Study of effect of anti-diarrheal medicinal plants on enteropathogenic *Escherichia coli* induced interleukin-8 secretion by intestinal epithelial cells

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Abstract

Diarrhea is a major health concern in developing countries with enteropathogenic *Escherichia coli* (EPEC) being a leading cause of infantile diarrhea. Much of the pathology of EPEC infection is due to the inflammatory responses of infected intestinal epithelium through secretion of pro-inflammatory cytokines such as interleukin (IL)-8. With medicinal plants gaining popularity as prospective antidiarrheal agents, we aimed to evaluate the effect of anti-diarrheal medicinal plants on secretion of IL-8 by epithelial cells in response to EPEC infection. The effect of the decoctions of four anti-diarrheal medicinal plants viz. *Aegle marmelos, Cyperus rotundus, Psidium guajava* and *Zingiber officinale* was studied on secretion of IL-8 by a human colon adenocarcinoma cell line, HT-29 infected with *E. coli* E2348/69. Two protocols were used viz. pre-incubation and post-incubation. The data obtained demonstrated that out of the four plants used, only *P. guajava* decreased secretion of IL-8 in the post-incubation protocol although in the pre-incubation protocol an increase was observed. A similar increase was seen with *C. rotundus* in the pre-incubation protocol. No effect on IL-8 secretion was observed with *A. marmelos* and *Z. officinale* in both protocols and with *C. rotundus* in the post-incubation protocol. The post-incubation protocol, in terms of clinical relevance, indicates the effect of the plant decoctions when used as treatment. Hence *P. guajava* may be effective in controlling the acute inflammatory response of the intestinal epithelial cells in response to EPEC infection.

Introduction

Diarrheal diseases are a major health concern in developing countries with an estimated 1.8 million deaths per annum.[1] Despite improvements in public health and economic well being, it remains an important clinical problem in developed countries as well.[2] It is estimated that infectious diarrhea will remain a cause of global health concern in the next 2-3 decades.[3]

Diarrhea is an etiologically diverse condition caused by a variety of enteric pathogens.[4] *E. coli* is recognized to be a common cause of gastroenteritis and accounts for nearly 30% of total diarrheal pathogens in some regions.[5] There are seven different categories of diarrheagenic *E. coli* strains based on epidemiology, clinical syndromes, and virulence properties.[6] The enteropathogenic *E. coli* (EPEC) is an important category that is a leading cause of infantile diarrhea in developing countries.[7]

EPEC colonize the intestinal epithelial surface and cause histopathological mucosal changes, the physiological end results of which are disruption of the intestinal epithelial barrier, alterations in intestinal transport, and inflammation.[8] Much of the pathology of EPEC infection has been linked to inflammatory responses by the infected epithelium which occurs through activation of the nuclear transcription factor NF-κB. Since EPEC is a non-invasive pathogen, NF-κB is activated by soluble factors secreted or shed by the pathogen or translocated type three secretory system-dependent effectors.[9] Activation of NF-κB in turn promotes the expression of proinflammatory cytokines such as interleukin (IL)-8[10,11] which result in substantial recruitment of neutrophils and other PMNs to the site of *in vivo* infection.[12] Although the inflammatory response is not the mechanism that initiates EPEC mediated diarrhea, it may contribute to the duration and severity of the diarrheal response.[8] The PMNs and other inflammatory cells recruited to the infected intestine also cause considerable tissue damage through the release of toxic inflammatory mediators.[13]

In recent years, medicinal plants have gained popularity as prospective antidiarrheal agents, with a large numbers of studies being published in the past decade.[14] Whilst a few studies have reported antimicrobial activity, a majority have focused on physiological diarrhea and thereby reporting their effect on intestinal motility in experimental models. Hence, we studied the activity of selected antidiarrheal medicinal plants against pathogenicity of infectious diarrhea, including parameters such as colonization of epithelial cells and production and action of enterotoxins.[15-18] In the present study we aim to screen medicinal plants for their effect on inflammatory responses to infections by enteric pathogens. These plants, if active, may prove to be an effective therapy towards control of the intestinal inflammation associated with infectious forms of diarrhea.

