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ORIGINAL PAPER

In vitro efficacy of phytotherapeutics suggested for prevention and therapy of urinary tract infections

Julian Marcon[1] · Sören Schubert[2] · Christian G. Stief[1] · Giuseppe Magistro[1]

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Abstract

Purpose To analyse the therapeutic efficacy of various phytotherapeutics and their antimicrobial compounds with regard to strain specificity and dose dependence.

Methods A representative strain collection of 40 uropathogenic bacteria isolated from complicated and uncomplicated urinary tract infection was subjected to various virulence assays (bacterial growth, mannose-sensitive agglutination, and motility) to determine the therapeutic impact of various compounds with antimicrobial activity. We tested proanthocyanidins (PAC), D-mannose, rosemary extract (Canephron®), and isothiocyanates (Angocin®).

Results D-mannose efficiently blocked the adhesive properties of all type 1 fimbriae-positive isolates in low concentration (0.2%), but showed no bacteriostatic effect. PAC also actively blocked agglutination, but the concentration varied considerably among isolates. *Escherichia coli* required the highest concentration (10%), while *Enterobacter cloacae* responded to low concentrations (0.1%). Allyl isothiocyanates not only impaired agglutination in all tested isolates, but also had a dramatic impact on flagella-mediated motility in *Escherichia coli, Klebsiella pneumoniae,* and *Proteus mirabilis* ($p < 0.001$). The administration of rosemary extracts revealed a strong bacteriostatic effect in growth assays. All tested strains were strongly inhibited by the addition of 10 µg/ml or 1 µg/ml of purified rosemary extractions with the exception of *Serratia marcescens. Morganella morganii* responded only to 10 µg/ml.

Conclusion Phytotherapeutics and small-molecular compounds like mannosides have the potential to become an integral part in a multi-modal treatment concept for the treatment and prevention of urinary tract infections. Their efficiency can be optimised when strain specificities and therapeutic concentrations are taken into account.

Keywords Urinary tract infections · Phytotherapeutics · Prevention · Therapy · Uropathogenic *Escherichia coli* (UPEC)

Introduction

Antimicrobial resistance (AMR) has emerged as one of the most challenging and threatening problems to public health on a global scale. If we do not change our practice today, we might be on the verge of a pre-antibiotic era scenario. Projections predict an alarming trend, with AMR as the number #1 death cause in the near future [1]. This especially holds true for the management of urinary tract infections (UTI), which are among the most common bacterial infections in clinical practice [2, 3]. Its socio-economic relevance is mirrored by the staggering financial burden to health care systems worldwide. Annual societal costs of 2.4 billion US dollars are caused by 1.3 million emergency room visits and 6.8 million office visits in the US alone [4]. Uropathogenic *Escherichia coli* (UPEC) are the main causative pathogens isolated from about 80% of uncomplicated UTIs [5]. Globally, the resistance rates for the treatment of UPEC are seriously high. Resistance rates for common antibiotic drugs, such as ciprofloxacin, trimethoprim/sulfamethoxazole, and aminopenicillins, reached 45, 48.2, and 50.4%, respectively [6]. Once, the antibiotic treatment of UTI was considered an easy and safe matter, but the rising AMR renders the management more and more challenging.

Now, we need to endorse action plans to prevent this critical development. The four most important cornerstones include the design of novel antibiotic agents, the

✉ Giuseppe Magistro
Giuseppe.Magistro@med.uni-muenchen.de

[1] Department of Urology, Ludwig-Maximilians-University, Marchioninistrasse 15, 81377 Munich, Germany

[2] Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Munich, Germany

development of alternative antimicrobial strategies, effective hygiene and preventive measures, and, finally, the concept of antimicrobial stewardship [7–11]. There has been a considerable developmental void for novel antibiotic agents in the last 3 decades. Consequently, we are in an urgent need for alternatives. Non-antibiotic approaches for the treatment or the prevention of UTI comprise a plethora of concepts. They are an integral part of current guideline recommendations now [12]. Hormonal agents, the use of probiotics, food supplements to modify the urine composition, the design of vaccines and immune-stimulatory compounds, intravesical instillations, and phytotherapeutics have been evaluated [13, 14].

