International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
www.florajournal.com
IJHM 2020; 8(5): 116-124
Received: 19-07-2020
Accepted: 21-08-2020

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# Effects of over-the-counter herbal preparations on biofilm formation by the urinary tract pathogen *Staphylococcus saprophyticus*

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**Abstract**
*Staphylococcus saprophyticus* is a Gram-positive bacterium that causes urinary tract infections in children, adolescent and adult women, and older adults fitted with catheters. A key virulence factor is its ability to form biofilms on the uroepithelium and implanted devices. Biofilm formation in laboratory cultures of *S. saprophyticus* was measured after growth in polystyrene plates and staining with crystal violet. Biofilm formation by the type strain ATCC 15305 in enriched P medium or an artificial urine medium was inhibited by commercially-available samples of green tea extract, Uva ursi, turmeric, and cranberry but varied from one supplier to another. It was not inhibited by many other herbal preparations. Although biofilm formation was not reduced by purified gallic acid, catechin, epigallocatechin gallate, or arbutin, it was inhibited by purified curcumin. These results indicate that some caution should be used in selecting herbal preparations to treat urinary tract infections.

**Keywords:** Biofilm formation, herbal medicine, plant extracts, *Staphylococcus saprophyticus*

## 1. Introduction
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 [1-4]. The primary etiological agents are the Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* and the Gram-positive bacteria *Staphylococcus saprophyticus*, *Enterococcus faecalis*, and *Staphylococcus aureus* [5-7]. *S. saprophyticus*, an example of the coagulase-negative staphylococci (CoNS), is most commonly associated with community-acquired UTIs in young women [8-9] but it can also cause catheter-associated nosocomial infections [10].
Two key virulence factors for*S. Saprophyticus* are synthesis of the enzyme urease, anamidohydrolase (EC 3.5.1.5) that catalyzes the hydrolysis of urea to form ammonium ions and carbonic acid, and production of surface molecules that allow the bacteria to attach to host cells and form biofilms. In the case of urease activity, the ammonium ions raise the urinary pH and leads to the formation of urinary stones and catheter encrustations [11-12]. In the case of attachment and biofilm formation, the binding of the bacteria to uroepithelial cellsor implanted catheters allows them to evade host immunological responses and resist antibiotics [13-15]. *S. saprophyticus* can form several key surface proteins that may contribute to biofilm formation including an adhesive and autolytic protein called Aas [16], a collagen-binding protein designated SdrI [17], a surface associated lipase called Ssp [18], and a fibronectin-binding surface adhesin called UafB [19]. It also can make several surface polysaccharides including a capsular polysaccharide [20] and a polysaccharide intercellular adhesin (PIA) containing *N*-acetylglucosamine that is synthesized by the products of the *icaADBC* genes [21-25].
Although most UTIs can be treated with antibiotics [26], resistant microorganisms are frequently recovered from infected individuals [27] and recurrent infections are common [28]. Because antibiotics can be relatively expensive and may require prescriptions from physicians, there is a great deal of interest in alternative approaches to preventing or treating UTIs [29-33]. These include the use of over-the-counter plant preparations such as those derived from fruits like cranberries or various herbs [34-37]. A previous study indicated that the urease activity in *S. saprophyticus* could be inhibited by some over-the-counters herbal preparations that are often used for the treatment of urinary tract infections [67]. Green tea extract and *Arctostaphylos Uva ursi* (Uva ursi) extract reduced urease activity by more than 75% and slowed the increase in pH that occurs in artificial urine medium. However, many other preparation were weakly inhibitory or had no effect. Other investigators have found that cranberry extract [39], curcumin [40], or various natural products [41] may inhibit biofilm formation in *Staphylococcus* or other bacteria. The purpose of this project was to describe the effects of a wide range of herbal preparations on biofilm formation by *S. saprophyticus*.

