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CONTENTS

Detection of dengue virus RNA from pericardial and ascitic fluids by reverse transcriptase polymerase chain reaction (RT-PCR)	1
<i>Khin Saw Aye, Min Thein, Aye Aye Win, Mu Mu Shwe, Tin Tin Han, Thazin Myint, Khin Than Maw, Kyaw Zin Wai & Kyu Kyu Khin</i>	
The antimicrobial activities of the extracts of <i>Terminalia catappa</i> Linn. (Banda) on some pathogenic microorganisms.....	6
<i>Khin Khin Aye, Thet Thet Mar, Zaw Myint, Mie Mie Nwe, Khin Nwe Oo & Lwin Zar Maw</i>	
Diagnostic accuracy of DCIP test (dichlorophenol indolphenol precipitation) and NESTROFT (Naked Eye Single Tube Red-cell Osmotic Fragility Test) in haemoglobin E screening.....	10
<i>Aung Kyaw Kyaw, Tin Tin Thein, Zaw Win Tun, Khin Moe Aung, Saw Myat Thwe, Mie Mie Lwin, Su Su Hlaing & Ne Win</i>	
The burden of caregiving in caregivers of stroke patients attending the Out-patient Follow-up Clinic of Neuromedical Unit, Yangon General Hospital.....	15
<i>Han Win, Thant Zin Soe, Yae Chan, Myat Myat Thu & Myo Myo Kyaw</i>	
Isolation and identification of Japanese encephalitis virus from piglets in Dike Oo pig farm.....	21
<i>Aung Zaw Latt, Hlaing Myat Thu, Kay Thi Aye, Khin Sandar Aye, Khin Khin Oo, Hla Myo Thu & Sutee Yoksan</i>	
Detection of influenza and parainfluenza viruses in children with acute respiratory infection attending Yangon Children Hospital.....	26
<i>Htin Lin, Hlaing Myat Thu, Kyu Kyu Khin, Khin Thet Wai, Win Mar Oo, Khin Mar Aye, Nila Zaw & Hay Mar Win</i>	
Acute and subacute toxicity of <i>Millingtonia hortensis</i> Linn. f. (Aykayit) leaves in animal model.....	32
<i>Khine Khine Lwin, Mu Mu Sein Myint, May Aye Than, Min Min Myint Thu, Thaung Hla, Khin Tar Yar Myint, Aung Myint & Ei Ei Soe</i>	
Determination of radionuclide level in cigarette available in Myanmar.....	38
<i>Win Thaw Tar Lwin, Tin Oo, Tin Myo Myo Win, Htet Nandar Aung, Su Mon, Ni Ni Than, Thin Thin War, Si Thu Soe Naing & Ni Ni Win</i>	

Association between the use of insecticide-treated nets (ITNs) and parasitaemia and presence of malaria antibody in Thanbyuzayat Township, Mon State.....	44
<i>Khin Myo Aye, Ye Htut, Myat Phone Kyaw, Khin Thet Wai, Myat Htut Nyunt, Thura, Soe Soe Han, Ni Ni Zaw & Moe Thida</i>	
Establishment and evaluation of in-house one stage prothrombin time test.....	51
<i>Myat Mon Oo, Moh Moh Htun, Kyaw Soe, Minn Minn Myint Thu, Kyaw Oo, Tin Tin San, Kyaw Kyaw Aung, Khin Khin Aye & Win Mar</i>	
The diuretic effect of <i>Hydrocotyle umbellata</i> Linn. on rat model.....	57
<i>May Aye Than, Than Than Lwin, Mu Mu Sein Myint, Mar Mar Myint, San San Myint, Aung Aung Maw, Nu Nu Win & Ei Ei Soe</i>	
Short Report: Coverage and side-effects of oral cholera (Dukoral) vaccine in Department of Medical Research (Lower Myanmar).....	63
<i>Khin Nwe Oo, Wah Wah Aung, Hlaing Myat Thu, Moh Moh Htun, Khin Saw Aye, Myat Phone Kyaw, Zaw Myint & Khin Thet Wai</i>	

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**Detection of dengue virus RNA from pericardial and ascitic fluids
by reverse transcriptase polymerase chain reaction (RT-PCR)**

**Khin Saw Aye, *Min Thein, *Aye Aye Win, *Mu Mu Shwe, *Tin Tin Han,
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A total of 7 fatal cases of clinically suspected dengue shock syndrome (DSS) was autopsied at Yangon Children Hospital in 2010 to identify dengue viral antigen and antibody from aspirated cardiac blood and dengue virus RNA from pericardial and ascitic fluids. The ages of the children were varied from 7 months to 7 years. Six-female and only one-male cases were collected. NS1 dengue viral antigen and viral antibodies of IgG and IgM were determined by SD Dengue Duo test. Dengue virus RNAs were detected by reverse transcriptase polymerase chain reaction (RT-PCR). Results of dengue viral antigen and antibodies showed that four cases were both IgG and IgM positive and one case was NS1 and IgM positive. NS1 and both antibodies were positive in one case and negative in one case. Out of 7 cases, 3 cases were RT-PCR positive from body fluids. Dengue virus serotype 2 was found in two cases, one in ascitic fluid and one in pericardial fluid. Dengue virus serotype 1 was found in one case of ascitic fluid. These findings are the part of pathology study on DSS autopsy cases and will be continued by detection of dengue virus, viral antigens, viral RNA extraction from paraffin embedded tissues and cryo-tissues and RT-PCR from fresh frozen tissues of main organs.

INTRODUCTION

Dengue is a major public health problem in tropical and subtropical countries [1, 2]. Each of the four serotypes of dengue virus (DENV-1 to DENV-4) is able to cause disease. Clinically apparent disease varies in severity from mild undifferentiated fever through to more severe syndromes, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF is a vasculopathy characterized by capillary leakage and haematological deregulation, which in severe cases can result in life-threatening hypovolemic shock (DSS). There are no licensed vaccines or specific therapies for dengue, and patient management relies on good supportive care.

There are several reasons why early and accurate diagnosis of dengue is important.

First, an early and accurate diagnosis can assist in patient management by directing clinical attention to the appearance of major warning signs of severe or even life-threatening complications, e.g. rapidly rising hematocrit, poor peripheral perfusion. Second, an accurate dengue diagnosis prevents unnecessary and possibly expensive antibiotic usage. Third, prompt diagnosis of index cases can facilitate vector control activities in the community so as to mitigate further transmission. Lastly, the expanded use of accurate dengue diagnostics provides important data on the epidemiology and health burden of dengue and in doing so can inform and guide public health policy, particularly as dengue vaccines and antivirals make their way through development pipelines. Commercial ELISA tests that detect the DENV NS1 protein in plasma/sera have provided a new avenue for

diagnosing dengue [3-10]. The detection of NS1 on rapid lateral flow point-of-care tests offers an even faster route to a presumptive dengue diagnosis [11]. As more point of care rapid diagnostic tests (RDT) for dengue, particularly those targeting NS1, reaches the marketplace their prices will likely drop to the point they are affordable for use in even resource limited health-care settings. The purpose of the current study was to confirm the diagnosis of DHF by using commercially available, dengue RDTs (SD Dengue Duo) in aspirated cardiac blood and RT-PCR for identification of dengue viral serotype from pericardial fluid and ascitic fluid.

The polymerase chain reaction (PCR) technique has been applied widely for the rapid and sensitive detection of many infectious agents including dengue viruses. A number of reverse transcription PCR (RT-PCR) procedures to detect and to identify dengue serotypes in clinical specimens have been reported (Deubel, *et al.*, 1990; Henchal, *et al.*, 1991; Morita, *et al.*, 1991; Lanciotti, *et al.*, 1992; Chang, *et al.*, 1994; Seah, *et al.*, 1995a,b; Yenchitso-manus, *et al.*, 1996; Meiyu, *et al.*, 1997; Sudiro, *et al.*, 1998; Harris, *et al.*, 1998; Laue, *et al.*, 1999; Houn, *et al.*, 2001; Callahan, *et al.*, 2001).

These PCR methods vary somewhat in terms of gene regions of the genome amplified, methods to detect the RT-PCR products, and methods of virus typing. RT-PCR for dengue serotypes was performed from clinical samples of blood, saliva, urine and tissues but there were no findings from ascitic and pericardial fluids.

MATERIALS AND METHODS

Patients

Seven autopsies of clinically suspected DHF/DSS cases were collected following the designated protocol, at Yangon Children Hospital (YCH) from April to August 2010. Autopsies were conducted as soon as possible after death in order to minimize autolysis

and post-mortem artifacts. Subjects were placed in the mortuary cold room of YCH, the internal temperature of which was kept at 4-8°C. Age, sex, date of onset of fever, date of hospitalization and date of death were recorded for each study subject. During autopsy, gross observations, weight and measurements of each organ were also recorded.

Ethical consideration

Ethical clearance was approved by Institutional Ethical Review Committee, Department of Medical Research (LM).

Sample collection

Autopsy tissues were collected from various organs from each subject, including bone marrow, lymph nodes, heart, lungs, stomach, small and large intestine, spleen, diaphragm, liver, gall bladder, adrenals, kidney and brain. Collection of body fluids such as cardiac blood aspiration, measuring the amount of pericardial, pleural and ascities fluids were done. Fresh frozen tissues and body fluids were kept in dry ice during autopsy and later transported to the laboratory of DMR (LM) where samples were stored in -80°C.

Dengue Duo test

SD BIOLINE Dengue Duo rapid tests were performed from serum of aspirated cardiac blood according to the manufacturer's instructions.

The brief descriptions of the test procedure for SD BIOLINE Dengue NS1 Ag were: the test device was removed from the foil pouch, and placed it on a flat and dry surface, then 3 drops (about 100 µl) of serum with a disposable dropper were added into the sample well, test result was interpreted at 15-20 minutes. Procedures of the test for SD BIOLINE Dengue IgG/ IgM were: all kit components and specimen were allowed to keep at room temperature prior to testing, removed the test from foil pouch and placed it on a flat, dry surface, then 10 µl of specimen with capillary pipette were added into the square sample well marked "S", and four

drops (about 90-120 µl) of assay diluent were added to the assay well round shaped. The test was interpreted at 15-20 minutes. For interpretation of the test, the presence of each once color line within the result window indicates negative result. The presence of two color lines ('T' band and 'C' band) within the result indicates a positive result. If the color line is not visible within the result window after performing the test, the result is considered invalid. It is recommended that the specimen be retested.

Extraction of viral RNA

Dengue viral RNA was extracted from pericardial and ascitic fluids with QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Oligonucleotide primers and RT-PCR

The specific DNA primers were designed to cover E region of dengue virus and these antisense primers were tagged with SP6 or T7 bacteriophage promoter at 5'end. To amplify PCR product of all four dengue serotypes, the following four oligonucleotide primer pairs specific for each serotype were used (Table 1). This RT-PCR used the superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) to synthesize the first strand cDNA with dengue specific anti-sense primers SP6D1R, T7D2R, SP6D3R and T7D4R for Den1, Den2, Den3 and Den4 serotype, respectively.

Table 1. PCR primers for amplification of the four serotypes of dengue virus

Primer	Sequences	Product size (bp)
D1L	5'-GGG GCT TCA ACA TCC CAA GAG -3'	174
SP6D1R	5'-ATT TAG GTG ACA CTA TAG ACC AGA CGT TTG GAT TTC TGT CG-3'	
D2L	5'-ATC CAA ATG TCA TCA GGA AAC-3'	205
T7D2R	5'-TAA TAC GAC TCA CTA TAG GAG AGC CGT CCC CTT CAT ATT-3'	
D3L	5'-CAA TGT GCTTGA ATA CCT TTG T-3'	228
SP6D3R	5'-ATT TAG GTG ACA CTA TAG AAG CCT CAA TGT TGA CAG GCT CC-3'	
D4L	5'-GGA CAA CAG TGG TGA AAG TCA-3'	162
T7D4R	5'-TAA TAC GAC TCA CTA TAG GGG TTA CAC TGT TGG TAT TCT CA-3'	

The PCR product was then amplified from cDNA by using primers showed in Table 1. Each PCR cycle consisted of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. PCR products were electrophoresed on 1.5% agarose gels, and visualized by ethidium bromide staining.

RESULTS

Patients and autopsies

Autopsy specimens from 7 patients were available for the study. Clinical and laboratory data were summarized in the Table 2.

Table 2. Clinical and laboratory data of autopsy cases

Age/ Sex	DOA	DOE	H. stay (day)	A. date	ICT result	PCR result DV serotype
7 yrs /F	7-4	8-4	1	8-4	IgG & M	(-)ve
7 ms/ F	11-5	13-5	3	14-5	NS1, Ig M	(-)ve
7 yrs/F	13-5	14-5	1	14-5	IgG & M	DV-2
1.5 yrs/F	23-6	25-6	3	25-6	NS1, IgG & M	(-)ve
1.6 yrs/F	24-6	24-6	1	25-6	IgG & M	(-)ve
2 yrs//M	28-6	29-6	2	29-6	IgG & M	DV-2
5 yrs/F	20-8	20-8	1	20-8	(-) ve	DV-1

DOA=Date of admission DOE=Date of expired
H. stay= Hospital stay A. date= Autopsy date
ICT=Immunochromatography test (Dengue DUO test)
DV=Dengue virus, M=Male, F=Female
DOA & DOE were in 2010.

The ages of the patients varied from 7 months to 7 years. Six were females and only 1 was male children. All the patients went through two clinical stages; febrile and shock, before succumbing to the disease. Hospital stay was varied from 1 to 3 days. The patients died within 24 hours (4 cases), 48 hours (1 case) and 3 days (2 cases). All cases (100%) presented with hepatomegaly and ascites, however, they had neither jaundice nor splenomegaly.

Immunochromatography (ICT)

Dengue DUO results of dengue viral antigen and antibodies showed that four cases were

both IgG and IgM positive and one case was NS1 and IgM positive. NS1 and both antibodies were positive in one case (Fig.1) and negative in one case.

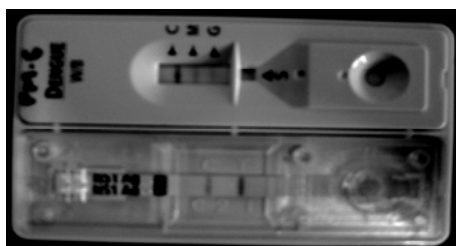


Fig. 1. Dengue DUO immunochromatography (ICT) test

RT-PCR results

Out of 7 cases, 3 cases were RT-PCR positive from body fluids. Dengue virus serotype 2 was found in two cases, one in ascitic fluid and one in pericardial fluid. Dengue virus serotype 1 was found in one case of ascitic fluid.

DISCUSSION

The emergence of dengue infection has become an important issue of great public health concern due to the recent incursion and continued transmission of dengue virus. Sporadic cases of DHF and DSS in both adults and children, require effective surveillance programs to identify areas of transmission and to enable the immediate implementation of procedures aimed at reducing transmission to humans, including vector control and vaccination. Identification of dengue virus from clinical specimens is also crucial role in early diagnosis of DF. In this study, RT-PCR assay in body fluids was successfully developed by a nested and multiplex technique that simultaneously detected four different dengue serotypes, including DV1, DV2, DV3 and DV4, in a one-tube reaction. This assay has proven to be specific and sensitive and decreases the risks of laboratory contamination [12].

Viral RNA is labile and is subject to rapid degradation by the presence of RNase and by duration of storage and/or storage

conditions. Consequently, the use of RNA extracted from virus-infected culture material is often unreliable, in particular among different laboratories. So we have carefully arranged sample collection, preservation, transportation and storage. Autopsy was performed within 5 hours after the patient expired while the body was kept in a cool room. Under sterile conditions pericardial and ascitic fluids were aspirated by disposable syringes, kept and transported with sterile tubes in dry ice and stored at -80°C up to test. RT-PCR for dengue serotypes were performed and results obtained from clinical samples of blood, saliva, urine and tissues [13-16] but none obtained from ascitic and pericardial fluids before. The study on liver, lung, spleen, brain, kidney, bone marrow and heart of 29 suspected fatal dengue cases, dengue virus (DENV) were isolated from these samples and inoculated into C6/36 cells and identified DV by nested RT-PCR to detect viral RNA [14].

Autopsy tissues from 18 Myanmar children believed to have died of dengue hemorrhagic fever were stored in -70°C for 20 years and were tested for the presence of dengue virus RNA by reverse transcription-polymerase chain reaction (RT-PCR) [16]. Dengue RNA was detected in serum, urine and saliva samples of 2 patients and suggested that urine and saliva could be used as alternative samples for early diagnosis of dengue virus infection when blood samples are difficult to obtain, e.g., in newborns and patients with hemorrhagic syndromes [13]. In conclusion, we have established RT-PCR procedures that have potential to be used in a routine laboratory to detect and identify dengue virus serotypes. These findings are also helpful for future studies on localization of dengue virus in human tissues.

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encouragements to our research. We are obliged to Pediatric Dengue Vaccine Initiative (PDVI)/International Vaccine Institute (IVI) for financial support.

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**The antimicrobial activities of the extracts of
Terminalia catappa Linn. (Banda) on some pathogenic microorganisms**

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The antimicrobial activity of petroleum ether, chloroform and methanolic extracts of dried roots of *Terminalia catappa* Linn. was tested on *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio cholerae*, *Salmonella typhi* and *Shigella dysenteriae* by agar disc diffusion method. The minimum inhibitory concentration (MIC) of the plant extracts were determined by tube serial dilution method. The chloroform extract and methanolic extract of *Terminalia catappa* Linn. showed prominent antimicrobial activity, while petroleum ether extract showed no antimicrobial activity on tested bacteria. The MIC of methanolic extract exhibited 0.065 mg/ml on *Vibrio cholerae* 01 and 0.125 mg/ml on other tested bacteria.

INTRODUCTION

Infectious diseases are the leading cause of death worldwide. Antibiotic resistance has become a global concern. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multi-drug-resistant pathogens. Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases [1]. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity.

Terminalia catappa Linn. family Combretaceae, is commonly known as Banda

(or) India almond (or) tropical almond and Myanmar name (Burmese Script)-ဗာဒံတော. It is widely grown in tropical regions of the world as an ornamental tree, grown for the deep shade its large leaves provide. Trees of this genus are known especially as a source of secondary metabolites, e.g. cyclic triterpenes and their derivatives, flavonoids, tannins, and other aromatics. Some of these substances have antifungal, antibacterial, anticancer and hepatoprotective indications.

The bark is used as mild diuretic and cardiogenic. In Taiwan, fallen leaves are used to treat liver diseases. In Suriname, a tea made from the leaves is prescribed against dysentery and diarrhea. The leaves contain agents for prevention of cancers and antioxidants as well as anti-clastogenic characteristics. Fruit contains antiasthmatic compound and antidiabetic activity [2].

Evidently, there are limited studies in Myanmar on the antimicrobial properties of *Terminalia catappa* Linn. This study was carried out to determine the *in vitro* antimicrobial activity of the roots of

Terminalia catappa Linn. against some pathogenic microorganisms that cause common infectious diseases in Myanmar.

Objectives

- To determine the antimicrobial activity of the petroleum ether, chloroform and methanolic extracts of the roots of *Terminalia catappa* Linn. on some bacteria
- To find out the minimum inhibitory concentration (MIC) of these extracts

MATERIALS AND METHODS

This was a laboratory-based study carried out at the Department of Medical Research (Lower Myanmar). The roots of the *Terminalia catappa* Linn. (Banda) tested were obtained from Yangon area. The samples were collected from May to September, 2008. Strains of bacteria used in this study were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio cholerae*, *Salmonella typhi* and *Shigella dysenteriae*. The bacterial cultures were obtained from Bacteriology Research Division, Department of Medical Research (Lower Myanmar). Petroleum ether, chloroform and methanol were used as solvents.

Preparation of extract

The collected roots were cleaned and dried under shade for 7 days and crushed into coarse powder. The powder thus obtained (5 gm) was extracted with different solvents (petroleum ether, chloroform and methanol) successively. The coarse powder was first extracted with petroleum ether (60-80°C) using Soxhlet apparatus. The extraction was continued for 10 hours. After the complete extraction, these extracts were filtered and kept in oven at 40°C for 24 hours to evaporate the alcohol from it. Dark brown and greenish black residues were obtained. The rest was then successively extracted with chloroform and methanol. The dried petroleum ether, chloroform and methanolic extracts were refrigerated until use.

Two milligrams of these residues were dissolved in 1ml of solvent and this solution was used as the extract.

Antimicrobial activity determination

Escherichia coli, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio cholerae*, *Salmonella typhi* and *Shigella dysenteriae*. were used for antimicrobial tests.

Agar disc diffusion method was used to determine the zone of inhibition of three extracts of *Terminalia catappa* [3]. Filter paper discs of 7 mm in diameter were punched from No. 3 Whatman filter papers. The discs were sterilized by autoclaving for one hour, followed by dry heat at 60°C for one hour. The discs were impregnated with concentrated extracts (1 mg/disc) and allowed to be dried at room temperature. The bacterial broth suspensions were streaked evenly three times onto the surface of the nutrient agar with sterile cotton swab. After drying the inoculums (5 mins), the dried discs were placed on the agar. Filter paper discs with solvents (petroleum ether, chloroform and methanol) were used as the solvent control. Standard antibiotic discs (tetracycline-10 µg, norfloxacin-10 µg, ciprofloxacin-5 µg, gentamicin-10 µg) were used as controls. The plates were incubated immediately or within 30 mins after inoculation. After overnight incubation, inhibition zone diameters including 7 mm discs were measured. The experiment was repeated for three times.