Especially, the role of phytotherapeutics remains still elusive. Although certain underlying modes of action have been postulated, results have not been convincing in the clinical evaluation. For example, cranberry extracts have a proposed efficacy due to its high content of proanthocyanidins (PAC). These compounds interfere with the most prevalent and important adhesins of UPEC. Therefore, they appeared to be effective for the prevention of UTI when administered on a daily basis. In a first Cochrane review, it was concluded that cranberry products can significantly reduce the incidence of recurrent UTIs over 12 months (RR 0.65, 95% CI 0.46–0.90) [15]. However, a more recent Cochrane meta-analysis, including more clinical studies, withdrew the former recommendation due to the lack of positive trials [16]. The optimal formulations, dosages, or regimens have not been determined yet. Similarly, the spectrum of pathogens that might respond to treatment has not been identified. Exemplarily, not all uropathogenic bacteria express the potential targets addressed by proanthocyanidins.

This lack of information applies to almost all tested phytotherapeutics. This prompted us to evaluate the efficacy of various phytopharmaceuticals in a representative collection of different uropathogenic species isolated from complicated and uncomplicated UTIs. In this first in vitro study, we determined the impact of different compounds on relevant virulence features. When phytotherapeutics are considered, we need to first assure that the right targets are hit. These results are supposed to give first insights into the potential spectrum of pathogens appropriate for specific phytotherapeutic compounds.

Methods

Bacterial strains used in the present work were isolated from uncomplicated and complicated UTIs. The strain collection included *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter cloacae, Serratia marcescens, Citrobacter freundii, Morganella morganii,* and *Pseudomonas aeruginosa*. We subjected five isolates of each species to different virulence assays.

In this study, we tested proanthocyanidins, D-mannose, rosemary extract, allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), and phenylethyl isothiocyanate (PITC). The composition of the phytomedical compound Angocin® consists of 38% AITC, 50% BITC and 12% PITC. Concentrations were applied as indicated. The purified substances were all purchased from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

Three relevant virulence mechanisms for the development of UTI were assessed. We investigated adhesive properties mediated by type 1 fimbriae, flagellum-mediated motility, and bacterial growth. All experimental settings were performed according to previously published protocols [17, 18]. Briefly, mannose-sensitive adhesion of bacteria was assayed by the ability to agglutinate yeast cells (*Saccharomyces cerevisiae*) on glass slides [19]. The outcome is evaluated microscopically by the presence of agglutinated cells or loose cells (Fig. 1). Motility was analysed using 0.3% Luria–Bertani soft agar plates. A late logarithmic phase culture (optical density at 600 nm = 1.0; OD_{600nm}) was stabbed into the middle of a soft agar plate and incubated at 37 °C. Motility was quantified by measuring the diameter of motile bacteria after 8 h of incubation (Fig. 2a). The soft agar plates were supplemented with 5 µM AITC, 7 µM BITC, and 8 µM PITC. Growth curves were assessed in triplicates of 50 ml cultures in 250 ml Erlenmeyer flasks. Overnight cultures were incubated in 50 ml fresh Luria–Bertani medium to a starting OD_{600nm} of 0.05 and the optical density was recorded every 20 min. We used a rich medium to ensure optimal growth conditions and focused on the exponential growth phase, which is known to represent the fastest growth kinetics.

The selection of assays and inhibitory compounds was the results of an exploratory screening in concert with the published literature. Published data are mainly restricted to single species. In this manuscript, we evaluated the impact on various strains of different origin. Our aim was to highlight clearly defined effects. Data with an inconsistent outcome were not presented. All experiments were performed

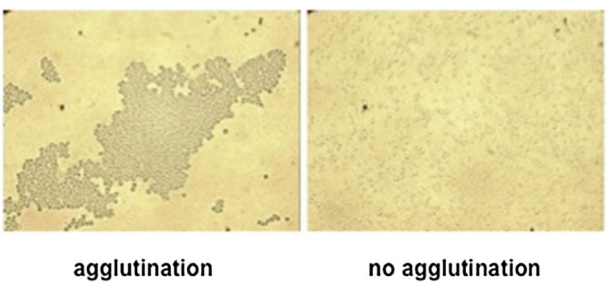

Fig. 1 Mannose-sensitive agglutination mediated by type 1 fimbriae. Microscopically examination of agglutinated cells

