DOI: 10.5138/09750185.1832

International Journal of Phytomedicine

2018;10(1):30-38

Original Research Article

Antifibrotic effect of *pluchea sagitallis* (lam.) Cabrera aqueous extract in grx cell lineage

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Abstract

Liver fibrosis is a complex disease that is caused by inappropriate tissue repair due to the deposition of connective tissue. When a chronic lesion affects the liver, regenerative response fails and hepatocytes are replaced with abundant extracellular matrix (ECM). The imbalance between production and degradation of ECM will result in the accumulation of proteins that change normal liver architecture, and thus its functionality. The main source of ECM is the activated hepatic stellate cell (HSC). In order, to clarify possible therapeutic approaches to the disease, this work aimed to evaluate the possible antifibrotic action of *Pluchea sagitallis* (Lam.) Cabrera on an activated HSC immortalized lineage (GRX). Our results demonstrated that the P. sagittalis aqueous extract at 0.039 and 0.078 mg/mL concentrations was able to reduce cell growth and proliferation. Regarding to oxidative stress evaluation, there was no statistically significant difference between the treated group and the control. Staining with OilRed-O (ORO) showed a statistically significant increase in intracellular lipid content after 5 days of treatment, exerting in vitro effect on the GRX phenotypic change of activated towards the quiescent state. These results were confirmed by colorimetric quantification of lipid content. Regarding the TGF-β1 and collagen production, there were no statistically significant differences observed between the groups. In conclusion, the *P. sagittalis* aqueous extract reduces the growth and proliferation of GRX cells and induces the reversal of activated towards a quiescent phenotype. There was no decrease in cell proliferation either by necrosis or by apoptosis via activation of the senescence. Thus, our data suggest that the extract showed an antifibrotic effect, possibly by activating phenotype reversal.

Keywords: hepatic fibrosis; hepatic stellate cell; Pluchea sagitallis

Introductionn

Hepatic fibrosis, a disease with highly complex etiology, is caused by inappropriate tissue repair due to the deposition of connective tissue, resulting in chronic liver damage. It may be caused by alcohol consumption, chronic viral hepatitis, and autoimmune diseases, among others. When there is inappropriate fibrosis control, the result can be a progression to cirrhosis [1] . When an acute injury affects the liver, parenchymal cells are regenerated and this process is associated with the inflammatory response and limited deposition of extracellular matrix (ECM). However, after chronic damage, the regenerative response fails and hepatocytes are replaced by excessive ECM mainly made of collagen of types I, III and IV, fibronectin, elastin, laminin and proteoglycans (Duval et al., 2014a). The main sources of ECM are the hepatic stellate cells (HSCs) [2] , which constitute around 15% of total liver cells [3] . Discovered in 1876 by Kuppfer and located within the perisinusoidal space of Disse, the HSCs contain fat globules and are responsible for storing approximately 80% of vitamin A [4] [5] [6] . These cells play an important role in the fibrogenesis that occurs in chronic lesions of hepatic tissue [7] . The HSCs activation process, when there is phenotypic change from the quiescent state to an activated state, can be divided into initiation, perpetuation and resolution, and is an early event in hepatic fibrogenesis. Several paracrine stimulation of damaged hepatocytes and other neigh-

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Received: 28 Jan 2016, Accepted: 18 May 2016