MIC determination

The minimum inhibitory concentration (MIC) of the plant extracts was determined by tube serial dilution method. In brief, 1 ml of sterile nutrient broth was used to determine the minimum inhibitory concentration (MIC) of the extracts. Stock solutions of chloroform and methanolic extracts, having 2 mg/ml conc. were used. Sterile dilutions in nutrient broth were done by using the stock solution to get the lowest conc. of 0.0078125 mg/ml. One milliliter of nutrient broth was pipetted into tube 2 through 10 for *Escherichia coli* organisms

to be tested. The nutrient broth test tubes were autoclaved for 15 min. The extract solution (1ml) was added into tubes 1 and 2. The contents of tube 2 were mixed well and 1ml was transferred to tube 3. This procedure was continued till tube 9, discarding 1ml from tube 9. Tube 10 served as the positive control (broth plus inoculum). For ethanol extract, the additional tube with broth plus ethanol was included for solvent control. The bacterial broth suspension of 200 μ l was then transferred into the test tubes (broth, broth plus extract, and extract solution). Then, the test tubes were incubated at 37°C for 24 hrs. After incubation, the last tube with no growth of the organism was taken as MIC expressed in mg/ml of the extract. The lowest concentration inhibiting microbial growth was considered to be MIC. The experiment was repeated three times.

Bacteriostatic or bactericidal determination

To determine whether the extract was bacteriostatic or bactericidal, the test tube serial dilution method was used. Tubes with the highest dilution showing no visible turbidity were taken as bacteriostatic concentrations. To measure bactericidal concentration, broth cultures from tubes were subcultured onto agar plates and allowed to dry at room temperature. The plates were incubated at 37°C for 24 hrs. After incubation, the highest dilution showing no growth was taken as the bactericidal concentration.

RESULTS AND DISCUSSION

The chloroform extract as well as methanolic extract exhibited prominent antimicrobial activity against all seven strains of microorganisms used in this study (Fig.1). The chloroform extract showed prominent antimicrobial activity against *S. aureus*, *Bacillus cereus*, *Vibrio cholerae* O1, *Salmonella typhi* and *Shigella dysenteriae*. Methanolic extract showed a prominent antimicrobial activity against all tested microorganisms (Table 1). None of the

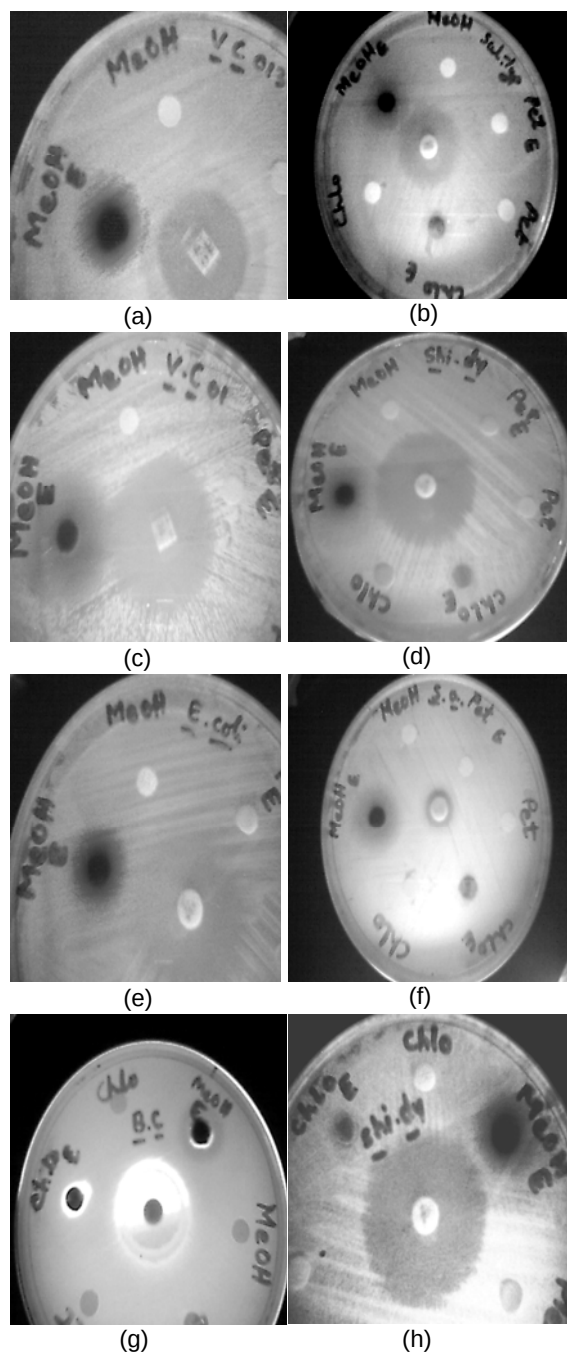


Fig. 1. The antimicrobial activity of extracts on (a) *Vibrio cholerae* O139 (b) *Salmonella typhi* (c) *Vibrio cholerae* O1 (d) *Shigella dysenteriae* (e) *Escherichia coli* (f) *Staphylococcus aureus* (g) *Bacillus cereus* (h) *Shigella dysenteriae*

solvent control exhibited antimicrobial activity.

Table 2 shows the average value of MIC in mg/ml of various extracts of *T. catappa* root used in this study. The MIC of chloroform extract against *Bacillus*

Table 1. The antimicrobial activity of different extracts of *Terminalia catappa* Linn.

Sr. No.	Tested bacteria	Average zone of inhibition (diameter in mm)		
		Petroleum ether extract	Chloroform extract	Methanolic extract
1	<i>Escherichia coli</i>	0	0	10
2	<i>Staphylococcus aureus</i>	0	10	17
3	<i>Bacillus cereus</i>	0	10	11
4	<i>Vibrio cholerae</i> O1	0	9	22
5	<i>Vibrio cholerae</i> O139	0	0	10
6	<i>Salmonella typhi</i>	0	9	12
7	<i>Shigella dysenteriae</i>	0	9	11

Table 2. The average minimum inhibitory concentration (MIC) in mg/ml of two extracts of *Terminalia catappa* Linn.

Sr. No.	Strain of microorganisms	MIC (mg/ml)	
		Chloroform extract	Methanol extract
1	<i>Escherichia coli</i>	Not tested	0.125
2	<i>Staphylococcus aureus</i>	0.5	0.125
3	<i>Bacillus cereus</i>	0.125	0.125
4	<i>Vibrio cholerae</i> O1	0.125	0.0625
5	<i>Vibrio cholerae</i> O139	Not tested	0.125
6	<i>Salmonella typhi</i>	0.125	0.125
7	<i>Shigella dysenteriae</i>	0.125	0.125

cereus, *Vibrio cholerae* O1, *Salmonella typhi* and *Shigella dysenteriae* were 0.125 mg/ml. The methanolic extract exhibited MIC 0.0625 mg/ml against *Vibrio cholerae* O1 and 0.125 mg/ml against other tested microorganisms. The petroleum ether extract showed no antimicrobial activity. The methanolic extract was more potent compared to the chloroform extract. The methanolic extract showed good antimicrobial activity against all tested microorganisms. Further research is needed to identify the active antimicrobial compounds of extracts which showed promising antimicrobial activities.

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Diagnostic accuracy of DCIP test (dichlorophenol indolphenol precipitation) and NESTROFT (Naked Eye Single Tube Red-cell Osmotic Fragility test) in haemoglobin E screening

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Various screening methods are used for detection of the HbE. It is important to find out the reliable test which is also cost effective in screening large population for the prevention of severe anaemia. This study was conducted to determine the accuracy of the dichlorophenol indolphenol precipitation (DCIP) test and NESROFT (Naked Eye Single Tube Red-cell Osmotic Fragility Test) for detection of Hb E disease. In a cross sectional, laboratory-based descriptive study, a total of 155 high school students were selected by simple random sampling and DCIP test and NESTROFT were employed as screening tools. Haemoglobin electrophoresis (iso-electric focusing method) was used as a confirmatory method for diagnosis of Hb E. The diagnostic accuracy parameters of the tests; sensitivity and specificity were 92%, 96.92% in DCIP test and 84%, 70% in NESTROFT. The positive predictive value and negative predictive value of DCIP test were 85.19% and 98.44% as well as 35% and 95.79% for the NESTROFT. When combined DCIP test and NESTROFT were used as the screening tools, they had more preferable sensitivity of 96%. Comparing the above result indicates that the combined method could be used as an effective preliminary screening among the Myanmar population.

INTRODUCTION

Haemoglobin E, HbE ($\alpha_2\beta_2^{26Glu-Lys}$), is one of the commonest types of abnormal unstable haemoglobin which occur due to the mutation in the β globin gene causing substitution of glutamic acid for lysine at position 26 of β globin gene. They mainly affect people who have originated from Southeast Asia. Most countries have an uneven distribution of the carrier rate of the abnormal haemoglobin. The prevalence of carrier is highest in Thailand, and the overall prevalence in Thailand and Myanmar is 14-15%. Researchers found the carrier rates for abnormal haemoglobin among Kayin pregnant mothers were beta thalassaemia trait (8.9%), alpha thalassaemia trait (6%) and Hb E (6%) [1]. By newborn cord blood screening in North Okkalapa General Hospital, HbF band alone

(83.12%), HbEF band (8.12%), HbFH band (7.48%) and HbEFH band (1.3%) were detected [2]. HbE thalassaemia can cause severe clinical manifestations of the disease when combined with one of the former abnormal genes and most are clinically asymptomatic (HbE trait). Health education, carrier screening and premarital counseling remain the best ways to reduce disease incidence with potentially significant financial savings and social and health benefits.

Various screening tools are used for detection of abnormal haemoglobin such as electronic blood cell counts, high performance liquid chromatography, mean corpuscular volume (MCV), Naked Eye Single Tube Red-cell Osmotic Fragility test (NESTROFT) and dichlorophenol indolphenol precipitation test (DCIP) [3, 4, 5]. It is important to evaluate the simple and

effective screening strategy for detection in large population. Screening for carriers of thalassaemia and sickle cell disorders was established in the developed countries. In Thailand, combined DCIP test and OFT are validated as screening tool. For detection of thalassaemia trait and haemoglobin E, the NESTROFT is the most reliable test of population screening in Myanmar. The purpose of this study was to determine the accuracy of the Naked Eye Single Tube Red-cell Osmotic Fragility test (NESTROFT) and dichlorophenol indolphenol precipitation test (DCIP) for detection of HbE.

MATERIALS AND METHODS

This field and laboratory-based, cross-sectional descriptive study was done at the Pathology Research Division, Department of Medical Research (Upper Myanmar) and National Health Laboratory, Department of Health. A total of 155 high school students within the age of 14 -19 years were selected by simple random sampling procedure.

Then, five mililiters of blood were taken after getting informed consents from their guardian after explaining the purpose, risks and benefits of the research. Both Naked Eye Single Tube Red-cell Osmotic fragility test (NESTROFT) and dichlorophenol indolphenol (DCIP) precipitation test were done as screening tools and haemoglobin electrophoresis by iso-electric focusing method (IEF) was used for confirmation of all samples. The principle of the NESTROFT was based on the limit of hypotonicity which the red cells withstand. This test was done by adding 20 microliters of fresh whole blood to two mililiters of 0.35% buffered saline in a test tube at room temperature and looking for cloudiness in twenty minutes.

For the DCIP test, KKU-DCIP Reagent Kit (Drew-Bio, Thailand, Co. Ltd) was used. The principle is that the amino replacement taken place at the $\alpha_1\beta_1$ junction that leads to the disassociation of dimmers into cor-

responding monomers and liberates reactive sulhydryl (-SH) group. The oxidation of sulhydryl group and DCIP dye results in precipitation of the haemoglobin E molecule. Twenty microliters of ethylene diamine tetrachloro acetic acid anti-coagulated whole blood and 2 mililiters of KKU-DCIP Reagent were mixed and incubated in waterbath preset at 37°C for 15 minutes. Then, twenty microliters of clearing solution were added to this test tube and stand for 2-3 minutes at room temperature.

After that, the result of precipitation was recorded and the turbid solution (precipitation) was taken as the test positive. Positive and negative control samples were tested by all methods of this study. Firstly, haemoglobin lysate was prepared and haemoglobin electrophoresis was run on polyacralamide gel by using iso-electric focusing method as the confirmation test. Data analysis was done by OpenEpi Version 2 software.

RESULTS

Among the 155 cases of high school students, 23 cases (14.8%) were Hb E carrier, Hb AE, and two cases (1.3%) were Hb E β thalassaemia (Hb EF). Not only 27 cases were positive on the screening tool, DCIP test, but also 60 cases were positive to NESTROFT in this study. If concurrent combined test was considered as screening program, 65 cases had given positive results (Table 1).

Table 1. Results of screening test and confirmation test

Total number of cases	DCIP test		NESTROFT		Combined		Confirmation test	
	+	-	+	-	+	-	+	-
155	27	128	60	95	65	90	25	130

+=Positive -=Negative

In spite of 27 cases showing positive result using DCIP as a single screening tool, only 23 samples had haemoglobin E pattern

against the electrophoresis reading and four subjects with positive DCIP test were normal haemoglobin pattern, Hb AA₂. Only two cases of HbE carriers showed negative result. The sensitivity and specificity of this test in using haemoglobin E screening were 92% and 96.92%, respectively. The accuracy of this test was 96.13%. The positive predictive value and the negative predictive value were 85.19% and 96.13%, respectively. Then, the likelihood ratio of a positive test was 29.9. By screening with the NESTROFT, 60 subjects had positive result. Only 21 subjects of positive NESTROFT had positive band in IEF test. Besides, four samples of haemoglobin E carrier (Hb AE) were negative in this screening. Therefore, NESTROFT was positive with 21/25 confirmed Hb E disease and 39/130 confirmed test negative samples. For that reason, the parameters of this screening test evaluation were sensitivity (84%), specificity (70%), positive predictive value (35%), negative predictive value (95.79%), diagnostic accuracy (72.26%) and likelihood ratio of a positive test (2.8).

Table 2. Diagnostic accuracy parameters of the screening tests

Test	Sensitivity	Specificity	Predictive value		Accuracy
			(+)	(-)	
DCIP test (%)	92	96.92	85.19	96.13	96.13
NESTROFT (%)	84	70	35	95.79	72.26
Combined test (%)	96	68.46	36.92	98.89	72.9

Statistics- OpenEpi software

Using above two tests as concurrent combined test, DCIP and NESTROFT, in preliminary screening for haemoglobin E, 65 cases were positive. Among these positive cases, 22 cases were positive on both tests and 38 cases showed positive with NESTROFT but not with DCIP test and five cases showed positive on DCIP test only and negative on NESTROFT. 24/65 cases (37%) of concurrent combined test positive were Hb E disease and 41/65 cases (63%) were normal. Therefore, the sensitivity, specificity, diagnostic accuracy, positive

predictive value, negative predictive value of this combined screening tool were 96%, 68.46%, 72.9%, 36.92%, 98.89%, respectively. The likelihood ratio of a positive test was also 3.04 (Table 2).

DISCUSSION

The carrier rates of haemoglobin E (Hb E trait) 14.8% and Hb E β thalassaemia (HbEF) 1.3% were detected in this study. Thus, the prevalence rate is similar to that of Thailand (14-15%) (1 in 9 Thailand births, HbE carrier). Then, one study described that the prevalence rate of haemoglobin E among Kayin pregnant mother was 6% [1]. One hospital-based study in Myanmar also found that 8.12% of newborn cord blood and 1.3% of cord blood had HbEF band and HbEFH band, respectively [2]. As a result of high prevalence of unstable haemoglobin, the consequent disability associated with this disorder becomes a burden to the health system and the whole population needs to be informed of their carrier status to prevent the severe thalassaemia. Screening of abnormal haemoglobin among the pregnant mother and reproductive-aged adult is important in a public health service. Because they can have an affected child due to the nature of disease which is genetic disorder and this approach reduces the number of affected births.

In this study, the diagnostic test evaluation parameters of NESTROFT were sensitivity (84%), specificity (70%), positive predictive value (35%) and negative predictive value (95.79%). One study regarding the validity of NESTROFT in Myanmar stated that positive predictive value and specificity of 100%, negative predictive value of 83.33% and sensitivity of 95.2% of the test [6]. A study on screening for the carriers of thalassemias and abnormal hemoglobins at the community level in Thailand described that sensitivity and specificity of Osmotic Fragility (OF) test were 89.5% and 93.3%, respectively [3]. Another study expressed that the diagnosis accuracy parameters of test were 69.12% sensitivity and

80% specificity [7]. Thus, the diagnostic accuracy parameters described that the precision of the test is not good and so there is a need to do the research regarding the reproducibility and precision of the test.

Regarding the accuracy of DCIP test, the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy were 92%, 96.92%, 85.19%, 98.44% and 96.13%, respectively, whereas a study in Thailand described that the sensitivity and specificity of DCIP test were 100% [3]. One study of reassessment of a simple chemical method using DCIP for screening for haemoglobin E described that the sensitivity and specificity of this test were 100% and 92%, respectively [8]. By using DCIP test as a screening tool for haemoglobin E among Thai pregnant women, the sensitivity and specificity of the screening test were 100% and 97.2%, respectively [9]. Therefore, the results were more or less similar to other studies.

Taking into consideration the result of this study, the concurrent combined DCIP test and NESTROFT showed the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of the test were 96%, 68.46%, 35%, 95.79% and 72.26%, respectively. Some researchers expressed that the combined OF and DCIP tests detected the carriers of the thalassemia, ie, alphathalassemia, beta-thalassemia, and hemoglobin E with a sensitivity of 100%, specificity of 87.1%, positive predictive value of 84.5%, and negative predictive value of 100%, which show more effectiveness than these values for the standard method based on red blood cell counts [10].

One study, to evaluate the simple screening strategy, using both tests as a preliminary screening tool, for the carriers of thalassaemia and haemoglobin E in the rural communities in Southeast Asia described the sensitivity and the negative predictive value of 100% and the specificity of 69.8% and the positive predictive value of 77.2% of the combine test [4]. The validation of osmotic fragility test and dichlorophenol indolphenol

precipitation test was also expressed via the sensitivity and specificity of 100% and 79.7%, respectively [11].

A study of screening for haemoglobin disorder among Thai population described that the combined osmotic fragility test and DCIP test is an effective method as a screening tool with the sensitivity of 100% and specificity of 97% [9]. The combination of DCIP test and mean corpuscular value (MCV<80fl) was found preferable to the combined DCIP and OF test because the combination of DCIP test and MCV yielding higher specificity results. The sensitivity and specificity for the combined DCIP and MCV were 99.43% as well as 98.93% and for the combined DCIP and OF test, the sensitivity was 99.43% and specificity was 79.29%, respectively [7].

According to the Table 2, combined test showed positive predictive value of 36.92% and highest positive predictive value of 85.19% expressed in DCIP test. However, the positive predictive value of the screening test, NESTROFT, was 35%. Thus, there is a need to find out the simple screening tool for detection of unstable haemoglobin. Regarding the precision of the two combined test, researchers expressed as the results assessed by two medical scientists were exactly the same with 93.3% accuracy in comparison to the confirmatory methods [11]. The screening system by local staff at three community hospitals found a sensitivity of 98.1-100% and a specificity of 65.4-88.4% with positive predictive values of 75.0-86.9% and negative predictive values of 98.1-100% [4].

By comparing the above results in this study, DCIP test had the highest accuracy and the most effectiveness for screening of Hb E in community.

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The burden of caregiving in caregivers of stroke patients attending the Out-patient Follow-up Clinic of Neuromedical Unit, Yangon General Hospital

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Stroke is one of the most disabling chronic diseases. The majority of stroke survivors live at home and are supported by informal caregivers. The aim of this study was to describe the level and nature of burden of caregiving experienced by caregivers of stroke patients, and to identify both patient's and caregiver's characteristics that influence the caregiver burden. A cross-sectional descriptive study was conducted at the Out-patient Follow-up Clinic of Neuromedical Unit, Yangon General Hospital during September 2008 to August 2009. The study subjects were 100 stroke patients with at least six months duration and their caregivers. The level and nature of burden of caregiving was assessed with Sense of Competence Questionnaire (SCQ). Mean total SCQ score of burden was 61.2 (score range of good to bad 27-108). The burden score concerning the consequences of involvement in care for personal life of caregivers (subscale 3 of SCQ) was higher (standardized mean 2.66) compared to other subscales (subscale 1=2.14, subscale 2=2.1). Higher level of burden is associated with patient age >65 years ($p=0.04$), disability ($p=0.01$), and handicap ($p=0.01$). Among the caregiver characteristics, higher level of burden is related to age >40 years ($p=0.04$), poor physical health ($p=0.04$) and poor mental health ($p=0.001$) status of caregivers. Advancing age in patients and caregivers, high dependency of patients, and poor health-related quality of life of caregivers identify caregivers at risk of high level of burden who require social support.

INTRODUCTION

Stroke is one of the most disabling chronic diseases [1]. The majority of stroke survivors live at home using long-term informal care which is provided by relatives and family members [2]. While these caregivers themselves have to cope with the devastating effects that stroke had on their partner, an increasing amount of demands is made on them. They need, for example, to provide emotional support or assist the patient in activities of daily living. Consequently, caregivers may experience unacceptably high levels of burden, leading to isolation and exhaustion [3]. Initial gains in rehabilitation are more effectively maintained if the family is healthy, involved, and supportive [4]. The burden of

caregiving can only be partly predicted from stroke severity or patient's dependency in performance of daily activities [5]. But, characteristics of caregivers themselves are also important, and include physical and psychosocial health problems [3, 6]. Knowledge of the relative contributions of various patient and caregiver characteristics to the level of the burden may be useful for identification of caregivers at risk and may enhance the understanding of how caregiver burden can be alleviated best, either by specific care for the patient or by providing support and relief for caregivers especially in resource-constrained settings.

Objectives

- To describe the level and nature of burden of caregiving as experienced by stroke patient's caregivers

- To identify the patient and caregiver characteristics that influence the caregiver burden

MATERIALS AND METHODS

Study design

A cross-sectional descriptive and hospital-based study was carried out.

