Title: The Pharmaceutical Industry and Natural Products: Historical Status and New Trends

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Abstract: Owing to the high diversity of terrestrial and marine organisms, natural products (NPs) (secondary metabolites) are some of the most successful source of drug leads for the treatment of many diseases and illnesses. In the 1990s, advancements in automation (High-Throughput Screening) and isolation technologies resulted in the surge in research on natural products both in the field of human health and agriculture. These strategies and techniques generated a substantial shift towards this *'green Eldorado,'* a real *'Green Rush'* between 1990 and 2000. However, in the early 2000s most of the big Pharmas terminated their HTS and bioprospecting endeavours but to date, the low productivity of combichem and rational drug design is silently positioning pharmacognosy back on the rails and natural product discovery is remerging as a reputable source of current drugs on the market.

Meanwhile, the World Health Organization (WHO) has come to the realisation of the importance of biodiversity which would be able to offer affordable, therapeutic solutions to the majority of the world population. The preservation of the world's biodiversity and its access is a critical issue which could hamper a serene utilisation of natural products in the developing world with herbal-based phytopharmaceuticals representing a significant share of the total world pharmaceutical market. This review presents an industrial perspective discussing natural product drug discovery, lead research, botanicals, pro-drugs, synergy effects, drugs interactions with botanicals, traditional medicines, reverse pharmacognosy and presents the difficulties in accessing biodiversity.

Keywords: Drug discovery, high-throughput screening (HTS), biodiversity, pharmaceutical industry, Access and Benefit Sharing.

Introduction

Natural products (NPs), commonly referred to as '*secondary metabolites*' (the end-products of gene-expression) are an essential, reputable source of successful drug leads which originate from Earth's bio-diverse flora and fauna. Since more than 95% of the world's biodiversity has not been evaluated (known biodiversity is estimated at 2 million species of plants, animals, fungi and micro-organisms and alike) for any biological activity, the challenge is how to efficiently and effectively access and valorise this natural chemical diversity (Colegate and Molyneux; Dewick 2009; Mishra and Tiwari 2011). Undoubtedly, natural products have been produced, as a result of millions of years of evolution of terrestrial and marine organisms adapting to various abiotic and biotic stresses. They are therefore encoded to be bioactive, for ages they have been used as medicines and today, they continue to be a reservoir of potential drugs (Lamottke et al. 2011).

The oldest records for the usage of medicinal plants dates back to 2400 B.C. on clay tablets (Mesopotamia), (Attinger 2008) 1534 B.C.; the Ebers Papyrus (9th year of Amenhotep 1 reign), and the Chinese Materia Medica, document written by Li Shizhen in 1578 (Zheng 1988). From, ~300 B.C. (Theophrastus) who dealt with medicinal herbs to the isolation of morphine around 1804 by Sertürner (Sertürner 1817), NPs have been the forefront of medicine to treat human disease (Dias et al. 2012). With the advancements in the field of chemistry at the dawn of the 19th century, plants were examined vigilantly to fathom their therapeutic potential (Beutler 2009). Historically, apothecaries and then pharmaceutical companies utilized plant extracts to produce relatively crude therapeutic formulations. In the mid-20th century, drug formulations of partly purified NPs became typical

prior to single molecule medicines (Mishra and Tiwari 2011). Following the discovery of the well-known antibiotic, penicillin (**1**) (Figure 1), many drug breakthroughs from microbial sources occurred and with the advances in diving techniques (improved SCUBA technologies) in the 1970s, subsequently opened the ocean as an overlooked source of NPs (Blunt et al. 2011). Chemical synthesis, shifted the focus of drug discovery efforts from nature to the laboratory bench in the late 1980s (Cragg and Newman 2013). Of the 1135 new drugs approved from 1981-2010, 50% were of NP origin (natural, derivatives and analogues) (Cragg 2007; Schmitt et al. 2011; Newman and Cragg 2012) with most of the chemical diversity nearly or completely absent from current small molecule-based screening libraries provided by combichem (Bauer et al. 2010). Well known examples include the widely used breast cancer drug, paclitaxel (**2**) (Taxol®), isolated from the bark of the Pacific Yew, *Taxus brevifolia* (Dewick 2009) and trabectedin (**3**) (Yondelis®) isolated from the sea squirt, *Ecteinascidia turbinata* (currently completing Phase III studies in the US) (Cuevas and Francesch 2009; Cragg and Newman 2013) which provided the first marine anticancer drug to be approved in Europe after cytarabine (**4**) (1969) (Mayer et al. 2010). Mevastatin (**5**) produced by *Penicillium citrinum* led to synthetic statins exemplified by atorvastatin (**6**) which is the best-selling blockbuster drug in Pharma history. Statins which lower cholesterol levels are frequently used as *"everyday medication"* (Verpoorte et al. 2005) and in some countries there is a shift towards purchasing this kind of medicine without prescription. Several other NPs or NP-derived drugs including ziconotide (**7**) (conopeptide), exenatide (**8**) (oligopeptide) and ixabepilone (**9**) (epothilone derivative) are other examples of current FDA approved drugs (Data available at http://www.accessdata.fda.gov). In this review we wish to discuss new trends, the future of natural products (as single-molecule entities and botanical extracts) from a Pharmaceutical Industry perspective.

Cragg and Newman have extensively reviewed NPs, semi-synthetic NPs and nature inspired molecules which are currently approved by the Food and Drug Administration (FDA) (Cragg et al. 1997; Newman et al. 2003; Newman and Cragg 2007; Newman 2008; Cragg and Newman 2013). In a recent review, Newman and Cragg have shown that 34 % of current drugs (where *N* = unmodified NP, 6 %; *NB* = a NP botanical and *ND* = a modified NP, 28 %) are NP inspired or derived. Sixty-six percent (where *S*/N* = a synthetic compound with a NP pharmacophore, 11 %; *S** = a synthetic compound with a NP pharmacophore, 5 %; *S/NM* = a synthetic compound showing competitive inhibition of the natural product substrate, 14 % and *S* = a synthetic compound with no natural product conception, 36 %) of NPs, NP inspired in the form of semi-synthetic/modified drug are the basis of current drugs on the market (Cragg and Newman 2013). In a study by Koehn and co-authors, they examined the worldwide patent trends between 1984–2003 in NP discovery (Koehn and Carter 2005).

According to the authors statistics, there was a period of increasing patent activity through the 1980s (as the investigation of NPs as sources of drugs reached its peak in the Western pharmaceutical industry), a slight decline from 1990 to 1999 and an increase in activity between 2000–2003 (Koehn and Carter 2005). Certainly, there are numerous NPs that have been patented however patenting would be required prior to publication and potential usage.

