Kandiah Memorial Graduateship Award - 2006

Local antibiotic and potential toxic principles of *Borassus flabellifer* L.

A. A. P. Keerthi

Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda.

Palmyrah palm (*Borassus flabellifer* L.) is widely spread in the landscape of North East Sri Lanka. Flabelliferins are a group of steroidal saponins contained in palmyrah fruit and flour. Depending on the carbohydrate moiety attached to hydroxyl group in position 3 of the β-sitosterol, flabelliferins vary in their structure and the bioactive properties. More than 16 fabelliferins have been isolated during the past 8 years. The content and composition of natural occurrence of the flabelliferins varies due to many factors, *viz.* genetics, location, climatic and edaphic factors. Studying the biologically active compounds of palmyrah could increase utilization of palmyrah products.

Our studies were directed to investigate the effect of flabelliferin B (F_B) on bacteria and infected wounds as a local antibiotic, effect of unidentified flabelliferin on lethality to mosquito larvae, isolation of a highly haemolytic and neurotoxic flabelliferin and deducing the mechanism of action of the principles of palmyrah flour causing neurotoxicity Throughout our studies flabelliferins were isolated according to the method described earlier by Nikawala *et al.* and modified by Darshika *et al.* Flabelliferins which associate with the fluorescent carotenoid binder (mainly phytofluene) were separated from the binder employing silica gel $G_{60}PF_{254}$ (1mm thickness) circular chromatotron disk, using propanol: methanol 1:1 mixture. Pure flabelliferins were obtained as white hydroscopic powders after solvent evaporation followed by freeze-drying.

An *E. coli* strain ATCC 25922 was obtained from the Medical Research Institute (MRI). Purified F_B with and without the UV binder was tested for anti-bacterial activity using Bauer-Kirby plate method by a 24-hour zone diameter. Results confirmed that F_B with the UV binder was more potent than without binder. For the controls and blanks inhibition zone was not observed. The IC_{50} for the F_B was obtained as 3.06 μmol ml^{-1} (13.3 mg in 5 ml). From all F_B liquid medium experiments, the colony counts and the visible spectra showed that there was complete inhibition above 9.22 μmol ml^{-1}. F_B with the UV binder was not soluble in the culture medium and results could not be obtained. Furthermore in the presence of the UV binder, combined precipitation of bacteria and adduct occurred and some bacteria were viable. Evidence from Bauer-Kirby plate method even though it is subject to diffusional and solubility problems emphasized the role of the UV binder in elevated anti-bacterial activity. The non-polar UV binder can alter the total polarity of the complex in order to penetrate the phospholipid bilayer very effectively. This facilitates the action of universal anti-bacterial carbohydrate moiety (β-Glc and 1,2 and 1,4 α-Rha) anti-bacterial site to kill the bacteria. According to Bauer-Kirby plate method calculations, F_B together with the UV compound (in hydrophobic adduct form) was about three times more powerful than F_B without binder.

For the animal experiments male Wistar rats (age group 5 to 7 months) were obtained from the MRI. After an acclimatization period, the hairs on the back of animals were removed. Two deep incisions were made in both test and control. Wounds were treated using 0.15 μmol of F_B every 12 hours. Wound swabs were obtained and subjected to microbiological examination. No allergic reactions, wound healing delaying factors or toxic effects were observed. The eye test, which is more sensitive for toxicity was next performed on Wistar rats and New Zealand white rabbits. Veterinary doctors subjected rabbits to ophthalmoscopic observations. No toxic effects were observed at 3.8 mmol of F_B per eye. At the same level human volunteers showed no allergenicity on applying F_B in saline on the skin.

Ethical clearance was obtained from the University of Sri Jayewardenepura Ethical Review Committee and the Colombo South Teaching Hospital (CSTH) Ethical Committee to conduct a trial at the hospital. F_B with the binder 1.4 μmol g^{-1} (2%) in white soft paraffin was prepared as an ointment. Fourteen patients were selected from the dermatology clinic, the OPD and the wound clinic. In 4 double blind trial the test group was treated only with F_B after cleaning the wound with hypertonic saline. Oral antibiotics were not administered. Wound measurements, swabs for microorganisms, and photographs were obtained. The controls were treated according to normal hospital procedure using metrogel, betadine ointment, soframicin containing metronidazole, iodine and furamycetin sulphate as antibacterial agents. The rate of re-epithelialization was measured and the mean wound area decline per week was 23.8% for test and 17.5% for controls. These results were not statistically significant probably due to inherent variation from patient to patient but clinically significant as F_B was at least good as the normal treatments currently available in the hospital. Furthermore F_B appears to act as an antibacterial agent, wound debridment agent to remove necrotic tissue from wound and a cleanser to promote the wound healing (i.e. multiple effects). This is the most exciting feature of the F_B, which yields different multiple effects from one single formula.

