tachment 1.

REVISED

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date AUGUST 10, 1988
AUGUST 10, 1989

Principal Investigator Dr A.K. Azad Chowdhury Trainee Investigator (if any) [illegible]

Application No. PCC/3/88 Supporting Agency (if Non-ICDDR,B) PCC-ICDDR,B-Bill Mahathali, Phoenix

Title of Study "studies on anti-shigella activity [illegible] and effect in experimental shigella [illegible] and effect in human volunteers"

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).

Source of Population:
(a) Ill subjects — Yes (No)
(b) Non-ill subjects — (Yes) No
(c) Minors or persons under guardianship — Yes (No)

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)

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)

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 Does not apply

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
D/A 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 Cttee. for review.

(PTO)

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

Principal Investigator

Trainee

SECTION I: RESEARCH PROTOCOL

1. Title : "Studies on anti-shigella activity of garlic extract and allicin in experimental shigellosis in animals and the determination of maximum tolerable single-dose of the aqueous extract in human volunteers".

2. Principal Investigator : Professor A.K. Azad Chowdhury
Department of Pharmacy, University of Dhaka.

3. Co-Investigators : 1. Dr. Zia Uddin Ahmed, Senior Scientist, ICDDR,B
2. Professor A.K. Azad Khan
Director, Research and Development,
BIRDEM, Dhaka
3. Dr. Muniruddin Ahmed
Associate Professor
Department of Pharmacy, University of Dhaka

4. Starting date : May 01, 1988

5. Completion date : April 30, 1990

6. Total direct cost : US$ 15,208.00

7. Scientific Program Head: This protocol has been approved by the Laboratory Sciences Division.

Signature of Chairman
Dept. of Pharmacy, Dhaka University

Date:

Signature of Associate Director, LSD

Date: July 18, 1988

8. Abstract summary

In a recently concluded study under a ICDDR,B and BMRC sponsored protocol, we found that an aqueous extract of garlic or it's active constituent, allicin, inhibited the *in vitro* growth of a number of multiply drug resistant strains of *Shigella flexneri*, *Shigella dysenteriae* type 1 and *Shigella sonnei*. The *in vivo* anti-shigella activity of the aqueous extract and allicin was studied in a rabbit model of experimental shigellosis using a well-characterized highly virulent strain of *Shigella flexneri* Y (strain SH-4). Most of the infected rabbits (4 out of 5) died within 36 hours of bacteria feeding (dose 10^{11} cfu) when left untreated by any of the agents (control). On the contrary, the aqueous extract of garlic or allicin when orally administered to the infected rabbits (aqueous extract: 7.5 ml/kg of body weight, loading dose followed by 1.5 ml/kg of body weight every 8 hour for 5 days or allicin: loading dose 50 mg/kg body weight followed by 15 mg/kg body weight 8 hourly for 5 days) protected all the animals (10 rabbits out 10) from the fatal attack. The anti-shigella activity was detected in the sera of the rabbits that received the aqueous extract or allicin through the oral route. These results are now being prepared for publication.

The promising results obtained in the previous study merit an extension of the work to (i) the rabbit model of experimental shigellosis using other serotypes of *Shigella flexneri*, (ii) the monkey model of experimental shigellosis using *Shigella dysenteriae* type 1 (the rabbit model is not responsive to this

serotype) and (iii) determination of maximum tolerable dose of the aqueous extract in healthy adult volunteers to obtain information that may be helpful in a possible clinical trial of the extract in human shigellosis.

SECTION II: RESEARCH PLAN

A. INTRODUCTION

1. Objective

To contribute towards the development of a herbal drug which will be easy to use and efficacious in the treatment of shigellosis.

2. Background

About 80-85% of the bacillary dysentery cases in Bangladesh is due to shigella infection (1). Since 1970, the treatment of shigellosis has become difficult because of the emergence of strains resistant to a number of drugs such as tetracycline, chloramphenicol, streptomycin and cotrimoxazole (1-3). Ampicillin and nalidixic acid were then left as the drugs of choice in the treatment of shigellosis (2, 4). Development of resistance to these drugs have also been reported recently (4-6). Newly emerged multiply drug-resistant strains have been causing outbreaks of shigellosis in recent years in Bangladesh (4, 7). Hence, research on antimicrobial drugs that may be effective against multiply-drug resistant strains of *Shigella* will be of interest.