boring cells can initiate HSCs activation. The activated stellate cells are characterized by increased contractility, retinoid loss, chemotaxis, proliferation, ECM degradation capacity and secretion of pro-inflammatory cytokines. TGF-β1 produced by liver cells represents a potent fibrogenic signal, since it increases the production of type I collagen and other matrix constituents, such as fibronectin and proteoglycans. Remarkably, this new ECM configuration induces a new fibrogenic stimulus that is responsible for exacerbating fibrosis (Duval et al., 2014a). The GRX cell line, which represents the activated HSC phenotype, has been used as an important tool for the study of the physiology of HSC and liver fibrosis [8] [9] . The Pluchea sagitallis (Lam.) Cabrera, which belongs to the Asteraceae family, is a native plant of South America, popularly known as "quitoco", "madre cravo" or "tabacarana" [10] . Important properties associated with the Pluchea gender, have been described including antioxidant activity, and anti-inflammatory and anti-ulcerogenic potential. Phytochemical studies with P. sagitallis identified several bioactive compounds, including flavonoids, phenols, terpenes, tannins, alkaloids and saponins [11] [12] [13] . Regarding to the anti-inflammatory property of P. sagitallis, pharmacological studies showed that the aqueous extract has anti-inflammatory activity that is correlated with a reduction of free radicals [14] . The antifibrotic properties of medicinal plants have been mainly reported mainly in models of in vitro and in vivo liver fibrosis. There are two ways in which these plants, together with their bioactive compounds, may act in reducing liver fibrosis: via inhibition of HSC activation and via reduction of ECM deposition . The advantages of using medicinal plants as possible antifibrotic agents include high safety, cost-effectiveness and versatility . Thus, the Pluchea gender seems to be a promising source of material on the research of new therapeutic drugs [15].

Material and Methods

Materials

P. sagitallis leaves were collected from the Protection Center and Nature Conservation Pro-Mata, at the town of São Francisco de Paula, Rio Grande do Sul, Brazil, during December 2014

Preparation of the aqueous extract of Pluchea sagitallis

Aqueous extracts were prepared with P. sagitallis leaves. The aqueous extracts were obtained by adapting the methodology used by [16] . Initially, 2 g of in nature plant leaves in a properly tagged Becker were selected. After cleaning was performed with distilled water, with subsequent drying and, thereafter, 10 mL of distilled water were added and then this solution was placed in a water bath (80˚C) for 30 min. The aqueous extract was filtered and centrifuged at 5000 rpm for 10 min and after was divided into aliquots of 500 uL in 1.5 mL Eppendorf tubes and stored at -20˚C

Cell culture

The murine GRX cell line 16 was obtained from the Rio de Janeiro Cell Bank (Federal University, Rio de Janeiro, Brazil). The GRX cell line was obtained from liver granuloma in mice of C3h line / HeN infected with Schistosoma mansoni cercariae [17] [18] . Under standard cultivation conditions, GRX cells express the myofibroblast phenotype, proliferative and ECM producer [19] [20] . Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 2g/L HEPES buffer, 3.7g/L NaHCO3 and 1% penicillin and streptomycin (Invitrogen) and incubated at 37˚C in a humidified atmosphere with 5% CO_2 .

Treatment with Pluchea sagitallis aqueous extract

The aqueous extract of P. sagitallis was used in concentrations of 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2,5 mg/mL. Based on previous studies, GRX cells were incubated and the analyses were performed after 5 days of treatment. all experiments were repeated three times and in triplicates [21] .

Viability and cell growth

Cellular viability and growth were assessed by Tripan blue dye exclusion. The number of cells was determined by the hemocytometer. GRX cells were seeded into 24-well plates (3 x 10^3 cells/well) and treated with P. sagitallis aqueous extract (as described above) for 5 days to evaluate the antifibrotic activity. The control group received only DMEM and 5% SFB for 5 days.

Cell toxicity by release of lactate dehydrogenase

We used the determination of lactate dehydrogenase (LDH) in supernatants of cultures as compared to the control group in order to evaluate the cytotoxicity of P. sagitallis aqueous extract in GRX cells. The LDH activity was measured by colorimetric assay [22] . Control of cell lysis was checked using 5% Tween.

Oxidative stress assessment

Oxidative stress was assessed by the concentration of MDA (malondialdehyde) in the supernatant of cell cultures by Thiobarbituric Acid Reactive Substances (TBARS) assay kit (Cayman Chemical Company). The TBARS is a well-established method for screening and monitoring of lipid peroxidation. The results are expressed in μM per 1.000 cells.

