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The Influence of Resveratrol on Swarming Differentiation and Expression of Some Virulence Factors of *Proteus Vulgaris*

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Abstract

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin with anti-inflammatory and antioxidant activities. It is produced naturally by several plants especially the roots of the Japanese Knotweed when under attack by pathogens such as bacteria or fungi. In this study we have confirmed that resveratrol has activity against Proteus vulgaris, an important pathogen infecting the urinary tract by investigating its effect on swarming and some virulence factor expression(haemolysin and urease). Swarming inhibition was limited on Luria Bertani agar with and without resveratrol and then bacteria was harvested to assay cell length and the producing of haemolysin and urease. Resveratrol significantly inhibited swarming and virulence factor expression but its effect on growth rate was not significant.

Keywords: Resveratrol, proteus vulgaris, phytoalexin, haemolysin, urease.

Introduction

Proteus vulgaris is a rod-shaped, Gram negative bacterium that inhabits in the intestine and cause intestinal tracts infection of humans and animals. It can also be found in soil, water and fecal matter. It is grouped with the enterobacteriaceae and as an opportunistic pathogen of human, it is known to cause urinary tract infections and wound infections[1]. The individuals has suffering hardship from urinary tract infections caused by *Proteus spp*. often develop bacteriuria, cystitis, kidney and bladder stones, catheter obstruction due to stone encrustation, acute pyelonephritis, and fever[2]. Various potential virulence factors including haemolysin, swarming, adhesions, proteases, and ureases, possibly responsible for the pathogencity of *P .vulgaris*. The expression of virulence factors and the ability to invasion the human urothelial cells is coordinately up regulated during swarming[3-5].

Resveratrol is a phytoalexin found in different plants like Peanuts, Cranberries, Grapes, Strawberries and wine, and some other botanical sources. The discovery of resveratrol was appeared in 1940, but the first study to show the useful effects of resveratrol on human health was conducted in the 1990. Since then, many papers have been published annually elucidating the interest of this molecule. Resveratrol has a wide range of biological activities and consequently it has many various targets and mechanisms of action. Resveratrol can block or slow the progression of several diseases; including cardiovascular, carcinogenic and neurodegenerative diseases[6,7]. Moreover, resveratrol has anti-inflammatory[8], antioxidant, and antimicrobial properties[8,9].

During the last century, antimicrobial agents have extraordinarily reduced the threats associated with infectious diseases. The use of these drugs, concerted with improvements in sanitation, housing and nutrition and the existence of comprehensive immunization programs, has permitted a radical reduction of untreatable infectious diseases, often fatal, contributing to increased life expectancy. However, the adaptation of microorganisms to antibiotics causes reproduction and persistence of drug resistance, currently a major public health problem, therefore it is urgent to discover new drugs gifted with antimicrobial activity[10, 11].

In recent years, an reproducible interest has been biologically active compounds including antioxidants from plants and other natural sources[12, 13]. Thus, resveratrol, in addition to the biological activities qualified above, has been the subject of study for its ability to stopped the growth of some pathogenic microorganisms such as Gram-positive and Gram-negative bacteria[14,15]. So, this study aimed to detect the effective resveratrol on the growth, swarming and virulence factor expression of *P. vulgaris*.

Material and Methods

Resveratrol Resveratrol was purchased from Indo fine Chemical Company. The purity of this commercial compound is 99%.

Bacterial isolate and growth conditions. *P. vulgaris*, which used in this study was isolated from a Patient with a urinary tract infection and identified by biochemical tests[16]. For the bacterial growth assay, *P. vulgaris* was cultured overnight at 37◦C in Luria Bertani(LB)broth, then diluted 1 in 100 in LB containing various concentrations of resveratrol (0,10,20,30,40,50, 60µg/ml) and the growth rate was monitored at 1h intervals. For swarming differentiation and virulence factor assays, LB agar plates containing various concentrations of resveratrol were inoculated centrally with 5µl of an overnight culture of *P. vulgaris* and incubated at 37◦C for 7h. Bacterial cells taken from swarming plates were suspended in 5ml of PBS; these cells were then used for morphology studies after Gram's staining, assays for urease and haemolysin production[4].

Swarming assay: The swarming migration distance assay was determined as described previously[4,5]. Briefly, an overnight *P. vulgaris* culture (5µl) was inoculated centrally onto LB swarming agar plates (2 %w/v) with different concentrations of resveratrol (0,10,20, 30,40,50,60µg/ml). The plates were incubated at 37ºC and the swarming migration distance was determined by checking the swarm fronts of the bacterial cells at 7h after inoculation.

