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THUJA OCCIDENTALIS MOTHER TINCTURE ALLEVIATE THE COX-2 EXPRESSION IN INTESTINAL MUCOSA OF THE TNBS-INDUCED ULCERATIVE COLITIS MODEL IN MICE

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Abstract: Inflammatory bowel diseases (IBD) is a great concern worldwide. New drugs are continuously being developed and combined in order to manage the symptoms and treatment of ulcerative colitis (UC). Moreover, studies show a new approach on plant derived extracts to be used in the treatment of IBD, being less invasive and having less secondary effects. Our aim was to investigate the COX-2 activity level in colonic tissue, through the immunohistochemistry (IHC) technique, and thus prove the anti-inflammatory effect of *Thuja occidentalis mother tincture (MT)* in oral administration in corresponding doses (5, 25, and 50 mg/kg of body weight). The intra-rectal administration of TNBS resulted in an increase in cyclooxygenase-2 (COX-2) expression in comparison to control. The IHC results showed that the expression of COX-2 inflammatory marker decreased significantly following the oral administration of three doses of *T. occidentalis* MT (5, 25, and 50 mg/kg of body weight), in dose dependent manner.

Keywords: colitis, COX-2, anti-inflammatory, Thuja occidentalis MT

INTRODUCTION

Inflammatory Bowel Diseases (IBD) usually describes two conditions that cause inflammation of the gastrointestinal tract: Ulcerative colitis and Crohn's disease. For the sake of our research we will concentrate on ulcerative colitis (UC). Ulcerative colitis is a chronic condition that involves the inflammation of the colon and rectum. The inflammation severity varies with edema, hemorrahage and ulceration along the affected area (Camacho-Barquero, 2007). Ulcerative colitis is usually limited to the mucosal layer of the gastrointestinal tract, beginning in the rectum where inflammation activity is at its highest (Dávalos, 2004). Rectum involvement with proximal extension is found in 95% of patients. Characteristics histological findings show polymorphonuclear leukocytes and mononuclear cells in addition to distortion and depletion of mucosal glands including goblet cells, and crypt abscesses presence (Camacho-Barquero, 2007). Consequently symptoms include abdominal pain, lower abdominal cramping and bloody mucus diarrhea (Camacho-Barquero, 2007) (Dávalos, 2004).

Genetic predisposition and environmental factors are said to play a role in the predisposition for this condition. Treatment modulations differ depending on the severity of the disease. Drugs are continuously being developed and combined in order to manage the symptoms of UC. Oral sulfasalazine, 5-aminosalicylic acid medications alongside steroid enemas and corticosteroids have been used in milder cases. In moderate to severe cases, Azathioprine and 6-mercaptopurine and infliximab were found to be more effective in the management in the treatment of UC. However, both were associated with high risk of lymphomas, and hepatosplenic T cell lymphoma respectively. Historically, plants have been used as remedy to many ailments. In attempts to determine those that result in a superior response in IBD or specifically ulcerative colitis, different plants have been studied for their anti-oxidant, anti-inflammatory, and antiulcerogenic effects on different diseases. Many herbal remedies showed improvement, symptom management including lesser toxicity and side effects such as *Triticum aestivum*, *Boswellia serrata*, *absinthium*, or *Tripterygium wilfordii*.

Thuja occidentalis also known as "Arbor vitae" or "white cedar" is a coniferous tree indigenous to Europe and North America. Thuja was utilized in folk medicine as a remedy for enuresis, cystitis, rheumatism and autoimmune diseases including psoriasis. Nowadays, it is used as dilution or mother tincture in homeopathic medicine alone or in combination with other plants such as *Echinaceea purpurea* and *Baptisia tinctoria*. The most valuable component in the plant is its richness in essential oils that make up 1.4-4%. These essential oils include Borneol, Camphene, Fenchone, Limonene, Myricene, Terpine, Terpinolene, Thujone, and Thujylalcohol. In addition to Coumarins, Flavonoids, tannic acid and polysaccharides and proteins are also important components found in *Thuja occidentalis*. These components provide the plant with its antioxidant, antibacterial, anti-inflammatory, emmenagogic diuretic and several other pharmacological and therapeutic properties.