The antimicrobial activity of garlic and it's active constituent allicin (diallyl thiosulfonate) against a number of bacteria have been reported (8-13). To the best of our knowledge, no such reports have been published on *Shigella*. It was also reported that the garlic extract is active against clinically resistant strains of *Staphylococcus*, *Escherichia*, *Proteus* and *Pseudomonas* (14). Chinese workers recently reported successful treatment of

cryptococcal meningitis, a serious fungal disease, b[illegible] a. intramuscular and or intravenous administration of garlic extract 15). The antifungal activity was detectable in the sera of healthy human volunteers after oral administration of garlic extract (16). Recently, it was reported that the garlic extract is also active against different strains of *Mycobacterium* (17). It was suggested that the extract may have a potential for use in the treatment of tuberculosis in conjunction with other anti tubercular drugs (17).

In a recently concluded study, we observed (manuscript in preparation) that the aqueous extract of garlic and allicin each had significant *in vitro* anti shigella activity against a number of multiply drug-resistant strains (resistant to ampicillin, nalidixic acid, cotrimoxazole, tetracycline, chloramphenicol) of *Shigella dysenteriae* type 1, *Shigella flexneri* and *Shigella sonnei*. The minimum inhibitory concentration (MIC) of the extract and allicin against *Shigella flexneri* Y were 5 and 0.4 mg/ml, respectively.

In the same study it was also shown that the aqueous extract and allicin each possessed *in vivo* anti-shigella activity in a rabbit model of experimental shigellosis. Each of these drugs, the extract or allicin, when administered through per oral route; aqueous extract at a dose of 7.5 ml/kg of body weight as a loading dose, followed by 1.5 ml/kg of body weight every eight hour for five days allicin 50 mg/kg of body weight as loading dose followed by 15 mg/kg of body weight 8 hourly for 5 days;

prevented the death of rabbits infected with a lethal dose of *Shigella flexneri* Y (SH-4) in all the cases, (10 out of 10 infected rabbits survived). On the contrary, the survival of identically infected rabbits which did not receive any drugs (control group) was only 25% (1 out of 5 infected rabbits survived the fatal attack of shigellosis). The anti-shigella activity against *Shigella flexneri* was also observed in the sera of the rabbits after oral administration of the extract/allicin. The LD_{50}, values (per oral route) were determined in mice and rats and these were found to be 100 g/kg for each species. The LD_{50} value (per oral route) of allicin in mice was 200 mg/kg. The values when compared with those of effective dose (ED_{50}), indicated that the drug seemed to have therapeutic potential.

3. Rationale

Newly emerged multiply-drug resistant (resistant to ampicillin, nalidixic acid, cotrimoxazole, tetracycline, chloramphenicol and streptomycin) strains of *Shigella* have been causing outbreaks of shigellosis in Bangladesh in recent years (4, 7). Hence, there is a need for studying the anti shigella drugs active against such resistant strains. In this context, the promising results of the anti shigella activity of an aqueous extract of garlic and allicin merit extension. We wish to extend (i) the rabbit model of shigellosis to include additional 2-3 most commonly isolated serotypes of *Shigella flexneri* and (ii) use the monkey model of shigellosis to study the therapeutic effect of the drugs against *S. dysenteriae* 1. The monkey model is chosen because of its close resemblence to human shigellosis and the fact that *S.*

dysenteriae 1 cannot cause disease in rabbits. Finally, (iii) the aqueous extract needs to be administered to healthy adult human volunteers to ascertain the maximum tolerated dose which could be useful in clinical trials if conducted at some stage. At least three doses will be given to three groups, one dose once to every individual in a group. This shall indicate what is the maximum tolerated dose of garlic extract which can be ingested without a burning sensation in the stomach, vomitting, anorexia and other discomforts. The study of the Biochemical parameters of the body fluids of the volunteers will reveal whether the aqueous extract has any adverse effects. The aqueous extract was not found to have any adverse reactions in patients in China (15) and in adult healthy volunteers (16) in the U.S.A. Hence this portion of the study is an extension the work already carried out in China and the U.S.A. Only difference is that the present work is planned in healthy adult Bangladeshi volunteers whose bodyweight, nutritional status, disease profile, ethnic components are different from those of the Chinese and the Americans. The anti-shigella activity will also be determined in the sera of the volunteers who will receive the garlic extract orally. If the drug is found to be safe in a single dose regime, studies using multiple therapeutic doses (that will be calculated on the basis of the animal studies) for five days in the healthy volunteers shall be initiated at a later stage not within the framework of this protocol.

