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*Original Article*

# Bacteriostatic effect of *Echeveria* extracts on diarrheagenic *E. coli* pathotypes and non-cytotoxicity on human Caco-2 cells

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## Abstract

Introduction: Diarrheagenic *Escherichia coli* pathotypes are important aetiological agents of diarrhoeal illness among children from less developed areas, worldwide. Diarrheagenic *E. coli* pathotypes strains are increasingly becoming drug resistant, thus effective and accessible therapeutic alternatives are required for their treatment; herbal extracts may be a potential alternative. Aims: to evaluate *Echeveria craigiana, E. kimnachii*, and *E. subrigida* methanol extracts antibacterial effect on six diarrheagenic *E. coli* reference strains and on human colorectal adenocarcinoma cells viability and cytokine production.

Methodology: Diarrheagenic *E. coli* pathotypes reference strains: typical enteropathogenic E2348/69, enterotoxigenic H10407, enterohaemorrhagic O157:H7/EDL933, enteroinvasive E11, diffusely adherent C18451-A, and enteroaggregative 042 *E. coli*. *E craigiana, E. kimnachii*, and *E. subrigida* leaves, collected at Sinaloa, Mexico, were freeze-dried and macerated in methanol solvent. Antibacterial activity was determined by a novel method developed in our laboratory, bacterial oxygen consumption by polarographic oxygen electrode technique and membrane integrity by two methods (live/dead and protein leakage assays). Colorectal adenocarcinoma cells viability by MTT assay and cytokine production using a Cytometric Bead Array kit.

Results: Extracts concentrations of 100 µg/mL and 5-hour incubation, reduced more than 93% the growth of all diarrheagenic *E. coli* pathotypes tested strains and significantly decreased bacterial oxygen consumption, like bacteriostatic antibiotics. After 24-hour incubation methanol extracts had a differential antibacterial effect on each diarrheagenic *E. coli* pathotypes strain. *Echeveria* extracts did not have any effect on viability and cytokine production of colorectal adenocarcinoma cells.

Conclusions: *Echeveria* methanol extracts have a bacteriostatic effect on all diarrheagenic *E. coli* pathotypes strains, thus potentially they could be used as antibacterial agents on diarrheagenic *E. coli* pathotypes-contaminated products and on patients with diarrheagenic *E. coli* pathotypes infections.

**Key words:** Diarrheagenic *E. coli* pathotypes; *Echeveria* spp.; methanol extracts; antibacterial effect; non-cytotoxicity; Caco-2 cells.

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## Introduction

Diarrhoeal diseases are an important cause of morbidity and mortality among children under five years old in less developed areas of Latin America, Asia, and sub-Saharan Africa [1-3]. The diarrhoeal syndrome is considered the second cause of morbidity and mortality among Mexican children aged less than five years and from 1 to 4 years old, respectively [4].

Diarrheagenic *Escherichia coli* pathotypes (DEP) are one of the main aetiological diarrhoeal agents among children and travellers visiting developing countries (traveller's diarrhoea) worldwide [5-7]. Furthermore, some of these pathotypes have been associated with 2.9 to 6.7% of deaths among children < 5 years, particularly in low-income and middle-income countries [8]. DEP are *E. coli* strains that have acquired diverse virulence factors by horizontal gene transfer. Based on these factors and their pathogenic mechanisms, DEP have been classified into six pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin-producing *E. coli* (STEC), which encompasses the

enterohaemorrhagic *E. coli* (EHEC) group. Except for EIEC strains, all other DEP strains are not invasive. DEP infections have been associated with acute and persistent diarrhoea (> 14 days), growth delays, and stunting that in turn can lead to long-term cognitive impairment [9-12]. It has been reported that DEP strains in Mexico are the main aetiological agents of acute diarrhoea in children requiring hospitalization, above rotavirus, *Salmonella enterica*, and *Shigella* spp. [6].

Although bacterial gastroenteritis is initially treated mainly through oral rehydration therapy (ORT) and nutritional support, ORT does not reduce DEP shedding or duration and severity of DEP illness. Therefore, antimicrobial therapy may be of value for some DEP diarrhoeal episodes. Antimicrobial resistance and multidrug resistance DEP strains (including to antibiotics commonly used to treat bacterial diarrhoea illness) have been isolated from children with diarrhoea, worldwide [13-17]. Furthermore, annual deaths due to antimicrobial resistance is projected to reach as high as 10 million by the year 2050 worldwide [18]. Consequently, effective and accessible therapeutic alternatives for the treatment of drug resistance bacteria are required, hence natural products like plant extracts could be a good therapeutic alternative. Antibacterial activity of plant extract is usually tested *in vitro*, using rich bacterial growth media. However, testing the antibacterial effect of plant extracts on bacterial growth into media culture for human cells is necessary, since their virulence factors are activated under these conditions [19]. Furthermore, it is also important to determine the cytotoxicity effects of plant extract on human cells (using cell lines) and toxicity on animal models, to provide preliminary scientific evidence whether they are safe or not for human treatment.

