



Chemical and Biological Screening Approaches to Phytopharmaceuticals

Jemilat Aliyu Ibrahim, Henry Omoregie Egharevba* and Karniyus Shingu Gamaniel

National Institute for Pharmaceutical Research and Development (NIPRD), Industrial Layout Idu, PMB 21 Garki, Abuja, Nigeria

ABSTRACT

The global demand for phytopharmaceutical products is on the upward trend and will probably continue to rise in the next few decades. This demand is fuelled by the growing acceptability, availability and affordability and the growing scientific evidence of efficacy. However, while great progress is being made in research and development of these products in the developed world, very little progress has been made in research, development and documentation of possible leads/products in developing countries of Africa. The challenges range from dearth of capacity to develop implement appropriate research protocols and tools. This article is an attempt toward providing a guide to the chemical and biological screening approaches in the research and development of phytopharmaceuticals. Approaches towards achieving quality products that meets basic regulatory requirements are discussed.

Keywords: Chemical; Biological; Screening approaches; Phytopharmaceuticals; Herbal products; Medicinal plants; Research and development; Quality parameters

INTRODUCTION

The research, development and utilization of traditional medicines and natural substances to address the health challenges is of major strategic and developmental interest especially in the developing countries of Africa, Asia and South America [1-3]. However, research and development approaches into traditional medicines in these countries, especially in Africa, highly fragmented and suffers from poor coordination and strategic investment. Growing scientific evidence in recent times suggest significant contribution of traditional herbal preparations to ameliorating adverse health conditions leading to reduction of excessive mortality, morbidity and disability due to diseases such as malaria, chikungunya and dengue, African sleeping sickness, leishmaniasis, Chagas disease, tuberculosis, sickle-cell anaemia, hypertension, diabetes, HIV/AIDS, and neurobehavioral and mental disorders [1-6]. Consequently, WHO estimates that about 80% of the population in developing countries uses traditional medicine as the first line of treatment [5-7]. Based on the accessibility, affordability, cultural acceptability and presumed efficacy of traditional medicines, particularly traditional African medicines (TAMs), WHO encourages member states, especially African states, to promote and integrate traditional medical practices into their health care delivery systems. The World Health Assembly (WHA) in Resolution WHA42.43 and several others recommended the inclusion of herbal medicine in the health care delivery system, and develop technical guidelines and standard methodologies for research as well as frameworks for their selection and inclusion in healthcare systems [8-11].

Plants have been a source of over 60% of all drugs in modern use, and traditional medical practices that use plant material (herbal medicine) have been good sources of drug leads for many decades [4,6,12-16]. The majority of drugs currently available in the market are natural products, or compounds derived from, or inspired by plant-based natural products. Natural compounds also offer the structural diversity that is not rivalled by the creativity or synthetic ingenuity of medicinal chemists [13,17]. Approximately one-third of the top-selling drugs in the world are natural products or natural product derivatives, indicating a higher hit rate for natural products compared to synthetic chemicals. Furthermore, despite the

billions of dollars currently being spent on drug discovery and development by the pharmaceutical companies, fewer and fewer new drugs are being approved for clinical use, fuelling the urgent search for drug leads from alternative medicine such as herbal medicine. Well documented practices like the traditional Chinese Medicine (TCM) has yielded a number of drug and drug candidates, including Artemisinin (i), Figure 1 the active ingredient of the *Artemisia annua* plant used for treating malaria, which was the subject of the 2015 Nobel Prize in medicine [17,18]. Other drugs/drug leads from traditional medicine include the alkaloids quinine (ii) and cinchonine (iii) from the Peruvian tree *Cinchona officinalis* (Rubiaceae) and other *Cinchona* spp. used for treating malaria, nicotine (iv) from *Nicotiana tabacum* L. (Solanaceae), gedunin (v) from the neem tree *Azadiracta indica*, thymol (vi) from *Ocimum gratissimum* L., rotenone from the Mexican yam bean or Jicama plant, *Pachyrhizus erosus*, taxol (vii) from *Taxus bravifolia*, canthin-6-one (viii), berberine (ix) and chelerythrine (x) from *Z. zanthoxyloides* Watchman. Chemical compounds from plant sources make good drug candidates because they evolved as components of biological systems.