[Insert Figure 1 Here]

NP research at the industry level

Worldwide pharmaceutical R&D spending, increased from US $10 billion to US $30 billion over the same period, however the overall trend in the 1990s showed a gradual decline (Koehn and Carter 2005). In a study by Butler and co-workers they documented that 34 NP-based drugs were launched between 1998-2007, (Butler 2005; McWilliams 2006) with 6 of them based on lead compounds from plants or marine macro-organisms. Thirty-six plant-derived compounds and 10 marine-derived compounds were in oncology clinical trials which were derivatives of 31 different lead NPs. However, in the last 25 years, of the 877 novel medicines developed between 1981 and 2002, 6 % were NPs and 27 % were NP derivatives indicating that they play an important source of novel leads for the production of therapeutic drugs (Newman et al. 2003; Yuliana et al. 2011). A different set of statistics was provided by Harvey and co-authors, which include data on drugs based on NPs from preclinical development through to pre-registration. One-hundred and eight plant-derived compounds, 24 animal (primarily marine) compounds, and 61 semisynthetic compounds out of 225 NPs were in development (Harvey 2008; Kingston 2011). A total of 26 plant-based drugs were approved/launched during 2000–2006 (Saklani and Kutty 2008). Even though there are numerous examples of NPs that have reached the market, NP extracts (botanicals or phyto-pharmaceuticals) also play an essential role in therapy (examples will be discussed later in this review). The current market is expanding as we are now seeking natural, traditional medicines which are available at relative low costs (Lawson 2013). Botanical therapeutics can be sold in the form of dietary supplements, drugs, or botanical drugs (Schmidt et al. 2008) and can eventually hold the status of current registered pharmaceuticals through regulatory offices such as the FDA if they surpass clinical trials and demonstrate efficacy and safety.

Why did many of Big Pharma's terminate their natural product programs?

There are evidences to suggest that there has been a significant decrease in the number of approved drugs by the FDA over the last 20 years (originally 45 approved in 1990 to 21 in 2010) (Kingston 2011). To date, pharmaceutical companies are under scrutiny due to the gradual decline and pressure to increase the number of new drugs on the market, and as a result in the last decade, many pharmaceutical companies have abandoned their natural product drug discovery programs (Table 1) (Dickson and Gagnon 2004; McChesney et al. 2007). Reasons for this included: lead compounds are available only in extremely small quantities, difficulties in sourcing/harvesting samples, extensive synthetic routes and development times resulting in poor yields, impracticality of scale-up, difficulties in the isolation and/or purification procedures, high toxicity of the active compound, ecological and legal considerations, government policies, lack of infrastructure and insufficient capital investment (Paterson and Anderson 2005; Bhatnagar and Se-Kwon 2010; Lamottke et al. 2011; Thomas and Johannes 2011). Companies including, Bristol Myers Squibb, Merck, Johnson & Johnson, Pfizer, GlaxoSmithKline gradually terminated or no longer maintained their screening programs whilst others, such as Novartis have continued their programs. Most NP discovery programs are confined to academic research within universities and start-ups (Ortholand and Ganesan 2004; Beutler 2009) mainly focusing on microorganisms (Sheridan 2012). The list of such companies is quoted by Sheridan.

Though there are some well-known examples of NP derived compounds which have made it through the arduous drug discovery process it is important to mention those that have failed. Rifalazil (**10**) was evaluated by ActivBiotics, and failed in a Phase III trial for the treatment of the intermittent claudication associated with peripheral arterial disease (Mishra and Tiwari 2011). Contulakin G (**11**) (marine cone snail) was an orphan drug designated lead of Cognetix that had completed Phase Ib clinical trials against chronic and intractable pains but was placed on hold until further funding was made available (Mishra and Tiwari 2011). In January 2005, Viprinex®, a defibrinogenating agent extracted from the Malayan pit viper venom (used for many years in Germany as for deep vein thrombosis and embolism) was fast-tracked for the treatment of acute ischemic stroke. However, Neurobiological Technologies evaluated Viprinex® in Phase III clinical trials and it failed to show benefits in patients suffering from acute ischemic stroke; in January 2009 the Viprinex® program was terminated. Almost half of the existing pharmaceuticals today are inspired by NPs and in our opinion, this trend will continue with a substantial amount of NP derived (leads or extracts) which will successfully reach the market in the future (Lamottke et al. 2011). Miller an co-authors stated, that if it is assumed that 60,000 species of plants have been screened to yield the 135 known drugs (at the time), then the 240,000-290,000 (or even a

higher figure, to date) the remaining plant species could be expected to yield 540 to 653 new drug candidates (Farnsworth 1990; Newman et al. 2003). In reality, though 60,000 may have been included in screening programs, most were evaluated against a limited number of disease targets, so many of the 60,000 plant species may still have some chance of yielding additional discoveries (Miller 2011). In addition to their role as drugs, NPs are frequently used as molecular probes to identify disease relevant targets and this aspect also justifies pursuing NP research at the industry level (Schmitt et al. 2011).

Current status of natural product (NP) research

Botanicals (Medicinal Plants and Herbs) – Potential sources of medicine

Herbal phytopharmaceuticals which have reached US $60 billion, with annual growth rates of 5% to 15% represent a significant share of the total world pharmaceutical market (Naoghare and Song 2010). The current increase may be due to the interest of phytopharmaceuticals in psychosomatic, metabolic and minor disorders. To some people, synthetic drugs cause harmful side effects and are expensive to purchase in comparison with traditional herbal products, even if "natural" is not always correlated with "harmlessness." They have been widely used as medicines, dietary products, and nutritional supplements since ancient times. Herbal medicines are rich in bioactives that are beneficial for human health. Modern and herbal medicines are not actually divergent to one another since more than 50% of currently marketed drugs are more or less derived from Earth's biodiversity (Newman and Cragg 2012; Cragg and Newman 2013). Medicinal plants or herbs thereof are essential for more than 70% of the world's population that do not have access to Western medicine, therefore traditional medicine is highly recommended by the WHO. This was endorsed in the 2008 'Beijing declaration' http://www.who.int/medicines/areas/traditional/congress/beijing_declaration/en. Traditional medicine is particularly well suited to local conditions and represents an initiative for the future of health in developing countries even if the vast majority of physicians have limited understanding of the bioactive molecules present in the extracts. Some countries like China, India, Germany still teach phytotherapy in medical schools and practice herbal medicine. Botanicals could also be a solution for industrialised countries facing dramatic increases in health costs due to *'single molecule'* medicine. It is estimated that around 140,000+ Australians are admitted to hospital every year because of problems associated with iatrogenic side effects of Western single molecule medicines (Mackay 1998). Natural products found in medicinal plants can efficiently mitigate the side effects of serious illnesses, for example, plants can alleviate the effects of onco-chemotherapy or radiotherapy.

