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Original article

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Running title

*A. lappa* attenuates steatohepatitis-associated hepatocarcinogenesis

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Abstract

Non-alcoholic steatohepatitis (NASH) is considered growing risk factor for hepatocellular carcinoma development in high income countries. Diet- and chemically-induced rodent models have been applied for the translational study of NASH-associated hepatocarcinogenesis due to their morphological and molecular similarities to the corresponding human disease. *Arctium lappa* L. (burdock) root tea has been extensively consumed in Traditional Chinese Medicine due to its potential therapeutic properties. Indeed, the bioactive compounds of *A. lappa* root, as the polyphenols, have already showed antioxidant and anti-inflammatory properties in different *in vivo* and *in vitro* bioassays. In this study, we investigated whether burdock root ethanolic extract (BRE) administration attenuates NASH-associated hepatocarcinogenesis. Eight-week-old male male Wistar rats received choline-deficient high-fat diet for 8 weeks and thioacetamide for 4 weeks in order to induce NASH and preneoplastic glutathione-S-transferase pi (GST-P) preneoplastic foci. Subsequently, rats were treated with BRE (100 or 200 mg/kg body weight) or vehicle by oral gavage for 2 weeks. BRE presented high levels of chlorogenic and caffeic acids and its administration reduced total fatty acid and lipid hydroperoxide levels, while increasing the activities of antioxidant superoxide dismutase and catalase enzymes in the liver. Furthermore, burdock intervention diminished the size of GST-P remodeling preneoplastic lesions and displayed a trend on reducing hepatocyte proliferation (Ki-67) inside them. These findings suggest that short-term exposure to BRE alleviated preneoplastic lesion development in NASH-associated hepatocarcinogenesis.

**Key words:** Burdock (*Arctium lappa* L.); non-alcoholic steatohepatitis (NASH); rat hepatocarcinogenesis; GST-P+ preneoplastic lesions; chlorogenic acid.

1. Introduction
The hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related deaths (8.5 deaths per 100,000 people), reflecting the worldwide relevance of this malignant neoplasia. There is growing evidence on the importance of non-alcoholic steatohepatitis (NASH), the advanced stage of non-alcoholic fatty liver disease (NAFLD), as a predisposing factor for HCC development, mainly in high income countries of the Western world. In the last decade, NASH-attributable HCC cases in the US increased nearly by 60%, being the second major cause for liver transplantation. The pathogenesis of NASH follows the “two-hit theory” and is closely related to metabolic disorders, as the highly prevalent obesity and/or diabetes. These conditions favor triglyceride accumulation that leads to fatty liver (considered the “first hit”). Subsequently, the lipotoxicity triggers inflammatory response (the “second hit”), gradually allowing the emergence of liver fibrosis/cirrhosis, a favorable background for HCC.

Considering that there are no approved therapies for NASH, the preclinical and translational investigation of therapeutic and/or preventive strategies is relevant. For this purpose, genetically modified, chemically- and diet- induced models are rodent models are available. These models resemble some of the physiological, metabolic, histological, transcriptomic traits and clinical endpoints of the corresponding human disease. The combination of chemically- and diet- induced regimens is commonly applied. High fat diet (HFD) interventions increase free fatty acid (FFA) availability, closely resembling the “first hit” of human pathology. When HFD is combined to methionine–choline-deficiency, fat β-oxidation and very low-density lipoprotein (VLDL) production and secretion are impaired, aggravating steatosis and inflammation (the “second hit”). Dietary interventions may also be associated to repetitive chemical insult, as thioacetamide (TAA) and carbon tetrachloride (CCl₄) administrations. After TAA liver metabolism, highly reactive sulfoxide (TASO) and sulf dioxide (TASO₂) metabolites are generated, promoting necro inflammatory (the “second hit”) and fibrotic/cirrhotic responses. In addition to steatohepatitis, TAA-mediated oxidative damage may contribute to the development of preneoplastic foci positive for glutathione-
S-transferase pi (GST-P), considered preneoplastic lesions (PNLs) and biomarkers of early hepatocarcinogenesis.\cite{9,10} PNL emergence in NASH context enable the screening of preventive/therapeutic agents of hepatocarcinogenesis, including medicinal plants applied in alternative medicine.

