

Assessment of effectiveness and safety of *Ibicella lutea* extract in the control of experimental *Proteus mirabilis* urinary tract infection

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Abstract

Introduction: *Proteus mirabilis* is an important cause of complicated urinary tract infections (UTI). Like many other microorganisms, *P. mirabilis* has acquired resistance to many antibiotics. Due to the serious effects associated with uropathogenic *P. mirabilis* and the problems related to the use of antibiotics, alternative strategies for its control must be developed. Previously, we studied the effect of *Ibicella lutea* extract, a South American indigenous plant, on *in vitro* uropathogenicity of *P. mirabilis*. We observed that *I. lutea* extract had an effect on various attributes associated with *P. mirabilis* urovirulence. The objective of this study was to assess *I. lutea* extract against UTI by *P. mirabilis*.

Methodology: This study was based on the effect of *I. lutea* extract to prevent or treat *P. mirabilis* experimental UTI in mice and the influence of this administration on the normal intestinal flora. Also, we studied the toxicity, mutagenicity, and antimutagenicity of the extract.

Results: In this study, while *I. lutea* administration showed an effect in the prevention and treatment of UTI in the mouse, the intestinal microflora did not change. The *I. lutea* extract was neither toxic nor mutagenic although the extract showed antimutagenic properties.

Conclusion: These findings suggest that the administration of *I. lutea* extract could represent an interesting new strategy to control *P. mirabilis* UTI.

Key words: urinary tract infection, *Proteus mirabilis*, *Ibicella lutea*, toxicity, mutagenicity.

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Introduction

Urinary tract infections (UTI) are among the most common infections in humans and represent the most frequent urologic disease affecting the bladder and kidneys [1]. *Proteus mirabilis*, a motile Gram-negative bacterium, is one of the principal cause of UTI in catheterized patients and those with urinary tract abnormalities. Individuals suffering from UTI caused by *P. mirabilis* often develop bacteriuria, cystitis, kidney and bladder stones, catheter obstruction due to stone encrustation, acute pyelonephritis, and fever [2].

The preventive therapeutics in UTI include the use of antibiotic treatments. Antibiotic-resistant bacterial strains are increasingly detected and thus new drugs are required [3]. Finding new antimicrobial agents with novel mechanisms is essential and extensively pursued in antibacterial drug discovery [4]. Natural products are still important as a source of great potential for drug discovery. Plants have been the largest natural source

of new drugs, though only less than 5% of known plants have been chemically characterized. Extracts from plants can have significant value in antimicrobial research as they may inhibit bacterial growth by different mechanisms or interfere with different bacterial virulence attributes.

In a previous work, we studied the extract of *Ibicella lutea*, a South American indigenous plant, and its effect on growth, virulence, and biofilm formation of uropathogenic *P. mirabilis* [5]. We observed that *I. lutea* extract had an effect on bacterial growth rate, bacterial morphology, 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 prevention or treatment of *P. mirabilis* UTI.

In general, *in vitro* results cannot be simply extrapolated to *in vivo* conditions for antimicrobial drugs [6]; therefore, data generated from both systems are necessary and irreplaceable.

Due to the lack of knowledge concerning potential toxicity and adequate usage, medicinal herbs are often incorrectly used, thereby not yielding the desired effect. Consequently, when a possible new antimicrobial agent is found, the next logical step is to assess its safety. The identification of chemicals or compounds capable of inducing mutations is crucial in safety assessment since mutagenic compounds can potentially induce cancer [7].

In this study, and based on our previous encouraging results, we examined the potential role of *I. lutea* extract to treat and prevent *P. mirabilis* experimental UTI in an animal model and the influence of this administration on the normal intestinal flora. We also studied toxicity, mutagenicity, and antimutagenicity of the crude chloroform extract of the aerial parts of *I. lutea* using different approaches.

Methodology

Bacterial strain, media and growth conditions

P. mirabilis strain Pr2921 was isolated from a symptomatic UTI of an adult woman (Montevideo, Uruguay) [8]. Pr2921 was stored at -80°C in Luria-Bertani (LB) broth supplemented with 12% (v/v) glycerol and grown aerobically at 37°C. One mutant strain, *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) strain TA98 (his D3052, rfa, uvrB, pKM101) [9], was used in the Ames test. This strain was maintained on nutrient agar at 3°C ± 1°C. The bacteria were inoculated in nutrient broth (NB) and incubated at 37°C for 18 to 24 hours before performing the test.

