

Full Length Research Paper

***Hemidesmus indicus* R. Br. root extracts reduce *Salmonella typhimurium* - induced inflammation in rat intestine by repressing its type three secretory proteins**

Sarita Das and S. Niranjali Devaraj*

Department of Biochemistry, University of Madras, Guindy Campus, Chennai-600 025, India.

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Emergence of multidrug resistant bacterial strains urged the investigation for alternative medicines. The present study was designed to evaluate the mode of action of the crude methanolic extract of *Hemidesmus indicus* root against *Salmonella typhimurium* both *in vivo* and *in vitro*. Pounded root was soaked in methanol for 7 days in room temperature with three solvent changes. The extract was pooled together, distilled and concentrated. An insoluble fraction comprising terpenoides, steroids and fatty acids, abbreviated as ME1 (methanol extract 1) was isolated from the crude methanol extract of *H. indicus* root (MHI with tannins and glycosides as the major constituents) during extraction. This study deals with the effect of these crude extracts (ME1 and MHI) against *S. typhimurium*-induced pathogenesis. *In vivo* ileal loop model in rat, *in vitro* organ culture and *in vivo* multiplication of *S. typhimurium* were used in mouse model (n=6). Both ME1 and MHI treated bacteria had 3 - 4 fold less initial attachment to murine intestinal epithelium in comparison to the wild type bacteria. The *in vivo* multiplication rate of wild *S. typhimurium* in the liver and spleen of mice was 100 fold higher, which was considerably less for MHI treated bacteria and negligible for ME1 treated *S. typhimurium*. The rat ileum infected with wild bacteria was severely inflamed; whereas the ileum infected with ME1 treated *S. typhimurium* was almost normal in appearance. The rats pre administered with MHI and then infected with wild type bacteria had normal villi, which proved its protective role. The *in vivo* activity of the extracts was further confirmed *in vitro* by using various simulating growth conditions, which mimic host environment. The type III secretory proteins (TTSPs) were isolated from wild as well as extract treated *S. typhimurium* and compared. Both the extracts inhibited the secretory proteins encoded by SPI-1 (involved in invasion and enteritis) as well as SPI-2 (involved in intracellular survival and multiplication) considerably. Differential activity of different constituents was observed to protect rat intestine as MHI had prophylactic and ME1 had therapeutic activity.

Key words: *Hemidesmus indicus* root, ME1, MHI, SPI-1, SPI-2, *Salmonella typhimurium*, type III secretory proteins (TTSPs).

INTRODUCTION

Salmonella enterica serotype Typhimurium (*Salmonella typhimurium*) is a principal etiological agent of gastro-enteritis in humans and produces a systemic illness in mice that serves as a model for human typhoid fever (Carter and Collins, 1974). Gram-negative pathogens employ complex syringe-like macromolecular structures termed type III secretion systems (TTSS) to facilitate

translocation of effector proteins into the cytoplasm of host cells (Hueck, 1998; Kubori et al., 1998; Kubori et al., 2000). These effector proteins are key virulence factors required for *Salmonella* intestinal invasion and the induction of fluid secretion and inflammatory response (Wallis and Galysov, 2000). *Salmonella* possess 2 such type III secretion systems for secreting virulence-associated effector proteins, named TTSS-1 and TTSS-2, which are structurally similar but functionally different (Hueck, 1998). TTSS-1 is involved with invasion and enteritis (Galan, 1999), whereas TTSS-2 is involved with intracel-

*Correspondence author. E-mail: niranjali@yahoo.com. Tel: 91-44-22351269. Fax: 91-44-22352494.

lular survival and replication (Hensel et al., 1998; Hensel, 2000). Invasion of entero-cytes and M cells results in villus blunting and loss of absorptive surfaces, a characteristic pathology of enteritis (Wallis and Galyov, 2000), and mutations in TTSS-1 greatly reduce enteropathogenicity in calves (Watson et al., 1998; Ahmer et al., 1999; Tsolis et al., 1999).

For centuries, indigenous plants have been used in herbal medicine for curing various diseases, including enteritis (Otshudi et al., 1999; Essawi and Srour, 2000). *Hemidesmus indicus* R. Br. (English: Indian Sarsaparilla, Sanskrit: Anantamul) (Family: Asclepiadaceae) is a well known medicinal plant, used for a variety of diseases. According to Ayurveda, it is used for rheumatism, leprosy, impotency and skin infections. The root has a sweet taste and pleasant smell due to the presence of an essential oil. The antiulcerogenic property (Anoop and Jegadeesan, 2003) and anti-thrombotic and antioxidant activity of *H. indicus* root extract have been reported previously (Mary et al., 2003). Most of the studies for antibacterial activity of natural products had dealt with the growth inhibitory effect, using simple techniques like disc diffusion, agar well diffusion or minimum inhibitory concentration (MIC) determination. We had reported the antienterobacterial activity (Das and Devaraj, 2006a) and the protective effect of glycosides of *H. indicus* root against *S. typhimurium*-induced cytotoxicity in host cells (Das and Devaraj, 2006b). The present investigation represents the effect of *H. indicus* root extract on *S. typhimurium* at its protein expression level.

MATERIAL AND METHODS

Bacterial maintenance and storage

Clinical isolates (CI) of *S. typhimurium* were procured from Christian Medical College; Vellore, India and standard strain of *S. typhimurium* (MTCC 98) was collected from Institute of Microbial Technology, Chandigarh, India. For routine use, the cultures were maintained on Nutrient agar (Himedia, Mumbai) plates. For long-term storage, glycerol stocks were prepared and stored at -20°C .