Essential oils also represent alternatives to antibiotics due to its resistance properties and are alternatives to expensive treatments with unfavourable risk, benefits and cost. For example, recently in Brazil a new anti-inflammatory phytopharmaceutical was developed based on an extract from the leaves of *Cordia verbenaceous* standardised in -humulene (**12**) (Acheflan®) (Matias et al. 2013) and is now supplanting established synthetic anti-inflammatory drugs on the local market. This product is based on the traditional usage of this Brazilian medicinal plant utilising an evidence-based phytotherapy approach. There are currently several FDA approved botanicals available in the global market including Veregen® (**13**) (Tea catechins) for the treatment of external genital and perianal warts (Chen et al. 2008) and Fulyzaq® (**14**) (extract from the red sap of the *Croton lechleri* for the treatment of HIV diarrhoea. Sativex® a titrated extract containing -9-tetrahydrocannabinol (**15**) (psychoactive) and cannabidiol (**16**) (anti-inflammatory) has been approved since 2005 in many countries (eg. Canada, The United Kingdom, Germany and New Zealand). This botanical prescription drug is an oromucosal spray cannabinoid medicine for the treatment of spasticity due to multiple sclerosis and neuropathic breads of various origins. Marinol® (**17**) (dronabinol) and Cesamet® (**18**) (nabilone) are on the North-American market for the treatment of vomiting and nausea associated with the chemotherapy of cancers. More recently, in 2012 the Dutch Medicines Evaluation Board approved a dry extract of *Dioscorea nipponica*, a traditional Chinese botanical to relief headache, muscle pain and cramps (Gilbert 2012). This was the first time that a TCM product (Traditional Chinese Medicine) was introduced in a European Union country. The inclusion of traditional medicines in the development of 21st century treatment paradigms can facilitate economical accessibility, convenience and acceptability.

Single-molecule NPs and medicinal extracts

As mentioned, plants have been the inspiration for an important number of current drugs today on the market. Though, part of the drug discovery process is based on *serendipity*, important discoveries have been initiated on the traditional usage of medicinal plants and subsequent isolation of their bioactive constituents. Substantial synergy and benefits for the development of improved medicines and new drugs can arise from linking these powerful analytics to robust ethno-medicinal and ethno-botanical studies of traditional medicines (Ngo et al. 2013). Evidence on the traditional usage of medicinal plants still represents a source for drug discovery and this knowledge and its implication in drug development are today well defined in the framework of biodiversity laws even if they are not always easy to implement.

The usage, most importantly the efficacy and safety of traditional medicines can be validated by clinical studies formulated according to the traditional preparation or on standardized extracts. From a pharmacological viewpoint, in many cases, the clinical efficacy of a given plant is not always explainable by the presence of a single active NP. In essence, the usage of traditional preparations (eg. tea, decoction, tincture) corresponds to the intake of highly complex mixtures of NPs that potentially may have mode of actions on multiple targets (Gertsch 2011). This becomes complicated when the preparations consist of a mixture of varying herbs as in the case of Traditional Chinese medicine (TCM) (Buriani et al. 2012). In the perspective of the drug discovery process, the development of drug leads ensures the beneficial effects of such herbal preparations to be valuable from a global health perspective. The pharmerging markets (Pharmergings are defined as "*emerging countries in the pharmaceutical world market"*) will double their expenditure on pharmaceuticals, growing to $150-165 Bn by 2016, and driven by rising incomes, continued low cost for drugs, and government sponsored programs will aim to increase access to medicines in developing countries (Kleinrock 2014). Such observations have led to the development of drugs that instead of being pure NPs, are actually plant-based extracts with defined composition. Such NP extracts are often referred to as phytopharmaceuticals (EU) or botanicals (USA) (Chen et al. 2008; Hoffman and Kishter 2013). Their status varies from country to country based on the health claims which are made, and can be registered either in the form of dietary supplements or as drugs *per se* if clinical studies are performed and registration is approved. As previously mentioned, a recent example is the approval of Veregen® (an enriched extract of tea polyphenols), for the treatment of genital warts linked to human papilloma viruses (HPV) (Scheinfeld 2008). The same extract is currently under clinical trials against various cancers as both a preventative and as a direct agent (Newman and Cragg 2012).

Formulating NP extracts is crucial in a pharmaceutical perspective for the cure, treatment or prevention of diseases (botanicals) but also from a food industry viewpoint in obtaining positive health profiles (eg. nutraceutical functional food) (Wolfender et al. 2011). The notable concerns, especially when dealing with plant extracts is the presence of potential pesticides and heavy metals as per the requirements of the Good Agricultural Practices (GAP) (Zhang et al. 2010). Toxic constituents (eg. hepatotoxic compounds such as pyrrolizidine alkaloids or aristolochic acids) (Stickel et al. 2005; Chen et al. 2012) and the risks associated with additional drug interactions must be identified beforehand prior to human administration. Plants that can be used in such preparations should be Generally Recognized As Safe (GRAS), a classification that is recognized by regulation authorities such as the American Food and Drug Administration (FDA), the European Medicines Agency (EMA) or European Food and Safety Authority (EFSA) (Nicoletti 2012). In order to safeguard a

continuous and approvable therapeutic effect the chemical composition of such multifaceted plant-based NP extracts has to be authenticated. Standardization procedures currently exist and are based on the quantification of the active principle(s) (when they are known), the detection of chemical marker(s) for assessing the correct botanical origin of the plant material, the acquisition of the complete metabolite profile (metabolome) and a comprehensive estimation of the biological variability of the extracts (van der Kooy et al. 2009). In addition, depending on the nature of the plant material used, various analytical validations have to be performed to ensure the absence of toxic or allergenic compounds (Ribnicky et al. 2008). These procedures ensure the quality of the botanical in terms of its composition and safety for human consumption. Unlike a pure compound the pharmacological mode of action of such a multicomponent mixture is far more complicated to ascertain and takes into account the bioavailability of the constituents, the presence of pro-drugs and the likelihood of synergetic effects (Wagner and Ulrich-Merzenich 2009).

