# DISCOVERING FUTURE CURES *from* PHYTOCHEMISTRY TO METABOLOMICS

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## ABSTRACT

Eukaryotes such as higher plants have evolved to produce a diverse range of low-molecular-weight compounds known as secondary metabolites or phytochemicals, that can be used as food and feed additives, flavours, fragrances, cosmetics, agrochemicals and pharmaceuticals. The chemical diversity of plants is more complex than any chemical library made by humans, and the plant kingdom therefore represents an enormous reservoir of valuable molecules just waiting to be discovered. There are approximately 298,000 species of higher plants, less than 10% of which have been chemically characterized to any extent**.** The majority of plant species are found in tropical rain forests. Extracts and infusions containing natural products from plants have historically been a major source of pharmaceutical ingredients, often comprising mixtures of several bioactive compounds with complex synergistic effects. The first secondary metabolite isolated from plants was morphine (over 200 years ago) and many other natural compounds have since become important pharmaceuticals in modern society. It is often difficult to find synthetic substitutes with the same efficacy and specificity as natural compounds. Consequently, molecules derived from plants make up a sizeable proportion of current high-value drugs, and also provide many new lead compounds for industrial applications. Many companies are undergoing a renaissance in their interest in plant-derived compounds, especially the pharmaceutical industry looking for new drugs, and the cosmetics industry which uses plant extracts, oils and even plant cell cultures in some products. The high structural diversity and complexity of secondary metabolites still pose technical challenges for a phytochemist since the chemical characteristics of the plant compounds vary to a very high degree, for example in the degree of polarity and solubility. Typically such compounds accumulate at low levels in plant tissues and

production is strongly correlated to specific vegetative stages. Thus the process of finding, extracting, isolating and purifying these phytochemicals is time-consuming and can be a daunting and challenging task. Further many plants that produce high-value secondary metabolites are difficult to cultivate or are endangered because of over-harvesting and deforestation activities. However, the last decade has seen tremendous development in technology and the birth of new, innovative methods that are now accessible for use by the current generation of researchers. In some cases, what was impossible to do then has become almost routine now. Armed with these new advantages and strength, the continuous exploration of plant biodiversity, using novel and innovative bioassays to evaluate natural compounds for their beneficial effects, is therefore of great scientific, medical and bioeconomic importance.

In this inaugural lecture, some highlights of my journey and discoveries made, together with my colleagues and students that I have worked with over the last 15 years of natural products research, will be presented. Our research endeavours which began with a mainly reductionist perpective, in accordance with the then popular drug discovery approaches used by big pharmas and academic laboratories all over the world, has now mellowed into a more holistic approach, utilizing 'omics' technologies. Sample size and loss of biological activity due to the reductionist approach of disturbing the inherent synergism of a plant metabolome, has always been a delimiting factor in phytochemistry studies. We believe that systems biology, in particular metabolomics/metabonomics, holds greater promise in our efforts to gain a deeper understanding of a plant metabolome's effects on a disease state or on other biological perturbations.

## INTRODUCTION

"What is a Weed - A Plant whose Virtues have yet to be Discovered"

*Robert Frost*

Plants are virtually an endless supply of potential cures for humanity. Historically, plants have formed the oldest basis for medicines and inspired many new ones to treat many debilitating diseases and relieve human sufferings. They have been used as medicinal agents, first only on a folkloric basis, but as chemists moved from myth and mystery to establishing basis using modern scientific methods, the true properties of natural extracts from plants began to be uncovered. Many of the early discoveries are now backed with scientific basis and some have been developed into single agent drugs. Notable examples of compounds from folkloric plants that have been particularly important for human medicine include morphine from Opium Poppy (*Papaver somniferum*), aspirin from the White Willow Tree (*Salix alba vulgaris*), tubocurarin from the Amazonia 'curare' (*Chondrodendron tomentosum*) and the famous vinca alkaloid duo, vinblastine and vincristine, from the Madagascar Periwinkle (*Catharanthus roseus*) (Figure 1).


**Figure 1** Amazonia curare *Chondrodendron tomentosum* (left) and Madagascar periwinkle *Catharanthus roseus* (middle), the botanical source of tubocurarin (muscle relaxant) and vincristine (anticancer), respectively

Novel bioactive phytochemicals are important feedstock for potential development of new pharmaceuticals and the rich biodiversity of the tropical forest plants holds great promise for the discovery of such compounds. The reason for this can probably be explained by their high chemical diversity which is unsurpassed by any other approaches used in drug discovery and development. Bioactive phytochemicals serve as useful chemical leads, to be refined synthetically by a medicinal chemist who creates analogues with enhanced potency, with better selectivity or specificity, and without the unwanted adverse side effects.

The approach to drug discovery using plants as a source has a historical justification i.e plants have consistently yielded important new pharmaceuticals. It also has a chemical rationale in that the natural product molecules provide templates for drug design. Finally, the biochemical rationale of using plants as a source is based upon the accepted understanding that the co-existence of plants with other occupants within the same ecosystem demands that they produce an armemantarium of defense substances, many of which would most likely be of a novel phenotype. It is the direct result of the natural selection during evolution and competition between the species that has produced many powerful, biologically active natural product compounds. The challenge now for us as Earth dwellers is to protect, conserve and sustainably harvest the benefits it can provide to humanity.

The chemical diversity of plants is more complex than any chemical library made by humans, and the plant kingdom therefore represents an enormous reservoir of valuable molecules just waiting to be discovered. The detailed exploration of plant biodiversity and the use of novel and innovative bioassays to evaluate natural compounds for their beneficial effects is therefore of great scientific, medical and bioeconomic importance. Many companies

are undergoing a renaissance in their interest in plant-derived compounds, especially the pharmaceutical industry looking for new drugs, and the cosmetics industry which uses plant extracts, oils and even plant cell cultures in some products. However, many plants that produce high-value secondary metabolites are difficult to cultivate or are endangered because of over-harvesting. The chemical synthesis of plant-derived compounds is usually uneconomical because the complex stereospecific structures are difficult to replicate. Typically such compounds accumulate at low levels in plant tissues and production is strongly correlated to specific vegetative stages, which makes isolation time consuming and expensive. Sustainable and cost-effective production systems for high-value plant-derived compounds must therefore be developed, and the best outcome can be achieved by integrating creative and multidisciplinary approaches into more sustainable production chains featuring cutting-edge innovative technologies.

## PHYTOCHEMICALS AND STRUCTURAL DIVERSITY

Plants are like a chemical factory where a wide range of organic substances are manufactured. A plant cell produces two types of metabolites. The first, the primary metabolites, such as carbohydrates, proteins and lipids, are directly involved in growth and metabolism. The other type is the secondary metabolites which are the end products of primary metabolism, such as, alkaloids, sterols, terpenoids, flavonoids, saponins, lignins and tannins. These secondary metabolites are not involved in the normal growth, development or reproduction of the plants, but are believed to function as a defense mechanism against predators, parasites and diseases. Due to this, secondary metabolites are accumulated by plants cells in smaller quantities than primary metabolites. Further,

their biosynthesis occurs only in specialized cells and at a particular development stage. The distribution of these metabolites in plants vary from one family to another. As shown in Figure 2, secondary metabolites are not made *via* a single pathway. Specific pathways biosynthesize different classes of compounds and the occurrence of the compounds may be restricted to a particular taxonomic group which forms the basis of the study of chemotaxonomy. The high structural diversity and complexity of the secondary metabolites also mean that the chemical characteristics of these phytochemicals are going to be very different with respect to the degree of polarity and solubility. Thus, the process of finding, extracting, isolating and purifying these phytochemicals can sometimes be a daunting and challenging task.

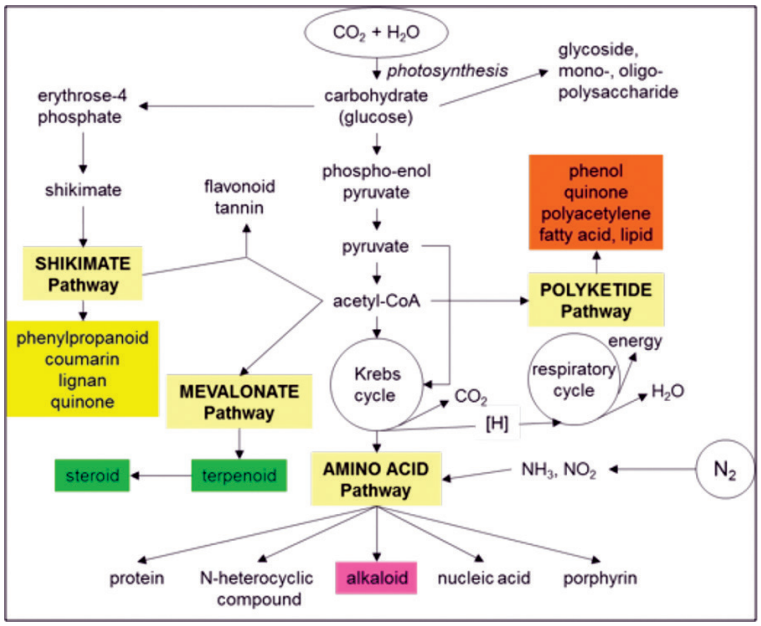


**Figure 2** Biosynthetic pathways to secondary metabolites

## APPROACHES IN NATURAL PRODUCTS RESEARCH

The use of natural products as a source of or as molecular templates for novel structures in drug discovery is still alive and well. Although combinatorial chemistry techniques have succeeded as methods of optimizing structures, it has produced only one *de novo* new chemical entity (NCE), reported in the public domain, since its first entry into the drug discovery scene 25 years ago (Newman & Cragg, 2007). In many areas of drug discovery research, specifically for cancer-treatment and new anti-infectives, natural products' continued influence is very clear, as can be seen from the high number of 'natural product mimics' approved as drugs for these diseases. This proves that the exploration of nature's 'treasure trove of small molecules' for novel active agents that may serve as leads and scaffolds into much needed efficacious drugs, has not been exhausted and should in fact be further expanded.

A major objective of natural products research is the preclinical development of bioactive natural products and their analogues. Searching for lead molecules from plants will usually involve following a straightforward information-led chemotaxonomic approach or following leads from ethnobotanical or ethnomedicinal uses of the plant. Often times too, the search for bioactive molecules is solely based on serendipity which results from curiosity driven random screening of plants extracts. In general, the more logical ethnomedicinal-led approach, where empirical information serves as a guide to select potential plants for study, seem to have produced better outcomes. This approach involves developing and optimizing suitable functional assays and evaluation of the medicinal plant extracts for the relevant biological activity. Once activity is validated, a bioassay-guided approach is adopted in the isolation and purification of the active constituents before culminating in the

elucidation of the chemical structure and stereochemistry using high resolution spectroscopic methods, in particular Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy.

The early part of the drug discovery process encompasses lead identification right up to lead optimization, i.e determination of the structure-activity relationships (SARs) of the molecule. Lead identification is made more efficient through the use of rational drug design which guides medicinal chemists in their decisions regarding the next logical synthetic step. It combines knowledge and skills from the fields of cheminformatics, molecular modeling and structural bioinformatics and demands in-depth understanding of the physico-chemical properties of the three-dimensional molecule (Adam, 2005). Meanwhile, lead optimization aims at enhancing the most promising compounds to improve effectiveness, diminish toxicity or increase absorption. Many of the technologies for lead discovery overlap with those for lead optimization as researchers attempt to incorporate the best drug characteristics early in the process. In designing a drug, attempts are made to find a ligand (the putative drug) that will interact favorably with a receptor that represents the target site. Based on the information that is available, either ligand-based or receptor-based molecular design methods may be used. The ligand-based approach is applicable when the structure of the receptor site is unknown but a series of compounds have been identified that exert the activity of interest. Using structurally similar compounds with high activity, with no activity and with a range of intermediate activities, the approach attempts to identify a pharmacophore *via* recognition site mapping prior to ligand design.

Meanwhile, the receptor-based approach applies when a reliable model of the receptor site is available, as from X-ray diffraction, NMR, or homology modeling. Ligands that will interact favorably

at the site are designed, aided by computational molecular docking experiments. SAR is the relationship between the chemical or three-dimensional structure of a molecule and its biological activity. The analysis of SAR enables the determination of the chemical groups responsible for evoking a target biological effect in the organism. This allows modification of the effect or the potency of a bioactive compound (typically a drug) by changing its chemical structure. Medicinal chemists use the techniques of chemical synthesis to insert new chemical groups into the biomedical compound and test the modifications for their biological effects. Further refinements *via* real and virtual experiments are then used to derive mathematical relationships between the chemical structure and the biological activity i.e quantitative structure-activity relationships (QSAR) that give greater insights into the molecular interactions that are expected to occur in the biological systems.

