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In vitro Anti-inflammatory and Protective Effects of ibidì® on Intestinal Epithelial Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Author SV designed the study, performed the statistical analysis, wrote the protocol, and wrote the manuscript. Author LP performed in vitro experiments and author FDS performed ELISA assay and RT-PCR analysis. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: To define the putative anti-inflammatory and cytotoxic effects of ibidì®, a new phytotherapeutic formulation composed of three extracts: *Punica granatum* L, pericarpum; *Boswellia serrata* Roxb., resina; *Curcuma longa* L,. rhizome, using Caco-2 cells, an in vitro model of human intestinal epithelium.
Methodology: cytotoxicity and capacity of ibidì® to induce cell proliferation were assessed respectively by 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide (XTT) assay and nucleotide 5-bromo-2'-deoxyuridine (BrdU) incorporation. Cell migration was evaluated by scratch wound assay. COX-2, IL-6, IL-8 and MCP-1 protein levels were measured in the supernatant of cells stimulated with or without TNF-alfa or IL-1 beta in presence or in absence of ibidì® using ELISA assays. Finally, the influence of ibidì® on the integrity, paracellular permeability, and viability of Caco-2 cell monolayers was monitored by measuring the transepithelial electrical resistance (TEER) in presence or in absence of TNF-α stimulation.
Results: No dose-response toxicity was observed after 48 h incubation with ibidì®. Interestingly the cell proliferation rate was generally lower in presence of ibidì® than vehicle at all concentrations tested, while ibidì® had no effects on cell migration. Ibidì® markedly inhibited TNF-alfa-induced production of IL-8 at all concentrations tested in a dose-response manner, while that of IL-6 and MCP-1 only at highest ibidì® concentrations. Importantly ibidì® in a range of concentration between 145 and 9 µg/ml

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not only abrogated TNF-alfa-dependent TEER depression, but also promoted higher resistance values than untreated cells.
Conclusion: These data demonstrate that ibidì® exerts anti-inflammatory and protective effects on intestinal epithelial cells by blocking the production of IL-8, IL-6 and MCP-1, and unveil that the synergism of the three extracts regulates epithelial barrier function.

Keywords: Punica granatum pericarpium; Curcuma longa; Boswellia serrata; epithelial barrier function; trans-epithelial resistance.

1. INTRODUCTION

ibidì® is a new phytotherapeutic formulation composed by three extracts:*Punica granatum* L, pericarpum; *Boswellia serrata* Roxb., resina; *Curcuma longa* L,. rhizome. The biological properties of each extract, widely described in the literature, have led to higher consumption of products containing these plants over the years. In many parts of the world the Punica granatum fruit rind is considered helpful for the treatment of malaria and cardiovascular disorders, and in the prevention of some inflammatory-mediated diseases including cancer [1-3]. The plant Boswellia serrata is mainly widespread in India, its rind contains several chemical constituents including alkaloids, terpenoids, tannins, phenols, saponins and pentacyclic triterpenes, considered beneficial in the treatment of inflammatory diseases such as arthritis, osteoarthritis and intestinal diseases [4]. Indeed, clinical studies demonstrated that patients suffering from chronic colitis treated with Boswellia serrata resin showed amelioration of colitis [5-7]. Similar findings were observed in vivo experimental models of colitis after the treatment not only with Boswellia serrata, but also with Punica granatum extracts suggesting both extracts could be used as new therapeutic adjuvant combination for the treatment of inflammatory bowel disease (IBD) [8-12]. Curcuma longa, belonging to Zingiberaceae family, is a perennial plant characterized by ovate or cylindrical rhizomes, which are often branched and brownish-yellow in color and rich of essential oil, alkaloid, starch grain and yellow matter curcumin. It is commonly used in China and India as analgesic, antibacterial, antioxidant, expectorant and flavouring agent [13]. Pilot clinical studies have reported that the use of curcumin in IBD improved patient's symptoms and reduced the consumption of corticosteroids and with remission maintenance [14,15]. Indeed, a diet enriched with curcumin prevented intestinal inflammation in induced experimental colitis. However, the anti-inflammatory properties of all three extracts are still under investigation [16]. I*n vitro* and *in vivo* studies have showed that individually Boswellia serrata, Punica granatum and Curcuma longa were able to reduce the activation of NF-kB pathway, the expression levels of COX-2 and IL-8 induced by TNF-alpha and Interleukin-beta (IL-1beta) [12,17-19], crucial in leading to immune responses and inflammation. Based on these evidences, recently a new product, ibidì®, has been introduced in the market. It is the first formulation containing a mixture of Punica granatum pericarpium, Curcuma longa, and Boswellia serrata, designed to capitalise the anti-inflammatory effects of a concomitant administration of more extracts in the resolution of intestinal inflammation through synergistic effect. A growing body of studies indicates that the combination of medicinal herbs may exert synergistic effects enhancing their therapeutic properties [20-22]. Although the mechanisms underlying this synergism is not completely clear, new mixture of bioactive herbal extracts may pave the way for the possible development of new phytomedicines or adjuvant therapies useful in clinical practice as ibidì®. However, so far there are no evidences on the effects of ibidì® on intestinal mucosa, therefore the aims of this study were to explore in vitro model of human intestinal epithelium: i) cytotoxicity of ibidì®; ii) the capacity of ibidì® to induce cellular proliferation and migration; iii) the capacity of ibidì® to modulate an

