

Wound Healing Properties of *Ziziphus jujuba* Mill. leaves

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Abstract

A dry ethanolic extract was obtained from *Ziziphus jujuba* Mill. leaves. To select the best solvent and extraction method for flavones and polyphenol-carboxylic acids, initially was applied a multiple linear regression model with the method and solvents as dummy variables, which indicated the largest positive influence on extraction for ethanol 70% and the largest negative effect for sonication at room temperature. Then, was dropped out the sonication method and was applied a simple linear regression model with the solvent as an independent variable for reflux extraction. The extract embedded in simple ointment (10% w/w) was evaluated in rat for wound healing activity by topical application for a period of 12 days. The animal experiment was carried out using Cicatrizin (a known commercial ointment) as a positive control. The animals treated with *Z. jujuba* leaf extract showed a healing of 82.25% compared to the beginning of the experiment. The healing effect was similar to Cicatrizin (83.10%) and slightly higher, but not statistically significant from the one seen in the negative control group (76.2%, $p=0.259$) at the end of the study. Complete healing occurred after 18 days in both groups, without obvious scars and after 20 days in the control group.

Keywords: *Ziziphus jujuba* Mill., ethanolic extract, wound healing activity

1. Introduction

Complex wound healing process consist in replacing affected cellular structures and tissue layers by new, healthy cells and tissues. This process has 3 distinct phases (inflammatory, proliferation and remodeling) and comprises dynamic phenomena: chemotaxis, phagocytosis, neocollagenesis, collagen degradation and collagen remodeling. Angiogenesis, epithelization and the production of new glycosaminoglycans and proteoglycans are very important for the wound healing [1, 2, 3, 4]. In present the researchers in the field of wound healing are considering the internal and external factors that influence the reparation of damaged tissue. Laser and nonlaser techniques, are being investigated to increase the rate of wound healing [5, 6]. In order to enhance the healing wounds, growth factor TGF- β 3, hyperbaric oxygen, platelet-rich plasma or erythropoietin have been used successfully in recent years [7, 8, 9, 10, 11, 12]. It has also been shown that stem cells, especially the adipose-derived ones, can improve the wound healing process [13, 14]. A wound healing favorable factor is improving the nutritional status

of the patient [15, 16]. Phytotherapy plays an important role in the healing process of wounds. Pine bark from the Turkish pine (*Pinus brutia* Ten.) was investigated in this sense with positive results in a rat model [17]. Other species known since ancient times for their healing properties are those belonging to genus *Aloe*. Regarding their potential curative effects, *Aloe arborescens* Mill. seems to be more potent as compared with *Aloe vera* (L.) Burm.f. [18]. Known for their healing properties are also *Calendula officinalis* L. [19], *Hypericum perforatum* L. [20] and *Symphytum officinale* L. [21]. *Ziziphus jujuba* Mill. (jujube, red date, Chinese date) from the Rhamnaceae family is a promising plant with healing properties. The traditional healers of Bastar region use the dried leaves and powdered bark to dress wounds [22]. The species of this genus are used medicinally in India, China and Japan for their putative hypoglycemic, sedative, anticancer, hepatoprotective and anti-inflammatory properties [23-27]. Regarding the chemical composition of *Ziziphus jujuba* leaves, the presence of saponins [28, 29], triterpenic acids (ceanothic acid, epiceanothic acid, ceanothenic acid, aliphilic acid, maslinic acid, zizyberanolic acid, 2-hydroxyursolic acid, betulinic acid, and oleanolic [30, 31] and flavonoids (quercetin-3-O-rutinoside) [31] has been reported. which exhibited multiple activities, such as antimicrobial [32], anti-inflammatory [33] and wound healing effects [34].

