RJM

Ruhuna Journal of Medicine

The Official Journal of the Faculty of Medicine
University of Ruhuna Galle, Sri Lanka
www.medi.ruh.ac.lk/journal

July 2014 *Volume 02* *Number 01*

Clock Tower, Galle Fort, Sri Lanka

RJM
Ruhuna Journal of Medicine

Proceedings of Faculty of Medicine Academic Session (FMAS) -2014, University of Ruhuna

Organizing / Editorial Committee

Cover picture

Dr. Peter Anthonisz who had saved the Fort from bubonic plague in 1922 has foresee the importance of Galle Fort as a protective barrier against natural elements. His efforts to save the ramparts from demolition by the British had lead the far-reaching consequences, for the Fort was the only part of Galle town to be proected from the 2004 tsunami. Dedication of this Clock Tower in later years to his name is rather justifiable.

Source: *Galle Fort: World Heritage Site* By Mark Thompson and Karl Steinberg
Cover page designed by Dr. Channa Yahathugoda
Photograph by Dr. Janaka Ruben

RJM
Ruhuna Journal of Medicine

Foreword

The future brings us many opportunities to improve our lives, but none so exciting as advances in Medicine. As doctors and scientists gain more knowledge about the human body they will find new ways to fight disease and conquer pain. The methods may take advantage of the process by which body defends itself, keeping us healthy in natural ways. No one should ever have to suffer prolonged pain or illness.

"*Hey! We're headed for the future.*" Neil Diamond sang in the 80s, and those words seem to echo the obsession society had at that time with technological advances the future world bring. A 1982 children's book called "*World of Tomorrow – Health and Medicine*" was full of predictions about medical care in the future. I won't go into detail about them all – you can read on each page for your self – but overall, it is pretty interesting how accurate some of the predictions were.

This year we have selected five completed higher degree projects and five abstracts to fill the 2nd FMAS programme. The research works done by our own academics and students cover quite a large spectrum from molecules to living things. In this convention they will get a chance to foretell some scientific predictions for the future. Therefore selecting "Enhanced Care Through Research" as the theme of FMAS-2014 is rather justifiable.

I am very humbled to witness and take part in the development of the FMAS to international standards by inviting world renowned scientists to share their experiences at the 2014 convention. Professor Tissa Kappagoda's key note address on "T*he Practice of Medicine in the 21st Century- Emphasizing a new kind of science*" will surely blow our minds. Subsequently, the audience will be introduced to new dimensions of cardiac health by Professor Kappagoda and his team.

I wish all said activities will uplift our academic profiles and bring much needed international recognition to our Faculty and the University. I express my sincere gratitude to all stakeholders of the FMAS-2014 and wish FMAS-2014 every success.

Dr. T. Channa Yahathugoda
Chairperson

RJM
Ruhuna Journal of Medicine

Message from the Vice Chancellor

First of all on behalf of University of Ruhuna, I as the Vice Chancellor, extend my warmest congratulations and sincere thanks to the Dean of the Faculty of Medicine, Chairman and the members of the organizing committee for organizing Faculty of Medicine Academic Sessions for the 2nd consecutive year. Therefore I send this message with great pleasure for the proceedings of the FMAS – 2014. I firmly believe this event will be an intellectual platform for academics, scholars, researchers and practitioners from diverse domains of medicine. Further I believe Ruhuna Journal of Medicine (RJM) will be a significant source of up to date information about medical research in the country. I suppose that the proceedings of the FMAS – 2014 which will be published in RJM will reflect the breadth of the research being conducted by our academics in the medical field.

As the Vice Chancellor, I am proud that our academics have continuously worked toward raising the bar in terms of quality and depth of research done and broadening the scope of the research work to ensure maximum impact across various subject areas. We have much to be proud of a record of research in a number of areas that have impacted positively on the development of the country, our graduates have gone on to serve at the highest levels within and outside the country and University of Ruhuna has grown in terms of student enrolment, staff complement and visibility among the universities in the country as well as in Asia. There is much more to be done, but I do believe that we have now established a strong base from which we can continue to develop. As University of Ruhuna looks at its future, particularly in times of limited financing and increased competition, Faculty of Medicine Academic Sessions and RJM must serve as important platforms to cultivate even more vibrant research culture within our academics. My warmest congratulations again!

Professor Gamini Senanayake
Vice Chancellor,
University of Ruhuna

RJM
Ruhuna Journal of Medicine

Message from the Dean

I am most happy to witness the 2nd Academic sessions of the Faculty of Medicine and send this message of best wishes. The 1st Academic session was held in 2013 and I was fortunate to be the Chief organizer of that event. This year event is an improved version of the last year event and includes a keynote address delivered by a world renowned scientist Prof Tissa Kappagoda.

Higher education institutes are essentially academic institutes and they are expected to contribute to the forward march of the own institution and, in general, the society. Being a medical faculty, an additional contribution must be made to improve the current patient and health care facilities.

Faculty of Medicine, University of Ruhuna has made significant advances in the recent past. Three new academic streams have been introduced and the intake of medical undergraduates has been increased to meet the national demand. Academics, however, despite heavy workload constantly engage in productive research projects.

Faculty of Medicine is on the top when the success rate of registered higher degrees is considered. Most of the registered higher degrees get completed within the allocated time period and Faculty Academic sessions provide a platform to disseminate those findings.

Finally, I wish all the success for this year Academic sessions and hope that participants will enjoy the proceedings.

Prof Sarath Lekamwasam
Dean,
Faculty of Medicine

Studies on selected bioactivities of *Gymnema lactiferum* leaf, *Canthium coromandelicum* leaf and Palmyrah *Pinnatu*

Thushari Bandara

Medical Laboratory Science Degree Program, Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka

ABSTRACT

This study was conducted to assess some bioactivities of *Gymnema lactiferum* var. *lactiferum* and hypocholesterolaemic effects of *Canthium coromandelicum* leaf and palmyrah *pinnatu*. *G. lactiferum* leaf showed significant hypoglycaemic and hypocholesterolaemic effects on a rat model and patients with type 2 diabetes mellitus. *Canthium coromandelicum* leaf on Wistar rats showed a significant decline in serum total cholesterol levels. Isolated pectin from the leaf could maintain the decline further. High molecular weight pectin appears to be responsible for the hypocholesterolaemic effect. *Pinnatu* did not show any hypocholesterolaemic effect on Wistar rats. It is proposed that the hypocholesterolaemic effect of fresh palmyrah fruit pulp is due to still unknown factors that are destroyed in the *pinnatu* manufacturing process. Pectin appears not to be a major factor.

Corresponding author: Thushari Bandara

<wvthush@yahoo.com>

This study was performed in University of Sri Jayewardenepura and the results were included in a thesis with five published papers and eight oral communications for a M. Phil degree with the University of Sri Jayewardenepura. The thesis defense examination was held on 9th April 2008.

Background

There has been a close relationship between man and plants throughout the development of civilization. In spite of providing food and shelter plants also serve to prevent or cure diseases (1). The use of plants for healing purposes predates human history and it has contributed much for modern medicine (2). At present there is a renewed interest towards the consumption of herbs. According to the World Health Organization (WHO), about 80% of people worldwide depend on herbal medicine for their primary health care needs (3). However, many herbal remedies that are used today have not undergone careful scientific investigations. Even though plant medicine comprises of natural substances they also can produce undesirable effects similar to what the regular drugs do (4). Some plants are highly toxic while some plant constituents react with other drugs or compounds in foods resulting overdoses and other harmful effects. The effectiveness of the plant medicines varies with the genetic and ecological differences as well. Contamination, adulteration and misidentification are the other main problems associated with these therapies (2). This does not mean that herbs do not work; it that we do not know for sure if all of them work or how well they work. Therefore it is of paramount importance to conduct research on medicinal plants and their products to evaluate their clinical efficacy and to determine their unfavorable effects if any by rigorous scientific approaches. This study was undertaken to evaluate some bioactivities of dried leaf suspensions of two medicinal plants namely *G. lactiferum var. lactiferum* and *Canthium coromendelicum* and the dried palmyrah fruit pulp called "*pinnatu*".

Study 1: Gymnema lactiferum var. lactiferum: is a twining/straggling plant which belongs to the family *Asclepiadaceae* (milk weed family). This plant is distributed in Assam, Malay Peninsula, Malaysia and in Sri Lanka (5). The identity of *G. lactiferum* has been subjected to controversy particularly with respect to its Sinhala names. In Sinhala it is known as 'Kurincha' (5,6). However, *Gymnema sylvestre* (Sinhala - Masbedda, Muva kiri-vel or Binnuga) is also known as 'Kurincha'. Kiri-anguna is also confused with kurincha and according to Ayurveda Pharmacopoeia (1961); Kiri-anguna and kurincha are very much similar plants (7). Leaves of the plant are used in salads, curries and herbal gruel. *G. lactiferum leaves have been used* as a supportive treatment for diabetes mellitus by the people in Jaffna, Sri Lanka for several decades. It has been further reported that this treatment could produce some favorable effects on lipid profiles as well. Based on these evidence this prospective study was designed to evaluate the hypoglycaemic and dislipidaemic effects of *G. lactiferum* leaves. Leaf powder was prepared from the plants grown in Jaffna, with organic compost avoiding the use of synthetic fertilizers, insecticides and fungicides. The plant was identified at the National Herbarium, Sri Lanka with reference to specimen no 1652. Mature leaves were washed and dried hygienically under sunlight for 4-5 days within an enclosed place covered with a glass roof. After grinding and sieving the dried leaves, leaf powder was packaged into 100g packets. *G. lactiferum* leaf powder contained 14.8% ash, 2.8% digestible carbohydrates, 63.6% insoluble dietary fibre (IDF), 1.3% soluble dietary fibre (SDF), 0.5% pectin, 15.3% protein and 3.3% calcium on dry weight. Calcium content was considerably higher than most of the green leafy vegetables consumed in Sri Lanka (8).

Acute and chronic effects of G. lactiferum leaf powder on glycaemic and lipidaemic status of type 2 diabetes mellitus Long Evans rats: This study was carried out on male Long Evans rats inbred in the research division, Bangladesh Institute for Research and Rehabilitation of Diabetes, Endocrine and Metabolic disorders (BIRDEM). Type 2 diabetes mellitus was induced by a single intraperitoneal injection of streptozotocin (STZ) at a dose of 90 mg/kg body weight (b.w.)/10 mL pH 4.5 citrate buffer (0.1 M) to the 48 h old rat pups (9). Experiments were performed after three months of STZ injection after confirming the presence of diabetes. To study the acute effects, rats were fasted for 12 h with free access to water and fasting blood samples were taken from the tip of tail. *G. lactiferum* leaf powder (1.25 g/kg b.w in 10 mL of water) was administered to diabetic rats orally, 30 min before a glucose load (2.5 g/kg b.w) Control rats were fed with an equal volume of deionized water. A positive control group was fed with an oral glibenclamide dose (5 mg/kg b.w./ 10 mL water). Blood samples were drawn after 60 min and 105 min and were analyzed for glucose (by enzymatic GOD-PAP, Randox Laboratories Ltd, UK). To study the chronic effects, diabetic rats were divided into three groups (n=8 each) and vehicle group was given water, Standard drug treated group received glibenclamide at a dose of 5 mg/kg b.w. /day for four weeks and the test group-was treated with *G.lactiferum* leaf powder suspension at a dose of 1.25 g/kg b.w. for four weeks. Body weights of the rats were measured in each week. Blood was drawn on day zero, 7, 14 and 21 by amputation of the tail tip under mild ether anesthesia. On the 28th day the animals were sacrificed and blood samples were collected. The serum glucose levels were determined (by enzymatic GOD-PAP, Randox Laboratories Ltd, UK) from the 7th, 14th and 21st day samples. Serum total cholesterol (Cholesterol oxidase/ peroxidase, CHOD-PAP), triglycerides (GPO-PA method, Randox Lab, UK) HDL-cholesterol (CHOD-PAP method, Randox Lab, UK), LDL-cholesterol (10), glucose and insulin [using rat insulin ELISA kit (11)] levels were determined on the day zero and 28.

G. lactiferum leaf powder did not show any acute effect on blood glucose levels of type 2 diabetic rats on glucose challenge test. Administration of leaf powder suspension for 28 consecutive days resulted in a gradual reduction in serum fasting glucose levels (15.5% and 37.3% reduction on the day14 and 28

respectively) and on the day 28 the levels had become significantly lowered (p=0.001) compared to day zero as shown on table 1. Serum total cholesterol levels of the *Gymnema* treated group were reduced significantly by 9.01% (p=0.015) as shown in table 2. Serum TG level and LDL- cholesterol levels were reduced by 23.5% (p=0.465) and 12.3% (p=0.189) respectively in the *Gymnema* treated group. HDL-cholesterol levels were increased by 39.42% (p=0.165). Results indicate improvement of lipid profiles in type 2 rats (table3).

Serum creatinine concentrations did not changed significantly throughout the study period among the different groups whereas serum ALT levels reduced significantly in glibenclamide treated group (p=0.014) while the values of other groups remained steady. The liver glycogen contents were not significantly different (p=1.000) between different groups. Fasting serum insulin content did not change in *Gymnema* treated group (p=0.893) whereas a 26.3% increase was observed in glibenclamide treated group (p=0.612).

Table 1: Effect of *G. lactiferum* leaf powder on glycaemic status of type 2 diabetic rats

Group	*Fasting serum glucose levels (mmol/L) Mean ±SD*				
	Day 0	Day 7	Day 14	Day 21	Day 28
Water	9.03 ± 0.68	9.13 ± 0.90	8.54 ± 1.63	7.81 ± 1.99	7.79 ± 1.82
Glibenclamide	9.50 ± 1.15	9.82 ± 1.52	10.30 ± 1.15	10.33 ± 4.07	7.19 ± 1.50
G. lactiferum	8.95 ± 1.36	10.18 ± 4.23	7.76 ± 3.54	8.75 ± 0.86	5.56* ± 0.72

*p – 0.001 in comparison to day 0 (as determined by the paired Student's t-test). Six animals were included in each group

Table 2: Effect of *G. lactiferum* leaf powder on serum total cholesterol and HDL levels

Group	*Serum levels (mg/dL) Mean ± SD*			
	Total Cholesterol		**HDL**	
	Day 0	Day 28	Day 0	Day 28
Water	67.00 ± 3.58	69.67 ± 13.77	29.33 ± 3.72	34.83 ± 8.70
Glibenclamide	70.83 ± 10.96	66.00 ± 9.90	31.50 ± 6.95	32.33 ± 7.15
G. lactiferum	76.00 ± 7.18	69.00* ± 6.07	27.67 ± 4.67	36.67 ± 10.13

*p – 0.015 in comparison to day 0 (as determined by the paired Student's t-test). Six animals were included in each group

Table 3: Effect of *G. lactiferum* leaf powder on serum LDL and TG levels

Group	*Serum levels (mg/dL) Mean ± SD*			
	LDL		**TG**	
	Day 0	Day 28	Day 0	Day 28
Water	19.37 ± 7.76	19.93 ± 7.95	91.50 ± 20.03	74.67 ± 22.32
Glibenclamide	20.23 ± 4.77	20.80 ± 6.90	95.50 ± 26.22	64.33* ±14.94
G. lactiferum	28.17 ± 12.91	14.90 ± 11.06	100.83 ± 21.78	87.00 ± 43.29

*p – 0.018 in comparison to day 0 (as determined by the paired Student's t-test). Six animals were included in each group

Next the effect of *G. lactiferum* leaf powder on intestinal carbohydrate digestion/absorption was studied by means of the gut absorption technique using six segments of the gastro intestinal tract (12). Percentages of sucrose remaining in the whole GIT in the control and test groups (Mean SD) after 60 min was 41.46 ± 5.34 vs 94.51 ± 6.62 (p = 0.000) and after 120 min it was 3.39 ± 1.29 vs 24.16 ± 7.47 (p = 0.021). The amounts of sucrose remaining in the stomach in the *Gymnema* treated group were found to be significantly higher than control after 30, 60 and 120 min time intervals (p= 0.034, p= 0.000 and p = 0.023 respectively). The quantity of sucrose remained in the stomach after 60 min of the test group was not differ in comparison to the amount after 30 min (85.03 ± 6.24 vs 86.16 ±4.81, p= 0.89). The control group showed a significant reduction in the remaining sucrose quantity in the stomach after 60 min (21.40 ± 4.35 vs 57.40 ± 10.67, p = 0.01). Sucrose levels at different time intervals are shown in figure 1. On the other hand, the quantity of sucrose in the upper intestine remained without a significant change in the test group at 30 min and 60 min time intervals (2.66 ±0.95 vs 2.00 ±0.81, p = 0.61), but this showed a significant reduction in the control group (10.46 ± 1.43 vs 4.79 ± 0.83, p = 0.01). Blood glucose analysis after 30 min of simultaneous sucrose administration gave a 21.1% increment of blood glucose concentrations in the *Gymnema* treated group in comparison to 50.1% increment in the control group. After 60 min the increment was 52.4% in the test group where as 35.4% in the control group. Interestingly after 2 h that was 26.2% in test and 5.5% in the control groups. However, the blood glucose levels of the test group were not significantly different (p = 0.627, p = 0.522 and p = 0.233 for 30 min, 60 min and 120 min respectively) in comparison to the control.

Further, the effect of *G. lactiferum* on intestinal glucose absorption was studied by using gut perfusion technique (13). Results of this experiment showed a significant reduction (p=0.040) in glucose absorption in the presence of *Gymnema* (total amount of glucose absorbed for 30 min) in comparison to control (82.1 mmol/L ± 75.2 vs 135.5mmol/L ± 121.0). Results are summarized in the table 4 and figure 2.

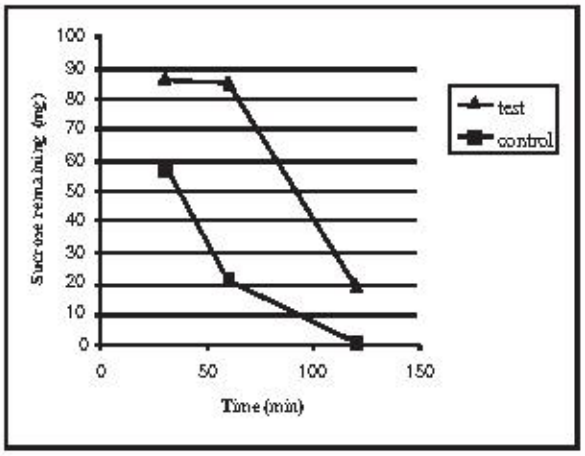

Figure1: Comparison of quantity of sucrose remaining in stomach at different time intervals

Effect of G. lactiferum leaf powder on glycaemic and lipidaemic status of patients with type 2 diabetic mellitus: Patients were recruited from the Out Patient Department of BIRDEM hospital. Type 2 diabetic mellitus patients (moderately controlled diabetics) with hypercholesterolaemia, aged 30-60 years were included. Exclusion criteria included the patients of more than 60 years of age, patients suffering from serious recurrent illnesses requiring systemic treatment and patients suffering from any other endocrinological diseases (hypo or hyperthyroid patients, patients with diabetes insipidus). Diabetes was diagnosed according to WHO criteria established in 1999 and hypercholesterolaemia was detected according to their lipid profile levels. The test group (n=12) consumed *G. lactiferum* leaf powder twice daily (3.5 g x 2) for one month. The control group consisted of 14 patients with type 2 diabetes mellitus and hypercholesterolaemia. The body weight, waist circumference and hip circumference of the patients were recorded at the beginning and at the end of the study period. Patient's complaints on twingling sensation of limbs, vertigo, headache, gastric discomfort, generalized weakness, constipation, nausea, drowsiness, dryness of mouth, anorexia and flatulence were recorded in each of their visits to see if there were adverse effects due to consumption of leaf powder. On the day zero the patient reported in

the fasting condition. Fasting blood samples (10 mL) were drawn from the antecubital vein. Then the patients (test group) received *G. lactiferum* leaf powder (3.5 g) dissolved in 30 mL of water. They were requested to swallow the suspension and then have breakfast according to their diet charts. Next blood sample was drawn at 1 h and 2 h and then they were provided with the other doses of *G. lactiferum leaf* powder (each individual dose wrapped in a paper pack and sealed in a polythene bag) for the rest of the week. They were requested to take the preparation just before breakfast and just before dinner every day for one month. From the fasting sample 1mL was transferred to an EDTA containing tube for measurement of HbA_{1c} and the rest of the blood was centrifuged. The level of HbA_{1c} was estimated by using by VARIANT Hemoglobin A_{1c} program (BIO-RAD, USA). Serum samples on the first and last day of the experiment were analyzed for fasting glucose, total cholesterol, TG, HDL- cholesterol, SGPT and creatinine as described above.

The body weight of the *Gymnema* treated group had reduced by 0.74% at the end of the experiment in comparison to their day zero values (66.92 ± 12.11 vs 67.42 ± 12.57, p = 0.089) and 0.39% reduction of the same parameter was observed in the control group (62.57 ± 10.84 vs 62.87 ± 11.31, p = 0.204). The leaf powder did not produce any acute effect on blood glucose levels of type 2 diabetic patients. However, there were significant reductions (p = 0.026 for 60 min and p = 0.022 for 120 min) in post prandial serum glucose levels in the *Gymnema* treated group on the day 29 in comparison to day zero. On the day 29, the fasting blood sugar (FBS) levels of the *Gymnema* treated group was reduced significantly (p=0.002) with 18.15% reduction compared to the control group (table 5). Patients of both groups were on their conventional oral hypoglycaemic drug doses. Though the study period was four weeks, there was a significant (p=0.012) reduction (11.6%) in HbA_{1c} levels in comparison to day zero values in the *Gymnema* treated group (6.99 ± 0.93 vs 7.91 ± 1.72). However, such reduction was not observed in the control group (7.39 ± 1.24 vs 7.58±1.68, p = 0.316). The serum total cholesterol levels of the *Gymnema* treated group reduced significantly (p = 0.004) by the 29th day (table 6). There was a 12.3% reduction in comparison to the day zero value. However, the serum HDL levels remained steady (p = 0.417) throughout the study period. As shown in the table 7, there was a significant reduction (p = 0.023) in the serum LDL levels in the *Gymnema* treated group on day 29 in comparison to day zero. The reduction was 15.5%. However, this therapy could not produce any significant reduction (p = 0.380) in serum TG levels **(table 7)**. Serum ALT (p = 0.261) and creatinine (p = 0.797) levels remained steady in both groups throughout the study period. No complaints were made by the patients while consuming the leaf powder. Some patients had a history of generalized weakness, gastric discomfort and constipation prior to the treatment. All the above complaints improved and gradually subsided while being fed on the leaf powder.