inflammatory response induced by TNF-alpha and Il-1beta, and finally iv) the capacity of ibidì® to regulate epithelial barrier function.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

ibidì® and its preparation. Punica granatum pericarpium (20% purity), Curcuma longa (65% purity) and Boswellia serrata (95% purity) were kindly provided by CRISTALFARMA S.r.l (Milan, Italy). Each extract was weighted individually and combined together, on the basis of data present in the literature, in the following proportions: 5 parts Punica granatum pericarpium, 1 part Curcuma longa and 2 parts Boswellia serrata.

The mixture was resuspended in 10% dimethyl sulfoxide (DMSO). Concentration range from 582.6 to 2.3 µg/ml of ibidì® were tested and the corresponding concentrations of each extract are reported in the Table 1 Reagents. Tumor necrosis factor alpha (TNF-α) (>98% purity), Epidermal cell growth factor (ECGF) (>98% purity) and Interleukin-1 beta (IL-1β) (>98% purity) were respectively from Invitrogen and R&D system.

Table 1. Concentrations of ibidì® tested in the study and respective concentrations of each extract

ibidì® (µg/mL)	*Punica granatum* (µg/mL)	*Curcuma longa* (µg/mL)	*Boswellia serrata* (µg/mL)
582.6	100	20	40
291.3	50	10	20
145.6	25	5	10
72.8	12.5	2.5	5
36.4	6.3	1.3	2.5
18.2	3.1	0.6	1.3
9.1	1.6	0.3	0.6
4.5	0.8	0.2	0.3
2.3	0.4	0.1	0.2

2.2 *In vitro* Model

Caco-2 cells are an human colon cancer cell line which reproduces many morphological and functional similarities to the human small intestinal epithelial cells when the cells are grown as polarized cells on permeable support. Indeed, since these cells express several drug transporters and enzymes, they are frequently used to test general properties of drugs. However, Caco-2 cells are widely used also to test the cytotoxicity of drugs and the effects of compounds on the intestinal permeability [23].

2.3 Cytotoxicity Assay

The cell viability was assessed by the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) into orange colored formazan product by the mitochondrial respiratory enzymes with a cell proliferation kit (AppliChem GmbH, Darmstadt) used according to the manufacturer's instructions [24].

To this purpose, 1.5 x 10^4 of Caco-2 had been plated into individual wells of tissue-culture microtiter plates with 96 wells. Following an incubation period of 18 hours with the complete culture medium, (DMEM (Gibco) supplemented with 10% foetal bovine serum, 1mM of sodium pyruvate, 2 mM of L-glutamine and 100 µg/mL of penicillin/streptomycin), the cells were washed with phosphate buffered saline (PBS) pH= 7.4 and subsequently treated with 0.1 mL of medium, respectively containing the 9 different concentrations of ibidì® (Table.1), together with the positive control containing DMSO (20%) and the vehicle made up of complete medium plus the different concentrations of DMSO as used to resuspend ibidì®. All incubations were carried out at 37± 1.5 °C in a humid atmosphere (5.0 ± 0.5% of CO_2). At the end of the 24 hours incubation period, the cells were analysed at the microscope for any morphological changes and then treated by adding 50 µL of XTT reaction solution to each well. After a further 24 hours of incubation, a spectrophotometric reading at a wavelength of 540 nm was performed. The absorbance values were normalised to the values of absorbance of the complete medium without the addition of DMSO, and reported as a % of mitochondrial activity.