## MOLECULE HUNTING IN THE RAINFOREST

Systematic botanists estimate that there are approximately 298,000 species of higher plants (Lawrence, 1951; Mora *et al.,* 2011). About 1%, roughly 3000, has been utilized for food and of these 3000, about 150 have been commercially cultivated. However, the vast majority of caloric intake derives from only 20 species of plants. These plants represent the basis upon which the world's population is fed, representing a very narrow foundation supporting the world's human population. On the other hand, less than 10% of the higher plants have been chemically characterized to any extent. This is despite the fact that, approximately 10,000 of the world's plants have documented medicinal use, and in modern medicine, roughly 150 to 200 of these plant materials are incorporated into pharmaceutical applications (Newman and Cragg, 2012). This is still a very small percentage of all higher plants, i.e "just the tip of the iceberg".

There are many more potentially important discoveries in the plant kingdom to be exploited for pharmaceutical applications.

Meanwhile, it has been estimated that approximately half of the species of higher plants are found in the tropical forests of the world (Sanjay 2007). Malaysia has 14,000 of those species in her tropical rainforest. Years ago, Burkill (1935) had reported the medicinal use of 1,300 of these species. It would not altogether be a surprise, if a similar inventory is carried out in the present time, that the real number of medicinal plants that could be found in the rainforest is much higher. Thus, the rainforest is a haven for phytochemists who are basically 'molecule hunters', in search of plants with compounds that challenge their structure elucidation skills and could be future lead molecules for future development into something useful. The remaining sections of this book attempt to give a brief account of the exploration and some of the discoveries made over the course of 15 years of research.

## Resolving Ambiguity of Flacourtiaceae Species

My phytochemical journey began with several rainforest plants which, under the Cronquist's classification, were classified as belonging to the family Flacourtiaceae (Chase *et al.,* 2002). Common Flacourtiaceae species include the chalmoogra (*Hydnocarpus kurzii*) and rukam fruit (*Flacourtia rukam*) trees (Figure 3). This family of plants was regarded as a 'dumping ground' for odd and anomolous genera. Therefore, the chemistry of these plants were unpredictable and presented a fertile field for research, holding great promise for discovery of interesting structures, just waiting to be identified. Our interest to chemically investigate the plants was not purely to look for novel and bioactive metabolites, but to also enrich the chemotaxonomic knowledge surrounding this heterogenous family of plants. Chemotaxonomy is the scientific

discipline of accumulating information on the distribution of secondary metabolites with a view of using this information to throw light onto systematic or phylogenetic relationships among extant taxa, usually of higher plants.

**Figure 3** Some Flacourtiaceae species found in Malaysia; (from left) *Hynocarpus kurzii, Flacourtia rukam, Scolopia spinosa*

As it turned out, our work on *Scolopia spinosa* (Khozirah & Waterman, 1994a) and *Homalium longifolium* (Khozirah & Waterman, 1995; 1996) can be considered to be of significant value to the field of chemotaxonomy as it provided proof that these two genera shared similar chemistry and therefore are taxonomicaly related. Of the novel metabolites obtained from them most proved to possess a central core of 2-(13-glucopyranosyloxy)-5-hydroxybenzyl alcohol, the position of glycosylation being confirmed by NMR experiments which showed both the anomeric H-1' of the sugar and H-7 of the benzyl alcohol coupling to C-2 (Figure 4). The sugar moiety was generally partially esterified with usually benzoic acid and occasionally, a *trans-para-coumaric* acid in *H. longifolium,* or (*S*)-aleprolic acid in *S. spinosa.*

Further series of esterifications to C-7 of the benzyl alcohol added more variability to the structures. Notable examples of the esterifying acids include 1-hydroxy-6-oxocyclohex-2-en-l-carboxylic acid in scoloposide-D and a 1,2,3-trihydroxy-6-oxocyclo-hexane-l-carboxylic acid in scoloposide-E. In a further

modification the 2-hydroxyl was esterified with the aleprolic acid esterifying group that is so common in *S. spinosa*. Meanwhile *H. longifolium* elaborated C-7 esterifications with 1,2,6-trihydroxy-5-oxocyclohex-3-en-l-carboxylic and isocoumarinic acids. For the former, an X-ray study resolved the stereochemistry of the three hydroxyls, all on the same face of the cyclohexenoic acid with the 2 and 6 hydroxyls in a pseudoequatorial configuration and the 1-hydroxyl pseudoaxial. In addition to this, several aberrant glycosides were also isolated with a unique C~5 unit linked to the glucose at C-1. The origins of this moiety is still unsolved. Interestingly, *S. spinosa* and *H. longifolium* have now been transfered to Salicaceae, where other 2,5-hydroxybenzyl alcohol-bearing genera such as *Poliothrysis*, *Xylosma* and *Phyllobotryon* are now placed. As a family, the Flacourtiaceae is now defunct and many of its 800 species have been transfered to other families, notably, Salicaceae, Samydaceae and Achariaceae in the molecular phylogeny-based classification.

Another Flacourtiaceae studied, *Casearia clarkei*, yielded podophyllotoxin-type lignans as major constituents (Khozirah and Waterman, 1994b), It did not elaborate any of the previous glucosides or the typical diterpenoids nor coumarins associated with the genus. This finding was rather unexpected since this class of lignans are characteristic constituents of the *Podophyllum* species (Berberidaceae), better known as the American Mayapple. The anticancer drugs, etoposide and teniposide, used in chemotherapy for lung cancer, lymphomas and genital tumors, were inspired from this classs of lignans. At the moment *C. clarkei* has still to be reclassified to a new family.

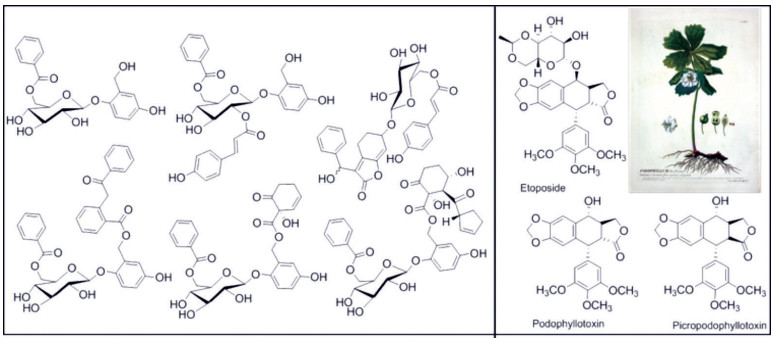


**Figure 4** Glucosides of 2,5-hydroxybenzyl alcohols isolated from *Homalium longifolium* and *Scolopia spinosa* (left) and podophyllotoxins from *Casearia clarkei*

The knowledge and experience gained from working on the Flacourtiaceae provided a platform that launched me into the rest of my forays into the structural world of secondary metabolites. Together with a band of 'molecule hunters' consisting of research associates and students of the Laboratory of Natural Products of the Institute of Bioscience, we continued the search for novel and new structures that could be lying in wait in the dense and rich tropical rain forests of Malaysia which included the Endau Rompin State Park and Royal Belum Forest Reserve. Highlights of some of our findings are presented in the following sections.

## Cytotoxic Xanthones of *Garcinia*

*Garcinia* is the largest genus of the Guttiferae family. They are usually small to medium dioecious fruit trees, characterized by a hard timber and the bark typically exudes a sulphur-yellow latex. Several species are famous for their edible fruits such as the 'manggis' (*G. mangostana*) and 'asam gelugor' (*G. atroviridis*) in Malaysia, and the 'asam kandis' (*G. cowa*) in Indonesia. The barks of several species are also the source of gamboges, a natural yellow

pigment used in dyeing. Ethnomedicinally certain *Garcinia* species are used to heal wounds, skin diseases or used internally to treat bilious conditions, diarrhea and dysentery (Burkill, 1966). Fifty of the 400 species of *Garcinia* distributed within the palaeotropical regions are found in Malaysia and many of them are endemic (Willis, 1973; Whitmore, 1983). Chemotaxonomically, *Garcinia* is known to be a prolific producer of cytotoxic xanthones, of which, the chemical structures range from simple to the more complex caged- and phloroglucinol-types (Sultanbawa, 1980; Bennet & Lee, 1989). Caged xanthones and phloroglucinols represent a challenging class of natural products in terms of both isolation and characterization. The total synthesis of the caged structure remains one of the unconquered challenges of organic synthesis which is still being pursued by many medicinal chemistry groups due to their potent antitumour activity.

During the course of our exploration of tropical rainforest species we collected and screened a number of *Garcinia* species for cytotoxity against several cell-lines. From these, several species i.e *G. cantleyana* (Figure 5), *G. penangiana*, *G. urophylla* and *G. cowa*, were found to be consistently active in the bioassays, which fueled our interest to subject them to further scrutiny of their bioactive constituents. Chromatographic separation and isolation of the secondary metabolites yielded a whole series of polyprenylated and caged-xanthones as well as depsidones. Full characterization of the novel structures made extensive use of NMR and mass spectroscopy.

**Figure 5** The leaves, bark and fruit of *Garcinia cantleyana*

The leaves and bark of the *G. cantleyana* yielded several new caged-xanthonoids cantleyanone A to D and 7-hydroxyforbesione, in addition to the common deoxygaudichaudione A and gaudichaudione H (Figure 6). The prenyl moieties on the non-caged section of these xanthonoids were in the free form or oxidized to the dihydrobenzofuran or benzofuran moieties (Khalid *et al.,* 2007). The biosynthetic pathway for the formation of cantelyanones B, D and F from gaudichaudione H were also proposed (Figure 7). Caged xanthones were also obtained from *G. urophylla viz* the new 7-hydroxydesoxymorellin together with gaudichaudione H. Other than these, polyprenylated xanthones were also a common feature of all three species. These included penangianaxanthone, macluraxanthone C, gerontoxanthone C and cudratricusxanthone H (Rozida *et al.,* 2007; Mat Lip *et al.,* 2009)

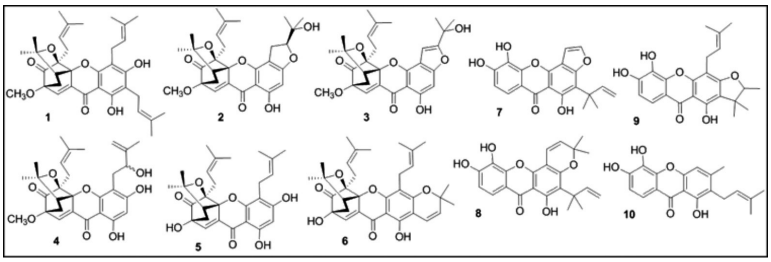

**Figure 6** Caged xanthones and polyprenylated xanthones from *Garcinia* species
**(1-4)** Cantleyanones A to D, **(5)** 7-hydroxyforbesione, **(6)** 7-hydroxydesoxymorellin, **(7)** penangianaxanthone, **(8)** cudratricusxanthone H, **(9)** gerontoxanthone C, **(10)** macluraxanthone C

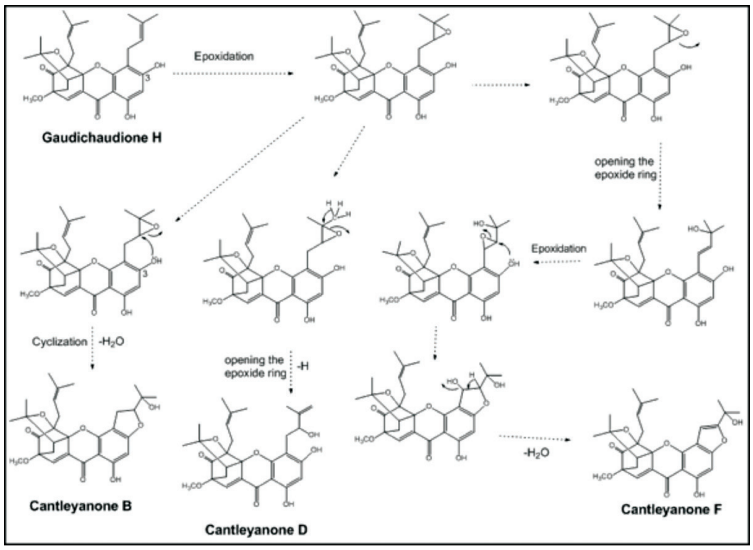


**Figure 7** Proposed biosynthetic pathway of cantleyanones B, D and F from gaudichaudione H

As we had anticipated, many of the xanthones, particularly, the caged-xanthonoids tested significantly positive for cytotoxity against several cancer cell lines. A structure-activity-relationship (SAR) analysis of the caged-xanthones clearly revealed that caged ring-B, a peri-hydroxyl group on ring-A, were an essential structural feature for bioactivity. This important finding was in accordance with other hypotheses (Cao *et al.,* 1998; Mackeen *et al.,* 2000). The C8-8a double bond was also thought to be important for bioactivity (Zhang *et al.,* 2004). However, as indicated by the weak cytotoxic activity exhibited by cantleyanone A, prenylation on C-2, appeared to significantly reduce the cytotoxic activity of these xanthones. Further work on these complex xanthones was not pursued due to the limited quantities that were successfuly isolated from their natural sources. Sadly, this is a reality which is a delimiting factor in natural products research. Total synthesis or other creative methods of obtaining adequate material for further biological and preclinical investigations are the obvious answer to this since sourcing from nature is just not sustainable in the long term.

