c) qRT-PCR respectively, in WOT, KC and 80 and 160 mg/ml *PB* treated groups at different periods of time (0, 3, 5 and 7 days).(d) Smad 2/3 phosphorylated and (e) ERK 1/2 phosphorylated analyzed by immunohistochemistry in WOT, KC and 80 and 160 mg/ml Bacopa treated groups at different periods of time (0, 3, 5 and 7 days). Values are expressed as mean±SEM of at least 6 animals per group. *p< 0.05 versus control as indicated by analysis of variance (ANOVA) and post hoc Tukey's test.

3. Discussion

It has been discovered that medicinal resources obtained from plants play an important role in the management of skin disorders [17,18]. The work presented was done to demonstrate the *in vitro* and *in vivo* wound healing activity and the mechanisms and putative molecular pathways exerted by Bacopa extract. Our findings suggest that 160 mg/ml *Polyphenolic compounds from Bacopa procumbens* accelerate the wound healing

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process, regulating the activation of key molecules such as TGF-β1 and ERK 1/2 and the synthesis of collagen, improving the mechanical properties of the scar.

There are few reports on the chemical composition of *Bacopa procumbens* Pathak et al. (2005) and González-Cortazar et al. (2019) reported arbutin in pure ethanol- and a hydroalcoholic mixture-derived extracts, respectively. The same compound was identified in the TAE of the present study. Additionally, Gonzalez-Cortazar et al. (2019) documented the presence of shikimic acid derivatives [19,20]].

The major components such as naringenin, paeoniflorin and rutinoside have an anti-inflammatory and antioxidant effect; also, paeoniflorin regulate the apoptosis in a foot wound healing in diabetic rats [21–23]. Sharath et al., 2010, suggested that B. monniera extract (BME) and Bacoside-A compound also reduce epithelization time and increase tensile strength; however, all these components could improve only one phase [24,25]. NF3 extract from *Radix astragali* and *Radix rehmanniae* improved the healing of diabetic wounds. In vitro, NF3 extract had a proliferation and angiogenic effects that reduce NO production [26].

The results demonstrated by MTT and Western blot assays that the Aqueous fraction at 10 μg/ml induced fibroblast proliferation at earlier times and this effect decreased in later times, suggesting a fine regulation of this process, considered as an important factor in dermis regeneration [27]. Similar results were found with hydroalcoholic extract of *Plinia peruviana* at 100 μg/ml, inducing a fibroblast proliferative effect at 24 h that decreased after 48 h. However, P. peruviana extract at 200 μg/ml had a toxic effect [28]. Instead, Aqueous fraction of *B. procumbens* at 10 μg/ml induced an increment of PCNA protein, displaying 60, 110 and 100 % of proliferation at 24, 48 and 72 h. Alerico et al. in 2015 showed that *Achyrocline satureioides* ethanolic extract using 1 μg/ml stimulated keratinocyte and fibroblast proliferation by MTT assay and by the expression of the proliferative Ki-67 at 24 h, however, the authors did not show the effect after 24 h; we found that Aqueous fraction increase proliferation in a gradual manner and at 72 h the proliferative effect decreased, while in control fibroblast without treatment, proliferation increased slowly having a maximal effect at least 72 h later, suggesting that Aqueous fraction accelerate the process [29].