2.4 Cell Proliferation Assay

The capacity of ibidì® to induce cell proliferation was assessed by the BrdU incorporation test, a quantitative colorimetric assay based on the ability of the nucleotide 5-bromo-2'-deoxyuridine (BrdU) to bind DNA during the replication phase. the incorporation of BrdU. 5 x 10^4 cells were plated in 96 wells. After 16 hours, cells were stimulated with ibidì® at different concentrations (see table 1), with the vehicle and with ECGF 100 ng/ml, in medium without foetal bovine serum. All incubations were carried out at 37± 1.5 °C in a humid atmosphere (5.0 ± 0.5% of CO_2). After 24 hours, cells were treated with 10 µM BrdU and subsequently incubated for 2 hours. At the end of incubation, the medium was removed and the cells were fixed with 200 µL of FixDenat solution. After a further incubation period of 30 minutes at room temperature (15-25 °C), the FixDenat solution was removed and replaced by 100 µL of anti-BrdU antibody labeled with peroxidase (anti-BrdU POD) (1:100) provided in the kit. After 90 minutes, the cells were washed 3 times with 300 µL of PBS, and incubated with 100 µL of substrate (provided in the kit Roche) for 5-30 minutes, at room temperature [25]. Subsequently, the absorbance values were acquired.

2.5 Scratch Assay

The effects of ibidì® on cell migration was carried out using a scratch assay which measures cell ability to restore confluence after an area has been injured as reported previously [26]. The scratch assay was performed using µ-Dish 35-mm culture inserts (Ibidi) according to the manufacturer's protocols. In brief, 5 x 10^4 Caco-2 cells were seeded into each well of culture inserts and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 24 hours the culture inserts were gently removed using sterile tweezers, a scratch was made in the monolayers, and the cells were incubated for 2 hours at 37°C with mitomycin C (20µg/ml) (Sigma) in order to inhibit the DNA division and to evaluate exclusively cell migratory capacity. Then the cells were co-incubated for 48h with ibidì® at different concentrations, (ECGF) (100ng/ml) or medium alone. Photographs of the wounded area were taken immediately before stimulation (0 hour time point) and after 24–48 hours to monitor the closure of the wounded area. The percentage wound closure was calculated as [(Area initial - Area final) / Area initial] x 100.

2.6 Trans-epithelial Electric Resistance (TEER)

Cells were plated at a density of 5 x 10^4 per well in 24-well transwell filters with a membrane pore size of 3.0 µm (Corning Costar, Cambridge, MA) as reported previously[26]. Trans-epithelial electric resistance (TEER) was measured using a Millicell-ERS epithelial volt ohmmeter (World Precision Instruments, New Haven, CT). Readings were taken daily for 10 days before and after 72 hours of both after apical and basolateral stimulation with TNF-α (25 ng/ml, Invitrogen), ibidì® at different concentrations and medium alone as control. The TEER values were calculated by subtracting the blank values from the sample. The percentage of change in the TEER calculated with respect to time 0 was evaluated for each concentration.

2.7 Secretion of Pro-inflammatory Mediators

After the stimulation of Caco-2 for 24 hours in the presence of different concentrations of ibidì®. with and without TNF-alpha 25 ng/ml or IL-1beta (25 ng/ml), the culture supernatants were collected and stored at -80°C, while the cells were processed by extracting the total RNA.Interleukin 6 (IL-6), Interleukin 8 (IL-8) and Monocyte Chemoattractant Protein-1 (MCP-1) secretions were evaluated using the ELISA assay, according to the manufacturer's instructions for the kits used (R&D system) [27-29]. The same system was used to quantify the levels of COX-2 (R&D system).

2.8 Transcription of IL-8

The total RNA extraction was performed using the Qiagen RNeasy Mini Kit. RT-PCR Syber green was used to quantify the level of IL-8 messenger RNA. Glyceraldeide-3-phosphate dehydrogenase gene (GAPDH) was used as housekeeping gene. Gene expression values were normalized to the expression value of GAPDH RNA and calculated on the basis of the comparative cycle threshold Ct method. The primers used were: IL-8: (sense 5'ACTGAGAGTGATTGAGAGTGGAC3'; antisense 5'AACCCTCTGCACCCAGTTTTC3'); GAPDH (sense 5'CATGAGAAGTATGACAACAGCCT3'; antisense 3'AGTCCTTCCACGATACCAAAGT5')

2.9 Statistical Analysis

Data were analyzed using GraphPad4 software and expressed as mean ± SEM. Unpaired Student's t test and U-Mann Whitney were used for comparison between two groups. Statistical significance was set at P =.05 or less of P =.05

3. RESULTS

3.1 Ibidi Does Not Induce Dose-response Toxicity

As it can be seen in Fig. 1, ibidì® at all the concentrations tested maintained mitochondrial activity at 100% in a way that was comparable to the vehicle, represented only by the culture medium with DMSO at the same concentrations of ibidì®, whereas the DMSO at 20% (used as a positive control) drastically reduced cell viability.

