Original Article

Effect of *Ibicella lutea* on uropathogenic *Proteus mirabilis* growth, virulence, and biofilm formation

Vanessa Sosa and Pablo Zunino

Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable (IIBCE), Avenida Italia 3318, CP11600, Montevideo, Uruguay

Abstract

Background: *Proteus mirabilis*, an important uropathogen that can cause complicated urinary tract infections (UTI), has emerged as a therapeutic problem following mutations that compromise the use of antimicrobial drugs. Due to the serious effects associated with uropathogenic *P. mirabilis* and the problems related to the use of antibiotics, it is necessary to develop alternative strategies for its control. The objective of this study was to assess the effect of *Ibicella lutea* extract, a South American indigenous plant, on growth, virulence and biofilm production of uropathogenic *P. mirabilis*.

Methodology: This study was based on the extract generation and the assessment of its effect on bacterial features related to virulence. These assays involved determination of antibacterial activity, swarming motility, Western blot to assess expression of fimbriae and flagella, biofilms formation, haemagglutination, haemolysis, and electron microscopy.

Results and Conclusions: *I. lutea* extract had an effect on bacterial growth rate and bacterial morphology. It also affected *P. mirabilis* swarming differentiation, hemagglutination and biofilm formation on glass and polystyrene. These findings suggest that *I. lutea* may have a role as an agent for the control of *P. mirabilis* UTI.

Keywords: *Ibicella lutea*; Prevention; *Proteus mirabilis*; urinary tract infection; vegetal extract

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Introduction

Urinary tract infections (UTI) are among the most common infections affecting humans and represent a serious health problem for millions of people each year. Nearly 20% of women who have a UTI will have another and 30% of those will have yet another one. *Proteus mirabilis* is an important opportunistic uropathogen, frequently isolated from catheterized patients or individuals with structural abnormalities of the urinary tract [1].

Although *P. mirabilis* does not commonly cause UTI in the normal host, it can cause severe UTI particularly in patients with urinary catheters or in people with structural abnormalities of the urinary tract [1]. It shows a predilection for the upper urinary tract where it can cause severe histological damage and it is frequently associated with bladder and kidney stone formation [2]. Several potential *P. mirabilis* virulence factors related to UTI have been described, including fimbrial-mediated adherence to the uroepithelium, swarming motility mediated by flagella, outer-membrane protein expression related to iron uptake, cell invasiveness, urease and hemolysin production, and iron acquisition [3].

UTI is commonly managed with antibiotic therapy. In general, such regimes are successful and infections can be resolved without complications. However, the antibiotics are expensive, can result in adverse effects, and may lead to bacterial resistance [4]. In general, bacteria have the genetic ability to acquire and transmit resistance to drugs used as therapeutic agents. Consequently, new infections can occur in hospitals resulting in high mortality rates.

Recently, the general acceptance of traditional medicine for health care and the development of microbial resistance to several available antibiotics have led researchers to investigate the activity of medicinal plants against infectious diseases [5-9]. The World Health Organization (WHO) reported that about 80% of the world's population depends primarily on traditional medicine that mainly involves the use of plant extracts [8]. The screening of plant extracts and plant products has shown that higher plants represent a potential source of new anti-infective agents. For instance, cranberry has long

been of interest for its beneficial effects in preventing UTI [11-13]. Howell *et al.* [16] determined that proanthocyanidins isolated from the cranberry fruit inhibit P-fimbrial adhesion *in vitro*, and thus may be the compounds responsible for the beneficial effect on UTI prevention. The urine of humans who consumed cranberry juice cocktail also exhibited anti-adhesion activity [12], which suggests that a certain level of absorption occurred and that bioactive proanthocyanidins and/or their metabolites have been excreted in the urine to inhibit adhesion.

The Latin-American flora is very diverse and includes a number of plants that are used in natural medicine for treating tropical diseases, including bacterial infections [6].

