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Efficacy of Earthworm Against Rheumatic Fever

Rahman, Md. Mijanur

University of Rajshahi

EFFICACY OF EARTHWORM AGAINST RHEUMATIC FEVER

Ph.D Dissertation

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Ecology Research Laboratory
Department of Zoology
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A Thesis Submitted for the Degree of Doctor of Philosophy to the Department of Zoology, Faculty of Life and Earth Science, University of Rajshahi, Bangladesh

Ecology Research Laboratory
Department of Zoology
University of Rajshahi
Bangladesh

December 2006

DEDICATED
TO
MY
BELOVED
PARENTS

DECLARATION

I declare that the work submitted as the thesis entitled "Efficacy of Earthworm Against Rheumatic Fever" to the Department of Zoology, University of Rajshahi, Rajshahi, Bangladesh for the degree of Doctor of Philosophy is the result of my own investigation carried out under the supervision of Professor Dr. Md. Sarwar Jahan, Director, Institute of Environmental Science, University of Rajshahi, Bangladesh. The thesis or part of it has not been submitted to any other University or Institution for any other degree or diploma. To the best of my knowledge and belief, it does not contain any material previously published or written by any other except when due reference is made in the text of the thesis.

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CERTIFICATE

This is to certify that the thesis **"Efficacy of Earthworm Against Rheumatic Fever"** submitted by Mr. Md. Mijanur Rahman for the award of the degree of Doctor of Philosophy to the Department of Zoology of the University of Rajshahi, is based on the results of his own research work carried out under my supervision as a PhD. research fellow. The thesis or part thereof has not been previously presented for any diploma or degree except due references wherever needed.

It becomes to me that Mr. Rahman has made some distinct contribution through this original research work in the concerned field of learning. I gladly recommend him to submit the thesis to the University for Ph.D. degree.

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ABBREVIATION

RF	:	Rheumatic fever
RHD	:	Rheumatic heart disease
ARF	:	Acute rheumatic fever
P	:	Patients
ASO	:	Anti-streptolysin 'O'
ESR	:	Erythrocyte sedimentation rate
ASP	:	Anti-streptococcal polysaccharide
ASK	:	Anti-streptokinase
CRP	:	c-reactive protein
ECG	:	Electrocardiograph
S	:	Saturated
HCF	:	Carbohydrate high fibres
mM	:	Milli mole
CHD	:	Coronary heart disease
TLC	:	Thin layer chromatography
PTLC	:	Preparatory thin layer chromatography
R_f	:	Front relation value

ABSTRACT

Rheumatic fever is a chronic lethal disease which affects human at the age of 5-22 years. The disease if not managed properly in time it provokes heart diseases or valvular heart diseases after 10-15 years. There is no specific or selected therapy for the treatment or management of rheumatic fever.

Earthworms had been used as the potential source of medicine for treatment of many diseases related to rheumatic fever effectively from ancient time. Accordingly, it was thought that earthworm tissue might contain certain effective medicinal compounds to cure rheumatic fever and investigations were performed *in vitro* (Laboratory test) and *in vivo* (clinically). First of all earthworm's extract was obtained from dry powder of one of the most common indigenous species by using successively ten different solvents through continuous hot percolation process for extraction. Diethyl ether, chloroform, methanol, ethanol, propanol, butanol, acetic acid, acetone, xylol and morpholine extracted out 2.89±1.23, 3.32±1.36, 16.25±3.15, 4.62±1.73, 0.86±0.29, 0.93±0.36, 1.15±0.49, 1.09±0.46, 0.76±0.38, and 0.80±0.18 in percent by weight (mg) of the dry powder of earthworm tissue.

Biochemical analysis disclosed that the total extract (32.64±4.75%) contained 59.52±9.96% crude protein, 4.82±1.36% crude fat, 4.67±2.10% crude carbohydrate, 5.65±2.38% total ash, 2.54±0.99% crude fibre and 0.048±0.018% steroids. TLC and PTLC revealed that 100 mg of earthworm protein contained seven essential amino acids, cysteine (1.64±0.72 mg), histidine (3.23±1.00mg), lysine (6.55±1.09mg), methionine (2.40±0.60mg), phenylalanine (5.25±1.01mg), tryptophan (2.11±0.55mg) and valine (4.22±1.02mg).

As the extraction was achieved to test the medicinal efficacy of earthworm extract it was obligatory to test the toxicity of the compounds. Toxicity test on guineapig (*Cavia porcellus*) showed that earthworm extracts obtained had no toxic or adverse effect.

Diethyl ether, chloroform, methanol, ethanol, propanol, butanol, acetic acid, acetone, xylol and morpholine extracts inhibited 12.50±2.20mm, 12.86±3.72mm, 14.60±4.00mm, 14.93±2.74mm, 1.50±0.77mm, 8.13±2.65mm, 14.70±3.12mm, 2.20±1.12mm, 1.46±0.50mm and 1.60±0.67mm respectively when applied on *ß-haemolytica streptococcus* culture *in vitro.*

Primarily each of the ten extracts was orally administrated on five rheumatic fever patients for six weeks to test their medicinal efficacy. It was found that only methanolic and ethanolic extracts were able to play vital role in curing the patients and the rest did not response positively.

Later on the effective i.e. methanolic and ethanolic ones were applied on rheumatic fever patients along with a control group treated with neutral sugar depending on the availability. For each treatment 20 patients were considered in course of the study. Methanolic and ethanolic extracts cure 55% and 70% patients respectively. This study shows that the earthworms tissue may be used as a source of an effective biomedicine for treatment of rheumatic fever. Further intensive experiments are obligatory for extraction, identification and separation of effective compounds and clinical trials shall have to be performed for ascertaining this biomedicine as an effective remedy for rheumatic fever.

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CHAPTER ONE

GENERAL INTRODUCTION

CHAPTER 1
GENERAL INTRODUCTION

The medicinal histories of earthworms are not new. It is used as medicine in many countries as found in many pharmacopoeias like Chinese Pharmacopoeia, Indian Pharmacopoeia, Vietnam Pharmacopoeia, Hamdard Pharmacopoeia of Eastern Medicine etc. for thousands of years (Fu-Zhen and Shi-Wei, 1982). It is used successfully on many critical and complicated diseases (Sabine, 1991). Earthworms are used as therapeutic drugs can be read in a book on Chinese medicine of the ancient "China, Shen Nong's herbal" (Fuxia *et al,* 1992).

Japanese Scholar Professor Shan Hangren discovered enzymatic functions of extract from earthworms and for this he was honoured with United Nation Science Conference Award in 1978. This extracted drug is included as a new medicine for thrombus, embolism and infarction in the Chinese Pharmacopoeia in 2000 (Ansari, 2001).

The medicines prepared from earthworms are used to cure stone in urinary bladder, jaundice, liver related problems, pyorrhea, piles, rheumatism or gout, diarrhoea, antipyretic, weakness of pregnancy, sexual impotency, anti-asthmatic, epilepsy, anti-bronchitis, anti-spasmodic, diuretic and detoxic, anti-hypertensive, spermatocides *in vitro* and *in vivo,* anti-viral, anti-tumour, hyperglycemic, hernia, chronic cough, diphtheria, anti-inflammatory, thrombosis, fibrin and infarct, hair grow, testing pregnancy, carcinogenic properties, wounds, chronic boils, induced oedema and anty-fungal (Stephenson, 1930; Bristowf, 1932; Shukla, 1950; Hasenbein, 1951;

Gerch, 1954; Wahid and Siddique, 1961; Weisbach, 1962; Said, 1971; Renolds and Renolds, 1972; Yegnanarayan *et al.*, 1987, 1988; Julka, 1988; Mihara *et al.*, 1991a, b; Sabine, 1991; Fuxia *et al.*, 1992; Ismail *et al.*, 1992; Qingsui, 1995; Edwards and Bohlen, 1996; Bundy *et al.*, 2001).

Earthworms were used variously as medicines in the past. Hamudullah Mustafi of Qazwin in `Naizat-ul-Qutub' (1340) and Dameri in `Hayat-ul-Haiwan (1371) tell us of medicines from earthworms to cure various diseases (Kotpal, 1998). In Vietnam, Korea, India, Pakistan and other Asian countries earthworms are valuable sources of medicine. In Vietnam the earthworm is the major ingredient in a formula amazingly called "Miracle Medicine" that can save life in sixty minutes (Qingsui, 1995).

It is known that rheumatic fever (RF) is a very serious type of disease of childhood and adolescence (Macleod, 1948). For instance, several minority groups of the pacific Islands still suffer from a very high prevalence of rheumatic heart disease (RHD) in comparison to general population. In New Zealand, most cases of RHD were reported from the northern part of North Island, where Maori and Polynesian populations were concentrated (Neutze, 1988 in Rouf, 1997). In Australia, RF and RHD persist only among aboriginal communities living in the Northern Territory (MacDonald and Walker, 1989). The prevalence of RHD tends to be higher among the urban poor than among rural poor. It ranges from 8.5 to 11 cases per 1,000 individuals in the largest cities of Africa, Asia, and Latin America, whereas in rural areas RHD prevalence does not exceed 3.5 per thousand on an average (Toma, 1985). Annual Incidences of Rheumatic Fever in selected areas per 1000 peoples are as follows:

England and Wales (1963) 4.7, Baltimore (United States) (1964) 5.3, Denmark (1970) 0.7, Singapore (1971) 9.2, Cyprus (1972) 27-43 person, Hong Kong (1972) 23, Czechoslovakia (1972) 8.5, Iran (1973) 5-100; Kuwait (1983) 19.6, Auckland (New Zealand) (1984) 7.6, Hawaii (United States) (1976-80) 14.4, Salt Lake country (United States) (1985) 18.1 (Majeed *et al.*, 1987 in Rouf, 1997). Again prevalences of Rheumatic Heart Disease in school age children in different areas are as follows:

Africa

Algeria (1970) 15.0, Nigeria (1970) 3.0, Egypt (1973) 10.0, Morocco (1973) 9.9, South Africa (1975) 6.9, Cote D'Ivoire (1985) 10.

Latin America

Brazil (1968-70s) 1.6-6.8, Montevideo, Uruguay (1970-1985) 10.0, La Paz,, Bolivia (1973) 17.0, Mexico City, Mexico (1977) 8.5, San Juan, Puerto Rico (1980) 1.9, Caracas, Venezuela (1985) 10.0, Porto Allegre, Brazil (1985) 10.0, Sao Paulo, Brazil (1985) 10.0.

Asia

Tokyo, Japan (1966) 0.3; Taiwan, Republic of China (1970) 1.4; India (1970) 6.0-11.0; Pakistan (1970s) 1.8-11.00; Thailand (1974) 1.2-2.1; China (1979); Monglia (nd) 3.5; New Dilhi, India (1985) 11; & Bangladesh (1988-96) 3.6 (WHO, 1988 in Rouf *et al.*, 1997)

The prevalence of this disease (RF & RHD) is very high in Bangladesh as it is a disease of people living in overcrowded unhygienic and poor socioeconomic conditions (Annonymous, 1995). According to

Rouf (1997) in Bangladesh about 500000 patients were suffering from RF & RHD of which about 15% would need cardiac surgery within next 10 to 15 years. Moreover about 40% of all the cardiac patients in National Institute of Cardiovascular Diseases (NICVD) belong to RHD and 60-80% of cardiac surgery patients were of RHD origin. But there are patients coming from higher socioeconomic groups also. Patients of this group usually take excessive cold drinks, ice creams, cakes, rich and fatty foods etc. at the early age.

Rheumatic fever is a syndrome due to non-suppurative inflammatory sequel of group A, β-haemolytic streptococcal pharyngitis occurring usually after 2 to 3 weeks later. The disease is manifested by arthritis, carditis, erythema marginetum, subcutaneous nodules, fever and supporting evidence of resent streptococcal infection in 5-22 years age group. If not treated properly the syndrome develops as Rheumatic Heart Disease later on (Macleoid 1948; Goldstein *et al.*, 1968; Cairns, 1988;Taranta and Markawitz, 1989; Rouf *et al.*, 1991; Haque *et al.*, 1992, 1993 and 1993; Annonymous, 1992 and 1992, 1996, 1998, 2005; Dean *et al.*, 1993; Nahar, 1994; Khan *et al.*, 1994; Begum *et al.*, 1994; Ahmed *et al.*, 1996; Rouf, 1996 and 1997; Rouf *et al.*, 1997; Jalil, 1997; Zaman *et al.*, 1998; Ahmed *et al.*, 2005).

The rank of heart disease was second to diarrhoeal disease in Worldwide prevalence of infection communicable disease, (Annonymous, 1994; Kumar and Clark, 1995), but after controlling of diarrhoeal and infectious diseases the prevalence rank is first in the worldwide (Shahidullah, 1997).

Diagnosis of Rheumatic Fever

Clinical Manifestations

Because of the unavailability of test that allows a specific diagnosis of acute rheumatic fever, it may continue to depend on the criteria for diagnosis that were formulated by Jones (1944) which have come into widespread use in a modified form. The revised Jones Criteria was widely used as a guidance of diagnosis of RF during the last decade. At last American Heart Association has updated the criteria for the diagnosis of initial attack of RF.

Salient features of RF are grouped into major and minor manifestations. The clinical signs which were most useful diagnostically were designated major manifestation and other less characteristic findings were termed as minor manifestations.

Major criteria: Major criteria included carditis, polyarthritis, chorea, subcutaneous nodules and erythemamarginatum.

Carditis is the most serious manifestations of RF because it may cause death during the acute attack or cause residual valvular damage with permanent disability and late mortality. Carditis appear usually within first 2 to 3 weeks of the attack and seldom later. Carditis occurs in 40 to 50% of the patients with initial attack. It usually involves the **endocardium, myocardium** and **pericardium** to varying degrees. Pericarditis occurs in 5 to 10% of patients with ARF (Acute rheumatic fever). Patients with pericaditis develop chest pain and fever, pericardial rub and pericardial effusion. Myocarditis is an important feature of ARF. It is difficult to diagnose

clinically. It is suggested by tachycardia and cardiac enlargement. Other symptoms include dyspnoea and oedema. Gallop rhythm is common physical finding. Endocarditis gives rise to heart murmur.

Polyarthritis is the most frequent but benign major manifestation of RF occurring in about 75% of patients. Joint involvement occur early and pain is more marked than swelling. It may affect several joints one after another and is called "migrating" or 'migratory' polyarthritis. The knee joints are most frequently affected (75%) followed by the ankles (50%), wrists, hips and small joints of the feet (each 12-15%), shoulders and small joints of the hand (7-8%). If left untreated the joint swelling disappear spontaneously in 3 to 4 weeks time leaving no permanent deformities of joints.

Sydenham's chorea is a neurological disorder consisting of involuntary movements, muscular weakness and emotional disturbances. The movements are abrupt, purposeless, non-repetitive and disappear during sleep. It usually involves the hands and face. Speech may become slurred. The emotional manifestations include crying smiling or restlessness. It is usually associated with carditis. It is unusual after puberty not occur in adult and is the only manifestations with a marked sex preference. Chorea is twice as frequent in girls as in boys.

Subcutaneous Nodules appears after few weeks of illness and usually associated with carditis. The nodules are firm, painless and move freely over the underlying skin. They vary from a few millimeters to 2 cm. They last one or more weeks but never more than a month. They are seen over the extensor surfaces of elbows, knees, wrists, and in the occipital region.

Erythemamarginatum occurs in the early stage of ARF. It may persist or may appear later or may appear for the first time during convalescence. It is also associated with carditis. It is transient, non-pruritic skin rash, pink coloured affecting the trunk and proximal part of limbs but never face. It consists of patches with pale centre and raised margin and may appear or disappear in few hours.

Minor Criteria: Arthralgia is joint pain without sign of inflammation and usually affects more than one joint called polyarthralgia. Fever is always present at the initial stage of polyarthritis but absent in chorea. Fever is remittent in type and rarely exceeds 39°C. Abdominal pain may occur with congestive heart failure due to distension of liver. Anorexia, nausea and vomiting may occur either due to heart failure or salicylate toxicity. Epitasis may also occur.

Duration of initial attack of RF ranges from 6 weeks to 3 months. But in patients with severe carditis it may prolonged to six months. It is shortest in attacks with arthritis.

The diagnosis of acute RF requires the presence of two major criteria or of one major and two minor criteria indicates a high probability or acute rheumatic fever, if supported by evidence of proceeding group A streptococcal infection.

Laboratory Test for Rheumatic Fever

Although there is no selected laboratory test for specific diagnosis of RF, but three kinds of laboratory test are useful for the diagnosis of rheumatic fever, test for evidence of recent streptococcal infection, systemic inflammation and evaluate cardiac involvement. These are

Identification of Organism by Culture and Non-culture Methods

i. Most widely detected throughout the world. In RF throat swab culture may be 90% positive.

ii. Measurements of antibodies against or streptococcal infection that are Anti-Streptolysin O (ASO), Anti-Streptokinase (ASK) Anti-Streptococcal Polysaccharide (ASP), etc.

ASO: ASO titre is most widely detected throughout the world. In RF 80% patients showed high ASO titre within 3 months of the onset of the disease. The normal limit of ASO titre may differ according to the year, season and age. The mean titre of ASO in 85% of healthy persons can be taken as normal limit. Usually in healthy population the range of ASO titre varies from 50 to 200 unit/ml. The antigen test is commercially available.

ASP: It is thought to have cross immunity between cardiac muscles or tissues and streptococcal polysaccharides according to many researchers. The ASP level is determined by passive haemagglutination. 15% of the controlled children show a high titre. In RF with severe carditis the tritre may be very high. It is also very high (about 85%) in acute glomerulonephritis. Rising titre may be helpful for diagnosis.

ASK: This antibody is determined by passive hemagglutination method using type 'O' human red cells treated with formalin and tannic acid. This test kit is available commercially (Kinase kit). In acute RF 81% of patients show high titre within 3 months of the onset. Recently sensitive gelatin particles are used instead of human 'O' red blood cells.

Tests for Evidence of Systemic Inflammation

Rheumatic fever is an inflammatory disease. Presence and degree of systemic inflammation can be measured by acute phase reactants of which erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are most commonly used. Neither of these tests is specific for rheumatic fever but they are very sensitive and useful supportive evidence. Both ESR and CRP reflect the magnitude of the inflammatory process and are useful to find out if the inflammatory process is still going on after the symptoms and signs have subsided.

Tests to Evaluate Cardiac Involvement

Radiography of the heart, ECG, Echocardiography and Doppler studies are the methods most often used to detect heart involvement in Rheumatic Fever (Islam e*t al.*, 1998).

