Attachment 1.

Date March 15, 1989

ETHICAL REVIEW COMMITTEE, ICDDR,B.

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Principal Investigator Dr.Kamaluddin Ahmad Trainee Investigator (if any)

Application No. PCC/1/88 (REVISED) Supporting Agency (if Non-ICDDR,B) PCC-Collaborati

Title of Study Anti-shigella drug from plant extract: Project status: funding.

( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population: NA
   (a) Ill subjects Yes (No)
   (b) Non-ill subjects Yes (No)
   (c) Minors or persons under guardianship Yes (No)
2. Does the study involve:
   (a) Physical risks to the subjects Yes (No)
   (b) Social Risks Yes (No)
   (c) Psychological risks to subjects Yes (No)
   (d) Discomfort to subjects Yes (No)
   (e) Invasion of privacy Yes (No)
   (f) Disclosure of information damaging to subject or others Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) Yes (No)
   (b) Use of fetal tissue or abortus Yes (No)
   (c) Use of organs or body fluids Yes (No)
4. Are subjects clearly informed about:
   (a) Nature and purposes of study Yes (No)
   (b) Procedures to be followed including alternatives used Yes (No)
   (c) Physical risks Yes (No)
   (d) Sensitive questions Yes (No)
   (e) Benefits to be derived Yes (No)
   (f) Right to refuse to participate or to withdraw from study Yes (No)
   (g) Confidential handling of data Yes (No)
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes (No)
5. Will signed consent form be required:
   (a) From subjects Yes (No)
   (b) From parent or guardian (if subjects are minors) Yes (No)
6. Will precautions be taken to protect anonymity of subjects Yes (No)
7. Check documents being submitted herewith to Committee:
   - X Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   - ✓ Protocol (Required)
   - ✓ Abstract Summary (Required)
   - X Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   - X Informed consent form for subjects
   - X Informed consent form for parent or guardian
   - ✓ Procedure for maintaining confidentiality
   - X Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:

1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

(PTO)

e agree to obtain approval of the Ethical Review Committee for any changes nvolving the rights and welfare of subjects before making such change.

Principal Investigator

Trainee

FORMAT FOR PREPARATION OF BMRC & ICDDR,B COLLABORATIVE PROTOCOLS

1. Title : Anti-*Shigella* drug from plant extract.

2. Principal Investigator : Dr. Kamaluddin Ahmad

   Co-Investigator : Dr. Khaleda Haider, ICDDR,B
   Dr. Khurshid Jahan, D.U.
   Dr. Khorshed Alam Chowdhury, ICDDR,B.

3. Starting date : February 1988

4. Completion Date : January 1989

5. Total Direct Cost : Tk. 371,000.00

6. ABSTRACT

We have learnt from folk medicine anecdotes that the juice of the leaves of *Euphorbia hirta* is occasionally used in the treatment of diarrhoea and bloody dysentery. This led to find out sinn *in vitro* tests of the activities of the juice against many diarrhoeagenic organisms.

Our interest has deepened whem we found that the juice is effective against *Shigella dysenteriae* type 1 that is resistant to a number of antibiotics, including nalidixic acid. In view of the fact that many strain of *Shigella* resistant to common antibiotics are being encountered in the field we consider extremely important that this herbal drug be developed. It may not be out of place to mention that the crude preparation was used to treat 21 *Shigella* patients (age 1-4 yrs) infected with resistant *Shigella*. All of them became "culture netgative" within 2-4 days. These patients

also underwent various biochemical tests for liver and kidney functions which remained unaffected by the drug, which constituted of alcoholic extracts of 100 gr of fresh leaves a day. The alcohol was distilled off from the extract and the latter was prepared as aqueous suspension for oral administration.

In this protocol, we wish to undertake research leading to purification of the active principle(s), its (their) toxicity and finally efficacy in experimental shigellosis in animals (when such models are available).

The procedure for isolation will be application of biochemical separation methods with in vitro trials for activity at every stage of purification. The tentative procedure would be:

1. Extraction of fresh leaves of *E. hirta* with 95% alcohol.

2. Concentrate of the extract

3. Attempts to prepare the active substance from the associated impurities by extraction in different solvents at different pH and application of defferent biochemical separation techniques as indicated during the progress of the work (e.g. different kinds of chromatography).

The active sfubstance will be tested for functional group so that derivatives can be made for further purification. Biochemical separation methods to be used will be determined by the results of preceding procedures. Characterization will be done following chemical analytical tools. The isolated material will be used for standard toxicity test using rats.

