Protective Effect of Hydroalcoholic Olive Leaf Extract on Experimental Model of Colitis in Rat: Involvement of Nitrergic and Opioidergic Systems

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The aim of the present study is to investigate the possible protective effect of dry olive leaf extract (OLE) against acetic acid-induced ulcerative colitis in rats, as well as the probable modulatory effect of nitrergic and opioidergic systems on this protective impact. Olive leaf extract was administered (250, 500 and 750 mg/kg) orally for two successive days, starting from the colitis induction. To assess the involvement of nitrergic and opioidergic systems in the possible protective effect of OLE, L-NG-Nitroarginine Methyl Ester (10 mg/kg) and naltrexone (5 mg/kg) intraperitoneal (i.p.) were applied 30 min before administration of the extract for two successive days, respectively. Colonics was investigated 48 h following induction through macroscopic, histological and biochemical analyses. Olive leaf extract dose-dependently attenuated acetic acid-provoked chronic intestinal inflammation. The extract significantly reduces the severity of the ulcerative lesions and ameliorated macroscopic and microscopic scores. These observations were accompanied by a significant reduction in the elevated amounts of TNF-α and interleukin-2 markers. Moreover, both systems blockage reversed protective effects of OLE in the rat inflammatory bowel disease model. These findings demonstrated, for the first time, a possible role for nitrergic and opioidergic systems in the aforementioned protective effect, and the extract probably exerted its impact increasing nitric oxide and opioid tones. Copyright © 2014 John Wiley & Sons, Ltd.

INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by recurrent inflammation and disruption of gut wall resulting from leukocyte infiltration and excessive generation of inflammatory mediators and oxidants. The two major types of IBD are ulcerative colitis and Crohn disease. These inflammatory bowel diseases are now recognized to be caused by crosstalk between a variety of factors. It is now known that inflammatory cytokines such as interleukin (IL)-1, IL-2, IL-6 and interferon gamma are upregulated at focal lesions induced by dietary antigen and/or intestinal bacteria (Nagura et al., 2001; Ludwiczek et al., 2004). Secretion of tumour necrosis factor-α (TNF-α) by epithelial cells may also represent a crucial event in the pathogenesis of IBD (Hagar et al., 2007). It has been suggested that inflammation of mucosa impairs antioxidant defence mechanisms and exposes tissue to oxidative attack imposed by infiltrating macrophages and neutrophils. Increased oxidative and nitrosative stress and decreased antioxidant defenses are reported in colonic mucosal biopsies of patients with IBD (McCafferty, 2000).

Nitric oxide (NO) regulates major epithelial functions involved in host defence such as mucus production and epithelial fluid secretion. Nitric oxide has also been found to alter the cytokine profile released by macrophages so that following a T helper 1 (Th1) response, Th1-associated cytokines are down-regulated and T helper 2 (Th2) cytokines are favoured (Huang et al., 1998). Nitric oxide has many well-documented antiinflammatory effects in the gastrointestinal tract (Wallace and Miller, 2000). Endothelial NO synthase appears to be a homeostatic regulator of numerous essential functions of the gastrointestinal mucosa, such as maintenance of adequate perfusion (Moncada, 1992), and regulation of microvascular and epithelial permeability (Alican and Kubès, 1996). There is also evidence for the involvement of oxidative stress and profound alterations in the biosynthesis of the free radical NO from L-arginine in the pathogenesis of colitis (La et al., 2003). Furthermore, it has been identified both in the studies held on patients with ulcerative colitis and experimental colitis that inducible NO synthase (iNOS) is upregulated in the colon and the levels of citrulline, nitrate and nitrite of the end metabolites of NO pathway are increased in blood, urine and rectal tissues (Macnaughton et al., 1998; Wallace and Miller 2000).
Intestinal opioid receptors (ORs) have now been found to be widely expressed in peripheral tissues including the gastrointestinal tract. Intestinal ORs are involved in regulation of motility, intestinal secretion and bowel transit time (Wood and Galligan 2004). The expression of μ-ORs is upregulated in ileal and colonic enteric neurons, and the immunocytes and muscosa of IBD patients, a process driven in part by inflammatory cytokines (Philippe et al., 2006). Evidence supports a role for endogenous opioid peptides (enkephalins and endorphins) in the development and or persistence of inflammation (Pol and Puig, 2004). Opioids represent a major part of their impact on the immune response by modulating cytokine production. Studies of the effects of endogenous and exogenous opioids have shown that the opioids possess the capacity to modify the expression of a large number of cytokines and cytokine receptors. Opioids selectively promote proinflammatory or antiinflammatory effects depending on the involvement of μ-ORs. Opioids are central participants in the inflammatory response in the brain and in the periphery (Rogers and Peterson, 2003).