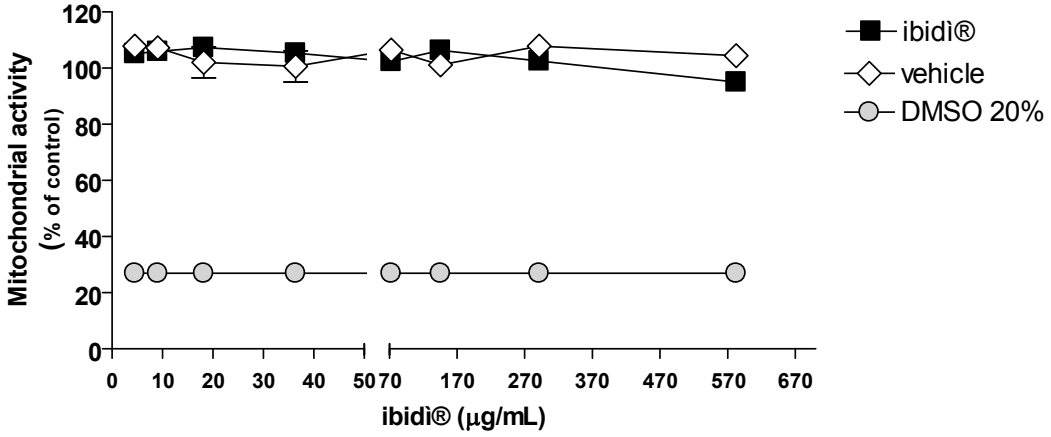


Fig. 1. Cell viability of Caco-2 cells after stimulation at different concentrations of ibidì®

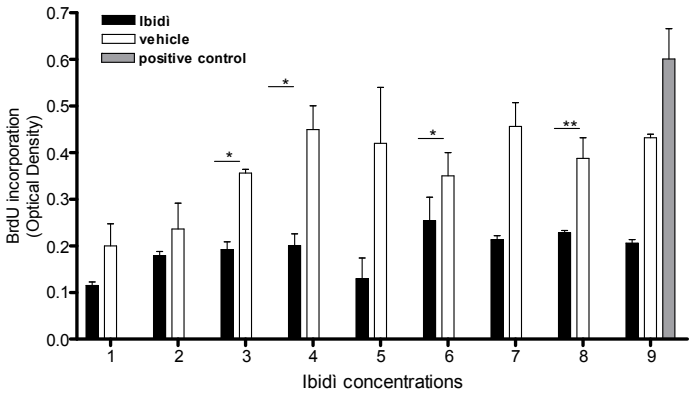
3.2 ibidì® Does Not Induce Proliferation or Migration of Intestinal Epithelial Cells

ibidì® did not promote cell proliferation of Caco-2 cells. On the contrary, at all concentrations tested the levels of BrdU incorporation were lower in the cells treated with ibidì® than those treated with vehicle (Fig. 2). In particular, ibidì® at concentrations of 145.6 µg/mL and below displayed a statistically significant inhibitory effects on epithelial cell growth compared to vehicle and EGF factor (positive control). Next, to assess whether ibidì® had effects on epithelial cell migration, the cells at 100% confluency were incubated in the presence of a mitosis inhibitor (mitomycin C, 20µg/ml) for 2 h at 37°C. Then a lesion was created with a fixed area value for all the cells and stimulated in absence or in presence of ibidì® (582.6 µg/ml or 145.65 µg/ml) for 48 hours. Healing of the injured area was assessed at time 0, 24 and 48 hours from stimulation. As can be seen in Figure 3, ibidì® at all the concentrations tested did not affect cell migration. Indeed, after blocking of proliferation, the lesion area treated with ibidì® remained unchanged (0% of initial area) during the observation period. Only the cells stimulated with the EGF factor (positive control) or with the vehicle showed after 24 and 48 h progressive wound closure as highlighted by red dashes lines (Fig. 3). Similar results were observed after stimulation of cells with low concentrations of ibidì® (data not shown).

3.3 ibidì® Reduces the Levels of IL-8, IL-6 and MCP-1

In order to verify whether the mixture of the three extracts maintained their anti-inflammatory properties, Caco-2 cells were stimulated with TNF-α in presence or absence of ibidì®, and then the levels of pro-inflammatory mediators in the supernatants were evaluated. At all concentrations tested ibidì® markedly reduced the levels of IL-8 (**P=.001), even if at low concentrations the power of ibidì® to revert the TNF-a induced IL-8 levels appeared slightly weaker (Fig.4). In line with protein levels, ibidì® reduced significantly the transcript levels of IL-8 (*P*=.05) compared to TNF-α (data not shown). Although not significant, ibidì® modulated IL-6 protein levels induced by TNF-α only at highest concentration (582.6 µg/ml) as reported

in (Fig. 5), while no changes in IL-6 expression were found with other doses (data not shown). Similarly to IL-6, ibidi® was able to reduce the expression of MCP-1 chemokine but only at the highest concentration (582.6 µg/ml) (Fig. 6).



