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Effects of resveratrol on the urease-positive urinary tract pathogens *Staphylococcus saprophyticus* and *Proteus mirabilis*

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Abstract
Resveratrol (3, 5, 4'-trihydroxy-*Trans*-stilbene) is a polyphenolic plant metabolite with antibacterial, antioxidant, and anti-inflammatory activities. *Staphylococcus saprophyticus* and *Proteus mirabilis* were inhibited by pure resveratrol with minimum inhibitory concentrations of 250 to 500 µg ml^{-1}. Urease activity in cell-free extracts and whole cells was partially inhibited by resveratrol but a concentration of 5 mmol l^{-1} was required to reduce it by more than 60%. Inhibition was competitive for *S. saprophyticus* but noncompetitive for *P. mirabilis*. Addition of resveratrol to the growth medium did not affect the specific activity of urease or prevent the increase in pH that occurs as a result of urease activity. Resveratrol reduced biofilm formation by *S. saprophyticus* at concentrations >125 µg ml^{-1} and partially inhibited it by *P. mirabilis* in rich media containing urea. While resveratrol has some potential for the treatment of urinary tract infections, it may need to be modified or combined with other urease inhibitors.

Keywords: *Proteus mirabilis*, resveratrol, *Staphylococcus saprophyticus*, urease, urinary tract pathogen

1. Introduction
Resveratrol (3, 4', 5-trihydroxy stilbene) is a naturally-occurring polyphenolic compound found in over 70 different plant species including grapes, peanuts, and berries [1]. The *Trans* isomer is the more stable biologically active form. This phytoalexin has been reported to have antibacterial activity towards both Gram-positive and Gram-negative bacteria as well as antifungal activity [2-6]. Resveratrol has antioxidant activity and can prevent the formation of biofilms [7-8]. It has been found to have anti-inflammatory, anti-carcinogenic, and Neuro protective activities and said to be useful for the treatment of cancer, diabetes, and coronary heart disease [1, 9-10]. Several studies have indicated that it also may be appropriate useful for some disorders of the renal system including cystitis of the bladder and prostatitis [11-14].
Urinary tract infections (UTIs) commonly occur in infants and small children, in adolescent and adult women, and in patients or older adults fitted with catheters [15-17]. The primary causes are the Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* and the Gram-positive bacteria *Staphylococcus saprophyticus*, *Enterococcus faecalis*, and *Staphylococcus aureus* [18-19]. A key virulence factor for *P. mirabilis* and *S. saprophyticus* is the enzyme urease, which catalyzes the hydrolysis of urea to form ammonia and carbonic acid [20-22]. The ammonia raises the urinary pH and leads to the formation of urinary stones and the encrustation of catheters [23]. Other virulence factors include the formation of flagella that allow movement within the urinary tract, the assembly of surface structures (pili or fimbriae) that permit adherence to host cells, the synthesis of specific hemolysins or cytotoxins that damage host cells, the release of siderophores that promote iron accumulation, the production of osmotically-compatible solutes that prevent dehydration, and the ability to form biofilms on the uroepithelium or urinary catheters [19]. Wang *et al.* [24] showed that resveratrol inhibited swarming and the formation of virulence factors in *P. mirabilis* and Torzewska and Rozsalski [25] found that it could inhibit urease activity and crystal formation by this bacterium. I have now looked in more detail at the effects of resveratrol on urease activity and biofilm formation for both *S. saprophyticus* and *P. mirabilis*.

2. Materials and methods
2.1 Chemicals and herbal extracts
Pure *trans*-resveratrol (FW = 228.24 g mol^{-1}) was obtained from Bulk Supplements

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(Henderson, NV, USA). Two commercial liquid preparations of resveratrol (Herbasway Laboratories Red Wine Alternative and Nature's Answer Alcohol-Free Resveratrol Reserve) were purchased from online distributors.

2.2 Bacterial strains, growth media, and growth conditions
S. saprophyticus strains ATCC 15305 was obtained from the American Type Culture Collection (Manassas, VA, USA). *Proteus mirabilis* strain BB2000 was part of a microbiology teaching collection. Bacteria were maintained on Difco™ tryptic soy agar (Becton, Dickinson and Company, Sparks, MD, USA). Liquid P medium pH 7.3 for *S. saprophyticus* was prepared as described by Gatermann *et al.* [21] and contained per liter: 10 g peptone, 5 g yeast extract, 1 g Na_2HPO_4, and 1 g D-glucose. Liquid LB medium for *P. mirabilis* contained per liter: 10 g tryptone, 5 g yeast extract, and 10 g NaCl; where necessary, it was supplemented with 100 mmol l^{-1} urea to induce urease activity. Mueller Hinton broth and urea broth were prepared from Difco™ dehydrated media as directed by the manufacturer. The artificial urine medium (AUM) described by Minuth *et al.* [26] contained per liter: 0.65 g $CaCl_2.2\ H_2O$, 0.65 g $MgCl_2.6\ H_2O$, 4.6 g NaCl, 2.3 g Na_2SO_4, 2.8 g KH_2PO_4, 1.6 g KCl, 1.0 g NH_4Cl, 12 g urea, 1.1 g creatinine, and 10 g tryptic soy broth. The medium was adjusted to pH 6.5 and sterilized by vacuum filtration through membrane filters (PES membrane, 0.22 µm pores, Genesee Scientific, El Cajon, CA, USA,). 50 mmol l^{-1} MES (2-(N-morpholino) ethane sulfonic acid) pH 6.5 was added to larger liquid cultures to improve the buffering capacity of the medium and increase the growth yields. Liquid cultures were routinely grown at 37 °C in flasks containing less than 10% of the total volume of medium and shaken at 250 rev min^{-1}. Turbidities in sidearm nephelometer flasks were measured in a Klett-Summerson colorimeter with a red (660 nm) filter.

