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Immuno-Modulatory Effects of Phytomedicines Evaluated Using Omics Approaches

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1. Introduction

The advent of the omics area has created new research systems, including genomics, proteomics, metabolomics, as well as the associated bio-informatics science, and databases. At the same time, progress in traditional western medical research has reached a bottleneck, as single compound drugs are costly to create, synthesize, or engineer. If we are to see real and sustained progress in research, we must find approaches to utilize traditional remedies to develop advanced medicines.

Instrumental systems for transcriptome, including the microarray of different messenger RNA, microRNA and other non-gene sequence-related RNA products, have already been developed; functional genomics studies using these systems have been remarkably successful. However, the candidate genes involved in specific functions often need further verifications for revealing their roles in the signal pathways. The difficulty may also arise from the high variety and seemingly unrelated responsive genes and complex signaling or regulatory systems involved.

Proteomic analysis has its disadvantages, although two-dimensional (2-D) gels can display viable candidate proteins for study. Up to two thousand proteins of biological systems can often be analyzed in sensitive 2-D gel systems. There are other proteins, such as cytokines or chemokines of most leukocyte cells are expressed at relatively low levels, and often are not detectable by 2-D gels. More sensitive methods, such as LC/MS and other fractionation systems, need to be used for such cases.

Metabolomics faces an even greater challenge: a 2-D or one run display of the components of a metabolome has not been defined and cannot be systematically evaluated. Therefore, sequential analyses, e.g. the LC/MS followed by NMR, were developed to address the "overall" or more comprehensive picture of metabolomes.

Several phyto-medicinal studies, including some in traditional Chinese herbal medicine (TCM), have been considered as metabolome investigations. New strategies employing omics approaches may be especially useful for phytomedicinal research, as conventional phytomedicines often employ multiple components and they often are believed to interact with multiple molecular targets related to cellular and physiological (e.g., immune-modulatory) effects. In order to successfully evaluate the effects of phytomedicines, various omics approaches are being systematically combined. New computational and cross-

disciplinary analyses will be required for most experimental biology studies. Some examples of systematic and technical considerations, in terms of research into the immune-modulatory and anti-inflammatory effects using the omics approaches, are addressed in this brief review.

1.1 Importance of systems biology and bioinformatics

Scientists investigate medicinal plants in search of regulatory genes and metabolites that can affect, modulate or upgrade the biological and metabolic processes, which in turn can confer specific physiological or pharmacological functions. Recently, various high-output technologies, including genomics, transcriptomics, proteomics and metabolomics, are employed in such research effort [1-3]. Bioinformatics and systems biology approaches are considered by many as needed to organize, manage, process, and understand the vast amounts of data obtained in various omics studies [4-8]. In addition, systems biology is aimed at understanding complex biology by integrating omics data from various sources for network analysis, for evaluating the holistic system as a whole, as experimental results from omics studies are most often not obtained or isolated as a single set of data points or evens [9]. By analyzing the omics data, bioinformatics tools can help upgrade new approaches for classifying and authenticating potential medicinal plants, identifying new bioactive phytochemicals or compounds, and even improving medicinal plant species or cultivars that can tolerate stressful environmental challenges.

The human immune system, as we currently conceptualize it, is under the tight control of a complex network of regulatory genes, RNAs, modulatory proteins and stimulatory metabolites. Past studies have often focused on understanding the roles of specific genes in immune responses. To associate expression changes with immunological conditions such as suppression, cancer, or autoimmunity, we can investigate the interrelationship of the up- and down-regulation of genes or proteins patterns. Using microarray analysis and comparative genomics, Hutton et al. [10] have identified genes and their regulatory elements responsible for maintenance, differentiation, and the general functioning of specific immune systems. In addition, most of the expression pattern of genes is related to the biological role and effects of the products of genes, and a similar statement may be made for protein expression [11]. Taken together, evaluation of gene and protein expression profiles may lead us to identify links between specific genes or proteins and the associated specific immuno-modularory effects. Moreover, omics technologies may also be employed to address our views of the often-used concepts in immunology, such as: molecular dynamics in response to specific stimulations or alterations of the molecular state of targeted specific cells, in the hypothesis-driven research approach [12]. For instance, in the drug discovery process, pharmaceutical companies have used various microarray systems as screening tools to eliminate compounds that have molecular indications of toxicities before preclinical and clinical testing [13]. In basic research, omics technologies have continually improved our understanding on how drugs can regulate the immune system as well as of a variety of issues in mechanistic or hypothesis-driven research [14-17]. The data obtained from these studies not only may have significant impact on the future directions of those specific lines of research but also may improve our understanding of the specific immuno-modulatory regulation of given drugs.

Bioinformatics is the application of computational tools for biological sciences; its major aim is the management and interpretation of biological data [18]. It has been an essential tool for

fully integrating and multi-disciplinary understanding the processes in various biological areas [19]. Among them, understanding omics data requires both common statistical and machine-learning methods, because the data are usually in high-dimensional form and complexity. On the other hand, as compared with other biomedical and agricultural areas, the study of omics and its use for research into medicinal plants are still in its infant stage. Given the demand for studies on immuno-modulatory effects of herbal medicines, this chapter introduces and summarizes the applications of some omics approaches and specific bioinformatics tools for investigating phytomedicines.

1.2 Omics technologies

The technology platforms generally used in systems biology research, including transcriptomics, proteomics and metabolomics, have enabled us to study living systems from a holistic or integrative perspective through revealing profiles of multitudinous biochemical components (Figure 1); it also opens up a unique opportunity to reinvestigate phytomedicines [20]. The revolution of genomics research and technology development has yielded complete or draft DNA sequence maps for a spectrum of species including human, mouse and a serious of model organisms. Having the genomic data available, many new 'drug-able' targets based on transcriptomics study have been identified, opening up new insights into explanations of biological systems at a global scale. Additionally, through proteomics, we are witnessing the development of wonderful and multi-application tools for studying various signaling or mechanism systems at the level of proteins and protein–protein interactions [20, 21]. In the meantime, studies on glycol-biology and bioactive polysaccharides are making great leaps in glycomics research; similarly, studies on regulation and metabolic control of a spectrum of lipids are creating new approaches for "lipidomics". The recent wave of data from genomics and proteomics has precipitated the measurement of increasingly a group or spectrum of elements to provide a systems approach, especially at the level of metabolites and for the field of metabolomics.