Cell–cell and cell–matrix interactions are relevant for wound healing process; in natural wound repair key molecules such as fibronectin and collagen, promotes signaling for cell adhesion and cell migration, and it serves as a binding site for an important number of growth factors [30–32]. Our results suggest that *Bacopa* improved cell adhesion at 30 min and interestingly after this time the adhesive effect slowly decreased; Raimoidin et al., 2000 showed that *Sedum telephium* and its polysaccharide fraction, inhibited cell adhesion in fibroblast, whereas the flavonoids fraction does not contribute to this effect [33]. On the other hand, Atropa belladonna L. aqueous extract at 1% induces in human dermal fibroblast, galectin, and fibronectin expression, but did not stimulate any transition from fibroblast to myofibroblast [34]. O di Martino et al., 2017 demonstrated in tissue explants, that Hibiscus syriacus ethanolic extract that contains flavonoids and coumarins, induces fibronectin and filaggrin expression [35], suggesting that this kind of components could be essential for the adhesion effect. We also showed, using scratch assays, that Aqueous enhanced fibroblast migration at 48 h, using 10 μg/ml of aqueous fraction; similar effect was found with P. cyanescens extract [27] and with *Stewertia koreana* ethyl acetate extract increasing the migration of human fibroblasts [36]. Talekar et al., 2017, showed that a 3 μg/ml formulation based on an aqueous extract of *Vitex negundo L., Emblica officinalis Gaertn, and Tridax procumbens L*, having an important content of flavonoids, induced keratinocytes, and fibroblast migration [37]. In contrast, *P. peruviana* hydroalcoholic extract did not affect the migration rate at 24 h, although the extract had cryoprotective and antioxidant effects [28].

Skin fibroblast differentiates to myofibroblast, by inducing the actin gene expression to acquire some contractile proprieties of smooth cells and produce wound contraction. Moreover, the participation of myofibroblast is also related to composition, organization, and mechanical proprieties of ECM [38]. The results showed that *B. procumbens* extract

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induced myofibroblast differentiation at 24 h, drastically diminishing the effect at 72 h. These results suggest that *B. procumbens* extract finely regulates the wound contraction activity, an important fact to lead non-healing wounds [39]. Water et al., 2013, have suggested that myofibroblast probably sense alteration in the mechanical microenvironment and translate these into changes in gene expression to produce a proper contraction, and in the other hand, it is well known that temporally limited myofibroblast function is necessary for normal acute wound healing [40].

We demonstrated that topical treatment of wounds using 160 mg/ml of *Polyphenolic compounds from Bacopa procumbens*, accelerate the wound reduction rate at least in 48 h, compared to WOT or KC groups, this effect was statistically significant in all evaluated times, suggesting that *PB* acts in inflammatory, proliferative and remodeling phases. Süntar et al., 2013 demonstrated that apigenin flavonoid improved wound healing after 12 days; Sharath et al., 2010, using Bacoside-A isolated from *B. monniera* found that its improved healing after 16 days [24,41]. The results give strong evidence to suggest that *PB* has a much better wound healing action, enhancing and accelerating the process to produce a scar with the highest quality compared to other plant extracts using a similar excisional model. In fact, our results also demonstrated that *PB* had a better effect than KitoCell (KC), a patented drug used for wound care. While *PB* hydrogel treatment initiate its modulating action very early (three days), the wound care effects of the commercial product (KC) were observed until day seven, strongly suggesting that KC, in contrast to *PB*, function mainly at the end of wound repair, modulating the tissue remodeling phase. Wound healing process involves cell proliferation and extracellular matrix production, resulting in re-epithelialization [25]; we demonstrated that *PB* produce a faster and better ECM deposition, in comparison to WOT or KC. During the wound healing process, type III collagen is the first produced; it acts as a scaffolding bridge into the wound, later type I collagen is produced to construct in junction with type III, a solid support [42]. We evidence by PicroSirus red staining and by immune-histochemical methods that *Polyphenolic compounds from Bacopa procumbens* improve the dynamics of collagen replacement, deposition, and arrangement. The arrangement of type I collagen fibers tended to be parallel to the surface of the wounds, similar to normal skin arrangement. Meanwhile, in the WOT or KC groups the collagen fibrils constituted mainly by collagen type III, were still aligned at random at similar experimental times that *PB* treatments. Murthy et al. 2013, showed that after 10 days post-wound in "wound incision model", rats treated with *B. monniera* extract increased the hydroxyproline content, relating it to the total collagen content, but they did not demonstrate its structuration within the tissue. The correct distribution of collagen in the skin contributes to restoring skin functionality and strength of rupture4. We found that after 24 days of wound healing, the tensile strength within the scar increased by 22.56% in the group treated with Bacopa compared to WOT group, suggesting a better functional performance. *Acalypa indica* has been shown to have flavonoids and glycosides that accelerate wound contraction and increased tensile strength due to increased collagen deposition during the day 10 post-wound [43]. Phytochemical and HPLC analyses of *PB* showed phenolic components, strongly suggesting this could be responsible for the wound repair effects

