Chapter 8
Metabolomic Study of Chemo-preventive Phytochemicals and Their Therapeutic Prospects

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Abstract The survival rates of cancer patients are decreasing over the years which are possibly due to selection of poor conventional anti-cancer drugs. At present complementary and alternate medicines (CAM) are high on demand as they show few or rather zero side-effects. Metabolomic analysis is considered to be a complex as well as efficient bridge between CAM and plants and its therapeutic possibilities as optimized and subscribed medicines. Metabolomics, considered to be the smallest domain comprises approximately 5000 metabolites, is chemically and physically more complex as compared to genomics (30,000 genes) and proteomics (100,000 or more proteins) associated with anti-cancer properties as it involves diverse groups of biological molecules. Stress on the cellular activity is inflicted directly by changes in the metabolome of an organism which underlines the importance of metabolomics in disease diagnosis and drug discovery. The present chapter focuses on the recent progresses in metabolomics which have transformed it to become a robust systems biology tool in studying both chemical and biochemical events that contribute to the cancer prevention activities of plant preparations or their bioactive components. Variations in the metabolome of cancer cell lines on treatment with plant extracts are also discussed. The current status of metabolic engineering efforts is highlighted for *in vitro* production of different chemo-preventive compounds viz. paclitaxel, geraniol, methyl cinnamate, Δ^9-tetrahydrocannabinol, etc. in their respective medicinal plants. The aim of present chapter is to explore the feasibility of metabolomic analysis of potent anticancer

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plants in order to search for the lead chemo-preventive compounds and also, to integrate the traditional cancer therapy with metabolomic profiling for the cure of cancer.

Keywords Cancer cell lines · CAM · Metabolomics · Phytochemicals · Screening

8.1 Introduction

Cancer is the leading cause of death worldwide, accounting for 8.8 million deaths in 2015 (Forouzanfar et al. 2016). According to WHO, the global burden of Cancer is predicted to increase by 50% to 15 million by 2020. In the present, scenario the search for novel chemo-preventive bioactive compounds have gathered momentum considering the dose limiting toxicity of conventional cancer treatment approaches. Complementary and alternative medicines (CAM) derived from plants are used in cancer treatment as these show reduced adverse side effects (Ravishankar and Shukla 2007). Treatment of cancer with Ayurveda, the traditional Indian medicine (TIM) dates back to seventh century BC, where early stages of cancer was treated using herbal medicines by Dhanwanthri and Atreya (Ravishankar and Shukla 2007; Poornima and Efferth 2016). In Ayurveda, cancer is described as an inflammatory and non-inflammatory swelling called as *Granthi* (minor neoplasm) or *Arbuda* (major neoplasm) (Patwardhan et al. 2005).

The approach or methodology used by the pharmaceutical companies for production of plant-derived drugs for the past decades was too expensive or time-consuming or both at the same time. Consequently, the former approaches have been regarded as the major bottleneck in exploiting the therapeutic potential of plants (Kim et al. 2010). Metabolomics have proved to be a promising tool as a new strategy for detection of bio-active compounds and such new strategies was the need of the hour to get natural products research out of its deadlock (Rochfort 2005; Verpoorte et al. 2005; Merzenich et al. 2007; Trenerry and Rochfort 2010). It is an 'omics' science that gives an overview of the metabolites present in a biological system. Its exposure under different conditions provides a better understanding about the biochemical reaction, disease development, selection of bio-markers, and patho-physiological interactions in the biological system (Sumner et al. 2003; Kim et al. 2010; Tomita and Kami 2012; Zhang et al. 2015). The downstream expression of the genome, transcriptome, proteome is represented by the metabolites thereby closely reflecting the phenotype of an organism at a specific time. Moreover, assessing the metabolite variation in cells on treatment with plant extracts or plant-derived compounds not only help us to monitor response of the cells but also to detect the activities of the bio-active compounds (Kim et al. 2010). The aim of present chapter is to explore the feasibility of metabolomic analysis of potent anticancer plants in order to search for lead chemo-preventive compounds and also to integrate the traditional cancer therapy with metabolomic profiling for the cure of cancer.

8.2 Metabolomics in Cancer Research and Diagnosis

Metabolomic approaches have brought metabolomics into the forefront of cancer research. The existing diagnostic modalities are not only expensive but also less sensitive in tumour detection (Soga et al. 2011). Conventional methodologies for detecting hepato-cellular carcinoma are by measuring serum-level of α-fetoprotein along with ultrasound imaging (Nicholson and Lindon 2008). However, other liver diseases may also result in high blood levels of α-fetoprotein resembling that of hepato-cellular carcinoma and thereby making α-fetoprotein an insensitive biomarker (Sumner et al. 2003; Robert and Morvan 2013). High-throughput metabolomics enables us to screen an array of biomarkers by comparing a diseased individual with healthy individuals (Fig. 8.1). Emerging metabolomics approaches have led to identification of biomarkers and associated candidate gene which is to be regulated for a particular type of cancer.

