

Metabolomics: A Tool for Anticancer Lead-Finding from Natural Products

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Abstract

Natural products have been the source of many drugs used in modern therapeutics, and particularly in the case of anticancer drugs, more than 50% originally came from natural products. Their importance as a source of leads for new drugs therefore cannot be underestimated. However, due to the painstaking way of conventional lead-finding, the attention towards natural products has been deviated in the last decades. A new strategy for the detection of active compounds is necessary to get natural product research out of its stalemate. Metabolomics, with its holistic approach and the possibility it provides for the simultaneous detection of all sorts of metabolites, has the potential to be instrumental for this new

approach. Therefore, this review aims at providing examples that illustrate the possibilities of using metabolomics as a tool to find active compounds from natural products, specifically anticancer drugs. Two different methods, *in silico* and *in situ*, have been introduced as possible approaches. Current methods to detect anticancer activity in natural products have been briefly reviewed and compared in the first section, and various applications of metabolomics in cancer research are mentioned as they can provide comprehensive information of cancer metabolites utilized in the *in situ* approach. Metabolomics will certainly improve the efficiency of lead-finding from natural products and thus reinstate this prolific source of potential anticancer drugs.

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Introduction

Natural products and particularly plants have long been a productive source of lead molecules or even drugs themselves. Specifically in the case of anticancer drugs, the fact that more than 50% of the modern oncological drugs are derived from natural products speaks for itself [1].

The traditional way of finding a lead from natural products is based on bioactivity-guided fractionation. For this approach, it is a prerequisite to have a well-established bioassay system which has high-throughput capacity and reliability. The procedure of finding a new active compound is very painstaking since it takes many separation and isolation steps until an active pure compound is finally obtained from the crude extracts. The final structure elucidation may even be a more difficult task as often very complex structures are found. However, the major problem is not only the process, but rather the fact that the bioassays are usually designed to detect single targets. When, as is commonly the case, the activity is due to a mix-

ture of compounds and their synergistic effect, it is not easy to find novel leads [2,3].

In the last decades, pharmaceutical companies and researchers diverted their attention from plants and microorganisms, turning instead to combinatorial chemistry. The reason for this change in attitude towards natural product research was not the conviction that there was no potential in this field, but rather disenchantment with the ratio of hits versus effort/time and expense. The problem clearly was the methodology or approach which was used, that was too expensive or time-consuming or both at the same time. A new strategy for the detection of the active compounds is necessary to get natural product research out of its stalemate. The decreasing number of novel drugs every year makes for a renewed interest in developing novel approaches. As other reviews have already suggested [2–5], a new approach to find a lead compound from natural products has to be developed. One of the promising tools for new strategies is the use of metabolomics. Metabolomics has been applied in

many fields in plant science and natural products chemistry, including the quality control of medicinal plants [6–8], the monitoring of the biochemical response of plants to diverse stress conditions [9–11], and the search for differences between genetically modified plants and controls [12, 13], among others. This is possible through the general overview of metabolites provided by the metabolomic analysis of organisms submitted to different conditions and consequently allows a better understanding of the biochemical status of biological systems [14].

Metabolomics of human biofluids or tissues, often referred to as “Metabonomics”, has been used for the analysis of the metabolic or physiological state of biological systems to provide reliable diagnosis and markers of diseases or toxicity [15, 16].

Metabolites are the end product of gene expression and enzyme activity of organisms. The metabolome thus reflects the state of an ongoing biological situation, and the changes in metabolite concentration and/or composition may describe the actual biochemical status of a system better than genomics and proteomics [14]. Measuring metabolite changes resulting from the response of cells upon treatment (with natural products or pure compounds) may help us not only to monitor the response of the cells but also eventually to detect activities of the treated materials themselves. However, to date, the metabolomic approach is more focused on finding system phenomena while the detection of lead compounds is still in a very premature state. Our intention in this review is to explore the feasibility of using metabolomic analysis in the search for lead compounds from natural sources. Due to the amount of existing literature and the importance of the issue, we have restricted this to anticancer lead-finding.

A general overview of methods which are currently used for anticancer activity, measurement, and metabolomic applications in the cancer research field are summarized in the first two sections.

Among many analytical platforms utilized in metabolomic research, NMR has some important advantages over other techniques, the most prominent of which are the broad spectrum of metabolites detected, ease of quantitation, its reproducibility, and straightforward metabolite identification [17]. Particularly in metabolomic analysis of biofluids, urine, blood, and culture extracts, NMR has an edge over other methods, since it allows the introduction of samples directly into the instrument with very simple sample-preparation procedures [18]. Moreover, solid samples from tissues can be directly analyzed using MAS (Magic angle spinning) NMR [19]. Thus, NMR is considered as a method of choice in metabolomic analysis of biofluids (metabonomics). Metabolomics in cancer research mostly deals with cell cultures and animal tissues for which NMR is undoubtedly the most useful and popular method of analysis, therefore our review will give special attention to the application of NMR-based metabolomics in cancer research.

