TABLE 2 : ESTIMATION OF SPARFLOXACIN IN PHARMACEUTICAL FORMULATIONS.

Sample	Labelled amount (mg)	Amount found (mg)		Percent recovery of the proposed method*
		Reported method[10]	Proposed method	
1	200	198.9	199.4	99.7
2	200	199.5	199.0	99.5

*Average of six determinations.

The optical characteristics such as, Beer's law limits, Sandell's sensitivity, molar extinction coefficient, correlation coefficient, % relative standard deviation and % range of error (0.05 and 0.01 confidence limits) were calculated and the results were summarized in Table 1.

The results showed that the present method have reasonable precision. Comparison of the results obtained with the proposed and the reference method for dosage forms (Table 2) confirm the suitability of this method for pharmaceutical dosage forms. Interference studies revealed that the common excipients and other additives usually present in the dosage form did not interfere in the proposed method. In conclusion the proposed method is simple, rapid, and sensitive with the reasonable precision and accuracy and it can be used for the determination of SFC in bulk as well as in its pharmaceutical formulations.

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Investigation of the Antidiarrhoeal Activity of *Holarrhena antidysenterica*

POONAM G. DASWANI, TANNAZ J. BIRDI*, D. S. ANTARKAR[1] AND N. H. ANTIA.

The Foundation For Medical Research,

84-A, R. G. Thadani Marg, Worli, Mumbai–400 018.

[1]3, Nanak Society, 444A, Manmala Tank Road, Matunga (W), Mumbai–400 016.

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***Holarrhena antidysenterica* (L)–Apocyanaceae, well known for its antidiarrhoeal activity was studied for its effect on diarrhoeagenic *Escherichia coli*. Different dilutions of the decoction of the plant were assayed for its effect on the adherence and toxin production of 2 groups of *E.coli*-**

*For correspondence

enteropathogenic (EPEC) and enterotoxigenic (ETEC.) Adherence per se was not affected though disruption of the characteristic 'microcolonies' of EPEC on HEp-2 cell line was observed. The decoction was more effective in inhibiting stable toxin production as compared with labile toxin production.

Diarrhoeal diseases pose a significant health threat globally, infections diarrhoea being the most common disease syndrome worldwide (for a review on infectious diarrhoea see Carroll and Reimer). A variety of pathogens like bacteria, viruses and parasties are responsible for the different clinical syndromes such as acute watery, bloody and persistent diarrhoea.

Recently there has been a tremendous increase in research of medicinal plants. With emphasis being on scientific validation, a large number of plants are being biologically and chemically evaluated for their acclaimed properties. *Holarrhena antidysenterica* (L) (Kutaja-Wall) Apocynaceae, is a common plant widely cited in literature for its medicinal values. Various parts of this tree viz. bank, root, stem and seeds are known to have various medicinal properties[2], including antidiarrhoeal activity. The preparation, Kutajarishta, the main ingredient of which is *H. antidysenterica*, has been shown to exhibit antiamoebic activity[3]. We have studied the effect of a decoction of *H. antidysenterica* root bark on diarrhoeagenic *E. coli*. The biological assays[4,5,6] used in the present study target important stages in the pathogenesis of diarrhoea i.e. colonization of the intestinal mucosa and the production of enterotoxins. This element distinguishes the present work from the earlier studies which almost entirely focus on the antimicorbial profile[7] and the gastrointestinal motility[7,8].

Fresh root bark of *Holarrhena antidysenterica* was collected by Vaidya Antarkar from Ratnagiri district, Maharashtra State, in June 1995. All the experiments were performed from the inner portion of the same plant batch. For each experiment, a decoction was prepared by boiling 1g of dried material in 16 ml distilled water till the volume was reduced to 4 ml. The decoction was then centrifuged at 2,500 rpm for 10 min and filtered through a membrane of 0.22 μ pore size before diluting it 1:2, 1:10, 1:100 and 1:1000 in appropriate media as indicated in each experiment. The three strains of *E.coli* (obtained from Center for Disease Control (CDC), Atlanta) used in the study were a) EPCE B – 170; (0111:NH) exhibiting localized adherence on HEp-2 cell line b) ETEC TX1; (078:H12), heat stable toxin producer and c) ETEC B831-2, (unknown) heat labile toxin producer.