8.2.1 Metabolomics of Medicinal Plants

As vast number of primary and secondary metabolites having the potential therapeutic importance are synthesized by medicinal plant. The medicinal plant-based drugs and the use of metabolomics study is of paramount importance in the cancer therapy. Several secondary metabolites (about 2,00,000) from plants have been explored (Sumner et al. 2003). For example, ~5000 secondary metabolites have been derived from *Arabidopsis thaliana* and approximately 1500 and 2500

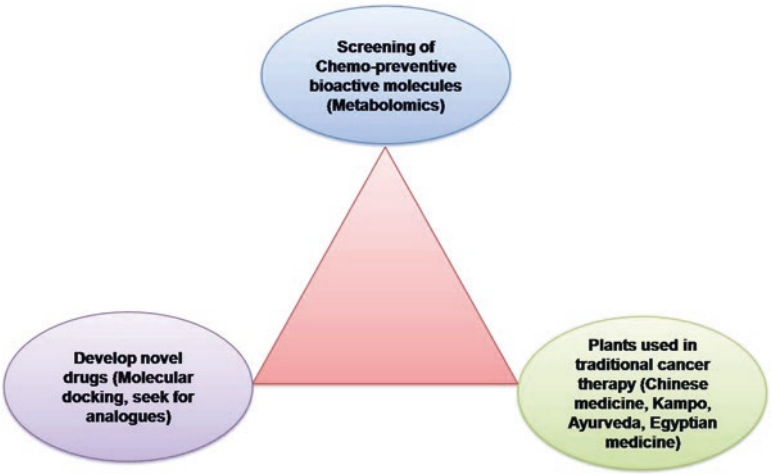

Fig. 8.1 Integrating traditional knowledge and high throughput metabolic approaches for discovering novel anticancer drugs

from microorganisms and animals, respectively. Different genomics-based 'phyto-chemical arrays (genome, transcriptome, proteome and metabolome)' have been established for measurement and analysis of several aspects including metabolite profiling in plants. Potential anticancer activity have been reported to be present in plant secondary metabolites like paclitaxel (taxol), camptothecin (irinotecan, topotecan) and podophyllotoxins (etoposide, teniposide) etc. (Schattka et al. 2011). Hence, medicinal plants or natural products are being considered as alternative application. It includes metabolite fingerprinting, which can be applied in different aspects like qualitative and quantitative analysis of target compound, identification of a set of compounds, quantification of all metabolites and rapid analysis of metabolites. This study has given rise to special emphasis on phyto-medicine research. It can be very useful in shifting the paradigm in drug discovery and development from natural resources. An overview of strategies pertaining to the enhancement of bioavailabity of the chemo-preventive bioactive molecule(s) from their respective plants is shown in Fig. 8.2 (Wang et al. 2012) (Table 8.1).

8.2.2 *Metabolomes of Cancerous Cell Lines Vary on Treatment with Natural Products*

Metabolomics have been applied to study the phytochemical induced chemo-preventive influences in carcinoma cells. Dose-dependent metabolic changes in breast cancer cells were revealed by NMR-based metabolomics on treatment with

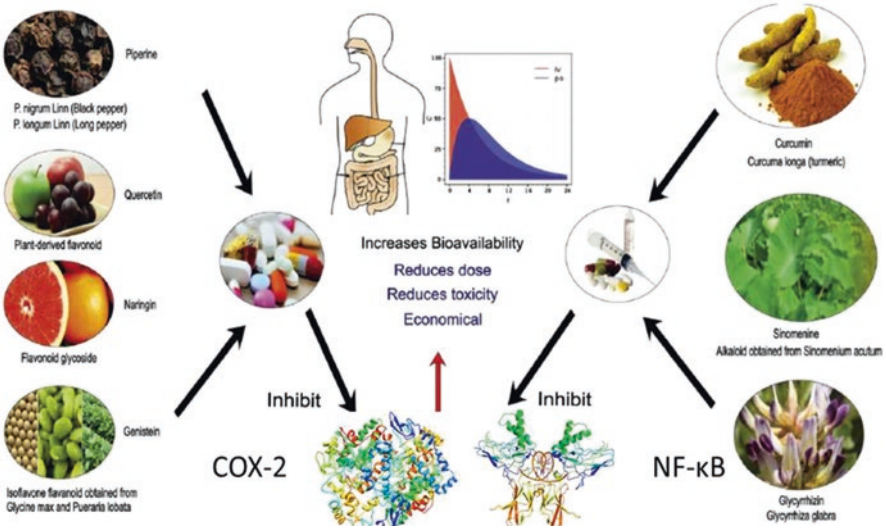

Fig. 8.2 Enhancing bioavailability of potent chemo-preventive bioactive compounds (Modified from Wang et al. 2012)