Current Methods for Anticancer Activity Measurements

The initial screening methods used to find anticancer leads in natural products are mostly either cell-based or mechanism-based *in vitro* assays. Cell-based assays usually evaluate the cytotoxicity of plant extracts on cell lines derived from major human neoplasmas. This type of test is routinely conducted with some of 60 cell lines representing 9 distinct types of tumors, which were established by NCI in 1990 [20]. Thanks to the amount of work

dedicated to the identification of molecular targets in these cell lines, mechanism-based assays have been established [21]. The targets that have been detected are at different levels: genes (*p53*, *Ras* gene) or RNA levels (tyrosine kinase, phosphatases) and enzyme activity (proteasome inhibition assay, histone deacetylase inhibition assay, DT-diaphorase) [22]. Using these *in vitro* assays, many natural products have been tested and new leads have been identified in the last decades [21, 23, 24].

These assays are quite simple, relatively fast, robust, and easy to set up but often too permissive, resulting in false-positive or false-negative results [22]. To overcome this problem, attempts have been made to develop multicellular tumor spheroid models which have intermediate complexity between *in vivo* models and *in vitro* monolayer cultures rather than monolayer culture models. This model has a great potential as it mimics the structural and functional properties of normal and tumor tissues much better, bridging the gap between cell-based assays and animal studies [25]. However, the application of this model to high-throughput screening in anticancer lead-finding has still to be validated. Notwithstanding, this *in vitro* assay is designed to select compounds for secondary and more comprehensive *in vivo* testing to ensure that only a small number of compounds with the best chances of success will make it through to the following, increasingly expensive phases of the drug development process.

There are plenty of reviews which deal with the state-of-the-art methods for detecting anticancer activity in natural products, including, among others, an excellent article by Kinghorn and his team [24, 26–30]. To date, more than 85 000 compounds have been screened using *in vitro* assays [22], and judging by scientific journals, these still seem to be the major tools used for this objective.

Application of Metabolomics in Cancer Research

It is quite obvious that monitoring chemical components of tumor cells can provide a better understanding of the complex biochemical process occurring in a cancer. Malignant cells undergo significant changes in their metabolism including a redistribution of metabolic networks, and these changes can result in a metabolic snapshot which is clearly different from that of normal cells [31]. Metabolomic analysis has been applied in many different fields in cancer research including the detection of biomarkers for diagnosis [32], the monitoring of drug response [33], and treatments or prediction of their toxicity [34]. It should be also strongly emphasized that one of the major benefits of metabolomic analysis is that metabolic profiling can usually be achieved using urine or plasma samples apart from the tumor cell itself, increasing the possibility of carrying out large-scale research in a noninvasive manner [35]. Some of the applications of metabolomic studies of cancer cells or samples obtained from cancer patients are described in the following section.

Diagnosis (Biomarkers)

Biomarkers are widely used in clinical medicine for the purpose of diagnosis or prediction of disease. Finding a new biomarker or, better still, a quantitative biomarker is of great importance in the development of sensitive and specific tests to detect the presence of malignant tumors. NMR-based metabolomics particularly is considered to be a very fast and noninvasive method for the identification of new biomarkers for clinical diagnosis [36]. Indeed, several metabolites have been detected to be metabolic bio-

markers of tumors thanks to the application of an NMR-based metabolomic approach. In breast cancer cells, one of the best applications of metabolomics in cancer diagnosis so far, the level of total choline-containing compounds (tCho) was found to be increased, whilst the levels of glycerophosphocholine and glucose were decreased as compared to benign tumors and healthy tissue [37–39]. This result is well reflected in the study by Yang et al. [40], who showed that the metabolic activity of several pathways was increased in breast cancer epithelial cell lines, leading to the upregulation of fatty acid synthesis among other changes. Similarly to breast cancer, prostate cancer cells exhibit a distinct metabolic profile characterized by high tCho and phosphocholine levels, along with an increase in the glycolytic products lactate and alanine [39,41]. Another good example is provided by the metabolomic study of liver cancer by Yang et al. [42]. They demonstrated that it was possible to distinguish between cancer patients and healthy volunteers, and even more, they were able to distinguish between patients with hepatocirrhosis and hepatitis from patients with liver cancer, with an HPLC-based analysis of urine samples. In this study, several metabolites having *cis*-diol structures together with typical nucleotide markers (pseudouridine, dihydrouridine) proved to be useful for the differentiation of patients' status [42].