Pro-drugs in natural product extracts

In plants many NPs exist in the form of conjugates with sugar moieties (called glycosides). In this way, plants store key secondary metabolites which are often involved in defense. These processes have been optimized though evolutionary processes as many of the glycosides are activated upon cell disruption to yield highly active defense compounds (*eg.* glucosinolates and cyanogenetic glycosides) (Bruneton 2009). Frequently, the glycosides are not active directly on therapeutic targets however they can become bioactively efficient upon metabolization. The glycosides are not active directly on therapeutic targets only when they are subjected to an enzymatic process activating the pro-drug. Another example includes laxative herbs (*eg.* Aloe) anthrones which are present in the form of *C*-glycosides that reach the intestine intact, and are hydrolyzed in the intestine in a reductive environment to reach their target in the form of anthrones (Bruneton 2009). Plant extracts may thus contain bioactive NPs in the form of pro-drugs and in some cases these compounds can provide optimized derivatives for reaching therapeutic targets. In several cases, NPs *per se* have surpassed evolution to be active in ecological interactions and have been optimized for human usage, this explains why an important proportion of NPs are required to be chemically modified for optimal efficacy with reduced toxicological effects (Newman and Cragg 2012). In addition, chemical modifications of NPs can result in the enhancement of the biological activity (based on an established pharmacophore), leading to a new entity. It is important to mention that the structure of a “new” natural product cannot be patented *per se*, only the extraction/purification processes and/or the use and/or its application. In other words a patent office will grant a patent, on a chemical structure which

was naturally pre-existing in nature only through the extraction process or industrial application which have to be truly inventive (Anonymous 1973).

Synergy: The "totum" effect!

The longstanding, successful usage of herbal drug combinations in traditional medicine makes it necessary to discover a rationale for the pharmacological and therapeutic superiority of many NPs in comparison to isolated single molecules (Wagner and Ulrich-Merzenich 2009). In this respect, due to the concomitant presence of the bioactive NPs in natural product extracts, synergistic effects are likely to occur. These properties have been disputed claiming superior pharmacological effects of mixtures of compounds in botanicals over mono-substances. Such effects, as well as poly-pharmacology (a given molecule binds to different targets) (Gertsch 2011) are however difficult to prove experimentally. Synergistic effects, can result in the following: *i)* the constituents of a NP extract affects different targets *ii)* they can interact with one another to improve the solubility and thereby enhancing the bioavailability of one or several substances of an NP extract and *iii)* compounds may also have their efficacy enhanced with agents that antagonize mechanisms of resistance (Wagner and Ulrich-Merzenich 2009).

The verification of a given synergistic effect can be achieved by comparing the pharmacological effects of the mono-substances versus the combination of substances by analyzing isobole curves based on data from several dose combinations. The analysis of such curves enables for the discrimination between simple additive effects, antagonistic interactions or real synergism with potentiated or over-additive effects (Berenbaum 1989; Yang et al. 2013). Synergistic effects have been studied, for example, the combination of ginkgolide A (**19**) and B (**20**), two compounds known to have anti-PAF effects in phytopharmaceutical preparations of *Ginkgo biloba*. The study of the isobole curves with different doses of these compounds demonstrated clear synergetic effects (Wagner 2005). Many of these types of studies as well as multi-target effects [summarized in reference (Wagner 2011)] has presented evidence for the therapeutic superiority of plant extracts over single isolated constituents, as well as their bioequivalence with synthetic chemotherapeutics. Despite evolutionary clues to molecular synergism in nature, sound experimental data is lacking and new concepts such as poly-pharmacology and network pharmacology are emerging in the context of the pharmacology of botanical drugs (Gertsch 2011). Depending on the molecular machinery, synergisms can be produced by differential (eg. allosteric) ligand interactions in one single protein or downstream effects. Different drugs may act synergistically simply by partially inhibiting different nodes in a given biological network that leads to gene expression (Gertsch 2011).

Poly-pharmacology and synergisms are creating the next paradigm in NP drug discovery. Complex aspects (multi-component mixtures acting in complex biological systems) may be tackled by emerging systems biology approaches (Wang et al. 2005; Fitzgerald et al. 2006; Verpoorte 2012). In particular, *"omics"* approaches have been used recently, more extensively for the study of TCMs (Buriani et al. 2012). For example, NMR based metabolomics has been applied to the study of human biological responses to chamomile tea ingestion. The strategy enabled the characterization of the metabolic effects of chamomile ingestion despite the high degree of variation from genetic and environmental sources (Wang et al. 2005). This study highlighted markers related to the ingestion of herbs and demonstrated the potential of such an approach in this emerging field. To date, and despite the rapid development of systems biology only a few studies have been published, but comprehensive approaches that combine phytoprofiling and metabotyping are now emerging (Xie et al. 2013). From a clinical viewpoint, systems biology approaches guided by Chinese medicine have revealed new markers for sub-typing rheumatoid arthritis patients (van Wietmarschen et al. 2009) and similar strategies have also been applied for studying effects of TCM for complex diseases. Altogether, such holistic approaches might provide evidence of efficacy of personalized medicine, which is intrinsically linked to the usage of traditional medicine by healers (Verpoorte et al. 2005), such studies might ultimately lead to evidence-based phytotherapy and provide a means to differentiate placebo effects from existing pharmacological efficacy.

The interaction of drugs with botanical extracts

While extracts and multicomponent mixtures are often argued to have higher efficacy than single constituents in the context of phytotherapy, the alternative is that the ingestion of these complex mixtures have greater probability to lead to drug-drug interactions, and in this context many herbs or botanicals are at risk (Posadzki et al. 2013). For example, phyto-preparations containing *Hypericum perforatum* (Saint John's Wort) have demonstrated to cause multiple drug interactions through induction of the cytochrome P450 enzyme CYP3A4 with drugs such as cyclosporin, indinavir, simvastatin, fexofenadine, and digoxin (Hammerness et al. 2003). The metabolism of these drugs increases, resulting in a decrease in the concentration and thus has negatively impacted clinical effects. In this case, the principal constituents thought to be responsible is hyperforin (**21**) which is the bioactive NP partly responsible for the pharmacological *in vitro* effects that have explained the anti-depressant activity of the NP extract (Muller et al. 2001). In the *in vitro* pharmacological assays, the extract was found to be more active than hyperforin (**21**) alone, and some companies have marketed Saint John's Wort

extracts with low amount of hyperforin (**21**) to reduce such interactions whilst still claiming good clinical efficacy (Woelk 2000).

