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A combination of NMR and liquid chromatography to characterize the protective effects of *Rhus tripartita* extracts on ethanol-induced toxicity and inflammation on intestinal cells

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Graphical abstract

Highlights

- *R. tripartita* extracts were assessed for their antioxidant and phytochemical properties
- Ethanol cytotoxicity was partially reversed by co-administration of the plant extracts
- Plant extracts exhibit anti-inflammatory potential

ABSTRACT

Consumption of ethanol may have severe effects on human organs and tissues and lead to acute and chronic inflammation of internal organs. The present study aims at investigating the potential protective effects of three different extracts prepared from the leaves, root, and stem of the sumac, *Rhus tripartita*, against ethanol-induced toxicity and inflammation using intestinal cells as a cell culture system, *in vitro* model of the intestinal mucosa. The results showed an induction of cytotoxicity by ethanol, which was partially reversed by co-administration of the plant extracts. As part of investigating the cellular response and the mechanism of toxicity, the role of reduced thiols and glutathione-S-transferases were assessed. In addition, intestinal cells were artificially imposed to an inflammation state and the anti-inflammatory effect of the extracts was estimated by determination of interleukin-8. Finally, a detailed characterization of the contents of the three plant extracts by high resolution Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry revealed significant differences in their chemical compositions.

ABBREVIATIONS

ACP – acid phosphatase

ALP – alkaline phosphatases

DMSO – dimethylsulfoxide

EGCG – epigallocatechin-3-gallate

GIT – gastrointestinal tract

GST – glutathione-S-transferase

IL-8 – interleukin 8

MTT – 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NR – neutral red

PBS – phosphate buffered saline

ROS – reactive oxygen species

Keywords: *Rhus tripartita* extracts, NMR, LC-MS, ethanol toxicity, antioxidant, anti-inflammatory

1. Introduction

Although alcohol, some 50 years ago, was not considered as toxic, Charles S. Lieber doubted this notion and pointed out alcohol toxicity to human organs [1]. Consumption of ethanol (EtOH) is a common habit in many humans and may result in severe adverse effects on organs and tissues e.g. the liver and the gastrointestinal tract (GIT). Due to its small size and the presence of a hydroxyl group, EtOH is soluble in both aqueous and lipid environments, thus allowing it to pass freely from body fluids into cells. EtOH mainly passes from the stomach and intestine mucosa to the blood and then to the liver, where most of it is metabolized. EtOH oxidation is initiated by alcohol dehydrogenases (ADH) in hepatocytes, which convert it into acetaldehyde, a potent metabolite, which upon entering into the mitochondria is oxidized to acetate by aldehyde dehydrogenases (ALDH). Other pathways of EtOH metabolism are performed by cytochrome P450 enzymes, which have been found to be induced in people who chronically consume alcohol, the fatty acid ethyl ester synthase and also in the peroxisomes via the activity of catalase. Other important enzymes related to EtOH metabolism are the glutathione-S-transferases (GSTs), which are antioxidant enzymes that

couple reduced glutathione (GSH) to xenobiotics, drugs or metabolites (e.g. acetaldehyde) to detoxify them.

The toxicity of EtOH has been attributed to the induction of oxidative stress. Oxidative stress is the detrimental side effect of oxidative metabolism, resulting from the formation of reactive oxygen species (ROS) in amounts that exceed the antioxidant (enzymatic and non-enzymatic) capacity of the cells. These antioxidant protective mechanisms include the cellular thiol redox state, thus the thiol reducing buffer comprising of molecules with redox-active sulfhydryl moieties (e.g. GSH, cysteine and thioredoxin) as well as enzymatic systems (e.g. catalase, glutathione reductase and peroxidase etc.) that modulate ROS levels [2, 3]. Alternatively, a variety of bioactive food components display antioxidant properties and/or anti-inflammatory effects, *i.e*. polyunsaturated fatty acids, phenolic compounds, vitamins that may be found e.g. in fruits, vegetables, grains, wine, and chocolate.

Intestinal inflammation is a vital protective process, which is crucial to maintain gut integrity and functioning, and requires a continuous crosstalk between different cell types present in the gut. Soluble mediators such as cyto/chemokines, eicosanoids, nitric oxide and growth factors play a significant role in the signaling and progress of inflammation, which if deregulated and becoming chronic, may cause severe disorders such as inflammatory bowel diseases and increase cancer risks. Treatment of such diseases has been of great medicinal interest for long time.

Plants that are not foodstuffs have also been used for medical purposes since a long time. *Rhus tripartita* is a local presaharan Tunisian plant located both in the semi-arid and, especially, the arid zone and its fruits can be consumed after mixing with water. *R. tripartita* is considered as a medicinal plant that has been used for a long time in Tunisian traditional medicine as an anti-diarrheal agent [4-6]. The phytochemicals (phenolic compounds, total flavonoids, condensed tannins) are responsible for the anticarcinogenic, antithrombotic and

anti-inflammatory medicinal properties of the plant [7]. Several plant extracts have been shown to have pharmacologic properties with applications to many disease models including EtOH-induced hepatotoxicity [8-10]. We have previously shown that treatment of rats with *R. tripartita* extracts provided a dose-dependent protection against EtOH-induced ulcer [11].

In the present study, we investigated, *in vitro*, the influence of EtOH on the intestinal mucosa using a cell culture system and examined the antioxidant and anti-inflammatory potential of stem, root and leaves extracts of *R. tripartita*. *In vitro* cell models increase our understanding of mechanisms and actions in human tissues and human colon carcinoma Caco-2 cells are the most widely used and validated cell culture systems to study the intestinal passage and transport mechanisms [12, 13]. The cellular viability, thiol-related parameters, GST activity were assessed to examine the potential preventive effects of plant extracts to the adverse effects of EtOH on intestinal cells. Furthermore, we imposed cells to an inflammatory stimulus [14] and the impact of plant extracts on the secretion of IL-8, a pro-inflammatory cytokine, was determined. Finally, a detailed characterization of the metabolic content of each of the three extracts was performed by combining NMR and mass spectrometry measurements.

2. Materials and Methods

2.1. Chemicals

All assay reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest analytical grade. Water was purified by a Milli-Q system (Millipore Corp., Darmstadt, DE).