Table 4: Percentage glucose absorption and SEM

Time (min)	*% Glucose Absorption*	
	Krebs solution	Krebs +leaf powder
5	16.02 ± 5.13	5.84* ± 2.23
10	10.01 ± 3.45	9.65 ± 3.31
15	10.01 ± 4.16	7.18 ± 3.21
20	10.82 ± 4.19	6.71 ± 2.82
25	10.28 ± 2.77	8.45 ± 2.80
30	11.03 ± 3.19	7.67 ± 2.02

*p=0.017 in comparison to Krebs solution

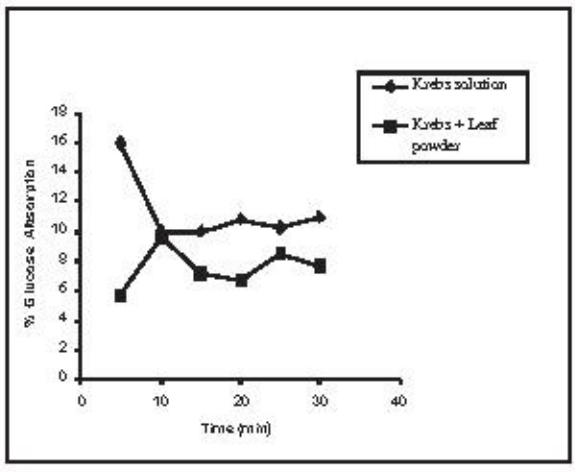

Figure 2: Effect of *G. lactiferum* leaf powder on the upper intestinal glucose absorption profile

Table 5: Effect of *G. lactiferum* leaf powder on FBS levels of patients with type 2 diabetes mellitus

Group	*Fasting serum glucose levels (mmol/L) Mean ± SD*		
	Day 0	Day 15	Day 29
Gymnema treated group (n=12)	8.05 ± 1.79	7.19 ± 1.56	6.59* ± 1.28
Control (n=14)	7.47 ±1.84	7.12 ± 1.61	6.84 ± 2.16

*p–0.002 in comparison to day 0

Table 6: Effect of *G. lactiferum* leaf powder on serum total cholesterol and HDL levels

Group	*Serum levels (mg/dL) (Mean ± SD)*			
	Total Cholesterol		**HDL**	
	Day 0	Day 29	Day 0	Day 29
Gymnema group (n=12)	235.83 ± 26.37	206.75* ± 35.74	36.82 ± 7.44	34.67 ± 6.15
Control (n=14)	235.71 ± 32.86	218.29 ± 38.36	37.54 ± 7.40	35.17 ± 8.23

* p–0.004 in comparison to day zero

Table 7: Effect of *G. lactiferum* leaf powder on serum LDL and TG levels

Group	*Serum levels mg/dL Mean ± SD*			
	LDL		**TG**	
	Day 0	Day 29	Day 0	Day 29
Gymnema group (n=12)	155 ± 20	131* ± 33	222.75 ± 81.10	207.17 ±105.41
Control (n=14)	155 ± 36	140 ± 44	218.14 ± 104.19	213.93 ± 99.88

*p–0.023 in comparison to day 0

Previous studies on the bioactivity of *G. lactiferum* are scarce. The hypoglycaemic activity of *G. sylvestre* is extensively studied. It has been suggested that the hypoglycaemic effects of *G. sylvestre* due to activation of the enzymes that are responsible for the utilization of glucose and stimulation of the glycogen synthesis (14) and stimulation and stimulation of insulin secretion by repair or regeneration of pancraeatic beta cells (15). In the present study *G. lactiferum* did not produce any significant acute effect on glucose challenge in diabetic rats. This is unlike what is reported about the *G. sylvestre*. According to Rokeya *et al.* (1999) *G. sylvestre* with a simultaneous glucose load, showed a significant reduction of serum glucose

concentration in both type 1 and type 2 diabetic rats (16). In the chronic study on type 2 diabetic model rats, although it was observed that body weight of the rats increased in all the groups; the increments were not significant. Hence, it can be concluded that *G. lactiferum* does not have any effect on degradation of depot fat and it can maintain the body weight in the type 2 diabetic state. Feeding of leaf powder suspension caused a gradual reduction in FBS levels of type 2 diabetic rats and the reduction became significant (p=0.001) after a period of 28 days. The effect of long term consumption of *G. sylvestre* on FBS has been extensively studied and the most potent principle was found to be *Gymnemic* acid IV (17).Glycogen metabolism in the liver regulates the blood glucose level. The regulation of glycogenesis and glycogenolysis in the liver is central to the regulation of glucose homeostasis. Liver glycogen level was estimated to determine if *G. lactiferum* leaf powder could increase liver glycogen content thereby exerting a hypoglycemic action. In the present study there was no significant effect ($p = 1.000$) on glycogen deposition in the liver in diabetic rats. This may reflect the absence of an effect by the leaf components on insulin secretion. As reported by Shanmugasundarum *et al.* (1983), treatment with *G. sylvestre extract* for twelve weeks had significantly increased ($p < 0.001$) liver glycogen contents of alloxan-induced diabetic rabbits (14). Unlike *G. sylvestre*, *G. lactiferum* did not increase the serum insulin levels. It is evident that the hypoglycaemic effect of *G. lactiferum* was brought about by some extra pancreatic factors, which are not related to the level of serum insulin. However, any factor which can either trigger insulin activity or which can overcome insulin resistance may be contributory to the hypoglycaemic effect.

Dyslipidaemia, particularly low levels of serum HDL-cholesterol and high levels of serum total cholesterol, TG and LDL-cholesterol, are important risk factors for atherosclerotic complications of diabetes mellitus (18). Hypercholesterolaemia and hypertriglyceridaemia have been reported to occur in STZ diabetic rats (19). Insulin deficiency fails to activate the enzyme lipoprotein lipase and causes hypertriglyceridaemia. Therefore it was considered that estimating serum cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol levels was relevant. A significant ($p = 0.015$) decline was shown in serum total cholesterol but not in serum HDL ($p = 0.165$), LDL ($p = 0.189$) or TG ($p = 0.465$) in the *G. lactiferum* treated group. There are few reports on the effect of *G. sylvestre* on lipid metabolism. Terasawa *et al.* reported that the serum TG and total cholesterol levels tended to be slightly lowered in obese but not in lean rats receiving long-term administration of a leaf extract of *G. sylvestre* (20). According to Nakamura *et al.* the fecal excretion of steroids and bile acids is increased in rats by oral administration of gymnemic acids contained in *G. sylvestre* leaves (21).

An attempt was made to study the effect of *G. lactiferum* on intestinal carbohydrate digestion and absorption by using the gut absorption technique, using sucrose. When the leaf powder was given by gavage simultaneously with sucrose solution to rats, it suppressed the resulting rise of serum glucose concentration and increased the unabsorbed sucrose content throughout the gastrointestinal tract. The amount of sucrose remaining in the stomach was significantly high in all three time intervals ($p = 0.034$, $p = 0.000$ and $p = 0.023$ for 30 min, 60 min and 120 min respectively). The results suggest an effect of *G. lactiferum* on gastric emptying and gastric motility. Though the postprandial blood glucose levels were not significantly affected there was a delay in the rise of postprandial glucose concentrations in the treated group, indicating a delay in glucose absorption or its prevention up to some extent. These effects might be associated with the inhibition of intestinal disaccharidase activity (as observed with alpha glucosidase inhibitors).

Invivo glucose absorption by the upper part of the small intestine was studied. A significant inhibition was observed ($p = 0.017$) only in the first fraction (for the first five minutes). The total amount of glucose absorbed over a period of 30 min of perfusion was significantly ($p =$

0.041) reduced in comparison to the control. Shimizu *et al.* has reported that some gymnemic acids from the leaves of *G. sylvestre* can suppress the glucose absorption in the small intestine of guinea pigs (22).

G. lactiferum is eaten as food and therefore should not pose a problem with regard to toxicity. The unaffected serum ALT (alanine transaminase, p = 0.807) and serum creatinine test (p = 0.148) support this conclusion with respect to liver and kidney function.

Based on these results there was enough confidence to test the *G. lactiferum* leaf on diabetic patients. Type 2 diabetic patients with hypercholesterolaemia who were not on hypocholesterolaemic or any other antidislipideamic drugs were included in the study. Patients of both test and control group were on their conventional oral hypoglycaemic drug (not insulin). There were problems with patients in that a double blind experiment was not possible to be conducted. Patients were aware of the tests and control groups. Despite this, after 28 days of treatment there was a significant (p = 0.002) decline in FBS levels. (This was seen in the animal model too). This indicates the added advantage of *G. lactiferum* therapy on the glycaemic status of the patients. Similar effects by *G. sylvestre* were reported by Shanmugasundarum *et al.* (15) and Balasubramanium *et al.* (23). Further, like in the case of rats there was no significant effect on post prandial glucose levels. Since the length of the study was four weeks, HbA_{1c} was measured only over this period of time. Despite the high t ½ of HbA_{1c} this parameter showed a significant decline (p = 0.012). Diabetic patients were tested for lipidaemic effects before and after the administration of *G. lactiferum* leaf powder suspension for 28 days. Serum total cholesterol concentrations (p = 0.004) and LDL (p=0.023) declined significantly, but there was no significant change in HDL (p = 0.417) and in TG (p=0.380). According to Balasubramanium *et al.* (23) serum total cholesterol, TG and free fatty acid levels of mild diabetics have shown significant declines on the ingestion of *G. sylvestre* leaf powder over a period of only seven days. Studies on serum ALT (p = 0.261) and creatinine (p = 0.797) indicated no adverse effects on liver and kidney of diabetic patients as in the case of animal experiments.

Study 2. Hypocholesterolaemic activity of Canthium coromandelicum

C. coromandelicum is a gregarious plant which belongs to the family Rubiaceae (24). The plant is known as 'Kara' in Sinhala. It grows in the western parts of the Indian Peninsula from Konkon southwards to Sri Lanka. In Sri Lanka it is commonly found in low country especially in the dry zone (24). Edible leaves of the plant were collected from Belihuloya in Sabaragamuwa province. Pressed samples of the vegetative parts were identified at the Department of Botany, Faculty of Science, University of Sri Jayewardenepura. Edible leaves were oven dried at 65-70˚C for 4-5 h and ground into a fine powder. The leaf contained 6.3% ash, 67.2% insoluble dietary fibre, 3.2% SDF and 10.2% proteins on dry weight. The amount of fibre was high and depending on the sample a high variation in pectin content was observed (21-45.2% SDF). The effect of the whole leaf powder was tested on male Wistar rats. Control group was fed on WHO recommended rat and mouse breeding feed (25) and test group was fed with a *C. coromandelicum* leaf powder incorporated diet. Modifications were done in the test diet in order to make the test diet isocaloric and with the same amount of insoluble dietary fibre to the control. Serum total cholesterol levels of the animals were estimated on the day zero, 31 and after seven weeks of the commencement of the experiment using CHOD-PAP kit. During the next four weeks the test rats were fed with SDF (pectin) extracted from the same amount of *C. coromandelicum* leaf powder. The amount of SDF added to test diet was balanced with grass powder. At the end of the four weeks serum total cholesterol levels of the animals were estimated. On feeding the whole leaf associated diet for seven weeks the serum total cholesterol levels of the rats in the test group were lowered significantly (p = 0.049). Separated pectin from *C. coromandelicum* leaf maintained (p = 0.025) the low cholesterol levels for the following four

weeks showing that pectin was likely to be one causative agent for the hypocholesterolaemic effect (table 8). It is known that the high molecular weight pectin can exert a hypocholesterolaemic effect (26). Sepharose gel chromatography confirmed that the molecular weight of pectin was extremely high, equal or more than two million Daltons. The leaf was found to contain 21.0% to 45.2% pectin in its soluble dietary fibre. These findings suggested that high molecular weight pectins are at least partly responsible for the hypocholesterolaemic effect of *C. coromandelicum* leaf. According to literature, dietary fibre can contribute to lowering of serum cholesterol by multiple mechanisms. High viscosity dietary fibre increases the viscosity of digesta in the upper gastrointestinal tract. This can interrupt the digestion and absorption of lipids by altering the rate of diffusion and the activity of some hydrolytic enzymes (27). Dietary fibre especially SDF binds with bile salts and prevents re-absorption *via* entero-hepatic circulation. This results in an increase in bile salt excretion in feces thus a lowering of serum cholesterol levels (28, 29). Further, the fermented products of SDF especially pectin inhibit HMG CoA reductase, the key regulatory enzyme of cholesterol synthesis in the liver and other tissues. This produces short chain carboxylic acids such as propionic and butyric acid by gut bacteria (30, 31), which are the inhibitory agents. High molecular weight pectin can effectively bind with cholesterol and bile salts, resulting in a reduction in serum cholesterol levels (26).

Table 8: Effect of *C. coromandelicum* leaf powder and isolated soluble dietary fibre on serum total cholesterol levels of Wistar rats

	Cholesterol (mg/ dL) Mean SD							
	Day 0		Day 31		After 7 weeks		After 11 weeks	
Group								
Test (n=6)	82.37	8.01	66.46	4.87	61.93[1]	8.62	76.37[2]	5.62
Control(n=6)	82.87	7.04	70.40	6.62	72.69	8.06	91.75	13.17

[1]p– 0.049, [2] p–0.025 in comparison to control. During the last four weeks the test rats were fed with soluble dietary fibre extracted from the same amount of *C. coromandelicum* leaf powder

Study 3: Palmyrah Pinnatu

In the last part of the study the effect of dried palmyrah fruit pulp (PFP) [*Pinnatu]* was tested on the serum total cholesterol levels of Wistar rats. *Pinnatu* is a popular snack fruit leather) among the Tamil people especially in the North and East of Sri Lanka. *Pinnatu:* was prepared under strict hygienic conditions, at the Industrial Technology Institute, from a bulk sample of PFP from the fruits obtained from Kalpitiya and Hambantota. Fruit pulp was layered (up to 1 cm thickness) on a piece of cloth and dried at 65°-67°C using the Mitchel dryer. When the first layer was dried the second PFP layer was layered on top of it and similarly dried. In this manner the whole sample was dried in successive layers (six layers) for 50 h in order to prepare brownish, sticky, chewing gum like '*pinnatu*'. The cholesterol lowering effect of *pinnatu* on Wistar rats was not significant ($p = 0.10$, $p = 0.50$ and $p = 0.79$ for day 14, 28 and 42 respectively). This is different to what had been reported earlier (32) on PFP. It was suggested that destruction of some active principle was taking place during the preparation or storage of *pinnatu*. As pectin was suspected to be the causative agent for the cholesterol lowering effect, the hydrolysis of pectin could have been the reason for the loss of hypocholesterolaemic activity in *pinnatu*. This postulate was supported by the gel chromatography elution profiles of *pinnatu* SDF and pectin. The next step was to attempt to prepare *pinnatu* while preserving pectin by

deactivating the activities of pectinases. This was done by heating of freshly extracted PFP as early as possible at 70-80˚C for 15 min at pH 2.5 and readjusting pH up to 4.6. The sepharose gel chromatography pattern of the isolated pectin of the modified *pinnatu* showed no degradation of pectin. Therefore it was evident that the pectinase activity had been successfully inhibited. However, on testing this modified *pinnatu* similarly on the Wistar rats, did not resulted in any hypocholesterolaemic ($p = 0.85$) activity. It is proposed that the hypocholesterolaemic effect of fresh palmyrah fruit pulp is due to still unknown factors that are destroyed in the *pinnatu* manufacturing process. Pectin appears not to be the major factor.

Conclusion

Gymnema lactiferum var. lactiferum has hypoglycaemic and hypocholesterolaemic effects on rat models and humans with diabetes mellitus and has potential as a functional food. *Canthium coromandelicum* has a hypocholesterolaemic effect caused partly or totally by pectin. It has potential as a functional food. Preparation of *pinnatu* destroys the hypocholesterolaemic effect of palmyrah fruit pulp. The major causative substance for lowering cholesterol has not been identified. High molecular weight pectins are not the only causative agent responsible for the lipid lowering activity.

Acknowledgement

Author is grateful to Emeritus Prof. E. R Jansz and Prof. Sagarika Ekanayake (University of Sri Jayewardenepura), Emeritus Prof. K. Balasubramanium (University of Jaffna), Prof Liaquat Ali and Prof. Begum Rokeya (Bangladesh Institute for Research and Rehabilitation of Diabetes Endocrine and Metabolic disorders) for their guidance, supervision and enormous support. Research grants from International Program In Chemical Sciences (IPICS-SRI-07) and Asian Network of Research on Anti-diabetic Plants (ANRAP) are also acknowledged.

References

1. Gilani AH (2002). Medicinal value of food plants. *Diabetic Endocrine Journal* **30(1):**8.
2. Vickers A and Zollman C (1999). ABC of complementary medicine: Herbal Medicine. *British Medical Journal* **319:**1050-1053.
3. Chakrabarthi S and Mukharjee B (2006). Plant antidiabetics: an overview. *Proceedings of 5th ANRAP International Seminar*, Published by ANRAP, Kuala Lumpur, Malaysia, 8-10 Nov 2006.
4. Nick H, Mashour MD, George I, Lin MD, Williams H and Frishman MD (1998). Herbal medicine for the treatment of cardiovascular diseases. *Archives of International Medicine* **158**:2225-2234.
5. Dasanayake MD and Fosberg FR (1983). A Revised Hand Book to the Flora of Ceylon. Vol. IV. Amering publishers Co. Pvt. Ltd., New Delhi. pp 97-98.
6. The Sri Lankan Forester (The Ceylon Forester) (1974), Vol. XI, No 3 and 4. Published by Sri Lanka Forest Department. pp 94.
7. Ayurveda Phamacopia (1961), Vol. I. part 3. Published by the Department of Ayurveda, Colombo, Sri Lanka. pp 114.
8. Perera WDA, Jayasekara PM and Thaha SZ (1989). Table of food composition for use in Sri Lanka. Published by UNICEF.
9. Bonner-Weir S, Trent DF, Honey RN and Weir GC (1981). Responses of Neonatal Rat Islets to Streptozotocin, Limited B-cell Regeneration and Hyperglycaemia. *Diabetes* **30**:64-69.
10. Friedewald WT, Levy RI and Fedrickson DS (1972). Estimation of the concentration of Low-density Lipoprotein Cholesterol in plasma, without use of the preparative Ultracentrifuge. *Clinical Chemistry* **18**:499-502.
11. Kratzsch J, Ackermann W, Leliacker H, Berch W and Keller E (1990). A sensitive sandwich enzyme immunoassay for measurement of insulin on microtitre plates. *Experimental and Clinical Endocrinology* **95**:229-236.
12. Goto Y, Yamada K, Ohyama T, Matsuo T, Odaka H and Ikeda H (1995). An alpha-glucosidase inhibitor AO-128 retards carbohydrate absorption in rats and humans. *Diabetic Research and Clinical Practice* **28**:81-87.

13. Swintosky JV and Pogonowska E (1982). The *in-situ* rat gut technique. *Pharmacy International* 163-164.

14. Shanmugasundarum KR, Paneerselvam C, Samudram P and Shanmugasundarum ERB (1983). Enzyme changes and glucose utilization in diabetic Rabbits: The effect of *Gymnema sylvestre*, R.Br. *Journal of Ethnopharmocology* 7:205-234.

15. Shanmugasundaram ERB, Gopinath KL, Shanmugasundaram KR and Rajendran M (1990). Possible regeneration of islets of langerhans in streptozotocin diabetic rats given *Gymnema sylvestre* leaf extracts. *Journal of Ethnopharmacology* **30:**265-279.

16. Rokeya B, Nahar N, Ali L, Hassan Z, Nur-e-Alam M, Chowdhury NS, Azadkhan AK and Mosihuzzaman M (1999) Effects of five medicinal plants on blood glucose levels in non-diabetic and diabetic model rats. *Diabetic Research* **34:**219-228.

17. Sugihara, Yoshitaka, Nojima, Hiroshi, Matsuda, Hisashi, Murakami, Toshiyoki, Yoshikawa, Masayuki, Kimura and Ikuko (2000). Antihyperglycaemic effect of GA IV, a compound derived from *Gymnema sylvestre* leaves in streptozotocin diabetic mice. *Journal of Asian Natural products Research* **2**(4):321-32.

18. Laakso M and Lehto S (1997). Epidemiology of macrovascular disease in diabetes. *Diabetes Research* **5:**294-315.

19. Sharma SR, Dwivedi SK and Swarup D (1997). Hypoglycemic, antihyperglycemic and hypolipidemic activities of *Caesalpinia bonducella*. *Journal of Ethnopharmacology* **58:**39-44.

20. Terasawa H, Miyoshi M and Imoto T (1994). Effects of long-term administration of *Gymnema sylvestre* water extract on variation of body weights, plasma glucose, serum triglycerides, total cholesterol and insulin in Wistar fatty rats. *Yonago Acta Med* **37:** 117-127.

21. Nakamura Y, Tsumura Y, Tonogai Y and Shibata T (1999). Fecal steroid excretion is increased in rats by oral administration Gymnemic acids contained in *Gymnema sylvestre* leaves. *Journal of Nutrition* **129:**1214-1222.

22. Shimizu K, Iino A, Nakajima J, Tanak K, Nakajyo S, Urakawa N, Atsushi M, Wada T and Yamashita C (1997). Suppression of glucose absorption by some fractions extracted from *Gymnema sylvestre*. *Journal of Veterinary and Medical Sciences* **59(4):**245-251.

23. Balasubramanium K, Arsaratnam V, Nagwswaran A, Anushiyanthan S and Muganthan N (1992). Studies on the effect of *Gymnema sylvestre* on Diabetics. *Journal of National Science Council Sri Lanka* **20**(1):81-89.

24. The Wealth of India (1950). A dictionary of Indian raw materials and Industrial products. Raw materials. Vol II. Council of Scientific and Industrial Research. India. Delhi.

25. Sabourdy MA (1988). Breeding and care of laboratory animals WHO/lab. 88.1:45.

26. Judd PA and Truswell AS (1985). The hypocholesterolaemic effect of pectin in rats. *British Journal of Nutrition* **53**:409-425.

27. Topping DL, Oakenful D, Trimble RP and Illman RJ (1998). A viscous fibre (methyl cellulose) lower blood glucose and plasma triglycerides and increases the liver glycogen independently of volatile fatty acids production in rats. *British Journal of Nutrition* **59:**21-30.

28. Arjamardi BHJ, Nathani AS and Reeves RB (1992). Dietary Soluble Fibre and cholesterol alter serum cholesterol concentrations, hepatic portal venous short chain fatty acids concentrations and faecal sterol excretion in rats, *Journal of Nutrition* **122:**246-253.

29. Vasudevan DM and Sreekumari S (2005). Text book of Biochemistry (for medical students) 4th Edition, Published by Jaypee Brothers Medical Publishers (P) Ltd. pp 319-320.

30. Bush RS and Milligan LP (1971). Study of mechanisms of inhibition of ketogenesis by propionate in bovine liver. *Canadian Journal of Animal Sciences* **51:** 121-127.

31. Bhagavan NV (2002). Medical Biochemistry. 4th Edition, Harcourt/Academic Press. pp 149-151.

32. Pathberiya LG (2005). Studies on the hypocholesterolaemic effect, *in-vitro* antioxidant capacity and carotenoids of Palmyrah fruit pulp. M.Phil. Thesis, University of Sri Jayewardenepura, Sri Lanka.

Relationship of serum leptin with body composition and historical risk factors of cardisease in premenopausal women aged 25 to 50 years

Nanayakkara J[1], Lekamwasam S[2], Liyanage C[3]

Departments of [1]Pharmacology, [2]Medicine and [3]Nuclear Medicine, Faculty of Medicine, University of Ruhuna, Sri Lanka

The dietary habits of people changed from plant-based diet to high fat, energy densed animal-based food. In addition, with the advancement of science people became less physicaly active. These significant changes of life style led to a pandemic of obesity in the world. Initially, obesity was considered a problem mainly confined to high-income countries. The trend has changed and, at present, overweight and obesity are on the rise in low and middle-income countries, especially among the urban population. In Sri Lanka the prevalence of obesity and its complications are increasing at an alarming rate (Tudawe, 2000; Wijewardene, at al 2005).

Corresponding author: J Nanayakkara

<nanajeeva@yahoo.com>

Leptin

Leptin hormone is a cytokine which is composed of 167 amino acids secreted by the adipose tissues and secretion is regulated by the Ob (Lep) gene located on chromosome 7 in humans. During the past fifteen years many studies have been conducted related to leptin, and now it is evident that leptin is not merely an appetite regulator but also involved in a wide range of metabolic actions in the human body.

Leptin is mainly secreted by white adipocytes in the body. In addition, it is also secreted by the brown adipose tissues, gastric epithelium and syncytiotrophoblasts of placenta, ovaries, skeletal muscle, stomach, mammary epithelial cells, bone marrow, pituitary and liver. Leptin circulates in serum in free and bound forms. (Auwerx & Staels, 1998). The mechanism of the regulation of leptin secretion in the adipose tissue is not fully understood.

Relationship of serum leptin with body composition and anthropometry

In this study, the relationship between body composition and serum leptin was studied and results revealed significant coorelations between serum leptin and weight, BMI, hip and, waist circumfernces and waist-hip ratio. Of all the anthropometric measurements studied, BMI was the best predictor of the serum leptin in this group of women. Although regression model excluded hip and waist circumferences as weak predictors in the presence of the BMI, these measurements also were able to predict serum leptin to the almost similar degree. Adjusting these correlations for the possible confounders such as age, average energy intake and level of physical activity did not change the results substantially and therefore it can be postulated that there is a genuine relationship between serum leptin and BMI.

Hip and waist circumferences represent the central fat mass, while BMI is more reflective of the total fat mass in the body and includes the peripheral fat tissue in which leptin is actively produced. This might explain why BMI and total fat which are measures of global adiposity of the body showed marginally better

correlations with serum leptin than the other anthropometric measurements which represent the regional adiposity.

Furthermore, when women were categorized according to their BMI, the highest mean serum leptin level was found in the highest BMI category (BMI >30.1) while the lowest mean serum leptin level was found in the lowest BMI category (BMI 18-22.5). These leptin values did not change when they were adjusted for age, total physical activity or average energy intake.

In this study sample, serum leptin concentration significantly and positively correlated with all the measurements of fat mass except the percentage abdominal fat content. Of these, the strongest correlation was seen between total fat mass and serum leptin. These findings are in accordance with the previous studies conducted on the relationship between serum leptin and fat mass of the body (Jun, *et al., 2010;* Shimizu, *et al.*, 1997). Jun *et al* (2010) found a significant correlation between serum leptin and fat mass ($r = 0.67$, $P < 0.0001$). In these studies, total fat mass, more than the regional fat content, was the main determinant of serum leptin. This fact is supported by the findings of Montague et al (1997), who also reported that leptin hormone is mainly produced by adipocytes and production is more prominent in subcutaneous fat than in visceral fat depots (Montague, et al., 1997).

A previous study conducted in the USA on Black and White males and females revealed a higher leptin concentration in Black females compared to White and total percentage body fat which again is a measure of global adiposity to have the highest correlation with leptin level (Constance E Ruhl et al., 2004). They have revealed percentage body fat which was estimated using DXA, showed the highest correlation with leptin ($r^2 = 0.56$ for both sexes). In the same study total body fat and BMI showed higher correlations with serum leptin than the visceral fat or measures of regional adiposity.

