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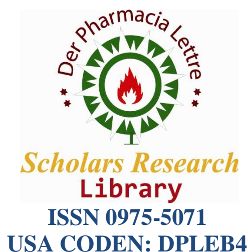

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Effect of ethanolic extract of *Scoparia dulcis* leaves on the virulence factors of uropathogenic *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Medicinal plants have been the source of various therapeutic agents since the ages. Among one of them is Scoparia dulcis, a commonly found herb in India that has been recognized for its immense potential in the treatment of diabetes. Though the plant has been used only for treating diabetes in general, it is also known to possess antibacterial properties on its own. In this study, we determined the effect of S.dulcis extract on major UTI (Urinary Tract Infections) causing bacteria Escherchia coli and Staphylococcus aureus. The effect of the plant extract on various virulence factors that are responsible for the pathogenicity of the uropathogens was analyzed. The bioactive compounds from the plant were extracted using ethanol. Virulence factors such as haemolysis, haemagglutination, proteolysis, lipolysis, cell surface hydrophobicity and gelatinase production were studied. It was observed that the extract inhibited almost all the virulence factors of both the pathogens. Based on the study it can be concluded that Scoparia dulcis is a potent inhibitor of both E.coli and S.aureus that affects their virulent factors that are involved in haemolysis, hameagglutination, proteolysis, lipolysis and gelatinase production.In future, the study can be extended to in vivo studies which will help in determining the efficiency of the plant extract in being used as a commercial drug against urinary tract infections.

Keywords: *Scoparia dulcis, Escherichia coli, Staphylococcus aureus,* uropathogens, virulent factors.

INTRODUCTION

Scoparia dulcis is an erect perennial herb that has serrated leaves and white flowers, measuring up to 0.5 meter in height after attaining full growth [1].The plant belongs to the neotropic regions that include South and Central America, Mexican lowlands, Caribbean islands and Southern Florida .in India, Taiwan, Brazil and Nigeria. Fresh or dried *Scoparia dulcis* plants have been traditionally used as remedies for stomach troubles, hypertension, diabetes, bronchitis and as analgesic [2]. The antimicrobial and antifungal effects of chloroform or methanol fractions of *Scoparia dulcis* have already been established. The plant extracts have been proved to be inhibitory for different bacteria like *Salmonella typhii, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Proteus vulgaris* and fungal strains such as *Candida albicans, Aspergillus niger, and Fusarium oxysporum* [3].

The pathogens causing urinary tract infections (UTI) are called as uropathogens. UTI has been found to be the second most widespread disease in hospitalized patients [4]. It is also known to be the most common bacterial infections that occur in people of all age groups. The occurrence of UTI is more common in women than in men and reports state that about 35% of healthy women possess the symptoms of urinary tract infection [5].

Among the pathogens that cause this disease, *Escherichia coli* is the most common species [6]. *Escherichia coli* are natural flora that is found in the bowel area of various species of animals and birds. Certain *E.coli* strains are capable of causing diseases due to the presence of virulence factors. The toxins that are synthesized by these bacteria for acquisition of pathogenic nature include colicin, enterotoxin, verotoxin and many others [7].

Staphylococcus aureus has been found to be the second most common bacteria that are involved in the process of urinary tract infections [8]. It is also reported to be the second most prevailing pathogen in the UTIs by Manikandan *et al.* [9] in India. These infections are seen to affect women in a much higher ratio when compared to men [10]. *S.aureus* is a common opportunistic pathogen that attacks both immune competent and immunocompromised persons, thus frequently resulting in high morbidity and with complications, which constitute challenges to health care institutions [11].

The other species that are responsible for causing these infections are *Proteus, Pseudomonas, Klebsiella* etc., [4].Our study focuses on the potential of *S.dulcis* extract to affect various virulent factors of the uropathogenic strains by conducting several comparative virulent tests.

There are several antibiotics available for urinary infections but the major problem is the emerging resistance of the uropathogens to the drugs [4].Hence it has become necessary to design novel drugs for the elimination of uropathogens.