We searched for the proliferative effects by measuring PCNA expression, the results suggest that *PB* improved cell proliferation in earlier times, while it is downregulated in the late phase, suggesting that *PB* extract modulates the proliferative process in the replacing tissue, avoiding the excess of dermal fibrosis and scarring, which are known to produce hypertrophic and keloid scars [44]. This reduction of the proliferative effect in the late phase could be related to the reduction of the inflammatory phase in the group treated with Bacopa, correlating it with the induction of collagen synthesis at day 3.

TGF-β represents a key molecule that regulates many events around the complete wound healing process [45,46]. It is shown that that *PB* treatment stimulates an earlier expression of TGF-β1, and its expression is turned off during resolution phase. Zhang et al. 2012, have suggested by in vitro studies that extract from *Radix astragali and Radix rehmanniae* induced over expression of TGF-β1 [47]. Suh et al., in 2003 reported that some

synthetic triterpenoids could mimic TGF-β1action in macrophages, activating the Smad2 pathway [48]. Using specific IHCs assay, the results suggested that Bacopa extract activated both TGF-β1 pathway, the canonical activating Smad2/3 complex, and ERK 1/2 phosphorylation: this complex regulated cell proliferation and differentiation, cytoskeletal structure, apoptosis, and other biological reaction [16,49]. Kim et al. 2017, showed *in vitro* that *S. chamaejasme* extract induced the proliferation of keratinocytes by activating ERK and Akt signaling; the extract from this plant also induced mRNA expression of collagen type I and III *in vitro*, suggesting that these pathways were involved in the wound healing activity [50]. The in vivo results demonstrated that *PB* treatment regulates ERK 1/2 activation in earlier phases, similarly as the TGF-β1 expression, strongly suggesting that *PB* could activate MAPK pathway, resulting in the improvement of wound healing effects; the results are in concordance with Kim findings, even though they only showed ERK activation *in vitro* model.

The specific interaction between *Polyphenolic compounds from Bacopa procumbens* with its putative receptor(s) is currently in progress. Also, works in progress are a focus on the identification and purification of the active compound (s) responsible for the wound healing effect.

4. Materials and Methods

4.1 Preparation of aquoethanolic extract and hydrogel formulation

The *Bacopa procumbens* (Mill.) Greenm plant was harvested from the state of Hidalgo, Mexico; and identified with the voucher specimen number 1972 (Herbolaria IZTA-Flora Útil-Facultad de Estudios Superiores Iztacala-UNAM). We followed the methods of Gómez et al 2016, the whole plant was dried, and ground; 40 g of powdery were extracted with aquoethanolic solution 50:50 (600 ml) at 76 °C for 4 h for three times using a reflux system, and finally it was lyophilized, the percentage yield was 30% w/w [11]. Total aquoethanolic extract (TAE) was obtained after removal of solvent under vacuum at 50 °C. Using 10 g of TAE, secondary extractions were carried out, resuspending it in 100 ml each, of water, hexane, or chloroform for 2 h at room temperature. Then, each fraction was filtered and concentrated using a rotary evaporator under vacuum conditions to obtain aqueous (9.187g, Aq); n-hexane (0.61g, Hx) and chloroform (0.203 g, Chl) fractions, respectively. All dried extracts were stored at 4 °C.