Extract preparation

Roots were collected, authenticated by botanist Dr. S. K. Dash, College of Pharmaceutical Sciences, Berhampur, Orissa; a voucher specimen was stored in the departmental herbarium (No. - 11/2001). Cool percolation method was followed for extract preparation (Das and Devaraj, 2006a). Briefly, the powdered root was soaked in methanol (Fischer Scientific Chemicals) for 7 days with three solvent changes, pooled together and concentrated. During concentration a fatty insoluble material separated, which was named as ME1 (methanolic extract 1) and the remaining crude extract was named as MHI (methanolic extract of *H. indicus*). Then the extracts were placed in a rotary evaporator to vaporize residual solvent and then stored in desiccator at 4°C . Phytochemical tests were carried out using lab protocol to find the main constituents present in each fraction. Before each assay, ME1 was mixed with olive oil and MHI was mixed with 1% Carboxy Methyl Cellulose (AR SRL, India) and hot water to prepare a homogeneous mixture of different dilution and used in our study.

Dose fixation and experimental design

Wild *S. typhimurium* was incubated with various concentrations of ME1/MHI overnight in static condition and optimum doses were fixed for both the extracts, which were neither too low nor too high, but there were appreciable growth and good number of treated bacteria ($>70\%$) was available for their character study. The optimum doses were fixed as 500 and 1000 $\mu\text{g/ml}$ for ME1 and MHI, respectively. Prior to each experiment, *S. typhimurium* was grown in Luria-Bertani (LB) broth (Difco) and added to fresh LB broth containing ME1/MHI, one set without extract served as control for wild type bacteria, incubated to either early log phase or stationary phase depending on the study. In all the *in vivo* experiments, equal numbers of wild and treated bacteria were used after harvesting and suspending them in sterile PBS/saline. In all the *in vitro* experiments, the fresh LB mediums without and with extracts were inoculated with equal number of bacteria and secretory proteins of wild and treated bacteria were isolated from their respective growth media.

Adherence assay

The method of Baumler et al. (1996) was followed. Briefly, *in vitro* intestine organ culture (IOC) was used in murine model to find out the effect of the extracts on adherence to host intestine. Wild and MHI/ME1 treated bacterial cells were harvested from 1 ml of static LB culture and resuspended in Dulbecco's minimum essential medium (DMEM) (Himedia, Mumbai). Small intestines were removed from 24 h starved (to avoid fecal contamination), 8-week old Swiss albino mice (procured from King Institute of Preventive Medicine, Chennai, approved by the Institute's Animal Ethics Committee, IAEC No.01/006/2002) after sacrificing them by cervical dislocation and placed in Petri dishes containing DMEM. Intestines were ligated at the distal end and filled with 1 ml bacterial suspension (10^9 colony forming units/ml) (CFUs/ml). The proximal end was also ligated with silk thread, incubated for 45 min at 37°C and $5\% \text{CO}_2$. Then the intestine was cut open at both ends, rinsed with PBS, opened longitudinally and washed 3 times with PBS (10 ml) to remove the non-adherent bacteria. Six separate 3 cm sections were homogenized in 5 ml of PBS for wild and treated bacteria infected intestine and aliquots were plated on MacConkey agar (Himedia, Mumbai) after adequate dilution and incubated overnight at 37°C . The percentage of bacterial recovery was calculated with respect to the initial inoculum (infection dose) and compared.

In vivo multiplication of *S. typhimurium* in liver and spleen of mouse

The method of Baumler et al. (1997) was followed. Briefly, 10^4 wild and MHI/ME1 treated *S. typhimurium* were injected intraperitoneally into 6-8 week old Swiss albino mice (20-30 g) (six in each group) and observed for 4 days. On the 5th day animals were sacrificed and the liver and spleen were separated. Equal weights (100 mg) of liver and spleen tissue from the animals administered with wild and extract treated bacteria were homogenized in 5 ml of PBS and the aliquots were plated on differential MacConkey agar medium, incubated overnight and the CFUs/gm tissue recovered were recorded.

Rat ileal loop model

The method of Aziz et al. (1968) was followed. Briefly, male Wistar rats (150-200 g) were obtained from King Institute of Preventive Medicine, Guindy, Chennai. The animals were fasted for 24 h prior to the experiment to avoid fecal debris accumulation in the intestine. The animals were anesthetized by using chloroform.

After making a small incision in the abdominal region, the small intestine was exposed and ileal loops (5 cm) were prepared by careful tying with silk thread. 0.3 ml of bacterial suspension (10^9 CFUs/ml) of wild and MHI/ME1 treated *S. typhimurium* were prepared in sterile saline and injected into separate loops. Loop injected with only saline served as control. Then the intestine was placed back in the abdominal cavity and the incision was closed by continuous suturing. After 8 h, the animals were sacrificed and the abdominal cavity was reopened, the appearance of the segments was recorded. The intestinal loops were also infected with wild *S. typhimurium* in rats pre administered with MHI or ME1 at a dose of 1000 and 500 mg/KG body weight respectively for 1 h. The segments were fixed in FAA fixative (formaldehyde, glacial acetic acid, 70% alcohol in a ratio of 10:15:75 v/v/v) (Fischer Scientific Chemicals) and processed for histological examination. The intestinal segments were cut into 1-2 μ m sections, stained with haematoxylin and eosin.