2.2. Plant material: extraction process and preparation of extracts for *in vitro* experiments.

R. tripartita (Ucria) were collected from Djebel Thelja, Gafsa, state of Tunisia (Tunisia; latitude 34°24'N, longitude 7°55'E). The leaves, roots stems were cut in slices, oven-dried at 40 °C and grounded. Grounded tissues (50 g) were extracted with 300 ml of methanol/water (1:1) solution for 24h at room temperature, under continuous stirring. The solution was filtered and centrifuged at 4,500 *g* for 15 min and the supernatant was lyophilized and stored at -20 °C until use.

Dried extracts of each tissue were dissolved immediately before use in HBSS at a concentration of 12.8 mg/ml, filtered (0.22 µm diameter) and diluted to the chosen concentration ± EtOH in HBSS for cell culture experiments.

2.3. Antioxidant properties of plant extracts

The dried extracts of *R. tripartita* stems, roots an leaves were re-suspended ddH_2O, filtered and assayed for total polyphenols, flavonoids, tannins, anthocyanins, ferric ions and ABTS radical scavenging capacity as follows.

Total polyphenols were determined by a modification of the Folin assay [15]. 50 µl appropriately diluted (in ddH_2O) plant extracts were mixed with 50 µl, 4x diluted (in ddH_2O), Folin reagent and 50 µl 1.89 M Na_2CO_3. After 40 minutes incubation at RT, absorbance was measured at 765 nm against a reagent blank (with ddH_2O instead of sample). The net absorbance was converted to equivalents of gallic acid from a linear standard curve (0-250 µM).

Total flavonoids were determined by their reaction with aluminum trichloride [16]. 100 µl appropriately diluted (in ddH_2O) plant extracts were mixed with 50 µl 2% (w/w) $NaNO_2$. After 10 minutes incubation at RT, 50 µl 7.5% $AlCl_3$ and 50 µl 3.5 N NaOH were added.

Mixtures were agitated and incubated at RT for 10 minutes. Absorbance was measured at 500 nm against a reagent blank (with ddH_2O instead of sample). The net absorbance was converted to equivalents of catechin from a linear standard curve (0-500 μM).

Total tannins were assayed by their reaction with vanillin under acidic conditions [17]. 100 μl appropriately diluted (in ddH_2O) plant extracts were mixed with 100 μl 4% vanillin (in absolute methanol) and 50 μl 100% H_2SO_4. The mixtures were incubated at RT for 10 minutes and absorbance was measured at 500 nm against a reagent blank (with ddH_2O instead of sample). The net absorbance was converted to equivalents of catechin from a linear standard curve (0-500 μM).

The determination of total anthocyanins is based on their pH dependent transformations [18]. Briefly, 1 volume of appropriately diluted sample extract was mixed with 0.5 volume 0.5 M HCl or 0.4 M acetic acid pH 4.5 to reach pH 1 or 4.5, respectively. Absorbances at 520 and 700 nm were measured after 15 min of incubation at RT and the net absorbance difference is calculated as follows: $A = (A_{520}\text{-}A_{700})_{pH1.0} - (A_{520}\text{-}A_{700})_{pH4.5}$. The content of total anthocyanins was expressed as mg cyanidin 3-glucoside equivalents (CGE, MW 449.2) per g extract using the molar absorption coefficient of 26,900 L mol^{-1} cm^{-1} (of cyanidin 3-glucoside).

Ferric reducing antioxidant power (FRAP) was determined by the reduction of ferric to ferrous ions that react with 2,4,6-tri-pyridyl-s-triazine (TPTZ) to form an absorbing complex at 595 nm [19]. 100 μl appropriately diluted (in ddH_2O) plant extracts were mixed with 100 μl 300 mM acetic acid: 10 mM TPTZ (initially dissolved at 100 mM in methanol and diluted to 10 mM with 40 mM HCl):0.54% $FeCl_3.6H_2O$ in a ratio of 10:1:1. The mixtures were incubated at RT for 10 minutes and absorbance was measured at 595 nm against a reagent blank (with ddH_2O instead of sample). The net absorbance was converted to equivalents of Fe^{+2} from a linear standard curve of ferrous sulfate heptahydrate (0-250 μM).

ABTS radical cation ($ABTS^{\bullet+}$) scavenging capacity was assayed by a modified version of the assay conditions of Valyova *et al.*[16] $ABTS^{\bullet+}$ was prepared by mixing equal volumes of 7 mM ABTS and 2.5 mM potassium persulfate and 12h in the dark at RT incubation. $ABTS^{\bullet+}$ solution was diluted appropriately with ddH_2O and 100 µl were mixed with 100 µl ddH_2O as reagent blank, which should give an absorbance of ~0.7 at 734 nm. 100 µl from samples were also mixed 100 µl appropriately diluted $ABTS^{\bullet+}$ solution. The % $ABTS^{\bullet+}$ scavenging was calculated by the following equation: $100x(A_{reagent\ blank}-A_{sample})/A_{reagent\ blank}$ and expressed as nmoles of equivalents of gallic acid $ABTS^{\bullet+}$ scavenging capacity from a linear standard curve of gallic acid and % $ABTS^{\bullet+}$ scavenging.

2.4. Metabolic characterization of plant extracts

Dried stem, root and leaf extracts from *R. tripartita* were assayed in triplicate for their metabolite content by NMR spectroscopy and LC-MS. For NMR, samples were analyzed on a Bruker AVANCE III HD 600 NMR Spectrometer (Bruker BioSpin, Karlsruhe, Germany) operating at a proton frequency of 600.13 MHz and equipped with a 1.7 mm TCI-cryoprobe ($^{1}H/^{13}C/^{15}N$), using a 1D ^{1}H Nuclear Overhauser Effect SpectroscopY (NOESY) with water suppression (Bruker *noesygppr1d* pulse sequence). The 1D NOESY was acquired using a 10 ms mixing time, a 7200 Hz spectral width, a 2.28 s acquisition time, a 4 s relaxation delay and 128 scans resulting in a total acquisition time of 14 min.

To assign the resonances, 2D homonuclear ^{1}H-^{1}H TOtal COrrelation spectroscopY (TOCSY) and heteronuclear ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) experiments were recorded on the same NMR system. To shorten the acquisition time of TOCSY and HSQC experiments a Non-Uniform Sampling (NUS) algorithm (Multi-dimensional Decomposition [20]) was used. The principle of NUS is to acquire only a subset

of data points in a semi-random manner to reduce significantly the acquisition time. For both experiments the amount of sparse sampling was set to 40%.