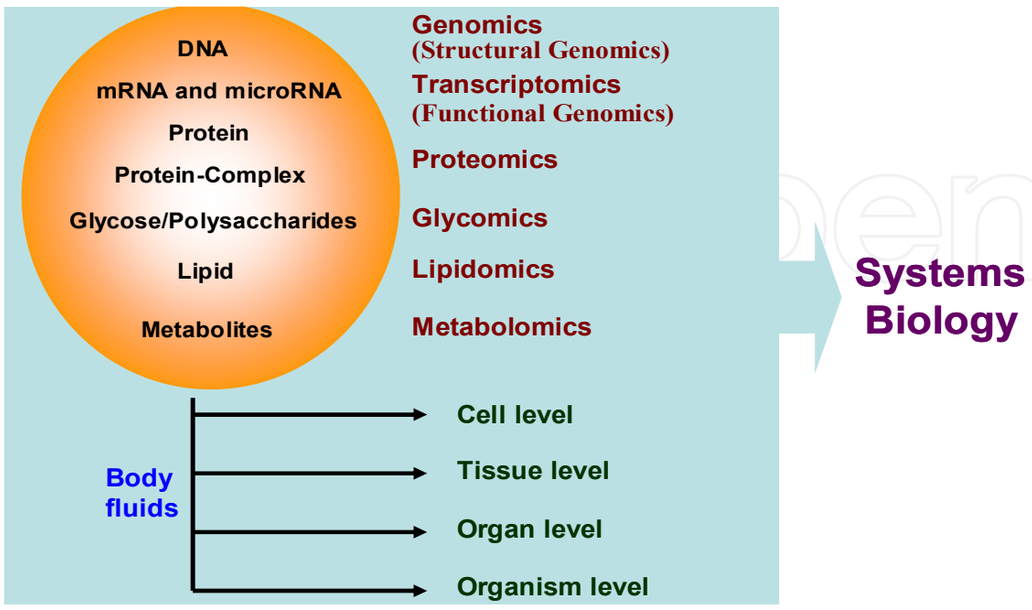

Fig. 1. The different levels of measurement in a systems biology approach.

Although omics are defined in several different ways today, in our opinion these systems provide "an integrated approach to study biological systems not only for the intracellular, but also at the cellular, organic and the whole body or organismic levels or networks, through measuring and integrating the genomic, proteomic and metabolic data" in a global consideration manner (Figure 1) [20, 22, 23].

In the search for new phyto-medicines, the necessary purification of single active components has been in general successful, whereas synergetic effects of mixtures of components (e.g., crude plant extracts) remain difficult to evaluate. Utilizing omics technology, scientists hope to develop methods and models to detect and observe the effects of complex mixtures such as various plant tissue extracts traditionally used in herbal medicines. This applies especially to approaches employing metabolomics which adress comprehensive phytochemical profiling, bioactivity phenotyping, sophisticated bio-organic chemistry instrumentation, and new cross-talk experimental designs. This scenario needs not only advancement in natural product research, but also revolutionary strategy in the development of molecular pharmacology-based herbal medicines [20].

1.3 Phytomics

The term "Phytomics" has been previously created to the "omics-based approach" for studying chemical compositions in plant (Kung PC et al., 2003), specifically: using bioinformatics and/or statistics to address qualitative and quantitative aspects of chemical compositions or profiles of the plant metabolites of our interest; or to develop databases for addressing such aspects [24].

2. Transcriptomics study on medicinal plant research

2.1 Application of DNA microarrays in toxicogenomics, pharmacogenomics and functional genomics studies of bioactivities from medicinal plants

Recent advances in genomics-based identification of responsive gene clusters, gene families or gene polymorphisms associated, with immune system dysfunction have helped to address some basic issues in immunology, and have begun to expand our understanding of immune-related disease processes [13]. The application of omics technologies in toxicological research (toxicogenomics) provided new insights into mechanisms of action, as well as data likely to be useful for risk assessment [13, 25]. Gene chips or microarrays are already employed in immunotoxicology research to identify biochemical pathways that are altered by specific chemical exposures. For example, trichothecene mycotoxin deoxynivalenol has been shown in mice to modulate splenic early responsive genes, which are functionally related to immunity, inflammation and chemotaxis [15, 26], indicating the importance of innate immune systems, including macrophages, granulocytes, neutrophils and various soluble mediators released in the inflammatory response activated by the hexachlorobenzene treatment. For basic research, a number of mechanistic studies have been performed towards gaining a comprehensive understanding of the immunomodulatory properties of potential new drugs or drug leads. Thymic atrophy, for instance, appears to be mediated in part by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced apoptosis [27]. Using an apoptosis-specific cDNA array combined with promoter analyses, specific and novel gene targets have been shown to enhance negative selection in the thymus and thus result in TCDD-induced thymic atrophy [16]. In a separate study, cDNA microarray analyses were utilized to evaluate the TCDD regulation of Fas ligand (FasL) promoter activity through modulation via NF-κB in thymic stromal cells and the subsequent initiation of the apoptotic pathway in thymic T cells [28].

During the past decade there has been a paradigm shift from utilizing single-target drugs to multi-target drugs [29, 30]. The concept of multi-targeted therapy was once believed to better represent the conventional herbal medicine treatments that often employ multi-component plant tissue extracts as natural products mixtures. However, very few phyto-medicinal products have clear or systematic documentations comparable to that of chemically synthesized drugs as single chemical compound. This situation has hampered our ability to predict precise or specific molecular targets, signaling or action mechanisms of activity, and possible side effects of "herbal drug" products [30]. With these requirements for botanical and clinical uses, a validated genomics and metabolomics approach in combination can be applied to quantify specific chemical markers and, subsequently, to obtain chemically standardized extracts [31]. In addition, researchers have witnessed a wide range of molecular mechanisms governing various cellular and tissue behaviors. The genomics approach with integrations of large and diverse sources of gene, protein and metabolite expression information will assist in making comprehensive and integrated predictions about the pharmacological effects of plant natural products [32].

While numerous laboratories use genomics in their investigation of underlying mechanisms of immunotoxicity, few have employed genomic analyses as a screening tool. Many differentially expressed genes are known to play a role in apoptosis, host defense, cell growth and differentiation, and trafficking of specific cells in body fluid systems. In the spleen, these may include the up-regulation of IL-18, lymphotoxin B receptor, and colony-stimulating factor receptor, and down-regulation of RANTES and histocompatibility antigens [15, 33-35]. In the thymus, gene changes included the down-regulation of nuclear factor of activated T cells, interferon gamma receptor, and T cell transcription factor 7, and the up-regulation of caspase 1 and ApoE. These findings are consistent with alterations previously observed in specific immune functions [34, 36] and could further expand our knowledge at gene regulation level.

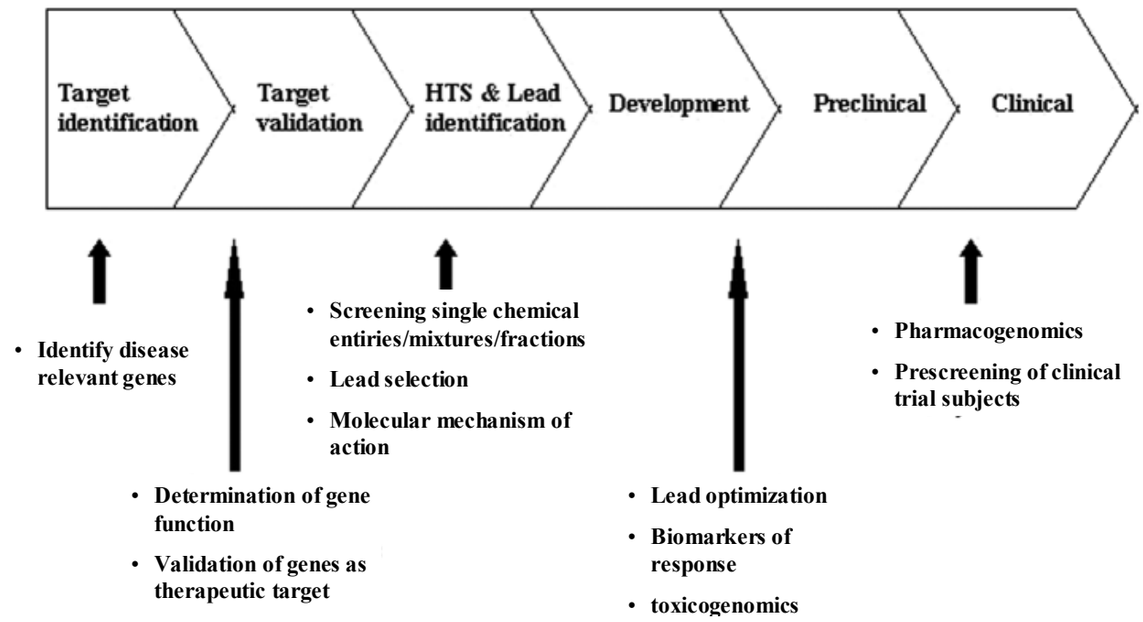

Fig. 2. DNA microarray applications in natural product drug discovery and development [37].