Isolation of secretory proteins

Isolation of SPI-1 encoded proteins (high osmolar and low oxygen condition)

The method of Kaniga et al. (1995) was followed. Briefly, an overnight culture of *S. typhimurium* was diluted 1:50 with fresh LB broth (40 ml containing 0.3 M NaCl) without and with MHI/ME1 and grown under O_2 tension condition (5% CO_2 incubator). Bacterial cells were harvested at an early log phase (after 4 h). The cell-free culture supernatant was precipitated with 10% TCA (w/v) (Fischer Scientific Chemicals), incubated on ice for 1 h and centrifuged at 10,000 rpm for 20 min. The pellet was washed with 1 ml cold acetone (Fischer Scientific Chemicals), centrifuged again at 10,000 rpm for 20 min. Then the pellet was air-dried and suspended in 0.1 ml of PBS containing 77mM Tris (SRL, India) (pH 7.4) and stored at $-20^\circ C$ until used. Secretory proteins, isolated from the wild, MHI and ME1 treated culture of both standard and CI of *S. typhimurium* were subjected to 10% SDS-PAGE (Laemmli, 1970) (BioRad Apparatus, Chemicals from SRL, India) and stained with 0.25% CBB R-250 (SRL, India).

Isolation of SPI-1 encoded proteins (pH shift method)

The method of Deafler, (1999) was followed. Briefly, overnight grown bacteria (at pH 7.4) were diluted 1:50 in fresh LB broth of pH 6.0 without and with the presence of MHI/ME1, allowed to grow up to O.D 0.4 - 0.5, cells were spun down, resuspended in fresh LB or DMEM medium of pH 8.0 and incubated for 1 h. The secretory proteins were isolated and processed as mentioned before (Kaniga et al., 1995).

Optimum period for protein secretion

This specific experiment was carried out to find out the duration of SPI-1-encoded protein secretion, which in turn can help us to understand the periodic episode of enteritis. Hence, secretory proteins were isolated from *S. typhimurium* with and without the presence of MHI/ME1, at 2, 4, 8 and 16 h intervals (Kaniga et al., 1995).

Isolation of secretory proteins from bacteria growing at acidic pH

An overnight culture at pH 7.4 was diluted (1:50) with fresh LB broth without and with MHI/ME1, at pH 4.5 and incubated for 4 h under static condition. Secretory proteins were isolated and processed as

mentioned above (Kaniga et al., 1995).

Isolation of SPI-2 encoded proteins

The method of Beuzon et al. (1999) was followed. Briefly, intracellular salts medium (ISM) containing 170 mM -2 [N-Morpholino] ethane-sulfonic acids (MES) at pH 5.0 or 7.0, 0.5 mM $MgSO_4$, 1 μ M $CaCl_2$, 6 mM K_2SO_4 , 5 mM NH_4Cl , 5 mM NaCl, 0.4% glucose and 2 μ g/ml of Nicotinic acid (low Mg^{2+} MEM medium) was used to isolate the SPI-2 encoded proteins of *S. typhimurium*. Bacteria culture were grown overnight in 40 ml of low Mg^{2+} MEM medium at pH 7.0/5.0 with and without the presence of MHI/ME1, cooled on ice and centrifuged at 7,000 g for 10 min at $4^\circ C$. The secretory proteins were isolated and processed as mentioned above (Kaniga et al., 1995).

RESULTS

Blockage of adherence to murine intestine in *H. indicus* root extract treated *S. typhimurium*

Adherence is followed by colonization and disease establishment. When this prime step is blocked, subsequent steps of pathogenesis are also blocked. Wild bacteria adhered tightly to the murine intestine to withstand repeated PBS washes. Our results showed that both ME1/MHI were capable of blocking 3-4 fold of bacterial adherence (Figure 1).

Reduction of *in vivo* multiplication in *H. indicus* root extract treated *S. typhimurium*

Wild bacteria proliferated 100 fold in liver and spleen, which was 3-fold less in both liver and spleen for MHI treated *S. typhimurium*. It was almost 500-fold less in liver and 10-fold less in spleen for ME1 treated bacteria (Figure 2).

Protective effect of *H. indicus* root extract against *S. typhimurium*-induced inflammation in rat intestine

Normal villi architecture was depicted in Figure 3a. The ileal loop infected with wild *S. typhimurium* showed exudates in the lumen containing polymorphs, RBC and fibrin threads with ulceration, shortened villi and exudative covering (Figure 3b). The loop infected with MHI treated *S. typhimurium* had a slightly shortened villi, minimal exudates, mild edema and inflammatory cells (Figure 3c). The histopathology of the ileum infected with ME1 treated *S. typhimurium* showed regenerated and hyperplastic glands with no inflammatory reaction (Figure 3d). In order to verify the prophylactic or therapeutic action of the extracts, the ileal loops of rats pre administered with MHI (1000 mg/KG body wt.) and ME1 (500 mg/KG body wt.) were also infected with wild type *S. typhimurium*. MHI pre administered rats had almost normal villi with mild edema and only little fibrinous exudates (Figure 3e), whereas in ME1 pre administered rat, the ileum had more cellular

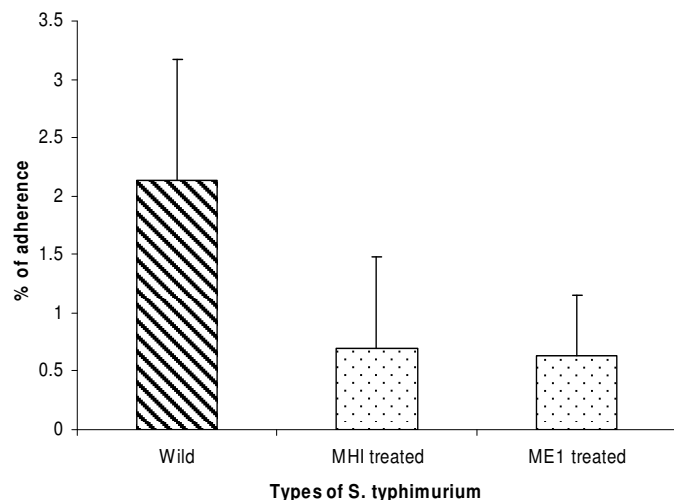


Figure 1. Adherence assay of wild and treated *S. typhimurium* in murine *in vitro* organ culture (IOC) model. Mice intestines were infected with either wild or MHI/ME1 treated *S. typhimurium* for 45 min; the number of adhered or invaded bacteria was retrieved by plating aliquots of homogenized intestine after adequate dilution and compared. Values represent the mean \pm SD of three different sets of experiments.