Traditional herbal medicines have been widely used to treat infectious diseases, because they contain antimicrobial compounds that protect against pathogens. Mesoamerican pre-Hispanic cultures describe the use of plants and herbs, including *Echeveria* species, a genus endemic from Mexico, in the treatment of innumerable diseases [20]. *E. leucotricha*, *E. craigiana*, *E. kimnachii*, and *E. subrigida* methanol extracts have shown antibacterial activity *in vitro* against several bacterial strains [21-23], but so far have not been tested on DEP isolates. The aims of this study were: 1) to develop a novel method for testing antibacterial activity, 2) to use this method to evaluate the antibacterial activity of methanol extracts of *E. craigiana, E. kimnachii,* and *E. subrigida* on six different DEP reference strains, 3) to determine based on their mechanism of action, to which class, bactericidal or bacteriostatic, *Echeveria* methanol extracts belong, and 4) to determine the effect of the three *Echeveria* methanol extracts on Caco-2 cell viability and cytokines production.

## Methodology

*Plant material and collection sites*

*Echeveria craigiana* was collected at "El Zapote" community, Choix, Sinaloa, located at 1050 meters above sea level (MASL), 26°46'03" N, 108°08'34" W; *Echeveria kimnachii,* at south of the "Estancia de los García", Culiacan, Sinaloa, at 450 MASL, 24°21'45" N, 107°01'05" W; while, *Echeveria subrigida,* near "El Palmito" town, Concordia, Sinaloa, at 2000 MASL, 23°34'06" N, 105°50'53" W. Preserved voucher specimens or exsiccata of *E. craigiana, E. kimnachii,* and *E. subrigida* make part of the permanent collection of the Agronomy Faculty herbarium, Autonomous University of Sinaloa.

*Bacterial strains*

Reference strains of each diarrheagenic *E. coli* pathotype were included in this study, from the Molecular Biomedicine Department *E. coli* collection. ETEC H10407 (O78:H11:K80) was recovered from faeces of a Bangladeshi adult, with cholera-like symptoms [24]. Typical EPEC E2348/69 (O127:H6), EAEC 042 (O44:H18), and DAEC C1845 (O75:NM), all three strains were isolated from diarrheagenic faeces of children from United Kingdom, Peru, and United States, respectively [25-27]. EIEC E11 (O124:NM), also registered as CDC EDL 1284 (929-78), was isolated from human faeces, in the United States [28]. *E. coli* O157:H7 strain EDL933 (ATCC 43895) was recovered from ground beef, during an EHEC human outbreak in the United States [29].

*Preparation of Echeveria leaves methanol extracts*

The leaves moisture content of *E. craigiana, E. kimnachii,* and *E. subrigida* was 95.43%, 96.43%, and 94.70%, respectively. Methanol extracts of each *Echeveria* species were prepared by maceration [22]. Briefly, 20 g of powdered freeze-dried leaves (freeze dryer machine, VirTis Company, New York, US) were mixed with 400 mL of methanol (1:20 w/v), macerates were incubated at 37 °C with shaking at 150 rpm for three days. Extracts were concentrated using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) and then freeze-dried. The yield of dried extract (w/w) was 26.02%, 40.02%, and 18.57% for *E. craigiana, E. kimnachii,* and *E. subrigida*, respectively.

*Development of an antibacterial activity method*

The novel developed method encompasses a tube dilution assay, bacteria growth until mid-log phase in DMEM-HG (Dulbecco's Modified Eagle Medium-High Glucose) medium, and CFU (colony forming units) counts by the drop plate technique, which was designated as the tube dilution bacterial-mid-log phase method (TUDBAL).

Bacterial culture in Dulbecco's Modified Eagle Medium-High Glucose

Bacteria were streaked onto MacConkey agar and incubated for 18 hours at 37 °C. Next day half of a colony of each reference strain was taken and placed into 1 mL of injectable water, boiled, and placed on ice. Bacterial lysates were characterized by two multiplex PCR for the identification of characteristic loci of each DEP strain [30,31]. The remaining half colony was resuspended in 3 mL of Luria-Bertani (LB) broth (Conda Laboratories, Madrid, Spain) and incubated overnight at 37 °C under static conditions. Then 250 µL of the overnight bacterial culture was inoculated into 4.75 mL of DMEM-HG (Sigma-Aldrich, St. Louis, US) and incubated until mid-log phase. The bacterial enumeration (CFU/mL) was carried out by the drop plate technique. Briefly, each hour 100 µL of bacterial suspension was taken, and then ten-fold serial dilutions in 0.85% sterile saline solution (SS) were done up to a dilution factor of $10^{-6}$ by triplicate. Tryptic soy agar (TSA; MCD LAB, Oaxaca, Mexico) plates were divided into six sections by drawing a line with a marker on the bottom of the plate, then 10 µL of each $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions (by triplicate) were dispensed onto in each section of the tryptic soy agar plate [32]. After the drops on the agar were absorbed, the plates were incubated at inverted positions. Enumeration of DEP viable bacteria were done after 18-20 hours at 37 °C. Total counts of CFU of duplicate experiments were averaged, the total count was scaled up and the viable bacteria counts were expressed as CFU/mL.