Applications of DNA microarray technologies in herbal drug research may be classified into three major areas. Firstly, it can be used in pharmacodynamics to aid the discovery of new diagnostic indicators and biomarkers for therapeutic response, elucidation of molecular mechanisms of herbal action, its formulations or its phytochemical components, and in identification and validation of new molecular targets for herbal drug development (Figure 2) [30], [37]. Secondly, it is applicable in toxicogenomics for predicting side effects of a medicinal herb or phytomedicine lead drug during preclinical activity and safety studies, conferring drug safety or resistance [38]. Thirdly, it is useful for botanical or plant identification and authentication of crude plant materials as part of an effort and regulatory system for standardization and quality control [39]. Given these considerations, DNA microarrays may thus offer powerful predictive functions at different stages of a typical drug/phytomedicine discovery pipeline.

2.2 Immuno-modulatory effects of different phyto-compounds/candidate phytomedicines

With the increased demand for validated herbal products for medicinal use comes the need to better understand the molecular mechanisms of their biological activities. Although many reputed herbal drugs are investigated at the molecular level, it remains difficult to realize the exact targets of individual phytochemical components and how these molecules together or independently can contribute to specific immuno-modulatory effects. Here, we discuss findings from some of the recent studies on microarray-based gene expression aimed at elucidating immune-regulatory mechanisms of pure phytochemicals as well as specific herbal extracts.

2.2.1 Purified compounds or specific phytochemical groups

The Chinese medicinal herb root *Tripterygium hypoglaucum* has been subjected to cDNA microarrays containing 3000 human genes (derived from a leukocyte cDNA library) in order to study its role in apoptosis-inducing activity of plant alkaloids. Apoptosis induced by these *T. hypoglaucum* alkaloids was shown to be mediated through c-myc and NF-kappa B signaling pathways [40]. In an animal model of aged rat, gene chip (Rat Genome U34A) analysis was applied to evaluate the gene regulatory pattern of *Epimedium* flavonoids in immune homeostasis. *Epimedium* flavonoids were found to reverse the "abnormal" or aging changes, allowing reconstruction of a beneficial equilibrium in gene expression and thus further remodeling of the immunohomeostasis in the aged rat [41]. Taken together, results from these studies indicate that the expression pattern characterized by up-regulation of specific apoptosis-promoting genes and down-regulation of certain apoptosis-inhibiting genes can be considered as important genomic background of an immunohomeostasis imbalance [30].

A traditional Chinese medicinal (TCM) herb prescription, Si-Jun-Zi decoction (SJZD), has been administered in a clinical setting to patients with disorders of the digestive system. Previous studies have indicated that the polysaccharides of SJZD are active components of the phyto-extract mixture in improving gastrointestinal function and immunity [42]. SJZD polysaccharides also had a protective effect and enhanced re-epithelialization on wounded IEC-6 cells. To further elucidate this effect at the molecular level, an oligonucleotide microarray was employed to study differential gene expression of SJZD-treated IEC-6 cells. There was, indeed, increased expression of genes encoding for ion channels and transporters, known as critical to cell migration and restoration of wounded cells,

suggesting a mechanism for re-epithelialization as well as improved immunity [43]. These studies demonstrate the useful approach of functional genomics for research into modernization of TCM.

Shikonin and its derivatives, from the TCM-claimed medicinal herb *Lithospermum erythrorhizon*, have been shown to possess numerous beneficial pharmacological properties, including anti-inflammatory and antitumor properties [44, 45]. In our previous report, shikonin was shown to confer a potent bioactivity on suppression of TNF-α promoter activity [46]. Additionally, shikonin was found to mediate cytokine expression through inactivation of the RNA-activated protein kinase (PKR) pathway [46]. It was also suggested from the reports that regulation of TNF-α pre-mRNA splicing may constitute a promising target for future anti-inflammatory application [47]. Moreover, the functional genomic (DNA microarray) analysis on the cellular immunological effects of shikonin effectively distinguished the complex and specific bioactivities of this phyto-compound in human monocytes [48]. Further, ubiquitin pathway regulator, e.g., Rad23A, was also identified as possible key regulators for this shikonin effect [48]. A transcriptomics approach has therefore been instrumental in screening immune-modulatory effects of noteworthy phytocompounds. These studies have set useful examples for future systematization of key traditional herbal medicine-derived phytomedicines.

2.2.2 Medicinal herbal extracts

Screening of the human genome for TNF-α-inducible genes has been used to identify the anti-inflammatory effects of 5-Loxin, a standardized Boswellia serrata extract, in microvascular endothelial cells [49, 50]. It was shown that 113 out of the 522 TNF-α-induced genes were responsive to 5-Loxin treatment. These genes are directly or apparently related to inflammation, cell adhesion, and proteolysis. These robust 5-Loxin-sensitive candidate genes were subjected to further evaluation for molecular signaling, and this processing led to the suggestion of the primary 5-Loxin-sensitive TNF-α-inducible pathways. Mechanistically, 5-Loxin can completely inhibit VCAM-1 expression, and TNF-α can cause inflammation by strongly up-regulating the expression of this adhesion molecule VCAM-1. [49].

Recently, genomics analysis has also evolved for evaluation of the efficacy of bioactive chemicals in natural health products as therapeutics, for instance, with regard to the alleviation of specific inflammatory activities in human airway epithelial cells [33]. In addition, one application of gene expression profiling in this research field may be the growing appreciation for the multiple and pivotal roles played by various dendritic cells (DCs) in initiating and regulating a spectrum of immune responses. These cells are responsible for recognizing and processing various antigens and their ultimate presentation to specific immune cell (e.g., T cells) systems [51-53]. It has been well established that DCs present in the epidermis (Langerhans cells (LCs)) are required for the presentation of chemical allergens at the skin's surface, as well as for skin sensitization [54, 55]. Investigations by Enk and Katz [56] have revealed that topical exposure of mice to chemical allergens, but not to a non-sensitizing skin irritant, caused numerous changes in expression of cytokines and chemokines by LC and local epidermal cells. Among these changes recorded following allergen treatment was a rapid increase in LC expression of mRNA for interleukin-1β (IL-1β), a cutaneous cytokine necessary for the regulation of LC function and for skin sensitization [56-59]. These results concluded that changes in the expressions of IL-1β by LC in response to chemical allergens might hence provide a practical and efficient *in vitro* approach for identifying skin-sensitizing chemicals [60].

