Determination of *Teloschistes flavicans* (sw) norm anti-inflammatory activity

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ABSTRACT

Background: Lichens produce a variety of substances that possesses pharmacological actions. However, rare products are submitted to rigorous scientific tests or have the risk potential or side effects evaluated. The lack of medical and sanitary control, absence of accurate botanical identification or purity certification, founded in diverse natural products, may represent great danger to population health. This work aimed to evaluate toxic effects and anti-inflammatory action *in vivo* of *Teloschistes flavicans* (Sw.) Norm. (TFN) unrefined extracts, as well as determinate its main constituents. **Methods:** The carrageenan induced paw edema and cotton pellet implant induced granuloma methods were utilized, besides a classic acute toxicity test. TFN acetone extract inhibited carrageenan paw edema on 60, 120, and 180 min (inhibition percentiles of 45.03%, 60.59% and 41.72%). **Results:** TFN ethereal (inhibition percentiles of 23.95% and 29.01%) and chloroform (inhibition percentiles of 28.8% and 22.04%) extracts inhibited edema on 120 and 180 min. None of the extract inhibited the granuloma development. None of the extract caused death or other acute toxicity signs. Vicanicine (60.26% in ethereal extract and 51.17% in acetone extract), parietine (9.60% in ethereal extract and 15.38% on second), falacinol (0.78% in ether and 14.95% in acetone) and very low concentration of falacinal (0.15% in ethereal extract and 3.32% in acetone extract) were detected in the medicine. **Conclusions:** The tested extracts have antiedematogenic activity, but are not effective on subchronic inflammation. The extracts do not present toxic effects in administered doses.

Key words: Anti-inflammatory activity, lichen substances, *Teloschistes flavicans*

INTRODUCTION

Lichens are living beings that are resulting of a fungus (nonchlorophilled = micobiont) and an algae (chlorofilled = photobiont) association, whose metabolites have proved economic utilization in popular medicines. Lichens were used during millenniums. Dioscoride, army surgeon of Nero, used the "Doctrine of Signals" where lichens were employed according to its likeness with the illness, or according to the affected organ. In this way, the long filaments of *Usneas barbata* were indicated for capillary treatments, and *Xanthoria parietina*, because its intense yellow color was indicated in the jaundice cases.[1,2]

References about lichen utilization in popular therapeutics shows that fragments of *Pseudevernia furfuracea* were found in Egyptian XVII dynasty vases, about 1700 B.C. This species owns effective compounds with properties against pathogenic microorganisms, notably gram positive bacteria. Moreover, *P. furfuracea* also was employed in embalmed of corpses (mummification). Lichens, not only on old Egypt, also were of wide utilization in odoriferous substances (essential oils) production or degradation products that generate appreciated perfumes. Nowadays still they are used as perfumes fixatives, notably in France.[1,3,4]

European, Japanese, and Indian lichens were widely used on a series of treatments. *Cetraria islandica*, or Icelandic moss, is used against tuberculosis bacilli, and owns high content of carbon hydrates, and others nutritious that recover hectic patients. As this species, many other can be cited: *Lobaria pulmonaria*, *Peltigera canine*, and *P. aphthosa,*

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that eradicate pulmonary infections, cure dogs and snakes bites, and eruptions in the mouth (aphtha), respectively.[3]

From the 40s, the first referring studies about lichenic biological activities were arising. Burkholder and colleagues on 1940s initiated these studies, elaborating a work for detection of lichenic compounds with antibacterial activity. Bustinza, disciple of Fleming, also was one of the precursors in this investigation theme, studying the antimicrobial efficiency of lichens derived compounds, notably the usnic acid and their isomers.[5,6]

Several researches used brute and purified lichen compound extracts, demonstrating other lichen activities: antibacterial (some *Pneumococcus* species and *Staphylococcus*), fungicide (some filamentous fungi), antimycobacterial,[7-16] antispasmodic and spasmolithic,[17] histaminergic,[18] hypotensive, hypoglycemic, neuromuscular, and analgesic of leprapinic acid extracted from *Lepraria* sp.[19]