**Table 1** Cytotoxic activity of *Garcinia* xanthones

| Compounds | $IC_{50}$ µg/ml* | | | |
|---|---|---|---|---|
| | MDA-MB-231 | CaOV-3 | HeLa-60 | MCF-7 |
| Cantleyanone A | 9.67 ± 1.53 | 13.83 ± 1.26 | 20.67 ± 3.06 | 24.33 ± 1.53 |
| 7-hydroxyforbesione | 2.17 ± 0.31 | 0.28 ± 0.07 | 0.22 ± 0.03 | 0.42 ± 0.08 |
| Cantleyanaxanthone | 25.50 ± 1.32 | 27.50 ± 0.87 | 13.33 ± 0.58 | 17.67 ± 1.53 |
| Cantleyanone B | 6.23 ± 0.93 | 0.28 ± 0.04 | 0.43 ± 0.10 | 0.83 ± 0.07 |
| Cantleyanone C | 6.70 ± 0.36 | 0.44 ± 0.06 | 0.48 ± 0.07 | 1.48 ± 0.37 |
| Cantleyanone D | 17.17 ± 0.76 | 3.47 ± 1.23 | 2.8 ± 0.3 | 4.40 ± 0.46 |
| Deoxygaudichaudione A | 0.44 ± 0.02 | 0.44 ± 0.02 | 0.34 ± 0.03 | 0.38 ± 0.06 |
| Gaudichaudione H | nd | nd | nd | 7.6±1.4 |

MDA-MB-231: Human breast cancer (Re-), CaOV-3: Human ovarian cancer , HeLa60: Human cervical cancer, MCF-7: Human -hormone dependent breast cancer cell lines

*Results are expressed as $IC_{50}$ values (µM) ± SD of three experiments performed in triplicate,

## Saponins and Sapindaceae

The Sapindaceae, vernacularly known as the Soap-Nut family, is a family of plants that elaborates saponins as its most abundant secondary metabolites. It is also famous for the edible fruits from some of its species such as *Nephelium lappaceum* Linn. (rambutan), *N. mutabile* Bl. (pulasan), *N. malaiense* Griff. (mata kucing) and *Litchi chinensis* Sonn. (litchi) (Hsuan, 1969). Our study focused on one of its lesser known genus, *Xerospermum*. Six *Xerospermum* species, collectively known as 'rambutan pachat', were recorded in Malaysia but during our collection trips we found only two i.e. X. *noronhianum* and *X. laevigatum* (Corner, 1988). The fruits of the species are also edible, the pulp sweet and pleasant although rather scanty. An infusion of the pulped stone of 'rambutan pachat' is prescribed as a drink to cure very severe or 'griping' stomach pains, and a decoction of the leaves is used if the previous treatment fails (Burkill, 1966). We were however not able to find any records of chemical or biological studies on the *Xerospermum* species. Our interest in the genus was based on the positive results shown by its aqueous methanolic extracts in a preliminary screening for anti-acetylcholinesterase and anti-butyrylcholinesterase activities. These two enzymes are implicated in Alzheimer's disease.

The word "saponins" implies a group of soap-like natural surfactants which form long-lasting bubbles on shaking of an aqueous solution. This chemical definition is a general term for surface-active glycosides of triterpenoids and steroids (triterpenoidal saponins and steroidal saponins). Saponins are characterized by a skeleton derived from the 30-carbon precursor, oxidosqualene, to which glycosyl residues are attached (Vincken *et al*., 2007). Saponins having one sugar moiety are called monodesmosides, and those having two sugar moieties at different positions are called bisdesmosides (or bidesmosides) (Ikan, 1999).

The separation and characterization of saponins are especially challenging, as are other similar very polar metabolites. It demands strong technical skills, excellent chromatographic facilities and high-end spectroscopic instruments. More often than not, strong perseverance on the part of the researcher will also be needed in solving the many technical difficulties that will arise. We were lucky to have such a student to carry out the tasks of purifying and characterizing the *Xerospermum* saponins. Her conscientious efforts succeeded in purifying twenty compounds from the plant which proved to be long and tedious. Extensive use of 1D and 2D NMR spectroscopy were used to elucidate the atom connectivities of the triterpenoids and sponins, while LC-MS/MS$^n$ was employed to determine the molecular weights and sequences of sugar linkages in the bidesmosides (Tan et al, 2008). These compounds, consisting of a variety of triterpenoids, flavonoid glycosides, phenolic acids and three bidesmosidic oleanane saponins i.e xerospermoside A, B and C (Figure 8), were the constituents present in the plant extract which tested positive for anti-cholinesterase and anti-butyrulnesterase, two enzymes implicated for Alzheimer disease. Although the extract was originally potent, the compounds were moderate or inactive when tested individually. This was not surprising as it is well known that the total biological effect of a plant could be due to synergistic effects. Synergism is a positive interaction created when two or more substances combine and exert an effect that is more than the sum of their individual effects (Aiyegoro and Okoh, 2009).

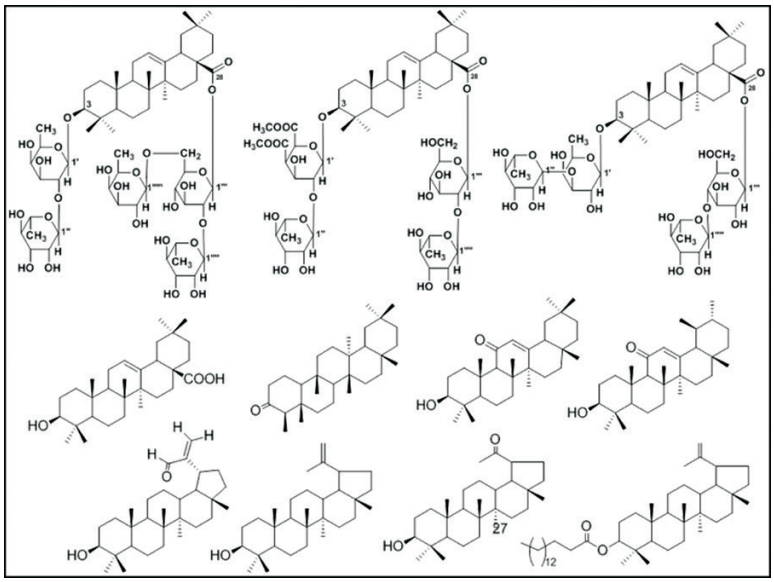

**Figure 8** Xerospermosides and various triterpenoids from *Xerospermum noronhianum*

## The Five-leaf Chaste-Tree and Gouty Pains

*Vitex negundo* is a popular medicinal plant in many Asian countries. Known as the five-leaved chaste tree, juice made from its purplish colored young leaves commonly serves as a treatment for sprained ankles, arthritic pain and similar injuries (Burkill, 1966). The leaves produce a cooling effect that eases pain and swelling and are therefore used in aromatic baths. The Malays also use the species as an ulam and for culinary purposes where the young leaves are an essential ingredient for preparing 'nasi lemuni', a traditional rice dish. In herbal medicine, *V. negundo* is regarded as an effective analgesic herb which alleviates pain and reduces edema.

A methanolic extract prepared in our lab tested significantly positive in our antioxidant assays, giving more than 95%

inhibition in both the 1,2-diphenyl-2-picrylhydrazyl (DDPH) and xanthine oxidase superoxide (XOD) scavenging assays, at a test concentration of 250 μg/ml sample. Xanthine oxidase (XO) is the enzyme that catalyzes the conversion of hypoxanthine-xanthine-uric acid, which are the terminal biochemical reactions of the purine degradation pathway. Therefore, any defects in purine metabolism results in an increase in the level of uric acid. This eventually leads to the deposition of sodium hydrogen urate monohydrate crystals, from the hyperuricaemic body fluids, in joints, resulting in gout, the most common cause of inflammatory arthritis in men over the age of 40 years.

With our interest now triggered we pursued this bioactivity in the extract further in a bioassay-guided approach, to isolate the secondary metabolites that might be responsible for the pain-relieving properties. Interphasing chromatographic separation and bioassaying the resultant column fractions repeatedly guided the isolation of several metabolites from the bioactive column fractions. The metabolites consisted of flavonoids, iridoids and lignans (Figure 9). The XOD scavenging property of the metabolites was particularly interesting as it indicated the metabolites that could be responsible for the pain-relieving activity (Table 2). Although the flavonoid luteolin showed more potent activity in the antioxidant assays, the overall bioactivity of the whole plant extract may have been due to synergy with the iridoids since these metabolites were also active in the XOD scavenging assay. Incidentally, *V. negundo* and luteolin have also been reported to reduce uric acid levels, hence the plant's use in alleviating gouty arthritic pain. Our study served to add more evidence and support to these findings.

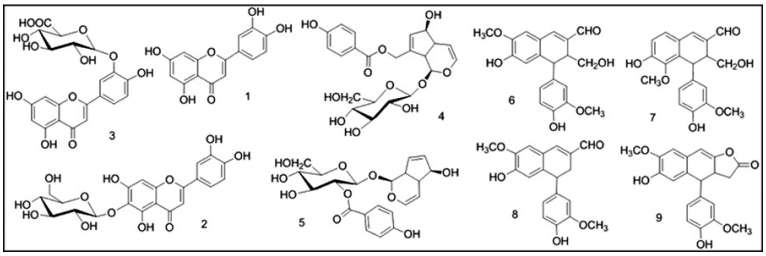

**Figure 9** Iridoid glucosides, flavonoids and lignans from *Vitex negundo* (**1**) luteolin, (**2**) isoorientin, (**3**) luteoline-3-O-glucoronide, (**4**) agnuside, (**5**) 2-p-hydroxybenzoyl mussaenosidic acid, (**6**) isovitedoin A, (**7**) vitedoin A, (**8**) vitrofolal E, (**9**) negundins

**Table 2** Inhibitory effects of the selected compounds of *Vitex negundo* on xanthine oxidase, DPPH radical and XO superoxide scavenging activities

| Compound | % inhibition | | |
|---|---|---|---|
| | **Xanthine oxidase (XO) (100 μg/ml)** | **DPPH radical scavenging (250 μg/ml)** | **XO Superoxide scavenging (250 μg/ml)** |
| Luteolin | 98.20 ± 0.02 | 98.45 ± 0.02 | 100.00 ± 0.01 |
| Isoorientin | 46.39 ± 0.26 | 93.15 ± 0.02 | 90.45 ± 0.02 |
| Agnuside | 59.90 ± 0.25 | 94.52 ± 0.01 | 71.57 ± 0.01 |
| Isovitedoin A | 66.45 ± 0.20 | 96.71 ± 0.01 | 71.40 ± 0.01 |
| 2'-p- hydroxybenzoyl mussaenosidic acid | 22.02 ± 0.32 | 9.14 ± 0.04 | 65.83 ± 0.02 |
| Allopurinol | 92.80 ± 0.21 | - | - |
| Ascorbic Acid | - | 98.09 ± 0.02 | 85.65 ± 0.01 |

*All values are mean ± S.D.*

## Chemical Marking Kachip Fatimah

*Labisia pumila* is the Latin name for the popular malay herb 'kacip Fatimah'. The estrogenic properties of *L. pumila* are very well known and have been, to a certain extent, supported by numerous biological studies (Jamal *et al.,* 1998). *L. pumila* has long been used in traditional medicine by the indigenous communities in the Malay archipelago, for treating pre- and post-partum complications, menstrual disorders, dysentery, rheumatism and flatulence, in addition to being used as a health tonic to regain vigor (Jaganath, 2000). In recent years, commercial products containing this herb have emerged in the Malaysian market for the purpose of enhancing vitality and libido (Latiff, 1997; Singh *et al.,* 2009). The popularity of the herb provided an impetus for studies on the biological properties of the herb viz. antibacterial (Karimi *et al.,* 2011, anti-inflammatory (Ibrahim et al., 1996; Rasadah *et al.,* 2001), antioxidant (Mohamad *et al.,* 2009), anti-ageing (Choi *et al.,* 2010) and cardioprotective (Mohamad and Nazaimoon, 2009). Yet, very little is known about the phytochemistry of sucn an important medicinal species. This gap in information and the medicinal importance as well as its economic promise, prompted our investigation of its uncharted secondary metabolites.

