Scopoletin reduces intracellular survival of *Salmonella typhi* within U937 human macrophage cell line *in vitro*

Dhruba Acharya¹*, Bikash Bogati¹ and Prabodh Risal²

¹Department of Microbiology, Kathmandu University School of Medical Sciences, Kavre, Nepal
²Department of Biochemistry, Kathmandu University School of Medical Sciences, Kavre, Nepal.

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The pathogenic role of *Salmonella* infection in the development of human diseases and the impact of resistance on the clinical outcome stimulated the search for newer treatments and natural products that could provide alternative therapies against typhoid fever caused by this intracellular pathogen. Scopoletin has been reported for its anti-inflammatory activity and various other mechanisms underlying intracellular signaling pathways. We aim to determine the *in vitro* effect of scopoletin in intracellular survival of *S. typhi* in U937 human macrophage cell line. The LDH releasing assay was used to assess the cytotoxicity of scopoletin and quantified colorimetrically with a CytoTox96 LDH-release kit (Promega, USA). To investigate whether scopoletin affects *S. typhi* survival within macrophages, U937 cells were incubated with and without scopoletin for various time intervals and measured survival rate counting viable cells. Statistical significance was set at p < 0.05. No cytotoxicity was observed to U937 cells below 50 µg/ml of scopoletin. The intracellular replication of *S. typhi* within all U937 cells showed a similar pattern until 6 h of incubation. But there was significant change in the number of viable cells when counted after 10 h (p < 0.05, t-Test) that suggest the difference in the bacterial survival within U937 cells between scopoletin treated and untreated experiments. Scopoletin could have intracellular activity to decrease the bacterial replication which could be beneficial for the treatment of intracellular replicating pathogens including *S. typhi*.

**Key words:** Scopoletin, typhoid fever, U937 cells.

**INTRODUCTION**

Typhoid fever, which is a systemic infection caused by *Salmonella enterica* serovar *typhi* (*S. typhi*) is a major health problem in developing countries, including Nepal (Parry et al., 2002). Over the past decade, increasing antibiotic resistance in *S. typhi* has lead to a shift in the antibiotics used against this organism from chloramphenicol and ampicillin to cotrimoxazole, fluoroquinolones (ofloxacin, ciprofloxacin), and third generation cephalosporins (ceftriaxone, cefotaxime). Moreover, *S. typhi* resistant to third generation cephalosporins has been documented from various parts of South Asia which leaves no alternative for another class of antibiotic as treatment option so far (Capoor et al., 2007; Saha et al., 1999).

The pathogenic role of *Salmonella* infection in the development of human diseases and the impact of resistance on the clinical outcome stimulated the search for newer treatments and natural products that could provide alternative therapies against typhoid fever caused by this intracellular pathogen. Previous study has shown the possibility of gatifloxacin as the alternative therapy (Pokherel et al., 2006) however, the demand of more and more drugs from plant sources is continuously increasing. It is therefore essential to evaluate plants of medicinal value systematically for various ailments that are used in traditional medicine.

Scopoletin is a coumarin compound found in the root of plants in the genus *Scopolia* (Figure 1) (Cowan, 1999). Studies have shown that it has anti-ulcer and anti-secretory properties (Mahattanadul et al., 2011). Scopoletin has been reported for its anti-inflammatory activity and various other mechanisms underlying intracellular signaling pathways (Panda and Kar, 2006; Lee et al., 2006). However, to date, studies to understand the affect of scopoletin in intracellular survival of pathogenic microorganisms are lacking. So, we aimed to determine its possible involvement in the intracellular survival of *S. typhi* within human U937 macrophage cell...
MATERIALS AND METHODS

Scopoletin preparation

Scopoletin powder was obtained from Department of Clinical Pathology, Siriraj Hospital. The powder was dissolved with distilled water in various concentrations as required for individual test procedures.

