Attachment 1.
(FACE SHEET)

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date May 02, 1991
December 18, 1991

Principal Investigator PROF. K. AHMAD
Application No. PCC/001/91
Title of Study SEARCH FOR HERBAL MEDICINE FOR MULTIPLE DRUG RESISTANT SHIGELLOSIS

Trained Investigator (if any) _____
Supporting Agency (if Non-ICDDR,B) _____
Project status:
( ) 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:
   (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:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ 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)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ 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 Ctte. for review.

This is a laboratory study involving no human subjects. This need not go to E.R.C.

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

Z. Ahmad

A-032081

ICDDR,B

SECTION I : RESEARCH PROTOCOL

Revised.

1. Title : Purification and characterisation of antishigella active principles from herbs.

2. Principal Investigator : Professor Kamaluddin Ahmad, Ph.D
Bangladesh Institute of Herbal Medicine

3. Co - P.I. : Professor K. Jahan, MBBS, Ph.D, MPH

4. Co-investigators : Ms. Khaleda Haider, Ph.D, ICDDR-B

5. Consultant : Professor Demissie Habte, MD

6. Starting date : January 01, 1992 or as soon as approved

7. Completion date : December 31, 1993

8. Total Direct Cost : Tk. ~~7,55,700.00~~ 7,72,200.00

9. Recommendations

(a) K Ahmad
Bangladesh Institute of Herbal Medicine (BIHM)
11.12.91

(b) Acting Head, Laboratory Science Division, ICDDR,B

10. Abstract summary:

This is a study on the purification and chemical nature and biochemical properties of antibacterial compounds from Hirta Linn. It has been confirmed that these compounds are effective against all shigella sp. including strains that are resistant to multiple chemotherapeutic agents including nalidixic acid in invitro studies. Their MIC will be determined. Study of the effect of these substances for any toxic effect in animals (rats and mice) will be made.

Studies on Herbal Medicine

Isolation and characterisation of Active Principles of E. hirta against multiple antibiotic resistant Shigellosis.

In preliminary investigation it has been demonstrated that extracts of E. hirta Linn have remarkable activities in inhibiting the growth of various species of Shigella, more specifically Shigella dysenteriae Type 1 that are resistant to most of the common antibioties including nalidixic acid.

It is recalled that the plant E. hirta has been mentioned for its use in the treatment for dysenteriae in various literature however cursorily (6,7,8). In view of the incidence of multiple antibiotics resistant Shigellosis in Bangladesh (1,2,3,4) it is important that the active principles of extracts of E. hirta be identified, characterised and evaluated for its biological activity, and toxicity when administered to animals (rats and mice).

Procedure:

Both fresh and sun dried leaves of the plant will be collected and extracted with various non-polar and polar organic solvents in soxhlets. The extracts will be concentrated and tested for activity against the cultures of multiple resistant S. antidysenteria Type 1 in standard procedure (9). On finding of activities in the solvent fractions, active principles will be separated by column chromatography trying various solvents and packing materials for column such as alumina, celite, silica gel, agar etc. Simultaneous monitoring of the active principles will be done microbiologically. The active principles will then be subjected to thin layer chromatography again using deferent solvent systems using silica gels as coating materials. Various bands in the thin layer plates will then be collected, extracted with various solvents for the recovery of active principles. When suitable amount of the active principles are obtained, they will be subjected to column chromatography once again for purification. The active principles will be tested for their stablity in various condition of PH and for heat

stability. Ultraviolet and infrared spectroscopy of the active principles for various functional groups - such as carbonyl, carboxyl, unsaturation etc.

Derivatives of the active substance will be prepared depending on the functional groups identified in UV and infrared spectroscopy and chemical tests. It is expected that following the above procedure supplemented if required by other biochemical separation techniques such as molecular sieving, the active principles will be obtained in sufficiently pure condition for chemical charecterisation.