Genome-wide analysis has been adopted as a less selective approach for measuring the holistic or global changes in gene expression [17, 61-64]. It can be anticipated that the activation and functional maturation of DCs, as drastic cellular activities, are likely to be associated with changes in the levels of a spectrum of gene expressions that are responsible for: (a) intracellular metabolic processes; (b) control of cell motility (including those regulating intercellular communication and interactions with the tissue matrix); (c) cytokine and chemokine production, and (d) cell growth regulation and survival [17, 61]. Results of our previous studies on the immune-modulatory effects of a phytocompound mixture, extracted from the butanol fraction (BF) of a stem and leaf (S+L) extract of *Echinacea Purpurea* ([BF/S+L/Ep]), suggest that [BF/S+L/Ep] can effectively modulate DC mobility *in vivo* and related cellular physiology in the mouse immune system. In addition, [BF/S+L/Ep] modulated cell adhesion-, cell mobility-, cytokine- and NF-κB signaling- related activities in primary cultures of mouse DCs [17]. Similar study of Wang et al [60], have further shown that genes expressed in [BF/S+L/Ep]-treated human DCs revealed a key-signaling network involving a number of immune-modulatory molecules and lead to the activation of a downstream molecule, adenylate cyclase 8. These examples show that genomics approaches can be usefully employed for predicting candidate target molecules in future translational studies of phytochemicals, phytocompound mixtures, and medicinal herbal extracts.

2.3 Use of cDNA microarray/ expression sequence tags (ESTs) for evaluating bioactivities of medicinal plants

A transcriptome is the set of all detectable RNA molecules, including mRNA, tRNA, rRNA, and non-coding RNAs (e.g., siRNA, microRNA) produced in a group of test cells or tissues. By using the advanced transcriptomics, an organism's entire transcriptome can now be effectively analyzed for many experimental systems. Technically, transcriptomics is a technology to reveal genome-wide gene expression profiles, patterns, integrated or segregated features or networks describing a global view or analysis of gene expression activities of the genome at the mRNA or regulatory RNA levels. These technologies comprise cDNA-AFLP, SAGE, cDNA microarray (or gene chip), oligonucleotide-microarray, and microRNA microarray [2]. Microarrays also have been used to detect gene expression changes of medicinal plants in a variety of developmental stages, geographic locations, natural growth environments, and/or cultivation conditions [2]. In phytomics studies, studies have aimed to identify the responsive genes that are regulated by active medicinal compounds, anti-pathogen infection, or adaptation to harsh environment [65].

To design appropriate probe sequences for a DNA microarrays efficiently, we need to consider the genome sequence information for a specific organism in its entirety or with a definable set or subset. However, since only very limitted genomes of medicinal plants have currently been sequenced, one alternative is to gather the necessary transcriptome information, by generating or making use of existing expression sequence tags (ESTs) [66, 67]. Increasing numbers of EST libraries from medicinal plants such as *Panax quinquefolius* [68], *Huperzia serrata* [69], *P. Notoginseng* [70], *Rehmannia glutinosa* [71], and *Catharanthus roseus* [72] have been recently obtained. An automatic system for large scale EST sequence retrieval, assembly, and functional and pathway analyses has been established [73]. This system has been successfully applied to analyzing both plant [74] and animal EST sequences [73, 75]. These EST and annotation systems have provided a good foundation for design of suitable arrays for representative genomes or focused transcriptomics, hence providing valuable information for genomic research into phytomedicine.

3. Proteomics studies on the research into medicinal plants

3.1 Use and advancement of analytical and instrumentation systems: Two-dimensional gel electrophoresis (2-DE), electrospray ionization, matrix-assisted laser desorption/ionization and surface-enhanced laser desorp

The Nobel Prize in Chemistry for 2002 was shared between scientists from two research expertise: mass spectrometry (MS) and nuclear magnetic resonance (NMR). These revolutionary breakthroughs have allowed chemical biology to become one of the most significant scientific disciplines in recent years. Scientists can now rapidly and reliably identify most proteins in a relatively small sample and readily produce three-dimensional display and/or images of expressed protein molecules with highly resolution. With these advancements, various experimental approaches and technologies were developed to obtain a better understanding of proteins and their regulatory effects on molecular and cellular functions of various biological systems [76, 77]. Among them, technologies including two-dimensional gel electrophoresis (2-DE) analysis [78, 79], matrix-assisted laser desorption/ionization (MALDI)- time-of-flight (TOF) [80] and Surface-Enhanced Laser Desorption/Ionization (SELDI)-TOF MS [81] have been broadly used in proteomics studies on the research of medicinal plants.

3.2 Application of proteomics for research into traditional herbal medicine

Proteomics technologies were applied to simultaneously study the function, organization, diversity, and the dynamic variety of total or a subset of proteins at the cellular or tissue levels [21]. The current integrative approach used in proteomics is in line with the practice and holistic philosophy of traditional Chinese medicine (TCM). Recent advances in multidimensional liquid chromatography, coupled with free-flow electrophoresis and capillary electrophoresis-based separation techniques, make it possible in separation of hundreds or even thousands of protein components in some medical plants [82, 83]. We may able now to explore an increased understanding of such complex mixtures and the reputed medicinal effects at the cellular and molecular levels through proteomics studies; it holds a key to the big demand for modernization and internationalization of a number of traditional phyto-medicines [83]. In this article, some of the proteomics approaches in TCM research and development are addressed, highlighting the application in mechanistic investigation of specific phytomedicines.

Panax ginseng and *Panax quinquefolius* are two of the valued herbs widely used in TCM. Conventional separation methods were unable to distinguish the different plant parts (main root, lateral roots, rhizome head and epidermal tissues) between these two species. On the other hand, when 2-DE maps were employed, plant tissue samples containing distinct or common protein species (spots) can be easily discriminated or distinguished. Clearly, these potential protein biomarkers may also facilitate the identification processes for various medicinal plants that may be difficult to identify morphologically or anatomically [84].

Numerous herbal medicines have been reported to have immunomodulatory and anti-tumor effects in cancer cells [85-87]. Recent biological and pharmaceutical researches have shown that diosgenyl saponins may exert a large variety of biological functions, with a potential for use in cancer chemoprevention [88]. By using 2-DE, tryptic in-gel digestion and MALDI-TOF MS analysis, Wang *et al.* [89] suggested that dioscin, a saponin extracted from *Polygonatum zanlanscianense* Pamp., exhibited cytotoxicity towards human myeloblast leukemia HL-60 cells. This proteomics analysis also revealed that the expression of

mitochondria-associated proteins was substantially altered in HL-60 cells upon dioscin treatment, suggesting that mitochondria were the major cellular and organelle target of dioscin cytotoxicity. Moreover, the results indicated that other pathways were likely also involved in detected dioscin cytotoxicity, including phosphorylation-based cellular signaling, RNA-related protein synthesis, and oxidative stress processes. The study demonstrated the benefits of using a proteomics approach in anticancer phytomedicine research [90].