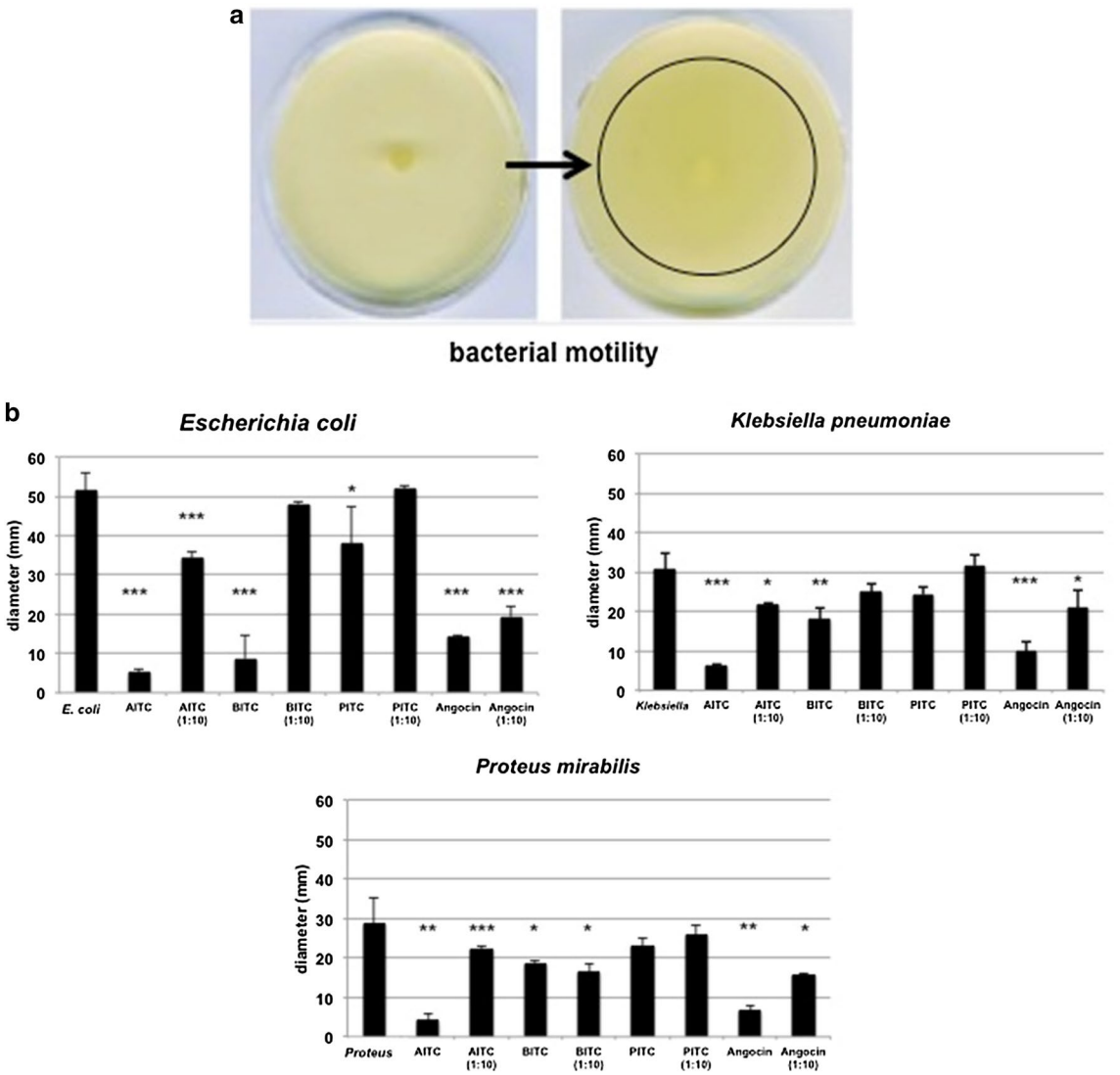

Fig. 2 Flagella-mediated motility. The front of swarming bacteria can be measured after spotting bacteria on the centre of a soft agar plate. Influence of isothiocyanates on the motility of uropathogenic bacteria. Allyl isothiocyanates had the strongest impact on flagella-mediated motility in *Escherichia coli, Klebsiella pneumoniae,* and *Proteus mirabilis*. Allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), phenylethyl isothiocyanate (PITC), Angocin® (38% AITC: 50% BITC: 12% PITC). $*p<0.05$, $**p<0.01$, $***p<0.001$

in duplicates and repeated at least three times. For statistical analysis, a paired *t* test or the Mann–Whitney *U* test was performed and results were considered statistically significant if the *p* value was lower than 0.05.