From clinical trials on traditional medicines to bioactive natural products and lead compounds (Reverse pharmacognosy)

In the field of drug discovery all information related to traditional medicine, clinical trials on NP extracts are potential sources for finding new targets by utilizing reverse pharmacology methodologies or explaining the mode of action of specific botanicals. Reverse pharmacology also known as *"target based drug discovery"* became popular after the sequencing of the human genome which allowed for the rapid cloning and synthesis of large quantities of purified proteins. In this context *"Bedside to Bench"* approaches in combinations with systems biology may also be of great interest and the application of a combination of ethno-pharmacological know-how with modern *in silico* tools may lead to the discovery of new NPs (Rollinger et al. 2006; Rollinger 2011). Gene differential expression technologies in mechanistic studies of NP-derived drugs have also shown to be of significant potential (Chen and Jiang 2012). At this level, systems biology approaches (Verpoorte et al. 2005; Verpoorte et al. 2009) and *in silico* tools may assist in the drug discovery process of the studies aimed at explaining the mode of action of traditional preparations. For example, clinical trials on GRAS botanicals with established traditional usage and well-defined composition may be conducted to demonstrate evidence of efficacy. Systems biology studies on well-defined cohorts should reveal biomarkers associated to the therapeutic effects. Furthermore, target discovery approaches may be conducted to understand the mode of action of NP extracts and the information of the active principles of the extracts may be retrieved by *in vitro* assays. Another approach is to use *"virtual screening"* software which uses existing libraries of compounds to test panels of targets (eg. antitumor) with the aim of identifying selectivity and specific pharmacological activities (Lauro et al. 2012). Ultimately, this strategy should guide the drug discovery process in finding or developing new NPs as monosubstances or formulate phytoextracts possessing ideal chemical profiles.

Current methodologies for assessing biologically active natural products

Complexity of natural extracts and lead identification

Unlike classical medicinal or combinatorial chemistry compounds, NPs are reputedly difficult to screen and to advance in the R&D pipeline. The molecular complexity of most of the bioactive NPs (*number of asymmetric*

centres, functional groups etc.) often discourages the researcher since their total or semi-synthesis is often not a trivial task. When the accurately identified sample enters the laboratory, its composition can differ according to edaphic and climatic parameters, seasonal variation, and may be contaminated by endophytic micro-organisms (*eg*. endophytic fungi residing in plants). Both terrestrial and marine organisms can contribute and/or modify its chemical composition, due to abiotic and biotic stresses which can be problematic when attempting to re-isolate a class of novel NPs and these aspects should be taken into consideration more often with acuity (Kusari et al. 2012). Undoubtedly, in industry HTS automation is the likely means of targeting bioactivity whilst in many academic institutions due to limitations in funding, HTS is not generally used *in-house* and often requires outsourcing. Of course, utilizing modern techniques of HTS at least in theory, can lead to the identification of NCEs and hits but the serendipity behind analyzing a less uncommon terrestrial or marine organism should not be overlooked (Michael et al. 2008; Frearson and Collie 2009; Macarrón et al. 2011).

The chemodiversity of NPs is much larger than those of synthetic compounds (Feher and Schmidt 2003) and when a screening campaign does not provide any result with classical organic compounds, NPs represents the final opportunity to address this issue. NPs play a significant biological role in terrestrial and marine organisms, and have evolved to interact with enzymes, receptors and ionic channels. They have the reputable advantage of being born to be active in living cells, able to cross membranes, interfere with enzymes or even act against parasites. It is noteworthy to mention that many human pharmaceutical targets have equivalent systems in the organisms studied for their NP content. Natural products belong to chemical families that have been known since the 19th century, but the number of possible combinations is unlimited and minor chemical differences may have significant pharmacological differences. Unquestionably, NPs are not designed for all targets but since many are defence products, they probably address vital and universal processes; this can explain why cancer drug discovery lures most of the research funds than in any other therapeutic area (Dančík et al. 2010).

High-throughput screening (HTS) versus evidenced-based traditional medicine

From the beginning of the 19th century to the 1980s, NP discovery has relied mainly on clinical, pharmacological observations, traditional knowledge, well documented usage (evidenced-based phytotherapy) and/or serendipity. Bioassays performed in the 20th century on small animals and isolated organs provided the vast majority of the chemical entities currently on the pharmaceutical market today. HTS was rising from 1,000 assays to 200,000 assays per day by the middle of the 2000s. Crude extracts were effectively evaluated in 96 and subsequently 384 well plates where the *"target enzymes/receptors"* ushered the combinatorial chemistry

screening methodologies. The subsequent lack of efficacy of the combichem libraries then led to the use of carefully chosen CC libraries that were built around NP skeletons such as the work carried out by Nicolaou in 1999-2000 and Waldmann from 2002 onwards. When Big Pharmas started their discovery programs based on HTS techniques, NPs provided the bioactive diversity, with hundreds of thousands of structurally diverse compounds readily available for robotic automation. This technological revolution helped all the Big Pharmas to shift to this new paradigm. Research started from molecular targets, to the cell, to groups of cells, to isolated organs, to small animals, to larger animals and then finally to patients. The random and systematic evaluation of huge libraries of chemicals likely to modulate a specific biological target is the principle of HTS (David and Ausseil 2014). The series of biological responses of chemical compounds or fractions after a HTS screening campaign produces *"hits."* These results are then controlled, confirmed and validated in order to be amenable in the drug discovery program. *"Hits"* will then be optimised by classical medicinal chemistry reactions in order to become *"leads,"* which then enter into preclinical development. HTS represents several advantages as it is fast and avoids the me-too approach, since this approach can provide unexpected and potentially original active compounds. The very small scale of the assays allows for hundreds of thousands of experiments to be performed within a single day but at this molecular level it is also a disadvantage since the interactions of small molecules with a receptor, an enzyme, and an ionic channel are only the very first steps in the discovery process. Therefore, hits are easily obtained but they are meaningless, valueless and void at the patient scale. As additional, novel elaborate assays for example using, animal/human organs are generated, a significant amount of time and resources would still be inevitably invested. This broad funnelling effect is named attrition and only around 1 in a million of the evaluated molecules in fact escapes this attrition and reaches the market through to FDA approval. Pre-fractionated NP extract libraries are also used in HTS and are demonstrating interesting activities in a wide variety of screens/diseases with novel agents being found from plants, microbes and marine organisms. Eldridge *et. al.,* (2002), describe their HTS process, in which each sample is separated by a parallel 4-channel preparative HPLC into 200 fractions that are then analysed by a parallel 8-channel LC-ELSD-MS. The authors state that 60 % of the analysed fractions contain detectable compounds with 1 to 5 compounds per fraction. A total of 36 fractions containing detectable compounds can be made, and these fractions are collectively called "the library" from which smaller, more focused libraries are drawn for screening and rapid purification, however this leads to a substantial increase in expenditure (Eldridge et al. 2002).