According to Rouf (1997), Haque *et al.* (1994) in Bangladesh about 500000 (patients were suffering from RF&RHD of which about 15% would need cardiac surgery within next 10 to 15 years. Moreover, about 40% of all the cardiac patients in National Institute of Cardiovascular Diseases (NICVD) belonged to RHD and 60-80% of cardiac surgery patients were of RHD origin. According to Murshed (2000) WHO had taken the following preventive strategies: i) Primary Prevention (ii) Secondary prevention (iii) High risk strategy

i) Primary Prevention

Control of hypertension, control of lipids and cholesterol, cessation of smoking, behavior changes in curtailing aggressive, anxious temperament

etc. in apparently unattacked but (prone on grounds of risk factors to coronary heart disease) consist of primary prevention.

a) Exercise: The best kind of 'fitness' was considered to be the endurance fitness: the ability to do prolonged work without fatigue which had to do with the body's overall health, the health of the heart, the lungs, the entire cardiovascular system and the other organs, as well as the muscles. The best kind of exercise is aerobic exercise that demands oxygen and forces the body to process more air with less effort, the heart grows stronger, pumps more blood with each stroke, reducing the number of strokes necessary.

b) Dietary Management: The main elements in dietary management involves reduction of fat and cholesterol intake and the substitution of polyunsaturated (P) for saturated (S) fats to achieve a P/S ratio of approximately 2/1, generous intake of vegetables and other fibers rich food, including maintenance of ideal body weight, physical activity and a life-style conducive to healthful living. A reduction of 20-30% in serum cholesterol can be expected in most subjects with high carbohydrate-high fibers (HCF) and low fat diets. Along with the decrease, there is a 10% increase of high-density lipoprotein (HDL) which is of protective value against coronary heart disease. For management of hypercholesterolaemia, a high carbohydrate-high fibres (HCF) and low fat diet, providing 55-60% of energy as carbohydrate, 15-20% protein, 20-30% fat, less than 300 mg cholesterol, and about 50g dietary fibers (20-30g/1000 Kcal. is recommended in tropical countries about 15% of fat may be adequate with increase of carbohydrate to 60-70%.

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ii) Secondary Prevention

a) Chemotherapy: Secondary prevention is the measures adopted when the subject is apparently ill with coronary heart condition. To prevent a major episode of myocardial infarction or cerebro-vascular stroke, therapeutic prophylaxis has been suggested through drugs, which are prostaglandin inhibitors like aspirin. Prostaglandin is known to play a role in creating cardiac spasm which aspirin prevents. Tiny blood cells called platelets stick together when artery tissues are damaged causing blood clots, which is the major cause of most heart attacks. Aspirin acts by blocking platelet aggregation and the initiation of the coagulation process. It works by reducing the clotting capability of blood, so clots are less likely to form in the body and block small blood vessels of the heart or brain causing death. Continuing attention to the clotting mechanism, aspirin had emerged also as a useful drug in the treatment of coronary attack and cerebral thrombosis.

b) Blood Pressure: Just by decreasing levels of 2-3 mm Hg can hypertension be more efficiently tackled. Limitation of intake of dietary salt, regular physical exercise and weight control should be carried out to stabilize blood pressure.

c) Physical Activity: Apart from aerobic and anaerobic exercises, just walking couple of mile everyday can go a long way in reducing heart diseases.

d) Control of Diabetes: Diabetes together with heart disease contributes more fatalities to patients with coronary conditions. So it is essential to check blood sugar levels and keep it at bay.

iii) High Risk Strategy

Those that are at high risk of contracting coronary heart diseases (CHD) are identified. Those who are at special risk are: smokers, obese, diabetics and elderly post-menopausal women. They are brought together for special sessions where they are informed of the risks that they face. They are given options to rectify their conditions and these participants discuss among themselves their experiences. Behavioral therapy in such a way has brought excellent results in target groups with high risk.

If the RF is controlled or checked at its initial attack, there would not be any risk of RHD.

There is no selected therapy for treatment or management of rheumatic fever. Some antibiotics, hormones are used with conservative managements with their side effects, toxicity and adverse reaction but their treatment times are very long and risky. Extraction or formulation of certain medicinal compound for combating such lethal diseases is the earnest need for the salvation of humankind.

So far knowledge goes there is no scientific medicinal application of earthworms on rheumatic fever as well as on any other disease in Bangladesh though the animals are the sources of medicinal compounds for treatment of various diseases mentioned above. Accordingly, it was assumed that such an effective animal as the source of medicinal compounds for curing various diseases may also serve as the source of some effective medicine for mitigating the suffering of people from Rheumatic Fever and consequently Rheumatic Heart diseases may be restricted. Keeping the above concept in mind the present research programme was chalked out to study the efficacy of earthworm against rheumatic fever.

OBJECTIVES

In the light of the above back ground . specific objectives of the study were to:

i) extract substances from dry tissue of some indigenous and easily available earthworm species;

ii) test toxicity of the extract;

iii) determine the components such as proteins, fats, carbohydrates, amino acids etc. of the earthworm species;

iv) assess the antimicrobial activity *in vitro* on *β-haemolytic streptococcus;*

v) assess the clinical efficacy on patients suffering from rheumatic fever *in vivo.*

CHAPTER TWO

EXTRACTION BIOCHEMICAL ANALYSIS AND TOXICITY OF EARTHWORM EXTRACT

CHAPTER 2
EXTRACTION, BIOCHEMICAL ANALYSIS AND TOXICITY OF EARTHWORM EXTRACT

INTRODUCTION

There are some methods for collection of earthworm such as (i) hand sorting methods (ii) soil washing methods (iii) electric methods (iv) chemical methods (v) heat extraction methods (vi) vibration methods (Edwards and Bohlen, 1996). Handsorting method is more popular than other methods for collection of earthworms, because the handling procedure of this method is very easy and about to free from toxic effect on earthworms.

Earthworms tissues contain high amount of protein, crude fiber, total ash, mineral and essential amino acids like alanine, argine, aspertic acid, cysteine, cystinine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenelalanine, proleine, serine, threonine, tryptophan, tyrosine and valine (Lowrence and Millar, 1945; Neilson, 1965; McInroy, 1971; Fosgate and Babb, 1972; Hansen and Czochanska, 1974; Hori *et al.*, 1974; Voogt *et al.*, 1975; Schulz and Graff, 1977; Sabine, 1978, 1981,1988, 1991; Yoshida and Hoshii, 1978; Taboga, 1980; Graff, 1981 and 1982; Hartenstein, 1981; Fu-Zhen and Shi-Wei, 1982; Hilton, 1983; Guerrero, 1983; Tacon *et al.*, 1983; Edwards, 1985; Fieldson *et al.*, 1985; Alawdeen and Ismail, 1986; Pulandiran and Ismail, 1987;

Yegnanarayan *et al.*, 1987; Edwards and Niedered, 1988; Julka, 1988; Nguyen and Furest, 1988; Rumseier *et al.*, 1989 and 1998; Furest *et al.*, 1989; Morgan and Morgan, 1990; Fuxia *et al.*, 1991; Guerrero, 1991; Ibanez *et al.*, 1993; Morgan *et al.*, 1993; Stürzenbaum *et al.*, 1994; Abbott, 1994; Willuhm *et al.*, 1994; Qingsui, 1995; Edwards and Bohlen, 1996; Ismail, 1997; Mehrotra, 1997; Sample *et al.*, 1999; Jin, 2000; Ohta *et al.*, 2000; Ansari, 2001; Bundy *et al.*, 2001; Davoli *et al.*, 2002; Li, 2004; Annonymous, 2005).

Reported and documented side effect of a natural medicine or its mixture, and its closely related species, constituent of the drugs and its preparation/finished products should be taken into account when decisions are made about the need for new pharmacological or toxicological studies. Suggested tests include immunotoxicity (e.g. test for allergic reactions) carcinogenicity, reproductive toxicity and genotoxicity (Annonymous, 2000).

There are some researchers who reported that earthworms are non toxic, (Naya and Kotake, 1967; McInroy, 1971; Hansen and Czochanska, 1974; Edwards and Lofty, 1977; Schulz and Graff, 1977; Yoshida and Hoshii, 1978; Harwood and Sabine, 1978; Taboga, 1980; Fu-Zhen and Shi-Wei, 1982 and 1992; Kale, 1986; Julka, 1988; Fuxia *et al.*, 1992; Qingsui, 1995; Ismail, 1997; Ansari, 2001). Before widespread use of earthworms in animal or human nutrition it could be contemplated, work will be needed to allay fears that they might carry disease or contain toxic

residues (Patel and Patel, 1959; Puttarudriah and Sastry, 1961; Dhennin *et al.*, 1963; Edwards and Lofty, 1977; Veeresh and Rajagopal, 1981; Sabine, 1991; Kotpal, 1998). Such as the presence of *salmonella* in *E. foetida* (Brown and Mitchell 1981), *Ascaridia galli* in chichens (Edwards and Lofty, 1972; Augustine and Lund, 1974) and *Ascaris suum* in pigs (Jakovljevic, 1975) and the vector of the foot and mouth disease (Dhennin *et al.,* 1963; Hagues *et al.,* 1980; Ireland, 1983; Julka, 1988). *Megascolids australis* contains and/or accumulated toxic substances like Lead, Cadmium, Chromium, Copper, Nickel, Mercury and Tin (Gish and Christensen, 1973; Hartenstein *et al.,* 1980; Suzuki *et al.,* 1980b; Beyer, 1981; Ireland, 1983; Josef *et al.,* 1992; Ramseier *et al.,* 1989 and 1998; Morgan *et al.,* 1993; Stűrzenbaum *et al.*, 1994; Gruber *et al.*, 2000).

On the basis of the above information it was assumed that appropriate methods for collection of earthworms for extraction of body constituents of the animal should be ensured for the first time. Secondly, to achieve appropriate earthworm extracts of medicinal importance possible extraction menustruum and methods should be explored cautiously. And finally toxicity test of the earthworm extracts to be obtained is essential. Accordingly, an attempt was made for obtaining extracts of some indigenous and easily available earthworm species of Bangladesh following the biochemical analysis and toxicity test.

MATERIALS AND METHODS

Collection of Earthworms

Uninfected adult individuals of one of the abundant indigenous species of earthworms were collected from garbage, compost pit, house hold drainage where organic matter and moister were sufficient by applying digging and hand sorting methods suggested by Edwards and Lofty (1977) from Rajshahi University campus and different areas of Rajshahi City Corporation for extraction, biochemical analysis and toxicity study.

Washing and Killing of Earthworms

Earthworms were washed in distilled water and kept in deep freezer (Model no. GPT18) for killing.

Drying and Powdering

Earthworms were dried in oven at 600c and then powdered in grinder (Hamandista).

Solvents as Menstruum

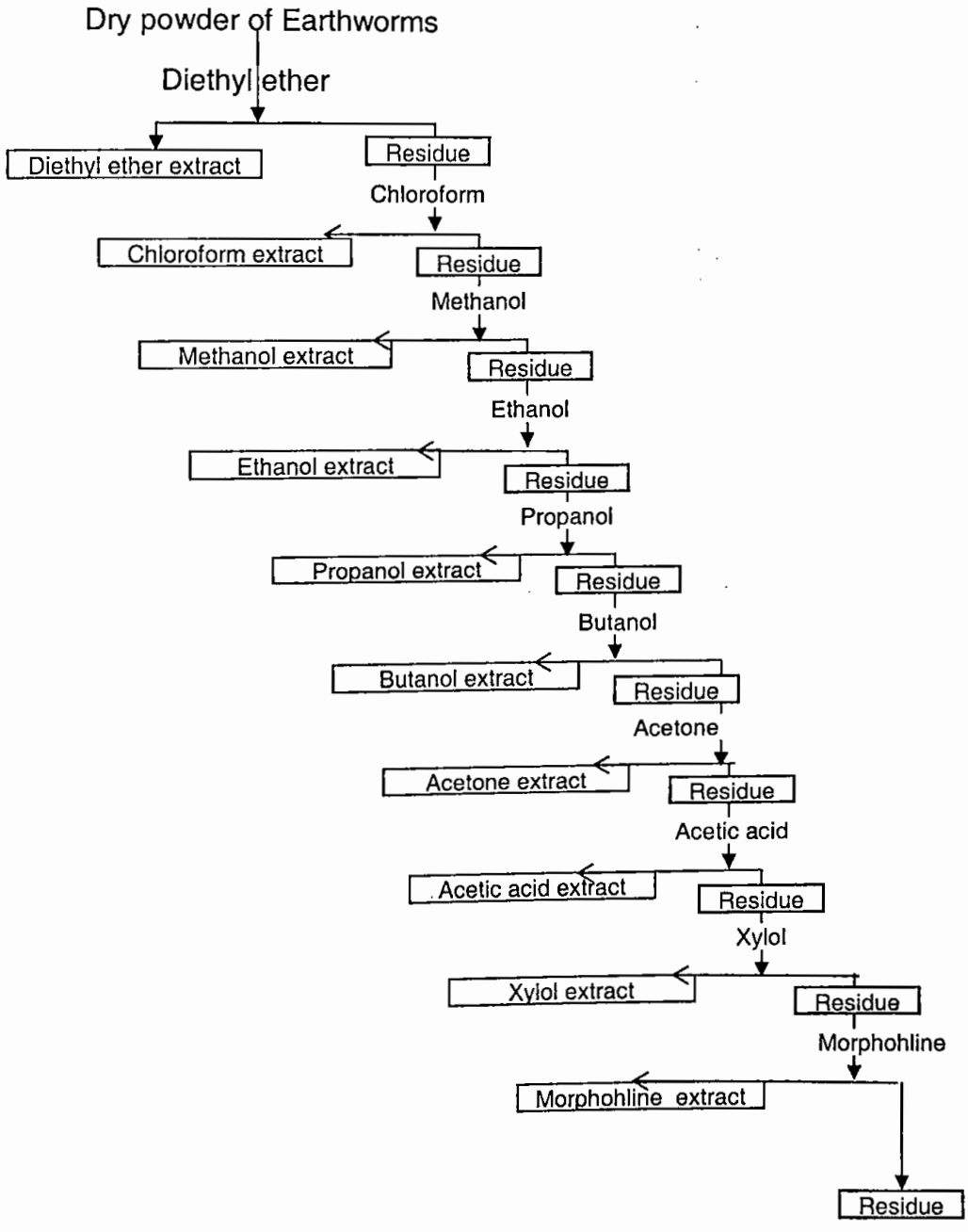
Diethyl ether, Chloroform, Methanol, Ethanol, Propanol, Butanol, Acetic acid, Acetone, Xylol and Morpholine were used as solvent for extraction. In every stage solvents were separated using Rotary Vacuum Evaporator, Electric Balance (Model No. GC12V, Made in Japan, d=0.001g) was used for taking weight.

Extraction of Earthworms

An amount of 90 gm dry course powder of Earthworm was taken in a piece of clean muslin cloth to make thimble packet and placed in Soxhlate

chamber. The extraction procedure was repeated for thirty times and average value with standard deviation for each replication was recorded. These works were done in the Ecology Research Laboratory, Department of Zoology, University of Rajshahi, Bangladesh, by applying continuous hot percolation process following Gupta (1991).

Flow Diagram of Extraction Procedure



Solvents separation from solution was done by Rotary Vacuum Evaporation and heat controlled oven. Carbohydrates were estimated by Enthrone Method (in Jayaraman, 1985). Extraction and calculation of fats and total lipids were performed following Bligh and Dyer Methods (Jayaraman, 1985).

Preparation of Crude Protein Extraction

1. Preparation of Fat Free Meals

In order to purify protein from earthworm extracts in biologically active form, all the operations were performed at 4^0C. First the dried earthworm extracts were taken in a mortar and pounded uniformly into fine powder. Then powder was added to pre-cooled petroleum ether (40^0-60^0C) and homogenized. The desired temperature was maintained by putting ice in the outer chamber of the vat. The oily extract was then kept in beaker at 4^0C for an hour with occasional stirring. The homogenate was then filtered through a muslin cloth. The process was repeated again by adding pre-cooled petroleum ether. The filtrate was then further clarified by centrifugation at 3000 rpm for ten minutes. The precipitate obtained after centrifugation were collected, air-dried at room temperature, and used for extraction of protein.

2. Methods of Crude Protein Extraction

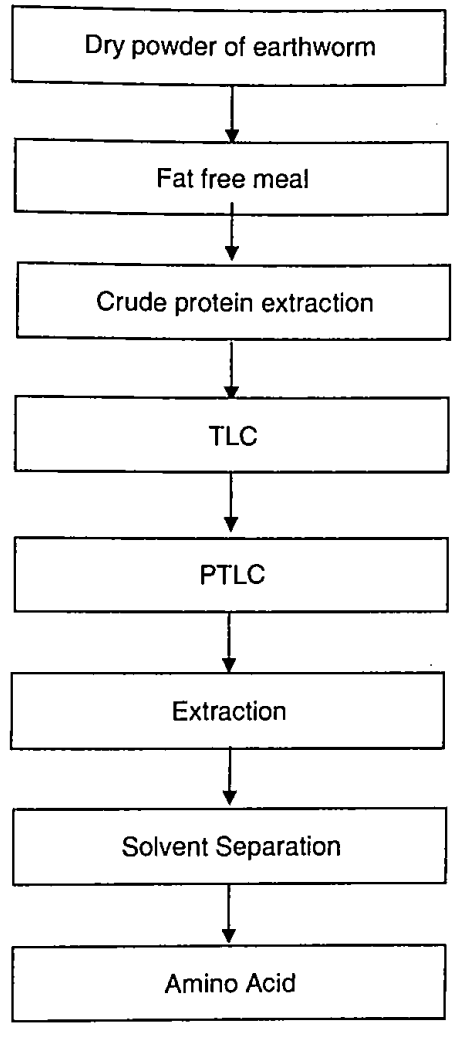
The fat free dry powder was mixed uniformly with pre-cooled distilled water containing 1% CH_3 COOH (6 ml/gm meal) in a beaker and kept overnight

at 4^0C with occasional stirring. The suspension was then filtered through a muslin cloth in the cooled room. The filtrate was collected and clarified further by centrifugation at 8×10^3 rpm, for 20 minutes. The clear supernatant was collected and adjusted to 100% saturation by adding solid ammonium sulfate. The ammonium sulfate precipitate was then centrifuged at 8×10^3 rpm for 20 minutes again. The precipitate was collected, dissolved in minimum volume of pre-cooled deionised water and dialyzed against cold distilled water for 12 hours with three changes and against 10 mM Tris-HCl buffer, pH 8.4 for 24 hours at 4^0C. After centrifugation the clear supernatant was preserved in the deep freezer for experimental purposes.

Calculation of Protein

For the calculation of protein clear supernatant sample were applied in Micro-Kjeldahl's Distillation unit. On the average most proteins contain 16% nitrogen in their composition. In other words, I mg nitrogen equals 6.25 gm protein i.e. Amount of Protein = estimated amount of Nitrogen ×6.25 (Jayaraman, 1985).

Determination and separation diagram of amino acids from crude protein.



Separation of Protein

TLC and PTLC were carried out by using silica gel G coated plate, 96% ethanol: water (7:3 v/v) as solvent to determine amino acids concentrated Ammonium hydroxide and iodine vapours were used for stabilization of

colours. All essential amino acids separation and calculation had done following standard chart of Chromatographic separation where,

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the Solvent}}$$ (Jayaraman, 1985).