Comprehensive phytochemical investigation on *L. pumila* leaf material revealed it to contain alkylresorcinols and 1,4-benzoquinone-phenol dimers (Figure 10), as characteristic constituents, plus other common metabolites such as triterpenoids, flavonoids, flavans, phenolic acids and sterols. The structures of these compounds were established on the basis of 1D and 2D NMR spectroscopy techniques and mass spectrometry. In the characterization of the dimers, labisiaquinones A and B, a slight problem was encountered in the double bond assignments for the alkyl chains. This was resolved by chemical derivatization (ozonolysis) followed by

ESIMS analysis of the reaction products (Figure 11). Several of the alkylresorcinols, were significantly cytotoxic towards the human prostate (PC-3), colon (HCT-116) and breast (MCF-7) cancer cell lines. The actoxyresorcinols exhibited strongest cytotoxic activity at equipotent submicromolar growth inhibition ($GI_{50}$) in the tested cancer cell lines, in fact more potent than doxorubin (Table 3). More importantly, they exhibited cancer-type selectivity against PC-3 and HCT-116 cells at the TGI and $LC_{50}$ levels. The 1,4-benzoquinone dimers however were not cytotoxic. Nevertheless, similar long chain alkylphenols/benzoquinones (monmeric, dimeric, and conjugated forms) have been reported from other genera in the Myrsinaceae family viz. *Ardisia, Embelia* and *Maesa*. The 1,4-benzoquinone dimers and alkylresorcinols will be useful as chemical markers in the chemical fingerprinting of *L. pumila* raw material and extracts. These are much needed for quality control purposes of the myriad of commercial products containing 'kachip Fatimah' that are flooding the Malaysian market.

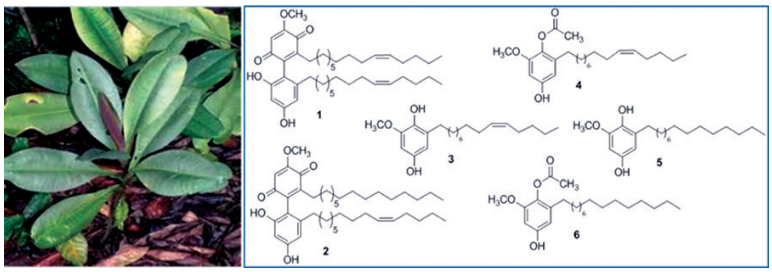

**Figure 10** *Labisia pumila* and its chemical markers: Labisiaquinone A (**1**), Labisiaquinone B (**2**), 5-(pentadec-10*Z*-enyl)resorcinol (**3**), 1-O-methyl-6-acetoxy-5-(pentadec-10*Z*-enyl)resorcinol (**4**), 5-pentadecylresorcinol (**5**), 1-O-methyl-6-acetoxy-5-pentadecylresorcinol (**6**)

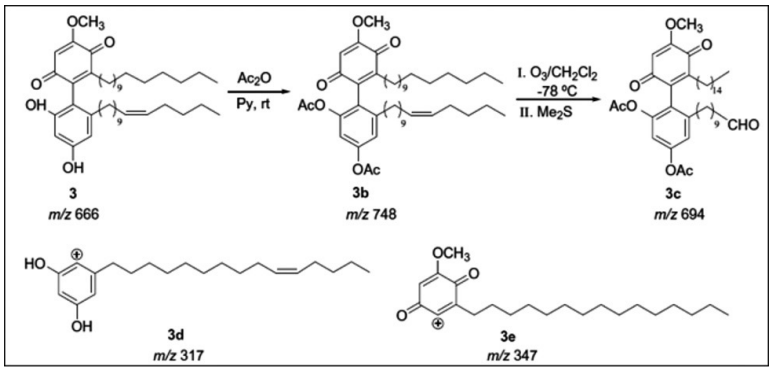


**Figure 11** Alkenyl resorcinol (3d), alkyl benzoquinone (3e) cation fragments and aldeyde adduct formation by ozonolysis of an acetylated analogue of labisiaquinone B

**Table 3** *In vitro* cytotoxic activity of acetoxy resorcinols 4 and 6 towards cancer cell lines

| Compound | Growth inhibitory parameters (μM) | | | |
|---|---|---|---|---|
| | Cell line | $GI_{50}$ | TGI | $LC_{50}$ |
| 1-O-methyl-6-acetoxy-5-(pentadec-10*Z*-enyl) resorcinol (**4**) | PC-3 | 0.3 ± 0.0 | 1.2 ± 0.0 | 8.0 ± 1.7 |
| | HCT-116 | 0.3 ± 0.0 | 1.0 ± 0.1 | 7.7 ± 6.4 |
| | MCF-7 | 0.4 ± 0.1 | 15.7 ± 2.1 | 41.3 ± 2.3 |
| 1-O-methyl-6-acetoxy-5-pentadecylresorcinol (**6**) | PC-3 | 0.4 ± 0.1 | 1.6 ± 0.4 | 10.0 ± 4.4 |
| | HCT-116 | 0.3 ± 0.0 | 1.6 ± 0.3 | 12.0 ± 8.7 |
| | MCF-7 | 0.5 ± 0.1 | 15.0 ± 1.7 | 40.0 ± 0.0 |
| Doxorubin | PC-3 | 0.7 ± 0.1 | 4.2 ± 0.2 | 20.0 ± 0.5 |
| | HCT-116 | 0.6 ± 0.1 | 2.0 ± 0.2 | 5.0 ± 0.5 |
| | MCF-7 | 0.6 ± 0.1 | 1.9 ± 2.2 | 5.5 ± 0.5 |

*PC-3: human prostate, HCT-116: colon, MCF-7: breast. The cells were treated for 96 h with at least four different concentrations of compounds ranging from 0.1 to 100 lM. MTT assay (Mosmann, 1983) was used to calculate GI50, TGI and LC50 values (expressed in lM). Values are mean of three independent experiments and errors represent the SD values. a Doxorubicin was used as a positive control*

## Hanging Out with Clubmosses and Filmy Ferns

Club mosses (Lycopodiaceae) and filmy ferns (Hymenophyllaceae) are two primitive sub-divisions of vascular plants, native to the moist tropical mountains and northen hemisphere. The club moss is a small sized, low growing evergreen plant, possessing a ground hugging stem that can reach lengths of up to four feet when fully grown. It is characterized by dense spirals of yellow green colored leaves. Meanwhile, ferns are distinctive-looking plants with many leaf stalks growing from a central base. Hundreds of leaflets grow along the stalks, further divided into subleaf or pinnules in some species. Depending on species, these fronds can grow up to 12 feet long, as in tree ferns, or only a fraction of an inch, as in filmy ferns. Ferns and lycophytes were long considered as mystical plants, because people could not understand how they could reproduce without ever producing a flower, a fruit or a seed. They are naturally beautiful visually appealing plants and many are sold as garden hanging plants. Club mosses and ferns have many medicinal uses, some dating way back to ancient times. Disorders like kidney stones and urinary tract infections were once treated using remedies that contained both the spores and the whole club moss plant, while the leaves and rhizomes of the maidenhair fern are used for coughs, asthma and pleurisy.

Our phytochemical study on several Lycophytes (*Huperzia* spp) and the filmy fern (*Hymenophyllum javanicum* Spreng) yielded several novel/new and known compounds. The work was very challenging due to the small amount of material that could be obtained for the species from our forages into the tropical rain forest. Thus the identification of these structures was carried out in collaboration with our Japanese counterparts who are equipped with better high end research facilities. *H. javanicum* elaborated hemiterpene glucosides i.e Javanicosides A-D, the hemiterpene

being a 2-hydroxymethyl-3,4-dihydroxy-1-butene (Figure 12). The glucose moiety was esterified on various positions by phenylacetic acid derivatives, which is responsible for the hot and bitter taste of the fern (Figure 13).

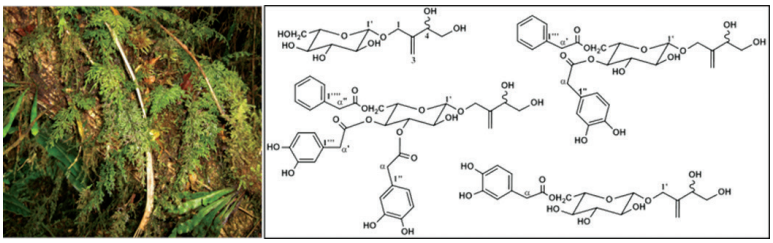

**Figure 12** Javanicosides from *Hymenophyllum javanicum*

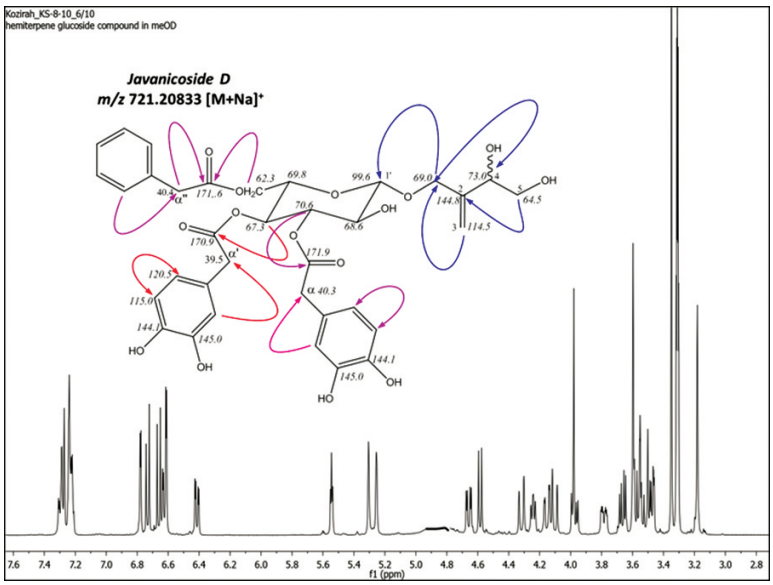


**Figure 13** $^{1}$H NMR spectrum Javanicoside D and selected correlations observed in the 2D NMR HMBC experiment run at 500MHz in deuterated methanol

The club mosses *(Huperzia carinata* and *H. nummularifolia*) (Figure 14) showed significant inhibition of the enzyme acetylcholinesterase (AChE). Further phytochemical investigation however yielded mainly serratane triterpenoids. The presence of minor amounts of lycopodium-type alkaloids, notably huperzine A, was detected *via* LCMS/MS in *H. carinata* (Figure 15). Huperzine A is a highly specific and potent inhibitor of acetylcholinesterase (AChE). Its presence in the extract explained the anti-AChE activity shown by the extract. The biological activity of the serratanes has yet to be tested.

