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Effect of certain phytochemicals on *Aeromonas hydrophila*

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This study was aimed at investigating effect of different phytochemicals on growth of *Aermonas hydrophila*. Susceptibility of *A. hydrophila* to seven different phytocompounds was evaluated by broth dilution assay. Among all the test compounds curcumin with a minimum inhibitory concentration of ~175 μg/ml was found to be most effective against *A. hydrophila*, followed by tannic acid. Gallic acid failed to show any effect on growth of the test organism. A growth curve study in absence and presence of curcumin was also made. Since *A. hydrophila* is viewed as a challenging and notorious food-borne pathogen, and difficult to control owing to its resistance to many antibiotics, it is required to find novel approaches to control it. Our study found curcumin to be effective against this organism as a bacteriostatic agent at microgram concentrations.

Keywords: Curcumin, Tannic acid, Bacteriostatic, Minimum inhibitory concentration (MIC), Food-borne pathogen.

Aeromonas hydrophila is one of the most challenging, ubiquitous and opportunistic food borne pathogens. Due to its capacity to grow even at low temperatures (temperature range: -0.1 to 37ºC) it has major role in spoilage of packaged foods (Adams and Moss, 1995). It causes various diseases like endophthalmitis, gastroenteritis, cellulitis, meningitis, diarrhoea, etc. This gram-negative rod is also resistant to many antibiotics; hence it has become one of the most notorious foodborne pathogens to handle. It is required to develop novel approaches for controlling this organism (Patel *et al.,* 2010). Most significant feature with regard to any threat *A. hydrophila* may pose in foods is its ability to grow down to chill temperatures.

A. hydrophila is found to be associated with variety of extraintestinal infections like peritonitis, cholangitis, skin and soft tissue infections, pneumonia, meningitis, heamolytic uremic syndrome, myonecrosis, bacteremia, septicemia, eczema, and ocular infections. When *A. hydrophila* crosses the blood-occular barrier to reach the eye via blood stream, it causes a sight-threatening condition known as endogeneous endophthalmitis (Sohn *et al.,* 2007; Mukhopadhyay *et al.,* 2008). Ljungh *et al.* (1977) found *A. hydrophila* as causative agent of acute diarrhoeal disease.

A. hydrophila shows resistance to antibiotics such as cabenicillin, vancomycin, ampicillin, cephalothin, riphampicin, penicillin, cefoxitin, sulbactam, erythomycincefoxitin, bacitracin, and trimethoprim (http://en.wikipedia.org/wiki/Aeromonas-hydrophila; Palu *et al.,* 2006). Aforementioned properties of *A. hydrophila* justify consideration of this pathogen as worthy of attention as it is getting infamous for spoilage of various packaged food products and also in causing gastrointestinal infections. Controlling this organism with conventional chemo-therapeutic agents is not easy due to its resistance to many of them. Hence it is necessary to find novel leads or to evaluate known but hitherto untested agents against this organism. More investigation is warranted to control the growth of this challenging pathogen in order to avoid spoilage of frozen packaged food products and preclude various gastrointestinal problems associated with *A. hydrophila* infection (Patel *et al.,* 2010).

We challenged *A. hydrophila* with various phytochemicals- gallic acid, quercetin, rutin, caffeine, curcumin, tocopherol, and tannic acid- to see their effect on its growth. Phytochemicals are structurally distinct from microbially derived antibiotic natural products, it is likely that this chemical uniqueness will give rise to classes of antibacterials which have modes of action distinct from existing compounds (Gibbons, 2008). Gallic acid and tannin in extracts of *Syzygium cumini* bark has been suggested to be responsible for its antibacterial activity (Sharma *et al.,* 2009). Mingyu and Zhuting (2008) reported quercetin in lotus leaves as a component that may be a potential antibacterial agent. Gallic acid and quercetin have also been indicated as antibacterial constituents in *S. cumini* seed extracts (Kothari, 2011).

Materials and Methods

Materials

Test organism: *Aeromonas hydrophila* (MTCC 1739) was procured from Microbial Type Culture Collection, Chandigarh, India.

Phytochemicals: Quercetin, tannic acid (S-d fine chemicals, Mumbai), caffeine, rutin, curcumin (Central drug house, Mumbai), gallic acid (SRL, Mumbai), and DL- alpha-tocopherol acetate (Merck, Mumbai).

