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Optimization of Aqueous Extraction and Biological Activity of *Harpagophytum procumbens* Root on *Ex Vivo* Rat Colon Inflammatory Model

Marcello Locatelli,[1] Claudio Ferrante,[1]* Simone Carradori,[1] Daniela Secci,[2] Lidia Leporini,[1] Annalisa Chiavaroli,[1] Sheila Leone,[1] Lucia Recinella,[1] Giustino Orlando,[1] Sara Martinotti,[1] Luigi Brunetti,[1] Michele Vacca[1] and Luigi Menghini[1]

[1]Department of Pharmacy, G. D'Annunzio University Chieti-Pescara, Via dei Vestini 31, 66100, Chieti, Italy
[2]Department of Drug Chemistry and Technologies, Sapienza University of Rome, P.le A. Moro 5, 00185, Rome, Italy

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***Harpagophytum procumbens* has a long story of use for the treatment of inflammatory diseases. Considering both the antiinflammatory effects of *H. procumbens* in multiple tissues and the stability of harpagoside in artificial intestinal fluid, the aim of the present study was to explore the possible protective role of a microwave-assisted aqueous Harpagophytum extract (1–1000 μg/mL) on mouse myoblast C2C12 and human colorectal adenocarcinoma HCT116 cell lines, and isolated rat colon specimens challenged with lipopolysaccharide (LPS), a validated *ex vivo* model of acute ulcerative colitis. In this context, we evaluated the effects on C2C12 and HCT116 viability, and on LPS-induced production of serotonin (5-HT), tumor necrosis factor (TNF)-α, prostaglandin (PG)E_2 and 8-iso-prostaglandin (8-iso-PG)$F_{2\alpha}$. Harpagophytum extract was well tolerated by C2C12 cells, while reduced HCT116 colon cancer cell viability. On the other hand, Harpagophytum extract reduced H_2O_2-induced (1 mM) reactive oxygen species (ROS) production, in both cell lines, and inhibited LPS-induced colon production of PGE_2, 8-iso-$PGF_{2\alpha}$, 5-HT and TNFα.**

Concluding, we demonstrated the efficacy of a microwave-assisted Harpagophytum aqueous extract in modulating the inflammatory, oxidative stress and immune response in an experimental model of inflammatory bowel diseases (IBD), thus suggesting a rational use of Harpagophytum in the management and prevention of ulcerative colitis in humans. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: Harpagophytum procumbens; microwave-assisted extraction; serotonin; TNFα; PGE_2; 8-iso-$PGF_{2\alpha}$; ROS; viability.

INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic disorders of the colonic mucosa (ulcerative colitis), whose etiology is strictly related to increased oxidative stress (Koutroubakis *et al.*, 2004; Rezaie *et al.*, 2007; Achitei *et al.*, 2013). On the other hand, antioxidant/antiinflammatory herbal extracts were able to contrast oxidative stress-related IBD symptoms (Chung *et al.*, 2007; Lenoir *et al.*, 2012). *Harpagophytum procumbens*, traditionally known as devil's claw or Harpagophytum, is a plant species that has been long used for the treatment of inflammatory diseases, including degenerative rheumatoid arthritis, osteoarthritis and tendonitis (Chantre *et al.*, 2000), kidney inflammation (Kaszkin *et al.*, 2004) and heart disease (Circosta *et al.*, 1984; Costa De Pasquale *et al.*, 1985). The major chemical constituents of Harpagophytum are iridoid glycosides (primarily harpagoside, harpagide and procumbide), sugars (mainly the tetrasaccharide stachyose), triterpenoids (oleanolic and ursolic acid), phytosterols (primarily β-sitosterol), aromatic acids (caffeic, cinnamic and chlorogenic acids) and flavonoids such as luteolin and kaempferol. Harpagoside, harpagide and procumbide, found in the tubers of the plant, appear to be the most therapeutically important constituents. Secondary storage tubers contain twice as much harpagoside as the taproot (Bradley, 1992).

To improve extraction efficiency, microwave-assisted extraction has been developed as green and alternative procedure that provides a high reproducibility, significant reduction in organic solvent waste, time and temperature, and lower energy consumption. Recent microwave-assisted extraction studies indicated that large dielectric constant solvents, such as water and ethanol as well as mixture of them, absorb strongly microwave energy furnishing fast heating of the sample and providing better extraction efficiency. In this paper, microwave-assisted extraction (MAE) was applied to the extraction of *H. procumbens* for the first time, because microwave irradiation could enhance molecular interactions between the solvent and the target compounds. Moreover, our interest aimed at validating the traditional procedure of decoction in water to easily obtain a harpagoside-enriched extract from *H. procumbens* root.

* Correspondence to: Prof. Claudio Ferrante, Dipartimento di Farmacia, Università 'G. D'Annunzio' Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy.
E-mail: claudio.ferrante@unich.it

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In order to monitor the extraction parameters, including time, solvent-to-material ratio and solvent type, HPLC-PDA has been widely used. Therefore, in this paper, we used an HPLC-PDA protocol to evaluate and optimize the extraction of selected secondary metabolites, with particular attention to harpagoside, in order to provide valuable information for *H. procumbens* MAE analysis with satisfactory separation, good resolution and high sensitivity.

The rationale for the traditional use is consistent, albeit in part, with the down-regulation of multiple pathways involved in inflammation, including ROS, cytokines and prostaglandin production (Huang *et al.*, 2006; Grant *et al.*, 2009; Fiebich *et al.*, 2012; Schaffer *et al.*, 2013; Schaffer *et al.*, 2016 Günther *et al.*, 2006). On the other hand, the possible hepatic cytotoxic effects induced by Harpagophytum and harpagoside should be taken in consideration (Biazi *et al.*, 2016). Considering both the capability of *H. procumbens* to contrast inflammatory stimuli in multiple tissues (Schaffer *et al.*, 2013), and the stability of harpagoside in artificial intestinal fluid (Chrubasik *et al.*, 2000), the aim of the present study was to explore the possible protective role of the plant microwave-assisted aqueous extract on mouse myoblast C2C12 and human colorectal adenocarcinoma HCT116 cell lines, and isolated rat colon specimens treated with lipopolysaccharide (LPS), a validated *ex vivo* model of acute ulcerative colitis (Bahar *et al.*, 2012; Menghini *et al.*, 2016). In this context, we assayed the protective role of a *H. procumbens* microwave-assisted extract by measuring the activities of different biomarkers of colon inflammation and lipid peroxidation such as ROS, serotonin (5-HT), prostaglandin (PG)E_2 and 8-iso-prostaglandin (8-iso-PG)$F_{2\alpha}$ (Nagib *et al.*, 2013; Motavallian *et al.*, 2013; Regmi *et al.*, 2014). In addition, we investigated the immune response modulatory effects of the plant, by measuring the gene expression of tumor necrosis factor (TNF)-α, a cytokine playing a key role in colon epithelium damage (Feghali and Wright, 1997; Lee *et al.*, 2010).

MATERIAL AND METHODS

Chemicals and standards

Harpagoside, as chemical standard used for the HPLC-PDA analyses (purity ≥95%), was obtained from Sigma-Aldrich (Milan, Italy). Methanol and acetonitrile (HPLC-grade) were purchased from Sigma-Aldrich (Milan, Italy), while HPLC-grade acetic acid was bought from Carlo Erba Reagents (Milan, Italy). Double distilled water (Milli-Q system, Millipore, Bedford, USA) was used.

Plant materials

Samples of dry root of *H. procumbens* DC. ex Meisn. were purchased in a local market in Namibia. It consists in round shape slices with variable diameter (10–50 mm) and thickness (3–15 mm). The thinner slices present margins partially rolled, while the thicker slices are wrinkled. The cortex is always present, with evident longitudinal wrinkles. The section is brown, with pale color in the central cylinder while cortex is dark brown. Following water reconstitution, slices rapidly return to stretch regular shape, showing the evident cambial zone and radial xylematic boundles see (Supporting Information).