Table 8.1 Some major secondary metabolites derived from plants with potent anticancer properties

Major constituent	Molecular target	Plant source	References
Boswellic acid	↓leukocyte elastase, ↓5-LOX	*Boswellia serrata*	Safayhi et al. (1992, 1995) and Ammon et al. (1993)
Curcumin	↓NF-κB, ↓AP-1, ↓Egr-1, ↓COX-2, ↓LOX, ↓iNOS, ↓MMP-9, ↓uPA,↓ TNF	*Curcuma longa*	Bharti et al. (2003) and Villas-Boas et al. (2005)
Flavopiridol	↓CDK1, ↓CDK 2, ↓CDK 4, ↓CDK 7, ↑TNF, ↑doxo-rubicin, ↑etoposide	*Dysoxylumbi nectariferum*	Losiewicz et al. (1994) and De Azevedo et al. (1996)
Guggulsterone	↓iNOS, ↓IAP, ↓Bfl-1/A1, ↓bcl-2, ↓cFLIP, ↓cyclin D1, ↓c-myc, ↓MMP-9, ↓COX-2, ↓VEGF	*Commiphora mukul*	Aggrawal (2004) and Shishodia and Aggrawal (2004)
Resveratrol	↑p21, ↑p53, ↑Bax, ↓cyclin D1, ↓cyclin E, ↓bcl-2, ↓bcl-XL, ↓cIAPs, ↓NF-κB, ↓AP-1, ↓Egr-1, ↓IκB α-kinase, ↓JNK, ↓MAPK, ↓Akt, ↓PKC, ↓PKD, ↓COX-2, ↓5-LOX, ↓VEGF, ↓IL-1, ↓IL-6, ↓IL-8	*Vitis vinifera*	Manna et al. (2000), Banerjee et al. (2002), Estrov et al. (2003) and Agrarwal et al. (2004)
Zerumbone	↓COX-2, ↓IL-1β	*Zingiber zerumbet*	Kitayama et al. (1999) and Muakami et al. (2003)

↓ = Down regulation; ↑ = Upregulation

curcumin which is an active chemo-preventive curcuminoid present in the rhizome of *Curcuma longa* (Xie et al. 2015). Robert and Morvan (2013) reported increased glutathione level in breast cancer cells at low dose of curcumin and decreased glutathione level at high dose, suggesting that glutathione biosynthesis was up-regulated at low dose while the consumption of glutathione elevated at high dose. In addition, the effects of curcumin treatment on lipid metabolism, including accumulation of polyunsaturated fatty acids and decrease of glyerophospholipids, were also observed (Xie et al. 2015). In another investigation, the chemo-preventive effect of American ginseng on progression of colorectal carcinogenesis in genetically modified mouse was reported (Noorolahi et al. 2016). Gas chromatography time-of-flight mass spectrometry (GC-TOFMS) and liquid chromatography time-of-flight mass spectrometry (LC-TOFMS) analysis of the serum shows significant alteration in the metabolites *viz.* amino acids, carbohydrates, fatty acids and organic acids which were attenuated by American ginseng and simultaneous histo-pathological improvement along with reduced tumor initiation, progression and gut inflammation (Noorolahi et al. 2016). Moreover, intestinal tissues of ginseng treated mice shows reduction of anti-inflammatory cytokines. Besides, ginseng extract independently exhibits chemo-preventive effects by anti-oxidant and anti-inflammatory mechanisms as it induces alteration of metabolites involved in inflammation and oxidation *viz.* tryptophan, arachidonic acid, glutamate, docosahexanoate and fructose (Noorolahi et al. 2016).

Metabolomic studies of *Aloe vera* extract on cancerous lymphoma cells showed altered levels of amino acids when compared with the untreated cells (Yagi et al. 2003). Glycoproteins present in the *A. vera* extract was described to be anti-ulceric and anti-tumor and observed to increase the rate of proliferation of normal human dermal cells (Singh et al. 2000; Yagi et al. 2003). A substance named *Aloin*, an anthroquine andthe main ingredient of *A. vera* has been shown to possess anticancer potential activities, as it blocks signal transducers and is an activator of transcription III activation by inhibiting effect on detoxification and are associated with carcinogen metabolism. The microsomal and cytosolic proteins were increased in the *A. vera* treated mice indicating the possibility of its involvement in the initiation of protein synthesis (Wang and Chen 2013).

