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FULL ARTICLE

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Protective effects of *Cotoneaster integerrimus* on *in vitro* and *ex-vivo* models of H_2O_2-induced lactate dehydrogenase activity in HCT116 cell and on lipopolysaccharide-induced inflammation in rat colon

Gokhan Zengin[1] | Claudio Ferrante[2] | Luigi Menghini[2] | Giustino Orlando[2] | Luigi Brunetti[2] | Lucia Recinella[2] | Annalisa Chiavaroli[2] | Sheila Leone[2] | Maurizio Ronci[3] | Muhammad Zakariyyah Aumeeruddy[4] | Mohamad Fawzi Mahomoodally[4]

[1]Faculty of Science, Department of Biology, Selcuk University, Konya, Turkey

[2]Department of Pharmacy, University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy

[3]Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy

[4]Faculty of Science, Department of Health Sciences, University of Mauritius, Réduit, Mauritius

Correspondence
Gokhan Zengin, Department of Biology, Faculty of Science, Selcuk University, Campus, 42250, Konya, Turkey.
Email: gokhanzengin@selcuk.edu.tr

Abstract

The present study evaluated the biological potential of methanol and aqueous extracts of the twigs and fruits of *Cotoneaster integerrimus* Medik. Lethality bioassays performed on *Artemia salina* showed that aqueous and methanol *C. integerrimus* extracts were non-toxic in the concentration range (0.1–20 mg/ml), with a LC50 ≥ 2.5 mg/ml, for each single extract. The protective effect of the extracts was assessed *in vitro* against hydrogen peroxide-induced lactate dehydrogenase (LDH) activity and tumor necrosis factor (TNF)α gene expression in colon cancer HCT116 cell line. All the extracts downregulated (H_2O_2)-induced TNFα gene expression, in HCT116. By contrast, it was observed that the lipopolysaccharide (LPS)-induced increase in colon nitrite, prostaglandin E_2, and 8-iso-$PGF_{2\alpha}$ levels were counteracted mostly by the methanol twig extract. The present study showed protective effects induced by *C. integerrimus in vitro* and *ex vivo*, thus supporting potential application in the management of chronic inflammatory diseases.

Practical applications

In the present study, protective effects of *C. integerrimus* are highlighted using *in vitro* and *ex-vivo* models of hydrogen peroxide-induced LDH activity in HCT116 cell and on LPS-induced inflammation in rat colon. Based on our results, this edible and traditionally used species could be considered as a valuable source of natural agents to combat inflammatory diseases, particularly ulcerative colitis. Results amassed herein advocates for further bioprospection of this species that could open new avenues for the development of nutraceuticals and functional foods geared toward the management of chronic inflammatory diseases.

KEYWORDS

antioxidant, *Cotoneaster integerrimus*, inflammation, natural agents, oxidative stress

Abbreviations: 5HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; IBD, inflammatory bowel disease; LDH, lactate dehydrogenase; NO, nitric oxide; PMA, phorbol myristate acetate; ROS, reactive oxygen species.

1 | INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) are byproduct molecules related to aerobic cell metabolism (Ismail et al., 2017). They are normally produced in limited amount within the body, in order to pro-homeostatic regulation of gene expression, signal transduction, and receptor activation. However, excess production beyond the level of endogenous antioxidants results in oxidative stress which plays a pathogenic role in chronic inflammatory diseases. ROS results in the synthesis and secretion of pro-inflammatory cytokines which initiate the inflammatory processes (Hussain et al., 2016). During an inflammatory reaction, macrophages secrete a number of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), and other inflammatory mediators including nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Zhu et al., 2018). Considering the pivotal role displayed by these pro-oxidant and pro-inflammatory cytokines, each of them could be considered as a key target in the development of anti-inflammatory molecules.

The genus *Cotoneaster*, belonging to the Rosaceae family, comprises 686 species names as recorded in *The Plant List* (http://www.theplantlist.org/); of these 278 are accepted species names, 98 synonyms, and 310 still unassessed. *Cotoneaster* species consists of woody plants, varying in height from 0.2 m prostrate shrubs to 15–20 m trees, and occur all over Europe, North Africa, and the temperate parts of Asia excluding Japan (Bartish, Hylmö, & Nybom, 2001). In Turkey, the genus *Cotoneaster* is represented by eight species and plants belonging this genus are commonly called as "Dağ muşmulası or Tavşan elması" in the different regions of Anatolia (Uysal et al., 2016; Zengin, Uysal, Gunes, & Aktumsek, 2014). In addition, the *Cotoneaster* species are widely used as culinary plants in different countries including Turkey (Cakilcioglu & Turkoglu, 2010).

The medicinal uses of *Cotoneaster* species have been reported across the world. Ethnobotanical surveys carried out in Pakistan, India, Turkey, Lebanon, and Iran revealed its use as an expectorant (Cakilcioglu & Turkoglu, 2010), astringent (Gairola, Sharma, & Bedi, 2014), and in the management of diabetes (Polat, Cakilcioglu, & Satıl, 2013), scurvy (Baydoun, Chalak, Dalleh, & Arnold, 2015), neonatal jaundice (Heydari et al., 2016), cuts, wounds, diarrhea (Singh, Husain, Agnihotri, Pande, & Khatoon, 2014), hypertension (Ahmad et al., 2015), digestive problems (Khan et al., 2015), jaundice, cough, constipation, and also used as an emetic and diuretic (Sadeghi, Kuhestani, Abdollahi, & Mahmood, 2014).

Multiple studies have investigated the protective effects of extracts of members of this genus including the antioxidant, anticancer, hepatoprotective, antidiabetic, and antidyslipidemic activities of *C. horizontalis* (Mohamed, Sokkar, El-Gindi, Zeinab, & Alfishawy, 2012; Sokkar et al., 2013), antioxidant activity of *C. zabelii*, *C. splendens*, *C. bullatus*, *C. divaricatus*, *C. hjelmqvistii*, and *C. lucidus* (Kicel et al., 2016), anticholinesterase and antioxidant potential of *C. meyeri* and *C. morulus* (Ekin, Gokbulut, Aydin, Donmez, & Orhan, 2016), antioxidant, antibacterial, anticholinesterase, antityrosinase, antiamylase, and antiglucosidase activity of *C. nummularia* (Ekin et al., 2016; Zengin et al., 2014).