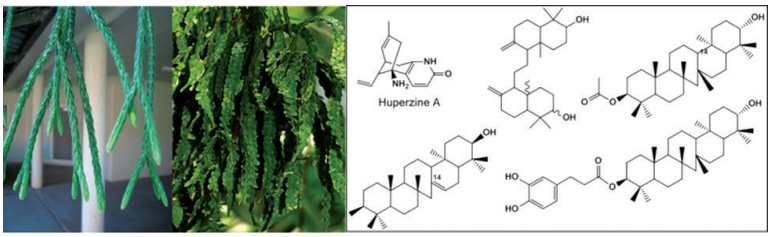


**Figure 14** Some phytochemical constituents isolated from *Huperzia* species; *H. carinata* (left), *H. nummularifolia* (right)

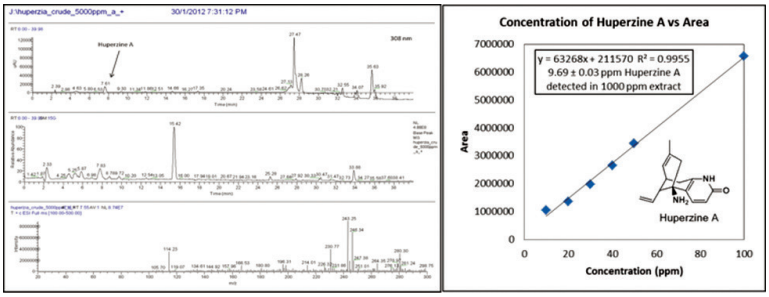


**Figure 15** LCMS qualitative and quantitative analyses of Huperzine A in methanolic extract of *Huperzia carinata*

## Discovering tHGA, a Natural LO Inhibitor

Late in 2002, a young and enthusiastic Indonesian student approached our group to pursue her Masters in Natural Products Chemistry. She brought with her several local herbs to screen for her project. Among them was a, then, little known 'ulam', which the locals called 'setenggek burung', in Latin *Melicope ptelefolia* Champ ex Benth (syn. *Melicope lunu-akenda*). Among the traditional uses of the species were as a digestive and an appetizer (Loi, 1977) and to treat menstrual pains and for healing wounds, fever and rheumatism (Perry & Metzger, 1980). The literature reported fungicidal (Kumar *et al*., 1990) and antibacterial (Manandhar *et al*., 1985; Rasadah & Zakaria, 1988) properties but there were no studies to support its use in alleviating inflammation.

**Figure 16** *Melicope ptelefolia Champ* ex Benth aerial parts and flowers (left). Chemical structure of 2,4,6-trihydroxygeranylacetophenone or tHGA (right)

The leaf crude extract and solvent fractions of the species were subjected to preliminary screening for antioxidant and anti-inflammatory activities. Although both the hexane and dichloromethane fractions were strongly antioxidant, it was the activity of the dichloromethane fraction in the lipoxygenase (LO) enzyme inhibition assay that caught our interest due to its direct relevance to inflammation and asthma.

LO is a group of closely related non-heme, iron containing dioxygenases catalyzing arachidonic acid metabolism. The enzyme inserts molecular oxygen at the fifth carbon, forming 5-hydroxyeicosatetraenoic acid (5-HETE). It then catalyzes a dehydration reaction, forming the unstable epoxide intermediate, leukotriene (LT) A4 which is then further metabolized to cysteinyl leukotrienes (CysLTs) by LTC4 synthase (Dahlén, 2006). It is beneficial to suppress production of CysLTs because these lipid mediators exhibit proinflammatory characteristics whereby they have been demonstrated to promote airway eosinophilia, increased mucus hypersecretion and potent bronchoconstriction and tissue remodeling in asthmatic individuals (Busse and Kraft, 2005). Over the last decade, LO inhibitors and leukotriene (LT) antagonists have become targets for rational drug design and discovery of mechanism-based drugs for treating chronic inflammatory diseases such as allergic asthma, psoriasis and rheumatoid arthritis.

Encouraged by these early findings, our group embarked on an exhaustive phytochemical investigation of the plant which resulted in the isolation and characterization of twenty secondary metabolites from the species (Khozirah *et al.,* 2006 & 2011a; Abas *et al.,* 2006). More significantly, utilizing a bioassay-guided approach, our efforts culminated in the identification of a class of prenylated acylphloroglucinols, as the bioactive constituents responsible for the LO inhibition, in particular 2,4,6-trihydroxy-3-geranylacetophenone (tHGA). This small molecule significantly inhibited human PBML 5-LO product synthesis, specifically suppressing the formation of CysLTs with an $IC_{50}$ value of 1.80 mM, with no cell toxicity effects (Khozirah *et al.,* 2011b). Since tHGA showed moderate DPPH activity and tested negative as a metal-chelator, it seemed that tHGA possibly acted *via* a competitive (non-redox) mechanism in its interaction with 5-LO. Non-redox type LO inhibitors have been shown to have fewer side effects than redox or iron-chelating agents (Dahlén, 2006).

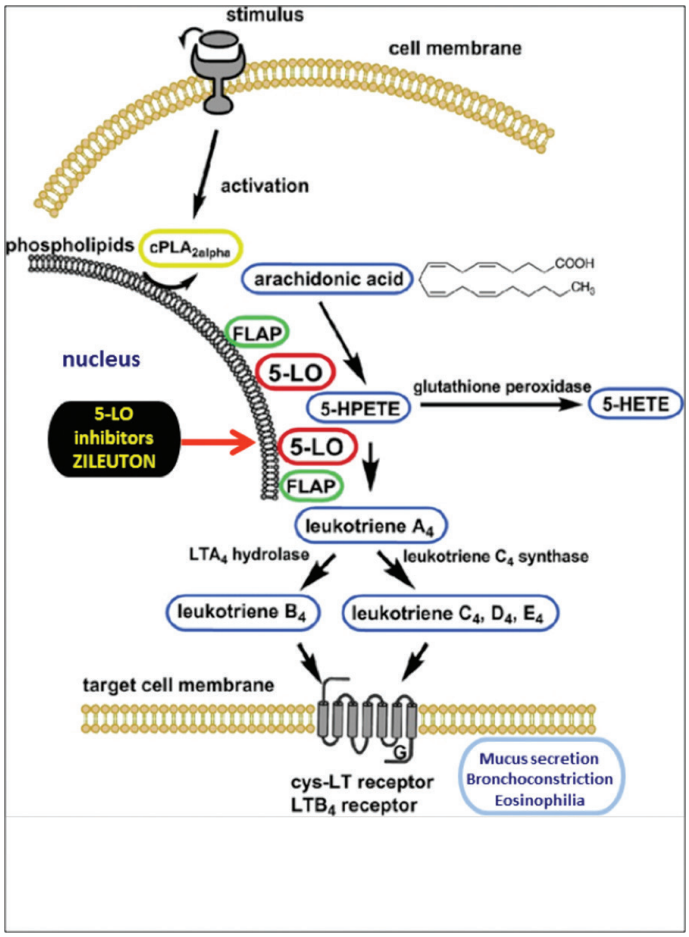


**Figure 17** Overview of pathways involved in biosynthesis of leukotrienes. Arachidonic acid is released from cellular phospholipids by phospholipase $A_2$ ($PLA_2$) and converted by 5-LO and FLAP to $LTA_4$. $LTA_4$ is transformed by either $LTA_4H$ or $LTC_4S$ into two classes of compounds, $LTB_4$ and cysteinyl leukotrienes ($LTC_4$, $LTD_4$, and $LTE_4$). Cysteinyl leukotrienes bind two distinct receptors, $CysLT_1$ and $CysLT_2$. $LTB_4$ acts at specific receptors that are designated $BLT_1$ and $BLT_2$ (GGT: γ-glutamyl transpeptidase, DP: dipeptidase). Leukotriene modifiers include both 5-lipoxygenase inhibitors and cysteinyl leukotriene antagonists (Source: Steinhilber *et al.,* 2010)

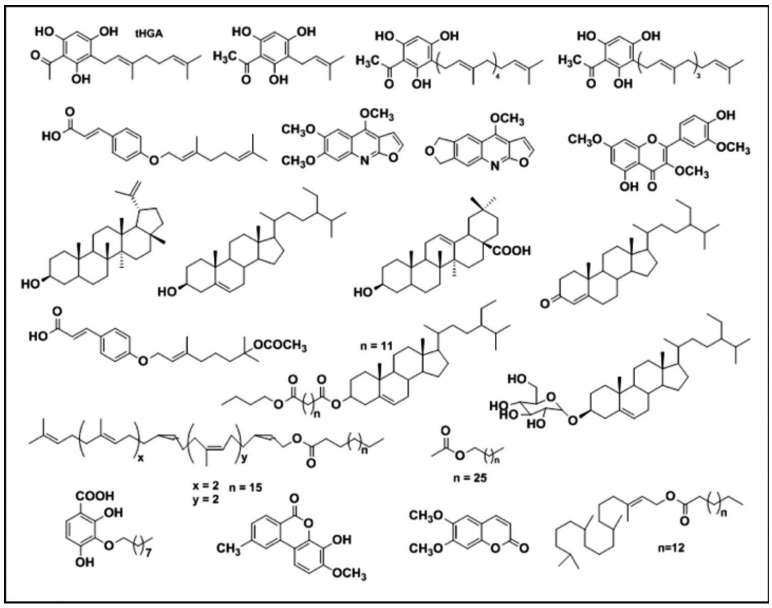


**Figure 18** Secondary metabolites of *Melicope ptelefolia* showing a wide variety of compound classes

Further tests on cyclooxygenases (COX) indicated that the compound acted via a dual LO/CO inhibitory mechanism, with greater selectivity for 5-LO and COX-2 ($IC_{50}$ 0.40 μM). These types of phloroglucinols, therefore, are promising candidates for further drug development studies since dual inhibitors of both LO and COX enzymatic pathways are expected to be more effective as anti-inflammatory agents and are expected to have better safety profiles. Thus. a more concerted effort to study the pharmacology of tHGA in experimental animal models of asthma was initiated in collaboration with another pharmacology group in UPM's Faculty of Medicine and Allied Health. Bulk synthesis of tHGA was initiated to provide gram quantities of the compound for the animal studies.

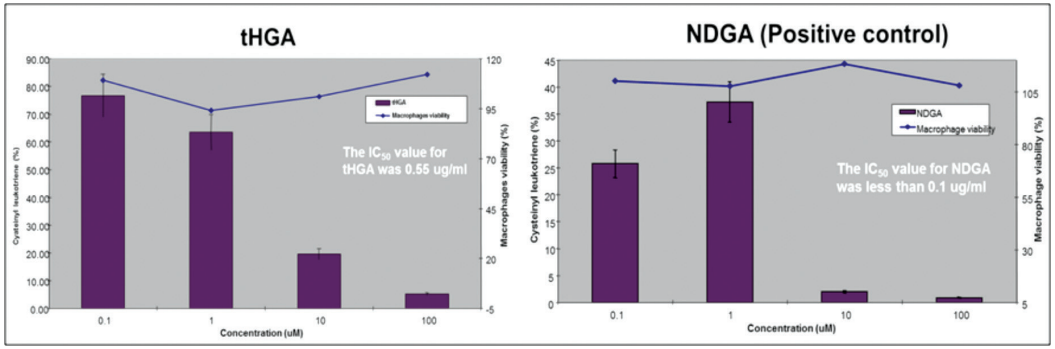


**Figure 19** Effects of (a) tHGA (1) and (b) NDGA on LTC4 release from calcium ionophore stimulated mouse macrophages. Inhibition percentages are expressed as mean values ± S.D. of three experiments

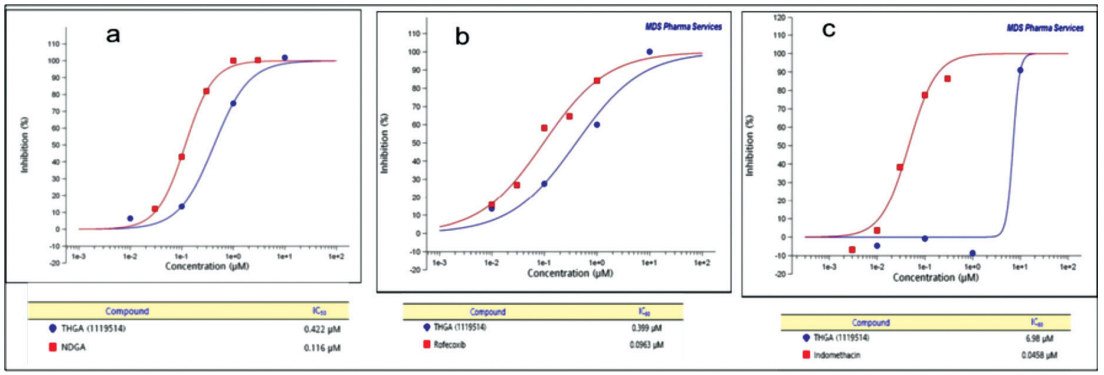


**Figure 20** a) Inhibition of human PBML 5-LOX by tHGA compared to NDGA b) Inhibition of human PBML COX-2 by tHGA compared to rofecoxib c) Inhibition of human PBML COX-1 by tHGA compared to indomethacin. (n=2)

It is not the intention to detail out the ensuing pharmacology aspects of the work on tHGA in this section since the excellent work that followed ours deserves a different platform for discussion. Suffice for us to mention here that, in summary, our findings regarding tHGA's inhibitory effects upon cysLT synthesis *in vitro* have been confirmed in an established acute model of murine allergic airway inflammation. Doses as low as 2 mg/kg are therapeutically effective in alleviating all common parameters of pulmonary dysfunction in the animal model and it is possible that these effects are due solely to the inhibition of 5-LO enzyme activity and subsequent synthesis of cysLTs (Ismail *et al.,* 2012). What is more exciting to highlight is that the efficacy of tHGA seems to rival that of zileuton, the only 5-LO inhibitor approved for clinical use currently. In contrast to tHGA, zileuton is a N-hydroxyurea derivative, and thus, most likely acts a iron-chelator at the active site of 5-LO, thereby blocking the enzyme's redox potential (Dahlén, 2006). Nevertheless, better understanding of its effects upon other proinflammatory mediators and their signaling pathways is still needed before the true potential of tHGA as an anti-asthma drug lead is established. Experiments are already underway to evaluate the effects of tHGA upon tissue remodeling in chronic models that better mimic human asthma.