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## 2. Materials and Methods

### 2.1. Bacterial strains and growth conditions

*S. saprophyticus* strains ATCC 15305, ATCC 35552, and ATCC 49907 were obtained from the American Type Culture Collection (Manassas, VA, USA). Bacteria were maintained on Difco™ tryptic soy agar (Becton, Dickinson and Company) and cultured at 37 ºC. Liquid P medium was prepared as described by Gatermann *et al*. [11] and contained per liter: 10 g peptone, 5 g yeast extract, 1 g $Na_2HPO_4$, and 1 g D-glucose. Liquid Luria-Bertani (LB) medium contained per liter: 10 g tryptone, 5 g yeast extract, and 10 g NaCl [42]. Liquid BHI + sucrose medium contained per liter: 37 g BBL brain heart infusion broth powder (Becton, Dickinson and Company) and 10 g sucrose. The defined staphylococcus medium was based on that of Townsend and Wilkinson [43] as previously described [44]. The artificial urine medium (AUM) used in these studies was based on the one described by Minuth *et al*. [45] and contained per liter: 0.65 g $CaCl_2.2\ H_2O$, 0.65 g $MgCl_2.6H_2O$, 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 pH was adjusted to 6.5and the solution sterilized by filtration. All liquid cultures were grown at 37 ºC and shaken at 250 rpm in flasks containing less than 10% of the total volume as medium.

### 2.2. Over-the-counter herbal preparations and purified chemicals

Table 1 lists the over-the-counter plant preparations initially tested as potential inhibitors of biofilm formation by *S. saprophyticus*. They were obtained from local natural food stores in the United States or from internet suppliers as alcohol-free products wherever possible. Purified (+) catechin, (-) epigallocatechin gallate, gallic acid, arbutin, and curcumin were obtained from Sigma-Aldrich and hydroquinone was purchased from Chemsavers. All of the chemicals were dissolved in distilled water except for curcumin which was dissolved in dimethylsulfoxide.

### 2.3. Biofilm assays

Biofilm formation was determined using a modification of previously-described methods [46-47]. Bacteria were grown overnight at 37 ºC with aeration in the test medium to be used. The cells were diluted 1/100 into 10 to 15 ml portions of the test medium containing 1/2 serial dilutions or small volumes of the extract or compound to be studied. Because a microtiter plate reader was not available, 2 ml of the bacterial suspensions were added to 4 to 5 replicate wells in24-well sterile polystyrene cell culture plates (TrueLineLab.com)and incubated for 24 h at 37 ºC. 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 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,

**Table 1:** Over-the-counter plant preparations used in these studies

| Name | Primary Plant Sources | Recommended Use |
|---|---|---|
| Sprouts Green Tea Herbal Supplement | *Camellia sinensis* | 30 drops (1 ml) in 6 ounces (180 ml) of cold or hot water |
| Nature's Answer Uva Ursi | *Arctostaphylos uva-ursi* | 28 drops (ml) in water |
| Native Remedies UTI Clear Herbal Supplement | Buchu (*Agathosma*sp.)<br>Bearberry (*Arctostaphylos* sp.)<br>Yarrow (*Achillea millefolium*)<br>Oatstraw (*Avena sativa*)<br>St. John's wort (*Hypericum perforatum*)<br>Echinacea (*Echinacea* sp.)<br>Bilberry (*Vaccinium* sp.) | 15 drops (0.5 ml) |
| Wish Garden UTI Herbal Supplement | *Arctostaphylos uva-ursi*<br>*Usnea* lichen<br>*Grindelia robusta*,<br>Cleavers aerials (*Galium aparine*)<br>Horsetails aerials (*Equisetum* sp.)<br>Corn silk (*Zea mays*)<br>Shepard'spurse aerials (*Capsella bursa-pastoris*) | 3 ml in water |
| Herb Pharm Horseradish Extract | *Armoracia rusticana* | 30-40 drops in water |
| Nature's Answer Cranberry Extract | *Vaccinium macrocarpon* | 56 drops (2 ml) in water |
| Nature's Answer Ginger Extract | *Zingerbar officinale* | 28 drops (1 ml) in water |
| Herb Pharm Horehound Herbal Supplement | *Marrubium vulgare* | 40 drops (1.5 ml) in 2 ounces (60 ml) of water |
| Kyloric Aged Garlic Extract | *Allium sativum* | 1/4 teaspoon (1.23 ml) |
| Nature's Answer Blueberry Fruit Supplement | *Vaccinium corymbosum* | 28 drops (1 ml) in water |
| Herb Pharm Goldenseal Respiratory System | *Hydrastis canadensis* | 20 drops (0.7 ml) |
| Herb Pharm Broccoli Extract | *Brassica oleracea* | 30 drops in water or juice |
| Nature's Answer *Gingko biloba* Extract | *Gingko biloba* | 28-56 drops (1 -2 ml) in water |
| Solaray Turmeric | *Curcuma longa* | 30 drops (1 ml) in 4 ounces |

| | | |
|---|---|---|
| Full spectrum Extract | | (120 ml) of water or juice |
| Herb Pharm<br>Cilantro Extract | *Coriandrum sativum* | 1 dropper (0.7 ml) in 2 ounces of water |
| Nature's Answer<br>Horsetail Extract | *Equisetum arvense* | 30 drops (1 ml) in 4 ounces<br>(120 ml) of water or juice |

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 preparation of interest, a portion of each cell suspension was incubated at 37 ºC for 24 hr and the absorbance at 600 nm determined. All experiments were done at least twice.