As a further investigation on this genus, we aimed to evaluate the biological potential of *Cotoneaster integerrimus* Medik. One of our previous studies (Uysal et al., 2016) evaluated the antimicrobial, antioxidant, antimutagenic, and the enzyme inhibitory properties of this species, particularly twig and fruit extracts, against key enzymes involved in the pathology of chronic diseases. Nonetheless, other biological potential of twig and fruit extracts of this plant are yet to be investigated. Also, *ex vivo* studies on the genus *Cotoneaster* is still limited. In this context, the present study aimed to evaluate the *in vitro* biological effects of *C. integerrimus* water and methanol fruit and twig extracts on H_2O_2-induced lactate dehydrogenase (LDH) activity, TNFα gene expression and wound healing effects, in HCT116 cells. Additionally, we evaluated the protective effects of the extracts against lipopolysaccharide (LPS)-induced production of nitrite, PGE_2 and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), tumor necrosis factor (TNF)-α and interleukin (IL)-6 gene expression, and the 5-HIAA/5-HT ratio in isolated rat colon specimens, *ex vivo*.

2 | MATERIALS AND METHODS

2.1 | Plant material and extractions

C. integerrimus was collected from Kayseri-Turkey (Kayseri-Hisarcik, dry slopes) during the end of flowering season (2014). Taxonomic identification of plant material and extraction procedure were performed as previously reported (Uysal et al., 2016).

2.2 | Artemia salina lethality bioassay

Artemia salina cysts were hatched in oxygenated artificial sea aqueous (1 g cysts/L). After 24 hr, brine shrimp larvae were gently transferred with a pipette in 6-well plate containing 2 ml of *C. integerrimus* extracts at different concentrations (0.1–20 mg/ml) in artificial sea aqueous. Ten larvae per well were incubated at 25–28°C for 24 hr. After 24 hr, the number of living napulii were counted under light microscope and compared to control untreated group.

2.3 | *In vitro* studies

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 humidified incubator with 5% CO_2 at 37°C. The detailed procedure is described in our previous paper (Locatelli et al., 2017).

To assess the basal cytotoxicity of aqueous and methanol *C. integerrimus* extracts, a viability test was performed on 96 microwell plates, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, as previously described (Menghini et al., 2018). Effects on cell viability were evaluated in comparison to untreated control group.

Lactate dehydrogenase (LDH) activity was measured by evaluating the consumption of NADH in 20 mM HEPES-K^+ (pH 7.2), 0.05% bovine serum albumin, 20 μM NADH, and 2 mM pyruvate using a microplate reader (excitation 340 nm, emission 460 nm) according to manufacturer's protocol (Sigma-Aldrich).

TNFα gene expression was evaluated as previously reported (Ferrante et al., 2017).

Finally, we tested extracts on HCT116 cell line, in wound healing experimental paradigm. Cell migration was determined using the scratch wound healing assay with slight modification (Ju, Kwak, Hao, & Yang, 2012). HCT116 cells (6×10^3 cells/well) were seeded on 6-well plastic plates. Cells monolayer were preliminarily treated with a proliferation inhibitor mitomycin C (Sigma-Aldrich) at the non-toxic concentration of 5 μM, in order to exclude the effect of cell proliferation (Taniguchi et al., 2018). After 2 hr on cells in the confluence interval 85%–90%, a wound was generated by scratching the cell monolayer using a 0–200 μl pipette tip. A gentle wash with PBS was performed twice to remove suspended and damaged cells. Cells were incubated in serum-free media supplemented with *C. integerrimus* extracts at the non-toxic concentration of 100 μg/ml. Cell migration was followed capturing at least 3 microscope images/well at time 0, 24, and 48 hr. An inverted light microscope Leika equipped with Nikon 5100 camera was used to capture image at 4× magnification. The quantification of scratch area with no cells were quantified using Image-J software (NIH). Using GraphPad software, mean data at T0, 24, and 48 hr were calculated for untreated control and cotoneaster treated-pharmacological groups and expressed as percentage variation with reference to relative 100% of at 0 hr.

2.4 | *Ex vivo* studies

Colon specimens were obtained as residual material from vehicle-treated male adult Sprague-Dawley rats randomized in our previous experiments approved by Local Ethical Committee (University "G. d'Annunzio" of Chieti-Pescara) and Italian Health Ministry (Italian Health Ministry N. 880, delivered on 24th 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 humidified incubator with 5% CO_2 at 37°C for 4 hr, 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 aqueous and methanol *C. integerrimus* extract (100 μg/ml). Tissue supernatants were collected, and the PGE_2 and 8-iso-$PGF_{2\alpha}$ levels (ng/mg wet tissue) were measured by radioimmunoassay, as previously reported (Chiavaroli et al., 2010; Locatelli et al., 2018; Menghini et al., 2016). Additionally, tissue supernatant was assayed for nitrite determination by Griess assay, as previously described (Zengin et al., 2017).

On the contrary, individual colon specimens were dissected and subjected to extractive procedures to evaluate 5-HT and 5HIAA (ng/mg wet tissue) through HPLC coupled to electrochemical detection, as previously reported (Brunetti et al., 2014; Ferrante et al., 2016).

2.5 | Statistical analysis

Experimental data were analyzed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Means ± *SEM* were compared through one-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test. As for gene expression analysis, 1.00 (calibrator sample) was considered the theoretical mean for the comparison. As regards to *Artemia salina* lethality bioassay, results were expressed as percentage of mortality calculated as: ((T–S)/T) × 100. T is the total number of incubated larvae and S is the number of survival napulii. Living nauplii were considered those exhibiting light activating movements during 10 s of observation. For each experimental condition, two replicates per plate were performed and experimental triplicates were performed in separate plates.

Statistical significance was accepted at $p < 0.05$

3 | RESULTS

3.1 | Effects of C. integerrimus extracts on Artemia salina lethality test

The *C. integerrimus* extracts did not reveal any toxicity in the concentration range (0.1–20 mg/ml), with a LC50 ≥ 2.5 mg/ml, for each single extract.

3.2 | Effects of C. integerrimus extracts on HCT116 cell line viability

Additionally, we observed that *Cotoneaster* extracts (10–1,000 μg/ml) exerted an inhibitory effect on HCT116 cell viability starting from the concentration of 150 μg/ml (data not shown).

3.3 | Effects of C. integerrimus extracts on HCT116 LDH activity

In the present study, hydrogen peroxide treatment induced an increase in LDH activity (31.21 milliunit/ml) compared to the control group (16.81 milliunit/ml). We observed that the aqueous fruit extract was most effective in reducing LDH activity (19.47 milliunit/ml) followed by the methanol fruit extract (22.80 milliunit/ml), methanol twig extract (23.01 milliunit/ml), and aqueous twig extract (23.15 milliunit/ml) (ANOVA, $p < 0.001$; post hoc, $^*p < 0.05$; $^{**}p < 0.01$ vs. hydrogen peroxide).