2. Materials and Methods

A dry leaf extract was obtained from *Ziziphus jujuba* leaves. Three solvents (ethanol 70% v/v, ethanol 50% v/v and purified water) and two extraction methods (refluxation and sonication at room temperature) were investigated to select the best extraction solvent and method for flavones and polyphenolcarboxylic acids (PCA), which were spectrophotometrically assayed by known methods. The extract was made from finely ground leaves (sieve IV) of indigenous plant harvested from Research Institute for Fruit Growing Pitesti, in June 2015. The powder was refluxed with ethanol 70% three times (1:10, m/v), and the resulted solutions were pooled and concentrated at 60 °C using a rotary evaporator (Ingos RVO 004). The concentrated solution was freeze-dried at - 55 °C (using a Scanvac CoolSafe Freeze Dryer). For the non-clinical assessment, the extract was embedded in simple ointment (10% w/w). For this experiment, three groups of male Wistar rats were used (n=10 per group), weighing 200 ± 10 g. Animals were kept in laboratory conditions for 2 days to get used to their new habitat (experimental room temperature 23 ± 2 °C, humidity of 40-50%, artificial lighting, alternating 12 hours light / 12 hour dark). Food regimen consisted of feeding at 8:00 a.m. and 17:00 p.m. and water *ad libitum* in bottles. The experiment was carried out in compliance to the provisions of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and the implementing Law no. 43/2014 on the protection of animals used for scientific purposes. The animals had hair shaved on the dorsal area. After ether anesthesia, wounds were produced by means of a device consisting of a metal disc with a diameter of 1 cm that was heated in water with 5% NaCl at 105°C. The disc was heated and applied to the dorsal shaved area and maintained for 10 seconds [35, 36, 37]. The animals were distributed by randomization method in batches of 10 animals and were treated as follows:

Lot 1 – the control group, untreated;

Lot 2 – the group treated with Cicatrizin (positive control group)

Lot 3 – the group treated with the dried leaf extract obtained from *Ziziphus jujuba* leaves embedded in simple ointment (10% w/w).

The treatment was applied once daily, for 12 days. The evolution of wounds was observed at every two days by measuring the treated surfaces (in mm²) in comparison with those of the negative and positive control groups. During the study, the clinical condition of animals was monitored [38].

Statistical analysis. Computations were carried out in the statistical and programming environment R, versions 3.2.0, using the „car“ [39], „MASS [40]“, QuantPsyc [41] and „gvlma“ [42] packages. In order to choose the solvent and the extraction method we have initially applied a multiple regression model with the extraction conditions (reflux, sonication) and solvents (water, ethanol 50%, ethanol 70%) codified as dummy variables. This model has indicated the greatest positive influence on the extraction for ethanol 70%, and the largest negative influence for ultrasonication. Because of the negative effect of ultrasonication, we have eliminated this variable from the selection variable list and built a new, simple linear regression model with solvent as the independent variable (equivalent to an one-way ANOVA). The same approach was used to select the solvent and the extraction method for flavones. The normality of residual distribution was assessed visually by examining quantile-quantile plots and histograms of the studentized residuals. To evaluate the assumption of homoscedasticity the Breusch-Pagan test, scale-location and residuals versus leverage plots were employed. Besides, a global validation of the model assumption was performed using the “gvlma” R package. Statistical evaluation of the non-clinical results was done by "t" student test and ANOVA.

3. Results and discussions

In the case of PCAs, the initial regression model (evaluating simultaneously the solvent and extraction method) indicated a negative effect of ultrasonication (at room temperature) when compared with reflux (at hot temperature) and an efficacy somewhat better of ethanol 50% in comparison with ethanol 70% and water. The standardized coefficients (all statistically significant, $p < 0.001$) for ethanol 50%, ethanol 70% and ultrasonication were 0.46, 0.36 and -0.80. In the second model, ethanol 70% was slightly superior to ethanol 50%, far from reaching the conventional threshold of statistical significance ($p = 0.4723$); instead, water had a negative effect on the extraction yield when compared with alcoholic solvents ($p = 0.002$). The standardized coefficients for ethanol 70% and water (compared with ethanol 50%) were 0.27 and -1.29. Taking into consideration that ethanol 70% had a coefficient higher than ethanol 50% (although not statistically significant) and considering the limited statistical power (by increasing the number of samples tested, it might reach statistical significance) we have selected ethanol 70% as the extraction solvent. The variation of the PCA concentration as a function of solvent and the extraction method is shown graphically in Figure 1.