Study area and study period

The study was carried out at Out-patient Follow-up Clinic of Neuromedical Unit, Yangon General Hospital during September 2008 to August 2009.

Study population

The study population consisted of currently non-institutionalized patients who had been hospitalized previously because of stroke at least six months earlier and their caregivers.

Working definitions

Stroke – defined by the World Health Organization as a condition characterized by rapidly developing symptoms and signs of a focal brain lesion, with symptoms lasting for more than 24 hours with no apparent cause other than that of vascular origin

Caregiver – most responsible person for caring the patient among family members

Sample size determination and sampling procedure

Assuming that proportion of caregivers who had the burden (p)=0.6, Z =1.96 at 95% CI, and precision (d)=0.1, required sample size was 92. Patients and their caregivers who were willing to give consent were recruited till the required sample size was accomplished. In this study, a total of 100 patients (and caregivers) were recruited.

Data collection

Clinical data on acute stroke were taken from the medical records. Patients were interviewed by trained research assistants using a structured questionnaire. If patients were not communicative because of cognitive, speech or language disorders,

proxy interviews were done with their caregivers. Data on caregivers themselves were collected by means of a self-report questionnaire. While the research assistants interviewed the patient, caregivers filled in this self-report questionnaire.

Data were collected on burden of caregiving and potentially explanatory factors of this burden. Explanatory factors were studied in terms of (1) background characteristics of patients (such as age, sex, monthly family income), clinical characteristics (type, and localization of stroke), and handicap (2) characteristics of caregivers: age, sex, education, relationship with the patient, and health-related quality of life (physical and mental health).

Characteristics of patients

Type of stroke (infarction or hemorrhage) and localization of stroke (left or right hemisphere) were assessed on clinical grounds and CT data. Disability in activities of daily living (ADL) was assessed with Barthel Index Score [7] and handicap with the modified Rankin Scale [8].

Characteristics of caregivers

Quality of life was assessed with Short Form 36 (SF-36), a widely used and validated generic health-related quality of life instrument. It comprised 8 subscales: physical functioning, role physical, bodily pain, general health, vitality, social function, role emotional and mental health. These 8 subscales were combined into 2 summary health status measures, physical health and mental health.

Burden of caregiving

The level and the specific nature of the burden of caregiving as experienced by partners of stroke patients were assessed with the Sense of Competence Questionnaire (SCQ). The SCQ was arranged into 3 subscales: (1) satisfaction with the impaired person as a recipient of care (7 items); (2) satisfaction with one's own performance as a caregiver (12 items); and (3) consequences of involvement in care for the personal life

of the caregiver (8 items). Each item was scored on 4-point rating scale. The burden score was determined by summation of the item scores; the total score ranges from 27 (no burden) to 108 (severe burden). Reliability of the total SCQ score was good (Chronbach's α coefficient=0.83; interclass correlation coefficient=0.93) [9].

Data analysis

Data analysis was done with SPSS version 13.0 software. The level and the specific nature of burden were described by the total mean and the subscale means of the SCQ. Since the SCQ contains 3 subscale with various numbers of items, standardized means were calculated for comparison of the subscale scores (standardized mean= mean subscale score/ number of subscale items). Factors influencing the caregiver burden were identified at a univariate level using Student's 't' test and ANOVA as appropriate.

Ethical consideration

This study was approved by the Institutional Ethical Review Committee, Department of Medical Research (Lower Myanmar).

RESULTS

Background characteristics of study population

Characteristics of patients

Median age of the patients was 65.5 years (range, 24 to 89 years), and most patients were males (55%). Forty-one patients (41%) had only primary education, and 63% were married. Median monthly family income was 60,000 kyats (range, 20000-500,000 kyats). There were 91 cases of cerebral infarcts and 9 intracerebral hemorrhages. As concerned with lesion location, 53 had left-hemisphere and 47 had right-hemisphere lesions. Altogether, 44 patients were severely disabled in ADL, whereas about two thirds of the patients (67%) were substantially handicapped in terms of care dependency.

Characteristics of caregivers

The median age of caregivers was 40 years (range 28 to 73 years), mostly females (75%). Twenty-five percent had only primary education, 62% were dependents, and 56% were married. Regarding the relationship to the patient, 51% were sons/daughters, 15% spouses, and 14% siblings.

Level and nature of burden of caregiving

Table 1 presents the level and nature of burden of caregiving as experienced by partners of stroke patients. The mean total SCQ score of burden was 61.2 (range, 39-79). The burden scores concerning the consequences of involvement in care for the personal life of the caregiver (subscale 3 of the SCQ) were higher (standardized mean= 2.66) compared with the scores on subscale 1 (standardized mean=2.14) and subscale 2 (standardized mean=2.1). The mean item score was 2.27.

Table 1. Level and nature of burden of caregiving assessed with the sense of competence questionnaire (SCQ)

Burden of caregiving, SCQ	Mean $\pm$ SD	Standardized mean*
Dissatisfaction with patient (Subscale 1; 7 items)	15.04 $\pm$ 2.69	2.14
Dissatisfaction with own performance (Subscale 2; 12 items)	24.90 $\pm$ 4.11	2.1
Consequences for personal life (Subscale 3; 8 items)	21.28 $\pm$ 2.7	2.66
Total level of burden	61.22 $\pm$ 7.99	2.27

Standardized mean=mean subscale score/ number of subscale items

Factors influencing the caregiver burden

Characteristics of patients

The univariate analysis showed that higher level of burden was associated with patient age more than 65 years ($p=0.04$). But there were no relationship between other socio-demographic and clinical characteristics, and caregiver's burden.

Patients' functional health problems in terms of disability in ADL ($p=0.01$), and handicap ($p=0.01$) also appeared to be associated with a higher burden among caregivers (Table 2).

Table 2. Differences in patient characteristics in relation to mean total score of caregivers' burden (based on sense of competence questionnaire)

Patient characteristics	Differentiation	No. of patients	Mean±SD	p
Age	≤65 years	50	59.62±6.87	0.04
	>65 years	50	62.82±8.7	
Sex	Male	55	62.18±7.65	0.18
	Female	45	60.04±8.33	
Education	≤Primary	41	61.78±6.74	0.56
	>Primary	59	60.83±8.80	
Family income (kyat)	≤60000	46	60.89±8.21	0.39
	>60000	54	59.60±6.55	
Disease type	Infarct	91	61.51±7.81	0.26
	Hemorrhage	9	58.33±9.71	
Disease site	Left	53	60.89±9.01	0.66
	Right	47	61.60±6.75	
Barthel index score	<40 (severe)	44	61.59±7.89	0.01
Disability	≥40 (mild to moderate)	56	57.44±8.56	
Rankin scale	<3 (mild)	33	58.33±7.53	0.01
Handicap	≥3 (moderate to severe)	67	63.03±8.7	

Table 3. Differences in caregiver characteristics in relation to mean total score of caregivers' burden (based on sense of competence questionnaire)

Characteristics of caregiver	Differentiation	No. of caregiver	Mean±SD	P
Age (years)	≤40	48	59.45±8.09	0.04
	>40	52	62.79±7.64	
Sex	Male	25	60.24±7.66	0.48
	Female	75	61.55±8.13	
Education	≤Primary	25	64.20±7.22	0.28
	>Primary	75	62.23±8.04	
Occupation	Full-time job	26	60.51±6.7	0.53
	Part-time job	12	59.3±9.61	
	Dependent	62	58.62±6.87	
Relationship with patient	Husband/ wife	15	62.67±6.72	0.26
	Son/ daughter	51	59.51±7.49	
	Sibling	14	61.86±6.01	
	Others	20	58.50±7.49	
Physical health score	≤248 (poor)	43	63.30±9.15	0.04
	>248 (good)	57	59.65±6.66	
Mental health score	≤280 (poor)	45	64.69±7.55	0.001
	>280 (good)	55	58.38±7.25	

Characteristics of caregivers

Univariate analysis revealed a significant association between age of the caregiver more than 40 years ($p=0.04$), poor physical health ($p=0.04$) and poor mental health ($p=0.001$) status of caregiver, and high level

of perceived burden. Other characteristics such as sex, education, occupation, and relationship with patient were not found to be associated with caregiver burden (Table 3).

DISCUSSION

Stroke rehabilitation needs to shift from a patient-focused approach to a combined patient- and caregiver-focused approach because these individuals are central in preserving rehabilitation gains and long-term well-being of stroke survivors [10]. But the determinants of caregiving burden and the needs of caregivers remain poorly understood.

In this study, mean total SCQ score of burden was 61.2 (score range of good to bad, 27-108). The burden scores were found to be moderate but higher than the burden scores of the caregivers (47.1) in one study of stroke patients. Caregivers of stroke patients were especially burdened by consequences of caregiving for their own personal life (subscale 3). Similar finding was also observed in the above study [11].

Advancing age of both patients and caregivers were related to greater caregiver burden. It was in agreement with the study by Emily McCullagh, *et al.* [12]. In addition, they found that age of the patient and caregivers were also related to quality of life of the caregivers. Disability and handicap (patient's dependency in performance of daily activities) was found to be a determinant of caregiver burden in this study. It was consistent with the findings from other studies [5, 12]. But gender, type and localization of stroke were not associated with the caregiver burden. Also the family income was not found to be a predictor of caregiver burden. It means that amount of care provided by caregivers was not associated with "out of pocket expenditure" in caregiving.

Poor health-related quality of life (both physical and mental health) of caregivers was related to greater caregiver burden. This association was found in other stroke

studies as well [11, 13, 14]. However, studies among caregivers of dementia patients did not demonstrate a relationship between caregivers' physical problems and the burden of caregiving they perceived [15, 16]. When the differences in health problems of dementia patients (ie., behavioral problems) and stroke patients (ie., physical disability) are considered, caregivers' physical health problems may become important when patients need assistance in the basic activities of daily living, such as getting out of bed.

Conclusion

This study may help to identify caregivers who are at risk for high levels of burden. Advancing age of patients and caregivers, high dependency of patients, and poor health-related quality of life (both physical and mental) of caregivers are the factors which influence the caregiver burden.

It also indicates that while the contribution made by caregivers of stroke patients to their care is substantial, this does not happen without consequences for their own health and interference with their overall lives. Appropriate advice and support may preserve informal caregiving which eventually enables patients to live for a longer period in the community.

Limitations of the study

- Firstly, generalization of our findings may be affected by the selection of patients who had been hospitalized. Since hospitalized patients have shown to be patients with more severe stroke, a community-based study might have revealed lower levels of caregiving burden.
- Secondly, the study included the stroke patients with at least 6 months duration. This period may not be sufficient enough to detect the burden. For example, post stroke patients were more likely to develop dementia after years which make caregiving more difficult.

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Isolation and identification of Japanese encephalitis virus from piglets in Dike Oo pig farm

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Japanese encephalitis is a vector-borne zoonotic disease caused by the bite of *Culex* mosquitoes. Japanese encephalitis virus circulates among the wild birds, and is transmitted to porcine, equine and human hosts. Isolation of JE virus is very rarely possible from other hosts rather than from piglets and *Culex* mosquitoes. Two hundred blood samples were collected from 6-14 weeks old piglets in Dike Oo pig farm. Sera were separated and virus isolation was done by using LLC-MK2 cells. Isolated JE virus was identified by immunofluorescent assay (IFA) using fluorescein isothiocyanate conjugated (FITC) JE monoclonal antibodies. For IFA positive tissue culture samples, RNA extraction was done and characterized further by molecular analysis. The results showed that isolated JE virus is genotype III. This study was the first molecular characterization of Myanmar JE virus from pigs.

INTRODUCTION

Japanese encephalitis virus (JEV) is the most important cause of epidemic encephalitis worldwide, with an estimated 35,000 to 50,000 cases and 10,000 deaths annually [1]. The case fatality rate of JE virus infections is approximately 25%, with 50% of survivors developing permanent neurological and psychiatric sequelae [2]. JEV is transmitted between vertebrate hosts by *Culex* mosquitoes, principally *Culex tritaeniorhynchus*. Pigs and wild birds are important natural hosts for JE virus, and they serve as amplifying or bridging hosts that transmit the virus to humans [1].

In Myanmar, in 1982, a study on vector, amplifier, and human infection with JEV was done in a Yangon community (Dawbon Township). JEV infection was detected in 52.1% of the pigs. The known JEV vector mosquito species, especially *Culex tritaeniorhynchus*, was found in the study area but no concurrent human JEV infections were detected [3]. An investigation on JEV

infection in Bogalay Township, Myanmar was done in 1999. JEV antibodies were detected in 33% of the pigs. They also found *Culex* vector mosquitoes especially *C. vishnui* followed by *C. tritaeniorhynchus* in the vicinity. JE virus antibodies were not detected among the villagers during the study [4]. JE virus can be isolated from humans, mosquitoes and swine. Because of transient viraemia and low titers, isolation from human blood is usually unsuccessful. However, it is occasionally isolated from CSF and necropsy samples [5].

The molecular aspects of JEV

It is an RNA virus of the genus *Flavivirus*, family *Flaviviridae*. It has a genome of 11 kb which encodes for three structural and seven nonstructural proteins. The envelope (E) protein is the most important among the structural proteins because it has neutralizing properties. According to phylogenetic analysis, there are five known genotypes of JEV worldwide based on the envelope (E) gene sequence [6].

In 2006, JE outbreak in China, isolation and sequencing of JE virus was attempted from CSF and mosquitoes. Eleven sequences were obtained and further analysis showed genotypes I and III [7]. JE virus was isolated from humans and human brain in Chiang Mai, northern Thailand in 1964 and 1982, respectively. They were found to be genotype III. However, in 1984, a 3-year survey (2003-2005) in pigs which studied changes in JE virus genotype pattern showed genotype I [8].

JE is a vaccine preventable disease and currently used vaccines are derived from JEV strain representative of genotype III. Clinical trials with genotype III vaccines have demonstrated effectiveness in areas where heterologous JEV genotypes cause human disease, supporting the hypothesis that immunity induced by genotype III vaccines protects against infection with JEV belonging to other genotypes [9]. JE virus was isolated and identified from the brain of a dead horse from Bahtoo, Myanmar in 1977. The isolate was confirmed by the WHO Reference Center in Poona, India [10]. The aim of this study was to optimize the methods for isolation and identification of JE virus from pigs in the laboratory. This would support the surveillance of JE virus by detecting circulating strains, changing circulating strains and emergence of new strains.

MATERIALS AND METHODS

Study site

A large pig farm in Dike Oo which is about 70 miles from Yangon was targeted for sample collection.

Sample collection and processing

Blood samples were collected from piglets between the ages of 8 to 14 weeks because the maternal antibody wanes at the age of 8 weeks, and at the age of 5-6 months most of the pigs were JE antibody positive. Each piglet was held in a dorsal recumbence position, strapped in a V-shaped cradle with the neck extended straight, and front legs

held back along the chest by an assistant. Two to three milliliters of blood sample were collected from anterior vena cava of each piglet. Two hundred blood samples were collected and they were transported to the laboratory of Virology Research Division of the Department of Medical Research (Lower Myanmar) in an ice box. After serum separation, sample tubes were properly labeled and kept at -80°C.

Sample transport

These serum samples were transported to the Center for Vaccine Development, Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus, Thailand. During transportation, samples were kept in a dry-ice box.

Isolation of Japanese encephalitis virus in LLC-MK2 cell

LLC-MK2 cells grown in modified eagles medium (MEM) with 10% fetal calf serum (FCS) in T-flasks and incubated at 37°C in 5% CO₂ incubator for 5 days were used. After discarding the media, the cell sheet was rinsed with phosphate buffered saline (PBS) pH 7.5. Trypsin-versene solution was added to loosen the cells to cell connection for 30-60 seconds.

After that, the solution was discarded and the cells were rinsed off with fresh medium by pipetting. Cell suspension was collected and cell number was counted by haemocytometer. Then, total cell count was calculated from cell number counted and dilution factors. The specified amount of cell suspension was mixed with fresh growth medium to give a final seeding concentration of 1.2×10^5 cells/ml. By using an automatic syringe, 2 ml of cell suspension were dispensed into each screw-capped test tube and were incubated for 5 days. One hundred microliters of each pig sera were used for inoculating each culture tube. After absorption for 90 mins, maintenance media (MEM with 2% FCS) was added, 2 ml per tube. After incubation for 7 days, tissue culture fluid (TCF) was

collected and reinoculated into new MK2 cells for the second passage with 300-500 µl/ sample of inoculum.

Virus identification

The harvested cells were spotted onto clean glass slides and fixed in cold acetone for 10 minutes. Virus detection was carried out by indirect immunofluorescent staining test (IFAT) using anti-*Flavivirus* monoclonal antibody (4G2) and anti-JE monoclonal antibody as the first antibodies and, FITC conjugated goat anti-mouse IgG as the second antibody. At each step, the slides were incubated at 37°C for 45 min and the slides were gently washed with PBS for 10 mins and left to air dry before the second antibody was added. After incubation, the slides were washed and mounted under cover slips before examination of the fluorescence staining under the fluorescence microscope. Each sample was passaged twice and was identified by IFAT after every passage.

Gene sequencing

RNA extraction was done from a supernatant of JEV positive cell culture, after the first passage, according to manufacturer's protocol as well as RNA reverse transcription-PCR (RT-PCR) [TPersonal (Biometra)]. RT-PCR was performed on 4 µl of cDNA template by using 2.5 units of Ampli-Taq Gold DNA Polymerase [RobusTTTMII RT-PCR Kit (Finnzymes)]. Overlapping of JEV E gene fragments was amplified with 2 sets of primers: Ea forward primer, Ea reverse primer; and Eb forward primer and Eb reverse primer, respectively [11]. The 1,414 nucleotides generated partial sequences of the JEV E gene that were compiled by using Sequence-Alignment Editor software version 5.0.9; pairwise genetic distances were calculated with MEGA software version 4.0 (Fig. 1).

RESULTS

One JE virus strain was isolated after the second passage of 200 pig serum samples in LLC-MK2 cells. The isolated

JEV sequence was analyzed with a group of known 36 JEV strain sequences from Asia and Australia. A phylogenetic tree was generated, and this new JEV isolate fits into the genotype III cluster (Fig. 1). GIII cluster includes JEV from Vietnam, China, Japan, Thailand, Philippines, Taiwan and Nepal. The overall identity of Myanmar JEV E gene sequence is 99.58% identical with Beijing, China strain.

DISCUSSION

The first evidence of the existence of Japanese encephalitis (JE) in Myanmar was highlighted in 1968 by the Central Epidemiology Unit of the Department of Health when sero-survey was done in Yangon population. The first confirmed outbreak of the disease in humans was in Tachileik in 1974 and another outbreak occurred in Lashio during the following year 1975. The outbreak occurred in horses at the animal breeding center and fodder farm of Bahtoo Myo in 1977.

Evidence of JE virus infection in humans and horses was confirmed, and the virus was first isolated from the brain of a dead horse. Small outbreaks and sporadic case reports continued, and non-epidemic seroprevalence studies showed that 16% were seen in Yangon with higher rates in some other States and Divisions. Entomological survey revealed that *Culex* vector mosquitoes were found to be distributed throughout the country. Seroprevalence study among the source of JE virus, pigs, in Dawbon and Insein jail farm showed 52.1% and 97.9% positivity, respectively [12]. Previous studies highlighted JE is endemic in many parts of Myanmar and the existence of source, vectors and seed virus means outbreak can occur in Myanmar population at any time.