8.2.3 Metabolomics Approaches for Investigation of Chemo-preventive Phytochemicals

8.2.3.1 Selection and Preparation of Sample

Based on the aims of metabolomics investigation, plants (extracts), bio fluids of animal models (treated *in vitro* with chemo-preventive plant extracts), tissue and cell extracts can be chosen for identifying bioactive phytochemicals *in vitro* and examining the impact of phytochemicals on metabolism on animal system (Fig. 8.3). In general, a power analysis should be conducted to ensure that a sufficient number of samples are included and the data can be statistically validated. In order to minimise or avoid the formation of new chemical species or degradation of metabolites during and after sample collection, the integrity of chemical composition in acquired samples, experimental techniques, storage conditions viz. (snap freezing in liquid nitrogen, quenching in preservation solution or freeze clamping) should be optimized (Villas-Boas et al. 2005; Wang and Chen 2013).

Preparation of the sample for the analytical platform is the key for success in metabolomic analysis, especially for MS-based approach. Metabolomic analysis of phytochemicals mediated chemoprevention demands ideal sample preparation strategies so as to efficiently extract small-molecule chemo-preventive phytochemicals from plants, human materials, and animals and also remove incompatible matrices such as salts and macromolecules. The chemical and physical properties of sample determine the extraction and preparation techniques to be applied. Liquid-liquid extractions, super-critical fluid extraction, solid-phase extraction, micro-wave assisted extraction, protein precipitation and dialysis are the widely applied (Dettmer et al. 2007). The obstacles for detecting phytochemicals in MS-based analysis is not only the concentration of bio-active compounds in samples but also their non-optimal performance in MS systems such as insufficient ionization in MS and poor retention in Liquid chromatography (Zhou et al. 2012). Chemical derivatization is an effective approach in this regard as it enhances the chromatographic and spectroscopic performance of these phyto-chemicals (Halket et al. 2005). This approach is

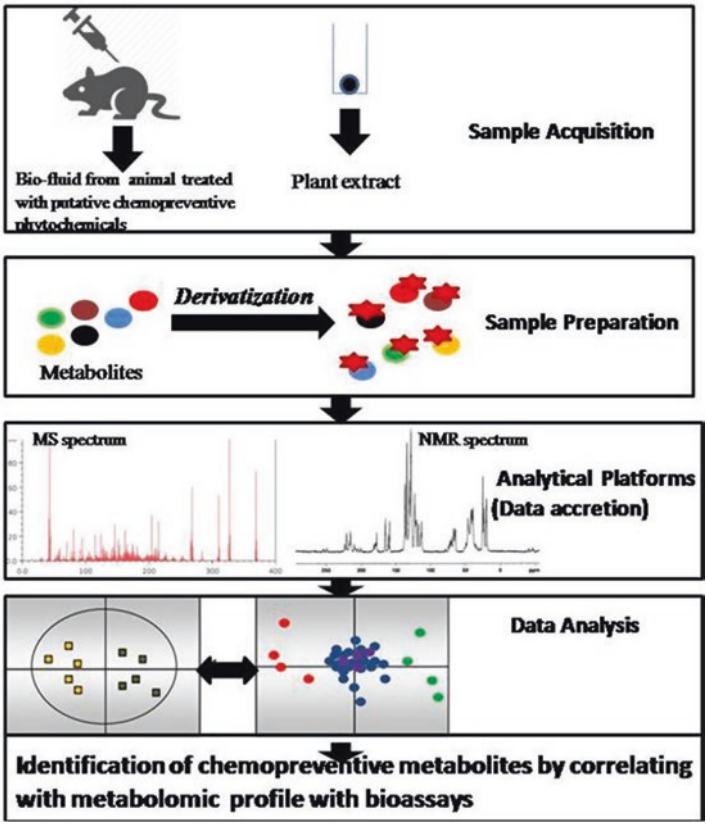

Fig. 8.3 Workflow of metabolomic approach for identification of chemo-preventive phytochemicals. Sample preparation by derivatization of metabolites making the metabolites compatible for analytical platform (Wang et al. 2013)

widely applied in gas chromatography GC-MS analysis as it improves the separation, detectability and sensitivity of metabolite detection. In recent years, chemical derivatization is widely applied in LC-MS analysis (Halket et al. 2005; Wang and Chen 2013). Derivatization reactions are designed based on the functional groups such as amino, carboxyl, carbonyl, and hydroxylmoieties in the metabolites. The two most desired outcomes of this approach are increased hydrophobicity and chargeability (Santa 2011).