Measurement of growth rates: Overnight *P. vulgaris* culture was diluted 1:100 in fresh LB broth consisting different concentrations of resveratrol (0, 10, 20, 30, 40, 50,60µg/ml). We also used other high concentrations of resveratrol (0.5, 1, 1.5,2, and 2.5, 3mg/ml) to determine the effect on the growth inhibition of *P .vulgaris*. The growth rate was monitored as OD_{600} at 1h intervals[5].

Measurement of cell length: Measurement of cell length was determined as described[5,17]. Briefly, 150µl of stationary-phase of bacterial LB cultures were diffusion onto LB agar plates without or with appropriate resveratrol and incubated at 37°C for different times. After incubation, cells from the entire surface of agar plates were harvested by washing into 5 ml of PBS. Bacterial cells were fixed constantly and subjected to gram stain (Ward's Science, USA), tested by light microscopy (Carl Zeiss, Germany) at a magnification of 100X, and digitalized by using a digital camera. The lengths of 100 cells in each sample were determined, and the average was calculated.

Haemolysin production assay: Haemolysin production was carried out by inoculating in blood agar medium containing 2% washed horse erythrocytes with bacterial cells taken after suspended in 5ml of PBS then incubated at 37◦C for 24h. The appearance of clear zone around the colonies referred to a complete hemolysis(β-hemolysis). The appearance of greenish zone around the colonies referred to a partial hemolysis (α-hemolysis), whereas no change of zone referred to non-hemolysis (γ-hemolysis)[4,18,19].

Urease production assay: The arrangement preparation of cells for urease assay was determined as described previously[3], in this test we inoculated the urea slant from bacterial suspension by streaking the entire slant surface, incubated the tubes with loosened caps at 37ºC then color change of medium was examined after 16h incubation. Urease production was indicated by changed medium color into pink color[20].

Statistical analysis: All the results present the average of three independent experiments. The data were presented as mean and analyzed by one-way analysis of variance with $P < 0.05$ being significant, calculated using the Graph Pad Prism 5 statistical software[21].

Results and Discussion

Inhibition of *P. vulgaris* swarming by resveratrol: After overnight incubation, the swarming behavior of *P. vulgaris* was monitored and we found that resveratrol has the ability to block the swarming migration of *P. vulgaris* in a dose dependent of the manner concentration of resveratrol product, (figure-1). The swarming behavior was significantly inhibited at concentrations as low as 20µg/ml and was suppressed completely at 60 µg/ml, (figure-1,2). The inhibitory effect of resveratrol on swarming might appear from a toxic effect on bacteria. To test this possibility, an overnight culture of *P. vulgaris* was inoculated into LB containing various concentrations of resveratrol and the growth rate of bacteria was monitored as shown in, figure-3. The growth rate of *P. vulgaris* was inhibited slightly but not significantly because it grew in all tubes regardless of whether resveratrol was present or not, indicating that resveratrol could inhibit swarming but not the growth in *P. vulgaris*.

Inhibition of cell length and virulence factor expression in *P. vulgaris* by resveratrol: Cell morphology was monitored after inoculation of an overnight culture of *P. vulgaris* onto LB swarming plates consisting various concentrations of resveratrol. As demonstrate in, figure-4, in the cases without of resveratrol product, the swarming cells were longer than the bacterial cells in the existence of resveratrol at the concentration 60µg/ml, suggesting that swarming differentiation was inhibited. The inhibition of differentiation started to be observed at a resveratrol concentration of 20µg/ml. Very few elongated swarming cells were observed at a resveratrol concentration of 50µg/ml. As the resveratrol concentration was increased to 60µg/ml, only short vegetative cells were observed. These results pointed that swarming differentiation of *P .vulgaris* was indeed inhibited by high concentrations of resveratrol, figure-5.

To study the production of haemolysin and urease in which it was also influenced by resveratrol, these products (haemolysin and urease) in *P. vulgaris* taken from LB agar plates consisting different concentrations of resveratrol that was determined, the production of virulence factors was not affected significantly at low resveratrol concentrations (0-40µg/ml) but was inhibited in the presence of increasing concentrations(50 and 60µg/ml), As shown in, figure-6.