H. procumbens root material was ground using a mixer grinder to a fine powder (FULL), passing through a 40 mesh to obtain a uniform granulometry and was stored in a vacuum box in the dark at 4°C until use. A part of dry plant material was manually divided into cortical cylinder (R-C) and central cylinder (IN) and powdered.

Plant extract

Microwave-assisted extraction was performed using an automatic Biotage Initiator™ 2.0 (Uppsala, Sweden), 2.45-GHz high-frequency microwaves, power range 0–300 W, as previously reported (Mollica *et al.*, 2016). An IR sensor probe controlled strictly the internal vial temperature. Plant powder (about 500 mg) was protected from the light and placed in a 20-mL sealed vessel suitable for an automatic single-mode microwave reactor. Then, 20 mL of the corresponding solvent (water or water:ethanol 50:50, *v*:*v*) was added to the sample to form a brown suspension. Microwave-assisted extraction was carried out, after a prestirring of 5 s, heating the sample by microwave irradiation for 2, 4, 6, 8, 10 or 15 min at 40, 60, 80, 100 or 120°C (± 1°C), followed by cooling with pressurized air. The suspension was then filtered through a 0.2-μm syringe filter (Sigma-Aldrich, Milan, Italy), and the extraction solvent was directly injected into the HPLC system. Each extraction was performed in triplicate.

Microwave-assisted extracts from the same batch powder were freshly prepared the day of the experiment, sterilized through 0.2-μm syringe filter (Sigma-Aldrich, Milan, Italy) and immediately used for biological tests.

HPLC-PDA analyses

Extracts of *H. procumbens* were analyzed for the harpagoside quantitative determination using a reversed phase HPLC-PDA in gradient elution mode. Analyses were carried out by using a Waters liquid chromatograph equipped with a photodiode array detector, a C18 reversed-phase column (Prodigy ODS(3), 4.6 × 150 mm, 5 μm; Phemomenex, Torrance, CA), an *on-line* degasser (Biotech 4-CH degasi compact, LabService, Anzola Emilia, Italy) and a column oven set at 30°C (± 1°C). The gradient elution was achieved by a solution of water–acetonitrile (93:7 ratio, with 3% of acetic acid) as initial conditions. The complete separation was achieved in 60 min by means of a validated method (Locatelli *et al.*, 2017; Zengin *et al.*, 2016a) and herein applied after evaluation of analytical performances and the absence of matrix interferences specifically for the harpagoside quantification.

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In vitro studies

C2C12 and HCT116 cells were cultured in DMEM (Euroclone) supplemented with 10% (*v*:*v*) heat-inactivated fetal bovine serum and 1.2% (*v*:*v*) penicillin G/streptomycin in 75 cm^2 tissue culture flask (n = 5 individual culture flasks for each condition). The cultured cells were maintained in a humidified incubator with 5% CO_2 at 37°C. For cell differentiation, C2C12 and HCT116 cell suspensions at a density of 1 × 10^6 cells/mL were treated with various doses (10, 50 and 100 ng/mL) of phorbol myristate acetate (PMA, Fluka) for 24 or 48 h (induction phase). Thereafter, the PMA-treated cells were washed twice with ice-cold pH 7.4 phosphate buffer solution (PBS) to remove PMA and non-adherent cells, whereas the adherent cells were further maintained for 48 h (recovery phase). Morphology of cells was examined under an inverted phase-contrast microscope (Sintiprungrat *et al.*, 2010). To assess the basal cytotoxicity of *H. procumbens*, a viability test was performed on 96-microwell plates, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Cells were incubated with extracts (ranging concentration 100–1000 μg/mL) for 24 h. Ten microliters of MTT (5 mg/mL) was added to each well and incubated for 3 h. The formazan dye formed was extracted with dimethyl sulfoxide and absorbance recorded as previously described (Menghini *et al.*, 2011). Effects on cell viability were evaluated in comparison to untreated control group.

Reactive oxygen species generation

Reactive oxygen species generation was assessed using a ROS-sensitive fluorescence indicator, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). When DCFH-DA is introduced into viable cells, it penetrates the cell and becomes deacetylated by intracellular esterases to form DCFH, which can react quantitatively with ROS within the cell and be converted to 2′,7′-dichlorofluorescein (DCF), which is detected by a fluorescence spectrophotometer. To determine intracellular effects on ROS production, cells were seeded in a black 96-well plate (1.5 × 10^4 cells/well) in medium containing scalar concentration of extracts. Immediately after seeding, the cells were stimulated for 1 h with H_2O_2 (1 mM). After the cells were incubated with DCFH-DA (20 μM) for 30 min, the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, using a fluorescence microplate reader.

Ex vivo studies

Male adult Sprague–Dawley rats (200–250 g) were housed in Plexiglas cages (40 cm × 25 cm × 15 cm), two rats per cage, in climatized colony rooms (22 ± 1°C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00–19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Rats were fed a standard laboratory diet (3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g). Housing conditions and experimentation procedures were strictly in accordance with the European Union ethical regulations on the care of animals for scientific research. According to the recognized ethical principles of 'Replacement, Refinement and Reduction of Animals in Research', colon specimens were obtained as residual material from vehicle-treated rats randomized in our previous experiments approved by Local Ethical Committee (University 'G. d'Annunzio' of Chieti-Pescara) and Italian Health Ministry (Project N. 880 definitely approved by Italian Health Ministry on 24 August 2015).

Rats were sacrificed by CO_2 inhalation (100% CO_2 at a flow rate of 20% of the chamber volume per min), and colon specimens were immediately collected and maintained in a humidified incubator with 5% CO_2 at 37°C for 4 h, in RPMI buffer with added bacterial LPS (10 μg/mL) (incubation period). During the incubation period, tissues were treated with scalar sub-toxic concentrations of microwave-assisted aqueous *H. procumbens* extract (100–1000 μg/mL). The efficacy of the extract was evaluated in comparison with sulfasalazine (2 mg/mL), that is used as a reference drug and whose efficacy could be partially related to reduced activity of cyclooxygenase and lipoxygenase. Tissue perfusates were collected and PGE_2 and 8-iso-$PGF_{2\alpha}$ levels (ng/mg wet tissue) were measured by radioimmunoassay (RIA), as previously reported (Chiavaroli *et al.*, 2010; Menghini *et al.*, 2010; Verratti *et al.*, 2011). Briefly, specific anti-8-iso-PGF2α and anti-PGE_2 were developed in the rabbit; the cross-reactivity against other prostanoids is <0.3%. One hundred microliters of prostaglandin standard or sample was incubated overnight at 4°C with the ^{3}H-prostaglandin (3000 cpm/tube; NEN) and antibody (final dilution: 1:120 000; kindly provided by Prof. G. Ciabattoni), in a volume of 1.5 mL of 0.025 M phosphate buffer. Free and antibody-bound prostaglandins were separated by the addition of 100 μL 5% bovine serum albumin and 100 μL 3% charcoal suspension, followed by centrifuging for 10 min at 4000 ×g at 5°C and decanting off the supernatants into scintillation fluid (Ultima Gold™, Perkin Elmer) for β emission counting. The detection limit of the assay method is 0.6 pg/mL. On the other hand, individual colon specimens were dissected and subjected to extractive procedures to evaluate 5-HT steady-state level (ng/mg wet tissue) and TNFα gene expression, as previously reported (Brunetti *et al.*, 2012; Brunetti *et al.*, 2013; Giorgioni *et al.*, 2010). As regards 5-HT analysis, tissues were homogenized in ice bath for 2 min with Potter–Elvehjem homogenizer in 1 mL of 0.05 N perchloric acid containing 0.004% sodium EDTA and 0.010% sodium bisulfite. Thereafter, samples were analyzed by HPLC coupled to electrochemical detection consisting of ESA Coulochem III detector equipped with ESA 5014B analytical cell. Finally, as regards to TNFα gene expression, colon specimens were dissected and stored in RNAlater solution (Ambion, Austin, TX) at −20°C until further processed. Total RNA was extracted from the hypothalamus and brown adipose tissue (BAT) using TRI Reagent (Sigma-Aldrich, St. Louis, MO). One microgram of total RNA extracted from each sample in a 20-μL reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reactions were incubated in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) initially at 25°C for