Different approaches have been used to analyze the generally anti-inflammatory potential of plants and plant-derived compounds which include acute and chronic inflammatory models. The acute models, which include both *in vitro* and *in vivo* studies, are designed to assess the effect of the plants on secretion of pro-inflammatory cytokines such as IL-8, changes in vascular permeability, leukocyte migration and chemotaxis, macrophage activity, and measurement of rat paw edema.[19] Therefore, in the present study we have evaluated the *in vitro* activity of hot aqueous extracts (decoction) of four selected antidiarrheal medicinal plants *i.e. Aegle marmelos* (L.) Correa (Family Rutaceae), *Cyperus rotundus* Linn., (Family Cyperaceae), *Psidium guajava* L. (Family Myrtaceae) and *Zingiber officinale* (Roscoe) (Family Zingiberaceae) against secretion of the pro-inflammatory cytokine IL-8 by HT-29 cells in response to EPEC infection. These plants were chosen on the basis of an ethonobotanical survey in the Parinche valley, near Pune, Maharashtra, India.[20] All four plants are widely used in indigenous systems of medicine across the world for their various medicinal properties. They are widely used for treatment of diarrhea and have been reported to have immuno-modulatory properties including anti-inflammatory activity.[21-24]

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Key words: *Aegle marmelos, Cyperus rotundus, Psidium guajava, Zingiber officinale,* IL-8, enteropathogenic *Escherichia coli.*

Contributions: BS carried out the laboratory studies, analyzed the data and prepared the manuscript; PT collected the plant materials, authenticated them and obtained voucher specimen numbers; TJB has overall responsibility for the study.

Conflict of interest: the authors report no conflicts of interest.

Received for publication: 17 May 2011.
Revision received: 28 September 2011.
Accepted for publication: 21 October 2011.

Licensee PAGEPress, Italy
Alternative Medicine Studies 2011; 1:e16
doi:10.4081/ams.2011.e16

Materials and Methods

Media, reagents, plastic ware and instrumentation

The bacteriological media were purchased from HiMedia laboratory, Mumbai, India. Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were procured from GibcoBRL, UK. The Quantikine human CXCL8/IL-8 immunoassay kit was purchased from R&D Systems, USA. All chemicals were from SD Fine Chemicals, Mumbai. The 24-well flat bottomed tissue culture plates were purchased from Nunclon, Denmark, and the 55 mm diameter tissue culture plates were obtained from Tarsons, Kolkata, India.

Bacterial strain

For the present study, EPEC strain E2348/69, serotype 0127:H6 (kindly provided by Dr. S. Knutton, Institute of Child Health, University of Birmingham, Birmingham, UK) was used. The bacterial strain was stored at -80°C in brain heart infusion (BHI) containing 20% glycerol. For each assay a frozen stock of the bacterial strain was revived in BHI.

Cell culture

The human colon adenocarcinoma cell line, HT-29, was obtained from the National Centre for Cell Sciences, Pune, India. The cell line was maintained by passage every 4-5 days in DMEM supplemented with 10% FCS, at 37°C in 5% CO_2 atmosphere.

Plant material

The unripe fruits of *A. marmelos*, rhizomes of *C. rotundus* and *Z. officinale* and the leaves of *P. guajava* were collected from the Parinche valley, about 53 km south east of the city of Pune in the state of Maharashtra, India. The plant materials were authenticated by Dr. P. Tetali, Naoroji Godrej Centre for Plant Research (NGCPR). Voucher specimens of *A. marmelos, C. rotundus* and *P. guajava* were deposited at the Botanical Survey of India (BSI), Western Circle, Pune, Maharashtra, India, and that of *Z. officinale* at the herbarium at the NGCPR (Table 1). The plant materials were shade dried and powdered, and stored at 4°C until used.

Preparation of extract

Crude aqueous extracts (decoctions) were used for the study since this represents the nearest form to traditional preparations. The decoctions were prepared as described in the Ayurvedic text:[25] 1 g of the powdered plant materials were boiled in 16 mL double distilled water till the volume reduced to 4 mL. The decoctions were centrifuged and filtered through a 0.22 μm membrane before use. To replicate field conditions, each assay was performed with freshly prepared decoctions. The decoctions were diluted 1:1000, 1:100, 1:20 and 1:10 in DMEM for each experiment and have been referred to as 0.1%, 1%, 5%, and 10%, respectively throughout the text.