Existence of significant variations of body fat distribution among different ethnicities is well known. At the beginning of the current study we have anticipated a different behavior of serum leptin with fat mass due to the variation of body fat distribution between Caucasians and South Asians. A study conducted in Texas, USA on South Asian and Caucasian males revealed a significant ethnic variation in body fat distribution (Chandalia, *et al.*, 2007). Even though there were no differences in BMI, waist or hip circumferences between the groups, South Asians had a higher total body fat and subcutaneous fat mass. Intraperitoneal fat masses were virtually identical in both South Asian and Caucasian males. Truncal skin fold thicknesses were higher in South Asians. Furthermore, South Asian men had higher plasma concentrations of leptin than Caucasians and the adipocyte cell size was significantly higher in the South Asian group (Chandalia, et al., 2007). Similar findings were also reported by Shimizu, *et al.* They found that subcutaenious fat in the abdominal region is one of the main sources of serum leptin showing a positive correlatation with serum leptin level in obese as well as non-obese females (Shimizu, *et al.*, 1997). Similar study conducted in Canada has also shown that Asian Indian men and women have higher leptin concentrations than Caucasian men and women (Jessica Smith, *et al.*, 2006). These data further reinforce findings of the current study in which a stronger correlation was found between serum leptin and BMI as well as total fat mass.

From above studies it can be postulated that although South Asians females have a significantly higher leptin level than its USA counterparts, subcutaneous fat tissue still is the major source of serum leptin. Therefore, irrespective of ethnicity, the stronger association between serum leptin and surrogate indices of total fat mass is still maintained. The significance of higher serum leptin level observed among Asians needs to be addressed. This may be clinically relevant since variations of body fat distribution and serum leptin level may contribute to increased insulin resistance observed among South Asians (Chandalia, *et al.*, 2007). Apart from the role of regulation of appetite, serum leptin acts as a mediator in maintaining body homeostasis. Furthermore role of leptin in a wide range of pathological

processes which determine the morbidity and mortality of disease processes and the contribution leptin makes to these outcome measures among Asians need to be explored.

Although we found a strong correlation between BMI and leptin, BMI explained only a smaller variation of leptin. The remaining 82% variation of serum leptin may be related to genetics but needs further studies to confirm.

Validity of BMI, hip and waist circumferences as surrogate measures of obesity in middle aged Sri Lankan premenopausal females

In general, obesity reflects body fat content and there is no reliable way to assess the body fat content, clinically. Current practice is to use either body mass index (BMI) or waist circumference (WC) as surrogate measurements of percentage body fat (BF%). This practice has been challenged due to the discovery of variations of fat mass in Asian population in comparison to European counterparts According to the findings of this study the current practice of defining obesity (i.e. BMI >30) has several drawbacks. One important fact is that BMI is a poor correlate of BF%. Although marginally better, WC also showed a poor correlation with BF%. While WC explained 24% of the BF% variation, BMI could explain only 17% variation in the BF%. This was evident further when BMI in general showed low sensitivity and specificity values. Due to this, there is a high possibility of missing actual individuals with obesity.

BMI cut-off values and corresponding sensitivity, specificity positive and negative predictive values are shown in Table 1.

Table 1: Cut-off values of BMI, hip and waist circumferences and corresponding sensitivity, specificity positive and negative predictive values.

Variable	*Cut –off values*	*Sensitivity*	*Specificity*	*PPV*	*NPV*
BMI (Kg/m^2)	30	32.7	95.8	97.1	24.7
	25	56.7	70.8	89.4	27.4
	24.5	61.5	70.8	90.1	29.8
	24	67.3	70.8	90.9	33.3
Hip circumference (cm)	92	68.3	75.0	92.2	35.3
Waist circumference (cm)	78	71.2	75.0	92.5	37.5

PPV - Positive predictive value
NPV - Negative predictive value

A trade off was seen between the sensitivity and specificity values of different BMI thresholds. According to findings of this study BMI 24 was the most appropriate value to determine obesity. Reduction of the BMI cut-off value from 30 to 24 improved the sensitivity at the expense of specificity. It is interesting to note that HC of 92 and WC of 78 also gave sensitivity, specificity and predictive values similar to BMI of 24. This indicates that any of these thresholds can be used to detect obesity with equal validity and only the practicality of the technique would

determine its use in both community and clinical settings. Most of the body measurements are correlated with BF%, significantly, they are not good predictors of obesity. The BMI, HC and WC thresholds that are in current use need to be redefined to suit the local subjects. We have suggested alternative cut-off values to be applied on middle aged premenopausal females.

Relationship of serum leptin with serum lipids (total cholesterol, HDL, LDL), fasting blood glucose and blood pressure

In the current study, serum leptin showed no significant correlations with lipids (total cholesterol, HDL, LDL, triglycerides) or fasting blood glucose. However, there was a statistically significant inverse correlation between serum leptin level and mean arterial blood pressure in subjects with systolic blood pressure >40 mmHg ($r = -0.45$, $P < 0.05$). Similar type of a positive correlation also seen in subjects with a BMI > 30 ($r = 0.32$, $P > 0.05$).

In normotensive subjects as well as subjects with diastolic blood pressure <90 mmHg and subjects with BMI <30 there was no statistically significant correlations between serum leptin and blood pressure.

Even though animal studies have found a clear association between serum leptin and blood pressure (Hiraoka *et al.*, 1997 and Ogawa *et al.*, 1998) data on human studies are not very conclusive. Findings of the current study are on par with findings of a Chinese study which also did not find an association between serum leptin and blood pressure (Wang, *et al.*, 1999). Findings of a another study conducted in Poland on a group of normotensive subjects and patients with essential hypertension also failed to find an association between serum leptin and blood pressure (Kokot *et al.*, 1999). Similar study conducted in the USA on a group of men who had untreated mild hypertension has initially found a correlation between serum leptin and hypertension but the association became insignificant when it was controlled for the BMI of the subjects (Narkiewicz *et al.*, 1999).

Some human studies however have reported an association between serum leptin and blood pressure. A study conducted in Japan on normotensive and hypertensive subjects showed a higher leptin level in the hypertensive group even after adjusting for the age, BMI and gender of the subjects (Agata *et al.*, 1997). Another study conducted in Turkey on lean, obese/ overweight essential hypertensives and age, gender, waist/ hip ratio and BMI matched group of normotensives demonstrated a strong association between serum leptin and hypertension. Moreover, plasma leptin levels correlated, significantly, with plasma angiotensinogen levels in this study group (Uçkaya *et al.*, 1999). Furthermore, Maria et al (2003), found a significant correlation between serum leptin and blood pressure and this association was independent of BMI (Maria, *et al.*, 2003). This particular study used 24-hour ambulatory BP monitoring and this may have an effect on the outcome of the study.

In the current study there were no statistically significant associations between serum leptin and lipid levels. A previous study using a group of hyperlipidaemic patients and a normal control group found no statistically significant relationship between serum leptin and lipoproteins in the normal group as well as in patients with combined hyperlipidaemia (Haluzik, *et al.*, 1999). Findings of the current study is concordant with another case-control study conducted in Turkey on a group of lean females with polycystic ovarian syndrome (PCOS), obese PCOS females and normal females. This study also found no significant association between serum leptin and lipoproteins (Erel *et al.*, 2003).

In the current study, serum leptin and fasting blood glucose levels showed no significant correlation with each other. This finding is consistent with findings of Chen *et al.*, who investigated the effects of internal change of plasma glucose and serum insulin levels on serum leptin concentrations in normal subjects and type II diabetics (Chen, *et al.*, 1999). A cross-sectional study conducted in Italy on a sample of obese and non-obese males and females, however showed a direct correlation

between serum leptin and fasting plasma glucose and this was evident only in the obese group and not in the control group (Corica *el al.*, 1999).

In the current study there was a significant difference between the mean leptin values of patients with and without metabolic syndrome. Subjects with metabolic syndrome had a higher mean leptin value than the subjects who did not have the metabolic syndrome. Leptin resistance that is seen in obese individuals may explain the higher leptin levels in patients with metabolic syndrome. Renal and sympathetic actions which are preserved in the leptin resistance status may play an important role in pathogenesis of metabolic syndrome (Marcelo & Kamal, 2003).

Similar results were observed in a study conducted in Taiwan (*Wen-Cheng Li, et al., 2011*). In this study increased leptin level was considered a predictor of metabolic syndrome in men as well as in women. Paul, *et al.* (2005) conclude that leptin can predict the development of metabolic syndrome which is characterized by dyslipidemia, glucose intolerance, insulin resistance and hypertension.

These findings may have a significant clinical implication since leptin can be utilized as a surrogate marker to predict metabolic syndrome. In the present study insulin resistance was not studied and that can be identified as a limitation in understanding the behavior of leptin among obese women.

Relationship of serum leptin with current diet and physical activity

In the current study correlations of serum leptin with current diet and physical activity were investigated. Serum leptin was negatively, and significantly, correlated with average energy intake and protein intake. Adjusting these correlations for age, did not change the results materially. But when they were controlled for both age and BMI, correlations between serum leptin and average energy and protein intakes became statistically insignificant. Therefore, it can be postulated that the inverse correlation between serum leptin and average energy intake is dependent on the BMI of the subject.

A previous study conducted in the USA found no association between serum leptin and dietary patterns (Vijay, *et al.*, 2009). Another study from the USA on a group of males with metabolic syndrome also showed no correlation between baseline intake of total energy, protein, carbohydrates, and cholesterol level with the plasma leptin concentration (Janne *et al.*, 2001). Considering these data it can be postulated that association between diet and serum leptin is not conclusive and more studies should be done to clarify this association. Also whether the association between serum leptin and diet has a geographical variation is worth considering.

There was no relationship between serum leptin and physical activity seen in this study and there was no significant difference in the mean leptin levels of subjects with different degree of physical activity. A population-based cross-sectional study has revealed a negative association between serum leptin and physical activity previously (Franks *et al.*, 2003). Another study conducted on ob/ ob mice has also further supported the findings of the Franks, *et al.* (2003) (Hwa *et al.*, 1997). In contrast, a cross-sectional study involving Pima Indian children in the USA has revealed a positive correlation between leptin levels and the physical activity (Salbe *et al.*, 1997). Malgorzata, *et al.* (2011) showed a significant difference in serum leptin level in physically active group of school children compared to physically inactive counterparts.

When the interaction between the physical activity and energy intake was assessed, the highest mean leptin value was seen among those physically least active and in the lowest energy intake category. The lowest mean leptin was seen among those physically very active and in the highest tertile of the energy intake. The differences were not statistically significant probably due to the limited sample size in this study and further studies should be done to examine this interaction in more detail.

Relationship of the components of body composition and historical risk factors of cardiovascular diseases (blood pressure, serum lipids, blood sugar)

Correlations of the components of body composition and historical risk factors of cardiovascular diseases were investigated in this study. Total fat mass showed significant correlations with total cholesterol, LDL, triglycerides, cholesterol/ high density lipoproteins ratio, BMI, mean systolic and diastolic blood pressure and fasting plasma glucose. Furthermore, truncal fat mass correlated, significantly, with total cholesterol, LDL, BMI, mean systolic blood pressure and fasting plasma glucose. These values did not change significantly when adjusted for age, total physical activity and average energy.

Abdominal adiposity plays a vital role in pathogenesis of cardiovascular diseases. Association between truncal fat and cardiovascular diseases is well known and current study also showed significant correlations between truncal fat and total cholesterol, LDL, systolic blood pressure and fasting plasma glucose.

In the present study lean mass positively correlated with total cholesterol, LDL, BMI and systolic blood pressure. In general when compared to fat mass, lean mass is considered a protective factor in cardiovascular disease. Our study showed significant correlations between muscle mass and important cardiovascular risk factors like total cholesterol, LDL, and systolic blood pressure. This needs to be further studied since it is an unexplored finding and very few studies have been conducted to address this issue.

Bone mineral concentration (BMC) and Bone mineral density (BMD) significantly correlated with total cholesterol, LDLC, and BMI. Furthermore BMC also correlated with systolic blood pressure in the current study. Most of the studies conducted in this area have revealed an inverse association between bone mass and cardiovascular risk (Lone *et al.*, 2004). In the current study correlations between BMC and BMD with total cholesterol, LDLC and systolic blood pressure became insignificant once they were controlled for the weight of the subject.

These findings have important clinical implications since therapeutic life style modifications on cardiovascular diseases target the fat mass. In prevention and therapeutic management of cardiovascular diseases, therapeutic procedures should be focused on reduction of BMI since most of the correlations with components of body composition and cardiovascular risk factors operated through BMI.

Associations described above have clear clinical implications. They help to understand the pathogenesis of NCDs and also emphasize the importance of therapeutic life style modifications which target the truncal obesity in the management of cardiovascular diseases. Even in lean individuals, abdominal adiposity plays a vital role in the pathogenesis of cardiovascular diseases.

The effects of dietary and physical activity modifications on serum leptin level and historical risk factors of cardiovascular diseases

This intervention examined the effect of diet and physical activity on CV risk factors as well as on leptin. The main focus of the intervention was to determine the behaviour of leptin during the intervention and to determine whether the effects of intervention were mediated through the action of leptin. Although therapeutic life style modifications are known to reduce CV risk profile, the exact mechanism of such effect is not well established.

The intervention resulted in significant reductions of leptin, BMI, FBS, systolic and diastolic BPs, total cholesterol, triglyceride, LDL cholesterol, Framingham score and 10-year CV risk in the Intervention group. When between-groups differences (between the Intervention and Control groups) were considered, except leptin all the other changes remained significant.

The observed changes of CV risk factors following the intervention were not related to leptin changes. They showed a stronger association with BMI change during the intervention. BMI showed a better association than hip and waist circumferences when explaining the effects of the intervention. Therefore it can also be inferred that the benefits of the intervention on CV risk factors are not mediated through leptin but more related to variation of BMI of the subjects.

According to the findings of the current study, the effect of physical activity combined with dietary modification on leptin was inconclusive. Although there was a significant reduction of leptin level after the intervention, the difference between the intervention and control groups was not significant. This occurred as there was a non-significant reduction of leptin among controls during the study period. Previous studies on leptin and life style modifications have generated conflicting results.

A study conducted in the USA showed that long-term changes in lifestyle consisting of decreased intake of dietary fat and increased physical activity reduced plasma leptin concentration (Janne, *et al.*, 2001). They also detected a significant difference of leptin levels between intervention and control groups following the intervention.

Louis Pérusse, *et al.* (1997) reported the effect of physical activity intervention on a group of sedentary adult men and women for 20 weeks duration and reported considerable inter-individual differences in the leptin level in response to acute and long-term effects of exercise; some individuals showed either increase or reduction in leptin, while others showed almost no change. On average, leptin levels were not significantly changed by the physical activity intervention.

The Phase II study was designed to understand the underlying mechanism of the cardiovascular (CV) benefits the intervention caused. During the planning of the study, it was felt that life style interventions may bring CV benefits either through their direct effects on body proportions such as BMI, hip or waist circumferences or may modify determinants of energy consumption or appetite such as leptin. According to the current study the change in BMI was the most plausible explanation for the beneficial effects of the intervention. BMI showed stronger correlations (r) and coefficients of determinations (r^2) than other body measurements such as HC or WC or leptin. Also there was no correlation between changes of serum leptin and BMI during the intervention. Hence, our findings indicate that the beneficial effect of the intervention is probably mediated through its direct effects on BMI than on leptin. A unit change of BMI was associated with significant changes in many CV risk factors and these changes were independent of the baseline BMI value.

There were notable and significant changes in the control group as well. Systolic and diastolic blood pressures, total cholesterol, triglycerides and HDL values significantly increased at the end of the study period. Framingham risk score and ten-year risk of cardiovascular event among controls did not change during the period of study. Significant weight gain seen in controls may be due to continuation of their normal dietary habits and less involvement in the physical activity.

Involvement of serum leptin in pathogenesis of hypertension and metabolic syndrome is well known. One clinical application of the findings of the current study is that low intensity physical activity or dietary intervention may not be effective in curtailing the pathological effects of leptin in disease processes. When deciding the dietary and physical activity interventions in the management of cardiovascular diseases and metabolic syndrome it is important to consider the intensity and the duration of the physical activity in order to achieve the desired therapeutic goals. According to the findings of the current study cardiovascular risk reduction achieved following the dietary and physical activity interventions, may operates through BMI.

References

1. Agata J, Masuda A, Takada M et al. 1997 High plasma immunoreactive leptin levels in essential hypertension. *Am J Hypertens* 1997;**10**:11711174.

2. Andrew Mente Fahad Razak Stefan Blankenberg Vlad Vuksan, A. Darlene Davis Ruby Miller Koon Teo Hertzel Gerstein Arya M. Sharma Salim Yusuf, Sonia S. Anand 2010 Ethnic Variation in Adiponectin and Leptin Levels and Their Association With Adiposity and Insulin Resistance Diabetes Care July 2010 vol. **33** No.7, 1629-1634.

3. Auwerx J Staels B. 1998 Leptin. *Lancet* **351**:737-42.

4. Chandalia M, Lin P, Seenivasan T, Livingston EH, Snell PG, et al. (2007) Insulin Resistance and Body Fat Distribution in South Asian Men Compared to Caucasian Men. PLoS ONE 2(8): e812. Doi:10.1371/journal.pone.

5. Chen S-C Kochan JP Campfield A Burn P Smeyne RJ. Splice 1999 variants of the ob receptor gene are differentially expressed in brain and peripheral tissues of mice. *J Recept Signal Transm R* 1999;**19**:245-266.

6. Constance E, Ruhl James, E Everhart. Leptin concentrations in the United States: relations with demographic and anthropometric measures 2001. *American Journal of Clinical Nutrition*, September 2001;**74**(3):295-301.

7. Corica F, Allegra A, Corsonello A, Buemi M, Calapai G, Ruello A, Nicita Mauro V, Ceruso D. Relationship between plasma leptin levels and the tumor necrosis factor-alpha system in obese subjects. *Int J Obes Relat Metab Disord* 1999 Apr;**23**(4):355-60.

8. Erel CT, Senturk LM, Kaleli S, Gezer A, Baysal B, Tasan E. Is serum leptin level regulated by thyroid functions, lipid metabolism and insulin resistance in polycystic ovary syndrome? *Gynecol Endocrinol.* 2003 Jun;**17**(3):223-9.

9. Franks PW, Farooqi IS, Luan J, Wong MY, Halsall I, O'Rahilly S, Wareham NJ. Does physical activity energy expenditure explain the between-individual variation in plasma leptin concentrations after adjusting for differences in body composition? *J Clin Endocrinol Metab* 2003;**88**:3258-3263.

10. Haluzik M, Fiedler J, Nedvidkova J, Ceska R. Serum leptin concentration in patients with combined hyperlipidaemia: Relationship to serum lipids and lipoproteins. *Physiol Res* 1999;**48**:363-368.

11. Hiraoka J, Hosoda K, Ogawa Y, et al. 1997. Augmentation of obese (ob) gene expression and leptin secretion in obese spontaneously hypertensive rats (obese SHR or Koletsky rats). *Biochem Biophys Res Commun* 1997;**231**:582585.

12. Hwa JJ, Fawzi AB, Graziano MP, Ghibaudi L, Williams P, Van HeekM, Davis H, Rudinski M, Sybertz E, Strader CD. Leptin increases energy expenditure and selectively promotes fat metabolism in ob/ obmice. *Am J Physiol Regul Integr Comp Physiol* 1997;**272**:R1204R1209.

13. Janne E Reseland, Sigmund A Anderssen, Kari Solvoll, Ingvar Hjermann, Petter Urdal, Ingar Holme, Christian A Drevon. Effect of long-term changes in diet and exercise on plasma leptin concentrations. *Am J Clin Nutr* February 2001;**73**(2):240-245.

14. Jessica Smith, Maha Al-Amril, Allan Sniderman1 and Katherine Cianflone. Leptin and adiponectin in relation to body fat percentage, waist to hip ratio and the apoB/apoA1 ratio in Asian Indian and Caucasian men and women. Nutrition & Metabolism 2006;**3**:18 doi:10.1186/1743-7075-3-18).

15. Jun I, Tsuyoshi T, Yoshihiro S, Hideo M. Serum Leptin Concentration Positively Correlates with Body Weight and Total Fat Mass in Postmenopausal Japanese Women with Osteoarthritis of the Knee 2011 *Arthritis* Volume 2011(2011), Article ID 580632, 6 pages doi:10.1155/2011/580632.

16. Kauter K, Ball M, Kearney P, Tellam R, McFarlane JR. 2000 Adrenaline insulin and glucagon do not have acute effects on plasma leptin levels in sheep: development and characterization of an ovine leptin. *ELISA Journal of Endocrinology* 2000;**166**:127135.

17. Kokot F, Adamczak M, Wiecek A, Cieplok J. 1999 Does leptin play a role in the pathogenesis of essential hypertension? *Kidney Blood Press Res* 1999;**22**:154160.

18. Lone Jørgensen, Oddmund Joakimsen, Gro K. Rosvold Berntsen, Ivar Heuch Bjarne K. Jacobsen Low Bone Mineral Density Is Related to Echogenic Carotid Artery Plaques: A Population-based Study *Am. J. Epidemiol* 2004;**160**(6):549-556.

19. Louis Pérusse Gregory Collier Jacques Gagnon Arthur S. Leon D. C. Rao James S. Skinner Jack H. Wilmore André Nadeau Paul Z. Zimmet Claude Bouchard Acute and chronic effects of exercise on leptin levels in humans. *Journal of Applied Physiology* July 1, 1997;**83**(1):5-10.

20. Malgorzata Plonka, A Toton-Morys, P Adamski, A Suder, W Bielanski, M J Dobrzanska, A Kaminska, B Piorecka, J Glodzik. Association of the Physical Activity with leptin blood serum level, body mass indices and obesity in schoolgirls. *Journal of Physiology and Pharmacology* 2011;**62**(6):647-56.

21. Marcelo LGC, Kamal R. Role of leptin cardiovascular and endocrine complecations in metabolic syndrome Diabetes, obesity and metabolism 2003;**8**:603-610.

22. Maria TG, Maria RM, Enzo B, Rosanna DV, Graziano R, Marina M, Michele N, Sergio S, Giovanni D. Association between Serum Leptin Levels and 24-Hour Blood Pressure in Obese Women *Obesity Research* 2003;**11**:549555.

23. Metabolic Syndrome Independently of Obesity. *Obesity Research* 2005;**13**:14761484.

24. Montague CT, Prins JB, Sanders L, Digby JE, O'Rahilly S. 1997 Depot- and sex-specific differences in human leptin mRNA expression: implications for the control of regional fat distribution Diabetes **46**:342-7.

25. Narkiewicz K, Somers VK, Mos L, Kato M, Accurso V Palatini, P1999. An independent relationship between plasma leptin and heart rate in untreated patients with essential hypertension. *Journal Hypertens* 1999;**17**:245249.

26. Ogawa Y, Masuzaki H, Aizawa M et al. 1998 Blood pressure elevation in transgenic mice overexpressing leptin, the obese gene product. *Journal Hypertens* 1998;**16**[Suppl2]: 7.on 12 August 2011.

27. Panarotto D, Ardilouze JL, Tessier D, Maheux P. The degree of hyperinsulinaemia and impaired glucose tolerance predicts plasma leptin concentrations in women only: a new exploratory paradigm 2000 Aug;**49**(8):1055-62.

28. Paul WF, Soren B, Jianan L, Ulf Ekelund, Mushtaquar R, Sadaf F, Ian H, Stephen Rahilly Nicholas. Leptin Predicts a Worsening of the Features of the Metabolic Syndrome Independently of Obesity. *Obesity Research* 2005;**13**:14761484.

29. Shimizu H, Shimomura Y, Hayashi R, Ohtani K, Sato N, Futawatari T, Mori M. Serum leptin concentration is associated with total body fat mass, but not abdominal fat distribution 1997 *International Journal on Obesity* July 1997;**21**(7):536-541.

30. Tudawe I (unpublished draft 2000)). The cost of diet-related disease in Sri Lanka. Food and Nutrition Bulletin 2001;**22**(4).

31. Ückaya G, Ozata M, Sonmez A, et al. 1999 Plasma leptin levels strongly correlate with plasma renin activity in patients with essential hypertension. *Horm Metab Res* 1999;**31**:435-438.

32. Vijay Ganji, Mohammad RK, Erin McCarthy. Serum leptin concentrations are not related to dietary patterns but are related to sex, age, body mass index, serum triacylglycerol, serum insulin, and plasma glucose in the US population *Nutrition ans Metabolism* 2009;**6**:3.

33. Wang G, Tang J, Chen M. Association of serum leptin concentrations with blood pressure 199 Zhonghua Yi Xue Za Zhi. 1999 Sep;**79**(9):664-7.

34. Wang J, Thornton JC, Russell M, Burastero S, Heymsfield S, Pierson RN Jr: Asians have lower body mass index (BMI) but higher percent body fat than do whites: comparisons of anthropometric measurements. *Am J Clin Nutr* 1994, **60**(1):23-28.

35. Wen-Cheng Li Kuang-Yu Hsia I-Chuan Chen Yu-Che Chang Shih-Hao Wang Kuan-Han Wu Serum leptin is associated with cardiometabolic risk and predicts metabolic syndrome in Taiwanese adults. *Cardiovascular Diabetology* 2011;**10**:36.

Anti-inflammatory natural products from plants used in traditional medicine: A mass spectrometric approach for fast screening and discovery

Napagoda MT

Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka

ABSTRACT

The traditional use of *Plectranthus zeylanicus* and *Munronia pinnata* as anti-inflammatory remedy was rationalized by the current study. Extremely potent 5-LO / mPGES-1 inhibitory activities were observed for the lipophilic extracts of the two plant species and the bioactive constituents were characterised by a rapid and convenient mass spectrometric approach.