2.3 Minimum inhibitory concentrations for resveratrol
S. saprophyticus strain ATCC 15305 or *P. mirabilis* strain BB2000 was grown overnight at 37 °C in 5 ml of P medium pH 7.3 or LB broth, respectively. The cultures were diluted 1 / 50 or 1 / 100 in sterile 0.85% (w / v) NaCl. A 10% (w/v) solution of resveratrol in dimethylsulfoxide (DMSO) was serially diluted into either Mueller Hinton broth or artificial urine medium to give test solutions containing 2000 µg ml^{-1} (8.76 mmol l^{-1}), 1000 µg ml^{-1} (4.38 mmol l-1), 500 µg ml^{-1} (2.19 mmol l^{-1}), 250 µg ml^{-1} (1.09 mmol l^{-1}), 125 µg ml^{-1} (0.54 mmol l^{-1}), 62.5 µg ml^{-1} (0.27 mmol l^{-1}), or 31 µg ml^{-1} (0.14 mmol l^{-1}). Three replicate 1 ml cultures at each concentration were inoculated with 50 µl of a diluted cell suspension and the tubes incubated at 37 °C for 24 hrs. The turbidity of each culture was determined at 600 nm in a Shimadzu UV-160 spectrophotometer and the average compared to cultures with no resveratrol. The minimum inhibitory concentration was defined as that concentration which inhibited growth by more than 90%.

2.4 Preparation of cell extracts
S. saprophyticus or *P. mirabilis* was grown to late exponential phase (75 to 100 Klett Units) in a total of 900 ml of P medium or in a total of 600 ml of LB medium containing 100 mmol l^{-1} urea at 37 °C with aeration. The bacteria were harvested by centrifugation at 10,000 *g* in a Bio-Lion XC-H165 centrifuge at room temperature, washed once with 0.85% (w/v) NaCl, and stored as a pellet at -20 °C. The cells were thawed and re suspended in 30 ml of urease re suspension buffer (50 mmol l^{-1} HEPES, 1 mmol l^{-1} Na_2EDTA, pH 7.5). The bacteria were combined with 0.1 mm glass beads and disrupted by 5 one-min cycles in a Bead-Beater® (Biospec Products, Inc., Bartlesville, OK, USA). After allowing most of the beads to settle, the remaining beads, unbroken cells, and debris were removed by centrifugation in a Bio-Lion XC-H165 centrifuge at room temperature for 5 min at 2,000 *g* and then for 10 min at 10,000 *g*. The supernatant fraction was saved as the cell extract and stored at -20 °C. Because the extract of *S. saprophyticus* was particularly active, it was diluted 1 / 3 with urease re suspension buffer before further use.

2.5 Preparation of whole cell suspensions
S. saprophyticus or *P. mirabilis* were grown to late exponential phase (75 to 100 Klett Units) in 10 ml of P medium pH 7.3 or LB medium containing 100 mmol l^{-1} urea at 37 °C with aeration. The bacteria were harvested by centrifugation at 10,000 *g* in a Bio-Lion XC-H165 centrifuge at room temperature, washed once with 0.85% (w / v) NaCl, and re suspended in 0.85% NaCl to give a suspension equal to 100 Klett Units. The suspensions were kept on ice until used in the urease assays.

2.6 Urease assays
Urease activity was determined using a colorimetric assay in which ammonium formation was measured by the phenol-hypochlorite method [27]. The absorbance of reactions was determined at 625 nm in a Shimadzu U-160 spectrophotometer and the ammonium concentrations calculated from an NH_4Cl standard curve. Samples of a *S. saprophyticus* or *P. mirabilis* cell extract or a whole bacterial cell suspension were added to a 50 mmol l^{-1} HEPES, 1 mmol l^{-1} Na_2EDTA, 25 mmol l^{-1} urea assay buffer, pH 7.5, without or with a particular amount of inhibitor, to give a total volume of 2000 µl (2.0 ml). Because DMSO is itself an inhibitor of urease activity [28], the resveratrol was dissolved in ethanol instead. In most cases, 10 to 20 µl of a cell extract were added to the 2.0 ml reaction mixture. For whole cell assays, 200 µl of exponential-phase cells were added to a 2.0 ml reaction mixture. After 30 to 90 min at 37 °C, three replicate 100 µl samples were removed and added to 1.5 ml of Solution A (1.0 g phenol, 5 mg sodium nitroprusside [sodium nitroferricyanide (III)] in 100 ml water). 1.5 ml of Solution B (0.5 g NaOH, 870 µl sodium hypochlorite in 100 ml water) were immediately added and the solution rapidly mixed. When all of the samples were collected, they were incubated at 37ºC for 30 min and the absorbance at 625 nm determined. All of the data reported were collected within the linear range of activity and replicate assays varies by <10%. Results were normally expressed as a percent of the control reaction ± one standard deviation of the replicate assays. All inhibition experiments were done at least twice.