An important essential oil found in this plant is Thujone ($C_{10}H_{16}O$). It is a monoterpene with a ketonic structure, found in nature in two diastereomeric

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(epimeric) forms: (−)-α-thujone and (+)-β-thujone. Its concentration depends on the extraction method used. In this study distillation based techniques were used to produce a mother tincture. MT was used to assess both its in vitro and in vivo antioxidant and anti-inflammatory by measuring the COX-2 activity and to validating it as a potential phyto-therapeutic treatment for IBD.

MATERIALS AND METHODS

Thuja Occidentalis Mother Tincture Preparation and Characterisation

The method of obtaining and characterizing the components of Thuja extract were shown in our previous work (Stan et al, 2019).

Animals and Experimental Method

The project was authorized according to emergency order (OG) no. 42/2004 (the National Sanitary Veterinary and Food Safety Authority (ANSVSA) authorization no. 001/12.08.2016). The experiment took place at Western University Vasile Goldis, Arad. Animals used in this experiment were raised in the animal facility of the university. The experiment was conducted on CD1 adult male mice weighing between 25 and 30 g. Colitis was induced using intra-rectal administration of 1 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS) dissolved in 0.1 mL of 50% ethano. Before surgical interventions the food was withdrawn a day prior and surgical intervention was performed according to the International Ethical Guidelines for Animal Care. 100 mice were selected for the purpose of this study. The mice were divided into 5 groups. Based on previous published reports three different does of *T. occidentalis* MT were selected 5, 25, and 50 mg MT/kg of body weight for this experiment. (Stan, 2019).

Groups	Type	Day 1	Over 7 days
Group 1	Control	70–80 ml 50% ethanol intra-rectally	300 mL 5% ethanol gavage solution/day
Group 2	TNBS	50 mg TNBS/kg of BW intra-rectally	5% ethanol gavage solution/day
Group 3	TNBS + 5 mg T. occidentalis MT/kg of BW	50 mg TNBS/kg of BW intra-rectally	5% ethanol gavage + 5 mg dry substance T. occidentalis MT/kg of BW /day
Group 4	TNBS + 25 mg T. occidentalis MT/kg of BW	50 mg TNBS/kg of BW intra-rectally	5% ethanol gavage + 25 mg dry substance T. occidentalis MT/kg BW /day
Group 5	TNBS + 50 mg T. occidentalis MT/kg of BW	50 mg TNBS/kg of BW intra-rectally	5% ethanol gavage + 50 mg dry substance T. occidentalis MT/kg of BW/day

Tissue samples were taken on day 9, for histopathological analysis, fixed in 4% formalin in Phosphate buffer in paraffin, processed by histological technique and cut to 5 µm using a Leica RM2125 RTS microtome (Leica Microsystems GmbH, Wetzlar, Germany).

Immunohistochemistry

Slides were deparaffinized in xylene for 2 times, 5 min each. Then they were transferred to 100% alcohol, for 2 times, 3 min each, and then transferred once through 95%, 70% and 50% alcohols respectively for 3 min each. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 solution in methanol at room temperature for 10 min following the rinse with PBS for 2 times, 5 minutes each. Appropriately diluted primary antibody (100 µl) was added to the sections on the slides and incubated in a humidified chamber at room temperature for 1 h. Polyclonal antibody COX-2 with dilution 1:200 (Santa Cruz, USA) was used as primary antibody. The control sections were processed by the substitution of primary antibodies with immunoglobulins for marked antibodies, used under the same conditions as primary antibodies. 100 µl DAB substrate solution (freshly made just before use: 0.05% DAB - 0.015% H2O2 in PBS) was applied to the sections on the slides to reveal the color of antibody staining. The tissue slides were cleared in 3 times of xylene and coverslip using a mounting solution. The mounted slides can be stored at room temperature permanently. The color of the antibody staining in the tissue sections were then observed under microscopy, Olympus BX43 optical microscope and captured with the help of the Olympus XC30 digital camera and the Olympus Cell Dimension software.

RESULTS AND DISCUSSIONS

The intra-rectal administration of TNBS resulted in an increase in immune-histochemical expression of inflammation marker cyclooxygenase-2 (COX-2) compared to control (Figure 1b). Following oral administration of three doses of *T. occidentalis* MT (5, 25, and 50 mg/kg of body weight) expression of COX-2 inflammatory marker decreased significantly in dose dependent manner. Figure 1e which received 50 mg tincture/kg of body weight dose shows remarkable decrease COX-2 almost resembling the control group figure 1a.