	ibidì® µg/mL)
1	582.6
2	291.3
3	145.6
4	72.8
5	36.4
6	18.2
7	9.1
8	4.5
9	2.3

Fig. 2. Quantification of BrdU incorporation in Caco-2 cells after stimulation at different concentrations of ibidì®

Optical density values were reported as average of three replicated for each sample

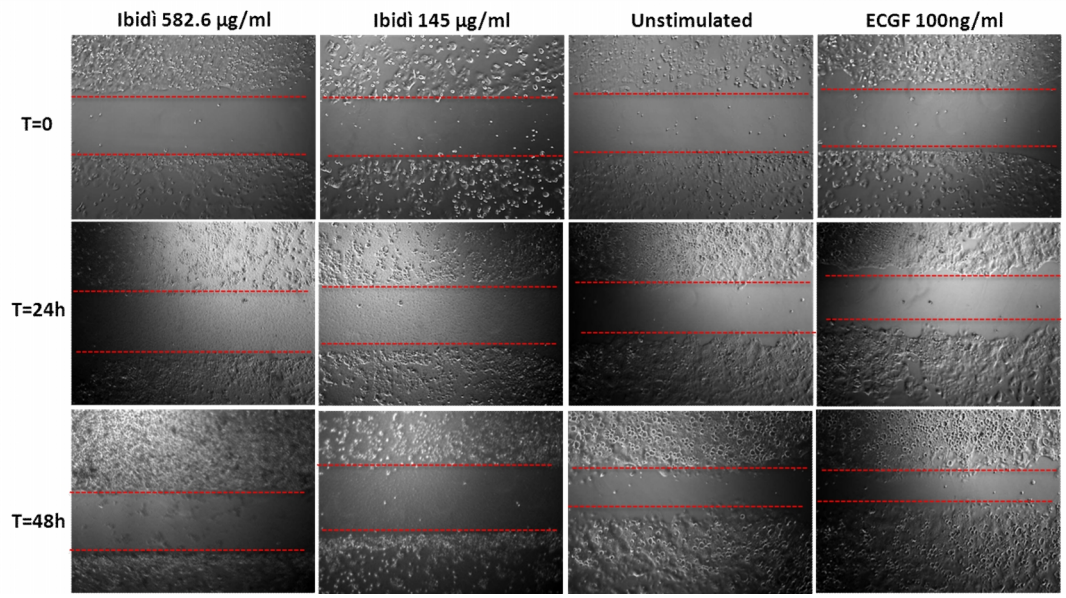


Fig. 3. Wound healing on Caco-2 cell monolayer in response to ibidì® stimulation

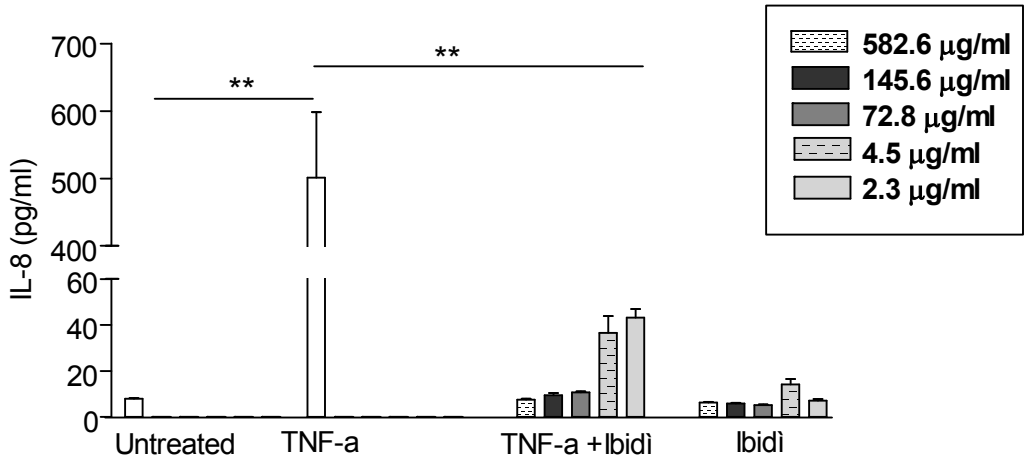


Fig. 4. Effects of ibidì® on the secreted levels of TNF-a-induced IL-8
***P=.001*

3.4 ibidì® Modulates COX-2 Expression

Next we evaluated the ability of ibidì® to inhibit the production of COX-2, which is considered an important enzyme involved in the formation of inflammatory mediators. To this end, we stimulated Caco-2 cells for 24h with IL-1β in presence or in absence of ibidì® at different doses and then we measured COX-2 levels. Ibidì® at 145µg/ml strongly reverted IL-1beta-induced COX-2 levels (*P= .0269 IL-1beta vs IL-1beta Ibidì®; *P=.0026 IL-1beta vs untreated), while alone at all concentrations tested Ibidì® showed no effects in promoting COX-2 expression. Indeed, no significant differences were found between ibidì® alone and only culture medium (unstimulated cells) (Fig.7).