Extracts antibacterial activity on DEP refence strains

The antibacterial activity of each *Echeveria* extract was evaluated against all six DEP reference strains by the novel TUDBAL method. One mL of each bacterial culture at mid-log phase (as above) was washed twice in SS, the pellet was resuspended in 1 mL of SS and bacterial concentrations were determined by a spectrometer (BIO-RAD SmartSpec 3000, Hercules, US) at a wavelength of 600 nm. All six bacterial concentrations were adjusted to $6 \times 10^3$ CFU/mL with DMEM-HG medium. Fifty µL of bacterial suspension were taken and added to polypropylene tubes (1.5 mL) containing 50 µL of *Echeveria* spp. extracts at concentrations of 2, 20, and 200 µg/mL in 0.1% dimethyl sulfoxide (DMSO), by duplicate and incubated at 37 °C, for 1.25, 2.5, and 5 hours; tubes containing 50 µL of 0.1% DMSO or DMEM-HG and 50 µL of bacterial suspension were used as negative controls. Then, cultures were centrifuged at 10,000 rpm/1 minute, bacterial pellets were resuspended in 1 mL of SS and bacterial enumeration was done as previously described. Also, the antibacterial effect of the extracts at concentrations of 100 µg/mL, was evaluated at 24 hours, using the protocol previously described.

*Echeveria spp. methanol extracts effect on bacterial membrane integrity*

After treatment with *Echeveria* extracts, DEP strains membrane integrity was evaluated by two methods. Briefly, mid-log phase of each DEP strain was harvested by centrifugation at 10,000 rpm/1 minute, washed with SS, and bacterial concentrations were determined, as previously described, to obtain 6 x $10^6$ CFU/mL of DMEM-HG. Then, to three tubes containing 250 µL of each *Echeveria* methanol extract at a concentration of 200 µg/mL, 250 µL of each DEP suspension ($6 \times 10^6$ CFU/mL) was added and incubated at 37 °C for 5 hours. After incubation, bacterial suspensions were centrifuged (10,000 rpm/1 minute) and the protein concentration of all supernatants was determined by Bradford dye-binding method (Bio-Rad protein assay, Hercules, US). Bacteria pellets were washed and resuspended in 400 µL of SS and incubated with 2 µL of PI (propidium iodide) at a concentration of 50 µg/mL (Biotium, Fremont, US) and 3 µL of 5 mM SYTO green (Molecular Probes®, Life Technologies, Eugene, US), for 15 min in the dark, at room temperature [33]. Flow cytometric analysis was performed by running the suspensions on a FACS Calibur cytometer (Becton Dickinson, San Diego, US), with 535 nm and 620 nm channels for SYTO green and PI fluorescence detection, respectively. Tubes containing non-heat treated and heat- treated (incubated at 80 °C for 2 minutes) bacterial suspensions, both without any *Echeveria* methanol extracts, were used as negative and positive controls, respectively. This experiment was conducted twice.

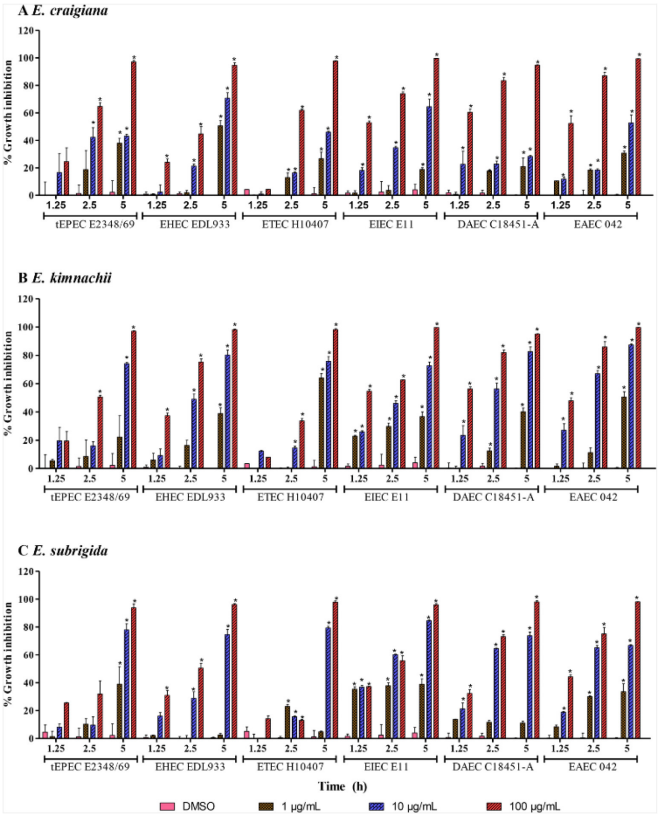
*Bacterial oxygen consumption analysis*

As previously, mid-log phase DEP bacterial were harvested by centrifugation and resuspended in