2D ^{1}H-^{1}H TOCSY spectra (Bruker *dipsi2esfbgpph* pulse sequence) were recorded using a DIPSI-2 mixing sequence and with the following parameters: a 285 ms acquisition time, a 65 ms mixing time, a 7200 Hz spectral width and a 2 s relaxation delay. A total of 16 transients were averaged for each of the 512 increments during t1, corresponding to a total acquisition time of 2h and 45 minutes. 2D ^{1}H-^{13}C HSQC experiments (Bruker *hsqcetgpsp* pulse sequence) were carried out using a 65 ms acquisition time with GARP ^{13}C decoupling and a 1.5 s relaxation delay. A total of 64 transients were averaged for each of the 256 increments resulting in an acquisition time of 3 hours and 35 minutes.

For LC-MS, dried stem, root and leaf samples were re-suspended in triplicate in 30 µl methanol:water (1:3) per mg of powdered and analyzed on a Thermo Scientific Dionex Ultimate RSLC 3000 system coupled with a Thermo Q Exactive mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, U.K.). For each run 4 µL were injected onto a Thermo Scientific Hypersil Gold column (2.1 x 100 mm, 1.9 µm particles) and separated at 40 °C with a flow rate of 250 µL/min and a gradient from 0.1 % formic acid in water (A) to 0.1 % formic acid in methanol (B). The flow was 100 % A for 1 min, followed by a 10 min gradient to B, and was constant for 5 min before reverting over 1 min back to A and re-equilibrating for another 3 min before the next injection. MS acquisition was started at 0.1 min. MS detection for the main set of runs was in the positive/negative ion switching mode, at *m/z* 100-1000 and 140,000 nominal resolution. Spray voltage was set to 4.0 kV and -3.5 kV, respectively, sheath gas to 40, auxillary gas to 15, capillary temperature to 300 °C, the S lens value to 100, ion fill time to 200 ms and AGC target was 1E6 ions. LC-MS/MS data were collected under the same conditions, but one ion mode a time, MS at *m/z* 120-900 with the resolution set to 35,000, MS/MS resolution to 17,000 on the five highest signals each (data

dependent acquisition; exclusion time 10 s) and collision energy (HCD) of 30 and 60. The data were converted into mzML format divided into positive and negative ion data using a batch processing script running ProteoWizard 3.0.7665. An in-house XCMS / Camera script was used for alignment of the MS-only data into one raw matrix per ion form (csv format). This matrix was imported into the Matlab based DIMS_Pipeline_1_4beta, in which a blank filter and a sample filter (100 % in at least one group) were applied. This output was searched against the KEGG, BioCyc / *A. thaliana*, and Lipid Maps databases using our MI-Pack annotation software (3 ppm error margin) [21]. Some annotations were improved by comparison of collected MS/MS data with authentic spectra (MassBank, www.massbank.jp/?lang=en).

2.5. Cell culture and experimental design.

Human colon carcinoma Caco-2 cells (clone 1; passage 10–30; from Dr. M. Rescigno, University of Milano, IT) were seeded on multiwell plates (12 or 48 well plates from Corning-Costar, Corning, NY) pre-coated with type I collagen (Sigma-Aldrich) at 60,000 cells/cm^2, as in Martirosyan *et al*. [22]. Cells formed mono-cultures, which were used as confluent, fully differentiated after 21 days. Cultures were washed twice with phosphate buffered saline (PBS) and for the ethanol (EtOH) toxicity experiments, 10% EtOH (v/v) in HBSS was added in the presence or absence of different concentrations of the plant extracts; cells were then incubated for 1h at 37 °C.

For the inflammation induction experiments, 21 days fully differentiated Caco-2 cells were incubated for 1h at 37 °C with each plant extract at different concentrations in DMEM medium and following the medium was replaced by fresh DMEM with the inflammatory cocktail, consisting of IL-1β, TNF-α, IFN-γ and LPS (25 ng/ml, 50 ng/ml, 50 ng/ml and 1 μg/ml, respectively) for 23h [14]. After incubation the medium was collected, centrifuged at

10,000 *g* and the IL-8 concentration was assessed by an ELISA kit (BD Biosciences Pharmingen, San Diego, CA) and normalized per protein amount of the cells as described elsewhere [22].

2.6. Determination of cellular viability, metabolic activity, ATP levels.

Caco-2 cells were grown in 48-well plates for 21 days and were incubated with HBSS ± 10% EtOH ± extract for 1h at 37 °C. The cell viability and metabolic activity were assessed via the neutral red (NR) lysosomal accumulation and the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) formazan crystals formation, respectively, while, ATP levels were determined by a luminescent-based assay. The treated cells, after washing with PBS, were incubated with:

(i) NR (0.75 ml at 0.33 mg/ml, 3h). After washings NR was extracted in 50% ethanol:1% (v/v) acetic acid and absorbance was measured at 540 nm.

(ii) MTT (0.36 ml at 0.8 mg/ml, 2h). After washings the formazan crystals were solubilised in 0.3 ml DMSO:ethanol:0.9 M NaOH (1/1/1, v/v/v) and absorbance was measured at 540 nm.

(iii) HBSS (0.15 ml) for 15 min and then with the luciferin/luciferase substrates (0.15 ml). Luminescence was measured for 20 min according to manufacturer's instructions (Promega).

2.7. Determination of reduced thiols and enzyme activities.

Caco-2 cells were grown in 12-well plates for 21 days and then incubated with HBSS ± 10% EtOH ± extract for 1h at 37 °C and washed with PBS and homogenized by scraping in 500 μM phenylmethylsulfonylfluoride, via sonication. The homogenate was cleared from cell debris by centrifugation at 12,000 *g* for 10 min and the supernatants were collected and

assayed for protein content [23] and oxidative stress related parameters as follows. The same procedure for the homogenization and oxidative stress markers determination was performed also for samples used for the inflammation experiments, which were also grown either in 12- or 48-well plates.

GST activity was assayed by the kinetics of the formation of the complex S-(DNP)GS by the reaction of GSH with 1-chloro-2,4-dinitrobenzene in the presence of GST at 340 nm [24].

Alkaline (ALP) and acid (ACP) phosphatases activities were determined by their transformation of p-nitrophenol phosphate in acetic pH 4.5 or boric acid pH 9.8 buffers, respectively, into p-nitrophenol, whose absorbance at 405 nm was measured after an alkalinization.

The total pool of reduced thiols was quantified (due to sample availability and also as a holistic more reliable marker of thiol redox state than specific thiols) by their reaction with the aldrithiol reagent in 12 M formamide 50 mM acetic acid pH 4.5 buffer and the absorbance of the formed complex at 325 nm [25].

