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Antibacterial Activity of *Oenothera rosea* (L 'Hér) Leaf Extracts

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Authors' Contributions

*RGF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscrip**t.** PTG managed the analyses of the study. RRM carried out the experiments and obtained his Master of Sciences degree. RQL managed the organic chemistry and the literature searches of the study. All authors read and approved the final manuscript.*

Research Article

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ABSTRACT

Aims: To determine the antibacterial effect of *Oenothera rosea* against *Escherichia coli*, *Salmonella enteritidis* and *Vibrio cholerae*.
Study Design: *In vitro* antibacterial study.
Place and Duration of Study: Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Departamento de Microbiología e Inmunología and Departamento de Química, San Nicolás de los Garza, NL. México, from June 2010 to June 2011.
Methodology: The antibacterial *in vitro* effect of methanol and aqueous extracts of the Mexican plant *O. rosea* against strains of *E. coli*, *S. enteritidis* and *V. cholerae* was evaluated in liquid medium by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay.
Results: Methanol and aqueous extracts significantly inhibited growth of all bacterium strains tested. The methanol extract caused up to 55%, 66% and 87% growth *against E. coli*, *S. enteritidis* and *V. cholerae*, respectively, whereas the aqueous extract induced up to 54%, 69% and 88% bacterial growth inhibition, respectively. Methanol and aqueous

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vehicle controls did not alter bacterial growth.
Conclusion: The observed antibacterial effect of *O. rosea* extracts may be of benefit as an adjuvant treatment of diseases caused by the studied enterobacteria.

Keywords: Oenothera rosea; antibacterial; enterobacteria; Mexican plants.

1. INTRODUCTION

Over the last decade, the interest in natural products for use in the agricultural, food and pharmaceutical industries has been renewed. Recently, scientists have focused in searching for new drugs from natural products (Cowan, 1999), since there is a continuous need to discover new antibacterial substances with diverse chemical structures and novel mechanisms of action. Another important concern is the development of antibiotics resistance in the clinics (Erturk et al., 2006) and the increase in the incidence of new and re-emerging infectious diseases, particularly, in developing countries; hence, it is necessary to provide affordable health care in these countries to a greater number of people (Goud et al., 2005).

Oenothera rosea is a Mexican plant commonly known as "hit grass", which belongs to the family *Onagraceae*. It has been traditionally used as a treatment against cough, diarrhea and skin infections (Andrade-Cetto, 2009) and it has characteristics that makes it ideal for the study of biological activity because of previous reports indicating that the plant produces phenolic compounds, flavonoids, and coumarins, which have shown cytotoxic and anti-inflammatory activity (Meckes et al., 2004), but its antibacterial potential has been only evaluated against *Neisseria gonorrhoeae* with minimum inhibitory concentrations ≥256 µg/mL (Cybulska et al., 2011). In addition, the species *Oenothera biennis* (common evening primrose) was reported to possess antibacterial activity against *Streptococcus mutans* (Matsumoto-Nakano et al., 2011).

The present study was undertaken to determine the *in vitro* antibacterial effect of *O. rosea* methanol and aqueous extracts on *E. coli, S. enteritidis* and *V. cholerae* growth. These microorganisms were selected because they are clinically relevant. *E. coli* is a major component of the normal human intestinal flora, but the enterotoxigenic, enteroinvasive, enteropathogenic and enterohemorrhagic pathotypes are frequently associated with diarrhea and other pathologies (Nataro and Koper, 1998). Although most patients may recover within 10 days, in some of them, particularly children and the elderly, the infection can be life-threatening (Coia, 1998). *Salmonella enterididitidis* is another important public health problem worldwide, particularly when eggs are eaten raw or undercooked. It can cause fever, abdominal cramps, and severe diarrhea. In the elderly, infants, and immunocompromised individuals (*Salmonella* infections are a complication in HIV-infected people) (Fernandez-Guerrero, 1997; Center for Disease Control, 1992; Cohen et al., 1987), the infection can be fatal unless antibiotic treatment is properly provided (Chiu et al., 2002). On the other hand, *V. cholerae* is the causative agent of cholera and represents a great public health problem, particularly in developing countries. It causes acute diarrheal disease and about 120,000 deaths every year (Kitaoka et al., 2011); if left untreated, the cholera death rate may reach up to 50% in a few hours to days after onset of the disease (Fournier and Quilici, 2007).