Dosage of Earthworm Extract for Toxicity Test

Healthy guineapig(*Cavia porcellus*) was used as the test animal for the experiment of toxicity test, extract amounting 160 mg/kg body weight/day was administrated orally following Pulandiran and Ismail (1987) per individual test animal.

Collection of Blood from Test Animals

Blood was collected from test animal (Guinaepig) by the Butterfly Baby needle (Baby skull vein baby needle) before and after the application of the extract.

Collected blood was preserved in test tube with anticoagulant. Blood test was done following "*Practical Pathology*" (Khalaque, 1996).

RESULTS AND OBSERVATION

For the extraction of earthworms ten solvents were used successively and the results are presented in Fig. 2.1 and appendix Table 1. A total of 32.68±4.75% of dry weight of earthworm was obtained as crude extracts when successively treated by the selected solvents. The solvents Diethyl ether, Chloroform, Methanol, Ethanol, Propanol, Butanol, Acetic acid, Acetone, Xylol and Morpholine extracted out 2.89±1.23, 3.32±1.36, 16.25±3.15, 4.62±1.73, 0.86±0.29, 0.93±0.36, 1.09±0.46, 1.15±0.49, 0.76±0.38, and 0.80±0.18 percents of dry weight respectively.

Freshly dry earthworm's extract contained 59.52±9.96%, 4.82±1.36%, 4.67±2.10%, 5.65±2.38%, 2.54±0.99% and 0.048±0.018%, crude proteins, crude fats, crude carbohydrates, total ash, crude fibre and steroids respectively (Fig. 2.1 and Appendix table 2). The crude proteins were analyzed and seven essential amino acids were detected such as cysteine (1.64±0.72%), histidine (3.23±1.00%), lysine (6.55±1.09%), methionine (2.40±0.60%), phenelalanine (5.25±1.01%) trytophan (2.11±0.55%), and valine (4.22±1.02%) from the extracted earthworm proteins (Fig. 2.1 and Appendix table 3). To find out the toxic effect of earthworm extract healthy Guineapig (*Cavia porcellus*) was used as the test animal. Single dose amounting 160mg/kg body weight/day was administrated orally. Before application of drugs, physical appearance of test animal, haematological test, serological and urine test reports were recorded. After application of extracts test animals were continuously monitored and physical appearance, haematological, serological and urine tests reports were compared with normal physiological value (Table 2.1).

Figure 2.1 Extraction and separation diagram of dry earthworm powder by using various solvents with results (percent by weight).

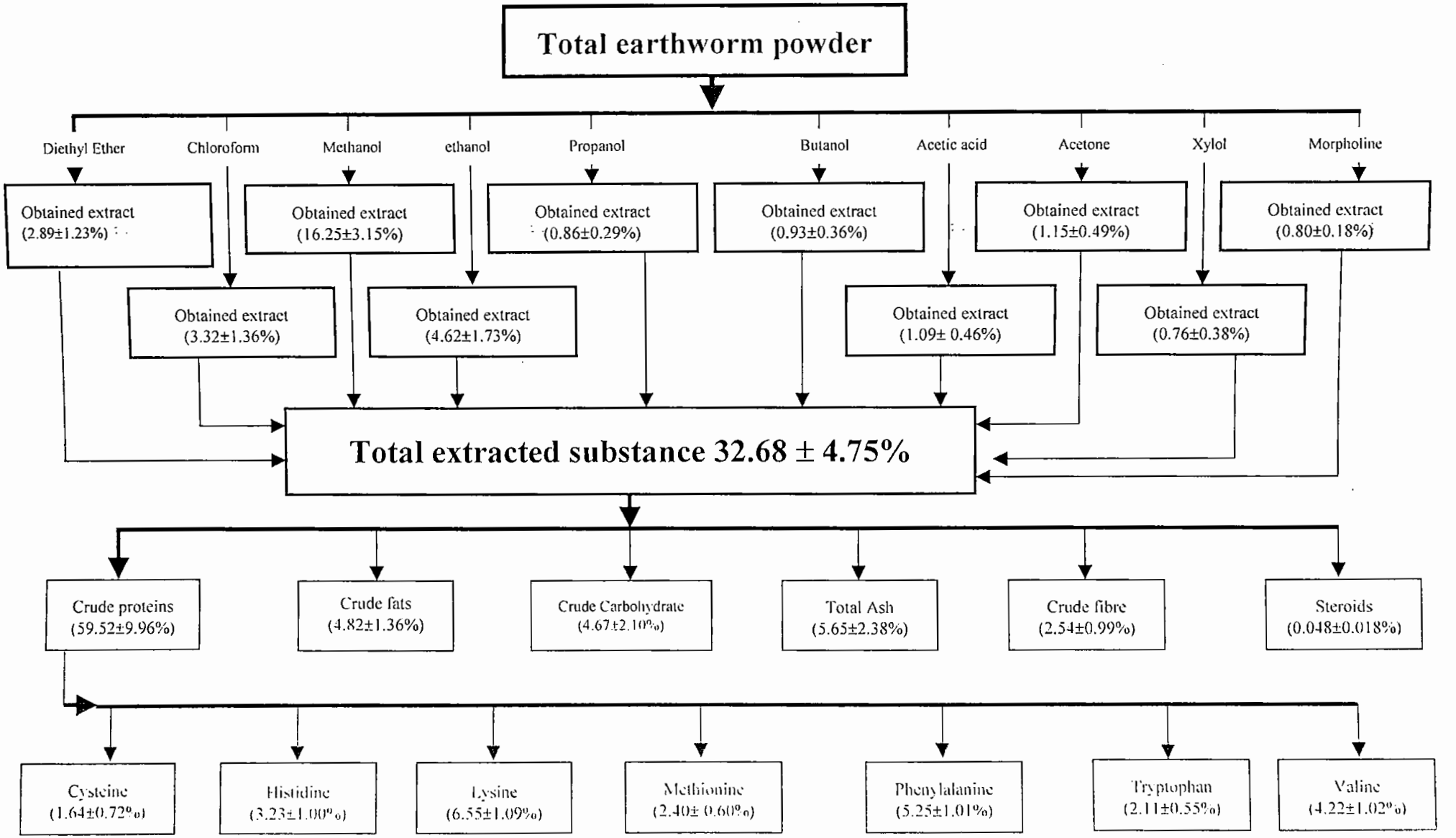


Table 2.1 Haematological serological and urine test report of test animal (*Cavia porcellus*) before and after application of earthworm extracts obtained by using different solvents

Name of the test		N.P.V.		Solvents applied for extraction									
				Diethyl ether	Chloroform	Methanol	Ethanol	Propanol	Butanol	Acetone	Acetic acid	Xylol	Morpholine
ESR(Westergreen Methods)		0-12mm	B	8	7	10	6	7	10	7	8	6	9
			A	8	7	10	7	8	9	8	3	6	9
Hb%(Cyn Methods)		13-18gm	B	14	13	14	13	15	17	16	14	16	16
			A	14	17	15	13	16	14	14	13	16	14
Total count of RBC		4.5-5.5x10^{12}	B	5x10^{12}	5x10^{12}	5x10^{12}	5.1x10^{12}	5x10^{12}	5x10^{12}	5x10^{12}	4.9x10^{12}	5x10^{12}	5x10^{12}
			A	4.5x10^{12}	4.5x10^{12}	4.5x10^{12}	4.5x10^{12}	5x10^{12}	4x10^{12}	4.5x10^{12}	4.5x10^{12}	5x10^{12}	4.5x10^{12}
Total count of WBC		4-11x10^{3}	B	8x10^{3}	6x10^{3}	6x10^{3}	4x10^{3}	5x10^{3}	7x10^{3}	5x10^{3}	5x10^{3}	5x10^{3}	5x10^{3}
			A	6x10^{3}	8x10^{3}	6x10^{3}	7x10^{3}	8x10^{3}	5x10^{3}	5x10^{3}	6x10^{3}	8x10^{3}	4x10^{3}
Differential count of WBC	Nutrophil	40-70%	B	65%	60%	45%	65%	70%	50%	60%	50%	70%	55%
			A	65%	65%	65%	55%	50%	60%	65%	60%	70%	50%
	Lymphocyte	20-40%	B	30%	35%	35%	40%	35%	32%	30%	32%	35%	37%
			A	32%	33%	38%	38%	35%	35%	36%	30%	34%	34%
	Esnophil	1-6%	B	4%	2%	2%	2%	3%	3%	3%	2%	3%	3%
			A	3%	3%	4%	2%	3%	3%	3%	2%	4%	3%
	Monocyte	2-10%	B	5%	8%	2%	3%	5%	5%	5%	4%	7%	5%
			A	9%	7%	5%	6%	7%	8%	3%	4%	6%	5%
	Basophil	0-1	B	-	-	-	-	-	-	-	-	-	-
			A	-	-	-	-	-	-	-	-	-	-
Serum Bilirubin		0.4-1.2mg/dl	B	0.4	0.6	0.6	0.7	0.5	0.8	0.9	0.4	0.7	0.8
			A	0.60	0.9	0.7	1.00	0.70	0.50	0.8	0.9	0.90	0.85
SGOT		≥38-u/l	B	25	27	25	28	30	32	35	30	25	28
			A	30	32	33	32	37	30	35	30	30	35
SGPT		≥12u/l	B	7	10	5	10	12	6	7	10	8	6
			A	7	5	5	10	12	6	7	10	8	6
Serum Uric Acid		2.5-7mg/dl	B	4	5	3	4	3	4	3	3	3	3
			A	3.50	3	4.00	3.00	3.00	3.50	3.55	2.52	4.00	4.25
Serum Creatinine		<1.4mg/dl	B	0.41	0.20	0.25	0.62	0.83	0.24	0.51	0.51	0.59	0.50
			A	0.61	0.52	0.55	0.62	0.30	0.54	0.55	0.52	0.75	0.50
Urine for Routine Examination	Appearance	Clear	B	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
			A	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
	Colour	Straw	B	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw
			A	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw
	Albumine	Nil	B	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
			A	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	Sediment	Nil	B	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
			A	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	Sugar	Nil	B	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
			A	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

N.P.V.= Normal Physiological Value, B= Before Application, A= After Application

DISCUSSION

Sabine (1991) review the works covering the value of earthworms produced as a source of food primarily proteins and drugs. He stated that the chemical composition of any potentiality should be considered in a number of ways the content of macro and micro-nutrients, the contents of specific organic compounds and the content of any possible hazardous materials, either natural constituents or contaminant. This idea had been justified by a lot of works so far carried out by different workers in determining the presence and quantity of various organic compounds in earthworm's tissues. Various workers found that earthworm's tissues contain crude proteins (45-71%), fats (2.3-11.60%) crude fiber (0.60%), total ash (5.20-11.36%), carbohydrate (3.30-21.0%), minerals and 9-18 kinds of essential amino acids like alanine (5.4-6.0%), argine (6.1-7.3%), aspertic acid (10.3-11.0%), cysteine (1.8-3.8%), cystinine (1.40-1.6%), glutamic acid (13.2-15.4%), glycine (4.3-4.8%), histidine (2.0-3.8%), isoleucine (4.5-5.3%), leucine (5.3-8.2%), lysine (6.6-7.5%), methionine (1.5-2.0%), phenelalanine (3.5-5.1%), proleine (5.3%), serine (4.7-5.8%), threonine (4.7-6.0%), tryptophan (2.1%), tyrosine (2.2-4.6%) and valine (4.4-5.1%) (Sabine, 1978, 1981,1988, 1991; Graff, 1982; Hartenstein, 1981; Fu-Zhen and Shi-Wei, 1982; Guerrero, 1983; Alawdeen and Ismail, 1986; Pulandiran and Ismail, 1987; yegnanarayan *et al.*, 1987; Julka, 1988; Rumseier *et al.*, 1989; Furest *et al.*, 1989; Fuxia *et al.*, 1991;

Fletcher, 1991; Guerrero, 1991; Stűrzenbaum *et al.,* 1994; Abbott, 1994; Willuhm *et al.*, 1994; Qingsui, 1995; Edwards and Bohlen, 1996; Mehrotra, 1997; Ismail, 1997; Ramseier *et al.*, 1998; Sample *et al.*, 1999; Jin, 2000; Ohta *et al.*, 2000; Ansari, 2001; Davoli *et al.*, 2002; Annonymous, 2005). During the present investigation only the adult individuals of an indigenous and easily available species of earthworm were considered for the extraction. Solvents *viz.* Diethyl ether, Chloroform, Methanol, Ethanol, Propanol, Butanol, Acetic acid, Acetone, Xylol, and Morpholine were used successively. Methanol was found as the most effective solvent which extracted out 16.25±3.15% followed by ethanol extracting 4.62±1.73% and Xylol was found as the least effective extracting 0.76±0.38% weight of dry earthworm's tissues. The tissues contained crude protein (59.52 ±9.97%), crude fats (4.82±1.36%), Carbohydrate (4.67±2.10%), total ash (5.65±2.38%), crude fibre (2.54±0.99%), steroid (0.048±0.018%). These obtained values are mostly within the ranges as mentioned above by different workers.

Again the past records revealed that so far knowledge goes 9-18 kinds of essential amino acids are present in earthworm's tissue in various percentages on the basis of dry weight of the worms. Presently, it was found that 59.52 ±9.97% by weight of crude protein contents only seven kinds of amino acids as compared with the estimations made by some of the previous workers and presented in table 2.2.

Table 2.2 Amino acid composition of earthworm protein (mg/100mg of protein)

Amino acid	McInroy (1971)	Taboga (1980)	Sabine (1981)	Graff (1981)	Present study
Cysteine	1.8	1.8	3.8	1.4	1.64±0.72
Histidine	2.2	3.8	2.6	2.3	3.23±1.00
Lysine	6.6	7.3	7.1	7.5	6.55±1.09
Methionine	1.5	2.0	3.6	1.8	2.40±0.60
Phenelalanine	4.0	5.1	3.7	3.5	5.25±1.01
Tryptophan	-	2.1	-	-	2.11±0.55
Valine	5.1	4.4	4.9	5.2	4.22±1.02

Though there is considerable variation in the contents of individual amino acids of earthworm protein, nevertheless the values obtained presently are within the ranges obtained by different laboratories. Moreover these sorts of variation could be due to variations in analytical techniques between different laboratories, species of earthworms. This is also supported by the findings of Alawdeen and Ismail (1986) that carbohydrate, protein, fat and ash content of *L. mauritii* from different stages that were in juveniles, carbohydrate 0.90±0.26%, protein 45.50±6.20%, Lipids 15.60±0.80%, ash 6.13±0.12%, in non-clitellates, carbohydrate 0.79±0.18%, protein 50.46±5.70%, Lipids 8.66±1.80% and clitellates Carbohydrate 0.99±0.27%, protein 48.13±6.60%, lipid 11.50±2.50%, ash 5.42±0.32%. Moreover clitellates records more carbohydrates (6.6%), the non clitellates more proteins (30.72%) and juveniles more lipids (0.85%) of *P. excavatus* (Ismail, 1997).

Dhennin *et al.* (1963) reported earthworm as the vector of foot and mouth disease of domesticated animals Odum (1971) that earthworms are just passive agents or reservoirs hosts. On the other hand there are some researchers who reported that earthworms are non toxic, (Naya and Kotake, 1967; Edwards and Lofty, 1977; Harwood and Sabine, 1978; Fu-Zhen and Shi-Wei, 1982 and 1992; MacDonald, 1983; Julka, 1988; Fuxia *et al.*, 1992; Qingsui, 1995; Ismail, 1997; Ansari, 2001). During present observation hematological toxicity, hepatological toxicity, immunotoxicity, carcinogencity(in blood), glomerulotoxicity and physical appearance were observed and results proved that it was non toxic (Table 2.1). The results clearly indicate that there was no toxic effect of extract of the indigenous earthworm species on the test animal *Cavia porcellus.*

Biochemical analyses for determining earthworms' tissue contents were carried out using the mixture of all the extracts obtained by applying considered solvents successively. It might be so happen that analyses of individual samples separately may result variedly. Again, only one pair of test animals was considered for extracts obtained by each solvent to determine the toxicity. Toxicity test is very difficult and depends on the physiology of individual test animal to some extent. So for confirmation of the results further detail experimentations are suggested.

CHAPTER THREE

APPLICATION OF EARTHWORM EXTRACT *IN VITRO* AND *IN VIVO*

CHAPTER 3
APPLICATION OF EARTHWORM EXTACT *IN VITRO* AND *IN VIVO*

INTRODUCTION

Earthworms are known to be associated with medicine since ancient times to cure various human diseases. In Indian Unani System of Medicine, preparations from dried worms, when applied externally, have been used in treating wounds, chronic boils, piles, sore throat, hernia and impotency and when taken internally, they are useful in curing chronic cough, diphtheria, jaundice, rheumatic pains, T. B., bronchitis, facial paralysis and impotency (Julka, 1988; Edwards and Bohlen 1996; Kotpal, 1998). Folk histories of the medicinal value of earthworms, dating from at least as far back as 1340 A.D. and covering a range of diseases from pyorrhea to postpartum weakness, from small pox to jaundice to rheumatism (Reynolds and Reynolds, 1972 in Sabine, 1991). With modern scientific methods, the antiviral and antitumour medicines have been extracted from earthworms, and ointment (extracted from earthworm) was used to treat herpes zoster, eczema. The latter research shows that the extraction of earthworm has strong immuno-competence and antitumour ability with the rate of restraining tumour. After 20 days from the day of inoculation blood was drown from the tail, a smear was prepared to classify and test the function of MO phagocytes. The results of the experiment showed that the

average weight of the spleen of mice in the experimental group was obviously lighter than that of mice in the placebo group. The average number of peripheral blood lymph cells increased notably compared to that of placebo group. It was proved that the antitumour medicine, the extraction from earthworms, can raise immuno-competence and resistance to tumour and inhibit the activity of inoculated tumour cells (Fu-zhen and Shi-wei; 1982 Ismail *et al.,* 1992). According to Traditional Chinese Medicine, the earthworms possess anti-pyretic, antispasmodic, diuretic and detoxic effects, etc. (Fuxia *et al.,* 1992). During the 70s, Professor Shan Hongren discovered enzymatic functions of extract from earthworms and for this he was honored with the United Nations Science Conference Award in 1978. In 1997 a product named plasmin made from earthworms was approved by the Chinese government as a new medicine. In 1997 plasmin was endorsed by the China Gerontology Foundation, and a year later it was endorsed by the China Gerontology Association Rehabilitation Committee. In 1999, China Medical Society decided to make plasmin a key product to be promoted all over China. In the same year it was registered by the China Supervisory and Administrative Bureau as a class two nationally protected TCM formula, and in 2000 it was included in the China National Pharmacopoeia (Ansari, 2001).

Long tong Capsule is a new medicine for heart diseases extracted from natural life body of special species of earthworms (Dilong) which proved that there exist thrombus dissolves anti-coagulation and enzyme

activator (Annonymous, 1995). DiLong powder (*Pheritima*) has a significant analgesic and antipyretic effects, reducing blood pressure, anti-coagulant and thrombotic effects can rapidly enhance the activity of general plasminogen activator, strengthening immunity in shortening the inflammation period and may promote wound recovery, anticancer effects (Mihara *et al.*, 1991a,b).