Another disadvantage of HTS versus approaches based on careful selection on well documented traditional usage is the narrowness of the screening. In evidence-based phytotherapy it is likely that the molecular and

pharmacological mechanism will be identified, however when working on NP extracts it is not possible to use a *magic-wand* to isolate every compound present. For example, a single plant extract can contain from hundreds to thousands of structurally different compounds belonging to various compound classes (Verpoorte et al. 2005). Evidence-based phytotherapy is a complementary approach which can provide reliable research orientations by the valorisation of plants traditionally for a long time in the market for which bioactive principles or standardized extracts can be valorised with less risk of toxicity compared to classical HTS based on serendipity. The financial investments necessary to market a new chemical entity (NCE) are substantial and many pharmaceutical groups are relying on the historical, well known active principles on the traditional usage of medicinal agents which represents a valid and reputable source of evidence (Helmstädter and Staiger 2013).

The most important factors for improving the drug discovery process are the enhancement of predictability and confidence of the target (Bunnage 2011). Initially, the methodology began with phenotypic screening, to HTS and then to no phenotypic screening but target based screening in combi-chem/HTS finally to HTS and high content screening (Swinney and Anthony 2011). HTS automation allows for the understanding of a large number of pharmacological experiments on very basic models. Before beginning screening on the basic assay, the next step would be to utilize more sophisticated assays and finally the delivery of a clinical evaluation on humans has to be selected and fully anticipated. This scheduling is required, otherwise the project will reach a bottleneck, this is why high content screenings are appreciated in order to save time-consuming steps. One example includes the zebrafish model which provides many advantages for drug discovery screening programs. The zebrafish, *Danio rerio*, is a small, tropical freshwater fish from India whose embryos are usable as *in vivo* HTS models. It has been nicknamed the *"vertebrate drosophila,"* since it is genetically tractable as a model of developmental biology and the living embryos can be used in the HTS screening in simple and reliable drug discovery programs. Several hundreds of eggs are produced by female, the cost of the embryos is very low and the development is efficient. The zebrafish model is the perfect tool to run High Content Throughput Screening (Challal et al. 2012). The embryos are transparent and it is possible to study the effect of a molecule on different organs at the same time on the living animal and hundreds of transgenic lines are available (Novodvorsky et al. 2013). Zebrafish is an efficient model not only for the drug discovery process but also for target validation, toxicity studies and drug optimization (Li et al. 2012).

Limitations in natural product discovery

Chemotaxonomy

Natural organisms and in particular plants are often collected by the researchers and unlike the medicinal chemists who purchase their starting materials from an *e*-catalogue, the NP researchers' collects the plants themselves or through botanists. When carrying out HTS, collections (terrestrial or marine) need to be substantial to provide optimal sampling. According to chemotaxonomy, a vast systematic diversity within samples will offer a qualitatively vast chemodiversity and a high taxonomic difference between two samples will provide high chemical diversity. However, taxonomy has its limitations especially when dealing with endophytic organisms (eg. bacteria and fungi) residing on terrestrial and marine organisms (eg. plants and algae). If this is the case, it is likely that that a proportion of the NPs isolated from plants are actually products of interactions between microbes in and around the plant than from the plant itself. Working on marine organisms is even more complicated due to the logistics in sample collection, recollection, and taxonomical identification. Most of the time marine organisms are unique biocenosis (biological communities) for example; two organisms living one meter a part from each other can present different chemical compositions.

In one example, Jing and co-workers, isolated three anthracenediones from an unidentified endophytic fungus, colonising mangroves (Jiang et al. 2000). Another example includes the isolation of 4 cytohalasins and 11 novel sesquiterpenoids from the cultures of the mitosporic fungus, *Geniculosporium* sp., an endophyte associated with the red alga *Polysiphonia* sp. (Krohn et al. 2005), therefore consideration should be taken with taxonomical identification. Methods for the isolation and identification of endophytes are well documented by (Zhang et al. 2006).

Obtaining the correct "*Latin name"* for a collected organism or determining the correct scientific name is not trivial. The most difficult task is to source a terrestrial or marine organism in the wild with the correct *genus* and *species*. Herbarium vouchers need to be prepared, since DNA analysis has not yet replaced herbarium samples. Field collections and botanical identification cannot be automated unlike biological screening and combinatorial chemistry (Miller 2011). Implementation of HTS requires only small amounts of plant material, but very quickly, additional amounts will be required for *"hit"* confirmations and even larger quantities for further pharmacological assays and preparation of analogues by medicinal chemistry. Further studies, very dependent on plant recollection are often disappointing since anthropic pressure can lead within only a few weeks to an irreversible loss of organisms and habitats. Respecting the protected species, avoiding duplication in libraries, collecting the correct plant species which are not sterile since identification is based on flower and fruit architecture are noteworthy tasks. The precise scientific nomenclature associated with a collection enables a

fruitful usage of databases; for example the Dictionary of Natural Products (www.dnp.chemnetbase.com) which correlates plant names and chemical content. Valid names and systematic botany could be perceived as limitations but are nevertheless precious for the NP chemist (Erkens 2011). Taxonomy is cumbersome due to the intricate issues of synonymy which add complications to the use of databases and bibliographic references. Systematic botany appears to be out-dated in the midst of the modern *"omics"* techniques but its role is still substantial. The disinterest for systematic botany and the poor renewal of specialists is somewhat alarming as plant taxonomists themselves are an endangered species!

Access and benefit sharing issues

When collecting plants, botanical identification is not the only factor to take into consideration. Respect of rights of the land owner, of diverse laws (protected species), phytosanitary and customs regulations are key and crucial aspects. A significant change occurred on the 29th of December 1993, upon the entry in the application of the Convention on Biological Diversity which moved the genetic resources from common heritage of mankind to the sovereignty of the states where they live. The Convention on Biological Diversity (CBD) was an agreement signed in June, 1992 by the international community in Rio de Janeiro, Brazil. The three objectives of the CBD are to conserve the biodiversity, to sustainably use the genetic resources and to share the benefits arising from the use of these resources in a fair and equitable manner. Article 2 of the CBD, defines biodiversity as –"*the variability among living organisms from all sources of terrestrial, marine and other aquatic ecosystems and ecological complexes*." This includes ecosystem diversity, species diversity between and within species. The scope of the CBD applies only to genetic resources according to Article 2: "Genetic resources" defined as "*any material of plant, animal, microbial or other origin containing functional units of heredity of actual or potential value*." In the 1990s, it was apparent to the Big Pharma industries that the national claims about the genetic resources will overflow to the biological resources. These latter resources being the NPs, the secondary metabolites are valued by pharmaceutical and cosmetics enterprises. This situation created juridical uncertainty which diverted most of the Pharma sector from NP discovery programs. In the late 1990s many pharmaceutical companies decommissioned their NP discovery programs or merged. Further details are presented in Table 1 below.