An unidentified flabelliferin had mosquito larvaecidal activity on *Aedes albopictus*, *Aedes egyptii* and *Culex quinquifaciatus* larvae, in different molting stages, were tested for lethality. Ethanolic extracts (free from solvents) as well as MPLC fractionated compounds were used in larval assays. Five grams of palmyrah flour samples obtained from Kalpitiya were extracted with different solvents after homogenization. Weights of each fraction were obtained and subjected to larval assay. The results of the present study indicate that the ethanolic extract had the highest larvaecidal activity with LD_{50} ranging from 60mg L^{-1} to 76mg L^{-1} depending on the larval stage. The MPLC separations were conducted to break down the compound mixtures to single compounds. Samples were subjected to mosquito larvae assay. Fraction 38 resulted in one single compound causing larvaecidal effect. The mass spectroscopic studies confirmed aglycone attached Glc Rha-Rha moieties with linear sequence.

The extract was lethal to all larvae tested except for *Culex quinquifaciatus* larvae that have longer breathing tube. Arrangement of the flabelliferin molecules on the surface of the water altered the surface activity and the larvae, which were unable to adhere to the surface water or penetrate the saponin layer died. A catfish (*Poecilia reticulata*) were exposed to 2.66 mg/ml solvent free methanol extract in order to test the effect of the extract on the free aquatic environment, the gill breather (*Multi-spotted glasstail guppy*) and lung breather. It was observed that the time of death was 15 min for *Multi-spotted glass tail guppy* and 60 min for *Poecilia reticulata*. This is probably due to the well-known effects of saponins on gill and lung membranes. Therefore, it is clear that, this particular toxin cannot be used in the free aquatic environment but is good enough for vases and drains etc. where the dengue mosquito breeds. The electron microscopic studies eliminated the possible clastrogenic effects which can lead to early pupation.

Our next experiments were related to isolation of highly haemolytic flabelliferin from palmyrah flour. Normally saponin molecules are reported as heamolysis causing, but these molecules were 8 fold more haemolytic when compared to other flabelliferins. The partial structure of the compound using mass spectroscopic studies confirmed the presence of β-sitosterol or isomer attached one Glc and five Rha units.

Palmyrah flour has been reported to be neurotoxic. The neurotoxicity can be eliminated by dry heat at 80 °C for 45 min. Isolated volatiles were subjected to GC-MS analysis. Decanoic acid (Capric acid), undecanoic acid, 2-butenedioic acid and their esters were identified in the volatiles. None of these compounds has been previously reported as neurotoxins. Animals were exposed to liberated volatiles upon heating, and the experiment confirmed the absence of toxic volatiles. Further, some ninhydrin positive spots were absent after dry heat treatment. Therefore it is possible that the neurotoxic effect is probably based in a complex. The complex of amine-flabelliferin-phytofluene had been found to be mildly neurotoxic. Its molecular weight is consistent with the unidentified toxin by Greig. Our ongoing research is aimed at elucidating the structure and mechanism of the neurotoxic principle.

Adamantane House Hall Hire Charges

PPGL Siriwardene Auditorium and J N O Fernando Hall will be hired for Seminars, Workshops etc. Hire charges are as follows:

PPGL Siriwardene Auditorium (with Multimedia)
Rs. 10,000/- (1 day with Air Conditioning)
Rs. 8,000/- (1 day without Air Conditioning)

J N O Fernando Hall
Rs. 5,000/- (1 day with Multimedia)

I.Chem. C Benevolent Fund - Benefits

Beneficiaries comprise Institute Members who have paid membership fees for Life as well as Graduateship Course Lectures/ Teaching Assistants, who contribute regularly towards the Fund.
Benefits are given below:

1. Death Donation of Rs 15,000 to next of kin for funeral expenses **or** Rs 10,000 donation after reaching 75 years, at the specific request of the member
2. Medical Expense for critical illness / accidents involving heavy expenditure (Max-Rs 25,000)
3. Contribution toward 50% of international travel expense of young Chemists attending Conferences abroad (Max-Rs 25,000)
4. Such other benefits as may be determined by the Council from time to time