Media and cell culture and subculture condition

U937 (Human macrophage cell line) was obtained from the American Type Culture Collection (ATCC, Manassas, Va.). All media and reagents for tissue culture were obtained from GIBCO Laboratories (Life Technologies, Inc., USA). U937 cells were maintained in RPMI 1640 medium (RPMI). All the medium were supplemented with 10% heat inactivated (30 min, 65°C) fetal bovine serum (FBS; Hyclone, USA). The culture was kept under a 5% CO₂ atmosphere at 37°C, in 25 cm² tissue culture flask (Costar, USA) in a volume of 15 ml. Differentiation of U937 to macrophage-like cells were activated by phorbol 12-myristate 13-acetate (PMA; Sigma, USA) to stimulate differentiation to macrophage-like cell line before exposure to S. typhi. U937 cells were harvested by centrifugation and suspended in medium. Then, cells suspension was diluted to a concentration of approximately 5 × 10⁵ cells/ml. PMA was added to obtain a final concentration of 50 ng/ml and 5 × 10⁵ cells/ml and seeded into a 24 well-tissue culture plate (Costar, USA). After incubation of activated U937 under a 5% CO₂ atmosphere at 37°C for 1 day, cells culture medium were replaced with fresh medium and further incubated overnight for infection study. In every cell culture viability and cell counting was performed by adding an equal volume of 0.4% trypan blue dye (GIBCO, USA) and counted by hemocytometer.

Cell cytotoxicity assay

Differentiated U937 were co-cultured with various concentrations of scopoletin (10, 50 and 100 µg/ml). After incubation for 24 h, the culture supernatant and cell lysates were collected for analysis. Cytotoxicity was quantified colorimetrically with a CytoTox96 LDH-release kit (Promega, USA). In brief, after the 24 h incubation of herb with U937 cells, 50 µl of the substrate was added. The plate was incubated for 30 min protecting from light. 50 µl stop solution was added to each well. LDH was measured by using ELISA reader. The percentage of cytotoxicity was calculated using the formula as follows:

% Cytotoxicity = {(Experimental – Effector Spontaneous – Target Spontaneous) / (Target Maximum – Target Spontaneous)} x 100

In which spontaneous release is the amount of LDH activity in the supernatant of untreated cells (no herb added) and total release is the activity in macrophage lysates. Maximum releasing LDH was calculated by using 0.5% triton X-100.

Intracellular survival assay

U937 activated cells at the density of 2 × 10⁵ cells per well were seeded and incubated at 37°C for 48 h. Cells were recovered with 500 µl 1X RPMI plus 10% FBS and incubated at 37°C for 24 h. S. typhi with the cell density of 1 × 10⁸ CFU/ml was prepared from the stationary phage during growth in LB broth. Bacterial suspension with MOI 20 (40 µl) was added to each well and incubated for 30 min. Monolayer was washed 3 times with PBS. Fresh complete medium containing 100 µg/ml gentamycin was added in each well. At different time points after infection: 0, 1, 2, and 6 h, the monolayer s were washed twice and lysed by the addition of 100 µl of PBS containing 1% triton X-100 per well. The plate was
Figure 2. Cytotoxicity of U937 macrophage cell line induced by treating with various concentrations of scopoletin.

incubated for 15 min at room temperature. The enumeration of bacteria were performed by plating suitable 2 fold dilution onto Trypticase Soy Broth (TSB) agar plates and incubated at 37°C for 24 h. The experiment was performed in triplicate.

Intracellular survival assay of *S. typhi* was again performed with scopoletin treated U937 cell line according to Kim et al. (2008). U937 cells were incubated with 50 µg/ml scopoletin for 4 h before bacterial infection, and then *S. typhi* (MOI 20) were deposited onto U937 cells and incubated at 37°C for 1, 2, 6, and 10 h. At every allocated time of incubation, the cells were lysed, and the numbers of viable intracellular bacteria were determined. For viable cell counting at different time points, the infected monolayers were washed 3 times with PBS and then lysed with distilled water. Aliquots of lysates were plated onto LB agar to assess bacterial colony forming units (CFUs).

Statistical analysis

The results obtained were expressed as mean +/- SD for the number of experiments. Student’s t-test was used to make statistical comparisons between groups. Results of p < 0.05 were considered to represent a statistically significant difference.