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10 min, then at 37°C for 120 min and finally at 85°C for 5 s. Gene expression was determined by quantitative real-time PCR using TaqMan probe-based chemistry (Applied Biosystems, Foster City, CA, USA). The real-time PCR was carried out in triplicate ~~for each cDNA sample in relation to each of the investigated genes~~. Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Means ± S.E.M. were determined for each experimental group and analyzed by one-way analysis of variance (ANOVA), followed by Newman–Keuls comparison multiple test. Statistical significance was set at $p < 0.05$. As regards gene expression analysis, the comparative $2^{-\Delta\Delta Ct}$ method was used to quantify the relative abundance of mRNA and then to determine the relative changes in individual gene expression (relative quantification) (Livak and Schmittgen, 2001). Finally, as regards the animals randomized for each experimental group, the number was calculated on the basis of the 'Resource Equation' N = (E + T)/T (10 ≤ E ≤ 20) (Charan and Kantharia, 2013), according to the guidelines suggested by the 'National Centre for the Replacement, Refinement and Reduction of Animals in Research' (NC3RS) and reported on the following web site: https://www.nc3rs.org.uk/experimental-designstatistics. In particular, N is the number of animals per treated group. E represents the degrees of freedom of the ANOVA. T is the number of treatments. Considering that E values should be between 10 and 20, the animal number N for *ex vivo* analysis was chosen in accordance to an E value of 20. Being E = 18 and *T* = 6, for the evaluation, N was 4.

RESULTS

Design and optimization of the MAE of this plant were performed keeping in mind the impact of different parameters (temperature, time, solvent, material-to-solvent ratio) on the recovery of its main metabolite (harpagoside) used as quality marker, as also requested by its Ph. Eur. 5.0 monograph. The selection of the most appropriate solvent for the selective extraction of the target compounds from the sample is an essential step for the development of any extraction method. As a non-toxic and pollution-free extraction solvent, water is widely used for natural product recovery. It has been observed that the addition of amounts of ethanol could enhance the extraction yield of the target compounds from the sample. Therefore, a mixture of ethanol/water 50:50 (*v:v*) was also chosen as the extraction solvent for comparison. Other extraction conditions were fixed as the solid to liquid ratio of 0.5: 20 (g/mL) and the irradiation power.

On the basis of literature data and considering the chemical nature of this hydrophilic monoglycoside, we carried out a preliminary MAE on Harpagophytum roots with water as polar solvent (solvent volume = 20 mL, material amount ~ 500 mg, extraction time = 10 min) to evaluate the best extraction temperature ranging from 40 to 120°C.

The recovery of harpagoside increased till around 100°C with possible degradation phenomena at higher temperature (Fig. 1A). Successively, we performed a time-dependent MAE at two discrete temperatures (80 and 100°C) to study the harpagoside saturation curve in this extract from 0 to 15 min. As reported in Fig. 1B, the curves reached a plateau in the range 6–10 min being the best aqueous extraction conditions temperature of 80°C and time of 8 min. Finally, we applied these parameters to select the best solvent for this extraction and the different localization of the metabolite in the root. As extrapolated from Fig. 1C, the mixture water/ethanol 50:50 was comparable to water alone in terms of harpagoside recovery, whereas this secondary metabolite was present in slightly higher concentration in the cortical cylinder (R-C) with respect to the central cylinder (IN) of the root. Moreover, we also evaluated another plant material-to-solvent ratio (250 mg of powder in 20 mL of extraction solvent) without obtaining an improvement of the harpagoside recovery from powdered root (data not shown). As shown in Fig. 1, the extraction recoveries to reach a plateau after 6 min at 80°C, and the obtained concentrations (as percentage, *w: w*) fulfill with Pharmacopoeia indications that report that *H. procumbens* must contain not less than 1.2% of harpagoside. In the HPLC-PDA analyses, the chromatographic run for the quantitative analyses was set to 60 min in order to resolve the harpagoside from the other secondary metabolites that could interfere with the final determination due to the overlapping of wavelengths used for quantitative purposes. Additionally, a good linearity was observed in the range 0.25–50 μg/mL, as reported by Zengin *et al.* (2016b).

Then, we performed *in vitro* and *ex vivo* studies to evaluate the efficacy of the described microwave-assisted aqueous Harpagophytum extract in modulating oxidative stress and inflammatory pathways, involved in IBD. The aqueous extract was chosen on the basis of a high harpagoside content and lower cost and toxicity of the solvent with respect to aqueous/ethanol (50:50, *v:v*).

Our *in vitro* study showed that the microwave-assisted aqueous Harpagophytum extract was well tolerated by C2C12 cell line in the range (100–1000 μg/mL) (Fig. 2), as observed for other aqueous extracts from other plant material (Paduch *et al.* 2015). Moreover, we observed a protective effect exerted by Harpagophytum extract (100–1000 μg/mL) as revealed by the significant concentration-dependent reduction of H_2O_2-induced (1 mM) ROS production (Fig. 3). However, we registered a significant concentration-dependent inhibitory effect on HCT116 colon cancer cell viability, following Harpagophytum treatment, and a concentration-dependent reduction of basal H_2O_2-induced ROS production (Figs 4, 5). Previously, Biazi and coworkers (2009) observed an inhibitory effect of HepG2/C3A hepatic cancer cell viability following the treatment with either harpagoside at a concentration of 700 μM or hydroalcoholic Harpagophytum extract (250 μg/mL). The same authors related the cytotoxic effect induced by ~~aqueous~~ Harpagophytum extract treatment to the possible inhibition of the metabolic pathways involved in the reduction of MTT in formazan.