Results

Screening for anti-adhesive compounds

In our first screening, we investigated the response to various available phytotherapeutics and small molecules like D-mannose in an agglutination assay (Table 1). D-mannose in various concentrations (0.2%, 2%, 10%) was always able to inhibit agglutination of *E. coli.* Purified PAC, as the active compound in cranberry extracts, was able to interfere with agglutination only using the 10% solution. Among the isothiocyanates, only AITC efficiently blocked agglutination at low concentrations, whereas both BITC and PITC required higher doses of 10%. The combination of all isothiocyanates, as present in the phytomedical preparation Angocin®, displayed a strong inhibitory effect for every concentration applied. Various *Enterobacteriaceae* express type 1 fimbriae, but their role for the development of UTIs is unclear. We included additional type 1 fimbriae-positive isolates with the ability to agglutinate yeast cells. As observed for *E. coli*, D-mannose efficiently blocked agglutination in *Klebsiella pneumoniae, Serratia marcescens,* and *Enterobacter*

Table 1 Inhibition of agglutination mediated by type 1 fimbriae

	Escherichia coli	*Klebsiella pneumoniae*	*Serratia marcescens*	*Enterobacter cloacae*
Supplements (concentration, %)				
D-mannose				
0.2%	+++	+++	+++	+++
2%	+++	+++	+++	+++
10%	+++	+++	+++	+++
Cranberry (PAC)				
0.1%	---	---	---	+++
1%	---	---	+++	+++
10%	+++	+++	+++	+++
AITC				
0.1%	+++	---	---	+++
1%	+++	+++	+++	+++
10%	+++	+++	+++	+++
BITC				
0.1%	---	---	---	---
1%	---	---	+++	---
10%	+++	+++	+++	---
PITC				
0.1%	---	---	---	---
1%	---	+++	+++	---
10%	+++	+++	+++	---
AITC:BITC:PITC (Angocin®)				
0.1%	+++	---	---	+++
1%	+++	+++	+++	+++
10%	+++	+++	+++	+++

In this qualitative assay, the agglutination is determined microscopically

+++ positive inhibition; — no inhibition

cloacae, as well. However, their response to PAC and isothiocyanates differed considerably compared to *E. coli*. With regard to PAC, *Klebsiella pneumoniae* reacted similarly to *E. coli*. However, *Serratia marcescens* and *Enterobacter cloacae* were responsive to lower concentrations. Regarding the response to isothiocyanates, *Enterobacter cloacae* demonstrated inhibition with all dosages of AITC. However, neither BITC nor PITC showed an inhibitory effect. The mixed preparation of isothiocyanates blocked agglutination efficiently. *Klebsiella pneumoniae* and *Serratia marcescens* responded to AITC in higher concentrations. Only the highly concentrated blocking solution with 10% of BITC was efficient in *Klebsiella pneumoniae.* For *Serratia marcescens,* 1% of BITC was sufficient. In the case of PITC, the 1% blocking solution showed an inhibitory effect in *Klebsiella pneumoniae* and *Serratia marcescens.* Correspondingly, the 1% solution of the mixed isothiocyanate compound blocked agglutination activity. This first descriptive screening suggests that the overall response to anti-adhesive compounds depends on the specific species. A dose-dependant inhibitory effect can be observed, but this was different for every species.

Screening for substances interfering with motility

The ability to move to a more favourable niche is another relevant feature associated with bacterial virulence. Some uropathogens express the so-called flagella to ascend within the urinary tract [20]. Flagella-mediated motility can be assayed by spotting bacteria on a soft agar plate and by measuring the diameter of moving bacteria (Fig. 2a). We investigated the effect of isothiocyanates on motility in *E. coli*, *Klebsiella pneumoniae,* and *Proteus mirabilis* (Fig. 2b). *E. coli* displayed the strongest impairment by 5 µM of purified AITC, with a 90% reduction of motility ($p < 0.001$). For the 1:10 dilution, this effect was diminished to 33% ($p < 0.001$). The application of 7 µM BITC yielded a strong inhibitory effect, with an 83% decrease of motility ($p < 0.001$). Supplementation with 8 µM PITC led to a reduction of 25% ($p < 0.05$). The mixed preparation of