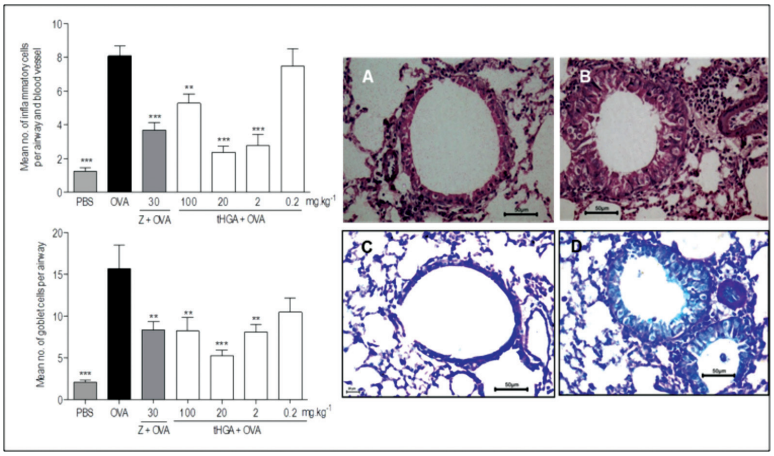


**Figure 21** Acute airway inflammation in OVA-treated mice – lung pathology following tHGA injections. Lung tissue (upper and lower lobes of left lung) of PBS-treated control mice (A, C) and OVA-sensitized/challenged mice (B, D) was fixed in 10% buffered formalin and stained with hematoxylin and eosin (A, B) or Periodic Acid Schiff (C, D) and examined by light microscopy. Bar=100 µm. Quantitative analysis of inflammatory cell infiltration (E) and goblet cell metaplasia (F). Data are expressed as mean±SEM. Significant difference from OVA-sensitized and challenged mice, ** Pb0.01, ***Pb0.001. Z+OVA = zileuton-treated group

## UNPICKING MOLECULES TO CREATE BETTER ANALOGUES

Drug discovery is an iterative process of lead discovery, i.e. isolation of bioactive lead compounds from natural sources, coupled with lead improvement, which involves rational design and synthesis of new analogues to improve pharmacological profiles. After selection of a new lead, drug development continues outside of the academic laboratories through preclinical studies comprising toxicology, formulation and production, followed by clinical trials. In the academic laboratory, drug design and structure modification

employs several tools to identify the optimum chemotherapeutic agent: (a) structure-activity relationship (SAR) studies including both qualitative and quantitative SAR, (b) mechanism of action studies including drug receptor interactions and specific enzyme inhibitions, (c) drug metabolism studies including identification of bioactive metabolites and blocking of metabolic inactivation, (d) molecular modeling studies including determination of 3D pharmacophores, and (e) combinatorial chemistry, including creation of peptide and nonpeptide libraries to generate new leads.

## Enhancing the Anti-inflammatory Effects of tHGA

After the discovery of the drug-like molecule, tHGA, we further ventured into an SAR study of the acylphloroglucinol structural-core, employing real and *in silico* docking experiments, to understand more about how such a molecule binds in the active site of the human 5-LO enzyme. The ligand-receptor interaction of tHGA was investigated via molecular docking simulation studies (Khozirah *et al.,* 2011b). The results provided a preliminary insight into the way tHGA could be binding with the 5-LO enzyme. The hydroxy groups on the phloroglucinol moiety seemed to be an essential feature, where the hydroxy groups formed hydrogen bonds to the carboxylate group of isoleucine residue in the active pocket of the enzyme. Strong Van der Waals interaction of the hydrophobic geranyl side chain of tHGA with several amino acid residues in the active site also indicated this to be an essential feature for bioactivity.

We had also observed that arachidonic acid and our compounds had similar structural features i.e a combination of a phloroglucinol core structure with a hydrophilic acyl group and a hydrophobic geranyl group. The observed inhibitory action of these compounds on 5-LO indicated that the lipophilic geranyl group is important for the pharmacological activity. The only difference between these

compounds was the nature of the acyl substituent. This motivated us to carry out the synthesis of tHGA analogues where our strategy was straightforward, i.e modify the acyl group of the tHGA template but preserve the geranyl moiety that resembles the hydrophobic tail of arachidonic acid (Figure 22).

A series of tHGA analogues were successfully prepared and tested for their *in vitro* anti-inflammatory activities against soybean 15-LO (Weckler, 2009). The synthesized analogues exhibited moderate to excellent inhibitory activity in as dose-dependent manner, giving $IC_{50}$ values ranging from10.3 to 27.6 μM. The SAR study suggested that the elongation of the aliphatic chain of acyl moieties (3c and 3e) and introduction of aromatic moiety (**3g**) significantly improved inhibitory activity by 30 to 50%, as compared to tHGA.

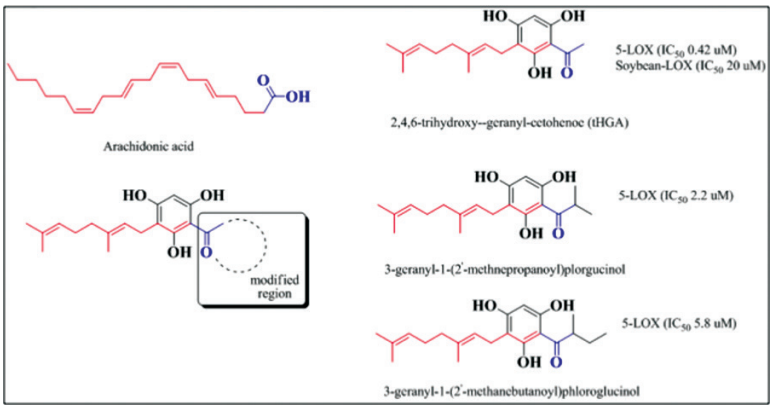


**Figure 22** $IC_{50}$ values of naturally active LOX inhibitors in which the structure features are similar to that of arachidonic acid

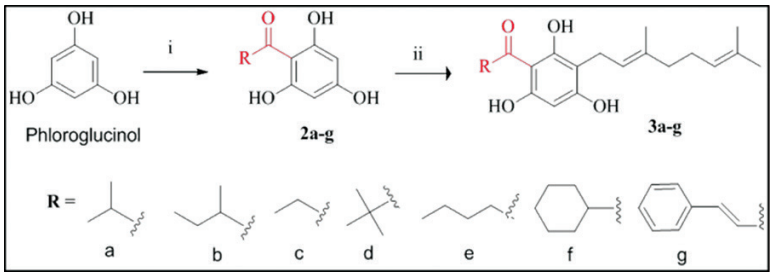


**Figure 23** Synthesis of analogues by two step synthesis from phloroglucinol by Friedel–Crafts acylation (R) followed by geranylation of the electron-rich benzene ring. Reagents and conditions: (i) Acyl chloride, anhydrous aluminium chloride, hydrochloric acid, dichloromethane; (ii) Geranyl bromide ($C_{10}H_{17}Br$), anhydrous potassium carbonate, dry methanol, reflux 8 hrs.

**Table 4** Anti-inflammatory activities of phloroglucinol (**1**), tHGA analogues (**3a, 3c, 3e-g**), tHGA and NDGA on soybean 15-LOX enzyme

| Compound | % Inhibition (100 µg/mL) | $IC_{50}$ value (µM) Mean ± SEM |
|---|---|---|
| **1** | 23.2 ± 2.2 | > 100 |
| **3a** | 90.3 ± 4.1 | 27.61 ± 3.6 |
| **3c** | 94.4 ± 3.0 | 12.32 ± 0.6 |
| **3e** | 90.3 ± 4.4 | 10.31 ± 1.5 |
| **3f** | 88.8 ± 5.9 | 26.31 ± 1.3 |
| **3g** | 90.8 ± 7.6 | 15.20 ± 1.2 |
| tHGA | 94.3 ± 3.4 | 23.61 ± 1.7 |
| NDGA | 100 ± 0.0 | ± 0.0 |

*NDGA (Nordihydroguaiaretic acid) as reference compound*

We further carried out molecular docking studies to obtain better insight on the molecular interaction of the compounds in the active site, in the hope of identifying important structural features that influence the ligand-protein interactions between the tHGA analogues and the enzyme. The 5-LO protein structure retrieved from the Brookhaven Protein Data Bank (PDB) and Discovery Studio® 3.1 (Accelrys, San Diego, USA) was employed for the docking simulation and analysis. The binding energy of the bioactive analogues obtained from the computer simulations correlated well with the bioassay results. Molecular docking studies revealed that the bioactive compounds occupied the receptor site by aligning itself in the same maner as (9*Z*,11*E*)-13(*R*)-hydroperoxy-9,11-octadecadienoic acid (13-HPOD), the original co-crystallized ligand in the ligand-protein complex crystal structure. Thus, it is suggested that the enzyme inhibition could be through competitive inhibition. The significant improvement in the bioactivities of these acylphloroglucinol analogues are worthy of further pharmacological evaluation and lead optimization as potential LO inhibitors. Further work is being planned to further evaluate the inhibitory effects of the bioactive tHGA analogues on experimental animal models of inflammation.

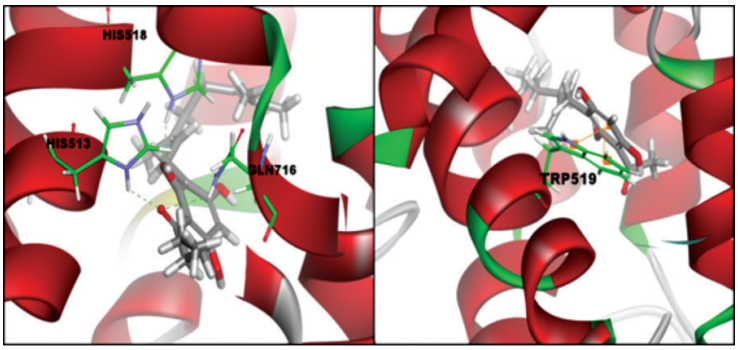


**Figure 24** Three-dimensional (3D) docking model of binding interaction of the compound with amino acid residues: (a) Compound **3e**; (b) tHGA; The atom colouring for the compounds is the following: carbons in grey, oxygen in red, nitrogen in blue, hydrogen in white and amino acid in green color. The orange line indicates the ϖ-interaction, while the green line indicates the hydrogen-bonding interactions

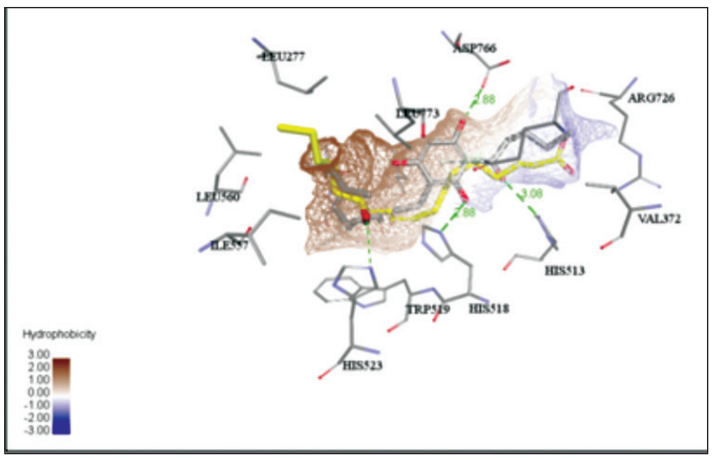


**Figure 25** Three-dimensional (3D) docking model of the overlaying pose between the most active compound **3e** with the *(9Z, 11E)-13(R)-hydroperoxy-9,11-octadecadienoic acid* (13-HPOD) as LOX substrate. The grey color represents compound **3e**, while yellow sticks represents 13-HPOD, with distance indicated in angstroms, (Å)

## MEETING THE METABOLOMICS CHALLENGE

Metabolomics is an approach which provides an essentially unbiased, comprehensive qualitative and quantitative overview of the metabolites present in an organism. It is often used in plant research. Meanwhile, metabonomics is used when refering to the same approach but on non-plants, to quantitatively measure the metabolic composition of body fluids following a response to pathophysiological stimuli. Metabolic analysis can be divided into four areas, i.e. target compound analysis (the quantification of specific metabolites), metabolic profiling (quantitative and qualitative determination of a group of related compounds or of specific metabolic pathways, metabolomics (qualitative and quantitative analysis of all metabolites) and metabolomic fingerprinting (sample classification by rapid global analysis). It uses several powerful platforms for data acquisition, such as, nuclear magnetic resonance (NMR) and LCMS spectroscopy, in combination with multivariate analysis to discern discriminating phenotype patterns and to gain holistic insights on the biological response resulting from external perturbations.