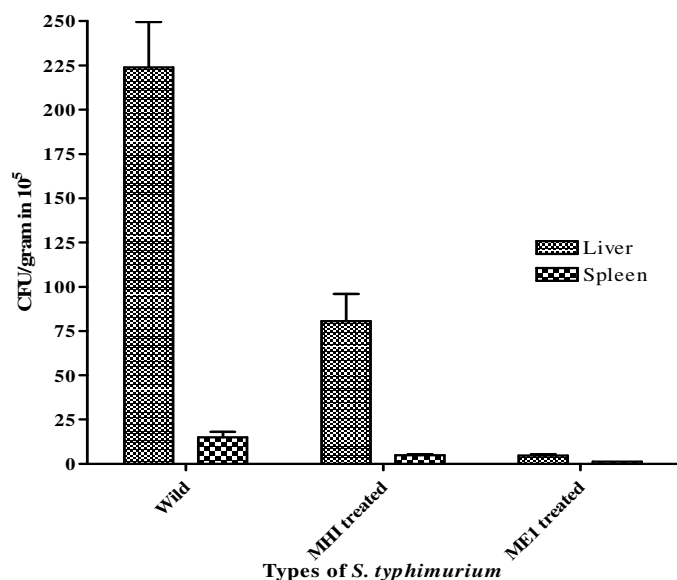


Figure 2. *In vivo* multiplication assay of *S. typhimurium* in liver and spleen of mouse. (n=5) Mouse were infected intraperitoneally and observed for 5 days and on day 5, sacrificed and their liver and spleen were separated out. 100mg of each tissue were homogenized in 5ml of PBS, aliquots were plated and incubated overnight and the CFU/gram bacteria recovery was calculated multiplying 10 X total bacteria in 5ml tissue suspension. Values represent the mean \pm SD of three different sets of experiments.

exudates, shortened villi, congestion and inflammatory infiltrates (Figure 3f).

Repression of SPI-1 encoded TTSPs by *H. indicus* root extract

Clinical isolate strain had high level of protein expression in comparison to the standard strain of *S. typhimurium*. Though most of the proteins were repressed in presence of ME1, a missing protein band at \sim 80 kDa was observed in MHI/ME1 treated bacteria (Figure 4, lane 2,3 and 6,7), which can be Salmonella invasion protein A (SipA).

Intestinal pathogens cross the hostile acidic environment of the stomach and then express their virulence proteins at alkaline pH of intestine. Shift in pH induces invasion-associated gene expression (Daefler, 1999). So, a mimicking growth condition was used to isolate SPI-1 encoded TTSPs. Though in LB medium (pH 8.0), no considerable difference was noticed between the wild and extract treated bacteria (Figure 5, lane 1, 2 and 3), in the nutrient rich tissue culture medium, DMEM (pH 8.0), the wild *S. typhimurium* expressed many proteins at higher level (Figure 5, lane 4). In MHI treated bacteria (Figure 5, lane 5), the protein expression was less and it was negligible in ME1 treated bacteria (Figure 5, lane 6).

The secretory proteins isolated at different time intervals showed that at 2 h only wild type bacteria had mild level of protein secretion (Figure 6a) and there was no protein secretion in MHI/ME1 treated *S. typhimurium* at 2 h. ME1 was more effective than MHI at 4 h (Figure 6b) as it repressed major invasins (\sim 80, \sim 66 and \sim 43 kDa proteins), which could be SipA, SipB and SipC, respectively. The secretory proteins of MHI treated *S. typhimurium* were greatly reduced at 8 h (Figure 6c). Around 16 h, the secretory proteins were completely inhibited (Figure 6d).

Repression of SPI-2 encoded TTSPs by *H. indicus* root extract

Proteins were isolated from bacteria grown in acidic pH. In standard strain proteins were much more (Figure 7, lane 1), in comparison to the clinical isolate, but, in both the strains, the protein expression was repressed appreciably in presence of MHI/ME1 (Figure 7, lane 2, 3 and 6,7).

The TTSPs encoded by SPI-2 were induced by growing the wild and MHI/ME1 treated bacteria in low Mg^{2+} containing ISM-MES medium. There was no difference in protein expression among the wild and treated bacteria grown at pH 7.0 (Figure 8, lanes 4, 5 and 6), but the bacteria grown at pH 5.0, which induces SPI-2 had considerable protein repression in MHI/ME1 treated bacteria (Figure 8, lanes 2 and 3) in comparison to wild type bacteria (Figure 8, lane 1).