2.8. Statistical analysis.

Statistical analysis was performed using two-way analysis of variance (ANOVA) for post hoc pairwise comparisons with Bonferroni multiple comparisons correction to compare significance over the HBSS control in the absence/presence of plant extract or 10% EtOH in the presence of plant extract, by means of the GraphPad prism program. Results were expressed as means ± SD of percentage of the HBSS control and considered statistically significant when the $P<0.05$.

3. Results and Discussion

3.1. Antioxidant and phytochemical properties of *R. tripartita* extracts

The extracts from stems, roots and leaves of *R. tripartita* were analyzed for their phytochemical components using biochemical approaches for each category of antioxidants moieties (**Table 1**). All extracts are rich in amounts of antioxidant compounds, which are responsible for their ferric ions and radical scavenging properties. Comparing the three extracts, the root seems more potent in antioxidant capacity, while the leaf and stem extracts are of similar antioxidant potential.

Table 1. Phytochemical and antioxidant properties of the *R. tripartita* extracts.

	Polyphenols	**Flavonoids**	**Tannins**	**Anthocyanins**	**FRAP**	**$ABTS^{\bullet+}$ scavenging**
Leaf	5375 ± 290	154 ± 12	318 ± 38	0.254 ± 0.05	2872 ± 204	1.52 ± 0.08
Root	7968 ± 366	1114 ± 73	670 ± 24	0.251 ± 0.018	4890 ± 161	2.26 ± 0.14
Stem	6647 ± 185	495 ± 23	363 ± 10	0.079 ± 0.002	3025 ± 52	1.57 ± 0.13

Total polyphenols were expressed as equivalents of nmoles gallic acid per mg dried extract. Total flavonoids and tannins were expressed as equivalents of nmoles catechin per mg dried extract. Total anthocyanins were expressed as mg cyanidin 3-glucoside equivalents per g extract. Ferric reducing antioxidant power (FRAP) was expressed as equivalents of Fe^{+2} per mg dried extract. $ABTS^{\bullet+}$ cation scavenging capacity was expressed as equivalents of scavenging capacity in nmoles gallic acid per mg dried extract.
Data represent means ± SD (N=3, n=5, $P<0.001$).

Combining NMR and LC-MS measurements we were able to detect specific metabolites in the three extracts studied. Annotated NMR metabolites are illustrated in **Figure 1**, while the numerous peaks putatively annotated by LC-MS are summarized in the Venn diagrams for both positive and negative ion modes of detection (**Figure 2**) and in detail in supplementary (**Supplementary Tables 1 and 2, Supplementary Figure 1**).

NMR analysis for metabolites characterization confirms the aforementioned trend for the antioxidant properties as the intensity of the phenolic region (6 to 9 ppm), which is directly linked to the total amount of material, higher in the root extract (**Figure 1**). Root samples are also characterized by much increased acetic acid and very low amount of shikimic acid compared to the other two extracts (**Figure 1, B spectrum**). Shikimic acid is an important

transitional biochemical metabolite involved in the metabolism of amino acids (in particular aromatic amino acids), tannins and alkaloid. The stem extract is clearly the most complex based on the NMR spectrum, and is mainly defined by high carbohydrates (especially fructose that has only been detected within this extract by NMR) and amino acids (**Figure 1, A spectrum**), and cytosine has only been detected in this extract. Finally, the leaf extract (**Figure 1, C spectrum**) is distinguished by very high shikimic acid amount and very low gallic acid content. Gallic acid is a phenolic acid, present in most plants and well known as an antioxidant agent.