All studies above have demonstrated that the level of several metabolites appeared to be important biomarkers (quantitative biomarkers) in the differentiation of diseased and normal conditions. On the other hand, these biomarkers can also be used to monitor the efficacy of a treatment, for instance a decrease in the tCho signal in the $^1\text{H-NMR}$ spectra can be associated to the positive response to chemotherapy or radiation in breast, prostate, and brain tumors. Metabolites which were found to be biomarkers in different tumors are listed in an excellent review on this topic [43].

For a biomarker to be really useful, it is essential for it to be detectable in the early stages of the development of a malignant tumor. In cancer, the early detection is very important since it can greatly increase the chance of total remission. Metabolomic analysis has also proved to be useful for this purpose. Odunsi et al. [44] reported the adoption of a metabolomic approach to discriminate between women with epithelial ovarian cancer (EOC) and healthy controls by analyzing their blood serum samples. $^1\text{H-NMR}$ spectra showed substantial differences between blood samples of the EOC patients and healthy subjects, and the consequent PCA analysis of NMR data showed a clear separation of all sera from the patients with malignant tumors, pre-menopausal women, and patients with benign ovarian disease. This clearly proves that the metabolomic analysis of blood samples has a great potential as a novel strategy for the early detection of epithelial ovarian cancer.

Many of these applications have a weakness consisting in the limited number of samples which were used and there might be sample-to-sample variation. This may prove to be an obstacle for the definition of biomarkers in different tumor types and pathological stages of the tumor. Thus, the first requisite for this application to be generalized is that a sufficient number of samples is analyzed and that the techniques are fully standardized in order to improve the reproducibility of these technologies. Quantitative biomarkers or general pattern recognition of tumor cells or samples from cancer patients would be a good way for diagnosis and prediction of disease.

Toxicity assessment

Metabolomics is a useful tool to monitor early changes in metabolic pathways and therefore be used to predict an anticancer agent's toxicity at an early stage [34]. There are not that many reports on studies made on this aspect in cancer research, but the use of metabolomics in toxicity assessment of drug treatment has been explored opening the possibility for its application to toxicity prediction in cancer therapy. Robertson et al. [45] evaluated the feasibility of applying this technology to screen the toxicity in male rats exposed to toxic agents. Spectra changes were observed shortly after exposure to toxic agents and during the recovery phases. PCA analysis revealed that the metabolites from the samples of all different toxin-treated rats were well clustered in localized regions, which also identified the treated groups but not the control groups. The results demonstrated that this metabolomic approach could readily distinguish the onset and reversal of toxicity with good agreement with clinical biochemistry.

The early diagnosis of toxicity is very necessary in cancer therapy. This is the case of anthracyclines, which are known to have a potential cardiotoxic effect [35]. The toxicity is cumulative, dose-related, and irreversible, thus, to monitor the early signs of cardiotoxicity will give an opportunity of early intervention, allowing attenuation or abrogation of the cardiac dysfunction.

Response to treatment

Perhaps the most important contribution which metabolomics can make to the search for an anticancer lead from natural products is providing the means to monitor the effects of anticancer drugs. Cancer is a highly complex disease and abnormalities appear in several genes, proteins, and biochemical pathways. Thus, monitoring all interconnected cellular networks will provide much more information than the use of single molecular markers. This technology can provide a view of all metabolic changes occurring after exposure to a certain anticancer agent. The resulting data provide a view of perturbations which might occur in a large number of tissues. These results can then be used to decide whether to continue the treatment based on the observation of the effectiveness of the drug and eventually to shed more light on its mechanism of action [46].

The application of $^1\text{H-NMR}$ to the prediction of resistance or response to chemotherapy by a neuroblastoma was reported by Lindskog et al. [33]. Several cytotoxic drugs such as cisplatin, etoposide, and irinotecan were used to treat cell lines with sensitivity to these, and the cells were examined by $^1\text{H-NMR}$ (● Fig. 1). Cytotoxic drug treatment was found to produce an increase in methylene and polyunsaturated fatty acid levels and a decrease in choline levels in drug-sensitive neuroblastoma cells. Methylene/choline ratio correlated with cell death and increased in cisplatin-treated drug sensitive cells but not in drug-resistant cell lines, indicating that this ratio can be used as an indicator of cytotoxic responses. A similar study by Morse et al. [47] was performed on breast tumor models. The treatment with docetaxel, an antimicrotubule agent, decreased phosphocholine (PC) levels significantly and increased glycerophosphocholine (GPC) levels in the cell extracts.