[Insert Table 1 Here]

To improve this unfavourable situation, the international community united in Nagoya in October, 2010 to clarify access to biological and genetic resources. The Nagoya Protocol (NP) covering access to genetic and non-genetic resources was agreed as an international instrument. NPs will contribute to the conservation and sustainable use of biodiversity through the fair and equitable sharing of benefits arising from their uses which in turn, will result in livelihood gains to both developing and Western countries.

All public or private researchers will be required to seek Prior Informed Consent (PIC) and to negotiate a Mutually Agreed Term (MAT) with the representative of the source country. According to the Aichi objectives, these regulations will have to be implemented in the party states by 2015. Anyone entering in R&D processes will require a PIC and a MAT negotiated with the source country about conditions of Access and Benefit Sharing. According to Article 2, R&D on NPs is clearly covered by this new regulation as per, (Article 2c: *"Utilization of genetic resources"* defined as *"conducting research and development on the genetic and/or biochemical composition, including through the application of biotechnology"*). One should expect flexible accessibility to rules to be realistic and beneficial to all parties. Some biodiversity-rich countries have already implemented national regulations, but their practical applications and interpretation are often cumbersome. There is a disparity between theoretical regulations and their everyday applicability (Kingston 2011). Stringent rules on accessing genetic resources may have counterproductive and paradoxical effects for conservation and research even locally (Gilbert 2010). There is generally a huge asymmetry between expectations of Benefit Sharing of biodiversity-rich countries and the possible benefit sharing of the academic or industrial users in the fields of pharmacy and cosmetology. Often enough, access permits to biodiversity-rich countries are fairly difficult to negotiate and require several years of uncertain processes. The issue and suspicion about bio-prospecting can be explained by historical standing points of exploitation of national, natural resources drifting from colonial times. The seminal contract between Merck and INBio in 1991 was often mentioned as an example to follow, but no commercial results and returns on investments occurred. This contract has received critics later by some NGOs for insufficient local consultations. The improbable large turnover provided by some unique NPs like taxoids unfortunately does not reflect the reality of possible returns from pharmaceutical industries. In the academic sector also, the probability of such success is extremely low. For example, the NCI worked for decades on 114,000 extracts from 12,000 different species to find only paclitaxel and camptothecin (Kingston 2011). Some very important questions remain unanswered: there is no clear cut between the commodities (outside the scope) and the resources included in the scope, the possible retroactivity by date of usage. Access and Benefit Sharing national laws will be implemented, the impact on NP drug discovery

research activities, on innovation and business activities in the near future. Adequate regulations are expected to be taken in order to make this international legislative process effective and conducive towards achieving the access, benefit sharing and conservation of biodiversity objectives.

Future directions in natural product research

Natural product research is still advancing even though it is difficult to maintain long-term expensive R&D programs, especially in the pharmaceutical industry, but this will likely survive and develop in academic universities (Beutler 2009). There are great expectations, on both the pharmaceutical industry and to some extent, academic institutes to identify the elusive, *'magic bullet'*, however it is likely that natural drugs may evolved from single-molecule NPs to well-defined enriched bioactive extracts. As technologies for NPs advances, analyses of limited amounts of compounds present in extracts for biological screening will be possible due to increases in both the sensitivity and dynamic linear range (Hong 2011; Schmitt et al. 2011). Advances in ultra-sensistive analytics for the rapid identification of novel bioactive NPs and sophisticated NMR structure prediction software will continue to improve the efficieny of the NP discovery process (Baker 2007). Natural product studies have an image of sophisticated and often hyphenated techniques that often fails when dealing with complex mixtures. This can be overcome by emerging fields such as metabolomics which deals with mixtures and uses the power of multivariate statistics to identify potential biomarkers (eg. new or a unique class of natural products) (Roessner 2011). Data mining approaches to identify bioactive NPs in mixtures, from a library of terrestrial and marine organisms are currently being developed and will be essential for the development of effective multiple-agent drugs from traditional medicines (Ngo et al. 2013). Another aspect will be the assessment of the variation of the metabolome of body fluids in the framework of clinical trials, involving complex phytopharmaceutical or herbal preparations to completely assess the efficacy of such therapies though a systems biology approach (van Wietmarschen et al. 2009; Wang and Chen 2013).

Conclusions

The industrial usage of dietary supplements, botanicals or phytopharmaceuticals in Western countries and traditional plants in developing countries is expanding readily as well as emerging technologies (eg. metabolomics) and the advent of sensitive analytics will ensure the quality control of these products on the market. Progress in traditional medicine, drug control based research on DNA analyses and gene differential

expression is providing a new frontier. The WHO is aware of the importance of the world's terrestrial and marine biodiversity, especially with regards to medicinal plants which have a track record of offering unique and affordable therapeutic solutions for minor disorders and sometime even major diseases like malaria to both developing and Western countries. Seventy percent of people rely on plants (either as mono-substances or botanical extracts), most of the time, this is not by ideological choice but by economic reasons. Modern techniques for the quality control of traditional drugs will continue to validate the positive benefits, costs-risk ratio of such therapies (Jiang et al. 2010) and, DNA analyses and gene differential expression techniques will hopefully actively develop in pharmerging and less advanced countries. Pharmergings are defined as *"emerging countries in the pharmaceutical world market."* Translational medicine, evidence-based phytotherapy and research focussing on NP-mixtures will provide new insight and innovation. These fields will develop while pharmaceutical productivity is facing a crisis with continued rising R&D costs and reduced drug approvals. Therefore the historical *'Green Rush'* for new chemical entities from biodiversity represented by HTS of natural products between 1990 and 2000 represents only a minor aspect of utilisation and valorisation of these substances in human health. In fact, most of the big pharmas have terminated their HTS and bioprospecting programs. Nowadays, the overall productivity of the big pharma industries is declining despite investment into new technologies such as combichem, rational drug and biotechnologies, but silently NP research is renewing especially through academic sector collaborations. When "blue-chip" companies closed their bioprospecting programs they kept an eye on start-ups and universities.