Apoptosis and senescence assay

The GRX cells apoptosis and senescence was assessed by immunofluorescence microscopy (x200). Cells were stained with DAPI (Invitrogen, Inc., Carlsbad, CA, USA) to assess nuclear changes or modifications of cells undergoing apoptosis. After treatment, the supernatant was removed and cells were washed three times with PBS and fixed with 4% paraformaldehyde. Next, cells were washed again with PBS, permeabilized with 0.5% Triton X-100 for 30 min and DAPI was added. After 2 min the dye was removed, and the last wash was performed with PBS. Finally, nuclear morphometric analysis (NMA) was performed to quantitatively evaluate the proportion of cells in senescence, apoptosis or nuclear irregularities within this population of in vitro cells [23] . DAPI staining reveal that apoptotic cells shows nuclei with chromatin condensation and apoptotic bodies.

Detection of lipid droplets by Oil Red-O staining

Cells were stained with Oil Red-O (ORO) (Sigma) [24] on day 5 to show cell morphology and lipid accumulation. Af- ter fixing cells with 10% formaldehyde, ORO (0.35g in 60% isopropanol) was briefly added for 15 min. Intracellular lipid droplets were examined using an inverted light microscope (BestScope, China).

Quantification of lipid accumulation

The procedure is also based on ORO staining of intracellular lipid droplets (Sigma Chemical Co., St. Louis, Mo). Cells were briefly fixed with perchloric acid and incubated with ORO dissolved in propylene glycol (2mg/mL) for 2h. The ORO within the lipid droplets was extracted using isopropanol. The absorbance was read at 492 nm using an ELISA plate reader. Each sample was normalized to 100.000 cells.

TGF- β1 Quantification

TGF-β1 concentration was measured, in cell supernatant, on day 5, using a commercially available ELISA kit (R&D Systems, Minneapolis, MN). Results were calculated on a standard curve concentration and multiplied for the dilution factor. TGF-β1 levels were expressed as nanograms per milliliter per 1.000 cells.

Measurement of collagen content

Collagen content in GRX cells was measured using Picro Sirius Red on day 5. Picro Sirius Red was added to cell supernatant to form a collagen-dye complex. After centrifugation, unbound dye was removed and collagen-dye complex dissolved in NaOH. The absorbance was measured at 540 nm in an ELISA plate reader. Each sample was normalized to 1.000 cells [25] . Results were calculated on a standard curve concentration. Collagen levels were expressed as the ratio of milligrams of collagen and cells number.

Statistics

Data are reported as mean ± SD. Each experiment was performed at least three independent times and in triplicates. Statistical testing was performed with Prism 5 software. Results were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. The level of significance was set at $p < 0.05$.

Results

Viability and cell growth

After 5 days of treatment with P. sagitallis aqueous extract, cell viability of GRX lineage was evaluated at 0.039, 0.078, 0.15625, 0.3125, 0.625, 1.25 and 2.5 mg/mL concentrations on cell viability of GRX lineage. A significant decrease in cell proliferation was observed with increasing concentrations of the aqueous extract (Fig. 1).

Cell toxicity by release of lactate dehydrogenase

In order to evaluate whether the reduction in number of cells treated with P. sagitallis aqueous extract was due to cytotoxicity, the lactate dehydrogenase (LDH) released was quantified in the culture medium. Among the concentrations tested, there was a statistically significant difference at 1.25 and 2.5mg/mL concentrations compared to the control, indicating cytotoxicity (Fig. 2). Thus, 0.039 and 0.078 mg/mL concentrations of P. sagitallis aqueous extract were selected for this study because lower concentrations had already presented an antiproliferative effect.

Oxidative stress assessment

Oxidative stress can damage the cell and, consequently, decrease cellular proliferation, allowing to evaluate MDA formation by MDA by TBARS assay. Our results showed that there was no statistically significant difference between the treated groups and the control group (Fig. 3).