The edible *Arctium lappa* L. plant, popularly known as burdock, has been extensively cultivated for over 3,000 years in Asia, being promoted as a nutritive and functional food in Traditional Chinese Medicine (TCM).\cite{11} In TCM, the *A. lappa* L. root tea has been used because of its potential therapeutic properties.\cite{11} Indeed, there are many bioactive compounds in the roots of this herb, mainly phenolic compounds, such as lignans and hydroxycinnamic acids, including chlorogenic and caffeic acids.\cite{12,13} This exotic plant or its isolated compounds have already demonstrated antioxidant and anti-inflammatory properties in different *in vivo* and *in vitro* bioassays.\cite{12-14} *A. lappa* L. root extracts were found to protect the liver against acute alcohol and carbon tetrachloride (CCl₄), acetaminophen and CCl₄, or cadmium-induced damage in rats, while *A. lappa* L. root-isolated polyphenol arctigenin triggered cytotoxicity in HCC cell line (HepG2).\cite{15-18} Nonetheless, further studies are warranted to unveil the potential protective effects of this popular herb in chronic liver disease models. Accordingly, the present study was set up to evaluate for the first time the beneficial effects of burdock root extract administration on NASH-associated hepatocarcinogenesis in male rats.

2. Materials and methods

2.1 Burdock roots and ethanolic extract

The burdock (*Arctium lappa* L.) roots were purchased from Chamel Agronegócios (Campo Largo - Brazil). After harvesting, roots were dried in a heater at 40°C, crushed and stored at -20°C. The extraction methodology used herein was similar to previous studies.\cite{13,17} Briefly, ~2.2 Kg of the dried and crushed roots were distributed in glass vials (200 g/vial), submitted to 3 extractions with
alcohol 90% for 20 days at room temperature and protected from direct light. Afterwards, the extract was filtered, and alcohol was removed by a rotary evaporator (Fisatom, Brazil) under 50°C with reduced pressure in order to concentrate the solution. Finally, the concentrated ethanolic extract of burdock roots was lyophilized and stored at -20°C protected from light.

2.2 Phenolic acid profile

Analysis of free phenolic acids was performed in triplicate as previously described. The extraction was carried out by mixing 1 g of the extract in 4 mL of methanol:water (50:50 v/v, pH 2.0) followed by mechanical stirring for 1 hour and centrifugation (5000×g) for 10 min. The supernatant was collected (extract 1). Then, 4 mL of acetone:water (70:30; v/v) was added to the residue and the mechanical stirring and centrifugation steps were repeated. The supernatant was collected (extract 2). Next, 3 mL of the supernatants (extract 1 and extract 2) were mixed and transferred to a 1.5 mL vial for chromatographic injection. The analyses were performed in a high-performance liquid chromatograph (Alliance Waters™ model 2690/5) with a Waters™ photodiode array detector model 2996 (270, 310, 325 and 370 nm) and 2 connected columns, Thermo BDS HYPERSIL C18 (50x4.6 mm; 2.4μm) and Thermo BDS HYPERSIL C18 (100x4.6 mm; 2.4μm) at 30°C. Conditions included a flow rate 1.2 mL/min in elution mode gradient using an aqueous solution of phosphoric acid (1.5 mL/L) (Phase A) and acetonitrile (Phase B), an injection volume of 5 µL and a run time of 30 min. Gradient conditions included phase B in 5% at initial time and at 12.00 min the acetonitrile concentration was increased to 12%, at 18.00 min to 20%, and at 20.00 min to 50% acetonitrile. The acetonitrile concentration was maintained at 5% until 25.00 min and then returned to the initial condition (5%). External standards were used for the identification and quantification of the phenolic acids.

2.3 Experimental design
Eight-week-old male Wistar rats (*Rattus norvegicus*) were randomly allocated into 3 experimental groups (G1 to G3, n=9 rats per group) and were submitted to a NASH-associated hepatocarcinogenesis model. Rats received a choline-deficient and high fat diet (CDHFD) (*i.e.* 35% total fat and 54% trans fatty enriched, Rhoster-SP, Brazil) *ad libitum* for 8 weeks (Figure 1). In addition, after the first month of dietary intervention, the animals received intraperitoneal (i.p.) injections of TAA [200 mg/Kg of body weight (b.wt.) in 0.9% saline, 3x/week] (Sigma-Aldrich, USA) for 4 weeks (Figure 1). Upon NASH-associated hepatocarcinogenesis, some groups were treated with 100 (G2) or 200 mg/Kg b.wt (G3) of lyophilized burdock root ethanolic extract (BRE) by daily oral gavage for 2 weeks, or just received distilled water and 5% dimethylsulfoxide (DMSO) vehicles (G1) (Figure 1). During burdock treatment, rats were fed basal diet (Nuvital, Nuvilab-Brazil). Considering that there are no studies that estimate the daily human intake of burdock roots, the selected doses were based on a previous *in vivo* study that showed the absence of liver toxicity in this dose range.17