Discussion: The emergence of bacterial strains that showed resistance to different antibiotics affect a major threat to public health. As a consequence for all of these situation, there is renewed interest in antibacterial targets which, by attenuating virulence, disrupt the capacity of pathogenic bacteria to cause infection[11]. The suggest of the present study was to examine the effect of plant extract (resveratrol) against uropathogenic *P. vulgaris*.

In this study, we found that resveratrol has the ability to inhibit *P. vulgaris* swarming significantly at lower concentration of 20 µg/ ml and inhibited swarming completely at 60µg/ml concentration (figure-1, 2). Also, it had the ability to suppress the production of virulence factors(haemolysin and urease) at concentrations of 50 and 60µg/ml (figure-6) but it did not significantly affect in the growth of bacteria at the concentrations up to 60 µg/ml (figure-3). While the resveratrol did not affect the viability of *P. vulgaris* at the concentration of 3mg/ml (data not shown). Based on this finding we concluded that the swarming ability of *P. vulgaris* is correlated with its ability to express virulence factors and these results were similar to those represented by Allison *et al.*[3, 22]; where it has been shown that swarming differentiation of *P. mirabilis* and expression of virulence factors, such as urease, haemolysin and protease, are coordinately regulated in *P. mirabilis*.

The possible mechanism by which resveratrol could inhibit *P. vulgaris* swarming and virulence factor expression is by acting as an inhibitor compound for bacterial quorum sensing(QS). QS is the term used for the phenomenon of cell to cell communication in bacteria using excrete chemical signaling molecules called auto inducers. As environmental conditions often change rapidly, bacteria need to respond quickly in order to survive. QS is the regulation of gene expression in response to fluctuations in cell-population density and it enables bacteria to coordinate their behavior. Gram-positive and Gram-negative bacteria use quorum sensing communication circuits to regulate a differ array of physiological activities. These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and bio film formation[23,24]. So, QS is considered a new target for antimicrobial therapy.

The continuing development of multiple-drug-resistant strains of bacteria has necessitated to finding new strategies for treating bacterial infections and the discovery of this wide spectrum of organisms use quorum sensing to control virulence factor production makes it an attractive target for antimicrobial therapy. Among all the possibilities to stopped the QS activity, the use of anti-QS compounds could be of great interest to prevent the bacterial infections[25,26]. Such anti pathogenic compounds, in otherwise the antibacterial compounds, didn't killed the bacteria or stop their growth and are assumed not to lead to the development of resistant strains[26,27]. Different mechanisms have been proposed to explain the interference of QS dependent processes by natural products. Some of these mechanisms are inhibition and stopping of signal molecule biosynthesis or AcylatedHomoserine lactones (AHL) QS auto inducers reception[28,29] and the enzymatic in activation and biodegradation of QS molecules[30]. Therefore, we can concluded that the inter eruption of QS control system has an anti-pathogenic effect and can be used in the treatment of bacterial infections. According to that, *Proteus spp.* warming and virulence factor expression are generally considered to be regulated through a QS system which requires the sensing and integration of different variety of environmental features, cell to cell and intracellular signals[31,32]. So the environmental factors changes or the presence of resveratrol in media of *P. vulgaris* has an effect on QS control system. The results from this study indicate that resveratrol has the probability to be an antimicrobial agent against *P. vulgaris* infection