The aim of the present study was to evaluate the *in vitro* antibacterial activity of methanol and aqueous extracts of *O. rosea* against *E. coli*, *S. enteritidis* and *V.. cholerae*, which are of clinical importance.

2. MATERIALS AND METHODS

2.1 Reagents, Culture Media and Microbial Strains

Sodium dodecyl sulfate (SDS), N, N-dimethylformamide (DMF) and 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). *E. coli* (ATCC 25922) and *V.. cholera* (ATCC 25870) were obtained from the American Type Culture Collection (Rockville, MD); *S. enteritidis* was a clinically important isolate obtained from chicken crude extracts and provided by Laboratorio de Bioquímica y Genética de Microorganismos, Departamento de Microbiología e Inmunología, Facultad de Ciencias Biológicas at Universidad Autónoma de Nuevo León. Brain heart infusion (BHI) was purchased from Remel (Lenexa, KS). Extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37ºC in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7.

2.2 Preparation of *O. rosea* Leaf Extracts

The plant material used in this study was obtained from a local market in downtown Monterrey, Nuevo Leon, Mexico and was identified as *O. rosea* by M.Sci. María del Consuelo González de la Rosa, Chief of the Herbarium of the Biological Sciences College at Autonomous University of Nuevo Leon. The aerial parts of *O. rosea* were dried in an oven at 40ºC, powdered using a Moulinex blender (Goldsmith 38, Colonia Polanco, Mexico DF) and stored. To prepare the methanol extract and the vehicle control, 100 ml of 100% methanol alone (methanol vehicle control) and methanol containing 10 grams of leaves powder were allowed to stand for 24 hours at room temperature. The resulting extract and vehicle control were centrifuged at 2800 rpm for 15 minutes and supernatants were placed in 1 mL Eppendorf tubes, previously weighted, after which they were dried under vacuum using a speed-vac concentrator (Savant Instruments Inc., Hicksville, NY). To prepare the aqueous extract and the vehicle control, 100 ml boiled water alone (aqueous vehicle control) or boiled water containing 10 grams of leaves powder were allowed to stand for 10 minutes, lyophilized (Labconco corporation, KC), and stored at -20ºC until use. The methanol and the aqueous extracts and the vehicle controls were suspended in sterile culture medium and then filter-sterilized through 0.22 μ-pore size diameter filters (Millipore, Bedford, MA).

2.3 Antibacterial Activity of *O. rosea* Extracts

Aqueous and methanol *O. rosea* leaf extracts were prepared to evaluate their *in vitro* antibacterial activity. We selected *E. coli, S. enteritidis* and *V. cholerae* species because they are clinically relevant, particularly in immunocompromised individuals. The percentage of microbial growth inhibition by *O. rosea* leaf extracts in liquid medium by a colorimetric technique (Gomez-Flores et al., 1995) was determined; for this, 100 μl of *E. coli* and *S. enteritidis* cultures were placed in 10 mL brain heart infusion culture medium (Becton Dickinson, Cockeysville, MD) or 10 mL of Luria-Bertani (LB) culture medium (Difco Laboratories, Detroit, MI) for *V. cholerae* cultures, and were incubated at 37ºC for 24 hour. Aliquots of 800 μL from these culture suspensions were taken, mixed with 200 μL of sterile glycerol and frozen at -70ºC, until use.

In order to evaluate the *in vitro* antibacterial activity of *O. rosea* extracts, bacteria frozen cultures were thawed at 4ºC and then they were activated by inoculating 10 µL of the bacteria suspensions in 1 mL of BHI medium for *E. coli* and *S. enteritidis* and LB medium for *V. cholera,* and incubated at 37ºC for 24 h. Next, bacterial concentration was determined in a Neubauer hematocytometer (Fisher Scientific Co., Pittsburgh, PA) and adjusted to $1x10^3$ cells/mL. To determine the antibacterial activity of the extracts, cell viability was measured by the MTT reduction assay (Gomez-Flores et al., 2007, 1995). MTT was prepared at a concentration of 5 mg/mL and sterilized by filtering through a 0.22-µm filter (Millipore, Carrigtwahill, CO, Ireland). Fifty microliters of the microbial suspensions were plated in their specific culture media, in flat-bottomed 96-well plates (Corning Incorporated, Corning, NY), in the presence or absence of serial dilutions (1:2) of the *O. rosea* leaf methanol and aqueous extracts (50 µL), tetracycline control (3 µg/mL; Lot # R32874, Research Organics, Cleveland, OH) and vehicle controls (methanol and culture medium). The vehicle controls were similarly processed as with plant methanol and aqueous extractions, but without plant material. Plates were then incubated for 6 hour at 37ºC, after which the tetrazolium salt MTT was added to all wells at a final concentration of 0.5 mg/mL and plates were incubated for 4 additional hours. At the end of the incubation period, 50 µL of extraction buffer were added to all wells and plates were incubated overnight at 37ºC. Optical densities resulting from dissolved formazan crystals were then read in a microplate reader (Beckman Coulter, Inc., Fullerton, CA) at 570 nm.