DMEM-HG medium at a final concentration of $6 \times 10^6$ CFU/mL; 5 mL of each bacterial suspension were incubated with 5 mL of *Echeveria* methanol extracts (200 µg/mL) at 37 °C for 5 hours. After treatment, bacteria were centrifuged at 4,500 rpm for 15 minutes, then pellets were resuspended in 200 µL of 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) pH 7.4, finally bacterial suspension protein concentrations were determined by Biuret method at 540 nm in a spectrophotometer (Beckman Coulter, Brea, US).

Bacterial oxygen consumption rates were measured by the polarographic oxygen electrode technique, using an oxygen meter (model 782, Warner/Strathkelvin Instruments, North Lanarkshire, Scotland) with a Clark electrode, at 37 °C, as previously described [34]. The reaction was performed in a water-jacketed chamber, containing 1 mL of an alive bacterial suspension in MES buffer with 10 mM glucose, which corresponds to a bacterial protein concentration of 5 mg. After three minutes, the reaction was stopped by adding cyanide (200 µM), a respiratory chain inhibitor, to block oxygen consumption.

**Figure 1.** Effect of different concentrations and time curse of *Echeveria* methanol extracts on diarrheagenic *Escherichia coli* pathotypes (DEP) growth (initial concentration 6 x $10^3$ CFU/mL).




Percentage of growth inhibition of typical enteropathogenic (tEPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), diffusely-adherent (DAEC) and enteroaggregative (EAEC) *E. coli* by methanol extracts of *E. craigiana* (A), *E. kimnachii* (B), and *E. subrigida* (C), at 1, 10, 100 µg/mL, after incubation for 1.25, 2.5, and 5 h at 37 ºC. Results are the percentage mean ± SEM (n= 6 per group) of growth inhibition calculated with respect to DEP growth, without treatment, in DMEM-HG. * $p < 0.05$ statistically significant compared to the control (DMEM-HG).

*Human colorectal Caco-2 cell viability assay*

Cell cytotoxicity was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [35]. Briefly, Caco-2 cells were seeded in 96-well plates (Corning, New York, US) at a concentration of $2 \times 10^4$ cells/mL in DMEM-HG supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Eugene, US) and 1% of antibiotic-antimycotic solution (10,000 U/mL penicillin, 10 mg/mL streptomycin, 25 µg/mL amphotericin B; Sigma-Aldrich, St. Louis, US). *Echeveria* methanol extracts, at concentrations of 1, 10, and 100 µg/mL were prepared using antibiotic-free DMEM-HG 0.05% DMSO solution. Cells were grown for 48 hours, washed three times with phosphate buffered saline (PBS) pH 7.4, PBS was removed by "flicking" and dabbing out the residual PBS, then to three wells 100 µL of each *Echeveria* extracts, at concentrations of 1, 10, and 100 µg/mL, or vehicle (antibiotic-free DMEM-HG containing 0.05% DMSO), were added and incubated for 3 or 24 hours, in 5% $CO_2$, at 37 °C. The culture medium was discarded and 100 µL of 0.5 mg/mL MTT (Sigma-Aldrich, St. Louis, US) reagent added under dim light. The plate was incubated for 4 hours at 37 °C in the dark. The crystal product formed in each well was dissolved in 100 µL of DMSO and absorbance was determined at 540 nm using a microplate reader (Multiskan Bichromatic, Thermo Fisher Scientific, Waltham, US).

*Cytokine production by human colorectal Caco-2 cells*

Caco-2 cells were seeded ($2 \times 10^4$ cells/well in DMEM-HG supplemented with 10% fetal bovine serum) in 24-well plates (Corning, New York, US) and incubated to obtain an 80% of confluence. Cells were then treated with 200 µL of each methanol extract (1, 10, and 100 µg/mL) or vehicle (antibiotic-free DMEM-HG containing 0.05% DMSO) and incubated for 1, 3, and 6 hours at 37 °C, in 5% $CO_2$, assays were done by triplicate. After incubations, culture supernatants were collected and centrifuged at 14,000 rpm for 5 minutes and cytokines (IL-8, TNF-α, IL-1β, IL-6, IL-10, and IL-12p70) were quantitatively measured by FACS (BD FACSCalibur cytometer, San Diego, US) using a

human inflammatory cytokines kit (BD Cytometric Bead Array, Biosciences Pharmingen, San Diego, US), following manufacturer's instructions. The quantitative results were generated using the software FCAP Array™ version 3.0 (Soft Flow Hungary Ltd., Pécs, Hungary) and expressed as pg/mL.