8.2.3.2 Analytical Platform and Data Analysis of Metabolomics

Sophisticated platforms available viz. NMR, MS, infrared spectroscopy (Gamache et al. 2004) and electrochemical array (Schattka et al. 2011) enables us to detect chemo-preventive bioactive molecules in plants. Moreover, these methods unravel

the interactions between phytochemicals and biological systems. NMR and MS are the most widely used platforms for metabolomics analysis. Under electromagnetic field, MS determines mass to charge ratio of ions, while NMR measures the resonant frequency of nuclei. As compared to MS, NMR is less sensitive for detecting low abundance metabolites. Besides, NMR is non-destructive in nature and capable of providing more structural information. Chromatographic separation is not required for NMR analysis whereas majority of MS-based metabolomics approaches, GC, LC, capillary electrophoresis (CE) requires, prior to sample introduction and mass detection in MS system. Volatile metabolites or the metabolites which becomes volatile on derivatization are excellently suited for analysis in GC platform and polar compounds separation is performed by capillary electrophoresis (Wolfender et al. 2005). Metabolites in the biological sample are better compatible with LC platforms as compared to the other MS-based metabolomics approaches. Ultra-high pressure liquid chromatography (UPLC/UHPLC) is preferred over high pressure liquid chromatography as UPLC has features which use small particles, faster flow rate and high pressure as compared to HPLC thereby improving the chromatographic resolution and reduced running time in LC system (Wang et al. 2011). On elution from GC, CE or LC the analytes are subjected to ionization for detection by mass detectors in MS-based chemical analysis. Data analysis of NMR and MS-based metabolomics approaches is the crucial step for annotation of metabolites in metabolomic studies. NMR data comprises chemical shift and signal intensity, whereas GC-MS and LC-MS data comprise retention time (RT), mass to charge ratio, and signal intensity. In order to conduct metabolomic analysis, these data are deconvoluted to suitable data matrix so as to perform multivariate data analysis.

8.2.3.3 Metabolomics Based Identification of Bioactive Phytochemicals

Bioassay-based screening of crude plant extracts followed by fractionation and purification of chemo-preventive phytochemicals are the initial strategies to be adopted for identification of potent bioactive phytochemicals. The two types of high-throughput bioassay approaches for screening candidate chemo-preventive phytochemicals are; (i) cell-based approach for determining anti-proliferative and cytotoxicity against variety of cancer cell-lines such as 60 human cancer cell-lines of National Cancer Institute (NCI) representing nine human cancers (Shoemaker 2006); (ii) mechanism-based approach in which signalling molecules responsible for regulating proliferation and apoptosis of cancerous cells (*Ras, p53, bcl-2*) or enzymes (such as histone deacetylase, DT-diapharose) are used as biomarkers of screening assays (Kinghorn et al. 2003; Holbeck 2004). This is followed by validation on animal models and human trials. Fractionation and purification of bioactivity-guided compounds is a daunting task considering the chemical properties of the phytochemicals and the mechanism of bioactivity. Moreover, if chemo-preventive phytochemicals acts synergistically, unravelling the bioactivity of pure phytochemicals by bioassays becomes challenging (Rochfort 2005). Advanced analytical platforms such as NMR and MS-based metabolomics, which are widely used, is capable

qualitatively and quantitatively defining chemical composition of multiple plant extracts and identifying major differences among them through PCA modelling. A number of studies have illustrated the application of metabolomics approach for the identification of chemo-preventive phytochemicals from potent anticancer plants (Yuliana et al. 2011; Xie et al. 2015; Noorolahi et al. 2016).

8.3 Conclusions and Future Prospects

Sophisticated and powerful analytical platforms used in metabolomics have the capacity to measure numerous chemicals simultaneously and detect subtle differences among sample groups. Integration of traditional knowledge of cancer therapy with metabolomics provides a holistic approach for discovering novel candidates for anticancer drugs from plant extracts and elucidating the mechanism of action of potent chemo-preventive phytochemicals on regulation of cancer in biological systems. However, this field of study is still in its infancy as majority of metabolomics studies on the metabolic effects of chemo-preventive phytochemicals remain in the observational level. To elucidate the structure of the potent chemo-preventive phytochemicals, chemical analogues can be developed for finding novel anticancer drugs. Metabolomics is a promising domain for finding lead compounds considering the great need for novel chemo-preventive phytochemicals.

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