2.7 Protein assays
Protein concentrations of the cell extracts and whole cell suspensions were determined by the Bradford bicinchoninic method [29] using bovine serum albumin as the standard.

2.8 Analysis of phenolic compounds
The concentrations of phenolic compounds in the two commercial preparations of resveratrol were determined using a previously-described method [30]. The preparations were diluted 1 / 100 or 1 / 300 with water and varying volumes of the diluted sample (5 µl, 10 µl, 20 µl, 50 µl, or 100 µl) were combined with water to give a total sample volume of 100 µl in a 12 x 75 glass tube. 800 µl of 5% (w / v) sodium carbonate

(Na_2CO_3) were added, followed by 100 µl of the Folin-Ciocalteu reagent. The reagent from Sigma Aldrich (P9252) was diluted 1/2 with water before use. The samples were mixed and incubated at 37 °C for 20 minutes. The absorbance of each solution was measured in a 1.5 ml semi-micro plastic cuvette at 750 nm in spectrophotometer. The absorbance values falling within the linear region of a resveratrol standard curve were converted to specific amounts and adjusted to concentrations. Two to four values were then averaged to get the mean concentration for the product tested.

2.9 Inhibition of bacterial growth in liquid cultures by resveratrol

S. saprophyticus strain ATCC 15305 or *P. mirabilis* strain BB2000 was grown overnight in P medium pH 7.3 or LB medium at 37 °C. Bacteria were diluted 1/100 into artificial urine medium pH 6.5 supplemented with 50 mmol l^{-1} MES pH 6.5 containing 0.25 mmol l^{-1} (57 µl^{-1}), 0.50 mmol l^{-1} (114 µg ml^{-1}), or 1.0 mmol l^{-1} (228 µg ml^{-1}) pure resveratrol. The cultures were incubated with aeration at 37 °C for 12 hr and the turbidities measured periodically. At the end of the growth cycle, the cultures were centrifuged for 10 min at 10,000 *g* in a Bio-Lion XC-H165 centrifuge at room temperature and the pH of the culture fluid determined.

2.10 Biofilm assays

Biofilm formation was determined using a modification of a previously-described method [31]. *S. saprophyticus* strain ATCC 15305 or *P. mirabilis* strain BB2000 was grown overnight at 37ºC in 5 ml of P medium pH 7.3 or LB broth, respectively. The cells were diluted 1 / 100 into 12 ml portions of the test medium containing 1 / 2 serial dilutions of resveratrol in DMSO. Because a microlitre plate reader was not available, 2 ml of the bacterial suspensions were added to 5 replicate wells in 24-well sterile polystyrene cell culture plates (TrueLineLab.com) and incubated for 24 h at 37 °C. The medium and unbound bacteria were removed by inverting the plates and dipping them three times in water. After draining on paper towels, 150 µl of 0.1% crystal violet were added to each well and allowed to sit for 20 min. The excess stain was removed by inverting the plates and dipping them three times in water. After draining on paper towels and drying overnight, 1000 µl of 30% acetic acid were added to each well and allowed to sit for 20 min. The absorbances of the solutions were determined at 590 nm in a Shimadzu U-160 spectrophotometer. When the crystal violet solution was very dark, it was diluted 1 / 4 in 30% acetic acid. To confirm that the bacteria could grow in the medium containing the concentration of resveratrol of interest, a portion of each cell suspension was incubated at 37 °C for 24 hr and the absorbance at 600 nm determined. All biofilm experiments were done at least twice.

3. Results

3.1 Minimum inhibitory concentrations for resveratrol

The minimum inhibitory concentrations (MIC) for resveratrol dissolved in DMSO were determined for *S. saprophyticus* and *P. mirabilis* in both standard Mueller Hinton broth and artificial urine medium. Based on the growth after 24 hr, the MIC for *S. saprophyticus* was 250 µg ml^{-1} (1.09 mmol l^{-1}) in both media while that for *P. mirabilis* was 500 µg ml^{-1} (2.18 mmol l^{-1}). Neither bacterium was very sensitive to resveratrol, although *P. mirabilis* was somewhat more resistant than *S. saprophyticus*.

3.2 Inhibition of urease activity in cell extracts by resveratrol

To determine if resveratrol can inhibit the urease activity from *S. saprophyticus* or *P. mirabilis*, cell-free extracts were prepared from each bacterium and tested for urease activity using a colorimetric assay in the presence of increasing concentrations of resveratrol. Because DMSO is itself an inhibitor of urease activity [28], the resveratrol was dissolved in 95% ethanol. The results were very similar in that activity gradually decreased at concentrations up to 5 mmol l^{-1} or 1140 µg ml^{-1} (Figure 1). The maximum inhibition observed was about 70%. Higher concentrations of resveratrol could not be tested because of its insolubility and color in the aqueous solutions.