4. Metabolomics study on the research of medicinal plants

Metabolomics, including both targeted and global metabolite profiling strategies, is rapidly becoming a popular and powerful approach of choice across a broad range of medical and biological sciences including systems biology, drug discovery, and molecular and cell biology [24]. Specifically for human metabolites, it is believed that at least 3,000 metabolites that are essential for normal growth and development (primary metabolites) and >2000 secondary metabolites that are not essential for growth and development but may help fight off infection and other forms of stress on the body [91]. In addition, metabolomics are now being generally considered a vital component of the systems biology approach, in which it can reflect and connect the genotypes with diverse yet specific phenotypes of specific types of cells, tissues, or organs [91]. Within the past decade, the number of publications of metabolomics-related research articles has increased from roughly 40 in 2002 to 100, 170, 200 and >250 articles in the years 2004, 2005, 2006 and 2007, respectively. Now it is estimated that >300 articles, with a general aim or study on metabolomics were published annually in 2010. Owing to its remarkable versatility, metabolomics is rapidly becoming a universal tool and key component in medical research [24]. Combined with genomics and proteomics technologies, systems biology research using metabolomics investigates characteristic molecular signatures for disease diagnosis, prognosis, and therapeutics [92]. This section reviews the recent developments in technology platforms and experimental approaches for metabolomics studies in the research of immunomodulatory properties of potential medicinal plants.

4.1 Use of GC-MS, LC- MS, FT-IR and NMR technologies

Currently, the term 'metabolomics' often can be used interchangeably with "metabolite profiling" because the type of one-step, two dimensional exhibition analysis used in genomics and proteonomics experiments is not possible at the present time, as the complexity of chemicals in most biological systems, especially in plants, is highly diversified and can be enormous [93]. The two basic approaches in metabolomics can be classified the targeted- and the global metabolite analyses. Targeted metabolite analysis, (or metabolite profiling), as the name implies, targets mainly a subset of metabolites in test sample, instead of a complete, global metabolome analysis, often by using a particular set of analytic technique(s) such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS), and yields an estimate of quantity [94]. Metabolomics approaches using GC–MS, LC–MS, or 2D NMR are effective tools for quality control of medicinal plants or herbal medicine products [95, 96]. As shown in Figure 3 [24], key aspects of the technology were assembled in many research institutions as "core labs/facilities" in the metabolomics approach for herbal medicine or other integrated research interest. Various other technical systems, methodologies or techniques, including

thin layer chromatography (TLC), Fourier transform infrared spectroscopy (FT-IR), Raman spectroscopy and NMR [97-99] are also important research facilities in the metabolite analysis arsenal.

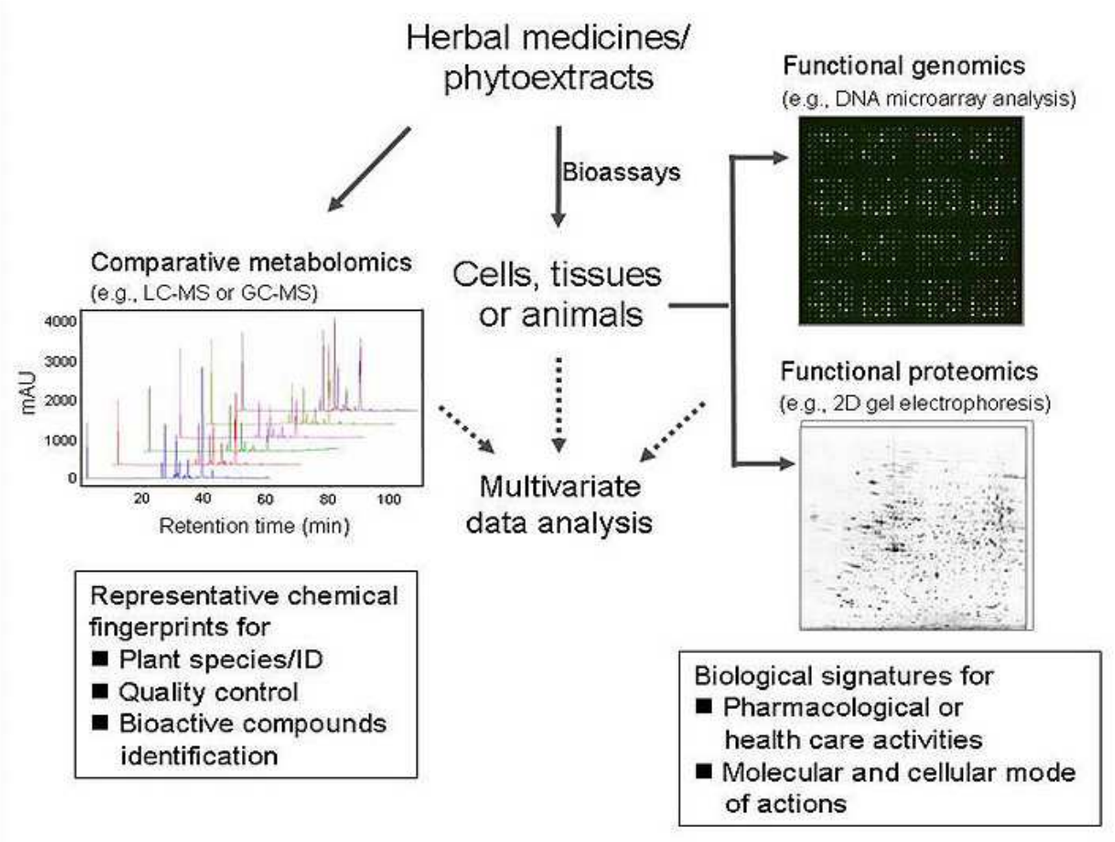

Fig. 3. Key features of metabolomics technologies employed for research into phytomedicines [24].

Mass spectrometry is currently the most broadly applied technology in metabolomic studies. Among the variety of MS techniques, GC-MS has long been popularly used in metabolite profiling of plant extracts [100, 101]. Rapid, high-resolution 2D GC x GC-TOF MS has been employed in the phenotyping of natural rice variants [102] as well as for efficient quality control or analysis of herbal medicines [95]. Recently, capillary electrophoresis-MS has also been developed as a metabolomics tool, capable of simultaneously analyzing over 1,000 charged chemical species, a technique that is expected to create a number of obvious applications in processing and characterization of various biological samples [95]. A shotgun approach using MALDI-TOF/TOF MS has recently been established for rapid analysis of negatively charged metabolites in mammalian tissues to: (a) facilitate the detection of low-abundant metabolites such as cAMP, cGMP, and IP3; and (b) discriminate isomeric molecular species [103]. In addition, novel instrumentation/equipment set ups developed recently, such as Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-MS), represents a quantum jump in the new capabilities of mass spectrometers for metabolite analysis. Due to the exceptionally high resolution of these instruments,

metabolites with mass differences of less than 2 ppm can now be separated on a chromatographic time scale (Van der Greef *et al.*, 2004). The accurate results obtained can help reveal elemental compositions, which often enable unequivocal metabolite identification.

Remarkable recent developments in analytical biochemistry regarding the detection and characterization of compounds with small molecular mass, such as MS and high-field NMR coupled with user-friendly multivariate statistics, have led to highly efficient systems for comprehensive analysis of the metabolite data matrices generated by metabolomics experiments [104]. One-dimensional (1D) NMR spectrometry has shown its capability for high-output analysis and classification of chemically similar groups of test samples. At the same time, the large numbers of overlapping peaks generated by such method may also hinder in some case accurate identification of specific metabolites. Recently, a replacement for the 1D ^{1}H NMR spectroscopic technology also has been developed: a two-dimensional (2D) ^{1}H–^{13}C NMR strategy (fast metabolite quantification, FMQ, by NMR), was developed for analyzing metabolites as multivariate statistical objects [105].