Corresponding author: MT Napagoda

Introduction

Natural products (secondary metabolites) are low molecular weight compounds made by living organisms (plants, animals, microorganisms), and have been exploited for treating and preventing diseases since ancient times. Despite the recent interest in other drug discovery approaches such as molecular modeling, combinatorial chemistry etc., natural products still play a major role as new clinical candidates and drugs (1). About half of the drugs currently in clinical use are based on natural product scaffolds (2,3).

Although plants and their products have been systematically used in Sri Lanka for treating illnesses for over thousand years. Sri Lankan flora has not yet been adequately studied phytochemically or pharmacologically.

The isolation and structural elucidation of novel natural products from medicinal plants and investigations on their bioactivities is rewarding, however, the interest in this field is dramatically declining due to the long persisting problems associated with conventional natural products isolation approaches. The requirement for large scale extractions, as well as laborious isolation and purification methods which are highly technically demanding are no longer cost-effective and time-effective, thus hindering the chemical profiling of medicinal plants, and thereby the validation of their uses in traditional medicine.

Over the years, mass spectrometry (MS) has increasingly become an analytical tool of choice in the field of natural products chemistry owing to its high throughput nature, quantitative capability and the facility to integrate with chromatographic separation methods. These advanced hyphenated spectrometric techniques afford for a rapid identification and characterisation of secondary metabolites without the necessity of isolation and purification, and detailed information on their metabolic profiles can be obtained with a minimal amount of material (4). Therefore, the present study was focused on the application of MS-based rapid screening strategies to characterise bioactive metabolites in two popular Sri Lankan medicinal plants; *Plectranthus zeylanicus* (Iruveriya) and *Munronia pinnata* (Binkohomba), that are neither phytochemically nor pharmacologically evaluated yet.

Plectranthus zeylanicus Benth is a perennial herb of the family Lamiaceae with aromatic,

astringent and stomachic properties and is used in folk medicine in decoctions for fevers, dysentery, diarrhoea, vomiting and thirst (5,6). Furthermore, it is used as a constituent of various ayurvedic and traditional medicinal preparations (Kalkaya, Prameha, Kvathaya) and the plant is described to be effective in combating asthma, common cold, varieties of fever, cough, leucoderma, diarrhea, chronic ulcers etc (7). *Munronia pinnata* (Wall) Theob (Family Meliaceae) is a small herb which is considered as a rare species but is also distributed in several other Asian countries (8). It is considered to be one of the most expensive plant materials (US$ 50-110/kg) used in traditional medicine in Sri Lanka (9). In Sri Lankan folk medicine, the plant is a major ingredient of decoctions and powders used for the treatment of fever, dysentery, skin diseases, purification of blood upon snake bites and malaria (10,11). According to the pharmacopoeia, it exhibits wound purifying, anthelmintic, carminative and laxative properties, it improves digestive power, reduces dermatitis, promotes lactation, destroys worms and interestingly, it is also used for the treatment of polyuria, cough and edema (7,11). Despite the therapeutic importance, the scientific evidences are insufficient to rationalize the reported ethnopharmacological use of these two plant species.

Prostaglandins (PG) and leukotrienes (LTs) are formed from arachidonic acid (AA) and act as important mediators of inflammation, allergy and pain (12). LTs contribute to various inflammatory and allergic reactions in the pathophysiology of asthma, allergic rhinitis, atherosclerosis, cancer, etc (13). 5-lipoxygenase (5-LO) that catalyzes the first two key steps in LT biosynthesis from AA is considered as a valuable drug target (14,15). Among the PGs, the PGE_2 is formed from AA under inflammatory conditions essentially by cyclooxygenase (COX)-2 coupled to microsomal PGE_2 synthase (mPGES)-1 (16). Dual pharmacological intervention with both LT and PGE_2 biosynthesis proposes a strong therapeutic benefit in inflammatory diseases. In fact, plant-derived natural products have been reported to dually suppress 5-LO and mPGES-1 activity (17,18) which rationalizes these pro-inflammatory enzymes as functional targets for anti-inflammatory phytomedicine.

Therefore the present study was undertaken to evaluate the anti-inflammatory mode of action of *P. zeylanicus* and *M. pinnata* and characterise the related secondary metabolites.

Materials and Methods

Plant Material

P.zeylanicus was collected in Nittambuwa (Gampaha district) while *M. pinnata* was collected in Weerasuriyakanda (Gampaha district) and Algama (Kegalle district) in 2011/2012. The plants were identified by the author and authenticated by comparing herbarium specimens at the National herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka.

Preparation of crude extracts

The plant materials (whole plants) were dried, powdered (13 g of *P. zeylanicus*, 15 g of *M. pinnata*) and successively extracted with 600 ml of *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol. Besides, 3g of powdered materials were extracted in 300 ml of 70% methanolwater. The solvents were evaporated and the crude extracts were then subjected to the bioactivity studies.

Evaluation of Bioactivity

5 - Lipoxygenase (5-LO) activity in intact neutrophils

Human neutrophils were isolated from leucocyte concentrates obtained from the University Hospital Jena, Germany. The neutrophils were treated as described (19) and were preincubated for 15 min at 37°C with test compounds or vehicle (0.1% DMSO), and incubated for 10 min at 37°C with the Ca^{2+}-ionophore A23187 (2.5 μM) plus 20 μM AA. Thereafter the 5-LO products formation (LTB_4 and its trans-isomers, 5-H(P)ETE) were analyzed by HPLC as described (19). Cysteinyl-LTs C_4, D_4 and E_4 and oxidation products of LTB_4 were not determined.

5-LO activity in cell-free assays (purified 5-LO)

E.coli (BL21) was transformed with pT3-5-LO plasmid and recombinant 5-LO protein was expressed and partially purified as described (20). Aliquots of semi-purified 5-LO were immediately diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added. Samples were pre-incubated with the test compounds or vehicle (0.1% DMSO) and the 5-LO inhibition was detected as described (20).

Determination of mPGES-1 activity

A549 cells were prepared and the microsomal fraction was obtained (21). The resuspended microsomal membranes were preincubated with the test compounds or vehicle (DMSO). After 15 min, PGE_2 formation was initiated by addition of PGH_2 (20 μM). After 1 min at 4°C, the reaction was terminated, and PGE_2 was separated by solid-phase extraction and analyzed by RP-HPLC as described (21).

DPPH assay

The radical scavenger capability was assessed by measuring the reduction of the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as described (22). The absorbance was recorded at 520 nm after 30 min incubation of the test samples (100 il of 5, 25, 50 μg/ml) with DPPH in ethanol (50 μM , 100 μl) under gentle shaking in the dark. Ascorbic acid and L-cysteine were used as reference compounds. All analyses were performed in triplicates.

Measurement of reactive oxygen species in neutrophils

Neutrophils were preincubated with test compounds (or 0.1% DMSO as vehicle) for 15 min. Then, the peroxide-sensitive fluorescence dye 2', 7'- dichlorodihydrofluorescein diacetate (DCF - DA, 1 μg/ml) and $CaCl_2$ (1 mM) were added 2 min prior to the addition of phorbol myristate acetate (PMA, 0.1 μM). The fluorescence emission at 530 nm was measured after excitation at 485 nm in a thermally controlled (37°C) NOVO star microplate reader (BMG Lab technologies GmbH, Offenburg, Germany).

Statistical analysis

Data are expressed as mean ± S.E. IC_{50} values were calculated by nonlinear regression using Graph Pad Prism software one site binding competition. Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni/Tukey-Kramer post-hoc test for multiple comparisons respectively. A p value < 0.05 (*) was considered significant.

Phytochemical screening

Bioassay-guided fractionation

M. pinnata hexane extract (130 mg) and *P. zeylanicus hexane and DCM extracts (230 mg and 120 mg respectively)* were fractionated over a silica gel column. The samples were eluted with *n*-hexane, different mixtures of EtOAC in *n*-hexane (3%, 5%, 10%, 15%, 25%, 35%, 50%, 75%, 100%) and methanol, successively, yielding 11 fractions. The collected fractions were evaporated and then subjected to bioactivity assays and LC-MS analysis.

Liquid Chromatography coupled Mass Spectrometric (LC-MS) analysis

n-Hexane and DCM extracts of *P. zeylanicus and n*-hexane extract of *M. pinnata* and the fractions obtained thereof were analyzed on LTQ-Orbitrap instrument (Thermo Fisher, San Jose, CA) with electrospray ionization (ESI) /atmospheric pressure chemical ionization (APCI). 15 μl aliquots of the diluted samples (10 μg/ml in EtOAc) were injected and separated by liquid chromatography by DionexAcclaim® RSLC 120 C18 column (2.1 × 150 mm packed with 2.2 μm, 120 Å). Reversed phase UPLC gradient separations were performed using water and methanol as mobile phases. The ESI/APCI conditions were optimized to generate full scan and collision-induced dissociation (CID) mass spectra with 30,000 and 7500 resolutions, respectively. The full scan

mass spectra were recorded in the *m/z* range 100-2000. CID mass spectra were obtained at different collision energies between 1 and 55 eV.

Molecular formula identification

Following a published method (23), the molecular formula were identified by isotope pattern and fragmentation tree analysis. The fragmentation tree that explains the data best is calculated by an optimization algorithm.

Gas Chromatography coupled Mass Spectrometric (GC-MS) analysis

GC-MS analysis of the crude extracts and the fractions of interest was carried out on a gas chromatograph HP6890 (Agilent, CA, USA) connected to a MS02 mass spectrometer from Micromass (Waters, Manchester, UK) with EI 70 eV equipped with ZB5ms column (30 m × 0.25 mm, 0.25 µm film thickness; Phenomenex, CA, USA).

Results

Bioactvity and Phytochemistry of P. zeylanicus

Evaluation of 5-LO inhibitory activities of *P. zeylanicus*

P. zeylanicus extracts (100 µg/ml) that were prepared by using *n*-hexane or DCM as solvent strongly inhibited 5-LO activity in A23187-stimulated neutrophils (inhibition >85%), whereas extracts obtained by the use of hydrophilic solvents, (water or methanol) were comparably ineffective (Figure 1A). More detailed concentration-response studies using this cell-based assay revealed IC_{50} values of 6.6 and 12 µg/ml for *P. zeylanicus* extracts prepared with *n*-hexane and DCM, respectively (Figure 1B). The synthetic reference inhibitor zileuton (approved as anti-asthmatic drug) blocked 5-LO activity with IC_{50}=0.13µg/ml. The unspecific detrimental effects of the extracts on the viability of neutrophils can be excluded based on the ability of the cells to prevent trypan blue uptake in the presence of 10 or 100 µg/ml.

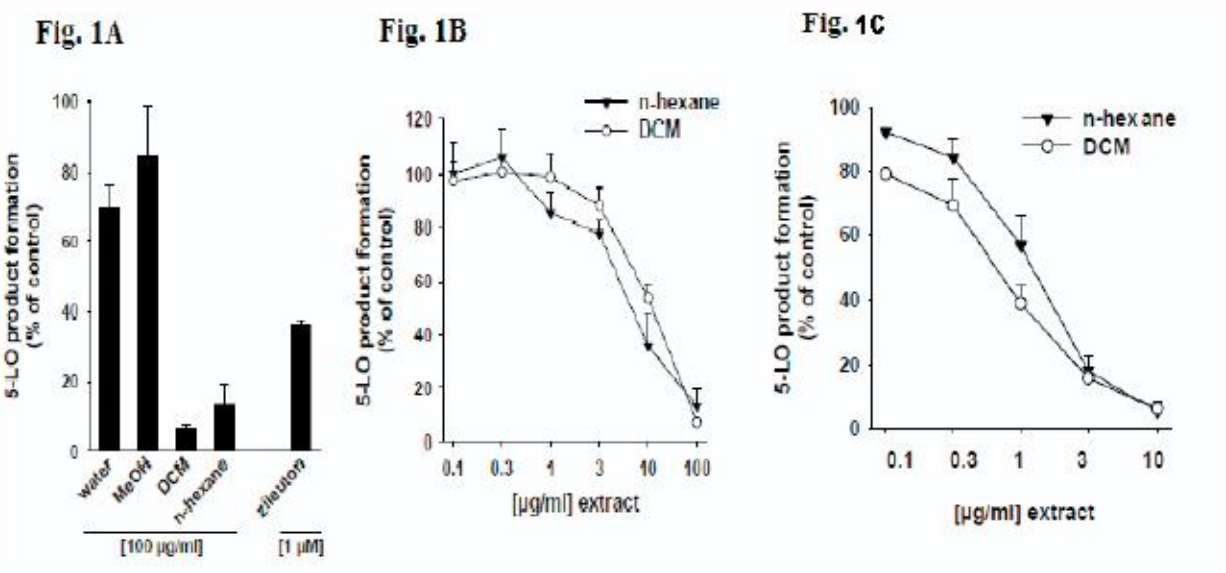

Figure 1: Inhibition of 5-LO activity by *P. zeylanicus* extracts: (A), (B) in intact human neutrophils and (C) in cell free assay. Data are given as mean +/-S.E.M., n = 3-4.

Suppression of 5-LO product synthesis in the cell may be caused by diverse mechanisms, others than interference with the 5-LO enzyme activity, thus to investigate whether or not the extracts directly inhibit 5-LO activity, a cell-free assay was applied. *n*-hexane and DCM extracts of *P. zeylanicus* caused concentration-dependent inhibition of cellular 5-LO with IC_{50} = 1.2 and 0.7 μg/ml, respectively (**Figure 1C**). For zileuton, the IC_{50} value was determined at 0.11 μg/ml.

Evaluation of radical scavenging properties and suppression of ROS formation in neutrophils

The majority of natural products from plant origin that suppress 5-LO activity may confer their inhibitory action by unselective antioxidant reactions as they reduce the active-site iron, decompose 5-LO-activating lipid hydroperoxides or scavenge intermediate fatty acid radicals within LT synthesis (18). To investigate whether such unselective antioxidant properties may account also for 5-LO inhibition by *P. zeylanicus* extracts, radical scavenging properties were assessed using the cell-free DPPH assay. In contrast to the reference antioxidants, the *n*-hexane or DCM extracts of *P. zeylanicus* were not able to significantly reduce radical formation, suggesting that 5-LO inhibition is not mediated by a redox-based mechanism. In accordance with the DPPH assay, neither the *n*-hexane nor the DCM extract of *P. zeylanicus* caused significant inhibition of ROS formation in neutrophils stimulated with the bacterial peptide fMLP.

Bioassay-guided separation of the *P. zeylanicus* extracts

To get more insights into the identity of the ingredient and composition of the extracts that might be responsible for the potent inhibition of 5-LO, the *n*-hexane and DCM extracts were separated by liquid column chromatography into 11 fractions that were analyzed for inhibition of isolated 5-LO in the cell-free assay at concentrations of 1 and 10 μg/ml, each. Out of the 11 fractions (F) of the *n*-hexane extract, F-6, F-7, F-8, F-9, F-10 and F-11 at a concentration of 10 μg/ml inhibited 5-LO activity by > 50%, and only F-9 and F-10 were active at 1 μg/mL (> 50% 5-LO inhibition). For the DCM extract, F-6, F-7, F-9, F-10 and F-11 were active at 10 μg/ml concentration in the 5-LO cell free assay, but none of these fractions showed significant 5-LO inhibitory activity at 1 μg/ml.

GC-MS analysis

Highly potent 5-LO inhibitory F-9 and F-10 from the *n*-hexane extract of *P. zeylanicus*, were subjected to a phytochemical screening by GC-MS and UPLC-MS. The GC-MS analysis of F-9 and F-10 revealed 13 and 12 components respectively, identified by comparison of their experimental mass spectrum with those recorded in the NIST MS Search 2.0 and Adams mass spectrum libraries as well as by comparison with the respective standards (Figure 2).

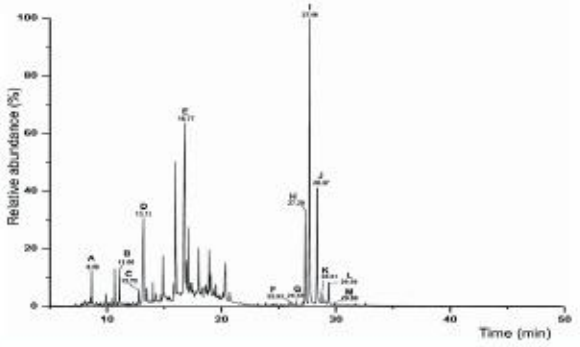

Figure 2: Characterisation of constituents of bioactive fraction F-9 of the *n*-hexane extract of *P. zeylanicus* by GC-MS

Total ion chromatograph of F-9 of the *n*-hexane extract of *P. zeylanicus* and its identified compounds. **A**: Eudesm-7(11)-en-4-ol, **B**: hexadecanoic acid, **C**: phytol, **D**: 9,12,15-octadecatrienoic acid, **E**: callitrisic acid, **F**: Cholest-5-en-3β-ol, **G**: ergosta-5,22-dien-3β-ol, **H**: campesterol, **I**: stigmasterol, **J**: β- sitosterol, **K**: β-amyrin, **L**: α-amyrin, **M**: stigmast-4-en-3-one.

UPLC - MS analysis

Analysis of F-9 by UPLC-MS revealed two uncommon compounds (denoted as compound **A** and **B**, Figure **3A**) and two common phytosterol derivatives were also detected in the TIC.

Only compound **B** yielded a substantial peak in the TIC of F-10.

The accurate mass measurements of compound **B** using the Orbitrap instrument and the search in the METLIN database suggested the presence of coleone P ($C_{22}H_{30}O_6$, Figure **3B**) in both F-9 and F-10. The identity of coleone P was further confirmed by its fragmentation pattern, which agrees with literature data[26]. In addition, the peak at *m/z* 383.20648 which corresponds to compound **A**, fits well with the molecular formula $C_{20}H_{30}O_7$ (mass accuracy of 0.131 ppm) suggesting the presence of cinncassiol A / cinncassiol C3, a diterpenoid which has been isolated from the family Lauraceae.

Fig. 3A

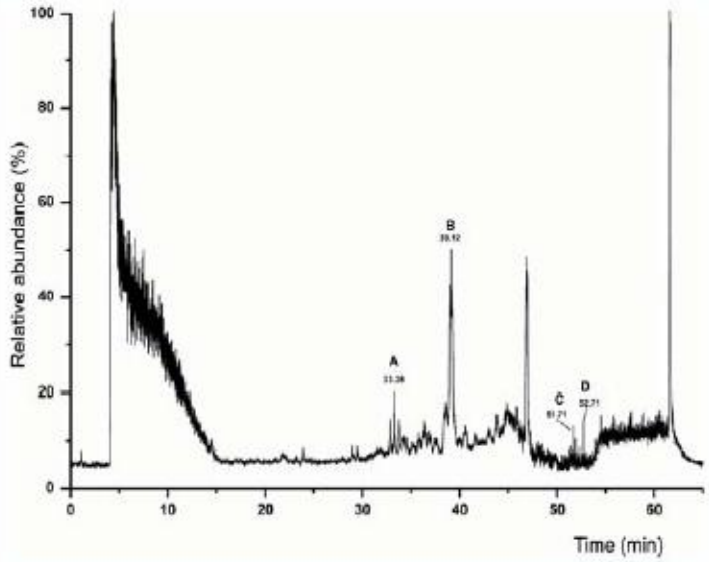

Fig. 3B

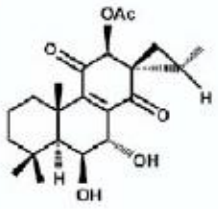

Figure 3: Identification of constituents of bioactive fractions of the *n*-hexane extract of *P. zeylanicus* by UPLC-MS

(**A**) Total ion chromatogram of F-9 of the hexane extract of *P. zeylanicus* and its identified compounds. **A**: cinncassiol A/ cinncassiol C3, **B**: coleone P, **C**: stigmasterol, **D**: stigmasterol-5, 22, 25-trien-3-β-ol

(**B**) Chemical structure of coleone P.

Bioactivity and Phytochemistry of *M. pinnata*

Evaluation of 5-LO and mPGES-1 inhibition

A potent inhibition of 5-LO activity in neutrophils was observed for the *n*-hexane and DCM extracts of *M. pinnata* (10 and 100 µg/ml, **Figure 4A**), whereas methanol and water extracts were almost ineffective and reduced 5-LO activity only by 15 and 16% at 100 µg/ml, respectively (Figure 4A). More detailed concentration-response studies using this cell-based assay revealed an IC_{50} value of 8.7 µg/ml for the *n*-hexane extract (Figure **4B**).

n-hexane and DCM extracts (at 10 µg/ml), efficiently blocked 5-LO activity in the cell free assay while extracts prepared with water or methanol were much less effective (Figure 5A). As shown in **Figure 5B**, the *n*-hexane extract of

M. pinnata caused potent and concentration-dependent inhibition of 5-LO activity with IC_{50} = 0.48 µg/ml. For zileuton, the IC_{50} value was determined at 0.11 µg/ml. As *n*-hexane extract appeared to be most interesting, the potential of this extract to interfere also with the formation of the pro-inflammatory PGE_2 produced by mPGES-1 was investigated.

The extract potently and concentration-dependently inhibited the enzymatic transformation of PGH_2 to PGE_2, catalyzed by mPGES-1. The IC_{50} value was determined at 1.0 µg/ml (Figure 5C), which is even slightly lower than that the IC_{50} for MK886 (1.3 µg/ml), a well-recognized mPGES-1 inhibitor, used as control (not shown).

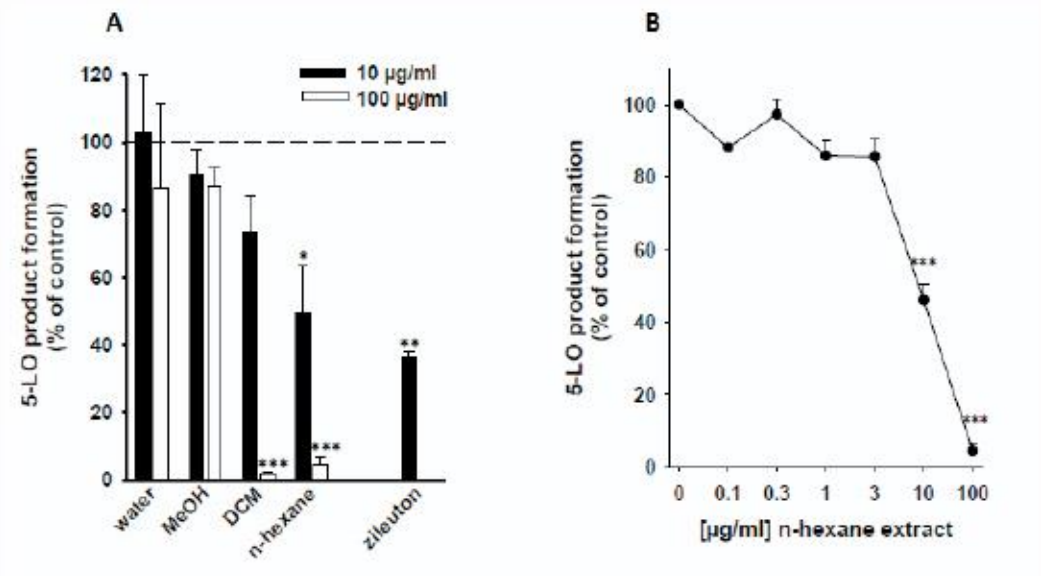

Figure 4: Inhibition of 5-LO activity in intact neutrophils (A) Inhibition of 5-LO activity by various extracts of *M. pinnata*. (B) Concentration-response analysis for the *n*-hexane extract of *M. pinnata*. Data are given as mean +/- S.E.M., n = 3-4.

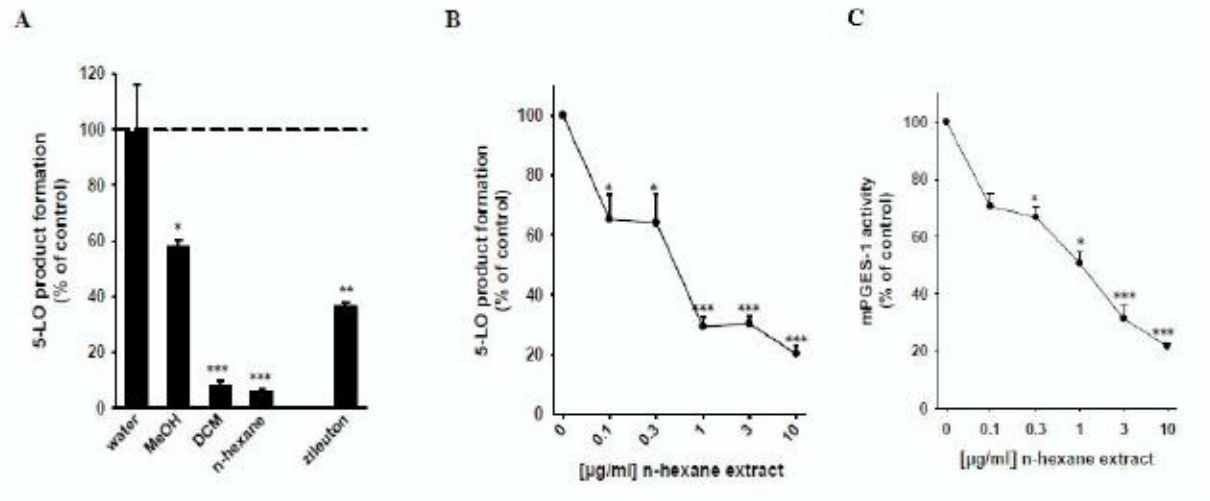

Figure 5: Inhibition of 5-LO activity in a cell-free assay and inhibition of mPGES-1, (A) Inhibition of 5-LO by various extracts (10 µg/ml) of *M. pinnata* or zileuton (3 µM) (B) Concentration-response analysis for the *n*-hexane extract. (C) Inhibition of mPGES-1 by the *n*-hexane extract of *M. pinnata*. Data are given as mean +/- S.E.M., n = 3-4.