3.4 | Effects of C. integerrimus extracts on HCT116 cell line viability

The present study also revealed that *Cotoneaster* extracts inhibited colon nitrite levels (Figure 1), as an index of free radical production. The increase in nitrite level induced by LPS (102.61 mmol/g wet tissue), compared to the control (73.84 mmol/g wet tissue), was

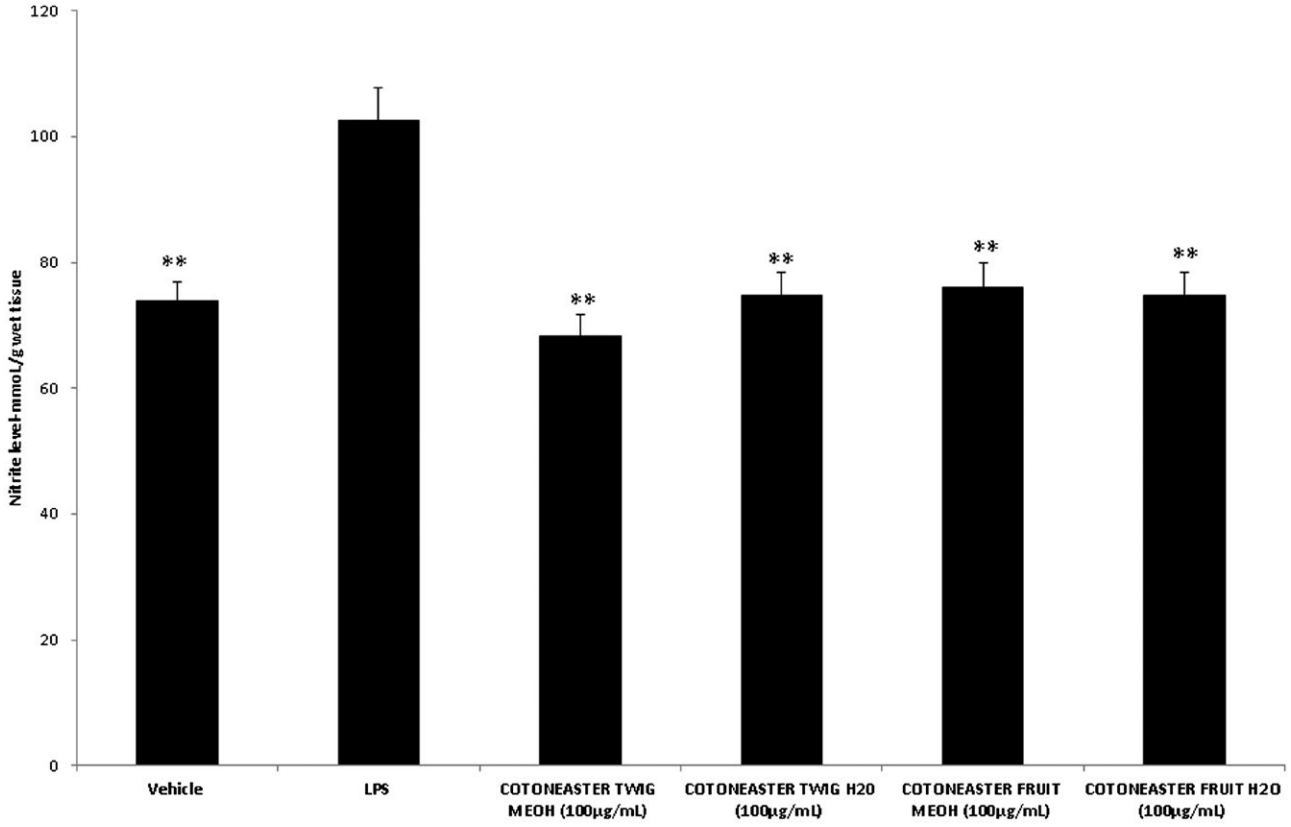

FIGURE 1 Effect of *C. integerrimus* aqueous and methanol extracts (100 µg/ml) on LPS-induced nitrite level (mmoL/g wet tissue) in rat colon specimens. ANOVA, $p < 0.001$; post hoc, $^{**}p < 0.01$ versus LPS

counteracted mostly by the methanol twig extract of *Cotoneaster* which resulted in increased nitrite level (68.33 mmol/g wet tissue). The aqueous twig and fruit extracts were equally effective in reducing LPS-induced nitrite production (74.71 and 74.72 mmol/g wet tissue, respectively) while the methanol fruit extract was less effective (76.08 mmol/g wet tissue) (ANOVA, $p < 0.001$; post hoc, $^{**}p < 0.01$ vs. LPS).

3.5 | Effects of C. integerrimus extracts on 8-iso-$PGF_{2\alpha}$, PGE2, and 5-HT colon level

In analogy with the effect exerted on nitrite production, the *C. integerrimus* extracts displayed inhibitory effect on isoprostane production (Figure 2), consistently with the strict relationship between lipid peroxidation and nitrosative stress (Tsikas, 2017). The 8-iso-$PGF_{2\alpha}$ level was increased in the LPS treatment group (9.97 pg/mg wet tissue) compared to the control group (6.36 pg/mg wet tissue). However, *C. integerrimus* extracts tended to suppress the increase in the 8-iso-$PGF_{2\alpha}$ level. Especially, the level was markedly decreased by the methanol twig extract (6.05 pg/mg wet tissue) which was most effective followed by the aqueous twig extract (6.45 pg/mg wet tissue), methanol fruit extract (6.75 pg/mg wet tissue), while the aqueous fruit extract was least effective (6.86 pg/mg wet tissue) (ANOVA, $p < 0.001$; post hoc, $^{*}p < 0.05$; $^{**}p < 0.01$ vs. LPS).

LPS stimulus was also effective in increasing colon PGE_2 level (12.06 pg/mg wet tissue) compared to the control group (9.18 pg/mg wet tissue). This increase was counteracted by the *Cotoneaster* extracts with the twig extracts (methanol: 6.73 pg/mg wet tissue; aqueous: 6.99 pg/mg wet tissue) being more effective than the fruit extracts (aqueous: 9.55 pg/mg wet tissue; methanol: 9.79 pg/mg wet tissue) (ANOVA, $p < 0.001$; post hoc, $^{*}p < 0.05$; $^{**}p < 0.01$ vs. LPS).