For adherence[4] strain B-170 ($5X10^8$/ml) was incubated onto a culture of HEp-2 cells (Source – National Center for Cell Science, Pune, India) on glass coverslips in presence of the different dilutions of decoction in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, USA) for 3 h at 37°. Non-adherent bacteria were washed off, coverslips fixed in 10% (v/v) formal-saline and stained with 0.1% (v/v) toluidine blue. Each dilution was carried out in duplicate. *E-coli* incubated on the HEp-2 cells in DMEM alone served as control. Under a light microscope, percentage HEp-2 showing adherence (having>5 *E.coli*), the average number of *E.coli* adhering/cell and the formation of bacterial microcolonies on HEp-2 cells in the absence and presence of the decoction were noted.

Production of heat labile toxin (LT) by *E.coli* B831-2 was measured by a modified ganglioside monosialic acid enzyme linked immunosorbent assay (GM-1 ELISA)[5]. *E.coli* were incubated in casamino acid yeast extract broth (CAYE, Himedia, India) alone (control) and in the presence of the different dilutions of decoction. After a 24 and 72 h incubation, the bacterial culture was washed, sonicated, the protein concentration adjusted to 50 ng/ml and added to wells of an ELISA plate precoated with GM-1 (1.5 μm/ml). A 1:200 and a 1:300 dilution of the primary (anticholera toxin, Sigma, USA) and secondary antibody (peroxidase labelled swine antirabbit Ig, Dako, Denmark) were used respectively. The reaction was developed using o-phenylene – diamine (OPD) and terminated using 2.5 N H_2SO_4. The intensity of the colour developed was read at 492 nm in a Titertek Multiscan Plus ELISA reader. Cholera toxin (Sigma, USA) and sonicate (50 ng/ml protein) of a non labile toxin producing bacteria were used as the positive and negative controls respectively.

In the suckling mouse assay[6] for stable toxin (ST) culture supernatants of *E.coli*. TX1 grown in CAYE alone (control) and in presence of the dilutions of decoction in CAYE were inoculated intragastrically in 2-3 d old Swiss white suckling mice. Following a 3 h incubation at room temperature, the neonatal mice were sacrificed and the ratio of their gut weight to the remaining carcass weight calculated. The results were interpreted as follows[6]:

If the ratio in the less than 0.074, it was interpreted as

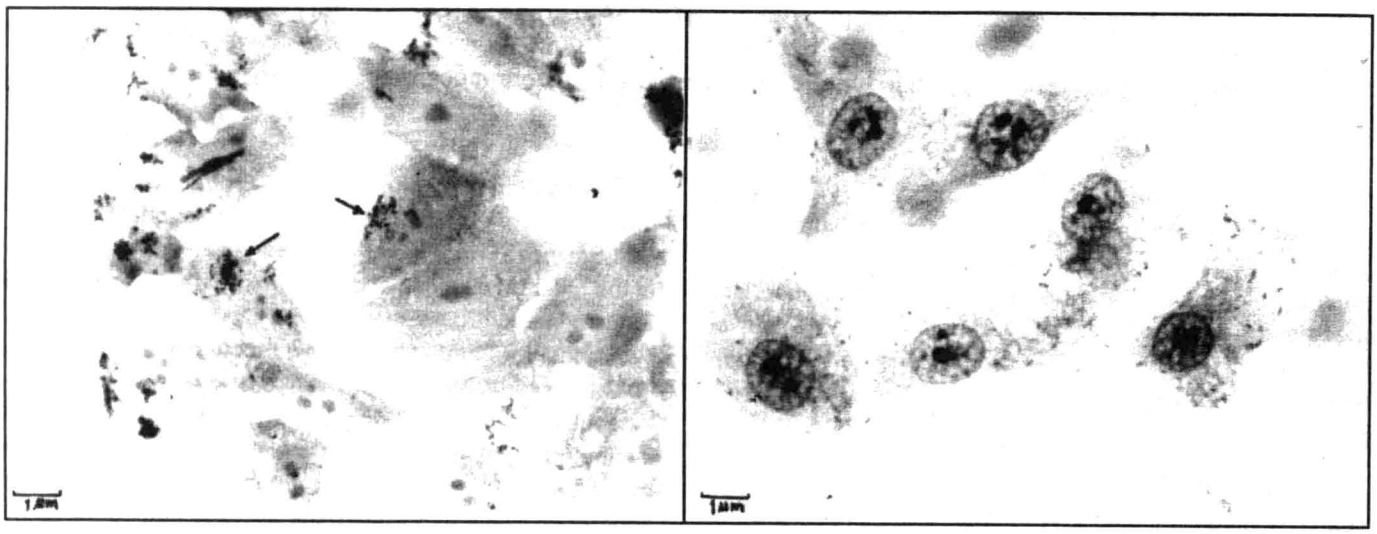

Fig. 1. Effect of *H. antidysenterica* on adherence of EPEC on HEp-2 cells.