The dry weight of the decoctions was recorded to determine the concentration of the extracts in each dilution. The qualitative phytochemical analysis was performed using standard methods.[26]

IL-8 secretion by intestinal epithelial cells in response to EPEC infection

The effect of the decoctions on the inflammatory response of intestinal epithelial cells to infection by EPEC was measured by estimation of IL-8 secreted by HT-29 cells in response to *E. coli* E2348/69 supernatant by IL-8 enzyme linked immunosorbent assay (ELISA).[9] Briefly, *E. coli* E2348/69 was grown overnight in BHI at 37°C. The culture supernatant obtained by centrifugation at 2,000 rpm was stored at -20°C till used for the assay. For each assay, 0.2 mL of the thawed culture supernatant of *E. coli* E2348/69 was added directly to HT-29 cells (5×10^5/well) grown overnight in 24-well tissue culture plates in a total assay volume of 0.5 mL containing 0.3 mL DMEM with 10% FCS. Following incubation for 18-20 h, the culture supernatant was collected and centrifuged at 14,000 rpm. The supernatant were either immediately estimated for IL-8 using a Quantikine IL-8 immunoassay kit as per the manufacturer's instructions or stored at -20ºC for estimation of IL-8 at a later date. The optical density was measured at 450 nm (reference 540 nm) on an ELISA plate reader (Labsystems, Finland).

The IL-8 assay was performed using two different protocols: pre-incubation protocol and post-incubation protocol. In the pre-incubation protocol the HT-29 cells were incubated with different concentrations of the plant decoctions for 3 h prior to addition of the EPEC supernatant whereas in the post-incubation protocol the plant decoctions were added into the assay system 3 h after the addition of the EPEC supernatant to the HT-29 cells.

Statistical analysis

The results have been expressed as the mean ± standard error of the percentage values of the test groups relative to control (100%) from three independent experiments. Data were analyzed by analysis of variance (ANOVA) and Dunnett's post test. A value of $P\leq0.05$ was considered to be statistically significant. The EC_{50} values, wherever applicable, were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve. All statistical analyses were performed using the software Prism 4.0 (GraphPad Software, Inc., USA).

Results

Phytochemical analysis

The percentage yield (mg/mL) for each decoction with respect to the respective starting material is shown in Table 1. The qualitative phytochemical analysis revealed the presence of constituents such as carbohydrates, flavonoids and tannins in the decoctions of all four plants. While saponins were detected in the decoctions of *A. marmelos, C. rotundus* and *P. guajava*, phytosterols and anthraquinone glycosides were detected in the decoction of *A. marmelos* only (Table 2).

Effect on IL-8 secretion in response to EPEC infection

The effect of the decoctions on EPEC induced secretion of IL-8 by HT-29 cells is shown in Figures 1-4. The baseline secretion of IL-8 by HT-29 cells in the culture medium was estimated to be 56.55±5.12 pg/mL whereas the secretion of IL-8 in presence of EPEC supernatant increased to 477.53±8.84 pg/mL (represented as 100%). The decoctions of *A. marmelos* and *Z. officinale* had no effect on EPEC induced secretion of IL-8 by the HT-29 cells in either of the two protocols used (Figures 1 and 4, respectively) whereas the decoction of *C. rotundus* had no effect in the post-incubation protocol (Figure 2). In comparison the decoction of *P. guajava* resulted in a significant decrease (EC_{50} value

Table 1. Details of the plant material.

Herbarium N.	Botanical name	Common name	Family name	Part Used	% yield of decoction (w/w)
BSI-124675	*A. marmelos*	Wood Apple, Bengal Quince	Rutaceae	Unripe fruit pulp	20.4±2.11
BSI-124666	*C. rotundus*	Nutgrass	Cyperaceae	Rhizomes	20.9±1.12
BSI-124672	*P. guajava*	Guava	Myrtaceae	Leaves	10.8±0.5
NGCPR-642	*Z. officinale*	Ginger	Zingiberaceae	Rhizomes	8.84±0.01

2.09±0.78%) in EPEC induced IL-8 secretion in the post-incubation protocol with a maximum decrease at 10% concentration (Figure 3). On the contrary, in the pre-incubation protocol the decoctions of both *C. rotundus* and *P. guajava* resulted in a significant increase in EPEC induced IL-8 secretion (Figures 2 and 3, respectively).