The phenolic profile of TAE was characterizing using chromatographic analysis coupled MS detector following the method proposed by Muñoz-Bernal et al. (2021). The HPLC system was an Agilent Infinity Series 1290 LC system with an Agilent 6500 Series Q-TOF MS. A Zorbax Eclipse plus C18 column (50 mm x 2.1 mm, 1.8 µm) (Agilent® Technologies, Santa Clara, CA, USA) was used at 25 °C for the separations a flow rate of 0.4 mL/min. Following elution program was used: 0-1 min 10 %, 1-4 min 30 %, 4-6 min 38 %, 6-8 min 60 %, 8-8.5 min 60 %, 8.5-9 min 10 %. Samples were filtered through 0.45 µm nylon filters and the injection volume was 1 µL. Conditions of Q-TOF MS were: ESI in negative mode, nitrogen as drying gas at 340 °C with a flow rate of 13 L/min; pressure of nebulizer was set at 60 psi, capillary voltage 175 V and the scanning mass to charge range of the Q-TOF mass analyzer was 100-3200 m/z. The identification of phenolic compounds was performed using retention times, UV/Vis spectra, and mass spectra from QTOF-MS using the Mass Hunter Qualitative software version B.07.00.

Using 10 g of TAE, secondary extractions were carried out, resuspending it in 100 ml each, of water, hexane, or chloroform for 2 h at room temperature. Then, each fraction was filtered and concentrated using a rotary evaporator under vacuum conditions to obtain aqueous (9.187g, Aq); n-hexane (0.61g, Hx) and chloroform (0.203 g, Chl) fractions, respectively. All dried extracts were stored at 4 °C.

For the in vivo assay TAE was included in a hydrogel (water, carbopol 0.7%, glycerin 1%, hydantoin, methylchloroisothiazolinone, and methylisothiazolinone 0.2% and triethanolamine 1%) at 80 mg/ml and 160 mg/ml final concentration.

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4.2 In vitro model

4.2.1 Cell culture

The mouse embryonic fibroblast cell line NIH 3T3 (3T3), obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), was grown in Dulbecco's Minimal Essential Medium (DMEM), supplemented with 10 % heat-inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were maintained at 37 °C, in a 5 % CO2 humidified atmosphere, and passaged after being detached from culture dishes P60 with 0.05% trypsin and 0.002% EDTA solution. Cells at 80–90% confluence were used for seeding and experiments.

4.2.2 Cell viability assay

3T3 fibroblasts were incubated with TAE, Hx, Chl or Aq for 24, 48 and 72 h, using 0.5, 1, 10, 50, 100 and 200 µg/ml, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays were used to determined cell viability. Cell proliferation was determined using crystal violet staining.

4.2.3 In vitro scratch wound-healing assay

Fibroblasts were seeded until 90-95% and cell cycle were arrest with mitomycin (10µg/ml) (MITOLEM, Leremy). Briefly, in the middle of the cell monolayer, a scratch was made. Then, cultures were incubated with the different concentrations of Aq (1, 10, 50, 100 and 200 µg/ml) for 48 h. Scratch wound closure was analyzed, capturing microscope images, and analyzed with the software Image Pro Plus.

4.2.4 Cell adhesion assay

Ninety-six-well plates were coated with serum purified fibronectin (10 mg/ml), incubated overnight at 4 °C for 12 h and blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at 4 °C for 2 h. Fibroblasts (104 cells/well) were incubated with different concentrations (1, 5, 10, 100 and 200 µg/ml) of Aq during 30, 60, 120 and 180 min. Adhered fibroblasts were fixed with paraformaldehyde (4%) and stained with 0.1% crystal violet (Sigma-Aldrich, Munich, Germany).