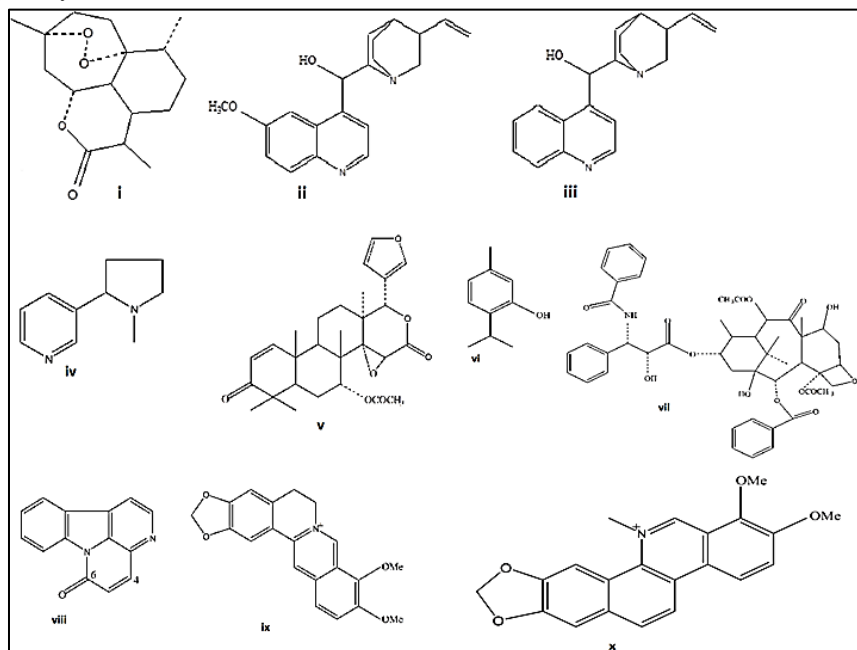


Figure 1: Traditional chinese medicine (TCM) has yielded a number of drug and drug candidates

Modern research and development approach to drug discovery including herbal products requires standardizes protocol, practices and ethics inherent in good agricultural practice (GAP), good laboratory practice (GLP), good manufacturing practice (GMP), good clinical practice (GCP), etc. This is aimed at achieving acceptable standardized products. Critical to all these practices are the chemical and biological screening approaches that established not only the safety and efficacy of the particular products but also their quality or required standards. Herbal drug and cosmetic products like synthetic drug products are composed of active chemicals known as the active pharmaceutical ingredients (APIs) and excipients which are the additives and medium used in formulation. These excipients are themselves chemicals. Hence the interplay of chemicals and biological activity to which all drugs owes their status and identity, leads to the inevitability of chemical and biological processes in determining the safety, efficacy and quality of any drug product. That is, a drug will be considered a failure or substandard primarily if it does not meet its ascribed status or quality through the process of chemical and biological screening. In other words, if we must refer to such substance as drug, then it will qualify as fake drug and if it is a herbal product, then it would be known and called a fake herbal medicine. Due to the seemingly more complex nature of herbal medicine with when compared to synthetic medicine, the subject of standardization of herbal medicine remains a burning issue among professionals and practitioners. This paper is an attempt to highlight biological and chemical screening approach to phytopharmaceuticals towards achieving standardized products and ensuring acceptable quality, especially in Traditional Africa Medicine (TAM) for effective integration into the conventional orthodox healthcare practice.