Evaluation of radical scavenging properties and suppression of ROS formation in neutrophils

The *n*-hexane extract of *M. pinnata* up to 50 µg/ml is not able to significantly reduce radical formation while it did not cause significant inhibition of ROS formation. Of interest, extracts based on water, methanol or DCM were able to reduce ROS formation, with the methanol extract being most potent (54.7 +/- 6.9% inhibition).

Bioassay-guided separation of the *M. pinnata* *n*-hexane extract

Early attempts of phytochemical screening of *M. pinnata* either failed (12) or provided incomplete information[27]. In order to get more insights into the identity of secondary metabolites that are responsible for the potent inhibition of 5-LO and mPGES-1, the *n*-hexane extract was fractionated and the resulted 11 fractions were analyzed for inhibition of isolated 5-LO and mPGES-1 in the cell-free assays at 1 and 10 µg/ml, each. Out of the 11 fractions (F) of the *n*-hexane extract, F-5, F-6, F-7, F-8, F-9 and F-11 at a concentration of 10 µg/ml inhibited 5-LO activity as well as mPGES-1 by >50%. Among these fractions, F-6, F-7, F-8, and F-9 were significantly active at 1 µg/ml for 5-LO whereas F-6, F-7, and F-8 inhibited mPGES-1 significantly at 1 µg/ml. These data imply a good correlation of the fractions for dual inhibition of 5-LO and mPGES-1.

Identification of constituents of the *n*-hexane extract and its bioactive fractions GC-MS analysis

The *n*-hexane extract and the fractions F-6 and F-8, which displayed high 5-LO and mPGES-1 inhibitory activities, were subjected to a phytochemical screening by GC-MS and UPLC-MS. The GC-MS analysis of the *n*-hexane crude extract led to the identification of 19 components (**Figure 6**). Among the above identified compounds, 12 compounds were detected in F-6 and only 3 compounds in F-8 after analysis.

LC-MS analysis

The accurate mass measurements and the subsequent database search in METLIN suggested several compounds for the peaks denoted as compounds **A**, **B**, **C** and **D** present in F-6 (**Figure 7**). Compound **A**, with a *m/z* value of 477.33337, fits the molecular formula of $C_{30}H_{46}O_3Na$ with a mass accuracy of -1.145 ppm. The comprehensive analysis of the fragmentation pattern suggested the most possible structure for the compound **A** as ganoderiol F. The accurate mass measurements of compound **B** from the Orbitrap instrument suggested the molecular composition of $C_{30}H_{50}O_3Na$ (*m/z* 481.36490) with a mass accuracy of -0.658 ppm. The database search and the analysis of CID spectra proposed compound **B** to be most likely the triterpenoids, **conicasterol C or theonellasterol E according to its characteristic fragmentation pattern.** In addition, two common phytosterol derivatives were also detected in the TIC and (compound **C and D**) and were proposed as stigmastentriol and tigmasterol, respectively.

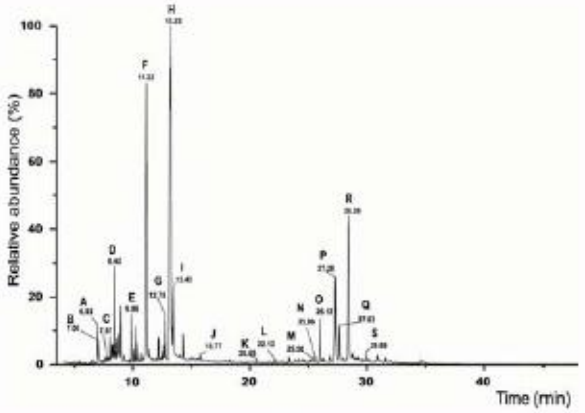

Figure 6: Total ion chromatograph of the *n*-hexane extract of *M. pinnata* and its identified compounds. **A**: β-caryophyllene, **B**: isocaryophyllene, **C**: dodecanoic acid, **D**: caryophyllene oxide, **E**: neophytadiene, **F**: hexadecanoic acid, **G**: phytol, **H**: 9,12 - octadecadienoic acid, **I**: octadecanoic acid, **J**: 4,8,12,16 tetramethylheptadecan-4-olide, **K**: heptacosane, **L**: squalene, **M**: stigmastan-3,

5-diene, **N**: hentriacontane, **O**: á-tocopherol, **P**: campesterol, **Q**: stigmasterol, **R**: β-sitosterol, **S**: stigmast-4-en-3-one.

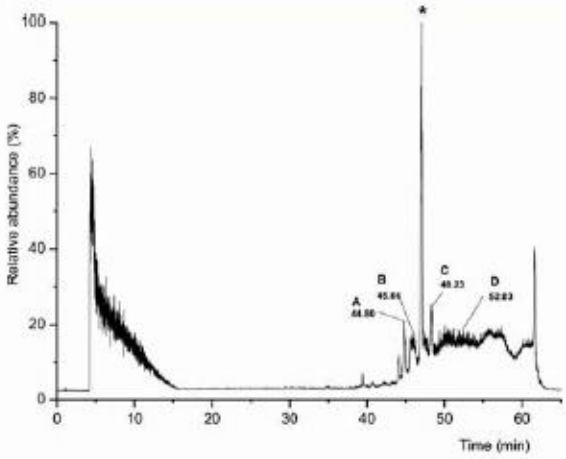

Figure 7: Total ion chromatograph of F-6 of the *n*-hexane extract of *M. pinnata* when analyzed with the ESI source and the presumed compounds. A: ganoderiol F, B: conicasterol **C**/ theonellasterol, **C**: stigmastentriol, **D:** stigmasterol (the peak denoted as " *" is due to erucylamide, a contaminant in the LC system).

Discussion

Here the attempts were made to (I) rationalize and validate the traditional use of the medicinal plants *P. zeylanicus* and *M. pinnata* as anti-inflammatory remedies by analysis of their ability to interfere with 5-LO/mPGES-1 activity and (II), to identify relevant constituents of the bioactive fractions. In fact, *n*-hexane or DCM extracts of *P. zeylanicus* and *n*-hexane extract of *M. pinnata* caused direct and potent inhibition of human 5-LO and suppressed the biosynthesis 5-LO products in isolated human neutrophils while the latter inhibited the human mPGES-1 as well. Notably, these extracts exhibited no significant radical scavenging or antioxidant activities in cell-free (DPPH assay) or cell-based (ROS generation in neutrophils) test systems, and failed to reduce neutrophil viability. Hence, the lipophilic *P. zeylanicus* and *M. pinnata* extracts contain nonredox-related principles that specifically interact with 5-LO supporting an anti-inflammatory potential. Instead of performing extensive chromatographic separations and isolation procedures aiming to reveal potential bioactive constituents by applying traditional phytochemical analysis, rapid and convenient chromatographic/MS approaches were employed for compound identification.

Since the *P. zeylanicus* and *M. pinnata* are widely used in traditional medicine in Sri Lanka to alleviate the pathological conditions caused by inflammation (6,7,10) the emphasis was given on inhibition of 5-LO and/or mPGES-1 as potential underlying mode of action. In fact, 5-LO as key enzyme in the biosynthesis of the pro-inflammatory LTs (14) is explored as a drug target for many inflammatory disorders (28). A large number of plants and their extracts and/or specific secondary metabolites thereof have been reported that are capable of suppressing the biosynthesis of 5-LO products. Such interference with 5-LO activity is considered as basis for the anti-inflammatory features of the respective plants (and medicinal preparations thereof) in folk medicine. However, many of these investigations lacked sufficient and detailed experimentation and the 5-LO inhibitory potencies of the extracts often turned out to be comparably low (IC_{50} values of approx. 20-80 μg/ml)(29). Even for extracts of the gum resin of *Boswellia serrata,* which is considered as potent 5-LO inhibiting natural product and thus frequently used as anti-inflammatory remedy (30), IC_{50} values of 8.4-30 μg/ml were determined (31). In direct comparison to these potencies, the results obtained with the *n*-hexane and DCM extracts of *P. zeylanicus* (IC_{50} of 0.7-12 μg/ml) and *n*-hexane extract of *M. pinnata* (IC_{50} of 0.48 -8.7 μg/ml) are remarkable and suggest a high pharmacological potential for intervention with 5-LO-related disorders.

Previous studies showed that plant derived 5-LO inhibitors such as hyperforin, myrtucommulone, boswellic acids etc, also inhibit the activity of mPGES-1 (17,34), and such dual suppression of two major pro-inflammatory pathways might be beneficial for effective and safe therapy. PGE_2 is considered as major mediator of inflammation and pain, and non-steroidal anti-inflammatory drugs are assumed to confer their anti-inflammatory effect essentially via suppression of PGE_2 biosynthesis

(12). In fact, the *n*-hexane extract of *M. pinnata* and also the fractions F-6 to F-9 effectively repressed the activity of mPGES-1 with IC_{50} = 1 µg/ml. To the best of knowledge, no other medicinal plant-derived extract has been reported thus far with such high potency against mPGES-1, and also the well-recognized synthetic mPGES-1 inhibitor MK886 (IC_{50} = 1.3 µg/ml) was not superior. Therefore, the high efficiency of *M. pinnata* against 5-LO and mPGES-1 in vitro might be of pharmacological relevance and provides a rationale for its use as anti-inflammatory use in folk medicine.

Efficient chromatographic and MS techniques were utilized in the current study on the putative bioactive constituents in restricted fractions of the *n*-hexane extracts of the two plant species. Tandem mass spectrometry was employed here and the accurate mass measurements data were used to tentatively identify the compound in the active fractions. This approach is novel in the fact that it allows for fast data de-replication and will give the researchers a hint on the class of compounds. The amounts of compounds that are able to assay using MS/MS experiments is typically much smaller and it can work on mixtures.

The current study provides new insights towards phytochemicals of *P. zeylanicus*, and among the identified compounds in the most active fractions, some have been proposed to possess in vitro and in vivo anti-inflammatory properties (35,36). Among the identified constituents in the active fractions of the *n*-hexane extract of *P. zeylanicus,* coleone P is of particular interest. The genus *Plectranthus* is rich in coleone-type diterpenoids which exert several biological activities such as anticancer and antimicrobial activities (37,38,39). Coleone P has been isolated from the *Plectranthus* species *P. caninus* (26), and current spectral analysis proposes the presence of this compound in the active fractions F-9 and F-10 of the *P. zeylanicus* *n*-hexane extract. Besides coleone P, cinncassiol A/ C3 as well as callitrisic acid were identified for which the knowledge regarding bioactivities is rare. Furthermore, the pentacyclic triterpenes α- and β-amyrin which have displayed anti-inflammatory activity in in vitro and in vivo models are present in the active fractions (35).

The chemical profiling of *M. pinnata* has been hindered for many years mainly due to the dearth of plant materials for large scale extraction and isolation procedures. However, the current study has unveiled the phytochemistry of this medicinal plant for the first time. Among the identified constituents in the *n*-hexane extract by GC-MS, â-caryophyllene and caryophyllene oxide might be of interest. Anti-inflammatory activity of â-caryophyllene was revealed in models of acute (carrageenan-induced) inflammation[40] while caryophyllene oxide exhibited significant cytotoxicity against the human cancer cell lines [41]. **Furthermore,** the presence of á-tocopherol could be correlated to the 5-LO inhibition as it has displayed a potent inhibition by selective and tight binding to 5-LO (42).

The LC-MS analysis of F-6 and of F-8 of the *n*-hexane extract of *M. pinnata* suggests the presence of some interesting compounds, however, the available tandem mass spectral data which resulted from poor fragmentation of precursor ions, are insufficient for a conclusion. Particularly, the tentative identification of ganoderiol F is of interest as this triterpenoid was reported to exhibit strong anti-HIV-1 protease activity (43) as well as *in vivo* antitumor effects (44). Further optimization of LC-MS/MS conditions in planned follow-up studies may permit confirmation of the identified structures.

Although it is reasonable to correlate the antiinflammatory activities of *P. zeylanicus* and *M. pinnata* to the presence of the identified compounds supported also on literature reports, there are several unknown compounds that are not in any database which might also contribute to the bioactivity. Therefore, the planned expansion of this study towards the identification of unknown compounds in the active fraction with the use of fragmentation tree alignments (23) will provide better insights into the chemical profiles of the above plants. Thereafter, further experiments with synthesized compounds will be worthwhile for a better understanding of their bioactivities, in particular with respect to the inhibition of 5-LO and mPGES-1.

Conclusion

By demonstrating potent inhibition of 5-LO/mPGES-1 activity by lipophilic extracts of *P. zeylanicus* and *M. pinnata* in different biological test systems, the traditional use of *the above plants* in Sri Lanka for the treatment of inflammatory conditions could be explained. The phytochemical analysis of the bioactive fractions *n*-hexane extracts of *P. zeylanicus* by MS techniques led to the identification of coleone P for the first time in this plant, along with other thus far unknown or potentially interesting constituents for which anti-inflammatory activity has been proposed. The phytochemistry of *M. pinnata* was revealed for the first time and several secondary metabolites with known anti-inflammatory properties were identified by mass spectrometric techniques. The solid platform laid by our study will be indispensable for further phytochemical and bioactivity research on these popular and valuable medicinal plants in the future. The current data may stimulate for more detailed preclinical analysis of the pharmacological properties of *P. zeylanicus* and *M. pinnata* that may further support its therapeutic potential in the treatment of inflammatory disorders.

Acknowledgements

The author sincerely acknowledge the International Max Planck Research School for the PhD fellowship (2010-2013). The study was conducted under the supervision of Dr. Aleš Svatoš (Research Group Mass Spectrometry/Proteomics, Max Planck Institute for Chemical Ecology, Jena, Germany), Prof. Oliver Werz (Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Germany) and Prof. Sebastian Boecker (Chair for Bioinformatics, Friedrich-Schiller-University Jena, Germany). The author expressed her sincere thanks to Jana Gerstmeier, Andreas Koeberle, Sandra Wesely, Sven Popella (Institute of Pharmacy, Friedrich-Schiller-University), Sybille Lorenz (Max Planck Institute for Chemical Ecology) and Kerstin Scheubert (Department of Bioinformatics, Friedrich-Schiller-University) for their contribution to this study.

References

1. Butler MS. The role of natural product chemistry in drug discovery. *Journal of Natural Products* 2004;**67**:2141-2153.
2. Newman DJ, Cragg GM,. Natural products as sources of new drugs over the last 25 years. *Journal of natural products* 2007;**70**(3):461-477.
3. Harvey AL. Natural products in drug discovery. *Drug Discovery Today* 2008;**13**(19/20): 894-901.
4. Sarker SD, Nahar L. An introduction to natural products isolation. *Methods in molecular biology* 2012; **864**:1-25.
5. Arambewela L, Wijesinghe A. Sri Lankan medicinal plant Monographs and analysis - Vol-11 *Plectranthus zeylanicus*, 2006; National Science Foundation, Sri Lanka.
6. Jayaweera DMA. Medicinal plants (Indigenous and exotic) used in Ceylon, Part **2**. 1982; National Science Council, Sri Lanka 108-109.
7. Ayurveda Pharmacopeia**1**. Part (1). 1979; Department of Ayurveda, Sri Lanka.
8. Dassanayake MD, Fosberg FR. A Revised Handbook to the Flora of Ceylon. Vol. **3**. 1981; Amerind Publ. Co. Ltd. India.
9. Dharmadasa RM, Hettiarachchi PL, Premakumara GAS. Geographical distribution and conservation of a rare medicinal plant *Munronia pinnata* (Wall) Theob (Meliacea) in Sri Lanka. *Bangladesh Journal of Plant Taxonomy* 2011;**18**:39-49.
10. Jayaweera DMA. Medicinal plants (Indigenous and exotic) used in Ceylon, Part **4**, 1982; National Science Council, Sri Lanka.
11. Arambewela L, Wijesinghe A. Sri Lankan medicinal plant Monographs and analysis- Vol-12 *Munronia Pumila.* 2006: National Science Foundation, Sri Lanka.
12. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001;**294**:1871-1875.
13. Werz O, Steinhilber D. Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacology and Therapeutics* 2006;**112**:701-718.

14. Radmark O, Werz O, Steinhilber D, Samuelsson B. 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends in Biochemical Sciences* 2007;**32**:332-341.

15. Pergola C, Werz O. 5-Lipoxygenase inhibitors: a review of recent developments and patents. *Expert Opinion on Therapeutic Patents* 2010;**20**(3):355-375.

16. Samuelsson B, Morgenstern R, Jakobsson PJ. Membrane prostaglandin E synthase-1: a novel therapeutic target. *Pharmacological Reviews* 2007;**59**:207-224.

17. Koeberle A, Werz O. Inhibitors of the microsomal prostaglandin E(2) synthase-1 as alternative to non-steroidal anti-inflammatory drugs (NSAIDs) - a critical review. *Current Medicinal Chemistry* 2009;**16**:4274-4296.

18. Werz O. Inhibition of 5-lipoxygenase product synthesis by natural compounds of plant origin, *Planta Medica* 2007;**73**(13):1331-1357.

19. Pergola C, Dodt G, Rossi A, Neunhoeffer E, Lawrenz B, Hinnak H, Samuelsson B, Rådmark O, Sautebin L, Werz O. ERK-mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for gender differences in inflammation and asthma. Proceedings of the National Academy of Sciences of the United States of America 2008;**105**:19881-19886.

20. Fischer L, Szellas D, Radmark O, Steinhilber D, Werz O. Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors. *FASEB Journal* 2003;**17**:949-951.

21. Koeberle A, Siemoneit U, Buehring U, Northoff H, Laufer S, Albrecht W, Werz O. Licofelone suppresses prostaglandin E2 formation by interference with the inducible microsomal prostaglandin E2 synthase-1. *Journal of Pharmacology and Experimental Therapeutics* 2008;**326**:975-982.

22. Blois MS. Antioxidant Determinations by the Use of a Stable Free Radical, *Nature* 1958;**181**:1199-1200.

23. Rasche F, Scheubert K, Hufsky F, Zichner T, Kai M, Svatoš A., Böcker S. Identifying the unknowns by aligning fragmentation trees. *Analytical Chemistry* 2012;**84**(7):3417-3426.

24. Werz O, Steinhilber D. Development of 5-lipoxygenase inhibitors-lessons from cellular enzyme regulation. *Biochemical Pharmacology* 2005;**70**:327-333.

25. Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, Summers JB, Brooks DW. 5-lipoxygenase inhibitory activity of zileuton. *Journal of Pharmacology and Experimental Therapeutics* 1991;**256**(3):929-937.

26. Arihara S, Ruedi P, Eugster CH. Neue spiro-Cyclopropyl-cyclohexendion-Diterpene: Coleone M, N, P, Q, R sowie Barbatusin aus *Plectranthus caninus* ROTH und Coleon 0 aus *Coleus somaliensis S.* MOOR. *Helvetica Chimica Acta* 1975;**58**(2):343-356.

27. Munasinghe MLAMS. Phytochemical screening of *Munronia pinnata* (Wall) Theob. *Ayurveda Sameekshava* 2002;**1**:164-171.

28. Peters-Golden M, Henderson WR. Leukotrienes. *The New England Journal of Medicine* 2007;**357**:1841-1854.

29. Schneider I, Bucar F. Lipoxygenase inhibitors from natural plant sources. Part 1: Medicinal plants with inhibitory activity on arachidonate 5-lipoxygenase and 5-lipoxygenase/ cyclooxygenase. *Phytotheraphy Research* 2005;**19**(2):81-102

30. Ammon HP. Boswellic acids in chronic inflammatory diseases. *Planta Medica* 2006; **72**(12):1100-1116.

31. Wildfeuer A, Neu IS, Safayhi H, Metzger G, Wehrmann M, Vogel U, Ammon HP. Effects of boswellic acids extracted from an herbal medicine on the biosynthesis of leukotrienes and the course of experimental autoimmune encephalomyelitis. *Arzneimittelforschung* 1998;**48**(6):668-674.

32. Eugster PJ, Guillarme D, Rudaz S, Veuthey JL, Carrupt PA, Wolfender JL. Ultra high pressure liquid chromatography for crude plant extract profiling, *Journal of AOAC International* 2011;**94**(1):51-69.

33. Perry RH, Cooks RG, Noll RJ, Orbitrap mass spectrometry: instrumentation, ion motion and applications. *Mass Spectrometry Reviews* 2008;**27**(6):661-699.

34. Bauer J, Koeberle A, Dehm F, Pollastro F, Appendino G, Northoff H, Rossi A, Sautebin L, Werz O. Arzanol, a prenylated heterodimeric

phloroglucinyl pyrone, inhibits eicosanoid biosynthesis and exhibits anti-inflammatory efficacy in vivo. *Biochemical Pharmacology* 2011;**81**:259-68.

35. Kweifio-Okai G, Macrides TA. Antilipoxygenase activity of amyrin triterpenes. Research communications in chemical pathology and pharmacology 1992;**78**(3):367-372.
36. Prieto-Garcia JM, Recio MC, Giner RM. Anti-inflammatory activity of beta-sitosterol in a model of oxazolone-induced contact delayed type hypersensitivity. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 2006;**6**(4):57-62.
37. Marques CG, Pedro M, Simões MFA, Nascimento MSJ, Pinto MMM, Rodríguez B. Effect of abietane diterpenes from *Plectranthus grandidentatus* on the growth of human cancer cell lines. *Planta Medica* 2002;**68**(9):839-840.
38. Xing X, Wu H, Wang X, Huang Y, Li Q, Li C, Yang Y, Liu Y, Liu J. Inhibition of tumor cell proliferation by Coleon C. *Journal of Chemotheraphy* 2008;**20**(2):238-245.
39. Wellsow J, Grayer RJ, Veitch NC, Kokubun T, Lelli R, Kite GC, Simmonds MSJ, Insect-antifeedant and antibacterial activity of diterpenoids from species of *Plectranthus*. *Phytochemistry* 2006;**67**:18181825.
40. Gertsch J, Leonti M, Raduner S, Racz I, Chen JZ, Xie XQ, Altmann, KH, Karsak M, Zimmer A. Beta-caryophyllene is a dietary cannabinoid. *Proceedings of the National Academy of Sciences of the United States of America* 2008;**105**:9099-9104.
41. Jun JN, Mosaddik A, Moon JY, Jang KC, Lee DS, Kwang SA, Cho SK. Cytotoxic Activity of caryophyllene oxide isolated from Jeju guava (*Psidium cattleianum* Sabine) leaf. *Records of Natural Products* 2011;**5**:242-246.
42. Reddanna P, Rao MK, Reddy CC. Inhibition of 5-lipoxygenase by vitamin E. *FEBS Letters* 1985;**193**:39-43.
43. el-Mekkawy S, Meselhy MR, Nakamura N, Tezuka Y, Hattori M, Kakiuchi N, Shimotohno K, Kawahata T, Otake T. Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum*. *Phytochemistry* 1998;**49**:1651-1657.
44. Gao JJ, Hirakawa A, Min BS, Nakamura N, Hattori M. *In vivo* antitumor effects of bitter principles from the antlered form of fruiting bodies of *Ganoderma lucidum*. *Journal of Natural Medicines* 2006;**60**:42-48.

The effect of c-KIT and NPM 1 mutations on myeloblast proliferation and differentiation

Kurukula Arachchi Chandana Wickramaratne

Senior Lecturer, Department of Pathology, Faculty of Medicine, University of Ruhuna, Sri Lanka

Corresponding author: KAC Wickramaratne
<chandana.wickramaratne@gmail.com>

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of malignancies arising from myeloid stem cell (1,2). The outcome of AML is ominous most of the time. The prognosis and treatment of AML (and other leukemias as well) is dependent on many genetic mutations and chromosomal aberrations present with the disease. The classification of AML is also based on morphology, cytochemistry and genetic markers. Except for the recurrently defined chromosomal aberrations such as t(9;21), t(15;17), inv(1)6, t(9;11), the majority (more than 50%) of AML carries normal karyotype with no underlying recurrent genetic abnormality (1,2). Two novel mutations identified in some haematological and non haematological neoplasia include mutations in c-KIT gene and NPM 1 gene.

c-KIT is a proto-oncogene which encodes a transmembrane tyrosine kinase III receptor (3,4). With its ligand stem cell factor, c-KIT receptor has been shown to be important for cell growth, function and survival of cells. It belongs to the same family of receptors, in Monocyte Colony Stimulating Factor (M-CSF), PDGF and FLT 3. It is expressed in many different human cells including normal haemopoietic precursor cells, mast cells, germ cells and melanocytes etc. Its activation leads to a cascade of phosphorylations in the cytoplasm. It has been implicated in many none haemopoietic malignancies including cancers in breast, lung, ovaries etc (3,4,5,6,). Almost all primary systemic mastocytosis patients show c-KIT mutations specially one named as c-KIT 816 (D816V). Over-expression of c-KIT receptor in myeloblasts was observed in 60-80% of AML. Point mutations of c-KIT gene were exclusively associated with Core Binding Factor AML (CBF-AML) which constitutes about 33-45% of AML and showed higher relapse rate. This could be related to direct effect of mutated c-KIT gene. The relapse and worse prognosis in AML sub types with inv (16) and t (8;21) were associated with c-KIT mutations even though these subtypes fall in to good prognosis category (5-12).