In contrast, the aqueous fruit extract was most effective in stimulating serotonin turnover, evaluated as 5HIAA/5-HT ratio, displaying a ratio value of 6.46 compared to LPS treatment (0.23) and control group (1.19), in isolated colon specimens. The methanol twig, methanol fruit, and aqueous twig extracts were less effective in stimulating 5HIAA/5-HT ratio, displaying ratio values of 0.51, 0.29, and 0.28, respectively (Figure 3) (ANOVA, $p < 0.001$; post hoc, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. LPS).

3.6 | Effects of C. integerrimus extracts on TNFα gene expression and wound healing in HCT116 cell line

The effects of methanol and aqueous *C. integerrimus* extracts on TNFα gene expression in hydrogen peroxide-challenged HCT116 have also been studied. We observed a downregulation of TNFα gene expression following extract treatment (Figure 4) (ANOVA, $p < 0.001$; post hoc, $^{*}p < 0.05$, $^{***}p < 0.001$ vs. hydrogen peroxide treated group).

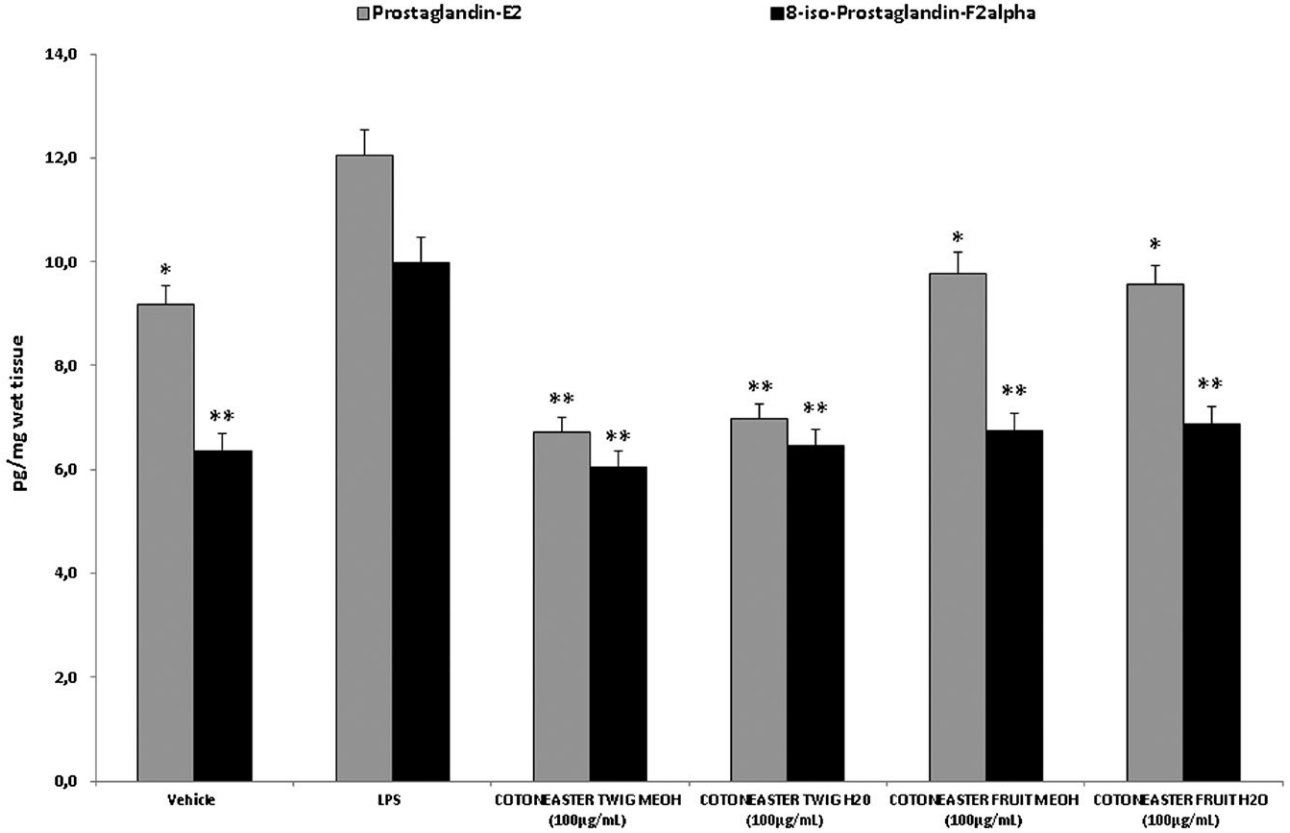

FIGURE 2 Effect of *C. integerrimus* aqueous and methanol extracts (100 µg/ml) on LPS-induced prostaglandin E_2 (PGE_2) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) level (pg/mg wet tissue) in rat colon specimens. ANOVA, $p < 0.001$; post hoc, $^{*}p < 0.05$; $^{**}p < 0.01$ versus LPS

Finally, we assayed the potential capability of *Cotoneaster* extracts to promote wound healing in the same cell line, finding a null effect (Figure 5).

4 | DISCUSSION

As a preliminary approach to evaluate potential toxicity, *C. integerrimus* extracts, in the concentration range (0.1–20 mg/ml), were tested on brine shrimp mortality. It is a typical and general bioassay that could give information on bioactivity of complex plant extracts evaluated as lethality induced on the brine shrimp, *Artemia salina*. This organism is commonly used to investigate a varieties of biological and toxicological activities of plant extracts and is considered, at least partially, predictive of cytotoxicity (Ohikhena, Wintola, & Afolayan, 2016). Experimental procedure was conducted following previous published data, with slight modification (Taviano et al., 2013).

The resulting LC50 value has been indicatory to choose the extract concentration range (10–1,000 µg/ml) for the subsequent evaluation of the effects on human colon cancer-derived HCT116 cell line viability (MTT test).

As previously reported, HCT116 cell line, which is characterized by a low grade of differentiation, was highly sensitive to the cytotoxic effects of xenobiotics, including herbal extracts (Locatelli et al., 2017). The viability test indicated the following biocompatibility range (10–100 µg/ml), corresponding to a cell viability ≥70% compared to vehicle-treated cells. Considering these findings, the subsequent pharmacological tests were performed at the upper concentration tolerated by the cell line (100 µg/ml).