*Ibicella lute*a (Lindl.) Van Eselt. (Martyniaceae Stampf.) is a "quasi-carnivorous" native plant of America and was introduced as an ornamental in North America and Europe [14]. It is commonly known for having the seed capsule body covered with short spines and for its unpleasant smell. In Uruguay, where the plant grows freely, it is called Cuerno del Diablo (Devil's horn) and its infusion is used in popular medicine for the treatment of eye and skin infections. In an earlier study, the antimicrobial activity of the aqueous and ethanolic extracts of *I. lutea* was assessed and the aqueous extract showed activity against some Gram positive and negative pathogens determined by an agar-diffusion method [6]. Several extracts (H_2O, EtOH:H_2O 70:30, EtOH, CH_2Cl_2:MeOH 3:1, acetone and $CHCl_3$) of *I. lutea* aerial parts were tested for antibacterial activity and it was concluded that the most active was the chloroformic one [15]. According to these authors, the extract was active against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus.*

The aim of this study was to evaluate the role of *I. lutea* chloroform extract obtained from the aerial parts of the plant to interfere with growth, virulence and biofilm formation of uropathogenic *P. mirabilis.*

Materials and methods

Bacterial strain, media, culture conditions

P. mirabilis strains Pr2921, Pr6515, Pr783 and Pr990 were isolated from symptomatic UTI of adult women (Montevideo, Uruguay). These strains possess MR/P, PMF, and UCA fimbriae, flagella and produce hemolysin [16-20]. *P. mirabilis* strains were stored at -80 ºC in Luria-Bertani (LB) broth supplemented with 12% (v/v) glycerol and grown aerobically at 37 ºC. All media were from Difco Laboratories (Detroit, Michigan) and all chemicals were reagent grade (Sigma Chemical Co). Ovine erythrocytes were from Biokey Laboratory (Uruguay), and rat erythrocytes were from IIBCE. All *in vitro* assays were performed in media supplemented with *I. lutea* extract and in media without vegetal extract but containing water/ethanol (70:30, control).

Plant material

I. lutea was collected from Canelones, Uruguay, and identified by Mario Piaggio in the Botanic Garden of Montevideo (Government of Montevideo, Uruguay). A voucher specimen was deposited in the herbarium of the Botanic Garden bearing the number MVJB 26512.

Aerial parts of *I. lutea* (1000 g) were air-dried in the dark. Once dried, the plant material was ground, extracted by maceration for 48 hours with $CHCl_3$, and the solvent was vacuum evaporated (oil obtained, 21 g). Next, solutions were prepared in 30% (v/v) ethanol and further dilutions were made in the same solvent to obtain the required extract concentrations for the different assays.

Determination of antimicrobial activity

Extract of *I. lutea* was tested for antimicrobial activity using two techniques of diffusion on solid media [5].

For the agar diffusion method, agar was inoculated with *P. mirabilis* strains, allowed to solidify, and then wells were punched in Petri dishes. The wells were filled with different concentrations of the extract (0.5, 1.0, 1.5, 2.0 mg), and distilled water/ethanol (70:30) and kanamycin (40 μg ml^{-1}) were used as respectively negative and positive controls. The plates were incubated at 37°C for 24 hours.

For the disk diffusion method, 0.5 ml of *P. mirabilis* strains suspensions (0.5 McFarland) were spread on a NB agar plate to avoid swarming motility. After drying, sterile 5 mm diameter disks soaked with 20 μl of different concentrations of *I. lutea* extract (0.1, 0.2 and 0.3 mg) were placed on the agar surface. Disks containing distilled water/ethanol (70:30) and kanamycin (40 μg ml^{-1}) were used as respectively negative and positive controls and the plates were incubated at 37°C for 24 hours.

All tests were performed in duplicate and the antibacterial activity was calculated by measuring the diameter of the inhibition zone.

Minimal inhibitory concentration (MIC)

The determination of the MIC value against *P. mirabilis* was performed using the Ericcson and Sherris [21] broth-dilution method.

The antibacterial activity also was tested using the dilution method in broth. In these experiments, *P. mirabilis* strains suspensions containing 10^6 colony forming units (CFU) ml^{-1} were added to LB containing serial twofold dilutions (ranging from 0.1 to 1 mg ml^{-1}) of the *I. lutea* extract in glass test tubes. The tubes were incubated aerobically at 37°C for 24 hours and bacterial growth was indicated by the presence of turbidity and a pellet on the well bottom. The MIC was the lowest concentration of the sample that prevented visible growth. The samples were examined in duplicate in three separate experiments.