Experimental animal studies demonstrated antiatherogenic, antiinflammatory, hypoglycemic and hypcholesterolemic effects of olive tree leaf (Olea europea L.), all of these positive effects were at least partly related to its antioxidative action (El and Karakaya, 2009). It was shown that a total olive leaf extract (OLE) had an antioxidant activity higher than those of vitamins C and E, because of the synergy between flavonoids, oleuropeosides and substituted phenols (Garcia et al., 2000). Orally applied OLE had a significant protective effect in hepatic oxidative stress and that OLE inhibited the inflammatory response (Wang et al., 2008). Oleuropein is the main constituent of olive leaf, which thought to be responsible for its pharmacological effects. It has remarkable antioxidant activity in vitro (Speroni et al., 1998), as do other constituents of olive leaf (Briante et al., 2002). In vitro, oleuropein and its major metabolite, hydroxytyrosol, exhibited a range of pharmacological properties, antiinflammatory effects (Miles et al., 2005), scavenging of free radicals in addition to inhibition of 5-lipoxygenase and 12-lipoxygenase (Visioli et al., 2002). Oleuropein and hydroxytyrosol enhanced NO production by mouse macrophages (Visioli et al., 1998). In acetic acid-induced writhing in mice, oleuropein-rich and hydroxytyrosol-rich extracts reduced significantly the number of writhing, which is associated with the release of endogenous substances including serotonin, histamine, prostaglandin and bradykinin (Collier et al., 1968).

Assuming the antiinflammatory and antioxidative properties of OLE and its main constitutes and that inflammation of mucosa impairs antioxidant defence mechanisms in IBD, we have attempted to show its probable protective impact on acetic acid-induced ulcerative colitis in rats. Moreover, we demonstrate if nitrigeric or opioidergic systems take part in this healing power.

Table 1. Percentage (%) of the main constituents of olive leaf extract (in 1 g).

<table>
<thead>
<tr>
<th>Main components of the OLE</th>
<th>Amount (%)</th>
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<tbody>
<tr>
<td>Oleuropein</td>
<td>71.2</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>9.88</td>
</tr>
<tr>
<td>Hydroxytyrosol</td>
<td>0.97</td>
</tr>
<tr>
<td>Tyrosol</td>
<td>0.74</td>
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</table>

OLE, olive leaf extract.

ASSESSMENT OF COLONIC DAMAGE

Forty-eight hours following induction of colitis, animals were euthanized. In an ice bath, distal colons were cut open, cleansed gently with normal saline, and macroscopic scores were determined. Subsequently, colons were cut into two same pieces, one for histopathologic assessment (kept in 5 ml of 10% formalin) and the other for analysis of biochemical markers.

**Determination of ulcer index**

Macroscopic scoring was performed under a magnifying glass by an independent observer according to the following criteria: 0, intact epithelium with no damage; 1, localized hyperemia but no ulcer; 2, linear ulcer with no significant inflammation; 3, linear ulcer with inflammation at one site; 4, two or more sites of ulcer and inflammation; 5, two or more sites of ulcer and inflammation extending over 1 cm (Morris et al., 1989).