B. SPECIFIC AIMS

1. To extend the study of *in vivo* anti-shigella activity of the aqueous extract of garlic and allicin to rabbit model of experimental shigellosis using additional 2-3 most commonly isolated serotypes of *Shigella flexneri*. The dose range for therapeutic effect (E.D. 50) of the extract shall also be determined: The dose regime which protected the infected rabbits from fatalities in our previous study shall be used as a guideline. The other doses shall be 1/2 and 1/4 of that dose (the doses used in the previous study were aqueous extract: 7.5 ml/kg of bodyweight as a loading dose followed by 1.5 ml/kg bodyweight every eight hourly for 5 days; allicin: loading dose of 50 mg/kg or body weight followed by 15 mg/kg of body weight 8 hourly for 5 days).

2. To extend the study to monkey model of shigellosis using strains of *Shigella flexneri* Y (strain SH-4) and *Shigella dysenteriae* type 1, to determine the therapeutic efficacy and toxicity of the agents in the monkeys.

3. To study the effect of multiple therapeutic dose of the garlic extract for a longer period on the biochemical parameters (SGOT, SGPT, serum alkaline phosphatase, BUN, serum creatinine) of the blood and the histological properties of liver and kidney in animals (rabbits and monkeys).

4. To establish the maximum tolerable dose of the aqueous extract in healthy adult volunteers. Three groups of volunteers each group consisting of three persons, will receive three different doses (0.125 ml/kg i.e. 7.5 ml/person, 0.25 ml/kg i.e. 12.5 ml/person and 0.5 ml/kg i.e. 25 ml/person). The discomfort such as burning sensation in the stomach, nausea, light headedness if experienced by the volunteers will be recorded. Levels of various toxicity indicator enzymes in the blood will be determined following oral administration of the extract.

C. METHODS

1. PREPARATION OF THE AQUEOUS EXTRACT OF GARLIC

The peeled and crushed garlic cloves (500 g) will be extracted with 150 ml of water (to a total volume 500 ml). The material will be filtered through a moslin cloth and the filtrate will be centrifuged at 2000 xg for 20 minutes. The supernatant will be sterilized by passing it through a millipore filter paper (0.45 u) and will be stored at $4^{o}C$ for use within a maximum period of six days.

2. ISOLATION OF ALLICIN

Allicin will be extracted according to the literature method (18). In the method the, ground garlic cloves (500 g) was extracted with ethyl alcohol (100 ml x 3). The combined alcohol extract was evaporated to dryness *in vacuo*. To the oily residue, water (200 ml) was added and the resulting suspension was

distilled till the volume was reduced to one-third. The distillate was extracted with ether (100 ml x 3). The combined ether extract was evaporated *in vacuo* to yield an oil which was mainly allicin (structure established by U.V., I.R. and N.M.R. spectroscopies)

3. *IN VIVO* ANTI-SHIGELLA ACTIVITY

(a) RABBIT MODEL OF EXPERIMENTAL SHIGELLOSIS

The model was originally developed for *V. cholerae* by Spira *et al* (19) and modified by Cray *et al* (20). By following the modifications suggested by Cray *et al* (19), the model was extended to *Shigella flexneri* serotypes 6 and Y by Dr. David Sack and Dr. Zia Ahmed (21). In our previous study, we used the model successfully to assess the therapeutic potential of the extract or allicin. The model involved fasting the rabbits for 40 hours. During this period they were allowed to have only drinking water containing tetracycline (1 mg/ml) *ad libitum*. After the period, the rabbits received (i) cimetidine (50 mg/kg body weight, i.v. at time 0), (ii) 5% sodium bicarbonate solution (15 mlx 2, per oral, at 15 min and 30 min), (iii) an inoculum of the virulent strain of *Shigella flexneri* Y (10^{11} cells suspended in BHI broth) per oral and (iv) tincture opium (2 ml i.p.). After these treatments, normal food and water were allowed. By conditioning rabbits in this manner and by using *Shigella flexneri* 6, it was observed that the organism colonized in the G.I. tract, caused death to most of the animals and left protective immunity to the surviving animals. In our proposed studies, the model will be