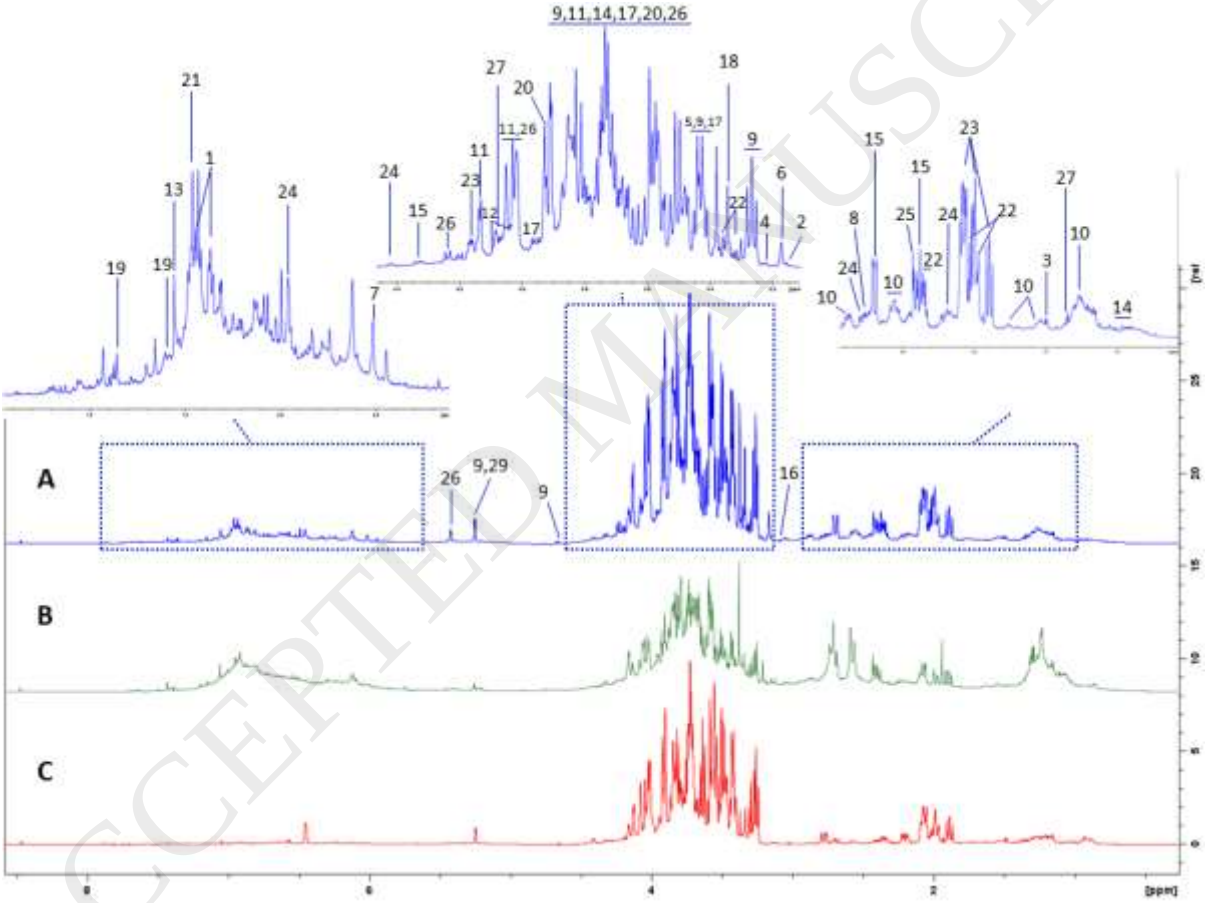

Figure 1. ^{1}H NMR spectra from the analysis of stem (A), root (B), and leaf (C) extracts. Numbers correspond to the metabolites identified. 1: 3,4 dihydroxymandelic acid, 2: Acetylcarnitine, 3: Alanine, 4: Betaine, 5: Carnitine, 6: Choline, 7: Cytosine, 8: Cystathionine, 9: D-Glucose, 10: Fatty Acids, 11: Fructose, 12: Gluconic acid, 13: Gallic acid, 14: Isoleucine, 15: Malic acid, 16: Malonic acid, 17: Mannose, 18: Methanol, 19: N,N-

Dimethylaniline, 20: N-acetylglucosamine, 21: Phenol , 22: Proline, 23: Quinic acid, 24: Shikimic acid, 25: Succinic acid, 26: Sucrose, 27: Threonine, 28: Threonic acid, 29: Xylose

The LC-MS runs resulted in final matrices with 8717 features (defined by a unique combination of *m/z* value and retention time) in the positive and 5691 features in the negative ion mode. Only relatively few features appear in all three extracts, which is partly due to the XCMS settings filtering out weaker signals in the extracts concerned, but clear differences were already expected from the LC-MS profiles (**Supplementary figure 1**). There is a good overlap in the two ion modes (**Figure 2**). Although signal intensity does not necessarily correspond to compound concentration, it is noticeable that quite a few of the most intense signals are flavonoids or polyphenols. The most intense feature in negative ion mode and second-most intense in the positive ion mode has been annotated as a gambiriin, a catechin (**Supplementary tables 1 and 2**). This signal is higher in stem than in root extract though and therefore not a major cause of the activity. A good number of other features strongest in root extract have been annotated as catechins (flavonoids) and other antioxidants, e.g. the glycosylated flavonoids phlorizin and tribuloside, or procyanidin C1 (cinnamtannin A1). Examining only a subset of features, those that are KEGG annotated in all three extracts and present in both positive and negative ion modes, we find just four different flavonoids and one tannin, but 16 different phenolic compounds and several (acenaphthene-1,2-diol, 4-hydroxymandelate, 4-hydroxyaminoquinoline N-oxide, 2-(acetamidomethylene)succinate) known reducing agents of NAD^+ or $NADP^+$ (**Supplementary Table 3**). While the individual annotations would be difficult to confirm at this point (basically requiring authentic standards), it is clear that many different, potentially active compounds are present in the extracts, including some of the most intense signals.

The current dataset is too small to allow a statistical correlation between compound classes and activities; it would require labor-intense assay-driven purifications to determine the most active compounds in this mix. A relatively small overlap with the NMR results was expected, as this is a technique mostly complementary to reversed-phase LC-MS.

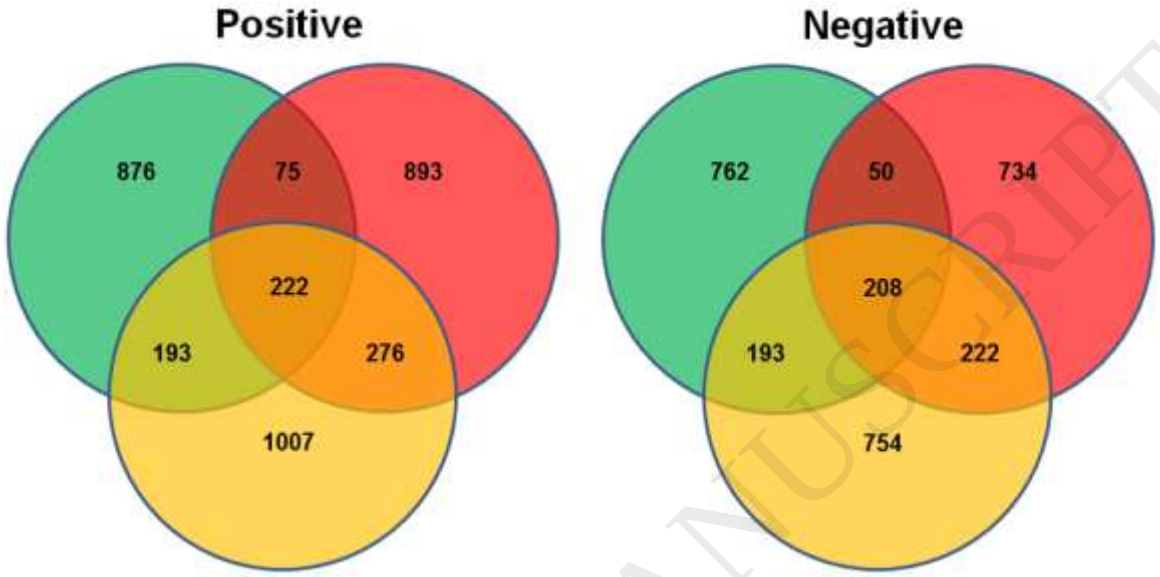

Figure 2. Venn diagrams of metabolites putatively annotated by LC-MS in positive and negative ion modes for stem (yellow), root (red) and leaf (green) extracts.

3.2. Cytoprotective effects of *R. tripartita* extracts on EtOH-induced stress

Initially the cytotoxicity of EtOH was evaluated by the lysosomal accumulation of the neutral red (NR) dye (**Figure 3**), and 10% (v/v) EtOH was chosen as a working condition for the following experiments as a concentration that is in the order of magnitude used in experiments in this cell line [26, 27]. Furthermore, the plant extracts used were also tested by NR for possible toxicity, and were found not to be toxic upon 1h exposure in saline (*i.e.* HBSS) and even up to 24h in culture medium (*i.e.* DMEM). This was necessary to establish the range of non toxic concentrations of the extracts for our experiments within the chosen range (0.8-6.4 mg extract/ml).