Discussion

The anti-inflammatory activity of four anti-diarrheal medicinal plants used in traditional medicine, *i.e. A. marmelos, C. rotundus, P. guajava* and *Z. officinale* were assessed against EPEC induced secretion of IL-8 by intestinal epithelial cells. Analysis of the results of the study shows that the decoctions of *A. marmelos* and *Z. officinale* had no effect on EPEC induced IL-8 secretion by HT-29 cells in both pre- and post-incubation protocols. On the other hand, the IL-8 secretion by HT-29 cells was increased in the pre-incubation protocol by decoctions of *C. rotundus* and *P. guajava* and inhibited in the post-incubation protocol by the decoction of *P. guajava*.

Extracts from several plants have been reported to inhibit IL-8 secretion from epithelial cells.[27-29] Our observations in the post-incubation protocol on the inhibitory activity of aqueous extract of *P. guajava* leaves on IL-8 secretion from epithelial cells confirm the results reported by Peng *et al.*[30] who used a similar protocol. However, in contrast to the observation made in the present study in which the decoction of *Z. officinale* showed no inhibition of IL-8 secretion, several studies have reported on the inhibitory effect of the ethanolic,[31] 50% ethanolic[32] and methanolic[33] extracts of *Z. officinale* on either IL-8 or IL-8 and NF-κB modulation *in vivo* and *in vitro*. These differences in observations could be due to the use of aqueous decoction in the present study compared to the use of organic extracts by other workers. Major bioactive constituents from *Z. officinale* such as [6]-shogaol, [6]-gingerol, [8]-gingerol, and [10]-gingerol,[34] and constituents such as *ar*-curcumene and α-pinene isolated from volatile oil,[35] have also been reported to have an inhibitory effect on IL-8. However, these belong to the class of constituents that are not soluble in water. In addition, the differences in the ecotype and/or variety of the *Z. officinale* used could also be responsible for the observed difference in the activity.

Inhibition of IL-8 secretion by epithelial cells has been attributed to polyphenolic compounds present in the extracts.[36] Different modes of action have been proposed for their inhibitory activity on IL-8 secretion which include: i) inhibition of inhibitory factor IKK

Table 2. Results for the qualitative phytochemical analysis of the decoctions of the plants used for the study.

Phytochemical Constituent	Phytochemical Test	Inference			
		A. marmelos	*C. rotundus*	*P. guajava*	*Z. officinale*
Carbohydrates	Molisch's test	+	+	+	+
Reducing sugars	Fehling's test	+	+	+	+
	Benedict's test	+	+	+	+
Starch	Iodine test	+	+	-	+
Phytosterols	Salkowski's test	+	-	-	-
Cardiac glycosides	Legal's test	-	-	-	-
Anthraquinone glycosides	Modified Borntrager's test	+	-	-	-
Saponins	Foam test	+	+	+	-
Flavonoids	Shinoda's test	+	+	+	+
Tannins	Ferric chloride test	+	+	+	+
	Lead acetate test	+	+	+	+
Alkaloids	Wagner's test	+	-	+	-

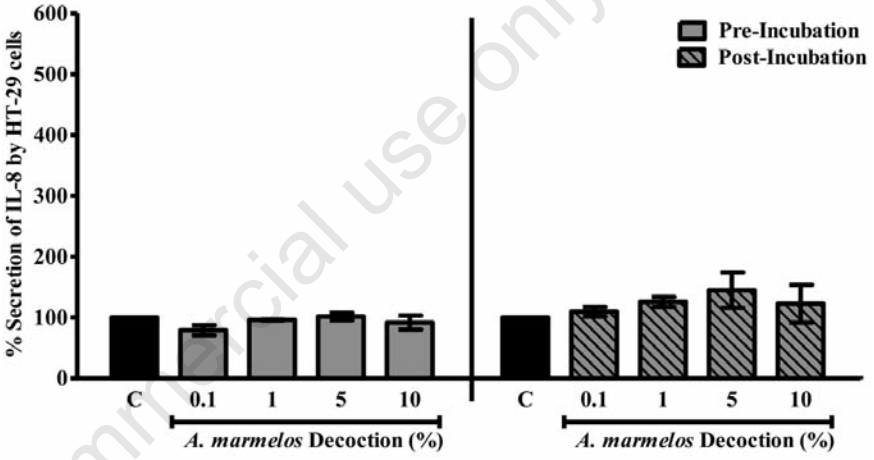

Figure 1. Effect of *A. marmelos* on IL-8 secretion by HT-29 cells in response to EPEC infection in the pre-incubation and the post-incubation protocols. Values represent mean ± standard error (n=3) of percentage secretion of IL-8 by HT-29 cells in presence of different concentrations of the decoction relative to control (100%).