All media were from Difco Laboratories (Detroit, MI, USA) and all chemicals were reagent grade (Sigma Chemical, Milwaukee, WI, USA).

All *in vitro* assays were performed in media supplemented with *I. lutea* extract as indicated in each assay 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.

The extract had been previously produced for *in vitro* studies reported in our previous work [5]. 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 CHCl₃, and the solvent was vacuum evaporated (oil obtained, 21 g). Then solutions were prepared in 30% (v/v) aqueous ethanol and further dilutions were made in the same solvent to obtain the required extract concentrations for the different assays.

UTI model in the mouse

Two-month-old female CD-1 mice weighing about 20 g were used. The animals were provided with food pellets and tap water *ad libitum* and their environmental conditions were kept constant. To assess the potential preventive or therapeutic effect of *I. lutea* extract, a well-established ascending UTI model in the mouse was used [8,10]. This experiment was conducted in accordance with procedures authorized by IIBCE, Montevideo, Uruguay. Briefly, bacteria for these assays were grown at 37°C for 48 hours in LB broth. Prior to transurethral challenge, mice had been anesthetized with xylazine (10 mg/kg) and ketamine (50 mg/kg), and their bladders were emptied by gentle abdominal massage. Using this technique, mice were anesthetized for approximately 40 minutes. A sterile 25 mm long soft polyethylene catheter (outer diameter 0.61 mm) was gently inserted into the bladder through the urethra. A 30 gauge needle attached to a tuberculin syringe containing the Pr2921 bacterial suspension (10⁸ colony-forming units (CFU)/mL) was inserted into the catheter lumen and 0.05 mL of the bacterial suspension was infused into the bladder over 30 seconds to avoid vesicourethral reflux. The catheter was removed immediately after the mouse challenge and the experimental mice were housed in conventional plastic cages. The mice were euthanized by cervical dislocation at seven days after transurethral challenge, and the bladder and kidneys of each animal were removed and homogenized in 10 mL of PBS using a Stomacher 80 Lab Blender (Seward, Worthing, UK). Viable bacterial counts were done on LB with 2% agar and without NaCl to avoid swarming motility.

Preventive assays

One group of ten mice was daily orally administered with of *I. lutea* extract (100 µL, 0.2 mg/mouse) by gavage. Another group of ten animals was treated in the same way with 100 µL of the vehicle of the *I. lutea* extract (30% ethanol). Both groups of mice were then transurethrally challenged with Pr2921 three days upon the initial treatment and

subsequently killed by cervical dislocation seven days after the challenge with *P. mirabilis* Pr2921.

Therapeutic assays

Three groups of 12 mice were challenged via the transurethral route with Pr2921. Five hours after the challenge, two groups of mice were orally treated with 100 μ L of *I. lutea* extract (0.2 and 0.5 mg/mouse) while the third group was treated with 30% ethanol only.

Animals were treated daily with *I. lutea* extract or 30% ethanol for five days, and killed by cervical dislocation seven days after the challenge.

Influence of I. lutea extract administration on the normal faecal flora

Qualitative and quantitative analysis of the faecal flora was performed by bacterial counts using fresh stools [11]. Briefly, dilutions of stools in PBS were inoculated on the following agar media: blood agar for total aerobes and anaerobes, MacConkey's agar for detection of *Enterobacteriaceae*, and MRS agar for lactic acid bacteria (LAB) (Difco Laboratories, Detroit, MI, USA). Aerobic cultures were incubated at 37°C for 72 hours. Media for anaerobic bacteria were incubated at 37°C in an anaerobic chamber up to five days. The number of bacteria was expressed as log₁₀ CFU/g of stools.

Brine shrimp lethality bioassay

Artemia salina eggs were incubated in artificial seawater (3.6 g NaCl, 1.5g Mg₂SO₄, 0.5 g NaHCO₃ in 100 mL distilled water, pH 9) at 28°C under tungsten light. After incubation for 24 hours, nauplii were collected with a Pasteur pipette. The *I. lutea* extract (triplicate) was serially diluted (0.5, 1, 2, 5, and 10 mg/mL) in artificial seawater. Between 10 \pm 2 nauplii were added to each set of microwells containing the samples and the cultures were further incubated at 28°C for 24 hours [12]. Controls containing 30% ethanol (negative control) and containing potassium dichromate (K₂Cr₂O₄, positive control) were included on each set of experiments.