MATERIALS AND METHODS

Preparation of leaf extract

Healthy, disease- free leaves of *S.dulcis* were isolated from the plant. The leaves were primarily washed in running tap water for 10 minutes and rinsed again in de-ionized water for a few minutes. After this, the leaves were washed using Tween 20 detergent to remove any surface dust. Then surface sterilization was done using sodium hypochlorite (1%) solution for few seconds and rinsed thoroughly with distilled water. The sterilized leaves were shade dried at $27 \pm 2\ ^{o}C$ for a few days. After drying the leaves were grinded to powder and stored for future use in a sealed container, in a cool, dark and dry environment [12].

The leaf extract was prepared by the method of Devendra *et al*., [13]. The dried leaf powder was soaked in ethanol at a concentration of 2 ml/g of powder for 2 days. The soaked powder was filtered using Whatman No.1 filter paper. The filtrate was dried to evaporate the solvent. A rotary vacuum evaporator was used for drying the filtrate while the powder was allowed to dry separately. A dark green paste was obtained after drying and this paste was re-suspended in DMSO.

Virulence tests

The uropathogens were obtained as clinical isolates from Madurai Medical College.

Haemolysis test

This test is performed to test the ability of the *S.dulcis* extract- treated bacteria to haemolyse the blood cells [14]. 5% blood agar was poured into the sterile petri plates. After solidification, the bacterial cultures were streaked onto the blood agar plates. The plates were incubated at 37˚C for 24 hrs. The plates were observed for haemolysis after the incubation period.

Haemagglutination test

This test is performed to test the ability of the *S.dulcis* extract- treated bacteria to agglutinate the red blood cells of higher organisms by rock slide method [14].

Gelatinase test

This test was performed to check the ability of the *S.dulcis* extract- treated bacteria to produce the enzyme gelatinase. Gelatinase enzyme is capable of liquefying the gelatin gel [14]. The sterilized gelatin agar (about 5 ml) was poured into the test tubes. The tubes were then inoculated with the pure and *S.dulcis* extract- treated cultures of *E.coli and S.aureus.* The gelatin tubes were then incubated at 37˚C for 24 hrs. After 24 hrs, the tubes were checked for any liquefaction. However before that, the tubes were kept in refrigerator at 4˚C for few minutes and then analysed for liquefaction.

Proteolytic test
This test is performed to check the proteolytic activity of the *S.dulcis* extract- treated bacteria [15]. The milk agar medium was poured into 4 petriplates (20 ml in each plate) and allowed to solidify for a few minutes. After solidification, both the pure broth cultures and *S.dulcis* extract-treated cultures of *E.coli and S. aureus* were inoculated into the plates. The inoculation was done by making a single streak at the center of the plate by using a sterile inoculation loop. The plates were incubated at 37˚C for 24 hrs.

Lipolytic test
This test is performed to check the lipolytic activity of the *S.dulcis* extract- treated bacteria. The lipolytic test was done using a modified method described by Md.Javed *et al*., [15].The tween 20 medium was poured into 4 petriplates under aseptic conditions. The medium was allowed to solidify. After solidification, agar wells were made using sterile microtips. The wells contained a positive control, negative control and extract-treated broth cultures of both the uropathogens in 3 different extract concentrations (25µl, 50µl and 100µl extract per 100 µl broth cultures). The plates were allowed for incubation at 37˚C for 24 hrs. After 24 hrs, the plates were observed for zones of clearance around the wells. The positive control is the antibiotic (chloramphenicol) treated uropathogen culture [15].

Salt aggregation test
The SAT (Salt Aggregation Test) is done to verify the ability of *S.dulcis* extract- treated bacteria to attach to the cell surfaces [14]. Sixteen slides were taken and cleaned properly using ethanol. One loopful of uropathogenic suspension (both pure and *S.dulcis* extract treated culture) in phosphate buffer was added to the slides followed by an equal volume of ferric chloride solution of 4 different molarities. The molarities that were taken were 0.4M, 0.8M, 1.2M and 2.4M. The slides were then rotated for a few minutes. Any clumping that is observed , will be taken as an indicator of a positive test.