Specific aims are:

1. To purify active principles in E. hirta responsible for antishigella activity.

2. To prepare adequate amount of the active substances for chemical characterisation.

Objectives:

1. The objectives of the study is to develop herbal medicine for shigellosis resistant to multiple antibiotics and other chemotherapeutic agents. To study the chemistry and biochemistry of the substances responsible for this antibacterial activity of the herbs.

Introduction and background :

Shigellosis, also known as blood dysentery, is one of the very serious problems afflicting a large number of people of many countries, specially of the developing countries. It has been estimated at ICDDR,B treatment centre at Dhaka mortality of patients was 3.5 percent for those under one year and 0.3 percent for all other age group including adults. However, mortality rate as high as 6 percent has also been reported in epidemics. This has become a menace of utmost severity for the children of Bangladesh now that watery diarrhoea is being managed by ORT.

The bacillary and amoebic forms of the dysentery were first distinguished by Kiyoshi when Japan was struck by epidemics in 1897. Shigellosis is caused by infection by different species of the Shigella bacilli such as S. flexneri, S. dysenteriae type 1, S. boydii and S. sonnei. Among them S. flexneri and S. dysenteriae type 1 are more frequent, the latter being more difficult to treat.

Antimicrobial therapy has been effective in the treatment of shigellosis but the infective bacilli were found to develop resistance to drugs in use within a short period of time. Sulphadiazine was used during the forties. But quite soon it proved ineffective. Then came tetracycline and others. By 1952 eighty percent of shigella isolates were found resistant to sulphanomides, tetracycline and chloromphenicol. Ampicillin became the drug of choice in the 1970's when Cotrimoxazole (trimethoprim sulphamethaxazole) was also found to be useful. Now isolates are seen resistant to all these antibacterials and more-including nalidixic acid. (1-4)

Cont'd.....P/5.

According to ICDDR,B findings 90% of S. dysenteriae type 1 isolates are from patients who are seen at Clinical Research Centre are now resistant to ampicillin and cotrimoxazole. Over 30 percent of s.flexneri isolates are now resistant to nalidixic acid which until recently had been a drug of choice. More recent findings are that almost 90 percent of S. dysenteriae type 1 are resistant to nalidixic acid (1-4). There is thus a crying need for new antimicrobial therapy against these multiple resistant shigellae.

One must remember that when antibiotics are available for the treatment of shigellosis they are very expensive and beyond affordability of the poor population of Bangladesh who are the major victims of this scourge. A cheap easily available antibacterial is the need of our people.

We wondered if any or some of the myriads of plants that make up the flora of Bangladesh could be useful in the treatment of shigellosis. After all, many of great drugs of modern medicine had their origin in plants - aspirin from willow bark, emetine from ipecac, atropine from belladona, quinine from cinchona and so on. There is a report that traditional chinese medicine is useful in treating sulphadrug resistant shigella infection in Fujian Province, China (5).

While we have located quite a few plants with promise, for becoming useful as anti-shigella drugs, we have concentrated our studies on Euphorbia hirta Linn. This plant had received only passing mention in books of indigenous herbal medicines (6,7,8). It is a shurb usually found in the grounds of old buildings in the rainy season. It is not much seen in dry season or in farm lands.

We have demonstrated in vitro the presence of at least six different active antimicrobial substances in the leaves of this plant. They are effective in vitro against shigella strains (all species) including those with multiple resistance to most antibiotics including nalidixic acid and even pivamdinocillin.

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Addendum -

Previous Work

Sundried powered leaves of E. hirta were extracted with petrol ether (40-60c°) in soxhlets. The petrol ether extract showed no antibacterial activity. The residue in the thimble was then extracted with diethyl ether. This ether extract showed rather mild activity which we left out for later studies. The residue after ether extraction was tested for activity against some partially resistant strains of shigella following Bauer, AW etal 1966 AM J Clin path, 45, 493-496. The results are shown in Table - 1.