The available literatures so far consulted reveal that earthworm extracts have enough medicinal effects on autoimmune, cardiovascular diseases, infarct and embolism diseases etc. which are closely related with rheumatic fever.

Rheumatic fever is a very complex type of cardiovascular and valvular diseases and there is no selected therapy for treatment. Some antibiotic and steroids have been used with their side effects and toxic effects with long-term treatment. Through the previous chapter (biochemical analysis) it has been established that the earthworms (indigenous species) contain lysine, methionine, cysteine, tryptophan, histidine, phenylalanine, steroids, carbohydrates, fats and others.

The above discussions suggest that the earthworm extract containing various compounds might play some vital roles in curing rheumatic fever. So the present investigation was designed to assess the medicinal value of earthworm *in vitro* and *in vivo* along with by placebo (controlled) on Rheumatic fever.

MATERIALS AND METHODS

In vitro and *In vivo* tests were performed to assess the medicinal efficacy of earthworm's extract.

In Vitro or Laboratory Test

The laboratory tests of earthworm extracts on the *ß-haemolytica streptococcus* were done in the Laboratory 2 (Microbiology and Biotechnology) Department of Pharmacy, University of Rajshahi, Bangladesh.

Principle

The susceptibility of the micro-organism to antimicrobial agents may be measured *in vitro* by utilizing agar diffusion technique. Dried filter paper discs containing the test materials are usually applied to the test plates containing the culture of micro organisms.

Experiments on several filter paper discs were carried out simultaneously. The dried discs absorb water from the agar medium and the test material was dissolved. Then the test material migrated through the adjacent agar medium according the physical law that governs diffusion of the molecules through an agar gel. As a result there was gradual change of drug (test material) concentration in the agar surrounding each disc. Activities of the test sample were expressed by measuring, the zone of inhibition around the area. The zone of inhibition was effected by various factors e.g. by the growth rate of the micro-

organisms, also by the rate of diffusion of drug through the agar gel. The diameter of the inhibition zone was usually measured to understand the extent of inhibition in different concentration.

Test *In Vitro*: Range of Antimicrobial Activity

To detect the antimicrobial activity of extracted substances newly obtained by applying solvents *viz.* Diethyl ether, Chloroform, Methanol, Ethanol, Propanol, Butanol, Acetone, Acetic acid, Xylol, and Morpholine in course of this study were applied individually *in vitro* against rheumatic fever causing microbe *β-haemolyticus*. Details of the techniques adopted are as follows.

Preparation of the Media: Nutrient Agar

Compositions

Name of the ingredients	Amounts
Meat extract	3 gm
Peptone from meat	5 gm
NaCl	5 gm
Agar	20 gm
Distilled water	1000 ml

The above ingredients were taken thoroughly mixed and the p^H was adjusted to 7±0.2. The resulting solution was transferred in *equal portions* (100 ml) into 10 different 250 ml conical flask. The conical flasks were autoclaved at 120°C under a pressure of 15 lb/sq inch for 20 minutes. Then flasks were removed from the autoclave and placed on water bath at 50°C.

Preparation of the Culture

A small bottle containing 10 ml sterile nutrient broth was taken and the test *β-haemolyticus* from the pure culture were transferred to this bottle with the help of an inoculation loop in an aseptic condition. After inoculation the bottle was subjected to incubation at 37±1°C for 24 to 72 hours to provide sufficient time and temperature for the growth of the test organisms.

To 100 ml of the nutrient agar, 1 ml of the prepared culture was added and was mixed thoroughly with shaking a 25 ml portion of this culture was poured into a Petridish and in order to facilitate a homogeneous distribution of the test organisms the Petridish was rotated several times, first in clockwise direction and then in anti-clock-wise direction. The media were poured into Petridish on a level horizontal surface. So as to give uniform depth of approximately 4 mm. the Petridish was kept undisturbed for about 15 minutes during which it was solidified. After complete solidification of the media 4-5 holes were made inside it with the help of a borer. Just before using plates with lids agar were placed in an incubator (25°C) for about 10-15 minutes until the excess of surface moisture was lost by evaporation. There should be no droplets of moisture on the surface of the media or on the Petridish plate cover. Then applied the extracts for observing their antibacterial activities. The experiments were replicated for thirty times.

In Vivo or Clinical Application

The earthworm extracts were applied on each patient suffering from rheumatic fever for 42 days. In course of the period July' 2003 to May' 2006 a total of 110 (50 for preliminary test +20 for methanolic extract + 20 for ethanolic extract + 20 for control *viz.* neutral sugar) patients were considered for the experimentation depending on the availability.

Dosages were determined as 4 gm (2gm+0+2gm) per day (orally) following Annonymous, 2005.

Symptoms and results of pathological investigations of the patients were recorded before and after the application of extract following the modified John's Criteria (Taranta and Markowitz, 1989).

RESULTS AND OBSERVATIONS

Experiments were conducted *in vitro* and *in vivo* with a view to examine the medicinal effect of earthworm on *ß- haemolytica streptococus* in the laboratory and on quite a good number of patients suffering from rheumatic fever respectively during the period of July 2003 to May 2006.

For the determination of bactericidal effects of extracted substances from dried earthworm powder by using solvents like Diethyl ether, Chloroform, Methanol, Ethanol, Propanol, Butanol, Acetic acid, Acetone, Xylol and Morpholine were applied *in vitro* on culture media of *β-haemolytica steptococcus* under the laboratory condition. The maximum zone of inhibition (14.93± 2.74 mm) was obtained for ethanolic extract followed by methanolic (14.60±4.00mm) extract and the minimum (1.46±0.50 mm) inhibition zone for xylol as the solvent (Table 3.1 and Appendix table 4).

Table 3.1 Bactericidal effect of substances extracted successively by using various solvents from dried earthworms (Powder) on β-haemolyticus

Solvents / No. of Observation	Diethyl ether	Chloroform	Methanol	Ethanol	Propanol	Butanol	Acetic acid	Acetone	Xylol	Morpholine
30	12.50±2.20	12.86±3.72	14.60±4.00	14.93±2.74	1.50±0.77	8.13±2.65	14.70±3.12	2.20±1.12	1.46±0.50	1.60±0.67

The extracted substances obtained by applying various solvents were applied on the patients suffering from Rheumatic fever *in vivo* and the results are presented in appendix table 5. The diethyl ether, chloroform, propanol, butanol, acetic acid, acetone, xylol and morpholine extracted substance were not effective on rheumatic fever, because none of those patients indicated any sort of improvement in their signs, symptoms and investigation results. Whereas 03 (three) out of 05 (five) patients came round within 35 to 42 days when treated with methanolic extract and similar results were obtained in case of ethanolic extract (Appendix table 5).

The encouraging results (60% recovery of methanolic and ethanolic extracted substances) inspired the worker to apply the methanolic and ethanolic extracted substances on rheumatic fever patients independently with control group (treated with neutral sugar as placebo) and the obtained results are presented in table 3.2 and appendix table 6, 7 and 8.

Table 3.2 Rheumatic fever patients treated by earthworm extracts (methanolic and ethanolic) and placebo (Neutral Sugar)

Drugs	Dosages and route of administration	Number of Patients treated	Number of patients recovered	Percentage Recovery
Methanol	2+0+2 mg, orally	20	11	55
Ethanol	2+0+2 mg, orally	20	14	70
Placebo	2+0+2 mg, orally	20	00	00

The table shows that ethanolic extracts cured 70% patients within 35-45 days and the recovery is 55% in case of methanolic extracts. Signs and

symptoms of the considered patients were recorded through keen and constant observations in course of treatment. Pathological investigations *viz.* determination of ASO titre (Anti-Streptolysin 'O') ESR (Erythrocyte Sedimentation rate) were performed after every seven days. Figures (3.1, 3.2; 3.3 and 3.4) are presented here to depict the decreasing trends of ASO titre values and ESR values.

The figures revealed that ASO titre and ESR values decreased around to 30-50 % of the initial values within 7-14 days. Then the decreasing rate was slow and steady but the values became more or less normal within 28 days in case of methanolic extract and 28-42 days in case of ethanolic extract in most of the cases.

Figure 3.1 Graphical representation of diminishing trends of ASO-titre results of patients in course of treatment by using methanolic extract of earthworms.

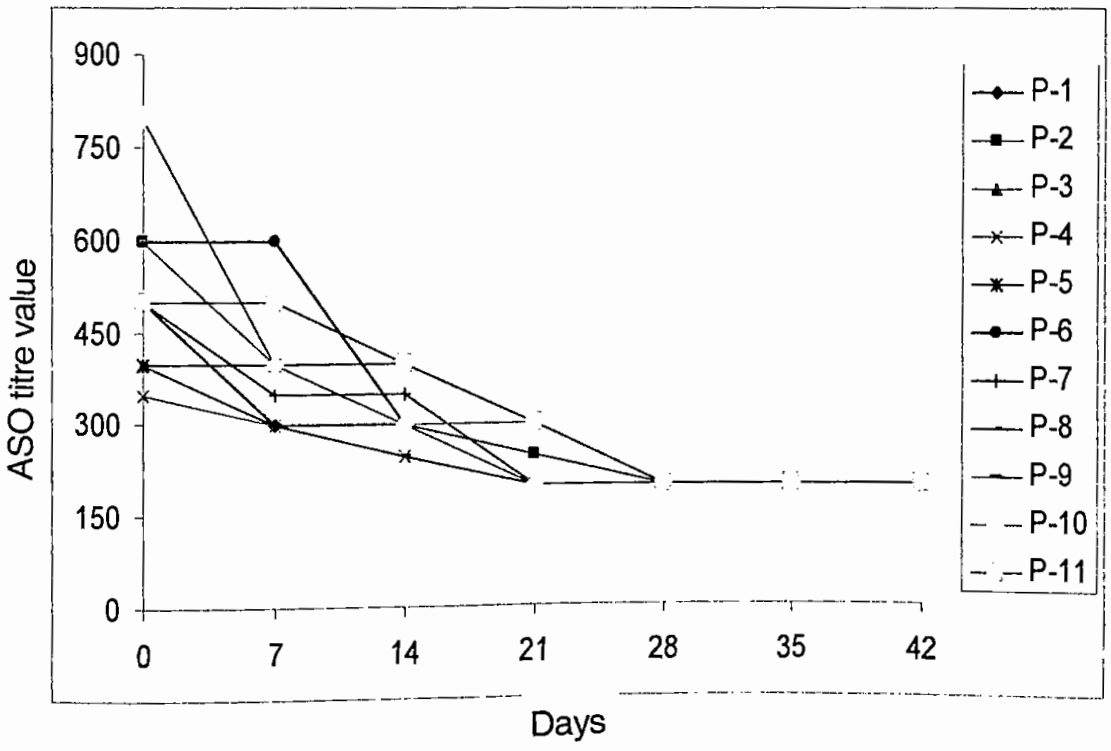


Figure 3.2 Graphical representation of diminishing trends of ESR results of patients in course of treatment by using methanolic extract of earthworms.

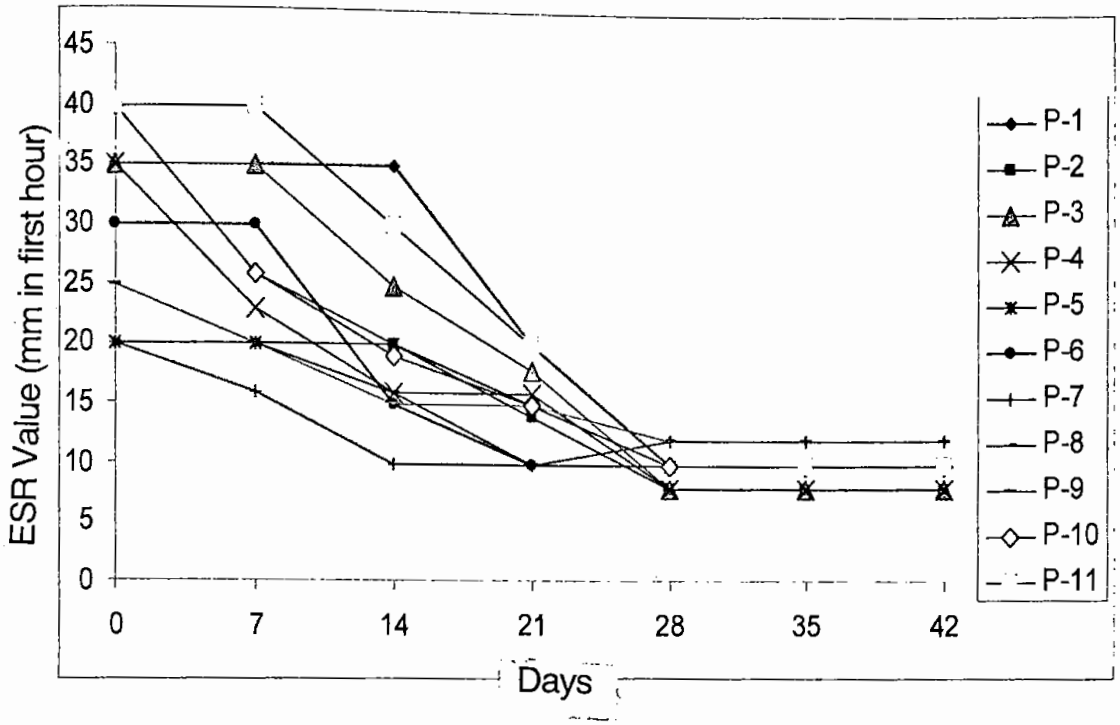


Figure 3.3 Graphical representation of diminishing trends of ASO-titre results of patients in course of treatment by using ethanolic extract of earthworms.

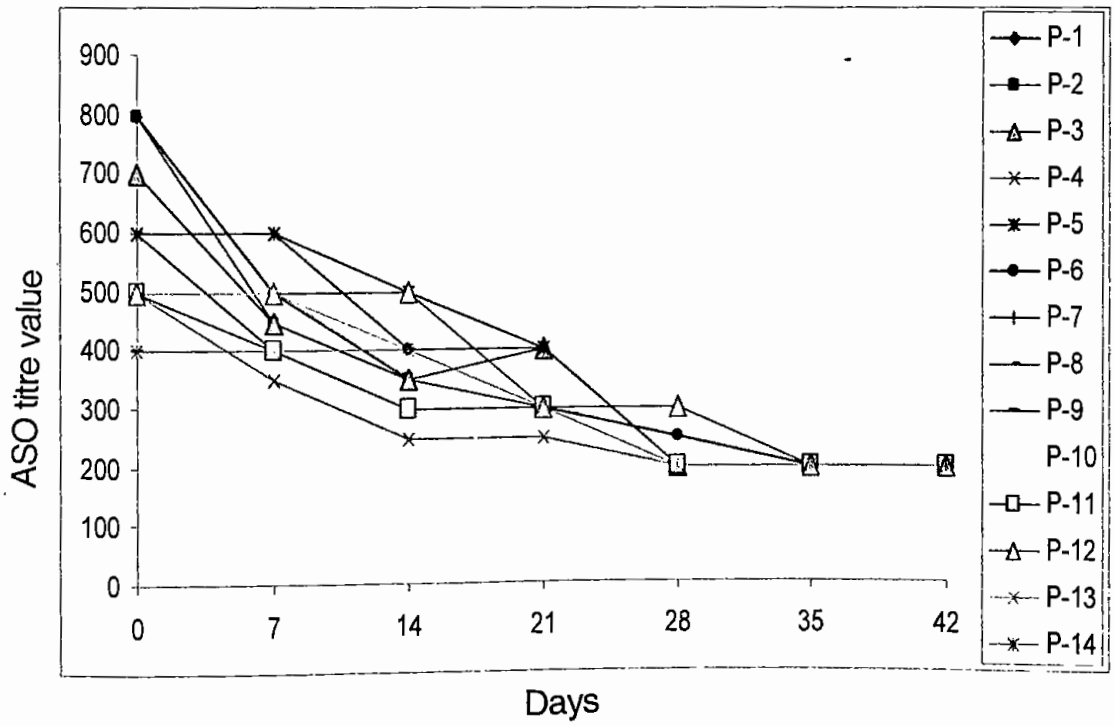
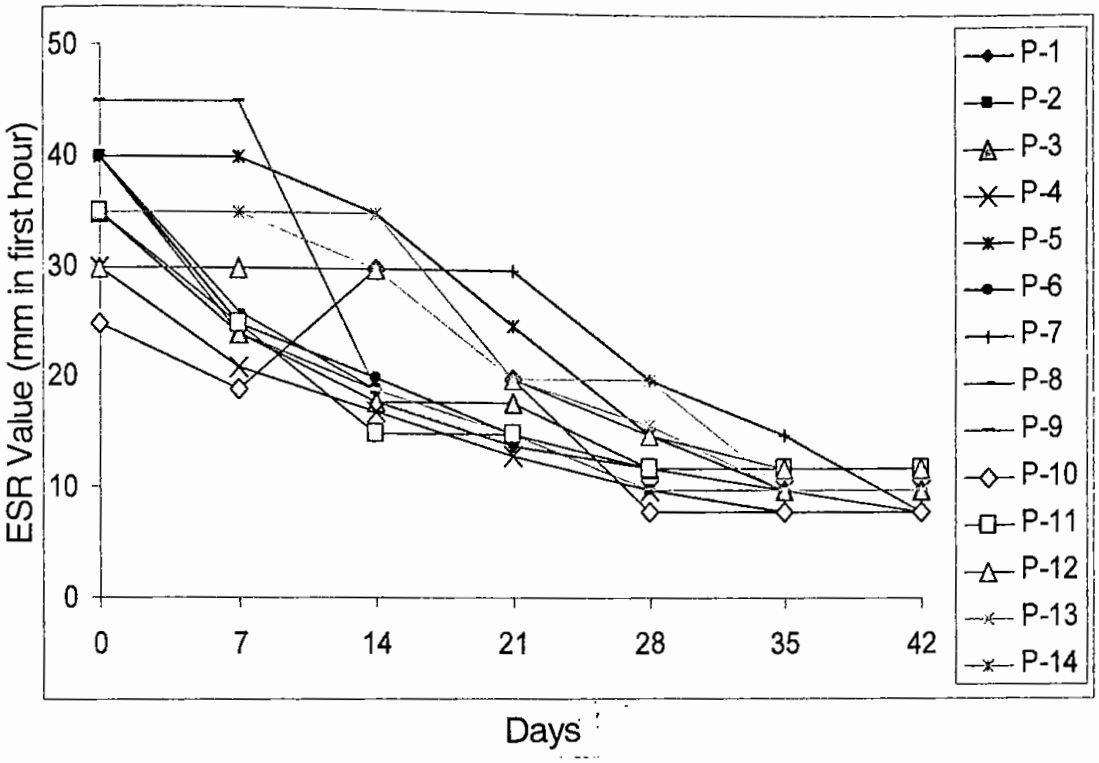


Figure 3.4 Graphical representation of diminishing trends of ESR results of patients in course of treatment by using ethanolic extract of earthworms.