used similarly (using adult albino rabbits each weighing 2 kg approximately. For each agent five rabbits will be used) but 2-3 most commonly isolated strains (from clinical cases of shigellosis) of *Shigella flexneri* will be used instead of *Shigella flexneri* Y (strain SH-4). Treatment with the aqueous extract or allicin will be started from 8 hours after inoculum feeding. Different doses of aqueous extract of garlic/allicin will be used to find out E.D. 50. The control group will receive distilled water in place of the agent. The statistical treatments on the data shall be done to find out the significance of antibacterial activity and toxicity of the agents.

(b) MONKEY MODEL

In the model, the monkeys will be fed with some well characterized virulent strains of *Shigella flexneri* or *Shigella dysenteriae* type 1 according to the standard methods (22). After the appearance of symptoms of shigellosis the animals will be treated with the aqueous extract/allicin (doses shall be equipvalent to what has been used for rabbit) (through the per oral route). The frequency and volume of stool and body temperature will be recorded and rectal swab cultures (once everyday) will be done on MacConkey and SS agar plates. The anti-shigella activity of the sera of the monkeys, 30 and 60 minutes after oral administration of the agents, will be determined.

(c) HEALTHY ADULT VOLUNTEER STUDY

(i) Selection criteria: Nine healthy adult male volunteers will be selected for the study after medical examination by a physician not associated with the project. Informed consent will be obtained from the volunteers prior to their inclusion in the study.

(ii) Place of study: The volunteer study will be conducted at the facilities of the BIRDEM hospital. The trial will be carried out under the overall supervision of Dr. A.K. Azad Khan, Professor of the Department of Gastroenterology, IPGM&R, Dhaka now on deputation in BIRDEM as its Research and Development Director.

(iii) The agents and the dose: Only the aqueous extract of garlic will be orally administered to the volunteers. The dose of the extract will be adjusted according to the body weight of the subject and keeping in view the reported safe (15) dose as a rough guidelines. The three doses, 0.125 ml/kg (7.25 ml/person); 0.25 ml/kg (12.5 ml/person) and 0.5 ml/kg (25 ml/person) will be given to nine persons of three groups. These doses may also be adjusted according to the ED_{50} values to be obtained from rabbit experiment. The normal bio-medical parameters such as (i) Heart rate, (ii) E.C.G., (iii) Blood pressure, (iv) Body temperature, (v) Feeling, etc. will be recorded before and after the administration of the garlic extract. On the third day preceding the administration of the agent the T.C., D.C., Hb% and E.S.R. will be determined. SGPT, SGOT, serum alkaline phosphatase,

blood urea nitrogen and serum creatinine will also be determined. The stool and urine of the volunteers will be subjected to routine examination. Discomfort such as burning sensation in the stomach, nausea, light headedness, if felt by the volunteers shall also be recorded.

(iv) Duration of the hospital confinement: Each of the volunteers will be confined to the hospital for one day for observations and studies, following the administration of garlic extract.

(d) TOXICOLOGICAL STUDIES

Toxicity studies will be extended to monkeys using standard methods (22). The chronic toxicity study shall be studied after multiple therapeutic doses over a longer period of time. Studies on the blood and biochemical parameters of the volunteers after administration of the aqueous extract of garlic shall also reveal the toxicities, if any, of the agent.

D. SIGNIFICANCE OF THE WORK

This study of anti-shigella activity of the aqueous extract of garlic and allicin in the rabbit and monkey model may be of significance in the development of a treatment against shigellosis by using this edible herb. The volunteer trial to determine the maximum tolerable dose of the aqueous extract is a pre-requisite for clinical trial of the agent.

E and F. FACILITIES REQUIRED AND COLLABORATIVE ARRANGEMENTS

The general operational strategy will be similar to that of the last protocol (Title: Shigella drugs from Garlic, 'Ashok' and 'Neem' sponsored by PCC, ICDDR,B and BMRC Collaborative Research Programme May 1987 to 30th April 1988). Monkeys shall be procured by the principal investigator. The ICDDR,B is expected to lend some cages for the monkeys and assist in training his workers in monkey handling and feeding procedures.

