TABLE 2: ESTIMATION OF SPARFLOXACIN IN PHARMACEUTICAL FORMULATIONS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Labelled amount (mg)</th>
<th>Amount found (mg)</th>
<th>Percent recovery of the proposed method*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reported method*</td>
<td>Proposed method*</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>198.9</td>
<td>199.4</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>199.5</td>
<td>199.0</td>
</tr>
</tbody>
</table>

*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

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Holarrhena antidysenterica (L)–Apocynaceae, 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.

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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 decocion 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 parasities 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) (Kutajawall) Apocynaceae, is a common plant widely cited in literature for its medicinal values. Various parts of this tree viz. bark, root, stem and seeds are known to have various medicinal properties, including antidiarrhoeal activity. The preparation, Kutajarisha, the main ingredient of which is H. antidysenterica, has been shown to exhibit antimicrobial activity, and the production of enterotoxins. This element distinguishes the present work from the earlier studies which almost entirely focus on the antimicrobial profile, and the gastrointestinal motility.

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 μm 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) EPEC 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 strain B-170 (5X10⁶/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 decocion in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco BRL, USA) for 3 h at 37°C. 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 decocion 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). E.coli were incubated in casaminio acid yeast extract broth (CAYE, Himedia, India) alone (control) and in the presence of the different dilutions of decocion. 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 μg/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-di amine (OPD) and terminated using 2.5 N H₂SO₄. The intensity of the colour developed was read at 492 nm in a Titerpak 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 for stable toxin (ST) culture supernatants of E.coli, TX1 grown in CAYE alone (control) and in presence of the dilutions of decocion 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:

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

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

Ratio of gut weight / carcass weight of neonatal Swiss White mice inoculated intragastrically 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≤ 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. antidiaesenterica 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. 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 leading to the subversion of host cell functions and signalling pathways. Hence the disruption of EPEC microcolonies on HEp-2 by H. antidiaesenterica 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. Thus the inhibition of stable toxin by H. antidiaesenterica 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 root decoction also had antidiarrhoeal activity. However, it prevented the production of both the toxins but had no effect on adherence. Thus both Holarrhena antidiaesenterica 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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