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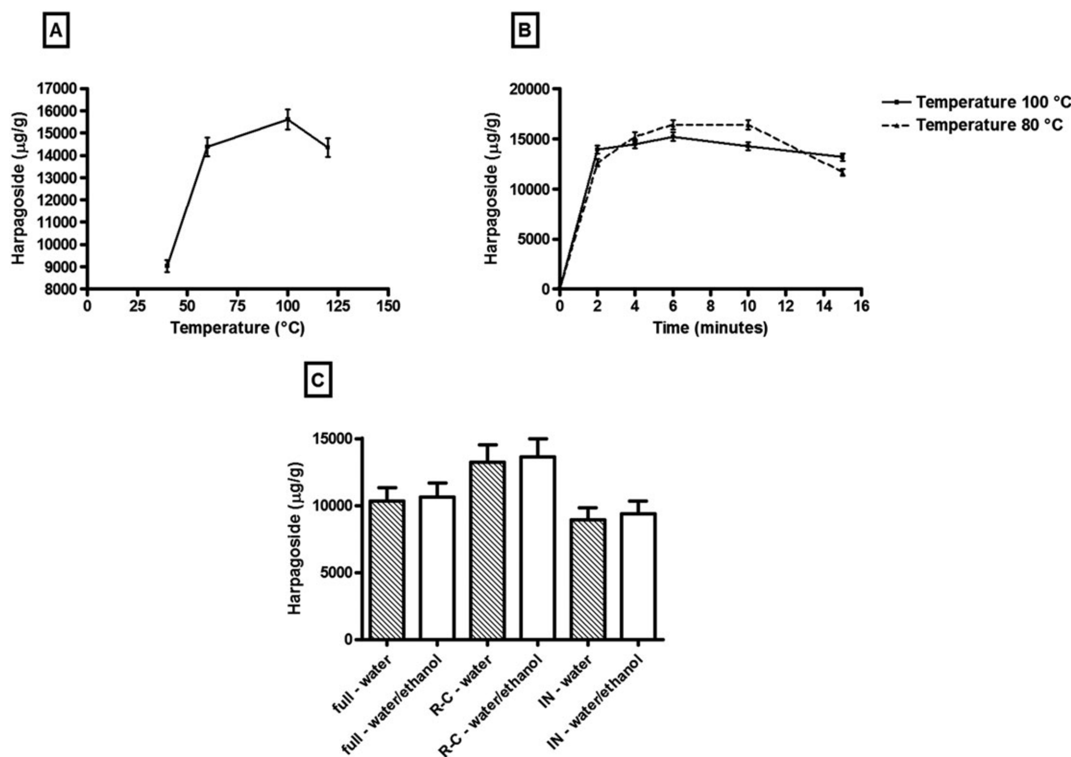
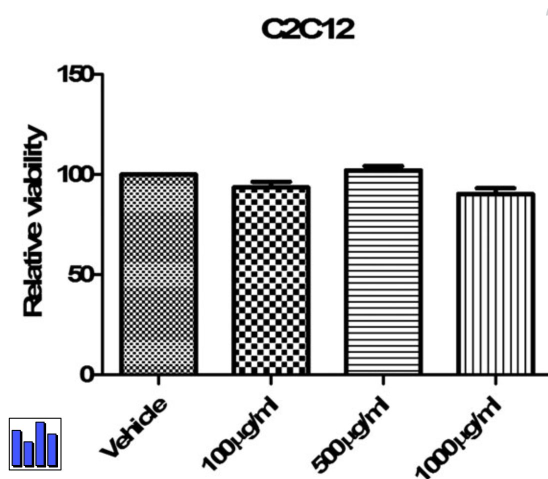


Figure 1. Recovery of harpagoside following microwave-assisted extraction of Harpagophytum roots at temperature ranging from 40 to 120°C (section a). Time-dependent microwave-assisted extraction at two discrete temperatures (80 and 100°C) to study the harpagoside saturation curve (section B). Solvent-dependent microwave-assisted extraction of harpagoside at 80°C for 8 min (section C) from whole root (FULL), central cylinder (IN) or cortical cylinder (R-C).



Q9 **Figure 2.** Effect of microwave-assisted aqueous Harpagophytum extract (100–1000 μg/mL) on C2C12 cell line viability.

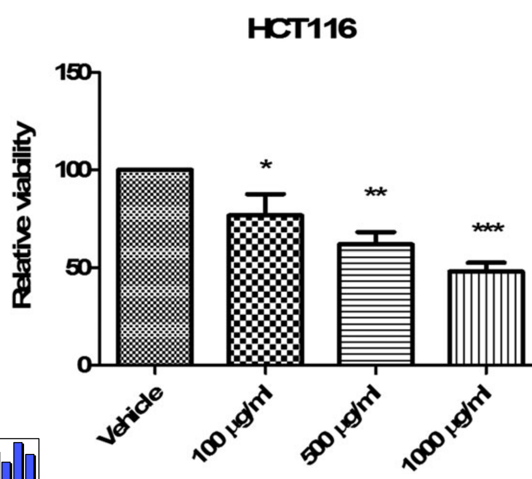


Figure 3. Effect of microwave-assisted aqueous Harpagophytum extract (100–1000 μg/mL) on H_2O_2 ROS production in C2C12 cell line. ANOVA, $p < 0.001$, *post hoc* $***p < 0.001$ *versus* H_2O_2-treated group.

Alcoholic Harpagophytum extract at concentration exceeding 500 μg/mL also inhibited human monocytic THP-1 cell viability (Hostanska *et al.*, 2014). On the other hand Schopohl *et al.* (2016) showed that only undifferentiated THP-1 cells were sensitive to the cytotoxic effects of harpagoside. In addition, Romiti *et al.* (2009) found no significant alteration of HK-2 cell line viability, following Harpagophytum extract (40–400 μg/mL) treatment. Their extracts were dissolved in dimethyl sulfoxide. These findings could indicate significant different sensitivities of the considered cell lines to Harpagophytum (Biazi *et al.*, 2016). In this context, our findings showing a different sensitivity between C2C12 and HCT116 cells, following Harpagophytum treatment, could be related to a different response to the metabolic effects induced by the extract. Considering the findings of Schopohl *et al.* (2016), we cannot exclude that the sensitivity of HCT116 to the cytotoxic effects of Harpagophytum could also depend on the low grade of differentiation (Ieta *et al.*, 2008).

The preliminary *in vitro* test was used as a valuable index of effective doses to define the concentration for colon tissue treatment. In the *ex vivo* experiments, Harpagophytum treatment (100–1000 μg/mL) was able

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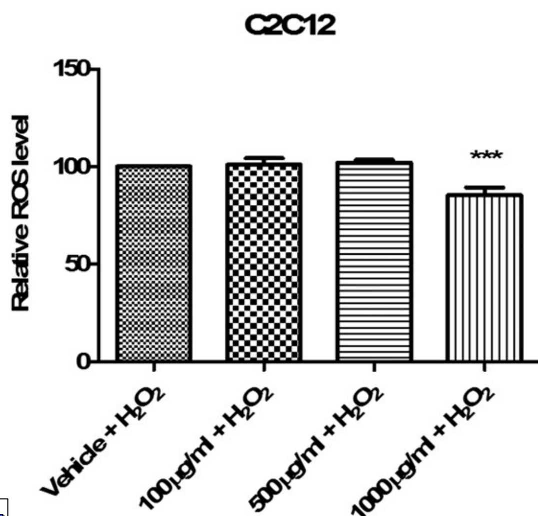


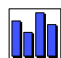
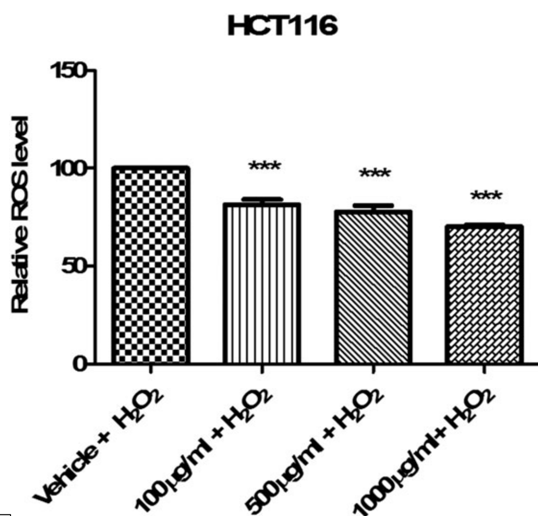


Figure 4. Effect of microwave-assisted aqueous Harpagophytum extract (100–1000 μg/mL) on HCT116 cell line viability. ANOVA, $p < 0.001$, *post hoc* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* H_2O_2-treated group.