For evaluation based on microscopical (histologic) characters, the tissue was fixed in phosphate-buffered formaldehyde, embedded in paraffin and 5-mm sections were prepared. The tissue was stained with haematoxylin and eosin and evaluated by light microscopy, being scored in a blinded manner by an expert pathologist. A validated histological grading scale was used; each of the individual parameters estimated was graded 0–3 (inflammation severity, inflammation extent and crypt damage) 0, no change; 1, mild; 2, moderate; 3, severe (Murthy et al., 1993). The evaluated parameters were erosion, ulceration, mucosal necrosis, haemorrhage of mucosa, lamina propria and submucosal edema, and inflammatory cell infiltration. The severity of changes was subjectively graded and compared with controls. Histological evaluation and scoring was carried out using a Zeiss® microscope equipped with an Olympus® colour video camera for digital imaging.

**Biochemical assays**

**Determination of inflammatory mediators.** Colonic levels of TNF-α and IL-2 were determined with an enzyme linked dimmunsorbent assay (ELISA kit) (Enzo Life Sciences, Lorrach/Germany). For the measurement of inflammatory cytokines, the colon was dissected out and homogenized in 50 mmol/L ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% of hexadecyltrimethylammonium bromide. Afterwards, homogenates were centrifuged at 4000 rpm for 20 min at 4 °C, and supernatants were separated and kept at −80 °C until analysis.

**Data and statistical analysis**

All values are expressed as means ±standard error SPSS (version 19.0, Chicago, USA). One-way analysis of variance was employed for analysing the data, followed by Tukey’s test for multiple comparisons. Significance ascribed when p < 0.05.

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**RESULTS**

**Macroscopic and histopathological scores**

As can be seen in Fig. 1(A), the IBD group had significantly higher scores (p < 0.001), and in the extract-treated group with the lowest dose (250 mg/kg), macroscopic scores were significantly higher (p < 0.05). On the other hand, the extract (250, 500 and 750 mg/kg) dose-dependently reduced the scores compared with IBD, p < 0.01, p < 0.001 and p < 0.001, respectively. The co-administration of L-NAME (10 mg/kg) with the highest dose of the extract (750 mg/kg) reversed the protective effect markedly (p < 0.01) (Fig. 2(A2)). Likewise, treatment with naltrexone (5 mg/kg) also reversed the protective effect in a significant manner (p < 0.01) (Fig. 2(B2)). The groups treated with either L-NAME or naltrexone alone had not shown any protective impact (Fig. 1(B)) and showing loss of mucosal architecture with ulceration and acute inflammatory cell infiltration (Fig. 2(A1) and (B1)), respectively.

In the sham-treated group (Fig. 3(A)), the histological features of the colon were typical of normal large bowel architecture with normal mucosal glands and intact epithelial surface, in contrast with acetic acid-treated rats with mucosal haemorrhage, severe inflammatory cell