Broth dilution assay: Muller-Hinton borth (HiMedia, Mumbai), dimethylsulfoxide (DMSO; Merck), gentamicin (HiMedia).

Antibacterial susceptibility test

MIC (minimum inhibitory concentration) determination was carried out using microbroth dilution method as per NCCLS guidelines (Jorgensen and Turnidge, 2003). Assay was performed in 96-well microtitre plates. Total volume of the assay system in each well was kept 200 µL. Muller-Hinton broth was used as growth medium. Inoculum density of the test organism was adjusted to that of 0.5 McFarland standard. Broth was dispensed into wells of microtitre plate followed by addition of test compound and inoculum. Test compounds (all reconstituted in DMSO, except gallic acid which was prepared in water) were serially diluted into each of the wells. A DMSO control was included in all assays (Wadhwani *et al.*, 2009). Gentamicin served as a positive control. Plates were incubated at 35ºC for 16-20 h, before being read at 655 nm in a plate reader (BIORAD 680). MIC was recorded as the lowest concentration at which no growth was observed. Concentration at which growth was inhibited by 50% was recorded as IC_{50} value. In case of those phytochemicals which were able to inhibit test organism's growth, subculturing was done on sterile nutrient agar plate from the wells showing inhibition so as to check whether the phytochemical in question is bacteriostatic or bactericidal. All experiments were performed in triplicate.

Growth curve

Two sets of test tubes containing Mueller-Hinton broth (5 ml) were prepared, one was having curcumin (70 µg/ml), whereas another was with equal volume of DMSO (in which curcumin was prepared) but no curcumin. Both sets were inoculated with *A. hydrophila* suspension of same density (OD equal to that of 0.5 McFarland standard) followed by incubation at 35ºC. Growth was monitored at regular time intervals up to 8 h by measuring OD at 625 nm.

Results and Discussion

Among all the compounds tested, curcumin proved to be most effective at inhibiting growth of *A. hydrophila.* It was able to inhibit growth of the test organism by 55% and ~80% at 80 and 175 µg/ml concentration respectively (Table 1). Thus curcumin can be said to have a IC_{50} slightly below 80 µg/ml and MIC at ~175 µg/ml. The concentration at which 80 % or greater diminution of growth compared with that of the control occurs is usually recorded as the MIC (Jorgensen and Turnidge, 2003). Curcumin was able to inhibit growth of *A. hydrophila* completely at 230 µg/ml. When content from the well corresponding to this concentration of curcumin inoculated with *A. hydrophila* was spread onto a sterile nutrient agar plate, the organism was able to resume its growth following overnight incubation at 35ºC indicating the effect of curcumin being bacteriostatic. When *A. hydrophila* was challenged with a sub-MIC concentration of curcumin (70 µg/ml), it achieved lesser growth as compared to control (Figure 1), suggesting a decreased growth rate in presence of curcumin.

Curcumin, a dietary polyphenolic compound, has been shown to have a potent antibacterial activity against a number of pathogenic bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus*. Rai *et al.* (2008) found that curcumin induced filamentation in the *Bacillus subtilis,* suggesting that it inhibits bacterial cytokinesis. Further they showed that curcumin strongly inhibited the formation of the cytokinetic Z-ring in *B. subtilis* without detectably affecting the segregation and organization of the nucleoids. Curcumin inhibited the assembly of FtsZ protofilaments and also increased the GTPase activity of FtsZ. Curcumin reduced the bundling of FtsZ protofilaments *in vitro.* Their results indicated that the perturbation of the GTPase activity of FtsZ assembly is lethal to bacteria and that curcumin inhibits bacterial cell proliferation by inhibiting the assembly dynamics of FtsZ in the Z-ring.

#Table 1. Result of broth dilution assay of different phytochemicals

Test compound	Concentration (µg/ml)	Inhibition (%) of *A. hydrophila* (Mean±SD)
Curcumin	80	55±0.0
	175	79±0.41
	230	100±0.0
Tannic acid	200	52±0.73
	500	75±0.70
Rutin	560	36±0.37
Quercetin	500	34±0.88
Caffeine	450	26±0.23
Tocopherol	250	16±0.95

#All compounds were tested at different concentrations in the range 50-500 µg/ml. In case of rutin highest concentration applied was 560 µg/ml. Results are shown for only those at which IC_{50}/MIC was found or whatever inhibition caused at highest test concentration.