Apoptosis and senescence assay

Regarding the evaluation of apoptosis and senescence measured by DAPI, there was no statistically significant difference between treated groups and the control group Thereby apoptotic and senescent cells were not detected in the studied groups (Fig. 4 A, B, C, D, E).

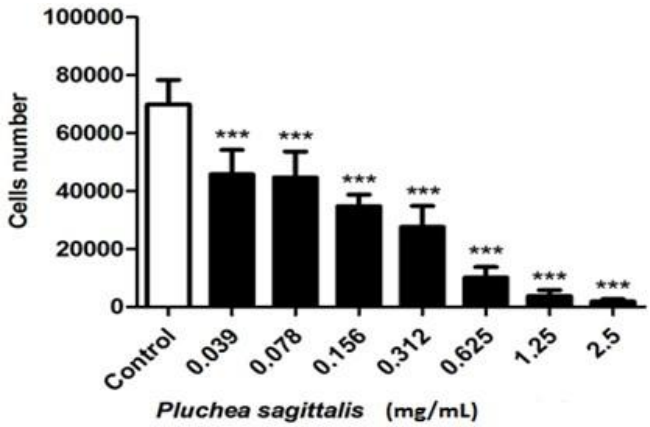

Figure 1 Effect ofPluchea sagitallis in GRX cells growth, assessed by direct counting onNeubaue´r s Chamber. Cells were treated with P.sagitallis aqueous extract for 5 days. Data represent the mean ± SD (n=4). Results are expressed as cellnumber per well. ***p<0.01 compared

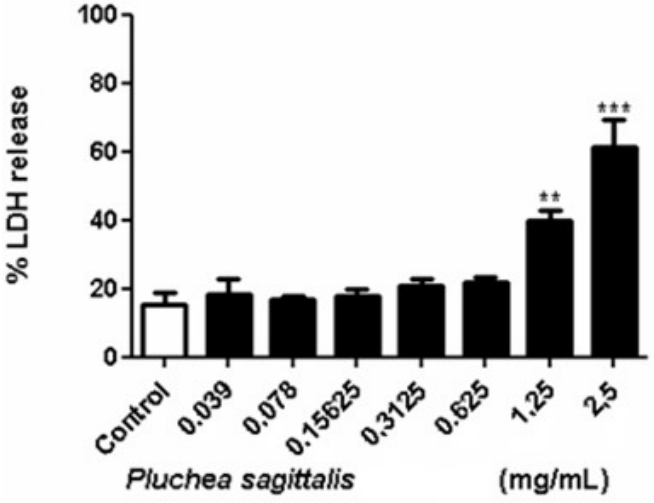

Figure 2 Effect of PlucheaSagitallis in lactate dehydrogenase (LDH) release on cell supernatant after 5 days. Data represent the mean ± SD (n=4). Results are expressed aspercentage of LDH. ** p<0.01 and ***p< 0,001 compared to control.

Detection of lipid droplets by Oil Red-O staining and quantification of lipid accumulation

When activated, stellate hepatic cells alter their phenotype and, therefore, increase their proliferation rate. In order to verify whether antiproliferative mechanism of P. sagitallis aqueous extract could be via phenotype regression, we assessed their lipid content. Control cells, which did not receive any treatment, had preserved their myofibroblastic phenotype (Fig. 5A). However, cells that were treated with P. sagitallis aqueous extract at 0.039 and 0.078 mg/mL concentrations showed a reversal of activated to quiescent phenotype (Fig. 5B, C).

The phenotype reversal was confirmed by colorimetric quantification of intracellular lipid content. There was a statistically significant increase in intracellular lipid content at 0.078 mg/mL concentration of P. sagitallis aqueous extract when compared to the control (Fig. 6).

TGF-β1 Quantification

There was no statistically significant difference in the amount of TGF-β1 between the treated groups and the control group after 5 days of treatment (Fig. 7).