Herbal Drugs and Cosmetics

Generally, the distinction between drug and cosmetic is blurred and almost a matter of semantics. This is because every so called cosmetic has a drug component and is expected to exert a drug-like effect on the skin or superficial tissues to which they are usually applied. By definition "herbal drugs" otherwise known as "phyto-medicine" connotes plants or plant parts, which have a medicinal or biological or pharmacological activities or the combination of these activities, and have been

converted into phytodrugs or cosmetics through some research, development and manufacturing processes. It denotes that the product is not formulated from pure and singular compounds as the Active Pharmaceutical Ingredient (API). Hence herbal drugs or cosmetics uses active crude extracts, fractions and sub-fraction which contain multiple compound that may be working in synergies [9]. Derived or isolated compounds in the processed state such as extract derivatives or even isolated purified compounds or mixtures of compounds are, as a rule, not included in the definition. Plants/herbs are highly valued all over the world as a rich source of therapeutic agent/s for cure and management of diseases with estimates of over 35,000 species of plants used for medicinal purposes all over the world [19]. Africa is endowed with a rich diversity of flora and it is estimated to contain 68,000 plant species and of which 35,000 species are endemic to the continent. The active principles for the development of many drugs in current use came from natural compounds derivable from plants of African origin. Due to the poor research culture, the practice of TAM has remained largely empirical, undocumented and to great extent embedded in superstition. Only an estimated 10% of Africa's rich biodiversity has any documented medicinal use, with the remaining 90% yet to be discovered or documented. WHO estimated that about 85% of traditional medicine involves the use of plants or plants extracts [20] and the main source of all cosmetics used before the advent of synthetics substance were all from plants or plant made products [21]. And also, many drugs presently prescribed by physicians are either directly isolated from plants or artificially modified versions of natural products. Ten to twenty five percent of pharmaceutical commodities e.g. prescription drugs prescribed in conventional western medicine contains one or more ingredients derived from plants, a natural product [22].

Secondary Metabolites from Plants

A natural product is simply defined as a small molecule that is produced by a biological source e.g. plants, animals etc., and it is sometimes synonymously referred to as secondary metabolites. Hence, natural product research is focuses on the chemical properties, biosynthesis and biological functions of secondary metabolites [23]. These secondary metabolites which are responsible for the biological activities and therefore the active pharmaceutical ingredients in herbal drugs, determines the value of a particular medicinal plant. These active secondary metabolites usually belongs to diverse classes of organic molecules and macromolecules such as alkaloids, saponins, flavonoids, terpenoids, anthraquinones, sterols, cardiac glycosides, phenolics, phlobatannins, tannins, polyphenolics, volatile oils (essential oil components), etc. The major macromolecules usually include proteins, peptides, carbohydrates, fats/oils, etc. Biological screening for efficacy or chemical screening of biologically active plant substance have been carried out by several workers in US, China and other parts of the world, and has almost become a routine in recent times in the developed countries due to the desire to integrate herbal medicine with orthodox medicine. Statistics has it that by 2007, about 3563 extracts and 5,000 single compounds from 3,000 traditional herbal medicines (THMs) had been collected by the Chinese in TCM [24], about 114,000 extracts had been screened from an estimated 35,000 plant samples against a number of tumor systems as early as before the 1990s, by the United States [25], over 139,000 diverse array of natural products which are potential candidates for drug development have been collected in the recent past few decades [24]. Finally, between 1981 and 2006, 47.1% of a total of 155 clinically approved anticancer drugs were derived from nature in North America, Europe and Japan market after passing the required chemical and biological tests [26].

Approaches to Herbal Drug and Cosmetic Discovery

Generally, new drug discovery involves the identification of biologically active chemical entities called New Chemical Entities (NCEs), and these NCEs can be sourced synthetically (chemical libraries) or naturally from sources like plants, marine, fungi etc. With advancement in chemical techniques, single chemical entities, which are judged to be more consistent and easier to quantify, is said to be specific in their therapeutic focus than natural products such as phytopharmaceuticals with several chemical entities. This led to use of conventional methods where biology and chemistry are combined for bioassay-guided isolation, structure determination and mechanism elucidation, etc. (Figure 2 and Table 1), which have led to the identification and characterization of individual chemical entities from plants that are now used for single compound drugs [27]. However, several researchers have documented that bioactivities of a plants extract which were initially active may disappear when fractionated into individual chemical entities [28-30].