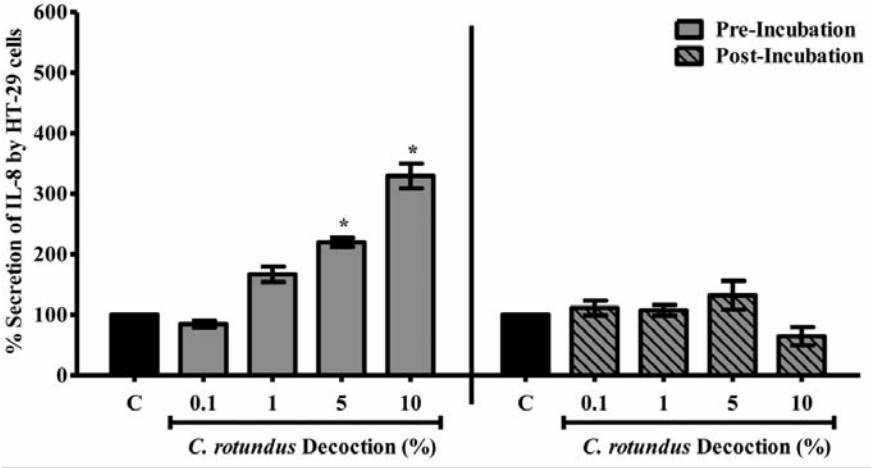

Figure 2. Effect of *C. rotundus* on IL-8 secretion by HT-29 cells in response to EPEC infection in the pre-incubation and the post-incubation protocols. Values represent mean ± standard error (n=3) of percentage secretion of IL-8 by HT-29 cells in presence of different concentrations of the decoction relative to control (100%). * P<0.05.

activity, *e.g.* curcumin;[37] and ii) inhibition of MAPK activation, *e.g. Punica granatum* fruit extract.[38] It is known that different polyphenolic compounds influence different molecular targets and pathways to exhibit their anti-inflammatory activities.[39] Though polyphenolic compounds such as tannins and flavonoids were detected in the crude decoctions of all four plants, only the decoction of *P. guajava* leaves inhibited IL-8 secretion by HT-29 cells.

Since EPEC is a non-invasive pathogen, the activation of NF-κB has been linked to soluble factors secreted or shed by the pathogen or translocated type three secretory system-dependent effectors.[9] Much of the pathology of EPEC infection which is responsible for triggering IL-8 release from epithelial cells has been linked to flagellin, the flagellar structural protein. Flagellin interaction with Toll-like receptors (TLR)-5 results in activation of the NF-κB which in turn promotes the expression of IL-8.[9] The increase in IL-8 secretion by HT-29 cells in the presence of the decoctions of *C. rotundus* and *P. guajava* in the pre-incubation protocol may be due to components that are similar to flagellins structurally and/or functionally which may be interacting with these receptors and inducing expression of IL-8 mRNA. The effect could be similar to that observed with green tea extract (GTE) which was reported to induce *de novo* synthesis of IL-8 in Caco-2 cells.[40] Interestingly, Netsch *et al.* reported that GTE, while inducing expression of IL-8 mRNA, specifically inhibited its extracellular secretion.[40] Similarly, the decoction of *P. guajava*, in addition to components that induce expression of IL-8 mRNA, may also contain constituents that may be specifically inhibiting the extracellular secretion of IL-8. This could probably be the reason for the inhibition of IL-8 secretion observed in the post-incubation protocol even when IL-8 expression is induced. However, no decrease in extracellular secretion of IL-8 in the pre-incubation protocol following induction of IL-8 mRNA expression could be due to the removal of the decoction from the assay system prior to addition of EPEC supernatant resulting in washing off the components responsible for inhibiting the extracellular secretion of IL-8.

It is interesting to note that the *P. guajava* decoction had a biphasic effect on expression of IL-8 mRNA in the pre-incubation protocol. There was a dose dependent increase in secretion of IL-8 with maximum secretion at a 5% concentration. However, at a 10% concentration of decoction the IL-8 secretion was lesser than that at 5%, though it was still much higher than the control. The observed biphasic activity may probably be due to possible interaction between constituents of the crude extract. Such biphasic activities of plant constituents have been reported previously.[41-44] However, as explained before, since the secretion of IL-8 was specifically inhibited by certain plant constituents in the post-incubation protocol in a dose dependent manner, the biphasic effect on expression of IL-8 mRNA was not apparent.