DISCUSSION

Numerous workers worked on the medicinal application of earthworms on various sorts of diseases since early fourteenth century. Researchers like Weisbach (1962), Julka (1988), Vohra and Khan (1978), Edwards and Bohlen (1996), and Kotpal (1998) reported that drugs prepared from earthworms are very effective on rheumatism or gout, inflammatory activity, induced oedema and hypertensiveness etc. Though the above mentioned diseases are closely related with rheumatic fever but there is no available literature regarding the application of drugs prepared from earthworms specifically for getting rid of rheumatic fever. Medicinal effectiveness of earthworm tissue contains on various diseases inspired to verify their efficacy against rheumatic fever.

Antimicrobial activity of earthworm extracts obtained by applying solvents *viz.* Diethyl ether, Chloroform, Methanol, Ethanol, Propanol, Butanol, Acetic acid, Acetone, Xylol and Morpholine on *β-haemolyticus,* rheumatic fever causing microbes was tested under laboratory condition. It was found that the ethanolic extract attained the highest rank in inhibiting *β-haemolyticus* followed by methanolic extract and extracted substances by using acetic acid and acetone occupied the third and fourth highest ranks respectively. These results indicate that earthworm extracts might be effective in recovering the rheumatic fever.

Earthworm extracts were applied on patients suffering from rheumatic fever, symptoms and results of pathological investigations were

recorded before and after application of extracts following the modified John's Criteria (Taranta and Markowitz, 1989). The diethyl ether, chloroform, propanol, butanol, acetone, acetic acid, xylol and morpholine extracted substances were not effective on rheumatic fever, because none of those patients indicated any sort of improvement in their sign, symptoms and investigation results. Whereas 03 (three) out of 05 (five) patients cured within 35 to 42 days when treated with methanolic extract and similar result was obtained in case of ethanolic extract. The encouraging results (60% recovery of methanolic and ethanolic extracted substances) inspired the worker to apply the methanolic and ethanolic extracted substances on rheumatic fever patients independently with control group (treated with neutral sugar as placebo). The results showed that ethanolic extract recovery rate was seventy percent within 35-42 days and it was fifty five percent in case of methanolic extract. Whereas there was no improvements of the patients treated with placebo.

It was observed that both the drugs were able to control ASO values rapidly in course of two to three weeks of treatment to fifty percent of the values achieved due to diseased condition.

Though the results could not be compared due to lack of available literature yet it is expected that the report will suggest some major attempts to search for some effective remedy for rheumatic fever.

CHAPTER FOUR

GENERAL DISCUSSION

CHAPTER 4
GENERAL DISCUSSION

A lot of researchers carried out research on the therapeutic application of earthworm extracts on various sorts of diseases like stone in bladder (Stephenson, 1930), piles (Julka, 1988), small pox (Bristowf, 1932), rheumatism (Weisbach, 1962), impotence, bronchitis (Renolds and Renolds, 1972), antiviral, anti-tumor herpes zoster, eczema, ulcer, antipyretic (Sabine, 1991), anti-spasmodic, diuretic and de-toxic effects, cerebral infarct, cerebrovascular disease, psychosomatic, ischemic, antithrombotic, fibrinolytic, pulmonary thrombosis, pulmonary embolism, secondary stroke, reduce blood sugar, cerebral thrombus, cerebral embolism, angitis, coronary heart disease, myocardial infarction, Hyperplasmineria, varicose, arteriosclerosis, platelet hypercoagulability, muscle relaxation, anti- hypertensive (Qingsui, 1995), high blood lipoprotein, high blood viscosity (Ansari, 2001), nervous debility (Said, 1971) etc. Though many of that disease are closely related with rheumatic fever but there is no available literature regarding the use of drugs prepared from earthworm specifically for curing rheumatic fever.

During the present time only the adult individuals of an indigenous and easily available species of earthworm were collected by hand sorting methods, killed within deep freezer, homogenized by distilled water and considered for the extraction. A good number of solvents *viz.* Diethyl ether, Chloroform, Methanol, Ethanol, Propanol, Butanol, Acetic acid, Acetone,

Xylol and Morpholine were used successively for extraction of dry earthworm tissues. Among all the above mentioned solvents Methanol and Ethanol were proved as the most effective solvents which extracted out 16.25±3.15% and 4.62±1.73% by weight of dry earthworm tissues respectively. On the other hand Xylol was as less effective menstruum in extracting only 0.76±0.38% by weight of dry earthworms tissues.

Dry earthworm powder contained crude proteins (59.52±9.96%), fats (4.82±1.36%), carbohydrates (4.67±2.10%), total ash (5.65±2.38%), fibre (2.54±0.99%) and steroids (0.048±0.018%) by weight. The obtained results are supported by the findings of Lawrence and Millar (1945), McInroy (1971), Fosgate and Babb (1972), Guerrero (1983), Hilton (1983), Tacon *et al.* (1983), Alawdeen and Ismail (1986), Sabine (1991) and Mehrotra (1997). Considerable information on biochemical composition has been reported by (McInroy, 1971; Schulz and Graff, 1977; Sabine, 1978, 1991; Julka, 1988; Graff, 1982 Edwards and Bohlen, 1996; and Ismail, 1997) that the tissues of earthworm contain high amount of protein. Essential amino acid spectrum for earthworm tissues, as reported by these different authors, compares well with those from other currently used sources of animal feed protein. There is good evidence that the mean amount of essential amino acids recorded are very adequate for a good animal feed when compared with the recommendation of FAO/WHO, all of which are important components of animal food and it has very medicinal value. In addition earthworm tissues contain a preponderance of long-chain fatty acids (Edwards and Bohlen, 1996). Sabine (1991) review the

works covering the value of earthworms produced as a source of food primarily proteins and drugs. Fu-zhen and Shi-wei (1982) determined eighteen kinds of amino acids like alanine (5.4-6.0%), argine (6.1-7.3%), aspartic acid (10.3-11.0%), cysteine (1.40-1.6%), cystenine (1.8-3.8%), glutamic acid (13.2-15.4%), glycine (4.3-4.8%), histidine (2.0-3.8%), isoleucine (4.5-5.3%), leucine (5.3-8.2%), lysine (6.6-7.5%), methionine (1.5-2.0%), phenelalanine (3.5-5.1%), proleine (5.3%), serine (4.7-5.8%), threonine (4.7-6.0%), tryptophan (2.1%), tyrosine (2.2-4.6%) and valine (4.4-5.1%) in various percentages on the basis of dry weight of the worms of which eight are essential for human being. In dry matter McInroy (1971) have done biochemical analysis from *Eisenia foetida* and the results were essential amino acids arganine (6.1%), cysteine (1.8%), histidine (2.2%), isoleucine (4.6%), lyscine (6.6%), leucine (8.1%), methionine (1.5%), phenelalanine (4.0%), threonine (5.3%) and valine (5.1%). Taboga (1980) have done biochemical analysis from mixture of *Eisenia foetida* and *Lumbricus rubellus* and the results were crude protein (62.71%), fat (2.3%), amino acids alanine (5.4%), arginine (7.3%), aspartic acid (10.5%), cysteine (1.8%), glutamic acid (13.2%), glycine (4.3%), histidine (3.8%), isoleocine (5.3%), leucine (5.3%), lysine (7.3%), methionine (2.0%), phenylalanine (5.1%), proline (5.3%), serine (5.8%), threonine (6.0%), trytophan (2.1%), tyrosine (4.6%) and valine (4.4%). Sabine (1978) find out crude protein (62.64%), fat (7.10%), ash (8.10%) and Sabine (1981) find out amino acid arginine (6.8%), cysteine (3.8%), glycine (4.8%), histidine (2.0%), phenelalaline (3.7%), serine (4.7%), threonine (4.8%), tyrosine (2.2%), valine (4.9%) from *Eisenia foetida*. Fosgate and

Babb (1972) analyzed *Eisenia foetida* for gross nutrients content and found crude protein (58.20%), crude fat (2.8%), carbohydrate (3.30%). Schulz and Graff (1977) showed that *Eisenia foetida* contain crude protein (66.30%), fat (7.9%), carbohydrate (14.2%), ash (11.6%). Graff (1981) analyzed amino acid from proteins of *Eisenia foetida* and showed that alanine (6.0%), arginine (6.1%), aspartic acid (11.0%), cysteine (1.40%), glutamic acid (15.4%), histidine (2.3%), Isoleucine (4.70%), Leucine (8.2%), Lysine (7.5%), methionine (1.8%), Phenelalanine (3.5%), Serine (4.8%), threonine (4.7%). Hartenstein (1981) estimated that earthworm tissues contain crude protein (65%), fat (9%) and carbohydrate (21%). Presently, it was found that 59.52±9.96% by weight of crude proteins contained only seven amino acids like cysteine (1.64±0.72%), histidine (3.23±1.00%), lysine (6.55±1.09%), methionine (2.40±0.60%), phenelalanine (5.25±1.01%), trytophan (2.11±0.55%) and valine (4.22±1.02%).

The above discussion revealed that the earthworm tissues contain crude proteins, fats, carbohydrates, total ash, fibres and steroids variously in percent by weight depending on the species and life stages. Till then there are definite ranges for each of the compounds. Likewise the amino acid contents of the crude proteins in earthworms vary in number and amount depending on the species but lies within the gross range for earthworms. Presently, the considered earthworm species contained only seven essential amino acids *viz.* cystine, histidine, lysine, methionine,

phenelalanine, trytophan and valine. These results are in accordance with the results of the previous researchers.

Reported and documented side effect of a natural medicine or its mixture, and its closely related species, constituent of the drugs and its preparation/finished products should be taken into account when decisions are made about the need for new pharmacological or toxicological studies. The absence of any reported or documented side effect is not an absolute assurance of safety of natural medicines. However a full range of toxicological test may not be necessary. Tests which examine effects that are difficult or even impossible to detect clinically should be encouraged. Suggested tests include immunotoxicity (e.g. test for allergic reactions) carcinoganicity, reproductive toxicity and genotoxicity (Annonymous, 2000). Before use of earthworm extracts in animal or human either as nutrient or drugs it is indispensable to be assured that the extracts are absolutely safe and have no effective side effect.

During the present investigation earthworm extracts were applied (160mg/kg body weight/day) on test animal for the determination of toxicity test and found there was no adverse effect of earthworm extracts on the test animals *Cavia porcellus*. It is also supported by the findings of Edwards and Lofty (1977), Harwood and Sabine (1978), Fu-Zhen and Shi-wei (1982), Julka (1988), Sabine (1988), Fletcher (1991), Qingsui (1995), Ismail (1997) and Ansari (2001). Moreover Chinese Medical Society applied "Boluoke Capsule", a product of earthworm extract on 1560 patients suffering from ischemic cerebrovascular diseases in 16 hospitals

and found that Boluoke Capsule was a promising new anti-thrombotic drug with no obvious toxic or adverse effects, worthy of extensive application (Qingsui, 1995 and www. Health King- 2004). Thus it can be concluded that drugs prepared from earthworm's tissues are recommendable as non-toxic natural medicine without any adverse side effects.

Rheumatic fever is a very complicated disease closely related with rheumatism, tonsillitis which ultimately develops to valvular heart diseases is caused by *β-haemolytica streptococcus.* It is the consequence of auto-immune disorder initiated by throat infection with a group A *β-haemolytica streptococcus.* Fu-zhen and Shi-wei, (1982) pointed out that earthworm tissues extracts play vital role in controlling auto-immune incompetence. Accordingly, it was thought that earthworm extracts might have some anti-microbial properties which may act against bacteria like *β-haemolytica streptococcus.* And experiments were conducted with the earthworm extracts obtained by using the solvents namely Diethyl ether, Chloroform, Methanol, Ethanol, Propanol, Butanol, Acetic acid, Acetone, Xylol and Morpholine on *β-haemolyticus.* Diethyl ether, Chloroform, Methanol, Ethanol, and acetic acid treated extracts were found effective in controlling the microbes effectively *in vitro.* Efficacy of any anti-microbial elements may differ depending on the application procedure (*in vitro* and *in vivo*) keeping those idea all the ten extracts were administrated orally (2gm+0+2gm) daily independently on 50 rheumatic fever patients and found that only methanolic and ethanolic extracts were clinically effective. Though Diethyl ether, Chloroform and acetic acids treated extracts were

effective in inhibiting *β-haemolytica streptococcus* in laboratory tests but did not response while administrated clinically. Which factors were responsible for such different action could not be explained at this moment. It needs further intensive and careful examination.

Later on methanolic and ethnolic extracted substances were given to forty patients (20 for each extract) along with another twenty patients (controlled) to examine the degree of effectiveness of the extracts. Ethanolic extract cured 70% within 35-42 days and methanolic one cured 55% patients within 28 days of treatment. The patients treated as controlled with placebo drugs (neutral sugar) did not show any development. ASO titre and ESR values were dropped by more or less 50% by the second week of treatment in case of majority of the patients and then slowly decreased to attend the normality by 40th day (Figure no. 3.1, 3.2 and 3.3, 3.4).

The present investigation is certainly a preliminary exercise for finding out effective biomedicine to cure rheumatic fever patients. Before application of it further intensive experiments are obligatory for extraction, identification and separation of effective compounds in one hand. On the other hand clinical efficacy shall have to be determined through application of the natural drugs on considerable number of rheumatic fever patients.

CHAPTER FIVE

CONCLUSSION AND RECOMMENDATION

CHAPTER 5
CONCLUSSION AND RECOMMENDATION

The present investigation was conducted with a view to detect effective and safe medicinal compounds for management of rheumatic fever from tissues of only adult individuals of an indigenous earthworm species. Earthworm extracts were applied clinically on rheumatic fever patients with prognostic symptoms like carditis, tonsillitis, polyarthrytis, erythemamargenatum etc. Along with these symptoms the pathological reports showed that the patients were very much susceptible to valvular heart diseases. Ethanolic and methanolic extracts responded positively to cure 70% and 50% patients respectively which inspire to conduct further intensive research.

The study was consequence of an initial attempt in searching natural remedy for a very obnoxious disease, rheumatic fever and leads to recommend the following points:

i) In order to find out more effective but specific compounds earthworms of all age groups of every species available in the country should be considered.

ii) Habitat of experimental healthy earthworms must be free from all sorts of pollution.

iii) Extracted medicinal active compounds from earthworms tissue should be identified with their chemical structures for industrial production.

iv) Rheumatic fever, a complicated disease provokes valvular heart diseases can not be cured within short time by applying the traditional medicines. So it requires both extensive and intensive research and clinical trials repeatedly to determine the specific extract with proper dose.

v) For the development of the drug Government and non-Government organizations shall have to contribute effectively.

vi) For salvation of human kind from rheumatic fever a pilot project should be implemented to carry out constant research for extraction of effective medicinal compound from earthworms' tissues and its application on rheumatic fever patients in registered hospitals.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abbott, I. 1994. Distribution of the native earthworm fauna of Australia a content-wide perspective. *Aus. Jour. Sol. Research*, **32**:117-126.

Agustine, P.C. and Lund, E.E. 1974. The fate of eggs and larvae of *Ascarida gath* in earthworms, *Avian. Dis.*, **18**: 394-398.

Ahmed, J., Rouf, M.A. and Hassan, M.M. 1996. Grouping and Typing of Beta Haemolytic Streptococcus Group A isolated from Throat of Patients. *Ban. Jour. Patho.* **11**: No.-1.

Ahmed, J., Zaman, M.M. and Hassan, M.M.M. 2005. Prevalence of rheumatic fever and rheumatic heart diseases in a rural population of Bangladesh. *Tropical Doctor,* **35**:160-161.

Alawdeen, S.S. and Ismail, S.A. 1986. Stage of growth as a factor in harvesting the earthworm, *Lampito mauritii* Kingerg. *Proc. Nat. Sem. Org. Waste Utiliz. Vermicomp. Part B:Verms and Vermicomposting* (Desh, M.C., Senapari, B.K. and Mishra, P.C., eds). Sambalpur University, Orissa, 122-127 pp.

Annonymous, 1992a. American Heart Association Writing Group. Guidelines for the diagnosis of Rheumatic fever: Jones Criteria, Updated 1992, Circulation 1993: **87**: 302-307.

Annonymous, 1992b. Prevention of rheumatic fever. Report of a WHO Expert Committee, Geneva, *World Health Organization* (*WHO Technical Report* Series 342).

Annonymous, 1994. Brochure for National Centre for Control of Rheumatic Fever and Heart Disease, A survey report of *MoHFW.* 181p.

Annonymous, 1995. A new medicine for heart diseases containing enzyme activator extracted from earthworm. *Research and Development Centre for Health Protection*, Nanjing. China. 135pp

Annonymous, 1996. Medicines from the wild: TRAFFIC examines impact on North American wildlife. *TRAFFIC USA.* **15**(13): 1.

Annonymous, 1998. Quality control methods for medicinal plant materials, *World Health Organization*, Geneva. 89PP.

Annonymous, 2000. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. *World Health Organization*, Genera. WHO/EDM/TRM/2000. **1**: 73.

Annonymous, 2005. Prevalence of Rheumative Ferver and Rheumatic Heart Disease in an Urban Population of Bangladesh, *Final Report on APW. (WHO, Bangladesh)* NCCRF/RHD, Bangladesh, 78pp.

Ansari, M.T. 2001. Possible fibrino(geno)lytic Activity in an Earthworm Extract : Development of a Trial Protocol to Identify Initial Efficacy and Safety and Further Clinical Development. For the Degree of Master, *Medical Science at the University of Hong Kong,* 121-130.

Begum, U.H.N., Haque, K.M.H.S.S. and Rouf, M.A. 1994. Clinical Profile of RF and RHD in 5-15 Years old school children: A school survey Report. *MURMUP.* **3**(6-7): 5 p.

Beyer, W.N. 1981. Metals and terrestrial earthworms (Annelida : Oligochaera). In *Workshop on the role of earthworms in the stabilisation of organic residues.* (Appelhof, M. compiler), Vol. **I**, *Proc. Beech Leaf Press,* Kalamazoo, MI, 137-150pp.

Bristowf, H.S. 1932. Insects and other invertebrates for human consumption in Siam. *Trans Entomol. Soc (London),* **80:** 387-404.

Brown, B.A. and Mitchell, M.J. 1981. Role of the earthworm, *Eisenia foetida,* in afffecting survival of *Salmonella entermidis* ser. *Typhimurium. Pedobiologia,* **21**: 434-438.

Bundy, J.G., Osborn, D, Weeks, J.M., Lindon, J.C. and Nicholson, J.K. 2001. Approach to the investigation of coelomic fluid biochemistry in earthworms under toxic stress. Published by *Elsevier Science, Biological Chemistry, Biomedical Science Division*, Imperial college of Science. Medicine and Technology, Sir Alexander Fleming Building, South Kensington, London. **20**: 594-620.

Cairns, L.M. 1988. The Immunology of Rheumatic Fever. *New Zealand Medical Journal.* **10**: 388-91.