RESULTS AND DISCUSSION

Virulence factors of the bacteria play a major role in determining whether an organism will invade the urinary tract and the level of infection acquired. Haemagglutination is a very important concept in terms of virulent factors of uropathogens. The colonization of the bacteria occurs due to the ability of adherence of bacterial cells to the urogenital tract. It further helps in the invasion of the urinary tract by the bacteria [14].

The rock slide method indicated that agglutination occured in non-treated cultures while it was inhibited in ethanolic *S.dulcis* extract- treated culture for both the strains *E.coli* and *S.aureus* (Fig 1). *S.dulcis* extract is capable of affecting the haemagglutination property of the UTI causing organisms probably by interfering with the activity of bacterial pili. The bacterial appendages that are responsible for haemagglutination are pili or fimbriae [16].

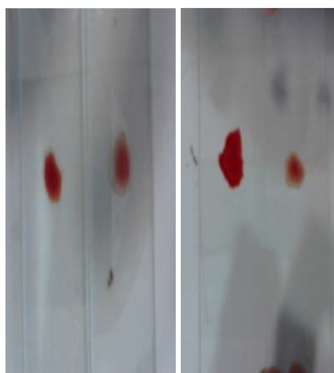

Fig 1 Haemagglutination observed for non treated and extract treated cultures of *E.coli* and *S.aureus*

Haemolysis takes place through hameolysin in the bacterial cells. This haemolysin is present in clinically important i.e more virulent strains of bacteria that cause serious UTI cases in humans. Haemolysin affects the host cell by causing inflammation of tisssues, injury of tissues and impairs the immune system [14]. According to the reports by Alexeeva *et al.,* [17] it was found that the haemolysin production is regulated by the the iron limitation or iron uptake mechansim. *Scoparia dulcis* extract inhibited haemolysis in both the strains (Figure 2).

Figure 2; Haemolysis observed for non treated and extract treated cultures of *E.coli* and *S.aureus*

Proteolysis takes place with the help of protease enzyme in the bacterial cells. This protease is responsible for hydrolyzing the peptide bonds and therefore breaking the proteins into their constituent aminoacid [18]. Milk agar was used to provide the protein necessary for the uropathogens to proteolyse. Multiple proteolytic events are organized into pathways. These events are also involved in pathogenesis, bacterial adaptation and biofilm formation [19].The non treated cultures of both the strains were proteolytic (Figure 3).The ethanolic *S.dulcis* extract- treated culture were deprived of their proteolytic capacity.

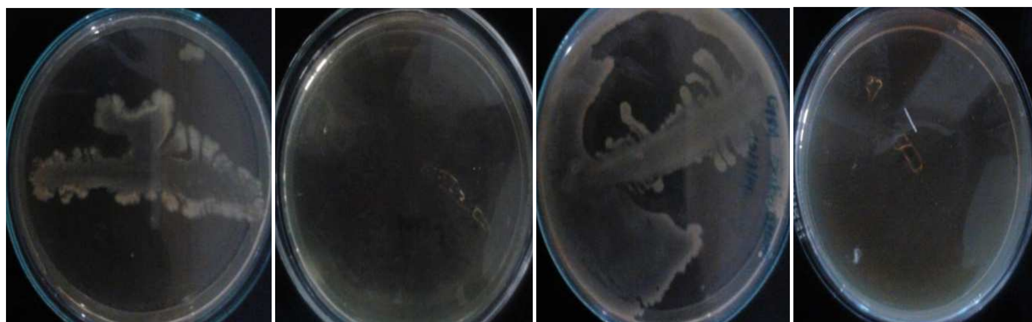

Figure 3; Proteolytic activity observed for non treated and extract treated cultures of *E.coli* and *S.aureus*