3.5 ibidì® Enforces Trans-epithelial Electrical Resistance

To verify whether the anti-inflammatory properties of ibidì® had effects also on the intestinal barrier function, trans-epithelial resistance was measured before and after 72 h of stimulation with TNF-α in the presence of ibidì® at different concentrations. Interestingly ibidì® alone in the range from 582.6 to 72.4 µg/ml enforced TEER values. In particular, after 72 h of stimulation ibidì® at the concentrations 145.6 and 72.4 µg/ml significantly enhanced the values of TEER compared to medium alone (*P*= .01 ibidì® 145 vs control; *P*=.01 ibidì® 72.4 vs control) (Fig.8), whereas no differences have been observed at lower doses. In addition, in the same range of concentrations (from 145 to 72.4 µg/ml) ibidì® was able to strongly revert TNF-α-dependent TEER changes. However the most efficient activity of ibidì® was found at 145.6 µg/ml showing 80% of TEER recovery compared to TNF-α (*P*=.01). Finally, no effects on TNF-α-dependent TEER alterations were seen at lower concentration of ibidì® (4.5-2.27 µg/ml).

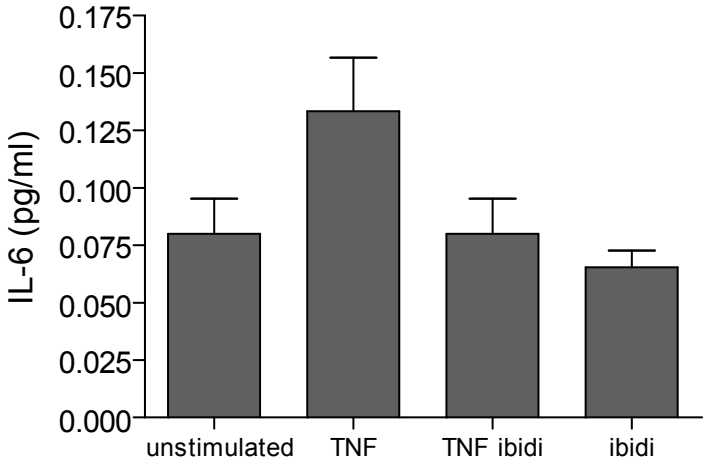


Fig. 5. Effects of ibidì® (582.6 µg/ml) on the secreted levels of TNF-a-induced IL-6

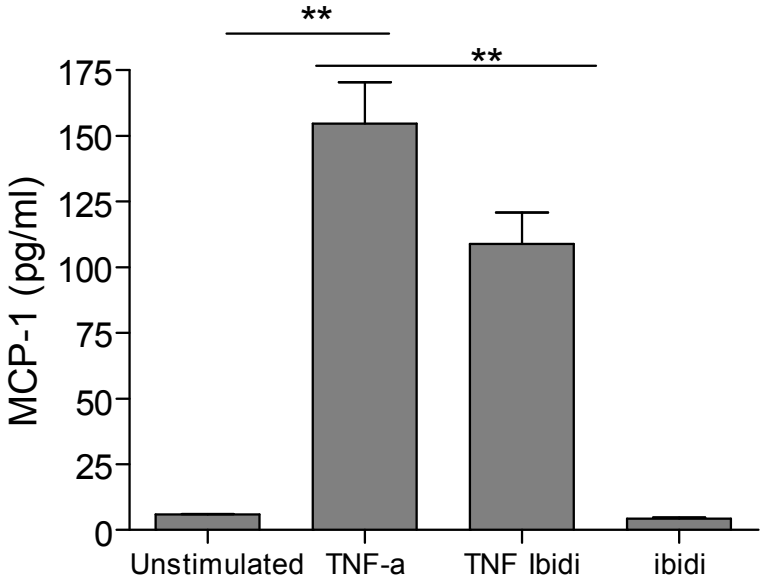


Fig. 6. Effects of ibidì® (582.6 µg/ml) on the secreted levels of TNF-a-induced MCP-1
****P=.001**