Davoli, C., Marconi, A., Serafino, A., Lannoni, C., Marcheggiano, A. and Ravagnan, G. 2002. Expression of nerve growth factor-life polypeptides and immuno-reactivity related to the two types of neurotrophin receptors in earthworm tissues. *Institute of Neurobiology and Molicular Medicine and Gastro enterology Unit, Department of Clinical Sciences.* La Saponza. Rome (Italy), **59**(3): 527-539.

Dean, T., Jamison, W., Mosley, H., Athony, R., Jose, M., Babajilla, L. 1993. Disease control priority in Developing countries. *A World Bank Book. Oxford University Press.* 223pp.

Dhennin, L., de Balsac, H.H., Verge, J. and Dhennin, L. 1963. Investigations on the role of *Lumbricus terrestris* in the experimental transmission of foot and mouth disease virus, *Bull. Acad. Vet. France,* **36**: 153-155.

Edwards, C.A. 1985. Production of feed protein from animal wastes by earthworms. *Phil. Trans. Roy. Soc. London,* **310**: 299-307.

Edwards, C.A. and Lofty, J.R. 1972. *Biology of earthworms.* Chapman and Hall Ltd., London. 283pp.

Edwards, C.A. and Bohlen, P.J. 1977. *Biology of Earthworms, 2nd ed.* Chapman & Hall, London, 380pp.

Edwards, C.A. and Lofty, J.R. 1977. *Biology of earthworms,* 2nd edition. Chapman and Hall, London. xviii: 333 pp.

Edwards, C.A. and Niederer, A. 1988. The production and processing of earthworm protein, in *Earthworms in Waste and Environmental Management,* (eds C.A. Edwards and E.F. Neuhauser), SPB Acad. *Publ., The Hague,* The Netherlands. 334pp.

Edwards, C.A. and Bohlen, P.J. 1996. *Biology of Earthworms.* Published by Chapman and Hall, 2-6 Boundary Row, London SEI8HN UK. 2nd edition, 380pp.

Fieldson, R.S., Billington, R.S. and Audsley, E. 1985. A study of the economic feasibility of on-farm vermiculture with centralized processing of worked waste to convert animal wastes to horticultural compost divisional note, DN. 1265, *National Institute of Agricultural Engineering. Silsoe,* U.K. 312pp.

Fletcher, K.E. 1991. The utilization of organic wastes by earthworms (Earthworms as animal feed protein), *Journal of Agricultural Zoology Reviews.* **9**(4): 245-246.

Fosgate, O.T. and Babb, M.R. 1972. Biodegradation of animal wastes by *Lumbricus terrestris. Journal of Dairy Science* **55**, 870-872.

Furest, A., Nguyen, Q.H. and Nguyen, L. 1989. Effect of Sulfur Amino Acid on GSH-S transterase in the common Earthworms (*Lumbricus terrestris*). *Presented at the Fifth International Brighton,* England. 73pp.

Fuxia, Z., Baozhu, G., Huiyan, W. and Guangzong, C. 1991. *Studies on the spermatocidal effect on the earthworm extract and its effective constituents.* M.C.H. Hospital and Family Planning Institute of Shaanxi Porvince, Xian, Peoples Republic of China. 340-351.

Fuxia, Z., Baozhu, G., Huiyan, W., Guangzong, C. 1992. Studies on the spermatocidal effecs of the earthworm extract and its effective constituents. *Management and conservation of soil fauna.* M.C.H. Hospital and family planning Institute of Shaanxi. Province, Xian, China. **8.8**: 683-687.

Fu-Zhen, H. and Shi-wei, Z. 1982. Studies on the distribution of earthworm and its comprehensive utilization in China. *Management and conservation of Soil Fauna. Academia Sinica,* Shaanxi, China, **8.2**: 635-641.

Fu-Zhen, H and Shi.-Wei, Z, 1992. Studies on the distributions of earthworm its Comprehensive utilization in China, *Northwestern Institute of Soil and Water conservation, Academia Sinica*, shaanxi, 640-641.

Gersch, M. 1954. Effect of carcinogenic hydrocarbons on the skin of earthworms. *Naturwissenschafl,* **41**: 337.

Gish, C.D. and Christensen, R.E. 1973. Cadmium, nickel, lead and zinc in earthworm from road side soil. *Environ Sci. Technol,* **7:** 1060-1062.

Goldstein, I.P., Rebeyrotte, and Parlebas, J. 1968. Isolation from heart valve of glycopeptides which share immunological properties with streptococcus haemolyticus group A polysacharide, *Nature.* **219**:866-8.

Graff, O. 1981. Pereliminary experiments of vermicomposting of different waste material using *Eudrilus engeniae* Kingberg. In *Workshop on the Role of Earthworms in the Stabilizastion of Organic Residues.* (M. Appelhof, compiler), *Proc. Beech Leaf Press, Kalamazoo,* MI, **1**:179-191.

Graff, O. 1982. Vergleich der Regenwurmarten *Eisenia foetida* und *Eudrilus eugeniae* hinsichtilch ihrer Eignung zur Proteingewinnung, aus Abfallstoffen. *Pedobiologia,* **23**: 277-82.

Gruber, C. Sturzenbaum, S., Gehrig, P., Sack, R., Hanziker, P., Berger, B. and Dallinger, R. 2000. Isolation and Characterization of a Self-sufficient one domain protein (Cd) Metiothionein from *Eisenia foetida.* Institute of Zoologie and Limnologie, der Universitat Innsbruck, *Austria. L. Biochem.* **267**:573-582.

Guerrero, R.D. 1983. The culture and use of *Perionyx excavatus* as a protein resource in he Philippines. In *Earthworm Ecology : from Darwin to Vermiculture* (J.E. Satchell, Ed.), *Chapman and Hall*, London, 309-313.

Guerrero, R.D. 1991. The culture and use of *Perionyx excavatus* as a protein resource in the Philippines. *Agricultural Zoology Reviews.* **26**(4): 309-313.

Gupta, A.K. 1991. *Introduction to Pharmaceutics-1*, CBS Publishers, New Dilhi, India, **1**: 164pp.

Hagues, M.K., Lepp, N.W. and Phipps, D.A. 1980. Aerial heavy metal pollution and terrestrial ecosystems, *Adv. Ecol. Res.*, **11**: 218-237.

Hansen R.P. and Czochanska, Z. 1974a. Occurrence of Triglycerides in earthworms. *Lipids,* **9**: 363-364.

Hansen, R.P. and Czochanska, Z. 1974b, Earthworm lipids: Occurrence of phytanic, Pristanic and 4, 8, 12 trimethyl-tridecanoic acids, *Lipids,* **9**: 825-827.

Haque, K.M.H.S.S., Haque, Z., Rouf, M.A., Mahmud, R.S., Faruq, Q.O., Islam, N., Khan, S.M.B., Hasan, F., Taher, A., Shah, M.N., Alam, S., Osman, M., Khan, A., Islam, M., Nahar, S. Rahman, A.Z.Z. and Arasaki, Y. 1992. Epidemiology of Rheumatic Fever and Rheumatic Heart Disease. Observation in 15,798 Children of rural and urban areas, Published by *IEDCR, Ministry of Health and Family Welfare, Government of the People's Republic of Bangladesh*, 35pp.

Haque, K.M.H.S.S., Haque, Z., Rouf, M.A., Mahmud, R.S., Faruq, Q.O., Islam, N., Khan, S.M.B., Hasan, F., Taher, A., Shah, M.N., Alam, S., Osman, M., Khan, A., Islam, M., Nahar, S. Rahman, A.Z.Z. and Arasaki,Y. 1993a. Clinical Profile of Acute Rheumatic Fever- A Study of Ninety cases. *MURMUR.* NCCRF/HD. **1**(2-4): 4.

Haque, K.M.H.S.S., Haque, Z., Rouf, M.A., Mahmud, R.S., Faruq, Q.O., Islam, N., Khan, S.M.B., Hasan, F., Taher, A., Shah, M.N., Alam, S., Osman, M., Khan, A., Islam, M., Nahar, S. Rahman, A.Z.Z. and Arasaki, Y. 1993b. Management of acute rheumatic fever, a study of ninety cases, *MURMUR.* NCCRF/HD. **1**(2-4): 6.

Haque, Z., Khan, A., Shah, T.M.N.N., Osman, M., Islam, M.M. and Nahar, S. 1994. 3rd National Scientific Conference on Rheumatic Fever and heart Diseases will be held in Dhaka in may 1994. Organized by National Centre for Rheumatic Fever and Heart Disease, Shere-E-Banglanagar, Dhaka. *MURMUR*, **2**(4):6.

Harteinstein, R. 1981. Use of *Eisenia foetida* in organic recycling based on Laboratory experiments. In workshop on the Role of Earthworm in the Stabilization of Organic Residue (M. Appelhof, compiler,. *Proc. Beech. Leaf Press,* Kalamazoo. MI. **1**:155-165.

Harteinstein, R., Neuhauser, E.F. and Collier, J. 1980. Accumulation of heavy metals in the earthworm *Eisenia foetida, J. Environ. Qual.,* **9**: 23-26.

Harwood, M. and Sabine, J.R. 1978. The nutritive value of worm meal. *Proc. 2nd Austr. Poult. Stockfeed Conv., Sydney,* 164-171.

Hasenbein, G. 1951. A pregnancy test on earthworms. *Arch. Gynakol.* **181**: 5-28.

Hilton, J.W. 1983. Potential of freeze-dried worm meal as a replacement for fish meal in trout diet formulations. *Aquaculture,* **32:** 277-283.

Hori, M., Kondon, K., Yoshida, T., Konishi, E. and Minami, S. 1974. Studies of antipyretic components in the Japanese earthworm. *Biochem Pharmacol.,* **23**: 1583-1590.

Ibanez, I.A., Herrera, C.A., Belazques, L.A. and Hebel, P. 1993. Nutritional and toxicological evaluation on rats of earthworm *(Eisenia foetida)* meal as a protein source for animal feed. *Anim. Feed. Sci. Tech.,* **42**(1-2): 165-72.

Ireland, M.P. 1983. Heavy metal uptake and tissue distribution in earthworms. In *Earthworm ecology* (Satchell, J.E. ed), *Chapman and Hall, London.* 247-265.

Islam, M.N., Mahmud, R.S., Hossain, M and Faruq, Q.O. 1998. "*DOCTORS' MANUAL*" Pilot Project for Prevention and Control of Rheumatic Fever and Rheumatic Heart Disease in Bangladesh. *MoHFW Bangladesh and JICA. Published by Scientific and Publication Committee of the Pilot Project.* **1**: 24.

Ismail, S.A. 1997. "*Vermicology*" The Biology of earthworm Orient Langman Ltd. India. 92pp.

Ismail, S.A., Pulandiran, K. and Yegnanarayan, R. 1992. Anti inflammatory activity of earthworm extracts. *Soil. Biol. Biochem.,* **24**: 1253-1254.

Jakovljevic, D.D. 1975. Some aspects of the epizootology and economical significance of ascariasis in swine. *Acta. Vet. Beogr.,* **25**: 315-325.

Jalil, M.K. 1997. Rheumative Ferver and Rheumatic Heart Disease, *Batjor and Redrog Barta,* NCCRF/RHD. **16:**5.

Jayaraman, J. 1985. Laboratory Manual in Biochemistry. *Wiley Eastern Ltd.* New Delhi, India. 178pp.

Jin, 2000. Lumbrokinase is beneficial to the treatment of cerebral infraction. *Journal of National Library of Medicine,* **120**:220-247.

Jones, T.D. 1944. The diagnosis of rheumatic fever. *J. Am. Med. Assoc.*, **126**, 481.

Josef, J., Islam, S.A. and Sharif, S.D. 1992. Modified continuous flow respirometter to measure to effect of tannery effluents on the respiration of earthworms, *Bull. environ. Contam. Toxicol.,* **49**:490-496.

Julka, J.M. and Senapti, B.K. 1987. Earthworms (Oligochaeta : Annelida) of Orissa, India. *Rec. Zool. Surv. India, Occ.* **92**: 1-49.

Julka, J.M. 1988. The Fauna of India and the Adjacent Countries, *Pub. by the Director Zoological Survey of India,* Calcutta, India, 365pp.

Kale, R.D. 1986. Earthworm feed for poultry and aquaculture. In: *Proc. Nat Sem Org Waste Utiliz Vermicomp Part B: Verms and Vermicomposting* (Dash, M.C., Senapati, B.K., and Mishra, P.C. eds), *Sambalpur University.* Orrisa. 137-145pp.

Khalaque, M.A. 1996. *Practical Pathology,* 5th edition. Eureka Pablication Ltd. Dhaka Bangladesh. 243pp.

Khan, A., Faruq, N.S., Nahar, S., Mahmud, R.S., Hasin, F., Nishi, Arashaki, Y., Haque, K.M.H.S.S., Shah, A.T.M.N.N. and Islam, M.M. 1994. Presentation pattern of RF/RHD. Patients at a rural Health complex Dhaka, RF/RHD control project MURMUR. **3**(6-7):4.

Khan, A., Faruq, N.S., Nahar, S., Mahmud, R.S., Hasin, F., Nishi, Arashaki, Y., Haque, K.M.H.S.S., Shah, A.T.M.N.N. and Islam, M.M. 1994. Demographic pattern of RF and RHD cases at the National Centre of RF and RHD control project. *MARMUR,* **3**(6-7):2.

Khan, S.M.B., Rauf, M.A. and Hassan, M.M.M. 1994. Presentation pattern of RF/RHD Patients at a rural Health complex Dhaka, RF/RHD control project *MURMUR.* **3**(6-7): 4.

Kotpal, R.L. 1998. *Annelida.* Rastogi Publications, Merut, India, 11th edition, **5**: 263pp.

Kumar and Clark, 1995. *Clinical Medicine,* 3rd Edition, ELBS, Landon, 1205pp .

Lawrence, R.D. and Millar, H.R. 1945. Protein content of earthworms. *Nature, Land.,* **155**(39): 517.

Li K. 2004. Bio-products from Earthworms. www.2004:Bio-products from Earthworms.

MacDonald, D.W. 1983. Predation on earthworms by terrestrial vertebrates, in *Earthworm Ecology from Darwin to Vermiculture* (ed. J.E. Satchell), *Chapman & Hall,* London, 393-414.

MacDonald, K.T. and Walker, A.C. 1989. Rheumatic Heart Disease in Aboriginal Children in the Northern Territory. *Medical Journal of Australia.* 150:503.

Macleoid , J. 1948. Davidson's Principal and Practice of Medicine, *14th Edition* , *ELBS.* 155pp.

McInroy, D. 1971. Evaluation of the earthworm *Eisenia foetida* as food for man and domestic animals. *Feedstuffs,* **43:** 37-47.

Mehrotra, V.S. 1997. *Vermiculture,* National Council of Educational Research and Training, Sri Aurobindo Marg, New Delhi, 110016, India, 25-26.

Mihara, H., Sumi, H., Mizumoto, H., Yoneta, T., Ikeda, R., Seiki, M. and Maruyama, M. 1991a. Oral administration of earthworm powder as a possible thrombolytic therapy. In : *Recent advances in thrombosis and fibrinolysis* (Tanaka, K. ed). *Academic Press,* 287-298pp.

Mihara, H., Sumi, H., Yoneta, T., Mizumoto, H., Ikeda, R., Seiki, M. and Maruyama, M. 1991b. A novel fibrinolytic enzyme extracted from the earthworm *Lumbricus rubellus. Japanese Journal of Physiology,* **41**: 461-472.

Morgan, J.E. and Morgan, A.J. 1990. The distribution of Cadmium, Copper, Lead, Zinc and Calcium in the tissues of the earthworm *Lumbricus rubellus* sampled from one uncontaminated and 4 polluted soils. *Oecologia.* **84**:559-566.

Morgan, A.J., Morgan, J.E. Turner, M. Winters, C. and Yarwood, A. 1993. Metal relationships of earthworms. In *Ecotoxicology of Metrals in Invertebrates* (Dallinger, R. and Rainbow, P.S., eds.) *Lewis Publishers, Boea Raton, FL,* USA. 333-358.

Murshed , M. 2000. Rheumatic Fever and Heart Diseases, Yearly Health Situation Report, Published by *IEDCR, Ministry of Health and Family Welfare, Government of the People's Republic of Bangladesh,* 180-185.

Nahar, S. 1994. Rheumatic Arthritis and Rheumatic Fever is not same. NCCRF/HD, *J. MURMUR,* **6-7**:1.

Naya, Y. and Kotake, M. 1967. Undersuchung der freien Fettsauren, der freien Sterine, der sterinester und der Glyzeride in Regenwurm (*Lumbricus spencer*) *Bull. Chem. Soi. Jpn.,* **40**: 880-884.

Neutze, J.M. 1988. Rheumatic Fever and Rheumatic Heart Disease in the Western Pacific Region. *New Zealand Medical Journal.* **101**:404-6.

Nguyen, Q.H. and Furest, A. 1988. Acute Toxicity of Cadmium and Zinc in the Earthworms (*Lumbricus terrestris*). *Biological Trace Element Research*, **18**: 81-83.

Nielson, R.L. 1965. Presence of plant growth substances in earthworms demonstrated by paper chromatography and the Went Pea test. *Nature,* **208:** 1113.

Odum, E.P. 1971. *Fundamentals of Ecology,* W.B. Saunders Company, Philadelphia, London, Toronto; Toppan Company Ltd., Tokyo, Japan. 574 pp.

Ohta, N., Shioda, S., Sekizawa, Y., Nakai, Y. and Kobayashi, H. 2000. Sites of expression of mRNA for lysenin, a protein isolated from the coelomic fluid of the earthworm *Eisenia foetida, Research Laboratory, Zenyaku Kogyo Co., Nerim-Ku, Tokyo,* Japan. 155: 121-133.

Patel, H.K. and Patel, R.M. 1959. Preliminary observations on the control of earthworms by soapnut (*Sapindus laurifaulius* Vahl) extract. *Indian J. Ent.,* **21**:251-255.

Pulandiran, K. and Ismail, S.A. 1987. Anti-Inflammatory activity of total earthworm extract in Rats. *Indian. J. Pharmae.,* **19**: 221-224.

Puttarudriah, M. and Sastry, K.S.S. 1961. A preliminary study of earthworm damage to crop growth. *Mysore Agric. J.,* **36**:2-11.

Qingsui, C. 1995. Phase III clinical trail of Boluoke Capsule in treating on 1560 patients, ischemic cerebrovascular disease. *Health protection J. Biochem.*, Nanjing, China. 150:273-86.

Ramseier, S., Haerdi, W., Honsberger, P., Martin, M., Cuendet, G. and Tarradellas, J. 1989. Bioaccumulation of Cadmium by *Lumbricus terrestris, Toxicol. Environ. Chem.* **22**:189-196.

Ramseier, S., Deshusses, J. and Haerdi, W. 1998. Cadmium speciation studies in the intestine of *Lumbricus terrestris* by electrophoresis of metal proteins complexes. *Department of Inorganic, Analytical and Applied Chemistry, University of Geneva*, Switzerland. **431**(3):437-42.