However, JE is a vaccine preventable disease and currently used vaccines are derived from JEV strain representative of genotype III [9]. There are five genotypes

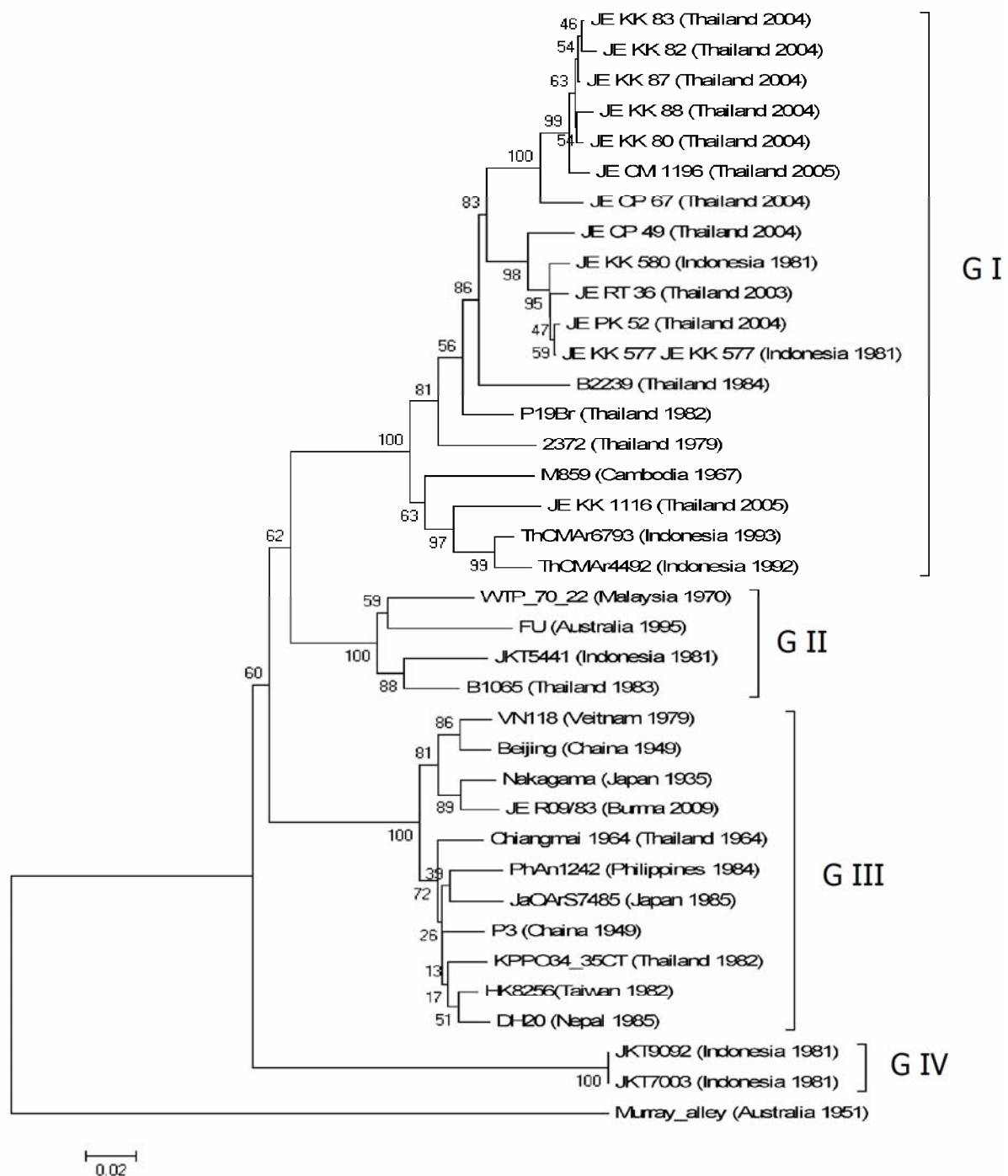


Fig. 1. Phylogenetic relationship of Japanese encephalitis viruses done by E gene (1500 nt) analysis by MEGA version 4 software, using the neighbor-joining (p-distance) method. The length of the tree branches indicates the percentage of divergence; the percentage of successful bootstrap replicates is specified at the nodes (1,000 replicates). Murray valley virus prototype sequence was included to root the tree.

found to be distributed all over southern Asia. Each clade contains genetically closely related forms and genotypes II and IV are associated with south endemic forms [3]. However, genotype pattern of endemic

JEV may change with time by evolution of virus strains within the country or introduction of strain from other neighboring countries. Genotype I (strain K9P05) contains strains from northern Thailand,

Cambodia and Korea. Genotype II (strain FU) comprises of virus strains from southern Thailand, Malaysia, Indonesia and northern Australia. Genotype III (strain JaOArS982) was identified in Japan, China, Taiwan and the Philippines. Genotype IV (strain JKT6468) was found in Indonesia and an isolate originated in Malaysia may represent the fifth genotype. Genotypes I and III are associated with north epidemics.

Thus, conducting surveillance in the form of genotypic studies at the sentinels can detect circulating strains, changing strains and emergence of new JEV strains is essential because the currently used vaccine based on genotype III may not be effective against new emerging JEV strains.

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Detection of influenza and parainfluenza viruses in children with acute respiratory infection attending Yangon Children Hospital

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This study involved the detection of influenza viruses and parainfluenza viruses (PIVs) in children with acute respiratory infection (ARI) attending Yangon Children Hospital (YCH) from September 2009 to August 2010. It aimed to determine the positivity rate of each of these viruses in ARI cases attending YCH. Throat swabs were taken from ARI cases attending Out-Patient Department of that hospital and were inoculated in Madin-Darby Canine Kidney cell lines for virus isolation. After the second passage, all tissue culture fluids were screened for a group of respiratory viruses by indirect immunofluorescence using screening antibody. Respiratory virus-positive tissue culture fluids proceeded to the third passage and the fourth passage. After the fourth passage, influenza and parainfluenza viruses were identified with specific monoclonal antibodies. Of 135 throat swab samples, 109 samples (81%) showed respiratory virus-positive with influenza virus-A (7.4%), influenza virus-B (7.4%), PIV-1 (9.6%), PIV-2 (6.7%) and PIV-3 (5.9%). Dual infection of influenza virus-A and PIV-3 was detected in one case, influenza virus-B and PIV-1 in one case, influenza virus-B and PIV-3 in one case and PIV-1 and PIV-3 in one case. Most cases were between 2 months and 3 years of age presenting mainly with cough (100%), fever (60-100%) and rhinorrhoea (60-85%). This study highlights the role of influenza and parainfluenza viruses in ARI cases. The obtained information will be important for management of ARI cases in the hospital.

INTRODUCTION

Acute respiratory infection (ARI) is an important cause of morbidity and mortality in children especially in infants and young children. About two million children die worldwide yearly due to ARI. The incidence of ARI in children under 5 years of age is about 151 million with 5 million new episodes each year in both developing countries and industrialized countries [1]. In Myanmar, ARI is one of the major health problems of the country. It has been ranked as the nineteenth priority disease of Myanmar National Health Plan for the years 2006 to 2011 [2].

Among many clinical presentations of ARI, pneumonia, bronchiolitis and acute laryngitis are known to be a threat to the children's lives [3]. Pneumonia accounts for about 21% of all deaths in children who are less than 5 years of age [1]. About 10% of all childhood pneumonia is associated with influenza virus [4]. WHO estimates that 140 million infants are at high risk of influenza-pneumonia and other outcomes of the influenza virus. Acute laryngotrachobronchitis or croup is another cause of childhood death in ARI cases. It is responsible for 10% to 25% of lower respiratory infections in pre school children [5]. The disease is frequently caused by parainfluenza viruses

especially type 1 [6, 7]. Besides, parainfluenza viruses are the second leading cause of serious respiratory illness immediately after respiratory syncytial virus [1].

In Myanmar, Under 5 Mortality Rate (U5MR) in urban population is 64.15 per 1000 live births in 2006 and 62.1 per 1000 live births in 2007 indicating that U5MR is gradually reduced in Myanmar in the recent years [8]. However, U5MR is expected to be reduced to 52 per 1000 live births by the year 2011 and beyond [9].

To meet the expected target, prevalence and case fatality rate of main killers like ARI must be reduced. Viruses are the main causes of ARI in children [10, 11]. Thus, the positivity rate of important viruses should be studied yearly among ARI cases. This study was conducted with the aim to determine the positivity rate of influenza viruses and parainfluenza viruses in children with acute respiratory infection attending the Yangon Children Hospital.

MATERIALS AND METHODS

Study population and sample size

This study was a cross-sectional descriptive study conducted at the Out Patient Department of Yangon Children Hospital and Virology Research Division of Department of Medical Research (Lower Myanmar). A total of 135 acute respiratory cases attending the Out-Patient Department of Yangon Children Hospital were recruited in this study from September 2009 to August 2010. Children of age up to 5 years, of both sexes, with sudden onset of cough or shortness of breath or coryza were included in this study.

Ethical consideration

Approval for the conduct of this study was obtained from the Institutional Ethical Review Committee of Department of Medical Research (Lower Myanmar).

Specimen collection and processing

The history and samples (throat swabs) were taken from clinically suspected ARI cases at

the Out-Patient Department of Yangon Children Hospital. The swabs were placed into the tubes containing 2 ml of Viral Transport Media (VTM). Each tube containing specimen was labeled with the specimen identification and patient identification. The tubes were vertically placed in an ice box and transported to the laboratory of Virology Research Division within 3 hours after collecting specimen.

At the laboratory, the swabs inside the VTM tubes were removed. The VTM tubes were centrifuged. Then, the fluid was filtered and poured into the vials. The vials were labeled and stored at -80°C until virus isolation was done.

Virus isolation and identification

The fluid from throat swab samples was inoculated into monolayer Madin-Darby Canine Kidney cell lines for virus isolation. Cytopathic effect was examined daily under the inverted microscope.

Virus isolation was done up to the fourth passage each passage taking 7 days. After the second passage, all tissue culture fluids were screened for a group of respiratory viruses (influenza virus type A and B, parainfluenza virus type 1, 2 and 3, adenovirus and respiratory syncytial viruses) by indirect immunofluorescent assay kits (Respiratory Panel 1, Chemicon) using screening antibody as the first antibody and FITC conjugated anti-mouse IgG as the second antibody. Only respiratory virus-positive tissue culture fluids were proceeded to the third passage and the fourth passage. After the fourth passage, influenza and parainfluenza viruses were identified by indirect immunofluorescent kits using specific monoclonal antibodies and FITC conjugated anti-mouse IgG. Both positive control slides and negative control slides were also stained and examined alongside the test slide.

Data entry and analysis

Data entry and analysis were done by using Statistical Package for Social Science (SPSS) version 15.0.

RESULTS

When 135 samples, after the second passage, were screened for a group of respiratory viruses, 109 samples (81%) showed respiratory virus-positive and 26 samples (19%) were respiratory virus-negative.

Table 1. Types of respiratory virus in ARI cases

Type of respiratory virus	Number of cases	% of ARI cases
Single infection of influenza virus or PIV	50	37
Influenza virus- type A	10	7.4
Influenza virus- type B	10	7.4
PIV-1	13	9.6
PIV-2	9	6.7
PIV-3	8	5.9
Dual infection of influenza and/or PIV	4	2.8
Influenza A & PIV-3	1	0.7
Influenza B & PIV-1	1	0.7
Influenza B & PIV-3	1	0.7
PIV-1 & PIV-3	1	0.7
No infection of influenza virus or PIV	55	40.7
No infection of respiratory virus (influenza, PIV, adenovirus and RSV)	26	19.5
Total	135	100

When 109 respiratory-positive tissue culture fluids, after the fourth passage, were tested with specific monoclonal antibodies, 50 samples (37%) showed single infection of influenza virus or parainfluenza virus, and 4 samples (2.8%) showed dual infection of influenza virus and/or parainfluenza virus. Remaining 55 samples (40.7%) were negative for respiratory viruses. Of 50 single infection cases, influenza viruses were isolated in 20 cases (14.8%) and parainfluenza viruses were isolated in 30 cases (21.1%). Among 20 influenza virus-positive cases, 10 cases (7.4%) showed influenza virus-A positive and 10 cases (7.4%) showed influenza virus-B positive. Among 30 PIV positive cases, 13 cases (9.6%) showed PIV-1 positive, 9 cases (6.7%) showed PIV-2 positive and 8 cases (5.9%) showed PIV-3 positive. Among 4 dual infection cases, both influenza virus-A and PIV-3 were identified in one case, influenza virus-B and PIV-1 in one case,

influenza virus-B and PIV-3 in one case, and PIV-1 and PIV-3 in one case (Table 1).

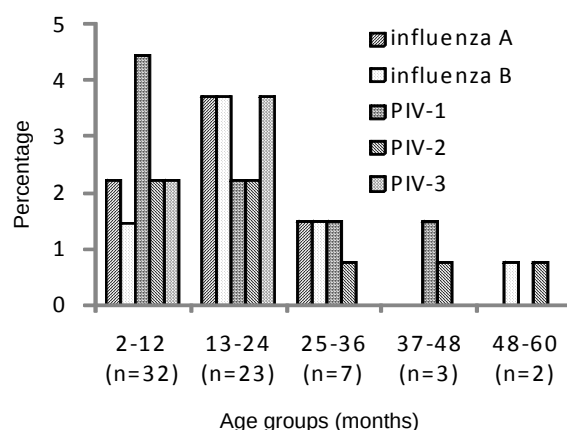


Fig. 1. Distribution of influenza and PIV single infection cases by age group

Most of the single infection cases of influenza virus type A and B, parainfluenza virus type 1, 2 and 3 were found in the age group between 2 to 36 months of age (Fig. 1).

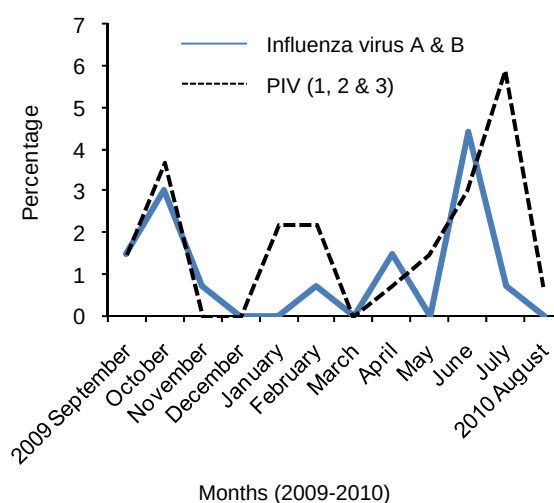


Fig. 2. Monthly distribution of influenza and PIV single infection cases in a period from September 2009 to August 2010

In a period from September 2009 to August 2010, majority of influenza viruses were isolated in October 2009 and June 2010. Majority of parainfluenza viruses were isolated in October 2009 and July 2010 (Fig. 2).

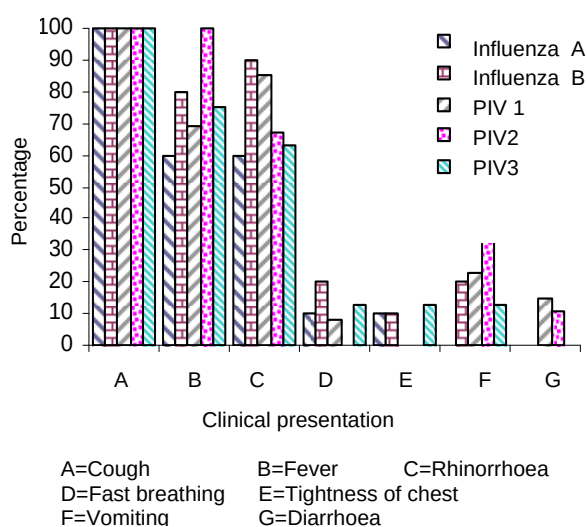


Fig. 3. Percentage of clinical presentations of influenza virus and PIV single infection cases

Most of the single infection cases of influenza and parainfluenza virus presented with cough (100%), fever (60-100%) and rhinorrhoea (60-90%). Some of the infected cases presented with fast breathing (0-20%), tightness of chest (0-13%), vomiting (0-33%) and diarrhoea (0-15%) (Fig. 3).

DISCUSSION

When 135 samples, after the second passage, were screened for a group of respiratory viruses (influenza viruses, parainfluenza viruses, adenovirus and respiratory syncytial virus), 109 samples (81%) showed respiratory virus-positive. Thus, viruses can be considered as main pathogens of ARIs in children up to five years of age. Similar finding was also found in a study conducted by Cabell, *et al.* in Mexico, in which virus infections were detected in 87% cases, as sole agents in 65% cases and as mixed infections with bacteria in 22% cases. In this present study, bacteria pathogens have not been identified, so mixed infections of respiratory virus with bacteria cannot be excluded in these screening positive cases [11].

The remaining 26 samples (29%) showed respiratory virus-negative indicating that they were negative for influenza virus

type A and B, parainfluenza virus type 1, 2 and 3, adenoviruses and respiratory syncytial virus since the screening antibody used in this study was the mixture of monoclonal antibodies to those 7 viruses. Thus, these samples could be positive for other respiratory viruses such as rhinoviruses, enteroviruses, human metapneumovirus, human bocavirus and human coronavirus [12, 13, 14, 15]. It is possible for the negative cases to have bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis* and *Haemophilus influenza* as causal agents. It was reported that bacteria species accounted for 56% of ARI cases attending OPD of the Yangon Children Hospital [16].

When influenza viruses and parainfluenza viruses were identified from screening positive cases, 50 cases (37%) were found as single infection of influenza virus (14.8%) and of parainfluenza virus (22.2%). However, mixed infection with other respiratory viruses such as adenovirus, respiratory syncytial virus and rhinovirus could not be excluded in the single infection cases.

Positivity rates of influenza virus type A and type B in this study were similar each being 7.4% of ARI cases. Go Hasegawa, *et al.* conducted an epidemiological study of influenza virus in Yangon from 2003 to 2004. They found that influenza A was detected in 21.6% and influenza B in 1% of patients with influenza like illness. According to these data, positivity rate of influenza virus type A was much higher than that of type B in the year 2003-2004 [17]. However, in a study conducted in central China in 2008-2009, influenza virus-A was detected in 97 ARI cases while influenza virus-B in 91 cases [18]. Those results showed that the ratio of influenza virus A-infected cases to influenza virus B-infected cases was approximately 1:1 which is in agreement with this present study.

Of 30 single infection cases of parainfluenza viruses, PIV-1 was detected in 13 cases (9.6%), PIV-2 in 9 cases (6.7%) and PIV-3

in 8 cases (5.9%). Fe[`], *et al.* conducted an epidemiological study in Brazil and the frequency of PIV-3, PIV-1 and PIV-2 was found to be 84%, 12% and 4%, respectively. In that study, PIV-3 was found to be the most prevalent and PIV-1 was the least prevalent [19]. However, in a study in central China, proportion of PIV-1 was higher than other types detected in mixed infections [18].

In the present study, proportion of PIV-1 was the highest among PIV single infection cases and proportion of PIV-3 was the lowest. Single infection cases of influenza virus in this study were more frequent in October 2009 and June 2010 (Influenza virus A in October 2009, Influenza virus B in June 2010). In October 2009 and July 2010, single infection cases of PIV were more frequently detected. Thus, most single infection cases of influenza and of parainfluenza viruses were detected in October 2009, June and July 2010. In other months, only a few cases of these viruses were detected. However, it cannot be assumed that influenza viruses and parainfluenza viruses are predominant in these months among ARI cases since ARI cases caused by organisms other than influenza and parainfluenza viruses prevalent during these months. The obvious finding is that frequency of ARI cases including these single infection cases was high in rainy season especially in June and July. Dapat, *et al.* conducted a study on epidemiology of influenza virus in Myanmar from 2005 to 2007 and found that seasonality of both influenza virus A and B coincided in the rainy seasons from June to August which is in agreement to this present study [20].

When the presenting symptoms of influenza and PIV cases were analyzed, cough, fever and rhinorrhoea were found to be the major symptoms of influenza and PIV cases. However, in this study, ARI caused by other organisms also presented with cough (100%), fever (76%) and rhinorrhoea (70%). Thus, ARI with cough and fever or rhinorrhoea are not strongly suggestive of

influenza virus infection or PIV infection. However, influenza and PIV infections can be considered as a differential diagnosis of ARI cases.

Most of the single infection cases of influenza and parainfluenza viruses presented with the symptoms described above but only fast breathing was found in 0 to 20% cases, tightness of chest in 0 to 13%, vomiting in 0 to 33% and diarrhoea in 0 to 15% of these cases. This data revealed that most of the influenza virus infection cases and PIV infection cases had mild symptoms and did not require antibiotic treatment immediately. In this study, four cases of mixed infections (dual infections) were found in the screening positive cases. In the similar study conducted in Central China from 2008 to 2009, mixed infections accounted for 36.1% of ARI cases with most frequent combinations being influenza virus-A and influenza virus-B [18]. In the present study, mixed infections (dual infections) accounted for 2.8% of ARI cases. The combination of influenza A and PIV -3 was detected in one case (0.7%), influenza-B and PIV-1 in one case (0.7%), influenza-B and PIV-3 in one case (0.7%) and PIV-1 and PIV-3 in one case (0.7%). All four cases presented with cough, fever and rhinorrhoea. Fast breathing was found in two cases which were clinically diagnosed as pneumonia. Significant association between dual infection and severe illness was not found in this study.

This study highlights the role of influenza and parainfluenza viruses in ARI cases. The information from this study will be important for management of ARI cases in the hospital. Further studies will be needed to characterize these viruses in order to support data for vaccine production.

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**Acute and subacute toxicity of *Millingtonia hortensis* Linn. f.
(Aykayit) leaves in animal model**

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Millingtonia hortensis Linn. f. (Aykayit) (Family-Bignoniaceae) is a commonly used medicinal plant for the treatment of hypertension in Myanmar. The present study was done to determine the phytochemical constituents, acute and subacute toxicity of *Millingtonia hortensis* Linn. f. dried leaves powder. Acute toxicity study of the dried leaves powder of this plant was carried out in albino mice by using oral route. In subacute toxicity study, the dried leave powder of this plant at the doses of 3 g/kg and 5 g/kg was administered orally to the albino rats daily for 3 months. At the end of 3 months, all the rats were sacrificed. Their blood samples were collected and tested for haematological and biochemical parameters. The internal organs were removed and collected for histopathological studies. It was found that the dried leaves powder contained alkaloids, flavonoids, glycosides, tannin, steroids, phenol, saponin, resin, carbohydrate and amino acid. In the acute toxicity study, it was found that the dried leaves powder was not toxic up to the maximum feasible dose of 8 g/kg. In the subacute toxicity study, the dried leaves powder showed no significant changes in body weights, hematological and biochemical (blood urea, liver function test) parameters when compared with those of the control group. Histopathological studies of the internal organs of the rats showed no pathological changes. In conclusion, it was found that *Millingtonia hortensis* Linn. f. dried leaves powder did not possess any acute toxic effect in the mice and subacute toxic effect in the rats.

INTRODUCTION

Millingtonia hortensis Linn. f. (Aykayit) (Family-Bignoniaceae) is a medicinal plant which widely grows in Myanmar, Thailand and India. Its English name is Indian Cork Tree or Tree Jasmine. This medicinal plant has been used for a long time in Thailand, Myanmar, India and Indonesia. The leaves and roots of *Millingtonia hortensis* Linn. f. are known to be useful for dizziness, muscle stiffness, pyrexia, hypertension etc. [1, 2]. Jetty and Iyengar [3] reported that aqueous extract of *Millingtonia hortensis* Linn. f. leaves showed antimicrobial activity against *E. Coli*, *Salmonella typhimurium* and other

gram negative organisms. Aqueous extract of the bark of this plant showed anti-proliferative activity on colon cancer cell lines [4].