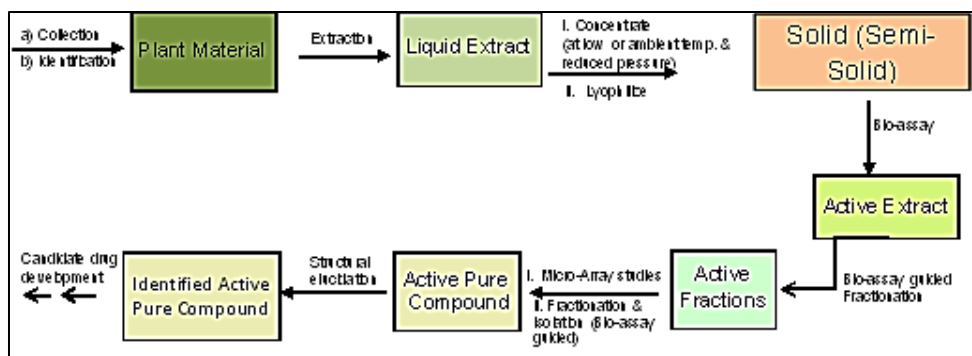


Figure 2: Schematic representation of modern approach to drug discovery from medicinal plants

Table 1: Approach to modern drug discovery from medicinal plants

Steps	Activity	Expected Output
1	A survey of medicinal plants traditionally used to treat of disease of interest	Establishment of a database of medicinal plants used to treat disease of interest
2	Establishment of relevant <i>in vivo</i> and <i>in vitro</i> models of screening selected plants for disease of interest	Rapid screening of medicinal plants extracts used to treat targeted disease through high throughput screening - usually <i>in vitro</i> models
3	Bioassay guided fractionation and isolation of active compounds from lead medicinal plants	Isolation and characterization of targeted bioactive compounds responsible for observed activity from selected medicinal plant extracts and fractions
4	Synthesis of analogues and derivatives	Chemical and biological screening of analogues or derivatives to determine identity, purity, yield, and efficacy
5	Preparation or formulation of phytomedicines/drug and effective delivery systems of the new candidate drug	Development of a candidate phytomedicines/drug
6	Preclinical studies of developed drugs	Establishment of safety and efficacy of phytomedicines/ drug in animals
7	Phase II clinical trial	Establishment of safety and efficacy of phytomedicines/ drug in humans

Information from traditional knowledge have speed up the drug discovery process as it has reduced the impediments that usually accompanied drug discovery and development in terms of time and money spent, and also in occurrence of toxicity when following the conventional method of random screening and chemical synthesis. Usage of botanical sources as starting point in the drug development program is associated with few specific advantages and disadvantage, for instance: Knowledge of historical use by local confers the assumption that the plant will likely be safe and efficacious compared to plants without historical usage. Novel molecules which are active can be derived or modified from the original compound due to limitation of the original compound. Concerns over pressure that would be exert on the natural resources from over-exploitation. Intellectual property rights protection and the adoption of conservation and biodiversity (CBD) guidelines on access and benefit sharing of genetic resources by most countries tend to impede the pace of discovery process. An estimated 250,000 species of higher plants have reportedly been screened for biological and phytochemical activities respectively [31].

Biological and Chemical Screening Approaches

Generally speaking, screening of Herbals is usually for the purposes of research and development, quality control and development of standards, as well as regulatory requirements. Biological and chemical screening of herbs progressed from the research and development viewpoint towards development of standards for quality control and regulatory purposes. Chemical and biological screenings generally focus on identity, quality, safety and efficacy [8]. Biological screening involves the use of various biological systems, techniques and methods, which may include mechanical equipment, electronic and optical instruments, behavioural observation, *in vitro* and *in vivo* assays, mathematical modelling, etc. Chemical screening deploys majorly chromatographic and spectroscopic techniques as well as reaction chemistry to determine material identity, composition and behaviour. However, in drug discovery or quality monitoring, biological and chemical screening goes almost paripassu.