Maia[20] refer to atranorin, main *Cladina dendroides* compound, as an excellent antiphlogistic agent. Actually, there is a considerable knowledge about inhibitory ability of these compounds against fungi and Gram-positive and negative bacteria.[15,16,21]

Usnic acid is one of the lichen compounds more studied and effective in lupus vulgaris treatment,[22] furunculosis and impetigo.[23,24] Immunostimulating activity was verified to d usnic acid and three active depsides, in allergic contact dermatitis, caused by the lichen mushroom portion, of which were extracted the compounds.[25] Usnic acid has the effect against *S. aureus*, besides an inhibitory effect on surfaces biofilm formation, and an anti-inflammatory effect, similar to ibuprofen.[26]

Despite some lichenic substances as evernic, estitic, fumarprotocetraric, lobaric, and salazinic acids are immunologically active,[25] other can cause allergy in natural form (when present in lichen), or as part of some cosmetic (e.g., fixative perfume).[27]

The growing market of natural products and the renewed interest on general phytotherapy makes necessary to review the sale situation of phytomedicine products, which can be acquired, surprisingly, in fairs, supermarkets, natural products stores, drugstores or industries, and in varied forms, since product in rude state (drought plant) until even tablets, liquid mixtures, or capsules. Besides that, the search for natural origin therapeutic products, aiming the cure of many diseases, is nowadays objective of researchers that investigate plants and animals active principles. Many phytomedicines, however, are sold and used based only on popular tradition and justify its recommendation in puerile suppositions. Rare products were submitted to rigorous scientific tests and, in spite of exhibiting recognized therapeutic properties, many products did not have the risk potential or side effects evaluated. This lack of medical and sanitary control, absence of accurate botanical identification or purity certification, founded in diverse phytomedicines, may represent great danger to population health.[28,29]

There is a rich lichenic mycotic in Brazil, with a large range of bioactive properties. The study of these compounds will enable not only a larger knowledge of natural resources, but also the potentiality for its sustained use.

The goal of this research was to evaluate toxic effects and anti-inflammatory action *in vivo* of *Teloschistes flavicans* (Sw.) Norm. unrefined extracts, as well as determinate its constituents chromatographically.

MATERIALS AND METHODS

Collection, storage and identification

For this work we used lichen *T. flavicans* (Sw.) Norm., removed from trees and shrubs cortex in Venturosa city, a small city in Pernambuco state, Brazil.

Organic extracts obtainment

Lichen dry thallus was submitted to the extraction in a cold exhaustion system, using different solvents in an eluotropic series: diethyl ether, chloroform, and acetone, all with analytic purity certified by Merck®.

Obtained organic extracts were evaporated in ambient temperature and kept in desiccator until constant weight for later utilization in the pharmacological rehearsals.

Chromatographic rehearsals

Organic extracts (1 mg/ml) and purified substances (0.1 mg/ml) were submitted to a high performance liquid chromatography (HPLC) in a UV detector (254 nm) coupled chromatograph (Hitachi). Conditions analysis were reversed phase column C18, 1.0 ml/min flow, 0.04 attenuation, methanol/water/acetic acid (80:19,5:0,5 v/v) mobile phase, in isocratic system.[30] Isolated and purified substances of *T. flavicans* brute extracts were considered standard to identification of chromatograms peaks. These substances were obtained in a series of crystallizations.

Crystals were resuspended in benzene and applied to preparative silica gel plates 60 Merck PF254+366. Chromatoplates were developed in ascending benzene. The development of chromatograms was preceded by viewing under UV light. The development of chromatograms was preceded by viewing under short and long UV, and

evidenced bands were removed from plate, eluted in benzene, vacuum filtered, and taken to evaporation until new crystals formation.

Resultant crystals were dissolved in benzene and applied in three chromatographic silica gel 60 columns (0.063 - 0.200 mm), one for each recrystallization, from different extracts. The visualized fractions were separated by staining and deposited in test tubes. Each fraction was evaporated to dryness.