Another interesting application, monitoring P-containing metabolites by ^{31}P NMR, allowed the detection of phospholipid metabolism changes since it allows the quantitative analysis of phosphate metabolites in cancer cells [48]. This provides information concerning key components of energy and phospholipids metabolism. Human colon cancer cells were treated with a novel anti-

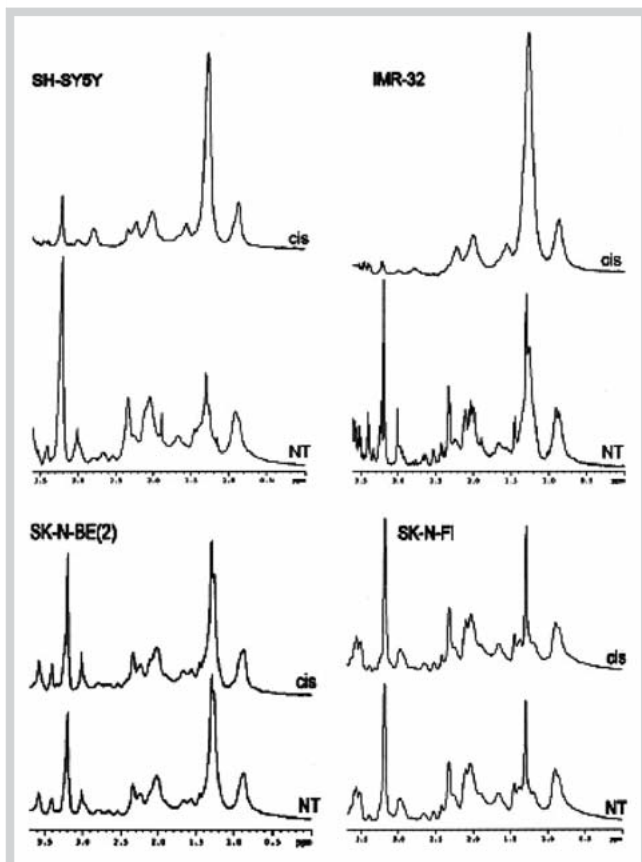


Fig. 1 ^1H -NMR spectra of cytotoxic drug-sensitive and -resistant neuroblastoma cell lines. Four neuroblastoma cell lines [drug-sensitive SH-SY5Y and IMR-32 cell lines, drug-resistant phenotypes SK-N-FI, SKN-BE (2)] incubated without (NT) or with cisplatin (cis, 5 μM) for 48 hours. Adapted from [33].

cancer drug: 17-allylamino,17-demethoxygeldanamycin, and ^{31}P -NMR spectroscopy allowed the screening of surrogate markers of drug activity. The increased phosphomonoesters/phosphodiester ratio was found to correlate with a favorable tumor response, indicating that this ratio can be used as a surrogate marker of response. A similar study was conducted on breast cancer cells by Sterin et al. [49]. Several anticancer agents were tested on the breast cancer cell lines showing different hormonal response and metastatic potential. The ^{31}P NMR spectra of perfused cells revealed a correlation between the mode of action of anticancer drugs and the observed changes in cell metabolic profiles. Briefly, cells treated with antimicrotubule drugs (paclitaxel, vincristine, colchicine) showed a notorious increase in intracellular glycerophosphorylcholine (GPC) while other drugs (methotrexate and doxorubicin) did not affect their levels whatsoever. This study showed that ^{31}P -NMR can be used as a diagnostic tool for decisions about treatment of cancers and a new approach to understand the mechanism of action of anticancer drugs. These studies suggest that tumor metabolic profile changes could be helpful in predicting the response to anticancer treatments faster than current methods (e.g., imaging).

Recently, Chung and Griffiths [50] published a review on the characteristic metabolic profiles of cancer cells by NMR after responding to different anticancer treatments with all kinds of drugs from conventional cytotoxic drugs to novel anticancer

drugs. This may be very useful to obtain an overview of the different effects of drugs on a cancer cell metabolome.

Metabolomics: Tool for Finding Anticancer Activities

Metabolomics has been applied in many fields in natural products including the study of the metabolism and biosynthetic pathways under different conditions [14, 51]. However, so far, its potential in the detection of active compounds (new leads) still remains untapped. Recently several attempts have been made [6, 52–54], and these have shown the feasibility of using metabolomics for this purpose.

Detecting activities in combination with metabolomics can be performed using two different approaches, *in silico* and *in situ*. Using the *in silico* approach a correlation between two different data sets – e.g., metabolomics and bioactivity results – is sought with the help of bioinformatics. On the other hand, the *in situ* approach consists in the monitoring of the metabolic changes of systems, e.g., tumor cells, microorganism cultures, upon the treatment with tested materials (plant extracts or single pure compound).

Even though most applications reported so far [6, 52, 53] do not refer to anticancer drug research, there is no reason not to adapt this method to this purpose. Therefore, the techniques/approaches which have been applied in other fields of natural products lead-finding will be reviewed.