Gelatinase production has been observed as a virulence factor in various disease causing bacteria. This gelatinase belongs to a diverse group of proteinase called as metallo protease or metallo-endopeptidase. Gelatinase not only hydrolyses the gelatin but also several other compounds such as pheromones, casein, collagen etc. The bacterial gelatinase breaks down the gelatin to smaller units such as proteins, peptides and aminoacids. These smaller units can cross the cell membrane and can be utilized by the bacteria [20]. Gelatinase production occurs in humans also. This gelatinase is capable of breaking down the extracellular matrix. It is used in several processes such as embryo development, tissue repairing, reproduction etc. Abnormal production causes diseases such as arthritis and promotes metastasis in malignant tumors [20]. Thus impairing the production of abnormal levels of gelatinase has become a major concern in the area of cancer research. These enzymes have become drug targets now.

The non treated cultures of both the uropathogens liquefied the gelatin agar while the extract treated cultures were not able to liquefy gelatin (Figure 4). From this test, it is concluded that the *S.dulcis* extract is capable of impairing the gelatinase production in the uropathogens *E.coli* and *S.aureus*. Thus there is some agent capable of inhibiting the production of bacterial gelatinase, in the extract. This study could be further extended to screen for compounds capable of inhibiting the production of human gelatinase and thus produce a valuable drug in treating cancer.

Figure 4 Gelatinase activity observed for non treated and extract treated cultures of *E.coli* and *S.aureus*

The lipolytic activity of bacteria takes place with the help of lipase enzyme. This enzyme is involved in the hydrolysis of triacylglycerol to glycerol and fatty acids [21]. Moreover lipase is an economically important enzyme and so there are studies that are involved in the optimization of lipase production by bacteria [22]. Complete inhibition of the lipolytic activity of the strains occured.

The salt aggregation test can also be called as the cell surface hydrophobicity test [14]. Cell surface hydrophobicity is also responsible for the biofilm formation in bacteria and cells that are more hydrophobic in nature have more capability to form the biofilm. This virulence factor is being studied to exploit this capability for biodegradation [23].The aggregation results for SAT are presented in table 1.

Table 1 The observation for SAT for *E.coli* and *S.aureus*

Molarity of ferric chloride(M)	*E.coli*	*S.aureus*
0.4	-	-
0.8	-	-
1.2	-	-
2.4	+	+

The SAT test indicates the hydrophobicity of the cells. This is another main virulence factor that helps the bacterial cells to attach to the host cells such as epithelia cells [14]. In our study, it was found that the *S.dulcis* extract affected the cell surface hydrophobicity of the bacterial cells upto a molarity of 1.2M. However at a higher molarity of 2.4M there was visible aggregation of the cells in both treated and non treated cells.

Previous reports on the phytochemical screening of the ethanolic extract of the plant indicated the presence of tannins, flavonoids, phenolic compounds and terpenoids. These compounds contributed to the antibacterial activity of the plant extract on few Gram negative and Gram positive bacteria [12]. Reports also indicate the multiple expression of the virulence factors which act synergistically in establishment of UTI [14]. The ability of the extract to inhibit multiple virulence factors of both the uropathogenic strains establishes its role as a potent antibacterial compound. Farook et al [24] have demonstrated the inhibition of urolithiasis by the extracts of *Scoparia dulcis*. Hence our result provides further scientific support to the studies on activity of *Scoparia dulcis* on UTI.

CONCLUSION

From this study it was found that the ethanolic extract of *Scoparia dulcis* was capable of affecting six different virulence factors of *E.coli* and *S.aureus*. These factors include the haemolysis, haemagglutination, gelatinase production, lipolysis, proteolysis and cell surface hydrophobicity. Thus the plant is a potent inhibitor of UTI causing pathogens and so can be used to design novel drugs for the disease. This might help to encounter the problem of antibacterial resistance in the field of medicine. Further investigations on the characterization of the compounds in the extract would lead to the identification of the active principle involved in the inhibition of the virulence factors of the pathogenic strains.

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