Light microscopy photographs showing a) Microcolonies (→)of B170 on HEp-2 cells following incubation for 3 h at 37° in the absence of *H.antidysenterica* decoction. b) Disrupted microcolonies on incubation of B170 in the presence of 1:10 dilution of *H. antidysenterica* decoction. Bar represents 0.27 μm. Magnification X600. Staining method used was monochrome staining with 0.1 % toluidine blue.

negative. Ratio in the range of 0.075 to 0.082 were regarded as intermediate and ratio above 0.083 were considered positive. Negative controls included suckling mice inoculated with CAYE broth alone and CAYE + 1:10 dilution of decoction. For each assay the values obtained in presence of the decoction at each dilution was compared with that obtained with *E.coli* in media alone. Results were analyzed using the Student's t-test. $P<0.05$ was considered to be significant.

TABLE 1: EFFECT OF *H. ANTIDYSENTERICA* ON THE STABLE TOXIN PRODUCTION OF ETEC.

Inoculated supernate of *E.coli.* TX1 incubated in	**Ratio of Gut weight / Carcass weight**	**Interpretation**
CAYE (Control)	0.104±0.005	POSITIVE
CAYE +1:2 H.a	0.064±0.003*	NEGATIVE
CAYE +1:10 H.a	0.071±0.014*	NEGATIVE
CAYE +1:100 H.a	0.082±0.007*	INDETERMINATE
CAYE +1:1000 H.a	0.081±0.013	INDETERMINATE
Negative controls inoculated		
CAYE	0.045±0.009	NEGATIVE
CAYE+1:10 H.a	0.040±0.013	NEGATIVE

Ratio of gut weight / carcass weight of neonatal Swiss White mice inoculated intragastricaly with *E.coli* TX1 in the sucking mouse assay following a 24 h incubation at 37° in casamino acid yeast extract (CAYE) alone (control) and in different dilutions of *H. antidysenterica* (H.a) decoction in CAYE media (CAYE + H.a.). Values represent the Mean±SD of three individual experiments. Interpretation of the ratio values as stated in the text*. Indicated statistically significant at $P \leq 0.05$.

In the HEp-2 adherence assay, the characteristic EPEC microcolonies (fig.1) were not seen following incubation of B-170 on the Hep-2 cells in the presence of 1:2 and 1:10 dilution of *H. antidysenterica* decoction. Other parameters viz. Percentage Hep-2 showing adherence and *E.coli*/cell ratio remained unchanged (data not shown). The labile toxin production was not significantly affected at 24 and 72 h intervals (data not shown). In comparison, the decoction was more effective in inhibiting stable toxin production, the inhibition being directly proportional to the dilution of decoction (Table 1).

The attaching and effacing (A/E) histopathology is the hallmark of EPEC infections and this characteristic feature observed in intestinal biopsy specimens from patients or infected animals is reproduced upon infection of EPEC to tissue cultured cells *in vitro*[9]. The A/E lesion formation is a multistep process involving three stages – localised adherence (characterised by the formation of bacterial microcolonies), signal transduction and intimate adherence[10] leading to the subversion of host cell functions and signalling pathways. Hence the disruption of EPEC microcolonies on HEp-2 by *H. antidysenterica* as indicated in the present study may probably prevent the A/E histopathology and avert the bacteria from the opportunity to establish intimate contact with host cells and thus prevent then from initiating the disease process.

The stable toxin (STa) production is associated with intestinal secretion due to elevation of guanylate monophosphate[11]. Thus the inhibition of stable toxin by *H. antidysenterica* may prevent intestinal secretion and hence modify the diarrhoeal pathogenesis thereby decreasing the virulence of ETEC.

Using a similar approach we have earlier reported that *Cyperus rotundus*[12] root decoction also had antidiarrhoeal activity. However, it prevented the production of both the toxins but had no effect on adherence. Thus both *Holarrhena antidysenterica* and *Cyperus rotundus* probably exert their antidiarrhoeal activity by acting at different stages of the disease process; the modes of action differing thereby highlighting the requirement for multiple screening assays.

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