It was reported that watery extract of leaves of this plant showed antifungal activity against some *Candida* organisms [5]. Decoction of the leaves and root bark of this plant have been used traditionally by local people to treat hypertension in Myanmar traditional medicine. It was also obtained in some Myanmar traditional medicine formulations for hypertension. The root bark is commonly used among Myanmar people as an anti-alcohol agent [6].

Yee Yee Tin reported that the watery extract of the leaves and root of *Millingtonia hortensis* Linn. f. showed hypotensive effect in dog model. It was also reported that the acute toxicity study of watery extract of this plant's leaves was done in mice and no toxicity was observed [7]. Despite the wide spread use of this plant, a few scientific studies have been undertaken to investigate the safety of this plant. Moreover, there is no scientific report of toxicity studies of the dried leaves powder of *Millingtonia hortensis* Linn. f. in Myanmar. So, this study was done to investigate the acute and subacute toxicities of dried leaves powder of *Millingtonia hortensis* Linn. f. in animal model as well as its phytochemical constituents.

MATERIALS AND METHODS

Study design

This study was the experimental study in mice and rats. Controlled parallel study design for toxicity studies was used.

Site of study

This study was done in Pharmacology Research Division, Department of Medical Research (Lower Myanmar).

Plant materials

The leaves of *Millingtonia hortensis* Linn. f. were collected from Mandalay. The leaves were identified by a botanist in Department of Botany, Yangon University. The leaves were carefully washed with tap water and they were air-dried under shade. Then, the air-dried leaves were made into powder by using grinding machine.

Phytochemical and physicochemical studies

Phytochemical study of the dried leaves powder of *Millingtonia hortensis* Linn. f. was done qualitatively for the detection of alkaloids, flavonoids, glycosides, steroids, saponins, tannins, resin, amino acids, carbohydrate, phenol and cyanogenic glycoside by using the method of Physicochemical Standard of Unani Formulations [8] and

Harborne [9]. The physicochemical study of the dried leaves powder of this plant was done by using the method of WHO [10].

Acute toxicity study

The acute toxicity study was done to determine the degree of toxicity to administration of the drug and to find out the median lethal dose (LD₅₀) by using the method of Litchfield and Wilcoxon [11].

Forty albino mice (ddy strain) of both sexes weighing (25-35 g) from Laboratory Animal Services Division, Department of Medical Research (Lower Myanmar), were used in this study. The mice were randomly divided into 4 groups and each group contained 10 mice. Each group of mice was placed separately in each mouse cage. They were fasted for 18 hours but allowed free access to water before the experiment. The dried leaves powder of *Millingtonia hortensis* Linn. f. was suspended in distilled water. The mice in group I were given distilled water orally and served as the control group. Group II, III and IV were given orally with 2, 4, 8 g/kg body weight of the dried leaves powder of this plant, respectively. Then, the mice were allowed free access to water and standard pellet diet *ad libitum*. Then, they were observed for general conditions, behavioral changes, motor activities as well as mortality daily up to 2 weeks.

Subacute toxicity study

The subacute toxicity study of the dried leaves powder of *Millingtonia hortensis* Linn. f. was done by using the method described in Sethi [12]. Thirty adult albino rats (Wistar strain) of both sexes weighing 170-250 gm from Laboratory Animal Services Division, DMR (LM) were used in this study. They were randomly divided into 3 groups and each group contained 10 rats. Each group of rats was placed separately in each rat cage. Two dose levels (3 g/kg body weight and 5 g/kg body weight) of the dried leaves powder of *Millingtonia hortensis* (suspended in distilled water) were used in this study. The rats in group I were given distilled water

through oral route and served as the control group. Group II and group III were given 3g/kg b.wt (ie. 20 times that of human dose) and 5g/kg b.wt (ie. 33 times that of human dose) of the dried leaves powder of this plant, respectively through oral route daily for 3 months. The rats were allowed free access to water and standard pellet diet *ad libitum*. They were observed for general conditions, behavioral changes, motor activities as well as mortality daily for 3 months. Body weights were measured weekly.

At the end of the experimental period (i.e. 3 months), all the rats were anesthetized with chloroform and blood samples were immediately collected from the hearts by cardiac puncture for haematological (blood for complete picture and platelet count) and biochemical analysis such as liver function tests [serum bilirubin, serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) and serum alkaline phosphatase (ALP)] and blood urea. Autopsy was performed to examine gross pathological lesions of the internal organs. Then, the internal organs such as brain, heart, lung, liver, stomach, small intestine, pancreas, spleen and kidneys were removed, weighed and preserved in 10% formalin solution. Tissues slides were prepared and stained with hematoxylin and eosin and histopathological examinations were performed by the pathologist.

Data analysis

The data were expressed as mean $\pm$ SD. The statistical differences between the means of treatment group and control group were tested by using Student 't' test. The difference at $p < 0.05$ was considered statistically significant.

RESULTS

Phytochemical and physicochemical studies

The phytochemical study of the dried leaves powder of *Millingtonia hortensis* Linn. f. showed the presence of alkaloids, flavonoids, glycosides, steroids, saponin, tannins,

amino acids, phenol, carbohydrate and resin. Cyanogenic glycoside was not detected. Physicochemical tests of the dried leaves powder of *Millingtonia hortensis* Linn. f. showed moisture content (6.2%), swelling index (1.4 cm), foaming index (<100) and total ash (8.4%). Extracts values were found to be ethanolic extract (21.7%), chloroform extract (5.8%), petroleum ether extract (3.5%) and watery extract (36.4%). Heavy metals like arsenic, lead, mercury and cadmium were not detected by using color test.

Acute toxicity study

There were no lethality or any signs of toxicity observed in the mice after oral administration of the dried leaves powder of *Millingtonia hortensis* Linn. f. at the doses of 2 g/kg, 4 g/kg and 8 g/kg.

Subacute toxicity study

(1) Effect of the dried leaves powder of *Millingtonia hortensis* Linn. f. on body weights and organ weights of the rats

The results are shown in mean $\pm$ SD. The average initial body weights of the control rats and the rats treated with the dried leaves powder of *Millingtonia hortensis* Linn. f. at the doses of 3 g/kg and 5 g/kg were 216.8 $\pm$ 33.36 g, 217 $\pm$ 25.54 g and 198.7 $\pm$ 15.14 g, respectively. At the end of 3 months, the average final body weights of the control rats and the rats treated with the dried leaves powder of this plant at the doses of 3 g/kg and 5 g/kg were 223.75 $\pm$ 26.2 g, 253.29 $\pm$ 44.37 g and 264.7 $\pm$ 55.58 g, respectively.

It was found that there were no significant differences in the average body weights between the rats treated with 3g/kg and 5g/kg dose of the dried leaves powder of this plant and the control group. No lethality and no toxicity signs were observed throughout the experimental period. There were no significant differences in the average organ weights between the rats treated with 3 g/kg and 5 g/kg dose of the dried leaves powder and the control group. The results are shown in Table 1.

Table 1. Changes in the organ weights (in gram) of the rats in the control group and groups receiving the dried leaves powder of *Millingtonia hortensis* Linn. f.

Organs	Dose of dried leaves powder of this plant		
	Group I (control)	Group II (Dried leaves powder (3 g/kg))	Group III (Dried leaves powder (5 g/kg))
Brain	1.58 ±0.33	1.63 ±0.22	1.70 ±0.16
Lung	1.13 ±0.31	1.12 ±0.13	1.31 ±0.36
Heart	0.88 ±0.21	0.87 ±0.16	0.85 ±0.16
Liver	10.16 ±3.86	10.29 ±2.78	9.67 ±2.19
Stomach	2.14 ±0.36	1.92 ±0.14	1.89 ±0.33
Intestine	0.53 ±0.28	0.62 ±0.29	0.67 ±0.23
Pancreas	0.61 ±0.52	0.37 ±0.2	0.44 ±0.37
Spleen	0.70 ±0.19	0.74 ±0.24	0.65 ±0.09
Kidney	2.39 ±0.73	2.42 ±0.71	2.07 ±0.47

Sample size for each group (n=10)

The data were expressed in mean±SD. Statistical comparisons were made between the values of the control and the groups receiving the dried leaves powder of *Millingtonia hortensis* Linn. f.

(2) Effect of the dried leaves powder of *Millingtonia hortensis* Linn. f. on haematological parameters

There were no significant differences in haemoglobin level, white blood cell count and platelets count between the rats treated with 3 g/kg and 5 g/kg doses of the dried leaves powder of *Millingtonia hortensis* Linn. f. and the control group. These results were also within the normal ranges [13]. The results are shown in Table 2. The data were expressed in mean±SD. Statistical comparisons were made between the values of the control and the groups receiving the dried leaves powder of *Millingtonia hortensis* Linn. f.

(3) Effect of the dried leaves powder of *Millingtonia hortensis* Linn. f. on biochemical parameters

The results are shown in Table 3. There were no significant differences in serum bilirubin, serum alanine aminotransferase

Table 2. Effect of the dried leaves powder of *Millingtonia hortensis* Linn. f. on haematological parameters of the rats

Parameters	Dose of dried leaves powder of this plant			
	Group I (control)	Group II (3 g/kg)	Group III (5 g/kg)	Normal range
Haemoglobin level (g/dl)	15.74 ±1.24	14.80 ±0.6	15.08 ±1.07	11.4-19.2
Total WBC count ($\times 10^3/\mu\text{l}$)	9.43 ±2.53	7.08 ±3.2	8.30 ±2.68	5-25
Neutrophil (%)	15.22 ±6.51	20.22 ±7.12	19.00 ±4.37	9-34
Lymphocyte (%)	78.11 ±6.66	75.89 ±8.49	75.30 ±4.35	65-84
Monocyte (%)	6.67 ±2.40	3±1	4.40 ±1.17	0-6
Eosinophil (%)	0	0.33 ±0.50	1.50 ±0.97	0-6
Platelet count ($\times 10^3/\mu\text{l}$)	957.38 ±185.78	956.86 ±94.59	856.75 ±144.56	500-1000

Sample size for each group (n=10)

(ALT), serum aspartate aminotransferase (AST) and serum alkaline phosphatase (ALP) and blood urea between the rats treated with 3g/kg and 5g/kg doses of the dried leaves powder of *Millingtonia hortensis* Linn. f. and the control group.

Table 3. Effect of the dried leaves powder of *Millingtonia hortensis* Linn. f. on biochemical parameters of the rat

Biochemical parameters	Dose of the dried leaves powder of the plant		
	Group I (control)	Group II (3 g/kg)	Group III (5 g/kg)
Total serum bilirubin ($\mu\text{mol/l}$)	13.8±1.99	13±2.96	13.6±3.03
Serum aspartate amino transferase (AST) (IU/L)	198±46.86	161.22±30.33	177.1±45.49
Serum alanine aminotransferase (ALT) (IU/L)	147±25.84	101.67±79.13	138.1±47.17
Serum alkaline phosphatase (ALP) ($\mu\text{mol/l}$)	182.9±33.01	210.44±27.51	207.8±27.54
Blood urea (mg/dl)	32.5±9.16	36.0±7.12	40.1±12.31

Sample size for each group (n=10)

The data were expressed in mean±SD. Statistical comparisons were made between the values of the control and the groups receiving the dried leaves powder of *Millingtonia hortensis* Linn. f.

(4) Effect of the dried leaves powder of *Millingtonia hortensis* Linn. f. on histology of internal organs

The gross examinations of the internal organs of both the control and the group of rats treated with the dried leaves powder of *Millingtonia hortensis* Linn. f. were found to be normal. In histopathological examinations of the rats, the tissues of the livers and kidneys of 3 out of 10 rats treated with the low dose (3 g/kg) of the dried leaves powder of *Millingtonia hortensis* Linn. f. showed mild congestions. The kidney tissues of 3 out of 10 rats treated with the high dose (5 g/kg) of the dried leaves powder of this plant showed mild congestions. It was also noted that the liver tissue of 2 out of 10 rats of the control group showed mild congestion. Apart from these findings, no pathological lesions were found in the tissues of brain, lung, heart, liver, stomach, intestine, pancreas, spleen and kidney of the control and treated groups of rats.

DISCUSSION

Millingtonia hortensis Linn. f. (Aykayit) is one of the most commonly used medicinal plants for the treatment of hypertension in Myanmar. Myanmar people also eat fresh leaves or boiled leaves of this plant as salad. The present study was carried out to assess the safety of the dried leaves powder of *Millingtonia hortensis* Linn. f. In the phytochemical study, it was found that the dried leaves powder of this plant did not contain toxic plant compound like cyanogenic glycoside.

Yee Yee Tin [7] reported that watery extract of the leaves of *Millingtonia hortensis* Linn. f. did not produce acute toxic effect in the albino mice through oral route. Bhakuni, *et al.* [14] observed that 50% ethanolic extract of the leaves of this plant showed no acute toxic effect in albino mice by intraperitoneal route.

In these toxicity studies, the oral route of administration was used because it is the

intended route for use in human. In acute toxicity study, it was observed that the oral administration of the dried leaves powder of *Millingtonia hortensis* Linn. f. was not toxic in mice up to the maximum feasible dose of 8 g/kg. This result suggests that median lethal dose (LD₅₀) of the dried leaves powder of this plant was above 8 g/kg. It indicates that the dried leaves powder of this plant is practically non toxic [15]. In the subacute toxicity study, the dried leaves powder of *Millingtonia hortensis* Linn. f. did not produce any toxic effect and lethality among the tested rats when two doses of the dried leaves powder (3 g/kg and 5 g/kg) were administered orally to the rats daily for 3 months. The dried leaves powder at the doses of 3 g/kg and 5 g/kg showed no significant changes in body weights and organ weights when compared with those of the control group.

Concerning the studies of haematological and biochemical parameters, there were no significant changes in haematological parameters and the biochemical parameters between the groups of the rats given with 3g/kg and 5g/kg of the dried leaves powder of this plant and the control group.

The most widely used criteria of the toxic action of a substance in rats are reduction in the rate of body weight gain, detection of gross and histological abnormalities in the organs, change in organ weights and increase in mortality rate. Total serum bilirubin concentration and liver enzymes activities are useful indicators for liver dysfunction. Blood urea is useful as an indicator of renal function. [13]. It was observed that the results of biochemical parameters of the rats treated with the dried leaves powder of *Millingtonia hortensis* Linn. f. showed no damage to liver and kidney.

In the present study, the histopathological studies of the tissue samples taken from selected organs of the rats treated with the dried leaves powder of this plant and the control group of rats showed no pathological lesions. The results indicated that

oral administration of the dried leaves powder of this plant to the rats daily for 3 months showed no toxic effects.

Therefore, it can be concluded that the dried leaves powder of *Millingtonia hortensis* Linn. f. did not produce any acute toxic effect in mice and subacute toxic effect in the rats .

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Determination of radionuclide level in cigarette available in Myanmar

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Exposure to radon (chemical symbol Rn) and its daughter is the second leading cause of lung cancer after smoking. When the tobacco leaves were burnt as cigarette, radon and its daughter naturally found in the leaves were altered. Tobacco is especially effective at absorbing radioactive elements from phosphate fertilizers, and also from naturally occurring radiation in the soil, air, and water. A descriptive study was carried out to determine the radionuclide level in cigarettes, cheroots and cigars available in Myanmar by using solid state nuclear track detector (SSNTD) LR115 type I film. Five different brands of cigarette, cheroot and 2 different brands of cigar (each sample of a brand) total 12 samples from Yangon market were coded and analyzed. The calculated values of radon concentration of cigarette, cheroot and cigar samples varied from 232.20 Bqm^{-3} to 71.20 Bqm^{-3} . The annual effective doses varied from 3.99 mSvyr^{-1} to 1.22 mSvyr^{-1} in cigarette, cheroot and cigar samples, respectively. Only one cigarette brand was higher than the others; but the effective dose of all 12 samples as at lower limit level of 5 mSvyr^{-1} . No risk of radiation exposure from tested 12 samples of cigarettes, cheroots and cigars to the workers as well as to community. International Commission on Radiological Protection (ICRP-2007) has recommended the limit to be 5 mSvyr^{-1} received from radon and its progeny above which it can be health hazard.

INTRODUCTION

Radon decays from uranium that has been in the ground since the times the earth was formed. Radon is a colorless, odorless, tasteless, invisible gas that is produced by the decay of naturally occurring uranium and radium in the environmental soil, ground water and air. Traces of ^{238}U , ^{226}Ra and ^{228}Th present in the soil were absorbed into the plants and can be found in the leaves. Some air borne radioactivity are deposited on the leaves. When the tobacco leaves were burnt as cigarette, radon and its daughter naturally found in the leaves were altered and are the ultimate cause of lung cancer in smokers. Radon emits alpha particles and produces several solid radioactive products called radon daughters or progeny [1].

The activities of the isotopes of radium,

^{226}Ra and ^{228}Ra , in the tobacco leaves reflected their origin from the soil by root uptake rather than from fertilizers used in the tobacco cultivation. ^{210}Pb originated from the air and was deposited onto the tobacco leaves and trapped by the trichomes. The trichomes of the leaves concentrate the polonium, which persists when tobacco is dried and processed. The alpha emitters ^{210}Po and ^{210}Pb are highly concentrated on tobacco trichomes and insoluble particles in cigarette smoke [2].

Alpha particle radiation has a very destructive effect on animal tissue because virtually all of its very high ionizing energy is expended within the tissue. Due to its double positive charge, limited range in tissue and enormously high energy, an alpha particle can produce huge numbers of ion pairs in substances with which it interacts. DNA chromosome damaged by alpha

particle radiation is much greater, by 100 times than by exposure of DNA to other types of radiation. Measurements of the whole lungs of smokers and ex-smokers show that the inhaled ^{210}Po is retained in the lower lung [3].

During the 17 years since the Surgeon General's first report on smoking, intense research activity has been focused on the carcinogenic potential of the tar component of cigarette smoke. The Surgeon General has warned that radon is the second leading cause of lung cancer in the United States. The U.S. Environmental Protection Agency provides radon risk comparison charts for people who smoke and those who have never smoked [4].

The Office of Radiation, Chemical & Biological Safety at Michigan State University reports that the radiation level for the same smoker was as high as 800 chest X-rays per year. The radioactivity in tobacco leaves collected from 15 different regions of Greece before cigarette production was studied in order to find any association between the uptake of the naturally occurring radio nuclides [5].

Several researchers from various countries have studied about radon concentration in various samples and various places. In 1988, Khan AJ, *et al.* found out the comparison of the radon contents in tobacco, tea and tooth powder using CR-39 nuclear track detector that the result of the radon concentration varied from $89.91 \pm 3.31 \text{ mBql}^{-1}$ to $14.06 \pm 1.4 \text{ mBql}^{-1}$ [6]. In 2001, Peres AC and Hiromoto G measured the concentrations of these two radio-nuclides ^{210}Pb and ^{210}Po were determined in eight of the most frequently sold cigarette brands produced in Brazil. The collective effective dose resulting from the use of cigarettes produced in Brazil per year is estimated to be $1.5 \times 10^4 \text{ man-Sv}$ [7]. In 2003, MA Misdaq, K Flata studied that the Uranium (^{238}U) and Thorium (^{232}Th) contents were measured inside various tobacco samples by using method based on determining detection efficiencies of CR-39 and LR-115 type II

solid state nuclear track detectors (SSNTD) for the emitted alpha particles and the result of the radon concentration varied from $205.98 \pm 10.3 \text{ mBql}^{-1}$ to $555.60 \pm 3.6 \text{ mBql}^{-1}$ [8].

In 2003, Ashraf EM Khater showed that due to the relatively high activity concentration of ^{210}Po and ^{210}Pb that are found in tobacco and its products, cigarette smoking highly increases the internal intake of both radio nuclides and their concentrations in the lung tissues [9].

Exposure to radon (chemical symbol Rn) and its daughter is the second leading cause of lung cancer after smoking. In Myanmar, one study found out the radon contents in some Myanmar cheroots have been estimated using LR 115 type II SSNTD that the result of the annual effective dose varied from 3.79 mSvyr^{-1} to 1.52 mSvyr^{-1} [10]. The goal of the present investigation study was to determine the radionuclide level of radon and its daughter in cigarettes, cheroots and cigars available in Myanmar by using solid state nuclear track detector (SSNTD) LR115 type I film that is sensitive to alpha particles.

Solid state nuclear track detector is one of the most fascinating nuclear particles detection techniques, developed to date. It has been found to be equally useful in basic and applied research work, particularly for scientists, engineers and technologists in the developing countries. That detector is small in size, flexible, inexpensive, efficient and free from sophisticated electronics and dead time problem. The plastic track detector LR115 type I film is a cellulose nitrate, red dyed film, manufactured by KODAK, France. The sensitive surface for alpha particles, red dyed, is $10 \mu\text{m}$ thickness of cellulose nitrate and the base is $100 \mu\text{m}$ polyester. When an alpha particle hits the film it causes localized damage to the molecular structure of the cellulose nitrate layer [11].

General objective

- To determine the radio-nuclides level among the popular brands of the

cigarettes, cheroots and cigars samples available in Myanmar

Specific objectives

- To detect alpha particles in cigarettes, cheroots and cigars samples by using solid state nuclear track detector LR 115 type I film
- To measure the radon concentration of the cigarettes, cheroots and cigars samples
- To compare the annual effective dose of cigarettes, cheroots and cigars samples

MATERIALS AND METHODS

Study design

Laboratory-based descriptive study

Study area

Radiation Toxicology Research Division, Department of Medical Research (Lower Myanmar)

Study population

Imported cigarettes, cheroots and cigars found in the popular brands of the Yangon market

Sample collection

To measure the radon concentration and annual effective dose, five different popular brands of cigarette, cheroot and two different brands of cigar (each samples of a brand) total 12 samples from Yangon market were coded and analyzed.