2.4 Statistical Analysis

The results were expressed as mean ± SEM of triplicate determinations from three independent experiments. Level of significance was assessed by Dunnet's *t* test.

3. RESULTS AND DISCUSSION

3.1 Antibacterial Activity of *O. rosea* Leaf Extracts

O. rosea methanol extract caused significant 34 ± 9 ($P = .01$), 47 ± 13 ($P = .01$), 55 ± 6 ($P = .05$) and 53 ± 6 ($P = .05$) percents growth inhibition of *E. coli* at 0.5 (minimal inhibitory concentration, MIC), 1, 2 and 4 mg/mL respectively) (Fig. 1), and the aqueous extract caused significant ($P = .05$) 21 ± 3, 28 ± 3, 35 ± 7, 44 ± 7 and 54 ± 10 percents growth inhibition of *E. coli* at 0.25 (MIC), 0.5, 1, 2 and 4 mg/mL respectively) (Fig. 2). Tetracycline caused 91%, 90% and 88% growth inhibition of *E. coli, S. enteritidis* and *V. cholera*, respectively, at 3 µg/ml.

In addition, *O. rosea* methanol extract caused significant ($P = .05$) 12 ± 3, 23 ± 4, 45 ± 8, 63 ± 4, 66 ± 7, and 66 ± 6 percents growth inhibition of *S. enteritidis* at 0.125 (MIC), 0.25, 0.5, 1, 2 and 4 mg/mL respectively) (Fig. 1); the aqueous extract also caused significant ($P = .05$) 6 ± 1, 15 ± 3, 24 ± 5, 35 ± 11, 46 ± 16, and 69 ± 5 percents growth inhibition of *S. enteritidis* at 0.125 (MIC), 0.25, 0.5, 1, 2 and 4 mg/mL respectively) (Fig. 2).

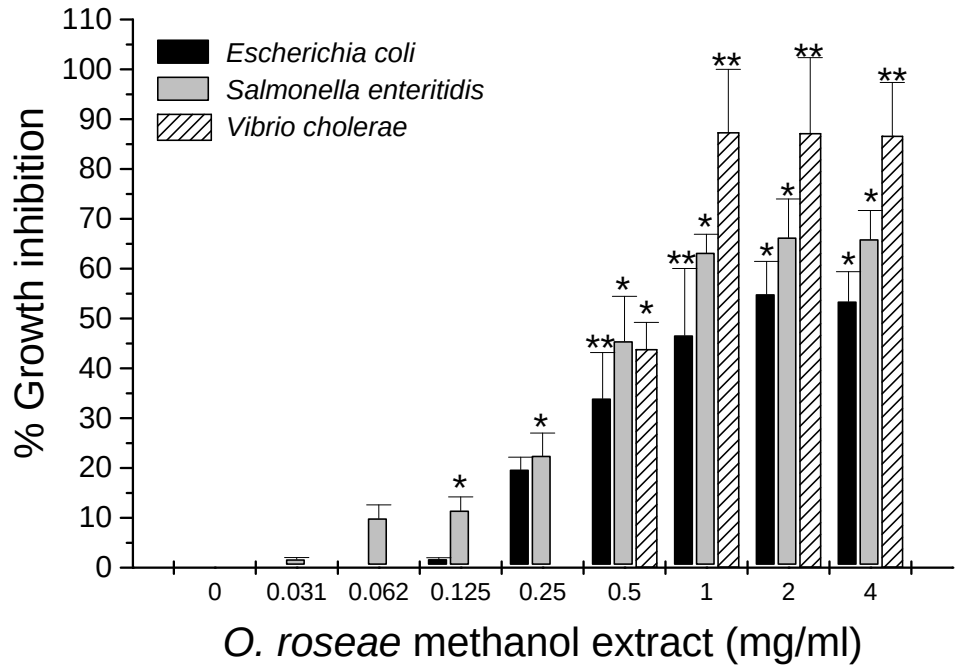

Fig. 1. Antibacterial effect of *O. rosea* methanol extract.