The application of the "-omic-" technologies have led to a change in paradigms towards the application of complex mixtures in medicine. The combined technologies in phytogenomics, proteomics and metabolomics substantially increase the number of proteins/genes/metabolites that can be detected simultaneously and have the potential to relate complex mixtures to complex effects in the form of gene/protein expression/metabolite profiles. These technologies have great potential for the chemical and pharmacological standardization and providing proof of the toxicological potential and the efficacy of a medicinal plant extract.

## Centella's Story

One of my mother's favorite ulam is 'pegaga'. In fact, most of the Malays in this country will know this little perennial and evergreen creeper, which to some is a secret to being 'awet muda' i.e youthful (Burkill, 1966). The plant is also featured in Ayurvedic and Traditional Chinese Medicine systems as a tonic for maintaining youthfulness, longevity and enhancing memory as well as a herbal remedy for a wide range of applications which include treatment of skin diseases, varicose veins, arthritis and wound healing (De Padua and Bunyapraphatsara, 1999). Currently, pegaga extract is added to cosmetics for its anti-ageing properties, specifically for ameliorating lines and wrinkles, prophylactically treating loss of elasticity (Oblong and Bissett, 1997), improving keratinocytes differentiation and for enhancing epidermal functionality. It is also a dermatologically effective phytotherapeutic agent that has been used in the form of complex homeopathic preparations, such as an ointment for external application. This plant apparently has the ability to inhibit a group of enzymes that break down collagen while simultanously increasing the rate at which collagen is synthesized, which is thought to underlie the increased wound healing rate (which has been proven in animal research). This is thought to be the reason why *pegaga* is used as a skin tightening agent as any increase in collagen synthesis will cause a firmness of the skin. Apart from these uses, the Malays also believe that *pegaga* has excellent anti-diabetic properties for which some scientific evidence has been reported in recent years (Kabir *et al,* 2014).

**Figure 26** Different varieties of pegaga. Left – *Centella asiatica* var 1, top middle – *C. asiatica* var 2, top right – *C. asiatica* var 3, bottom middle – *Hydrocotyle bonariensis*, bottom right – *H. sibthorpiodes*

Several varieties of *Pegaga* are known, *viz. C. asiatica* (pegaga kampong), *Hydrocotyle bonariensis* (pegaga nyonya) and *H. sibthorpioides* (pegaga embun) (Figure 26). Although *C. asiatica* seems to be related to the *Hydrocotyle* species, and has been classified in the same subfamily as *Hydrocotyle bonariensis* and *H. sibthorpioides* Lam., its morphological, anatomical, palynological and phytochemical characteristics retain it in the genus *Centella* (Downie *et al.,* 1996). All of the varieties are edible, but some are more favored over others for a certain use. For example pengaga nyonya is good for juicing and pegaga kampong and embun to make 'kerabu', a healthy traditional salad. Despite its popular use, a great deal of uncertainly still exist with regards to the differences between the *Pegaga* varieties, which present problems in the quality control and standardization of the plant material for use in downstream products. It raises questions such as, which pegaga variety is the

right or most effective one for a specific use? Do all the varieties work indiscriminately or are the effects different for each variety? If this is true what are the differences in the metabolome of each variety that acts as the discriminating factor in their biological properties? In an attempt to answer these questions, our group embarked on an NMR-based metabolomics study of the three distinctive varieties of pegaga.

Metabolite fingerprinting using $^{1}$H NMR in combination with multivariate data analysis, (PCA and HCA), allowed discrimination of the *Pegaga* varieties into three clusters (Maulidiani et al., 2012). The study revealed that asiaticoside and madecassoside along with chlorogenic acids were the metabolites contributing to the separation of *C. asiatica, H. bonariensis* and *H. sibthorpiodes* extracts (Figures 27 – 29). The two triterpene glycosides were only detected in *C. asiatica* extracts but not in the *H. bonariensis,* and *H. sibthorpioides* extracts. These compounds are well known to exhibit important biological activities, including antioxidant, anticancer and anti-inflammation (Al-Saeedi et al., 2011; Wan et al., 2012). Thus, we established the phytochemical relationship between the varieties, which could be applied in the selection of varieties for specific phytomedicinal use and to establish the standard fingerprint targeted for this purpose.

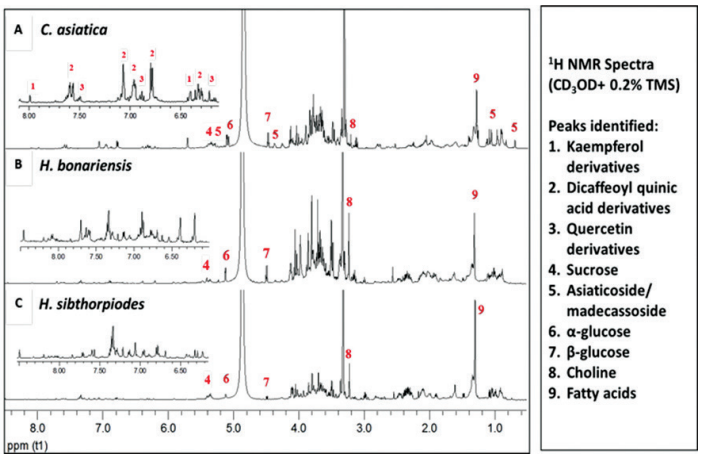


**Figure 27** $^{1}$H NMR Spectra ($CD_3OD$+ 0.2% TMS) of methanolic extract of *H. sibthorpioides* (**A**), *H. bonariensis* (**B**), and *C. asiatica* (**C**). Identified signals: 1. kaempferol derivatives; 2. dicaffeoyl quinic acid derivatives; 3. quercetin derivatives; 4. sucrose; 5. asiaticoside/madecassoside; 6. α-glucose; 7. β-glucose; 8. choline; 9. fatty acids

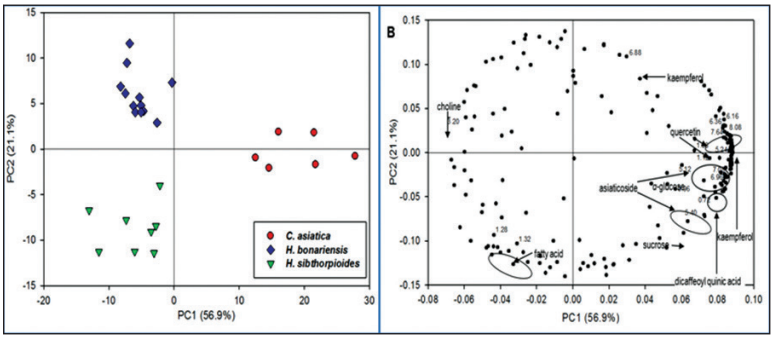


**Figure 28** Two dimensional score (**A**) and loading (**B**) plots of the principal component analysis (PCA) separated by PC1 and PC2 of the methanolic extracts of *C. asiatica, H. bonariensis* and *H. sibthorpioides*. Score plot (**A**) shows the discrimination of three *Pegaga* varieties. Loading plots (**B**) indicate the $^{1}$H NMR signals of compounds that are responsible for the separation of the three *Pegaga* varieties including sucrose at $\delta_H$ 5.4 (d, *J* = 4.0 Hz); asiaticoside at 5.24 (t, *J*= 3.5 Hz), 1.12 (s), 1.08 (s), 0.72 (*s*); dicaffeoyl quinic acid derivatives at 7.08 (d, *J*= 2.0 Hz), 6.96 (dd, *J*= 8.0, 2.0 Hz); kaempferol derivatives at 8.08 (d, *J*=8.0 Hz), 6.88 (d, *J*=8.0 Hz); and quercetin derivatives at 7.64 (dd, *J*=8.5, 2.0 Hz), 6.36 (d, *J*=1.5 Hz), 6.16 (d, *J*=2 Hz)

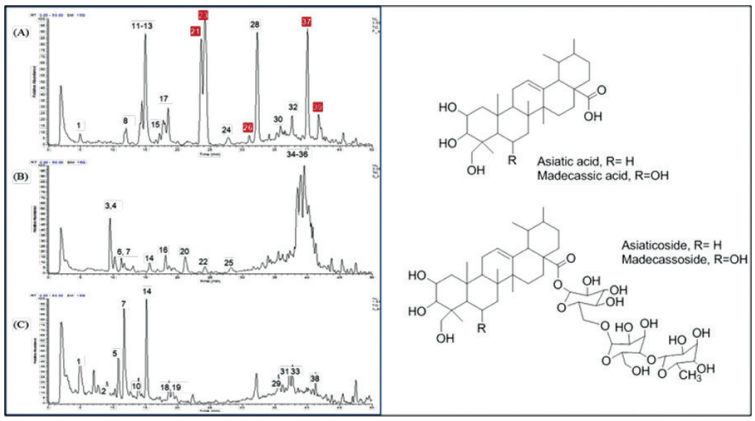


**Figure 29** LC-DAD-ESIMS/MS (-ve ion mode) TIC profiles of the ethanolic extracts of (A) *C. asiatica*, (B) *Hydrocotyle bonariensis*, and (C) *H. sibthorpioides.* (Madecassoside (21) Asiaticoside B (23) Centellasaponin A (26) Asiaticoside (28) Madecassic acid (37) Asiatic acid (39)

Pushing the limits a little further, we carried out a metabonomics study on pegaga species to understand their anti-diabetic effects. One of the traditional medicinal uses of *pegaga* is in treating diabetes mellitus (Semenya et al., 2012). Several scientific studies have also reported the anti-hyperglycemic activity of pegaga in diabetic rat models (Zheng et al., 2011; Kabir et al., 2014). Our question was which *pegaga* variety exerts the effect?

For the study, an obese-diabetic rat model was developed using non-genetic out-bred Sprague–Dawley rats fed with high fat diet and diabetes-induced with a low-dose of streptozotocin followed by treatment with the plant extract (Figure 30). Subsequently, the metabolic changes in the biofluids of the rats fed with the plant extract were followed by analyzing the $^{1}$H NMR spectrum of their urine and blood samples.

A PCA-model was fitted to the bucketed NMR spectra of urine samples collected from normal (nd) and obese rats before injection with STZ (ob, initial), after injection with STZ (obdb, baseline), and from the plant extract-treated obdb groups (*C. asiatica* and *H. bonariensis*) as well as metformin-treated obdb group (middle and final). The individual score plots final timepoints (blue) and trajectory changes from initial (red), base (green), middle (yellow) to final timepoints, resulting from treatments with metformin and plant extracts are shown in Figure 31.

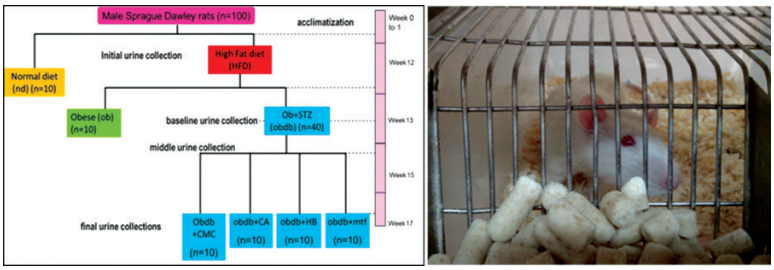


**Figure 30** Experimental design for Type 2 diabetes mellitus animal model study to evaluate anti-diabetic effects of *C. asiatica* and *H. bonariensis*

The movement of the trajectory from the initial to the left (**A**) along the first principal component after diabetes-induction indicated significant metabolic changes and/or metabolic disturbances in the animals, clearly reflecting the effect of the STZ injection. Following treatments with *C. asiatica* extract (**B**) it was observed that the trajectory showed a reversal of the treated rats back to normal state whereas treatment with *H. bonariensis* (**C**) did not show a similar reversal. This indicated that long term treatment with *C. asiatica* extract restored the metabolic disturbances back to the normal state whereas a similar effect was not shown in the treatment

with *H. bonariensis* extract. Further, it appeared that long term treatment with metformin **(D)** seemed to exert a different effect on the metabolic conditions of the obdb rats. Although glucose levels were lowered, creatinine levels had somehow been elevated!