Data collection method

In this study, the Can technique was used. Although there are many techniques to find out the alpha detection, the Can technique is more convenient than other methods. In this technique the known amount (25 gm) of the samples was placed in plastic cans. Solid state nuclear track detector LR115 type I film about (1cm x 1cm) was fixed on the bottom of the lid of each can with tape so that sensitive side of the detector faced the specimen. The cans were tightly closed from the top and sealed. The geometrical

parameters were: plastic can with diameter 6.8 cm, height 9.9 cm, volume 360 cm³.

At the end of the exposure time (30 days), SSNTD LR-115 type I film was removed from the lid of plastic can and subjected to a chemical etching process. For etching of the LR-115 detectors, 2.5 N NaOH solutions at 60°C for 25 min constant (temperature was kept with an accuracy of $\pm 1^\circ\text{C}$ and without stirring) were used. The detectors were rinsed, dried and the tracks produced by alpha particles were observed and counted with optical microscope. Then alpha track density was calculated for each detector [11, 12].

RESULTS

LR-115 detectors for background were etched under corresponding identical conditions. To reduce the statistical errors, alpha tracks were counted for different fifty views and the alpha track densities were calculated.

Total number of tracks	= 5
Average number of tracks	= $5/50 = 0.10$
$\Sigma\delta^2$	= 4.05
$\sigma = \frac{\sqrt{\Sigma\delta^2}}{N}$	= 0.04

Number of tracks	= 0.10 ± 0.04
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Background number of tracks	= 0.10 ± 0.04
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SE (standard error) $\sqrt{\Sigma\delta^2 / N}$, where σ is the standard deviation and N is the number of observation (50 fields of view).

The background of the alpha track density for SSNTD LR-115 type I was 0.10 ± 0.04 tracks (Fig. 1).



Fig. 1. Photomicrograph of alpha tracks in LR 115 type I for cigarette C₁ sample with 40X objective

Table 1. No. of tracks in LR 115 type I film for cigarette sample (C₁) (exposure time=30 days) 2.5N NaOH at etching temperature 60°C and etching time 25 mins

Sr.no	Number of tracks	$\delta = (n - n_i)$	δ^2
1	1	0.10	0.01
2	2	1.10	1.21
3	1	0.10	0.01
4	-	-	-
5	1	1.10	1.21
6	-	-	-
7	-	-	-
8	1	1.10	1.21
9	2	1.10	1.21
10	-	-	-
11	1	1.10	1.21
12	-	-	-
13	2	1.10	1.21
14	1	0.10	0.01
15	1	0.10	0.01
16	-	-	-
17	-	-	-
18	1	0.10	0.01
19	1	0.10	0.01
20	-	-	-
21	2	1.10	1.21
22	2	1.10	1.21
23	1	0.10	0.01
24	1	0.10	0.01
25	-	-	-
26	1	0.10	0.01
27	-	-	-
28	1	0.10	0.01
29	2	1.10	1.21
30	-	-	-
31	1	0.10	0.01
32	1	0.10	0.01
33	-	-	-
34	2	1.10	1.21
35	-	-	-
36	1	0.10	0.01
37	2	1.10	1.21
38	1	0.10	0.01
39	2	1.10	1.21
40	-	-	-
41	2	1.10	1.21
42	1	0.10	0.01
43	2	1.10	1.21
44	-	-	-
45	2	1.10	1.21
46	1	0.10	0.01
47	-	-	-
48	-	-	-
49	-	-	-
50	2	1.10	1.21
Total	45		15.92

According to Table 1, the net number of track of the cigarette sample (C₁) was calculated.

$$\begin{aligned}
 \text{Total number of tracks} &= 45 \\
 \text{Average number of tracks} &= 45/50=0.90 \\
 \Sigma\delta^2 &= 15.92 \\
 \sigma &= \frac{\sqrt{\Sigma\delta^2}}{N} = 0.32 \\
 \text{Average number of tracks} &= 0.90\pm0.32 \\
 \text{Background number of tracks} &= 0.10\pm0.04 \\
 \text{Thus, net number of tracks} &= 0.80\pm0.26
 \end{aligned}$$

The alpha track density of cigarette (C₁) sample was calculated by using the track density equation.

$$\begin{aligned}
 \text{Alpha track density} &= \frac{\text{Net number of tracks}}{\text{Microscopic view area} \times \text{exposure time}} \\
 &= 2.64\pm0.22 \text{ tracks cm}^{-2}\text{day}^{-1} \text{ (cigarette sample, C}_1\text{)}
 \end{aligned}$$

The net number of track, the alpha track densities of five different popular brands of cigarette, cheroot and two different brands of cigar (each samples of a brand) total 12 samples were calculated with the same calculation of the cigarette sample (C₁).

Table 2. The comparison of the net number of track, alpha track densities, and radon concentration of the cigarette, cheroot and cigar

Sr. no.	Code no.	Total no. of tracks	Net no. of tracks	Alpha track density (tracks cm ⁻² day ⁻¹)	Radon concentration (Bqm ⁻³)
1.	C ₁	45	0.80±0.26	2.64±0.22	131.80±10.56
2.	C ₂	43	0.76±0.07	2.51±0.24	125.40±11.88
3.	C₃	76	1.41±1.22	4.64±0.37	232.20±18.48
4.	C ₄	35	0.60±0.05	1.98±0.18	99.00±9.24
5.	C ₅	51	0.92±0.09	3.04±0.30	151.80±15.18
6.	C ₆	37	0.64±0.06	2.11±0.20	105.60±9.80
7.	C ₇	43	0.76±0.07	2.51±0.24	125.40±11.88
8.	C ₈	70	1.30±0.11	4.30±0.36	215.20±17.80
9.	C ₉	27	0.43±0.05	1.42±0.16	71.20±7.90
10.	C ₁₀	59	1.08±0.09	3.56±0.32	177.80±15.80
11.	C ₁₁	35	0.60±0.05	1.98±0.18	99.00±9.24
12.	C ₁₂	39	0.68±0.06	2.25±0.21	112.20±10.56

To find the radon concentration in cigarettes cheroots and cigars (the present study) calibration factors used is 0.02 track cm⁻² day⁻¹=1 Bqm⁻³ (Muramatsu-1999).

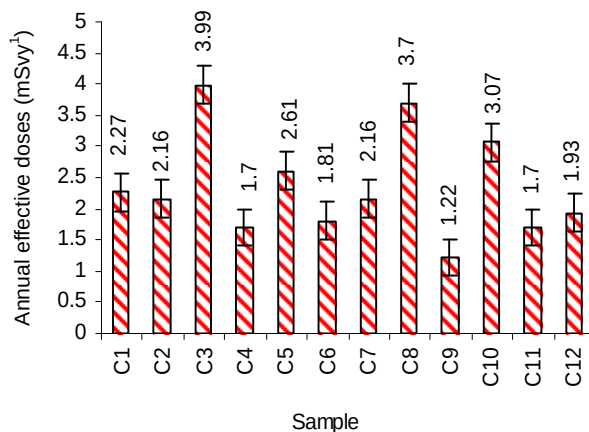


Fig. 2. The histograms of the observed annual effective dose vs. samples

And then, annual effective doses from all these samples were calculated using Publication International Commission on Radiological Protection (ICRP-2007) where $1\text{Bqm-3} = 0.0172\text{ mSvyr}^{-1}$.

The comparison of the net number of track, the alpha track densities and radon concentration of cigarette, cheroot and cigar (total 12) samples is shown in Table 2. The comparison of the annual effective doses for all those samples is shown in Fig.2.

DISCUSSION

By using the LR115 type I film detector, the minimum alpha track density ($1.42 \pm 0.16\text{ track cm}^{-2}\text{ day}^{-1}$) and the maximum alpha track density ($4.64 \pm 0.37\text{ track cm}^{-2}\text{ day}^{-1}$ in sample C₃) were found in cigarette, cheroot and cigar samples. The maximum alpha track density was found in sample C₃ (Classy) as illustrated in Table 2.

The measurements indicate moderate to some high level of radon concentration in cigarette, cheroot and cigar samples using LR 115 I detectors. The calculated values of radon concentration of cigarette, cheroot and cigar samples varied from $232.20 \pm 18.40\text{ Bqm}^{-3}$ to $71.20 \pm 7.80\text{ Bqm}^{-3}$. The annual effective doses varied from 3.9 mSvyr^{-1} to 1.22 mSvyr^{-1} in cigarette, cheroot and cigar samples, respectively. In comparison with the measured results in the

five different brands of cigarette, cheroot and two different brands of cigar (total 12 samples), as it was expected more than the other, the higher radon concentration was found in cigarette C₃ (Classy).

Conclusion

In this study it was found that no risk of radiation exposure from tested 12 samples of cigarettes, cheroots and cigars to the workers as well as to community. Only the cigarette C₃ (Classic) is higher than the other but radon concentration and annual effective dose of 12 samples were at lower limit level of 5mSv . International Commission on Radiological Protection (2007-ICRP 60) and National Commission on Radiological Protection (NCRP 115) have recommended the limit to be 5mSvyr^{-1} received from radon and its progeny above which it can be health hazard.

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Association between the use of insecticide-treated nets (ITNs) and parasitaemia and presence of malaria antibody in Thanbyuzayat Township, Mon State

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A cross-sectional community-based study was conducted in six villages of Thanbyuzayat Township, Mon State in July 2010 to detect association between the insecticide-treated nets (ITNs) usage, parasitaemia and merozoite antibody. Fifty-two percent of 183 adult population used ITN regularly and most of them were aware of ITNs through health personnels. They used ITN to prevent mosquito bite and malaria, but only 29% could buy ITN. The unpleasant smell of ITNs was complaint in 14.8%. About 22.1% of ITN users washed their nets every 4-6 months and only 7% retreated their nets after washing. Fever suspected as malaria during last year and two days before study were present in 25.3% of ITN users, 69.6% in net users and 63.2% in non-users, respectively. Malaria parasite was not detected in ITN group, but 18.3% was detected in non-ITN group (nets and non-users). The presence of antibody detected by rapid diagnostic test (RDT) were 45.5% among ITN usage of up to 6 months and 54.5% among more than 6 months users ($p=0.03$). It was also cross-checked by ELISA and the results were found to be similar. In non-ITN group, 52.2% was positive by RDT and 46% by ELISA, respectively. There was no association between parasitaemia and antibody. However, kappa agreement between RDT and ELISA was 0.66 ($p=0.000$). It indicated that the use of ITN has no effect on the development of acquired immunity to malaria and also confirmed the protective effect of ITN.

INTRODUCTION

Malaria constitutes one of the largest global health problems. Current WHO initiatives in malaria control such as Roll Back Malaria (RBM) emphasized the use of insecticide-treated nets (ITNs) as one of the key strategies for malaria prevention and control in sub-Saharan Africa [1]. Although the vast majority of deaths caused by malaria worldwide occur in Africa, it is also largely present in South America and Southeast Asia. There are at least 300 million acute cases of malaria each year globally, resulting in more than one million deaths [2].

Malaria is still also a priority public health problem for Myanmar. One of the objectives of the National Strategic Plan

Malaria (2010-2015) states that, by 2015, at least 80% of the people in high and moderate risk villages in 180 priority townships will be protected against malaria by using insecticide treated nets/long-lasting insecticidal nets [3]. ITN reduces contact between the mosquito vector and the human host. If ITN is used widely in a community, it reduces the population of mosquitoes which is reflected in a reduction of the plasma antibody levels among the bed net users [4].

Several independent seroepidemiological studies have demonstrated that there is a significant association between high IgG1 and/or IgG3 antibody ratios against glutamate-rich protein (GLURP) and merozoite surface protein (MSP3) and protection against clinical *P. falciparum* malaria [5].

The benefit of using ITNs in Africa has been shown through carefully controlled efficacy trials, resulting in a pooled estimate of a 20% reduction in all cause mortality in children aged under 5 years [6].

The functional background for malaria immunity is not fully understood, but antibodies are generally accepted markers of infection [7]. Following infection with any of the four species of *Plasmodium*, specific antibodies are produced, in virtually all individuals, one or two weeks after initial infection and persist for three to six months after parasite clearance. These antibodies may persist for months or years in semi-immune patients in endemic countries where reinfection is frequent. However, in a non-immune patient, treated for a single infection, antibody levels fall more rapidly and may be undetectable by three to six months [2]. Antibody detection is definitely not a substitute for blood film examination in the diagnosis of an acute attack of malaria and is mainly used in screening of prospective blood donors to avoid transfusion-transmitted malaria [8], diagnosis of fever of unknown origin especially in patients inadequately treated for malaria and epidemiological studies [9].

Antibodies specific for merozoite antigens have been shown to inhibit both erythrocyte invasion and parasite growth *in vitro* [10]. The ELISA (enzyme linked immunosorbent assay) format and immunochromatographic format (rapid test) to detect antibody of malaria are available recently. Although IFAT (immunofluorescence antibody test) is the gold standard for malaria serology, it is time-consuming, requires fluorescence microscopy and trained technicians. ELISA and malaria antibody rapid test are easy to perform, more practical alternative to IFAT and ELISA test kit shows a good correlation with IFAT for analytical and clinical parameters [11]. In Myanmar, currently, there was no known study to assess the association between the use of ITNs and parasitaemia and presence of malaria antibody. Acquired protective immunity

to malaria requires constant boosting through natural exposure to malaria parasites and wanes in the absence of such exposure [12].

So, could the use of ITN effect on the development of acquired immunity to malaria or not? By conducting this study, it can elicit antibody responses among the study population and usefulness of serological diagnosis in malaria control programs. Because acquisition and maintenance of antimalarial antibodies depend on exposure to malaria infection, such antibodies might be used as proxy measures of transmission intensity [13].

Objective

- To compare the parasitaemia and the presence of malaria antibody among insecticide-treated nets (ITN) users, untreated net users and non-users in Thanbyuzayat Township, Mon State.

Specific objectives

- To explore the knowledge status and opinions on and the utilization of bednets, insecticide-treated nets (ITN), untreated bed nets among the study population.
- To detect the association between malaria antibody and bednet use.
- To detect the association between levels of parasitaemia and bednet use.
- To detect the association between the parasitaemia and antibody among the study population.

MATERIALS AND METHODS

A cross-sectional community-based study was carried out in six villages of Thanbyuzayat Township, Mon State using multistage stratified cluster sampling two ways stratification technique focusing on the bednets, insecticide-treated nets (ITNs) among the adult population. Intravenous blood specimen (3 cc) was taken for serum separation for ELISA. The finger tip blood was also collected for rapid malaria

antibody test and peripheral blood smear (thick and thin film) for microscopy.

Parasite densities were measured by counting asexual parasites against 300 WBCs in the thick film and parasitaemia was calculated as follows:

Parasite = $\frac{\text{number of parasites counted} \times 6000}{\text{density}/\mu\text{l number of leucocytes counted}}$ [14]

A blood film was recorded as negative after examining 200 thick blood smear fields. Antibody levels were detected by malaria *P.f/P.v* (*Plasmodium falciparum*, *Plasmodium vivax*) antibody rapid diagnostic test kit (RDT) (capture recombinant merozoite protein (MSP) and cross-checked by commercial malaria antibody detection ELISA (recombinant MSP) according to their instructions.

The SD malaria *P.f/ P.v* antibody test is a qualitative immunochromatographic rapid test for detection of all isotypes (IgG, IgM, IgA) specific to *P.f/ P.v* simultaneously in human serum, plasma or whole blood. Both the test lines and control line in result window are not visible before applying any samples. The control line is used for procedural control. The tests results were interpreted within 10 minutes.

The SD malaria ELISA is also for qualitative detection of malaria antibodies *P.f/ P.v* in human serum or plasma. The procedure was done according to instructions. Before using, they were brought to room temperature (15~30°C). The absorbance of each well at a wavelength of 450 nm was read within 30 minutes. The mean absorbance of the negative controls was calculated, then the cut-off value was calculated by adding 0.100. Test results were classified as follows:

- i. $A(\text{sample}) < \text{cut-off} = \text{Malaria antibody negative}$
- ii. $A(\text{sample}) \geq \text{cut-off} = \text{Malaria antibody positive}$

Negative result meant there was no detectable malaria antibody and positive result meant the presence of detectable

malaria antibody. The test did not distinguish between IgG and IgM, or between antibodies to *P.f/ P.v*.

Data analysis was done by using SPSS 11.5, Chi-square test was used and $p < 0.05$ was considered as statistically significant.

Ethical clearance was obtained from the Ethical Committee of the Department of Medical Research (Lower Myanmar).

RESULTS

The knowledge status, opinions and utilization of bednets among the study population

Among 183 adult population, 52% used ITN regularly from which 97.9% were already factory-treated net, 37.7% used conventional bednets and 10.4% were non-users. About 69.4% had heard of ITN and most of them were learned about ITNs from health personnels, but only 29% could afford to buy ITNs. ITNs were obtained from local health staff (85.1%) and 13.8% from non-governmental organization. The range of family income per month was 9,000-2,000,000 kyats and their mean income per month was 164,417.58 kyats. Most study population had passed primary education.

The range of household members was 1-12 and about 60% were 3-5 (Fig.1). About 42% of ITN-users had two ITNs per home, 27.4% had three ITNs, 17.9% had one ITN, 10.5% had four ITNs and 2.1% had six ITNs per home, respectively. In (non-ITN) net-users 40.6% had one net per home, 37.7% had two nets, 13% had three nets, 4.3% had four nets and 4.3% had five nets, respectively. The numbers of persons sleeping under ITN were three persons per net (24.2%), two per net (21.1%), four per net (18.9%). In (non-ITN) net-users, the numbers of persons sleeping under conventional nets were five persons per net (29%), three per net (23.2%), four per net (15.9%) and two per net (11.6%).

About 80.3% of respondents in the study reported protection against mosquito

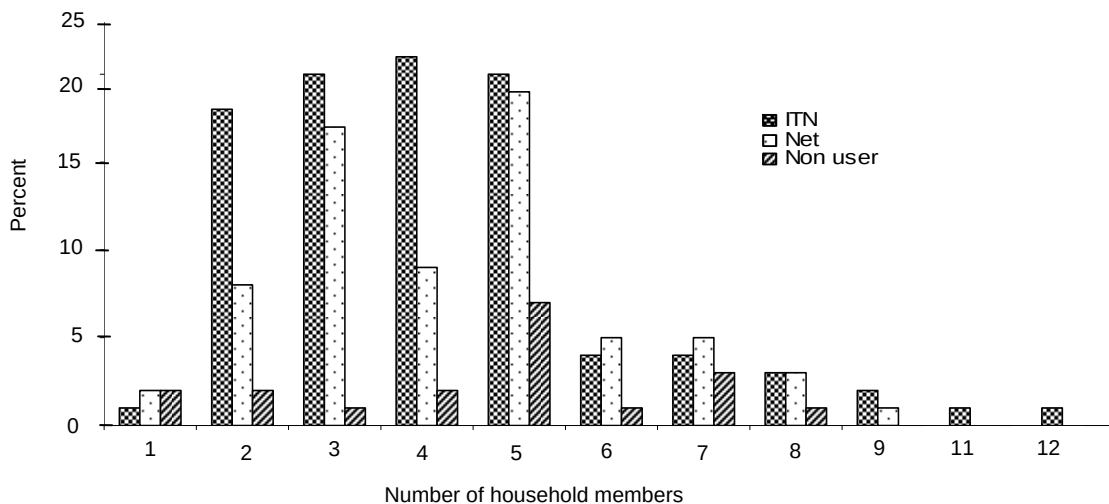


Fig. 1. Household members in different groups

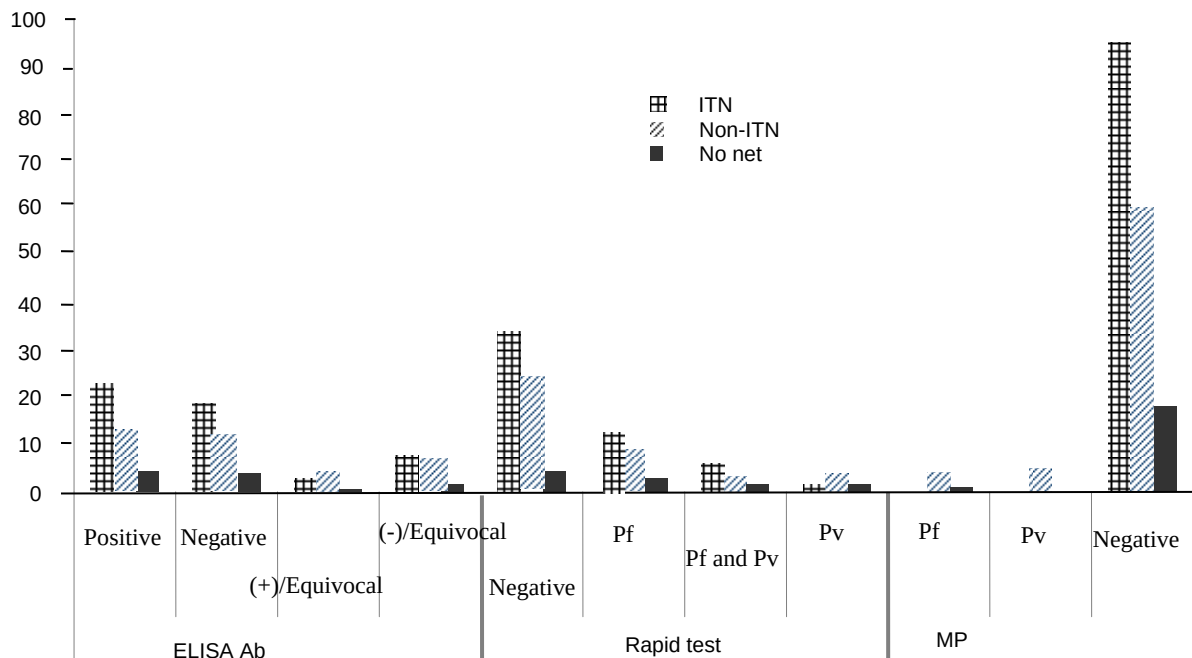


Fig. 2. Antibody test result in study population

nuisance as the primary reason for sleeping under a net. About 58.5% said they used nets to prevent malaria. Other reasons included protection against diseases (13.1%) particular dengue, protection against insects, protection against wind and the cold (13.1%). The unpleasant smell of ITNs was complaint in 14.8%.