Screening Approach in Research and Development of Phytopharmaceuticals

Research and development of a herbal drug is usually different from those of a synthetic medicine whose active ingredient is known [9,10]. Recent approach to herbal medicine development follows random selection and screening of medicinal plant which has been selected from knowledge of traditional use or related literature information, and taken through conventional methods that combines biology and chemistry to determine active extract, fractions and isolated compounds, which are structurally elucidated and characterized (Table 1). Hence development is usually bioassay guided. Due to the fact that the

plants and their active principles are usually unknown and has to be subjected to systematic study, and modern technologies have evolved the automated high throughput screening (HTS) that allow hundreds of thousands of extracts and fraction or isolates to be screened for bioactivity within a reasonably short time, other fast screening *in vitro* methodologies that use enzyme immunoassays, gene cloning, tissue culture, etc. for bioactivity have been developed and incorporated in to HTS for use in the fields and laboratories. Chemical screening is important to establish the source and nature of the source of biological activity, quality parameters for raw materials and finished product, and develop or modify existing methodologies for better yield. Chemical screening of crude extracts and fractions are qualitative and quantitative. It include solubility studies (including extraction study), compounds classification studies (usually called phytochemical screening), ash and moisture contents, profile of heavy metals and mineral components, extract profiling and elucidation, etc. using classical approach and analytical tools like the TLC, HPLC, UV-VIS, FTIR, MS NMR and AAS. These chemical screening approaches combine spectroscopic and chromatographic principles in analytical instruments which couple chromatographic equipment which help in the separation of chemical constituents based on chromatographic principles to spectroscopic equipment for the spectroscopic or spectrometric elucidation. For proteins and other charged groups, electrophoretic methods are deployed [9,10]. The unique features of chemical entities of natural origin pose a string of challenges for medicinal chemists who try to develop analogues, either to improve the absorption or to reduce the toxicity and improve upon efficacy which is often achieved by addition or deletion of selected functional groups.

Before proceeding to the clinical phases of development, pharmacological and toxicological studies are usually carried out in animals (lower primates) to determine safety and efficacy. Modern approach tries to eliminate animal study through tissue culture study, enzyme and protein receptor cloning and other biotech techniques etc., due to animal's rights issues. Ethical requirement for studies involving animals requires that the researchers must obtain clearance from Ethics Review Committee or Boards. A simplified approach to biological and chemical screening in R&D of herbal drugs and cosmetics after plant selection, identification include.

Biological Screening

Activity screening includes safety/toxicity and efficacy:

The tremendous progress made in life sciences has resulted in the definition of many pathological processes and mechanisms of drug action. This advancement has led to the establishment of various molecular and cellular bioassays in conjunction with High throughput screening (HTS) methods. HTS decreases the amount of testing compound required such that only microgram quantities are needed. This is advantageous for certain natural products that are difficult to isolate and purify, and permits compounds that are difficult to synthesize to be readily assayed. Thus, due to resource limitation, it is always better to begin screening of extracts and fractions with *in vitro* assays to establish activity. There are high throughput *in vitro* screening methodologies that involve the use of cell and/or tissue culture, enzyme-binding ability etc., to determine antimicrobial, cytotoxicity, and other pharmacological activities. *In vivo* studies involve the use of whole animal and/or whole organ. *In vivo* study usually consumes more resources and is recommended after the *in vitro* assay or where there is no facility for the *in vitro* assay. This is because result of *in vitro* study could serve as guide for the *in vivo* study. The use of animals must be approved by the animal ethics review committee of the research organization to ensure ethical handling of animals. *In vivo* toxicity studies usually extend from acute to chronic toxicity studies. Results obtained from both studies are used in drug formulation and subsequent studies.

Microscopic examination and determination: Though this not be activity related it is relevant for material identification and authentication. This usually applies to starting raw material before extraction is done, and helps to determine phenotypic characteristics such as appearance and physical description of the parts used, presence of adulterants such as insects and microbial contaminants e.g., pollens, molds, as well as stones and sand, etc., thus enhancing quality.