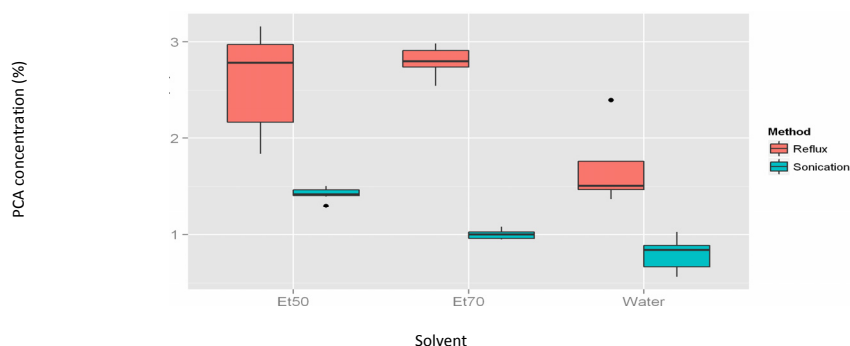


Fig. 1. Variation of the PCA concentration in the extractive solutions depending on solvent and extraction method.

For the flavone derivatives, the initial regression model (assessing simultaneously the solvent and extraction method) has also indicated a negative effect of ultrasonication (at room temperature) in comparison with reflux (hot temperature) and a decreased efficacy of alcoholic solvents (ethanol 50%, ethanol 70%) in comparison with water (but the negative coefficients were not statistically significant). The standardized coefficients for ethanol 50%, ethanol 70% and ultrasonication were -0.05, -0.13 and -0.81, respectively. In the second model (evaluating the solvent effect in the case of reflux as an extraction method), ethanol 70% was slightly superior to ethanol 50%, while water was slightly inferior to ethanol 50%, but the coefficients did not reach the conventional threshold of statistical significance (0.05). The standardized coefficients for ethanol 70% and water (in comparison with ethanol 50%) were of 0.71 and -0.36, respectively. The variability recorded experimentally was higher than in the case of PCAs and the applied model explained less than a quarter of the variability seen ($R^2=0.239$, R^2 ajustat=0.144). Considering that ethanol 70% was not different from the statistical significance perspective in influencing the extraction yield as compared with ethanol 50%, but also that the p value of the coefficient ($p=0.159$) indicated a trend towards statistical significance, and with a view to extracting simultaneously the PCAs, ethanol 70% was selected as an extraction solvent. The variation of flavone concentration as a function of solvent and the extraction method is shown graphically in Figure 2.

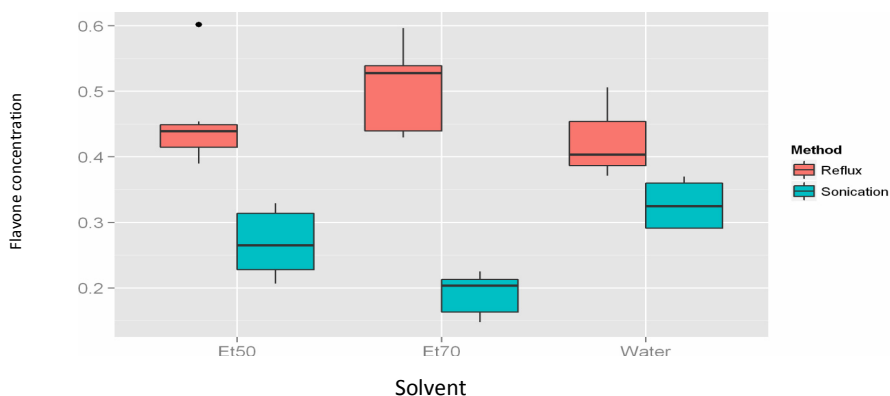


Fig. 2. Variation of the flavone concentration in the extractive solutions depending on solvent and extraction method.