To this regard, we evaluated the effect of aqueous and methanol fruit and twig *C. integerrimus* extracts on LDH activity in HCT116 cell line following hydrogen peroxide treatment (Figure 6). LDH is a cytoplasmic cellular enzyme which, when increased in the serum, serves as an indicator of cell lysis or breakdown of cell integrity induced by pathological conditions. LDH is raised in a number of pathological conditions such as hematological disorders, liver disease, malignancies, tissue infarction, congestive cardiac failure, and various respiratory conditions (Faruqi, Wilmot, Wright, & Morice, 2012; Madole, Dilip, Mamatha, & Ankur, 2016). The reduction of LDH activity after challenging HCT116 cells with the extracts, supports a possible protective effects on inflamed colon.

To test this hypothesis, we performed a subsequent panel of experiments on isolated rat colon treated with LPS, a validated *ex vivo* experimental paradigm to evaluate the efficacy of drugs and extracts on oxidative and inflammatory pathways involved in ulcerative colitis (Locatelli et al., 2017; Menghini et al., 2018).

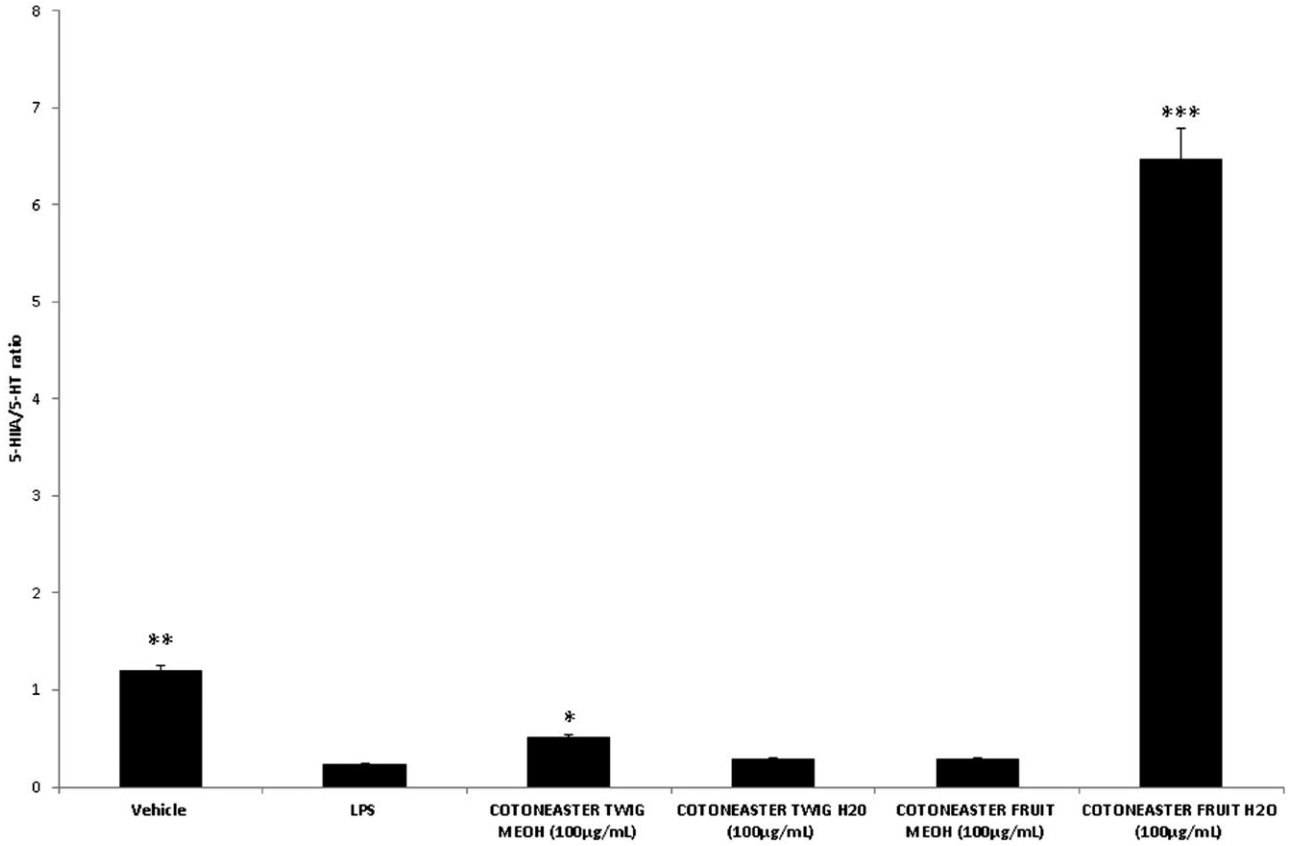

FIGURE 3 Effect of *C. integerrimus* aqueous and methanol extracts (100 µg/ml) on 5HIIA/5-HT ratio in rat colon specimens challenged with LPS. ANOVA, $p < 0.001$; post hoc, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus LPS

To this regard, estimation of nitrite level is a useful marker of the synthesis of chronic inflammatory diseases, including ulcerative colitis (Goggins et al., 2001). NO is a well-known free radical which can react with a variety of biomolecules in body fluids and tissues. These interactions produce a number of oxidation products including nitrite, nitrate, nitrosyl (NO-heme) species, and S- and N-nitroso products. The level of tissue NO-related substances could be considered as a valuable index of inducible NO synthase (iNOS) activity during inflammation (Saijo et al., 2010). The reduction of nitrite level following extract treatment supports protective role in the colon. This finding is in agreement with the reduction of LDH activity and could indicate a minor grade of lipid peroxidation in the colon membrane, as confirmed by the blunting effect on LPS-induced F2-isoprostane level, in colon specimens.

F2-isoprostanes are prostaglandin-like molecules produced by peroxidation reactions induced by oxidative and nitrosative stress on membrane-bound arachidonic acid. These molecules are stable, robust, and detectable in various types of body fluids including plasma, bile, bronchial lavage fluid, cerebrospinal fluid, and urine. Quantification of these molecules has long been considered a valid tool to evaluate lipid peroxidation-induced damages. Currently, F2-isoprostanes are the most commonly used markers to measure oxidative stress *in vivo*. In particular, 8-isoprostaglandin F2α (8-iso-$PGF_{2\alpha}$) is the best studied F2-isoprostane (Mure et al., 2015; van't Erve et al., 2016).

Alongside with the assessment of *C. integerrimus* extract activity on oxidative stress biomarkers, we also investigated the activity of methanol and aqueous fruit and twig extracts on the activity of key inflammatory cytokines involved in pathophysiology of ulcerative colitis, including PGE_2, 5-HT, and TNFα.