isothiocyanates decreased motility by 73% in its undiluted form and by 62% in a 1:10 dilution (both, $p < 0.001$). In *Klebsiella pneumoniae,* AITC was again the strongest inhibitor, showing a motility reduction of 79% ($p < 0.001$). BITC impaired motility by 41% ($p < 0.01$). PITC had no inhibitory effect at all. The Angocin®-like preparation impaired motility by 67% ($p < 0.001$). A similar pattern was also revealed for *Proteus mirabilis.* AITC and BITC decreased motility by 86% ($p < 0.01$) and 38% ($p < 0.05$), respectively. No impairment was detected using PITC. Mixed isothiocyanates reduced motility by 77% ($p < 0.01$). Isothiocyanates turned out to have a strong impact on flagella-mediated motility. The pattern was similar in various species, with AITC displaying the strongest inhibitory effect.

Screening for bacteriostatic agents

The next virulence assay evaluated the inhibitory effect on bacterial growth. We tested the influence of D-mannose and rosemary extracts in *E. coli, Proteus mirabilis, Enterobacter cloacae, Serratia marcescens, Citrobacter freundii, Morganella morganii,* and *Pseudomonas aeruginosa* (Fig. 3). We started to analyse the impact of D-mannose to rule out that our observations made for bacterial agglutination were due to a possible bacteriostatic effect. As depicted in Fig. 3, D-mannose did not affect bacterial growth of the screened isolates at all. The next step was the investigation of rosemary extracts, as they are proposed to mediate various antimicrobial effects [22]. The concentrations were 10 µg/ml and 1 µg/ml. With the only exception of *Serratia marcescens,* all included isolates were significantly impaired when the highest concentration of 10 µg/ml was applied. Even 1 µg/ml of rosemary extract was sufficient to inhibit bacterial growth in most strains, excluding *Serratia marcescens and Morganella morganii.* Again, our results do not speak for a consistent reaction to phytotherapeutics. Our observations rather suggest a differentiated response depending on the target and the concentration of the agent.

Discussion

One of the main questions to be answered is how to tackle the problem of AMR. To overcome this obstacle, we need to act on various levels. The development of novel antibiotic agents is one important mainstay, but it still remains a formidable task. Recently, the novel antibiotic zoliflodacin has been tested successfully for the oral treatment of urogenital gonorrhoea caused by antibiotic-resistant *Neisseria gonorrhoeae* in a phase 2 trial [23]. Novel antimicrobial combinations including ceftolozane/tazobactam, ceftazidime/avibactam, and meropenem/vaborbactam are additional examples that the efforts made in the last years are bearing fruits [24].

Another important aspect is the introduction of efficient vaccines and immune-stimulatory agents as a preventive measure. Uro-Vaxom® and Urovac®/StroVac® have been shown to reduce the UTI recurrence rate compared to placebo in clinical trials and meta-analyses [9, 25]. Newly designed vaccines, such as ExPEC4, are emerging and entering the stage of clinical evaluation [26]. The search for alternative antimicrobial compounds is attracting more and more attention. We strive for equally effective approaches without the risk of inducing antibiotic resistance mechanisms. The concept of anti-virulence treatment addresses the most important virulence factors of pathogens and turns them into strongly attenuated bacteria. Adhesion of the pathogen is one of the first essential steps in the pathogenesis of infectious diseases. In UPEC, up to 13 diverse fimbrial systems can be expressed [20]. Fimbriae are complex microbial structures on the surface with the ability to bind specifically to the host epithelium. Type 1 fimbriae are among the most important adhesins in UPEC. They mediate the first attachment to the urothelium by binding to mannosylated uroplakins. Mannose derivatives are known to block type 1 fimbriae [21]. They proved to be promising candidates for the treatment and prevention in the murine model of UTI [21, 27, 28]. Experimental data even suggest that the action of mannosides is not restricted to the urinary tract. They also appear to selectively deplete type 1 fimbriae-positive UPEC from the intestinal microbiota. These experimental results have also been confirmed in clinical trials. Daily administration of 2 g of D-mannose was equally effective as the antibiotic prophylaxis with nitrofurantoin regarding risk reduction of recurrent UTIs [29]. Our data support the concept of anti-virulence treatment using mannosides. No bacteriostatic or bactericidal effects were observed in the strains tested in this study. However, it was highly effective as a type 1 fimbriae-blocking compound. Our results in concert with published data suggest that, by the inactivation of relevant virulence factors of a pathogen, we are able to efficiently treat and prevent bacterial infections.