About 22% of ITN users washed their nets every 4-6 months, 18.9% washed every 2-3 months, 13% washed every month, 12.6% washed every 15 days but only 7% retreated their nets after washing. Two respondents could not remember. About 52.6% of ITN

users went to sleep at about 9 pm and 27.4% at about 8 pm. In net users, 36.2% went to sleep at about 9 pm and 23.2% at about 8 pm.

Parasitaemia and antibody in different population

About 25.3% of ITN users, 69.6% in net users and 63.2% in non-users gave the history of malaria last year and/ or history of fever within 48 hours. Malaria parasite was not detected in ITN group, but 18.3% was detected in non-ITN group (nets and non-users). The lowest parasite density of the microscopy positive cases was 39 p/μl and

the highest was 26849.5 p/μl. The number of *P. falciparum* positive was 5 (i.e. 80% in net users and 20% in non-users) and *P. vivax* was 5 (i.e. 100% in net users). There was no mixed infection. *P. ovale* and *P. malariae* were also not detected in this study (Fig. 2).

Association between RDT antibody positivity and type of bednet use

In this study, 33.88% was malaria antibody negative in ITN group, 23.5% and 4.37% in non-ITN groups (nets and non-users), respectively. *P. falciparum* malaria antibody was positive in 10.38 % of ITN groups and 6.56 % and 2.73 % in non-ITN groups. For *P. vivax* malaria antibody, 1.64% was positive in ITN groups whereas 3.83% and 1.64% in non-ITN groups. In *P. falciparum* and *P. vivax* malaria antibody, 6.01% were found in ITN groups, 3.28% and 1.64% in non-ITN groups.

Association between ELISA antibody positivity and type of bednet use

In this study, 18.58% was negative in ITN group, 13.11% and 3.83% in non-ITN groups (nets and non-users), respectively. About 22.95% was positive in ITN groups whereas 12.57% and 4.37% were positive in non-ITN groups (net and non-users), respectively. There was no significant association between parasitaemia and antibody (i.e. $p=0.143$ for parasitemia and malaria antibody test kit; $p=0.281$ for parasitemia and ELISA). However, kappa agreement between RDT and ELISA was 0.66 ($p=0.000$).

Table 1. Malaria antibody test kit (RDT) and ELISA result crosstabulation

		ELISA result								Total	
		(+) (+)		(-) (-)		(+) / (Equi)		(-) / (Equi)			
		No.	%	No.	%	No.	%	No.	%	No.	%
Malaria antibody test kit (RDT)	(-)	16	22	59	91	9	64	29	94	113	61.7
	Pf	29	40	4	6.2	2	14	1	3.2	36	19.7
	Pf&Pv	19	26	0	0	1	7.1	0	0	20	10.9
	PV	8	11	2	3.1	2	14	1	3.2	13	7.1
	Invalid	1	1.4	0	0	0	0	0	0	1	0.5
Total		73	100	65	100	14	100	31	100	183	100

+ = positive, - = negative, Equi = equivocal

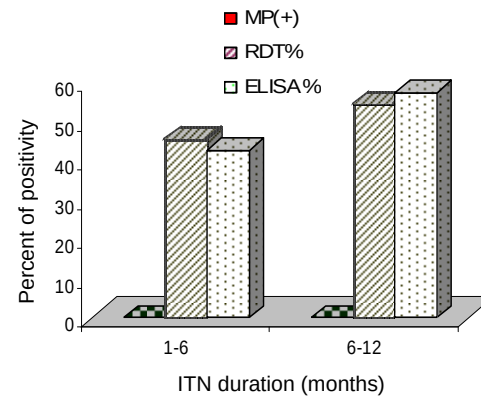


Fig. 3. The parasitaemia and antibody among the insecticide-treated nets population

The presence of antibody detected by rapid diagnostic test (RDT) were 45.5% among ITN usage of up to 6 months and 54.5% among more than 6 months users ($p=0.03$) (Fig. 3). It was also cross-checked by ELISA and the results were found to be more or less similar (Table 1). In non-ITN group, 52.2% were positive by RDT and 46% by ELISA, respectively.

DISCUSSION

Insecticide-treated nets (ITNs) protect users from mosquito bites, thereby preventing transmissions of mosquito-borne pathogens. The use of ITNs has been adopted as a standard method for malaria vector control [15]. Impressive results had been achieved in lowering malaria cases using ITNs. It was shown an association of 60% reduction in both clinical malaria and severe anaemia in ITN users of western Kenya [16]. The protection against parasitemia and febrile illness was observed when anti-MSP1-19kD antibodies were present [17].

This study relied on the collection of self-reported information. The results from this study suggested that bed-nets were used throughout the year and no seasonal variations in the compliance with ITNs. The average household members were 3-5 and had at least one to two per nets at their home. The unscheduled frequent washing by users to keep nets clean, unwarring of insecticide loses and low retreatment compliance cause constraint to get the

effectiveness of ITN utilization. Net washing data have not yet been validated properly and it was possible that house-holders exaggerate the reported frequency of net washing. Answers to the question on frequency of washing gave a wide range of answers. About 22% of ITN users washed their nets every 4-6 months, 18.9% washed every 2-3 months, 13% washed every month, 12.6% washed every 15 days but only 7% retreated their nets after washing. Based on WHO recommendation, it is generally believed that washing of long lasting (LLINs) below 20 washes has no effect on their efficacy [18].

In this study, most of the respondents reported that ITNs offered protection against mosquitoes and malaria, indicating awareness about ITNs and malaria but only 29% could afford to buy ITN. ITN user was generally more effective in reducing febrile episodes ($p=0.011$) than non-ITN users. Malaria parasite was not detected in ITN group, but 18.3% was detected in non-ITN group (nets and non-users). The antibodies detected by malaria antibody test kit (RDT) were 45.5%, 54.5% among ITN users who slept with ITNs up to 6 months and more than 6 months ($p=0.03$), respectively.

It was cross-checked by ELISA and the results were found to be more or less similar ($p=0.023$). Therefore, antibody could be maintained for certain periods even in the absence of infection (malaria parasite was not found) in ITN users. The absence of infection in ITN users might also be due to below the detection limit of microscopy. In non-ITN group, 52.2% were malaria antibody positive by RDT and 46% by ELISA, respectively.

The reason for malaria antibody detection in non-ITN group could be due to their long stay in malaria endemic area and past malaria infection. There was no significant association between parasitaemia and antibody determined by malaria antibody test kit (RDT) ($p=0.139$) and by ELISA ($p=0.144$). In ITN groups, there was no detection of parasite but presence of the

malaria antibody as described above. The kappa agreement between RDT and ELISA was 0.66 ($p=0.000$). This finding supported that malaria antibody RDT was a useful tool to assess the malaria antibody in local community alternative to ELISA method, the latter was unsuitable in the field where there was no electricity, ELISA reader, etc.

This study could indicate that the use of ITN has no effect on the development of acquired immunity to malaria and also confirmed the protective effect of ITN.

Conclusion

Insecticide-treated nets (ITNs) have been one of the major tools in the global fight against the burden of malaria. In this study, ITNs reduced febrile episodes compared to non-ITN groups and it can develop antibody even in the absence of infection. Therefore, ITN should be encouraged and promoted to use especially in malaria endemic areas and monitoring of malaria transmission using serological measures should be continued as antibodies can persist for months or years after infection.

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Establishment and evaluation of in-house one stage prothrombin time test

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One stage prothrombin time test (PTT) is one of the diagnostic coagulation assays and specifically to thromboplastin reagents suitable for use in thromboplastin- based coagulation. The aim of this study was to establish the production of in-house human brain tissue thromboplastin reagent by extraction of fresh brain tissue of human autopsy case. The protein content of in-house thromboplastin reagent is 1mg/ml by Lowry's method and the value of which is the same as reference reagent. The in-house reagent was established, standardized and compared with reference thrombospan-LS reagent in this study. Thirty-six apparently healthy persons and twenty patients with liver cirrhosis were tested for prothrombin time (PT) by using these reagents. International sensitivity index (ISI) of the in-house reagent was 0.93 which is calculated by standard calibration curve. The mean PTT result of in-house reagent was 13.9 seconds in normal person and 23 seconds in liver cirrhotic patients. Normal reference PTT result is 11-16 seconds. The mean international normalize ratio (INR) result of in-house reagent was 1 in normal person and 1.6 in cirrhotic liver patient. The coefficient of variation (CV) was 6.2% in normal person and 21.3% in liver cirrhotic patients. By using in-house thromboplastin reagent, the PT results is the same as commercial test kit. The sensitivity of in-house reagent was 95% and specificity was 100%. The cost of in-house reagent is lower than the price of reference reagent. Therefore, this reagent is suitable for use in coagulation assays for monitoring of anti-coagulant therapy.

INTRODUCTION

One stage prothrombin time test (PTT) is one of the diagnostic tests for coagulation disorders and specifically used as thromboplastin reagents for thromboplastin-based coagulation. Prothrombin time assay has been used extensively for determining blood coagulation factor deficiencies (Factor V, VII, X) associated with the extrinsic pathway, screening before surgical operation, monitoring the anticoagulant therapy such as coumarin, wafarin, vitamin K deficiency and chronic liver diseases [1, 2].

Thromboplastins are tissue extracts obtained from brain and placenta of human and can be extracted from brain and lung of rabbit. Human tissue thromboplastin extract is more sensitive than the animal tissue extract [3]. The commercially produced thromboplastins of animal origin are commonly used in many diagnostic laboratories. Human brain thromboplastin is no longer used for prothrombin time test because of the potential danger of transmission of viral infections.

Recombinant tissue factor (r-TF) is currently used for conventional thromboplastin in the prothrombin time test [4]. The calibration

and standardization of any types of thromboplastin reagents should be needed in all laboratories by using the control WHO reference thromboplastins reagent. The test thromboplastin reagent must be calibrated against the reference thromboplastin of same species by international sensitivity index (ISI) and international normalized ratio (INR) methods [2]. The primary performance criterias of thromboplastin reagents in PTT assays are the coagulation time of a normal plasma sample, the sensitivity of the reagent and the lot-to-lot reproducibility. Standard result of PTT is 14 seconds (11-16 sec) by using reference thromboplastin reagent. The sensitivity of this reagent is calculated by comparison of PT results between normal plasma and standard abnormal samples [1].

This study was carried out to evaluate the in-house human tissue thromboplastin reagent extracted from normal fresh brain tissue. This test is a basic coagulation screening test for the diagnosis of congenital and acquired coagulation disorders [5].

MATERIALS AND METHODS

The study was carried out in 40 apparently healthy blood donors with 20-40 years age group from the Blood Bank of Yangon General Hospital (YGH). The blood samples of 20 cirrhotic patients were collected from Liver Unit of Defense Services Liver Hospital (DSLH). A part of fresh brain tissue was collected from human autopsy case in Forensic Department of YGH.

Sample collection

This study was performed after getting approval from the Ethical Review Committee of DMR (LM). The brain tissue was collected after taking consent from parents or close relatives of deceased subjects. The brain tissue was put into the plastic box containing 0.9% normal saline (N/S) and sent to Pathology Research Division, DMR (LM) as soon as possible to extract thromboplastin reagent. For normal control, blood

samples were collected from blood donors from National Blood Bank and for abnormal control, samples were collected from ultrasound approved liver cirrhosis patients from October 2009 to September 2010. From all subjects, 3 ml of venous blood were collected into the silicon glass tube contained 0.3 ml of 3.2% of trisodium citrate solution and kept in crushed ice [6].

Extraction of in-house thromboplastin reagent from brain

A freshly obtained human brain was freed of its membranes and blood vessels. After washing, a measured weight of it was emulsified in 9 g/l NaCl, with the help of a blending machine. The emulsion was centrifuged at 3000 rpm for 30 mins and supernatant was collected. The protein content was estimated by Lowry's method. The final protein concentration of in-house tissue thromboplastin reagent was 1 mg/ml in one stage prothrombin time test and standard commercial test kit [7]. The doubling dilutions up to 1 in 8 were performed in saline and the dilution which was given the shortest time in Quick's one-stage test was chosen. The final product was diluted with one-tenth volume of barbitone buffer, pH 7.35. The extract was aliquoted in 2-ml each volumes in small screw-capped bottles which was then stored at -20°C until used. A satisfactory extract gave a clotting time in the Quick test of 11-16 sec with normal plasma [3]. Every test must be added normal control.

One stage prothrombin time test procedure

Platelet-poor plasmas (PPP) were prepared by centrifugation at 3000 rpm for 10 min at 4°C. PT of the in-house brain thromboplastin reagent was compared with PT of the reference thrombospan-LS reagent [8] using pooled normal plasma as well as a number of factor-deficient plasmas [5]. These two brain thromboplastin reagents were pre-warmed at 37°C water-bath for 10 minutes. Citrated plasma (PPP) 0.1 ml and thromboplastin 0.1 ml were mixed in glass tube and placed in water-bath. After the mixture was

waited for 1-3 min to warm, 0.1 ml of warmed 25 mmol CaCl₂ was added and the contents of the tube were mixed. The stopwatch was started and recorded the end-point of clotting. Each test was carried out in duplicate on both the patient's and the normal plasma.

Standardization of calibration curve

PT results of normal subjects and cirrhotic liver patients using in-house tissue thromboplastin reagent were compared with the reference thrombospan-LS reagent (control reference reagent) and drew calibration curve. The logarithms of the PT results were plotted on arithmetic graph paper by using the reference thrombospan-LS reagent on vertical axis (y) and the in-house thromboplastin reagent on the horizontal axis (x). The relationship between the two thromboplastin reagents was determined by the slope of the line.

The ISI of in-house tissue thromboplastin reagent was calculated with the following formula [9]:

$$\text{ISI of in-house} = \frac{\text{ISI of reference thromboplastin}}{\text{slope}}$$

Reference value of ISI is between 1–1.4 [10]. The INR is the ratio of a patient's prothrombin time to a normal (control) sample, raised to the power of the ISI value for the analytical system used.

$$\text{INR} = \left[\frac{\text{PT test}}{\text{PT normal}} \right]^{\text{ISI}}$$

INR = International normalized ratio
ISI = International sensitivity index

According to this system, the PT result is transformed mathematically to the INR which is the basis for standardization of the report [11]. The sensitivity and specificity of in-house thromboplastin reagent was determined by following [12]:

$$\text{Test sensitivity in disease} = \frac{\text{True positive results}}{\text{True positive results} + \text{False negative results}} \times 100$$

$$\text{Test specificity in disease} = \frac{\text{True negative results}}{\text{True negative results} + \text{False positive results}} \times 100$$

$$\text{Coefficient of variation} = \frac{\text{Standard deviation}}{\text{Mean}}$$

RESULTS

The PT results (in seconds) were converted to their logarithms which were plotted on arithmetic graph paper. The value of slope was 0.93 as in Fig.1.

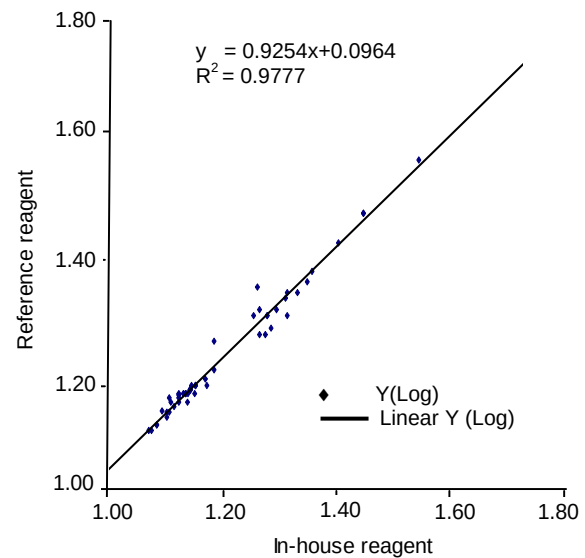


Fig. 1. Calibration of in-house thromboplastin reagent

$$\begin{aligned} \text{ISI of in-house throm-} &= \text{ISI of reference} \\ \text{boplastin reagent} & \quad \text{thrombospan-LS} \times \text{slope} \\ &= 1 \times 0.93 \\ &= 0.93 \end{aligned}$$

ISI of in-house thromboplastin reagent was found to be 0.93 and closed to 1.0. Mean value of PT, INR and CV of in-house brain thromboplastin reagent and reference thrombospan LS reagent in samples is shown in Table 1.

Table 1. Mean value of PT, INR and CV of in-house brain thromboplastin reagent and reference thrombospan LS reagent

Type of samples	PT		INR		CV	
	A	B	A	B	A	B
Normal	13.9 ±0.9	14.3 ±0.8	1.0 ±0.1	1.0 ±0.1	6.2%	5.7%
Cirrhosis patients	23.0 ±5.3	22.8 ±5.1	1.6 ±0.3	1.6 ±0.4	21.3%	22.2%

A= In-house, B= Reference, values=mean± SD
PT = Prothrombin time SD= Standard deviation
INR= International normalized ratio
CV = Coefficient of variation

Table 2. The sensitivity and specificity of the in-house thromboplastin reagent

		In-house reagent		Total results
		Patients (+)	Normal (-)	
Reference reagent	Patients (+)	19	1	20
	Normal (-)	0	36	36
Total results		19	37	56

$$\begin{aligned}\text{Sensitivity} &= \frac{\text{True positive results}}{\text{True positive results} + \text{False negative results}} \times 100 \\ &= \frac{20}{20+1} \times 100 \\ &= 95\%\end{aligned}$$

$$\begin{aligned}\text{Specificity} &= \frac{\text{True negative results}}{\text{True negative results} + \text{False positive results}} \times 100 \\ &= \frac{36}{36+0} \times 100 \\ &= 100\%\end{aligned}$$

The in-house thromboplastin reagent was compared with the reference thrombospan-LS reagent by using positive and negative plasmas tested by one stage prothrombin time. The sensitivity and specificity of in-house reagent were obtained by comparing the results of positive and negative plasmas [12]. The sensitivity of in-house reagent was 95% and specificity was 100% (Table 2).

DISCUSSION

The prothrombin time is most commonly useful for determination of coagulation system and it is always measured from plasma. It is also used to determine the clotting tendency of blood in patients with warfarin therapy, liver damage, and vitamin K deficiency [10]. The prolonged PT may be caused by some clinical conditions such as liver disease, vitamin K deficiency or a coagulation factor deficiency [13]. The human brain thromboplastin reagents are suitable for the use in prothrombin time assays [1]. In this study, the in-house thromboplastin reagent was obtained by extracting from human brain of deceased

cases. The in-house brain thrombolastin reagent preparation which had been calibrated against the reference thrombospan-LS reagent was used and supplied with an ISI and a table converting PT to INR [14].

In this study, the in-house reagent was emphasized in convert to INR values. The results were analyzed using reference ISI and in-house calibrated ISI values. The PT results of in-house reagent are compared with a true target value of reference. Attempts to improve performance of the INR include the use of reference ISI values [15]. This working reference has been calibrated against an internationally accepted standard reference preparation which has an ISI value of 1 [4, 16]. ISI of in-house thromboplastin reagent was found to be 0.93 and closed to 1. So, in-house human brain thromboplastin reagent is applicable for PTT.

In this study, the in-house reagent was compared with the reference thrombospan-LS reagent by using PT-INR results of normal healthy persons and prolonged plasmas of liver cirrhotic patients. Twenty out of 39 liver cirrhosis patients were obtained the prolonged PT by using two reagents. Thirty-six out of 40 blood donors had normal PT. In comparison, with these two reagents, PT-INR results of the participants were nearly equal in average. Mean INR of in-house reagent was similar to reference reagent. Our study demonstrated that PT-INR of in-house reagent was approximately similar to PT-INR of reference reagent.

The INR was calculated from the data provided by using the ISI and the mean normal prothrombin time. INR is widely used to monitor oral anticoagulation and to evaluate patients with coagulation disorders [17]. Since 1983, INR has been used to standardize prothrombin time results of patients on oral anticoagulants [18]. These results were in a very good agreement in patients with INR values close to 1, but increasingly poorer agreement was evident with longer PT times [19]. In this study,

the CV of mean INR obtained by comparing with both two reagents was nearly similar either normal plasmas or prolonged liver cirrhosis plasmas. The normal range for the INR was 0.8-1.2 [10]. Positive INR value of thrombospan-LS reference reagent was ≥ 1.2 [8].

The sensitivity and specificity of in-house reagent were 95% and 100%, respectively. So, sensitivity and specificity of the in-house reagent are good as the reference thrombospan-LS reagent test kit. In the comparison of the in-house thromboplastin reagent and the reference thrombospan-LS reagent, we obtained fairly good correlation ($R^2=0.98$). The use of human thromboplastin reagent was the first priority to introduce INR system for evaluation of multiple coagulation factors depletions [20].

Conclusion

In our study, the quality of in-house thromboplastin reagent is good enough for determination of PTT compared the reference thrombospan-LS reagent. This study demonstrated that the in-house reagent is cheaper than the reference reagent and achievable for PT test. These results suggested that the use of in-house human thromboplastin reagent is suitable in coagulation assays for monitoring of anti-coagulant therapy.