PGE2 represents the main product of cyclooxygenase 2 (COX-2) conversion of arachidonic acid. Particularly, PGE_2 long been involved in colon epithelium inflammation and damage (Feghali & Wright, 1997). Accordingly with the present findings (Figure 2), the reduced levels of PGE_2 could account for the anti-inflammatory effects induced by the *C. integerrimus* extracts (Figure 2).

Serotonin (5-hydroxytryptamine, 5-HT) is a monoaminergic neurotransmitter synthesized in raphe nuclei, in the central nervous system. Additionally, 5-HT has long been considered a pro-inflammatory cytokine, particularly in inflamed colon (Regmi, Park, Ku, & Kim, 2014), possibly via 5-HT3 receptor activation (Mousavizadeh, Rahimian, Fakhfouri, Aslani, & Ghafourifar, 2009). To this regard, we have previously reported that anti-inflammatory herbal extracts reduced LPS-induced 5-HT levels, *ex vivo* (Locatelli et al., 2017; Menghini et al., 2016, 2018).

Actually, the higher stimulatory effect exerted by fruit aqueous extract on serotonin turnover could be related, albeit partially, to its content in benzoic acid (Batshaw et al., 1988).

On the contrary, from the present findings, higher activity of the twig extracts in suppressing the LPS-induced nitrite, PGE_2,

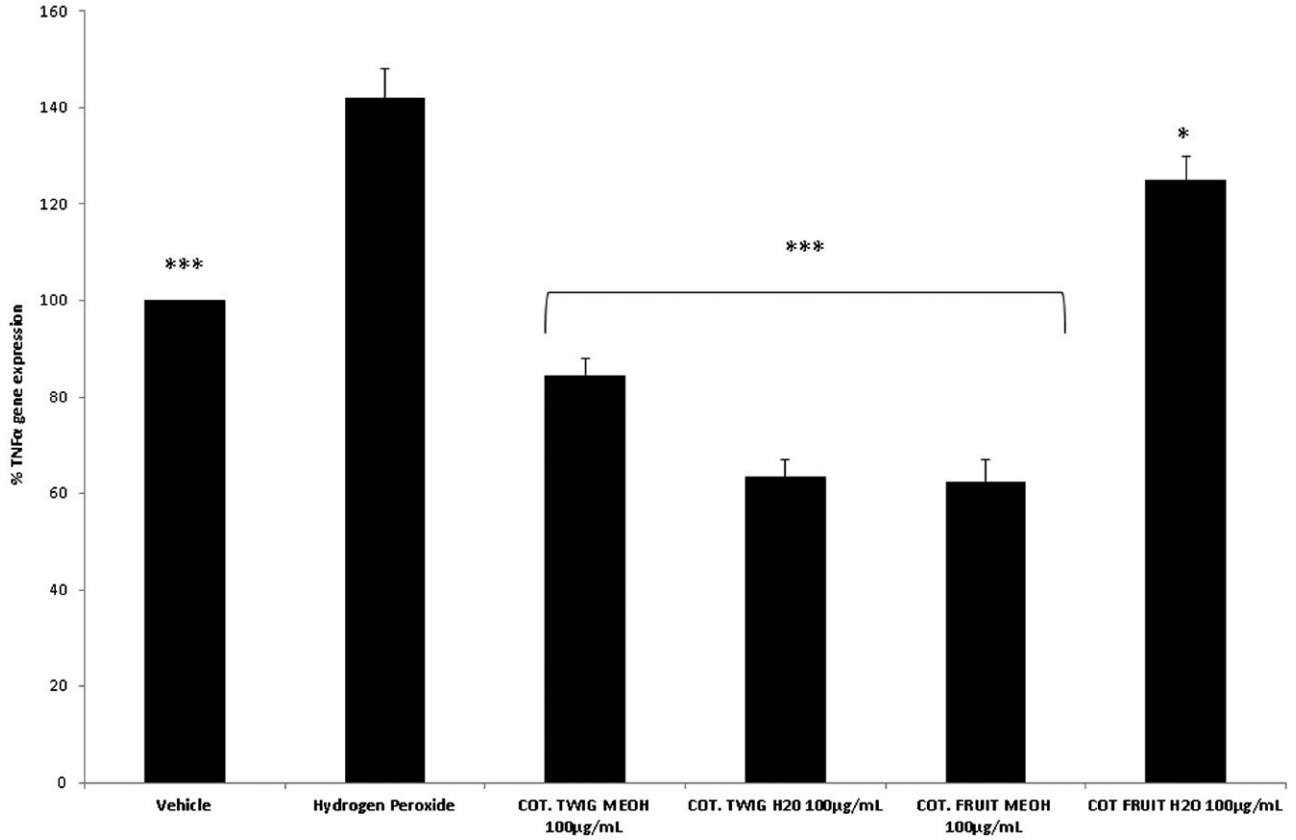

FIGURE 4 Effect of *C. integerrimus* aqueous and methanol extracts (100 µg/ml) on TNFα gene expression in HCT116 cell line challenged with hydrogen peroxide. ANOVA, $p < 0.001$; post hoc, $^{*}p < 0.05$, $^{***}p < 0.001$ versus hydrogen peroxide treated group