Following treatments, rats were euthanized by exsanguination under isoflurane-induced anesthesia. At necropsy, blood samples were collected using heparinized syringes, centrifuged for 10 min (1503×g) and serum was stored at -20°C. The liver as well as retroperitoneal and epididymal fat tissues of each animal were weighed. Relative liver and fat tissue weights were calculated. Liver fragments were fixed in phosphate-buffered formalin or snap-frozen in liquid nitrogen with storage at -80°C. The animals were kept in a room with ventilation, relative humidity, controlled temperature and light/dark cycle 12:12. This study has been approved by the Committee on Bioethics of the School of Veterinary Medicine and Animal Science of the University of São Paulo (Protocol number 3132/2013) and all animals received humane care according to previous established criteria.20

2.4 Histopathology and collagen morphometry
Formalin-fixed liver tissue samples were embedded in paraffin and 5 µm sections were stained with hematoxylin and eosin (HE) for steatosis (0-3), hepatocellular ballooning (0-2) and lobular inflammation (0-3) scoring as previously described.\textsuperscript{21} The non-alcoholic fatty liver disease (NAFLD) activity score (NAS) was calculated by the sum of these scores (0-8).\textsuperscript{21} Fibrosis scoring and collagen morphometric analysis were performed in Sirius Red-stained sections.\textsuperscript{21-22} For collagen deposition analysis, 10 random fields (20× objective) were selected per section, and the area of collagen fibers was calculated using Image-Pro Plus 4.5 software (Media Cybernetics, USA). Results were expressed as percentage of the total liver area analyzed.

\textbf{2.5 Immunohistochemistry}

Immunoreactivity for GST-P (i.e. PNL marker)\textsuperscript{10} or GST-P/Ki-67 (i.e. proliferation marker) double stain were evaluated. Briefly, 5 µm liver sections on silane-treated slides were deparaffinized, re-hydrated and subjected to antigen retrieval in 0.01 M citrate buffer (pH 6.0) at 120°C for 5 min in a Pascal Pressure Chamber (Dako Cytomation, Denmark), endogenous peroxidase blockade with 1% H\textsubscript{2}O\textsubscript{2} in phosphate-buffered saline (PBS) for 15 min and low-fat milk treatment for 60 min. Subsequently, for GST-P, the slides were incubated with anti-GST-P antibody (#311, 1:1000 dilution, MBL, Japan) in a humidified chamber for 60 min at 4°C and with an anti-rat horseradish peroxidase (HRP) polymer (Thermo Fisher Scientific, USA) for 30 min. The reaction was visualized with 3’3 diaminobenzidine (DAB) chromogen. For GST-P/Ki-67 double stain, the slides were firstly incubated with rabbit polyclonal anti-Ki-67 antibody (ab6667, 1:100 dilution, Abcam, UK) in a humidified chamber overnight at 4°C, with the HRP polymer for 20 min, and the reaction was visualized with DAB. Then, the same slides were incubated with anti-GST-P antibody for 60 min at 4°C, with an anti-rat alkaline phosphatase (AP) polymer (Dako Cytomation, USA) for 20 min, and the reaction was visualized with Permanent Red (PR) chromogen. All slides were counterstained with Harris’ hematoxylin.
The GST-P⁺ PNLs were identified classified into persistent or remodeling, according to border regularity and uniformity of staining as previously established. The incidence was calculated, the number of lesions/liver area were analyzed (cm²), and the size of these lesions and the % area of section occupied by PNLs were assessed. Cell proliferation (Ki-67) was evaluated in double-stained sections. For PNLs, all Ki-67⁺ (DAB) hepatocytes into the lesions were counted and divided by the PNL area (mm²) (PR). For cell proliferation in hepatocytes surrounding PNLs, 10 random fields (40× objective) were assessed, Ki-67⁺ hepatocytes were counted and divided by the liver area analyzed (mm²).

2.6 Analysis of serum aminotransaminases, triglycerides and cholesterol

Alanine (ALT) and aspartate (AST) aminotransferases, triglycerides and cholesterol were measured in serum by a bench-top dry chemistry analyzer (Vet-Test 8008 QBC Analyzer, IDEXX Laboratories Ltd, UK).