The efficacy of the material for treating experimental shigellosis will depend on the availability of Monkey models for the purpose for which another protocol will be submitted at a later stage.

7. REVIEWS

a. Ethical Review Committee : _____

b. Research Review Committee: _____

c. Director's signature and remark, if any : _____

8.

i) Signature of Scientific Program Head (ICDDR,B) : _____ 20/3/88

ii) Signature of Heads of Collaborative collaborative departments/institute : _____ 21/3/88

iii) ICDDR,B Ethical Review Committee : _____

iv) PCC Research Review Committee : _____

Chairman
Dept. of Biochemistry
Dacca University

Approved/Not approved

SECTION II : RESEARCH PLAN

A. INTRODUCTION

1. Objectives

To isolate, purify and characterize the active ingredients of the plant extract which will show antibacterial activity against shigellae.

2. Background Information

Shigellosis is a major cause of morbidity and mortality in children in the developing countries (1) Antimicrobial therapy has been widely used to treat shigellosis. The gradual acquiring of drug resistances in *Shigella* strains has promted the search for newer and more effective drugs. Although many antibiotics have been used effectively in the past for the treatment of shigellosis, most of these are now ineffective, because of complete or partial resistance to them.Sulphadiazine was the drug of choice for treatment of shigellosis back in 1940s, and it was followed by tetracycline. By 1952, 80% of *Shigella* strains were resistant to sulphonamides (2) and by 1958, a majority exhibited multiple resistance to sulphonamides streptomycin, tetracycline, and chloramphenicol. With the development of multiple-drug resistance among *Shigella* species, ampicillin became the drug of choice in early 1970s. However, resistance of *Shigella* to ampicillin has been reported by many workers from various parts of the world (3,4). With

the ampicillin resistant strains, trimethoprim-sulphamethoxazole was tried and found to be effective and suitable drug for the treatment of shigellosis (5,6). However, during the last few years, there are reports of the emergence of trimethoprim-sulphamethoxazole-resistant strains among the clinical isolates of *Shigella* (7,8,9). Nalidixic acid has then been used successfully in many places for the treatment of shigellosis caused by multiple-resistant strains. However, emergence of resistance to this drug has also been observed in Bangladesh. (10). It is vital, at this stage, to search for new antimicrobial agents against shigellosis.

Drug of plant origin have been shown to have promising antibacterial activity against various bacteria (11,12,13,14,15). Preliminary investigation, on the biological activity *in vitro* of the crude extract of *E. hirta* (20 mg/disc) on multiple drug resistant *Shigella dysenteriae* type 1 strains (resistant to ampicillin, chloramphenicol, streptomycin, tetracycline, trimethoprim-sulphamethoxazole, and nalidixic acid) was done at ICDDR,B by way of collaboration with University of Dhaka. *In vivo* efficacy of the crude extract was also tested in 21 patients of shigellosis following BMRC ethical clearance with encouraging results at the University of Dhaka. Stool examination of these patients and isolation of *Shigella* organisms was also done at ICDDR,B laboratory (unpublished

observation). However, no detailed study on antibacterial activity of herbal medicine specially on *Shigella* has been reported. Thus this study will be carried out with an aim to develop an Anti-Shigella drug from plant extract.

3. Rationale

Shigellosis due to multiple drug resistant *Shigella* strains has become a great public health problem in developing countries. It is a urgent need to develope antimicrobial drug which will be active against multiple drug resistent shigellae. Prospective activity of the herbal drugs as an antimicrobial agents lead us to study the antibacterial activity of these drug against shigellae and to isolate, and characterize the active component of the plant extrct of *Euphorbia hirta*.

B. SPECIFIC AIMS

1. To isolate, purify and characterize the active components of the plant extract which has antimicrobial activity against multiple drug resistant shigellae strains.

2. To study the in vitro antibacterial activity of the extracts of the plant on eight multiple drug resistant shigellae strains (two from each species).

3. To determine the acute and chronic toxicities of the plant extracts in animal model (rats).

C. METHODS AND MATERIALS

1. Bacterial strains

Eight multiple drug resistant *Shigella* strains (two from each species) which are biochemically and serologically characterized, are stored in trypticase soy broth with 0.3% yeast extract and 15% glycerol at -70°C, at ICDDR,B will be used in this study.

2. Preparation of the plant extract :

The leaves (fresh) of the plant Euphorbia hista will be extracted by 95% alcohol, the extract will then be concentrated in vacuo. The active crude extract will be purified using different biochemical separation techniques following at each step its antimicrobial activity.