## 3. Results

### 3.1. Effect of growth medium on biofilm formation by *S. saprophyticus* strains

To prepare for these experiments, *S. saprophyticus*strains ATCC 15305, ATCC 35552, and ATCC 49907 were grown in various mediaand tested for biofilm formation using the crystal violet staining assay. ATCC 15305 is the *S. saprophyticus* type strain and strains ATCC 35552 and ATCC 49907 are control samples used in various clinical testing systems. Fig. 1 shows the means and standard deviations of the absorbances of the crystal violet solutions obtained for the three strains in five different media. *S. saprophyticus* strains ATCC 15305 and ATCC 49907 gave similar results but strain 35552 showed consistently less biofilm formation. The amounts of biofilm formed by strains ATCC 15305 and 49907 were highest in P medium, BHI medium containing 10% sucrose, the defined *Staphylococcus* medium, and artificial urine medium. The amounts of biofilm were lower when the bacteria were grown in LB medium and in tryptic soy broth/yeast extract medium. In general, there plicate wells in each set varied by less than 10%. However, there were some larger variations when larger stained aggregates were lost during the washing step and some daily variations between experiments. Because of the intrinsic variability in the biofilm assays, uninoculated medium and untreated control cultures were included in all experiments. For further experiments, *S. saprophyticus* ATCC 15305 was used as the test strain and enriched P medium and artificial urine medium chosen as the primary test media.

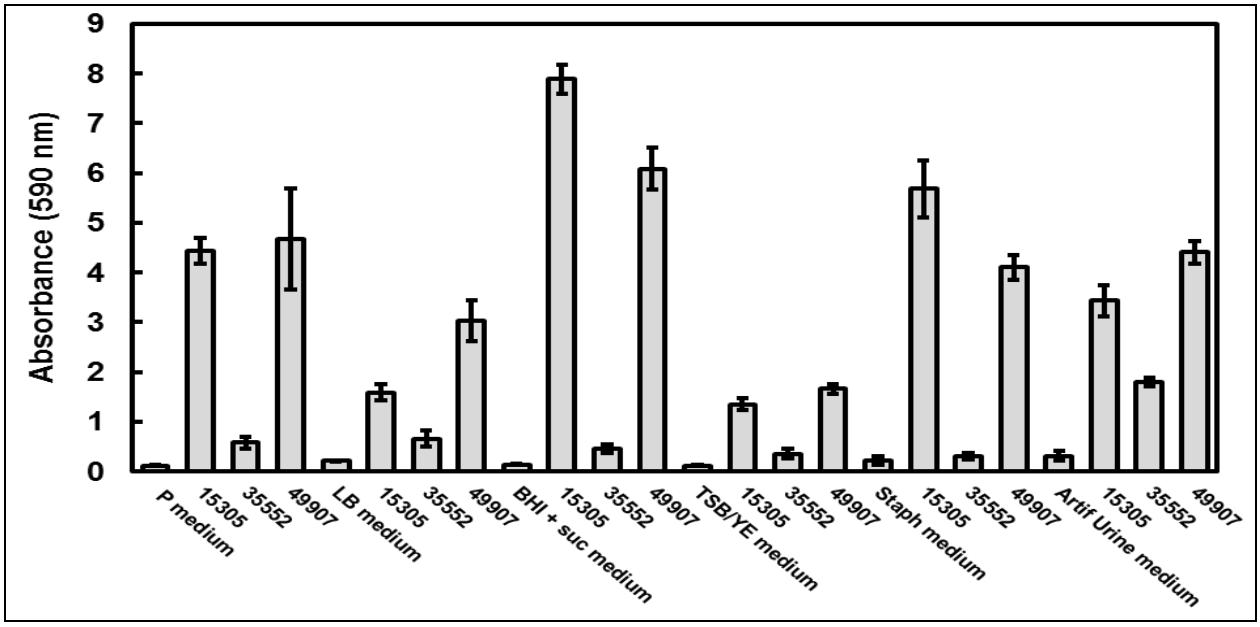


**Fig 1:** Biofilm formation by *Staphylococcus saprophyticus* strains ATCC 15305, ATCC 35552, and ATCC 49907 in five different media. Bars show the mean ± one standard deviation of the crystal violet absorbances of five replicates of each strain in each medium. The control values for the uninoculated media are given before each set of strains.