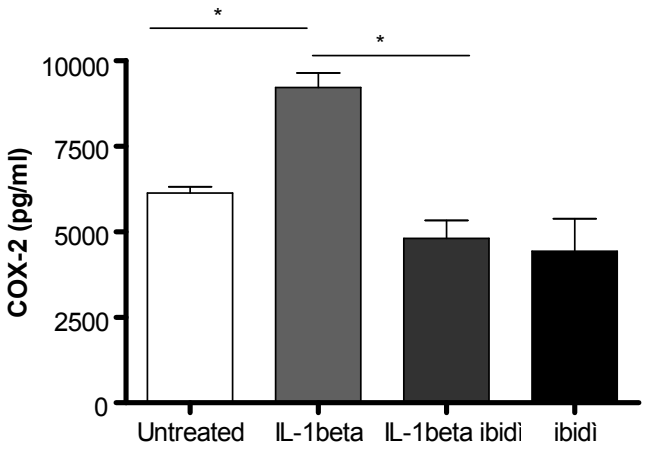


Fig. 7. Effects of ibidì® (145µg/ml) on COX-2 expression induced by IL-1β in Caco-2 cells

*(*P= .0269 IL-1beta vs IL-1beta Ibidì®; *P=.0026 IL-1beta vs untreated)*

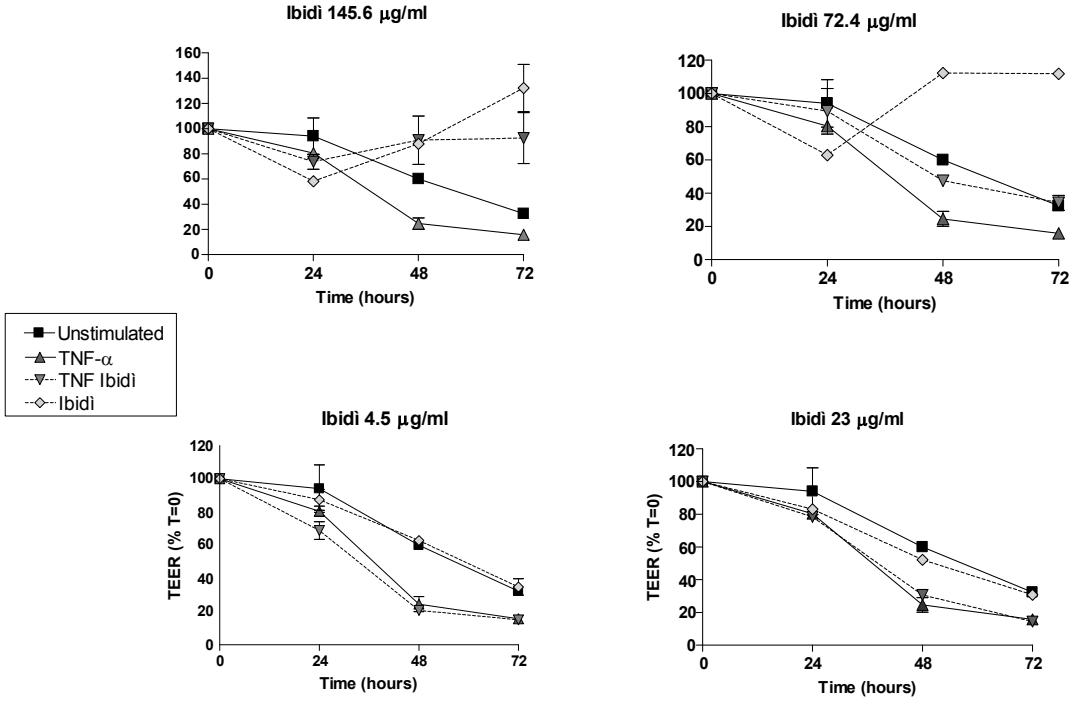


Fig. 8. Effects of ibidì® on epithelial barrier function

Confluent Caco-2 cells were incubated with TNF-α (25 ng/ml) in the presence or absence of ibidì®. Values of TEER were measured over 72 hours of stimulation with TNF-α, and normalized on time 0. These data are representative of three independent experiments

4. DISCUSSION

ibidì® is a phytotherapeutic product composed by three extracts in the following ratios 5 parts of Punica granatum pericarpium, 1 part of Curcuma longa and 2 parts Boswellia serrata.