Percentage of mortality was calculated according to the following formula: $M_{mct} = (N_{Mm}/N_0)*100$, where: M_{mct} is mortality of individuals in time t, N_{Mm} is average number of dead individuals, and N_0 is initial number of living individuals at the test start.

Ames test

In the Ames test, *S. Typhimurium* TA98 was genotyped according to Maron and Ames [9] to ensure the purity of the strain.

Toxicity assay

To examine the toxic effects on *S. Typhimurium* TA98, a diluted *I. lutea* extract (1x10⁻⁵ - 2x10⁻⁵ mg) was added to overnight-cultured *S. Typhimurium* TA98 (0.1 mL). The mixture was preincubated at 37°C for five minutes before it was diluted with phosphate buffer, and the mixture was then poured onto NA plates. The plates were incubated at 37°C for two days, and the number of colonies was counted [13]. The *I. lutea* extract was then tested for its mutagenic/antimutagenic potency exclusively in the nontoxic concentration range.

Mutagenicity assay

The mutagenic effect of *I. lutea* extract was assayed according to the Ames test using *S. Typhimurium* TA98 [9]. The tested strain was cultured overnight in NB for 12 hours. Different concentrations of *I. lutea* extract (0.5, 1, and 2 mg/plate) were added to 2 mL of top agar and 0.1 mL of bacterial culture and then poured onto a plate containing minimum agar. The plates were incubated at 37°C for 48 hours, and the His⁺ revertant colonies were manually counted.

All experiments were analyzed in triplicate with at least two replicates. A sample was considered to be mutagenic when the number of revertant colonies was at least twice the negative control yield and showed a significant response in analysis of variance. The mutagen used as a positive control was 4-nitro-*o*-phenylenediamine (NPD, 5 μ g/plate), which is a direct-acting mutagen.

Antimutagenicity test

The antimutagenic effect of *I. lutea* extract was assessed using the Ames *Salmonella* mutagenicity test. The NDP mutagen or 2-aminofluorene (2-AF), which is an indirect-acting mutagen (5 μ g/plate), were added to the mixture of *I. lutea* extract (0.1, 0.2, and 0.4 mg/mL) and bacterial culture (0.1 mL) with S9 mix (0.5 mL). The mutagenicity of each mutagen in the absence of extract is defined as 100%. The number of His⁺ revertants (after subtracting the spontaneous reversions) induced by direct and indirect mutagens tested without extract was considered as 100%.

The calculation of % inhibition was done according to the formula given by Ong *et al.* [14]: % inhibition = $[1-(T/M)]/100$ where T is the number of revertants per plate in the presence of the mutagen and test sample, and M is the number of revertants per plate in the positive control. The number of spontaneous revertants was subtracted from the numerator and denominator.

Acute toxicity test

To assess the acute toxicity of *I. lutea* extract, a well-established model in the rat was used [15]. This experiment was conducted in accordance with procedures authorized by IIBCE, Montevideo, Uruguay. Male and female (nulliparous and non-pregnant) Sprague–Dawley (SD) rats were approximately 9-10 weeks old on the day of dosing. All animals were housed individually in suspended, stainless steel, wire-mesh cages in animal rooms maintained at a temperature of 18-26°C, and a relative humidity of 30-70%. Animal rooms were artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle. Tap water and pellet feed was available *ad libitum* except during the fasting period.

Rats were fasted approximately 18 hours prior to dosing. The *I. lutea* extract was administered to five male and five female rats in a single oral dose by gavage at a dose of 2,000 mg/kg body weight at a volume of 10 mL/kg body weight. Food was returned to the animals approximately three to four hours after dosing. Individual weights were determined at the time of fasting (Day 1), shortly before the test substance was administered (Day 0), and on test days 1, 2, 4, 7, and 14. The animals were observed for clinical signs of toxicity while handled before and after fasting, once during the first 30 minutes after dosing, at least two more times within four hours after dosing, and daily thereafter. Observations for mortality and signs of illness, injury, or abnormal behaviour were conducted twice daily. Surviving

animals were sacrificed and given a complete gross pathology examination.