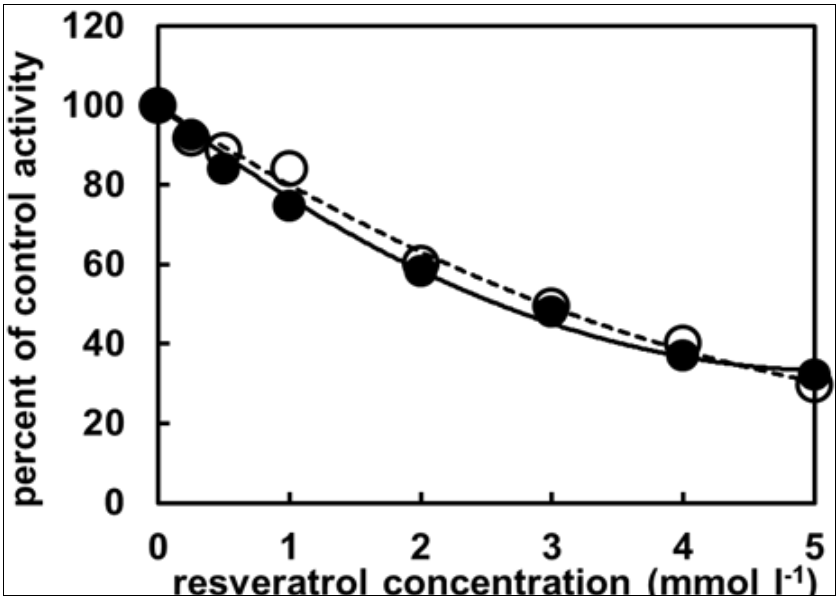


Fig 1: Inhibition of urease activity in cell-free extracts of *S. saprophyticus* (●) and *P. mirabilis* (○) by resveratrol. The extract from *S. saprophyticus* was diluted 1 / 3 before use and 10 µl added to a standard 2.0 ml reaction mixture. The extract from *P. mirabilis* was not diluted and 100 µl added to a standard reaction mixture. All reactions were run at 37 °C for 30 min. Data points shows the average values for three replicate assays that varied by <10%. The activities of the control reactions for *S. saprophyticus* and *P. mirabilis* were 1138 nmol min^{-1} ml^{-1} and 112.7 nmol min^{-1} ml^{-1}, respectively

To extend these results, the kinetics of urease activity in the cell-free extract of each bacterium were determined by varying the urea concentration from 1.25 to 25 mmol l^{-1} in the presence of 0, 0.5 mmol l^{-1} (114 µg ml^{-1}), or 1.0 mmol l^{-1} (228 µg ml^{-1}) resveratrol. The results were analyzed as Line weaver-Burke plots of 1 / *V* and a function of 1 / [*S*]. In case of the extract from *S. saprophyticus*, the results indicated a pattern of simple competitive inhibition in which the V_{max} remained the same and K_m was increased (Figure 2A). In the case of the extract from *P. mirabilis*, the results indicated a pattern of noncompetitive inhibition in which V_{max} decreased and Km remained the same (Figure 2B).

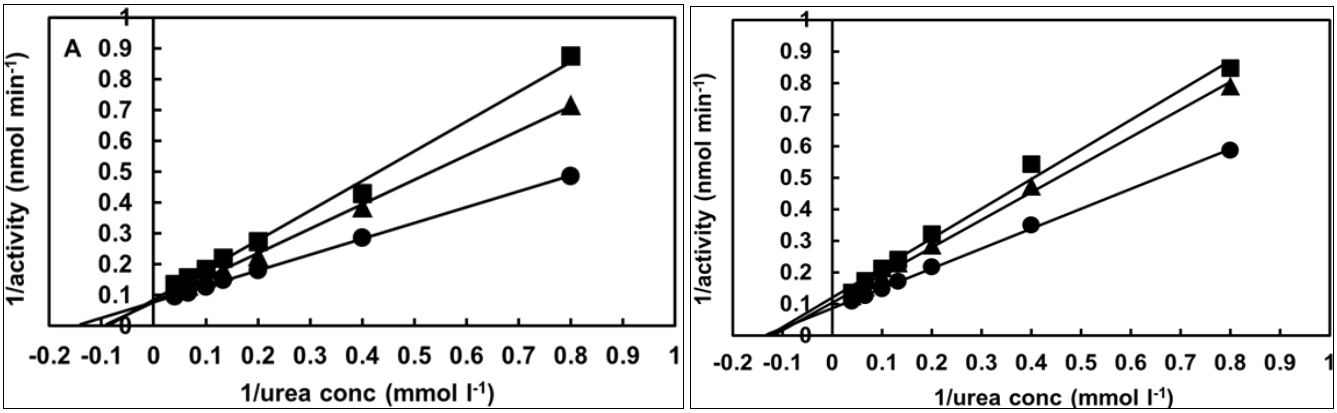
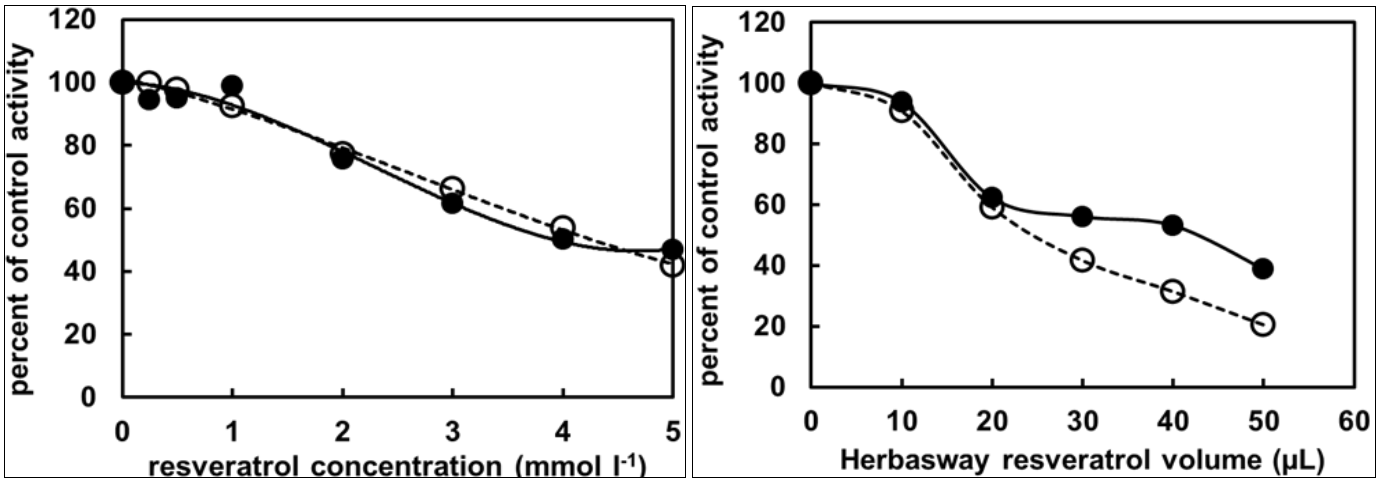