4.2.5 Western blotting analysis

Fibroblasts were treated with 50 µg/ml of the Aq for 24, 48 and 72 h, in 2% FBS fresh medium. The cells were resuspended in ice-cold lysis buffer (10 mM/L Tris-HCL, 5 mM EDTA, 150 mM/L NaCl, 0.1 % SDS, 1% NP-40, 1% sodium deoxycholate) containing a protease inhibitor cocktail (1 mml/L phenylmethyl sulfonyl fluoride, 100 µg/L leupeptin, and 2 µg/L aprotinin). The protein concentrations were determined using Bradford protein quantification assay (Biorad). Protein lysates (30 µg) were subjected to electrophoresis on 10% SDS- polyacrylamide gels and transferred to 0.45 µm nitrocellulose membranes. The membranes were stained with 0.2 % Ponceau S red to ensure equal protein loading. After blocking with 5% milk in PBS- Tween, membranes were incubated with primary rabbit antibodies against α -SMA (1:200, Santa Cruz, CA), PCNA (1:200, Santa Cruz, CA) or β- actin (1:1500, Santa Cruz, CA) for 2 h. Membranes were finally incubated with horseradish peroxidase-conjugate secondary antibody (1:200), and the immunocomplexes were visualized by ECL Western blotting detection reagents. All experiments were performed by triplicate.

4.3 In vivo skin wound model

4.3.1 Animals

220-240 g male Wistar rats were maintained at 26 °C under 12:12 hours light/dark cycle. The animal received chow and water ad libitum. This experimental work followed the guidelines of the Norma Oficial Mexicana Guide for the use and care of laboratory animals (NOM-062-ZOO-1999) and the disposal of biological residues (NOM-087-ECOL-1995). The ethics committee of ENMyH postgraduate section approved the experimental procedure of this study (approval number CBE009/2019).

The animals were divided into four randomized groups: untreated group (WOT), control group using KitosCell®(KC), CellPharma [9], and two experimental Bacopa treated groups using 80 mg/ml and 160 mg/ml TAE. The animals were anesthetized, the dorsal region was chemically shaved (Veet cream, Reckitt Benckiser), harvested and four complete thickness excision surgeries of 1 cm2 were performed12. The wounds were

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treated daily, topically with 100 µl per wound of 8% KC or with 80 and 160 mg/ml of Bacopa extract hydrogel. The healings were observed and photographed by a digital camera 16 MPX at 15 cm away using a pedestal, the measuring immediately after surgery and at 3, 5 and 7 days were performed using a Vernier caliper; after photographed digitization, the wound area was measured using Image Pro plus software (Media Cybernatics, Inc. USA) to calculate the percent of wound reduction, then the formula % of wound reduction= 100 - [(final areaX100)/initial area] was applied. At 3, 5 and 7 days, wound lesions were removed with adjacent healthy skin, fixed in 4% buffered paraformaldehyde; embedded in paraffin and 5 µm thickness sectioned, and then were stained with hematoxylin & eosin, Trichome Masson, PicroSirius-Red reagent (Abcam, USA) using standard protocols13. Finally, tissue samples from the six rats of each experimental group were examined, and analyzed in three different areas from three consecutive sections and photographed using the Olympus DP74 system (Olympus, Tokyo, Japan) or using a polarizing light microscope (Nikon, Tokyo, Japan)

For tensile test, WOT, KC and 160 mg/ml animal groups were sacrificed at 24 day and the wound healing skin tissue was cut with a dumbbell shape using a custom-made metal template. The tensile test was performed using the Texture Analyzer ta-xt2/1 (Stable Microsystems, Vienna Cour, England) using a 100N load cell at a constant strain rate of 1 mm/sec