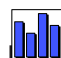
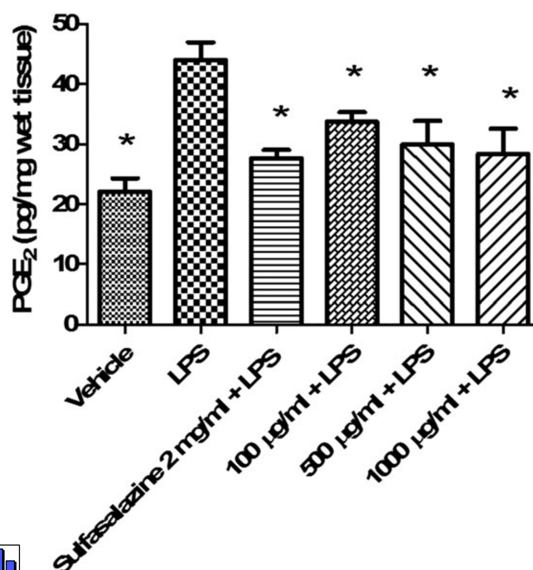


Figure 5. Effect of microwave-assisted aqueous Harpagophytum extract (100–1000 μg/mL) on H_2O_2 ROS production in HCT116 cell line. ANOVA, $p < 0.001$, *post hoc* $^{***}p < 0.001$ *versus* H_2O_2-treated group.

to significantly inhibit LPS-induced production of PGE_2, 8-iso-$PGF_{2\alpha}$, 5-HT and TNFα (Figs 6–9). The inhibitory effect was concentration independent, while the efficacy was comparable with the reference drug, sulfasalazine (2 mg/mL). On the other hand, Fiebich *et al.* (2012) showed a concentration-dependent (100–500 μg/mL) inhibition of LPS-stimulated production of PGE_2 and TNFα, induced by a hydroalcoholic Harpagophytum extract. In this context, our findings of concentration-independent inhibition of the tested inflammatory and stress oxidative mediators could depend on the employed *ex vivo* experimental paradigm.

DISCUSSION

Overproduction of reactive oxygen/nitrogen species (ROS/RNS) has long been involved in disruptive



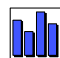
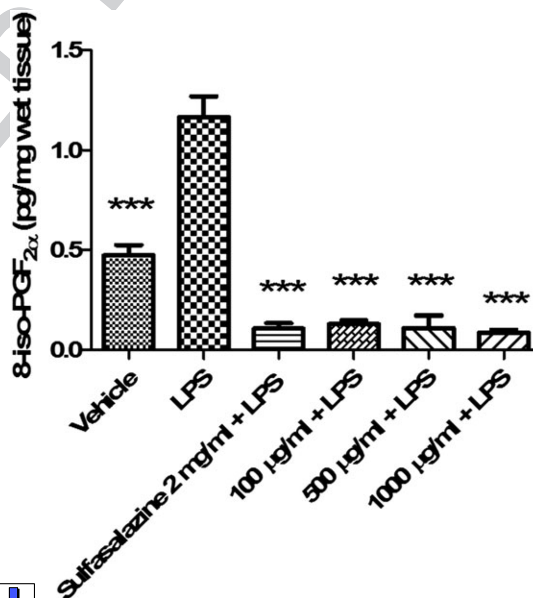


Figure 6. Effect of microwave-assisted aqueous Harpagophytum extract (100–1000 μg/mL) on prostaglandin E2 (PGE_2) levels (pg/mg wet tissue). ANOVA, $p < 0.05$, *post hoc* $^{*}p < 0.05$ *versus* LPS-treated group.



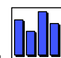


Figure 7. Effect of microwave-assisted aqueous Harpagophytum extract (100–1000 μg/mL) on 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) levels (pg/mg wet tissue). ANOVA, $p < 0.0001$, *post hoc* $^{***}p < 0.001$ *versus* LPS-treated group.

peroxidation reactions on cellular substrates such as proteins, lipids and nucleic acids (Uttara *et al.*, 2009). In particular, lipid peroxidation has been recognized in the onset of chronic diseases, including IBDs (Achitei *et al.*, 2013). The effects of ROS/RNS, mainly produced by macrophages and neutrophils, include neutrophils recruitment at the inflamed tissues (Kruidenier and Verspaget, 2002; Fialkow *et al.*, 2007). 8-Iso-PGF2α, an isomer of classic prostaglandins deriving from ROS/RNS peroxidation of membrane arachidonic acid, has been long considered a stable marker of oxidative stress, *in vivo* (Praticò, 2002). The observed antioxidant effect, as revealed by the inhibitory effect on ROS and isoprostane production, could be related to different

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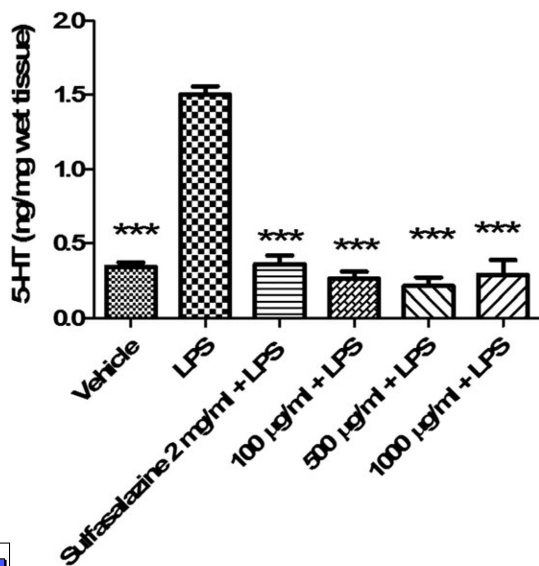


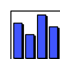
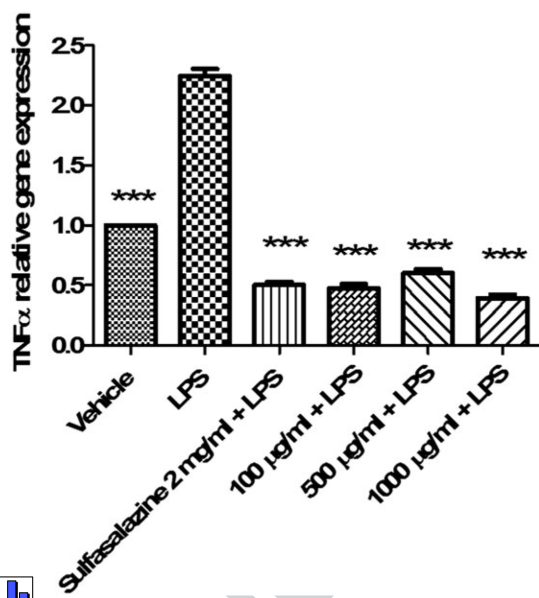


Figure 8. Effect of microwave-assisted aqueous Harpagophytum extract (100–1000 µg/mL) on serotonin (5-HT) levels (ng/mg wet tissue). ANOVA, $p < 0.0001$, *post hoc* *** $p < 0.001$ *versus* LPS-treated group.



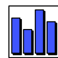


Figure 9. Effect of microwave-assisted aqueous Harpagophytum extract (100–1000 µg/mL) on tumor necrosis factor α (TNFα) and gene expression. ANOVA, $p < 0.0001$, *post hoc* ***$p < 0.001$ *versus* LPS-treated group.