*Statistical analysis*

Data were expressed as mean ± standard error. One-way analyses of variance and Dunnet or Tukey test were performed to determine significant differences ($p < 0.05$, two tailed) among groups on GraphPad Prism version 5 (San Diego, US).

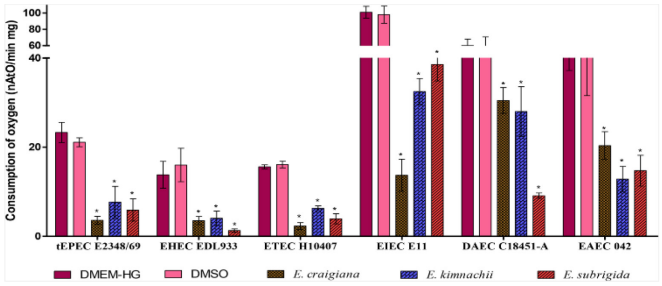
## Results

*Antibacterial activity of Echeveria spp. methanol extracts evaluated by TUDBAL method*

The antibacterial effect of *E. craigiana*, *E. kimnachii*, and *E. subrigida* methanol extracts on DEP strains, was evaluated at three different concentrations 1, 10, and 100 µg/mL of methanol extracts and incubation times of 1.25-, 2.5-, and 5-hours at 37 °C. As illustrated in Figure 1, all three *Echeveria* extracts have the most significantly antibacterial effect on the six tested DEP reference strains, at concentrations of 100 µg/mL and 5-hour incubation time, and bacterial percentage growth inhibition ranging from 94.5% to 99.7%. E.g., under these condition *E. craigiana* and *E. kimnachii* extracts inhibited the growth of tEPEC 97% while *E. subrigida* extract 93.7%, in contrast, *E. subrigida* extract inhibited DAEC growth by 98% *E. craigiana* and *E. kimnachii* extracts ≈ 95%. However, a heterogenous effect was observed on the reduction on DEP growth (ranging from 28.3-87.4%), after 5-hour incubation with 10 µg/mL of the *Echeveria* methanol extracts (Figure 1).

In contrast, when DEP reference strains were incubated with 100 µg/mL of *Echeveria* methanol extracts for 24-hour, a differential antibacterial effect of each *Echeveria* extracts on each DEP strains were observed (Table 1). E.g., *E. subrigida* methanol extract reduced more than 92% the growth of five DEP strains, whereas *E. craigiana* methanol extract had a differential reduction on the growth of four DEP strains ranging from 39.12% to 99.99%. *E. kimnachii* methanol extract only significantly reduced the growth of EIEC and ETEC. Moreover, neither of the three *Echeveria* extracts inhibited DAEC growth after 24-hour incubation time.

**Figure 2.** Rate of oxygen consumption by diarrheagenic *Escherichia coli* pathotypes after incubation with *Echeveria craigiana*, *E. kimnachii*, and *E. subrigida* methanol extracts.




Oxygen consumption by typical enteropathogenic (tEPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), diffusely-adherent (DAEC) and enteroaggregative (EAEC) *E. coli*, at initial concentration 6 x $10^6$ CFU/mL, treated with 100 µg/mL of methanol extracts of *E. craigiana*, *E. kimnachii*, and *E. subrigida* and untreated (dimethyl sulfoxide, DMSO, and DMEM-HG), after incubation for 5 hours at 37 ºC. Data are expressed as mean ± SEM (n = 4 per group). * $p < 0.05$, significant difference compared to the control (DMEM-HG).

*Effect of Echeveria species methanol extracts on DEP oxygen consumption, membrane integrity and bacterial death*

The effect of the three *Echeveria* methanol extracts on DEP oxygen consumption rates was similar at concentrations of 100 µg/mL and 5-hour incubation time at 37 °C, since in comparison with untreated DEP strains, a significant 3- to10-fold reduction in the rate of oxygen consumption was observed on DEP strains treated with *Echeveria* extracts (Figure 2). In contrast, non-cytotoxic effects were observed on DEP strains after treatment with *Echeveria* extracts, based on

**Table 1.** Effect of 100 µg/mL of *Echeveria* methanol extracts on diarrheagenic *Escherichia coli* pathotypes (DEP) growth (initial concentration 6 x $10^3$ CFU/mL) after incubation for 24 hours at 37 ºC.