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**Figure 1.** Extent of colonic damage according to macroscopic scores in acetic acid-treated rats. * significantly different from sham (p < 0.05), ** ** (p < 0.001), ## significantly different from inflammatory bowel disease (p < 0.05), ## (p < 0.01) and ### (p < 0.001), & significantly different from the extract (750 mg/kg) (p < 0.05) and && (p < 0.01).
infiltration, submucosal edema, loss of mucosal architecture with ulceration, crypt abscess formation and acute inflammatory cell infiltration (Fig. 3(B)). When compared with the sham-treated group, intracolonic administration of acetic acid increased the macroscopic damage score \( p < 0.001 \) (Fig. 1(A)) and extensive hemorrhagic, ulcerated damage and transmural necrosis to the distal colon. The architecture of the crypt was distorted, and the lamina propria was thickened in periphery of distorted crypts, especially in the basal areas. The colonic damage as determined histologically paralleled that of macroscopically visible damage (Table 2). Histopathological features in groups of rats treated with the extract (250, 500 and 750 mg/kg), there was only slight submucosal edema, minimal subepithelial hemorrhage, mild inflammatory cell infiltration and regenerated epithelium with normal gland architecture, and decreased number of inflammatory cells in lamina propria. Extract-treated rats showed less severe ulceration and less edema (Fig. 3(D)). Thus, the colonic damage score was reduced from virtually 4.6 in the control colitis group to 1.6 in the group treated with the greatest dose of the extract (Fig. 1(A)).
ultrastructural studies of colonic tissue provided evidence that the extract administration resulted in reduced inflammation. Treatment of rats with the extract (250, 500 and 750 mg/kg) resulted in a significant decrease in the extent and severity of injury of the large intestine \( p < 0.01 \) and \( p < 0.001 \), respectively, as evidenced by macroscopic damage score (Fig. 1(A)) as well as histopathological assessment (Fig. 3(D), Table 2) and strongly prevented propagation of colitis. In colitis rats treated with the extract (250 mg/kg), slight recovery of microvilli in some epithelial cells was observed. The greater inhibitory effect was achieved using the doses (500 and 750 mg/kg), which indicated a reduction of neutrophil infiltration in colonic tissues. It was comparable with dexamethasone-treated rats (Fig. 3(C)).

### Biochemical markers

**Effect on colonic TNF-\( \alpha \) and colonic interleukin-2 concentrations.** TNF-\( \alpha \) level as depicted in Fig. 4(A) reduced in a dose-dependent manner in extract-treated groups (250, 500 and 750 mg/kg) \( p < 0.01 \), \( p < 0.001 \) and \( p < 0.001 \), respectively. In contrast, colonic damage by acetic acid administration was also indicated by an increase of the proinflammatory cytokine TNF-\( \alpha \) (\( p < 0.001 \)). Dexamethasone pretreatment (1 mg/kg) markedly diminished TNF-\( \alpha \) level in colonic mucosa in comparison with control group (\( p < 0.001 \)). Treatment of the rats with L-NAME or naltrexone reversed the extract (750 mg/kg) protective effect, and the figure for TNF-\( \alpha \) was profoundly higher. L-NAME and naltrexone groups had high level of TNF-\( \alpha \) as well (Figure 4(B)).

**As presented in Fig. 5, the IL-2 level was significantly lower only in the extract-treated groups (500 and 750 mg/kg) \( p < 0.001 \) and \( p < 0.001 \), respectively. In the other groups, the marker increased in a significant way.**

### DISCUSSION

In this study, we examined the effect of OLE on acetic acid-induced IBD in rats. We also evaluated the involvement of nitrergic/opioidergic systems, probable modulatory effect, on the protective effect of OLE.

**Table 2. Effects of olive leaf extract on histopathologic scoring of acetic acid-induced colitis in rats**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Histopathologic scores</th>
</tr>
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<tr>
<td>Total</td>
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</tr>
<tr>
<td>Saline (sham)</td>
<td>0.33 ± 0.21</td>
</tr>
<tr>
<td>Acetic acid (control)</td>
<td>9.50 ± 0.34</td>
</tr>
<tr>
<td>Extract (250 mg/kg)</td>
<td>7.33 ± 0.21*</td>
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<tr>
<td>Extract (500 mg/kg)</td>
<td>5.24 ± 0.40***</td>
</tr>
<tr>
<td>Extract (750 mg/kg)</td>
<td>3.67 ± 0.21***</td>
</tr>
<tr>
<td>Dexamethasone (1 mg/kg)</td>
<td>0.50 ± 0.17</td>
</tr>
<tr>
<td>L-NAME (10 mg/kg)</td>
<td>9.58 ± 0.33</td>
</tr>
<tr>
<td>L-NAME + extract (750 mg/kg)</td>
<td>9.48 ± 0.29</td>
</tr>
<tr>
<td>Naltrexone (5 mg/kg)</td>
<td>9.47 ± 0.32</td>
</tr>
<tr>
<td>Naltroxane + extract (750 mg/kg)</td>
<td>9.35 ± 0.29</td>
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L-NAME, L-NG-Nitroarginine Methyl Ester.