Phytotherapeutics might also contain antimicrobial compounds with therapeutic potential. As explained above for the use of cranberry products, they are not recommended due to the lack of positive trials. Our results demonstrate that PAC as the active compound is efficiently impairing the adhesive ability of bacteria. However, we clearly show that this effect is strain specificity and dose dependence. Only type 1 fimbriae-positive strains are responsive. With regard to UPEC, high concentrations were required for complete inhibition, whereas, in *Enterobacter cloacae,* low concentrations were sufficient. In clinical trials, the bacterial spectrum was rarely taken into consideration. With UPEC as the most common pathogen isolated from UTI, our data suggest that the concentration of PAC reached in the urine of patients with recurrent UTI might have been insufficient.

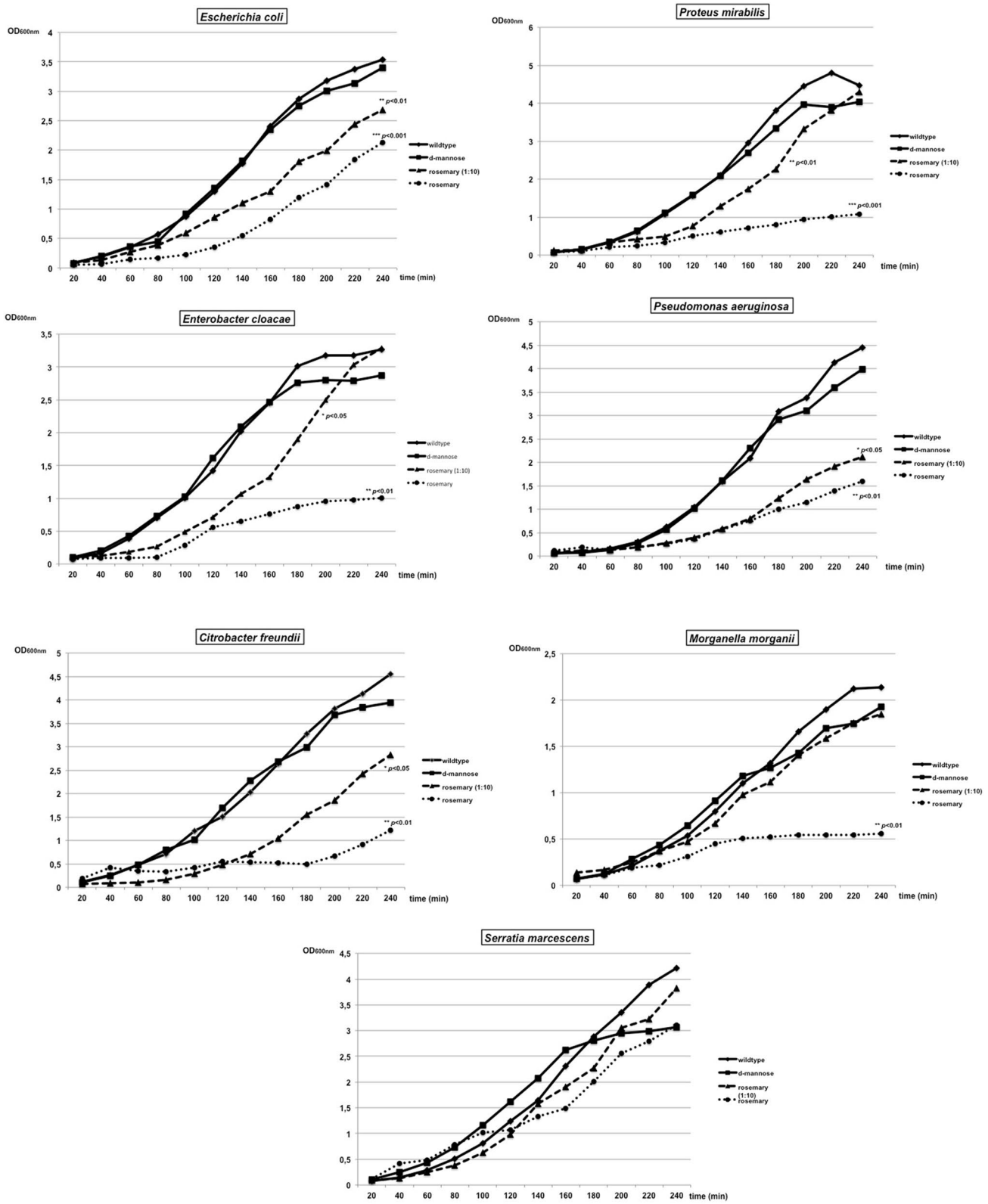

Fig. 3 Bacteriostatic effect of rosemary extracts. Impact of D-Mannose and rosemary extracts on the growth kinetics of various uropathogenic bacteria. OD_{600nm} (optical density at 600 nm). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Further RCTs are warranted to evaluate higher dosages of standardised PAC preparations. Phytotherapeutics containing rosemary extracts have recently been analysed in two RCTs for the treatment of uncomplicated cystitis [30, 31]. In the study by Wagenlehner et al., herbal treatment was non-inferior to the antibiotic therapy with fosfomycin, but higher rates of pyelonephritis were detected. Furthermore, Sabadash et al. reported that the addition of phytotherapeutics (Canephron®) to fluoroquinolones (ofloxacin) provided better relief of bothersome cystitis symptoms compared to fluoroquinolones only. Furthermore, they also reduced the rate of recurrent infection. The exact mechanism underlying this observation is not clear. Our results pinpoint towards a bacteriostatic effect of rosemary extracts, which again is strain specificity and dose dependence. All tested isolates responded to purified rosemary extracts except *Serratia marcescens*. For *Morganella morganii,* a bacteriostatic effect was also confirmed, but only at higher concentrations. Another interesting herbal combination containing nasturtium and horseradish indicated clinical efficacy for the treatment and prevention of recurrent UTIs [32, 33]. The high concentration of isothiocyanates is considered to play a relevant role for its therapeutic potential. We investigated the impact of isothiocyanates on flagella-mediated motility. Bacterial motility is another relevant virulence mechanism for the successful ascension and colonisation of the urinary tract [34–36]. Results of the present study provide evidence for a significant impact on motility. Especially, the addition of AITC showed the strongest reduction of bacterial motility in UPEC, *Klebsiella pneumoniae* and *Proteus mirabilis*. The antimicrobial properties of AITC also appear to have an activity against biofilms, which anticipates a potential impact on catheter-associated UTIs [37].