Chromatographic obtained compounds were submitted to reactions to confirm identification of their chemical nature. Sodium bisulfite saturated solution, potassium carbonate solution (2%), potassium hydroxide (0.5 N) and ferric chloride in ethanol (5%) were utilized.[31] The same samples were analyzed in infra red (Brucker IFS66) by KBr tablet technical, with 4.0/cm output resolution.

Animals

Swiss white mice (25 – 30 g) and Wistar rats (200 – 300 g) kept in controlled temperature (23 ± 2ºC) environment under a 12 h clear - dark cycles, with *ad libitum* food and water, on biotery of Physiology and Pharmacology Department of Federal University of Pernambuco, Brazil. All the steps of this study followed principles for laboratory animal use and care, according to Brazilian College of Animal Experimentation (COBEA).

Acute toxicity

The acute toxicity was determined by Lorke's Method.[32] Five groups of mice (both sexes; 20 – 30 g; n = 10 animals/group), submitted to 18 h - fasting were used. These received three growing doses (0,1; 1; 2 g/kg, p.o.) of TFN acetone extract. Control group received saline solution 0.9% (35 ml/kg, p.o.). The groups in study were meticulously observed during the 30, 60, 120, 240, and 360 min and later, every 24 h, for 14 days. That observation period allows verifying the appearance of tardy effect, as well as the reversible or irreversible effects in surviving animals and to determine, accurately, in case happens, the death moment. At the end of rehearsal, surviving animals were sacrificed through deep ethereal anesthesia and were accomplished macroscopic examinations of the following organs: heart, lungs, kidneys, liver, stomach, ovaries, uteruses, and testicles. The mortality percentage was observed by 72 h and DL50 was made calculations, according to the method of Probito.

Pharmacological essays – inflammation experimental models

Carrageenan paw edema

Five groups (n = 05) of Wistar rats (200 – 300 g) received saline 0.9% (5 ml/kg; p.o.), indomethacin (10 mg/kg; p.o.), ethereal, chloroform, or acetone extracts of TFN (100 mg/kg; p.o.), respectively. One hour after treatment, 0.05 ml carrageenan solution (1%) was injected in subplantar region of the animal's right hind paw.

Paw volume was plethismographically measured before (V_0) and 3 h after carrageenan injection (V_3). Paw volume variation was calculated in cubic millimeters, by the difference $V_3 - V_0$. The antiphlogistic potential of organic extracts and indomethacin was expressed by inhibition percentage (%), calculated according to the formula % = 1 - VT/VC × 100, where VT and VC are the variation measures of treaty and control groups, respectively, according to the methodology described by Winter *et al.*[33]

Granulomatous lesion induced by cotton pellet

In this essay, five groups (n = 05) of Wistar rats (200 – 300 g) were submitted to a classic method described by Winter and Poter in 1957.[34] Each animal received two sterile cotton pellet implants (10 ± 1 mg), under ethereal anesthesia, in subcutaneous tissue. The implant was placed in axillary and inguinal right regions. The groups received, in daily administration, saline 0.9% (5 ml/kg; p.o.), indomethacin (1.5 mg/kg; p.o.), ethereal, chloroform, and acetone extracts of TFN (100 mg/kg; p.o.), respectively, during 7 days, counted from the pellets implantation day. In the eighth day, all the animals were sacrificed and pellets withdrawn. Each pellet was dissected and dry in stove (70ºC) until obtainment of constant weight. Pellets' weights variation was stipulated by the difference between weights before and after the implantation. Granulomatous reaction inhibition percentage (%) was calculated by the formula % = 1 - PS/PI × 100, in which, PS is pellets weight after the drying and PI is the pellets weight before the implantation.

RESULTS AND DISCUSSION

Many chemical studies about *T. flavicans* were made using collected species in India. For these species, many natural compounds separated by column chromatography were reported: vicanicine, parietine (physcion), and teloquistine (falacinol).[35,36] Rajagopalan e Seshadri[37] reported extraction of falacinal and parietine (1:4), but not teloquistine from *T. flavicans* collected in India. Vicanicine like substance was extracted from *T. flavicans*.[38,39]

Initially, we compared the number of substances present on brute extract with data from literature. The Rf values were too close because of the similarity that occurs on antraquinones isolated. This fact hampered their isolation and purification. Antraquinones differs ones and others by carbonyl chains position. Despite that, it was possible to observe four bands in a pattern that suggests falacinol,

falacinal, vicanicine, and parietine. This pattern is similar to that described previously.[31]

Ancient data showed the presence of telosquistine, falacinal, parietine, and vincanicine.[40-42] Our chromatographic (CCD) data also revealed the presence of these substances in the used material [Figures 1 and 2].