Some experimental studies support the hypothesis that these fusion proteins impair myeloid differentiation and expand haemopoietic stem cell pool. Some of these mutations specially 816 when present in Chronic Myeloid Leukemia (CML) showed increased resistance and treatment failure to Imatinib Mesylate, the current best therapy available for CML (13,14).

Therefore, study of effects of these mutations on myeloblasts could be important to understand disease outcome and to assess possibilities for therapeutic interventions by blocking mutated protein as it is one major research in leukaemia therapeutics (15-19).

On the other hand, nucleophosmin (nucleoplasmin) (NPM) is a nucleocytoplasmic protein found in both normal and tumour cells (20,21). It was shown in studies that a mutation in NPM (NPM 1) is more abundant in nuclei of tumour cells than normal cells (20,21). Mitogenic activation or growth of cells is associated with increased nuclephosmin protein in nucleus in many folds. NPM regulates p 53 and tumour suppressor pathways both positively and negatively. It is involved in ribosome

biogenesis and centrosome duplication as well (20,21,22).

The NPM 1 mutation has been associated with different haemopoietic malignancies by virtue of its involvement in translocations. Recently in 35.2% of primary AML, it was shown aberrant cytoplasmic localization of nucleophosmin protein. It was associated with many different subtypes of AML and interestingly most were of normal karyotype (20-23). AML with recurrent genetic abnormalities and secondary AML were devoid of NPM1 mutations thus cytoplasmic localization of nucleophosmin (22,23,24).

The immortalized murine myeloblast (32D) cells have been widely used in research and assessed for stability in in-vitro cultures. These cells are easy to manipulate in nucloefection procedures. The characteristics of the 32D cells are well defined (25,26).

Considering these facts, it can be argued that C-KIT mutations and NPM 1 mutation can play a significant role in leukemogenesis. Therfore, this study was planned to assess effects of these mutations on two most important features in tumourgenesis; excessive uncontrolled proliferation and inhibition of differentiation. To study effects of genetic mutations, 32D cells are used in many researches showing its applicability in laboratory setting thus selected in this study.

Objectives

Objectives of this study was to assess differences in proliferation, differentiation and autonomous proliferative capacity of normal and c-KIT & NPM 1 mutated murine myeloblasts.

Method

Immortalized murine myeloblasts (32D cells) were selected for the study. The 32 D cells are derived and established from long term bone marrow cultures of C3H/HeJ mice infected with Friend of Murine Leukaemia virus. These cells are immortalized and are constitutively dependent on IL 3 to proliferate continuously and on G-CSF to differentiate in to neutrophils. In the absence of growth factors these cells decline proliferative capacity and undergo apoptotic death within 24 hours of culturing in the absence of growth factors (IL 3 & G-CSF).Unmutated (wild type 32 D cells) were selected as negative control.

Using plasmid vectors and mutated gene sequence available, murine myeloblasts were nucleofected with c-KIT and NPM 1 mutations using standard protocol. Wild type 32 D cells were mixed with nucleofection media containing highly purified plasmids and were incubated providing standard conditions.

After completion of nucleofection, cells from each nucleofection group were transferred to culture dishes. Culture dishes were incubated under standard conditions and isolation of clones were done under strict sterile conditions avoiding mixing or contamination.

The eukaryotic green fluorescence protein (EGFP) gene was used in the procedure of nucleofection to verify success of the procedure.

All the mutated and unmutated murine myeloblasts (32D cells) were propagated and cultured using standard technique. At the end of the initial incubation phase, cultures were assessed for positivity of green fluorescence under fluorescent microscope. Sets of cell cones positive were considered successful and further propagated in RPMI 1640 (Rosewell Park Memorial Institute) media (by Cambrex Bio Science - Walkersville, Inc.) with antibiotics (penicillin and streptomycin) to avoid contaminant bacterial and fungal growth. The media was mixed with G-CSF and or IL 3 (depending on assay) and foetal calf serum to provide required basic factors for cell survival and propagation. The most stable and cell count optimal, clones with adequate surviving cells were selected and transferred to 25cm^3 corning culture flasks with angled neck (CORNING Flasks, polystyrene, sterile) with RPMI media to perform proliferation and incubation assay.

All the clones incubated over night at 37^{0}C with 5% CO_2 and other standard conditions. Differentiation assay and cell count were performed using standard techniques at the beginning of each day, in each cultured clones.

A cytospin preparation was prepared every day and stained with Giemsa method using standard protocol and kept labelled with mutation and the date.

After taking the cell count every morning, a standard quantity of cells (1.5 x 10^6/ml RPMI solution) re transferred for culture propagation to be continued overnight. Excess cells and media were removed, destroyed and discarded using standard protocol. Thus every day the proliferation started with the same cell number as per the unit volume of media.

The different clones propagated in the study are given in the table 1 with abbreviations.

The clones in proliferation assay were propagated only with IL 3 as the growth factor. The clones were propagated for 7 days. Cell counting was performed every morning using Casey cell counter. The cells were observed for fungal or bacterial growths and for dead cells using the inverted microscope.

The differentiation assay was performed in the absence of IL 3 but with G-CSF as the growth factor. Cell counting, cytospin preparation and replacement of media continued.

To assess autonomous proliferation ability clones were propagated in the absence of both IL 3 and G-CSF.

When more dead cells are present in clones, they were removed using density gradient cell sorting system using standard protocol (with lymphoprep solution) to minimize negative effect of dead cell constituents on cell proliferation.

Presence of mutations in research cell clones was confirmed using following three techniques.

1. Examination under UV fluorescent microscope for positive green fluorescence (Figure 1).
2. Flowcytometry using C-KIT antibodies to check expression level with fluorescence activated cell sorting (FACS) system. This confirms expression of C-KIT gene in cell cones.
3. Western blot analysis was used to assess degree of expression of nucleophosmin. A control phoenix cells (murine fibroblasts) with NPM 1 mutation were taken as the control. Cells from each selected clone were lysed to extract proteins and purified (to retain nucleophosmin). The protein blots prepared were incubated with radiolabeled antinucleophosmin antibodies and X-ray exposure films were taken to confirm expression of NPM 1 in selected clones.

Table 1: Clones of 32 D cells and abbreviations used

Clone number	*Clone*	*Abbreviation used*
1,9,17,25	32 D cells – negative control – wild type	32D WT
2,10,18,26	32 D cells with EGFP nucleofection	32D EGFP
3,11,19,27	32 D cells with wild type C-KIT gene	32D KT WT
4,12,20,28	32 D cells with wild type NPM1 gene	32D NPM1 WT
5,13,21,29	32 D cells with mutated NPM 1 gene	32D NPM1MUT
6,14,22,30	32 D cells with C-KIT mutations – insertion/deletion 1	KIT ID 1
7,15,23,31	32 D cells with C-KIT mutations – insertion/deletion 2	KIT ID 2
8,16,24,32	32 D cells with C-KIT 816 mutation	KIT 816

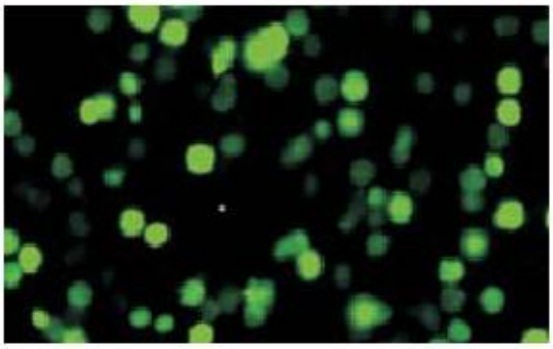

Figure 1: Demonstration of green fluorescence of nucleofected cells.

Results

Proliferation assays

The proliferation assay showed an almost similar proliferation pattern and rate in all the cell lines. There was no difference in rate of proliferation observed in wild type over mutated ones. Table 2 shows the proliferation of different cell clones over 10 days in culture media.

Table 2: Proliferation assay - results are given number of cells X 10^9/ml media

	Day of the Culture									
Cell clone	*D1*	*D2*	*D3*	*D4*	*D5*	*D6*	*D7*	*D8*	*D9*	*D10*
1	3.2	4.5	6.4	6.7	9	8	8.5	8	8.8	8.3
2	3.4	6	6.5	6.9	8.2	7.8	8.4	7	6.3	8
3	6	9.6	3.5	5.6	5.4	9	8	8.3	8.3	8
4	3.3	6.3	5.4	6.5	7.4	8.6	9	8.7	7.8	8
5	9.4	5.2	9.6	10	13	10	12	9.6	9.5	10
6	5.5	4	8.3	13	6.6	9.4	8	9.3	7.8	7.6
7	5	6	5.2	10	7.6	12	7.4	8	9.6	8.2
8	3.2	5	7.8	6.7	10	9.7	9	9.8	8.9	8
9	3	7.4	6.2	9.1	9.4	11	7.6	9.5	8.6	9
10	3.8	6.9	5.2	7.5	7.8	10	7	7	7	8.8
11	2.9	8.8	8.3	8	9.9	9.5	10	10	8.5	8.9
12	2.7	5.8	6.4	6	9.2	9	6.7	7	7.4	7
13	2.8	5	6.3	5.8	10	9.4	8.5	9	7.9	8.4
14	5.8	5.7	7.8	7.5	10	10	7.5	8.2	7.9	7
15	4	6.8	4.3	7.7	9	9.7	9.2	8.4	9	10
16	2	6.8	5.5	9	8.6	9.8	7.9	6.4	8	7.5

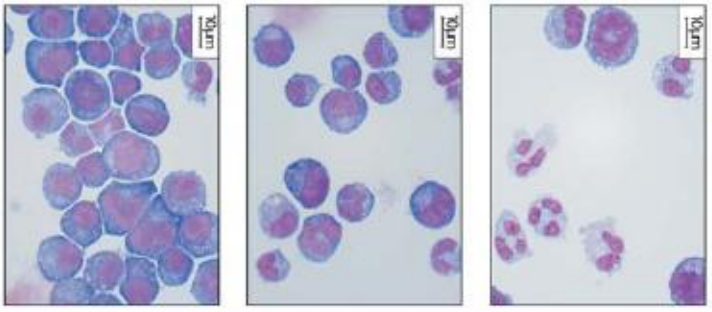

Figure 2: 32D cells in different stages of differentiation assay.
A. 32D cells with c-KIT 816 mutation on day 2, **B.** 32D cells with c-KIT mutation on day 10, **C.** Wild type 32D cells in day 10

Differentiation assay

Interestingly, the differentiation assay showed impaired differentiation compared to 32D WT cells. This was reflected by the presence of large percentage of blasts compared to 32D WT even towards the end of the assay. Specially the cell cone with c-KIT 816 showed highest difference compared to wild type. Even on day 12, mutated clones showed over 50% blasts in the presence of 100% differentiation in to neutrophils in wild type clones by day 10. Figure 2 shows different 32D cells at different stages of differentiation assay.

Interestingly, both mutated and wild type clones failed to sustain autonomous proliferation in the absence of growth factors and undergo complete apoptosis by day 2. FACS analysis of cell cones showed c-KIT positivity. However, the signals were weak, could be related to the degree of expression.

In addition, more apoptotic cells were noted in wild type clones while less apoptosis was noted in mutated clones in differential assay. However, apoptosis was not assayed in the study to ascertain exact details.

Discussion

The proliferation of wild type cells and mutated cells showed almost equal degree and rate of proliferation in this study. The 32D cells used in the study are immortalized blasts having enormous capacity to proliferate thus, the proliferation assay may not be sensitive enough to identify the differences. The insensitive nature could have been the reason for absence of an observable difference in proliferation assay. Therefore, more sensitive assays (such as H3 - Thymidine incorporation) are needed to ascertain differences in proliferation at molecular level.

Surprisingly both wild type and mutated ones failed to sustain proliferation beyond two days without growth factors. This needs further evaluation to ascertain the exact nature of activity of the mutations studied and need for additional supportive mutations.

In this study, the most conclusive results are obtained in differentiation assay. Those support the effect of c-KIT mutation in propagation of leukemia by inhibiting differentiation. Even at the last day of the assay, mutated clones had a significant quantity of undifferentiated blasts in the culture. In c-KIT 816 clone, it was more than 50%. The clones with pure wild type cells and wild type gene had shown rapid differentiation and marked apoptosis within a few days after commencement of assay. This could be utilized to explain ominous nature of the c-KIT mutation in leukaemias.

However, the expression of c-Kit appears weak in clones studied. If the expression of c-KIT was optimal the results could have been more decisive.

The NPM 1 mutation however, failed to show any influence either on proliferation or differentiation of myeloblasts.

Acknowledgements

Prof. Dr. Bob Löwenberg (Head, Department of Haematology) and Dr Peter Valk (Head, Molecular Diagnostics Unit of Department of Haematology) of Erasmus University Medical Centre, Rotterdam, The Netherlands for provision of training, supervision, guidance and funds.

References

1. Neame PB, Soamboonsrup P, Browman GP, Meyer RM, Benger A, Wilson WE, Walker IR, Saeed N, McBride JA. Classifying Acute Leukaemia by Immunophenotyping: A Combined FAB-Immunologic Classification of AML. *Blood* 1986 Dec;**68**(6):1355-62.
2. Bain BJ. Leukaemia diagnosis. 3rd ed, Blackwell Science 2003, Oxford, UK.

3. Boissan M, Feger F, Guillosson JJ, Arock M. c-KIT and c-Kit mutations in mastocytosis and other haematological diseases. *J Leukoc Biol.* 2000;**67**(2):135-48.

4. Piao X, Bernstein A. A point mutation in the catalytic domain of C-KIT induces growth factor independence, tumorigenicity and differentiation of mast cells. *Blood* 1996;**87**(8): 3117-23.

5. Crosier PS, Ricciardi ST, Hall LR, Vitas MR, Clark SC, Crosier KE. Expression of isoforms of the human receptor kinase c-KIT in leukemic cell lines and acute myeloid leukaemia. *Blood* 1993;**82**(4):1151-8.

6. Yanagihori H, Oyama N, Nakamura K, Kaneko F. c-KIT mutations in patients with childhood-onset mastocytosis and genotype-phenotype correlation. *J Mol Diagn* 2005;7(2):252-7.

7. Zhou G, Wang YY, Yin T, Bai X, Liang W, Xu F, Liu Y, Chen Z, and Chen S. High frequency gain of function mutation and/ or over-expression of proto-oncogene c-KIT as major contributor to the leukaemogenesis in human acute myeloid leukaemia with t(9;21). *Blood* 2004;**104**:4322-4323.

8. Hongyo T, Li T, Syaifudin M, Baskar R, Ikeda H, Kanakura Y, Aozasa K, Nomura T. Specific c-KIT mutations in sinonasal Natural Killer/ T-Cell lymphoma in China and Japan. *Cancer Res* 2000;**60**(9):2345-7.

9. Yavuz AS, Lipsky PE, Yavuz S, Metcalfe DD, Akin C. Evidence of the involvement of a haemopoietic progenitor cell in systemic mastocytosis from single cell analysis of mutation sin teh c-KIT gene. *Blood* 2002;**100**(2):661-5.

10. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y. Gain of function mutations of C-Kit in human gastrointestinal stromal tumours. *Science* 1998;**279**(5350):577-80.

11. Wang YY, Zhou GB, Yin T, Chen B, Shi JY, Liang WX, Jin XL, You JH, Yang G, Shen ZX, Chen J, Xiong SM, Chen GQ, Xu F, Liu YW, Chen Z, Chen SJ.PNAS, Jan 2005; **102**:1104-1109. AML-ETO and C-KIT mutations/ over expression in t(8;21) leukaemia: Implications in stepwise leukaemogenesis and response to Gleevec. Proc Natl Acad Sci U SA. 2005;**102**(4):1104-9.

12. Kitayama H, Tsujimura T, Matsumura I, Oritani K, Ikeda H, Ishikawa J, Okabe M Suzuki M, Yamamura K, Matsuzawa Y, Kitamura Y, Kanakura Y. Neoplastic transformation of normal haemopietic cells by onstitutively activating mutations of C-KIT receptor tyrosine kinase. *Blood* 1996;**88**(3):995-1004.

13. Kindler T, Breitenbuecher F, Marx A, Beck J, Hess G, Weinkauf B, Duyster J, Peschel C, Kirkpatrick CJ, Theobald M, Gschaidmeier H, Huber C, Fischer T. Efficacy and safety of imatinib in adult patients with c-kit-positive acute myeloid leukaemia. *Blood* 2004;**103** (10):3644-54.

14. Nanri T, Matsuno N, Kawakita T, Mitsuya H, Asou N. Imatinib mesylate for refractory acute myeloblastic leukemia harboring inv(16) and a C-KIT exon 8 mutation. *Leukemia* 2005;**19**(9):1673-5.

15. Cairoli R, Beghini A, Grillo G, Nadali G, Elice F, Ripamonti CB, Colapietro P, Nichelatti M, Pezzetti L, Lunghi M, Cuneo A, Viola A, Ferrara F, Lazzarino M, Rodeghiero F, Pizzolo G, Larizza L, Morra E. Prognostic impact of c-KIT mutations in core binding factor leukaemias: an Italian retrospective study. *Blood* 2005;**107**(9):3463-8.

16. Beghini A, Larizza L, Cairoli R, Morra E. c-kit activating mutations and mast cell proliferation in human leukemia. *Blood* 1998;**92**(2):701-2.

17. Wadleigh M, DeAngelo DJ, Griffin JD, Stone RM. After chronic myelogenous leukaemia: tyrosine kinase inhibitors in other haematologic malignancies. *Blood* 2005; **105**(1):22-30.

18. Heinrich MC, Blanke CD, Druker BJ, Corless CL. Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies. *J Clin Oncol* 2002 Mar 15;**20**(6):1692-703.

19. Growney JD, Clark JJ, Adelsperger J, Stone R, Fabbro D, Griffin JD, Gilliland DG. Activation mutations of human c-KIT resistant to imatinib mesylate are sensitive to the tyrosine kinase inhibitor PKC412. Blood. 2005;**106**(2):721-4.

20. Grisendi S, Bernardi R, Rossi M, Cheng K, Khandker L, Manova K, Pandolfi PP. Role of nucleophosmin in embryonic development and tumorigenesis. Nature. 2005;**437** (7055):147-53.

21. Alcalay M, Tiacci E, Bergomas R, Bigerna B, Venturini E, Minardi SP, Meani N, Diverio D, Bernard L, Tizzoni L, Volorio S, Luzi L, Colombo E, Lo Coco F,Mecucci C, Falini B, Pelicci PG. Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. *Blood* 2005;**106**(3):899-902.

22. Cazzaniga G, Dell'Oro MG, Mecucci C, Giarin E, Masetti R, Rossi V, Locatelli F, Martelli MF, Basso G, Pession A, Biondi A, Falini B. Nucleophosmin mutations in childhood acute myelogenous leukaemia with normal karyotype. *Blood* 2005;**106**(4): 1419-22.

23. Boissel N, Renneville A, Biggio V, Philippe N, Thomas X, Cayuela JM, Terre C, Tigaud I, Castaigne S, Raffoux E, De Botton S, Fenaux P, Dombret H, Preudhomme C. Prevalence, clinical profile, and prognosis of NPM mutations in AML with normal karyotype. *Blood* 2005:**106**:3618-3620.

24. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D. Growth of factor dependent haemopoietic precursor cell lines. *J Exp Med* 1980;**152**:1036-1047.

25. Valtieri M, Tweardy DJ, Caracciolo D, Johnson K, Mavilio F, Altmann S, Santoli D, Rovera G. Cytokine dependent granulocytic differentiation-regulation of proliferative and differentiative responses in murine progenitor cell line. *J Immunol* 1987;**138**:3829-3835.

26. Metcalf D. Multi-CSF dependent colony formation by cells of murine haemopoietic cell lines: specificity and action of multi-CSF. *Blood* 1985;**65**:357-362.

The heterogeneity of the RNA degradation exosome in *Sulfolobus*

Chamindri Witharana

Department of

Introduction

RNA is necessary for protein synthesis and for gene regulation in all living organisms. Most RNA molecules are transcribed as precursors which are then maturated by ribonucleases (RNases) and RNA modification enzymes. Often large multiprotein complexes are responsible for the maturation and for the degradation of various RNA molecules in the cell. The general mechanisms of RNA processing and degradation are highly conserved and include endonucleolytic cleavages, posttranscriptional modification at the 3'-end (RNA tailing) and exoribonucleolytic degradation or trimming in 3'-5' direction or in 5'-3' direction (for recent reviews see Refs (1-3). Controlled RNA degradation as a part of the posttranscriptional gene regulation is especially important for unicellular, prokaryotic microorganisms, which are exposed to changing environmental conditions. Prokaryotes are a highly heterogeneous group which include bacteria and Archaea. Archaea show morphological similarities to bacteria, but are closely related phylogenetically to eukarya than to bacteria (4). They also show a strong similarity to eukarya at the molecular level, for example in the mechanisms of replication, transcription and translation. Archaeal mRNA, however, is more similar to bacterial mRNA: it is generally intron-less and lacks a long stabilising poly (A)-tail at the 3'-end as well as a methylguanosine cap at the 5'-end. In accordance with this, the mechanisms of RNA degradation in Archaea show strong similarities to those operating in bacteria (2). For example, the archaeal protein complex named exosome is structurally and functionally similar to the bacterial polynucleotide phosphorylase (PNPase) (5-8). The bacterial PNPase and the archaeal exosome show structural similarities to the eukaryotic nine-subunit exosome (9), but there are important functional differences between the eukaryotic and prokaryotic exosome-like machineries. The activity of the eukaryotic nine subunit exosome is due to a tenth subunit with similarity to the bacterial RNase R, while its PNPase-like, nine-subunit core is catalytically inactive (10). The eukaryotic ten-subunit exosome that functions as a hydrolytic 3'-5' exoribonuclease and an endoribonuclease (11,12) is essential and participates in various RNA processing and RNA degrading pathways both in the nucleus and in the cytoplasm (13,14). Eukaryotic RNAs are intended for exoribonucleolytic 3'-5' degradation by the addition of short poly (A) tails. This destabilizing polyadenylation is performed by specialized protein complexes that are different from the eukaryotic exosome (15,16). In contrast, both the bacterial PNPase and the archaeal nine subunit exosome are phosphorolytic 3'-5' exoribonucleases, which also can use NDPs to synthesize destabilizing, heteropolymeric RNA tails, which are used as loading platforms for exo-ribonucleases (6,17,19). In Archaea lacking the exosome, RNA is not post-transcriptionally modified at the 3'-end (19). In these exosome-less Archaea, RNA is either exoribonucleolytically degraded in 3'-5' direction by a homologue of bacterial RNase R (in halophiles) or seems to be exclusively degraded in 5'-3' direction by a homologue of the bacterial RNase J (in some methanogenic Archaea) (20,21). Like in Bacteria, in Archaea degradation in 5'-3' direction is inhibited by a 5'-triphosphate and is performed by RNase J homologues (22,23). The endoribonucleolytic mechanisms, which are of central importance for RNA degradation in Bacteria (3,24) are still not explored in Archaea. The recently described, endonucleolytically

active RNase J homologues in methanogens are good candidates for principle endoribonucleases in the third domain of life (21). The existence of the archaeal exosome was proposed by bioinformatic analyses based on its similarity to the eukaryotic exosome (25) and was verified by co-immunoprecipitation from the thermoacidophilic archaeon *Sulfolobus solfataricus* (26). Later, its existence *in vivo* was verified for two further archaeal species, *Methanotermobacter thermoautotrophicus* and *Thermococcus kodakarensis* (27,28). Major components of the archaeal exosome are the orthologs of the eukaryotic exosomal subunits Rrp41, Rrp42, Rrp4 and Csl4 forming the nine-subunit form of the archaeal complex, and the archaeal DnaG protein, the function of which in respect to RNA is still unknown. The structure and the catalytic mechanism of the reconstituted archaeal nine-subunit exosome are well understood. It is built of a phosphorolytically active hexametric ring containing the subunits Rrp41 (harbouring the active centre) and Rrp42, to which a trimeric cap of the RNA-binding proteins Rrp4 and/or Csl4 is bound. It performs metal-dependent phosphorolysis of RNA in the presence of inorganic phosphate (Pi) and Mg_2Cl, and synthesizes RNA using NDPs without a template (5-8, 29-31).