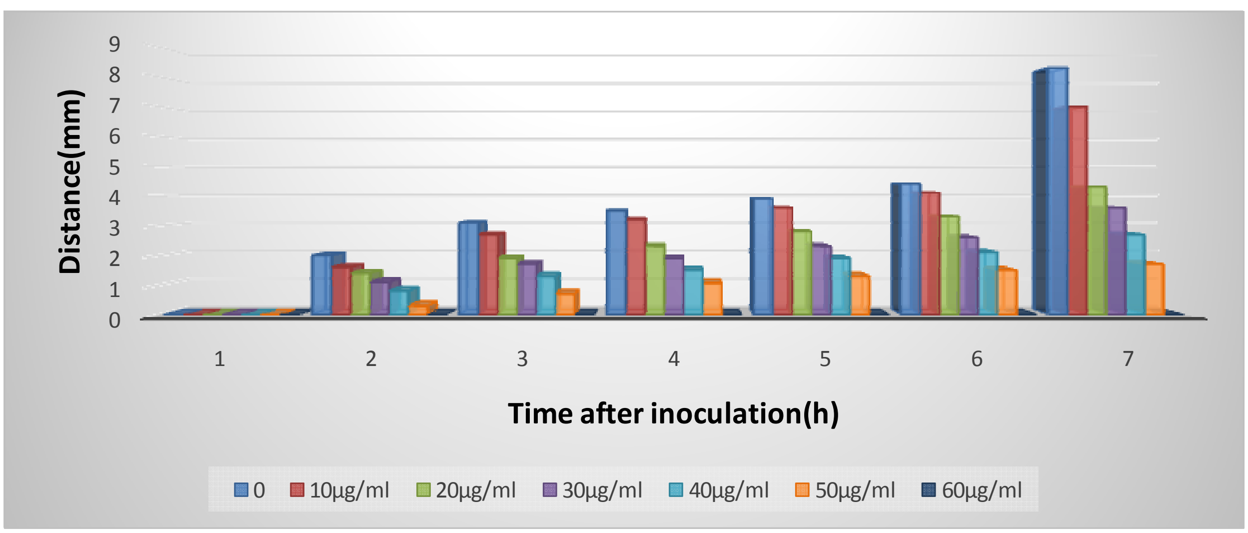
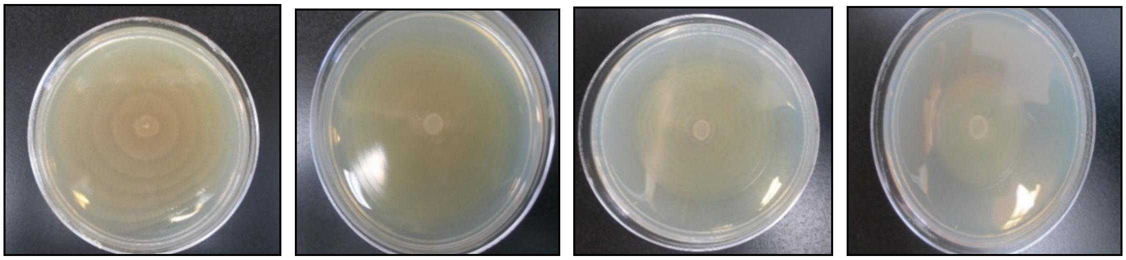
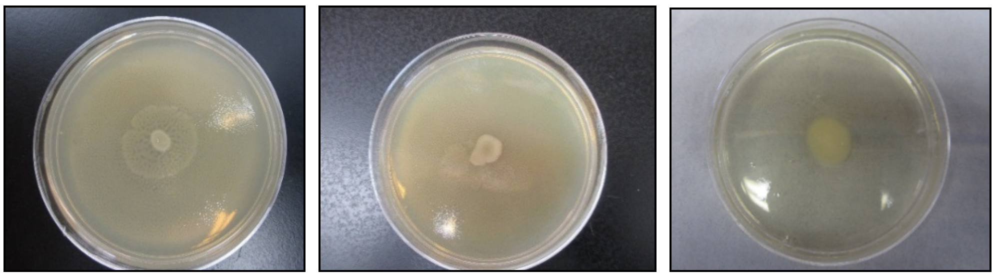


Figure-1

Effect of resveratrol on the swarming of *P.vulgaris*.The histogram shows the migration distance of *P. vulgaris* in the presence of various concentrations of resveratrol(0, 10, 20, 30, 40, 50,60 µg/ml). The data represent the average of three independent experiments and the differences are significant (P value <0.05)



0 10µg/ml 20µg/ml 30µg/ml



40µg/m 50µg/ml 60µg/ml

Figure-2

Halo images of swarming plates consisting of different concentrations of resveratrol (0, 10, 20, 30, 40, 50, 60 µg/ml) at 7h after inoculation