concomitant mechanisms. On one side, Harpagophytum tincture (100 mg/mL in 66% ethanol) and DMSO extract inhibit myeloperoxidase (MPO) activity, a biomarker of neutrophil infiltration (Grant *et al.*, 2009). The same authors also observed a direct scavenging activity. On the other hand, Harpagophytum extract (ethyl acetate fraction) treatment could also stimulate catalase activity, a well-established anti-oxidative marker (Schaffer *et al.*, 2016). 5-HT pro-inflammatory role in IBDs has been previously suggested (Regmi *et al.*, 2014), possibly involving the activation of 5-HT3 receptors (Mousavizadeh *et al.*, 2009). Actually, our findings of reduced 5-HT steady-state level, in the rat colon, following aqueous Harpagophytum extract treatment, are consistent with the possible activation of growth hormone secretagogue receptor (GHSR) (Torres-Fuentes *et al.*, 2014). Previously, we observed that the feeding-stimulating hormone ghrelin, the endogenous GHSR agonist, and the anorexigenic hormone obestatin, which antagonizes the same receptor, were able to inhibit and stimulate depolarization-induced 5-HT release, respectively, from isolated presynaptic endings (synaptosomes), *ex vivo* (Brunetti *et al.*, 2002; Brunetti *et al.*, 2009; Brunetti *et al.*, 2010). In despite of a more detailed assessment of pharmacological modulation of neurotransmitter release, given by synaptosome experimental paradigm, several studies have confirmed that steady-state monoamine concentrations also prove to be a valuable index of neurotransmitter activity in multiple brain areas and at any given time point (Bungo *et al.*, 2009; Clark *et al.*, 2006). In this context, the reduction of colon 5-HT level following Harpagophytum treatment could be related to reduced serotonergic signaling, in the colon. In addition, we observed an inhibitory effect of Harpagophytum treatment on PGE_2 level and TNFα gene expression. TNFα and PGE_2 are two cytokines which have long been involved in colon epithelium inflammation and damage (Feghali and Wright, 1997; Lee *et al.*, 2010; Nagib *et al.*, 2013). Actually, our findings of reduced TNFα and PGE_2 activity following Harpagophytum treatment are in agreement with the reported inhibitory effects of the hydroalcoholic extract on TNFα and PGE_2 production in LPS-stimulated human monocytes (Fiebich *et al.*, 2012). The same authors suggested that Harpagophytum could act, at least in part, via inhibition of activator protein-1 (AP1)-mediated transcription of TNFα and cyclooxygenase- (COX-2) genes, without affecting the NFkB and the MAP kinase pathway. Activator protein-1 is a transcription factor that upregulates pro-inflammatory genes deeply involved in the pathogenesis of IBD, which could represent a key target for the development of new drugs for IBD treatment (Moriyama *et al.*, 2008). The inhibitory effects on the production of the tested pro-inflammatory mediators support a rationale use of homemade water extract by decoction of Harpagophytum in the management of the clinical symptoms related to IBDs. In addition, the protective effects on colon tissue induced by Harpagophytum treatment, in our *ex vivo* experimental model of IBD (Menghini *et al.*, 2016), are consistent with the inhibitory effects on COX-2 and PGE_2 activity exerted by ethanol Harpagophytum extract on isolated porcine skin, *ex vivo* (Ouitas and Heard, 2009). The possible use of the plant in IBDs could be also hypothesized based on the stability of harpagoside, in artificial intestinal fluid (Chrubasik *et al.*, 2000). However, the transition of aqueous Harpagophytum extract through the stomach could lead to loss of biological activity (Soulimani *et al.*, 1994). Finally, considering the inherent limitations of the *in vitro* and *ex vivo* experimental paradigms and the lack of analog scientific papers, as regards Harpagophytum efficacy in IBDs, further studies, comparing different analytical methods and experimental paradigms, including *in vivo* studies, for detection and quantification of oxidative stress, inflammation and immune response biomarkers are required for an accurate evaluation and validation of plant efficacy.

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Conflict of Interest

Authors declare no financial/commercial conflicts of interest.

REFERENCES

Achitei D, Ciobica A, Balan G, Gologan E, Stanciu C, Stefanescu G. 2013. Different profile of peripheral antioxidant enzymes and lipid peroxidation in active and non-active inflammatory bowel disease patients. *Dig Dis Sci* **58**: 1244–1249.

Bahar B, O'Doherty JV, Hayes M, Sweeney T. 2012. Extracts of brown seaweeds can attenuate the bacterial lipopolysaccharide-induced pro-inflammatory response in the porcine colon ex vivo. *J Anim Sci* **90**(Suppl 4): 464–468.

Biazi BI, D'Epiro GF, Zanetti TA, de Oliveira MT, Ribeiro LR, Mantovani MS. 2016. Risk assessment via metabolism and cell growth inhibition in a HepG2/C3A cell line upon treatment with Arpadol and its active component Harpagoside. *Phytother Res* . https://doi.org/10.1002/ptr.5757.

Bradley PR (Ed). 1992. British Herbal Compendium **1**. British Herbal Medicine Association: Dorset, UK.

Brunetti L, Recinella L, Orlando G, Michelotto B, Di Nisio C, Vacca M. 2002. Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. *Eur J Pharmacol* **454**(2–3): 189–192.

Brunetti L, Leone S, Orlando G, *et al.* 2009. Effects of obestatin on feeding and body weight after standard or cafeteria diet in the rat. *Peptides* **30**: 1323–1327.

Brunetti L, Di Nisio C, Recinella L, *et al.* 2010. Obestatin inhibits dopamine release in rat hypothalamus. *Eur J Pharmacol* **641**(2–3): 142–147.

Brunetti L, Orlando G, Ferrante C, *et al.* 2013. Orexigenic effects of omentin-1 related to decreased CART and CRH gene expression and increased norepinephrine synthesis and release in the hypothalamus. *Peptides* **44**: 66–74.

Brunetti L, Recinella L, Di Nisio C, *et al.* 2012. Effects of visfatin/PBEF/NAMPT on feeding behaviour and hypothalamic neuromodulators in the rat. *J Biol Regul Homeost Agents* **26**: 295–302.

Bungo T, Shiraishi J, Yanagita K, Ohta Y, Fujita M. 2009. Effect of nociceptin/orphanin FQ on feeding behavior and hypothalamic neuropeptide expression in layer-type chicks. *Gen Comp Endocrinol* **163**: 47–51.

Chantre P, Cappelaere A, Leblan D, Guedon D, Vandermander J, Fournie B. 2000. Efficacy and tolerance of *Harpagophytum procumbens* versus diacerhein in treatment of osteoarthritis. *Phytomedicine* **7**: 177–183.

Charan J, Kantharia ND. 2013. How to calculate sample size in animal studies? *J Pharmacol Pharmacother* **4**: 303–306.

Chiavaroli A, Brunetti L, Orlando G, *et al.* 2010. Resveratrol inhibits isoprostane production in young and aged rat brain. *J Biol Regul Homeost Agents* **24**: 441–446.

Chrubasik S, Sporer F, Dillmann-Marschner R, Friedmann A, Wink M. 2000. Physicochemical properties of harpagoside and its in vitro release from *Harpagophytum procumbens* extract tablets. *Phytomedicine* **6**: 469–473.

Circosta C, Occhiuto F, Ragusa S, *et al.* 1984. A drug used in traditional medicine: *Harpagophytum procumbens* DC. II. Cardiovascular activity. *J Ethnopharmacol* **11**: 259–274.

Clark KA, MohanKumar SMJ, Kasturi BS, MohanKumar PS. 2006. Effects of central and systemic administration of leptin on neurotransmitter concentrations in specific areas of the hypothalamus. *Am J Physiol Integr Comp Physiol* **290**: 306–312.

Costa De Pasquale R, Busa G, Circosta C, *et al.* 1985. A drug used in traditional medicine: *Harpagophytum procumbens* DC. III. Effects on hyperkinetic ventricular arrhythmias by reperfusion. *J Ethnopharmacol* **13**: 193–199.

Feghali CA, Wright TM. 1997. Cytokines in acute and chronic inflammation. *Front Biosci* **2**: 12–26.

Fialkow L, Wang Y, Downey GP. 2007. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radic Biol Med* **42**: 153–164.

Fiebich BL, Muñoz E, Rose T, Weiss G, McGregor GP. 2012. Molecular targets of the antiinflammatory *Harpagophytum procumbens* (devil's claw): inhibition of TNFα and COX-2 gene expression by preventing activation of AP-1. *Phytother Res* **26**: 806–811.

Giorgioni G, Claudi F, Ruggieri S, *et al.* 2010. Design, synthesis, and preliminary pharmacological evaluation of new imidazolinones as L-DOPA prodrugs. *Bioorg Med Chem* **18**: 1834–1843.

Grant L, McBean DE, Fyfe L, Warnock AM. 2009. The inhibition of free radical generation by preparations of *Harpagophytum procumbens* in vitro. *Phytother Res* **23**(1): 104–110.