The evolution of wound healing in our experiment is shown in table 1 and figures 3, 4, 5 and 6. The negative control animals presented initially a surface area burn of 94.1 mm²; after 4 days of treatment it decreased to 66.1 mm², after 10 days it reached 37.5 mm², and after 12 days treatment it reached 21.9 mm², corresponding to a healing extent of 76.72% as compared to the initial surface. Complete healing in the negative control occurred after 20 days.

The animals treated with the dried leaf extract obtained from *Ziziphus jujuba* embedded in simple ointment (10% w/w) showed a healing extent of 33.01% after four days of treatment, and of 65.91% after ten days. At the end of the experiment they were healed in proportion of 82.25% as compared to the beginning of the experiment. Complete healing occurred after 18 days. The animals treated with Cicatrizin positive control showed a healing of 28.67% after four days of treatment, 67.71% after ten days and 83.10% after 12 days. Complete healing occurred after 18 days. Although at day 12 of the experiment the difference in the healing extent between the three groups was not statistically significant, this might be related to the Romanian Biotechnological Letters, Vol. 21, No. 5, 2016

small sample size in each lot. As shown in table 1, the two active products (the tested extract incorporated in simple ointment and the reference product Cicatrizin) accelerated wound healing in the first 6 days, whereas after this interval the difference between the active treatment groups and the negative control diminishes and become statistically non-significant. The effect observed for the first 6 days may be relevant though, because it is known that in the case of burn wounds, the extent of the burn is one of the most relevant risk factors, larger burns having a stronger tendency to develop infection at the wound site [43].

Table 1. The evolution of wound healing effect

Group	Wound area (mm ²)						
	$\bar{X} \pm SD$						
	day 1	day 2	day 4	day 6	day 8	day 10	day 12
Control	94.1±15.98	83.8±10.62	66.1±5.56	67.2±17.81	48.6±13.14	37.5±16.17	21.9±15.09
E%	-	10.94	29.75	28.58	48.35	60.14	76.72
Ziziphus ointment	93±12.53	72.4±12.11*	62.3±11.54	52.4±13.40	42.4±10.9	31.7±11.35	16.5±5.23
E%	-	22.15	33.01	43.65	54.40	65.91	82.25
Cicatrizin	85.8±17.79	70.8±15.44*	61.2±12.89	45.6±13.93**	38.8±12.29	27.7±7.51	14.5±7.38
E%	-	17.48	28.67	46.85	54.77	67.71	83.10
F (ANOVA)	0.83616	3.02623	0.59998	5.29262	1.66436	0.34097	1.41913
P (ANOVA)	0.44429	0.06519	0.55598	0.0115	0.20815	0.7141	0.2594

Statistical significance : *p<0.05, **p<0.01 compared to initial.