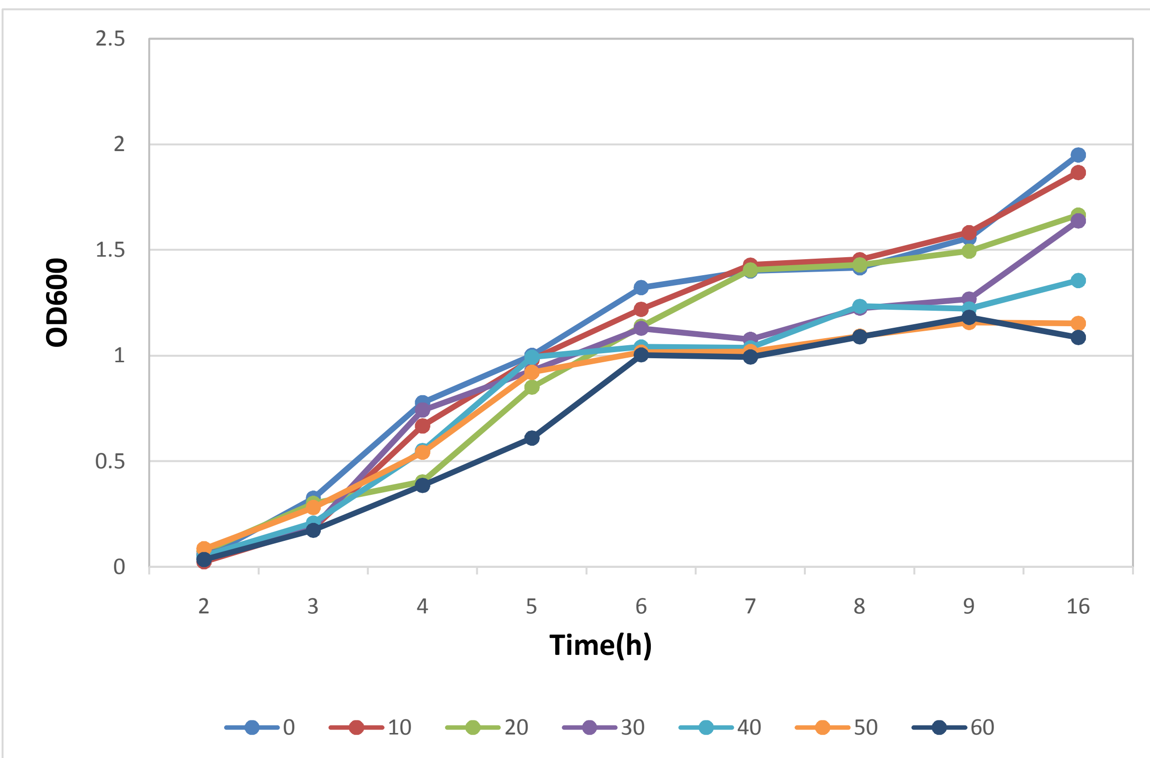


Figure-3

Effect of resveratrol on the growth of *P.vulgaris*.OD_{600} was measured overtime in the presence of different concentrations of resveratrol (0, 10, 20, 30, 40, 50, 60 µg/ml). The data represent the average of three independent experiments, there is no significant difference between concentrations (P value >0.05)

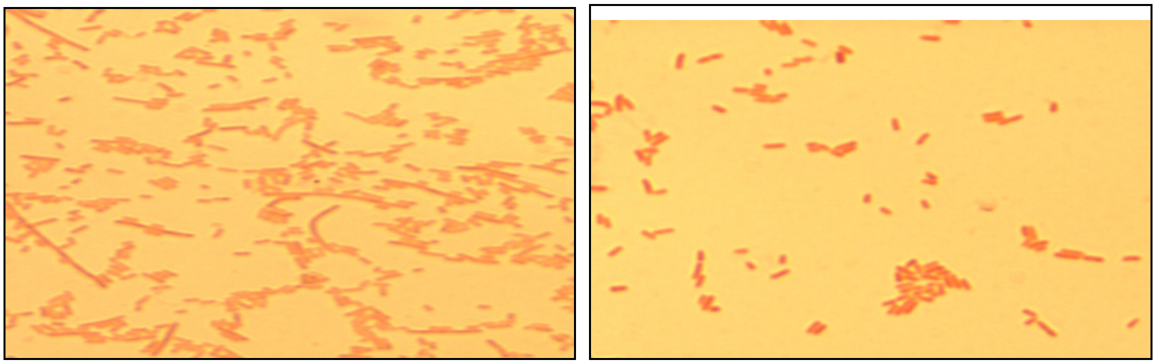


Figure-4
Microscopic observation of *P.vulgaris* isolated from the LB plates without resveratrol (-) and with resveratrol 60ug/ml(+)