It is hypothesized that in terms of clinical relevance the results of the pre-incubation protocol indicate a preventive effect of the plant decoctions on the host whereas the post-incubation protocol indicates the effect of the plant decoctions when used as treatment. The results, therefore, indicate that amongst the plants tested, while none of them may be effective as preventive measures, *P. guajava* can be used for the treatment of inflammatory diarrheal episodes. Our previous studies with these four plants, however, have demonstrated their efficacy against other parameters of infectious forms of diarrhea, such as colonization of epithelial cells, and production and action of enterotoxins[15-18] which still render them effective for treatment of diarrhea.

In conclusion, it may be stated that of the four antidiarrheal medicinal plants used in the present study, only *P. guajava* is effective in controlling the acute inflammatory response of the intestinal epithelial cells in response to EPEC infection.

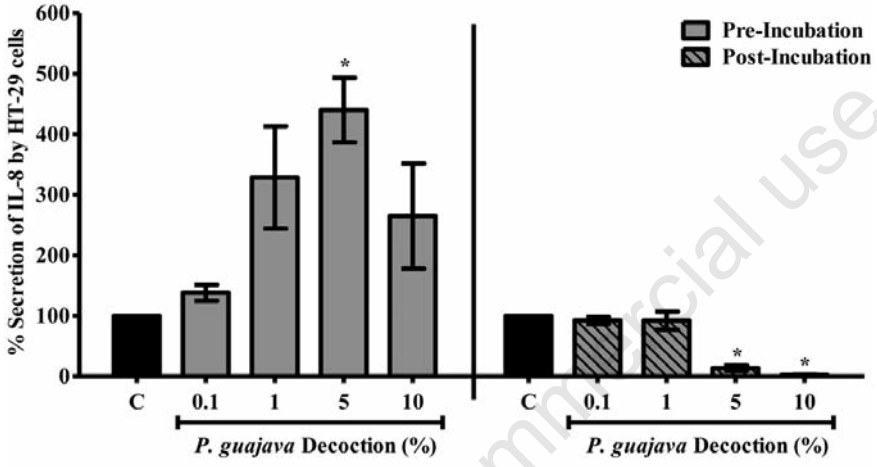

Figure 3. Effect of *P. guajava* on IL-8 secretion by HT-29 cells in response to EPEC infection in the pre-incubation and the post-incubation protocols. Values represent mean ± standard error (n=3) of percentage secretion of IL-8 by HT-29 cells in presence of different concentrations of the decoction relative to control (100%). * P<0.05

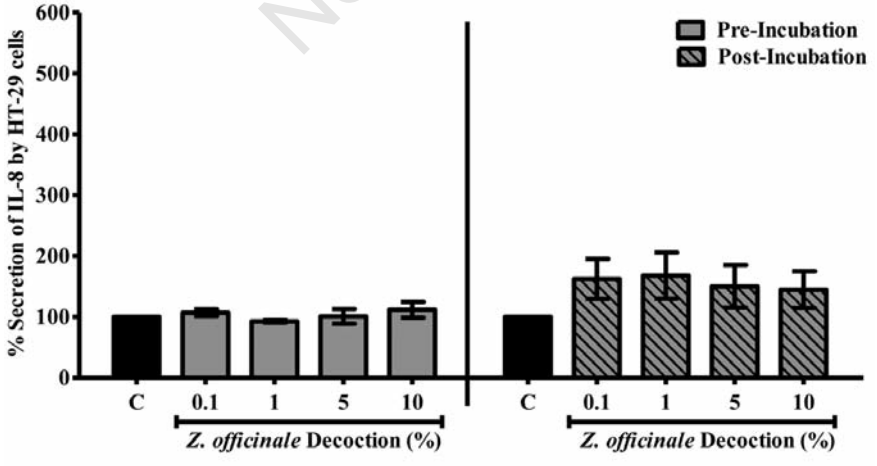

Figure 4. Effect of *Z. officinale* on IL-8 secretion by HT-29 cells in response to EPEC infection in the pre-incubation and the post-incubation protocols. Values represent mean ± standard error (n=3) of percentage secretion of IL-8 by HT-29 cells in presence of different concentrations of the decoction relative to control (100%).

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