Reynolds, J.W. and Reynolds, W.M. 1972. Earthworms in medicine, *Amer. J. Nursing,* **72**:1273.

Rouf, M.A. 1996. Magnitude of the Problems of Streptococcal sore throat in Bangladesh. *Ban. Jour. Patho.* **11**(2):6.

Rouf, M.A. 1997. Pathogenesis of Rheumative Ferver. *MURMUR,* NCCRF/HD, **5**(17):1.

Rouf, M.A., Khan, H.I.L.R. and Hassan, M.M.M. 1991. Prevalence of streptococcus beta haemolyticus among school children of Dhaka city, *Chest and heart bulleitn,* **XV**(1):3.

Rouf, M.A. Khan, H.I.L.R. and Hassan, M.M.M. 1997. Rheumative Ferver and Rheumatic Heart Disease in Industrial and Developing countries, *MURMUR,* NCCRF/RHD, Bangladesh, **5**(16):4.

Sabine, J.R. 1978. The nutritive value of earthworm meal, in *Utilization of Soil Organisms in sludge Management,* (ed. R. Hartenstein), *Natl. Tech. Inf. Services.* PB286932, Springfield, **V**: 285-96.

Sabine, J.R. 1981. Vermiculture as an option for resource recovery in the intensive animal industries. *Proc. Workshop on the Role of Earthworms in the Stabilization of Organic Residues,* (ed. M. Appelhof), *Beach Leaf Press*, Kalamazoo, Michigan, **I**: 241-54.

Sabine, J.R. 1988. Vermiculture : bring on the future. In *Earthworms in Waste and Environmental management* (C.A. Edwards and E.F. Neuhauser, Eds), *SPB Academic Publishing. The Hague, The Netherlands,* 3-6.

Sabine, J.R. 1991. Earthworms as a source of food and drugs. *Earthworms Ecology. Chapman and Hall Ltd.* **24**: 285-295.

Said, M. 1971. *Hamdard Pharmacopoeia of Eastern Medicine.* Medical Research Group. Hamdard Laboratories (Waqf) Pakistan. 554 pp.

Sample, B.E., Suter, G.W., Beauchamp, J.J., Efromson, R.A., 1999. Literature -derived bioaccumulation for earthworms: Development and validation. *Journal of TCMI*, **14**(2): 132-134.

Schulz, E. and Graff, O. 1977. Zur Berwertung von Regenwurmmehl aus *Eisenia foetida* als Eiweissfuttermittel. *Landb. Forsch. Vilkr.,* **27:** 216-18.

Shahidullah, M. 1997. Prevention of Heart Disease, *Batjor and Redrog Barta*, NCCRF/RHD, **5**(15): 1.

Shukla, M.R. 1950. *Unani Chikitsa Sagar* (Hindi). 1st edition. Motilal Banarsidass, Varanasi. 383-485.

Stephenson, J. 1930. *The Oligochasta.*Oxford university Press, London. 977pp.

Stürzenbaum, S.R., Kille, P. and Morgan, A.J. 1994. The Identification, Cloning and characterization of earthworm metallothionein. *J. Biol. Chem.* **269**(40): 24688-24691.

Suzuki, K.T., Yamamura, M. and Mori, T. 1980b. Metabolic fate of earthworm cadmium binding proteins in rats, *Arch. Environ. Contam. Toxicol,* **9**:519-531.

Taboga, L. 1980. The nutritive value of earthworms for chickens. *Br. Poult. Sci.,* **21**: 405-410.

Tacon, A.G.J., Stafford, E.A. and Edwards, C.A. 1983. A preliminary investigation of the nutritive value of three terrestrial lumbricid worms for rainbow trout. *Aquaculture,* **35:** 187-199.

Taranta, A.R. and Markowitz, M. 1989, *Rheumatic Fever,* Kluwer Academic Publishers, Dordrecht, Boston, London, 2nd. Edt., 95pp.

Toma, S. 1985. "Cost-Effective Control of Rheumatic Fever in community: *Health Policy,* **5**:159-64.

Veeresh, G.K. and Rajagopal, D. 1981. Annelids as pests of paddy in Karnataka. *Newsl. Soil. Biol. Ecol.,* **2**(1): 1-2.

Vohora, S.B. and Khan, M.SY. 1978. *Animal origin drugs used in Unani medicine,* Institute of History of Medicine and Medical Research, Tughlaqabad, New Delhi, 137pp.

Voogt, P.A., van Rhee, J.W.A. and Zandee, D.I. 1975. What about squalence in the earthworm *Lumbricus terrestris Comp. Biochem. Physioil.,* **50B**: 511- 513.

Wahid, A. and Siddiqui, H.H. 1961. A *Survey of Drugs.* 2nd edition. Institute of History of Medicine and Medical Research, New Delhi, 16-17pp.

Weisbach, W.W. 1962. Regenwürmer und Essbnare Erde. *Biol. Jaarb. Dodonea.*, **30:** 225-38.

Willuhm, J., Schmitt-Wrede, H.P., Greven, H. and Wunderlich, F. 1994. CDNA cloning of a cadmium-inducible messenger-RNA encoding a novel cysteine-rich, nonmetallothionein 25-KDa protein in an enchytracid earthworm. *J. Biol. Chem.* **269**:24688-24691.

Yegnanarayan, R., Sethi, P.P., Rajhans, P.K., Pulandiran, K. and Ismail, S.A. 1987. Anti-inflammatory activity of total earthworm extracts in rats. *Ind. J. Pharmac.*, **19**: 221-224.

Yegnanarayan, R., Ismail, S.A. and Shortri, D.S. 1988. Anti-inflammatory activity of two earthworm potions in Carrageenan pedal oedema test in rats. *Ind. J. Physiol. Pharmac.*, **32**: 72-74.

Yoshida, M. and Hoshii, H. 1978. Nutritional value of earthworms for poultry feed. *Jpn. Poult. Sci.*, **15**(6): 308-11.

Zaman, M.M., Yoshiika, N., Ahmed, J. Hassan, M.M.M., Rauf, M.A. and Haque, K.M.H.S.S. 1998. Does Rheumatic Fever occur usually between the ages of 5 to 15 years? *J. Cardiol*, **66**: 17-21.

APPENDICES

Appendix table 1 Estimation of earthworm extracts (solid) by using various solvents successively in percent of dry weight

Observation No	Diethyl ether	Chloroform	Methanol	Ethanol	Propanol	Butanol	Acetic acid	Acetone	Xylol	Morpholine
1	3.32	3.84	20.20	5.20	0.33	0.53	1.00	1.05	1.00	0.53
2	2.40	2.48	17.50	7.44	1.00	0.73	0.53	1.53	0.43	1.05
3	3.20	2.18	16.00	6.64	0.42	0.60	2.00	1.00	0.36	0.80
4	1.25	2.48	15.53	7.20	0.94	0.80	0.53	0.90	0.63	1.00
5	1.71	1.62	20.19	5.40	0.88	0.70	1.05	2.63	0.36	0.53
6	4.10	3.38	17.17	4.40	0.70	0.99	1.53	1.36	1.53	1.00
7	2.04	4.22	18.18	3.20	1.20	1.20	1.00	1.00	1.00	0.63
8	3.00	4.00	17.17	4.00	1.00	2.63	0.88	1.12	0.95	0.70
9	1.00	1.20	15.18	3.20	1.11	1.00	0.94	0.53	2.00	0.80
10	2.22	2.20	13.23	2.94	1.20	1.00	0.99	1.12	1.05	1.05
11	2.42	3.40	12.22	3.60	0.68	0.53	0.94	1.00	1.50	0.80
12	0.94	5.20	24.17	4.04	0.70	0.90	0.90	1.05	0.90	1.00
13	3.20	1.60	12.12	5.04	0.33	1.00	0.89	0.55	0.99	1.05
14	4.20	4.20	14.17	4.20	0.43	1.12	0.90	0.70	0.96	0.98
15	2.54	5.54	16.16	5.00	0.84	1.05	2.63	0.90	0.60	0.90
16	3.62	2.88	20.48	2.30	0.53	0.73	1.12	0.84	0.63	1.05
17	2.40	3.48	13.40	2.94	1.20	0.83	1.00	0.94	0.43	1.00
18	1.11	2.78	14.00	3.00	1.42	0.73	0.53	2.63	0.30	0.90
19	1.70	2.94	16.16	3.00	0.90	0.94	0.73	1.00	0.63	0.88
20	2.80	3.11	17.42	4.30	0.40	0.99	1.70	0.90	0.69	0.40
21	4.04	6.12	13.48	4.40	1.20	0.94	1.05	2.00	0.66	0.60
22	3.30	7.20	15.20	4.40	1.02	0.90	1.12	1.00	0.70	0.70
23	2.60	2.46	12.12	2.60	1.11	0.70	0.85	1.05	0.71	0.80
24	2.22	3.46	16.06	2.60	0.80	0.86	0.80	1.53	0.72	0.70
25	4.30	2.05	14.14	5.50	1.11	0.95	1.53	1.05	0.43	0.53
26	3.20	3.07	13.12	6.20	0.94	0.90	0.90	1.00	0.36	0.62
27	2.60	2.46	24.24	5.60	0.99	0.70	1.90	0.90	0.63	0.70
28	3.84	3.05	17.25	6.20	0.99	0.99	0.88	1.25	0.53	0.70
29	5.40	4.50	16.26	4.20	0.77	0.99	0.94	0.94	0.63	0.80
30	6.20	2.70	15.20	10.12	0.68	1.00	0.96	1.05	0.74	0.86
M±SD	2.89±1.23	3.32±1.36	16.25±3.15	4.62±1.73	0.86±0.29	0.93±0.36	1.09±0.46	1.15±0.49	0.76±0.38	0.80±0.18

Appendix table 1 Estimation of earthworm extracts (solid) by using various solvents successively in percent of dry weight

Observation No	Diethyl ether	Chloroform	Methanol	Ethanol	Propanol	Butanol	Acetic acid	Acetone	Xylol	Morpholine
1	3.32	3.84	20.20	5.20	0.33	0.53	1.00	1.05	1.00	0.53
2	2.40	2.48	17.50	7.44	1.00	0.73	0.53	1.53	0.43	1.05
3	3.20	2.18	16.00	6.64	0.42	0.60	2.00	1.00	0.36	0.80
4	1.25	2.48	15.53	7.20	0.94	0.80	0.53	0.90	0.63	1.00
5	1.71	1.62	20.19	5.40	0.88	0.70	1.05	2.63	0.36	0.53
6	4.10	3.38	17.17	4.40	0.70	0.99	1.53	1.36	1.53	1.00
7	2.04	4.22	18.18	3.20	1.20	1.20	1.00	1.00	1.00	0.63
8	3.00	4.00	17.17	4.00	1.00	2.63	0.88	1.12	0.95	0.70
9	1.00	1.20	15.18	3.20	1.11	1.00	0.94	0.53	2.00	0.80
10	2.22	2.20	13.23	2.94	1.20	1.00	0.99	1.12	1.05	1.05
11	2.42	3.40	12.22	3.60	0.68	0.53	0.94	1.00	1.50	0.80
12	0.94	5.20	24.17	4.04	0.70	0.90	0.90	1.05	0.90	1.00
13	3.20	1.60	12.12	5.04	0.33	1.00	0.89	0.55	0.99	1.05
14	4.20	4.20	14.17	4.20	0.43	1.12	0.90	0.70	0.96	0.98
15	2.54	5.54	16.16	5.00	0.84	1.05	2.63	0.90	0.60	0.90
16	3.62	2.88	20.48	2.30	0.53	0.73	1.12	0.84	0.63	1.05
17	2.40	3.48	13.40	2.94	1.20	0.83	1.00	0.94	0.43	1.00
18	1.11	2.78	14.00	3.00	1.42	0.73	0.53	2.63	0.30	0.90
19	1.70	2.94	16.16	3.00	0.90	0.94	0.73	1.00	0.63	0.88
20	2.80	3.11	17.42	4.30	0.40	0.99	1.70	0.90	0.69	0.40
21	4.04	6.12	13.48	4.40	1.20	0.94	1.05	2.00	0.66	0.60
22	3.30	7.20	15.20	4.40	1.02	0.90	1.12	1.00	0.70	0.70
23	2.60	2.46	12.12	2.60	1.11	0.70	0.85	1.05	0.71	0.80
24	2.22	3.46	16.06	2.60	0.80	0.86	0.80	1.53	0.72	0.70
25	4.30	2.05	14.14	5.50	1.11	0.95	1.53	1.05	0.43	0.53
26	3.20	3.07	13.12	6.20	0.94	0.90	0.90	1.00	0.36	0.62
27	2.60	2.46	24.24	5.60	0.99	0.70	1.90	0.90	0.63	0.70
28	3.84	3.05	17.25	6.20	0.99	0.99	0.88	1.25	0.53	0.70
29	5.40	4.50	16.26	4.20	0.77	0.99	0.94	0.94	0.63	0.80
30	6.20	2.70	15.20	10.12	0.68	1.00	0.96	1.05	0.74	0.86
M±SD	2.89±1.23	3.32±1.36	16.25±3.15	4.62±1.73	0.86±0.29	0.93±0.36	1.09±0.46	1.15±0.49	0.76±0.38	0.80±0.18

Appendix table 2 **Crude protein, crude fat, carbohydrates, total ash, crude fibre and steroids contents of earthworms in percent by dry weight of earthworm extract.**

Obs. No.	Crude protein	Crude fat	Carbohydrates	Total ash	Crude fibre	Steroids
1	72.29	6.20	5.60	5.19	2.92	0.004
2	67.02	4.20	2.42	10.19	1.20	0.006
3	58.00	5.58	3.44	11.11	3.40	0.004
4	60.00	4.98	6.07	5.72	2.43	0.003
5	66.62	6.06	2.94	8.80	2.12	0.004
6	65.70	7.12	3.90	6.12	3.12	0.007
7	62.66	2.83	4.42	9.42	4.00	0.009
8	75.00	4.44	10.10	5.80	0.60	0.006
9	45.92	3.43	10.12	7.47	3.21	0.007
10	5058	5.53	4.12	4.10	2.92	0.002
11	45.94	4.20	2.32	3.40	1.92	0.004
12	60.62	3.92	1.09	3.00	2.20	0.006
13	75.20	4.39	3.64	3.00	2.42	0.004
14	45.95	4.47	3.46	5.82	3.00	0.005
15	50.70	3.37	4.64	6.06	1.60	0.006
16	66.66	2.93	3.99	4.40	2.60	0.008
17	60.67	4.45	4.12	5.40	4.20	0.003
18	58.70	6.00	5.00	3.30	3.20	0.004
19	48.20	5.20	5.25	7.00	4.20	0.008
20	45.40	4.20	6.66	8.12	4.40	0.005
21	60.58	3.90	4.46	2.22	4.20	0.004
22	47.47	2.30	6.60	3.89	2.00	0.007
23	64.60	3.40	4.08	4.42	1.00	0.004
24	60.48	7.00	2.88	6.60	2.00	0.003
25	45.45	6.60	3.30	7.20	1.80	0.002
26	75.40	5.20	4.40	8.00	2.00	0.003
27	70.60	4.44	8.00	4.20	1.90	0.004
28	60.65	7.20	6.60	3.99	2.00	0.006
29	48.48	7.00	2.20	4.22	1.80	0.004
30	70.00	4.20	4.30	1.46	2.00	0.003
M±SD	59.52±9.96	4.82±1.36	4.67±2.10	5.65±2.38	2.54±0.99	0.048±0.018

Appendix table 3 Essential amino acid compositions of earthworm's protein (mg per 100 mg of protern)

Obs. No.	Cysteine	Histidine	Lysine	Methionine	Phenylalanine	Tryptophan	Valine
1	1.70	3.60	7.40	2.12	5.00	2.20	4.12
2	1.00	3.70	6.94	2.00	4.26	2.12	4.50
3	1.70	3.12	7.22	2.42	4.88	1.88	3.88
4	2.00	3.00	6.87	3.00	5.80	1.90	3.88
5	1.60	4.12	6.78	4.12	4.66	2.00	3.58
6	0.84	4.44	7.12	1.00	4.44	3.12	5.40
7	0.90	5.04	6.87	1.42	4.26	1.20	4.46
8	1.20	2.20	6.66	2.12	5.26	1.40	4.64
9	3.00	4.12	7.78	2.18	6.12	2.00	3.84
10	4.10	2.44	7.78	2.10	4.44	3.04	4.40
11	1.00	4.00	5.58	2.42	5.80	2.40	3.40
12	0.94	1.20	7.46	2.44	4.68	1.68	2.00
13	0.64	2.88	5.64	2.42	5.80	1.90	6.00
14	1.78	2.98	4.22	3.12	4.40	1.70	5.80
15	2.00	3.78	8.25	2.19	5.80	2.88	6.82
16	2.40	4.40	5.26	2.33	3.49	2.20	3.20
17	2.48	4.20	5.12	2.54	4.94	2.30	3.44
18	1.00	1.22	5.55	3.42	6.78	2.22	4.12
19	1.70	2.48	4.20	2.32	7.20	1.78	3.80
20	1.58	2.44	4.88	1.88	5.40	1.48	2.40
21	2.00	3.88	7.88	3.12	6.06	3.44	3.44
22	1.70	3.40	6.42	2.88	5.00	2.40	4.80
23	1.48	4.58	7.44	2.32	6.12	2.12	4.12
24	1.48	2.22	8.00	1.88	7.00	1.99	3.88
25	2.00	2.22	6.60	2.66	4.20	2.12	3.88
26	1.64	4.44	6.74	2.46	5.12	3.00	4.48
27	0.88	1.99	7.20	3.00	6.00	1.66	5.42
28	2.17	2.99	6.22	3.00	7.00	2.20	5.20
29	1.70	2.50	6.48	1.88	4.12	1.87	4.70
30	0.88	3.40	60.6	1.92	3.52	1.12	3.20
M±SD	1.64±0.72	3.23±1.00	6.55±1.09	2.40±0.60	5.25±1.01	2.11±0.55	4.22±1.02

Appendix table 4 Bactericidal effect of substances extract successively by using various solvents from dried earthworms (Powder) on β-haemolyticus