In vitro, the amounts of Pi, NDPs and Mg_2Cl determine the direction of the reaction (32). However, little is known however about the regulation of the functions of the archaeal exosome *in vivo*. The function of the RNA-binding cap was investigated *in vitro* using recombinant exosomes of several archaea belonging to the genera *Sulfolobus*, *Pyrococcus* and *Archaeoglobus*. So far, homomeric caps composed of Rrp4 or Csl4 were studied in detail. Generally, the presence of Rrp4 or Csl4 increases RNA binding and the efficiency of RNA degradation and RNA synthesis by the archaea exosome (7,8,18, 32-34). Recently we have shown that Rrp4 and Csl4 confer different substrate specificities to the archaeal exosome and that Rrp4 strongly prefers poly(A). Although *S. solfataricus* does not have poly(A) tails, poly(A) stretches are present in the heteropolymeric RNA tails synthesized by the exosome (19). Furthermore, the GC content of the *S. solfataricus* genome is 37% and short poly(A) stretches are present in its mRNAs. These poly(A) stretches are most probably the determinants recognized by the Rrp4-containing exosome, since the presence of a naturally occurring, adenine-rich RNA tail enhanced the degradation of a synthetic, heteropolymeric RNA by the exosome carrying a homomeric cap built of Rrp4 but not of Csl4. Although it was shown *in vitro* that the reconstituted exosome of Archaeoglobus fulgidus can carry a heteromeric cap containing Rrp4 and Csl4 (7), the cap composition of the archaeal exosome was not studied *in vivo* so far. Changes in the composition of the exosome may influence not only its substrate specificity, but also the interaction with other proteins and even its subcellular localization. The majority of the active site containing subunit Rrp41 is localized at the periphery of the *S. solfataricus* cell and is detectable in the insoluble fraction of a cell-free extract. DnaG was also detected at the cell periphery and is essentially insoluble. The aim of this work was to analyze the composition of the soluble and the insoluble exosomes. We found that the exosome contains heteromeric, Rrp4 and Csl4 containing caps *in vivo*, and that exosomes with different sedimentation behaviours differ in their composition.

Material and Methods

Cell growth and fractionation experiments

S. solfataricus P2 was grown in a 10 l fermenter at 75°C in a rich medium under air supply as described. Cells (470 mg wet pellet) were re-suspended in 1 ml lysis buffer containing 20 mM MES (2-(N-morpholino)ethanesulfonic acid), pH 6.5, 0.5 mM EDTA, 1 mM PMSF, 2 mM DTT, DNase I and 1 mM protease inhibitor cocktail (Sigma Aldrich, Germany), and sonified. After lysis, 500 mM ammonium chloride and 10 mM magnesium acetate were added and the cell free extract was clarified by centrifugation at 2500 g for 20 min at 4°C. To obtain S100 and P100 fractions, the supernatant was subjected to ultra centrifugation at 100,000 g for 1 h at 4°C. Prior to analysis, the pellet

fraction was re-suspended in 1 ml of lysis buffer containing 500 mM ammonium chloride and 10 mM $MgCl_2$. Membranes were removed at 13,000 g. Fractionation in 15-70% sucrose density gradient containing 500 mM ammonium chloride and 10 mM magnesium acetate was performed.

SDS-PAGE and Western blot analysis

Proteins were separated in 12% SDS-PAGE and silver stained, or blotted onto a nitrocellulose membrane and hybridized with rabbit sera directed against Rrp41 (26), DnaG (18), Rrp4 and Csl4 (Davids Biotechnologie GmbH, Regensburg, Germany). The secondary antibody was anti-rabbit IgG conjugated with peroxidise (Pierce), and the Lumi-Light Western blotting substrate (Roche Diagnostics GmbH) was used for detection. The bands were quantified using Peqlab Fusion SL 4 instrument and the corresponding software.

Co-immunoprecipitation and depletion experiments

Co-immunoprecipitation and depletion experiments with polyclonal antibodies covalently coupled to protein A-Sepharose were performed as previously described (18). We used 800 ml of cell-free extract or 600 ml density gradient fractions (pooled were 300 ml of fractions 6 and 7 or fractions 12 and 13) and 0.04 g of beads coupled with antibodies in 150 ml phosphate buffered saline were used.

Reconstitution of exosomal complexes

PCR was performed with genomic DNA of S. *solfataricus*, the amplificates were cloned in pDrive (Qiagen) and re-cloned between the XhoI and NdeI restriction sites of the pET-15b expression vector. Rrp4 deletion variants as well as the full-length subunits of the exosome Rrp41, Rrp42 (6) and Rrp4 (29) were over expressed in *Escherichia coli* BL21 (DE3) and purified by Ni-NTA affinity chromatography. To analyze the interaction of deletion variants of Rrp4 with the hexameric ring, the purified subunits were mixed together in nearly equimolar amounts and were dialysed at room temperature in a buffer containing either 150 mM or 500 mM NaCl, 10 mM Tris pH 7, 5 mM $MgCl_2$, 0.5 mM EDTA, 5% Glycerol, 0.05% Tween 20, 0.2 mM DTT for 20 min. Then co-immunoprecipitation with Rrp41-specific antibodies followed by SDS-PAGE analysis was performed.

Results

Differences in the composition of the soluble and the insoluble exosomes

Previously we have shown by immunofluorescence that the majority of the *S. solfataricus* exosome is localized at the periphery of the cell. Consistent with this, only a minor part of Rrp41 was detected in the soluble, supernatant fraction (S100) and DnaG was detected only in the insoluble, pellet fraction (P100) after ultracentrifugation of the cell-free extract at 100,000 g. So far, the RNA-binding subunits of the exosome Rrp4 and Csl4 were not investigated in this respect. To address the question whether there are differences in the composition of the RNA binding caps of the soluble and the insoluble exosomes, antibodies were raised against recombinant Rrp4 and Csl4 of *S. solfataricus* and were used in quantitative Western blot analyses of the S100 and P100 fractions. DnaG and Rrp41 were also included in the analyses. In three independent experiments, 21 (+ or -) 3% of Rrp4 and 23 (+ or -) 3% of Rrp41 were detected in the S100 fraction. In contrast, Csl4 and DnaG were detected in the P100 fraction only (Figure 1A). Since the sensitivities of the antibodies used for detection of Csl4 and DnaG are lower than the sensitivities of the anti-Rrp41 and anti-Rrp4 antibodies (not shown), this result does not necessarily imply different subunit contents of the soluble and the insoluble exosomes, but confirms that the majority of the exosome is insoluble. To directly compare the compositions of the soluble and the insoluble exosomes, we decided to purify the exosome from the S100 and the P100 fractions by co-immunoprecipitation. The isolation of the exosome from the S100 fraction succeeded (see below), but we were not able to

immunoprecipitate the complex from the P100 fraction. Therefore we decided to use sucrose density gradient fractions and to compare the composition of exosomal complexes with different sedimentation behaviours. The majority of the exosome sediments with the membranes, but some exosome is also present in fractions of low density (soluble exosome) and in the middle of the gradient (part of the insoluble exosome co-sedimenting with ribosomal subunits). Since immunoprecipitation of the exosome from the membrane containing fraction 19 failed, we decided to compare the soluble exosome from fraction 6 to the insoluble exosome from fraction 12. We first removed the heavy membrane fraction with the Sla proteins and the associated exosome by centrifugation of the crude extract at 13,000 g. The supernatant containing the soluble components of the extract and particles with high molecular weight like the ribosomal subunits was then fractionated through a sucrose density gradient, and the fractions were analyzed for the presence of exosomal subunits by Western blot hybridization (Figure 1C). In agreement with the data in figure 1A, Rrp41 and Rrp4 were detected in fractions corresponding to soluble and insoluble components of the extract, while DnaG and Csl4 were detected only in the fractions with insoluble components. Using Rrp41-specific antibodies, we were able to isolate the soluble exosome from fraction 6 and the insoluble exosome from fraction 12 (Figure 2). SDSPAGE analysis revealed the presence of following proteins in the elution fraction containing the soluble exosome: Rrp4, Rrp41, Rrp42, DnaG, and a protein larger than DnaG, which was identified as EF1-alpha by mass spectrometry (Figure 2A). The identity of DnaG was also confirmed by mass spectrometry. Figure 2B shows that the insoluble exosome from fraction 12 contains no EF1-alpha. A control experiment with a pre-immune serum was also performed using fraction 6 of the density gradient. No EF1-alpha and no DnaG were detected, neither by SDS-PAGE and silver staining, nor by mass spectrometry analysis (not shown).

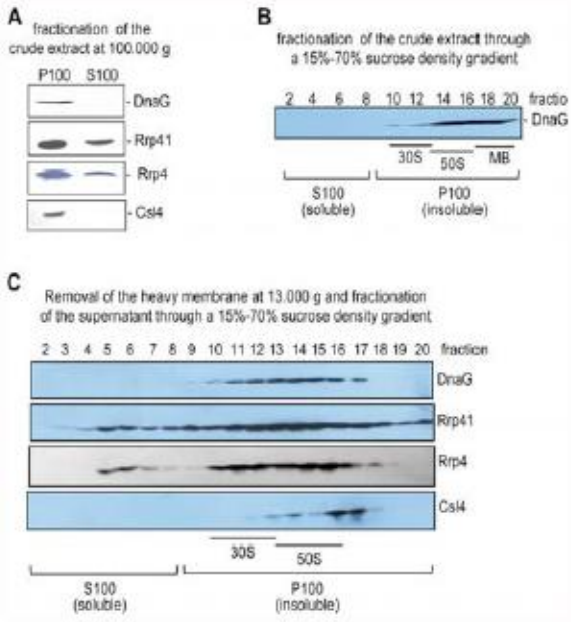

Figure 1: Detection of DnaG, Rrp41, Rrp4 and Csl4 in fractions of the *S. solfataricus* cell-free extract by Western blot analysis A) Western blot analysis of S100 and P100 fractions. Equal volume of the S100 and the P100 fractions were separated in 12% SDS-PAGE, blotted and hybridized with sera directed against the exosomal subunits indicated on the right side of the panels. B) Schematic representation of the sedimentation of the small (30S) and large (50S) ribosomal subunits, and of membranes (MB) with surface layer proteins in fractions of a sucrose density gradient with 500 mM salt. Shown is also the relationship between sucrose density gradient fractions and S100 and P100 fractions. The sedimentation of the exosome is shown on the example of DnaG detected by Western blotting of selected fractions. Fractionated was the crude extract C). The cell-free extract was subjected to low speed centrifugation to remove the membranes with the surface layer proteins and the associated exosome. The supernatant was fractionated through the sucrose density gradient and the fractions were analyzed for the presence of DnaG, Rrp41, Rrp4 and Csl4 by Western blot hybridization. The analyzed fractions are given above the panels the detected proteins are marked on the right side. The relationship between density gradient fractions, S100 and P100, and the sedimentation of the ribosomal subunits is given below the panels.

Since the exosomes from fractions 6 and 12 represent a minority of the exosome in *S. solfataricus* cells, questions arise about their functional relevance. To address this, we performed activity assays with the co-immunoprecipitated complexes. The assays were performed directly with the proteins bound to the protein A-Sepharose beads (18). Lanes 7 and 8 in figure 2C show that the soluble and the insoluble exosomes can degrade RNA. Thus, although present in low amounts, the soluble exosome is active. We verified that the exosome is a major RNA degrading enzyme in the soluble fraction of S. *solfataricus*: The RNA degrading activity of fraction 6 disappeared after depletion of the exosome by three rounds of co-immunoprecipitation with Rrp41-directed antibodies (Figure 2C, lanes 1 and 2). Similar results were obtained for the polyadenylation activity of fraction 6 and for fraction 12 (not shown). A comparison between figure 2A and B shows that in relation to the hexameric core composed of Rrp41 and Rrp42, more DnaG is present in the insoluble exosome from fraction 12 than in the soluble exosome from fraction 6. Csl4 was not clearly detected in the silver stained gels, but it was possible to detect it by Western blot hybridization. To analyze the differences in the composition of the RNA-binding caps of the soluble and the insoluble exosomes, the elution fractions shown in figure 2A and B were hybridized simultaneously with Rrp4-and Csl4-directed antibodies. Figure 2D shows that in relation to Rrp4, the insoluble exosome contains higher amounts of Csl4 than the soluble exosome. DnaG and Csl4 were not detected by Western blot analysis in the S100 fraction (Figure 1A) and in fractions of low sucrose density like fraction 6 (Figure 1C), but were co-immunoprecipitated from those fractions (Figure 2A and D). This shows that the amounts of DnaG and Csl4 in S100 and in fraction 6 were under the limit of detection in the Western blot analysis, but it was possible to enrich these proteins during the immunoprecipitation procedure. In summary, Figure 2 shows that the soluble and the insoluble exosomes differ in their DnaG content and in the composition of the RNA-binding caps. The data also suggests an interaction between the soluble exosome and EF1-alpha. Both the soluble and the insoluble exosomes can degrade RNA, but their functional relevance is still unexplored.

To confirm an interaction between the soluble exosome and EF1-alpha by an independent experiment, we purified the exosome from the S100 fraction using DnaG-specific antibodies. The immunoprecipitated exosome contained Csl4, Rrp4, Rrp41, Rrp42, DnaG and EF1-alpha (Figure 3A). The identities of DnaG and EF1-alpha were confirmed by mass spectrometry. We assumed that only a part of the exosomal complexes present in the soluble fraction contain DnaG. Therefore, we decided to remove the DnaG containing exosome from the S100 fraction by three rounds of immunoprecipitation with the DnaG-specific antibodies, and then to purify the DnaG-less exosome from the depleted S100 fraction using Rrp41-specific antibodies. Figure 3B shows the Western blot analysis of the flow through (depleted S100 fraction) after each.

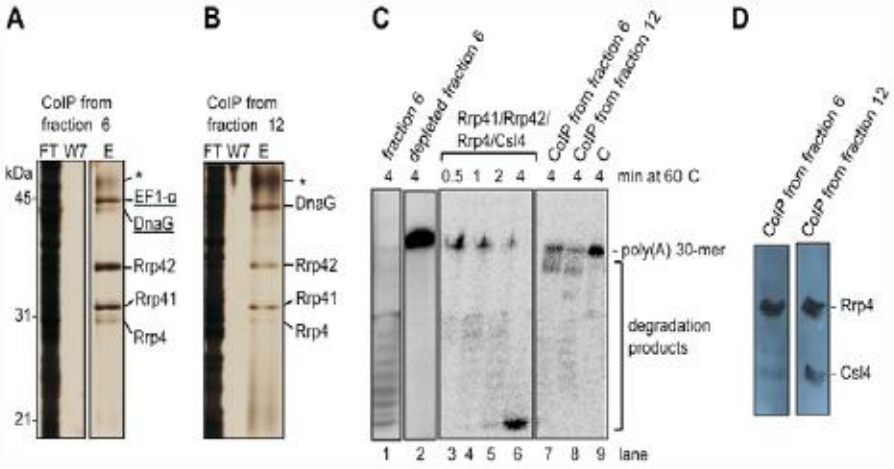

Figure 2: Exosomal complexes with different sedimentation behaviours are active and differ in their composition. A) and B) Silver stained SDS-gels showing proteins purified by coimmunoprecipitation (CoIP) with Rrp41-specific antibodies from different sucrose density gradient fractions. A) The soluble exosome was purified from fraction 6. B) The insoluble exosome was purified from fraction 12. FT, flow-through; W7, last, seventh washing fraction; E, elution fraction. The migration of marker proteins is marked (in kDa). Underlined proteins were identified by mass spectrometry, bands with known migration behaviour are marked with the names of the respective proteins. The band corresponding to antibodies is marked with an asterisk. C) Phosphor images of degradation assays with fraction 6 (lane 1), depleted fraction 6 (the flow-through after three rounds of immunoprecipitation of the exosome with Rrp41-specific antibodies (18); lane 2), exosomes reconstituted by mixing of equimolar amounts of Rrp41, Rrp42 and the RNA-binding proteins Rrp4 and Csl4 (lanes 3e6), the co-immunoprecipitated exosome from fraction 6 (lane 7), the co-immunoprecipitated exosome from fraction 12 (lane 8), and water (negative control C, lane 9), as indicated above the panels. The incubation time in minutes (min) is also indicated. The 50-labelled 30-meric poly(A) RNA and the degradation products are marked on the right side. D) Western blot analysis of the elution fractions are shown in A) and B). To estimate the relative amounts of Csl4 and Rrp4, the membranes were hybridized simultaneously with Csl4- and Rrp4-directed antibodies. The detected proteins are marked on the right side.

Immunoprecipitation step. The removal of the exosome with DnaG specific antibodies led to a strong decrease in the intensity of the Rrp41 signal. The remaining exosome was purified with Rrp41- specific antibodies. The SDS-PAGE analysis of the elution fraction (Figure 3C) revealed bands with similar migration when compared to Figure 3A. The presence of Csl4 and DnaG in the elution fraction shown in Figure 3C was confirmed by mass spectrometry. The band of low intensity above DnaG was identified as TIP49 protein, showing that EF1-alpha was already removed together with the exosome during the depletion with the DnaG-specific antibodies. This result points to the possibility that the soluble exosome interacts with different proteins. TIP49 was not detected as a minor protein in the EF1-alpha containing gel slices analyzed by mass spectrometry. This protein was also not found in the corresponding gel slices in a control experiment with the pre-immune serum (not shown).

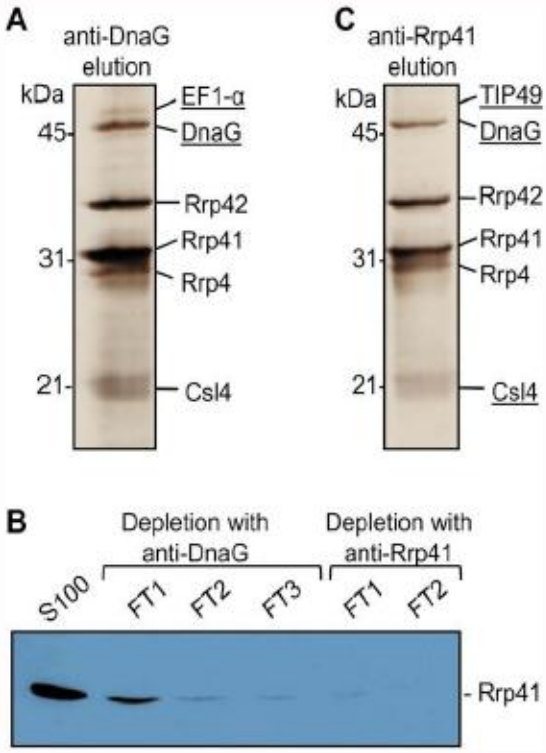

Figure 3: Co-immunoprecipitation (CoIP) of EF1-alpha with the soluble exosome using DnaG-specific antibodies. The S100 fraction was subjected to three rounds of CoIP with DnaG-specific antibodies (anti-DnaG). Subsequently, two rounds of CoIP with Rrp41- specific antibodies (anti-Rrp41) were performed. A) Silver stained SDS-gels showing the proteins, which were co-precipitated during the first CoIP round with anti-DnaG (anti-DnaG elution). B) Western blot analysis of the flow-through fractions (FT) after each CoIP round. The antibodies used for depletion by CoIP are marked above the panels. The FT fractions (depleted S100) were separated on a 12% SDS-PAGE and hybridized with anti-Rrp41. The detected Rrp41 is marked on the right side of the panel. C) Silver stained SDS-gels showing the

proteins, which were co-precipitated during the first CoIP round with anti-Rrp41 (anti-Rrp41 elution). For further description see Figure 2B.

Figure 3 shows that, it was not possible to completely remove the DnaG-containing exosome from the S100 fraction after three rounds of co-immunoprecipitation with DnaG specific antibodies. On the other hand, it strongly suggests that the most o the exosomes in the soluble fraction are associated with DnaG, since it was not possible to enrich exosomes without DnaG. Based on Figure 2 and 3, we conclude that DnaG and Csl4 are integral parts of the soluble exosome. Furthermore, the data supports the interaction between EF1-alpha and the exosome and suggests that DnaG and EF1-alpha are present together in the soluble exosome.

Rrp4 and Csl4 form heteromeric RNA-binding caps *in vivo*

Figure 2C suggests that exosomes with different sedimentation behaviours have differences in the composition of the RNA-binding cap. However, it was still not clear whether Rrp4 and Csl4 are present together in the exosome. The RNA-binding cap of the exosome of A. fulgidus can be composed of Rrp4, Csl4 or both Rrp4 and Csl4 *in vitro (7)*. However, the existence of archaeal exosomes with heteromeric RNA-binding caps containing Rrp4 and Csl4, was not shown *in vivo* so far. To study the *in vivo* composition of the RNA-binding cap, the exosome was immunoprecipitated with Rrp4-specific antibodies or with Csl4-specific antibodies from *S. solfataricus* cell-free extracts and from S100 fraction. The elution fractions were tested for the presence of Csl4 and Rrp4, respectively. To ensure detection of Csl4, 1.2 g of cells (wet pellet) were lysed in 1 ml of buffer, and the clarified cell-free extract (supernatant after centrifugation at 2500 g) or the corresponding S100 fraction was used for co-immunoprecipitation. The immunoprecipitated proteins were eluted in 40 ml, and the complete elution fraction was loaded. Figure 4A and B shows that Csl4 is coimmunoprecipitated together with the exosome when Rrp4- specific antibodies are used, and vice versa. The identities of Csl4 and Rrp4 were confirmed by mass spectrometry. Thus, the archaeal exosome contains heteromeric RNA-binding caps *in vivo*, and such caps are present in the soluble exosome. Since Rrp4 and Csl4 confer different substrate specificities to the exosome and may also be responsible for the interaction with different protein partners, it is interesting to know whether different RNA-binding caps exist *in vivo* consisting of Rrp4 only, Csl4 only, and Rrp4 and Csl4 in different relative amounts. We assumed that if heterogeneous RNA-binding caps are present, they should be differently enriched in immunoprecipitation experiments with Rrp4- or Csl4-directed antibodies. The elution fractions shown in Figure 4A and B suggest that similar relative amounts of Rrp4, Rrp41 and Rrp42 were isolated with the two different antibodies, but higher amounts of Csl4 were isolated with the Csl4 - directed antibodies than with the Rrp4-directed antibodies. Figure 4C confirms that different relative amounts of Rrp4 and Csl4 are immunoprecipitated from the S100 fraction when the two different antibodies are used. These results confirm that Rrp4 and Csl4 are parts of protein complexes *in vivo* and do not exist as monomers. From the results in Figure 4 we conclude that exosomes with heterogeneous RNA-binding caps are present in the soluble fraction of S. *solfataricus*.

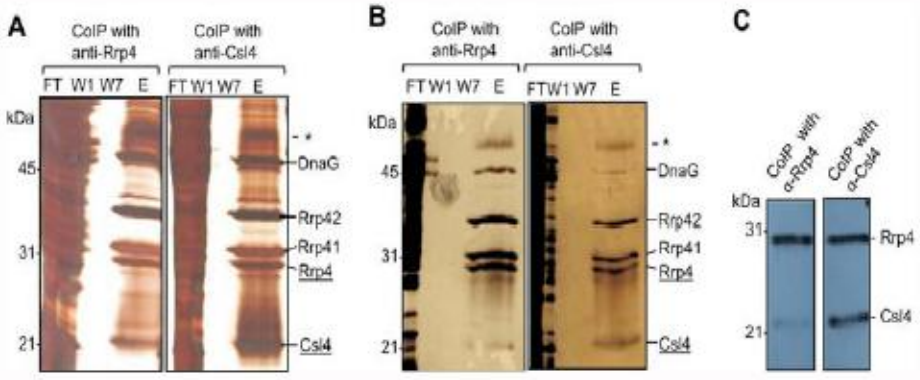

Figure 4: *In vivo* analysis of the RNA-binding cap of the *S. solfataricus* exosome. A) and B) show silver stained gels, C shows Western blot analysis. A) Co-immunoprecipitation (CoIP) experiments with anti-Rrp4 and anti-Csl4 antibodies from a clarified cell-free extract. B) CoIP with anti-Rrp4 and anti-Csl4 antibodies from S100 fractions. W1, first elution fraction. For further descriptions see Figure 2C. Western blot analysis of exosomes immunoprecipitated from S100 fractions with anti-Rrp4 and anti-Csl4 antibodies (marked above the panels). To estimate the relative amounts of Csl4 and Rrp4 in the immunoprecipitated complexes, the membranes were hybridized simultaneously with Csl4- and Rrp4-directed antibodies. The detected proteins are marked on the right side.