The new 'hyphenated' techniques that combine in assay sequence various forms of liquid chromatography with NMR, such as HPLC-SPENMR, have effectively improved the sensitivity of NMR analyses and can be employed to characterize both high- and low-abundant metabolites in complex crude plant extracts [106, 107].

4.2 Metabolomics research in medicinal chemistry studies

Diverse secondary plant metabolites are believed to have evolved through continuous interactions with challenging and predominantly hostile environments, including both abiotic and biotic stresses. When these features are coupled with characteristic species and agronomic differences, various phyto-chemicals as secondary metabolites generally can confer various specific bioactivities related to their biochemical structures [108]. These bioactivities apparently can help the host plants to defend specific plant pathogens and to reduce a spectrum of abiotic stresses, e.g., drought, heat and saline conditions. Interestingly, these secondary plant metabolites often were also found to confer potent and valuable bioactivities for defending human sickness, including viral, cancerous and inflammatory diseases. Some well-known cancer chemotherapeutic drugs have been initially derived from plant secondary metabolites, such as paclitaxel (taxol), camptothecin (irinotecan, topotecan), and podophyllotoxins (etoposide, teniposide) [24, 109]. Recent re-recognization of the vast potential of plant secondary metabolites or natural products to serve as lead compounds for drug discovery and development, or as various general health care products, has renewed a lot of interest in pharmaceutical and nutraceutical research. *De novo* combinational chemistry has so far produced only a very limited number of novel drugs, the natural products and their derivatives are still considered by many scientists to be the primary source of leads for drug development [110]. In this area, the use of whole plants or their extracts as medicines gave way to the isolation of active phyto-compounds, beginning in the early 19th century with the isolation of morphine from opium. In such a reductionist approach, however, single active phytocompounds may often be not identifiable because of their low abundance in test plant extracts, or alternatively, a spectrum of pharmacological efficacy traditionally observed arises only as a synergistic action of the multiple but specific ingredients present in a single plant or even from a multiple medicinal plant formulation, as in TCM [111, 112].

To efficiently link the flood of experimental data and specific metabolites or general metabolite profiles information to biology and metabolism study systems, traditional bioinformatics is being combined with cheminformatics to generate a basic computational infrastructure for analysis of metabolomics [113, 114]. A number of metabolomics databases, some based on both chemical and biological/biochemical data, have been made publicly available [114]. The Human Metabolome Database (HMDB) is currently the largest and most complete database in breadth and depth, offering spectral, physico-chemical, clinical, biochemical, genomic, and metabolism information for a library of >2500 known human metabolites [115, 116]. Other databases include the BioMagResBank (BMRB) with an emphasis on NMR data (>270 pure compounds), the Madison Metabolomics Consortium Database (MMCD) which presents MS and/or NMR data on more than 10,000 metabolites [117], and the Golm Metabolome Database (GMD) which has been specifically designed for plant research and utilizes GC–MS data [118]. Additionally, Wishart [113] has reviewed the development of algorithms and innovations in informatics concerning data reduction, normalization, and alignment that offer sufficient biological insight into metabolic profiles.

4.3 Metabolomics approach applied to research into immuno-modulatory effects of phytomedicine

It is now generally accepted that chronic inflammation is a key factor in the development of many types of cancers. Natural products, especially from plants, were once popular choices in cancer therapeutics based on their immunosuppressive or anti-inflammatory effects [110, 119-121]. Recently, metabolomics has been effectively used to characterize and monitor carcinogenesis activities in mouse models [122]. In addressing oncology metabolomics, NMR was used to target biomarkers for prostate cancer by analyzing metabolites with anti-inflammatory effects in the development and progression of this cancer for better future management [123, 124]. This metabolomics approach has also been successfully implemented to monitor the metabolism in human brain, liver tumors, lymphomas, and colon cancers [125].

5. Comparative and bioinformatics tools for omics studies

5.1 Ingenuity (http://www.ingenuity.com/)

Functional genomics experimental approaches were employed in our previous studies on the modulatory effect of *Echinacea* plant extracts (e.g., the butanol-fractionated Leaf and Stem tissue extract designated as BF/S+L/Ep) on both mouse and human DCs [17, 63, 64]. Using the same defined phytochemical extracts in the study, we analyzed the genome-wide transcriptional response in the context of known functional activities and interrelationships among specific protein molecules and/or different cell phenotypes. Ingenuity systems, a structured network knowledge-based approach, provided us good tools and insight into the regulation of bone marrow-derived dendritic cell activities relevant to the body's immune system. Figure 5 shows candidate molecular networks revealed by clustering analysis of the representative genes involved in the BMDC response to [BF/S+L/Ep] treatment [17]. The prototypical cell was constructed from 37 representative genes that responded to treatment with [BF/S+L/Ep] *in vitro* from 4 hours to 12 hours. Genes whose expression was upregulated (more than doubled) are indicated in red, and those whose expression was downregulated (to less than half) are shown in green. Selected regions of the network highlight three groups of

genes. Group 1: Immune response-related genes. Group 2: Adhesion molecules and cytoskeleton; cell movement related genes. Group 3: Cell cycle, cell proliferation and apoptosis related genes. Gene networks were analyzed using the Ingenuity Pathways program.

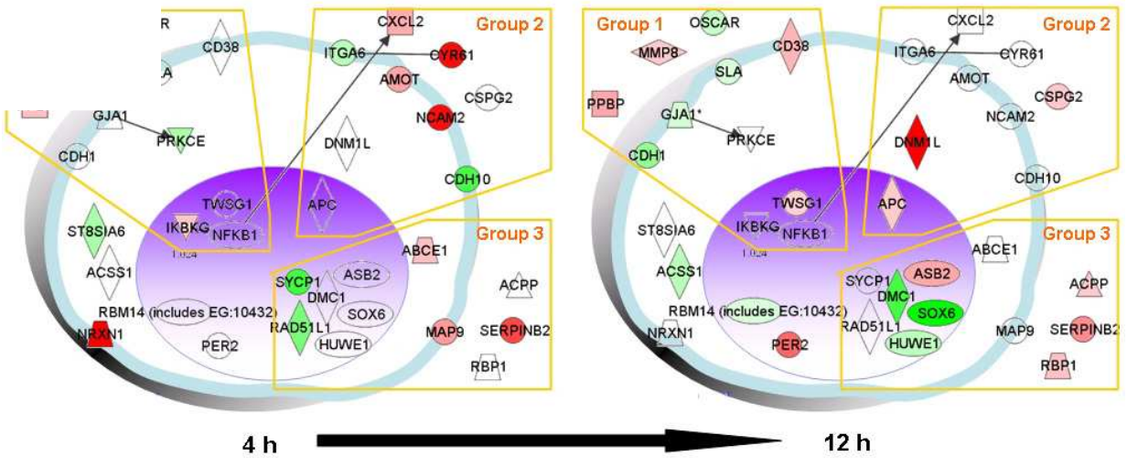

Fig. 5. Pathway analysis of representative genes that responded to [BF/S+L/Ep] treatment [17].