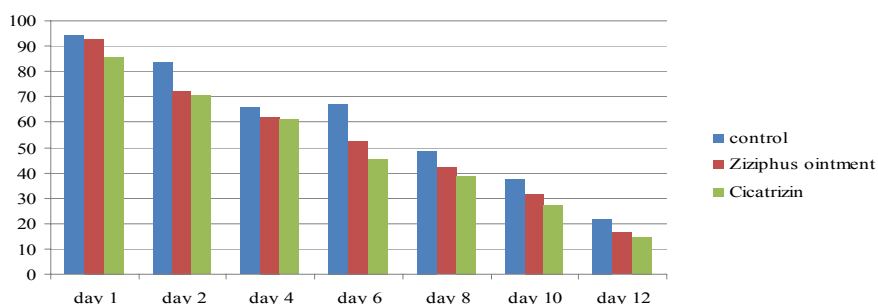


Fig. 3. Graphical representation regarding the evolution of wound healing

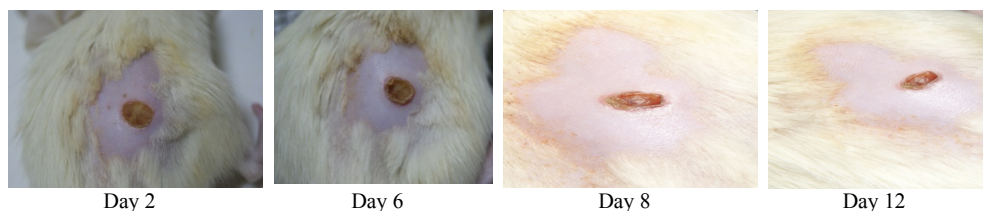


Fig. 4. The evolution of wound healing observed at controls across time

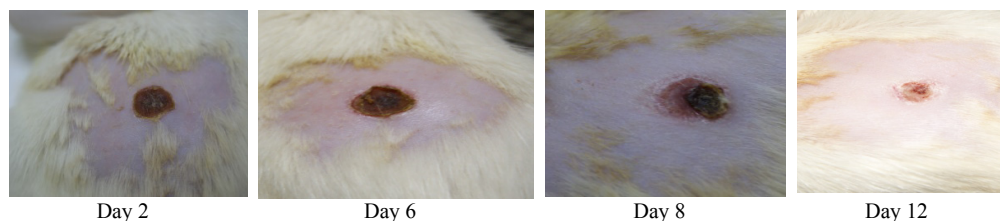


Fig. 5. The evolution of wound healing observed at the group treated with *Ziziphus* leaves extract across time

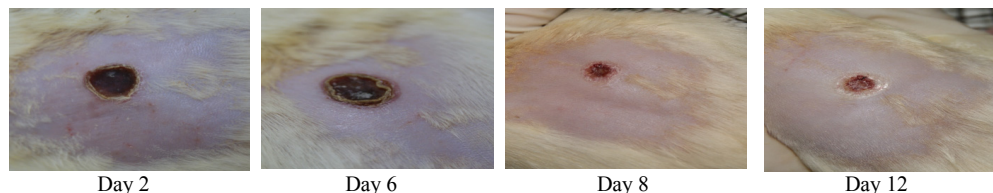


Fig. 6. The evolution of wound healing observed at the group treated with Cicatrizin across time

This *Ziziphus jujuba* activity of wound healing promotion observed on experimentally produced plagues on skin is probably related to its complex chemical composition (including flavonoids, triterpene acids, saponins). Recent researches have demonstrated that these constituents accelerate wound healing process. Kaempferol-3-O-rutinoside, for instance, which is found in *Z. jujuba* [44],) was reported to act by inducing the formation of filopodia and lamellipodia, raising the cellular levels of phosphorylated FAK and Akt and up-regulating Rac1-GTP [45]. Maslinic acid (identified in *Z. jujuba* [46]) has anti-inflammatory effects, inhibiting nuclear factor-kappa B activation and the phosphorylation of I κ B- α [47], possibly mediated by inhibition of iNOS and COX-2 expression [48]. Oleanolic acid is known for healing properties from several experimental studies [49-51].

4. Conclusions

In this study the action of a dry extract obtained from *Ziziphus jujuba* leaves embedded in simple ointment (10% w/w) was assessed in comparison to Cicatrizin used as a reference product (positive control). It was found that the ointment from *Ziziphus* leaf extract showed a wound healing activity similar to Cicatrizin. The healing occurred in both cases without obvious scars. The effect was significant in the first 6 days from the occurring of the wounds and initiation of treatment. The results obtained in this study may explain the traditional use of *Ziziphus jujuba* leaves for healing wounds.

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