### 3.2. Inhibition of biofilm formation by over-the-counter herbal preparations

To determine if over-the-counter plant preparations might affect biofilm formation by *S. saprophyticus*, an initial set of preparations was added at a concentration of 0.5% to either P medium or to artificial urine medium. The amounts of biofilm formation were assessed by crystal violet staining after 24 hr (Fig. 2). With the enriched P medium, the most inhibition occurred with the Nature's Answer Uva ursiextract and the Solaray turmeric extract. There was partial inhibition with the Wish Garden Herbs UTI preparation, but most of the other preparations did not reduce biofilm formation. In some cases, the crystal violet absorbance in the presence of the preparation was higher than that of the control. This appeared to be a growth effect because many of the samples contained glycerol, honey, or other carbon sources. With the artificial urine medium, there was good inhibition with the Sprouts Farmer's Market green tea extract and the Nature's Answer Uva ursi extract, and partial inhibition by the Wish Garden Herbs UTI preparation. Most of the other preparations had no inhibitory effect or resulted in an increase in the crystal violet absorbance.

### 3.3. Variations in the efficacy of over-the-counter plant preparations

One of potential issues in the use of over-the-counter herbal preparations for the treatment of urinary tract infections is that there is no standardization of their content. The amounts of

key leaf or root components may be indicated in mg per serving, but the concentrations of specific compounds or other additives such as glycerol are not indicated on the labels. To compare the properties of different green tea, Uva ursi, turmeric, and cranberry preparations, three samples of each type were tested as inhibitors of biofilm formation at a concentration of 0.25% in either P medium or artificial urine medium (Fig. 3). There were marked differences between similarly-named products from different suppliers and some increased the absorbance of the crystal violet solutions after staining of the biofilms rather than decreasing it. For the green tea extracts, the Sprouts Farmer's Market sample inhibited biofilm formation in artificial urine medium but not in P medium. The Botanic Choice and Horbaach green tea extracts did not inhibit biofilm formation in either medium. For the Uva ursi extracts, the Nature's Answer preparation inhibited biofilm formation in both P medium and artificial urine medium. The Honey Combs Laboratories and Hawaii Pharm preparations did not inhibit biofilm formation in either medium. For the turmeric (curcumin) extracts, the Solaray and Herb Pharm preparations inhibited biofilm formation in P medium but the Hawaii Pharm extract was not inhibitory. The Herb Pharm preparation was more intensely yellow in color than the others and also caused partial inhibition in artificial urine medium while the others had no effect. For the cranberry extracts, the Nature's Answer preparation shown in Figure 2 and the Herb Pharm sample had no inhibitory effect in either medium. The Botanic Choice sample was more intensely red in color than the other samples and reduced biofilm formation in P medium but not in artificial urine medium.