| Treatment | % Growth inhibition | | | | | |
|---|---|---|---|---|---|---|
| | EPEC | EHEC | ETEC | EIEC | DAEC | EAEC |
| *E. craigiana* | 0.00 ± 0.00 | 39.12 ± 9.70* | 67.94 ± 20.09* | 99.99 ± 0.01* | 0.00 ± 0.00 | 45.73 ± 12.03* |
| *E. kimnachii* | 0.00 ± 0.00 | 0.00 ± 0.00 | 99.90 ± 0.22* | 98.97 ± 1.35* | 2.15 ± 2.00 | 8.78 ± 7.50 |
| *E. subrigida* | 99.93 ± 0.09* | 99.97 ± 0.02* | 99.99 ± 0.01* | 93.46 ± 4.03* | 17.00 ± 10.52 | 92.93 ± 7.25* |

Growth inhibition percentage of typical enteropathogenic (tEPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), diffusely-adherent (DAEC) and enteroaggregative (EAEC) *E. coli* by methanol extracts of *E. craigiana*, *E. kimnachii*, and *E. subrigida*. Results are the percentage mean ± SEM (n = 6 per group) of growth inhibition calculated with respect to the bacterial growth on DMEM-HG. * $p < 0.05$, statistically significant compared to the control (DMEM-HG).

**Table 2.** Effect of *Echeveria craigiana*, *E. kimnachii*, and *E. subrigida* methanol extracts on diarrheagenic *E. coli* pathotypes membrane integrity and viability, after incubation for 5 hours at 37 °C.

| Treatments | *Escherichia coli* strains | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | tEPEC E2348/69 | | EHEC EDL933 | | ETEC H10407 | | EIEC E11 | | DAEC C18451-A | | EAEC 042 | |
| | MI | PI | MI | PI | MI | PI | MI | PI | MI | PI | MI | PI |
| DMEM-HG | 2.50 ± 0.31 | 95.16 ± 0.18 | 1.24 ± 0.06 | 94.00 ± 0.20 | 1.40 ± 0.28 | 95.66 ± 0.27 | 1.28 ± 0.22 | 97.90 ± 0.80 | 1.87 ± 1.24 | 94.04 ± 0.85 | 4.33 ± 0.46 | 95.94 ± 1.37 |
| DMSO | 2.44 ± 0.22 | 95.57 ± 1.10 | 1.17 ± 0.20 | 94.13 ± 1.56 | 1.35 ± 0.15 | 94.52 ± 1.55 | 1.61 ± 0.13 | 97.54 ± 0.91 | 1.63 ± 0.92 | 90.01 ± 6.70 | 4.23 ± 0.17 | 96.23 ± 1.16 |
| *E. craigiana* | 1.78 ± 0.64 | 97.29 ± 2.31 | 2.43 ± 0.81 | 98.51 ± 0.83 | 1.43 ± 0.41 | 99.46 ± 0.41 | 0.20 ± 0.09* | 99.63 ± 0.11 | 1.83 ± 0.95 | 98.48 ± 0.51 | 2.94 ± 0.55 | 98.40 ± 1.06 |
| *E. kimnachii* | 1.91 ± 0.89 | 98.50 ± 0.75 | 1.87 ± 0.67 | 96.76 ± 0.60 | 1.97 ± 0.98 | 96.49 ± 0.40 | 0.31 ± 0.01* | 99.45 ± 0.15 | 1.53 ± 1.27 | 97.09 ± 1.11 | 1.99 ± 0.38 | 97.84 ± 1.45 |
| *E. subrigida* | 1.97 ± 0.71 | 96.97 ± 0.39 | 2.76 ± 0.94 | 96.46 ± 0.90 | 1.75 ± 0.68 | 95.63 ± 1.92 | 0.54 ± 0.15* | 97.57 ± 0.17 | 1.80 ± 1.09 | 89.01 ± 5.95 | 2.05 ± 1.63 | 95.08 ± 2.84 |
| Heat | 8.23 ± 3.01* | 8.95 ± 3.72* | 8.08 ± 0.64* | 9.30 ± 3.48* | 5.58 ± 2.21* | 9.28 ± 3.55* | 5.02 ± 0.03* | 2.73 ± 0.25* | 9.34 ± 0.21* | 13.52 ± 8.48* | 10.02 ± 0.81* | 9.54 ± 6.27* |

The membrane integrity (MI) and viability of all six pathotypes (typical enteropathogenic (tEPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), diffusely-adherent (DAEC) and enteroaggregative (EAEC) *E. coli*) at initial concentration 6 x $10^6$ CFU/mL was determined by evaluating the amount of protein present on the supernatants and propidium iodine (PI) technique, respectively, after treatment with *Echeveria* spp. methanol extracts (*E. craigiana*, *E. kimnachii*, and *E. subrigida*), and without treatment in DMEM-HG and DMSO, after 5 hours/37 °C. Results are the mean ± SEM (n = 6 per group), * $p < 0.05$ statistically significant compared to the control (DMEM-HG).

bacterial viability (evaluated by propidium iodide) and membrane integrity (evaluated by protein concentration in the supernatant) assays (Table 2).