Measurement of collagen content

There was no statistically significant difference in collagen content between the treated groups and the control group after 5 days of treatment, (Fig. 8).

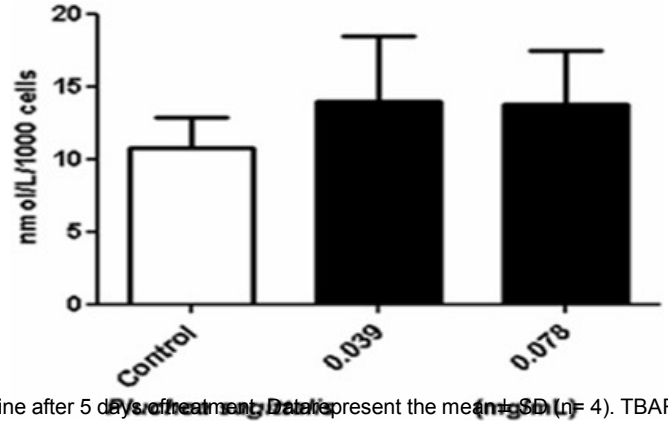

Figure 3 TBARS levels in GRX cell line after 5 days of treatment. Data represent the mean ± SD (n = 4). TBARS levels were the expressedper nanomol per1000 cells.

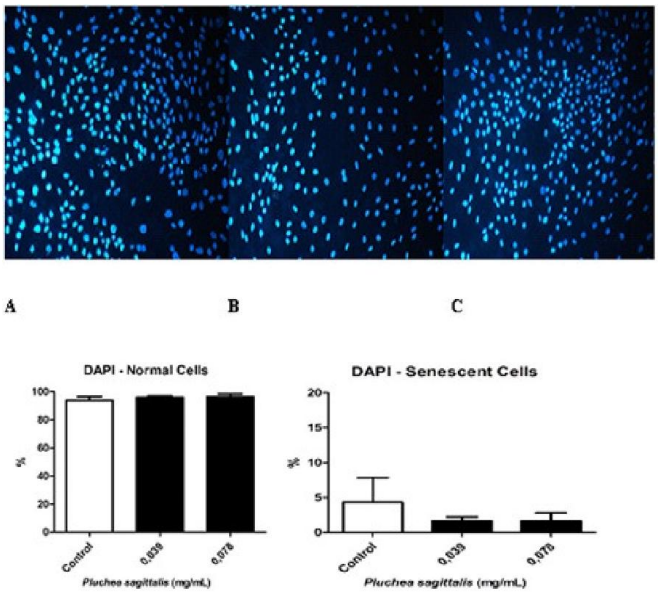

Figure 4 DAPI Staining to assess nuclearchanges or modifications ofcells undergoing apoptosis and senescence. A. Control; B. 0.039; C.0.078 mg/mL; D. Percentageof normal cells; E. percentage ofsenescent cells.

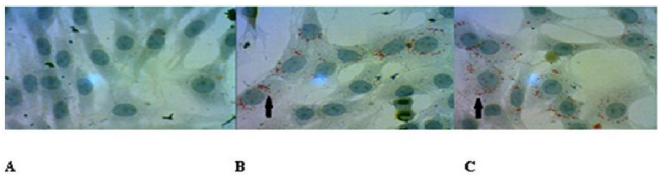

Figure 5 OilRed-O (ORO) staining and lipid quantitation of GRX cells at day 5.(A) Control cells; (B, C) cells treated with Pluchea sagitallisaqueous extract at 0.039 and 0.078 mg/mL,respectively. Bar length = 20 μm. The arrows show the lipid droplets in cellstreated with Pluchea sagitallis and absent in the control group.