The main objective of the current study was to perform a systematic analysis of available phytotherapeutics with antimicrobial properties and their impact on specific virulence and fitness factors in a representative strain collection of uropathogenic bacteria. It was not the goal to decipher the exact mechanism underlying the effects observed in our assays on a molecular level. This study is descriptive in its nature. Nevertheless, this is the first analysis highlighting the strain specificity and dose dependence of phytotherapeutics. Not all species are responsive to the bioactive compounds in herbal therapeutics. The various therapeutic concentrations have to be considered, as well. These are two relevant aspects that have been largely neglected in clinical trials, which might be one reason for the failure of most studies. Here, we attempted to correlate the in vitro activity with the positive outcomes of clinical trials. Certain limitations of this study need to be acknowledged. This is a descriptive study analysing the antimicrobial activity under standardised in vitro conditions in a qualitative way. It was not the main goal to determine the minimum inhibitory concentrations under our laboratory in vitro settings. We have to admit that the pharmacokinetics of the substances used in this study is still unclear. We still do not know the dosage necessary to achieve a therapeutic concentration in the urinary tract. Further studies are warranted to clarify this essential aspect. Furthermore, our study does not claim to be exhaustive. We focused on Gram-negative bacteria only, but are aware that also Gram-positive pathogens like *Enterococcus spp.* are relevant uropathogenic bacteria. Accordingly, the phytotherapeutics tested in our analysis are not available in every country and more herbal combinations are on the market registered as food supplements. It is important to stress that no recommendation for clinical practice can be formulated based on these in vitro data. We believe that the therapeutic potential of standardised phytotherapeutics can be optimised when they are applied more specifically. Of course, RCTs are necessary to determine the true clinical value of this approach.

Conclusions

With the challenging problem of AMR still rising, we need to act on various levels to check its progress. Phytotherapeutics and small-molecular compounds like mannosides have the potential to become an integral part in a multi-modal treatment concept for the treatment and prevention of UTI.

Author contributions JM: project development, data collection and analysis, and manuscript writing. SS: supervision. CGS: project development and supervision. GM: project development, data collection and analysis, and manuscript writing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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