*Significantly different from saline (\( p < 0.05 \)). *** (\( p < 0.001 \)).

In our study, OLE (250, 500 and 750 mg/kg) dose-dependently attenuated acetic acid-provoked chronic intestinal inflammation. The present investigation outlines the antiinflammatory and healing effects of OLE against experimental IBD confirmed by histological investigation. The extract profoundly improved macroscopic and histological scores of colonic injury. Rats in sham group showed intact mucosa and submucosa. On the basis of a validated histopathologic scoring system, treatment with OLE blocked propagation of colitis. In colitis rats treated with the extract (250 mg/kg), slight recovery of microvilli in some epithelial cells was observed. The greater inhibitory effect was achieved using the doses (500 and 750 mg/kg), which indicated a reduction of neutrophil infiltration in colonic tissues. It was comparable with dexamethasone-treated rats (Fig. 3(C)).

Figure 4. Extent of colonic damage according to microscopic scores in acetic acid-treated rats. TNF-\( \alpha \) level in colon (Pg/ml). *** significantly different from sham (\( p < 0.001 \)). ## significantly different from inflammatory bowel disease (\( p < 0.01 \)) and ### (\( p < 0.001 \)). &&& significantly different from the extract (750 mg/kg) (\( p < 0.001 \)).

*Phytother. Res. (2014)*
Virtually consistent with our study, the study of Giner et al. (2013) is in agreement with our experiment in which oral administration of oleuropein notably attenuated dextran sulphate sodium induced experimental colitis in mice, by reducing neutrophil infiltration, production of NO, IL-1β, IL-6 and TNF-α expression in colonic tissue. Olive oil diets in mice also exerted a noteworthy beneficial effect in chronic dextran sulphate sodium induced colitis by cytokine modulation and COX-2 and iNOS reduction and supplementation of the diet with hydroxytyrosol may improve chronic colitis through increases in mucous and bicarbonate secretions on epithelia but also decreases cytokine secretion and neutrophil adherence and increases the level of prostaglandin (Wallace and Miller, 2000). Some researchers have determined that it is possible to prevent mucosal inflammation through NO inhibition (iNOS) (Rachmilewitz et al., 1995). Consistent with our study, L-NAME treatment (50 mg/kg) for 7 days (i.r.) and (i.p.) does not have any protective effect on the colonic injury (2,4,6-trinitrobenzenesulfonic acid) in rats (Vardarci et al., 2003).

Olive leaf extract could potentiate the antinociceptive property of morphine subeffective dose and suppress low-dose morphine hyperalgesia in rats through Ca<sup>2+</sup> channel blocking activity (Esmaeili-Mahani et al., 2010). Oleuropein prevents the development of morphine antinociceptive tolerance through inhibition of morphine-induced L-type calcium channel overexpression (Zare et al., 2012). Olive leaf extract (200, 300 and 500 mg/kg) and oleuropein relieved the development of morphine physical dependence in rats (Esmaeili-Mahani and Zare, 2013). Exogenous opioids have been shown to exert an anti-inflammatory effect in mouse IBD models, whereas μ-OR knockout mice show increased susceptibility to colitis (Philippe et al., 2006).

The results of this study demonstrate that OLE probably decreased intestinal damage through the antioxidant and antiinflammatory effects of polyphenols, mainly, oleuropein and hydroxytyrosol. In addition, both systems blockage reversed protective effects of OLE. According the aforementioned information, we suggested that OLE through increasing NO tune probably through raising endothelial NO synthase or inhibiting iNOS activity could be involved in its protective effect. Also, enhancing opioid tone could take part improving the intestinal damage. Further studies will be needed in future to explore the main active ingredient of OLE to be evaluated against ulcerative colitis and further explore their antiinflammatory and antioxidants mechanisms.

**Conflict of Interest**

The authors have declared that there is no conflict of interest.

**REFERENCES**


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

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