DISCUSSION

The intestinal epithelium remains intact for up to 2 h if

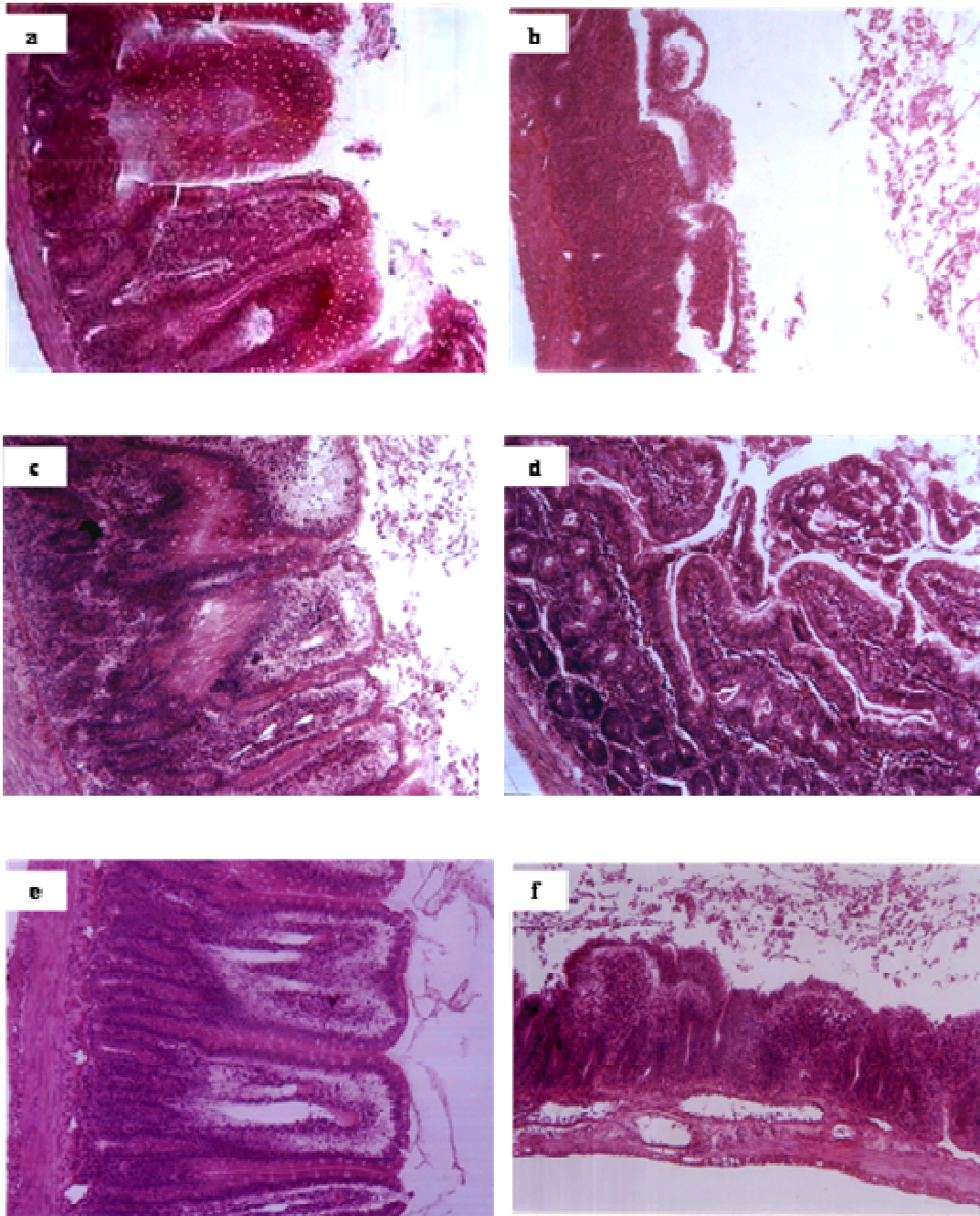


Figure 3. Rat ileal loop infected with wild and treated *S. typhimurium*. (a) normal villi, (b) infected with wild type bacteria showing damaged microvilli, (c) infected with MHI treated bacteria showing less damage and inflammation, (d) infected with ME1 treated bacteria showing almost normal architecture, (e) MHI pre administered rats infected with wild type bacteria showing almost normal architecture and (f) ME1 pre administered rats infected with wild type *S. typhimurium* showing damaged villi.

sections of murine small bowel are placed into tissue culture medium (Worton et al., 1989). A possible reason for strong binding of *S. typhimurium* may be the fact that this serotype expresses at least six different fimbriae (Baumler and Hefron, 1995), encoded by *lpfABCDE*, a fimbrial operon, which is involved in adhesion of this pa-

thogen to murine small intestine. The wild bacteria were recovered in larger number from the intestine in comparison to the MHI/ME1 treated bacteria could be due to adhesion, invasion or both (Baumler et al., 1996). This could be possible that the treated bacteria may be defective for either adherence/invasion or both.

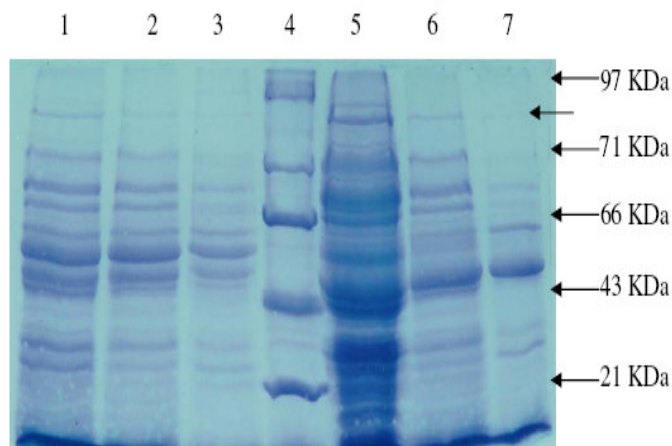


Figure 4. Secretory protein profile of wild, MHI and ME1 treated *S. typhimurium* (MTCC98 and CMC, CI). Overnight grown culture diluted 1:50 with high osmolar (0.3M NaCl) LB broth. Equal volume of fresh medium without and with MHI/ME1, incubated in oxygen limiting condition for 4h and the culture supernatant proteins were isolated by TCA (10% v/v) precipitation, washed with cool acetone and suspended in equal volume of PBS containing 77mM Tris, pH 8.0. Equal amounts of proteins were loaded into each well, separated by 10% SDS-PAGE and stained with CBB R-250. Arrows on figure indicate the repressed protein