Several evidences have demonstrated that individually these extracts exert anti-inflammatory properties modulating the production of pro-inflammatory mediators such as IL-6, which is a pleiotropic cytokine, IL-8 and MCP-1 chemokines, responsible for the recruitment of neutrophils and monocytes respectively, and induction of COX-2, an enzyme involved in the formation of inflammatory mediators of Prostaglandin series [18, 19]. Emerging data have been indicated these herbal extracts as novel adjuvants for the treatment of inflammatory bowel disease [30]. Indeed, the treatment with Boswellia serrata promoted healing of ulcers and the resolution of mucosal integrity in patients with chronic colitis [5]. Moreover, the therapy was safe and well tolerated in patients without serious adverse events. A brief pilot study conducted on 5 patients suffering from Crohn's Disease and 5 patients with ulcerative colitis, demonstrates that the intake of curcumin ameliorates clinical symptoms of patients [14]. Although so far there are no clinical studies providing the beneficial effects of the Punica treatment in IBD patients, data came from in vitro and in vivo studies using experimental model of colitis proved the beneficial antioxidant and anti-inflammatory properties also of Punica granatum [18, 30]. Therefore, the anti-inflammatory properties of the individual extract have suggested the formulation of a new adjuvant product for the treatment of IBD, ibidì®, designed to synergise the capabilities of Boswellia serrata, Curcuma longa and Punica granatum. However, there are no evidences about the use of the three extracts together, and on the effects that they may induce when used together, therefore the objectives of this study were to evaluate using an *in vitro* model of human intestinal epithelium the cytotoxic and anti-inflammatory effects of ibidì®. Data in the literature show that Punica granatum exerts its anti-inflammatory properties in a range of concentrations between 30-200 µg/mL, Curcumin in a range of 3.6-15 µg/ml, and Boswellia serrata around 100 µg/ml. Based on these evidences, we tested the effects of ibidì® on Caco-2 cells within a range from 582.6 to 2.3 µg/mL corresponding to a range from 100 to 0,3 µg/mL for Punica, a range from 20 to 0.07 for Curcuma and a range from 40 to 0.16 µg/mL for Boswellia. At all doses tested ibidì® resulted not toxic for intestinal epithelial cells. In fact, the mitochondrial activity used as a parameter of viability was unaffected at all the concentrations and with no difference if compared with the control. Furthermore, ibidì® did not induce cell proliferation, by contrast displayed inhibitory effect on cell growth. Similar inhibitory findings have been reported after stimulations with curcumin alone at concentrations higher than 9 µg/mL [18]. Interestingly, we found an inhibitory effect of ibidì® also at lower concentrations than 9 µg/mL of Curcuma thus indicating a potential synergistic effect of Curcuma with others extracts. However, the mechanisms by which ibidì® acts promoting cell cycle arrest remain to be investigated.

Since Boswellia serrata favoured the healing of ulcers in IBD patients, we next explored whether ibidì® promoted wound closure, but at all doses tested ibidì® showed no effects on mucosal healing. On the contrary ibidì® exerted potent anti-inflammatory effects in reducing the levels of IL-8, IL-6 and MCP-1(only at highest dose tested). These results confirm the anti-inflammatory properties of Boswellia serrata, Curcuma Longa and Punica granatum, and demonstrate that the mixture of the extracts enhances their power of action. Indeed, ibidì® at concentrations of 2.3 µg/ml corresponding to 0,4 µg/ml of Punica granatum, 0.08 µg/ml of Curcuma Longa and 0.16 µg/ml of Boswellia serrata, reduced markedly IL-8 production. Dysregulation of IL-8 levels has been proposed to contribute to IBD

pathogenesis leading to an aberrant recruitment of neutrophils in the intestinal mucosa [31]. The strong ability to suppress the production of IL-8 promotes ibidì® as adjuvant therapy for IBD. Besides the already known effects of three extracts, this study unveils the potential function of ibidì® in regulating intestinal epithelial permeability. At the concentrations of 145 and 72.8 µg/mL ibidì® not only reverts the changes in resistance induced by TNF-alpha, but also increases the base values of resistance compared to the unstimulated control. The value of trans-epithelial electrical resistance (TEER) is a parameter used to assess intestinal permeability since it is an indication of the level of integrity of the epithelial cells to form junction molecules (tight junctions), which are necessary for intestinal barrier function. Therefore, low resistance values are an indication of increased intestinal permeability, whereas higher values indicate strengthening of the barrier function. Although these data require further confirmation both in terms of protein and mRNA expression of the junction molecules following stimulation with ibidì®, indicate, in any case, that ibidì® in an *in vitro* system in a range of concentrations between 145-72.8 µg/mL acts on the intestinal epithelium by regulating barrier function.

5. CONCLUSION

ibidì®, a phytotherapic combination containing extracts from three different and selected medical plants, is safe and not toxic for the epithelial cells.

If these preliminary data should also be confirmed *in vivo*, ibidì® could be used clinically not only as an anti-inflammatory treatment, but also as an adjuvant therapy to regulate and strengthen intestinal permeability recurring in many diseases such as chronic inflammatory bowel diseases.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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