Günther M, Laufer S, Schmidt PC. 2006. High anti-inflammatory activity of harpagoside-enriched extracts obtained from solvent-modified super-and subcritical carbon dioxide extractions of the roots of *Harpagophytum procumbens*. *Phytochem Anal* **17**: 1–7.

Hostanska K, Melzer J, Rostock M, Suter A, Saller R. 2014. Alteration of anti-inflammatory activity of *Harpagophytum procumbens* (devil's claw) extract after external metabolic activation with S9 mix. *J Pharm Pharmacol* **66**: 1606–1614.

Huang TH, Tran VH, Duke RK, *et al.* 2006. Harpagoside suppresses lipopolysaccharide-induced iNOS and COX-2 expression through inhibition of NF-kappa B activation. *J Ethnopharmacol* **104**(1–2): 149–155.

Ieta K, Tanaka F, Haraguchi N, *et al.* 2008. Biological and genetic characteristics of tumor-initiating cells in colon cancer. *Ann Surg Oncol* **15**: 638–648.

Kaszkin M, Beck KF, Koch E, *et al.* 2004. Downregulation of iNOS expression in rat mesangial cells by special extracts of *Harpagophytum procumbens* derives from harpagoside-dependent and independent effects. *Phytomedicine* **11**(7–8): 585–595.

Koutroubakis IE, Malliaraki N, Dimoulios PD, Karmiris K, Castanas E, Kouroumalis EA. 2004. Decreased total and corrected antioxidant capacity in patients with inflammatory bowel disease. *Dig Dis Sci* **49**(9): 1433–1437.

Kruidenier L, Verspaget HW. 2002. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease—radicals or ridiculous? *Aliment Pharmacol Ther* **16**(12): 1997–2015.

Lee JS, Park SY, Thapa D, *et al.* 2010. *Grifola frondosa* water extract alleviates intestinal inflammation by suppressing TNF-alpha production and its signaling. *Exp Mol Med* **42**(2): 143–154.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)). *Method Methods* **25**: 402–408.

Locatelli M, Zengin G, Uysal A, *et al.* 2017. Multicomponent pattern and biological activities of seven Asphodeline taxa: potential sources of natural–functional ingredients for bioactive formulations. *J Enzyme Inhib Med Chem* **32**: 60–67.

Menghini L, Genovese S, Epifano F, Tirillini B, Ferrante C, Leporini L. 2010. Antiproliferative, protective and antioxidant effects of artichoke, dandelion, turmeric and rosemary extracts and their formulation. *Int J Immunopathol Pharmacol* **23**(2): 601–610.

Menghini L, Leporini L, Scanu N, *et al.* 2011. Effect of phytochemical concentrations on biological activities of cranberry extracts. *J Biol Regul Homeost Agents* **25**: 27–35.

Menghini L, Ferrante C, Leporini L, *et al.* 2016. A hydroalcoholic chamomile extract modulates inflammatory and immune response in HT29 cells and isolated rat colon. *Phytother Res* **30**(9): 1513–1518.

Mollica A, Locatelli M, Macedonio G, *et al.* 2016. Microwave-assisted extraction, HPLC analysis, and inhibitory effects on carbonic anhydrase I, II, VA, and VII isoforms of 14 blueberry Italian cultivars. *J Enzyme Inhib Med Chem* **31**: 1–6.

Nagib MM, Tadros MG, ElSayed MI, Khalifa AE. 2013. Anti-inflammatory and anti-oxidant activities of olmesartan medoxomil ameliorate experimental colitis in rats. *Toxicol Appl Pharmacol* **271**(1): 106–113.

Moriyama I, Ishihara S, Rumi MA, *et al.* 2008. Decoy oligodeoxynucleotide targeting activator protein-1 (AP-1) attenuates intestinal inflammation in murine experimental colitis. *Lab Invest* **88**(6): 652–663.

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Motavallian A, Minaiyan M, Rabbani M, Andalib S, Mahzouni P. 2013. Involvement of 5HT3 receptors in anti-inflammatory effects of Tropisetron on experimental TNBS-induced colitis in rat. *Bioimpacts* **3**(4): 169–176.
Ouitas NA, Heard CM. 2009. A novel ex vivo skin model for the assessment of the potential transcutaneous anti-inflammatory effect of topically applied *Harpagophytum procumbens* extract. *Int J Pharm* **376**(1–2): 63–68.
Paduch R, Wiater A, Locatelli M, Pleszczyńska M, Tomczyk M. 2015. Aqueous extracts of selected Potentilla species modulate biological activity of human normal colon cells. *Curr Drug Targets* **16**: 1495–1502.
Praticò D. 2002. Alzheimer's disease and oxygen radicals: new insights. *Biochem Pharmacol* **63**(4): 563–567.
Regmi SC, Park SY, Ku SK, Kim JA. 2014. Serotonin regulates innate immune responses of colon epithelial cells through Nox2-derived reactive oxygen species. *Free Radic Biol Med* **69**: 377–389.
Rezaie A, Parker RD, Abdollahi M. 2007. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Dig Dis Sci* **52**(9): 2015–2021.
Romiti N, Tramonti G, Corti A, Chieli E. 2009. Effects of devil's claw (*Harpagophytum procumbens*) on the multidrug transporter ABCB1/P-glycoprotein. *Phytomedicine* **16**: 1095–1100.
Schaffer LF, Peroza LR, Boligon AA, *et al.* 2013. *Harpagophytum procumbens* prevents oxidative stress and loss of cell viability in vitro. *Neurochem Res* **38**(11): 2256–2267.
Schaffer LF, de Freitas CM, Chiapinotto Ceretta AP, *et al.* 2016. *Harpagophytum procumbens* ethyl acetate fraction reduces fluphenazine-induced vacuous chewing movements and oxidative stress in rat brain. *Neurochem Res* **41**(5): 1170–1184.
Schopohl P, Grüneberg P, Melzig MF. 2016. The influence of harpagoside and harpagide on TNFα-secretion and cell adhesion molecule mRNA-expression in IFNγ/LPS-stimulated THP-1 cells. *Fitoterapia* **110**: 157–165.
Sintiprungrat K, Singhto N, Sinchaikul S, Chen ST, Thongboonkerd V. 2010. Alterations in cellular proteome and secretome upon differentiation from monocyte to macrophage by treatment with phorbol myristate acetate: insights into biological processes. *J Proteomics* **73**: 602–618.
Soulimani R, Younos C, Mortier F, Derrieu C. 1994. The role of stomachal digestion on the pharmacological activity of plant extracts, using as an example extracts of *Harpagophytum procumbens*. *Can J Physiol Pharmacol* **72**(12): 1532–1536.
Torres-Fuentes C, Theeuwes WF, McMullen MK, *et al.* 2014. Devil's claw to suppress appetite—ghrelin receptor modulation potential of a *Harpagophytum procumbens* root extract. *PLoS One* **9**(7): e103118. doi: https://doi.org/10.1371/journal.pone.0103118.
Uttara B, Singh AV, Zamboni P, Mahajan RT. 2009. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol* **7**(1): 65–74.
Verratti V, Brunetti L, Tenaglia R, *et al.* 2011. Physiological analysis of 8-ISO-PGF2 alpha: a homeostatic agent in superficial bladder cancer. *J Biol Regul Homeost Agents* **25**(1): 71–76.
Zengin G, Menghini L, Malatesta L, *et al.* 2016a. Comparative study of biological activities and multicomponent pattern of two wild Turkish species: *Asphodeline anatolica* and *Potentilla speciosa*. *J Enzyme Inhib Med Chem* **31**: 203–208.
Zengin G, Locatelli M, Ceylan R, Aktumsek A. 2016b. Anthraquinone profile, antioxidant and enzyme inhibitory effect of root extracts of eight Asphodeline taxa from Turkey: can Asphodeline roots be considered as a new source of natural compounds? *J Enzyme Inhib Med Chem* **31**: 754–759.