RESULTS

Cytotoxic effect of scopoletin in macrophage

There were no detectable cytotoxic effects of scopoletin at concentration with 10 and 50 µg/ml. The microscopic observation of the cell line showed no cytotoxicity till 50 µg/ml but the significant changes of cell morphology was observed with scopoletin 100µg/ml (microscopic picture not shown). With 100 µg/ml of scopoletin, the cytotoxicity of U937 cells was 82% whereas with 50 µg/ml, there was only 7% cytotoxicity as shown in Figure 2. The 50 µg/ml of the scopoletin was further used to determine the intracellular survival assay.

Effects of scopoletin on bacterial survival within macrophage

The results showed that the intracellular replication of *S. typhi* within all U937 cells showed a similar pattern until 6 h of incubation. But there was significant change in the number of viable cells when counted after 10 h of incubation (p < 0.05) as shown in Figure 3.

DISCUSSION

Medicinal plants have been used to treat a variety of disorders including inflammatory conditions, infections with microorganisms, cancer, allergy and other diseases (Cowan, 1999). Recently, the consumption of antibiotics has increased worldwide, causing serious problems such as multidrug-resistant bacteria, antibiotic misuse/overuse, antibiotic residues in food, etc. For these reasons, many researchers have attempted to find natural materials to replace antibiotics to treat bacterial infections.

Anticancer potential of scopoletin were demonstrated earlier from *in vitro* and *in vivo* studies. Cytotoxic activity towards cancer cells was reported (Bhattacharyya, 2010). Scopoletin was also reported to have anti-inflammatory effect through inhibiting the release of TNF-alpha, IL-1β, and IL6 (Kim et al., 2004). Therefore, in this study, we
focusing on the antibacterial activity of this coumarin compound with the objective of determining cytotoxicity and intracellular survival rate of *S. typhi* in *in vitro* model.

Scopoletin had moderate *in vitro* antibacterial activity against *Staphylococcus aureus* (Gram positive) in an earlier study, however, they didn’t observe any antibacterial activity against *E. coli* (Gram negative) (Ojala et al., 2000). So far no study has been performed to detect any antibacterial activity of scopoletin against *S. typhi* to our knowledge. We found moderate antibacterial activity of scopoletin against *S. typhi*. The extraction of this coumarin compound from various plant sources might be different leading to varied purity. In our study we used pure scopoletin which has been already extracted and purity was maintained. This could have made the difference.

*Salmonella* can prevent the induction or neutralize the action of antimicrobial effector mechanisms within macrophages and can therefore survive and multiply within phagosomes (Foster and Spector, 1995). Nitrogen oxide (NO) and hydrogen peroxide (H$_2$O$_2$) have been identified as major effector molecules produced by activated macrophages and are involved in the host defense against microorganisms and tumor cells (Gorelick et al, 1996). Although bacterial replication of *S. typhi* was not affected in human U937 macrophage cell line until 6 hours, the significant difference was observed after 10 h of incubation in our study. In *Salmonella* infections, previous study has proved the role of inducible nitrogen oxide synthases (iNOS) is controlling multiplication at later stages of infection without direct bacterial killing within macrophages (Cherayil and Antos, 2001). This has happened probably in our study. The later effect of scopoletin in intracellular survival of *S. typhi* within macrophage might be the involvement of iNOS. The increased and induced NO limits bacterial replication and helps in the clearance of intracellular bacteria by other bactericidal mechanisms including cytokine analysis and phagolysosome fusion within macrophages. However further study is required to clarify the mechanisms.

**Conclusion**

In conclusion, scopoletin has no cytotoxicity at concentration dependent manner for U937 human macrophage cell line. Scopoletin shows virulence reduction effects against intracellular bacteria *S. typhi*. Our findings suggest that scopoletin could be a possible alternative choice of medicinal plant to study further on *in vivo* cell signaling pathways to conclude remarkable findings instead of misusing or overusing antibiotics to treat intracellular bacterial infections.

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