In silico approach – correlation by chemometric methods

A good example of the successful application of this approach can be found in a recently published paper on the metabolomic study of *Galphimia glauca* [54]. *Galphimia glauca* is a Mexican plant which has been used in traditional medicine for the treatment of central nervous disorders. Six different collections of this plant from the whole Mexican area showed different degrees of sedative and anxiolytic activities when tested on animal models. Only two collections were highly active and the others did not show significant activity. Metabolic profiling of six collections was performed using ^1H -NMR and analyzed further by partial least square-discriminant Analysis (PLS-DA) using previous information on their activities (● Fig. 2). Interpretation of the PLS-DA loading plot clearly demonstrated that the signal strongly correlated with the above-mentioned activities, and this signal was identified as corresponding to galphimine (● Fig. 3). When targeted HPLC analysis was performed, it was proven that the two collections possessing strong activities contained a high amount of galphimine while both other (less active) samples did not.

Before this application, Roos et al. [6] had attempted to correlate metabolomes of St. John's wort extract with opioid receptor binding activity. In this study, they showed that NMR combined with multivariate data analysis could effectively predict the activity of preparations, based on a database they constructed. This was suitable for the quality control of preparations made of this plant since it provided a correlation with its efficacy, but they did not attempt to identify the corresponding active compound/s.

In these applications, the use of an appropriate chemometric tool is very critical since it determines the main output from the huge data sets. One of the useful methods to correlate two different data sets (even two different types of “omics” data) is the PLS/OPLS regression method. PLS is a method for relating two data matrices, X and Y, by a linear multivariate model, but goes beyond traditional regression in that it also models the structure of two data

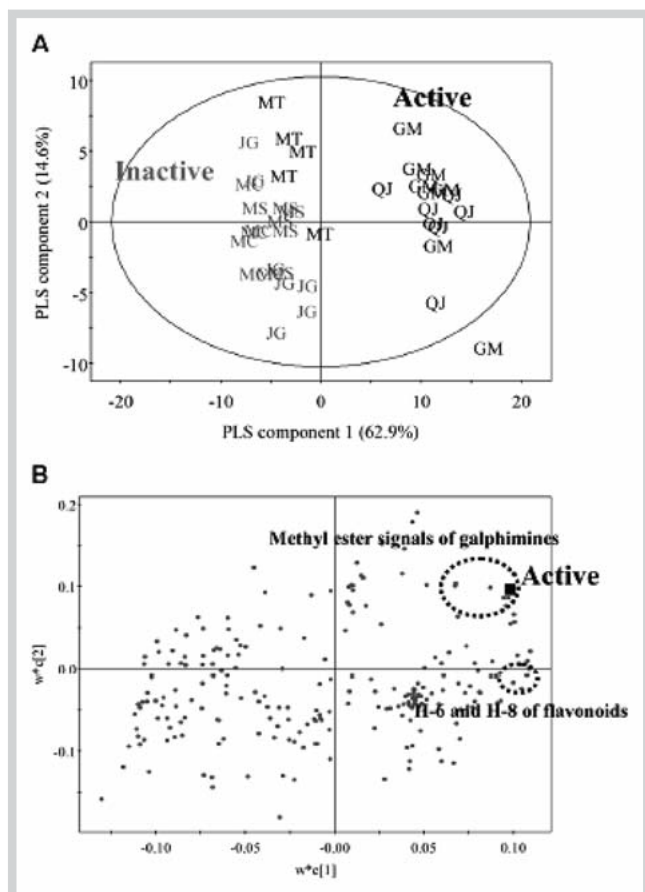


Fig. 2 Score (A) and loading (B) plots of PLS-DA models (PLS component 1 vs. PLS component 2) based on $^1\text{H-NMR}$ resonances and sedative activity of *G. glauca* crude extracts based on two classes, active and inactive plants. Active plant materials include GM, MT, and QJ. Inactive plant materials include MS, MC, and JG. Adapted from [54].

sets. It provides an approach to the quantitative modeling of the often complicated relationships between predictors, X (metabolome), and responses, Y (bioactivity) [55,56].

On the other hand, the OPLS method is designed to separate the variance of the data matrix X according to the variance of the data matrix Y, into three parts: the first part represents the variance that is related to Y, the second part, the interfering systematic variation not related (orthogonal) to Y, and the last part contains some residual variance not interfering with the prediction of Y. OPLS is an extension of PLS and has similar objectives to orthogonal signal correction but is integrated directly in the modeling, which allows an easier validation of orthogonal components [57]. The direct correlation and PLS-based approaches were also adopted by Rantalainen et al. [58] in an integrated analysis of NMR metabolic profiles and 2-D differential in gel electrophoresis (DIGE) proteomic data from a murine cancer xenograft model. Metabolites strongly correlating positively or negatively to proteins can be determined in this way. Other useful methods that can be used for this purpose are well described in the review [57,59].