Thin-layer chromatography (TLC) and bioautography

Bioautographic assays were used to locate antibacterial compounds [22]. *I. lutea* extract was spotted onto a silica gel TLC plate (Kieselgel 60 F254 0.2 mm, Merck). Spots were visualized by UV irradiations at 365 nm and TLC plates were dried overnight. Then, the plates were covered with 2 ml of soft medium (Brain Heart Infusion (BHI) with 0.6% agar) containing 10^5 CFU of *P. mirabilis* strains, incubated at 37°C for 24 hours and sprayed with a 2.5 mg ml^{-1} MTT solution (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) in PBS. Plates were incubated at 37°C for one hour in the dark for colour development.

Growth-rate determination

The bacterial growth determination was performed according to Carlberg [23]. Pr2921 suspensions [optical density at 600 nm (OD_{600}) = 0.015] were prepared and 100 µl of these suspensions were transferred to 10 ml LB and incubated at 37 ºC with shaking. The capability to grow in broth was monitored by serial viable bacterial counts on nutrient agar (NA). Three independent assays were performed and the following parameters were used to evaluate growth (24): doubling time [ln(2tm)/ln(R2/R1)] and specific growth rate [ln(R2/R1)/t] where R1 represents the viable bacterial count at time 1 (4 h), R2 the viable bacterial count at time 2 (7 h), t the interval time in hours, and tm the interval time in minutes.

The data were presented as mean ± standard deviation. Statistical differences were assessed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. Values of $P < 0.05$ were regarded as significant.

Western blotting

Whole-cell preparations obtained from 48-hour static cultures in LB supplemented with 1 mg ml^{-1} of *I. lutea* extract were run on SDS-PAGE and separated proteins were transferred to nitrocellulose membranes (Bio-Rad) as described by Towbin *et al.* [24]. Western immunoblots were performed using a 1:200 dilution in PBS-Tween 20 1% (w/v) skimmed milk of rabbit polyclonal immune sera raised against MrpA, PmfA, UcaA and native flagella [16, 17, 19, 25]. Isogenic fimbrial *mrp*A, *pmf*A and *uca*A mutants [16, 17, 20], were included in the Western blotting assays.

Hemagglutination and hemolysis

The hemagglutination and hemolytic activity were determined according to [26].

For hemagglutination, *P. mirabilis* strains were grown in LB supplemented with 1 mg ml^{-1} of *I. lutea* extract, washed and adjusted to a concentration of 10^{10} CFU ml^{-1} in PBS. Fifty µl of the bacterial suspension were mixed with an equal volume of a 3% ovine and rat erythrocyte suspensions in PBS. The assay was performed adding 1% mannose solution to the suspension.

Hemolytic titer was defined as the inverse of the last dilution at which erythrocyte pellets were no longer visible. With the aim of determining whether the tested products exert hemolysis per se, negative controls with no addition of *P. mirabilis* strains were also done. Differences of more than twofold in the hemolytic titers were considered significant.

Swarming behavior assay

The effect of the extract on swarming migration was assessed as described by Liaw *et al.* [27]. Briefly an overnight bacterial culture (5 µl) was inoculated centrally onto the surface of dry LB swarming agar plates without or with *I. lutea* at 0.5, 1.0 and 1.5 mg ml^{-1} which were then incubated at 37 ºC for 24 hours. The swarming migration distance was assayed by measures of the swarming areas fronts.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed according to Legnani-Fajardo *et al.*[19] to determine the effect of *I. lutea* (1 mg ml^{-1}) on Pr2921 morphology TEM. Pr2921 was incubated for 48 hours at 37°C in LB. After incubation, bacteria were washed and a suspension of Pr2921 was adsorbed to

Formvar-coated grids 300 mesh (Sigma), followed by staining on drops of 1% uranyl acetate. The screens were observed and photographed with a JOEL JEM-1010 model transmission electron microscope.