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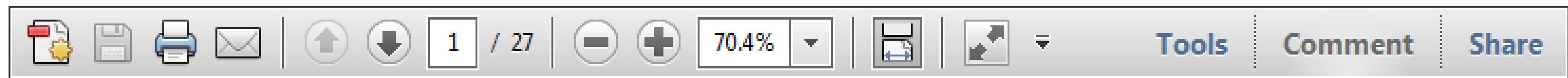

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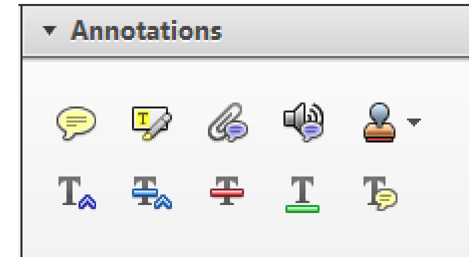

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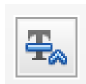

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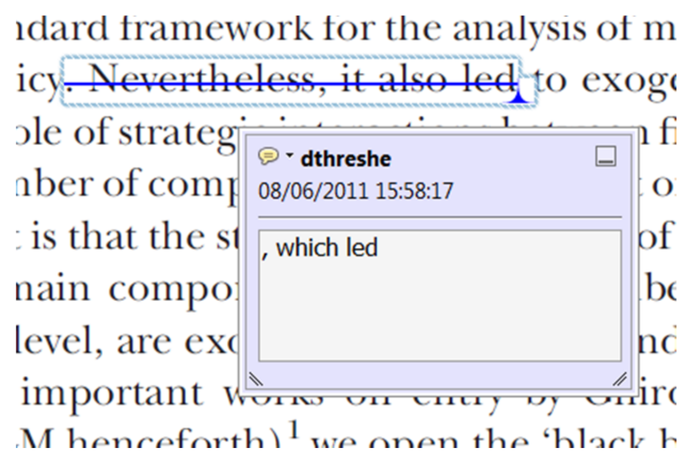

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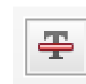

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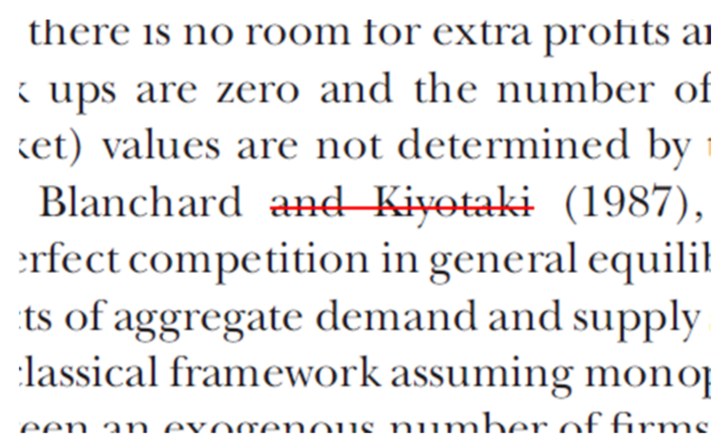
- Click on the Strikethrough (Del) icon in the Annotations section.



3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

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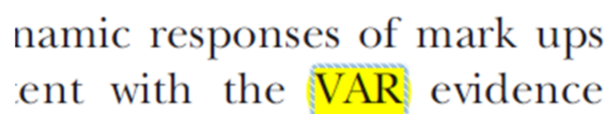

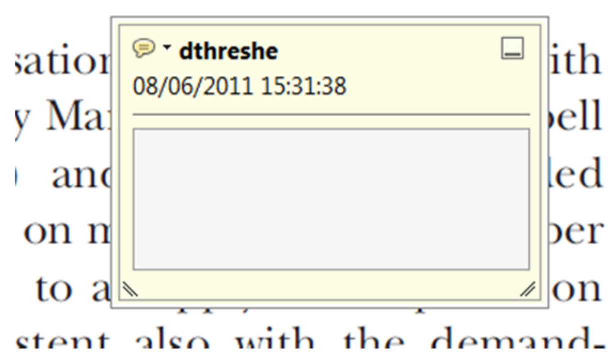

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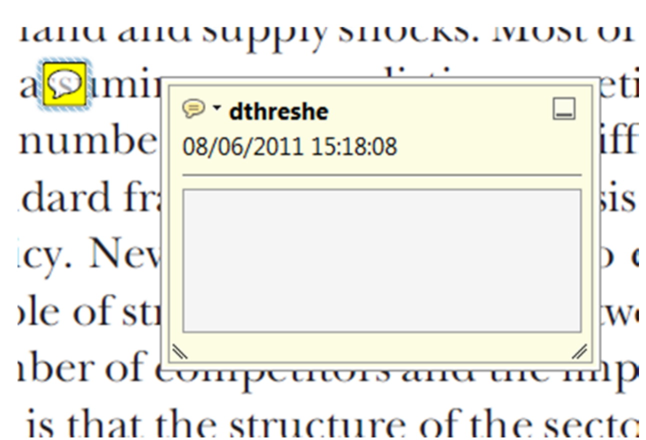

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5. Attach File Tool – for inserting large amounts of text or replacement figures.

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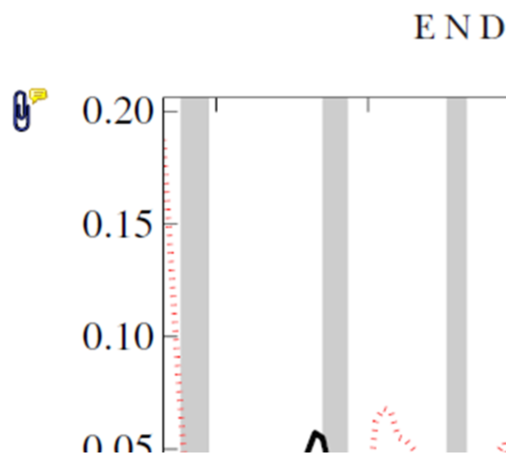

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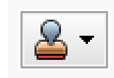

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- Click on the Add stamp icon in the Annotations section.
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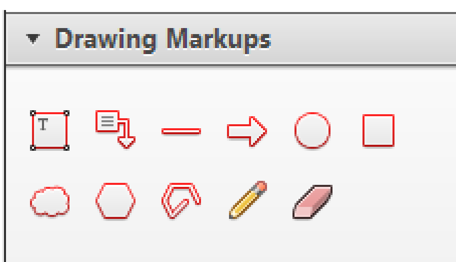

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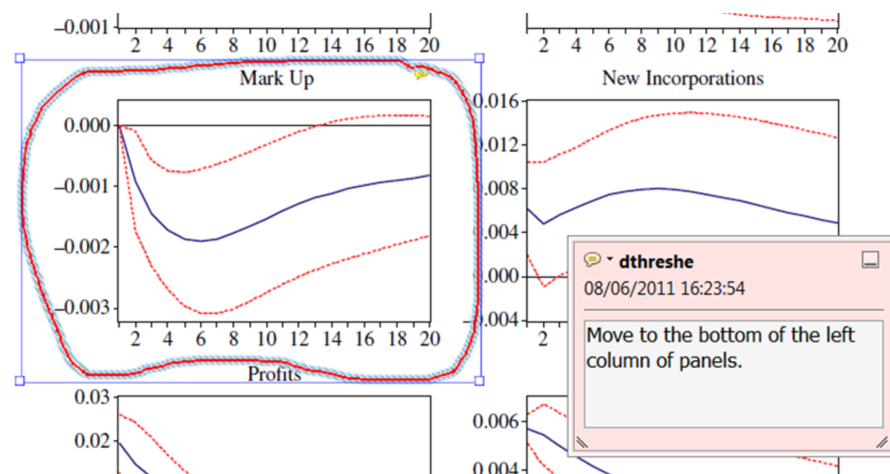

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