2.7 Analysis of liver triglycerides and fatty acids

Liver lipids were extracted from liver tissue (100 mg) by Folch’s method. For the quantification of triglycerides, 10 µL of each sample was added to 1 mL Triglyceride Working Reagent (Sigma-Aldrich, USA) in a cuvette. For this purpose, 10 µL water and 10 µL glycerol were used as blank and standard, respectively. All solutions were gently mixed and incubated for 5 min at 37°C. Absorbance was measured at 540 nm using a Varioskan Flash plate reader (Thermo Fisher Scientific, USA).

For total fatty acid determination, extracted lipids were reconstituted in 0.5 mL iso-octane. The quantification of fatty acids was performed by gas chromatography using a mass spectrometry detector (Agilent 7890 A GC System, Agilent technologies Inc., USA). A fused silica capillary column (J&W DB-23 Agilent 122-236; 60 mm x 250 mm inner diameter) was used for injection of 1
9 μL of sample. High-purity helium was used as carrier gas at a flow rate of 1.3 mL/min with a split injection of 50:1. The GC temperature increased gradually from 80°C to 175°C at a rate of 5°C/min, followed by another gradient of 3°C/min to 230°C for 5 min. The temperature of GC inlet and transfer line was 250°C and 280°C, respectively. GC-mass spectrometry was performed using 70 eV electron-ionization in scan acquisition and quantified by total ion current. The fatty acids were identified by the National Institute of Standards and Technology 11 database and their retention times were compared with those of 4 purified standard mixtures of fatty acid methyl esters (Sigma-Aldrich, USA). All mass spectra were acquired over the m/z range of 40-500. Samples were analyzed in triplicate.

2.8 Lipid hydroperoxide and antioxidant profiling

The lipid hydroperoxide levels were measured through hydroperoxide-mediated oxidation of Fe²⁺, with 100 μL of sample and 900 μL of a reaction mixture containing 250 μM FeSO₄, 25 mM H₂SO₄, 100 μM xylene orange and 4 mM butylated hydroxytoluene in 90% (v/v) methanol. The superoxide dismutase (SOD) activity was determined based on the ability of the enzyme to inhibit the reduction of nitro blue tetrazolium (NBT), which was generated by hydroxylamine in a medium containing phosphate buffer, 0.1 mM EDTA, 50 mM NTB, 78 mM NADH and 33 mM phenazine methosulfate. Catalase activity was determined in a solution containing 30 mM hydrogen peroxide and sodium phosphate buffer 50 mM, with 0.1 mM ethylenediamine tetra-acetic acid. For glutathione peroxidase (GSH-Px) activity, tert-butylhydroperoxide was used as substrate and the formation of oxidized glutathione was monitored spectrophotometrically through NADPH consumption. All determinations were performed using a Varioskan Flash plate reader at 340 nm (37°C) (Thermo Fisher Scientific, USA).

2.9 Statistical analysis
Incidence data were analyzed by Fisher’s exact test and data on scores were analyzed by Kruskal-Wallis tests. Other data were analyzed by One-way ANOVA or Kruskal-Wallis tests and post hoc Tukey tests, using Prism 6 software (GraphPad, USA), and considering $p<0.05$ as significant.

3. Results

3.1 Phenolic profile of A. lappa root extract

The phenolic content analysis of BRE revealed chlorogenic [535.00 ± 29.70 mg/100g of sample, data are mean ± standard deviation (S.D.)] and caffeic acid contents (47.00 ± 1.41 mg/100g). Representative chromatogram and UV spectra of phenolic acid determination is presented in Figure 2. These compounds are mainly located on the peel of burdock roots and possess high antioxidant activity.$^{29}$ The non-identified peaks presented in the UV spectra are similar to other lignans previously identified in burdock roots.$^{12,30,31}$ Compared to other A. lappa root extracts applied in previous bioassays, the present BRE displayed similar or higher phenolic acid content.$^{13,31}$

3.2 General findings, transaminases and histological analysis

All groups showed similar body weight gain and evolution during the whole experimental period (data not shown). Although a trend for increase in liver relative weight in G3 (BRE at 200 mg/Kg b.wt.) ($p=0.007$) was observed, none of the groups significantly differed in liver absolute weight as well as in both absolute and relative retroperitoneal, epididymal and total fat weights (Supplementary Table 1).