Lane 1 – wild type, Lane 2 - MHI (1000 µg/ml) treated, Lane 3 – ME1 (500 µg/ml) treated MTCC98. Lane 4 – Mol. wt. marker, Lane 5 – wild type, Lane 6 – MHI (1000 µg/ml) treated and Lane 7– ME1 (500 µg/ml) treated clinical isolate of *S. typhimurium*.

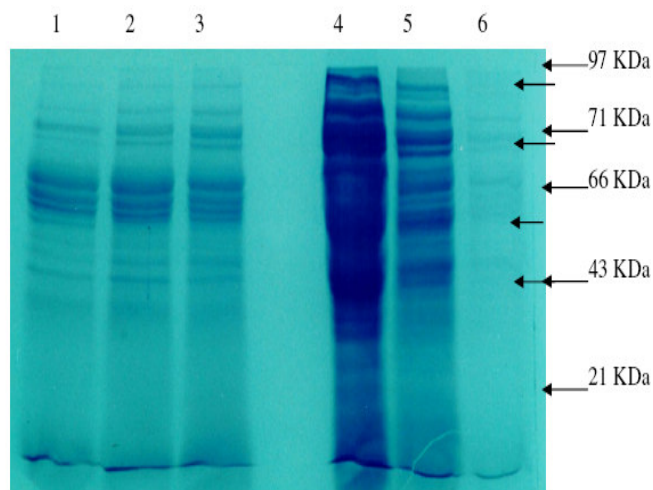


Figure 5. SPI-1 encoded protein profile of wild, MHI and ME1 treated *S. typhimurium*. Overnight grown culture diluted 1:50 with fresh LB medium (pH 6.0) without and with MHI/ME1, grown for 4h. Cells were collected and suspended in fresh LB broth and DMEM (pH 8.0) incubated 1h. The secretory proteins were isolated and separated as mentioned in figure 4. Arrows on figure indicate the repressed protein

Lane 1 – wild type, Lane 2 - MHI (1000 µg/ml) treated, Lane 3 – ME1 (500 µg/ml) treated *S. typhimurium* suspended in LB medium and Lane 4 – wild type, Lane 5 - MHI (1000 µg/ml) treated, Lane 6 – ME1 (500 µg/ml) treated *S. typhimurium* suspended in DMEM.

SPI-1 mutants were equally virulent as wild type *S. typhimurium* when injected intraperitoneally as SPI-1 encoded proteins are required for crossing the intestinal barrier after oral administration in order to cause a systemic disease in mice (Galan and Curtiss, 1989). Our first experiment established that both MHI and ME1 were capable of blocking adhesion and invasion processes encoded by SPI-1. In order to find the effect of the extracts on SPI-2, which is involved in intracellular multiplication and systemic dissemination (Shea et al., 1996; Hensel et al., 1998), intraperitoneal route was chosen for this study. We observed that the *in vivo* multiplication of *S. typhimurium* was also reduced for the extract (MHI/ME1) treated bacteria in comparison to wild type. But, ME1 was more effective than MHI in reducing the multiplication rate of *S. typhimurium* as the CFUs recovered from the liver and spleen of the mouse injected with ME1 treated *S. typhimurium* were negligible.

Ligated rat ileal loop models have been widely exploited to study the interaction of Salmonella with intestinal mucosa, *in vivo* (Vashisht et al., 1991). The strains that caused enteritis in primates induced enteropathogenic responses such as fluid secretion, intestinal inflammation and mucosal damage in rabbit ileal loops (Giannella et al., 1973; Wallis et al., 1986). The strains with reduced enteropathogenicity in bovine ligated ileal loops were attenuated in calves following oral challenge (Watson et al., 1998). Our results support the concept that different bioactive compounds have differential activity to combat pathogenesis, as MHI pre-coating helped in protecting the ileum and the ME1 treated bacteria were defective in causing inflammation. The major constituents were identified as triterpenoid, steroid and fatty acids etc in ME1 and tannin, flavonoid and glycosides etc in MHI. According to our previous report, pre-coating of host cells with glycolipids (major constituent of MHI) protected them from *S. typhimurium* inflicted pathogenesis in both intestinal epithelial and macrophage cell lines (Das and Devaraj, 2006b).

When the pathogen comes in contact with tissue culture cells, effector molecules either move to the surface of the bacterium forming appendages (Ginocchio et al., 1994) or get delivered into the host cell (Pettersson et al., 1996). The effector molecules bring changes in host cell function, which facilitate the pathogen's ability to survive and replicate (Rosqvist et al., 1990; Kenny et al., 1996; Menard et al., 1996). But, temperature, pH, growth phase and osmotic conditions are environmental cues known to induce type III secretion apparatus and effector molecules in various pathogens without the presence of host cells (Lee and Falkow, 1990; Hromockyj et al., 1992; Straley and Perry, 1995).