Biofilm formation assay

The effect of *I. lutea* extract on biofilms formation was performed according to Pitts *et al.* [28]. Bacteria were statically grown in polystyrene multi-well plates and in glass tubes containing unmodified LB or LB supplemented with *I. lutea* (1 and 2 mg ml^{-1}) for 48 h at 37°C. The culture was subsequently discarded and a 1% solution of crystal violet (CV) was added. Successive rinses with distilled water were performed 20 minutes afterward. The crystal violet in each well was solubilized in ethanol 95% and a spectrophotometer was used to measure the absorbance at 540 nm.

Three identical samples were used for each concentration. A Mann-Whitney one-tailed non-parametric analysis was used to compare biofilm formation with values of $P < 0.05$ regarded as significant.

Biofilm optical microscopy

Using the method proposed by Allison and colleagues [29], optical microscopy was used to observe the biofilm formed on cover slips. Bacteria were grown in tubes containing a glass slide submerged in unmodified LB and LB supplemented with *I. lutea* extract (1 mg ml^{-1}) during 48 hours at 37°C. Samples were fixed with methanol, stained with 1% solution of CV, and observed with an OLYMPUS BX61 microscope.

Autoaggregation assays

Overnight cultures of Pr2921 in the presence and absence of *I. lutea* extract were harvested by centrifugation, suspended in PBS, and normalized to an OD_{600} = 1.0. One milliliter of each culture was then transferred to a spectroscopy cuvette and vortexed for two minutes. The autoaggregation was determined by the mean decrease in OD_{600} over time and the rate of autoaggregation (% C) of Pr2921 was determined (% C = $[1-(DO_{t2}/DO_{t1})]*100$, where DO_{t1}, absorbance in time t1, DO_{t2} absorbance in the next time). The results were compared using Mann-Whitney one-tailed non-parametric analyses with values of $P < 0.05$ regarded as significant.

Table 1. Influence of *I. lutea* extract on Pr2921 growth parameters

Concentration of *I. lutea*	Specific growth rate (h)	Doubling time (min)
0 mg ml^{-1}	0.981 ± 0.061	42.64 ± 3.72
0.2 mg ml^{-1}	0.913 ± 0.068	45.71 ± 4.71
1.0 mg ml^{-1}	0.429 ± 0.028*	97.76 ± 8.89*

Data are means ± standard deviations of three independent assays. The *I. lutea* extract affected the specific growth rate and the bacterial doubling time of bacteria.
* $P < 0.05$, statistical differences were assessed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test.

Results

Effect of I. lutea *extract on* P. mirabilis *growth*

The plate diffusion techniques showed that *P. mirabilis* growth was not affected when different concentrations of the *I. lutea* extract were used. In addition, bioautography detection indicated that the crude extracts of *I. lutea* did not show activity against *P. mirabilis*. Finally, the MIC of *I. lutea* extract against *P. mirabilis* was evaluated and the results revealed that bacteria grew even at 1 mg ml^{-1}, the highest concentration used in the assay. However, at this concentration of extract the bacterial specific growth rate and doubling time were significantly affected. The specific growth rate significantly decreased and the bacterial doubling time significantly increased ($P = 2.5 \times 10^{-4}$ and $P = 2.2 \times 10^{-4}$, respectively) (Table 1). In contrast, 0.2 mg ml^{-1} concentrations of *I. lutea* extract did not significantly modify bacterial growth rate ($P = 0.35$) or doubling time ($P = 0.69$) of Pr2921.

Influence of I. lutea *extract on* P. mirabilis *virulence-related features*

When *P. mirabilis* strains were grown in LB supplemented with 1 mg ml^{-1} of *I. lutea* extract, the uropathogens expressed a lower titre of hemolysin activity (titre of 8) than the control (titre of 32).