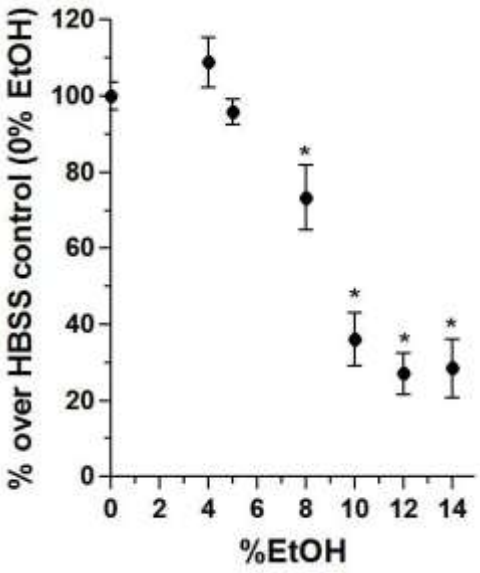

Figure 3. Cytotoxicity of EtOH on Caco-2 cells. Cytotoxicity was assayed by lysosomal accumulation of neutral red (NR) in fully differentiated confluent Caco-2 cells exposed for 1h to EtOH (0-14% v/v). Data represent means ± SD (N=3, n=5, P<0.001). *Samples significantly different from the HBSS control.

The cytotoxic effect upon exposure of fully differentiated confluent Caco-2 cells to EtOH was reverted upon addition of increasing concentrations of the plant extracts, with the root extract being the more potent, as shown by both NR and MTT tests (**Figure 4**). Considering the metabolic activity, it seems that the extracts stimulate the cellular metabolism, which is decreased (due to toxicity) by EtOH. This effect is mirrored in the decrease of ATP. However, considering ATP, all three extracts decrease intracellular ATP levels with the stem causing the minimum decrease.

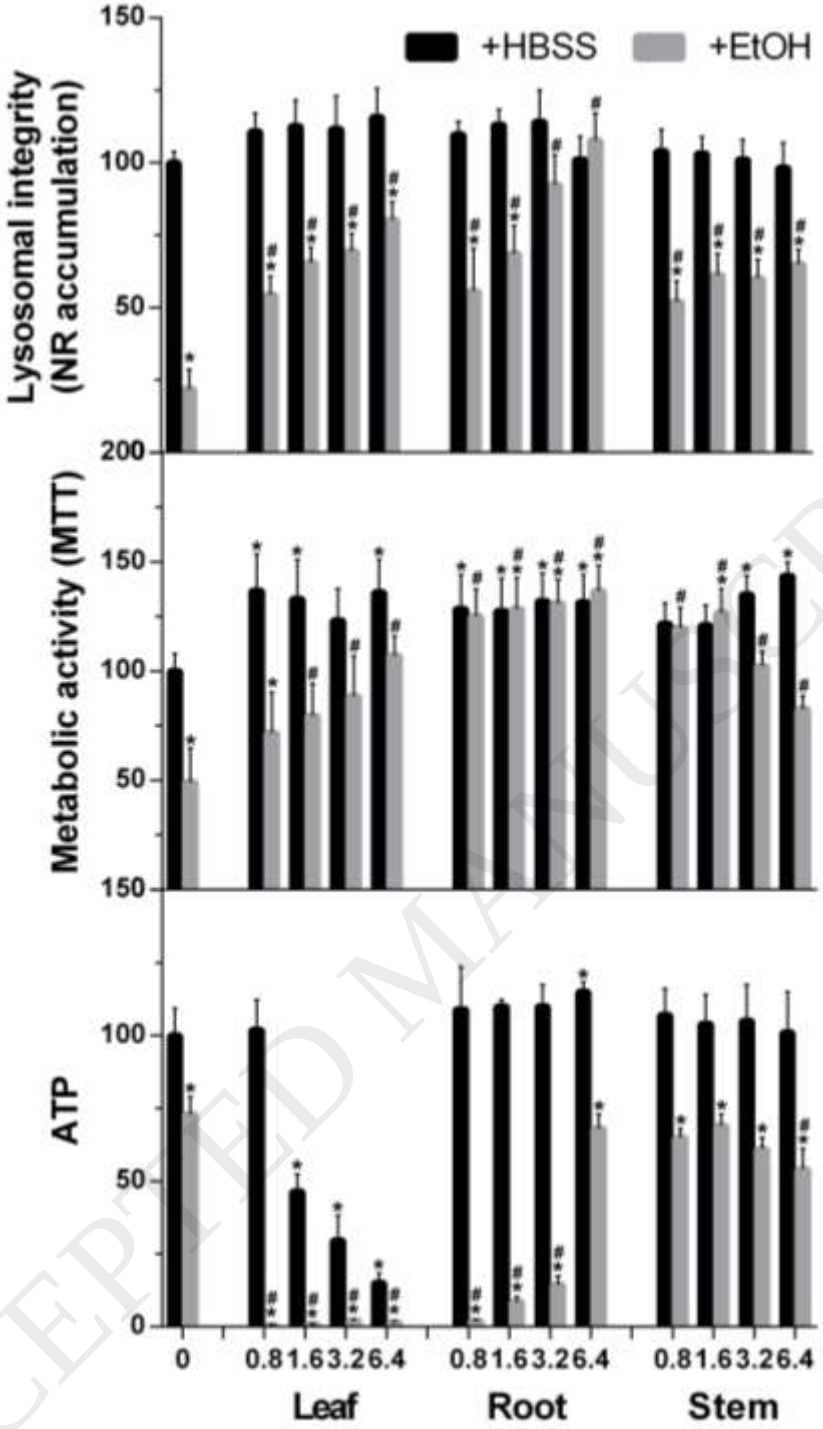

Figure 4. Protective effects of plant extracts on EtOH-induced cytotoxicity. Cytotoxicity was assayed by the lysosomal accumulation of neutral red (NR), metabolic activity (MTT crystal formation), and ATP levels in fully differentiated confluent Caco-2 cells exposed for 1h to EtOH (10% v/v) in the presence or absence of plant extracts (0-6.4 mg/ml). Data represent means ± SD (N=3, n=5) of values expressed as a percentage of the HBSS control. Samples

statistically significant were detected in the absence/presence of EtOH from the HBSS control (*) or only for the presence of EtOH over the 10% EtOH in the absence of extracts (#) using 2 way ANOVA ($p>0.05$).