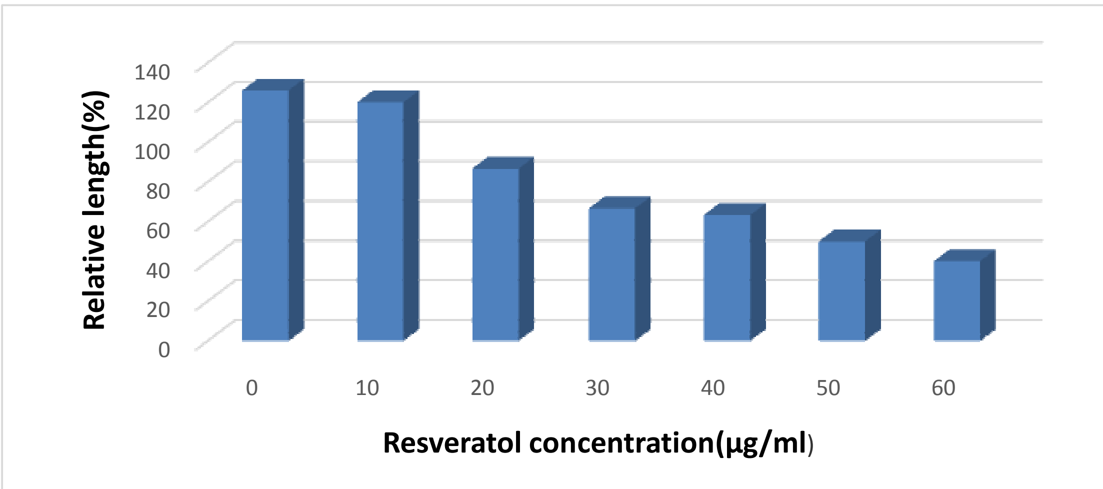


Figure-5
Effect of resveratrol on the cell length of *P.vulgaris*.The histogram shows the cell length of *P .vulgaris* in the presence of different concentrations of resveratrol (0, 10, 20, 30, 40, 50, 60µg/ml). The lengths of 100 cells in each sample were confirmed and the mean was calculated. The difference between concentrations is statistically significant (P value <0.05)

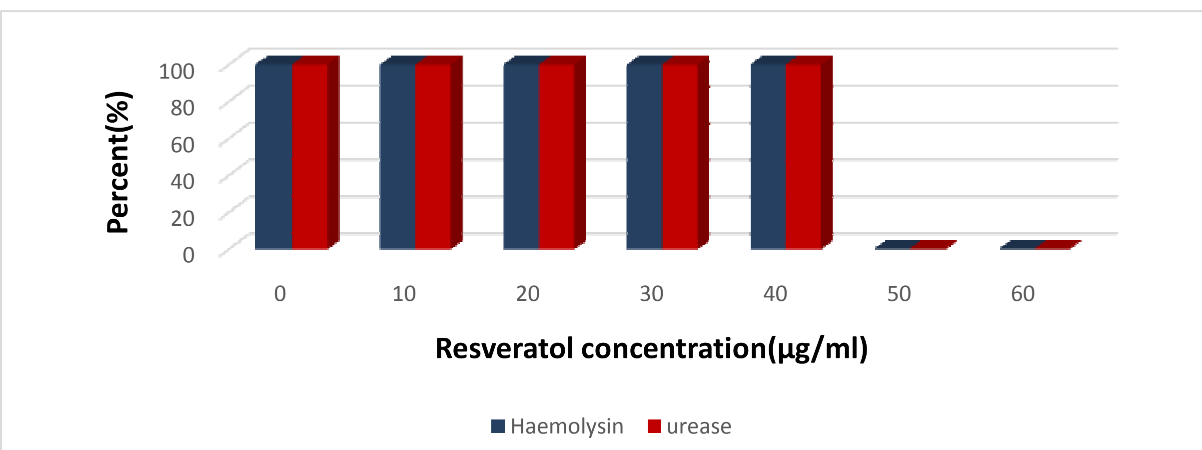


Figure-6
The influence of resveratrol on the expression of virulence factors in *P. vulgaris*. The histogram shows the production of haemolysin and urease at different concentrations of resveratrol (0, 10, 20, 30, 40, 50, 60µg/ml). The data present the mean of three independent experiments. The differences statistically significant (P value<0.05)

Conclusion

In this study we concluded that the swarming differentiation correlated with the ability of P. vulgaris to invade cells and to understanding the consequence of swarming motility inhibition and blocked the production of some virulence factors (haemolysis and urease enzymes by anti –swarming agent.

The high level of resveratrol concentration blocked the swarming migration of P. vulgaris bacteria and the surviving cells were more longer than bacterial cells in presence of high level concentration of this product. The effect of some anti-swarming agent on some virulence factors production was observed to cause marked reduction in our parameters.

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