*Effect of Echeveria species methanol extracts on human Caco-2 cells viability and cytokine production*

As illustrated in Figure 3, the three *Echeveria* methanol extracts at concentrations of 1, 10, and 100 µg/mL were found to be non-cytotoxic towards the Caco-2 cells after incubation times of 3- and 24-hours at 37 °C.

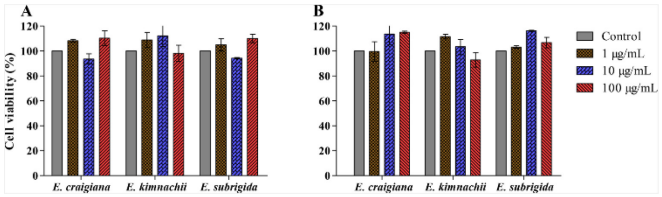
Culturing Caco-2 cells with 1, 10, and 100 µg/mL of the three different *Echeveria* methanol extracts or untreated Caco-2 cells, for 1-, 3-, and 6-hours at 37 °C, did not induce cytokine production of TNF-α, IL-1β, IL-6, IL-10, and IL-12p70, since all samples were under the kit limit of detection. On the other hand, Caco-2 cells cultured with and without the *Echeveria* methanol extracts induced similar IL-8 production (Table 3).

## Discussion

It has been suggested that plant extracts with antibacterial activities at concentration of 100 µg/mL or below can potentially be used as antibacterial agents [36]. In accordance, the three *Echeveria* methanol extracts, at concentrations of 10 and 100 µg/mL, had antibacterial activity against all DEP reference strains after 5-hour incubation at 37 °C, observing the highest bacterial growth inhibition percentage at 100 µg/mL.

It was concluded that *Echeveria* methanol extracts have a bacteriostatic effect rather than a bactericidal effect, based on the significantly decreased of oxygen

**Figure 3**. Effect of three concentrations of *Echeveria craigiana*, *E. kimnachii*, and *E. subrigida* methanol extracts (1 µg/mL, 10 µg/mL and 100 µg/mL) on Caco-2 cell growth at 3 hours (A) and 24 hours (B).




Data is expressed as mean ± SEM (n = 6 per group).

**Table 3.** Effect of *Echeveria* methanol extracts on interleukin-8 production by Caco-2 cells.

| Treatment | µg/mL | IL-8 (pg/mL) | | |
|---|---|---|---|---|
| | | 1 h | 3 h | 6 h |
| DMEM-HG | | 6.68 ± 3.29 | 6.73 ± 1.51 | 5.71 ± 1.25 |
| DMSO | | 8.23 ± 3.91 | 7.69 ± 1.45 | 5.91 ± 1.92 |
| *E. craigiana* | 1 | 5.87 ± 1.61 | 7.24 ± 1.59 | 8.78 ± 3.16 |
| | 10 | 1.99 ± 1.83 | 3.57 ± 1.28 | 9.50 ±1.96 |
| | 100 | 1.87 ± 1.64 | 4.84 ± 4.13 | 4.84 ± 3.60 |
| *E. kimnachii* | 1 | 4.26 ±1.65 | 8.22 ± 3.22 | 5.33 ± 0.75 |
| | 10 | 2.52 ± 2.36 | 3.85 ± 0.61 | 8.28 ± 2.53 |
| | 100 | 1.92 ±1.85 | 2.93 ±2.07 | 5.05 ± 3.52 |
| *E. subrigida* | 1 | 4.38 ±0.33 | 7.61 ±1.53 | 6.48 ± 4.46 |
| | 10 | 3.07 ±1.01 | 6.71 ± 1.47 | 6.50 ± 1.96 |
| | 100 | 3.39 ± 2.73 | 3.32 ± 2.95 | 3.93 ± 2.47 |

Data are expressed as mean ± SEM (n = 6 per group). One-way ANOVA and Dunnet post-test were performed.

consumption and maintenance of membrane integrity and permeability of all tested DEP strains, after incubation with the three *Echeveria* methanol extracts (at concentrations of 100 µg/mL, for 5 hours). It has been demonstrated that bacteriostatic antibiotics decrease oxygen consumption, while bactericidal antibiotics increase it [37,38]. On the other hand, bactericidal antibiotics also damage the bacterial membrane, resulting in both a very high number of propidium iodide-positive bacteria and increased concentrations of bacterial proteins in the supernatant [37,38]. The potential use of these *Echeveria* extracts in DEP-treatment is substantiated by the fact that the bacteriostatic sulfamethoxazole-trimethoprim (co-trimoxazole) is the antibiotic of choice for treatment of severe cases of DEP infection, in less developed regions of the world [39]. Furthermore, bacteriostatic compounds are the antibiotic choice for treatment of EHEC or STEC infections; since the use of bactericidal antibiotics, particularly β-lactams, for their treatment have been associated with the subsequent development of haemolytic uremic syndrome [40].