It has to be noted that in such correlations between activity in *in vivo* assays and the metabolome of extracts, the compounds found to correlate to activity not necessarily are active in itself, they could also be pro-drugs or take part in synergistic activity. This is in fact a major advantage of this approach if compared

with the classical bioassay-guided isolation of active compounds in which such compounds will not be found.

In situ approach – metabolic footprinting/fingerprinting

Numerous different terms have been used for metabolomic analysis, e.g., metabonomic analysis, metabolic profiling, metabolic fingerprinting, and metabolic footprinting [60]. Among these, metabolic footprinting (exometabolomic) is often applied to the measurement of all extracellular metabolites present, for example, in a culture media. The identified compounds are either metabolites which were secreted by the cells into the medium or medium components biochemically transformed by the organism. On the other hand, metabolic fingerprinting measures a large number of intracellular metabolites detected by a selected analytical technique in defined conditions [60]. Due to the fact that the separation of extracellular media is much easier than any other culture media, metabolic footprinting of microorganisms has been amongst the frontier research fields [61]. Even though metabolic footprinting represents only a fraction of the extracellular contents, it has a tight relationship with the intracellular metabolism, thus it can provide important information and a picture of the intracellular metabolic status.

If this technique is to be used to measure the anticancer activity of natural products, the main strategies to study this should be based on the comparison of the metabolic footprint/fingerprint of cancer cells before/after treatment. We have already seen that metabolomic analysis can be used as a tool to monitor the response of anticancer drugs in tumor cells or the patients' response to anticancer treatment. A similar method could be used to monitor activities as their metabolic profiles can be directly compared to those of positive controls. In this way, biomarkers of activity or the pattern of activity could be detected and subsequently could be identified.

This approach has appeared in the work of Zhi Biao-Yi et al. [52], in which the effect of different antibiotics with different modes of action on the microbial metabolomics was studied. Their results allowed them to conclude that dihydrocurcurbitacin F-25-O-acetate, a major constituent of the Chinese plant *Hemsleya pengxianensis*, showed antimicrobial activity. The metabolome of a *Staphylococcus aureus* culture treated with a plant extract, dihydrocurcurbitacin F-25-O-acetate, and several known antibiotics (streptomycin, vancomycin, erythromycin, rifampicin, etc.) were compared. PCA analysis revealed that dihydrocurcurbitacin F-25-O-acetate was the component responsible for the main antimicrobial activity on *Staphylococcus aureus* in *H. pengxianensis* through its ability to inhibit cell wall synthesis, as in the case of vancomycin (● Fig. 4).

Similar studies aimed at seeking to discover the mode of action behind antibacterial [53], antifungal [62], and anti-inflammatory activities have been published [63].

One of the advantages of this approach is that it can also be used to predict the mode of actions and not only to select active samples. In order to find an anticancer agent, it is essential that the metabolic footprint/fingerprint of the target cancer cells is analyzed first so that the metabolic footprint/fingerprint of the same cancer cells after treatment with plant extracts can be compared to detect changes. In NMR-based metabolomic analysis, metadata of two different measurements can be easily compared to find changes if existent. One of the possible ways to achieve this is to subtract the obtained spectra, thus revealing the signals that have changed. For this purpose, HSQC (heteronuclear single quantum coherence) spectroscopy is very useful. In this case, as