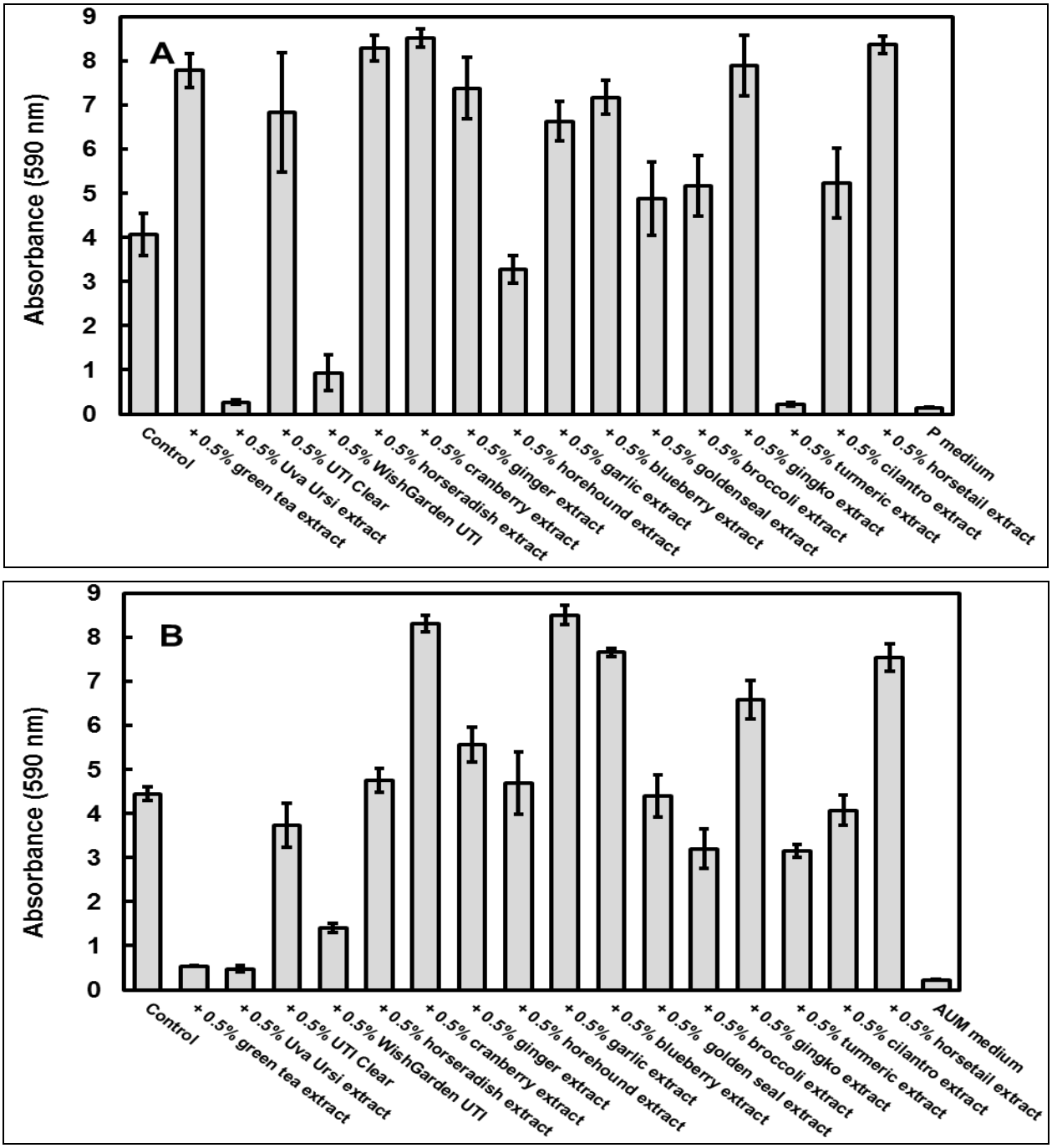


**Fig 2:** Effect of over-the-counter herbal preparations on biofilm formation by *S. saprophyticus* strain ATCC 15305. Bars show the mean ± one standard deviation of the crystal violet absorbances of five replicates in P Medium (Panel A) or artificial urine medium (AUM, Panel B) containing 0.5% of each plant preparation. The brand names of the products and their plant sources are listed in Table 1.

### 3.4. Determination of minimum inhibitory concentrations

To extend these results, serial 1/2 dilutions of the most effective preparations were tested as inhibitors of biofilm formation in P medium or artificial urine medium. The results for the Nature's Answer Uva ursi extract are shown in Fig.4 as an example. With P medium, there was a sharp transition between inhibitory and non-inhibitory concentrations; with artificial urine medium, there was a more gradual effect on biofilm formation. Other preparations showed similar differences. A minimum inhibitory concentration (MIC) was determined for each preparation, where the MIC was defined as the percentage of the extract needed to reduce the crystal violet absorbance to less than 25% of the control value. The results are summarized in Table 2.