4.3.2 Immunohistochemistry

Protein detection was performed using the PolyDetector HRP / DAB Mouse / Rabbit Detection System (Bio SB, USA). Tissues were rinsed with PBS and epitope retrieval was performed in a pressure cooker using the Immune Retrivever Citrate Solution (Bio SB, USA). Slides were cooled to room temperature and incubated in Peroxidase Block quenching buffer (Bio SB, USA) for 20 min to block endogenous peroxidase activity. After sections were washed with PBS, nonspecific binding sites were blocked for 30 min with BSA, Cohn fraction V, pH 7.0; then, sections were incubated 16-18 h at 4 °C with primary antibodies: mouse monoclonal anti-Collagen type I (Calbiochem, USA); PCNA (Santa Cruz Biotechnology, USA); TGF◎1(Santa Cruz Biotechnology, USA); Smad 2/3-p (Santa Cruz Biotechnology, USA) and ERK1/2-p (Santa Cruz Biotechnology, USA) all diluted 1:100. Following three washes with PBS, samples were incubated for 30 min with secondary antibody (PolyDetector HRP label; Bio SB, USA), counterstained with hematoxylin and mounted in GVA-mount reagent (Zymed, USA). Negative controls without primary antibody were carried out. The slides from each experimental group from three consecutive sections were observed and photographed using the Olympus system (supplementary figure1-5). Quantification of expression protein was performed by pixel measure using the Image-Pro Premier software (Media Cybernatics, Inc. USA).

4.3.3 Quantitative real-time RT-PCR

Total RNA was extracted from normal and wound tissues using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). For reverse transcription, SuperScript II Reverse Transcriptase kit (Invitrogen, USA) was used to obtain cDNAs for qRT-PCR assays. Primers used for qRT-PCR were collagen type I 900 nM forward 5′-CAACCTGGATGCCATCAAGG-3′ and reverse 300 nM 5′-ATCGGTCATGCTCTCTCCAAA-3′; TGF◎1 900 nM forward 5′-GCAGTGGCTGAACCAAGGAG-3′ and 300 nM reverse 5′-TCGGTTCATGTC-ATGGATGG-3′, GADPH 50 nM forward 5′-CACCACCAACTGCTTAGCCC-3′ and reverse 50 nM 5′-TCTGAGTGGCAGTGATGGCA-3′. All reactions were performed using SYBR-Green PCR master mix (Applied Biosystems, USA) in 7300 Real-Time PCR System (Applied Biosystems, USA). As an internal control, glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was used in parallel to the target genes. Normalization and fold changes were calculated using ◎◎Ct method.

4.4 Statistical Analysis

Statistical significance was analyzed using ANOVA post hoc Turkey 's test. All analyses were performed using Graph Pad Prism software version 7.0.

5. Conclusions

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All the results presented, suggest that 160 mg /ml Bacopa extract treatment improve and accelerate wound healing process regulating more than one event of different wound phase process, it reduces the inflammatory phase, modulate TGFβ1 action, promoting a proliferative effect, also enhancing collagen synthesis and correct deposition, resulting in an optimal physiological and mechanical proprieties of scar tissue repaired.

Supplementary Materials: The following supporting information can be downloaded at:

Figure S1: Collagen type I expression. Representative microphotographs of collagen type I protein by immunohistochemistry detection on wound tissue at different periods of time (0, 3, 5 and 7 days) in WOT, KC, and 80 and 160 mg/ml of *PB*.

Figure S2: PCNA expression. Representative microphotographs of PCNA protein by immunohistochemistry detection on wound tissue at different periods of time (0, 3, 5 and 7 days) in WOT, KC, and 80 and 160 mg/ml of *PB*

Figure S3: TGFβ-1 expression. Representative microphotographs of TGFβ-1 protein expression from wound tissues were analyzed by immunohistochemistry at different periods of time (0, 3, 5 and 7 days) in WOT, KC, and 80 and 160 mg/ml of *PB*.

Figure S4: SMAD 2/3-p expression. Representative microphotographs of SMAD 2/3 phosphorylated protein by immunohistochemistry detection on wound tissue at different periods of time (0, 3, 5 and 7 days) in WOT, KC, and 80 and 160 mg/ml of *PB*.

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Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

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Sample Availability: Samples of the compounds are available from the authors.

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