Fig 2. Kinetic analysis of urease activity in cell-free extracts of *S. saprophyticus* (panel A) and *P. mirabilis* (panel B). The reactions contained 0 (●), 0.5 mmol l^{-1} (▲), or 1.0 mmol l^{-1} (■) of pure resveratrol. The extract from *S. saprophyticus* was diluted 1 / 3 before use and 20 µl added to a standard 2.0 ml reaction mixture. The extract from *P. mirabilis* was diluted 2 / 3 before use and 15 µl added to a standard reaction mixture. All reactions were run at 37ºC for 30 min. Data points shows the average values for three replicate assays that varied by <10%. The activities of the control reactions for *S. saprophyticus* and *P. mirabilis* were 1053 nmol min^{-1} ml^{-1} and 1228 nmol min^{-1} ml^{-1}, respectively

3.3 Inhibition of urease activity in whole cells

The effects of pure resveratrol and two commercial preparations on the urease activity in whole cells of *S. saprophyticus* and *P. mirabilis* were determined. With pure resveratrol, there was again a gradual decrease in activity at concentrations up to 5 mmol l^{-1} or 1140 µg ml^{-1} (Figure 3A). The pattern of inhibition for the two bacteria was similar and the maximum inhibition observed was about 60%. Higher concentrations could not be tested because of the insolubility of the resveratrol.

Because many people use commercial preparations of resveratrol which are available in pharmacies or health food stores and from internet sources as liquids or capsules, two examples of liquid preparations were also tested as inhibitors of urease activity. With the Herb sway Laboratories Red Wine Alternative liquid product, there was a gradual decrease in urease activity for both *S. saprophyticus* and *P. mirabilis* (Figure 3B). The enzyme from *P. mirabilis* was more sensitive using inhibitor volumes up to 50 µl in a standard 2.0 ml reaction mixture. This preparation led to the formation of an orange color when samples of the reactions were combined with the reagents used to detect ammonium formation, this color gradually faded and was replaced by the typical blue color. The Nature's Answer Alcohol-Free Resveratrol Reserve liquid product was quite viscous and had to be diluted 1 / 3 with water before use as an inhibitor. In this case, the enzyme from *S. saprophyticus* was slightly stimulated by the preparation while the enzyme from *P. mirabilis* was gradually inhibited (Figure 3C). Again, an orange color initially appeared when samples of the reaction mixtures were combined with the reagent used to detect ammonium formation.