Results

Effect of *I. lutea* extract using the UTI model in the mouse

To investigate the potential preventive effect of the *I. lutea* extract against UTI, we treated one group of mice daily with *I. lutea* extract through the oral route, as explained in the Methodology section. *P. mirabilis* CFU recovered from the bladder and kidneys of treated and non-treated animals with the *I. lutea* extract are shown in Figure 1. The number of viable *P. mirabilis* recovered from the kidneys of mice treated with *I. lutea* was significantly lower than the count recovered from the kidneys of non-treated mice ($P < 0.05$). No significant differences were detected in the bladder CFU of both groups at 0.2 mg/mice exposure ($P > 0.05$).

Figure 1. Evaluation of the protection of *I. lutea* extract against UTI caused by *P. mirabilis*. Data are expressed as \log_{10} CFU per kidneys or bladder. The median (-) is indicated for each group. The range of detection in this assay is 10^2 to 10^9 CFU per kidney or bladder. P values were determined by the Mann-Whitney nonparametric test when comparing the control group (without extract, white dots) and the group treated with the *I. lutea* extract (black dots).

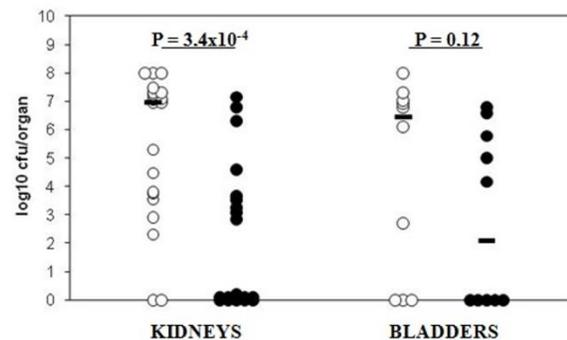
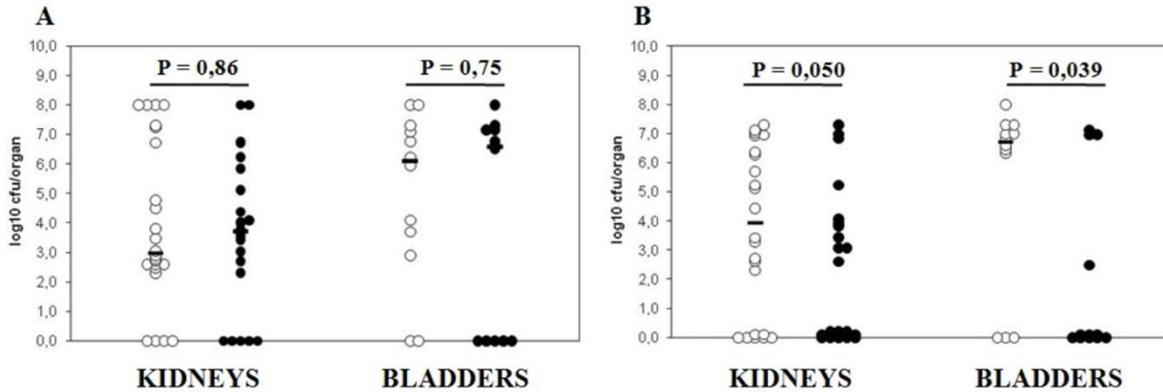


Figure 2. Evaluation of the possible therapeutic effect of *I. lutea* extract against UTI caused by *P. mirabilis*. Data are expressed as log₁₀ CFU per kidneys or bladders. The median (-) is indicated for each group. The range of detection in this assay is 10² to 10⁹ CFU per kidney or bladder. *P* values were determined by the Mann-Whitney nonparametric test when comparing the control group (without extract, white) and the group treated (with extract, black) with 0.2 mg/mouse (Fig. 2A) and 0.5 mg/mouse (Fig. 2B) of *I. lutea* extract.



The ability of *I. lutea* extract to treat the mice already challenged with *P. mirabilis* is shown in Figure 2. No significant differences were detected in kidney and bladder bacterial counts of treated and non-treated mice when a 0.2 mg/mouse *I. lutea* dose was used (*P* > 0.05); however, a therapeutic effect of *I. lutea* could be observed when the mice were treated with a 0.5 mg/mouse extract dose. *P. mirabilis* CFU recovered from the kidneys and bladders of treated animals were significantly lower than CFU recovered from the organs of non-treated mice (*P* < 0.05).