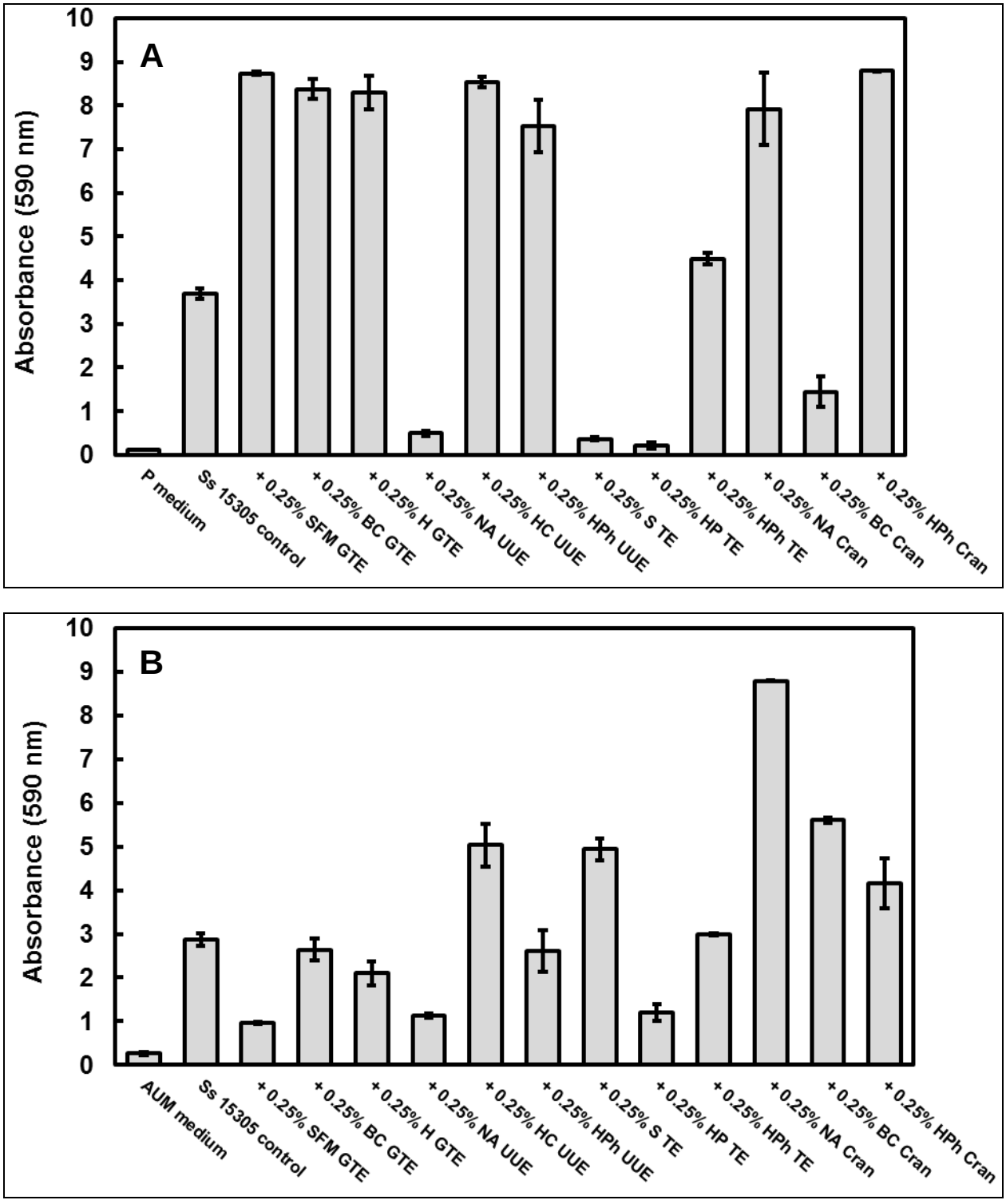


**Fig 3:** Effect of different samples of green tea extract, Uva ursi extract, turmeric extract, and cranberry extract on biofilm formation by *S. saprophyticus* strain 15305 in P medium (Panel A) or artificial urine medium (Panel B). Each sample was tested at a concentration of 0.25%. The samples were Sprouts Farmer's Market green tea extract (SFM GTE), Botanic Choice green tea extract (BC GTE), Horbaach green tea extract (H GTE), Nature's Answer Uva ursi extract (NA UUE), Honey Combs Uva ursi extract (HC UUE), Hawaii Pharm Uva ursi extra (HPh UUE), Solaray turmeric extract (S TE), Herb Pharm turmeric extract (HP TE), Hawaii Pharm turmeric extract (HPh TE), Nature's Answer cranberry extract (NA Cran), Botanic Choice cranberry extract (BC Cran), and Hawaii Pharm cranberry extract (HPh Cran). Bars show the mean ± one standard deviation of the crystal violet absorbances of four to five replicates.