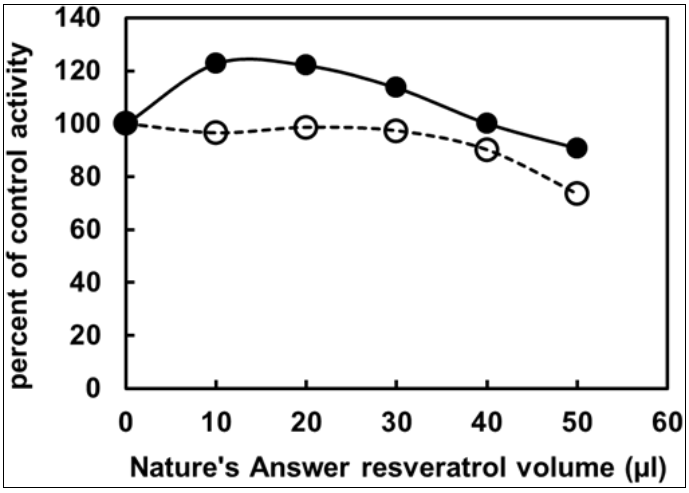


Fig 3: Inhibition of urease activity in whole cells of *S. saprophyticus* (●) and *P. mirabilis* (○) by resveratrol. Panel a shows the results for a pure sample of resveratrol dissolved in ethanol. Washed exponential-phase cells (200 µl) were added to a standard 2.0 ml reaction mixture and the reactions run at 37ºC for 90 min for *S. saprophyticus* or 60 min for *P. mirabilis*. Three replicate 100 µl samples were removed and tested for ammonium formation. Data points shows the average values for the replicate assays which that varied by <10%. The activity of the control reaction for *S. saprophyticus* was 21.8 nmol min^{-1} ml^{-1} and that for *P. mirabilis* was 13.3 nmol min^{-1} ml^{-1}. Panel B shows the results for reactions in which 0 to 50 µl of the Herbasway liquid preparation were added to the standard reaction mixture. Washed exponential-phase cells (200 µl) were added to a standard 2.0 ml reaction mixture and the reactions run at 37ºC for 45 min for *S. saprophyticus* or 60 min for *P. mirabilis*. The activities of the control reaction for *S. saprophyticus* was 36.7 nmol min^{-1} ml^{-1} and that for *P. mirabilis* was 13.1 nmol min^{-1} ml^{-1}. Panel C shows the results for reactions in which 0 to 50 µl of a 1 / 3 dilution of the Nature's Answer liquid preparation were added to the standard reaction mixture. Washed exponential-phase cells (200 µl) were added to a standard 2.0 ml reaction mixture and the reactions run at 37 °C for 45 min for *S. saprophyticus* or 60 min for *P. mirabilis*. The activities of the control reaction for *S. saprophyticus* was 33.8 nmol min^{-1} ml^{-1} and that for *P. mirabilis* was 25.2 nmol min^{-1} ml^{-1}

The actual amounts of resveratrol in the commercial preparations is not indicated on the labels so the concentrations were determined using a previously-described reaction with the Folin-Ciocalteu reaction [30] and a resveratrol standard curve. The Herbasway preparation gave a value of 224 mmol l^{-1}, so 50 µl in a standard 2000 µl reaction mixture would have an estimated concentration of 5.6 mmol l^{-1}. The Nature's Answer preparation gave a value of 462 mmol l^{-1}, so 50 µl of a 1/3 dilution in a standard 2000 µl reaction mixture would have an estimated concentration of 3.8 mmol l^{-1}. The varying effects of these preparations on urease activity compared to pure resveratrol may be due to other unidentified components in the products.

3.4 Effects of resveratrol on the growth and pH of cultures of *S. saprophyticus* and *P. mirabilis*

The presence of urease activity in bacterial cultures containing urea can lead to an increase in pH. To determine if addition of resveratrol to the culture medium affects the formation of urease in these bacteria, the cells were first grown in rich media without or with 125 µg ml^{-1} (0.54 mmol l^{-1}) resveratrol. The specific activity of urease was determined using the whole cell assay with exponential phase cells (Table 1). As expected, the formation of urease in *S. saprophyticus* was constitutive and did not require the presence of urea. The specific activity was not affected by the presence of resveratrol. The formation of urease in *P. mirabilis* was induced by the presence of urea but again the specific activity was not affected by the presence of resveratrol.

Table 1: Specific activities of urease in *Staphylococcus saprophyticus* and *Proteus mirabilis*

Bacterium	Medium	Resveratrol	Nmole min^{-1} (mg protein)$^{-1}$
S. saprophyticus	P medium pH 7.3	0	531
S. saprophyticus	P medium pH 7.3	125 µg ml^{-1}	525
P. mirabilis	LB	0	2.2
P. mirabilis	LB	125 µg ml^{-1}	1.0
P. mirabilis	LB + 100 mmol l^{-1} urea	0	266
P. mirabilis	LB + 100 mmol l^{-1} urea	125 µg ml^{-1}	261

To determine if resveratrol can affect the pH in media containing urea, *S. saprophyticus* and *P. mirabilis* that had been grown overnight in P medium pH 7.3 or LB broth were used to inoculate 3 ml portions of a standard urea broth containing 0, 62.5 µg ml^{-1} (0.27 mmol l^{-1}), 125 µg ml^{-1} (0.54 mmol l^{-1}), or 250 µg ml^{-1} (1.09 mmol l^{-1}) pure resveratrol. This is a phosphate-buffered medium containing 0.1 g l^{-1} yeast extract, 20 g l^{-1} urea (0.33 mole l^{-1}), and phenol red as a pH indicator [32]. The breakdown of urea to form of ammonium can raise the pH and lead to formation of an intense pink color. All of the cultures turned pink within 24 hr indicating no significant inhibition of urease activity.