Obs. No.	Diethyl ether	Chloroform	Methanol	Ethanol	Propanol	Butanol	Acetic acid	Acetone	Xylol	Morpholine
1	12	8	10	15	1	7	12	4	1	1
2	10	7	12	13	2	6	18	1	1	2
3	13	10	15	12	3	5	17	3	1	1
4	15	12	20	17	4	7	10	1	1	1
5	17	13	18	18	2	8	15	2	1	2
6	10	15	20	14	3	10	12	3	1	3
7	13	8	12	15	1	7	10	4	2	2
8	12	7	10	16	2	8	15	1	2	2
9	10	12	20	18	2	9	12	3	2	1
10	8	7	12	16	1	7	17	1	1	3
11	7	8	10	17	2	8	16	2	2	2
12	13	12	20	18	1	9	15	4	1	1
13	13	13	12	12	1	7	10	1	2	3
14	15	15	10	18	2	8	12	1	1	1
15	14	14	20	10	1	9	14	2	2	2
16	13	15	12	12	1	10	16	3	2	1
17	15	16	10	18	2	12	17	3	2	2
18	10	8	16	10	1	10	13	2	1	2
19	12	16	16	13	1	17	10	1	1	1
20	13	17	10	18	2	10	18	1	1	1
21	15	15	17	12	1	13	20	2	2	2
22	11	15	12	16	1	5	15	3	1	1
23	13	12	10	18	2	6	16	4	2	2
24	14	17	17	17	1	8	18	1	1	1
25	15	17	17	14	1	5	10	2	1	2
26	14	18	18	15	2	6	12	1	1	1
27	12	19	20	16	1	8	18	1	1	2
28	11	12	20	18	1	6	18	2	2	1
29	12	18	10	12	1	4	20	4	2	2
30	13	10	12	10	1	8	15	1	2	1
M±SD	12.50±2.20	12.86±3.72	14.60±4.00	14.93±2.74	1.50±0.77	8.13±2.65	14.70±3.12	2.20±1.12	1.46±0.50	1.60±0.60

Appendix table 5 Effect of earthworm extracts on patients suffering from Rheumatic Fever (*in vivo*)

Solv.	Sign and symptoms	Investigation	Results						
			After 7 days	After 14 days	After 21 days	After 21 days	After 28 days	After 35 days	After 42 days
Diethyl ether	Shifting pain, tonsillitis, decrease mean pressure	ASO-Titre600, ESR40 mm CRP positive	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, throat, pain	ASOTitre800 ESR-35 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Heart palpitation, decrease mean pressure	ASO-Titre400, ESR-30 mm	No change	No change	No change	No change	No change	No change	No change
	Joint pain, rheumatic nodules	ASO-Titre,500, ESR-35 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Heart palpitation, decrease mean pressure	ASO-Titre400, ESR-30 mm	No change	No change	No change	No change	No change	No change	No change
Chloroform	Carditis, tonsillitis shifting pain	ASO-Titre500, ESR-25 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, rheumatic nodules	ASO-Titre600, ESR-27 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Shifting pain, tonsillitis,	ASO-Titre500, ESR-40 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Palpitation of heart, tonsillitis, fever, weakness	ASO-Titre700, ESR-32 mm	No change	No change	No change	No change	No change	No change	No change
	Shifting pain, tonsillitis, abnormal mean pressure	ASO-Titre400, ESR-32 mm	No change	No change	No change	No change	No change	No change	No change
Methanol	Poly arthritis, rheumatic nodules	ASO-Titre600, ESR-27 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Shifting pain, tonsillitis,	ASO-Titre 500, ESR-40 mm	ASO-T. 500, ESR-26mm	ASO-T. 400, ESR-19mm	ASO-T. 300 ESR-15mm	ASO-T. 300, ESR-13mm	ASO-T. 200, ESR-12mm	ASO-T. 200, ESR-10mm	ASO-T. 200, ESR-12mm
	Palpitation of heart, tonsillitis, fever, weakness	ASO-Titre700, ESR-32 mm	ASO-T. 500, ESR-26mm	ASO-T. 500, ESR-26mm	ASO-T. 400, ESR-26mm	ASO-T. 300, ESR-26mm	ASO-T. 300, ESR-26mm	ASO-T. 200, ESR-26mm	ASO-T. 200, ESR-26mm

Solv.	Sign and symptoms	Investigation	Results						
			After 7 days	After 14 days	After 21 days	After 21 days	After 28 days	After 35 days	After 42 days
	Shifting pain, tonsillitis, abnormal mean pressure	ASO-Titre400, ESR-32 mm	ASO-T. 400, ESR-26mm	ASO-T. 400, ESR-20mm	ASO-T. 300, ESR-15mm	ASO-T. 300, ESR-15mm	ASO-T. 300, ESR-15mm	ASO-T. 200, ESR-10mm	ASO-T. 200, ESR-10mm
	Poly arthritis, throat, pain	ASO-Titre600, ESR-36 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
Ethanol	Joint pain, rheumatic nodules	ASO-Titre500, ESR-30 mm	ASO-T. 400, ESR-15mm	ASO-T. 400, ESR-15mm	ASO-T. 300, ESR-13mm	ASO-T. 300, ESR-13mm	ASO-T. 300, ESR-15mm	ASO-T. 200, ESR-12mm	ASO-T. 200, ESR-10mm
	Heart palpitation, decrease mean pressure	ASO-Titre700, ESR-25 mm	ASO-T. 500, ESR-20mm	ASO-T. 400, ESR-20mm	ASO-T. 400, ESR-15mm	ASO-T. 300, ESR-12mm	ASO-T. 300, ESR-12mm	ASO-T. 300, ESR-10mm	ASO-T. 200, ESR-10mm
	Carditis, tonsillitis, shifting pain	ASO-Titre600, ESR-20 mm	ASO-T. 400, ESR-20mm	ASO-T. 300, ESR-16mm	ASO-T. 300, ESR-15mm	ASO-T. 300, ESR-16mm	ASO-T. 300, ESR-15mm	ASO-T. 200, ESR-13mm	ASO-T. 200, ESR-10mm
	Poly arthritis, rheumatic nodules	ASO-Titre400, ESR-18 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Shifting pain, tonsillitis,	ASO-Titre500, ESR-32 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
Propanol	Poly arthritis, throat, pain	ASO-Titre300, ESR-27 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Joint pain, rheumatic nodules	ASO-Titre500, ESR-15 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Heart palpitation, decrease mean pressure	ASO-Titre300, ESR-32 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Abnormal mean pressure, tonsillitis Shifting pain	ASO-Titre400, ESR-35 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, abnormal mean pressure	ASO-Titre600, ESR-30 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Shifting pain, tonsillitis, abnormal heart sound	ASO-Titre800, ESR-40 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Rheumatic nodules tonsillitis fever weakness	ASO-Titre400, ESR-30 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve

	Sign and symptoms	Investigation	Results						
			After 7 days	After 14 days	After 21 days	After 21 days	After 28 days	After 35 days	After 42 days
Solv.	Shifting pain, tonsillitis, heart sound murmur	ASO-Titre500, ESR-27 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, throat pain,	ASO-Titre400, ESR-28 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Joint pain, rheumatic nodules	ASO-Titre500, ESR-35 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
Butanol	Heart palpitation, decrease mean pressure	ASO-Titre600, ESR-37 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Carditis, tonsillitis shifting pain	ASO-Titre600, ESR-23 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, rheumatic nodules	ASO-Titre700, ESR-36 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, rheumatic nodules	ASO-Titre600, ESR-27 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Shifting pain, tonsillitis,	ASO-Titre500, ESR-40 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
Acetone	Palpitation of heart tonsillitis, fever, weakness	ASO-Titre700, ESR-32 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Shifting pain, tonsillitis, abnormal mean pressure	ASO-Titre400, ESR-32 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, throat pain,	ASO-Titre600, ESR-36 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Joint pain, rheumatic nodules	ASO-Titre500, ESR-30 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Heart palpitation, decrease mean pressure	ASO-Titre700, ESR-25 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve

Solv.	Sign and symptoms	Investigation	Results						
			After 7 days	After 14 days	After 21 days	After 21 days	After 28 days	After 35 days	After 42 days
Acetic acid	Carditis, tonsillitis, shifting pain	ASO-Titre600, ESR-20 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, rheumatic nodules	ASO-Titre400, ESR-18 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Carditis tonsillitis, fever, weakness	ASO-Titre600, ESR-32 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Shifting pain, tonsillitis, abnormal mean pressure	ASO-Titre500, ESR-35 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Abnormal mean pressure, tonsillitis, fever, weakness	ASO-Titre500, ESR-32 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
Xylol	Shifting pain, tonsillitis, abnormal heart sound	ASO-Titre700, ESR-30 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, throat, pain	ASO-Titre300, ESR-27 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Carditis, tonsillitis, fever, weakness	ASO-Titre600, ESR-32 mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Shifting pain, tonsillitis, abnormal mean pressure	ASO-Titre500, ESR-35mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Abnormal mean pressure, tonsillitis, fever, weakness	ASO-Titre500, ESR-32mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
Morpholine	Shifting pain, tonsillitis, heart sound murmur	ASO-Titre700, ESR-30mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Poly arthritis, throat pain,	ASO-Titre300, ESR-27mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Joint pain, rheumatic nodules	ASO-Titre500, ESR-15mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Heart palpitation, short mean pressure	ASO-Titre300, ESR-32mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve
	Abnormal mean pressure, tonsillitis, shifting pain	ASO-Titre400, ESR-35mm	No improve	No improve	No improve	No improve	No improve	No improve	No improve

Appendix table 6 Effect of Methanolic extract on patients suffering from Rheumatics fever (*in vivo*)

Sl. No.	Signs and Symptoms	Pathological Investigation	Results					
			After 7 days	21	After 21days	After 28 days	After 35 days	After 42 days
1.	Tonsillitis, chest pain	ASO-titre 300, ESR-35mm	No change	No change	ASO-T. 200 ESR-20mm	ASO-T. 200 ESR-10mm	Normal	"
2.	Tonsillitis, arthritis, carditis	ASO-titre 600, ESR-40mm	ASO-T. 400 ESR-26mm	ASO-T. 300 ESR-20mm	ASO-T. 250 ESR-14mm	ASO-T. 200 ESR-8mm	Normal	Normal
3.	Tonsiilitis, chest pain, shifting pain	ASO titre-500, ESR-39mm	No change	No change	No change	No change	No change	No change
4.	Shifting pain arthritis breathlessness	ASO-T. 300, ESR-40mm CRP Positive	No change	No change	No change	No change	No change	No change
5.	Tonsillitis, arthritis carditis	ESR-35mm, ASO-T. 400	No change	ASO-T. 300 ESR-25mm	ASO-T. 300 ESR-18mm	ASO-T. 200 ESR-8mm	Normal	Normal
6.	Tonsillitis, joint pain ankle pain	ESR-35mm, ASO-T. 350	ASO-T. 300 ESR-23mm	ESR-16mm ASO-T. 250	ESR-16mm ASO-T. 200	ESR-8mm ASO-T. 200	Normal	Normal
7.	Tonsillitis, chest pain, shifting pain	ASO titre-400, ESR-20mm CRP Negative	ASO-T. 400 ESR-20mm	ASO-T. 400 ESR-20mm	ASO-T. 300 ESR-15mm	ASO-T. 200 ESR-10mm	Normal	Normal
8.	Tonsillitis, chest pain, shifting pain	ESR-30mm, ASO-T. 600	No change	ESR-15mm ASO-T. 300	ESR-10mm ASO-T. 200	Normal	Normal	Normal
9.	Tonsillitis, arthritis carditis	ASO titre-200, ESR-25mm, ECG-Prolonged p-r interval	No change	No change	No change	No change	No change	No change
10.	Tonsillitis, arthritis shifting pain	ESR-20mm, ASO-T. 500	ESR-16mm ASO-T. 350	ESR-10mm ASO-T. 350	ESR-10mm ASO-T. 200	Normal	Normal	Normal
11.	Carditis, tonsillitis weakness	ASO titre-400, ESR-25mm	No changes	No changes	No changes	No changes	No changes	No changes
12.	Tonsillitis, carditis, shifting pain	ASO titre-500, ESR-20mm	No changes	No changes	No changes	No changes	No changes	"
13.	Breathlessness, toncilitis, carditis	ASO titre-400, ESR-30mm	No changes	No changes	No changes	No changes	No changes	"
14.	Tonsillitis, carditis, arthritis	ESR-20mm, ASO-T. 400	ESR-20mm ASO-T. 300	ESR-16mm ASO-T. 250	ESR-10mm ASO-T. 200	ESR-10mm ASO-T. 200	Normal	Normal
15.	Shifting joint pain, tonsillitis, carditis	ESR-25mm, ASO-T. 600 CRP-positive	ESR-20mm ASO-T. 400	ESR-15mm ASO-T. 300	ESR-15mm ASO-T. 300	Normal	Normal	Normal
16.	Tonsillitis, arthritis, shifting joint pain	ESR-40mm, ASO-T. 800	ESR-26mm ASO-T. 400	ESR-19mm ASO-T. 300	ESR-15mm ASO-T. 200	ESR-12mm ASO-T. 200	Normal	Normal
17.	Arthritis, carditis, shifting big joints pain	ASO-T. 400, ESR-30mm	No change	No change	No change	No change	No change	No change
18.	Tonsillitis, carditis	ASO-T. 500, ESR-25mm	No changes	No changes	No changes	No changes	No changes	"
19.	Tonsillitis, shifting pain, carditis	ESR-40mm, ASO-T. 500	No change	ESR-30mm ASO-T. 400	ESR-20mm ASO-T. 300	ESR-10mm ASO-T. 200	Normal	Normal
20.	Tonsillitis, rheumatic nodules, shifting pain	ASO-T. 400, ESR-25mm	No change	No change	No change	No change	No change	No change

Appendix table 8 Effect of Placebo Drugs (Neutral sugar) on patients suffering from rheumatics fever

Sl. No.	Signs and Symptoms	Pathological Investigation	Results					
			After 7 days	21	After 21days	After 28 days	After 35 days	After 42 days
1.	Tonsillitis chest pain arthritis	ASO-titre 500, ESR-35mm	No change	No change	No change	No change	No change	No change
2.	Tonsillitis, carditis	ASO- title 300, ESR-40mm	No change	No change	No change	No change	No change	No change
3.	Chest pain, shifting joint pain	ASO titre-500, ESR-30mm	No change	No change	No change	No change	No change	No change
4.	Shifting arthritis, tonsillitis, Breathlessness	ASO-title 300 ESR-25mm CRP Positive	No change	No change	No change	No change	No change	No change
5.	Tonsillitis, carditis joint pain and inflamm	ESR-34mm, ASO-T. 400	No change	No change	No change	No change	No change	No change
6.	Tonsillitis, ankle pain, arthritis	ESR-30mm, ASO-T. 350	ESR-23, ASO-275	No change	No change	No change	No change	No change
7.	Tonsillitis, chest pain, rheumatic nodules	ASO titre-500, ESR-20mm CRP Negative	No change	No change	No change	No change	No change	No change
8.	Tonsillitis, carditis, shifting pain	ESR-30mm, ASO-T. 400	No change	No change	No change	No change	No change	No change
9.	Tonsillitis, arthritis sydenham choeria shifting pain	ASO titre-200, ESR-25mm ECG-Prolonged p-r interval	No change	No change	No change	No change	No change	No change
10.	Tonsillitis, arthritis	ESR-20mm, ASO-T. 500	No change	No change	No change	No change	No change	No change
11.	Carditis, tonsillitis	ASO titre-500, ESR-25	No changes	No changes	No changes	No changes	No changes	No changes
12.	Tonsillitis shifting pain weakness	ASO titre- 400 ESR-20mm	No changes	No changes	No changes	No changes	No changes	"
13.	Breathlessness tonsillitis, carditis	ASO titre-700, ESR-30mm	No changes	No changes	No changes	No changes	No changes	"
14.	Tonsillitis, carditis, arthritis	ESR-20mm, ASO-T. 700	No change	No change	No change	No change	No change	No change
15.	Shifting arthritis, tonsillitis, carditis	ESR-25mm, ASO-T. 500 CRP-positive	No change	No change	No change	No change	No change	No change
16.	Tonsillitis, carditis, arthritis, shifting joint pain	ESR-40mm, ASO-T. 600	No change	No change	No change	No change	No change	No change
17.	Arthritis, carditis shifting pain	ASO-T. 400, ESR-33mm	No change	No change	No change	No change	No change	No change
18.	Tonsillitis, Carditis	ASO-T. 400, ESR-20mm	No changes	No changes	No changes	No changes	No changes	"
19.	Tonsillitis, shifting big joints pain, carditis	ESR-40mm, ASO-T. 600	No change	No change	No change	No change	No change	No change
20.	Tonsillitis, rheumatic nodules	ASO-T. 400, ESR-25mm	No change	No change	No change	No change	No change	No change

Appendix table 8 Effect of Placebo Drugs (Neutral sugar) on patients suffering from rheumatics fever

Sl. No.	Signs and Symptoms	Pathological Investigation	Results					
			After 7 days	21	After 21days	After 28 days	After 35 days	After 42 days
1.	Tonsillitis chest pain arthritis	ASO-titre 500, ESR-35mm	No change	No change	No change	No change	No change	No change
2.	Tonsillitis, carditis	ASO- title 300, ESR-40mm	No change	No change	No change	No change	No change	No change
3.	Chest pain, shifting joint pain	ASO titre-500, ESR-30mm	No change	No change	No change	No change	No change	No change
4.	Shifting arthritis, tonsillitis, Breathlessness	ASO-title 300 ESR-25mm CRP Positive	No change	No change	No change	No change	No change	No change
5.	Tonsillitis, carditis joint pain and inflamm	ESR-34mm, ASO-T. 400	No change	No change	No change	No change	No change	No change
6.	Tonsillitis, ankle pain, arthritis	ESR-30mm, ASO-T. 350	ESR-23, ASO-275	No change	No change	No change	No change	No change
7.	Tonsillitis, chest pain, rheumatic nodules	ASO titre-500, ESR-20mm CRP Negative	No change	No change	No change	No change	No change	No change
8.	Tonsillitis, carditis, shifting pain	ESR-30mm, ASO-T. 400	No change	No change	No change	No change	No change	No change
9.	Tonsillitis, arthritis sydenham choeria shifting pain	ASO titre-200, ESR-25mm ECG-Prolonged p-r interval	No change	No change	No change	No change	No change	No change
10.	Tonsillitis, arthritis	ESR-20mm, ASO-T. 500	No change	No change	No change	No change	No change	No change
11.	Carditis, tonsillitis	ASO titre-500, ESR-25	No changes	No changes	No changes	No changes	No changes	No changes
12.	Tonsillitis shifting pain weakness	ASO titre- 400 ESR-20mm	No changes	No changes	No changes	No changes	No changes	"
13.	Breathlessness tonsillitis, carditis	ASO titre-700, ESR-30mm	No changes	No changes	No changes	No changes	No changes	"
14.	Tonsillitis, carditis, arthritis	ESR-20mm, ASO-T. 700	No change	No change	No change	No change	No change	No change
15.	Shifting arthritis, tonsillitis, carditis	ESR-25mm, ASO-T. 500 CRP-positive	No change	No change	No change	No change	No change	No change
16.	Tonsillitis, carditis, arthritis, shifting joint pain	ESR-40mm, ASO-T. 600	No change	No change	No change	No change	No change	No change
17.	Arthritis, carditis shifting pain	ASO-T. 400, ESR-33mm	No change	No change	No change	No change	No change	No change
18.	Tonsillitis, Carditis	ASO-T. 400, ESR-20mm	No changes	No changes	No changes	No changes	No changes	"
19.	Tonsillitis, shifting big joints pain, carditis	ESR-40mm, ASO-T. 600	No change	No change	No change	No change	No change	No change
20.	Tonsillitis, rheumatic nodules	ASO-T. 400, ESR-25mm	No change	No change	No change	No change	No change	No change