5.2 Metacore™ (http://www.genego.com/metacore.php)

MetaCore™ is another integrated knowledge database and software suite for pathway analysis of experimental data and gene lists. In the research of phytomedicines, it has also been used to evaluate the possible hierarchical control of microRNA expression from mouse tissues in order to identify trends of miRNA and mRNA expressions in response to targeted phytomedicinal treatment. Utilizing Metacore software, a prototypical network was constructed from 6 representative microRNAs that responded to treatment *in vivo* with specific phyto-chemicals (Figure 6). All selected microRNAs were found to be down-regulated to less than half of the untreated levels, and are shown with blue circles.

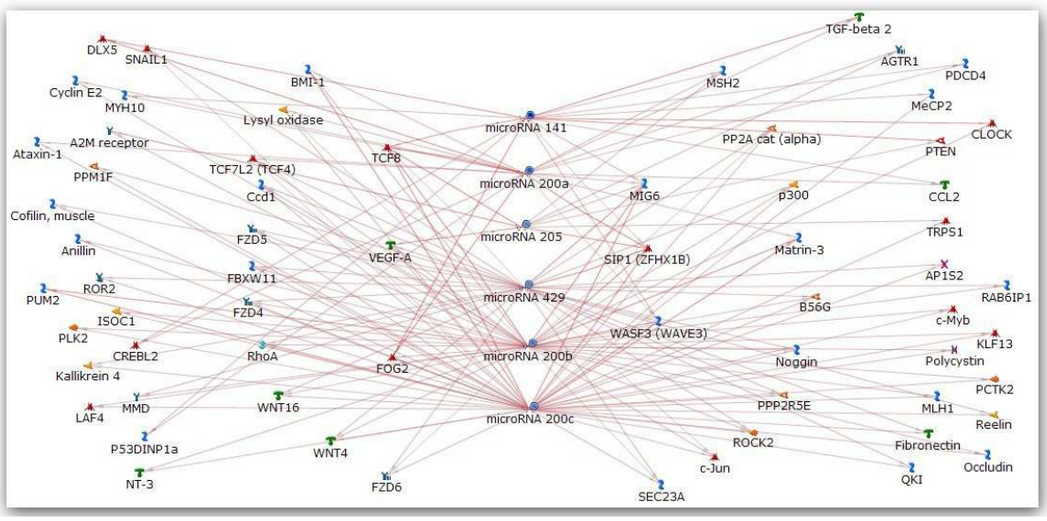

Fig. 6. Pathway/network analysis of representative microRNAs which are responsive *in vivo* to a specific single phytocompound treatment in inflammed mouse tissues.

Specifically, connections (hits) within 6 microRNAs were employed as the parameter for this specific search. Arrows indicate the cross talks among five key molecules/pathways, (TCF8, VEGF-A, FOG2, MIG6, SIP1 and WASF3), and there are postulated to be regulated by treatment with a specific phyto-chemical from TCM formulation.

5.3 TRANTHPATH (http://www.gene-regulation.com/index.html)

TRANSPATH, a database system about gene regulatory networks, combines encyclopedic information on signal transduction with tools for visualization and analysis. By integrating with TRANSFAC, a database about transcription factors and their DNA binding sites, TRANSPATH can predict putative signaling pathways from ligand to target genes and their products, which may themselves be involved in a regulatory action.

For studying specific imumodulatory effect of herbal medicine, the possible signaling pathways, networks or potential interactions among the responsive genes/target molecules in DCs treated with *Echinacea* extracts [BF/S+L/Ep] was assessed by using such Transpath software. This bioinformatics analysis has predicted a key-signaling network involving a number of immune-modulatory molecules leading to the activation of a very important downstream regulatory molecule, the adenylate cyclase 8, effectively in regulating cAMP levels in mammalian cells. This analysis indicated two postulated key molecules/pathways, Adenylate cyclase (AC8) and calmudulin (CaM), responsive to the *Echinacea* extracts (Figure 7).

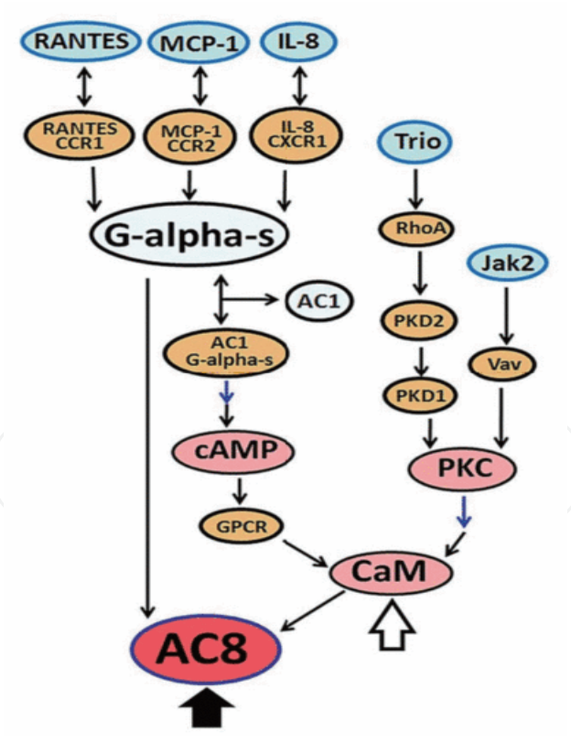

Fig. 7. Bioinformatics analysis of [BF/S+L/Ep] bioactivity and the underlying candidate molecular signaling networks in human DCs. The 20 genes that were up- or down-regulated at least 5-fold over controls were analyzed. Specifically, connections (hits) within 7 genes were employed as the parameter for the current search [63].

5.4 KEGG (http://www.kegg.jp/kegg/)

KEGG (*Kyoto Encyclopedia of Genes and Genomes*) is a multi-functional bioinformatics resource for linking genomes to metabolic activities. It consists of 16 main databases and has been widely used as a reference knowledge base for biological interpretation of large-scale datasets generated by sequencing and other high-throughput experimental technologies. Among these databases, the KEGG DRUG database contains crude drugs (consisting of multiple chemical compounds) and formulas (consisting of multiple crude drugs) in the Traditional Chinese Medicine (TCM). In addition, KEGG PATHWAY and KEGG ENVIRON are also being organized to interpret and correlate relationships between genomic and chemical information of various natural products/metabolites from plants. For example, the biosynthetic pathway of stilbenoids, a group of phenolic compounds, was provided for revealing specific molecular interaction and different reaction networks (Figure 8). Although the knowledge on biosynthetic pathways of plant natural products is in general largely incomplete, the genomic information is expected to provide clues to missing enzymes and reactions for biosynthesis of specific plant secondary metabolites, the source for future modernized phytomedicines, either as pure compounds, fractionated phytochemical mixtures, or as crude plant extracts. Moreover, the genomic information may also uncover the architecture of biosynthetic pathways for generating chemical diversity of natural products.