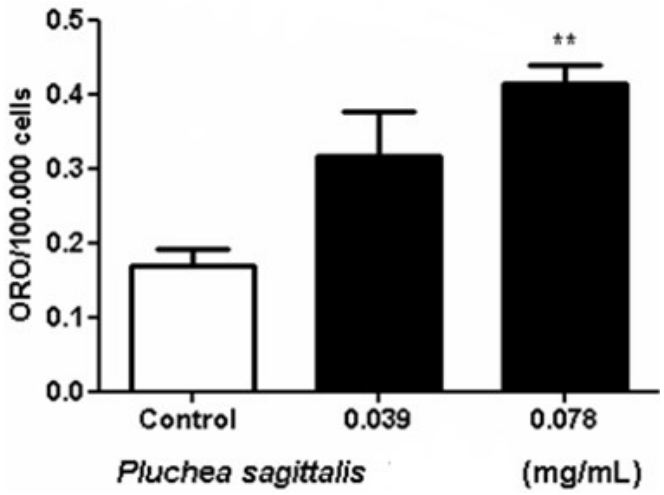

Figure 6 Specific lipid content expressedspectrophotometrically as the ratio of absorbance value obtained for ORO andthe cell number. Results are expressed as mean ± SD. ** **p<0.01, comparedto control.

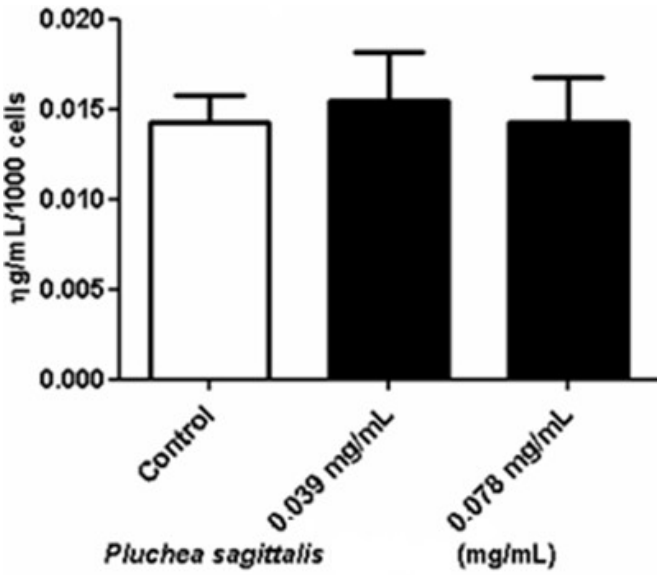

Figure 7 ELISA assay of TGF-β1 in cell supernatantof 5 days treatment. Datarepresent the mean ± SD (n=4). TGF-β1 levels are expressed as nanogramsper milliliter per 1000 cells.

Discussion

Liver fibrosis is a multifactorial process feature by an imbalance of components of ECM synthesis and degradation, resulting in proteins accumulation that change normal liver architecture and, consequently, their functionality. GRX cells represent an interesting study of liver fibrosis model because in basal culture conditions exhibit the myofibroblastic fenotype [26] . This study demonstrated that P. sagitallis aqueous extract decreased GRX cells proliferation rate at the tested concentrations. Initially, we evaluated the possible cytotoxic action of the aqueous extract. We found that at 1.25 and 2.5 mg/mL concentrations the extract was toxic to cells, as it caused cell necrosis. This was evidenced by the significant increase of LDH in the cell culture supernatant. Based on these results, we decided to investigate the cellular mechanism involved in the antiproliferative effect of the P. sagitallis aqueous extract at 0.039 and 0.078 mg/mL concentrations. Oxidative stress is present in many liver diseases. This process happens due to an imbalance between oxidants and antioxidants, resulting in an excessive increase of free radicals.