P. mirabilis strains were also grown under conditions that favored expression of MR/P, UCA and PMF fimbriae, in the presence of *I. lutea* extract. Results of Western blotting using immune polyclonal antisera indicated that *P. mirabilis* fimbriae

Figure 1. Halo images of swarming plates containing different concentrations of *I. lutea* extract (0, 0.5, 1 and 1.5 mg ml^{-1}), 24 h after inoculation. Aliquots (5 µl) of Pr2921 culture were inoculated onto the center of LB swarming agar plates without or with the indicated *I. lutea* extract and representative plates were photographed. As it can be seen, the *I. lutea* extract interfered with the swarming differentiation of Pr2921 in a dose-dependent manner.

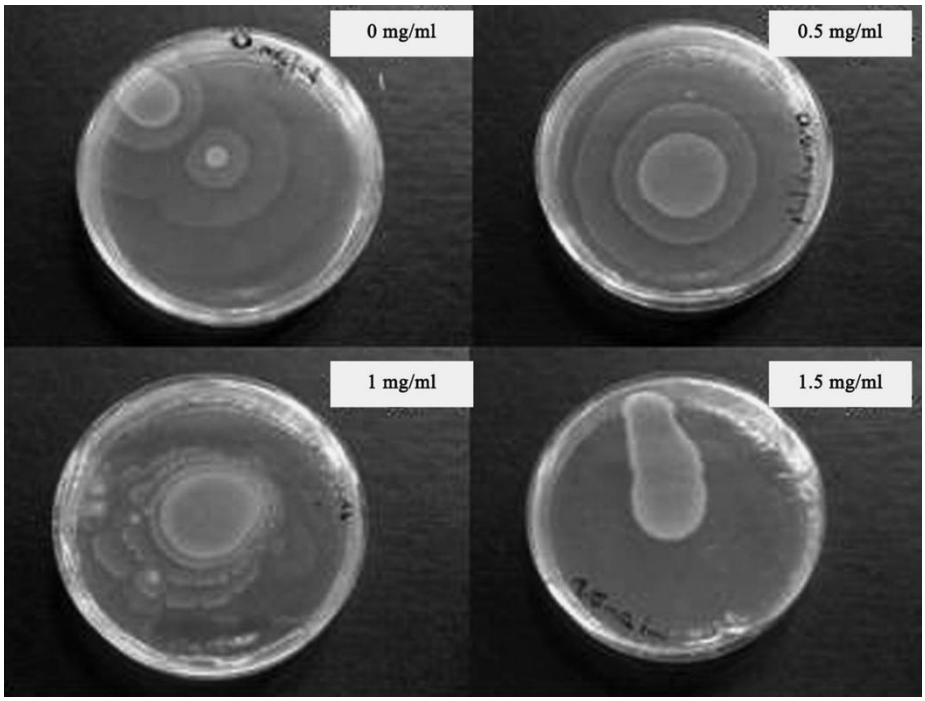
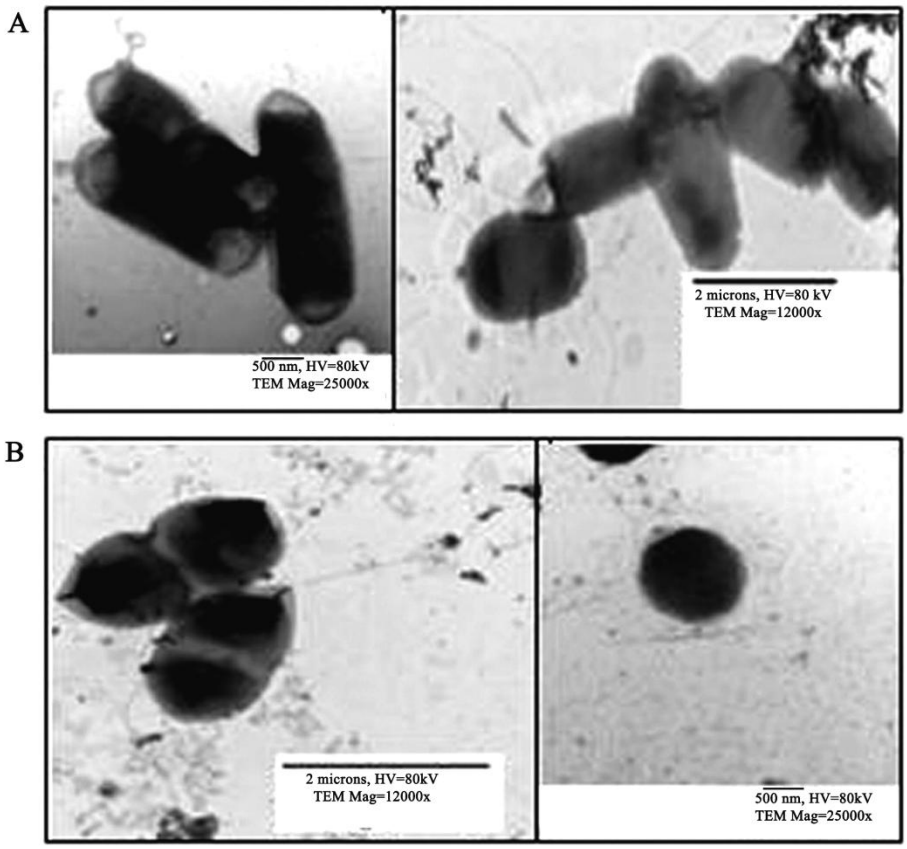