To quantify these results, *S. saprophyticus* or *P. mirabilis* was grown in 10 ml portions of artificial urine medium pH 6.5 supplemented with 50 mmol l^{-1} MES. The cultures were supplemented with 0, 0.25 mmol l^{-1} (57 µg ml^{-1}), 0.5 mmol l^{-1} (114 µg ml^{-1}), or 1.0 mmol l^{-1} (228 µg ml^{-1}) pure resveratrol. The turbidities of the cultures were determined every hour

and the pH measured at the end of the growth cycle. For *S. saprophyticus* in artificial urine medium, there was a small increase in the lag phase as the resveratrol concentration was increased and a somewhat lower yield (Fig. 4 panel A). In 1.0 mmol l^{-1} (228 µg ml^{-1}) resveratrol, which was close to the MIC of 250 µg ml^{-1}, growth was noticeably reduced. However, the final pH of all of the cultures was about 8.5. For *P. mirabilis* in artificial urine medium, the results were similar but growth in 1.0 mmol l^{-1} resveratrol was not reduced as much as seen with *S. saprophyticus* (Figure 4 panel B). The final pH in these cultures was consistently about 9.0.

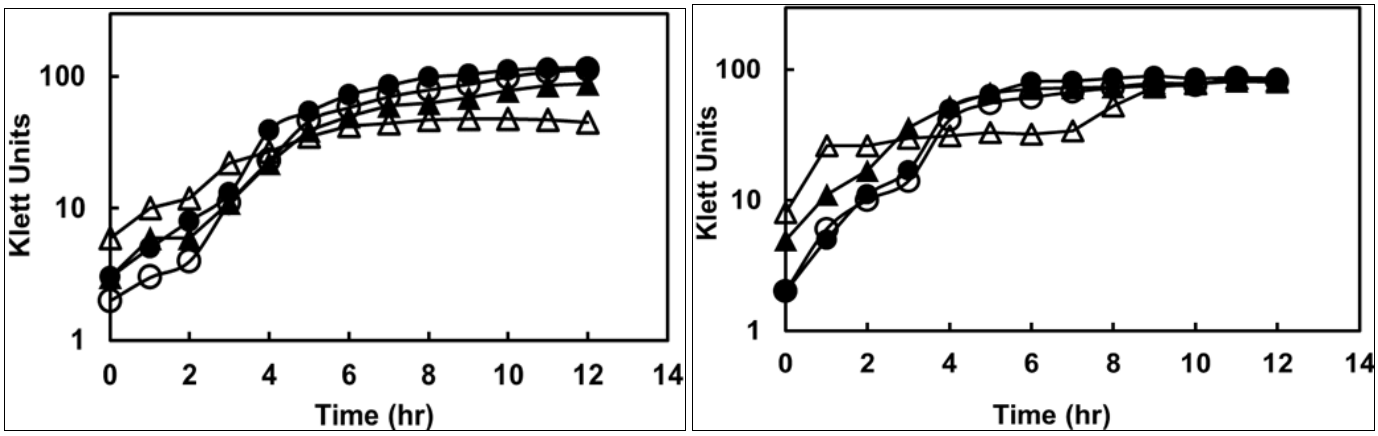


Fig 4: Effect of resveratrol on the growth of *S. saprophyticus* (panel A) and *P. mirabilis* (panel B) in artificial culture medium pH 6.5 containing 50 mmol l^{-1} MES. The cultures contain 0 (●), 0.25 mmol l^{-1} (○), 0.5 mmol l^{-1} (▲), or 1.0 mmol l^{-1} (△) pure resveratrol.

3.5 Effect of resveratrol on biofilm formation by *S. saprophyticus* and *P. mirabilis*

Another key virulence factor for *S. saprophyticus* and *P. mirabilis* is their ability to form biofilms on the urinary epithelium or urinary catheters. To determine if resveratrol can affect biofilm formation, the bacteria were grown in polystyrene plates and tested for biofilm formation with a standard crystal violet assay. For *S. saprophyticus*, addition of 125 µg ml^{-1} resveratrol to cultures in P medium pH 7.3 did reduce biofilm formation (Figure 5 panel A). Addition of 250 µg ml^{-1} had a more dramatic effect, although this was primarily due to an inhibition of growth. Biofilm formation for cultures grown in artificial urine medium pH 6.5 was noticeably less and only affected at the two highest concentrations of resveratrol. For *P. mirabilis*, biofilm formation in LB broth was reduced only at the highest concentration of 250 µg ml^{-1} (Figure 5 panel B). Biofilm formation in LB broth containing 100 mmol l^{-1} of urea was consistently lower and was gradually reduced as the resveratrol concentration was increased. There was very little biofilm formation when these bacteria were grown in artificial urine medium.