At necropsy, livers of all groups showed similar macroscopic appearance, presenting irregular surfaces and yellowish appearance (Supplementary Figure 1). As expected, the analysis of HE and Sirius red-stained liver sections of rats submitted to NASH-associated
hepatocarcinogenesis (G1-G3) revealed liver steatosis, hepatocyte ballooning, lobular inflammation and fibrosis (Supplementary Figure 1), typical features of NASH.\textsuperscript{21} The scoring of these parameters, as well as collagen morphometry in Sirius red-stained sections, did not show a significant difference among the experimental groups (Supplementary Table 2). In addition, animals displayed similar ALT and AST serum levels in all experimental groups (Supplementary Table 1).

3.3 \textit{A. lappa root decreases total fatty acids in the liver}

Despite of unaltered serum cholesterol levels as well as unmodified serum and liver triglycerides amounts (Supplementary Table 1), both BRE doses (G2 and G3) decreased liver total fat acids levels (p<0.001) (Figure 3). In particular, burdock treatment decreased hepatic levels of palmitic (C16:0), linoleic (18:2 n-6) and arachidonic acids (C20:4 n-6) (p<0.001, for all) (Figure 3), while stearic (C18:0) and oleic acid (C18:1 n-9) remained unaffected. Both treatments led to similar reductions, not differing from each other.

3.3 \textit{A. lappa root decreases oxidative stress in the liver}

Enhanced oxidative stress plays a pivotal role in both steatohepatitis and liver carcinogenesis, contributing to NAFLD promotion and preneoplastic lesion development.\textsuperscript{32,33} Burdock treatment significantly decreased lipid hydroperoxide levels in the liver (p=0.003) (Figure 6), which are byproducts of fatty acid oxidization and well-accepted as oxidative stress markers. Moreover, both BRE treatments (100 and 200 mg/Kg) enhanced the activity of antioxidant SOD (p=0.020) and catalase enzymes in the liver (p=0.040), respectively (Figure 4), while treatments did not alter GSH-Px activity. SOD and catalase are endogenous antioxidant enzymes responsible for neutralizing reactive species, as TAA metabolites and other byproducts of lipotoxicity.

3.5 \textit{A. lappa root attenuates GST-P positive PNL development}
During rat hepatocarcinogenesis, GST-P expression is a classical and reliable biomarker for identification, quantification and measuring of both persistent and remodeling PNL\textsuperscript{23} (Figure 4). Although BRE 200 mg/Kg treatment (G3) tended to reduce the incidence of persistent and remodeling PNL compared to control group (G1), respectively, no statistical differences were observed among groups [Persistent: G1 = 9/9 (100%) vs. G2 = 8/9 (88.9%) vs. G3 = 6/9 (66.7%); values are the proportion of affected animals] [Remodeling: G1 = 9/9 (100%) vs. G2 = 8/9 (88.9%) vs. G3 = 7/9 (77.8%)]. Burdock intervention did not alter the number of both persistent and remodeling PNL per cm\textsuperscript{2} [Persistent: G1 = 6.41 ± 2.21 vs. G2 = 7.93 ± 2.84 vs. G3 = 6.69 ± 1.48] [Remodeling: G1 = 4.05 ± 1.35 vs. G2 = 5.07 ± 3.49 vs. G3 = 6.09 ± 3.86]. Nonetheless, burdock, in both doses, reduced the size of remodeling GST-P positive PNL (p=0.008) (Figure 5). Furthermore, BRE 100 mg/kg treatment (G3) decreased the area of liver section occupied by remodeling PNL (p=0.023) (Figure 5).

In line to reduced PNL size, it was found that BRE at 100 and 200 mg/Kg b.wt. presented a trend on decreasing hepatocyte proliferation (Ki-67) inside remodeling PNL (p=0.059), leading to 48% and 75% reductions, respectively (Figure 6). BRE 200 mg/kg treatment (G3) also reduced hepatocyte proliferation outside PNL by 38%, yet not being statistically relevant (p=0.09) (Figure 6).