Also, the effect of *I. lutea* extract on faecal total aerobic and anaerobic bacteria, LAB, and *Enterobacteriaceae* was assessed. Changes in bacterial counts between the beginning and the end of the administration of *I. lutea* extract were assessed. Mean CFU of faecal bacteria is shown in Table 1. In general, faecal bacterial count was not affected by *I. lutea* extract consumption (*P* > 0.05).

Table 1. Effect of *I. lutea* extract on the composition of faecal flora of treated mice. Bacterial counts are expressed as mean ± SD log₁₀ CFU/gr.

	Organism			
	Total aerobes	Total anaerob	LAB	<i>Enterobacteriaceae</i>
Pre-treatment	9.02 ± 0.73	7.54 ± 0.20	8.67 ± 0.46	7.02 ± 0.72
Post-treatment	9.10 ± 0.15	8.29 ± 0.36	8.90 ± 0.22	8.01 ± 0.20

LAB : Lactic acid bacteria

Table 2. Toxicity of *I. lutea* extract on brine shrimp larvae. Values are showed as the means of three independent experiments

	Concentration	Larvae mortality (%)
<i>I. lutea</i>	0.5 mg/mL	3
	1 mg/mL	6
	2 mg/mL	6
	5 mg/mL	7
	10 mg/mL	5
Potassium dichromate (K ₂ Cr ₂ O ₄)	12.5 mg/mL	53
	25 mg/mL	70
	50 mg/mL	77

Artemia salina test

The cytotoxic assays were conducted using the *Artemia salina* lethality test. The results obtained are shown in Table 2. *I. lutea* extract did not show cytotoxic activity at concentration values lower than 10 mg/mL.

Ames test

The *I. lutea* extract exhibited a non-toxic effect on *S. Typhimurium* TA98 strain at the dose of 2 mg/plate (*P* > 0.05).

When the Ames test was used to detect possible mutagenicity of *I. lutea* extract, the tested sample did not exhibit mutagenic properties on *S. Typhimurium* strain TA98 (Table 3).

Table 3. Mutagenicity relation of *I. lutea* extract toward *S. Typhimurium* TA98. Data are means \pm SD of three plates. The number of spontaneous revertants was determined in assays without *I. lutea* extract. NPD was used as a positive control. The mutagenicity relation was determined according the following formula: mutagenicity relation: His⁺ revertant per plate/His⁺ spontaneous revertant.

<i>I. lutea</i> doses (mg/plate)	Mutagenicity relation
0.5	0.90 \pm 0.22
1.0	1.04 \pm 0.43
2.0	1.04 \pm 0.23

The protective action of *I. lutea* extract against the mutagenicity of NPD and 2-AF was evaluated by the Ames test using *S. Typhimurium* TA98, as presented in Table 4. According to the Ames parameters, when prevention percent ranges between 25-40%, the antimutagenesis effect is considered medium, and when this prevention percentage is higher than 40%, the antimutagenesis effect of the test sample is strong [16,17]. In this study, *I. lutea* extract was able to inhibit the mutagenesis NPD and 2-AF, indicating that the extract showed antimutagenic properties.

Acute toxicity

When acute toxicity of the *I. lutea* extract in rats was assessed, all animals gained weight and no adverse clinical signs were observed during the 14 days following oral administration of *I. lutea* extract at a dose of 2,000 mg/kg (data not shown). Additionally, no gross lesions were found in any of the organs at necropsy (data not shown). These results demonstrated that *I. lutea* extract was not acutely toxic at 2,000 mg/kg body weight.

Table 4. Inhibitory effect of *I. lutea* extract against the mutagenicity of NPD and 2-AF to *S. thyphimurium* TA98. The calculation of % inhibition was done according to the formula: % inhibition = $[1-(T/M)]/100$ where T is the number of revertants per plate in the presence of mutagen and test sample and M is the number of revertants per plate in positive control.

Mutagen	<i>I. lutea</i> doses (mg/plate)	% Inhibition	Antimutagenic effect
NDP	0.5	6.4	Weak
	1	32.5	Moderate
	2	42.0	Strong
2-AF	0.1	29.0	Moderate
	0.2	49.0	Strong
	0.4	48.0	Strong

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, but resistance to these drugs by microorganisms has increased. Recently, the exploitation of wild plants for medicinal purposes has gained acceptance worldwide. The botanical bioactive agents have evolved to protect the plant and therefore may be useful to prevent or treat infections in animals. Because many of these compounds are excreted through the renal system, they could be particularly useful as urinary antiseptics [18].