A more thorough determination of the reduced thiol pool as well as thiol-related EtOH metabolizing enzymes (e.g. GST) would provide a more detailed picture of the oxidative effects of EtOH on intestinal cells and their response over co-administration of plant extract material (**Figure 5**).

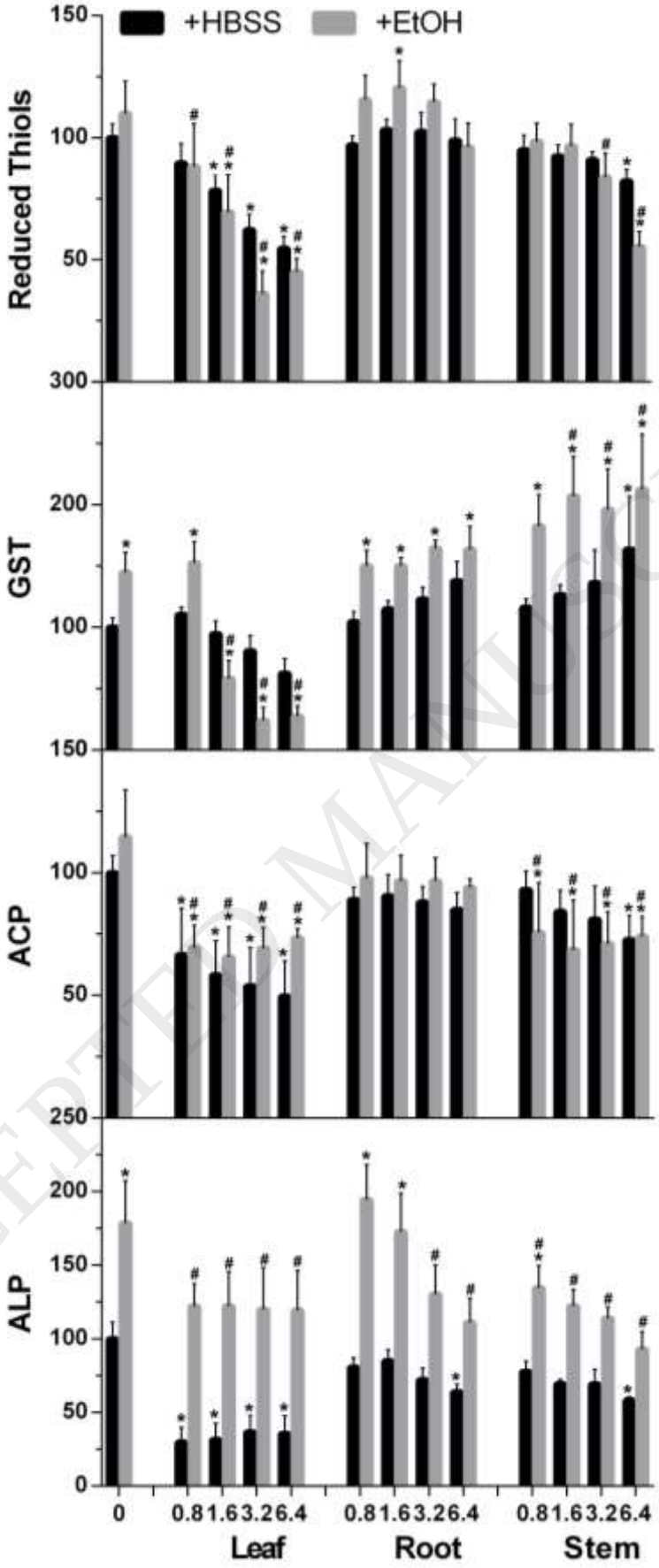

Figure 5. Impact of plant extracts on thiol redox state, glutathione-S-transferases and phosphatases of fully differentiated confluent Caco-2 cells. Reduced thiols, glutathione-S-transferase (GST), and acid (ACP) and alkaline (ALP) phosphatases activities were assayed in confluent Caco-2 cells exposed for 1h to EtOH (10% v/v) in the presence or absence of plant extracts (0-6.4 mg/ml). Data represent means ± SD (N=3, n=5) of values expressed as a percentage of the HBSS control. Samples statistically significant were detected in the absence/presence of EtOH from the HBSS control (*) or only for the presence of EtOH over the 10% EtOH in the absence of extracts (#) using 2 way ANOVA ($p>0.05$).

EtOH was shown to induce a significant increase in the activities of GST and ALP, while there was no significant change in reduced thiols content and ACP activity. The increase of GST activity by EtOH has also been observed *in vivo* in rats [8, 9] and could be attributed to a response of the cells against the possible production of acetaldehyde upon oxidation of EtOH. Furthermore, ALP activity has also been detected to be increased due to EtOH in other human colon carcinoma cell lines [28] and rats *in vivo* [29], while in humans its increase has been associated with certain medical conditions such as liver-associated diseases [30]. Although the function of this elevated level remains not clear, in the intestinal cells, a comparable increase in the activity of ALP was observed upon exposure of Caco-2 cells to silver nanoparticles[31].

The treatment with various plant extracts resulted in different cell responses. The administration of the leaf extract in the absence of EtOH, decreased reduced thiol levels and ACP and ALP activities, as those of GST but at higher doses. For GST, a dual behavior (initial increase followed by decrease at high concentrations of the extract) was observed upon co-administration of EtOH together with the extract. The root extract did not alter the levels of GST, but in the case of co-administration with EtOH, it increased GST more than

the leaf extract. In contrast to the leaf, the root extract decreased less the ACP and ALP activities in the absence of EtOH and increased them more in the presence of EtOH. Finally, the stem extract did not decrease significantly reduced thiols, but increased GST in the presence of EtOH more than the other extracts, and showed a similar action with the root extract for ACP and ALP activities (with less increase in the presence of EtOH).

In other studies, the effect of EtOH and acetaldehyde on the integrity and functions of the tight junctions in intestinal cells was also assessed [32, 33]. The disruption of the tight junctions barrier would allow increase in epithelial permeability to hydrophilic substances in the gastric and the intestinal lumen as well as possible passage of toxic substances. In our experiments, we detected both a 70% decrease in the transepithelial electrical resistance (TEER) and the paracellular passage of phenol red of 4.6 fold and 16 fold from apical to basolateral and basolateral to apical, respectively due to EtOH toxicity (data not shown). *In vitro*, EtOH-induced toxicity results in loss of cellular integrity in intestinal cells as shown by ZO-1 expression, paracellular dye passage and transepithelial electrical resistance (TEER) decrease [26, 27]. In this context, alcohol may change the pharmacokinetics and bioavailability of drugs through changes in gastrointestinal system as shown for opioid and other drugs [34, 35] and the impact of ethanol metabolism and drug interactions outside of the liver should be taken into account.