Synthesis of thirty-three proteins of *S. typhimurium* was increased in response to oxygen limitation (Spector et al., 1986). Low oxygen concentration inside host tissue (intestinal lumen or other tissues) augments invasion-associated gene expression. A growth medium similar to host intestine (high osmolar and low oxygen tension) was

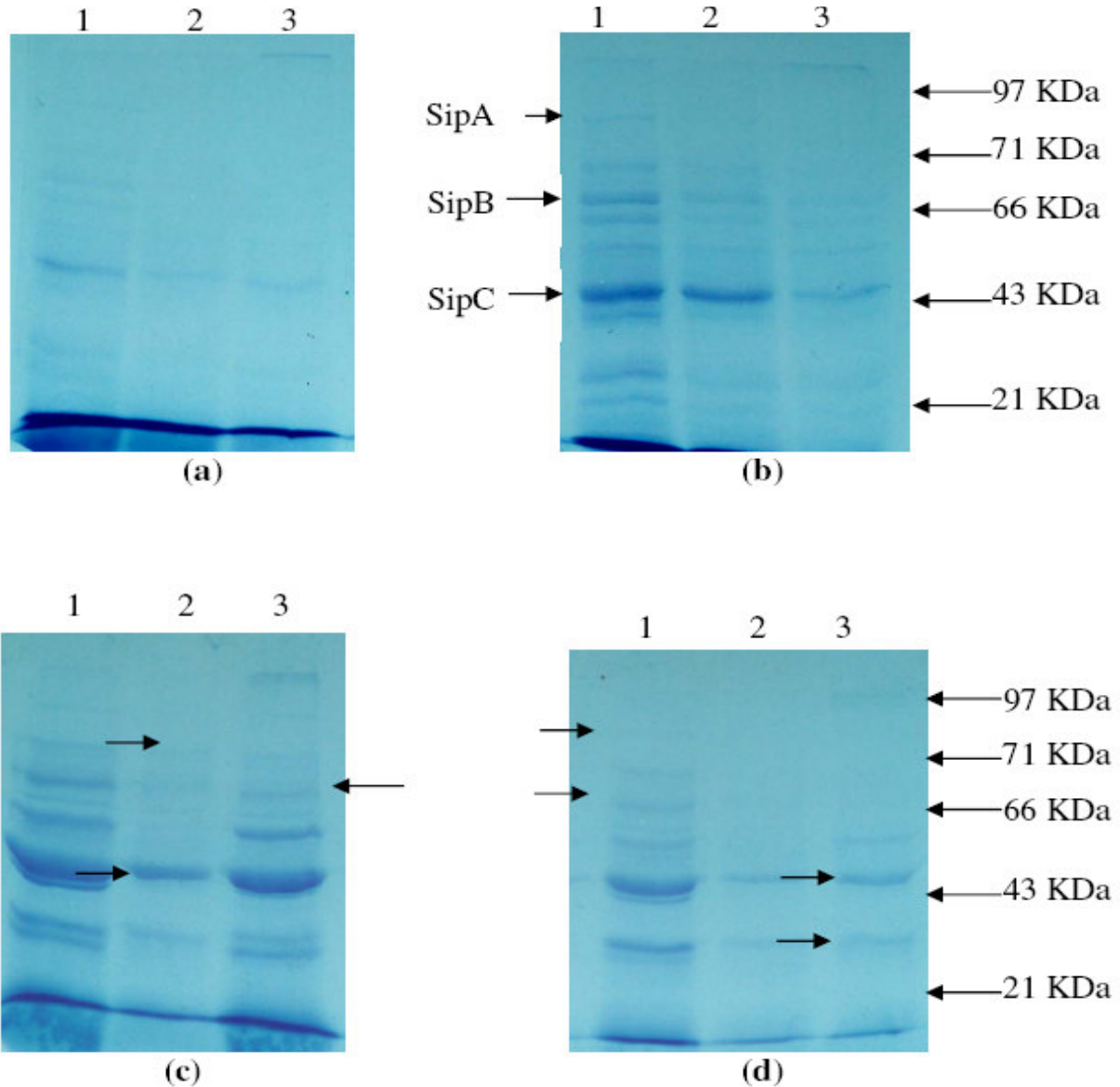


Figure 6. Secretory protein profile of wild, MHI and ME1 treated *S. typhimurium* at different time intervals. Same methodology followed as mentioned in Figure 4. (a) at 2h, (b) at 4h, (c) at 8h and (d) at 16h. Arrows on figure indicate the repressed protein

Lane 1 – wild type, Lane 2 - MHI (1000 µg/ml) treated, Lane 3 – ME1 (500 µg/ml) treated *S. typhimurium*.

used for the culture of wild and extract treated *S. typhimurium* and the proteins secreted into the growth medium were isolated and separated by SDS-PAGE. We used various growth conditions (high osmolarity, oxygen limiting and pH shift) as environmental cues to induce the SPI-1 encoded gene expression. Under all conditions, the *H. indicus* root extract inhibited TTSPs expression including a protein at ~ 80 kDa, which might be SipA that is necessary for actin polymerization and intimate host bacterial interaction (Zhou et al., 1999). SipA mutants were attenuated in bovine intestinal models with impaired invasion ability, reduced fluid accumulation as compared to wild type strain (Zhang et al., 2002). Loss of inflamma-

tion in rat ileum infected with MHI/ME1 treated *S. typhimurium* could be due to repression of SPI-1 encoded invasion-associated proteins in MHI/ME1 treated *S. typhimurium*.