Figure 2. TEM of Pr2921 cells stained with uranyl acetate grown in unmodified medium (2A) and after treatment with 1 mg ml^{-1} of *I. lutea* extract (2B). The *I. lutea* extract had an effect on the morphology of bacteria.



expression was not modified by exposure to *I. lutea* extract. Hemagglutination was inhibited when bacteria grew in the presence of *I. lutea* extract. This effect was observed when hemagglutination was performed using erythrocytes of rat and ewe and in the presence and absence of mannose.

The ability of *P. mirabilis* strains to express flagella in the presence of *I. lutea* extract was assessed with SDS-PAGE and Western blotting using a polyclonal rabbit anti-native flagella antiserum. It was determined that flagella expression by *P. mirabilis* strains was not modified.

To test the effect of *I. lutea* extract on Pr2921 swarming differentiation, bacteria were inoculated onto the centre of LB swarming agar plates containing different concentrations of extract, and the migration distance of the bacteria was measured (Fig. 1). This assay showed that *I. lutea* extract significantly altered swarming differentiation at 1 mg ml^{-1} and completely inhibited swarming differentiation at 1.5 mg ml^{-1}.

TEM technique showed that the *I. lutea* extract also had an effect on the morphology of Pr2921. *I. lutea* extract-treated Pr2921 cells revealed smaller cellular elongation (Fig. 2B) compared with ovoid for the control (Fig. 2A). The treated cells exhibited an atypical appearance and the contents of some were seen as depleted (observed as less dense compared to non-treated cells). It was observed that flagella of Pr2921 had a normal appearance in the presence of 1mg ml^{-1} *I. lutea* extract (Fig. 2B).

I. lutea *extract inhibited Pr2921 biofilm formation on glass and polystyrene*

The I. lutea extract was added to LB at two different concentrations to determine its inhibitory effect on biofilm formation by Pr2921. The formation of biofilm was significantly inhibited when *I. lutea* extract was used at a concentration of at least 1 mg ml^{-1} ($P < 0.05$) (Table 2).

Optical microscopy confirmed the inhibitory effect of *I. lutea* extract on Pr2921 biofilm formation. The smears of untreated Pr2921 showed attached cells while no adhered cells were seen when the smears were prepared in presence of *I. lutea* extract.

Pr2921 did not show a significant increase in auto-aggregation index values when was grown the bacteria in presence of 1 and 2 mg ml^{-1} of *I. lutea* extract ($P = 0.066$ and $P = 1.000$, respectively).

Discussion

UTIs are among the most common infections in humans and represent the most common urologic disease affecting the bladder and kidneys. Traditionally, UTIs are treated with antibacterial drugs. Even though pharmaceutical industries have produced a number of new antibiotics, they are expensive and can have adverse effects. Furthermore, resistance to these drugs by microorganisms has increased. In the last decades, plants have been a valuable source of natural products for the promotion of human health [7]. Because many of these compounds are renally excreted, several botanical products could be particularly useful as urinary antiseptics [9].