SECTION III: BUDGET

YEAR 1

1. Personnel services

Name	Time required	Honorarium/Salary P.A.
Dr. A.K. Azad Chowdhury Principal Investigator	20%	Tk. 6000x12 = 72,000.00
Dr. Zia Uddin Ahmed Co-Investigator	5%	-
Dr. A.K. Azad Khan Co-Investigator	5%	-
Dr. Muniruddin Ahmed Co-Investigator	5%	Tk. 2000x12 = 24,000.00
Research Fellow (to be appointed)	100%	Tk. 4000x12 = 48,000.00
Laboratory Assistant	20%	Tk. 1000x12 = 12,000.00
Animal House Attendant	10%	Tk. 500x12 = 6,000.00

2. Supplies

(a) Bacteriological media, disposable Petri dishes, syringes and glass ware	Tk.	25,000.00
(b) Chemical and reagents and biochemical tests	Tk.	40,000.00
(c) Animals:		
Monkeys Tk. 2000x25 = 50,000.00 Rabbits Tk. 400x50 = 20,000.00 Rats Tk. 25x50 = 1,250.00 Mice Tk. 12x25 = 300.00		
Tk. 71,550.00	Tk.	71,550.00
(d) Animal feed, drugs and maintenance	Tk.	30,000.00
c/o. Page total	Tk.	3,28,550.00

YEAR 1 (continued)

b/f Page total	Tk.	3,28,550.00
3. Volunteer study (including missed salary or wages of the volunteers), hospital charges, clinical tests	Tk.	50,000.00
4. Transport and conveyance	Tk.	15,000.00
5. Overhead, contingency and stationary	Tk.	20,000.00
Grand total	Tk.	4,13,550.00
	=	US$ 12,923.00

YEAR 2

1. Personnel services (same as year 1)	Tk.	1,62,600.00
2. Supplies		
a) . Chemicals, reagents, expendables	Tk.	20,000.00
b) Animals (mainly maintenance)	Tk.	45,000.00
3. Transport	Tk.	15,000.00
4. Overhead, contingency, stationery	Tk.	12,000.00
Total Tk.		2,57,000.00
		= US$ 8,031.00

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CONSENT FORM

Garlic has shown significant promise in the treatment of experimental shigellosis in a rabbit model (manuscript in preparation). We wish to study in the future it's usefulness in human dysentery caused by *Shigella*. Before using this in human subjects, it is imperative that a formal safety study be done in healthy adult volunteers. Garlic at a dose of 30 g/person administered once has been shown to be free from any adverse effects in healthy adult volunteers[1]. It has also been orally and intravenously administered to patients with cryptococcal meningitis at a daily dose of 18 g and 80 to 100 mg, respectively, with no adverse effects. In our toxicity studies in mice, rats and rabbits, the above doses were found to be completely safe.

If you wish to participate in the present safety study of garlic extract you will be given 25 ml of the extract (contents of 25 garlic cloves) to drink. A 5 ml sample of blood will be taken from your anticubital vein twice, 30 and 60 min after drinking the extract. A sample of your urine will also be collected for analysis of the biochemical parameters and also for the determination of anti-shigella activity. You will be required to stay in the hospital for a maximum period of 24 h for routine observation. You may withdraw yourself from the study any time you wish. Your hospital treatment at BIRDEM will not be influenced in any way by your decision to withdraw. Complete

confidentiality of your health records shall be maintained. We shall be ready to answer any questions regarding the trial.

If you agree to participate in the trial please put your signature below.

Signature of the investigator

Date:

Signature of the volunteer

Address:

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(papers enclosed)

Date Name Weight No.

Pentagastrin ug -(ml)

	Periods	Gastric content (ml)	Free acid	Total acid
BASAL	T_0			
	T_{15}			
	T_{30}			
	T_{45}			
	T_{60}			
	T_{75}			
STIMULATED	T_{90}			
	T_{105}			
	T_{120}			
	T_{135}			

BASAL ACID OUTPUT mmol H+/hour

MAXIMUM ACID OUTPUT mmol H+/hour