Chromatogram shows four main peaks, in which the ascending order of retention times are (1) falacinol, (2) falacinal, (3) vicanicine, and (4) parietine. This presentation is according to Huneck and Yoshimura.[31] According to peaks areas, on both chromatograms [Figure 1], it is possible to indicate the vicanicine – the major substance in the evaluated extracts (60.26% in ethereal extract and 51.17% in acetone extract); parietine presented 9.60% on first extract and 15.38% on second; falacinol presented 0.78% in ether and 14.95% in acetone; only very low concentration of falacinal was detected (0.15% in ethereal extract and 3.32% in acetone extract) [Figure 1].

Isolated and purified substances were used as standard for HPLC [Figure 2]. The substances were identified as parietine (retention time 30.55 min), with violet KOH positive reaction, by reaction between OH (parietine) and potassium, resulting in a colored reaction. It is noteworthy that other substances found did not demonstrate such reaction. The second substance (retention time 23.04 min) presented a characteristic vicanicine peak. The third substance (retention time 9.74 min) was identified as falacinol. Infrared analyses confirmed HPLC obtained data and information were backed by Huneck and Yoshimura.[31]

TFN acetone extract, orally administered, until the dose of 2 g/kg, did not provoke death, as well as no toxicity indication in the animals; similar data for atranorin, lichenic substance that possesses strong inhibitor power against inflammation.[20]

TFN ethereal, chloroform, and acetone extracts (100 mg/kg; p.o.) exhibited significant ($P<0.05$) anti-edematogenic activity when compared to control group. TFN acetone extract exhibited significant ($P<0.05$) anti-edematogenic activity in 60, 120, and 180 min periods, after edema induction. Acetone extract group paw volumes (ml), 0.372 ± 0.129, 0.538 ± 0.079, and 0.918 ± 0.100, reached inhibition percentiles of 45.03%, 60.59%, and 41.72%, respectively. Chloroform extract group (100 mg/kg; p.o.) exhibited significant ($P<0.05$) antiedematogenic activity in 120 and 180 min periods, after carrageenan administration. The edema volumes observed for that group, 0.972 ± 0.204 and 1.228 ± 0.278, correspond to an inhibition percentile of 28.8% and 22.04%, respectively. The ethereal extract (100 mg/kg; p.o.) inhibited ($P<0.05$) the edema in 120 and 180 min periods. The edema volumes for that group, 1.038 ± 0.095 and 1.118 ± 0.076, correspond to an inhibition percentile of 23.95% and 29.01%, respectively. The control group edema volume (ml) registered in 60, 120, and 180 min intervals were 0.6767 ± 0.247, 1.3650 ± 0.2369, and 1.5750 ± 0.1740, respectively [Table 1]. Table 2 demonstrates the effects of *T. flavicans* (Sw.) Norm. brute extracts and indomethacin on cotton pellets induced granulomatous lesion. None of the extracts exhibited effect on granuloma development. Indomethacin exhibited significant reduction in the inflammation process.

CONCLUSIONS

The ethereal, chloroform, and acetone extracts of *T. flavicans* (Sw.) Norm. have antiedematogenic activity. However, these extracts are not effective on subchronic