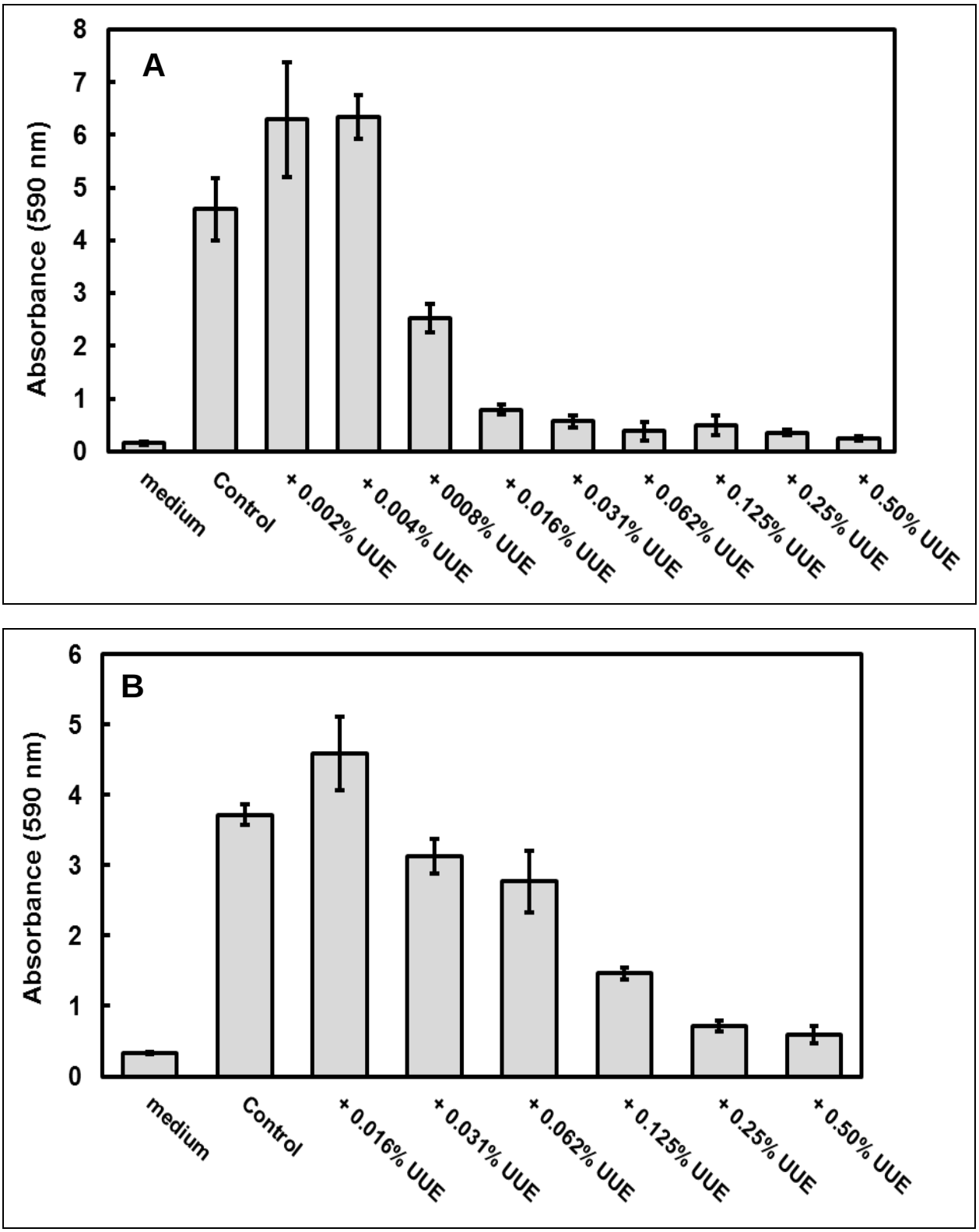


**Fig 4:** Effect of decreasing concentrations of the Nature's Answer Uva ursi extract on biofilm formation by *S. saprophyticus* strain ATCC 15395 in P medium (Panel A) or artificial urine medium (AUM, Panel B. Bars show the mean ± one standard deviation of the crystal violet absorbances of four to five replicates.

### 3.5. Inhibition of biofilm formation by specific chemicals

The plant extracts used in these experiments contain a wide range of organic compounds. Green tea extract contains catechin, epigallocatechin, and other compounds related to gallic acid [48]. Uva ursi extract contains many phenolic compounds [49] while turmeric extract from the roots of *Curcuma longa* contains curcumin as its primary active ingredient [50]. Cranberry extracts contain polyphenols, proanthocyanidins and anthocyanins [51]. The clinical effects of Uva ursi extracts have been attributed to arbutin (4-hydroxyphenyl-β-D-glucopyranoside. Arbutin undergoes hydrolysis to yield free D-glucose and hydroquinone, which may then undergo oxidation to form 1,4-benzoquinone (*p*-benzoquinone) [52-54]. When (+) catechin, (-) epigallocatechin gallate, gallic acid, arbutin, and hydroquinone were tested as inhibitors of biofilm formation at concentrations up to 1 mg/ml, there was some inhibition of growth or formation of dark colored products at the highest concentrations but no inhibition of biofilm formation as measured by crystal violet staining. On the other hand, purified curcumin did inhibit

biofilm formation in P medium with a minimum inhibitory concentration of8µg/ml. Consistent with the studies with the turmeric extract, curcumin did not inhibit biofilm formation in artificial urine medium.