4. Discussion

The present study evaluated the protective effects of edible \textit{Arctium lappa} root, widely applied in TCM, on a short-term diet- and chemically- induced model of NASH-associated hepatocarcinogenesis in rats. Considering the popular belief on the beneficial properties of this
millenary herb and, recently, the antiproliferative, anti-inflammatory and antioxidant properties described in vivo and in vitro\textsuperscript{11-14}, the authors hypothesized that A. lappa root extract treatment would alleviate NASH development and attenuate PNL development. In summary, A. lappa root extract reduced total fatty acids in the liver (including palmitic, linoleic, and arachidonic acids), diminished oxidative stress and increased the activity of antioxidant SOD and catalase enzymes. Ultimately, A. lappa root reduced the size of GST-P positive remodeling PNL that developed in NASH context. The phenolic analysis of BRE used revealed the presence of caffeic acid and, especially, high levels of chlorogenic acid.

In human and murine diet-NAFLD models, the increase in total fatty acid levels is positively correlated to liver lipogenesis and play critical roles in the establishment and progression of this disease.\textsuperscript{7,8,34} In vitro data from HCC cell lines (HepG2 and Huh7) suggest that palmitic acid increases fatty acid uptake via upregulation of peroxisome proliferator-activated receptor gamma (PPARγ) and cluster of differentiation 36 (CD36) protein expression, subsequently leading to oxidative and endoplasmic reticulum (ER) stress.\textsuperscript{35} Moreover, the increase in the levels of arachidonic and linoleic acid metabolites has been proposed as a biomarker for different stages of NAFLD.\textsuperscript{36,37} In contrast, chlorogenic acid administration (100 mg/Kg b.wt., for 15 weeks) is known to reduce PPARγ and CD36 mRNA expression and hence to attenuate steatosis in vivo.\textsuperscript{38} In addition, caffeic acid treatment by gavage (12 mg/Kg b.wt., for \textasciitilde7 weeks) led to diminished levels of fatty acids in the liver, kidney and serum in an alcohol-induced steatosis rat model.\textsuperscript{39} Thus, although both burdock doses did not decrease cholesterol, triglyceride quantities and steatosis score upon subacute exposure (2 weeks), the decrease in hepatic fatty acid levels may be attributed to the polyphenols in burdock root ethanolic extract, especially the abundantly present chlorogenic acid. Longer-term treatments with polyphenol-rich burdock extract could alleviate lipid accumulation (therefore, the “first hit”) in NASH-submitted rats, yet this warrants further investigation.
Hepatic lipid overloading is also proposed to disrupt of the redox balance, contributing to accumulation of reactive oxygen species (ROS), then abrogating antioxidant response. In CDHFD and TAA-induced NASH rat model, lipid accumulation and TAA metabolism are responsible for fatty acid oxidization, generating lipid hydroperoxides. A. lappa root interventions decreased the levels of these byproducts in the liver. Moreover, the ethanolic extract of this root enhanced SOD and catalase activities. The polyphenols, including the hydroxycinnamic acids present in burdock roots, can trigger the antioxidant response element (ARE)/ Nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, leading to enhanced gene expression of many antioxidant agents, including catalase and SOD. In addition, these compounds may exert antioxidant activity due to their electron-donating groups, thereby directly neutralizing reactive oxygen species and lipid hydroperoxides. Chlorogenic and caffeic acids isolated from burdock root peel indeed displayed significant free radical scavenging activity in vitro. Thus, burdock treatments are likely to reduce oxidative stress in the liver by increasing endogenous antioxidant agents. Reduced oxidative stress is proposed to prevent the "second hit" necessary to NAFLD progression.

In the rat model used in this study as well as in the corresponding human disease, the context of steatohepatitis is a permissive environment for preneoplastic lesion emergence and progression. Both A. lappa root treatments, showing no dose-response effect, only reduced the size of remodeling GST-P positive PNL. In keeping with these findings, BRE treatment presented a trend for reduction in hepatocyte proliferation (Ki-67) inside these lesions. Sustained cell proliferation inside PNL, which is a classical hallmark of cancer, could lead to lesion growth and may predispose to neoplastic transformation. Indeed, there are many molecular differences between persistent and remodeling PNL, and remodeling PNL tend to disappear. Nonetheless, global gene expression showed that early PNL displays similar gene expression patterns compared to HCC, thereby highlighting the potential importance of these lesions during the early stages of rat
hepatocarcinogenesis. A reduction in PNL size therefore indicates a direct modulation of early hepatocarcinogenesis by short-term burdock treatments (2 weeks). Long-term or medium-term interventions could unravel beneficial effects of burdock on persistent PNL and HCC, and further investigations are warranted.