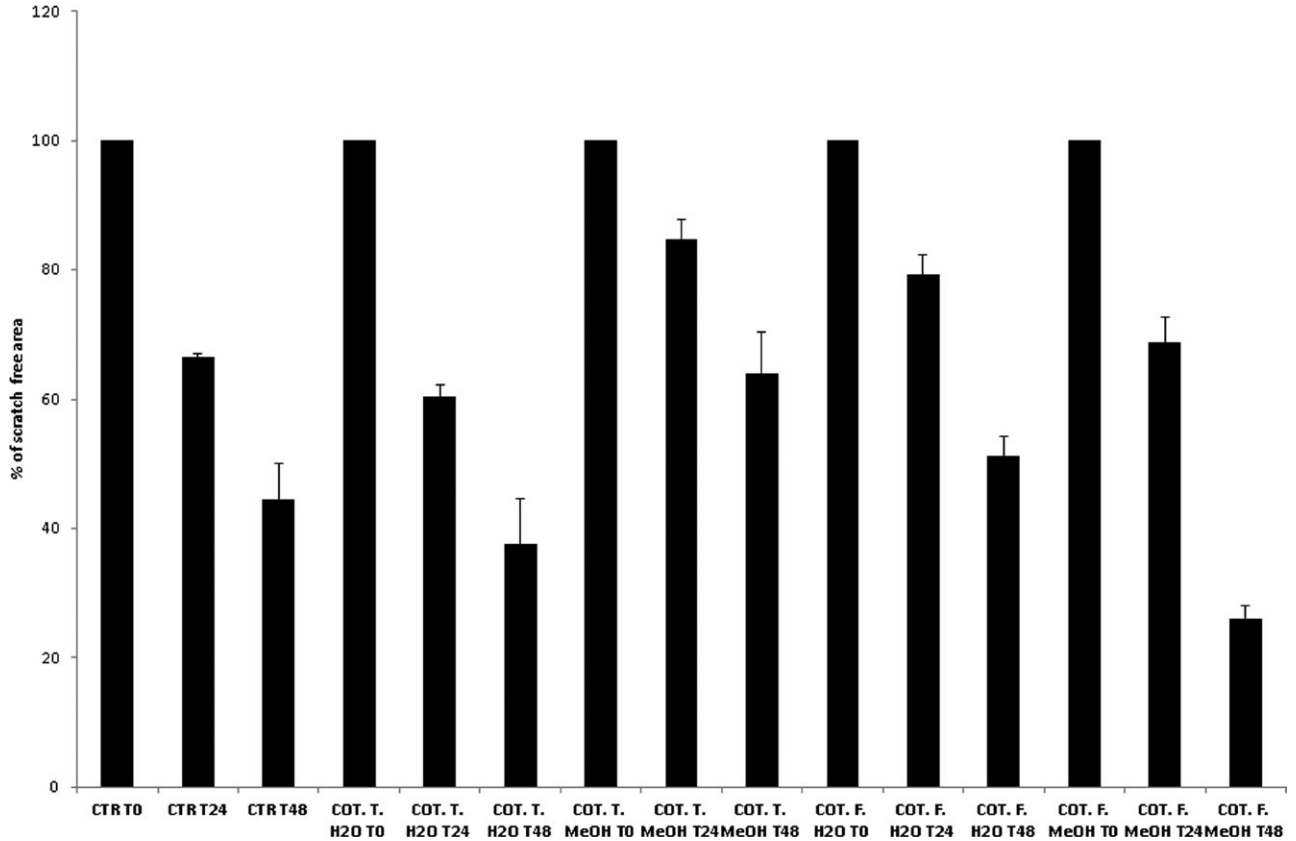

FIGURE 5 Effect of *C. integerrimus* aqueous and methanol extracts (100 µg/ml) on HCT116 cells migration

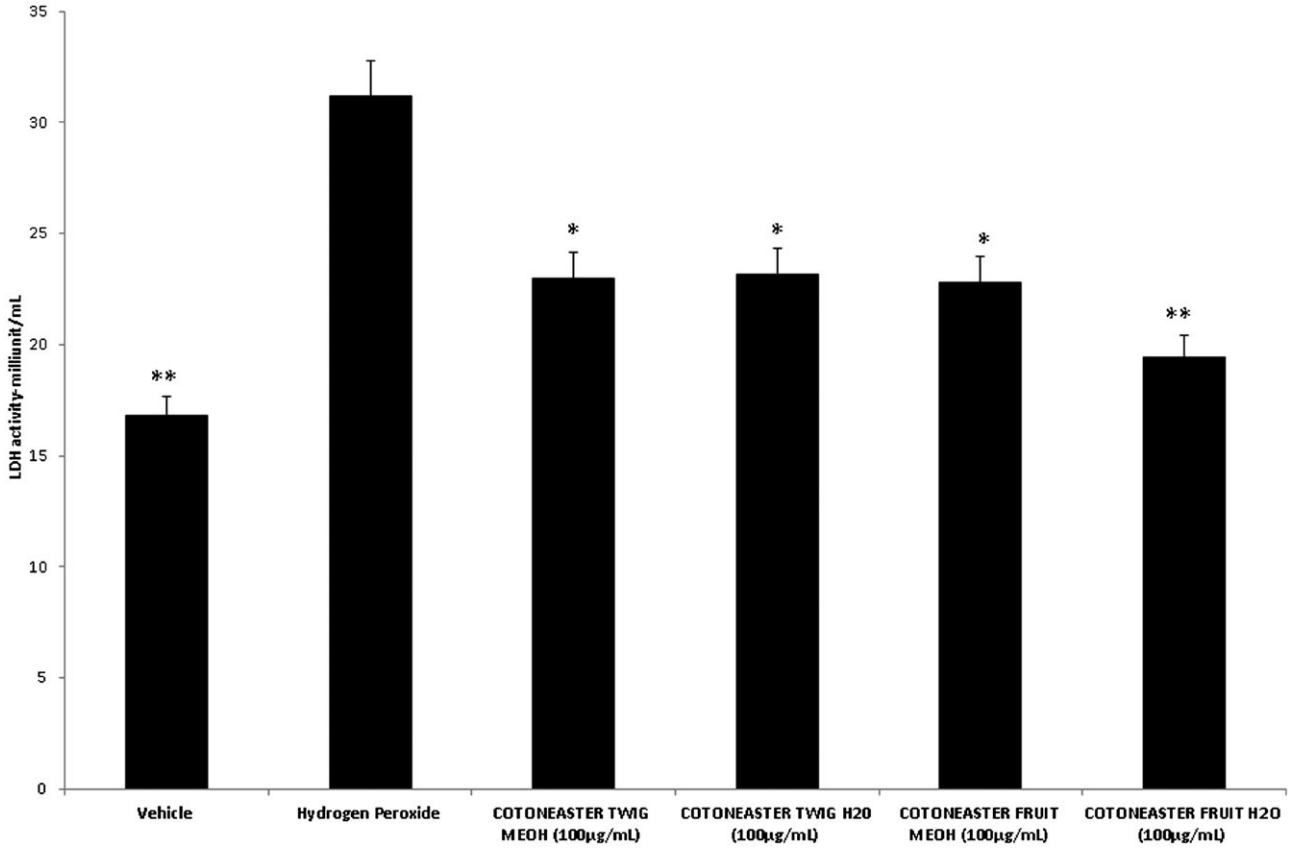

FIGURE 6 Effect of *C. integerrimus* aqueous and methanol extracts (100 µg/ml) on H_2O_2-induced LDH activity in HCT116 cells. ANOVA, $p < 0.001$; post hoc, $^{*}p < 0.05$; $^{**}p < 0.01$ versus hydrogen peroxide

and 8-iso-$PGF_{2\alpha}$ production compared to the fruit extracts can be deduced. This effect could be attributed to its plethora of bioactive compounds. Indeed, a previous study by Uysal et al. (2016) found that both the methanol and aqueous extract of the twigs of *C. integerrimus* displayed higher total phenolic and flavonoid content compared to its fruit extracts (Table 1). In addition, RP-HPLC analysis revealed a number of phenolic compounds in the twig extract which were absent in the fruit extract, including protocatechuic acid, ferulic acid, hesperidin, eriodictyol, and apigenin. Also, the twig extract was rich in chlorogenic acid and (-)-epicatechin (Table 2). These bioactive compounds can act in combinations to produce the overall activity of the twigs. Indeed, previous studies