In *S. enterica*, acidic pH has been previously identified as an environmental stimulus for the secretion of SPI-2 encoded proteins (Beuzon et al., 1999; Klein and Jones, 2001; Nikolaus et al., 2001). *S. typhimurium* resides inside a vesicle throughout its intracellular life. Once internalized, the pH of the phagosome goes down to 4-5 and this condition activates the expression of SPI-2 encoded proteins, which are essential for the growth and multiplication of *S. typhimurium* inside the macrophage.

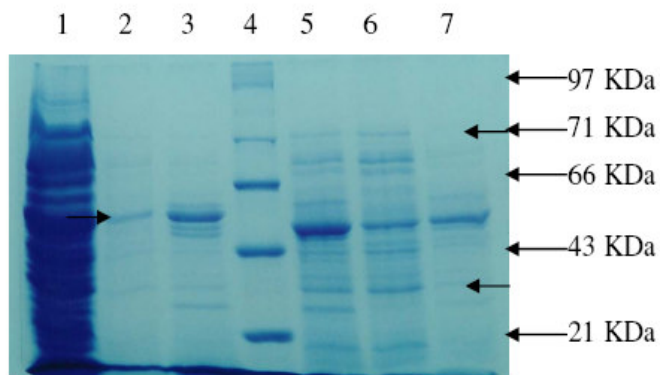


Figure 7. Overnight grown culture in LB broth at pH 7.4, diluted 1:50 with fresh medium without and with MHI/ME1, pH 4.5 and grown for 4h. Secretory proteins were isolated and separated as mentioned in Figure 4. Arrows on figure indicate the repressed protein

Lane 1 – wild type, Lane 2 - MHI (1000 µg/ml) treated, Lane 3 – ME1 (500 µg/ml) treated MTCC98. Lane 4 – Mol. wt. marker, Lane 5 – wild type, Lane 6 – MHI (1000 µg/ml) treated and Lane 7 – ME1 (500 µg/ml) treated clinical isolate of *S. typhimurium*.

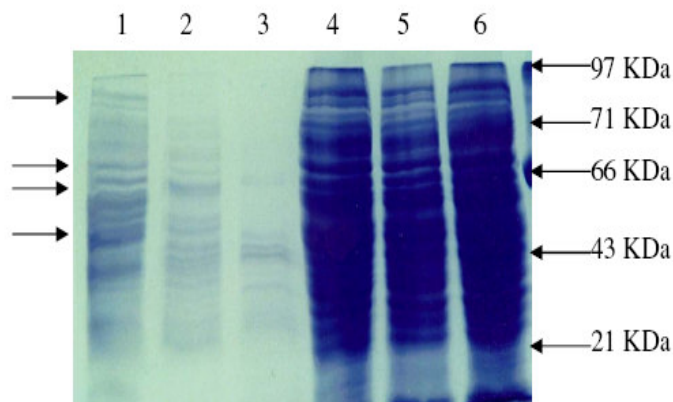


Figure 8. SPI-2 encoded protein profile of wild, MHI and ME1 treated *S. typhimurium*. Overnight grown culture at pH 7.4, diluted in fresh low Mg^{2+} ISM-MES at pH 5.0 and 7.0, incubated overnight and the secretory proteins isolated and separated as mentioned in figure 4.

Lane 1 – wild type, Lane 2 - MHI (1000 µg/ml) treated, Lane 3 – ME1 (500µg/ml) treated *S. typhimurium* grown at pH 5.0 and Lane 4 – wild type, Lane 5- MHI ml) treated and Lane 6 – ME1 (500 µg/ml) treated *S. typhimurium* grown at pH 7.0. Arrows on figure indicate repressed protein

So, a similar growth medium was used to mimic the intracellular environment and the proteins thus isolated from the MHI/ME1 treated and wild *S. typhimurium* were compared. Reduced protein expression in treated bacteria would be the reason for loss of *in vivo* multiplication in MHI/ME1 treated *S. typhimurium*.

To establish intracellular niche, *Salmonella* needs to

differentiate between the extra cellular and intracellular location. Divalent cations act as an extra cellular signal to regulate virulence gene expression (Garcia-Vescovi et al., 1996). To strictly co-ordinate expression of the two type III secretory systems (TTSSs), these bacteria utilize Mg^{2+} concentration to sense a sub cellular locality (Pegues et al., 1995; Groisman, 2000). During the invasion process, bacteria are subject to high Mg^{2+} concentrations outside the cell compared to inside host cell vacuoles (Groisman, 1998) and thus growth in medium containing a low Mg^{2+} concentration induces expression of several SPI-2 genes (Deiwick et al., 1999) as the mediator(s) of this up regulation, PhoP/PhoQ two-component regulatory system, responds to Mg^{2+} limiting conditions (Garcia-Vescovi et al., 1996; Groisman, 2000) by down regulating SPI-1 gene expression (Bajah et al., 1996). Our results proved that the SPI-2 encoded proteins were inhibited in the presence of *H. indicus* root extract, which would be the reason for reduced CFUs/ml recovery from the liver and spleen of the mice infected with treated bacteria in comparison to the wild type.

Conclusion

The protein expression of both SPI-1 and SPI-2 was considerably reduced in presence of the extracts. Antibiotics kill the pathogenic bacteria along with the nonpathogenic probiotics, thereby disturbing the normal body flora, which facilitates opportunistic organisms to flare up and infect the host. But, natural products like *H. indicus* root extract, which target the type three secretory systems of pathogenic bacteria can be used more safely. So, *H. indicus* root extract can be used as an effective remedy or as a supplementary medicine for *S. typhimurium*-induced inflammation. Further research can be conducted to find out its effect against other enterobacterial pathogenesis and to prime immunity with these attenuated bacteria.

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