Chemical Screening

Common approach to chemical screening to establish the chemical constituents of plants or plants extracts follows the sequence of:

Extraction and solubility study:

Extraction is the process of solubilising constituent compounds from the plant material with the use of solvents which may be organic or aqueous in nature. The filtered extract solution is usually concentrated to dryness at low temperature under reduced pressure to obtain the dry extract. Simple extraction methods include solvent extraction, and hydro distillation. Different techniques and instrumentation of solvent extraction are available which may include maceration, soxhlet extraction, counter-current extraction, etc. The yield of extract is determined to know the suitability of a particular method or solvent. Also the solubility of the targeted extract, fractions or isolated compound is of paramount interest to the scientist. This is because the extract or compound is usually needed in solution before other analyses including bioactivity screening can be conducted. Major solvents for solubility determination include methanol, ethanol, propanol, chloroform, ethyl

acetate, carbontetrachloride, dichloromethane, benzene, cyclohexane, n-hexane, tetrachloromethane, n-butanol, acetic acid, acetone, dimethylsulfoxide (DMSO), Tween 80 and water, etc.

Phytochemical screening to determine classes of secondary metabolites: This used colour change reaction and complexation chemistry to establish classes of organic components present in the plant extract. Phytochemical screening may help to redirect efforts by revealing interesting classes of compound known for pharmacological activities such as alkaloids, triterpenoids, saponins, cardiac glycosides, tannins, anthraquinones, etc. When such classes are revealed, researchers usually direct their efforts towards isolating novel compounds of interest.

Bioassay guided chromatographic fractionation and isolation of targeted compounds: Isolation and purification of compounds from the extract is achieved through components separation. Chromatographic techniques such as liquid chromatography (LC), gas chromatography (GC), thin layer chromatography (TLC), etc. are used to carry out component separation before purification and characterization of the compounds. Some of these chromatographic techniques have been integrated with detectors that use spectroscopic principles in a hyphenated method. Examples include gas chromatography mass spectrometry (GCMS), liquid chromatography mass spectrometry (LCMS), high performance liquid chromatography (HPLC), etc. The frequently used laboratory technique is the column chromatography and HPLC.

Chemical profiling: This employs chromatographic and spectroscopic techniques such as TLC, HPLC, GCMS, UV-VIS, FTIR, NMR, etc., to establish a finger-print or profile of a particular extract. Sometimes such finger-print or profile are established with reference to a marker compound identified in the plant. **Structural elucidation:** The chemical structure of isolated compounds which are active or could serve as markers compounds for the plant are done by combination of spectroscopic techniques such as UV-VIS, IR, MS and NMR. These techniques reveals the nature of compounds including the sub-molecular level such as nature of functional groups, type and number of protons, carbons, oxygen and nitrogen atoms, molecular mass, etc., and the bonding interactions between these atomic moieties. Such information enables the organic chemists to determine the chemical structure of the particular compound. **Physicochemical characterization:** The physicochemical parameters of are used to establish material's or compound's identity due to their uniqueness. Examples include parameters like freezing point, melting point and boiling points, colour, crystal shape, natural form, smell, solubility, volatility, texture, behaviour when exposed to air and moisture, etc. Physicochemical properties are usually determined for raw materials, isolated compounds and finished products to define their unique identity which will serve as reference for determining adulteration or counterfeits. **Proximate and elemental analyses:** This is mostly done for raw materials or extracts to determine their ash and moisture contents as well as mineral composition especially the level of heavy metals such as lead, cadmium, mercury, chromium, vanadium, arsenic, zinc, iron, copper, tin, etc. The ash content is a measure of the inorganic or mineral components in the plant while moisture content is a measure of hygroscopicity of the dry material. The hygroscopicity of the dry material is a pointer to the extent it could be stored or exposed to moisture without growing molds or supporting microbial growth. The African Pharmacopeia (1986) recommended moisture content for vegetable drugs is 8-14% [32]. Proximate and elemental analysis is important for establishing the quality and safety of raw material to be used. The elemental analysis which is usually done with the aid of atomic absorption spectrophotometry (AAS) reveals the levels of heavy metals which may be detrimental when high amount is consumed, as well as essential and mineral elements like sodium, magnesium, calcium, potassium, etc.