In a previous work, we studied the effect of *I. lutea* extract on *in vitro* uropathogenicity of *P. mirabilis* [5]. We observed that *I. lutea* extract had a significant effect on various attributes associated with *P. mirabilis* urovirulence, including bacterial growth rate, bacterial morphology, swarming differentiation, hemagglutination, and biofilm formation on glass and polystyrene; however, the extract did not exert a significant antibacterial effect.

Complex environmental conditions *in vivo*, such as osmolarity, pH and nutrient or substrate availability, differ from those in laboratory cultures [19]. Since environmental conditions affect on bacterial growth, metabolism, and regulation of gene expression, it is necessary to evaluate the effect of antibacterial agents through *in vivo* approaches after *in vitro* assays.

In the present study, we analyzed the effect of the *I. lutea* extract on UTI in an experimental mouse model. The results demonstrated the effectiveness of the *I. lutea* extract to prevent UTI in a dose-effect manner.

Previously, we had observed that *I. lutea* extract had an effect on the mannose-resistant hemagglutination capacity of different *P. mirabilis* strains [5]. According to these results, we hypothesized that the inhibited adherence to erythrocyte receptors indicate that *I. lutea* could also interfere with renal tissue colonization mediated by adherence to other eukaryotic cell receptors.

A similar situation was seen when epithelial cells from the urinary tract of mice were given cranberry juice and tested for adherence. Over a 14-day period, the mice were given cranberry juice, a product effective in the prevention and management of UTI, instead of water. When compared with control cells, cranberry juice reduced bacterial adherence by 80% in mice cell preparations [20]. In addition, it is probable that the extract has a role in affecting other bacterial features related to urovirulence that could be inferred from our previous *in vitro* results, such as altered growth rate, diminished ability to form biofilms, or affected swarming differentiation.

The intestinal microbiota performs important metabolic and immunological functions and acts as a biological barrier against pathogens. In this study, we evaluated whether the intestinal flora was altered after the supply of *I. lutea* extract and showed that the numbers of faecal bacteria of treated mice did not change during this study. Kontiokari *et al.* [21] examined the effect of continuous daily cranberry juice consumption on the normal colonic bacterial flora and no effect was perceptible.

The *I. lutea* showed no significant toxicity when the brine shrimp test was used. The results found by the brine shrimp assay indicate that the extract has an LC₅₀ value higher than 20 µg/mL, the recommended cutoff point for the detection of cytotoxic activity [20]. This means that *I. lutea* might not be toxic to humans.

The Ames bacterial test done with mutagenic *S. Typhimurium*, has been used with good success to screen mutagenic and antimutagenic properties of different agents [9]. The Ames *Salmonella* assay has been validated and tested with known mutagens. It has also been shown to work as a mutagenicity assay in different laboratories and yield results comparable to *in vivo* effects [22,23]. In this assay, no mutagenic

activity was detected when the *I. lutea* extract was evaluated, indicating that it is not mutagenic.

I. lutea extract was able to inhibit the mutagenesis of NPD and 2-AF. According to these results, it may be inferred that the extract contains chemical compounds capable of inhibiting the mutagenicity of direct-acting and indirect-acting mutagens. The mechanism by which the antimutagens in the *I. lutea* extract inhibited the mutagenicity is not known, but we suggest that these antimutagens may interact with some specific enzymes in the liver homogenates that are necessary for the activation of chemical mutagens.

In the present study, the Ames test was also performed to consider an anticancer effect using *S. Typhimurium*. Interestingly, *I. lutea* extract displayed unexpected antimutagenesis effects.

Oral acute toxicity of *I. lutea* extract was also evaluated. There was no indication that *I. lutea* extract was acutely toxic following oral gavage in rats using a 2,000 mg/kg dose. All the rats survived the experiment period and no biologically significant differences in body weights, weight gain, or feed consumption were observed.

The results obtained through *in vivo* and *in vitro* approaches suggest that *I. lutea* extract might represent an interesting new strategy to prevent or treat UTI, if consumed orally.

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