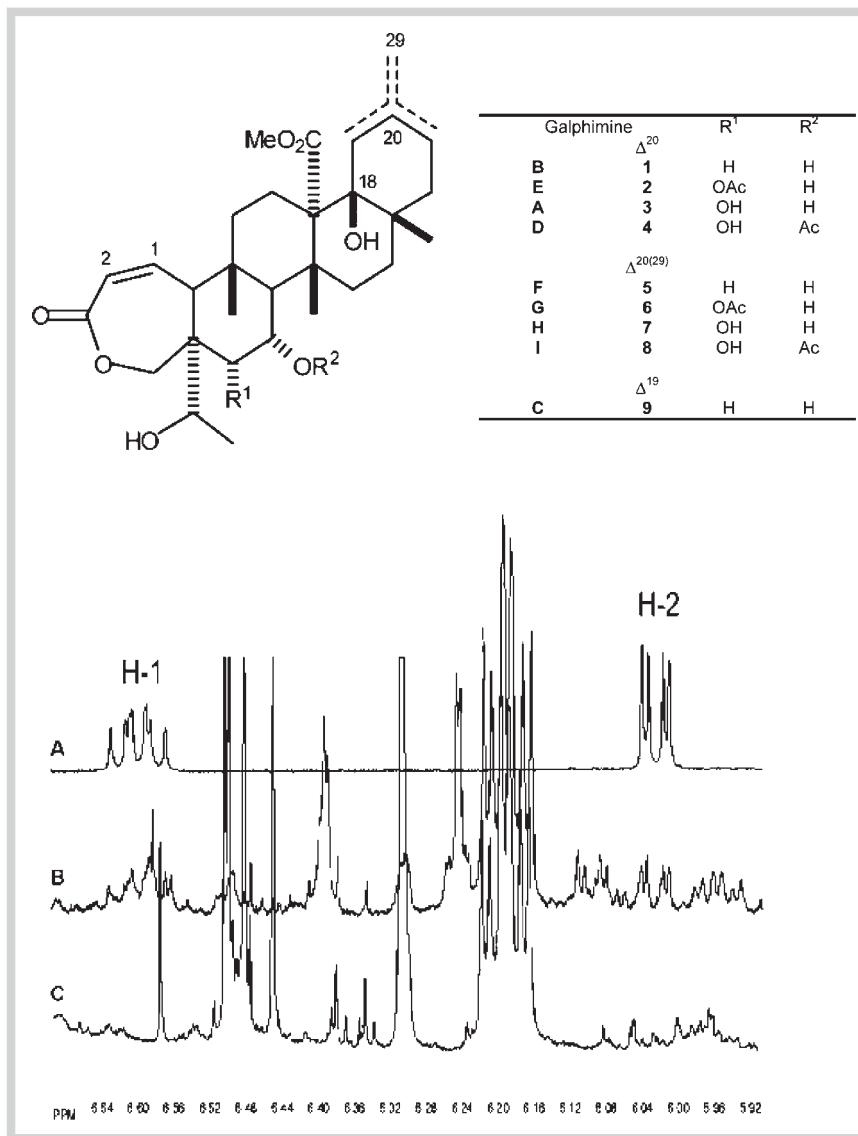


Fig. 3 Structure of galphimine series and $^1\text{H-NMR}$ spectra of: **A** resonances for vinylic protons H-1 and H-2 of an HPLC peak containing the diastereomeric pair of galphimines B and F, **B** an MeOH crude extract from GM sample (active) showing the resonances for H-1 ($\delta = 6.55\text{--}6.63$) and H-2 ($\delta = 6.00\text{--}6.05$) for the galphimine series and **C** a MeOH crude extract from one individual of JG sample (inactive) without galphimines. Adapted from [54].

the ^{13}C chemical shift is relatively insensitive to the change of pH and concentration of samples, it is much more reliable and reproducible to use this rather than $^1\text{H-NMR}$ [64]. Recently this technique was used for the comparison of two different species of *Ilex* and revealed that the signals of saponins constituted the main difference between the species (● Fig. 5) [65].

In addition, HSQC can be used for a quick screen of the presence of certain metabolites within an extract (a mixture) by comparison of the HSQC spectra of mixtures of known reference compounds [66].

To increase the efficiency of activity screening, the material to be tested from the plant also has to be defined. Usually, the screening material – plant extracts or fractions of extracts – were used for initial testing. In conventional extraction methods, plants or parts of plants are extracted with organic solvent/s, and dried extracts are subjected to screening. Depending on the properties of extraction solvent, the metabolome of the extract will vary, and consequently its activity will surely also differ. It is even possible that some methods may not extract the active metabolites efficiently resulting in low concentrations which may not be sufficient to produce any desirable cellular effect. To improve the potential of finding active compounds and at the same time to reduce elaborated fractionation steps, alternative fast extraction

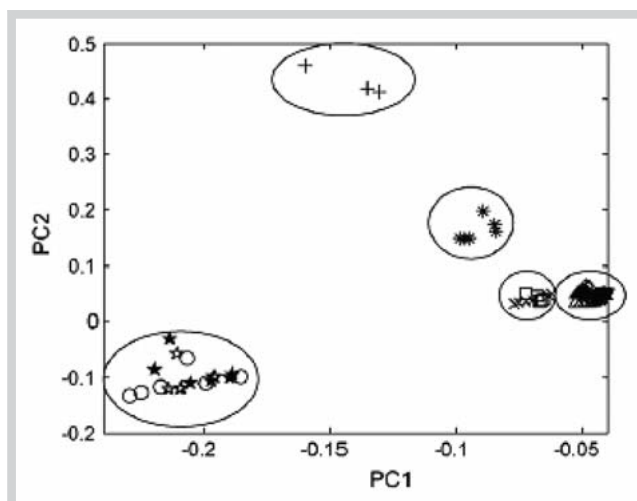


Fig. 4 PCA scatter plot of HPLC profile of controls and cultures treated with different drugs and extract of *Hemsleya pengxianensis* W.J. Chang [controls (+); norfloxacin (×); acheomycin (◇); linclozolin (▲); cefataxime (*); vancomycin (○); rifampicin (□); erythromycin (Δ); chloromycetin (▽); streptomycin (▼); dihydrocucurbitacin F-25-O-acetate (★); extract of *Hemsleya pengxianensis* (✱)]. Adapted from [52].