Table - 1

Species:	Susceptibility pattern of						
Shigella dysenteriae type 1 Strain No.	AM	NA	TE	SXT	C	GM	MEC
2633(16)*	R	S	R	R	R	S	S
2552 (20)*	R	R	R	R	R	S	S
2606 (25)*	R	R	R	R	R	-	S
Shigella flexneri (18)*	R	S	R	R	R	S	-
Shigella boydii No. 2155 (11)*	R	S	-	R	-	-	-
Shigella Sonnei (46)*	R	-	-	R	-	-	-

AM, ampicillin; NA, nalidixic acid; TE, tetracycline; SXT, cotrimoxazole; C, chloromycetin; GM, gentamycin; MEC, Pivamdinocillin.

* Inhibition zone diameter in mm shown in parenthesis, Crude material in filter paper discs about 10 mg. The shigella strains are from ICDDR B.

N.B: Resistant inhibition zone diameter in mm against ATCC E.coli 25922 for various antibictics are AM/11, NA/13, TE/14, SXT/10, C/12, GM/12 and MEC/20.

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Antimicrobial activity of diethyl ether and alcohol extracts of Euphorbia hirta, resistant to almost all antibacterials, namely, tetracycline, ampicillin, chloramphenicol, cotrimoxazole and nalidixic acid.

Test organism	Diameter of inhibition Zone (mm)	
Shigella dysenteriae type 1	Diethyl ether extracted fraction	Alcohol extracted fraction
4487	12	20
4562	±	18
4563	10	25
17(18)	-	20

The test organisms were obtained from ICDDR,B. The amount of crude drug per disc was 10 mg.

The extract obtained as above was taken to dryness in and then taken up in n-hexane for column chromatography. The column was packed with crude agar (Difco) and fine agar (Marck) 3:1. The running solvents were successively n-hexane, diethyl ether, ethanol and acetic acid. There was no antibecterial activity in the n-hexane eluate, while the other three solvents carried considerable antimicrobial activity.

The solvents from each of three active fractions were removed and each of the three residues so obtained has been further separated in thin layer chromatography. The chromatoplates were made of silicagel G. of 0.5 mm thickness and activated by heating at 120°c for 1 hour.

The developing solvent for the ascending T.L.C of the material from diethyl ether was chloroform, methanol, and ethyl acetate in the ration 5:2:3.

For the same from alcohol it was toluene, ethyl acetate and acetic acid 7:3:5, and for the material from acetic acid it was acetic acid itself. The samples for TLC were prepared as 1 percent solution in the respective solvents. The samples were applied in several spots along a line about 2 cm above the lower edge of the plate.

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After development of the TLC, the plates were taken out and air dried. The developed air dried plates were then covered with another plate except for a strip on one side which had the track of movement of one sample spot. The exposed area was then sprayed with conc. sulphuric acid. The covered portion of the plate not sprayed by sulphuric acid were scrapped by a clean spatula. The scrappings were then treated with acetic acid to dissolve out the active substances if any and then filtered. The filtrate (acetic acid) was then dried over waterbath and tested for antimicrobial activity against shigella organisms.

The results of the preparation by TLC are shown in Table-3. Two active fractions were found in each of the three portions from the coloumn chromatography.

The acetic acid of the acetic acid extracts from TLC plates was removed by drying over waterbath and then dissolved in alcohol before the microbiological tests were done. Solvents are removed from the disc in all tests, and there were controls for all tests.

Working with other eluate from the column, the two active fractions had rf values of 0.86 and 0.66 when the mobile solvent was choroform methanol ethyl acetate, 5:2:3.

Working the second fraction, namely, eluate with alcohol in column chromatography the two active spots had rf value of 0.86 and 0.5 when the solvent system was Toluene ethylacetate acetic acid, 12:3:5.

Finally the acetic acid fraction gave two active spots with rf values of 0.25 and 0.70 when the solvent system was only acetic acid.

Thus we have six active fractions which are active against multiple resistant shigella, (Tables-3,4 and 5). Further investigation with tests, will show us which fraction will be of greater interest.