3. Susceptibility tests of the antimicrobial agent(s):

a) Antimicorbial susceptibility tests will be performed on the multiple drug resistant *Shigella* strains using standard method of Kirby Bauer method (16). The antimicrobial agents in discs (BBL) which will be used in this study are ampicillin, chloramphenicol, tetracycline, streptomycin, trimethoprim-sulphamethoxazole nalidixic acid, Kanamycin, gentamycin, norfloxacin, and Pevmecillinum. The active component of the plant extract.will be soaked in sterile discs and will also be used against the

Shigella strains. A disc, soaked in the solvent used for the plant extract, will be used as blank control.

b) MIC: Minimum inhibitory concentration of the active component of the plant extract will be done by serial tube dilution method of Seligman *et al* (17).

4. Toxicity Tests

i) Determination of LD_{50} in mg/kg by oral administration in mouse will ascertain the acute toxicity of the test material.

ii) Toxicity test using animal model: Sub-chronic toxicity test of the active component of the plant extract will be performed using rat model. Twenty young rats will be divided into four equal groups (5 in each). Groups 1-3 will receive active component of plant extract in high, medium and low doses to be determined on the basis of *in vitro* activity, vis-a-vis, known antibiotics. Group 4 will receive sterile solvent used for the active principle under consideration which will act as control. All twenty rats will be fed the respective materials on day 1 by means of a special syringe. The animal will then be allowed to live on normal food ;and water for 90 days. On

90 days all the experimental rats will be sacrificed and a routine postmortem will be performed. The organs/tissues that will be collected in 10% buffered formalin for histopathological examination are : liver, kidney, spleen, heart, brain pancreas, testis, ovary and skeletal muscle. Gross and microscopic changes of these organs will be recorded. Any toxic effects, i.e. degeneration, necrosis, hemorrhages or neoplastic lesion will be recorded. These lesion will be compared with the control rats. For evaluation of pathological changes, a grading system of 0-3 (No significant lesion, minimal, mild and severe lesion) will be used to express severity of the changes observed. Groups of rats fed with plant material will then be compared to the control group using chi-square analysis.

D. SIGNIFICANCE

Treatment of shigellosis due to multiple drug resistant shigellae, by a low cost, easily available, herbal medicine shall be a great benefit to the public health problem of the developing countries. The purification of the active principle will help us find out about its chemical action, mode of action and probable toxicity on short and long term use. It may lead us to devicing new structure for development of still newer drugs.

E. FACILITIES REQUIRED

General facilities required for isolation and characterization of the active component of the plant product will be done at the laboratory of the department of Biochemistry, Dhaka University and the newly established Bangladesh Institute of Herbal Medicine.

F. COLLABORATIVE ARRANGEMENTS

ICDDR,B shall provide all the facilities required for *in vitro* testing of the antimicrobial activity of the active component of the plant extract and toxicity tests on animal model.

FLOW SHEET OF THE PROCEDURES

Fresh leaves of *E. hirta* extracted with
equal volume of 95 percent alcohol in blendor.
Test for antimicrobial activity against *Shigella* spp.

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Evaporate off the alcohol in
a rotary evaporator

↓

Add water to make an equal suspension.
Determine pH

↓

Take one portion (Fraction 1) to acidic pH 5
and another (Fraction 2) to alkaline pH 8

↓

Extract fraction 1 and fraction 2 with benzene

↓

Test the extract for antimicrobial activity
against *Shigella* spp.

↓

Chromatograph the active fraction in a
column (alumine-celite) using benzene as the
running solvent and collect the eluent in
a fraction collector

↓

Identify the active portion of the
eluent by anti-microbial test on *Shigella*

↓

Remove the solvent from the active portion

↓

Do thin layer chromatography for
further purification.
Do MIC of the active component on shigellae
and toxicity test on rats.

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SECTION III : BUDGET

1. Personnel Services

Name	Position	% Time required	Taka Per month	Taka Per Year
Prof. Kamal Ahmad	Prof.Dept. Biochemistry	30%	5,000	60,000
Dr. Khaleda Haider	Asstt.Scientist ICDDR,B	10%	-	-
Dr.Khurshid Jahan	Assoc.Prof. INFS, DU.	30%	3,000	36,000
A Research Assistant to be appointed		100%	3,500	42,000
A Research Fellow to be appointed		100%	3,000	30,000
A Lab Attendant to be appointed		100%	2,500	30,000

2. Supplies:

Media, Equipment
Petridishes, Chemicals 60,000

3. Animals 100,000

4. Conveyance and Transport 8,000

5. Printing and reproduction 5,000

TOTAL TAKA 371,000