Approach to Screening of Phytopharmaceuticals for Quality and Regulatory Requirement

Another objective of chemical and biological approaches to screening of phytopharmaceuticals is for the preparation of monographs of indigenous herbal drugs, and attainment of regulatory requirements for registration as drugs by concerned regulatory bodies such as United States FDA and the ISO. Local drug regulators in Africa like the Nigerian National Agency for Food and Drugs administration and Control (NAFDAC) also require certain standards to be met for any phytopharmaceuticals entry the Nigerian market. Quality control is founded on the questions of Identity – that is, is the herb the one it should be? Purity – that is, are there contaminants?, Quantity of content – that is, is the amount of active ingredient within the defined limits?

Essentially due to the multi-component composition of herbal drugs/cosmetics, they are prone to material variation caused by geographical source, harvest time and method, season of harvest, species variation and similarity, etc. These possible challenges create the need for standardization of raw materials and herbal drugs products (finished products). Biological and chemical screening approach in quality assurance and establishments of standards for phytopharmaceuticals seeks to establish set parameter for quality evaluation based on safety, efficacy and composition. While biological screening addresses validation of safety (microbial contaminants) and efficacy, chemical screening addresses validation of constituent composition (usually through marker compounds, spectroscopic or chromatographic profiles, etc.) and presence of high levels of poisonous heavy metals and chemical contaminants such as pesticide and herbicide residues. Biological screenings are mostly designed for rapid testing using rapid test kits and microwell plates or automated HTS for multiple sample handling. HTS used microgram quantities or less which is desired for compounds that are difficult to isolate and synthesis. HTS is also able to analyse multiple samples sometimes running into hundreds of thousands, thus reducing the average time

required for analysis. Chemical screening use standardized methods available in published pharmacopeias to establish chemical profiles and presence of marker compounds. Spectroscopic techniques like AAS are used to determine presence of harmful heavy metal contaminants. For volatile substances, pesticide and herbicide residues, GCMS, LCMS and related techniques are applied.

Common quality parameters include ash content and moisture content, heavy metals pollutants, microbial contaminants and aflatoxin, pesticide and herbicide residues, radioactive contamination and presence of marker compounds or peaks.

CONCLUSION

The demand and amount of phytopharmaceuticals entry the global market is on a steady rise obviously due to the growing acceptance. China, Korea, India and Brazil remains the world leader in research and production of medicinal plant products. It is on record that between 1911 and 2000, the Chinese were able to isolate different bioactive plant compounds like alkaloid, steroid, triterpene, limonoid, etc. through bioassay guided approach [33]. The large number of medicinal plant yet to be evaluated and documented in Africa and other developing countries will benefit from the chemical and biological screening approach discussed in here. If Africa is to become a major player with its vast biodiversity resources, it must begin the process of research and development of these resources through an aggressive programme that ensures quality products through the processes enumerated in this paper. Currently, possible biological activity of a herbal product/herbal extract can be speculated on base on its phytochemical composition. For example:

Plant polysaccharides are known to exhibit stimulating or suppressing effect on the immune system e.g. *Ganoderma lucidum* (LeysserFr.)Karst, *Cordyceps sinensis* (Berk.) Sacc [34,35]. Phenolic compounds like Flavonoids, which include flavones, isoflavone, flavonols, flavonones, and xanthenes, and non-flavonoid like lignin, stilbenes have been shown to possess strong antioxidant, anti-inflammatory, antiproliferative, and antiaging activities [36-38]. Amino acids and proteins in herbs are usually regarded as natural nutritional supplements for patients recovering from diseases [39].

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