E. coli, S. enteritidis and V. cholerae culture suspensions were incubated in the presence or absence of various concentrations of O. rosea methanol extract, after which growth was measured colorimetrically, as explained in the text.

*Data represent means ± SEM of triplicate determinations from three independent experiments. **P = .01, *P = .05 compared with O. rosea extract-untreated control. Optical density at 570 nm for untreated cells was 1.12 ± 0.062.*

Furthermore, *O. rosea* methanol extract caused significant 44 ± 5 (*P* = .05), 87 ± 12 (*P* = .01), 87 ± 15 (*P* = .01), and 87 ± 11 (*P* = .01) percents growth inhibition of *V. cholerae* at 0.5 (MIC), 1, 2 and 4 mg/mL respectively) (Fig. 1), whereas the aqueous extract caused significant 24 ± 3 (*P* = .05), 13 ± 1 (*P* = .05), 23 ± 2 (*P* = .05), 25 ± 4 (*P* = .05), 25 ± 7 (*P* = .05), 86 ± 6 (*P* = .01), 88 ± 12 (*P* = .01), and 87 ± 11 (*P* = .01) percents growth inhibition of *V. cholerae* at 0.031 (MIC), 0.062, 0.125, 0.25, 0.5, 1, 2 and 4 mg/mL respectively) (Fig. 2). Methanol vehicle control caused not significant (*P* = 0.2) 28% *V. cholerae* and 21% *E. coli* growth inhibition only at 1mg/ml (Fig. 3), as compared with culture medium alone; aqueous vehicle control did not alter bacterial growth.

Medicinal plants have become part of alternative medicine worldwide because of their potential health benefits. These plants can be ingested or directly applied to treat infections (Rojas et al., 2006) and this may be useful to overcome the increased resistance of microorganisms to conventional antibiotics from bacteria and fungi (Chinedum, 2005).

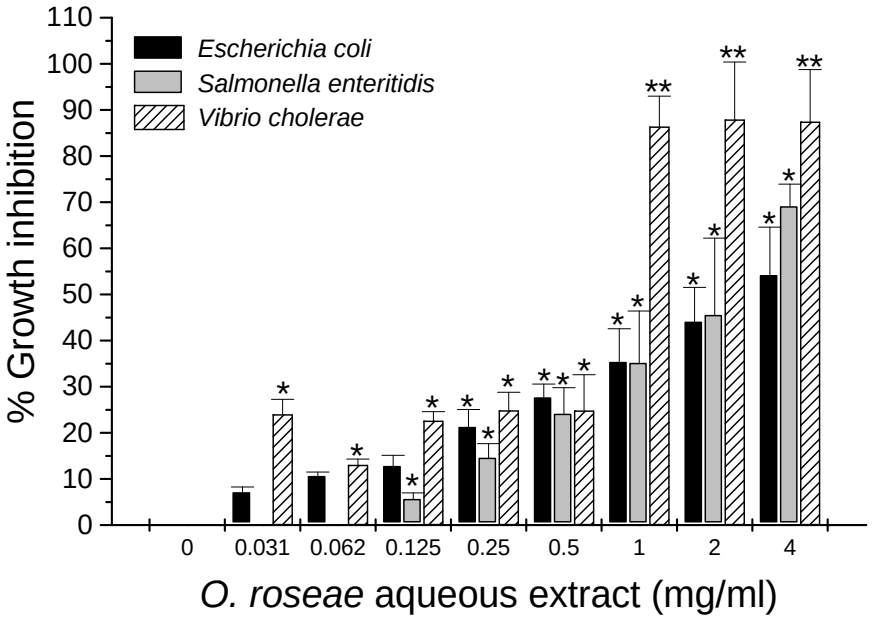

Fig. 2. Antibacterial effect of *O. rosea* aqueous extract.

E. coli, S. enteritidis and V. cholerae culture suspensions were incubated in the presence or absence of various concentrations of O. rosea aqueous extract, after which growth was measured colorimetrically, as explained in the text.

*Data represent means ± SEM of triplicate determinations from three independent experiments. **P = .01, *P = .05 compared with O. rosea extract-untreated control. Optical density at 570 nm for untreated cells was 1.11 ± 0.068.*

Compounds synthesized by plants have a broad therapeutic potential due to their chemical structures, for which the evaluation of their biological activity is important to develop new products with pharmacological potential and to validate treatments traditionally used by the Mexican population and other people from developing countries (Rodriguez-Fragoso et al., 2008).