The purpose of the present study was to investigate bioactivity of aerial part extracts of *I. lutea* extract, a plant native of America, against uropathogenic *P. mirabilis* for prevention and treatment UTI, and to explore its potential mechanisms of action.

Most assays were done using several uropathogenic *P. mirabilis* strains of clinical origin and the results were similar in all cases. Nonetheless, more laborious experiments were conducted only with *P. mirabilis* strain Pr2921 since this strain has been extensively characterized in our laboratory [16-18, 20].

The vegetal extract was evaluated at a maximum concentration of 1 mg/ml; thus higher concentrations were not considered relevant for the evaluation of its bioactivity [30].

First, the antimicrobial activity of *I. lutea* extract was studied and it was found to have neither a bactericidal nor bacteriostatic role potentially associated with the prevention or treatment of UTI caused by *P. mirabilis*. These results were similar to those reported by Ahuja *et al.* [13] and Labrecque *et al.* [28] who observed that cranberry juice concentrate had neither antibacterial activity on uropathogenic *E. coli* nor on *Porphyromonas gingivalis*. The results were also similar to those obtained by Wang *et al.* [31] who suggested that the inhibitory effect of resveratrol, a naturally occurring phytoalexin, on *P. mirabilis* swarming and virulence factors expression is unlikely to be due to its inhibitory effect on bacterial growth.

Since it was suggested that the bacterial growth rate may be related to uropathogenicity [32], the potential influence of *I. lutea* extract on Pr2921 growth dynamics was analyzed and it could be observed that the extract at 1 mg ml^{-1} induced a significantly decreased Pr2921 growth rate. There

was a growth effect of Pr2921; however, it was neither bactericidal nor bacteriostatic. Taking into account these results, a concentration of 1 mg ml^{-1} was selected for further analyses.

dose-dependent manner. Wang *et al.* [31] evaluated the effect of resveratrol on swarming of *P. mirabilis* and they observed that this compound also inhibited *P. mirabilis* swarming in a dose-dependent manner.

Table 2. Effect of *I. lutea* extract on biofilm formation by Pr2921 in polystyrene and glass and on auto-aggregation.

Concentration of *I. lutea*	Biofilm quantification		Auto-aggregation
	Polystyrene	Glass	
0 mg ml^{-1}	1.529 ± 0.600	0.623 ± 0.294	8.95 ± 6.34
1 mg ml^{-1}	0.236 ± 0.020*	0.007 ± 0.003*	7.31 ± 2.90
2 mg ml^{-1}	0.450 ± 0.163*	0.045 ± 0.035*	11.96 ± 2.59

Results are presented as the means ± standard deviations of three independent assays. The *I. lutea* extract inhibited biofilm formation but not affected the auto-aggregation.
*significantly different from control at $P < 0.05$ assessed by Mann-Whitney non-parametric analysis.
No significant differences between the two concentrations of extract *I. lutea* were observed.

Different assays were performed to determine whether potentially *P. mirabilis* virulence factors, including hemolysin, hemagglutinin, flagella and fimbriae, were affected by the presence of *I. lutea* extract.

Hemolysin, which is cytotoxic for cultured urinary tract epithelial cells, has been correlated with the ability of bacteria to invade cells [26]. Under the presence of *I. lutea* extract, Pr2921 expressed a lower titre of hemolysin activity than untreated bacteria.

An additional assessed virulence factor was the expression of fimbriae, which facilitate attachment to epithelial cell surfaces and play an important role in the initiation of UTIs [3]. Various natural compounds, including proanthocyanidins isolated from cranberry fruit, resulted in a markedly decreased fimbriation by uropathogenic *E. coli* strains [11, 33]. In *P. mirabilis* it was demonstrated that MR/P, PMF and UCA fimbriae enhance the establishment of UTI. When we assessed the expression of these fimbriae in Pr2921 cultured in the presence of *I. lutea*, we observed that expression of Pr2921 fimbriae remained unaltered.