Collectively, the findings of this pioneering study indicate that short-term exposure to chlorogenic acid-rich BRE reduces hepatic fatty acid accumulation and, particularly, alleviate PNL development in NASH-associated hepatocarcinogenesis. Medium or long-term interventions with A. lappa root extract are proposed in order to elicit more pronounced effects on pathogenesis of this disease. To the best of our knowledge, this is the first work to present evidence on the beneficial effects of a TCM edible plant on a NASH disease model.

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7. Disclosure statement

No competing financial interests exist.
Figure captions

Figure 1. Experimental design. For details, see “Materials and Methods” section.

Figure 2. Chromatogram and UV spectra of the free phenolics present in burdock root ethanolic extract (BRE). Peak identifications - peak 1: chlorogenic acid; peak 2: caffeic acid. Chromatogram extracted at 325 nm.

Figure 3. Effects of burdock root ethanolic extract (BRE) administration on total fatty acid, palmitic, linoleic, arachidonic, stearic and oleic acid hepatic levels in NASH-associated hepatocarcinogenesis. Values are mean ± S.D. All groups (n=9 rats/group) received choline-deficient high-fat diet (CDHFD) for 8 weeks and thioacetamide (TAA, 200 mg/Kg b.wt., 3x/week, i.p. for 4 weeks). Thereafter, rats received (G1) 5% DMSO vehicle, (G2) 100 or (G3) 200 mg/Kg b.wt. of BRE (gavage, for 2 weeks). Data were analyzed by ANOVA and post hoc Tukey’s tests (p<0.05).

Figure 4. Effects of burdock root ethanolic extract (BRE) administration on lipid hydroperoxide levels and glutathione peroxidase (GSH-Px), catalase and superoxide dismutase (SOD) activities in NASH-associated hepatocarcinogenesis. Values correspond to mean ± S.D. All groups (n=9 rats/group) received choline-deficient high-fat diet (CDHFD) for 8 weeks and thioacetamide (TAA, 200 mg/Kg b.wt., i.p., 3x/week, for 4 weeks). Thereafter, rats received (G1) 5% DMSO vehicle, (G2) 100 or (G3) 200 mg/Kg b.wt. of BRE (gavage, for 2 weeks). Data were analyzed by ANOVA and post hoc Tukey’s tests (p<0.05).

Figure 5. Representative photomicrographs of (A) remodeling (rPNL) and (B) persistent (pPNL) GST-P⁺ preneoplastic lesions (PNL) (scale bar = 200 μm). Effects of burdock root ethanolic extract (BRE) administration on size and liver area occupied by PNL in NASH-associated hepatocarcinogenesis. Values are mean ± S.D. All groups (n=9 rats/group) received choline-deficient high-fat diet (CDHFD) for 8 weeks and thioacetamide (TAA, 200 mg/Kg b.wt., i.p. 3x/week,
for 4 weeks). Thereafter, rats received (G1) 5% DMSO vehicle, (G2) 100 or (G3) 200 mg/Kg b.wt. of BRE (gavage, for 2 weeks). Data were analyzed by ANOVA and post hoc Tukey's tests (p<0.05).

**Figure 6.** Effects of burdock root ethanolic extract (BRE) administration on hepatocyte proliferation (Ki-67) in surrounding tissue and remodeling preneoplastic lesions (PNL) in NASH-associated hepatocarcinogenesis. [Scale bars: surrounding tissue (20 μm); Remodeling PNL G1 (100 μm); G2 and G3 (50 μm)]. Values are mean ± S.D. All groups (n=9 rats/group) received choline-deficient high-fat diet (CDHFD) for 8 weeks and thioacetamide (TAA, 200 mg/Kg b.wt., i.p., 3x/week, for 4 weeks). Thereafter, rats received (G1) 5% DMSO vehicle, (G2) 100 or (G3) 200 mg/Kg b.wt. of BRE (gavage, for 2 weeks). Data were analyzed by ANOVA (p<0.05).

**Supplementary Figure 1.** Representative macroscopic appearance and photomicrographs of HE and Sirius red–stained sections of experimental groups (scale bar = 50 μm). All groups (n=9 rats/group) received choline-deficient high-fat diet (CDHFD) for 8 weeks and thioacetamide (TAA, 200 mg/Kg b.wt., i.p., 3x/week, for 4 weeks). Thereafter, rats received (G1) 5% DMSO vehicle, (G2) 100 or (G3) 200 mg/Kg b.wt. of BRE (gavage, for 2 weeks).

**References**