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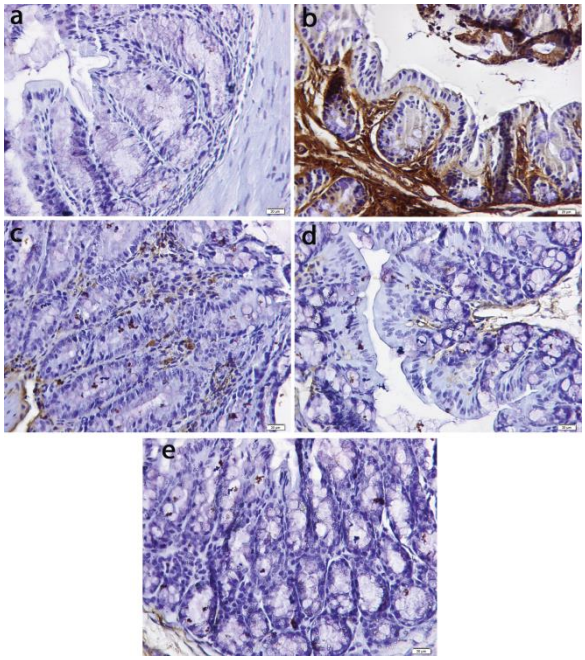

Fig. 1. Appearance of COX-2 inflammatory marker in intestinal mucosa in experimental groups: (a) Control; (b) TNBS; (c) TNBS + 5 mg Thuja occidentalis MT/kg of body weight; (d) TNBS + 25 mg Thuja occidentalis MT/kg of body weight; (e) TNBS + 50 mg Thuja occidentalis MT/kg of body weight.

Our results are in agreements with our previous study where we found the same pattern for Il-6 and TNF- α (Stan, 2019).

Extensive research has been done over the years to promote phytotherapy in treatment of a variety of diseases. Plant extracts have been used in multiple fields of medicine, nutrition and cosmetics as they demonstrate lesser side effects than traditional drugs. Traditionally, plant extracts have been used because of their anti-oxidative and anti-inflammatory properties including their ability to strengthen the immune system. The present study in addition to the previous one has been done to demonstrate the anti-oxidative and anti-inflammatory effects of *Thuja occidentalis.* in inflammatory bowel disease specifically ulcerative colitis. This study is a follow-up of a previous one which is an experimental model of colitis that was induced in CD1 mice intra-rectally using 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Stan et al 2019).

This study in combination with the previous one reflects the benefits of the therapeutic properties of *T. occidentalis* MT for the treatment of ulcerative colitis. The two studies combined give a thorough in vitro and in vivo assessment of the *Thuja* plant extract and its therapeutic effects in IBD. However, further clinical research should be carried out to assess its safety, administration and side effects on humans.

Moreover, other studies have approached the natural treatment of UC through plant extracts. Youjim kim et al, 2017 have shown that Isothiocyanate-enriched moringa seed extract (*Moringa oleifera* Lam.) alleviates DSS induced ulcerative colitis symptoms in mice, through its anti-inflammatory and antioxidant activities. Other extracts, as Dandelion root extract protects NCM460 colonic cells and relieves experimental mouse colitis (Ding et al, 2018), while a Brassica extract was effective in alleviating signs of ulcerative colitis in mice (Ding et al, 2018). The therapeutic effect and mechanism of proanthocyanidins isolated from grape seed (GSPE) were investigated for their activity in the treatment of recurrent ulcerative colitis (UC) in rats (Ding et al, 2018).

Thus, natural products, in the treatment of ulcerative colitis, have been the subject of interest of many studies. In our study, the therapeutic properties of *T. occidentalis* MT were reflected clearly in oral administration of medium and high doses, which inhibited the inflammatory process induced by TNBS. This experiment can be a cornerstone for further clinical research in the formulation of a therapeutic drug for the treatment of IBD patients.

Cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) are cyclooxygenase enzymes that convert arachidonic acid to inflammatory and other physiological mediators (Agoff et al, 2000).Our results show a dose-dependent decrease of COX-2 expression in colonic tissue after TNBS-ulcerative colitis induction, being in agreement with other studies. Thus, other natural compounds as curcumin of *Curcuma longa* (Camacho-Barquero et al, 2007) and glycoprotein of *Gardenia jasminoides* (Oh &Lim, 2006) had the same pattern for COX-2 in UC experiments.

Nevertheless, we pave the way to new researches regarding *Thuja occidentalis* MT pharmacodynamic abilities, in hope that through our study we find a way to alleviate the colonic damages of ulcerative colitis.

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