Plant antibiotics are not currently used in a health program because of their low activity, unless their MICs are in the range of 0.1 to 1 mg/mL (Tegos et al., 2002; Drusano, 2004); thus, the results of the present study may be an indication of an important antibiotic activity of *O. rosea* extracts. Novel antimicrobial activity of plant extracts is an alternative to be considered as a result of the increasing resistance of microorganisms, mostly bacteria, to antibiotics (Russell, 2000; Moreillon, 2000). Because of this, isolation and evaluation of potential natural antibiotic agents, particularly, *O. rosea* leaves, may lead to the discovery of antibiotics for which bacteria and other organisms are susceptible (Diallo et al., 1999).

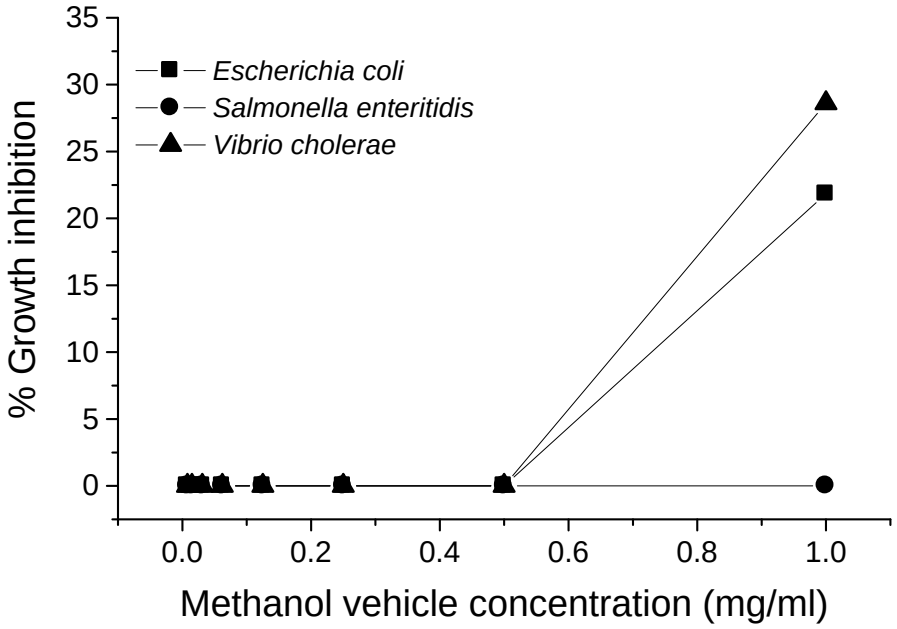

Fig. 3. Antibacterial effect of methanol vehicle.

E. coli, S. enteritidis and V. cholerae culture suspensions were incubated in the presence or absence of various concentrations of the methanol vehicle, similarly prepared as with the methanol plant extract, after which growth was measured colorimetrically, as explained in the text.
Data represent means ± SEM of triplicate determinations from three independent experiments, compared with culture medium control. Optical density at 570 nm for untreated cells was 1.03 ± 0.12.

The higher antibacterial activity of the aqueous extracts, compared with the methanol ones, may be related to alterations of the active compound (s) present in the fresh plant after the methanol extraction and because of the water soluble nature of the anionic substances which are naturally occurring in most plant materials (Darout *et al.*, 2000; El Astal *et al.*, 2005). This may be of relevance since aqueous infusions are commonly administered or ingested by many cultures to treat diverse maladies.

4. CONCLUSION

To our knowledge, this is the first report showing that *O. rosea* leaf extracts inhibit *E. coli*, *S. enteritidis* and *Vibrio cholera* growth. The respective observed MICs were 0.5 mg/mL, 0.125 mg/mL and 0.5 mg/mL for the methanol extract, and 0.25 mg/mL, 0.125 mg/mL and 0.031 mg/mL for the aqueous extract, with the order of potency of *S. enteritidis* > *E. coli* = *Vibrio cholera* for the methanol extract and *Vibrio cholera* > *S. enteritidis* > *E. coli* for the aqueous extract. There are still a number of plant compounds that remain to be evaluated at the molecular, cellular and physiological levels for their potential to treat human diseases. Further studies are underway to evaluate the *O. rosea* leaf extracts and active compounds in an *in vivo* model of infection, and to characterize the antibacterial active compound (s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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