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Table - 3

The table shows the activity of TLC fractions of diethyl ether eluate against the resistant strains of shigellae :

Organisms Shigella	R-type	Fractions	Weight of the material (mg)	Zone of inhibitions (mm)
T-4563	ACT SXT	# 3	6.9	21
(S.dyst.1)		# 6	5.9	10
T-4487	ACT SXT NA	# 3	6.0	18
(S.dyst.1)		# 6	5.5	11
T-17(18)	Sensitive	# 3	6.0	13
(S.dyst.1)		# 6	5.5	12
2155	-	# 3	6.0	10
(S.boydii)		# 6	5.2	9
2315	-	# 3	5.0	10
(S.flexneri)		# 6	5.5	8

Cont'd.....P/10

Table - 4

The table shows the activity of TLC fractions of 'alcohol eluate' against the resistant strains of shigellae.

Organisms Shigellae	Fractions	Weight of the material (mg)	Zone of inhibitions (mm)
T-4487	# 2	7.5	20
(S.dyst.1)	# 4	5.2	10
T-17(18)	# 2	7.5	21
(S.dyst.1)	# 4	5.2	20
2155	# 2	7.5	10
(S.boydii)	# 4	5.2	11
2315	# 2	7.5	17
(S.flexneri)	# 4	5.2	9

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Table - 5

The table shows the activity of TLC fractions of " acetic acid eluate " against the resistant of shigella strains.

Organisms Shigellae	Fractions	Weight of the material	Zone of inhibitions
T-3563	# 1	5mg	16mm
(S.dyst.1)	# 3	5.2mg	15mm
T-4487	# 1	5mg	12mm
(S.dyst. 1)	# 3	5.2mg	14mm
T-17(18)	# 1	5mg	12mm
(S.dyst. 1)	# 3	5.2mg	11mm
2155	# 1	4.9mg	10mm
(S.boydii)	# 3	5mg	20mm
2315	# 1	4.9mg	9mm
(S.flexheri)	# 3	5mg	8mm.

It is to be noted that while metabolites (which we call antibiotics) from some microorganisms are found effective in microgram quantities against the growth of other microorganisms the antimicrobial substances from higher plants need not be effective in microgram quantities for use in therapy. The critical matter is the safety of the effective dose, the margin between effective and toxic dose. In the present case of E. hirta, we have only impure preparations so far and they are effective in petri-dish culture in quantities ranging between 5 to 7 mg. It would not be surprising if after further purification the activity could be demonstrated using microgram quantities. Use of the crude products in indigenous medicine is a common practice (6,7,8).

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ıe proper use of medicinal plants is a necessity, not a luxury. One f the elements critical to the success of primary health care is the vailability and use of appropriate dugs. The Alma-Ata Conference on rimary Health Care recommended to governments that they give high riority to the utilization of traditional medicine, including the in- orporation of proven traditional remedies into their national drug olicies and regulations. In the 13 years since Alma-Ata, decision-mak- rs in the health sector of many countries have taken the first steps, mong them the identification of locally available plants or plant xtracts that could usefully be added to national lists of drugs for se in primary health care, and which could even replace some pharma- eutical preparations that need to be purchased and imported.

WHO

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REFERENCES:

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DETAILED BUDGET

Item of expenditure/ budget heads	Rate per month	Amount Tk.
A. Personnel Costs:		
P.I. (1)	4,500x1x12	54,000.00
Co - P.I. (1)	4,000x1x12	48,000.00
Biochemist (1)	4,500x1x12	54,000.00
Asstt. Biochemist (1)	3,500x1x12	42,000.00
Asstt. Microbiologist (1)	3,500x1x12	42,000.00
Lab. Assistant (2)	3,000x2x12	72,000.00
B. Equipment:		
T.L.C equipment		1,00,000.00
Glasswares		75,000.00
C. Supplies:		
Chemicals & Solvents and plants		1,00,000.00
Stationary & Pringing		10,000.00
D. Transportation:		40,000.00
E. Contingency:		50,000.00
		6,87,000.00
Add overhead 10% of the above costs:		68,700.00
	Total Taka =	7,55,700.00

Total US Dollars = 19,377/-

1 US Dollar = Taka 39.00