**Table 2:** Minimum inhibitory concentrations of various herbal preparations for biofilm formation by *Staphylococcus saprophyticus*

| Preparation | P medium | Artificial Urine Medium |
|---|---|---|
| Sprouts Farmer's Market green tea extract | none | 0.25% |
| Nature's Answer<br>Uva ursi extract | 0.16% | 0.25% |
| Herb Pharm<br>turmeric extract | 0.125% | >0.5% |
| Botanic Choice<br>cranberry extract | 0.25% | none |

## 4. Discussion

Because urinary tract infections are so common, there has been great interest in finding new treatments for them including the use of herbal preparations. For such preparations to be effective, they must be safe, inexpensive, and target the specific pathogens involved. The goal of this project was to evaluate the effects of a wide range of herbal preparations on biofilm formation by the Gram-positive uropathogen *Staphylococcus saprophyticus*. The data shown indicated that there were variations in biofilm formation as measured by the crystal violet staining assay with the strain of this bacterium tested and with the growth medium used. Several recent studies have indicated there are important differences among strains of *S. saprophyticus* with respect to virulence genes and the proteins they encode [55-56]. These experiments focused on the type strain (ATCC 15305) but they cannot exclude the possibility that other strains may vary.

Of the various herbal preparations tested, the most inhibition of biofilm formation occurred with green tea extract, Uva ursi extract, turmeric extract, and cranberry extract. Most of the other samples had no inhibitory effect or actually stimulated biofilm formation. In some cases, inhibition of biofilm formation occurred in enriched P medium but not in the artificial urine medium used. The Nature's Answer Uva ursi preparation was the most effective one in that in reduced biofilm formation in both media. Samples of the four most effective herbal extractsobtained from different suppliers varied in their effects on biofilm formation. Some had no inhibitory activity and only the two turmeric (curcumin) preparations from Solaray and Herb Pharm both reduced biofilm formation inP medium. Of the commercially available purified compounds found in various herbal preparations, only curcumin reduced biofilm formation. These results were consistent with previous studies on the effects of green tea extract [57-58], curcumin [59-60], or cranberry [39] on cell adhesion or biofilm formation by other staphylococci. The effects of Uva ursi (*Arctostaphylos uva-ursi*) extracts on biofilm formation by *S. saprophyticus* has not been previously described but it has been reported to increase biofilm formation by *Escherichia coli* [61].

It is been suggested that urease activity in *Staphylococcus* can contribute to biofilm formation [62]. Urease activity in *S. saprophyticus* is constitutivein bacteria grown in both P medium and artificial urine medium and can be inhibited by specific chemicals such as acetohydroxamic acid and fluorofamide [63]. In experiments parallel to those reported here, there was no inhibition of biofilm formation in *S. saprophyticus* ATCC 15305 by acetohydroxamic acid (up to 400 µg/ml) or fluorofamide (up to 50 µg/ml). While some of the herbal extracts used in these studies such as green tea extract and Uva ursi extract could also inhibit urease activity (38), this did not interfere with their inhibition of biofilm formation. The relationship between these two virulence factors is thus still unclear.

Commercially-available herbal preparations are complex mixtures and it is not yet clear how some of them can inhibit biofilm formation by *S. saprophyticus*. Most recent studies of biofilm formation in staphylococci have focused on the polysaccharide intercellular adhesin (PIA) containing *N*-acetylglucosamine synthesized by the products of the *icaADBC* genes [21-25]. The complete genomic sequence of *S. saprophyticus* has been determined [64] and reported to contain these four genes [23, 65] and to form the polysaccharide intercellular adhesin [66]. While transcriptional analysis of these genes and characterization of the polysaccharide was beyond the scope of this project, it would be an important line for future investigations.

## 5. Conclusion

These experiments showed that biofilm formation by *Staphylococcus saprophyticus* in laboratory cultures can be inhibited by some over-the-counter herbal preparations but not others. Preparations with similar names and plant sources varied in their effectiveness. Some caution thus should be used in selecting a product to treat a urinary tract infection.

## 6. Acknowledgement

I thank Jacob Ramsey for his help with the preliminary biofilm experiments on *S. saprophyticus* as an undergraduate student at the West campus of Arizona State University.

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