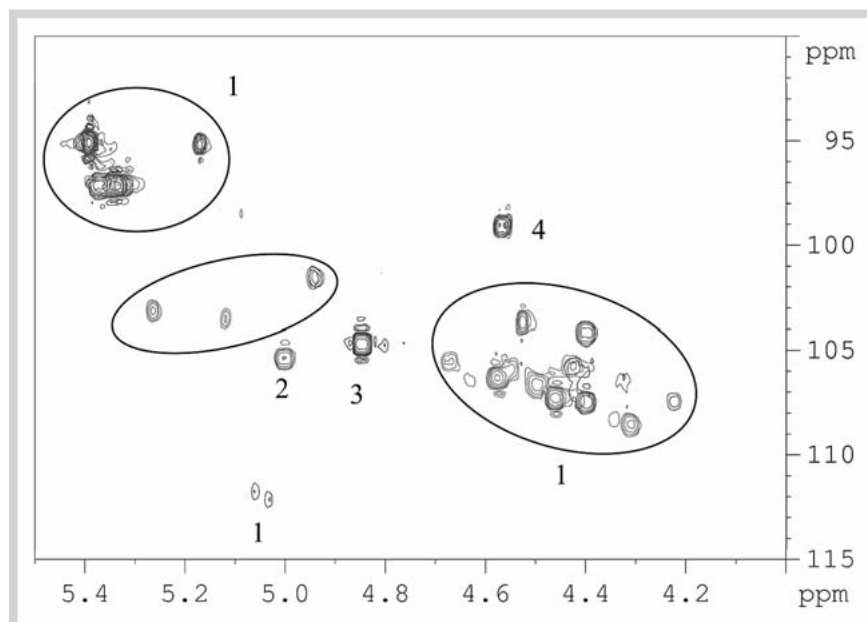


Fig. 5 HSQC spectra of *I. brevicuspis* (black) and *I. dumosa* var. *guaranina* (gray) in the range of δ 4.0 – δ 5.5 of ^1H and δ 90 – δ 115 of ^{13}C for HSQC.

1: HSQC correlation between anomeric protons and carbons of sugars in saponins, 2: HSQC correlation between glucosyl H-1 and C-1 rutin, 3: HSQC correlation between H-1 and C-1 of arbutin, 4: HSQC correlation between rhamnosyl H-1 and C-1 of rutin. Adapted from [65].

methods should also be considered. This can be accomplished by combining different solvents in one system, applying gradients of polarity and extracting them consecutively in an on-line system. This approach has been developed in our lab and has proved to be very successful allowing the extraction of metabolites ranging from very nonpolar to polar compounds from one single plant sample (unpublished results).

Perspectives

Metabolomics is a very promising tool to use for the detection of activities from natural products as it has been used in many similar fields, allowing the accumulation of vast experience. What should not be underestimated from the start, is the importance of the interpretation of the biological information revealed by this powerful technique. Therefore, the first requirement is that the obtained data are reliable and reproducible. As many researchers have mentioned, drawing conclusions on the basis of small experiments is very risky [46]. To obtain reliable information from an experiment, a sufficient number of samples have to be analyzed. Furthermore, the communication with metadata obtained from other experiments or groups is only possible if the firm standardization of all processes, from the sample preparation to the measurement and data analysis, needs to be done beforehand. At the same time, if all data have been measured under the same conditions, particularly NMR data can be stored forever and whenever new data is generated, this can be included for use in a new data analysis.

Another important aspect to consider is the assay system required for this approach which has to be fast and simple and suitable to be applied to high-throughput screening. An NMR-based technique – but also other techniques which are used in the metabolomic field – has a great potential for a high-throughput application. Some obstacles are still present, namely the need for fast metabolite identification. The improvements made to NMR sensitivity tend to make fast identification feasible, particularly in mixtures. On the other hand, the existence of public databases of metabolites will again contribute to their identification.

Lastly, whichever compound determined in principle to be active will still have to be submitted to testing on a more solid model system in order to confirm this activity. However, this feature can improve the efficiency and provide shortcuts in the bioassay guided lead-finding research from natural products, thus recovering this prolific source of potential anticancer drugs. Moreover, it can offer a chance to reveal the complex mechanism and synergism of metabolites.

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