MR/P fimbriae *P. mirabilis* differentiates from other fimbriae by its capability to agglutinate erythrocytes in the presence of mannose [16]. When bacterial cultures were agglutinated with ovine and rat erythrocytes in an assay designed for mannose-resistant fimbriae, *I. lutea* extract inhibited hemagglutination with both types of red cells. These results suggest that the *I. lutea* extract may contain compounds that interfere with bacterial adhesion to eukaryotic cells and may be useful for the prevention of the urinary tract colonization.

When the effect of *I. lutea* extract on Pr2921 swarming differentiation was tested, it could be observed that Pr2921 swarming was inhibited in a

Expression of functional flagella has been recognized as a virulence factor in bacteria and motility dependent on flagella has been considered a means of dissemination for the bacterium, thus favoring infection. The ability of Pr2921 to synthesize flagella under the presence of *I. lutea* extract was assessed by Western blotting using a polyclonal rabbit anti-flagella antiserum and by TEM. It was observed that the expression of Pr2921 fimbriae was unaltered by *I. lutea* extract.

Other potential effects of *I. lutea* extract would include modifications of cellular morphology. TEM techniques revealed that the untreated cultured cells remained unaltered, while *I. lutea* extract apparently was associated with the loss of bacteria elongation and content depletion. This effect may be due to damage of the cell wall and alterations in the cytoplasmic membrane permeability, resulting in the loss of cytosol.

Biofilms formed by pathogenic *P. mirabilis* strains can pose serious problems to human health, such as urinary catheters obstruction [34]. There are several reports about natural compounds that inhibit biofilm formation [7, 35, 36]. In the present study, we evaluated the influence of *I. lutea* extract on biofilm formation by quantification with CV, a colorimetric method widely used since it is inexpensive [28]. In the present study, it could be seen that the *I. lutea* extract prevented the formation of *P. mirabilis* biofilm on different surfaces. The inhibition of swarming differentiation could be related to this effect since motility may be important for initial cell attachment, for example in *E. coli* biofilm formation [28].

Finally, the auto-aggregation of Pr2921 in presence of *I. lutea* extract was evaluated. According to Rickard *et al*. [37], bacterial aggregation proceeds

in the form of a succession of adhesion and multiplication events. When the auto-aggregation of Pr2921 was assessed in the presence of *I. lutea* extract, it was observed that it remained unaltered. These results indicated that inhibition of biofilm formation was not related to Pr2921 auto-aggregation.

To our knowledge, this is the first report that describes the effects of *I. lutea* extract on different features related to the virulence of uropathogenic *P. mirabilis*. In previous studies, a stearic acid glycoside [11-O-(6´-O-acetyl-β-Dglucopyranosyl) stearic acid], two dammarane triterpenes (3-acetyl-24- epi-polacandrin and 1,3-diacetyl-24-epi-polacandrin), a 20S, 24 R-epoxy-3α, 12β, 25-trihydroxydammarane and a flavonoid apigenin were isolated from *I. lutea*. According to Cerdeiras *et al.* [15] and Simirgiotis *et al.* [38], the stearic acid glycoside and the 20 S, 24 R- epoxy-3a-acetoxy-1b, 12 b, 25- trihydroxydammarane were responsible for the antibacterial activity of the extract against a group of bacterial isolates.

It was considered that the use of the whole extract against *P. mirabilis* urovirulence-related attributes allowed for the evaluation of the activity of the complex mixture of compounds present in the plant. It is important to take into account that the extract did not show any direct antibacterial effect against *P. mirabilis* and that different compounds or even combinations between them are likely responsible for the interference of the *I. lutea* extract with different bacterial pathogenesis features.

The results of our study suggest that *I. lutea* extract could have an additional use as the basis for a novel strategy to prevent or treat UTI. At present, investigations are in progress to determine the degree of toxicity of *I. lutea* extract *in vitro* and *in vivo* and to analyze the effects induced by the oral administration of *I. lutea* extract in an experimental model of UTI in the mouse.

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Corresponding Author
Dr. Pablo Zunino
Avenida Italia 3318, CP11600 Montevideo, Uruguay
Tel.: +598 (2) 4871616, fax: +598 (2) 4861417
E-mail: pablo@iibce.edu.uy

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