Discussion

Our approach to purify the archaeal exosome using antibodies directed against different subunits and using different protein fractions was helpful to uncover the heterogeneity in the composition of this protein complex in *S. solfataricus*. We found that the content of the soluble exosome differs from the content of the insoluble exosome which sediments in the middle of sucrose density gradients together with ribosomal subunits. Since the majority of the exosome co-sediments with membranes, the complexes analyzed in this work represent a minor part of the exosome of *S. solfataricus*. Nevertheless this minor part should be of functional importance, since both the soluble and the insoluble exosomes were active. The functions of archaeal exosomes with different sedimentation properties are still not explored, but it can be speculated that the soluble exosome is important for the metabolism of mRNAs and tRNAs present in the soluble fraction, while the exosome which co-sediments with ribosomal subunits may be involved in rRNA processing. In this respect, the differences in the protein composition of the soluble and the insoluble exosomes may reflect the need to interact with different RNA substrates and different accessory proteins. So far the archaeal nine-subunit exosome was intensely studied *in vitro*, but little is known about its interactions with other proteins. Association of the archaeal DnaG protein with the exosome was reported for several archaeal species (18,27,28), but the function of DnaG in this context remains unclear. Recently it was shown that DnaG exhibits primase activity and it was proposed that archaea harbour both an eukarya-like and a bacteria-like primase. This might explain the high conservation of DnaG in archaea, even in species lacking the exosome. Nevertheless, DnaG seems to be of fundamental importance for the archaeal exosome. Our results show that although it is present in lower amount in soluble exosomes of *S. solfataricus* than in exosomes of higher sedimentation coefficient, the majority or all soluble exosomes contain DnaG. Accordingly to our data, DnaG is the major interaction partner of the exosome with higher sedimentation coefficient, while the soluble exosome interacts with additional proteins. The copurification of EF1-alpha with the soluble exosome in three biologically independent experiments, using two different sera (Rrp41-specific and DnaG-specific) and different protein fractions (S100 or sucrose density gradient fraction), strongly suggests a functional interaction between EF1-alpha and the protein complex. The co-purification of TIP49 with the residual soluble exosome after depletion with DnaG-specific antibodies indicates that the soluble exosome may interact with different minor protein partners. The specificity of the co-purification of the exosome and TIP49 remains to be verified. Interaction of the exosome with other proteins was reported for several archaeal species. The DnaG-containing exosome was detected in a high molecular mass complex with the tRNA-intron endonuclease in *M. thermoautotrophicus (27)*. On the other hand, it was co-purified with an rRNA biogenesis factor (FAU-1 protein) and a protein with unknown function (TK0790) in *T. kodakarensis (28)*. In another study, the activity of the *Pyrococcus abyssi* exosome was shown to be affected *in vitro* by two RNA binding proteins, PaSBDS and PaNip7, and it was shown that PaNip7 associates with the exosome in the absence of RNA. Our data strongly support the interaction between the exosome and EF1-alpha in S. *solfataricus*. The archaeal EF1-alpha is involved in translational elongation and

termination, and mRNA surveillance pathways, suggesting that it may link the release of mRNA from the ribosome to its tailing and/or degradation by the exosome. The simultaneous presence of Rrp4 and Csl4 in the RNA-binding cap of the archaeal exosome *in vivo* probably ensures its interaction with different transcripts and protein partners. Different Rrp4/Csl4 stoichiometries in different fractions of the exosome probably influence the functions of the complex. It was shown for Archaeoglobus, Pyrococcus and *Sulfolobus*, that Rrp4 and Csl4 differently influence the interaction of the exosome with RNA. Little is known, however, about the *in vivo* stoichiometry of these subunits in the heterogeneous exosomal complexes and about the specific function of these proteins and their individual domains. The comparison of exosomes isolated with anti-Rrp4 and anti-Csl4 antibodies suggests that in both cases, when compared to the amounts of Rrp41 and Rrp42 forming the catalytic hexamer, quite similar amounts of Rrp4, but very different amounts of Csl4 were isolated (Figure 4). Since the presence of monomeric Csl4 was excluded, the high amount of Csl4 isolated with the anti-Csl4 antibodies is probably a part of the co-precipitated exosome. In such a case, however, our results are not compatible with a trimeric structure of the RNA-binding cap which is composed of Rrp4 only, Csl4 only or Rrp4 and Csl4 in different stoichiometries. Such a trimeric structure implies that enrichment of Csl4 by co-immunoprecipitation should result in less Rrp4 in the elution fraction and vice versa. The results shown in Figure 4 rather imply that a small amount of the exosome with the usual Rrp41/Rrp42/Rrp4 stoichiometry but containing an excess of Csl4 is enriched by co-immunoprecipitation with Csl4-specific antibodies. The exosome which is immunoprecipitated with Rrp4-specific antibodies seems to contain Rrp41, Rrp42 and Rrp4 in similar amounts and Csl4 in very low amounts, resembling the reconstituted *S. solfataricus* exosome. The possibility that some Csl4 aggregates, which do not interact with the exosome, were co-precipitated with the Csl4-specific antibodies should be excluded, since Csl4-poor exosomes and Csl4-rich exosomes were also purified with Rrp41-antibodies (see Figure 2). Thus, Csl4-rich exosomes and Csl4-poor exosomes exist *in vivo* and can be separated in sucrose density gradients. Based on this, it is tempting to speculate that individual domains in the RNA-binding cap of the exosome are involved in the regulation of the dual function of the exosome as an exoribonuclease and an RNA tailing enzyme in archaea.

Conclusions

In this study we showed the existence of RNA exosomes with heterogeneous composition in the archaeon, S. *solfataricus*. The different relative amounts of DnaG and Csl4 found in the soluble and the insoluble exosomes suggest that these proteins determine the sedimentation properties of the complex. We also found that although present in lower amounts, DnaG is an integral part of the soluble exosome, and that the soluble exosome interacts with EF1- alpha. The presence of heteromeric, Rrp4 and Csl4 containing, RNA binding caps *in vivo*, the different relative amounts of Rrp4 and Csl4 in soluble and insoluble exosomes, and the different effects of DKHRrp4 on the activities of the hexameric ring *in vitro* strongly suggest that the RNA-binding cap is involved in the regulation of the functions of this RNA-degrading and RNA-tailing protein complex.

Acknowledgements

This work was supported by DFG, grant Kl563/27-1 and IRTG "Enzymes and multi-enzyme complexes acting on nucleic acids".

References

1. C Condon. Maturation and degradation of RNA in bacteria, *Curr. Opin. Microbiol.* (2007);**10**:271-278.
2. E Evguenieva-Hackenberg, G. Klug, RNA degradation in Archaea and Gramnegative bacteria different from Escherichia coli, Prog. *Mol. Biol. Transl. Sci.* 2009;**85**:275-317.
3. JG Belasco. All things must pass: contrasts and commonalities in eukaryotic and bacterial mRNA decay. *Nat. Rev. Mol. Cell. Biol.* 2010;**11**:467-478.

4. CR Woese, GE Fox, Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci. U.S.A.* 1977;**74**:5088-5090.

5. E Evguenieva-Hackenberg, The archaeal exosome, Adv. Exp. Med. Biol. 702 (2010) 29e38.

6. E Lorentzen, P Walter, S Fribourg, E Evguenieva-Hackenberg, G Klug, E Conti. The archaeal exosome core is a hexameric ring structure with three catalytic subunits, *Nat. Struct. Mol. Biol.* 2005;**12**:575-581.

7. K Büttner, K Wenig, KP Hopfner. Structural framework for the mechanism of archaeal exosomes in RNA processing, *Mol. Cell* 2005;**20**:461-471.

8. CR Ramos, CL Oliveira, IL Torriani, CC Oliveira. The Pyrococcus exosome complex: structural and functional characterization. *J. Biol. Chem.* 2006;**281**;6751e6759.

9. Q Liu, JC Greimann, CD Lima. Reconstitution, activities, and structure of the eukaryotic RNA exosome, Cell 127 (2006) 1223e1237 Erratum in: *Cell* 2007;**131**:188-189.

10. A Dziembowski, E Lorentzen, E Conti, B Séraphin. A single subunit, Dis3 is essentially responsible for yeast exosome core activity. *Nat. Struct. Mol. Biol.* 2007;**14**:15-22.

11. A Lebreton, R Tomecki, A Dziembowski, B Séraphin. Endonucleolytic RNA cleavage by a eukaryotic exosome, *Nature* 2008;**456**:993-996.

12. D Schaeffer, B Tsanova, A Barbas, FP Reis, EG Dastidar, M Sanchez-Rotunno, CM Arraiano, A van Hoof. The exosome contains domains with specific endoribonuclease, exoribonuclease and cytoplasmic mRNA decay activities. *Nat. Struct. Mol. Biol.* 2009;**16**:56-62.

13. P Mitchell, E Petfalski, A Shevchenko, M Mann, D Tollervey. The exosome: a conserved eukaryotic RNA processing complex containing multiple 30/50 exoribonucleases, *Cell* 1997;**91**:457-466.

14. C Allmang, J Kufel, G Chanfreau, P Mitchell, E Petfalski, D Tollervey. Functions of the exosome in rRNA, snoRNA and snRNA synthesis. *EMBO J* 1999;**18**:5399-5410.

15. J LaCava, J Houseley, C Saveanu, E Petfalski, E Thompson, A Jacquier, D Tollervey, RNA degradation by the exosome is promoted by a nuclear polyadenylation complex, *Cell* 2005;**12**:713-724.

16. S Vanácová, J Wolf, G Martin, D Blank, S Dettwiler, A Friedlein, H Langen, G Keith, W Keller. A new yeast poly(A) polymerase complex involved in RNA quality control. *PLoS Biol.* 2005;**6**:189.

17. BK Mohanty, SR Kushner. Polynucleotide phosphorylase functions both as a 3'-5' exonuclease and a poly(A) polymerase in Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.* 2000;**97**:11966-11967.

18. P Walter, F Klein, E Lorentzen, A Ilchmann, G Klug, E Evguenieva-Hackenberg, Characterization of native and reconstituted exosome complexes from the hyperthermophilic archaeon *Sulfolobus solfataricus*, *Mol. Microbiol.* 2006;**62**:1076-1089.

19. V Portnoy, E Evguenieva-Hackenberg, F Klein, P Walter, E Lorentzen, G Klug, G Schuster. RNA polyadenylation in Archaea: not observed in Haloferax while the exosome polynucleotidylates RNA in *Sulfolobus*, *EMBO Rep.* 2005;**6**:188-1193.

20. V Portnoy, G Schuster, RNA polyadenylation and degradation in different Archaea: roles of the exosome and RNase R. *Nucleic Acids Res.* 2006;**34**:5923-5931.

21. S Levy, V Portnoy, J Admon, G Schuster. Distinct activities of several RNase J proteins in methanogenic archaea. *RNA Biol.* 2011;**8**:1073-1083.

22. N Mathy, L Bénard, O Pellegrini, R Daou, T Wen, C Condon, 5'-to-3' exoribonuclease activity in bacteria: role of RNase J1 in rRNA maturation and 5'stability of mRNA. *Cell* 2007;**129**:681-692.

23. D Hasenöhrl, R Konrat, U Bläsi, Identification of an RNase J ortholog in *Sulfolobus solfataricus*: implications for 50-to-30 directional decay and 5'-end protection of mRNA in Crenarchaeota. *RNA* 2011;**17**:99-107.

24. K Shahbabian, A Jamalli, L Zig, H Putzer, RNase Y, A Novel. Endoribonuclease, initiates riboswitch turnover in Bacillus subtilis. *EMBO J.* 2009;**28**:3523-3533.

25. EV Koonin, YI Wolf, L Aravind. Prediction of the archaeal exosome and its connections with the proteasome and the translation and transcription machineries by a comparative-genomic approach. *Genome Res.* 2001;**11**:240-252.

26. E Evguenieva-Hackenberg, P Walter, E Hochleitner, F Lottspeich, G Klug. An exosome-like complex in *Sulfolobus solfataricus*. *EMBO Rep*. 2003;4:889-893.

27. MH Farhoud, HJ Wessels, PJ Steenbakkers, S Mattijssen, RA Wevers, BG van Engelen, MS Jetten, JA Smeitink, LP van den Heuvel, JT Keltjens. Protein complexes in the archaeon Methanothermobacter thermautotrophicus analyzed by blue native/ SDS-PAGE and mass spectrometry. *Mol. Cell. Proteomics* 2005;4:1653-1663.

28. Z Li, TJ Santangelo, L Cubo nová, JN Reeve, Z Kelman. Affinity purification of an archaeal DNA replication protein network. *MBio* 2010;1:00221-002210.

29. E Lorentzen, A Dziembowski, D Lindner, B Seraphin, E Conti. RNA channelling by the archaeal exosome. *EMBO Rep*. 2007;8:470-476.

30. MV Navarro, CC Oliveira, NI Zanchin, BG Guimarães. Insights into the mechanism of progressive RNA degradation by the archaeal exosome. *J. Biol. Chem*. 2008;283:14120-14131.

31. C Lu, F Ding, A Ke. Crystal structure of the *S. solfataricus* archaeal exosome reveals conformational flexibility in the RNA-binding ring. *PLoS One* 2010;5.

32. E Evguenieva-Hackenberg, V Roppelt, P Finsterseifer, G Klug, Rrp4 and Csl4 are needed for efficient degradation but not for polyadenylation of synthetic and natural RNA by the archaeal exosome. *Biochemistry* 2008;47:13158-13168.

Knowledge and attitudes related to Dementia among in ward patients aged more than 50 years

Kumarasinghe WAA[1], Rajapakse IH[2], Samarasinghe LNP[3], Dissanayake As[4]

[1]*Nursing student, Faculty of Medicine, University of Ruhuna, Sri Lanka*
[2]*Lecturer, Department of Psychiatry, Faculty of Medicine, University of Ruhuna, Sri Lanka*
[3]*Registrar, Professorial Psychiatry Unit, Teaching Hospital Karapitiya, Galle, Sri Lanka*
[4]*Senior Lecturer, Department of Medicine, Faculty of Medicine, University of Ruhuna, Sri Lanka*

Background
Sri Lanka has a rapidly aging population with a high prevalence of dementia. Elders are dependent on an intergenerational care system. Deficient knowledge about nature, causes and availability of treatment are barriers to improved outcomes.

Aim
Gain an in-depth understanding on what cognitive impairment meant, its impact, coping strategies among a cohort of in hospital patients aged more than 50 years

Methods
A semi structured questionnaire was used on 200 participants. Descriptive statistics were used to analyse findings

Results
Males constituted 44%. Fifty-seven percent were in the 50-60 year age group. More than 40% had received education up to Ordinary Level examination and 55% were from social class 5. Approximately half acknowledged 'loss of recent memories' affecting old people. Only 59% recognized this as an illness and not part of normal aging. Thus treatment was sought only when symptoms were severe from western, ayurvedic and traditional practitioners. Mental health services were used very late. Approximately 60% felt that those with dementia exhibited disruptive behaviour. Stigma led to 39% stating they will be ashamed to have someone in the household with dementia. Ninety percent felt being involved in religious activities improved the symptoms. Fifty percent felt those with dementia should continue to live at home, whilst 35% wanted specific places for them. Fifteen percent stated they had no wish to live if they got dementia.

Conclusions
Elders did not recognize dementia as an illness requiring treatment. Mental health services were underutilized due to general ignorance, stigma and prejudices. A wider awareness of dementia and early utilization of mental health services need to be promoted to meet the challenge of caring for an ever increasing elderly population with dementia.

A survey of breakfast practices, meal skipping and related factors among adolescent school children in Galle Municipality area

Silva APH[1], Wijesinghe Cj[2]

[1]*BSc Nursing Degree Programme, Faculty of Medicine, University of Ruhuna, Sri Lanka.*
[2]*Department of Community Medicine, Faculty of Medicine, University of Ruhuna, Sri Lanka.*

Introduction
Adolescence is a challenging period of an individual's life. The nutrition and dietary habits of adolescents are particularly important not only for today's health but also to prevent future development of non-communicable diseases. Skipping meals is a common unhealthy dietary behaviour seen among adolescents.

Aim
To assess the patterns of meal skipping, breakfast practices and associated factors among adolescent school children in Galle Municipality area.

Method
A descriptive, cross-sectional study was conducted among 360 randomly selected adolescents from grade 9-11, in schools of Galle Municipality area. Data were collected using a self-administered questionnaire to identify selected dietary habits and analyzed using SPSS statistical software package.

Results
Breakfast was the most commonly skipped meal among adolescent school children in the sample. Only 58.6% of the respondents reported having breakfast daily, whereas 11.9% of them missed breakfast daily and 29.5% had breakfast irregularly. Of those who had breakfast daily or irregularly, 22.7% purchased it from school canteen, food/pastry shops or street vendors. Skipping breakfast was significantly higher among female adolescents (23.2%) compared to males (16.4%, $p<0.05$). The residential area (urban/ rural), residential place (home/ hostel/ boarding place) or mode of travelling to school was not significantly associated with missing breakfast. In contrast, lunch and dinner patterns were more regular; only 0.8% and 2.2% skipping these meals daily, respectively.

Conclusion
The adolescent school children in Galle Municipality area seem to have unhealthy breakfast practices, which in turn will affect their health and nutritional status. Promoting healthy eating habits should be given a priority in school health programmes.

Key words: breakfast practices, dietary habits, adolescents, Galle.

A comparison between Pyrogallol Red and Sulphosalicylic acid turbidimetric methods for protein estimation in Cerebrospinal fluid

Gunaratna KAPD[1], Siriwardhana ID[2], Dayanath BKTP[3]

[1]Medical Laboratory Sciences Degree Programme, Faculty of Medicine, University of Ruhuna, Sri Lanka
[2]Department of Pathology, Faculty of Medicine, University of Ruhuna, Sri Lanka
[3]Teaching Hospital Karapitiya, Galle, Sri Lanka.

Introduction and Objectives
The estimation of protein in cerebrospinal fluid (CSF) is important in certain disease conditions. The sensitivity of the Biuret method which is the standard method for protein estimation is not sufficient to be used for CSF specimens. This has lead to the development of various assay techniques for this purpose. The laboratory of Teaching Hospital, Karapitiya employs two such methods; the Pyrogallol red (PGR) method in the day laboratory and Sulphosalicylic acid turbidimetric method (SSA) in the on-call laboratory. The aim of this study was to compare these two methods.

Methods
Linearity and reproducibility were evaluated using solutions with known albumin concentrations while correlation was tested using retained CSF specimens.

Results
Linearity: The PGR method gave a linear response up to 200 mg/dL while the SSA method was linear up to 300 mg/dL. Reproducibility: the inter-assay CVs at 40 mg/dL were 9.94% and 5.09% for the PGR and SSA methods respectively. Reproducibility was superior at 120 mg/dL for both methods with CVs of 5.72% and 3.55% for PGR and SSA methods respectively. Correlation: Though a positive correlation (r) of 0.9077 (r^2=0.824) was observed there was a significant difference between the values obtained using the two methods and this was confirmed using the simple t-test ($p < .0.0001$). The equation of [PGR] = 0.435 x [SSA] + 0.693 was derived through linear regression.

Conclusions
The significant difference observed between the results obtained using the two techniques discredits the use of the two methods within the same laboratory. Further studies needs to be done to determine the best test method to be used in routine practice.

Epidemiological and clinical characteristics of dengue during epidemic and non-epidemic periods in southern Sri Lanka- lessons learned from 2007 and 2012-2013

Bodinayake CK[1], Tillekeratne LG[2], Nagahawatte A[3], Devasiri IV[4], KodikaraArachchi W[5], Ostbye T[6], Gubler DJ[7], Woods CW[8], Reller Me[9]

[1]Department of Medicine, University of Ruhuna, Galle, Sri Lanka.
[2]Duke Global Health Institute, Durham, USA.
[3]Department of Microbiology, University of Ruhuna, Galle, Sri Lanka.
[4]Department of Pediatrics, University of Ruhuna, Galle, Sri Lanka
[5]Teaching Hospital Karapitiya, Galle, Sri Lanka.
[6]Community and Family Medicine, Duke University School of Medicine, Durham, USA.
[7]Emerging Infectious Diseases, Duke- National University of Singapore, Singapore.
[8]Medicine, Duke University School of Medicine, Durham, USA,
[9]Pathology University School of Medicine, Baltimore, USA.

Objectives
We evaluated epidemiological, clinical characteristics of dengue, comparing two similar studies performed at Teaching Hospital Karapitiya (THK) Galle during outbreak (2012-2013) and without outbreak (2007).

Methods
Two cross-sectional studies have been conducted at THK from March - October 2007 and June 2012 - February 2013. Adults and children with acute fever (tympanic >100.4°F, documented >102°F), with no obvious bacterial focus, have been enrolled within 48 hours of admission. Data and blood have been collected at enrollment and 2-4 weeks later. Acute dengue was confirmed by paired IgG and IgM ELISA, PCR or viral isolation.

Results
In 2007 total of 1079 patients (61.2% male, median age 30.7 years) and in 2012 /2013 total 409 patients (64.3% male, median age 25.2 years) have been enrolled. Acute dengue was confirmed in 54 (6.3%) in2007and 188 (46.0%) in 2012-2013. Acute dengue had leukopenia (WBC <4.0x10^3/µl, p<0.01) and thrombocytopenia (platelets <100x10^3/µl, p<0.01) than non-dengue in both studies. Total 3 (2007) and 7 (20012-2013) patients met criteria for dengue hemorrhagic fever. In 2007, clinical diagnosis sensitivity, specificity and positive predictive value were 14.0%; (95% CI 11.6 - 16.4), 97.8% (95% CI 96.8 - 98.9) and 30.4% (95% CI 27.2 - 33.6) respectively. In 2012/2013 the corresponding values were 64.4%; 95% CI 57.1 - 71.2), 71.5%; 95% CI 65.1 - 77.4) and 65.8% (95% CI 58.4 - 72.6). Serotypes DEN 2-4 isolated in 2007, changed to DEN-1 (94.6%), DEN-4 (5.4%) in 2012/2013. Sero- prevalence increased from 50.9% (2007) to 83.7% 2 (2012/2013).

Conclusions
Dengue was a major cause of febrile hospital admissions during 2012-2013. DEN 1 caused the 2012-2013 epidemic. Sensitivity of clinical diagnosis and positive predictive value significantly improved during 2012-2013.

Evaluation of new, non-cyanide and simple method for blood haemoglobin estimation

Sirajul Muneera MY[1], Wickramaratne KAC[2]

[1]Medical Laboratory Science Degree Programme, Faculty of Medicine, University of Ruhuna, Sri Lanka.
[2]Department of Pathology, Faculty of Medicine, University of Ruhuna, Sri Lanka.

Introduction
Haemoglobin estimation is an important test. The standard Haemoglobin (Hb) estimation method is Cyanmethaemoglobin (HiCN) method. The Drabkin reagent used in HiCN method contains cyanide compounds which is hazardous for both environment and laboratory personnel.

Aim
To assess accuracy of Hb estimation by using distilled water as the reagent compared to the HiCN method.

Materials and Method
Retained 95 EDTA blood samples received at the Haematology laboratory, Teaching Hospital, Karapitiya were included in this study. The Hb value of each sample was estimated by standard HiCN method and using the new method (performed by using distilled water as the reagent). In Drabkin method 10 – 20 minutes are needed to develop stable Hb pigment for the estimation thus readings are taken at 20 minutes. Using new method, Hb concentration of all the samples were estimated immediately and after 20 minutes. Correlation studies and paired t test were taken to statistical analysis.

Result
A good correlation was observed between the cyanmethaemoglobin method and the new method. Paired t test values of immediate absorbance and absorbance at 20 minutes of the new method showed no significant difference from values obtained by standard Cyanmethaemoglobin method (immediate absorbance - P = 0.819, Absorbance at 20 minutes - P = 0.941).

Discussion and Conclusion
This study shows statistical agreement of results generated by new method against the standard. The new method doesn't require any reagent or toxic chemicals. However appropriate test validation study is needed to verify the applicability of new method for routine testing before it is recommended.

RJM
Ruhuna Journal of Medicine

Sub-committees of Faculty of Medicine Academic Session (FMAS) - 2014, University of Ruhuna

Steering Committee
Prof. Sarath Lekamwasam
Dr. Channa Yahathugoda
Dr. A.S. Dissanayake

Journal Committee
Prof. C. Liyanage (PIC)
Prof. Kamani Jayatilleke
Dr. Sampath Gunawardena (Editor-in-Chief)
Dr. I.D. Siriwardhena
Dr. A.J.P.M. Jayawardena
Dr. R.P. Hewawasam

Opening Ceremony Committee
Dr. K.G. Imendra (PIC)
Dr. M .Rodrigo
Dr. C. Wijesinghe
Dr. H. Rajapakse
Dr. L .Probodha
Dr. J. Ruben

Food & Beverages and Registration Desk Committee
Dr. A. Jayasekera (PIC)
Dr. G. Alwis
Dr. J. Lenora
Dr. G. Wijeratne
Mr. Thilina Abeygunawardene

Panel of Evaluators
Prof. C. Liyanage
Prof. Sarath Lekamwasam
Prof. Lakmini Mudduwa
Prof T.P .Weerarathna

Invitation & Decoration Committee
Dr. Chandana Wickremarathne (PIC)
Dr. Eisha Waidyarathne
Dr. J. Warusahennadi
Dr. J. Nanayakkara
Dr. J. Ruben

Technical Sessions Committee
Dr. M .Hettiarachchi (PIC)
Dr. K.D. Mahinda
Mr. K.T.S. Pushpakumara
Mr. Darshana
Mr. Upawansa
Mr. Pathirana

Registration Desk Committee
Dr. G. Liyanage (PIC)
Dr. T. Ponnamperuma
Dr. Nayani Weerasinghe
Dr. ATIM Amarasinghe
Ms. Sujeeva