TABLE 1 Total phenolic and flavonoid contents of the extracts (mean ± *SD*) (Uysal et al., 2016)*

Part	Solvent	Total phenolics (mg GAEs/g extract)**	Total flavonoids (mg REs/g extract)***
Twig	Methanol	115.15 ± 1.39a	16.29 ± 0.43a
	water	96.98 ± 0.97b	6.02 ± 0.16b
Fruit	Methanol	38.47 ± 0.57d	2.03 ± 0.20d
	water	42.70 ± 0.61c	3.96 ± 0.15c

*Different letters (a, b, c and d) in the extracts indicate significant difference ($p < 0.05$). **GAEs, gallic acid equivalents. ***REs, rutin equivalents.

(Chen & Wu, 2014; Hwang, Kim, Park, Lee, & Kim, 2014; Wang & Cao, 2014; Yang et al., 2015) found that chlorogenic acid and epicatechin inhibited NO and PGE_2 production and the expression of COX-2 and iNOS, and also blunted pro-inflammatory cytokines, including IL-1β and TNFα and other pro-inflammatory cytokine, including IL-6. An imbalance of pro-inflammatory cytokines such as TNFα has long been involved in modulating inflammatory response, in colon (Feghali & Wright, 1997; Lee et al., 2010; Sakthivel & Guruvayoorappan, 2014).

In this context, we performed a further set of experiments on HCT116 to evaluate the effects of the extracts on H_2O_2-induced TNFα gene expression. Our findings of reduced TNFα gene expression in hydrogen peroxide-challenged HCT116 cells following extract treatment, besides corroborating the reported previous studies, further support the protective role of *C. integerrimus* extracts in the gut.

TNFα has been recently reported to stimulate HCT116 cell migration, in an experimental wound healing test (Lee et al., 2018).

Considering the downregulating effect induced by the extracts on TNFα gene expression in HCT116 cells, we also explored the effects of *C. integerrimus* extracts in an experimental model of wound healing. We observed that all the extracts were ineffective in modifying the spontaneous cell migration up to 48 hr following treatment.

Taken together, our findings suggest that *C. integerrimus* extracts could display preventive tissue damage effects, as revealed by the

TABLE 2 Phenolic components in the solvent extracts from *C. integerrimus* (mg/g extract) (mean ± *SD*) (Uysal et al., 2016)*

No	Phenolic components	Twig-methanol	Twig-aqueous	Fruit-methanol	Fruit-aqueous
1	Gallic acid	0.04 ± 0.001a	nd	0.04 ± 0.01b	nd
2	Protocatechuic acid	0.68 ± 0.04b	0.89 ± 0.04a	nd	nd
3	(+)- Catechin	3.95 ± 0.02a	2.27 ± 0.12b	0.06 ± 0.01c	nd
4	*p*-Hydroxybenzoic acid	2.51 ± 0.03b	2.37 ± 0.03c	2.90 ± 0.03a	1.40 ± 0.02d
5	Chlorogenic acid	6.81 ± 0.08b	8.29 ± 0.08a	4.54 ± 0.11c	3.99 ± 0.08d
6	Caffeic acid	1.45 ± 0.04b	1.58 ± 0.04a	0.87 ± 0.02c	0.83 ± 0.02c
7	(−)- Epicatechin	19.05 ± 1.15b	32.89 ± 1.13a	11.36 ± 0.24c	9.27 ± 1.19d
8	Syringic acid	nd	nd	nd	nd
9	Vanilin	nd	nd	nd	nd
10	*p*-Coumaric acid	0.11 ± 0.01b	0.29 ± 0.01a	0.03 ± 0.01d	0.06 ± 0.01c
11	Ferulic acid	4.48 ± 0.02b	9.26 ± 0.26a	nd	nd
12	Sinapic acid	nd	nd	nd	nd
13	Benzoic acid	nd	nd	0.69 ± 0.01b	0.95 ± 0.01a
14	*o*-Coumaric acid	0.01 ± 0.01c	0.09 ± 0.01a	0.03 ± 0.01c	0.07 ± 0.01b
15	Rutin	0.21 ± 0.01b	nd	0.29 ± 0.01a	0.31 ± 0.01a
16	Hesperidin	0.14 ± 0.01a	0.15 ± 0.01a	nd	nd
17	Rosmarinic acid	nd	nd	nd	nd
18	Eriodictyol	0.61 ± 0.02a	0.10 ± 0.01b	nd	nd
19	*trans*-Cinnamic acid	nd	0.14 ± 0.01a	nd	0.05 ± 0.01b
20	Quercetin	0.94 ± 0.02a	nd	nd	0.07 ± 0.01b
21	Luteolin	nd	nd	nd	nd
22	Kaempferol	nd	nd	nd	0.17 ± 0.02
23	Apigenin	nd	0.30 ± 0.01	nd	nd

*Different letters (a, b, c, and d) in the extracts indicate significant difference ($p < 0.05$). nd, not detected.

decreased activity of all tested markers of inflammation and oxidative stress. On the contrary, the null effect on wound healing test ruled out a possible role of the extracts in modifying migration and invasion capacities of HCT116 human colon cancer cells.

In conclusion, we report for the first time the protective role of *C. integerrimus* on H_2O_2 and LPS-induced toxicity model of ulcerative colitis. The protective effect exerted by *C. integerrimus* could be related, albeit partially, to its downregulating effects of multiple pro-inflammatory biomarkers involved in ulcerative colitis. This study adds a new insight to the *ex vivo* pharmacological properties of *C. integerrimus*. However, validation using *in vivo* models is required to ensure the safety, quality, and efficacy of *C. integerrimus* before it can be used in the treatment and/or management of inflammation-related diseases in humans, particularly ulcerative colitis.

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CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

ORCID

Gokhan Zengin https://orcid.org/0000-0001-6548-7823
Mohamad Fawzi Mahomoodally https://orcid.org/0000-0003-3962-8666

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