Plant extracts are multicomponent systems, and some have bioactive effects such as hepatoprotective and antioxidant properties *in vivo* against EtOH-induced toxicity [9, 10]. For *R. tripatita*, we have previously shown that the root bark extract protects over EtOH-induced ulcer in rats,[11] while others demonstrated its antifungal and antibacterial properties [4, 6].

3.3. Anti-inflammatory properties of *R. tripartita* extracts

Intestinal epithelial cells play an important role in the immunological inflammatory response of the gut especially after shock and trauma. EtOH toxicity is a significant contributing factor and may increase the appearance of posttraumatic complications. EtOH has been shown to induce inflammatory responses in both *in vitro* and *in vivo* models. Amin *et al.*[36] showed that low (<2%) EtOH concentrations induce pro-inflammatory cytokine (TNFα and IL-6) release and barrier dysfunction. However, in our effort to detect IL-8 after 1h exposure to EtOH and a total 24h post EtOH exposure, no detectable amount of IL-8 was present in the culture medium of Caco-2 cells. Therefore, to estimate if the plant extracts could have an anti-inflammatory effect, we imposed the cells to a severe inflammatory condition using an inflammatory cocktail [14]. As shown by the secreted IL-8 level, a decrease was observed upon the administration of the EGGC, as positive control, but also in a dose-dependent manner when the plant extracts were administered (**Figure 6**). This clearly shows the anti-inflammatory protective role of the extracts. The leaf extract seems to be the more potent followed by the root extract, while the least anti-inflammatory is the stem extract.

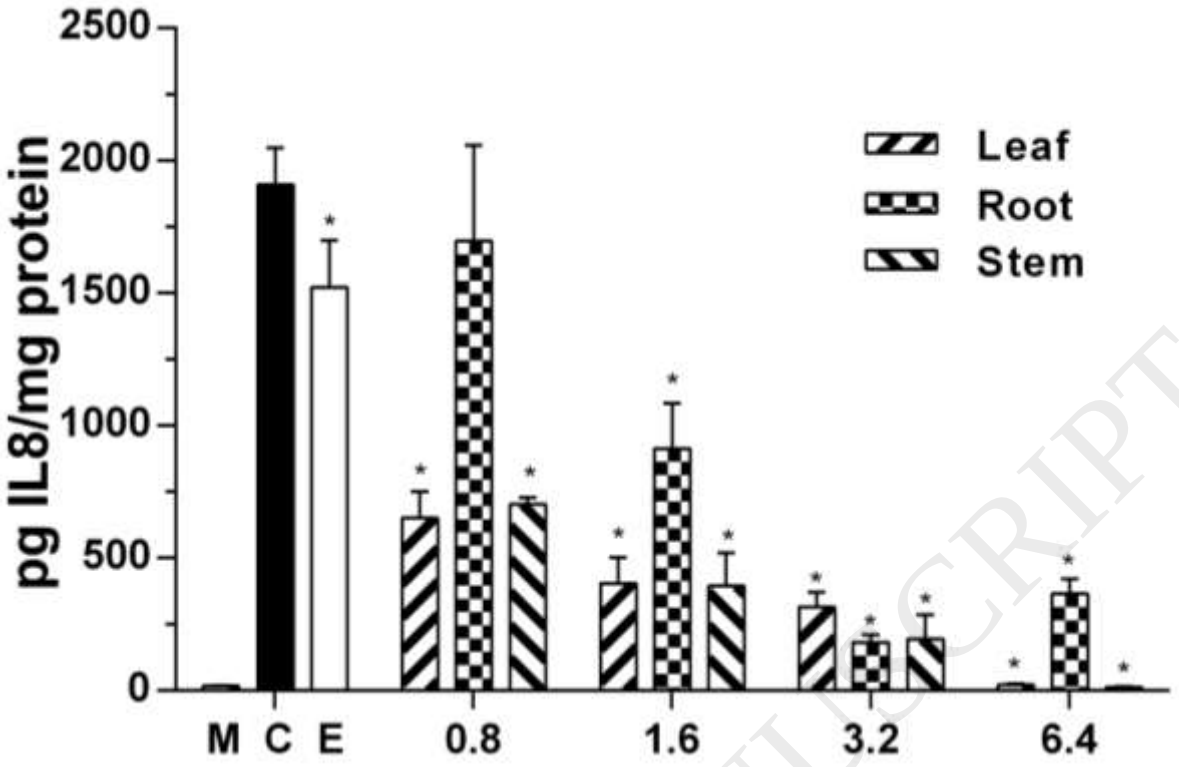

Figure 6. Preventive anti-inflammatory effects of the plant extracts. IL-8 levels secreted by Caco-2 cells incubated for 1h to plant extracts (0.8-6.4 mg/ml) and then exposed for the following 23h to an inflammatory cocktail, in the absence of the extract. Data represent means ± SEM (N=3, n=4, P<0.001) of values expressed as a pg IL-8 normalized per mg cell protein. *Samples significantly different from the corresponding cocktail (C) in the absence of extract. EGGC (E) was used as a control of anti-inflammatory vegetal substances, while cells were grown also in medium in the absence of inflammatory cocktail (M) as a negative control for induction of inflammation.

4. Conclusion

This research study showed, for the first time, the chemical composition of *R. tripartita* different tissues and their protective effects on EtOH-induced cytotoxicity and inflammation on intestinal cells. The root, leaf and stem extracts of *R. tripartita* showed high amounts of antioxidant compounds, with the root extract having the higher antioxidant capacity. The

aforementioned observations are strengthen by the putative annotation of compounds by NMR and LC-MS. The extracts protected dose-dependently fully differentiated confluent Caco-2 cells against EtOH-induced cytotoxicity and stimulated the cellular metabolism that was decreased upon EtOH exposure. The extracts had different effects on reduced thiols contents and on GST, ALP and ACP activities in the absence or presence of EtOH. These different cellular responses could be attributed to differences between the extracts' contents and/or to a possible interaction of EtOH with the pharmacokinetics of the extracts. The extracts of *R. tripartita* showed a powerfull anti-inflammatory activity as revealed by a dose-dependent decrease in the levels of IL-8.

Conflict of interest

The authors declare that they have no conflicts of interest concerning this article.

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