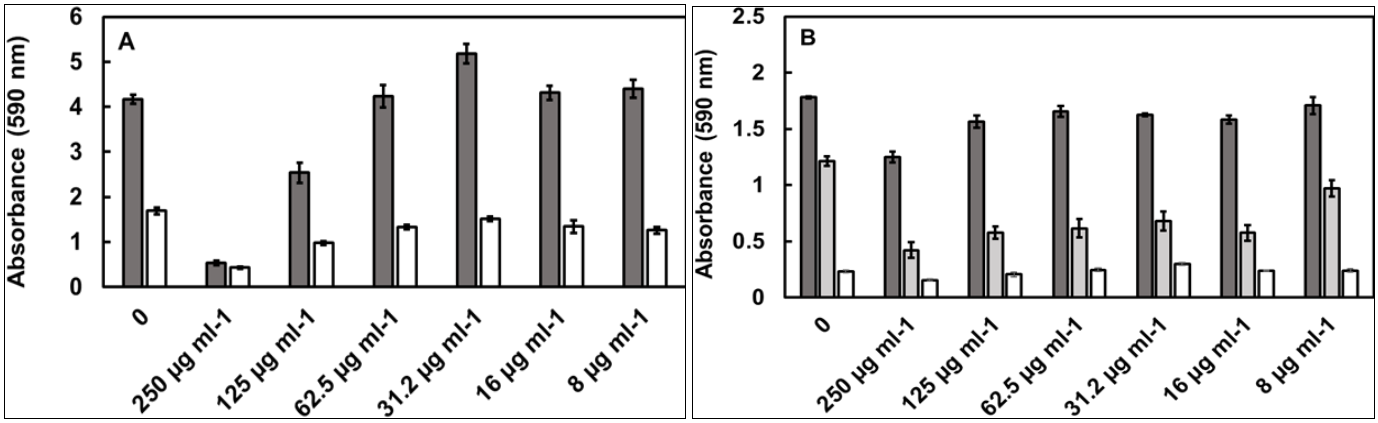


Fig 5: Biofilm formation by *S. saprophyticus* (panel A) and *P. mirabilis* (panel B). Data show the means and standard deviations of absorbance due to crystal violet binding to 5 replicate wells at each concentration of resveratrol. For panel A, the media were P medium pH 7.3 (dark gray bars) and artificial urine medium (white bars). For panel B, the media were LB (dark gray bars), LB containing 100 mmol l^{-1} urea (light gray bars), and artificial urine medium (white bars)

4. Discussion

There is a growing interest in the health benefits of medicinal plants, particularly those from South Asia and East Asia [33-34]. These plants contain chemicals with a wide range of chemical structures including terpenoids, phenolic compounds, alkaloids, and quinones, some of which can act as urease inhibitors [35]. Although resveratrol has been reported to have a wide range of health benefits [1], it has been less commonly studied as a treatment for urinary tract infections. It was found to inhibit urease activity in *P. mirabilis* [24-25] and to inhibit biofilm formation and other virulence factors in urease-negative uropathogenic *Escherichia coli* [8]. The experiments described here extend research in this area by focusing on the effects of resveratrol on both *S. saprophyticus* and *P. mirabilis*. Although the minimum inhibitory concentrations for these bacteria were relatively high and similar to those values previously reported [2-3, 5], resveratrol could inhibit urease activity in both cell extracts and whole cells. However, relatively high concentrations were required to inhibit activity more than 60%. While the ureases from these bacteria share

some common features including multiple subunits and the presence of Ni^{2+} at the active site [20], the inhibition kinetics were competitive for *S. saprophyticus* but noncompetitive for *P. mirabilis*. Resveratrol did not affect the formation of urease in these bacteria, which was constitutive for *S. saprophyticus* and inducible by urea for *P. mirabilis*. The inhibition of urease activity by resveratrol in whole cells was not sufficient to prevent the increase in pH that normally occurs as a result of urease activity. Resveratrol had limited effects on biofilm formation by these bacteria both in rich medium and artificial urine medium. The inhibition was most dose-dependent for *P. mirabilis* grown in LB medium containing urea.

Resveratrol is often promoted for its potential health benefits and widely available without prescription from online distributors and pharmacies [36-38]. Its value for the treatment of urinary tract infections is not well documented and may be due primarily to its general effects as an antioxidant or inhibitor of biofilm formation. The two commercial preparations tested here were found to be less effective than pure resveratrol as inhibitors of urease activity in whole cells. Two major limitations in the use of resveratrol are its limited binding to target proteins and low solubility and bioavailability in aqueous solutions. Kataria and Khatkar have studied the binding of natural phenolic compounds to urease and made a series of resveratrol derivatives with higher inhibitory activity [39-40]. These have not yet been tested with whole bacterial cells. Various investigators have attempted to improve the bioavailability of resveratrol by incorporating it into liposomes or nanoparticles or by combining it with proteins such as fibrinogen [41-42]. Many new derivatives of resveratrol with different ring substituents have also been synthesized by other investigators [43]. It will be important in future studies to test these compounds against the ureases from *S. saprophyticus* and *P. mirabilis* in both laboratory and animal model systems [44-45].

5. Conclusions

The experiments described here indicate that the pure resveratrol has some antimicrobial activity against the urinary tract pathogens *S. saprophyticus* and *P. mirabilis*. While it can inhibit urease activity in cell extracts and whole cells, it does not inhibit urease formation or prevent the increase in pH that occurs as a result of urease activity in media containing urea. It should therefore be used with caution as an over-the-counter treatment for urinary tract infections.

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7. Contributor Role

All of the experiments were designed and performed by Charles E. Deutch as was preparation of the manuscript.

8. Conflicts of Interest

The author declares there are no conflicts of interest.

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