A detailed analysis of the PCA loadings showed that long term treatment with *C. asiatica* extract could reverse glucose, lipid, tricarboxylic acid cycle, and amino acid metabolic disorders back towards normal state. It is interesting to note also that long term treatment with metformin had altered some of the metabolism of the animal models. However, this study is just a brief look into the metabonomics of the *in vivo* effects. The observations made in this study have answered some of our initial questions but, at the same time, it has raised new ones which deserve more thorough pharmacological studies. As far as our objectives are concerned, the results have provided evidence that *C. asiatica* has anti-hyperglycemic and anti-antihyperlipidemic effects, and may perhaps be very beneficial for use in managing the complications arising from diabetes mellitus. Metabolomics, and in this particular case, NMR-based, has been proven to be a very useful and holistic approach towards providing scientific evidence of the use and value of medicinal plants.

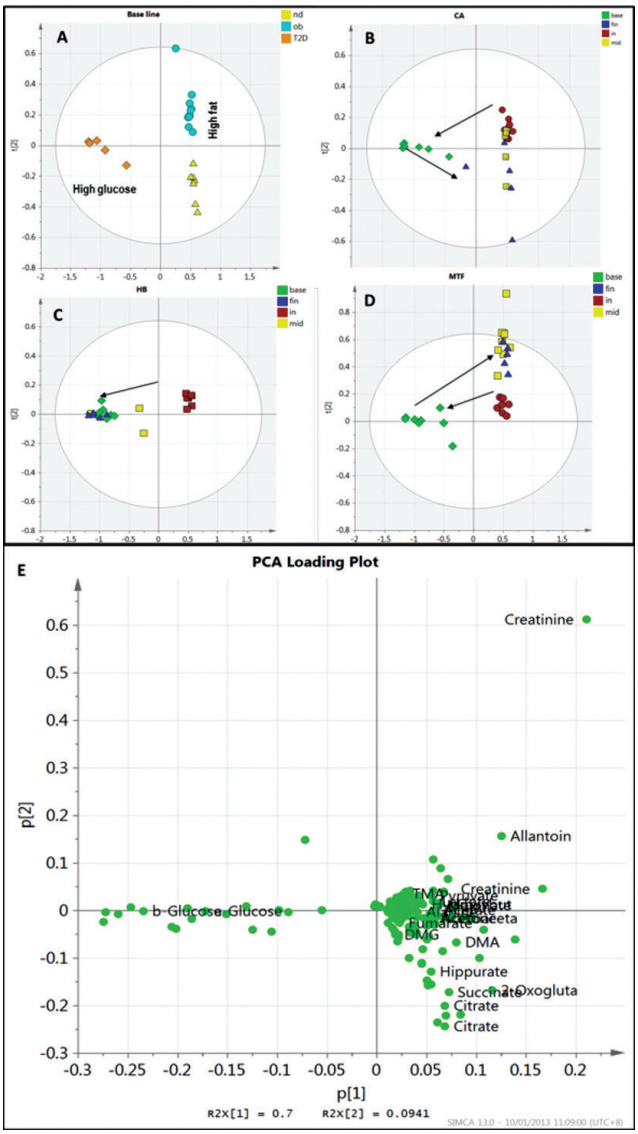


**Figure 31** PCA score for (a) control, trajectory (b) treatment with metformin, (c) *C. asiatica* and (d) *H. bonariensis* extracts and (e) PCA loading plots of the $^{1}$H NMR data

## BACK TO THE FUTURE

A renaissance in the exploitation of nature´s resources has been witnessed in the last few years because plants still provide a vast resource of structural complex and diverse molecules with a broad range of desired functions, many of which still remain elusive unless suitable assays can be developed to identify them. It is evident that natural products research is interdisciplinary and requires that collaboration exists between different scientific disciplines, including botany, biochemistry, pharmacognosy, pharmacology, phytochemistry, medicine, toxicology and biotechnology. Pharmacognosy, based for many years in botanical methodology, embraced phytochemistry and the new, powerful and sensitive methods of analysis for identifying, characterising and determining chemical structures of natural products. The parallel developments of sensitive methods for assessing biological activities enables research to focus on the active constituents of plants. The creative approaches that can be used alongside these facilities provide an excellent platform for researchers in the various disciplines to congregate and work together better in pursuit of the main goal of finding future cures. It must be recognized that there are still numerous plants of value which might be brought to commerce for the benefit of mankind and our goal is to find them, demonstrate their safety and efficacy. To realize this great promise effectively, we need a "systems" approach which applies careful coordination of the entire process of discovery and development. Finally, natural products research as a whole is enriched by both phytochemistry and metabolomics approaches as useful tools and will remain an exciting subject for research. It cannot be denied that the identification of structural diversity and biological activity of natural products are essential routes to finding new pharmaceutical leads. Sustainable and cost-effective production systems for high-value plant-derived

compounds must be developed, and the best outcome can be achieved by integrating scientifically-based approaches and sound methodologies into more sustainable production chains featuring cutting-edge innovative technologies. Deforestation issues and ecosystem changes are current threats which will still be there in the immediate future. Humankind is in a race against time before these valuable resources are forever lost. Thus, its exploration needs to continue, especially for the discovery of new molecules and potential drug leads to alleviate or cure current and future diseases.

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## BIOGRAPHY

**Professor Dr Khozirah Shaari** was born in 1961, in Telok Intan, Perak, but spent most of her school days in St. Annes Convent in Kulim, Kedah (1967-1972), then at the Convent Secondary School (CSS), Butterworth, Seberang Perai (1973-1975), and later, at the Alor Star Technical School (ASTECH) (1976-1977). She later pursued her GCE A Levels at Loughborough Technical College in England (1979-1980) and then her BSc Honours degree in Chemistry at the University of Swansea (now Univeristy of Wales, Swansea), in Wales (1981-1984). In 1984, she came home to work, first as a secondary school teacher and later as a tutor during which time she also pursued her Masters degree in Phytochemistry at University Malaya, Kuala Lumpur. After completing her Masters there, she assumed a position as Research Officer at the Forest Research Institute Malaysia (FRIM) in Kepong where she worked until the middle of 2001. During this time she obtained her PhD in Phytochemistry from University of Strathclyde in Glasgow, Scotland (1992-1994).

Professor Khozirah Shaari first began her career as a researcher in the Chemistry Division, FRIM where she acquired most of her research skills and build her expertise in Phytochemistry. After receiving her PhD she was promoted to Senior Research Officer where she contributed significantly in the planning and setting up of the Medicinal Plants Division in FRIM. In those early days, she helped establish laboratories and recruited and trained a young natural products team together with Dr Azizol Abd Kadir who was then the Division Director. Her experience in instrument operations during her PhD studies helped her in the establishment and the operations of the Nuclear Magnetic Resonance Spectroscopy Unit which housed the first 300MHz Bruker NMR in Malaysia. The instrument is still in full operations to this day.

Professor Khozirah Shaari started her career as an academician in Universiti Putra Malaysia (UPM), Serdang in 2001, as an Associate Professor at the Department of Chemistry, Faculty of Science. Her move to UPM was motivated by the need to improve her knowledge in fundamental science and contribute more to talent development for the nation. Her teaching duties comprises teaching Organic Chemistry and Spectroscopy courses to students at both undergraduate and postgraduate levels. On several occasions she was also involved in teaching Research Methodology to postgraduate students.

In research, Professor Khozirah Shaari is, to this day, an active researcher in the Laboratory of Natural Products (LHS), at the Institute of Bioscience (IBS). She has been affiliated with the Institute as an Associate Researcher since 2001, making major contributions to the establishment of the laboratories, units and facilities at LHS. She has supervised and co-supervised many postgraduate students of both Masters and PhD levels as well as undergraduate students in their final year projects. She was also involved in co-supervising a number of postgraduate students from other national universities. Her research interests center around Phytochemistry or Natural Products Chemistry, working closely with collaborators in Biomedical Science, Immunology and Pharmacology. Subjects of studies range from plants, marine natural products and microbes. In recent years her interests have expanded to applications of phytochemical and spectroscopic methods in systems biology research, in particular metabolomics and metabonomics. In her research career she has led and is leading numerous reseach projects involving a total of close to RM7 million in research funds. Over the years she has authored and co-authored about 120 papers in refereed journals. Currently she has attained a H-index of 15 and 1005 total citations. She was made a full Professor in 2010 after serving UPM for 9 years.

Her expertise in the field of Natural Products Chemistry is recognized nationally and internationally. She has been invited to give lectures at seminars and conferences as well as to provide input as a Resource Person for various technical workshops. As a reviewer she sits on the Editorial Board of Phytochemical Analysis, a Wiley publication with an impact factor of 2.45 (2014). As an expert panel member she has been invited as research grant proposal assessor at the Ministry of Agriculture (MOA) and Ministry of Science, Technology and Innovation (MOSTI). She has also served as internal reviewer of postgraduate dissertations for UPM and as external reviewer for postgraduate students at several national Universities i.e University Malaya (UM), Universiti Institute Technlogy Malaysia (UITM), Universiti Sains Malaysia (USM), Universiti Teknologi Malaysia (UTM), Universiti Kebangsaan Malaysia (UKM) and International Medical University (IMU). As an administrator, she was appointed to the Research Management Center (RMC) in 2010 as the Deputy Director of the Research Grants Division (BGP) and later in 2014 as the Director for Research at RMC until the present day.

Professor Khozirah Shaari is also an active member in the Malaysian Natural Products Society (MNPS) of which she has been the Vice-President for three consecutive terms, until 2016. Together with UPM and MNPS she has presided over the organization of various seminars, conferences and workshops, at national and international levels. Internationally she is on the board of the Asian Network of Research on Antidiabetic Plants (ANRAP) and a constituent member of the Africa, Asia and South America (AFASSA) group that co-ordinates activities of several networks involved in natural products research. She is also a member of the Royal Society of Chemistry (UK), RSC Malaysia and the Asian Phytochemical Society (APS).

Her dream for the future is to continue contributing her knowledge and expertise to young researchers and to help raise Natural Products Research in this country to greater heights.

## ACKNOWLEDGEMENT

*In the name of Allah the Most Gracious and the Most Merciful.*

Alhamdulillah, all praises be to Allah SWT. I am most grateful to Him for all His Blessings and Guidance throughout my humble journey and for enriching my life with the knowledge that he has bestowed upon me.

First, I thank the Universiti Putra Malaysia for giving me this career opportunity and a chance to contribute to the Nation's future generations through my academic activities over the last 15 years.

Greatest appreciation to my mentor Prof. Peter G. Waterman, for introducing me to phytochemistry and for having taught me all I needed to know back then about elucidating structures and operating a complex instrument like the Nuclear Magnetic Resonance Spectroscopy. I remember your advice to always face my challenges with a positive mind and perseverance. Nothing is impossible. My deepest appreciation too to Prof. Nordin Hj Lajis, for his constant belief in my capabilities and who has encouraged and supported me since the first day I joined UPM. I have enjoyed working with you and I hope to have the opportunity to work and learn more from you in the coming years.

Thanks to all my good friends and colleagues, and students who have given me so much in return for the little that I could give them. There are so many of you that if I were to list all of you, it would take up many more pages of this little book. You know who you are and thank you so much for being there with me as my friend.

Thanks also to all my colleagues and staff of the Research Management Center (RMC) at the office of the Deputy Vice Chancellor for Research and Innovation, the Chemistry Department at the Faculty of Science, the Laboratory of Natural Products at the

Institute of Bioscience, for their support and assistance in fulfilling my responsibilities. Special thanks too to the Committee members who helped organize this Inaugural Lecture.

Finally, to my beloved Mak, Puan Hajjah Ramlah Hj Sirat, my undying gratitude for all that you have done for us, your children. And to my beloved Arwah Bapak Allahyarham Shaari bin Ibrahim, you are constantly in my prayers. I miss your presence and great wisdom.

Last but not least to my husband Shaari Jantan, thank you for standing by me through stormy weathers and hardships, and sharing my joys and sorrows. Thank you for the five 'natural products' that we made together: Amien, Aisyah, Irsyad, Alia and Wahida. They are the most precious.

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