Chemically, curcumin is a bis-α,β-unsaturated β-diketone (commonly called a diferuloylmethane) (Anand *et al.,* 2007). It has an interesting structure with two phenolic groups and one active methylene function, which are potential sites for attaching biomolecules. Curcumin bioconjugates have been reported to possess antibacterial activity (Kumar *et al.,* 2001). Curcumin has also been reported as a promising antifungal (Martins *et al.,* 2009), as well antiprotozoal (Cui *et al.,* 2007) agent. Use of curcumin as an antimicrobial finish owing to its bactericidal properties on dyed textiles was reported by Han and Yang (2005). Curcumin was reported to attenuate the virulence of *Pseudomonas aeruginosa* by inhibiting the virulence factors such as biofilm formation, pyocyanin biosynthesis, elastase/protease activity, and acyl homoserine lactone production (Rudrappa and Bais, 2008). Curcumin is known for its ability to bind a variety of proteins and inhibit the activity of various kinases. It is believed to be safe even when consumed at a daily dose of 12 g for 3 months (Goel *et al*., 2008).

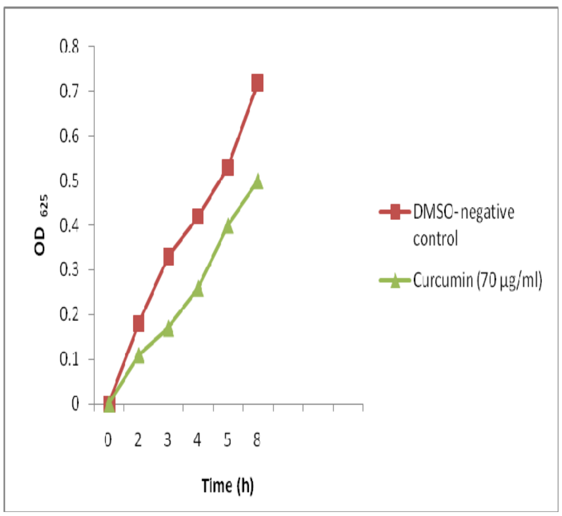

Figure 1. Effect of curcumin at sub-MIC concentration on *A. hydrophila*

Tannic acid was second most effective compound against *A. hydrophila*. Its IC_{50} is little less than 200 µg/ml, and MIC can be said to be slightly above 500 µg/ml (Table 1). Tannic acid was reported to inhibit the growth of intestinal bacteria, and its inhibitory effect was suggested to be due to its strong iron binding capacity (Chung *et al*., 1998). Tannic acid is an important gallotannin belonging to a hydrolysable class. It was found to inhibit growth of various strains of *Staphylococcus aureus* at 250-1000 mg/l, and was suggested as a potential adjuvant agent against *S. aureus* skin infections treated with β-lactam antibiotics (Akiyama *et al*., 2001). Tannic acid was reported to have an inhibitory effect on cellulolytic bacteria at concentrations ≤ 45µg/ml. It is suggested that the site of action of tannins on sensitive microorganisms is primarily the cell envelope (Henis *et al*., 1964).

Except curcumin and tannic acid no other test compound inhibited *A. hydrophila* to any notable extent at concentrations tested. Gallic acid failed to exert any effect on *A. hydrophila* even at highest concentration (500 µg/ml) tested. Inability of gallic acid to inhibit bacterial growth to a significant extent was also reported by Chung *et al*. (1998) and Henis *et al*. (1964), which may be attributed to its low iron-binding capacity. Rutin, caffeine, tocopherol, and quercetin were able to inhibit the growth of *A. hydrophila* up to certain extent, but none was found to have an IC_{50} below 500 µg/ml, making them not an attractive candidate for control of this pathogen.

Since *A. hydrophila* is viewed as a challenging and notorious food-borne pathogen, and difficult to control owing to its resistance to many antibiotics, it is required to find novel approaches to control it. Our study found curcumin to be effective against this organism as a bacteriostatic agent at microgram concentrations. Curcumin is already a widely used food ingredient. It can be introduced at appropriate concentration in those food preparations in which *A. hydrophila* most commonly establishes itself. It can also be used as a supplementary therapeutic agent in people suffering from *Aeromonas* infections. We suggest more such phytochemicals should be tested against this and other pathogenic microorganisms in order to find better therapeutic alternatives.

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