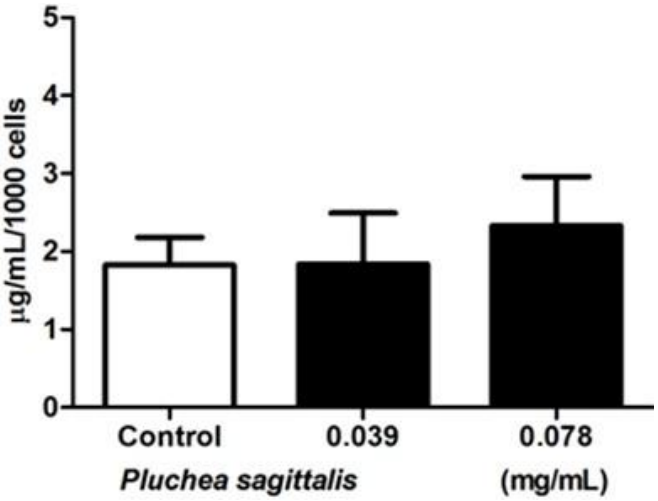

Figure 8 Total collagen content in cell supernatant of 5days treatment. Datarepresent the mean ± SD (n=4). Results are expressed as microgramsper milliliter / 1000 cells

This fact leads to the oxidation of biomolecules with consequent loss of biological functions and homeostatic imbalances, ending in a powerful oxidative damage to cells and tissues The hepatic damage can lead to an inflammatory condition which activates hepatic stellate cells, initiating the fibrotic process [27] . Our results showed no statistically significant differences in oxidative stress among treated and control groups, demonstrating that the antiproliferative effect did not occur via oxidative stress. Apoptosis plays an important role in the homeostasis of the liver cells, considering that many diseases that affect their cells are associated with increased apoptosis in hepatocytes, in order to protect against organ inflammation. Therefore, programmed cell death causes the hepatocytes die without causing a potentially damaging inflammatory response (Neuman, 2001). Our results demonstrated that the aqueous extract treatment did not increase the cell death by apoptosis, as well as not changing the senescent cells number, which could decrease cell proliferation by disrupting the cell cycle. The GRX cell proliferation is related to the myofibroblastic phenotype. Therefore, we histologically analyzed the possible reversal of the phenotype and evaluated the intracellular lipid content. Our results showed an accumulation in intracellular lipid content as evidenced by ORO staining and confirmed by intracellular lipids quantification. For this reason, the P. sagitallis aqueous extract acts as a potent inducer of quiescent phenotype. This effect may explain the antiproliferative action of the plant. TGF-β1 is the major pro-fibrotic cytokine in chronic hepatic injury. This mediator activates the HSCs, resulting in increased cell proliferation and ECM deposition [28] . Increased TGF-β1 levels represent a potent fibrogenic signal and its increase is associated with increased collagen production [29] .Ours results showed concentrations of TGF-β1, -***when analyzed in supernatant GRX cell line, no statistically significant difference between the groups treated with P. sagitallis aqueous extract and the control group. The same occurred when we assessed the collagen concentration. Although these results show no differences between the groups, there was consistency, since TGF-β1 and collagen are related to each other. According to Ye & Dan (2010), the collagen expression and production are decreased when TGF-β1 synthesis decreases and, consequently, so does the fibrotic process. Our results showed no significant decrease in these parameters, probably because the incubation time of the cells and the studied doses, despite decreasing cell proliferation and GRX cells phenotype reversion were not sufficient to decrease TGF-β1 and collagen synthesis. In conclusion, this study demonstrated for the first time a possible antiproliferative effect of P. sagitallis in an in vitro model of liver fibrosis. The results showed that the plant aqueous extract 0.078 mg/mL concentrations, was able to induce reversion from quiescent to activated phenotype in GRX cell line. Based on our findings, this study suggests a possible role for the P. sagitallis aqueous extract in liver fibrosis treatment. The findings highlight the importance of further research in this area towards a more efficient and effective treatment. A better understanding of fibrogenesis inhibition and HSCs pathways activation and deactivation are still challenges to be unraveled, not only have a better understanding of disease pathogenesis, but also to validate the therapeutic use of medicinal plants in liver fibrosis.

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