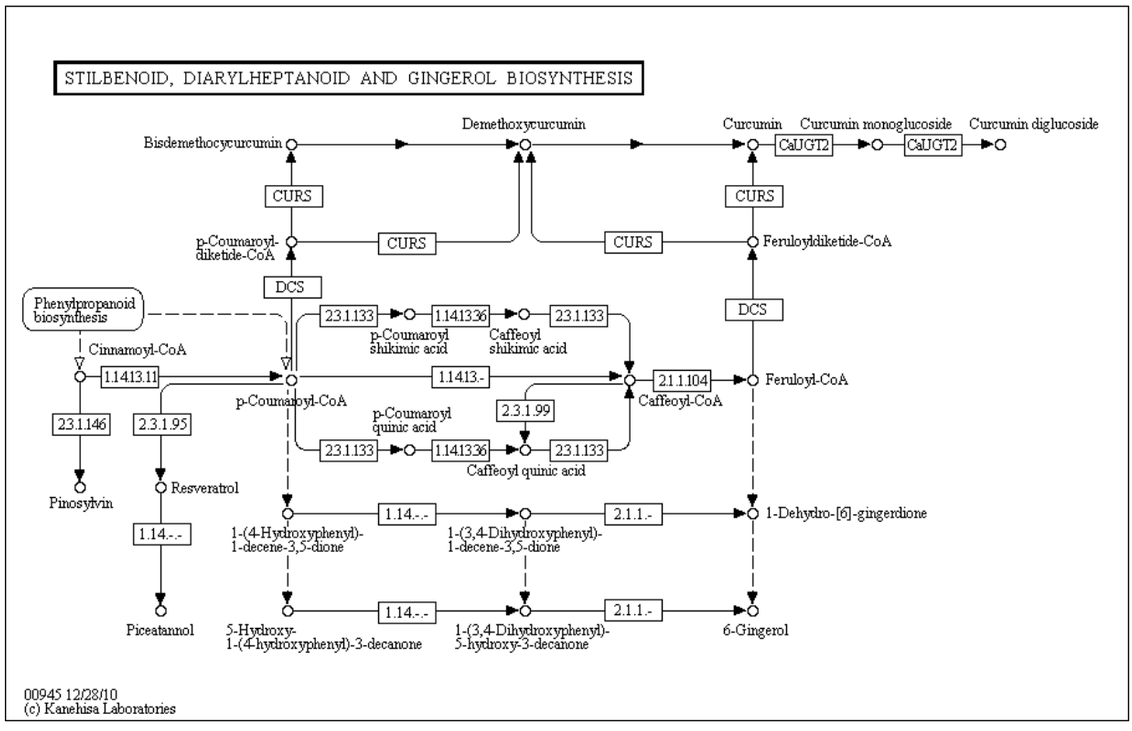

Fig. 8. Stilbenoid, diarylheptanoid and gingerol biosynthetic networks cited in KEGG. (Adopted from http://www.kegg.jp/kegg/pathway/map/map00945.html)

6. Challenges and perspectives

Traditionally, the pharmaceutical research and industries have focused on evaluating or monitoring individual gene, proteins as the target or basis for identifying new drugs. The

quest for single molecules to modify single key factors in a disease process is now recognized as may not be able to provide a solution for a spectrum of diseases in which multiple cell types, target molecules and/or multiple pathways are known or believed to contribute to the diseases. Herbal extracts/mixtures as conventional phytomedicines may represent the combinational chemistry of the nature of traditional medicines, and encompass a vast repertoire of chemical entities that may have anecdotally and empirically found through long human culture history to confer a complex and yet integrated effect on numerous cellular components and functions, effecting a medicinal activity. Various traditional herbal drugs may thus have good potential for re-invention and newly found use in the multi-target approach in treating various diseases. However, such potential of herbal drugs is undermined by difficulties in standardization, and the pharmacodynamics and pharmacokinetics studies of these multicomponent plant extract mixtures. Microarray analysis of gene expression profiles may be useful for elucidating such complex molecular mechanisms and networks underlying the multi-target pharmacological functions of herbal extracts and phytomedicine mixtures. Research into the patterns of gene expression at a range of stages during the treatment process may reveal key targets and mechanisms and help to identify biomarkers of either adverse- or favorable response. A positive correlation between the transcriptional response induced by a putative or candidate herbal drug and the database profile of an existing pharmaceutical or therapeutic agent as a single chemical may provide us insight into the target specificity, mechanism of action, as well as in facilitating analysis of signaling pathways downstream of the specific target. This information could in turn be used to interpret possible bioactivity, function or effectiveness of test phytomedicines. In addition, various DNA, RNA or protein microarrays may also be used for bioactivity-guided fractionation of herbal extracts, thereby narrowing in the active principles delivering the desired or observed effect. Microarrays may also improve the power for selection of biological targets and lead compounds up or down the drug discovery pipeline. Once useful transcriptome or/and proteome data from herbal drug candidates can be correlated with *in vivo* bioactivity or preclinical or "existing clinical" (as in some TCM) response outcomes (biomarkers) in defined biological systems, the best candidates can then be selected for further drug development [30].

Although some DNA microarrays have already offered impressive potential for pharmacodynamics and toxigenomics applications, they are still being considered as in an exploratory stage and the data obtained from them will need validation by other biological experiments. Bioinformatics and statistical tools have a major role to play in analysis of the microarray results, whereby data from multiple experiments can and may need to be integrated to address complex biological activities, functions or effects. Another factor currently limiting microarray application is the cost of this technology [30]. The challenge we face today is to develop or construct standardized, sensitive, reproducible microarray platforms, databases and visualization methods for expression profiles that are affordable to most research scientists. With the use or development of improved, uniform and sophisticated experimental designs, data management systems [126, 127], statistical tools and upgraded algorithms for data analysis [128, 129], DNA microarrays hopefully can be more optimally used in herbal drug research. In spite of the vast potential offered by microarray and the related functional genomics and proteomics technology, the importance of integrating various *in vitro* biological assays, cell culture-based and *in vivo* animal experimental systems cannot be ignored. Comprehensive strategy integrating information from diverse scientific experiments and technologies are expected to benefit and lead to molecule and cell evidence-based phytomedicines.

The integration of information from genomics, proteomics, and metabolomics is hoped to provide solid evidence-based rationales for systematic development of various modern phytomedicines, on top of the foundations of various traditional medicine cultures. The search for specific, active single phytocompound may also be expedited when various metabolomics approaches are combined with a comprehensive array of bioactivity assay systems using standardized and normalizable mammalian cell, tissue and animal models.
Wherea a "complete metabolome-exhibition" system is currently not available, HPLC-, GC- and LC/MS-based metabolite-profiling systems, alone or in combination, may already offer a good description or authentication tool for comparative and qualitative analyses and definition of the unique, distinctive, or combinational profile features of the conventional herbal medicine formulations, as elegantly demonstrated recently by W. Lam et al (2010) [112]. These and the improved or newly developed metabolomics technologies in linkage may also be usefully applied to discovery and development of new phytomedicines, as single phyto-chemicals or their mixtures, or as fractions or the whole preparation of the crude extracts of various medicinal plant tissues. Our challenges together, as scientists and health care-takers are to coordinate and integrate our intellectual thrusts, talents and efforts to address and target specific medical and medicinal research areas, e.g., for anti-inflammation and related chronic or cancerous diseases, for future research and development of advanced phytomedicines, may be to be pursued more effectively as an international program.

7. References

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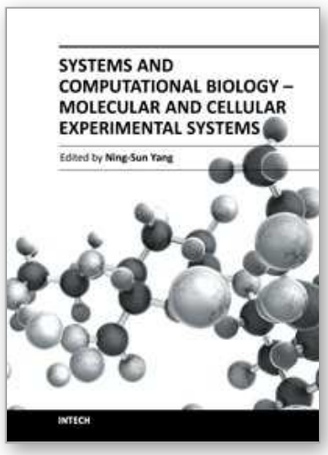

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