It is important to mention that the novel method (TUDBAL) allowed to establish for the first time the antibacterial effect of plant extracts on fast-growing bacteria as *E. coli,* at short incubation times. The TUDBAL method, in comparison with most methods to evaluate the antibacterial activity of extracts [22,23,41,42], has several advantages: 1) bacteria are growth in cell culture media that activate virulence factors mimicking the human physiological condictiones, 2) bacteria are evaluated in mid-log phase, synchronizing their growth, thus extracts antibacterial effect can be evaluated at short periods of time, less than 24 hours, plus CFU counts are highly reliable and reproducible, and 3) CFU counts was determined by the drop plate technique which is less time consuming and less expensive than traditional CFU counts techniques. Furthermore, TUDBAL method could be used to evaluate the effect of plant extracts on the expression or not of bacterial virulence genes, as well [19].

After 24-hour incubation time of the six DEP reference strains with *E. subrigida, E. craigiana,* and *E. kimnachii* methanol extracts (100 µg/mL), a significantly reduction on the growth of five, four, and two tested DEPs, respectively, was observed. Suggesting that *E. subrigida* methanol extract is the best antibacterial candidate to eliminated DEP strains.

It has been well established that plant extracts antibacterial activities are due to the presence of several secondary metabolites: e.g., flavonoids, tannins, terpenes/sterol, saponins, coumarins, free anthracenics, and organic acids. So far, some of these compounds have been identified in methanol extracts of *Echeveria* species as: kaempferol-3-*O*-glucoside in *E. subrigida,* epigallocatechin gallate in *E. kimnachii,* and lupeol in both *E. craigiana* and *E. kimnachii* [43]. These compounds isolated from other plants have shown antibacterial activities against Gram-positive and Gram-negative bacteria [44-46], furthermore, other components of plants extract, as essential oils, as those from *Atalantia sessiflora,* have antibacterial and antiparasitic activities [47], but their mechanisms of action are unknown.

On the other hand, after culturing Caco-2 cells with the three *Echeveria* methanol extracts, at concentrations of 1, 10, and 100 µg/mL, were found to be non-cytotoxic towards the Caco-2 cells and their cytokine profiles were alike to untreated Caco-2 cells. *E. craigiana*, *E. kimnachii*, and *E. subrigida* methanol extracts are not cytotoxic, since plant extracts with LC50 at concentrations of 20 µg/mL or below are considered cytotoxic [48]. Furthermore, *Echeveria* methanol extracts did not induced the production of TNF-α and IL-1β cytokines that disrupt intestinal epithelial tight junctions [49,50]. In contrast, essential oils from *Lavandula* species have indeed a cytotoxic effect on Caco-2 cells, affecting cells morphology and tight junctions [51]. Moreover, IL-8 was the only cytokine produced by treated and untreated Caco-2 cells, which secretion levels were similar in both groups; supporting the observation that IL-8 is produced by Caco-2 cells even in absence of a clear stimulus [52]. Together, these results clearly highlight that, at least *in vitro*, *Echeveria* methanol extracts do not have cytotoxic effect or induce cytokines production of human intestinal epithelial cells; suggesting that *Echeveria* methanol extracts may not be harmful *in vivo.* Consequently, the three *Echeveria* methanol extracts need to be tested in acute and chronic toxicity animal models.

Fresh and ready to eat food products are the main vehicle of DEP infections, so they are one of the most common agents associated with foodborne outbreaks due to DEP, worldwide [53,54]. In Mexico, it has been reported that raw lettuce, non-pasteurized cheeses, and ready to eat foods, as raw spinach salads and chili sauces, are contaminated with DEP strains in enough quantities to cause disease [12,55-58]. The increasing number of foodborne outbreaks due to DEP, has raised awareness for interventions to eliminate DEP and other human pathogens from fresh products.

## Conclusions

Together, our results reveal that methanol extracts of *E. craigiana*, *E. kimnachii,* and *E. subrigida* have a bacteriostatic effect on tEPEC, EHEC, ETEC, EIEC, DAEC, and EAEC reference strains, non-cytotoxic effect on Caco-2 cells, and did not induce cytokine production by Caco-2 cells. Therefore, *Echeveria* methanol extracts, particularly *E. subrigida* extract, may be used as a natural antibacterial agent to remove DEP from contaminated vegetables and fruits and could be a safe alternative to treat DEP infected patients. Nevertheless, testing of *Echeveria* methanol extracts in acute and chronic toxicity animal models, is essential to provide scientific evidence whether *Echeveria* methanol extracts are safe or not for human treatment.

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