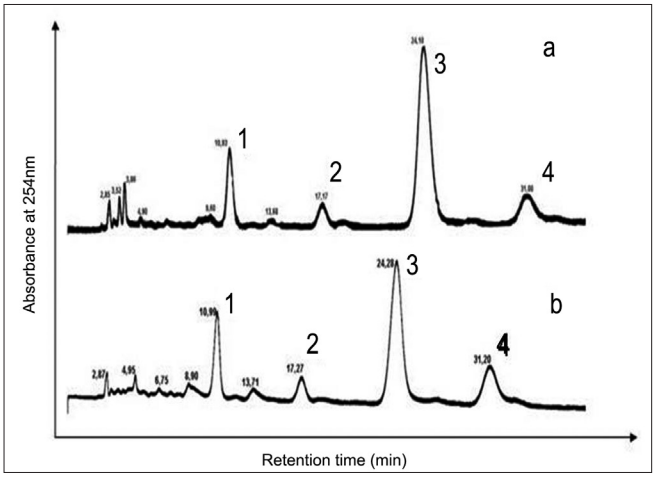

Figure 1: HPLC of *T. flavicans* brute extracts: (a) ethereal extract and (b) acetone extract; (1, 2, 3, 4) suggested peaks are falacinol, falacinal, vicanicine, and parietine, respectively.

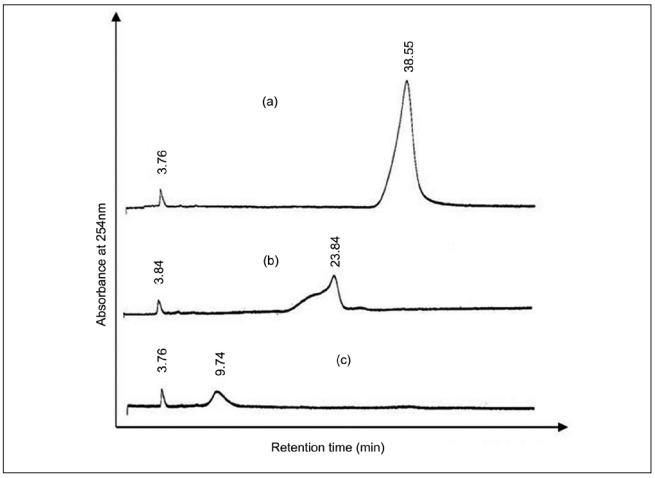

Figure 2: HPLC of *T. flavicans* purified substances: (a) parietine; (b) vicanicine; (c) falacinol.

Table 1: Effect of different *T. flavicans* organic extracts on carrageenan paw edema

Extracts/control	Paw volume (ml) in different time ranges			
	30 min	60 min	120 min	180 min
	X ± SD	X ± SD	X ± SD	X ± SD
Saline (5 ml/kg; p.o.)		0.6767 ± 0.2470	1.3650 ± 0.2369	1.5750 ± 0.1740
Indomethacine (10 mg/kg; p.o.)		0.4150 ± 0.0817 (38.67%)*	0.6033 ± 0.1108 (55.80%)*	0.7767 ± 0.1591 (50.68%)*
Ethereal	0.506 ± 0.102	0.608 ± 0.072 (10.58%) NS	1.038 ± 0.095 (23.95%)*	1.118 ± 0.075 (29.01%)*
Chloroformic	0.464 ± 0.220	0.688 ± 0.207 NS	0.972 ± 0.204 (28.8%)*	1.228 ± 0.278 (22.04%)*
Acetone	0.404 ± 0.122	0.372 ± 0.129 (45.03%)*	0.538 ± 0.079 (60.59%)*	0.918 ± 0.100 (41.72%)*

Percentile of inhibition with significance; *$P < 0.05$; NS = not significant. ANOVA followed by Bonferroni's Multiple Comparison Test.

Table 2: Antiphlogistic action of different organic extracts of *T. flavicans* (100 mg/kg) in lesion granulomatosa induced by cotton pellets

Extracts/control	Granulomatous lesion mean ± SD
Saline (5 ml/kg; p.o.)	70.64 ± 8.75
Indomethacin (1.5 mg/kg; p.o.)	51.71 ± 8.78 (26.79%)*
Ethereal	73.92 ± 4.66 NS
Chloroform	88.3 ± 8.93 NS
Acetone	85.16 ± 8.11 NS

Percentile of inhibition with significance; *$P < 0.05$; NS = not significant. ANOVA followed by Bonferroni's Multiple Comparison Test.

inflammation. The extracts do not present toxic effects in administered doses.

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