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The diuretic effect of *Hydrocotyle umbellata* Linn. on rat model

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The aim of this study was to evaluate the diuretic activity of *Hydrocotyle umbellata* Linn. The plants were collected from Yangon area and were extracted in Pharmacology Research Division. The cross-over study design was used to test diuretic efficacy. Six albino rats with body weight (200-260 gm) were used in this study. Firstly, the rats were given 0.9% NaCl (normal saline) 5 ml/100 gm body weight and served as control group. Then, urine output was collected at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The same procedures were conducted on the same six rats with watery extract 1.5 gm/kg, 3 gm/kg and 6 gm/kg body weight and standard furosemide 40 mg/kg body weight after one week rest. Urine volume was measured and urinary electrolytes (sodium and potassium) were determined by using digital flame analyzer. The watery extract 3 gm/kg and 6 gm/kg body weight treated groups showed significant increase in mean urine volume ($p < 0.05$) at 2 hr, 3 hr and 4 hr. Standard furosemide 40 mg/kg body weight given group showed significant increase in mean urine volume ($p < 0.05$) at 3 hr, 4 hr and 5 hr. Significant urinary sodium and potassium excretion was found with watery extract of *Hydrocotyle umbellata* Linn. at 1.5 gm/kg, 3 gm/kg and 6 gm/kg body weight dose and standard furosemide treated group ($p < 0.0005$ to $p < 0.05$) when compared with that of control. It is concluded that watery extract of *Hydrocotyle umbellata* Linn. has diuretic activity.

INTRODUCTION

Diuretics play an important role in treatment of fluid overload, like acute and chronic renal failure, congestive cardiac failure, cirrhosis of liver and also as an antihypertensive agent. A number of diuretics like mannitol, thiazides and furosemide are used in clinical practice. Still there is a need for more effective diuretic with less side effect. Myanmar population have been using many medicinal plants claimed to have diuretic activity. They are Kyaukpayon, Kyahin, Htigayon 'Kon', Thanmanaing Kyaukmanaing and Taungzebyu [1]. *Hydrocotyle umbellata* Linn. belongs to the family Apiaceae/Umbelliferae (carrot). It is an aquatic plant that thrives in wet, sandy habitat. Its English common names are many flower marsh pennywort, dollar weed and water pennywort. It is native to north

America and parts of south America and used by many U.S native American tribes for respiratory ailments [2].

It has been introduced in Myanmar since a few years and called (ထိုင်းဝမ်မြင်းခွံ). Literature survey revealed that the plant extract has yet not been screened for its traditional diuretic activity in experimental animals. Therefore, the present study was carried out to evaluate its potential diuretic activity.

General objective

- To study the diuretic effect of *Hydrocotyle umbellata* Linn. on animal model

Specific objectives

- To carry out watery extraction
- To find out the diuretic effect of watery extract of *Hydrocotyle umbellata* Linn. on albino rats

- To determine the urinary electrolyte composition (Na^+ and K^+) during diuresis

MATERIALS AND METHODS

Materials

Materials used are metabolic cages, 18 gauge intragastric dosing cannula, isotonic saline solution and digital flame analyzer.

Study area

The plant extraction was done in Pharmacology Research Division and the diuretic efficacy was carried out in Laboratory Animal Services, DMR (Lower Myanmar).

Study design

Cross-over study design was used in this study.

Sample size

Six female albino rats with body weight (b.w) (200-260 gm) were used in the study.

Preparation of extract

The plants were collected within the Yangon Division and botanically identified and authenticated by a taxonomist from Botany Department, Yangon University. It was confirmed as *Hydrocotyle umbellata* Linn. The plants were washed thoroughly with water to remove dust and dried in the shade. They were then cut into pieces and powdered with the blender to obtain crude powder. The crude powder (100 gm) was put into 2 liters conical flask and mixed with 1 L of distilled water and heated at 70°C for 6 hours. It was then allowed to cool at room temperature and filtered using cheese cloth and the filtrate was evaporated to be dryness on a boiling water bath. The dried extract obtained was kept in a desiccator [3].

Diuretic effect of *H. umbellata*

Six female albino rats weighing 200-260 gm were used in the study. Each rat was given 0.9% NaCl solution (normal saline), 3 different doses of watery extract of *H. umbellata* and furosemide at 1 week interval for each dose. Rats were fasted and

deprived of water for 18 hrs before the experiment. Firstly, the rats were given normal saline 5 ml/100 gm b.w and served as control group. After administration of normal saline, rats were then placed back in metabolic cages individually and urine output was collected at 1 hr, 2 hr, 3 hr, 4 hr, and 5 hr, respectively. The same procedures were conducted on the same six rats with watery extract 1.5 gm/kg, 3 gm/kg and 6 gm/kg b.w and standard furosemide 40 mg/kg b.w. Urine volume was measured and urinary electrolytes (sodium and potassium) were determined by using digital flame analyzer.

Data analysis

The results were shown in mean $\pm$ standard deviation. Student 't' test was used for calculation of statistical significance between control and treated groups. $p < 0.05$ was considered as significance. The method used for calculation of diuretic action in this experiment was according to the Rosenthale, *et al.* [4]. The urine volume excreted over 5 hours collection period was expressed as the % of administered water load. Diuretic action was calculated as follows.

$$\begin{aligned} \text{Urinary excretion} &= \frac{\text{Volume of urine collected in 5 hours}}{\text{Volume of fluid administered}} \times 100\% \\ \text{Diuretic activity} &= \frac{\text{Urinary excretion in test group}}{\text{Urinary excretion in control group}} \times 100\% \\ &= \frac{\% \text{ excretion in test group}}{\% \text{ excretion in control group}} \end{aligned}$$

Diuretic effect is presented if the ratio is more than one.

RESULTS

The results of mean urine volume (ml) in control group were 2.03 ± 1.88 , 1.33 ± 0.38 , 0.48 ± 0.38 , 0.19 ± 0.32 , 0.51 ± 0.59 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The results of cumulative mean urine volume (ml) in control group were 2.03 ± 1.88 ,

3.36±2.22, 3.84±2.35, 4.04±2.66, 4.55±2.49 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively (Fig. 1).

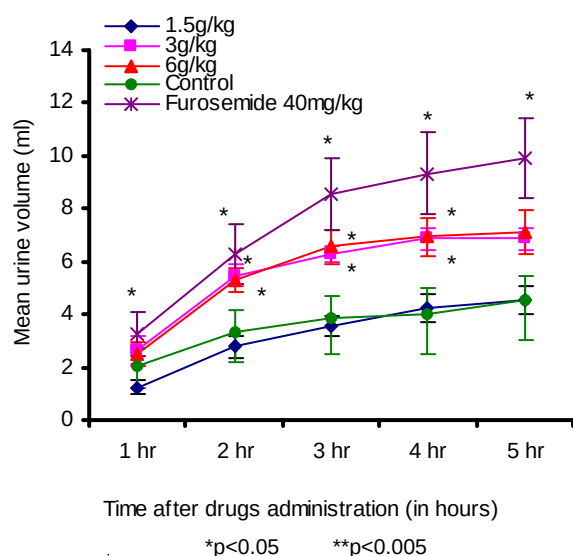


Fig.1. Statistical comparison of cumulated urine volumes among control group, furosemide group and three doses of aqueous extract groups

The results of mean urine volume (ml) in 1.5 gm/kg receiving group were 1.23±0.68, 1.53±0.94, 0.81±0.45, 0.68±1.11, 0.29±0.55 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The results of cumulative mean urine volume (ml) in this group were 1.23±0.68, 2.77±1.07, 3.58±0.96, 4.26±1.26, 4.55±1.24 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The mean urine volume was not significantly increased in this dose. The results of mean urine volume (ml) in 3 gm/kg receiving group were 2.67±1.47, 2.82±0.63, 0.8±0.72, 0.57±0.5, at 1 hr, 2 hr, 3 hr and 4 hr, respectively. The results of cumulative mean urine volume (ml) in this group were 2.67±1.47, 5.48±1.24, 6.28±0.87, 6.85±1.11, 6.85±1.11 at 1 hr, 2 hrs, 3 hrs, 4 hrs and 5 hrs, respectively. 'p' values were p<0.05 at 2 hr, 3 hr and 4 hr, respectively (Fig. 1).

The results of mean urine volume (ml) in 6 gm/kg receiving group were 2.5±1.18, 2.8±0.56, 1.25±0.8, 0.37±0.75, 0.22±0.53 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The results of cumulative mean urine volume (ml) in this group were 2.5±1.18,

5.31±1.27, 6.56±1.81, 6.93±2.02, 7.14±2.36 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were p<0.05 at 2 hr, 3 hr and 4 hr, respectively. The results of mean urine volume (ml) in furosemide (40 mg/kg) receiving group were 3.23±2.37, 3.02±1.56, 2.31±0.8, 0.77±0.82, 0.58±0.97 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The results of cumulative mean urine volume (ml) in this group were 3.23±2.37, 6.25±3.23, 8.56±3.87, 9.32±4.35, 9.905±4.32 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were p<0.05 at 3 hr, 4 hr and 5 hr, respectively (Fig. 1).

The cumulative mean amount of urinary electrolyte (Na⁺) (mg/L) in control group were 6.53±4.65, 11.28±6.13, 12.93±6.93, 13.71±8.16, 15.61±7.33 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The cumulative mean amount of urinary electrolyte (Na⁺) (mg/L) in 1.5 gm/kg receiving group were 11.48±7.1, 20.09±10.56, 26.74±11.85, 28.92±11.45, 31.44±12.35 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The mean amount of urinary sodium was not significantly greater than that of control group in this dose. The cumulative mean amount of urinary electrolyte (Na⁺) (mg/L) in 3 gm/kg receiving group were 25.8±14.45, 57.91±10.09, 67.77±10.83, 74.48±12.23, 74.48±12.29 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were p<0.005 at 2 hr and p<0.0005 at 3 hr, 4 hr and 5 hr, respectively (Fig. 2).

The cumulative mean amount of urinary electrolyte (Na⁺) (mg/L) in 6 gm/kg receiving group were 30.58±14.95, 67.86±16.2, 83.99±22.62, 89.94±25.49, 92.86±29.69 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were p<0.05 at 1 hr, 5 hr and p<0.005 at 3 hr, 4 hr and p<0.0005 at 2 hr, respectively. The cumulative mean amount of urinary electrolyte (Na⁺) (mg/L) in furosemide 40 mg/kg receiving group were 25.71±20.15, 54.47±29.95, 76.66±36.03, 81.37±37.39, 86.34±37.84 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were p<0.05 at 2 hr, 3 hr, 4 hr and 5 hr, respectively (Fig. 2).

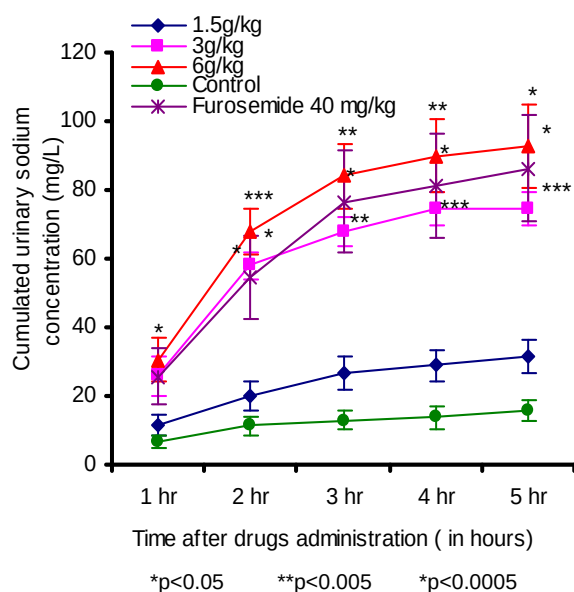


Fig. 2. Statistical comparison of cumulated urinary sodium concentrations among control group, furosemide group and three doses of aqueous extract groups

The cumulative mean amount of urinary electrolyte (K^+) (mg/L) in control group were 2.85 ± 2.35 , 4.69 ± 2.98 , 5.33 ± 3.13 , 5.64 ± 3.61 , 6.25 ± 3.58 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. The cumulative mean amount of urinary electrolyte (K^+) (mg/L) in 1.5 gm/kg receiving group were 5.68 ± 3.24 , 13.72 ± 6.01 , 17.6 ± 5.58 , 21.17 ± 7.87 , 22.47 ± 7.48 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were $p < 0.05$ at 2 hr and $p < 0.005$ at 3 hr, 4 hr and 5 hr, respectively. The cumulative mean amount of urinary electrolyte (K^+) (mg/L) in 3 gm/kg receiving group were 13.62 ± 7.81 , 29.28 ± 7.44 , 33.64 ± 6.37 , 36.65 ± 7.69 , 36.65 ± 7.69 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were $p < 0.05$ at 1 hr, $p < 0.005$ at 2 hr and $p < 0.0005$ at 3 hr, 4 hr and 5 hr, respectively (Fig. 3).

The cumulative mean amount of urinary electrolyte (K^+) (mg/L) in 6gm/kg receiving group were 12.85 ± 6.47 , 28.74 ± 8.49 , 35.44 ± 11.31 , 37.1 ± 11.23 , 38.31 ± 13.48 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were $p < 0.05$ at 1 hr and $p < 0.005$ at 2 hr, 3 hr, 4 hr and 5 hr, respectively. The cumulative mean amount of urinary electrolyte (K^+) (mg/L) in furosemide 40 mg/kg receiving group were 10.71 ± 8.25 , $20.97 \pm$

11.34 , 28.84 ± 14.02 , 30.62 ± 14.73 , 32.6 ± 4.55 at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr, respectively. 'p' values were $p < 0.05$ at 2 hr, 3 hr, 4 hr and 5 hr, respectively (Fig. 3).

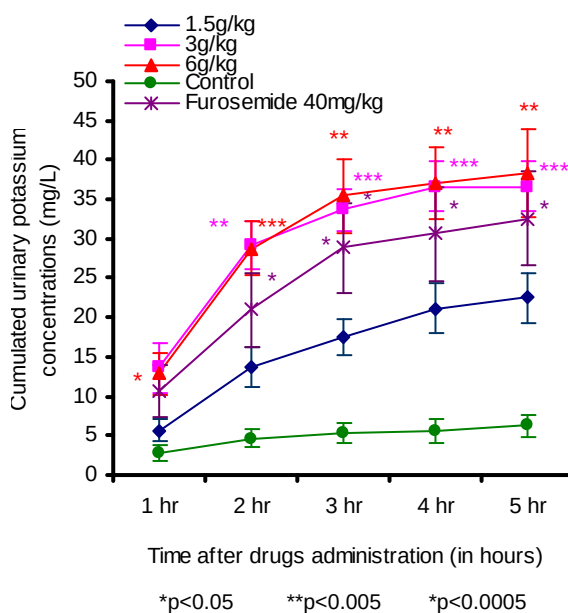


Fig. 3. Statistical comparison of cumulated urinary potassium concentrations among control group, furosemide group and three doses of aqueous extract groups

The mean diuretic responses of aqueous extract 1.5 gm/kg treated group was 0.97, that of 3 gm/kg treated group was 1.54, 6 gm/kg treated group was 1.41 and standard furosemide given group was 1.95, respectively. Watery extracts 3 gm/kg, 6gm/kg and furosemide receiving groups showed diuretic activity after fifth hour of drug administration because the activity is more than '1' [4].

DISCUSSION

Diuresis is defined as an increase in the rate of urine formation. It may be induced by copious intake of beverages (water), by pathological states or by the action of certain chemical agents. Such chemical agents are referred to as diuretics. The main site of action of these agents is in the kidney regardless of the cause of formation of urine [5]. The test available for assessing diuretics has been based on the effect of water and electrolyte excretion in rats.

Many researchers used various methods to assess diuretic activity in laboratory rats. The important factor in assessing diuretic activity is to induce diuresis by water loading. Without water loading, urine flow in rats is only 0.5 ml/100 g/hr, which is inconveniently low to determine the urine volume and electrolytes excretion after administration of test agent. Some used water as loading agent and some used 0.9% NaCl, 10% acacia, 0.3% NaCl and 2% polyethylene glycol. In this study, 0.9% NaCl was used as loading agent. To test diuretic activity, urine volume as well as amount of sodium and potassium excreted are important parameters. Tested animals loaded with 2.5 ml/100 g of 0.9% sodium chloride were adequate both for sodium and water excretion [6]. But, in this experiment, 5 ml/100 gm of 0.9% NaCl was used according to Kar K [5]. The period of collection of urine are varied among researchers. The method used in this experiment was according to the Rosenthale, *et al.* [4], in which the collection period for diuretic activity was 5 hours.

The test substances are given in solution or suspension by various routes. The route and nature of fluid load will influence the diuresis. Bare JE [7] stated that an orally administered load will be more completely excreted than with an intraperitoneally load. Diuretics are given orally to human. Therefore, the extract of *H. umbellata* was given orally to rats. In this study, it was noted that watery extract of *H. umbellata* didn't cause the increase in urine output at 1.5 gm/kg dose but it showed the significant increase in urine output at 3 gm/kg and 6 gm/kg dose as compared to that of control. In this study, furosemide increased the excretion of Na^+ and K^+ . Furosemide acts by inhibiting electrolytes reabsorption in the thick ascending limb of the loop of Henle by inhibiting $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter (co-transporter system) [8].

The tested extract significantly increased urine concentration of sodium except at 1.5 gm/kg dose. It also significantly

increased urine concentration of potassium at all dose levels. It may be due to a high sodium and potassium content of this extract [9]. The pattern of urinary excretion of the extract is similar to thiazide and furosemide diuretics but does not have potassium sparing effect like spironolactone. In a previous work in our laboratory, the preliminary phytochemical analysis of the tested plant revealed the presence of glycosides, flavonoids, steroids, triterpen, polyphenols, saponins, carbohydrates and reducing sugar [9]. At present, it is not known which compounds are responsible for the diuretic, natriuretic and kaliuretic activities of *H. umbellata*. The literature review supported that the diuretic properties of other plant extracts were attributed to their content of flavonoids and glycosides [10, 11].

Conclusion

These results demonstrated that watery extract of *Hydrocotyle umbellata* Linn. has a significant diuretic effect because of increased urinary electrolyte excretion with significant increase in the urine output. It is recommended to determine the acute, subacute toxicity study for the safety of *Hydrocotyle umbellata* Linn. to introduce clinical trial on human being and to isolate and structural elucidation of active diuretic agents from *Hydrocotyle umbellata* Linn.

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SHORT REPORT

Coverage and side-effects of oral cholera (Dukoral) vaccine in Department of Medical Research (Lower Myanmar)

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Orally administered cholera vaccines represent a potential new tool to prevent or control cholera outbreaks. The two-dose oral killed rBS-WC cholera vaccine is well tolerated and provides significant protection against cholera. Field trials conducted in Bangladesh and Peru [1, 2] showed that this vaccine confers a high level of protection: 85% after the second dose and still about 62% protection two years later, in adult vaccinees. Protection among children less than 5 years is high during the first 6 months and then decreases dramatically.

Cholera is endemic in our country with high seasonal variations. Cases usually occur during the beginning of rainy season from May to June. Although there have been no large outbreaks reported during the past few years, number in tenths of culture positive cholera cases have been reported from different townships all year round. When the rectal swab samples of April and May from North Okkalapa General Hospital were confirmed in National Health Laboratory, 35 isolates were positive for both serotypes (Inaba & Ogawa) of *V. cholerae* 01. During this year, in Yangon Division, tested/culture positive cholera have been reported as: 35/56 (Inaba>Ogawa serotype), 71/226 (Inaba=Ogawa) and 51/109 (Ogawa>Inaba) in March, April and May (up to 15th), respectively (personal communication, Bacteriology Section, NHL). Some of the divisions of DMR have to handle stool samples from which *V. cholerae* can be isolated. Therefore, the two-dose killed oral vaccine against cholera and ETEC diarrhea

(Dukoral) was studied among our staff and their relatives in Department of Medical Research (Lower Myanmar) with an aim to assess acceptability, coverage and side effects of the vaccination.

Vaccine, one dose (3 ml) contains: *Vibrio cholera* 01 Inaba and Ogawa, Classical and El Tor strains approximately 1×10^{11} vibrios (heat/formalin inactivated) cholera toxin B subunit 1 mg, sodium dihydrogenphosphate, disodium hydrogen phosphate, sodium chloride and sterile water [3]. Since the vaccine is 99 acid labile, each dose was provided with a sachet of sodium hydrogen carbonate. Buffer solution was prepared by dissolving the sachet in 150 ml of water. The full dose of vaccine was mixed with 40, 75 and 150 ml of buffer solution, for persons 2-4 years, 5-11 years, and over 11 years, respectively.

This study was conducted from 7th May to 21st June 2009. In this department, the total numbers of staff and their relatives are 950 (583 females and 367 males). The two doses of vaccine were given at 7-day interval. The age, sex, volume of vaccine ingested by each individual were recorded at the time of first visit. The side effects were interviewed at the time of taking second dose.

Totally 1,664 doses were administered and vaccine coverage was: 849 (517 females and 332 males)/950 (89%) for first dose and 815 (492 females and 323 males)/950 (85.8%) for second dose. Among our staff, 101 (10.6%) had not received any dose of the vaccine. Among 849 vaccinees (383 staff

and 466 relatives), there were 14 and 20 drop-outs, respectively, and their complaints were diarrhoea and itchiness.

All the vaccinees who had taken 2nd dose were interviewed for acceptability, 779 (95.6%) said they liked the taste and took the 2nd dose. The remaining 36 (21 females and 15 males) (3.8%) had some side effects such as abdominal pain (31), dizziness (28), abdominal discomfort (20), loose motion (19), nausea and vomiting (9), headache (9) and non-specific complaints, including nausea, weakness and loss of appetite by seven.

In this study, vaccine coverage of the total numbers of staff and their relatives were 89% for first dose and 85.8% for second dose. Among our vaccinees, only 3.8% reported side-effects following its ingestion. It gives a high level of protection: 100% protection one year later, in studied

vaccinees where there was still no cholera cases reported during May to July 2010.

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