One important aspect is the preservation of biodiversity and access to organisms, in particular in biodiversity rich countries which constitute a key issue which could hamper a serene utilisation of natural products to develop new drugs (as pure compounds or extracts) in developing countries (Genilloud 2012). Nevertheless, researchers in public and private sectors need juridical security. Access to samples in particular in biodiverse rich countries is problematic and ironically, in the meantime the losses of biodiversity under overexploitation and anthropic pressures have never been so dramatic. Natural products are of high intrinsic value, but they carry many risk factors such as legal access, supply and re-supply, identification of activity, intellectual properties and the value chain is long and uncertain. One of the major challenges is to implement fair, reliable, simple, and transparent access regulations. The forthcoming implementation of the Nagoya Protocol should break the vicious circle of unrealistic expectations and conditions of accessibility to biodiversity for researchers.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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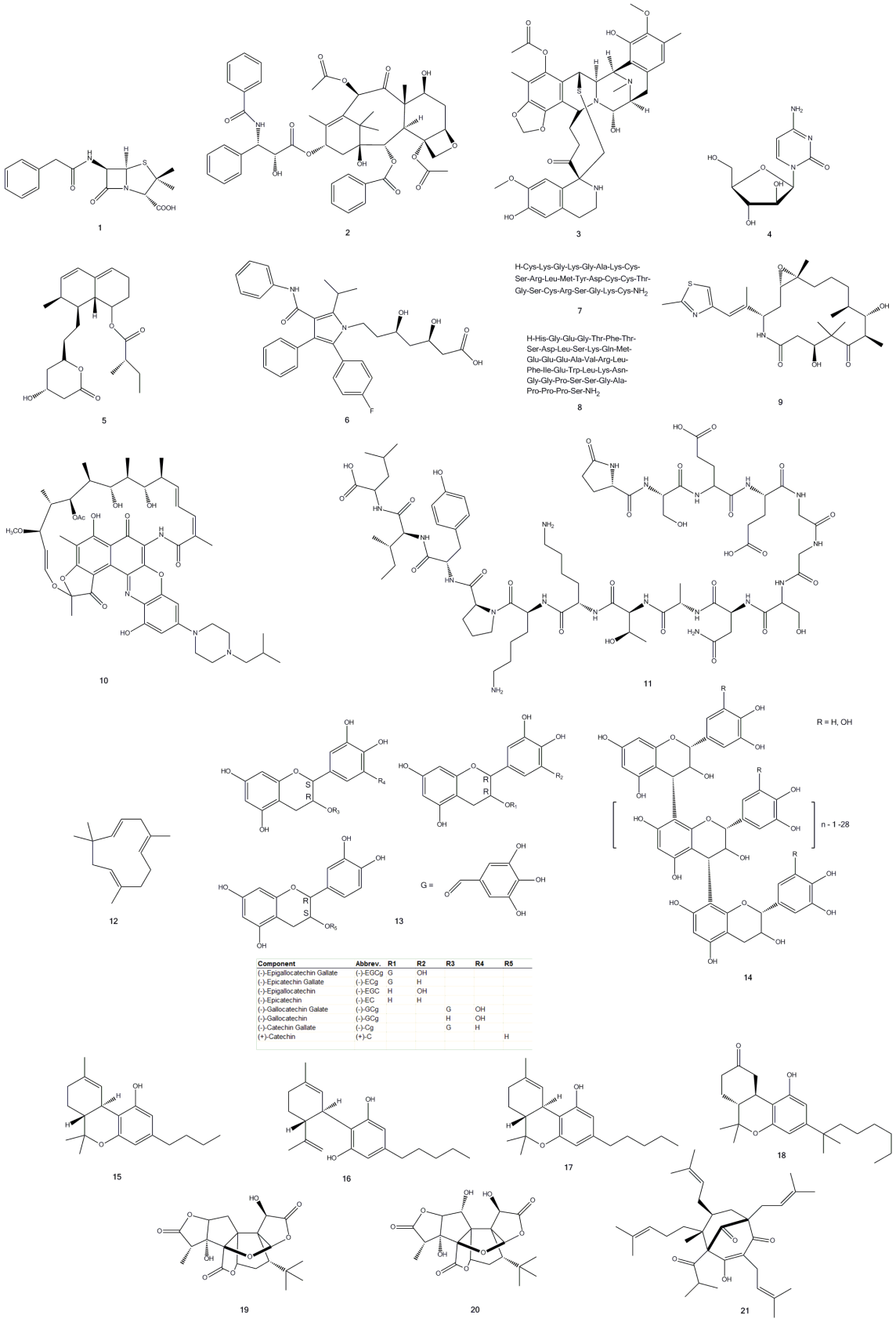
Figures

Figure 1: Structures of well-known natural products.



Component	Abbrev.	R1	R2	R3	R4	R5
(-)-Epigallocatechin Gallate	(-)-EGCg	G	OH			
(-)-Epicatechin Gallate	(-)-ECg	G	H			
(-)-Epigallocatechin	(-)-EGC	H	OH			
(-)-Epicatechin	(-)-EC	H	H			
(-)-Gallocatechin Galate	(-)-GCg			G	OH	
(-)-Gallocatechin	(-)-GCg			H	OH	
(-)-Catechin Gallate	(-)-Cg			G	H	
(+)-Catechin	(+)-C					H

Table 1:

Big/medium Pharma Companies which have currently ceased (between 2000-2013) or are still in bioprospecting. All of these companies (and companies which were subsequently absorbed) were active in bioprospecting in the 1990s'. (To the best of the authors' knowledge, non-official corporate information).

Arrest	**Continuation**
Abbott	Dabur
Astellas	Eisai
Bayer	Novartis
Boehringer Ingelheim	Otsuka
Bristol-Myers Squibb	Pierre Fabre
Daiichi Sankyo	Piramal
Eli Lilly	
GlaxoSmithKline	
Johnson & Johnson	
Kyowa Hakko	
Merck Sharp & Dohme	
Novo Nordisk	
Pfizer	
Roche	
Sanofi	
Servier	
Takeda	

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Title:
The pharmaceutical industry and natural products: historical status and new trends

Date:
2015-04-01

Citation:
David, B., Wolfender, J. -L. & Dias, D. A. (2015). The pharmaceutical industry and natural products: historical status and new trends. PHYTOCHEMISTRY REVIEWS, 14 (2), pp.299-315. https://doi.org/10.1007/s11101-014-9367-z.

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